Cellules stromales mésenchymateuses comme vecteurs cellulaires de nanoparticules : un nouvel outil thérapeutique des tumeurs cérébrales

Mathilde Roger

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Année 2010
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Thèse DE DOCTORAT
Spécialité : NEUROSCIENCES

ECOLE DOCTORALE : BIOLOGIE SANTE

Présentée et soutenue publiquement
Le 30 novembre 2010
à Angers

par **Mathilde ROGER**

Devant le jury ci-dessous :

Dr Christine Vauthier — Rapporteur
Directeur de recherche, CNRS UMR 8612, Université Paris-Sud

Pr Edwige Petit — Rapporteur
Professeur des universités, UMR 6232, Université de Caen

Pr Pascale Jeannin — Examinateur
Professeur des universités, UMR_S892, Université d'Angers

Dr Anne Clavreul — Co-Encadrante de thèse
Ingénieur hospitalier, INSERM U646, Angers

Pr Phillipe MENEI — Directeur de thèse
Professeur des universités/ Praticien Hospitalier, INSERM U646, Angers

INSERM U646, Ingénierie de la Vectorisation Particulaire
IBT, 10 rue André Boquel, 49100 Angers, France

ED 502 - BS

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J'espère que ce travail est à la hauteur de

Celui que tu aurais pu accomplir.

Pas un jour ne passe sans que je ne pense à toi

Et à tous nos moments partagés…

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ABBREVIATIONS

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5-FU : 5 -Fluorouracile

BHE : barrière hémato-encéphalique

BMP-2 : « bone morphogenetic protein-2 »

BT-SCs : « brain tumor stem-like cells »

CAFs : « cancer associated fibroblasts »

CED : « convection enhanced delivery »

CSE : cellules souches embryonnaires

CSMs : cellules souches mésenchymateuses

CSNs : cellules souches nerveuses

CSTs : cellules souches tumorales

EGFR : « epidermal growth factor receptor »

ERO : Espèces réactives de l'oxygène

Fc-diOH : ferrociphenol

FDA*:* « Food and Drug Administration»

FSP : « fibroblast surface protein »

GASCs : « Glioblastoma associated stromal cells »

GB : gliobastome

GM-CSF : « Granulocyte Macrophage Colony-Stimulating Factor »

GVHD : « graft-versus host disease »

hADSC : « human adipocytes derived-stem cells”

HIF-1 : « Hypoxia-inducible factor-1 »

IDH1 : isocitrate déshydrogénase 1

IFN-β : interféron-β

IL : Interleukin

l'IL-13Rα2 : « Interleukin-12 receptor α2 »

MCP-1 : « macrophage chemoattractant protein-1 »

MEC : matrice extracellulaire

MGMT : O6-méthyl transférase

MIAMI : « Marrow-Isolated Adult Multilineage Inducible »,

MMPs : métalloprotéases matricielles

MO : moelle osseuse

MS : microsphères

NCLs : nanocapsules lipidiques

NG2 : « neuron-glia 2 »

NLSs : nanoparticules solides lipidiques

NPs : nanoparticules

NT-3 : « neurotrophin-3 »

(ODN)-CpG : OligoDésoxyNucléotides

PDGF-BB : « platelet-derived growth factor BB»

PDGFR : « platelet derived-growth factor receptor»

PDGFR-β : « platelet derived growth factor receptor »

Pgp : glycoprotéine P

PLA : acide lactiques

PLGA : acides lactiques et glycoliques

PMM 2.1.2 : poly(méthylidène malonate 2.1.2)

RTK : récepteurs à tyrosine kinase

SDF-1 alpha : « stromal cell-derived factor-1 alpha »

SNC : système nerveux central

SVF : sérum de veau fœtal

TAAs : « tumor associated antigens »

TGF-ss1 : « transforming growth factor-ss1 »

TGFβ2 : « transforming growth factor beta 2 »

TRAIL : « Tumor necrosis factor-related apoptosis-inducing ligand »

VEGF : « vascular endothelial growth factor »

α-SMA : « α-smooth muscle actin »,

INTRODUCTION GENERALE

-1- Les gliomes malins

Les gliomes malins ou tumeurs gliales malignes sont des tumeurs primitives du système nerveux central (SNC). Avec une incidence de 5 à 7 cas pour 100 000 personnes en France chaque année, elles représentent plus de 60% des tumeurs intracrâniennes chez l'adulte. Ces tumeurs gliales peuvent être classées en fonction de leur type histologique. En 2007, l'OMS a mis à jour sa classification (1) (Tableau 1). Elle distingue, parmi les gliomes adultes, les astrocytomes (grade I, II, III ou IV), les oligodendrogliomes (grade II ou III) et les oligoastrocytomes (grade II ou III). Les tumeurs gliales de haut grade de malignité (III et IV) sont souvent regroupées sous le terme de gliomes malins. La forme la plus commune mais aussi la plus agressive est l'astrocytome de grade IV, aussi appelé gliobastome (GB, 2 à 3 cas pour 100 000 personnes) (2). Le traitement standard optimisé des GB consiste en une exérèse chirurgicale suivie d'une radiothérapie et d'une chimiothérapie concomitante et adjuvante par Témozolomide. Cependant, le pronostic des patients est particulièrement mauvais avec une médiane de survie de 9 mois pour toute la population en intention de traitement et de 14,6 mois pour les patients traités (3, 4).

Tableau 1 : classification de l'OMS des gliomes

Type histologique	*Grade*
Astrocytaire	Grade I : Astrocytomes pilocytiques Grade II : Astrocytomes diffus Grade III : Astrocytomes anaplasiques Grade IV : Glioblastomes
Oligodendrocytaire	Grade II Grade III
Mixte	Grade II : Oligoastrocytomes mixtes Grade III : Oligoastrocytomes anaplasiques

-2- Développement de thérapies ciblées et personnalisées pour les gliomes malins

Compte tenu de l'hétérogénéité intratumorale et de la variabilité inter-patient des gliomes malins, diverses voies de recherche sont développées pour concevoir des thérapies ciblées et personnalisées (5, 6). Ces voies de recherche décrites ci-après de façon non exhaustive consiste principalement à caractériser la biologie et le microenvironnement des gliomes malins, comprendre les mécanismes de récidives et à rechercher des marqueurs génomiques, transcriptomiques et/ou épigénétiques capables de fournir une information sur le pronostic et la sensibilité aux traitements pour développer des thérapies personnalisées.

-2.1- Caractérisation de la biologie des gliomes malins

Plusieurs études en cours cherchent à identifier les molécules qui contrôlent le cycle cellulaire, la résistance à la mort cellulaire, la motilité des cellules, la formation de nouveaux vaisseaux et la reconnaissance du système immunitaire afin de les cibler spécifiquement. Par exemple, il a été montré l'importance des récepteurs à tyrosine kinase (RTK) tels que l'EGFR (« epidermal growth factor receptor ») et le PDGFR (« platelet derived-growth factor receptor) (7) et de leurs voies de signalisation (PI3K/Akt/mTOR, Ras/MAPK, protéine kinase C) (8-10) dans le développement et la croissance des gliomes. De nombreux inhibiteurs ciblant ces récepteurs et leurs voies de signalisation ont ainsi été développés ou étudiés et plusieurs sont actuellement évalués en essais cliniques (6, 11).

-2.2- Caractérisation du microenvironnement des gliomes

Aujourd'hui, il est évident que le microenvironnement tumoral incluant le réseau vasculaire, la matrice extracellulaire (MEC) et divers types cellulaires (cellules immunitaires, cellules endothéliales et cellules stromales), a un rôle très important dans la progression des tumeurs en général.

L'angiogénèse est reconnue comme un élément clé de la progression des GB et a été particulièrement étudiée (12). La formation de vaisseaux permet aux cellules tumorales de se fournir en nutriments et oxygène mais aussi d'éliminer des déchets métaboliques. L'inhibition de l'angiogénèse tumorale est aujourd'hui une stratégie thérapeutique très attractive (13, 14). Les bases moléculaires de la prolifération vasculaire dans les GB sont complexes. Les facteurs les plus importants incluent HIF-1 (Hypoxia-inducible factor-1), le VEGF (« vascular endothelial growth factor ») et les angiopoiétines. Des inhibiteurs directs et indirects de l'expression de HIF-1 et en particulier de la sous-unité HIF-1α sont testés en clinique (15). De plus, le traitement utilisant le bevacuzimab®, un anticorps monoclonal qui se lie au VEGF et bloque ainsi la liaison du VEGF à ses récepteurs entrainant une inhibition de l'angiogenèse, est au cœur de nombreux essais cliniques (16).

D'autres études s'intéressent au processus d'invasion des GB. L'invasion du parenchyme cérébral par les cellules tumorales est gouvernée par une interaction coordonnée de composants intracellulaires et extracellulaires : les constituants de la MEC, les protéases (comme les métalloprotéases matricielles, MMPs) ou les récepteurs à la surface des cellules tumorales (principalement les intégrines et les RTK). Plusieurs essais cliniques utilisant des antagonistes d'intégrines (Cilengitide) (17) et des inhibiteurs des MMPs (Marimastat, Metastat et Prinomastat) (18) ont lieu pour traiter les gliomes malins.

Enfin, le statut immunitaire du microenvironnement tumoral des gliomes est également étudié. Il est clairement reconnu que les gliomes développent différents mécanismes pour échapper au système immunitaire (19).

Un de ces mécanismes correspond à la sécrétion de molécules immunosuppressives dont le « transforming growth factor beta 2 » (TGFβ2). Le TGFβ2 est une molécule connue pour diminuer l'activation des cellules effectrices du système immunitaire comme les cellules NK, les lymphocytes T cytotoxiques ou les lymphocytes B. Le TGFβ2 inhibe également la maturation des cellules présentatrices d'antigène telles que les cellules microgliales et les cellules dendritiques. Enfin, le TGFβ2 stimule la croissance tumorale en favorisant l'angiogenèse (20). L'inhibition de ce facteur est donc une stratégie séduisante. Une étude de phase I/II utilisant un oligonucléotide antisens l'AP 12009, qui bloque la production du TGFβ2 a ainsi permis d'augmenter la médiane de survie des patients par rapport au traitement classique (21).

Une immunothérapie locale *via* les OligoDésoxyNucléotides (ODN)-CpG perfusés au sein de la tumeur a également été développée par le Pr Antoine Carpentier pour stimuler l'immunité au sein de la tumeur cérébrale (22). Les ODN-CpG imitent l'effet de l'ADN bactérien et sont de puissants activateurs du système immunitaire. Un essai de phase II national et multicentrique est actuellement proposé à des patients nouvellement diagnostiqués.

D'autres stratégies utilisent des vaccins périphériques pour stimuler la réaction immunitaire anti-tumorale. A Angers, une étude de phase I coordonnée par le Pr Philippe Menei a été réalisée sur des patients porteurs d'un gliome malin pour évaluer la tolérance et la faisabilité d'une vaccination par cellules tumorales autologues irradiées associée à une microperfusion locale de GM-CSF

(« Granulocyte Macrophage Colony-Stimulating Factor »). Le traitement a été bien toléré et trois patients sur les 5 vaccinés ont eu une survie inhabituellement longue (23).

Une étude de phase I avec un vaccin dendritique DCVax-Cerveau produit par la société Biotherapeutics a également permis d'obtenir des résultats encourageants avec 63% de survivants à 2 ans contre 26% avec le traitement standard. Ce vaccin emploie les cellules dendritiques autologues qui sont mélangées avec des antigènes de tumeur (24). Un autre vaccin actuellement très utilisé est le CDX-110 développé par la société Celldex therapeutics qui cible l'EGFRvIII. L'EGFRvIII, un variant du récepteur à l'EGF, est exprimé dans 25% des GB (25). Ce vaccin en complément du traitement standard (Radio-chimiothérapie avec Témozolomide suivie de Témozolomide adjuvant) a presque doublé les médianes de survie (26).

-2.3- Compréhension des mécanismes de récidive

Les récidives surviennent dans près de 90% des cas à,2 cm autour du site initial malgré la radio/chimiothérapie (27, 28). Les mécanismes de cette récidive sont actuellement explorés. Il a été identifié dans la masse tumorale des gliomes, un petit nombre de cellules dites cellules souches tumorales (CSTs ou BT-SCs pour « brain tumor stem-like cells »)). Ces CSTs résistent aux traitements agressifs de radiothérapie et de chimiothérapie et sont tenues pour responsable des récidives des gliomes malins. Différentes stratégies sont développées pour viser ces CSTs telles que l'inhibition de la migration de ces cellules vers la tumeur, l'amélioration de la différenciation des CSTs, la radiosensibilisation, le traitement direct de la niche cellulaire des CSTs (la zone sous-ventriculaire), la virothérapie oncolitique, la vaccination (ICT-107) et le blocage des molécules d'adhésion (29).

Récemment, Glas *et al.* (2010) ont isolés des cellules tumorales infiltrantes par primocultures de biopsies issues de la cavité de résection de GB. Ces cellules présentent un profil moléculaire et des réponses à l'irradiation et aux substances chimiothérapeutiques *in vitro* distincts des cellules tumorales présentes dans le centre de la tumeur (30). L'étude de ces cellules pourrait ouvrir de nouvelles voies de recherche pour une optimisation de la thérapie du gliome.

D'autre part, suite au projet Gliome Grand-Ouest « du centre de la tumeur vers la périphérie » coordonné par le Pr Philippe Menei, une population cellulaire originale a été isolée à partir de primocultures de la zone macroscopiquement normale, prélevée à 2 cm de la cavité d'exérèse du GB. Cette population cellulaire présente des caractéristiques très proches des « cancer associated fibroblasts » (CAFs) décrits dans le stroma des carcinomes. Comme les CAFs, ces cellules qui ont été appelées GASCs (« Glioblastoma associated stromal cells »), ont un génome normal mais possèdent des modifications transcriptionnelles qui favorisent la prolifération de lignées gliales tumorales *in vitro* et *in vivo*. Ces cellules pourraient également représenter une cible potentielle nouvelle pour le traitement des gliomes malins.

-2.4- <u>Recherche des marqueurs génomiques, transcriptomiques et/ou épigénétiques</u>

Les marqueurs génomiques, transcriptomiques et/ou épigénétiques pourraient fournir une information sur le pronostic et la sensibilité aux traitements pour développer des thérapies personnalisées. De nombreuses aberrations chromosomiques sont retrouvées dans les GB. Les plus fréquentes incluent le gain du chromosome 7, la perte du bras court du chromosome 9 et la perte du

chromosome 10 (31). Compte-tenu de l'hétérogénéité génomique des GB, aucun marqueur pronostic robuste lié aux altérations du nombre de copies de l'ADN n'a encore pu être mis en évidence. Seule la mutation somatique du gène isocitrate déshydrogénase 1 (IDH1) constitue une réelle avancée en ce domaine et semble permettre des répercussions en clinique (32). En effet, une mutation du gène IDH1, en position 132 sur le bras long du chromosome 2, 2q32, est fréquente dans les GB secondaires et en particulier dans les astrocytomes de bas grade et les oligodendrogliomes. Sa présence serait de bon pronostic. De plus, cette mutation n'a pas été détectée dans les autres types de tumeurs (tumeurs gastro-intestinales, mélanomes, cancers de la vessie, du sein, du poumon, du pancréas, de la prostate, colorectal, ovarien, ou carcinomes thyroïdiens) suggérant un rôle unique de cette mutation dans le développement des gliomes. (33).

Des études transcriptomiques des gliomes malins ont également permis de déceler de nouveaux sous-types moléculaires qui semblent influencer la progression tumorale, la réponse aux traitements, ainsi que le pronostic (34-37). Par exemple, Philipps et ses collaborateurs ont définis trois sous-groupes : « pro-neuraux », « proliférants » et « mésenchymateux » (34). Cette sous-classification est fortement associée au grade et à la survie des patients. L'entité des gliomes « pro-neuraux », est de « bon pronostic » et regroupe la plupart des tumeurs de grade III quelles que soient leurs morphologies (oligodendrogliale ou astrocytaire). En revanche, les entités des gliomes « proliférants » et « mésenchymateux », sont de « mauvais pronostic » et rassemblent une majorité de GB, respectivement avec et sans nécrose.

Des études sur les régulations épigénétiques et plus particulièrement celles liées à l'état de méthylation de l'ADN ont aussi permis de montrer l'importance du

statut de méthylation du promoteur de l'ADN O6-méthyl transférase (MGMT), enzyme de réparation de l'ADN, comme facteur prédictif de réponse positive à la chimiothérapie par Témozolomide (4, 38).

-3- Développement de stratégies pour apporter les molécules thérapeutiques dans les gliomes malins

En parallèle du développement de thérapies ciblées et personnalisées, il est nécessaire de trouver de nouvelles stratégies globales capables de vectoriser les agents thérapeutiques dans la tumeur cérébrale. L'apport de traitements est limité par plusieurs obstacles :

D'une part, le SNC reste un site privilégié, en effet la présence de la barrière hémato-encéphalique (BHE) qui sépare le sang du parenchyme cérébral (39), limite l'entrée d'agents extérieurs présents dans la circulation sanguine, ce qui inclus aussi les molécules thérapeutiques. Très peu de molécules peuvent traverser les cellules endothéliales de la BHE, qui sont reliées entre elles par des jonctions serrées (40). Seules les molécules présentant des propriétés physico-chimiques particulières, notamment une faible masse moléculaire et un fort caractère lipophile, sont capables de traverser cette barrière biologique (41).

D'autre part, les gliomes malins ont un caractère extrêmement infiltrant. La résection chirurgicale est « macroscopiquement » complète, laissant des cellules tumorales infiltrées dans les parois de la cavité d'exérèse. Ce sont ces cellules qui infiltrent le parenchyme sain et qui sont à l'origine des récidives. La diffusion de molécules thérapeutiques dans le parenchyme cérébrale est très faible due en partie à la forte pression de fluide intercellulaire dans les tumeurs (42-44). Il est donc

difficile de traiter ces cellules tumorales infiltrées. De plus, la BHE surexprime les pompes d'efflux, telles que la glycoprotéine P (Pgp). La Pgp est capable d'effluer les molécules thérapeutiques hors du cerveau provoquant ainsi un phénomène de résistance (45).

Enfin, les molécules injectées ont un faible index thérapeutique. En effet, Les cellules tumorales s'abritent derrière un tissu extrêmement fragile et sensible aux agressions comme la radiothérapie ou certains antimitotiques. Les doses de principe actif injectées sont donc limitées afin de détruire les cellules tumorales sans détruire les cellules nerveuses normales.

Pour optimiser la thérapie des gliomes malins, il est donc nécessaire de mettre aux points des stratégies pouvant améliorer la biodistribution et l'index thérapeutique du principe actif. Différentes stratégies ont été développées telles que la CED « convection enhanced delivery », les virus oncolytiques, les systèmes polymériques, les nanotechnologies, et les cellules transfectées dont les cellules souches. Dans cette introduction, nous allons principalement parler de l'apport de ces trois dernières stratégies dans la thérapie du gliome.

-3.1- Les biotechnologies

3.1.1. Les implants et microsphères

Depuis une vingtaine d'année, les avancées effectuées dans le domaine des biotechnologies laissent entrevoir de nouvelles perspectives. C'est le cas, notamment des implants polymériques qui peuvent être déposés après résection de la tumeur dans la cavité formée. Approuvé depuis 1996, l'implant nommé Gliadel® est utilisé dans le traitement du gliome. Cet implant est composé d'un polymère polyanhydride, l'acide poly[1,3-bis(carb-oxyphenoxy)propane-co-sebacic] et est

chargé en carmustine (BCNU) permettant ainsi une libération de façon prolongée du principe actif après implantation (46, 47). L'expérience française de l'utilisation de cet implant a montré une bonne tolérance et une augmentation de la médiane de survie avec association d'une chimiothérapie au Témozolomide (48). Cependant, bien que très intéressante, cette technique pose le problème de la faible diffusion du principe actif. De plus, le dosage du principe actif est limité par la taille de l'implant.

D'autres stratégies utilisant les polymères ont aussi été testées. Des microsphères (MS) d'acide poly(lactic-co-glycolic) (PLGA) libérant du 5-Fluorouracile (5-FU) ont montré des résultats encourageants lors de deux essais cliniques réalisées au sein du CHU d'Angers (49-51). L'essai randomisé multicentrique de phase IIb a montré une amélioration de la médiane de survie (15.2 mois) des patients traités avec une combinaison radiothérapie/MS-5-FU en comparaison avec le groupe radiothérapie seule (12.3 mois), bien que ces résultats n'aient pas été significativement différents. Cette faible amélioration de la médiane de survie peut-être due à une dégradation trop rapide de polymère. En effet, les microsphères libèrent le 5-FU *in situ* seulement pendant 20 jours et sont totalement dégradées après 2 mois. D'autres essais ont été réalisés chez le rat en changeant le polymère afin d'obtenir une dégradation plus lente, tel que le poly(méthylidène malonate 2.1.2) (PMM 2.1.2) (52). Malgré une amélioration de la survie des animaux, il a été montré que ce polymère était très toxique (53).

3.1.2. Les nanovecteurs

Aujourd'hui, de nombreuses autres stratégies ont été évaluées avec des systèmes qui sont devenus de plus en plus petits pour atteindre l'échelle nanométrique. Ces nanovecteurs de 10 à 1000 nm peuvent encapsuler de nombreux agents thérapeutiques tels que des drogues (54-56), des radionucléides (57) ou de l' ADN (58, 59). Ces systèmes (Figure 1) sont principalement constitués de polymères ou de lipides. On distingue :

- les liposomes : vésicule artificielle formée par une ou plusieurs bicouches lipidiques, de nature phospholipidique renfermant un compartiment aqueux (60),
- les micelles polymères : structure cœur-noyau formée par un arrangement spontané de polymères amphiphiles (61),
- les nanoparticules (NPs) : soit constituées d'un couronne polymérique ou lipidique et d'un cœur huileux ou aqueux (nanocapsule)(62), soit constituées d'une matrice polymérique ou lipidiques (nanosphères)(63),
- les dendrimères : complexes composés d'une répétition de molécules polymères (64),
- les nanocristaux : agrégats de molécules pouvant être combinées en une forme cristalline (65)

L'agent thérapeutique encapsulé dans ces systèmes est ainsi protégé d'une dégradation prématurée et peut être libéré de façon contrôlée.

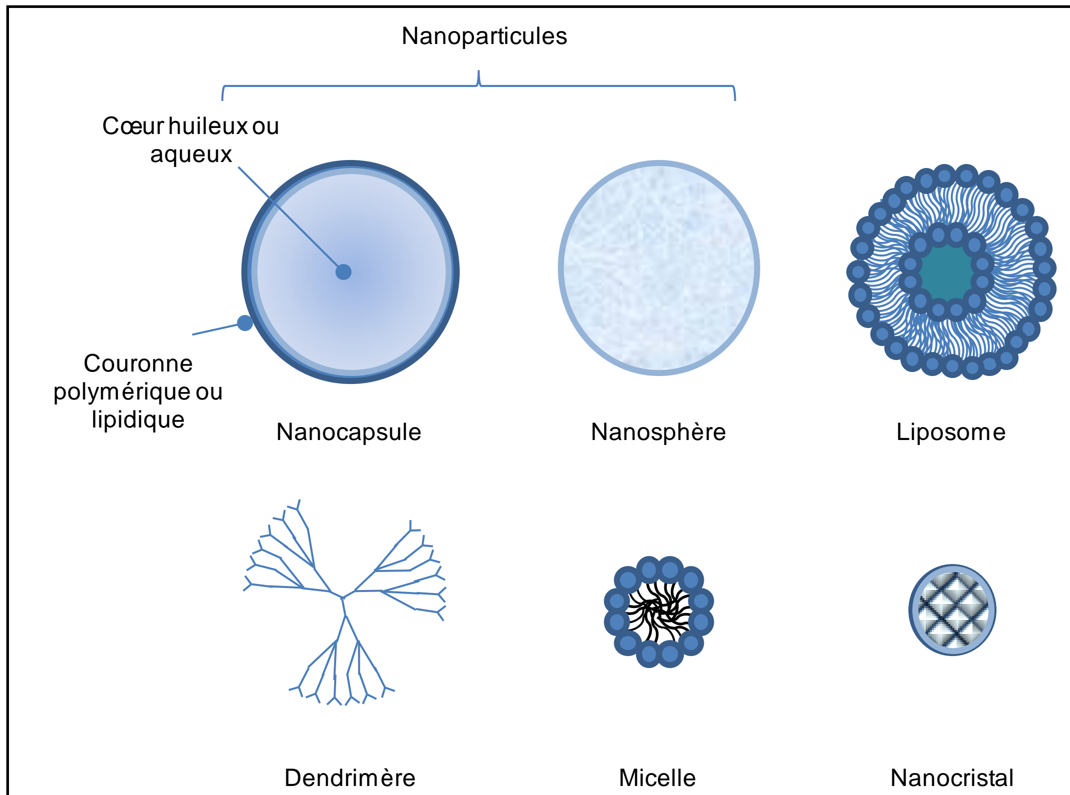

Figure 1 : Schéma des différents systèmes nanoparticulaires.

Seuls les liposomes ont été testés actuellement en clinique dans la thérapie du gliome. Une étude de phase II a ainsi montré une amélioration de la survie des patients traités avec des liposomes chargés en doxorubicine en combinaison avec un traitement au Témozolomide (66). Cependant, les systèmes liposomes présentent des problèmes de stabilité. Les systèmes nanoparticulaires qui sont plus stables par rapport aux liposomes sont en pleine expansion.

3.1.3. Les nanoparticules

Parmi les systèmes nanoparticulaires on distingue les NPs polymériques et les NPs lipidiques. Principalement formulées avec des co-polymères synthétiques, les NPs polymériques ont déjà été largement étudiées et utilisées (67, 68). Deux principaux sous groupes constituent les NPs lipidiques : les NPs solides lipidiques

(NLSs) et les nanocapsules lipidiques (NCLs). Les NLSs sont composées d'une matrice lipidique solide stabilisée avec des surfactants (69) et les NCLs développées au sein de l'INSERM U646 (70) sont constituées d'un cœur huileux entouré de surfactants lipophiles et hydrophiles. Des NPs polymériques et des NCLs délivrant différentes drogues ont déjà montré des résultats encourageants dans la thérapie du gliome expérimental (54, 71-74). Cependant, l'utilisation des systèmes nanoparticulaires comme celle des nanocarriers en général est limitée par le manque de spécificité de ces vecteurs qui est indispensable pour obtenir une thérapie optimale sans majoration de la toxicité systémique.

Pour permettre un ciblage actif des cellules tumorales, différentes stratégies ont été développées. Parmi elles, on distingue l'utilisation de NPs magnétiques. Ces NPs magnétiques chargées en principe actif permettent une délivrance spécifique grâce à l'utilisation d'un champ magnétique externe guidant les NPs (75). Zhao *et al.* (76) ont montré qu'une injection intraveineuse de NPs magnétiques de paclitaxel chez des rats porteurs du gliome C6 suivi d'un ciblage magnétique de 0.5T entrainait une augmentation de l'accumulation du paclitaxel de 6 à 14 fois par rapport à une traitement avec du paclitaxel libre. De plus, la survie des animaux ainsi traités était significativement augmentée.

D'autre part, le greffage de ligands ou d'anticorps dirigés contre des antigènes associés aux tumeurs (TAAs « tumor associated antigens ») cérébrales sur les NPs a été réalisé. Par exemple, des NPs polymériques chargées en BCNU ont été greffées avec de la transferrine pour permettre le passage de la BHE et le ciblage des cellules tumorales qui sur-expriment le récepteur de la transferrine. Des NPs conjuguées avec l'anticorps anti-EGFRvIII ont également été développées (77). D'autres TAAs pourraient être ciblés tels que l'IL-13Rα2 (« Interleukin-12 receptor

α2 »). Ce récepteur est surexprimé dans 82% des GB (78-80). Des résultats encourageants ont été obtenus avec IL13-PE38 et IL13PE38QQR (Cintredekine-Besudotox), des protéines de recombinaison conçues pour détecter et se lier aux récepteurs IL-13 sur la surface des cellules malignes de gliome (81, 82). Bien que ces stratégies ciblant les cellules tumorales *via* les TAAs soient intéressantes, elles sont limitées par le fait que les gliomes sont des tumeurs très hétérogènes et qu'aucun TAA spécifique des gliomes malins n'a été à ce jour identifié.

-3.2- <u>Les cellules souches adultes</u>

Une alternative pour cibler les cellules tumorales quelque soit leur expression de TAAs pourrait être l'utilisation de cellules ayant un tropisme naturel vers les cellules tumorales. Ces cellules attirées par l'environnement tumoral permettraient une stratégie ciblée vers les foyers tumoraux et les cellules infiltrantes tout en préservant les zones saines. Les cellules souches nerveuses (CSNs) et les cellules souches mésenchymateuses (CSMs) possèdent cette capacité. Après injection intracérébrale en contralatérale ou par voie intraveineuse, ces cellules migrent spontanément vers les gliomes (83, 84).

Les CSNs ont été mises en évidence dans le système nerveux en développement mais également dans des régions du cerveau adulte des mammifères : le gyrus denté de l'hippocampe, la zone sous-ventriculaire du plancher du IVe ventricule et dans le bulbe olfactif (85-88). Ces cellules possèdent des activités d'auto-renouvellement et sont capables de générer différents types de cellules à l'intérieur du SNC (neurones, astrocytes, et oligodendrocytes) et d'autres types cellulaires en fonction des conditions environnementales. Leur propriété migratrice vers les tumeurs cérébrales et leur possibilité de disséminer des

molécules bioactives ou des vecteurs viraux sont maintenant bien connues (89). Les implications thérapeutiques en neuro-oncologie sont grandes, mais la préparation de quantité suffisante de CSNs autologues pour une application clinique représente actuellement un challenge technique. Des CSNs allogéniques provenant de cerveaux fœtaux ou adultes ont été considérées comme des substituts possibles pour les CSNs autologues (90). Cependant, plusieurs raisons limitent l'utilisation de ces sources dans le cadre d'une application clinique telles que les problèmes éthiques et l'induction d'un rejet immunitaire.

En revanche, les CSMs, qui peuvent être isolées à partir de la moelle osseuse (MO) ou du sang pourraient rendre réaliste cette stratégie thérapeutique. Les CSMs constituent avec les cellules souches hématopoïétiques les deux principaux types de cellules souches de la MO. Représentant approximativement 0.001-0.01% du nombre total de cellules de la MO, les CSMs humaines peuvent être isolées par adhésivité différentielle, et multipliées en grand nombre *in vitro* (91, 92). Ces cellules sont des précurseurs multipotents qui sont à l'origine des cellules osseuses, cartilagineuses, stromales et adipocytaires (92, 93). Des études récentes indiquent également que les CSMs peuvent se différencier dans d'autres types cellulaires tels que des myoblastes cardiaques (94), des cellules endothéliales (95), des hépatocytes (96), des neurones et des cellules gliales (97-100).

Compte tenu de leur rôle dans la régulation et la production du tissu hématopoiétique et de leurs propriétés immunosuppressives, les CSMs ont été utilisées en clinique pour réduire la durée d'aplasie postgreffe lors de greffes de cellules souches hématopoïétiques et pour limiter le risque de réaction aiguë du greffon contre l'hôte dans les allogreffes (« graft-versus host disease », GVHD) (101). De plus, de part leur multipotence, les CSMs ont été utilisées dans le domaine

de la réparation cellulaire, en orthopédie pour la reconstruction osseuse et cartilagineuse, en chirurgie plastique reconstructive pour le tissu adipeux, en cardiologie pour la réparation du tissu musculaire myocardique après un infarctus, en neurologie pour la réparation de tissu cérébral après une ischémie cérébrale ou une maladie neurodégénérative (102).

La découverte du tropisme des CSMs vers les tumeurs a fait émerger une nouvelle utilisation de ces cellules comme véhicules d'agents thérapeutiques dans les foyers tumoraux. La migration des CSMs a été montrée aussi bien pour les tumeurs périphériques que pour les tumeurs du SNC (103-107). Concernant les tumeurs cérébrales, il a été démontré, à partir de modèles animaux de gliomes (syngénique ou xénogreffe), que les CSMs étaient capables de se disperser de façon homogène tout autour dans la tumeur, s'arrêtant à l'interface tissu sain-tumeur après leur injection soit dans la tumeur, soit à distance de la tumeur, dans l'hémisphère controlatéral ou par voie intraveineuse (108-110). Les mécanismes et les facteurs responsables du tropisme des CSMs vers une tumeur cérébrale restent à élucider. Plusieurs études ont montré le rôle de facteurs solubles produits par les cellules de gliome pour induire la migration des CSMs tels que des chimiokines (MCP-1, « macrophage chemoattractant protein-1 » (111) ; SDF-1 alpha, « stromal cell-derived factor-1 alpha » (104)), des facteurs de croissance (PDGF-BB, « platelet-derived growth factor BB) (112, 113) ou des cytokines angiogéniques (VEGF-A,; IL-8 ; TGF-ss1, « transforming growth factor-ss1 » ; NT-3 « neurotrophin-3 ») (114). Une étude récente indique aussi l'importance de l'expression de MMP1 par les CSMs pour leur migration tumorale (115).

Le devenir des CSMs dans l'environnement tumoral des gliomes est également obscur. Bexell et ses collaborateurs ont montré que les CSMs injectées

dans une tumeur intracérébrale intégraient la paroi des vaisseaux tumoraux et exprimaient des marqueurs associés aux péricytes telles que α-SMA (« α-smooth muscle actin »), NG2 (« neuron-glia 2 ») et PDGFR-β (« platelet derived growth factor receptor ») (116). Une autre étude a décrit la capacité des CSMs à se différencier en CAFs dans un milieu conditionné de gliome *in vitro* (117). Concernant la fonction biologique des CSMs dans l'environnement du gliome, peu d'études ont été réalisées et les résultats obtenus sont contradictoires (118). Il a été observé que les CSMs favorisaient la croissance tumorale, la diminuaient ou n'avaient aucun effet (84, 116, 119, 120).

Néanmoins, la capacité des CSMs à se diriger vers les tumeurs cérébrales a encouragé l'investigation des CSMs comme véhicules pour des virus oncolytiques (121-123) et des gènes toxiques ou immunostimulateurs (84, 124-133). Ces essais ont été réalisés, par exemple, avec le gène de la thymidine kinase du virus de l'herpés (HSV) qui transforme la prodrogue non toxique, le ganciclovir, en un métabolite toxique, entraînant une diminution significative de la masse tumorale et une augmentation du nombre de long survivants (124-127). Les gènes des interleukines-2, 12 et 18 et de l'interféron-β (IFN-β) ont aussi été transférés dans des CSMs *via* l'utilisation de virus permettant, quand ces cellules sont implantées dans une tumeur cérébrale établie, une augmentation de survie des animaux traités (84, 128-130). Enfin, l'injection de MSCs transfectées pour sécréter le facteur TRAIL (« Tumor necrosis factor-related apoptosis-inducing ligand ») au sein de tumeurs cérébrales a montré une forte inhibition des tumeurs. Des analyses immunohistochimiques des tumeurs ont montré une apoptose des cellules tumorales 8 fois plus importantes par rapport aux groupes non traités (131-133). Cependant, l'utilisation de virus pour modifier génétiquement les CSMs présente de nombreux

inconvénients tels que des risques de toxicité, d'immunogénéicité, de mutation et un fort coût de production (134). Un substitut possible au virus pourrait être les nanoparticules. En effet, elles offrent diverses avantages par rapport aux vecteurs viraux : une sécurité biologique, un faible risque immunogène ainsi qu'un faible coût et une production aisée.

Les CSMs présentent donc un potentiel important en tant que vecteurs thérapeutiques pour le traitement des gliomes. La facilité d'obtention des CSMs par rapport aux CSNs, permet d'envisager le prélèvement, la manipulation et la greffe des propres cellules d'un patient donné, éliminant ainsi tous les problèmes d'immunocompatibilité et de sécurité biologique.

-4-Objectif du travail de thèse :

L'objectif de ce travail de thèse a été le développement d'un nouvel outil thérapeutique des gliomes malins en combinant les NPs et les CSMs. Les CSMs permettraient d'apporter un ciblage tumoral spécifique et les NPs une protection et une libération contrôlée de l'agent thérapeutique. La figure 2 représente une schématisation de cette stratégie.

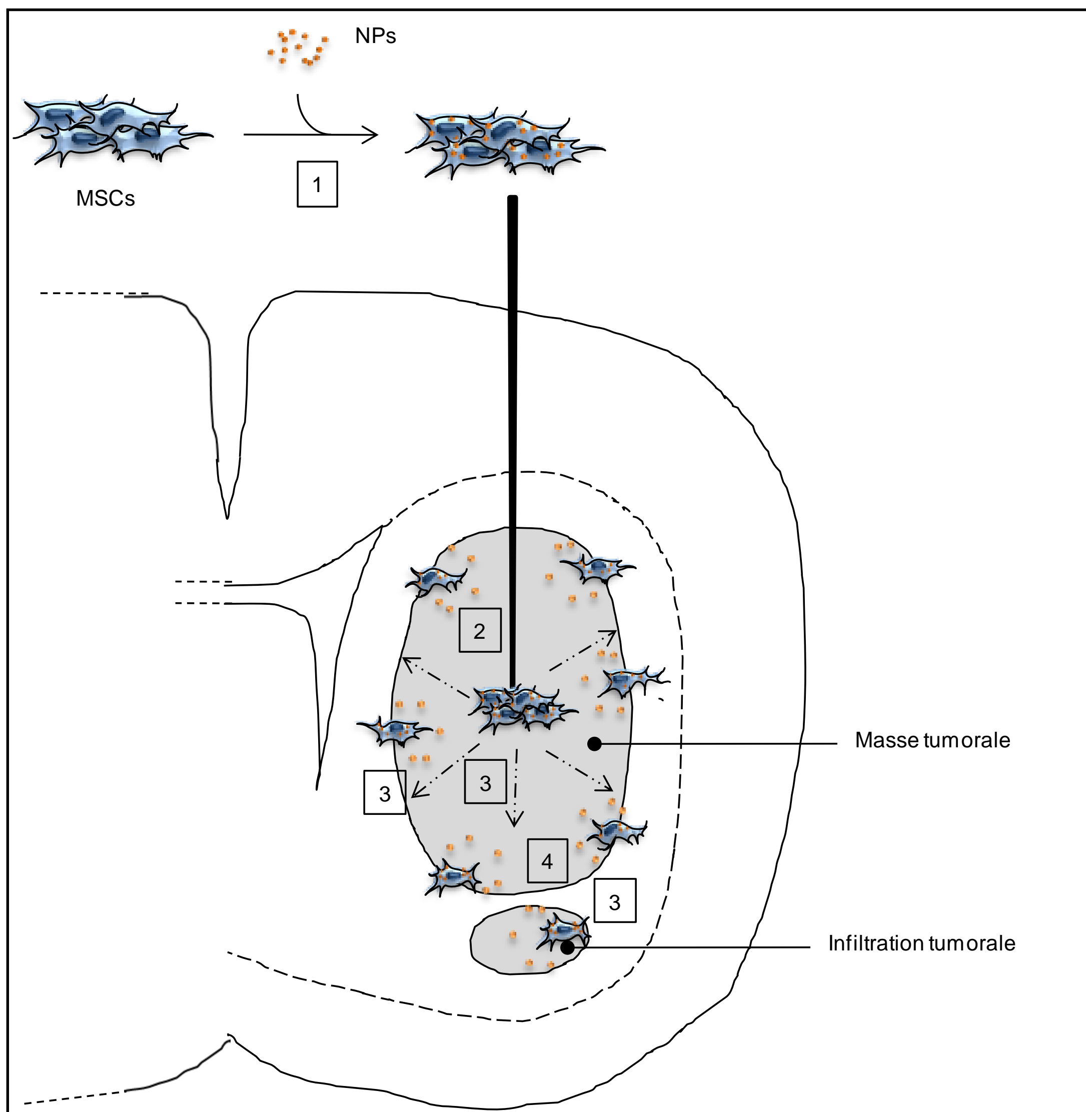

Figure 2 : Représentation schématique de l'utilisation des CSMs pour délivrer des NPs au sein d'une tumeur cérébrale. 1. Incorporation de NPs chargées en principe actif dans les CSMs *in vitro*. 2. Injection intratumorale des CSMs ayant internalisées les NPs au sein d'une tumeur cérébrale. 3. Migration et distribution des CSMs à la périphérie de la tumeur, y compris dans les infiltrats tumoraux. 4. Libération de l'agent thérapeutique.

Pour réaliser ce travail, une sous population de CSMs humaines, les cellules MIAMI pour « Marrow-Isolated Adult Multilineage Inducible », a été choisie comme vecteur cellulaire. Dans le laboratoire du Pr. Paul Schiller (Université de Miami, USA), ces cellules ont pu être isolées dans des conditions particulières et brevetées, à partir de la moelle osseuse vertébrale de sujets humains, hommes et femmes de différents âges (3 à 72 ans) et multipliée en conditions d'hypoxie (99). Cette sous-population exprime des marqueurs de cellules souches embryonnaires (CSE). Elle est capable de se différencier en précurseurs neuraux et en cellules présentant des propriétés électro physiologiques similaires aux neurones. Ces cellules ont montré des résultats prometteurs dans la médecine régénérative (100). Deux types de vecteurs nanoparticulaires ont été évalués, les NPs de PLA et les NCLs. Ces NPs ont été choisies pour leur biocompatibilité (135, 136), leur importante capacité d'encapsulation et de libération contrôlée (54, 67). De plus, grâce à leur petite taille, ces NPs peuvent être incorporées par les cellules sans agents de transfection (137-139). Ces NPs ont déjà montré des résultats encourageant dans la thérapie du gliome expérimental (54, 71-74).

La première partie de cette thèse est consacrée à une revue bibliographique sur l'utilisation des NPs dans la thérapie du gliome et les stratégies utilisées pour permettre un ciblage spécifique du gliome. L'apport des cellules souches adultes dans ce domaine sera discuté.

La seconde partie, organisée en trois chapitres porte sur le travail expérimental réalisé au cours de cette thèse. Le premier chapitre traite de la capacité des cellules MIAMI à incorporer des NPs de PLA et des NCLs tout en conservant leurs propriétés de cellules souches. Le chapitre suivant présente les résultats

d'efficacité thérapeutique de cellules MIAMI ayant internalisées des NCLs de ferrociphenol (Fc-diOH), un dérivé du tamoxifène, dans le modèle hétérotopique de la lignée de gliome U87MG chez la souris nude. Le troisième et dernier chapitre expérimental décrit l'interaction des cellules MIAMI avec les cellules de gliome *in vitro* et *in vivo*.

Enfin une discussion générale commente l'ensemble des résultats et élargie sur les perspectives de ce travail.

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Combinations of drug-loaded nanoparticle systems and adult stem cells as future perspectives for glioma therapy

Mathilde Roger [a], Anne Clavreul [a, b], Marie-Claire Venier-Julienne [a], Catherine Passirani [a], Claudia N. Montero-Menei [a], Philippe Menei [a,b]

[a] INSERM Unit 646, Ingénierie de la Vectorisation Particulaire, 10, rue André Bocquel, Université d'Angers, 49100 Angers, France

[b] Département de Neurochirurgie, Centre Hospitalier Universitaire d'Angers, 4 rue Larrey, 49100 Angers, France

Corresponding author:

Tel +33241354822

Fax +33241735853

E-mail address: PhMenei@chu-angers.fr

Abstract

The prognosis of patients with malignant glioma remains extremely poor, despite surgery and improvements in radio- and chemo-therapies. Nanotechnologies hold great promise in glioma therapy as they protect the therapeutic agent and allow its sustained release. However, new paradigms permitting tumor-specific targeting and extensive intratumoral distribution must be developed to efficiently deliver nanoparticles. Modifications and functionalizations of nanoparticles have been developed to specifically track tumor cells. However, these nanoparticles have yielded few clinical results due to intra-patient heterogeneity and inter-patient variability. Stem cells with a specific tropism for brain tumors could be used as delivery vehicles for nanoparticles. Indeed, these cells have a natural tendency to migrate and distribute within the tumor mass and they can also incorporate nanoparticles. Stem cell therapy combined with nanotechnology could be a promising tool to efficiently deliver drugs to brain tumors.

Keywords: glioma, nanoparticles, adult stem cells, targeting.

Introduction

Malignant gliomas are primitive brain tumors of the central nervous system (CNS). The most common and malignant subtype is the glioblastoma (GB), which is characterized by aggressive invasion and diffuse infiltration of tumor cells into the surrounding brain tissues. Standard treatment is surgical resection followed by fractionated radiotherapy (XRT) with concomitant and adjuvant chemotherapy with Temozolomide (TMZ) [1, 2]. Although this treatment improves the median overall survival from 6 to 14.6 months, GBs remain a lethal tumor.

Numerous chemotherapeutic drugs affecting cell division or DNA synthesis have been tested to improve the prognosis of GB, including alkylating agents (Busulfan, Bicnu, Carboplatine, Carmustine, Cisplatine, Lomustine, Oxaliplatine, TMZ), topoisomerase inhibitors (Irinotecan [CPT-11], Topotecan), anthracyclines (Doxorubicine), and antimitotic agents (Vincristine, Taxanes) [3]. Furthermore, new innovative anticancer drugs targeting receptor tyrosine kinases (RTKs) —epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR)— and their downstream signaling pathways —PI3k/Akt/mTOR, Ras/mitogen-activated protein kinase, protein kinase C— have also been developed and used in clinical trials [4]. However, the greatest obstacle in the treatment of brain tumors with these drugs is often not drug potency, but the physical barriers that render the usual circulatory routes of delivery ineffective [5]. The delivery of drugs to brain tumors is limited by the presence of the blood-brain barrier (BBB). This physical barrier is characterized by tight intracellular junctions (*zona occludens*) [6] with an absence of fenestrations. Thus, the BBB prevents the uptake of all large molecules and more than 98% of pharmaceutical small-molecule drugs [7]. Furthermore, the BBB expresses high levels of drug efflux

pumps such as P-glycoprotein (P-gp), which actively remove chemotherapeutic drugs from the brain [8]. In addition, diffusion in the brain parenchyma is very weak, which is partially due to the high intercellular fluid pressure in tumors [9, 10]. Finally, brain tissue is highly sensitive, so only limited doses of therapeutic agents can be used.

Many strategies have been developed to overcome these obstacles. Among these, drug delivery nanosystems have been produced to protect therapeutic agents and improve their biodistribution and therapeutic index. These systems include mainly polymer or lipid-based carriers such as liposomes, nanoparticles (NPs) including nanospheres and nanocapsules, micelles, dendrimers, nanocrystals, and nanogolds (Figure 1).

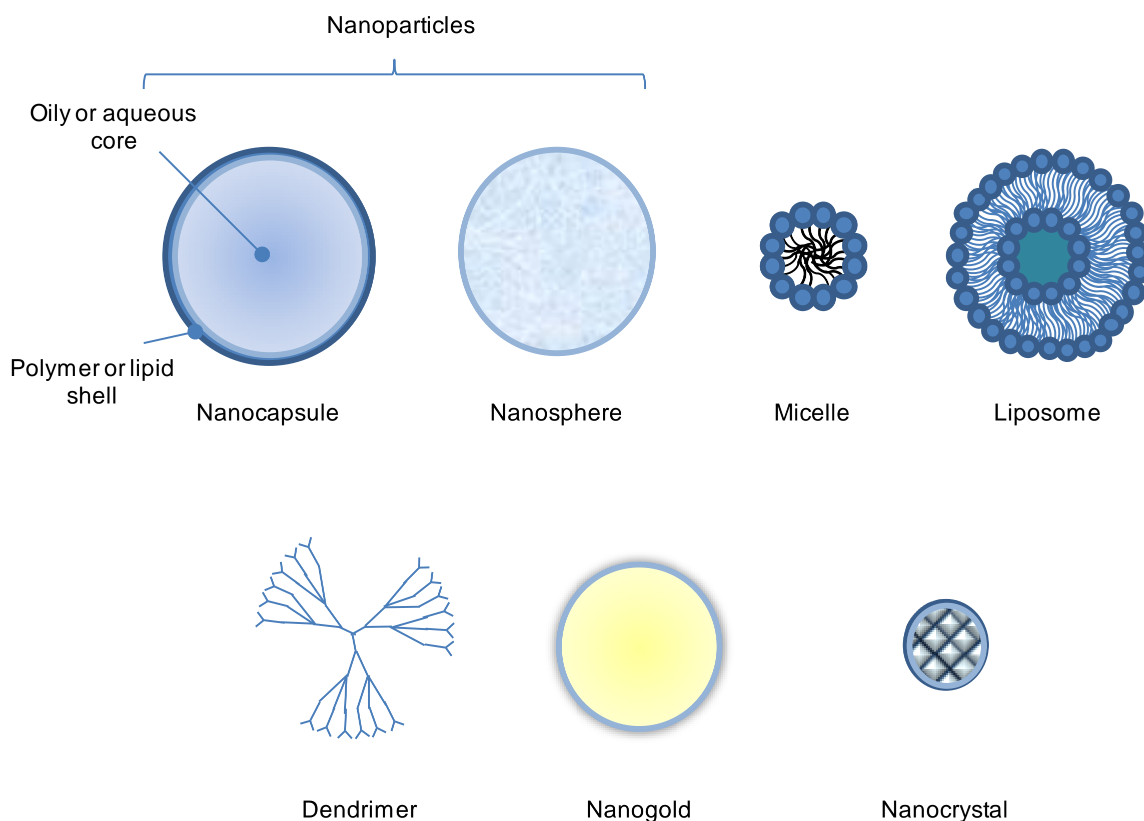

Figure 1: Schematic structure of principal drug-delivery systems.

In this review, we shall focus on the use of drug-loaded NPs in brain therapy and discuss the strategies allowing their specific targeting. In particular, the potential use of stem cells to deliver these NPs will be described.

1. Drug-loaded nanoparticle systems and glioma therapy

NPs are polymer or lipid-based carriers that range from 10 to 1000 nm that can carry multiple therapeutic agents such as drugs [11-13], radionuclides [14] or DNA [15, 16]. The therapeutic agent can be entrapped in, adsorbed or chemically coupled onto the NP surface. In this way, NPs can protect therapeutic agents from chemical or enzymatic degradation and allow their sustained and controlled release.

1.1. Polymer NPs

Polymer NPs are usually formulated using hydrophobic synthetic polymers and copolymers such as polylactide-polyglycolide (PLA, PLGA), polyacrylates, and polycaprolactones or natural polymers such as albumin, gelatin, alginate, collagen, and chitosan. Polymer NPs provide a sustained delivery release [17]. PLGA and PLA are the most investigated NPs for drug delivery [18, 19]. Degradation of these polymers can be altered by changing the block copolymer composition and molecular weight [20]. Hence, the release of an encapsulated therapeutic agent can be adjusted to last from days to months. Polymer NPs are mainly formulated using emulsion/solvent evaporation or solvent displacement techniques [18]. Using these methods, a variety of therapeutic agents including low molecular weight lipophilic or hydrophilic drugs and high molecular weight DNA or antisense oligonucleotides can be encapsulated in polymer NPs [16, 21].

1.2. Lipid NPs

During the past decade, lipid systems have emerged as a new generation of nanocarriers. These NPs have a low toxicity compared with polymer particles,

because they are composed of physiological lipids. There are two main types of lipid NPs: solid lipid NPs (SLNs) [22], and lipid nanocapsules (LNCs) [23]. SLNs are composed of a solid lipid matrix stabilized by surfactants. These lipid NPs are usually produced by high-pressure homogenization [24], solvent emulsification-evaporation or diffusion [25], and microemulsion [26]. LNCs are composed of a liquid, oily core (medium-chain triglycerides) surrounded by hydrophilic (PEG_{660}-hydroxystearate) and lipophilic (phosphatidylethanolamine and phosphatidylcholine) surfactants. LNCs have the advantages of being prepared without organic solvent using a low-energy process [27]. Formulation of these nanocapsules is achieved using the phase-inversion temperature method (PIT method) [23]. Lipophilic or amphiphilic molecules can be incorporated into either the oil core or the shell of LNCs (patent WO02688000).

1.3. NPs in glioma therapy

Polymer and lipid NPs have been engineered for drug delivery to brain tumors (Table 1). Systemic or direct delivery to the CNS has been used to administer these drug-loaded NPs and various approaches have been evaluated for their selective targeting of brain tumors.

1.3.1. Systemic NP delivery

The greatest advantage of using systemic drug-loaded NP delivery approaches is their non invasive nature. The most commonly used method for administration of NPs into the body is the intravenous (iv) route. Systemic delivery is nevertheless limited due to the nonspecific uptake of NPs by the mononuclear phagocyte system (MPS) as well as problems in penetrating the BBB. To avoid macrophage uptake, reduced particle sizes (< 100 nm) and surface modifications

with hydrophilic polymers such as poly(ethylenglycol) (PEG) have been developed [28]. Covalent grafting, entrapment, or adsoption of PEG chains onto NPs give a hydrophilic surface which reduces macrophage recognition and complement activation, thereby prolonging NP circulation-time. In addition, conjugation of pegylated NPs with cationic bovine serum albumin (CBSA) has been developed to allow crossing of the BBB. CBSA shows a good accumulation profile in brain, and has great potential in targeting the brain through absorptive mediated transcytosis [29]. As expected, aclarubicin-loaded albumin-conjugated pegylated NPs had much higher accumulation within brain tumors than NPs without conjugated CBSA following iv injection [30]. Specific surfactants such as polysorbate 80 have also been used to help bypass the BBB. Polysorbate 80 adsorbs apolipoproteins B and E allowing receptor-mediated endocytosis by the brain capillary endothelial cells [31]. In a number of rat studies, polysorbate 80-NPs carrying doxorubicine [32-37], gemcitabine [38], or indomethacin [39] can cross the intact BBB and reach therapeutic levels in the brain thereby extending survival in glioblastoma models. Furthermore, polysorbate 80 and PEG are able to inhibit the P-gp and reverse multi-drug resistance [40, 41].

1.3.2. Direct NP delivery

To circumvent the BBB, NPs can be administered directly into the brain *via* a bolus injection. This has the advantage of delivering much higher concentrations of drug-loaded NPs to the brain. However, a major problem is the slower movement of NPs within the brain due to limited diffusion coefficients and backflow of the injection. This is because of the closely packed arrangement of cells in both the gray and white matter microenvironments [42]. Convection-enhanced-delivery (CED) has been used

to overcome these problems. Using an external pressure gradient inducing fluid convection in the brain *via* a surgically implanted catheter, this method allows a greater volume of distribution to be achieved compared with diffusion alone [43, 44]. A combination of nanotechnology with the CED technique has shown promising results in 9L experimental glioma in rats. Ferrociphenol (Fc-diOH) and Paclitaxel LNCs injected by CED and combined with external radiotherapy resulted in an increase of rat median survival time [45, 46]. However, this approach has some disadvantages such as long infusion times inducing infections, potential intracranial hypertension, and unpredictable drug distribution.

1.3.3. Approaches for selective brain tumor-targeting

Passive and active targeting strategies can be used for drug-loaded NPs. Passive targeting is based on the characteristics of brain tumor tissues such as hypervascularization, defective vascular architecture, and a deficient lymphatic drainage system. These features lead to preferential accumulation and retention of NPs in tumor tissues rather than normal tissues *via* the “enhanced permeability and retention (EPR) effect” [47]. The EPR effect is more pronounced for long-circulating NPs that avoid MPS uptake and rapid clearance from the circulation [17]. Indeed, iv injection of PEG-coated hexadecylcyanoacrylate NPs in Fisher rats with intracranial 9L gliosarcoma concentrated 3.1 times higher within the gliosarcoma, compared with non-PEG-coated NPs [48]. Passive targeting can be increased by using superparamagnetic NPs which can be concentrated within the tumor tissue by local application of an external magnetic field thereby sparing unaffected tissues and organs [49]. Indeed, iv injection of magnetic paclitaxel NPs into C6 glioma-bearing rats and magnetic targeting with a 0.5T magnet induced a 6- to 14-fold increase of

drug content within implanted glioma compared with free paclitaxel [50]. Furthermore, the survival of glioma-bearing rats was significantly prolonged after therapy with the magnetic paclitaxel NPs.

Active targeting consists of functionalizing the drug-loaded NP surface with ligands, or antibodies to specifically target the tumor endothelium and tumor-associated antigens (TAAs). For example, Kang *et al*, [51] have selected a transferrin (Tf) ligand that binds to the Tf receptor which is overexpressed on the brain capillary endothelium [52] and on the surface of proliferating brain tumor cells [53]. Investigation of biodistribution by single photon emission computed tomography of 99Tc-labeled NPs showed that Tf-coated PLA NPs concentrate in the brain with no radioactive foci outside the brain. Furthermore, Tf-coated PLA NPs loaded with BCNU had stronger cytotoxicity and prolonged the survival time of C6 tumor-bearing rats. Recently, Hadjipanayis *et al.*[54] used an antibody against the TAA EGFRvIII which is expressed in 25% of GBs but not in the normal brain [55]. They showed that iron oxide NPs conjugated to this antibody can provide selective magnetic resonance imaging contrast enhancement and targeted therapy of infiltrative GB cells after CED. Other TAAs such as interleukin-13 receptor alpha 2 (IL-13Rα2) and IL-4R [56-58] may be also attractive to specifically target tumor cells . Cytotoxins composed of IL-13 or IL-4 and a mutated form of Pseudomonas exotoxin produced to target IL-13Rα2 and IL-4R have shown promising results in glioma therapy [59]. However, due to the intra-tumor heterogeneity and the inter-patient variability, it is difficult to target all tumor cells with these TAA targeting approaches.

Table 1 : Drug-loaded NP system applications in orthotopic glioma model

Particular vector	Modification	Therapeutic encapsulation	Animal model	Glioma cells	Route of administration	Results	Ref
Systemic delivery							
CBSA-NPs	PEG-NPs	Aclarubin	Wistar rats	C6	iv	Higher survival times	[30]
PBCA-NPs	Polysorbate-80 NPs	Doxorubicine	Wistar rats	101/8	iv	No toxicty	[32]
						Higher survival times; over 20% of long-term remission	[33]
						Accumulation of NP in the tumor site	[34]
						Higher survival times; 35% of long-survivors	[36]
						Tumor size reduction	[35]
		Gemtabine	Sprague Dawley rats	C6	iv	Tumor growth inhibition; higher survival times	[38]
PLGA-NPs		Doxorubicine	Wistar rats	101/8	iv	Higher survival times; 40% of long-term remission	[37]
PCL-NPs		Indomethacin	Wistar rats	C6	ip	No toxicity; tumor size reduction; higher survival times	[39]
Local delivery							
LNCs	None	Fc-diOH + radiotherapy	Fisher rats	9L	CED	Higher survival times; 10.5% of long survivors	[46]
	None	Paclitaxel + radiotherapy	Fisher rats	9L	CED	Higher survival times; 10% of long survivors	[45]
Specific delivery							
Magnetic NPs	None	Paclitaxel	Sprague Dawley rats	C6	iv + 0.5 T magnet	Accumulation of NPs in tumor site; higher survival times	[50]
PLA-NPs	Tf- NPs	BCNU	Sprague Dawley rats	C6	stereotaxic	Accumulation of NP in tumor sit; higher survival times	[51]

Abbreviations: PBCA: polybutylcyanoacrylate; PLGA: copoly lactic acid/glycolic acid; PCL: poly(ε-caprolactone); CBSA: cationic bovine serum albumin; LNC: lipid nanocapsule; Fc-diOH: ferrociphenol; CED: convenction enhanced delivery; PLA: poly lactic acid; Tf: transferrin

2. Adult stem cells and glioma therapy

The special tropism of stem cells such as neural stem cells (NSCs) and mesenchymal stem cells (MSCs) suggests that they are potential candidates as therapeutic cellular vectors in glioma therapy (Tables 2 and 3).

2.1. Neural stem cells

NSCs were the first cells to be envisaged as therapeutic cellular vectors. Adult NSCs are self-renewing, proliferative, multipotent cells that give rise to the different neuroectodermal lineages of the CNS, including the many different neuronal and glial subtypes. NSCs can be isolated from two major neurogenic regions of the adult mammalian brain: the subgranular zone (SGZ) of the dentate gyrus and the anterior area of the subventricular zone (SVZ) along the ventricle [60]. As the preparation of sufficient amounts of autologous NSCs for clinical applications remains technically challenging, fetal brain, adult allogeneic brain, and embryonic stem (ES) cells are being considered as possible substitutes for autologous NSCs [61]. Aboody *et al.*, were the first to report that NSCs display extensive tropism for experimental intracranial gliomas in adult rodents [62]. These results were confirmed by Benedetti *et al.*, published at the same time [63]. In experimental glioma models, strong antitumor effects were reported with intracranial administration of gene-modified NSCs expressing cytokines [64-66], tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) [67-69], toxic molecules [70-75], a proliferation inhibitor, the naturally occurring fragment of human metalloproteinase 2, PEX [76], anti-angiogenic agents [77, 78], or cytokines combined with toxic molecules [75] (Table 2). NSCs have also been used as cell carriers for replication, transportation, and local release of intact oncolytic adenoviruses into tumors [79]. A clinical trial is currently in process to

evaluate the safety of genetically-modified embryonic NSCs expressing the suicide gene (cytosine deaminase) combined with oral administration of 5-Fluorocytosine (NCT01172964, clinical trial.gov). However, clinical applications of NSCs will undoubtedly be limited by logistic and ethical problems associated with their isolation and by potential immunological incompatibility due to the requirement for allogenic transplantation.

Table 2 : NSCs in glioma therapy

Adult stem cells	Animal model	Glioma cells	Therapeutic transgene/modification	Route of administration	Results	Ref
			Cytokines			
mNPC	C57BL1/6 mice	GL261	IL-4	Intratumoral	86% extended survival	[63]
				Intracranial mixed with glioma cells	71% extended survival	
rSR14A NPC	Sprague Dawley rats	C6	IL-4	Intratumoral	65% of extended survival	
				Intracranial mixed with glioma cells	50% extended survival	
mNSC	CB57BL/6 mice	GL26	IL-12	Intratumoral	80% of tumor reduction	[64]
hNSC	Sprague Dawley rats	C6	IL-12	Intratumoral	Higher survival times; tumor growth reduction	[65]
mBM-NSC	C57BL/6 mice	GL26	IL-23	Intratumoral	Higher survival times; 60% of long survivors	[66]
			Apoptosis induced ligand			
mNPC	Nude mice	Gl36	TRAIL	Intratumoral	Tumor growth reduction	[67]
mNSC	Nude mice	U343MG	TRAIL	Intracranial mixed with glioma cells	50% reduction of tumor growth; higher survival times	[68]
mC17.2-NPC	Nude mice	U87MG	TRAIL	Intracranial mixed with glioma cells	Tumor growth reduction; higher survival times	[69]
			Suicide gene			
mC17.2-NSC	Nude mice	CNS-1	CD + i.p. injection of 5-FC	Intracranial mixed with glioma cells	80% reduction of tumor growth	[62]
rST14A-NPC	Sprague Dawley rats	C6	CD + ip injection of 5-FC	Intracranial mixed with glioma cells	50% reduction of tumor growth	[70]
rNSC	Sprague Dawley rats	C6	TK + ip injection of GC	Intracranial mixed with glioma cells	Higher survival times; tumor growth reduction	[71]
rNSC	Nude mice	C6	TK + ip injection of GC	Intracranial mixed with glioma cells	No tumor development	[72]
rNSC	Sprague Dawley rats	C6	TK + ip injection of GC	Intratumoral	Total tumor regression for 66% animals	
rNSC	Sprague Dawley rats	C6	TK + ip injection of GC	Intratumoral	Tumor growth reduction; higher survival times.	[73]

hNT2-NPC	Nude mice	U87MG	TK + ip injection of GC	Contralateral	Tumor growth inhibition; higher survival times	[74]
Proliferation inhibitor						
HB1.F3	Nude mice	U87MG	PEX	Intratumoral	90% reduction of tumor growth	[76]
Anti-angiogenic agent						
mNSC	C57BL/6 mice	Gl261	endostatin	Intratumoral	65% reduction of tumor growth	[77]
hNSC	SCID mice	Gli36	aaTSP-1	Intratumoral	Tumor growth inhibition; higher survival times	[78]
Cytokine + Suicide gene						
hHB1-NSC	Nude mice	U251MG	IFN-β + CD + ip injection of 5-FC	Iv	Tumor growth inhibition; higher survival times	[75]
Virus delivery carrier						
hNSC	Nude mice	U87MG	Oncolytic adenovirus	Intratumoral	Not tested; good result in subcutaneous model	[79]

Abbreviations: mNPC: mouse neural progenitor cell; rNPC: rat NPC; hNPC: human NPC; NSC: neural stem cell; IL: interleukin; TRAIL: tumor necrosis factor-related qpoptosis-inducing ligand; CD: cytosine deaminase; ip: intraperitoneal injection; iv: intravenous injection; 5-FC: 5-fluorocytosine; TK: thymidine kinase; GC: ganciclovir; aaTSP-1: anti-angiogenic thrombospondin protein; IFNβ: Interferon β

2.2 Mesenchymal stem cells

Compared with NSCs, MSCs (also called mesenchymal stromal cells) have the advantage of being easily propagated *in vitro* and implantation of autologous MSCs into patients with malignant glioma has less ethical problems. Although MSCs were originally isolated from bone marrow [80, 81], similar populations are reported in other tissues. Human MSCs have been isolated from adipose tissue [82], umbilical cord blood [83-86], peripheral blood [87, 88], connective tissues of the dermis, and skeletal muscle [89]. MSCs are defined by their plastic adherent growth and subsequent expansion under specific culture conditions, by a panel of non-specific surface antigens (CD29, CD44, CD90, CD73) and by their propensity to give rise to adipocytes, chondrocytes, and osteoblasts.

Different subpopulations of MSCs have been identified. Schiller *et al.* isolated a unique subpopulation of human MSCs named Marrow-Isolated Adult Multilineage Inducible (MIAMI) cells, using specific culture conditions such as low oxygen tension (3%). These cells possess a unique molecular phenotype and express embryonic stem cell markers. MIAMI cells derived from donors of different ages (3-72 years) are capable of *in vitro* differentiation into cell lineages derived from all three germ layers, particularly into neuron-like cells [90, 91]. This developmental program requires a multi-step process involving their sequential differentiation into NSCs, neural/neuronal progenitors, and finally neuron-like cells with electrophysiological characteristics that are similar to those observed in neurons.

As with NSCs, the ability of MSCs to migrate to areas of injury and tumors has encouraged investigation of their therapeutic potential. After intratumoral or contralateral injection of MSCs into mice bearing glioma, MSCs are found all around the tumor, at the border between the tumor mass and the brain parenchyma (Figure

2). Furthermore, MSCs follow tumor infiltrations. MSCs migrate and localize around glioma cells *via* processes involving different factors such as stromal-derived factor-1 [92], platelet-derived growth factor [93], epidermal growth factor [94], matrix metalloproteinase-1 [95], and macrophage chemoattractant protein-1 [96].

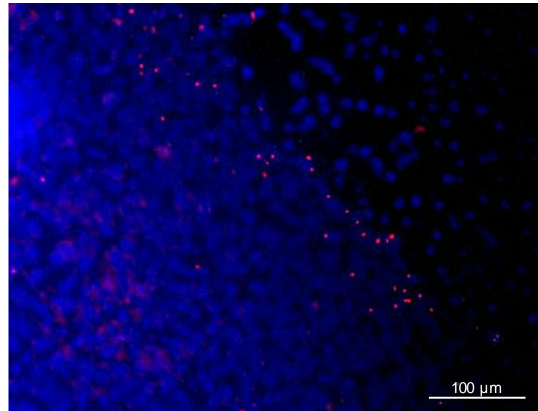

Figure 2 : The human glioma cell line U87MG from a female donor was injected at day 0 into the striatum of female nude mice. At day 6, MIAMI cells derived from a male donor were injected intratumorally and their migratory behavior towards the U87MG cells was detected at day 13 by Y-chromosome labeling with FISH technique (red spot).

As described for NSCs, MSCs modified genetically with viruses expressing immunostimulators or toxic molecules have been shown to impede tumor growth in animal models [97-108]. MSCs have also been used to deliver intact oncolytic adenoviruses into tumors [109, 110] (Table 3). Due to their ease of isolation, MSCs are a promising tool as therapeutic gene-delivery vectors. However, until now, no clinical trials have been performed using these cells. This may be because of the viral vectors used for the therapeutic gene delivery. Indeed, these viral carriers have a number of disadvantages, including risks of toxicity, immunogenicity, insertional mutagenesis, and high manufacturing costs [111, 112]. Another reason may be the conflicting data concerning the use of MSCs in cell-based therapies (see concluding remarks).

Table 3 : MSCs in glioma therapy

Adult stem cells	Animal model	Glioma cells	Therapeutic transgene/modification	Route of administration	Results	Ref
			Cytokines			
rMSC	Fisher rats	9L	IL-2	Intracranial mixed with glioma cells	Tumor growth reduction; higher survival times	[97]
mMSC	C57B16 mice	Ast11.9-2	IL12	Intracranial mixed with glioma cells	Higher survival times	[98]
hMSC	Nude mice	U87MG	IFNβ	Intratumoral	Higher survival times	[99]
rMSC	Sprague Dawley rats	C6	IL-18	Intratumoral	Tumor growth inhibition; higher survival times	[100]
rMSC	Sprague Dawley rats	N32	IL-7	Intratumoral + IFNγ immunization	Tumor growth regression	[101]
			Apoptosis induced ligand			
hUCB-MSC	Nude mice	U87MG	TRAIL	Intratumoral	Tumor growth inhibition; higher survival times.	[102]
hMSC	Nude mice	U87MG	TRAIL	Intratumoral	81% reduction of tumor growth; 55% extended survival	[103]
hMSC	Nude mice	Gli36	TRAIL	Intracranial mixed with glioma cells	Tumor mass reduction; higher survival times	[104]
			Suicide gene			
rBM-TIC	Fisher rats	9L	TK + ip injection of GC	Intratumoral	Higher survival times; total tumor regression for 60% of animals	[105]
hAC-MSC	Nude mice	U87MG	TK + ip injection of GC	Contralateral	Reduction of tumor growth	[106]
rMSC	Nude mice	9L	TK + ip injection of GC	Contralateral	Tumor growth inhibition	[107]
rMSC	Sprague Dawley rats	C6	TK + ip injection of GC	Intracranial mixed with glioma cells	Tumor growth reduction; higher survival times	[108]
rMSC	Sprague Dawley rats	C6	TK + ip injection of GC	Intrathecal	Tumor growth reduction, higher survival times	
			Virus delivery carrier: oncolytic			

hMSC	Nude mice	U87MG	adenovirus	Intratumoral	Not tested	[109]
ADSC	Nude mice	U87MG	adenovirus	Intratumoral	Tumor growth inhibition; higher survival times	[110]
ADSC	Nude mice	U87MG	adenovirus	Intracerebral mixed with glioma cells	Tumor growth reduction; higher survival times	[110]

Abbreviations: rMSC: rat mesenchymal stromal cell; mMSC: mouse MSC; hMSC: human MSC; IL: interleukin; IFNβ: interferon β; UCB: umbilical cord blood; TRAIL: tumor necrosis factor-related apoptosis-inducing ligand; BM-TIC: bone marrow-tumor infiltrating cells; TK: thymidine kinase; ip: intraperitoneal injection; GC: ganciclovir; hAC-MSC: human artificial chromosome-MSC; ADSC: adipocyte derived stromal cell

3. Combinations of drug-loaded NPs and adult stem cells

As previously described, NPs and stem cells are promising tools to treat brain tumors. However, combining their use could have additional advantages. Currently, NPs can be incorporated into cells and this technology has been already evaluated to manipulate and track stem cells [113].

3.1. Incorporation of NP into cells and intracellular trafficking

Interactions between nanocarriers and cells have already been studied. Nanocarriers can be incorporated into cells using transfection agents which are mostly cationic, positively charged molecules such as poly-L lysine or polyethyleneimine [114, 115]. However, these transfection agents are toxic and have not been clinically approved. More recently, spontaneous incorporation of NPs into cells has been described without the use of any transfection agents [116-123]. Futhermore, NPs have been shown to enter inside cells *via* passive transport [124] and active endocytosis [118, 125, 126]. Three endocytosis pathways can be used according to the size of the NP: clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis [127]. Once inside the cells, NPs are usually transported to the endo-lysosomal system, where they are destroyed. PLA and PLGA NPs as well as LNCs are able to escape the lysosomal compartment by disrupting the integrity of the lysosome membrane. In the acidic pH of secondary endosomes, the surface charge of PLGA or PLA-NPs changes from anionic to cationic resulting in a local NP-membrane interaction and escape of NPs into the cytoplasm [120, 121]. For LNCs, the hypothesis is that the hydroxystearate-PEG of the Solutol, which constitutes the most external phase of LNCs, has a lysosomotropic property and therefore destabilizes the lysosome membrane [128].

This endo-lysosomal escape leads to NP accumulation in the cytoplasm. Figure 3 illustrates the incorporation, trafficking, and cytoplasmic accumulation of NPs within cells.

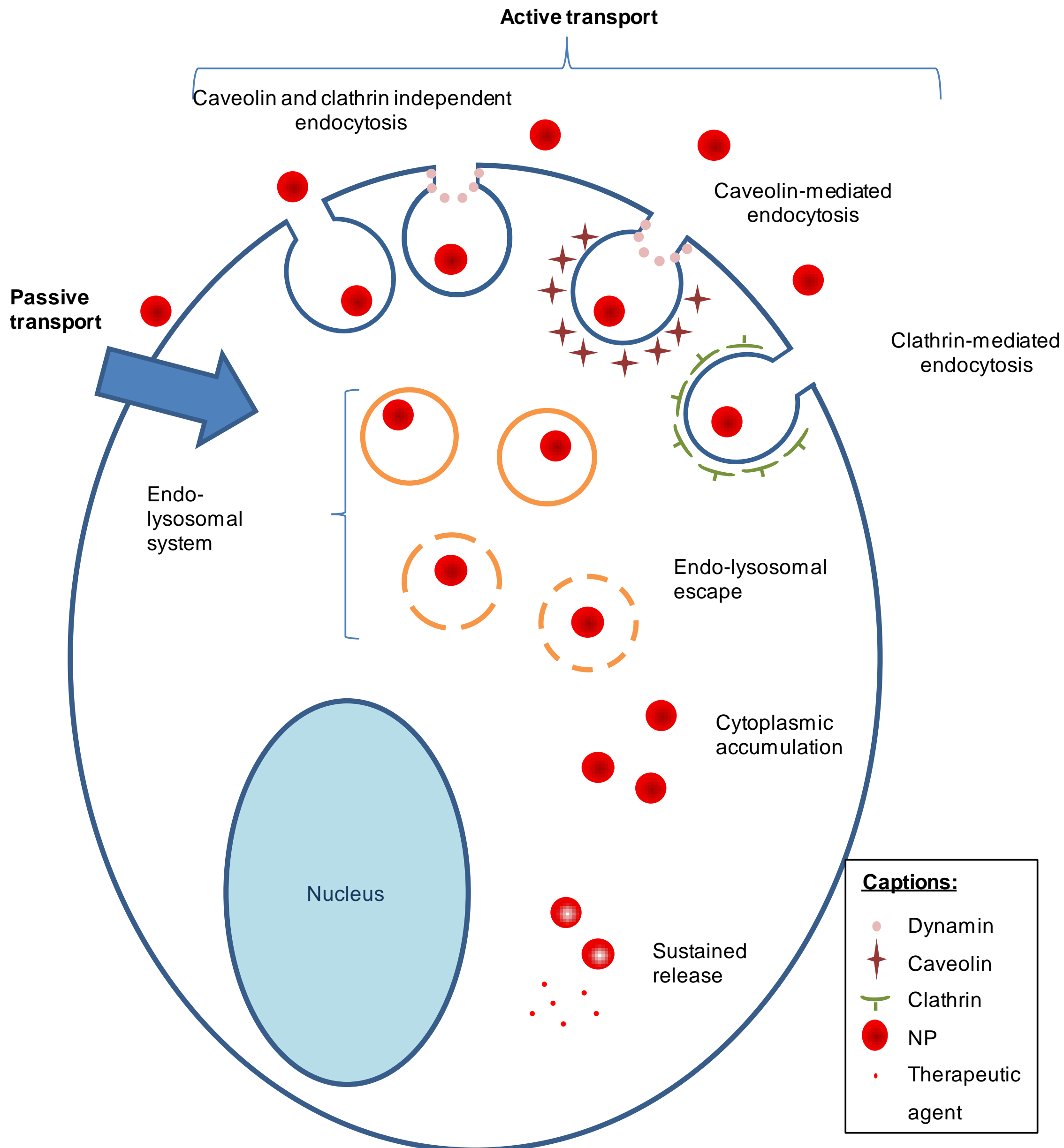

Figure 3 : Schematic representation of incorporation and trafficking of NPs within cells.

3.2. Applications of NP systems with stem cells

The applications of NP systems with stem cells include long-term labeling of stem cells with superparamagnetic iron oxide (SPIO) NPs, fluorochrome-loaded NPs, or quantum dots to monitor their fate and regenerative potential [129-134] (Table 4). The migratory behavior of stem cells towards glioma *in vivo* has been routinely demonstrated with this approach [135-139]. NP systems have also been used for gene transfection. For example, biodegradable polymer NPs have been developed to deliver the VEGF gene to human MSCs and human ESC-derived cells to promote angiogenesis [140]. Another application of NPs with stem cells is intracellular delivery of growth factors to induce osteogenic or chondrogenic differentiation of stem cells [141-147]. Stem cells were also transfected using NPs encapsulated with plasmid DNA encoding bone morphogenetic protein-2 (BMP-2) to induce their ondotogenic or osteogenic differentiation [148, 149].

Table 4 : Vector applications of NPs and stem-cells

Applications	Encapsulated agent	Cell	ref
Cell labeling	Quantum dots	ADSCs	[129, 130]
		MSCs	[131, 132, 134]
	Fluorochrome loaded NPs	MSCs	[133]
Cell tracking	superparamagnetic iron oxide	NSCs	[135]
		MSCs	[136-139]
Enhance angiogenesis	Growth factor: VEGF	MSCs	[140]
Osteogenic differentiation	Growth factor: bFGF, BMP-2, BMP-2/BMP-7	MSCs	[141-145]
	DNA: BMP-2 gene vector	MSCs	[148]
Chondrogenic differentiation	Growth factor: TGF-beta1, TGF-beta 3	ADSCs	[146]
		MSCs	[147]
Odontogic differentiation	DNA: BMP-2 gene vector	Dental pulp stem cells	[149]

Abbreviations: bFGF: basic fibroblast growth factor; BMP: bone morphogenetic protein; TGF: transforming growth factor; VEGF: vascular endothelial growth factor; ADSC: adipocyte derived stromal cells.

3.3. Future perspectives

The combination of stem cells and drug-loaded NPs for therapeutic applications in glioma therapy is a promising strategy. This concept is schematically presented in Figure 4 as well as the properties of NPs and stem cells necessary for optimal combination.

We recently proved this concept by combining MIAMI cells with a subpopulation of MSCs with PLA-NPs and LNCs to target brain tumors [123]. MIAMI cells were selected as NP cellular carriers because they are easy to obtain from patients and easy to handle *in vitro*. Moreover, as stated above, MIAMI cells can differentiate into neuron-like cells thus representing autologous human cell populations for tissue regeneration of skeletal and nervous system disorders. We showed that MIAMI cells mainly localized at the border between tumor cells and normal brain parenchyma following intratumoral or contralateral injection into mice with U87MG glioma. PLA-NPs and LNCs were chosen for their biocompatibility

properties; the degradation products of PLA-NPs are none cytotoxic and biodegradable [150] whereas LNCs are prepared with FDA-approved constituents using a solvent-free and low energy process [27]. Furthermore, PLA-NPs and LNCs have a considerable drug encapsulation capacity and exhibit sustained-release properties at the site of action [11, 151]. As a result of their small size, PLA-NPs and LNCs can also be incorporated into cells without any transfection agents and can escape from the endo-lysosomal compartment [117-119]. We showed that PLA-NPs and LNCs can be efficiently internalized into MIAMI cells while cell viability and differentiation are not affected. Furthermore, these NP-loaded cells were able to migrate towards the U87MG experimental human glioma model [123]. The therapeutic efficacy of MIAMI cells carrying drug loaded NPs was evaluated by using the cytotoxic compound Fc-diOH [1,1-di(4-hydroxyphenyl)-2-ferrocenylbut-1-ene], an analog of 4-hydroxy-tamoxifen (personal communication). We showed that MIAMI cells loaded with Fc-diOH-LNCs were toxic to the U87MG glioma cell line both *in vitro* and *in vivo*. This indicates that MIAMI cells are able to internalize drug-loaded NPs and deliver the cytotoxic agent into the tumor. Agents other than drugs could be encapsulated into NPs, such as DNA to transfer genes into the tumor or miRNA to repress the translation of mRNAs.

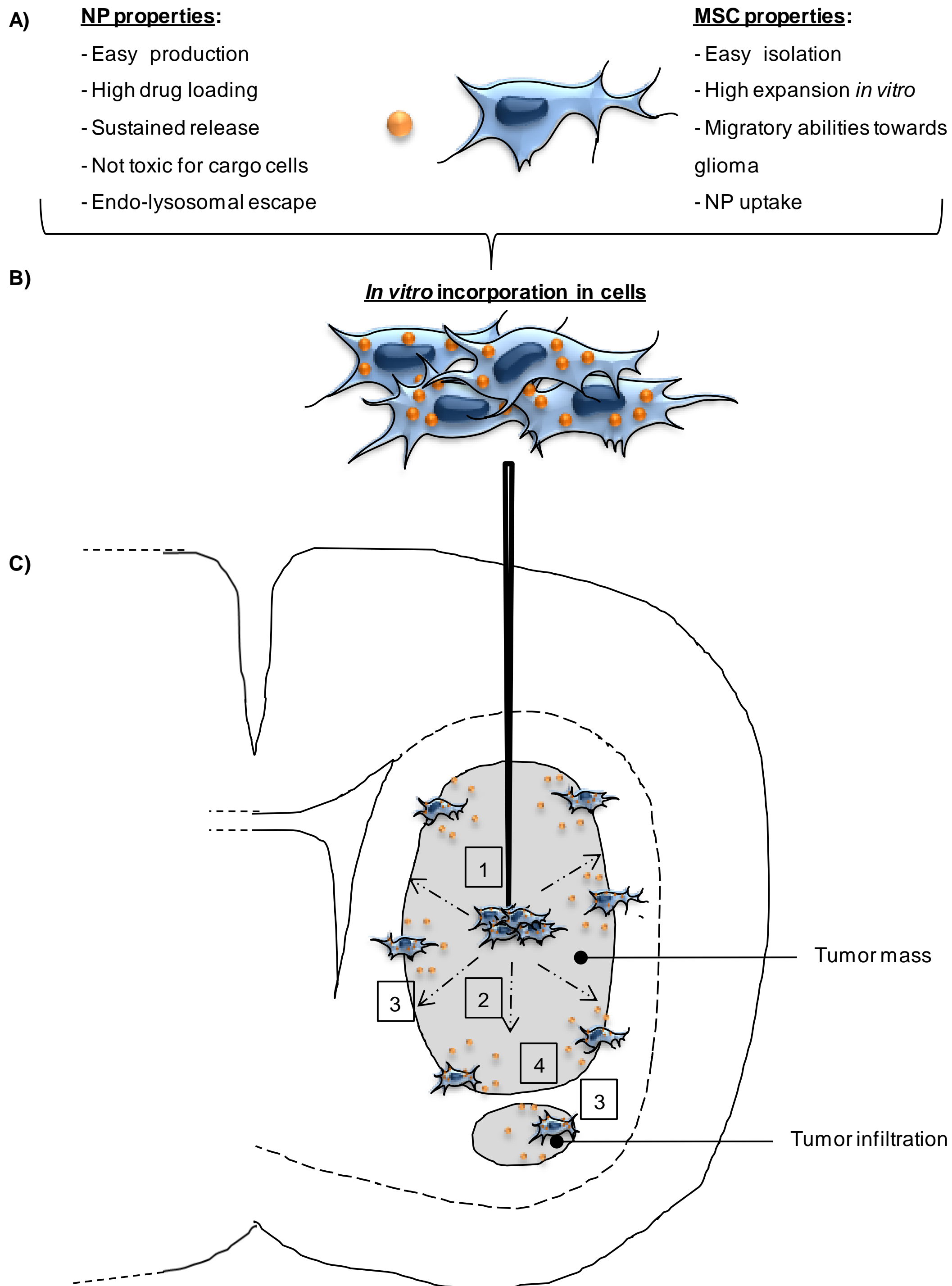

Figure 4 : Schematic representation of NP delivery by stem cells within a brain tumor. A) Properties of NPs and stem cells for optimal combination. B) Before intratumoral injection, NPs should be incorporated into cells *in vitro*. C) Step-by-step delivery strategy: 1. Intratumoral injection of MSCs loaded with NPs into the tumor mass. 2. Migration of MSCs loaded with NPs. 3. MSC distribution at the border between normal brain parenchyma and the tumor mass, including tumor infiltrations. 4. Release of the therapeutic agent.

Concluding remarks

The use of stem cells, in particular MSCs as cellular carriers is a promising therapeutic strategy to deliver specific drug-loaded NPs. However, the interaction between MSCs and tumor cells should be determined. Currently, several studies highlight that caution should be used in the therapeutic exploitation of MSCs for malignant conditions. Indeed, the exact biological function of MSCs in brain tumors and peripheral tumors is still unclear [152]. Some studies indicate that MSCs promote tumor development either by providing a niche for cancer stem cells, through impairing immune surveillance or by differentiation into cancer-associated fibroblast-like cells [153-155]. Other studies have demonstrated an opposite effect [97, 156] or no effect at all [104, 157-159]. Before using MSCs as cellular carriers, the fate of these cells within the brain tumor still needs to be carefully evaluated.

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TRAVAIL EXPERIMENTAL

CHAPITRE 1 :

Etude de l'incorporation de systèmes nanoparticulaires par les cellules MIAMI et suivi des propriétés souches des cellules chargées.

ETUDE DE L'INCORPORATION DE SYSTEMES NANOPARTICULAIRES PAR LES CELLULES MIAMI ET SUIVI DES PROPRIETES SOUCHES DES CELLULES CHARGEES

L'objectif de cette première étude était d'obtenir la preuve de concept sur la faisabilité d'un nouvel outil thérapeutique utilisant des CSMs pour véhiculer des NPs au sein d'une tumeur cérébrale. Une sous-population de CSMs, les cellules MIAMI, et deux types de NPs (NPs polymériques de PLA et NCLs) ont été utilisés pour fabriquer cet outil.

Afin de valider notre stratégie, diverses conditions devaient être atteintes :

- L'incorporation efficace des NPs par les cellules MIAMI
- La rétention des NPs après incorporation dans les cellules
- La non-toxicité des NPs pour les cellules chargées
- La conservation du potentiel souche des cellules MIAMI après leur incorporation de NPs
- La capacité des cellules chargées à migrer au sein d'une tumeur gliale *in vivo*

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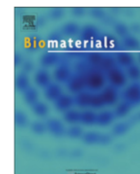

Mesenchymal stem cells as cellular vehicles for delivery of nanoparticles to brain tumors

Mathilde Roger [a], Anne Clavreul [a,b], Marie-Claire Venier-Julienne [a], Catherine Passirani [a], Laurence Sindji [a], Paul Schiller [c,d], Claudia Montero-Menei [a], Philippe Menei [a,b,*]

[a] INSERM Unit 646, Ingénierie de la Vectorisation Particulaire, 10 rue André Bocquel, Université d'Angers, 49100 Angers, France
[b] Département de Neurochirurgie, Centre Hospitalier Universitaire d'Angers, 4 rue Larrey, 49100 Angers, France
[c] Departments of Biochemistry/Molecular Biology and Medicine, University of Miami Miller School of Medicine, Miami, FL 33125-1693, USA
[d] Geriatric Research, Education and Clinical Center (GRECC) and Research Service, VA Medical Center; and Departments of Medicine and Biochemistry & Molecular Biology, and Stem Cell Institute, University of Miami Miller School of Medicine, Miami, FL 33125-1693, USA

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ABSTRACT

The prognosis of patients with malignant glioma remains extremely poor, despite surgery and improvements in radio- and chemo-therapies. Nanotechnologies represent great promise in glioma therapy as they protect therapeutic agent and allow its sustained release. However, new paradigms allowing tumor specific targeting and extensive intratumoral distribution must be developed to efficiently deliver nanoparticles (NPs). Knowing the tropism of mesenchymal stem cells (MSCs) for brain tumors, the aim of this study was to obtain the proof of concept that these cells can be used as NP delivery vehicles. Two types of NPs loaded with coumarin-6 were investigated: poly-lactic acid NPs (PLA-NPs) and lipid nanocapsules (LNCs). The results show that these NPs can be efficiently internalized into MSCs while cell viability and differentiation are not affected. Furthermore, these NP-loaded cells were able to migrate toward an experimental human glioma model. These data suggest that MSCs can serve as cellular carriers for NPs in brain tumors.

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1. Introduction

Malignant glioma has a poor prognosis despite aggressive treatment using surgery, radiotherapy and chemotherapy [1,2]. The difficulty with cancer treatments, especially with brain, is to achieve an effective delivery of therapeutic agents to the tumor and to the infiltrating tumor cells. Many promising treatments for glioma involve nanoparticles (NPs). NPs are defined as solid colloidal particles of matricial (nanospheres) or vesicular type (nanocapsules) ranging in size from 10 to 1000 nm. They are generally constituted of biodegradable and non-biodegradable polymers or lipids. NPs can carry multiple therapeutic agents such as drugs [3–5], radionucleides [6] or DNA [7,8]. The therapeutic agent can be entrapped in, adsorbed or chemically coupled onto their surface. These NPs protect the therapeutic agent from a premature degradation and allow its sustained and controlled release. Although NPs represent promising carriers for drug delivery to glioma [4,9,10], an efficient NP delivery to brain tumor is a central mandate of this treatment. The main limitation is the obstacle of the blood–brain barrier (BBB), which separates the blood from the cerebral parenchyma [11]. Active brain-targeting strategies to cross BBB have been investigated such as the conjugation of lipid nanocapsules (LNCs) to OX26 monoclonal antibodies which can then recognise the transferrin receptor localized principally on the brain capillary endothelium [12]. However, this active targeting leads to a low NP accumulation in the brain. To bypass the BBB, direct intratumoral NP delivery using convection-enhanced-delivery (CED) has been used. This method, using an external pressure gradient inducing fluid convection in the brain via a surgically implanted catheter, allows greater volume distribution to be achieved in comparison to diffusion alone [13,14]. A combination of nanotechnology with the CED technique showed promising results in 9L experimental glioma in rats [15,16]. However, this approach presented some disadvantages such as long infusion times inducing infections, potential intracranial hypertensions and unpredictable drug distribution.

Another alternative to enhance NP delivery in brain tumor is the use of cellular vectors that have endogenous tumor-homing activity and can thereby chaperone NP delivery *in vivo*. In this regard, mesenchymal stem cells also called multipotent mesenchymal stromal cells (MSCs) which have a tendency to distribute at the site of tumors could be potential candidates [17,18]. Following intra-arterial

* Corresponding author. INSERM Unit 646, Ingénierie de la Vectorisation Particulaire, 10 rue André Bocquel, Université d'Angers, 49100 Angers, France. Tel.: +33241354822; fax: +33241735853.
E-mail address: phmenei@chu-angers.fr (P. Menei).

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and intracranial injections, gene modified MSCs, have shown therapeutic effects in several preclinical models of gliomas [19–23]. MSCs can serve as cell carriers for the replication, transportation, and local release of intact oncolytic adenoviruses into tumors [24,25].

A unique subpopulation of human MSCs named "marrow-isolated adult multilineage inducible" (MIAMI) cells, has been isolated and amplified using particular culture conditions as low oxygen tension (3%) that mimic the MSC niche [26]. These cells possess a unique molecular phenotype and are also capable of differentiating *in vitro* into cell lineages derived from all three germ layers and particularly into immature neurons [26]. Furthermore, MIAMI cells can be easily obtained from patients and proliferate extensively without evidence of senescence or loss of differentiation potential and thus may represent an ideal candidate for cellular therapies [27].

In this study, we determined if these cells can be used as NP delivery vehicles in brain tumor therapy. Two different kinds of NPs were investigated to evaluate the feasibility of this approach: poly-lactic acid NPs (PLA-NPs) and LNCs. These two types of NPs are non-cytotoxic and their use as therapeutic vectors in glioma showed promising results. In this work, we evaluated the cellular uptake and retention of these two types of NPs by MIAMI cells. Furthermore, the effect of PLA-NP and LNC uptake on viability, differentiation, and *in vivo* migration capacity of MIAMI cells was assessed.

2. Materials and methods

2.1. NP materials

50:50 PLA, $M_W = 160$ kDa was kindly provided by Dr X. GARRIC (Institut des Biomolécules Max Mousseron, Université de Montpellier I, CNRS UMR 5247, France). Polyvinyl alcohol (PVA, Mowiol® 4-88, 88% hydrolysed) was obtained from Kuraray Specialities Europe (Frankfurt, Germany). Acetone was purchased from Carlo Erba Regenti® (Rodano, Italy). Dicholoromethane and coumarin-6, were purchased from Sigma–Aldrich (St. Quentin Fallavier, France).

The lipophilic Labrafac® CC (caprylic-capric acid triglycerides) was kindly provided by Gattefosse S.A. (Saint-Priest, France). Lipoïd® S75-3 (soybean lecithin at 69% of phosphatidylcholine) and Solutol® HS15 (a mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate) were a gift from Lipoïd Gmbh and BASF (Ludwigshafen, Germany), respectively.

2.2. PLA-NP and LNC formulations

PLA-NPs containing coumarin-6 were prepared using a single emulsion–solvent evaporation technique [28]. PLA polymer (200 mg) and coumarin-6 (0.1 mg) were dissolved in 17.5 mL of dichloromethane and 17.5 mL of acetone. The mixture was emulsified into 200 mL of 0.5% (w/v) PVA solution using a high-pressure homogenizer (ALM2®, Guérin, Mauzé, France). Emulsification was carried out for 40 s at 150 bar. Dichloromethane and acetone were removed under reduced pressure in a rotary evaporator. To separate NPs from non-encapsulated coumarin-6 and PVA excess, NP suspension was then centrifuged for 20 min at 21,000 g and 25 min at 19, 000 g at 4 °C, and collected in 5 mL of sterile water for injection. Finally, PLA-NPs were sterilized by filtration (0.2 μm).

To formulate coumarin-6-LNCs, coumarin-6 was dissolved in acetone (1.25 mg/mL) and this solution was added in Labrafac® (400 μL in 2418 mg of Labrafac). This mixture was finally agitated under magnetic stirring up to 50 °C to eliminate acetone. LNCs were prepared according to the phase-inversion temperature method [29]. Briefly, Solutol® HS15 (9.7% w/w), Lipoid® (1.5% w/w), Labrafac® (24.2% w/w), NaCl (1.8% w/w) and water (62.8% w/w) were mixed and heated under magnetic stirring up to 95 °C. Three cycles of progressive heating and cooling between 95 °C and 60 °C were then carried out and followed by an irreversible shock induced by dilution with 12.5 mL of 2 °C deionised water added to the mixture at 80–85 °C. Finally, LNCs were sterilized by filtration (0.2 μm).

To determine NP concentration, 1 mL of LNCs and PLA-NPs were freeze-drying lyophilized (Lyovac GT2, SRK Systemtechnik, Reistatd-Godellau, Allemagne) for 24 h to obtain a dry powder.

2.3. PLA-NP and LNC characterization

2.3.1. Particle size and surface charge

LNCs and PLA-NPs were characterized for size, polydispersity index and charge distribution by dynamic light scattering using a Zetasizer® Nano Series DTS 1060 (Malvern Instruments S.A., Worcestershire, UK). LNCs were diluted 1:60 (v/v) in deionised water in order to ensure a convenient scattered intensity on the detector.

2.3.2. Scanning electron microscopy

The surface morphology of PLA-NPs was observed by scanning electron microscopy (SEM, 6301F, JEOL, Japan). Samples were prepared from NP suspension. A drop of PLA-NPs was dripped onto silicium wafer and dried overnight.

2.3.3. In vitro release of coumarin-6

In vitro release of coumarin-6 from NP was studied to ensure that coumarin-6 estimated in MSCs was due to the uptake of NPs and not to the uptake of free coumarin-6 that might have been released from the NPs during the experiment. Coumarin-6 is a lipophilic dye strongly fluorescent in solvent solution where it emits in the green at 518 nm. According to its hydrophobic structure, this dye precipitates in aqueous solution reducing drastically its fluorescence emission at 518 nm [30]. This property was used to evaluate *in vitro* release of coumarin-6 from PLA-NPs and LNCs. NPs were suspended in DPBS (Dulbecco's Phosphate Buffered Saline, Lonza, Verviers, Belgium) and incubated at 37 °C in an orbital shaker. At designated time points, fluorescence of samples was measured by a microplate reader (Fluoroskan Ascent FL, Labsystem, Cergy-Pontoise, France) with excitation wavelength at 480 nm and emission wavelength at 518 nm. This fluorescence was compared to the fluorescence obtained directly after NP formulation which is considered to be 100%. A decrease of the fluorescence indicated a release of coumarin-6 from NPs.

2.4. Cell culture

MIAMI cells were isolated by differential adhesion of iliac crest aspirate of a male human post-mortem organ donor (protocol agreed by the French Agency of Biomedecine) and cultured *in vitro* following the conditions described by D'Ippolito and its collaborators [26]. Briefly, MIAMI cells were plated at low density (100 cell/cm^2) on fibronectin-coated dishes (10 ng/mL, Sigma–Aldrich) in Dulbecco's modified Eagle's medium-low glucose (DMEM-LG, Gibco, Cergy-Pontoise, France) supplemented with 3% fetal bovine serum (FBS, Hyclone, PerbioScience, Bredières, France) and 1% antibiotics (Sigma–Aldrich). Cells were expanded at low density (<30% confluency) in a humidified incubator at 37 °C in an atmosphere of 3% O_2/5% CO_2/ 92% N_2. These cells are negative for hematopoietic lineage markers (CD45, CD34) while they are positive for CD13, CD29, CD44, CD73, CD90, CD105, CD140b, CD164 and CD166.

The human glioma cell line U87MG was provided by ATCC (LGC Promochem, Molsheim, France) and comes from a female donor. Cells were maintained in minimum essential medium (Eagle) with Earls's BSS (Lonza) containing 10% FBS (Lonza), 0.1 mM non-essential amino acids (Lonza), 1.5 g/L sodium bicarbonate (Lonza), 1 mM sodium pyruvate (Lonza) and 1% antibiotics (Sigma–Aldrich) in humidified incubator gassed with 5% CO_2 (37 °C) until reaching 80% confluency.

2.5. NP uptake studies

To determine the effect of NP concentration on uptake, 5×10^4 MIAMI cells were incubated in HBSS (Lonza) with NPs in the concentration range of 0–200 μg/mL for PLA-NPs and 0–2 mg/mL for LNCs for 1 h at 37 °C. In a separate experiment, to study the effect of incubation time on NP uptake, 5×10^4 MIAMI cells were incubated in HBSS with 100 μg/mL of PLA-NPs and 1 mg/mL of LNCs for 30 min, 1 h and 2 h at 37 °C. At the end of the incubation period, cells were washed two times with DPBS and extracellular fluorescence was quenched with trypan blue [31]. After washing, the cells were fixed in 2% formaldehyde and analyzed using a FACScan flow cytometer with CellQuest Software (BD Biosciences).

2.6. Cell viability and proliferation assay

In vitro cell viability experiments were performed as follows. After incubation with native and with various diluted concentrations of NP suspension (0–200 μg/mL for PLA-NPs and 0–50 mg/mL for LNCs) in HBSS for 1 h at 37 °C, cells were incubated with fresh HBSS containing 100 μl of combined MTS/PMS solution (CellTiter 96® AQ_{ueous} non-radioactive cell proliferation assay kit, Promega, Charbonnieres, France) for 3 h at 37 °C. For proliferation assay, the cells were treated with 200 μg/mL of PLA-NPs or 1 mg/mL of LNCs for 1 h at 37 °C in HBSS and were allowed to grow in expansion medium for 1, 3 and 7 days followed by incubation with MTS/PMS solution. The soluble formazan generated by the live cells was proportional to the number of live cells and the absorbance at 490 nm was recorded using a microplate reader (Multiskan Ascent, Labsystem).

2.7. In vitro differentiation

Experiments evaluating the effect of NP uptake on MIAMI cell differentiation were performed as follows. MIAMI cells were incubated with 200 μg/mL of PLA-NPs or 1 mg/mL of LNCs for 1 h at 37 °C followed by DPBS wash, and then cultured in adipogenic or osteogenic medium for 21 days. Adipogenic medium consists of DMEM-LG

supplemented with 3-isobutyl-1-methylxanthine (0.5 mM), dexamethasone (1 μM), insulin (10 μg/mL), indomethacin (100 μM) purchased from Sigma–Aldrich, 1% antibiotics, and 10% FBS. Osteogenic medium consists of alpha-MEM (Lonza) supplemented with ascorbic acid (100 μM), beta-glycerophosphate (10 mM), purchased from Sigma–Aldrich, dexamethasone (10 nM), 1% antibiotics, and 10% FBS. Medium changes were carried out twice weekly. Adipogenic differentiation was assessed by the cellular accumulation of neutral lipid vacuoles after cells were fixed with 4% paraformaldehyde (PFA, Sigma–Aldrich) pH 7.4 and stained with oil-red O. For evaluation of osteogenic differentiation, cells were fixed with 70% EtOH and stained with alizarin red to detect calcium deposits.

2.8. Intracellular retention studies

The retention of PLA-NPs and LNCs in MIAMI cells was followed using flow cytometry and fluorescent microscopy. MIAMI cells were plated in 6-well plates and in 8-well labtek chambers at the concentration of 5×10^4 cells per well and 1×10^4 cells per well, respectively. After 24 h, MIAMI cells were incubated with 200 μg/mL of PLA-NPs or 1 mg/mL of LNCs in HBSS, 1 h at 37 °C. After incubation, cells were washed twice with DPBS and re-covered with DMEM-LG free-serum. At designated time intervals, cells were analyzed by flow cytometry as described above and by fluorescent microscopy. For fluorescent microscopy analysis, cells were washed and stained with Lysotracker Red (Molecular Probes, Invitrogen, Cergy-pontoise, France), 30 min at room temperature, fixed 5 min with 4% PFA pH 7.4 and nuclei were counterstained with 4′, 6-diamino-2′-phenylindole dihydrochloride (DAPI, Sigma–Aldrich). Slides were mounted in a fluorescent mounting medium (Dako) and analyzed on a fluorescence microscope (Axioscope® 2 optical, Zeiss, Le Pecq, Allemagne).

2.9. Intracerebral glioma model

Female athymic Swiss nude mice, ranging in age from 8 to 9 weeks were obtained from Charles River (L'Arbresle, France). The experiments were conducted according to the French Minister of Agriculture and the European Communities Council Directive of 24 November 1986 (86/609/EEC). Animals were anesthetized by an intraperitoneal injection of xylazine (50 mg/kg body weight) and ketamine (10 mg/kg body weight). Using a stereotactic frame (David Kopf instruments, Tujunga, CA, USA) and a Hamilton syringe, a 5 μl dose of HBSS with Ca^{2+} and Mg^{2+} containing 5×10^4 U87MG cells was injected into the striatum of the mouse at Day 0. Coordinates used for intracerebral injection were 2 mm lateral, 0.5 mm anterior to Bregma, and 3 mm deep from the outer border of the cranium. At Day 6, 0.3×10^6 MIAMI cells, loaded or not with 200 μg/mL of PLA-NPs or with 1 mg/mL of LNCs, were stereotaxically injected in the tumor at the same coordinates. Mice were killed 7 days after MIAMI cell injection and brains were snap-frozen in isopentane cooled by liquid nitrogen and stored at −80 °C.

2.10. Brain staining

Brain coronal sections were cut at 10 μm and collected onto silanized slides. MIAMI cells were detected by the fluorescence in situ hybridization (FISH) technique using a Y-chromosome probe. The DNA probe is complementary to the highly repetitive human satellite III sequences close to the centromeric region of the human Y-chromosome DYZ1 locus (CEPY) and labeled with the fluorochrome SpectrumOrange (Vysis, Abbott Molecular, Rungis, France). Frozen sections were air-dried, rehydrated in DPBS and fixed 10 min in 4% PFA pH 7.4 at 4 °C. Nuclei were denatured for 5 min using 70% Formamide (Sigma–Aldrich)/2× Standard Saline Citrate (SSC, Vysis) at 73 °C and dehydrated next in gradient alcohol bathes (EtOH 70%, 80%, 90% and 100%), 2 min each. Y-chromosome probe was diluted with hybridization buffer according to manufacturer instructions and dropped on slides, covered with coverslip and sealed with rubber cement. The hybridization was performed in 3 steps: 10 min at 73 °C to denature the DNA and the probe, 2 min at 4 °C to fix the denaturation and overnight in a humid chamber at 45 °C to hybridize DNA with the probe. After 3 washes for 10 min in 50% formamide/2X SSC baths and 5 min in 2X SSC/0.1% NP-40, nuclei were counterstained in DAPI II with antifade (Vysis) and slides were analyzed on a fluorescence microscope (Axioscope® 2 optical). Coumarin-6 NPs were analyzed on adjacent frozen sections. Sections were air-dried, rehydrated in DPBS and fixed 10 min in 4% PFA pH 7.4 at 4 °C. After washing, nuclei were counterstained in DAPI II with antifade (Vysis) and slides were analyzed on a fluorescence microscope (Axioscope® 2 optical).

2.11. Statistical analysis

Result is given as mean ± standard error of mean (SEM). To describe statistical differences, the Dunnet test was used. Differences were considered statistically significant for $P < 0.05$.

3. Results

3.1. PLA-NP and LNC characterization

Particle size, polydispersity index and zeta potential of coumarin-6-loaded PLA-NPs and LNCs are presented in Table 1. Coumarin-6-PLA-NPs produced by an emulsion–solvent evaporation technique have a mean diameter of 136 ± 1 nm with a polydispersity index of 0.040. They present a regular spherical shape according to the SEM study (Fig. 1). LNCs formulated by the phase-inversion temperature method have a mean diameter of 88 ± 2 nm with a polydispersity index of 0.060. For both NPs, the zeta potential was negative, −2.11 ± 0.2 mV and −3.7 ± 0.9 mV for PLA-NPs and LNCs, respectively.

The *in vitro* release of coumarin-6 from NPs was followed using the fluorescent property of this molecule. A decrease of the fluorescence of the sample under study compared to fresh NPs indicates a release of coumarin-6. The results showed that only 2% of the dye was released from PLA-NPs during a 4 h-period at 37 °C and no release from LNCs was observed probably due to its high affinity for the oily core (data not shown).

3.2. Cellular NP uptake

Since our goal is to develop particles to be used with cells, the toxicity of various concentrations of PLA-NPs and LNCs on MIAMI cells was quantified. For PLA-NPs, the cell viability was not affected even at the concentration of 200 μg/mL (Fig. 2A). For LNCs, toxicity was only observed for concentrations superior to 2 mg/mL (Fig. 2B).

Secondly, to perform an optimal intracellular uptake, the effect of incubation time and NP concentration on the uptake by MIAMI cells was assessed. For doses of 200 μg/mL of PLA-NPs and 1 mg/mL of LNCs, results show that the NP uptake was dependent on the incubation time. The uptake was first detected at the 30 min time point and increased gradually with the incubation time (Fig. 3A). For PLA-NPs almost all the cells had incorporated NPs within the 30 min-period but they continued to incorporate more NPs until the end of the experiment. The NP uptake also gradually increased depending on the NP concentration in the medium for an incubation time of 1 h (Fig. 3B). Again, for the PLA-NPs even if all the cells had incorporated the NPs at the lowest concentration, the NP uptake per cell continued to increase in a concentration dependent manner.

3.3. Intracellular retention of NPs

The intracellular retention of NPs was studied by flow cytometry and fluorescence microscopy (Fig. 4). Flow cytometry analysis indicated that, for PLA-NPs, more than 95% of the cells were fluorescent after 3 days, although the mean fluorescence decreased (Fig. 4A). For LNCs, the mean fluorescence and the number of positive cells declined during time to reach only 46% of positive cells after 3 days (Fig. 4A). These results were confirmed by fluorescence microscopy (Fig. 4B). After 3 and 7 days, cells loaded with PLA-NPs still contained coumarin-6 while MIAMI cells loaded with

Table 1
Physicochemical characteristics of coumarin-6-PLA-NPs and coumarin-6-LNCs (mean of 3 samples ± SEM).

	Mean particle size (nm)	Polydispersity PDI	Zeta potential (mV)
Coumarin-6-PLA-NPs	136 ± 1	0.04 ± 0.005	−2.11 ± 0.2
Coumarin-6-LNCs	88 ± 2	0.06 ± 0.01	−3.7 ± 0.9

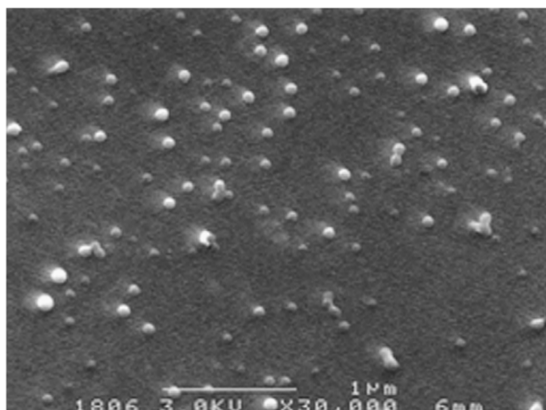

Fig. 1. SEM images of PLA-NPs.

LNCs were not all coumarin-6 fluorescent. PLA-NPs formed aggregates observed as large spots while LNCs were diffused and covered the whole cytoplasm of cells. The decrease of fluorescence is probably due to the proliferation of cells. Indeed the density of cells increased with time and the multiplication of cells could thus dilute the NPs.

3.4. Effect of NP uptake on proliferation and differentiation of MIAMI cells

Cell viability of unloaded and NP-loaded MAMI cells is presented in Fig. 5A. Internalisation of PLA-NPs and LNCs in MIAMI cells delayed cell proliferation in the first 3 days. However, 7 days after NP internalization, NP-loaded MIAMI cells showed a renewal of proliferation suggesting that PLA-NPs and LNCs are non-cytotoxic. Within this line, MIAMI cells loaded with PLA-NPs and LNCs were able to differentiate into osteoblasts and adipocytes, similarly to unloaded MIAMI cells (Fig. 5B).

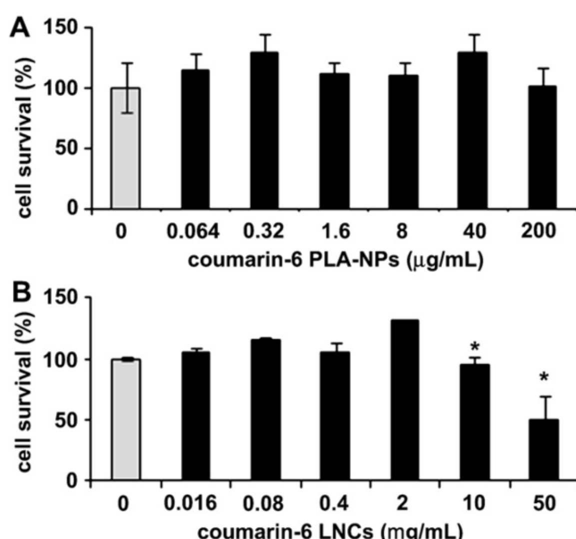

Fig. 2. Cell viability analysis of MIAMI cells after 1 h of incubation with 0–200 μg/mL for PLA-NPs and 0–5 mg/mL for LNCs. Cell survival was expressed in percentage by comparison with cell survival of MIAMI cells without NPs, considered as 100%. Data are expressed as the mean of four wells ± SEM. (*$P < 0.05$).

3.5. Capacity of MIAMI cells to carry NPs in an in vivo glioma model

Unloaded and NP-loaded MIAMI cells, derived from a male donor, were grafted into female nude mice, carrying the U87MG tumor providing from a female donor. MIAMI cell migration pattern was analyzed 7 days after intratumoral MIAMI cell injection. FISH for the Y-chromosome was performed to specifically label MIAMI cells. Unloaded MIAMI cells were largely distributed at the border zone between tumor and normal parenchyma (Fig. 6A). Furthermore, these cells followed infiltrated tumor cells. No Y-chromosome-positive cells were seen in the normal brain. NP-loaded MIAMI cells presented a similar migration pattern and no difference was observed between MIAMI cells containing PLA-NPs or LNCs (Fig. 6B). Furthermore, analysis of adjacent sections showed that a green fluorescence corresponding to coumarin-6 was present around the brain tumor consistent with the localization of MIAMI cells.

4. Discussion

In recent years, significant efforts have been devoted to develop nanocarrier systems to improve drug or gene delivery for brain tumor treatments [3,32–35]. However, new paradigms allowing tumor specific targeting and extensive intratumoral distribution must be developed for delivering NPs. The use of cellular vehicles with homing property to brain tumors could be an alternative to distribute NPs. Several cellular vehicles can be suggested such as macrophages and stem cells. Macrophages have the ability to migrate in and out of intracranial tumors and distribute to other tumor cells within the CNS [36]. Alizadeh et al. [37], recently studied the uptake of cyclodextrin-based NPs into a murine glioma model and showed that these NPs are preferentially taken-up by tumor-associated macrophages. After NP incorporation, these cells distributed within and around the tumor mass indicating that macrophages could vehicle NPs. MSCs are also able to migrate toward brain tumors and thus represent another potential candidate [18]. Moreover, MSCs possess the potential of converting to tissue types of other lineages, both within and across germ lines [38].

Several studies described the therapeutic effects of MSCs carrying virotherapeutic agents [25] or producing anticancer agents following their genetic modification with viral vectors in brain tumor [19,20,22,23,39]. The use of virus has a number of disadvantages including risk of toxicity, immunogenicity, insertional mutagenesis and high manufacturing costs [40]. Nanotechnology offers several advantages over viral vectors, including biological security, low risk of immunogenicity as well as low cost and easy production. This technology has been already evaluated to manipulate and track MSCs [41]. For example, internalization of magnetic NPs and quantum dots in MSCs has been used for long-term labeling of MSCs and monitoring their fate and their regenerative potential [42,43]. Furthermore, NPs have been used to deliver vascular endothelial growth factor (VEGF) to human MSCs to enhance angiogenesis [44]. To our knowledge, the use of MSCs to carry drug delivery NPs into brain tumors has not been described.

In this study, we were particularly interested in MIAMI cells, a unique subpopulation of non-transformed non-immortalized adult human marrow stem cells capable of differentiating into cell lineages derived from all three germ layers [27]. These cells are easy to obtain from patients and to handle *in vitro*.

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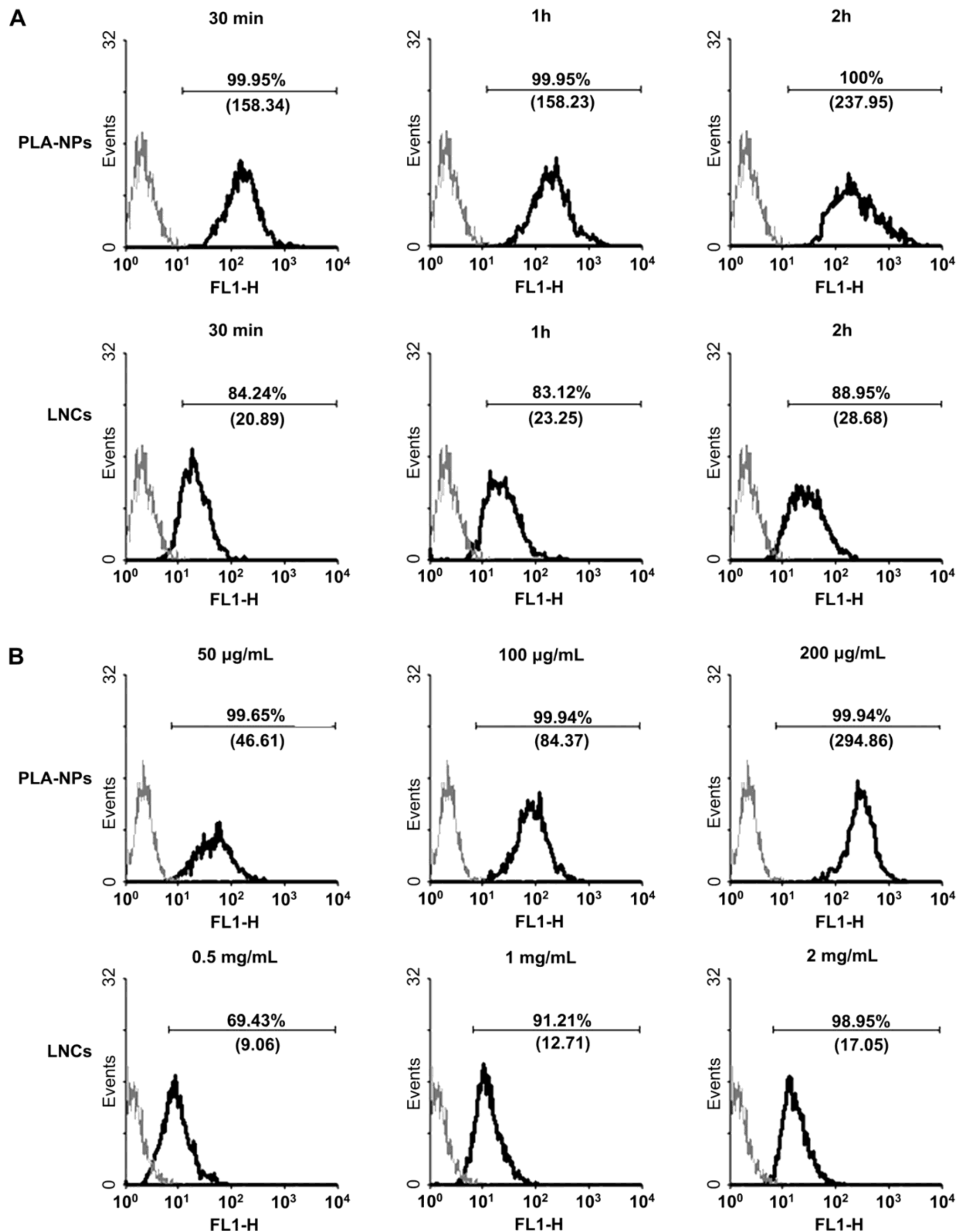

Fig. 3. Time- and dose-dependent cellular uptake of PLA-NPs and LNCs in MIAMI cells detected by flow cytometry. A) For time-dependent uptake, MIAMI cells were incubated with 100 μg/mL of PLA-NPs or 1 mg/mL of LNCs for the indicated time (0 h, 30 min, 1 h, and 2 h). B) For dose-dependent uptake, MIAMI cells were incubated 1 h with 50, 100 or 200 μg/mL of PLA-NPs and 0.5, 1 or 2 mg/mL of LNCs. The mean fluorescent intensity of NP-treated cells was noted in the parentheses and the number of positively labeled cells was represented as the percentage of total counted cells in each panel. Grey histogram represents control cells without NP.

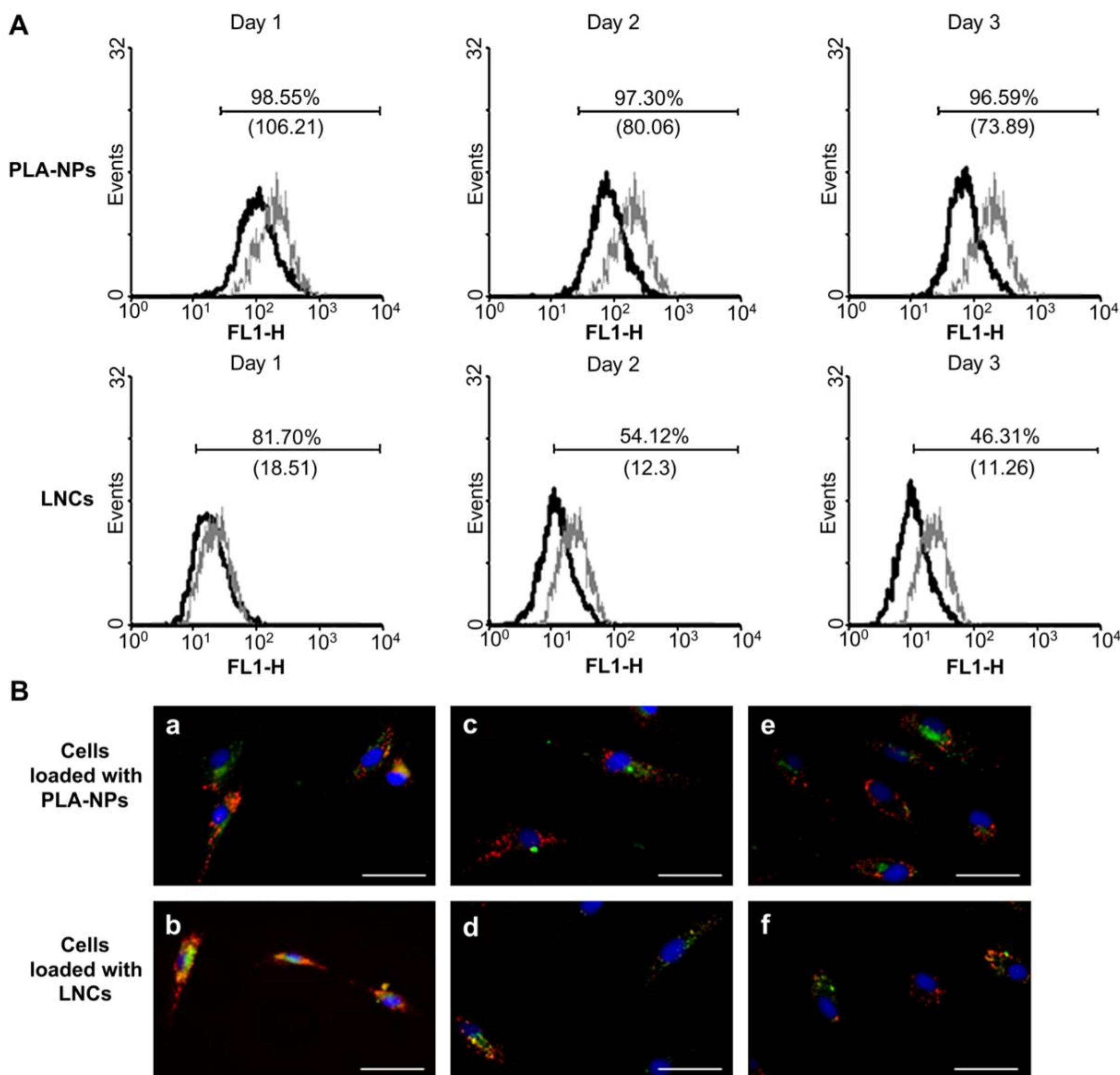

Fig. 4. Cellular retention of PLA-NPs and LNCs in MIAMI cells detected by flow cytometry (A) and fluorescent microscopy (B). MIAMI cells were incubated 1 h at 37 °C with 100 µg/mL of PLA-NPs and 1 mg/mL of LNCs. After washes cells were harvested and analyzed after different times. A) The mean fluorescent intensity of NP-treated cells was noted in the parentheses and the number of positively labeled cells was represented as the percentage of total counted cells in each panel. The grey histogram represents intracellular NPs retention at Day 0. B) Fluorescent microscopy images of MIAMI cells loaded with PLA-NPs or with LNCs at Day 0 (a and b), Day 3 (c and d) and Day 7 (e and f). Cells were stained with Dapi for nuclei (blue) and Lysotracker for lysosomes (red). The green fluorescence represents coumarin-6 (scale bar = 50 µm).

Furthermore, these cells are able to differentiate into neuron-like cells thus representing autologous human cell populations for tissue regeneration of skeletal and nervous system disorders [45]. We have evaluated the ability of MIAMI cells to migrate toward glioma by using the U87MG experimental human glioma in nude mice. Male-derived MIAMI cells were tracked by the detection of the Y-chromosome with the FISH technique, which is only expressed by these cells in this model. We showed that when MIAMI cells were implanted directly into the tumor, they localized mainly at the border between tumor cells and normal brain parenchyma (Fig. 6A). This peri-tumoral distribution of MIAMI cells is in agreement with previous studies using MSCs [19,20].

We investigated two different NPs: PLA-NPs and LNCs. These NPs were chosen for their biocompatibility properties. On one hand the degradation products of PLA-NPs are non-cytotoxic and biodegradable [46] and on the other hand LNCs are prepared with FDA-approved constituents and by a solvent-free and low energy process [47]. Furthermore, PLA-NPs and LNCs provide considerable drug encapsulation capacity and also exhibit sustained release functions at the site of action [3,10]. Moreover, as a result of their small size, PLA-NPs and LNCs can be incorporated by cells without any transfection agents [48–50]. To assess the intracellular uptake of PLA-NPs and LNCs in MIAMI cells, coumarin-6, a lipophilic fluorescent dye, was encapsulated in these NPs. This dye has been shown to serve as

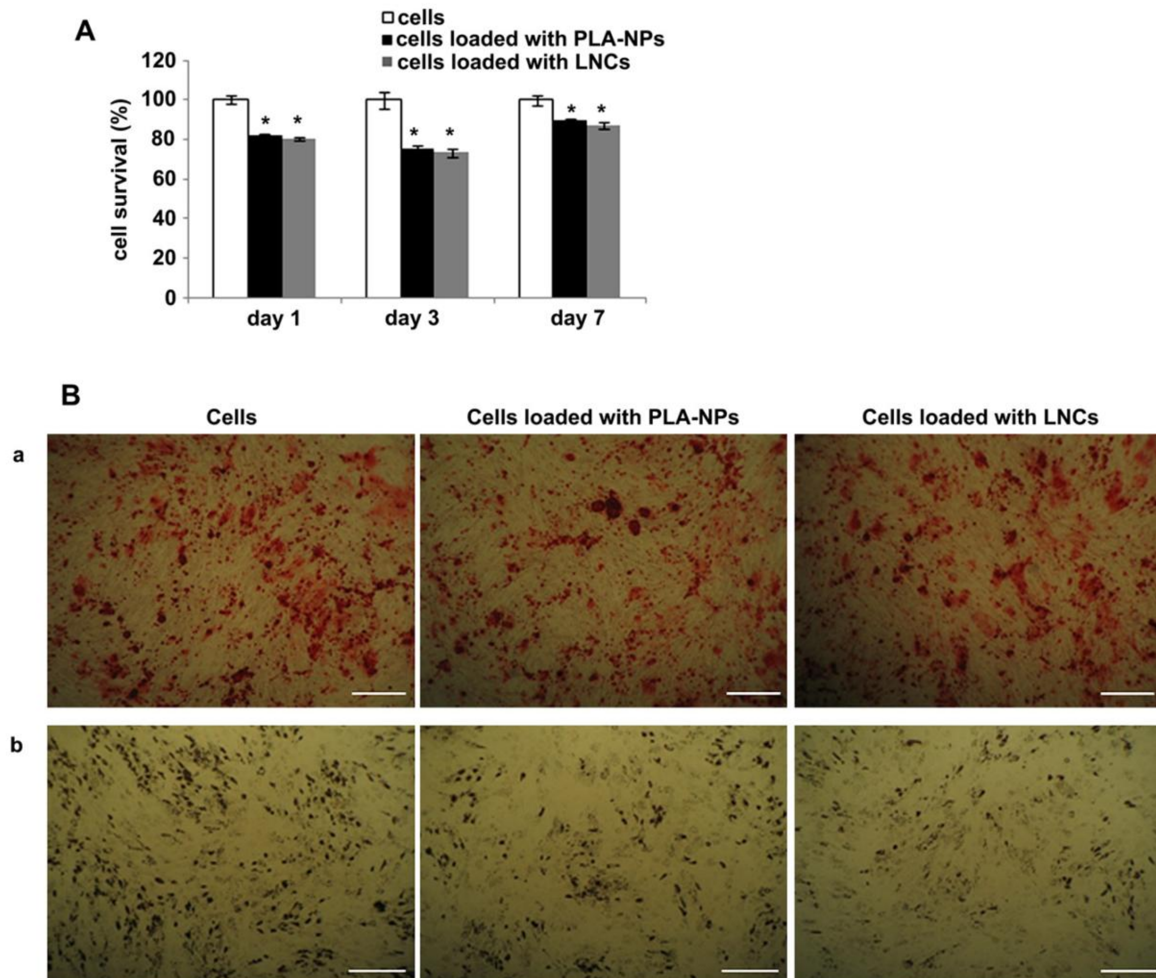

Fig. 5. A) Analysis of NP uptake effect on MIAMI cell survival. MIAMI cell were incubated 1 h at 37 °C with 100 μg/mL of PLA-NPs and 1 mg/mL of LNCs. After 1, 3 and 7 days, cell survival was determined and expressed in percentage by comparison with cell survival of MIAMI cells without NPs, considered as 100%. Data are expressed as the mean of four wells ± SEM. (*$P < 0.05$). B) Analysis of NP uptake effect on MIAMI cells differentiation in osteoblasts (a) and adipocytes (b). MIAMI cells differentiated in the absence of NPs served as control. Alizarin Red S staining was used to visualize mineral opposites at day 21(a). Oil-Red O staining was used to visualize lipid vacuoles in cells cultured under adipogenic conditions at day 21 (b). (Scale bar = 500 μm).

a useful probe for studying NP uptake, retention and distribution *in vivo* and *in vitro* [51]. Since only a negligible quantity of coumarin-6 was liberated from NPs, coumarin-6 uptake by MIAMI cells corresponded to PLA-NP or LNC uptake and not to free coumarin-6.

We showed that MIAMI cells can efficiently internalize coumarin-6-PLA-NPs and coumarin-6-LNCs. This uptake was concentration and time-dependent and doses of 200 μg/mL for PLA-NPs and 1 mg/mL for LNCs with an incubation time of 1 h at 37 °C allowed an uptake of NPs in more than 80% of MIAMI cells. In addition, NPs stay in cells after incorporation and internalization of PLA-NPs or LNCs did not inhibit the proliferation and differentiation of MIAMI cells into osteoblasts and adipocytes. Moreover, MIAMI cells loaded with NPs can migrate and distribute around the tumor mass as unloaded cells. Due to the high temperature incubation used in the FISH protocol, we could not investigate the co-localization of coumarin-6-NPs with Y-chromosome positive MIAMI cells. However, analysis of adjacent sections suggested a selective distribution of coumarin-6-containing NPs around the tumor in the same way as MIAMI cells. Furthermore, *in vitro* analysis of intracellular NP retention in MIAMI cells indicated that PLA-NPs and LNCs remained at least 7 days in MIAMI cells. It is interesting to note that MIAMI cells localize in the border of the tumor. Infiltrating tumor cells also localize in the same area and after resection these cells are usually at the origin of tumor recurrence.

This study shows that MIAMI cells combined with NPs represent a promising tool as cell delivery systems in brain tumor therapy. However, the interaction between MIAMI cells and tumor cells should be determined. Actually, several studies underline that caution should be used in the therapeutic exploitation of MSCs in the context of malignant conditions. In fact, the exact biological function of MSCs in brain tumors as in peripheral tumors is still unclear. Some studies indicated that MSCs promote tumor development either by providing the niche of cancer stem cells, by impairing immune surveillance or by differentiation in cancer-

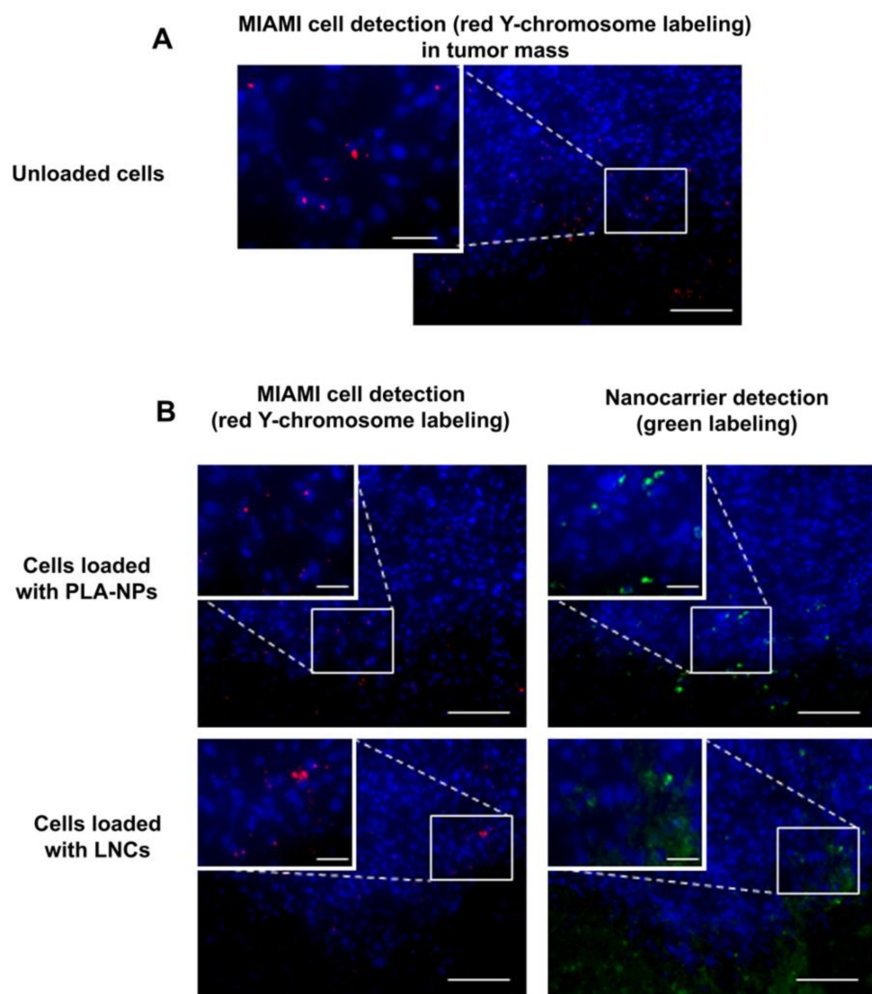

Fig. 6. Fluorescent microscopy images of tissue sections after intratumoral injection of MIAMI cells unloaded or loaded with PLA-NPs or with LNCs in brain mice. A) *In vivo* migratory capacity of unloaded MIAMI cells 7 days after implantation. MIAMI cells were detected by red Y-chromosome labeling. B) In vivo migratory capacity of MIAMI cells loaded with PLA-NPs or LNCs. MIAMI cells were detected by red Y-chromosome labeling and NP detection is revealed on adjacent sections by green labeling. Nuclei were stained with Dapi. (Scale bar = 200 μm and 50 μm for magnification).

associated fibroblast-like cells. Other studies have demonstrated an opposite effect [52]. In a future study, the effect of MIAMI cells on glioma growth will be evaluated as well as the fate of MIAMI cells after intratumoral injection.

5. Conclusion

This study demonstrates that MIAMI cells can serve as cellular carriers for NPs in brain tumors. These cells distribute around the tumor after their direct tumoral injection. This approach could be useful to track infiltrating cells after glioma resection and for inoperable brain tumor. Therapeutic agent encapsulation in NPs is currently in progress. It has been shown that local irradiation might promote specificity of migration and engraftment of MSCs [53]. Thus, the combination of MIAMI cells carrying radiosensitiser-loaded NPs such as paclitaxel or ferrocifen could be interesting. Due to the distribution of MIAMI cells at the border between tumor and parenchyma, another alternative could be the encapsulation of therapeutic agents targeting the tumor microenvironment as anti-angiogenic factors.

Acknowledgments

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Appendix

Figures with essential color discrimination. Figs. 4–6 in this article are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.07.048.

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CHAPITRE 2 :

Evaluation de l’efficacité thérapeutique d’un principe actif encapsulé dans des nanocapsules lipidiques et délivré par des cellules MIAMI au sein d’une tumeur gliale

EVALUATION DE L'EFFICACITE THERAPEUTIQUE D'UN PRINCIPE ACTIF ENCAPSULE DANS DES NANOCAPSULES LIPIDIQUES ET DELIVRE PAR DES CELLULES MIAMI AU SEIN D'UNE TUMEUR GLIALE

Lors de notre première étude, nous avons obtenu la preuve de concept d'un nouvel outil thérapeutique qui utilise la capacité migratrice des cellules MIAMI pour véhiculer des NPs dans une tumeur cérébrale. Cependant, cette stratégie est-elle efficace quand les NPs sont chargées en principe actif ?

Dans cette seconde étude, nous avons encapsulé dans des NCLs, un dérivé du tamoxifène le Fc-diOH. Dans un premier temps, nous avons contrôlé que les cellules MIAMI n'étaient pas sensibles aux NCLs de Fc-diOH, critère indispensable pour les utiliser en tant que cellules cargos. Dans un second temps, nous avons évalué la toxicité des NCLs de Fc-diOH délivrées par les cellules MIAMI *in vitro* et *in vivo* dans le modèle de gliome humain U87MG.

Ferrociphenol loaded lipid nanocapsules deliver by mesenchymal stem cells in brain tumor therapy

Mathilde Roger [a]***, Anne Clavreul*** [a, b]***, Trinh Huynh***[a]***, Catherine Passirani*** [a]***, Paul C. Schiller*** [c]***, Anne Vessières***[d]***, Claudia N. Montero-Menei*** [a]***, Philippe Menei*** [a,b]

[a] **INSERM Unit 646, Ingénierie de la Vectorisation Particulaire, 10, rue André Bocquel, 49100 Angers, Université d'Angers, France**

[b] **Département de Neurochirurgie, Centre Hospitalier Universitaire d'Angers, 4 rue Larrey, 49100 Angers, France**

[c] **Departments of Biochemistry/ molecular Biology and Medicine, University of Miami Miller School of Medicine, Miami, FL 33125-1693, USA**

[d] **CNRS-UMR 7223, ENSCP, 11, rue Pierre et Marie Curie, 75231 PARIS, Cedex 05, FRANCE**

Corresponding author:

Tel +33241735846

Fax +33241735853

E-mail address : PhMenei@chu-angers.fr

Abstract

The prognosis of patients with malignant glioma remains extremely poor despite surgery and improvements in radio- and chemo-therapies. We have previously shown that a subpopulation of mesenchymal stromal cells (MSCs), named “marrow-isolated adult multilineage inducible” (MIAMI cells), which possess migratory abilities towards brain tumor, were able to incorporate efficiently lipid nanocapsules (LNCs) without modification of their stemness properties and their migration capacity. In this study, we assessed the cytotoxic effect of MIAMI cells, loaded with LNCs containing an organometallic complex ferrociphenol (Fc-diOH), to treat brain tumor. The results showed that MIAMI cells were able to internalize Fc-diOH-LNCs and this internalization did not induced MIAMI cell death. Furthermore, Fc-diOH-LNC loaded MIAMI cells, induced a cytotoxic effect on U87MG glioma cells *in vitro*. This cytotoxic effect was validated *in vivo* after intratumoral injection of Fc-diOH-LNC loaded MIAMI cells in the heterotopic U87MG glioma model in nude mice. These promising results open up a new field of treatment combining cellular vehicles and nanoparticles to treat brain tumor.

Key words mesenchymal stromal cells, glioma, lipid nanocapsules, ferrociphenol

1. Introduction

Malignant glioma have a poor prognosis despite aggressive treatment using surgery, radiotherapy and chemotherapy (1, 2). The difficulty with cancer treatments, especially with brain tumors, is to achieve an effective delivery of therapeutic agents to the tumor site and to the infiltrating tumor cells. Many promising treatments involve nanoparticle (NP) systems for brain tumor therapy (3-7). These systems protect the therapeutic agent from a premature degradation and allow its sustained and controlled release. Several studies showed the interest of NPs for drug delivery to glioma (8-10). Among these systems, lipid nanocapsules (LNCs) which are developed in our laboratory (11) represent a promising tool. LNCs are prepared with FDA-approved constituents and by a solvent-free and low energy process (12). LNCs provide considerable drug encapsulation capacity and also exhibit sustained-release functions at the site of action (4). However, new paradigms allowing their tumor specific targeting and their extensive intratumoral distribution must be developed. Among them, an alternative to enhance NP delivery in brain tumor is the use of cellular vectors that have endogenous tumor-homing activity and can thereby chaperone NP delivery *in vivo*. In this regard, mesenchymal stromal cells (MSCs) which have a tendency to distribute at the site of tumors could be potential candidates (13, 14).

A subpopulation of human MSCs named ”marrow-isolated adult multilineage inducible” (MIAMI) cells, have been isolated and amplified using particular culture conditions as low oxygen tension (3%) that mimic the MSC niche (15). These cells possess a unique molecular phenotype and are also capable of differentiating *in vitro* into cell lineages derived from all three germ layers and particularly into immature neurons (15, 16). Furthermore, MIAMI cells can be easily obtained from patients and

proliferate extensively without evidence of senescence or loss of differentiation potential and thus may represent an ideal candidate for cellular therapies (17).

Recently, we obtained the proof of concept that MIAMI cells can be used as LNC delivery vehicles (18). The results showed that these NPs can be efficiently internalized into MIAMI cells while cell viability and differentiation were not affected. Furthermore, these LNC-loaded cells were able to migrate toward the U87MG experimental human glioma model.

In the current study, we evaluated the cytotoxic effect of MIAMI cells carrying drug-loaded LNCs in a glioma heterotopic model. In particular, 1,1-di(4-hydroxyphenyl)-2-ferrocenylbut-1-ene, also called ferrociphenol (Fc-diOH; Figure 1), an organometallic complex analog of 4-hydroxy-tamoxifen was encapsulated in LNCs. It has been shown that the modification of polyphenols with a ferrocenyl group can dramatically enhance their cytotoxicity. Indeed, the Fc-diOH cytotoxicity was shown on breast tumor cell lines (19, 20) and melanoma cell lines (21). Furthermore, LNCs loaded with Fc-diOH were tested on 9L rat glioma cell lines and revealed a specific toxicity on glioma cells but not on astrocytes (22, 23). In these studies, this compound showed high levels of *in vitro* antiproliferative activity associated with weak IC_{50} (22, 24).

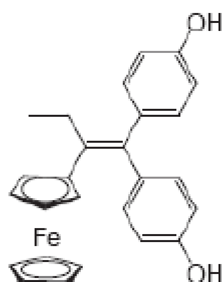


Figure 1: Chemical formulae of Fc-diOH molecule, 2-ferrocenyl-1,1-bis(4-hydroxyphenyl)-but-1-ene (MMr= 424.4 g mol−1).

2. Materials and Methods

2.1. NP materials

Fc-diOH was prepared by McMurry coupling (25). The lipophilic Labrafac® CC (caprylic-capric acid triglycerides) was kindly provided by Gattefosse S.A. (Saint-Priest, France). Lipoïd® S75-3 (soybean lecithin at 69% of phosphatidylcholine) and Solutol® HS15 (a mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate) were a gift from Lipoïd Gmbh and BASF (Ludwigshafen, Germany), respectively. NaCl was obtained from Prolabo (Fontenay-sous Bois, France). Deionised water was obtained from a Milli-Q plus system (Millipore, Paris, France).

2.2. LNC formulations

LNCs were prepared according to the phase-inversion temperature method (11). Briefly, Solutol® HS15 (9.7% w/w), Lipoid® (1.5% w/w), Labrafac® (24.2% w/w), NaCl (1.8% w/w) and water (62.8% w/w) were mixed and heated under magnetic stirring up to 95°C. Three cycles of progressive heating and cooling between 95°C and 60°C were then carried out and followed by an irreversible shock induced by dilution with 12.5 mL of 2°C deionised water added to the mixture at 80–85°C. Finally, LNCs were sterilizated by 0.2 µm filtration. To formulate Fc-diOH-LNCs, Fc-diOH was dissolved in Labrafac® (40 mg/mL) under sonication before formulation (22).

2.3. LNC characterization

2.3.1. Particle size and surface charge

LNCs were characterized for size, polydispersity index and charge distribution by dynamic light scattering using a Zetasizer® Nano Series DTS 1060 (Malvern

Instruments S.A., Worcestershire, UK). LNCs were diluted 1:60 (v/v) in deionised water in order to ensure a convenient scattered intensity on the detector.

2.3.2 Drug loading

Because of the orange color of the anticancer drug, the Fc-diOH payload was determined by spectrophotometry at 450 nm after dissolving LNCs in solvent mixtures. Drug loading was calculated as previously described (22). Briefly, a part of the formulation of each batch was filtrated using a Minisart® 0.1 µm filter (Sartorius). Three samples of each batch of Fc-diOH-loaded nanocarriers (filtrated and non-filtrated) were prepared by dissolving 250 mg of nanocarriers in 2.25 ml of 22/67/11 (v/v/v) acetone/THF/water solution. Quantification was achieved by comparing the absorbency of ferrocenyl derivative samples to a calibration curve made with blank nanocarriers and Fc-diOH ethanol/THF/water solution. Mean drug payload was calculated in milligrams of drug per milliliter of LNC dispersion.

2.4. Cell culture

MIAMI cells were isolated by differential adhesion of iliac crest aspirates of a human post-mortem organ donor (protocol agreed by the French Agency of Biomedecine) and cultured *in vitro* following the conditions described by D'Ippolito and its collaborators (15). Briefly, cells were plated at a density of 10^5 cells/cm^2 in DMEM-low glucose media (DMEM-LG, Gibco, Cergy Pontoise, France), containing 3% fetal bovine serum (FBS, Hyclone, PerbioScience, Bredières, France) and antibiotics (Sigma-Aldrich, St. Quentin Fallavier, France) on a fibronectin (FN, 10 ng/mL, Sigma) substrate, under low oxygen conditions (3% O_2, 5% CO_2 and 92% N_2). Fourteen days after the initial plating, non-adherent cells were removed. Pooled colonies of

adherent cells were selected and plated at low density for expansion (100 cells/cm²) on a FN substrate. Cells were expanded in DMEM-LG with 3% FBS at low density, <40% confluency in a humidified incubator at 37°C in an atmosphere of 3% O_2/5% CO_2/92% N_2. These cells are negative for hematopoietic lineage markers (CD45, CD34) while they are positive for CD13, CD29, CD44, CD73, CD90, CD105, CD140b, CD164 and CD166. They are also capable of differentiating *in vitro* in adipocytes and osteoblasts.

The human glioma cell line U87MG was provided by ATCC (LGC Promochem, Molsheim, France). Cells were maintained in minimum essential medium (Eagle) with Earls's BSS (Lonza, Verviers, Belgium) containing 10% FBS (Lonza), 0.1 mM non-essential amino acids (Lonza), 1.5 g/L sodium bicarbonate (Lonza), 1 mM sodium pyruvate (Lonza) and 1% antibiotics (Sigma-Aldrich) in humidified incubator gassed with 5% CO_2 (37°C) until reaching 80% confluency.

2.5. Cell viability

In vitro cell viability experiments were performed as follows. MIAMI or U87MG cells were plated in 24-well plates at 2.5 x 10^4 cell/mL. After 48 h, 0-700 µL/mL of blank LNCs or Fc-diOH LNCs in HBSS (Lonza) were added for 1 h at 37°C. Cells were washed in DPBS (Lonza) and culture medium specific for each cellular type was added. After three days, cells were incubated with fresh HBSS containing 100 µl of combined MTS/PMS solution (CellTiter 96® AQ_{ueous} non-radioactive cell proliferation assay kit, Promega, Charbonnieres, France) for 3 h at 37°C. The soluble formazan generated by the live cells was proportional to the number of live cells and the absorbance at 490 nm was recorded using a microplate reader (Multiskan Ascent, Labsystem).

2.6. *In vitro* and *in vivo* toxicity of Fc-diOH LNC-loaded MIAMI cells on U87MG cells

MIAMI cells were incubated 1 h with 700 µL/mL of Fc-diOH LNCs in HBSS which was equivalent to 1.84 mg/mL of Fc-diOH.

In vitro toxicity of Fc-diOH LNC-loaded MIAMI cells on U87MG cells was performed by coculture experiments using 0.4 µm pore size Transwell plates (Millipore, Guyancourt, France). U87MG cells (2.5×10^4 cells) were filled into the lower compartment. After 48 h, 2.5×10^4, $1{,}25 \times 10^5$ or 2.5×10^5 MIAMI cells loaded or not with Fc-diOH-LNCs were added to the upper compartment. Three days later, inserts were removed and a MTS test was performed as previously described.

In vivo toxicity of Fc-diOH LNC-loaded MIAMI cells on U87MG cells was performed in a heterotopic glioma model. Female athymic Swiss nude mice, ranging in age from 8-9 weeks were obtained from Charles River (L'Arbresle, France). The experiments were conducted according to the French Minister of Agriculture and the European Communities Council Directive of 24 November 1986 (86/609/EEC). For tumor growth analysis, animals received subcutaneous injections (s.c.) of 0.5×10^6 U87MG cells into the right thigh. The length and width of each tumor were regularly measured using a digital caliper, and tumor volume was calculated as follows: $(\pi/6) \times width^2 (l) \times length (L)$. When tumor volume reached 500 ± 50 mm^3 mice implanted with U87MG cells were treated by an intratumorale injection (100 µl) of the following treatments: HBSS saline solution ($n = 10$), 0.5×10^6 MIAMI cells ($n = 10$) and Fc-diOH LNC-loaded MIAMI cells ($n = 10$). Tumor volume was calculated every day. At day 15 after the second injection mice were sacrificed and the weight of each tumor was evaluated.

2.8. Statistical analysis

Results are given as mean ± standard error of mean (SEM). To describe statistical differences with controls, the Dunnet test was used. For tumor growth analysis a two way ANOVA was tested to compare curve profiles. Differences were considered statistically significant for $P < 0.05$.

3. Results

3.1. Fc-diOH LNC characterization

Table 1: Physicochemical characteristics of blank LNCs and Fc-diOH-LNCs (mean of three samples ± SEM)

	Mean particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)	Drug loading (mg/mL of suspension)
Blank LNCs	95.09 ± 1.58	0.0615 ± 0.0005	-5.135 ± 0.875	-
Fc-diOH-LNCs	75.64 ± 2.28	0.0290 ± 0.0066	-7.256 ± 2.121	2.62 ± 0.06

Particle size, polydispersity index and zeta potential of blanks LNCs and Fc-diOH-LNCs are presented in Table 1. NPs formulated by the phase-inversion temperature method have a mean diameter of 95.09 ± 1.58 nm and 75.64 ± 2.28 nm for blank NPs and Fc-diOH-loaded NPs, respectively, with a polydispersity index of 0.0615 and 0.0290. In both cases, the zeta potential was negative, -5.135 ± 0.875 mV and -7.256 ± 2.121 mV for blank and loaded LNCs, respectively. The drug loading of Fc-diOH LNCs was 2.62 ± 0.06 mg/mL of LNC suspension.

3.2. *In vitro* survival of MIAMI cells and U87MG cells after Fc-diOH-LNC internalization

Three days after uptake of Fc-diOH-LNCs, less than 65% of U87MG cells survived whatever the concentration used. On the contrary, MIAMI cells were not sensitive to Fc-diOH-LNC uptake (Figure 2). Blank LNCs were not toxic, except for high concentration (700µL/mL).

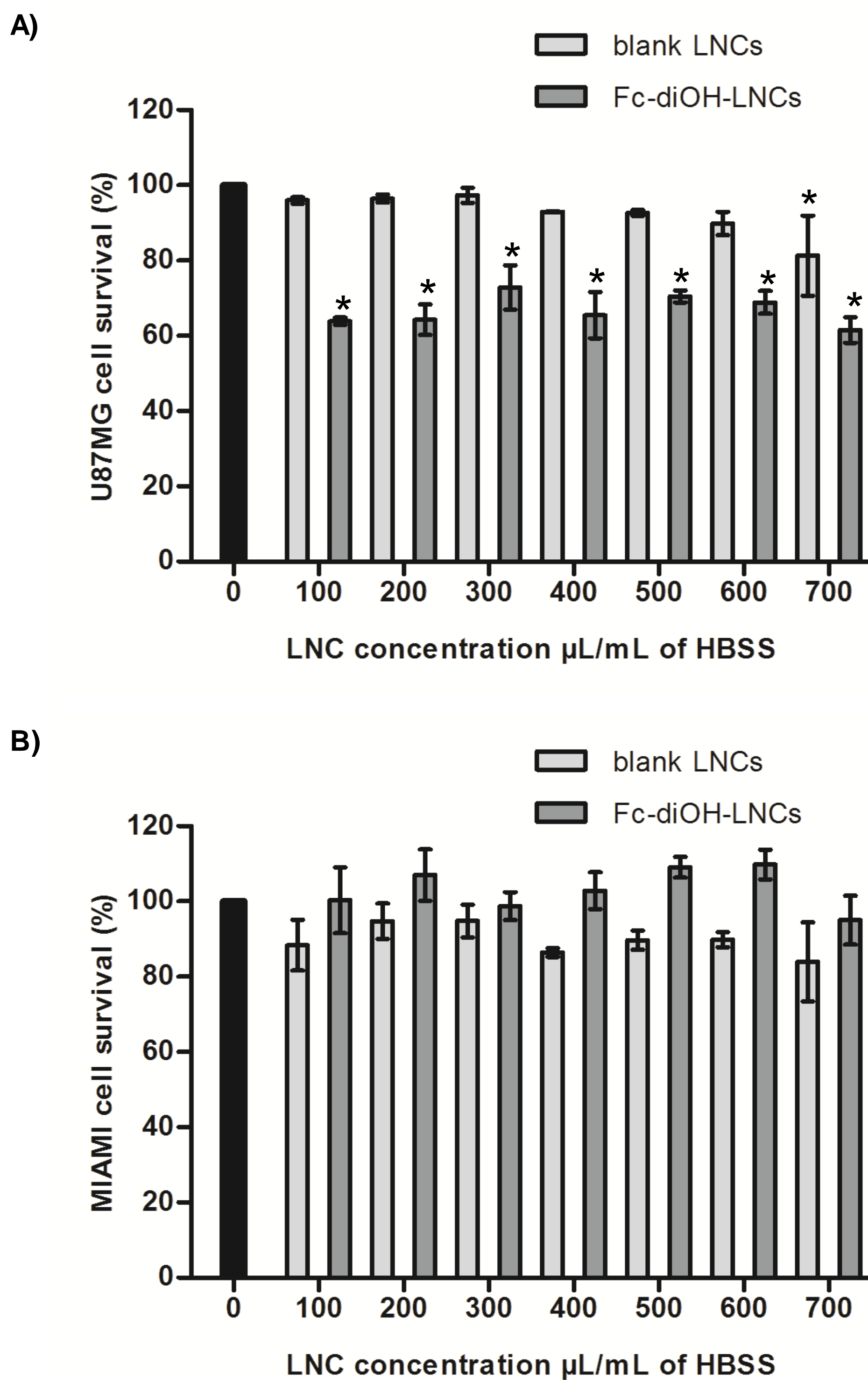
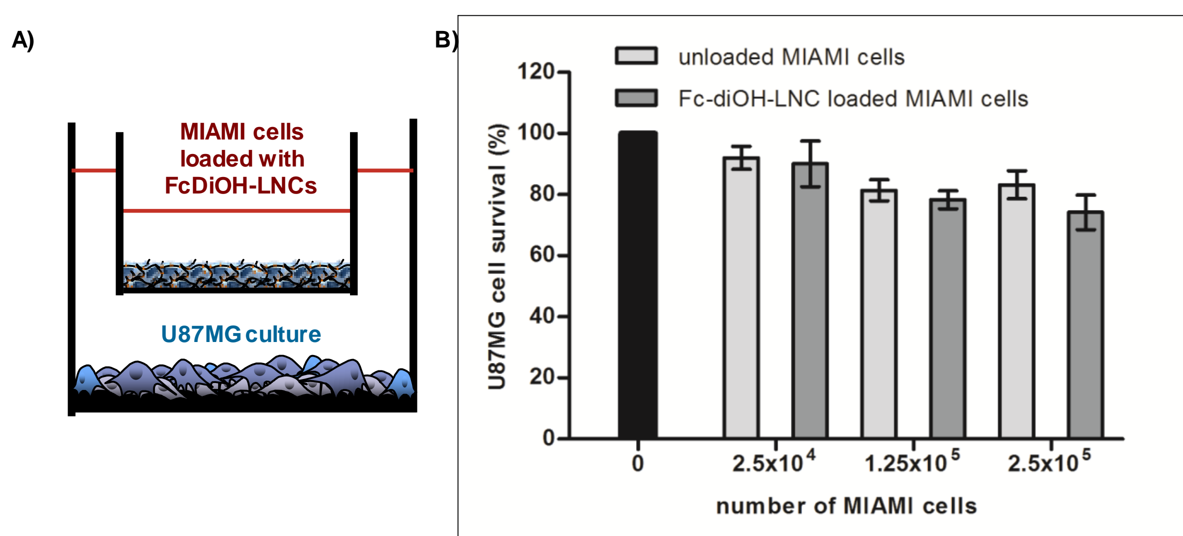


Figure 2: Cell viability analysis of U87MG cells (A) and MIAMI cells (B) three days after incorporation of various concentrations of blank LNCs and Fc-diOH LNCs. Cell survival was expressed in percentage by comparison with survival of cells without NPs, considered as 100%. Data are expressed as the mean of four wells ± SEM (n=3) (**P* < 0.05, versus control without NP).

3.3. *In vitro* toxicity of Fc-diOH LNC-loaded MIAMI cells on U87MG cells

After three days of coculture, Fc-diOH LNC-loaded MIAMI cells induced a significant dose-dependent inhibition of the proliferation of U87MG cells (Figure 3). Unloaded MIAMI cells also induced a cell growth inhibition but this effect was not dose-dependent (Figure 3).



<u>Figure 3</u> : A) Schematic principle of coculture experiments to assess the toxicity of Fc-diOH LNCs deliver by MIAMI cells on U87MG culture. The size insert -0.4µm- allows the passage of LNCs.

B) Cell viability analysis of U87MG glioma cells three days after coculture with MIAMI cells unloaded or loaded with Fc-diOH LNCs. Three doses of MIAMI cells were tested. Cell survival was expressed in percentage by comparison with U87MG cell survival without MIAMI cells, considered as 100%. Data are expressed as the mean of four wells ±SEM (n=3) (*$P < 0.05$, versus control without MIAMI cells).

3.4. *In vivo* tumor growth effect of Fc-diOH LNC-loaded MIAMI cells

The U87MG heterotopic (s.c). glioma model was used to evaluate the efficacy of Fc-diOH LNC-loaded MIAMI cells. After tumors had reached 500 ± 50 mm^3, we performed comparative efficacy studies by dividing animals into three groups according to the treatment they received: the control groups received HBSS solution or unloaded MIAMI cells and the treated group received Fc-diOH LNC-loaded MIAMI

cells (Figure 4A). Intratumoral injection of Fc-diOH LNC-loaded MIAMI cells resulted in significantly stronger tumor volume reduction that the injection of HBSS or unloaded MIAMI cells (Figure 4A). Analysis of the tumor mass at the end of the experiment confirmed these results (Figure 4B).

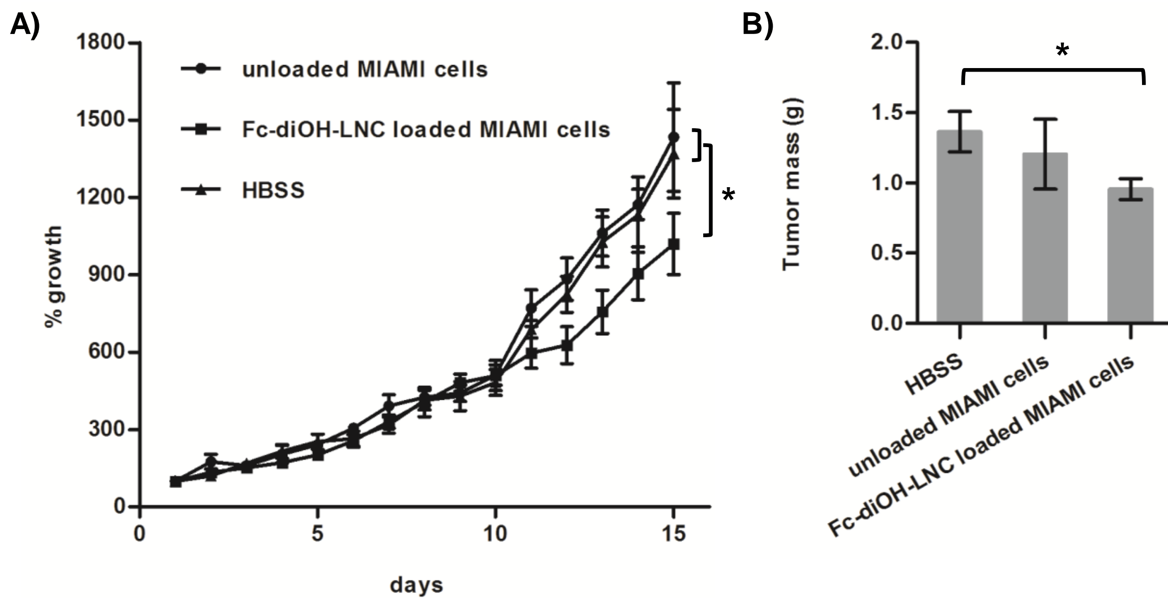


Figure 4: *In vivo* effects of Fc-diOH LNC-loaded MIAMI cell on the growth of the heterotopic U87MG glioma model. (A) was an estimation of tumor growth after treatment injection, in % calculated by tumor size measurements ± SEM. (B) represents values of tumor mass weighted at Day 15 ± SEM. * $P < 0.05$

4. Discussion

In recent years, significant efforts have been devoted to develop nanocarrier systems to improve drug or gene delivery for brain tumor treatments (3-7). MSCs which have been shown to distribute at the site of tumor, could be potential candidates (13, 14) to allow tumor specific targeting and extensive intratumoral distribution. Indeed, the combination of NPs and stem cells has been already used. Recently, we obtained the proof of concept that a subpopulation of MSCs, MIAMI cells, can be used as NP delivery vehicles. MIAMI cells were able to internalize efficiently PLA NPs and LNCs without affecting cell viability.

In the current study, we determined the cytotoxicity of MIAMI cells loaded with Fc-diOH LNCs on the U87MG glioma cell line *in vitro* and *in vivo*. Fc-diOH is one of the most active molecules of a new class of organometallic drugs, showing *in vitro* anti-proliferative effects on breast cell lines and the 9L glioma cell line (19, 22). As Fc-diOH is very hydrophobic, this compound was encapsulated in LNCs (22). Fc-diOH LNCs showed low toxicity levels when in contact with healthy cells but the cytostatic activity was very effective on rat 9L glioma cells *in vitro* and *in vivo* (22, 23, 26).

In this study, we also found that Fc-diOH LNCs were toxic on the human U87MG cells and no cytostatic activity was found on MIAMI cells. This result confirmed that Fc-diOH has no cytostatic activity with healthy cells, conferring a functional specificity of this compound on tumor cells. To determine if Fc-diOH LNC-loaded MIAMI cells had a cytotoxic effect on U87MG cells, Transwell membrane inserts with 0.4 µm pore size were used to separate MIAMI cells from U87MG cells and to allow the diffusion of Fc-diOH LNCs. We observed that Fc-diOH LNC-loaded MIAMI cells were able to decrease the viability of U87MG cells in a dose dependent

manner. This result suggests that MIAMI cells are able to deliver Fc-diOH or Fc-diOH LNCs in the medium. A reduction of the number of U87MG cells was also observed with unloaded MIAMI cells but less strongly than Fc-diOH LNC-loaded MIAMI cells. However, this inhibitory effect observed *in vitro* was not confirmed *in vivo*. MSCs have been already reported to have inhibitory effects on tumor cells (27, 28) but other groups also reported that MSCs could enhance the tumor growth (29-31) or did not affect the tumor growth (32-35).

The cytotoxic effect of Fc-diOH LNC-loaded MIAMI cells was validated *in vivo* in the heterotopic U87MG glioma model. Intratumoral injection of Fc-diOH LNC-loaded MIAMI cells slowed down significantly the glioma growth in comparison with intratumoral injection of HBSS or unloaded MIAMI cells. In our study, we did not compared the effect of Fc-diOH LNC-loaded-MIAMI cells with Fc-diOH-LNCs alone because we did not know the exact dose of Fc-diOH-LNCs internalized in MIAMI cells. The efficiency of Fc-diOH-LNCs had already been tested in another heteroptopic glioma model,the 9L glioma and similar results to ours had been obtained. Indeed, the tumor growth slowed down without tumor regression. However, in our study, the dose of Fc-diOH injected may be slower. Future studies are need to determine the dose of the drug loaded in a MIAMI cell to know the therapeutic index of this new NP delivery system (22). The precise mechanism underlying the Fc-diOH-LNC cytotoxicity is not fully understood and is still discussed. Vessieres et al. (20) suggested that organometallic ferrocifens induce a significant ROS production in tumor cell lines associated with cell cycle arrest and senescence which can explain why we did not observe tumor regression.

Altogether, these results showed that MIAMI cells were able to deliver drug-loaded LNCs within a tumor mass. The efficacy of this strategy in an orthotopic

glioma model should be confirmed. In addition, this strategy could also be evaluated in combination with external radiotherapy (23). Indeed, it has been shown that local irradiation might promote specificity of migration and engraftment of MSCs (36) and that the Fc-diOH action could be potentiated by external radiotherapy(23).

5. Conclusion

The combination of NPs and stem cells has been already used to deliver growth factors to enhance angiogenesis (VEGF) (37) and to induce osteogenic (bFGF, BMP-2, BMP-2/BMP-7) or chondrogenic differentiation (TGF-beta1, THF-beta3) of stem cells (38-44). We demonstrated for the first time in this study, that MSCs can also be used to deliver therapeutic agent-loaded NPs in a tumor mass. The efficacy of this approach was showed both *in vitro* and *in vivo*. To resume, the combination of cellular carriers and NPs promises new treatments of malignant gliomas.

Acknowledgments

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CHAPITRE 3 :

Caractérisation des interactions entre les cellules MIAMI et les cellules de gliome, étude *in vitro* et *in vivo*

CARACTERISATION DES INTERACTIONS ENTRE LES CELLULES MIAMI ET LES CELLULES GLIALES, ETUDE *IN VITRO* ET *IN VIVO*

Nous avons donc validé l'utilisation des cellules MIAMI pour véhiculer des NPs chargées en principe actif dans un contexte thérapeutique. Cependant, plusieurs études soulignent que les CSMs pourraient promouvoir la croissance tumorale limitant leur utilisation en clinique. Qu'en est-il pour les cellules MIAMI ?

Dans cette dernière étude, nous avons analysé l'interaction des cellules MIAMI avec les cellules gliales tumorales *in vitro* et *in vivo*. Cette étude est essentielle pour affirmer la sureté de ce vecteur cellulaire. Ainsi, nous avons analysé l'effet des cellules MIAMI sur la prolifération de plusieurs lignées de gliomes humains en réalisant des cocultures *in vitro*. Cette analyse a été également réalisée *in vivo* dans deux modèles orthotopiques de gliomes humains (U87MG et LAB1) chez la souris nude. De plus, nous avons étudié le devenir des cellules MIAMI dans un environnement de tumeur cérébrale. En particulier, nous avons analysé leur capacité de différenciation en cellules endothéliales, en péricytes, en astrocytes et en CAFs ainsi que leur capacité de prolifération.

In vitro and _in vivo_ interactions between Marrow-Isolated Adult Multilineage Inducibe (MIAMI) cells and glioma cells

Mathilde Roger [a], Anne Clavreul [a, b], , Laurence Sindji [a], Adrianna Gomez [c], Agnès Chassevent [d], Paul C. Schiller [c,e], Claudia N. Montero-Menei [a], Philippe Menei [a,b]

[a]INSERM Unit 646, Ingénierie de la Vectorisation Particulaire, 10 rue André Bocquel, Université d'Angers, 49100 Angers, France

[b]Département de Neurochirurgie, Centre Hospitalier Universitaire d'Angers, 4 rue Larrey, 49100 Angers, France

[c]Geriatric Research, Education and Clinical Center (GRECC) and Research Service, VA Medical Center, Miami, FL 33125-1693, USA

[d] Centre Paul Papin, CRLCC, 2, rue Moll, 49933 Angers, France

[e]Departments of Medicine and Biochemistry & Molecular Biology, and Geriatrics and Interdisciplinary Stem Cell Institute,s University of Miami Miller School of Medicine, Miami, FL 33125-1693, USA

Corresponding author

Tel +33241354822

Fax +33241735853

E-mail address: Ph.Menei@chu-angers.fr

Abstract

The prognosis of patients with malignant glioma remains extremely poor, despite surgery and improvements in radio- and chemo-therapies. Recently, we showed that marrow-isolated adult mutilineage inducible (MIAMI) cells, a subpopulation of human mesenchymal stem cells (MSCs), can serve as cellular carriers for nanoparticles in brain tumors. However, for a clinical purpose, the safety of the culture of MIAMI cells and their interaction with glioma cells must be evaluated. In this study, we characterized MIAMI cells extracted from iliac crest. Furthermore, we analyzed their glioma migration, their effect on the proliferation of different glioma cells lines and their fate in a brain tumor environment. Results showed that MIAMI cells can be isolated and expanded safely. Furthermore, these cells were able to migrate specifically toward the orthotopic U87MG glioma model and did not influence its growth. In this model, MIAMI cells did not give rise to endothelial-like, pericyte-like, CAF-like nor astrocyte-like cells but a fraction of MIAMI cells was found in a proliferate state. Nevertheless, these proliferating MIAMI cells did not influence the survival of U87MG bearing mice. Despite these positive results, it is important to highlight that the effects of MIAMI cells may be glioma dependant. MIAMI cells were not found to migrate in the orthotopic Lab1 glioma model and can induce the proliferation of other glioma cell lines. Supplementary studies are need before the therapeutic exploitation of MIAMI cells in the context of glioma conditions.

Key words mesenchymal stromal cells, glioma, cancer associated fibroblasts, tumor microenvironment

Introduction

Glioblastoma (GB) is a highly infiltrative tumor with a recurrence rate of 90% in the first few centimeters of the resection cavity, even in cases of complete tumor resection and adjuvant chemo/radiotherapy (1-3). After optimal treatment, median survival is around 14 months and less than 10% of patients survive more than five years (4).

The failure of existing treatment is partly due to the inability to target tumor cells infiltrating the brain tissues surrounding the tumor. The discovery of the special tropism of neural stem cells (NSCs) and adult multipotent mesenchymal stromal cells (MSCs) for brain tumors opens up a novel area of research. These cells distribute throughout the primary tumor bed and migrate toward the widely outgrowing tumor microsatellites after intratumoral implantation. Moreover, when NSCs or MSCs are implanted intracranially at sites distant from the tumor, they migrate through the normal parenchyma and localize within the tumor in the peri-tumoral zone (5-7). Furthermore, recent studies, indicate that NSCs and MSCs may be located within the vascular niche where brain tumor stem-like cells (BT-SCs) also reside (8-11). This behavior of NSCs and MSCs has been exploited as a tumor-targeting strategy for glioma gene therapy. Strong antitumor effects have been reported following intracranial administration of gene-modified NSCs or MSCs expressing immunostimulator or toxic molecules in experimental glioma models (12-22). However, the clinical application of NSCs will be limited undoubtedly by logistic and ethical problems associated with their isolation and by potential immunologic incompatibility due to the requirement for allogenic transplantation. On the contrary, MSCs have the advantage of being easily isolated then propagated *in vitro* and the

implantation of autologous MSCs into patients with malignant glioma is ethically unproblematic.

A unique subpopulation of non-transformed non-immortalized adult human MSCs named marrow-isolated adult multilineage inducible (MIAMI) cells have been isolated from vertebral aspirates, using particular culture conditions as low oxygen tension (3%) to mimic the *in vivo* niche (23). MIAMI cells are a homogenous population capable of differentiating into cell lineages derived from all three germ layers and particularly into immature neurons exhibiting neuronal ionic channel activity *in vitro* (24). We recently showed that pre-treatment of MIAMI cells with epidermal growth factor and basic fibroblast growth factor enhanced their neural specification and the response to neuronal commitment *in vitro* (25). These cells, which can be easily obtained from patients, may represent an ideal candidate for cellular therapies. Recently, we showed that MIAMI cells isolated from iliac crest aspirates can serve as cellular carriers for nanoparticles (NPs) in brain tumors (26). NPs can carry multiple therapeutic agents such as drugs (27-29), radionucleides (30) or DNA (31, 32), hence MIAMI cells carrying NPs constitute an appealing therapeutic strategy for brain tumors. However, several studies underline that caution should be used in the therapeutic exploitation of MSCs in the context of malignant conditions (33). In fact, MSCs could participate in tumor growth and metastasis, either by providing the niche of BT-SCs, by impairing immune surveillance or by differentiation into cancer-associated fibroblast (CAF)-like cells (33). In order to use MIAMI cells in tumor-targeted therapy, it is therefore warranted to study their interaction with glioma cells.

In the current study, we characterized MIAMI cells isolated from fresh and frozen iliac crest aspirates of human post-mortem organ donors aged between 65 and 68 years. We furthermore studied their migratory behaviour after implantation using the U87MG

and Lab1 experimental glioma models, and evaluated their interaction with different glioma cell lines *in vitro* and *in vivo*. In particular, we assessed their effect on glioma proliferation and their fate in a brain tumor environment.

Materials and Methods

Cell culture

MIAMI cells were isolated by differential adhesion of fresh or frozen iliac crest aspirates of human male post-mortem organ donors (median age = 66 years) (protocol agreed by the French Agency of Biomedecine) and cultured *in vitro* following the conditions described by D'Ippolito and its collaborators (34). Briefly, cells were plated at a density of 10^5 cells/cm^2 in DMEM-low glucose media (DMEM-LG, Gibco, Cergy Pontoise, France), containing 3% fetal bovine serum (FBS, Hyclone, PerbioScience, Bredières, France) and antibiotics (Sigma-Aldrich, St. Quentin Fallavier, France) on a fibronectin (FN, 10 ng/mL, Sigma) substrate, under low oxygen conditions (3% O_2, 5% CO_2 and 92% N_2). Fourteen days after the initial plating, non-adherent cells were removed. Pooled colonies of adherent cells were selected and plated at low density for expansion (100 cells/cm^2) on a FN substrate. Cells were expanded in DMEM-LG with 3% FBS at low density, <40% confluency in a humidified incubator at 37°C in an atmosphere of 3% O_2/5% CO_2/92% N_2.

Seven human glioma cell lines were provided by ATCC (A172, HS683, U118MG, U138MG, U87MG; LGC Promochem, Molsheim, France) and our laboratory (Lab1, Lab2; Table 1). Cells were maintained in DMEM-HG (Lonza, Verviers France) containing 10% FBS (Lonza), and 1% antibiotics (Sigma-Aldrich) in humidified incubator gassed with 5% CO_2 (37°C) until reaching 80% confluency.

Table 1: Cell line histology

Cell lines	Histology	Tumorigenicity of nude mice
A172	GBM (ATCC description)	No
HS683	Glioma (ATCC description)	No
U118MG	GBM (ATCC description)	Rejected
U138MG	GBM (ATCC description)	No
U87MG	GBM, astrocytoma (ATCC description)	Yes
Lab1	Recurent primary GBM	Yes
Lab2	Primary GBM	Not tested

Phenotypic analysis by flow cytometry

Using an indirect labeling method, MIAMI cells suspended in PBS containing 5% FCS and 0.02% NaN_3 were incubated on ice for 1 h with primary antibodies against CD34, CD45, CD13, CD29, CD44, CD49b, CD49c, CD49e, CD63, CD73, CD90, CD105, CD140b, CD164, CD166 and SSEA4 (stage-specific embryonic antigen 4), and isotype control antibodies. Antibodies were from BD Biosciences (Le Pont de Claix, France) except the antibodies against CD105, and SSEA4 which were from R/D Systems (Lille, France) and Millipore (Guyancourt, France), respectively. Following incubation, the cells were washed and further stained with fluorescein isothiocyanate (FITC)-conjugated goat $F(ab')_2$ anti-mouse immunoglobulin (Dako, Trappes, France) for 30 min on ice. After washing, the cells were fixed in 2% formaldehyde. The stained cells were analysed using a FACScan flow cytometer with CellQuest Software (BD Biosciences, Le Pont-de-Claix, France**).**

Total RNA extraction and real-time RT-PCR

Total RNA extraction. Total MIAMI cell RNA was isolated using the RNAqueous®-4PCR kit (Ambion, Austin, TX) following the manufacturer's specifications. RNA

concentrations were determined with the Nanodrop ND-1000 Spectrophotometer (Nanodrop, Wilmington, DE).

Reverse transcription. cDNA was prepared from 2 µg purified RNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA). Then, cDNA was purified using a Qiaquick PCR purification Kit (Qiagen, Valancia, CA). Purified cDNA was aliquoted and stored at -20°C.

*Real-time RT-PCR***.** The relative expression levels of genes were determined with SYBR^{TM}Green PCR Master Mix (Agilent Technologies, Santa Clara, California) and *EF1α* (Elongation Factor 1α) as housekeeping gene. Real-time RT-PCR was performed with the Mx3005P Multiplex Quantitative PCR System (Stratagene, La Jolla, CA), according to manufacturer's protocol. Triplicate reactions were carried out for each data point. Details of the primers and the GenBank accession numbers are given in Table 2. All genes transcripts were quantified by the ΔΔCt method (35).

Table 2: Primers used in the experimental studies

Gene	Full Name	Accesion Number	Primer Pair Sequence
EF1α	Elongation Factor 1α	NM_001402	F=5'-AGGTGATTATCCTGAACCATCC-3' R=5'-AAAGGTGGATAGTCTGAGAAGC-3'
SOX2	SRY(Sex Determining Region)-box	NM_003106	F=5'-CCCCGGCGGCAATAGCA-3' R=5'-TCGGCGCCGGGGAGATACAT-3'
NANOG	Nanog homeobox	NM_024865	F=5'-TTGGTGATGAAGATGTATTCGT-3' R=5'-ACAGAGCCAAAAACGGTAAG-3'
OCT4a	Octamer-binding transcription factor 4a	NM_002701	F=5'-TGGAGAAGGAGAAGCTGGAGCAAAA-3' R=5'-GGCAGATGGTCGTTTGGCTGAATA-3'
h-TERT	Human Telomerase Reverse Transcriptase	NM_198253	F=5'-TCTGGGATGCGAACGGGC-3' R=5'-TCCGGCTCAGGGGCAGC-3'
SSEA-4 synthase	Stage-Specific Embryonic Antigen-4 synthase	NM_174971	F=5'-GTGGTGCCTGGGCCTCTATG-3' R=5'-TTGGCCTGCCCTTCAACAAT-3'

In vitro differentiation

Adipogenesis.

Cells were seeded at a concentration of 1 x 10^4 cells per well in 24-well plates. Two days later, adipogenic medium containing DMEM-LG supplemented with IBMX (0.5 mM), dexamethasone (1 µM), insulin (10 µg/mL), indomethacin (100 µM) purchased from Sigma-Aldrich, 1% antibiotics, and 10% FBS, was added. The cells were grown for 3 weeks, with medium replacement twice a week. Adipogenesis was detected by oil-red O staining.

Osteogenesis.

Cells were seeded at a concentration of 2 x 10^4 cells per well in 24-well plates. The next day, osteogenic medium containing α-MEM (Lonza) supplemented with ascorbic acid (100 µM), β-glycerophosphate (10 mM), purchased from Sigma-Aldrich, dexamethasone (10 nM), 1% antibiotics, and 10% FBS, was added. The cells were grown for 3 weeks, with medium replacement twice a week. Osteogenic differentiation was detected by alizarin red staining.

In vitro proliferation assay

We assessed the effect of MIAMI cells on the proliferation of glioma cells using aneuploid glioma cell lines (A172, U118MG, U138MG, HS683, Lab1 and Lab2) to distinguish tumor cells from diploid MIAMI cells. Glioma cell lines were seeded at 10^5 per well in 6-well plates and cultured in DMEM supplemented with 10% FCS and 1% antibiotics. After 24 h, 10^5 MIAMI cells were added into the wells. The cells were co-cultured for 3 days and then harvested. Vindelov’s protocol was used to stain DNA (36) and flow cytometry was performed with a FACScan flow cytometer (BD Biosciences). DNA index (DI) and the percentage of cell populations identified on the

basis of different DNA contents were calculated with Modfit version 5.2 software (Verity Software House, Topsham, Maine). The DNA diploid peak was located on DNA histograms, according to an external standardization procedure using normal human lymphocytes and an internal standardization procedure using trout red blood cells. DI was calculated as the ratio of the mean DNA content of tumor cells to the mean DNA content of diploid cells. Cases with a DI value of 1 were classified as DNA-diploid and cases with DI values < or > 1 were classified as aneuploid. Cell cycle analysis was carried out on the aneuploid glioma cells and the proliferation index (PI) of these cells was evaluated as S+G2M%. This experiment was performed for each cell line in triplicate.

***In vivo* experiments**

Female athymic Swiss nude mice, ranging in age from 8-9 weeks were obtained from Charles River (L'Arbresle, France). The experiments were conducted according to the French Minister of Agriculture and the European Communities Council Directive of 24 November 1986 (86/609/EEC). Animals were anesthetized by an intra-peritoneal injection of xylazine (50 mg/kg body weight) and ketamine (10 mg/kg body weight) and positioned in a Kopf stereotaxic instrument.

Two glioma model were used in this study: U87MG and Lab1. Both glioma cells provided from female human donors and were inoculated at day 0 in mice striatum [coordinates: posterior to bregma (AP), -0.5 mm; lateral (L), +2 mm; deep from the outer border of the cranium (D), -3 mm]. Six days after tumor cells inoculation MIAMI cells provide from male donor or a saline solution were injected into the tumor as the same coordinate than glioma cells or in the contralateral hemisphere to the tumor mass (coordinates: AP, -0.5 mm; L, -2 mm; D, -3 mm).

To assess the effect of MIAMI cells on tumor growth, mice were implanted in the striatum at day 0 with 5×10^4 U87MG cells (n = 20) or 2×10^4 Lab1 cells (n = 14) in 5 µl HBSS with Ca^{2+} and Mg^{2+}. At day 6, 0.3×10^6 MIAMI cells in 5 µl HBSS with Ca^{2+} and Mg^{2+} or 5 µl HBSS with Ca^{2+} and Mg^{2+} (negative control) were injected in the tumor at the same coordinates. Animals were sacrificed when they lost more than 10% of weight.

To study the distribution ability of MIAMI cells in the tumor mass, mice were implanted intracranially at day 0 with 5×10^4 U87MG cells (n = 6) or 2×10^4 Lab1 cells (n = 3) as described below. At day 6, 0.3×10^6 MIAMI cells were injected in the tumor at the same coordinates. Mice were killed 7 days after MIAMI cell injection.

To study the migratory behavior of MIAMI cells toward U87MG glioma model, mice were implanted intracranially at day 0 with 5×10^4 U87MG cells (n = 6) as described below. At day 6, 0.3×10^6 MIAMI cells were injected in the contralateral hemisphere to the tumor mass (coordinates: AP, -0.5 mm; L, -2 mm; D, -3 mm). Mice were killed 3 or 7 days after MIAMI cell injection.

To analyze the fate of MIAMI cells *in vivo*, 0.3×10^6 MIAMI cells were injected in the striatum of healthy mice (n = 3) or in mice bearing an intracranial U87MG glioma (day 6 after U87MG cell injection) (n = 6) (coordinates: AP, -0.5 mm; L, +2 mm; D, -3 mm). Mice were killed 7 days after MIAMI cell injection.

Mice brains were snap-frozen in isopentane cooled by liquid nitrogen and stored at -80°C. Brain coronal sections were cut at 10 µm and collected onto silanized slides.

Fluorescent in situ hybridization

MIAMI cells were detected by the fluorescence in situ hybridization (FISH) technique using a Y-chromosome probe. The DNA probe is complementary to the highly

repetitive human satellite III sequences close to the centromeric region of the human Y-chromosome DYZ1 locus (CEPY) and labeled with the fluorochrome SpectrumOrange (Vysis, Abbott Molecular, Rungis, France). Frozen sections were air-dried, rehydrated in DPBS and fixed 10 min in 4% PFA pH 7.4 at 4°C. Nuclei were denatured for 5 min using 70% Formamide (Sigma-Aldrich)/2x standard citrate saline (SSC, Vysis) at 73°C and dehydrated next in gradient alcohol bathes (EtOH 70%, 80%, 90% and 100%), 2 min each. Y-chromosome probe was diluted with hybridization buffer according to manufacturer instructions and dropped on slides, covered with coverslip and sealed with rubber cement. The hybridization was performed in 3 steps: 10 min at 73°C to denature the DNA and the probe, 2 min at 4°C to fix the denaturation and overnight in a humid chamber at 45°C to hybridize DNA with the probe. After 3 washes for 10 min in 50% formamide/2X SSC baths and 5 min in 2X SSC/0.1%NP-40, nuclei were counterstained in DAPI II with antifade (Vysis).

Immunofluorescence

Briefly, cryo-sections were air-dried, rehydrated in PBS and fixed 10 minutes in 4% Paraformaldehyde. In order to block nonspecific binding, sections were incubated in PBS containing 4% BSA and 10% normal goat serum. Incubations with isotype controls and primary antibodies against endothelial cells (human and mouse CD31, BD Biosciences), astrocytes (GFAP, glial fibrillary acidic protein, BD Biosciences), pericytes (desmin, Dako), CAFs (α-SMA, alpha-smooth muscle actin, R&D Systems, Lille, France and vimentin, Dako), and proliferative cells (Ki-67, Dako) were performed overnight at 4°. Primary antibodies were detected using biotinylated secondary antibody and amplified using streptavidine-FITC (Dako). After washes,

MIAMI cells were detected by the FISH technique using a Y-chromosome probe as described above.

Image analysis

Brain cryo-sections from 3 mice injected intracerebrally with MIAMI cells and 3 U87MG glioma-bearing mice injected intratumorally with MIAMI cells were analyzed on a fluorescence microscope (Axioscope® 2 optical, Zeiss, Le Pecq, germany) and a confocal microscope (LSM 700, Zeiss). Quantification of labeled MIAMI cells (Y^+) and double labeled MIAMI cells (Y^+/Ki-67^+, Y^+/α-SMA^+, Y^+/$vimentin^+$, Y^+/$desmin^+$, Y^+/$GFAP^+$ and Y^+/$CD31^+$) was performed using a computerized image analysis system (MetaView, Roper Scientific, Evry, France). Results were expressed as the mean percentage of double labeling MIAMI cells of a representative slice in 3 animals ± standard error of the mean (SEM).

Statistical analysis

Results are given as mean ± SEM. To describe statistical differences, Dunnet and Student's *t* test were used. The Kaplan-Meier method was used to plot animal survival. Statistical significance was calculated using the log-rank test (Mantel-Cox Test). Differences were considered statistically significant for $P < 0.05$.

Results

MIAMI cell characterization

Using FACS analysis (Figure1A), the expression of surface markers known to be associated with MSCs were analyzed on MIAMI cells isolated from fresh or frozen iliac crest aspirates from post-mortem donnors. MIAMI cells are negative for hematopoietic lineage CD45 and CD34 markers while they are positive for CD13, CD29, CD44, CD49b, c and d, CD63, CD73, CD90, CD105, CD140b, CD164 and CD166. No difference in marker expression was observed between MIAMI cells isolated from fresh and frozen iliac crest aspirates (data not shown). Moreover, MIAMI cells expressed transcripts for embryonic stem cell markers involved in pluripotency such as *Oct-4a*, *nanog* and *SSEA-4 synthase* (Figure 1B) and a weak expression of *h-TERT*. *SOX2* expression was not detected in MIAMI cells. MIAMI cells produced and mineralized an extracellular matrix, and made bone nodules *in vitro* after osteoblastic differentiation and showed adipose tissue-forming capacity and accumulated large amounts of triglycerides in their cytoplasm under adipoblastic condition (Figure1C).

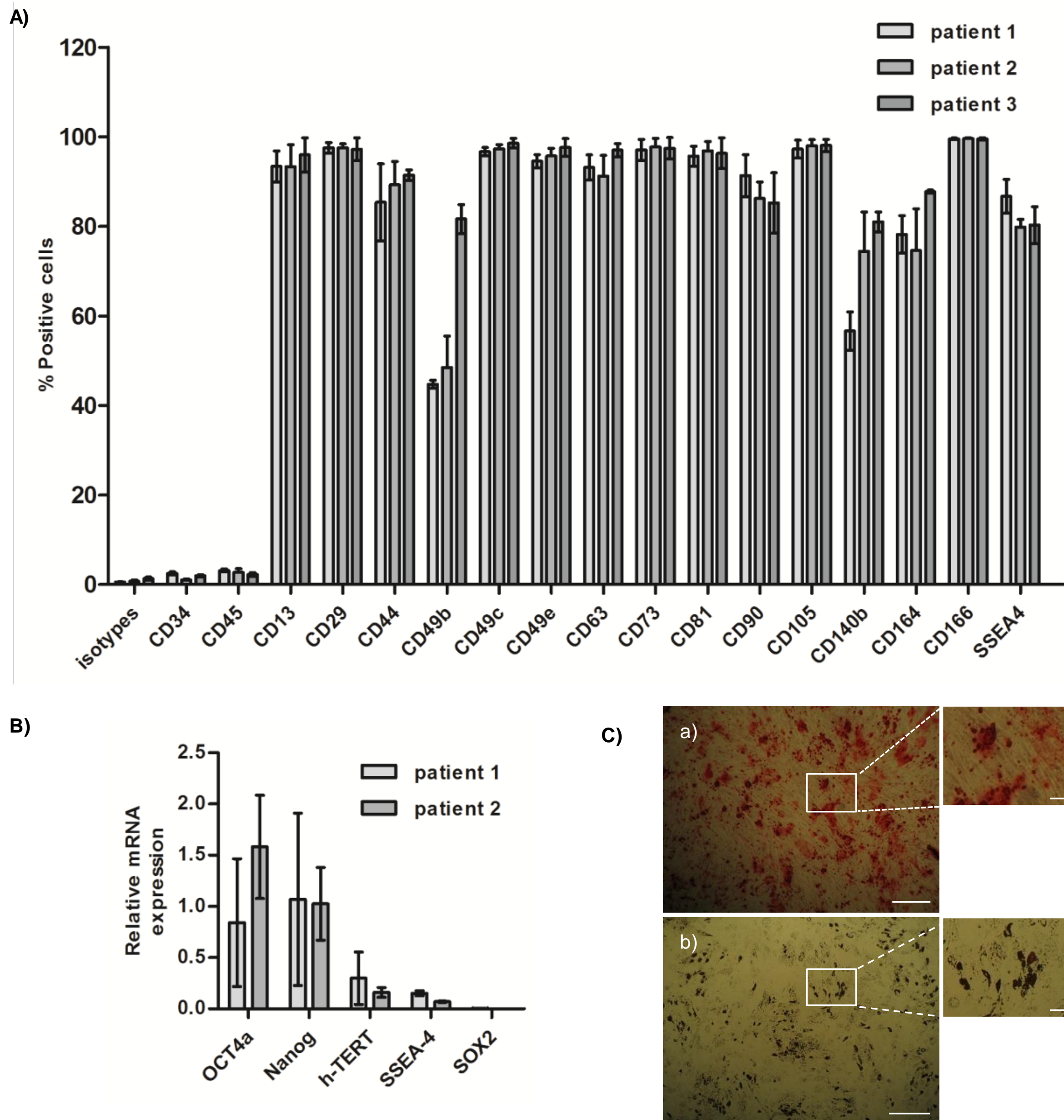

Figure 1 : A) FACS analysis expression of surface markers of MIAMI cells isolated from fresh iliac crest aspirates of three human post-mortem organ donors. Data are expressed as the mean of % of positive cells ±SEM of three independent experiments (median age = 66 years).

B) mRNA expression profile of *OCT-4a*, *Nanog*, *h-TERT*, *SSEA-4 synthase* and *SOX2* in MIAMI cells from 2 patients. Total RNA was reverse transcribed into cDNA, and real-time RT-PCR was performed using specific primers. The conversion of Ct values in relative quantities was performed with the delta-Ct method (see 'Materials and Methods'). Data are presented as the mean ± SEM of three independent experiments (mRNA expression was considered negative when the Ct score was > 33 cycles).

C) MIAMI cell differentiation into osteoblasts (a) and adipocytes (b). Alizarin Red S staining was used to visualize mineral opposites at day 21 (a). Oil Red O staining was used to visualize lipid vacuoles in cells cultured under adipogenic conditions at day 21 (b). (Scale bar = 500 μm and 200 μm for magnifications)

Glioma model and migratory behavior of MIAMI cells

MIAMI cells derived from a male donor were injected intratumorally six days after the injection of U87MG and Lab1 glioma cells from female donors in the striatum of female nude mice. The migratory behavior of MIAMI cells toward glioma cells was detected by FISH for the Y chromosome at day 7 (Figure 2A). The results showed that in the U87MG glioma, MIAMI cells were largely distributed at the border zone between tumor and normal parenchyma and followed tumor infiltrates (Figure 2B). No Y chromosome-positive MIAMI cells were observed in the opposite hemisphere (data not shown). On the contrary, after injection in the Lab1 model, MIAMI cells did not distribute themselves in the tumor mass and remained at the injection site (Figure 2C). These result indicated a specific distribution of MIAMI cells within the U87MG glioma model.

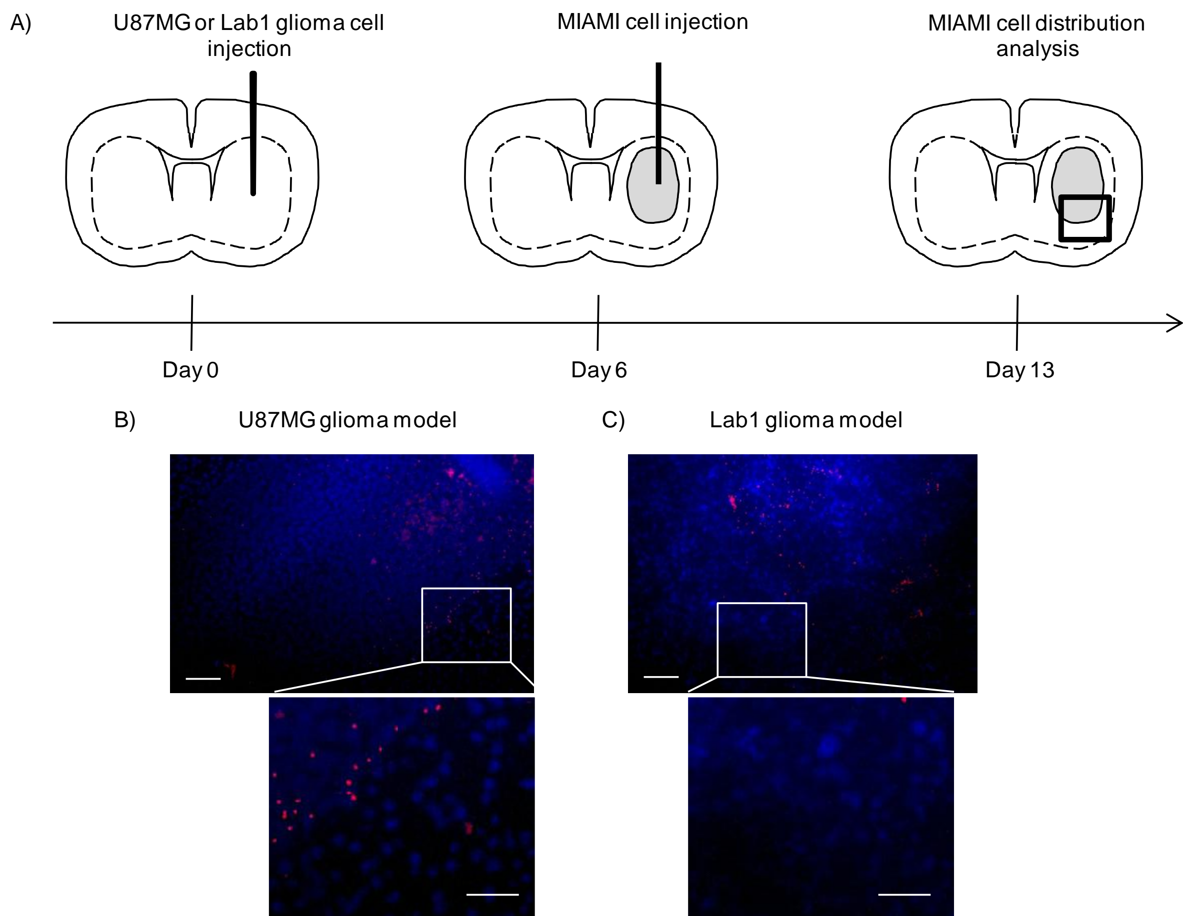

Figure 2: Fluorescent microscopy images of tissue sections after intratumoral injection of MIAMI cells in U87MG or Lab1 glioma-bearing mice. A) Schematic representation of the experimental model. B) MIAMI cells were distributed at the border of the tumor seven days after their intratumoral injection in the U87MG glioma model. C) MIAMI cells remained at the injection site after their intratumoral injection in the LAB1 glioma model. MIAMI cells were detected by red Y-chromosome labeling. Nuclei were stained with Dapi. (Scale bar = 100 µm and 50 µm for magnifications).

The migration of MIAMI cells toward the U87MG glioma model was also confirmed when these cells were inoculated into the contralateral hemisphere. By 3 and 7 days after their injection, MIAMI cells migrated away from the initial injection site toward the tumor and were located in the peri-tumoral zone (Figure 3). Some MIAMI cells still remained at the injection site (Figure 3).

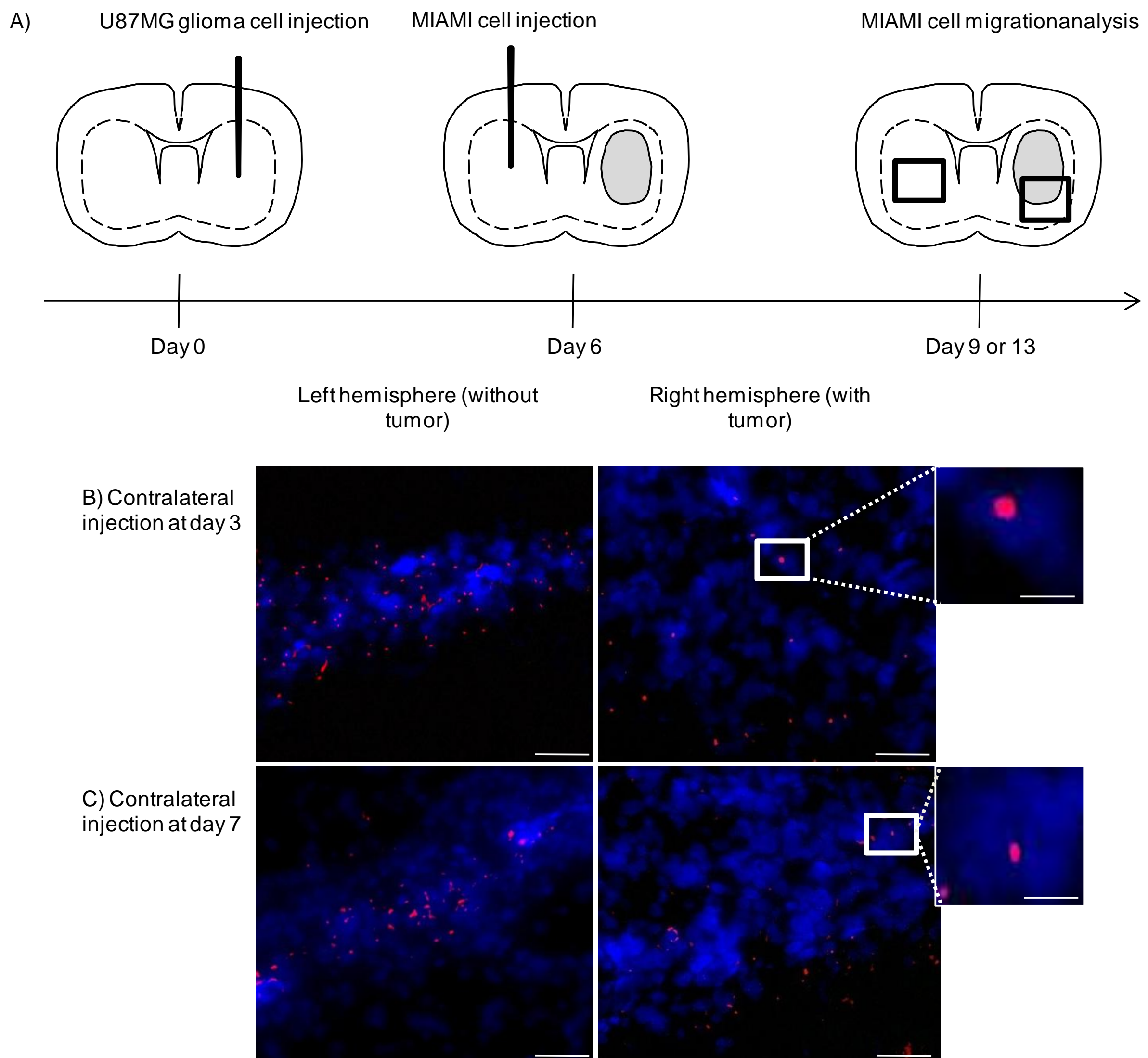

Figure 3: Fluorescent microscopy images of tissue sections after contralateral injection of MIAMI cells in U87MG glioma-bearing mice. A) Schematic representation of the experimental model. Three days (B) and 7 days (C) after MIAMI cell injection in the contralateral hemisphere to tumor, MIAMI cells migrated and localized within the tumor mass. MIAMI cells were detected by red Y-chromosome labeling. Nuclei were stained with Dapi. (Scale bar = 200 µm and 50 µm for magnifications).

Effect of MIAMI cells on the proliferation of glioma cell lines *in vitro* and *in vivo*

We studied the effect of MIAMI cells on the proliferative activity of several aneuploid glioma cell lines *in vitro* (Figure 4A). A172 and U118MG cell lines co-cultured for 72 h. with MIAMI cells displayed significantly increased growth, whereas, the proliferation of Lab2 and U138MG cell lines co-cultured with MIAMI cells were inhibited. No significant difference was observed for HS683 and Lab1 cell lines. U87MG cell line which was diploid was not tested. These data suggest that MIAMI cells have heterogeneous effects on the proliferation of glioma cells *in vitro*.

We next examined the *in vivo* effect of MIAMI cells on the growth of two tumorigenic glioma cell lines, U87MG and Lab1 using a nude mice orthotopic xenograft model (Figure 4B). U87MG glioma-bearing mice and Lab1 glioma-bearing mice had a median survival of 30 $\pm$ 2.1 days and 20 $\pm$ 2.3 days respectively. This survival was not statistically different from the one observed with mice which received intratumoral injection of MIAMI cells (29.5 $\pm$ 2.5 days and 20 $\pm$ 1.5 days for U87MG glioma-bearing mice and Lab1 glioma-bearing mice respectively). These results indicate that intratumoral injection of MIAMI cells do not influence the growth of glioma cells *in vivo*.

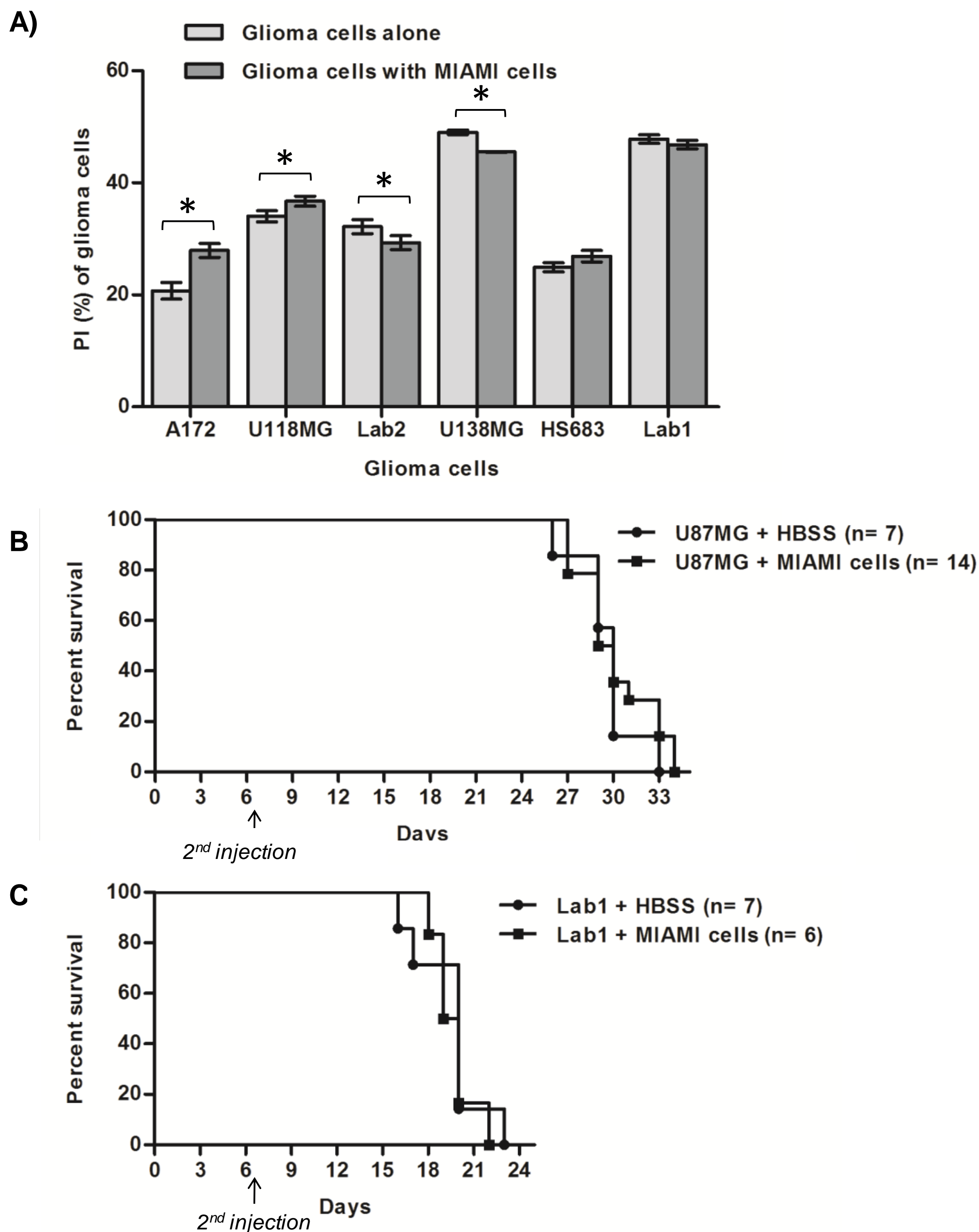

Figure 4: Effect of MIAMI cells on the proliferation of glioma cell lines *in vitro* and *in vivo.* (A) Direct co-culture assay of MIAMI cells and aneuploid glioma cell lines. The DNA content of glioma cells and MIAMI cells was analyzed by flow cytometry and the PI of aneuploid cells alone or co-cultured with MIAMI cells was calculated. The proliferation of A172 and U118MG cell lines co-cultured for 72 h with MIAMI cells increased, whereas, Lab2 and U138MG cell lines decreased. No significant difference was observed for HS683 and Lab1 cell lines (means ± SEM from triplicate wells) *, $P < 0.05$ versus

glioma cells alone. B and C) Kaplan-Meier graphs showed that the survival of U87MG (B) or Lab1 (C) glioma-bearing mice which received an intratumoral injection of 0.3 x 10^6 MIAMI cells at day 6 after the intracerebral glioma cell inoculation were not different of the survival of control animals.

Fate of MIAMI cells in glioma environment

The fate of MIAMI cells in the U87MG glioma environment was compared with MIAMI cells intracranially injected in healthy mice 7 days post-injection (Figure 5). For MIAMI cell proliferation study, the number of double-labeled Ki-67 and Y chromosome-positive MIAMI cells in U87MG glioma was calculated. A fraction of MIAMI cells within the tumor (19.5 ± 3.6 %) was found in a proliferative state around the tumor. For MIAMI cell differentiation analysis, the expression of astrocyte (GFAP), endothelial cell (CD31), pericyte (desmin) and cancer associated fibroblast (CAFs) (α-SMA, vimentin) associated markers were assessed on Y-positive MIAMI cells. Most of the MIAMI cells expressed α-SMA and vimentin markers in the U87MG glioma (87.7 ± 6.3 % and 92.9 ± 1.4%, respectively). Confocal microscopy analysis indicated that MIAMI cells did not seem to express GFAP. However, it is difficult to ensure this no expression since MIAMI cells were principally localized in the glial scar which is $GFAP^+$. MIAMI cells did not express desmin and human CD31. However, 36 ± 2 % of MIAMI cells were closed to mice endothelial cells within the U87MG glioma environment. No significant differences were observed in the expression of all the marker analysed by MIAMI cells injected in a healthy brain.

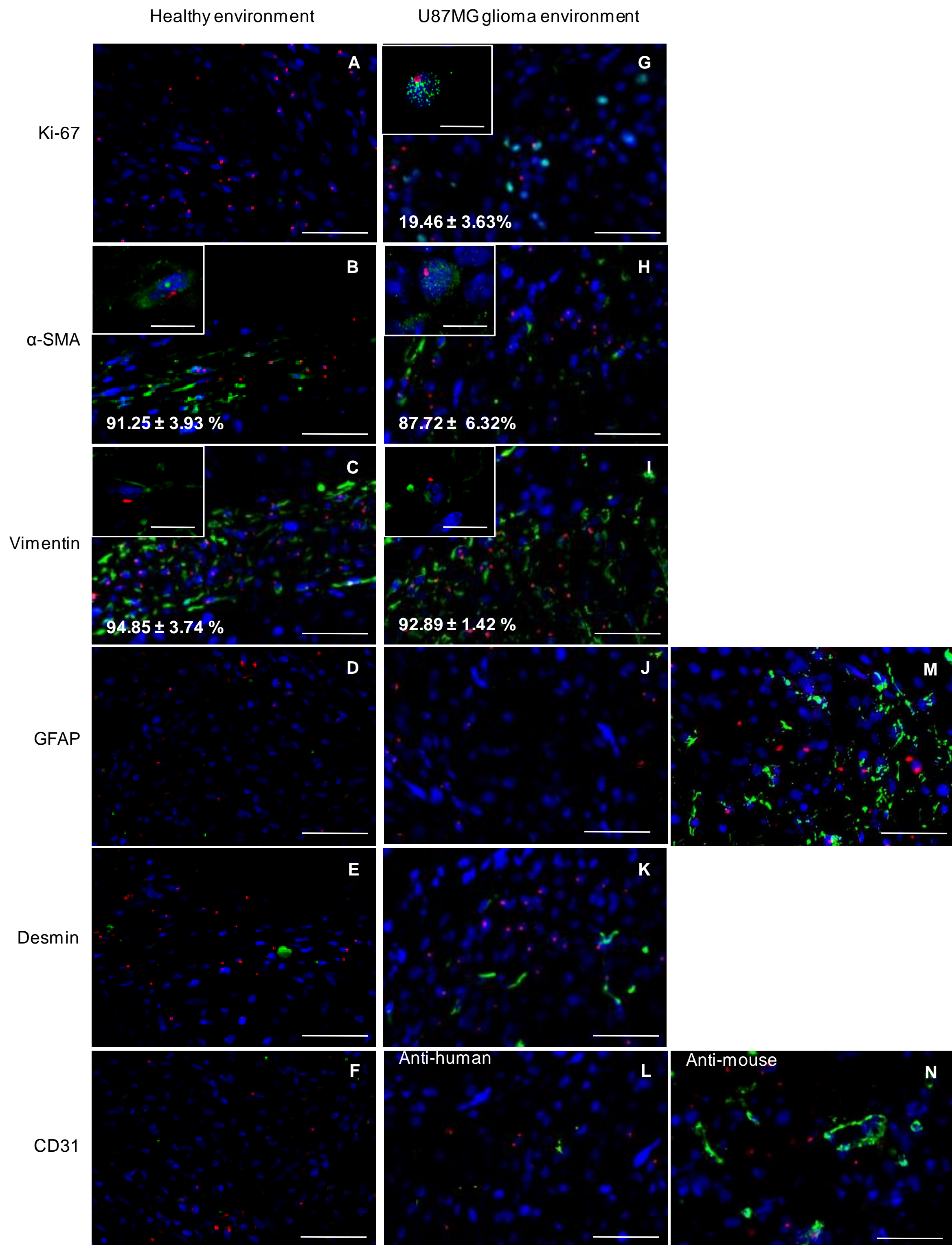

Figure 5: Analysis of differentiation of MIAMI cells in the healthy (A-F) and U87MG glioma environments (G-N). MIAMI cells were detected by red Y-chromosome labeling and markers (Ki-67, α-SMA, vimentin, GFAP, desmin or mouse and human CD31) were detected in green. Nuclei were stained with Dapi. On the contrary with the healthy environment, 20% of MIAMI cells expressed Ki-67 in the glioma environment (G). After injection in healthy or glioma environment, MIAMI cells expressed

α-SMA (B, H) and vimentin (C, I) markers, and did not express GFAP (D, J), desmin (E, K) and human CD31(F, L). Note the localized of MIAMI cells in the glial scar (M) and close to mice endothelial cells (N). Examples of co-stained cells were enlarged in photo corner. The percentage of co-stained cells calculated was given on the picture. (Scale bar = 100 μm and 10 μm for magnification).

Discussion

MIAMI cells have been shown to represent an attractive autologous human cell population for tissue regeneration of skeletal and nervous system disorders (24). Recently, we showed that these cells can internalize NPs and migrate toward an experimental human glioma model serving as targeted cellular carriers for NPs providing an anti-tumoral therapeutic strategy (26). In order to implement this approach in a clinical setting, the ease to isolate and expand MIAMI cells as well as their glioma tropism and their interaction with glioma cells must be evaluated.

In this study, we showed that MIAMI cells can be extracted from fresh or frozen iliac crest aspirates of human post-mortem organ donors aged between 65 and 68 years. This is of great importance for clinical trials in patients with glioma since the median age at diagnosis in primary GB is 64 years (37). Furthermore, we observed that fresh and frozen iliac crest-derived MIAMI cells had a similar pattern of marker expression (data not shown). Thus, it makes it possible to store aliquots of a patient iliac crest aspirate to avoid long-term *ex-vivo* culture of MIAMI cells and to design a treatment regimen where MIAMI cells are injected at different time-points. Several groups have published results showing spontaneous transformation of human MSCs *in vitro* (38-40) whereas other groups reported no transformation of MSCs after long-term culture (41, 42). In this study, experiments were done with MIAMI cells under passage 6. At this passage, the cells were diploid and a weak expression of RNA *h-TERT* was observed. Furthermore, intracranial injection of MIAMI cells in nude mice did not lead to tumor formation after 6 months (data not shown). All these observations indicate no malignant transformation of MIAMI cells *in vitro* and *in vivo*. It is important to note that recent studies highlight that the

spontaneous transformation of human MSCs was due to the MSC culture contamination with human immortalized cell lines (43).

As described for MSCs (44, 45), we showed that MIAMI cells were able to migrate to and disperse throughout the tumor mass in the peri-tumoral zone when they were injected intratumorally or in the contralateral hemisphere of mice bearing the U87MG glioma. We noted that this behavior seemed to be glioma dependant. Indeed, after injection of MIAMI cells in Lab1 glioma-bearing mice, MIAMI cells remain within the injection point, without distribution in the tumor mass. This result indicates the specificity of the distribution of MIAMI cells toward U87MG cells. It furthermore points out that for a clinical purpose, the screening of factors produced by glioma cells to allow the migration of MIAMI cells is necessary. Several factors mediating the tropism of human MSCs to human glioma cells have already been described such as epidermal growth factor (46), platelet-derived growth factor-BB (47 , 48) and stromal-derived fator-1 (49). The comparison of the factors produced by U87MG and Lab1 cells may be interesting to define those who are essential for MIAMI cell glioma tropism.

An issue of controversy is whether MSCs may increase cancer growth or not (33). Yu *et al.* (50) showed into BALB/c nude mice that subcutaneous or intracranial co-injection of MSCs derived from human adipose tissues (hADSCs) together with U87MG cells promoted glioma outgrowth. In the same way, Nakamizo et al. (13) observed that nude mice bearing U87MG xenografts that received hMSC-β-gal survived for shorter times than did mice who received saline treatments. On the contrary, Nakamura *et al.* (12) observed that intra-tumoral injection of rat MSCs 3

days after tumor inoculation caused significant inhibition of 9L tumor growth and increased the survival of 9L glioma-bearing Fisher 344 rats. Other groups have published that MSCs did not promote growth of glioblastoma cell lines *in vitro* and *in vivo* (51, 52). For example, after intratumorally injection of rat MSCs on day 2 into N29 or N32 tumor–bearing rats, Bexell *et al* did not observed significant difference between animals that received MSCs compared to animals that received control medium (51). Moreover, Kucerova *et al.* (52) did not show effect of hADSCs on the growth of human glioblastoma cells 8MGBA *in vitro* and *in vivo*. In the current study, we analyzed *in vitro*, the effect of MIAMI cells on the proliferation of a panel of aneuploid glioma cell lines. We showed that this effect was glioma cell line-dependant. MIAMI cells increased (A172 and U118MG cells), decreased (Lab2 and U138MG cells) or had no effect (Lab1 and HS683 cells) on the growth of glioma cells. The effect of MIAMI cells on the glioma cell proliferation was performed *in vivo* with two tumorigenic glioma cell lines (Lab1 and U87MG). The results showed that MIAMI cell grafting into these tumor did not influence the survival of Lab1 or U87MG glioma-bearing mice. Since the other glioma cell lines tested *in vitro* were not tumorigenic or induced an immune reject in the nude mice, they were not analysed *in vivo*. MSCs were also shown to increase the metastatic potential of breast cancer cells rather than significant tumor growth support (53). Using an invasion chamber, we observed that conditioned medium from MIAMI cells was not able to induce the invasion of U87MG cells. By contrast, a strong invasion of U87MG cells was observed toward culture medium supplemented with serum (data not shown).

In a glioma environment, it has been shown that MSCs give rise to pericyte-like cells contributing to stabilization of tumor vessel walls (51). In this study, we

observed that only 36% of MIAMI cells were close to mouse endothelial cells. Furthermore, no co-localisation of MIAMI cells with the pericyte marker, desmin, was detected nor expression of human CD31 by MIAMI cells. Mishra *et al.* (54) observed that hMSCs exposed to U87MG conditioned medium over a prolonged period of time assume a CAF-like phenotype. Accordingly, we observed that MIAMI cells exposed to U87MG conditioned medium for 7 or 28 days increased CAF associated marker expression (data not shown). The expression of some of these markers (αSMA and vimentin) was confirmed *in vivo*. However, no difference in expression of these markers was observed between MIAMI cells injected in a healthy environment or in the U87MG glioma environment. In addition, in contrast with CAFs which play an important role in the growth of epithelial solid tumors (55, 56), MIAMI cells did not exhibit this functional property. In fact, intratumoral injection of MSCs in Lab1 or U87MG glioma-bearing mice did not promote glioma outgrowth. MSCs have also been shown to differentiate into astrocytes after their injection into neonatal mouse brains (57). In this study, we did not observe the expression of the astrocyte marker GFAP by MIAMI cells grafted into healthy mice or into U87MG glioma-bearing mice. On the other hand, we observed that 20% of MIAMI cells expressed Ki-67 in the U87MG environment. No MIAMI cells were found in a proliferative state in the healthy environment suggesting that factors produced by U87MG cells induced the proliferation of MIAMI cells. Nevertheless, this proliferation had no impact on the survival of U87MG-bearing mice.

To conclude, this study demonstrated that MIAMI cells can be cultured safely for a clinical application. These cells were easy to extract and expand from iliac crest aspirates allowing autologous grafts. We showed that MIAMI cells were able to

migrate toward the orthotopic U87MG glioma model and did not influence its growth. Furthermore, in this model, MIAMI cells did not give rise to endothelial-like, pericyte-like, CAF-like or astrocyte-like cells but a low proportion was in a proliferative state. However, these MIAMI cell properties seemed to be glioma dependant and careful attention will be necessary for clinical purpose. It will be interesting to understand why MIAMI cells have different behavior depending on glioma cells used. Subgroups of BT-SCs were recently identified which may have distinct role in the development and progression of malignant glioma (58, 59) We can assume that in function of BT-SCs present in the tumor, MIAMI cells could have different effect on glioma growth. Moreover, the proliferation of MIAMI cells in the glioma environment should not be ignored. The glioma factors involved in this proliferation and the fate of the proliferating cells must be elucidated before the use of MIAMI cells in clinical applications.

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DISCUSSION GENERALE

Face au faible pronostic des patients atteints de GB, il est nécessaire de mettre en place de nouveaux outils thérapeutiques. Ces nouveaux outils doivent faire face à trois principales limites :

- Le passage de la BHE qui isole le SNC du reste de l'organisme et qui ne laisse passer que des molécules de petites tailles et liposolubles (1).
- Le ciblage des cellules infiltrantes restées après exérèse de la tumeur qui sont en partie à l'origine de la récidive dans les 2 cm du site initial (2, 3).
- Le tissu cérébral extrêmement fragile qui entoure les cellules cancéreuses et limite les doses de traitements injectées.

Durant ces dernières années, de nombreux efforts ont été mis en place pour développer des systèmes nanoparticulaires délivrant des molécules thérapeutiques au sein des tumeurs cérébrales (4-8). Ces systèmes permettent d'augmenter la quantité de principe actif délivrée et une meilleure diffusion du traitement. Cependant, le ciblage spécifique de ces systèmes dans les tumeurs cérébrales représente leur principale limite. Différentes stratégies ont été utilisées telles que le greffage de ligands ou d'anticorps dirigés contre les TAAs afin d'augmenter l'internalisation dans les cellules tumorales. Cependant, compte tenu de l'hétérogénéité intra-tumorale et de la variabilité inter-patients, il est nécessaire de développer des stratégies permettant de cibler les cellules tumorales quel que soit leur phénotype. Une alternative est l'utilisation de vecteurs cellulaires possédant un tropisme vers les tumeurs cérébrales pour transporter les NPs et les distribuer dans la masse tumorale. Les CSMs représentent des candidates idéales de part leur facilité d'isolement et leur capacité à migrer vers les gliomes (9-12). De plus, ces cellules ont déjà été utilisées après modification par des virus pour libérer des

molécules immunostimulatrices ou toxiques ou encore pour véhiculer des virus oncolytiques au sein du foyer tumoral (11, 12).

Ce travail de thèse à donc consister à valider la possibilité d'utiliser des CSMs pour véhiculer des NPs dans une tumeur cérébrale. La combinaison NPs/CSMs a déjà été utilisée pour manipuler ou suivre les CSMs (13). Par exemple, l'internalisation de NPs d'oxyde de fer ou des « quantum Dots » dans des CSMs a permis de marquer et de suivre le devenir et le potentiel régénératif de ces cellules (14-21). Il a été également rapporté que les NPs pouvaient être une alternative à la transfection génique par les virus. Cette stratégie a pu être exploitée pour la délivrance du VEGF par des CSMs, entrainant ainsi une augmentation de l'angiogénèse (22). L'utilisation de CSMs ayant internalisées des NPs chargées en facteurs de croissance ou en ADN plasmidique codant par exemple pour la « bone morphogenetic protein-2 » (BMP-2) a aussi été exploitée pour induire la différentiation ostéogénique ou chondrogénique des CSMs (23-29). L'utilisation des CSMs pour véhiculer des NPs chargées en molécules thérapeutiques dans un contexte tumoral n'a jamais été réalisée et constitue l'originalité de ce projet de thèse.

-1- Les outils utilisés

Afin de réaliser ce travail de thèse, différents outils ont été choisis ; les cellules et les nanovecteurs. Dans un modèle idéal, chacun de ces acteurs doit grâce à ses propriétés permettre d'obtenir une combinaison efficace (Tableau 1). Les critères clés que doivent posséder les cellules sont une capacité de migration spécifique vers les cellules de gliomes et une incorporation efficace des nanovecteurs *in vitro*. De plus, en vue d'une utilisation clinique, ces cellules ne doivent pas interférer avec la croissance tumorale. En ce qui concerne les nanovecteurs, ceux-ci doivent protéger et libérer le principe actif de façon contrôlée, et surtout, ils ne doivent pas modifier la survie et les propriétés des cellules cargos. Enfin, un isolement et une amplification faciles des cellules ainsi qu'une production des nanovecteurs à faible coût et à grande échelle sont des points positifs dans cette stratégie.

Tableau 1 : Caractéristiques nécessaires aux cellules et aux nanovecteurs pour une combinaison efficace.

Cellules	Nanovecteurs
Isolement aisé	Production aisée
Forte expansion *in vitro*	Fort taux d'encapsulation
Capacité de migration vers les tumeurs cérébrales	Libération contrôlée
Ne pas influencer la croissance tumorale	Non toxique pour la cellule cargo
Capacité à incorporer le nanovecteur	Echappement endolysosomal

-1.1- Les nanovecteurs

Les systèmes nanoparticulaires développés actuellement sont d'une grande diversité de composition et de structure leur permettant une grande modularité. La première contrainte imposée pour notre stratégie était d'utiliser un vecteur d'une taille inférieure à 300 nm afin de permettre une incorporation dans les cellules sans agents de transfection. Deux types de NPs ont été utilisés dans ce projet, des NPs de PLA

et des NCLs. La 6-coumarine, une molécule fluorescente a été utilisée comme molécule modèle pour l'encapsulation et pour visualiser l'incorporation des NPs par les cellules MIAMI.

-1.1.1- *Les NP polymériques*

Les NPs polymériques présentent plusieurs avantages :

- Généralement préparées par simple ou double émulsion, les NPs polymériques peuvent encapsuler des principes actifs à la fois hydrophiles et lipophiles (30).

- Les polymères les plus utilisés sont le PLGA (acides lactiques et glycoliques) et le PLA qui sont biocompatibles et biodégradables. En effet, les produits de dégradation de ces polymères sont très facilement métabolisés et éliminés dans l'organisme. De plus ils sont approuvés par la *Food and Drug Administration* (FDA).

- La proportion de monomère d'acide lactique dans le polymère permet de contrôler la libération du principe actif en conférant aux NPs une dégradation plus ou moins lente. Plus le polymère contient de monomère d'acide lactique, plus il sera stable. Ainsi, le PLA qui est constitué uniquement d'acide lactique est plus stable que le PLGA qui est composé d'acide lactique et d'acide glycolique.

- Les NPs polymériques ont aussi montré un échappement à la dégradation endolysosomale après incorporation dans les cellules. En effet, une fois dans les endosomes secondaires où le pH est très acide, la charge de surface des NPs de PLGA change. Les NPs initialement anioniques deviennent cationiques et interagissent avec la membrane de l'endosome pour la rompre (31, 32).

De nombreuses études ont déjà utilisé ce type de polymère, notamment dans la thérapie du gliome (33, 34). Au sein du laboratoire, une formulation des NPs de PLGA avait été déjà mise au point par simple émulsion évaporation de solvant (35).

Nous avons modifié cette formulation pour obtenir des NPs monodisperses d'une taille d'environ 120 nm (117,26 ± 0.4 nm). Cependant, les premières expériences réalisées avec un PLGA (37.5/25) ont montré une dégradation précoce du polymère et une libération anticipée du principe actif modèle utilisé. Il est important que les NPs ne soient pas dégradées trop rapidement pour permettre l'incorporation dans les CSMs et laisser le temps aux CSMs de les transporter jusqu'aux cellules tumorales.

Notre choix c'est alors tourné vers un polymère plus hydrophobe et plus stable, le PLA. Nous avons ainsi obtenu des NPs de PLA chargées en 6-coumarine d'une taille de 135.9 ± 1.2 nm avec un index de polydispersité de 0.040 et un potentiel zêta négatif de -2.11 ± 0.2 mV. Nous avons vérifié que les NPs de PLA ne libéraient pas la 6-coumarine à 37°C sur une période de 8 h.

Il est à noter que la production de ces NPs est très longue, nécessite du matériel spécifique (notamment l'homogénéisateur à filière) et possède des problèmes de rendement de production. En effet, lors de la production des NPs de PLA, deux centrifugations étaient réalisées pour concentrer les NPs et éliminer l'excès de PVA qui est toxique pour les cellules (36). Ces centrifugations entraînaient une perte de 90% de NPs. Ce problème de rendement de production est largement décrit dans la littérature. D'autres méthodes de concentration des NPs peuvent être réalisées telles que la filtration, la dialyse ou l'ultrafiltration (37). Cependant, chaque nouveau protocole de production nécessite une nouvelle caractérisation des NPs.

-1.1.2- *Les NCLs*

Une technologie brevetée au sein du laboratoire a aussi été utilisée dans ce travail de thèse, les NCLs. Comme les NPs polymériques, les NCLs présentent plusieurs avantages :

- Les NCLs sont formulées grâce à un procédé original sans solvant organique utilisant des excipients biocompatibles tous approuvés par la FDA ainsi qu'un faible besoin d'énergie (38). Ce procédé repose sur une série d'inversions de phase entre une émulsion huile dans eau (H/E) et une émulsion eau dans huile (E/H) suite à une augmentation et une diminution de la température du milieu réactionnel.

- Suivant la proportion d'excipient utilisée, différentes tailles de NCLs peuvent être formulées (39, 40).

- Ces nanocapsules ont déjà permis d'encapsuler différents anticancéreux (41) tels que le paclitaxel (42), l'étoposide (43) et des dérivés du tamoxifène (44).

- Les NCLs possèdent également la propriété d'inhiber la P-gp grâce à la présence du Solutol®HS15 (43, 45). L'inhibition de la P-gp surexprimée par les cellules tumorales (46) permet de limiter l'efflux des nanovecteurs après incorporation par les cellules.

- Les NCLs ont aussi montré comme les NPs polymériques un échappement à la dégradation endolysosomale. En effet, il est supposé que l'hydroxy-stéarate de PEG présent dans le solutol qui constitue la phase la plus externe des NCLs possède des propriétés lysosomotropiques ce qui entrainerait une déstabilisation de la membrane du lysosome pour la rompre (47).

Nous avons produit des NCLs chargées en 6-coumarine monodisperses de 88.1 ± 2.2 nm avec un index de polydispersité de 0.060 et un potentiel zêta négatif de -3.7 ± 0.9 mV.

Contrairement au NPs de PLA, la formulation des NCLs est très simple, ne nécessite aucun matériel sophistiqué et est très rapide. De plus, avec ces NCLs, il n'y a pas de problème de rendement de production puisque après formulation, les

NCLs sont prêtes à être utilisées. Des travaux sont actuellement en cours au laboratoire pour permettre la production à grande échelle de ces NPs.

-1.2- Les cellules : le choix des cellules MIAMI

Dans ce travail de thèse, nous nous sommes particulièrement intéressés aux cellules MIAMI (48). Il s'agit d'une sous-population de CSMs adultes, obtenue et caractérisée dans le laboratoire de P. Schiller (Université de Miami, FL), après prélèvement de MO vertébrale. Ces cellules sont isolées et cultivées dans des conditions similaires à leur niche *in vivo* ; en conditions hypoxiques et à faible confluence sur substrat de fibronectine.

Ces cellules ne posent pas de problème éthique contrairement aux cellules fœtales ou embryonnaires. De plus, des greffes autologues en vue d'une application clinique peuvent être envisagées. L'isolement aisé de ces cellules est aussi un avantage par rapport aux CSNs, qui aurait également pu être des candidates dans le développement de cet outil thérapeutique.

Les cellules MIAMI possèdent un phénotype moléculaire unique et expriment des marqueurs de CSE. Ces cellules sont aussi capables de se différencier *in vitro* en cellules des trois feuillets embryonnaires et notamment vers le phénotype neuronal (49). Au sein de l'INSERM U646, une population de cellules similaire a été obtenue par adhésion différentielle de prélèvement de MO issue de la crête iliaque de donneurs post-mortem en collaboration avec le Dr JP Jacob (Coordination Hospitalière, CHU, Angers) et le Dr C Montero-Menei (INSERM U646). Ce sont ces cellules, également appelées cellules MIAMI, qui ont été utilisées dans ce projet de thèse.

Elles ont été extraites à partir d'isolats frais de la crête iliaque de donneurs ayant une moyenne d'âge de 66 ans. Ceci est particulièrement important dans la mise en place de notre outil thérapeutique, puisque la médiane d'âge des patients atteints de GB est de 64 ans (50). Ces cellules MIAMI expriment un panel de marqueurs associés aux CSMs tels que CD73, CD105, CD29 et CD90 et partagent leur capacité de différenciation en ostéoblastes et en adipocytes. De plus, elles expriment des marqueurs de CSE comme *nanog*, *SSEA4* et *OCT4a*. Notons de plus, que ces cellules, peuvent être extraites à partir d'isolats qui ont été préalablement congelés. Ceci est très intéressant puisque cela permet, en vue d'une utilisation clinique, de faire des aliquots d'isolats de la crête iliaque d'un patient donné permettant l'utilisation de CSMs plusieurs fois dans le temps sans avoir à les maintenir pendant de longue période en culture. Nos études ont été réalisées avec des cellules MIAMI qui étaient à un passage inférieur à 6. A ce passage, les cellules étaient diploïdes et exprimaient faiblement le marqueur *h-TERT* dont l'expression est associée à la transformation des cellules. De plus, l'injection intracérébrale des cellules MIAMI chez la souris nude n'a pas conduit à la formation de tumeurs après 6 mois. L'ensemble de ces résultats indiquent que les cellules MIAMI ne semblent pas se transformer *in vitro* et *in vivo*. Ceci est indispensable pour envisager leur utilisation en clinique. Des études ont rapporté une transformation spontanée des CSMs *in vitro* (51-53). Cependant, des données récentes soulèvent que cette transformation n'est pas due aux CSMs proprement dites mais à une contamination croisée de la culture avec des lignées établies. Ces résultats soulignent la vigilance qui doit être réalisée lorsque la culture de lignées cellulaires et de primocultures s'effectuent en même temps (54).

-2- Les cellules MIAMI : un vecteur cellulaire pour véhiculer des NPs au sein de tumeurs cérébrales

Dans un premier temps, ce travail de thèse s'est intéressé à déterminer si les cellules MIAMI étaient des candidates efficaces pour véhiculer des NPs au sein d'une tumeur cérébrale. Elles devaient présenter des capacités de migration vers une tumeur cérébrale et des capacités d'internalisation des NPs.

-2.1- Analyse du tropisme des cellules MIAMI vers une tumeur cérébrale

Pour étudier le tropisme des cellules MIAMI vers une tumeur cérébrale, nous avons tout d'abord mis en place un modèle orthotopique de la lignée de gliome humain U87MG chez la souris nude femelle. Ce modèle consistait en une injection intracérébrale des cellules U87MG qui sont issues d'un individu de sexe féminin. Après 6 jours, les cellules MIAMI issues d'un individu de sexe masculin étaient injectées soit en intratumorale ou en controlatéral. Les souris étaient sacrifiées 7 jours plus tard et les cellules MIAMI étaient détectées *via* le marquage du chromosome Y par la technique FISH. Cette technique est très intéressante car elle permet de détecter les cellules sans avoir à les modifier avant leur injection. De nombreuses études ont modifié les CSMs *in vitro* pour pouvoir les détecter *in vivo.* Les principales méthodes de modifications des CSMs sont le marquage par un fluorochrome tel que le SP-diI (55) ou le DAPI (56), la transfection avec un vecteur GFP (green fluorescent protein) (10) ou l'internalisation de NPs d'oxyde de fer (57). Cependant, l'utilisation de ces méthodes nécessite de vérifier si les propriétés des CSMs sont conservées après leur modification.

Par ce modèle, nous avons montré que les cellules MIAMI, après leur injection intratumorale ou contralatérale, se distribuaient à la limite entre la masse tumorale et le parenchyme sain tout autour de la tumeur (Figure 1). De plus, aucune cellule MIAMI, n'a été observée dans le tissu sain autour de la tumeur. Ces résultats sont en accord avec ceux obtenus dans la littérature (10, 55). La capacité des CSMs à migrer vers une tumeur cérébrale a été validée dans des modèles syngéniques et non syngéniques chez le rat (lignées 9L et C6) (10, 58-61) ainsi que dans plusieurs modèles de xénogreffes de lignées humaines de gliome chez la souris immunodéprimée (lignée U87MG, lignée Glio 36)(55, 62-67). De plus, ce tropisme tumoral a été montré avec des CSMs d'origines variées : moelle osseuse (55, 63, 65), sang de cordons ombilicaux (62) ou tissus adipeux (66).

Notons cependant, que nous n'avons pas observé de migration des cellules MIAMI après leur injection intratumorale dans le modèle expérimental du gliome humain Lab1. Les cellules Lab1 sont issues d'une récidive d'un GB chez une patiente opérée au Département de Neurochirurgie à Angers. A notre connaissance, il n'a pas été rapporté de modèles expérimentaux de tumeurs cérébrales dans lesquels les CSMs ne migraient pas. Actuellement, comme nous l'avons mentionné dans l'introduction de ce manuscrit, plusieurs facteurs produits par les gliomes sont impliqués dans le tropisme tumoral des CSMs tels que MCP-1(68), PDGF-BB (69 , 70), EGF (71), VEGF A, IL-8, TGF-ss1 et NT-3 (72). La comparaison des facteurs produits dans le modèle Lab1 avec ceux produits dans le modèle U87MG pourrait permettre l'identification des facteurs essentiels permettant la migration des cellules MIAMI.

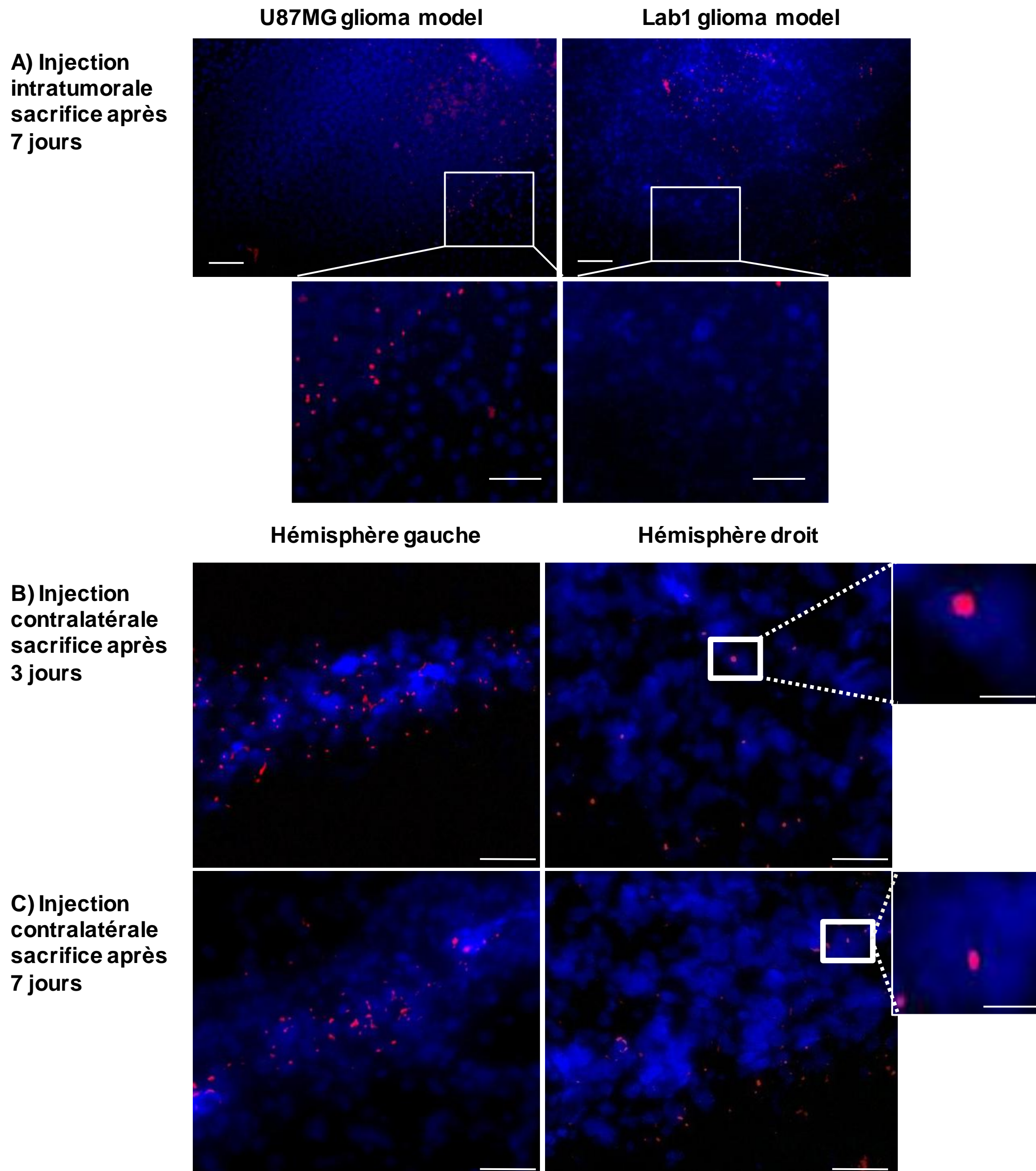


Figure 1: Images de microscopie fluorescente de coupes de cerveaux 7 jours après l'injection intratumorale (A) dans un souris nude porteuse d'une tumeur gliale intracérébrale U87MG ou Lab1, et 3 jours (B) ou 7 jours (C) après injection contralatérale de cellules MIAMI dans une souris nude porteuse d'une tumeur gliale intracérébrale U87MG. Détection des cellules MIAMI par le marquage du chromosome Y (marquage rouge). Les noyaux sont contre-colorés en bleu (échelle = 100 µm et 50 µm pour les agrandissements pour A; et 200 µm et 10 µm pour les agrandissements pour B et C).

-2.2- Analyse de l'internalisation et de la rétention des NPs de PLA et des NCLs par les cellules MIAMI

Suite à l'utilisation de NPs de PLA et de NCLs chargées en 6-coumarine, nous avons montré que les cellules MIAMI étaient capables d'internaliser efficacement les NPs (80% des cellules MIAMI sont chargées après 1 h d'incubation avec 200 µg/mL de NPs de PLA et 1 mg/mL de NCLs). De plus, les cellules MIAMI conservent les NPs après 3 et 7 jours de culture (Figure 2).

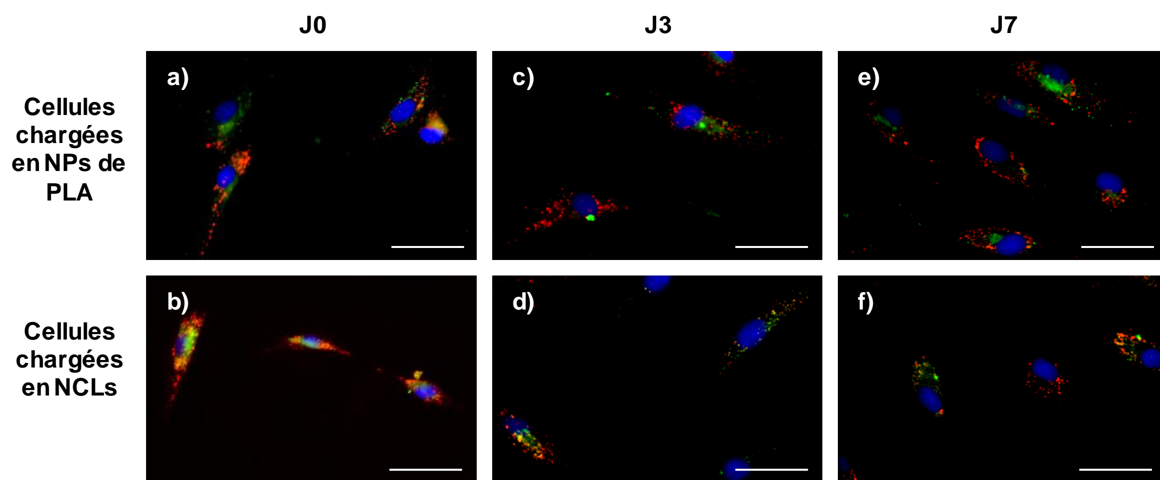
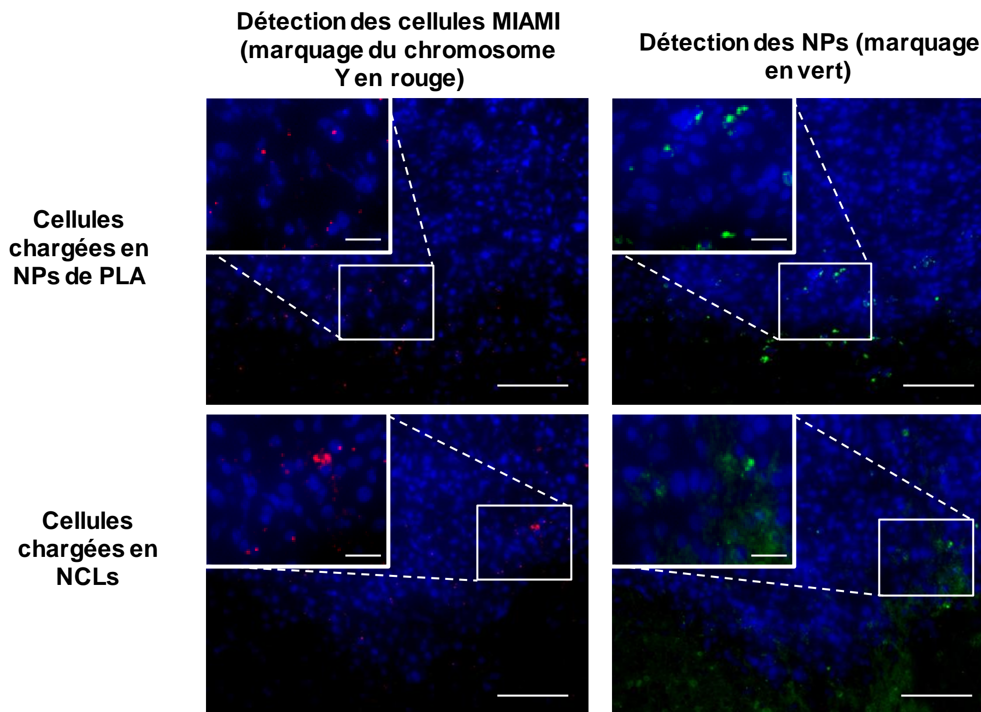


Figure 2 : Images en microscopie fluorescente de cellules MIAMI chargées avec des NPs de PLA ou des NCLs à J0, J3 et J7. Le noyau des cellules est coloré en bleu par le DAPI, les lysosomes en rouge (marquage Lysotracker) et la fluorescence verte représente la 6-coumarine (échelle = 50 µm).

Bien que les cellules MIAMI soient capables d'incorporer et de conserver les NPs, pour être un bon vecteur cellulaire, elles doivent aussi conserver leurs propriétés souches. Nous avons ainsi observé que la prolifération et les capacités de différenciation en adipocytes et en ostéoblastes des cellules MIAMI chargées en NPs de PLA ou en NCLs n'étaient pas affectées. De plus, les cellules MIAMI chargées en NPs migraient et se distribuaient de façon péritumorale après leur injection intratumorale. L'analyse de coupes adjacentes à aussi montré une colocalisation de

la 6-coumarine avec les cellules MIAMI (Figure 3). Cependant, il est à noter que bien que la 6-coumarine soit visible, sa localisation dans la cellule ne peut pas être garantie. En effet, due aux fortes températures utilisées lors de la technique FISH, il n'est pas possible de colocaliser les cellules MIAMI et le fluorochrome sur une même coupe ; il est donc difficile de préciser si la 6-coumarine est toujours présente dans les cellules MIAMI. Il n'est pas non plus possible de distinguer les NPs et d'observer si la 6-coumarine est toujours encapsulée. Néanmoins, la localisation de la 6-coumarine reste très intéressante puisqu'elle se situe dans la région péritumorale là où à lieu généralement la récidive.



<u>Figure 3</u> : Images de microscopie fluorescente de coupes de cerveaux 7 jours après l'injection intratumorale de cellules MIAMI dans une souris nude porteuse d'une tumeur gliale intracérébrale U87MG. Détection des cellules MIAMI par le marquage du chromosome Y (marquage rouge). Sur les coupes adjacentes, la fluorescence verte correspond à la 6-coumarine. Les noyaux sont contre-colorés en bleu (échelle = 200 µm et 50 µm pour les agrandissements).

-3- Combinaison NCLs/ cellules MIAMI : une association efficace pour délivrer du Fc-diOH

La preuve de concept de l'outil thérapeutique combinant les cellules MIAMI et les NPs étant validée, nous avons testé l'efficacité de cet outil sur le traitement du gliome en faisant véhiculer des NPs chargées d'un principe actif par les cellules MIAMI. Seule les NCLs ont été utilisées car, bien que nous ayons montré une incorporation efficace des NPs de PLA, ces NPs ne rentraient pas dans le cahier des charges que nous nous étions imposés. En particulier, comme indiqué précédemment, leur production n'est pas aisée.

Nous avons choisi le Fc-diOH (Figure 4) comme principe actif. Ce composé appartient à la famille des organométalliques et est un dérivé du tamoxifène où l'un des groupements phényl est substitué par un groupement ferrocène [Fe(C5H5)2] potentiellement cytotoxique. Le Fc-diOH est synthétisé au sein du laboratoire du Pr Jouen (UMR CNRS 7223, Ecole Nationale Supérieure de Chimie de Paris).

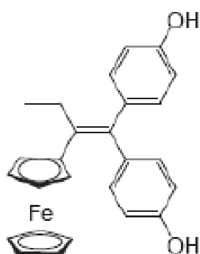


Figure 4 : Formule chimique de la molécule de Fc-diOH, 2-ferrocenyl-1,1-bis(4-hydroxyphenyl)-but-1-ene (MMr = 424.4 g mol−1).

Le Fc-diOH a déjà montré son efficacité dans le cancer du sein et de la peau, par un effet anti-oestrogènique sur des lignées possédant le récepteur aux œstrogènes, ainsi qu'un effet cytotoxique sur des lignées hormone-indépendantes

(73-75) et sur des cellules de mélanome (76). L'encapsulation du Fc-diOH a aussi été effectuée dans des nanosphères et des nanocapsules de PEG/PLA sans altération de l'effet cytotoxique sur des lignées du cancer du sein (77). Au sein de l'INSERM U646, Emilie Allard a mis au point une formulation de NCLs chargées en Fc-diOH au cours de sa thèse. Ces NCLs de Fc-diOH ont montré leur efficacité *in vitro* et *in vivo* dans un modèle expérimental hétérotopique de gliome 9L chez le rat (44, 78, 79).

Les NCLs formulées dans notre étude ont une taille de 75.64 ± 2.28 nm avec un index de polydispersité de 0.0290 et un potentiel zêta de -7.256 ± 2.121 mV. Enfin, elles contiennent 2.62 ± 0.06 mg de Fc-diOH /mL de suspension de NCLs.

Dans un premier temps, nous avons évalué la sensibilité des cellules U87MG et des cellules MIAMI, après 1 h d'incubation avec des NCLs chargées en Fc-diOH. Après lavages, les cellules ont été remises en culture pendant 3 jours et la viabilité des cellules a été analysée *via* un test MTS. Nous avons ainsi montré que ces NCLs étaient toxiques pour la lignée U87MG *in vitro*. Cependant, contrairement aux résultats obtenus sur les cellules 9L, nous n'avons pas pu déterminer l'IC_{50}. En effet, même aux plus fortes doses testées, 60% des cellules sont encore vivantes après 3 jours de culture. Ce résultat montre une résistance de cellules U87MG face à cette molécule. Il est possible que la toxicité augmente au cours du temps. Cependant, les cellules U87MG en culture forment des sphéroïdes après plusieurs jours de culture. Le test MTS est un test efficace pour évaluer la survie cellulaire de culture en monocouche, mais il s'incorpore très mal dans les amas de cellules tels que les sphéroïdes ce qui induit des résultats erronés. Nous n'avons donc pas pu évaluer la toxicité après une durée supérieure à 3 jours. D'autres tests de survie cellulaire devraient être plus adaptés tels que l'incorporation de BrdU ou de thymidine tritiée.

En parallèle, nous avons montré que les NCLs de Fc-diOH n'étaient pas toxiques pour les cellules MIAMI *in vitro*. Nous avons donc des nanovecteurs toxiques pour les cellules de gliome mais n'affectant pas les cellules cargos ce qui rentre dans le cahier des charges que nous nous étions fixés.

Dans un deuxième temps, nous avons évalué si les cellules MIAMI chargées en NCLs de Fc-diOH pouvaient induire la mort cellulaire de la lignée U87MG. Pour cela, nous avons utilisé un modèle de coculture avec un système d'insert. Ce système permet de cultiver deux types cellulaires de part et d'autre d'une membrane. Le compartiment inférieur contient des cellules U87MG adhérées et les cellules MIAMI en suspension ont été ajoutées dans le compartiment supérieur. La membrane qui sépare les 2 compartiments contient des pores de 0,4 µm permettant ainsi le passage de NCLs ou du Fc-diOH, mais pas le passage des cellules d'un compartiment à l'autre. Grâce à ce modèle, nous avons montré une diminution du nombre de cellules U87MG après culture avec des cellules MIAMI chargées. Cette toxicité augmente avec la dose cellules MIAMI chargées déposée c'est à dire avec la quantité de NCLs de Fc-diOH.

Enfin, nous avons évalué cette stratégie *in vivo* dans un modèle sous-cutané de la lignée U87MG chez la souris nude. Bien que ce modèle ne reflète pas l'environnement cérébral, il était rapide à mettre en place pour évaluer la toxicité des cellules MIAMI chargées en NCLs de Fc-DiOH. Après développement de la tumeur sur le flanc des souris, nous avons procédé à trois traitements intratumoraux : injection d'une solution saline (HBSS, contrôle négatif), injection de cellules MIAMI non chargées (afin de vérifier que ces cellules n'influencent pas la croissance tumorale) et injection de cellules MIAMI chargées en NCLs de Fc-diOH. Notons tout d'abord, que le traitement avec l'HBSS ou avec des cellules non chargées en NCLs

entraînait une augmentation du volume tumoral de 1400% après 15 jours de traitement. Pour les souris traitées avec des cellules MIAMI chargées en NCLs de Fc-diOH, à partir du 11ème jour, la croissance tumorale ralentissait pour atteindre une augmentation de 1000% à la fin de l'expérience. L'analyse des masses tumorales en fin d'expérience a confirmé ces résultats avec une masse moyenne de 1.36 ± 0.38 g pour le groupe HBSS et seulement de 0.95 ± 0.22 g pour le groupe ayant reçu des cellules MIAMI chargées en NCLs de Fc-diOH (Figure 5).

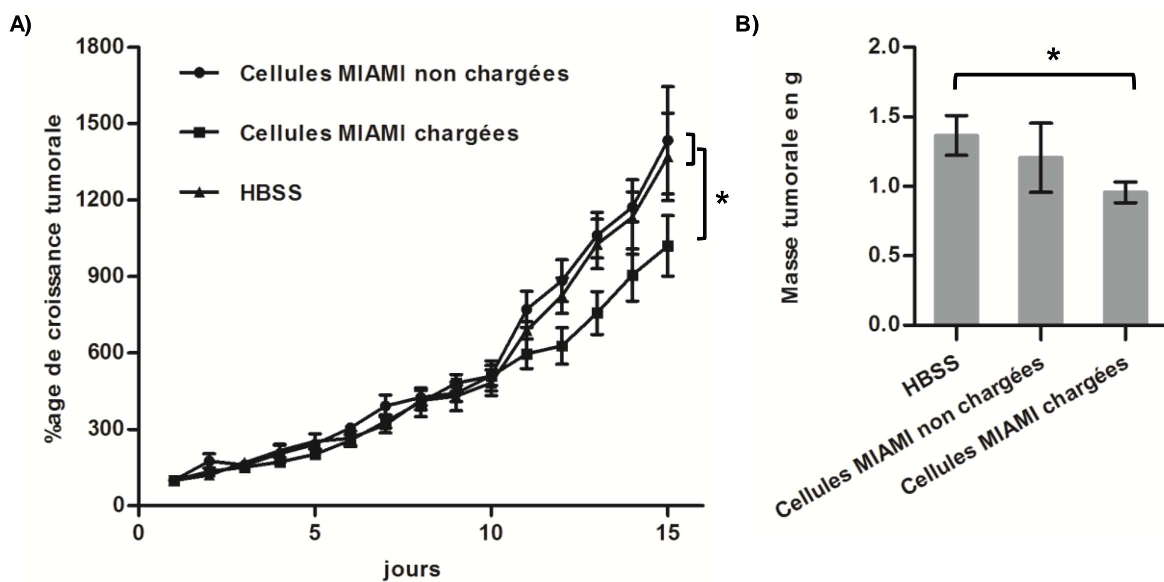


Figure 5 : Effet de l'injection intratumorale des cellules MIAMI chargées en NCLs de Fc-diOH sur la croissance tumorale du modèle hétérotopique de la lignée U87MG chez la souris nude. A) Pourcentage de la croissance tumorale. La taille de la tumeur est mesurée tous les jours et les résultats sont exprimés en pourcentage ± SEM par rapport à la taille de la tumeur mesurée le jour de l'injection du traitement. B) Masse tumorale 15 jours après le traitement (poids ± SEM). * $P < 0.05$.

Dans le modèle hétérotopique du gliome 9L chez le rat, des résultats similaires ont été observés après injection intratumorale de NCLs de Fc-diOH (44). En effet, un ralentissement de la croissance a été mis en évidence, mais sans régression de la tumeur. Ceci est probablement lié au mécanisme d'action du Fc-

diOH. En effet, il a été suggéré que le Fc-diOH induisait une production importante d'ERO (espèces réactives de l'oxygène) dans les lignées cellulaires tumorales associée à un arrêt du cycle cellulaire et à la sénescence (75). Dans le modèle orthotopique du gliome 9L, les NCLs de Fc-diOH n'ont pas montré d'effet sur la survie des animaux. Cependant une augmentation de la médiane de survie et du nombre de longs survivants a été observé en combinaison avec une radiothérapie externe (79). Une étude ultérieure sera réalisée pour évaluer l'efficacité de notre outil thérapeutique dans le modèle orthotopique de la lignée U87MG chez la souris nude en association ou non avec une radiothérapie externe.

-4-Les cellules MIAMI un vecteur cellulaire sûr ?

Les cellules MIAMI semblent donc très intéressantes pour délivrer des NPs cytotoxiques au sein de tumeurs cérébrales. Cependant, en vue d'une utilisation clinique des cellules MIAMI, il est impératif d'analyser leur fonction et leur devenir au contact d'une tumeur cérébrale.

-4.1- Fonction des cellules MIAMI au contact des cellules gliales tumorales

La fonction biologique exacte des CSMs au sein des tumeurs est encore inconnue (80). Concernant les tumeurs cérébrales, peu d'études ont été réalisées et les résultats obtenus sont contradictoires. Yu *et al.* (81) ont montré que la co-injection, sous-cutanée ou par voie intracrânienne, de CSMs humaines provenant de tissus adipeux avec les cellules U87MG dans des souris nude induisait une augmentation de la croissance du gliome U87MG. De la même façon, Nakamizo *et al.* (55), ont observé dans un modèle de xénogreffe chez la souris nude (U87MG), qu'après injection de MSCs humaines, les animaux survivaient moins longtemps que les animaux contrôles. Au contraire, Nakamura *et al.* (10) ont observé que l'injection intratumorale de CSMs de rat dans le modèle syngénique orthotopique du gliome 9L provoquait une inhibition significative de la croissance tumorale et augmentait la survie des rats porteurs du gliome 9L. D'autres groupes ont publié que les CSMs ne favorisaient pas la croissance de lignées cellulaires de glioblastome *in vitro* et *in vivo* (82, 83).

Nous avons analysé *in vitro*, l'effet des cellules MIAMI sur la prolifération d'un panel de lignées de gliome aneuploïdes (Figure 6A). L'utilisation de lignées aneuploïdes permet de réaliser des cocultures directes avec les cellules MIAMI qui

sont diploïdes. Nous avons montré que cet effet était différent en fonction de la lignée testée. En effet, les cellules MIAMI soit augmentaient (cellules A172 et U118 MG), soit diminuaient (cellules Lab2 et U138MG) ou n'avaient aucun effet (cellules LAB1 et HS683) sur la croissance des cellules de gliome. L'effet des cellules MIAMI sur la lignée U87MG n'a pas pu être réalisé du fait que ces cellules sont majoritairement diploïdes. L'étude de l'effet des cellules MIAMI sur la prolifération de deux lignées de gliome tumorigènes chez la souris nude (Lab1 et U87MG) a également été analysée *in vivo*. L'injection intratumorale de cellules MIAMI n'avait pas d'influence sur la survie de souris porteuses du gliome Lab1 ou U87MG en intracérébral (Figure 6B). Les autres lignés de gliome testées *in vitro*, n'ont pas pu être évalué *in vivo*. En effet ces cellules ne sont pas tumorigènes chez la souris nude ou induisent un rejet immunitaire.

Une étude de Karnoub *et al.* (84) a montré que les CSMs pouvaient augmenter le potentiel métastatique des cellules cancéreuses du sein sans influencer leur croissance. En utilisant une chambre d'invasion, nous avons observé que le milieu conditionné de cellules MIAMI n'était pas capable d'induire l'invasion des cellules U87MG (Figure 6C). Par contre, une forte invasion des cellules U87MG a été observée lorsque le compartiment inférieur de la chambre contenait du milieu de culture avec du sérum.

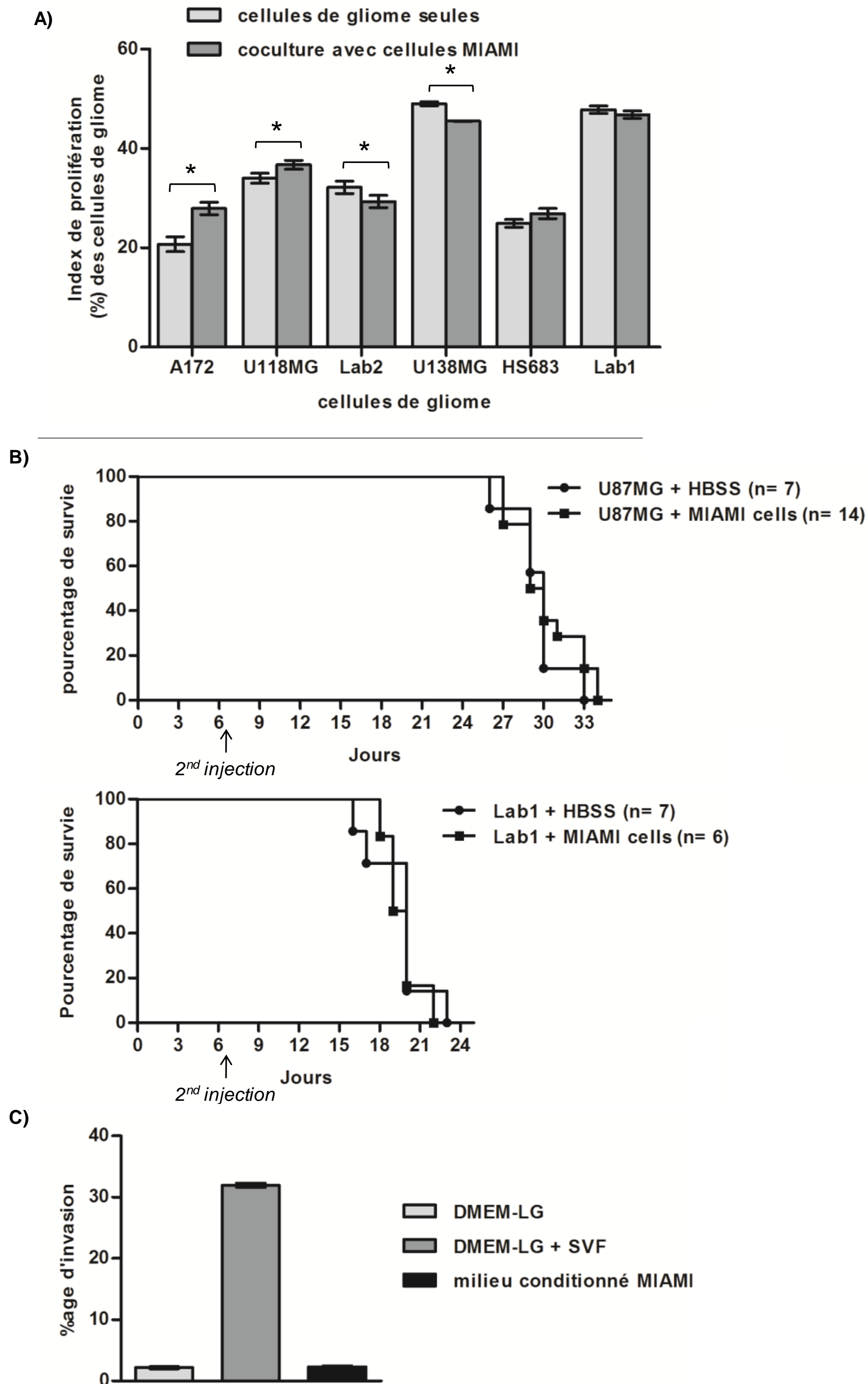


Figure 6 : Evaluation de l'effet des cellules sur la prolifération *in vitro* et *in vivo* et l'invasion des cellules de gliomes. A) coculture directe des cellules MIAMI avec les cellules de gliome aneuploïdes. Le contenu en ADN des cellules de gliome et des cellules MIAMI a été analysé par cytométrie en flux

et l'index de prolifération des cellules aneuploïdes seules ou cultivées avec des cellules MIAMI a été calculé *versus* (moyennes ± SEM, triplicate). *, $P < 0.05$

B) Courbes de survie de Kaplan-Meier après injection intratumorale d'HBSS ou de cellules MIAMI dans les modèles U87MG et Lab 1.

C) Analyse de l'invasion des cellules U87MG in vitro vers un milieu contrôle négatif, sans sérum (DMEM-LG), un milieu contrôle positif (DMEM-LG + SVF) et un milieu conditionné de cellules MIAMI. Les résultats sont exprimés en % age de cellules invasives par rapport au nombre de cellules déposées. (moyennes ± SEM, n=2, triplicate). * $P < 0.05$.

-4.2- Devenir des cellules MIAMI au contact des cellules gliales tumorales

Bexell *et al*. (82) ont montré que les CSMs s'incorporaient dans les vaisseaux tumoraux et présentaient des caractéristiques similaires aux péricytes dans un modèle orthotopique de gliome de rat (82). Cependant, ils n'ont pas observé d'influence des CSMs sur la densité des vaisseaux tumoraux ni sur la survie des animaux porteurs du gliome. Nous avons constaté qu'après injection intratumorale des cellules MIAMI dans le modèle orthotopique du gliome humain U87MG chez la souris nude, 30% des cellules MIAMI étaient proches des vaisseaux tumoraux identifiés *via* le marquage du CD31 murin (Figure 7). Aucune colocalisation des cellules MIAMI avec le marqueur des péricytes, desmine, a été détectée, ni l'expression de CD31 humain par les cellules MIAMI. Cette différence de résultats avec Bexell *et al*. peut être due à l'utilisation d'un modèle de xénogreffe plutôt qu'un modèle syngénique.

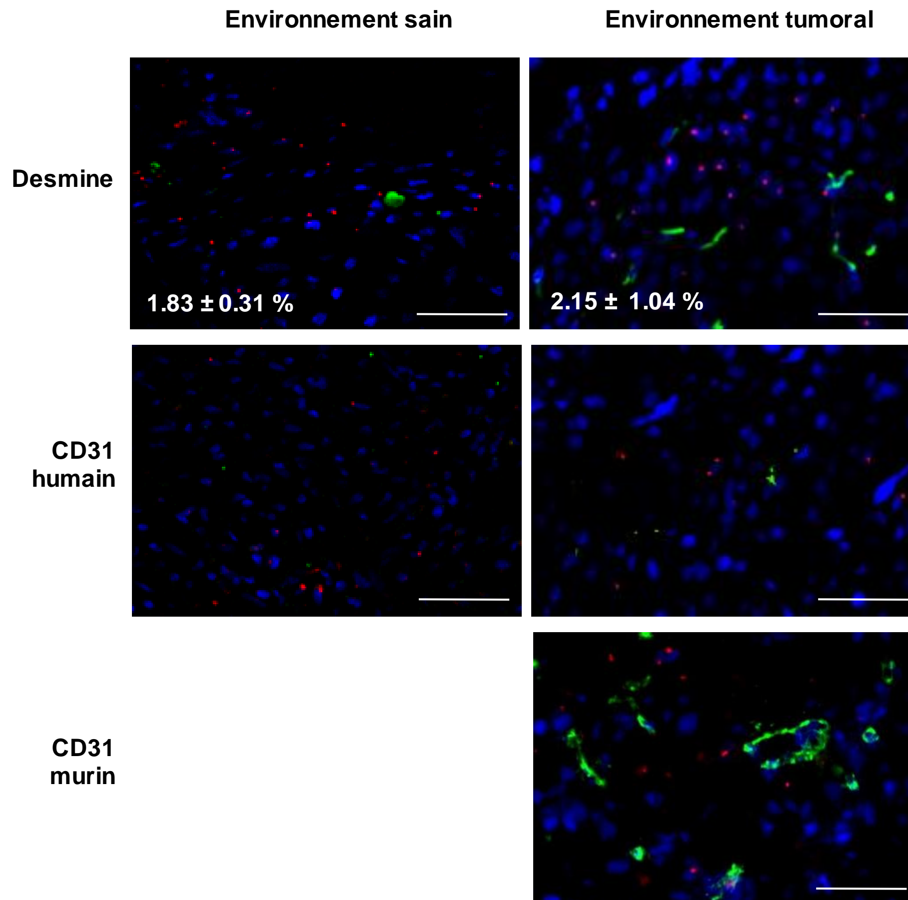


Figure 7 : Analyse de la différenciation en péricytes des cellules MIAMI au contact des cellules gliales tumorales U87MG. Images de microscopie à fluorescence de sections de cerveaux de souris après co-marquage du chromosome Y (marquage rouge) et des marqueurs desmine, CD31 humain et CD31 murin (marquage vert). Les noyaux sont contre colorés au DAPI en bleu. (Échelle = 100 µm et 10 µm pour les agrandissements).

Plusieurs études sur des tumeurs périphériques (carcinome du sein, mélanome) ont montré que les CSMs pouvaient se différencier en cellules similaires aux CAFs au contact de cellules tumorales et ainsi promouvoir la croissance tumorale (81, 84-88). Mishra *et al.* (88) ont observé que des CSMs humaines exposées au milieu conditionné de la lignée de gliome U87MG présentaient un phénotype de type CAF avec une augmentation de l'expression de α-SMA, de la vimentine et de FSP (« fibroblast surface protein »). Comme Mishra *et al.*, nous

avons également constaté une augmentation de l'expression de plusieurs marqueurs associés aux CAFs comme α-SMA, FSP et PFGFRβ lorsque les cellules MIAMI étaient incubées pendant 7 jours avec le milieu conditionné de la lignée U87MG (Figure 8A). *In vivo*, 90% des cellules MIAMI injectées dans un environnement cérébral sain ou dans l'environnement tumoral U87MG exprimaient la vimentine et l'α-SMA (Figure 8B). La technique d'analyse utilisée ne nous a pas permis de définir si des différences d'expression existaient entre ces deux types d'environnement. Il est à noter qu'il n'existe pas de marqueurs spécifiques des CAFs. α-SMA est le marqueur le plus couramment utilisé pour l'identification des CAFs. Ces cellules sont essentiellement définies par leur capacité à augmenter la croissance des cellules épithéliales des tumeurs solides (89, 90). Les cellules MIAMI ne semblent pas partager cette propriété fonctionnelle. En effet, l'injection intratumorale de ces cellules dans les deux modèles de gliomes humains analysés (Lab1 et U87MG) n'affecte pas la croissance tumorale.

Kopen *et al*. (91) ont montré que les CSMs se différenciaient en astrocytes après leur injection dans le cerveau de souris nouveau-nées. Nous n'avons pas observé d'expression du marqueur des astrocytes, GFAP, par les cellules MIAMI, injectées dans un environnement cérébral sain ni dans l'environnement tumoral U87MG (Figure 8B).

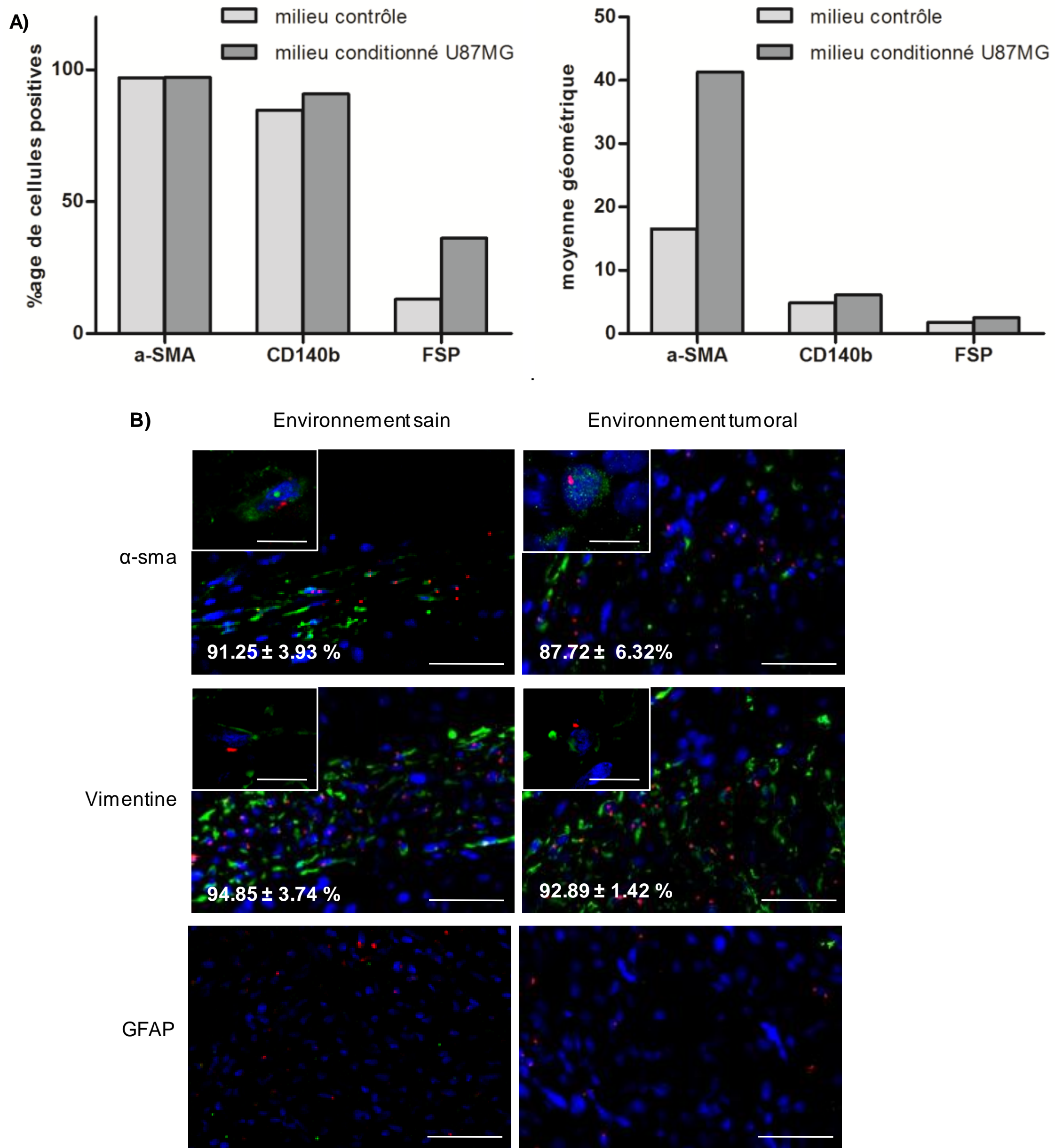


Figure 8 : Analyse de la différentiation en CAF et en astrocytes des cellules MIAMI au contact des cellules gliales tumorales U87MG. A) Analyse par cytométrie de flux des marqueurs α-sma, CD140b et FSP par les cellules MIAMI cultivées 7 jours dans un milieu contrôle ou dans un milieu conditionné du gliome U87MG. B) Images de microscopie à fluorescence de sections de cerveaux de souris après co-marquage du chromosome Y (marquage rouge) et des marqueurs α-sma, vimentine et GFAP (marquage vert). Les noyaux sont contre colorés au DAPI en bleu. (Échelle = 100 µm et 10 µm pour les agrandissements).

L'ensemble de ces résultats indiquent que les cellules MIAMI injectées dans le gliome U87MG ne semblent pas se différencier en cellules endothéliales, en astrocytes, en péricytes ni en CAFs. Notons cependant, que 20% des cellules MIAMI exprimaient le marqueur de prolifération, Ki-67 dans l'environnement tumoral U87MG (Figure 9A). Aucune expression de ce marqueur par les cellules MIAMI injectées dans un environnement cérébral sain n'a été détecté. L'augmentation de Ki-67 a également été observée *in vitro* lorsque les cellules MIAMI étaient incubées 7 jours dans le milieu conditionné de la lignée U87MG (Figure 9B). Néanmoins, l'état prolifératif des cellules MIAMI n'a pas interféré avec la survie des souris porteuses de la tumeur U87MG.

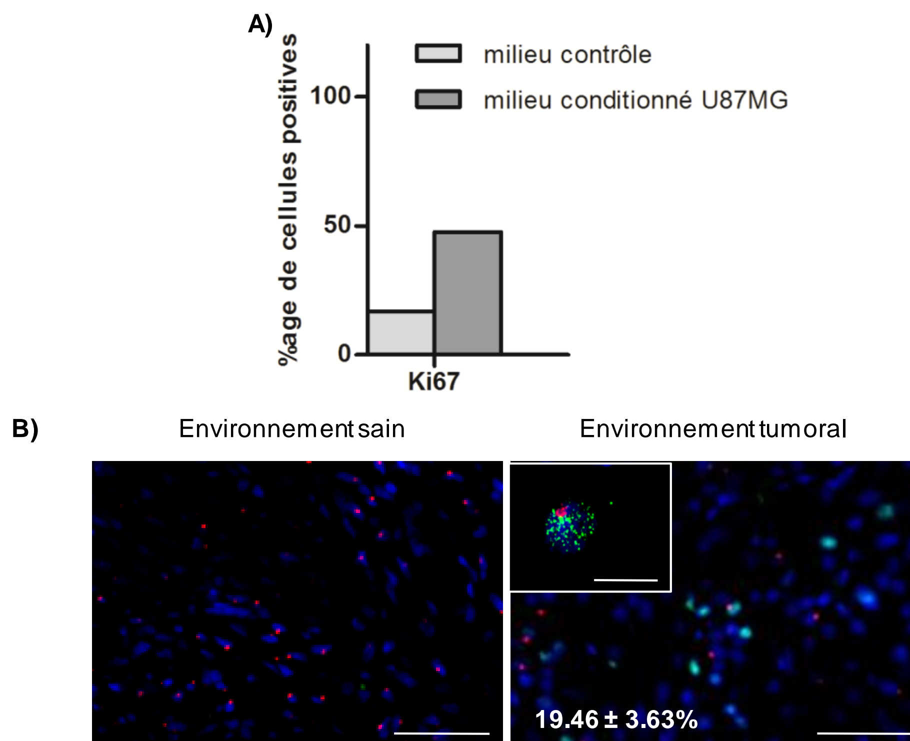


Figure 9 : Analyse de la prolifération des cellules MIAMI au contact des cellules gliales tumorales U87MG. A) Analyse par cytométrie de flux du marqueur Ki-67 par les cellules MIAMI cultivées 7 jours dans un milieu contrôle ou dans un milieu conditionné du gliome U87MG. B) Images de microscopie à fluorescence de sections de cerveaux de souris après co-marquage du chromosome Y (marquage rouge) et du marqueur Ki-67 (marquage vert). Les noyaux sont contre colorés au DAPI en bleu. (Échelle = 100 µm et 10 µm pour les agrandissements).

CONCLUSION ET PERSPECTIVES

Dans ce travail de thèse, nous avons développé un nouvel outil thérapeutique des tumeurs cérébrales en combinant à la fois les cellules MIAMI et les NPs chargées en principe actif. Grâce à la capacité de migration des cellules MIAMI vers la tumeur cérébrale, cette stratégie permet à la fois un ciblage des cellules tumorales et une distribution dans la tumeur. De plus, les NPs permettent d'apporter le principe actif en grande quantité, de le protéger ainsi que de le libérer de façon contrôlé. Cette stratégie permet donc de contourner plusieurs obstacles qui limitent actuellement le traitement des gliomes.

Nous avons validé cet outil thérapeutique dans un modèle hétérotopique du gliome humain U87MG chez la souris nude avec des cellules MIAMI chargées en NPs de Fc-diOH. D'autre part, la sureté de l'utilisation des cellules MIAMI en tant que vecteurs cellulaires a été vérifiée. Nous avons ainsi montré que les cellules MIAMI pouvaient être cultivées de façon sécurisée sans engendrer leur transformation. De plus, l'injection intratumorale de ces cellules dans deux modèles orthotopiques de gliomes humains (U87MG et Lab 1) chez la souris nude n'interfèrait pas avec la survie des animaux.

L'ensemble de ces résultats indiquent que les cellules MIAMI représentent un vecteur cellulaire idéal pour véhiculer les NPs. Toutefois, plusieurs études complémentaires sont nécessaires avant de pouvoir utiliser cet outil thérapeutique en clinique :

- Tout d'abord, cette stratégie doit être validée par l'injection de cellules MIAMI chargées en NCLs de Fc-diOH dans un modèle orthotopique de tumeur cérébrale. Jusqu'à présent, aucun système nanoparticulaire n'a montré d'efficacité dans des modèles intracrâniens. Cette stratégie pourrait être plus efficace en apportant une meilleure distribution du principe actif dans la masse tumorale. D'autre part, une

combinaison avec une radiothérapie externe pourrait être intéressante. En effet, d'une part le Fc-diOH est une molécule radiosensible dont l'effet est potentialisé après radiothérapie (79). D'autre part, il a été montré que l'irradiation locale pouvait promouvoir la spécificité de migration des CSMs et leur intégration dans les tissus (92). D'autres molécules thérapeutiques pourraient également être testées. Par exemple, la localisation des cellules MIAMI proches des vaisseaux tumoraux permet d'envisager l'encapsulation de facteur anti-angiogéniques tels que le Budésonide, un corticostéroïde capable d'inhiber l'expression du VEGF (93). D'autre part, l'encapsulation de molécules inhibant l'invasion des cellules tumorales comme des antagonistes d'intégrines ou des inhibiteurs des MMPs est très séduisante de part la distribution des cellules MIAMI à la limite entre la masse tumorale et le parenchyme sain.

- Notons aussi, que le modèle utilisé pour tester notre outil thérapeutique est un modèle de xénogreffe de cellules humaines chez la souris nude. L'avantage de ce modèle est de pouvoir travailler avec du matériel humain. Cependant, dans ce modèle, la tumeur se développe dans un environnement immunodéprimé et dans un stroma murin qui ne représentent pas la réalité. L'utilisation de modèles expérimentaux syngéniques doit être envisagée pour étudier l'influence du stroma tumoral sur l'efficacité de cet outil thérapeutique. Certains modèles, comme le modèle F98 chez le rat (94) et des modèles de GB spontanés chez le chien (95) qui présentent l'avantage d'être très proches des GB humains (signes cliniques, dévelopement tumoral et pronostic) et qui permettraient de réaliser des greffes autologues pourraient être intéressants.

- De plus, en vue d'une utilisation clinique des cellules MIAMI, la voie d'administration de ces cellules doit être évaluée. Dans ce travail de thèse, les

cellules MIAMI ont été injectées en intratumorale. Cette voie d'injection pourrait être envisageable dans un protocole clinique dans lequel les cellules seraient injectées dans la cavité tumorale après exérèse de la tumeur pour cibler les cellules tumorales infiltrées dans le parenchyme sain. Cependant, pour les tumeurs non opérables ou si plusieurs injections de cellules MIAMI doivent être effectuées, la voie intraveineuse semble très séduisante. La capacité de migration des CSMs après injection dans la circulation sanguine chez des animaux porteurs de tumeurs cérébrale a déjà été envisagé (55), mais qu'en est-il pour les cellules MIAMI ? Des études de biodistribution après administration des cellules MIAMI dans la circulation sanguine chez des animaux porteurs d'une tumeur cérébrale pourraient répondre à cette question. L'accumulation des cellules MIAMI dans la tumeur cérébrale et dans différents organes tels que les poumons, le foie ou la rate serait déterminée. De plus, la dose de Fc-diOH incorporé par cellule MIAMI devra être évaluée après incorporation des NCLs. En effet, pour la mise en place d'un protocole clinique, la quantité de cellules MIAMI nécessaire pour obtenir une dose efficace devra être déterminée.

- Dans un contexte clinique, les protocoles utilisés pour fabriquer cet outil thérapeutique doivent également être optimisés. Par exemple, la lyophylisation des NCLs pourrait être envisagée pour une meilleure conservation des formulations et une reconstitution extemporanée des NPs (96). En ce qui concerne la culture des cellules MIAMI, des substituts du sérum de veau fœtal (SVF) dans le milieu de culture comme le sérum humain autologue (97) ou du lysat plaquettaire humain (98) doivent être testés. En effet, le SVF qui possède des propriétés antioxydantes, fournit aux cellules les nutriments essentiels pour leur croissance mais présente des inconvénients tels que le déclenchement de réactions immunitaires *in vivo* et la

présence possible d'agents infectieux dont les prions (encéphalopathie spongiforme bovine) qui peuvent causer la maladie de Creutzfeldt-Jackob.

- Enfin, il est nécessaire de continuer à caractériser les interactions des cellules MIAMI avec les cellules gliales tumorales. En particulier, nous avons observé que la migration des cellules MIAMI était dépendante de la lignée de gliome utilisée. Nous avons remarqué une migration des cellules MIAMI dans le modèle de gliome U87MG mais pas dans le modèle Lab1. Il est indispensable dans un contexte clinique d'identifier les facteurs essentiels pour le tropisme tumoral des cellules MIAMI et donc de notre outil thérapeutique. Cette identification pourrait être réalisée *via* la comparaison des facteurs produits par les lignées U87MG et Lab1. L'identification du ou des facteur(s) permettrait le développement d'une thérapie personnalisée. D'autre part, même si nous n'avons pas observé d'effet des cellules MIAMI sur la croissance tumorale des deux modèles de gliomes humains analysés *in vivo*, Lab1 et U87MG, nous avons constaté que sur certaines lignées, les cellules MIAMI pouvaient augmenter leur croissance *in vitro*. D'un point de vue clinique, il faut donc rester prudent. Il serait intéressant de déterminer si ces lignées se distinguent par la présence ou l'absence de CSTs. En effet, il a été montré que ces cellules étaient susceptibles d'initier et d'entretenir le pool cellulaire tumoral (99, 100). de plus, des sous-groupes de CSTs ont été identifiés pouvant avoir un rôle différent sur la croissance tumorale (101). Nous pouvons ainsi supposer qu'en fonction du couple CSTs/cellules MIAMI, l'effet des cellules MIAMI sur la croissance tumorale soit différent. Enfin, l'observation de la prolifération des cellules MIAMI dans un environnement tumoral ne doit pas être négligée. Il est important de vérifier *in vitro* et *in vivo* que ces cellules, après leur incubation dans un milieu conditionné de gliome, ne soient pas capables de se transformer ni de promouvoir la croissance tumorale.

Récemment, nous avons identifié une nouvelle population stromale dans la zone péritumorale du GB appelée GASCs, qui augment la croissance des lignées de gliomes humains. Ces cellules qui possèdent des caractéristiques mésenchymateuses pourraient dérivées des CSMs.

Ce travail de thèse explore ainsi une nouvelle voie de recherche sur la thérapie du gliome. L'ensemble des études complémentaires précédemment citées, permettrait d'optimiser et de valider ce nouvel outil thérapeutique des tumeurs cérébrales. Cet outil représente donc un nouvel espoir pour cibler spécifiquement les foyers tumoraux.

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ANNEXES

MATHILDE ROGER
26 ans
4 rue de l'Aubépin
49100 Angers, France
Email : mathilde.roger@yahoo.fr
Tél: +339 52 78 93 17

EXPERIENCE PROFESSIONNELLE

2007: **Doctorante en neuroscience** (Angers, France)
Cellules stromales mésenchymateuses comme vecteurs cellulaire de nanoparticules: un nouvel outil thérapeutique des tumeurs cérébrales
Directeur de thèse: Pr Philippe MENEI
INSERM U646, Université d'Angers, Angers, France, Prof. **J-P BENOIT**

2006: Stage pratique au sein de l'unité INSERM U646 (Angers, France)
Les cellules stromales mésenchymateuses dans la thérapie du gliome
Encadrants: Pr Philippe MENEI et Dr Anne CLAVREUL.

2005: Stage pratique au sein de l'unité INSERM U646 (Angers, France)
Mécanismes d'échappement à la réponse immunitaire du glioma F98
Encadrants: Pr Philippe MENEI et Dr Anne CLAVREUL.

2003: Stage bibliographique au sein de l'unité INSERM U533 (Nantes, France).
Les cellules souches dans la thérapie cardiaque
Encadrant: Pr Patricia LEMARCHAND

SCOLARITE

2007: **Doctorat de neuroscience** (Angers, France)
2005-2007: Master recherche en biologie moléculaire et cellulaire (Angers, France)
2002-2005: Licence de biologie moléculaire et cellulaire (Angers, France)
2001: Baccalauréat Scientifique spécialité mathématiques (La Flèche, France).

COMPETENCES TECHNIQUES

Biologie moléculaire et biochimie:
- Extraction d'ARN, PCR et RT-PCR
- Dosage protéique
- Western Blot et Elisa

Biologie cellulaire et immunologie:
- Culture cellulaire: rat, souris, humaine (lignées cellulaires et cultures primaires)
- Immunocytochimie et immunohistochimie
- Hybridation in situ fluorescente
- Cytometrie en flux (analyse de marqueurs de surface et incorporation intracellulaire de nanoparticules)
- Test de prolifération
- Protocole de différenciation de cellules souches (neurogénèse, adipogénèse, osteogenénèse)
- Tests de migration et d'invasion
- Microscopie optique, à fluorescence, confocale et analyses d'images

Expérimentation animale:
- Isolation de cellules stromales mésenchymateuse de rat
- Implantation intracérébrale par stéréoaxie de modèles de gliome chez le rat et la souris
- Injection sous cutanée d'un modèle de gliome chez la souris

Nanotechnologie:
- Formulation et caractérisation de nanocapsules lipidiques et de nanoparticules polymériques
- Etude d'internalisation de nanoparticules dans des cellules stromales mésenchymateuses

ENSEIGNEMENT ET ENCADREMENT

Enseignement:

- 96 heures/an pendant les 3 années de doctorat à des étudiants de licence (disciplines: biologie cellulaire et immunologie)
- Séminaire à des étudiants de master recherche

Encadrement d'étudiants:

- 3 étudiants de pharmacie en stage d'initiation à la recherche (5 semaines chacun)
  - Optimisation d'une formulation de nanoparticules de PLGA
  - Optimisation d'une formulation de nanoparticules de PLA
  - Mise au point d'un co-marquage technique FISH et immunofluorescence
- 3 étudiants en licence professionnelle ou en BTS (11 semaines, 10 semaines et 4 semaines)
  - Devenir des cellules stromales mésenchymateuses au sein d'un modèle orthotopique de tumeur cérébrale
  - Expérimentation animale (injection par stéréotaxie et sous-cutanée)

COMPETENCES LINGUISTIQUE ET INFORMATIQUE

Langue: Anglais

Office use: Pack office, Photoshop, EndNote, Ascent Multiscan et Fluoroscan, WinMDI, Prism5, Zen 2009 light edition.

PRODUCTIONS SCIENTIFIQUES

Publications:

- **M. Roger**, A. Clavreul, M.-C. Venier-Julienne, C. Passirani, L. Sindji, P. C. Schiller, C. N. Montero-Menei et P. Menei , Mesenchymal stem cells as cellular vehicles for delivery of nanoparticles to brain tumors. **Biomaterials**, (2010), Nov;31(32):8393-401.
- **M. Roger**, A. Clavreul, C. Passirani et P. Menei , Ferrociphenol loaded lipid nanocapsules deliver by mesenchymal stem cells in brain tumor therapy. *En cours.*
- **M. Roger**, A. Clavreul, L. Sindji, P. C. Schiller, C. N. Montero-Menei et P. Menei, *In vitro* and *in vivo* interactions between Marrow-isolated adult multilineage inducibe (MIAMI) cells and glioma cells. *En cours.*
- **M. Roger**, A. Clavreul, M.-C. Venier-Julienne, C. Passirani, N. Montero-Menei et P. Menei , Drug-loaded nanoparticle systems and adult stem cells as strategies for glioma therapy, review. *En cours.*

Communications orales:

- Séminaire à l'université de Miami, Florida, 2008 (Paul C. Schiller laboratory)
  - Présentation du projet de thèse (Anglais)
- 5ème journée de l'IFR132, 2010 (Angers, France). Prix de la meilleure communication orale
  - Mesenchymal stromal cells as cellular vehicles for delivery of nanoparticles to brain tumors. **Mathilde Roger**, Anne Clavreul, Marie-Claire Venier-Julienne, Catherine Passirani, Laurence Sindji, Paul C. Schiller, Claudia N. Montero-Menei, Philippe Menei

Posters:

- Mesenchymal stromal cells and tumor microenvironment: an update, 2009 (Montpellier, France)
  - Mesenchymal stromal cells as cellular vehicles for delivery of nanoparticles to brain tumors. **Mathilde Roger,** Anne Clavreul, Marie-Claire Venier-Julienne, Laurence Sindji, Paul C. Schiller, Claudia N. Montero-Menei, Philippe Menei
- 5ème congrés national des cellules souches, 2009 (Nantes, France)
  - Mesenchymal stromal cells as cellular vehicles for delivery of nanoparticles to brain tumors. **Mathilde Roger**, Anne Clavreul, Marie-Claire Venier-Julienne, Laurence Sindji, Paul C. Schiller, Claudia N. Montero-Menei, Philippe Menei
- "Autour de la cellule souche", 2010 (Bordeaux, France)
  - Mesenchymal stromal cells as cellular vehicles for delivery of nanoparticles to brain tumors. **Mathilde Roger**, Anne Clavreul, Marie-Claire Venier-Julienne, Catherine Passirani, Laurence Sindji, Paul C. Schiller, Claudia N. Montero-Menei, Philippe Menei

Résumé : Ce travail de thèse a pour objectif le développement d'un nouvel outil thérapeutique des tumeurs cérébrales en utilisant le tropisme tumoral des cellules stromales mésenchymateuses (CSMs) pour véhiculer et distribuer des nanoparticules (NPs) chargées en principe actif. Une sous-population de CSMs humaines extraites à partir de la moelle osseuse de la crête iliaque de patients, les cellules MIAMI (« Marrow-Isolated Adult Multilineage Inducible") et deux types de NPs [NPs polymériques et nanocapsules lipides (NCLs)] ont été utilisés pour montrer la preuve de concept de cet outil thérapeutique. Nous avons montré que les cellules MIAMI incorporent efficacement ces deux types de NPs sans modification de leur potentiel souche. De plus, ces cellules sont capables de véhiculer et de distribuer les NPs *in vivo* au sein d'une tumeur cérébrale. La toxicité de ce nouvel outil a été validée *in vitro* et *in vivo* dans le modèle du gliome humain U87MG en utilisant des cellules MIAMI chargées en NCLs de ferrociphenol. La sureté de l'utilisation des cellules MIAMI, en tant que vecteurs cellulaires, a également été étudiée. Nous avons montré que les cellules MIAMI n'étaient pas capables de se transformer *in vitro* et *in vivo*. Cependant, l'interaction des cellules MIAMI avec les cellules gliales tumorales semble être gliome dépendant suggérant que des investigations supplémentaires doivent être réalisées pour s'assurer de la sureté des cellules MIAMI. L'ensemble de ce travail de thèse a montré que les cellules MIAMI combinées à des NPs pour délivrer spécifiquement un principe actif au sein d'une tumeur cérébrale constituent un outil prometteur dans la thérapie du gliome.

Mots clés : Gliome, Cellules stromales mésenchymateuses, Vecteurs cellulaires, Nanoparticules, Ferrociphenol.

Abstract : The aim of this study is to develop a new therapeutic tool for brain tumors using the migratory capacity of mesenchymal stromal cells (MSCs) towards tumor to vehicle and distribute therapeutic nanoparticles (NPs). A subpopulation of human MSCs extracted from patient iliac crest bone marrow, MIAMI cells (« Marrow-Isolated Adult Multilineage Inducible"), and two types of NPs [polymer NPs and lipid nanocapsules (LNCs)] were used to show the proof of concept of this therapeutic tool. We have shown that MIAMI cells efficiently incorporate these NPs, without modification of their stemness properties. Moreover, MIAMI cells were able to vehicle and distribute NPs i*n vivo* within the brain tumor. The toxicity of this new tool has been validated *in vitro* and *in vivo* in the human U87MG glioma model with MIAMI cells loaded with ferrociphenol-LNCs. The safety of the use of MIAMI cells, as cellular vectors, was also studied. We have shown that MIAMI cells were not able of transformation *in vitro* and *in vivo*. However, the interaction between MIAMI cells and glioma cells seemed glioma dependant suggesting that supplementary studies may be done to ensure the safety of MIAMI cells. Taken together, this work demonstrates that MIAMI cells combined with NPs to deliver specifically a drug within a brain tumor constitute a new promising tool in glioma therapy.

Keywords : Glioma, Mesenchymal stromal cells, Cellular vector, Nanoparticles, Ferrociphenol.