UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Mémoire de Maîtrise en médecine N° 3427
Master Thesis

Influence of tumor cells on mesenchymal stem cells in lung carcinoma

Student
Joanna Vuille

Tutor
Prof. Ivan Stamenkovic
Institut de pathologie, IPA, CHUV

Co-tutor
Dr. Giulia Fregni, Postdoctoral fellow
IPA, CHUV

Expert
Prof. Tatiana Petrova
Département d'oncologie fondamentale, UNIL

Lausanne, December 2016

Acknowledgments

Premiers mots lus, derniers écrits: je désire les utiliser pour remercier chaleureusement mes deux tuteurs qui ont suivi l'évolution de ce travail du début à la fin. Je remercie le Professeur Ivan Stamenkovic de m'avoir permis de découvrir le monde passionnant de la recherche dans des conditions positives et dynamiques et d'avoir suivi avec autant d'attention mon travail. Je tiens à le remercier particulièrement pour sa confiance et pour ses conseils sur mon parcours futur. Un merci chaleureux à Giulia Fregni pour son enthousiasme contagieux concernant son travail, sa supervision attentive et tout ce qu'elle m'a appris.

Après tous les moments passés au laboratoire, je souhaite également adresser mes remerciements à tous les membres de l'équipe. Un merci particulier à Patricia Martin, pour son aide précieuse et son sourire, et à Sabine Waeber, pour les semaines passées à ses côtés, durant lesquelles j'ai acquis une base essentielle pour ce travail, et pour nos discussions scientifiques et personnelles.

De plus, parce qu'au même titre qu'une cellule, j'évolue dans un microenvironnement, je souhaite remercier ici mon entourage si précieux pour son rôle primordial dans tout projet que j'entreprends : ma famille pour la joie quotidienne de sa présence et son soutien inconditionnel, en particulier mon père pour sa patience inestimable et sa précision scientifique, mes amis dont les intérêts variés m'apportent rire et inspiration, et Simon pour sa présence à la fois apaisante et stimulante, pour nos expériences partagées et pour son amour.

Enfin, un *mulțumesc* plein de douceur à Rodica, grand-mère passionnante et sensible, qui m'a transmis une tendresse infinie et une curiosité pour tout ce qui nous entoure.

TABLE OF CONTENTS

GLOSSARY

AC	adenocarcinoma
ADAMTS12	a disintegrin and metalloprotease with thrombospondin domains
BST2	bone marrow stromal antigen 2
CD	cluster of differentiation
CHI3L1	chitinase-3-like 1
CSC	cancer stem cells
D	direct [co-culture]
ECM	extracellular matrix
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FIGF	c-fos-induced growth factor
GJA1	gap junction protein α1
GREM1	gremlin 1
IL	interleukin
IMDM	Iscove's modified Dulbecco's media
ITGA11	integrin subunit α11
LOX	lysyl-oxidase
LOXL2	lysyl-oxidase like 2
MSC	mesenchymal stem cells
MX2	myxovirus resistance 2
N-MSC	normal adjacent tissue-isolated mesenchymal stem cells
NEAA	non-essential amino acids
NSCLC	non-small-cell lung cancer
PDGF	platelet-derived growth factor
NSG	NOD-SCID common-γKO [mouse]
PS	penicillin streptomycin
qRT-PCR	quantitative real-time polymerase chain reaction
SCC	squamous cell carcinoma
SCLC	small-cell lung cancer
T-MSC	tumor tissue-isolated mesenchymal stem cells
TIC	tumor initiating cells
TW	transwell [co-culture]
VEGF	vascular endothelial growth factor

ABSTRACT

Context and aim:

Mesenchymal stem cells (MSC) are multipotent cells displaying a variety of roles. MSC in the lung tumor stroma (T-MSC) have been found to be functionally different from MSC isolated from normal adjacent tissue (N-MSC) and to increase the metastatic potential of the tumor.

We hypothesized that tumor cells can induce N-MSC evolution into T-MSC. Our group previously observed that T-MSC highly expressed various genes. We selected 11 genes involved in angiogenesis, immunomodulation or which were the most highly induced in T-MSC. The aim of the study was to analyze the expression of the 11 genes in N-MSC from lung carcinoma patients, cultured with and without tumor-initiating cells (TIC).

Methods:

Human N-MSC, T-MSC, and TIC were isolated from squamous cell carcinoma. N-MSC and paired TIC were co-cultivated at different ratios, in direct and transwell co-culture and studied at various incubation times. After co-culture, we analyzed the phenotype of N-MSC by fluorescence-activated cell sorting. Furthermore, the expression of the 11 genes of N-MSC assessed by real-time PCR was compared with that of paired T-MSC.

Results:

After co-culture, N-MSC kept a similar phenotype to the control N-MSC. By contrast, the gene expression of N-MSC was modulated by TIC. Three distinct patterns were found :

i) Genes showing early induction of expression, in direct and transwell co-culture, according to N-MSC : TIC ratio.
ii) Genes showing late induction of expression, in direct and transwell co-culture, according to N-MSC : TIC ratio.
iii) Genes showing no direct modulation by TIC

Conclusions:

TIC can modulate the expression of the 11 selected genes in N-MSC in three distinct ways. Among the inducible genes, the degree of up-regulation was dependent on N-MSC : TIC ratio and appeared in both direct and transwell co-cultures, suggesting a mechanism induced by soluble factors secreted by TIC. TIC are at least partially responsible for the evolution of N-MSC into T-MSC.

Keywords:

Mesenchymal stem cell, tumor microenvironment, lung carcinoma.

1. INTRODUCTION

Lung carcinoma

Lung carcinoma is currently the leading cause of cancer-related mortality in both genders worldwide[1] due to its high incidence, poor response to therapy and high metastatic proclivity. Lung tumors arise from cells of the respiratory epithelium and are divided into two major classes: small-cell lung cancer (SCLC, shown in figure 1A) and non-small-cell lung cancer (NSCLC). This latter class, which accounts for 85% of lung cancers[2], includes three principal histological subtypes[3]: adenocarcinoma (AC, 40% of all lung cancers, fig. 1B), squamous cell carcinoma (SCC, 25%, fig. 1C), large cell carcinoma (10%, fig. 1D) and mixed subtypes (5%). In addition to their histological characteristics (figure 1), the subtypes show distinct molecular profiles, suggesting a probable different pathogenic evolution.

Our current work focuses on SCC, also known as epidermoid carcinoma, which is initiated in bronchial epithelial cells that have undergone squamous metaplasia in response to chronic irritation by diverse toxins. Tobacco use is by far the most important risk factor for this tumor subtype. Pulmonary SCC, similar to most solid cancers, evolves through sequential preneoplastic stages: hyperplasia, metaplasia, dysplasia, and finally carcinoma *in situ.* This multistep process, which is directly correlated to tobacco exposure[4], is driven by consecutive genetic mutations and epigenetic modulation[5, 6].

Lung carcinoma has been intensely studied in recent years but the precise mechanisms underlying its development remain incompletely understood. However, interactions between lung cancer cells and cells of the tumor microenvironment seem to play an important role.

Although lung cancer incidence rates have been declining for the last 20 years[7], more than 220,000 newly diagnosed patients of both sexes were reported in the United States in 2015 with a 5-year relative survival rate of only 17%[8]. There are at least two major explanations for this poor prognosis: the difficulty to diagnose lung cancer in its early stages and the lack of effective therapies for advanced disease. First-line treatment includes surgical resection, chemotherapy and radiation therapy, alone or in combination but the poor response emphasizes the need for new strategies to improve the outcome of the disease.

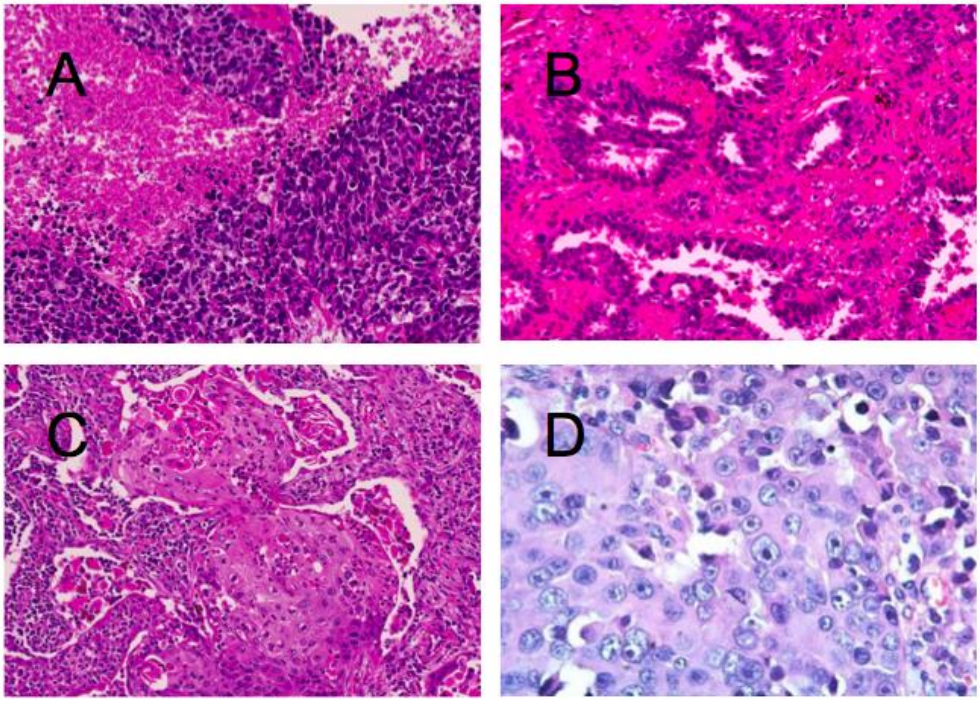

Figure 1: Characteristic histology of lung carcinoma subtypes. A. Small cell lung carcinoma: round small cells with scant cytoplasm form compact cellular sheets frequently accompanied by necrosis. **B.** Lung adenocarcinoma: glandular architecture and secretory vacuoles, sometimes with mucus, are key components. **C.** SCC: squamous cell differentiation is evident by the presence of keratin pearls. **D.** Large cell carcinoma: poorly differentiated tumor composed of large polygonal cells with prominent nucleoli.

Images in panels A, B and C are courtesy of Dr. I. Letovanec. Image in panel D was taken from the address « http://cqmsjt.com/files6/large-cell-lung-carcinoma.html » (October 2016).

Alternative approaches including immunotherapy and a variety of targeted therapies have been and continue to be applied, often providing limited clinical benefit with prolonged survival. Higher specificity of targeted approaches can provide more potent therapeutic effects with lower systemic toxicity due to limited damage to healthy cells. Several clinical trials have demonstrated their potential in lung cancer even at metastatic stages[9, 10]. The aim of immunotherapy is to stimulate the immune response to tumor cells but also to block mechanisms whereby tumor cells evade immune surveillance. Targeted therapies are based on the identification of specific « driver » mutations, most often in oncogenes, to which tumor cells may become “addicted”. The mutated proteins, which may be signaling molecules or cell surface receptors can then be targeted by new drugs or antibodies that do not recognize the wild type counterparts. As both strategies require in-depth knowledge of lung

cancer pathogenesis, it is crucial to precisely elucidate not only the properties of the tumor cell subpopulations that determine tumor heterogeneity, but also the function of cells that compose the tumor microenvironment on which the tumor relies for its progression.

Lung cancer cells and tumor microenvironment

Different tissues display diverse levels of regenerative capacity, depending in part on their stem cell content and whether the stem cells are constantly engaged in the cell cycle (e.g. bone marrow and intestinal epithelium) or whether they remain dormant but responsive to stimuli that include inflammation and injury (e.g. liver and kidney). In both cases, stem cell activity is strictly regulated. If one cell incurs a mutation in an oncogene and/or a tumor suppressor gene (provided it is permissive for oncogenic events), it may no longer respond to cell cycle regulatory cues. The cell may then display relentless division possibly leading to the initiation of a tumor. As the tumor develops, its component cells become heterogeneous, displaying diverse properties, including a variety of differentiation stages. Two main hypotheses regarding the mechanisms underlying tumor heterogeneity have been proposed: the stochastic model and the cancer stem cell model. According to the first model, every tumor cell has more or less the same intrinsic potential to initiate tumor growth but its fate with respect to division, differentiation and survival is unpredictable. By contrast, the cancer stem cell model suggests a hierarchical organization of this heterogeneity[11]. At the top of the hierarchy is a subpopulation of poorly differentiated cells, known as cancer stem cells (CSC) that display a high degree of plasticity. They possess self-renewal and tumor initiating capacity and give rise to differentiated, non-tumorigenic cells. These cells may also display resistance to conventional anti-cancer drugs, although this may not be the case in all tumor types. Results from several research groups support the CSC model in lung carcinoma[12, 13]. However, global tumor heterogeneity depends not only on the heterogeneity among the tumors cells but also on the host tissue microenvironment.

Chronic inflammation, due to the continuous exposure of lung epithelium to inhaled toxins (e.g. tobacco smoke), is a key component in the development of lung carcinoma, continuously recruiting different cell types that likely interact with cancer cells[14]. Together with extracellular matrix (ECM), blood vessels and secreted molecules, these cells constitute the tumor microenvironment. Cells in the lung carcinoma stroma include ECM-producing cancer-associated fibroblasts, endothelial cells, pericytes, pulmonary epithelial cells, hematopoietic cells, including neutrophils, B and T-cells, macrophages, and mesenchymal stem cells (MSC)[15]. Figure 2 illustrates part of this complex cellular network, which evolves concomitantly to tumor growth.

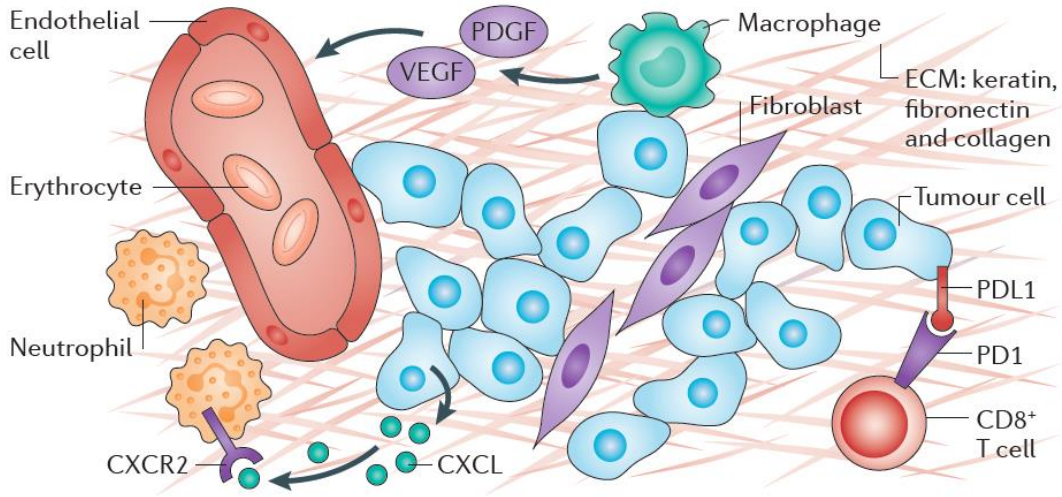

Figure 2: Lung tumor microenvironment[1]. A multitude of cells interact in the tumor, and participate in determining the characteristics of lung cancer. A dynamic ECM, containing secreted growth factors, chemokines and cytokines, provides a scaffold that connects fibroblasts, immune cells and tumor cells. Angiogenesis provides new vessels helping recruit various immune cells, including neutrophils, macrophages and T and B lymphocytes. Tumor cells can modulate the immune response by producing soluble factors that recruit leukocyte subsets (secreted CXCL that bind to CXCR2) and cell surface receptors that recognize ligands on immune cells (PD1-PDL1). An important component not shown in this figure is constituted by MSC.

CXCL : CXC-chemokine ligand. CXCR2 : CXC-chemokine receptor 2. ECM : extracellular matrix. PD1 : programmed cell death 1. PDL1 : programmed cell death 1 ligand. PDGF : platelet-derived growth factor. VEGF : vascular endothelial growth factor.

Figure taken from : Zhao Chen et al., "Non-Small-Cell Lung Cancers: A Heterogeneous Set of Diseases," *Nature Reviews Cancer* 14, no. 8 (August 2014).

Dvorak suggested in 1986 that tumors can be regarded as « wounds that never heal », arguing that tumor tissues share multiple properties with injury-associated tissues. Similar to the stroma of any damaged tissue, tumor stroma undergoes complex remodeling that includes hemostasis, inflammation, humoral and cellular immune responses, angiogenesis, production and deposition of connective tissue[16]. Over the next thirty years numerous studies validated this notion and also revealed a more malicious role of the tumor microenvironment: its pro-disseminating action. Cell-cell interactions and soluble factors can promote tissue invasion and metastasis[17]. Metastasis is a multistep process, including local invasion, intravasation of tumor cells into the lymphatic or blood vessels, circulation to distant organs, extravasation and survival in a new stromal environment that may have been conditioned to provide « pre-metastatic niches »[18]. Tumor stroma participates in virtually all steps of this process[14, 19], including ECM degradation, angiogenesis, invasion, protection during circulation in blood vessels and preparation of secondary tumor sites. Although numerous

players and secreted proteins are known to be present in the tumor microenvironment, their precise roles remain to be elucidated.

In our work, we focused on MSC because of their interaction with both tumor and immune cells.

Mesenchymal Stem Cells

Mesenchymal stem cells are multipotent cells that display self-renewal and the capacity to differentiate into a variety of mesenchymal cell lineages (figure 3), contributing to the maintenance of connective tissues. The current definition of MSC includes three properties[20]:

1) MSC must adhere to plastic material in standard *in vitro* culture;
2) After isolation, MSC express CD73, CD90, and CD105 and are *lineage* negative, which implies the absence of lineage markers CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR surface antigens;
3) MSC are able to differentiate *in vitro* into osteoblasts, chondroblasts and adipocytes.

Mesenchymal stem cells are mostly found in the bone marrow, where they co-exist with hematopoietic stem cells and represent less than 0.01% of the total cellular content[21]. However, MSC have been identified in many other locations including adipose tissue, peripheral blood and lung. Beside their ubiquity, MSC are multitasking cells, playing a role in angiogenesis[22], immunomodulation[23], tissue regeneration and maintenance of the stem niche in bone marrow (table 1). They display a tropism for inflamed tissues[24], endowing them with a particular scientific appeal: they have already been employed as tools in replacement therapies and some novel strategies using them as vectors to deliver anti-cancer drugs into the tumor[25].

MSC have been found in the primary tumor microenvironment of several cancers including lung carcinoma, probably recruited through inflammation[24] and/or tumor cell-derived chemokines. Although their number in the microenvironment is very limited, between 0,01% and 1% of all cells[26], these multipotent cells are an important component of the cellular network associated with tumor progression. The precise mechanism that underlies their involvement in tumor pathogenesis remains incompletely elucidated and their role in promoting or counteracting cancer progression is still controversial[27, 28], which could be attributed to context-dependence (e.g. microenvironment, involved organ, tumor stage) of their effects.

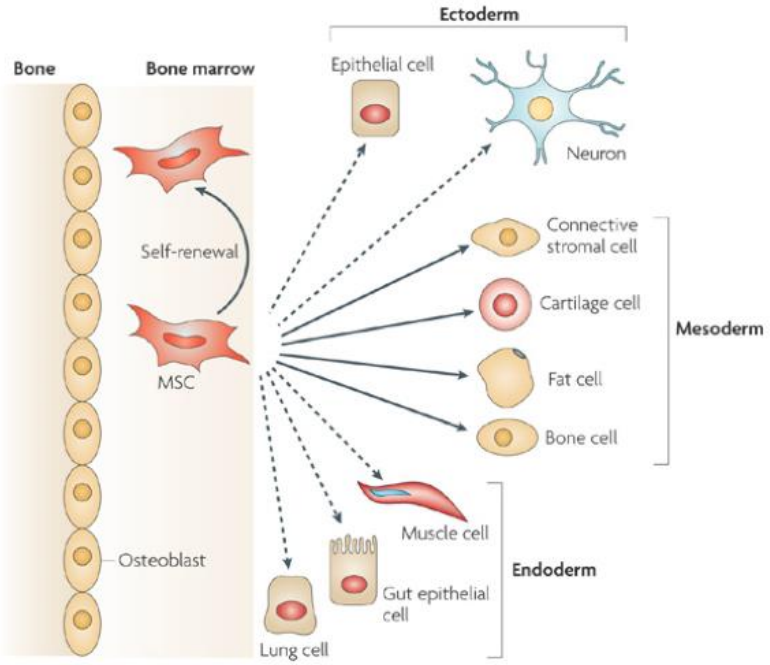

Figure 3: Differentiation potential of MSC. MSC display self-renewal capacity. They differentiate into all mesodermal-derived cells, such as adipose cell, chondroblast and osteoblast. Differentiation into cells from unrelated germline lineages has also been reported by several studies.

Figure taken from : Antonio Uccelli et al., "Mesenchymal stem cells in health and disease," *Nature Reviews Immunology* 8, (September 2008).

Several groups including ours made the observation that MSC isolated from lung cancer patients display specific functional and molecular properties[29]. We used 9 pairs of MSC coming from 9 different lung carcinoma patients (4 SCC, 3 AC, 2 SCLC), isolated from tumor tissue (T-MSC) and from the paired macroscopically normal adjacent tissue (N-MSC) and we compared their expression profiles. Our data (unpublished) revealed a distinct gene expression profile between N-MSC and T-MSC. Moreover, for 3 SCC patients, we were able to isolate and expand paired cancer cells from the tumor samples that we used to assess the influence of MSC on tumor cell growth and invasion. For this, we used a model of NOD-SCID common-γKO (NSG) mice and co-injected the human lung tumor cells with their paired MSC beneath the kidney capsule. We found that MSC co-injection increased the metastatic potential of paired tumor cells. Moreover, with T-MSC co-injection, the metastatic tumor burden was significantly higher, especially in liver and lung, while the tumor growth at the site of injection was not affected.

In the present work, using material from the 3 SCC patients, we further analyzed the changes in expression of selected genes from N-MSC and T-MSC cultured alone and from N-MSC co-cultured with their paired tumor-cells at different time points and cell ratios. Our hypothesis was that tumor cells could modulate gene expression in MSC and possibly convert N-MSC to T-MSC. Thus, we assessed the potential of primary tumor cells to induce some of the genes that we found to be highly expressed in T-MSC and that are likely involved in their metastatic potential and immune modulating functions. Table 2 lists the 11 genes on which we focused, based on the level of their expression

and/or their known physiological roles. This analysis should allow a better understanding of the educational role of tumor cells towards their microenvironment, particularly regarding MSC, and may uncover new targets for the control of tumor growth and metastasis.

Angiogenesis	Several studies showed that *in vivo* MSC-induced tumor growth promotion includes angiogenesis. This neovascularization may be stimulated through secretion of pro-angiogenic factors and through differentiation of MSC into pericytes and endothelial cells[28, 30]. However, others found an inhibitory effect of MSC on angiogenesis[15].
Immunomodulation	MSC display immunosuppressive properties[31] that alter immune reactions against malignant cells. Thus, MSC provide an immune-favored environment for tumor cells[23, 27].
Intra-tumor differentiation	MSC display their pluripotency within the tumor, with multilineage differentiation capability[29]. When they reach the tumor, MSC can differentiate into cancer-associated fibroblasts, macrophages or endothelial cells[32].
Tumor cell proliferation	MSC provide a stromal support system that favor tumor growth *in vivo*[33]. Several MSC secreted cytokines (e.g. IL-6 and IL-8), growth factors and chemokines recognize corresponding receptors on tumor cells, leading to tumor progression[27].
Migration capacity	MSC display chemotactic properties in response to the release of damage signals and are mobilized toward injured tissues. Tumor cells secrete various soluble factors such as TNFα and other cytokines that stimulate MSC tumor migration[24].
Hematopoietic stem cell niche	Hematopoietic stem cells and MSC coexist closely in these niches, which are architectural units within the bone marrow, with hematopoietic and skeletal homeostatic functions. MSC provide key cellular components for these niches and are thought to regulate hematopoietic stem cell quiescence and mobilization through secretion of chemokines and growth factors[34].
Regenerative function	MSC are a key component of regeneration of injured tissues, i.e. clearly distinct from the immune-modulated scarring process. MSC play two roles: they stimulate tissue-specific stem cells through secretion of growth factors and differentiate into cells of diverse mesenchymal lineages[34].

Table 1: MSC functions and effects on physiological processes.

Gene	Functions and roles in tumor microenvironment
ADAMTS12 A disintegrin and metalloprotease with thrombospondin domains	Effects of ADAMTS12 include matrix-metalloproteinase action and regulation of a specific integrin that links cells to the ECM. Although several experiments suggest an anti-tumorigenic potential of ADAMTS12[35], others shed light on the invasive phenotype that ADAMTS12 confers on trophoblastic cells to penetrate maternal tissues through regulation of the integrin [36]. Since this invasion shares several molecular and mechanistic features with metastatic carcinoma, ADAMTS12 may play a role in metastasis and ECM regulation through its non proteolytic activity.
BST2 Bone marrow stromal antigen 2	This cell surface protein is expressed in differentiated B cells but its functional role remains elusive. Published data demonstrated its elevated expression in various solid tumor cells that exhibit an invasive phenotype[37, 38].
CHI3L1 Chitinase-3-like 1	CHI3L1 is an activity-lacking enzyme in mammals and its biological function still remains elusive. However elevated serum levels of this protein, found in numerous tumors including NSCLC, are associated with poor prognosis[39]. CHI3L1 has been observed to induce angiogenesis and attract macrophages, leading to a higher metastatic potential[40].
FIGF c-fos-induced growth factor or Vascular Endothelial Growth Factor D	FIGF is a ligand for an endothelial-specific receptor tyrosine kinase, leading to stimulation of lymphangiogenesis. In NSCLC, FIGF was detected in both tumor cells and stromal cells. A correlation has been shown between the expression of FIGF at the leading edge of NSCLC and lymph node metastasis[41].
GJA1 Gap junction protein α1	GJA1 is a component of gap junctions. Researchers found evidence that GJA1 expression possibly regulates invasion and metastasis through interactions between tumor cells and the stroma[42].

GREM1 Gremlin 1	As member of the bone morphogenic protein antagonist family, GREM1 might play a role in organogenesis, tissue differentiation and angiogenesis. GREM1 overexpression in lung AC, but not in SCC, has been correlated with enhanced tumor proliferation, suggesting an oncogenic role of GREM1 through an unknown mechanism[43]. Contrasting results in other tumors may indicate a tissue-specific function of gremlin.
IL-6 Interleukin 6	This cytokine is involved in inflammation and B-cell maturation. IL-6-mediated inflammation may contribute to NSCLC-related morbidity and mortality, through debilitating complications including anemia and cachexia[44]. Furthermore, IL-6 can induce tumor epithelial-mesenchymal transition (EMT), a potentially key step in the acquisition of a pro-metastatic phenotype for a tumor cell[45].
ITGA11 Integrin subunit α11	When it dimerizes with the ß1 integrin subunit, the α11 integrin chain forms one of the four collagen receptors. It has been found to be highly expressed in NSCLC-associated stroma and to promote tumor growth[46]. In addition, a strong association between ITGA11, collagen stiffness, and cancer cell metastasis has been reported[47].
LOX Lysyl-oxidase	These family members of copper-dependent amine-oxidases can crosslink collagen and elastin, playing an important role in ECM remodeling. LOX expression is enhanced by NSCLC-associated hypoxia[48]. Although LOX shown several antitumor effects, LOX family oxidases have been observed to promote tumorigenesis and metastasis through active remodeling of the tumor microenvironment and are also actively involved in the process of EMT undergone by tumor cells[49].
LOXL2 Lysyl-oxidase like 2	
MX2 Myxovirus resistance 2	This member of the GTPase family has a cytoplasmic and a nuclear form and has an HIV-1 restriction function. MX2 was shown to be involved in regulating nucleocytoplasmic transport and cell cycle progression[50]. Its expression is enhanced by interferon-alpha[51] and was shown to be up-regulated in lung adenocarcinoma cells[52].

Table 2: Functions of 11 selected genes that we found to be highly expressed in T-MSC and analyzed in the present work.

2. Materials and methods

N-MSC and tumor cell isolation from fresh patient samples and cell culture

Tumor-associated MSC (T-MSC) and tumor initiating cells (TIC) were isolated in the lab from 3 squamous cell carcinoma (SCC) samples, #21, #26 and #32, removed by surgical resection at the Centre Universitaire Hospitalier Vaudois (CHUV) with the agreement of the Swiss ethic committee (project authorization n° 131/12). Normal tissue-associated MSC (N-MSC) were obtained from paired macroscopically normal adjacent tissues.

To isolate MSC, tissue samples were mechanically and enzymatically disrupted into small pieces and dissociated in Iscove's Modified Dulbecco's Media (IMDM, Gibco) supplemented with Collagenase II and IV (0,5 mg/mL, Gibco) and DNAse (0,1 mg/mL, Roche) for 2 hours at 37°C. The resulting single cell bulk was cultured one night in MSC medium: IMDM (Gibco), supplemented with 10% fetal calf serum (FCS ; Gibco), 1% penicillin streptomycin (PS) (Gibco), 1% non-essential amino acids (NEAA)(Gibco) and 10 ng/mL PDGF (Prospec). The next day, the medium was changed and only adherent cells were kept in culture.

TIC were obtained culturing single cell tumor bulk, as spheres in ultra-low attachment flasks (Corning, Falcon) and in KO medium: IMDM medium (Gibco) completed with 20% knockout serum (Gibco), 10 ng/mL LIF (Millipore), 10 ng/mL recombinant human EGF (Invitrogen), 10 ng/mL recombinant human bEGF (Invitrogen) and 1% PS (Gibco). Cell tumorigenicity was verified by injecting them in the kidney capsule of NSG mice at low numbers (1000-3000 cells).

Tumor initiating cells and N-MSC co-culture

N-MSC cells were co-cultured with TIC in direct and transwell culture conditions (figure 5) at different ratios between N-MSC and TIC (2:1 ; 1:1 ; 1:2 ; 1:5 respectively) and studied at different incubation time (2 or 3 days; 5 days ; 7 days).

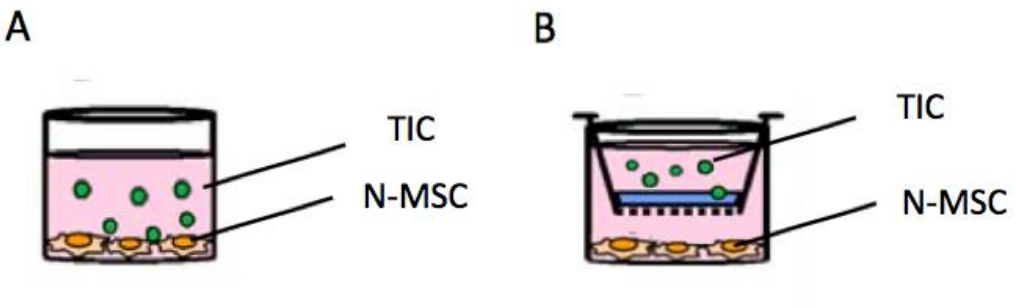

Figure 5: The two different co-culture types. **A**: In the direct co-culture type, TIC and N-MSC can establish cell-cell contacts. **B**: In the transwell co-culture, TIC and N-MSC are separated, and soluble factors are the only possible link between the two compartments.

- For the direct co-culture, N-MSC (100,000 cells/dish) were seeded at passage 5 onto 10 centimeter-diameter Petri dish (Corning, Falcon), and TIC were added according to the four different ratios. As controls, we used T-MSC and N-MSC cultured alone. All culture conditions were tested at each time point. Cells were cultured in MSC medium, half of which was refreshed at day 3 and 5.
- In transwell co-culture, N-MSC (20.000 cells/well) were seeded at passage 5 onto six-well plates (Costar, Corning incorporated). TIC were seeded into 1,0 µm-pore insert of PET-membrane (Corning, Falcon) to obtain the four different ratios. As controls, we used T-MSC and N-MSC cultured alone. All culture conditions were tested at each time point. Cells were cultured in MSC medium, half of which was refreshed at day 3 and 5.

Isolation of N-MSC after co-culture

N-MSC cultured in transwell systems were isolated by collecting cells from the lower compartment after removal of the upper chamber containing TIC. N-MSC were then trypsinized (Clonetics), washed twice in PBS, snap frozen and stored at -80°C until RNA extraction.

The isolation of N-MSC from direct co-culture needed a supplementary step to remove TIC. According to previous results showing CD45 expression by tumor cells in our sphere culture condition, TIC were removed after trypsinization (Clonetics), using anti-CD45 magnetic beads (Miltenyi biotec), following manufacturer instructions. CD45- cells, assumed to be only N-MSC, were recovered and washed twice in PBS, immediately snap-frozen and stored at -80°C.

Fluorescence-activated cell sorting (FACS) analysis of N-MSC and TIC after isolation from direct co-cultures

After removal of tumor initiating cells from direct co-cultures, 20.000 N-MSC from each direct co-culture condition were analyzed by FACS in order to verify the purity of CD45- collected cells. Cells were stained 25 minutes at 4°C with « human MSC phenotyping kit » (Miltenyi) containing a cocktail of antibodies for the analysis of MSC phenotype: FITC-conjugated mouse anti-human CD90, PE-conjugated rat anti-human CD105, PerCP-Cy5,5-conjugated mix of antibodies targeting CD14, CD20, CD34 and CD45, and APC-conjugated mouse anti-human CD73. To exclude dead cells, they were also stained with fixable viability dyes (LIVE/DEAD®, Life Technology). After antibody staining, cells were washed once in PBS and fixed in PFA 4%. Cell phenotypes were then acquired using FACS Gallios (Beckman Coulter) and results analyzed by FlowJo software.

N-MSC cultured alone at each time condition and TIC from #21 collected from the upper compartment of transwell co-culture at day 7 were used as control and analyzed by FACS using the same reagents and protocol as described above.

mRNA Expression and cDNA synthesis

Total RNA was extracted from MSC using the RNeasy mini Kit (Qiagen), following the standard manufacturer protocol and stored at -80°C.

For each sample, 500 ng of cDNA were synthesized by reverse transcription using M-MLV Reverse Transcriptase (Promega) according to manufacturer instructions, and stored at -20°C.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Levels of gene expression were determined using the comparative method (C_T) with cDNA and samples analyzed in triplicates. Quantitative RT-PCR amplification was performed using TaqMan Universal PCR mastermix or SYBR® Green mix (Applied Biosystems). SYBR® Green primer sequences for the quantification of the 11 selected genes are listed in table 3. PP1A (protein phosphatase 1 ; TaqMan probe, Hs99999904_m1) was used as housekeeping gene.

	Forward	Reverse
ADAMTS12	5'- ATTGTTGTGGTTCGGCTCATT -3'	5'- AGGTCACTCTTGGGATTGATACT -3'
BST2	5'- CACACTGTGATGGCCCTAATG -3'	5'- GTCCGCGATTCTCACGCTT -3'
CHI3L1	5'- GTGAAGGCGTCTCAAACAGG -3'	5'- GAAGCGGTCAAGGGCATCT -3'
FIGF	5'- ATGGACCAGTGAAGCGATCAT -3'	5'- GTTCCTCCAAACTAGAAGCAGC -3'
GJA1	5'- GGTGACTGGAGCGCCTTAG -3'	5'- GCGCACATGAGAGATTGGGA -3'
GREM1	5'- CGGAGCGCAAATACCTGAAG -3'	5'- GGTTGATGATGGTGCGACTGT -3'
IL-6	5'- ACTCACCTCTTCAGAACGAATTG -3'	5'- CCATCTTTGGAAGGTTCAGGTTG -3'
ITGA11	5'- GTGGCAATAAGTGGCTGGTC -3'	5'- GTTCCCGTGGATCACTGGAC -3'
LOX	5'- CGGCGGAGGAAAACTGTCT -3'	5'- TCGGCTGGGTAAGAAATCTGA -3'
LOXL2	5'- GGGTGGAGGTGTACTATGATGG -3'	5'- CTTGCCGTAGGAGGAGCTG -3'
MX2	5'- CAGAGGCAGCGGAATCGTAA -3'	5'- TGAAGCTCTAGCTCGGTGTTC -3'

Table 3: SYBR Green primer sequences used for gene quantification by qRT-PCR.

3. Results

In previous comparisons between mRNA expression profiles of paired N-MSC and T-MSC samples by microarray, we observed that tumor-associated MSC display up-regulated expression of numerous genes. Aiming to evaluate the role of the tumor cells in this up-regulation, we analyzed the expression profile of a selection of 11 of these genes in N-MSC after culture with tumor initiating cells (TIC) from the same patient, in different conditions.

Phenotype of Cells Isolated from Direct Co-Cultures

CD45- cells exhibit the same phenotype as MSC alone

To validate the purity of our MSC isolation after negative selection for CD45, we analyzed the phenotype of CD45- cells and compared the results to MSC cultured alone as a control. Figure 6 shows the expression profiles of one representative analysis performed at day 7 on 3 types of cells:

- N-MSC cultured alone (see figure 6, bottom panel);
- CD45- cells, assumed to be MSC, isolated from direct co-culture at 1:5 N-MSC : TIC ratio (fig. 6, middle panel)
- TIC, forming spheres in culture, collected from the upper compartment of transwell co-culture at 1:5 ratio from patient #21 (fig. 6, upper panel).

Consistent with the MSC phenotype and similar to MSC cultured alone, CD45- isolated cells were positive for CD105 (SH2, endoglin), CD73 (SH3) and CD90 (Thy-1) expression, and did not express leukocyte or hematopoietic stem cell markers (CD34, CD45, HLA-DR, CD14 or CD11b, CD79a or CD19), congruent with the definition of the *lineage negativity* of the MSC.

By contrast, sphere-derived tumor cells showed a clearly distinct phenotype: they were negative for MSC markers and positive for *lineage* markers.

Thus, we assumed that the isolated MSC collections were pure, without CD45+ sphere-derived contaminating tumor cells.

The entire FACS analysis is reported in the appendices (figures I, II and III). It is of note that for some conditions we observed low expression of *lineage* markers. These results were in accordance with the bone marrow MSC phenotype that displayed a similar low expression (data not shown) of one or more of the *lineage* makers. The markers responsible for this up-regulation are currently under investigation.

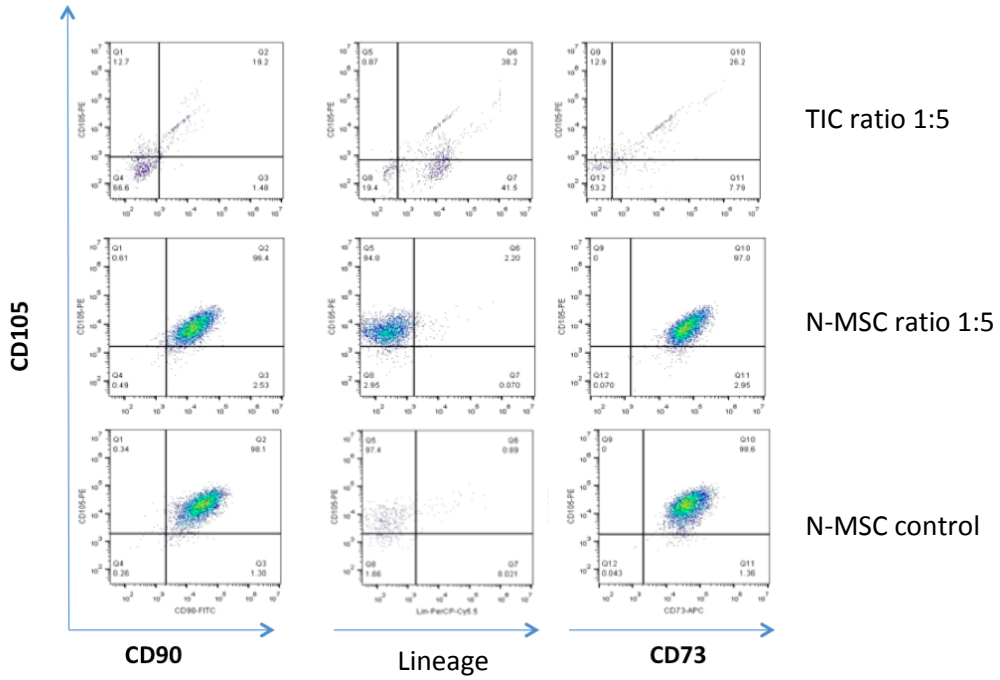

Figure 6: Phenotype of isolated cells. FACS analysis compared phenotypes of sphere-derived TIC cells (upper panel), assumed N-MSC isolated from co-culture (middle panel) and N-MSC cultured alone (lower panel) at day 7 (patient #21). The signal lying in the diagonal (upper panel) is likely due to auto-fluorescence of tumor cells.

Impact of lung carcinoma TIC on MSC expression profile

Compared to N-MSC alone, T-MSC display up-regulation of the 11 selected genes after 7 days of co-culture

We first sought to verify the differential expression of the 11 selected genes between N-MSC and T-MSC cultured alone: ADAMTS12; BST2; CHI3L1; FIGF; GJA1; GREM1; IL6; ITGA11; LOX; LOXL2; MX2.

The up-regulation in T-MSC, displayed in black in figures 7, 8 and 9, was indeed validated for every condition, with the exception of a few genes from sample #32, and mostly at day 3. Relative gene expression was always normalized to the corresponding gene expression level in N-MSC, displayed in red, cultured alone under comparable conditions.

TIC co-culture-dependent modulation of MSC gene expression

Our results suggest that expression of some of the selected genes is modulated by the presence of tumor cells. We observed differential regulation of the expression of these genes in terms of time of induction and N-MSC : TIC ratio. To refine our results, we sorted the 11 genes according the way in which their expression was modulated:

1. Genes showing early induction of expression
2. Genes showing late induction of expression
3. Genes showing no direct modulation by tumor cells

The expression profile of several genes in MSC was induced early by TIC

The first category of genes includes ADAMTS12, BST2, IL6 and MX2 (figure 7). These genes displayed increased expression in both direct and transwell co-cultures with a strong correlation between the number of tumor cells, as measured by the N-MSC : TIC ratio, and the transcript expression level. Interestingly, the level of expression of some of the genes (e.g. IL6) in N-MSC in presence of tumor cells at a 1:5 ratio exceeded that in T-MSC alone. This up-regulation was already present at day 3 and remained unchanged up to day 7, without significant differences between direct and indirect co-cultures.

As an exception to this induction, in some conditions the expression of ADAMTS12 in MSC from patient #32 did not increase and even decreased: e.g. TW co-culture at day 3. It is of note that this gene was less differentially expressed between #32 T-MSC and #32 N-MSC compared to MSC derived from the other patients.

The expression profile of each gene is described below.

ADAMTS12

Expression of ADAMTS12 displayed a slight but constant difference between control T-MSC and N-MSC, with a 1.5 to 3-fold higher expression in T-MSC. Following MSC-TIC co-culture, it was clearly induced in N-MSC from patient 21, especially at day 7, and from patient 26 already at day 2. Direct and TW co-cultures were largely comparable.

BST2

The differential expression of BST2 between T-MSC and N-MSC was more pronounced than for ADAMTS12 and reached a 150-fold difference in patient #26 at day 2 of direct co-culture. Patients #21 and #32 displayed lower differential expression ranges: 7 to 14 and 2 to 4-fold respectively. For all patients, we observed induced expression at early time points, correlating with the increase in N-

MSC : TIC ratios. Interestingly, the highest induction was seen at day 3 for two patients, at a level similar to T-MSC expression.

IL6

The up-regulation of IL6 transcripts in T-MSC compared to N-MSC was between 1 and 3-fold. We observed strong tumor-induced expression in N-MSC, at early time points. The induction was highly dependent on the N-MSC : TIC ratio, and at a 1:5 ratio in all conditions, except for patient #26 at day 7 direct, the induced level in N-MSC exceeded the expression in T-MSC alone.

MX2

T-MSC expression of the MX2 gene was 5-fold higher than in N-MSC, except for MSC from patient 32, where a minor differential expression was observed. Following co-culture of cells from patients #26 and #32, we observed a clear induction according to the ratio of N-MSC to TIC. Interestingly, induced expression levels comparable to those in T-MSC alone were observed in MSC from patient #26. Patient #21 MSC also showed induced up-regulation but only at day 3. In general, comparable results were obtained from direct and TW co-cultures.

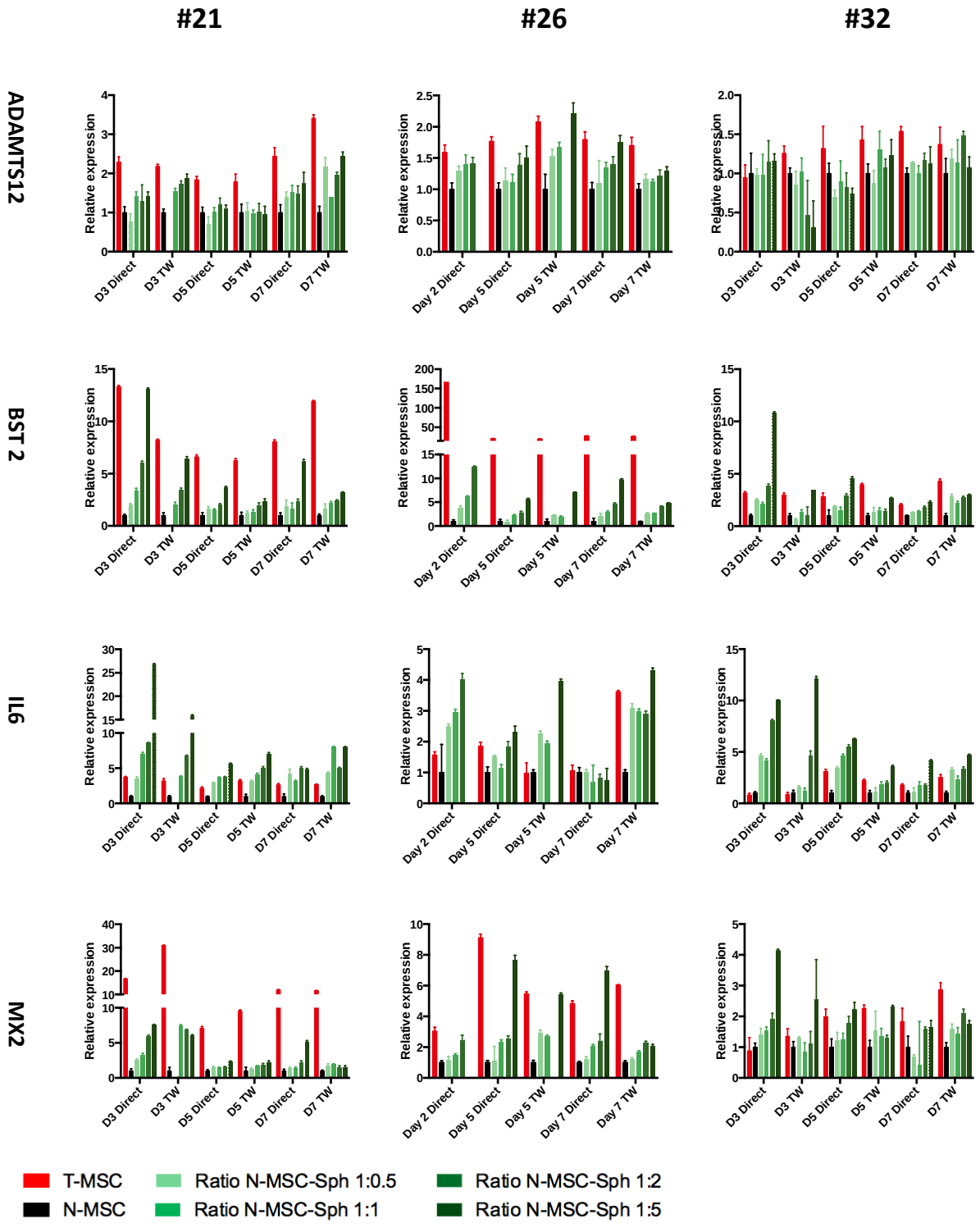

Figure 7: Relative expression of ADAMTS12, BST2, IL6 and MX2 genes in N-MSC after co-culture with TIC. Expression was always normalized to the expression level of N-MSC cultured alone in comparable conditions (same time point and co-culture type). For all of these genes, we observed up-regulated expression that correlated with tumor cell quantity in culture. For several conditions (e.g. IL6 #26 ratio 1:5), up-regulated levels were comparable to expression in T-MSC alone.

The expression profile of several genes in MSC was up-regulated by TIC after a latency period

This second category of genes includes GJA1, LOX and LOXL2. These genes displayed stable expression during the first days of co-culture and an increased level after 5 or 7 days, in correlation with the quantity of tumor cells in culture (figure 8). The highest difference between T-MSC and N-MSC alone was observed in patient #21, while in patients #26 and #32 the difference was slight or even inversed (e.g. LOX from patient #32). However, up-regulation occurred after 5 or 7 days of co-culture, particularly for LOX and LOXL2 at day 7.

MSC from patient #32 did not display this « late modulation »: none of the 3 genes were up-regulated even at day 7.

Below is the description of the expression of each gene in MSC from the 3 patients:

GJA1

The expression of GJA1 was quite stable: all N-MSC co-cultured with tumor cells had a similar expression level to that of control N-MSC. This gene seemed to be slightly up-regulated only by tumor cells from patient #26, with comparable induction between direct and TW co-cultures.

LOX

Expression of LOX was mildly modulated upwards after 5 (patient #26) or 7 days (patients #21 and #32) in both types of co-culture. In patient #32, where no difference in expression between N-MSC and T-MSC was observed, expression was stable, with the exception of day 7 when up-regulation occurred in the transwell co-culture.

LOXL2

LOXL2 had a similar induction of expression as LOX. Up-regulation was observed after 5 or 7 days, particularly in patient #26, where expression levels at 1:5 N-MSC : TIC ratio reached those of T-MSC. Interestingly, LOXL2 expression by MSC in patient #32 was not modified by TICs.

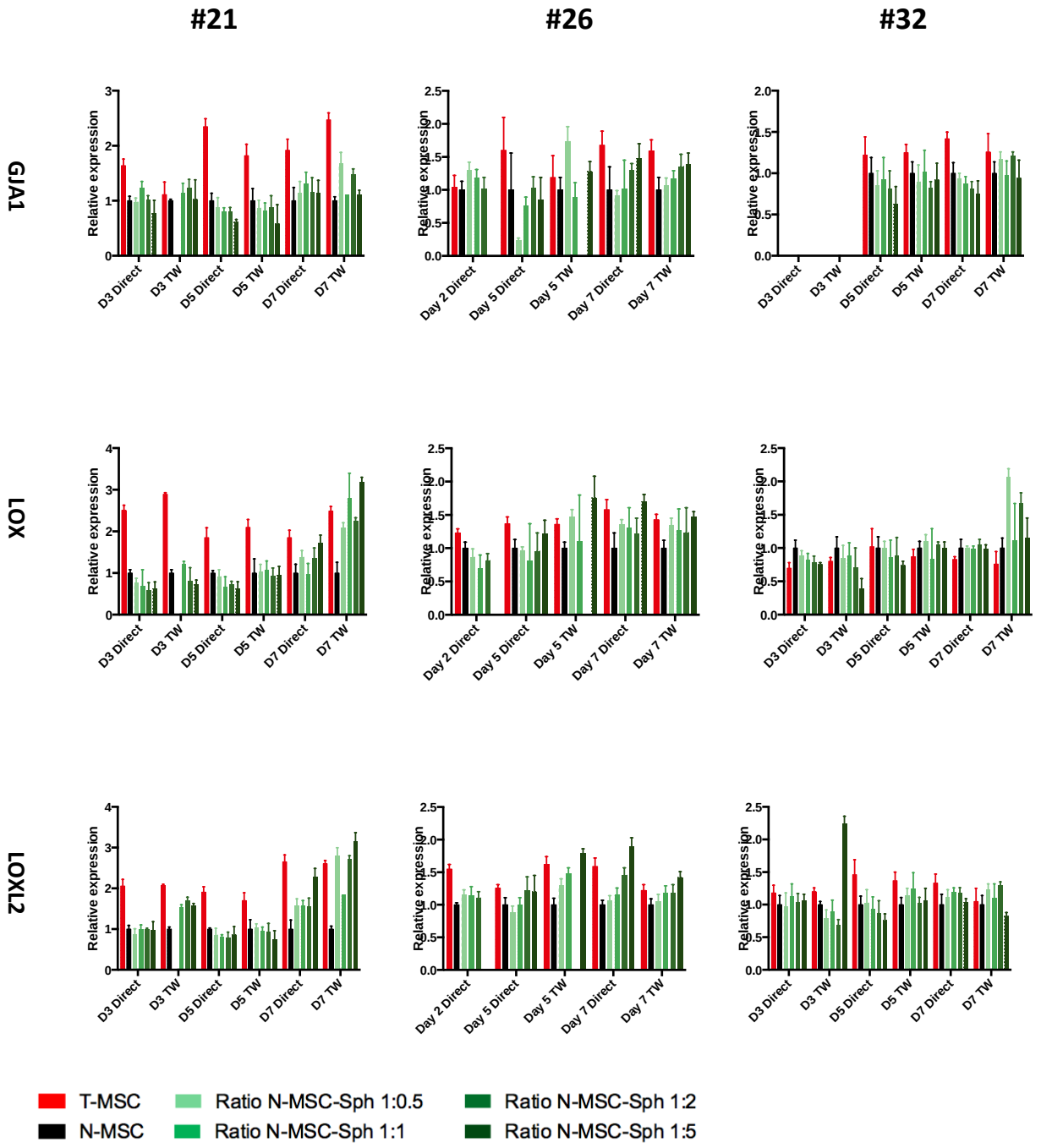

Figure 8: Relative expression of GJA1, LOX and LOXL2 genes in N-MSC after co-culture with TIC. Expression was always normalized to the expression level of N-MSC cultured alone in comparable conditions (same time point and co-culture type). The 3 genes showed a stable expression level during the first days of co-culture. The expression was induced after 5 or 7 days and according to N-MSC : TIC ratio.

The expression of several genes in MSC was not induced by TIC

The third category includes CHI3L1, FIGF, GREM1 and ITGA11 and is depicted in figure 9. For these 4 genes, no induction of expression occurred after MSC-tumor cell co-culture. The transcript level either remained stable or was reduced by the co-culture (e.g. ITGA11 in patients #21 and #26).

It is of note that these 4 genes were highly differentially expressed between N-MSC and T-MSC alone, particularly for CHI3L1 with more than 150-fold higher expression in T-MSC from patient #21.

Below is the description of the expression profile of each gene:

CHI3L1

This gene was not up-regulated following the MSC-TIC co-culture. Instead, according to N-MSC : TIC ratio a general reduced expression was observed in both types of co-culture and especially in MSC from patients #21 and #26.

FIGF

FIGF expression was largely stable regardless of the time point and co-culture type. Interestingly, we observed more variable expression in patient #32, where T-MSC expression was very close that in N-MSC alone.

GREM1

The presence of TIC did not influence GREM1 expression, which remained stable and comparable to that in N-MSCs alone. The single exception to this plateau was a strong increase in MSC from patient #32 at day 3 in transwell co-culture, where the expression at 1:5 N-MSC : TIC ratio was comparable to that in T-MSC alone.

ITGA11

Co-culture did not increase ITGA11 expression in MSC. In two patients #21 and #26, we even observed a small “down-regulation” after co-culture with tumor cells. Similar to GREM1, co-culture in patient #32 at day 3 in transwell conditions was an exception, with a strong expression level at the highest N-MSC : TIC ratio.

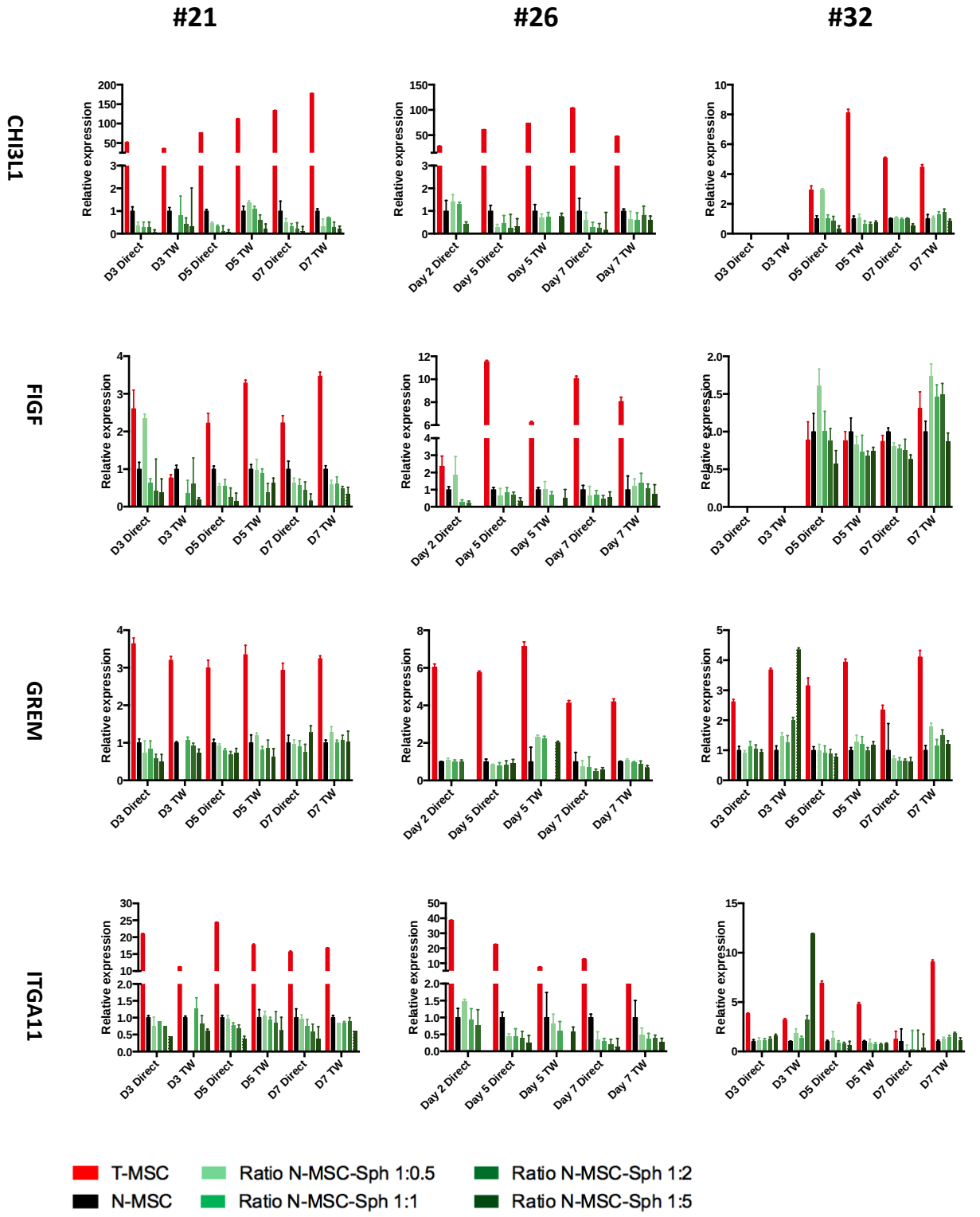

Figure 9: Relative expression of CHI3L1, FIGF, GREM1 and ITGA11 genes in N-MSC after co-culture with TIC. Expression was always normalized to the expression level in N-MSC cultured alone in comparable conditions (same time point and co-culture type). None of the 4 genes showed increased expression following co-culture with TIC.

4. Discussion

Previous studies have investigated the role of MSC in tumor microenvironment and their various effects on angiogenesis, immune modulation, metastasis and chronic inflammation. Gottschling et al. described the presence of MSC endowed with specific functional properties in NSCLC[29]. A similar observation in our lab highlighted that T-MSC display a distinct transcriptome from that of N-MSC, and were associated with an increased metastatic potential of primary tumor cells when co-injected in NSG mice.

In the present study, we focused on 11 genes that were found to be up-regulated in T-MSC compared to N-MSC. Some of them, including ADAMTS12, BST2, GREM1, ITGA11, LOX, LOXL2, IL6 and FIGF are known to be involved in metastasis or immune modulation, whereas others, including CHI3L1, MX2, GJA1, whose role is not fully elucidated, were among the most highly up-regulated genes.

To address our hypothesis that resident lung or bone marrow-derived MSC could be directed by the tumor to acquire a T-MSC profile, we analyzed the expression levels of the 11 selected genes following co-culture with tumor cells. In addition, to determine whether the modulation was dependent on cell-cell contact or soluble factors, we established direct and transwell co-cultures.

TIC induced gene modulation in MSC

FACS analysis showed that CD45- cells isolated from direct co-cultures had a similar phenotype to that of MSC cultured alone. We thus concluded that we did not have tumor cell contamination and could proceed to RNA isolation and gene expression analysis.

The results of transcriptome analysis following co-culture allowed stratification of the genes in 3 categories:

a) Genes showing early up-regulation: ADAMTS12, BST2, IL6 and MX2.

b) Genes showing late up-regulation: GJA1, LOX, LOXL2.

c) Genes showing no direct modulated expression by tumor cells: CHI3L1, GREM1, ITGA11, FIGF.

The first two categories displayed clear induction of gene transcription following co-culture with TIC and the highest relative expression of the genes, sometimes comparable to T-MSC expression levels, especially at 1:5 N-MSC : TIC ratio. Interestingly, the action of TIC occurred in both direct and transwell co-cultures, supporting the assumption of a paracrine action of TIC-derived soluble factors.

These could be secreted proteins alone, protein complexes, or proteins transported in exosomes or microvesicles. Shedding vesicles (< 1µm) and exosomes (40-100 nm) derived from late endosomes, containing heterogeneous material, are biologically active bodies and are recognized as a possible way for tumor cells to interact with their microenvironment[19, 53]. Irrespective of the form in which they are exported, these proteins can trigger signals that induce transcription of target genes in MSC cultured in a distinct compartment from that of TIC.

In the first category, TIC induced rapid up-regulation of the genes, in proportion to their ratio with N-MSC, detectable already at day 3. The second category includes genes that were also up-regulated by TIC co-culture but with a longer latency, as transcriptional modulation occurred only after 5 or 7 days of co-culture. This latency period is possibly due to various mechanisms, at intra- or extracellular levels:

1) TIC may need to receive a signal from MSC in order to secrete the modulating factor. Such activation-dependent expression of a modulating factor could potentially explain the latency in contrast to constitutive expression of an inducing factor.

2) We can hypothesize that genes with latent expression are not accessed by their transcription factors, meaning that their expression requires epigenetic regulation such as histone modifications or DNA demethylation. These processes require time to render the gene promoter accessible.

3) A possible slowdown in gene expression may arise at the very first step of transcription, when the transcription factor binds to the promoter region. It has been shown that the dynamics of transcriptional activation are correlated to the affinity of the transcription factors for their target sequences[54].

Thus, the observed latency may potentially be explained by various mechanisms. If the genes within this class are found to unequivocally increase the metastatic potential, in-depth investigation would be warranted to elucidate the precise underlying inducing pathways.

The last category of genes was not induced by TIC either in direct or in transwell co-culture. The increased expression of these genes in T-MSC compared to N-MSC therefore does not appear to be induced by TIC. This may be explained by the experimental setting: the *in vivo* microenvironment is far more complex and involves more than only two types of cells communicating with one another. Other actors may play a role, such as inflammatory leukocytes and environmental conditions (e.g. hypoxia, tissue pH, etc.). For example, Wei at al. showed that hypoxia could increase LOX-expression

in NSCLC[48]. We must also mention that we cannot rule out latency beyond 7 days, with a possible induction after a longer period of co-culture.

Interestingly, two exceptions to the lack of modulation were found in this category of genes: both occurred in patient #32 at day 3 in a transwell co-culture and concerned GREM1 and ITGA11 genes. For both genes, up-regulation was congruent with the N-MSC : TIC ratio and the highest ratio (1:5) gave rise to levels exceeding that in T-MSC. A possible explanation may be that the up-regulation happened at day 3 in the transwell co-culture but was subsequently silenced by high cell confluence leading to the inhibition of gene expression. However, this observation was made only in TW co-culture suggesting that there may be soluble factors, which promote expression of these genes that may be silenced by signals from transmembrane proteins at TIC surface. An imbalance in favor of this potential inhibition, triggered by cell-cell contact, may lead to absence of overexpression in direct co-culture.

Our experiments showed that MSC can be directly affected by TIC resulting in the up-regulation of genes involved in metastasis and immune modulation. Since this study was based only on RNA level assessment, a next step would be to validate the differences between expression of the genes in T-MSC and N-MSC at the protein level.

We suggest that soluble factors are likely involved in the induction of genes expression in N-MSC by tumor cells. The precise identity of these factors, however, remains to be elucidated. Further experiments will be relevant to identify these factors and their mechanisms of action.

This study focused on only one side of the bidirectional crosstalk that exists between TIC and MSC. Because the observed modifications of the MSC transcriptome may be relevant for tumor evolution, it would be interesting to evaluate the effects of MSC co-culture on tumor cell expression profiles. Actions of MSC on TIC have been investigated in previous studies concluding that this relationship leads to enhanced migration capacity and tumor growth[33, 55, 56]. Indeed, we also observed higher metastatic tumor content in mice in which tumor cells were co-injected with N- and T-MSC (data not shown).

Other studies aimed to characterize MSC in lung cancer. Gottschling et al. drew attention to the fact that NSCLC-associated MSC display particular molecular and functional properties[29]. The original approach of our study consists in the more physiological experimental design using paired primary samples of human N-MSC and TIC. Moreover, since cells used for the *in vitro* co-cultures come from the same patient, *in vitro* observations may provide a simple means to draw correlations with clinical data.

TIC-induced modulation is patient-dependent.

Inter-individual variability was also appreciated. Patient #26 and patient #21 followed a similar profile. In these patients, we observed marked differential expression level between T-MSC and N-MSC of most of the studied genes and a distinct classification of each gene in the early up-regulated, late up-regulated or no modulated groups. These distinctions between the three classes have blurred boundaries for patient #32, where several genes showed only slight, and sometimes undetectable, up-regulation in T-MSC compared to N-MSC (#32, LOX and #32, FIGF).

Interestingly, compared to other patients the up-regulation observed in patient #26 is more distinct for most of the genes. Since every co-culture includes N-MSC and TIC coming from the same patient, it is possible that the modulation observed is dependent on the relative sensitivity of N-MSC specific to each patient, a more or less aggressive behavior of the TIC and/or a specific interaction between the two cell types. To answer this question, it might be of interest to create artificial co-culture couples, mixing N-MSC from one patient with TIC from another or with tumor cell lines.

In vitro MSC modulation intensity and clinical tumor aggressiveness: a clear correlation?

We have observed the presence of a distinct inter-individual pattern of MSC transcription associated with TIC. An interesting study would be the analysis of the link between clinical outcome and the intensity of MSC modulation, to confirm or deny an association between *in vitro* results and patient evolution.

In case of a clear relationship, the next step would be to test whether any of selected genes could be used as a marker, which could help to predict the behavior of tumor cells, and thus the aggressiveness and/or the metastatic potential of the tumor. To reach this goal, a combination of genes or a gene signature, would most likely be more informative and helpful than any single gene. Further studies are required to evaluate the implication of the different selected genes on these processes and their potential role as markers.

Limitations

A limitation in our study is the lack of information about the heterogeneity of MSC populations. At least a fraction of our MSC populations may have begun to differentiate along a define lineage, thereby losing their pluripotency. Although FACS analysis suggests relative phenotypic homogeneity, MSC may well display functional heterogeneity. Indeed, Gottschling et al. showed that, upon

exposure to tumor cell-conditioned medium, N-MSC acquired expression of alpha-smooth muscle actin, a major feature of differentiated cancer-associated fibroblasts[29]. Since we did not evaluate this parameter, we cannot rule out this type of differentiation and must keep in mind that it may contribute to the observed modulation of the MSC transcriptome.

Two other points have to be underlined. The first concerns the results of qRT-PCR, since some of them display a relatively small gap. However, we focused on the general trend and on relative expression, more than on one particular result. In addition, many of our observations concerning gene up-regulation were supported by previous assays done in our lab or observed by others[29]. The second concern is related to the experimental frame, since one can argue that MSC, which normally represent a very small population of the microenvironment, were in our assays incubated at artificially high concentrations.

Concluding remarks

With the present study, we were able to highlight several of the modulations MSC undergo in response to tumor cell presence. Indeed, we demonstrated that co-cultures of TIC and N-MSC induce overexpression by N-MSC of several genes involved in metastasis and immune modulation. Thus, we concluded that tumor cells are at least partially responsible for the modification of N-MSC gene expression. We also propose a paracrine mechanism through secretion of factors by TIC for the observed gene up-regulation. To evaluate the safety and efficacy of stem cell use in anti-cancer therapy, the precise effect of MSC on tumor cells needs to be investigated in-depth. Our assay provides several elements for a detailed characterization and a better understanding of their action. In addition, our results support the concept of the “educational” role of tumor cells towards its microenvironment. Further studies are needed to evaluate the precise *in vivo* effects of the observed gene up-regulation in MSC, but the first results show a correlation between up-regulation of a few of these genes and an increased metastatic potential. In case of positive validation of this hypothesis, strategies aiming to block this modulation are of prime interest, either directly with an antibody targeting the MSC product or with an inhibitor acting at an earlier stage (e.g. blocking the inducing factors produced by TIC).

Regarding the second axis to decrease lung cancer mortality – the inefficiency of early detection - a correlation between *in vitro* modulation and clinical outcome may help identify new markers and possibly original detection methods. This could further increase the sensitivity of detection, allowing diagnosis at earlier stages and improving prognosis.

APPENDICES

APPENDIX 1 Patient #21

Figure I: Phenotype of #21 MSC after selection, analyzed by FACS.

MSC were negatively selected. Phenotypes displayed a clear similarity between MSC cultured alone and isolated from the different co-culture ratios and at different days. Cells were positive for CD90, CD105 and CD73, with a slightly decreased expression of CD105 at day 7 in all conditions.

Although MSC were supposed to be *lineage* negative, they were distinct from the negative control. This little positivity was similar to the one that we observed in MSC isolated from bone marrow after culture (data not shown), suggesting a likely induction of *lineage* genes *in vitro*.

APPENDIX 2

Patient #26

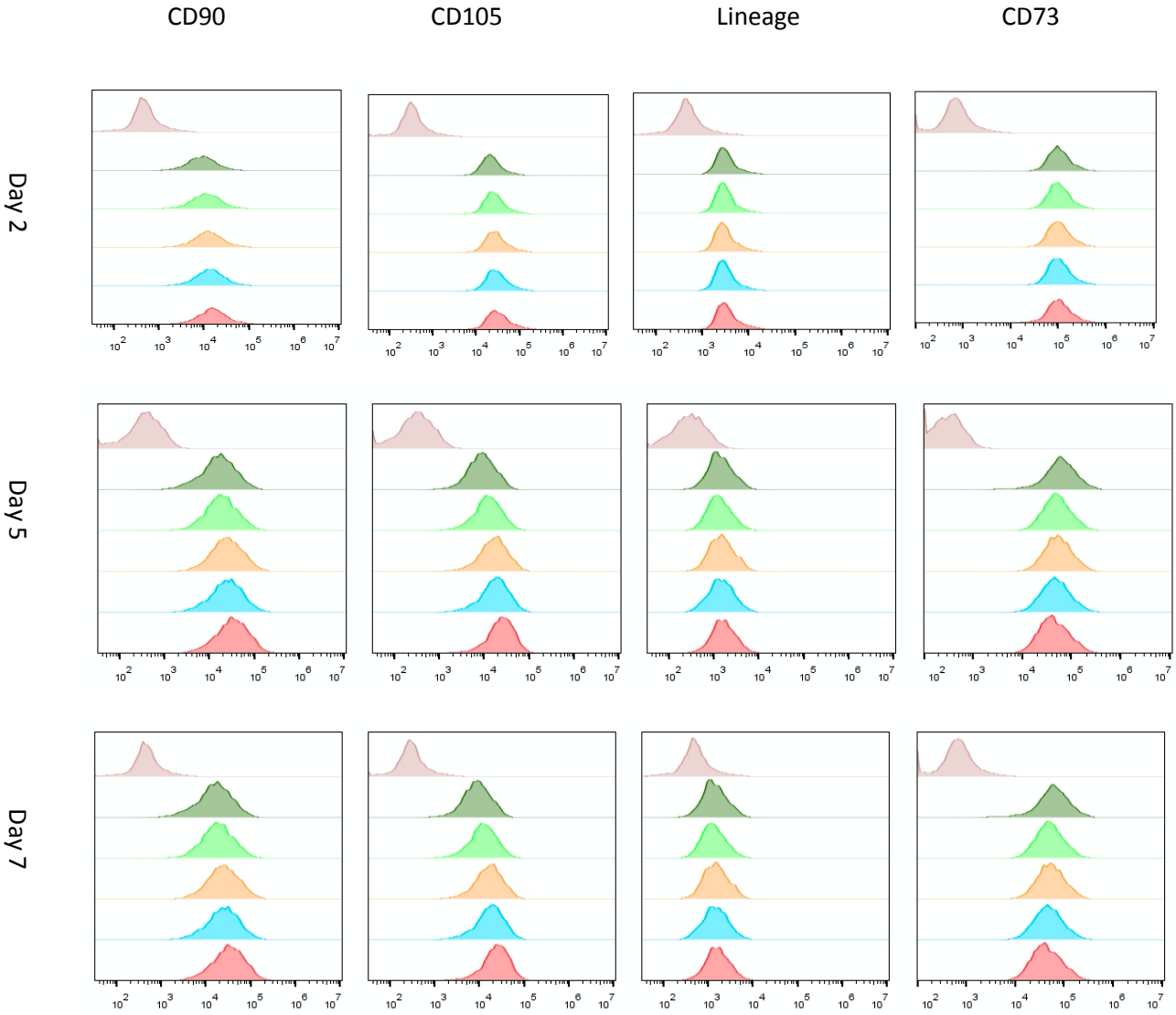

Figure II: Phenotype of patient #26 MSC after selection.

MSC phenotype was comparable between MSC cultured alone and after CD45 negative selection from all direct co-culture conditions.

APPENDIX 3

Patient #32

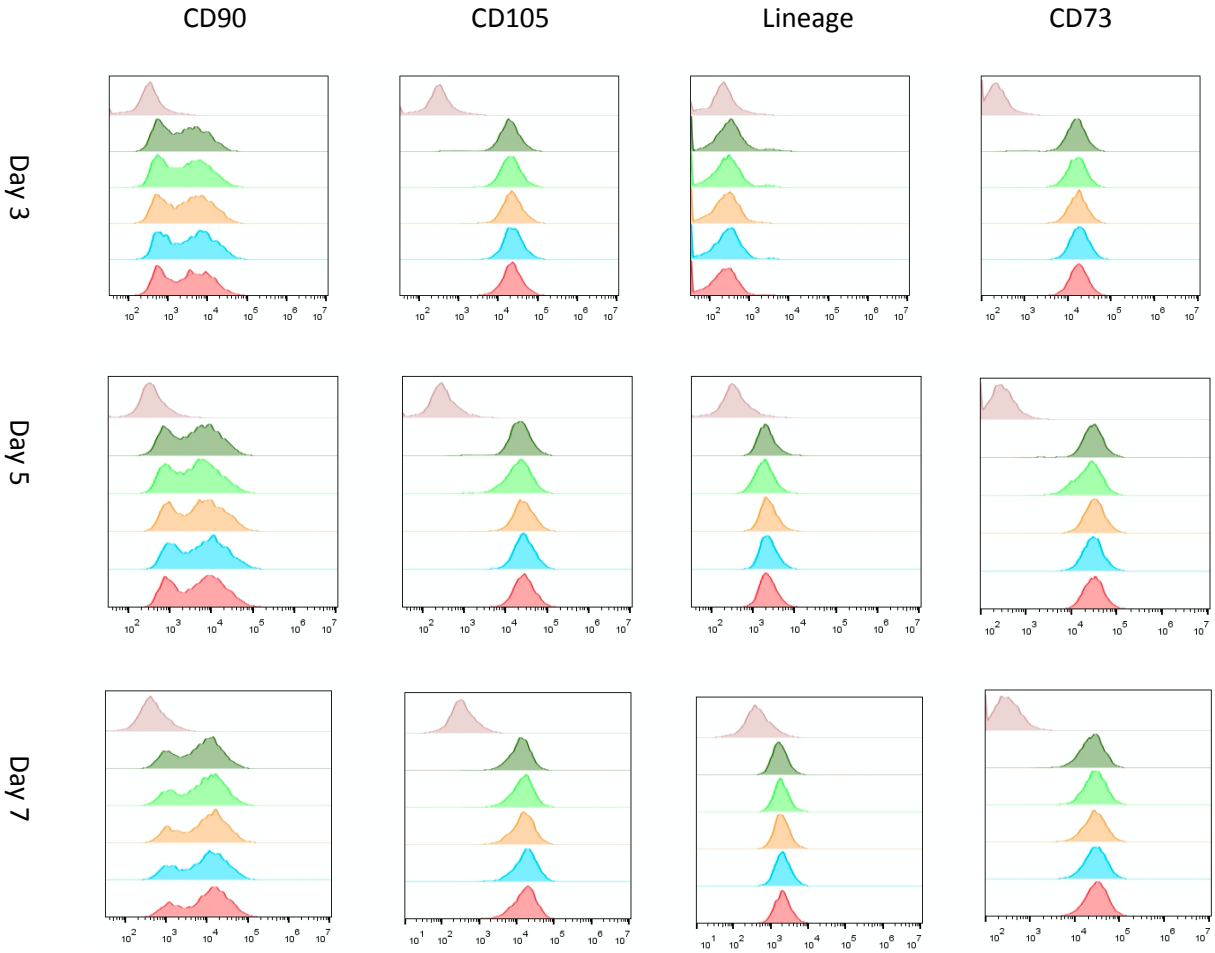

Figure III: Phenotype of patient #32 MSC after selection.

As for patients #21 and #26, MSC phenotype was comparable in all conditions and similar to control MSC cultured alone. The double peak observed for the CD90 attested to the likely heterogeneity of the MSC population of patient #32.

APPENDIX 4

Patient #32

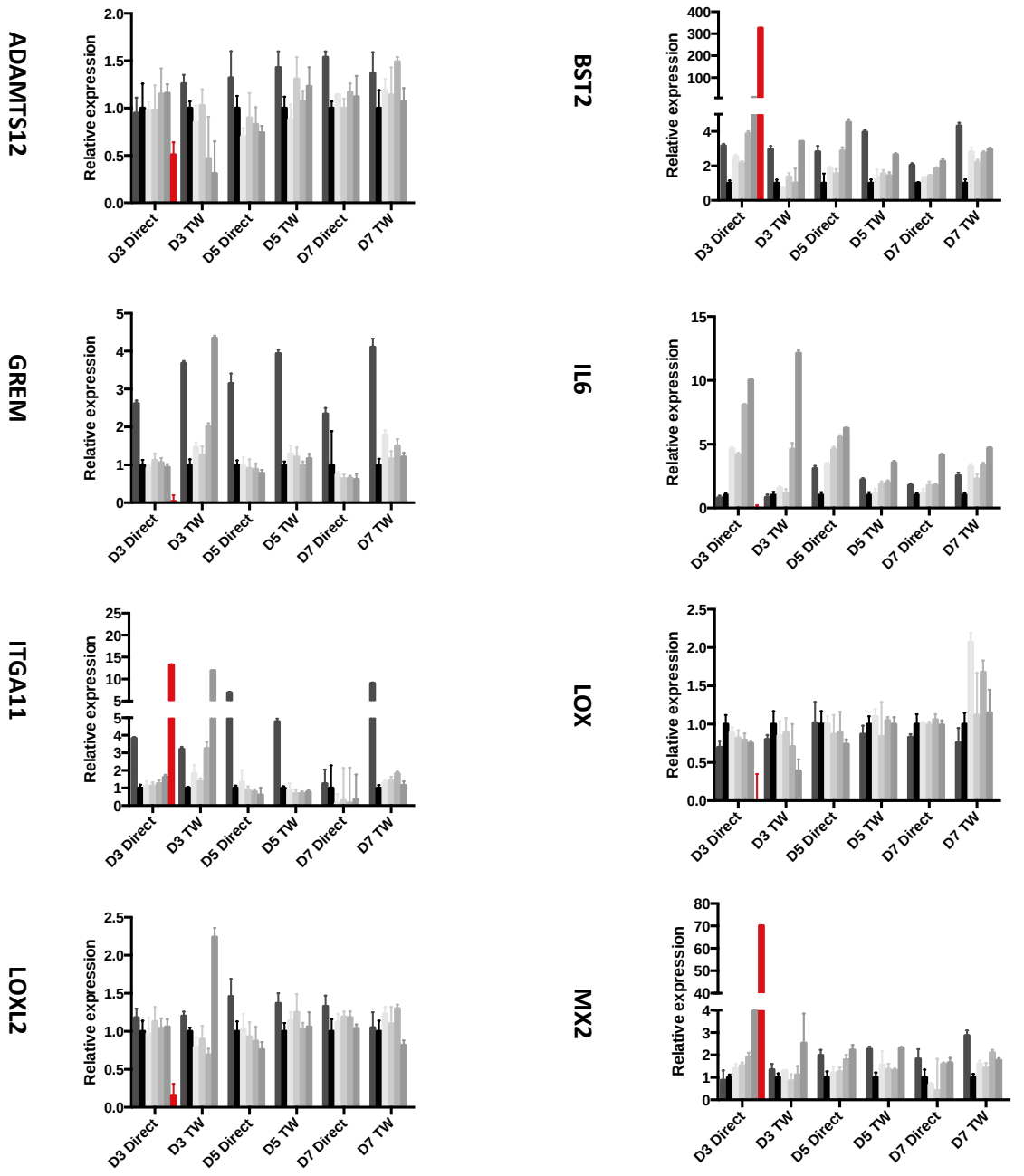

Figure IV: Relative gene expression in N-MSC and TIC in co-cultures (Patient #32).

TIC were collected from transwell co-culture at day 3.

TIC displayed a distinct gene expression compared to either N-MSC in co-culture or control MSC. They highly overexpressed BST2, ITGA11 and MX2 compared to MSC.

References

[1] J Ferlay et al., "Cancer Incidence and Mortality Worldwide: Sources, Methods and Major Patterns in GLOBOCAN 2012," *International Journal of Cancer* 136, no. 5 (March 1, 2015): E359–86, doi:10.1002/ijc.29210.
[2] "Lung and Bronchus Cancer, CSR 1975-2013 - sect_15_lung_bronchus.pdf," accessed September 9, 2016, http://seer.cancer.gov/csr/1975_2013/results_merged/sect_15_lung_bronchus.pdf.
[3] V Kumar, A Abbas , J Aster, "Robbins Basic Pathology," 9th edition (Philadelphia, PA: Elsevier/Saunders, 2013, n.d.).
[4] O Auerbach, EC Hammond, and L Garfinkel, "Changes in Bronchial Epithelium in Relation to Cigarette Smoking, 1955–1960 vs. 1970–1977," *New England Journal of Medicine* 300, no. 8 (février 1979): 381–86, doi:10.1056/NEJM197902223000801.
[5] L Thiberville et al., "Evidence of Cumulative Gene Losses with Progression of Premalignant Epithelial Lesions to Carcinoma of the Bronchus," *Cancer Research* 55, no. 22 (November 15, 1995): 5133–39.
[6] Y Sekido, KM Fong, and JD Minna, "Molecular Genetics of Lung Cancer," *Annual Review of Medicine* 54, no. 1 (2003): 73–87, doi:10.1146/annurev.med.54.101601.152202.
[7] RL Siegel, KD Miller, and A Jemal, "Cancer Statistics, 2015," *CA: A Cancer Journal for Clinicians* 65, no. 1 (January 1, 2015): 5–29, doi:10.3322/caac.21254.
[8] "Cancer Facts & Figures 2015 - Acspc-044552.pdf," accessed March 28, 2016, http://www.cancer.org/acs/groups/content/@editorial/documents/document/acspc-044552.pdf.
[9] Y Wang, G Schmid-Bindert, and C Zhou, "Erlotinib in the Treatment of Advanced Non-Small Cell Lung Cancer: An Update for Clinicians," *Therapeutic Advances in Medical Oncology* 4, no. 1 (January 2012): 19–29, doi:10.1177/1758834011427927.
[10] E Lasalvia-Prisco et al., "Randomized Phase II Clinical Trial of Chemo-Immunotherapy in Advanced Nonsmall Cell Lung Cancer," *Biologics : Targets & Therapy* 2, no. 3 (September 2008): 555–61.
[11] P Dalerba, RW Cho, and MF Clarke, "Cancer Stem Cells: Models and Concepts," *Annual Review of Medicine* 58, no. 1 (2007): 267–84, doi:10.1146/annurev.med.58.062105.204854.
[12] M Alamgeer et al., "Cancer Stem Cells in Lung Cancer: Evidence and Controversies," *Respirology (Carlton, Vic.)* 18, no. 5 (July 2013): 757–64, doi:10.1111/resp.12094.
[13] L MacDonagh et al., "Lung Cancer Stem Cells: The Root of Resistance," *Cancer Letters* 372, no. 2 (March 28, 2016): 147–56, doi:10.1016/j.canlet.2016.01.012.
[14] SL Wood et al., "The Role of the Tumor-Microenvironment in Lung Cancer-Metastasis and Its Relationship to Potential Therapeutic Targets," *Cancer Treatment Reviews* 40, no. 4 (mai 2014): 558–66, doi:10.1016/j.ctrv.2013.10.001.
[15] R Liu et al., "Mesenchymal Stem Cells in Lung Cancer Tumor Microenvironment: Their Biological Properties, Influence on Tumor Growth and Therapeutic Implications," *Cancer Letters* 353, no. 2 (October 28, 2014): 145–52, doi:10.1016/j.canlet.2014.07.047.
[16] HF Dvorak, "Tumors: Wounds That Do Not Heal--Redux," *Cancer Immunology Research* 3, no. 1 (January 2015): 1–11, doi:10.1158/2326-6066.CIR-14-0209.
[17] D Hanahan and LM Coussens, "Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment," *Cancer Cell* 21, no. 3 (March 20, 2012): 309–22, doi:10.1016/j.ccr.2012.02.022.
[18] B Psaila and D Lyden, "The Metastatic Niche: Adapting the Foreign Soil," *Nature Reviews. Cancer* 9, no. 4 (April 2009): 285–93, doi:10.1038/nrc2621.
[19] DF Quail and JA Joyce, "Microenvironmental Regulation of Tumor Progression and Metastasis," *Nature Medicine* 19, no. 11 (November 2013): 1423–37, doi:10.1038/nm.3394.
[20] M. Dominici et al., "Minimal Criteria for Defining Multipotent Mesenchymal Stromal Cells. The International Society for Cellular Therapy Position Statement," *Cytotherapy* 8, no. 4 (2006): 315–17, doi:10.1080/14653240600855905.

[21] MF Pittenger et al., "Multilineage Potential of Adult Human Mesenchymal Stem Cells," *Science* 284, no. 5411 (April 2, 1999): 143–47, doi:10.1126/science.284.5411.143.
[22] M Kéramidas et al., "The Dual Effect of Mesenchymal Stem Cells on Tumour Growth and Tumour Angiogenesis," *Stem Cell Research & Therapy* 4, no. 2 (April 29, 2013): 41, doi:10.1186/scrt195.
[23] J Stagg and J Galipeau, "Mechanisms of Immune Modulation by Mesenchymal Stromal Cells and Clinical Translation," *Current Molecular Medicine* 13, no. 5 (May 1, 2013): 856–67, doi:10.2174/1566524011313050016.
[24] E Spaeth et al., "Inflammation and Tumor Microenvironments: Defining the Migratory Itinerary of Mesenchymal Stem Cells," *Gene Therapy* 15, no. 10 (avril 2008): 730–38, doi:10.1038/gt.2008.39.
[25] KS Aboody, J Najbauer, and MK Danks, "Stem and Progenitor Cell-Mediated Tumor Selective Gene Therapy," *Gene Therapy* 15, no. 10 (March 27, 2008): 739–52, doi:10.1038/gt.2008.41.
[26] G Bassi et al., "CCR2-Dependent Recruitment of Macrophages by Tumor Educated Mesenchymal Stromal Cells Promotes Tumor Development and Is Mimicked by TNF-Alpha," *Cell Stem Cell* 11, no. 6 (December 7, 2012): 812–24, doi:10.1016/j.stem.2012.08.013.
[27] BG Cuiffo and AE Karnoub, "Mesenchymal Stem Cells in Tumor Development," *Cell Adhesion & Migration* 6, no. 3 (May 1, 2012): 220–30, doi:10.4161/cam.20875.
[28] CMF Gomes, "The Dual Role of Mesenchymal Stem Cells in Tumor Progression," *Stem Cell Research & Therapy* 4, no. 2 (April 29, 2013): 42, doi:10.1186/scrt189.
[29] S Gottschling et al., "Mesenchymal Stem Cells in Non-Small Cell Lung cancer—Different from Others? Insights from Comparative Molecular and Functional Analyses," *Lung Cancer* 80, no. 1 (April 1, 2013): 19–29, doi:10.1016/j.lungcan.2012.12.015.
[30] K Suzuki et al., "Mesenchymal Stromal Cells Promote Tumor Growth through the Enhancement of Neovascularization," *Molecular Medicine* 17, no. 7–8 (2011): 579–87, doi:10.2119/molmed.2010.00157.
[31] JG Casado, R Tarazona, and FM Sanchez-Margallo, "NK and MSCs Crosstalk: The Sense of Immunomodulation and Their Sensitivity," *Stem Cell Reviews and Reports* 9, no. 2 (February 10, 2013): 184–89, doi:10.1007/s12015-013-9430-y.
[32] J Guan and J Chen, "Mesenchymal Stem Cells in the Tumor Microenvironment," *Biomedical Reports* 1, no. 4 (July 2013): 517–21, doi:10.3892/br.2013.103.
[33] W Zhu et al., "Mesenchymal Stem Cells Derived from Bone Marrow Favor Tumor Cell Growth in Vivo," *Experimental and Molecular Pathology* 80, no. 3 (juin 2006): 267–74, doi:10.1016/j.yexmp.2005.07.004.
[34] M Valtieri and A Sorrentino, "The Mesenchymal Stromal Cell Contribution to Homeostasis," *Journal of Cellular Physiology* 217, no. 2 (November 1, 2008): 296–300, doi:10.1002/jcp.21521.
[35] M Llamazares et al., "The ADAMTS12 Metalloproteinase Exhibits Anti-Tumorigenic Properties through Modulation of the Ras-Dependent ERK Signalling Pathway," *Journal of Cell Science* 120, no. 20 (October 15, 2007): 3544–52, doi:10.1242/jcs.005751.
[36] AG Beristain, H Zhu, and PCK Leung, "Regulated Expression of ADAMTS-12 in Human Trophoblastic Cells: A Role for ADAMTS-12 in Epithelial Cell Invasion?," *PLoS ONE* 6, no. 4 (April 11, 2011), doi:10.1371/journal.pone.0018473.
[37] J Walter-Yohrling et al., "Identification of Genes Expressed in Malignant Cells That Promote Invasion," *Cancer Research* 63, no. 24 (December 15, 2003): 8939–47.
[38] EH Yi et al., "BST-2 Is a Potential Activator of Invasion and Migration in Tamoxifen-Resistant Breast Cancer Cells," *Biochemical and Biophysical Research Communications* 435, no. 4 (juin 2013): 2, doi:10.1016/j.bbrc.2013.05.043.
[39] XW Wang et al., "Increased Expression of Chitinase 3-like 1 Is a Prognosis Marker for Non-Small Cell Lung Cancer Correlated with Tumor Angiogenesis," *Tumor Biology* 36, no. 2 (October 12, 2014): 901–7, doi:10.1007/s13277-014-2690-6.
[40] M Kawada et al., "Chitinase 3-like 1 Promotes Macrophage Recruitment and Angiogenesis in Colorectal Cancer," *Oncogene* 31, no. 26 (June 28, 2012): 3111–23, doi:10.1038/onc.2011.498.
[41] Y Feng et al., "Expression of VEGF-C and VEGF-D as Significant Markers for Assessment of Lymphangiogenesis and Lymph Node Metastasis in Non-Small Cell Lung Cancer," *The Anatomical*

Record: Advances in Integrative Anatomy and Evolutionary Biology 293, no. 5 (mai 2010): 802–12, doi:10.1002/ar.21096.
[42] JL Solan, SR Hingorani, and PD Lampe, "Changes in Connexin43 Expression and Localization During Pancreatic Cancer Progression," *The Journal of Membrane Biology* 245, no. 5–6 (June 2012): 255–62, doi:10.1007/s00232-012-9446-2.
[43] MS Mulvihill et al., "Gremlin Is Overexpressed in Lung Adenocarcinoma and Increases Cell Growth and Proliferation in Normal Lung Cells," *PLoS ONE* 7, no. 8 (August 1, 2012), doi:10.1371/journal.pone.0042264.
[44] TJ Bayliss et al., "A Humanized Anti-IL-6 Antibody (ALD518) in Non-Small Cell Lung Cancer," *Expert Opinion on Biological Therapy* 11, no. 12 (décembre 2011): 1663–68, doi:10.1517/14712598.2011.627850.
[45] W Chen et al., "The CCL2/CCR2 Axis Enhances IL-6-Induced Epithelial-Mesenchymal Transition by Cooperatively Activating STAT3-Twist Signaling," *Tumor Biology* 36, no. 2 (October 16, 2014): 973–81, doi:10.1007/s13277-014-2717-z.
[46] CQ Zhu et al., "Integrin α11 Regulates IGF2 Expression in Fibroblasts to Enhance Tumorigenicity of Human Non-Small-Cell Lung Cancer Cells," *Proceedings of the National Academy of Sciences of the United States of America* 104, no. 28 (July 10, 2007): 11754–59, doi:10.1073/pnas.0703040104.
[47] R Navab et al., "Integrin α11β1 Regulates Cancer Stromal Stiffness and Promotes Tumorigenicity and Metastasis in Non-Small Cell Lung Cancer," *Oncogene* 35, no. 15 (April 14, 2016): 1899–1908, doi:10.1038/onc.2015.254.
[48] L Wei et al., "Lysyl Oxidase May Play a Critical Role in Hypoxia-Induced NSCLC Cells Invasion and Migration," *Cancer Biotherapy & Radiopharmaceuticals* 27, no. 10 (December 2012): 672–77, doi:10.1089/cbr.2012.1241.
[49] Q Xiao and G Ge, "Lysyl Oxidase, Extracellular Matrix Remodeling and Cancer Metastasis," *Cancer Microenvironment* 5, no. 3 (April 13, 2012): 261–73, doi:10.1007/s12307-012-0105-z.
[50] MC King, G Raposo, and MA Lemmon, "Inhibition of Nuclear Import and Cell-Cycle Progression by Mutated Forms of the Dynamin-like GTPase MxB," *Proceedings of the National Academy of Sciences of the United States of America* 101, no. 24 (June 15, 2004): 8957–62, doi:10.1073/pnas.0403167101.
[51] K Melén et al., "Human MxB Protein, an Interferon-A-Inducible GTPase, Contains a Nuclear Targeting Signal and Is Localized in the Heterochromatin Region beneath the Nuclear Envelope," *Journal of Biological Chemistry* 271, no. 38 (September 20, 1996): 23478–86, doi:10.1074/jbc.271.38.23478.
[52] S Cal et al., "Identification, Characterization, and Intracellular Processing of ADAM-TS12, a Novel Human Disintegrin with a Complex Structural Organization Involving Multiple Thrombospondin-1 Repeats," *Journal of Biological Chemistry* 276, no. 21 (May 25, 2001): 17932–40, doi:10.1074/jbc.M100534200.
[53] H Peinado, S Lavotshkin, and D Lyden, "The Secreted Factors Responsible for Pre-Metastatic Niche Formation: Old Sayings and New Thoughts," *Seminars in Cancer Biology*, The Biology of Cancer Metastasis, 21, no. 2 (avril 2011): 139–46, doi:10.1016/j.semcancer.2011.01.002.
[54] E Segal and J Widom, "From DNA Sequence to Transcriptional Behavior: A Quantitative Approach," *Nature Reviews. Genetics* 10, no. 7 (July 2009): 443–56, doi:10.1038/nrg2591.
[55] S Liu et al., "Breast Cancer Stem Cells Are Regulated by Mesenchymal Stem Cells through Cytokine Networks," *Cancer Research* 71, no. 2 (January 15, 2011): 614–24, doi:10.1158/0008-5472.CAN-10-0538.
[56] K McLean et al., "Human Ovarian Carcinoma–associated Mesenchymal Stem Cells Regulate Cancer Stem Cells and Tumorigenesis via Altered BMP Production," *The Journal of Clinical Investigation* 121, no. 121(8) (August 1, 2011): 3206–19, doi:10.1172/JCI45273.