Transfert de mitochondries des cellules souches mésenchymateuses aux cellules souches de glioblastome :
effets sur le métabolisme et la résistance à la chimiothérapie
Jean Nakhle

► To cite this version:

HAL Id: tel-03510337
https://tel.archives-ouvertes.fr/tel-03510337
Submitted on 4 Jan 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
THÈSE POUR OBTENIR LE GRADE DE DOCTEUR
DE L’UNIVERSITÉ DE MONTPELLIER

En Biologie Santé

École Doctorale Sciences Chimiques et Biologiques pour la Santé (CBS2 № 168)

Unités de recherche INSERM UMR1183 – CNRS UMR5535

Transfert de mitochondries des cellules souches mésenchymateuses aux cellules souches de glioblastome :
Effets sur le métabolisme et la résistance aux chimiothérapies

Présentée par Jean NAKHLE
Le 15 décembre 2020

Sous la direction de Marie-Luce VIGNAIS, CR
et Valérie DARDALHON, CR

Devant le jury composé de

Hugues DUFFAU, Prof., IGF, CNRS, INSERM, Univ. Montpellier, Montpellier, France
Norbert BAKALARA, Prof., CBMN, CNRS, Univ. Bordeaux, Bordeaux, France
Thierry VIROLLE, DR, IBV, CNRS, INSERM, Univ. Côte d’Azur, Nice, France
Anne-Marie RODRIGUEZ, CR, IMRB, INSERM, Univ. Paris-Est, Créteil, France
Laetitia LINARES, CR, IRCM, INSERM, Univ. Montpellier, Montpellier, France

Président
Rapporteur
Rapporteur
Examineur
Examineur
TO MADELEINE & ELIE
TO ANGELA & ANTHONY

TO BEIRUT
ACKNOWLEDGEMENTS

Since research is the “teamiest of team sports”, I would like to acknowledge the contributions of the people who shared my day-to-day life for the last three years and without whom this piece of work would not have come to fruition; the people who shaped the “young researcher” that I am today and whose lessons I will carry for the remainder of my career; the people who helped me blossom as a human.

Marie-Luce VIGNAIS, my thesis director, who took a chance on the 23-year-old Lebanese student who had just hopped off the plane, for her scientific supervision, her rigor, her integrity, her work ethic, her thoughtfulness and her presence on the daily. Everything I have learnt over the course of my thesis, I owe it to you.

Valérie DARDALHON and Naomi TAYLOR, who welcomed me and stood by me in my time of need. This work would not have been possible if it weren’t thanks to you.

Norbert BAKALARA and Thierry VIROLLE, who reviewed this manuscript, for their time and their constructive comments. Your critical eye enriched my work and I learned a lot from your expertise during our many exchanges.

Anne-Marie RODRIGUEZ, who offered me the opportunity to participate in my very first review article (and the collaboration lives on). Your knowledge on mitochondrial transfers taught me a lot.

Hugues DUFFAU, who invited me into his operation room. You showed me the most riveting and the most impressive thing I have ever witnessed.

Laetitia LINARES, who gave me important pieces of advice during my thesis committee. I look forward to your input during my defense.

Sabine GERBAL-CHALOIN, Martine DAUJAT-CHAVANIEU and Philippe BRIOLOTTI, who helped me every single day and passed on their immense knowledge, theoretical and practical, to me. It will be difficult to find colleagues and office neighbors like you.

Christian JORGENSEN and Jean-Marc BRONDELLO, who welcomed me within the group.

Daouda MOUSTAPHA ABBA MOUSSA, who helped me in my (many) Seahorse experiments. I will miss our weekly appointments and your good spirit.

Adel BOUGHLITA, who performed the time-lapse microscopy and took the amazing video.

Nicolas BUISINE, who performed the RNA-seq analyses. Your mind never ceases to amaze me.

Lara GALES and Florian BELLVERT, who performed the Mass Spectrometry experiments. Thank you for your professionalism.

Yalda HEKMATSHOAR, Tülin ÖZKAN and Kateřina LNĚNIČKOVÁ, the dream team, who became family.

All my IRMB colleagues, who made the laboratory feel like home.
**Summary**
Glioblastomas are heterogeneous tumors with high metabolic plasticity. Their poor prognosis is linked to glioblastoma stem cells (GSCs) which provide resistance to therapy, in particular to temozolomide (TMZ). It is worsened with the recruitment of mesenchymal stem cells (MSCs) to the tumor microenvironment. We show that, following tunneling nanotube interactions, MSCs transfer mitochondria to GSCs. We found that MSC mitochondria modify the metabolic response of GSCs to TMZ, by increasing their OXPHOS and their production of metabolites linked to the TCA cycle, the pentose phosphate pathway and the pyrimidine/purine synthesis. A RNA-seq analysis revealed that MSC mitochondria also disrupt the GSC transcriptional response to TMZ, leading to the expression of genes related to DNA damage and cell cycle. These observations were linked to enhanced GSC survival to TMZ. Together, our data show that the mitochondria transfers that originate from cells of the tumor microenvironment can modify the response of cancer cells to therapy, at both levels of cellular metabolism and gene expression.

**Keywords**
Mitochondria, metabolism, metabolites, glioblastoma stem cells, mesenchymal stem cells, tumor microenvironment, mass spectrometry, RNA-seq, mitochondrial DNA

**Résumé**
Les glioblastomes sont des tumeurs hétérogènes à haute plasticité métabolique. Leur mauvais pronostic est lié aux cellules souches de glioblastome (GSC) qui offrent une résistance au traitement, en particulier au témozolomide (TMZ). Cette résistance est aggravée par le recrutement de cellules souches mésenchymateuses (CSM) dans le microenvironnement tumoral. Nous montrons que, suite à des interactions de type nanotubes, les MSC transfèrent des mitochondries aux GSC. Nous avons constaté que les mitochondries de MSC modifient la réponse métabolique des GSC au TMZ, en augmentant leur OXPHOS et leur production de métabolites liés au cycle de Krebs ainsi qu’aux voies de synthèse du pentose phosphate, des pyrimidines et des purines. Une analyse RNA-seq a révélé que les mitochondries de MSC perturbent également la réponse transcriptionnelle des GSC en réponse au TMZ, conduisant à l’expression de gènes liés aux dommages à l’ADN et au cycle cellulaire. Ces observations ont été liées à une survie accrue des GSC au TMZ. Nos données montrent que les transferts de mitochondries qui proviennent des cellules du microenvironnement tumoral peuvent modifier la réponse des cellules cancéreuses à la thérapie, tant au niveau du métabolisme cellulaire que de l'expression des gènes.

**Mots-clés**
Mitochondries, métabolisme, métabolites, cellules souches de glioblastome, cellules souches mésenchymateuses, microenvironnement tumoral, spectrométrie de masse, RNA-seq, ADN mitochondrial
# Table of Contents

**Acknowledgements** 3

**Summary** 4

**Table of Contents** 5

**Abbreviations** 7

**Thesis Project** 10

  - **General Background** 11

**Introduction** 14

**Chapter 1: Mitochondria Transfer via Tunneling Nanotubes from Mesenchymal Stem Cells to Target Cells** 15

  1. **Mesenchymal Stem Cells** 18
     1.1. **Definition** 18
     1.2. **Mesenchymal Stem Cell Homing to Tumor Sites** 19
     1.3. **Mesenchymal Stem Cell Pro-Tumorigenic Role** 20
        1.3.1. **Angiogenesis** 20
        1.3.2. **Metastasis** 21
        1.3.3. **Immunosuppression** 22
        1.3.4. **Drug Resistance** 23

**Review 1: Intercellular Mitochondria Trafficking Highlighting the Dual Role of Mesenchymal Stem Cells As Both Sensors and Rescuers of Tissue Injury** 25

**Chapter 2: Role of Metabolism in Cancer Drug Resistance** 35

**Review 2: The Role of Metabolism and Tunneling Nanotube-Mediated Intercellular Mitochondria Exchange in Cancer Drug Resistance** 37

**Review 3: Multifaceted Roles of Mitochondrial Components and Metabolites in Metabolic Diseases and Cancer** 61

**Chapter 3: Glioblastoma** 92

  1. **Introduction** 93
  2. **Glioblastoma Classification** 95
     2.1. **Genetic Subclasses** 95
     2.2. **Transcriptional Subclasses** 97
        2.2.1. **Implication in Glioblastoma Heterogeneity** 98
     2.3. **Methylation-Based Subclasses** 99
  3. **Glioblastoma Stem Cells** 101
     3.1. **Glioblastoma Stem Cell Discovery** 101
     3.2. **Glioblastoma Stem Cell Plasticity** 101
     3.3. **Glioblastoma Stem Cell Isolation and Enrichment in vitro** 102
     3.4. **Glioblastoma Stem Cell Models in vivo** 104
     3.5. **Glioblastoma Stem Cell Model Limitations** 105
  4. **Glioblastoma Microenvironment** 108
     4.1. **Brain Compartment** 109
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2.</td>
<td>Vasculature</td>
<td></td>
</tr>
<tr>
<td>4.3.</td>
<td>Immune Cells</td>
<td></td>
</tr>
<tr>
<td>4.4.</td>
<td>Mesenchymal Stem Cells</td>
<td></td>
</tr>
<tr>
<td>4.4.1.</td>
<td>Mesenchymal Stem Cell Recruitment to the Glioblastoma Microenvironment</td>
<td></td>
</tr>
<tr>
<td>4.4.2.</td>
<td>Role of Mesenchymal Stem Cells in Glioblastoma Progression and Resistance</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Glioblastoma Metabolism</td>
<td></td>
</tr>
<tr>
<td>5.1.</td>
<td>Glucose Metabolism</td>
<td></td>
</tr>
<tr>
<td>5.2.</td>
<td>Krebs Cycle</td>
<td></td>
</tr>
<tr>
<td>5.3.</td>
<td>Glutamine Metabolism</td>
<td></td>
</tr>
<tr>
<td>5.4.</td>
<td>Lipid Metabolism</td>
<td></td>
</tr>
<tr>
<td>5.5.</td>
<td>Link Between Tumor Microenvironment, Molecular Signatures and Metabolic Phenotypes of Glioblastoma</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Glioblastoma Resistance to Therapy</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Tumoral Network in Glioblastoma</td>
<td></td>
</tr>
<tr>
<td>7.1.</td>
<td>Long-Distance Intercellular Communication in the Brain</td>
<td></td>
</tr>
<tr>
<td>7.2.</td>
<td>Long-Distance Intercellular Communication in Glioblastoma</td>
<td></td>
</tr>
<tr>
<td>7.2.1.</td>
<td>Tunneling Nanotubes (TNTs)</td>
<td></td>
</tr>
<tr>
<td>7.2.2.</td>
<td>Tumor Microtubes (TMs)</td>
<td></td>
</tr>
<tr>
<td>RESULTS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Article 1: Acquisition of Exogenous MSC Mitochondria Modifies the Metabolic and Functional Response of Glioblastoma Stem Cells to Temozolomide Treatment</td>
<td>134</td>
<td></td>
</tr>
<tr>
<td>Article 2: Methods for Simultaneous and Quantitative Isolation of Mitochondrial DNA, Nuclear DNA and RNA from Mammalian Cells</td>
<td>178</td>
<td></td>
</tr>
<tr>
<td>DISCUSSION &amp; PERSPECTIVES</td>
<td></td>
<td>186</td>
</tr>
<tr>
<td>MATERIALS &amp; METHODS</td>
<td></td>
<td>197</td>
</tr>
<tr>
<td>MitoCeption</td>
<td></td>
<td>198</td>
</tr>
<tr>
<td>Seahorse Analysis</td>
<td></td>
<td>199</td>
</tr>
<tr>
<td>FACS</td>
<td></td>
<td>203</td>
</tr>
<tr>
<td>Mass Spectrometry</td>
<td></td>
<td>204</td>
</tr>
<tr>
<td>Nucleic Acid Purification – TRIzol Method</td>
<td></td>
<td>206</td>
</tr>
<tr>
<td>Nucleic Acid Purification – QIAGEN COLUMN Method</td>
<td></td>
<td>207</td>
</tr>
<tr>
<td>RNA-Sequencing Analysis</td>
<td></td>
<td>208</td>
</tr>
<tr>
<td>RESUME EN FRANÇAIS</td>
<td></td>
<td>211</td>
</tr>
<tr>
<td>ANNEX</td>
<td></td>
<td>218</td>
</tr>
<tr>
<td>Book Chapter: Tunneling Nanotubes (TNTs): Cell-to-Cell Communication</td>
<td>219</td>
<td></td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td></td>
<td>229</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

ACBP: Acyl coenzyme A binding protein
ACC: Acetyl coenzyme A carboxylase
Acetyl-CoA: Acetyl coenzyme A
ACLY: ATP citrate lyase
ACSS: Acetyl coenzyme A synthetase
Ang1: Angiopoietin 1
ASCT2: Solute carrier family 1 member 5
ATP: Adenosine triphosphate
ATRX: Alpha-thalassemia/mental Retardation syndrome, X-linked
CALD1: Caldesmon
CCL2: C-C motif chemokine ligand 2
CD105: Endoglin cell surface marker
CD133: Prominin 1
CD15: Fucosyltransferase 4
CD39: Ectonucleoside triphosphate diphosphohydrolase 1
CD44: Hematopoietic cell E- and L-selectin ligand
CD73: 5’-nucleotidase cell surface antigen
CD90: Thy-1 cell surface antigen
CDK: Cyclin-dependent kinase
CDKN: Cyclin-dependent kinase inhibitor
CEP: Circulating endothelial progenitor cells
CIMP: CpG island methylator phenotype
CNN1: Calponin 1
CPT1: Carnitine palmitoyl transferase 1
CRYAB: alpha-Crystallin B
CSPG4/NG2: Chondroitin sulfate proteoglycan 4/Neural glial antigen 2
CTGF: Connective tissue growth factor
Cx43: Connexin 43
CXCL12: C-X-C motif chemokine ligand 12
CXCR4: C-X-C motif chemokine receptor 4
DNA: Deoxyribonucleic acid
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
EMT: Epithelial-to-mesenchymal transition
ERK: Extracellular regulated kinase
FABP7: Fatty acid binding protein 7
FASN: Fatty acid synthase
FGF2: Fibroblast growth factor 2
FSP/S100A4: Fibroblast specific protein/S100 calcium-binding protein A4
GAP43: Growth-associated protein 43
GASC: Glioma-associated stem cells
GCSF: Granulocyte colony-stimulating factor
GLN: Glutamine
GLS: Glutaminase
GLUT: Glucose transporter
GS: Glutamine synthase
GSEA: Gene set enrichment analysis
HGF: Hepatocyte growth factor
HK: Hexokinase
IDH: Isocitrate dehydrogenase
IFN: Interferon
IL: Interleukin
JAK: Janus Kinase
L1CAM: L1 cell adhesion molecule
LAG3: Lymphocyte activating 3
LDHA: Lactate dehydrogenase A
MCP1: Monocyte chemoattractant protein 1
MDM2: Mouse double minute 2
MGMT: O^6^-methylguanine-DNA methyltransferase
mTOR: Mechanistic target of rapamycin kinase
MYC: Avian myelocytomatosis viral oncogene homolog
NF-κB: Nuclear factor kappa B
NF1: Neurofibromin 1
NLGN3: Neuroligin 3
NMR: Nuclear magnetic resonance
OLIG2: Oligodendrocyte transcription factor 2
PC: Pyruvate carboxylase
PD-L1: Programmed cell death ligand 1
PD1: Programmed cell death 1
PDGFRA: Platelet derived growth factor receptor alpha
PDH: Pyruvate dehydrogenase
PDP: Pyruvate dehydrogenase phosphatase
PDX: Patient-derived xenograft
PFKP: Phosphofructokinase 1, platelet isoform
PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PIK3R1: Phosphoinositide-3-kinase regulatory subunit 1
PK: Pyruvate kinase
PKM2: pyruvate kinase isoform M2
POU3F2: POU class 3 homebox 2
PTEN: Phosphatase and tensin homolog
RAS: Rat sarcoma viral oncogene homolog
RB1: Retinoblastoma 1
RNA: Ribonucleic acid
RTK: Receptor tyrosine kinase
SALL2: Spalt like transcription factor 2
SDF1: Stromal cell-derived factor 1
SLC1A5: Solute carrier family 1 member 5
SOX2: SRY-box transcription factor 2
STAT: Signal transducer and activator of transcription
TCA: Tricarboxylic acid
TERT: Telomerase reverse transcriptase
TGF-β: Transforming growth factor beta
TIGIT: T cell immunoreceptor with Ig and ITIM domains
TM: Tumor microtube
TNT: Tunneling nanotube
TP53: Tumor protein P53
TTYH1: Tweety homolog 1
VASP: Vasodilator stimulated phosphoprotein
VEGF: Vascular endothelial growth factor
ZEB1: Zinc finger E-box binding homeobox 1
THESIS PROJECT

MITOCHONDRIA TRANSFER FROM MESENCHYMAL STEM CELLS TO GLIOBLASTOMA STEM CELLS:
EFFECTS ON GLIOBLASTOMA METABOLISM AND RESISTANCE TO THERAPY
GENERAL BACKGROUND

Although the combined cancer death rate steadily dropped from 1991 to 2015 by a total of 26% (Siegel et al., 2018), resistance to cancer therapy is still a major obstacle in the way of effective and lasting treatment, resulting in relapse, metastasis and reduced overall survival. Many mechanisms, intrinsic and extrinsic alike, have been described to factor into this resistance. Recently, intra-tumoral heterogeneity has garnered growing attention as a modulator of the response to therapy, or impediment thereof (Marusyk et al., 2020). It’s becoming increasingly evident that tumors don’t act as masses of homogenous malignant cells, but rather as complex, bona fide organs in dynamic progression through time and space, which enhances the fitness of the tumor and shapes its resistance (Greaves, 2015).

Lineage tracing and cell ablation experiments demonstrated that many tumors exhibit a peculiar population of self-renewing cells, dubbed cancer stem cells. Evidence from xenografts in immunodeficient mice indicated that cancer stem cells have the capacity to regenerate tumors evocative of the tumor of origin, give rise to different cell types and survive many commonly used cancer treatments. Consequently, cancer stem cells are directly implicated in drug resistance and are highly predictive of the patient’s overall survival. This model illustrates tumors as hierarchically organized malignant tissues where cancer stem cells represent the pinnacle of the hierarchy and sustain the long-term repopulation of the neoplasm (Batlle and Clevers, 2017).

In addition, heterogeneity arises from the fact that the tumor microenvironment harbors a plethora non-malignant cell types recruited to the tumor site, namely cancer-associated fibroblasts, mesenchymal stem cells and immune cells (Quail and Joyce, 2017; Chen and Song, 2019; Wolf et al., 2019). Reports state that the non-malignant tumor stroma constitutes up to 90% of a given tumor volume and is correlated with worse prognosis (Lou, 2016). The interaction of a tumor cell with its surroundings highly modifies its fate, thereby playing a role in the acquisition of drug resistance, as cells of the same genetic background can respond differently to the same cellular insults depending on their microenvironment.
Tunneling nanotubes have recently emerged as a new means of intercellular communication. Tunneling nanotubes are thin (diameter < 1 μm), actin-based cytoplasmic extensions connecting non-adjacent cells over long distances (> 100 μm) (Vignais et al., 2019). The biological signals that trigger tunneling nanotube formation include cellular stress induced by genotoxic chemotherapeutic agents (Desir et al., 2016; Moschoi et al., 2016; Victoria et al., 2016). Importantly, tunneling nanotubes imply a continuity in plasma membrane and cytoplasm between the connected cells, therefore changing our current paradigm of the cell limited by its own plasma membrane (Baker, 2017). Tunneling nanotubes thereby allow the intercellular transport of cellular components ranging from ions to whole organelles like mitochondria (Vignais et al., 2019). Tunneling nanotube-mediated mitochondria transfers were both observed in vitro and in vivo in murine models, with the injection of fluorescently-labeled MSCs (Islam et al., 2012) as well as with C57BL/6Nsu9DsRed2 mice harboring fluorescently-labeled mitochondria (Tan et al., 2015). Tunneling nanotube-mediated mitochondria transfers were shown to have both metabolic and functional effects on the recipient cells. In the context of cancer, exogenous mitochondria acquisition was reported to increase drug resistance. In the past few years, mesenchymal stem cells were described to connect with surrounding cells via tunneling nanotubes, leading to mitochondria transfer to these target cells and to biological effects such as cellular metabolic reprogramming, protection against tissue injury and resistance to chemotherapy (Caicedo et al., 2015; Moschoi et al., 2016; Rodriguez et al., 2018; Hekmatshoar et al., 2018; Nakhle et al., 2020; Pinto et al., 2020).

Mitochondria are considered as a metabolic hub where the various metabolic pathways converge including glycolysis, TCA cycle, glutaminolysis, pentose phosphate pathway and lipid metabolism. In this context, functional alterations of such metabolic pathways were directly linked to cancer cell drug resistance (Hekmatshoar et al., 2018). In addition, the production of TCA cycle metabolites also contribute to the epigenetic regulation of the cancer cell gene expression, as shown for succinate, fumarate, 2-hydroxyglutarate and a-ketoglutarate through the activities as DNA and histone demethylases (Tsukada et al., 2006; Xiao et al., 2012; Killian et al., 2013; Letouzé et al., 2013; Nakhle et al., 2020). The established role of mitochondria in tumor progression and resistance to therapy can account for the beneficial
anticancer effects achieved by mitochondria inhibitors like metformin (Sanchez-Alvarez et al., 2013) or by mitochondrial-biogenesis-inhibiting antibiotics (e.g. doxycycline, azithromycin) (Lamb et al., 2015). It also opens the way for innovative therapeutic strategies based on metabolic synthetic lethality for example (Navarro et al., 2016). Nonetheless, central questions remain, therefore warranting further fine-tuned studies.

My thesis project focuses on glioblastoma, a devastating primary brain tumor. Patients are treated by tumor resection followed by radiation and chemotherapy with alkylating agent temozolomide. However, resistance to temozolomide treatment appears quickly, mainly due to glioblastoma stem cells present in the tumor. Glioblastoma stem cells are characterized by the expression of stemness markers (including OLIG2, NESTIN, NANOG, CD133, SOX2). They form neurospheres in vitro and are able to generate full GBM tumors following xenograft in mice. Glioblastoma resistance to temozolomide is described to depend on various mechanisms, including overexpression of MGMT (O6-methylguanine methyltransferase), of efflux proteins MDR1 (Multi Drug Resistance 1) and ABCG2 (ATP-binding cassette subfamily G member 2) as well as modified glioblastoma metabolism (Velpula et al., 2012; Shinojima et al., 2013; Fan et al., 2013; Osuka and Van Meir, 2017; MacLeod et al., 2019; Garnier et al., 2019). Interestingly, glioblastoma stem cells were also reported, both in vitro and in vivo, to engage in a functional network of direct cell-cell connections that contributed to glioblastoma resistance to radiotherapy (Osswald et al., 2015).

Mesenchymal stem cell recruitment to the glioblastoma microenvironment has been observed in resected glioblastoma tumors. Importantly, their presence inversely correlated with patient survival (Hossain et al., 2015; Shahar et al., 2017). It was further confirmed in glioblastoma stem cell orthotopic xenograft models, a process promoted by glioblastoma stem cell-secreted TGF-β (Velpula et al., 2012; Shinojima et al., 2013).
INTRODUCTION
CHAPTER 1:  
MITOCHONDRIA TRANSFER VIA TUNNELING NANOTUBES  
FROM MESENCHYMAL STEM CELLS  
TO TARGET CELLS
Solid tumors are massively heterogeneous tissues. This heterogeneity arises from tumor cells themselves and, at their apex, cancer stem cells believed to be responsible for tumor progression and therapy failure. Neoplastic tissues also include non-tumorous cells. These comprise residing mesenchymal, epithelial and endothelial cells, as well as recruited immune and mesenchymal stem cells. Albeit non-cancerous, these cells often exhibit an abnormal phenotype and consequently foster tumor progression, metastasis and drug resistance.

Cell communication within the tumor, both between cancer cells themselves as well as between cancer cells and their neighbors, is now fully acknowledged as widely used by the tumor to grow and circumvent therapeutic treatments. In the past decades, intercellular communication was believed to heavily rely on secreted cytokines, chemokines, metabolites and extracellular vesicles. In the past few years, however, a new means of cell-cell communication through tunneling nanotubes was shown to enable cells to connect to their far-off counterparts and to transfer them biological cargos, ranging from ions and microRNAs to whole organelles like mitochondria, as it will be detailed in this chapter. This donation is qualified as horizontal, to distinguish it from the vertical donation from a parental cell to its offspring during mitosis. This tunneling nanotube-mediated trafficking occurs from cells of the microenvironment to cancer cells, thereby modifying their functional properties and response to therapy. Conversely, it also occurs in the opposite direction, from cancer cells to non-malignant cells of the tumor microenvironment, as cancer cells hijack their surroundings for their own sake.

Mitochondria have been the most extensively-studied tunneling nanotube cargo thus far. This stems from the extent of the biological effects of transported mitochondria, both on the metabolic signature and on the functional capacities of recipient cells. Mitochondria transfer was observed amongst both normal and cancer cells, in both physiological and pathological circumstances. A vast majority of studies focused on mitochondria transport originating from mesenchymal stem cells and targeting a wide panel of acceptor cells including, but not limited to, cardiomyocytes, endothelial cells, alveolar epithelial cells, renal tubular cells, macrophages as well as cancer cells such as acute myeloid leukemia cells, breast cancer cells and glioblastoma stem cells. From a practical point of view, mitochondria transfer detection was performed using mitochondria-specific fluorescent vital dyes and viral constructs. In
heterologous systems, human mitochondria could be detected with antibodies specific for human mitochondria. At the genetic level, mitochondria originating from different donors could be detected and their concentration evaluated on the basis of the single nucleotide polymorphisms present in the mitochondrial DNA.

The occurrence of tunneling nanotubes in tumors and the ensuing intercellular mitochondria trafficking are bringing about a radical turmoil in the current paradigm of the intercellular communications that take place in tumors. It now clearly appears that the discovery of tunneling nanotubes, and of cargos transported from one cell to the other through these connections, bring a novel understanding of the cancer cell biology that takes place within tumors. As this cargo trafficking has consequences on tumor progression and resistance to therapy, the challenge will now be to exploit this new knowledge to conceive and develop novel anticancer therapeutic strategies.

This chapter details the biological outcomes of mitochondria transfer implicating mesenchymal stem cells, in both damaged tissues and tumors. It also discusses the mechanisms underlying this process and the fate of internalized mitochondria in recipient cells (Review 1: Rodriguez, Nakhe et al., 2018).
1. **Mesenchymal Stem Cells**

1.1. **Definition**

Mesenchymal stem/stromal cells are adult multipotent stem cells endowed with high self-renewal ability and extensive differentiation potential (Ridge et al., 2017; Timaner et al., 2020; Koliaraki et al., 2020). Mesenchymal stem cells were first identified, in the late 1960s, as a bone-marrow derived, spindle-shaped cell subpopulation capable of differentiating into fibrous tissue and bone stroma. They were then named “fibroblast-like cells” (Friedenstein et al., 1966). Only in the early 1990s, Caplan and collaborators termed them “mesenchymal stem cells”, in respect to their specific properties, notably, their proliferative and multi-lineage differentiation potentials (Caplan, 1991). However, confusion arose regarding the exact definition of mesenchymal stem cells, which made comparisons among studies published until the 2000s difficult. This led to a new homogenized terminology and a minimum consensus for defining mesenchymal stem cells, as proposed by the International Society for Cellular Therapy (Horwitz et al., 2005; Dominici et al., 2006). The minimal criteria for defining mesenchymal stem/stromal cells can currently be summarized as follows (Keating, 2012) *(Figure 1)*:

1. an adherence capacity onto plastic,
2. a high proliferative potential,
3. a tri-lineage differentiation *in vitro* into adipocytes (Oil Red lipid vesicle staining), chondrocytes (Alcian Blue staining or collagen type II-specific immunohistochemical staining) and osteoblasts (Alizarin Red or von Kossa staining),
4. cell-surface expression markers: positive for CD105 (endoglin), CD73 (ecto-50-nucleotidase), and CD90 (Thy1); negative for CD45 (protein tyrosine phosphatase), CD19 (B-lymphocyte antigen 19), CD79 (transmembrane protein forming a complex with the B-Cell Receptor BCR), CD14 (differentiation antigen of monocytes related to LPS binding), CD11b (subunit of the heterodimeric integrin alpha-M beta-2) and HLA-DR (MHC class II cell surface receptor).

The classical and most widely-used human mesenchymal stem cell sources are the bone marrow, the adipose tissue and the umbilical cord. More recently, Wharton’s jelly, the
amnion, the chorion and the umbilical cord blood were proposed as alternative mesenchymal stem cell sources (Bajetto et al., 2020).

**FIGURE 1. MESENCHYMAL STEM CELL CHARACTERIZATION.**
The plastic-adherent cellular fraction of many tissues contains stromal progenitor cells, known as colony forming unit fibroblasts (CFU-Fs), that reside in proximity of blood vessels. When cultured under the appropriate conditions, colonies derived from single CFU-Fs can be expanded in vitro without losing their multipotency. These cultured cells are classically referred to as mesenchymal stem cells. The hallmark that defines mesenchymal stem cells is their capacity to differentiate into 3 lineages: osteoblasts, adipocytes and chondrocytes, when placed under inductive stimuli. (Nombela-Arrieta et al., 2011)

1.2. **MESENCHYMAL STEM CELL HOMING TO TUMOR SITES**
Mesenchymal stem cells display a high tropism to wounds and injured tissues, where they promote healing and regeneration (Caplan and Dennis, 2006; Islam et al., 2012; Rustad and Gurtner, 2012). Tumors, which can be generally assimilated to chronic wounds, also recruit mesenchymal stem cells to support cancer progression and resistance to therapy (Velpula et al., 2012; Shinojima et al., 2013; Hill et al., 2017, 2020). Such mesenchymal stem cell
recruitment to tumor sites has been described in various cancer types including colorectal (O’Malley et al., 2016; Nishikawa et al., 2019; Vangala et al., 2019), pancreatic (Jing et al., 2014), breast (Karnoub et al., 2007; Hill et al., 2020), gastric (Donnelly et al., 2013; Kasashima et al., 2016) and ovarian (Li et al., 2015) cancers as well as glioma (Birnbaum et al., 2007; Doucette et al., 2011; Velpula et al., 2012; Shinojima et al., 2013; Behnan et al., 2014; Thomas et al., 2018). The mechanisms of mesenchymal stem cell homing to tumors are yet to be fully elucidated; however, it is likely that the factors that promote mesenchymal stem cell tropism to tumors are similar to those implicated in the recruitment of other accessory cells (e.g., bone marrow-derived immune cells). Among these are: growth factors such as EGF, β-FGF, HGF and IGF-1; angiogenic factors such as VEGF and HIF-1α; chemokines such as CCL2, CCL5, CCL22 and CXCL12; and inflammatory cytokines such as TNF-α, TGF-β, IL-1β, IL-8 (Ridge et al., 2017; Timaner et al., 2020). The extensive recruitment of mesenchymal stem cells to different tumor sites suggest that it may have an important contribution to tumor fate.

1.3. **Mesenchymal Stem Cell Pro-Tumorigenic Role**

1.3.1. Angiogenesis

Mesenchymal stem cells secrete high levels of pro-angiogenic growth factors such as VEGF and β-FGF, as well as cytokines including IL-6, IL-8, TGF-β and Ang-1, thereby promoting tumor angiogenesis (Du et al., 2016). For instance, pancreatic carcinoma-residing mesenchymal stem cells were shown to produce high VEGF levels, which induced angiogenesis and enhanced micro-vessel density within the tumor (Beckermann et al., 2008). In addition, IL-6 and Ang-1, secreted by mesenchymal stem cells in colorectal carcinoma, activated the AKT and ERK pathways in tumor endothelial cells, leading to their recruitment for tumor vessel formation (Huang et al., 2013; O’Malley et al., 2016). In another report, melanoma cells educated mesenchymal stem cells via VEGF secretion towards creating capillary-like structures and vascular-like networks *in vitro*, therefore promoting tumor vasculature through vasculogenic mimicry (Vartanian et al., 2016). Finally, mesenchymal stem cells were suggested to actually be resting fibroblasts that could undergo transformation into activated cancer-associated fibroblasts endowed with high angiogenic capacity, by secreting IL-6, IL-8, TGF-β, VEGF and CXCL12 (Kalluri, 2016; Koliaraki et al., 2020) (Figure 2).
Mesenchymal stem cells were also demonstrated to be implicated in tumor vasculature by stabilizing pre-existing blood vessels. Mesenchymal stem cell-produced Ang-1 was indeed shown to enhance the endothelial cell expression of cell-to-cell junction proteins such as occludin, thus reducing blood vessel leakiness (Zacharek et al., 2007). In addition, glioma cells were described to induce mesenchymal stem cell differentiation into functional pericytes, that incorporated into tumor vessel walls and supported their integrity (Bexell, 2009; Dhar et al., 2010; Caplan, 2017). Therefore, studies are currently seeking to block the mesenchymal stem cell angiogenic activities in order to improve anti-angiogenic therapy (Timaner et al., 2020).

**Figure 2. Mesenchymal Cell Activation into Cancer-Associated Fibroblasts.** Different mesenchymal cell populations, including mesenchymal stem cells, can be activated into cancer-associated fibroblasts following exposure to tumor-mediated inductive stimuli. Cancer-associated fibroblasts exert a multitude of pro-tumorigenic functions, via effector molecule production or direct cell-to-cell contacts. (Koliaraki et al., 2020)

### 1.3.2. Metastasis

Several studies have demonstrated the mesenchymal stem implication in cancer cell epithelial-to-mesenchymal transition, migration, invasion and metastasis (El-Haibi et al., 2012; Fregni et al., 2018; Zhang et al., 2018). These effects are mediated by several mesenchymal stem cell-secreted growth factors and cytokines. Among these factors are C-C and C-X-C motif chemokines that promote cancer cell migration (Halpern et al., 2011; Sarvaiya et al., 2013; Swamydas et al., 2013; Yu et al., 2017), extracellular matrix-modulating factors...
such as lysyl oxidase (El-Haibi et al., 2012), and growth factors such as TGF-β, FGF, HGF and EGF that promote epithelial-to-mesenchymal transition and invasiveness of tumor cells (McAndrews, 2015; Berger et al., 2016; Hill et al., 2020).

1.3.3. Immunosuppression

Mesenchymal stem cells are known for their immunomodulatory capacities, as they constitute key regulators of both innate and adaptive immune responses. Within the tumor microenvironment, mesenchymal stem cells promote extensive immunosuppression and foster anti-tumoral immune evasion, mainly by producing soluble factors and mediators such as TGF-β, IFN-γ, TNF-α, prostaglandin E2, IL-1, IL-4 and IL-6, as well as by directly interacting with various immune cells, including macrophages, T cells, B cells and NK cells (Rivera-Cruz et al., 2017; Poggi, 2018). For instance, mesenchymal stem cell-secreted prostaglandin E2 was shown to induce macrophage-mediated production of the anti-inflammatory factor IL-10, which inhibited T cell activation and proliferation (Németh et al., 2009). In addition, mesenchymal stem cells secreted high levels of immunosuppressive TGF-β, thus inducing T cell suppression in breast cancer (Niu et al., 2017). Moreover, mesenchymal stem cells were described to induce the skewing of pro-inflammatory Th1 cells towards an anti-inflammatory Th2 phenotype, thus minimizing anti-tumoral immune cell activation (Bai et al., 2009; Fiorina et al., 2009). Besides the immunosuppressive effects on T cells, mesenchymal stem cells were also demonstrated to attenuate B-cell-mediated adaptive immune response, by inducing B-cell cell cycle arrest (Uccelli et al., 2008) and reducing B-cell antibody production (Asari et al., 2009; Ungerer et al., 2014). Taken together, mesenchymal stem cells strongly inhibit T- and B-cell mediated adaptive immune response, which is well exploited by cancer cells.

In addition to adaptive immunity suppression, mesenchymal stem cells also inhibit innate immune cells, thereby diminishing first-line anti-tumoral responses. In particular, mesenchymal stem cells were described to inhibit NK cell proliferation and IFN-γ production, mainly by prostaglandin E2 and IL-6 secretion, thus impairing their anti-cancer activity (Galland et al, 2017). In addition, mesenchymal stem cells directly inhibit macrophage activity. For instance, mesenchymal stem cell-conditioned medium was shown to decrease macrophage phagocytic capacities, subsequently promoting a pro-tumorigenic macrophage phenotype (Chen et al., 2018). Mesenchymal stem cells were also described to reprogram
macrophages towards the pro-tumorigenic, anti-inflammatory M2 phenotype, resulting in higher levels of immune-inhibitory IL-10 secretion (Vasandan et al., 2016).

1.3.4. Drug resistance
Multiple studies have demonstrated that the mesenchymal stem cell pro-tumorigenic activities were strengthened in response to therapy, therefore being at the root of tumor regrowth and resistance (Houthuijzen et al., 2012). For example, in hematological malignancies such as chronic myeloid leukemia, mesenchymal stem cells reduced imatinib-mediated cell death by inhibiting caspase-3 activity in a CXCL12-CXCR4-dependent manner (Vianello et al., 2010). Other cytoprotective activities were demonstrated in chronic lymphoid leukemia, where resistance to forodesine was attributed to mesenchymal stem cell-induced RNA and protein synthesis increase (Balakrishnan et al., 2010). Mesenchymal stem cells were also shown to promote resistance in solid tumors, for instance, to paclitaxel and cisplatin (Scherzed et al., 2011; Roodhart et al., 2011). Strikingly, mesenchymal stem cells were also shown to either transform into cancer stem cells or to support cancer stem cell niches, thus contributing to drug resistance (Skolekova et al., 2016; Melzer et al., 2017; Timaner et al., 2018).
**Figure 3. Pro-Tumorigenic Processes Fostered by Mesenchymal Stem Cells.**

**Angiogenesis:** Mesenchymal stem cells promote angiogenesis via the production of pro-angiogenic factors, including VEGF, β-FGF and IL-6. In addition, they support vasculogenesis by recruiting circulating endothelial progenitor cells (CEPs) and stabilize blood vessels by differentiating into pericytes.

**Metastasis:** Mesenchymal stem cells secrete factors that promote epithelial-to-mesenchymal transition (EMT), such as CCL5, TGF-β and IFN-γ. Moreover, mesenchymal stem cells differentiate into cancer stem cells (CSCs) or cancer-associated fibroblasts (CAFs), which enhance the migratory and metastatic potential of cancer cells.

**Immunosuppression:** Mesenchymal stem cells inhibit Th1 cells, B cells and NK cells and, on the other hand, activate T regulatory cells (Tregs) and Th2 cells, thus dampening the anti-tumoral immune response. Mesenchymal stem cells also educate macrophages towards the anti-inflammatory M2 phenotype.

**Drug resistance:** Mesenchymal stem cells shield cancer cells against therapy-induced cell death. In addition, they protect resistant CSCs or directly trans-differentiate into CSCs, thereby enriching the CSC niche. (Timaner et al., 2020)
Intercellular mitochondria trafficking highlighting the dual role of mesenchymal stem cells as both sensors and rescuers of tissue injury

Anne-Marie Rodriguez, Jean Nakhle, Emmanuel Griessinger and Marie-Luce Vignais

Introduction

Mitochondria likely represent the most complex organelles found in the cytosol of eukaryotic cells, with regard to their structural organization and the diversity of their functions. One of their peculiarities is to originate from a bacterial ancestor, by an endosymbiosis that occurred more than 1.5 billion years ago [1]. From these ancient bacteria, mitochondria retained several structural features, notably their inner membrane. Indeed, mitochondria contain both an inner and an outer membrane. Whereas the mitochondria outer membrane, that separates the inside of the organelle from the rest of the cell, is a phospholipid bilayer membrane similar to that of the eukaryotic cell own membrane, the mitochondria inner membrane shares lipid components like cardiolipin with bacteria membranes, reminiscent of its prokaryotic origin. In addition, they contain a circular genome harboring a genetic code different from that of nuclear DNA [2]. As a result, they can replicate their DNA and divide, within the cells, independently of cell division. In addition, mitochondria exert a broad range of important functions in the cells. They are commonly considered as the powerhouse of the cells, devoted to convert nutrients into energy to fuel the cellular biological activities, as they produce most of the cell demands in adenosine triphosphate (ATP) through oxidative phosphorylation. Mitochondria are involved in numerous anaerobic and catabolic processes. Overall, they regulate a broad range of cellular functions, including specific metabolic pathways activation, tissue temperature maintenance, calcium signaling and cell death induction. Therefore, they contribute to cell adaptation to physiological and pathological environmental changes. Understanding the complexity of mitochondria functions became a challenging area of research over the past few years, with implications in the fields of regenerative medicine, oncology and immunology. This stems from the discovery that the mitochondria-dependent metabolic reprogramming controls a wealth of different functions: the self-renewal and differentiation capacities of mesenchymal stem cells (MSCs) and embryonic (ES) stem cells [3–5], the secretion of inflammatory cytokines by immune cells such as macrophages [6–8] and dendritic cells [8–10] and also the malignant properties of cancer cells [11,12].

Mitochondria recently attracted a renewed attention from the scientific community, as either the whole organelles, the mitochondrial genome or other mitochondrial components were shown to translocate between cells, thus providing intercellular signaling cues able to (i) alert the recipient cells of a danger situation [13], (ii) restore their biological functions [14] or, in the case of cancer cells, (iii) modify their functional capacities and response to therapy [15,16]. In conditions of severe tissue damage, multiple mitochondrial elements, including mitochondrial DNA (mtDNA), N-formyl peptides, ATP or cardiolipin, were shown to be liberated from the dying cells to the surrounding tissue and to the bloodstream [17,18]. These mitochondrial products are recognized as damage associated molecular patterns (DAMPs) by specific receptors on immune cells and consequently trigger innate and adaptive inflammatory responses [19,20]. In addition, whole mitochondria can also be transferred between a broad diversity of cell types by the means of specialized structures including tunneling nanotubes (TNTs) [16,21] or microvesicles (MVs) [21,22]. A large part of the studies in this field focused on mesenchymal stem
cells (MSCs) and their ability to communicate through organelle exchange with their surrounding environment following tissue injury or during cancer progression [23]. Here, we review the biological consequences of the mitochondria transfer between MSCs and differentiated cells, in damaged tissues as well as in tumors. We report both situations that is MSCs as either mitochondria donors or receivers. Finally, we discuss the mechanisms underlying this process, the fate of the transferred organelles in the recipient cells and the potential therapeutic applications of mitochondrial exchanges as means to repair damaged organs or treat mitochondrial inherited diseases and cancer.

**Mitochondria, released by MSCs, as pro-survival effectors**

Mesenchymal stem cells hold great promise for regenerative medicine due to their plasticity, their pro-angiogenic and anti-apoptotic functions and their immune-regulatory properties [24,25]. Although most of the beneficial effects exerted by MSCs have primarily been ascribed to paracrine mechanisms [26], this concept recently evolved with the discovery of the outstanding capacity of MSCs to share their mitochondria with target cells, resulting in the protection of the recipient cells from tissue injury. In this section, we will discuss to what extent the mitochondria delivery from MSCs to the recipient cells can be beneficial for wound healing processes and immune regulation and, on the other hand, be deleterious for the organism in the case of cancer.

**MSC-mediated mitochondria transfer in cell rescue**

Ten years ago, Spees and collaborators provided the first evidence that mitochondria or mtDNA could translocate from MSCs to mammalian cells harboring nonfunctional mitochondria, due to their lack of effective oxidative phosphorylation [27]. This pioneer study was revealing how, through such a phenomenon, MSCs could restore aerobic respiration in mtDNA-depleted human lung alveolar epithelial A549 \( \rho^0 \) cells. In the following years, the mitochondria donor capacity of MSCs was confirmed by several laboratories worldwide as well as the physiological significance of this phenomenon. On the whole, these studies indicate that MSCs preferentially release their mitochondria to suffering or damaged cells and that this process results in the rescue of the recipient cells from cell death by preserving their energy metabolism. Through coculture settings, mitochondria transfer was observed to occur in vitro from MSCs to various kinds of differentiated cells, including cardiomyocytes [28,29], endothelial cells [30], bronchial epithelial cells [31], corneal epithelial cells [32] and neuronal cells [33]. These studies showed that the mitochondria transfer from MSCs conferred protection against apoptosis to damaged cells following exposure to several stressor stimuli such as ischemic/reperfusion injury [28,30,32,33], oxygen/glucose deprivation [30] and tobacco smoke exposure [31]. Mitochondria donation by MSCs was invariably found to improve the survival of the injured cells and to increase their respiratory function and ATP production. The rescue of the cellular bioenergetics of the differentiated cells required the delivery of functional respiring mitochondria by the MSCs as shown by the loss of the cytoprotective function of mtDNA-depleted MSCs (\( \rho^0 \) cells) [29,30].

The transfer of mitochondria from MSCs to differentiated cells was also observed in animal models for tissue injuries such as ischemic heart [34], injured lung through exposure to LPS [35], rotenone [36] or cigarette-smoke [31] and rotenone-treated cornea [32]. These in vitro studies substantiated the initial in vitro coculture observations and confirmed that engrafted MSCs can transfer mitochondria to damaged cells, resulting for these cells in a pro-survival outcome through the OXPHOS-dependent restoration of their ATP production.

Finally, MSCs were demonstrated to have the capacity to reprogram fully differentiated mouse cardiomyocytes back to a cardiac progenitor-like state, in a process that relied on the mitochondrial transfer from MSCs [29]. In these settings, the mitochondria transfer from MSCs was observed to improve the survival of the mature cardiomyocytes in vitro, which is a prerequisite for their reprogramming back to a progenitor state.

**MSC-mediated mitochondria transfer in inflammation**

The therapeutic benefits of MSCs have been partly attributed to their immunosuppressive properties and their ability to regulate the functions of many cell types, from the innate and adaptive immune systems, such as dendritic cells [37–39], T-lymphocytes [40,41] and also macrophages [42–44], these latter cells playing a critical role in tissue repair by ensuring the clearance of dying cells and cell debris through phagocytosis. Two main groups of macrophages, differing by their pro- or anti-inflammatory phenotypes, co-exist within the healing wound. Following tissue damage, macrophages initially adopt a pro-inflammatory phenotype M1, that is then switched towards an anti-inflammatory pro-healing M2 phenotype at the time of resolution of inflammation [45–47]. MSCs are known to favor the macrophage differentiation towards an anti-inflammatory/pro-healing M2 phenotype [42–44]. More recently, the transfer of mitochondria from MSCs to macrophages was observed to occur both in vitro and in vivo and shown to drive phenotypic changes in the macrophages [48–50]. In particular, Jackson and colleagues as well as Morrison and colleagues provided evidence that the mitochondria conveyed by MSCs, in the context of the Acute Respiratory Distress Syndrome (ARDS), increased the oxidative phosphorylation of the recipient macrophages and then stimulate their phagocytic activity [48,49] and their differentiation towards a M2 anti-inflammatory phenotype [50]. In addition, the inhibition of this mitochondria transfer was shown to abrogate the antimicrobial effects of MSCs following their engraftment in mice suffering from bacterial pneumonia (ARDs), supporting the importance of this process in the regulation of macrophage functions and bacteria clearance [49].

Interestingly, the transfer of mitochondria to macrophages does not solely occur from healthy but also from damaged MSCs [51]. In this latter context, this process was proposed as a mechanism allowing stem cells to get rid of their deleterious organelles to improve their own survival, although it could also be envisioned as a means of alerting macrophages of danger situations [51], as discussed in section II.

**MSC-mediated mitochondria transfer in tumor progression**

The recent research efforts to better understand the cross-talk between cancer cells and their microenvironment identified mitochondria transfer as a process contributing to the tumor
development and progression. In a fashion comparable to that observed in the context of tissue repair, MSCs were shown to deliver mitochondria to various kinds of malignant cells, including those from breast and ovarian cancer, melanoma, acute myeloid leukemia and glioblastoma [52–55], resulting in induced invasiveness and resistance to chemotherapy.

The seminal work reporting on the horizontal mitochondria transfer was actually performed on A549 lung adenocarcinoma cells [27]. These mitochondria acceptor cells were $\rho^0$ cells, harboring a defective mitochondrial DNA (mtDNA) after chronic ethidium bromide treatment and, as a consequence, having an inoperative respiratory chain and respiration. These $\rho^0$ cells rely on glycolysis and are dependent of exogenous supplementation of pyruvate and uridine in the culture medium (auxotrophy). After the mitochondria transfer, evidenced by the detection in the acceptor cells of the mtDNA from the donor cells, the A549 cells recovered a respiratory function and an oxidative metabolism while they lost their auxotrophy [27]. Other $\rho^0$ cells, including melanoma and breast solid tumor cells, have been shown to lose tumor latency compared to the parental mitochondrial competent cells. It was nicely demonstrated that mitochondrial transfer from the tumor microenvironment toward these $\rho^0$ cells could fully restore their respiration and invasiveness pattern [53]. Using C57BL/6Nsu9-DsRed2 mice that express a red fluorescent protein in their mitochondria, Neuzil and collaborators recently established the transfer of whole mitochondria from the host animal towards the injected B16 $\rho^0$ mouse melanoma cells [56]. It is worth mentioning that the permanent recovery of the mitochondrial function of the $\rho^0$ cells was achieved using donor and recipient cells either from the same murine species [53] or from different species (human and mouse) [27], suggesting a lack of species barrier for this particular phenomenon. However, long-lasting acquisition of exogenous mitochondria was not reported for other non-$\rho^0$ cancer cell models. It will require further investigation to determine whether the auxotrophic status of the $\rho^0$ cells constitutes a selection pressure and leads to mitochondria acquisition mechanisms different from those of the other cell systems described so far.

Independently of the auxotrophic issue of $\rho^0$ cells, a higher tumorigenicity upon mitochondrial transfer was also observed for mitochondrial competent leukemic and bladder cancer cells [54,57]. The recipient cancer cells for the mitochondria exchange displayed a higher tumorigenic potential. Besides, the mitochondria recipient cells were consistently shown to display an increased oxidative phosphorylation (OXPHOS) phenotype in the cell systems independently investigated [30,35,54,58,59].

**Mitochondria transferred to MSCs as sensors for tissue homeostasis**

As detailed above, mitochondria trafficking from MSCs to differentiated cells and their biological outcomes have been reported by several laboratories. Conversely, mitochondria can also be transferred in the reverse orientation i.e. from fully differentiated cells to MSCs. Although this has been much less studied so far, current in vitro studies provide evidence that mitochondria released by differentiated cells can be captured by MSCs and that this process contributes to the maintenance of tissue homeostasis. In this section, we will provide an overview on how mitochondrial transfer promotes an adaptive response of recipient MSCs to face up to micro-environmental demands. We will distinguish the effects of mitochondria transferred from either "healthy" or "damaged" differentiated cells.

**Mitochondria transferred from healthy differentiated cells**

Several studies indicate that MSCs or progenitor cells co-cultured with fully differentiated cells can acquire the phenotype of these differentiated interacting cells, as shown for renal tubular cells and cardiomyocytes, and that this phenomenon is accompanied by mitochondrial transfer from the differentiated cells [60,61]. Although the actual role of the exogenous mitochondria in the acquired phenotype of the MSCs was not assessed in these studies, it is likely that the transfer of the external mitochondria provides to stem cells a means to regulate their metabolism, that was shown to be essential for their plasticity [62,63]. In particular, the exogenous supply of mitochondria is expected to favor the metabolic switch of the MSCs from glycolysis to oxidative phosphorylation, a process that was reported to be required for their differentiation [64–68]. In addition, mitochondria transferred from vascular smooth muscle cells (VSMCs) to MSCs were found to stimulate their proliferation in vitro. The actual role of the transferred VSMC mitochondria in promoting MSC proliferation was based on the fact that both the inhibition of this process and the transfer of mitochondria with impaired respiratory function, by long-term ethidium bromide treatment ($\rho^0$), abrogated this effect [69]. Although the mechanisms underlying the proliferative effects of transferred mitochondria have not been addressed in this study, exogenous mitochondria are expected to modulate the bioenergetics of MSCs, known as a key regulator of their growth [70–72].

**Mitochondria transferred from “damaged” differentiated cells**

Beyond their role as energy producers and cell metabolism regulators, mitochondria are capable of sensing the cellular stress generated following tissue injury and of relaying danger signals, at both the intracellular and intercellular levels [73]. Following acute tissue injury, damaged cells release various mitochondrial components in the bloodstream. These components are recognized as damage associated molecular patterns (DAMPs) by innate immune cells (including neutrophils and monocytes/macrophages) through their interactions with specific pattern recognition receptors [17,74]. As a result, mitochondrial DAMPs elicit a sterile immune response (i.e. in the absence of any microorganisms). Mitochondrial DAMPs include mtDNA, N-formyl peptides, mitochondrial proteins, such as TFAM and cardiolipin, and ATP [14,74]. In addition, recent findings indicate that mitochondria, as whole organelles, can also act as DAMPs following acute injury [13] or inflammation [75]. In particular, our laboratory reported that the transfer of mitochondria from apoptotic endothelial or cardiac cells to MSCs constitute a signaling messenger that triggers the cytoprotective response of the MSCs, contributing to their supporting action for the injured cells [13]. We found that the conveyed organelles were degraded by MSCs through a mitophagy process involving the stress-inducible heme oxygenase-1 (HO-1)
signaling pathway, associated with the stimulation of mitochondrial biogenesis. As a result, MSCs enhanced the donation of their own mitochondria towards the damaged cells to rescue them. Importantly, this cross-talk signaling was shown to depend on reactive oxygen species (ROS) produced by the damaged cells since a ROS scavenger abrogated both the mitochondrial transfer from the injured cells to the MSCs and the MSC rescuing function [13]. The molecular mechanisms whereby the mitochondrial ROS produced by injured cells activate the cytoprotective functions of MSCs remain to be carefully investigated. It is worth considering, though, that mitochondrial ROS are known to stimulate autophagy processes [76–78] and to regulate the HO-1 signaling pathway [76,77,79–81].

Mechanisms of intercellular mitochondria transfer

As described above, MSCs clearly demonstrate a capacity to exchange mitochondria with a diversity of cell types. For this, they can either form tunneling nanotubes (TNTs) and/or extracellular vesicles (EVs) as it will be discussed in this section. It is worth noting that this represents a novel property for MSCs that were, up to now, mostly characterized for their interactions with neighboring cells through secreted cytokines [82–84].

Mitochondrial transfer through tunneling nanotubes

Tunneling nanotubes were first described in 2004 by the two groups of Gerdes [85] and Davis [86]. Ever since, the interest in TNTs and the number of cell types shown to be involved in such intercellular connections has been steadily growing. TNTs are long tubular structures, connecting cells together, that can reach lengths of several hundreds of microns with diameters from 50 to 1 500 nm [87]. Actually, the fact that TNTs involve a continuity in plasma membrane and cytoplasm between the connected cells is radically changing our conception of the cell, limited by its own plasma membrane [88]. MSCs are particularly prone to engage in TNT connections and mitochondria donation to different cell types, including cardiomyocytes [29,59,89], lung epithelial cells [31,35,36], renal tubular cells [60], vascular smooth muscle cells [69], corneal epithelial cells [32] and macrophages [49] (Table 1). Apart from their role in tissue homeostasis, MSCs are also known to be recruited to tumors [90,91]. TNT-mediated mitochondria transfer was actually observed between MSCs and different cancer cells, including breast and ovarian cancers, melanoma, acute myeloid leukemia as well as glioblastoma as shown in Figure 1 [52–55].

Connexin 43 and Miro-1 (RHOT1) were shown to control TNT formation and the TNT-mediated mitochondria exchange, respectively [35,36]. Connexin 43 (Cx43), also known as GAP Junction Alpha-1 protein (GJA1), is a transmembrane protein that assembles as hexamers to form connexons. Two connexons from two neighboring cells can then dock end-to-end to establish the intercellular channel, called GAP junction, that allows the direct cell-to-cell transfer of small molecules (Goodenough and Paul, 2009). Cx43 was first described to be implicated in the formation of TNTs and the establishment of GAP junctions between the two connecting cells in a murine model of LPS-mediated lung injury [35]. In fact, while Cx43-expressing human MSCs were able to rescue the injured pulmonary alveolar epithelial cells via TNT formation, Cx43-depleted MSCs were unable to form TNTs and, consequently, could not reduce lung injury [35]. Miro-1, an outer mitochondrial membrane Rho-GTPase, was also shown to play an important role in mitochondria trafficking through TNTs. In neurons, Miro-1 is known to interact with the adaptor proteins TRAK1-2 and with mitofusins 1–2, resulting in the recruitment of kinesin motor proteins to the mitochondria and their shuttling along the axons and dendrites microtubules [92,93]. In models of either rotenone or ovalbumin-induced airway injury, MSCs overexpressing Miro-1 transferred higher amounts of

### Table 1. Cell types involved in TNT-mediated mitochondria transfer with MSC and biological outcome.

<table>
<thead>
<tr>
<th>References</th>
<th>Cell types involved in TNTs with MSCs</th>
<th>Cargo transferred</th>
<th>Biological Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spees et al. (2006)</td>
<td>A549 / mouse lung epithelial cells</td>
<td>Mitochondria/mtDNA</td>
<td>Aerobic respiration restoration</td>
</tr>
<tr>
<td>Plotnikov et al. (2008)</td>
<td>Rat cardiomyocytes</td>
<td>Mitochondria</td>
<td>Restoration of cardiomyocytes bioenergetics</td>
</tr>
<tr>
<td>Plotnikov et al. (2010)</td>
<td>Rat renal tubular cells</td>
<td>Mitochondria</td>
<td>MSC differentiation into kidney tubular cells</td>
</tr>
<tr>
<td>Acquistapace et al. (2011)</td>
<td>Adult cardiomyocytes</td>
<td>Mitochondria</td>
<td>Conversion to progenitor-like state by metabolic</td>
</tr>
<tr>
<td>Islam et al. (2012)</td>
<td>Damaged murine alveolar epithelial cells</td>
<td>Mitochondria</td>
<td>Tissue repair (ATP level restoration, pulmonary surfactant secretion)</td>
</tr>
<tr>
<td>Vallabhaneni et al. (2012)</td>
<td>Vascular smooth muscle cells</td>
<td>Mitochondria</td>
<td>Increase in MSC proliferation</td>
</tr>
<tr>
<td>Pasquier et al. (2013)</td>
<td>Human ovarian and breast cancer cell lines</td>
<td>Mitochondria</td>
<td>Increased doxorubicin chemoresistance</td>
</tr>
<tr>
<td>Ahmad et al. (2014)</td>
<td>Stressed murine lung epithelial cells</td>
<td>Mitochondria</td>
<td>Lung injury repair and mouse survival</td>
</tr>
<tr>
<td>Li et al. (2014)</td>
<td>Lung epithelial cells exposed to cigarette smoke</td>
<td>Mitochondria</td>
<td>Decrease in cigarette-smoke-induced alveolar damage</td>
</tr>
<tr>
<td>Liu et al. (2014)</td>
<td>Human umbilical vein endothelial cells (HUVEC)</td>
<td>Mitochondria</td>
<td>Injury rescue, increased oxygen consumption</td>
</tr>
<tr>
<td>Caicedo et al. (2015)</td>
<td>MDA-MB-231 breast cancer cells</td>
<td>Mitochondria</td>
<td>Increased OXPHOS, ATP production, invasion &amp; proliferation</td>
</tr>
<tr>
<td>Han et al. (2015)</td>
<td>Ischemic H9c2 rat cardiomyocytes</td>
<td>Mitochondria</td>
<td>Apoptosis decrease, mitochondrial function restoration</td>
</tr>
<tr>
<td>Tan et al. (2015)</td>
<td>Murine B16 / melanoma and 4T1 / breast carcinoma</td>
<td>Mitochondria</td>
<td>Restored respiratory functions, increased tumor-initiation</td>
</tr>
<tr>
<td>Jackson et al. (2016)</td>
<td>Macrophages</td>
<td>Mitochondria</td>
<td>Enhanced macrophage phagocytosis and improved</td>
</tr>
<tr>
<td>Jiang et al. (2016)</td>
<td>Corneal epithelial cells</td>
<td>Mitochondria</td>
<td>Protection against oxidative-stress-induced mitochondrial damage</td>
</tr>
<tr>
<td>Moschoi et al. (2016)</td>
<td>Acute Myeloid Leukemia cells</td>
<td>Mitochondria</td>
<td>Increased chemoresistance to ARA-C</td>
</tr>
<tr>
<td>Zhang et al. (2016)</td>
<td>Cardiomyocytes</td>
<td>Mitochondria</td>
<td>Rescue of anthracycline-induced myopathy</td>
</tr>
<tr>
<td>Nzigou Mombo et al. (2017)</td>
<td>Glioblastoma stem cells</td>
<td>Mitochondria</td>
<td></td>
</tr>
<tr>
<td>Sanchez et al. (2017)</td>
<td>Wharton’s jelly mesenchymal stem cells</td>
<td>Mitochondria</td>
<td></td>
</tr>
</tbody>
</table>
mitochondria to the injured murine alveolar epithelial cells and induced more efficient tissue repair than the wild-type MSCs. Conversely, Miro-1-knocked-down MSCs lost their mitochondria transfer capacity and healing ability [36]. Besides, iPS-MSCs expressing high Miro-1 concentrations also demonstrated a higher rate of mitochondria trafficking, leading to the rescue of anthracycline-mediated cardiomyopathy [59].

Mitochondrial transfer through microvesicles

Intercellular trafficking can also rely on extracellular vesicles. These comprise microvesicles and exosomes. Microvesicles are circular fragments, with diameters from 0.1 to 1 μm, that are shed through blebbing and budding processes from lipid-raft-enriched cell membranes. Exosomes, on the other hand, are smaller fragments (40 to 150 nm in diameter) derived from endosomal cell membranes that originate from multivesicular bodies (MVBs). Whereas, due to their smaller size, exosomes can only load mitochondrial DNA, miRNAs, and small proteins like cytokines and chemokines, microvesicles do have the capacity to carry and transport whole mitochondria [94]. Mitochondria transfer through microvesicles has been less extensively described than through TNTs. It was first reported by Islam et al. in 2012 [35] for damaged murine lung alveolar epithelial cells. Interestingly, the authors reported that, in this cell system, the transfer of MSC mitochondria to the lung epithelial cells could occur through both TNTs and microvesicles. Other cell types were since demonstrated to acquire MSC mitochondria via microvesicles, notably macrophages [50,51] and renal tubular epithelial cells [95] (Table 2).

Figure 1. Tunneling nanotube (TNT) formation and mitochondria transfer from mesenchymal stem cells (MSC) to glioblastoma stem cells (GSC). MSCs and GSCs were labeled beforehand, resp. with red MitoTracker CMXRos and green CellTracker CMFDA. The coculture was performed for 48h and fluorescence microscopy was performed on the fixed cells, using a Leica SPE confocal microscope. A TNT connection is observed between the two cell types. The lower frame shows a different MitoTracker and CellTracker exposure that allows the observation of MSC mitochondria (in red) observed inside the TNT and the glioblastoma cell. Scale bar = 50 μm.

Table 2. Cell types involved in microvesicle-mediated mitochondria transfer with MSC and biological outcome.

<table>
<thead>
<tr>
<th>References</th>
<th>Cell types involved in microvesicles trafficking with MSCs</th>
<th>Cargo transferred</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Islam et al. (2012)</td>
<td>Damaged murine alveolar epithelial cells</td>
<td>Mitochondria</td>
<td>Increased ATP production, pulmonary surfactant secretion, lung injury repair</td>
</tr>
<tr>
<td>Phinney et al. (2015)</td>
<td>Macrophages</td>
<td>Mitochondria</td>
<td>Enhanced bioenergetics</td>
</tr>
<tr>
<td>Jackson et al. (2016)</td>
<td>Monocyte-derived macrophages</td>
<td>Mitochondria</td>
<td>Enhanced bioenergetics and macrophage phagocytosis</td>
</tr>
<tr>
<td>Morrison et al. (2017)</td>
<td>Macrophages</td>
<td>Mitochondria</td>
<td>Enhanced bioenergetics and macrophage phagocytosis, lung injury repair</td>
</tr>
</tbody>
</table>
It is remarkable that some cells, like lung epithelial cells and macrophages, can simultaneously employ both mechanisms of mitochondrial transfer, TNTs being apparently more potent in the case of macrophages [35,49]. In fact, following a cytochalasin B pre-treatment at concentrations that block TNT formation without affecting endocytosis, Jackson et al. reported that macrophages acquire MSC mitochondria simultaneously through a TNT-mediated, contact-dependent mechanism and, less intensively, through contact-independent mechanisms.

Transfer of free mitochondria
Beyond TNTs and microvesicles, cell extrusions could constitute an alternative process allowing the conveyance of mitochondria between cells. Although the occurrence of such a phenomenon is yet to be documented for intercellular mitochondria trafficking involving MSCs, several studies indicate that functional mitochondria can be released, during inflammation, as free organelles by different cell types such as platelets [96] to activate neutrophils, or by TNF-α-induced necroptotic cells to activate macrophages and dendritic cells [75]. Importantly, the occurrence of this phenomenon was also detected in vivo [96], pointing out the need to further study this mitochondria transfer mechanism and determine whether it is also relevant for MSC-related mitochondria exchange.

Cells have also been described to internalize free mitochondria, isolated in vitro beforehand. This phenomenon, dubbed "mitochondrial transformation", was first described in 1982 by Clark and Shay. They observed that, by simple co-incubation, chloramphenicol-(CAP)- and efrapeptin-(EF)-sensitive mammalian cells were able to incorporate mitochondria, purified beforehand from CAP- and EF-resistant cells [97]. Ever since, many cells have been reported to undergo mitochondrial transformation [55,98–102]. This process is believed to occur via macropinocytosis. In fact, pre-treating cells with EIPA, a macropinocytosis inhibitor, impeded the mitochondria uptake in a dose-dependent manner whereas chlorpromazine, a clathrin-mediated endocytosis inhibitor, had no effect [98,100].

Mitochondrial transformation depends on the integrity of the outer mitochondrial membrane and the presence of intact fusion proteins that conduct effective mitochondrion-cell interactions [100,101]. For instance, Díaz-Cardallo and colleagues reported that mitochondria acquisition in chemo-resistant U87MG glioblastoma cells is syncytin-mediated. In fact, the outer mitochondrial membrane harbors both HERV-W-E1 (syncytin-1) and HERV-FRD1 (syncytin-2) and their respective receptors ASC2 and MFSD2 in order to accomplish successful mitochondrial transformation, which was blocked by anti-syncytin 1–2 antibodies [101]. Mitochondria uptake was also described in vivo in a rabbit ischemia model. Cardiomyocytes were able to internalize free autologous mitochondria transplanted at the ischemia site, resulting in the reduction of the infarct size and the increase of contractility and ATP production [103]. Following the efficiency of mitochondria transplantation on cardioprotection in animal models, the first clinical application was conducted on five pediatric patients suffering from myocardial ischemia-reperfusion injury, leading to a significant improvement in their myocardial systolic function [104].

Unsolved questions and perspectives
As outlined above, the whole process of intercellular mitochondria trafficking, notably through TNTs, constitutes a novel biological concept [88]. It is of great interest both for the sake of a holistic comprehension of the cellular interactions taking place in tissues and for the possible clinical applications of this discovery. As this research domain is still in its early phases of study, a number of key questions obviously remain to be answered. For instance, it is unknown whether the mitochondria destined to be exchanged undergo a pre-selection process. It could be either the selection of "healthy" mitochondria originating from "healthy" cells and targeted to damaged cells, resulting in the repair of these cells or, conversely, mitochondria endowed with a lowered membrane potential released from cells of an injured tissue. Mitochondria are also known to undergo the dynamic process of fusion/fission [105,106]. So far, it is unclear to what extent this process interferes with intercellular mitochondria trafficking. However, mitochondria dynamics were reported to be affected by exogenous mitochondria delivery in vitro [13,107] and to be closely linked to mitophagy, mitochondria biogenesis and motility [13,81,108]. Consequently, this mitochondria fusion/fission process is predicted to play a critical role in the fate of the conveyed organelles and of their genome. Persistence of the mitochondria and their mtDNA, following their transfer from Whitson's Jelly MSCs (WJ MSCs), was recently reported by Chuang and colleagues, in cybrids harboring mitochondrial DNA mutations for the MERRF (Myoclonus epilepsy associated with ragged-red fibers) syndrome [107]. The authors reported that exogenous mitochondria correct the MERRF phenotype, in the long-term, through their fusion with the recipient mutated mitochondria. This led to mtDNA heteroplasmy and, as a consequence, to a reduction in the mutant mtDNA load in the MERRF cybrids [107].

A number of questions also arise about the cellular mechanisms enabling the mitochondria trafficking from one cell to the other. In particular, it still to be determined whether a given cell type exclusively uses TNTs, microvesicles or cell extrusion for mitochondrial transfer or whether it can accommodate all three. In addition, further investigation will be required to assess the effects of the mitochondria state per se or of more general factors, like the microenvironment conditions, in promoting either one of these mitochondria transfer mechanisms.

A robust knowledge of the processes that allow intercellular mitochondria transfers is expected to nurture clinical applications in a number of medical fields. First of all, the discovery of mitochondria transfer originating from MSCs is anticipated to open new avenues for the optimization of MSC-based therapies, notably for degenerative diseases associated with impaired mitochondrial functions. These include diseases affecting the central nervous system (i.e. Alzheimer and Parkinson diseases) [109,110], the cardiovascular system (myocardial infarction/ischemia) [29,30] and the lung (chronic obstructive pulmonary disease) [31].

Beyond the treatment of degenerative pathologies, the mitochondrial donor capacity of MSCs could be exploited for other clinical purposes. It could notably be extended to human diseases characterized by mitochondrial DNA mutations, as suggested by the recent findings on the MERRF syndrome [107].
This maternally inherited disease affects the nervous system and skeletal muscles. It is associated with a point mutation in the mitochondrial tRNALys encoding gene, leading to severe defects in protein synthesis, to oxidative stress and impaired OXPHOS [111]. In their recent study, Chuang and al. reported that the delivery of healthy MSC mitochondria rescued the phenotype of the MERRF cybrids by reducing the oxidative stress and improving mitochondrial bioenergetics [107].

Finally, targeting the mitochondria transfer process, with the goal of inhibiting it rather than activating it, also appears as a promising adjuvant approach to combat cancer progression. It would be very instructive to be able to establish links between the occurrence of mitochondria transfers within tumors and clinical criteria such as cancer progression, relapse or overall patient survival. Nevertheless, it was established that mitochondria transfers lead to drug resistance, as shown for solid tumors and leukemia, therefore contributing to the general process of environment-mediated drug resistance (EM-DR) [52,54]. It is worth noting that this mitochondria-related EM-DR is further increased by chemotherapeutic agents like doxorubicine, cytarabine or etoposide [52,54,57,59,112] while other conventional chemotherapies, such as vincristine or cytoplasm that target the cytoskeleton, do not demonstrate these effects [54,112]. Overall, these data suggest the targeting of the intercellular mitochondria transfer as an attractive novel adjuvant approach to improve current anticancer regimens. 

Conclusion

The mitochondria transfer occurring between cells, notably to and from MSCs, constitutes a fascinating and still largely unexplored process with expected pleiotropic effects on diseases including degenerative diseases, cancers and mitochondrial inherited pathologies. Novel insights on the mechanisms involved in these mitochondria exchanges will be valuable to pharmacologically stimulate or abrogate these organelles exchanges and, accordingly, to develop novel therapeutic approaches to regenerate damaged organs, treat defective mitochondria-related diseases and curb cancer progression.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank the Montpellier RIO imaging facility (MRI) for providing adequate environment for confocal microscopy. AMR and M.L.V are staff scientists from the French National Institute of Health and Medical Research (INSERM) and the National Center for scientific research (CNRS), respectively. J.N. is supported by a PhD fellowship from the French Ministry of Research. This work was supported by grants from the Ligue Contre le Cancer – Comité de l’Aude and Association pour la Recherche et l’Etude des Maladies Cardiovasculaires (AREMCAR).

References


Jackson MV, Morrison TJ, Doherty DF, et al. Mitochondrial transfer via tunneling nanotubes is an important mechanism by which mesenchymal stem cells enhance macrophage phagocytosis in the in vitro and in vivo models of ARDS. Stem Cells Dev. 2016;25:2210–2223. doi:10.1089/stem.2016.2243.


Schwarz TL. Mitochondrial trafficking in nerves. Cold Spring Harb Perspect Biol [Internet]. 2013;5. doi:10.1101/cshperspect.a011304. PMID:23732472


CHAPTER 2:
ROLE OF METABOLISM IN CANCER DRUG RESISTANCE
Cancer cells sustain metabolic reprogramming whereby the metabolic flux via conventional metabolic pathways is modified in order to generate ATP, maintain redox balance and produce biomass. Metabolic reprogramming is a consequence of mutations that can either target the mitochondrial DNA or genes encoding key metabolic enzymes. Tumor cells actively engage both in glycolysis (and its branching pathways) and in TCA cycle metabolism. Glycolysis supports cell proliferation by generating metabolic intermediates that can be funneled into multiple biosynthetic pathways, such as the pentose phosphate pathway and the one-carbon metabolism, which sustains nucleotide synthesis, NADPH production and methylation reactions. TCA cycle generates metabolites that shunt into amino acid, lipid, nucleotide and heme synthesis (cataplerosis). In return, the TCA cycle is replenished in carbons in order to stay functional (anaplerosis). Several anaplerotic reactions are employed by cancer cells, including the oxidation of glutamine into α-ketoglutarate, the catabolism of branched-chain amino acids into succinyl-CoA, and the carboxylation of pyruvate into oxaloacetate. TCA cycle also produces NADH and FADH2 that are regenerated to NAD+ and FAD by the mitochondrial electron transport chain. Besides the anabolic role of the TCA cycle, it also generates oncometabolites, as a consequence of mutations in mitochondria DNA or in TCA cycle enzymes, that drive tumor growth. In particular, loss-of-function mutations in succinate dehydrogenase and fumarate hydratase result in succinate and fumarate accumulation, respectively. Consequently, these metabolites inhibit α-ketoglutarate-dependent dioxygenases involved in histone and DNA methylation, thereby inducing epigenetic modifications that contribute to malignant transformation.

A thorough understanding of the metabolic pathways allowing cancer cells to enhance their growth rate and invasiveness, and to counteract therapy, will be paramount in order to build an integrated view of the hallmark metabolic features of cancer and to envision effective therapeutic strategies. In this chapter, I discuss how cancer cell modify their metabolism as a shield against therapy. First, I will focus on the role of the recently-discovered mitochondria transfers and of mutations targeting major metabolic pathway enzymes (REVIEW 2: HEKMATSHOAR, NAKHLE ET AL., 2018). Then, I will give an overview on the critical involvement of mitochondrial DNA mutations as well as mitochondrial metabolites/components in metabolic diseases (REVIEW 3: NAKHLE ET AL., 2020).
The role of metabolism and tunneling nanotube-mediated intercellular mitochondria exchange in cancer drug resistance

Yalda Hekmatshoar1,2, Jean Nakhle1,3, Mireille Galloni1 and Marie-Luce Vignais1

1IRMB, INSERM, CNRS, Université de Montpellier, Montpellier, France; 2Department of Medical Biology, School of Medicine, Ankara University, Ankara, Turkey; 3IGMM, CNRS Université de Montpellier, Montpellier, France

Correspondence: Marie-Luce Vignais (marie-luce.vignais@inserm.fr)

Mechanisms of drug resistance

Resistance to cancer therapy is still a major obstacle for effective and lasting treatment, resulting in relapse, metastasis and reduced overall survival. Many mechanisms have been described that foster this resistance, including both cell autonomous (or intrinsic) and extrinsic processes, the latter greatly resulting from the tumor microenvironment (TME) complexity [1,2]. It is indeed becoming increasingly evident that tumors do not behave as masses of homogeneous malignant cells, but rather as complex, full-fledged ‘organs’ in dynamic progression through time and space, resulting in enhanced tumor fitness and resistance to therapy [3,4].

Drug resistance intrinsic processes

Understanding the drug resistance molecular mechanisms is more crucial than ever in order to achieve effective and long-lasting cancer therapy. The mechanisms of drug resistance include drug transporters, DNA damage repair (DDR) and genomic instability, apoptosis inhibition and metabolic adaptation [5,6]. Unfortunately, these mechanisms often overlap in the context of cancer, adding an extra layer of complexity that often precludes the full deciphering of all resistance causes.
Drug efflux
Transporters are one of the most significant players in multidrug resistance. ATP-binding cassette (ABC) proteins are pumps responsible for the active transport of various substrates, notably xenobiotics, across cell membranes. ABC transporters are minimally formed by two transmembrane domains that recognize and transport substrates across the membrane through conformational changes, in addition to two nucleotide-binding domains that hydrolyze ATP into ADP. ABC proteins include (a) P-glycoprotein (P-gp) also known as Multi-Drug Resistance protein 1 (MDR1) or ATP-Binding Cassette subfamily B member 1 (ABCB1), (b) Multidrug Resistance-Associated Protein 1 (MRP1), (c) Breast Cancer Resistance Protein (BCRP) also known as ATP-Binding Cassette subfamily G member 2 (ABCG2) and (d) Lung Resistance-related Protein (LRP) also known as Major Vault Protein (MVP) [7]. Efflux proteins like MDR1, MRP1, ABCG2 and MVP expel drugs outside the cell, thus decreasing their intracellular concentration and contributing to chemoresistance. Overexpression of efflux proteins has been described in many hematological and solid malignancies, such as leukemia, neuroblastoma, lung, breast and ovarian cancers [6]. On the other hand, underexpression of influx proteins like Organic Cation Transporter-1 (Oct-1) results in an insufficient drug uptake and, consequently, in chemoresistance as well [8,9].

DDR and genomic instability
Repair of DNA damage is another molecular mechanism accounting for drug resistance. DNA repair is a biological process whereby DNA damage is recognized and corrected by the cell DDR machinery. Increased levels of endogenous reactive oxygen species (ROS), ultraviolet (UV) radiation, mutagenic chemicals and chemotherapeutic agents have been shown to cause an accumulation of DNA alterations. Accordingly, DNA repair mechanisms, such as Base Excision Repair (BER), Nucleotide Excision Repair (NER) and Mismatch Repair (MMR) are triggered to repair the damaged DNA and thus contribute to resistance to DNA-targeting drugs [10]. Efficient NER is essential for DNA repair induced by DNA-damaging therapies, such as platinum-based drugs. Excision Repair Cross-Complementing 1 (ERCC1) is one of the most important NER enzymes. Very low levels of ERCC1 are found in cisplatin-sensitive cancers, such as testicular cancers. In contrast, ERCC1 overexpression has been correlated with a poor chemotherapy clinical response in numerous cancer types, including non-small cell lung carcinoma (NSCLC), melanoma, gastric and ovarian cancers [5]. MMR is responsible for the maintenance of genomic integrity, and experiments have shown that MMR deficiency is linked with increased tolerance to DNA-damaging drugs like cisplatin [5]. Another DNA repair protein, O6-methylguanine-DNA-methyltransferase (MGMT) reduces the mutagenic effects of DNA-alkylating agents such as temozolomide (TMZ), thus playing a crucial role in TMZ resistance in Glioblastoma Multiforme (GBM) patients [11].

Genomic instability may arise following cancer therapy, leading to additional mutations and conferring further drug resistance. These include alterations to the drug target, such as mutations or changes in expression levels, or emergence of new driver mutations that help bypass the effects of the drug. For instance, overexpression of the androgen receptor (AR) in prostate cancers confers acquired resistance to standard androgen deprivation therapy such as testosterone-lowering drugs or AR antagonists. Likewise, patients with high initial response to Epidermal Growth Factor Receptor (EGFR) inhibitors in NSCLCs acquire resistance mostly due to a secondary EGFR mutation [5].

Inhibition of apoptosis and metabolic adaptation
As will be further discussed below, mitochondria play an active and central role in cancer drug resistance as they constitute a hub for several molecular mechanisms such as apoptosis and metabolic reprogramming [12,13]. Apoptosis provides an important mechanism for the maintenance of cellular homeostasis under physiological conditions. BCL2 family proteins are mostly known for their role in regulating apoptosis. The BCL2 family comprises anti-apoptotic proteins, such as BCL2, BCL-XL and MCL1, that promote cell survival as well as pro-apoptotic proteins, such as BID, BIM, BAD, BAX and BAK, that are required to initiate cell death. Down-regulation of the pro-apoptotic genes was reported to play a critical role in drug resistance for various cancers such as chronic lymphoblastic leukemia, head and neck and ovarian cancers. On the other hand, up-regulation of the anti-apoptotic genes was correlated with chemoresistance in myeloma, melanoma, mesothelioma, laryngeal, pancreatic, ovarian and prostate cancers [6,14,15].

Alterations of cancer cell metabolism can affect cancer cell proliferation, differentiation, apoptosis and response to therapy, through the intervention of several metabolic pathways. Intriguingly, metabolic reprogramming in cancer cells was also observed following many chemotherapies, which was further linked to resistance.
to these therapies [16,17]. In this review, we will discuss the role of cancer cell metabolism in acquired drug resistance, focusing on major metabolic pathway enzymes, on metabolites secreted within tumors and on the newly discovered TNT-mediated mitochondria transfers.

**Drug resistance extrinsic processes: a consequence of intratumoral heterogeneity**

Intratumoral heterogeneity mainly stems from three key factors. Firstly, spatially and/or temporally distant tumor regions can correspond to genetically distinct subclones that originated through branching evolution [18]. This was demonstrated with the advent of high-throughput sequencing, such as whole-exome sequencing (WES) and whole-genome sequencing (WGS). A cell may acquire a mutation that will give rise to a clone endowed with a survival advantage over surrounding cells lacking this mutation. Additional mutations may accumulate afterwards, providing further fitness to other cells within the clone. These driver mutations translate into a plethora of phenotypic manifestations, thus contributing to functional heterogeneity. Secondly, many tumors exhibit a particular population of self-renewing cells, known as cancer stem cells (CSCs), as evidenced by lineage tracing and cell ablation experiments, that can be isolated from patients. It is increasingly evident that tumors are hierarchically organized malignant tissues where CSCs represent the apex of the hierarchy and sustain the long-term repopulation of the tumor [19]. Evidences from xenograft experiments in immunodeficient mice indicate that CSCs have the capacity to (a) regenerate tumors evocative of the heterogeneity of the tumor of origin, (b) give rise to cells at different differentiation states and (c) survive many commonly used cancer treatments, which makes CSCs highly predictive of the patient’s overall survival [20]. Finally, a third layer of heterogeneity arises from the fact that the TME harbors many non-malignant cells, including cancer-associated fibroblasts (CAFs), mesenchymal stem cells (MSCs) and immune cells that are recruited to the tumor site [3,21]. The interactions of tumor cells with their microenvironment highly modify their fate and, thereby, their acquisition of drug resistance. Intercellular communications are prominent contributors to tumor heterogeneity, strongly underlying therapeutic failure and tumor relapse.

**Cellular communications within the TME**

In this chapter, we provide a general overview of the different means of intercellular communication implicated in drug resistance within the tumor niche, namely soluble factors and extracellular vesicles (EVs), with a special focus on the recently discovered tunneling nanotubes (TNTs). The interest in TNTs has been steadily growing over the years due to the biological cargos they transport and their subsequent biological effects on the recipient cell, mitochondria being the most widely studied cargos thus far (Table 1). We further discuss the contribution of the transferred mitochondria to the deregulation of cancer cell metabolism and subsequent resistance to therapy.

**Soluble factors**

It has been long recognized that the intercellular communications within the tumor niche heavily rely on soluble factors like chemokines and cytokines [53,54]. Among them, Transforming Growth Factor-β (TGF-β) plays a pivotal role in cancer progression and drug resistance. TGF-β promotes epithelial-to-mesenchymal transition (EMT), angiogenesis and tumor-cell escape from immune surveillance. Accordingly, high TGF-β levels correlate with poor prognosis for cancer patients [55].

**Extracellular vesicles**

Cells secrete many heterogeneous vesicles, commonly referred to as EVs, that differ in their biogenesis, morphology and size as well as in the molecular cargos they carry. EVs are circular fragments of lipid bilayers, mainly comprising sphingolipids, cholesterol and ceramide [56]. Based on their sizes and biogenesis mechanisms, EV populations can be categorized into (a) small exosomes (40–150 nm in diameter) originating from endocytosis and able to carry only small molecules like proteins, mitochondrial DNA (mtDNA) and micro RNAs (miRNAs), or (b) bigger microvesicles (0.1–1 μm in diameter) directly deriving from the plasma membrane by blebbing and budding and capable of engulfing bigger cargos like entire mitochondria [56]. Larger membrane-derived vesicles that transfer oncogenic signals have also been described as a distinct EV category, known as oncosomes (1–10 μm in diameter) [57–59].
<table>
<thead>
<tr>
<th>Publications</th>
<th>Donor cells</th>
<th>Target cells</th>
<th>Metabolic and functional outcome</th>
<th>Cellular insults</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onfelt et al. [22]</td>
<td>Monocyte-derived macrophages</td>
<td>Human monocyte-derived macrophages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Koyanagi et al. [23]</td>
<td>Endothelial progenitor cells</td>
<td>Neonatal rat cardiomyocytes</td>
<td>Acquisition of a cardiomyogenic phenotype</td>
<td></td>
</tr>
<tr>
<td>Spees et al. [24]*</td>
<td>MSCs</td>
<td>A549 ρ0 lung epithelial cells</td>
<td>Restoration of aerobic respiration</td>
<td>mtDNA damage</td>
</tr>
<tr>
<td>Plotnikov et al. [25]</td>
<td>MSCs</td>
<td>Rat cardiomyocytes</td>
<td>Restoration of cardiomyocytes' bioenergetics</td>
<td></td>
</tr>
<tr>
<td>Plotnikov et al. [26]</td>
<td>MSCs</td>
<td>Rat renal tubular cells</td>
<td>MSCs differentiation into kidney tubular cells</td>
<td></td>
</tr>
<tr>
<td>Wang et al. [27]</td>
<td>Rat hippocampal neurons and astrocytes</td>
<td>Rat hippocampal neurons</td>
<td>Metabolic reprogramming into a progenitor-like state</td>
<td></td>
</tr>
<tr>
<td>Acquistapace et al. [28]</td>
<td>MSCs</td>
<td>Adult cardiomyocytes</td>
<td>Metabolic reprogramming into a progenitor-like state</td>
<td></td>
</tr>
<tr>
<td>Islam et al. [29]</td>
<td>MSCs</td>
<td>Damaged murine alveolar epithelial cells</td>
<td>Restoration of ATP levels</td>
<td>LPS-induced lung injury</td>
</tr>
<tr>
<td>Lou et al. [30]</td>
<td>Human primary cancer cells</td>
<td>Human primary cancer cells</td>
<td>Increased mouse survival</td>
<td></td>
</tr>
<tr>
<td>Vallabhaneni et al. [31]</td>
<td>MSCs</td>
<td>Vascular smooth muscle cells</td>
<td>Increase in MSCs proliferation</td>
<td></td>
</tr>
<tr>
<td>Pasquier et al. [32]</td>
<td>MSCs</td>
<td>Human ovarian and breast cancer cell lines</td>
<td>Resistance to doxorubicin</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>Ahmad et al. [33]</td>
<td>MSCs</td>
<td>Stressed murine lung epithelial cells</td>
<td>Decrease in apoptosis and inflammatory cell infiltration</td>
<td>Rotenone-induced airway injury</td>
</tr>
<tr>
<td>Li et al. [34]</td>
<td>MSCs</td>
<td>Lung epithelial cells</td>
<td>Decrease in alveolar damage</td>
<td>Cigarette smoke</td>
</tr>
<tr>
<td>Liu et al. [35]</td>
<td>MSCs</td>
<td>HUVEC</td>
<td>Increased oxygen consumption and cell viability</td>
<td>Ischemic damage</td>
</tr>
<tr>
<td>Caicedo et al. [36]</td>
<td>MSCs</td>
<td>MDA-MB-231 breast cancer cells</td>
<td>Increase in OXPHOS and ATP production, decrease in glycolysis and lactate production</td>
<td></td>
</tr>
<tr>
<td>Wang and Gerdes [37]</td>
<td>Healthy PC12 cells</td>
<td>Stressed PC12 cells</td>
<td>Decrease in apoptosis</td>
<td>UV light</td>
</tr>
<tr>
<td>Tan et al. [38]*</td>
<td>MSCs</td>
<td>ρ0 B16 melanoma and 4T1 breast carcinoma</td>
<td>Restoration of respiratory functions, increase in tumor-initiating capacity</td>
<td>mtDNA damage</td>
</tr>
<tr>
<td>Han et al. [39]</td>
<td>MSCs</td>
<td>Ischemic H9c2 rat cardiomyocytes</td>
<td>Decrease in apoptosis, restoration of mitochondrial function</td>
<td>Ischemic damage</td>
</tr>
<tr>
<td>Hayakawa et al. [40]</td>
<td>Astrocytes</td>
<td>Neurons</td>
<td>Restoration of ATP levels and viability</td>
<td>Ischemic damage</td>
</tr>
</tbody>
</table>

Continued
Cancer cell-derived EVs were described to play an active role in intercellular communication within the TME [59–61]. Several studies demonstrated an EV-mediated horizontal propagation of tumor-promoting proteins like mutant Epithelial Growth Factor Receptor (EGFR) [57,62] and of nucleic acids such as double-stranded DNA [63], mRNAs [62] and miRNAs [64,65] from tumor cells to non-malignant cells of the TME. Tumor-derived EVs have been shown to modify the TME in various ways. First, they participate in the remodeling of the extracellular matrix (ECM) by transferring metalloproteases like MT1-MMP, MMP9, MMP2 and MMP14 [66–68]. Furthermore, tumor-derived EVs can functionally reprogram normal fibroblasts of the TME into CAFs [69,70]. This reprogramming is mediated by TGF-β1 in the case of mesothelioma cells [69,70], or fibronectin 1 (FN1) and transglutaminase (tTG) in breast cancer and glioblastoma cells [71]. Secondly, EVs play an important role in angiogenesis as they were shown to engulf canonical pro-angiogenic factors like interleukin-6 (IL-6), Vascular Endothelial Growth Factor (VEGF) and EGFR [57,62,72], as well as other recently discovered molecules that participate in angiogenesis, such as miR-210 [73] and miR-9 [74]. Thirdly, from an immunological perspective, tumor-derived EVs can impede dendritic cell maturation and natural killer (NK) cell activation, thereby promoting immune tolerance [75]. In addition, they educate macrophages into a pro-tumor phenotype with a pro-inflammatory secretion profile [75]. TGF-β-carrying EVs can also increase regulatory T cell proliferation and induce effector T cell apoptosis, thus creating an immuno-tolerant niche [75].

### Table 1 Cell types involved in TNT-mediated mitochondria transfer and biological outcomes

<table>
<thead>
<tr>
<th>Publications</th>
<th>Donor cells</th>
<th>Target cells</th>
<th>Metabolic and functional outcome</th>
<th>Cellular insults</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jackson et al. [41]</td>
<td>MSCs</td>
<td>Mouse and human macrophages</td>
<td>Increased basal respiration and mitochondrial ATP turnover</td>
<td>E. coli pneumonia</td>
</tr>
<tr>
<td>Jiang et al. [42]</td>
<td>MSCs</td>
<td>Corneal epithelial cells</td>
<td>Increase in OXPHOS, protection against mitochondrial damage</td>
<td>Rotenone-induced oxidative stress</td>
</tr>
<tr>
<td>Moschoi et al. [43]</td>
<td>MSCs</td>
<td>AML cells</td>
<td>Increase in OXPHOS and ATP production</td>
<td>Cytarabine (ARA-C)</td>
</tr>
<tr>
<td>Zhang et al. [44]</td>
<td>MSCs</td>
<td>Cardiomyocytes</td>
<td>Increase in OXPHOS and ATP production</td>
<td>Anthracycline-induced cardiomyopathy</td>
</tr>
<tr>
<td>Lu et al. [45]</td>
<td>T24 bladder cancer cells</td>
<td>RT4 bladder cancer cells</td>
<td>Increase in invasiveness</td>
<td></td>
</tr>
<tr>
<td>Mahrouf-Yorgov et al. [46]</td>
<td>Cardiomyocytes; HUVECs</td>
<td>MSCs</td>
<td>Activation of autophagy, HO-1 and mitochondrial biogenesis</td>
<td>Ischemic damage</td>
</tr>
<tr>
<td>Marlein et al. [47]</td>
<td>MSCs</td>
<td>AML blasts</td>
<td>Increase in OXPHOS and ATP production</td>
<td></td>
</tr>
<tr>
<td>Neigou Mombbo et al. [48]</td>
<td>MSCs</td>
<td>Glioblastoma stem cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sanchez et al. [49]</td>
<td>Wharton’s jelly MSCs</td>
<td>Wharton’s jelly MSCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Babenko et al. [50]</td>
<td>MSCs</td>
<td>Astrocytes and PC12 cells</td>
<td>Increase in OXPHOS, decrease in glycolysis</td>
<td>Ischemic damage</td>
</tr>
<tr>
<td>Guo et al. [51]</td>
<td>Uninfected MARC-145 cells</td>
<td>PRRSV-infected MARC-145 cells</td>
<td>Decrease in apoptosis and necrosis</td>
<td>PRRSV infection</td>
</tr>
<tr>
<td>Wang et al. [52]</td>
<td>Jurkat and human T-ALL cells</td>
<td>MSCs</td>
<td>Decrease in ROS levels</td>
<td>Chemo therapy (ARA-C; MTX)</td>
</tr>
</tbody>
</table>

*TNTs were shown to contain either mitochondria or mtDNA [24] or mtDNA only [38].

Cancer cell-derived EVs were described to play an active role in intercellular communication within the TME [59–61]. Several studies demonstrated an EV-mediated horizontal propagation of tumor-promoting proteins like mutant Epithelial Growth Factor Receptor (EGFR) [57,62] and of nucleic acids such as double-stranded DNA [63], mRNAs [62] and miRNAs [64,65] from tumor cells to non-malignant cells of the TME. Tumor-derived EVs have been shown to modify the TME in various ways. First, they participate in the remodeling of the extracellular matrix (ECM) by transferring metalloproteases like MT1-MMP, MMP9, MMP2 and MMP14 [66–68]. Furthermore, tumor-derived EVs can functionally reprogram normal fibroblasts of the TME into CAFs [69,70]. This reprogramming is mediated by TGF-β1 in the case of mesothelioma cells [69,70], or fibronectin 1 (FN1) and transglutaminase (tTG) in breast cancer and glioblastoma cells [71]. Secondly, EVs play an important role in angiogenesis as they were shown to engulf canonical pro-angiogenic factors like interleukin-6 (IL-6), Vascular Endothelial Growth Factor (VEGF) and EGFR [57,62,72], as well as other recently discovered molecules that participate in angiogenesis, such as miR-210 [73] and miR-9 [74]. Thirdly, from an immunological perspective, tumor-derived EVs can impede dendritic cell maturation and natural killer (NK) cell activation, thereby promoting immune tolerance [75]. In addition, they educate macrophages into a pro-tumor phenotype with a pro-inflammatory secretion profile [75]. TGF-β-carrying EVs can also increase regulatory T cell proliferation and induce effector T cell apoptosis, thus creating an immuno-tolerant niche [75].
Finally, EVs are also implicated in drug insensitivity as they were shown to propagate docetaxel resistance from resistant to sensitive breast cancer cells [76] as well as gefitinib resistance in NSCLC [77].

Oncosomes are cancer-derived EVs with an atypically large size (>1000 times larger than standard exosomes) [59]. In the context of gliomas, cancer cells expressing the truncated oncogenic EGFR variant EGFRvIII were capable of horizontally transferring it to neighboring normal cells via oncosomes. Oncosomes merged with the plasma membrane of the recipient cells, thereby inducing their transformation [57]. Large oncosomes were also observed to be shed from the membrane of prostate cancer cells that have acquired an amoeboid migration phenotype and to transfer Cav-1, a biomarker of metastatic prostate cancer. These cancer-derived EVs activated the Akt signaling pathway in the recipient neighboring tumor cells stimulating both their proliferation and migration capacities [78,79]. Likewise, both estrogen receptor alpha (ERα) positive and negative human breast cancer cell lines were shown to produce oncosome-like vesicles [58]. The capacity to shed large oncosome-like blebs was also shown for CAFs but not for benign tissue fibroblasts [65].

**Tunneling nanotubes**

TNTs were first described in 2004 in two elegant studies in both human and murine immune cells [22] and in the pheochromocytoma-derived PC12 cancer cell line [80]. TNTs are transient cytoplasmic extensions connecting non-adjacent cells that can span impressive lengths of several hundreds of micrometers, with diameters ranging from 50 to 1500 nm [81]. Remarkably, TNTs are characterized by a continuity of both the plasma membrane and the cytoplasm of the two connected cells allowing trafficking of much bigger cargos than gap junctions. This strikingly challenges our current definition of cells, delimited by their own plasma membrane, and even of tissues with this TNT-based unanticipated level of intercellular communication [82].

Two main mechanisms of TNT formation were proposed by Zurzolo and collaborators, both requiring active cell motion and cytoskeleton remodeling [83,84]. In the first model, known as the actin-driven mechanism, one cell extends a filopodia-like protrusion toward another distant cell. Membrane fusion at the contact site gives rise to the TNT structure. In the second model, known as the cell dislodgement mechanism, membrane fusion occurs between two cells in close physical contact. Their subsequent migration in opposite directions elongates a TNT connection. Connexin 43 (Cx43), also known as GAP Junction Alpha-1 protein (GJA1), was also demonstrated to be required for the establishment of GAP junctions between the two connecting cells, at the site of cell–cell physical contact [29].

TNTs can transport a multitude of cellular signals that range from ions (e.g. Ca$^{2+}$) [85,86] and small molecules (e.g. miRNAs) to entire organelles (e.g. mitochondria) [22–51,87] (Table 1). Other transported cargos include organelle-derived vesicles (early endosomes, endoplasmic reticulum, Golgi apparatus and lysosomes) [27,85] and plasma membrane elements [80]. TNTs also allow the transfer of pathogens like bacteria [88], viruses [89,90] and prions [91], thus leading to the spreading of the corresponding pathologies.

TNTs were originally observed in co-culture experiments in vitro [22,80], but also rapidly reported to actually occur in vivo [29,33,84,92] with the primary observation made in resected solid tumors from pleural mesothelioma and lung carcinoma [30]. Several cancer cells were described to engage into efficient cell–cell communications via TNTs including colon [93], breast [32,36,94], ovarian [32,95,96] and bladder [45] cancer cells, in addition to pheochromocytoma [80,97,98], malignant mesothelioma [30,99,100], laryngeal squamous cell carcinoma [101], osteosarcoma (OS) [96] and astrocytoma cells [86].

TNTs do not only link cells of the same type as it is the case for astrocytomas [86], but they were also described to occur between healthy and transformed cells as it was observed for human ovarian epithelial cells and ovarian cancer-derived SKOV3 cells and for murine stromal osteoblasts MC3T3 and K7M2 OS cell lines [96]. Moreover, TNTs can connect cancer cells with cells of their microenvironment, as shown for HeLa cells and NRK fibroblasts [102]. A large number of studies focused on TNT connections between cancer cells and MSCs [87], key components of the TME [103,104]. These include breast and ovarian cancer cells [32], as well as acute myeloid leukemia [43,105], melanoma [38], glioblastoma [48] and breast cancer cells [36].

It is worthwhile mentioning that TNTs do not only occur in pathological or malignant conditions, as TNTs were also shown to participate in cell–cell communications in physiological conditions, such as development and immunity [87].

**Tunneling nanotubes-mediated intercellular mitochondria transfer**

Mitochondria are the TNT cargos most widely studied thus far. This can be explained by the magnitude of the biological effects of the transported mitochondria, on the metabolism and functional properties of the receiving...
cells. Additionally, from a practical perspective, the wide range of available experimental tools made it easier to track mitochondria trafficking, both in vivo and in vitro, albeit with some limitations [106].

Mitochondria are structurally dynamic, double-membrane organelles that play a wide range of essential cellular functions. As the site of oxidative phosphorylation (OXPHOS), mitochondria convert energy-rich molecules into adenosine triphosphate (ATP) via a series of redox reactions through the different complexes of the electron transport chain (ETC). Beyond being the powerhouse of the cell, they regulate many other biological processes including cell metabolism, apoptosis, calcium signaling and cellular temperature.

The vast majority of mitochondrial proteins are encoded by the nuclear genome, translated by cytosolic ribosomes and subsequently imported into the mitochondria [107]. Nevertheless, mitochondria still maintain a small genome, essential for their respiratory function. The human mitochondrial genome (mtDNA) is a 16.5 kb, double-stranded, circular DNA encoding 13 polypeptides of the mitochondrial respiratory chain, in addition to 2 rRNAs and 22 tRNAs involved in mitochondrial protein synthesis. Moreover, it contains a non-coding regulatory region called the displacement loop (D-loop). The D-loop contains a replication origin and transcription regulatory sites. Overall, the D-loop integrates nuclear-encoded events into the regulation of transcription and replication of the mtDNA [13]. Depending on the cell type, mitochondria contain a variable number of mtDNA molecules, with an average of 5 molecules per mitochondrion [108].

Because the donor cell mtDNA is transferred along with the mitochondria in the TNTs, it is worth mentioning that the mitochondria-recipient cells will harbor both the donor cell mtDNA and their own. These mtDNAs can be genetic variants i.e. intact in the normal cells and mutated in cancer cells. From a practical standpoint, the heteroplasmy originating from either different mtDNA haplotypes (for different donors) or based on mtDNA mutations (i.e. for cancer vs. normal cells) allows for tracking of the transferred mitochondria [36,48].

The occurrence of TNT-mediated mitochondria transfer was reported both between cancer cells and between cancer cells and normal cells of the microenvironment such as MSCs [109]. This was observed in many different cancer types, including squamous cell carcinoma [101,110], mesothelioma [30], acute myeloid leukemia (AML) [43,47,111], breast [32,36,94], bladder [45] and ovarian cancers [32,95] as well as in glioblastoma (Figure 1) [48].

Figure 1. Transfer of mitochondria between MSCs and glioblastoma stem cells (GSCs).
MSCs were labeled with a red MitoTracker and GSCs with a green CellTracker before the co-culture. Imaging of live cells (Zeiss LSM 700) was performed 24 h after the beginning of the co-culture. GSCs are observed as green-stained cells, while the MSCs mitochondria networks are seen in red. In the frame with higher exposure, the MSC mitochondria (red) are observed inside the GSC-MSC TNT and inside the GSC. Scale bar: 50 μm. Asterisks: MSC mitochondria.
The most observed outcome of mitochondria uptake is a survival advantage for the recipient cell. For cancer cells, several studies reported that the acquisition of mitochondria endowed them with a survival benefit, thus enhancing their regrowth potential and increasing their chemoresistance as further detailed in the next part of this review. Briefly, the mitochondria transfer from endothelial cells to MCF7 breast cancer cells increased their resistance to doxorubicin [32]. Similar results were observed for hematopoietic malignancies such as AML [43]. AML cells were shown to take up functional mitochondria derived from bone marrow stromal cells after co-culture, leading to resistance to cytarabine (ARA-C) treatment [43]. In line with these studies, MSC mitochondria were able to increase the proliferation and invasion capacities of the MDA-MB-231 breast cancer cell line [36].

A direct effect of the TNT-mediated transfer of mitochondria is the modification of the target cell energetic metabolism, with increased OXPHOS at the expense of glycolysis in the various cell systems studied [36,38,43,112].

In addition to the ‘inbound’ horizontal cargo transfers to cancer cells described above for mitochondria, TNT-mediated ‘outbound’ transfers from cancer cells can also play a role in drug resistance as it was shown with the detoxifying role of chemotherapeutic-loaded lysosomal vesicles from leukemic cells [113].

**Role of intercellular mitochondria and mtDNA transfer in cancer cell drug resistance**

As described above, cancer cells were recently described to acquire mitochondria from normal cells of the TME, through TNT-mediated intercellular communications. The transferred mitochondria act as signaling cues and have pleiotropic effects in the recipient cells, due to the role of mitochondria as both an energy provider and a metabolic hub. In that sense, the acquisition of mitochondria via TNTs not only increases the OXPHOS and ATP production of the target cells but also indirectly affects their general metabolism. This has functional consequences for cancer cells as it enhances their proliferative and migratory properties as well as their capacity to develop resistance to therapeutic treatment as outlined below.

**TNT-mediated mitochondria transfer**

The horizontal transfer of mitochondria was first described in the A549 ρ0 lung adenocarcinoma-derived cell line [24]. A549 ρ0 cells lack mtDNA due to a chronic exposure to ethidium bromide. Consequently, they are incapable of aerobic respiration, thereby relying on glycolysis and fermentation. After a four-day co-culture of A549 ρ0 cells with human mesenchymal stem cells (hMSCs), the A549 ρ0 cells acquired hMSC mitochondria, as proved by the detection of hMSC mtDNA in these cells, and consequently recovered their respiratory function and oxidative metabolism [24]. Other OXPHOS-incompetent cells, including B16 ρ0 melanoma and 4T1 ρ0 breast carcinoma cancer cell lines, were shown to display a lower tumorigenic and metastatic potential compared with their OXPHOS-competent parental cells [38]. Following the injection of these ρ0 cells in a mitochondria-competent microenvironment in vivo, horizontal mitochondria transfer from the TME toward these ρ0 cells restored their respiration and capacity to form tumors [38,112]. It is worth noting that in the above-mentioned studies the requirement of TNTs for the transfer of mitochondria was not formally proved.

Resistance to doxorubicin was also attributed to ‘inbound’ mitochondria transfer from endothelial cells to MCF7 breast cancer cells [32]. In addition, Moschoi et al. showed that AML cells can internalize functional bone marrow stromal cell-derived mitochondria in co-cultures. This process increased the AML cells’ mitochondrial mass by up to 14% and protected them against the cytotoxic effects of the nucleoside analog ARA-C as shown both in vitro and in vivo [43]. Horizontal mitochondria transfer was also observed in Leukemia-Initiating Cells (LICs), a small leukemic subpopulation involved in chemoresistance and cancer relapse [43].

TNT-mediated mitochondria transfer was also reported in stressed pheochromocytoma-derived PC12 cells after exposure to UV radiation [37]. These cells had lost cytochrome c and were on the verge of undergoing apoptosis, as marked by caspase-3 activation. In co-culture experiments, TNTs formed between healthy and stressed PC12 cells, they allowed mitochondria transfer and rescued the stressed cells from apoptosis, highlighting a novel TNT-mediated survival mechanism [37].

Besides receiving mitochondria, cancer cells can also employ an ‘outbound’ mitochondria transfer to modify and educate their microenvironment, thus favoring tumor progression. In this particular context, cancer cells can transfer their mitochondria to normal stromal cells of their microenvironment, thereby modifying the
microenvironment cytokine secretion pattern. B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cells were actually shown to use TNTs to signal to MSCs and to induce pro-inflammatory cytokine secretion [105]. T cell acute lymphoblastic leukemia (T-ALL) cells also use the outbound mitochondria transfer process to escape chemotherapy-induced intracellular oxidative stress [52]. Jurkat cells exposed to ARA-C or methotrexate exchange mitochondria both ways with MSCs. However, the TNT-mediated bidirectional mitochondria transfer occurs mostly from the Jurkat cells towards the MSCs. As a consequence, it reduces ROS levels inside the Jurkat cells, thus promoting their survival [52].

**mtDNA mutations**

Cancer cells often harbor alterations in their mtDNA, including mutations, deletions or changes in mtDNA copy number, leading to mitochondrial dysfunction. Interestingly, mtDNA modifications do not completely impede mitochondrial functions. Instead, they were shown to rewire cancer cell behavior through retrograde mitochondria-to-nucleus signaling, also known as mitochondrial stress signaling, that is considered a cellular adaptation mechanism to mitochondrial stress through metabolic reprogramming [12,13].

In mammalian cells, this mitochondrial retrograde signaling was linked with tumorigenesis as modifications in mtDNA content were shown to contribute to tumor progression and correlated with poor prognosis in different tumor types like gastric, hepatocellular, colorectal as well as lung, prostate and breast cancers [12,114]. Some mtDNA mutations were demonstrated to increase ROS levels, thus inducing Akt-, MAPK- and HIF-1α-mediated signaling pathways, which are associated with chemoresistance and increased metastatic potential [115,116].

The partial (ρ−) or total (ρ0) ethidium bromide-induced mtDNA depletion demonstrated the effects of mitochondrial dysfunction in cancer drug resistance as the ρ− and ρ0 prostate cancer cells were found less sensitive to paclitaxel [117] and N-(4-hydroxyphenyl) retinamide [118] than the parental cells. In line with these findings, ρ0 HeLa cells and mtDNA-depleted breast cancer cells were shown to be insensitive, respectively, to adriamycin [119] and to hormone therapy [120]. Likewise, hepatoma cells deprived of mtDNA were shown to be less sensitive to paraquat, doxorubicin and menadione [121]. In addition to these mtDNA depletion effects, it is worth mentioning that increased mtDNA content can also lead to acquired drug resistance, as shown for docetaxel-resistant head and neck cancer cells [122].

The molecular mechanisms underlying mtDNA-mediated drug resistance are still widely unknown. mtDNA-depleted hepatocarcinoma cells were shown to be chemoresistant to doxorubicin, cisplatin and to the DNA topoisomerase I inhibitor SN-38 [123], a phenotype that was associated with an up-regulation of Multi-Drug Resistance gene 1 (MDR1), and MRPI and MRP2, all three proteins involved in multidrug resistance [123]. Overexpression of MDR1 was also observed upon mtDNA depletion in colon cancer cells [124]. In addition, the ability of doxorubicin, cisplatin and SN-38 to induce apoptosis was reduced in mtDNA-depleted cells when compared with parental cells, as evidenced by the decreased BAX/BCL2 expression ratio and increased survivin level [123]. mtDNA-depleted murine rhabdomyoblasts were also shown to become resistant to staurosporine-mediated apoptosis through profound modifications of the apoptotic machinery, including sequestration of the pro-apoptotic factors BID, BAX and BAD, overexpression of the anti-apoptotic proteins BCL2 and BCL-XL, and reduced activation of caspases 3, 9, 8 [125].

**mtDNA transferred via EVs**

TNTs allow the trafficking of mitochondria (with their endogenous mtDNA) between cancer cells and cells of their microenvironment. Recent studies demonstrated that exosomes containing mtDNA, but not the full mitochondria, also affect cancer progression and response to therapy as shown in various cancer types. Indeed, the EV-mediated transfer of mtDNA from CAFs to hormonal therapy-resistant (HTR) metastatic breast cancer cells resulted in OXPHOS restoration and led to an exit from therapy-induced quiescence, thus promoting drug resistance both in vitro and in vivo [126]. Moreover, Philley et al. reported the presence of cancer-derived exosomes containing mtDNA and mitochondrial fusion protein mitofusin (MFN) in the serum of prostate cancer patients as well as in the tumor itself, an observation that was associated with prostate cancer progression [127].

Taken together, these observations corroborate the notion of tumor cell metabolic plasticity and illustrate the remarkable capacity of cancer cells to overcome dire circumstances by modifying their metabolism notably through TNT-mediated mitochondria transfer.
Role of cancer cell metabolic pathways in drug resistance

Cancer cells are confronted with widely changing conditions within the tumor and can adapt to drastic micro-environments including hypoxia, low nutrient supplies and acidosis, all the while sustaining high proliferation rates and developing migratory phenotypes. This outstanding metabolic plasticity has an impact on responses to therapeutic treatments, and indeed therapy resistance has been consistently linked to the overexpression and activation of core metabolic enzymes (Figure 2 and Table 2).

Figure 2. Metabolic pathways and enzymes involved in drug resistance.
Glycolysis
Cancer cells import massive amounts of glucose and preferentially use fermentative glycolysis for energy production rather than OXPHOS even in normoxic conditions. This is known as the ‘Warburg effect’, which ultimately leads to high lactate secretion and cancer tissue acidification. The overexpression of several glycolysis enzymes has been shown to contribute to tumor progression and resistance to therapy as discussed below.

The first, irreversible, step of glycolysis is the conversion of glucose into G6P catalyzed by hexokinases. High expression of hexokinase 2 (HK2) has been correlated with resistance to chemotherapy in glioblastoma, pancreatic and ovarian cancers [128,147,148]. HK2 has also been linked to tamoxifen resistance in breast cancer-derived cell lines [149]. In the A2780 cisplatin-sensitive ovarian cancer cell line, overexpression of HK2 triggered cisplatin resistance, while, conversely, knocking down HK2 increased sensitivity to cisplatin in AC2780/CP70 cisplatin-resistant cells. Furthermore, HK2 overexpression enhanced cisplatin-induced ERK1/2 phosphorylation and autophagy, while HK2 knockdown reduced these two effects, both known to contribute to cisplatin resistance in ovarian cancer cells. This HK2-mediated resistance to cisplatin was further confirmed in murine ovarian tumor xenograft models [128].

In step 4 of glycolysis, fructose-1,6-bisphosphate is cleaved by aldolases into two C3 metabolites, DHAP and GA3P. High expression of fructose-bisphosphate aldolase A (ALDOA) has been associated with drug resistance in OS, leukemia and colorectal cancers (CRCs) [130,135,150]. In OS, ALDOA has been linked to resistance to platinum analogs, and overexpression of ALDOA in the MG-63 low ALDOA-expressing OS cell line was indeed found to increase in vitro cell invasion, expression of the MMP-2 metalloproteinase and resistance to cisplatin-induced apoptosis. Conversely, the inhibition of ALDOA expression in the U-2 high ALDOA-expressing OS cell line reversed all three phenotypes, notably with an increased sensitivity to cisplatin-induced

<table>
<thead>
<tr>
<th>Drug classes</th>
<th>Drugs</th>
<th>Cancers</th>
<th>Enzymes</th>
<th>TNT transfer</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA damaging agents</td>
<td>Cisplatin</td>
<td>Ovarian cancer</td>
<td>HK2</td>
<td>[128]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>OS</td>
<td>G6PD</td>
<td>[129]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALDO</td>
<td>PKM2</td>
<td>[130]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lung adenocarcinoma</td>
<td>ENO1</td>
<td>[131]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carboplatin</td>
<td>PKM2</td>
<td>[132]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxaliplatin</td>
<td>CRC</td>
<td>[133]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gastric adenocarcinoma</td>
<td>FASN</td>
<td>[134]</td>
<td></td>
</tr>
<tr>
<td>DNA intercalating agents</td>
<td>Doxorubicin</td>
<td>Colon cancer</td>
<td>G6PD</td>
<td>Mitochondria</td>
<td>[135]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Breast cancer</td>
<td>G6PD</td>
<td>[32]</td>
<td></td>
</tr>
<tr>
<td>DNA alkylating agents</td>
<td>Mitomycin</td>
<td>Gastric adenocarcinoma</td>
<td>PGK1</td>
<td>[136]</td>
<td></td>
</tr>
<tr>
<td>Nucleoside analogs</td>
<td>Fluorouracil</td>
<td>CRC</td>
<td>ALDO</td>
<td>[137]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gastric adenocarcinoma</td>
<td>LDHA</td>
<td>[138]</td>
<td></td>
</tr>
<tr>
<td>Cytarabine (ARA-C)</td>
<td>Acute myeloid leukemia</td>
<td>6PGD</td>
<td>Mitochondria</td>
<td>[139]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G6PD</td>
<td>FASN</td>
<td>[140]</td>
<td></td>
</tr>
<tr>
<td>Topoisomerase inhibitors</td>
<td>SN38</td>
<td>CRC</td>
<td>ACL</td>
<td>[141]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Daunorubicin</td>
<td>Acute myeloid leukemia</td>
<td>6PGD</td>
<td>[142]</td>
<td></td>
</tr>
<tr>
<td>Growth factor receptor inhibitors</td>
<td>CTX</td>
<td>Ewing’s sarcoma</td>
<td>LDHA</td>
<td>[143]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Imatinib</td>
<td>CML</td>
<td>TK</td>
<td>[144]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sorafenib</td>
<td>Liver cancer</td>
<td>SCD1</td>
<td>[145]</td>
<td></td>
</tr>
<tr>
<td>Microtubule damaging agents</td>
<td>Paclitaxel</td>
<td>Breast cancer</td>
<td>LDHA</td>
<td>[146]</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 Metabolic enzymes involved in cancer cell drug resistance

© 2018 The Author(s). Published by Portland Press Limited on behalf of the Biochemical Society
apoptosis [130]. In CRC cells, ALDOA expression was linked to cell proliferation, invasion and spheroid formation as well as to resistance to 5-fluorouracil (5-FU) and oxaliplatin chemotherapy. Furthermore, its expression was found up-regulated in hypoxia-cultured CRC-derived cell lines when compared with cells cultured in normoxic conditions [135].

**Phosphoglycerate kinases** (PGK) are ATP-generating enzymes that catalyze step 7 of the glycolysis reactions. PGK1 overexpression was reported to increase tumor growth and metastasis in gastric, pancreatic, prostate, breast and ovarian cancers. Simultaneous suppression of PGK1 and treatment with either 5-FU or mitomycin significantly increased the drug response of the human gastric 23132/87 (ACC409) adenocarcinoma cell line, when compared with the drug treatment alone [138].

**Enolases** (ENO) are lyases that perform the last but one step of glycolysis that is formation of phosphoenol pyruvate. High ENO1 expression has been correlated with lung, prostate and gastric cancer progression and recurrence [132,151,152]. High ENO1 levels detected in gastric cancer patients were correlated to lower patient survival. Glycolysis was found enhanced and ENO1 overexpressed in two cisplatin-resistant human gastric cancer cell lines, BGC823/DDP (with acquired cisplatin resistance) and MGC803 (intrinsically resistant to cisplatin). On the other hand, siRNA silencing of ENO1 decreased glycolysis and lowered resistance to cisplatin treatment [132].

In the final, irreversible step of glycolysis, **pyruvate kinases** (PKs) lead to the formation of the key metabolite pyruvate and to the generation of ATP. High levels of the PKM2 muscle isozyme were shown to be responsible for higher glucose metabolism and carboplatin resistance in A549/R and PC9/R carboplatin-resistant NSCLC cell lines. SiRNA-mediated inhibition of PKM2 resensitized A549/R and PC9/R cells to carboplatin therapy. Conversely, PKM2 overexpression in the parental carboplatin-sensitive A549 and PC9 cells induced resistance to carboplatin. Metformin, which is known to indirectly down-regulate PKM2 expression, partially impaired the resistance of A549/R and PC9/R cells to carboplatin [134]. Metformin was also found instrumental in enhancing sensitivity to cisplatin in OS stem cells that exhibit PKM2-dependent cisplatin resistance [131].

**Lactate metabolism**

In fermentative glycolysis, a glucose catabolic pathway favored by many cancer cells, lactate dehydrogenases (LDHs) catalyze the reversible conversion of pyruvate into lactate. In different cancers, including breast and colon cancers and Ewing sarcoma, high LDHA expression has been correlated to therapy resistance [139,143,146]. LDHA protein levels and activity were found to be higher and to contribute to taxol resistance in taxol-resistant MDA-MB-435 breast cancer-derived cells when compared with the parental taxol-sensitive cell line, while LDHA knockdown increased their sensitivity to the drug. Interestingly, a higher sensitivity to the LDHA inhibitor oxamate was observed in taxol-resistant MDA-MB-435 cells and the combined treatment with taxol and oxamate exhibited synergistic effects in inducing apoptosis in these cells [146]. LDHA expression was also found up-regulated in 5-FU-resistant DLD-1 colon cancer-derived cells as a result of the lowered expression of the miR-34a microRNA. Overexpressing miR-34a in 5-FU-resistant DLD-1 cells reduced LDHA expression and resensitized the cells to 5-FU [139]. As another example, the up-regulation of LDHA was shown to be involved in cetuximab (CTX) resistance in Ewing sarcoma. In CTX-resistant Ewing sarcoma tissues and in the CTX-resistant A673 cell line, LDHA expression, glucose consumption and lactate production were found to be higher than in the CTX-sensitive A673 cells. Both siRNA-mediated inhibition of LDHA expression and oxamate-based inhibition of LDHA activity increased CTX therapeutic efficiency in CTX-resistant cells [143].

High lactate production and export in cancerous tissues contribute to the extracellular acidic pH shift observed in solid tumors. As a consequence of tumor acidosis, drug properties change and drug absorption is impaired, thus reducing chemotherapeutic efficiency (reviewed in [153,154]). Lactate can also be used by cancer cells as a substrate and primary source of energy, instead of glucose, as shown in the MDA-MB-436 triple negative breast cancer cell line. The lactate metabolic switch rendered these cells resistant to PI3K/mTOR inhibitors. The nuclear receptor ERRα (Estrogen-Related Receptor alpha) was reported to regulate the expression of proteins involved in lactate metabolism. Accordingly, both the genetic and pharmacological inhibitions of ERRα activity were found to impede lactate oxidation and to increase the efficiency of PI3K/mTOR inhibitors in vitro and in vivo [155]. In cervical cancer-derived cell lines, lactate was reported to modulate the cellular DNA damage response processes. Both L- and D-lactate enantiomers inhibited histone deacetylases and increased chromatin accessibility in the HeLa, Ca Ski and C33A cervical carcinoma cells, thereby enhancing DNA repair. The increase in the activity of the DDR machinery resulted in acquired resistance to the
chemotherapeutic agents neocarzinostatin, doxorubicin and cisplatin. The role of lactate in this process was confirmed by pharmacological inhibition of the lactate transporter MCT4, which prevented DNA repair and resensitized the cancer cells to chemotherapy [156].

Pentose phosphate pathway

The oxidative branch of the pentose phosphate pathway (PPP) produces ribose-5-phosphate from glucose-6-phosphate (G6P) while generating nicotinamide adenine dinucleotide phosphate (NADPH), G6P being a common substrate for the two branching-out glycolysis and PPP pathways (Figure 2). While ribose-5-phosphate constitutes an important substrate for DNA synthesis, the reducing activity of NADPH contributes to the generation of glutathione (GSH) and therefore to redox buffering, thus preventing ROS-induced cell damage. The PPP has been shown to support cancer cell proliferation and to be enhanced in various cancers, including breast, kidney and liver cancers [157–159]. High PPP activity was also linked to tumor cells’ resistance to chemotherapy by various mechanisms, as detailed below.

Glucose-6-phosphate dehydrogenase (G6PD) is a key rate-limiting enzyme of the oxidative branch of PPP that converts G6P into 6-phosphoglucono-δ-lactone (6PGL). G6PD activity was found increased in the doxorubicin-resistant HT29-DX colon cancer cell line. This phenotype was associated with an increased concentration of GSH as well as an increased expression of the multidrug resistance proteins MRP1 and MRP2. The forced expression of G6PD in HT29 doxorubicin-sensitive cells led to an acquired doxorubicin resistance, with increased GSH levels. The G6PD inhibitors dehydroepiandrosterone (DHEA) and 6-aminonicotinamide (6-AN) were shown to decrease GSH levels both in resistant HT29-DX and in G6PD overexpressing HT29 cells and to reverse their resistance to doxorubicin [137]. Similarly, cisplatin-resistant C13 ovarian cancer-derived cells were reported to exhibit increased G6PD expression and enzymatic activity. Combining either DHEA or 6-AN treatment with cisplatin therapy had a synergistic effect and helped overcome C13 cells’ cisplatin resistance [129].

6-phosphogluconate dehydrogenase (6PGD) is another major NADPH-producing enzyme of the PPP. The growth of the MV4.11 AML cell line was reported to highly depend on the NADPH produced by 6PGD. The knockdown of 6PGD in MV4.11 cells resistant to the topoisomerase inhibitor daunorubicin and to the nucleoside analog cytarabine enhanced their response to these therapies. Moreover, it circumvented the stromal cell-mediated resistance to quizartinib, a specific inhibitor of the oncogenic FLT3 mutant kinase [140].

Lastly, transketolase (TK), a rate-limiting enzyme of the non-oxidative branch of the PPP, was associated with drug resistance in chronic myeloid leukemia (CML). Imatinib-resistant murine BaF3 and human LAMA-84-R CML cell lines exhibited high HIF-1α expression levels. As a consequence, the oxidative PPP-mediated ribose synthesis was reduced, accompanied by TK activation as a compensatory mechanism. Oxythiamine, a TK inhibitor, enhanced tumor cell sensitivity to imatinib as shown both in patient CML primary cell cultures exhibiting blast transformation and in tumor xenografts [144].

Lipid metabolism

Most cancer cells require lipids to sustain their high proliferation rates, survival and metastatic spreading. Blocking lipid metabolism pathways, including lipid de novo synthesis, lipolysis and lipid storage in lipid droplets, was indeed reported beneficial for anticancer therapies [160,161].

Lipids are obtained through the uptake and consumption of exogenous lipids or through the activation of lipid endogenous de novo synthesis. When oxygen and rich extracellular nutrients like glucose and glutamine are available, cancer cells synthesize lipids de novo, predominantly through the fatty acids (FAs) synthesis pathway. In starvation conditions, cancer cells increase their uptake of extracellular lipids for growth [161–163]. The specific lipid metabolic pathways used by cancer cells actually depend both on their original cell type and on their interactions with the microenvironment [164]. Lipid metabolism reprogramming has been correlated to cancer progression and resistance to therapy through different mechanisms. Due to the high metabolic demands and proliferation rates of cancer cells, the expression and activation of several lipogenic enzymes are high in these cells, as detailed below.

The ATP citrate lyase (ACL), the first-step rate-limiting enzyme for de novo lipogenesis, was found up-regulated in glioblastomas and ovarian cancers where its inhibition with hydroxycitrate and siRNA knockdown, respectively, led to suppression of cancer cell migration and to cell cycle arrest [165,166]. ACL up-regulation was found to be linked to cancer cell chemoresistance as shown for metastatic CRC cells in comparison with chemo-naive CRC cells. Lentiviral overexpression of ACL in chemo-naive CRC cells induced
Fatty acid synthase (FASN) is also a key enzyme for de novo fatty acid biosynthesis, responsible for the synthesis of palmitate. FASN overexpression in cancer cells is correlated with poor prognosis and with a higher risk of relapse. It is responsible for resistance to anticancer therapy via various mechanisms as outlined below. It was shown to block the production of tumor necrosis factor-α (TNF-α) and doxorubicin-induced ceramide production as well as to inhibit caspase 8 production, thereby preventing apoptosis in the MCF7 breast cancer cell line [167]. Correlation between FASN overexpression and resistance to the nucleoside analog gemcitabine was established in several studies, notably for pancreatic cancer. In the Panc-1, Mia-PaCa-2 and BxPC-3 pancreatic cancer cell lines, FASN overexpression induced resistance to gemcitabine and to radiation therapy, an observation corroborated by the fact that FASN knockdown lowered resistance to the treatments [141]. Likewise, increased FASN expression was associated with disease progression in pancreatic cancer mouse models and with poor patient survival. Combination of the orlistat FASN inhibitor with gemcitabine did increase pancreatic cell sensitivity to gemcitabine by inducing endoplasmic reticulum (ER) stress-mediated apoptosis [168].

FASN expression is regulated by many transcriptional regulators, including c-Myc, liver X receptors (NR1H2 and NR1H3) and SREBP1 [169]. It is also controlled by MACC1 (Metastasis-associated in colon cancer 1) whose expression was correlated to that of FASN in gastric cancers in vivo and in vitro and to poor patient prognosis. Direct inhibition of FASN by either the synthetic C75 FASN inhibitor or by FASN siRNA was indeed shown to suppress endogenous fatty acid metabolism and to decrease cell proliferation as well as oxaliplatin resistance in both the gastric adenocarcinoma poorly differentiated BGC-823 and in the well-differentiated MKN-28 cell lines, in spite of MACC1 overexpression in these cells [136].

Recent studies demonstrated the role of stearoyl-CoA desaturase (SCD1), an enzyme involved in the conversion of saturated into monounsaturated FAs, in cancer cell biosynthesis of membrane phospholipids and energy-storing lipids. Overexpression of SCD1 was correlated with tumor progression, survival and chemoresistance in lung, liver and pancreatic cancers. SCD1 inhibition was shown to revert resistance to cisplatin and sorafenib chemotherapies in diverse CSCs from lung, breast, hepatocellular carcinomas and ovarian cancers [133,145,170,171]. SCD1 was also found to up-regulate YAP/TAZ activity and Wnt/β-catenin signaling in lung adenocarcinoma [172]. Increased levels of SCD1 were correlated with poor lung adenocarcinoma prognosis. Furthermore, cisplatin-based therapies were shown to further induce the expression of SCD1 and of CSCs markers such as CD24, CD133, CD44 and SOX2 [133]. Interestingly, the combined treatment with cisplatin and the SCD1 inhibitor MF-438 reduced the expression of stem cell markers and induced cell cycle arrest, apoptosis, ER stress and autophagy [133].

Finally, lipid droplets (LDs) formation is an emerging mechanism of drug resistance [173]. Resistance of the T47D progesterone receptor-positive breast cancer cell line to the microtubule-stabilizing drug docetaxel was reported to be due to progestin-induced LD accumulation [174]. Progestin also promoted the expression of SCD1 and altered the fatty acids profile of T47D cells. LD played a cancer cell protective role by enwrapping docetaxel molecules, thus inhibiting the drug effect. Importantly, chemical inhibition of SCD1 decreased LD formation and increased sensitivity to docetaxel [174]. A similar drug sequestration mechanism leading to AML cell resistance was also described for the aminopeptidase inhibitor CHR2863 prodrug [175]. In addition, LD accumulation was recently demonstrated to impede caspase activation and ER stress responses in CRC cell lines, thus promoting their resistance to 5-FU and oxaliplatin both in vitro and in vivo. Moreover, LD accumulation decreased CD8+ T cell infiltration and immunogenic cell death, both in patients and in murine xenograft models. The drug resistance phenotype was effectively reversed by inhibition of LD biogenesis, as shown in CRC cell lines and in tumor-bearing mice [176].

MSCs were also reported to be potent contributors to lipid-based chemotherapy resistance [177]. Following treatment with platinum analogs, MSCs were shown to secrete two distinct polysaturated fatty acids (PIFAs): 12-oxo-5,8,10-heptadecatrienoic acid (KHT) and hexadeca-4,7,10,13-tetraenoic acid (16:4(n-3)). Minute quantities of these platinum-induced PIFAs promoted chemoresistance to cisplatin, oxaliplatin and carboplatin in vivo, but not to other chemotherapeutic agents such as 5-FU, irinotecan, paclitaxel or doxorubicin. Interestingly, blocking the cyclooxygenase-1/thromboxane synthase pathway prevented PIFA formation and, consequently, MSC-induced resistance [177].
Novel mitochondria and metabolism-targeted strategies to overpower drug resistance

Drug resistance can be developed by tumor cells independently of the acquisition of additional mutations. This is related to the inherent biological and metabolic plasticity of the resistant cancer cells. It actually provides a window for effective therapies, as outlined below. As expected, these novel therapeutic strategies target notably the functions of mitochondria as they constitute a major cell metabolic hub.

Targeting mitochondria

Mitochondria can be targeted by many different small bioactive molecules. These were called ‘mitocans’ (a contraction of mitochondria and cancer) by J. Neuzil on the basis of their selectivity for cancer cells. Mitocans can target different mitochondria components involved in the functioning of mitochondria membranes, TCA cycle, electron transport chain or mtDNA [178]. They include paclitaxel, etoposide, vinorelbine, ceramide, lonidamine or betulinic acid, which, by their action on different mitochondrial targets, all lead to cytochrome C release and cancer cell apoptosis [179]. The use of such mitocans raises the acute question of their selectivity for cancer cell mitochondria. Their specific targeting is an obvious concern and a major therapeutic challenge because affecting cancer cell mitochondria while sparing the normal cell mitochondria is required to provide efficient treatment with minor toxic side effects [179]. An alternative mitochondria-targeting strategy relies on the use of antibiotics, based on the mitochondria bacterial ancestry, as supported by the endosymbiotic theory. Because of the striking evolutionary resemblance of mitochondria with prokaryotes, the effects of antibiotics on the functionality of eukaryotic cell mitochondria actually raise concerns, especially for the interpretation of gene-expression studies based on the use of antibiotics such as tetracyclines and doxycycline [180]. Nonetheless, the use of antibiotics can be valuable to target cancer cell mitochondria. It is the case for tigecycline, which was shown to inhibit the translation of the mitochondrial cytochrome c oxidases I and II and accordingly to reduce cancer cell mitochondrial respiration and survival in CML [181]. Likewise, several classes of mitochondria-targeted antibiotics, including erythromycins, tetracyclines and glycyclines, were reported to affect cancer cell survival for cancers affecting different organs (breast, DCIS, ovarian, prostate, lung, pancreatic, melanoma and glioblastoma) with little effect on normal cells [182].

Targeting ROS production

Cancer cells are often subject to increased ROS production, which contributes to genetic instability and tumor progression. This led to efforts aiming at diminishing the production of ROS with the goal of controlling tumor growth. However, the general view changed in the past few years, leading to changes in therapeutic approaches. Instead of diminishing ROS production, a more up-to-date approach aims at taking into account this new metabolic state of the cells linked to therapeutic susceptibility and, on the contrary, at further enhancing ROS accumulation [183]. The goal of such an ROS-inducing therapy is to reach a threshold of ROS concentrations beyond which the toxic effects of ROS override their supportive effects for tumor progression so as to eventually induce cancer cell death [184]. Normal cells with intrinsically lower ROS levels might be expected to be spared by such treatments. This was nicely shown in transformed ovarian epithelial cells and in fludarabine-resistant chronic lymphocytic leukemia cells, both endowed with elevated ROS levels that rendered them more sensitive to treatment with β-phenylethyl isothiocyanate (PEITC). PEITC further increased their ROS cellular concentrations which was instrumental for the resulting massive cell death [185,186].

Targeting tumor drug addiction

A novel phenomenon called cancer cell ‘drug addiction’ was recently reported. This phenomenon can occur for cells that were subjected to therapeutic treatment and that became resistant to this treatment. These cells are viable in the presence of the lethal chemical they became resistant to. Interestingly, they die if this chemical is removed. This was shown for human melanoma cells depending on the BRAF(V600E) mutant protein [187]. Stuart and colleagues developed human-patient-derived xenografts of BRAF-mutated melanoma cells that were submitted to continuous treatment with vemurafenib to generate a model of drug-resistant tumors. These tumors were characterized by high ERK1/2 activation. In these xenografts, the withdrawal of vemurafenib led to an increase in ERK1/2 activation and to tumor regression in mice. This first observation suggested that discontinuing a chemotherapy could provide a therapeutic approach to selectively kill therapy-resistant cells and that intermittent rather than continuous treatment might be more beneficial to patients [187].
reported using an unbiased CRISPR-Cas9 knockout screen on BRAF-inhibitor resistant melanoma cells that pointed out to a role of ERK2, JUNB and FRA1 on cell death upon treatment withdrawal. In particular, the ERK2-induced expression upon therapy withdrawal was observed across several cell lines and independently of the drug resistance mechanisms [188]. These reported observations suggest possible therapeutic strategies, based on alternating therapeutic treatments, that will need to be precisely scheduled to effectively yield long-term benefits.

Targeting uncovered drug sensitivity of tumor-resistant cells
Resistance of cancer cells to therapy has been associated to their high mesenchymal state. Schreiber and collaborators carried out a screen across a vast panel of such high mesenchymal cancer cell lines with the goal of finding common vulnerabilities in these cells amenable to drug therapy [189]. Very interestingly, these studies led to the discovery that these resistant cancer cells are characterized by a specific lipid metabolism leading to the increased synthesis of polyunsaturated lipids and to the toxic accumulation of their hydroperoxide derivatives. Furthermore, the glutathione peroxidase GPX4 was shown to play a central role in the inhibition of ferroptosis, a cell death mechanism induced by lipid peroxides, thus allowing the survival of these cells [189]. In a follow-up study, the loss of GPX4 function was demonstrated to induce the ferroptotic death of drug-resistant cancer cells over a wide panel of cancer types and corresponding therapeutic treatments [190]. Interestingly, concomitant treatment of BT474 line breast cancer cells with lapatinib (an inhibitor of EGFR and HER2 tyrosine kinases) and GPX4 inhibitors did not show therapeutic synergy, confirming that the GPX4 dependence is a feature of the chemotherapy-resistant cells [190]. The present study therefore provides a novel paradigm, pointing out that apoptosis-resistant cancer cells can nonetheless be steered to cell death via apoptosis-independent and ferroptosis-dependent mechanisms. It also suggests that spaced out treatments, within a defined time window, with chemotherapeutic agents and GPX4 inhibitors might be a valuable therapeutic protocol enabling an enhanced efficacy in destroying the reservoir of resistant cells while sparing the non-cancerous ferroptosis-insensitive cell population.

Targeting interdependent pathways through synthetic lethality
Many cancer cells rely on fermentative glycolysis as a main energy provider. However, therapeutic treatments, notably antiangiogenic treatments with tyrosine kinase inhibitors (TKIs) can reprogram this metabolism towards mitochondria-based respiration and ATP production, a metabolic pathway that becomes then required for the cancer cell survival [191]. Treatment of PyMT breast tumors with mitochondria inhibitors (phenformin or ME344) alone had no effect on tumor growth. However, mitochondria inhibitors worked synergistically with TKI inhibitors (nintedanib and regorafenib) to control tumor growth. These observations paved the way for the development of synthetic lethality protocols, concomitantly targeting tumor angiogenesis through TKIs and OXPHOS through specific inhibitors of the respiratory chain [191]. Similar findings were obtained by Gottlieb and collaborators for the treatment of CML with c-Abl-specific TKIs. Using stable-isotope-assisted metabolomics, they identified oxidative metabolism as a key feature for CML survival. They further demonstrated that concomitant treatment of CML with the TKI imatinib and the mitochondrial inhibitor tigecycline killed the leukemic stem cells responsible for disease recurrence [181]. Similarly, as shown in lymphoma mouse models, cancer cells that undergo therapy-induced senescence also undergo metabolic reprogramming that can be exploited to increase therapeutic efficacy. These cells were shown to display increased glucose usage as well as increased sensitivity to glucose blockade, providing a means for inducing cancer cell death by glucose deprivation [192]. Thus, exploiting the synthetic lethality of several cellular pathways by concomitant treatments should enhance therapeutic efficacy.

Conclusion
Overall, current data indicate that the cancer cell metabolic plasticity plays a key role in acquired tumor resistance to therapy. As expected, mitochondria activity is a main culprit in these resistance phenomena as it is central to many metabolic pathways. The observation that functionally different mitochondria can be exchanged through TNTs between tumor cells or between normal cells of the microenvironment and tumor cells broadens the panel of mechanisms that lead to cancer cell metabolic reprogramming. Furthermore, integrating the various mechanisms leading to cancer cell drug resistance allows to envision novel therapeutic strategies that will provide the move forward to efficiently block cancer progression.
Abbreviations

2PGA, 3-phospho-D-glyceroyl phosphate; 3PGA, 3-phospho-D-glycerate; 5-FU, 5-fluorouracil; 6-AN, 6-aminonicotinamide; 6PG, 6-phosphogluconate; 6PGD (6PGDH), 6-phosphoglucuronate dehydrogenase; 6PGL, 6-phosphogluronolactone; ABC, ATP-binding cassette; ABCB1, ATP-binding cassette subfamily B member 1; ABCG2, ATP-binding cassette subfamily G member 2; ACC1, acetyl-CoA carboxylase-1; Acetyl CoA, acetyl coenzyme A; ACL, ATP citrate lyase; ADP, adenosine diphosphate; AKT, protein kinase B; ALDOA, aldolase A; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; AR, androgen receptor; ARA-C, cytarabine; ATP, adenosine triphosphate; Bad, Bcl-2-associated death promoter; Bak, Bcl-2 homologous antagonist/killer; Bax, BCL2 associated X, apoptosis regulator; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra large; BCRP, breast cancer resistance protein; BER, base excision repair; Bid, BH3 interacting domain death agonist; Bim, Bcl-2 interacting mediator of cell death; BRAF, B-Raf proto-oncogene, serine/threonine kinase; c-Abl, Abelson murine leukemia viral oncogene; c-Myc, MYC - Myc proto-oncogene protein; C75, 4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid) is a synthetic fatty acid synthase (FASN) inhibitor; CAFs, cancer-associated fibroblasts; Cav-1, caveolin-1; CTX, cetuximab; CML, chronic myeloid leukemia; CRC, colorectal cancer; CRIPR-Cas9, CRISPR-associated protein-9 nuclease (Cas9); CSCs, cancer stem cells; Cx43, connexin 43; D-loop, displacement loop; DCIS, ductal carcinoma in situ; DDR, DNA damage repair; DHAP, dihydroxyacetone phosphate; DHEA, dehydroepiandrosterone; DNA, deoxyribonucleic acid; ECM, extracellular matrix; EGFR, epithelial growth factor receptor; EMT, epithelial-to-mesenchymal transition; ENU, Enolase 1; ER, endoplasmic reticulum; ERCC1, excision repair cross-complementing 1; ERK1 (MAPK3), mitogen-activated protein kinase 3; ERα, estrogen receptor alpha; ETC, electron transport chain; EVs, extracellular vesicles; F6P, fructose 6-phosphate; FAs, fatty acids; FASN, fatty acid synthase; FLT3, Fms-like tyrosine kinase 3; FN1, Fibronectin 1; FRA1, Fos-related antigen 1; G6P, glucose-6-phosphate; G6PD (G6PDH), glucose-6-phosphate dehydrogenase; GA3P, glyceraldehyde 3-phosphate; GBM, glioblastoma multiforme; GJA1, GAP junction alpha-1 protein; GPX4, glutathione peroxidase 4; GST, glutathione; HIF-1α, hypoxia-inducible factor 1-alpha; HK2, hexokinase isofrom 2; hMSCs, human mesenchymal stem cells; HTR, hormonal therapy-resistant; IL-6, interleukin-6; JUNB, JunB Proto-Oncogene; LDHA, lactate dehydrogenase A; LICs, leukemia-initiating cells; LRP, lung resistance-related protein; MACC1, metastasis-associated in colon cancer 1; Malonyl CoA, malonyl coenzyme A; MAPK, mitogen-activated protein kinase; Mcl-1, myeloid cell leukemia factor 1; MCT4, monocarboxylate transporter 4; MDR1, multi-drug resistance protein 1; MF-438, stearoyl-CoA desaturase 1; MPP2, matrix metallopeptidase 2; MPP9, matrix metallopeptidase 9; MMP14, matrix metallopeptidase 14; MMR, mismatch repair; mRNAs, messenger RNAs; MRP1, multidrug resistance-associated protein 1; MRP2, multidrug resistance-associated protein 2; MSCs, mesenchymal stem/stromal cells; MT1-MMP, membrane type 1 metalloprotease; mtdNA, mitochondrial deoxyribonucleic acid; MUFA, monounsaturated fatty acids; MVP, major vault protein; NADPH, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NER, nucleotide excision repair; NK, natural killer; NR1H2, nuclear receptor subfamily 1 group H member 2; NR1H3, Nuclear receptor subfamily 1 group H member 3; NSCLC, non-small cell lung carcinoma; OS, osteosarcoma; OXPHOS, oxidative phosphorylation; P-gp, P-glycoprotein; PEITC, β-phenylethyl isothiocyanate; PEP, phosphoenolpyruvate; PFK1, phosphofructokinase-1; PGK, phosphoglycerate kinase isofrom 2; pHAs, polysaturated fatty acids; PKM2, pyruvate kinase isozymes M2; PPP, pentose phosphate pathway; PyMT, polyoma middle T oncogene; R5P, ribose 5-phosphate; RLS5, ribulose-5-phosphate; ROS, reactive oxygen species; rRNAs, ribosomal RNA; SCD1, stearyl-CoA desaturase-1; SN-38, 7-ethyl-10-hydroxy-comptothecin, potent metabolite of irinotecan; SREBP1, sterol regulatory element-binding protein 1; TCA, tricarboxylic acid; TGF-β, transforming growth factor-β; TK, transketolase; TK1, tyrosine kinase inhibitors; TME, tumor microenvironment; TMZ, temozolomide; TNF-α, tumor necrosis factor-α; TNTs, tunneling nanotubes; tRNAs, transfer ribonucleic acid; tTG, transglutaminase; UV, ultraviolet; VEGF, vascular endothelial growth factor; WES, whole-exome sequencing; WGS, whole-genome sequencing; YAP/TAZ, YAP (Yes-associated protein) and TAZ (transcriptional co-activator with PDZ-binding motif); p63 cells, eukaryotic cells devoid of mitochondrial DNA; KHT, 12-oxo-5,8,10-heptadecatrienoic acid; 16:4 (n-3) acid, hexadec-4,7,10,13-tetraenoic acid; 1,3-BPGA, 1,3-bisphosphoglycerate; F1,6BP, fructose 1,6-bisphosphate; Oct-1, organic cation transporter-1.

Author contribution

All the four authors contributed to the writing of the review and to the design of the figures and tables.

© 2018 The Author(s). Published by Portland Press Limited on behalf of the Biochemical Society
Funding
J.N. is supported by a PhD fellowship from the French Ministry of Research. Y.H. was supported by an Erasmus fellowship (Ankara University, Turkey). This work was supported by a grant from the Ligue Contre le Cancer-Comité de l’Aude.

Acknowledgements
We thank the Montpellier RIO imaging facility (MRI) for providing adequate environment for confocal microscopy. M.G. and M.L.V. are staff scientists from the University of Montpellier and the National Center for scientific research (CNRS), respectively.

Competing Interests
The Authors declare that there are no competing interests associated with the manuscript.

References


33 Ahmad, T., Mukherjee, S., Pattaik, B., Kumar, M., Singh, S., Kumar, M. et al. (2014) Miro1 regulates intercellular mitochondrial transport & enhances mesenchymal stem cell rescue efficacy. EMBO J. 33, 994–1010


5342


Zhuang, G., Wu, X., Jiang, Z., Kasman, I., Yao, J., Guan, Y. et al. (2012) Tumour-secreted miR-9 promotes endothelial cell migration and angiogenesis by activating the JAK-STAT pathway. EMBO J. 31, 3515–3525 https://doi.org/10.1038/emboj.2012.183


Multifaceted Roles of Mitochondrial Components and Metabolites in Metabolic Diseases and Cancer

Jean Nakhle 1,2, Anne-Marie Rodriguez 3,4,5,6,* and Marie-Luce Vignais 1,*

1 Institute for Regenerative Medicine & Biotherapy (IRMB), INSERM, University of Montpellier, F-34090 Montpellier, France; jean.nakhle@umontpellier.fr
2 Institute of Molecular Genetics of Montpellier (IGMM), CNRS, University of Montpellier, F-34090 Montpellier, France
3 University of Paris Est Créteil, INSERM, IMRB, F-94010 Créteil, France
4 EnvA, IMRB, F-94700 Maisons-Alfort, France
5 EFS, Mondor Institute for Biomedical Research (IMRB), F-94010 Créteil, France
6 AP-HP, Hopital Mondor, Service d’histologie, F-94010 Créteil, France
* Correspondence: anne-marie.rodriguez@inserm.fr (A.-M.R.); marie-luce.vignais@inserm.fr (M.-L.V.)

Received: 30 May 2020; Accepted: 17 June 2020; Published: 20 June 2020

Abstract: Mitochondria are essential cellular components that ensure physiological metabolic functions. They provide energy in the form of adenosine triphosphate (ATP) through the electron transport chain (ETC). They also constitute a metabolic hub in which metabolites are used and processed, notably through the tricarboxylic acid (TCA) cycle. These newly generated metabolites have the capacity to feed other cellular metabolic pathways; modify cellular functions; and, ultimately, generate specific phenotypes. Mitochondria also provide intracellular signaling cues through reactive oxygen species (ROS) production. As expected with such a central cellular role, mitochondrial dysfunctions have been linked to many different diseases. The origins of some of these diseases could be pinpointed to specific mutations in both mitochondrial- and nuclear-encoded genes. In addition to their impressive intracellular tasks, mitochondria also provide intercellular signaling as they can be exchanged between cells, with resulting effects ranging from repair of damaged cells to strengthened progression and chemo-resistance of cancer cells. Several therapeutic options can now be envisioned to rescue mitochondria-defective cells. They include gene therapy for both mitochondrial and nuclear defective genes. Transferring exogenous mitochondria to target cells is also a whole new area of investigation. Finally, supplementing targeted metabolites, possibly through microbiota transplantation, appears as another therapeutic approach full of promises.

Keywords: mitochondria; electron transport chain (ETC); tricarboxylic acid (TCA) cycle; mitochondrial DNA (mtDNA); metabolism; mitochondria exchange; microbiota; metabolites; cancer; therapy

1. Introduction

Mitochondria are descendants from alpha-proteobacteria that survived after their endocytosis by eukaryotic progenitors, more than 1.5 billion years ago. These bacterial ancestors became symbiotic with their host cells and gradually gave rise to the permanent organelles found today in almost all eukaryotic cells [1]. As a vestige of their bacterial endosymbiotic origin, mitochondria have retained their double membranes and circular genome [2]. However, this mitochondrial DNA (mtDNA) has been dramatically reduced over evolution, rendering mitochondria dependent on the nucleus for the expression of the vast majority of their proteins [2]. As a result, the biogenesis and functionality of mitochondria are tightly regulated by the continuous crosstalk with the nucleus [3].
assigned to mitochondria is their capacity to produce high amounts of ATP, from glucose breakdown, through oxidative phosphorylation (OXPHOS). OXPHOS-dependent ATP synthesis is supported by the mitochondrial electron transport chain (ETC), composed of protein complexes and organic molecules, which conveys electrons to molecular oxygen and creates the electrochemical gradient needed for the functioning of ATP synthase [4]. In tight connection with the ETC, metabolites are produced in the mitochondria through the tricarboxylic acid (TCA) cycle. The TCA cycle is composed of eight enzymes catalyzing the chemical breakdown of carbohydrates, fats, and proteins to produce either ATP or building blocks for the synthesis of nucleic acids, amino-acids, and lipids.

Beyond their role as energy and building-block producers, mitochondria also act as signaling organelles that govern cell fate by regulating essential biological processes, such as cell growth, differentiation, and apoptosis, as well as Ca\textsuperscript{2+} and redox homeostasis. Mitochondria are thus implicated in essential physiological and pathophysiological processes including tissue healing, inflammation, and cancer [5–8]. The complexity and diversity of mitochondrial functions are reflected by their wide-ranging proteome of more than 1000 proteins [9,10]. The already-extensive panel of mitochondrial functions has been further increased following the recent discovery of their capacity to translocate to other cells and to alter their behavior [11,12]. This signaling role of mitochondria is also supported by their recently-documented capacity to release metabolites as well as mitochondrial fragments, also called damage-associated molecular patterns (DAMPs) [13–15].

The severity of the diseases caused by mitochondrial dysfunctions confirms the critical importance of mitochondria for living cells. These diseases can originate from genetic mutations directly affecting the ETC activity or other mitochondrial functions. However, they can also be owing to unbalanced production of metabolites and mitochondrial components. The challenging identification of mitochondrial dysfunctions will need to be overcome in order to develop therapeutic strategies for the related metabolic, inflammatory, and malignant pathologies [14,16–18].

Here, we present an overview of recent findings on the critical involvement of mitochondrial metabolites/compounds in the signaling functions of mitochondria and on the emergence of novel therapies for diseases caused by mitochondrial dysfunctions.

2. Role of the Mitochondrial ETC and OXPHOS in Physiology and Disease

The respiratory machinery responsible for OXPHOS is located in the inner mitochondrial membrane. It includes the four complexes (I–IV) of the electron transport chain (ETC), the free-electron carriers ubiquinone and cytochrome c, and the ATP synthase (complex V) that produces OXPHOS-dependent ATP (Figure 1). The ETC machinery is encoded by both the nuclear DNA (nDNA) (80 proteins) and mitochondrial DNA (mtDNA) (13 proteins). Nuclear-encoded proteins (roughly 70% of total mitochondrial proteins) also have functions in ETC supercomplex (or respirasome) assembly [19] and in mtDNA replication and repair [20]. Therefore, nuclear and mitochondrial gene expressions need to be tightly coordinated to ensure effective mitochondrial functioning. Alterations in either result in mitochondrial defects, which makes their treatment particularly challenging. Mitochondrial DNA exhibits high mutation rates owing to (1) direct exposure to ETC-derived reactive oxygen species (ROS), (2) asymmetric replication leading to higher exposure of the single H-strand, (3) intramitochondrial dNTP pool imbalance favoring dGTP incorporation, and (4) low-efficiency DNA repair mechanisms [21]. Mitochondrial DNA mutations range from point mutations to large-scale DNA rearrangements. They can be found in cells together with their wild-type counterpart, a feature called heteroplasmy. Exceeding the critical heteroplasmy thresholds causes mutant mtDNA to trigger mitochondria-related pathologies [22].

Mitochondrial diseases are characterized by genetic alterations that primarily compromise oxidative phosphorylation (OXPHOS) and OXPHOS-dependent ATP generation. Mitochondrial diseases thus affect organs that mainly rely on OXPHOS for energy supply, including the eye, ear, liver, kidney, heart, and skeletal muscles, as well as the brain. These diseases are associated with syndromes such as CPEO (chronic progressive external ophthalmoplegia), KSS (Kearns–Sayre syndrome), LHON (Leber hereditary optic neuropathy), MIDD (maternally inherited diabetes and deafness),
Mitochondrial disorders play a pivotal role in tumorigenesis by orchestrating metabolic reprogramming, apoptosis, and ROS signaling at virtually all stages, from tumor initiation to metastasis, and mtDNA mutations have been proposed as drivers/modifiers of tumorigenesis in many cancers [24–26]. Sequencing of mtDNAs from over 2000 tumor samples across over 30 tumor types revealed important features of the mitochondrial mutational signature [27,28]. These large-scale studies identified intrinsic replicative errors, characterized by an asymmetric bias towards C→T and T→C transitions on the mtDNA heavy and light strands respectively, as the most prominent source of mtDNA mutations, overpowering the 4% caused by ROS exposure. While neutral missense mutations were found to gradually drift towards homoplasmy, deleterious frameshift or nonsense mutations,

Figure 1. Oxidative phosphorylation (OXPHOS) through the electron transport chain (ETC) and adenosine triphosphate (ATP) production. OXPHOS is the process leading to ATP synthesis through the transport of electrons released by the oxidation of nicotinamide adenine dinucleotide (NADH) and succinate generated by the tricarboxylic acid (TCA) cycle. The ETC is composed of four complexes: NADH–ubiquinone oxidoreductase (complex I), succinate–CoQ oxidoreductase (complex II), ubiquinol–cytochrome c oxidoreductase (complex III), and cytochrome c oxidase (complex IV), and of the free-electron carriers ubiquinone (CoQ10) and cytochrome c (CytC). Electrons are transferred through the ETC and finally to oxygen (O₂) (dashed lines). This electron transfer is accompanied by a flow of protons (H⁺) from the mitochondrial matrix into the intermembrane space, across complexes I, III, and IV. The generated transmembrane electrochemical proton gradient allows the ATP synthase (complex V) to produce ATP. Also shown is the dihydroorotate dehydrogenase (DHODH), which participates in the electron transfer process through the oxidation of dihydroorotate to orotate, leading to de novo pyrimidine synthesis.

3. Effects of Mutations in the Subunits of Complexes I and II of the ETC in Cancer

Mitochondrial disorders play a pivotal role in tumorigenesis by orchestrating metabolic reprogramming, apoptosis, and ROS signaling at virtually all stages, from tumor initiation to metastasis, and mtDNA mutations have been proposed as drivers/modifiers of tumorigenesis in many cancers [24–26]. Sequencing of mtDNAs from over 2000 tumor samples across over 30 tumor types revealed important features of the mitochondrial mutational signature [27,28]. These large-scale studies identified intrinsic replicative errors, characterized by an asymmetric bias towards C→T and T→C transitions on the mtDNA heavy and light strands respectively, as the most prominent source of mtDNA mutations, overpowering the 4% caused by ROS exposure. While neutral missense mutations were found to gradually drift towards homoplasmy, deleterious frameshift or nonsense mutations,
often resulting in truncated proteins, were almost exclusively heteroplasmic, thus highlighting a selective pressure in cancer cells to retain residual mitochondrial functionality [27,28].

3.1. Complex I Mutation-Induced ROS Production Promotes Tumorigenesis through Phosphatidylinositol 3-Kinase (PI3K)/Protein Kinase B (AKT) Signaling, Hypoxia-Inducible Factor 1 Alpha (HIF1α) Stabilization, and NADPH-Oxidase 1 (NOX1) Signaling

Complex I of the ETC transfers electrons from NADH produced in the TCA cycle and from the β-oxidation of fatty acids to ubiquinone. This reaction is accompanied by the translocation of four protons from the mitochondrial matrix into the intermembrane space, which generates a transmembrane electrochemical gradient and drives final ATP production by complex V (Figure 1). Complex I is the largest (1 MDa) and most elaborate complex of the ETC. X-ray crystallography revealed a structure with two L-shaped arms: a peripheral arm that catalyzes the redox reaction and an inner membrane-embedded arm containing the proton-translocating machinery. Complex I is composed of 14 conserved core subunits, encoded by both the mitochondrial and nuclear DNAs, and of at least 30 additional nuclear-encoded accessory subunits. Half the core subunits (ND1, ND2, ND3, ND4, ND4L, ND5, ND6) are mitochondria-encoded and constitute the membrane-embedded arm, while the other half (NDUF1, NDUF1V, NDUF2, NDUF3, NDUF8, NDUF7) are nuclear-encoded and constitute the peripheral arm [19,29,30]. Complex I is the main target of the currently-identified mtDNA mutations. Taking into account both mtDNA and nDNA origins, mutations are found in all 14 catalytic core subunits, in 13 accessory subunits, and in at least 11 assembly factors [31]. They have been described in cancers [32] as diverse as head and neck [33,34], breast [27,35,36], thyroid [37–39], prostate [40], renal [39,41], and hepatocellular cancers [42]. The technique of transmitochondrial cybrids, which consists of repopulating ρ0 cells (depleted of their mtDNA) with exogenous mitochondria, has been widely used to establish the role of mtDNA variants in pathologies, independent of nDNA backgrounds [43]. Transmitochondrial cybrid studies thus showed that complex I mutants can exhibit both pro- and anti-tumorigenic effects in cancer, in an OXPHOS- and ROS-dependent fashion, as detailed below.

3.1.1. PI3K/AKT Signaling

The ND5 m.12418insA frameshift mutation, found in colorectal cancers, leads to a truncated ND5 protein, which destabilizes the assembly of the membrane-embedded arm of complex I [44,45]. As shown in 143B osteosarcoma transmitochondrial cybrids, the effects of this mutant highly depended on its heteroplasmatic level. While a 72% ND5 mutant heteroplasmy still retained 46% residual complex I activity, ND5 mutant homoplasmy (96%) led to loss of complex I function [44]. These effects were associated, as expected, with a gradual decrease of OXPHOS-dependent oxygen consumption and ATP production [46]. Heteroplasmic cybrids exhibited increased levels of mitochondria-specific ROS (but not of intracellular ROS), which correlated with the overexpression of the catalase, the glutathione peroxidase 4 (Gpx4), and the Cu-Zn superoxide dismutase (SOD1) anti-oxidative enzymes. This enhanced ROS production also activated the PI3K/AKT signaling pathway and the expression of downstream genes encoding the hypoxia-inducible factor 1 alpha (HIF1α) and the anti-apoptotic proteins B-cell lymphoma-extra large (BCL-XL) and induced myeloid leukemia cell differentiation protein (MCL1) (Figure 2) [47]. Osteosarcoma cybrids harboring a heteroplasmic ND5 mutation were thus endowed with resistance to oxidative stress and, subsequently, enhanced tumorigenicity upon subcutaneous injection in nude mice [46,47]. ND5-mutant homoplasmic cybrids, however, showed increased levels in both mitochondrial and intracellular ROS, which led to apoptosis and prevented tumor formation in vivo [46].
The ND1m.3571insC frameshift mutation, observed in thyroid oncocylic carcinoma, also generates a truncated ND1 protein, which leads to complex I disassembly [48]. Transmitochondrial osteosarcoma cybrids expressing this ND1 mutant totally lost their complex I activity and their basal oxygen consumption, revealing a dramatic OXPHOS deficit [49]. Complex I dysfunction also induced the accumulation of NADH, an allosteric inhibitor of α-ketoglutarate dehydrogenase, which led to increased α-ketoglutarate levels and to HIF1α destabilization (Figure 2). These osteosarcoma cybrids did not form tumors in vivo [49,50]. Another ND1 mutation (missense m.3460G>A, A52T) showed a weaker phenotype as homoplasmic osteosarcoma cybrids retained 50% of complex I activity, with only a partial reduction in basal oxygen consumption [49]. These mutants were still able to form tumors upon xenograft in mice, a tumorigenic potential linked to cytoplasmic ROS accumulation and HIF1α stabilization [49]. A third type of mutation (m.4776G>A; A103T), found in the ND2 subunit of complex I, in head and neck squamous cell carcinoma, also illustrates the role of ROS accumulation and HIF1α stabilization in tumorigenesis [33,34]. This mutation, which impaired cell respiration and increased ROS production, led to a feedback metabolic signaling involving pyruvate dehydrogenase (PDH) and pyruvate dehydrogenase kinase 2 (PDK2) [34]. ROS-induced PDK2 activity resulted in increased pyruvate, HIF1α activation and tumor growth (Figure 2) [34]. Finally, the highly-metastatic capacities of murine Lewis lung carcinoma and fibrosarcoma were attributed to the missense m.13997G>A (P25L) and frameshift m.13885insC mutations, respectively, in the mtDNA ND6-encoding gene, as further confirmed in cybrids [51]. The increased metastatic capacity of these complex I mutants was also

**Figure 2.** Effects of complex I mutations. (1) As a result of complete loss of complex I, NADH inhibits the TCA cycle enzyme α-ketoglutarate dehydrogenase (αKGDH). This increases α-ketoglutarate levels and results in HIF1α inhibition. (2-4) Complex I mutations increase reactive oxygen species (ROS) production. (2) ROS activate pyruvate dehydrogenase kinase (PDK2), which inhibits pyruvate dehydrogenase (PDH). Pyruvate accumulation activates HIF1α. (3) ROS activate the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/hypoxia-inducible factor 1 alpha (HIF1α) signaling pathway. (4) Mitochondrial ROS activate the NADPH oxidase, which produces cytoplasmic superoxide. ROS lead to genomic instability via double-strand breaks (DSBs) and activate the cellular anti-oxidant responses.
associated with a higher ROS production, which led to high levels of HIF1α, of the anti-apoptotic MCL-1 protein, and of the vascular endothelial growth factor (VEGF), thus nurturing neoangiogenesis. Consistent with this role of ROS, pretreatment of highly-metastatic cybrids with the ROS scavenger N-acetylcysteine (NAC) decreased MCL-1 expression in vitro and reduced metastasis in vivo [51].

3.1.3. NOX1 Signaling

NADPH-oxidase 1 (NOX1) activity was also shown to contribute to tumorigenesis, together with increased mitochondrial ROS, as a result of complex I dysfunction [52]. The various osteosarcoma cybrids analyzed, carrying the m.3460G>A/MT-ND1 (A52T), m.11778G>A/MT-ND4 (R340H), and m.14484T>C/MT-ND6 (M64V) point mutations, respectively, exhibited decreased OXPHOS and ATP production. Their enhanced tumorigenicity, upon xenograft in nude mice, was associated with increased levels of both mitochondrial and cytoplasmic ROS as well as increased activity of NOX1, resulting in the production of superoxide and hydrogen peroxide, major sources of cytoplasmic ROS (Figure 2) [52]. Overall, mutations in complex I appear to exert anti-tumorigenic effects only upon complete abolishment of complex I function, which prevents ROS production. Milder mutations, which preserve some complex I activity, enhance ROS production and stimulate tumor growth [46,49,52].

3.2. OXPHOS-Harmful Complex I Mutations Elicit Metabolic Compensation

3.2.1. Shift towards Glycolysis

Several studies reported that OXPHOS dysfunction, as a consequence of complex I impairment, leads to a metabolic shift towards glycolysis, which enables cells to maintain cell growth and ATP production [33,34,46,49,52,53]. This metabolic shift was reported in transmitochondrial osteosarcoma cybrids harboring the m.12418insA/MT-ND5 mutation. Increasing the mutational load from 72% of mutant ND5 in heteroplasmic cybrids to 96% in homoplasmic cybrids gradually decreased respiration (from 53% to 17% of wild type (WT)) and OXPHOS-linked ATP production (from 21% to 2% of WT), while it concomitantly increased glucose uptake and lactate production (from 28% to 56% of WT) [46]. Likewise, as shown for another subunit of complex I, osteosarcoma cells harboring the severe m.3571insC/MT-ND1 mutation showed increased glucose uptake and lactate production, while the milder m.3460G>A/MT-ND1 mutant had a glucose metabolism similar to that of the wild-type cells [49]. This glycolytic shift observed in transmitochondrial osteosarcoma cybrids was ascribed to HIF1α activation [50]. HIF1α stimulated the expression of the glucose transporters 1 (SLC2A1) and 3 (SLC2A3) as well as a series of glycolytic enzymes, including phosphofructokinase (PFKP), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK1), and lactate dehydrogenase (LDHA) [50]. Taken together, these data indicate that complex I mutations could induce a Warburg-like effect in cancer cells, fostering tumor growth.

3.2.2. Shift towards Complex II-Dependent Succinate Oxidation

Beyond the Warburg effect, a metabolic shift towards complex II-dependent succinate oxidation was recently described as an adaptive mechanism to compensate for OXPHOS dysfunction in high-grade prostate cancers, mutant for the ND1 subunit of complex I [54]. As a response to the inhibition of glutamate and malate oxidation and to the subsequent electron transport loss across complex I, ND1-mutant cancer cells were found to produce ATP by aerobic respiration through complex II-mediated succinate oxidation [54]. This metabolic reprogramming was associated with shorter patient survival, highlighting the critical role of complex II and succinate in malignancies associated with complex I dysfunction [54].

3.2.3. Shift towards Serine Catabolism

The catabolism of serine, a precursor for nucleic acids, proteins, and fatty acids [55,56], was also shown to maintain OXPHOS activity in colon cancer cells with mutant complex I, by producing NADH.
and feeding it to the ETC [57]. Higher NADH levels than expected could be reached through serine catabolism because of the insensitivity to NADH concentrations of MTHFD2 (methylene tetrahydrofolate dehydrogenase 2), the enzyme involved in this conversion [57]. Overall, these findings demonstrate the various ways tumor cells manage to get around complex I dysfunction to maintain efficient OXPHOS.

3.2.4. Complex I Loss Alleviates Complex V Dysfunction

By applying a genome-wide CRISPR screening on human K562 chronic myeloid leukemia cells concomitantly treated with oligomycin, an inhibitor of complex V, Mootha and colleagues identified a series of both synthetic lethal mutants and suppressors showing that complex I mutations can alleviate complex V dysfunction [58]. Mechanistically, concomitant inhibition of complexes I and V was accompanied by an increased reductive carboxylation of α-ketoglutarate [58]. Experimental depletion of cytosolic NADPH, known to drive reductive pathways, suppressed the protective effect of complex I loss against complex V inactivation, thus supporting the role of reductive metabolism in these effects [58].

3.3. Complex II Dysfunction Induces Tumorigenicity via Succinate Accumulation

Complex II, also known as succinate dehydrogenase (SDH) and succinate ubiquinone oxidoreductase, has the unique property of linking both the TCA cycle and the ETC by coupling succinate oxidation to fumarate within the TCA cycle with ubiquinone (coenzyme Q10) reduction to ubiquinol in the ETC. Complex II is composed of four subunits solely encoded by nuclear genes (SDHA, SDHB, SDHC, and SDHD). SDHA oxidizes succinate to fumarate, while reducing FAD to FADH2. Electrons from FADH2 are then transferred sequentially to SDHB and to ubiquinone at the inner membrane-embedded site formed by SDHC and SDHD. Contrary to complexes I, III, and IV, complex II-mediated electron transport is not accompanied by proton translocation into the mitochondrial intermembrane space [19,59].

Deleterious mutations in any of the four subunits of complex II decrease SDH activity and result in abnormal accumulation of succinate, as observed in complex II-mutated cells in vitro and in the extracellular fluids (plasma, urine, saliva, and feces) of complex II-deficient patients. Complex II mutations, principally affecting SDHB and SDHD subunits, have been associated with various cancers including hereditary paraganglioma and pheochromocytoma (i.e., neuroendocrine tumors of the paraganglionic tissue), gastrointestinal stromal tumors [60], and renal cell carcinoma [61]. Therefore, SDH has now been defined as a tumor suppressor and succinate as an oncometabolite [62,63].

3.3.1. Complex II Mutations Inhibit 2-Oxoglutarate-Dependent Dioxygenases

The R22X nonsense SDHD mutation found in hereditary paraganglioma and pheochromocytoma generates a truncated SDHD protein of 21 amino acids (instead of 159), resulting in the loss of complex II electron transfer and enzymatic activities and in the activation of the HIF1α signaling pathway [64,65]. The R22X nonsense SDHD mutation leads to succinate accumulation, which inhibits the activity of prolyl hydroxylase (PHD) and, consequently, induces HIF1α stabilization [65]. PHD is a 2-oxoglutarate (2-OG, also known as α-ketoglutarate)-dependent dioxygenase that, in normal settings, hydroxylates HIF1α and leads to its poly-ubiquitylation by the Von Hippel–Lindau protein (pVHL) complex and, ultimately, to its degradation by proteasomes [66] (Figure 3). Succinate is structurally similar to 2-OG. It thus acts as a competitive inhibitor for PHD and promotes HIF1α stabilization [67,68].

Succinate accumulation resulting from complex II mutations was shown to inhibit other 2-OG-dependent dioxygenases, including the Jumonji C (JmjC)-domain containing histone lysine demethylases (KDMs) [69] and the ten-eleven translocation (TET) family of DNA hydroxylases [70] (Figure 3). TET enzymes mediate DNA oxidative demethylation through 5-methylcytosine (5mC) hydroxylation into 5-hydroxy-methylcytosine (5hmC) [70,71]. Succinate accumulation was shown to reduce TET-induced levels of 5hmC in human embryonic kidney cells (HEK293T) with SDHA/B knockdown as well as in mice livers with transient SDHA knockdown [72]. In line with these observations, DNA hypermethylation was found in SDHx-mutated paraganglioma and
pheochromocytoma samples and in SDH-deficient gastrointestinal stromal samples, as a result of succinate inhibition of KDM and TET enzymes [73–75].

Figure 3. Role of TCA cycle intermediates in HIF1α stabilization and nuclear epigenetic modifications. Following its hydroxylation by prolyl-hydroxylases (PHDs), HIF1α is recognized by the von Hippel–Lindau (VHL) complex that targets it for proteasomal degradation. The inactivation of complex II of the ETC leads to ROS accumulation and inhibition of PHD activity by succinate, preventing HIF1α hydroxylation and degradation. Upon translocation to the nucleus, HIF1α binds to the hypoxic response element (HRE) and activates gene transcription, contributing to tumorigenesis. Complex II-deficient cells primarily rely on glutamine to fuel the TCA cycle and utilize oxaloacetate to generate aspartate for their biosynthetic pathways. The TCA cycle also produces metabolites that act as epigenetic modifiers. The α-ketoglutarate is a cofactor of 2-oxoglutarate-dependent dioxygenases, including the ten-eleven translocation (TET) family of DNA demethylases and the histone lysine demethylase (KDM) family. The metabolites 2-hydroxyglutarate, succinate, and fumarate, which are structurally similar to α-ketoglutarate, act as antagonists of TET- and KDM-catalyzed reactions.

3.3.2. The Hypermethylator Phenotype: A Double-Edged Sword for Complex II-Mutated Cancers

As mentioned above, SDH mutations can promote tumorigenesis by a succinate-mediated genome-wide epigenetic remodeling [74,76]. As shown in mouse SDHB-knockout chromaffin cells, increased succinate levels were associated with Cpg island hypermethylation in the promoters of genes encoding proteins such as the matrix-metalloprotease inhibitor SPOCK2, the metastasis suppressor DNAJA4, and the cell adhesion marker KRT19 [74], thus resulting in a derepression of epithelial-to-mesenchymal transition (EMT). Succinate accumulation following SDHB-knockdown also led to an EMT-like phenotype in a murine serous ovarian carcinoma cell line, which was attributed to H3K27 hypermethylation [77]. The now-established succinate-mediated “hypermethylator” phenotype was also recently shown to synergize with HIF2α in establishing a mesenchymal-like phenotype and enhancing the metastatic potential of SDHB-knockout mouse chromaffin cells in vivo [75].

Yet, the hypermethylator phenotype of succinate accumulation was also found to sensitize complex II-deficient cancer cells to chemotherapy, as shown in paragangliomas and pheochromocytomas following treatment with the alkylating agent temozolomide [78]. This was attributed to the decreased expression of the DNA repair enzyme O6-methylguanine-DNA methyltransferase (MGMT) consequent to its promoter hypermethylation, as detected in patient SDHB-mutated paraganglioma and pheochromocytoma metastasis [78,79]. Similar inhibition of DNA repair processes was reported for SDHB-deficient YUNK1 kidney cell lines as a result of histone lysine demethylase (KDM) inhibition [80].
Deficiency in homologous recombination rendered SDHB-knockdown cells sensitive to synthetic lethality with poly(ADP)-ribose polymerase (PARP) inhibitor drugs olaparib and BMN-673, as shown both in vitro and in vivo, thus suggesting potential therapies for renal cell cancer (HLRCC) [80]. Taken together, these studies indicate that the succinate-induced hypermethylator phenotype acts as a double-edged sword in the physiopathology of cancers harboring SDH mutations, as it can either promote or inhibit cancer progression, depending on the genes targeted for methylation.

3.4. Metabolic Compensation Following Complex II Mutations Affecting OXPHOS

3.4.1. Preferential Usage of Glucose and Glutamine

As complex II links the ETC to the TCA cycle, alterations of its functions expectedly result in important metabolic rewiring, found to support the bioenergetic needs of tumors. In particular, murine SDHB-deficient serous ovarian carcinoma cells were shown to exhibit an unbalanced TCA cycle, with low production of fumarate and malate, owing to high succinate levels. In addition, these complex II-deficient cells preferentially used glucose for anaerobic ATP production (through glycolysis) and glutamate for fueling the TCA cycle [77]. Similar preferential incorporation of glutamine carbons in the TCA cycle has been reported in murine SDHB-knockout chromaffin cells [81]. These data suggest that SDH-deficient cells rewire their central carbon metabolism through differential metabolite usage, with glucose as a source of ATP production and glutamine as a major fuel for the TCA cycle.

3.4.2. Dependence on Pyruvate Carboxylation

In addition to its effects on glucose and glutamine metabolism, complex II deficiency leads to alterations in the metabolism of aspartate, a major precursor for non-essential amino acid, protein, and nucleotide biosynthesis. Seminal studies elegantly demonstrated that SDH-deficient cells use oxaloacetate, generated by pyruvate carboxylation, to produce aspartate, while wild-type cells rely on acetyl-CoA originating from pyruvate oxidation. These results suggest that SDH-deficient cells switch from pyruvate oxidation to carboxylation to sustain their aspartate anabolic needs. These data were further confirmed by the growth arrest of SDH-deficient cells following pyruvate carboxylation inhibition [81,82]. Taken together, these results confirm previous studies on the role of the ETC for cell proliferation, owing to its support of the synthesis of aspartate [83,84].

3.4.3. CII$_{low}$, an Energy Consumption Regulator

An interesting study by the Neuzil team recently identified an alternative assembly of complex II, termed CII$_{low}$, and composed only of the SDHA subunit. This CII$_{low}$ complex, detected in patients with SDHB-mutated paragangliomas, was linked to poor survival [62]. Expression of CII$_{low}$ complex in SDHB-deficient cancer cells was associated with a decrease in de novo pyrimidine synthesis and cell proliferation along with an upregulation of catabolic and salvage pathways. The expression of such a CII$_{low}$ complex may thus result from the metabolic adaptation of SDHB-deficient cancer cells, which are less proliferative, but endowed with high invasive and metastatic capacities [62].

4. Role of Complex III of the ETC for Physiological Functions

4.1. Links between Complex III Activity in T$_{reg}$ Cells and Immune Function

As developed above, the diverse complexes of the electron transport chain (ETC) have an essential role for the effective coupling of mitochondrial respiration and energy production. Nonetheless, the defective activity of ETC complexes can have further functional consequences that depend on mitochondria-produced metabolites and reach beyond mere mitochondrial activity. T regulatory (T$_{reg}$) cells are a subset of CD$^{+}$ T cells characterized by a mitochondrial metabolism. Chandel and collaborators recently showed that the activity of complex III (ubiquinol-cytochrome c reductase) of the ETC is needed for the immune suppressive function of T$_{reg}$ cells and that
its ablation leads to fatal inflammatory disease in treated mice, through modified metabolite production [85]. Mice whose T<sub>reg</sub> cells are deficient in complex III were generated, by deleting the gene encoding the Rieske iron-sulfur protein (RISP), one of its essential subunits. Although T<sub>reg</sub> cell proliferation and survival, as well as Foxp3 expression, were maintained in these mice, their immune suppressive capacity was lost. Increased concentrations of the 2-hydroxyglutarate (2-HG) and succinate metabolites, which are known inhibitors of the TET-family of DNA demethylases (Figure 3), were found responsible for the DNA hypermethylation phenotype, for the altered gene expression in T<sub>reg</sub> cells and, eventually, for the disruption of their immune suppressive function [85].

4.2. Links between Complex III Activity in Haematopoietic Stem Cells and Haematopoiesis

Haematopoietic stem cells (HSCs) display a mainly glycolytic phenotype. However, the importance of HSC mitochondrial activity for hematopoiesis was demonstrated by the same team, by depleting complex III in murine fetal hematopoietic stem cells. As for RISP-null T<sub>reg</sub> cells, RISP-null fetal HSCs maintained their proliferation. However, HSC impaired respiration was accompanied by a decreased NAD<sup>+</sup>/NADH ratio and, as observed for RISP-null T<sub>reg</sub> cells, levels of the metabolites 2-hydroxyglutarate and succinate were increased, as that of fumarate. The functional consequences of RISP-dependent complex III depletion and the resulting unbalanced metabolite production were the inability of HSCs to generate multipotent progenitors, leading to mice anemia and fetal death [86].

4.3. Links between Complex III Activity in Endothelial Cells and Angiogenesis

Endothelial cells (ECs) also primarily use glycolysis for ATP production, vessel sprouting, and angiogenesis. However, in addition to glucose that fuels glycolysis, endothelial cells can also take up fatty acids and glutamine that fuel the TCA cycle and, further, the ETC. By antimycin inhibition of complex III in human umbilical vein endothelial cells (HUVECs) in vitro, Chandel and collaborators showed that complex III is necessary for angiogenesis through the maintenance of NAD<sup>+</sup>/NADH ratios, aspartate concentrations, and proliferation of endothelial cells. These effects of a dysfunctional complex III in reducing NAD<sup>+</sup>/NADH ratios, proliferation, and angiogenesis were confirmed in vivo in mice harboring a deletion of the Uqcrq gene, encoding the ubiquinol-binding protein QPC, a critical subunit of complex III. They were accompanied by a loss of postnatal retinal and lung angiogenesis, as well as melanoma angiogenesis in a B16-F10 melanoma model [87].

These studies highlighted the physiological consequences of a dysfunctional complex III of the mitochondrial ETC, for immunity, hematopoiesis, or angiogenesis. Some of these effects were linked to the overproduction of metabolites like 2-hydroxyglutarate and succinate, or fumarate, which interestingly appeared to be cell-type dependent, suggesting other levels of regulation.

5. Versatile Roles of Mitochondrial Components in Physiology and Disease

5.1. The Role of Ubiquinone (Coenzyme Q10), Activated by the Mevalonate Pathway, in Cancer

Ubiquinone, also known as coenzyme Q10 (CoQ10), is an important electron carrier located in the inner mitochondrial membrane, where it transfers electrons from complexes I and II to complex III in the electron transport chain (ETC) (Figure 1). Ubiquinone is thus involved in the regulation of oxidative stress and ROS production. Ubiquinone is also a downstream metabolite of the mevalonate pathway. The mevalonate pathway uses acetyl-CoA, derived from glucose, glutamine, and/or acetate metabolism, to produce mevalonate; farnesyl-pyrophosphate (FPP); and, thereafter, different metabolites including cholesterol and ubiquinone [88] (Figure 4). The mevalonate pathway is often upregulated in cancers, which leads to increased mitochondrial concentrations of CoQ10. Statin inhibition of the mevalonate pathway is beneficial and statin treatment has been correlated with tumor cell apoptosis and reduced mortality in diverse cancers, notably breast cancer, pancreatic adenocarcinoma, and hepatocellular carcinoma [88]. As shown for pancreatic ductal adenocarcinoma (PDAC) tumor cells, ubiquinone levels are lowered by statin treatment, resulting in increased oxidative
stress and ROS production. However, this oxidative stress triggers redox-active metabolic pathways aimed at lowering ROS levels, including the increased import of cystine for downstream glutathione production [89]. Therefore, the dysfunctional role of ubiquinone in the mitochondria of PDAC cells can be addressed by the concomitant targeting of the upstream mevalonate pathway (with statins) and of the metabolic glutathione-based compensation of excessive ROS production (with cystine/glutamate xCT antiporter inhibitors). As demonstrated in PDAC murine models, this effective approach triggers cancer cell death while sparing the mitochondrial functions [89].

Figure 4. The mevalonate and the fatty acid synthesis pathways. Acetyl-CoA, derived from glucose, glutamine, or citrate following its export to the cytosol, is converted through the mevalonate pathway into several metabolites including cholesterol and coenzyme Q10. Coenzyme Q10 transfers electrons from complexes I and II of the electron transport chain, as well as from dihydroorotate dehydrogenase (DHODH), to complex III. Acetyl-coA also acts a precursor for fatty acid synthesis, through its conversion to malonyl-CoA, and then to palmitate. The mevalonate pathway is represented in yellow boxes. The fatty acid synthesis pathway is represented in blue boxes. Dashed arrows represent multiple steps. HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; IPP, isopentenyl-diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl-diphosphate; TCA cycle, tricarboxylic acid cycle.

5.2. Changing Dogmas about the Mitochondrial Role of CPT1, in both Synthesis and Oxidation of Fatty Acids

Lipids are important metabolites for membrane building and, therefore, for cell proliferation. They also provide cellular energy, act as signaling entities, and are involved in intercellular communication. All these functions allow lipid metabolism to contribute to cancer progression [90]. Both activation of fatty acid (FA) synthesis and FA oxidation have been linked to cancer progression. As recently reviewed [90], enzymes involved in fatty acid β-oxidation were overexpressed in diverse cancers and their inhibition was shown to curb cancer progression. Such is the case for CPT1 (carnitine palmitoyltransferase 1), a protein associated with the outer mitochondrial membrane, allowing the transport of long-chain fatty acids into the mitochondrial matrix. CPT1-dependent transfer of long-chain acyl groups from coenzyme A to carnitine constitutes the rate-limiting enzymatic process for the oxidative degradation of fatty acids [91].

However, CPT1 is now demonstrated to affect cancer cell proliferation by mechanisms relying on anabolic FA synthesis rather than FA β-oxidation (FAO) [92]. This novel role for CPT1 was uncovered
by changing the tools to inhibit CPT1 activity. Instead of the etomoxir-dependent pharmacological inhibition of CPT1, CPT1 knock downs were performed [92]. Studying the effects of CPT1 using high concentrations of etomoxir was marred by its off-target inhibition of complex I of the electron transport chain, a feature that was reported for the BT549 breast cancer cell line [92] as well as for T cells [93–95]. CPT1 knock down in the BT549 breast cancer cell line demonstrated the role of CPT1 for cell proliferation, independently of FAO. CPT1 was actually found necessary for mitochondrial morphology maintenance, regulated mitochondrial lipid levels, polarized mitochondrial membrane, and efficient respiratory chain coupling [92]. As suggested by the authors, an important CPT1 function may thus be to provide long-chain fatty acids for anabolic processes in the mitochondria, needed for healthy cells and exploited in enhanced cancer cell proliferation [92].

5.3. Different Types of Mitochondria Linked to Lipid Metabolism

Attributing both antagonistic functions of FA synthesis and FA oxidation to mitochondria might be counter-intuitive. However, the recently-reported existence of metabolically-distinct mitochondrial subpopulations—one endowed with FA oxidation activity, the other involved in lipid droplet formation—could reconcile these two different mitochondrial activities [96,97]. A mitochondrial population associated with lipid droplets was characterized in brown adipose tissue (BAT). These peridroplet mitochondria (PDM) showed a high bioenergetic capacity that supported triacylglyceride (TAG) synthesis and lipid droplet formation. The protein composition of these mitochondria and the structure of their cristae appeared different from that of cytosolic mitochondria. They remained as an isolated pool different from cytosolic mitochondria owing to their low fusion-fission activity. In BAT cells isolated from cold-exposed mice, PDM abundance was reduced two fold in association with enhanced β-oxidation activity [97]. Mitochondria recruitment to lipid droplets is not limited to BAT, as also reported in mouse embryonic fibroblasts (MEFs) and in striated muscle upon nutrient deprivation, as reviewed by Benador et al. [96]. Lipid droplet accumulation has been associated with therapy resistance for various cancers, including glioma, colon, kidney, lung, and prostate cancers [98]. Whether PDM represent a population of mitochondria that can be targeted therapeutically in these pathologies will be worth investigating.

6. Mitochondria as Signaling Organelles: Functional Effects of Mitochondrial Exchange between Cells

Mitochondria are known to act as signaling organelles owing to their production of reactive oxygen species, structural compounds, and metabolites. As described above, metabolites produced by the TCA cycle can be used for the biosynthesis of macromolecules such as lipids, proteins, and nucleotides, as well as for epigenome modifications and post-translational protein changes [99]. As a result, mitochondria regulate a wide range of biological functions, including survival, growth, and differentiation.

However, the effects of mitochondria are not restricted to the cells that originally produced them, as we now know that, quite unexpectedly, mitochondria can translocate between cells. By their capacity to get transferred between cells, mitochondria can thus provide signaling cues to other cells. Numerous studies have now documented the diverse effects generated by intercellular mitochondria transfers and shown that they depend on the physiological/pathophysiological conditions and, more specifically, on the state of both mitochondria-provider and -recipient cells. The parts below provide an overview of the functional effects of intercellular mitochondria transfers in tissue repair, inflammation, and cancer progression, with a focus on the role of metabolites and structural compounds generated in the mitochondria-recipient cells.

6.1. Biological Effects of Intercellular Mitochondria Transfers

The past two decades revealed that mitochondria constantly communicate with both the cytosol and the nucleus under normal and stress conditions, thus eliciting adaptive cellular biological responses. More recently, the concept that mitochondria could exert biological effects outside from
their original cells emerged from the observation that whole mitochondria, or parts of mitochondria, can translocate from one cell to the other. One example of intercellular mitochondrial communication is provided by the crosstalk of dying cells, following tissue injury, with the innate immune system of recipient cells, through the release of mitochondrial molecules including mitochondrial DNA (mtDNA), N-formyl peptides, ATP, or cardiolipin [100,101]. These mitochondrial products, recognized as damage-associated molecular patterns (DAMPs) by specific receptors on innate immune cells, are capable of directly activating the innate immune system and triggering adaptive inflammatory responses [102,103]. In addition to the crosstalk mediated by mitochondrial fragments, cells have been shown to communicate with each other by exchanging whole mitochondria.

Intercellular transfers of whole mitochondria have been reported to occur both in vitro and in vivo, and in both physiological and pathological conditions. This phenomenon is involved in several biological processes such as tissue repair, inflammation, and cancer progression (see reviews [11,12,104,105]). Mitochondria can translocate from one cell to the other by different modalities. These include thin membrane channels called tunneling nanotubes (TNTs) that ensure the connection and the mitochondria transfer between mitochondria-donor and -acceptor cells [11,12,104,105]. Mitochondria can also be transferred through their release in the extracellular space, either encapsulated inside microvesicles or as free organelles [104,106,107]. Importantly, the biological effects promoted by intercellular mitochondria transfers are highly dependent on the state of both mitochondria-donor and mitochondria-recipient cells. The outcome of these transfers can be beneficial, for instance, in tissue healing; on the other hand, they can also enhance tumor progression [11,12,104,105].

The vast majority of the studies addressing the process of intercellular mitochondria transfers have been conducted on mesenchymal stem cells (MSCs) as donor or acceptor cells for the conveyed mitochondria. These studies revealed that, compared with other cells, MSCs demonstrate a high capacity to donate their mitochondria to neighboring cells. This capacity is attributed to high expression of Miro-1, an outer mitochondrial membrane Rho-GTPase that mediates mitochondria trafficking through TNTs [108–110]. Mitochondria donation from MSCs to damaged or cancer cells has invariably been shown to improve recipient cell survival (see reviews [11,12,104,105]). Therefore, this process has important consequences in tissue regeneration and in cancer progression and aggressiveness (resistance to chemotherapy). MSCs have also been reported to transfer mitochondria to immune cells including T cells and macrophages. In these settings, transferred mitochondria decreased inflammation by promoting recipient immune cell differentiation towards an anti-inflammatory phenotype [111–116]. On the contrary, the transfer to T cells of mitochondria originating from myeloid cells was shown to exacerbate inflammation and to lead to asthma aggravation [117]. In addition, mitochondria transfer from pro-inflammatory monocytes to endothelial cells was associated with vessel inflammation observed in cardiovascular diseases [118]. It is worth mentioning that intercellular mitochondria transfers can be bidirectional, eliciting distinct biological responses in the two communicating cells [119]. For instance, and as mentioned above, MSCs can transfer mitochondria to damaged cells, which results in improved survival. However, MSCs can also acquire mitochondria released from damaged cells. These mitochondria function as signaling organelles that alert MSCs of a danger situation and trigger an adaptive wound-healing response [119].

The presence in the bloodstream of free or membrane-encapsulated respiratory-competent mitochondria has also been recently reported. Although the biological impact of these circulating mitochondria has yet to be determined, a role in regulating systemic inflammation through their interactions with immune cells could be speculated [107,120,121]. This pro-inflammatory role of circulating mitochondria is supported by observations made in organs from deceased patients and used for allotransplantation. In particular, circulating whole mitochondria and mtDNA levels were strongly correlated with the inflammation initiated by neutrophil activation and early allograft dysfunction, as shown for liver transplantation [120]. Besides, the concentration of free mitochondria in the blood
of cancer patients correlated with metastasis, suggesting that circulating mitochondria are involved in cancer cell-to-cell communication processes [107].

6.2. OXPHOS Induced by Mitochondria Transfer in Tissue Repair and Cancer

The first discovered biological effect assigned to intercellular mitochondria transfers was their capacity to improve the survival of recipient cells, either damaged following a stress injury [122,123] or, for cancer cells, treated by chemotherapy [124]. Increased recipient cell survival was invariably correlated with enhanced OXPHOS activity and ATP production. The most convincing proof of the importance of functional OXPHOS restoration in the mitochondria-recipient cells was provided by studies using, as either mitochondria-donor or -recipient cells, \( \rho^0 \) cells, which are devoid of mtDNA and have an impaired respiratory chain. The pioneering work of Prockop and collaborators thus demonstrated the OXPHOS activity restoration in \( \rho^0 \) A549 lung adenocarcinoma cells owing to mitochondria transfer from cocultivated MSCs [125]. Likewise, Neuzil and collaborators found that mitochondria transfer occurred in vivo, from host stroma cells to engrafted \( \rho^0 \) melanoma or \( \rho^0 \) breast cancer cells, and that this process fully restored the respiration in the acceptor \( \rho^0 \) cancer cells [126,127].

Beside the studies conducted with \( \rho^0 \) cells, many works reported that mitochondria are transferred during cocultures from MSCs to damaged cells, including cardiomyocytes [122,128], endothelial cells [129], bronchial epithelial cells [130], corneal epithelial cells [131], and neuronal cells [132], or with leukemic, bladder, and breast cancer cells [133–135]. In addition, the physiological relevance of this process was strengthened by the in vivo demonstration of an increase in OXPHOS activity and ATP production following mitochondria transfer from mouse lung alveolar epithelial cells injured through exposure to LPS [123], to rotenone [110], or to cigarette smoke [130].

Finally, the prerequisite for transferred mitochondria to harbor functional respiration in order to exert their cytoprotective effects was reinforced by using \( \rho^0 \) MSCs, which were unable to protect damaged cells against apoptosis [122].

6.3. Metabolic Reprogramming of MSCs and Cancer Cells by Metabolites Supplied by Mitochondria Transfer

As mentioned above, the acquisition of functional mitochondria leads to the enhancement of OXPHOS activity and ATP production in mitochondria-recipient cells. This process is of critical importance in damaged and dying cells owing to the role of ATP in restoring cellular bioenergetics. Beyond its impact on ATP levels, OXPHOS activation may also reflect an activation of the TCA cycle with an increased generation of some metabolites, with the OXPHOS and the TCA cycle being tightly coordinated [14,99]. It is still poorly documented to what extent intercellular mitochondria transfers contribute to the metabolic reprogramming of recipient cells through enhanced production of TCA cycle metabolites. However, two recent works demonstrated that TCA cycle metabolites are involved in the signaling mediated by the transferred mitochondria in recipient cancer cells and MSCs. As shown by Neuzil’s group, \( \rho^0 \) melanoma and breast cancer cells lacking functional OXPHOS were unable to form tumors in mice. However, acquisition of respiratory-competent mitochondria from stromal cells allowed these \( \rho^0 \) malignant cells to become highly proliferative, leading to tumor formation and progression [127]. Mechanistically, the authors demonstrated that OXPHOS-mediated ATP production was not required for tumor progression, but that, instead, OXPHOS function was critical for the generation of orotate, which is an essential intermediate for pyrimidine de novo synthesis [136,137]. In particular, OXPHOS restoration in \( \rho^0 \) cells was found to re-activate the enzymatic activity of dihydroorotate dehydrogenase (DHODH), which is the ubiquinone-oxidoreductase responsible for orotate formation through dihydroorotate oxidation (Figure 1) [136,138]. As pyrimidine serves as building blocks for DNA replication and transcription, this explains why restoration of OXPHOS in \( \rho^0 \) cells is essential to sustain cell proliferation [137]. In the absence of a functional respiratory chain, as is the case in \( \rho^0 \) cells prior to intercellular mitochondria transfers, DHODH is unable to oxidize dihydroorotate, thus blocking pyrimidine synthesis and, as a result, arresting DNA replication and cell division [136].
Another example of the role of TCA cycle metabolites in mediating the effects of exogenous mitochondria is provided by recent observations from our laboratory indicating that the respiratory-competent mitochondria released by activated platelets can be engulfed by MSCs and can stimulate the MSC pro-angiogenic activity [139]. Citrate levels were increased in MSCs following platelet mitochondria transfer. Citrate was identified as a key metabolite initiating metabolic remodeling and functional alterations in recipient MSCs. Citrate was shown to stimulate recipient MSCs through its export to the cytosol where it fueled the ATP citrate lyase enzyme (ACLY), leading to the activation of fatty acid synthesis (Figure 4) and, subsequently, to the stimulation of the angiogenic activity of MSCs [139].

6.4. Role of DAMPs, Generated by the Transfer of Damaged Mitochondria, in Regulating Inflammation and MSC Activation

Following tissue injury, damaged cells have been reported to release their mitochondria to the extracellular environment [119,140,141]. As discussed below, the translocation of these damaged mitochondria to target cells, such as immune cells or reparative progenitors cells, functions as signaling cues, able to alert the rest of the body of a danger situation [140,142,143]. In contrast to the above-mentioned studies where transferred mitochondria needed to be fully functional to behave as pro-survival factors or as metabolite suppliers, mitochondria transferred from suffering cells mediate their effects by the means of mitochondrial fragments or DAMPs generated by recipient cells. Several DAMPs produced by recipient cells following the intercellular transfer of damaged mitochondria have been involved in inflammation [106,141]. One example is provided by the role of mitochondria released by activated platelets to neutrophils in the stimulation of the innate immune system [106]. Following their engulfment by neutrophils, platelet-derived mitochondria have been shown to have their membranes hydrolyzed by the phospholipase A2 enzyme, this mitochondria degradation leading to the production of various DAMPs, including lysophospholipids, fatty acids, and mtDNA, which are known to trigger leukocyte activation to have their membranes hydrolyzed by the phospholipase A2 enzyme [106].

More recently, Zhu and colleagues reported that both apoptotic and necrotic cells released whole mitochondria in the extracellular compartment [141]. Strikingly, the authors observed that the mitochondria released by the two types of dying cells did not support the same systemic effects following their internalization in cultivated macrophages, with mitochondria from apoptotic cells generating more inflammation than those derived from necrotic cells. Mechanistically, cardiolipin was identified, in the recipient macrophages, as the DAMP responsible for the activation of the inflammasome, a multi-protein complex able to detect danger signals and to trigger the secretion of the pro-inflammatory IL-1α and IL-18 cytokines [141]. How mitochondrial cardiolipin exerts its pro-inflammatory effects in these settings remains to be formally demonstrated. One possible explanation suggested by the authors is that the mitochondrial cardiolipin was externalized from the inner to the outer mitochondrial membrane in the apoptotic mitochondria. This conformational change would allow the binding of cardiolipin to the inflammasome sensor NLRP3 (NOD-, LRR-, and pyrin domain-containing protein 3), as previously reported [144,145].

Besides their role as pro-inflammatory mediators, mitochondria released by damaged cells can also be involved in tissue repair processes, following an injury. In particular, we recently reported that the mitochondria transfer from apoptotic endothelial or cardiac cells to MSCs constitutes a signaling messenger that triggers a cytoprotective response in the recipient MSCs, consisting of the enhanced donation of mitochondria by MSCs towards damaged cells to rescue them [119]. Interestingly, this study underlined that two DAMPs, namely reactive oxygen species (ROS) and heme, mediate the effects of damaged mitochondria in the recipient MSCs. These two DAMPS are involved at different levels of the cascade of events leading to the activation of the MSCs. First, our findings indicated that the ROS produced by damaged cells are critical regulators of the transfer of the mitochondria from the damaged cells towards MSCs, as the use of ROS scavenger abrogated both the mitochondrial transfer from the injured cells to the MSCs and the resulting MSC rescuing function [119]. The role of ROS signaling as
modulator of mitochondria transfer and its associated effects have also been reported in the context of cancer, between leukemic cells and bone marrow-derived MSCs [146]. The molecular mechanisms whereby mitochondrial ROS produced by injured cells activate the cytoprotective functions of MSCs remain to be fully investigated. However, one possible mechanism is that ROS produced in excess by damaged mitochondria are sensed by MSCs as the signal triggering the degradation of dysfunctional organelles, as previously reported in other experimental settings [147,148]. The second DAMP involved in the activation of the recipient MSCs was the mitochondrial heme, released in the cytosol following the degradation of the transferred damaged mitochondria. In response to the increased level of cytosolic free heme, which has powerful pro-oxidant and toxic capacities, MSCs were found to enhance their expression of the heme oxygenase I (HO-1) enzyme to catalyze the degradation of heme, thus showing the role of HO-1 signaling in the stimulation of the pro-healing properties of MSCs [119].

7. Mitochondria and Microbiota: Two Sources of Metabolites for Cell Metabolism and Functions

7.1. Endosymbiotic Origin of the Mitochondria

Mitochondria and their host eukaryotic cells have a now well-documented endosymbiotic history showing the bacterial origin of the mitochondria. Over time, this endosymbiosis resulted in gene transfers between the mitochondrial and nuclear genomes [9,149]. As described above, this concerns the genes encoding the various subunits of the complexes of the electron transport chain, which are found in both the nucleus and the mitochondria. Many other mitochondrial proteins are encoded in the nucleus, translated in the cytoplasm, and transported to mitochondria where they exert their biological functions. Reciprocally, through the metabolites they produce, mitochondria have the capacity to exert retrograde controls on nuclear gene expression. Such is the case for alpha-ketoglutarate, 2-hydroxyglutarate, succinate, and fumarate, which regulate nuclear gene expression via the activity of DNA demethylases (Figure 3) [14].

7.2. Role of the Gut Microbiota in Physiology and Disease

Bacteria are actually present in mammals, in particular in their gut. The complex set of bacteria constituting the gut microbiota provides essential functions for the host mammalian body, as shown by numerous publications in this rapidly evolving area of research [150–154]. Its effects are not restricted to organs located near the gut, but extended to all organs as shown by liquid chromatography–tandem mass spectrometry (LC–MS/MS) of samples collected from 29 organs from either germ-free (GF) or specific-pathogens-free (SPF) mice [155]. The gut microbiota contributes to metabolically balanced physiological conditions, including the regulation of the immune system. The gut microbiota was found to alter the efficacy of immunotherapies based on the blockade of programmed cell death 1 (PD-1) or its ligand (PD-L1). These immune effects were observed for cancers whose primary sites were distant from the gut, like melanoma, non-small cell lung carcinoma (NSCLC), and renal cell carcinoma (RCC) [154]. In NSCLC and RCC patients, the presence of Akkermansia muciniphila in their gut microbiota was associated to a better response to PD-1 blockade [156]. Santoni and collaborators proposed that the role of A. muciniphila in promoting an immune response to anti PD-1 treatment could be attributed to the short-chain fatty acids (SCFAs), mainly acetate and propionate, produced by these bacteria. These SCFAs can activate the G-protein-coupled receptors (GPRCs), GPR41 and GPR43, with expected downstream effects on both cancer cell apoptosis and immune response [157].

The composition of the gut microbiota has also been recently tightly linked to malignancies. While the interaction of fecal bacteria with human colorectal cancers was expected, much less expected was the persistent association of bacteria like Fusobacterium nucleatum and its associated gram-negative microbiota with the colon cancer metastatic cells, at sites distant from the primary tumor. This was the case, for instance, for metastases in liver, which was associated with tumor progression [154,158]. As pointed out by the authors, the findings that the bacteria Fusobacterium travel with the primary tumor
cells to their metastatic sites suggest that tumor microbiota might constitute an essential component of the tumor microenvironment [158].

7.3. Role of the Short-Chain Fatty Acids (SCFAs) Secreted by the Microbiota

Outside from physiological conditions, the gut microbiota can also either support or mitigate the metabolic functions of mammalian cells, in pathological conditions or when under metabolic stress. A lot of the effects of the microbiota, through its secreted metabolites, actually go through the functioning of their “distant cousins, the mitochondria” as recently reviewed by Agrawal and collaborators [150].

An important part of the metabolites circulating in mammals are not produced by the mitochondria-containing mammalian cells, but instead originate from the commensal bacteria found in the gut. These microbiota metabolites are mainly produced through the bacterial metabolism of dietary products and host molecules. These microbiota metabolites include lactate and the short-chain fatty acids (SCFA) acetate, propionate, and butyrate, in roughly a 3:1:1 ratio [150,152]. They provide the means for the metabolic interactions between the gut microbiota and the human host and play a key role for the overall metabolism in physiological and pathological conditions, as well as for the response to therapy treatments [150–152,159].

Acetate can be processed to acetyl-CoA through the enzymatic activity of the nucleocytosolic acetyl-CoA synthetase 2 (ACSS2). The overexpression of ACSS2 was observed in diverse cancers including glioblastoma and brain metastases, hepatocellular carcinoma, and breast and colorectal cancers and correlated with tumor progression both in humans and in murine models [160–162]. ACSS2-dependant production of Acetyl-CoA is used by the cells for lipid synthesis and acetylation of histones [161]. Therefore, as expected, high levels of acetyl-CoA lead to the increased acetylation of histones and, consequently, to the expression of genes, including those involved in cell growth. When nutrients are fully available, the acetyl-CoA comes primarily from the citrate produced from the TCA cycle in the mitochondria. However, in glioblastoma and brain metastases, the amount of the acetyl-CoA originating from acetate and feeding in the TCA cycle was substantially increased [160]. As shown by Gottlieb and collaborators [162], acetate can be processed to acetyl-CoA in conditions of low oxygen and low lipid concentrations, and thus can provide an alternative carbon source for the synthesis of fatty acids and cholesterol. In these metabolically stressed conditions, the upregulated expression of ACSS2 was responsible for cancer cell growth and survival by increasing lipid biomass. Therefore, there too, the acetate produced by the gut microbiota is expected to play a determinant role in supporting lipid biosynthesis and cancer progression.

7.4. Role of Other Microbiota-Secreted Metabolites

Outside from the SCFA major classes of microbiota-secreted metabolites, other metabolites produced by the gut bacteria from dietary products can influence the host cell metabolism. Such is the case for isovanillic acid 3-O-sulfate (IVAS), a metabolite produced by the microbiota upon consumption of the cyanidin 3-O-glucoside found in berries and that is detected in the blood. Houghton and coworkers showed that this compound increased the uptake of glucose and the metabolism of the differentiated human skeletal muscle myoblast line LHCN-M2 through an increased concentration and activity of the glucose transporter GLUT4, along with an activated PI3K/AKT signaling [153].

8. Current Therapeutic Approaches and Clinical Trials for the Treatment of Mitochondria Dysfunctions

As outlined above, diverse biological processes can account for mitochondrial dysfunctions with their load of adverse biological consequences. From the knowledge of the biological mechanisms at play, at least four types of strategies emerge for keeping control of diseases linked to mitochondrial dysfunctions:

1. directly target and repair the genes, encoded either by the mitochondrial or nuclear genomes, which are responsible for the defects;
2. use whole mitochondria to restore metabolic activities;
3. supplement the unbalanced production of metabolites from the deficient mitochondria by adding purified metabolites;
4. exploit the diversity of metabolites produced by the gut microbiota and supplement with a subset of this microbiota to provide the missing metabolites.

A number of therapeutic strategies, with their related clinical trials, have been considered in the past few years to counteract mitochondrial dysfunctions and their associated pathologies [163–165]. These therapies have as goals to restore the functioning of the electron transport chain, to reduce the overall oxidative stress, to supply the defective metabolites, and to enhance mitochondrial biogenesis. For most of them, however, the sought-after objective is to alleviate the symptoms rather than permanently cure the disease. The more advanced (phase III) clinical trials target genetic diseases such as mitochondrial myopathies, pyruvate dehydrogenase complex deficiency (PDCD), and Leber’s hereditary optic neuropathy (LHON) [165].

8.1. Gene Therapy for Both Mitochondrial and Nuclear-Encoded Proteins

One example of transient gene therapy is the expression of the wild-type and functional subunit 4 of NADH dehydrogenase (ND4), via adeno-associated virus (AAV) vectors and by intravitreal injection, in patients suffering from LHON disease in connection with the point mutation G11778A in their mitochondrial ND4 encoding gene [166]. The allotropic expression of the mitochondria-targeted ND4 in the retinal ganglion cell nuclei was followed by the import of the mitochondria-tagged ND4 protein in the mitochondria-imbedded complex I. This process restored complex I function and ATP production and preserved the visual function, as shown in both murine and rat models [167,168]. Parkinson’s disease has been linked to mitochondria dysfunction and to defective mitophagy-dependent mitochondria clearance in the substantia nigra pars compacta. This is notably owing to the non-functional ubiquitin ligase Parkin and PTEN-induced putative kinase 1 (PINK1) in connection with the numerous mutations found in the corresponding PARK2 and PINK1 genes in patients with Parkinson’s disease [169–171]. The conditions for the viral delivery of the wild-type genes PARK2 and PINK1 in the brain have been set up in preclinical murine and rat models. However, conclusive preclinical data showing a therapeutic effect for Parkinson disease phenotypes are still lacking, thus presently precluding gene therapy clinical trials [169].

8.2. Therapy by Mitochondria Replacement

As detailed above, the intercellular transfers of mitochondria from MSCs to damaged recipient cells have been shown to promote beneficial wound healing effects in a wide range of pathophysiological conditions. As a result, novel mitochondria-targeted therapeutic approaches have emerged, consisting of the replacement of dysfunctional mitochondria in the injured/diseased organs through the transplantation of exogenous functional mitochondria. Mitochondrial dysfunction, leading to decreased ATP production, increased oxidative stress, apoptosis, and loss of tissue function, is the hallmark of aging and of numerous pathologies, including those caused by ischemia injury, mtDNA mutations, and metabolic disorders [172]. As a matter of fact, mitochondria transplantation has been successfully tested in several animal models for mitochondrial diseases. In particular, this therapeutic approach has been invariably shown to significantly reduce hypoxic/ischemic insult and restore tissue function following myocardial infarction [173–176], acute kidney injury [177], stroke [178,179], spinal cord injury [180,181], or optic nerve crush leading to glaucoma [182] by improving the bioenergetics and cell survival and by decreasing oxidative stress and mitochondrial DNA damages. Similarly, mitochondria transplantation has been reported to exert beneficial effects in animal models for either metabolic syndromes, including diabetic ischemic heart [183] and non-alcoholic fatty liver [184], or for neurological disorders, such as Parkinson’s disease [185,186] and schizophrenia [187]. Beyond mitochondrial disease treatment, the transplantation of exogenous mitochondrial has been evaluated in the attempt to mitigate mitochondrial dysfunction of cancer cells.
This approach has been shown to attenuate the Warburg effect and to enhance the sensitivity to anti-tumoral treatments (i.e., chemotherapy or radiotherapy) in breast cancer [188] or glioma cells [189].

Overall, and similar to what is observed in the rescue of damaged cells following intercellular mitochondria transfer from MSCs, studies testing the therapeutic efficacy of mitochondria transplantation indicate that mitochondria need to be functional to exert their beneficial effects [178,182,188]. Strikingly, mitochondria used for therapeutic purposes may be of autologous, allogeneic, or xenogeneic origin, because, in all the cases, transplanted mitochondria have been reported to be non-immunogenic [178,185–190]. Although the mitochondrial transplantation procedure remains largely experimental, this technique has been successfully applied in the damaged hearts of five pediatric patients sharing ischemia-reperfusion-associated myocardial dysfunction [191].

8.3. Therapy by Metabolites Supplementation

A number of antioxidant molecules, such as quinone derivatives, are currently used for patients with mitochondrial diseases [165]. A water-soluble analog of coenzyme Q10, idebenone, was reported to improve the ATP production in the affected retinal ganglion cells from LHON patients and to improve the disease symptoms. Current efforts are devoted to modify these compounds so as to reduce their lipophilicity, with the goal of increasing their bioavailability. However, safety issues also need to be monitored as anti-oxidants such as N-acetylcysteine (NAC) and vitamin E are also likely to promote tumor progression [165].

Supplementation with coenzyme Q10 may also be a therapeutic option for patients suffering from heart failure, whose severity is associated with reduced levels of CoQ10. There have been numerous clinical trials testing CoQ10 supplementation for heart failure patients, notably based on the anti-oxidant capacity of CoQ10 and on the assumption that it could support the production of ATP in the defective cardiomyocytes, through the activation of the electron transport chain [192]. The Q-SYMBIO latest trial and longer (two-year end-point) to date suggested that the supplementation with CoQ10 reduced cardiovascular death, but will definitely need to be confirmed owing to the small number of patients tested [192].

Still, despite all current clinical trials, there are no FDA-approved treatments for curing mitochondrial diseases or even mitigating their effects [165].

8.4. Therapy by Metabolite-Producing Microbiota

Depending on the properties of the metabolites produced by specific microbiota, which can be either beneficial or harmful for humans, the corresponding microbiota can be therapeutically supplemented to the patients, or instead specifically removed with adapted antibodies. Current clinical trials actually include the supplementation of the microbiota of cancer patients undergoing strong antibiotic treatments prior to bone marrow transplantation [159]. Microbiota supplementation can be performed either by heterologous or homologous fecal microbiota transplant (FMT). The latter possibility can be used, for instance, with the microbiota of patients that can be stored during the patient microbiota-damaging treatment, such as radio- or chemo-therapy, in order to prevent further microbiota-related therapeutic side effects [159].

9. Concluding Remarks

While merely considered as the powerhouse of the cells for a long time, mitochondria have provoked great enthusiasm in the scientific community in this past decade. A wide number of recent investigations have recognized mitochondria as essential hubs governing cell fate, through the regulation of their bioenergetics and the production of various metabolites. On top of that, mitochondria were shown to have the capacity to translocate between cells. As a result, these fascinating organelles control multifaceted biological processes including wound healing and inflammation and their dysfunctions are associated with a broad range of diseases including inherited mitochondrial disorders and metabolic diseases such as diabetes and cancer. The full understanding of the fine metabolic mechanisms that allow mitochondria to
exert their biological/signaling effects will undoubtedly need further investigations. However, the rapid scientific progress in this field highlight the key role played by the metabolites produced through the tightly interconnected ETC and TCA cycles and by mitochondrial components like DAMPs. Although additional research is needed to identify the specific metabolites/mitochondrial components responsible for the given effects of mitochondria, several therapeutic options can now be envisioned to treat mitochondrial diseases and cancer and to promote wound healing in injured/degenerative tissues, by focusing attention on whole mitochondria as well as on the metabolites/compounds they produce.

**Author Contributions:** Writing, J.N., A.-M.R. and M.-L.V. Figure design with BioRender.com, J.N. All authors have read and agreed to the published version of the manuscript.

**Funding:** J.N. is supported by a PhD fellowship from the French Ministry of Research. This work was supported by a grant from the Ligue Nationale contre le Cancer-Comité du Gard and from the Fondation ARC pour la recherche sur le cancer (M.-L.V.).

**Acknowledgments:** A.-M.R. and M.-L.V. are staff scientists from the National Institute for Health and Medical Research (INSERM) and the National Center for scientific research (CNRS), respectively.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>ACSS2</td>
<td>Acetyl-CoA synthetase 2</td>
</tr>
<tr>
<td>AGC</td>
<td>Aspartate-glutamate carrier</td>
</tr>
<tr>
<td>aKG</td>
<td>Alpha-ketoglutarate</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ALA</td>
<td>Lipoic acid (1,2-dithiolane-3-pentanoic acid or thiocic acid)</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>BCL-XL</td>
<td>B-cell lymphoma-extra large</td>
</tr>
<tr>
<td>CoQ10</td>
<td>Coenzyme Q10</td>
</tr>
<tr>
<td>CPEO</td>
<td>Chronic progressive external ophthalmoplegia</td>
</tr>
<tr>
<td>CPT1</td>
<td>Carnitine palmitoyltransferase 1</td>
</tr>
<tr>
<td>DAMP</td>
<td>Mitochondrial damage-associated molecular patterns</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxyguanosine triphosphate</td>
</tr>
<tr>
<td>DHODH</td>
<td>Dihydroorotate dehydrogenase</td>
</tr>
<tr>
<td>DNAJA4</td>
<td>Dnaj heat shock protein family (Hsp40) member A4</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FAO</td>
<td>Fatty acids beta-oxidation</td>
</tr>
<tr>
<td>FMT</td>
<td>Fecal microbiota transplant</td>
</tr>
<tr>
<td>FOXP3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>FPP</td>
<td>Farnesyl pyrophosphate (or diphosphate)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GF</td>
<td>Germ-free</td>
</tr>
<tr>
<td>GOT2</td>
<td>Glutamate-oxaloacetate transaminase</td>
</tr>
<tr>
<td>Gpx4</td>
<td>Glutathione peroxidase 4</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>2-HG</td>
<td>2-hydroxyglutarate</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HLRC</td>
<td>Hereditary leiomyomatosis and renal cell cancer</td>
</tr>
<tr>
<td>5hmc</td>
<td>5-hydroxymethylcytosine</td>
</tr>
<tr>
<td>HMGCR</td>
<td>HMG-CoA reductase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme oxygenase-1</td>
</tr>
<tr>
<td>Jmjc</td>
<td>Jumonji C</td>
</tr>
<tr>
<td>KDM</td>
<td>Histone lysine demethylase</td>
</tr>
<tr>
<td>KRT19</td>
<td>Keratin 19</td>
</tr>
<tr>
<td>KSS</td>
<td>Kearns–Sayre syndrome</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography–tandem mass spectrometry</td>
</tr>
<tr>
<td>LDHA</td>
<td>Lactate dehydrogenase A</td>
</tr>
<tr>
<td>LHON</td>
<td>Leber’s hereditary optic neuropathy</td>
</tr>
<tr>
<td>5mc</td>
<td>5-methylcytosine</td>
</tr>
<tr>
<td>MCL1</td>
<td>Induced myeloid leukemia cell differentiation protein</td>
</tr>
<tr>
<td>MEFs</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MELAS</td>
<td>Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes</td>
</tr>
<tr>
<td>MERRF</td>
<td>Myoclonic epilepsy with ragged-red fibers</td>
</tr>
<tr>
<td>MGMT</td>
<td>O-6-methylguanine-DNA methyltransferase</td>
</tr>
<tr>
<td>MIDD</td>
<td>Maternally inherited diabetes and deafness</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>MTHFD</td>
<td>Methylene tetrahydrofolate dehydrogenase</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NARP</td>
<td>Syndrome neuropathy, ataxia, and retinitis pigmentosa</td>
</tr>
<tr>
<td>ND4</td>
<td>NADH dehydrogenase subunit 4</td>
</tr>
<tr>
<td>nDNA</td>
<td>Nuclear DNA</td>
</tr>
<tr>
<td>NOX</td>
<td>Nicotinamide adenine dinucleotide phosphate oxidase</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small-cell lung carcinoma</td>
</tr>
<tr>
<td>2-OG</td>
<td>2-oxoglutarate</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP)-ribose polymerase</td>
</tr>
<tr>
<td>PC</td>
<td>Pyruvate carboxylase</td>
</tr>
<tr>
<td>PD1</td>
<td>Programmed cell death 1</td>
</tr>
<tr>
<td>PDAC</td>
<td>Pancreatic ductal adenocarcinoma</td>
</tr>
<tr>
<td>PDCD</td>
<td>Pyruvate dehydrogenase complex deficiency</td>
</tr>
<tr>
<td>PDH</td>
<td>Pyruvate dehydrogenase</td>
</tr>
<tr>
<td>PDK2</td>
<td>Pyruvate dehydrogenase kinase 2</td>
</tr>
<tr>
<td>PDM</td>
<td>Peridroplet mitochondria</td>
</tr>
<tr>
<td>PFKP</td>
<td>Phosphofructokinase</td>
</tr>
<tr>
<td>PGK1</td>
<td>Phosphoglycerate kinase</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl hydroxylase</td>
</tr>
<tr>
<td>P3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN-induced putative kinase 1</td>
</tr>
<tr>
<td>PPP</td>
<td>Pentose phosphate pathway</td>
</tr>
<tr>
<td>pVHL</td>
<td>Von Hippel–Lindau protein</td>
</tr>
<tr>
<td>RCC</td>
<td>Renal cell carcinoma</td>
</tr>
<tr>
<td>RISP</td>
<td>Rieske iron-sulfur protein</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short-chain fatty acid</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>SLC2A</td>
<td>Solute carrier family 2</td>
</tr>
<tr>
<td>SMAD</td>
<td>Mothers against decapentaplegic homolog 1</td>
</tr>
<tr>
<td>SNAIL</td>
<td>Snail family transcriptional repressor</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific pathogens-free</td>
</tr>
<tr>
<td>SPOCK</td>
<td>SPARC (Osteonectin), Cwcv, and Kazal like domains proteoglycan</td>
</tr>
</tbody>
</table>
STAT  Signal transducer and activator of transcription
TAG  Triacylglyceride
TCA  Tricarboxylic acid
TET  Ten-eleven-translocation
TGFβ  Transforming growth factor beta
TNT  Tunneling nanotube
Treg  Regulatory T cell
VEGF  Vascular endothelial growth factor

References

7. de Souza Breda, C.N.; Davanzo, G.G.; Basso, P.J.; Saraiva Cândara, N.O.; Moraes-Vieira, P.M.M. Mitochondria as central hub of the immune system. *Redox Biol.* 2019, 26, 101255. [CrossRef]
21. Sharma, P.; Sampath, H. Mitochondrial DNA Integrity: Role in Health and Disease. *Cells* 2019, 8, 100. [CrossRef] [PubMed]


43. King, M.; Attardi, G. Human cells lacking mtDNA: Repopulation with exogenous mitochondria by
59. Rutter, J.; Winge, D.R.; Schi
55. Amelio, I.; Cutruzzol
56. Ducker, G.S.; Rabinowitz, J.D. One-Carbon Metabolism in Health and Disease. *Cell Metab.* 2017, 25, 27–42. [CrossRef]
49. Iommarini, L.; Kurelac, I.; Capristo, M.; Calvaruso, M.A.; Giorgio, V.; Carelli, V.; Baracca, A.; Tallini, G.;
55. Amelio, I.; Cutruzzol
56. Ducker, G.S.; Rabinowitz, J.D. One-Carbon Metabolism in Health and Disease. *Cell Metab.* 2017, 25, 27–42. [CrossRef]
49. Iommarini, L.; Kurelac, I.; Capristo, M.; Calvaruso, M.A.; Giorgio, V.; Carelli, V.; Baracca, A.; Tallini, G.;


75. Loriot, C.; Chougnet, C.; et al. SDHB mutations are associated with response to temozolomide in patients with metastatic phaeochromocytoma or paraganglioma. Int. J. Cancer 2014, 135, 2711–2720. [CrossRef]


86. Diebold, L.P.; Gil, H.J.; Gao, P.; Martinez, C.A.; Weinberg, S.E.; Chandel, N.S. Mitochondrial complex III is necessary for endothelial cell proliferation during angiogenesis. Nat. Metab. 2019, 1, 158–171. [CrossRef]


120. Pollara, J.; Edwards, R.W.; Lin, L.; Bendersky, V.A.; Brennan, T.V. Circulating mitochondria in deceased organ donors are associated with immune activation and early allograft dysfunction. *JCI Insight* 2018, 3. [CrossRef]
143. Islam, M.N.; Das, S.R.; Emin, M.T.; Wei, M.; Sun, L.; Westphalen, K.; Rowlands, D.J.; Quadri, S.K.; Bhatcha

**Int. J. Mol. Sci. 2020, 21, 4405**
152. Cani, P.D.; Van Hul, M.; Lefort, C.; Depommier, C.; Rastelli, M.; Everard, A. Microbial regulation of organismal energy homeostasis. Nat. Metab. 2019, 1, 34–46. [CrossRef]


CHAPTER 3:
GLIOBLASTOMA
1. **Introduction**

Glioblastoma is the most aggressive malignant brain neoplasm of glial lineage and the most prevalent central nervous system tumor overall (Darlix et al., 2017; Ostrom et al., 2019). In spite of the many advances towards a better understanding of its etiology, glioblastoma remains a conundrum in neuro-oncology (Aldape et al., 2019). The current standard of care for newly-diagnosed glioblastomas, the Stupp protocol (Stupp et al., 2005, 2009), consisting of maximal safe resection followed by radiotherapy with concomitant and adjuvant temozolomide chemotherapy, has increased the patients’ overall median survival estimates from 12.5 months (range 2.3 – 28) to 15.6 months (range 3.8 – 29.6) (Marenco-Hillembrand et al., 2020), with a median progression-free survival of 7.4 months (Kelly et al., 2017). However, glioblastoma management has remained unchanged for over a decade and fails to tackle the complex facets of the tumor. First, glioblastoma cells display a highly invasive nature and an unmatched infiltrative capacity into the surrounding brain parenchyma, thus making the maximal surgical resection unattainable. Second, the tremendous inter- and intra-tumoral heterogeneity within the glioblastoma microenvironment, along with the intricate interactions between cancer cells and their neighboring counterparts, are still widely underestimated and make it challenging to eradicate cancer cells. As a result, and despite the aforementioned aggressive first-line treatments, recurrence almost inevitably occurs, for which there is still no standard treatment protocol to date (Perrin et al., 2019) (Figure 1).

**Epidemiology**

According to the Central Brain Tumor Registry of the United States – which provides the most updated, population-based data on primary brain and other central nervous system tumors in the United States – glioblastoma accounted for 48.3% of all primary malignant brain tumors and for 57.3% of all gliomas. Glioblastoma was most common in older adults, with a median of 65 years old at diagnosis, and its incidence increased with age, with rates highest in individuals of 75 – 84 years old. Glioblastoma had the highest average annual age-adjusted incidence for malignant tumors (3.22 per 100,000 people), along with the lowest five-year relative survival (6.8%) (Ostrom et al., 2019). According to the French Brain Tumor Database, the national histological database of all primary central nervous system tumors in Metropolitan France, glioblastoma made up 49.3% of all neuroepithelial tissue tumors and
54.8% of all gliomas. The median age at diagnosis was 63 years old, and the average annual age-adjusted incidence rate was 3.33 per 100,000 people (Darlix et al., 2017). Both these epidemiological reports, however recent, were still based on the 2007 World Health Organization Classification of Tumors of the Central Nervous System (Louis et al., 2007), in which the sole consideration of histopathological criteria does not fully illustrate the brain tumor heterogeneity. The advent of the recently-revised classification (Louis et al., 2016), in which the inclusion of genetic and molecular features into the diagnosis of central nervous system tumors established their relevance in prognostic evaluation and disease management, should also be met with a new era of updated epidemiological studies.

**FIGURE 1. TREATMENT AND TUMOR RECURRENTENCE IN GLIOBLASTOMA.**

Upper panel: MRI scans of primary glioblastoma before treatment, after treatment following the Stupp protocol, and after recurrence. Middle panel: Cartoon rendering of clonal glioblastoma subpopulations at each of the three steps. The primary tumor is made up of several heterogeneous subclones. Tumor resection removes the tumor burden, and post-operative radio- and chemo-therapy manage to further eliminate some remaining subclones. However, a small fraction of tumor cells thwart therapy and generate a secondary tumor. Lower panel: Phylogenetic tree representation of the clonal evolution at each of the three steps. Line lengths are proportional to the number of mutations acquired between each clone. Branching represents the acquisition of divergent mutations. RISC: recurrence-initiating stem cell. (Osuka and Van Meir, 2017)
2. **Glioblastoma Classification**

Histologically similar glioblastomas might nevertheless elicit varying clinical outcomes and inconsistent responses to therapy. This could be partially owing to the glioblastoma heterogenous molecular patterns. Therefore, a thorough understanding of the molecular mechanisms underlying glioblastoma tumorigenesis is a requisite in order to design novel diagnostic and therapeutic strategies. Harnessing these breakthroughs could subsequently pave the way towards personalized medicine.

In this regard, several seminal efforts, based on large-scale and single-cell genomic and/or epigenomic profiling, defined glioblastoma classification into genetic-, transcription- or methylation-based subtypes (Lee et al., 2018; Stoyanov and Dzhenkov, 2018) (Figure 5).

2.1. **Genetic Subclasses**

Glioblastoma was the first cancer to be exhaustively analyzed by The Cancer Genome Atlas Research Network (The Cancer Genome Atlas Research Network, 2008). This comprehensive analysis revealed a large number of mutations in core tumor suppressor genes and oncogenes, as well as structural chromosomal rearrangements illustrated by somatic copy-number aberrations. Constructing an integrated view of the frequent genetic alterations in glioblastoma revealed a highly interconnected aberration network, converging in three critical signaling pathways, namely (a) RTK/RAS/PI3K, (b) p53 and (c) RB pathways (Figure 2).

(a) RTK/RAS/PI3K pathway aberrations were observed in 88% of glioblastoma samples, through *EGFR* mutation or amplification (18%), *NF1* mutation or deletion (21%), *PTEN* mutation or deletion (33%), *PIK3R1* mutation (10%) and *PIK3CA* mutation (7%).

(b) p53 pathway was shown to be affected in 87% of glioblastoma samples, including *CDKN2A* deletion (chromosome 9), *MDM2* amplification (chromosome 12) and *TP53* mutation or deletion (42%).

(c) RB pathway was altered in 77% of glioblastoma samples, via *CDKN2A/B* deletion (chromosome 9), *CDK4* amplification (chromosome 12), *CDK6* amplification (chromosome 7) and *RB1* mutation or deletion (11%).
The Cancer Genome Atlas scope was later expanded with the aim of constructing an even more elaborate landscape of somatic genomic alterations in glioblastoma (Brennan et al., 2013). On top of the previously-reported alterations, novel mutations were identified, such as PDGFRA (13%) belonging to the RAS/PI3K signaling pathway. Most importantly, they identified two distinct TERT mutations (60% and 24%) in glioblastoma samples, that were mutually-exclusive with ATRX mutations (the remaining 16%), suggesting a telomere maintenance activity either through TERT-mediated telomerase reactivation or ATRX-mediated telomere lengthening.

**FIGURE 2. CORE SIGNALING PATHWAY ALTERATIONS IN GlioBLASTOMA.**
Represented are the primary sequence aberrations and significant copy number alterations for the RTK/RAS/PI3K (a), p53 (b), and RB (c) signaling pathways. Red indicates an activating genetic alteration. Conversely, blue illustrates an inactivating alteration. Deeper color shades represent a higher alteration frequency. (The Cancer Genome Atlas Research Network, 2008)
2.2. TRANSCRIPTIONAL SUBCLASSES

An influential work by Verhaak et al. (Verhaak et al., 2010) classified glioblastoma tumors, based on an unsupervised clustering of their gene expression profiles in The Cancer Genome Atlas databases, into four distinct molecular subtypes: Classical, Mesenchymal, Pro-neural and Neural. Integrating these transcriptomic data with the previously-mentioned genomic classification (section 2.1.) revealed that these four subtypes were defined by unique somatic mutations and DNA copy-number aberrations. Interestingly, the expression patterns of the four glioblastoma subtypes were reminiscent of distinct neurodevelopmental cell types (FIGURE 3):

(a) The classical subtype was defined by a high-level amplification of EGFR with, in tandem, a lack in TP53, IDH, RB and PDGFRA mutations. Moreover, the classical subtype was characterized by a Notch and Sonic Hedgehog pathway activation. It also exhibited expression patterns strongly associated with an astrocytic signature.
(b) The mesenchymal subtype was predominantly defined by a NF1 deletion, in addition to AKT and NF-κB pathway activation. The mesenchymal subtype was also heavily enriched in astroglial signature.
(c) The pro-neural subtype displayed two major features: PDGFRA amplification and IDH1 point mutations, accompanied by TP53 loss or inactivation. The pro-neural subclass was enriched in oligodendrocytic signature.
(d) The neural subtype was recently proposed to arise from contaminations by normal neuronal cells, thus refining the molecular subtypes based on transcriptomic profiles to three instead of four (Wang et al., 2017).

Additionally, these subtypes hold an undeniable clinical relevance as they exhibited significantly different responses to standard-of-care treatments. The pro-neural subgroup proved to be associated with longer survival outcomes compared to other subgroups. However, both the classical and mesenchymal subtypes were more likely to benefit from aggressive therapy (Verhaak et al., 2010).
2.2.1. Implication in Glioblastoma Tumor Heterogeneity

Verhaak’s classification was adopted by a large number of studies, which viewed each individual glioblastoma tumor as belonging to one and only specific subtype. Nevertheless, recent studies challenged this idea, as they argued that glioblastoma tumors simultaneously encompassed all four different cellular states, and that Verhaak’s glioblastoma subtypes primarily reflected the most abundant cellular state within the tumor bulk, rather than its exclusive presence (Patel et al., 2014; Neftel et al., 2019). This discovery was made possible with the advent of single-cell RNA-sequencing, that revolutionized our understanding of complex biological systems, as opposed to standard bulk RNA-sequencing where only the strongest, most predominant signal is observed. In this sense, Neftel and colleagues elegantly documented that each individual glioblastoma tumor mapped into at least three distinct molecular subtypes as defined by Verhaak (Neftel et al., 2019). The inter-tumoral relative frequency of each subtype, however, considerably varied with mainly one dominant subpopulation in each tumor. Furthermore, these different cellular states were tightly linked to neurodevelopmental signatures. For instance, high-level EGFR amplification was associated with astrocyte-like (AC-like)-abundant tumors whereas high-level PDGFRA
amplification was observed in oligodendrocyte progenitor-like (OPC-like)-rich tumors. Notably, lineage tracing experiments showed that glioblastoma cells were capable of transitioning between the different cellular states, thus highlighting their tremendous plasticity (FIGURE 4).

**FIGURE 4. CELLULAR HIERARCHIES AND STEM-CELL MARKERS OF GliOBLASTOMA CELLULAR SUBTYPES.**
A. Single-cell RNA-sequencing identified that glioblastoma tumors primarily consisted of four malignant and highly plastic cellular states (as represented by full arrows). B. A representation of established glioblastoma stem cell marker density in each glioblastoma cellular state. (Suvà and Tirosh, 2020)

### 2.3. METHYLATION-BASED SUBCLASSES

Glioblastoma clustering based on DNA methylation profiles identified a distinct hypermethylated subtype, dubbed G-CIMP (Glioma-CpG island methylator phenotype) (Noushmehr et al., 2010). Hypermethylated tumors mostly belonged to the pro-neural subtype and were associated with longer patient survival. Out of the hypermethylated loci, the promoter of the *MGMT* gene, encoding the O\(^6\)-methylguanine-DNA methyltransferase (*MGMT*), was found to be methylated in 45% of glioblastomas. MGMT reverses the temozolomide-mediated DNA alkylation, thus rendering glioblastoma cells resistant to temozolomide chemotherapy. Therefore, *MGMT* silencing was associated with a prolonged overall and progression-free survival (Noushmehr et al., 2010). In this respect, *MGMT* promoter hypermethylation is considered a prognostic and a predictive marker of chemotherapy success.
Glioblastoma displays distinct molecular subtypes that differ in their genetic and epigenetic landscapes, and that are associated with specific transcriptional signatures reminiscent of neurodevelopmental cells. On this basis, glioblastoma can be classified into three main subtypes: pro-neural, mesenchymal and classical. Pro-neural glioblastoma exhibits an oligodendrocyte-like expression profile, along with PDGFRA overexpression and characteristic IDH1 mutations. Both mesenchymal and classical glioblastoma cells resemble astrocyte-like gene expression profiles. Mesenchymal glioblastoma is characterized by NF1 mutations and NF-κB activation. Classical glioblastoma, on the other hand, exhibits wild-type TP53 accompanied by EGFR mutation/amplification. On another note, IDH1 mutations dictate the CpG island methylator phenotype (G-CIMP) and, thus, hold prognostic and predictive relevance to temozolomide treatment efficiency. G-CIMP high tumors are associated with a better prognosis and longer survival than G-CIMP low tumors. Noteworthily, MGMT methylation, in conjunction with 1p/19q deletion, were described to sensitize glioblastoma cells to temozolomide. (DeCordova et al., 2020)
3. GLIOBLASTOMA STEM CELLS

3.1. GLIOBLASTOMA STEM CELL DISCOVERY

The first report describing the existence of cancer stem cells within brain tumors dates back to 2003, when Singh and colleagues isolated “Brain Tumor Stem Cells” from patient-resected tumors based on CD133 expression, a neural stem cell surface marker. Functionally, these CD133+ cells formed neurospheres in vitro and were endowed with an extensive proliferation capacity and an impressive self-renewal potential. Most importantly, CD133+ cells were multipotent, as they differentiated in culture into cells of various lineages, phenotypically resembling the tumor of origin (Singh et al., 2003). Remarkably, CD133+ cells were the only brain tumor fraction that exhibited in vivo tumorigenicity – a tremendous one for that matter – in immunodeficient mice xenografts. As few as 100 CD133+ cells generated a phenocopy of the parental tumor when injected into mice brains, whereas 100,000 CD133- cells did not develop any detectable tumor mass (Singh et al., 2004).

In addition to their tumor-initiating ability, subsequent studies provided strong evidence for the implication of glioblastoma stem cells in therapy resistance. For instance, the fraction of CD133-expressing glioblastoma cells was shown to be enriched after ionizing radiation, both in cell culture and in immunocompromised mice brains. CD133+ cells survived radiation doses that were lethal for CD133− cells, owing to a preferential DNA damage checkpoint response activation and to an increased DNA repair capacity (Bao et al., 2006). Moreover, primary CD133+ glioblastoma cell resistance to chemotherapy by temozolomide, carboplatin and paclitaxel, was attributed to an increased expression of drug efflux pumps (Liu et al., 2006).

3.2. GLIOBLASTOMA STEM CELL PLASTICITY

Functional flexibility is paramount in the cancer stem cell concept, in which a select subpopulation of tumor-promoting cells, endowed with stem-like characteristics, yields offspring that undergo expansion and differentiation in order to form the tumor bulk. While the cancer stem cell model increased our understanding of tumor evolution and intracellular heterogeneity, it also raised important questions concerning the dynamics within the cellular hierarchy, in other terms, the relationship between cancer stem cells and their differentiated progeny.
Amongst the factors regulating the self-renewal of glioblastoma-initiating cells, the ERK/miR-18a*/NOTCH-1 feed-forward loop was correlated with stemness, both in vitro and in vivo, by turning on the Sonic Hedgehog-NANOG regulatory network (Turchi et al., 2013). Moreover, comparing epigenetic signatures and gene expression profiles from stem-like, tumor-promoting glioblastoma cells (TPCs) versus differentiated glioblastoma cells (DGCs), Suvà and collaborators elegantly identified four transcription factors that are a requisite for the TPC state, namely POU3F2 (POU Class 3 Homebox 2), SALL2 (Spalt Like Transcription Factor 2), SOX2 and OLIG2. Strikingly, expressing these four factors in DGCs was able to reprogram DGCs into TPCs. This process, known as dedifferentiation, demonstrated a bidirectional plasticity and an interconvertibility between TPCs and DGCs through epigenetic reprogramming, rather than a unidirectional hierarchy (Suvà et al., 2014; Gronych et al., 2014; Nawy, 2014). Dedifferentiation of glioblastoma cells was also recently shown to depend on EGFR/ERK-mediated miR-199a-3p repression and EGR1 expression, as evidenced by their acquisition of stem-like properties such as stemness marker expression and tumorigenic potential (Almairac et al., 2020). Interestingly, glioblastoma cell dedifferentiation was observed following exposure to ionizing radiation in a survivin-dependent fashion. Considering that glioblastoma stem cells are at the core of therapy resistance, this dedifferentiation could potentiate glioblastoma recurrence after radiotherapy (Dahan et al., 2014).

3.3. Glioblastoma Stem Cell Isolation and Enrichment in Vitro

"Ce qui est simple est toujours faux. Ce qui ne l’est pas est inutilisable." – Paul Valéry

Ever since their discovery, efforts have been made in the fields of glioblastoma stem cell identification, isolation and propagation. To date, glioblastoma stem cell enrichment strategies are widely similar to those of their normal counterparts, i.e., of neural stem cells. These strategies consist of fluorescence- or magnetic-activated cell sorting based on specific cellular markers. The panel of glioblastoma stem cell isolation markers has also been extensively appropriated from normal neural stem cells. These include cell surface markers such as CD133, CD15, CD44, CD90, A2B5 and L1CAM, as well as intracellular proteins like SOX2, OLIG2, MYC, NESTIN and NANOG (Brescia et al., 2012; Guichet et al., 2013; Virolle, 2017; Gimple et al., 2019; Vieira de Castro et al., 2020).
Isolated glioblastoma stem cells are then grown in vitro in serum-free, growth factor-rich media, auspicious for stem cell discriminability, as one of three in vitro cultures: (1) neurospheres in non-adherent conditions (Dirks, 2008), (2) two-dimensional adherent cultures on poly-lysine/laminin-coating (Pollard et al., 2009) or (3) three-dimensional organoids (Hubert et al., 2016; Jacob et al., 2020; Pine et al., 2020) (FIGURE 6).

FIGURE 6. SOURCES AND IN VITRO CULTURE OF GlioBLASTOMA STEM CELLS.
Glioblastoma tissues can be resected from rodent models or patients (adult or pediatric). Glioblastoma stem cells are then isolated and maintained in culture in serum-free, growth factor-rich (EGF, FGF2) media. Glioblastoma stem cells can be expanded in suspension as neurospheres or organoids, as well as in an adherent monolayer. Clonal cell lines can be obtained, which allows for arrayed genetic or chemical screens. Moreover, glioblastoma stem cells can be engrafted on brain slices in order to mimic tumor-host interactions. (Robertson et al., 2019)

Neurosphere-forming assays are the most widely used glioblastoma stem cell cultures; notwithstanding, they present major caveats. First, only a small cell percentage within a neurosphere are bona fide glioblastoma stem cells, whereas the majority undergo spontaneous differentiation and/or apoptosis during serial passages. Thus, neurospheres do not reflect the accurate cell number endowed with true stemness or, for that matter, with
real *in vivo* tumor-initiating capacity. Second, neurosphere cultures inherently create a gradient of access to oxygen, nutrients and growth factors, to such an extent that the neurosphere center may become necrotic. Adherent cell monolayers allow to address both these issues; however, they fail to faithfully depict the tumor architecture and its heterogeneity. Recently, cerebral organoids have emerged in the field as a better alternative for *in vitro* cultures and as a substitute for *in vivo* models. Organoids are complex models that enable the growth of glioblastoma stem cells co-cultured with a wide range of other key players of the tumor microenvironment, thus properly imitating important features of the tumor bulk.

### 3.4. Glioblastoma Stem Cell Models *In Vivo*

"*All models are wrong but some are useful.*" – George E. P. Box

Whole-animal models are pivotal for glioblastoma research breakthrough, especially for preclinical testing of novel therapies; however, the lack of models that fittingly depict the biology of glioblastoma is still a major deterrent.

Autochthonous models are genetically-engineered models obtained by inducing genetic alterations *de novo* and, hence, portray the pathophysiological relevance of tumor initiation. Alternatively, glioblastoma stem cells, isolated beforehand, can be engrafted in recipient animals. The implantation can be carried out in the brain, i.e., in the organ relevant to the disease (orthotopic model), or in a more accessible location, typically in the flank (heterotopic model). The latter, however, is generally discouraged, because it does not adequately recapitulate the brain tumor microenvironment and its infiltrative nature. On another note, while xenograft models (i.e., the host and the implanted glioblastoma stem cells present different genetic backgrounds) require immunocompromised hosts, syngeneic models (i.e., the host has the same genetic background as the implanted glioblastoma stem cells) are a great asset to study the immune system implication in glioblastoma biology (Broekman et al., 2018; Gargiulo, 2018; Robertson et al., 2019).
In spite of their advantages, the aforementioned animal models inherently require preliminary glioblastoma stem cell culture – and hence, selection – *in vitro* and may lack human-specific glioblastoma characteristics. An elegant approach to bypass the preceding cell culture bias and to focus on patient-oriented glioblastoma modeling is to develop patient-derived orthotopic xenografts (PDX). PDX models are generated by transplanting freshly-resected patient biopsies into host brains, excluding any prior *ex vivo* culture. PDX models thus enable the existence of clonally-heterogenous cell populations, reminiscent of the parental tumor (Broekman et al., 2018; Gargiulo, 2018; Robertson et al., 2019).

Choosing the adequate glioblastoma animal model relies on a trade-off between accurately portraying the tumor complexity and obtaining clear, undisputable outputs. It is therefore highly dependent on the asked question.

### 3.5. Glioblastoma Stem Cell Model Limitations

While glioblastoma stem cell identification propelled the neuro-oncology research field forward and played an important role in designing therapeutic strategies to selectively eradicate these peculiar cells, it is equally as important to comprehend the limitations of the glioblastoma stem cell model (Gimple et al., 2019). First, the term glioblastoma “stem cell” does not allude, in any way, to the glioblastoma cell of origin (Box 1). Second, glioblastoma stem cells should not be solely defined on the basis of cell surface or other cellular marker expression, as these markers are not prerequisite nor specific to glioblastoma stem cell populations. CD133⁻ glioblastoma cells were in fact described, in some instances, to exhibit stem-like and tumorigenic features, albeit to a lesser extent than CD133⁺ cells (Beier et al., 2007; Joo et al., 2008); on the other hand, some CD133⁺ glioblastoma cells lacked stem-like characteristics (Ding et al., 2013). Third, the nomenclature of glioblastoma stem cells does not preclude their plastic nature. Glioblastoma stem cells can exist at different stages on the highly dynamic plasticity spectrum, which allows the interconversion between glioblastoma stem cells and more differentiated glioblastoma cells, depending on microenvironmental cues including, but not limited to, oxygen levels, nutrient availability, pH and neighboring cells.
The aforementioned limitations call for a series of functional criteria in order to confirm the glioblastoma stem cell identity, by demonstrating (1) their capacity to extensively proliferate and self-renew, (2) their propensity to initiate tumors upon serial transplantations and (3) their faculty to mimic the parental tumor heterogeneity (Figure 7).

**Figure 7. Functional Features of Glioblastoma Stem Cells.**
Glioblastoma stem cells are characterized on the basis of functional criteria such as sustained self-renewal, persistent proliferation and, most importantly, tumor-initiation ability upon secondary transplantation. Nevertheless, glioblastoma stem cells also share features with non-neoplastic stem cells including rare frequency, stem cell-marker expression and pluripotency. (Lathia et al., 2015)
The precise glioblastoma cell of origin remains ambiguous and controversial. Recent reports, however, seemed to favor neural stem cells, an immature cell population typically localized in the subventricular zone, that shares conspicuously similar transcriptional profiles with glioblastoma stem cells. These reports suggested that neural stem cells may be early precursors of glioblastoma onset (Alcantara Llaguno et al., 2015; Duan et al., 2015; Pollen et al., 2015; Bardella et al., 2016; Matarredona and Pastor, 2019). Whole-exome and single-cell sequencing of human glioblastoma tissues and their corresponding subventricular zone tissues revealed that normal subventricular zone tissues, far from the tumor site, harbored low-level glioblastoma driver mutations that were highly present in their matching tumors (Lee et al., 2018). These driver mutation-bearing neural stem cells were reported to migrate from the subventricular zone and to develop high-grade, malignant gliomas in distant brain regions, thus providing compelling evidence concerning the glioblastoma cell of origin (Lee et al., 2018; Zarco et al., 2019). In this model, transformed subventricular zone-derived neural stem cells give rise to a small population of self-renewing, multipotent glioblastoma stem cells that maintain tumor propagation and heterogeneity.

**Box 1. Glioblastoma Cell of Origin**

The precise glioblastoma cell of origin remains ambiguous and controversial. Recent reports, however, seemed to favor neural stem cells, an immature cell population typically localized in the subventricular zone, that shares conspicuously similar transcriptional profiles with glioblastoma stem cells. These reports suggested that neural stem cells may be early precursors of glioblastoma onset (Alcantara Llaguno et al., 2015; Duan et al., 2015; Pollen et al., 2015; Bardella et al., 2016; Matarredona and Pastor, 2019). Whole-exome and single-cell sequencing of human glioblastoma tissues and their corresponding subventricular zone tissues revealed that normal subventricular zone tissues, far from the tumor site, harbored low-level glioblastoma driver mutations that were highly present in their matching tumors (Lee et al., 2018). These driver mutation-bearing neural stem cells were reported to migrate from the subventricular zone and to develop high-grade, malignant gliomas in distant brain regions, thus providing compelling evidence concerning the glioblastoma cell of origin (Lee et al., 2018; Zarco et al., 2019). In this model, transformed subventricular zone-derived neural stem cells give rise to a small population of self-renewing, multipotent glioblastoma stem cells that maintain tumor propagation and heterogeneity.
4. **Glioblastoma Microenvironment**

Despite the term “multiforme” being dropped from the latest World Health Organization classification of central nervous system tumors, few solid tumors are as histologically “multiforme” as glioblastomas. The term “multiforme” is nonetheless still used in common vocabulary as a testament to the extent of histological varieties, as well as of inter- and intratumoral heterogeneities, of glioblastoma (Quail and Joyce, 2017; Perrin et al., 2019). In fact, glioblastoma cells grow in a dynamically remodeled microenvironment in which they manage to hijack surrounding cells, resident and infiltrating alike, for their own benefit (Figure 8). Strikingly, glioblastoma hardly ever disseminates outside the central nervous system niche, as shown by the extremely rare occurrence of extracranial metastases (Lun et al., 2011). Glioblastoma thus seems to prosper in the unique intracranial microenvironment, where it interacts with its various neighboring components, tucked behind the blood-brain barrier and benefiting from the privileged, semi-protected immune state of the brain.

![Figure 8. Heterogeneity of the Glioblastoma Tumor Microenvironment.](image)

Glioblastoma cells are embedded in a heterogenous microenvironment, not only composed of diverse non-neoplastic glial, stromal and immune cells, but also compartmentalized into distinct niches defined by their vasculature, metabolism and extracellular matrix. ECM, extracellular matrix; HA, hyaluronic acid; HAS, hyaluronan synthase; Hyal, hyaluronidase; MMP9, matrix metalloprotease-9; TAM, tumor-associated macrophages; VEGF, vascular endothelial growth factor. (Perrin et al., 2019)
4.1. **Brain Compartment**

Glioblastoma cells are intermingled with local astrocytes, oligodendrocytes and neurons (Figure 10).

**Astrocytes** play an important role in metabolic (glutamate) and fluid (ion and water) homeostasis as well as synapse formation and vascular blood flow. Importantly, astrocyte endfeet cover up to 99% of the brain cerebrovascular surface, thus contributing to the blood-brain barrier formation (Matias et al., 2018; Brandao et al., 2019). However, during glioblastoma genesis, invading cancer cells were shown to displace normal astrocytes from blood vessels, thereby disturbing the astrocyte-vascular interactions and rupturing the blood-brain barrier (Watkins et al., 2014). Additionally, astrocytes of the glioblastoma microenvironment were shown to undergo reactive astrogliosis, similar to that observed during central nervous system injury, whereby they secreted growth factors and cytokines that further promoted glioblastoma genesis, on top of becoming highly proliferative and invasive. For instance, reactive astrocyte-produced connective tissue growth factor (CTGF) exerted a paracrine effect on glioblastoma stem cells which activated their NF-κB signaling, thereby inducing ZEB1, an epithelial-to-mesenchymal transition transcription factor. Consequently, reactive astrocytes facilitated glioblastoma stem cell invasiveness (Edwards et al., 2011). Recently, reactive astrocytes were demonstrated to foster an immunosuppressive environment through the JAK/STAT-mediated secretion of anti-inflammatory cytokines, such as transforming growth factor beta (TGF-β), interleukin-10 (IL10) and granulocyte colony-stimulating factor (G-CSF). These results thus linked reactive astrocytes to glioblastoma immune evasion (Henrik Heiland et al., 2019).

**Neurons** have also been implicated in glioblastoma growth and invasion. Beside glioblastoma cell migration along axonal trajectories, neurons were shown to secrete neuroligin 3 (NLGN3), which exerted a mitogenic effect and promoted glioblastoma proliferation via PI3K/PTEN/AKT/mTOR pathway activation, both in culture and in patient-derived xenograft models. NLGN3 also induced NLGN3 feed-forward expression, which inversely correlated with patient survival (Venkatesh et al., 2015, 2017). On the other hand, neurons of the tumor-adjacent brain tissue were described to promote glioblastoma cell death. Neuronal
overexpression of programmed death-ligand 1 (PD-L1) was indeed shown to induce glioblastoma caspase-dependent apoptosis and, hence, was linked to favorable prognosis. Strikingly, neuronal PD-L1 expression was inversely correlated with that of glioblastoma cells, suggesting that PD-L1 expression in native brain neurons instigated a negative feedback signal that downregulated PD-L1 expression in tumor cells. Therefore, an impairment in such a regulatory pathway would foster glioblastoma immune surveillance evasion (Liu et al., 2013).

4.2. **Vasculature**

Glioblastoma is one of the most vascularized solid tumors. Extensive neo-angiogenesis as well as abnormal vasculature, as depicted by hyper-dilated and leaky vessels, are indeed a hallmark of glioblastoma, triggering brain oedema, interstitial pressure and inflammation. As a consequence, inconsistent oxygen delivery within the glioblastoma microenvironment leads to local hypoxic niches, which could develop into pseudopalisading necrosis, another hallmark of glioblastoma (Figure 9). These vasculature abnormalities were predominantly attributed to extremely high vascular endothelial growth factor (VEGF) levels (Dieterich et al., 2012). Strikingly, lineage tracing experiments in glioblastoma mouse models revealed that glioblastoma stem cells “supply their own blood” by trans-differentiating into pericytes and endothelial cells, thus becoming full-fledged vascular components (Ricci-Vitiani et al., 2010; Wang et al., 2010; Guichet et al., 2015).
**FIGURE 9. REGIONS OF THE GLIOBLASTOMA TUMOR MICROENVIRONMENT.**

(a) The necrotic core forms following extensive cell density increases or hypoxia-inducing vaso-occlusive events. (b) Pseudopalisades form following cell migration away from hypoxic niches, starting from the outer edge of cell-dense regions into healthy tissues at the infiltrating rim. (c) Glioblastoma is characterized by increased angiogenesis and hypervascularity; however, tumor-associated vasculature is poorly formed and leaky. (d) Glioblastoma cells invade along axon routes into the surrounding parenchyma. (e) Glioblastoma cells rapidly invade the perivascular niche to procure oxygen and nutrients. BBB, blood-brain barrier; ECM, extracellular matrix; TAM, tumor-associated macrophages. (Wolf et al., 2019)
4.3. **IMMUNE CELLS**

Although an insignificant amount of immune cells manage to infiltrate the brain parenchyma in healthy physiological conditions, immune cells are a substantial part of the glioblastoma microenvironment, remarkably constituting up to 40% of the tumor mass (Glass and Synowitz, 2014). Both macrophages and lymphocytes were observed in the glioblastoma microenvironment (**Figure 10**). This observation challenges the once-believed notion of an immune-privileged central nervous system, and rather underlines that an immune reaction within the brain is circumstantial.

**Macrophages** exist within the glioblastoma microenvironment as ontogenetically distinct populations. Flow cytometry analyses of human glioblastoma tissues indeed uncovered 40% of tumor-infiltrating macrophages and 20% of brain-resident microglia (Gabrusiewicz et al., 2016). Macrophages were long thought to exist along the linear M1 (pro-inflammatory, anti-tumoral) – M2 (anti-inflammatory, pro-tumoral) phenotypic spectrum; however, recent reports challenged this dogma. Increasing evidence indeed showed that macrophages were endowed with a dynamic phenotypic plasticity upon disease progression. Such was the case in orthotopic glioblastoma mouse models where M1 macrophages, that infiltrated the microenvironment at early stages of tumor development, underwent massive differentiation towards M2 macrophages in advanced stages of tumorigenesis (Ginhoux et al., 2016; De Vleeschouwe and Bergers, 2017). These pro-tumorigenic macrophages were shown to produce low levels of inflammatory cytokines and to lack T cell co-stimulation markers, which explained the poor T cell response in glioblastoma (Hussain et al., 2006). In addition, tumor-associated macrophages were shown to regulate glioblastoma stem cell pools, angiogenesis and invasion (Zhou et al., 2015; Quail and Joyce, 2017).

**Tumor-infiltrating lymphocytes** are less frequent than tumor-associated macrophages in the glioblastoma microenvironment. T cells were described to express LAG3, CD39, TIGIT and PD1, thus depicting an extremely exhausted phenotype (Woroniecka et al., 2018). Moreover, the vast majority of glioblastoma-infiltrating T cells were shown to be regulatory T cells, that promoted an immunosuppressive environment (Li et al., 2016).
The glioblastoma microenvironment is composed of diverse cellular components – ranging from infiltrating, peripherally-derived immune cells to specialized, resident cells – all of which contributing to tumor progression and resistance to therapy. (Quail and Joyce, 2017)

4.4. **Mesenchymal Stem Cells**

Contrary to most solid tumors, classical fibroblasts are not a substantive constituent of the glioblastoma microenvironment. Several reports, however, showed that mesenchymal stem cells were enriched in the glioblastoma perivascular niche (Bajetto et al., 2020; Clavreul and Menei, 2020) (Figure 11).

4.4.1. **Mesenchymal Stem Cell Recruitment to the Glioblastoma Microenvironment**

The recruitment of mesenchymal stem cells to the glioblastoma microenvironment was demonstrated in mouse xenograft models following orthotopic or systemic injection (Nakamizo et al., 2005; Doucette et al., 2011; Velpula et al., 2012; Shinojima et al., 2013; Behnan et al., 2014), and was further confirmed ex vivo (Behnan et al., 2014; Thomas et al., 2018; Sun et al., 2019).
The molecular mechanisms underlying the mesenchymal stem cell tropism to the glioblastoma microenvironment are not fully understood yet. Studies showed that glioblastoma secretion of TGF-β (Birnbaum et al., 2007; Shinojima et al., 2013), along with angiogenic factors including interleukin-8, neurotrophin-3 and vascular epithelial growth factor (Birnbaum et al., 2007), were responsible for the homing of mesenchymal stem cells to the tumor site. Several chemotactic factors were also shown to be implicated in this phenomenon, such as the monocyte chemoattractant protein-1 MCP-1/CCL2 and the stromal cell-derived factor-1 SDF-1/CXCL12 (Pavon et al., 2018; Thomas et al., 2018). Moreover, recruited mesenchymal stem cells were described to express the chemokine receptor CXCR4 (Behnan et al., 2014). Strikingly, mesenchymal stem cells of the glioblastoma microenvironment are not all homogenous and can thereby be classified into at least five distinct subtypes, regarding their origin, genetic pattern and marker expression: (1) those recruited from local brain sources, (2) those homed from the bone marrow, (3) those differentiated from glioblastoma stem cells, (4) those derived from an epithelial-to-mesenchymal transition of reactive astrocytes, and (5) those trans-differentiated from pericytes (Birnbaum et al., 2007; Behnan et al., 2014; Hossain et al., 2015; Svensson et al., 2017; Oliveira et al., 2018; Yi et al., 2018; Bajetto et al., 2020; Clavreul and Menei, 2020).

4.4.2. Role of Mesenchymal Stem Cells in Glioblastoma Progression and Resistance

Mesenchymal stem cells tightly communicate with other components of the tumor microenvironment, including glioblastoma stem cells – through both indirect (cytokines, exosomes and micro-vesicles) as well as direct (gap junctions and tunneling nanotubes) interactions – thereby actively participating in tumor progression and therapy resistance.

Mesenchymal stem cells appeared to enhance glioblastoma proliferation and tumorigenicity both in vitro and in vivo (Clavreul et al., 2012; Bourkoula et al., 2014; Behnan et al., 2014; Hossain et al., 2015; Figueroa et al., 2017; Oliveira et al., 2018; Tumangelova-Yuzeir et al., 2019; Sun et al., 2019; Salaud et al., 2020; Huang et al., 2020; Lim et al., 2020). For instance, subcutaneous injection into nude mice of mesenchymal stem cells isolated from the peritumoral region of human glioblastoma, together with U87MG glioblastoma cells, induced larger tumors than those generated by U87MG alone or together with non-glioblastoma-derived stromal cells (Clavreul et al., 2012). In addition, mesenchymal stem cells isolated from
human glioblastoma samples fostered glioblastoma stem cell proliferation and self-renewal in vitro, and increased their tumor-formation capacity when implanted in the flanks of nude mice (Hossain et al., 2015).

Mesenchymal stem cells were also shown to exert angiogenic functions within the glioblastoma niche (Kong et al., 2013; Clavreul et al., 2014; Zhang et al., 2018). Mesenchymal stem cells isolated from the glioblastoma peritumoral zone were indeed described to increase the number of small vessels when injected in the striatum of nude mice, in comparison with stromal cells derived from non-glioblastoma peripheral brain tissues. This angiogenic property was mediated by an increased secretion of angiogenic cytokines such as SDF-1/CXCL12 and HGF, and an overexpression of blood vessel-development proteins including CSPG4/NG2, CRYAB, CNN1, CALD1 and VASP (Clavreul et al., 2014). Recently, studies have shown that mesenchymal stem cells within the glioblastoma microenvironment existed in two distinct subpopulation, CD90\textsuperscript{high} and CD90\textsuperscript{low} (Svensson et al., 2017; Zhang et al., 2018). Interestingly, CD90\textsuperscript{low} mesenchymal stem cells were endowed with a strong angiogenic capacity, unlike their CD90\textsuperscript{high} counterparts, as evidenced by higher vessel formation and length. The angiogenic ability of CD90\textsuperscript{low} cells was correlated with an increased secretion of angiogenic factors, namely VEGF and IL-6 (Zhang et al., 2018).

Several reports also established a link between mesenchymal stem cells and glioblastoma invasiveness (He et al., 2018; Lim et al., 2017, 2018, 2020). For instance, glioblastoma-associated mesenchymal stem cells were shown to drive a pro-invasive extracellular matrix remodeling, through CCL2/JAK1-mediated collagen assembly and actomyosin contractility (Lim et al., 2018), as well as ERK/MAPK-mediated hyaluronic acid secretion (Lim et al., 2017). Recently, the same group demonstrated that mesenchymal stem cell-secreted complement component C5a increased ZEB1 expression through MAPK activation and, consequently, enhanced glioblastoma invasion into the surrounding parenchymal brain tissue (Lim et al., 2020).

In addition to their role in promoting tumor progression, mesenchymal stem cells also fostered glioblastoma drug resistance via the activation of the Wnt/β-catenin axis (Huang et al., 2020) and the IL-6/STAT3 pathway (Hossain et al., 2015). In this respect, the mesenchymal
stem cell percentage within the glioblastoma microenvironment was shown to augment following radiotherapy (Thomas et al., 2018) and was inversely correlated with patients’ overall survival (Bourkoula et al., 2014; Yoon et al., 2016; Shahar et al., 2017; He et al., 2018; Ius et al., 2018).

The host laboratory and others have previously demonstrated that mesenchymal stem cells have the propensity to interact through tunneling nanotubes and to transfer their mitochondria to glioblastoma stem cells in *in vitro* co-cultures (Nzigou Mombo et al., 2017; Salaud et al., 2020). However, the outcome of this mitochondria acquisition is still largely unknown. Regarding the aforementioned elements, mesenchymal stem cells are key players in glioblastoma aggressiveness, thus warranting further investigations.

**Figure 11. Mesenchymal Stem Cells in the Glioblastoma Tumor Microenvironment.**

Mesenchymal stem cells are an integral constituent of the microenvironment in both low-grade (LGG) and high-grade gliomas (HGG), where they are known as glioma-associated stem cells (GASCs). Mesenchymal stem cells may be recruited from the bone marrow or from local brain sources, and reside mostly around blood vessels. In culture, mesenchymal stem cells have a spindle-shaped morphology and adhere to plastic. They express characteristic surface antigens (CD73, CD90, CD105) and have the ability to differentiate into three cell types: adipocytes, chondrocytes and osteocytes. Moreover, they share phenotypic and functional properties with cancer-associated fibroblasts (CAFs), including FSP/S100A4 expression as well as soluble factor- and exosome-mediated tumor promotion. (Clavreul and Menei, 2020)
5. Glioblastoma Metabolism

Energetic metabolism deregulations are a hallmark of cancer (Hanahan and Weinberg, 2011). Glioblastoma metabolism has recently become a growing field of heightened interest, with the aim of identifying novel druggable metabolic biomarkers and developing innovative therapeutic approaches, in hopes to meet more success than those targeting genomic alterations. Like most cancers, glioblastoma is endowed with the faculty of remodeling its energetic metabolism for survival ends, namely ATP production, macromolecule biosynthesis and therapy evasion. In addition, glioblastoma stem cells are lodged in select niches, thus necessitating high metabolic plasticity in order to adjust to fluctuating oxygen levels and nutrient availabilities (Libby et al., 2018; Garnier et al., 2019; Zhou and Wahl, 2019) (Figure 12).

**Figure 12. Principal Metabolic Pathways of Glioblastoma Stem Cells.**
Glucose is taken up by GLUT transporters and is then converted into pyruvate through the glycolysis pathway. Glycolytic biproducts constitute precursors for other biosynthetic pathways such as the PPP as well as lipid and amino acid biosynthesis. Pyruvate is then either converted into lactate or into acetyl-CoA that fuels the TCA cycle. Glutamate is another key metabolite that can fuel the TCA cycle. Glutamate is also involved in many biosynthetic pathways, such as amino acid and lipid and glutathione biosynthesis. α-KG, α-ketoglutarate; FAS, Fatty acid synthase; FBP1, Fructose-1,6 bisphosphatase 1; GCL, glutamate-cysteine ligase; GLS, Glutaminase; GS, Glutamine synthetase; GLUT, Glucose transporter 1; HK2, Hexokinase 2; IDH, Isocitrate dehydrogenase; LDHA, Lactate dehydrogenase A; MCT, Monocarboxylate transporter; MK, Mevalonate kinase; OAA, Oxaloacetate; PC, Pyruvate carboxylase; PDH, Pyruvate dehydrogenase; PDK, Pyruvate dehydrogenase Kinase; PKM2, Pyruvate kinase M2; PPP, Pentose phosphate pathway; TCA, Tricarboxylic acid cycle. (Garnier et al., 2019)
5.1. **GLUCOSE METABOLISM**

Glucose, taken up by the cells through glucose transporters GLUT1 and GLUT3, is catabolized into pyruvate via a series of enzymatic reactions. Pyruvate acts as a major metabolic hub and a critical metabolic checkpoint. In fact, pyruvate can be converted into lactate by the lactate dehydrogenase (LDHA) and, subsequently, be shunted away from the Krebs cycle, which reflects a non-oxidative glucose metabolism. On the other hand, pyruvate can also be converted into acetyl coenzyme A (acetyl-CoA) by the pyruvate dehydrogenase (PDH) or into oxaloacetate by the pyruvate carboxylase (PC), in order to fuel and to replenish the Krebs cycle (also known as anaplerosis), respectively. Both these reactions belong to the glucose mitochondrial, oxidative metabolism.

Much like normal brain cells, glucose metabolism has been described to be significantly increased in glioblastoma (Flavahan et al., 2013). $^{13}$C-labelled nutrients in orthotopic murine models indeed demonstrated that glioblastoma stem cells used glucose carbons in order to produce both lactate as well as Krebs cycle metabolites, suggesting that glioblastoma stem cells perform both oxidative and non-oxidative glucose metabolisms (Marin-Valencia et al., 2012). These observations were further confirmed in $^{13}$C-glucose-infused glioblastoma surgical resections (Maher et al., 2012).

Glycolysis was demonstrated to be essential for glioblastoma tumorigenesis. Genome-wide transcriptomic analysis of patient-derived glioblastoma cells, as well as of glioblastoma stem cell lines, revealed a significant upregulation of glycolytic genes in hypoxic conditions, which contributed to their adaptation and survival, both in vitro and in vivo. Therefore, knockdown of several glycolytic genes including *PFKP*, encoding the phosphofructokinase 1 (PFK1), and *PDK1*, encoding the pyruvate dehydrogenase kinase 1 (PDK1), dramatically increased the survival of glioblastoma mouse xenografts (Sanzey et al., 2015). In this respect, glycolytic enzymes were often shown to be regulated by the activated oncogenic signaling pathways in glioblastoma. For instance, AKT activation, mediated by PTEN loss and EGFR-dependent PI3K activation, stabilized PFKP and upregulated its expression, both at the mRNA and protein levels, in human glioblastoma cell lines and in primary glioblastoma cells. Consequently, PFKP enhanced glycolysis, cell proliferation and tumor growth in mice models. In line with these
findings, PFKP expression in human glioblastoma specimens correlated with poor prognosis (Lee et al., 2017). Moreover, MYC overexpression in glioblastoma cell lines significantly increased the expression of their glycolytic enzymes, such as hexokinase 2 (HK2), pyruvate kinase isoform M2 (PKM2) and LDHA. This was accompanied by an increase in glucose dependence for cell growth. As a result, MYC-knockdown inhibited the expression of HK2, PKM2 and LDHA and significantly decreased cell proliferation (Tateishi et al., 2016). Finally, EGFR activation in glioblastoma cell lines was found to translocate PKM2 into the nucleus, where it mediated the c-Myc-dependent glycolysis activation and, in turn, cell proliferation. Accordingly, PKM2 expression in human glioblastomas inversely correlated with patient survival (Liang et al., 2016). Interestingly, treatment of C6 glioma cells with 7β-hydroxycholesterol was shown to reduce PKM affinity to its substrate, phosphoenolpyruvate, which preceded 7β-hydroxycholesterol-mediated cytostatic action (de Weille et al., 2013).

Taken together, these data illustrate the importance of the glycolytic pathway for glioblastoma growth and suggest that glycolysis inhibition may be of therapeutic value.

5.2. **KREBS CYCLE**

Although the aforementioned studies proposed glycolysis to be the major metabolic pathway in glioblastoma, others reported that glioblastoma stem cells preferably rely on oxidative phosphorylation and the Krebs cycle. For instance, glioblastoma stem cells were found to consume less glucose and to produce less lactate than their differentiated counterparts, hinting at a lower glycolytic metabolism. Glioblastoma stem cells were nevertheless able to achieve higher ATP levels, through enhanced mitochondrial respiratory and reserve capacities, suggesting that they mainly rely on oxidative phosphorylation for energy production. Noteworthily, this increased oxidative metabolism in glioblastoma stem cells was correlated with resistance to radiotherapy (Vlashi et al., 2011).

Interestingly, NMR microscopy on [U-¹³C] glucose-infused glioblastoma resections revealed that glycolysis-derived pyruvate accounted for less than 50% of the acetyl-CoA pool that fuels the TCA cycle (Maher et al., 2012). This observation suggests that other substrates contribute carbons to the TCA cycle intermediates in glioblastoma. Further studies within primary and
metastatic mouse orthotopic glioblastoma tumors demonstrated that it was actually acetate that contributed a significant carbon fraction into the Krebs cycle, through the action of acetyl-coA synthetase enzyme 2 (ACSS2). ACSS2 was consequently associated with a more aggressive glioma phenotype and its knockdown in glioblastoma cell lines resulted in cell death and neurosphere formation failure. Strikingly, circulating glutamine was not directly oxidized into the TCA cycle of glioblastoma cells. Instead, glioblastoma cells synthetized glutamine from astrocyte-produced glutamate, through the action of glutamine synthase (GS) (Mashimo et al., 2014).

5.3. **GLUTAMINE METABOLISM**

Glutamine enters the cell through its transporter ASCT2 (SLC1A5). It is then catabolized into glutamate via the action of glutaminase (GLS). Glutamate is a substantial metabolic hub: it can (a) replenish the TCA cycle intermediates through anaplerosis, (b) provide lipid biosynthetic precursors, (c) generate de novo reduced glutathione, and (d) participate in non-essential amino acid and nucleotide synthesis.

Glutamine concentrations were shown to be greater within the glioblastoma bulk as compared to the surrounding normal brain (Maher et al., 2012) and glutamine addiction has been proposed as a hallmark of glioblastoma (Obara-Michlewska and Szeliga, 2020). However, glutamine addiction has not been described in all glioblastoma cells. Recent studies, based on transcriptomic and metabolic phenotyping of primary glioblastoma cultures, indeed identified two glioblastoma subpopulations according to their glutamine usage: GLN$^{\text{high}}$ and GLN$^{\text{low}}$. Glutamine deprivation of GLN$^{\text{high}}$ cells hindered their in vitro and in vivo growth, notwithstanding their higher metabolic compensation by shuttling pyruvate into the TCA cycle. Interestingly, GLN$^{\text{high}}$ cells were shown to strictly belong to the mesenchymal glioblastoma subtype (Oizel et al., 2017).

As previously mentioned, circulating glutamine only marginally contributes to glioblastoma growth. Glutamine is rather supplied by astrocytes or synthetized in situ, from glutamate and ammonia, by glutamine synthase (GS)-expressing glioblastoma cells. GS expression inversely correlated with patient survival and was shown to be elevated in glioblastoma stem cells as
compared to their differentiated counterparts. In this case, GS-mediated glutamine synthesis did not replenish the TCA cycle through anaplerosis but, instead, fueled \textit{de novo} purine biosynthesis (Tardito et al., 2015).

Glutamine breakdown into glutamate, mediated by GLS, was also described to be essential for glioblastoma survival and therapy resistance. It was indeed demonstrated that increased GLS expression and, subsequently, glutamate levels, promoted glioblastoma resistance to mTOR inhibition. GLS inhibition synergistically potentiated the effect of mTOR inhibitors and reduced glioblastoma growth \textit{in vivo} (Tanaka et al., 2015).

5.4. **LIPID METABOLISM**

Lipids constitute an additional source of membrane building blocks and signaling molecules involved in malignant transformation, tumor progression and therapy resistance. In order to satisfy their needs, cancer cells can either take up exogenous lipids or perform \textit{de novo} lipogenesis from cytosolic acetyl-CoA. Cytosolic acetyl-CoA can either be generated from citrate \textit{via} ATP citrate lyase (ACLY), or from acetate \textit{via} acetyl-CoA synthetase (ACSS). Cytosolic acetyl-CoA is then carboxylated by acetyl-CoA carboxylase (ACC) to form malonyl-CoA, which in turn generates palmitate \textit{via} multi-step reactions catalyzed by fatty acid synthase (FASN).

\textit{De novo} lipogenesis was shown to be essential for glioblastoma growth and, as for glycolysis, it was described to be regulated by glioblastoma-relevant oncogenes and tumor suppressors. For instance, \textit{ex vivo} astrocyte malignant transformation following concomitant $BRAF^{V600E}$ introduction and $TP53$ and $PTEN$ deletion increased their ACSS2 expression compared to wild-type astrocytes, which elevated their cytosolic acetyl-CoA pool. ACSS2 was consequently associated with a more aggressive glioma phenotype and its knockdown in glioblastoma cell lines decreased their viability and neurosphere formation ability (Mashimo et al., 2014). In addition, glioblastoma cell lines expressing the constitutively-active $EGFR^{vIII}$ exhibited an enhanced \textit{de novo} lipogenesis and higher proliferation than their counterparts. Consequently, ACC inhibition, both genetic and pharmacological, blunted their \textit{de novo} lipogenesis and induced their apoptotic cell death (Jones et al., 2017). Similarly, FASN inhibition significantly
suppressed the de novo lipogenesis of glioma stem cells, which hindered their proliferation and migration capacities. Interestingly, FASN inhibition also decreased the expression of glioma stemness markers, such as NESTIN, SOX2 and CD133, suggesting that de novo lipogenesis, controlled by FASN, is necessary for glioma stem cell maintenance (Yasumoto et al., 2016). Interestingly, glioblastoma tissue clustering according to their tumorigenic potential, based on their single-cell RNA-sequencing signatures, revealed that tumors with high tumorigenicity exhibited a significant increase in lipid synthesis gene expressions (Saurty-Seerunghen et al., 2019).

Besides lipid synthesis, lipid oxidation also governs glioblastoma growth and survival. Indeed, human glioblastoma tissues expressed high levels of fatty acid oxidation enzymes, such as carnitine palmitoyl transferase (CPT1), and primary human glioblastoma cells primarily utilized fatty acids for their oxidative metabolism. As a consequence, etomoxir-mediated CPT1 inhibition slowed primary glioblastoma cell proliferation in vitro and increased the survival of syngeneic mouse models (Lin et al., 2017). Moreover, the slow-cycling glioblastoma stem-like subpopulation was demonstrated to store energy in the form of lipid droplets, that were specifically oxidized under glucose deprivation (Hoang-Minh et al., 2018; Taïb et al., 2019). This increased lipid content was owing to a high expression of fatty acid binding protein 7 (FABP7), a chaperone that mediates exogenous fatty acid uptake. FABP7 inhibition slowed glioblastoma growth and invasiveness both in vitro and in vivo, by abrogating their adaptation faculty to metabolic stress conditions (Hoang-Minh et al., 2018). Acyl-CoA-binding protein (ACBP), another fatty acid oxidation regulator, was also shown to be highly expressed in glioblastoma. ACBP supported tumor growth by increasing the availability of long-chain fatty acyl-CoAs to mitochondria, thus promoting fatty acid oxidation and shorter survival (Duman et al., 2019).

5.5. Link Between Tumor Microenvironment, Molecular Signatures and Metabolic Phenotypes of Glioblastoma

Both the tumor niche and the molecular signature of glioblastoma stem cells dictate their metabolic phenotype. For instance, glioblastoma stem cells of the pro-neural subtype are localized in highly vascularized tumor zones, where they have easy access to oxygen and
glucose. They are hence characterized by a strong glycolytic metabolism. Furthermore, these cells express high GS levels, allowing potent glutamine synthesis. On the other hand, glioblastoma stem cells belonging to the mesenchymal subtype reside in hypoxic niches where oxygen and glucose availabilities are tight. In order to overcome these harsh conditions, these cells display an important metabolic flexibility, as they can perform both glycolytic and oxidative metabolisms. Moreover, glioblastoma stem cells of the mesenchymal subtype do not express GS, which highlights their dependence on glutamine synthetized by their neighboring cells (Garnier et al., 2019).

Taking into account that one glioblastoma tumor encompasses glioblastoma stem cells of several subtypes (Neftel et al., 2019), these observations underline that, depending on their spatial distribution, (1) glioblastoma stem cells having different molecular signatures could exhibit similar metabolic profiles and, inversely, (2) glioblastoma stem cells of the same tumor could display different metabolic features. Importantly, these findings suggest that simultaneously inhibiting multiple bioenergetic pathways, e.g., a dual inhibition of glycolysis and oxidative phosphorylation, or a dual blockade of fatty acid synthesis and fatty acid oxidation, could be more effective in the clinic than targeting one single metabolic pathway.
6. **Glioblastoma Resistance to Therapy**

As previously mentioned, despite the aggressive first-line treatments, recurrence almost inevitably occurs. As detailed below, several factors were linked to glioblastoma resistance to therapy.

First, glioblastoma cells display a highly invasive nature and an unmatched infiltrative capacity into the surrounding brain tissue, thus making the optimal surgical resection unattainable. Single-cell RNA-sequencing analyses indeed identified a small fraction of infiltrating glioblastoma cells in peripheral regions to the core lesions. These cells were characterized by the overexpression of genes involved in interstitial matrix invasion and cell survival signaling. Infiltrating cells were also shown to remain in a non-proliferative state until recurrence (Darmanis et al., 2017).

Second, concomitant radiotherapy and chemotherapy aim to exterminate these remaining cells; however, the tremendous inter- and intra-tumoral heterogeneity within the glioblastoma microenvironment constitute a major hurdle (Marusyk et al., 2020). For instance, irradiation substantially alters the tumor microenvironment, by increasing ROS production, hypoxia, senescence and neuroinflammation as well as inducing long-term changes to the extracellular matrix, stem/progenitor cell frequency, immune cell infiltration and vasculature (Gupta and Burns, 2018). Similarly, temozolomide treatment activates inherent or acquired resistance mechanisms, including DNA repair (mismatch repair, base excision repair, MGMT-mediated repair), drug efflux (ABC transporters) and anti-apoptotic pathways (Arora and Somasundaram, 2019). As a result, therapy accumulates glioblastoma stem cells within the tumor microenvironment (Dahan et al., 2014; Lan et al., 2017) and exacerbates glioblastoma aggressiveness in a “what does not kill you makes you stronger” kind of way.

Third, acquired resistance is a direct consequence of the presence of glioblastoma stem cells within the tumor microenvironment. As previously mentioned, glioblastoma stem cells were shown to exhibit a preferential DNA damage checkpoint response activation and an increased DNA repair capacity (Bao et al., 2006), along with an increased expression of drug efflux
pumps (Liu et al., 2006). Moreover, these slow-cycling glioblastoma stem cells were described to evade anti-proliferative therapies and to subsequently give rise to a fast-cycling progenitor population, endowed with high self-maintenance and invasive faculties (Lan et al., 2017; Hoang-Minh et al., 2018; Sabelström et al., 2019).

Fourth, glioblastoma stem cells were recently shown to engage in intricate interactions with each other as well as with their non-malignant counterparts. Winkler and colleagues elegantly demonstrated that glioblastoma stem cells interconnect through long cellular extensions, thus creating a functional tumoral network. Glioblastoma stem cells integrated within this network were protected against cell death inflicted by radiotherapy and chemotherapy, compared to those remaining unconnected (Osswald et al., 2015; Weil et al., 2017). Tunneling nanotubes could play a part in this resistant network, thereby requiring further investigations.

Taken together, glioblastoma stem cells are likely the most relevant glioblastoma components underlying therapy resistance, owing to their intrinsic resistant nature and to their intercellular communication with surrounding cells.
7. TUMORAL NETWORK IN GLIOBLASTOMA

7.1. LONG-DISTANCE INTERCELLULAR COMMUNICATION IN THE BRAIN

Long-distance intercellular communications play a substantial role in the developing and the adult brain alike. For instance, the elaborate brain capacities are made possible thanks to complex neuronal circuits via their axons and dendrites (Osswald et al., 2019). In addition, astrocytes form gap junction-mediated multicellular networks in order to maintain microenvironmental homeostasis and to modulate neuronal activity (Halassa and Haydon, 2010). Likewise, neurogenesis is tightly regulated by gap junction-mediated neuronal stem cell networks within the neurogenic niches (Malmersjo et al., 2013). Recently, tunneling nanotubes within the brain were not only shown to be implicated in physiological conditions such as intercellular calcium waves and neurodevelopmental regulation (Wang et al., 2012), but increasing pieces of evidence also suggested their involvement in neurodegenerative disease onset by spreading pathogenic proteins such as α-synuclein (Abounit et al., 2016a), tau (Abounit et al., 2016b; Tardivel et al., 2016) and prions (Gousset et al., 2009; Victoria et al., 2016).

7.2. LONG-DISTANCE INTERCELLULAR COMMUNICATION IN GLIOBLASTOMA

In a similar fashion, a plethora of different cellular extensions have been described in glioblastoma, that mainly differ in their length, diameter, composition and stability over time (Osswald et al., 2019; Venkatesh and Lou, 2019; Pinto et al., 2020) (Figure 13).

7.2.1. Tunneling Nanotubes (TNTs)

TNTs are described as thin (diameter of 50 – 200 nm), fragile, short-lived cellular extensions, mostly made out of actin filaments, and mainly observed in vitro. Importantly, TNTs have a functional relevance as they allow the transfer of biological cargos between the connected cells (Osswald et al., 2019).

Several studies observed TNT-like structures between glioblastoma cell lines, but their involvement in cancer was not addressed. However, these studies reported that external stimuli, including oxidative stress, protein aggregate uptake or cocaine administration, were able to increase TNT formation (Carone et al., 2015; Ding et al., 2015). Recently though, both
U87 and T98G glioblastoma cells were shown to form an active network of TNT-mediated communication following oxidative stress and/or combined irradiation and temozolomide treatment. Interestingly, TNT formation enabled the unidirectional transport of MGMT from therapy-resistant to therapy-sensitive glioblastoma cells, thereby constituting a novel mechanism of resistance spreading in glioblastoma (Valdebenito et al., 2020).

TNT connections were also observed between glioblastoma cells and astrocytes; their outcomes, however, were somewhat contradictory. For instance, primary rat astrocytes were shown to transfer mitochondria through TNT connections to C6 rat glioma cells in *in vitro* co-cultures. In this case, TNTs significantly decreased glioma cell proliferation and sensitized them to oxidative stress, suggesting that TNTs could be an interesting way to inhibit glioma progression (Zhang and Zhang, 2015). On the other hand, mitochondria transfer *via* TNTs from human UP-010 astrocytes to human U87MG and to UP-007 glioblastoma cells promoted their proliferation and invasion capacities as well as their resistance to temozolomide, both in 2D and in 3D co-cultures (Civita et al., 2019). Strikingly, homo-cellular TNTs between U87MG glioblastoma cells were observed to be thicker, longer and more efficient in cargo transfer than homo-cellular TNTs between normal astrocytes or hetero-cellular TNTs between glioblastoma cells and astrocytes. The authors argue that these thicker TNTs could constitute useful targeted drug-delivery channels, facilitating drug distribution to hardly-accessible tumor niches (Formicola et al., 2019).

In addition, pericyte-derived TNTs were indeed demonstrated, both in *in vitro* cultures and in *ex vivo* glioblastoma slices, to bridge the gap between the walls of distant vessels or between close vessel sprouts. These data suggest that TNTs are also implicated in the earliest phases of tumor angiogenesis (Errede et al., 2018).

One major limitation in the field of TNT research is that most studies were conducted *in vitro*; hence, their relevance *in vivo* remains uncertain. This is largely due, on one hand, to the minute size and the short lifespan of TNTs, and on the other, to the lack of specific markers that unquestionably detect TNTs in the dense glioblastoma tissue. In this sense, even gentle tissue fixation techniques could disrupt TNT structures (Osswald et al., 2019; Venkatesh and Lou, 2019; Pinto et al., 2020).
7.2.2. Tumor Microtubes (TMs)

In contrast with TNTs, TMs are described as thicker (diameter of 1.7 μm), longer and more stable intercellular connections, containing both actin filaments and microtubules, and mainly observed in vivo (Osswald et al., 2019; Venkatesh and Lou, 2019; Pinto et al., 2020).

Glioblastoma was the first cancer reported to develop a functional and resistant TM network in an in vivo model (Osswald et al., 2015). Glioblastoma stem cells, isolated from patients with different glioma grades, were engrafted in nude mice brains with a hardened-glass window in their skull, through which the researchers could follow the tumor formation using longitudinal intravital two-photon microscopy. As the cells invaded, they formed long intra-tumoral protrusions, thereby developing a multicellular communicative network. Interestingly, highly interconnected tumors, which corresponded to higher glioma grades, were able to propagate irradiation-induced calcium ion fluxes through the TM network, thus becoming more resistant to irradiation (Osswald et al., 2015). Furthermore, TMs were shown to drive the repopulation of surgically-resected areas in glioblastoma mouse models (Weil et al., 2017). This observation made it evident that TMs exist in at least two different subtypes: (1) interconnecting TMs that, similarly to TNTs, are involved in multicellular network establishment and (2) invasive TMs that drive cell invasion (Osswald et al., 2019). These data unambiguously support the idea that cell-cell connections are a critical mechanism in treatment failure and tumor relapse. However, no intercellular organelle exchange could be observed through TMs yet. This could be explained by the presence in TMs of connexin 43, a monomeric component of gap junctions, that would not allow the transfer of biological cargos superior to their pore size of 1 kDa, contrary to TNTs that are usually open-ended (Pinto et al., 2020).

In many aspects, TMs resemble cellular extensions established by neurons and their progenitor cells, called neurites. For starters, TMs span several hundreds of micrometers and last for several months, just like neurite extensions. Second, TM formation was shown to depend on growth-associated protein 43 (GAP-43) and tweety-homolog 1 (Ttyh1), important proteins for neurite formation, regeneration and plasticity. Consistently, neurite growth cone-like structures were observed at the tips of extending TMs (Osswald et al., 2019). Third, TMs were demonstrated to be neuron post-synaptic targets, as axons can dock onto TMs in
order to generate synchronized calcium ion fluxes. In addition, depolarization of post-synaptic glioma cells fostered TM-dependent proliferation (Venkatesh et al., 2019) and invasion (Venkataramani et al., 2019). These data suggest that glioma cells form synaptic connections, or “dangerous liaisons”, with neurons via TMs, which accelerates tumor growth rate and lethality (Barria, 2019).

Taken together, it is possible to envision that glioblastoma networks comprise several types of cell-cell connections, that differ in size, composition and function: open-ended TNTs, synaptic-like TM connections and/or thick gap-junction-linked TM extensions (Pinto et al., 2020) (Figure 12). The discovery of long-distance connections between glioblastoma cells in vivo casts a light on a previously underappreciated means of tumoral communication. This is all the more critical considering that only 10 – 20% of a given tumor’s volume is made up of cancer cells. In other terms, cancer cells that may not be in a sufficiently-close proximity to exchange biological cues via cytokines or gap junctions can still communicate through a biological “social network” (Lou, 2016).
Glioblastoma cells (blue) establish a functional network encompassing different types of intercellular connections. Thin tunneling nanotubes (TNT, < 1 μm) connect glioblastoma cells and allow the transfer of biological cargos. In addition, astrocytes (yellow) form TNT-like structures and transfer their mitochondria to glioblastoma cells, which modifies their proliferation and their response to therapy. On the other hand, thick tumor microtubes (TM, > 1 μm) connect glioblastoma cells and contain both connexin 43 (Cx43) and growth-associated protein 43 (GAP-43). Glioblastoma cells can also form TMs that drive cell invasion. In addition, pre-synaptic neurons (orange) dock their axons onto TMs, which regulates the calcium ion fluxes within the glioblastoma network and fosters tumor growth and invasion. (Pinto et al., 2020)
RESULTS
WORKING HYPOTHESIS

The working hypothesis of my thesis is that the tunneling nanotube-mediated mitochondria transfer that occurs in the tumor microenvironment, from mesenchymal stem cells to glioblastoma stem cells, can affect glioblastoma metabolism and, subsequently, induce resistance to temozolomide. In order to answer this question, I developed my PhD project along 3 main axes:

Axis 1: I wanted to determine the metabolic effects of mesenchymal stem cell mitochondria acquisition on glioblastoma stem cells. First, I started by confirming, using mesenchymal stem cells from four different donors, preliminary data from the host laboratory showing tunneling nanotube connections between mesenchymal stem cells and glioblastoma stem cells. Second, in order to specifically study the effects of transferred mesenchymal stem cell mitochondria, regardless of the effects of cytokines secreted in cell co-cultures, I took advantage of the host laboratory’s own MitoCeption protocol (Caicedo et al., 2015; Nzigou Mombo et al., 2017), which allows the transfer of pre-isolated mitochondria to target cells. I further implemented this protocol and adapted it in order to prepare large quantities of glioblastoma stem cells, with quantified amounts of internalized mitochondria, to allow for thorough phenotypic analyses. Third, I confirmed the dose-response acquisition of mesenchymal stem cell mitochondria by glioblastoma stem cells, which enhanced their general energetic metabolism and proliferation.

Axis 2: Cancer cells can develop resistance to chemotherapy through metabolic reprogramming. I therefore asked whether mesenchymal stem cell mitochondria modified the metabolic response of glioblastoma stem cells to temozolomide. For this, I started by assessing the general energetic metabolism, upon temozolomide treatment, of glioblastoma stem cells that had acquired mesenchymal stem cell mitochondria. I then sought to determine if modifications in the overall energetic metabolism were accompanied by changes in nutrient usage and metabolite production. Metabolites are important molecules that not only provide building blocks for macromolecule synthesis, but also act as signaling cues that may induce transcriptional effects.
**Axis 3:** Finally, I wanted to determine if mesenchymal stem cell mitochondria conferred a survival advantage to glioblastoma stem cells against temozolomide chemotherapy. In order to answer this question, I first undertook experiments to measure both the survival and the cell death of MitoCepated glioblastoma stem cells following temozolomide treatment, in the same conditions where the metabolic modifications were observed. Second, I was intrigued as to the mitochondrial oxidative stress in this specific context, that could be a double-edged sword for cancer cells. On one hand, oxidative stress can push cells over the edge and induce their death; on the other, it can protect them, in part, by increasing the frequency of protective tunneling nanotube-formation. Last, I performed high-throughput RNA-sequencing analysis in order to determine the gene expression profile of glioblastoma stem cell following mitochondria acquisition and temozolomide treatment, with the goal to determine transcriptional modifications that might contribute to temozolomide resistance.

The presence of cellular networks mediated by tunneling nanotubes in tumors, along with the intercellular mitochondria transfers they entail, are creating a new paradigm in the cancer field. Tunneling nanotube-based mitochondria transfers are actually totally new means of intercellular communication that had not been anticipated. They have far-reaching implications for tumor progression and resistance to therapy and, therefore, call for novel designs of efficient therapeutic protocols. Glioblastoma patients have few therapeutic options and rapidly face therapy resistance, which results in limited survival once the disease is diagnosed. We propose that the tunneling nanotube-mediated mitochondria transfers from mesenchymal stem cells to glioblastoma stem cells participate in glioblastoma progression and acquired resistance to temozolomide, thus constituting an interesting avenue to explore. This work will be the subject of a soon-submitted article (**Article 1**).

In addition, I set up the experimental conditions for the reproducible extraction and the quantification of acquired mesenchymal stem cell mitochondrial DNA, on the basis of single nucleotide polymorphisms belonging to each donor. This work resulted in a publication as first author in BioTechniques (Nakhle et al., 2020) (**Article 2**).
ACQUISITION OF EXOGENOUS MSC MITOCHONDRIA MODIFIES THE METABOLIC AND FUNCTIONAL RESPONSE OF GLIOBLASTOMA STEM CELLS TO TEMOZOLOMIDE TREATMENT

Jean Nakhle¹²³ et al.

¹Institute for Regenerative Medicine & Biotherapy (IRMB), INSERM, Univ Montpellier, F-34090 Montpellier, France; ²Institute of Molecular Genetics of Montpellier (IGMM), CNRS, Univ Montpellier, F-34090 Montpellier, France; ³Institute of functional genomics (IGF), CNRS UMR5203, INSERM U1191, Univ Montpellier, F-34094 Montpellier, France

*Corresponding author
Marie-Luce Vignais
IGF, CNRS UMR5203, INSERM U1191, UM, 141 rue de la Cardonille
Montpellier F-34094 cedex 5,
France

Email: marie-luce.vignais@igf.cnrs.fr

Keywords
Mitochondria, metabolism, metabolites, glioblastoma stem cells (GSC), mesenchymal stem cells (MSC), tumor microenvironment, mass spectrometry, RNA-seq, mitochondrial DNA (mtDNA)
Graphical abstract

- GSCs acquire MSC mitochondria through dynamic TNT interactions
- MSC mitochondria trigger resistance of GSCs to TMZ
- MSC mitochondria alter the metabolic response of GSCs to TMZ by enhancing OXPHOS and production of metabolites linked to TCA, PPP and purine/pyrimidine synthesis.
- Prior MSC mitochondria acquisition reorients GSC gene expression in response to TMZ towards genes linked to DNA damage and cell cycle regulation
SUMMARY

Glioblastomas are heterogeneous tumors with high metabolic plasticity. Their poor prognosis is linked to glioblastoma stem cells (GSCs) which provide resistance to therapy, notably temozolomide (TMZ). It is worsened by mesenchymal stem cell (MSC) recruitment. We show that, following tunneling nanotube interactions, MSCs transfer mitochondria to GSCs. We find that MSC mitochondria modify the metabolic response of GSCs to TMZ by increasing the OXPHOS and production of metabolites linked to the TCA cycle, pentose phosphate and pyrimidine/purine synthesis pathways. MSC mitochondria also enhanced ROS production and survival of GSCs in response to TMZ. A RNA-seq analysis revealed that MSC mitochondria disrupt GSC transcriptional response to TMZ leading to gene expression related to DNA damage and cell cycle. Together, our data show that the acquisition of exogenous MSC mitochondria can modify the response of cancer cells to therapy, at the levels of cellular metabolism and gene expression.
INTRODUCTION

The development of resistance to chemotherapy is a major obstacle for effective and lasting treatment of cancer. This is an acute issue for the treatment of glioblastoma (GBM), which is an aggressive brain tumor, with limited patient survival. GBM patients are treated by tumor resection followed by radiotherapy and chemotherapy with alkylating agents like temozolomide (TMZ) (Ostermann et al., 2004; Stupp et al., 2005). However, resistance to TMZ treatment appears quickly, mainly due to glioblastoma stem cells (GSCs) present in the tumor (Osuka and Van Meir, 2017; MacLeod et al., 2019). GSCs are characterized by the expression of stemness markers including OLIG2, NESTIN, NANOG, CD133 and SOX2 (Lathia et al., 2015; Garnier et al., 2019). They generate full GBM tumors following xenograft in mice and can be cultured as neurospheres in vitro (Velpula, Dasari and Rao, 2012; Shinojima et al., 2013; Osuka and Van Meir, 2017; MacLeod et al., 2019).

The contribution of the tumor microenvironment (TME) in cancer progression and resistance to therapy has long been recognized, notably in glioblastoma, and through the role of cytokines/chemokines secreted by the CAFs (cancer associated fibroblasts) present in tumors (Broekman et al., 2018; Chen and Song, 2019). This role of the TME highlights the complex reciprocal interactions which take place between cancer cells and normal cells of their microenvironment and contribute to tumor progression and therapy resistance. Mesenchymal stem/stromal cells (MSCs) are a major source of CAFs (Mishra et al., 2008; Jung et al., 2013; Zhu et al., 2014; Weber et al., 2015; Chen and Song, 2019). The recruitment of MSCs to glioblastoma was shown in resected GBM tumors where their presence inversely correlates with patient survival (Hossain et al., 2015; Shahar et al., 2017). It was confirmed in GSC orthotopic xenograft models, where MSCs recruitment in the intracranial tumor was further promoted by glioma-secreted transforming growth factor beta (TGF-β) (Velpula, Dasari and Rao, 2012; Shinojima et al., 2013), angiogenic factors including interleukin-8 (IL-8), neurotrophin-3 (NT-3) and vascular epithelial growth factor (VEGF) (Birnbaum et al., 2007) and chemotactic factors such as the monocyte chemoattractant protein-1 (MCP-1/CCL2) and the stromal cell-derived factor-1 (SDF-1/CXCL12) (Pavon et al., 2018; Thomas et al., 2018).

Intercellular communication is also supported by direct physical cellular connections through tunneling nanotubes (TNTs) (Ariazi et al., 2017; Baker, 2017). TNTs are thin (less than 1 um) open membrane tubular structures, more precisely bundles of individual tunneling nanotubes (Sartori-Rupp et al., 2019), which allow long range (up to several hundred microns) intercellular connections. TNTs allow the intercellular trafficking, mostly along actin microfilaments, of various cargoes including mitochondria. TNT formation is enhanced by cellular stress, including ROS and ROS-inducing
chemotherapies (Rustom, 2016; Victoria et al., 2016; Marlein et al., 2017; Desir et al., 2018). TNTs were observed \textit{ex vivo} in 2D-cell cultures and organoids and \textit{in vivo} in human resected tumors and xenografts from a wide range of cancers including astrocytoma, mesothelioma, osteosarcoma, acute myeloid leukemia and carcinoma from diverse tissues (Lou et al., 2012; Antanavičiūtė et al., 2014; Thayanithy et al., 2014; Osswald et al., 2015; Marlein et al., 2017; Sáenz-de-Santa-María et al., 2017; Weil et al., 2017; Desir et al., 2018; Pinto et al., 2020). In glioma, TNT networks were shown to form both between cancer cells and between cancer cells and cells of the tumor microenvironment (Zhang and Zhang, 2015; Civita, M. Leite and Pilkington, 2019; Formicola et al., 2019; Valdebenito et al., 2020). Intercellular connections led to cancer cell plasticity and resistance to therapy (Osswald et al., 2015; Winkler and Wick, 2018; Venkatesh and Lou, 2019; Valdebenito et al., 2020).

MSCs were characterized for their capacity to make such TNT intercellular connections, notably with cancer cells. Transfers of mitochondria mediated by TNTs and originating from MSCs were reported both \textit{in vitro} and \textit{in vivo} in murine models (Islam et al., 2012; Ahmad et al., 2014; Berridge et al., 2016; Dong et al., 2017). TNT-mediated transfers of mitochondria were shown to have both metabolic and functional effects. MSC mitochondria transfer resulted in protection against tissue injury of the damaged target cells and, for cancer cells, in tumor progression and resistance to therapy as shown by us and others (Islam et al., 2012; Ahmad et al., 2014; Caicedo et al., 2015; Moschoi et al., 2016; Mahrouf-Yorgov et al., 2017; Hekmatshoar et al., 2018; Rodriguez et al., 2018; Nakhle, Rodriguez and Vignais, 2020; Pinto, Brou and Zurzolo, 2020).

Recent work demonstrates that the metabolic properties of tumors and of their microenvironment change during cancer progression and therapeutic treatment, which contributes to tumor cell proliferation and resistance to therapy (Cable et al., 2020; Faubert, Solmonson and DeBerardinis, 2020; Vasan, Werner and Chandel, 2020). Mitochondrial metabolism is central for this process as it provides key anabolic metabolites for macromolecule synthesis and cancer cell proliferation (Nakhle, Rodriguez and Vignais, 2020; Vasan, Werner and Chandel, 2020). In addition, the production of metabolites linked to the mitochondrial tricarboxylic acid (TCA) activity also contributes to epigenetic regulation of cancer cell gene expression, as shown for succinate, fumarate, 2-hydroxyglutarate and \(\alpha\)-ketoglutarate, through the activities of DNA and histone demethylases (Tsukada et al., 2006; Xiao et al., 2012; Killian et al., 2013; Letouzé et al., 2013; Nakhle, Rodriguez and Vignais, 2020). As observed for other cancer types, such as acute leukemia or pancreatic cancer, glioblastoma stem cells were reported to mainly rely on oxidative phosphorylation (Vlashi et al., 2011; Vlashi and Pajonk, 2015). In addition, fatty acid oxidation (FAO) and synthesis (FAS) as well as amino-acid metabolism are also associated with
glioblastoma tumorigenesis (Libby et al., 2018; Duman et al., 2019; Garnier et al., 2019; Saurty-Seerunghen et al., 2019; Cheng et al., 2020).

In this report, we used metabolic analyses to examine the effects of acquired MSC mitochondria on the response of glioblastoma stem cells to temozolomide (TMZ) treatment. We found that GSC oxidative phosphorylation (OXPHOS) and production of metabolites linked to the TCA cycle, pentose phosphate and pyrimidine/purine synthesis pathways were enhanced in these conditions. It was associated with GSC increased ROS production and survival in response to TMZ. Furthermore, our RNA-seq data demonstrated that the overall GSC transcriptional response to TMZ is altered upon prior acquisition of MSC mitochondria, with TMZ-induced regulation of a distinct set of genes linked to DNA damage and cell cycle regulation. Thus, our study uncovers fundamental metabolic and genomic changes in TMZ-treated GSCs, upon prior acquisition of MSC mitochondria, which have physiopathological and therapeutic outcomes.

RESULTS

Human glioblastoma stem cells (GSCs) and mesenchymal stem cells (MSCs) display dynamic interactions which lead to the acquisition of MSC mitochondria by GSCs

To test whether GSCs and MSCs could establish dynamic physical connections and exchange mitochondria, we set up cocultures of red MitoTracker-labeled MSCs and green CellTracker-labeled GSCs, which were analyzed by Incucyte time-lapse imaging (video-1 of images taken every 30 min for 34.5 hr). MSCs and GSCs demonstrated dynamic interactions, through TNT-like protrusions, which were maintained for up to 14 hours. Some of these connections led to the transfer of MSC mitochondria to the GSCs (Figure 1A, see enlarged frame). The acquisition of MSC mitochondria by GSCs was further confirmed by confocal microscopy, performed 24 hr after the beginning of the coculture. MSC mitochondria were identified both inside the TNTs connecting MSCs to GSCs and in the TNT-connected GSCs (Figure 1B).

To characterize the functional and metabolic effects of the acquired MSC mitochondria, our previously described Mitocception protocol was used (Caicedo et al., 2015; Nzigou Mombo et al., 2017; Nakhle et al., 2020). It is based on the quantitative transfer of pre-isolated MSC mitochondria to GSCs, whose extent is determined post hoc by the quantification of the MSC mitochondrial DNA (mtDNA) in the GSCs (Nakhle et al., 2020). This protocol allows the establishment of dose-response effects of acquired exogenous mitochondria. The biological effects of the acquisition of MSC mitochondria by GSCs were analyzed 72 hr after the mitochondria transfer by Mitocception (see time-line, Figure 2).
MSC mitochondria enhance GSC energetic metabolism and proliferation

To test the effects of MSC mitochondria on GSC energetic metabolism, Seahorse experiments were performed. The oxygen consumption rate (OCR) of GSCs increased with the acquisition of MSC mitochondria, in a dose-dependent fashion (Figure 3A, B). This increase in GSC OXPHOS was observed for basal respiration, respiration linked to ATP production and maximal respiration, with respectively 1.74, 1.78 and 2.12-fold increases for the most effective MSC mitochondria concentrations (Figure 3B). Likewise, the measure of the extracellular acidification rate (ECAR) demonstrated increases of basal glycolysis, maximal glycolytic capacity and acidification linked to lactate production, of respectively 1.69, 1.57 and 2.28 folds (Figure 3C, D). Plotting OCR vs ECAR values indicated concomitant increases in OXPHOS and glycolysis in GSCs following MSC mitochondria acquisition. It showed that the acquisition of MSC mitochondria not only enhanced OXPHOS, which is directly dependent on mitochondrial activity, but more generally the overall energy metabolism (Figure 3E).

This enhanced GSC energy metabolism was accompanied with a 1.21-fold increase in GSC cell number for the most effective MSC mitochondria amount, as observed 72 hr after the transfer of MSC mitochondria (Figure 3F). As the acquisition of exogenous mitochondria was previously associated with increased endogenous mitochondrial DNA concentrations (Caicedo et al., 2015; Moschoi et al., 2016), we checked whether a similar effect was observed in GSCs following the acquisition of MSC mitochondria. We measured total mtDNA concentrations at different time-points following the transfer of MSC mitochondria and observed a 1.98-fold increase at 48 hr (Figure 3G). This increased mtDNA concentration is not directly attributable to the acquired MSC mtDNA as it was not observed 24 hr after MSC mitochondria acquisition. Moreover, the amount of MSC mitochondria responsible for the highest effects on GSCs was determined on the basis of MSC mtDNA concentrations in GSCs, measured 24 hr after Mitoception. MSC mtDNA was estimated to 0.4 % of the endogenous GSC mtDNA (Figure S1). The specific detection of MSC mtDNA is based on the presence of SNPs, different between the MSC and GSC donors. It previously allowed us to demonstrate the quantitative transfer of MSC mitochondria to GSCs by the Mitoception protocol (Nakhle et al., 2020). Overall, our results showed that minute amounts of acquired MSC mitochondria led to metabolic reprogramming of GSCs.

MSC mitochondria modify the metabolic response of GSCs to temozolomide treatment

We then asked whether the acquired MSC mitochondria change the response of GSCs to the therapeutic DNA-alkylating agent temozolomide (TMZ). TMZ concentrations of 50 μM were used as they correspond to the concentrations reported in the tumor bulk and in the cerebrospinal fluid (Ostermann, 2004; Rosso et al., 2009) and as they yielded 50% cell death after 6-day GSC treatment (Figure S2). The effects of TMZ on GSC metabolism were tested in the presence of MSC mitochondria,
at their most effective concentrations as determined above. When OXPHOS was measured, TMZ alone showed no significant effects on GSC basal respiration, respiration linked to ATP production or maximal respiration (Figure 4A, B). However, TMZ further supported the enhancing effects of MSC mitochondria on GSC OXPHOS. Basal respiration was increased 1.24-fold compared to the control untreated GSCs versus 1.18-fold with MSC mitochondria alone, respiration linked to ATP production was increased 1.28-fold (vs 1.18-fold) and maximal respiration increased 1.46-fold (vs 1.26-fold) (Figure 4B). GSC glycolysis was not affected by TMZ treatment alone (Figure 4C, D). Contrary to OXPHOS, TMZ did not further enhance the effects of MSC mitochondria. Instead, TMZ diminished the effects of MSC mitochondria, as observed for GSC basal glycolysis (Figure 4D). These data showed that the acquisition of MSC mitochondria by GSCs disrupts their metabolic response to TMZ, notably by enhancing their OXPHOS.

We tested whether the enhanced OXPHOS was associated with increased mitochondrial mass. FACS analysis of MitoTracker-labeled GSCs showed an increase at 48 hr following either TMZ treatment or MSC mitochondria acquisition, which was enhanced by simultaneous treatment (1.27-fold compared to untreated GSCs) (Figure 4E). This effect of the simultaneous MSC mitochondria/TMZ treatment was maintained at 72 hr (1.12-fold increase) (Figure 4E). This increase in mitochondrial mass was associated with increased concentrations of COX IV, a protein from the inner mitochondrial membrane, of respectively 1.29 and 1.23-fold at 48 hr and 72 hr (Figure 4F).

**MSC mitochondria modify the usage and production of metabolites by GSCs in response to TMZ**

As the simultaneous treatments of GSCs with MSC mitochondria and TMZ altered their energetic metabolism, we tested their effects on the usage and production of metabolites linked to the TCA cycle and to metabolic pathways including glycolysis, pentose phosphate and pyrimidine/purine synthesis. We used the Biolog Mitoplate assays to measure the usage of 32 metabolic substrates (Figure S3A). Among these, we focused on the seven TCA substrates more highly used by the GSCs, set their total usage to 100 percent and measured how the usage of each (expressed as a percentage) varied, depending on the GSC treatments. The simultaneous treatment with MSC mitochondria and TMZ increased the consumption of cis-aconitate 2.2-fold while MSC mitochondria or TMZ alone had no detectable effects (Figure S3B). On the other hand, TMZ alone decreased the usage of succinate 1.5-fold, an effect that was reduced to a 1.1-fold decrease in the presence of MSC mitochondria. For L-malate, whereas TMZ alone had no statistically-significant effect, the simultaneous MSC mitochondria/TMZ treatments reduced its usage 1.4-fold (Figure S3B).

The metabolites produced by GSCs were measured by mass spectrometry (LC-HRMS) on GSC whole extracts. Simultaneous MSC mitochondria/TMZ treatments increased the cellular concentrations of
most metabolites of the TCA cycle: citrate (1.31-fold), cis-aconitate (1.23-fold), α-ketoglutarate (1.17-fold), fumarate (1.10-fold) and malate (1.17-fold), while TMZ alone had no detectable effect. Prior MSC mitochondria acquisition also abolished the 1.24-fold reduction in succinate production observed in response to TMZ (Figure 5A). MSC mitochondria also abolished the decreased production of metabolites of the pentose phosphate pathway observed in response to TMZ alone, i.e., sedoheptulose-7-phosphate (1.70-fold) and phosphoribosyl pyrophosphate (1.27-fold) (Figure 5B). In addition, the simultaneous MSC mitochondria/TMZ treatments increased 1.1-fold the production of pyridoxal-5-phosphate (P5P), otherwise unaffected by TMZ alone (Figure 5B). Whereas TMZ had no detectable effects on purine and pyrimidine production, the prior acquisition of MSC mitochondria increased the production of UMP (1.13-fold), UDP (1.12-fold) and UTP (1.24-fold) as well as of AMP (1.23-fold) and ATP (1.34-fold) (Figure 5B). These results confirmed that the acquisition of MSC mitochondria by GSCs changes their metabolic response to TMZ as observed for the production of a number of metabolites (albeit not all, see Figure S4).

**MSC mitochondria change the functional response of GSCs to TMZ**

As the cancer cell response to therapy is known to be tightly linked to metabolism, we tested GSC survival in response to TMZ at this early time-point (48 hr of TMZ treatment), i.e., in conditions similar to that of the metabolic analyses. At the TMZ concentration of 50 uM, which reduces survival to 50% at day 6 (Figure S2), a small but detectable increase in GSC cell death was observed, from 10% to 12.4% (p= 0.004, n=7). This TMZ effect was abolished when GSCs had acquired MSC mitochondria beforehand (Figure 6A). Likewise, GSC proliferation was diminished by TMZ (13.3 % cell number decrease), an effect that was lost in the presence of MSC mitochondria as the simultaneous MSC mitochondria/TMZ treatments led to 8.2 % GSC cell number increase (Figure 6B).

We tested whether these effects of MSC mitochondria on GSC metabolic and functional response to TMZ were correlated to modified mitochondrial ROS production, as assayed by MitoSOX staining. While TMZ alone had no detectable effects on ROS production, in the presence of MSC mitochondria, it increased ROS production 1.2-fold at 48 hr and up to 1.3-fold by 72 hr (Figure 6C). We checked whether this increase in ROS triggered GSCs to activate an anti-oxidative response by measuring SOD2 expression. While little or no effects on SOD2 concentrations were observed at 48 hr, SOD2 expression showed a 1.3-fold increase in GSCs simultaneously treated with MSC mitochondria and TMZ, an effect more pronounced than with either treatment alone (resp. 1.1-fold and 1.2-fold for TMZ and MSC mitochondria alone) (Figure 6D).
MSC mitochondria modify the TMZ-induced GSC gene expression pattern

We measured by RNA-Seq the effects of either TMZ treatment, MSC mitochondria acquisition or both on GSC gene expression pattern compared to the control untreated GSCs. Principal Components Analysis (PCA) clearly identified TMZ treatment as the major source of variance (30% of the total variance) while the effects of MSC mitochondria, projected on components 4 and 6 (respectively 9% and 7% of the total variance), were more limited (Figure 7A). Analysis of the gene expression heatmap led to similar conclusions (Figure 7B). Importantly, the effects of MSC mitochondria were different depending on whether or not they were combined with TMZ treatment, as no component was found to display a common “mitochondria” effect.

As shown from the differential analysis (DA), the number of differentially expressed genes (DE genes) varied strongly with the different treatments and ranged from 4 (CTRL vs Mitochondria) to 1515 (CTRL vs TMZ) (Figure 7B). Treatment with both TMZ and mitochondria resulted in a slightly lower number of DE genes compared to TMZ alone (1430 vs 1515). Examination of these two sets of DE genes showed an important overlap (1150 genes) with, nonetheless, significant qualitative differences. Indeed, 365 genes (24%) were specifically regulated in response to TMZ alone while another 280 genes (19%) were specifically regulated in the condition TMZ plus MSC mitochondria (Figure 7C), a result consistent with the PCA analysis (Figure 7A).

The results of the differential analysis were experimentally validated by RT-qPCR on a set of 6 genes (COL1A1, COL6A3, LIF, FAS, WDR63, INPP5D) differentially regulated under one or more conditions. For all 6 genes, the results obtained by RT-qPCR were highly correlated with those predicted by RNA-Seq (r=0.93) (Figure S5).

Gene ontology (GO) analysis showed that the CTRL vs TMZ and CTRL vs Mito-TMZ comparisons resulted in some very similar statistically over-represented GO terms (i.e. biological processes) (Figure 7D), as expected from the important overlap between the two data sets (Figure 7C). These common processes included regulation of transcription and DNA damage response. TMZ treatment was associated with additional GO terms such as "regulation of RNA metabolic process", "regulation of biosynthetic process" and "regulation of cellular macromolecule biosynthetic process". MSC mitochondria treatment on its own was not associated with any specific GO term, as expected from the very low number of corresponding genes (Figure 7C). Nonetheless, the prior acquisition of MSC mitochondria did have an important effect on TMZ regulation of GSC gene expression as the Mito-TMZ treatment triggered the regulation of expression of a whole new set of genes compared to TMZ alone. These processes were more specifically focused on DNA integrity and damage and on cell cycle control (Figure 7D). These were important results relative to our observation of the effects of the Mito-TMZ co-treatment on GSC survival and proliferation (Figure 6).
The genes were then clustered in various “synexpression” groups, according to their dynamic expression profiles in response to the treatment with TMZ, to MSC mitochondria or both (Figure 7E). These clusters revealed several types of biological response highlighting the complexity of the biological response following treatments. As expected, the “d_n_d” and “u_n_u” were the largest clusters and corresponded to genes whose expression was respectively repressed (down, 601 genes) and induced (up, 1041 genes) following TMZ and Mito-TMZ treatments, while MSC mitochondria alone had little effect (Figure 7E, left panels). The clusters “d_d_d” and “u_u_u” corresponded to sets of genes that were either down-regulated or upregulated in all three experimental conditions and only represented a fraction of the DE genes. Treatment with MSC mitochondria had weak (in terms of gene counts) but very specific effects. For a number of genes, treatment with MSC mitochondria counteracted the effect of TMZ, both in case of repression (“d_n_n”, 30 genes) and activation (“u_n_n”, 37 genes), while MSC mitochondria treatment had no effect on its own (Figure 7E). We also identified two additional sets of genes, corresponding to the “n_n_d” and “n_n_u” clusters, for which the effects of TMZ were observed only in the presence of MSC mitochondria, while each treatment alone had no effect (Figure 7E, right panels). We found no instance of genes with opposite regulation between TMZ and Mito-TMZ treatment (i.e., no cluster “d_n_u” or “u_n_d”). Altogether, these data obtained from RNA-seq analysis clearly showed a strong GSC transcriptional response to TMZ, but also highlighted very specific differences upon MSC mitochondria acquisition suggestive of a molecular switch induced by MSC mitochondria.

**DISCUSSION**

While they are central for tissue homeostasis, intercellular interactions also support rapid cell adaptation to external challenges. The recently characterized tunneling nanotubes contribute to such intercellular communication, notably by allowing intercellular trafficking of mitochondria. The acquisition of these exogenous mitochondria was shown to support resistance of cancer cells to therapy (Pasquier et al., 2013; Desir et al., 2016; Moschoi et al., 2016; Wang et al., 2018). However, the biological rationale for this acquired resistance was not determined. Here, we explored the biological effects of prior acquisition of MSC mitochondria on the response of human glioblastoma stem cells to TMZ treatment. By using large-scale analyses, we determined that the metabolic response of GSCs to TMZ is altered and that these changes affect different metabolic pathways, including the TCA cycle, the pentose phosphate pathway as well as pyrimidine and purine synthesis. In addition, our RNA-seq analysis revealed that the transcriptional response of GSCs to TMZ is largely modified by MSC mitochondria. Even though MSC mitochondria alone showed neglectable effects on GSC transcription,
they deeply modified the GSC response to TMZ by allowing the transcriptional regulation of new sets of genes, particularly linked to cell cycle progression and DNA damage.

To address these questions, we exploited our patented Mitoception protocol to quantitatively transfer human MSC mitochondria to GSCs (Caicedo et al., 2015; Nzigou Mombo et al., 2017; Nakhle et al., 2020). Our conclusions on the effects of the simultaneous MSC mitochondria acquisition and TMZ treatment on GSC metabolism were drawn from the set of complementary biochemical techniques of Seahorse, Biolog Mitoplates and mass spectrometry, which allowed us to determine their effects on GSC energy metabolism, metabolites consumption and production. Experiments were performed with mitochondria isolated from primary bone-marrow mesenchymal stem cells isolated from four donors, which generated similar effects. On the other hand, the human glioblastoma stem cells we used were primary cells grown as spheroids (clone Gli4) (Guichet et al., 2013). These spheroids can possibly contain both stem and more differentiated glioblastoma cells, in variable proportions, therefore accounting for some of the variations we observed (Wan et al., 2010; Mikhailova et al., 2018; Zhang et al., 2020). This variability was taken into account by our statistical analyses.

Our data indicate that small amounts of exogenous MSC mitochondria (0.4 % of the endogenous GSC mtDNA; Figure S1) were sufficient to trigger short-term effects in GSCs, as observed at 48 hr and 72 hr. This included increased OXPHOS and production of metabolites of the TCA cycle which both rely on mitochondrial activity. Our data suggest that the increased overall GSC mitochondrial activity is not directly attributable to these relatively few acquired MSC mitochondria but to increased concentrations of endogenous mitochondria, as supported by the observed increase in total mtDNA concentrations (Figure 3G) and in GSC mitochondrial mass (Figure 4E). The biochemical mechanisms leading to this increase in endogenous mitochondria concentrations still need to be determined. They are not expected to be specific for glioblastoma cells as similar findings were obtained for the breast cancer cell line MDA-MB-231 (Caicedo et al., 2015). They also seem independent of the process of exogenous mitochondria acquisition, here by the Mitoception protocol, as acquisition of MSC mitochondria by human T cells in coculture led to similar observations (Luz-Crawford et al., 2019).

The effects of MSC mitochondria, observed for GSC metabolism and gene expression, were also measurable for GSC survival and proliferation following TMZ treatment. Prior acquisition of MSC mitochondria abolished the TMZ-induced cell death observed at 48 hr of TMZ treatment (Figure 6A). This effect of TMZ on GSC cell death at 48 hr was low but did lead to 50% GSC cell death after 6 day-treatment (Figure S2). The rationale for studying the GSC response at 48 hr of TMZ treatment was to determine the early functional, metabolic and transcriptional responses of GSCs following acquisition
of MSC mitochondria and TMZ treatment, as presented here. We tried to establish the effects of MSC mitochondria on GSC survival after 6-day TMZ treatment (protocol with single mitochondria transfer and TMZ treatment). However, GSC survival measurements at day 6 led to conflicting data (not shown), which suggested that other, as yet unidentified, factors were also at play. Long-term effects of exogenous mitochondria on glioblastoma resistance to treatment might depend on reiterated acquisition of these mitochondria. To what extent this actually takes place in tumors will need further investigation. Still, our own data from time-lapse imaging showed renewed dynamic physical interactions leading to mitochondria transfers between MSCs and GSCs (Figure 1 and video 1). Moreover, our previous work on cocultures of human MSCs and T cells showed that T cells could physically interact and acquire mitochondria from MSCs in an iterative manner over time (Luz-Crawford et al., 2019). It suggests that such dynamic and iterative interactions could take place in tumors as well, leading to renewed acquisition of exogenous mitochondria and to the associated increased survival to therapeutic treatment.

Our metabolic analysis of GSCs following exogenous mitochondria acquisition, with or without TMZ treatment, demonstrated how the metabolic properties of cancer cells can evolve during cancer progression, due to the cumulative effects of their interactions with their microenvironment and of the cellular challenge induced by chemotherapy. In particular, we showed that the TCA, PPP and purine/pyrimidine pathways, all linked to biosynthetic processes and cell proliferation, were stimulated in these conditions. This metabolic plasticity of cancer cells, depending on the environmental cues and on the intrinsic progression of the tumor cells, undoubtedly constitutes a major current challenge for future therapeutic strategies (Faubert, Solmonson and DeBerardinis, 2020; Fendt, Frezza and Erez, 2020; Vasan, Werner and Chandel, 2020). We also observed increased ROS production by GSCs following MSC mitochondria acquisition and TMZ treatment. Although ROS can contribute to genetic instability and tumor progression, highly increased ROS concentrations were shown to contribute to cancer cell therapeutic susceptibility (Trachootham et al., 2006; Trachootham, J and P, 2009; Gill, Piskounova and Morrison, 2016; Hekmatshoar et al., 2018). This could open novel therapeutic strategies for evolving glioblastoma.

The RNA-seq analyses indicated that MSC mitochondria per se had little effect on GSC transcription while TMZ induced a strong transcriptional response in GSCs. However, MSC mitochondria deeply changed the transcriptional response of GSCs to TMZ. Interestingly, these changes targeted another set of genes, linked to DNA damage and cell cycle regulation. This suggested that the inhibiting effects of MSC mitochondria on TMZ-induced GSC cell death was not merely associated to the deregulation of the set of genes whose expression was modified by TMZ alone. Instead, MSC mitochondria plus TMZ
treatment appeared to trigger a molecular switch in GSCs which was correlated with TMZ resistance. Besides, the RNA-seq analyses did not uncover genes encoding enzymes linked to metabolic processes, i.e., TCA cycle, PPP, pyrimidine/purine production, which were enhanced in the conditions MSC mitochondria plus TMZ, as shown by metabolomics. As both the RNA-seq and mass spectrometry analyses provided snapshots of GSCs at a given time, it may be that they did not catch the two processes of metabolic enzyme gene expression and resulting production of metabolites if they occurred in distinct time-frames. Alternatively, the changes in metabolism which were observed through differences in metabolite production could be the consequences of cellular signaling triggered by processes other than gene expression regulation and occurring for instance through protein post-translational modifications.

Our study focused on mitochondria originating from MSCs as these cells are found in glioblastoma tumors where their presence is linked to an unfavorable prognosis (Velpula, Dasari and Rao, 2012; Shinojima et al., 2013; Hossain et al., 2015; Shahar et al., 2017). In addition, they were characterized, by us and others, for their capacity to share mitochondria with other cells (Acquistapace et al., 2011; Caicedo et al., 2015; Rodriguez et al., 2018). However, other cells of the glioma microenvironment like astrocytes can also form TNTs and transfer mitochondria to glioblastoma cells (Zhang and Zhang, 2015; Civita, M. Leite and Pilkington, 2019). More generally, the tumor microenvironment contains diverse cell types, including macrophages or endothelial cells, which can also exchange mitochondria with neighboring cells (Onfelt et al., 2006; Yasuda et al., 2011; Pasquier et al., 2013; Vignais et al., 2017). It remains to be determined whether these cells can also make connections and transfer mitochondria to glioblastoma cells. If so, it will be of interest to investigate if it leads to biological effects, in response to TMZ, similar to those we established for MSC mitochondria. This would undoubtedly further support the intricacy of the intercellular connecting network in GBM and demonstrate the importance of integrating the biology of GBM cells in the complexity of their microenvironment.

On a more general perspective, mitochondria intercellular transfer is now envisioned as a therapeutic tool of “mitochondria transplantation” for pathologies like myocardial infarction, acute kidney injury, stroke, spinal cord injury, or glaucoma with the rationale of restoring the failing energy metabolism (Masuzawa et al., 2013; Cowan et al., 2016; Huang et al., 2016; Kaza et al., 2017; Gollihue et al., 2018; Fang et al., 2019; Shin et al., 2019; Zhang et al., 2019; Jabbari et al., 2020; Nakhle, Rodriguez and Vignais, 2020; Nascimento-Dos-Santos et al., 2020). Clinical trials have also been initiated for ischemia-related-myocardial dysfunction (Emani et al., 2017). The results presented here on glioblastoma stem cells showed that the energy metabolism of these cells is also enhanced upon exogenous mitochondria acquisition. Besides, it showed that the sole acquisition of MSC mitochondria did not lead to any
detectable effects on GSC gene expression, as determined by large-scale RNA-seq analysis. However, quite unexpectedly, MSC mitochondria deeply altered GSC transcriptional response to the chemical agent TMZ. This could be considered as a warning when considering mitochondria-based therapy and additional therapeutic treatments. As our study suggests, be for spontaneous mitochondria exchange in the context of cancer or for mitochondria-based therapy, the effects of mitochondria exchange are expected to reach beyond the mere energy metabolism of the mitochondria recipient cells and should be fully considered for effective therapeutic strategies.

ACKNOWLEDGMENTS
Support from La Ligue Contre le Cancer (Comité de l’Aude and Comité du Gard), from ARC and from SATT-AxLR are gratefully acknowledged. We are also grateful to the MRI Imaging platform (Montpellier Bio campus), to the Métamontp metabolic platform (Montpellier Bio campus) for access to Seahorse XFe96 technology, to the IMRB genomic core facility (Créteil, France) for libraries construction and sequencing. MetaToul (Metabonomics & Fluxomics Facilities, Toulouse, France) and its staff member Lara Gales are gratefully acknowledged for carrying out metabolome analyses. MetaToul is part of the national infrastructure MetaboHUB-ANR-11-INBS-0010 (the French National infrastructure for metabolomics and fluxomics).

AUTHOR CONTRIBUTIONS
J.N. and ML.V. designed the study, J.N., T.Ö, A.B. and D.M.A.M. performed the experiments, N.B. performed the RNAseq analysis, J.N., S G-C., M.D-C., M.G., V.D., A-M.R., N.B. and ML.V. analyzed and interpreted the data, J.N., N.B. and ML.V. prepared the figures, A.B. prepared the video, J.N., N.B. and ML.V. wrote the manuscript and all authors reviewed and edited the final manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.
FIGURE LEGENDS

Figure 1. Dynamic interactions between human glioblastoma stem cells (GSCs) and mesenchymal stem cells (MSCs) lead to the acquisition of MSC mitochondria by the glioblastoma cells.

MSCs and GSCs were labeled with red MitoTracker and green CellTracker, respectively, prior to co-culture.

(A) Representative image of time-lapse Incucyte imaging.

(B) Confocal fluorescence microscopy imaging 24 hr after the beginning of the co-culture. Scale bars: left panel, 20 µm; right panel, 5 µm. Arrows: MSC mitochondria.

Figure 2. Scheme of the experimental workflow

For analyzing the effects of MSC mitochondria on GSCs, pre-isolated MSC mitochondria are transferred to GSCs by Mitoception (day 0). Twenty-four hr later (D1), GSCs are collected to quantify acquired MSC mitochondria (MSC mtDNA quantification) and to set functional assays, with or without temozolomide treatment (48 hr). All measures are performed at day 3 following the acquisition of MSC mitochondria (D3).

Figure 3. MSC mitochondria enhance GSC energetic metabolism and proliferation

MSC mitochondria (4 concentrations with 2-fold incremental increases) were transferred to GSCs by Mitoception and their effects on GSC functions were analyzed 72 hr later. Data were obtained with MSCs from 3 donors.

(A, B) Dose-response effect of MSC mitochondria on GSCs oxygen consumption rates (OCR).

(A) Representative plot of GSC OCR in basal conditions and after sequential addition of oligomycin, FCCP and rotenone/antimycin. Mean values and SEM are indicated (n=4).

(B) Tukey boxplots showing basal respiration (left), respiration linked to ATP production (middle) and maximal respiration (right). n = 18 from 4 independent experiments. One-way ANOVA, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

(C, D) Extracellular acidification rates (ECAR)

(C) Representative plot of GSC ECAR in basal conditions and after sequential addition of glucose, oligomycin, oxamate and 2-deoxyglucose. Mean values and SEM are indicated (n=6).

(D) Tukey boxplots showing basal glycolysis (left), glycolytic capacity (middle) and lactate acidification (right). n = 13 from 3 independent experiments. One-way ANOVA, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

(A-D) All values were normalized to GSC cell numbers.

(E) OCR vs ECAR of GSCs with MSC mitochondria

(F) Tukey boxplots showing GSCs proliferation. One-way ANOVA ***p ≤ 0.001.
(G) Total mtDNA content in GSCs with acquired MSC mitochondria. mtDNA concentrations are expressed relative to GSC genomic DNA. Mean ± SEM and multiple t tests, *p ≤ 0.05

Figure 4. MSC mitochondria modify the metabolic response of GSCs to temozolomide

MSC mitochondria were transferred to GSCs which were subsequently treated with temozolomide (TMZ, 50 μM).

(A, B) Effects of TMZ in the presence/absence of MSC mitochondria on GSCs oxygen consumption rates (OCR).

(A) Representative plot of GSC OCR in basal conditions, with MSC mitochondria, TMZ treatment or both, and after sequential addition of oligomycin, FCCP and rotenone/antimycin. Mean values and SEM are indicated (n=4).

(B) Tukey boxplots showing basal respiration (left), respiration linked to ATP production (middle) and maximal respiration (right). n = 25 from 4 independent experiments. One-way ANOVA, **p ≤ 0.01, ***p ≤ 0.001.

(C, D) Extracellular acidification rates (ECAR)

(C) Representative plot of GSC ECAR in basal conditions, with MSC mitochondria, TMZ treatment or both, and after sequential addition of glucose, oligomycin, oxamate and 2-deoxyglucose. Mean values and SEM are indicated (n=5).

(D) Tukey boxplots showing basal glycolysis (left), glycolytic capacity (middle) and lactate acidification (right). n = 25 from 4 independent experiments. One-way ANOVA, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

(A-D) All values were normalized to GSC cell numbers.

(E) GSC Mitochondrial mass. MitoTracker-labeled GSCs were analyzed by FACS, 48 hr (n=3) and 72 hr (n=7) following the acquisition of MSC mitochondria. Upper panels: representative experiment. Lower panels: relative MFI values represented as mean ± SEM. Two-tailed unpaired t tests, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

(F) Western blot for Cytochrome c oxidase IV (COX IV) and b-actin expression (MW markers in kDa). Quantification (n=3) represented as mean ± SEM. Two-tailed unpaired t tests, *p ≤ 0.05, **p ≤ 0.01.

Figure 5. MSC mitochondria modify GSC metabolite production in response to temozolomide

GSC metabolites production as analyzed by mass spectrometry. (A) TCA cycle metabolites. (B) Metabolites of the pentose phosphate and nucleotide synthesis pathways. Three independent experiments were performed, each in triplicate. Values were normalized to cell numbers. Each point corresponds to an individual culture and extraction. Median with interquartile range. Two-tailed unpaired t tests, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
Figure 6. MSC mitochondria change the functional response of GSCs to TMZ

(A) GSC cell death. GSCs were stained with Zombie violet and analyzed by flow cytometry. Left panel, representative data. Right panel, quantification from 7 independent experiments with mean and SEM values. Statistical analysis by Student’s t test, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

(B) GSC proliferation as measured cell counting (n=84 from 7 independent experiments). Tukey boxplots with two-tailed unpaired t tests, ***p ≤ 0.001.

(C) ROS production. GSCs were stained with MitoSox and analyzed by flow cytometry, 48 hr (left panels) and 72 hr (right panels) following the acquisition of MSC mitochondria. Representative data (upper panels) and quantification from independent experiments (n=3 for 48 hr; n=9 for 72 hr) with mean and SEM values. Statistical analysis by Student’s t test, *p ≤ 0.05, **p ≤ 0.01.

(D) Western blot for superoxide dismutase 2 (SOD2) and b-actin expression (MW markers in kDa). Quantification (n=3) represented as mean ± SEM. Two-tailed unpaired t tests, *p ≤ 0.05, **p ≤ 0.01.

Figure 7. MSC mitochondria modify the transcriptional response of GSCs to TMZ

RNA-seq data from GSCs treated (or not) with TMZ and/or MSC mitochondria (n=3).

(A) Principal Components Analysis (PCA). Left panel, the effect of TMZ projects on the major component PC 1. Right panels, effects of MSC mitochondria, which depend on TMZ, project on distinct components (PC4 and PC6).

(B) Heatmap of gene expression change.

(C) Differential Analysis.

(D) Biological processes affected (Gene ontology). Circle diameter refers to gene count and color to p-values.

(E) Synexpression groups based on normalized expression values.
STAR+METHODS

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Marie-Luce Vignais (marie-luce.vignais@inserm.fr).

Materials Availability

RNAseq sequences

METHOD DETAILS

Cell culture of glioblastoma stem cells and mesenchymal stem cells

Human primary glioblastoma stem cells (clone Gli4, Guichet et al., 2013) were grown as spheroids in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (Gibco 21331046), supplemented with L-Glutamine (2 mM) (Gibco 25030024), D-Glucose (0.3 %) (Sigma-Aldrich G8769), bovine insulin (0.002 %) (Sigma-Aldrich I1882), N-2 supplement (Gibco 17502048), B-27 supplement (Gibco 12587010), human EGF (10 ng/mL) (Miltenyi Biotec 130-097-750) and human FGF-2 (10 ng/mL) (Miltenyi Biotec 130-104-924).

Human mesenchymal stem cells (MSCs) were isolated from the bone marrow of healthy donors at the authorized cell therapy unit (Biotherapy Team of General Clinic Research Center, French health minister agreement TCG/04/0/008/AA) at the Grenoble University Hospital. MSCs were cultured in Minimum Essential Medium Eagle, alpha modification (Lonza Bioscience BE12-169F), supplemented with Fetal Bovine Serum (10 %) (Sigma-Aldrich F7524, lot BCBQ9326V), L-Glutamine (2 mM) (Gibco 25030024) and FGF-2 (1 ng/mL) (Miltenyi Biotec 130-104-924). MSCs from 4 donors were used in this study. All cells were cultured at 37°C with 5 % CO2 without antibiotics. Absence of mycoplasma contamination was verified with MycoAlert™ Mycoplasma Detection Kit (Lonza LT07-118).

Mitochondria preparation

MSCs (subconfluent cultures) were trypsined without EDTA (Gibco 15090046). MSCs (5 x 10^6) were then mechanically lysed in a buffer containing mannitol (200 mM) (Sigma-Aldrich M1902), saccharose (70 mM) (Sigma-Aldrich S0389), EDTA (1 mM) (Sigma-Aldrich E9884), HEPES (10 mM, pH = 7.4) (Sigma-Aldrich H3375) and 1X cocktail of protease and phosphatase inhibitors (Roche 04693159001), by using a syringe with 25- and 27-gauge needles. MSC mitochondria were isolated by two differential
centrifugations (10 min each) at 800 g and 8,000 g respectively, where mitochondria were collected respectively in the centrifugation supernatant and as a pellet. All steps of the mitochondria isolation were carried out at 0°C and the mitochondria pellet was finally resuspended in ice-cold mannitol isolation buffer and diluted in ice-cold GSC culture medium.

**Transfer of MSC mitochondria to GSCs by Mitoception**

The transfer of MSC mitochondria to GSCs was performed as previously described (Caicedo *et al.*, 2015; Nzigou Mombo *et al.*, 2017). Briefly, GSC spheroids were dissociated 24 hr prior to the mitochondria transfer. GSCs were seeded as single cells, as a confluent cell layer, the day of the Mitoception (5 x 10^5 cells/well of 24-well plates). MSC mitochondria were isolated immediately prior to the Mitoception. They were serially diluted in ice-cold GSC culture medium and added to the GSCs (close to the GSC cell layer). The culture plates were centrifuged at 3,000 rpm for 15 min. GSCs were incubated for 24 hr at 37°C in 5 % CO_2 prior to collecting the Mitocepted GSC cells for further analysis. The amounts of acquired MSC mitochondria were quantified on the basis of MSC mitochondrial DNA (mtDNA) PCR quantification. The serial dilutions of the MSC mitochondria preparations used for the Mitoception were made considering the number of target GSCs. A ratio of 1 to 16-32 between the number of MSCs used for the mitochondria preparation and the number of target GSCs was found optimal to provide the observed biological effects.

**Extracellular Flux Assays**

For all extracellular flux assays, GSCs were seeded at a density of 4 x 10^4 cells per well on Seahorse XFe96 culture microplates coated beforehand with poly-lysine (0.1 mg/ml) (Sigma-Aldrich P7280) and laminin (10 mg/ml) (Sigma-Aldrich L2020). The assay plates were spin-seeded for 5 min at 1,200 rpm and incubated at 37°C with CO_2 for 48 hr prior to performing the assay on the Seahorse Bioscience XFe96. Before the assay, cells were incubated in the assay medium for 1 hr at 37°C without CO_2. Sensor plates were calibrated overnight in XF calibration buffer at 37°C in atmospheric conditions without CO_2 supplementation.

**Oxygen Consumption Rate (OCR) Assays**

OCR measurements were performed in XF media (non-buffered DMEM) supplemented with glucose (10 mM) (Seahorse XF 103577-100), L-glutamine (2 mM) (Gibco 25030024) and sodium pyruvate (1 mM) (Seahorse XF 103578-100), under basal conditions and in response to mitochondrial inhibitors: oligomycin (1 uM) (Sigma-Aldrich O4876), FCCP (1 uM) (Sigma-Aldrich C2920), rotenone (100 nM) (Sigma-Aldrich R8875) and antimycin A (1 uM) (Sigma-Aldrich A8674).

**Extracellular Acidification Rate (ECAR) Assays**
ECAR measurements were performed in XF media supplemented with L-glutamine (2 mM) (Gibco 25030024) and sodium pyruvate (1 mM) (Seahorse XF 103578-100), in response to glucose (10 mM) (Seahorse XF 103577-100), oligomycin (1 uM) (Sigma-Aldrich O4876), oxamate (75 mM) (Sigma-Aldrich O2751) and 2-deoxyglucose (2-DG, 100 mM) (Sigma-Aldrich D8375).

For each biological replicate, at least four technical replicates were prepared. Oxygen consumption rates (OCR, pMoles/min) and extracellular acidification rates (ECAR, mpH/min) were measured every 6 min (3-minute mix and 3-minute measurement), 3 times for each condition. All measures were normalized to the number of cells counted in each well of the Seahorse XFe96 culture microplates at the end of the SeaHorse experiments, on the basis of Hoechst (Invitrogen H3570) nuclei labeling and automatic counting (Thermo Scientific Cellomics BioApplications-Image).

**Cell Proliferation**

Cell growth of GSCs upon acquisition of MSC mitochondria was determined by automated cell counting. In brief, GSCs were seeded at a density of 4 x 10^4 cells per well in 96-well plates, the day following Mitoception. Forty-eight hours later, cells were fixed in 4% formaldehyde (Sigma-Aldrich) and stained with Hoechst 33342 (5 mg/ml, Invitrogen H3570). Cell numbers were determined on the basis of the stained nuclei which were quantified automatically (Thermo Scientific Cellomics BioApplications-Image).

**Western Blot**

For GSC protein samples preparation, GSCs (2 x 10^5) were collected directly in Laemmli buffer (2X) (Bio-Rad 161-0747). After heating (95°C) for 5 minutes, protein samples (equivalent to 4 x 10^4 cells) were run using Bio-Rad Mini-Protean TGX 4-15% gradient gels (Bio-Rad 4561083) and transferred onto PVDF membranes (Bio-Rad 1704157). Membranes were blocked for 30 minutes in 5% skimmed milk in TBST at room temperature and incubated with primary antibodies overnight in 1% skimmed milk in TBST according to manufacturer suggested primary antibody dilutions. Membranes were then incubated with HRP-conjugated secondary antibodies at a 1:10,000 dilution, developed using Millipore ECL (WBKLS0500) and quantified using the BioRad ChemiDoc XRS instrument. Antibodies directed against COXIV (GeneTex GTX628901), SOD2 (GeneTex GTX116093) and β-actin (Abcam ab8226) were used.

**Flow Cytometry (FACS)**

GSCs (2.5 x 10^5 cells) were dissociated to single cells by pipetting prior to the labeling with MitoTracker Deep Red FM (250 nM, Molecular Probes, M22426), MitoSOX™ Red mitochondrial superoxide indicator (5 uM, Molecular Probes, M36008) and Zombie violet (1X, BioLegend, BLE423113).
Fluorescence-activated cell sorting was performed on a Gallios Beckman Coulter Flow Cytometer and analysis was done with the Kaluza software.

**Metabolite usage Mitoplates**

GSCs (8 x 10⁴ cells/well) were seeded in 96-well MitoPlate™ S-1 Microplates (Biolog). MitoPlates™ S-1 contain 3 sets of wells pre-coated with either cytoplasmic or mitochondrial substrates. Saponin (50 μg/ml) was used to permeabilize the GSCs. Substrate usage was determined on the basis of the initial usage rate measured, in each well, at 37°C with a Biolog OmniLog instrument.

**Mass Spectrometry Quantification**

Cells were seeded on coverslips in 6-well culture plates. The coverslips were coated beforehand with poly-lysine (0.1 mg/ml) (Sigma-Aldrich P7280) and a control with poly-lysine but without cells was included. The extraction was performed at -20°C in a solution (8 mL) of acetonitrile/methanol/water, 4:4:2 v/v containing formic acid (125 mM). IDMS (Isotope dilution mass spectrometry) was used for quantification. The analyses were carried out on an IC-MS platform with a liquid anion exchange chromatography Dionex™ ICS-5000+ Reagent-Free™ HPIC™ (Thermo Fisher Scientific™, Sunnyvale, CA, USA) system, coupled to a Thermo Scientific™ LTQ Orbitrap Velos™ mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a heated electrospray ionization probe.

Analytes were separated within 50 min, using a linear KOH gradient elution applied to an IonPac AS11-HC column (250 x 2 mm, Dionex) equipped with an AG11-HC guard column (50 x 2 mm, Dionex) at a flow rate of 0.38 ml/min. The gradient program was the following: equilibration with KOH (7 mM) for 1.0 min; then KOH ramp from 7 to 15 mM, 1–9.5 min; constant concentration 10.5 min; ramp to 45 mM in 10 min; ramp to 70 mM in 3 min; ramp to 100 mM in 0.1 min; constant concentration 8.9 min; drop to 7 mM in 0.5 min and equilibration at 7 mM KOH for 7.5 min. The column and autosampler temperatures were thermostated at 25°C and 4°C, respectively. The injected sample volume was 15 μl. Measures were performed in triplicates from separate cultures.

Mass detection was carried out in a negative electrospray ionization (ESI) mode at a resolution of 60 000 (at 400 m/z) in full-scan mode, with the following source parameters: the capillary temperature was 350 °C, the source heater temperature, 300 °C, the sheath gas flow rate, 50 a.u. (arbitrary unit), the auxiliary gas flow rate, 5 a.u., the S-Lens RF level, 60 %, and the source voltage, 2.75 kV. Data acquisition was performed using Thermo Scientific Xcalibur software. Metabolites were determined by extracting the exact mass with a tolerance of 5-10 ppm. Data processing: TraceFinder 4.1 software. Raw measures were normalized to cell numbers.
**Preparation of RNA samples**

RNA was isolated from GSCs (5 x 10^5 cells) using the QIAGEN RNeasy kit (Qiagen 74106) per manufacturer’s instructions, including the on-column DNase I digestion step using RNase-free DNase set (30 Kunitz units, Qiagen 79254), and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). RNA quality controls (including RIN values) were performed with a Tapestation (Agilent) while RNA concentrations were determined by Qubit fluorometric quantitation.

**Analysis of RNAseq data**

RNA integrity number (RIN) was also measured on an Agilent TapeStation 4200 system. Only RNA samples with a RIN > 8 were used for cDNA library construction. Libraries of polyA mRNA were generated using the TruSeq® Stranded mRNA Library Prep kit (Illumina) with double indexing using TruSeq RNA UD Illumina Indexes. RNA was reverse transcribed using SuperScript™ II (Invitrogen). Next generation sequencing was performed by 100*2 bp paired end reading with the NextSeq 500/550 High Output Kit v2.5 (300 Cycles) on a NextSeq 500 analyzer (all from Illumina) resulting in about 40 million raw reads.

**Data processing**

High quality reads were mapped on Hg19 human genome sequence (ftp.genome.ucsc.edu) with BOWTIE (Langmead et al., 2009) using stringent parameters (l = 45, 3’ = 5, n = 2, m = 1). Redundant reads were discarded. Read count on individual exons was carried out with INTERSECTBED (Bedtools software suite), and used to derive a gene level count table. Principal Component Analysis (PCA) was performed using the R prcomp function after variance stabilization transformation, as instructed by Anders and Huber (Anders and Huber, 2010). Genes with low read count (i.e. low expression level) were discarded, corresponding to ~ 40% of the entire gene set.

Differential analysis was carried out with DEseq (Anders and Huber, 2010), p-val ≤ 0.05. Heatmap of expression fold changes (in log2-scale) was computed with the heatmap.2 function provided in R, together with a custom color map.

Gene Ontology (GO) term enrichment analysis with GORILLA (Eden et al., 2009) was used to describe the functional categories affected by the various treatments and characterize the corresponding molecular phenotypes. Graphic representation was produced with a custom script (N.B) based on the MATPLOTLIB PYTHON library, in which circle size and color are proportional to the number of genes per functional category and the p-value, respectively.

Clustering was used to group together genes based on their transcriptional responses independently of their expression level (synexpression groups). To this end, normalized expression levels were computed following a simple transformation where expression values for each gene were centered and reduced (variance equal to 1 and mean centered at 0):
Each cluster was then named by a set of letters representing the normalized expression level measured for each treatment relative to CTRL: letter 'u' for 'up', 'd' for 'down' and 'n' for 'no difference'. For example, cluster u_n_u corresponds to genes with increased expression after TMZ treatment, no significant difference with MSC mitochondria and increased expression after MSC mitochondria/TMZ treatments (Figure 7E).

mRNA RT-qPCR
First-strand cDNA was synthetized from 500 ng of total RNA, using the M-MLV RT kit (Invitrogen 28025013) and random hexamers (Invitrogen N8080127). Real-time quantitative PCR was performed using the SYBR Green Master Mix (Roche 04887352001), with 250 nM primer concentrations and the LightCycler 480 instrument (Roche, Meylan, France), with the following program: 10 min at 95°C, 50 cycles of 10 s at 95°C, 15 s at 65°C and 15 s at 72°C. The last steps of PCR are performed to acquire the dissociation curve, validating the specificity of the PCR products. Each assay was performed in duplicate. Negative controls without template were added and the PCR efficiencies were checked by sample dilutions. The following primer sequences were used to amplify the cDNAs from:

Col1A1: COL1A1-F: 5’ – GATTCCCTGGACCTAAAGGTGC – 3’
       COL1A1-R: 5’ – AGCCTCTCCATCTTTGCCAGCA – 3’

Col6A3: COL6A3-F: 5’ – CCTGGTGTAACTGATGCTGCCA – 3’
       COL6A3-R: 5’ – AAGATGGCGTCCACCTTGGACT – 3’

LIF: LIF-F: 5’ – AGATCAGGAGCCAACCTGGCACA – 3’
     LIF-R: 5’ – GCCACATAGCTTGTCCAGGTTG – 3’

FAS: FAS-F: 5’ – GGACCCAGAATACCAAGTGCAG – 3’
     FAS-R: 5’ – GTTGCTGGTGAAGTGTGCATTCC – 3’

WDR63: WDR63-F: 5’ – CCACCTGAAAGATACCAGTCC – 3’
        WDR63-R: 5’ – AAGAAAGTCGACGGCTTCCGAAGCT – 3’

INPP5D: INPP5D-F: 5’ – TGTGACCGAGTCTCCTGGAAGT – 3’
        INPP5D-R: 5’ – GCCCTAAATGTGGAAGACAGG – 3’

Preparation and quantification of MSC mtDNA in GSCs
DNA from GSCs was prepared using a TRizol-based protocol as previously described (Nakhle et al., 2020). Briefly, GSCs (5 x 10⁵ cells) were homogenized in 700 μL TRizol reagent. After addition of chloroform, the upper aqueous phase was recovered and DNA was precipitated in the presence of glycogen (Invitrogen 10814010) and isopropyl alcohol.
Mitochondrial DNAs were quantified by PCR by using the SYBR Green Master PLUS Mix (Roche 03515885001) and the LightCycler 480 instrument (Roche, Meylan, France), with the following program: 10 min at 95 °C, 50 cycles of 10 s at 95 °C, 15 s at 67 °C and 15 s at 72 °C. Total mitochondrial DNA (mtDNA) was quantified by amplifying a DNA domain within the D-loop of mtDNA by using the following primers: Universal-F: 5'- TTA ACT CCA TTA GCA CC -3'; Universal-R: 5'- GAG GAT GGT GGT CAA GGG A -3' (Lyons et al., 2013). To specifically amplify mtDNA from MSCs (donor MSC119), the following set of primers was used: MSC-F: 5'-AAG CAA GTA CAA TCA ACC CC-3'; MSC-R: 5'-TTA AGG GTG GGT AGG TTT GTA GC-3' (Nakhle et al., 2020). To increase the specificity of the MSC primers for MSC mtDNA, by further diminishing their binding capacity to GSC mtDNA, an additional mismatch was introduced in the MSC primer sequences. This mismatch was found to decrease the efficacy of the initial PCR amplification cycles of the MSC mtDNA. This technically-based difference in the PCR cycle numbers (5.5 cycles) was taken into account in the calculation of the amount of MSC mtDNA in the GSCs following the transfer of MSC mitochondria by Mitoception.

**Imaging**

Cell imaging was performed on GSCs and MSCs respectively labeled, prior to the coculture, with the vital dyes Green CellTracker CMFDA (4 uM, Molecular Probes C2925) and Red MitoTracker CMXRos (500 nM, Molecular Probes M7512). The coculture was performed in αMEM/FCS 5% in 6-well plates. Imaging started 24 hr after the beginning of the coculture. Confocal fluorescence imaging was done on live cells with a Carl Zeiss LSM 5 live duo (LSM 510 META and 5 live) confocal laser system using a Zeiss 40X plan NeoFluar Oil objective. Time-lapse imaging was performed with an IncuCyte® S3 Live-Cell Analysis System with a 10X objective. Pictures were taken every 30 minutes for 72 hours, for Green CellTracker, Red MitoTracker and phase contrast, with acquisition times of 250 ms for the green channel and 400 ms for the red channel. Stacks of images were exported in Tagged Image File Format (TIFF) and treated with the ImageJ image processing program. Backgound was removed from the green and red channel image sequences by using the Substract Background function (Rolling ball radius: 50 pixels). Phase image sequences were first stabilized (Image Stabilizer Log Applier plugin). The same log transformation coefficients were applied to the green and the red sequences to align the fluorescent images to the phase (Image Stabilizer Log Applier plugin). The colors of the phase images were inverted and all three sequences inverted phase, green and red sequences were merged using the ImageJ Image Calculator command. After treatment, all image sequences were exported as .AVI files (compression: JPEG and Frame Rate: 4 fps).

**Quantification and statistical analysis**

Statistical analyses were carried out using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA).
Statistical assays were performed as described in each figure legend. Multiple samples were analyzed by one-way analysis of variance (ANOVA) and Tukey post-hoc test to evaluate statistical differences among the samples. Differences were considered statistically significant for $p < 0.05$ (\*\(P \leq 0.05\), \**\(P \leq 0.01\), \***\(P \leq 0.001\)). All data are presented as mean values with S.E.M.

**SUPPLEMENTARY VIDEO, FIGURES AND TABLES**

**VIDEO 1** Dynamic interactions between MSCs and GSCs leading to MSC mitochondria transfer to GSCs

**FIGURE S1** Quantification of MSC mtDNA in GSCs

**FIGURE S2** Survival of GSCs in response to TMZ treatment

**FIGURE S3** MSC mitochondria modify the usage of metabolites by GCSs in response to TMZ

**FIGURE S4** Metabolites produced by GSCs following MSC mitochondria acquisition and TMZ treatment

**FIGURE S5** RNAseq validation

**REFERENCES**


Figure 1
Isolation of mesenchymal stem cell mitochondria

Mitochondria transfer by MitoCeption

Quantification of mitochondria acquisition by qPCR

mtDNA

Energetic metabolism by Seahorse

Substrate usage by MitoPlates

Metabolite production by mass spectrometry

Mitochondrial mass, ROS production and viability by FACS

Gene expression by RNAseq

Dissociation of glioblastoma stem cell neurospheres

Temozolomide treatment

Mitochondria conditioning (72 hr)

Temozolomide treatment (48 h)
Figure 3

A. Oxygen Consumption Rate

B. Basal respiration vs. Maximal respiration

C. Extracellular Acidification Rate

D. Glycolysis vs. Glycolytic Capacity

E. OCR vs. ECAR

F. Cell number over time

G. Total mtDNA relative to genomic DNA over time
Figure 4

A. Oxygen Consumption Rate

B. Basal respiration

C. Extracellular Acidification Rate

D. Glycolysis

E. Mitochondrial Mass (48 hr)

F. Mitochondrial Mass (72 hr)

G. Respiration linked to ATP production

H. Maximal respiration

I. Respiration linked to ATP production

J. Relative Mean Fluorescence Intensity

K. Relative COX IV expression

L. Relative Lactate Acidification

M. Relative Mean Fluorescence Intensity

N. Relative COX IV expression

O. Relative Lactate Acidification

P. Oxygen Consumption Rate

Q. Basal respiration

R. Extracellular Acidification Rate

S. Glycolysis

T. Glycolytic capacity

U. Lactate acidification

V. Mitochondrial Mass (48 hr)

W. Mitochondrial Mass (72 hr)

X. COX IV (48 hr)

Y. COX IV (72 hr)
Figure 6

A

CTRL

TMZ

Mitoxantrone

Mitoxantrone + TMZ

Cell death

Cell number

Mitochondrial ROS (48 hr)

Mitochondrial ROS (72 hr)

Mitochondrial SOD2 (48 hr)

Mitochondrial SOD2 (72 hr)

Cell death (Zombie positive (%))

Cell number

Mitochondrial ROS

Mitochondrial SOD2

Mitochondrial SOD2

Cell death (Zombie positive (%))

Cell number

Mitochondrial ROS

Mitochondrial SOD2

Mitochondrial SOD2

Cell death (Zombie positive (%))

Cell number

Mitochondrial ROS

Mitochondrial SOD2

Mitochondrial SOD2

Cell death (Zombie positive (%))

Cell number

Mitochondrial ROS

Mitochondrial SOD2

Mitochondrial SOD2

Cell death (Zombie positive (%))

Cell number

Mitochondrial ROS

Mitochondrial SOD2

Mitochondrial SOD2
Figure 7

A

B

C

D

signal transduction involved in DNA integrity checkpoint
signal transduction involved in DNA damage checkpoint
signal transduction involved in cell cycle checkpoint
regulation of protein tyrosine kinase activity
regulation of extrinsic apoptotic signaling pathway
negative regulation of signal transduction
negative regulation of response to stimulus
negative regulation of proteolysis
negative regulation of peptide activity
negative regulation of G1/S transition
negative regulation of cell cycle G1/S phase
intrinsic apoptotic signaling by p53 mediator
apoptotic signaling pathway
regulation of nucleobase metabolism
negative regulation of apoptotic signaling pathway
response to abiotic stimulus
regulation of RNA metabolic process
negative regulation of developmental growth
DNA damage response, p53-related cell cycle arrest
response to mechanical stimulus
regulation of cellular macromolecule biosynthetic process
regulation of cellular biosynthetic process
regulation of macromolecule biosynthetic process
regulation of apoptotic signaling pathway
regulation of transcription, DNA-templated
regulation of nucleic acid-templated transcription
regulation of RNA biosynthetic process
DNA damage response, p53-related signal transduction
Quantification of MSC mtDNA in GSCs.
The detection of MSC mtDNA (donor MSC119) in GSCs (Gli4 clone) was determined by qPCR 24 hrs after the transfer of MSC mitochondria to GSCs by Mitoception. Shown are the relative concentrations of MSC mtDNA relative to GSC total mtDNA concentrations. Each dot corresponds to an individual GSC Mitocepted sample, which were obtained from 3 independent experiments. Relative MSC mtDNA quantification represented as median with interquartile range. Two-tailed unpaired t tests, \( **p \leq 0.01 \).
**Figure S2**

Survival of GSCs in response to TMZ treatment
GSC cell number as measured by cell counting following 6-day TMZ treatment at the indicated concentrations. TMZ was added at the time of GSC seeding in poly-D lysine coated wells. Four independent experiments, each with 4 replicates. Tukey boxplots with two-tailed unpaired t tests, ***p ≤ 0.001.
MSC mitochondria modify the usage of metabolites by GCSs in response to TMZ
(A) Metabolic substrate consumption of GSCs expressed as metabolite initial consumption rates (Biolog MitoPlates). Mean values ± SEM (4 independent experiments).
(B) Production of cis-aconitate, succinate and L-malate expressed as a percentage of all TCA metabolites produced in each experimental condition. Tukey boxplots with two-tailed unpaired t tests, *p ≤ 0.05, ***p ≤ 0.001.
Figure S4  Metabolites produced by GSCs following MSC mitochondria acquisition and TMZ treatment

A

B

C

D

E
Figure S5  RNAseq validation

Relative gene expression as determined by RNA-seq (upper panel, n = 3) and RT-qPCR (lower panel, n = 9)
In order to be able to quantify mesenchymal stem cell mitochondria acquisition on the basis of mitochondrial DNA single nucleotide polymorphisms, I was looking for a reproducible nucleic acid isolation method that allows to minimize the manipulation time and to save up the starting biological material. Therefore, I compared two different protocols using commercially-available, widely-used products: Invitrogen TRizol and Qiagen DNeasy columns. Invitrogen TRizol reagent is commercialized for RNA extraction in the upper, aqueous phase whereas Qiagen DNeasy columns are conceived for total DNA isolation. Much to my surprise, my results showed that both the aqueous phase in the TRizol-based method and the eluent in the Qiagen DNeasy columns allowed the simultaneous isolation of RNA, mitochondrial DNA and nuclear DNA from mammalian cells, which goes beyond the advertised properties of these protocols. These results could be of great interest for the numerous TRizol reagent and DNeasy column users, who are mostly unaware of these properties. It could be helpful for other researchers to have access to such technical information, which would save them a lot of time when starting their research project, in order to use the appropriate nucleic acid isolation method for their subsequent experiments. These results were published as a technical article in *BioTechniques*. 
Methods for simultaneous and quantitative isolation of mitochondrial DNA, nuclear DNA and RNA from mammalian cells

Jean Nakhle1,1,2, Tülin Özkan1,1,3,4, Kateřina Lneníčková1,4, Philippe Briolotti1 & Marie-Luce Vignais3,1

1 Institute for Regenerative Medicine & Biotherapy (IRMB), INSERM, Univ Montpellier, F-34090 Montpellier, France; 2 Institute of Molecular Genetics of Montpellier (IGMM), CNRS, Univ Montpellier, F-34090 Montpellier, France; 3 Faculty of Medicine, Department of Medical Biology, University of Ankara, Ankara, Turkey; 4 Department of Medical Chemistry & Biochemistry, Faculty of Medicine & Dentistry, Palacky University, Olomouc, Czech Republic; *Author for correspondence: marie-luce.vignais@inserm.fr; † Authors contributed equally.

ABSTRACT
The aim of this study was to assess two protocols for their capacities to simultaneously isolate RNA, mtDNA and ncDNA from mammalian cells. We compared the Invitrogen TRIzol-based method and Qiagen DNeasy columns, using the HepG2 cell line and human primary glioblastoma stem cells. Both methods allowed the isolation of all three types of nucleic acids and provided similar yields in mtDNA. However, the yield in ncDNA was more than tenfold higher on columns, as observed for both cell types. Conversely, the TRIzol method proved more reproducible and was the method of choice for isolating RNA from glioblastoma cells, as demonstrated for the housekeeping genes RPLP0 and RPS9.

METHOD SUMMARY
Here we compare two methods – Invitrogen TRIzol reagent and Qiagen DNeasy columns – for simultaneously extracting RNA, mtDNA and ncDNA from mammalian cells.

KEYWORDS:
mammalian cells • mitochondria • mitochondrial DNA (mtDNA) • nuclear DNA (ncDNA) • RNA

Mitochondria are endowed with essential cellular functions. Mitochondrial dysfunctions, which are caused by mutations in both ncDNA and mtDNA, are associated with a number of severe metabolic diseases [1–3]. Changes in mtDNA copy number have also been directly associated with aging and disease [4–9]. As demonstrated recently, mitochondria and their intrinsic mtDNA are also exchangeable between cells, resulting in the metabolic reprogramming of the recipient cells, in tissue repair and cancer progression [1,10–13]. This metabolic and functional reprogramming is also associated with modifications of the cellular gene expression pattern. In metabolic diseases originating from mtDNA mutations, the degree of heteroplasmy – the percentage of mutated mtDNA – is an essential criterion for the declaration of the pathology. For intercellular mitochondrial exchange, the concentration of exogenous mitochondria also determines the phenotype of the recipient cells. Therefore, in both cases, gene expression as measured by mRNA levels needs to be assessed as a function of the mtDNA concentrations in order to establish mechanistic links. This prompted us to identify robust methods to concurrently isolate the mtDNA and RNA in these eukaryotic cells. In addition, we were looking for a protocol that allowed the simultaneous isolation of RNA and DNA in order to save both the starting biological material and isolation time. A number of protocols have already been proposed to isolate RNA [14–16], mtDNA [15,17,18] or ncDNA [14,15,19] and most commercial kits are recommended for the exclusive isolation of either RNA or DNA. However, this is more costly and requires more biological material.

We assessed two experimental methods for nucleic acid isolation and tested their yields in mRNA, mtDNA and ncDNA. One of these methods was based on the Invitrogen TRIzol™ reagent, designed primarily for RNA isolation from the aqueous extraction phase [20]. The second method used the Qiagen DNeasy Blood & Tissue Kit, based on the known silica-adsorption properties of DNA [21] and designed for the rapid purification of total DNA (i.e., ncDNA and mtDNA). We tested whether the aqueous phase recovered in the TRIzol-based protocol also contained ncDNA and mtDNA and allowed their quantitative recovery. Conversely, we tested whether the DNeasy Blood & Tissue Kit also allowed the quantitative recovery of RNA. We performed these tests on the HepG2 adherent human hepatocyte cell line and on nonadherent human primary glioblastoma stem cells (GSCs) (clone Gli4) [22].

Materials & methods

Cell culture

The human hepatocyte cell line HepG2 was grown in Minimum Essential Medium Eagle, alpha modification (Lonza Bioscience BE12-169F), supplemented with 10% fetal bovine serum (Sigma-Aldrich F7524) and 2 mM L-glutamine (Gibco 25030024).
Human primary GSCs (clone GlI4 [22]) were grown as spheroids in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Gibco 21331046), supplemented with 2 mM L-glutamine (Gibco 25030024), 0.3% D-glucose (Sigma-Aldrich G8769), 0.002% bovine insulin (Sigma-Aldrich I1882), N-2 supplement (Gibco 17502048), B-27 supplement (Gibco 12587010), 10 ng/ml human EGF (Miltenyi Biotec 130-097-750) and 10 ng/ml human FGF-2 (Miltenyi Biotec 130-104-924).

Human mesenchymal stem cells (MSCs) were isolated from the bone marrow of healthy donors at the authorized cell therapy unit (Biotherapy Team of General Clinic Research Center, French health minister agreement TCG/04/0/008/AA) at the Grenoble University Hospital. MSCs were cultured in Minimum Essential Medium Eagle, alpha modification (Lonzia Bioscience), supplemented with 10% fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine (Gibco) and 1 ng/ml FGF-2 (Miltenyi Biotec). All cell types were cultured at 37°C in 5% CO2.

Nucleic acid isolation by the TRizol method & on column

Nucleic acids were isolated from 5 × 10^5 cells. The TRizol reagent (Invitrogen 15596026) procedure was performed according to the manufacturer's instructions. Briefly, cells were homogenized in 700 µl TRizol reagent. The solution was separated into the two phases, aqueous and organic, by adding 200 µl chloroform. The upper aqueous phase was gently recovered, without touching or aspirating the interphase, and transferred to a microtube containing 1.5 µl glycogen (Invitrogen 10814010). The nucleic acids contained in this phase were precipitated with 500 µl isopropyl alcohol, washed twice with 900 µl ice-cold 70% ethanol, air-dried and resuspended in RNase-free water. The centrifugations for collecting and rinsing the nucleic acid pellets were all performed at 12,000 × g, 4°C, for 15 min. Nucleic acid isolation with the Qiagen DNeasy Blood and Tissue kit (Qiagen 69506) was also performed according to the manufacturer's instructions. Briefly, cells were resuspended in 200 µl phosphate-buffered saline to which 20 µl proteinase K was then added. After the addition of 200 µl buffer AL, the samples were vortexed. Ethanol (100%, 200 µl) was then added and the samples vortexed again. The mixture was pipetted into a spin column which was then centrifuged at 6000 × g for 1 min on top of a collecting tube. The column was then rinsed twice, first with 500 µl buffer AW1 (centrifugation at 6000 × g for 1 min) and second with 500 µl buffer AW2 (centrifugation at 20,000 × g for 3 min). Nucleic acids were eluted with 200 µl buffer AE (centrifugation at 6000 × g for 1 min). All centrifugations were performed at room temperature. Nucleic acid concentrations and purity were assessed with a NanoDrop spectrophotometer.

Transfer of MSC mitochondria to GSCs by MitoCeption

The transfer of MSC mitochondria to GSCs was performed as previously described [23,24]. Briefly, GSC spheroids were dissociated 24 h prior to the mitochondrial transfer and seeded as single cells immediately before the mitochondria transfer. On the day of mitochondria transfer, MSCs (5 × 10^5) were trypsinized in the absence of EDTA (Gibco 15090046). MSCs were mechanically lysed using a syringe with 25- and 27-gauge needles. MSC mitochondria were isolated by two differential centrifugations of 10 min each, at 800 × g and 8000 × g respectively, using a buffer containing 200 mM mannitol (Sigma-Aldrich M1902), 70 mM saccharose (Sigma-Aldrich S0389), 1 mM EDTA (Sigma-Aldrich E9884), 10 mM HEPES (pH 7.4) (Sigma-Aldrich H3375) and 1× protease and phosphatase inhibitor cocktail (Roche, Meylan, France; 04693159001). MSC mitochondria were then added to the previously-seeded single-cell GSCs and centrifuged at 3000 rpm for 15 min. GSCs were incubated for 24 h at 37°C in 5% CO2.

qPCR (mtDNA & ncDNA)

Real-time quantitative PCR to quantify mtDNA and ncDNA was performed on 30 ng nucleic acids, directly after the nucleic acid isolation step, by using the SYBR Green Master Plus Mix (Roche 03515885001) and the LightCycler 480 instrument (Roche), with the following program: 10 min at 95°C, 50 cycles of 10 s at 95°C, 15 s at 67°C and 15 s at 72°C. ncDNA was quantified by using the following primers: β-globin-F: 5’-ACACAACTGTTCCGCTAGC-3’; β-globin-R: 5’-CCAACCTTATCCAGTTCA-3’, targeting the nuclear β-hemoglobin gene. Total mtDNA was quantified by amplifying a DNA domain within the D-loop of mtDNA by using the following primers: Universal-F: 5’-TTAATTCTCACCATTAGCACC-3’; Universal-R: 5’-GAGGATGTTGCTCAAGGGA-3’ [25]. To specifically amplify mtDNA from MScs (donor MSC19), the following set of primers was used: MSC-F: 5’-AAGCAAGTACAGCAATCAACCCC-3’; MSC-R: 5’-TTAAGGGTTGGTAGTTGTAGC-3’. The amplification efficiencies with the different sets of primers were verified with serial DNA dilutions.

mRNA quantification

RNAs were reverse transcribed using the M-MLV RT kit (Invitrogen 28025013) and random hexamers (Invitrogen N8080127). Real-time quantitative PCR was performed using the SYBR Green Master Mix (Roche 04887352001), with 250 nM primer concentrations and the LightCycler 480 instrument (Roche), with the following program: 10 min at 95°C, 50 cycles of 10 s at 95°C, 15 s at 65°C and 15 s at 72°C. The following primer sets were used for RPLP0 and RPS9: RPLP0-F 5’-TGCAACTATGCAATC-3’, RPLP0-R 5’-GCCTGACCTTCCCAGCA-3’, RPS9-F 5’-ATGAAAGACGGAGGTGTCCAC-3’, RPS9-R 5’-GATTACATCTGGGCTGAA-3’.

Statistical analysis

Data were analyzed using GraphPad Prism 7 (GraphPad Software Inc., CA, USA). All data are presented as mean values with standard deviations. Unpaired Student t-tests with Welch's correction were performed to compare the concentrations of mtDNA, ncDNA and mRNA. Differences were considered statistically significant for p < 0.05 (*p < 0.05, **p < 0.01, ***p < 0.001).
Results & discussion

Isolation of mtDNA & ncDNA

We first showed that the TRIzol-based method allowed the isolation of both nuclear and mitochondrial DNA from the HepG2 cell line (Figure 1A). Both ncDNA and mtDNA were isolated from the aqueous phase also containing RNA, which contributes to a simplified isolation procedure. Of note, we were unsuccessful in isolating DNA from the TRIzol organic phase as we encountered both low isolation yields and reproducibility with the two human cell lines we tested. Isolation of mtDNA from the TRIzol aqueous phase showed high reproducibility and the yield with the column method was only 1.7-fold higher than with the TRIzol method. However, the column appeared superior for ncDNA isolation, providing a 21.3-fold higher yield than the TRIzol method. Similar results were obtained when DNA was isolated from human primary GSCs (Gli4 clone [22]). While the mtDNA yields with the column were only slightly higher than with the TRIzol method (1.3-fold), ncDNA isolation using the column was 14.5-times more efficient than with the TRIzol method (Figure 1B).

Overall, these data show that both methods are highly reliable for quantitatively isolating mtDNA, while the column proved more efficient than the TRIzol-based method for ncDNA isolation.

Isolation of mRNA

We next checked whether the Qiagen DNeasy column procedure also allowed the isolation of mRNA. For this purpose, we prepared cDNA from the Qiagen DNeasy column samples, for both the HepG2 cells and the GSCs, and measured the expression of the two housekeeping genes RPLP0 and RPS9. The same was done with the samples prepared by the TRIzol method. Interestingly, the HepG2 samples prepared on the Qiagen DNeasy columns yielded mRNAs in amounts close to those obtained with the TRIzol-based isolation method, albeit with yield differences depending on the analyzed genes (Figure 2A). For GSCs, the mRNA recovery appeared higher with the Qiagen DNeasy columns (Figure 2B). However, the isolation efficiency appeared less reproducible than with the TRIzol procedure, with more widespread mRNA concentrations, as indicated by standard deviations that were on average twice as big for the column samples than for the TRIzol-derived samples. These measures indicated that both the TRIzol-based and column methods allow the concurrent isolation of mRNA, mtDNA and ncDNA. However, the TRIzol-based method is to be preferred whenever experimental priority needs to be given to mRNA and mtDNA.

Application of the TRIzol-based method to quantify exogenous mtDNA following mitochondrial intercellular transfer

One of our research goals is to determine changes of gene expression in GSCs following the acquisition of exogenous mitochondria from MSCs. On the basis of the above study, we opted for the TRIzol-based method because it provides efficient and reproducible
Figure 2. Comparison of the mRNA extraction yields by TRizol reagent and Qiagen DNeasy Blood and Tissue kit. Samples of (A) hepatocellular carcinoma cells (HepG2) and (B) human primary glioblastoma cells (Gli4) were used to extract nucleic acids, by using either the Invitrogen TRizol-based method \( n = 10 \) or the Qiagen DNeasy columns \( n = 10 \). cDNA was prepared and the relative concentrations of mRNA for the housekeeping genes RPLP0 and RPS9 were determined by qPCR. Each dot represents an independent extraction. Represented are the means ± standard deviation. Unpaired Student t tests with Welch’s correction were performed.

GSC: Glioblastoma stem cell.

isolation of mRNA as well as mtDNA (required to quantify the amounts of acquired exogenous mitochondria). qPCR quantification of the exogenous mtDNA is based on the recognition of single nucleotide polymorphisms specific to the cell donor [23,24]. We first validated the qPCR conditions for specifically detecting MSC mtDNA (and not GSC mtDNA). For this, we isolated DNA from both the GSCs (Gli4) and the MSCs (MSC119) using the TRizol-based method. We then performed serial dilutions of the MSC DNA (30 to \( 6 \times 10^{-2} \) ng) in a solution containing the GSC DNA (total DNA amount 30 ng) and PCR-amplified the MSC mtDNA. As shown by the linear regression, this provided the range of quantitative detection for MSC119 mtDNA in GSC-Gli4 by qPCR (Figure 3A). We then transferred mitochondria, isolated beforehand from MSCs (MSC119), to GSCs (clone Gli4) using the MitoCeption protocol [23,24]. Following the transfer, we used the TRizol-based method to isolate DNA from GSCs and determined the concentrations of MSC mtDNA by qPCR. This allowed us to demonstrate the dose–response acquisition of MSC mitochondria by the GSCs (Figure 3B). Additionally, the functional effects of MSC mitochondria acquisition were determined by quantifying mRNA concentrations and gene expression in these samples (data not shown). Overall, these data demonstrate that the TRizol-based method allows the simultaneous and quantitative isolation of both mtDNA and mRNA from mammalian cells, and from GSCs in particular, and can be exploited in the context of intercellular mitochondrial transfers.
Figure 3. Quantification of mesenchymal stem cell mtDNA following the transfer of mitochondria to glioblastoma stem cells. (A) The range of detection of MSC (donor MSC119) mtDNA in GSCs (Gli4 clone) was determined by qPCR quantification of MSC mtDNA, following serial dilutions of MSC DNA with GSC DNA, both isolated by the TRIzol method. Two independent experiments were performed. Data are presented on a semi-log scale and shown are the line and equation of interpolation best-fit. (B) MSC mitochondria were transferred in increasing amounts to GSCs by MitoCeption. The following day, total DNA was isolated by the TRIzol-based method and the relative concentrations of MSC mtDNA were determined by qPCR and by using the titration curve shown in (A). Shown are the measured concentrations of MSC mtDNA as a function of transferred MSC mitochondria, expressed as relative values. Each dot corresponds to an individual TRIzol extraction; data were obtained from four independent MitoCeption experiments. Data are presented on logarithmic scales (Log2) and shown are the line and equation of best-fit.

GSC: Glioblastoma stem cell; MSC: Mesenchymal stem cell.

As already observed [26–28], the respective yields in mitochondrial and nuclear DNAs are different depending on the DNA isolation method used. As the concentrations of mtDNA and ncDNA are often used to determine the number of mitochondria per cell, these method-related differences underline the caution required when drawing this type of conclusion.

Future perspective

The role played by mtDNA is beginning to emerge, in the fields of both mitochondrial intercellular exchanges and mitochondrial diseases (originating either from mutations in the mitochondrial DNA or from changes in mitochondrial DNA copy number). Studies in these fields need precise quantification of, first, the mtDNA concentrations of exogenous versus endogenous mitochondria and second, mutant versus wild-type mtDNA concentrations; therefore protocols enabling quantitative isolation are valuable. This further supports the need for robust and rapid mtDNA and mRNA isolation methods, as the characterization of the mtDNA sequence and copy number along with the gene expression pattern will be valuable for more accurate clinical evaluation. Studies on mtDNA characterization constitute an active and rapidly evolving field, as illustrated by novel technologies aiming at, for instance, single-cell mtDNA PCR detection [29,30]. In addition to qPCR, other technical approaches to determine mitochondrial DNA copy number have recently been proposed – for example, whole genome sequencing [31] – opening further possibilities for characterizing mitochondrial DNA sequences and copy number and, in the longer term, therapeutic manipulation of heteroplasmy [32–34].

Author contributions

The experiment was designed by J Nakhle, T Özkan and M Vignais and was undertaken by J Nakhle, T Özkan, K Lněničková and P Briolotti. The manuscript was prepared by J Nakhle, T Özkan and M Vignais. All authors had the opportunity to read and approve the manuscript.
Acknowledgments
We thank M Daujat-Chavanieus and S Gerbal-Chaolain for helpful discussions. The authors declare that there is no conflict of interest regarding the publication of this paper. No writing assistance was utilized in the production of this manuscript.

Financial & competing interests disclosure
This work was supported by grants from the Ligue Contre le Cancer-Comité du Gard and ARC. J Nakhlé was supported by a PhD fellowship from the French Ministry of Research (MESRI), T Özkan by a post-doctoral research fellowship from the Scientific and Technological Research Council of Turkey (TUBITAK) and K Lneníková by the Mobility Support project at UP, CZ.02.2.69/0.0/0.0/16_027/0008482. P Briolotti and M Vignais are staff scientists from INSERM and CNRS, respectively. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research
Human MSCs were isolated from bone marrow from healthy donors at the authorized cell therapy unit (Biotherapy Team of General Clinic Research Center, French health minister agreement TCG/04/0/008/AA) at the Grenoble University Hospital. The line of GSCs that was used was previously published.

Open access
This work is licensed under the Attribution-NonCommercial-NoDerivatives 4.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/4.0/

References
DISCUSSION & PERSPECTIVES
The medical bottleneck for glioblastoma treatment likely stems from a partial understanding of the tremendous intra-tumoral heterogeneity of this devastating malignant brain tumor, which highlights its inherent plasticity. The ability of glioblastoma stem cells to adapt their functioning to microenvironmental changes – especially, to benefit from and to subdue cells of their microenvironment in their own favor – may have been underestimated thus far. In particular, the cellular interactions of glioblastoma stem cells with their microenvironment may modify their metabolic features and, therefore, change their response to therapy. The mechanisms underlying this phenomenon certainly deserve more thorough investigation. In this study, we show that mesenchymal stem cell mitochondria modify the metabolic and functional response of glioblastoma stem cells to chemotherapy by temozolomide. In particular, mesenchymal stem cell mitochondria increased the oxidative phosphorylation of glioblastoma stem cells along with their production of metabolites belonging to the TCA, pentose phosphate and pyrimidine synthesis pathways. In addition, mesenchymal stem cell mitochondria changed the glioblastoma stem cell transcriptional pattern, especially the expression of genes related to cell cycle progression. These observations were linked to an enhanced survival of glioblastoma stem cells against cell death by temozolomide.

**Experimental model**

In our study, we used primary human glioblastoma stem cells of mesenchymal subtype that expressed several markers of neural precursors (CD133, CD15, NESTIN, OLIG2, SOX2) (Guichet et al., 2013). However, Verhaak and colleagues raised the question of the existence of several glioblastoma stem-like cell subpopulations, each giving rise to a distinct glioblastoma subtype (Verhaak et al., 2010). Later on, Neftel challenged this hypothesis, by demonstrating that each glioblastoma tumor contained cells belonging to different subtypes and endowed with high plasticity, i.e., that could transition between different cellular states in response to microenvironmental cues or to chemical insults (Neftel et al., 2019). Considering this high inter- and intra-tumoral heterogeneity in glioblastoma, it could be of interest to examine if the same results we observed using glioblastoma stem cells of mesenchymal subtype – namely, the metabolic and transcriptional modifications following dual mitochondrial acquisition and temozolomide treatment – could also be reproduced in glioblastoma stem cells belonging to other subtypes (pro-neural, classical). In addition, we used bone marrow mesenchymal stem cells of healthy donors. We plan to extend the study to mesenchymal
stem cells isolated from glioblastoma resected tumors. This could be of interest since mesenchymal stem cells of the glioblastoma microenvironment are not all homogenous, and could differ in their origin, genetic pattern, surface marker expression and function (Hossain et al. 2015; Svenssen et al., 2017; Zhang et al., 2018; Clavreul and Menei, 2020). For instance, CD90\textsuperscript{high} mesenchymal stem cells were shown to increase glioblastoma proliferation and migration, whereas CD90\textsuperscript{low} mesenchymal stem cells mainly participated in angiogenesis (Zhang et al., 2018). This would enable us to determine whether the mitochondria acquisition from different mesenchymal stem cells elicits different effects on recipient glioblastoma stem cells.

**Tunneling nanotube-formation and mitochondria transfer between mesenchymal stem cells and glioblastoma stem cells in co-culture**

We performed *in vitro* co-cultures between mesenchymal stem cells whose mitochondria were labeled with a red MitoTracker and glioblastoma stem cells labeled with a green CellTracker. Using confocal fluorescence microscopy, we showed that mesenchymal stem cells engaged in long cell-cell connections and transferred mitochondria to glioblastoma stem cells. This suggests that (1) the intercellular connections between mesenchymal stem cells and glioblastoma stem cells are open-ended and that (2) they accomplish a functional role, in this case, of cargo transfer. Both these conditions fit the definition of a tunneling nanotube (Pinto et al., 2020). Using time-lapse microscopy, we observe that this cell-cell interaction corresponds to the cell dislodgment model of tunneling nanotube biogenesis, where the mesenchymal stem cell and the glioblastoma stem cell, initially in close physical proximity, drifted apart and extended a thin membranous thread between them (Abounit et al., 2015). Interestingly, the interaction between both cells was very dynamic, as the intercellular connection was elongated (first tunneling nanotube), resorbed, then elongated once again (second tunneling nanotube). The total duration of this interaction was over 14 hours, with each tunneling nanotube lasting for about 5 hours. This lifespan is longer than what was previously described (Osswald et al., 2019). Several studies indeed reported that tunneling nanotubes were very transient structures, lasting for 4 – 7 minutes in the case of rat pheochromocytoma cells (Rustom et al., 2004; Bukoreshtliev et al., 2009) and 30 minutes for colon cancer cells (Lou et al., 2018). Very few tunneling nanotubes were observed to last longer than 2 hours (Bukoreshtliev et al., 2009; Lou et al., 2018).
The limitation of fluorescence microscopy in the tunneling nanotube research field, however, is that it only highlights the transport of the labeled cargo – i.e., of mitochondria – and does not consider other cargos that could be transferred through the same connections, including those that could be transferred in the opposite direction. This is all the more important since Zurzolo and collaborators elegantly demonstrated, using correlative light- and cryo-electron microscopy, that a single tunneling nanotube observed between neuronal cells by fluorescence microscopy is in fact made out of several individual tunneling nanotubes (iTNTs) tied together by N-cadherins (Sartori-Rupp et al., 2019). Noteworthily, the authors also showed that each iTNT contained one actin bundle organized in a highly-ordered fashion, conserving the same polarity all along the tube. This suggests that each iTNT only allows a unidirectional cargo transport. In other terms, iTNTs extending from opposing cells would have opposite polarities, thereby allowing a unidirectional transport each, but in opposite directions (Sartori-Rupp et al., 2019), which could explain the “bidirectional transfer” previously observed inside what was mistaken as a single TNT by fluorescence microscopy (Zhu et al., 2016). It would be interesting to examine the structure of TNTs between mesenchymal stem cells and glioblastoma stem cells (actin filaments vs. microtubules) as well as the motors involved in mitochondria transfer. While microtubules and their motors have been established as important factors for mitochondria trafficking (Melkov et al., 2018), increasing pieces of evidence indicate that mitochondria are able to interact with the actin cytoskeleton in many cell types, in a microtubule-independent mechanism (Quintero et al., 2009; Pathak et al., 2010; Sartori-Rupp et al., 2019).

**Mesenchymal stem cell mitochondria isolation and MitoCeption**

In order to determine the effect of mesenchymal stem cell mitochondria on glioblastoma stem cell metabolism, we took advantage of the laboratory’s MitoCeption protocol. MitoCeption consists of isolating mitochondria from a cell type A and transferring them to a cell type B by co-incubation (Caicedo et al., 2015). Internalization of isolated mitochondria is believed to occur via macropinocytosis (Kitani et al., 2014). The MitoCeption technique allows to determine the exclusive effects of mitochondria on the recipient cells without any intervention from other microenvironmental cues. In order to isolate mesenchymal stem cell mitochondria, we used a buffered, iso-osmotic monosaccharide solution containing mannitol and sucrose. This solution presents two main advantages in comparison to other
mitochondria isolation techniques, as it was shown (1) to conserve mitochondrial integrity, coupling and respiration and (2) to generate a better mitochondrial purification yield by limiting microsome and Golgi contaminations (Corcelli et al., 2010). We then confirmed the integrity of isolated mesenchymal stem cell mitochondria by Western Blot detection of TOM20 and COX IV, outer- and inner-mitochondrial membrane proteins, respectively (data not shown).

**Quantification of acquired mesenchymal stem cell mitochondria**

We showed that a minute amount of mesenchymal stem cell mitochondrial DNA (less than 1% compared to endogenous mitochondrial DNA) is sufficient to elicit major metabolic and functional effects in glioblastoma stem cells. This observation is comparable to previous studies that reported the internalization of 0.43% of mesenchymal stem cell mitochondrial DNA relative to endogenous mitochondrial DNA in MDA-MB-231 breast cancer cells (Caicedo et al., 2015). In addition, we show that mesenchymal stem cell mitochondria increase the total mitochondrial DNA content of glioblastoma stem cells at 48 hours by 2-fold. This is in line with previous studies reporting an increase of 1.13-fold (Moschoi et al., 2016) and 1.27-fold (Caicedo et al., 2015). Interestingly, Caicedo reported that this increase was not due to exogenous mitochondrial DNA; instead, it was due to increased amounts of endogenous DNA. This suggests that acquired mesenchymal stem cell mitochondria could possibly enhance endogenous mitochondrial DNA replication or inhibit its degradation (Caicedo et al., 2015).

The quantification of acquired mesenchymal stem cell mitochondria was undertaken on the basis of single nucleotide polymorphisms present in the mitochondrial DNA, as previously described (Caicedo et al., 2015; Nzigou Mombo et al., 2017). Mitochondrial DNA was isolated using the TRIzol reagent. We have previously demonstrated that the TRIzol-based nucleic acid isolation method allows the simultaneous and quantitative isolation of both mitochondrial and nuclear DNAs (in addition to RNA) from glioblastoma stem cells, and can be exploited in the context of mitochondria transfers (Nakhle et al., 2020). Expressing the amount of internalized exogenous mitochondria in terms of the amount of exogenous mitochondrial DNA inside the recipient cells is one of the most accurate techniques because it resolves two major issues. First, considering that the mitochondrial preparation is not 100% pure, expressing the amount of internalized exogenous mitochondria in terms of protein
concentration does not reflect the reality. Second, the efficiency of exogenous mitochondria acquisition is not known.

Enhancement of glioblastoma stem cell energetic metabolism following mesenchymal stem cell mitochondria acquisition

In line with previous reports (Caicedo et al., 2015; Tan et al., 2015; Moschoi et al., 2016; Dong et al., 2017), the acquisition of increasing doses of mesenchymal stem cell mitochondria increased the oxygen consumption rate of glioblastoma stem cells, reflecting an increase in oxidative phosphorylation. In addition, mesenchymal stem cell mitochondria increased the extracellular acidification rate of glioblastoma stem cells, suggesting an increase in glycolysis. To our knowledge, the only study reporting the effect of acquired mitochondria on the glycolysis of recipient cells comes from our own laboratory. In this case, however, mesenchymal stem cell mitochondria decreased the extracellular acidification rate of MDA-MB-231 breast cancer cells (Caicedo et al., 2015). This suggests that mesenchymal stem cell mitochondria do not always exert the same effect, which seems to be recipient cell-specific. Besides, we show that mesenchymal stem cell mitochondria also increased the cell number of glioblastoma stem cells. This observation is in agreement with previous reports that mesenchymal stem cell mitochondria enhanced the proliferation of target cells (Hekmatshoar et al., 2018).

Disruption of glioblastoma stem cell energetic metabolism following mesenchymal stem cell mitochondria acquisition and temozolomide treatment

We then sought to determine if the acquisition of mesenchymal stem cell mitochondria modifies the metabolic response of glioblastoma stem cells to temozolomide. To answer this question, we compared four conditions: (1) control glioblastoma stem cells, (2) glioblastoma stem cells treated with 50 μM temozolomide, (3) glioblastoma stem cells that acquired mesenchymal stem cell mitochondria at the optimal dose determined previously and, finally, (4) glioblastoma stem cells that acquired the optimal dose of mesenchymal stem cell mitochondria and that were treated with 50 μM temozolomide. We used 50 μM temozolomide because it is close to the area under concentration-time curve in the cerebrospinal fluid (31 ± 6.2 μM/h) achieved in the clinic for an administration of 200 mg/m²/day of temozolomide (Ostermann, 2004; Rosso et al., 2009; Gratas et al., 2014).
We show that while temozolomide slightly decreased the oxygen consumption of glioblastoma stem cells, it increased that of glioblastoma stem cells that had acquired mesenchymal stem cell mitochondria even further. Concomitantly, while temozolomide had no effect on the extracellular acidification of glioblastoma stem cells, it decreased that of glioblastoma stem cells that had acquired mesenchymal stem cell mitochondria. Interestingly, the increase in glioblastoma stem cell oxidative metabolism (OXPHOS-linked ATP levels and mitochondrial reserve capacity), accompanied by a decrease in their glycolytic activity (lactate production), have previously been linked to radiotherapy resistance (Vlashi et al., 2011). In addition, we show that this increase in oxygen consumption could be explained by an increase in mitochondrial mass. Interestingly, cancer stem cells exhibiting an increased mitochondrial mass and an enhanced oxygen consumption rate, in the case of breast cancer for instance, were shown to have a more invasive phenotype, but mechanistic links have not been drawn (LeBleu et al., 2014; Tan et al., 2016). The increase in mitochondrial mass could result from several factors, including (1) an enhanced mitochondrial biogenesis through PGC1α or (2) a lowered mitophagy. Our RNA-sequencing data, however, do not show a PGC1α overexpression in glioblastoma stem cells that had acquired mesenchymal stem cell mitochondria following temozolomide treatment in comparison to control glioblastoma stem cells, suggesting that this hypothesis is unlikely. Further investigations are required to confirm the implication of mitophagy. The increases in oxidative phosphorylation and in mitochondrial mass were also accompanied by an increase in mitochondrial reactive oxygen species production at 48 and 72 hours, followed by the establishment of an anti-oxidative response at 72 hours. Reactive oxygen species levels were long thought to be harmful and induce cell death, but increasing pieces of evidence are demonstrating that a slight increase in ROS levels is actually beneficial for cells, inducing their survival (Sies and Jones, 2020). This could support the mitochondria-mediated increase in glioblastoma stem cell survival against temozolomide. Oxidative stress is also known to increase tunneling nanotube formation and mitochondria transfer, thus participating even further in temozolomide resistance (Moschoi et al., 2016). Therefore, it could be interesting to check whether the tunneling nanotube formation frequency in increased in co-cultures between mesenchymal stem cells and glioblastoma stem cells upon temozolomide treatment.
Modifications of TCA cycle metabolite production following dual mitochondria acquisition and temozolomide treatment

As the dual mitochondria acquisition and temozolomide treatment disrupted both oxidative phosphorylation and glycolysis of glioblastoma stem cells, we assessed the effect of mesenchymal stem cell mitochondria on glioblastoma stem cell nutrient usage upon temozolomide treatment. We observed an increased usage of metabolites belonging to the oxidative branch of the TCA cycle (cis-aconitate), along with a decreased usage of those belonging to the reductive branch (succinate, malate) when glioblastoma cells that acquired mitochondria were treated with temozolomide. Concerning the differentially-produced metabolites of the TCA cycle, temozolomide treatment increased the production of metabolites belonging to the oxidative branch (citrate, cis-aconitate and α-ketoglutarate) and had no detectable effect on those belonging to the reductive branch (succinate, malate), in glioblastoma cells conditioned with mesenchymal stem cell mitochondria. Taken together, these results suggest that glioblastoma stem cells predominantly use the oxidative branch of the TCA cycle in both directions – i.e., oxidation and reductive carboxylation – when they receive mitochondria and temozolomide treatment. This could mean that, following temozolomide treatment, glioblastoma stem cells that had acquired mesenchymal stem cell mitochondria sought to slow down their TCA cycle, probably owing to an increased energy production and, thus, increased ATP/ADP ratios, which would reverse the reaction equilibrium. Moreover, employing reductive carboxylation could generate citrate for fatty acid synthesis, a key pathway implicated in glioblastoma drug resistance (Lin et al., 2017; Libby et al., 2018; Garnier et al., 2019; Duman et al., 2019; Saurty-Seerunghen et al., 2019; Taïb et al., 2019; Nakhle et al., 2020). In addition, TCA cycle metabolite production could contribute to the epigenetic regulation of glioblastoma gene expression. In fact, histone demethylases (KDMs) (Tsukada et al., 2006) and the ten eleven translocation (TET) family of DNA hydroxylases (Ito et al., 2010) are α-ketoglutarate-dependent dioxygenases. Therefore, KDM and TET enzymes are activated by α-ketoglutarate. On the other hand, succinate, which is structurally similar to α-ketoglutarate, acts as a competitive inhibitor of KDM and TET enzymes (Nakhle et al., 2020). Therefore, the observed increase in α-ketoglutarate production, along with the absence of effect on that of succinate, could activate DNA and histone demethylases, thereby enhancing the expression of genes implicated in resistance, including MGMT.
Modifications of pentose phosphate pathway and pyrimidine synthesis metabolite production following dual mitochondria acquisition and temozolomide treatment

We also report an increase in metabolite production related to the pentose phosphate pathway and to pyrimidine synthesis in glioblastoma stem cells following mesenchymal stem cell mitochondria acquisition and temozolomide treatment. The pentose phosphate pathway is an alternative anabolic pathway, generating ribose-5-phosphate and NADPH for nucleic acid and fatty acid synthesis. Studies have demonstrated that, glioblastoma stem cells relied the pentose phosphate pathway upon acute oxygenation which increased their proliferation (Kathagen et al., 2013). In addition, the pentose phosphate pathway was demonstrated to accelerate DNA synthesis and to detoxify intracellular reactive oxygen species in EGFR-activated glioblastoma, which was respectively associated with tumor growth and therapy resistance (Liu et al., 2019; Maurer et al., 2019). Besides, the inhibition of pyrimidine synthesis was shown to specifically target glioblastoma stem cells and to abrogate tumor formation (Laks et al., 2016). This suggests that the increased production of metabolites belonging to the pentose phosphate and pyrimidine synthesis pathways could play a role in temozolomide resistance and constitute new avenues for glioblastoma treatment by targeting these pathways.

Role of mitochondrial metabolism in glioblastoma temozolomide resistance

Several groups have previously reported metabolic changes of glioblastoma cells following temozolomide treatment. These studies indicated that mitochondria are key players in glioblastoma resistance to temozolomide (Oliva et al., 2010; St-Coeur et al., 2015). For instance, temozolomide-resistant glioblastoma cells exhibited changes in the composition and the enzymatic activity of the electron transport chain, including decreased levels of complexes I (electron transport chain entry) and V (ATP production) with increased levels of complexes II (another electron transport chain entry), III and cytochrome c oxidase (electron transfer) (Oliva et al., 2010, 2016, 2017). Moreover, temozolomide-resistant glioblastoma cells produced high levels of citrate and isocitrate, notorious metabolites for their role in chemoresistance establishment (St-Coeur et al., 2015). These data strongly suggest that mitochondrial metabolism plays an important role in the acquisition of temozolomide resistance.
Disruption of glioblastoma stem cell gene expression following mesenchymal stem cell mitochondria acquisition and temozolomide treatment

Changes in metabolism, and most specifically in cellular metabolite concentrations, are known to induce changes in the cellular gene expression profiles. Our RNA-sequencing data revealed that the dual mesenchymal stem cell mitochondria acquisition and temozolomide treatment disrupted the transcriptional pattern of glioblastoma stem cells. Strikingly, the effect of mitochondria alone is modest; however, it becomes strongly apparent when the cells are challenged with temozolomide. Specifically, dual mitochondria acquisition and temozolomide treatment implicated processes of cell cycle progression that were not observed for temozolomide treatment alone, including G1/S phase transition regulation and DNA damage checkpoint. Interestingly, while the expression of each individual gene belonging to these biological processes was not significantly different between both conditions, it was the association of all gene differences that induced the observed results.

Concluding remarks

Our study suggests that the metabolic signature of glioblastoma stem cells can change during cancer progression, following microenvironmental interactions and/or chemotherapy, which could lead to an increased cancer cell survival. The paramount role of glioblastoma stem cells in drug resistance calls for novel therapeutic strategies to annihilate this peculiar malignant population. The immense intrinsic glioblastoma stem cell inter- and intra-tumoral heterogeneities, combined with those dictated by the tumor microenvironment (cellular composition, vasculature, oxygenation, pH, nutrient availability), might actually command glioblastoma metabolic signatures and overpower onco-genetic events. In this respect, glioblastoma stem cell metabolic plasticity is still a hurdle for efficient and long-lasting treatment. Therefore, simultaneous blockade of multiple metabolic pathways (TCA cycle/pentose phosphate pathway/pyrimidine synthesis for instance), in combination with standard anticancer therapies (chemo-, radio- or immunotherapy), could hold encouraging therapeutic prospects. There is a crucial need to re-envision glioblastoma therapies, by exploring the role of the tumor microenvironment in promoting tumor progression and drug resistance, while factoring in the inherent glioblastoma heterogeneity. This can be achieved by using patient-derived glioblastoma cells rather than long-term cell lines, coupled with “normal”, cancer-associated key players of the tumor microenvironment. These advances
have the potential to reveal new resistance mechanisms that, if appropriately targeted, can hinder tumor growth. Studies should also integrate large-scale, multi-omics data, in order to identify new glioblastoma biomarkers, thus allowing the development of specifically-targeted therapies.
**MB buffer preparation**

MB: HEPES (pH 7.4) 10 mM For 100 mL: - 238.3 mg (MW = 238.3 g/L)
  EDTA 1 mM - 37.2 mg (MW = 372.24 g/L)
  Mannitol 210 mM - 3.826 g (MW = 182.17 g/L)
  Saccharose 70 mM - 2.396 g (MW = 342.3 g/L)

**Mesenchymal stem cell (MSC) mitochondria isolation**

- Adjust centrifuge temperature to 4 °C.
- Prepare one 1.5 mL microtube labeled “MB” containing 1 mL MB buffer + 100 μL protease inhibitor cocktail. Prepare two other 1.5 mL microtubes labeled “MSC” and “Mito”. Keep all microtubes on ice.
- Wash the MSCs with 2 mL trypsin-EDTA, then add 1 mL trypsin (no EDTA). Incubate for 5 – 10 min at 37 °C.
- Recover the MSCs by adding 10 mL αMEM/FBS 10%. Transfer to a 50 mL tube.
- Centrifuge at 1 200 rpm, 20 °C for 5 min. Discard the supernatant.
- Resuspend the cell pellet in 10 mL αMEM/FBS 10%. Count (5.10^5 MSCs are needed).
- Centrifuge at 1 200 rpm, 20 °C for 5 min. Discard the supernatant.
- Resuspend the cell pellet in 1 mL ice-cold PBS. Transfer to the “MSC” tube.
- Centrifuge at 1 200 rpm, 20°C for 5 min. Discard the supernatant.
- Resuspend the pellet in 200 μL MB buffer + protease inhibitors.
- Lyse cells with a syringe and a needle: 10x 25G + 10x 27G.
- Centrifuge at 800 g, 4°C for 10 min. Discard the pellet.
- Transfer the supernatant to the “Mito” tube.
- Centrifuge at 8 000 g, 4°C for 10 min. Discard the supernatant.
- Resuspend the mitochondria pellet in 100 μL MB buffer + protease inhibitors.
- Dilute the mitochondria to the desired concentrations using cold GSC proliferation medium.

**Transfer of isolated MSC mitochondria to GSCs (MitoCeption)**

- Add 20 μL of isolated mitochondria into the wells of the 96-well plate containing the GSCs, at the desired concentration. Add 20 μL GSC proliferation medium into the control wells. (Add the mitochondria slowly, close to the bottom of the well, covering its entire surface).
- Centrifuge the 96-well plate at 3 000 rpm, 4 °C for 15 min.
- Incubate at 37 °C.
**SEAHORSE ANALYSIS**

**Day 1 – Monday: GSC trypsinization (see MitoCeption protocol)**

**Day 2 – Tuesday: MitoCeption + Cell Culture Microplate coating**

1. **Cell Culture Microplate coating with poly-D-lysine and laminin**
   1. Prepare the poly-D-lysine solution at a final concentration of 0.1 mg/mL in sterile culture grade water.
   2. Add 20 μL poly-D-lysine per well. Incubate for 5 – 6 hours at 37 °C. Discard.
   3. Prepare the laminin solution at a final concentration of 10 μg/mL in sterile culture grade water.
   4. Add 20 μL laminin per well. Incubate overnight at 37 °C.

**Day 3 – Wednesday**

1. **Seeding GSCs on the Seahorse Cell Culture Microplate**
   1. Discard the laminin solution.
   2. Rinse with 200 μL PBS (or sterile water) per well.
   3. Let the plate dry for 20 min – 1 hour under the hood at room temperature.
   4. Collect the GSC neurospheres in 50 mL tubes. Dissociate the neurospheres mechanically with a P1000. Count on a Thoma hemocytometer.
   5. Centrifuge the GSCs at 1 200 rpm for 5 min.
   6. Add the required volume of proliferation medium in order to reach a final concentration of 200 000 GSCs/mL.
   7. Seed 200 μL = 40 000 GSCs per well.
   8. Centrifuge the plate at 450 rpm (short spin, just to reach the max speed. Brake = 0. Acceleration = 4).
   9. Turn the plate in the opposite direction. Centrifuge at 650 rpm (same).
   10. Incubate at 37 °C for 48 hours.

**Day 4 – Thursday**

1. **Turning on the Agilent Seahorse XFe96 Analyzer**
   1. Turn on the Analyzer.
   2. Launch the Wave application. Make sure the Analyzer is connected to the computer.
   3. Let the Analyzer warm up overnight (Seahorse has to be at 37°C).

2. **Sensor cartridge hydration (step 1 of 2)**
   1. Open the XFe96 Extracellular Flux Assay Kit, and remove the contents. Place the sensor cartridge upside down.
2. Fill each well of the utility plate with 200 μL of sterile water.
3. Lower the sensor cartridge onto the utility plate, submerging the sensors in the water.
4. Place assembled sensor cartridge and utility plate at 37 °C in a non-CO₂ incubator overnight.
5. Aliquot 20 mL of Seahorse XF calibrant into a 50 mL tube. Place it at 37 °C in a non-CO₂ incubator overnight.

Day 5 – Friday: Seahorse experiment

1. **Sensor cartridge hydration (step 2 of 2)**
   1. Remove the XF calibrant and assembled sensor cartridge with utility plate from the incubator.
   2. Place the sensor cartridge upside down.
   3. Discard the water from the utility plate.
   4. Add 200 μL of the pre-warmed XF calibrant per well of the utility plate.
   5. Lower the sensor cartridge onto the utility plate.
   6. Place assembled sensor cartridge with utility plate in a non-CO₂ 37 °C incubator for 45 – 60 min prior to loading the injection ports of the sensor cartridge.

2. **Seahorse medium preparation**
   1. **For OCR**
      Supplement the Seahorse XF Base Medium with:
      - Pyruvate 100 mM (final concentration = 1 mM)
      - L-Glutamine 200 mM (final concentration = 2 mM)
      - D-Glucose 1 M (final concentration = 10 mM)
      → For 10 mL XF Base Medium, add: 100 μL pyruvate, 100 μL L-glutamine and 100 μL D-glucose.

   2. **For ECAR**
      Supplement the Seahorse XF Base Medium with pyruvate and L-glutamine, without D-glucose (see OCR).
   3. Warm the assay medium to 37°C in a water bath until usage.
   4. Gently discard the proliferation medium in the cell culture microplate using a multichannel pipette.
   5. Add 180 μL Seahorse XF medium (OCR or ECAR) per well (check under the microscope that the cells didn’t detach).
   6. Incubate at 37 °C in a non-CO₂ incubator for 45 – 60 min.

3. **Drug preparation**
   1. Oligomycin and FCCP
Stock solution: 100 mM in DMSO
Intermediate dilution: 10 mM in Seahorse XF medium
Port (10X): 10 μM (3 μL + 3 mL Seahorse XF medium)
Well (1X): 1 μM

**Rotenone**
Stock solution: 1 mM in DMSO
Port (10X): 1 μM (3 μL + 3 mL Seahorse XF medium)
Well (1X): 100 nM

**Antimycin A**
Stock solution: 40 mM in DMSO
Port (10X): 10 μM (0.75 μL + 3 mL Seahorse XF medium)
Well (1X): 1 μM

**2-DG**
Port (10X): 1 M in Seahorse XF medium
Well (1X): 100 mM

**Etomoxir**
Stock solution: 10 mM in sterile water
Port (10X): 1 mM (300 μL + 2.7 mL Seahorse XF medium)
Well (1X): 100 μM

**Oxamate**
Port (10X): 750 mM (0.26 g + 3 mL Seahorse XF medium)
Well (1X): 75 mM

**BPTES**
Stock solution: 4 mM in DMSO
Port (10X): 30 μM (22.5 μL + 3 mL Seahorse XF medium)
Well (1X): 3 μM

**UK5099**
Stock solution: 10 mM in DMSO
Port (10X): 20 μM (6 μL + 3 mL Seahorse XF medium)
Well (1X): 2 μM

2. Load the inhibitors in ports A, B, C and D as follows:
3. Load the assembled sensor cartridge with utility plate into the Seahorse XFe analyzer. Run the equilibration protocol.
4. When the machine is ready, take out the utility plate and load the cell culture microplate. Launch the analysis.

5. PFA fixation and Hoechst staining
   1. Discard the Seahorse XF medium using a multichannel pipette.
   2. Add 100 μL PFA 4% per well. Incubate for 10 min at room temperature.
   3. Discard the PFA. Rinse with 100 μL PBS per well.
   4. Add 100 μL PBS per well. Place the plate at 4 °C until Cellomics microscopy.

   On day of Cellomics microscopy:
   5. Discard the PBS.
   6. Stain cells with 100 μL Hoechst 1/10,000 per well (1 μL Hoechst in 10 mL PBS) for 10 min at room temperature.
   7. Rinse with 100 μL PBS.

Poly-D-lysine: SIGMA P7280
Laminin: SIGMA L2020
Glucose: Seahorse XF 103577-100
Pyruvate: Seahorse XF 103578-100
2-DG: D8375
FCCP: C2920
BPTES: SIGMA SML0601
Etomoxir: SIGMA E1905
UK5099: SIGMA PZ0160
Oxamate: SIGMA O2751
Hoechst: Invitrogen H3570
GSC Staining for FACS

1. Collect GSC neurospheres in 1.5 mL microtubes. Dissociate mechanically by pipetting ~10 times with a P200 then a P1000.
2. Wash with 1 mL PBS.
3. Label single GSCs in 100 µL PBS for 20 min in the dark at RT.
4. Centrifuge at 1200 rpm for 5 min at RT. Discard the supernatant.
5. Wash with 1 mL PBS + FCS 5%. Repeat.
6. Add 250 µL PBS + FCS 5%.
7. Before each reading: Transfer labelled GSCs to FACS tubes. Dissociate mechanically by pipetting ~10 times with a P200.

MitoTracker Deep Red FM 644/665 (Invitrogen ref. M22426)

Stock concentration: 1 mM (50 µg in 94 µL DMSO)
Final concentration: 250 nM (1 µL in 4 mL PBS)

MitoSOX Red 510/580 (Invitrogen ref. M36008)

Stock concentration: 5 mM (50 µg in 13 µL DMSO)
Final concentration: 5 µM (1 µL in 1 mL PBS)

Zombie Violet 405/423 (Biolegend ref. 423113)

Stock concentration: 1000X (one vial in 100 µL DMSO)
Final concentration: 1X (1 µL in 1 mL PBS)
MASS SPECTROMETRY

1. Sample preparation

Extraction protocol applied for the sample preparation is extraction by cold solution of 8 mL: Acetonitrile / Methanol / Water, 4:4:2 v/v at -20°C, containing 125 mM formic acid v/v. Volume of internal standard (IDMS) used is 100 µL per sample. Recovery volume after evaporation: 200 µL with H2Omq. One injection per sample for central and energetic metabolism analysis.

Quality control

Conformity of mass spectrometry analysis according to EN-ARM-ANAL-013 MetaToul document:

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Theoretical Concentration, µmol/L (Recovery ± 20 %)</th>
<th>Experimental Concentration, µmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBP</td>
<td>0.674 ± 0.135</td>
<td>0.626</td>
</tr>
<tr>
<td>Rib 1P</td>
<td>0.428 ± 0.088</td>
<td>0.473</td>
</tr>
<tr>
<td>Oro</td>
<td>0.668 ± 0.132</td>
<td>0.601</td>
</tr>
</tbody>
</table>

This protocol describes the absolute quantification of intracellular metabolites by Ion Chromatography (IC) coupled to High Resolution Mass Spectrometry (HRMS). It includes both IC and MS settings.

LTQ Orbitrap Velos™ / Liquid anion exchange chromatography Dionex™ ICS-5000+ Reagent-Free™ HPIC™

Equipment used for analysis: The analyses were carried out on an IC-MS platform of a liquid anion exchange chromatography Dionex™ ICS-5000+ Reagent-Free™ HPIC™ (Thermo Fisher Scientific™, Sunnyvale, CA, USA) system, coupled to a Thermo Scientific™ LTQ Orbitrap Velos™ mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a heated electrospray ionization probe.

Liquid anion exchange chromatography was performed with the Thermo Scientific Dionex ICS-5000+ Reagent-Free HPIC system (Thermo Fisher Scientific) equipped with an eluent generator system (ICS-5000+EG, Dionex) for automatic base generation (KOH). Analytes were separated within 50 min, using a linear KOH gradient elution applied to an IonPac AS11-HC column (250 x 2 mm, Dionex) equipped with an AG11-HC guard column (50 x 2 mm, Dionex) at a flow rate of 0.38 ml/min. The gradient program was following: equilibration with 7 mM KOH during 1.0 min; then KOH ramp from 7 to 15 mM, 1–9.5 min; constant concentration 10.5 min; ramp to 45 mM in 10 min; ramp to 70 mM in 3 min; ramp to 100 mM in 0.1 min; constant concentration 8.9 min; drop to 7 mM in 0.5 min; and equilibration at 7 mM KOH for 7.5
The column and autosampler temperatures were thermostated at 25°C and 4°C, respectively. The injected sample volume was 15 µl. Measures were performed in triplicates from separate cultures.

Mass detection was carried out in a negative electrospray ionization (ESI) mode at a resolution of 60 000 (at 400 m/z) in full-scan mode, with the following source parameters: the capillary temperature was 350 °C, the source heater temperature, 300 °C, the sheath gas flow rate, 50 a.u. (arbitrary unit), the auxiliary gas flow rate, 5 a.u., the S-Lens RF level, 60 %, and the source voltage, 2.75 kV. Data acquisition was performed using Thermo Scientific Xcalibur software. Metabolites were determined by extracting the exact mass with a tolerance of 5 ppm.

Data processing: TraceFinder 4.1 software
NUCLEIC ACID PURIFICATION – TRIZOL METHOD

1. Lyse the cells in 700 μL TRIzol reagent. Pipet the lysate up and down several times to homogenize.
2. Add chloroform to the homogenized TRIzol solution. Adjust the chloroform volume according to the TRIzol volume (200 μL for 800 μL trizol, 140 μL for 500 μL trizol). Vortex for 15 seconds.
3. Incubate at room temperature for 3 minutes.
4. Centrifuge for 15 minutes at 12 000 x g at 4 °C.
5. Transfer the aqueous phase to 1.5 mL tubes containing 1.5 μL of glycogen. Avoid transferring any of the interphase or organic phase.
6. Add 500 μL of isopropanol to the aqueous phase. Vortex.
7. Incubate at room temperature for 10 minutes.
8. Centrifuge for 15 minutes at 12 000 x g at 4 °C. Discard the supernatant.
9. Resuspend the pellet in 900 μL of ice-cold 70 % ethanol. Vortex.
10. Incubate for 1 hour on ice at 4 °C.
11. Centrifuge for 10 minutes at 12 000 x g at 4 °C. Discard the supernatant.
12. Repeat steps 10-11 once.
13. Air dry the pellets for 10 – 15 minutes.
14. Resuspend the pellets in 20 – 30 μL of RNase-free water (depending on the size of the pellet).
15. Determine the nucleic acid extraction yield by measuring the absorbance with a NanoDrop spectrophotometer.
**TOTAL DNA PURIFICATION – QIAGEN DNEASY BLOOD AND TISSUE SPIN-COLUMN METHOD**

1. Centrifuge $5 \times 10^5$ cells for 5 min at 300 x g. Resuspend the pellet in 200 μL PBS. Add 20 μL proteinase K.
2. Add 200 μL Buffer AL (without added ethanol). Mix thoroughly by vortexing.
3. Add 200 μL ethanol (96 – 100%) to the sample. Mix thoroughly by vortexing.
4. Pipet the mixture into the DNeasy Mini spin column placed in a 2 mL collection tube. Centrifuge at 6000 x g for 1 min. Discard flow-through and collection tube.
5. Place the DNeasy Mini spin column in a new 2 mL collection tube. Add 500 μL Buffer AW1. Centrifuge for 1 min at 6000 x g. Discard flow-through and collection tube.
6. Place the DNeasy Mini spin column in a new 2 mL collection tube. Add 500 μL Buffer AW2. Centrifuge for 3 min at 20,000 x g to dry the DNeasy membrane. Discard flow-through and collection tube.
7. Place the DNeasy Mini spin column in a clean 1.5 mL (or 2 mL) microcentrifuge tube. Pipet 200 μL Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min. Centrifuge for 1 min at 6000 x g to elute.
8. For maximum DNA yield, repeat elution once as described in step 7.
RNA-SEQUENCING ANALYSIS

1. Sequence quality control
This step is an essential pre-requisite for processing data from all massive sequencing technologies. The quality of the sequences directly conditions the resolution and the quality (specificity and sensitivity) of subsequent steps. It is not uncommon to decide that you need to re-sequence one or several samples at the end of this quality control.

The quality of the recovered raw sequences was verified using the FastQC software. This tool provides a set of parameters allowing to obtain an overview of the quality of the sequences at each sequencing cycle. This helps to determine if there has been an operating problem that systematically affects all sequences produced. A high redundancy level (number of identical sequences) illustrates an experimental artifact, likely related to sample preparation for the sequencing. FastQC works on all operating systems (MS Windows, Mac OS, Linux) and is user friendly, thanks to its intuitive graphical interface.

2. Raw data analysis
Raw sequences are not directly exploitable. They must be transformed in order to obtain the gene expression levels. It is therefore necessary to evaluate the optimal parameters for the treatment of raw data in order to unveil biological results.

2.1. Read mapping on the genome
Sequence repositioning on the genome was carried out using the BOWTIE software. The reference genome used is the latest version to date, exhibiting the most common structural polymorphisms (available for download on the link ftp.genome.ucsc.edu).

The high quality of the sequences allowed the use of very stringent parameters ($l = 45$, $5' = 0$, $3' = 5$, $n = 2$, $m = 1$), which made it possible to maximize the specificity of this step.

2.2. Measurement of raw gene expression levels
The number of sequences positioned between the ends of each gene corresponds to the gross measurement of its expression level. This step was ensured by the INTERSECTBED software (from the Bedtools toolkit). Subsequently, we built a counting table that summarizes the expression level of each gene under all the experimental conditions. Differential analysis,
which searches for genes with significantly different expressions from one condition to another, relies on this counting table.

2.3. Normalization
Crude measurements of gene expression levels depend on several parameters. Besides the actual level of gene expression, these values also depend on several factors, the main one being the sequencing depth (i.e., the total number of unique sequences replaced on the genome, for each biological sample). The more sequences, the higher the level of expression. It is essential to artificially correct the raw values of gene expression by normalizing them to a constant sequence number between conditions. This step was performed by applying a simple scale factor, calculated according to the total number of reads from each bank.

2.4. Quality control by Principal Component Analysis (PCA)
The total variance (i.e., all sources of technical and biological variability) of the dataset was divided into different 'components', that were subsequently classified in decreasing order of importance. Each experiment was then projected onto a graph where each axis corresponds to a variance component. The first components represent the major sources of biological variability in the entire data set and, hence, must be associated with the experimental treatments. On the other hand, minor components must correspond to technical, technological or biological background noise. Moreover, the biological replicas must be grouped together.
The Principal Component Analysis is then a question of quality control, ensuring that the major observed effects are well associated with the treatments and, therefore, the RNA-sequencing data faithfully capture the expected biological response. In other terms, the measurement of gene expression by RNA-sequencing is not biased.
The Principal Component Analysis was carried out in the R software, using the prcomp function.

2.5. Data filtering
The purpose of this step is to exclude genes with a low expression level. In fact, below a certain threshold, the low read number associated with a gene is not representative of variations in its expression. This threshold is generally set by DEseq to exclude 40% of the
expressed genes. This "independent filter" step was automatically undertaken by the software for measuring differential gene expression (see below).

2.6. Differential Analysis (DA)

We used the standard tool for differential analysis of gene expression: the DEseq software in R (Anders and Huber, 2010). For each gene, DEseq performed an ANOVA analysis, which is based on the modeling of the number of "reads" associated with each gene by a negative binomial distribution. The latter has the advantage of better modeling the distribution of number of "reads" than a Poisson law, especially for moderately to weakly expressed genes. Each gene was then associated with an adjusted p-value. The lower the p-value, the more likely the gene is differentially expressed. The threshold of significance retained, p-val = 0.05, is the commonly used threshold.

Key conceptual steps of RNA-sequencing data analysis.
RÉSUMÉ EN FRANÇAIS
Bien que le taux de mortalité due au cancer ait diminué de 26% entre 1991 et 2015 (Siegel et al., 2018), la résistance aux thérapies reste un obstacle majeur pour un traitement efficace et durable, entraînant des rechutes, des métastases et une réduction de la survie globale des patients. De nombreux mécanismes, à la fois intrinsèques et extrinsèques aux tumeurs, ont été décrits comme responsables de cette résistance. Récemment, l'hétérogénéité intratumorale a attiré une attention accrue en tant que modulateur de la réponse à la thérapie, étant souvent à l'origine de la résistance (Marusyk et al., 2020). Il devient de plus en plus évident que les tumeurs n'agissent pas comme des masses de cellules malignes homogènes, mais plutôt comme des organes complexes en progression dynamique dans le temps et dans l'espace, ce qui augmente leur tumorigénicité et façonne leur résistance (Greaves, 2015).

Des expériences de traçage de lignées et d'ablation de cellules ont démontré que de nombreuses tumeurs présentent une population particulière de cellules auto-renouvelables, appelées cellules souches cancéreuses. Les preuves provenant de xénogreffes chez des souris immunodéficientes ont indiqué que les cellules souches cancéreuses ont la capacité de régénérer des tumeurs évoquant la tumeur d'origine, de donner naissance à différents types de cellules et de survivre à de nombreux traitements anti-cancéreux couramment utilisés en clinique. Par conséquent, les cellules souches cancéreuses sont directement impliquées dans la résistance aux médicaments et sont hautement prédictives de la survie globale des patients. Ce modèle illustre les tumeurs comme des tissus malins organisés de manière hiérarchique où les cellules souches cancéreuses représentent l'apogée de la hiérarchie et soutiennent le repeuplement à long terme du néoplasme (Batlle et Clevers, 2017).

De plus, l'hétérogénéité provient du fait que le microenvironnement tumoral abrite plusieurs types de cellules non malignes recrutées sur le site tumoral, à savoir les fibroblastes associés au cancer, les cellules souches mésenchymateuses et les cellules immunitaires (Quail et Joyce, 2017; Chen et Song, 2019 ; Wolf et al., 2019). Les études indiquent que le stroma tumoral non malin constitue jusqu'à 90% d'un volume tumoral donné et est corrélé à un pronostic défavorable (Lou, 2016). L'interaction d'une cellule tumorale avec son environnement modifie fortement son sort, jouant ainsi un rôle dans l'acquisition de la résistance aux médicaments. Ainsi, les cellules appartenant à un même patrimoine génétique peuvent
répondre différemment aux mêmes agressions cellulaires en fonction de leur microenvironnement.

Les interactions cellules-cellules de type « nanotubes » ont récemment été décrites comme un nouveau moyen de communication intercellulaire. Les nanotubes sont des extensions cytoplasmiques minces (diamètre < 1 μm) à base d’actine, reliant des cellules non adjacentes sur de longues distances (> 100 μm) (Vignais et al., 2019). Les signaux biologiques qui déclenchent la formation de nanotubes comprennent le stress cellulaire induit par des agents génotoxiques (Desir et al., 2016; Moschoi et al., 2016; Victoria et al., 2016). Surtout, les nanotubes impliquent une continuité dans la membrane plasique et le cytoplasme entre les cellules connectées, modifiant ainsi notre dogme actuel de la cellule limitée par sa propre membrane plasique (Baker, 2017). Les nanotubes permettent ainsi le transport intercellulaire de composants cellulaires allant des ions aux organelles entières comme les mitochondries (Vignais et al., 2019). Les transferts de mitochondries médiés par des nanotubes ont été observés in vitro et in vivo dans des modèles murins. Les transferts de mitochondries médiés par des nanotubes se sont avérés avoir des effets à la fois métaboliques et fonctionnels sur les cellules receveuses. Dans le contexte du cancer, l’acquisition de mitochondries exogènes augmente la résistance aux médicaments. Au cours des dernières années, les cellules souches mésenchymateuses ont été les plus profondément décrites pour se connecter aux cellules environnantes à travers des nanotubes, conduisant au transfert de mitochondries vers ces cellules cibles et à des effets biologiques tels que la reprogrammation métabolique, la protection contre les lésions tissulaires et la résistance à la chimiothérapie (Caicedo et al., 2015; Moschoi et al., 2016; Rodríguez et al., 2018; Hekmatshoar et al., 2018; Nakhle et al., 2020; Pinto et al., 2020).

Les mitochondries sont considérées comme un centre où convergent les différentes voies métaboliques, notamment la glycolyse, le cycle de Krebs, la glutaminolyse, la voie du pentose phosphate et le métabolisme des lipides. Dans ce contexte, les altérations fonctionnelles de ces voies métaboliques sont directement liées à la résistance des cellules cancéreuses aux thérapies (Hekmatshoar et al., 2018). En outre, la production de métabolites du cycle de Krebs contribue également à la régulation épigénétique des cellules cancéreuses, comme le montre le succinate, le fumarate, le 2-hydroxyglutarate et l’a-cétabutarate, par le biais d’inhibition
de l’activité des histones déméthylases (Tsukada et al., 2006; Xiao et al., 2012; Killian et al., 2013; Letouzé et al., 2013; Nakhle et al., 2020). Le rôle établi des mitochondries dans la progression tumorale et la résistance au traitement peut expliquer les effets anticancéreux bénéfiques obtenus par les inhibiteurs des mitochondries comme la metformine (Sanchez-Alvarez et al., 2013) ou par les antibiotiques inhibant la biogenèse mitochondriale (par exemple doxycycline, azithromycine) (Lamb et al., 2015). Elle ouvre également la voie à des stratégies thérapeutiques innovantes basées sur la létalité métabolique synthétique (Navarro et al., 2016). Néanmoins, des questions centrales demeurent, ce qui justifie de nouvelles études affinées.

Mon projet de thèse porte sur le glioblastome, une tumeur cérébrale primaire dévastatrice. Les patients sont traités par résection tumorale suivie d’une radiothérapie et d’une chimiothérapie avec un agent alkylant, le témozolomide. Cependant, la résistance au traitement par témozolomide apparaît rapidement, principalement en raison des cellules souches de glioblastome présentes dans la tumeur. Les cellules souches de glioblastome sont caractérisées par l’expression de marqueurs de souchitude (y compris OLIG2, NESTIN, NANOG, CD133, SOX2). Elles forment des neurosphères in vitro et sont capables de générer des tumeurs hétérogènes après xénogreffe chez la souris. La résistance du glioblastome au témozolomide est dépendante de divers mécanismes, y compris la surexpression de la MGMT (O⁶-methylguanine methyltransferase), des protéines d’efflux MDR1 (Multi Drug Resistance 1) et ABCG2 (ATP-binding cassette subfamily G member 2) ainsi que la modification du métabolisme (Velpula et al., 2012; Shinojima et al., 2013; Fan et al., 2013; Osuka et Van Meir, 2017; MacLeod et al., 2019; Garnier et al., 2019). A noter que les cellules souches de glioblastome ont également été décrites, à la fois in vitro et in vivo, à s’engager dans un réseau fonctionnel de connexions cellulaires de type nanotubes qui ont contribué à la résistance du glioblastome à la radiothérapie (Osswald et al., 2015). De plus, le recrutement des cellules souches mésenchymateuses dans le microenvironnement du glioblastome a été observé dans les tumeurs du glioblastome réséquées. Surtout, leur présence était inversement corrélée à la survie des patients (Hossain et al., 2015; Shahar et al., 2017). Il a également été confirmé dans des modèles de xénogreffe orthotopique de cellules souches de glioblastome, un processus médié par le TGF-β sécrété par les cellules souches de glioblastome (Velpula et al., 2012; Shinojima et al., 2013).
L'hypothèse de travail de ma thèse est que le transfert de mitochondries médié par les nanotubes, qui se produit dans le microenvironnement tumoral entre cellules souches mésenchymateuses et cellules souches du glioblastome, peut affecter le métabolisme du glioblastome et, par la suite, induire sa résistance au témozolomide. Afin de répondre à cette question, j’ai développé mon projet de thèse selon 3 axes principaux.

Premièrement, je voulais déterminer les effets métaboliques de l’acquisition de mitochondries de cellules souches mésenchymateuses sur les cellules souches de glioblastome. Tout d’abord, j’ai commencé par confirmer, en utilisant des cellules souches mésenchymateuses de quatre donneurs différents, les données préliminaires du laboratoire montrant des connexions de type nanotubes entre les cellules souches mésenchymateuses et les cellules souches de glioblastome en co-culture. Puis, afin d’étudier spécifiquement les effets des mitochondries de cellules souches mésenchymateuses transférées, indépendamment des effets des cytokines sécrétées dans les modèles de co-cultures cellulaires, j’ai profité du protocole de MitoCeption du laboratoire (Caicedo et al., 2015; Nzigou Mombo et al., 2017), qui permet le transfert de mitochondries pré-isolées vers des cellules cibles. J’ai mis en œuvre ce protocole et l’ai adapté afin de préparer de grandes quantités de cellules souches de glioblastome, avec des doses quantifiées de mitochondries internalisées, pour permettre des analyses phénotypiques approfondies. J’ai également mis en place les conditions expérimentales pour l’extraction reproductible et la quantification de l’ADN mitochondrial des cellules souches mésenchymateuses acquises, sur la base de polymorphismes nucléotidiques uniques appartenant à chaque donneur (Nakhle et al., 2020). J’ai pu ainsi démontrer un effet dose-réponse de l’acquisition de quantités croissantes de mitochondries sur les cellules souches de glioblastome, ce qui a amélioré leur métabolisme énergétique général (à la fois l’oxydation phosphorylante et la glycolyse) et leur prolifération.

Deuxièmement, sachant que les cellules souches tumorales peuvent développer des résistances à la chimiothérapie en modifiant leur métabolisme, je me suis interrogé si les mitochondries de cellules souches mésenchymateuses modifient la réponse métabolique des cellules souches de glioblastome au témozolomide. Pour cela, j’ai commencé par évaluer le métabolisme énergétique général, lors du traitement au témozolomide, des cellules souches de glioblastome ayant acquis des mitochondries de cellules souches mésenchymateuses. J’ai
trouvé que, quand les cellules souches de glioblastome sont traitées au témozolomide, les mitochondries de cellules souches mésenchymateuses augmentent leur oxydation phosphorylante davantage mais diminuent leur glycolyse. J'ai ensuite cherché à déterminer si les modifications du métabolisme énergétique global étaient accompagnées de changements dans l'utilisation des nutriments et la production de métabolites. J'ai pu montrer que les mitochondries des cellules souches mésenchymateuses modifient à la fois l'utilisation et la production de métabolites liés au cycle de Krebs, ainsi que la production de métabolites liés à la voie des pentoses phosphates et la synthèse des pyrimidines. Les métabolites sont des molécules importantes qui, non seulement fournissent des éléments de base pour la synthèse des macromolécules, mais agissent également comme des signaux de signalisation pouvant induire des effets transcriptionnels.

Enfin, je voulais déterminer si les mitochondries de cellules souches mésenchymateuses confèrent un avantage de survie aux cellules souches de glioblastome contre la chimiothérapie au témozolomide. Afin de répondre à cette question, j'ai d'abord entrepris des expériences pour mesurer à la fois la survie et la mort cellulaire des cellules souches du glioblastome ayant acquis des mitochondries suite au traitement au témozolomide, dans les mêmes conditions où les modifications métaboliques ont été observées. J'ai ainsi pu démontrer que l'acquisition de mitochondries de cellules souches mésenchymateuses augmentent la survie des cellules souches de glioblastome au traitement. Ensuite, j'ai été intrigué par le stress oxydatif mitochondrial dans ce contexte spécifique, qui pourrait être une arme à double tranchant pour les cellules cancéreuses. D'une part, le stress oxydatif peut provoquer leur mort cellulaire ; d'autre part, il peut protéger les cellules, en partie, en augmentant leur défenses anti-oxydatives et la fréquence de formation de nanotubes à effet protecteur. Enfin, j'ai effectué une analyse de séquençage d'ARN à haut débit afin de déterminer le profil d'expression génique des cellules souches de glioblastome après l'acquisition de mitochondries et le traitement au témozolomide, dans le but de déterminer les modifications transcriptionnelles qui pourraient contribuer à la résistance au témozolomide. J'ai ainsi mis en évidence que l'acquisition de mitochondries de cellules souches de glioblastome activent leur cycle cellulaire quand celles-ci sont traitées au témozolomide.
La présence de réseaux cellulaires médiés par des nanotubes dans les tumeurs, ainsi que les transferts intercellulaires de mitochondries qu'ils impliquent, créent un nouveau paradigme dans le domaine du cancer. Les transferts de mitochondries à base de nanotubes sont en fait des moyens totalement nouveaux de communication intercellulaire qui n'avaient pas été prévus. Ils ont des implications profondes pour la progression tumorale et la résistance au traitement et, par conséquent, nécessitent de nouvelles conceptions de protocoles thérapeutiques efficaces. Les patients atteints de glioblastome ont peu d'options thérapeutiques et font face rapidement à une résistance au traitement, ce qui se traduit par une survie limitée une fois la maladie diagnostiquée. Nous proposons que les transferts de mitochondries médiés par les nanotubes, des cellules souches mésenchymateuses du microenvironnement tumoral vers les cellules souches du glioblastome, participent à la progression du glioblastome et à la mise en place d'une résistance au témozolomide, constituant ainsi une piste intéressante à explorer.
Tunneling Nanotubes (TNTs): Intratumoral Cell-to-Cell Communication

Marie-Luce Vignais and Jean Nakhle, IRMB, INSERM, CNRS, University of Montpellier, Montpellier, France
Emmanuel Griessinger, INSERM, C3M, Nice, France; and University of Nice Sophia, Nice, France

© 2019 Elsevier Inc. All rights reserved.

Intercellular Connections in Tumors

Solid tumors and hematological malignancies are considerably heterogeneous tissues. This heterogeneity comes from the tumor cells themselves that include cancer stem cells (CSCs), believed to be responsible for tumor progression and recurrence following therapy, and cancer cells stratified at different stages of differentiation. Neoplastic tissues also include non-cancer cells. These comprise residing mesenchymal, epithelial and endothelial cells, as well as cells recruited by the tumor such as immune cells and mesenchymal stem cells (MSCs). Importantly, although non-cancerous, these cells nonetheless often present a modified and abnormal phenotype due to their location in the tumor microenvironment and consequently favor tumor progression, metastasis and resistance of the cancer cells to therapy.

Cell communication within the tumor, amongst cancer cells themselves and between cancer and non-cancer cells is now fully acknowledged as widely used by the tumor to grow and circumvent therapeutic treatments. In the last decades, this intercellular communication was believed to heavily rely on secreted cytokines/chemokines, metabolites and extracellular vesicles. In the past few years, a new means of cell-to-cell communication that uses tunneling nanotubes (TNTs) was shown to enable cells to connect to far-off cells and to transfer them biological cargos, ranging from ions to whole organelles, as it will be detailed in this chapter. This donation is qualified horizontal, to distinguish it from the vertical donation from a parental cell to its offspring during mitosis. The number of scientific publications describing this TNT-mediated new mode of communication between cells, including cancer cells, steadily increased since 2004, when they were initially described (Fig. 1). Importantly, TNTs involving cancer cells were also observed in situ, in patient resected solid tumors from both malignant pleural mesothelioma and lung adenocarcinoma, demonstrating their relevance in the cancer pathology.

The occurrence of TNTs in tumors and the ensuing intercellular trafficking are now bringing about a radical turmoil in the current paradigm of the intercellular communications that take place in tumors as TNTs guide and allow the dynamic fluxes of biological cargos, notably mitochondria, that are literally passed from the cytoplasm of the donor cell to that of the recipient cell. This TNT-mediated trafficking occurs from cells of the microenvironment to the cancer cells, modifying the functional properties and response to therapy of the tumor cells. It also occurs in the reverse direction, from the cancer cells to non-cancer cells of the tumor microenvironment, likely contributing to the observed changes in phenotype of these normal cells that ultimately further contribute to tumor progression and resistance to therapy.

General Features of TNT-Dependent Cell-to-Cell Exchanges

TNTs are long tubular structures, with diameters ranging from 50 and 1500 nm and lengths that can span several tens to hundreds of microns. The most important feature of TNTs is definitely the fact that they allow cytoplasm continuity between the connected cells.
Tunneling nanotubes are formed between different cell types and consequently enable the transport of cellular components between these cells. The transported cargos include a whole panel of cellular components, from ions, miRNAs, proteins and virus to whole organelles like lysosomes and mitochondria, as schematized in Fig. 2.

In this review, we provide a general overview of what is currently known about tunneling nanotubes: the cells involved, the cargos transported and the mechanisms that allow this intercellular trafficking. We further focus on the specific signals that induce the formation of nanotubes in tumors and on the biological outcome of these TNT-dependent communications, that ranges from metabolic reprogramming to induced survival in response to therapy. A special attention will be given to the TNT-mediated intercellular transfer of mitochondria as it has been the focus of intense scrutiny over the past few years and gives rise to remarkable biological effects in the recipient cells.

### Cell Types Involved in TNT Connections

Tunneling nanotubes were first described, in 2004, in pheochromocytoma-derived PC12 cancer cells and in normal immune cells. Ever since, the number of cell types described to undergo such connecting processes has grown to more than 50, comprising both normal and cancerous cells, as shown in Table 1. These include bladder, breast, colon, and ovarian cancer cells as well as laryngeal squamous cell carcinoma, malignant mesothelial, pheochromocytoma, and osteosarcoma cells. TNTs can provide connections between cells of the same type as for astrocytomas. TNT formation was also observed between normal and cancer cells, including human ovarian epithelial benign and cancer cells, murine stromal osteoblast and osteosarcoma cells, fibroblasts, and HeLa breast cancer cells. A number of the TNTs described between normal and cancer cells involve mesenchymal stem cells (MSCs), known to be recruited to tumor sites. MSCs are stem cells characterized by their capacity to differentiate along several lineages including...
<table>
<thead>
<tr>
<th>Authors</th>
<th>Cells involved in the tunneling nanotube connection</th>
<th>Transported cargoes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onfelt et al. (2004)</td>
<td>Human NK cells/EBV transformed human B-cells</td>
<td>GFP-tagged cell surface class I MHC</td>
</tr>
<tr>
<td></td>
<td>Human macrophages</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Human EBV transformed human B-cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Murine J774 macrophages</td>
<td></td>
</tr>
<tr>
<td>Rustom et al. (2004)</td>
<td>Rat pheochromocytoma PC12</td>
<td>Microvesicles</td>
</tr>
<tr>
<td></td>
<td>Human embryonic kidney (HEK)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normal rat kidney (NRK)</td>
<td></td>
</tr>
<tr>
<td>Castro et al. (2005)</td>
<td>Colon carcinoma cell line SW620</td>
<td>ND</td>
</tr>
<tr>
<td>Koyanagi et al. (2005)</td>
<td>Human endothelial progenitor/rat cardiomyocytes</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Watkins and Salter (2005)</td>
<td>Human dendritic cells/THP-1 cells</td>
<td>Calcium flux</td>
</tr>
<tr>
<td></td>
<td>Human THP-1 monocytes</td>
<td>Major-histocompatibility proteins (MHC class I)</td>
</tr>
<tr>
<td>Onfelt et al. (2006)</td>
<td>Human macrophages</td>
<td>Bacteria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mitochondria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vesicles (endosomes, lysosomes)</td>
</tr>
<tr>
<td>Chinnery et al. (2008)</td>
<td>Murine MHC class II dendritic cells</td>
<td>ND</td>
</tr>
<tr>
<td>Gurke et al. (2008)</td>
<td>Normal rat kidney cells (NRK)</td>
<td>Endocytic organelles</td>
</tr>
<tr>
<td>Sowinski et al. (2008)</td>
<td>Jurkat and primary T cells</td>
<td>HIV viral particles</td>
</tr>
<tr>
<td>Bukseshtiev et al. (2009)</td>
<td>Pheochromocytoma PC12 cells</td>
<td>Intracellular organelle</td>
</tr>
<tr>
<td>Eugenin et al. (2009)</td>
<td>Human macrophages</td>
<td>HIV viral particles</td>
</tr>
<tr>
<td>Plotnikov et al. (2010)</td>
<td>Human mesenchymal stromal cells/normal renal tubular cells</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Acquistapace et al. (2011)</td>
<td>Human mesenchymal stem cells/cardiomyocytes</td>
<td>Mitochondria and intracellular material</td>
</tr>
<tr>
<td>Domhan et al. (2011)</td>
<td>Human proximal tubular epithelial cells (RPTEC)</td>
<td>Microvesicles</td>
</tr>
<tr>
<td>Wang et al. (2011)</td>
<td>Rat hippocampal astrocytes and neurons</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mitochondria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Golgi fragments</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Endosomes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amyloid β</td>
</tr>
<tr>
<td>Yasuda et al. (2011)</td>
<td>Human umbilical vein endothelial cells (HUVEC)</td>
<td>Lysozymes</td>
</tr>
<tr>
<td>Islam et al. (2012)</td>
<td>Murine MSCs and alveolar epithelial cells</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Lou et al. (2012)</td>
<td>Human primary cancer cells</td>
<td>Mitochondria</td>
</tr>
<tr>
<td></td>
<td>Human mesothelial lines (MSTO-211H, VAMT, H-Meso)</td>
<td></td>
</tr>
<tr>
<td>Vallabhaneni et al. (2012)</td>
<td>Human MSCs and vascular smooth muscle cells</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Wittig et al. (2012)</td>
<td>Human retinal pigment epithelial (ARP-19) cells</td>
<td></td>
</tr>
<tr>
<td>Costanzo et al. (2013)</td>
<td>CAD cells</td>
<td>Htt aggregates</td>
</tr>
<tr>
<td>Pasquier et al. (2013)</td>
<td>Primary cerebellar granule neurons (CGNs)</td>
<td>Mitochondria</td>
</tr>
<tr>
<td></td>
<td>Human mesenchymal stem cells (MSCs)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Human endothelial cells (HECs)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Human ovarian cancer cells (SKOV3, OVCAR3, HTB-161)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Human breast cancer cells (MDA-MB-231 and MCF7)</td>
<td></td>
</tr>
<tr>
<td>Rainy et al. (2013)</td>
<td>Human B cells and T cells</td>
<td>Plasma membrane associated proteins (H-Ras)</td>
</tr>
<tr>
<td>Schiller et al. (2013)</td>
<td>HeLa</td>
<td>Transmembrane HLA-A2-EGFP protein</td>
</tr>
<tr>
<td>Ady et al. (2014)</td>
<td>VAMT (sarcomatoid mesothelioma cell line)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>H2052 (mesothelioma cell line)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MSTO-211H (derived from mesothelioma patient)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Met5A (immortalized mesothelioma cell line)</td>
<td></td>
</tr>
<tr>
<td>Ahmad et al. (2014)</td>
<td>Murine MSCs and lung epithelial cells</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Antanaviciute et al. (2014)</td>
<td>Laryngeal squamous cell carcinoma</td>
<td>Mitochondria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DAPI-positive vesicles</td>
</tr>
<tr>
<td>Figeac et al. (2014)</td>
<td>Murine cardiomyocytes and human MSCs</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Liu et al. (2014)</td>
<td>Human MSCs and umbilical vein endothelial cells (HUVEC)</td>
<td>MicroRNAs (miR-199a)</td>
</tr>
<tr>
<td>Thayanithy et al. (2014a,b)</td>
<td>Murine K7 M2 osteoscarcoma cells and MC3T3 osteoblasts</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ovarian epithelial cells and SKOV3 ovarian cancer cells</td>
<td></td>
</tr>
<tr>
<td>Thayanithy et al. (2014a,b)</td>
<td>Human biphasic mesothelioma MSTO-211H cells</td>
<td>Exosomes from other cells</td>
</tr>
<tr>
<td>Astarina et al. (2015)</td>
<td>Epithelial cells</td>
<td>Lipid droplets</td>
</tr>
<tr>
<td>Biran et al. (2015)</td>
<td>NK cells</td>
<td>Proteins</td>
</tr>
<tr>
<td>Burtey et al. (2015)</td>
<td>HeLa and NRK fibroblasts</td>
<td>TF-R (transferrin receptor), endosomes</td>
</tr>
<tr>
<td>Calcedo et al. (2015)</td>
<td>Human MSCs and breast cancer cell line MDA-MB-231</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Oswald et al. (2015)</td>
<td>Astrocytoma</td>
<td>Intercellular calcium waves (ICWs)</td>
</tr>
</tbody>
</table>

(Continued)
Table 1  TNT-connected cells and cargos—cont’d

<table>
<thead>
<tr>
<th>Authors</th>
<th>Cells involved in the tunneling nanotube connection</th>
<th>Transported cargoes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polak et al. (2015)</td>
<td>Human MSCs and acute lymphoblastic leukemia cells</td>
<td>Vital dyes</td>
</tr>
<tr>
<td></td>
<td>Human MSCs and B-cell precursors</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Zhu et al. (2015)</td>
<td>CAD neuronal cells</td>
<td>Prions</td>
</tr>
<tr>
<td>Ady et al. (2016)</td>
<td>Herpes simplex virus (NIV1066) infected and non-infected cells</td>
<td>Activated ganciclovir (Bystander effect)</td>
</tr>
<tr>
<td>Desir et al. (2016)</td>
<td>Chemoresistant ovarian cancer cells (SKOV3, C200)</td>
<td>Mitochondria</td>
</tr>
<tr>
<td></td>
<td>Resistant (SKOV3) and sensitive (A2780) ovarian cancer cells</td>
<td>Resistant ovarian cancer cells (SKOV3) and benign epithelial ovarian cells (IOSE)</td>
</tr>
<tr>
<td>Hashimoto et al. (2016)</td>
<td>Monocyte-derived macrophage</td>
<td>HIV-1</td>
</tr>
<tr>
<td>Hayakawa et al. (2016)</td>
<td>Astrocytes and neurons</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Jackson et al. (2016)</td>
<td>Human MSCs and macrophages</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Jiang et al. (2016)</td>
<td>Mesenchymal stem cells and corneal epithelial cells</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Moschoi et al. (2016)</td>
<td>BM-MSCs and acute myeloid leukemia cells</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Reichert et al. (2016)</td>
<td>Hematopoietic progenitors</td>
<td>CD133</td>
</tr>
<tr>
<td>Tardivel et al. (2016)</td>
<td>Neurons</td>
<td>Tau protein</td>
</tr>
<tr>
<td>Victoria et al. (2016)</td>
<td>Astrocytes and neurons</td>
<td>Prions</td>
</tr>
<tr>
<td>Zhang et al. (2016)</td>
<td>iPSC-MSCs, BM-MSCs and cardiomyocytes</td>
<td>Mitochondria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxidized phospholipids</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calreticulin</td>
</tr>
<tr>
<td>Claus et al. (2017)</td>
<td>Murine macrophage-like cells (J774A.1)</td>
<td>SAA1 protein</td>
</tr>
<tr>
<td>Dieriks et al. (2017)</td>
<td>Neuroblastoema cells (SH-SYSY)</td>
<td>α-Synuclein</td>
</tr>
<tr>
<td>de Rooij et al. (2017)</td>
<td>ALL cells and MSCs</td>
<td>Autophagosomes mitochondria ICAM1</td>
</tr>
<tr>
<td>Kumar et al. (2017)</td>
<td>Lung epithelial cells (infected and uninfected)</td>
<td>Mitochondria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ribosomes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Influenza virus proteins and genome</td>
</tr>
<tr>
<td>Lu et al. (2017)</td>
<td>Bladder cancer cells</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Mahrouf-Yorgov et al. (2017)</td>
<td>Cardiomyocytes and MSCs</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Marlein et al. (2017)</td>
<td>Bone marrow stromal cells and leukemic blasts</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Nzigou Mombo et al. (2017)</td>
<td>Human MSCs and glioblastoma stem cells</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Patheja and Sahu (2017)</td>
<td>Breast adenocarcinoma cells (MCF-7)</td>
<td>Cytoplasmic fragments</td>
</tr>
<tr>
<td>Sáenz-de-Santa-María et al. (2017)</td>
<td>Squamous cell carcinoma</td>
<td>Endosomal/lysosomal vesicles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Autophagosomes</td>
</tr>
<tr>
<td>Sanchez et al. (2017)</td>
<td>Wharton’s jelly mesenchymal stem cells</td>
<td>Mitochondria</td>
</tr>
</tbody>
</table>

Osteocytes, adipocytes and chondrocytes. They are found in many tissues, notably the bone marrow and the adipose tissue. They are recruited to the inflammatory tumor microenvironment where they have been shown to make TNT connections in vivo.

It is worth noting that the occurrence of TNTs is not limited to pathological and cancerous tissues. TNTs also constitute a means of intercellular connection in physiological conditions. This was shown for renal tubular, kidney and retinal pigment epithelial cells. TNT formation was also reported for connecting endothelial progenitor cells with endothelial cells and cardiac myocytes. Likewise, TNTs were described between hippocampal neurons and astrocytes. The immune system, notably macrophages, monocytes, dendritic cells, natural killer and B cells, appear particularly prone to use TNT-mediated communication, with measurable effects on the immune response. MSCs are also recruited to damaged or inflammatory tissues where they contribute to tissue repair. TNT-dependent connections have been observed between MSCs and diverse cell types such as renal tubular cells, cardiomyocytes, bronchial epithelial cells, macrophages, endothelial cells, and vascular smooth muscle cells. Even though TNT-mediated connections appear as a widespread process, not all cell types are endowed with this connecting capacity. For instance, astrocytic brain tumors can develop TNT connections that allow intercellular calcium waves and resistance to radiotherapy, but oligodendroglial tumors do not.
Beyond Humans and the rat and mouse mammalian models, it is worthwhile noting that TNTs were also described in *B. subtilis* and *E. coli* bacteria, in Drosophila and in the zebrafish, thus underlining the evolutionary conservation of this cell-to-cell connecting mechanism.

**Cargos Transported Between TNT-Connected Cells**

**Mitochondria**

Mitochondria have been the TNT cargos most extensively studied so far. This stems from the extent of the biological effects of the transported mitochondria, both on the metabolism and functional capacities of the target cells but also, and more practically, from the available tools to observe this mitochondria trafficking, both in vivo and in vitro. Detection of the mitochondria transfer was performed using fluorescent mitochondrial vital dyes and viral expression of GFP-tagged proteins targeting the mitochondria (Fig. 3). In heterologous systems, human mitochondria could be detected with antibodies specific for human mitochondrial organelles. At the genetic level, taking advantage of the SNPs present in the mitochondrial DNA (mtDNA), mitochondria originating from different donors could be detected and their concentration evaluated on the basis the mtDNA SNP quantification. In addition, and independently of TNTs, mitochondria (isolated beforehand) display the remarkable capacity to be directly internalized by cells in a macropinocytosis-like process. This provided a useful tool to analyze the biological effects of the transferred mitochondria in the target cells.

TNT and gap junction-mediated mitochondria trafficking involves both normal and cancer cells, and cells as diverse as renal tubular epithelial cells, endothelial cells, macrophages, neuronal cells, astrocytes, astrocytomias, laryngeal squamous cell carcinoma, and leukemia cells. A vast majority of studies so far focused on mitochondria transport originating from MSCs and targeting a whole series of different cells, notably cardiomyocytes, endothelial cells, pulmonary alveolar epithelial cells, renal tubular cells, macrophages as well as cancer cells such as acute myeloid leukemia cells, breast cancer cells and glioblastoma stem cells (Fig. 4).

**Other Cargos**

Lots of cargos, outside from mitochondria, can be transported between TNT-connected cells. This is the case for the lysosomal organelles. Transported cargos also include the prion and the Tau infectious proteins, possibly contributing to the related pathologies. The HIV virus was described to traffic through TNTs from infected to non-infected cells, thus precluding the need for a fully mature HIV virus to infect neighboring cells. The variety of TNT-transported cargos is amazingly large as, in addition to the above, it also includes microRNAs, lipid droplets and Ca²⁺ calcium ions.

**Mechanisms of TNT and Gap Junction-Dependent Cell-to-Cell Exchanges**

Gap junctions (GJCs) are intercellular channels that connect the cytoplasms of different cells and allow the exchange of molecules and ions between these cells. First observed in 1958, their role in cardiac tissue has been largely studied. GJCs are composed...
of two hemi-channels (one for each cell), each of them composed of a hexamer of transmembrane connexin proteins. The connexin 43 (Cx43) was shown, both in vitro and vivo, to play an essential role in the formation of the TNT and gap junction-dependent cell-to-cell connections and subsequent cargo trafficking. Since GJCs only allow passage of molecules smaller than ATP, their precise implication in the TNT-mediated intercellular transfer of organelles like mitochondria obviously deserves further investigation. Additional mechanisms for TNT-mediated intercellular cargo trafficking have also been described to involve M-Sec/TNFαip2 and the exocyst complex as well as various GTPases. However, the precise role of the different GTPases, notably Cdc42, RalA, Rab8, that have been analyzed so far for their involvement in TNT elongation and cargo trafficking, still needs to be fully clarified. Importantly though, the Rho GTPase Miro1 (also called RhoT1/2), well known for taking part with the Milton adapter protein and the kinesin molecular motor in mitochondria transport in axons, is now established to play a key role in the TNT and gap junction-dependent mitochondria transport. Cargo transport within the TNTs occurs along cytoskeleton fibers. The nature of this cytoskeleton depends on both the types of cells connected and the cargos transported. Both microtubules and actin microfilaments, either alone or together inside the same tunneling nanotubes, mediate the active cargo trafficking inside the TNTs.

### Signals That Regulate TNT Formation and Cargo Trafficking

Identifying the factors that promote, or inhibit, the formation of TNTs and the subsequent cargo trafficking is of great importance to fully understand the role of this process in tissue homeostasis and, most of all, in pathologies. Different genetic and chemical stresses initiate or stimulate TNT formation and intercellular organelle exchange. These stimuli can be classified in two categories of stress: those affecting the cellular energy metabolism and those related to cell defense in response to inflammation or DNA damage.

Mitochondrial deficiency is a cellular stimulus sufficient to trigger exchange of mitochondria between mitochondria-competent and deficient cells. For instance, lung carcinoma cells treated over long periods with the mitochondrial DNA damaging agent ethidium bromide have non-functional mitochondria but are still viable. These carcinoma cells trigger TNT formation from bone marrow MSCs toward the carcinoma cells leading to the exchange of functional mitochondria and to the recovery of mitochondrial function in the cancer cells. Mitochondrial chemical poisoning with agents such as rotenone or antimycin, that block the electron transport chain, also increases the mitochondria donation from untreated cells to the chemically treated cells. In a consistent manner, glucose starvation and oxygen deprivation also constitute inducer stimuli. Reactive oxygen species (ROS) that are produced during metabolic or physical stress conditions are also involved in the cross talk signaling between the requesting
and donor cells. Actually, the experimental increase in cellular ROS, upon cell exposure to hydrogen peroxide for instance, is a strong TNT inducer.

Alternatively, inflammatory signaling induced by bacterial lipopolysaccharides or TNFα treatment of the recipient cells are also strong stimuli identified as promoters of mitochondria exchange. In addition, a number of DNA damaging agents, mostly employed for cancer treatment, are also potent inducers of TNT-mediated mitochondria exchange. Zeocin, a DNA intercalating agent related to bleomycin, was found to increase by a factor of 10 the number of TNTs formed between renal proximal tubular epithelial cells. Cytarabine (ARA), a nucleoside analog used for the treatment of acute myeloid leukemia (AML), as well as the topoisomerase II inhibitor etoposide and the anthracycline doxorubicin were found to promote the acquisition of MSC mitochondria by AML cells.

Inhibition of the horizontal mitochondrial organelle exchange can be achieved by targeting different steps of the TNT exchange process including: (1) the initial stress signal produced by the cargo-requesting cells, (2) the plasma membrane protrusion formation, (3) the contact/docking between the TNT-involved cells, and (4) the “railway motorization” allowing the cargo trafficking along the cytoskeleton fibers contained within these TNTs. Stress signals leading to local ROS increase can be lessened with antioxidizing agents such as N-acetylcysteine. Cell directional polarization and TNT formation can be opposed by virtually all cytoskeleton inhibitors, including agents that either stabilize or depolymerize microtubules and actin microfilaments. Inhibiting the connexin mediated cell-cell docking or shielding the cargo-carrying cells with annexin-V, a protein that recognizes phosphatidylserines abnormally exposed extracellularly by the stressed cargo-requesting cells, can target the cell-cell docking. Finally, even when bridging TNTs have already been established, inhibiting the actin or microtubule-based molecular motors can block cargo trafficking between the donor and acceptor cells.

**TNT/Gap Junction-Dependent Connections in Cancer**

The occurrence of intercellular organelle exchange is established for solid tumors as well as for hematological malignancies, using immortalized cell lines or fresh primary cancer cells isolated from patients resected tumors. With regard to the cancer cells, organelle exchange can be either inbound (organelle intake by the cancer cell) or outbound (organelle unloading toward the cellular microenvironment). The tumor microenvironment, although not transformed and mutated, plays a direct role in cancer initiation, progression and response to therapeutic treatments. In particular, cells of the microenvironment interact with the cancer stem cells (CSC) present in the tumor and display instructive and protective functions to maintain CSC quiescent and immature properties. Cancer cells educate their microenvironment for the benefit of the CSCs and at the expense of the normal tissue adult stem cells present within the tissue. In this particular context of microenvironment remodeling, cancer cells can use the outbound transfer of mitochondria or lysosomes, to the non-cancer cells of the microenvironment and consequently modify their cytokine secretion pattern. Acute lymphoblastic leukemia cells use TNTs to signal to MSCs and drive the stroma secretion of the pro-inflammatory cytokines CXCL10, CCL2, and interleukin 8. Beyond the outbound transfer to modify their microenvironment, cancer cells can use this unloading system to detoxify during chemotherapy exposure. The outbound transfer of lysosomal vesicles containing the chemotherapeutic agents was shown to reduce the concentration of these toxic agents in leukemic cells.

Regarding the intake of mitochondria, its main reported outcome is a survival benefit for the recipient cells, for both normal or cancer cells. For instance, leukemic cancer stem cells that capture mitochondria during cytarabine exposure become more resistant to cell death and boosted with a long-term regrowth potential. In line with these observations, mitochondria horizontal transfer directly modifies the energetic metabolism of the recipient cells. Many cancer cells predominantly produce energy through a high rate of glycolysis, which converts glucose to pyruvate, followed by the production of lactic acid from this pyruvate. On the contrary, most normal cells use an alternative pyruvate metabolic pathway that takes place in the mitochondria where the oxidation of pyruvate ultimately leads to the phosphorylation of ADP in ATP, in a pathway called oxidative phosphorylation (OXPHOS). The complete eradication of OXPHOS for a cancer cell is a disadvantage. This can be achieved experimentally by a long-term exposure to mitochondrial DNA damaging agents, leading to cells named ρ0 cells. These OXPHOS-incompetent ρ0 cancer cells have a lower tumorigenic and metastatic potential compared to the mitochondria-competent parental cancer cells. Over time ρ0 cancer cells, placed in vivo within a mitochondria-competent surrounding environment, recover their OXPHOS activity through mitochondria horizontal transfer and retrieve their tumorigenic potential. Independently of the organelle exchange, different anticancer radiation or chemical therapies can switch cancer cell glycolytic metabolism toward an OXPHOS metabolism. In different cancer models, an OXPHOS metabolism was shown to confer cancer cells an increased drug resistance compared to a glycolytic metabolism. Antineoplastic agents stimulate intercellular mitochondria exchange, which further favors the OXPHOS metabolism in the recipient cancer cells. This survival benefit associated with the intake of exogenous organelles is observed not only for differentiated cancer cells but also for cancer stem cells. These observations, while questioning our long-standing view of cancer metabolism, offer new opportunities for developing innovative and more efficient therapeutic treatments. For instance, blocking the microenvironment-mediated drug resistance acquisition by interfering with the TNT-based intercellular organelle exchange combined with the inhibition of the OXPHOS metabolism is expected to improve the current treatment standards and tackle cancers at their roots.

Noteworthy, outside from cancer, TNT and gap junction-dependent cargo exchange also has important biological effects, resulting notably in tissue repair. As demonstrated in murine models of lipopolysaccharide and rotenone-induced damage of pulmonary alveoli, lung instillation of MSCs leads to in situ connections between the MSCs and the damaged pulmonary alveolar epithelial cells and to the subsequent transfer of MSC mitochondria, resulting in the regeneration of the affected alveoli and mice.
survival. In another context, that of E. coli-induced pneumonia (murine model), acquisition of MSC mitochondria by lung alveolar macrophages results in increased phagocytic activity and antimicrobial response. Consequently, in non-tumor injured tissues, therapeutic strategies would aim at increasing the mitochondria transfer occurrence and efficacy, both by increasing the local number of mitochondria donor cells in the wounded environment and by pharmacologically promoting cell–cell interactions and organelle trafficking from these cells.

**Prospective View**

The discovery of TNT-mediated organelle exchange provides new perspectives in the field of cancer cell biology. The discovery of this new mode of intercellular communication stems from the efforts of the scientific community, in the past two decades, to improve the physiological relevance of the studied cancer models. Although immortalized cancer cell lines were helpful to study the intrinsic cellular and molecular deregulations responsible for cancer cell transformation, these models were less suited for studying the biology of the primary tumors and their interactions with their microenvironment. For example, standard cancer cell lines are generally cultured in vitro with high glucose and oxygen concentrations, at least double their actual physiological concentrations. The recent advances in engineering organotypic co-culture systems, in 2- or 3-dimensions, as well as the development of humanized xenograft systems using immunodeficient mice, are prompting the discovery of new features in cancer biology.

It is worth mentioning, at this point, that most of our current knowledge of the TNT-mediated intercellular exchanges originates from in vitro observations. Even though TNT connections and mitochondria intercellular trafficking were also demonstrated to occur in vivo, it will be worthwhile extending these in vivo studies. For instance, it is presently unknown whether current anti-cancer therapies do increase TNT formation in vivo. A number of likewise unanswered questions remain to be explored, such as the amplitude of the TNT phenomenon in the different types of cancer, its frequency as well as at its prognostic value. The time scale during which cells remain biologically modified, following TNT-mediated mitochondria organelle acquisition, needs as well to be better assessed. It also remains to be determined whether there are “quality control” checkpoints for mitochondria donated by the donor cells as well as for those accepted by the recipient cells. It will most likely depend on both the donor and recipient cells biological properties. Furthermore, technical issues will need to be settled, notably the monitoring of chemical probes and the development of novel markers for selectively tracking the exchanged mitochondria organelles over longer periods of time.

As a conclusion, it now clearly appears that the discovery and characterization of TNT intercellular connections, and of cargos transported from one cell to the other through these connections, bring a novel understanding of the cancer cell biology that takes place within tumors. As this cargo trafficking has consequences on tumor progression and resistance to therapy, the challenge will now be to exploit this new knowledge to conceive and develop novel anticancer therapeutic strategies.

**References**


Further Reading


BIBLIOGRAPHY


stromal cells (TASC) to primary Glioblastoma cells. Biochemical and Biophysical Research Communications S0006291X20316995.


