Role of stroma and Wound Healing in carcinoma response to ionizing radiation

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Role of Stroma and Wound Healing in Carcinoma Response to Ionizing Radiation

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"Being a scientist means living on the borderline between your competence and your incompetence. If you always feel competent, you aren't doing your job."

Carlos Bustamante

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.

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ABBREVATIONS

ADAM	A Disintegrin and Metalloproteinase
bFGF	basic Fibroblast Growth Factor
BM	Basement Membrane
BMDCs	Bone Marrow-Derived Cells
CAF	Cancer-Associated Fibroblast
COX-2	Cyclooxygenase-2
COXIBs	COX-2 Inhibitors
CTGF	Connective Tissue Growth Factor
CXCL	CXC Chemokine Receptor
CXCR	CXC Chemokine Receptor
DNA	Deoxyribo Nucleic Acid
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial To Mesenchymal Transition
EMMPRIN	Extracellular Matrix Metalloproteinase Inducer
ERKs	Extracellular-Signal-Regulated Kinases
FAP	Fibroblast Activation Protein
FDA	Food and Drug Administration
FGFR	Fibroblast Growth Factor Receptor
FMT	Fibroblast to Myofibroblast Transdifferentiation
FSP-1	Fibroblast Specific Protein 1
G-CSF	Granulocyte Colony-Stimulating Factor
GAP	GTPase-Activating Proteins
GBM	Glioblastoma Multiforme
GDI	Guanine Nucleotide Dissociation Inhibitors
GEF	Guanine Exchange Factors
GTP	Guanosine-5'-Triphosphate
Gy	Gray
HCC	Hepatocellular Carcinoma
HGF	Hepatocyte Growth Factor
HNSCC	Head and Neck Squamous Cell Carcinomas
IGFs	Insulin-Like Growth Factors
IL	Interleukin
IL-1β	Interleukin 1 Beta
IFN γ	Interferon Gamma
IR	Ionizing Radiation
JNK	c-Jun N-Terminal Kinases
kDa	KiloDalton
LOH	Loss of Heterozygosity
LOX	Lysyl Oxidase
mAb	Monoclonal Antibody
mRNA	Messenger RNA (Ribonucleic acid)
mDia	Mammalian Diaphanous
MAPK	Mitogen-Activated Protein Kinases

MCP	Monocyte Chemoattractant Protein
MGMT	Methylguanine-DNA Methyltransferase
MLC	Myosin Light Chain
MMP	Matrix Metalloproteinases
MSC	Mesenchymal Stem Cell
MVB	Multivesicular Body
NF-κB	Nuclear Factor Kappa B
NO	Nitric Oxide
NSAIDs	Nonsteroidal Anti-Inflammatory Drugs
NSCLC	Non-Small-Cell Lung Cancer
p21waf	Cyclin-Dependent Kinase Inhibitor 1 or CDK-Interacting Protein 1
PAI	Plasminogen Activator Inhibitor
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PDGFR	Platelet-Derived Growth Factor Receptor
PGE_2	Prostaglandin E_2
PI3K	Phosphoinositide 3-Kinase
PMNs	Poly Morpho Nuclear Cells
PTEN	Phosphatase and Tensin Homolog
RCC	Renal Cell Carcinoma
ROCK	Rho-Associated Protein Kinase
RhoB-/-	RhoGTPase B Deficient
RILI	Radiation Induced Lung Injury
ROCK	Rho Associated Protein Kinase
ROS	Reactive Oxygen Species
R-Smad	Receptor-Regulated SMADs
RT	Radiation Therapy
SDF-1	Stromal-Derived Factor-1
siRNA	Small Interfering RNA
SOD	Superoxide Dismutase
STAT3	Signal Transducer and Activator Of Transcription 3
α-SMA	Alpha-Smooth Muscle Actin
TGF-β	Transforming Growth Factor-β
TGFβR	Transforming Growth Factor-β Receptor
TIMP	Tissue Inhibitor of Matrix Metalloproteinase
TK	Tyrosine Kinase
TKI	Tyrosine Kinase Inhibitor
TME	Tumor Microenvironment
TNF-α	Tumor Necrosis Factor-α
TNFR	Tumor Necrosis Factor Receptor
TSP	Thrombospondin
uPA	Urokinase Plasminogen Activator
uPAR	Urokinase Plasminogen Activator Receptor
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
WT	Wild Type
ZEB	Zinc finger E-box-Binding Homeobox

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Foreword

Wound healing and carcinogenesis are defined as complex, adaptive processes which are controlled by intricate communications between the host and the tissue microenvironment. During a normal wound healing process, regeneration and repair of a wound, depends on a variety of signals which coordinate the response to injury. These processes entail cell proliferation, survival, and migration which are controlled by growth factors, cytokines as well as inflammatory and angiogenic signals. These signals are derived from multiple intra and extracellular components embedded in the microenvironment of wounds and are also involved in cancer. Therefore, a number of phenotypic similarities are shared by wounds and cancers in cellular signaling and gene expression. These similarities between wound healing and carcinogenesis were first recognized by Haddow, and the notion that 'cancer are wounds that do not heal' was defined by Dvorak [1, 2]. Most recently genomic approaches revealed that wound healing and stromal signatures were able to predict for metastatic progression [3] and resistance to neo adjuvant chemotherapy in breast cancers [4]. This underscores the importance of microenvironment in tumor progression [5] and response to antitumor treatments and suggests that the radiation induced wound healing response could be responsible for tumor radio resistance.

Radiotherapy is the second most effective modality of cancer treatment after surgery and can be used either alone or in combination with chemotherapy. The main anti-tumor effect of radiation therapy is the induction of tumor cell death but recent findings suggest that radiotherapy also rapidly and persistently modifies the tissue microenvironment. These modifications affect cell phenotype, tissue metabolism, bidirectional exchanges and signalling events between cells [4]. While there is evidence indicating that these changes might contribute to the antitumor effects of radiotherapy, some clinical and experimental observations indicates that irradiated stroma might exert tumor-promoting effects [4].

It has been shown previously that in glioblastoma, radioresistance signals are transmitted by $\alpha 5\beta$ integrins 3, 5 ILK and then relayed by HIF-1α and RhoB [6, 7]. R Kolesnick and F Paris groups showed the importance of the vascular microenvironment in tumor response to radiotherapy [8, 9], which is further strengthened by the fact that under the influence of PI3K a portion of tumor cells within the perivascular niche presents an extended shutdown of the cell cycle in G2/M and becomes resistant to IR; while majority of the tumor cells die after radiation [10]. These data suggest the existence of a link between tumor microenvironment, radioresistance and cell cycle control after irradiation.

Indeed in a mouse model mimicking tumor recurrence after irradiation where the tumor bed is irradiated tumor recurrence seems to depend upon the development of immature blood vessels initiated by immobilization of circulating cells from bone marrow and MMP-9 induced matrix remodeling. [11]. These later data is in line with our previous work showing the importance of cell cycle regulation and activation of the PI3K/AKT pathway after irradiation [12-14].

MH. Barcellos-Hoff`s Group has indeed shown a major contribution of TGF-β1 produced by irradiated stroma to carcinogenesis [15-17] as high dose of radiotherapy is known to stimulate TGF-β1 production [18]. TGF-β1 is the prototype of pro-wounding molecules shown to be the main inducer of reactive stroma, by not only affecting chemotaxis of fibroblasts, but also their trans-differentiation into reactive fibroblasts, termed myofibroblasts [19]. TGF-β1 also regulates epithelial phenotype and has been especially described as a potent stimulatory molecule during the late phase of carcinogenesis and metastasis dissemination.

Beside TGF-β1 signal, the contribution of the Rho pathway to radiation response has been proposed by our group and others [20]. Rho GTPases are a family of signalling mediators implicated in regulating cytoskeletal dynamics, motility, cell division, and transcriptional regulation. Amonst various Rho GTPases, RhoB has been described as a determinant of intrinsic tumor radiosensitivity [21, 22]; which acts downstream of integrins alpha 3, 5 [23] and HIF-1α [24] in tumor cells. More specifically, RhoB expression is increased by a variety of extra-cellular stimuli which include irradiation, epidermal growth factor (EGF) and transforming growth factor β (TGF-β) [18, 25]. Most Rho proteins are modified by the covalent attachment of a geranylgeranyl group, but RhoB can exist in either a geranylgeranylated (RhoB-GG) or a farnesylated (RhoB-F) form. RhoB-F localizes to the cell membrane, modulates actin cytoskeleton, activates nuclear factor kappa B and promotes cell growth [26-28]. In contrast, RhoB-GG localizes to endosomes and induces cell apoptosis [26]. A role for RhoB in TGF-β induced cell responses such as epithelial-mesenchymal transition (EMT) and apoptosis was suggested by a series of DNA microarray studies, which showed that RhoB expression was upregulated by TGF-β in a variety of cell types such as keratinocytes, mouse mammary gland epithelial cells, hepatoma cells, and dermal fibroblasts [29]. TGF-β also stimulates actin stress fiber formation in Ras-transformed cells in a way which is associated with upregulation of RhoB [30, 31].

Moreover, our previous genomic studies allowed us to identify the members of the Rho/ROCK cascade involved and showed a preferential activation of RhoB and ROCK 1 in tissues and cells from fibrotic tissues [32, 33]. Infact pharmacological inhibition of Rho by statins prevents the development of pulmonary fibrosis and radiation induced intestinal fibrosis [34, 35] and reverses radiation induced fibrosis [36].

Studies on tumor radiation sensitivity have focused for about 2 decades on intrinsic tumor radiation sensitivity which highlighted the roles of the EGF/Ras/PI3k/AKT pathways in tumor radiation sensitivity. In parallel, several groups have also highlighted the role of tumor microenvironment of tumor radiosensitivity. Mostly these groups are focusing on anti angiogenic approaches which may potentially increase the normal tissue toxicities [37-40]. More recently our group have highlighted the importance of mitotic catastrophe in tumor response to radiotherapy, we demonstrated that agents targeting the G2/M check points are good candidates for radiosensitization [41, 42]. We have also showed that normal tissue response to radiotherapy can activate a wound healing response. The current project aims at merging our expertise on both normal tissue and tumor; studying the role of the tumor microenvironment on tumor cure after radiation. This work will enable us to provide information about the involvement of the wound healing response to radiation on tumor radiation sensitivity.

Furthermore, in recent decade a lot of work has been focused on circulating tumor cells (CTCs), which acts as a liquid biospsy and are detectable in many cancers [43] (including non-small cell lung cancer (NSCLC)). Detection of CTCs had been linked to prognostic significance [44, 45] in different cancers. Previously, not much work has been done on the effect of radiation therapy on CTC level related to tumor microenvironmental changes because of the lack of a suitable detection method, as there is no useful data available on CTCs in patients undergoing RT.

Currently, the only US FDA approved technique is CellSearch platform which uses epithelial cellular adhesion molecule EpCAM [46] for CTC detection. However, it lacks the detection of tumor cell clusters or “circulating tumor microemboli” (CTMs) [47] and can therefore, significantly underestimate total CTC numbers. Furthermore, CTCs in NSCLC often lose epithelial markers [48-50] allowing them to evade detection. Moreover, there were major inconsistencies between different methods. In samples where CellSearch detected few or no cells, manual counts typically detected abundant CTCs. These findings are consistent with another report indicating limited efficiency of CellSearch in NSCLC CTC detection [51]. Therefore we also evaluated and compared a newer PCR detection method developed by Charles Decreane at institute curie with CellSearch.

I. INTRODUCTION

Lung Cancer

Incidence and prevalence

Lung cancer has been the most common cancer in the world for several decades. According to the most recent statistics published (GLOBOCAN 2008) [52]. Worldwide 1,092,000 men and 516, 000 women were diagnosed with lung cancer which represents 12.7% of all new cancer cases. About 1.4 million people died from the disease, which represented 18% of all cancer deaths that year [53]. In 2010, the number of deaths from lung cancer worldwide increased to 1.5 million, representing 19% of all cancer death that year [54]. There are now fewer lung cancer cases in more developed regions (Northern America, Japan, Europe, and Australia/New Zealand) than in rest of the world. The statistics showed that there are 16.2% or 479,000 of all cancers in males in the developed countries as compared to 16.8% or 612,000 in developing countries. In both men and women, the incidence of lung cancer is low in persons under age 40 and increases up to age 70 or 75.

Causes

Lung cancer is often treacherous, as it may not produce any symptoms until the disease is well advanced. Approximately 7-10% of patients with lung cancer are asymptomatic, and their cancers are diagnosed incidentally after a chest radiograph performed for other reasons.

Causes of lung cancer include the following:

- Smoking (78% in men, 90% in women)
- Asbestos exposure
- Radon exposure
- Halogen ether exposure
- Chronic interstitial pneumonitis
- Inorganic arsenic exposure
- Radioisotope exposure, ionizing radiation
- Atmospheric pollution
- Chromium, nickel exposure
- Vinyl chloride

Classification of lung cancer

Recently a significant amendment in pathologic classification of lung cancer arose after the publication of a new lung adenocarcinoma classification in 2011, under the sponsorship of the American Thoracic Society (ATS), International Association for the Study of Lung Cancer (IASLC) and the European Respiratory Society (ERS). This new classification has paved the path for the role of personalized medicine for patients with lung cancer. This classification takes into account the importance of histologic classification and molecular testing in stratifying patients for specific therapies. This has been the central theme of this new classification.

This classification is divided into two modules based on the primary lung cancer diagnosis:

1) Small biopsy and cytology specimens for patients with advanced-stage lung cancer

Tables 1 and 2

2004 WHO Classification Including Updated IASLC/ATS/ERS Terminology	Morphology/Stains	IASLC/ATS/ERS Terminology
Adenocarcinoma	Morphologic adenocarcinoma patterns clearly present	Adenocarcinoma (describe identifiable patterns present)
Mixed subtype		
Acinar		
Papillary		
Solid		
Micropapillary		
Lepidic (nonmucinous)		Adenocarcinoma with lepidic pattern (if pure, add note: an invasive component cannot be excluded)
Lepidic (mucinous)		Invasive mucinous adenocarcinoma (describe patterns present; use term "mucinous adenocarcinoma with lepidic pattern" if pure lepidic pattern is present)
No 2004 WHO counterpart; most will be solid adenocarcinomas	Morphologic adenocarcinoma patterns not present (supported by special stains such as TTF-1)	NSCLC-favor adenocarcinoma
Squamous cell carcinoma	Morphologic squamous cell patterns clearly present	Squamous cell carcinoma
No 2004 WHO counterpart	Morphologic squamous cell patterns not present (supported by stains such as p40)	NSCLC-favor squamous cell carcinoma
Large-cell carcinoma	No clear adenocarcinoma, squamous, or neuroendocrine morphology or staining pattern	NSCLC-NOS

Table 1: Specific terminology and criteria for adenocarcinoma, squamous cell carcinoma, and NSCLC-NOS in small biopsies and cytology. IASLC/ATS/ERS, International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society; NOS, not otherwise specified; NSCLC, non–small-cell carcinoma; TTF-1, thyroid transcription factor 1. **Adapted from** [55, 56]

2004 WHO Classification	Small Biopsies/Cytology: IASLC/ATS/ERS
Small-cell carcinoma	Small-cell carcinoma
LCNEC	NSCLC with NE morphology and positive NE markers; possible LCNEC
LCNEM	NSCLC with NE morphology (negative NE markers). Comment: This is an NSCLC in which LCNEC is suspected, but stains failed to demonstrate NE differentiation
Adenosquamous carcinoma	Morphologic squamous cell and adenocarcinoma patterns present: NSCLC-NOS. Comment: Adenocarcinoma and squamous components are present and this could represent adenosquamous carcinoma
No 2004 WHO counterpart classification	Morphologic squamous cell or adenocarcinoma patterns not present but immunostains favor separate glandular and adenocarcinoma components, NSCLC-NOS (specify the results of the immunohistochemical stains and the interpretation). Comment: This could represent adenosquamous carcinoma
Sarcomatoid carcinoma	NSCLC with spindle and/or giant cell carcinoma (mention if adenocarcinoma or squamous carcinoma is present)

Table 2: IASLC/ATS/ERS classification for small biopsies/cytology comparing 2004 WHO. **Adapted from** [55, 56]

2) Resection specimens for early-stage patients who are eligible for surgical resection Table 3

Preinvasive lesions
Atypical adenomatous hyperplasia
Adenocarcinoma in situ (≤ 3 cm; formerly solitary BAC)
Nonmucinous
Mucinous
Mixed mucinous/nonmucinous
Minimally invasive adenocarcinoma (≤ 3 cm lepidic-predominant tumor with ≤ 5 mm invasion)
Nonmucinous
Mucinous
Mixed mucinous/nonmucinous
Invasive adenocarcinoma
Lepidic predominant (formerly nonmucinous BAC pattern with > 5 mm invasion)
Acinar predominant
Papillary predominant
Micropapillary predominant
Solid predominant with mucin production
Variants of invasive adenocarcinoma
Invasive mucinous adenocarcinoma (including formerly mucinous BAC)
Colloid
Fetal (low and high grade)
Enteric

Table 3: IASLC/ATS/ERS Classification of lung adenocarcinoma in resection specimens. **Adapted from** [55, 56]

An important point in this classification is the concept that personalized medicine in advanced lung cancer is determined by genetics and histology and that strategic tissue handling of small biopsies is critical for diagnosis and for molecular studies.

The majority of late stage lung cancer patients die within 18-months of diagnosis [52]. These subtype differerences can be attributed to the site of origin and patient characteristics, SCC being associated with smoking and originates from bronchial epithelial cells, whilst adenocarcinoma is mainly derived from alveolar/bronchial cells [57]. Principal sites for NSCLC metastasis are bone, brain, adrenal gland and the liver. Evidence states that these sites of preferential metastasis are determined by interactions between cancer-cell-surface proteins and capillary lining endothelial-cell receptors at distant sites. Although a uniformly accepted staging of lung cancer is crucial for management of the disease, a considerable proportion of patients with lung cancer show tumour spread at the time of diagnosis and 40% of patients with non-small-cell lung cancer have distant metastases at presentation [58].

Management of lung cancer

Surgery, chemotherapy, and radiation are the main stay treatment options for NSCLC. As most lung cancers cannot be cured with currently available therapeutic modalities, the appropriate application of skilled palliative care is an important part of the treatment of patients with NSCLC.

❖ *Surgery*

Surgery is the treatment of choice for stage I and stage II NSCLC. Several different types of surgery can be offered such as:

- Lobectomy – removing a section of the lung
- Pneumonectomy – removing the entire lung
- Wedge resection – removing part of a lobe

❖ *Chemotherapy*

At some point during the course of their illness, approximately 80% of all patients with lung cancer are considered for chemotherapy.

❖ *Radiation*

In the treatment of stage I and stage II NSCLC, radiation therapy alone is considered only when surgical resection is not possible [59]. Among those who are not candidates for surgery, radiation is a reasonable option for lung cancer treatment [60].

Radiotherapy

The radiotherapeutic approach for the eradication of malignant cells was first introduced during the late 19th century. Today radiotherapy is used as the primary therapy or in combination with surgery, chemotherapy, hormone therapy, and therapy with antibodies or a mixture of all strategies. Most cancer types can be treated with radiotherapy. Radiotherapy can be given either as a curative modality, adjuvant, or as a palliative treatment in lung cancer.

Non-small cell lung cancer (NSCLC) constitutes approximately 85% of all lung cancers. Roughly 1/3 of these patients have early stage disease (stages I and II) at the time of presentation and surgery is the preferred mode of treatment, whereas radiotherapy is reserved for medically inoperable patients. Radiotherapy is reserved as a palliative treatment for another 1/3 of patients presenting with disease at advance stage. The rest of the patients with locally advanced disease (stage III) and unresectable tumor, radical intent radiotherapy is the mainstay of treatment. A brief overview of treatment schematic for NSCLC is shown below (Figure 1), as clinical staging and histopathological grading greatly influence the choice of treatment modalities and outcome.

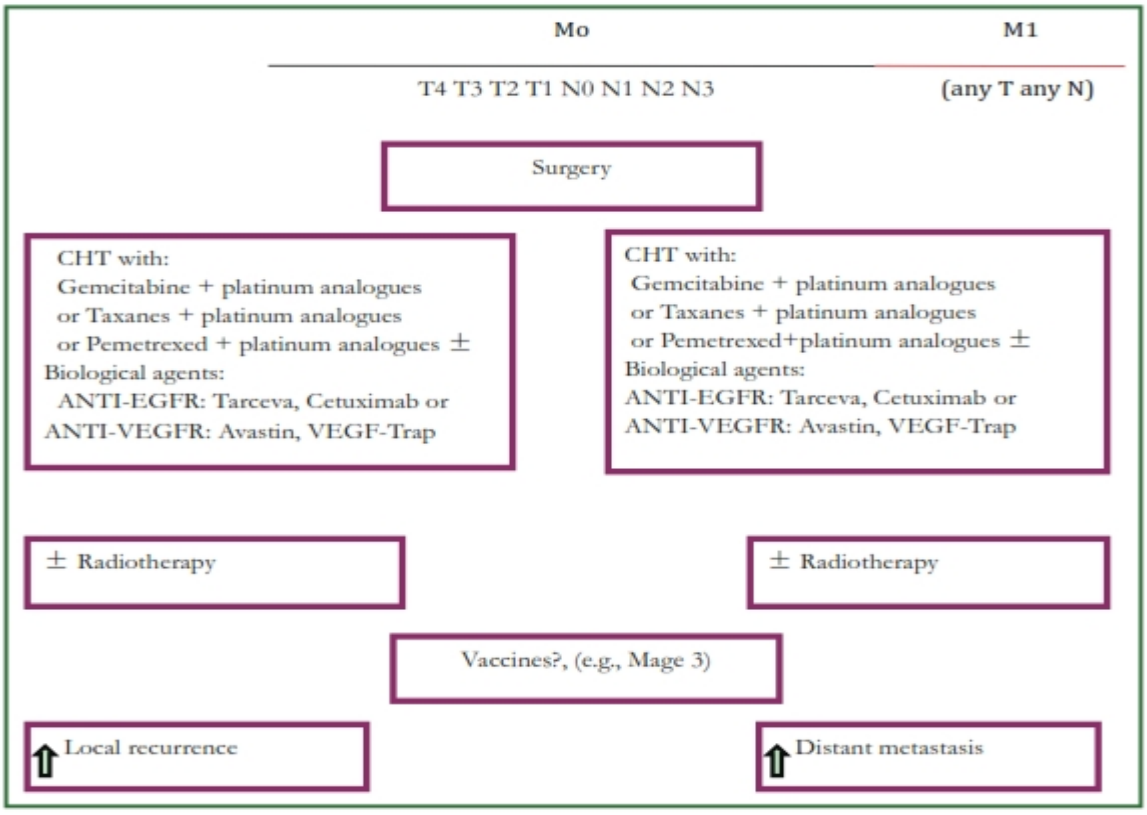


Figure 1: Treatment algorithm for NSCLC. (T, tumor; N, lymphnodes; M, metastasis).
Adapted from [61]

Mode of action

Radiation damage is a complex multi-faceted biological effect. Radiation damage occurs either through DNA damage caused directly by ionizing the atoms which make up the DNA chain or it can be a result of indirect ionization by ionization of water, forming free radicals, which then damages the DNA [62]. Several treatment specific factors (e.g., overall treatment time, total radiation dose, dose per fraction,) and patient-specific characteristics (e.g., performance status, disease stage, histology) are known to influence the probability of local tumor control by radiation therapy [63, 64]. It is well known that the biological response to radiotherapy influences the outcome of radiation therapy; e.g. clonogenic cell growth can occur following fractionation, and if the cancer is growing exceedingly fast then it may grow between each daily fraction of treatment.

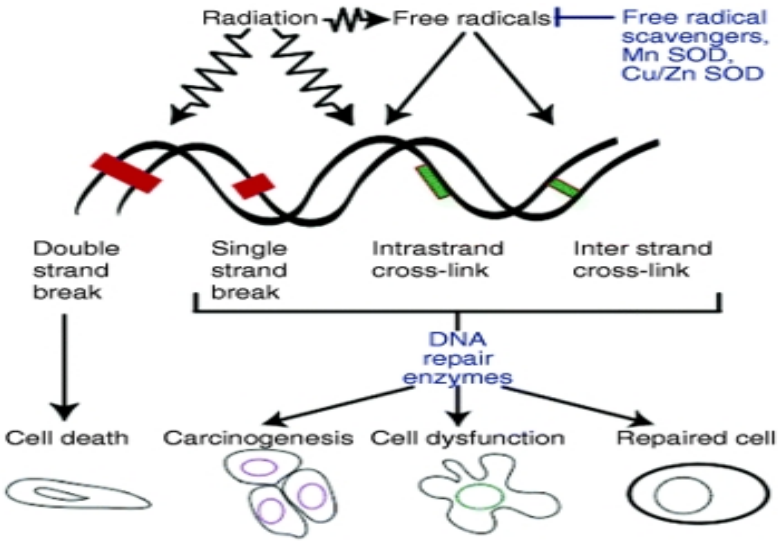


Figure 2: DNA damage due to radiotherapy. Radiation induces single and double stranded DNA breaks and DNA strand cross-linking. **Adapted from** [65]

Methods of delivery

Following methods are used in clinical practice to deliver radiotherapy, depending upon tumor size, location, patient status and stage of cancer

a) **Conventional external beam radiation therapy (2DXRT)** is delivered via two-dimensional beams using linear accelerator machines.

b) **Stereotactic radiation** is a specialized type of external beam radiation therapy. It uses focused radiation beams targeting a well-defined tumor using extremely detailed imaging scans. Radiation oncologists perform stereotactic treatments, often with the help of a neurosurgeon for tumors in the brain or spine. There are two types of stereotactic radiation. **Stereotactic radiosurgery** (SRS) is when radiation oncologists use a single or several stereotactic radiation treatments of the brain or spine. **Stereotactic body radiation therapy** (SBRT) refers to one or several stereotactic radiation treatments with the body, such as the lungs [66].

c) **Virtual simulation, 3-dimensional conformal radiation therapy, and intensity-modulated radiation therapy**

Radiation therapy has been revolutionized due to the ability to delineate tumors and adjacent normal structures in three dimensions using specialized CT and/or MRI scanners and planning software.

- **Virtual simulation**, the most basic form of planning, allows more accurate placement of radiation beams than is possible using conventional X-rays.
- **3-dimensional conformal radiation therapy (3DCRT)**, is an enhancement of virtual simulation, in which the profile of each radiation beam is shaped to fit the profile of the target. Thus the relative toxicity of radiation to the surrounding normal tissues is reduced, allowing a higher dose of radiation to be delivered to the tumor than conventional techniques would allow [67]
- **Intensity-modulated radiation therapy** (IMRT) is an advanced type of high-precision radiation that is the next generation of 3DCRT. IMRT also improves the ability to adapt to the treatment volume to concave tumor shapes, for example when the tumor is wrapped around a vulnerable structure such as the spinal cord or a major organ or blood vessel [68].

d) **Particle therapy** (proton therapy being one example), energetic ionizing particles (protons or carbon ions) are directed at the target tumor. Proton beams differ from photon beams mainly in the way they deposit energy in living tissue. Whereas photons deposit energy in small packets all along their path through tissue, protons deposit much of their energy at the end of their path (called the Bragg peak) and deposit less energy along the way (into the healthy tissue surrounding the target tissue). In theory, use of protons should reduce the exposure of normal tissue to radiation, possibly allowing the delivery of higher doses of radiation to a tumor [79].

e) **Brachytherapy** uses high-dose intracavitatory radiation or radioactive implants and is used to deliver radiotherapy directly to prostate tumours, soft tissue sarcoma, breast tumours and cervical tumours [69, 70].

f) **Radioisotope therapy (RIT)** is a form of targeted therapy. Targeting can be achieved by attaching the radioisotope to another molecule or antibody to guide it to the target tissue. The radioisotopes are delivered through infusion or ingestion. Examples are the infusion of metaiodobenzylguanidine (MIBG) to treat neuroblastoma, of oral iodine 131 to treat thyroid cancer or thyrotoxicosis, lutetium-177 and yttrium-90 to treat neuroendocrine tumors (peptide receptor radionuclide therapy) [71].

Side effects

Radiation therapy is associated with both acute toxicity and long-term sequel and depend on the radiosensitivity of the body sites being treated, the volume of normal tissue irradiated, the total dose and the rate of dose accumulation [74]. Side effects are most evident in rapidly proliferating tissues, such as the skin, mucosa and bone marrow, but may arise in almost any organ system Table 4. **Early effects** are related to the loss of stem cells in quickly renewing tissues (such as epithelial layers) or from inflammatory cytokine release (such as oedema, erythema, fatigue) [72]. Renewal of stem cells occurs either through division of surviving cells within the treatment area or through migration of stem cells from outside the treated area [75].

	Acute
General	*Anorexia, nausea, malaise and pain*
Skin	*Erythema, dry desquamation, pigmentation and hair loss*
Mucosa	*Oesophagitis and diarrhoea*
Bone	*Myelosuppression*
	Long-term
Skin	*Ischaemia, ulceration and telangiectasia*
Bone	*Necrosis and fracture*
Cardiovascular	*Atherosclerosis Pericardial fibrosis and cardiomyopathy*
Respiratory	*Radiation pneumonitis and lung fibrosis*
Gastrointestinal	*Xerostomia, sialitis, mouth ulceration, bowel stenosis, fistula and diarrhoea*
Genitourinary	*Cystitis, nephropathy, sexual dysfunction, dyspareunia, vaginal stenosis, infertility and menopause*
Nervous system	*Myelopathy, brain lesions, brainstem encephalopathy, sensorineural hearing loss and otitis media*

Table 4: Complications of radiotherapy. **Adapted from** [65]

Late effects occur due to processes that take months to years to develop, either because the tissue renews slowly due to chronic inflammatory processes, their relation to connective tissue cells or genomic damage. These include but not limited to fibrosis [73] and tissue necrosis. Patients undergoing radiotherapy display a large patient-to-patient variability in their risk of developing tissue reactions as normal body tissue varies in its response to radiation.

Tissue component	Principle role	Regulated by
Extracellular matrix (ECM)	Tissue structure — collagen	NO and TNFα, IFNγMMP and TIMP
Inflammatory cells	healing — removal of dead cells, prevention of infection	Cytokines growth factors and TGβ
Endothelial cells	blood vessels — capillaries, angiogenesis	VEGF and FGF
Fibroblasts	secretion of ECM proteins degradation of ECM proteins: MMP, TIMP	TGFβ and PDGF, EGF and FGF

Table 5: Tissue components affected by radiotherapy. **Adapted from** [65]

Radiotherapy protocols should balance the expected outcome of the radiotherapy with the expected side effects. More severe side effects may be tolerated following curative radiotherapy but are not acceptable for palliative radiotherapy.

Wound Healing

Injury to the tissues initiates an erudite repair process that restores the damage. The different processes involved in repair are tightly regulated and synchronized to restore the integrity of the affected tissue. Wound healing is a dynamic interactive process that involves a series of molecular and cellular events. All of which revolves around the reorganization of cells and extracellular matrix, involving biological signs to repair the tissue. Defects in wound repair frequently affects not only aged individuals, patients with diabetes or immunosuppression, but patients who receive chemotherapy or radiotherapy [76] are also affected; owing to the formation of hypertrophic scars and keloids. Malignant transformation is a particularly severe complication of non-healing wounds and a frequent event is the development of cancer in fibrotic tissue [77].

This suggests that common cellular and molecular mechanisms are active in wounds and in cancer tissue. Alexander Haddow suggested that "tumor production is a possible overhealing" [78]. A decade later Dvorak postulated that "tumors are wounds that do not heal" [1]. He further on clarified that the composition of the tumour stroma strongly resembles the granulation tissue of healing skin wounds. However, in contrast to healing wounds, the process is not self-limiting in cancer tissue, resulting in uncontrolled cell proliferation, invasion and metastasis. Recently, microarray technology revealed not only remarkable similarities between early wounds and cancer, but important differences are also highlighted [80-83]. So understanding the mechanisms of normal wound healing will be an asset in understanding tumor biology.

Stages of wound healing

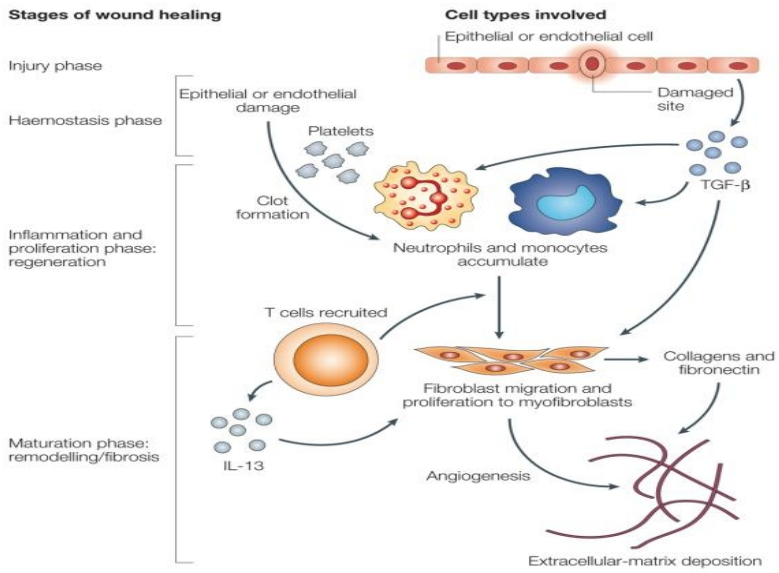


Figure 3: Stages of wound healing. **Adapted from** [84]

Wound healing of the tissue follows a specific time sequence (Figure 4), and this very complex repair processe is sub-divided into three phases that overlap in time and space. The normal wound healing response is depicted in (Figure 3 and 4). It represents a range of events that overlap in time and activity with one stage merging into the next [85, 86].

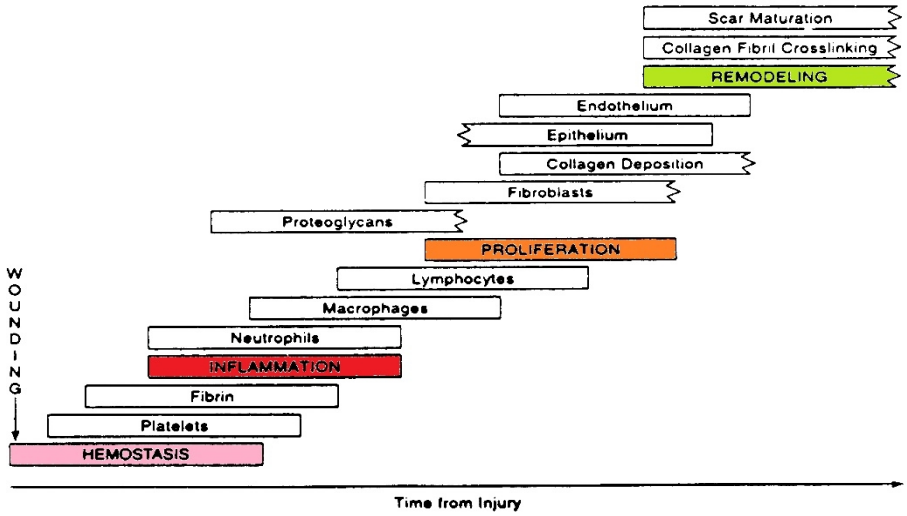


Figure 4: Normal wound healing requires multiple events, many of which occur simultaneously or overlap in time. **Adapted from** [87]

Phase I: Inflammation

The inflammatory phase is a period of active cellular migration and it takes place within the first three days post injury [88]. It accomplishes both hemostasis and formation of a temporary matrix on which fibroblasts and monocytes migrate [89]. At the time of wounding, hemostasis is achieved by the formation of a platelet plug [90]. Blood products released into the wound initiate the coagulation cascade. Platelets not only assist in clotting, but also release cytokines such as PDGF and TGF-β [86, 91], which act as chemotactic agents in the recruitment of inflammatory cells, epithelial cells and fibroblasts [92]. Blood clots consist predominantly of crosslinked fibrin and plasma fibronectin secreted primarily by fibroblasts but also include other components, such as the ECM proteins vitronectin and thrombospondins. The PMNs (macrophages, monocytes, neutrophils, and lymphocytes) are activated by the proinflammatory cytokines IL-1β, TNF-α and IFN-γ [93]. About two days after injury, macrophages remain the predominant cells in the wound. They are stimulated by the hypoxia in the wound and release IL-1 (an angiogenesis promotor) and bFGF (a chemotattractant for fibroblasts and endothelial cells). Worth noting here is that deposition of the fibrin and fibronectin matrix is an acute and transient event in normal wound repair, but a chronic event and a hallmark of most cancers [94].

Phase II: Proliferative phase (Tissue formation)

The phase of proliferation (formation of granulation tissue), occurs in the fourth to seventh day after the injury. It is characterized by angiogenesis, the proliferation of fibroblasts and the formation of collagen resulting in complete wound closure. It continues for at least 3 weeks, and the major event is the accelerated production of collagen, which is enhanced in the presence of PDGF and TGF-β [95]. Capillary formation continues, fibroblasts proliferate whereas mast cells and macrophages remain activated [96]. Angiogenesis is further stimulated by local hypoxic conditions which may be present [86]. Myofibroblasts also appear within the wound and stimulate contraction of the wound and reduce the distance that must be bridged by forming collagen [97]. In addition, the presence of growth hormone may stimulate enhanced collagen deposition by fibroblasts [98].

Phase III: Maturation and remodelling

The maturation stage begins around week three and lasts for 2 years. A subset of wound fibroblasts differentiates into myofibroblasts (Figure 5), which are responsible for wound contraction and for the deposition of additional matrix proteins. Collagen continues to mature with additional crosslink formation [86].

The acellular matrix is actively remodelled from a mainly type III collagen backbone to one which predominantly composed of type I collagen [99]. This process is carried out by matrix metalloproteinases (MMPs) which are secreted by fibroblasts, macrophages and endothelial cells [100]. To sum up, there is a reduction in the number of fibroblasts and macrophages, an increase in collagen content, and the wound regains most but not all of the strength of normal tissue.

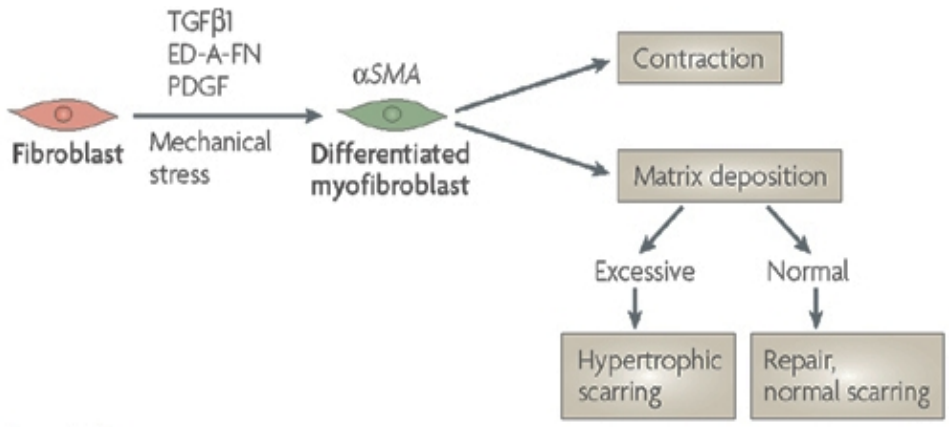


Figure 5: Myofibroblast differentiation in healing wounds. **Adapted from** [101]

Radiotherapy and Wound Healing

Despite the fact that radiation therapy is used to kill cancerous cells, it also damages healthy cells as well. This leads to many acute and chronic side effects. One such serious complication is difficulties in wound healing. The long-term effects of radiotherapy include but not limited to skin atrophy, microvascular damage and soft tissue fibrosis.

Radiotherapeutic injury is a multifaceted process that occurs in organised tissues consisting of a large number of interrelating cellular lineages, as well as a multitude of biologically active extracellular molecules. The radiotherapeutic response of normal tissues comprise of two partially interacting components. **The first** is a process that resembles the healing of traumatic wounds after perturbation by the radiation treatment. **The second** is a set of specific injuries that affect almost all cellular and extracellular components within the irradiated volume.

Radiotherapeutic injury and wound healing

The response of normal tissues radiation injury diverge in many instances from a traumatic wound healing response, yet many processes are similar and/or occur in a similar sequence. A major differential effect between radiation injury and traumatic wounds is the accumulating and repetitive nature. That's why the tissue irradiated at the beginning of the radiotherapy is very different from the tissue that is irradiated towards the end. During radiation therapy, the inflammatory phenomena does not dissipate within 24 hrs, thus leading to an accumulating response known as '**fractionated inflammatory insult'**. This Inflammation further aggravates the radiation response by augmenting endothelial dysfunction and by increasing the levels of cytokines and growth factors, such as TGF-β, thus delaying the process of re-epithelialisation.

Effect of radiotherapy on early wound matrix

High dose radiotherapy does not induce the rapid granulation tissue response that occurs in the milieu of acute traumatic wounds. Radiation inhibits fibrogenesis and angiogenesis in a dose and fractionation-dependent manner. Wound contraction can hasten the process of re-epithelialisation of traumatic wounds and suppression of the evolving wound matrix by radiation [102, 103] results in disruption of the actin microfilaments within wound fibroblasts. This could lead to impaired wound contraction (Figure 6).

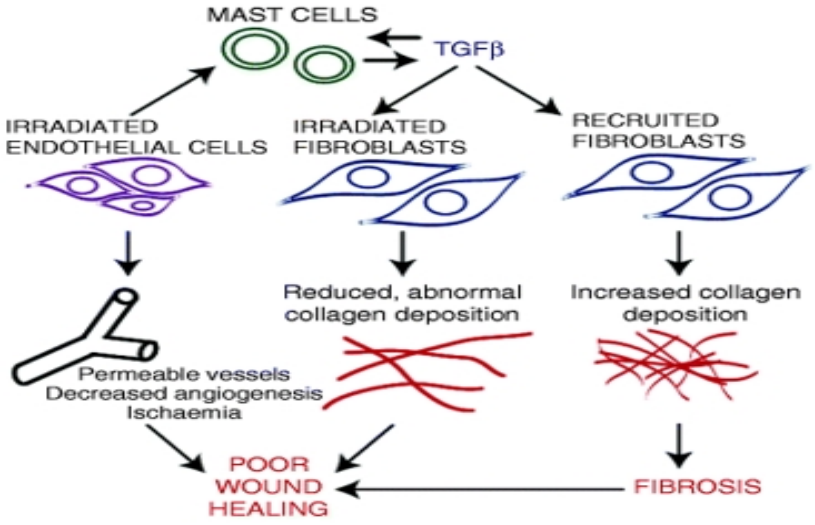


Figure 6: Cellular components involved in radiation induced poor wound healing.
Adapted from [65]

Delayed wound healing

Wound healing is orchestrated in an ordered sequence of cellular interactions. A key difference between wound healing and cancer is that wound healing is a self-limiting process; whereas, tumours continue to expand, evolve and spread Table 6. This difference is correlated with the alterations in components of the microenvironment. In wound healing, inflammation resolves once re-epithelialization is complete, but this is not the case during tumorigenesis. Radiation injury interrupts this highly controlled sequence of events, resulting in persistent inflammatory responses and delayed wound healing [104]. The early effects of radiation may be due to disruption of the inflammatory and proliferative phases. Late effects such as reactive fibrosis occur in face of radiation induced wound milieu formation and/or chronic inflammation, because high radiation doses can affect fibroblasts and other cells involved in tissue repair permanently.

It has also been suggested, that overexpression of TGF-β1 in irradiated tissues, induces fibroblast proliferation through an expansion of the progenitor fibroblast pool and premature differentiation of progenitor fibroblasts [105]. Pathological radiation fibrosis in humans is associated with increased collagen synthesis, altered remodelling and sequential activation of key fibrogenic growth factors and cytokines, including TGF β1 and CTGF. It is assumed that processes deregulated a few weeks post-radiotherapy are responsible for complications presenting decades later. It is likely that molecular pathways exist that are common to many fibrotic responses, and that these involve the signalling networks as discussed [106].

Cancer	Wound healing
Sustained proliferative signalling	Transient proliferative signalling
Evasion of growth suppressors	Transient evasion of growth suppression
Invasion and metastasis	Activation of cell migration without invasion or metastasis; basement membrane repair*
Enabling replicative immortality	No
Inducing angiogenesis‡	Yes
Resisting cell death	Transient decrease in terminal differentiation; transient increase in cell death

Table 6: Comparison between the hallmarks of cancer and wound healing.**Adapted from** [111]

* The absence of invasion and metastasis in wound healing is linked to the absence of epithelial–mesenchymal transition.

‡ In addition to angiogenesis, lymphangiogenesis is stimulated in both cancer and wound healing.

Microenvironment

Like the developmental processes and tumor growth, wound healing involves intricate and balanced interactions between cells and their microenvironment [107-109]. Through these interactions the cells are being directed to differentiate, proliferate or remain quiescent, and assume the architecture and function of that organ [107-110]. Wound healing and tumorigenesis are dynamic events that require interactions with a wide variety of different cell types, including epithelial cells, fibroblasts, endothelial cells and immune cells. Chemokines and cytokines that are released from epithelial cells during injury are very similar to the ones found in invading tumours. All this integral relationship occurs in the milieu of microenvironment of host and depicts the outcome of therapeutic modalities in cancer treatment.

Recently it has been proposed that tumor microenvironment displays a striking resemblance to the disrupted wound healing in normal tissues [111]. The tumor microenvironment was lately recognized as the product of a developing crosstalk between different cells types. So, understanding the biology of microenvironment is an important asset for future cancer treatment modalities such as radiotherapy.

Tumor microenvironment

The concept of the tumor microenvironment originated from Paget's "seed and soil" theory. It implies that tumor microenvironment is as important as tumor cell itself for metastasis initiation and progression. The tumor microenvironment is an emergent concept that defines the behavior of tumor importantly by the surrounding milieu along with the genetics of the tumor [112]. The tumor microenvironment is a vibrant network composed of the tumor cells, stromal tissue (immune cells, fibroblasts, cytokines, and vascular tissue), as well as the surrounding extracellular matrix. It is also characterized by unique properties namely hypoxia, low extracellular pH, low glucose concentration and necrosis.

The microenvironment of each organ and tissue develops to specifically support the function of those cells, while tumor microenvironments are subverted to promote tumor growth and expansion at the expense of normal tissue. Radiation therapy changes both, frequently in a manner that is reminiscent of processes associated with wound healing or inflammation. Defining the nature of the irradiated microenvironment provides a means of further targeting therapies directed towards inhibiting normal tissue toxicity or improving tumor control (Figure 7).

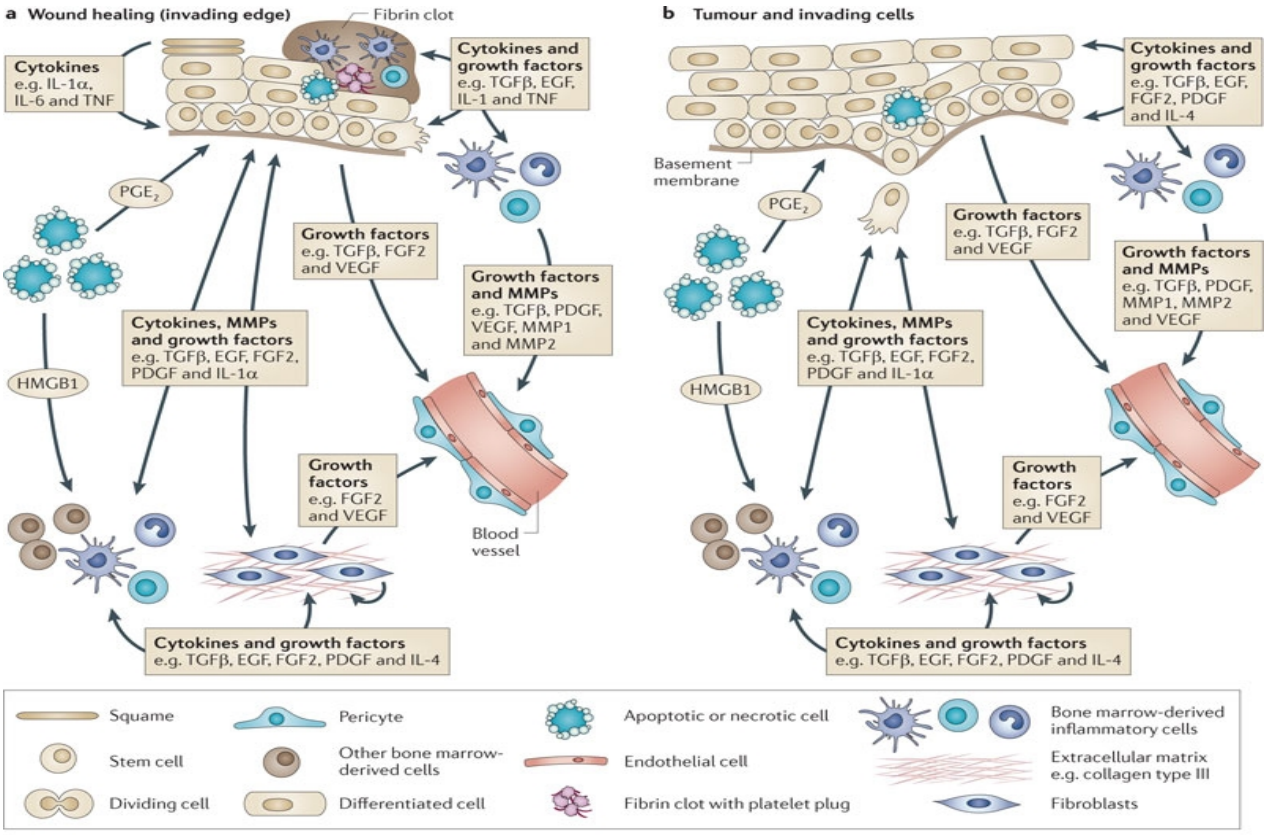


Figure 7: Comparison of the microenvironments of a healing wound and an invading tumour margin. **Adapted from** [111]

Overview of microenvironment components

Basically, the tumor microenvironment is composed, in addition to the tumor cells themselves, of resident cells such as fibroblasts and endothelial cells, infiltrating cells such as macrophages and lymphocytes and of released products of all these cells. Among these products are extra cellular matrix (ECM) components, growth factors, cytokines, chemokines antibodies, proteases, other types of enzymes and various metabolites. These molecules are released from the tumor cells themselves as well as from the non-tumor cells mentioned above. The cross-talk between tumor cells and microenvironmental factors may result in diametrically opposed effects which could either enhance or block tumor formation or progression.

Recent evidence indicate that premalignant tumor cells may in fact prime their own microenvironments, that is, form the metastatic niche in situ [113]. Tumor cells collaborate with local stromal cells to recruit myeloid cells and initiate the formation of a **metastatic niche.** Following extravasation and invasion at the secondary site, tumor cell survival and proliferation may be influenced by cell–cell and cell–matrix interactions in the metastatic niche. The disseminated tumor cell can successfully seed at metastatic lesion by evading the cell death signals, survive in the circulation and then effectively communicate with the stroma of the distant site.

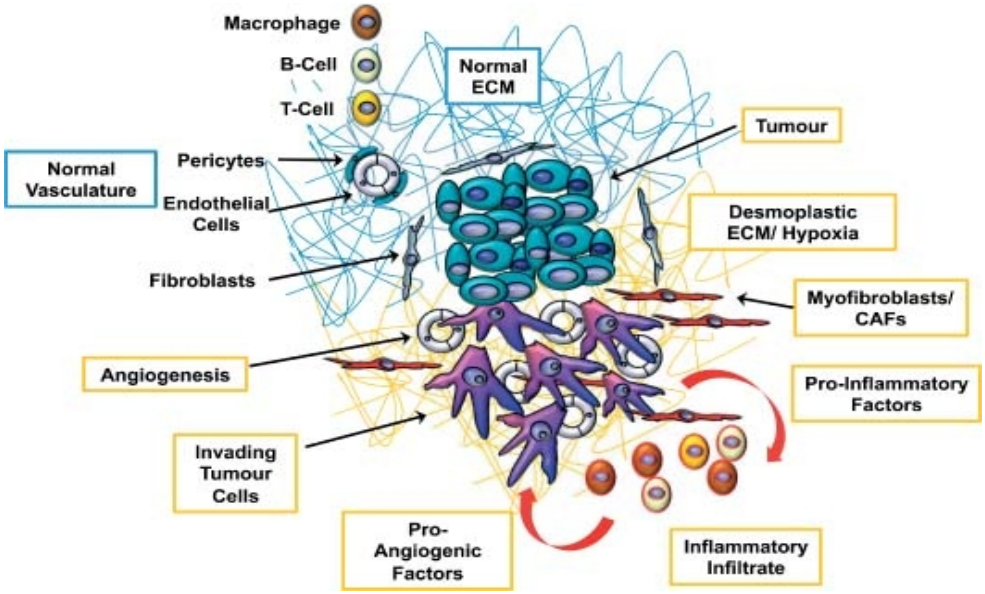


Figure 8: Changes to the normal microenvironment promote tumour invasion. **Adapted from** [114]

A) Tumor cells

Tumor cells are characterized by a variety of biological functions including deregulated proliferation, suppression of apoptosis, evasion of host immune response, neovascularization and metastatic spread to distant sites [115].

Tumor Cells normally survive in hypoxic environment [116, 117] through adaptive phenotype by virtue of their genetic, epigenetic instability and mutations [118]. Previous studies showed that cells grown in a tumor undergo a significantly higher rate of mutations than the same grown in cell culture [119]. This shows that the microenvironment of the tumor is responsible for inducing changes in the surrounding and intervening cells.

The micro-environment induce genetic and epigenetic instability in the cancer cells in several ways. One way, is the oxygen tension of the microenvironment which is generally characterized by series of hypoxia and reoxygenation. This leads to the formation of reactive oxygen species that induce damage by single and double strand DNA breaks, aberrant DNA synthesis, single and point mutations and amplifications, and so on. In addition, hypoxic conditions cause a deficiency in DNA damage repair in the form of decreased mismatch and decreased nucleotide excision repair [120]. These alterations produce tumor cells that have escaped regulatory control leading to an aggressive phenotype (Figure 9).

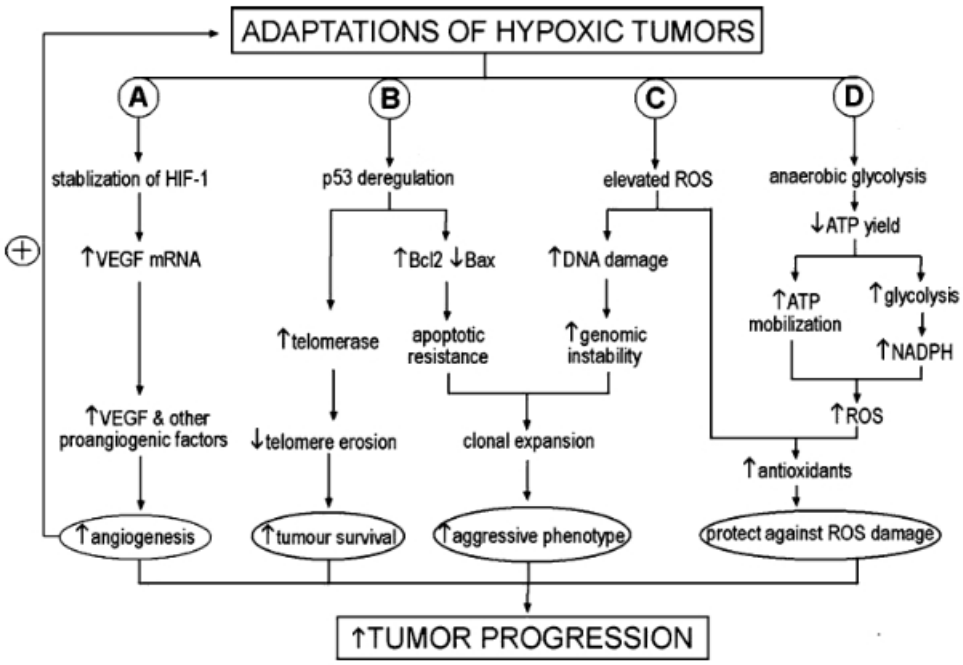


Figure 9: Hypoxic microenvironment regulation on tumor survival. **Adapted from** [120a]

Secondly, by regulating cancer genetics in relation to two gene classes. Class I genes such as the tumor suppressor genes and oncogenes that are mutated or deleted and controlled at the DNA level. Class II genes that have altered epigenetics, or a change in phenotype and are not changed at the DNA [121]. These cause the cells to be more aggressive and malignant. The microenvironment is thought to modify these Class II genes, particularly late in the course of cancer progression and metastasis.

B) Stromal tissue

Stroma is the supportive and connective tissue of the host tissue. It is composed of different cell types including fibroblasts, myofibroblasts, vascular and lymphovascular endothelial cells, and cells of the immune system such as macrophages. There is a bidirectional, dynamic, very intricate interaction between the cells of the stromal tissue and the cancer cells [122].

Fibroblasts

The wound-healing response of fibroblasts has many similarities to the activation of fibroblasts in the tumour stroma. Fibroblasts secrete soluble factors that act in a paracrine or autocrine fashion on the tumor cells resulting in a phenotype change that is more aggressive. These activated fibroblasts that interact with the tumor cells are called cancer associated fibroblasts CAFs [123, 124].

One very well studied soluble factor is TGF-β (transforming growth factor-β). The effects of TGF-β are complicated and can be either tumor suppressive or tumor promoting [125]. It is thought that this could be due to the fact that TGF-β acts both on epithelial and mesenchymal cells i.e. both the

tumor cells and the fibroblasts have receptors for TGF-β. The receptors for TGF-β on the epithelial cells can function in tumor suppression causing apoptosis and cytostasis by causing arrest of the cell cycle at G1, thus resulting in an inhibition of epithelial cell cycle progression. On the other hand, two molecular signaling pathways (SMAD and MAPK) have been identified in which TGF-β leads to an epithelial–mesenchymal transition (EMT) which results in tumor progression and metastasis [126, 127]. Other paracrine factors secreted by fibroblasts include EGF (epidermal growth factor), IGF (insulin like growth factor), FGF (fibroblast growth factor), and HGF (hepatocyte growth factor).

Other cells of the stromal tissue are also involved in promoting tumor proliferation and motility. For example, myoepithelial cells secrete CXCL14 and myofibroblasts secrete CXCL12 which both bind to receptors on epithelial cells which in turn cause enhanced proliferation and invasion. The receptor on the epithelial cell for CXCL12 has been defined as CXCR4 and in vivo and in vitro studies link this interaction to breast cancer metastasis [124].

EMT

Changes in epithelial cells and fibroblasts that are transient during wound healing can be sustained in tumors. The increase in epithelial cell migration and proliferation that is required for wound healing returns to normal on wound closure as the basement membrane is rebuilt. However, in tumors, those processes can continue unchecked; epithelial cells sustain oncogenic mutations that can result in immortalization, and they may undergo an epithelial–mesenchymal transition (EMT), which is associated with gaining the properties of cancer stem cells [128].

The transition from epithelial to mesenchymal cell produces a tumor cell with increased motility, the ability to penetrate the basement membrane, and the ability to form foci of cancer at distant locations in the body [127]. The epithelial-mesenchymal transition (EMT) is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal stem cells; these are multipotent stromal cells that can differentiate into a variety of cell types. EMT has also been shown to occur in embrogenesis, wound healing, organ fibrosis and in the initiation of metastasis for cancer progression. Furthermore, EMT is divided into three types. **Type 1 EMT** occurs during the life of an embryo and organogenesis, **Type 2 EMT** is involved in wound healing and tissue regeneration, and **Type 3 EMT** is that associated with cancer progression and metastasis [126]. The properties of the microenvironment have been shown to influence the propensity of the tumor cells to metastasize or penetrate the basement membrane [129].

C) Extracellular matrix

The extracellular matrix is composed of collagen, elastin, proteoglycans, and other specialized structural proteins that provide support to and division of the cells of the host tissue. Many of these components are actually synthesized by fibroblasts. However, in cancer, fibroblasts are transformed into myofibroblasts and secrete proteins that degrade this ECM. One of the first steps involved in formation of the tumor microenvironment is remodeling of the extracellular matrix. This is considered a seditious event in the formation of this dynamic network that supports tumor initiation and proliferation. The structure of the ECM is remodeled by enzymes called MMPs (matrix metalloproteinases) that are capable of hydrolyzing protein macromolecules. Many studies have shown that MMPs are overexpressed in human cancers [113]. These molecules are known to be pro-angiogenic and metastatic because the digestion of the ECM by proteases such as MMPs allow the entry of cancer cells into the host tissue and passage of tumor cells through the barrier of the host tissue as well as migration of endothelial cells into the matrix that results in neovascularization. In the process of degradation the MMPs also cause a release of growth factors that amplify the process of tumor growth and invasion.

The ECM contains other proteins such as integrins, which are cell surface receptors made up of various compositions of alpha and beta subunits responsible for binding components of the ECM and thus altering the structure of the ECM and transferring information between cells and the ECM.

In effect, integrins are the receptors for the tumor microenvironment of the cancer cell and then effect cell migration, proliferation, and metastasis via tyrosine kinases. In studies on the tumor microenvironment, integrins have been shown to promote angiogenesis, tumor proliferation, and metastasis [130]. As a part of the epithelial–mesenchymal transition described above, the subtypes of integrins found on the epithelial tumor cells change or undergo "**integrin switching**" which further potentiate the effects of TGF-β in regards to tumor proliferation and metastasis.

D) Cytokines chemokines and growth factors in microenvironment

There are striking similarities between the growth factors, cytokines and chemokines that are present in healing wounds and those present in tumours [131], but the kinetics of expression differ Table 7. In solid tumours, the same signalling pathways that are transiently upregulated to repair wounds are hijacked and activated constitutively.

Cytokines, chemokines and growth factors	Receptors	Functions in wounds	Functions in cancer
Growth factors			
EGF family (EGF, TGFα, HB-EGF, amphiregulin and heregulin)	EGFR, ERBB2 and ERBB4	Epidermal and mesenchymal regeneration; accelerates wound healing	Cancer cell invasion, macrophage signalling and autocrine growth of tumour cells
FGF family (FGF2)	FGFR1 and FGFR2	Early angiogenesis, fibroblast proliferation and re-epithelialization via keratinocyte migration	Angiogenesis and fibroblast proliferation
TGFβ family	TGFβR1 and TGFβR2	Attracts neutrophils and macrophages, mediates ECM deposition, angiogenesis, epithelial cell migration and wound healing	Tumour development, tumour cell invasion and metastasis
PDGF	PDGFR	Attracts neutrophils and macrophages, and mediates ECM deposition and angiogenesis. Stimulates wound healing when applied topically	Recruits inflammatory infiltrate and mediates angiogenesis and lymphangiogenesis
VEGF	VEGFR1–3	Angiogenesis	Tumour cell invasion and angiogenesis
Cytokines and chemokines			
IL-1α and IL-1β	IL-1R	Fibroblast and keratinocyte proliferation and neutrophil recruitment	Tumour cell proliferation, angiogenesis and inflammation
IL-6	IL-6R	Fibroblast proliferation and neutrophil recruitment	Tumour development, tumour cell invasion and metastasis
TNF	TNFR1 and TNFR2	Leukocyte infiltration	Tumour promotion or suppression
CXCL2 (also known as MIP2α and GROβ)	CXCR2	Epithelial proliferation	Recruits inflammatory infiltrate and migration
CXCL8 (also known as IL-8)	CXCR1, duffy and KSHV	Inflammation, wound contraction and epithelial proliferation	Angiogenesis, migration and invasion
CXCL12 (also known as SDF1α)	CXCR4 and KSHV	Angiogenesis	Migration, invasion and angiogenesis

CSF1, colony stimulating factor 1; CSF1R, CSF1 receptor; HB-EGF, heparin-binding ; MCP1, monocyte chemoattractant protein 1; MIP2α, macrophage inflammatory protein 2α; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; *The cytokines, chemokines and growth factors were included on the basis that they have been shown to influence both wound healing and tumour invasion or progression in vivo

Table 7: Cytokines, chemokines and growth factors that influence wound healing and tumour progression. **Adapted from** [111]

Communication between cells and their microenvironment occurs through a complex network of signals generated by cell-ECM and cell-cell adhesion and junctional molecules, as well as by collaboration between the epithelial, stromal and other organ-specific cell types. These ECM-molecules, together with the enzymes that remodel them, organize and sculpt tissues but also directly signal to the cells. The cells respond to both soluble and insoluble factors and in turn change their microenvironment in a fugue-like reciprocity, the end result of which is a magnificent and still somewhat mysterious integrated system that guides and allows maintenance of the differentiated state. If the microenvironment were not dominant, each cell would have its own way and the result would be either a uniform lump of similar fate or absolute chaos.

❖ *IL-6 and bFGF*

Interleukin (IL)-6 is one of the best-characterized pro-tumorigenic cytokines. It affects cell proliferation, survival, differentiation, migration, invasion, metastasis, angiogenesis, inflammation and metabolism [132] and is elevated in many cancers [133]. Major signal transducer downstream of IL-6 is STAT3 which acts as a key player linking inflammation and cancer [134-136]. IL-6 has a direct growth stimulatory effect on many cancer cells through several signaling pathways [137]. IL-6 family stimulates tumor invasion and migration (metastatic spread) through induction of EMT phenotype [138-140]. bFGF is a multipotential glycoprotein which exerts its mitogenic and angiogenic characteristics, through tissue remodelling, wound healing and neovascularization. The bFGF efficiently inhibits terminal differentiation of fibroblasts to myofibroblast, by a significant decrease of alpha–smooth muscle actin (α-SMA) positive cells [141]. The majority of human NSCLC cell lines produce elevated levels of bFGF which in turn stimulate the growth of these tumor cells by intracrine mechanisms [142-145]. bFGF is also regarded as one of the most specific and crucial regulators of angiogenesis [146, 147].

Tumor Microenvironment (cellular players)

The microenvironment of a tumor is a vital part of its structure, physiology and function. An essential unbalanced relationship between tumor and stromal cells is required for tumor cell growth, progression, and metastasis. Improved understanding of this interaction may provide valuable clinical targets for cancer management. Secreted proteins from tumor, stromal cells and non-malignant cells are dynamic participants in cancer progression. Apart from others cells such as macrophages, endothelial cells present in tumor microenvironment fibroblasts and its transformed forms still remains the most important determining factor for the fate of tumor and its response to radiotherapeutic interventions.

Fibroblasts

Fibroblasts are elongated, spindle shaped and metabolically active cells in many tissues in the body and are mainly responsible for the production and turnover of extracellular matrix (ECM) by expressing collagens, fibronectins, laminins, elastins, proteoglycans, integrins, matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs) and a host of other ECM proteins that are expressed in a tissue-specific manner [148, 149].

Fibroblasts activity and their consequent differentiation into myofibroblasts is dependent upon different growth factors, extracellular matrix components, and mechanical stress. Worth noticing is the expression of receptors for a number of cytokines including PDGF, TGFβ1, and TNFα by fibroblasts. The heterogeneous nature of the fibroblast phenotype derived from different anatomical locations had been described by Alvarez et al [150].

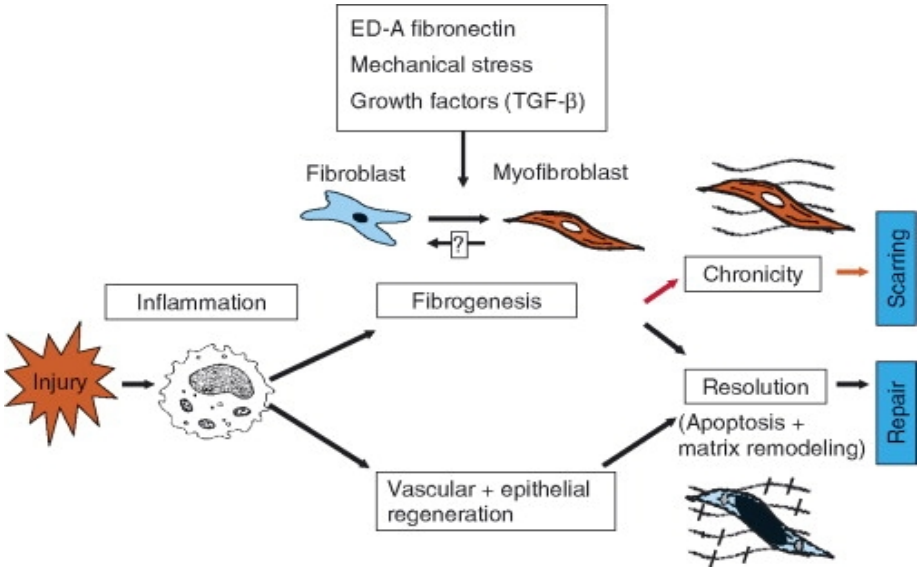


Figure 10: Generalized model to show the role of fibroblast in wound healing and scarring versus pathological fibrosis. **Adapted from** [151]

Fibroblast activity is crucial during processes of wound healing and inflammation. Under such conditions, fibroblasts are generally considered to be "activated". In particular, as the healing process progresses, fibroblasts turn on expression of afilamentous actin, alpa-smooth muscle actin (α-SMA), which enables them to exert contractile forces to close the wound. Local tissue contractility is mediated by focal adhesions between the activated fibroblasts called **myofibroblasts** and the ECM. After wound closure, the balance of MMPs and TIMPs secreted by fibroblasts is changed to favor ECM degradation (as opposed to synthesis) which leads to massive apoptosis of the myofibroblast population. Instead of deactivation, the myofibroblast population persists during fibrosis or tumorigenesis for reasons that are not clear. This aspect of fibroblasts can be regarded as bimodal. Because, in early tumorigenesis, they negatively regulate malignant progression, but in advance stages they are subverted to promote tumor growth-being referred to as cancer-associated fibroblasts (CAFs).

It is important to underline that a cross talk and reciprocal relations between tumor and its stroma are necessary for tumor formation and its progression. Recent advances showed that fibroblasts in stroma actively drive tumorigenesis and cancer progression [151-156], negating the previous notion that fibroblast behavior is dictated by the epithelium. Fibroblasts play important roles during tumor development, from the pre-neoplastic state until metastasis. In vitro co-culture and in vivo xenograft systems demonstrate that factors derived from tumour fibroblasts contribute to the transformation of immortalized epithelia [157]. Table 8 shows soluble factors from fibroblasts effecting tumor behaviour

Soluble factors	Cells expressed	Responding cells	Possible role
HGF and MSP	Fibroblasts	Epithelia	+ Proliferation + Transformation + Morphogenic
IGF-1, IGF-2	Fibroblast	Epithelia (breast)	− Apoptosis + Proliferation
EGF and TGF-α	Epithelia and fibroblasts	Epithelia	+ Proliferation + Morphogenic
TGF-β1, TGF-β2, TGF-β3	Epithelia and fibroblasts	Epithelia and fibroblasts	− Proliferation +/− Apoptosis + Morphogenic
FGF7/KGF	Fibroblast	Epithelia	+ Proliferation + Morphogenic
IL6, LIF, and oncostatin M	Fibroblast	Epithelia (colonic)	+ Proliferation + Transformation
FGF2	Fibroblast	Epithelia	+ Proliferation + Transformation
FGF10	Fibroblast	Epithelia	+ Proliferation
NGF	Fibroblast	Epithelia	+ Transformation
Stromal cell-derived factor 1α (CXCL12)	Fibroblast	Epithelia (glioblastoma)	+ Proliferation + Transformation
Wnt1, Wnt3	Fibroblast	Epithelia	+ Proliferation + Transformation
MMP-1, MMP-7	Fibroblast	ECM and growth-factor activation in the stroma affect epithelia	+/− Proliferation +/− Apoptosis + Morphogenic

Table 8: Regulation of epithelial growth, differentiation and apoptosis. **Adapted from** [157].

Myofibroblasts

During wound healing resident fibroblasts differentiate into myofibroblasts. The main feature of myofibroblastic differentiation, is the neo-expression of α-SMA. Myofibroblasts play a central role in closing the wound tissue by producing a strong contractile force [158].

In the tumor stroma they not only provide source of growth factors but are also involved in connective tissue remodelling. Indeed many myofibroblasts have been at the forefront of invasion in tumors [159]. Several authors have shown that the acquisition of myofibroblast features in lung fibroblasts is TGFβ1-mediated [160]. Although TGFβ1 upregulate α-SMA expression in a diverse range of fibroblasts [158, 160, 161], it only upregulates a subset of smooth muscle differentiation markers in non–smooth muscle cells [162].

CAF (carcinoma associated fibroblasts)

Once fibroblasts are recruited, activated, and accumulated to the tumor area, these transformed cells known as CAFs, myofibroblasts, reactive stromal fibroblasts, or tumor-associated fibroblasts, have distinctly different functional and morphological features from their normal counterparts [163]. It is gradually recognized that CAFs are heterogeneous in nature oriniginated from various sources. The locally resident stromal fibroblasts are considered a major source of CAFs and this process is called fibroblast to myofibroblast transdifferentiation (FMT) [164].

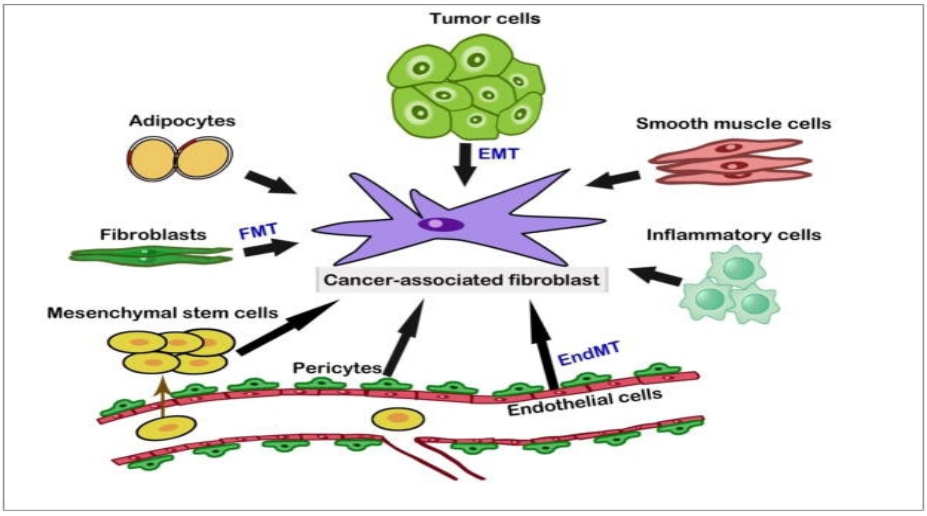


Fig 11: Possible origins of cancer-associated fibroblasts (CAFs) in TME.
Adapted from [165]

Apart from expression of α-SMA, several additional markers such as : vimentin, platelet-derived growth factor receptor alpha (PDGFR-α), platelet-derived growth factor receptor beta (PDGFR-β), fibroblast specific protein (FSP-1), and fibroblast activation protein (FAP) are used to identify CAFs [166-169]. Enhanced proliferation potential of CAF is the result of direct paracrine interactions with tumor cells. Under various contexts, CAFs produce a range of growth factors and cytokines that influence the behavior of the epithelium, including HGF, EGF, IGFs, IGFBPs, b-FGF, and TGFβ, to name a few [155]. *In vivo*, crosstalk between CAFs and malignant cells, within the tumor microenvironment, is not merely through paracrine signaling, but CAFs also actively engage other resident and distant stromal cell types including endothelial cells, bone marrow-derived cells (BMDCs) and inflammatory cells, in order to stimulate cancer growth, angiogenesis and metastatic spread [170].

Tumor Microenvironment (molecular players)

TGF β

Transforming growth factor-β (TGF-β) was discovered as a secreted protein that plays a vital role in enhancing tumor cell survival, invasion and metastasis by targeting immune cells, fibroblasts and myofibroblasts within the tumor microenvironment. It is a double edged multifunctional cytokine, that inhibits tumor growth at the early stage but promote tumor cell invasiveness and metastasis at later stage. TGFβ signalling is an important mediator not only of changes to the epithelial phenotype but also of changes in the stromal environment that are essential for tumor progression.

TGFβ1 superfamily include three mammalian TGF isoforms (TGFβ1, β2, β3), activin/inhibin, and bone morphogenic proteins. These three TGF-β isoforms are initially synthesized as a 75-kDa homodimer (pro-TGF-β). Pro-TGF-β is then cleaved in the Golgi to form the mature 25-kDa TGF-β homodimer [171]. These interact with latency associated proteins to form the small latent complex [171-173]. In the endoplasmic reticulum, a single latent TGF-β binding protein forms a disulfide bond with the TGF-β homodimer to form the large latent complex, allowing for targeted export to the extracellular matrix [172], where it is stored until its activation [174, 175]. Latent TGF-β is activated by several factors, including proteases [175, 176], thrombospondin 1 [177], reactive oxygen species [178], and integrins [179, 180]. This occurs through liberation from latency-associated proteins, degradation of latent TGF-β binding protein, or modification of latent complex conformation.

TGF-β signaling pathways

Once the ligand is activated, TGF-β signaling is mediated through Smad and non-Smad pathways to regulate transcription, translation, microRNA biogenesis, protein synthesis, and post-translational modifications [181-183]. ***In the Smad pathway***, the TGF-β ligand binds to the type 2 TGF-β receptor (TβRII) that recruits the type 1 TGF-β receptor (TβRI). These receptors dimerize and autophosphorylate serine/threonine residues, allowing for the phosphorylation of Smad2 and Smad3 by TβRI. The now activated Smad proteins dissociate from the Smad anchor for receptor activation (SARA) protein, hetero-oligomerize with Smad 4, and translocate to the nucleus, interacting with

numerous transcriptional co-regulators to facilitate target gene expression or repression [184, 185]. These canonical effectors can be classified as R-Smads (Smad2/3) and the co-Smad (Smad4). However, there are also inhibitor Smads (I-Smads; Smad6/7) that function to negatively regulate R-Smad activation and nuclear translocation. Overexpression of the I-Smads, particularly Smad 7, is common to pathologies in which TGF-β signaling is perturbed [186]. TGF-β also signals through a number of ***Non-Smad pathways***, including p38 MAPK, p42/p44 MAPK, c-Src, m-TOR, RhoGTPases, RAS, PI3K/Akt, protein phosphatase 2A (PP2A)/p70s6K, and JNK MAPK [183, 187-191].

TGF-β paradox

TGF-β expression has been studied in nearly all epithelial cancers, including, prostate, breast, lung, colorectal, pancreatic, and skin cancers [192]. Through these studies, it has become clear that TGF-β can function both as a tumor suppressor and a tumor promoter [193-196]. In benign epithelia and many early-stage tumors, TGF-β is a potent inducer of growth arrest. However, in advanced tumors, TGF-β signaling pathways are severely dysregulated. Rather than inhibiting carcinogenesis, TGF-β promotes tumor growth and progression at late stages [192-199]. This functional switch is known as the ***TGF-β paradox*** [195].

TGF-β is a key regulator of its own expression [200, 201]. There are a number of mechanisms whereby altered TGF-β signaling contributes to cell proliferation, and ultimately to the development of cancer and its spread. These include the inactivation of various components of the TGF-β receptor signaling system [202-204], including mutations of TβRII, TβRI, and the various Smad proteins that decrease sensitivity to the inhibitory effects of TGF-β. This receptor repression may correspond with compensatory induction of the TGF-β ligand, which is further complicated by data suggesting that TGF-β can function as a cell-autonomous tumor promoter through non Smad mechanisms [205].

TGF-β role in tumor progression

The effects of TGF-β are complicated [206]. In normal cells and cells of early tumor stages, TGF-β suppresses tumor growth through transcriptional control of genes affecting the cell cycle and induction of apoptosis [207]. As tumorigenesis develops, tumor cells frequently become resistant to TGF-β-mediated growth inhibition because of mutations of cell cycle regulators, such as p15 or p16, altered or loss of TGF-β signaling through genetic loss of TGF-β signaling components, such as Smad4, or downstream alteration of cross-talk signaling pathways, such as by activation of oncogenic Ras [192, 195, 197, 208, 209].

Tumor cells that lack inactivating mutations then undergo epithelial to mesenchymal transdifferentiation and are able to respond to TGF-β with increased migration, invasion and metastasis. It is thought that this could be explained in part by the fact that TGF-β acts both on epithelial and mesenchymal cells- *i.e.* both the tumor cells and the fibroblasts have receptors for TGF-β. The receptors for TGF-β on the epithelial cells can function in tumor suppression causing apoptosis and cytostasis by causing arrest of the cell cycle at G1, thus resulting in an inhibition of epithelial cell cycle progression. On the other hand, two molecular signaling pathways (Smad and MAPK) have been identified in which TGF-β leads to an epithelial–mesenchymal transition (EMT) resulting in tumor progression and metastasis [210, 211] (Figure 12).

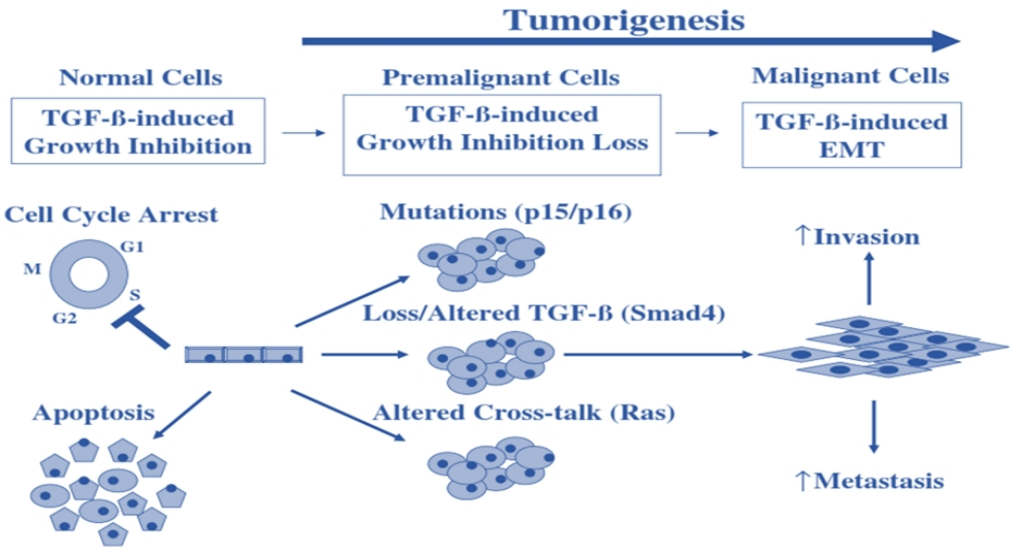


Figure 12: Transition of TGF-ß as a growth inhibitor to an inducer of epithelial to mesenchymal transition in tumorigenesis. **Adapted from** [212]

TGF β and EMT

Epithelial–mesenchymal transition (EMT) is a process that makes the epithelial cell to acquire a mesenchymal phenotype and is characterized by down-regulation of epithelial markers, such as E-cadherin and up-regulation of mesenchymal markers, such as vimentin, and fibronectin [213, 214]. In more-advanced disease stages, TGF-β induces EMT in cancer cells [215, 216]. In epithelial cell lines, TGF-β signalling directly activates the expression of the EMT transcription factors, Snail and Slug, ZEB1 and ZEB2, and Twist [217-219]. Smad-independent activation of Snail is a result of Ras/ERK activation [220, 221] and the nuclear translocation of the TβRI-ICD [222]. Thus both Smad-dependent and Smad-independent arms of TGF-β repression of E-cadherin in cancer cells promotes EMT [220, 221, 223, 224].

A well known fact observed *in vitro* is that, ionizing radiation (IR) not only kill cancer cells but enhance migration and invasion of surviving cancer cells through EMT [225-228]. This dual effect is associated with the different dose or dose rate acting on different targets. It had also been showed previously that IR with relatively higher dose (7.5–10 Gy) can induce invasive capability of cancer cells [226-228] and low dose or dose rate could promote angiogenesis [229]. TGF-β signaling played an important role in this process through Smad signaling [230-232] but, some studies had also showed that the secretion of matrix metalloproteinases [233] also play an equivalent role.

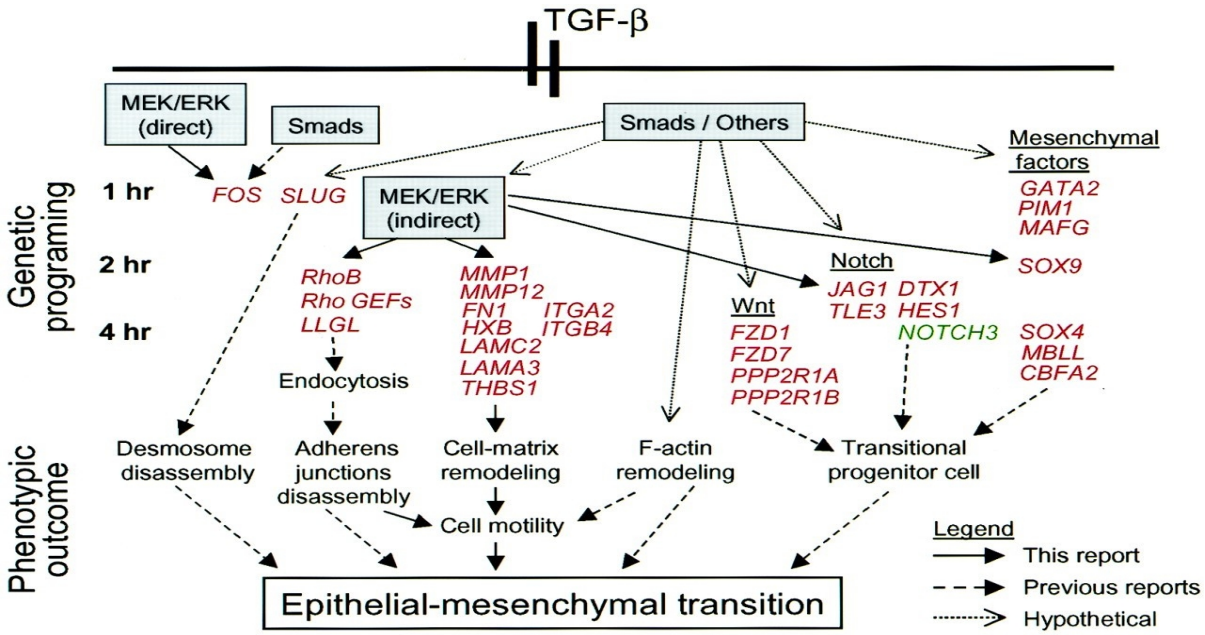


Figure 13: Signaling modules and genetic programs underlying EMTs induced by TGF-β.
Adapted from [234]

TGF-β also upregulates MMPs to promote invasion through proteolytic degradation and remodeling of the extracellular matrix [235]. In the tumor context, TGF-β upregulates expression of MMPs through at least two mechanisms. One mechanism is dependent on the TβRI-ICD, shown in prostate cancer, and the other is dependent on p38 being demonstrated in breast cancer [236, 237]. TGF-β may also promote metastasis by interrupting cell–cell adhesion [220, 225, 226], upregulating vimentin and MMPs [222, 226, 238], and attracting new vasculature.

❖ *TGF β induced EMT in driving the stem cell phenotype*

TGF-β induced EMT might be even more striking, because TGF-β induced EMT is thought to transform cells into a more "stem cell-like" phenotype. Induction of EMT either by TGF-β1 or its downstream targets, Snail or Twist, stimulated the expression of cell surface markers linked to cancer stem cells (CSCs) in immortalized human mammary epithelial cells (HMECs) [239]. In addition, TGF-β can differentiate CSCs into a multi-potential cell [240]. This TGF-β induced stem cell-like phenotype may be critical for tumor progression and metastasis. As it effects tumor cell dissemination and homing, as well as colony-initiating activity.

TGF β , RhoGTPases and fibroblasts

TGF-β also activates other signaling pathways that do not involve Smads. For example, TGF-β signaling activates the PI3K–Akt–mTOR pathway, and small GTPases (including Rho) leading to increased motility, invasion and changes in protein synthesis and actin reorganization. Functional interactions of cancer cells with stromal cells in the tumor microenvironment are important determinants for cancer progression. In contrast with its canonical role in regulating epithelial cell proliferation, TGF-β is considered a pro-growth signal with respect to the mesenchyme. Classically, in response to injury, the influx of granulocytes, platelets, leukocytes, and additional parenchymal cells increases the presence of TGF-β at the site of the wound [237, 241]. TGF-β then induces fibroblast proliferation, myofibroblast differentiation, and remodeling of the extracellular matrix [237, 241-244] and thus contributes to the generation of CAFs, providing a scenario for how TGF-β can contribute to cancer progression through effects on stromal fibroblasts. TGF-β induces expression of RhoA and RhoB through smad2/3 signaling during fibroblast-myofibroblast differentiation [245].

Therefore, TGF-β is heavily implicated in a variety of fibrous diseases [237] and may serve as an attractive therapeutic target in desmoplastic tumors; should the tumor epithelia be desensitized to the growth inhibitory effects of TGF-β.

Matrix Metalloproteinases (MMPs)

Tumor cells express a diverse array of proteins, including growth factors and ECM-degrading proteinases or prompting the host to secrete biomolecules that have the ability to degrade the matrix and adhesion molecules. The matrix degradation takes place when there is imbalance between the amount of active degradative enzymes and the proteinase inhibitors present in the matrix. Proteins secreted by tumor cell into the microenvironment are therefore involved in intercellular communication, cell adhesion, motility, and invasion.

Role of MMPs in ECM modulation

The matrix is subject to remodelling by proteinases, including metalloproteinases and plasmin/plasminogen family. An important factor in determining the extent of ECM accumulation is the balance between collagen synthesis and degradation [246-249]. Tumor invasion, metastasis, and angiogenesis require controlled degradation of ECM and associated with it is increased expression of matrix metalloproteinases (MMPs) [250, 251].

MMPs are a family of more than 25 zinc-dependent endopeptidases that are, metal dependent and share a common modular domain structure, collectively capable of degrading essentially all ECM components [251-253]. According to their substrate specificity and structure, members of the MMP gene family can be classified into subgroups of collagenases, stromelysins, gelatinases, membranetype MMPs, and other MMPs. A brief overview of different MMPs secretd by stromal cells is shown in (Figure 14). MMP-2 and MMP-9 are regarded as key enzymes for degrading type IV collagen during cancer invasion and metastasis [254]. MMP-2 is expressed constitutively in stromal cells whereas expression of MMP-9 is typically low or absent in normal quiescent tissues. However, MMP-9 is induced under stress conditions that require tissue remodeling such as development, wound healing, and tumor invasion [255]. Like other MMPs, the proteolytic activity of MMP-2/9 is regulated by several mechanisms, including gene transcription, pro enzyme activation, and inhibition by tissue inhibitors of the metalloproteinases (TIMPs).

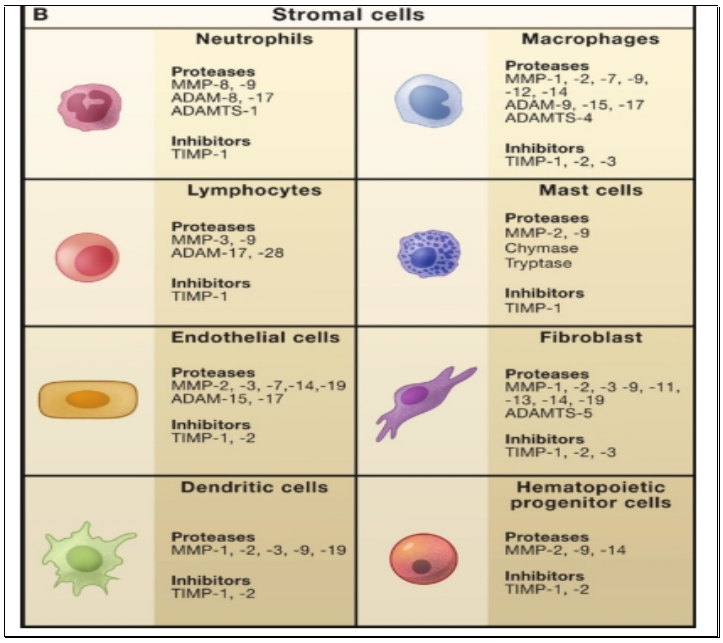


Figure 14: Expression pattern of proteinases and their physiological inhibitors in non-malignant stromal cells**. Adapted from** [256]

Most MMPs are secreted as latent precursors (zymogens) that are proteolytically activated in the extracellular space, with the exception of MMP-11 and MT1-MMP [251, 252, 257]. The activity of MMPs in extracellular space is specifically inhibited by tissue inhibitors of metalloproteinases (TIMPs), which bind to the highly conserved zinc binding site of active MMPs. The TIMP gene family consists of four structurally related members, TIMP-1, -2, -3, and -4 [258].

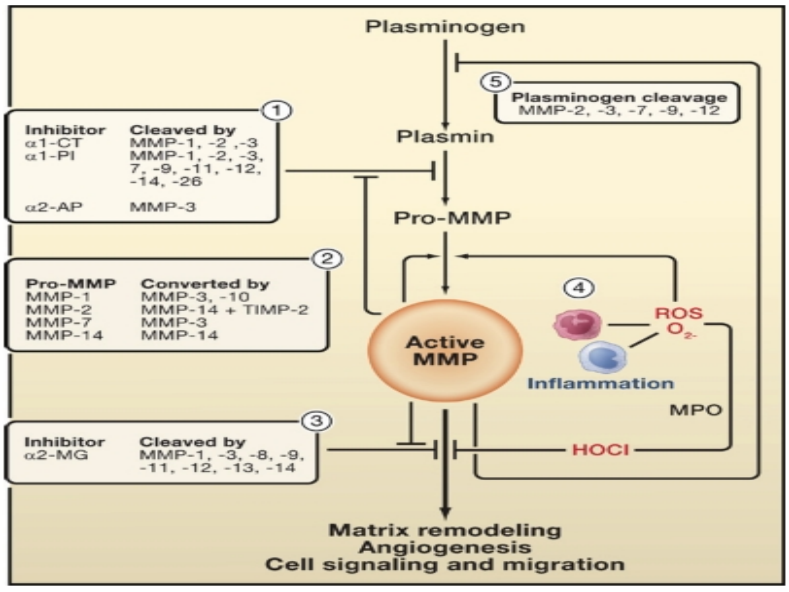


Figure 15: Proteolytic cascades regulate MMP function from inflammatory cells. **Adapted from** [256]

Expression of MMPs in cancer

Apart from tumor invasion degradation of stromal ECM is also considered essential in tumor-induced angiogenesis. MMPs have been shown to play an essential role in angiogenesis and tumor cell intravasation [259, 260] both of which are required for tumor cell growth and metastasis.

The expression of MMPs in tumors is regulated in a paracrine manner by growth factors and cytokines secreted by tumor infilltrating inflammatory cells as well as by tumor or stromal cells; recent studies have suggested continuous cross talk between tumor cells, stromal cells, and inflammatory cells during the invasion process [250, 251, 261, 262]. Indeed, recent studies of clinical tumor specimens provided evidence that most MMPs are generated by peritumoral fibroblasts in the stromal compartment, instead of tumor cells themselves. Peritumor stromal cells are indeed responsible for the production of MMP-1, MMP-3, MMP-11, and MMP-2 in breast, colon, lung, skin, and head and neck cancers [261-264].

Accordingly, tumor cell-derived factors that increase the expression of several MMPs in stromal cells have been purified. One of these factors, a tumor cell surface glycoprotein, extracellular matrix metalloproteinase inducer (EMMPRIN) [265-267] has been shown to induce the expression of MMP-1, MMP-3, and MMP-2 by fibroblasts [263, 265, 268, 269]. Therefore, EMMPRIN functions as an upstream modulator of MMP production in tumor local environment. EMMPRIN positive tumor cells stimulate neighbouring fibroblast cells to express MMPs and therefore facilitate tumor invasion and metastasis.

Regulation of MMP activity

Expression of most MMPs is normally low in tissues and is induced when remodeling of ECM is required. The complexity of the tumor microenvironment allows for a variety of regulatory cascades that determine the functions of the diverse MMPs expressed. Proteolytic activity of MMPs can be regulated at different levels: gene expression (which is primarily regulated at the transcriptional level), but there is also evidence about modulation of mRNA stability in response to growth factors and cytokines [270-273], compartmentalization, conversion from zymogen to active enzyme, and, finally, the presence of specific inhibitors (Figure 16). When judging the pathophysiological relevance of increased expression of proteinases in tumor tissues, the particular context is important, that is, whether endogenous inhibitors or activating (converting) enzymes in the microenvironment are present.

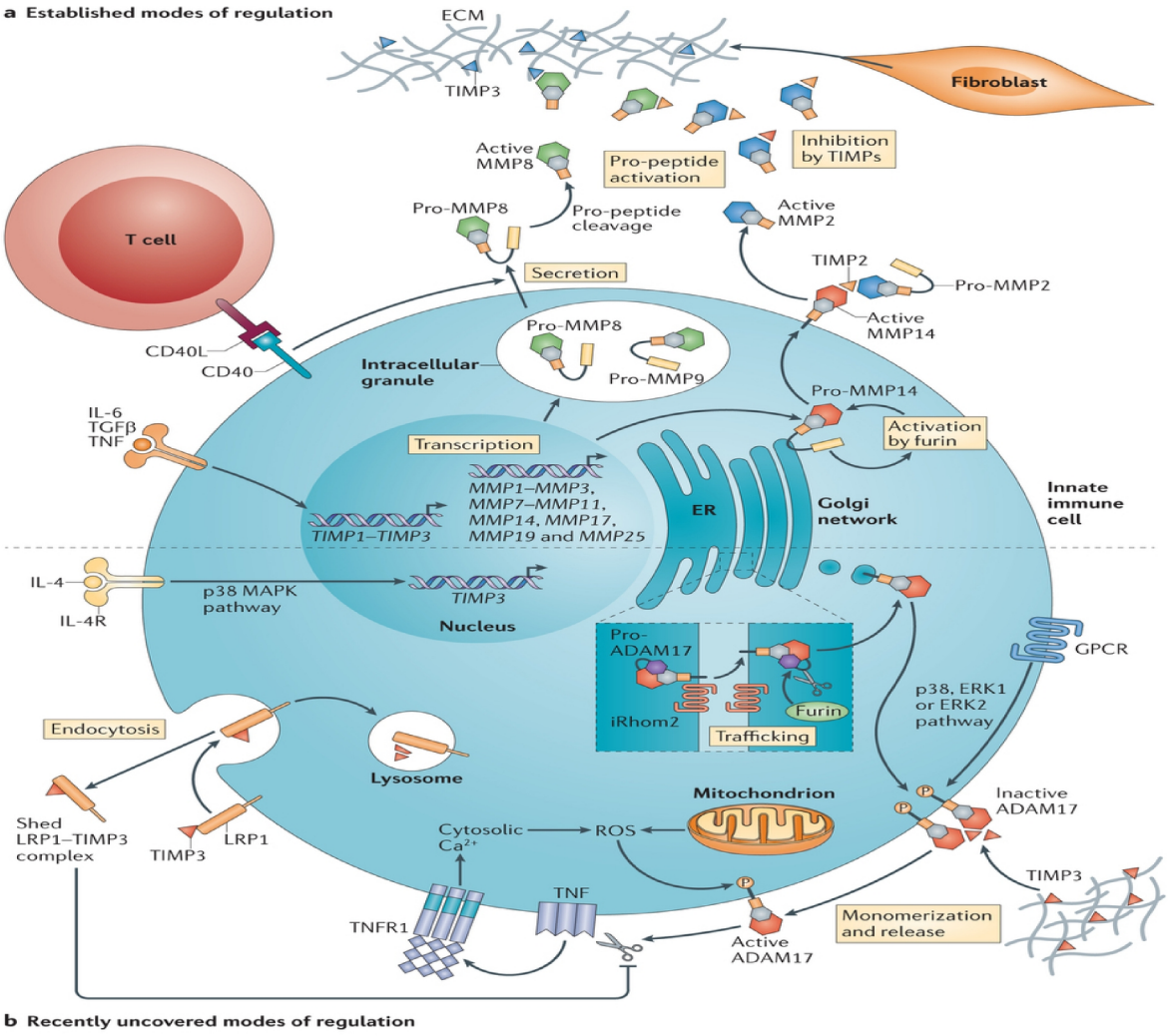


Figure 16: Established and recently uncovered modes of MMP, ADAM and TIMP regulation. Adapted from [273]

❖ *Role of AP-1 (transcription factors) in the regulation of MMP gene expression*

The activator protein 1 (AP-1) is a transcription factor which comprises of diverse homodimeric or heterodimeric combinations of proteins formed by members of the c-Fos, c-Jun, ATF and JDP families. It regulates gene expression in response to a variety of stimuli, including cytokines, growth factors, stress, inflammation, bacterial and viral infections [274-278]. This diversity in the composition of AP-1 complexes and furthermore their important role in transcriptional regulation places them at the heart of pathological signal relay particularly in the context of malignancy [278-280].

The biological function of transcription factor such as AP-1 is mediated by the direct regulation of gene expression. A single AP-1 element, which binds members of the AP-1 transcription factor family, is found at approximately −70 in the promoter region of each inducible MMP gene. Several MMPs, including MMP3 and MMP9, have long been known to be regulated by AP-1 in a variety of cellular contexts [281-283]. The activation of these transcription factors plays a pivotal role in metastasis due to their ability to induce the transcription of metastasis-related genes, including MMP-9 [284, 285].

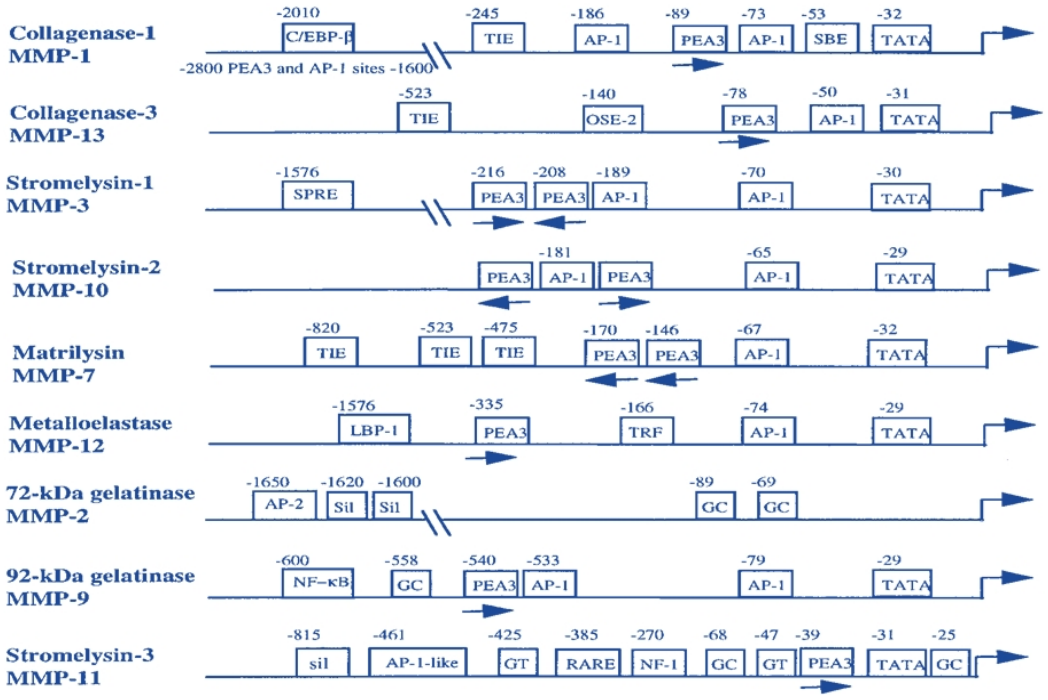


Figure 17: Regulatory elements of MMPs promoter. **Adapted from** [270]

Role of MMPs in EMT

Signals from the microenvironment that induce characteristics of the developmental EMT program can confer the ability to invade surrounding tissue and metastasize to distant sites [286]. In cancers, MMPs act through cleavage and release of bio-active molecules that inhibit apoptosis and stimulate invasion as well as through degradation of ECM components that prevent tumor cell growth [287]. MMPs also promote angiogenesis and modulate immune responses so as to block immune surveillance and to stimulate tumor growth [288].

Expression and activation of MMPs is at very low levels and is tightly regulated during normal tissue homeostasis [289]. MMP production and activation is rapidly induced during active tissue remodeling and in pathological conditions such as in cancers [290].

Increased expression of MMPs is predictive of tumor aggressiveness, metastasis and poor survival in many cancers including lung, prostate, colon, breast and pancreatic [291-296]. It is notable, however, that there is neither a single MMP that is consistently over-expressed in every tumor type nor a consistent pattern of MMP expression across the variety of human cancers [297]. The expression of MMPs in tumors reflects intrinsic tissue heterogeneity, as different tumors express distinct subsets of ECM components, cell surface receptors, and cell tissue interactions. More recent studies have shown that MMPs can induce EMT or EMT-related processes in cultured cells [298-302].

Extracellular functions of MMPs on tumor cells

a) Activation of growth factors and growth factor receptors

MMPs mediate a variety of biological effects on their surrounding tissue. MMPs alter the cellular microenvironment and influence tumor initiation and progression through release of bioactive fragments following degradation of ECM or latent extracellular growth factors or growth factor receptors.MMP-1, -2, -9, and MT1-MMP have been found to lead to increased signaling of TGF-β in melanoma cells, keratinocytes and osteoblasts [303-305]. MMPs can also activate factors that themselves induce EMT: the association between MMP-9 and CD44 is thought to lead to the processing and activation of TGF β [305], which is known to induce EMT in many tumor systems. MMPs may be critically involved in disrupting the balance between growth and antigrowth signals in the tumor microenvironment, as they potently influence the bioavailability or functionality of multiple important factors that regulate growth.

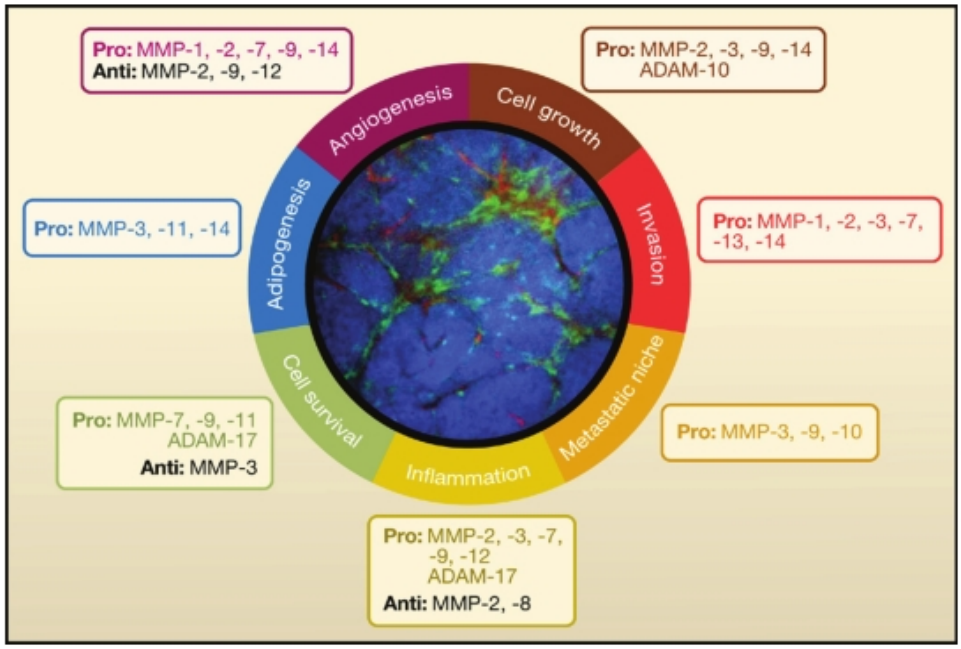


Figure 18: Modulation of the tumor microenvironment by MMPs. **Adapted from** [256]

b) TGF β proteolytic activation by MMPs

Transforming growth factor-β (TGF-β) pathway plays a fundamental role in tissue homeostasis. TGF-β is actively derived from an inactive pro form by proteolytic conversion with the help of furin or other proteinases such as MMP-9, which is usually expressed by inflammatory cells. Given the fact that tumor cells often attain non responsiveness to TGF-β, suggesting that proteolytic activation of TGF-β by MMPs has tumor-promoting effects. This is acquired by selectively driving stroma-mediated invasion and metastasis of the tumor.

TGF-β has also been reported to regulate the expression of MMP-9, particularly in epithelial and cancer cells. [306-310]. However, induction of MMP-9 by TGF-β is not a universal finding, and examples of down regulation of MMP-9 expression by TGF-β have been reported [311, 312]. The production of the endogenous inhibitors of MMPs, the TIMPs, is also regulated by growth factors. TGF-β increased the levels of TIMPs in fibroblasts [313].

c) MMPs and Integrins

MMPs also modulate cell–ECM interactions through alterations of integrin expression of several types of carcinoma [314], and MMP-induced alterations in integrin signaling can lead to upregulation of MMP expression, stimulating feedback loops [315, 316]. MMPs can also process and cleave the integrins themselves [317, 318].

d) Effects of differential expression of MMPs

Tumor-associated MMPs are produced by the tumor cells as well as by a variety of tumor-associated stromal cells, including fibroblasts, smooth muscle, and vascular cells and also by cells of the immune system [319]. Furthermore, MMPs can play opposing roles in tumor progression depending on the expression by tumor or stromal cells [320-323].

MMPs and metastatic niche formation

Some organs such as lung, liver, or bone are the favoured sites for metastases. Metastasis not only depends on EMT of the cancer cells for disseminating from the primary tumor but the formation of a receptive environment, a metastatic niche is also an important requirement. This niche is specifically suited for the engraftment of tumor at the distant organ. MMPs and other proteinases are significantly involved in the formation of a metastatic niche [324].

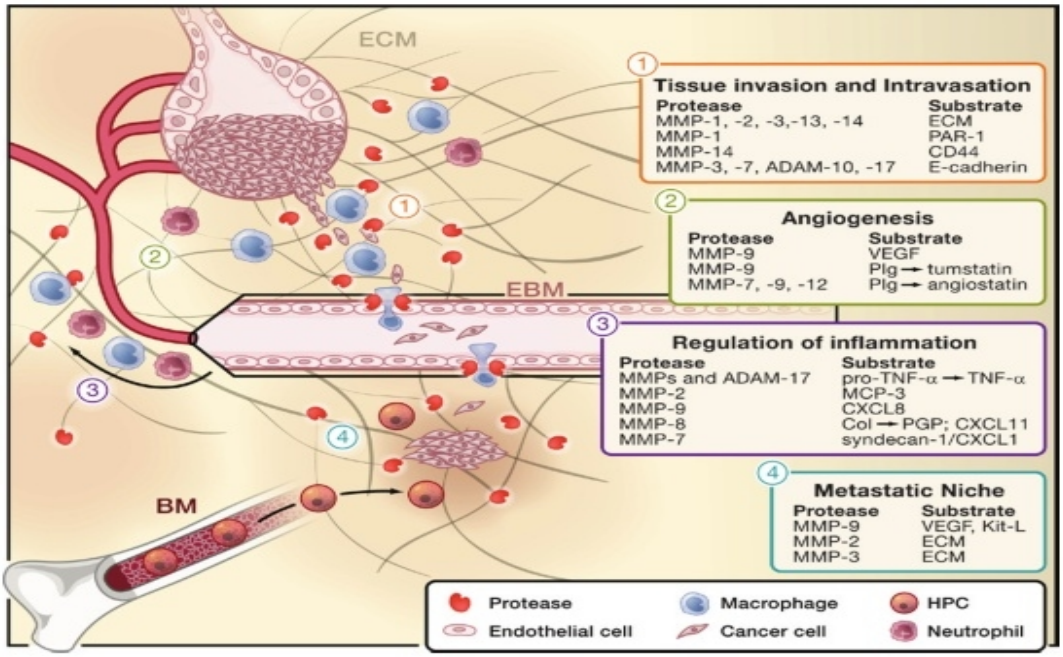


Figure 19: Multiple functions of MMPs in the tumor microenvironment. **Adapted from** [256]

TGF-β and TNF-α produced by tumor cells elicit the expression of chemokines by lung endothelium, which, in turn, mediate the directional migration of myeloid cells to these distant sites [325]. In fact, MMP-9 seems to be critical mediator for the formation of the metastatic niche [324], which is presumably linked with its ability to release VEGF and thereby support angiogenesis [326] (Figure 19).

RhoB

Rho-family GTPases are molecular switches that transmit extracellular signals to intracellular molecular pathways. The small GTPase RhoB is specifically implicated in the regulation of a these pathways. Previous studies on RhoB elucidate the dominant role of RhoB in cancer progression and show differential effects on on signaling pathways in the tumor stromal component. RHOB effects are stage and/or cell-type dependent and belongs to a novel class of "genes of recurrence" that have a dual role in metastasis and treatment resistance.

RhoB characteristics

The Rho family of GTPases consists of 22 Rho family members. Rho functions as a molecular switch in cellular processes such as cell adhesion, migration and cell cycle progression including cytokinesis [327,328]. The Rho GTPases are divided into six groups: Rho (RhoA, RhoB, RhoC), Rac (Rac1, Rac2, Rac3, RhoG), Cdc42 (Cdc42, TC10, TCL, Chp, Wrch-1), Rnd (Rnd1, Rnd2, Rnd3/RhoE), RhoBTB (RhoBTB1 and RhoBTB2) and Miro (Miro-1 and Miro-2) [329].

Mechanism of activation

Like other members of the Ras family of GTPases, most Rho proteins cycle between an active GTP-bound state and an inactive GDP-bound state (Figure 20).

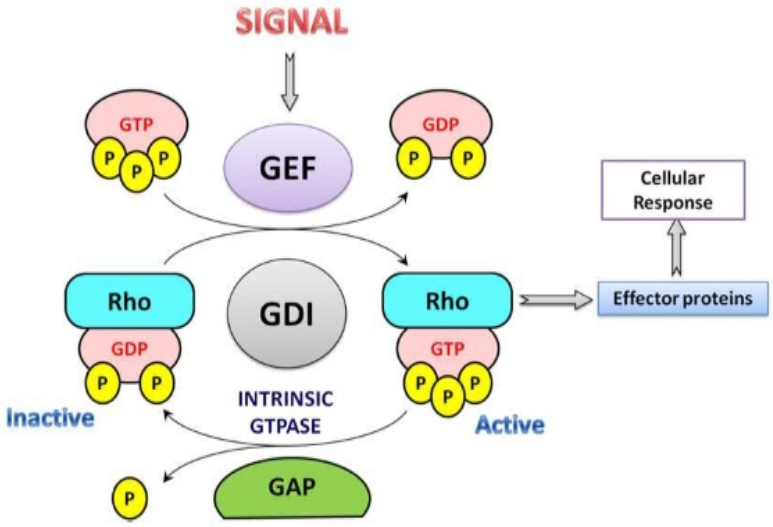


Figure 20: Basic Rho GTPase activation cycle. **Adapted from** [330]

This regulatory cycle is controlled by three distinct families of proteins, guanine exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). Upon GTP loading, a conformational change takes place that allows Rho proteins to interact with several downstream effectors that ultimately propagate the signal within the cell. Additional regulatory mechanisms such as serine phosphorylation might further contribute to the tight regulation of these proteins [331, 332].

The primary action of Rho is to induce a specific type of actin cytoskeleton in the cell by modulating local dynamics of microtubules (MTs). Typical actin cytoskeletons induced by Rho are stress fibers running in an interphase cell, and the contractile ring formed in a mitotic cell (Figure 21).

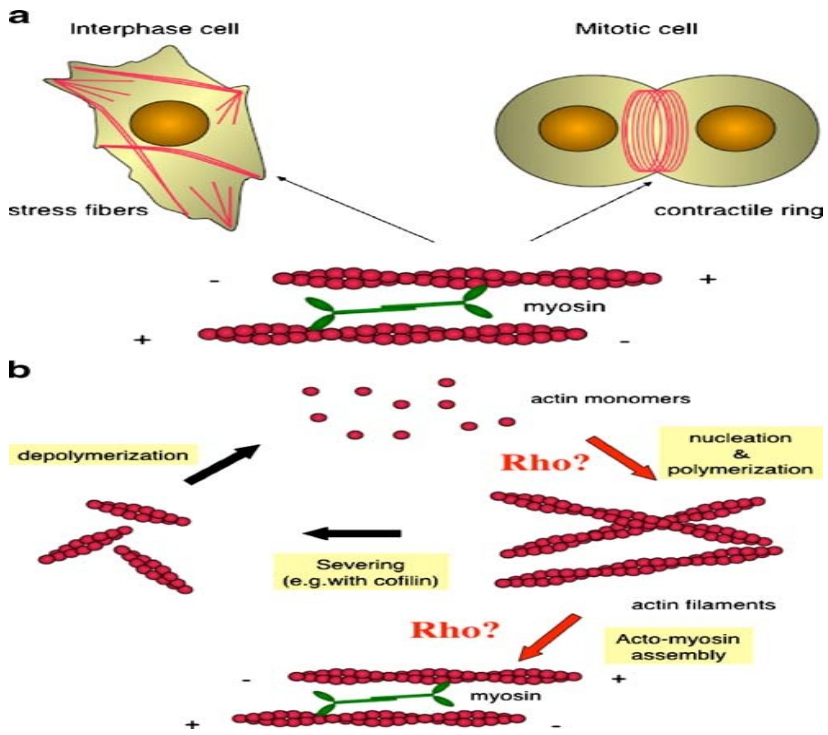


Figure 21: (a) Stress fibers and the contractile ring, (b) Presumed Rho regulated assembly of actin myosin bundles. **Adapted from** [333]

These actions of Rho are elicited by two major effectors; one is ROCK [334-336] and the other is mDia. The Rho pathway has a significant role in the malignant phenotype of cells [337] by inducing cell cycle progression, neovascularization, resistance to apoptotic stimuli, and tumour invasiveness and metastasis.

RhoB displays several properties which makes it functionally distinct from the other isoforms. First, it is an early response gene whose expression is regulated by a variety of extra-cellular stimuli including UV irradiation [338, 339], ionizing radiation [340, 341], PDGF [342-344], epidermal growth factor (EGF), transforming growth factor β (TGF-β) [345, 346] and reactive oxygen species [347, 348], which suggests that it may be a component of the cellular stress response [327, 349]. It also has a unique pattern of cellular distribution, been localized to the Golgi, endosomal vesicles, and the multivesicular body (MVB) [350, 351]. Several studies suggested a role for RhoB in endosome-mediated trafficking of growth factor receptors [351-354]. RhoB localizes to both the plasma membrane and the membrane of early and late endosomes [353, 355, 356].

❖ *Downstream mediators of Rho*

Rho kinase and mDia in combination induce actomyosin contraction in cells such as mDia produces actin filaments by actin nucleation whereas Rho kinase promotes filament cross-linking and the formation of stress fibers by increasing the phosphorylation of the myosin light chain (MLC) on serine 19. Role of RhoA and RhoB in malignant transformation [357] is opposite to each other but evidence shows that RhoB enhances the effects of RhoA or compensate for RhoA loss of function [358].

RhoB shows a unique pattern of posttranslational modification. As majority of Rho proteins are modified by the covalent attachment of a geranylgeranyl group, uniqueness of RhoB in that it can be in either a geranylgeranylated (RhoB-GG) or a farnesylated (RhoB-F) form. Geranylgeranylated and farnesylated RhoB perfoms different functions in cells. Farnesylated RhoB localizes to the cell membrane, promoting cell growth, activates NF-κB and mediates the effects of Ras on actin cytoskeleton [359-361]. In disparity, geranylgeranylated form of RhoB localizes to endosomes and induces cell apoptosis [359, 362]. It is generally believed that farnesylated RhoB promotes cell growth and is pro-oncogenic, whereas geranylgeranylated RhoB has anti-oncogenic [363] and pro-apoptotic effects [362] although some reports show no differential effects [364]. The antioncogenic effects of farnesyltransferase inhibitors are thought to result from an increase in the levels of geranylgeranylated RhoB [363].

When activated, RhoB triggers a signalling cascade promoting the activation of AKT and NF-κB survival pathways [365].

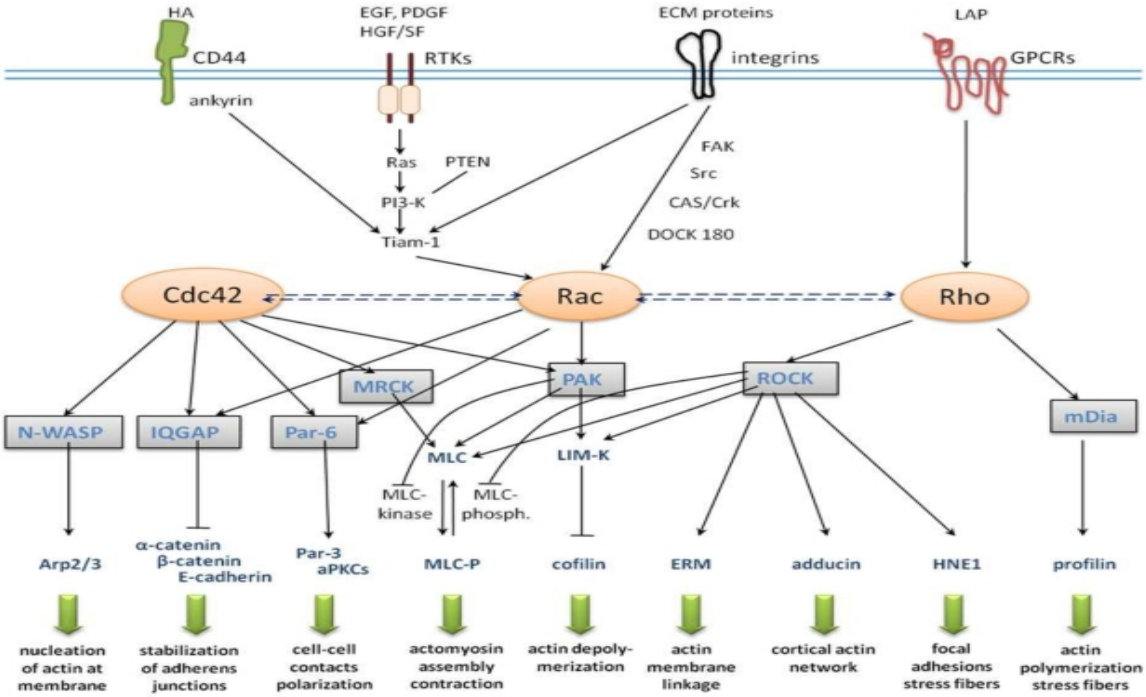


Figure 22: Regulators and effectors of the Rho GTPases. **Adapted from** [366]

Complexity in the role of RhoB:

❖ *RhoB as tumor suppressor*

Inspite antineoplastic effects of RhoB have been reported in some types of cancer, it has growth-promoting effects in numerous cell types. Several growth factors induced by hypoxia require RhoB and it also regulates endocytotic trafficking of pro-proliferative and anti-apoptotic kinases Src and Akt [367, 368]. The transformations mediated by Ras and Rho GTPases are similar in context and the pathways controlled by both form a complex signaling interlace that finally results in tumorigenesis[369-372]. Rho signaling is also necessary for aberrant growth induced by several oncogenic pathways [373-378].

Although early studies showed that RhoB has a positive role in cell growth, more recent studies indicate that RhoB seems to be involved in tumor suppression [379], since it is downregulated with tumor progression and functions by opposing the effect of other Rho GTPases [380]. Whereas expression of RhoA is upregulated upon progression of HNSCC, RhoB expression is detected in the normal epithelium and well-differentiated tumors, but becomes undetectable as tumors become less differentiated and invasive [381, 382]. However, little is known about the regulation of RhoB tumor-suppressive activity. Recently, it has been described that histone deacetylase 1 represses the small GTPase RhoB expression in a human non-small lung carcinoma cell line [383]. RhoB was also reported to inhibit Ras-induced invasion and metastasis [384, 385]. The exact mechanism whereby RhoB suppresses tumour growth and invasion is not clear, although its role in endosomal trafficking could be important.

❖ *RhoB as tumor promoter*

The overexpression of RhoB in Ras-transformed cells was shown also to induce a reversion of transformed phenotype, resulting in activation of p21Waf and inhibition of cell growth [380, 381]. RhoB effects are cell-type and/or are stage dependent, and in some cellular milieu, ectopic expression of RhoB promotes proliferation and transformation [386], however in others, high RhoB levels inhibit invasive and migratory cell behavior [387, 388]. This context-specificity has also been observed *in vivo* in some tumors [389], including lung cancer [390]. Because of these findings in lung cancer and in other tumors [380, 384, 391, 392], RhoB has been considered to function as a tumor suppressor [393].

Recently this prevailing view has been challenged by the unveiling of dominant host-derived effects in tumor vasculature elicited by RhoB [394]. Diego Luis-Ravelo et al showed that that ablation of RhoB levels in tumor cells dramatically decreased bone metastasis in an experimental model. In contrast, RhoB overexpression was associated with enhanced dissemination from the primary location and increased resistance to chemotherapy and radiation [395].

RhoB as a metastatic effector

RhoB is a strong metastatic effector with robust functional activities that also contributes to treatment resistance in lung cancer. Decreased levels of RhoB lead to high radiosensitization by enhanced tumor oxygenation in GBM and HeLa cells [396, 397] through HIF-1α downregulation [398] and altered DNA repair via integrins [399]. In contrast, in other models, genetic ablation of RhoB renders cells resistant to chemotherapeutic agents that target microtubules or DNA [393].

This seeming discrepancy could be explained by non-cell autonomous effects derived by host cell contacts [394] that markedly softened the longstanding perception of RhoB as a tumor-suppressor [386]. Kazerounian et al pointed out that this specificity could explain the obvious paradoxical observation [394]; that in early stages of lung tumorigenesis, loss of RhoB expression occurred between pre-invasive and invasive stages [390, 400]. Thus the various responses elicited by RhoB in a stage or subtype specific manner could be explained by the phenomenon that RhoB can recruit different effector proteins involved in intracellular trafficking to endosomal membranes, such as Dia1 [401] and PRK1 [402]. Kang et al identified a triple gene signature consisting of ROBO1, PITX1 and HDAC4 using a dual screening strategy of transcriptomic selection of genes with robust pro-metastatic activity [403]. Even though the individual contribution of each gene is irrelevant [404], this transcriptomic analysis also enabled researchers to identify RHOB as another top overexpressed gene as shown by [395] see Table 9 below

n°	probeset I	D	d.value	p.value	q.value	R.fold	Genename
1	207076_s_at		3,93	0,0002128	0,05033	7,63	ASS
2	220178_at		9,83	0,0000009	0,00988	5,08	NA
3	206924_at		5,67	0,0000108	0,01225	4,80	IL11
4	206698_at		4,65	0,0000530	0,02428	4,67	XK
5	204684_at		4,71	0,0000498	0,02405	4,45	NPTX1
6	208170_s_at		4,43	0,0000844	0,03147	4,26	TRIM31
7	205097_at		10,55	0,0000004	0,00988	4,23	SLC26A2
8	213194_at		5,62	0,0000121	0,01225	3,45	ROBO1
9	211980_at		4,20	0,0001230	0,03684	3,40	COL4A1
10	211981_at		4,02	0,0001764	0,04566	3,38	COL4A1
11	202238_s_at		4,35	0,0000965	0,03358	3,24	NNMT
12	202237_at		4,46	0,0000786	0,03086	2,79	NNMT
13	208502_s_at		3,99	0,0001872	0,04680	2,75	PITX1
14	212099_at		4,99	0,0000319	0,01798	2,68	RHOB
15	204971_at		4,69	0,0000503	0,02405	2,59	CSTA
16	206638_at		5,17	0,0000233	0,01556	2,58	HTR2B
17	204225_at		3,95	0,0002033	0,04949	2,48	HDAC4
18	217992_s_at		5,09	0,0000265	0,01619	2,43	EFHD2
19	218729_at		5,59	0,0000126	0,01225	2,37	LXN
20	203726_s_at		4,10	0,0001495	0,04176	2,37	LAMA3
21	221497_x_at		4,91	0,0000341	0,01877	2,31	EGLN1
22	206456_at		4,20	0,0001225	0,03684	2,18	GABRA5
23	219634_at		6,65	0,0000031	0,00988	2,12	CHST11
24	218872_at		4,34	0,0000992	0,03358	2,10	TSC
25	210719_s_at		5,66	0,0000117	0,01225	2,07	HMG20B
26	205462_s_at		5,74	0,0000099	0,01225	1,95	HPCAL1
27	219863_at		4,06	0,0001629	0,04426	1,94	HERC5
28	202679_at		5,50	0,0000139	0,01225	1,92	NPC1
29	220266_s_at		4,35	0,0000979	0,03358	1,88	KLF4
30	212288_at		4,24	0,0001140	0,03636	1,86	FNBP1
31	213223_at		4,41	0,0000862	0,03161	1,83	RPL28
32	210367_s_at		5,09	0,0000260	0,01619	1,80	PTGES
33	209587_at		4,10	0,0001499	0,04176	1,80	PITX1
34	207030_s_at		4,56	0,0000637	0,02697	1,78	CSRP2
35	204663_at		6,81	0,0000027	0,00988	1,78	ME3
36	212552_at		6,22	0,0000049	0,00988	1,68	HPCAL1
37	201250_s_at		4,29	0,0001073	0,03577	1,64	SLC2A1
38	202886_s_at		4,12	0,0001436	0,04159	1,58	PPP2R1B
39	218648_at		4,52	0,0000696	0,02835	1,56	TORC3
40	211126_s_at		3,90	0,0002244	0,05144	1,49	CSRP2

Table 9: Transcriptomic selection of relevant candidate genes. **Adapted from** [405]

In certain cell type, like prostate cancer, RhoB works as a tumor promoter not as a suppressor [406]. Sorting out the cell-type-specific bifunctional mechanisms and conditions of RhoB protein should help understanding the biological, physiological and pathological protein behaviour.

TGF β and RhoB axis

Rho proteins do not appear to be mutated in cancer cells, their expression is frequently elevated, signifying that Rho dysregulation promotes malignant phenotypes [380]. RhoB is the only member of the Rho small GTPases which is regulated at the transcriptional level. This regulation is important for its function due to the short half-life in the cell [380, 407]. TGF β has a vital role in this transcriptional modulation of Rho GTPases specially RhoB.

Non-Smad and Smad pathways in Rho GTPase activation by TGF β

Several studies have elaborated the role of MAP kinases or the phosphatidylinositol 3-kinase (PI3K) as downstream signaling effectors that cooperate with Rho GTPases or are activated by them to achieve TGFβ-induced cytoskeleton remodeling [408]. It also had been shown previously that Rho GTPases and Smad proteins played a significant role in long-term TGFβ induced actin cytoskeleton reorganization in various cell models [409-412]. Infact, their activation by TGFβ in fibroblasts is correlated with potent induction of α-SMA expression indicative of a myofibroblast phenotype [413] and its subsequent incorporation into microfilamentous structures.

RhoB /TGF β axis

Interestingly, the induction of RhoB gene transcription by TGF β shows a periodic pattern [411]. Here worth noting is that, Smad proteins specially Smad 3, in addition to serving as specific transcriptional activators of the RhoB gene, can also act as activators of Rho GTPase function [411, 414] and the regulation of actin dynamics [408].

Vasilaki E et al characterized that both the Smad as well as the MEK/ERK pathway are required for early RhoB gene induction by TGFβ. RhoB gene regulation by TGFβ-1 is a 2-phase process: in the first phase, ERK phosphorylation and Smad phosphorylation by the TGFβ-1 receptors coregulate the transcription of the RhoB gene. In the second phase, ERK phosphorylation is no longer required for RhoB gene transcription, which is now facilitated by Smad proteins alone or the cooperation of Smads with other factors bound to the RhoB promoter [408].

❖ Mechanisms of short-term and long-term actin cytoskeleton reorganization induced by TGF β*: Cross-talk between Rho GTPases and the TGFβ/Smad pathway*

With a combination of different studies a model of short- and long-term TGFβ-induced actin cytoskeleton reorganization in fibroblasts and other cell types has been proposed. This model is shown schematically in (Figure 23), and can be summarized as follows. In the **short-term** , TGFβ receptor activation by its ligand induces rapid activation of RhoA and RhoB GTPases (1), which is followed by activation of the ROCK/LIMK/cofilin pathway (2–4) and actin cytoskeleton restructuring (5),

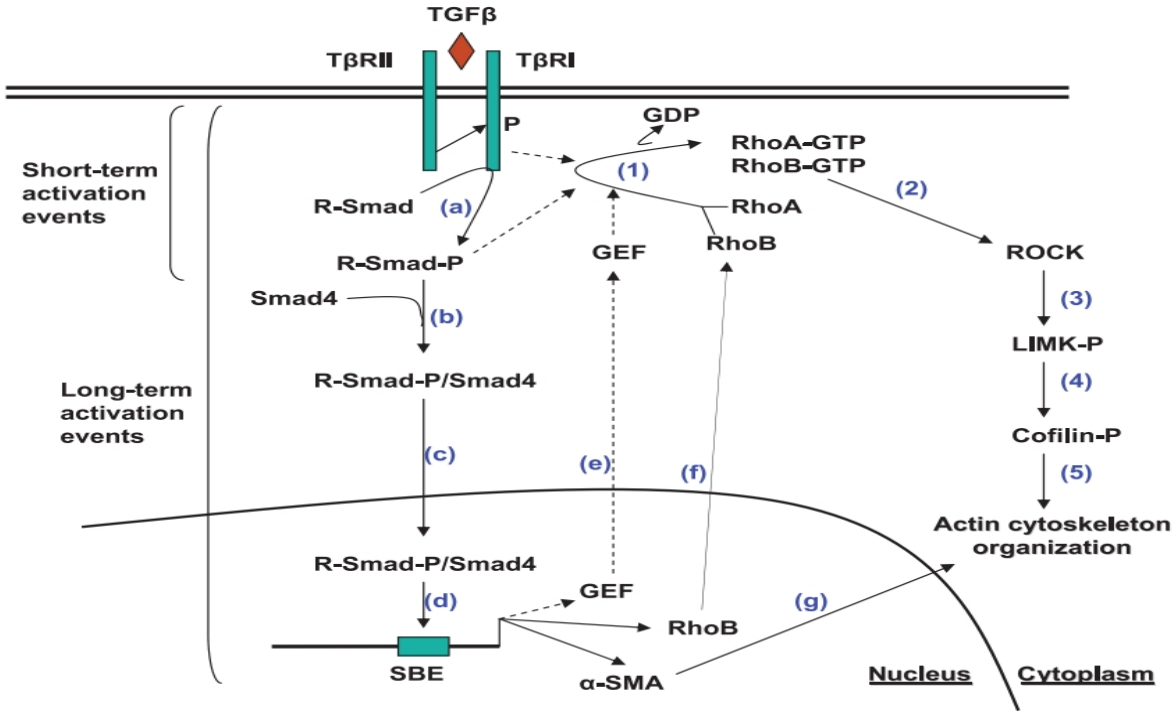


Figure 23: Mechanisms of short-term and long-term actin cytoskeleton reorganization induced by TGFβ in fibroblasts. **Adapted from** [408]

The **long-term** actin cytoskeleton response to TGFβ stimulation involves the Smad pathway and transcriptional activation events. TGFβ rapidly induces the phosphorylation of R-Smad proteins (a), this leads to the formation of R-Smad (P)–Smad4 complexes (b).These complexes are then translocated to the nucleus (c) and recruited to various TGFβ-responsive genes (d). RhoB and α-SMA genes are two of the important genes activated by this pathway. The preferential transcriptional activation of the RhoB gene over the RhoA gene strongly suggests an exclusive role of RhoB GTPase in long-term actin cytoskeleton reorganization by TGFβ (f). It is worth mentioning the role of Smad proteins in long-term Rho GTPase activity by various mechanisms (such as transcriptional activation of a gene coding for a Rho GEF (e).

Finally, in fibroblasts, the TGFβ–Smad pathway causes transcriptional activation of the α-SMA gene and its incorporation into the cytoskeleton (g), a process that is characteristic of a fibroblast to myofibroblast differentiation program [413, 415].

RhoB gene (but not the RhoA gene) is a direct transcriptional target of the TGFβ–Smad signal transduction pathway, and that expression of the RhoB gene as well as the activity of the RhoB promoter can be induced by TGFβ-regulated Smad proteins [416]. In fibroblasts, activation of RhoB gene expression by TGFβ is associated with the stimulation of α-SMA expression and microfilament incorporation, indicative of a fibroblast to myofibroblast differentiation program.

Therapeutic Targeting of Tumor Microenvironment

Wound healing is a process that allows cells to break normal constraints on their growth and cross boundaries. When a cell gain access to that program, that's become a good environment for cancer. Conventionally, the main treatment plan for cancer patients relies on debulking surgery, radiotherapy, chemotherapy with cytotoxic agents, to reduce tumor bulk. But more recently, modulation of tumor microenvironment has also become an acceptable therapeutic target. Lung microenvironment represents a unique setting in which lung carcinogenesis proceeds in collaboration with the soluble (cytokines, proteases, hormones, etc.), cellular (fibroblasts, inflammatory cells, endothelial cells, etc.) and structural (extracellular matrix or ECM), components of the microenvironment.

Utilizing proteomic approaches, the investigators have established that stromal cells in the tumor microenvironment do alter the tumor cell secretome, including proteins required for tumor growth and dissemination. Aptly targeting the tumor microenvironment is an emerging strategy that embraces distinctive potential for advancing the current state of lung cancer treatment (Figure 24).

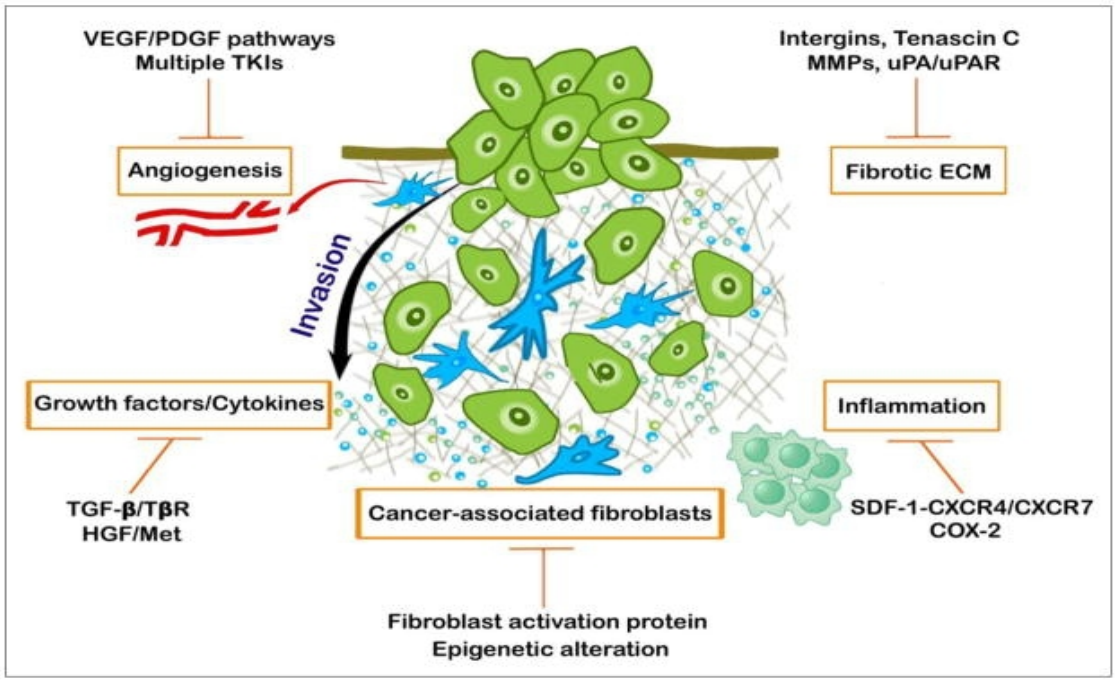


Figure 24: Several currently therapeutic strategies aim at the tumor microenvironment
Adapted from [417]

In general, these innovative treatment strategies can be summarized as follow: targeting stromal components (such as the ECM and CAFs), and interfering with growth factor/cytokine-mediated signaling.

1. The ECM antagonists

The function of integrin's as cellular adhesive and signaling molecule make of attractive target for cancer therapy. Cilengitide (a αvβ3 and αvβ5 antagonist) appeared to prolong survival in a phase II randomized clinical trial when combined with standard radiochemotherapy in patients with newly diagnosed glioblastoma regardless of methylguanine-DNA methyltransferase (MGMT) methylation status [418].

Secondly, strategies to target the uPA system have been adapted which comprise of inhibiting uPA/uPAR expression and activity, or blocking uPA-uPAR interaction. In preclinical studies, inhibition of uPA/uPAR with antibodies or small interfering RNA (siRNA) has caused a significant restraint in tumor cell growth and invasion [419, 420]. WILEX AG develops two small synthetic molecules specifically against uPA, the intravenous drug WX-UK1 and its oral pro-drug WX-671/Mesupron [421].

❖ *MMP inhibitors*

MMP inhibitors are promising since they play a role in both ECM remodeling and epithelial-CAF signaling. However, so far, no MMP inhibitors have been approved clinically for cancer treatment. Although preclinical studies testing the efficacy of MMP suppression in tumor models were encouraging, the results of clinical trials in cancer patients have been rather disappointing [422]. Several broad-spectrum MMP inhibitors (e.g., marimastat and tanomastat/BAY12-9566) have not shown significant efficacy in randomized phase III trials in patients with breast and ovarian cancer as they do not prolong overall progression free survival or exhibit dose-dependent toxicity [423, 424]. Table 10 shows MMP inhibitors in clinical development.

Inhibitor	Company	Structure	Specificity
Batimastat (BB-94)*	British Biotech	Peptidomimetic	Broad spectrum
Ilomastat (GM-6001)*	Glycomed	Peptidomimetic	Broad spectrum
Marimastat (BB-2516)	British Biotech; Schering-Plough	Peptidomimetic	Broad spectrum
Tanomastat (BAY-12-9566)	Bayer	Small molecule	Higher specificity towards MMP2, MMP3 and MMP9; does not target MMP1
Prinomastat (AG-3340)	Agouron; Pfizer	Small molecule	Broad spectrum
Metastat (COL-3)	Collagenex	Low-dose tetracycline derivative	Broad spectrum; higher specificity towards MMP2 and MMP9
Neovastat (AE-941)	Aeterna	Shark cartilage extract	Broad spectrum
BMS-275291	Bristol-Myers Squibb; Celltech	Small molecule	Broad spectrum
MMI-270B (CGS-27023A)	Novartis	Small molecule	Broad spectrum
Trocade (Ro-32-3555)	Roche	Peptidomimetic	MMP1, MMP8 and MMP13
MMI-166	Shionogi	Small molecule	Broad spectrum
*First-generation matrix metalloproteinase (MMP) inhibitor drugs were not orally bioavailable and were not advanced to phase III clinical trials.			

Table 10: MMP inhibitors in clinical development. **Adapted from** [425]

The other main cause of the failure of MMPIs in phase III clinical trials, aside from dose limiting side effects, can be the broad spectrum action of these drugs. There is now sufficient evidence that MMPs have complex roles some of which helps in disease progression while others are protective in nature. These “good” and “bad” roles are related to disease stage and genotype specific. Hence, optimum use of MMP inhibitors centers on identifying the harmful MMP activity and targeting it whereas sparing any non-contributory or beneficial activities. The resemblance in the structure of the catalytic domain of many MMPs has made, design of truly specific small molecule inhibitors, very difficult [426].

2. Anti-CAF therapy

CAFs have been an important therapeutic target because of their genetic stability as well as important role in tumor progression, which may be an important factor in reducing drug resistance [427]. Anti-CAF therapy has a triple effect by way of their anti-tumor qualities, anti-angiogenesis potencies, and the ability to increase tumor uptake of chemotherapeutic drugs.

Evidence that CAFs are distinct from normal fibroblasts is beginning to define these cells as potential targets for cancer therapy. Fibroblast activation protein (FAP) is a member of the serine protease family that is selectively expressed in the stromal fibroblasts associated with epithelial cancers and is expressed at low or undetectable levels in the resting fibroblasts of normal adult tissues. FAP is expressed in more than 90% of epithelial carcinomas, which makes it a promising target [428]. Potential therapeutic strategies include inhibiting FAP enzymatic activity or delivering drugs/toxins to the tumor stroma via antibodies or small-molecular inhibitors.

However, worth noting is that, CAFs also have significant individual differences in drug sensitivity similar to tumor cells. Therefore it is important to note that FAP inhibition may not halt the tumor cell growth in all cancer types [429].

So Gonda *et al.* also emphasize that Epigenetic interventions are also a very attractive candidate for tumor stroma–specific therapies by inhibition of DNA methyltransferase (DNMT1, DNMT3a, and DNMT3b) [430]. They may offer an opportunity to inhibit CAFs and foresee its prospects. These are summarized in Table 11

Proposed Mechanism	***Target***	***Drugs, drug classes***
CAF Differentiation	DNMT1	5-aza-2'-deoxycitidine
	FAPa	Sibrotozumab
CAF-Epithelial Interaction	HGF/Met	NK4, anti-HGF mAbs
	MMPs	Non-peptidic MMP inhibitors (*) TIMPs
	SDF1/CXCL2	AMD-3100
	Smo (Hedgehog pathway)	IPI-926
CAF-ECM Interaction	MMPs	(as above)
	Tenascin-C	Radioactive labeled antibody, siRNA
	PAI-1/uPAR	Radioactive labeled PAI, Å6
	FAPα	Sibrotozumab, small molecule inhibitors
CAF-Endothelial Interaction	PDGF-C	Antibodies used in synergy with anti-VEGF-A
CAF targeted anti-Inflammatory signaling	CD11b+ myeloid stromal cells (MDSCs) COX-2	CTL
CAF DNA methylation	DNMT1	5-aza-2'-deoxycitidine

*Examples of MMP inhibitors include Marimastat, Rebimastat (broad spectrum); Tanomastat (MMP2,3, 9); Prinomastat (MMP 2,3,9,13,14)

Table 11: Stroma targeted anti-cancer strategies. **Adapted from** [430]

3. Interfering with growth factor/cytokine-mediated crosstalk between CAFs and cancer cells

The signaling amongst CAFs and epithelial cancer cells propose multiple attractive targets for intervention. Discovering the taut interactions between tumor progression and angiogenesis or chronic inflammatory environment led to the intensive investigation of relevant growth factor/cytokine-mediated signaling pathways as targets for cancer therapy. Can be summarized as in Table 12

Mediators	Approach	Effect
Combating pro-tumorigenic factors		
CXCR4/SDF-1	Plerixafor (AMD3100) & T140 analogs, antagonists for the seven-transmembrane G-protein-coupled chemokine receptor	Disruption of CXCR4-mediated tumor cell adhesion to stromal cells; sensitization of cells to cytotoxic drugs
TGF-β	SM16, TGF-β type I receptor kinase inhibitor	Combined with adenoviral IFN-β, increased intratumoral leukocytes (macrophages, NK cells and $CD8^+$ cells)
	Antisense TGF-β in allogenic vaccine combined with GM-CSF on TAG plasmid	Reduction of tolerance and stimulation of immune responses
	Soluble TFG-β RII:Fc	Potent anti-metastatic activity
	SD-093 & LY580276, blockers of TGF-β catalytic activity	Blocking EMT and migration
VEGF	Avastin, a humanized antibody against VEGF-A	Targeting VEGF signaling and inhibition of angiogenesis
	Anti-VEGFR1 and R2 antibody	Blockade of the lung pre-metastatic niche; antiangiogenic and antimetastatic effect
	Sorafenib & sunitinib, TKIs	Vessel normalization; antiangiogenic effect
PDGF	Imatinib, a TKI targeting PDGFR	Targeting pericytes and CAFs in TME
HGF	Rilotumumab, a humanized monoclonal antibody against HGF	Inhibiting HGF signaling
IL-6	ALD518, a humanized anti-IL-6 monoclonal antibody Phase I/II NSCLC	Palliative treatment in Phase I/II NSCLC as IL-6 is correlated with poor prognosis, anemia and cachexia
FAP	Sibrotuzumab, a monoclonal antibody against FAP	FAP is highly expressed on stromal fibroblasts
Integrins	Etaracizumab, an integrin αvβ3 antagonist	Migration and proliferation
	CNT095, antagonist against αvβ3/αvβ5	Inhibition of tumor neoangiogenesis; effect on
	Volociximab, monoclonal antibody against α5β1 integrin	CAF-driven tumorigenesis
Increasing anti-tumorigenic factors		
IFN-γ	Type I on vaccinia/adenovirus (mouse/human models)	Activation of cytotoxic T cells and NK cells
	Type I + type III (IL-29)	Growth inhibition by cell cycle arrest and apoptosis
GM-CSF	1650-G vaccine using GM-CSF as adjuvant to autologous DCs	Antigen presentation; activation of immune response

Table 12: Targeting the microenvironment with secretory factors. **Adapted From** [431]

a. Disabling TGF-β signaling

Studies support targeting TGF-β signaling as an anti-cancer therapeutic strategy (Figure 25). Currently, therapeutic strategies against TGF-β can be divided into three approaches: i) prevention of TGF-ß synthesis by using antisense molecules; ii) inhibition of the ligand-receptor interaction by ligand traps (monoclonal antibodies and soluble receptors) and anti-receptor monoclonal antibodies; and iii) inhibition of the receptor mediated signaling cascade, using TGF-β receptor kinases inhibitors and aptamers [432]. For each of these approaches, several drugs have been developed and are either in non-clinical or in early stages of clinical investigation. Since TGF-β level is often elevated during tumor progression, inhibiting its synthesis has the potential to decrease TGF-β levels within the tumor microenvironment. Each agent in these categories has shown efficacy for halting tumor growth and dissemination in preclinical data [433-435]. These very encouraging results merit disabling TGF-β pathway as a therapeutic target.

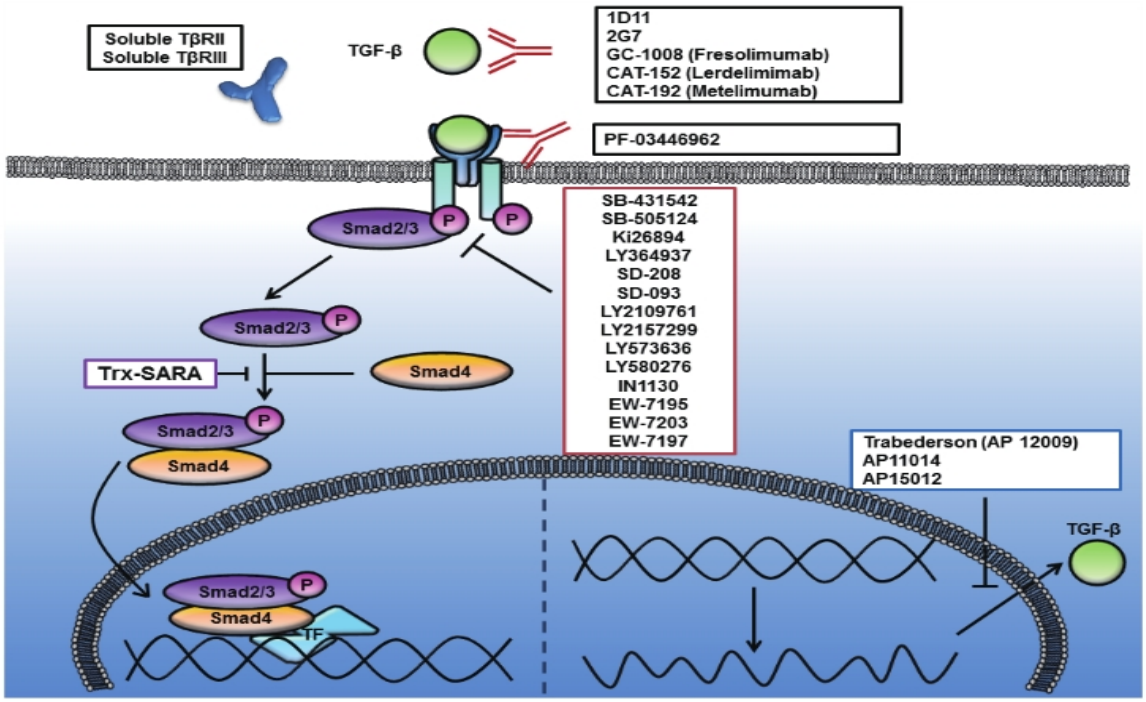


Figure 25: TGF β signaling pathway inhibitors under development for potential cancer therapy. (Blue line box) Antisense molecules inhibit TGFß synthesis; (black line box) monoclonal antibodies, soluble receptors and anti-receptor monoclonal antibodies inhibit ligand-receptor interaction; (Red line box) receptor kinase inhibitors and peptide aptamers (Purple line box) inhibit signal transduction**. Adapted from** [436]

b. Inhibiting the HGF/Met axis

Inhibiting the HGF/Met axis has become one of the hottest approaches in anticancer drug exploitation and is designed with distinct lines [437]. Pertaining to effective therapeutic results in the preclinical studies, some inhibitors have been selected for clinical development. Amongst these selective inhibitors AMG 102 (rilotumumab) and ARQ 197 (tivantinib) have been the most widely studied.

c. Stifling Angiogenesis

The strategy stifling angiogenesis is regarded as a desirable anticancer approach aimed at multifaceted rationale including enhancement of chemotherapeutic sensitivity and reduction of tumor burden and metastasis [438]. It has become the first clinical entity among all approaches for targeting the TME, due to this, some anti-angiogenesis drugs receiving FDA approval.

Bevacizumab (Avastin) (a recombinant humanized anti-VEGF mAb) has been approved for patients with advanced or metastatic renal cell carcinoma (RCC), colon cancer and NSCLC [439, 440] in combination with IFN-α or chemotherapy Several phase III studies for Ramucirumab (IMC-1121B) (a fully human mAb that specifically inhibits VEGFR-2) [441], in patients with NSCLC (NCT01168973), digestive tract adenocarcinoma (NCT01170663), hepatocellular carcinoma (HCC, NCT01140347), and breast cancer (NCT00703326) are also ongoing.

Secondly, inhibiting the PDGF pathway via human anti-PDGF/PDGFR mAb or highly specific anti-PDGFR small-molecular TK inhibitors (TKIs) can be an effective strategy. IMC-3G3 and MEDI-575 are the fully IgG1 and IgG2 mAb against PDGFRα. These are in Phase I trials in patients with advanced solid malignancies and lymphomas and shown good tolerance [442, 443].

Recently, studies have shown that some tumors may overcome inhibition of VEGF-mediated angiogenesis through up regulation of PDGF-C [444]. Keeping in view, multiple TKIs, targeting VEGFR, PDGFR and the other receptor TK (such as FGFR, FLT-3, and c-Kit), have grown into a novel class of antitumor drugs.

d. Neutralizing inflammatory response

The blockade of the SDF-1–CXCR4/CXCR7 axis has been designated as a potential additional target for standard neoadjuvant and adjuvant treatments. Some SDF-1/CXCR4 signaling antagonists, e.g., the anti-CXCR4 drugs plerixafor (AMD3100) and BKT140 (4F-benzoyl-TN14003), the anti-SDF-1 analog CTCE-9908 have shown significant inhibitory effects on primary tumor growth and metastasis in mouse tumor models [445-447].

Neutralization of the inflammatory response has long been tested with COX-2 inhibitors such as nonselective NSAIDs (e.g., aspirin, naproxen, and ibuprofen) and selective COX-2 inhibitors (COXIBs, e.g., celecoxib and etoricoxib). Currently many clinical trials utilizing COX-2 inhibitors alone or in combination with other drugs are in progress (e.g., NCT01111591, NCT01614301, and NCT00427999). However, various adverse effects associated with the use of NSAIDs and COXIBs, such as gastrointestinal complications, inhibition of platelet activation and high incidence of cardiovascular events associated with prolonged use of selective COX-2 inhibitor have limited their use. Also in patients with advanced NSCLC, no prolonged survival duration was observed after therapy using celecoxib plus docetaxel and carboplatin [448].

Persistently, more attention has been diverted to design therapeutic strategies to target the TME as well as tumor cells. Among these targeted agents, some have successfully become a clinical reality and are used as first-line drugs (such as bevacizumab); others are already in clinical trials, and many more are still in preclinical studies. As the complexity of relationship between the TME and tumor cells had unfolded the combination of two or more therapeutic agents may be more beneficial to cancer patients.

II. OBJECTIVES

Objectives

It is now becoming increasingly evident that ionizing radiation also induces modifications of the tumor microenvironment, which profoundly impact tumor biology [449]. This is particularly relevant to cancers relapsing after radiotherapy, which tend to develop into invasive and metastatic conditions with poor prognosis [450]. Paracrine interactions between carcinomas and their mesenchymal stroma are critical to tumor development and progression [451-453]. The molecular details of this cross-talk may provide some insight into the mechanisms by which epithelial cells and mesenchymal cells converse during injury repair in the adult lung.

Moreover, scarring signals activated by irradiation within tumor microenvironment could impair tumor response to irradiation and contribute to the emergence of residual disease responsible for local recurrence and metastasis. Furthermore these signals emitted by the tumor stroma could share similar molecular characteristics than those activated in radiation induced fibrosis. Deciphering these signals may offer novel therapeutic opportunities with differential effect protecting normal tissues and enhancing tumor response to radiation. Our previous studies mostly performed on human tissues and cells have shown the differential involvement of TGF β/Smad and Rho/ROCK/CTGF cascade in the mechanisms of development and maintenance of radiation induced intestinal fibrosis [33]. This activation could be a general mechanism for stromal activation after irradiation and constitute a common molecular denominator of wound healing response and desmoplastic reaction surrounding tumors and metastatic process.

In the present study, we proposed that scarring signals including TGF-β and RhoB that are activated by irradiation in the stroma, could enhance tumor aggressiveness after radiation therapy. Therefore, RhoB deficiency would indirectly enhance anti-tumor effect of radiation therapy.

In the first part, our objective was to investigate this crosstalk so we used *in vitro*, a co-culture model consisting of lung carcinoma cells (TC-1) and lung primary fibroblasts (Wt) .We also used RhoB deficient fibroblasts (RhoB-/-) to assess our hypothesis.

Secondly, we investigated the effect of irradiated microenvironnement on tumor growth *in vivo* and finally investigated possible release of Circulating Tumor Cells. We hypothesized that radiation-induced disruption of tumor architecture during and after RT could cause tumor cells escape into the the peripheral circulation. At the early stage of commencement of RT, when many tumor cells have sustained only minimal damage, release of such circulating tumor cells (CTCs), could be detected. These cells, when present, might contribute to the risk of distant metastases. We tried to answer the following questions utilizing a lung orthotopic model.

1- Does Local Irradiation of the stroma modify tumor up-take and growth?
2- Does irradiation of the tumor release CTC and/ or modify their Level?

III. MATERIALS AND METHODS

Materials and Methods

In vitro Experiments

1- Cells

C57BL6 (Wt) and RhoB-/- mice [454, 455] were used to isolate primary Lung Fibroblasts by enzymatic digestion (collagenase/trypsine) and cells were subculture in DMEM +Glutamine with 20% foetal calf serum, 50U penicillin /streptomycin, 1% Hepes, 10mg EGF, ITS.

TC-1 cells (Murine adenocarcinoma lung) were grown in RPMI 1640 medium with 10% fetal bovine serum or conditioned medium isolated from fibroblasts culture.

2- Chemicals

TGF β1 Inhibitor (SB 431542 used in 10 μM concentration) and MMP Inhibitor (O-Phenanthroline used in 100 μM) were both bought from Santa Cruz.

3- Irradiation

Wt and RhoB-/- Fibroblasts were grown to 80 % confluence, culture medium was replaced with FCS-free medium before irradiation. Fibroblasts were irradiated at 10 Gy with Cs137 (IBL-637 (CIS-BioInternational, France) gamma irradiator (662 keV photons) at 1Gy/Min. 24hours later conditioned medium (CM) from Wt and RhoB-/- cells was added to TC-1 cells and left for 24hrs more. Supernatants and cells were prepared for Western Blot analysis. The same is done as vice versa with TC-1 cells irradiated. See (Figure 26) for Experimental Scheme.

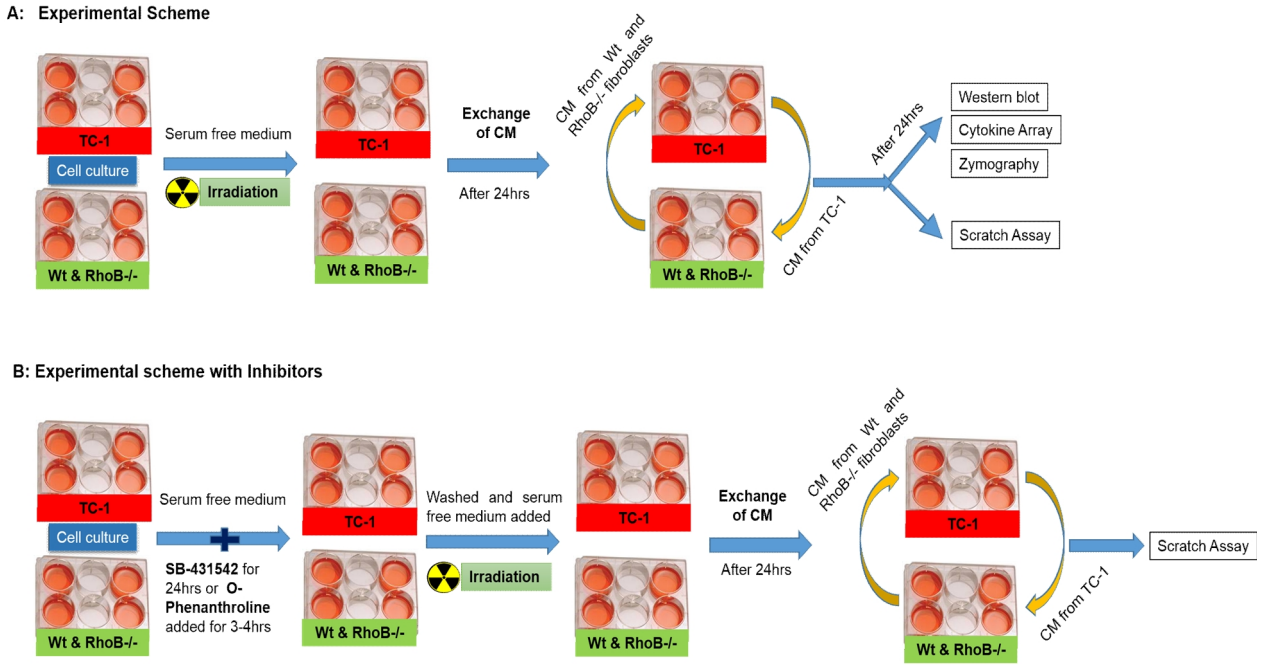


Figure 26: Experimental Scheme for *in vitro* coculture experiments.

4- Clonogenic assay

TC-1 cells were plated at different cellular density. 4hrs later, cells were irradiated at doses ranging from 0 to 10 Gy (Cesium=Cs137, 1 Gy/min, gamma irradiator IBL-637 from CIS-BioInternational, IBA, Saclay, France). Cells were incubated for 5–7 days under standard culture conditions and colonies were stained with crystal violet, washed and counted. The same is done with conditioned medium added from Wt and RhoB -/- fibroblasts. Colonies with more than 50 cells were counted and surviving fraction (SF) for each was calculated by dividing the number of cell clones by the number of cells plated and was normalized to the ratio of clonogenic survival of non-irradiated controls and clonogenic survival for each radiation dose.

5- Wound healing assay

Wild type and RhoB-/- Fibroblasts and TC-1 cells were grown to confluence in 6-well culture plates. Cells were irradiated at 10Gy. 24 hours later cell layers were wounded using a sterile 200 µl pipette tip and dead cells are washed out, then conditioned medium was added. Images are captured at 0hr with Nikon Phase contrast, Japan at 10X and plates were returned to the incubator to recover from wounding. After 24 Hrs culture plates were removed and monolayers were photographed again. Wound width was measured on hard copy prints of the images.

6- Electrophoresis and western blotting

Cell lysate are prepared utilizing RIPA lysis buffer (Sigma Biotech). Protein concentration was measured using a BioRad protein assay and electrophoresed. Primary antibodies were diluted in TBS-T solution (Vimentin (E-5) dil 1:1000 from Santa Cruz Biotechnology, Snail(C15D3) dil 1:1000, TGF β1(V) dil 1:250 all from Cell Signaling , α–SMA dil 1:500 from Abcam and GAPDH dil 1:5000 from Millipore) overnight at 4 ° C, washed next day in TBS-T and incubated with the horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody. The blots were developed using SuperSignal® West Pico Chemiluminiscent Substrate (Pierce Biotechnology, IL) according to the manufacturer's instructions. The chemiluminiscence signal from the membranes was detected and evaluated using G-box iChemi XT4 digital imaging device (Syngene Europe, Cambridge). Quantification was performed using Image J software. Relative proteins expression is normalized to the respective value for GAPDH.

7- Cytokine array analysis

The conditioned medium (undiluted) were collected, clarified by centrifugation at 1,200 rpm for 15 min and was probed for cytokine profile using the RayBio® Mouse Cytokine Antibody Array 3 and 4 kits according to the manufacturer's instructions (RayBiotech®; Norcross, GA, USA). Briefly,

the array membranes pretreated with cytokine antibodies was first blocked with blocking buffer for 1 hr and then incubated with 2 ml of serum free conditioned medium from TC 1 samples overnight at 4°C. Next day membranes were washed and incubated with biotin-conjugated anticytokine mix for 2 hr at room temperature. Membranes were washed again and incubated with horseradish peroxidase-conjugated streptavidin for 2 hr at room temperature. The membranes were developed using enhanced chemiluminescence solution. The chemiluminiscence signal from the membranes was detected and evaluated using G-box iChemi XT4 digital imaging device (Syngene Europe, Cambridge). Quantification was done by Genetools from syngene. Data were imported into an Excel spreadsheet, normalized against a control across membranes and final values were calculated.

8- Zymography

Zymography was performed on CM using Novex Zymogram Gels (Invitrogen) following the manufacturer's protocol as described previously [456]. Briefly Serum samples were mixed with Tris-Glycine SDS Sample Buffer (2X) 1:1 and separated using 10% zymography gel (Invitrogen) to document cleavage of gelatin by MMP-2 and MMP-9. Electrophoresis is done at Voltage: 125 V constant for 90 minutes. After electrophoresis, gels were incubated with 1X Zymogram Renaturing Buffer for 30 minutes at room temperature with gentle agitation. Then 1X Zymogram Developing Buffer is added to the gel to equilibrate the gel for 30 minutes at room temperature with gentle agitation. After that, fresh 1X Zymogram Developing Buffer was added to the gels. Gels were incubated at 37°C for at least overnight for maximum sensitivity. Zymogram gels are stained with the SimplyBlue™ Safestain and destained with milli Q until clear. Gelatinolytic activity was evident as clear bands against a dark blue background.

9- Statistical analysis

Statistical analysis was performed using Graphpad prism 5. Statistics were expressed as mean ± SEM and analyzed using the ANOVA and the Student Newman Keul's test with a p value <0.05 was considered significant.

In vivo Experiments

10-Cells

For *in vivo* experiments, human A549 lung adenocarcinoma cell line stably transfected with luciferase (A549luc) was purchased from Caliper Lifesciences Corp. Cells were cultured in RPMI 1640 medium with 10% fetal bovine serum. Cells were tested as luciferase positive using direct application of 2 mL of a 150 mg/ml solution of luciferine (Firefly Luciferin, Caliper Lifescience Corp, USA), followed by immediate bioluminescent imaging (IVIS system, Caliper Lifescience Corp).

11-Animals

Six-week-old female athymic mice were purchased from Janvier (Elevage Janvier, Mayenne, France), and kept in appropriate conditions. Once arrived in our animal facility, mice were divided in 3 groups, underwent xenografts implantation following the protocols described below (Figure 27), and subsequent bioluminescent imaging. Animals were then sampled at dedicated time point.

Primary tumour, lungs and heart were surgically harvested, fixed using a formalin-free fixative (Finefix, Milestone S.r.l., Sorisole, Italy), and embedded into paraffin. Histological examination was then performed. All animal experiments were approved by the local Ethics Committee (CEEA IRCIV/IGR nu26, registered with the French Ministry of Research), and were in compliance with the European Directive 86/609/CEE and French laws and regulation.

Mice were divided into 3 groups:

1) Group 1: act as control: athymic nude mice underwent orthotopic injection of A549luc cells in the parenchyma of the left (n = 10).

2) Group 2: animals underwent Orthotopic injection of A549luc cells in the parenchyma of the left lung (n = 10). After 2 weeks, Irradiation was performed, and CTC analysis was performed 24Hrs (n=5) and 1 week (n=5).

3) Group 3: mice were irradiated with 15Gy and orthotopic lung implantation of A549Luc was performed after 5 weeks then weekly CTC analysis was performed. In each group final comparative analysis with PCR and veridex was performed after 2 weeks. (n=10).

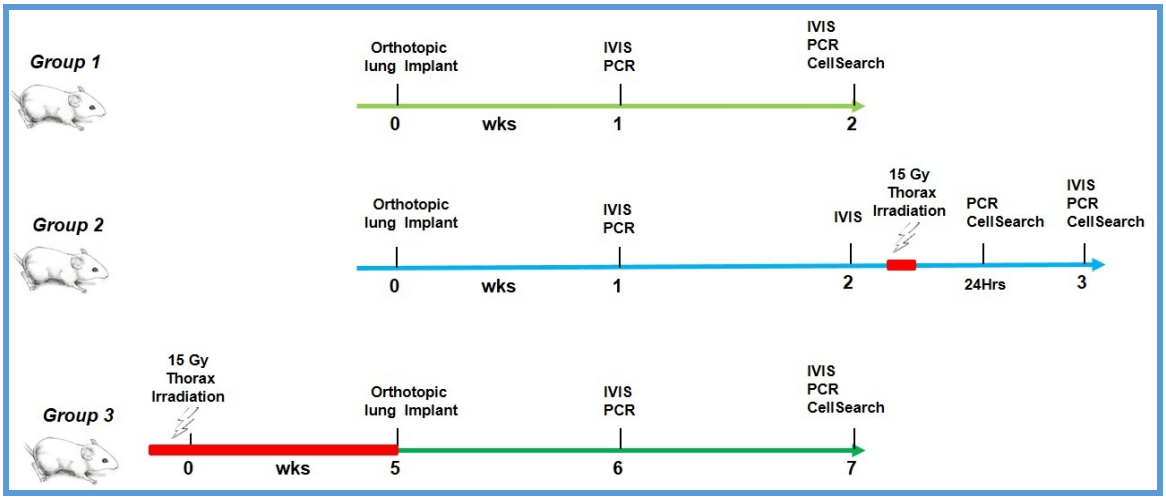


Figure 27: Experimental scheme for *in vivo* experiments

12-Tumorigenicity

Tumorigenicity was examined by injection of 2 x 10^6 cells / 200ul of PBS subcutaneous into the right flank of nude mice (n=3). Tumor size was measured twice a week. Tumor Volume (V) was calculated by the formula V=1/2 x length x $width^2$.

13-Transpleural orthotopic injection

Animals were anesthetized using Isofluorane inhalation and put in a position of right lateral decubitus. A 2-cm skin incision was performed below the left scapula, and a sharp dissection of the chest muscles was performed, in order to expose the costal layer. On observing the left lung motion through the pleura, 3 x 10^6 A549luc cells in a solution containing 20 µL of culture medium, and 20 µL of mouse sarcoma extracellular matrix (Matrigel) was directly injected through the intercostal space into the lung to a depth of 3 mm using a 29 G needle permanently attached to a 0.5 mL insulin syringe. The skin incision was closed by a 4-0 polypropylene suture [457].

14-Ionizing radiation

Ionizing radiation with X-Rays Tube Comet MXR-225/22 operating at 200 kV/15 mA was applied to the thorax; the area of irradiated tissue was approximately 2–2.5 cm2. The rest of the body above the base of neck and below the xyphoid process was shielded from radiation using lead blocks.

15-Bioluminescent imaging

Bioluminescence was detected weekly from luciferase expressing A549 cells (A549luc) after implantation into mice to monitor tumor growth. Luciferin was used as the substrate for the luciferase expressing tumour cells and injected intra peritoneally at a concentration of 150 mg/kg in PBS, 10 minutes before imaging. Mice were then anesthetized using 2% isofluorane and imaged using a cooled CCD camera (IVIS system, Caliper Lifesciences Corp, USA). Exposure times ranged from 2-3

minutes with Binning Factor:2, f Number:16, Filter Position:1 and Emission filter:Open. Images were quantified as photons/s using the Living Image software (Caliper Lifesciences Corp, USA).

16-CTC analysis in collaboration with C Decreane

❖ *Blood sampling*

For each time point in experimental schedule blood sampling for PCR analysis was done by retro orbital route (100µl) and jugular venous puncture (100µl). For endpoint analysis by veridex intra-cardiac blood sampling was done (600µl) . Every time blood was collected in EDTA tubes.

❖ *PCR*

Detection of CTCs by PCR was done at Institute Curie by Charles Decraene Group. Weekly 100µl of blood was collected in EDTA tubes after retro-orbital punctures.

❖ *CellSearch epithelial cell kit (Veridex LLC, USA)*

A Intracardiac blood puncture of 600µL was performed and was tested for CTC using the CellSearch system and a modified protocol based on the Study flowchart. Briefly, to identify human CTCs in mouse blood, each 600µL mice blood samples was mixed with 7 mL of healthy human blood. Then, each sample was automated enriched for cells expressing the epithelial-cell adhesion molecule (EPCAM) with antibody-coated magnetic beads, and cells were labeled with the fluorescent nucleic acid dye 49,6-diamidino-2-phenylindole dihydrochloride (DAPI). Fluorescently labeled monoclonal antibodies specific for leukocytes (CD45–allophycocyan) and epithelial cells (cytokeratin 8,18,19-phycoerythrin) were used to distinguish epithelial cells from leukocytes. Cell Enrichment and labeling were performed using the CellSearchH Autoprep. The identification and enumeration of CTCs was performed using the CellSearchH Analyzer II. CTCs were defined as nucleated cells lacking CD45 and expressing cytokeratin 8, 18, 19. As a negative control, a solution containing 600µL of non-tumor bearing mouse blood and healthy human blood was analysed. As a positive control, 50 and 500 A549luc cells were analysed in a solution containing 600µL of medium and 7 mL of healthy human blood [457].

17-Histopathological analysis

One week after hemodynamic studies, mice were killed by cervical dislocation; hearts and lungs were collected at dedicated time points. Organs were fixed in Finefix (Milestone medical, Italy), paraffin embedded and cut into 4 µm sections. Longitudinal sections were cut at the level of mid-horizontal plane of the heart. Sections were stained with Hematoxilin-Eosin-Saffranin (HES), Cytokeratin staining was done according to manufacturer protocol on parafin embedded blocks using monoclonal mouse anti-human cytokeratin 18 clone DC 10 from Dako, Denmark and examined using conventional light microscopy.

IV. RESULTS

Results

1. Paracrine signals secreted by fibroblasts do not modulate TC-1 radiosensitivity

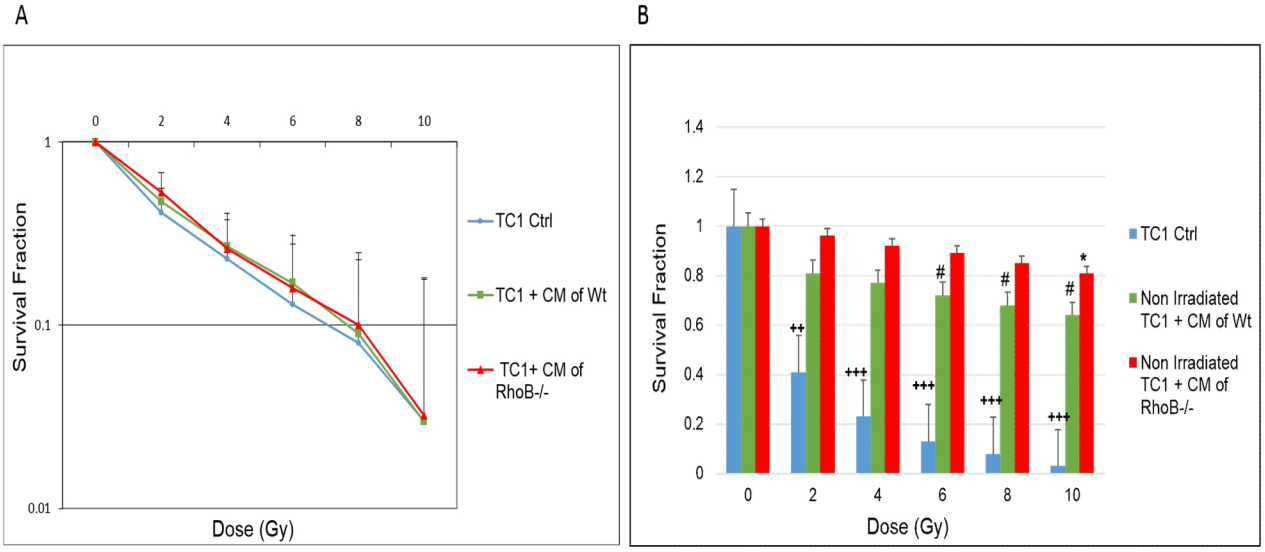


Figure 28: TC-1 Clonogenic Survival Curve: Paracrine signals secreted by fibroblasts do not modulate TC-1 radiosensitivity.

Irradiation has previously been associated with either cell survival or cell death promoting mechanisms. Thus, we tested whether co-culture would modify the effect of Ionizing radiation on TC-1 cells survival. To this aim we produced conditioned medium from Wt and RhoB -/- fibroblasts and used them to culture TC-1 in clonogenic survival assay. We observed that media conditioned by fibroblasts do not modify TC-1 intrinsic radiation sensitivity assessed by the survival fraction (SF) as determined by clonogenic assays (Figure 28A), suggesting that signals triggered by irradiation are stronger than survival signals triggered by fibroblasts.

Interestingly, CM isolated from irradiated Wt and RhoB-/- fibroblasts do alter clonogenicity and survival of non-irradiated TC-1, suggesting that paracrine signals produced by irradiated fibroblasts indeed operates as a cytoprotective mechanism that alter TC-1 clonogenic potential (Figure 28B).

2. Wound healing signals secreted by fibroblasts enhance TC-1 migration

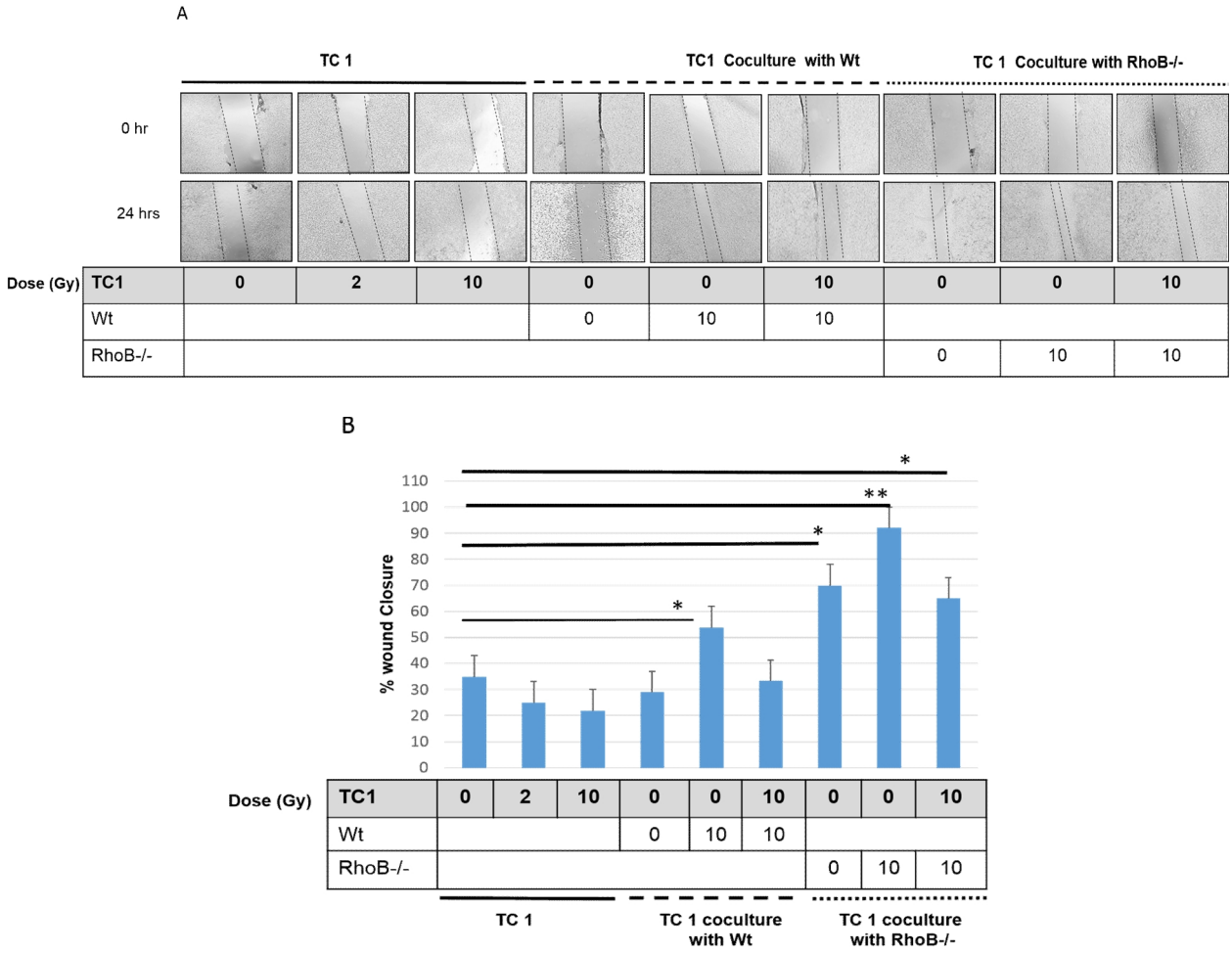


Figure 29: Wound healing signals secreted by fibroblasts enhance TC-1 migration.
Migratory potential of TC -1 cells was analyzed (A) and quantified (B) in various culture conditions at 0hr and 24 Hrs after wounding.

As it has been reported previously that the proliferative, invasive, and metastatic capacities of tumor cells can be augmented in the repopulated tumors that survive radiotherapy, therefore, we assessed whether irradiation modulated TC-1 cell motility. Our results showed that TC-1 migration was reduced with increasing dose of irradiation. Then, we showed that conditioned medium (CM) produced by non-irradiated Wt fibroblasts had no effect on TC-1 cell migration, whereas CM produced by 10 Gy irradiated fibroblasts significantly stimulated TC-1 cells migratory capability. Interestingly when both Wt and TC-1 cells are co-irradiated at 10 Gy and co-cultured, TC-1 cell migration returned to normal level.

Moreover, alongside we showed that CM produced by non-irradiated RhoB-/- fibroblasts increased TC-1 cell migration more significantly than Wt fibroblasts, suggesting that RhoB deficiency promote the production of pro-migratory signals. This stimulation is further enhanced by 10 Gy-irradiated RhoB-/- fibroblasts but repressed back to the control level when RhoB-/- fibroblasts and TC-1 are co-irradiated at 10 Gy (Figure 29 A, B). These results suggests that irradiation with doses of 10 Gy in a single-fractioned not only cause indirect death of tumor cells but furthermore, exposure of tumor fibroblasts to high radiation doses induces permanent DNA-damage responses and irreversible cellular senescence, which in turn might influence therapeutic upshots by the altered release of cytokines, chemokines, and growth factors.

3. TC-1 migration is mediated by MMP

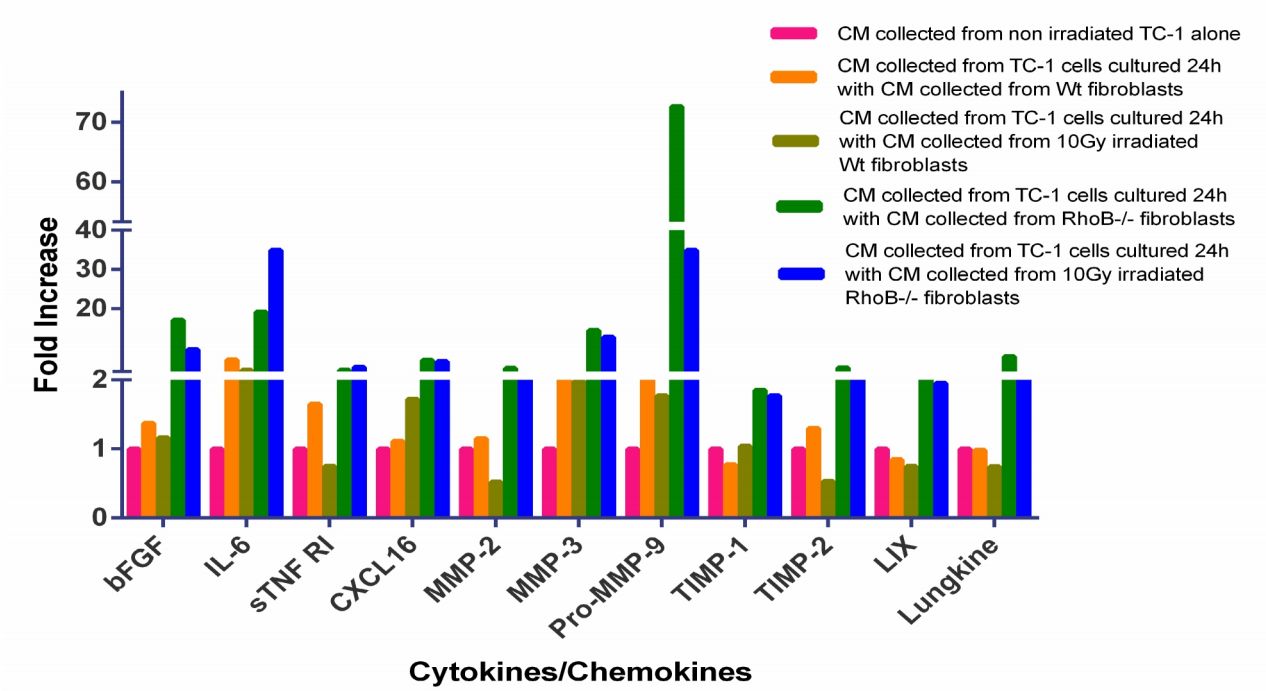


Figure 30A: Cytokine Array Analysis performed on TC-1 cell lysate to search for mediators of increase TC-1 invasiveness. Fold increase of significantly upregulated cytokines is shown.

Next we searched for the mediators of TC-1 cell migration and invasiveness. For this purpose we analyzed the proteome of TC-1 cultured alone or with CM produced by Wt and RhoB-/- fibroblasts irradiated or not. While most tested proteins (96) were not affected, production of IL-6, bFGF, CXCL 16, sTNF RI, MMP 2, MMP 3, Pro MMP 9 were significantly stimulated by the co-culture process (Figure 30A). Interestingly, CM from RhoB deficient fibroblasts modulated more proteins secreted by TC-1 than CM from Wt, with a marked increase in Pro MMP-9, MMP-3 and MMP-2.

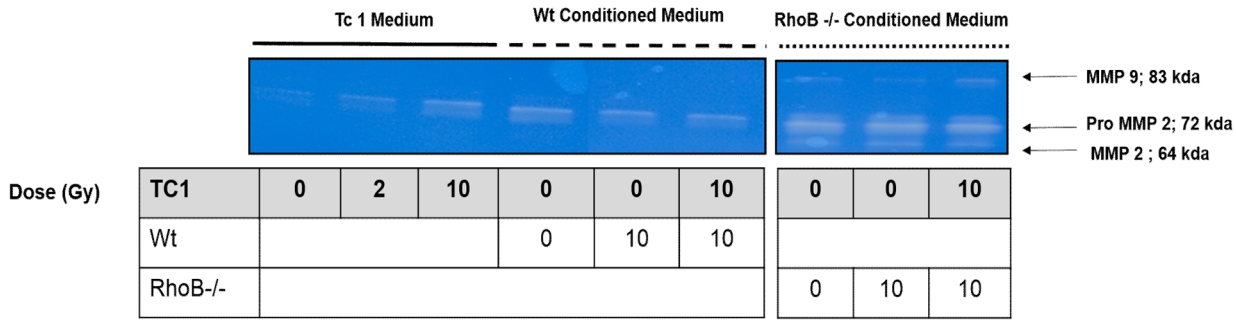


Figure 30B: MMP secretion from TC-1 cells. Zymography was performed on CM collected from TC1 cells 24hrs after coculture with Wt and RhoB-/- fibroblasts. Protease activity is visible as a clear band against a blue background.

Given that the expression of matrix metalloproteinases was upregulated in irradiated cells in CM from RhoB deficient fibroblasts, we next investigated the gelatinase activity of TC-1 cells by zymography. Under our experimental conditions, we observed two gelatinolytic bands representing gelatinase/active-MMP9 (MW 83 kDa) and gelatinase/active-MMP2 (MW 64 kDa). Our results show a weak secretion of MMP 2 and MMP 9 when TC-1 are cultured alone, slightly enhanced when TC-1 are irradiated (10 Gy). CM of Wt fibroblasts irradiated or not does not change the level and activity of MMP2/9 produced by TC-1, whereas CM from RhoB-/- fibroblasts irradiated or not significantly enhance level and activity of proMMP2, MMP2 and MMP9 (Figure 30B). This suggests that RhoB deficient fibroblasts secrete paracrine factors, that remain to be identified, but that are able to enhance MMPs secretion in TC-1.

It is worth noting that in RhoB deficient fibroblasts, the secretion of MMP2 and 9 was not modulated by irradiation nor by co-culture (Figure 30C), supporting the fact MMP induction was TC-1 mediated.

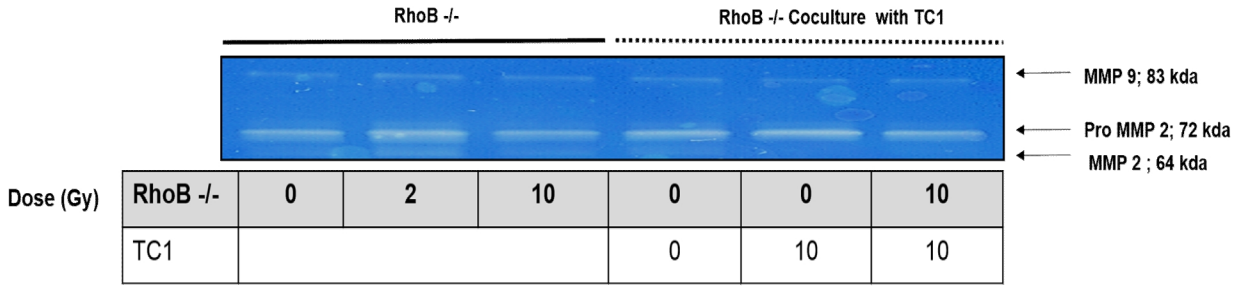


Figure 30C: MMP secretion from RhoB-/- Fibroblasts. Zymography was performed on CM collected from RhoB-/- fibroblasts 24hrs after coculture with TC-1 cells non Irradiated and irradiated at 10Gy. Protease activity is visible as a clear band against a blue background.

As MMP are known to degrade the extracellular matrix and is required for tumor cell invasion into surrounding tissue, we postulated that they could be involved in the enhanced migration of TC-1 and confirmed this hypothesis using the MMP inhibitor O-phenanthroline. Our results show that O-phenanthroline inhibited TC-1 migration cultured with CM medium produced by RhoB -/- fibroblasts irradiated or not (Figure 31).

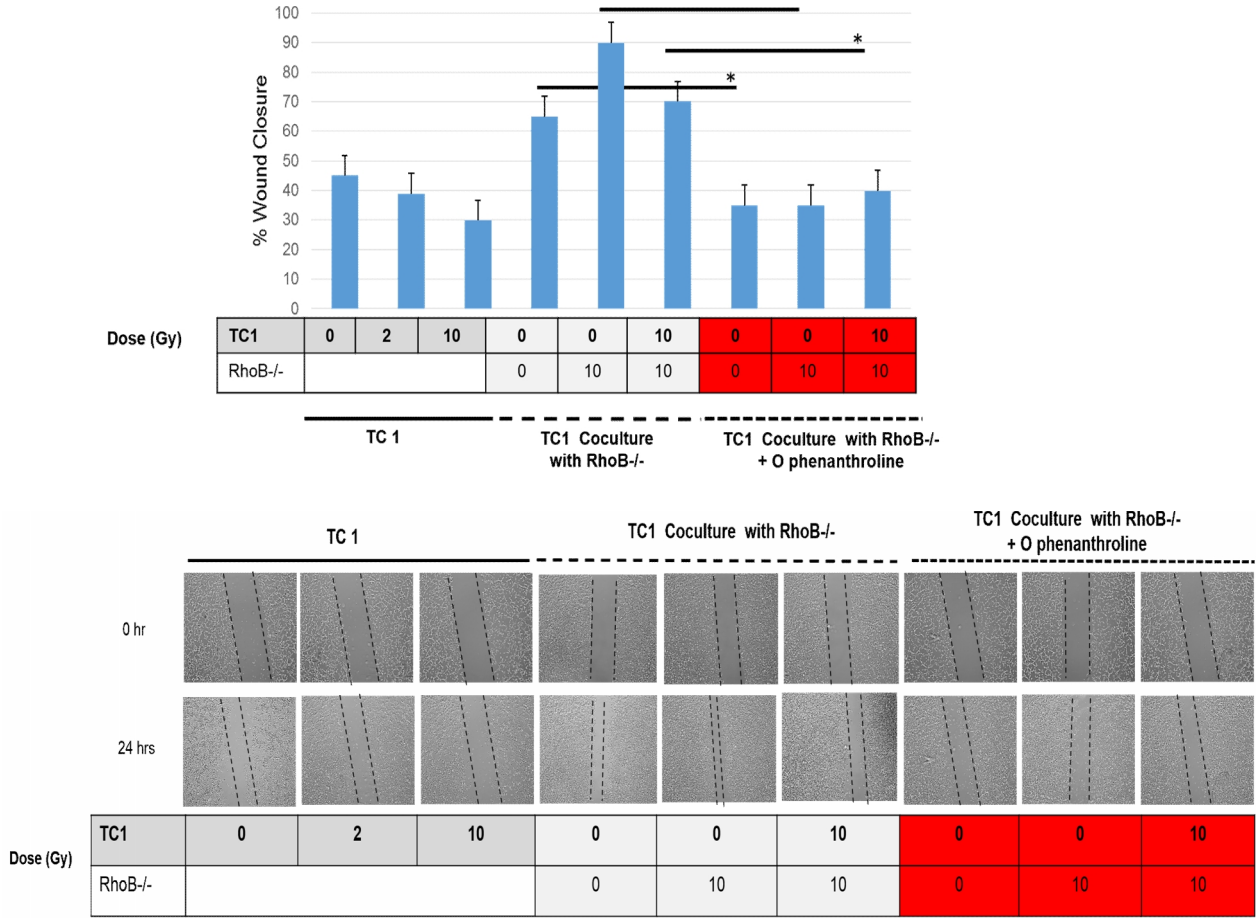


Figure 31: Modulation of TC-1 Migration by O-Phenanthroline: scratch assay after RhoB-/- fibroblasts incubation with MMP inhibitor. O-phenanthroline (100 µM).

4. Wt but not RhoB -/- fibroblasts enhance TC-1 invasiveness by secretion of TGF β1

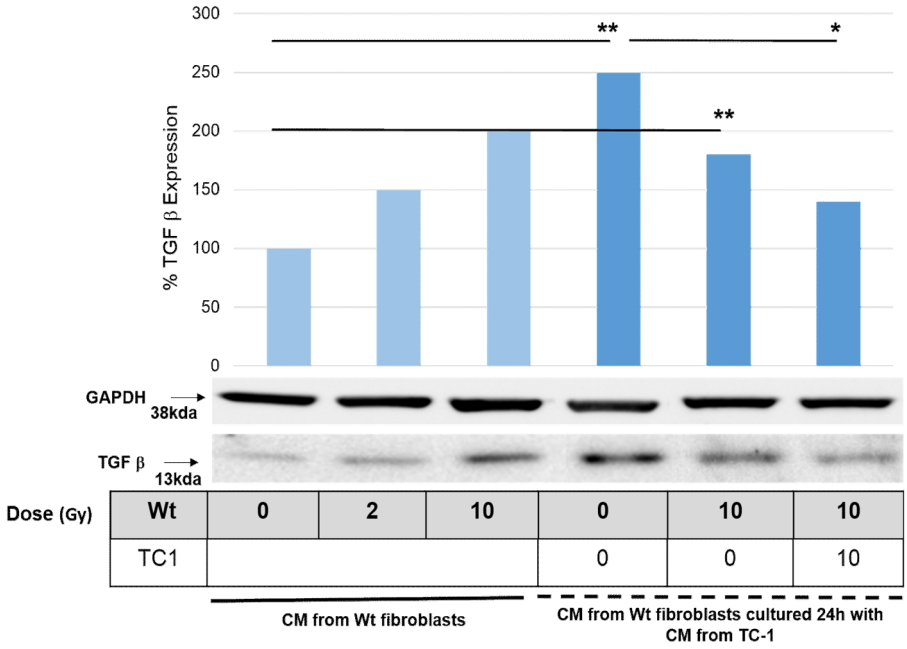


Figure 32: Effect of Irradiation and coculture on TGF β1 Expression in Wt fibroblasts. Whole cell lysate from Wt Fibroblasts were subjected to western blot using antibodies for TGF β1 (13 kDa).

Then we investigated the stimulatory pro-invasive mediators produced by Wt and RhoB-/- fibroblasts upon irradiation. TGF-β has emerged as a potent secreted factor that drives cancer progression, more importantly, as a potent inducer of epithelial plasticity leading to EMT. For this purpose we analysed TGF-β1 expression and found that TGF-β1 production was indeed stimulated by irradiation in a dose dependent manner in Wt fibroblast but not in RhoB-/- fibroblasts. When Wt fibroblasts were cultured with CM collected from TC-1, TGF-β1 production by Wt fibroblasts was further enhanced, whereas co-culture and co-irradiation of Wt at 10 Gy repressed TGF-β induction (Figure 32).

It was also observed that Co-culture and co-irradiation of RhoB-/- irrespective of the dose down regulate TGF-β induction whereas TC-1 irradiated at 10Gy induced TGF-β. (Figure 33). MMP secretion by TC-1 cells seemed to be the factor related for its own augumented motility.

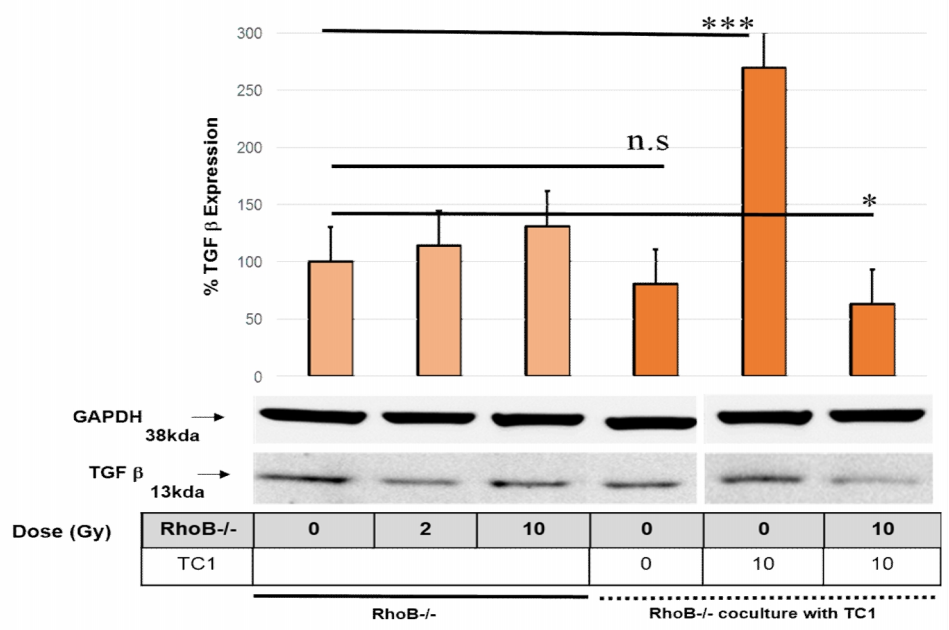
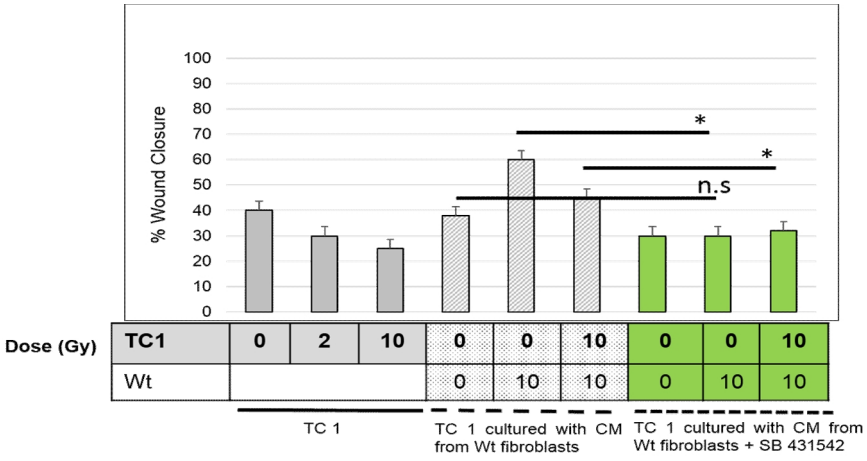


Figure 33: Effect of Irradiation and coculture on TGF β1 Expression in RhoB-/-fibroblasts. Whole cell lysate from RhoB-/- fibroblasts were subjected to western blot using antibodies for TGFβ1 (13 kDa).

We further confirmed the role of TGF-β1 in accelerated wound closure of TC-1 cells by utilizing SB-431542, a TGF-β inhibitor. We noticed that SB-431542 inhibited Wt fibroblasts induced TC-1 cell motility and delayed the wound closure (Figure 34) whereas TC-1 cell motility induced by RhoB -/- fibroblasts was not inhibited by SB-431542 (Figure 35). In inference, TGF-β1 is the main secretory factor inducing TC-1 cell migration by the Wt fibroblasts.



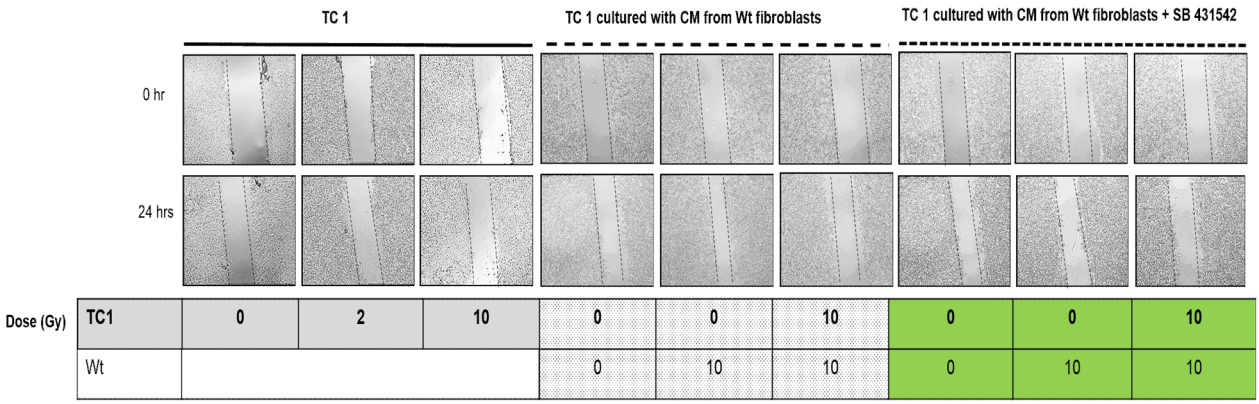


Figure 34: Modulation of TC-1 migration by SB 41542 (10μM) with Wt. Migratory potential of TC-1 cells was analyzed and quantified in various culture conditions at 0hr and 24 Hrs after wounding with Wt fibroblasts.

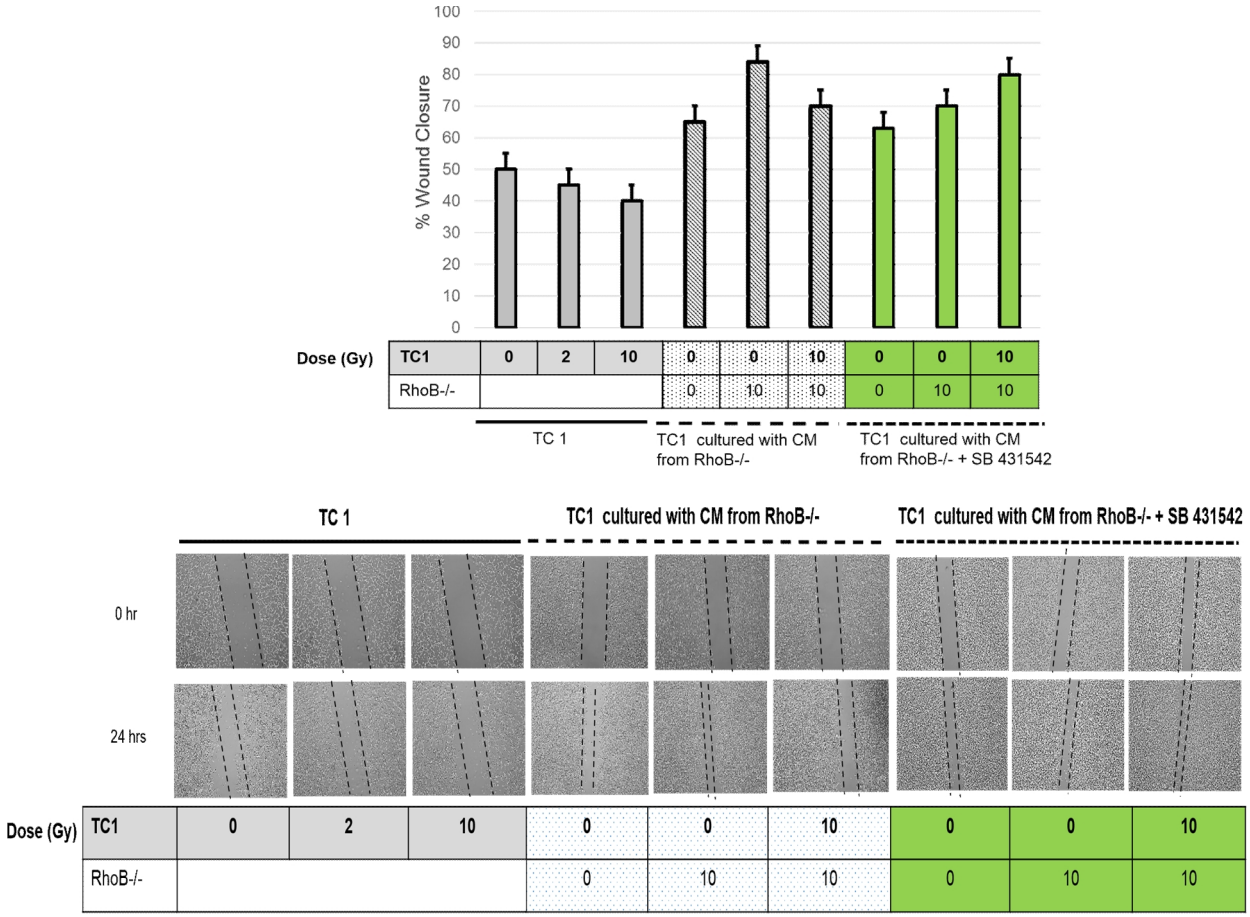


Figure 35: Modulation of TC-1 migration by SB 41542 (10μM) with RhoB-/-. Migratory potential of TC-1 cells was analyzed and quantified in various culture conditions at 0hr and 24 Hrs after wounding with RhoB-/- fibroblasts.

5. Irradiation and TC-1 stimulated the myofibroblastic differentiation in Wt fibroblasts

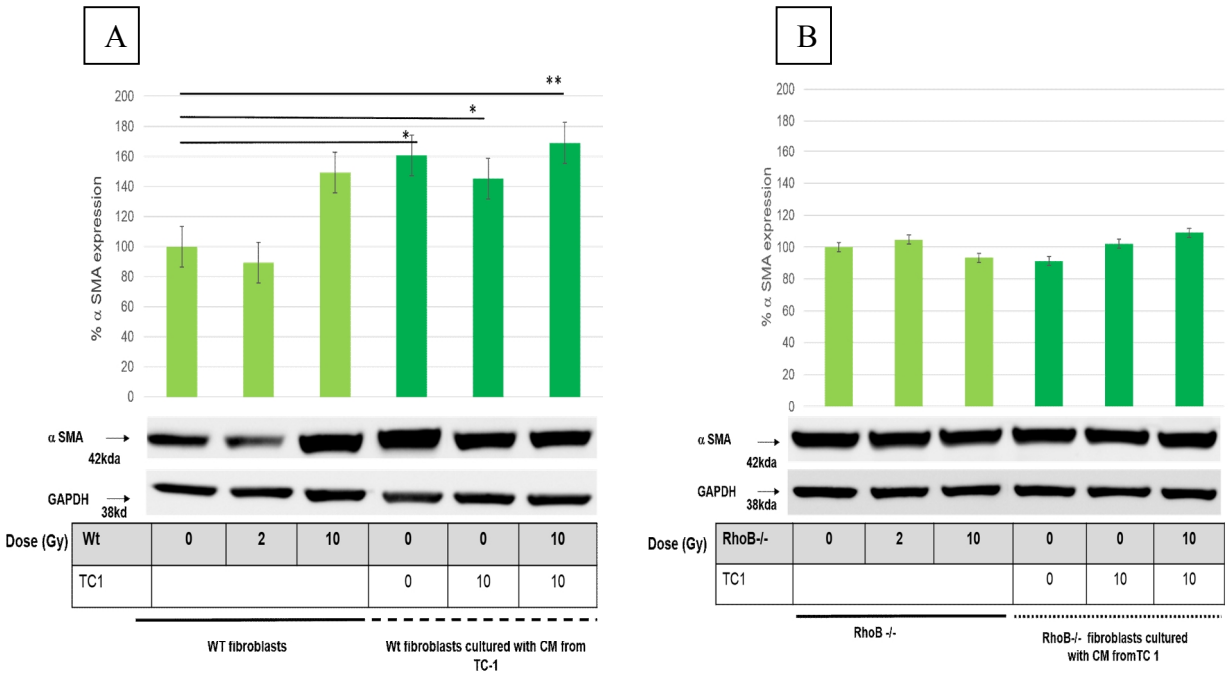


Figure 36: Effect of irradiation and coculture on α SMA expression in Wt and RhoB-/- fibroblasts. Whole cell lysate from Wt fibroblasts A) and RhoB -/- fibroblasts B) were subjected to western blot using antibodies for TGF β1 (13 kDa).

As we know that myofibroblast transdifferentiation (MTD) is associated with the tissue remodeling phase and indeed, this transdifferentiation can be induced by the inflammasome secreting cytokines and by chemokines, such as TGF-β. Therefore we used TGF β1 and α SMA expression to monitor the myofibroblastic differentiation in fibroblasts. Our results show that TGF β1 and α SMA expression increased with increasing dose of irradiation (Figure 32 and 36). Interestingly coculture of Wt fibroblasts with TC-1 also significantly increased TGF β and α SMA level (Figure 36A) suggesting that both irradiation and TC-1 tumor cells stimulated the myofibroblastic differentiation. α SMA expression remains unchanged in RhoB -/- fibroblasts in any conditions (Figure 36B), showing the relationship between RhoB deficiency and actin cytoskeleton, suggesting that RhoB deficiency would at least partly impair myofibroblastic differentiation.

6. TGF–β1 production induces EMT markers in TC-1

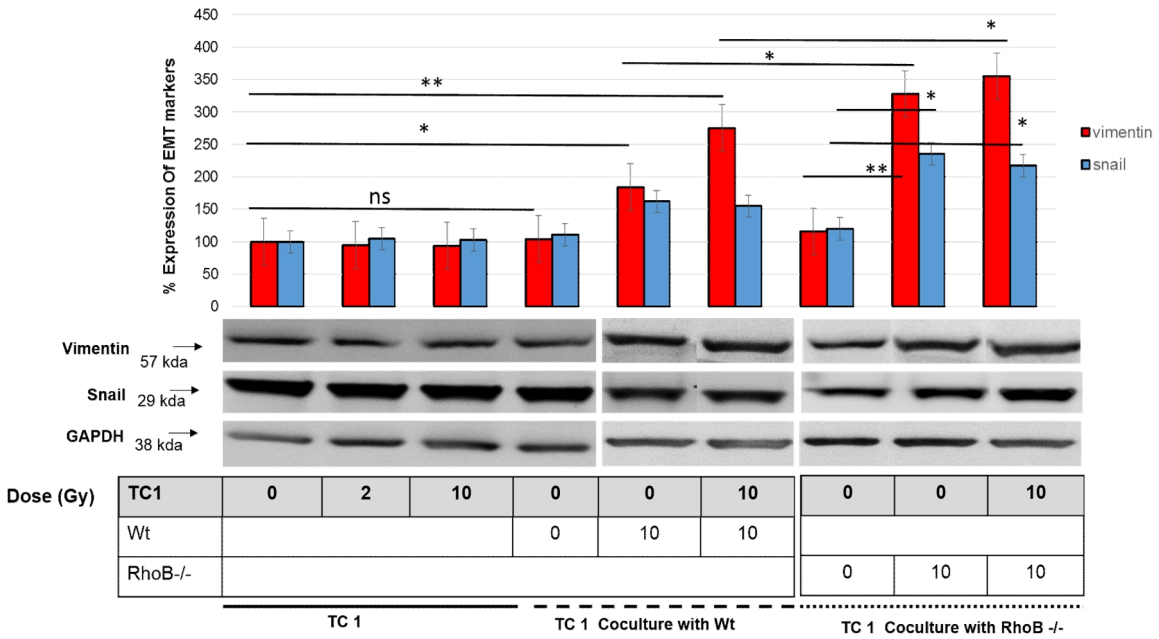


Figure 37: TC-1 enhanced migration is associated with induction of EMT markers: TC-1 lysate was subjected to western blot using antibodies for vimentin (57kDa) and snail (29kDa).

In order to investigate, whether the variation of migratory potential of TC-1 cells was associated with altered phenotype and induction of EMT, we analyzed vimentin and snail expression in carcinoma cells by western blot. Our results (Figure 37) showed that ionizing radiation does not promote vimentin and snail expression in TC-1 nor does the co-culture with Wt fibroblasts supporting the scratch assay's results (Figure 29). However, CM produced by 10 Gy irradiated fibroblasts stimulated both vimentin and snail protein expression in TC-1 suggesting induction of an EMT phenotype by paracrine factors secreted by irradiated fibroblasts. Surprisingly, co-irradiation of Wt fibroblast and TC-1 further enhanced vimentin expression but has no effect on snail, suggesting that the decreased migration observed by scratch assay was not associated with alteration of the EMT phenotype.

CM from RhoB-/- fibroblasts neither induce vimentin nor snail protein expression in TC-1 suggesting that the enhanced migration observed by scratch assay when both cell type are co-cultured is mediated by other mechanisms. However, when irradiated, RhoB-/- fibroblasts stimulate vimentin and snail in TC-1. As for Wt fibroblast, co-irradiation of RhoB-/- fibroblast and TC-1 further enhance vimentin expression but has no effect on snail (Figure 37). Taken together, these results indicate that distinct mechanisms are responsible for the enhanced cell invasion and various independent paracrine factors produced by irradiation of fibroblasts modulate migration and EMT phenotype.

Effect of Irradiation of host and tumor microenvironment (*in vivo* studies)

Next we carry out *in vivo* experiments to further explore the effects of irradiation of host and tumor microenvironment by utilizing tumor growth kinetics and variations in the release of CTCs.

7. Tumor growth kinetics and tumor bed effect (TBE)

To study tumor growth kinetics, we utilized bioluminescence imaging (BLI) according to the experimental timepoints as showed in materials and methods (Figure 27). We observed exponential growth of tumor in Group 1 till experimental end point at 2 weeks. Whereas in Group 2, tumor regresses in size 1 week post-irradiation (Figure 38).

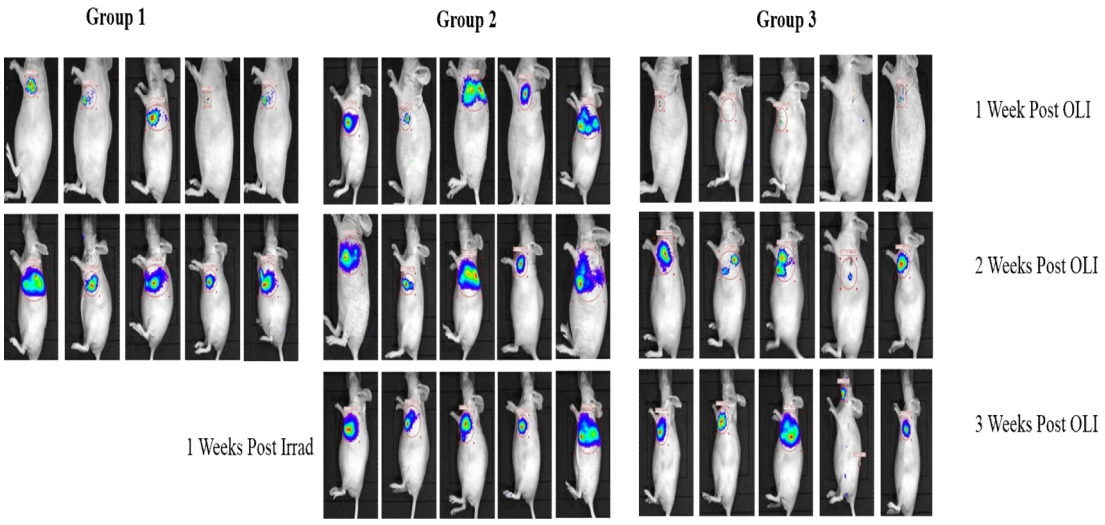


Figure 38: **Bioluminescence imaging (BLI) for 3 Groups** at different time points. OLI (Orthotopic Lung Implantation).

Experiments were intended to investigate the influence of time interval between lung irradiation and tumor cell transplantation on 3 different aspects of the tumor bed effect (TBE): tumor take, growth rate, and metastasis formation. TBE states that tumors implanted in pre-irradiated stroma grow more slowly as compared to tumors in non irradiated stroma and become more metastatic and radioresistant. True to TBE our results in Group 3 bioluminescence imaging showed delayed uptake of tumor cells as well as their slow growth rate, compared to control group 1. ($P<0.0003$) (Figure 39). But at the sametime tumor uptake was similar in all three groups and within experimental endpoints no metastasis were observed in group 3 implanted tumors. These results suggested that in this tumor system TBE effects tumor take, growth rate, and metastasis formation differently; but for all parameters, the effect perished with increasing time between irradiation and tumor cell transplantation.

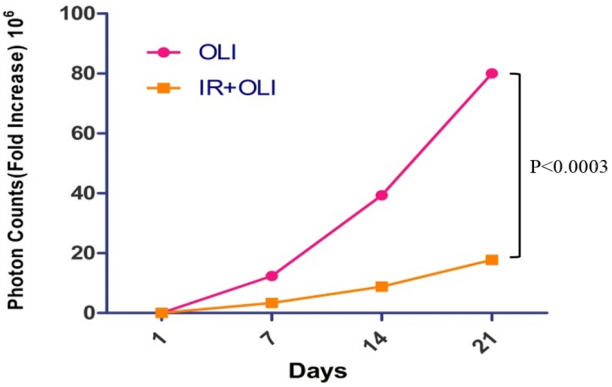


Figure 39 : **Tumor bed effect.** Fold Increase in BLI(Bioluminescence) was calculated as the proportion of BLI to baseline BLI (Day 0). Photon Counts from mice of each group were averaged. OLI Orthotopic Lung Implantation **Group 1**. IR +OLI Ionizing Radiation + Orthotopic Lung Implantation **Group 3**

It is worth noting that when there is a recurrence of tumor growing in an irradiated bed, there are two effects going on. First, there is a change in the vasculature of the normal tissue which will tend to produce a subclinical hypoxia. Secondly, there is a change in the genomics of the recurrent tumor which has undergone un-natural selection making more cells radioresistant. Either or both of these changes could make a tumour slower and more 'aggressive', i.e., resistant to treatment.

8. Gross and microscopic appearance of tumor

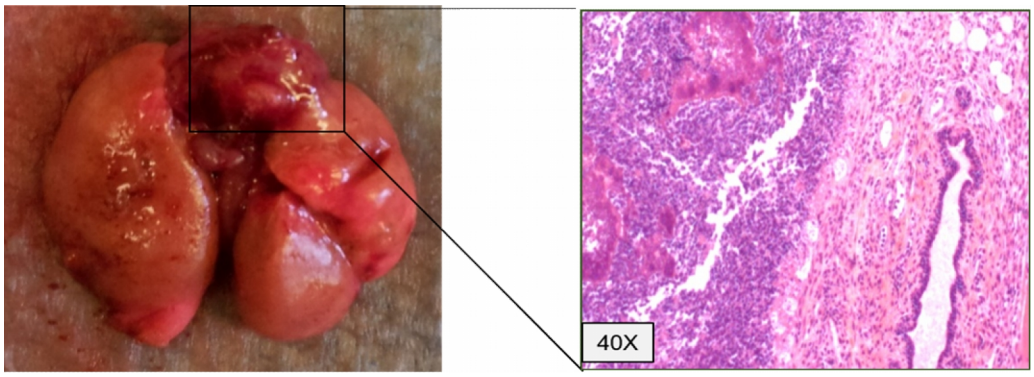
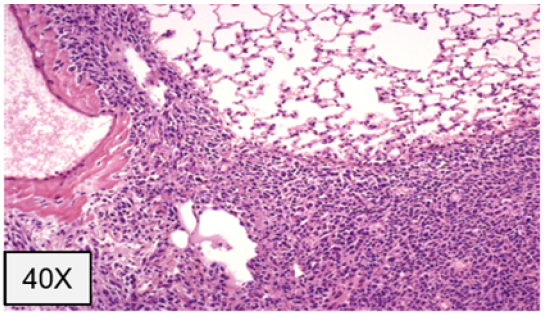


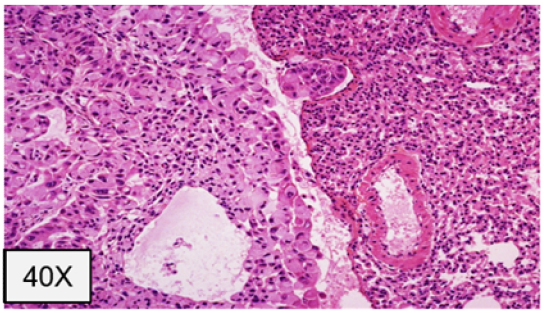
Figure 40: Gross and microscopic appearance (HES) of orthotopic lung implantation of A549 Luc+ cells after 2 weeks

Gross examination of implanted tumor showed that after 2 weeks of orthotopic implantation of A549 luc+ cells, the tumor is well localized to the apex of left lung with normal appearance of the rest of left lung i.e middle and caudal lobe. Right lung also appears free of any visible pathology. Histological appearance shows typical anaplastic tumor with darkly stained nuclei with abundant of mitotic figures. The tumor is well localized without any signs of breach in basement membrane of blood vessel and alveoli.

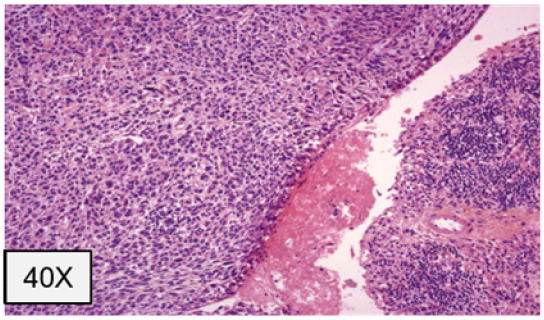
9. Histological appearnce of tumor and lung parenchyma in 3 Groups



Group1 2 weeks after orthotopic Tumor implantation in Lung



Group2 2 weeks after Irradiation of orthotopically implanted tumor



Group3 2 weeks after Orthotopic Tumor implantation in pre irradiated Lung Tissue

Figure 41: **HES staining and microscopic examination of Tumor + Lung Stroma in 3 groups**

No significant difference was observed in gross and microscopic examination of lung tissue bearing the tumor in the three groups. Microscopic examination of H/E stained sections showed that the architecture of the lung tissue is well preserved in all three groups apart from heterogenous spread of the tumor in between alveoli and vessels. In group 2, exudative infiltration was observed in blood vessel lumen due to leaky vessels after irradiation. Contrary to the second half of TBE definition for increase invasiveness; we neither observed any disruption in basement membrane of pulmonary vessels nor increased collagen deposition was appreciated in group 3 with pre irradiated bed.

10. CTC Enumerationin after orthotopic implantation and irradiation

Circulating tumor cells (CTCs) are thought to be potential prognostic indicator of invasion and metastasis. Comparison of CTCs detection by PCR and CellSearch enables us to show that PCR is more sensitive in detecting CTCs in peripheral blood of mice as compared to CellSearch.

In control Group 1 the median number of CTCs detected 2 weeks post Orthotopic implantation was 90 (range 0-2250/100µl of blood) in PCR assay and range 0-1935/600µl by CellSearch assay.

In Group 2, the median number of CTCs detected 2 weeks post Orthotopic Implantation was 50 (range 0-50/100µl of blood) in PCR and 0/600 µl of blood by CellSearch. 24hrs post irradiation median number of CTCs detected was 110 (range 0-1180/100µl of blood) by PCR and (range 0-316/600µl of blood) by CellSearch. 1week post-irradiation the median number was 900 ranging from 0-23300/100µl of blood by PCR and 0-1501/600µl of blood by CellSearch. CTCs were detectable in Group 1 and 2 within 1 week of implantation.

In Group 3, 2 weeks post orthotopic Implantation median number of CTCs detected was 35 ranging from 0-1940/100µl of blood by PCR and 0-805/600µl of blood by CellSearch.

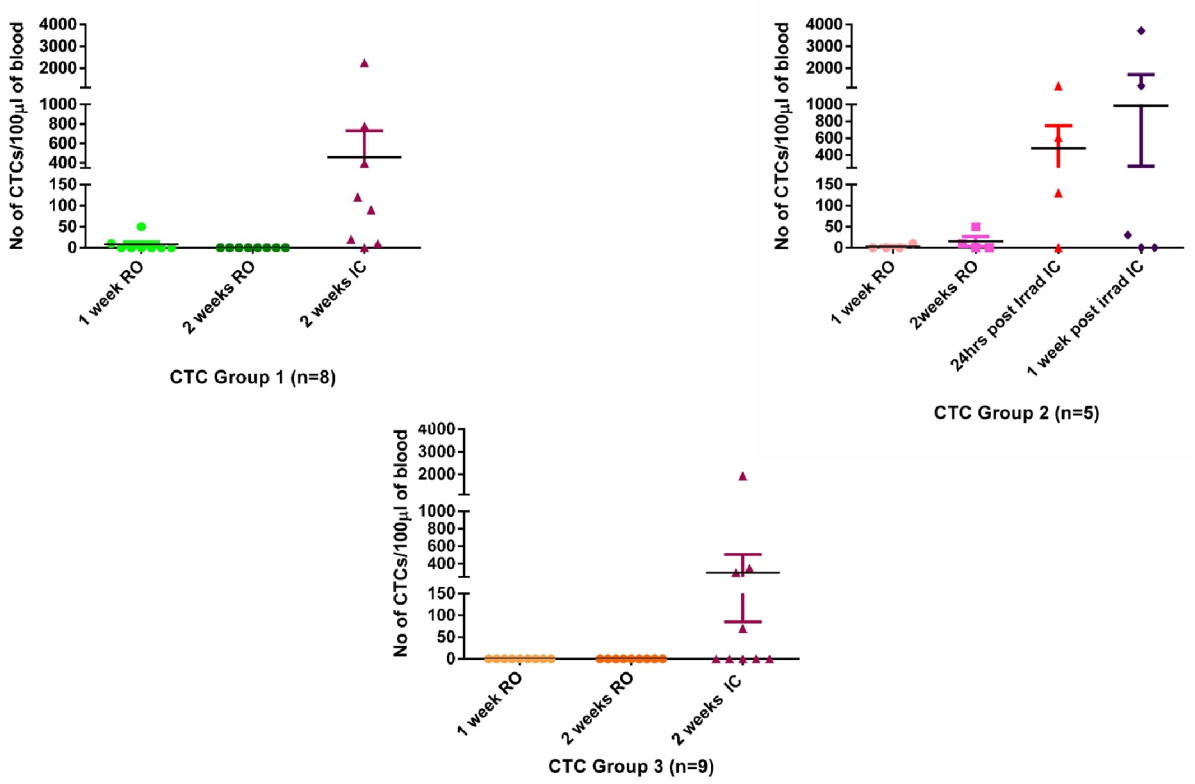


Figure 42*:* No of CTCs detected by PCR in 3 groups. RO- Retro orbital IC-Intracardiac

Our results showed that CTC number measured 2 weeks post engraftment in non-irradiated and irradiated lungs i.e Group 1 and 3 were similar suggesting, that the stromal remodelling induced by irradiation does not modulate tumor cell extravasation in the blood stream. However, when the tumor was irradiated a massive uptake of CTC in the blood stream was observed as early as 24h by intracardiac sampling which had increased after 1 week of irradiation.

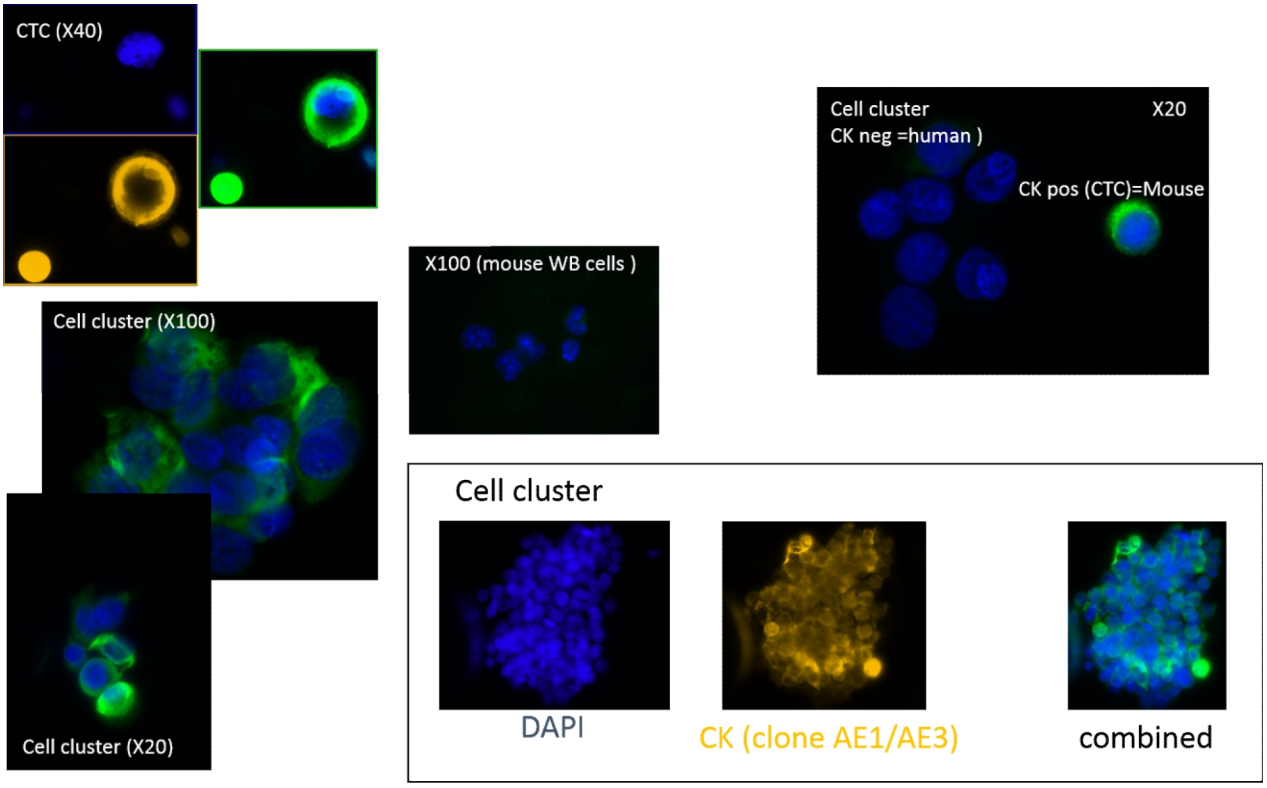
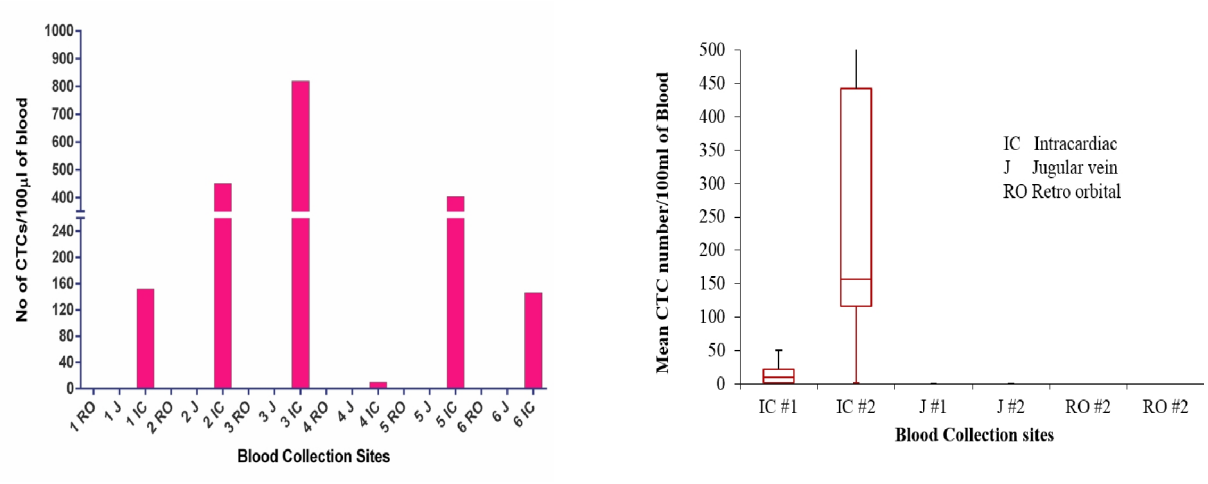


Figure 43: **Immunohistochemical analysis of mice CTCs clusters**. Strong positive cytokeratin staining.

11. Difference in CTCs detection by blood collection sites

At this point we assume that low number of CTCs detected by retro orbital route may be related to the site of blood sampling. For this porpose, we sampled jugular venous blood in addition to retro-orbital and intra-cardiac route. We observed that, no CTCs were detectable by either retro orbital and jugular venous routes but still at the same timepoint, intracardiac sampling reveals a number of CTCs (Figure 44).



A **B**

Figure 44: Comparison of CTCs count before A) and 24Hrs after irradiation B) between different blood collection sites. IC= Intracardiac, J= Jugular Vein, RO=Retro orbital

This leads us to probe for the potential entrapment of CTCs inside the heart chambers. For this porpose we did cytokeratin staining on the paraffin embedded sections of heart, which showed sparsely located tumor cells within red blood cells in heart chambers. This potentially confirmed our hypothesis. Cytokeratin staining also showed increase number of entrapped CTCs (in between red blood cells) in Group 2 post-irradiation (Figure 45B) as compared to group 1 and group 3 (Figure 45A) which is in accordance with our results depicted earlier (Figure 42).

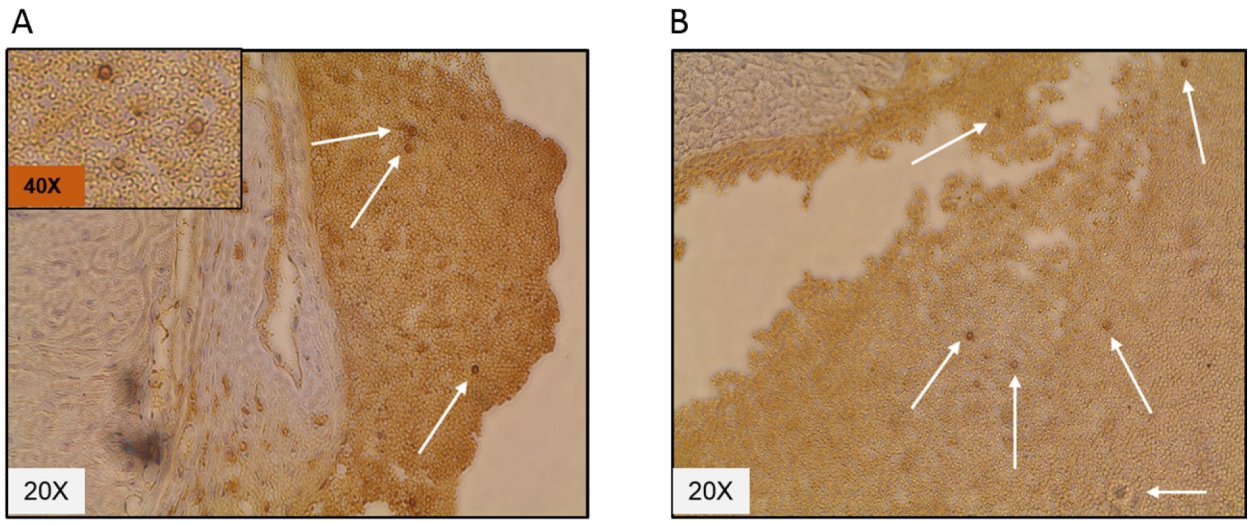


Figure 45: Cytokeratine staining: White arrows show intracardiac entraped circulating tumor cells. A) Group 1 and 3 B) Group 2.

12. Metastatic potential of CTCs:

Next an important question arises that whether this increase no of disseminated tumor cells after irradiation has the potential to form metastatic foci or not. For this purpose we injected 5 nude mice (recipient) with CTCs collected from 800µl of intracardiac blood sampled from 5 selected donor mice from Group 2 which have more than 1000 CTCs/100µl of blood. The mice were followed for 2 months for the formation of the tumor by bioluminescence imaging and we observed no detectable tumor formation in either lung or any other site during the time and all the mice remained healthy and alive.

Therefore, we established that, CTCs detected by either PCR or Cellsearch are epithelial in phenotype associated with the original tumor which are released into circulation because of acute inflammatory reaction (leaky vessels). So we can infer that either these circulating cells are not in EMT and are less likely to form metastatic niche either in receptive lung parenchyma or any other potential metastatic site such as bone marrow or their number is very low to initiate metastaic cascade.

V. DISCUSSION

Discussion

A tumor is a complex organ-like structure whose emergence, expansion and survival depends on interactions between multiple cell types including fibroblastic cells, endothelial cells, cells of hematopoietic origin and cancer cells themselves. The host-derived stromal cells play a critical role in all aspects of cancer biology including tumor growth, transformation, progression, and drug resistance. The complex interactions between stromal cells and cancer cells are of immense interest and in the first part of my thesis, we tried to explore the crosstalk between stromal and carcinoma cells after irradiation .We postulated that fibroblasts would promote TC-1 tumor migration after irradiation whereas deficiency in RhoB a protein described to be pro-fibrogenic would prevent it. Our data are different from our initial hypothesis as RhoB deficient fibroblasts enhanced TC-1 migration more potently than Wt fibroblasts do. RhoB deficient fibroblasts stimulated MMP secretion in TC-1 cells thus favoring their migration, whereas Wt fibroblasts stimulated TC-1 migration via TGF-β1 and induction of EMT in TC-1 cells (Figure 37). We also found that TC-1 intrinsic radiation sensitivity was not altered by conditioned-medium (CM) produced by Wt and RhoB deficient fibroblasts but that clonogenic potential of TC-1 was impaired by diffusible factors secreted by irradiated fibroblasts. Lastly, we found that co-irradiation of tumor cells and fibroblasts inhibited both TGF-β1 and MMP induction and repressed TC-1 cells migration.

Our results show that intrinsic radiosensitivity of carcinoma cells TC-1 is not altered by paracrine mediators secreted by non-irradiated stroma but show that the secretion of soluble factors by irradiated stromal cells promotes TC-1 migration and potential metastatic escape. This result is consistent with the literature [458, 459] and as expected we found that TGF-β1 was a stimulatory factors produced by irradiated Wt fibroblast. MH. Barcellos-Hoff's Group has demonstrated a major contribution for TGF-β1 produced by irradiated stroma to carcinogenesis [460]. TGF-β1 is also known to be the main inducer of reactive stroma and promote chemotaxis of fibroblasts and their transdifferentiation into myofibroblasts [461] by turning on expression of alpha-smooth muscle actin (αSMA). The presence of myofibroblasts-like cells, called CAF, in the tumor stroma may provide a source of growth factors, but could also be involved in connective tissue remodeling allowing expansion and invasion of tumors [462]. In addition, TGF-β1 immunosuppressive function may promote tumor growth and expansion by induction of immune anergy in tumor's stroma [463].

The use of primary lung fibroblasts isolated from RhoB deficient transgenic mice allowed us to identify activation of 3 major proteins in the proteome of TC-1 tumor cells cultured with conditioned media produced by these fibroblasts: MMP, bFGF and IL-6. Production of MMP 3, 9 and bFGF were specifically found to be stimulated in tumor cells when co-cultured with RhoB-/- fibroblasts but repressed when fibroblasts were irradiated. As suggested by the literature [464], we postulated that this increased expression of MMPs was associated with tumor invasion and indeed MMP inhibition in co-culture conditions repressed TC-1 migration as assessed in wound closure assay. Beside modulation of the migratory properties of cancer cells, RhoB -/- fibroblasts seemed to modulate both vascular remodeling and immune infiltration signals in tumor cells. Both MMP9 and bFGF are indeed involved in the regulation of vascular function after high doses of irradiation: MMP9 regulates vasculogenesis and recruitment of myeloid cells [465] whereas bFGF is a major pro-angiogenic factor [466, 467]. Our co-culture system does not allow direct assessment of the vascular and immune contribution: Therefore, additional *in vivo* experiments will be needed and are on going to conclude. However, the notion that stromal cells could promote an immunostimulating milieu is further supported by our simultaneous finding of IL-6 induction whose secretion is stimulated in TC-1 cells when they are cultured with medium conditioned by RhoB-/- fibroblasts and further enhanced when tumors cells are cultured in conditioned medium by irradiated RhoB-/- fibroblasts. IL-6 is also upregulated by coculture with wt fibroblast but to a lesser extent. IL-6 is one of the best-characterized pro-inflammatory cytokines [468, 469] and our results strongly suggest that after high dose of radiation stromal cells are able to modify the milieu and induce tumor cells to secrete factors able to alter immune infiltrate. Whether we can modify stromal response and peri-tumoral milieu to trigger immuno-stimulatory (IL-6) vs immune-suppressive (TGF-β) response needs to be investigated in detail but our results suggest that RhoB targeting could be a mean to achieve this goal.

More interestingly, our results bring a completely new vision and show that co-irradiation of tumor cells and fibroblasts abrogate migration of tumor cells. This is more relevant to the clinical situation and in accordance with clinical findings, but challenges previous publication including Ohuchida *et al*. and Hwang *et al.* papers [458, 459] showed that coculture with irradiated fibroblasts enhances the invasive potential of pancreatic cancer cells. They concluded that tumor/stroma interactions would stimulate metastasis after irradiation. Although we confirmed that irradiation of fibroblasts (irrespective of their genotype but via distinct mechanisms) promotes TC-1 invasive potential, this set-up is poorly relevant to the clinical situation, as stroma is never irradiated alone but on the contrary co-irradiation of tumors and surrounding stroma is always performed with sufficient safety margins.

Therefore and to stick to the clinical situation, we co-irradiated fibroblasts and TC-1 carcinoma cells and in that case our results bring to a different conclusion as we show that co-irradiation of tumor cells and fibroblasts repressed the pro-migratory signals, suggesting that conversely to what was previously described radiotherapy rather prevented metastatic spread than promoted it.

Secondly our *in vivo* experiments, although preliminary, tends to confirm the *in vitro* data showing that irradiated tumor bed does not stimulate tumor growth and escape. Experimental evidence shows that tumors growing within already irradiated tissue are predisposed to be more metastatic. This effect has been formerly known as **tumor bed effect** [470, 471]. The predominant concept is that the transformed vasculature and connective tissue in the tumor vicinity is less able to support tumor growth. This may have the unpredicted effect of creating more resistant tumours (due to increased hypoxia). However, experiments by Milas et al. [471, 472] reasonably suggested that the frequency of release of tumor cells into the circulation was similar in tumors in pre-irradiated and un-irradiated beds, and the increased metastasis observed in tumors embedded in pre-irradiated tissues was merely a secondary effect of the longer time period these tumors needed to reach a given volume [471, 472]. Also, a recent clinical study reported that recurrences after post-mastectomy radiation therapy are not associated with more aggressive local behaviours or reduced survival as compared to patients after mastectomy alone [473]. Thus, at this point this question remains open and debatable. In view of these observations we know only little about the cellular and molecular mechanisms mediating the increased metastatic capacity of experimental tumors developing within a pre-irradiated field [474-476].

The third part of my project was dedicated to CTC after radiotherapy. In animals treated with RT for NSCLC, our results showed that large numbers of viable tumor cells were frequently released into the circulation but these cells seemed to be entrapped into the cardiac cavity. The presence of CTCs in peripheral blood was confirmed by PCR, Cytokeratin Staining and CellSearch. The consistent pattern of RT-induced CTC increase in mice is clinically relevant for patients with already advance stage of disease as Tinhofer I et al demonstrate for the first time that radiation-induced migration and systemic spread of tumor cells might also occur in humans [477] and showed, the role of EGFR signalling, in the radiation-induced increase in CTC numbers. The beneficial role of radiotherapy in tumor control is well established and some relevant studies also suggest that radiation of the primary tumor might not only lead to tumor cell killing and growth inhibition but also increase the migratory and invasive potential of surviving radioresistant tumor cell subclones [478, 479]. It clearly establishes the principle that RT can cause shedding of viable tumor cells into the circulation.

When we investigated CTC release in the group 1 (Control) and group 3 (Pre irradiated) of mice with local and locally advanced tumor, we found same pattern of CTC release (Mean CTC count remains the same). Many factors could have been important contributors to these observations, including irradiated tumor volume, dose per fraction and the capacity of released CTCs to survive in the circulation. We believe that our findings open new areas of research and support the initiation of larger studies of CTC viability and release with different radiotherapy treatment schedules alone or in combination with chemotherapy but more experiments are needed to conclude.

CTCs that enter the circulation after initiation of RT [480] are most probably with mesenchymal marker expression. Yu et al [481] defined, that progression of breast cancer in patients correlates well with these mesenchymal CTCs and Park et al [482] described EMT of tumor cells implanted into mice as RT induced. During RT, acquisition of EMT phenotype could make CTCs more aggressive and harder to detect. The failure of the CellSearch platform to detect many of the CTCs that we counted by PCR may be due to the presence of CTMs and the downregulation of epithelial markers including EpCAM occurring during EMT induced during RT. Due to these factors the CellSearch methodology proved unusually insensitive. The abundance of tumor cell clusters or CTMs after RT is related to extensive tumor disruption and could have prognostic significance. Circulating tumor microemboli can be associated with adverse prognosis [483]. As it is well acknowledged that stromal cells play key role in tumor growth, the presence of non-tumor cells in clusters could also have significance in the process of metastasis.

Consistently with the results reported after surgery [484-487], the number of CTC increases in the blood stream after radiotherapy probably due to radiation-induced vascular injury induced or/and by EMT induction in tumor cells. The significance of these CTC to metastatic development is still under investigation but there is evidence for a metastasis-promoting effect of RT from animal studies [478, 488], for example Kaplan and others reported increased rates of lung metastasis in tumor-bearing mice treated with RT compared with sham-irradiated control [489, 490] but we had not been able to observe it in nude mice. Infact tumor microenvironment and host immune response play an important role in this discrepancy in results as primary lung tumors implanted orthotopically behave in different way as compared to subcutaneous implanted ones. However their genotype and phenotypic appearance remains the same.

Disseminated cells or CTCs from primary tumour may not immediately home to sites of future metastasis, but rather propagate to other microenvironments, mainly the lymph nodes and bone marrow. It remains a topic of debate that whether tumor cells actually metastasize to other organs through lymph nodes or their presence in draining lymphatics merely reflects their intrinsic invasiveness [491]. Cancer cells may acquire additional mutations in the supportive environment of lymph nodes that increase their metastatic tendency. This hypothesis can provide a rational explanation for several genomic studies showing limited similarities between primary tumours and disseminated cells [492, 493]. However, to date there is little experimental evidence supporting an indispensable role for intermediate stromal microenvironments in the metastatic cascade.

Passive entrapment may have a role in metastatic seeding but active adhesion and invasion is also essential for the successive establishment and growth of tumor [494]. Little is known of the early steps in this process. In some cases, proliferation occurs within the blood vessels [495] while in others, cancer cells extravagate first and then proliferate. Integrins expressed on the cancer cell surface are important mediator in this process.Thus the microenvironment can exert inhibitory effects on malignant cells. However, during their progression, tumors evade these inhibitory signals and instead exploit these stromal cells to their own benefit in the processes that result in inappropriate growth, invasion and metastasis.

In conclusion, stromal component of the tumor do secrete pro-migratory factors as function of their genotype. The pro-migratory action of wt fibroblasts is mainly TGF-β mediated whereas RhoB deficient fibroblasts stimulate MMP secretion. In addition and interestingly RhoB deficiency in the stroma enhanced tumor cell migration but simultaneously stimulated pro-inflammatory signals (IL-6) that would impact on immune recruitment and favor anti-tumor immune response. See (Figure 46) for summary of the findings.

Lastly, our results challenges the view that irradiated stroma would promote migration of carcinoma cells as we show that independently from their genotype co-irradiation of fibroblasts and carcinoma cells repressed carcinoma cell migration and confirmations studies are currently performed *in vivo*.

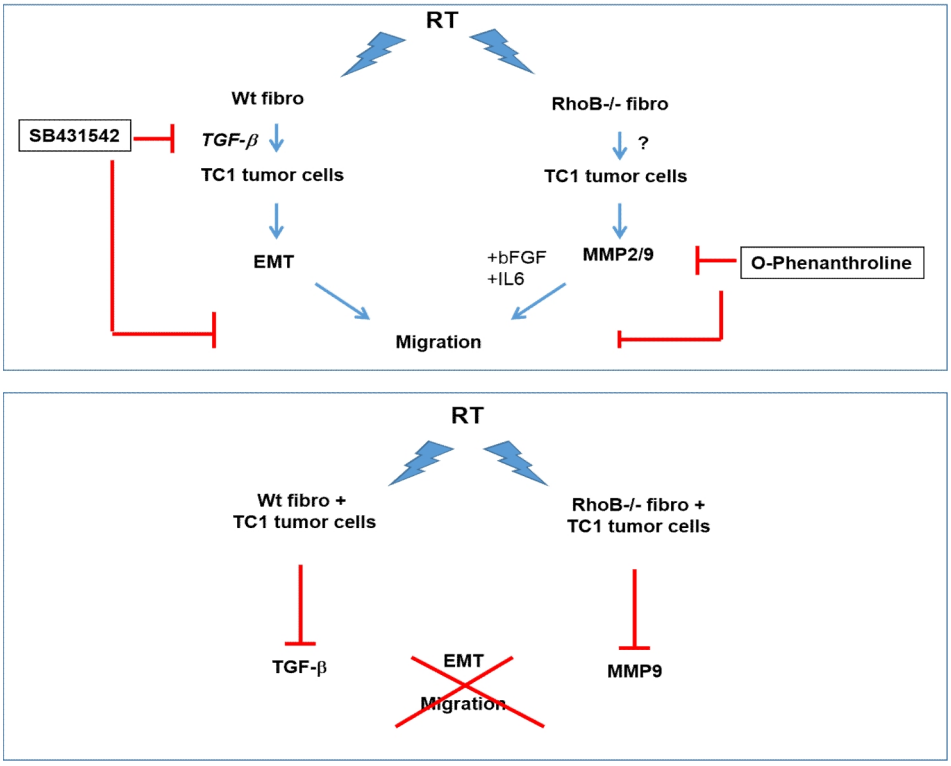


Figure 46: Summary of the findings.

Perspectives:

Researchers are increasingly "thinking outside the cancer cell" to provide insights into how other cell types and the tumor microenvironment (TME) support tumor growth and metastasis. These include normal host cells such as endothelial cells, fibroblasts, mesenchymal cells, and immune cells, at sites distant from and local to the site at which malignant transformation occurs.

The balance of these cellular interactions both determines the natural history of the cancer and influences its response to therapy. This active tumor-host dynamic has stimulated interest in the TME as a key target for cancer drugs. The microenvironment of a cancer is now perceived as an integral part of a tumor's "landscape" and physiology that functionally cannot be totally dissociated from what has traditionally been called "cancer." This includes distinctive microenvironmental conditions, like hypoxia, that result from the disordered vasculature characteristic of solid tumors.

Among potential non cancer cells in the TME, cancer stromal cells including fibroblastic cells, endothelial cells, and cells of hematopoietic origin have emerged as critical players in promoting tumor proliferation.

These cells participate in neovascularization, invasion, and metastasis as well as interacting with immune cells to "tilt the equilibrium toward a tolerogenic environment favoring the tumor cells."RhoB had been previously identified as a member of triple gene signature which confer strong prometastatic activity [496] and could impart resistance to treatment by its actions on microtubule regulations or by manipulating tumor host dependent MMPs secretion which modulates radiosensitivity [497]. Rho GTPase especially RhoB is involved in all stages during cancer progression. Its role goes beyond as just the regulator of cytoskeleton remodelling contributing to the cancer cell migration and invasion as it can affect tumour cells through gene transcription, intracellular transport of signalling molecules or modifying cancer cells interactions with surrounding stromal cells. However, the specific induction of RhoB by TGF-β and its requirement in TGF-β-induced migration [498] indicate that RhoB induction and signaling could be a target for therapeutic intervention in malignant invasive tumors.

It remains to be explored that if irradiated fibroblasts or CAFs contribute to tumor evolution post-RT, then which molecules and pathways are implicated in this regulation. Also, it remains unclear whether normal tissue fibroblasts and CAFs respond to RT in the same way, or if medium–high-doses (8–10 Gy) employ different effects than higher doses (15–20 Gy) in these cells [499]. As tumors contain different cellular components and they exert different responses at the same time provoked by RT. Only if we globally consider all events, in a system biology way of thinking, we could predict more precisely the ultimate consequences of the therapy. Dividing the responses endured by the different constituents of the tumor stroma under different RT-regimens will enable us to more effectively and efficiently choose the suitable treatment schedules in RT.

Apparently tumor bed or tumor irradiation can enhance tumor invasion or metastasis of experimental tumors but clinical evidence for this effect in human patients remains unclear. Literature in support or against such likely effect in patients is based majorly on retrospective non-randomized studies. At this point we can assume that in the primary tumor, a subpopulation of tumor cells acquire a mesenchymal phenotype during tumor progression by losing their epithelial properties (i.e. down regulate EpCAM and CK) through EMT and enter the circulation. These specific CTCs are believed to have stem cell characteristics. After extravasation into distant organs, these detached tumor cells (DTCs) re-express their epithelial properties through MET to form tumor cell clusters (micrometastasis). A subpopulation of CTCs, which may not be able to undergo EMT also disseminate through the circulation towards distant organs but lacks cancer stem cell characteristics and, therefore, does not form micrometastasis. These are the cells mainly detected by current CTC technologies, whereas the EMT induced CTCs are missed.Bearing in mind the clinical relevance of

such an effect, it can be important to plan prospective studies clearly addressing this issue in cancer patients treated.

It is widely attributed that the stromal components of solid neoplasms play vital roles in the overall responses of tumors to therapies. Radiation therapy is an exclusive therapeutic modality which targets a defined tumor volume indicating, that all cell types within the radiation field receive the given radiation dose. This insult incites the activation of cell death as well as cell damage programs that significantly influence the therapeutic outcomes. Consequently, it becomes a significance undertaking to uncover all potential side signals and events occurring during and after the course of radiation in stroma-tumor interactions that could influence tumor evolution and even the fate of distant metastasis.

Future Directions:

Radiation therapy is an established curative modality for epithelial cancers and is a vital component in many modern combined modality regimes. The tumor associated stromal cells are more stable genetically than tumor cells and therefore should be less prone to developing drug resistance [500]. The tumor stromal cells can also contribute to tumor chemoresistance [501-505] so targeting stromal cells or their products may be a viable strategy for overcoming the problem of drug resistance [505].

Crosstalk within the tumor microenvironment is not limited to paracrine signalling between activated fibroblasts and malignant cells but also occurs between different resident and distant stromal cell types. It would be exciting to understand how the tumor microenvironment manipulated by RT contributes to cancer progression. This knowledge would lead the way for the development of state-of-the-art strategies aimed at achieving a more effective anti-tumor microenvironment; mainly how soluble mediators, particularly cytokines and chemokines released by tumor and non-tumor cells in response to RT modify tumor growth and anti-tumor responses. However the pro- or anti-tumor effects of many cytokines or chemokines are mostly unknown. In fact, in a clinical setting, even the same mediator may act as a double-edged sword by exerting pro or anti-tumor effects. Hence, more information is needed before firmly concluding about the role of several cytokines in tumor development. A brief view of the different cells present in tumor microenvironment *in vivo* is shown in (Figure 47).

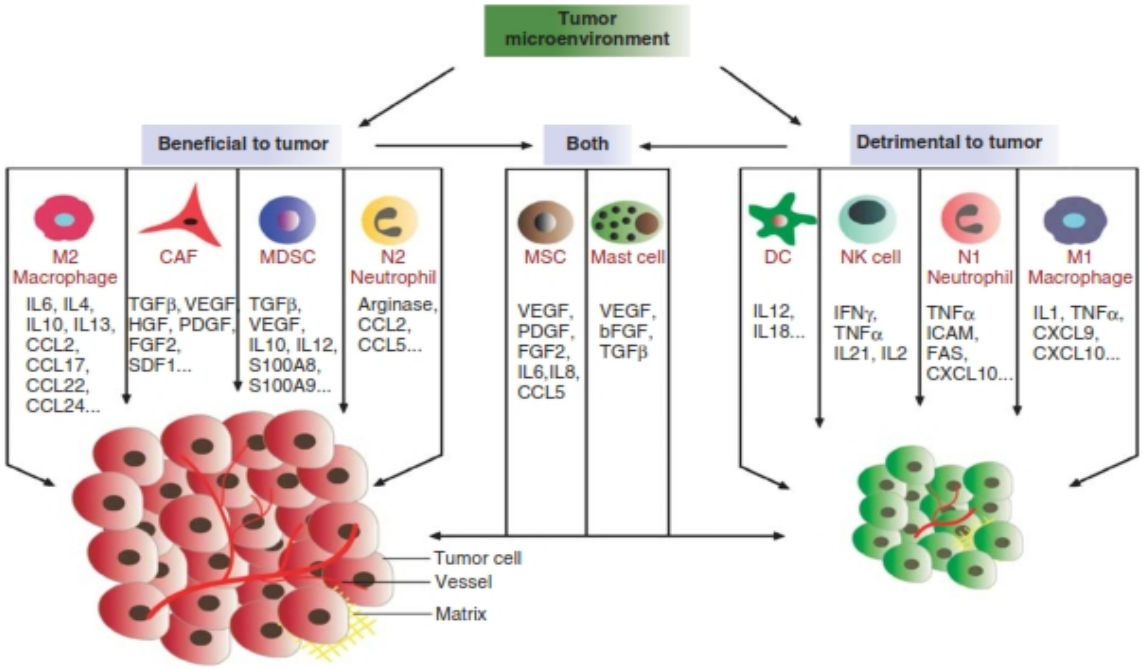


Figure 47: Tumor cell- host cell interaction. The tumor microenvironment is composed of a heterogeneous mixture of different stromal cells. **Adapted from** [506]

So, *in vivo* models will be invaluable for future translational research. Our study is amongst the first to study the effects of simultaneous irradiation of both tumor and stroma *invitro* but validation of these results using appropriate in vivo models will be valuable. Using *in vivo* models was a challenging part of this work but many obstacles hindered us from conducting invivo experiments in parallel with our invitro study **1)** MHC infection in our animal facilities restrain us from experiments for 6-8 months **2)** Difficulties related to the early death of TGF B1 knock out mice [507, 508] **3)** the inconvenience of models such as conditional inactivated TGFbRII mice [509]. We have planned to extend our results to the in vivo experiments by overcoming these difficulties and these experiments are currently ongoing using RhoB deficient animals and Cre/lox mice deficient and proficient for TGF-β signal.

Distant metastasis frequently imparts failure in loco-regionally advanced cancers after both surgery and RT. However, if CTC releases during RT give rise to metastasis, the knowledge of the nature and kinetics of this occurrence could allow us to develop new therapeutic strategies, such as early acceleration or intensification of RT, or novel combined therapeutic modalities including early trapping of CTC. Furthermore, RT-mobilized CTCs and CTMs signify not only prospective

therapeutic targets but a source of new diagnostic measures. Successive studies of CTC's molecular biology and analysis during therapy and follow up could give new insights in detecting therapeutically significant mutations in patients with inadequate biopsies. In the Future we will also seek associations between the cellular composition of CTMs, before and after treatment. These studies will also give definitive evidence on the association of CTCs with subsequent metastasis. We will also attempt to grow tumors derived from CTCs in nude mice.

Moreover, we will also try to identify the molecular elements and signaling pathways in the tumor microenvironment for each cancer type. In vitro identification of molecular components in the tumor microenvironment without contamination from the cell cytosol has been a challenge but it can be overcome by the latest improved techniques for enrichment and analysis. Next we will also plan to monitor the changes in the microenvironment molecular profile through tumor progression. This might be helpful for identifying cell or protein targets that have undergone modification. Least but not the least, the most important query will be to know, how the tumor microenvironment varies across different cancer types.

VI. BIBLIOGRAPHY

Bibliography

[1] Dvorak HF.**Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing.** N Engl J Med. 1986 Dec 25;315(26):1650-9.

[2] Haddow A .**Molecular repair, wound healing, and carcinogenesis: tumor production a possible overhealing?** Adv Cancer Res.1972 16: 181–234.

[3] Chang HY, Nuyten DS, Sneddon JB, Hastie T, Tibshirani R et al. **Robustness, scalability, and integration of a wound-response gene expression signature in predicting breast cancer survival.** Proc Natl Acad Sci U S A. 2005 Mar 8;102(10):3738-43.

[4] Farmer P, Bonnefoi H, Anderle P, Cameron D, Wirapati P et al. **A stroma-related gene signature predicts resistance to neoadjuvant chemotherapy in breast cancer.** Nat Med. 2009 Jan;15(1):68-74.

[5] Barcellos-Hofs MH, Park C, Wright EG. **Radiation and the microenvironment tumorigenesis and therapy.** Nat Rev Cancer.2005;5:867–875

[6] Monferran S, Skuli N, Delmas C, Favre G, Bonnet J et al. **Alphavbeta3 and alphavbeta5 integrins control glioma cell response to ionising radiation through ILK and RhoB.** Int J Cancer. 2008 Jul 15;123(2):357-64.

[7] Skuli N, Monferran S, Delmas C, Favre G, et al.**Alphavbeta3/alphavbeta5 integrins-FAK-RhoB: a novel pathway for hypoxia regulation in glioblastoma**. Cancer Res. 2009 Apr 15;69(8):3308-16.

[8] Garcia-Barros M, Paris F, Cordon-Cardo C, Lyden D, Rafii S et al. **Tumor response to radiotherapy regulated by endothelial cell apoptosis.** Science. 2003 May 16; 300 (5622):1155-9.

[9] Gaugler MH, Neunlist M, Bonnaud S, Aubert P, Benderitter M, Paris F. **Intestinal epithelial cell dysfunction is mediated by an endothelial-specific radiation-induced bystander effect.** Radiat Res. 2007 Feb;167(2):185-93.

[10] Hambardzumyan D, Becher OJ, Rosenblum MK, Pandolfi PP, Manova-Todorova K, Holland EC.**PI3K pathway regulates survival of cancer stem cells residing in the perivascular niche following radiation in medulloblastoma in vivo.** Genes Dev. 2008 Feb 15;22(4):436-48.

[11] Ahn GO, Brown JM. **Matrix metalloproteinase-9 is required for tumor vasculogenesis but not for angiogenesis: role of bone marrow-derived myelomonocytic cells**.Cancer Cell. 2008 Mar;13(3):193-205. doi: 10.1016/j.ccr.2007.11.032.

[12] Prevo R, Deutsch E, Sampson O, Diplexcito J et al. **Class I PI3 kinase inhibition by the pyridinylfuranopyrimidine inhibitor PI-103 enhances tumor radiosensitivity.** Cancer Res. 2008 Jul 15;68(14):5915-23.

[13] Cengel KA, Deutsch E, Stephens TC, Voong KR, Kao GD, Bernhard EJ. **Radiosensitizing effects of the prenyltransferase inhibitor AZD3409 against RAS mutated cell lines**. Cancer Biol Ther. 2006 Sep;5(9):1206-10.

[14] Wen B, Deutsch E, Marangoni E, Frascona V, Maggiorella L et al.**Tyrphostin AG 1024 modulates radiosensitivity in human breast cancer cells.**Br J Cancer. 2001 Dec 14;85(12):2017-21.

[15] Barcellos-Hoff MH, Ewan KB. **Transforming growth factor-beta and breast cancer: Mammary gland development.** Breast Cancer Res.2000;2(2):92-9.

[16] Barcellos-Hoff, M. H. **Radiation-induced transforming growth factor ß and subsequent extracellular matrix reorganization in murine mammary gland.** Cancer Res.1993; 53, 3880–3886.

[17] Barcellos-Hoff, M. H. & Ravani, SA.**Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells.** Cancer Res. 2000; 60, 1254–1260

[18] John Yarnolda, Marie-Catherine Vozenin Brotons. **Pathogenetic mechanisms in radiation fibrosis.** Radiother Oncol. 2010 Oct; 97(1):149-61.

[19] Lewis MP, Lygoe KA, Nystrom ML, Anderson WP, Speight PM, et al. **Tumour derived TGF-β1 modulates myofibroblast differentiation and promotes HGF/SFdependent invasion of squamous carcinoma cells**. Br.J.Cancer.2004; 90, 822–832.

[20] Jaffe AB, Hall A .**Rho GTPases: biochemistry and biology**. Annu. Rev. Cell Dev. Biol.2005; 21,247 –269.

[21] Ader I, Toulas C, Dalenc F, Delmas C, Bonnet J et al. **RhoB controls the 24 kDa FGF-2-induced radioresistance in HeLa cells by preventing post-mitotic cell death.** Oncogene. 2002 Sep 5; 21(39):5998-6006.

[22] Milia J, Teyssier F, Dalenc F, Ader I, Delmas C et al. **Farnesylated RhoB inhibits radiation-induced mitotic cell death and controls radiation-induced centrosome overduplication.** Cell Death Differ. 2005 May; 12(5):492-501.

[23] Monferran S, Skuli N, Delmas C, Favre G, Bonnet J et al. **Alphavbeta3 and alphavbeta5 integrins control glioma cell response to ionising radiation through ILK and RhoB.** Int J Cancer. 2008 Jul 15; 123(2):357-64.

[24] Skuli N, Monferran S, Delmas C, Favre G, Bonnet J et al.**Alphavbeta3/alphavbeta5 integrins-FAK-RhoB: a novel pathway for hypoxia regulation in glioblastoma**. Cancer Res. 2009 Apr 15; 69(8):3308-16.

[25] Fritz G, Kaina B. **RhoB encoding a UV-inducible Ras-related small GTP-binding protein is regulated by GTPases of the Rho family and independent of JNK, ERK, and p38 MAP kinase** .J Biol Chem.1997 ;272(49):30637–30644.

[26] Wherlock M, Gampel A, Futter C, Mellor H .**Farnesyltransferase inhibitors disrupt EGF receptor traffic through modulation of the RhoB GTPase.** J Cell Sci.2004; 117:3221–31.

[27] Prendergast GC, Khosravi-Far R, Solski PA, Kurzawa H, Lebowitz PF, et al . **Critical role of Rho in cell transformation by oncogenic Ras.**Oncogene.1995; 10:2289 –2296.

[28] Rodriguez PL, Sahay S, Olabisi OO, Whitehead IP.**ROCK I-mediated activation of NF-kappaB by RhoB**. Cell Signal.2007; 19: 2361–2369

[29] Verrecchia, F., Chu, M. L., and Mauviel, A. **Identification of novel TGF-beta/Smad gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach.** J. Biol.Chem.2001; 276, 17058–17062

[30] Moustakas A, Stournaras C.**Regulation of actin organisation by TGF-beta in H-ras-transformed fibroblasts.** J Cell Sci.1999; 112:1169–1179.

[31] Liu AX, Rane N, Liu JP, Prendergast GC.**RhoB is dispensable for mouse development, but it modifies susceptibility to tumor formation as well as cell adhesion and growth factor signaling in transformed cells.** Mol Cell Biol.2001; Oct;21(20):6906-12

[32] Vozenin-Brotons MC, Milliat F, Linard C, Strup C, François A et al. **Gene expression profile in human late radiation enteritis obtained by high-density cDNA array hybridization.** Radiat Res. 2004 Mar; 161(3):299-311

[33] V. Haydont, C. Bourgier, M.C. Vozenin-Brotons.**Rho/ROCK pathway as a molecular target for modulation of intestinal radiation-induced toxicity.**Br J Radiol, 80 (2007), pp. S32–S4

[34] Haydont V, Gilliot O, Rivera S, Bourgier C et al. **Successful mitigation of delayed intestinal radiation injury using pravastatin is not associated with acute injury improvement or tumor protection.** Int J Radiat Oncol Biol Phys.2007;Aug1;68(5):1471-82.

[35] Wang J, Boerma M, Fu Q, Kulkarni A, Fink LM, Hauer-Jensen M. **Simvastatin ameliorates radiation enteropathy development after localized, fractionated irradiation by a protein C-independent mechanism.** Int J Radiat Oncol Biol Phys. 2007 Aug 1; 68(5):1483-90.

[36] Haydont V, Bourgier C, Pocard M, Lusinchi A et al. **Pravastatin Inhibits the Rho/CCN2/ extracellular matrix cascade in human fibrosis explants and improves radiation induced intestinal fibrosis in rats.** Clin Cancer Res. 2007 Sep 15; 13(18 Pt 1):5331-40.

[37] Korpanty G, Smyth E, Carney DN.**Update on anti-angiogenic therapy in non-small cell lung cancer: Are we making progress?** J Thorac Dis. 2011 Mar; 3(1):19-29.

[38] Spigel DR, Hainsworth JD, Yardley DA, Raefsky E, Patton J et al.**Tracheoesophageal fistula formation in patients with lung cancer treated with chemoradiation and bevacizumab.** J Clin Oncol. 2010 Jan 1;28(1):43-8.

[39] Shih T1, Lindley C. Bevacizumab: an angiogenesis inhibitor for the treatment of solid malignancies. Clin Ther. 2006 Nov; 28(11):1779-802.

[40] Kuhn H, Hammerschmidt S, Wirtz H. Targeting tumorangiogenesis in lung cancer by suppression of VEGF and its receptor - results from clinical trials and novel experimental approaches. Curr Med Chem. 2007; 14(30):3157-65. Review.

[41] Tao Y, Leteur C, Calderaro J, Girdler F, Zhang P,et al . **The aurora B kinase inhibitor AZD1152 sensitizes cancer cells to fractionated irradiation and induces mitotic catastrophe.**Cell Cycle. 2009; Oct 1; 8(19):3172-81

[42] Tao Y, Leteur C, Yang C, Zhang P, Castedo M, Pierré A. **Radiosensitization by Chir-124, a selective CHK1 inhibitor: effects of p53 and cell cycle checkpoints.** Cell Cycle. 2009 Apr 15; 8(8):1196-205.

[43] Pantel K, Brakenhoff RH, Brandt B. **Detection, clinical relevance and specific biological properties of disseminating tumour cells.** Nat Rev Cancer 2008; 8:329-340.

[44] Wendel M, Bazhenova L, Boshuizen R, et al. **Fluid biopsy for circulating tumor cell identification in patients with early-and late-stage non-small cell lung cancer: A glimpse into lung cancer biology.** Phys Biol 2012; 9:016005.

[45] Krebs MG, Sloane R, Priest L, et al. **Evaluation and prognostic significance of circulating tumor cells in patients with non-small-cell lung cancer.** J Clin Oncol 2011; 29:1556-1563.

[46] Riethdorf S, Fritsche H, Muller V, et al. **Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: A validation study of the cellsearch system.** Clin Cancer Res 2007;13:920-928.

[47] Hou JM, Krebs MG, Lancashire L, et al. **Clinical significance and molecular characteristics of circulating tumor cells and circulating tumor microemboli in patients with small-cell lung cancer.** J Clin Oncol 2012; 30:525-532.

[48] Young R, Pailler E, Billiot F, et al. **Circulating tumor cells in lung cancer.** Acta Cytol 2012; 56:655-660.

[49] Taenzer A, Alix-Panabieres C, Wikman H, et al. **Circulating tumorderived biomarkers in lung cancer.** J Thorac Dis 2012; 4:448-449.

[50] Yu M, Bardia A, Wittner BS, et al. **Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition.**Science 2013;339:580-584.

[51] Allard WJ, Matera J, Miller MC, et al. **Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases.** Clin Cancer Res 2004; 10:6897-6904.

[52] The GLOBOCAN 2008 database. [Available from: http://globocan.iarc.fr]

[53] Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. **Global cancer statistics.** CA Cancer J Clin 2011; 61: 69–90.

[54] Lozano R, Naghavi M, Foreman K, et al. **Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010.** Lancet. 2012; 380: 2095–128.

[55] Travis WD, Brambilla E, Noguchi M, et al**. International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society international multidisciplinary classification of lung adenocarcinoma.**J Thoracic Oncol.2011 6:244-285.

[56] Cancer Genome Atlas Research Network, Hammerman PS, Hayes DN, et al. **Comprehensive genomic characterization of squamous cell lung cancers.** Nature.2012 489:519-525.

[57] Langer CJ, Besse B, Gualberto A, Brambilla E, Soria JC. **The evolving role of histology in the management of advanced non-small-cell lung cancer.** J Clin Oncol 2010; 28:5311–20

[58] Rami-Porta R, Crowley JJ, Goldstraw P. **The revised TNM staging system for lung cancer.** Ann Thorac Cardiovasc Surg 2009; 15: 4–9.

[59] Rowell NP, Williams CJ. **Radical radiotherapy for stage I/II non-small cell lung cancer in patients not sufficiently fit for or declining surgery (medically inoperable): a systematic review.** Thorax. Aug 2001; 56(8):628-38.

[60] Strand TE, Brunsvig PF, Johannessen DC, et al. **Potentially curative radiotherapy for non-small-cell lung cancer in Norway: a population-based study of survival**. Int J Radiat Oncol Biol Phys. May 1 2011; 80(1):133-41.

[61] Konstantinos Zarogoulidis, Paul Zarogoulidis, Kaid Darwiche, et al. **Treatment of non-small cell lung cancer (NSCLC)**. Thorac Dis 2013; 5(S4):S389-S396

[62] Perona R and Sanchez-Perez I: **Control of oncogenesis and cancer therapy resistance.** Br J Cancer 90: 573-577, 2004

[63] Brizel DM, Sibley Gs, prosnitz Lr, scher rL and Dewhirst M.**Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck.** Int J radiat Oncol Biol phys. 1997; 38: 285-289.

[64] Harrison LB, Chadha M, Hill RJ, Hu K and Shasha D: **Impact of tumor hypoxia and anemia on radiation therapy outcomes**.Oncologist.2002; 7: 492-508.

[65] Dormand, E.-L., Banwell, P. E. and Goodacre, T. E. **Radiotherapy and wound healing.** International Wound Journal.2005; 2: 112–127

[66] Bucci MK, Bevan A, Roach M. **Advances in radiation therapy: conventional to 3D, to IMRT, to 4D, and beyond**. CA Cancer J Clin. 2005; 55 (2): 117–34.

[67] Camphausen KA, Lawrence RC. "Principles of Radiation Therapy" in Pazdur R, Wagman LD, Camphausen KA, Hoskins WJ (Eds) **Cancer Management: A Multidisciplinary Approach. 11 ed. 2008.**

[68] Galvin JM, Ezzell G, Eisbrauch A, Yu C, Butler B et al. **Implementing IMRT in clinical practice: a joint document of the American Society for Therapeutic Radiology and Oncology and the American Association of Physicists in Medicine**. Int J Radiat Oncol Biol Phys. 2004; 58 (5): 1616–34.

[69] Thomas MR**. Brachytherapy: here today, gone tomorrow?** Heart. 2005 Jun; 91 Suppl 3:iii32-4.

[70] Kassis AI. **Therapeutic radionuclides: biophysical and radiobiologic principles. S**emin Nucl Med .2008.38:358-366.

[71] Ward JF. **DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation, and reparability**. Prog Nucleic Acid Res Mol Biol. 1988;35: 95-125.

[72] Smets EM, Garssen B, Schuster-Uitterhoeve AL and De Haes JC. **Fatigue in cancer patients.** Br J Cancer.1993; 68: 220-224.

[73] Robinson DW. **Surgical Problems in The Excision And Repair Of Radiated Tissue.** Plast Reconstr Surg.1975; 55: 41-49.

[74] Stone HB, Coleman CN, Anscher MS, McBride WH. **Effects of radiation on normal tissue: consequences and mechanisms.** Lancet Oncol 2003; 4(9):529–36.

[75] Suzuki K, Yamashita S. **Radiation-Induced Bystander Response: Mechanism and Clinical Implications**. Adv Wound Care (New Rochelle). 2014 Jan 1; 3(1):16-24

[76] Reed, B. R. & Clark, R. A. **Cutaneous tissue repair: Practical implications of current knowledge.** II. J. Am.Acad. Dermatol.1985; 13, 919–941.

[77] Dunham, L. J. **Cancer in man at site of prior benign lesion of skin or mucous membrane: a review.** Cancer Res.1972; 32, 1359–1374.

[78] Haddow, A. **Molecular repair, wound healing, and carcinogenesis: tumor production a possible overhealing?** Adv. Cancer Res.1972; 16, 181–234.

[79] Schulz-Ertner D, Jäkel O, Schlegel W. **Radiation therapy with charged particles**. Seminars in Radiation Oncology 2006; 16(4):249–259

[80] Iyer VR, Eisen MB, Ross DT, Schuler G, Moore T, et al. **The transcriptional program in the response of human fibroblasts to serum.** Science.1999; 283,83–87.

[81] Chang, HY. Sneddon JB, Alizadeh AA, Sood R, West RB et al. **Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds.** PLoS Biol.2004; 2, E7.

[82] Riss J, Khanna C, Koo S, Chandramouli GV, Yang HH et al. **Cancers as wounds that do not heal:differences and similarities between renal regeneration/repair and renal cell carcinoma.** Cancer Res. 2006 Jul 15; 66(14):7216-24.

[83] Pedersen TX, Leethanakul C, Patel V, Mitola D, Lund LR et al. **Laser capture micro dissection based in vivo genomic profiling of wound keratinocytes identifies similarities and differences to squamous cell carcinoma.** Oncogene. 2003 Jun 19; 22(25):3964-76

[84] Wynn TA. **Fibrotic disease and the T (H)1/T(H)2 paradigm.** Nat Rev Immunol. 2004 Aug; 4(8):583-94

[85] Ehrlichman, R.J., Seckel, B.R., Bryan, D.J. and Moschella C.J. **Common complications of wound healing.** Surg. Clin. North Am.1991; 71: 1323–1351.

[86] Springfield, D.S. **Surgical wound healing: A Multidisciplinary Treatment of Soft Tissue Sarcomas,** Chapter 5, pp. 81–98. Editors J. Verweij, H.M. Pinedo and H.D. Suit. Cancer Treat. Res.1993; 67: 81–98.

[87] Mast, B.A. **The skin In Wound Healing: Biochemical and Clinical Aspects**. Editors: I.K. Cohen, R.F. Diegelmann and W.J. Lindblad. W.B. Saunders, Philadelphia, 1992, p. 347.

[88] Nurden At, Nurden p, Sanchez M, Andia I and Anitua E.**Platelets and wound healing**. Front Biosci. 2008; 13: 3532-3548.

[89] Gieringer M, elliott K, Gosepath J and Naim R. **Keloids: fundamental principles and prospects (review)**. Mol Med rep. 2010; 3:13-20.

[90] Knighton Dr, hunt TK, thakral KK and Goodson Wh 3rd. **Role of platelets and fibrin in the healing sequence: an in vivo study of angiogenesis and collagen synthesis.** Ann surg.1982; 196: 379-388.

[91] Cromack, D.T., Porras-Reyes, B., Purdy, J.A., Pierce, G.F. and Mustoe, T.A. **Acceleration of tissue repair by transforming growth factor beta 1: identification of in vivo mechanism of action with radiotherapy-induced specific healing deficits.** Surgery.1993; 113: 36–42.

[92] Mazzucco L, Borzini P and Gope R. **Platelet-derived factors involved in tissue repair-from signal to function.** transfus Med rev .2010;24: 218-234.

[93] Eming SA, Krieg T and Davidson JM: **Inflammation in wound repair: molecular and cellular mechanisms.** J Invest Dermatol.2007; 127: 514-525.

[94] Sakai T, Johnson KJ, Murozono M, Sakai K, Magnuson MA et al. **Plasma fibronectin supports neuronal survival and reduces brain injury following transient focal cerebral ischemia but is not essential for skin wound healing and hemostasis.** Nature Med.2001; 7,324–330.

[95] Knighton Dr, Hunt TK, Thakral KK and Goodson Wh III: **Role of platelets and fibrin in the healing sequence: an in vivo study of angiogenesis and collagen synthesis.** Ann surg.1982; 196: 379-388.

[96] Drake, D.B. and Oishi, S.N. **Wound healing considerations in chemotherapy and radiation therapy.** Clin. Plast. Surg.1995; 22: 31–37.

[97] Mazzucco L, Borzini P and Gope R. **Platelet-derived factors involved in tissue repair-from signal to function.** transfus Med rev.2010; 24: 218-234.

[98] Jorgensen, P.H. and Andreassen, T.T. **Influence of biosynthetic human growth hormone on biomechanical properties of rat skin incisional wounds.** Acta Chirurg. Scand.1988; 154: 623.

[99] Muller K and Meineke V. **Radiation-induced alterations in cytokine production by skin cells.** Exp hematol.2007; 35: 96-104.

[100] Levenson SM, Geever eF, Crowley LV, Oates JF III, Berard CW and Rosen H. **The healing of rat skin wounds.** Ann surg.1965; 161: 293-308.

[101] Matthias Schäfer & Sabine Werner. **Cancer as an overhealing wound: an old hypothesis revisited.** Nature Reviews Molecular Cell Biology.2008; 9, 628-638.

[102] Yanase A, Ueda M, Kaneda T, Torii S, Matsuyama M. **Irradiation effects on wound contraction using a connective tissue model**. Ann Plast Surg 1993;30:435–440.

[103] Denham JW, Hauer-Jensen M, Kron T, Langberg CW. **Treatment-time-dependence models of early and delayed radiation injury in rat small intestine**. Int J Radiat Oncol Biol Phys. 2000 Oct 1; 48(3):871-87

[104] Dormand EL, Banwell PE, Goodacre TE: **Radiotherapy and wound healing**.Int Wound J 2005, 2:112–127.

[105] Rodemann HP, Bamberg M. **Cellular basis of radiation-induced fibrosis.** Radiother Oncol 1995; 35:83–90.

[106] Yarnold J, Brotons MC.**Pathogenetic mechanisms in radiation fibrosis.** Radiother Oncol. 2010 Oct; 97(1):149-61.

[107] Nelson CM, Bissell MJ. **Of extracellular matrix, scaffolds, and signaling: tissue architecture regulates development, homeostasis, and cancer**. Annu Rev Cell Dev Biol. 2006; 22:287–309.

[108] Hynes RO. **The extracellular matrix: not just pretty fibrils.** Science. 2009; 326:1216–9.

[109] Bornstein P, Sage EH. **Matricellular proteins: extracellular modulators of cell function.** Curr Opin Cell Biol. 2002; 14:608–16.

[110] Xu R, Boudreau A, Bissell MJ. **Tissue architecture and function: dynamic reciprocity via extra- and intra-cellular matrices.** Cancer Metastasis Rev. 2009; 28:167–76.

[111] Arwert EN, Hoste E, Watt FM. **Epithelial stem cells, wound healing and cancer.** Nat Rev Cancer. 2012 Feb 24; 12(3):170-80

[112] F. Mbeunkui, D.J. Johann. **Cancer and the tumor microenvironment: a review of an essential relationship**. Cancer Chemother Pharmacol. 2009; Mar; 63(4):571-82.

[113] Chin L.**The genetics of malignant melanoma: lessons from mouse and man.** Nat Rev Cancer. 2003 Aug; 3(8):559-70.

[114] Allen M, Louise Jones J. **Jekyll and Hyde: the role of the microenvironment on the progression of cancer**. J Pathol. 2011 Jan; 223(2):162-76.

[115] Hanahan D, Weinberg RA.**The hallmarks of cancer.** Cell. 2000 Jan 7; 100(1):57-70.

[116] I.P. Witz, O. Levy-Nissenbaum T**he tumor microenvironment in the post-PAGET era**. Cancer Lett. 2006 Oct 8; 242(1):1-10.

[117] C. Cuvier, A. Jang, R.P. Hill .**Exposure to hypoxia, glucose starvation and acidosis: effect on invasive capacity of murine tumor cells and correlation with cathepsin (L + B) secretion**.Clin Exp Metastasis. 1997; Jan; 15(1):19-25.

[118] R.S. Bindra, P.M. Glazer .**Genetic instability and the tumor microenvironment: towards the concept of microenvironment-induced mutagenesis**. Mutat Res. 2005 Jan 6; 569(1-2):75-85.

[119] T.Y. Reynolds, S. Rockwell, P.M. **Glazer Genetic instability induced by the tumor Microenvironment**.Cancer Res. 1996 Dec 15; 56(24):5754-7.

[120] C.L. Chang, G. Marra, D.P. Chauhan, H.T. Ha, K. Chang, L. Ricciardiella et al. **Oxidative stress inactivates the human DNA mismatch repair system**.Am J Physiol Cell Physiol. 2002 Jul; 283(1):C148-54.

[120a] Daruwalla J, Christophi C. **Hyperbaric oxygen therapy for malignancy: a review**. World J Surg. 2006 Dec; 30(12):2112-31.

[121] R. Sager **Expression genetics in cancer: shifting the focus from DNA to RNA** .Proc Natl Acad Sci USA. 1997 Feb 4; 94(3):952-5.

[122] Liotta LA, Kohn EC. **The microenvironment of the tumour-host interface.** Nature 2001; 411(6835):375e9.

[123] Franco OE, Shaw AK, Strand DW, Hayward SW. **Cancer associated fibroblasts in cancer pathogenesis.** Semin Cell Dev Biol 2010; 21(1):33e9.

[124] Bhowmick NA, Neilson EG, Moses HL. **Stromal fibroblasts in cancer initiation and progression.** Nature 2004; 432(7015):332e7.

[125] Akhurst RJ, Derynck R. **TGF-beta signaling in cancerea double-edged sword.**Trends Cell Biol 2001; 11(11):S44e51.

[126] Kalluri R, Weinberg RA. **The basics of epithelial-mesenchymal transition**. J Clin Invest 2009; 119(6):1420e8.

[127] B. Bierie, H.L. Moses.**Transforming growth factor beta (TGF-beta) and inflammation in cancer.**Cytokine Growth Factor Rev, 21 (1) (2010), pp. 49–59

[128] Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A et al. **The epithelial-mesenchymal transition generates cells with properties of stem cells.** Cell. 2008 May 16; 133(4):704-15.

[129] A.R. Anderson, A.M. Weaver, P.T. Cummings, V. Quaranta.**Tumor morphology and phenotypic evolution driven by selective pressure from the microenvironment.** Cell. 2006 Dec 1; 127(5):905-15.

[130] A. Alphonso, S.K. Alahari. **Stromal cells and integrins: conforming to the needs of the tumor microenvironment**. Neoplasia. 2009 Dec;11(12):1264-71.

[131] Pedersen TX, Leethanakul C, Patel V, Mitola D, Lund LR, et al. **Laser capture microdissection-based in vivo genomic profiling of wound keratinocytes identifies similarities and differences to squamous cell carcinoma.**Oncogene 22, 3964–3976 (2003).

[132] C. Garbers, H.M. Hermanns, F. Schaper, G. Muller-Newen, J. Grotzinger, S. Rose-John *et al.* **Plasticity and cross-talk of interleukin 6-type cytokines** .Cytokine Growth Factor Rev. 2012 Jun;23(3):85-97. doi: 10.1016/j.cytogfr.2012.04.001

[133] Y. Guo, F. Xu, T. Lu, Z. Duan, Z. Zhang.**Interleukin-6 signaling pathway in targeted therapy for cancer.** Cancer Treat Rev. 2012 Nov;38(7):904-10. doi: 10.1016/j.ctrv.2012.04.007.

[134] T. Kishimoto **IL-6: from its discovery to clinical applications**. Int Immunol. 2010 May;22(5):347-52. doi: 10.1093/intimm/dxq030.

[135] Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG et al. **Stat3 as an oncogene**. Cell. 1999 Aug 6;98(3):295-303.

[136] H. Yu, D. Pardoll, R. Jove .**STATs in cancer inflammation and immunity: a leading role for STAT3.** Nat Rev Cancer. 2009 Nov;9(11):798-809.

[137] T. Ara, Y.A. Declerck. **Interleukin-6 in bone metastasis and cancer progression**. Eur J Cancer. 2010 May;46(7):1223-31.

[138] N.J. Sullivan, A.K. Sasser, A.E. Axel, F. Vesuna, V. Raman et al.**Interleukin-6 induces an epithelial–mesenchymal transition phenotype in human breast cancer cells**. Oncogene. 2009 Aug 20;28(33):2940-7

[139] C. Huang, G. Yang, T. Jiang, G. Zhu, H. Li, Z. Qiu .**The effects and mechanisms of blockage of STAT3 signaling pathway on IL-6 inducing EMT in human pancreatic cancer cells in vitro.** Neoplasma. 2011;58(5):396-405.

[140] A. Yadav, B. Kumar, J. Datta, T.N. Teknos, P. Kumar. **IL-6 promotes head and neck tumor metastasis by inducing epithelial–mesenchymal transition via the JAK-STAT3-SNAIL signaling pathway.** Mol Cancer Res. 2011 Dec;9(12):1658-67.

[141] Tiede S. Ernst N. Bayat A. Paus R. Tronnier V. Zechel C. **Basic fibroblast growth factor: a potential new therapeutic tool for the treatment of hypertrophic and keloid scars**. Ann Anat. 2009;191:33.

[142] H. Kuhn, C. Köpff, J. Konrad, A. Riedel, C. Gessner, H. Wirtz .**Influence of basic fibroblast growth factor on the proliferation of non-small cell lung cancer cell lines**. Lung Cancer. 2004 May;44(2):167-74.

[143] M. Volm, R. Koomägi, J. Mattern, G. Stammler.**Prognostic value of basic fibroblast growth factor and its receptor (FGFR-1) in patients with non-small cell lung carcinomas**. Eur J Cancer. 1997 Apr;33(4):691-3.

[144] Y. Shou, T. Hirano, Y. Gong, Y. Kato, K. Yoshida, T. Ohira et al.**Influence of angiogenetic factors and matrix metalloproteinases upon tumour progression in non-small-cell lung cancer**. Br J Cancer. 2001 Nov 30;85(11):1706-12.

[145] A. Iwasaki, M. Kuwahara, Y. Yoshinaga, T. Shirakusa.**Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) levels, as prognostic indicators in NSCLC.** Eur J Cardiothorac Surg. 2004 Mar;25(3):443-8.

[146] Farhat FS1, Tfayli A, Fakhruddin N, Mahfouz R, Otrock ZK, Alameddine RS, et al **Expression, prognostic and predictive impact of VEGF and bFGF in non-small cell lung cancer**.Crit Rev Oncol Hematol. 2012 Nov;84(2):149-60.

[147] A. Dowlati, R. Gray, A.B. Sandler, J.H. Schiller, D.H. Johnson .**Cell adhesion molecules, vascular endothelial growth factor, and basic fibroblast growth factor in patients with non-small cell lung cancer treated with chemotherapy with or without bevacizumab—an Eastern Cooperative Oncology Group Study**. Clin Cancer Res. 2008 Mar 1;14(5):1407-12

[148] M. Jacob, L. Chang, E. Pure, **Fibroblast activation protein in remodeling tissues**. Curr Mol Med. 2012 Dec;12(10):1220-43.

[149] R. Kalluri. **Basement membranes: structure, assembly and role in tumour angiogenesis.** Nat Rev Cancer. 2003 Jun;3(6):422-33.

[150] Alvarez, R. J., Sun, M. J., Haverty, T. P., Iozzo, R. V., Myers, J. C., and Neilson, E. G. .**Biosynthetic and proliferative characteristics of tubulointerstitial fibroblasts probed with paracrine cytokines.** Kidney Int. 1992 Jan;41(1):14-23.

[151] Ian A. Darby, Tim D. Hewitson. **Fibroblast Differentiation in Wound Healing and Fibrosis**. Int Rev Cytol. 2007;257:143-79.

[152] Bhowmick NA, Moses HL.**Tumor-stroma interactions**. Curr Opin Genet Dev. 2005 Feb;15(1):97-101.

[153] N.A. Bhowmick, A. Chytil, D. Plieth, A.E. Gorska, N. Dumont et al. **TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia.**Science. 2004 Feb 6;303(5659):848-51.

[154] Hayward, S. W. et al. **Malignant transformation in a non tumorigenic human prostatic epithelial cell line.** Cancer Res. 61, 8135–8142 (2001).

[155] R. Kalluri, M. Zeisberg. **Fibroblasts in cancer**. Nat Rev Cancer. 2006 May;6(5):392-401.

[156] Yashiro M, Ikeda K, Tendo M, Ishikawa T, Hirakawa K. **Effect of organ-specific fibroblasts on proliferation and differentiation of breast cancer cells**. Breast Cancer Res Treat. 2005 Apr;90(3):307-13

[157] Bhowmick NA, Neilson EG, Moses HL. **Stromal fibroblasts in cancer initiation and progression.** Nature. 2004 Nov 18;432(7015):332-7.

[158] Desmoulière, A. Geinoz, F. Gabbiani, G. Gabbiani.**Transforming growth factor beta1 induces alpha smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts.** J Cell Biol. 1993 Jul;122(1):103-11.

[159] De Wever, O., and Mareel, M. **Role of tissue stroma in cancer cell invasion.** J Pathol. 2003 Jul;200(4):429-47.

[160] Evans, R. A., Tian, Y. C., Steadman, R., and Phillips, A. O.**TGF-beta1-mediated fibroblast-myofibroblast terminal differentiation-the role of Smad proteins.** Exp Cell Res. 2003 Jan 15;282(2):90-100.

[161] R. Masterson, T.D. Hewitson, K.J. Kelynack, M. Martic, L. Parry et al.**Relaxin downregulates renal fibroblast function and stimulates matrix remodelling in vitro.** Nephrol Dial Transplant. 2004 Mar;19(3):544-52.

[162] M.B. Hautmann, P.J. Adam, G.K. Owens.**Similarities and differences in smooth muscle alpha-actin induction by TGF-beta in smooth muscle versus non-smooth muscle cells**. Arterioscler Thromb Vasc Biol. 1999 Sep;19(9):2049-58.

[163] Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T et al. **Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion.** Cell. 2005;121:335–348

[164] Untergasser G, Gander R, Lilg C, Lepperdinger G, Plas E, Berger P. **Profiling molecular targets of TGF-β1 in prostate fibroblast-to-myofibroblast transdifferentiation**. Mech Ageing Dev.2005;126:59–69.

[165] Zhang J, Liu J. **Tumor stroma as targets for cancer therapy**. Pharmacol Ther. 2013 Feb;137(2):200-15. doi: 10.1016/j.pharmthera.2012.10.003.

[166] C. Anderberg, H. Li, L. Fredriksson, J. Andrae, C. Betsholtz et al. **Paracrine signaling by platelet-derived growth factor-CC promotes tumor growth by recruitment of cancer-associated fibroblasts**. Cancer Res. 2009 Jan 1;69(1):369-78.

[167] P. Micke, A. Ostman. Tumour-stroma interaction: cancer-associated fibroblasts as novel targets in anti-cancer therapy? Lung Cancer. 2004 Aug;45 Suppl 2:S163-75.

[168] H. Sugimoto, T.M. Mundel, M.W. Kieran, R. Kalluri. **Identification of fibroblast heterogeneity in the tumor microenvironment.** Cancer Biol Ther. 2006 Dec;5(12):1640-6.

[169] J.Paulsson, T.Sjoblom, P.Micke, F.Ponten,G.Landberg.**Prognostic significance of stromal platelet-derived growth factor beta-receptor expression in human breast cancer.** Am J Pathol. 2009 Jul;175(1):334-41.

[170] Marsh T, Pietras K, McAllister SS. **Fibroblasts as architects of cancer pathogenesis.** Biochim Biophys Acta. 2013 Jul;1832(7):1070-8

[171] Annes JP, Munger JS, Rifkin DB. **Making sense of latent TGFbeta activation.**J Cell Sci.2003;116(Pt 2):217–224.

[172] Rifkin DB. **Latent transforming growth factor-beta (TGF-beta) binding proteins: orchestrators of TGF-beta availability**.J Biol Chem.2005;280(9):7409–7412.

[173] Horiguchi K, Shirakihara T, Nakano A, et al. **Role of Ras signaling in the induction of snail by transforming growth factor-beta.** J Biol Chem.2009;284(1):245–253

[174] Isogai Z, Ono RN, Ushiro S, et al. **Latent transforming growth factor beta-binding protein 1 interacts with fibrillin and is a microfibril-associated protein.** J Biol Chem .2003;278(4):2750–2757.

[175] Taipale J, Miyazono K, Heldin CH,et al. **Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein**. J Cell Biol. 1994;124(1–2):171–181.

[176] Ge G,Greenspan DS . **BMP1 controls TGFbeta1 activation via cleavage of latent TGFbeta-binding protein**. J Cell Biol. 2006;175(1):111–120.

[177] Schultz-Cherry S, Murphy-Ullrich JE. **Thrombospondin causes activation of latent transforming growth factor-beta secreted by endothelial cells by a novel mechanism.** J Cell Biol. 1993;122(4):923–932.

[178] Barcellos-Hoff MH, Dix TA. **Redox-mediated activation of latent transforming growth factor-beta 1.** Mol Endocrinol. 1996;10(9):1077–1083.

[179] Munger JS,Huang X, Kawakatsu H,et al. **The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis.** Cell. 1999;96(3):319–328.

[180] Wipff PJ, Hinz B. **Integrins and the activation of latent transforming growth factor beta1 an intimate relationship.** Eur J Cell Biol. 2008;87(8–9):601–615.

[181] Hata A, Davis BN. **Control of microRNA biogenesis by TGFbeta signalling pathway—a novel role of Smads in the nucleus.**Cytokine Growth FactorRev.2009;20(5–6):517–521.

[182] Hussey GS, Chaudhury A, Dawson AE, et al. **Identification of an mRNP complex regulating tumorigenesis at the translational elongation step.**Mol Cell. 2011;41(4):419–431.

[183] Mu Y, Gudey SK, Landstrom M. **Non-Smad signaling pathways.** Cell Tissue Res. 2012;347(1):11–20.

[184] Shi Y, Massague J. **Mechanisms of TGF-beta signaling from cell membrane to the nucleus.**Cell.2003;113(6):685–700.

[185] Tsukazaki T, Chiang TA, Davison AF, et al. **SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor.** Cell. 1998;95(6):779–791.

[186] Yan X, Chen YG. **Smad7: not only a regulator, but also a cross-talk mediator of TGF-beta signalling.** Biochem J. 2011;434(1):1–10.

[187] Hong M, Wilkes MC, Penheiter SG, et al. **Non-Smad transforming growth factor-beta signaling regulated by focal adhesion kinase binding the p85 subunit of phosphatidylinositol 3-kinase**.J Biol Chem.2011;286(20):17841–17850.

[188] Kang JS, Liu C, Derynck R. **New regulatory mechanisms of TGF-beta receptor function.** Trends Cell Biol. 2009;19(8):385–394.

[189] Hartsough MT, Mulder KM. **Transforming growth factor beta activation of p44mapk in proliferating cultures of epithelial cells**. J Biol Chem 1995;270(13):7117–7124.

[190] Park SS, Eom YW, Kim EH, et al. **Involvement of c-Src kinase in the regulation of TGF-beta1-induced apoptosis**.Oncogene.2004;23(37):6272–6281.

[191] Yu L, Hebert MC, Zhang YE. **TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses.** EMBO J.2002;21(14):3749–3759.

[192] Padua D, Massague J. **Roles of TGFbeta in metastasis.** Cell Res. 2009;19(1):89–102.

[193] Akhurst RJ, Derynck R. **TGF-beta signaling in cancer—a double-edged sword.** Trends Cell Biol. 2001;11(11):S44–S51

[194] Inman GJ. **Switching TGFbeta from a tumor suppressor to a tumor promoter.** Curr Opin Genet Dev. 2011;21(1):93–99.

[195] Wendt MK, Tian M, Schiemann WP. **Deconstructing the mechanisms and consequences of TGF-beta-induced EMT during cancer progression.** Cell Tissue Res. 2012;347(1):85–101.

[196] Cui W, Fowlis DJ, Bryson S, et al. **TGFbeta1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice.** Cell. 1996;86(4):531–542.

[197] Pasche B. **Role of transforming growth factor beta in cancer.** J Cell Physiol. 2001;186(2):153–168

[198] Langenskiold M, Holmdahl L, Falk P, et al. **Increased TGF-beta 1 protein expression in patients with advanced colorectal cancer.**J Surg Oncol.2008;97(5):409–415.

[199] Zhao Z, Ma W, Zeng G, et al. **Preoperative serum levels of early prostate cancer antigen (EPCA) predict prostate cancer progression in patients undergoing radical prostatectomy.** Prostate. 2012;72(3):270–279

[200] Kim SJ, Angel P, Lafyatis R, et al. **Autoinduction of transforming growth factor beta 1 is mediated by the AP-1 complex.** Mol Cell Biol.1990;10(4):1492–1497.

[201] Yu N, Kozlowski JM, Park, II, et al. **Overexpression of transforming growth factor beta1 in malignant prostate cells is partly caused by a runaway of TGF-beta1 auto-induction mediated through a defective recruitment of protein phosphatase 2A by TGF-beta type I receptor.** Urology. 2010;76(6):1519.

[202] Zhang Q, Chen L, Helfand BT, et al. **TGF-beta regulates DNA methyltransferase expression in prostate cancer,correlates with aggressive capabilities ,and predicts disease recurrence.**PLoSOne.2011;6(9):e25168.

[203] Malkoski SP, Haeger SM, Cleaver TG, et al. **Loss of transforming growth factor beta type II receptor increases aggressive tumor behavior and reduces survival in lung adenocarcinoma and squamous cell carcinoma.**Clin Cancer Res. 2012;18(8):2173–2183.

[204] Zhao H, Shiina H, Greene KL, et al. **CpG methylation at promoter site -140 inactivates TGFbeta2 receptor gene in prostate cancer.** Cancer.2005;104(1):44–52.

[205] Bello-DeOcampo D, Tindall DJ. **TGF-betal/Smad signaling in prostate cancer.** Curr Drug Targets. 2003;4(3):197–207.

[206] R.J. Akhurst, R. Derynck.**TGF-beta signaling in cancer–a double-edged sword.**Trends Cell Biol, 11 (11) (2001), pp. S44–S51

[207] Shi Y, Massague J. **Mechanisms of TGF-beta signaling from cell membrane to the nucleus.**Cell.2003;113(6):685–700.

[208] Massague J. **TGF beta in Cancer**. Cell. 2008;134(2):215–230.

[209] Shin I, Bakin AV, Rodeck U, et al. **Transforming growth factor beta enhances epithelial cell survival via Akt-dependent regulation of FKHRL1**.Mol Biol Cell. 2001;12(11):3328–3339.

[210] R. Kalluri, R.A. Weinberg.**The basics of epithelial-mesenchymal transition**. J Clin Invest. 2009 Jun;119(6):1420-8.

[211] B. Bierie, H.L. Moses.**Transforming growth factor beta (TGF-beta) and inflammation in cancer**.Cytokine Growth Factor Rev, 21 (1) (2010), pp. 49–59

[212] Sonia B. Jakowlew .**Transforming growth factor-ß in cancer and metastasis.** Cancer Metastasis Rev. 2006 Sep;25(3):435-57.

[213] Kalluri R, Weinberg RA. **The basics of epithelial-mesenchymal transition.** J Clin Invest 2009;119:1420–1428.

[214] Voulgari A, Pintzas A. **Epithelial-mesenchymal transition in cancer metastasis: Mechanisms, markers and strategies to overcome drug resistance in the clinic**.Biochim Biophys Acta 2009;1796:75–90

[215] Han G, Lu SL, Li AG, et al. **Distinct mechanisms of TGF-beta1-mediated epithelial-to-mesenchymal transition and metastasis during skin carcinogenesis**.J Clin Invest. 2005; 115 (7) :1714–1723.

[216] Zavadil J, Cermak L, Soto-Nieves N, et al. **Integration of TGF-beta/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition**.EMBO J.2004;23(5):1155–1165.

[217] Peinado H, Olmeda D, Cano A. **Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype?** Nat Rev Cancer 2007; 7:415–428.

[218] Moustakas A, Heldin CH. **Induction of epithetlial mesenchymal transition by transforming growth factor beta**. Semin Cancer Biol 2012; 22:446–454.

[219] Vincent T, Neve EP, Johnson JR, et al. **A SNAIL1-SMAD3/4 transcriptional repressor complex promotes TGF-b mediated epithelial mesenchymal transition .** Nat Cell Biol 2009; 11:943–950.

[220] Horiguchi K, Shirakihara T, Nakano A, et al. **Role of Ras signaling in the induction of snail by transforming growth factor-beta.** J Biol Chem.2009;284(1):245–253.

[221] Peinado H, Quintanilla M, Cano A. **Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines:mechanisms for epithelial mesenchymal transitions.** J Biol Chem . 2003;278(23):21113–21123

[222] Mu Y, Sundar R, Thakur N, et al. **TRAF6 ubiquitinates TGFbeta type I receptor to promote its cleavage and nuclear translocation in cancer.**Nat Commun. 2011;2:330.

[223] Peinado H, Ballestar E, Esteller M, et al. **Snail mediates E-cadherin repression by the recruitment of the Sin3A/histone deacetylase 1 (HDAC1)/HDAC2 complex**.Mol Cell Biol. 2004;24(1):306–319.

[224] Vogelmann R, Nguyen-Tat MD, Giehl K, et al. **TGFbeta-induced downregulation of E-cadherin-based cell-cell adhesion depends on PI3-kinase and PTEN.**J Cell Sci.2005;118(Pt 20):4901–4912

[225] Madani I, De Neve W, Mareel M. **Does ionizing radiation stimulate cancer invasion and metastasis?** Bull Cancer 2008;95:292–300.

[226] Ho JN, Kang GY, Lee SS, et al. **Bcl-X(L) and STAT3 mediate malignant actions of gamma-irradiation in lung cancer cells.**Cancer Sci 2010;101:1417–1723.

[227] Tsukamoto H, Shibata K, Kajiyama H, et al. **Irradiation-induced epithelial-mesenchymal transition (EMT) related to invasive potential in endometrial carcinoma cells.** Gynecol Oncol 2007;107:500–504.

[228] Cheng JC, Chou CH, Kuo ML, et al. **Radiation-enhanced hepatocellular carcinoma cell invasion with MMP-9 expression through PI3K/Akt/NF-kappaB signal transduction pathway.**Oncogene 2006;25:7009–7018

[229] Sofia VI, Martins LR, Imaizumi N, et al. **Low doses of ionizing radiation promote tumor growth and metastasis by enhancing angiogenesis.** PLoS One 2010;5:e11222.

[230] Xu J, Lamouille S, Derynck R. **TGF-β-induced epithelial to mesenchymal transition**. Cell Res 2009; 19:156–172.

[231] Ikushima H, Miyazono K. **TGFβ signalling: a complex web in cancer progression.**Nat Rev Cancer 2010; 10:415–424

[232] Yong-Chun Zhou, D.Jun-Ye Liu. **Ionizing Radiation Promotes Migration and Invasion of Cancer Cells Through Transforming Growth Factor-Beta–Mediated Epithelial Mesenchymal Transition**.Int J Radiat Oncol Biol Phys. 2011 Dec 1;81(5):1530-7

[233] J.C. Cheng, C.H. Chou, M.L. Kuo et al.**Radiation-enhanced hepatocellular carcinoma cell invasion with MMP-9 expression through PI3K/Akt/NF-kappaB signal transduction pathway**. Oncogene. 2006 Nov 9;25(53):7009-18.

[234] Zavadil J, Bitzer M, Liang D, et al .**Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta.** Proc Natl Acad Sci U S A. 2001 Jun 5;98(12):6686-91.

[235] Gilles C, Newgreen DF, Sato H, et al. **Metalloproteases and Epithelial-to Mesenchymal Transition:Implications for Carcinoma Metastasis.**Austin,TX:Landes Bioscience; 2000.

[236] Kim ES, Kim MS, Moon A. **TGF-beta-induced upregulation of MMP-2 and MMP-9 depends on p38 MAPK, but not ERK signaling in MCF10A human breast epithelial cells.** Int J Oncol. 2004;25(5):1375–1382.

[237] Branton MH, Kopp JB. **TGF-beta and fibrosis**. Microbes Infect.1999;1(15):1349–1365.

[238] Zhang Q, Helfand BT, Jang TL, et al. **Nuclear factor-kappaB-mediated transforming growth factor-beta-induced expression of vimentin is an independent predictor of biochemical recurrence after radical prostatectomy.**ClinCancer Res.2009;15(10):3557–3567.

[239] Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. **The epithelial-mesenchymal transition generates cells with properties of stem cells**. Cell. 2008; 133: 704-15.

[240] Battula VL, Evans KW, Hollier BG, Shi Y, Marini FC, Ayyanan A, et al. **Epithelial-mesenchymal transition-derived cells exhibit multilineage differentiation potential similar to mesenchymal stem cells.** Stem Cells.2010; 28: 1435-45.

[241] Border WA, Noble NA. **Transforming growth factor beta in tissue fibrosis**.N Engl J Med. 1994;331(19):1286–1292.

[242] Midgley AC, Rogers M, Hallett MB, et al. **Transforming growth factorbeta1(TGF-beta1)-stimulated fibroblast to myofibroblast differentiation is mediated by hyaluronan (HA)-facilitated epidermal growth factor receptor (EGFR) and CD44 co-localization in lipid rafts.**J Biol Chem.2013;288(21):14824–14838.

[243] Xiao L, Du Y, Shen Y, et al. **TGF-beta 1 induced fibroblast proliferation is mediated by the FGF-2/ERK pathway.** Front Biosci (Landmark Ed) 2012;17:2667–2674

[244] Roberts AB, Sporn MB, Assoian RK, et al. **Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro.** Proc Natl Acad Sci U S A. 1986 Jun;83(12):4167-71.

[245] Vardouli L, Moustakas A, Stournaras C. **LIM-kinase 2 and cofilin phosphorylation mediate actin cytoskeleton reorganization induced by transforming growth factor-beta.** J Biol Chem. 2005;280:11448 –11457.

[246] Li, J., Zhang, Y. P., and Kirsner, R. S. **Angiogenesis in wound repair: Angiogenic growth factors and the extracellular matrix.** Microsc Res Tech. 2003 Jan 1;60(1):107-14.

[247] Yaguchi, T., Fukuda, Y., Ishizaki, M., and Yamanaka, N. **Immunohistochemical and gelatin zymography studies for matrix metalloproteinases in bleomycin-induced pulmonary fibrosis.** Pathol Int. 1998 Dec;48(12):954-63.

[248] Alexander, C. M., and Werb, Z. (1989). **Proteinases and extracellular matrix remodeling.** Curr Opin Cell Biol. 1989 Oct;1(5):974-82.

[249] Cheng S, Lovett DH. **Gelatinase A (MMP -2) is necessary and sufficient for renal tubular cell epithelial-mesenchymal transformation**. Am J Pathol. 2003 Jun;162(6):1937-49.

[250] Basset P, Okada A, Chenard MP, Kannan R, Stoll I et al. **Matrix metalloproteinases as stromal effectors of human carcinoma progression: therapeutical implications**. Matrix Biol. 1997 Mar;15(8-9):535-41.

[251] Johnsen, M., Lund, L. R., Romer, J., Almholt, K., Danø, K.**Cancer invasion and tissue remodeling: common themes in proteolytic matrix degradation.** Curr Opin Cell Biol. 1998 Oct;10(5):667-71

[252] Kähäri, VM., Saarialho-Kere U.**Matrix metalloproteinases in skin**. Exp **Dermatol**. 1997 Oct;6(5):199-213.

[253] Woessner, J. F, Parks, W. C. Mecham, R. P. **The matrix metalloproteinase family**. Matrix Metalloproteinases ,1-14 Academic Press San Diego.

[254] Björklund, M., Koivunen, E. **Gelatinase-mediated migration and invasion of cancer cells.** Biochim. Biochim Biophys Acta. 2005 May 25;1755(1):37-69

[255] Egeblad, M., Werb, Z. **New functions for the matrix metalloproteinases in cancer progression.** Nat.Rev.Cancer. 2002,2, 161–174.

[256] Kessenbrock K, Plaks V, Werb Z.**Matrix metalloproteinases: regulators of the tumor microenvironment.** Cell. 2010 Apr 2;141(1):52-67.

[257] Murphy, G., Knäuper, V. **Relating matrix metalloproteinase structure to function: why the 'hemopexin' domain?.** Matrix Biol. 1997 Mar;15(8-9):511-8.

[258] Gomez, D. E., Alonso, D. F., Yoshiji, H., and Thorgeirsson,U. P. **Tissue inhibitors of metalloproteinases: structure,regulation and biological functions.** Eur J Cell Biol. 1997 Oct;74(2):111-22.

[259] Kim, J., Yu, W., Kovalski, K., and Ossowski, L.**Requirement of specific proteases in cancer cell intravasation asrevealed by novel semiquantitative PCR-based assay**. Cell. 1998 Aug 7;94(3):353-62.

[260] Brooks, P. C., Silletti, S., von Schalscha, T. L, Friedlander, M.,and Cheresh, D. A. **Disruption of angiogenesis by PEX,a noncatalytic metalloproteinase fragment with integrin binding activity**. Cell. 1998 Feb 6;92(3):391-400.

[261] Uría, J. A., Ståhle-Bäckdahl, M., Seiki, M., Fueyo, A., López-Otín, C. **Regulation of collagenase-3 expression in human breast carcinomas is mediated by stromal-epithelial cell interactions.** Cancer Res. 1997 Nov 1;57(21):4882-8.

[262] Johansson, N., Vaalamo M., Grénman S., Hietanen S., Klemi P et al. **Collagenase-3 (MMP-13) is expressed by tumor cells in invasive vulvar squamous cell carcinomas.** Am J Pathol. 1999 Feb;154(2):469-80.

[263] Guo, H., Zucker, S., Gordon, M. K., Toole, B. P., and Biswas, C. **Stimulation of matrix metalloproteinase production by recombinant extracellular matrix metalloproteinase inducer from transfected Chinese hamster ovary cells.** J Biol Chem. 1997 Jan 3; 272(1):24-7.

[264] Atula, S., Grenman, R., Syrjänen, S. **Fibroblasts can modulate the phenotype of malignant epithelial cells in vitro.** Exp Cell Res. 1997 Aug 25; 235(1):180-7.

[265] Guo H, Zucker S, Gordon MK, Toole BP, Biswas C. **Stimulation of matrix metalloproteinase production by recombinant extracellular matrix metalloproteinase inducer from transfected Chinese hamster ovary cells**. J Biol Chem, 1997;272:24 – 27.

[266] Lim M, Martinez T, Jablons D, Cameron R, Guo H, Toole B, et al. **Tumorderived EMMPRIN (extracellular matrix metalloproteinase inducer) stimulates collagenase transcription through MAPK p38**. FEBS Lett, 1998;441:88 – 92.

[267] Biswas C, Zhang Y, DeCastro R, Guo H, Nakamura T, Kataoka H, et al.**The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily**. Cancer Res,1995;55:434 – 9.

[268] Kataoka H, DeCastro R, Zucker S, Biswas C. **Tumor cell-derived Collagenase stimulatory factor increases expression of interstitial collagenase,stromelysin, and 72-kDa gelatinase**. Cancer Res, 1993;53:3154 – 8.

[269] Sameshima T, Nabeshima K, Toole BP, Yokogami K, Okada Y, Goya T, et al. **Glioma cell extracellular matrix metalloproteinase inducer (EMMPRIN) (CD147)stimulates production of membrane-type matrix metalloproteinases and activated gelatinase A in co-cultures with brain-derived fibroblasts.** Cancer Lett, 2000;157:177 – 84.

[270] Westermarck J, Kähäri VM. **Regulation of matrix metalloproteinase expression in tumor invasion**. FASEB J. 1999 May;13(8):781-92.

[271] Johnsen, M., Lund, L. R., Romer, J., Almholt, K., Danø, K. **Cancer invasion and tissue remodeling: common themes in proteolytic matrix degradation**. Curr Opin Cell Biol. 1998 Oct;10(5):667-71.

[272] Atula, S., Grenman, R., Syrjänen, S. **Fibroblasts can modulate the phenotype of malignant epithelial cells in vitro.** Exp Cell Res. 1997 Aug 25;235(1):180-7.

[273] Khokha R, Murthy A, Weiss A. **Metalloproteinases and their natural inhibitors in inflammation and immunity.** Nat Rev Immunol. 2013 Sep;13(9):649-65

[274] Hess J, Angel P, Schorpp-Kistner M. "**AP-1 subunits: quarrel and harmony among siblings".** J Cell Sci. 2004 Dec 1;117(Pt 25):5965-73

[275] Lee, W., Mitchell, P. & Tjian, R. **Purified transcription factor AP1 interacts with TPA-inducible enhancer elements.** Cell. 1987 Jun 19;49(6):741-52

[276] Angel, P. et al. **Phorbol ester-inducible genes contain a common cis element recognized by a TPA modulated trans-acting factor.** Cell. 1987 Jun 19;49(6):729-39.

[277] Wisdom, R. **AP1: One switch for many signals.** Exp Cell Res. 1999 Nov 25;253(1):180-5.

[278] Eferl, R. & Wagner, E. F. **AP1: a double-edged sword in tumorigenesis**. Nat Rev Cancer. 2003 Nov;3(11):859-68.

[279] Angel, P. & Karin, M. **The role of Jun, Fos and the AP1 complex in cell-proliferation and transformation**. Biochim Biophys Acta. 1991 Dec 10;1072(2-3):129-57.

[280] Vlahopoulos SA, Logotheti S, Mikas D, Giarika A, Gorgoulis V, Zoumpourlis V.**The role of ATF-2 in oncogenesis.** Bioessays. 2008 Apr;30(4):314-27.

[281] Angel P, Imagawa M, Chiu R, Stein B, Imbra RJ, Rhamsdorf HJ et al. **Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor.** Cell. 1987 Jun 19;49(6):729-39.

[282] Lee W, Mitchell P, Tjian R. **Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements.**Cell. 1987 Jun 19;49(6):741-52.

[283] Benbow U, Brinckerhoff CE**. The AP-1 site and MMP gene regulation: what is all the fuss about?** Matrix Biol. 1997 Mar;15(8-9):519-26

[284] Woo JH, Park JW, Lee SH, et al. **Dykellic acid inhibits phorbol myristate acetate-induced matrix metalloproteinase-9 expression by inhibiting nuclear factor kappa B transcriptional activity.** Cancer Res.2003; 63:3430–3434.

[285] Aggarwal BB, Kumar A and Bharti AC.**Anticancer potential of curcumin: Preclinical and clinical studies.** Anticancer Res.2003;23:363–398.

[286] Thiery JP, Sleeman JP . **Complex networks orchestrate epithelial-mesenchymal transitions.** Nat Rev Mol Cell Biol. 2006 Feb;7(2):131-42.

[287] Sternlicht MD, Werb Z. **How matrix metalloproteinases regulate cell behavior**. Annu Rev Cell Dev Biol. 2001;17:463-516.

[288] Overall CM, Lopez-Otin C .**Strategies for MMP inhibition in cancer: innovations for the post-trial era.** Nat Rev Cancer. 2002 Sep;2(9):657-72.

[289] Matrisian LM. **Matrix metalloproteinase gene expression**. Ann N Y Acad Sci. 1994 Sep 6;732:42-50.

[290] Westermarck J, Kahari VM. **Regulation of matrix metalloproteinase expression in tumor invasion.** FASEB J. 1999 May;13(8):781-92.

[291] Jones LE, Humphreys MJ, Campbell F et al. **Comprehensive analysis of matrix metalloproteinase and tissue inhibitor expression in pancreatic cancer: increased expression of matrix metalloproteinase-7 predicts poor survival.** Clin Cancer Res. 2004 Apr 15;10(8):2832-45

[292] Liu D, Nakano J, Ishikawa S et al .**Overexpression of matrix metalloproteinase-7 (MMP-7) correlates with tumor proliferation,and a poor prognosis in non-small cell lung cancer.** Lung Cancer. 2007 Dec;58(3):384-91

[293] Morgia G, Falsaperla M, Malaponte G et al. **Matrix metalloproteinases as diagnostic (MMP-13) and prognostic (MMP2,MMP-9) markers of prostate cancer.** Urol Res. 2005 Feb;33(1):44-50

[294] Wu CY, Wu MS, Chiang EP et al .**Plasma matrix metalloproteinase-9 level is better than serum matrix metalloproteinase-9 level to predict gastric cancer evolution.** Clin Cancer Res. 2007 Apr 1;13(7):2054-60.

[295] Mook OR, Frederiks WM, Van Noorden CJ .**The role of gelatinases in colorectal cancer progression and metastasis.** Biochim Biophys Acta. 2004 Dec 17;1705(2):69-89.

[296] Somiari SB, Somiari RI, Heckman CM et al .**Circulating MMP2 and MMP9 in breast cancer—potential role in classification of patients into low risk, high risk, benign disease and breast cancer categories.** Int J Cancer. 2006 Sep 15;119(6):1403-11.

[297] Kerkela E, Saarialho-Kere U. **Matrix metalloproteinases in tumor progression: focus on basal and squamous cell skin cancer.** Exp Dermatol. 2003 Apr;12(2):109-25.

[298] Illman SA, Lehti K, Keski-Oja J et al .**Epilysin (MMP-28) induces TGF-beta mediated epithelial to mesenchymal transition in lung carcinoma cells**. J Cell Sci. 2006 Sep 15;119(Pt 18):3856-65.

[299] Radisky DC, Levy DD, Littlepage LE et al. **Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability.** Nature. 2005 Jul 7;436(7047):123-7.

[300] Lochter A, Sternlicht MD, Werb Z et al. **The significance of matrix metalloproteinases during early stages of tumor progression.**Ann N Y Acad Sci 857:180–193

[301] Noel A, Boulay A, Kebers F et al. **Demonstration in vivo that stromelysin-3 functions through its proteolytic activity.** Oncogene. 2000 Mar 16;19(12):1605-12.

[302] McGuire JK, Li Q, Parks WC .**Matrilysin (matrix metalloproteinase-7) mediates E-cadherin ectodomain shedding in injured lung epithelium.** Am J Pathol.2003; 162(6):1831–1843

[303] Karsdal MA, Larsen L, Engsig MT et al. **Matrix metalloproteinase-dependent activation of latent transforming growth factor-beta controls the conversion of osteoblasts into osteocytes by blocking osteoblast apoptosis.** J Biol Chem.2002;277(46):44061–44067

[304] Iida J, McCarthy JB.**Expression of collagenase-1 (MMP1) promotes melanoma growth through the generation of active transforming growth factor-beta.** Melanoma Res.2007; 17(4):205–213

[305] Massague, J. **TGFbeta in Cancer**. Cell. 2008 Jul 25;134(2):215-30..

[306] Farina AR, Coppa A, Tiberio A, Tacconelli A, Turco A, Colletta G, Gulino A, Mackay AR.**Transforming growth factor-β1 enhances the invasiveness of human MDA-MB-231 breast cancer cells by upregulating urokinase activity**. Int J Cancer.1998;75: 721–730.

[307] Shimizu S, Nishikawa Y, Kuroda K, Takagi S, Kozaki K, Hyuga S, Saga S, Matsuyama M.**Involvement of transforming growth factor beta1 in autocrine enhancement of gelatinase B secretion by murine metastatic colon carcinoma cells.** Cancer Res.1996; 56: 3366–3370.

[308] Duivenvoorden WCM, Hirte HW, Singh G. **Transforming growth factor β1 acts as an inducer of matrix metalloproteinase expression and activity in human bone-metabolizing cancer cells.** Clin Exp Metastasis. 1999 Feb;17(1):27-34.

[309] Sehgal I, Thompson TC. **Novel regulation of type IV collagenase (matrix metalloproteinase-9 and -2) activities by transforming growth factor-β1 in human prostate cancer cell lines**. Mol Biol Cell.1999; 10: 407–416.

[310] Festuccia C, Angellucci A, Gravina GL, Villanova I, Teti A, Abini A, Bologna M. **Osteoblast-derived TGF-β1 modulates matrix degrading protease expression and activity in prostate cancer cells.** Int J Cancer.2000; 85: 407–415.

[311] Lohi J, Lehti K, Westermarck J, Kahari VM, Keski-Oja J**. Regulation of membrane-type matrix metalloproteinase-1 expression by growth factors and phorbol 12-myristate 13-acetate.** Eur J Biochem.1996; 239: 239–247.

[312] Fang KC, Wolters PJ, Steinhoff M, Bidgol A, Blount JL, Caughey GH. **Mast cell expression of gelatinases A and B is regulated by kit ligand and TGF-beta.** J Immunol.1999; 162: 5528–5535.

[313] Overall CM, Sodek J. **Concanavalin A produces a matrix-degradative phenotype in human fibroblasts: induction and endogenous activation of collagenase, 72-kDa gelatinase, and pump-1 is accompanied by the suppression of the tissue inhibitor of matrix metalloproteinases**. J J Biol Chem. 1990 Dec 5;265(34):21141-51

[314] Impola U, Uitto VJ, Hietanen J et al. **Differential expression of matrilysin-1 (MMP-7), 92 kD gelatinase (MMP-9), and metalloelastase (MMP-12) in oral verrucous and squamous cell cancer**. J Pathol. 2004 Jan;202(1):14-22.

[315] Ellerbroek SM, Fishman DA, Kearns AS et al . **Ovarian carcinoma regulation of matrix metalloproteinase-2 and membrane type 1 matrix metalloproteinase through beta1 integrin.** Cancer Res. 1999 Apr 1;59(7):1635-41.

[316] Symowicz J, Adley BP, Gleason KJ et al. **Engagement of collagen-binding integrins promotes matrix metalloproteinase-9dependent E-cadherin ectodomain shedding in ovarian carcinoma cells.** Cancer Res.2007; 67(5):2030–2039

[317] Ratnikov BI, Rozanov DV, Postnova TI et al. **An alternative processing of integrin alpha(v) subunit in tumor cells by membrane type-1 matrix metalloproteinase**. J Biol Chem.2002; 277(9):7377–7385

[318] von Bredow DC, Nagle RB, Bowden GT et al .**Cleavage of beta 4 integrin by matrilysin.** Exp Cell Res.1997; 236(1):341–345

[319] Egeblad M, Werb Z .**New functions for the matrix metalloproteinases in cancer progression.** Nat Rev Cancer.2002; 2(3):161–174

[320] Sillanpaa S, Anttila M, Voutilainen K et al. **Prognostic significance of matrix metalloproteinase-9 (MMP-9) in epithelial ovarian cancer.** Gynecol Oncol.2007; 104(2):296–303

[321] Zhang Q, Chen X, Zhou J et al. **CD147, MMP-2, MMP-9 and MVD-CD34 are significant predictors of recurrence after liver transplantation in hepatocellular carcinoma patients.** Cancer Biol Ther. 2006 Jul;5(7):808-14

[322] Cardillo MR, Di Silverio F, Gentile V. **Quantitative immunohistochemical and in situ hybridization analysis of metalloproteinases in prostate cancer.** Anticancer Res.2006; 26(2A):973–982

[323] Ohtani H, Motohashi H, Sato H et al.**Dual over-expression pattern of membrane-type metalloproteinase-1 in cancer and stromal cells in human gastrointestinal carcinoma revealed by in situ hybridization and immunoelectron microscopy.** Int J Cancer.1996; 68(5):565–570

[324] Kaplan R.N., Riba R.D., Zacharoulis S., Bramley A.H., Vincent L., et al. **VEGFR1positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche.** Nature. 2005 Dec 8;438(7069):820-7.

[325] Hiratsuka, S., Watanabe, A., Aburatani, H., and Maru, Y. **Tumour mediated upregulation of chemoattractants And recruitment of myeloid cells predetermines lung metastasis**. Nat Cell Biol. 2006 Dec;8(12):1369-75

[326] Bergers G., Brekken R., McMahon G., Vu T.H., Itoh T., Tamaki K., Tanzawa et al.(2000). **Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis.** Nat Cell Biol. 2000 Oct;2(10):737-44.

[327] Jaffe, A. B., & Hall, A. (2005). Rho GTPases: biochemistry and biology. Annual Review of Cell and Developmental Biology, 21,247–269.

[328] Burridge, K., & Wennerberg, K. (2004). Rho and Rac take center stage. Cell, 116, 167–179.

[329] Wennerberg K, Der CJ. 2004. **Rho-family GTPases: it's not only Rac and Rho** . J Cell Sci.2004; 117:1301–1312.

[330] Teresa Gomez del Pulgar, Salvador A. Benitah, Pilar F. Valeron, Carolina Espina, and Juan Carlos Lacal.**Rho GTPase expression in tumourigenesis: evidence for a significant link.** Bioessays. 2005 Jun;27(6):602-13.

[331] Forget MA, Desrosiers RR, Gingras D, Beliveau R. **Phosphorylation states of Cdc42 and RhoA regulate their interactions with Rho GDP dissociation inhibitor and their extraction from biological membranes.** Biochem J. 2002 Jan 15;361(Pt 2):243-54.

[332] Ellerbroek SM, Wennerberg K, Burridge K.**Serine phosphorylation negatively regulates RhoA in vivo.** J Biol Chem.2003; 278:19023–19031.

[333] Shuh Narumiya & Masahiro Tanji &Toshimasa Ishizak . **Rho signaling, ROCK and mDia1, in transformation,metastasis and invasion.**Cancer Metastasis Rev.2009; 28:65–76

[334] Leung, T., Manser, E., Tan, L., & Lim, L. **A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membra**nes. J Biol Chem. 1995 Dec 8;270(49):29051-4.

[335] Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu,A., Fujita, A., et al.**The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase.** EMBO J. 1996 Apr 15;15(8):1885-93

[336] Matsui T., Amano M., Yamamoto T., Chihara K., Nakafuku M.et al. **Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho**. EMBO J. 1996 May 1;15(9):2208-16.

[337] Gaugler MH, Vereycken-Holler V, Squiban C, Vandamme M, Vozenin-Brotons MC, Benderitter M. **Pravastatin limits endothelial activation after irradiation and decreases the resulting inflammatory and thrombotic responses**. Radiat Res 2005;163:479–87.

[338] Canguilhem B, Pradines A, Baudouin C et al. R**hoB protects human keratinocytes from UVB-induced apoptosis through epidermal growth factor receptor signaling**. J Biol Chem.2005; 280:43257–63

[339] Fritz G, Kaina B .**Transcriptional activation of the small GTPase gene rhoB by genetoxic stress is regulated via CCAAT element**. Nucleic Acids Res.2001; 29:792–8

[340] Milia J, Teyssier F, Dalenc F et al. **Farnesylated RhoB inhibits radiationinduced mitotic cell death and controls radiation-induced centrosome overduplication**. Cell Death Differ.2005; 12:492–501

[341] Monferran S, Skuli N, Delmas C et al.**Alphavbeta3 and alphavbeta5 integrins control glioma cell response to ionising radiation through iLK and RhoB.** Int J Cancer.2008; 123:357–64

[342] Gampel A, Parker PJ, Mellor H . **Regulation of epidermal growth factor receptor traffic by the small GTPase RhoB**. Curr Biol.1999; 9: 955–958.

[343] Huang M, Duhadaway JB, Prendergast GC, Laury-Kleintop LD. **RhoB regulates PDGFR-beta trafficking and signaling in vascular smooth muscle cells**.Arterioscler Thromb Vasc Biol.2007; 27: 2597–2605.

[344] Lajoie-Mazenc I, Tovar D, Penary M, Lortal B, Allart S, et al.**MAP1A light chain-2 interacts with GTP-RhoB to control epidermal growth factor (EGF)-dependent EGF receptor signaling.** J Biol Chem.2008; 283: 4155–4164.

[345] Fritz G, Kaina B. **RhoB encoding a UV-inducible Ras-related small GTP-binding protein is regulated by GTPases of the Rho family and independent of JNK, ERK, and p38 MAP kinase**. J Biol Chem. 1997;272(49):30637–30644.

[346] Jahner D, Hunter T. **The ras-related gene rhoB is an immediate-early gene inducible by v-Fps, epidermal growth factor, and platelet-derived growth factor in rat fibroblasts.** Mol Cell Biol. 1991;11(7):3682–3690

[347] Fritz G, Kaina B, Aktories K .**The ras-related small GTP-binding protein RhoB is immediate-early inducible by DNA damaging treatments**. J Biol Chem.1995; 270: 25172–25177.

[348] Kajimoto H, Hashimoto K, Bonnet SN, Haromy A, Harry G, et al.**Oxygen activates the Rho/Rho-kinase pathway and induces RhoB and ROCK1 expression in human and rabbit ductus arteriosus by increasing mitochondriaderived reactive oxygen species: a newly recognized mechanism for sustaining ductal constriction**.Circulation. 2007 Apr 3;115(13):1777-88.

[349] Jahner D, Hunter T. **The ras-related gene rhoB is an immediate-early gene inducible by v-Fps, epidermal growth factor, and platelet-derived growth factor in rat fibroblasts.** Mol Cell Biol. 1991;11(7):3682–3690.

[350] Adamson P, Paterson HF, Hall A. **Intracellular localization of the P21rho proteins.**J Cell Biol. 1992;119(3):617–627.

[351] Wherlock M, Gampel A, Futter C, Mellor H. **Farnesyltransferase inhibitors disrupt EGF receptor traffic through modulation of the RhoB GTPase.** J Cell Sci. 2004;117(Pt 15):3221–3231.

[352] Sandilands E, Cans C, Fincham VJ, Brunton VG, Mellor H, et al. **RhoB and actin polymerization coordinate Src activation with endosome-mediated delivery to the membrane**. Dev Cell .2004;7: 855–869.

[353] Fernandez-Borja M, Janssen L, Verwoerd D, Hordijk P, Neefjes J .**RhoB regulates endosome transport by promoting actin assembly on endosomal membranes through Dia1.** J Cell Sci.2005 118: 2661–2670.

[354] Gampel A, Parker PJ, Mellor H. **Regulation of epidermal growth factor receptor traffic by the small GTPase rhoB**. Curr Biol. 1999;9(17):955–958

[355] Adamson P, Paterson HF, Hall A. **Intracellular localization of the p21rho proteins.** J. Cell Biol.1992;119:617–627

[356] Mellor J, Flynn P, Nobes CD, Hall A, Parker PJ. **PRK1 is targeted to endosomes by the small GTPase, RhoB.** J. Biol. Chem. 1998;273:4811–4814.

[357] Liu AX, Rane N, Liu JP, Prendergast GC. **RhoB is dispensable for mouse development, but it modifies susceptibility to tumor formation as well as cell adhesion and growth factor signaling in transformed cells.** Mol Cell Biol. 2001;21:6906 –6912.

[358] Ho TT, Merajver SD, Lapiere CM, Nusgens BV, Deroanne CF.**RhoA-GDP regulates RhoB protein stability. Potential involvement of RhoGDIalpha.** J Biol Chem. 2008;283:21588 – 21598.

[359] Wherlock M, Gampel A, Futter C, Mellor H. **Farnesyltransferase inhibitors disrupt EGF receptor traffic through modulation of the RhoB GTPase**. J Cell Sci. 2004;117:3221–31.

[360] Prendergast GC, Khosravi-Far R, Solski PA, Kurzawa H, Lebowitz PF,Der CJ. **Critical role of Rho in cell transformation by oncogenic Ras.**Oncogene. 1995;10:2289 –2296.

[361] Rodriguez PL, Sahay S, Olabisi OO, Whitehead IP. **ROCK I-mediated activation of NF-kappaB by RhoB**. Cell Signal. 2007;19:2361–2369.

[362] Liu A, Du W, Liu JP, Jessell TM, Prendergast GC. **RhoB alteration is necessary for apoptotic and antineoplastic responses to farnesyltransferase inhibitors**. Mol Cell Biol. 2000;20:6105– 6113.

[363] Zeng PY, N Rane, W Du, J Chintapalli, and GC Prendergast. **Role for RhoB and PRK in the suppression of epithelial cell transformation by farnesyltransferase inhibitors**. Oncogene. 2003;22:1124 –1134.

[364] Chen Z, Sun J, Pradines A, Favre G, Adnane J, Sebti SM. **Both farnesylated and geranylgeranylated RhoB inhibit malignant transformation and suppress human tumor growth in nude mice**. J Biol Chem. 2000;275: 17974–17978.

[365] Mazieres J, Tillement V, Allal C et al. **Geranylgeranylated, but not farnesylated, RhoB suppresses Ras transformation of NIH-3T3 cells**. Exp Cell Res.2005; 304:354–64

[366] Schmitz AA1, Govek EE, Böttner B, Van Aelst L.**Rho GTPases: signaling, migration, and invasion.** Exp Cell Res. 2000 Nov 25;261(1):1-12.

[367] Huang M, Duhadaway JB, Prendergast GC, Laury-Kleintop LD. **RhoB regulates PDGFR-beta trafficking and signaling in vascular smooth muscle cells.** Arterioscler Thromb Vasc Biol. 2007;27:2597–2605

[368] Filippi I, Naldini A, Carraro F. **Role of the hypoxic microenvironment in the antitumor activity of tyrosine kinase inhibitors.** Curr Med Chem.2011;18:2885–2892

[369] Van Aelst L, D'Souza-Schorey C. **Rho GTPases and signalling networks.** Genes Dev. 1997;11:2295–2322.

[370] Aznar S, Lacal JC. **Rho signals to cell growth and apoptosis**. Cancer Lett. 2001 Apr 10;165(1):1-10

[371] Aznar S, Lacal JC. **Searching new targets for anticancer drug design: the families of Ras and Rho GTPases and their effectors.** Prog Nucleic Acid Res Mol Biol. 2001;67:193-234..

[372] Schmitz AA, Govek EE, Bottner B, Van Aelst L. 2000. **Rho GTPases: signaling, migration, and invasion**. Exp Cell Res 261:1–12.

[373] Barone MV, Sepe L, Melillo RM, Mineo A, Santelli G, et al. **RET/PTC1 oncogene signaling in PC Cl 3 thyroid cells requires the small GTP-binding protein Rho.** Oncogene.2001; 20:6973–6982.

[374] Boerner JL, McManus MJ, Martin GS, Maihle NJ. **Rasindependent oncogenic transformation by an EGF-receptor mutant**.J Cell Sci.2000;113:935–942.

[375] Kim BC, Yi JY, Yi SJ, Shin IC, Ha KS, et al.**Rac GTPase activity is essential for EGF-induced mitogenesis.** Mol Cells.1998; 8:90–95.

[376] Royal I, Lamarche-Vane N, Lamorte L, Kaibuchi K, Park M.**Activation of cdc42, rac, PAK, and rho-kinase in response to hepatocyte growth factor differentially regulates epithelial cell colony spreading and dissociation.** Mol Biol Cell.2000; 11:1709–1725.

[377] Sachdev P, Jiang YX, Li W, Miki T, Maruta H, et al. **Differential requirement for Rho family GTPases in an oncogenic insulin-like growth factor-I receptor-induced cell transformation.** J Biol Chem.2001; 276:26461–26471.

[378] Whitehead IP, Zohn IE, Der CJ. **Rho GTPase-dependent transformation by G protein-coupled receptors.** Oncogene.2001; 20:1547–1555.

[379] Huang, M. and Prendergast, G.C. **RhoB in cancer suppression.** Histol Histopathol. 2006 Feb;21(2):213-8.

[380] Prendergast GC. **Actin' up: RhoB in cancer and apoptosis**. Nat Rev Cancer. 2001 Nov;1(2):162-8.

[381] Adnane J, Muro-Cacho C, Mathews L, Sebti SM, Munoz-Antonia T. **Suppression of rho B expression in invasive carcinoma from head and neck cancer patients.** Clin Cancer Res. 2002 Jul;8(7):2225-32.

[382] Mazieres J, Antonia T, Daste G, Muro-Cacho C, Berchery D, et al.**Loss of RhoB expression in human lung cancer progression.** Clin Cancer Res.2004; 10:2742–2750.

[383] Wang S, Yan-Neale Y, Fischer D, Zeremski M, Cai R, et al.**Histone deacetylase 1 represses the small GTPase RhoB expression in human nonsmall lung carcinoma cell line.** Oncogene.2003;22:6204–6213.

[384] Liu, A.X., Rane, N., Liu, J.P. and Prendergast, G.C. **RhoB is dispensable for mouse development, but it modifies susceptibility to tumor formation as well as cell adhesion and growth factor signaling in transformed cells**. Mol. Cell Biol.2001;21, 6906–6912.

[385] Jiang, K., Sun, J., Cheng, J., Djeu, J.Y., Wei, S. and Sebti, S.**Akt mediates Ras downregulation of RhoB, a suppressor of transformation, invasion, and metastasis.** Mol. Cell Biol.2004; 24,5565–5576.

[386] G.C. Prendergast, R. Khosravi-Far, P.A. Solski, H. Kurzawa, P.F. Lebowitz, C.J. Der.**Critical role of Rho in cell transformation by oncogenic Ras.** Oncogene. 1995 Jun 15;10(12):2289-96.

[387] E. Bousquet, J. Mazieres, M. Privat, V. Rizzati, A. Casanova et al. **Loss of RhoB expression promotes migration and invasion of human bronchial cells via activation of AKT1**. Cancer Res. 2009 Aug 1;69(15):6092-9.

[388] K. Jiang, J. Sun, J. Cheng, J.Y. Djeu, S. Wei et al.**Akt mediates Ras downregulation of RhoB, a suppressor of transformation, invasion, and metastasis.** Mol Cell Biol. 2004 Jun;24(12):5565-76.

[389] W. Du, G.C. Prendergast.**Geranylgeranylated RhoB mediates suppression of human tumor cell growth by farnesyltransferase inhibitors**. Cancer Res. 1999 Nov 1;59(21):5492-6.

[390] J. Mazieres, T. Antonia, G. Daste, C. Muro-Cacho, D. Berchery et al.**Loss of RhoB expression in human lung cancer progression**. Clin Cancer Res. 2004 Apr 15;10(8):2742-50.

[391] B. Couderc, A. Pradines, A. Rafii, M. Golzio, A. Deviers et al.**In vivo restoration of RhoB expression leads to ovarian tumor regression**. Cancer Gene Ther. 2008 Jul;15(7):456-64.

[392] A. Liu, G.J. Cerniglia, E.J. Bernhard, G.C. Prendergast.**RhoB is required to mediate apoptosis in neoplastically transformed cells after DNA damage.** Proc Natl Acad Sci U S A. 2001 May 22;98(11):6192-7

[393] J. Zhou, Y. Zhu, G. Zhang, N. Liu, L. Sun et al. **A distinct role of RhoB in gastric cancer suppression**. Int J Cancer. 2011 Mar 1;128(5):1057-68.

[394] S. Kazerounian, D. Gerald, M. Huang, Y.R. Chin, D. Udayakumar et al.**RhoB differentially controls Akt function in tumor cells and stromal endothelial cells during breast tumorigenesis**. Cancer Res. 2013 Jan 1;73(1):50-61

[395] Luis-Ravelo D, Antón I, Zandueta C. **RHOB influences lung adenocarcinoma metastasis and resistance in a host-sensitive manner.** Mol Oncol. 2014 Mar;8(2):196-206.

[396] Ader, C. Toulas, F. Dalenc, C. Delmas, J. Bonnet et al.**RhoB controls the 24 kDa FGF-2-induced radioresistance in HeLa cells by preventing post-mitotic cell death**. Oncogene. 2002 Sep 5;21(39):5998-6006.

[397] Ader, C. Delmas, J. Bonnet, P. Rochaix, G. Favre et al.**Inhibition of Rho pathways induces radiosensitization and oxygenation in human glioblastoma xenografts**. Oncogene. 2003 Dec 4;22(55):8861-9.

[398] N. Skuli, S. Monferran, C. Delmas, I. Lajoie-Mazenc, G. Favre et al.**Activation of RhoB by hypoxia controls hypoxia-inducible factor-1alpha stabilization through glycogen synthase kinase-3 in U87 glioblastoma cells**. Cancer Res. 2006 Jan 1;66(1):482-9.

[399] S. Monferran, N. Skuli, C. Delmas, G. Favre, J. Bonnet et al.**Alphavbeta3 and alphavbeta5 integrins control glioma cell response to ionising radiation through ILK and RhoB**. Int J Cancer. 2008 Jul 15;123(2):357-64. doi: 10.1002/ijc.23498.

[400] N. Sato, T. Fukui, T. Taniguchi, T. Yokoyama, M. Kondo et al.**RhoB is frequently downregulated in non-small-cell lung cancer and resides in the 2p24 homozygous deletion region of a lung cancer cell line.** Int J Cancer. 2007 Feb 1;120(3):543-51.

[401] M. Fernandez-Borja, L. Janssen, D. Verwoerd, P. Hordijk, J. Neefjes.**RhoB regulates endosome transport by promoting actin assembly on endosomal membranes through Dia1**. J Cell Sci. 2005 Jun 15;118(Pt 12):2661-70.

[402] Mellor H, Flynn P, Nobes CD, Hall A, Parker PJ.**PRK1 is targeted to endosomes by the small GTPase, RhoB.** J Biol Chem. 1998 Feb 27;273(9):4811-4.

[403] Y. Kang, P.M. Siegel, W. Shu, M. Drobnjak, S.M. Kakonen et al.**A multigenic program mediating breast cancer metastasis to bone**. Cancer Cell. 2003 Jun;3(6):537-49.

[404] Luis-Ravelo D, Antón I, Zandueta C, Valencia K, Ormazábal C et al. **A gene signature of bone metastatic colonization sensitizes for tumor-induced osteolysis and predicts survival in lung cancer**. Oncogene. 2013 Oct 28. doi: 10.1038/onc.2013.440. [Epub ahead of print]

[405] Luis-Ravelo D, Antón I, Zandueta C. **RHOB influences lung adenocarcinoma metastasis and resistance in a host-sensitive manner.** Mol Oncol. 2014 Mar;8(2):196-20

[406] Misao Yoneda , Yoshifumi S., Hirokawa. **RhoB enhances migration and MMP1 expression of prostate cancer DU145.** Exp Mol Pathol. 2010 Feb;88(1):90-5

[407] Zalcman G, Closson V, Linares-Cruz G, Lerebours F,Honore ´N et al. **Regulation of Ras-related RhoB protein expression during the cell cycle. Oncogene.** 1995 May 18;10(10):1935-45.

[408] Eleftheria Vasilaki , Elsa Papadimitriou , Virginia Tajadura et al.**Transcriptional regulation of the small GTPase RhoB gene by TGFβ-induced signaling pathways.** FASEB J. 2010 Mar;24(3):891-905. doi: 10.1096/fj.09-134742.

[409] Vardouli L, Vasilaki E, Papadimitriou E, Kardassis D, Stournaras C. **A novel mechanism of TGF β induced actin reorganization mediated by Smad proteins and Rho GTPases**. FEBS J. 2008 Aug;275(16):4074-87

[410] Edlund S, Landstrom M, Heldin CH & Aspenstrom P . **Transforming growth factor-beta-induced mobilization of actin cytoskeleton requires signaling by small GTPases Cdc42 and RhoA.** Mol Biol Cell. 2002 Mar;13(3):902-14.

[411] Vardouli L, Moustakas A & Stournaras C **. LIM-kinase 2 and cofilin phosphorylation mediate actin cytoskeleton reorganization induced by transforming growth factor-beta**. J Biol Chem. 2005 Mar 25;280(12):11448-57. Epub 2005 Jan 11.

[412] Vardouli L, Vasilaki E, Papadimitriou E, Kardassis D & Stournaras C. **A novel mechanism of TGFbeta-induced actin reorganization mediated by Smad proteins and Rho GTPases.** FEBS J. 2008 Aug;275(16):4074-87. doi: 10.1111/j.1742-4658.2008.06549.x

[413] Adler KB, Low RB, Leslie KO, Mitchell J & Evans JN .**Contractile cells in normal and fibrotic lung.** Lab Invest. 1989 Apr;60(4):473-85.

[414] Chen S, Crawford M, Day RM, Briones VR, Leader JE et al. **RhoA modulates Smad signaling during transforming growth factor-betainduced smooth muscle differentiation**. J Biol Chem. 2006 Jan 20;281(3):1765-70.

[415] Masszi A, Di Ciano C, Sirokmany G, Arthur WT,Rotstein OD et al .**Central role for Rho in TGF-beta1-induced alpha-smooth muscle actin expression during epithelial–mesenchymal transition.** Am J Physiol Renal Physiol. 2003 May;284(5):F911-24

[416] Adnane J, Seijo E, Chen Z, Bizouarn F, Leal M et al.**RhoB, not RhoA, represses the transcription of the transforming growth factor beta type II receptor by a mechanism involving activator protein 1**. J Biol Chem. 2002;277, 8500–8507.

[417] Fig Zhang J, Liu J. **Tumor stroma as targets for cancer therapy**. Pharmacol Ther. 2013 Feb;137(2):200-15. doi: 10.1016/j.pharmthera.2012.10.003.

[418] Nabors LB, Mikkelsen T, Hegi ME, Ye X, Batchelor T et al. **A safety run-in and randomized phase 2 study of cilengitide combined with chemoradiation for newly diagnosed glioblastoma (NABTT306)**Cancer. 2012 http://dx.doi.org.gate2.inist.fr/10.1002/cncr.27585.

[419] Kargiotis O, Chetty C, Gogineni V, Gondi CS, Pulukuri SM et al. **uPA/uPAR downregulation inhibits radiation-induced migration, invasion and angiogenesis in IOMM-Lee meningioma cells and decreases tumor growth in vivo**. Int J Oncol. 2008;33:937–947

[420] Duriseti S, Goetz DH, Hostetter DR, LeBeau AM, Wei Y, Craik CS. **Antagonistic anti-urokinase plasminogen activator receptor (uPAR) antibodies significantly inhibit uPAR-mediated cellular signaling and migration.** J Biol Chem. 2010;285:26878–26888.

[421] Meyer JE, Brocks C, Graefe H, Mala C, Thans N et al. **The Oral Serine Protease Inhibitor WX-671 - First Experience in Patients with Advanced Head and Neck Carcinoma**. Breast Care (Basel) 2008;3:20–24.

[422] Hua H, Li M, Luo T, Matrix metalloproteinases in tumorigenesis: an evolving paradigm. Cell Mol Life Sci 2011;68:3853-68.

[423] Sparano JA, Bernardo P, Stephenson P, Gradishar WJ, Ingle JN, Zucker S, Davidson NE. **Randomized phase III trial of marimastat versus placebo in patients with metastatic breast cancer who have responding or stable disease after first-line chemotherapy: Eastern Cooperative Oncology Group trial E2196**. J Clin Oncol. 2004;22:4683–4690.

[424] Hirte H, Vergote IB, Jeffrey JR, Grimshaw RN, Coppieters S et al. **A phase III randomized trial of BAY 12-9566 (tanomastat) as maintenance therapy in patients with advanced ovarian cancer responsive to primary surgery and paclitaxel/platinum containing chemotherapy: a National Cancer Institute of Canada Clinical Trials Group Study.** Gynecol Oncol. 2006;102:300–308.

[425] Overall CM, Kleifeld O. **Tumour microenvironment - opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy**. Nat Rev Cancer. 2006 Mar;6(3):227-39

[426] Overall CM, Kleifeld O. **Towards third generation matrix metalloproteinase inhibitors for cancer therapy**. Br J Cancer 2006;94:941–6.

[427] Cirri P, Chiarugi P. **Cancer-associated-fibroblasts and tumour cells: a diabolic liaison driving cancer progression.** Cancer Metastasis Rev. 2012;31:195–208.

[428] Rettig WJ, Garin-Chesa P, Healey JH. **Regulation and heteromeric structure of the fibroblast activation protein in normal and transformed cells of mesenchymal and neuroectodermal origin**. Cancer Res 1993;53:3327-35

[429] Liu R, Li H, Liu L, Yu J, Ren X. **Fibroblast activation protein: A potential therapeutic target in cancer.** Cancer Biol Ther. 2012;13:123–129.

[430] Gonda TA, Varro A, Wang TC, Tycko B. **Molecular biology of cancer-associated fibroblasts: can these cells be targeted in anti-cancer therapy?** Semin Cell Dev Biol. 2010 Feb;21(1):2-10.

[431] El-Nikhely N, Larzabal L, Seeger W, Calvo A, Savai R. **Tumor-stromal interactions in lung cancer: novel candidate targets for therapeutic intervention.** Expert Opin Investig Drugs. 2012 Aug;21(8):1107-22.

[432] Padua, D. and Massagué, J. **Roles of TGFß in metastasis**. Cell Res.2009 ; 19, 89-102.

[433] Tzai TS, Lin CI, Shiau AL, Wu CL. **Antisense oligonucleotide specific for transforming growth factor-β1 inhibit both in vitro and in vivo growth of MBT-2 murine bladder cancer.**Anticancer Res. 1998;18:1585–1589.

[434] Nam JS, Terabe M, Mamura M, Kang MJ, Chae Het al. **An anti-transforming growth factor beta antibody suppresses metastasis via cooperative effects on multiple cell compartments.** Cancer Res. 2008;68:3835–3843.

[435] Mazzocca A, Fransvea E, Lavezzari G, Antonaci S, Giannelli G. **Inhibition of transforming growth factor beta receptor I kinase blocks hepatocellular carcinoma growth through neo-angiogenesis regulation.** Hepatology. 2009;50:1140–1151.

[436] Sheen YY, Kim MJ, Park SA, Park SY, Nam JS. **Targeting the Transforming Growth Factor-β Signaling in Cancer Therapy.** Biomol Ther (Seoul). 2013 Sep 30;21(5):323-331.

[437] Matsumoto K, Nakamura T. **Hepatocyte growth factor and the Met system as a mediator of tumor-stromal interactions.** Int J Cancer. 2006;119:477–483.

[438] Lin MI, Sessa WC. **Antiangiogenic therapy: creating a unique "window" of opportunity.**Cancer Cell. 2004;6:529–531.

[439] Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J et al.**Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer**. N Engl J Med. 2004;350:2335–2342.

[440] Albiges L, Salem M, Rini B, Escudier B. **Vascular endothelial growth factor-targeted therapies in advanced renal cell carcinoma.** Hematol Oncol Clin North Am. 2011;25:813–833.

[441] Krupitskaya Y, Wakelee HA. **Ramucirumab, a fully human mAb to the transmembrane signaling tyrosine kinase VEGFR-2 for the potential treatment of cancer.** Curr Opin Investig Drugs. 2009;10:597–605.

[442] Shah GD, Loizos N, Youssoufian H, Schwartz JD, Rowinsky EK. **Rationale for the development of IMC-3G3, a fully human immunoglobulin G subclass 1 monoclonal antibody targeting the platelet-derived growth factor receptor alpha.** Cancer. 2010;116:1018–1026.

[443] Kono SA, Heasley LE, Doebele RC, Camidge DR. **Adding to the mix: fibroblast growth factor and platelet-derived growth factor receptor pathways as targets in non-small cell lung cancer.**Curr Cancer Drug Targets. 2012;12:107–123.

[444] Crawford Y, Kasman I, Yu L, Zhong C, Wu X et al. **PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment**. Cancer Cell. 2009;15:21–34.

[445] Tamamura H, Hori A, Kanzaki N, Hiramatsu K, Mizumoto M et al. **T140 analogs as CXCR4 antagonists identified as anti-metastatic agents in the treatment of breast cancer.** FEBS Lett. 2003;550:79–83.

[446] Smith MC, Luker KE, Garbow JR, Prior JL, Jackson E et al. **CXCR4 regulates growth of both primary and metastatic breast cancer.** Cancer Res. 2004;64:8604–8612.

[447] Kim SY, Lee CH, Midura BV, Yeung C, Mendoza A et al. **Inhibition of the CXCR4/CXCL12 chemokine pathway reduces the development of murine pulmonary metastases.** Clin Exp Metastasis. 2008;25:201–211.

[448] Groen HJ, Sietsma H, Vincent A, Hochstenbag MM, van Putten JW et al. **Randomized, placebo-controlled phase III study of docetaxel plus carboplatin with celecoxib and cyclooxygenase-2 expression as a biomarker for patients with advanced non-small-cell lung cancer**: the NVALT-4 study. J Clin Oncol. 2011;29:4320–4326.

[449] Barcellos-Hoff MH, Park C, Wright EG. **Radiation and the microenvironment tumorigenesis and therapy.** Nat Rev Cancer 2005;5: 867-75.

[450] Madani I, De Neve W, Mareel M. **Does ionizing radiation stimulate cancer invasion and metastasis?** Bull Cancer 2008;95: 292-300.

[451] Orimo A, Weinberg RA. **Stromal fibroblasts in cancer: a novel tumor-promoting cell type.** Cell Cycle. 2006 Aug;5(15):1597-601.

[452] Bhowmick NA, Neilson EG, Moses HL. **Stromal fibroblasts in cancer initiation and progression**. Nature. 2004 Nov 18;432(7015):332-7.

[453] Littlepage LE, Egeblad M, Werb Z. **Coevolution of cancer and stromal cellular responses.** Cancer Cell. 2005 Jun;7(6):499-500

[454] Liu AX, Rane N, Liu JP, Prendergast GC . **RhoB is dispensable for mouse development, but it modifies susceptibility to tumor formation as well as cell adhesion and growth factor signaling in transformed cells.** Mol Cell Biol. 2001 Oct;21(20):6906-12.

[455] Liu A-X, Du W, Liu J-P, Jessell T M, Prendergast G C. **RhoB alteration is necessary for the apoptotic and antineoplastic responses to farnesyltransferase inhibitors**. Mol Cell Biol. 2000 Aug;20(16):6105-13

[456] Hu, X., Beeton, C.**Detection of Functional Matrix Metalloproteinases by Zymography.** J Vis Exp. 2010 Nov 8;(45). pii: 2445. doi: 10.3791/2445.

[457] Mordant P, Loriot Y, Lahon B, Castier Y, Lese`che G, et al. **Bioluminescent Orthotopic Mouse Models of Human Localized Non-Small Cell Lung Cancer: Feasibility and Identification of Circulating Tumour Cells**. PLoS One. 2011;6(10):e26073.

[458] Ohuchida K, Mizumoto K, Murakami M, Qian LW, Sato N et al. **Radiation to stromal fibroblasts increases invasiveness of pancreatic cancer cells through tumor stromal interactions.** Cancer Res.2004; 64:3215-3222

[459] Hwang RF, Moore T, Arumugam T, Ramachandran V, Amos KD et al. **Cancer-associated stromal fibroblasts promote pancreatic tumor progression.** Cancer Res.2008;68 (3).918-926

[460] Verrecchia, F., Chu, M. L., Mauviel, A.**Identification of novel TGF-beta/Smad gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach.** J. Biol. Chem.2001; 276,17058-17062

[461] Barcellos-Hoff MH, Ewan KB. **Transforming growth factor-beta and breast cancer: Mammary gland development.** Breast Cancer Res.2000; :2(2):92-9. Epub 2000 Feb 21.

[462] De Wever O, Mareel M.Role of tissue stroma in cancer cell invasion. J. Pathol.2003;200, 429–447

[463] Principe DR, Doll JA, Bauer J, Jung B, Munshi HG.**TGF-β: Duality of Function Between Tumor Prevention and Carcinogenesis.** J Natl Cancer Inst.2014; Feb 1;106(2):djt369. doi: 10.1093/jnci/djt369.

[464] Johnsen M, Lund LR, Rømer J, Almholt K, Danø K. **Cancer invasion and tissue remodeling: common themes in proteolytic matrix degradation.** Curr Opin Cell Biol.1998 Oct;10(5):667-71.

[465] Ahn GO, Brown JM. **Matrix metalloproteinase-9 is required for tumor vasculogenesis but not for angiogenesis: role of bone marrow-derived myelomonocytic cells.** Cancer Cell.2008 Mar;13(3):193-205.

[466] Farhat FS, Tfayli A, Fakhruddin N, Mahfouz R, Otrock ZK, Alameddine RS, et al. **Expression, prognostic and predictive impact of VEGF and bFGF in non-small cell lung cancer.**Crit Rev Oncol Hematol. 2012 Nov;84(2):149-60.

[467] A. Dowlati, R. Gray, A.B. Sandler, J.H. Schiller, D.H. Johnson. **Cell adhesion molecules, vascular endothelial growth factor, and basic fibroblast growth factor in patients with non-small cell lung cancer treated with chemotherapy with or without bevacizumab—an Eastern Cooperative Oncology Group Study**. Clin Cancer Res. 2008 Mar 1;14(5):1407-12.

[468] C. Garbers, H.M. Hermanns, F. Schaper, G. Muller-Newen, J. Grotzinger, et al. **Plasticity and cross-talk of interleukin 6-type cytokines.** Cytokine Growth Factor Rev. 2012 Jun;23(3):85-97.

[469] Y. Guo, F. Xu, T. Lu, Z. Duan, Z. Zhang (2012) Interleukin-6 signaling pathway in targeted therapy for cancer .Cancer Treat Rev.2012 Nov;38(7):904-10.

[470] Milas L, Hirata H, Hunter N, Peters LJ. **Effect of radiation-induced injury of tumor bed stroma on metastatic spread of murine sarcomas and carcinomas**. Cancer Res 1988 ; 48 : 2116-20.

[471] Milas L, Hunter N, Peters LJ. **The tumor bed effect: dependence of tumor take, growth rate, and metastasis on the time interval between irradiation and tumor cell transplantation**. Int J Radiat Oncol Biol Phys 1987 ; 13 : 379-83.

[472] Milas L, Hirata H, Hunter N, Peters LJ. **Effect of radiation-induced injury of tumor bed stroma on metastatic spread of murine sarcomas and carcinomas**.Cancer Res 1988;48:2116–20.

[473] Woodward WA, Truong PT, Yu TK, et al. **Among women who experience a recurrence after postmastectomy radiation therapy irradiation is not associated with more aggressive local recurrence or reduced survival.**Breast Cancer Res Treat. 2010 Sep;123(2):597-605.

[474] Rofstad EK. **Microenvironment-induced cancer metastasis.** Int J Radiat Biol 2000 ; 76 : 589-605.

[475] Rofstad EK, Mathiesen B, Henriksen K, Kindem K, Galappathi K. **The tumor bed effect: increased metastatic dissemination from hypoxia-induced up-regulation of metastasis-promoting gene products**.Cancer Res 2005 ; 65 : 2387-96.

[476] Kozin SV, Winkler F, Garkavtsev I, Hicklin DJ, Jain RK, Boucher Y. **Human tumor xenografts recurring after radiotherapy are more sensitive to anti-vascular endothelial growth factor receptor-2 treatment than treatment-naive tumors**. Cancer Res 2007 ; 67 : 5076-82.

[477] Tinhofer I, Hristozova T, Stromberger C, Keilhoiz U, Budach V.**Monitoring of Circulating Tumor Cells and Their Expression of EGFR/Phospho-EGFR During Combined Radiotherapy Regimens in Locally Advanced Squamous Cell Carcinoma of the Head and Neck** .Int J Radiat Oncol Biol Phys. 2012 Aug 1;83(5):e685-90.

[478] Camphausen K, Moses MA, Beecken WD, et al. **Radiation therapy to a primary tumor accelerates metastatic growth in mice.** Cancer Res 2001;61:2207-2211.

[479] Biswas S, Guix M, Rinehart C, et al. **Inhibition of TGF-beta with neutralizing antibodies prevents radiation-induced acceleration of metastatic cancer progression.** J Clin Invest 2007;117:1305-1313.

[480] Martin OA, Anderson RL, Russell PA, Cox RA, Ivashkevich A et al. **Mobilization of viable tumor cells into the circulation during radiation therapy**. Int J Radiat Oncol Biol Phys. 2014 Feb 1;88(2):395-403.

[481] Yu M, Bardia A, Wittner BS, et al. **Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition**.Science 2013;339:580-584.

[482] Park JK, Jang SJ, Kang SW, et al. **Establishment of animal model for the analysis of cancer cell metastasis during radiotherapy.** Radiat Oncol 2012;7:153.

[483] Hou JM, Krebs MG, Lancashire L, et al. **Clinical significance and molecular characteristics of circulating tumor cells and circulating tumor microemboli in patients with small-cell lung cancer.** J Clin Oncol 2012;30:525-532.

[484] Rolle A, Günzel R, Pachmann U, Willen B, Höffken K, Pachmann K.**Increase in number of circulating disseminated epithelial cells after surgery for non-small cell lung cancer monitored by MAINTRAC® is a predictor for relapse: a preliminary report**. World J Surg Oncol 200;5:18.

[485] Aylwin JA. **Avoidable vascular spread in resection for bronchogenic carcinoma.** Thorax 1951;6:250–67.

[486] Cho Y, Hida Y, Kaga K, Kato H, Iizuka M, Kondo S. **Brain metastases secondary to tumor emboli from primary lung cancer during lobectomy.** Ann Thorac Surg 2008;86:312–3.

[487] Hashimoto M, Tanaka F, Yoneda K, Takuwa T, Matsumoto S et al.**Significant increase in circulating tumour cells in pulmonary venous blood during surgical manipulation in patients with primary lung cancer**. Interact Cardiovasc Thorac Surg. 2014 Mar 11. [Epub ahead of print]

[488] Rofstad EK, Mathiesen B, Galappathi K. **Increased metastatic dissemination in human melanoma xenografts after subcurative radiation treatment: Radiation-induced increase in fraction of hypoxic cells and hypoxia-induced up-regulation of urokinase-type plasminogen activator receptor.** Cancer Res 2004;64:13-18.

[489] Kaplan HS, Murphy ED. **The effect of local roentgen irradiation on the biological behavior of a transplantable mouse carcinoma; increased frequency of pulmonary metastasis.** J Natl Cancer Inst 1949;9:407-413.

[490] Sheldon PW, Fowler JF. **The effect of low-dose pre-operative x-irradiation of implanted mouse mammary carcinomas on local recurrence and metastasis.** Br J Cancer 1976;34:401-407.

[491] Das, S. & Skobe, M. **Lymphatic vessel activation in cancer**. Ann N Y Acad Sci. 2008;1131:235-41.

[492] Klein CA, Blankenstein TJ, Schmidt-Kittler O, Petronio M, Polzer B et al. **Genetic heterogeneity of single disseminated tumour cells in minimal residual cancer.** Lancet. 2002 Aug 31;360(9334):683-9.

[493] Schmidt-Kittler O, Ragg T, Daskalakis A, Granzow M, Ahr A, et al. **From latent disseminated cells to overt metastasis: genetic analysis of systemic breast cancer progression.** Proc Natl Acad Sci U S A. 2003 Jun 24;100(13):7737-42.

[494] Miles FL, Pruitt FL, van Golen KL, Cooper CR. **Stepping out of the flow: capillary extravasation in cancer metastasis.** Clin Exp Metastasis. 2008;25(4):305-24.

[495] Al-Mehdi AB, Tozawa K, Fisher AB, Shientag L, Lee A, Muschel RJ. **Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: a new model for metastasis.** Nat Med. 2000 Jan;6(1):100-2.

[496] Luis-Ravelo D. **RHOB influences lung adenocarcinoma metastasis and resistance in a host-sensitive manner** .Mol Oncol. 2013 Nov 12. pii: S1574-7891(13)00159-2.

[497] Nagaraju GP, Nalla AK, Gupta R, Mohanam S, Gujrati M,et al. **siRNA-mediated downregulation of MMP-9 and uPAR in combination with radiation induces G2/M cell cycle arrest in medulloblastoma.** Mol Cancer Res. 2011 Jan;9(1):51-66.

[498] Eleftheria Vasilaki et al.**Transcriptional regulation of the small GTPase RhoB gene by TGFβ-induced signaling pathways** .FASEB J March 2010 24:891-905;

[499] Brown JM, Koong AC. **High-dose single-fraction radiotherapy: exploiting a new biology?** Int J Radiat Oncol Biol Phys2008 ; 71 : 324-5.

[500] Kerbel RS. **Inhibition of tumor angiogenesis as a strategy to circumvent acquired resistance to anti-cancer therapeutic agents**. Bioessays 1991, 13:31-36.

[501] Loeffler M, Kruger JA, Niethammer AG, Reisfeld RA. **Targeting tumor-associated fibroblasts improves cancer chemotherapy by increasing intratumoral drug uptake.** J Clin Invest 2006,116:1955-1962.

[502] Matsunaga T, Takemoto N, Sato T, Takimoto R, Tanaka I et al. **Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia.** Nat Med 2003, 9:1158-1165.

[503] Kim DW, Huamani J, Fu A, Hallahan DE. **Molecular strategies targeting the host component of cancer to enhance tumor response to radiation therapy.** Int J Radiat Oncol Biol Phys 2006,64:38-46.

[504] Shain KH, Yarde DN, Meads MB, Huang M, Jove R et al. **Beta1 integrin adhesion enhances IL-6-mediated STAT3 signaling in myeloma cells: implications for microenvironment influence on tumor survival and proliferation**. Cancer Res 2009, 69:1009-1015.

[505] Meads MB, Gatenby RA, Dalton WS: **Environment-mediated drug resistance: a major contributor to minimal residual disease**. Nat Rev Cancer 2009, 9:665-674.

[506] El-Nikhely N, Larzabal L, Seeger W, Calvo A, Savai R. **Tumor-- stromal interactions in lung cancer: novel candidate targets for therapeutic intervention.** Expert Opin Investig Drugs. 2012 Aug;21(8):1107-22. doi: 10.1517/13543784.2012.693478

[507] Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ et al. **Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease**.Nature. 1992 Oct 22;359(6397):693-9.

[508] Kulkarni AB1, Huh CG, Becker D, Geiser A, Lyght M,et al. **Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death**. Proc Natl Acad Sci U S A. 1993 Jan 15;90(2):770-4.

[509] Chytil A, Magnuson MA, Wright CV, Moses HL. **Conditional inactivation of the TGF-beta type II receptor using Cre:Lox**. Genesis. 2002 Feb;32(2):73-5.

VII.ANNEX-Articles

Article 1: Co-irradiation of fibroblasts and carcinoma cells repress carcinoma cell migration.

This is an original article issued from my PhD Thesis work .This has been submitted to plosone and under review. We have assessed the crosstalk between stromal and carcinoma cells in response to radiotherapy by genetic modulation of the stroma and irradiation. Our results showed that fibroblasts irrespectively from their RhoB status do not modulate intrinsic radiosensitivity of TC-1 .Then we found that Wt and RhoB deficient fibroblasts stimulated TC-1 migration through distinct mechanisms respectively TGF-β1 and MMP-mediated. Lastly, we found that co-irradiation of fibroblasts and TC-1 abrogated the pro-migratory phenotype by repression of TGF-β and MMP secretion. This last result is highly relevant to the clinical situation and suggests that conversely to, the current view; irradiated stroma would not enhance carcinoma migration and could be manipulated to promote anti-tumor immune response.

Article 2: Ultra-high dose-rate FLASH radiotherapy allows dose escalation that cures leukemia without hematological toxicity

I have been actively involved in mice sampling and analyzing of data for this project. This article has been submitted and under review.

In this article we have investigated an innovation in radiotherapy that is not based on the use of pharmacological targeting of pathways and that increases leukemic cells eradication while decreasing hematopoietic toxicity. This novel modality of radiotherapy is based on high dose delivery at Ultra-High Dose-Rate (> 50 Gys Dose-Rate (0.04 Gy.s-1-1, FLASH) thereby contrasting with conventional (CDR) that can delivers high doses within minutes. To assess the efficacy of high dose-rates on living animals and on cancer, we performed total body irradiation (TBI) using a linear electron accelerator of 4.5 MeV (LINAC) capable of delivering high doses of irradiation with a wide range of dose-rates. We studied the differential effects of high doses of radiation delivered using CDR or FLASH on normal hematopoiesis and leukemia and showed that dose-rate is as important as doses in the beneficial or deleterious effects of radiotherapy.

Materials and Methods: Four wild-type strains of mice, C57BL/6J, 129/Sv, C3H and Balb/c, known to have various radiation sensitivities. Linear electron accelerator that can deliver high doses of radiation at conventional dose-rate (0.04 Gy.s-1) or in short pulses at high dose-rate (50 Gy.s -1). For the determination of chimerism in transplantation experiments, BM cells were stained with FITC- or Alexa Fluor®700-conjugated anti-CD45.2 antibody (clone104, BD Biosciences or eBioscience) and with PE-, PercpCy5.5- or APC-conjugated anti-CD45.1 antibodies (A20, eBioscience).

One humerus of each mouse was removed and immediately fixed in FineFIX (formalin-free fixative), then decalcified and embedded in paraffin FOR histology. For cell sorting, the hematopoietic stem/progenitor cell population (HSCP, Linor LSK cells (Linneg, Sca1+, c-Kit+neg, c-Kit+, Sca1) was used alongwith clonogenic cell survival assays and LTC-IC assays. Karyotyping and cytogenetic analysis was performed by means of the ISIS software (MetaSystems). At least, 40 metaphase spreads were analyzed for each experimental point. Murine leukemic cells that express the human MLL-ENL fusion protein were transduced with a lentivirus expressing GFP (MND-GFP lentivirus) and amplified in vivo in syngeneic 129/Sv mice.

Results: We observed that mice can survival increased with high doses of irradiation when delivered with FLASH irradiation, FLASH irradiation prevents lethal aplasia after high doses TBI. Also, mice developed normal hematopoiesis with functional HSCs 6 months after high doses TBI using FLASH irradiation. Most importantly, high doses TBI using FLASH irradiation can impair leukemic cell expansion in vivo

Discussion: In the present report, we show for the first time that total body irradiation with very high doses of ionizing radiation at high dose-rate allowed long-term survival of irradiated mice without evidence of any disease. This new modality of irradiation protected mice from acute hematological syndrome and enabled high dose escalation able to eradicate leukemic cells. The early and fast brought out of aplasia following FLASH irradiation was associated with an efficient HSC repopulation and required a physiological microenvironment.

In this study, together with the accompanying paper on lung, proposes an innovation in radiotherapy using FLASH. The FLASH enhancement of dose-rate (by >800 fold) has led to completely unexpected biological effects as it destroys solid tumors or leukemia without having any apparent adverse effects on normal tissues as shown for lung and hematopoiesis. These results demonstrate that the duration of radiation is at least as critical to normal tissue response than the dose of radiation, thus challenging the current views about the effects of high doses of radiation on living animals. Three research challenges emerge from these two studies. A physicist's challenge: fundamental physics experiments need to be done to incorporate the notion of duration of dose deposition at such high dose-rates. A biologist's challenge: biological investigations should explain why even after 20 Gy total body irradiation, FLASH radiotherapy has no toxic effect on one of the most radiosensitive tissue and why irradiated animals exhibit long-term survival without any evidence of disease. A physician's challenge: our results indicate an important new approach in radiotherapy that can be easily translated into clinics for non-invasive, reduced-toxicity and cost-effective cancer treatment.

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Co-irradiation of fibroblasts and carcinoma cells repress carcinoma cell migration.

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Abstract:	Stroma mediated wound healing signals may share similarities with the ones produced by tumor's microenvironment and their modulation may impact tumor response to the various anti-cancer treatment including radiation therapy. Therefore we conducted this study, to assess the crosstalk between stromal and carcinoma cells in response to radiotherapy by genetic modulation of the stroma and irradiation. We found that fibroblasts irrespectively from their RhoB status do not modulate intrinsic radiosensitivity of TC-1 but produce diffusible factors able to modify tumor cell fate. Then we found that Wt and RhoB deficient fibroblasts stimulated TC-1 migration through distinct mechanisms respectively TGF-β1 and MMP-mediated. Lastly, we found that co-irradiation of fibroblasts and TC-1 abrogated the pro-migratory phenotype by repression of TGF-β and MMP secretion. This last result is highly relevant to the clinical situation and suggests that conversely to, the current view; irradiated stroma would not enhance carcinoma migration and could be manipulated to promote anti-tumor immune response.
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recommendations of the Weatherall report, "The use of non-human primates in research." The relevant guidelines followed and the committee that approved the study should be identified in the ethics statement. If anesthesia, euthanasia or any kind of animal sacrifice is part of the study, please include briefly in your statement which substances and/or methods were applied. Please enter the name of your Institutional Animal Care and Use Committee (IACUC) or other relevant ethics board, and indicate whether they approved this research or granted a formal waiver of ethical approval. Also include an approval number if one was obtained. **Field Permit** Please indicate the name of the institution or the relevant body that granted permission.	

Co-irradiation of fibroblasts and carcinoma cells repress carcinoma cell migration.

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ABSTRACT (word count: 155)

Stroma mediated wound healing signals may share similarities with the ones produced by tumor's microenvironment and their modulation may impact tumor response to the various anti-cancer treatment including radiation therapy. Therefore we conducted this study, to assess the crosstalk between stromal and carcinoma cells in response to radiotherapy by genetic modulation of the stroma and irradiation. We found that fibroblasts irrespectively from their RhoB status do not modulate intrinsic radiosensitivity of TC-1 but produce diffusible factors able to modify tumor cell fate. Then we found that Wt and RhoB deficient fibroblasts stimulated TC-1 migration through distinct mechanisms respectively TGF-β1 and MMP-mediated. Lastly, we found that co-irradiation of fibroblasts and TC-1 abrogated the pro-migratory phenotype by repression of TGF-β and MMP secretion. This last result is highly relevant to the clinical situation and suggests that conversely to, the current view; irradiated stroma would not enhance carcinoma migration and could be manipulated to promote anti-tumor immune response.

Key words: Wound healing, NSCLC (Non Small Cell Lung Carcinoma), Rho, TGF-β 1, MMP, Microenvironment,

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None.

Author contributions:

AA; MCV: Conceived and designed the experiments:. AA: Performed the experiments. AA; MCV: Analysed the data. MCV, ED: Contributed reagents/materials/analysis tools. AA; MCV: Wrote the paper: ED: Made critical revision of manuscript.

INTRODUCTION

Wound healing and carcinogenesis are defined as complex, adaptive processes which are controlled by intricate communications between the host and the tissue microenvironment. During a normal wound healing process, regeneration and repair of a wound, depends on a variety of signals which coordinate the response to injury. These processes entail cell proliferation, survival, and migration which are controlled by growth factors, cytokines as well as inflammatory and angiogenic signals. These signals are derived from multiple intra and extracellular components embedded in the microenvironment of wounds and are also involved in cancer. Therefore, a number of phenotypic similarities are shared by wounds and cancers in cellular signaling and gene expression. These similarities between wound healing and carcinogenesis were first recognized by Haddow, and the notion that 'cancer are wounds that do not heal' was defined by Dvorak [1, 2].

Radiotherapy is the second most effective modality of cancer treatment after surgery and can be used, either alone or in combination with chemotherapy. The main anti-tumor effect of radiation therapy is the induction of tumor cell death but recent findings suggest that radiotherapy also rapidly and persistently modifies the tissue microenvironment. These modifications affect cell phenotype, tissue metabolism, bidirectional exchanges and signaling events between cells [3]. While there is evidence indicating that these changes might contribute to the antitumor effects of radiotherapy, some clinical and experimental observations indicates that irradiated stroma might exert tumor-promoting effects [3]. MH. Barcellos-Hoff`s Group has indeed shown a major contribution of TGF-β1 produced by irradiated stroma to carcinogenesis [4-6] and high dose of radiotherapy are known to stimulate TGFβ1 production [7]. TGF-β1 is the prototype of pro-wounding molecules shown to be the main inducer of reactive stroma, by not only affecting chemotaxis of fibroblasts, but also their trans-differentiation into reactive fibroblasts, termed myofibroblasts [8]. TGF-β1 also regulates epithelial phenotype and has been especially described as a potent stimulatory molecule during the late phase of carcinogenesis and metastasis dissemination.

Beside TGF-β1 signal, the contribution of the Rho pathway to radiation response has been proposed by our group and others [9]. Rho GTPases are a family of signaling mediators implicated in regulating cytoskeletal dynamics, motility, cell division, and transcriptional regulation. More specifically, RhoB expression is increased by a variety of extra-cellular stimuli which include irradiation, epidermal growth factor (EGF) and transforming growth factor β (TGF-β) [7, 10]. Most Rho proteins are modified by the covalent attachment of a geranylgeranyl group, but RhoB can exist in either a geranylgeranylated (RhoB-GG) or a farnesylated (RhoB-F) form. RhoB-F localizes to the cell membrane, modulates actin cytoskeleton, activates nuclear factor kappa B and promotes cell growth [11–13]. In contrast, RhoB-GG localizes to endosomes and induces cell apoptosis [11]. A role for RhoB in TGF-β induced cell responses (such as epithelial-mesenchymal transition (EMT) and apoptosis) was suggested by a series of DNA microarray studies, which showed that RhoB expression was upregulated by TGF-β in a variety of cell types such as keratinocytes, mouse mammary gland epithelial cells, hepatoma cells, and dermal

fibroblasts [14]. TGF-β also stimulates actin stress fiber formation in Ras-transformed cells in a way which is associated with upregulation of RhoB [15, 16]

In the present study, our hypothesis was that scarring signals including TGF-β and RhoB that are activated by irradiation in the stroma could enhance tumor aggressiveness after radiation therapy. Therefore, RhoB deficiency would indirectly enhance anti-tumor effect of radiation therapy. To investigate this crosstalk, we used *in vitro* a co-culture model consisting of lung carcinoma cells and lung primary fibroblasts. We also used RhoB deficient fibroblasts to assess our hypothesis. Interestingly we found that the intrinsic radiosensitivity of carcinoma cells was not modulated by paracrine factors secreted either by Wt or RhoB-/- fibroblasts. Irradiation of fibroblasts stimulated migration of carcinoma cells but co-irradiation of carcinoma and fibroblasts repressed the secretion of these pro-invasive signals by fibroblasts.

MATERIALS AND METHODS

1- Cells

C57BL6 (Wt) and RhoB-/- mice [16,17] were used to isolate primary Lung Fibroblasts by enzymatic digestion (collagenase/trypsine) and cells were subcultured in DMEM +Glutamine with 20% foetal calf serum , 50U penicillin /streptomycin ,1% Hepes,10mg EGF, ITS. RhoB deficiency was controlled and monitored by genotyping and western-blot. C57BL6 mice were purchased from Charles River laboratories, RhoB mice were obtained from Pr Prendergast GC laboratory. Mice were maintained in the animal care facilities of Institut Gustave-Roussy (agreement No. D94076-11). Authorization for experiments was obtained from the Comité d'Ethique en Expérimentation Animale Paris-11.

TC-1 cells (Murine adenocarcinoma lung) were grown in RPMI 1640 medium with 10% fetal bovine serum or conditioned medium isolated from fibroblasts culture.

2- Chemicals

The Inhibitor of TGF-β type I receptor (ALK5, ALK4 and ALK7), SB 431542 was used at 10 µM and the MMP Inhibitor, O-Phenanthroline at 100 µM. Both were bought from Santa Cruz.

3- Irradiation

Wt and RhoB-/- Fibroblasts were grown to 80 % confluence, culture medium was replaced with FCS-free medium before irradiation. Fibroblasts were irradiated with 2 and 10 Gy with Cs137 (IBL-637 (CIS-BioInternational, France) gamma irradiator (dose rate 1Gy/Min). 24 Hours later conditioned medium (CM) from Wt and RhoB-/- cells was collected and added to TC-1 cells and left for 24hrs more. Supernatants and cells were prepared for Western Blot analysis. The same is done as vice versa with TC-1 cells irradiated.

4- Clonogenic Assay

TC-1 cells are plated in 6 well plates at different concentrations in duplicate. Irradiation is done at 2 to 10Gy for TC-1 cells, 4hrs after seeding and cultured with conditioned medium from irradiated or non-irradiated Wt and RhoB-/- Fibroblasts or non-conditioned fresh culture media.

5- Wound Healing Assay

Wild type (Wt), RhoB-/- Fibroblasts and TC-1 cells were grown to confluence in 6-well culture plates. Cells were irradiated at 2 and 10Gy. 24 hours later cell layers were wounded using a sterile 200 µl pipette tip and dead cells are washed out, then conditioned medium was added. Images are captured at 0hr with Nikon Phase contrast, Japan at 10X and plates were returned to the incubator to recover from wounding. After 24 Hrs culture plates were removed and monolayers were photographed again. Wound width was measured on hard copy prints of the images.

6- Electrophoresis and Western-Blotting

Cell lysate are prepared with RIPA lysis buffer (Sigma Biotech). Protein concentration was measured using a BioRad protein assay and proteins were submitted to electrophoresis. Primary antibodies were diluted in TBS-T solution (Vimentin (E-5) dil 1:1000 from Santa Cruz Biotechnology , Snail (C15D3) 1:1000, TGF β1(V) 1:250 all from Cell Signaling , α SMA 1:500 from Abcam and GAPDH 1:5000 from Millipore overnight at 4° C, washed next day in TBS-T and incubated with the horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody. The blots are developed using SuperSignal® West Pico Chemiluminiscent Substrate (Pierce Biotechnology, IL) according to the manufacturer's instructions. The chemiluminiscence signal from the membranes was detected and evaluated using G-box iChemi XT4 digital imaging device (Syngene Europe, Cambridge). Quantification was performed using Image J software. Relative proteins expression are normalized to the respective value for GAPDH.

7- Cytokine Array Analysis

Conditioned medium (undiluted) was probed for secreted cytokine profiling using the RayBio® Mouse Cytokine Antibody Array 3 and 4 kits according to the manufacturer's instructions (RayBiotech®; Norcross, GA, USA). Quantification was done by Genetools from Syngene. Data were imported into an Excel spreadsheet, normalized against a control across membranes and final values were calculated

8- Zymography

Zymography was performed on CM using Novex Zymogram Gels (Invitrogen) following the manufacturer's protocol as described previously [18].

9- Statistical Analysis

Statistical analysis was performed using Graphpad prism 5. Statistical analysis were expressed as mean ± SEM and analyzed using the ANOVA and the Student Newman Keul's test with a p value < 0.05 was considered significant.

RESULTS

1. Paracrine Signals Secreted By Fibroblasts do not modulate TC-1 Radiosensitivity

To test whether co-culture would modify the effect of Ionizing radiation on TC-1 cells survival, we produced conditioned medium from Wt and RhoB -/- fibroblasts and used them to culture TC-1 in clonogenic survival assay. Fig 1A showed that media conditioned by fibroblasts do not modify TC-1 intrinsic radiation sensitivity assessed by clonogenic assays, suggesting that signals triggered by irradiation are stronger than survival signals triggered by fibroblasts.

Interestingly, CM isolated from irradiated Wt and RhoB-/- fibroblasts do alter clonogenicity and survival of non-irradiated TC-1, suggesting that paracrine signals are produced by irradiated fibroblasts and alter TC-1 clonogenic potential (Fig 1B).

2. Paracrine Signals secreted by fibroblasts enhance TC-1 migration

We assessed whether irradiation modulated TC-1 cell motility and showed that TC-1 migration reduced with increasing dose of irradiation. Then, we showed that conditioned medium (CM) produced by non-irradiated Wt fibroblasts had no effect on TC-1 cell migration, whereas CM produced by 10 Gy irradiated fibroblasts significantly stimulated TC-1 cells migratory capability. Interestingly when both Wt and TC-1 cells are 10Gy-co-irradiated and co-cultured, TC-1 cell migration returned to normal level.

Alongside we showed that CM produced by non-irradiated RhoB-/- fibroblasts increased TC-1 cell migration more significantly than Wt fibroblasts, suggesting that RhoB deficiency promote the production of pro-migratory signals. This stimulation is further enhanced by 10 Gy-irradiated RhoB-/- fibroblasts but repressed back to the control level when RhoB -/- fibroblasts and TC-1 are co-irradiated at 10 Gy (Fig 2A,B).

3. TC-1 migration is mediated by MMP.

To search for mediators of TC-1 cell invasiveness and migration, we investigated the proteome of TC-1 cultured alone or with CM produced by Wt and RhoB-/- fibroblasts irradiated or not. While most tested proteins (96) were not affected, production of IL-6, bFGF, CXCL 16, sTNF RI, MMP 2, MMP 3, Pro MMP 9 were significantly stimulated by the co-culture process (Fig 3A). Interestingly, CM from RhoB deficient fibroblasts modulated more proteins secreted by TC-1 than CM from Wt, with a marked increase in Pro MMP-9, MMP-3 and MMP-2.

We confirmed MMP induction by zymogram analysis. Our results show a weak secretion of MMP 2 and MMP 9 when TC-1 are cultured alone, slightly enhanced when TC-1 are irradiated (10 Gy). CM of Wt fibroblasts irradiated or not does not change the level and activity of MMP2/9 produced by TC-1, whereas CM from RhoB-/- Fibroblasts irradiated or not significantly enhance level and activity of proMMP2, MMP2 and MMP9 (Fig 3B). This

suggests that RhoB deficient fibroblasts secrete paracrine factors, that remain to be identified, but that are able to enhance MMPs secretion in TC-1. As MMP are known to degrade the extracellular matrix, we postulated that they could be involved in the enhanced migration of TC-1 and confirmed this hypothesis using the MMP inhibitor O-phenanthroline. Our results show that O-phenanthroline inhibited TC-1 migration cultured with CM medium produced by RhoB -/- fibroblasts irradiated or not (Fig 3C). Note that in RhoB deficient fibroblasts, the secretion of MMP2 and 9 was not modulated by irradiation nor by co-culture (Fig S 1), supporting the fact MMP induction was TC-1 mediated.

4. Wt but not RhoB -/- Fibroblasts enhance TC-1 invasiveness by secretion of TGF β1

Then we investigated the stimulatory the pro-invasive mediators produced by Wt and RhoB-/- fibroblasts upon irradiation and found that TGF-β1 production was indeed stimulated by irradiation in a dose dependent manner in Wt fibroblast but not in RhoB-/- fibroblasts. When Wt Fibroblasts were cultured with CM collected from TC-1, TGF-β1 production by Wt fibroblasts was further enhanced, whereas co-culture and co-irradiation of Wt at 10 Gy repressed TGF-β induction (Fig 4 A). We further confirmed the role of TGF-β1 in accelerated wound closure of TC-1 cells by utilizing SB-431542, a TGF-β inhibitor. We noticed that SB-431542 inhibited fibroblasts induced TC-1 cell motility and delayed the wound closure (Fig 4 B).

5. TGF-β1 production induces EMT markers in TC-1

Then, we investigated whether the variation of migratory potential of TC-1 cells was associated with altered phenotype and induction of two EMT makers, Vimentin and Snail, in carcinoma cells. Fig 5 showed that ionizing radiation does not promote Vimentin and Snail expression in TC-1 nor does the co-culture with Wt fibroblasts supporting the Scratch assay's results (Fig 2). However, CM produced by 10 Gy irradiated fibroblasts stimulated both Vimentin and Snail protein expression in TC-1 suggesting induction of an EMT phenotype by paracrine factors secreted by irradiated fibroblasts. Surprisingly, co-irradiation of Wt fibroblast and TC-1 further enhanced Vimentin expression but has no effect on Snail, suggesting that the decreased migration observed by scratch assay was not associated with alteration of the EMT phenotype.

CM from RhoB-/- fibroblasts do not induce Vimentin nor Snail protein expression in TC-1 suggesting that the enhanced migration observed by Scratch assay when both cell type are co-cultured is mediated by another mechanisms. However when irradiated, RhoB-/- fibroblasts stimulate Vimentin and Snail in TC-1. As for Wt fibroblast, co-irradiation of RhoB-/- fibroblast and TC-1 further enhance Vimentin expression but has no effect on Snail (Fig 5). These results suggest that various and independent paracrine factor are produced by irradiation of fibroblasts and independently modulate migration and EMT phenotype.

DISCUSSION

In our present study, we investigated the crosstalk between stromal and carcinoma cells after irradiation and postulated that fibroblasts would promote TC-1 tumor migration after irradiation whereas deficiency in RhoB a protein described to be profibrogenic would prevent it. Our data are different from our initial hypothesis as RhoB deficient fibroblasts enhanced TC-1 migration more potently than Wt fibroblasts do. RhoB deficient fibroblasts stimulated MMP secretion in TC-1 cells thus favoring their migration, whereas Wt fibroblasts stimulated TC-1 migration via TGF-β1 and induction of EMT in TC-1 cells (Fig 6). We also found that TC-1 intrinsic radiation sensitivity was not altered by conditioned-medium (CM) produced by Wt and RhoB deficient fibroblasts but that clonogenic potential of TC-1 was impaired by diffusible factors secreted by irradiated fibroblasts. Lastly, we found that co-irradiation of tumor cells and fibroblasts inhibited both TGF-β1 and MMP induction and repressed TC-1 cells migration.

Our results show that intrinsic radiosensitivity of carcinoma cells TC-1 is not altered by paracrine mediators secreted by non-irradiated stroma but show that the secretion of soluble factors by irradiated stromal cells promotes TC-1 migration and potential metastatic escape. This result is consistent with the literature [19, 20] and as expected we found that TGF-β1 was a stimulatory factors produced by irradiated Wt fibroblast. MH. Barcellos-Hoff's Group has demonstrated a major contribution for TGF-β1 produced by irradiated stroma to carcinogenesis [21]. TGF-β1 is also known to be the main inducer of reactive stroma and promote chemotaxis of fibroblasts and their transdifferentiation into myofibroblasts [22] by turning on expression of alpha-smooth muscle actin (αSMA). The presence of myofibroblasts-like cells, called CAF, in the tumor stroma may provide a source of growth factors, but could also be involved in connective tissue remodeling allowing expansion and invasion of tumors [23]. In addition, TGF-β1 immunosuppressive function may promote tumor growth and expansion by induction of immune anergy in tumor's stroma [24].

The use of primary lung fibroblasts isolated from RhoB deficient transgenic mice allowed us to identify activation of 3 major proteins in the proteome TC-1 tumor cells cultured with conditioned media produced by these fibroblasts: MMP, bFGF and IL-6. Production of MMP3, 9 and bFGF were specifically found to be stimulated in tumor cells when co-cultured with RhoB -/- fibroblasts but repressed when fibroblasts were irradiated. As suggested by the literature [25], we postulated that this increased expression of MMPs was associated with tumor invasion and indeed MMP inhibition in co-culture conditions repressed TC-1 migration as assessed in wound closure assay. Beside modulation of the migratory properties of cancer cells, RhoB -/- fibroblasts seemed to modulate both vascular remodeling and immune infiltration signals in tumor cells. Both MMP9 and bFGF are

indeed involved in the regulation of vascular function after high doses of irradiation: MMP9 regulates vasculogenesis and recruitment of myeloid cells [26] whereas bFGF is a major pro-angiogenic factor [27, 28]. Our co-culture system does not allow direct assessment of the vascular and immune contribution: Therefore, additional *in vivo* experiments will be needed to conclude. However, the notion that stromal cells could promote an immunostimulating milieu is further supported by our simultaneous finding of IL-6 induction whose secretion is stimulated in TC-1 cells when they are cultured with medium conditioned by RhoB-/- fibroblasts and further enhanced when tumors cells are cultured in conditioned medium by irradiated RhoB-/- fibroblasts. IL-6 is also upregulated by coculture with wt fibroblast but to a lesser extent. IL-6 is one of the best-characterized pro-inflammatory cytokines [29, 30] and our results strongly suggest that after high dose of radiation stromal cells are able to modify the milieu and induces tumor cells to secrete factors able to alter immune infiltrate. Whether we can modify stromal response and peri-tumoral milieu to trigger immuno-stimulatory (IL-6) vs immune-suppressive (TGF-β) response needs to be investigated in detail but our results suggest that RhoB targeting could be a mean to achieve this goal.

Lastly and more interestingly, our results bring a completely new vision and show that co-irradiation of tumor cells and fibroblasts abrogate migration of tumor cells. This is more relevant to the clinical situation and in accordance with clinical findings, but challenges previous publication including Ohuchida *et al*. and Hwang *et al.* papers [18, 19] who showed that coculture with irradiated fibroblasts enhances the invasive potential of pancreatic cancer cells. They concluded that tumor/stroma interactions would stimulate metastasis after irradiation. Although we confirmed that irradiation of fibroblasts (irrespectively from their genotype but via distinct mechanisms) promotes TC-1 invasive potential, this set-up is poorly relevant to the clinical situation, as stroma is never irradiated alone but on the contrary co-irradiation of tumors and surrounding stroma is always performed with sufficient safety margins. Therefore and to stick to the clinical situation, we co-irradiated fibroblasts and TC-1 carcinoma cells and in that case our results bring to a different conclusion as we show that co-irradiation of tumor cells and fibroblasts repressed the pro-migratory signals, suggesting that conversely to what was previously described radiotherapy rather prevented metastatic spread than promoted it.

In conclusion, stromal component of the tumor do secrete pro-migratory factors as function of their genotype. Wt fibroblasts pro-migratory action is mainly TGF-β mediated whereas RhoB deficient fibroblasts stimulate MMP secretion. In addition and interestingly RhoB deficiency in the stroma enhanced tumor cell migration but simultaneously stimulated pro-inflammatory signals (IL-6) that would impact on immune recruitment and favor anti-tumor immune response. Lastly, our results challenges the view that irradiated stroma would promote migration of carcinoma cells as we show that independently from their genotype co-irradiation of fibroblasts and carcinoma cells repressed carcinoma cell migration.

REFERENCES:

[1] Dvorak HF (1986) Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med ; 315: 1650–9.

[2] Haddow A (1972) Molecular repair, wound healing, and carcinogenesis: tumor production a possible overhealing? Adv Cancer Res ; 16: 181–234.

[3] Barcellos-Hofs MH, Park C, Wright EG (2005) Radiation and the microenvironment tumorigenesis and therapy. Nat Rev Cancer; 5:867–875

[4] Barcellos-Hoff MH, Ewan KB (2000) Transforming growth factor-beta and breast cancer: Mammary gland development. Breast Cancer Res.;2(2):92-9.

[5] Barcellos-Hoff, M. H (1993) Radiation-induced transforming growth factor ß and subsequent extracellular matrix reorganization in murine mammary gland. Cancer Res. 53,3880–3886.

[6] Barcellos-Hoff, M. H. & Ravani, SA. (2000). Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells. Cancer Res. 60, 1254–1260

[7] John Yarnolda, Marie-Catherine Vozenin Brotons (2010) Pathogenetic mechanisms in radiation fibrosis.Radiother Oncol. Oct;97(1):149-61

[8] Lewis MP, Lygoe KA, Nystrom ML, Anderson WP, Speight PM, et al (2004). Tumour derived TGF-b1 modulates myofibroblast differentiation and promotes HGF/SFdependent invasion of squamous carcinoma cells. Br.J.Cancer 90, 822–832.

[9] Jaffe AB, Hall A (2005) Rho GTPases:biochemistry and biology. Annu. Rev. Cell Dev. Biol.21, 247 –269.

[10] Fritz G, Kaina B (1997) RhoB encoding a UV-inducible Ras-related small GTP-binding protein is regulated by GTPases of the Rho family and independent of JNK, ERK, and p38 MAP kinase .J Biol Chem. ;272(49):30637–30644.

[11] Wherlock M, Gampel A, Futter C, Mellor H (2004)Farnesyltransferase inhibitors disrupt EGF receptor traffic through modulation of the RhoB GTPase. J Cell Sci.:117:3221–31.

[12] Prendergast GC, Khosravi-Far R, Solski PA, Kurzawa H, Lebowitz PF, et al (1995). Critical role of Rho in cell transformation by oncogenic Ras.Oncogene 10:2289 –2296.

[13] Rodriguez PL, Sahay S, Olabisi OO, Whitehead IP (2007) ROCK I-mediated activation of NF-kappaB by RhoB. Cell Signal;19:2361–2369

[14] Verrecchia, F., Chu, M. L., and Mauviel, A (2001) Identification of novel TGF-beta/Smad gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach. J. Biol.Chem : 276, 17058–17062

[15] Moustakas A, Stournaras C (1999) Regulation of actin organisation by TGF-beta in H-ras-transformed fibroblasts. J Cell Sci ;112:1169–1179.

[16] Liu AX, Rane N, Liu JP, Prendergast GC (2001) RhoB is dispensable for mouse development, but it modifies susceptibility to tumor formation as well as cell adhesion and growth factor signaling in transformed cells. Mol Cell Biol. Oct;21(20):6906-12

[17] Liu A-X, Du W, Liu J-P, Jessell T M, Prendergast G C (2000) RhoB alteration is necessary for the apoptotic and antineoplastic responses to farnesyltransferase inhibitors. Mol Cell Biol :20:6105–6113

[18] Hu, X., Beeton, C (2010) Detection of Functional Matrix Metalloproteinases by Zymography. J. Vis. Exp. Nov 8;(45). pii: 2445. doi: 10.3791/2445.

[19] Ohuchida K, Mizumoto K, Murakami M, Qian LW, Sato N et al (2004) Radiation to stromal fibroblasts increases invasiveness of pancreatic cancer cells through tumor stromal interactions. Cancer Res: 64:3215-3222

[20] Hwang RF, Moore T, Arumugam T, Ramachandran V, Amos KD et al (2008) Cancer-associated stromal fibroblasts promote pancreatic tumor progression.Cancer Res:68 (3).918-926

[21] Verrecchia, F., Chu, M. L., Mauviel, A. (2001) Identification of novel TGF-beta/Smad gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach. J. Biol. Chem. 276,17058-17062

[22]. Barcellos-Hoff MH, Ewan KB (2000) Transforming growth factor-beta and breast cancer: Mammary gland development. Breast Cancer Res :2(2):92-9. Epub 2000 Feb 21.

[23] De Wever O, Mareel M (2003) Role of tissue stroma in cancer cell invasion. J. Pathol:200, 429–447

[24] Principe DR, Doll JA, Bauer J, Jung B, Munshi HG (2014) TGF-β: Duality of Function Between Tumor Prevention and Carcinogenesis. J Natl Cancer Inst: Feb 1;106(2):djt369. doi: 10.1093/jnci/djt369.

[25] Johnsen M, Lund LR, Rømer J, Almholt K, Danø K (1998) Cancer invasion and tissue remodeling: common themes in proteolytic matrix degradation.Curr Opin Cell Biol. Oct;10(5):667-71.

[26] Ahn GO, Brown JM (2008) Matrix metalloproteinase-9 is required for tumor vasculogenesis but not for angiogenesis: role of bone marrow-derived myelomonocytic cells. Cancer Cell. Mar;13(3):193-205. doi: 10.1016/j.ccr.2007.11.032.

[27] Farhat FS, Tfayli A, Fakhruddin N, Mahfouz R, Otrock ZK, Alameddine RS, et al (2012) Expression, prognostic and predictive impact of VEGF and bFGF in non-small cell lung cancer.Crit Rev Oncol Hematol. Nov;84(2):149-60.

[28] A. Dowlati, R. Gray, A.B. Sandler, J.H. Schiller, D.H. Johnson (2008) Cell adhesion molecules, vascular endothelial growth factor, and basic fibroblast growth factor in patients with non-small cell lung cancer treated with chemotherapy with or without bevacizumab—an Eastern Cooperative Oncology Group StudyClin Cancer Res, 14, pp. 1407–1412

[29] C. Garbers, H.M. Hermanns, F. Schaper, G. Muller-Newen, J. Grotzinger, et al (2012). Plasticity and cross-talk of interleukin 6-type cytokines. Cytokine Growth Factor Rev Jun; 23(3):85-97. doi: 10.1016/j.cytogfr.2012.04.001

[30] Y. Guo, F. Xu, T. Lu, Z. Duan, Z. Zhang (2012) Interleukin-6 signaling pathway in targeted therapy for cancer .Cancer Treat Rev Nov;38(7):904-10. doi: 10.1016/j.ctrv.2012.04.007.

Figure Legends

Figure 1: TC-1 Clonogenic Survival Curve: Paracrine Signals Secreted By Fibroblasts do Not Modulate TC-1 intrinsic Radiation sensitivity. **A)** Media conditioned by fibroblasts do not modify TC-1 intrinsic radiation sensitivity. **B)** Conditioned Media (CM) isolated from irradiated Wt and RhoB-/- fibroblasts increase the clonogenic potential of non-irradiated TC-1 cells.

Figure 2: A and B) Paracrine Signals secreted by fibroblasts enhance TC-1 migration Migratory potential of TC -1 cells was analyzed **(A)** and quantified **(B)** in various culture conditions at 0 and 24 Hrs after wounding . Culture conditions are: cultured TC-1 non-Irradiated (0Gy) and irradiated at 2, 10 Gy alone or cultured using conditioned media from wt and RhoB -/- fibroblasts. Images are recored at x20 with Evo microscope. In all cases, differences were considered significant at: * P<0.05; ** P<0.01; *** P<0.001.

Figure 3: A) Cytokine Array Analysis : performed on TC-1 secretome to search for mediators of Increase TC-1 invasiveness. Significantly upregulated cytokines is shown.
B) MMP secretion from TC 1 cells . Zymography was performed on conditioned media collected from TC-1 cells 24hrs after culture with conditioned media collected from wt and RhoB-/- fibroblasts**. C) Modulation of TC-1 Migration by O-Phenanthroline** Migratory potential of TC-1 cells was analyzed and quantified in various culture conditions at 0 and 24 Hrs after wounding. Culture conditions are: CM from TC-1 non Irradiated (0Gy) and irradiated at 2, 10 Gy alone or cultured with CM from Wt fibroblasts with O-Phenanthroline. Images are acquired at x20 with Evos microscope. In all cases, differences were considered significant at: * P<0.05; ** P<0.01; *** P<0.001.

Figure 4: A) Effect of Irradiation and coculture on TGF β Expression in Wt fibroblasts . Whole cell lysate from Wt Fibroblasts was subjected to Western Blot using antibodies for TGF β1 (13 kDa). Histogram shows relative protein levels normalized to the intensity of the corresponding GAPDH values. In all cases, differences were considered significant at: * P<0.05; ** P<0.01; *** P<0.001. **B) Modulation of TC-1 migration by SB 41542.** Migratory potential of TC-1 cells was analyzed and quantified in various culture conditions at 0 and 24 Hrs after wounding. Culture conditions are: CM from TC-1 non Irradiated (0Gy) and irradiated at 2, 10 Gy alone or cultured with CM from Wt fibroblasts with SB41542. Images are acquired at x20 with Evos microscope. In all cases, differences were considered significant sat: * P<0.05; ** P<0.01; *** P<0.001.

Figure 5: TC-1 enhanced migration is associated with induction of EMT markers: TC-1 lysate was subjected to Western Blot using antibodies for Vimentin (57kDa) and Snail (29kDa) .Histograms show the

relative protein levels of each normalized to the intensity of the corresponding GAPDH values. In all cases, differences were considered significant at: * P<0.05; ** P<0.01; *** P<0.001.

Figure 6: Summary of the findings

Supplementary Figures

Figure S 1: MMP secretion from RhoB-/- Fibroblasts. Zymography was performed on conditioned media collected from RhoB-/- fibroblasts , 24hrs after co-culture with TC-1 cells.

Figure S 2: A) Effect of Irradiation and coculture on TGF β Expression in RhoB-/- fibroblasts . Whole cell lysate from RhoB-/- Fibroblasts was subjected to Western Blot using antibodies for TGF β1 (13 kDa). Histogram shows relative protein levels normalized to the intensity of the corresponding GAPDH values. In all cases, differences were considered significant at: * P<0.05; **B) Modulation of TC-1 migration by SB 41542.** Migratory potential of TC-1 cells was analyzed and quantified in various culture conditions at 0 and 24 Hrs after wounding. Culture conditions are: CM from TC-1 non Irradiated (0Gy) and irradiated at 2, 10 Gy alone or cultured with CM from RhoB -/-fibroblasts with SB41542. Images are acquired at x20 with Evos microscope. In all cases, differences were considered significant at: * P<0.05; ** P<0.01; *** P<0.001.

Figure 1 A

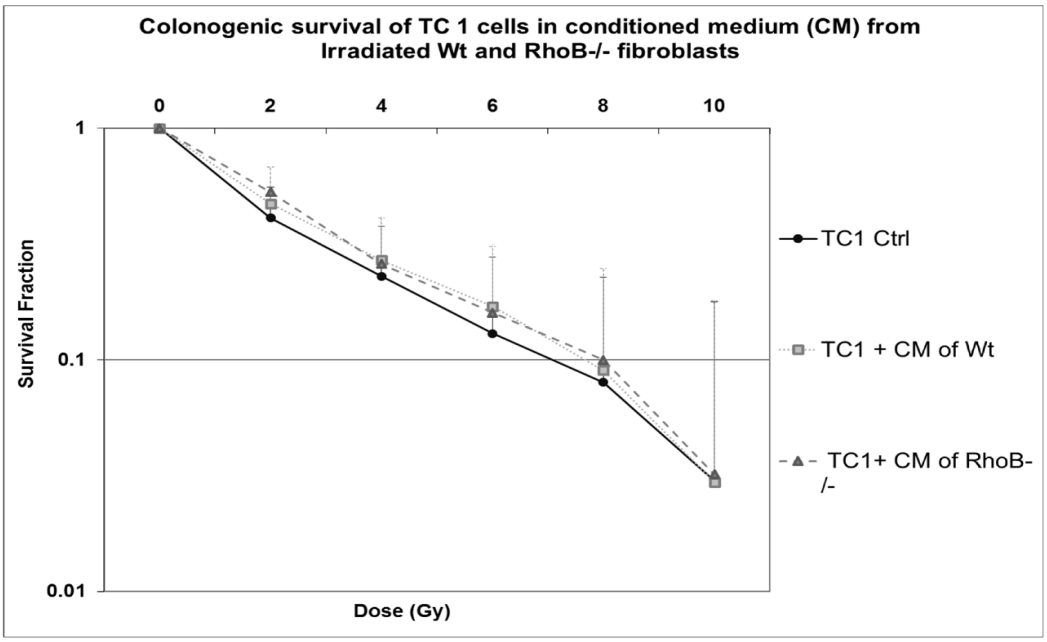


Figure 1 B

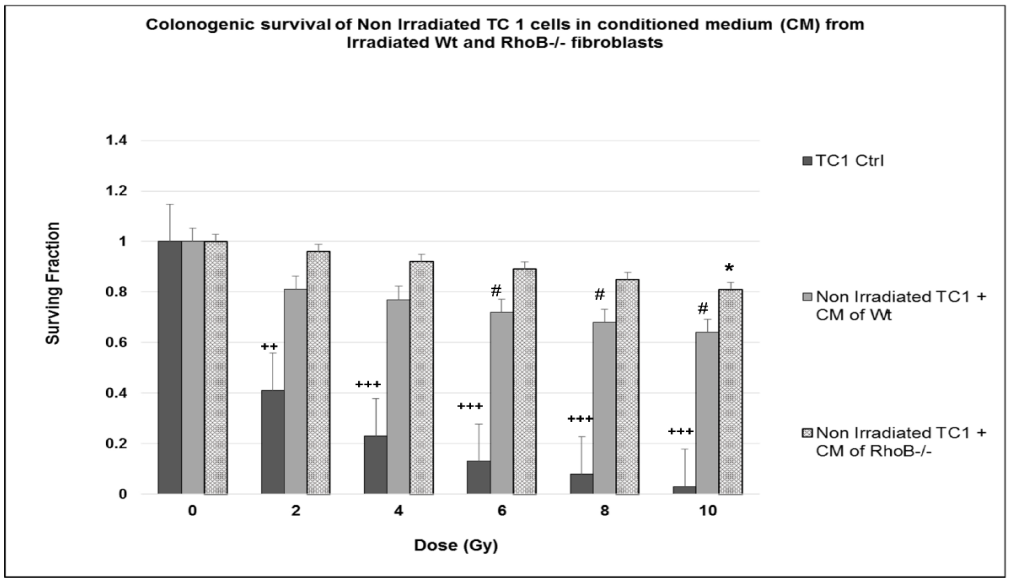
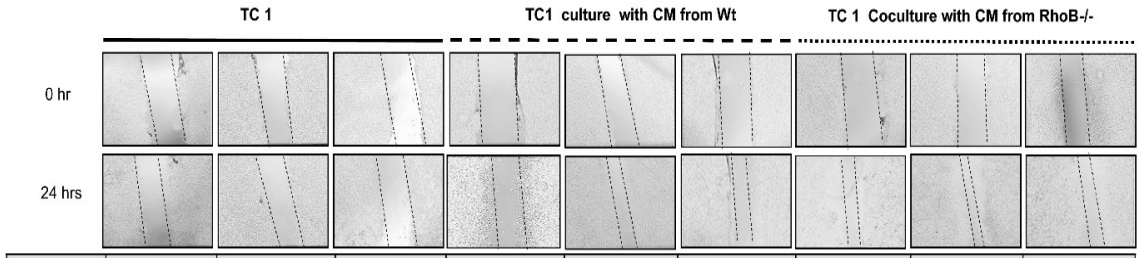
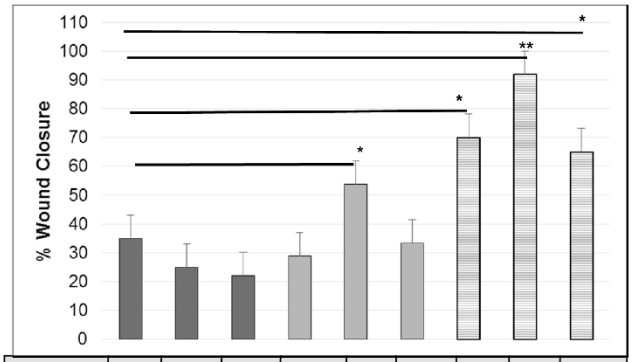


Figure 2 A



Radiation dosage (Gy)										
	TC1	0	2	10	0	0	10	0	0	10
	Wt				0	10	10			
	RhoB-/-							0	10	10

Figure 2 B : TC-1 migration



Radiation dosage (Gy)										
	TC1	0	2	10	0	0	10	0	0	10
	Wt				0	10	10			
	RhoB-/-							0	10	10

TC 1 | TC 1 coculture with Wt | TC 1 coculture with RhoB-/-

Figure 3 A: Study of TC-1 Proteome

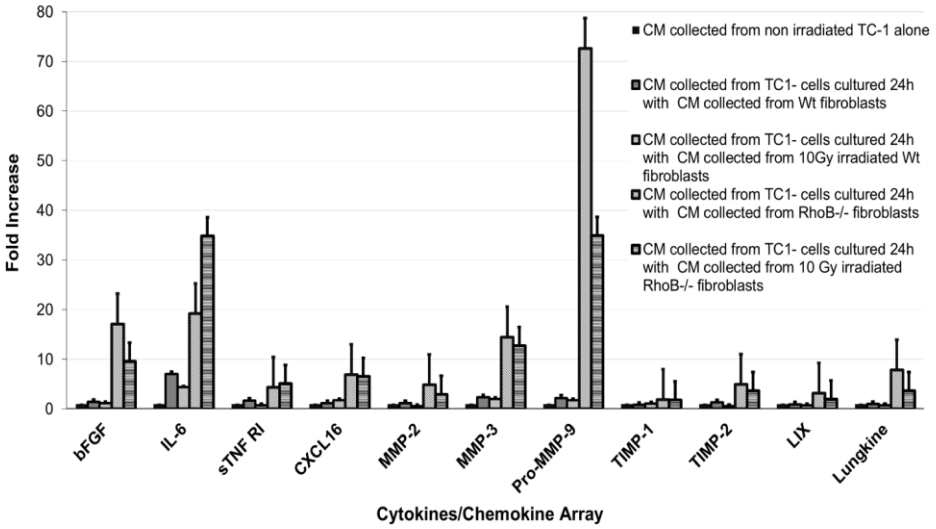


Figure 3 B

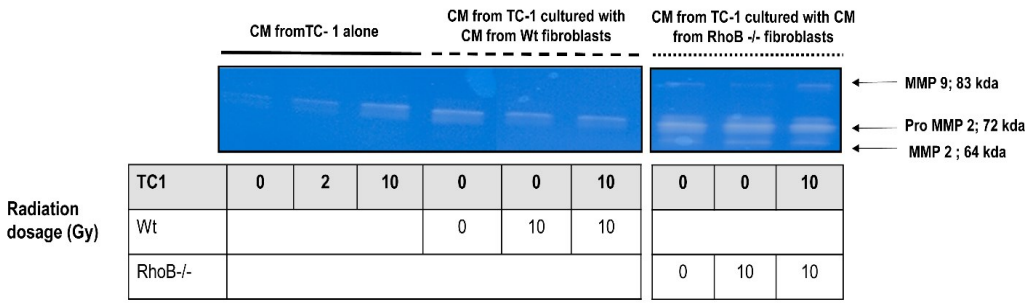
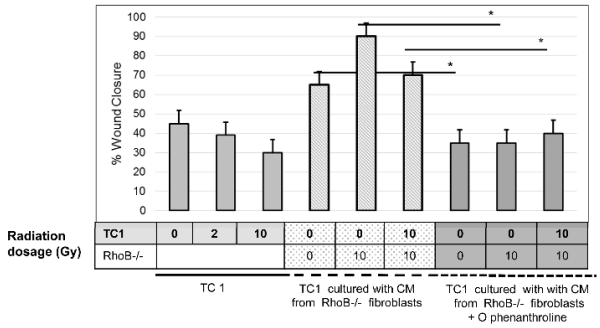


Figure 3 C



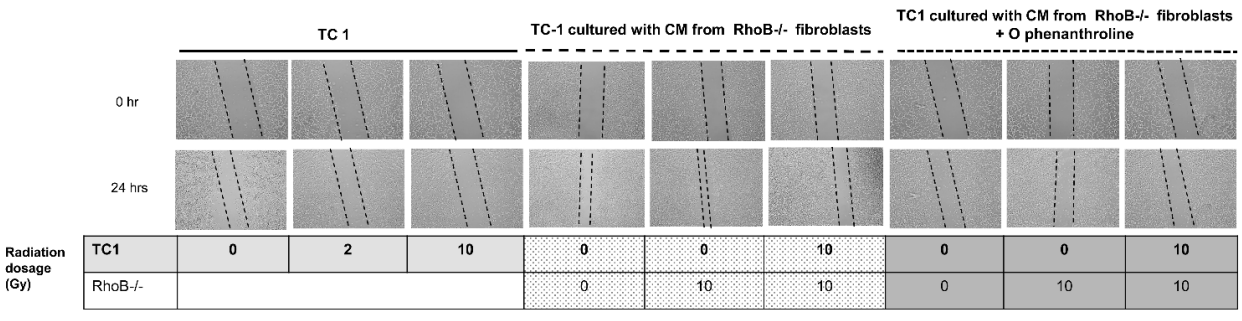


Figure 4 A

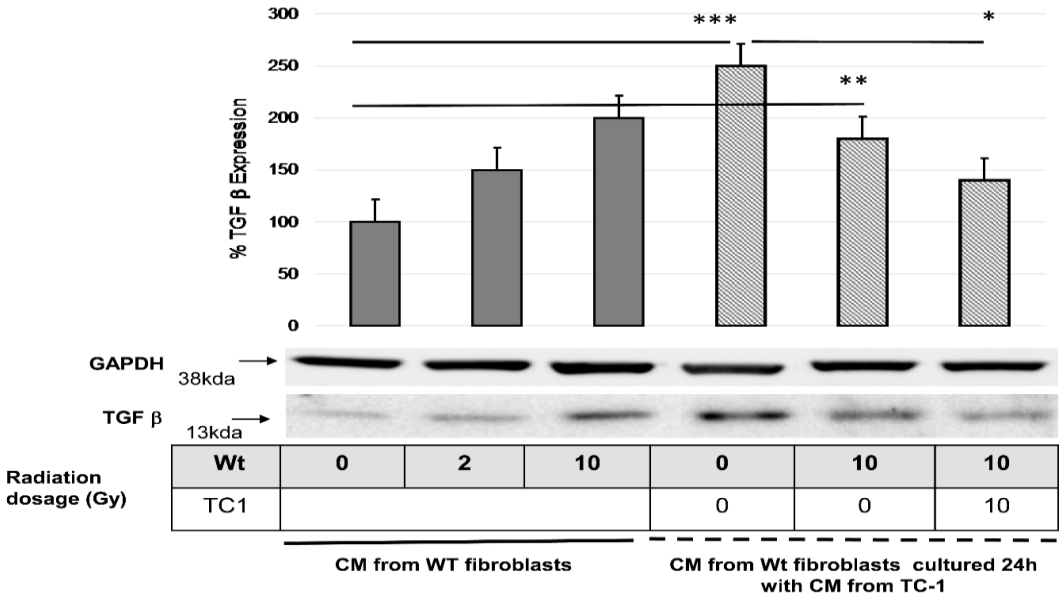


Figure 4 B

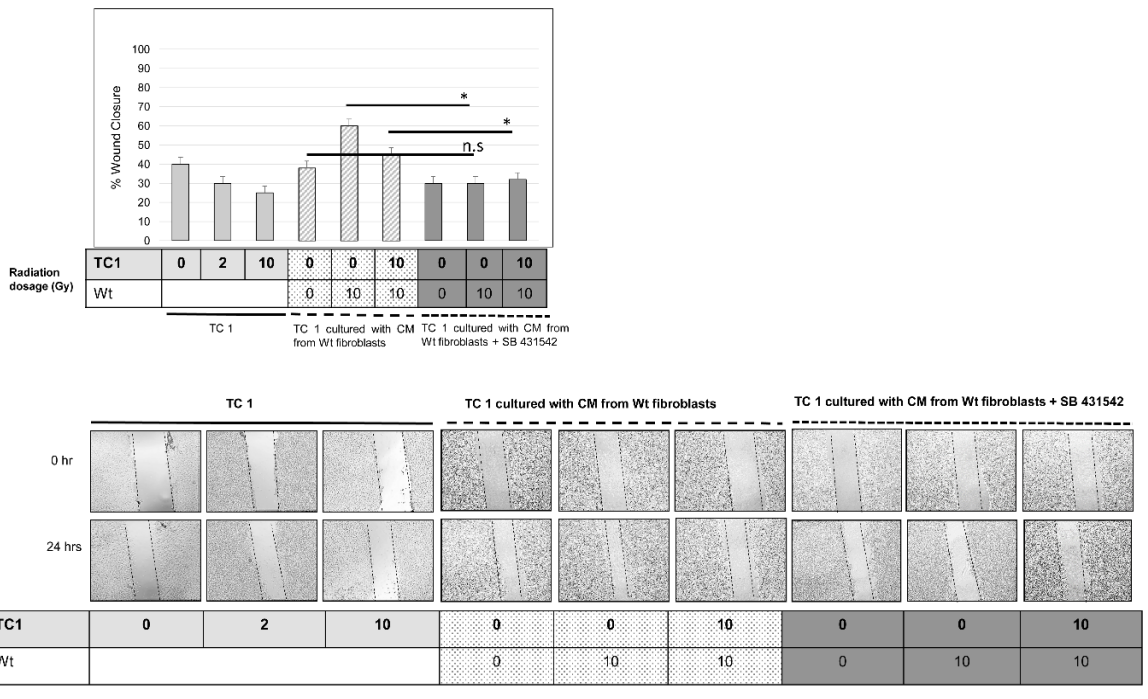


Figure 5 : Study on TC-1 cell lysate

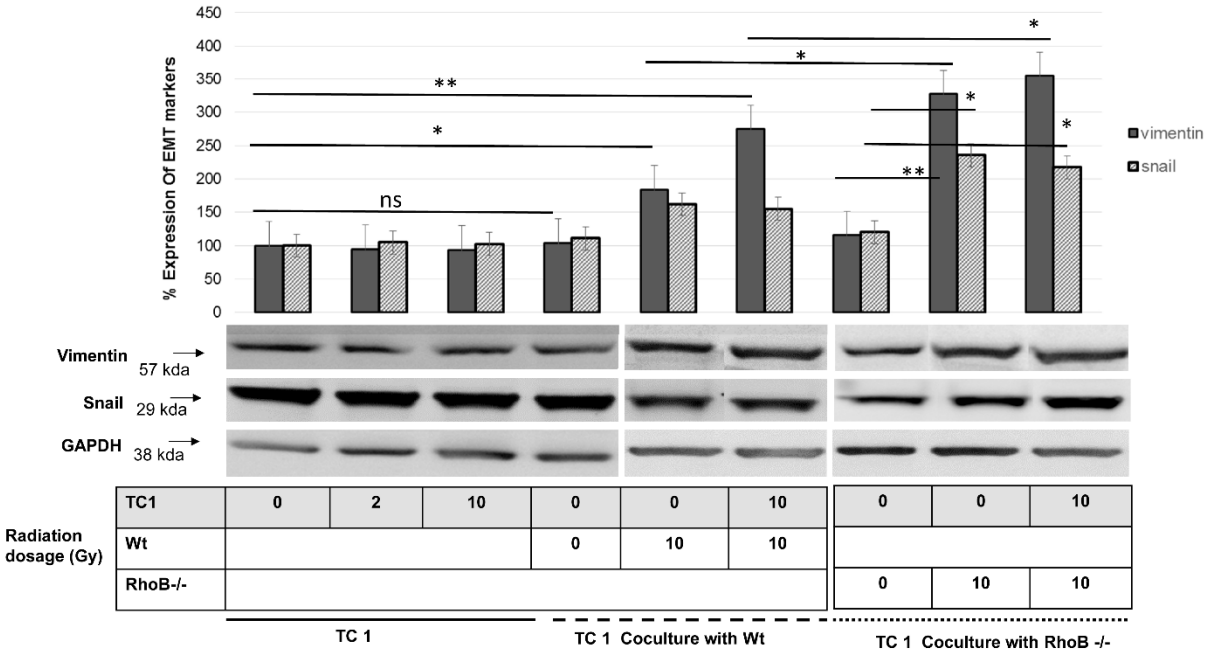
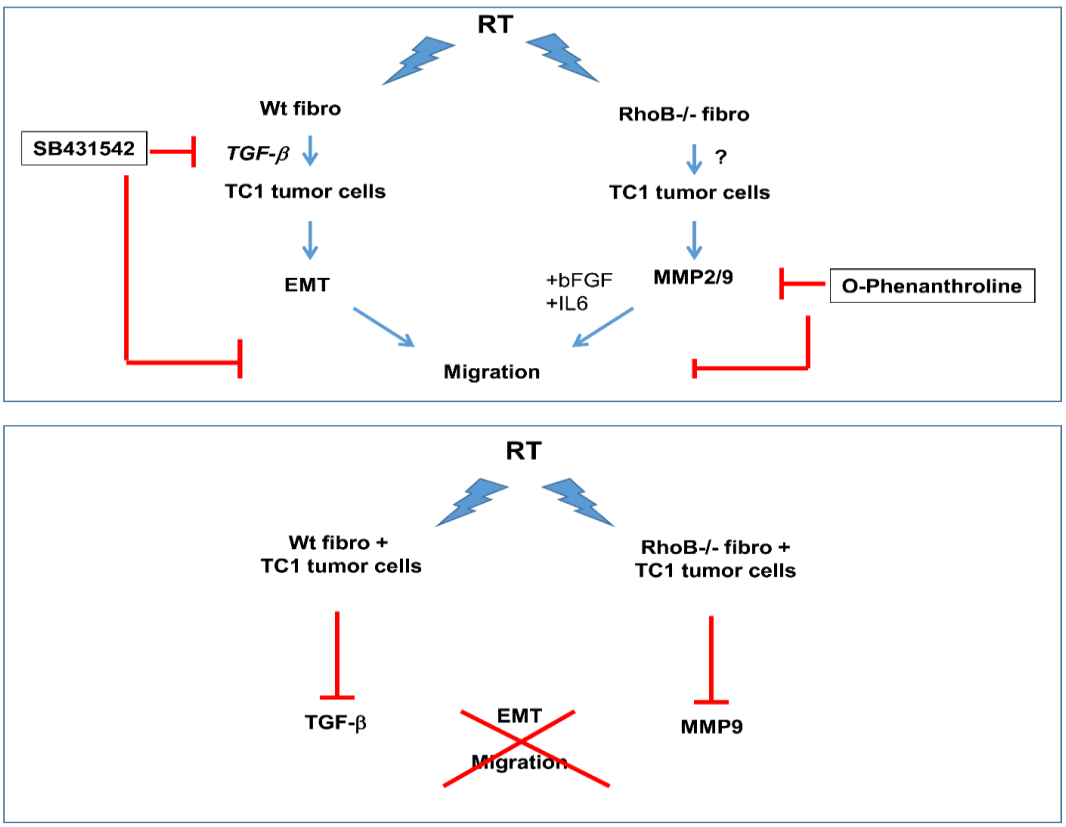
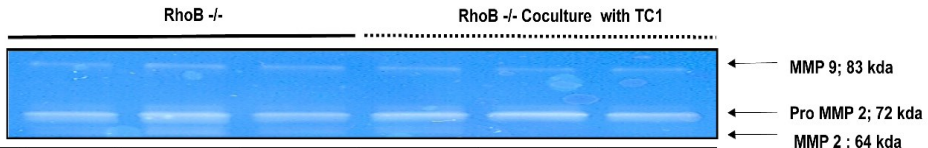


Figure 6

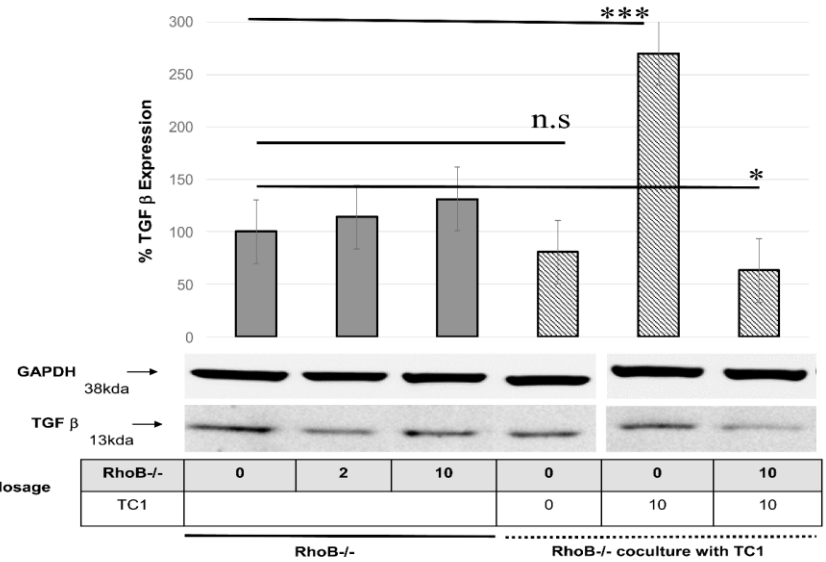


Supplementary Figure: S 1



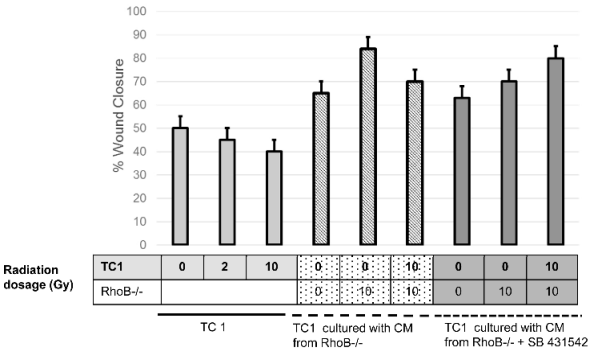
Radiation dosage (Gy)	RhoB -/-	0	2	10	0	0	10
	TC1				0	10	10

Supplementary Figure: S 2A

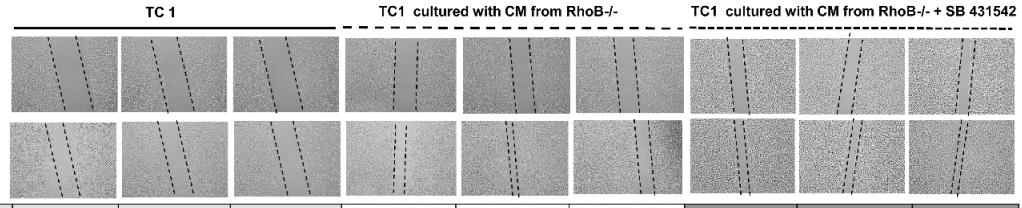


Radiation dosage (Gy)	RhoB-/-	0	2	10	0	0	10
	TC1				0	10	10

Supplementary Figure : S 2B



Radiation dosage (Gy)	TC1	0	2	10	0	0	10	0	0	10
	RhoB-/-				0	10	10	0	10	10



Radiation dosage (Gy)	TC1	0	2	10	0	0	10	0	0	10
	RhoB-/-				0	10	10	0	10	10

Ultra-high dose-rate FLASH radiotherapy allows dose escalation that cures leukemia without hematological toxicity

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One Sentence Summary: Total body irradiation at very high doses using ultra-high dose-rate can be used to cure leukemia while sparing normal hematopoiesis.

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Running title: Differential effect of FLASH irradiation on normal hematopoiesis and leukemia

Abbreviations: CDR: continuous, conventional dose-rate irradiation (0.04 $Gy.s^{-1}$); FLASH: pulsed, ultra-high dose-rate irradiation (50 $Gy.s^{-1}$); TBI: Total Body Irradiation.

Word count: 7699 words (all inclusive)

ABSTRACT

Radiotherapy efficiency is limited by adverse effects on normal tissues. In mice, total body irradiation (TBI) at high doses (> 10 Gray (Gy)) delivered at a conventional dose-rate (0.04 $Gy.s^{-1}$, CDR) results in lethal aplasia pinpointing hematopoiesis as a model system to study differential effects of doses and dose-rates of radiation on hematopoiesis and leukemia development. Here, we used an experimental linear electron accelerator that can deliver high doses of radiation in less than 500 ms by increasing the dose-rate above 50 $Gy.s^{-1}$ (FLASH). More than 70% of TBI-FLASH treated mice showed long-term survival without hematological disorders at doses up to 20 Gy whereas all TBI-CDR treated animals died at 10-12 Gy. Hematopoietic stem and progenitor cells (HSPCs) from TBI-FLASH treated mice showed an increased clonogenic potential whereas purified HSPCs FLASH- or CDR-irradiated *ex vivo* display similar radiosensitivities indicating that the effect of TBI-FLASH on HSPCs is not cell-autonomous. Compared to 8 Gy TBI-CDR, 16 Gy TBI-FLASH paradoxically elicited a faster recovery of hematopoietic stem cells (HSCs) and a decreased number of 53BP1 and γH2AX foci in irradiated HSCs. Six months after TBI, three-fold fewer HSPCs with chromosomal aberrations were found in FLASH-irradiated mice and primary or competitive transplantations showed that HSCs from 16 Gy TBI-FLASH treated mice could reconstitute hematopoiesis, without any hematological disease, as efficiently as HSCs from 8 Gy TBI-CDR treated mice. Complete remission of an aggressive murine myeloid leukemia was observed in more than 60% of mice irradiated with a single 16 Gy TBI-FLASH whereas 8 Gy TBI-CDR had no effect on leukemic progression. Finally, propagation of human primary leukemia in secondary recipient mice was dramatically delayed after 16 Gy TBI-FLASH of infiltrated primary mice. These combined results show that modulation of the irradiation dose-rate can allow high dose escalation for the impairment of leukemia while preserving hematopoiesis.

INTRODUCTION

Nearly half of all cancer patients will undergo radiotherapy. The beneficial effect of this treatment is limited by radioresistance of the cancer cells and by adverse effects of the ionizing radiation on the normal tissues surrounding the tumor (*1-4*). Over the past twenty years, progress in biology and physics together with the development of hypofractionated irradiation have broadened the therapeutic window and improved radiotherapy safety and accuracy (*5, 6*). Further improvement of the therapeutic window includes sensitizing the tumor cells or protecting the normal tissue and most of the research in this field was focused on the discovery of pharmacological or biological modulator combined with conventional radiotherapy (*7-11*) but, despite some progresses, toxicity occurrence still remains a major dose-limiting factor and impairs radiotherapy efficacy and prognosis.

The most radiosensitive normal tissues are the hematopoietic system and the gastrointestinal tract and tissue damage induced by ionizing radiation in these two tissues underlies deleterious side effects after irradiation (*12, 13*). The effects of radiation on the hematopoietic system have been largely investigated for radioprotection purpose after Hiroshima-Nagasaki (*14*) and well characterized as total body irradiation (TBI) is currently used with great success to myeloablate and immune suppress before allogeneic or autologous hematopoietic stem cell (HSC) transplantation (*15*). In addition, combined with chemotherapy, TBI can be used to destroy leukemic cells before stem cell transplantation but this acute myeloid leukemia treatment is poorly tolerated by patients and often failed due to residual radioresistant leukemic clones that remain and lead to relapse (*16*). Despite the widespread use of TBI in the preparation of patients for hematopoietic stem cell transplantation, the identification of pathways that will confer radiomitigating activity is an unmet challenge and very few therapeutic agents that can mitigate radiation-induced hematopoietic toxicity (*17*) while enhancing leukemic cells eradication have been developed.

Here, we have investigated an innovation in radiotherapy that is not based on the use of pharmacological targeting of pathways and that increases leukemic cells eradication while decreasing hematopoietic toxicity. This novel modality of radiotherapy is based on high dose delivery at Ultra-High Dose-Rate (> 50 $Gy.s^{-1}$, FLASH) thereby contrasting with Conventional Dose-Rate (0.04 $Gy.s^{-1}$, CDR) that can delivers high doses within minutes. To assess the efficacy of high dose-rates on living animals and on cancer, we performed total body irradiation (TBI) using a linear electron accelerator of 4.5 MeV (LINAC) capable of delivering high doses of irradiation with a wide range of dose-rates. We studied the differential effects of high doses of radiation delivered using CDR or FLASH on normal hematopoiesis and leukemia and showed that dose-rate is as important as doses in the beneficial or deleterious effects of radiotherapy.

RESULTS

Mice can survive to high doses of irradiation when delivered with FLASH irradiation

As standard dosimetric methods based on the measurement of the fluence of charged particles in air do not operate at high dose-rate (*18*), accurate control of the dose delivered was performed using chemical dosimeters based upon real-time, optical detection of radio-induced formation of the blue methyl viologen radical dication in aqueous solution (*19*) (Fig. S1-S2 and Data file S1).

We first determined survival of four strains of mice (C57BL/6J, 129/Sv, C3H and Balb/c), known to exhibit different radiosensitivities (*20*) after increasing doses of TBI delivered using CDR or FLASH. The median lethal dose (LD-50) of TBI-CDR treated mice was 10 Gy and the lethal dose (LD-100) was 12 Gy with mice dying between 7 and 15 days post TBI-CDR (Fig.1A). Death was mainly due to aplasia as bone marrow transplantation after a 16 Gy TBI-CDR resulted in survival of the irradiated mice (Fig. S3). In sharp contrast, when TBI-FLASH was

used, doses up to 20 Gy could be administered with more than 70% (C57BL/6J) and 100% (129/Sv and Balb/c) long-term survival (Fig. 1A). Although 20 Gy TBI-FLASH was lethal for 100% of C3H mice, these radiosensitive mice tolerated doses up to 16 Gy TBI-FLASH with 100% long-term survival (Fig. 1A). To ascertain that the protection induced by FLASH relied on high dose-rate, we performed dose-rate escalation and studied the effect of TBI using single doses of 20 Gy-FLASH delivered at 50, 10, 2, 0.4 and 0.04 $Gy.s^{-1}$and found 100% of lethality in mice irradiated at 2, 0.4 and 0.04 $Gy.s^{-1}$ while 10 $Gy.s^{-1}$ resulted in death of 1/3 of the animals and 50 $Gy.s^{-1}$was completely safe (Fig. 1B). These results indicated that TBI-FLASH allows irradiation at high doses without apparent adverse effects and that the duration of irradiation is a critical factor for survival.

FLASH irradiation prevents lethal aplasia after high doses TBI

In C57BL/6J mice, bone marrow cell counts began to decrease 6 h after TBI-CDR or TBI-FLASH, reached a lowest level 3 or 6 days later depending on the doses and returned to normal levels 6 days (8 Gy-CDR and 8 Gy-FLASH) or 15 days (16 Gy-FLASH) after TBI (Fig. 2A and Fig. S4). In contrast, a 16 Gy TBI-CDR resulted in lethal aplasia in all treated mice 6 days after irradiation (Fig. 2A and Fig. S4). Mature hematopoietic compartments showed similar kinetics of recovery after 8 Gy TBI-CDR or TBI-FLASH whereas an increase in $CD3^+$ T-cells and no increase in $GR1^+CD11b^+$ myeloid cells were observed during hematopoietic recovery after 16 Gy TBI-FLASH (Fig. 2B). Fifteen days after TBI-CDR or TBI-FLASH, the hematopoietic reconstitution showed a bias towards B lymphoid and erythroid compartments at the expense of the myeloid compartment (Fig. 2B). The LT-HSC compartment was restored six days after 8 or 16 Gy TBI-FLASH, whereas it was still highly diminished at day 6 following 8 Gy TBI-CDR, the LT-HSC recovery being apparent by day 15 (Fig. 2C). Finally, as one of the major adverse effects of irradiation is chromosomal damage (*21*), we studied the kinetics of DNA double-strand break (DSB) repair in LT-HSCs after TBI-CDR or TBI-FLASH by counting 53BP1 and

γH2AX foci. When mice were sacrificed immediately after irradiation, fewer 53BP1 and γH2AX foci were observed in LT-HSCs from 8 or 16 Gy TBI-FLASH mice compared with LT-HSCs from 8 or 16 Gy TBI-CDR mice (Fig. 2D and Fig. S5, left panel). Later time points showed similar kinetics of 53BP1 and γH2AX foci disappearance and thus similar kinetics of DNA repair in HSCs after TBI-FLASH or TBI-CDR (Fig. 2D and Fig. S5, right panels).

Finally, we studied any cell-autonomous responses of hematopoietic stem and progenitor cells (HSPCs) to TBI-CDR and TBI-FLASH. HSPCs were sorted either directly from TBI-CDR or TBI-FLASH treated mice or from sham-irradiated mice and then CDR- or FLASH-irradiated. Both HSPC groups were plated and compared in clonogenic assays. HSPCs from TBI-FLASH treated mice displayed a 1.6 reduced radiation sensitivity (D_{37}) compared to HSPCs from TBI-CDR treated mice (Fig. 2E, left panel) whereas sensitivities of *ex vivo* CDR- or FLASH-irradiated HSPCs were similar (Fig. 2E, right panel). These combined results indicate that 16 Gy TBI-FLASH has less adverse effects than 8 Gy TBI-CDR on HSC recovery and suggest potential microenvironmental components to HSPC radioprotection.

Normal hematopoiesis with functional HSCs 6 months after high doses TBI using FLASH irradiation

Four to six months after TBI-CDR or TBI-FLASH, irradiated mice appeared healthy with normal weights (Fig. S6) and sizes (not shown) and without evidence of any disease. In fact, the only phenotype observed in irradiated mice was a gradient of depigmentation as a function of the dose of FLASH irradiation (Fig. S7). Bone marrow cell counts and percentages of mature and progenitor hematopoietic cells and of ST-HSCs and LT-HSCs from 8 Gy CDR and 8 or 16 Gy FLASH irradiated mice were identical to those in sham-irradiated mice (Fig. 3A and Fig. S8). Cytogenetic analyses performed on HSPCs six months after TBI showed a 3-fold decrease of HSPCs with chromosomal aberrations in 8 or 16 Gy-FLASH-irradiated mice compared with 8 Gy-CDR irradiated mice (Fig. 3B). This result indicates that FLASH-irradiated mice reconstituted

their hematopoiesis with LT-HSCs that had less chromosomal rearrangements than CDR-irradiated reconstituting LT-HSCs. The LTC-IC frequency was similar in sham-irradiated mice, 8 Gy-CDR and 8 Gy- and 16 Gy-FLASH irradiated mice (Fig. S9) and primary transplantation of LSK from irradiated mice six months post 8 Gy TBI-CDR and 8 Gy and 16 Gy TBI-FLASH into irradiated recipients resulted in long-term survival of recipient mice without any observed hematological disorders (Fig. 3C, upper panels). Four months after these primary transplantations, bone marrow cell counts were normal and no significant difference in the percentage of mature and progenitor hematopoietic cells could be observed (Fig. S10-11). The percentage of ST-HSCs was not significantly different and a slight decrease in LT-HSCs could only be seen in mice reconstituted with LSK from 8 Gy TBI-CDR irradiated mice (Fig. 3C, lower panels). Finally, compared to HSCs from sham-irradiated mice, HSCs from 8 Gy TBI-CDR and 8 and 16 Gy TBI-FLASH treated mice showed a 1.5- to 2-fold decreased capacity to compete for hematopoietic reconstitution (Fig. 3D). These results show that, when mice are irradiated at 16 Gy TBI-FLASH, the reconstitution capacity of at least some HSCs is conserved but with a decreased efficiency compared to sham-irradiated HSCs.

High doses TBI using FLASH irradiation can impair leukemic cell expansion *in vivo*

As FLASH irradiation protects normal hematopoiesis from adverse effects of high doses of irradiation, we investigated whether leukemia progression could be controlled by dose escalation using FLASH irradiation. We used a mouse model of aggressive myeloid leukemia (*22*) (*23*) by transplanting $5x10^3$ GFP^+ MLL-ENL leukemic cells into sham-irradiated 129/Sv mice. This transplantation resulted in rapid development of leukemia with death following approximately 40 days post-transplantation. TBI at 8 Gy CDR, 8 Gy FLASH or 16 Gy FLASH was performed 6 and 15 days post-transplantation of MLL-ENL cells to study the effects of irradiation on the seeding phase and the invading phase of leukemia. Sham-irradiated, 8 Gy TBI-CDR and 8 Gy TBI-FLASH treated mice exhibited similar survival kinetics and died 42 to 52

days after injection of leukemic cells. In striking contrast, 16 Gy FLASH irradiation significantly extended the animals' life span by at least 3-fold in more than 60% of the animals (Fig. 4A). The remaining 40% mice died of severe aplasia but no GFP^+ MLL-ENL leukemic cells could be found in their bone marrow (our unpublished data). Three months after 16 Gy TBI-FLASH, no GFP^+ MLL-ENL leukemic cells could be detected in the surviving irradiated mice (Fig. S12). Significantly, their bone marrow was clear of leukemic cells (Fig. 4B, upper right panel) and bone marrow transplants from these mice never induced leukemia in secondary recipients (Fig. 4B, lower right panel), thus indicating a complete cure of this leukemia model after a single 16 Gy TBI-FLASH. TBI-FLASH effects on *in vivo* human leukemia development were studied by xenografts of primary human leukemia into NSG mice (*24*). As NSG mice are highly sensitive to irradiation due to their scid mutation (*25*), 8 Gy TBI-CDR or 16 Gy TBI-FLASH was performed after human leukemia spreading into NSG mice. Bone marrow from these irradiated NSG mice was then transplanted into non-conditioned secondary NSG mice 24 h post-TBI and leukemia development was assessed. All transplanted mice with bone marrow cells from sham-irradiated mice or 8 Gy CDR irradiated mice developed leukemia with more than 97% of leukemic cells in their bone marrow 7 weeks after transplantation (Fig. 4C, lower left and middle panels). In contrast, 7 weeks after transplantation, less than 4% of leukemic cells were detected in the bone marrow of 3/4 mice transplanted with bone marrow isolated from mice irradiated at 16 Gy TBI-FLASH (Fig. 4C, right panel) showing a delayed human leukemia development after 16 Gy TBI-FLASH. These results demonstrate that TBI-FLASH has differential outcomes on normal hematopoiesis and leukemia with successful impairment of leukemic cell expansion *in vivo*.

DISCUSSION

In the present report, we show for the first time that total body irradiation with very high doses of ionizing radiation at high dose-rate allowed long-term survival of irradiated mice without

evidence of any disease. This new modality of irradiation protected mice from acute hematological syndrome and enabled high dose escalation able to eradicate leukemic cells. The early and fast brought out of aplasia following FLASH irradiation was associated with an efficient HSC repopulation and required a physiological microenvironment. Moreover, compared to CDR irradiation, FLASH irradiation decreased the number of HSCs with chromosomal abnormalities and preserved long-term hematopoiesis homeostasis. These results suggest that dose-rate modulation and acceleration of the dose delivery can be used to increase the differential effects of high doses of irradiation between normal tissues and tumors with a threshold at 10 $Gy.s^{-1}$ and 2 s of radiation exposure beyond which the protection is lost.

Besides improvements in ballistics and imaging, radiotherapy technology has not changed significantly in the last 60 years and most modern accelerators still use technology akin to that for the first X-ray tubes developed half a century ago. The recent development of proton therapy facilities and the use of high LET ion exemplify progresses that improve radiotherapy and more innovative approaches such as property of coulombic decay displayed by Auger electron might bring new progresses in radiotherapy (*26, 27*) but are far to be translatable into clinics. In this line but using an easy to transfer technology and approach, we propose a completely novel modality of radiotherapy that display a major differential effect and is able to cancer cells while protecting normal tissue. FLASH radiotherapy is based on highly enhanced dose rates delivered with a 4.5 MeV experimental LINAC operated in a chopped electron mode. Built with enhanced power supply (hyperfrequency and electron delivery), this device can deliver radiation doses at a dose rate > 40 $Gy.s^{-1}$, thus contrasting dramatically with clinically supported accelerators (either photon or electron), which classically operate at 3-4 Gy $min^{-1}$and can be scale up to 0.4 $Gy.s^{-1}$in the case of Flattening Filter Free Linac (1400-2400 MU min^{-1}) (*28, 29*). Our results clearly indicate that such dose rates are not sufficient to trigger any differential protection of

mice against high doses TBI, the TBI threshold limit being set at 10 $Gy.s^{-1}$and 50 $Gy.s^{-1}$ being the most protective dose-rate.

The physical basis behind the protective effect of FLASH *in vivo* is complex and will need further investigations especially in order to include a novel parameter in dosimetric reconstruction program (such as Monte Carlo calculation) which is the duration of dose deposition. However, we found several biological evidences that might explain how FLASH displays its protective effect. First, cytogenetic analysis showed less chromosomal aberrations in HSC used to reconstitute long-term hematopoiesis when mice are TBI irradiated using FLASH versus CDR. This result is in accordance with previous reports (*30-34*). Experiments conducted with short pulses of X-rays on lymphocytes (*30*) or protons on human-hamster hybrid cells and skin cells (*31-33*) including micro-channel radiotherapy that operates at 200 $Gy.s^{-1}$ dose-rate in the micro-channel (*34*) produce less cytogenetic damages. These dat along with ours suggests that high dose-rate results in less double strand breaks in DNA and/or that cells irradiated at high dose-rate and harboring chromosomal aberrations are rapidly eliminated. Second, HSC protection induced by FLASH is not a cell autonomous property of HSC but required the bone marrow microenvironment. Micro-environmental contributions to the maintenance of life-long HSC function is well documented (for review see, (*35*)) and multiple cell types such as endothelial cells, CXCL12-abundant reticular (CAR) cells, $Nestin^{+}$ mesenchymal cells and osteoblasts can regulate HSC fate in terms of localization, quiescence, apoptosis, self renewal, motility and differentiation directly by physical interactions or indirectly by secretion of paracrine factors. We are currently exploring the contribution of these different cells and paracrine factors in the FLASH induced protection of HSC.

A major point of this study is the differential effect triggered by FLASH on leukemic cells versus normal HSC. Dose escalation up to 16 Gy destroys human leukemic cells and rescue more than 60% of the mice with MLL-ENL leukemia. Thus, leukemic cells are more sensitive to FLASH

than HSC and it is tempting to speculate that this higher sensitivity is due to their highly proliferative status. Histological and flow cytometry analysis performed on the 40% of dead mice revealed no leukemic cells in the bone marrow of 16-Gy-FLASH-irradiated mice but showed severe aplasia suggesting a threshold during leukemia progression after which HSC could not restore hematopoiesis after 16-Gy-FLASH irradiation. Altogether, our results suggest that HSC in a normal bone marrow microenvironment can cope with 16-Gy-FLASH irradiation and reconstitute hematopoiesis, whereas HSC that share space with too many leukemic cells in the bone marrow have a decreased capacity to restore hematopoiesis after a 16-Gy-FLASH irradiation. This decreased capacity of HSC can be due to a cell autonomous deficiency related to the presence of leukemic cells or/and to a modification of the bone marrow HSC microenvironment by the leukemic cells (*36*) that will not be permissive to FLASH irradiation. Additionally, death signals released by dying irradiated leukemic cells might negatively affect HSC repopulation capacity after 16-Gy-FLASH irradiation.

In summary, this study, together with the accompanying paper on lung, proposes an innovation in radiotherapy using FLASH. The FLASH enhancement of dose-rate (by >800 fold) has led to completely unexpected biological effects as it destroys solid tumors or leukemia without having any apparent adverse effects on normal tissues as shown for lung and hematopoiesis. These results demonstrate that the duration of radiation is at least as critical to normal tissue response than the dose of radiation, thus challenging the current views about the effects of high doses of radiation on living animals. Three research challenges emerge from these two studies. A physicist's challenge: fundamental physics experiments need to be done to incorporate the notion of duration of dose deposition at such high dose-rates. A biologist's challenge: biological investigations should explain why even after 20 Gy total body irradiation, FLASH radiotherapy has no toxic effect on one of the most radiosensitive tissue and why irradiated animals exhibit long-term survival without any evidence of disease. A physician's challenge: our results indicate

an important new approach in radiotherapy that can be easily translated into clinics for non-invasive, reduced-toxicity and cost-effective cancer treatment.

MATERIALS AND METHODS

Mice

Four wild-type strains of mice, C57BL/6J, 129/Sv, C3H and Balb/c, known to have various radiation sensitivities, were used.

Total Body Irradiation (TBI) of mice

We use an experimental linear electron accelerator that can deliver high doses of radiation at conventional dose-rate (0.04 $Gy.s^{-1}$) or in short pulses at high dose-rate (50 $Gy.s^{-1}$) for Total Body Irradiation (TBI) using single doses ranging from 8 to 20 Gy. Female C57BL/6J (B6-CD45.2) mice and congenic mice for the CD45 locus (B6-CD45.1), 129/Sv, C3H, Balb/c and NSG were purchased from Charles River Laboratories (L'Arbresle, France) at the age of 10-12 weeks, and maintained in the animal care facilities of Institut Curie (agreement No. B91471-108, Ministère de l'Agriculture, Paris, France) or Institut Gustave-Roussy (agreement No. D94076-11) and authorization for experiments was obtained from the *Comité d'Ethique en Expérimentation Animale Paris-11*. 129/Sv mice engrafted with murine leukemic cells (GFP^+ MLL-ENL) and NSG mice engrafted with a human leukemia (bi-clonal proB and monocytic leukemia, with a MLL-AF4 fusion) were total body irradiated using the 4.5 MeV LINAC operated in either the FLASH or CDR mode. Mice were anaesthetized with isoflurane and immobilized in a dorsal position on 100 x 170 mm lucite beds by rubber straps around the legs, and set in a vertical position at 880 mm from the electron output window at the exact center of the radiation field. A laser was used for precise positioning of mice. The homogeneity of the radiation field over the mouse body surface was better than 5%. The depth-dose distribution of radiation was determined and is shown in Fig. S1.

Daily follow-up of each animal was performed over the time course of the experiments and the mice's weight was monitored twice per week: short-term follow-up was to 15 days, intermediate term to 4 months; long-term to one year post-irradiation. Interestingly except hair greying (Fig. S7), all animals exposed to ≤ 20 Gy FLASH were in excellent general condition for up to one year post-irradiation.

Sample preparation

At specific time points post-irradiation, bone marrow (BM) cells were flushed out of the humerus, femurs, tibias and pelvis using a syringe filled with PBS without calcium and magnesium, and suspended in PBS without calcium and magnesium supplemented with 2% heat-inactivated bovine serum (Gibco®, Life Technologies,) and filtered through a 40 µm cell sieve, except for one humerus that was fixed for histopathological preparation. Peripheral blood samples (PB) were collected by retro-orbital bleeding with heparinized capillary tubes under isoflurane anesthesia. PB and isolated BM were depleted of red blood cells using NH_4Cl solution (STEMCELL Technologies).

Histology

One humerus of each mouse was removed and immediately fixed in FineFIX (formalin-free fixative), then decalcified and embedded in paraffin. Then 4 µm sections were made from the blocks using a conventional microtome and placed on Superfrost®Plus slides for staining. After drying at 37°C for at least 2 h, slides were stained by Hematoxylin-Eosin-Safran (HES), for standard histological analysis. Hematoxylin stains nucleus in purple, eosin stains cytoplasm in pink and safran stains collagen fibers in orange. Microscope observations were performed under bright field and photographs taken using a color camera.

Bone marrow cell phenotype and cell sorting

For the determination of chimerism in transplantation experiments, BM cells were stained with FITC- or Alexa Fluor®700-conjugated anti-CD45.2 antibody (clone104, BD Biosciences or eBioscience) and with PE-, PercpCy5.5- or APC-conjugated anti-CD45.1 antibodies (A20, eBioscience). For lymphocyte cell population analyses, the BM cells were stained with PECy7-conjugated anti-B220 (RA3-6B2, eBioscience) and APC-conjugated anti-CD3 antibodies (145-2C11, eBioscience). For granulocyte/monocyte analyses, BM cells were stained with PECy7-conjugated anti-CD11b (M1/70, eBioscience) and APC-conjugated anti-Gr1 antibodies (RB6-8C5, ebioscience). For erythroid cells analyses, BM cells were stained with PE-conjugated anti-CD71 (C2, BD Biosciences) and APC-conjugated anti-Ter119 antibodies (Ter119, BD Biosciences). For progenitors, MPP, ST-HSC and LT-HSC analyses, the BM cells were first labeled with a biotinylated lineage cell detection cocktail (Miltenyi Biotec) and revealed by streptavidin-eFluor®450 (48-43-17, eBioscience) staining to discriminate between the positive and negative lineages. For GMP, CMP and MEP analyses, cells were then labeled with FITC-conjugated anti-CD34 (RAM34, eBioscience), APC-eFluor®780-conjugated anti-c-Kit (2B8, eBioscience), PE-conjugated anti-Sca-1 (E13-161-7, BD Biosciences) and PECy7-conjugated anti-CD16/32 (clone 93, eBioscience) antibodies. For CLP analyses, cells were labeled with APCeFluor®780-conjugated anti-c-Kit, PE-conjugated anti-Sca-1 and PE-Cy7-conjugated anti-IL7Rα (A7R34, eBioscience) antibodies. For MPP-, ST- and LT-HSC analyses, cells were labeled with APC-eFluor®780-conjugated anti-c-Kit, PE-Cy7 conjugated anti-Sca-1 (D7, eBioscience), PE-conjugated anti-Flk2 (A2F10-1, eBioscience), FITC-conjugated anti-CD150 (9D1, eBioscience) and APC-conjugated anti-CD48 (BCMIH48.1, eBioscience) antibodies. Flow cytometry was performed with a BD™LSRII flow cytometer (BD Biosciences) and data were analyzed with the FlowJo software.

For sorting the hematopoietic stem/progenitor cell population (HSCP, Lin^{neg}, c-Kit^{+}, $Sca1^{+}$, $Flk2^{-}$) or LSK cells (Lin^{neg}, $Sca1^{+}$, c-Kit^{+}), BM cells were first depleted from hematopoietic mature cells

using a lineage cell depletion kit (Miltenyi Biotec), and then labeled with PE-Cy7-conjugated anti-Sca-1, APC-conjugated anti-c-Kit (2B8 eBioscience) and PE-conjugated anti-Flk2, and sorted using Moflo (Dako).

Immunofluorescence

10^3 cells sorted from the bone marrow of sham-irradiated, 8 Gy CDR-, 8 Gy and 16 Gy FLASH-irradiated mice were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 10 min at room temperature (RT), washed with PBS and deposited on Superfrost®Plus Gold poly-lysine coated slide. After 12 h at 37°C, cells were permeabilised with 0.2% Triton X-100 in PBS for 15 min and blocked for 1 h in PBS containing 0.2% TritonX-100, 2% Fetal Calf Serum (FCS), 2% Bovine Serum Albumin (BSA). Cells were incubated for 1 h at 37°C with mouse anti-phospho-histone H2AX (Ser139) (Millipore), or mouse anti-human Ki67 (BD Biosciences), or rabbit anti-53BP1 (Novus Biologicals), or rabbit anti-cleaved caspase-3 antibodies (Cell Signaling Technology, Ozyme) at a 1:500, 1:50, 1:500 or 1:100 dilution, respectively, in PBS supplemented with 0.2% Triton X-100, 2% FCS and 2% BSA. This was followed by incubation with a secondary anti-mouse antibody coupled to Alexa-Fluor®488 (Invitrogen™, Life Technologies) or anti-rabbit antibody coupled to Alexa-Fluor®594 (Invitrogen™) and counterstained with DAPI (1 $\mu g.ml^{-1}$). Coverslips were mounted in Fluorescent Mounting Medium (SouthernBiotech, Clinisciences). Image acquisition was performed with a Zeiss Axio Imager microscope driven by MetaSystems software or with a Leica confocal microscope SPE. The images were processed with ImageJ software (http://rsbweb.nih.gov/ij/).

Clonogenic cell survival assays and LTC-IC assays

Clonogenic assays were performed on HSPC cells isolated from bone marrow of CDR- and FLASH-irradiated mice at doses ranging from 0 to 16 Gy. HSPC cells were plated at 10^3 cells in 35 mm diameter petri dishes (3 dishes per dose) and cultured in Methocult™GF M3434 medium (STEMCELL Technologies) at 37°C and 5% CO_2 for 7-10 days to form hematopoietic progenitor

colonies (CFCs). Colonies were counted under a microscope. The surviving fraction was determined by dividing the number of colonies of the irradiated cells by the number of colonies of sham-irradiated cells and survival curves were fitted by a linear-quadratic model. These fits were used to determine the doses that induced 37% of survival (D_{37}) for the different irradiation conditions. To determine intrinsic radiosensitivity, HSPC cells isolated from bone marrow of sham-irradiated mice were then CDR or FLASH *ex vivo* irradiated using the same dose range and handled in the same manner as described above.

LTC-IC assays were performed on whole BM cells. Suitable numbers of cells ($7.5x10^3$ to $6x10^4$ cells/12 wells/dose) from sham-, 8 Gy CDR- and 8 Gy/16 Gy FLASH-irradiated mice were seeded in monolayers of MS5 cells plated in a 96-well plate ($2.5x10^3$ cells per well) and cultured in a MyeloCult™ M5300 medium supplemented with 1 µM hydrocortisone (STEMCELL Technologies) and 1% Penicillin-Streptomycin. The co-culture was maintained at 33°C, 5 % CO_2 for 4 weeks with weekly replacements of one-half of the medium. At the end of the culture period, the adherent and non-adherent cells were harvested from each individual well and assayed in methylcellulose cultures for CFCs (STEMCELL Technologies). The frequency of negative wells (no detectable CFCs) was then determined and the frequency of LTC-IC in the initial cell population was calculated by Poisson statistics as the reciprocal.

Cytogenetic analysis on BM cells

LSK cells were isolated from the bone marrow of sham-, 8 Gy CDR-, 8 Gy and 16 Gy FLASH-irradiated mice at 6 months post-TBI. Cells were plated in 6-well plates ($5x10^2$ cells per well, 3 wells per dose) and cultured for 6 days at 33°C, 5% CO_2 in StemSpan™ serum-free medium (STEMCELL Technologies) supplemented with mouse IL11 (100 $ng.ml^{-1}$) (STEMCELL Technologies), mouse Flt3L (100 $ng.ml^{-1}$), mouse SCF (50 $ng.ml^{-1}$) (Miltenyi Biotech), LDL solution (10 $ng.ml^{-1}$) (Sigma) and 1% Penicillin-Streptomycin. At the end of culture period, cells were exposed to 10 $\mu g.ml^{-1}$ colchicine (Sigma) for 3 h, collected, harvested and incubated in 75

mM KCl and FCS (1:6 v/v) for 15 min at 37°C, fixed in ethanol/acetic acid (3:1 v/v), spread on cold, clean slides and hybridized with the 21X-Cyte Mouse Multicolor FISH Probe Kit (MetaSystems,) following manufacturer's instructions. Briefly, slides were denatured in 0.07 N NaOH and then rinsed in solution of SSC and graded ethanol. Meanwhile, probe mix was denatured for 5 min at 75°C. Slides were then hybridized in a humidified chamber at 37°C for 72 h and counterstained with DAPI (1 $\mu g.ml^{-1}$). Metaphases were finally visualized and captured using an Axio Imager microscope Zeiss driven by MetaSystems hardware. Karyotyping and cytogenetic analysis was performed by means of the ISIS software (MetaSystems). At least, 40 metaphase spreads were analyzed for each experimental point.

Transplantations

Transplantations were performed in several ways. In brief, 24 h after TBI 8 Gy/16 Gy CDR- and 8 Gy/16 Gy FLASH-irradiated mice (CD45.1, CD45.2) were retro-orbitally transplanted with $5x10^5$ BM cells from sham-irradiated age–matched mice (CD45.1). Peripheral blood chimerism was assessed 3 months after transplantation.

Primary lethally irradiated recipient mice (CD45.2) were transplanted non-competitively with 10^4 LSK cells (CD45.1) from the bone marrow of 8 Gy CDR- or 8 Gy/16 Gy FLASH- irradiated mice 6 months post TBI. At 4 months post-transplantation, mice were examined. Bone marrow cell numbers were estimated by counting in Malassez chambers. The percentages of mature cells, progenitors including MPP, ST- and LT-HSCs in bone marrow were determined as described above.

Primary lethally irradiated recipient mice (CD45.2, CD45.1) were retro-orbitally transplanted competitively with $2.5x10^5$ CD45.1 competitors and $2.5x10^6$ (CD45.2) donor cells from 8 Gy CDR- and 8 Gy/16 Gy FLASH-irradiated mice 6 months post-TBI. Four months post-transplantation, primary recipient mice were sacrificed and the percentage of donor-derived (CD45.2) cells in the lineage negative cells was determined.

MLL-ENL leukemia and *in vivo* fluorescence imaging

Murine leukemic cells that express the human MLL-ENL fusion protein were transduced with a lentivirus expressing GFP (MND-GFP lentivirus) and amplified *in vivo* in syngeneic 129/Sv mice. Non-conditioned 129/Sv mice were transplanted with $5x10^3$ GFP^+ MLL-ENL leukemic cells and were then irradiated at 8 Gy CDR or 8/16 Gy FLASH at 6 days post-engraftment, i. e., during the period of leukemia latency, or at 15 days, i. e., just before the burst of leukemia. MLL-ENL leukemia growth was monitored using fluorescent tomography with an IVIS Spectrum® apparatus (Perkin-Elmer, Waltham, Massachusetts). Images were quantified as photons.s^{-1} using the LivingImage software (Caliper Life Sciences). Before imaging mice were shaved to avoid high GFP background signal. Four months post-TBI-FLASH, bone marrow of survival mice was harvested and more than $5x10^6$ cells were analyzed in flow cytometry to track GFP^+ MLL-ENL leukemic cells. $5x10^5$ of these bone marrow cells were transplanted into secondary 129/Sv recipient mice and their bone marrow cells were analyzed in flow cytometry 4 months after transplantation to verify the complete cure of leukemia.

Human primary leukemia

NOD.Cg-Prkdc(scid)Il2rγ(tm1Wjll)/SzJ (abbreviated NSG) mice (The Jackson Laboratory, Bar Harbor, USA) were housed in pathogen-free animal facilities at CEA, Fontenay-aux-Roses, France. Mice were anesthetized with isoflurane before intravenous injection of human leukemic cells. Experimental procedures were performed in compliance with the French Agriculture Ministry and local ethics committee regulations (Authorization number 12-015). Leukemia was allowed to grow until > 95% of BM was positive for human leukemic cells thus expressing either CD34 (clone 4H11 eBioscience) and CD19 (clone J3119 Beckman Coulter) or CD33 (clone D3HL60.251 Beckman Coulter). Mice were then exposed to 8 Gy CDR or 16 Gy FLASH. After 24 h BM was harvested and intra-femoraly retransplanted into naïve NSG mice. Leukemia growth was monitored by intra-femoral puncture.

Statistical methods.

Data from immunofluorescence experiments were analyzed using unpaired t-test (GraphPadPrism, GraphPad Software, Inc.). Phenotyping data were analyzed using Kruskal and Wallis test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Values of $p < 0.05$ were considered statistically significant. Unless otherwise stated, data are presented as Mean ± s.e.m. or median values. Student's *t*-tests or Kruskal-Wallis tests were used for statistical significance.

LIST OF SUPPLEMENTARY MATERIALS

http://www.sciencetranslationalmedicine.org/cgi/content/xxxx

- § S1. Linear electron accelerator set-up and dosimetry,
- Fig. S1. Setup for Total Body Irradiation of mice using the LINAC.
- Fig. S2. Time-dependent formation of the $MV^{\bullet 2+}$ radical and depth dose distribution.
- Fig. S3. Survival curves of 16 Gy CDR-TBI mice transplanted or not transplanted with $5x10^5$ hematopoietic cells 24 h after TBI.
- Fig. S4. Histochemistry of decalcified humerus of sham-irradiated and CDR or FLASH irradiated mice.
- Fig. S5. Kinetics of γH2AX foci in LT-HSCs isolated from 8 or 16 Gy TBI-CDR or TBI-FLASH mice.
- Fig. S6. Time-dependent evolution of the weight of mice after TBI-CDR or TBI-FLASH.
- Fig. S7. Picture of representative C57BL/6J mice 4 months post-CDR or -FLASH TBI.
- Fig. S8. Percentage of mature hematopoietic cells 6 months after TBI at 8 Gy CDR, 8 Gy FLASH or 16 Gy FLASH.
- Fig. S9. Fold change of LTC-IC frequency 6 months after TBI at 8 Gy CDR, 8 Gy FLASH or 16 Gy FLASH.
- Fig. S10. Chimerism of mature hematopoietic cells in bone marrow of CD45.2 recipient mice 4 months after transplantation of LSK from CD45.1 mice total body irradiated 6 months before the LSK transplantation.
- Fig. S11. Percentage of progenitor and mature hematopoietic cells in the recipient CD45.2 mice described in Fig. S10.
- Fig. S12. Representative IVIS imaging, 38 or 51 days after 8 Gy TBI-CDR or 16 Gy TBI-FLASH, of mice engrafted with $5x10^3$ GFP^+ MLL-ENL cells.

REFERENCES

1. H. B. Stone, C. N. Coleman, M. S. Anscher, W. H. McBride, Effects of radiation on normal tissue: consequences and mechanisms. *The lancet oncology* **4**, 529-536 (2003); published online EpubSep (
2. J. Yarnold, M. C. Brotons, Pathogenetic mechanisms in radiation fibrosis. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology* **97**, 149-161 (2010).
3. R. Baskar, K. A. Lee, R. Yeo, K. W. Yeoh, Cancer and radiation therapy: current advances and future directions. *International journal of medical sciences* **9**, 193-199 (2012).
4. P. Maier, F. Wenz, C. Herskind, Radioprotection of normal tissue cells. *Strahlentherapie und Onkologie*, Radioprotection of normal tissue cells (2014), epub.
5. D. De Ruysscher, C. Faivre-Finn, U. Nestle, C. W. Hurkmans, C. Le Pechoux, A. Price, S. Senan, European Organisation for Research and Treatment of Cancer recommendations for planning and delivery of high-dose, high-precision radiotherapy for lung cancer. *J Clin Oncol* **28**, 5301-5310 (2010).
6. E. C. Ko, K. Forsythe, M. Buckstein, J. Kao, B. S. Rosenstein, Radiobiological rationale and clinical implications of hypofractionated radiation therapy. *Cancer Radiother* **15**, 221-229 (2011).
7. J. B. Maxhimer, D. R. Soto-Pantoja, L. A. Ridnour, H. B. Shih, W. G. Degraff, M. Tsokos, D. A. Wink, J. S. Isenberg, D. D. Roberts, Radioprotection in normal tissue and delayed tumor growth by blockade of CD47 signaling. *Science translational medicine* **1**, 3ra7 (2009).
8. A. C. Begg, F. A. Stewart, C. Vens, Strategies to improve radiotherapy with targeted drugs. *Nat Rev Cancer* **11**, 239-253 (2011).
9. H. Geiger, S. A. Pawar, E. J. Kerschen, K. J. Nattamai, I. Hernandez, H. P. Liang, J. A. Fernandez, J. A. Cancelas, M. A. Ryan, O. Kustikova, A. Schambach, Q. Fu, J. Wang, L. M. Fink, K. U. Petersen, D. Zhou, J. H. Griffin, C. Baum, H. Weiler, M. Hauer-Jensen, Pharmacological targeting of the thrombomodulin-activated protein C pathway mitigates radiation toxicity. *Nature medicine* **18**, 1123-1129 (2012).
10. J. H. Kim, R. K. Thimmulappa, V. Kumar, W. Cui, S. Kumar, P. Kombairaju, H. Zhang, J. Margolick, W. Matsui, T. Macvittie, S. V. Malhotra, S. Biswal, NRF2-mediated Notch pathway activation enhances hematopoietic reconstitution following myelosuppressive radiation. *The Journal of clinical investigation* **124**, 730-741 (2014).
11. P. L. Doan, H. A. Himburg, K. Helms, J. L. Russell, E. Fixsen, M. Quarmyne, J. R. Harris, D. Deoliviera, J. M. Sullivan, N. J. Chao, D. G. Kirsch, J. P. Chute, Epidermal growth factor regulates hematopoietic regeneration after radiation injury. *Nature medicine* **19**, 295-304 (2013).
12. T. M. Fliedner, W. Nothdurft, W. Calvo, The development of radiation late effects to the bone marrow after single and chronic exposure. *International journal of radiation biology and related studies in physics, chemistry, and medicine* **49**, 35-46 (1986).
13. E. L. Travis, L. J. Peters, J. McNeill, H. D. Thames, Jr., C. Karolis, Effect of dose-rate on total body irradiation: lethality and pathologic findings. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology* **4**, 341-351 (1985).
14. T. Okita, Review of thirty years study of Hiroshima and Nagasaki atomic bomb survivors. II. Biological effects. A. Acute effects. *Journal of radiation research* **16 Suppl**, 49-66 (1975).

15. E. D. Thomas, H. L. Lochte, Jr., J. H. Cannon, O. D. Sahler, J. W. Ferrebee, Supralethal whole body irradiation and isologous marrow transplantation in man. *The Journal of clinical investigation* **38**, 1709-1716 (1959.
16. M. R. Litzow, W. S. Perez, J. P. Klein, B. J. Bolwell, B. Camitta, E. A. Copelan, R. P. Gale, S. A. Giralt, A. Keating, H. M. Lazarus, D. I. Marks, P. L. McCarthy, C. B. Miller, G. Milone, H. G. Prentice, J. A. Russell, K. R. Schultz, M. E. Trigg, D. J. Weisdorf, M. M. Horowitz, Comparison of outcome following allogeneic bone marrow transplantation with cyclophosphamide-total body irradiation versus busulphan-cyclophosphamide conditioning regimens for acute myelogenous leukaemia in first remission. *British journal of haematology* **119**, 1115-1124 (2002).
17. E. C. Guinan, C. M. Barbon, L. A. Kalish, K. Parmar, J. Kutok, C. J. Mancuso, L. Stoler-Barak, E. E. Suter, J. D. Russell, C. D. Palmer, L. C. Gallington, A. Voskertchian, J. A. Vergilio, G. Cole, K. Zhu, A. D'Andrea, R. Soiffer, J. P. Weiss, O. Levy, Bactericidal/permeability-increasing protein (rBPI21) and fluoroquinolone mitigate radiation-induced bone marrow aplasia and death. *Science translational medicine* **3**, 110ra118 (2011).
18. V. Favaudon, H. Tourbez, C. Houee-Levin, J. M. Lhoste, CO2.- radical induced cleavage of disulfide bonds in proteins. A gamma-ray and pulse radiolysis mechanistic investigation. *Biochemistry* **29**, 10978-10989 (1990).
19. T. N. Das, T. K. Ghanty, H. Pal, Reactions of methyl viologen dication (MV2+) with H atoms in aqueous solution: mechanism derived from pulse radiolysis measurements and a initio MO calculations. *J. Phys. Chem.* **A 107**, 5998-6006 (2003).
20. J. P. Williams, S. L. Brown, G. E. Georges, M. Hauer-Jensen, R. P. Hill, A. K. Huser, D. G. Kirsch, T. J. Macvittie, K. A. Mason, M. M. Medhora, J. E. Moulder, P. Okunieff, M. F. Otterson, M. E. Robbins, J. B. Smathers, W. H. McBride, Animal models for medical countermeasures to radiation exposure. *Radiation research* **173**, 557-578 (2010).
21. K. Suzuki, M. Ojima, S. Kodama, M. Watanabe, Radiation-induced DNA damage and delayed induced genomic instability. *Oncogene* **22**, 6988-6993 (2003).
22. C. Lavau, S. J. Szilvassy, R. Slany, M. L. Cleary, Immortalization and leukemic transformation of a myelomonocytic precursor by retrovirally transduced HRX-ENL. *The EMBO journal* **16**, 4226-4237 (1997).
23. A. Jaracz-Ros, D. Lewandowski, V. Barroca, C. Lavau, P. H. Romeo, MLL-ENL leukemia burden initiated in femoral diaphysis and preceded by mature B-cell depletion. *Haematologica* **96**, 1770-1778 (2011).
24. D. Bonnet, J. E. Dick, Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature medicine* **3**, 730-737 (1997).
25. K. A. Biedermann, J. R. Sun, A. J. Giaccia, L. M. Tosto, J. M. Brown, scid mutation in mice confers hypersensitivity to ionizing radiation and a deficiency in DNA double-strand break repair. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 1394-1397 (1991).
26. F. Trinter, M. S. Schoffler, H. K. Kim, F. P. Sturm, K. Cole, N. Neumann, A. Vredenborg, J. Williams, I. Bocharova, R. Guillemin, M. Simon, A. Belkacem, A. L. Landers, T. Weber, H. Schmidt-Bocking, R. Dorner, T. Jahnke, Resonant Auger decay driving intermolecular Coulombic decay in molecular dimers. *Nature* **505**, 664-666 (2014).
27. K. Gokhberg, P. Kolorenc, A. I. Kuleff, L. S. Cederbaum, Site- and energy-selective slow-electron production through intermolecular Coulombic decay. *Nature* **505**, 661-663 (2014).
28. M. Scorsetti, F. Alongi, S. Castiglioni, A. Clivio, A. Fogliata, F. Lobefalo, P. Mancosu, P. Navarria, V. Palumbo, C. Pellegrini, S. Pentimalli, G. Reggiori, A. M. Ascolese, A. Roggio, S. Arcangeli, A. Tozzi, E. Vanetti, L. Cozzi, Feasibility and early clinical

assessment of flattening filter free (FFF) based stereotactic body radiotherapy (SBRT) treatments. *Radiation oncology* **6**, 113 (2011).
29. B. M. Prendergast, M. C. Dobelbower, J. A. Bonner, R. A. Popple, C. J. Baden, D. J. Minnich, R. J. Cerfolio, S. A. Spencer, J. B. Fiveash, Stereotactic body radiation therapy (SBRT) for lung malignancies: preliminary toxicity results using a flattening filter-free linear accelerator operating at 2400 monitor units per minute. *Radiation oncology* **8**, 273 (2013).
30. T. Prempree, A. Michelsen, T. Merz, The repair time of chromosome breaks induced by pulsed x-rays on ultra-high dose-rate. *International journal of radiation biology and related studies in physics, chemistry, and medicine* **15**, 571-574 (1969).
31. S. Auer, V. Hable, C. Greubel, G. A. Drexler, T. E. Schmid, C. Belka, G. Dollinger, A. A. Friedl, Survival of tumor cells after proton irradiation with ultra-high dose rates. *Radiation oncology* **6**, 139 (2011).
32. T. E. Schmid, G. Dollinger, W. Beisker, V. Hable, C. Greubel, S. Auer, A. Mittag, A. Tarnok, A. A. Friedl, M. Molls, B. Roper, Differences in the kinetics of gamma-H2AX fluorescence decay after exposure to low and high LET radiation. *International journal of radiation biology* **86**, 682-691 (2010).
33. T. E. Schmid, G. Dollinger, V. Hable, C. Greubel, O. Zlobinskaya, D. Michalski, S. Auer, A. A. Friedl, E. Schmid, M. Molls, B. Roper, The effectiveness of 20 mev protons at nanosecond pulse lengths in producing chromosome aberrations in human-hamster hybrid cells. *Radiation research* **175**, 719-727 (2011).
34. O. Zlobinskaya, S. Girst, C. Greubel, V. Hable, C. Siebenwirth, D. W. Walsh, G. Multhoff, J. J. Wilkens, T. E. Schmid, G. Dollinger, Reduced side effects by proton microchannel radiotherapy: study in a human skin model. *Radiation and environmental biophysics* **52**, 123-133 (2013).
35. A. Ehninger, A. Trumpp, The bone marrow stem cell niche grows up: mesenchymal stem cells and macrophages move in. *The Journal of experimental medicine* **208**, 421-428 (2011).
36. K. Schepers, E. M. Pietras, D. Reynaud, J. Flach, M. Binnewies, T. Garg, A. J. Wagers, E. C. Hsiao, E. Passegue, Myeloproliferative neoplasia remodels the endosteal bone marrow niche into a self-reinforcing leukemic niche. *Cell stem cell* **13**, 285-299 (2013).

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Author contributions: P-H.R. and M-C.V. designed the project, analyzed data and wrote the manuscript. V.F. set up the LINAC facility and dosimetry, organized all the irradiations and wrote the manuscript. N.G., F.H., S.G.M., T.H.V.T. and D.L. organized, performed and analyzed most experiments and wrote the manuscript. S.R-M. performed initial experiments. M-C.V., T.H.V.T, A.A, B.P performed animal sampling and the daily follow-up of animals. R.M. was involved in dosimetry measurements. J.H. and J.B. provided support to perform the project and analyzed the results. F.P and B.U. organized, performed and analyzed experiments using NSG mice.

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FIGURES AND FIGURE LEGENDS

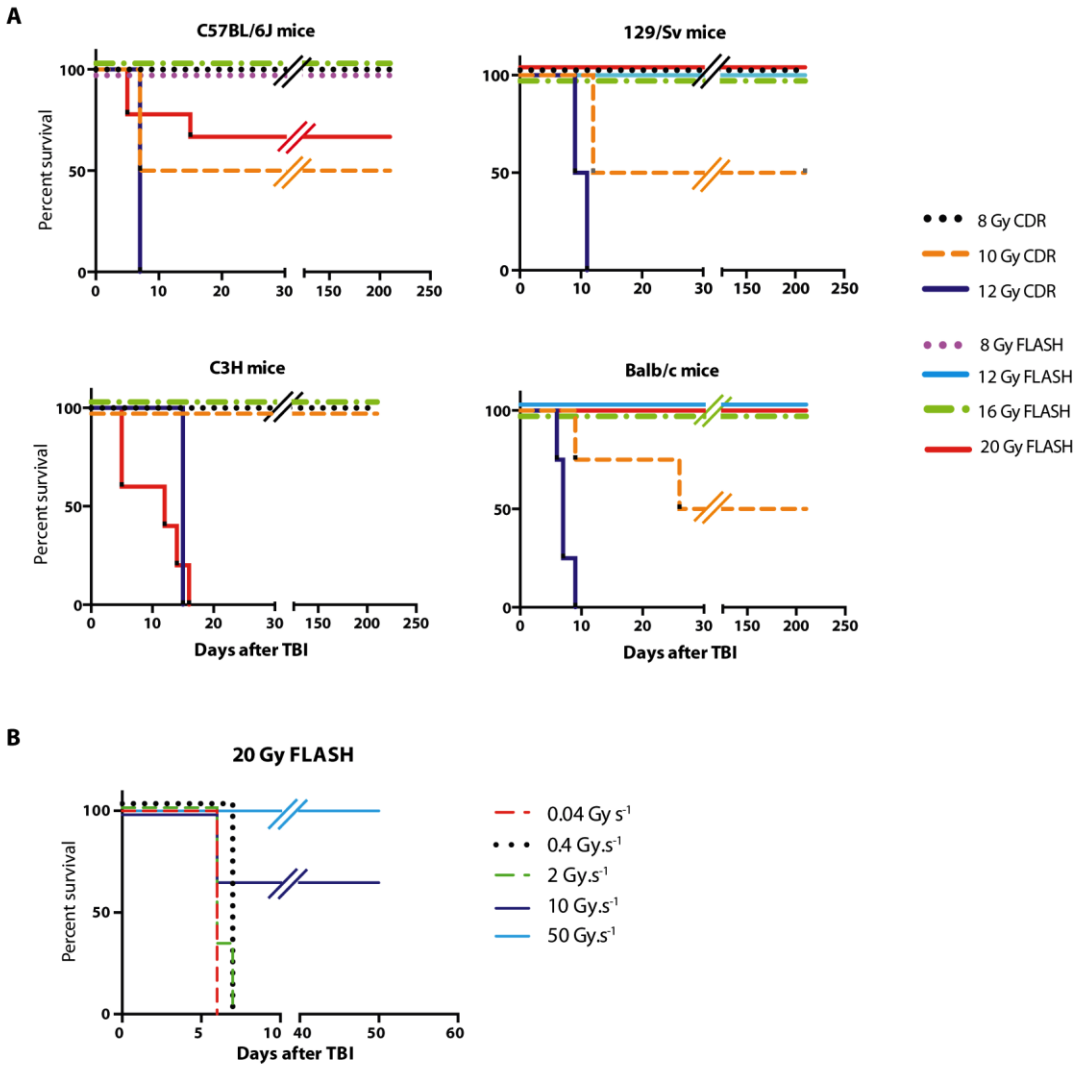

Fig. 1. Total body irradiation with FLASH allows high dose escalation without apparent adverse effects to health. (A) Survival of C57BL/6J (n = 91), 129/Sv (n = 32), C3H (n = 24) and Balb/c (n = 26) mice after TBI using increasing doses of CDR or FLASH irradiation. **(B)** Survival of C57BL/6J FLASH-irradiated at 20 Gy using the indicated dose-rate (n = 4 mice for each dose-rate).

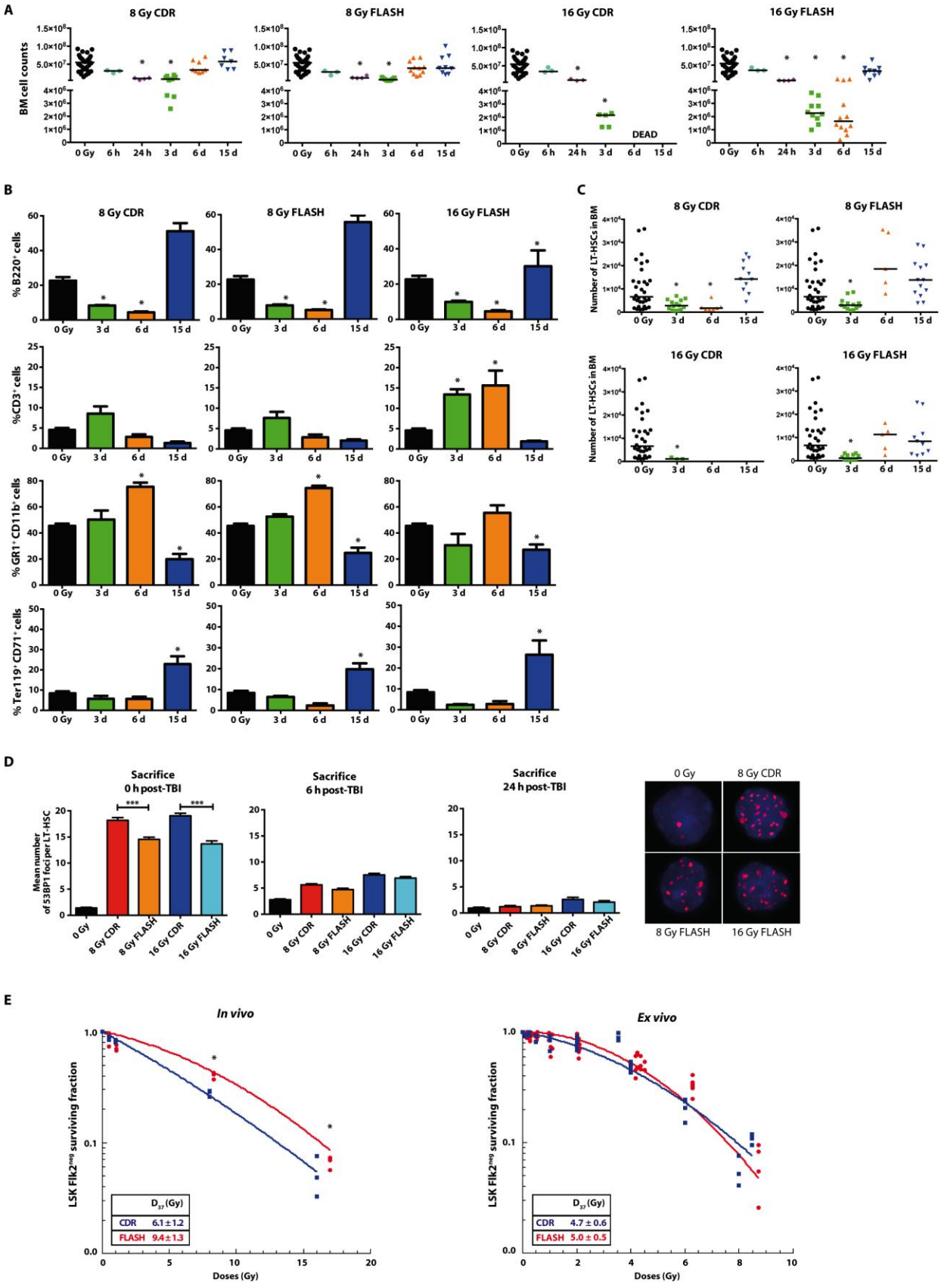
A
8 Gy CDR
8 Gy FLASH
16 Gy CDR
16 Gy FLASH
BM cell counts
DEAD
0 Gy
6 h
24 h
3 d
6 d
15 d
B
% B220+ cells
%CD3+ cells
% GR1+ CD11b+ cells
% Ter119+ CD71+ cells
C
Number of LT-HSCs in BM
D
Sacrifice 0 h post-TBI
Sacrifice 6 h post-TBI
Sacrifice 24 h post-TBI
Mean number of 53BP1 foci per LT-HSC
0 Gy
8 Gy CDR
8 Gy FLASH
16 Gy CDR
16 Gy FLASH
E
In vivo
Ex vivo
LSK Flk2neg surviving fraction
Doses (Gy)
D37 (Gy)
CDR 6.1 ± 1.2
FLASH 9.4 ± 1.3
CDR 4.7 ± 0.6
FLASH 5.0 ± 0.5

Fig. 2. Early haematopoietic recovery after TBI-CDR or TBI-FLASH of C57BL/6J mice. (A) Kinetics of bone marrow cell counts 6 h to 15 days after 8 or 16 Gy CDR-TBI or FLASH-TBI of C57BL/6J mice. **(B)** Kinetics of mature hematopoietic cells in C57BL/6J mice after TBI at 8 Gy CDR or 8 or 16 Gy-FLASH (3 experiments, n = 8 for each type of irradiation). **(C)** Kinetics of $LSKFlk2^{-}CD150^{+}CD48^{-}$ LT-HSC recovery in C57BL/6J mice after 8 or 16 Gy TBI-CDR or TBI-FLASH (3 experiments, n = 8 for each type of irradiation). **(D)** Kinetics of 53BP1 foci in $LSKFlk2^{-}CD150^{+}CD48^{-}$ LT-HSCs isolated from 8 Gy or 16 Gy TBI-CDR or TBI-FLASH mice (n = 120 cells analyzed for each experiment). Right pictures are images representative of 53BP1 foci in LT-HSCs after CDR or FLASH irradiation at different doses. **(E)** Clonogenic cell survival assays on HSPCs ($LSKFlk2^{-}$) isolated from TBI-CDR or TBI-FLASH mice (left panel) or clonogenic cell survival assays on HSPCs CDR or FLASH irradiated *ex vivo* (right panel). The different D_{37} are indicated for each irradiation condition (3 experiments) (Student's *t*-test, $^{*}P<0.01$).

When significant, difference between sham-irradiated and irradiated groups is indicated. **A**, Student's *t*-test, $^{*}P<0.01$, **B**, Kruskal-Wallis test, $^{*}P<0.05$, **C**, Kruskal-Wallis test, $^{*}P<0.05$, **D**, Student's *t*-test, $^{***}P<0.001$.

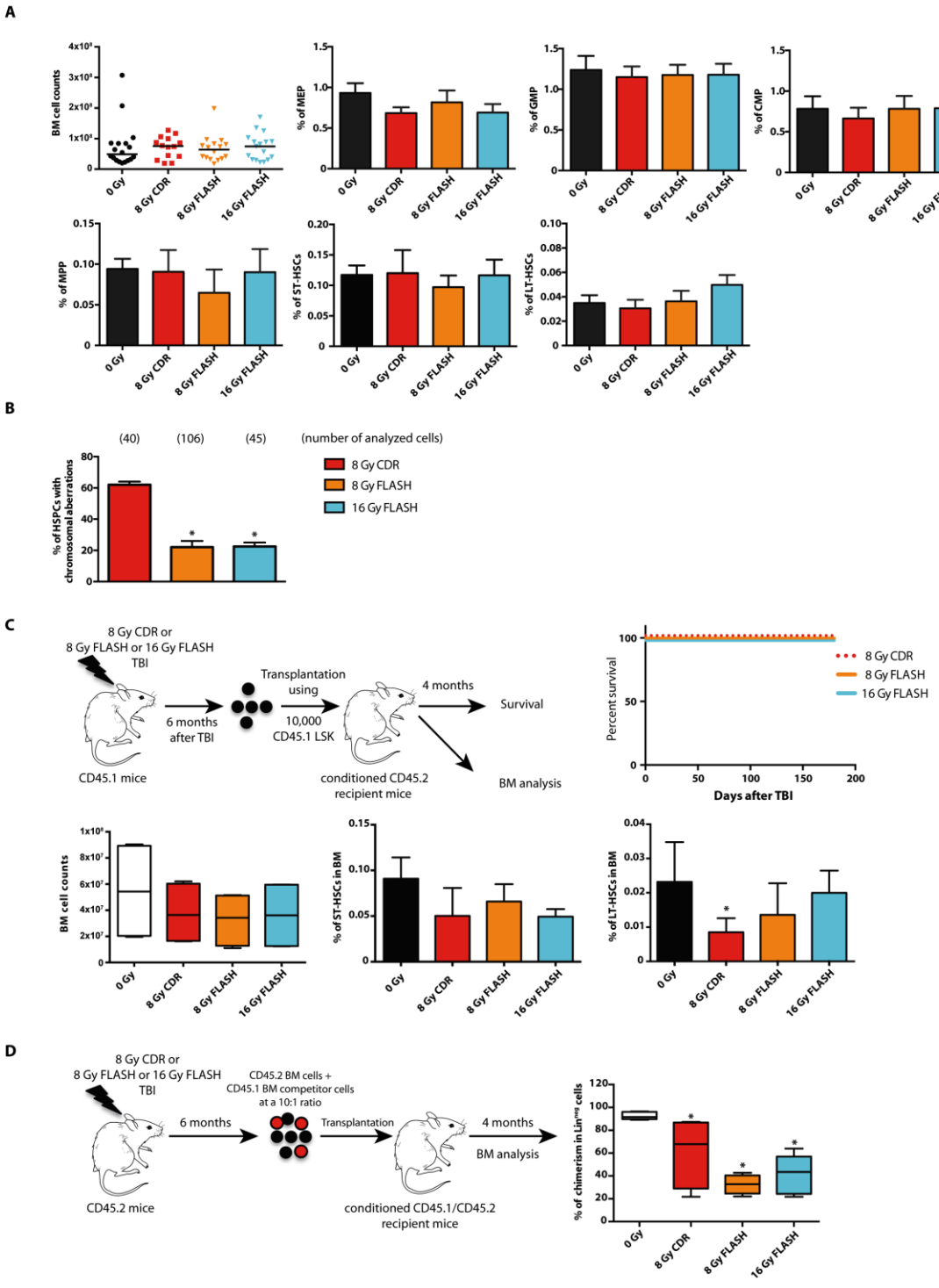

Fig. 3. Long-term effects of CDR and FLASH irradiations on hematopoiesis and hematopoietic stem cells. **(A)** Six months after TBI at 8 Gy CDR, 8 Gy FLASH or 16 Gy FLASH, bone marrow cell counts and percentages of MEP, GMP, CMP, MPP, ST-HSCs and

LT-HSCs (n = 6 mice for each type of irradiation). Differences between groups were not significant (Kruskal-Wallis test). **(B)** Percentage of bone marrow HSPCs with chromosomal aberrations 6 months after TBI at 8 Gy CDR, 8 Gy FLASH or 16 Gy FLASH (n = 3 experiments). Mean ± s.e.m. (Student's *t*-test, $^{*}P<0.05$). The number of cells blindly analyzed is indicated. **(C)** Schematic diagram showing the protocol. Survival of the recipient CD45.2 mice (upper right panel), bone marrow cell counts and percentage of ST-HSCs and LT-HSCs in the recipient CD45.2 mice (lower panels) (n = 4 recipient mice). When significant, difference between groups is indicated (Kruskal-Wallis test $^{*}P<0.05$). **(D)** Schematic diagram showing the protocol. Four months after transplantation, chimerism in the lin^{neg} bone marrow cells was determined (n = 4). Significant differences between sham-irradiated and irradiated groups are indicated (Kruskal-Wallis test $^{*}P<0.05$).

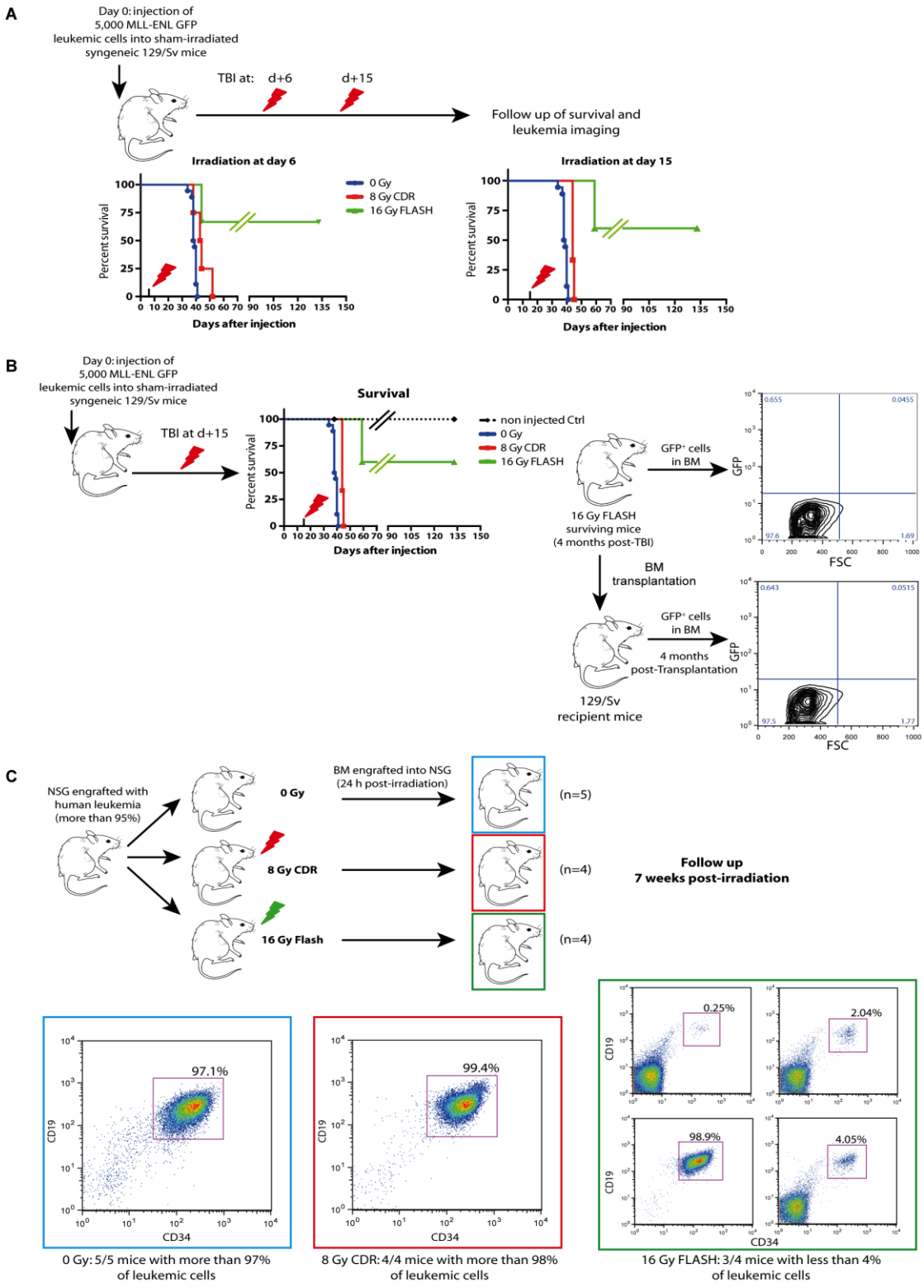

Fig. 4. FLASH radiotherapy allows dose escalation that has anti-leukemic effects on mouse and human leukemia. (A) Schematic diagram showing the protocol. Survival of mice

irradiated with the maximal CDR tolerated dose, *i.e.* 8 Gy CDR or 16 Gy FLASH, 6 days (initiation of leukemia) or 15 days (progression of leukemia) post-injection of GFP^+ MLL-ENL leukemic cells (n = 8 mice per group). **(B)** Four months after TBI, representative analysis of GFP^+ leukemic cells in surviving mice (upper right diagram). Bone marrow from surviving mice was transplanted into non-irradiated syngeneic 129/Sv mice. No leukemia developed and, 4 months after transplantation, a representative analysis of GFP^+ leukemic cells in bone marrow is shown (lower right diagram) (n = 4 mice for each experiments). **(C)** NSG mice were intravenously injected with $2x10^6$ human leukemic cells. After 8-10 weeks, human leukemia develops and mice were total body irradiated with 8 Gy CDR or 16 Gy FLASH. 24 h after irradiation, $5x10^5$ cells of total bone marrow were transplanted by intra-femoral route into NSG mice and progression of leukemia was studied 7 weeks after this injection.

Abstract

Wound healing and carcinogenesis are defined as complex, adaptive processes which are controlled by intricate communications between the host and the tissue microenvironment. A number of phenotypic similarities are shared by wounds and cancers in cellular signaling and gene expression. Radiotherapy is the second most effective modality of cancer treatment after surgery and can be used, either alone or in combination with chemotherapy. Recent findings suggest that radiotherapy apart from tumor cell death also rapidly and persistently modifies the tissue microenvironment. These modifications affect cell phenotype, tissue metabolism, bidirectional exchanges and signaling events between cells.

The complex interactions between stromal cells and cancer cells are of immense interest and in **The First Part of My Thesis**, I tried to explore the crosstalk between stromal and carcinoma cells in response to radiotherapy by genetic modulation of the stroma and irradiation. We found that fibroblasts, irrespective of their RhoB status, do not modulate intrinsic radiosensitivity of TC-1 but produce diffusible factors able to modify tumor cell fate. Then we found that Wt and RhoB deficient fibroblasts stimulated TC-1 migration through distinct mechanisms respectively, TGF-β1 and MMP-mediated. We also found that co-irradiation of fibroblasts and TC-1 abrogated the pro-migratory phenotype by repression of TGF-β and MMP secretion. This result is highly relevant to the clinical situation and suggests that conversely to, the current view; irradiated stroma would not enhance carcinoma migration and could be manipulated to promote anti-tumor immune response.

Secondly, our *in vivo* experiments, tends to confirm the *in vitro* data showing that irradiated tumor bed does not stimulate tumor growth and escape. Our results also challenges the view that irradiated stroma would promote migration of carcinoma cells as we show that independently from their genotype co-irradiation of fibroblasts and carcinoma cells repressed carcinoma cell migration and confirmations studies are currently performed *in vivo*.

The Third Part of My Project, was dedicated to investigate the effect on CTC release after radiotherapy. Consistently with the results reported after surgery , the number of CTC increases in the blood stream after radiotherapy probably due to radiation-induced vascular injury induced or/and by EMT induction in tumor cells but these cells seemed to be entrapped into the cardiac cavity. The significance of these CTC to metastatic development is still under investigation but there is evidence for a metastasis-promoting effect of RT from animal studies.

Thus the microenvironment can exert antagonist stimulatory or inhibitory effects on malignant cells.

Key words: Wound healing, NSCLC (Non Small Cell Lung Carcinoma), Rho, TGF-β 1, MMP, Microenvironment, CTCs (Circulating Tumor Cells) ,RT(Radiotherapy)

Résumé

Les phénomènes cicatriciels et de carcinogenèse partagent des points communs et peuvent être définis comme des processus complexes et adaptatif régulés par les interactions entre l'hôte et le microenvironnement tissulaire. Après la chirurgie, la radiothérapie est la seconde modalité la plus efficace dans le traitement du cancer et une approche thérapeutique multimodale impliquant chirurgie, radiothérapie et chimiothérapie est aujourd'hui classiquement utilisée. Des résultats récents suggèrent que, en plus des effets létaux sur les cellules tumorales, la radiothérapie modifie le microenvironnement tissulaire. Ces modifications affectent le phénotype cellulaire, le métabolisme des tissus, et les événements de signalisation entre les cellules.

Les interactions complexes entre les cellules stromales et les cellules cancéreuses suscitent beaucoup d'intérêt et dans **la première partie de ma thèse**, j'ai exploré les interactions entre stroma et cellules de carcinome en réponse à la radiothérapie par modulation génétique du stroma après irradiation. J'ai constaté que les fibroblastes, indépendamment de leur statut RhoB, ne modulaient pas la radiosensibilité intrinsèque des TC- 1, mais produisaient des facteurs diffusibles capables de modifier le devenir des cellules tumorales. Ensuite, j'ai constaté que fibroblastes sauvages et RhoB déficients stimulaient la migration des TC-1 par des mécanismes distincts, impliquant respectivement TGF- β1 et MMP. J'ai également constaté que la co-irradiation, des fibroblastes et des TC- 1, abrogait le phénotype pro-migratoire des TC-1 par répression de la sécrétion du TGF- β et des MMP. Alors que le protocole de co-irradiation utilisé mime la situation clinique, mes résultats sont en désaccord avec les publications récentes et suggèrent que le stroma irradié ne renforce pas la migration des cellules tumorales mais au contraire pourrait être manipulé pour promouvoir une réponse immunitaire anti-tumorale.

Deuxièmement, mes expériences in vivo, semblent confirmer les données obtenues in vitro et montrent que l'irradiation préalable du lit tumoral ne stimule ni croissance de la tumeur et ni sa dissemination. Nos résultats semblent montrer que l'irradiation du stroma ne favorise pas la migration des cellules de carcinome et ceci indépendamment de leur génotype.

La Troisième Partie De Mon Projet, a été consacrée à l'étude des cellules tumorale circulantes (CTC) après la radiothérapie. En accord avec les résultats rapportés après la chirurgie, le nombre de CTC augmente dans la circulation sanguine après radiothérapie probablement à cause des lésions vasculaires radio-induites ou/et par induction d'EMT dans les cellules tumorales. Néanmoins ces CTC semblent être piégées dans la cavité cardiaque. La signification de la présence de ces CTC pour le développement métastatique n'est pas élucidée mais on peut suspecter un effet promoteur de métastase.

Ainsi le microenvironnement pourrait avoir des effets antagonistes promoteurs ou inhibiteurs de malignité.

Mots clés: cicatrisation, NSCLC (cancer du poumon non à petites cellules) , Rho , TGF- β 1 , MMP, microenvironnement , CTC (cellules tumorales circulantes) , RT (radiothérapie)