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Regulation of pro-angiogenic and pro-inflammatory cytokines during epithelial-to-mesenchymal transitions associated to the metastatic progression of breast and lung tumor cells.

Régulation de cytokines pro-inflammatoires et pro-angiogènes au cours de la transition épithélio-mésenchymateuse associée à la progression métastatique de cellules tumorales mammaires et pulmonaires.

Mémoire présenté par

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en vue de l'obtention du grade de Docteur en sciences biomédicales et pharmaceutiques

Année académique 2014-2015

Remerciements

J'adresse en premier lieu toute ma gratitude au Docteur Christine Gilles qui a été mon mentor pendant ces quatre années de thèse. Christine, ton implication quotidienne et ta disponibilité hors norme m'ont énormément encouragée. Tu t'es patiemment attelée à m'aider à développer progressivement une compréhension nuancée de la littérature et un esprit critique. Je garde un excellent souvenir de nos discussions et de ton encadrement bienveillant pendant toute ma thèse. Merci !

Ma reconnaissance va également aux Professeurs Agnès Noël et Philippe Delvenne qui ont rendu ce projet possible en m'accueillant au sein de leur laboratoire respectif et ont consacré du temps à la relecture de ce manuscrit et des publications.

Je tiens ensuite à remercier le Docteur Michael Herfs. Michael, merci pour ta disponibilité, ton soutien considérable et la perpétuelle confiance que tu me portes. Merci aussi aux autres membres du Laboratoire de Pathologie Expérimentale avec qui j'ai eu la chance et la joie de travailler : Pascale Hubert, Patrick Roncarati et Estelle Dortu.

Merci à mes collègues de l'équipe EMT et tout particulièrement à Morgane Bourcy. Morgane, pour beaucoup de raisons, j'ai vraiment apprécié travailler avec toi. Je ne citerai ici que ton organisation impressionnante … et ton imagination désopilante ! Merci aussi aux autres sympathiques membres de l'équipe : Laïdya Syne et « les jeunes » : Justine Lambert et Marie-Emilie Francart. Ça a été pour moi très agréable de travailler au sein de cette « CG team » drôle et dynamique.

Il me tient aussi à cœur de remercier le Docteur Silvia Blacher, notre insolite analyste d'images, dont l'expertise a amplement bénéficié à mon travail. Merci Silvia pour ton travail efficace et pour ton enthousiasme débordant.

Je remercie évidement l'équipe des techniciens du laboratoire : Marie Dehuy, Guy Roland, Isabelle Dasoul, Emilie Feyereisen, Laurette Volders, Erika Konradowski et Nathalie Lefin, pour leur précieuse contribution technique bien sûr mais aussi pour leur souci de l'autre. Marie et Guy : c'est grâce à votre aide que je suis devenue capable d'aborder sereinement les expériences animales. Erika : un grand merci pour ton aide, toutes techniques confondues ! Isabelle et Emilie : merci pour l'énorme travail que vous accomplissez en histologie. Enfin, merci Laurette pour la génération de plasmides et merci Nathalie pour ton aide pour les qPCR.

Merci aussi à Natacha et Cédric, mes voisins de bureau, pour leur bonne humeur et la bonne ambiance de travail. Merci Natacha pour nos discussions, pour tes conseils amicaux et ta chaleureuse présence.

Je suis également très reconnaissante envers nos collaborateurs au sein du laboratoire d'histologie du Professeur Philippe Birembaut : Professeur Myriam Polette et Julien Lesage, qui ont partagé de précieux prélèvements humains et ont investi beaucoup de leur temps dans cette étude.

Je souhaite bien évidemment remercier mes amis et ma famille. Je commence par toi, Gaël, qui as vaillamment partagé le quotidien d'une doctorante stressée et plus que probablement stressante, avec beaucoup de patience et d'amour. Je remercie tout spécialement l'équipe des « Fruits en Folie » pour nos réunions sportives et moins sportives, toujours joyeuses et ressourçantes. Merci à Estelle pour son amitié inébranlable et sa présence chaleureuse. Merci à mes parents pour leur soutien inconditionnel et enfin à mon parrain pour son intérêt pour mes travaux et pour nos longues discussions enthousiasmées.

Pour terminer, je remercie tous les membres du Comité de thèse pour avoir suivi et guidé mon avancement annuellement. Enfin, j'adresse mes remerciements anticipés à tous les membres du jury, qui prendront le soin d'étudier mon manuscrit et d'y apporter leur critique finale et leur juste appréciation.

Abstract

Even though epithelial-to-mesenchymal transition (EMT) existence in cancer epithelial cells has been a topic of debate for a long time, it is now widely accepted that cancer cells undergo EMT-like changes, which are tightly regulated in time and space and endow cells with intrinsically enhanced invasive and survival capacities that might favor metastatic dissemination. Clinical observations showing that presence of EMT markers relates to a high histological grade, presence of lymph node metastasis and chemoresistance are corroborated by animal models and *in vitro* data demonstrating a role for EMT in invasion and intravasation. Nevertheless, although signaling cascades triggering EMT have been extensively studied, the impact of EMT on the crosstalk between tumor cells and the tumor microenvironment remains elusive. Metastatic dissemination indeed largely relies on tumor-stroma interactions. Tumor cells can secrete numerous soluble factors such as cytokines, chemokines and growth factors, some of which have been described as upregulated in cancer progression, even though the mechanisms of such an overexpression remain unclear. During this thesis, we aimed at characterizing the impact of EMT on the tumor-stroma crosstalk. Using *in vitro* models of cell lines inducible for EMT, we identified five EMT-induced soluble factors: Interleukin 8 (IL-8), Interleukin-6 (IL-6), soluble form of InterCellular Adhesion Molecule-1 (sICAM-1), Plasminogen Activator Inhibitor-1 (PAI-1) and Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF). These factors are partially regulated by Snail even though other transcription factors are most likely involved. We further demonstrated that EMT-derived soluble factors are pro-angiogenic. Finally, our preliminary data suggest that conditioned medium from EMT-positive cells stimulates the recruitment of myeloid-derived suppressor cells (MDSC). Taken together, our results show that EMT programs trigger the expression of soluble mediators in breast and lung cancer cells that stimulate angiogenesis and recruit MDSCs, which might in turn favor metastatic dissemination.

Résumé

Bien que l'existence de processus de transition épithélio-mésenchymateuse (TEM) dans les cellules épithéliales cancéreuses fût débattue pendant plusieurs années, il est aujourd'hui largement accepté que les cellules cancéreuses subissent des changements semblables à la TEM. Ces changements, étroitement régulés dans le temps et l'espace, confèrent aux cellules des propriétés intrinsèques invasives et de survie accrues qui pourraient faciliter la progression métastatique. Des observations cliniques montrant que la présence de marqueurs de la TEM est associée à un grade histologique élevé et à la présence de métastases ganglionnaires sont corroborées par des modèles animaux et *in vitro* démontrant le rôle de la TEM dans l'invasion et l'intravasation. Néanmoins, si les cascades de signalisation de la TEM ont fait l'objet d'études approfondies, la compréhension de l'impact de la TEM sur le dialogue entre les cellules cancéreuses et le microenvironnement tumoral est lacunaire. Les cellules cancéreuses peuvent en effet sécréter un grand nombre de facteurs solubles tels des cytokines, chémokines et facteurs de croissance. Certains sont augmentés lors de la progression cancéreuse, bien que les mécanismes impliqués dans leur surexpression par les cellules cancéreuses soient méconnus. Au cours de cette thèse, nous avons visé à caractériser l'impact de la TEM sur le dialogue établi entre les cellules tumorales et le stroma. À l'aide de modèles *in vitro*, nous avons identifié cinq facteurs solubles dont l'expression est régulée au cours de la TEM : l'interleukine-8 (IL-8), l'interleukine-6 (IL-6), la forme soluble de l'InterCellular Adhesion Molecule-1 (sICAM-1), le Plasminogen Activator Inhibitor-1 (PAI-1) et le Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF). L'expression de ces facteurs est en partie régulée par le facteur de transcription Snail. De plus, nous avons démontré que ces facteurs solubles exercent une activité angiogène *in vitro* et *in vivo*. Enfin, nos données préliminaires suggèrent que ces facteurs solubles recrutent des cellules immunosuppressives dérivées de la lignée myéloïde, les MDSC. Dans l'ensemble, nos résultats indiquent que la TEM induit l'expression de facteurs solubles par les cellules tumorales mammaires et pulmonaires, lesquels sont pro-angiogènes et pourraient recruter les MDSC, favorisant ainsi la mise ne place d'environnement permissif à la progression cancéreuse.

List of abbreviations

α-SMA	Alpha-smooth muscle actin
β-TrCP1	Beta-transducin repeat-containing protein
ANG	Angiopoietin
BC	Breast cancer
bHLH	Basic helix-loop-helix
BRCA1/2	Breast cancer
CK	Cytokeratin
COX2	Cyclooxygenase 2
CTBP	C-terminal binding protein
DAPI	4',6'-diamidino-2-phénylindole
DCIS	Ductal carcinoma in situ
ECM	Extracellular matrix
EGF(R)	Epidermal growth factor (receptor)
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
FBXL14	F-box and leucine-rich repeat protein 14
(b)FGF	(basic) Fibroblast growth factor
FSP1	Fibroblast specific protein 1
GM-CSF	Granulocyte monocyte-colony stimulating factor
GSK3β	Glycogen synthase kinase 3 beta
HβD	Human beta-defensin
HDAC	Histone deacetylase
HER2/Neu	Human epidermal growth factor receptor/neuroglioblastoma
HMGA2	High motility group A2
HUVEC	Human umbilical vein endothelial cell
IDC	Invasive ductal carcinoma
IL-6/8	Interleukin-6/8
ILC	Invasive lobular carcinoma

iNOS	Inducible nitric oxide synthase
KLF	Krüppel-like factor
LCIS	Lobular carcinoma in situ
LN	Lymph node
LTBP	Latent TGF-beta-binding protein
MDSC	Myeloid-derived suppressor cell
NES	Nuclear export signal
NF-κB	Nuclear factor kappa-B
NSCLC	Non-small cell lung carcinoma
(MT-)MMP	(membrane type) Matrix metalloproteinase
OS	Overall survival
PAI-1	Plasminogen activator inhibitor-1
PCNA	Proliferating cell nuclear antigen
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol-3 kinase
PlGF	Placental growth factor
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
ROS	Reactive oxygen species
SCC	Squamous cell carcinoma
sICAM-1	Soluble form of intercellular adhesion molecule-1
TIE	Tyrosine kinase with Immunoglobulin-like and EGF homology
TNBC	Triple-negative breast cancer
VEGF	Vascular endothelial growth factor
ZEB	Zinc finger E-box-binding homeobox

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Part I: EMT-regulated soluble factors mediate cancer-related angiogenesis and myeloid cell recruitment

Introduction

1 Breast cancer

1.1 Introduction: epidemiology and etiology

Breast cancer is a malignant disease involving the neoplastic transformation of mammary epithelial cells. As illustrated in Figure 1, these cells are normally arranged into acini/alveoli comprising milk-producing luminal epithelial cells supported by flat myoepithelial cells. Inside acini, secretions from luminal epithelial cells are collected in small intralobular ducts. These ducts drain milk to interlobular ducts, which are delimited by a mono-stratified cuboid epithelium and converge into large lactiferous canals that fuse at the nipple. Normal breast stroma contains fibroblasts and collagen chiefly but also fat, blood and lymphatic vessels.

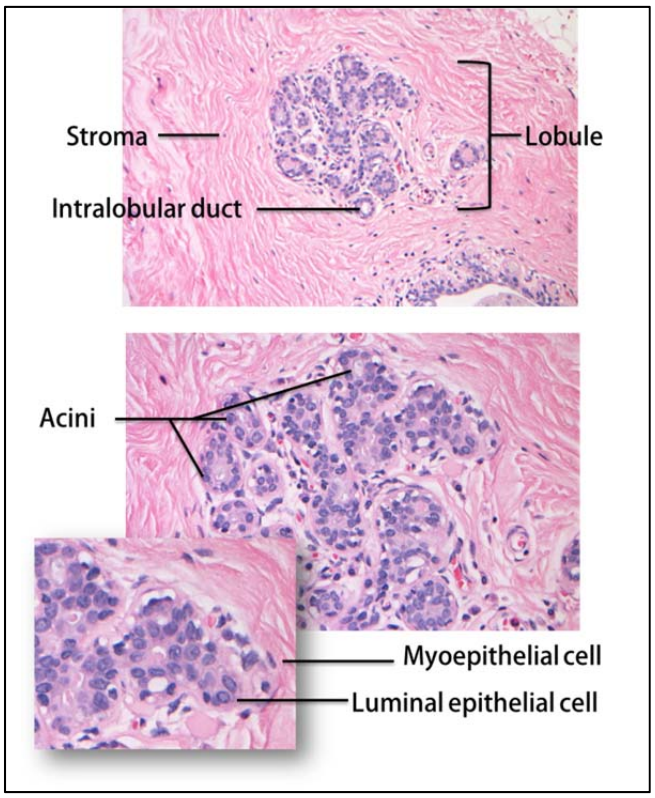

Figure 1 : Histology of the normal breast.

Representing 28,8% of global cancer incidence and 16,8% of cancer-related mortality in Europe (Youlden et al., 2012), breast cancer (BC) is by far the most common cancer in women and their leading cause of cancer-related death in Europe and also worldwide (Ferlay et al., 2013). In Belgium, over 10.000 new cases of invasive breast cancer were diagnosed in 2011 (Cancer, 2014).

Because of the strong association between early diagnosis and overall survival, many countries have developed population-based organized screening programs using mammography to detect breast cancer at asymptomatic stages. The Belgian screening program started in 2001 targeting 50-69 year-old women. Like in most European screening programs, women are screened every second year (Youlden et al., 2012). Systematic screening programs have successfully led to a reduction in mortality in several countries (Youlden et al., 2012).

Numerous risk factors of breast cancer include age (75% of breast cancers affect women over 50 but less than 10% affect women under 40), late first pregnancy, family history of cancer, menstrual history (i.e. early menarche or late menopause), and onset of benign breast disease such as fibrocystic changes or papilloma. Geographic factors also influence the probability to develop breast cancer, since breast cancer incidence is for example highest in North America. Obesity, oral contraception, exogenous estrogens, high fat diet, alcohol consumption and cigarette smoking are less well established risk factors (Kumar et al., 2007).

1.2 Histopathological classification

Breast cancers are primarily classified according to histopathological criteria such as basement membrane crossing/degradation and histological specialization. Non-invasive breast carcinomas are delimited by an intact basement membrane and comprise ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS). Invasive carcinomas are not contained by any basement membrane and invade adjacent stromal tissue. They include several histopathological types.

Invasive ductal carcinoma (IDC) is the most encountered invasive breast cancer histological type (Figure 2A, C) since it represents about 75% of all breast cancers (Farhat et al., 2010). It

is also known as scirrhous carcinoma, because of the high amount of fibrosis surrounding epithelial neoplastic cells, which leads to the formation of a hard palpable mass in clinics. The 'invasive ductal carcinoma' designation actually comprises every carcinoma that does not fit in other groups and is therefore very heterogeneous microscopically (see paragraph 1.3).

Invasive lobular carcinoma (ILC) represents 10-15% of invasive breast cancers and is characterized by a loss of E-cadherin expression (Morrogh et al., 2012) (Figure 2F) and a specific 'Indian file' infiltration pattern (Figure 2B, D) that is discussed in section 2: EMT and cancer.

Other types such as medullary carcinoma, colloid/mucinous carcinoma, tubular carcinoma, inflammatory carcinoma are less frequent (Kumar et al., 2007).

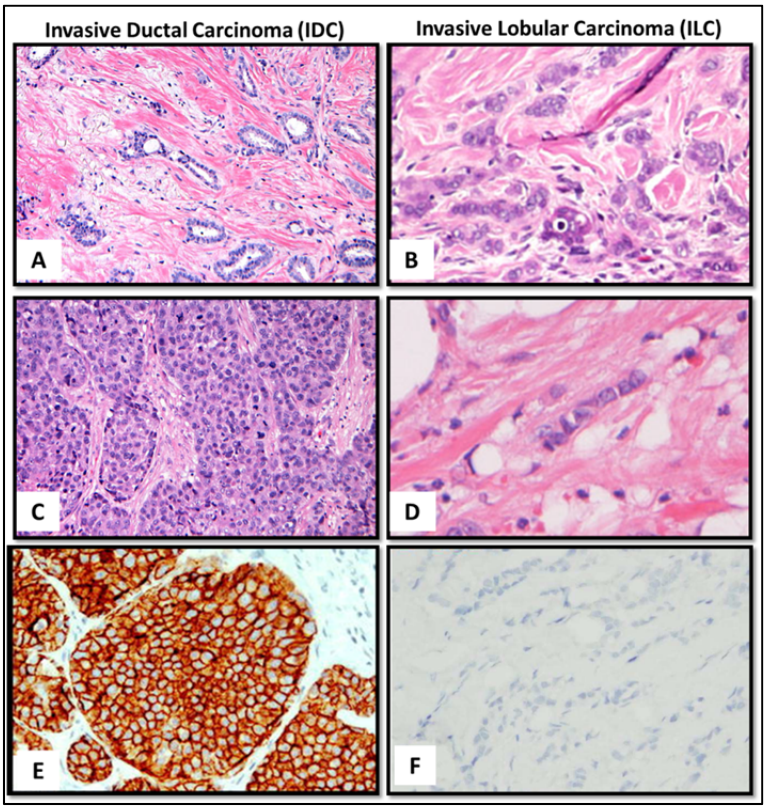

Figure 2 : Illustration of two main histological types of breast cancer. Microscopy images illustrating invasive ductal carcinoma (IDC) (A,C) and invasive lobular carcinoma (ILC) (B, D) morphology. (E, F) Immunostaining for E-cadherin in IDC (E) and ILC (F) (www.archivesofpathology.org and www.breastpathology.info).

1.3 Clinical course, prognosis factors and molecular classification

As they progress, invasive breast carcinomas develop adhesions to muscles or skin and sometimes trigger lymphedema. Clinically, spread initially manifests itself by lymph node involvement. Then, although no organ is exempt, distant blood-borne metastases preferentially affect lungs, bones, liver and adrenals. Brain, spleen and pituitary are occasionally involved. Metastases may appear very late after remission, sometimes 15 years later, but the longer the remission, the higher the chances are not to relapse.

Prognostic factors include:

- ***TNM staging of the cancer*** (where T stands for size of the primary tumor, N for lymph node involvement and M for distant metastasis);
- ***Histological type*** (IDC relates to a poorer prognosis than 'specialized' histological types);
- ***Histological grade***: the Nottingham score exploits three histological criteria (percentage of tubule formation, nuclear pleomorphism and mitotic index) to define three grades: well differentiated, moderately differentiated and poorly differentiated). The less differentiated the carcinoma, the poorer the prognosis;
- ***Hormone receptor status***: estrogen (ER) and/or progesterone receptor (PR) expression correlates with a better prognosis;
- ***Proliferative rate*** : high proliferative rates are associated to a poor prognosis;
- ***Aneuploidy***, which is associated with poor prognosis;
- ***Her2/Neu overexpression***: overexpression of this mutated, constitutively activated transmembrane EGF receptor is observed in about 15-25% of patients (Wolff et al., 2013) and often comes from genetic amplification. Her2/Neu overexpressing tumors are poorly differentiated tumors of high grade, with a high proliferative index and relatively chemoresistant (Burstein, 2005). They would relate to a poor prognosis (Kumar et al., 2007) until a specific treatment arose and dramatically improved outcomes (see paragraph 1.4).

Based on the TNM classification, the American Joint Committee of Cancer has defined five clinical stages, described in Table 1.

Table 1 : Clinical stages of breast cancer, corresponding pathological criteria and 5-year survival expectations (Kumar et al., 2007).

Clinical stage	Criteria	5-year survival
0	DCIS/LCIS	92%
I	Tumor size ≤ 2 cm	87%
II	Tumor size ≤ 5 cm and ≤ 3 involved LN OR Tumor size > 5 cm and LN-negative	75%
III	Tumor size ≤ 5 cm and > 3 LN OR Tumor size > 5 cm and LN-positive OR > 10 LN OR Involvement of ipsilateral LN OR Clinically inflammatory carcinoma	46%
IV	Distant metastases	13%

Because of the high heterogeneity of the disease, two patients with the same stage do not necessarily have the same outcome. Several studies based on gene profiling have aimed to identify gene expression signatures in order to classify breast carcinomas more accurately and succeeded in identifying five 'intrinsic" or "molecular" subtypes of breast cancer. In 2000, Perou and colleagues classified breast cancers into luminal A and B, HER2-positive and basal-like (Perou et al., 2000) (Figure 3). More recently, a claudin-low subtype has been identified (Prat et al., 2010).

As illustrated in Figure 3, luminal subtypes express the estrogen and progesterone receptors (the two other subtypes are negative for these two receptors). Luminal breast cancers are generally associated with a lower histological grade and a better outcome. In contrast to luminal A, the luminal B subtype is positive for HER2/Neu. It is also slightly more aggressive, with increased Nottingham score and proliferation index.

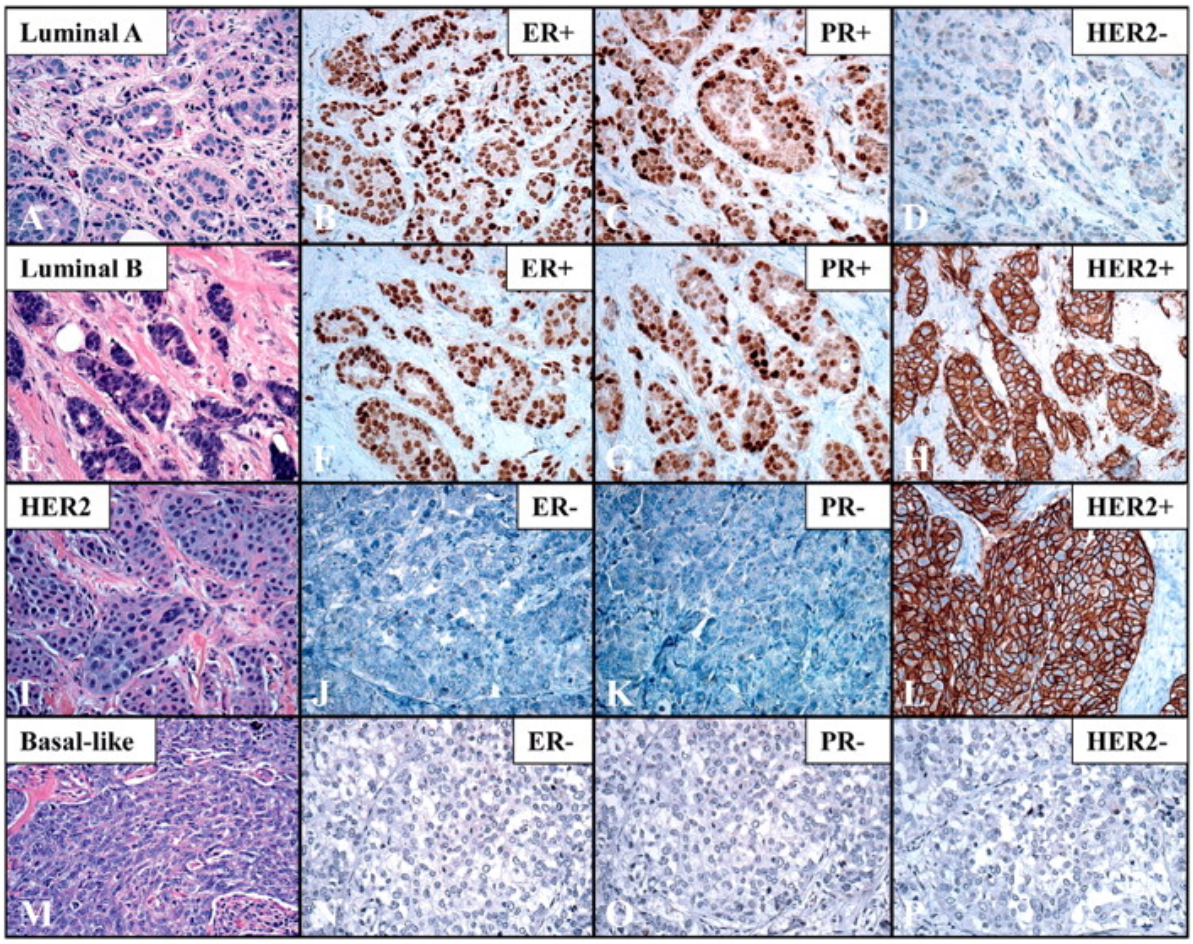

Figure 3 : Intrinsic/molecular subtypes of breast cancer microscopy images representing breast cancer subtypes morphology (left column) and immunohistochemistry for ER, PR and HER2 (right columns)(Sandhu et al., 2010).

Representing 15-25% of total breast cancers, the basal-like subtype has a genomic signature similar to that of normal myoepithelial mammary cells (Bertucci et al., 2012). Basal-like breast cancers are associated with a high histological grade, affect younger patients and generally involve frequent relapse and an overall poor prognosis (Bertucci et al., 2012). By immunohistochemistry, they have been characterized as follows: ER-negative, PR-negative and HER2-negative, cytokeratin (CK) 5/6-positive or epidermal growth factor receptor (EGFR)-positive. However, because a clinically validated threshold for CK5/6 and EGFR positivity is lacking and because immunohistochemistry is more relevant for routine diagnosis that RNA approach, the triple-negative criterion is preferred to the basal-like one in clinics (Kumar et al., 2007). The basal-like subtype is indeed encompassed by the triple-negative type of breast cancer (TNBC), which is ER-negative, PR-negative and HER2/Neu-

negative and to which the basal-like subtype is often assimilated (Figure 3). However, the overlap between the two entities is not perfect. TNBC indeed represent a heterogeneous group comprising basal-like and non-basal-like cancers (Bertucci et al., 2012). An example of non-basal-like TNBC is the claudin-low subtype (Prat et al., 2010).

1.4 Current treatments and clinical challenges

Whenever possible (in clinical stages I, II and some cases of stage III), surgical resection of the primary tumor is the first line of treatment in breast cancer. Surgery is sometimes preceded by chemotherapy to reduce the size of the tumor and can either be a mastectomy or breast-conserving surgery. The latter is accompanied by adjuvant radiotherapy to reduce the risk of local recurrence. Axillar lymph nodes are collected during surgery to assess nodal colonization by cancer cells and foresee further treatment strategy.

Lymph node involvement, molecular characteristics and histological grade of the primary tumor combined allow classification of patients in low, intermediate and high risks and condition systemic treatment strategy. When estrogen receptors are present at the surface of cancer cells, hormonal therapy is used alone or in combination with chemotherapy. Two kinds of drugs can inhibit estrogen-signaling and thereby estrogen-dependent tumor cell proliferation and survival: Tamoxifen and Aromatase inhibitors. While Tamoxifen competitively inhibits estrogen receptor function, aromatase inhibitors block the production of estrogens in tissues. Another surface marker that is selectively targeted in therapy is Her2/Neu. About 15-25% of breast cancer patients have HER2/Neu-positive cancer cells and can be treated with Trastuzumab. This humanized monoclonal antibody binds to the HER2/Neu receptor and impairs signaling, leading to the induction of apoptosis, the potentiation of chemotherapy and the reduction of VEGF release by cancer cells (Burstein, 2005; Wolff et al., 2013). Clinical studies have indeed revealed that patients treated with chemotherapy in combination with Trastuzumab had a longer overall survival (Burstein, 2005).

In contrast to patients with ER-positive or HER2/Neu-positive tumors, high risk patients (with high histological grade and/or triple negative cancer and/or lymph node involvement), only receive adjuvant chemotherapy as potential systemic treatment. Even though triple-

negative breast cancers (TNBC) initially respond well to either taxanes or anthracyclin-based drugs, they are associated with a short progression-free period. TNBC are indeed characterized by high rates of relapses within 3 years of follow up, which are often fatal (Bertucci et al., 2012). There is currently no standard treatment for metastatic TNBC, but various clinical studies are testing potential new drugs such as EGFR inhibitors and anti-VEGF drugs (as illustrated in Table 2). Anti-VEGF drugs are now approved for clinically advanced metastatic breast cancer, as detailed in section 3.1.4.

Table 2: Agents in clinical development for treatment of triple-negative breast cancer (Herold and Anders, 2013).

Therapeutic target/Drug class	Pharmacologic agent	Phase of clinical trial
EGFR	Cetuximab	II
VEGF/VEGFR	Bavacizumab	III
Poly (ADP-ribose) polymerase (PARP) inhibitors	Veliparib, carboplatin	II
Platinum salts	Cisplatin, carboplatin	II/III
HDAC (histone deacetylase) inhibitors	Vorinostat	II
Androgen receptor (AR)	Bicalutamide	II
Folate receptor (FR)	Vintafolide	II
MEK inhibitors	Trametinib	I/II
mTOR inhibitors	Everolimus	II
PI3K inhibitors	BKM-1200	I/II

2 Epithelial-to-mesenchymal transition (EMT) in cancer progression

2.1 EMTs: an introduction

Epithelial-to-mesenchymal transitions (EMTs) are known as programs transforming epithelial cells into mesenchymal derivatives (Kalluri and Weinberg, 2009; Thiery et al., 2009), endowing epithelial cells with motile and invasive properties. Historically, EMT changes were first reported in the chick embryo in the 1960s by Elizabeth Hay (Hay, 1968) and have since been described in various steps of development and in various physiological and pathological contexts. EMTs have been categorized accordingly in three types (Figure 4).

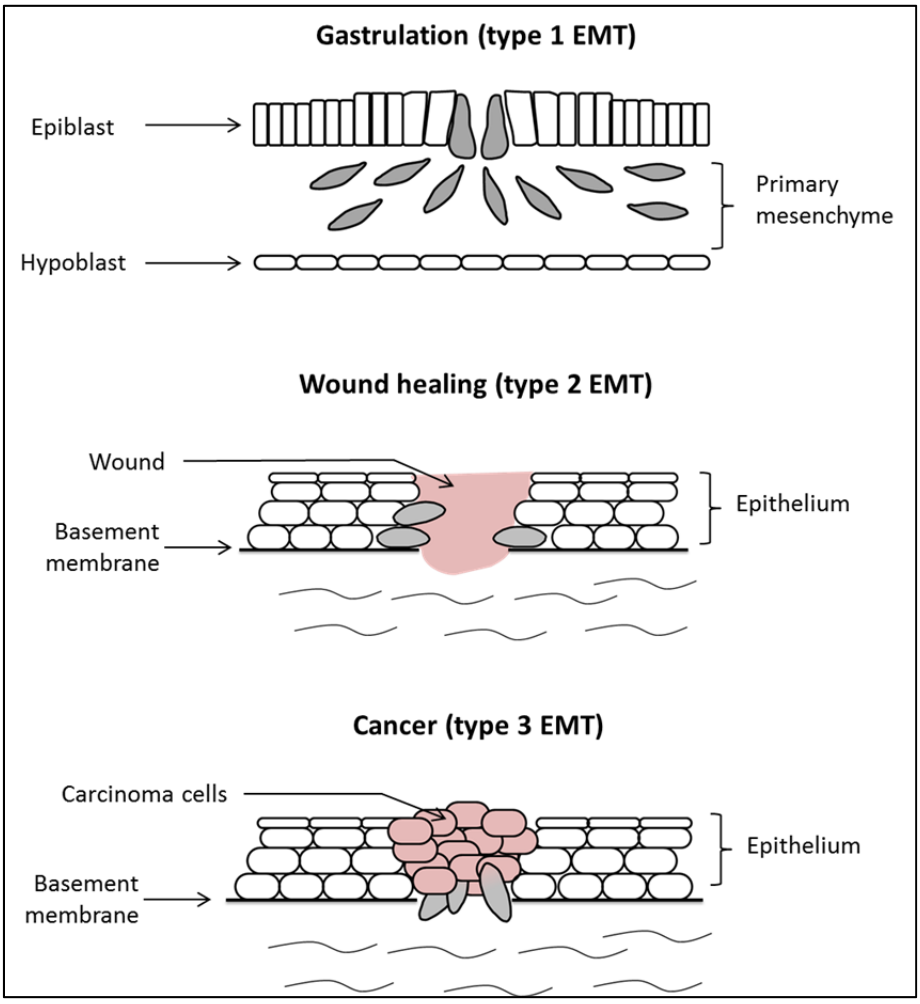

Figure 4 : Illustration of EMTs associated to gastrulation (type 1), to wound healing (type 2) and to cancer (type 3). EMT-undergoing/derived cells are represented in grey. In the representation of type 3 EMTs, cancer cells are in pink.

Developmental EMTs constitute **type 1 EMTs** (Kalluri and Weinberg, 2009). Primary EMTs occur at gastrulation: some cells from the epiblast undergo EMTs, migrate to the midline (forming the so-called primitive streak) and ingress, giving rise to the primary mesenchyme (Figure 4), that will later specialize into mesoderm and endoderm (Acloque et al., 2009). EMTs also occur during neural tube formation: upon tube invagination, cells nearest to the dorsal midline undergo EMTs and hence becoming the migratory neural crest cells. These cells subsequently differentiate into several cell types, often via a mesenchymal-to-epithelial transition (MET). During organogenesis, mesodermal and endodermal cells undergo several rounds of EMT-MET. For instance, cardiac valve formation requires three successive rows of EMT-MET (Acloque et al., 2009; Yang and Weinberg, 2008).

Type 2 EMTs (Figure 4) are repair-associated: they occur during wound healing, tissue regeneration and organ fibrosis. They are linked to inflammation: during wound healing, EMTs reverse when inflammation ends. Conversely, fibrosis arises when inflammation and EMTs persist. In the former case, cells at the border of a wound exhibit EMT changes, the importance of which has been emphasized by the delay of healing when molecular actors of EMTs are inhibited (Thiery and Sleeman, 2006). In the latter case, EMT-derived cells leave the epithelial compartment and irreversibly transform into fibroblasts (Kalluri and Weinberg, 2009). This may be observed in fibrosis of the kidney, liver, lung and intestine, in which a significant proportion of fibroblasts thus arise from tubular epithelial cells that have undergone EMTs (Kalluri and Weinberg, 2009).

Focus of this work, **type 3 EMTs** are cancer-related and occur in neoplastic cells, endowing them with pro-invasive and pro-metastatic properties (Figure 4). There are various degrees of EMTs, giving rise to diverse intermediate phenotypes (Figure 5) (Kalluri and Weinberg, 2009). Because of the tight regulation in time and space of EMTs, its observation in cancer has been a challenge and its very occurrence was debated for a while (Tarin et al., 2005; Thompson et al., 2005). Hence and also because of the partial and reversible nature of cancer-related EMT, the less categorical term ‘EMT-like changes’ is sometimes preferred. It is now widely accepted that EMT-like changes are triggered in cancer cells (Brabletz, 2012; Thiery and Lim, 2013; Thompson et al., 2005).

2.2 Molecular actors of EMTs

During EMT, epithelial cells undergo morphological as well as changes. Genes of which expression is altered during EMT are referred to as target genes and include components of adhesion structures, components of the extracellular matrix (ECM), elements of the cytoskeleton and other factors involved in cell migration, cell survival etc. Target genes may be regulated at the transcriptional level by several families of transcription factors involved in EMT and called EMT-TFs. Signaling cascades triggering expression of EMT-TFs and upstream extracellular inductors of EMT are also molecular actors of EMT and will be discussed.

2.2.1 Target genes of EMT

During EMTs, a variety of target genes are modulated, resulting in morphological and functional changes. Morphologically, EMT programs trigger a switch from a cobblestone shape with an apico-basal polarity to an elongated form. Functionally, EMT-derived cells express an enhanced ability to migrate and invade the surrounding extracellular matrix (ECM). These functional changes largely rely on alterations of cell-cell adhesion structures, of the cytoskeleton and on an enhanced ability to remodel the ECM. Some of the main variations in cell morphology, markers and functions that occur during EMTs are represented in Figure 5.

We have underneath categorized molecular targets of EMTs into four groups, as those involved in cell-cell adhesion, cytoskeleton and cell-ECM interactions, ECM remodeling and in cell cycle and survival.

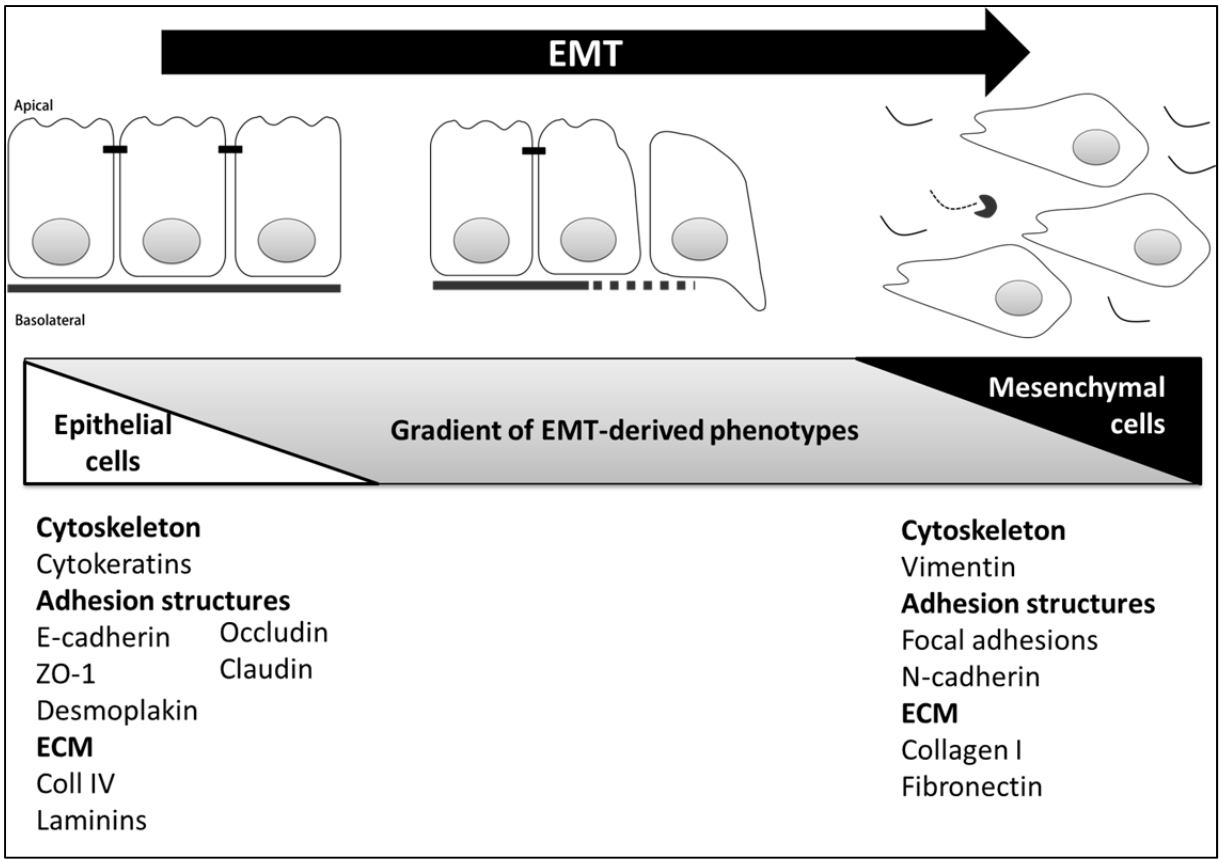

Figure 5 : Schematic representation of EMT-associated morphological changes (top) and list of some key EMT-target genes (bottom).

2.2.1.1 Cell-cell adhesion structures

Epithelial cells display an apico-basal polarity: they are in contact with a basement membrane and interact with one another through complex networks of adhesion structures forming a lateral belt and called tight junctions, adherens junctions and desmosomes. ***Tight junctions*** involve transmembrane Occludin and Claudins and cytoplasmic Zonula Occludens (ZO)-1, -2 and -3, which are anchored to the actin cytoskeleton. ***Adherens junctions*** are arranged into a lateral belt formed by homotypic interactions between E-cadherin, a 120 kDa calcium binding single-span transmembrane glycoprotein, which interacts, through adaptor molecules such as α- and β-catenins to the actin cytoskeleton as well. ***Desmosomes*** are formed by transmembrane desmosomal cadherins (desmoglein and desmocollin) anchored to cytokeratin intermediate filaments through adaptor proteins such as plakoglobin, desmoplakin and plakophilin (Xu et al., 2009).

EMTs trigger deep alterations of these cell-cell adhesion complexes partly through a transcriptional repression of several molecular actors of tight junctions (claudins and occludin), desmosomes (desmoplakin) and adherens junctions (E-cadherin) (De Wever et al., 2008). N-cadherin, a member of junctional proteins expressed in the neural plate, may on the other hand be over-expressed. In EMT-derived cells, adhesion between cells is rather mediated by focal adhesion points comprising compounds such as integrin $\alpha_v\beta_{6,}$ fascin and ILK (integrin linked kinase).

More than the loosening of epithelial cell-cell interactions, EMT also provokes the release from cell-cell complexes of molecules that may then initiate a specific pro-invasive signaling. For instance, β-catenin, once delocalized out of the adherens junctions, may translocate in the nucleus where it participates itself to the transcriptional regulation of target genes (De Wever et al., 2008).

2.2.1.2 Cytoskeleton

In epithelial cells, F-actin fibers are polarized around the nucleus. During EMTs, these fibers arrange into stress fibers, sometimes positive for α-SMA (smooth muscle actin) at the end of which focal adhesion points are located (De Wever et al., 2008). Regarding intermediate filaments, those of epithelial cells are made of cytokeratin. Upon EMT, cells decrease, or may lose in the case of complete EMTs, the expression of cytokeratin and instead express *de novo* fibroblastic intermediate filaments compounds such as vimentin and desmin (De Wever et al., 2008). EMT-derived cells also express FSP1 (fibroblast-specific protein 1 or S100A4), a calcium binding protein normally specifically expressed by fibroblasts and involved in tubulin polymerization.

2.2.1.3 Cell-ECM interaction and ECM remodeling

Epithelial cells typically produce components of the basement membrane such as laminin, at their basal surface. EMT-derived cells, on the other hand, rather secrete interstitial ECM compounds such as collagen I, fibronectin, SPARC (secreted protein acidic and rich in cysteine) and tenascin C (De Wever et al., 2008). EMT-derived cells are also able

to remodel the ECM thanks to their production of a variety of matrix metalloproteinases (MMPs). MMP-1, MMP-2, MMP-3, MMP-7 and MMP-14 are often expressed in EMT-derived cells for example (Bonnomet et al., 2008; De Wever et al., 2008; Nawrocki-Raby et al., 2003).

2.2.1.4 Cell cycle and survival

The transcription of multiple regulators of the cell cycle is affected during EMTs. For example, Cyclin D1 and 2 (Koay et al., 2012; Peinado et al., 2007) and PCNA (proliferating cell nuclear antigen) (Peinado et al., 2007) are regulated during EMT. INK4a and p21, which both promote progression through cell cycle, are also upregulated during EMT (Peinado et al., 2007). EMT programs have also been associated to cell survival. For example, human carcinoma cells that undergo EMT under genotoxic stress are protected from p53-mediated apoptosis (Peinado et al., 2007).

2.2.2 Transcription factors of EMTs (EMT-TFs)

Target genes of EMTs can be transcriptionally regulated by a variety of transcription factors called EMT-TFs. As best studied concerning CDH1 (encoding E-cadherin), many epithelial target genes of EMTs can be transcriptionally repressed by families of transcription factors such as the Snail family (Snail/Snail1 and Slug/Snail2), the ZEB family (ZEB1 and 2), the bHLH (basic helix loop helix) protein E47, or KLF8 for example. In their promoter, such target genes contain E-boxes, 5'-CACCTG-3' consensus sequences that can be directly bound by EMT-TFs Snail, Slug, ZEB1 or ZEB2. ZEB TFs interact with E-boxes via two Zinc finger domains, located at the two extremities of the proteins (Xu et al., 2009), while Zinc fingers in the Snail family are located on a single domain (for a detailed description of Snail TFs, see paragraph 2.2.2.1.1). E47, a bHLH TF, also binds E-boxes though with lower affinity compared to Snail TFs (Yang and Weinberg, 2008). Some transcription repressors such as KLF8 bind other sequences called GT boxes (de Herreros et al., 2010). Twist is another bHLH TF, which was found to directly repress E-cadherin expression (Vesuna et al., 2008) and to induce EMT in mammary epithelial cells (Yang et al., 2004a).

T-box transcription factor involved in mesoderm formation, Brachyury was recently added to the list of EMT-TFs (Fernando et al., 2010).

During this thesis, we have more particularly studied the expression of Snail and Slug transcription factors, which are therefore described in more details hereafter.

2.2.2.1 The Snail family

2.2.2.1.1 Structure and activity

Probably the most studied EMT-TF family, the Snail family of transcription factors is composed of Snail (Snail1), Slug (Snail2) and a third poorly characterized member called Smuc (Snail3) (de Herreros et al., 2010). Structurally, the proteins are composed of three main domains (Figure 6).

The highly conserved **C-terminal domain** contains four to six Zinc fingers, which allow fixation to E-boxes and repression of E-cadherin transcription, but also of other targets such as Claudin, Occludin or PTEN for example. Snail would bind E-boxes with higher affinity and would be more efficient to inhibiting transcription comparatively to Slug (de Herreros et al., 2010). The **N-terminal domain** in this family is much less conserved and is responsible for the regulation of TF activity. In Snail protein for example, The SNAG (Snail-Gfi1) domain (located in the N-terminal domain) interacts with co-repressors such as HDAC1 and 2 (histone deacetylases) via adaptor proteins (like mSin3A) (Peinado et al., 2004a). Snail N-terminal domain has also been shown to co-immunoprecipitate with Smad2/3 to form a complex, which is present at the CDH1 promoter when transcription of E-cadherin is repressed (Vincent et al., 2009). The N-terminal domain of Slug also enables Slug to interact with its partners (i.e. HDAC1/3 and CTBP [C-terminal Binding Protein] for instance) (Xu et al., 2009). The **central domain** is also variable amongst Snail family members and is responsible for protein stability, subcellular location and degradation, as detailed in the following section.

Snail is able not only to inhibit epithelial gene transcription but also to activate transcription of mesenchymal genes (for a review, see (Peinado et al., 2007)). Epithelial genes repressed by Snail include E-cadherin, claudins, occludin, desmoplakin, plakophilin, CK

17-20 (Xu et al., 2009). Snail-regulated genes can be either directly repressed upon Snail binding to their promoter or indirectly (Peinado et al., 2007). Conversely, Snail activates the expression of mesenchymal genes, although the underlying mechanisms are not understood. Vimentin, fibronectin, vitronectin, N-cadherin, collagen III and I, RhoB, PAI-1, MMP2 and MMP9 are target genes positively regulated by Snail (Xu et al., 2009). Whether Snail directly binds the promoter of those genes or act through other transcription factors is unclear. For example, MMP9 induction by Snail would involve MAPK signaling and SP1 and ETS transcription factors (Peinado et al., 2007). MMP2 regulation would be indirectly mediated by Snail through the ETS1 transcription factor.

Snail can thereby induce EMTs by modifying several components and functions of cells: cytoskeleton components, adhesion structures, production and remodeling of the extracellular matrix. Snail and Slug can also modulate transcription of genes involved in cell cycle, cell survival and apoptosis. They indeed bind promoters of genes such as p53, BRCA2 or BID (Peinado et al., 2007). Snail also specifically regulates Cyclin D2 and PCNA (Peinado et al., 2007).

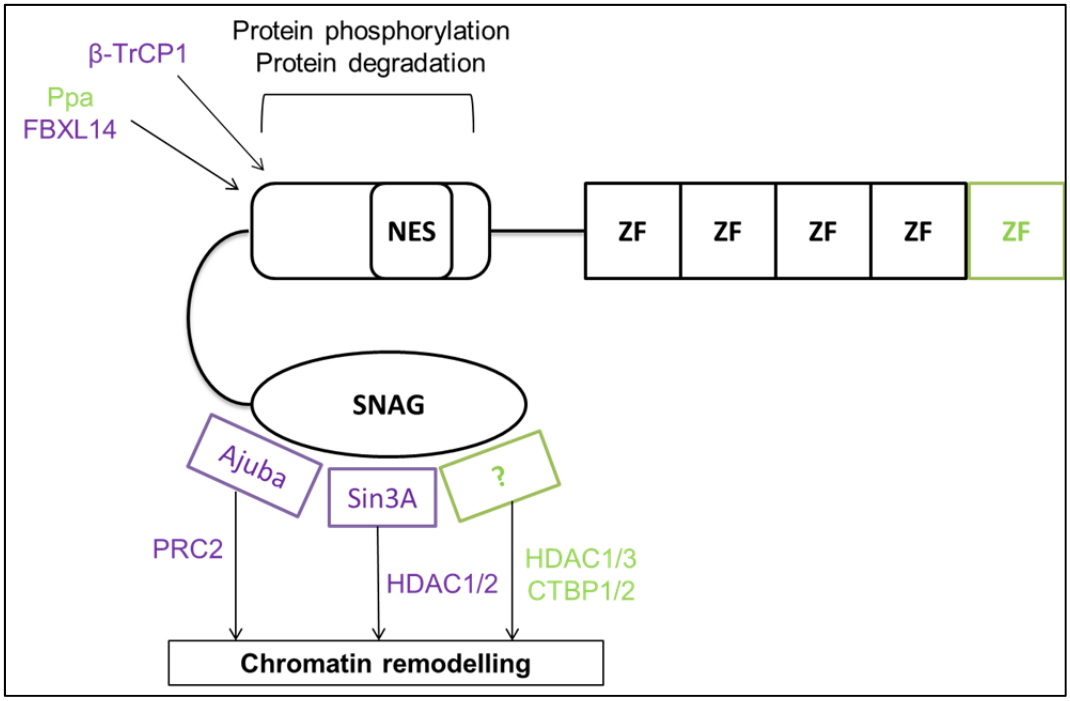

Figure 6 : The Snail family of transcription factors. Snail factors share a common three-domain-based structure with a N-terminal SNAG (Snail-Gfi1) domain, a central domain contain a NES (nuclear export signal) and a C-terminal domain contain Zinc fingers (ZF). Snail and Slug interact with specific partners (in purple and green, respectively). Slug contains one more ZF than Snail (in green).

2.2.2.1.2 Control of transcription factor expression and activity

Snail family factors can be induced by a variety of signals such as TGF-β, Notch, TNF-α, EGF, FGF, Wnt, sHh, SCF/c-kit, hypoxia, they can be inhibited by estrogens (Joannes et al., 2010; Sanchez-Tillo et al., 2012). Insight on how Snail expression is regulated at the transcriptional level is lacunar. Nonetheless, some positive and negative regulators of Snail transcription have been described. Indeed, Snail transcription can be enhanced by hypoxia-induced Notch (Sahlgren et al., 2008) or TGF-β-induced Smads. These interact with HMGA2 (High Mobility Group protein A2) that in turn increases Smad interaction with Snail promoter (Thuault et al., 2008). HGF-induced Egr1 protein also binds on Snail promoter to enhance its transcription (de Herreros et al., 2010). Notably, a positive cross-regulation between NF-κB and Snail has also been described. NF-κB is indeed an inducer of Snail expression whereas Snail increases NF-κB transcriptional activity (de Herreros et al., 2010). Moreover, NF-κB is repressed by E-cadherin. Therefore, when E-cadherin expression is decreased, NF-κB and Snail can mutually induce each other and cooperatively trigger EMTs. Finally, the induction of Snail and EMTs can also be triggered by oncogenic stress (by overexpression of Ras or ErbB2) (de Herreros et al., 2010). Conversely, Snail is a potent repressor of its own transcription: both directly upon binding to its own promoter, which contains an E-box and indirectly by repressing transcription of its enhancer Egr1.

Snail expression is additionally regulated at the protein level. Snail protein stability is indeed increased by EMT-signaling pathways such as Wnt, MAPK or PI3K, or NF-κB that modulate Snail phosphorylation by GSK3β (glycogen synthase kinase 3β) (de Herreros et al., 2010; Sanchez-Tillo et al., 2012). TNF-α signaling, for example, mediates PI3K/Akt-mediated inhibitory phosphorylation of GSK3β (Wang et al., 2013a). When GSK3β is active, it phosphorylates Snail in its central domain (Figure 6) inside the nucleus. This exposes a nuclear export signal (NES). Once in the cytoplasm, a second phosphorylation by the same kinase enables ubiquitination of Snail by β-TrCP1 (Beta-Transducin Repeat-Containing Protein) ubiquitin ligase, which triggers its degradation through the proteasome (Zhou et al., 2004). Snail can also be unbiquitinated by FBXL14 (F-box and leucine-rich repeat protein 14), in a phosphorylation-independent way (de Herreros et al., 2010) and has a short half-life of 20-45 minutes (de Herreros et al., 2010). Upon hypoxia, Snail is also stabilized on one hand, via Twist-mediated downregulation of FBXL14 ubiquitin ligase and on the other hand, via

HIF-1α (Hypoxia-inducible factor 1α)-mediated transcription of LOX (Lysil oxidase), which stabilizes Snail protein (Sahlgren et al., 2008). Inflammatory cytokines decrease Snail interaction with β-TrcP1 ubiquitin ligase by increasing NF-κB (de Herreros et al., 2010). At last, a very recent study has identified an additional ubiquitin ligase involved in Snail degradation, called FBXO11, which expression blocks Snail-induced EMT in breast cancer models (Zheng et al., 2014).

Slug regulation is much less described in the literature. In its central domain, Slug lacks all phosphorylation sites of Snail (Figure 6), yet its stability is controlled as well. In Xenopus, Partner of Paired (Ppa), the ortholog of FBXL14, can ubiquitinate Slug (Lander et al., 2011). It can also be ubiquitinated by mdm2 (mouse double minute 2) (Sanchez-Tillo et al., 2012). Slug activates its own transcription during neural crest formation by binding on E-boxes on its promoter (de Herreros et al., 2010). Slug expression is also activated by EGFR/Src/ERK signaling in bronchial cells (Joannes et al., 2014).

2.2.2.2 Regulation of EMT-TFs by microRNAs

Protein expression of EMT-TFs is also regulated by microRNAs (miRs), small non-coding RNAs that mainly inhibit protein translation (Ryan et al., 2010). MicroRNA implication in cancer is being extensively studied and most data linking EMT-TFs to miRs have been obtained in cancer cell lines.

Some miRs can be positively regulated during EMT. For instance, Twist upregulates miR-10b, which favors the migration and the invasion of breast cancer cells (Ma et al., 2007). Moreover, in tumors arising in mice deleted for p53 in the epidermis, miR-21 expression is associated with EMT features (Bornachea et al., 2012).

Conversely, EMT-TFs inhibit many miRs such as the miR-200 family, comprising miR-200a, b, c, miR-409 and miR-141, or other miRs (let-7, miR-203 or miR-429 for example). These miRs in turn decrease protein expression of EMT-TFs, creating a double regulatory loop maintaining either the epithelial or the mesenchymal phenotype (Bracken et al., 2008; DeCastro et al., 2013; Ding et al., 2013; Gregory et al., 2008; Li et al., 2009; Sun et al., 2014; Taube et al., 2013). Interactions between some miRs and EMT-TFs are illustrated in Figure 7.

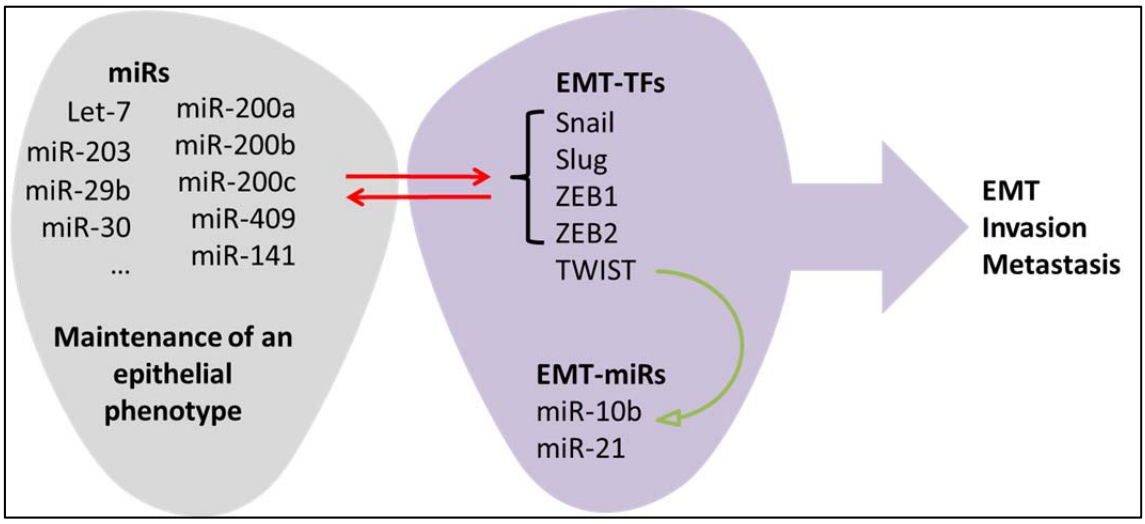

Figure 7: Representation of relationship between some miRs and EMT-TFs. On the left, miRs maintain an epithelial phenotype by repressing protein expression of EMT-TFs. On the right, EMT-TFs inhibit miRs to induce and maintain an EMT-derived phenotype. Red arrows represent negative regulation while the green arrow represents positive regulation.

2.2.2.3 Cross-regulation of EMT-TFs

EMT-TF families regulate one another in complex multifaceted networks. Generally, Snail and Twist families are upstream ZEB families: Snail has indeed been shown to increases protein levels of ZEB1 and ZEB2 expression via unknown mechanisms. Discrepancies exist between some studies pointing out an increase and others reporting a decrease of Twist by Snail according to the cellular context (Sanchez-Tillo et al., 2012). Snail and Twist have been shown to cooperatively increase ZEB1 during TGF-β-induced EMT (Dave et al., 2011). Globally, even though the complex interplay linking the different families of EMT-TFs is not comprehensively described, EMT-TFs seem to interact with one another in a context-dependent way to tightly regulate the induction and maintenance of the EMT phenotype.

2.2.3 Extracellular signals and signaling pathways

The expression of EMT-TFs is the converging point of diverse signaling cascades initiated by a variety of extracellular signals (Figure 8A), such as growth and survival factors (TGF-β …) or ligands binding to a tyrosine kinase receptor (EGF, FGF, HGF, PDGF …). Some pro-inflammatory cytokines like TNF-α (Wang et al., 2013a) or IL-6 (Sullivan et al., 2009) can also

induce EMT, which can also be activated by hypoxia, in part through Notch (Sahlgren et al., 2008) and by alteration in cell-cell interactions (Grosse-Steffen et al., 2012). Some well described EMT-inducing signaling pathways are illustrated in Figure 8A.

Because of the variety of EMT inducers and the complexity of EMT-inducing signaling pathways, we have focused on signaling cascades initiated by TGF-β and EGF, which we used to induce EMT-like changes in cell culture.

2.2.3.1 EGF signaling (Figure 8B)

The EGFR is part of a family of receptor tyrosine kinases (RTKs) comprising four members (EGFR/HER1/ErbB1, HER2/ErbB2/Neu, HER-3/ErbB3 and HER4/ErbB4) that form homo- or heterodimers when bound to their ligands (EGF, TGF-α and HB-EGF). Dimerization is followed by auto-phosphorylation of tyrosine residues. From then on, activated EGFR may interact with and activate secondary messengers such as phospholipase C-γ (PLC-γ), Ras and phosphatidylinositol-3 kinase (PI3K). Activated EGFR also phosphorylates STAT3, thereby enabling its dimerization, nuclear import and transcriptional activity. Nuclear EGFR has been detected in cancer cells and acts as a transcription co-factor upon binding to STAT3, although whether target genes affected by this nuclear complex comprise target genes of EMT or not is unknown (Brand et al., 2013; Lo et al., 2006).

The specific signaling pathways leading to EGFR-induced EMTs highly depend on the cellular context. In bronchial epithelial cells for instance, EGFR phosphorylation induces Slug expression via the Src/ERK signaling pathway (Joannes et al., 2014). In ovarian carcinoma cells, EGFR and IL-6R would cooperate via JAK2/STAT3 to induce EMTs (Colomiere et al., 2009). Indeed, several target genes of EMTs that are induced following EGF treatment, such as N-cadherin or vimentin, failed to be induced in the presence of a blocking anti-IL-6 antibody (Colomiere et al., 2009). In head and neck SCC cell lines, endothelial cell-derived EGF triggers EMTs via PI3K/Akt signaling pathway (Zhang et al., 2014). In Ras-transformed keratinocytes (HaCaT II-4 cells), combined treatment with EGF and TGF-β induces the phosphorylation of p38 and ERK MAPK (Freytag et al., 2010). At last, in the MCF10A breast epithelial cell line, ERK2 is necessary for EGF-induced migration (Shin et al., 2010).

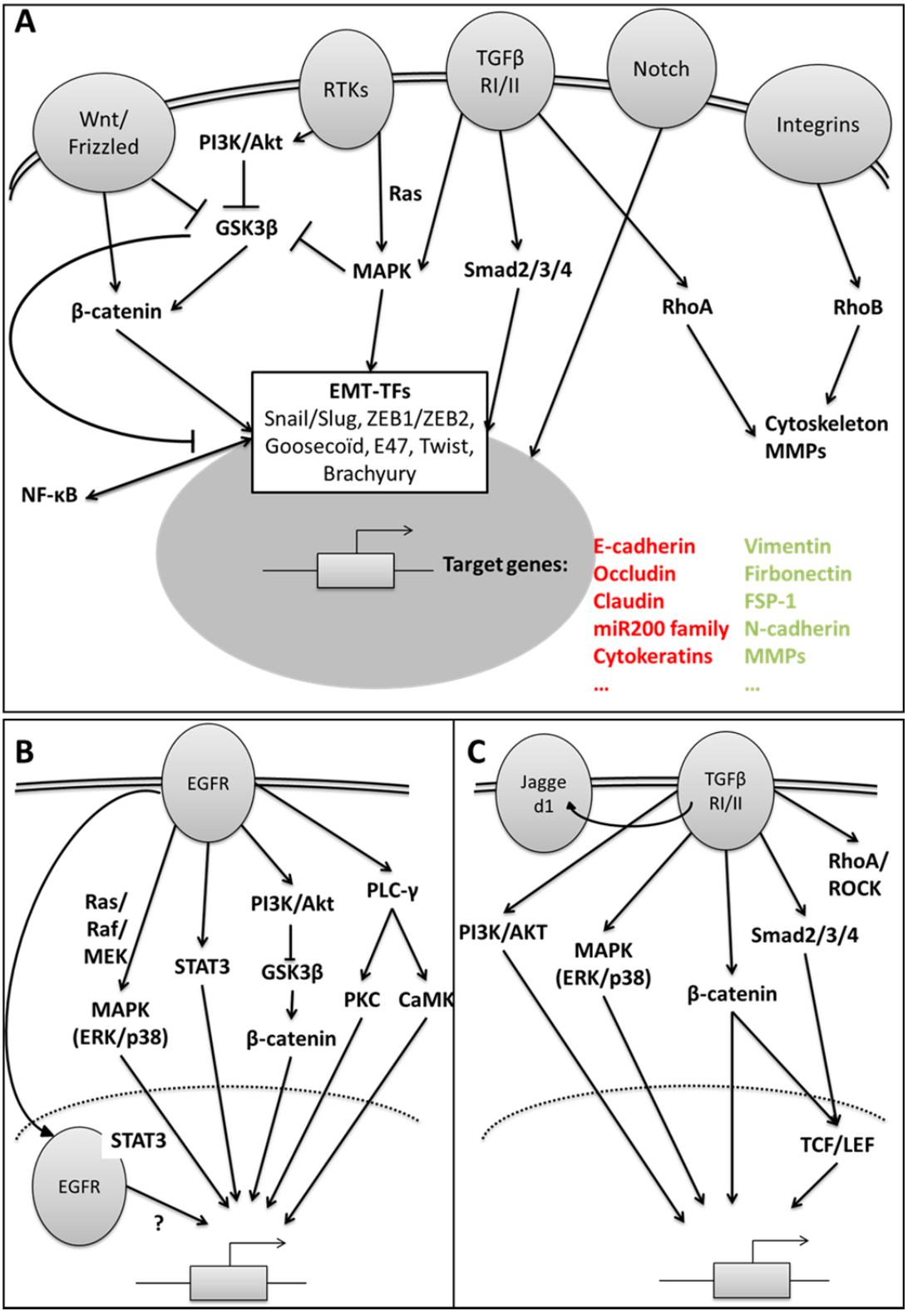

Figure 8 : EMT signaling pathways. (A) Schematic representation of some signaling pathways converging to activation and/or stabilization of EMT-TFs and to induction (in green) or repression (in red) of target genes. (B) Detailed representation of the EGFR pathway. (C) Detailed representation of the TGF-β pathway.

2.2.3.2 TGF-β signaling (Figure 8C)

Ligands for TGF-β receptor comprise TGF-β1, 2 and 3, which may be sequestered in the ECM in the form of homodimers bound to LTBP (latent TGF-β-binding protein). Freed ligands bind to TGF-βR2, resulting in the recruitment and the phosphorylation of TGF-βR1. TGF-βR3 can also be recruited. Canonical TGF-β signaling involves the phosphorylation of SMAD2 or 3, the recruitment of SMAD4 and translocation of the SMAD2/3/4 complex to the nucleus where it activates or represses transcription. Non-canonical TGF-β signaling involves pathways such as RhoA/ROCK, MAPK or PI3K/Akt (Pickup et al., 2013; Xu et al., 2009). Signaling cascades initiated by TGF-β have been studied in cancer and trigger a dual cell context-dependent effect. Indeed, TGF-β primarily inhibits cell proliferation in normal cells and early tumors but in advanced carcinomas, TGF-β signaling rather induces EMT changes (Pickup et al., 2013; Xu et al., 2009).

TGF-β-mediated EMTs depend on both canonical and non-canonical signaling that converges to EMT-TF Snail, Slug, ZEB1 or ZEB2 activation. Smad inactivation or depletion studies have pinpointed the importance of Smad-dependent TGF-β signaling in EMT (Xu et al., 2009). For instance, the loss of Smad3/4 inhibits EMT but loss of Smad2 induces EMTs because Smad3 binds better to Snail promoter (Xu et al., 2009). In addition, Smad3-dependent repression of Id1 (inhibitor of differentiation 1) decreases the expression of adhesion proteins such as E-cadherin and ZO-1 (Pickup et al., 2013). Among the non-canonical pathways triggering EMTs, which include the PI3K/AKT, MAPK and Rho/ROCK (Rho-associated protein kinase 1) pathways, the last one seems particularly important in single-cell migration, since inhibiting this pathway is able to switch cell to cohesive migration (Pickup et al., 2013).

It is noteworthy that several signaling pathways may cooperate to induce EMT. In lung cancer cells for example, the JAK2/STAT3 pathway is required for TGF-β-induced phosphorylation of Smad3, induction of Snail, MMPs and mesenchymal morphology (Liu et al., 2014).

2.3 EMTs in cancer

The actual occurrence of EMT-like changes in cancer has been a topic of debate for many years. Indeed, EMT studies are limited by the lack of markers and cancer cells from metastatic lesions are sometimes as differentiated as in the primary tumors (Christiansen and Rajasekaran, 2006).

In 2012, Thomas Brabletz gathered a large amount of observations to propose a model according to which there exist two types of routes leading to the formation of metastases: one route involves phenotypic plasticity of cancer cells and the other route would result from genetic alterations (Figure 9).

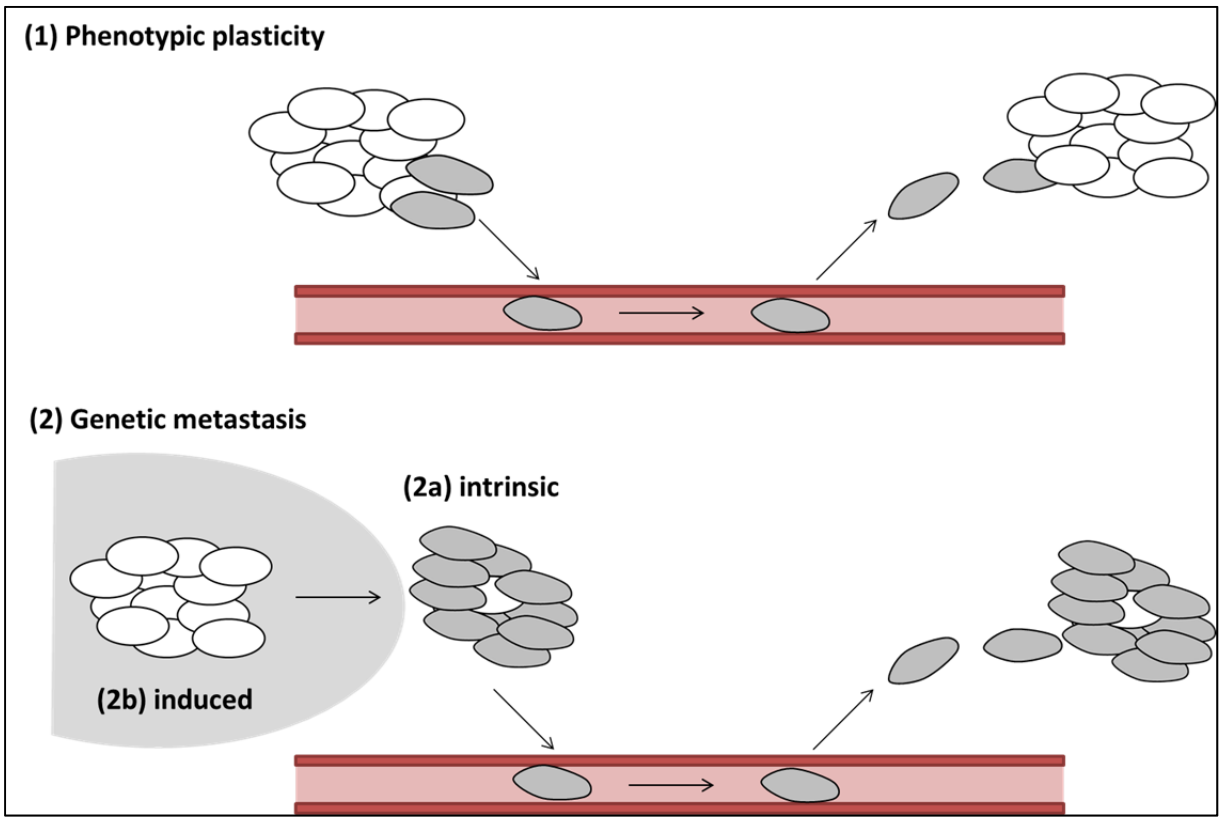

Figure 9 : Models of EMT-mediated metastatic dissemination as proposed by Thomas Brabletz (Brabletz, 2012). In the proposed type 1 metastatic dissemination, the phenotypic plasticity of cancer cells allows EMT induction, which favors the release of CTCs (circulating tumor cells) and the arrest at distant sites. The metastatic outgrowth is preceded by MET, for hypothetical reasons such as the overcoming of the EMT-related proliferative arrest. In the genetic type 2 metastatic dissemination, metastases are undifferentiated/mesenchymal-like, as a result of potential genetic alterations of cancer cells occurring in the primary tumor. These genetic alterations would be intrinsic or induced, for instance by several rounds of chemotherapy.

In the **first route**, cancer cells would acquire EMT-like traits and **phenotypic plasticity** under the influence of the micro-environment of the primary tumor. This would enable them to invade adjacent tissues, enter the blood stream and survive in the circulation.

Supporting this hypothesis of phenotypic plasticity induced within the primary tumors, a variety of micro-environmental factors in the primary tumor could trigger EMT-like changes in cancer cells. Many cell types from the host can provide cancer cells with EMT-inducing agents. Such cell types include for example, tumor-associated macrophages (Bonde et al., 2012), cancer-associated fibroblasts (through MMPs) (Giannoni et al., 2011) and myeloid-derived suppressor cells (MDSCs) (via TGF-β, EGF and HGF pathways) (Toh et al., 2011). All these cells secrete known potent EMT inducers including TGF-β, EGF or HGF. Macrophages indeed secrete TGF-β but are also an important source of EGF in breast cancer. They also accumulate in hypoxic areas. This combined with the described upregulation of EGFR by cancer cells under hypoxic conditions (Misra et al., 2012) suggest that EMT-like changes most likely occur within hypoxic zones on tumors. Neutrophil-derived elastase-mediated E-cadherin destruction has also been shown to trigger EMT in pancreatic tumor cell lines (Grosse-Steffen et al., 2012).

Still in Brabletz's model of phenotypic plasticity-induced metastasis, cancer cells would be able to revert to an epithelial phenotype in the metastatic niche, better suited for proliferation (Brabletz, 2012; Christiansen and Rajasekaran, 2006; Peinado et al., 2007) (Figure 9). This type of metastatic dissemination could account for the apparent discrepancies between the theory of EMT-undergoing cancer cells and the differentiated characteristics of some overt metastases. Thus, several works have demonstrated that cancer cells derived from EMTs are able to revert to an epithelial phenotype, which has led to the concept of "EMP" or epithelial-mesenchymal plasticity (Thompson and Haviv, 2011). In a mouse model for instance, breast carcinoma cells were shown to re-express E-cadherin in metastases (Bukholm et al., 2000). E-cadherin was furthermore found to be re-expressed in metastatic lesions from breast cancer patients (Bukholm et al., 2000). More recently, in a cohort of 16 metastatic breast cancer patients, a significant proportion of hepatic metastases displayed an increased E-cadherin expression compared to primary tumors (Chao et al., 2010). Moreover, after intravenous injection in mice of different cell lines, the epithelial-like cell lines appeared more able to form metastases compared to the

mesenchymal-like ones (Chaffer et al., 2006). Finally, in a transgenic mouse model of Twist-mediated EMTs in which Twist expression was inducible by Doxycyclin, the 'reversible EMT' settings (induced by topical treatment with Doxycyclin on the primary tumor) produced significantly more metastases than the 'irreversible EMT' settings (triggered by oral treatment with Doxycyclin) (Tsai et al., 2012).

Brabletz' proposed **second type of metastatic dissemination** involves **genetic alterations** inducing a stable EMT-derived and stem cell-like phenotype in cancer cells in the primary tumor, that would be kept in metastases (Brabletz, 2012). These genetic alterations could be either intrinsic or induced. One example of intrinsic genetic metastasis would be claudin-low breast cancer, in which cells from the primary tumor intrinsically display EMT and stem cell-like features characterized by high expression of EMT-TFs Slug and ZEB2 and low expression of miR-200 (Brabletz, 2012). Still according to Brabletz' theory, the induction of genetic alterations could also and most probably be the result of several rounds of chemotherapy for example. Supporting this last hypothesis, residual tumors after chemotherapy have been shown to be enriched in EMT and stem cell-related markers (Creighton et al., 2009).

2.3.1 Evidence of EMT occurrence in several cancer types: clinical observations

Markers of EMT-like changes have been observed in a variety of carcinomas, of which a non-exhaustive list is provided in Table 3.

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Table 3 : Examples of observations of EMT features in human cancers.

Cancer type	Markers of EMT	Tissue	Methods	Observations	Ref
Breast cancer	Vimentin, cytoplasmic E-cadherin	57 IDC	IHC[1]	EMT features are enriched in TNBC	(Sethi et al., 2011)
	28 markers	479 IDC and 12 carcino-sarcomas	IHC on small zones (TMA)[2]	EMT features are enriched in basal-like BC	(Sarrio et al., 2008)
	GES[3] (159 genes)	49 BC	Microarray	EMT gene expression signature relates to the claudin-low subtype	(Taube et al., 2010)
Cervical cancer	E-cadherin, β-catenin, cyclin D1	22 biopsies of cervical SCC	IHC	Most invasive tumors display a decrease in E-cadherin and β-catenin (in the cytoplasm) and express cyclin D	(Koay et al., 2012)
Colorectal cancer	Decrease in membrane E-cadherin and relocation of β-catenin	72 cases of PDAC[4]	IHC	Beta-catenin is located in the nucleus in dedifferentiated cells in the invasive front, but is located at the membrane in central parts of the tumors	(Brabletz et al., 2001)
Pancreatic cancer	Vimentin, fibronectin, E-cadherin, phospho-Erk	34 PDAC	IHC	EMT markers are associated to poor overall survival	(Javle et al., 2007)
Lung cancer	E-cadherin	87 NSCLC[5]	IHC	E-cadherin positivity is associated to a longer time to progression	(Yauch et al., 2005)
	Vimentin	295 NSCLC	IHC	Vimentin positivity is observed in 49.2% of samples and predicts occurrence of metastasis	(Dauphin et al., 2013)
Oral cancer	E-cadherin	973 oral SCC	Meta-analysis	Patients with reduced E-cadherin expression have poorer overall survival	(Luo et al., 2014)
Skin SCC	Vimentin, E-cadherin, Twist and	107 primary tumors and metastatic	IHC	Vimentin is an indicator of recurrence, disease-related death, T stage, perineural	(Toll et al., 2013)

	ZEB1	lymph nodes		infiltration, poor differentiation and desmoplasia. ZEB1 and Twist are more frequently expressed in metastatic SCC compared to non-metastatic ones.	
Ovarian cancer	Snail, E-cadherin	48 primary ovarian tumors and metastases		Snail expression relates to a poor prognosis	(Blechschmidt et al., 2008)
Cholangio-carcinoma	12 EMT-related proteins	117 tumors	TMA	E-cadherin and N-cadherin are independent prognostic factors. The cadherin switch is associated to a poor prognosis	(Nitta et al., 2014)

[1]IHC: immunohistochemistry. [2]TMA: tissue micro-array. [3]GES: gene expression signature. [4]PDAC; pancreatic. ductal adenocarcinoma. [5]NSCLC: non-small cell lung cancer

2.3.1.1 The case of breast cancer

While complete EMTs are hardly ever observed in breast cancer, EMT-like changes generating cells with an intermediate phenotype have been described in some specific entities such as ILC, basal-like and claudin-low breast cancers.

In ILC, EMT-like changes comprising loss of E-cadherin – as a result of a mutation (de Leeuw et al., 1997) (Figure 2F) – decreased expression of β-catenin at the membrane, expression of Twist and retention of cytokeratins are observed (Morrogh et al., 2012). This partial EMT is associated with a specific 'Indian file' pattern of invasion in the classic form of ILC (de Herreros et al., 2010) (Figure 2B, D).

In basal-like breast cancer, cells express EMT-related markers such as vimentin, N-cadherin or α-SMA, while retaining some epithelial features like E-cadherin and CK8/18 (Sarrio et al., 2008).

In claudin-low breast cancer, cells express Snail and Twist EMT-TFs and low levels of E-cadherin, occludin and claudins-3, -4 and -7 levels (Prat et al., 2010; Taube et al., 2010).

Representing less than 1% of breast carcinomas and associated to a poor prognosis, carcinosarcomas, also called metaplastic carcinomas, contain clear cut keratin-positive epithelial-like and vimentin-positive mesenchymal-like areas. Mesenchymal cells are supposed to arise from the same cellular origin as epithelial cells according to genetic studies and would derive from a complete EMT, with Snail and Slug expression and Akt and β-catenin pathway activity (de Herreros et al., 2010).

As illustrated in Table 3, a growing number of studies have demonstrated that EMT occurrence is associated to a poor prognosis in breast cancers as well as in many other types of cancer. Indeed, EMT traits have been associated with aggressive subtypes such as the TNBC subtype (Sethi et al., 2011), the basal-like subtype (Sarrio et al., 2008) or the claudin-low subtype, a non-basal-like subtype of TNBC (Taube et al., 2010). GES (gene expression signature) analysis in breast cancer patients furthermore unraveled that EMT signature correlates negatively with complete pathological response (as defined by the absence of residual cancer on hematoxylin and eosin evaluation of the complete resected breast) (Taube et al., 2010). Additionally, the presence of EMT-TFs would be associated to positive lymph node status (Blanco et al., 2002). Finally, a fast growing number of studies have reported enrichment of CTC expressing mesenchymal traits in breast cancer patients (Aktas et al., 2009; Kallergi et al., 2011; Yu et al., 2013b).

2.3.2 Repercussion of EMT in cancer progression: *in vitro* evidence and animal models

EMT-derived cancer cells acquire traits that might enhance their aptitude to invade adjacent tissues, to resist to chemotherapy and to generate CTCs. They also acquire transient expression of certain phenotypic properties of cancer stem cells. Some of the works indicating those EMT-related traits are briefly discussed below.

2.3.2.1 EMT provides cancer cells with enhanced migratory/invasive properties

EMT-like changes endow tumor cells with enhanced migratory and invasive properties. The role of EMT-TFs in this process has been reported for instance in a study demonstrating

that Slug promotes breast cancer cell migration (Chen et al., 2009). Also, ectopic expression and silencing experiments in cancer cells have shown that Snail promotes tumor cell invasiveness (Olmeda et al., 2007; Ota et al., 2009; Peinado et al., 2004a; Sun et al., 2008). Additionally, EMT-TFs facilitate cancer cell invasion by increasing the capacities of cancer cells to remodel the extracellular matrix. Ectopic expression of Snail and ZEB2 increases expression of matrix metalloproteinases (MMPs) such as MMP-1, MMP-2, MMP-7 and MT1-MMP (membrane type 1- MMP) in hepatocellular carcinoma cells (Miyoshi et al., 2004). Ectopic expression of Snail in breast cancer cells also triggers expression of MT1-MMP and MT2-MMP, thereby facilitating basement membrane transmigration (Ota et al., 2009).

2.3.2.2 EMT confers resistance to apoptosis and chemotherapy.

Several lines of evidence support that EMT-signaling pathways make cancer cells more resistant to apoptosis. First, EMT-derived cells secrete high amounts of growth and survival factors such as TGF-α and HB-EGF that constitutively activate the pro-survival EGFR pathway (Del Castillo et al., 2006). Second, ectopic expression EMT-TFs has been shown to decrease cell sensitivity to apoptosis-inducers. Thus, Snail decreases cell sensitivity to serum depletion and to TNF-α treatment (Vega et al., 2004), and inhibits TGF-β-induced apoptosis in hepatocytes (Franco et al., 2010). Similarly, ZEB2 expression reduces DNA damage-induced apoptosis in cancer cells (Sayan et al., 2009). At last, enrichment in mesenchymal features has been described in residual breast cancer cells after conventional chemotherapy (Creighton et al., 2009).

2.3.2.3 EMT promotes tumor cell escape from the primary tumor and the generation of circulating tumor cells (CTCs)

A growing number of mice models (Bonnomet et al., 2012; Gorges et al., 2012) have corroborated clinical studies showing that CTCs concomitantly express epithelial and mesenchymal markers (Aktas et al., 2009; Kallergi et al., 2011; Yu et al., 2013b). Moreover, the enrichment of EMT features (more specifically of the mesenchymal marker Plastin3) in CTCs would relate to a poorer prognosis in patients with colorectal cancer (Yokobori et al., 2013). Similarly, enrichment of mesenchymal markers in CTCs was found to correlate with

disease progression in a study involving eleven patients with metastatic breast cancer (Yu et al., 2013b). These results suggest that EMT-derived cells might be more fit to generate CTCs and also maybe to survive in the blood stream.

2.3.2.4 EMT triggers expression of cancer stem cell phenotypic properties.

EMT-like changes have been shown to partially overlap with the expression of stemness markers both in cancer specimens and in CTCs (Aktas et al., 2009; Bonnomet et al., 2010). More specifically, EMTs have been shown to provide cancer cells with several phenotypic properties expressed by cancer stem cells. Thus, ectopic expression of Snail and the resulting EMT-like changes trigger $CD44^{High}/CD24^{Low}$ stem cell phenotypic signature in immortalized mammary epithelial cells. These cells furthermore gain the ability to form mammospheres and to differentiate into any mammary epithelial cell type (myoepithelial or luminal) (Mani et al., 2008). Snail has also been shown to induce expression of stemness-promoting genes like Nanog and KLF4 (de Herreros et al., 2010). In colorectal carcinoma cells, ectopic expression of Snail likewise triggers stem cell-like phenotype acquisition by cancer cells (Hwang et al., 2011). Slug expression has been described to provide breast cancer cells with a stem cell-like phenotype including CD44 and Jagged1 expression, ERα decrease, invasiveness and mammosphere formation (Storci et al., 2010). These data suggest that EMTs, and more specifically EMT-TFs Slug and Snail, are associated to cancer stemness properties. The resistance to apoptosis described in EMT-derived cells combined to their stem cell-like properties might collectively account for the enrichment in EMT features in residual cancer cells remaining in tumor tissue after chemotherapy or hormonal therapy as pointed out by some authors (Creighton et al., 2009).

3 The tumor microenvironment and its role in metastatic dissemination

Beside the intrinsic effects of EMTs in cancer cells described above, emerging data from the past several years have suggested that EMT programs in cancer cells might alter the tumor microenvironment. Tumors are indeed composed of a vast array of cell types

including cancer cells but also host cells such as blood and lymphatic endothelial cells, bone marrow-derived cells, granulocytes, monocytes and several subpopulations of macrophages, lymphocytes, fibroblasts, mesenchymal stem cells, etc. The interplay between cancer cells and host cells and how it affects tumor dissemination has raised much interest for the past decades (Joyce and Pollard, 2009).

High levels of soluble factors can be secreted by cancer cells and recruit host cells, which provide a second source of soluble factors, creating a so-called cytokine storm that fuels cancer-related inflammation (Lazennec and Richmond, 2010) and could affect tumor growth, angiogenesis, senescence, EMT, metastasis formation and immune evasion (Lazennec and Richmond, 2010; Sarvaiya et al., 2013). As mentioned above, many of these soluble factors act as potent inducers of EMTs.

Alternatively, EMT events could directly act on the creation of the microenvironment by recruiting host cells in the primary tumors. This aspect remains largely uncovered and constitutes the basic hypothesis of our work, in which we addressed the matters of tumor angiogenesis and MDSC recruitment. The implication of these two aspects of the tumor microenvironment on cancer progression is therefore discussed below.

A growing body of evidence describing effects of EMT on the tumor microenvironment pertinently supports our hypothesis. In PDAC for instance, neutrophil infiltration of the tumor correlates with EMT features (as determined by ZEB1 expression and nuclear accumulation of β-catenin) (Grosse-Steffen et al., 2012). Also, Snail expression in keratinocytes triggers the recruitment of M2 macrophages and the activation of resident γδT T lymphocytes (Du et al., 2010). Furthermore, Snail expression in a murine model of melanoma triggers immunosuppression by the induction of T regulatory cells and by the partial activation of dendritic cells (DCs), which turns them into tolerogenic cells (Kudo-Saito et al., 2009). Pro-angiogenic functions have also been reported for EMT-TF Slug and Snail (Peinado et al., 2004b). Finally, recent works suggest EMT-derived cells produce soluble factors such as chemokines, which might affect the tumor microenvironment (Brysse et al., 2012; Reka et al., 2014).

3.1 Angiogenesis and cancer

3.1.1 Angiogenesis: introduction

Established during embryonic development, the blood vessel network carries hematopoietic cells all over the body, allowing immunosurveillance. It also provides tissues with nutrients and oxygen and collects waste.

Blood vessel formation is a stepwise process, initiated during embryogenesis by a phase of vasculogenesis during which endothelial cell precursors arrange into a 'vascular labyrinth'. Then, the branching and organization of the labyrinth, through a process called sprouting angiogenesis, gives rise to the adult blood system with arteries, veins and capillaries. The normal mature blood vessel wall is composed of a confluent monolayer of endothelial cells tightly adhering to one another via adhesion complexes involving VE-cadherin and claudins. Endothelial cells are covered by pericytes, with which they produce a common basement membrane that is essentially composed of collagen IV and laminin (Potente et al., 2011).

In the healthy adult, the blood vessel network is kept quiescent. Endothelial cells produce basal levels of autocrine maintenance factors such as VEGF, NOTCH, ANG-1 and FGFs, which keep the blood barrier intact. Pericytes inhibit endothelial cell proliferation and provide them with survival signals such as VEGF and ANG-1 (Carmeliet and Jain, 2011). Endothelial cells express hypoxia sensors (PHD2 [prolyl hydroxylase domain-containing protein 2] and HIF-2α [hypoxia-inducible factor 2α]), which enable them to modify blood vessel shape and adjust blood flow when needed (Carmeliet and Jain, 2011).

However, sprouting angiogenesis can be reactivated in wounds and malignant diseases. In the former, endothelial cells mediate perfusion-independent functions that support tissue growth and repair. In the latter, newly formed blood vessels supply the tumor with oxygen and nutrients but also allow tumor cell intravasation (Carmeliet and Jain, 2011). When an angiogenic signal is detected in a tumor, several steps lead to the sprouting and the assembling of new blood vessels (Figure 10). At first, pericytes detach from the basement membrane thanks to proteases, and endothelial cells loosen their cell-cell adhesion complexes. Then, and as the permeability of the vessel is increased, plasma proteins extravasate to form a provisional extracellular matrix along which the endothelial cells are

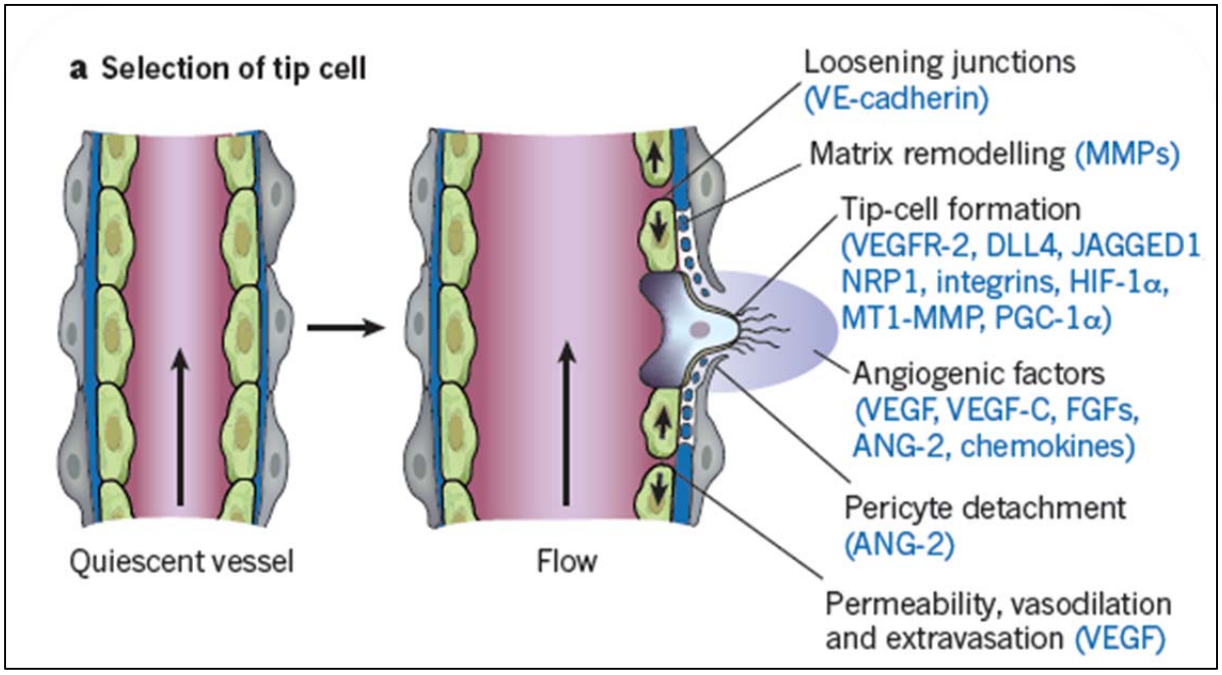
able to migrate. At this point, and because of a continuous crosstalk between endothelial cells, one cell is selected to become a specialized tip cell, equipped with filopodias and migrating so as to lead the way for the blood vessel branch in formation. The tip cell neighbors then become stalk cells that proliferate to form a growing tube behind the tip cell. Angiogenic response is over once all endothelial cells have gone back to quiescence and have been covered by pericytes (Carmeliet and Jain, 2011).

Other ways to produce new blood vessels have been described:

- Vasculogenesis, which involves the differentiation of endothelial precursor cells (EPC) recruited from the bone marrow and differentiate into endothelial cells;
- Intussusception, which is the splitting of one blood vessel into two;

And, specifically in tumor sites:

- Vessel co-option: defined by the growth of tumor cells around pre-existing vessels;
- Vascular mimicry, during which tumor cells line up in vessel-like shapes;
- And differentiation of tumor cells into endothelial cells (Carmeliet and Jain, 2011).



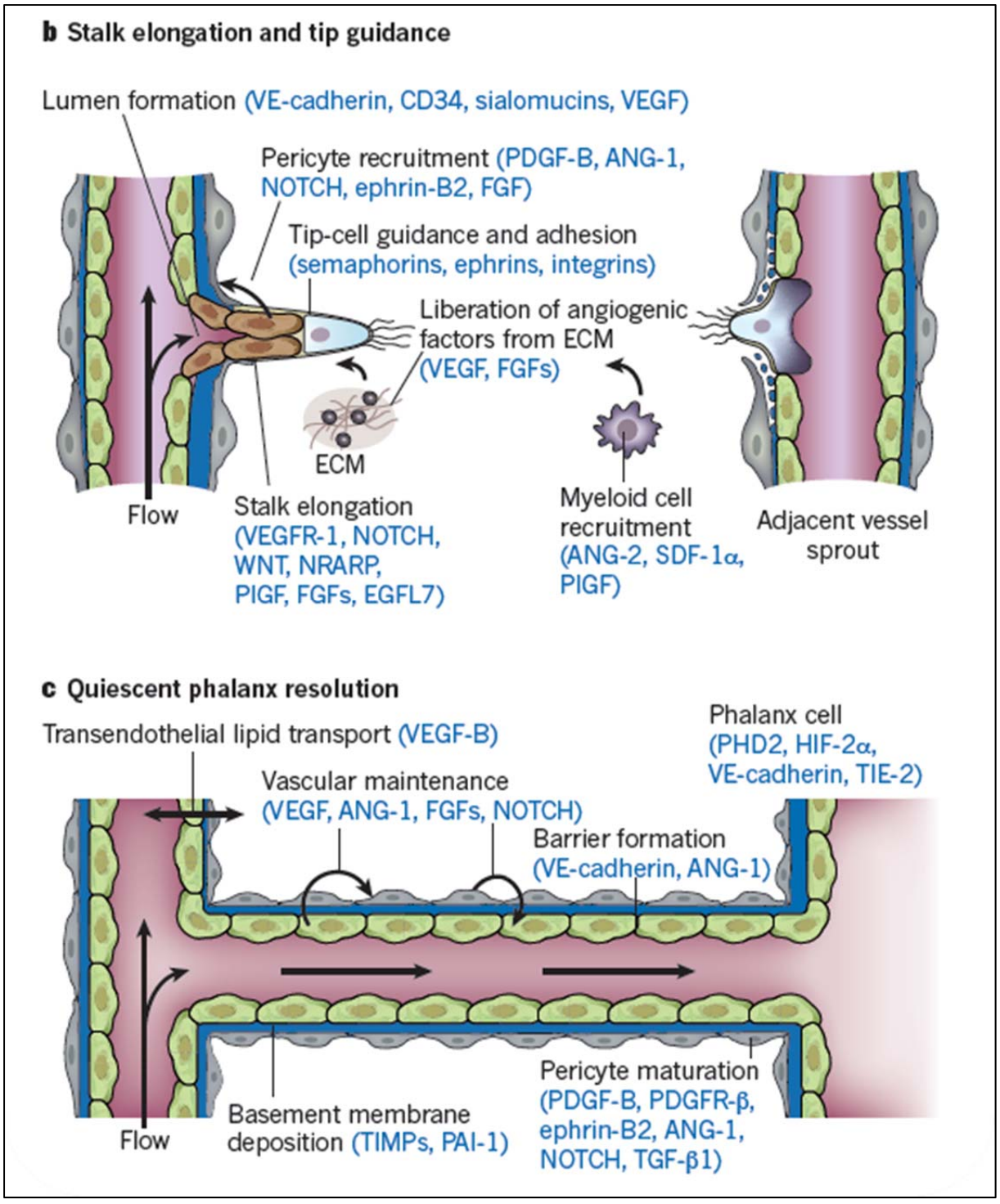


Figure 10 : Schematic representation of the multistep process of sprouting angiogenesis (Carmeliet and Jain, 2011). When a pro-angiogenic signal is detected, pericytes detach themselves from the basement membrane and a tip cell is selected based on continuous cross-talk between the tip cell and its neighbors, the stalk cells (a). The migrating tip cells lead the way for proliferative stalk elongation (b). The angiogenic response is complete once the newly-formed blood vessel is functional (blood flow is reestablished) and mature (covered in pericytes) (c). The endothelial cells are then all reverted to a stable, quiescent state, in which they are referred to a phalanx cells, as opposed to tip cells and stalk cells.

3.1.2 Modulation of angiogenesis

Molecular inducers of angiogenesis are numerous. Among them, we briefly describe here some compounds such as the VEGF (vascular endothelial growth factor) family, the FGF (fibroblast growth factor) family, proteases and chemokines (Carmeliet and Jain, 2011).

The VEGF family comprises five members called VEGF-A, B, C and D and PlGF (placental growth factor). VEGF-A, mostly referred to as VEGF, stimulates angiogenesis by binding to its receptor VEGFR2. High VEGF levels, originating from stromal or tumor cells, increase vascular branching and trigger abnormal vasculature formation whereas autocrine VEGF is responsible for blood vessel homeostasis (Figure 10) (Carmeliet and Jain, 2011).

Comprising 23 members, the FGF family is another widely described family of pro-angiogenic compounds (Figure 10). FGF2 (also known as bFGF) binds to tyrosine kinase receptors FGFR1 and FGFR2 expressed at the surface of endothelial cells and stimulates endothelial cell proliferation, migration and secretion of proteases such as uPA and MMPs. FGF2 is particularly involved in vessel maturation (Presta et al., 2009).

Proteases and their inhibitors can participate in the tumor angiogenic switch by releasing growth factors from the ECM. MMP-9/gelatinase B mediates release of VEGF-A in the RIP-Tag mouse model of pancreatic cancer (Bergers et al., 2000). Mice deficient for MMP-2/gelatinase A display a delayed angiogenic response to wounds (Bergers et al., 2000). PAI-1 (Plasminogen activator inhibitor-1), is an inhibitor of the two serine proteases that activate plasminogen: tPA (tissue plasminogen activator) and uPA (urokinase plasminogen activator). PAI-1 thereby inhibits fibrinolysis. Apart from this function, PAI-1 has been shown to contextually either stimulate or inhibit angiogenesis (Bajou et al., 2014; Bajou et al., 2004; Bajou et al., 1998). The effects of PAI-1 on vasculature are indeed affected namely by PAI-1 concentration, cellular source and binding partners. Regarding concentration, PAI-1 was demonstrated to promote tumor angiogenesis at physiological concentrations but when overexpressed, PAI-1 totally blocks tumor angiogenesis (Bajou et al., 2004). Concerning cellular source, it has been shown that PAI-1 deficiency in mice reduces tumor angiogenesis, which cannot be restored by PAI-1 overexpression by cancer cells (Bajou et al., 2004). At last, concerning the partners of PAI-1, it has recently been shown that, while PAI-1 deficiency

reduces tumor angiogenesis in melanoma B16F10 xenografts, PAI-1, when bound to 16K prolactin is actually necessary to mediate its anti-angiogenic effect (Bajou et al., 2014).

Regarding chemokines, CXC chemokines, characterized by the presence of two cysteine residues separated by one amino acid, are subdivided into pro-angiogenic or angiostatic according to whether they contain an ELR motif (a glutamic acid-leucin-arginin sequence) or not (Strieter et al., 1995) (Figure 10). CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL8, CXCL12 and CCL16 are angiogenic while CXCL4, CXCL9, CXCL10, CXCL11 and CXCL14 are anti-angiogenic (Sarvaiya et al., 2013). Angiogenic chemokines act upon binding to their receptors CXCR1, CXCR2 and CXCR4 expressed at the surface of endothelial cells. They promote endothelial cell migration, proliferation or capillary tube formation *in vitro* (Sarvaiya et al., 2013). Some chemokines play an additional indirect role on angiogenesis. CXCL8/IL-8 for instance can indirectly stimulate angiogenesis by recruiting neutrophils that promote angiogenesis namely by degrading matrix or releasing Bv8, a soluble myeloid-derived pro-angiogenic protein (Sarvaiya et al., 2013; Shojaei et al., 2008). Chemokines may also recruit other pro-angiogenic myeloid cells like macrophages, MDSC or dendritic cells (Sarvaiya et al., 2013).

Many other modulators of angiogenesis exist with more or less described mechanisms of action and include growth factors such as TGF-α (Schreiber et al., 1986), TGF-β (involved in vessel maturation), PDGF (platelet-derived growth factor)(Carmeliet and Jain, 2011), inflammatory compounds such as TNF-α (Leibovich et al., 1987) or IL-6 (Hernandez-Rodriguez et al., 2003; Middleton et al., 2014; Nagasaki et al., 2014), or even adhesion molecules such as integrins (Weis and Cheresh, 2011) or ICAM-1 (intercellular adhesion molecule) (Gho et al., 1999).

3.1.3 Angiogenesis in cancer

In tumors, rapid growth gives rise to large hypoxic areas at the source of massive pro-angiogenic factor secretion by hypoxic cancer cells. Excessive pro-angiogenic signaling generates an abnormal vasculature with leaky, tortuous vessels. Endothelial cells are poorly interconnected, the basement membrane is irregular, the arteriovenous differentiation is incomplete and pericytes are fewer and loosely attached; all of this facilitating tumor cell

intravasation. Tortuosity further impairs blood flow with, as a consequence, altered oxygen, nutrient and drug delivery to the tumor. Low oxygen increases pro-angiogenic factor secretion (Potente et al., 2011). At last, the hypoxic environment is a source of cancer cell stress that is believed to engender heterogeneity and therefore the appearance of more malignant cells (Potente et al., 2011).

Beside cancer cells, numerous cell types from the tumor microenvironment promote tumor angiogenesis, by releasing growth factors, cytokines and proteases (Joyce and Pollard, 2009). Tie-2 expressing monocytes (TEM) are located around tumor vessels and release pro-angiogenic factors. M2-polarized macrophages also stimulate angiogenesis, for instance via VEGF or PlGF production (Joyce and Pollard, 2009). Mast cells secrete proteases that liberate pro-angiogenic factors from the extracellular matrix such as FGF2 (Presta et al., 2009). Neutrophils may produce Bv8, a pro-angiogenic factor, as observed for example in tumors showing resistance to anti-VEGF treatment (Shojaei et al., 2008). They also have been shown to be a major source of pro-angiogenic MMP-9 in a mouse model of pancreatic cancer (Nozawa et al., 2006). CXCR4-positive bone marrow precursors stimulate angiogenesis (Potente et al., 2011). Cancer-associated fibroblasts have been suspected to stimulate tumor angiogenesis as well. They are indeed able to recruit endothelial progenitor cells (EPCs) and to produce angiogenic factors (Potente et al., 2011).

3.1.4 Anti-angiogenic treatments

Anti-VEGF (receptor) inhibitors have been clinically approved for advanced metastatic cancers. Anti-VEGF antibody (Bevacitumab, Avastin©) is used in combination with chemotherapy in metastatic breast cancer, and is also approved for other advanced metastatic cancers like non-small cell lung cancer, colorectal cancer and renal cell cancer (Potente et al., 2011). Anti-VEGF receptor tyrosine kinases are also used in clinics and include: Sunitinib (Sutent©), Pazopanib (Votrient©), Sorafenib (Nexavar©) and Vandetanib (Zactima©). Efficacy of VEGF (receptor) inhibitors relies on several effects: the inhibition of vessel expansion, the regression of pre-existing vessels, the sensitization of endothelial cells by removing VEGF pro-survival effect, and inhibition of VEGFR-positive tumor cell growth (Potente et al., 2011).

Anti-VEGF (receptor) inhibitors have also been tested in combination with chemotherapy in adjuvant settings, in micrometastatic diseases after surgical resection of the primary tumor, but failed to enhance overall survival. Hypotheses for this lack of effectiveness include the theory of angiogenic dormancy in micrometastatic lesions, which keeps metastatic cells insensitive to VEGF blockade, and the theory of vascular rebound upon treatment arrest (Carmeliet and Jain, 2011). VEGF (receptor) inhibition has even been suspected to increase hypoxia and trigger a metabolic pro-malignant switch in tumor cells (Carmeliet and Jain, 2011; Sounni et al., 2014).

Even though anti-angiogenic drugs inhibiting vessel growth by targeting VEGF were approved for eye disease and cancer, clinical effectiveness of such treatments is limited by the high frequency of drug resistance. Additionally, refractoriness is also encountered (Carmeliet and Jain, 2011). Resistance to treatment can be accounted for by several mechanisms including: VEGF-independent vessel growth, sprouting-independent vessel growth (such as vessel co-option or vascular mimicry), secretion of compensatory pro-angiogenic factors by stromal cells or tumor cell metabolic switch (Carmeliet and Jain, 2011; Potente et al., 2011; Sounni et al., 2014). Vessel destruction by VEGF inhibition can exacerbate hypoxia, which triggers recruitment of BMDC (bone marrow-derived cells) such as TEM, TAM, neutrophils, mast cells and MDSCs, see section 3.2), which in turn release pro-angiogenic compounds like VEGF, Bv8 or MMPs (Carmeliet and Jain, 2011).

With the clinically unsatisfactory effects of VEGF (receptor) inhibitors, other strategies have emerged to target tumor vasculature. Studies have targeted another angiogenic factor, PDGF, with disappointing results.

The strategy of vasculature normalization aims at regularizing tumor vessel structure (with low doses of anti-VEGF [receptor] inhibitors) rather than destroying them, in order to improve tumor perfusion, oxygenation and accessibility to drugs (Potente et al., 2011). A normal vessel wall should also be a more effective barrier to tumor cell intravasation (Potente et al., 2011). Moreover, preclinical studies have shown an enhanced response to chemotherapy and immunotherapy when combined with low-doses of anti-VEGF (receptors) inhibitors (Figure 11) (Huang et al., 2013; Huang et al., 2012).

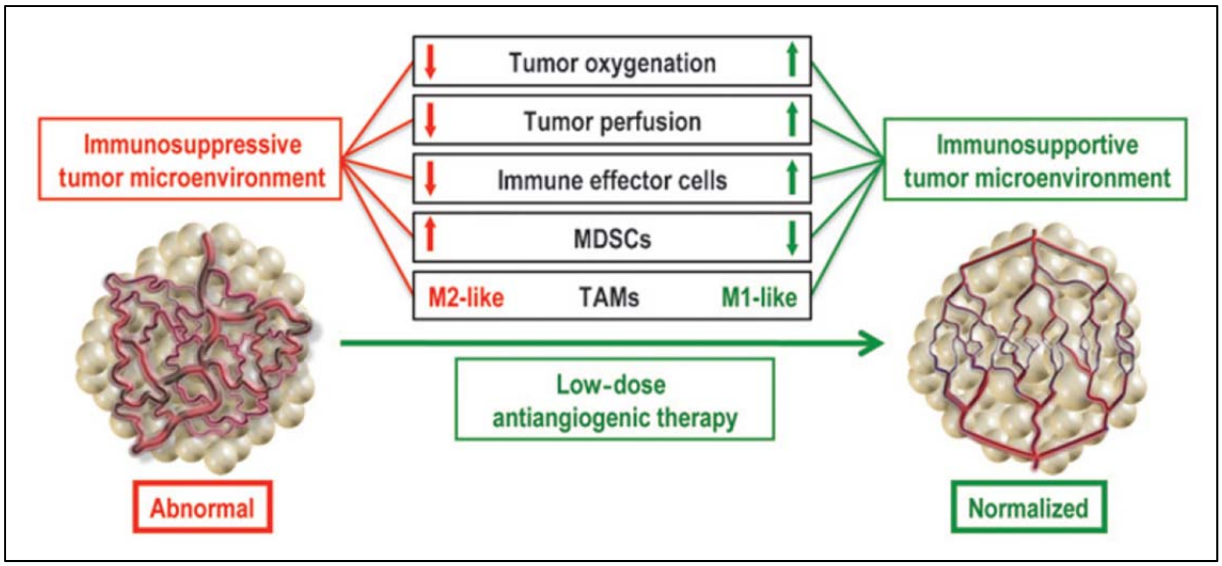


Figure 11 : The strategy of vasculature normalization. the aberrant tumor-related vasculature is associated to an immunosuppressive microenvironment, which could be turned into an immunosupportive milieu by vasculature normalization (Huang et al., 2013).

3.2 Myeloid-derived suppressor cells (MDSC)

3.2.1 MDSC: definition and markers

MDSCs are a heterogeneous population of immature myeloid cells including progenitors of granulocytes, monocytes and DCs, which all display a tolerogenic and immunosuppressive function. In the healthy individual, MDSCs are only present in the bone marrow. They accumulate in blood, spleen, lymph nodes and primary tumor in cancer patients (Gabrilovich and Nagaraj, 2009) where their number correlates negatively with prognosis and overall survival, making them a potential target for immunotherapy. MDSCs have also been involved in other diseases such as bacterial and parasitic infections, inflammatory and auto-immune diseases (Markowitz et al., 2013).

In mice, the cells share the neutrophil markers $CD11b^{+}$ $GR1^{+}$. However, and unlike neutrophils, MDSCs can be subdivided into $CD11b^{+}GR1^{hi}$ and $CD11b^{+}GR1^{low}$ MDSC. The transmembrane GR1 protein contains two nonexclusive epitopes that can be recognized by different antibodies: Ly6G and Ly6C. $CD11b^{+}GR1^{hi}$ MDSCs are $CD11b^{+}Ly6G^{hi}Ly6C^{-}$ and display a granulocytic morphology. They are precursors of granulocytes and are called granulocytic MDSC or PMN-MDSCs. On the other hand, $CD11b^{+}GR1^{low}$ MDSCs are $CD11b^{+}Ly6G^{-}Ly6C^{hi}$ and

are called monocytic MDSCs or Mo-MDSCs (Gabrilovich and Nagaraj, 2009) (Figure 12). Because Ly6G and Ly6C are shared with neutrophils and inflammatory monocytes respectively, there is currently no specific phenotypic marker for murine MDSCs even though a more accurate phenotypic classification include other markers leading to definition of MDSCs as $CD11b^{+}$ $GR1^{+}$ IL-4Rα^{+} $CD11c^{-}$ $F4/80^{+/-}$ cells (Solito et al., 2014). Therefore, MDSC phenotypic identification should be complemented by functional assays showing cell immunosuppressive activity.

In humans, MDSCs are defined by a somewhat more complex set of markers since they are $CD11b^{+}CD33^{+}$HLA-DR^{-} and $lineage^{-}$ (i.e. negative for markers of mature myeloid and lymphoid cells : CD3, CD19, CD14 and CD57) (Diaz-Montero et al., 2009; Gabrilovich and Nagaraj, 2009).

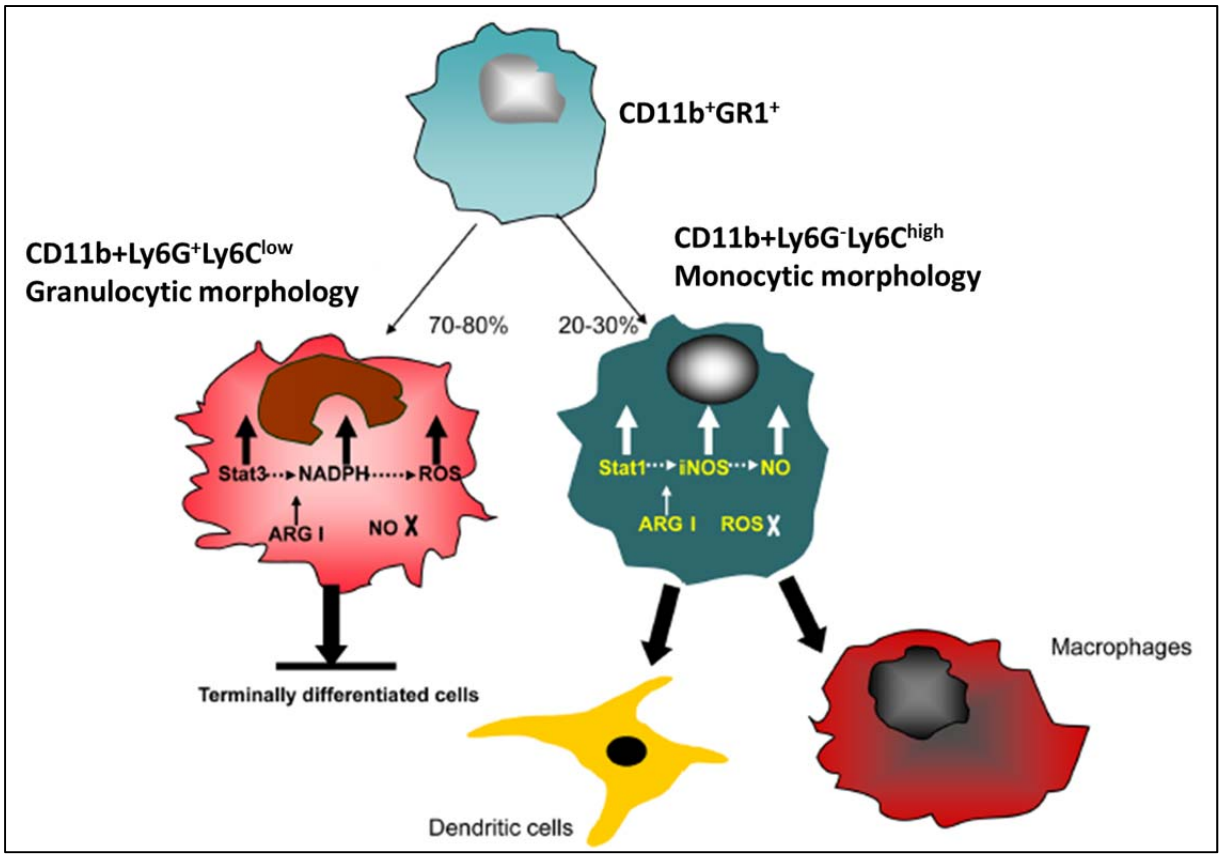


Figure 12 : Classification of murine MDSCs (Gabrilovich and Nagaraj, 2009). Total CD11b+GR1+ MDSC are divided into $CD11b^{+}Ly6G^{+}Ly6C^{low}$ granulocytic MDSCs (PMN-MDSCs) and $CD11b^{+}Ly6G^{-}Ly6C^{high}$ monocytic MDSCs (Mo-MDSCs). PMN-MDSCs express high levels of STAT3 and their immunosuppressive activity mainly relies on arginase and ROS production. Mo-MDSCs express STAT1 and high levels of iNOS. They also produce arginase but not ROS.

3.2.2 MDSCs in cancer

Several clinical studies have detected higher levels of circulating MDSCs in breast cancer patients compared to healthy volunteers. Levels of MDSCs in blood, spleen, lymph nodes and primary tumor have additionally been correlated to disease burden in patients with breast (Diaz-Montero et al., 2009) or gastric cancers (Wang et al., 2013b)) and to probability of death (Cole et al., 2010; Diaz-Montero et al., 2009; Markowitz et al., 2013).

A causal effect of MDSCs on cancer progression has been illustrated by mouse models in which MDSC depletion decreases tumor burden (Markowitz et al., 2013). In a model of experimentally-induced colitis-associated cancer, suppression of CXCR2 specifically in MDSC prevents their recruitment to inflammatory sites and delays colitis-associated tumorigenesis. Conversely, the addition of MDSCs accelerates tumor growth via inhibition of CD8+ T cell cytotoxic activity (Katoh et al., 2013). Data obtained in mouse models should however be interpreted with care, according to Diaz-Montero and colleagues. Indeed, in tumor-bearing mice and unlike to human patients, up to 50% of blood cells are MDSCs (this could be explained by the huge tumor burden in some mouse models). Moreover, while in mice there is a linear relationship between primary tumor size and circulating MDSC levels, this relationship is more complex in human (Diaz-Montero et al., 2009).

3.2.3 Immunosuppressive activity

MDSCs are potent pleiotropic tolerogenic and immunosuppressive cells: they can affect T cell proliferation and activation (as measured by IL-12 and IFN-γ production) (Diaz-Montero et al., 2009), induce regulatory T cell activity and inhibit Natural Killer cell activation. Molecular mechanisms of MDSC-mediated immunosuppression include arginase and iNOS (inducible Nitric Oxyde Synthase) expression. Other pathways such as ROS (reactive oxygen species) and peroxynitrite production are also involved (Gabrilovich and Nagaraj, 2009).

Arginase and iNOS inhibit T cell proliferation and function. Arginase converts L-arginin into urea and L-ornithine, leading to arginine depletion, responsible for CD3 T cell proliferation arrest. Indeed, L-arginin depletion causes a decrease in CD3ζ chain synthesis

and a decrease in cell-cycle controlling Cyclin D3 and Cyclin-Dependent Kinase 4 (CDK4) in T cells (Gabrilovich and Nagaraj, 2009). iNOS produces nitric oxide (NO), which inhibits T cell function via inhibition of JAK3 and STAT5, inhibition of MHC II expression and induction of apoptosis in T cells (Gabrilovich and Nagaraj, 2009).

Increased production of ROS by MDSCs has been described both in patients with cancer and in tumor-bearing mice (Gabrilovich and Nagaraj, 2009). At last, peroxynitrite ($ONOO^-$) is a very oxidant compound which, in contact with T-cells, nitrates CD8 and TCR, thereby abrogating T-cell response and ADCC (antigen-dependent cell-mediated cytotoxicity) (Gabrilovich and Nagaraj, 2009).

Some of the mechanisms cited above are subset-specific. For instance, PMN-MDSCs are ROS^{high} in contrast with Mo-MDSCs, which are ROS^{low}, but both population are equally efficient in inhibiting T cell proliferation (Gabrilovich and Nagaraj, 2009).

3.2.4 Mechanisms of MDSC accumulation and activation in cancer and therapeutic targeting of MDSCs

Despite the fact that MDSCs are consistently increased in primary tumors of cancer patients and in a variety of mouse models, mechanisms of MDSC recruitment are not totally understood. Nonetheless, some data imply CXCL5-CXCR2 and CXCL12-CXCR4 interactions in the recruitment of MDSCs (Joyce and Pollard, 2009). In addition, tumor cells produce several factors that can promote the expansion of MDSCs by stimulating myelopoiesis and inhibiting differentiation, among which COX2, prostaglandins, SCF, (G)M-CSF, IL-6 and VEGF. Soluble factor-mediated JAK/STAT3 signaling increases myeloid cell survival and prevents cell differentiation. Finally, tumor stroma releases factors such as IFN-γ, TLR ligands or TGF-β, which activate MDSC immunosuppressive activity via STAT6, STAT1 and NF-κB signaling (Gabrilovich and Nagaraj, 2009).

Because of their immunosuppressive activity, MDSCs are key players in metastatic progression and have been studied as potential therapeutic cellular targets. Forcing MDSC differentiation into DC or macrophages has been done by treating mice with vitamin A or vitamin D3. Alternatively, MDSC expansion could be blocked by inhibiting stem cell factor

(SCF) or VEGF. MDSC suppressive activity could be blocked by COX2 inhibitors (which prevent the production of PGE2, essential to arginase expression), or by phosphodiesterase-5 inhibitors (like sildenafil) that downregulate iNOS expression. Finally, some preclinical models suggest that specific chemotherapeutic drugs used in cancer would be able to eliminate MDSC, although whether this is a specific effect or a manifestation of the generally toxic effect of chemotherapeutic agents is unclear. For instance, Gemcitabin is able to decrease levels of MDSC in mice and improve effect of immunotherapy (Gabrilovich and Nagaraj, 2009). Doxorubicin is able to eliminate MDSCs in mice bearing overt mammary tumors (Alizadeh et al., 2014). Doxorubicin is more specifically able not only to induce apoptosis in PNM-MDSCs via ROS production, but also to alter the immunosuppressive activity of residual MDSCs (Alizadeh et al., 2014). Mouse models using antibody therapy have also shown promising results. On one hand, combined treatment with adoptively transferred CD8 cells and anti-GR1 antibody-mediated MDSC depletion triggered tumor regression (Markowitz et al., 2013). On the other hand, inhibition of MDSC recruitment to the tumor with anti-CCL5 blocking antibodies have also been tested (Markowitz et al., 2013).

Purpose and plan

Part 1: EMT-regulated soluble factors mediate cancer-related angiogenesis and myeloid cell recruitment

Linked publication:

***Suarez-Carmona M**, Bourcy M, Syne L, Blacher S, Hubert P, Erpicum C, Polette M, Noël A, Delvenne P, Birembeaux P and Gilles C, Epithelial-to-mesenchymal transition (EMT)-regulated soluble factors mediate tumor angiogenesis and myeloid cell recruitment. Journal of Pathology, under revision.*

Metastatic dissemination is a stepwise process involving the invasion of adjacent tissues by cancer cells, the intravasation in the blood and/or lymphatic system, the survival in the circulation and arrest at distant sites, before dormancy or direct proliferation to form overt metastases. Epithelial-to-mesenchymal transitions (EMTs) provides cancer cells with phenotypic properties such as increased invasive ability, resistance to apoptosis and stem cell-like traits, which enhance their capacity to overcome the barriers met throughout metastatic spread. EMT impact on metastasis is accordingly demonstrated by clinical observations, corroborated by *in vitro* data and animal models.

However, the metastatic dissemination largely relies on signaling networks between cancer cells and the host compartment, essentially composed of endothelial cells, cancer-associated fibroblasts and inflammatory cells. The overexpression of soluble factors by cancer cells has been shown to promote metastatic dissemination even though the mechanisms underlying such an overexpression of soluble compounds by cancer cells are elusive. Furthermore, while the impact of EMTs on intrinsically enhanced invasive properties of cancer cells has been extensively studied, the involvement of EMTs on the interplay between cancer cells and the stroma is still largely uncovered.

Since EMT-like changes are often observed at the invasive margin of cancer, at the tumor-stroma interface, **we hypothesized that EMT programs might modulate the secretory profile of cancer cells. EMT-regulated soluble factors could in turn chemo-attract**

host cells to the tumor, thereby elaborating a permissive microenvironment for cancer progression.

In line with that hypothesis, we established three sequential objectives. Our first objective was to characterize the secretory profile of EMT-negative and EMT-positive cell lines and to subsequently switch to *in vivo* models to extend our observations. We next studied the implication of EMT-associated transcription factors in the regulation of the identified soluble factors, so as to link them to specific EMT-related signaling cascades. Finally, we aimed at exploring the functional consequences that the EMT-associated soluble factors might trigger on the tumor microenvironment and the metastatic progression.

Our results are presented as five chapters, systematically preceded by a concise introduction and followed by a specific discussion.

Chapter 1: EMT phenotypes relate to the expression of a set of soluble factors in vitro and in vivo

In the first part of our work, we screened for the expression of soluble factors by EMT-negative and EMT-positive cells from breast and lung cancer *in vitro*. We also used an *in vivo* model of laser-capture microdissection of EMT-positive and EMT-negative zones of xenotransplanted tumors in mice.

Chapter 2: EMT-associated soluble factors are regulated by Snail family of transcription factors

Secondly, we linked the expression of EMT-modulated soluble factors to EMT-specific signaling cascades by examining the implication of Snail-family transcription factors Snail and Slug in the regulation of the soluble factors of interest.

Chapter 3: conditioned medium from EMT-positive cells stimulates angiogenesis

Thirdly, we studied the consequences of EMT-regulated soluble factors on the tumor microenvironment. We first demonstrated a contributing effect of conditioned medium from EMT-positive cells on angiogenesis using *in vitro* and *in vivo* and *ex vivo* models.

Chapter 4: Conditioned medium from EMT-positive cells recruits myeloid cells in vivo

Subsequently, we investigated on the effect of conditioned-medium of EMT-positive cells on inflammatory cell recruitment and rapidly focused on MDSCs. This last part of our work has provided promising yet preliminary results that we would like to validate with additional models.

Chapter 5: The presence of EMT features relates to increased angiogenesis and tumor infiltration by myeloid cells in human triple-negative breast cancers

Finally, we conducted a study on a triple-negative breast tumor bank in collaboration with Professors Myriam Polette and Philippe Birembaut from the laboratory Paul Bouin (INSERM UMR-S 903, Reims, France), in which we examined the potential association between EMT features, angiogenesis and recruitment of MDSCs to the tumor.

A general discussion is then presented after these four "results" chapters in the "discussion and perspectives" section, followed by the article under revision as mentioned above.

Part 2: DeltaNp63 isoform-mediated β-defensin family is associated with (lymph-) angiogenesis and poor outcome in patients with squamous cell carcinoma

Linked publications:

Suarez-Carmona M, *Hubert P, Delvenne P and Herfs M (2014) Defensins : « simple » antimicrobial peptides or broad-spectrum molecules ? Cytokines and Growth Factor Reviews [Epub ahead of print]*

Suarez-Carmona M*, Hubert P, Gonzalez A, Duray A, Roncarati P, Erpicum C, Boniver J, Castronovo V, Noel A, Saussez S, Peulen O, Delvenne P, Herfs M. (2014) ΔNp63 isoform-mediated β-defensin family up-regulation is associated with (lymph)angiogenesis and poor prognosis in patients with squamous cell carcinoma. Oncotarget 5(7): 1856-68.*

During this thesis and in parallel to the abovementioned work, I have had the opportunity to further perform a study that had been initiated during my master thesis in the laboratory of experimental pathology under the supervision of Dr Michael Herfs and Professor Philippe Delvenne. Our objective was to study the microenvironmental factors driving neoplastic progression in uterine cervix. Based on micro-array data available in the literature showing lower β-defensin 2 levels when ΔNp63 – a transcription factor expressed in developing pluristratified epithelia and overexpressed in cervical carcinomas – is decreased, we investigated the regulation of β-defensins (small antimicrobial peptides secreted by epithelial cells) by ΔNp63 and explored the functional consequences of such β-defensin overexpression by cancer cells on the tumor microenvironment.

In the first part of this work, we investigated the regulation of all defensins by all isoforms and isotypes of p63 and searched cervical and head and neck tumor banks for association between p63 expression and defensin secretion. Then, we analyzed the effect of each p63-regulated defensin on (lymph-) angiogenesis in several *in vitro* and *in vivo* models. We also looked for any association between p63 and (lymph-) angiogenesis in tumor banks. Finally, we studied the impact of p63 expression on patient overall survival. These results led to a publication as a first author, which will not be detailed in this document, but is annexed, after a brief description.

Results

1 EMT phenotypes associate with a high expression of a set of soluble factors *in vitro* and *in vivo*

1.1 Introduction

The metastatic dissemination of cancer largely relies on complex tumor-stroma crosstalk (Grivennikov and Karin, 2010; Hanahan and Weinberg, 2011). Cancer cells are indeed able to secrete high amounts of soluble factors such as cytokines and growth factors that favor the recruitment and the activation of host cells, which themselves constitute a second source of soluble factors. The interplay between cancer cells and host cells has been shown to twist the initially normal host response into a metastasis-promoting one, with for instance M2-polarized inflammation, excessive angiogenesis and immunosuppression (Grivennikov and Karin, 2010; Hanahan and Weinberg, 2011; Kudo-Saito et al., 2009).

EMT-like changes occurring in cancer cells are well-known to enhance invasive properties of cancer cells, but their impact on tumor-stroma interactions is poorly described. Because EMT-like changes are mostly observed in the invasive margins of tumors in close proximity of tumor stroma, we hypothesized that EMT programs might affect the secretory profile of cancer cells, hence the interactions between cancer cells and host cells. Accordingly, several studies suggest a role for EMT programs in the modulation of soluble factor production by cancer cells. For instance, IL-8 has been shown to be regulated during EMT in breast and colorectal cancer cell lines (Bates et al., 2004; Brysse et al., 2012; Palena et al., 2012). A previous study led in our laboratory also showed an association between EMT-signaling pathways and the overexpression of CCL2/MCP-1 in breast cancer cells (Mestdagt et al., 2006).

In this first part of our study, we analyzed the secretion of soluble factors in cell lines inducible for EMT and in “stable” EMT-positive and EMT-negative cell lines. We showed that EMT phenotype relate to the expression of five soluble factors: IL-8, IL-6, PAI-1, sICAM-1 and

GM-CSF. IL-6, IL-8 and PAI-1 are also enriched in EMT-positive cells in an *in vivo* model of human cell xenotransplantation in mice.

1.2 Results

1.1.1 EMT induction increases the mRNA expression and protein secretion of a variety of soluble factors

Two models of inducible EMT were used in order to analyze EMT implication in the regulation of soluble factors: the MDA-MB-468 human mammary adenocarcinoma cell line and the A549 human lung carcinoma cell line. Both cell lines were treated with EGF or TGF-β, respectively. The induction of EMT was assessed by observation of morphological changes and by RT-qPCR analysis showing decreased E-cadherin and increased vimentin mRNA expression (Figure 13A-D). The secretion of 36 different soluble factors by EMT-positive (EGF-treated) and EMT-negative (untreated) MDA-MB-468 cells was examined using a cytokine array (Figure 13E). This approach revealed that a consistent number of soluble molecules was increased upon EMT induction in the array (Figure 13F).

The five most overexpressed molecules identified from the array were Interleukin-8 (IL-8), Interleukin-6 (IL-6), soluble form of InterCellular Adhesion Molecule-1 (sICAM-1), Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF) and Plasminogen Activator Inhibitor-1 (PAI-1). This induction was confirmed in the MDA-MB-468 at the RNA and protein levels by RT-qPCR (Figure 14A) and ELISA (Figure 14B). It is worth mentioning that while IL-8, IL-6, GM-CSF and PAI-1 are secreted molecules, ICAM-1 can be either in a transmembrane form or soluble, due to both alternative splicing and protein shedding (Ramos et al., 2014). Primers recognizing all forms of ICAM-1 were used, while only the soluble form (sICAM-1) was detected by ELISA.

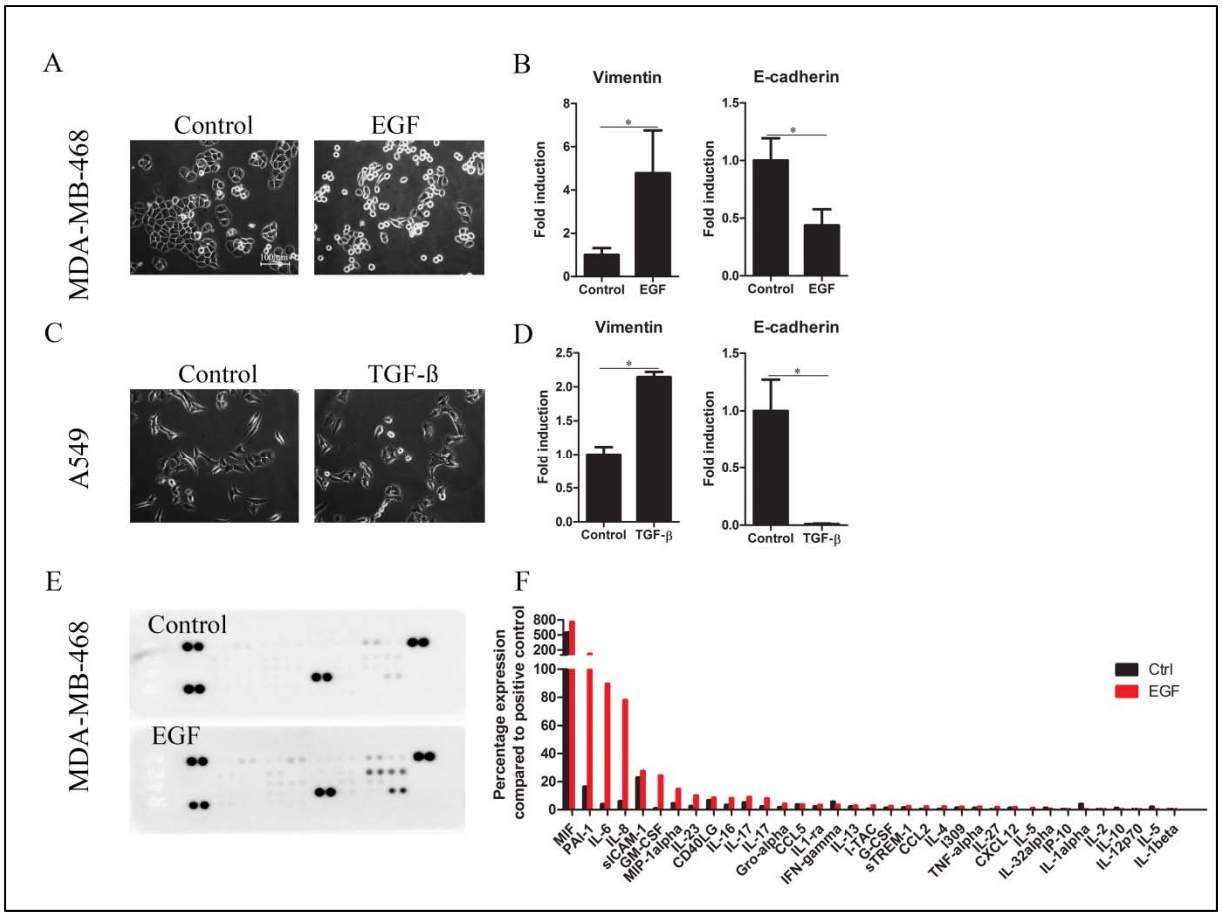


Figure 13 : Models of EMT induction in cancer cells lines and cytokine array. (A,C) Microscopy images illustrating morphological changes in MDA-MB-468 (A) and A549 (C) cells upon EGF or TGF-β treatment, respectively. (B, D) RT-qPCR analyzes of vimentin and E-cadherin in control or EGF-treated MDA-MB-468 cells (B) and in control or TGF-β-treated A549 cells (D). Data are expressed as fold induction in treated cells relative to the controls. (E) Membranes of the cytokine array performed on conditioned medium from control or EGF-treated MDA-MB-468 cells and (F) Histogram representing the relative expression of each tested soluble factor compared to the membrane internal positive control.

Similar results were obtained with the A549 cell line (Figure 14C-D), except for ICAM-1. ICAM-1 mRNA expression was unchanged upon EMT induction (Figure 14C) and sICAM-1 could not be detected in the supernatant (Figure 14D). Concerning PAI-1, it is interesting to note that in MDA-MB-468 cells, PAI-1 protein secretion could not be detected in either untreated conditioned medium or condition medium after Amicon filter-mediated concentration, but could only be detected after acetone precipitation of the cell supernatant.

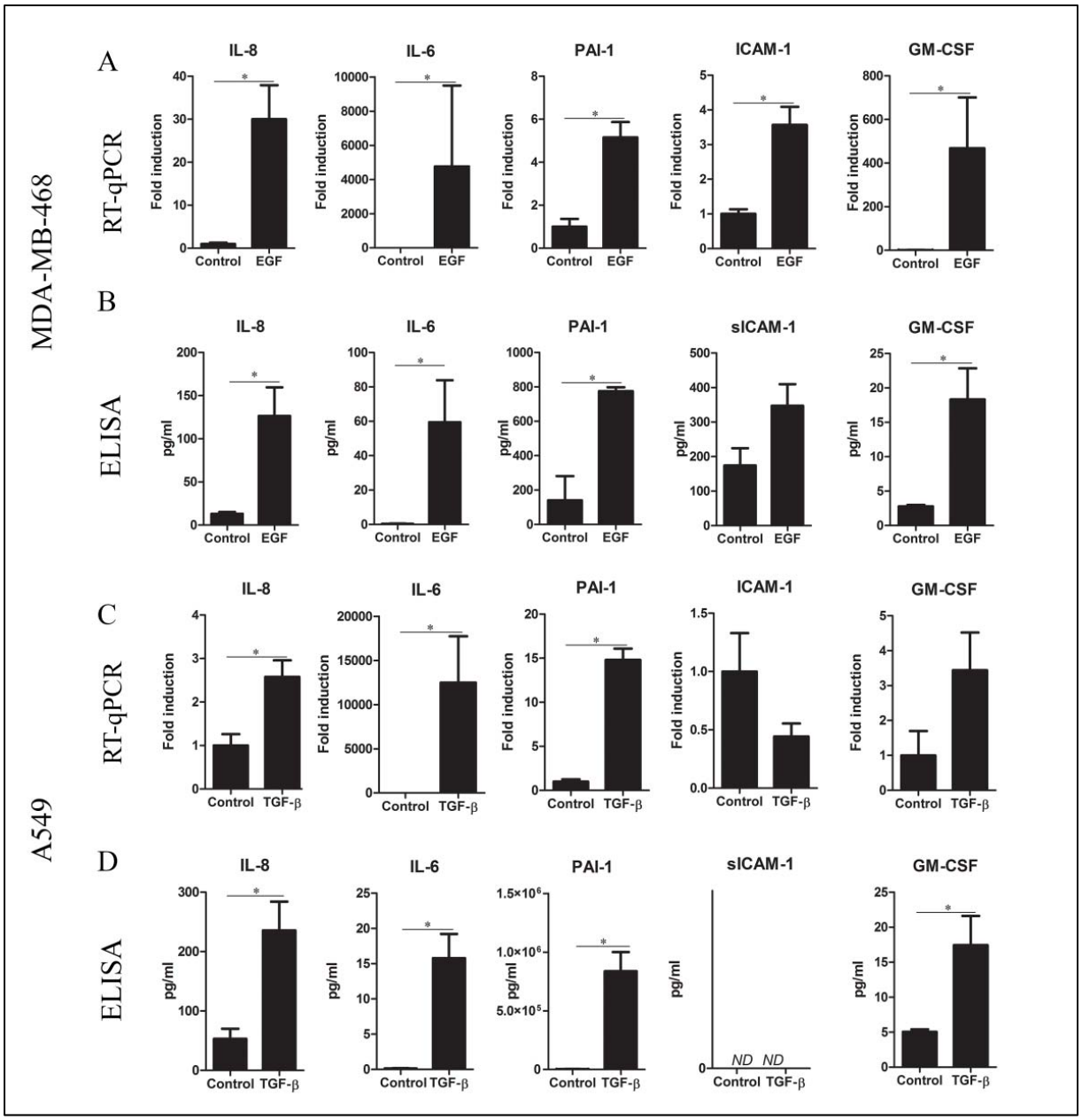


Figure 14: Validation of the results of the cytokine array in MDA-MB-468 and A549 cells. Analyzes of IL-8, IL-6, PAI-1, ICAM-1 and GM-CSF in control or EGF-treated MDA-MB-468 cells by (A) RT-qPCR or (B) ELISA and in control or TGF-β-treated A549 cells by (C) RT-qPCR or (D) ELISA. *: p>0.05.

1.1.2 EMT phenotype is associated with the expression of soluble factors

1.1.2.1 In vitro

Several cell lines well-known to express stable EMT-positive (i.e. vimentin positivity and E-cadherin negativity) and EMT-negative (i.e. vimentin negativity and E-cadherin positivity) characteristics were screened for mRNA expression of soluble factors. Supporting our results

on inducible cell lines, IL-8, IL-6, ICAM-1, PAI-1 and GM-CSF were expressed at a higher level in EMT-positive cell lines (MDA-MB-231, BT549 and Hs578t) compared to epithelial-like cell lines (MDF7 and T47D) (Figure 15A).

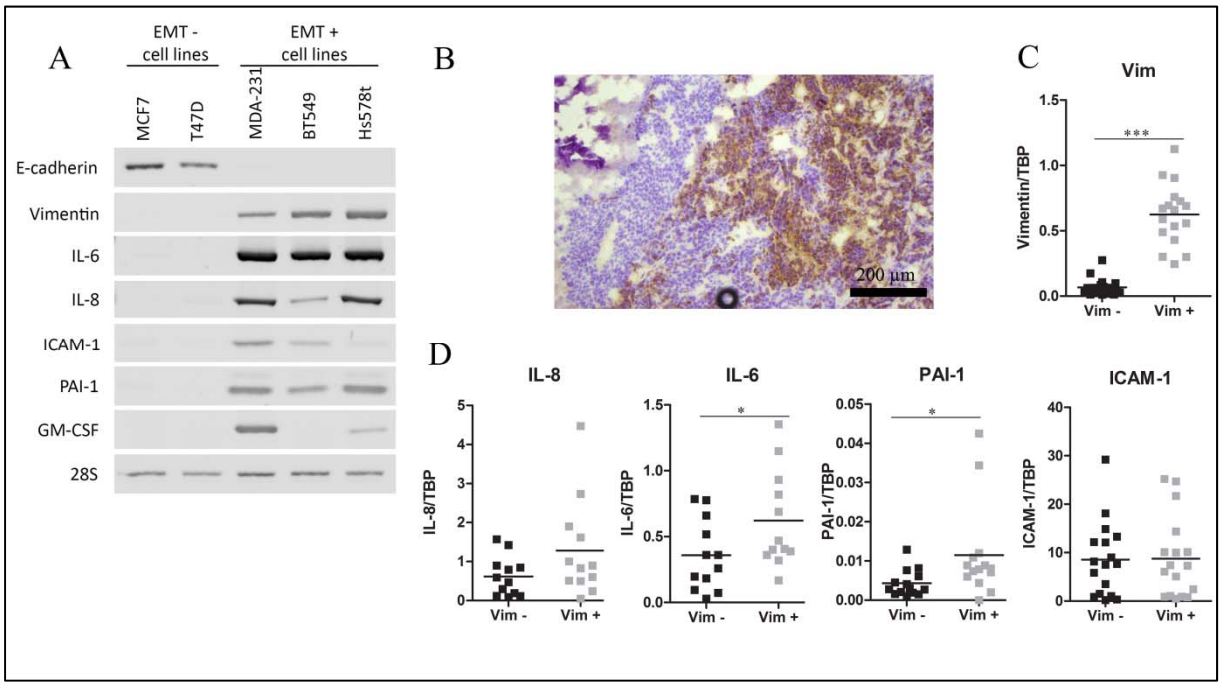


Figure 15: Screening for association between EMT features and soluble factors *in vitro* and *in vivo.* (A) Analyzes by endpoint RT-PCR of E-cadherin, vimentin, IL-8, IL-6, PAI-1, ICAM-1 and GM-CSF in a panel of well-known epithelial-like and EMT-derived human breast cancer cell lines. (B) Microscopy image of human vimentin specific immunostaining (brown) performed on a section of a MDA-MB-468-formed tumor in SCID mice. (C) RT-qPCR analysis of human vimentin on RNA extracted from microdissected vimentin-negative (Vim-) and vimentin-positive (Vim+) tumor areas. (D) RT-qPCR analyzes of human IL-8, IL-6, PAI-1 and ICAM-1 on RNA extracted from microdissected areas. *: p<0.05 and ***: p< 0.001.

1.1.2.2 In vivo

To extend these observations *in vivo*, we took advantage of the capacity of MDA-MB-468 cells to undergo EMT-like changes when subcutaneously injected in mice, as previously described in our laboratory (Bonnomet et al., 2012). Briefly, 2 x 10^6 cells were mixed with Matrigel and injected in seven-week-old immunodeficient SCID female mice. After 100 days, large tumors arose in which vimentin-expressing human cells could be specifically stained. Vimentin immunohistochemistry revealed the co-existence of areas containing either vimentin-positive or vimentin-negative cancer cells (Figure 15B). These areas were isolated by laser-capture microdissection (Figure 15C). Messenger RNA expression of IL-6 and PAI-1

appeared significantly higher in vimentin-positive areas compared to vimentin-negative ones as shown by RT-qPCR (Figure 15D). IL-8 mRNA expression also tended to be enriched in the vimentin-positive compartment (Figure 15D). No change in ICAM-1 mRNA expression was observed (Figure 15D). GM-CSF was not detectable in the *in vivo* situation, in accordance with an already low expression level *in vitro*.

1.3 Discussion

In this first part, we used several cellular systems to show that EMT phenotypes relate to increased expression of five soluble compounds (IL-8, IL-6, sICAM-1, PAI-1 and GM-CSF). These factors are consistently increased in two cell lines (MDA-MB-468 and A549) following different growth factor treatments (EGF and TGF-β respectively) and preferentially expressed in cell lines displaying endogenous EMT traits. IL-6 and PAI-1 mRNA expression is furthermore increased in EMT-positive zones of MDA-MB-468-formed tumors in mice.

Though enhanced cytokine expression in various cancer cells has been reported by many authors, our results are most pertinently supported by a few data linking EMT-signaling pathways and expression of soluble factors. Thus, the association between EMT features and IL-8 has already been reported by us and others in breast cancer cell lines (Brysse et al., 2012) or in colorectal carcinoma cell lines (Bates et al., 2004). Clinically, EMT features were found to be enriched in IL-8-positive hepatocellular carcinoma biopsies (Yu et al., 2013a). Data regarding the regulation of other soluble factors through EMT are scarce. PAI-1 expression has nevertheless been shown to be induced during EMT in Ras-transformed keratinocytes (HaCaT II-4) (Freytag et al., 2010) and has been identified as upregulated upon TGF-β-mediated EMT in A549 cells (Reka et al., 2014). Concerning GM-CSF, a very recent study revealed that mesenchymal-like breast cancer cells secrete high amounts of GM-CSF (Su et al., 2014). Regarding ICAM-1, the membrane bound form would be involved in accelerating TGF-β-induced EMT in renal cells, in the presence of ICAM-1 ligand LFA-1, which is expressed at the surface of monocytes (Li et al., 2011; Morishita et al., 2011). However, there is currently no data clearly linking sICAM-1 expression and EMT to our knowledge.

Inversely, certain chemokines have also been shown to act as EMT inducers. Independent studies have shown that IL-8 (Fernando et al., 2011; Li et al., 2012b; Palena et

al., 2012) and IL-6 (Sullivan et al., 2009) act as key mediators of EMT induction and maintenance in several cancer cell lines (from breast, lung, nasopharyngeal and pancreatic cancer) (Fernando et al., 2011). CXCR1, one of IL-8 receptors, is concomitantly increased with IL-8 in colorectal cell lines upon EMT induction (Bates et al., 2004). Also, a functional role of PAI-1 in the modulation of EMT programs has been reported in the Ras-induced EMT model of HaCaT II cells mentioned above (Freytag et al., 2010) as well as in a model of EMT-induced lung fibrosis (Senoo et al., 2010). In the latter, PAI-1 repression by siRNA inhibited TGF-β-triggered EMT in murine epithelial cells. These data imply that induction of IL-8, IL-6 and PAI-1 might create a positive regulatory loop to maintain an EMT-derived phenotype in cancer cells.

2 EMT-associated soluble factors are regulated by Snail family of transcription factors

2.1 Introduction

In a second part of our work, we aimed at more specifically linking the EMT-associated expression of soluble factors to well-known EMT-TFs. Therefore, MDA-MB-468 and A549 cells were screened for the expression of EMT-TF Snail, Slug, ZEB1, ZEB2, Twist and E47 (data not shown). Because their mRNA expression increased upon EMT induction in both inducible cell lines, we focused on Snail and Slug transcription factors.

2.2 Results

Slug and Snail involvement in the regulation of soluble factors was investigated by modulating their expression through the transfection of siRNA or expression vectors. Using independent siRNA against Snail and Slug to transfect MDA-MB-468 and A549 cells, we observed that Snail knockdown affected Slug expression and vice versa. We thus chose to knock both genes down (Figure 16A and Figure 17A) to evaluate the impact on the expression of our soluble factors of interest.

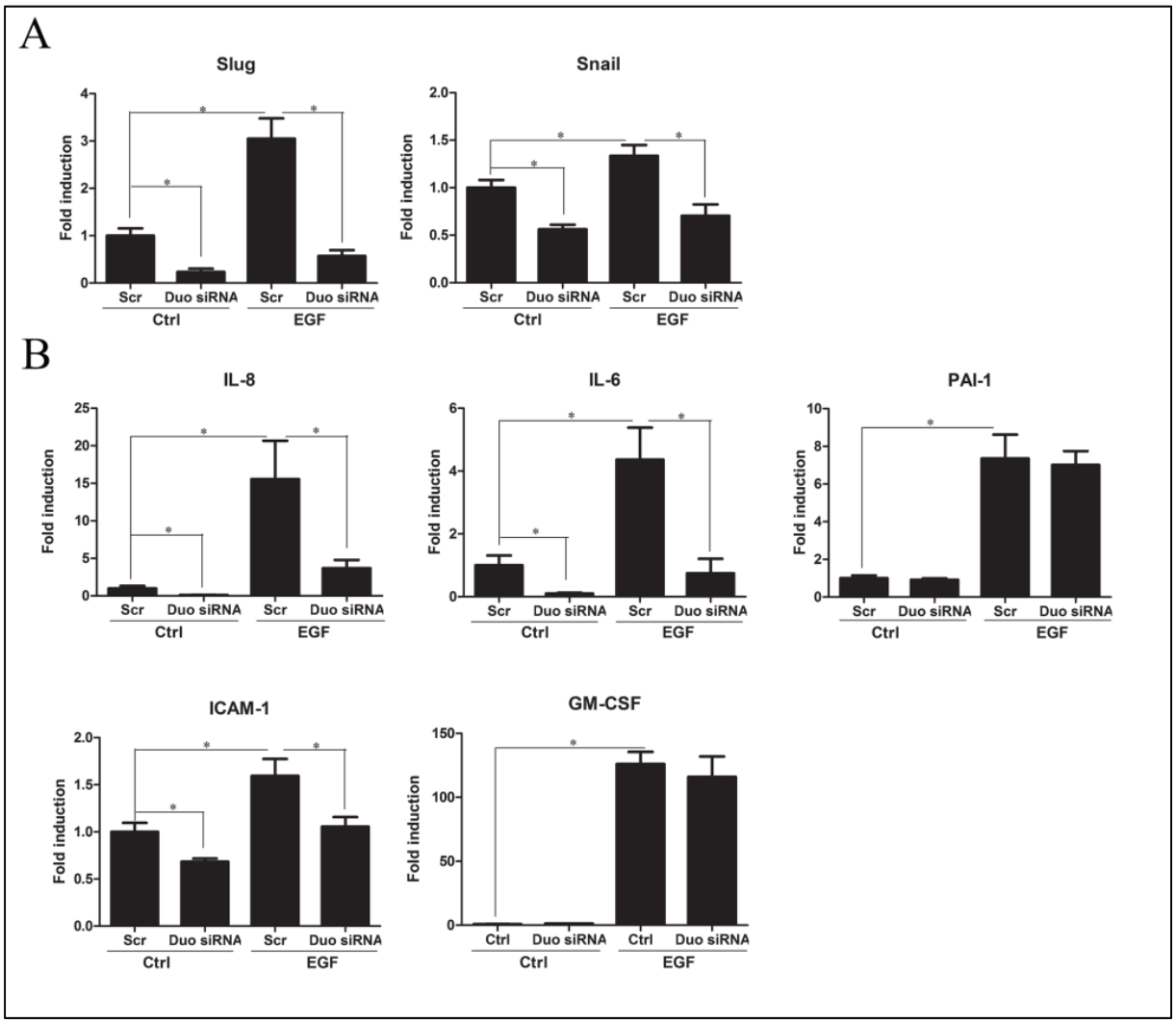


Figure 16: Regulation of soluble factors by combined repression of Slug and Snail in MDA-MB-468. RT-qPCR analyzes of (A) Slug and Snail, (B) IL-8, IL-6, PAI-1, ICAM-1 and GM-CSF in MDA-MB-468 cells transfected with a combination of Snail and Slug siRNA or a control siRNA. Data are expressed as fold induction relative to the non-treated control siRNA condition. *: p<0.05.

Combined repression of Slug and Snail significantly and strongly inhibited mRNA expression IL-8 and IL-6 in both untreated and EGF-treated MDA-MB-468 (Figure 16B) and in both untreated and TGF-β-treated A549 (Figure 17B). ICAM-1 mRNA expression was moderately affected only in MDA-MB-468 (Figure 16B). PAI-1 expression was unaffected (Figure 16B and Figure 17B). GM-CSF mRNA expression was inhibited in A549 cells (Figure 17B) but not in MDA-MB-468 cells (Figure 16B).

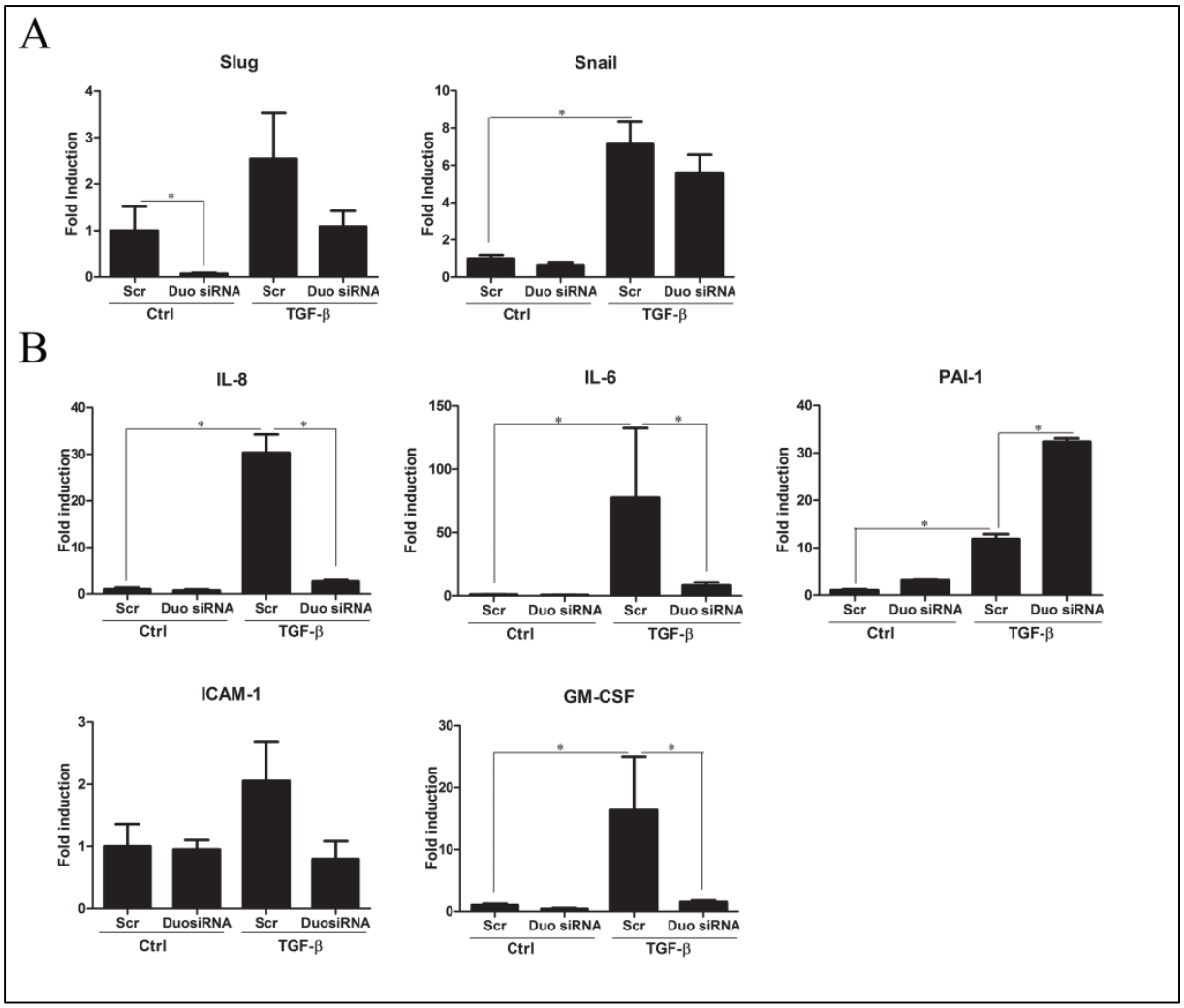


Figure 17: Regulation of soluble factors by combined repression of Slug and Snail in A549 cells. RT-qPCR analyzes of (A) Slug and Snail, (B) IL-8, IL-6, PAI-1, ICAM-1 and GM-CSF in A549 transfected with a combination of Snail and Slug siRNA or with a control siRNA. Data are expressed as fold induction relative to the non-treated control siRNA condition. *: $p<0.05$.

Inversely, we transfected expression vectors for snail and slug in MDA-MB-468 and A549 cells. However, such ectopic expression of either Slug or Snail failed to increase soluble factor expression (data not shown). These results were nonetheless challenging to interpret since while mRNA of Slug and Snail was significantly increased in the respective transfected cells, their protein expression was undetectable. Failure to detect Snail protein could be explained by its tightly regulated stability and its very short half-life (Zhou et al., 2004). Accordingly, Snail could be detected upon ectopic expression after treating transfected cells with lithium chloride, a GSK3β inhibitor (Figure 18A). We thus decided to transfect a degradation-resistant mutant form of Snail. Indeed, in a study specifically characterizing the

degradation pathway of Snail, Zhou and colleagues created a mutated form of Snail (termed Snail-6SA (Zhou et al., 2004)) that is insensitive to GSK3β phosphorylation and therefore degradation-resistant. Ectopic expression of that Snail mutant, in MDA-MB-468 and A549, led to a detectable increase of Snail protein expression (Figure 18B). Using this model, we could show that ectopic expression of Snail is sufficient to increase mRNA expression of all factors of interest, reaching significance for IL-6 and PAI-1 in MDA-MB-468 cells and for IL-6 and IL-8 in A549 cells (Figure 18C-D).

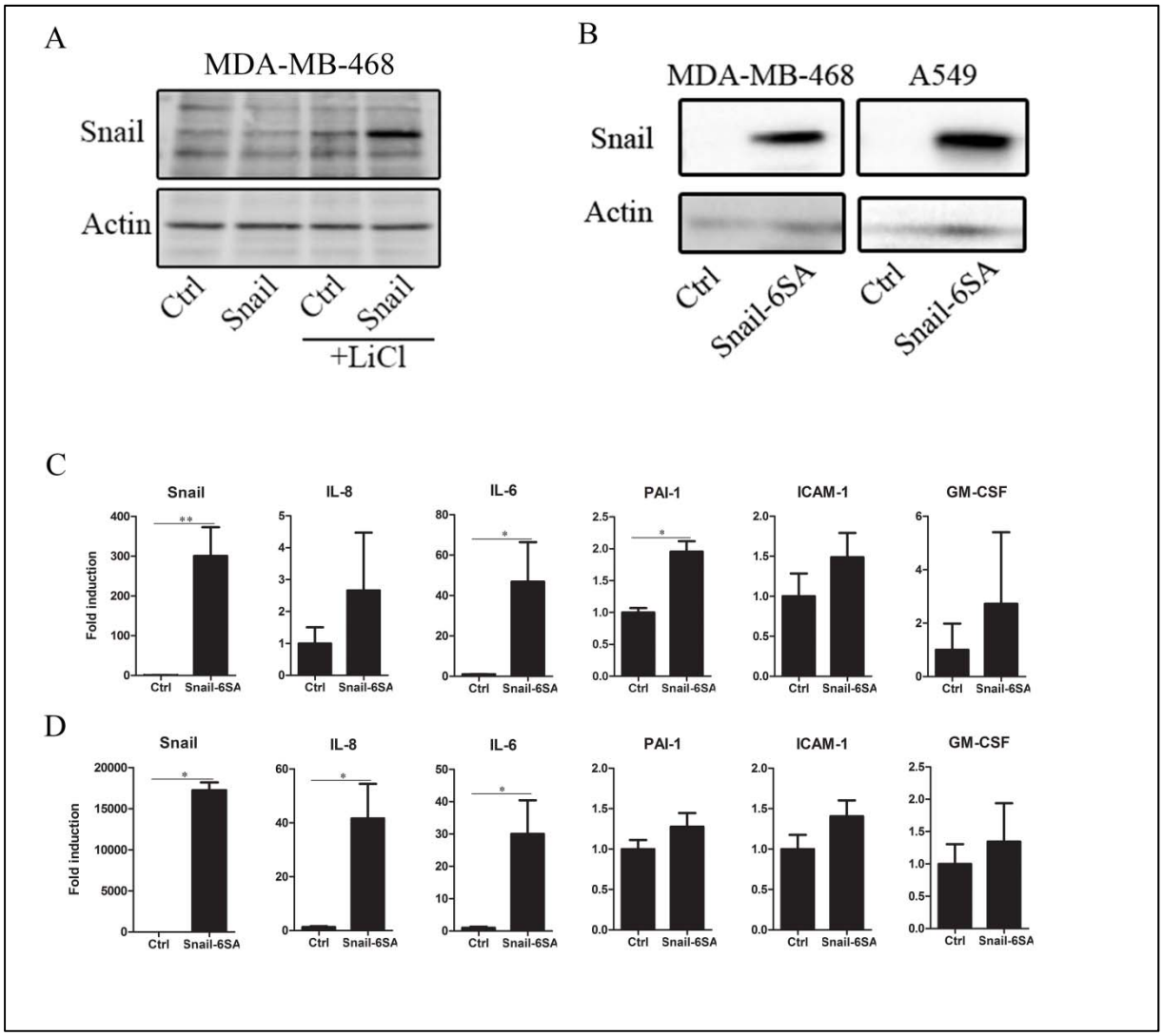


Figure 18: Regulation of soluble factors by ectopic expression of Snail EMT-TF. (A) Western blot analyzes of Snail in MDA-MB-468 transfected with a cDNA encoding Snail or with the corresponding empty vector (Ctrl), untreated or treated with lithium chloride (+LiCl) for four hours. (B) Western blot analyzes of Snail in MDA-MB-468 and A549 cells after transfection with a cDNA encoding the degradation-resistant mutant of Snail, Snail-6SA. (C-D) RT-qPCR analyzes of Snail, IL-8, IL-6, PAI-1, ICAM-1 and GM-CSF in (C) MDA-MB-468 and (D) A549 cells.

2.3 Discussion

Our findings indicating that EMT-associated cytokines are in part regulated by EMT-transcription factor Snail are in line with some published works. Ectopic expression of Snail in the canine renal MDCK (Madin-Darby canine kidney) cell line heightens IL-6 and IL-8 increase upon IL-1β stimulation (Lim et al., 2013) and Snail expression in head and neck carcinoma cells augments IL-6 and IL-8 expression (Lyons et al., 2008). Snail also regulates IL-8 in colorectal cancer cells (Hwang et al., 2011). Conversely, the functional inhibition of Snail by ectopic expression of a dominant negative form in breast cancer cells has been shown to decrease PAI-1 expression (Fabre-Guillevin et al., 2008). This apparently contrasts with our data showing no PAI-1 regulation after siRNA snail inhibition. Nevertheless, this study was performed using other cell systems and the particular signaling pathways involved in the modulation of a specific cytokine expression are indeed likely various and dependent on the cellular context. Other EMT-transcription factors such as Brachyury (Fernando et al., 2011) or Twist (Li et al., 2012a) have also been shown to promote IL-8 expression. Previous data of our laboratory have also shown an increased expression of IL-8 in breast cancer cell lines depending on EMT-induced ZO-1 delocalization from the membrane (Brysse et al., 2012).

Altogether, these results suggest a functional contribution of EMT pathways, and particularly of Snail, in the specific regulation of the five factors of interest, even though other transcription factors are most likely involved.

3 Conditioned medium from EMT-positive cells stimulate angiogenesis

3.1 Introduction

Based on the aforementioned results showing overexpression of IL-6, IL-8, PAI-1, ICAM-1 and GM-CSF upon EMT induction, we decided to investigate the functional consequences of such a combined overexpression of a set of soluble factors by cancer cells. Because IL-8 have a well described pro-angiogenic activity (Heidemann et al., 2003; Li et al., 2003) and because some data suggest an angiogenic function of sICAM-1 (Gho et al., 1999) and PAI-1 (Bajou et

al., 2004), we made the assumption that a combined overexpression of several pro-angiogenic cytokines should have a significant impact on angiogenesis.

3.2 Results

Conditioned media from control and EGF-treated MDA-MB-468 cells were tested for their chemotactic activity on primary endothelial cells (HUVEC) in a Transwell chemotaxis assay. Conditioned medium from EGF-treated cells significantly increased HUVEC cell migration compared to control cell-derived conditioned medium (Figure 19A). Any direct EGF-related effects were excluded by the extemporaneous addition of EGF, which did not affect HUVEC cell migration compared to conditioned medium from untreated cells (Figure 19A). To further characterize the involvement of each EMT-regulated soluble factor in HUVEC cell chemo-attraction, conditioned medium from EMT-positive cells was pre-incubated with a blocking antibody against each factor. IL-6 and PAI-1 appeared necessary for HUVEC cell migration (Figure 19B). Surprisingly, the blockade of IL-8 and sICAM-1, which are well-described angiogenic factors, did not impede HUVEC cell migration, though the efficiency of our blocking antibodies was validated using recombinant IL-8 and sICAM-1 proteins (Figure 19C-D). Taken together, these results suggest that IL-6 and PAI-1 are functional secreted mediators of the pro-angiogenic activity associated with EMT in our models.

Rat aortic ring assays were performed to assess the effect of conditioned mediums *ex vivo*. Computerized analysis of newly formed vessels sprouting from aortic rings cultured for 9 days with conditioned medium from either untreated or EGF-treated MDA-MB-468 cells revealed that the number of sprouting vessels is higher in the EGF condition. Indeed, in Figure 19F, the number of vessels detected at several distance points from the border of the aortic ring is plotted and the peak of the EGF condition is higher compared to the control condition. The area under the curve of Figure 19F, shown in Figure 19G, represents the total area occupied by spouting vessels, whatever the distance from the border of the aortic rings, which is higher in the EGF condition. Overall, the results show that conditioned medium from EGF-treated MDA-MB-468 significantly increases the *ex vivo* vessel sprouting (Figure

19E-G). Similarly to the sponge assay, EGF-related effects were excluded by the extemporaneous addition of EGF in the control conditioned medium (C+E).

To test the effect of conditioned medium from EMT-derived cells on angiogenesis *in vivo*, we used the gelfoam sponge assay, which is particularly well suited to study the impact of soluble compounds on the microenvironment. In this assay, sponges, previously soaked in concentrated conditioned medium of EMT-positive cells or EMT-negative cells and coated in diluted collagen, were subcutaneously inserted under the posterior epithelial sheet of C57BL/6 mice ears. Sponges were collected after 21 days to analyze angiogenesis. Blood vessels were detected by CD31 immunostaining (Figure 20A) and blood vessel density was analyzed by computer-assisted quantification as previously described (Lenoir et al., 2014).

Sponges soaked in conditioned medium from EGF-treated MDA-MB-468 were infiltrated by a significant higher number of blood vessels compared to sponges soaked into control conditioned medium (from untreated MDA-468) (Figure 20B). Direct EGF-related effects were excluded by the extemporaneous addition of EGF in control conditioned medium (Figure 20B). The experiments were reproduced in A549 cell line and yielded similar results (Figure 20C-D). EMT-induced cytokine secretion thus clearly associates with an enhanced angiogenic response.

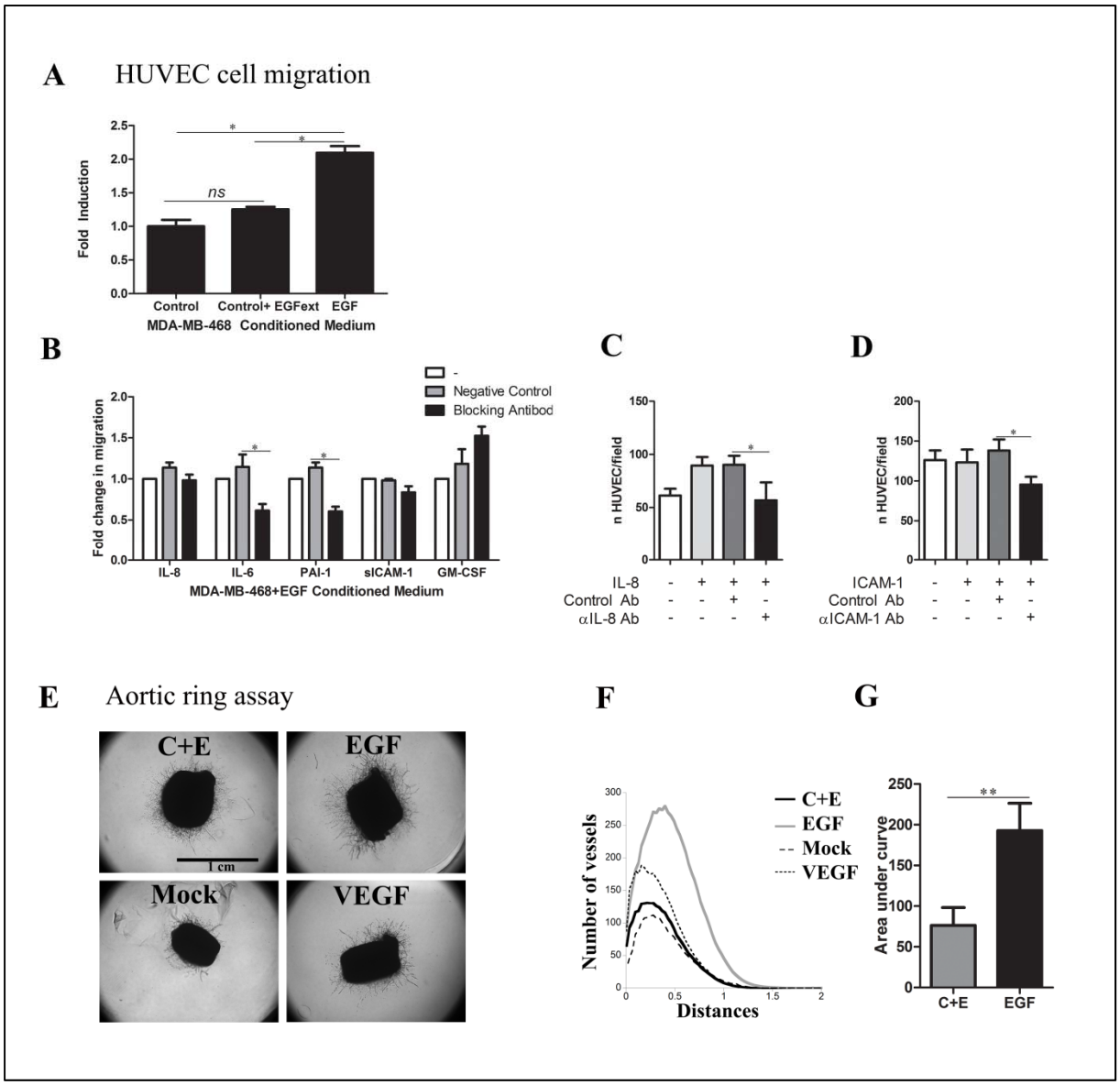


Figure 19: Effect of conditioned medium from EMT-derived cells on angiogenesis: *in vitro* and *ex vivo* assays. (A) HUVEC chemotaxis assay using conditioned medium from EGF-treated (EGF) or untreated (Control) MDA-MB-468. A condition using conditioned medium of untreated cells in which EGF was added extemporaneously (Control + EGF_{ext}) was performed to exclude any direct effect of EGF in HUVEC chemotaxis. (B) HUVEC chemotaxis assay using conditioned medium of EGF-treated MDA-MB-468 containing blocking antibodies for the factors of interest (IL-8, IL-6, PAI-1, sICAM-1 or GM-CSF) or the corresponding control antibodies (Negative control). (C-D) HUVEC chemotaxis assay using conditioned medium from untreated MDA-MB-468 with addition of recombinant (C) IL-8 or (D) sICAM-1 with or without (C) anti-IL-8 antibody (αIL-8 Ab) or control antibody (Control Ab) or with or without (D) anti-ICAM-1 antibody (αICAM-1) or control antibody (control Ab). (E) Microscopy images illustrating 9-day aortic rings accompanied by (F) results of the computer-assisted quantification of images shown in (E). (G) Area under the curve quantification graphs corresponding to analyzes shown in (F).

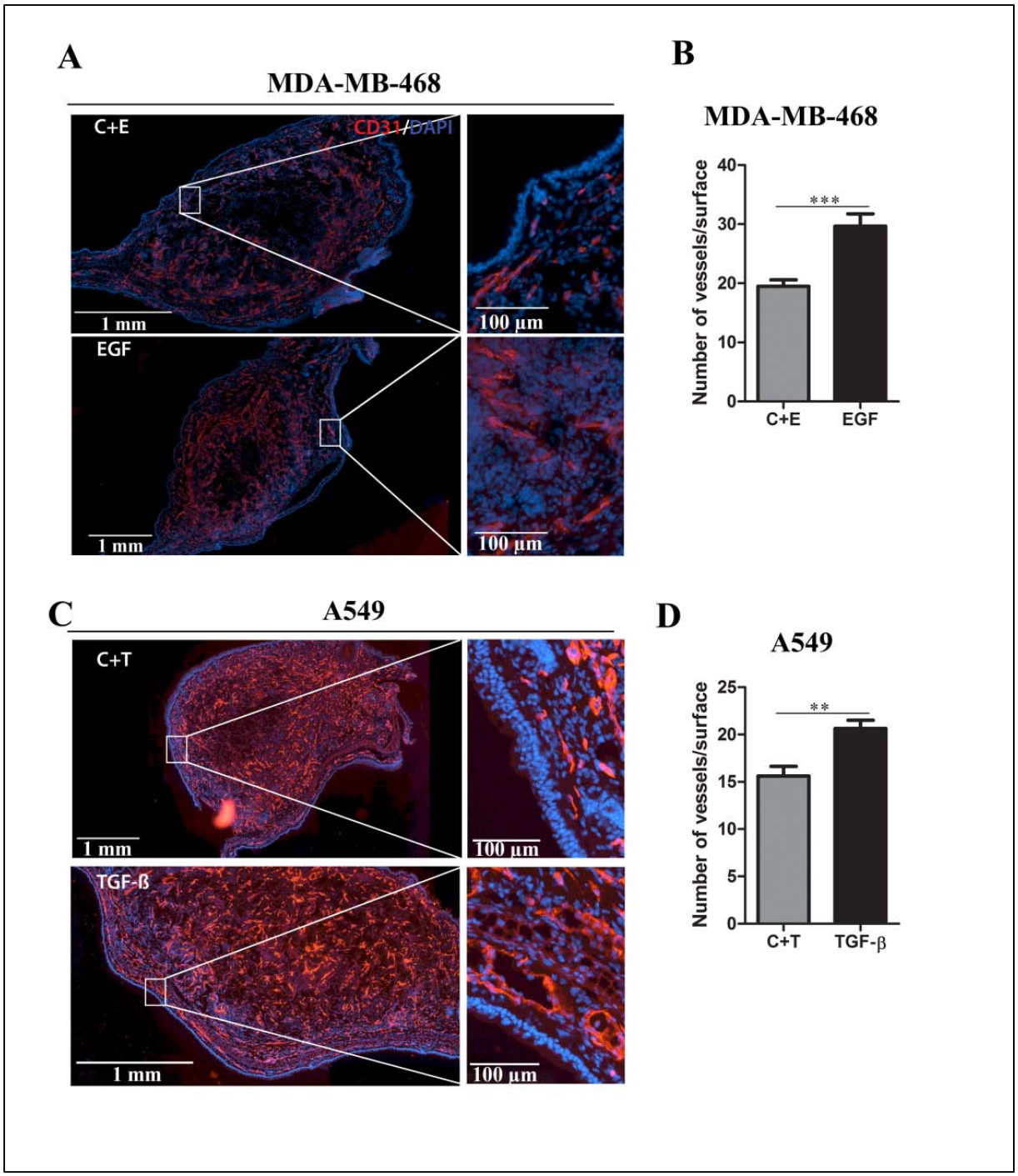


Figure 20: Effect of conditioned medium from EMT-derived cells on angiogenesis in the sponge assay. (A, C) Immunofluorescent staining for CD31 (in red, labeling blood vessels) on ear sections containing 3 week-sponges soaked in conditioned medium of (A) non-treated (C+E) or EGF-treated MDA-MB-468 (EGF) or (C) non-treated (C+T) or TGF-β-treated A549 (TGF-β). (A) EGF or (C) TGF-β was added to the control conditioned medium to exclude EGF/TGF-β-related effects. DAPI (in blue) is used to label nuclei. (B, D) Results of the computer-assisted quantification of the CD31 staining as shown in A or C representing blood vessel density. (B) Pooled results of three independent mice experiments leading to n = 14 (C+E) and n= 15 (EGF) are shown for MDA-468. (D) Pooled results of two independent experiments leading to n=10 (C+T) and n=11 (TGF-β) are shown for A-549.

The sponge assay model has been successfully developed in our laboratory to study lymphatic vessel recruitment (Lenoir et al., 2014). We thus took this opportunity to study the potential infiltration of the sponge by lymphatic vessels in response of conditioned medium from EMT-induced or EMT-negative MDA-468. Infiltration by lymphatic vessels was limited compared to infiltration by blood vessels and no statistical difference could be found between EMT-positive and negative conditions (Figure 21).

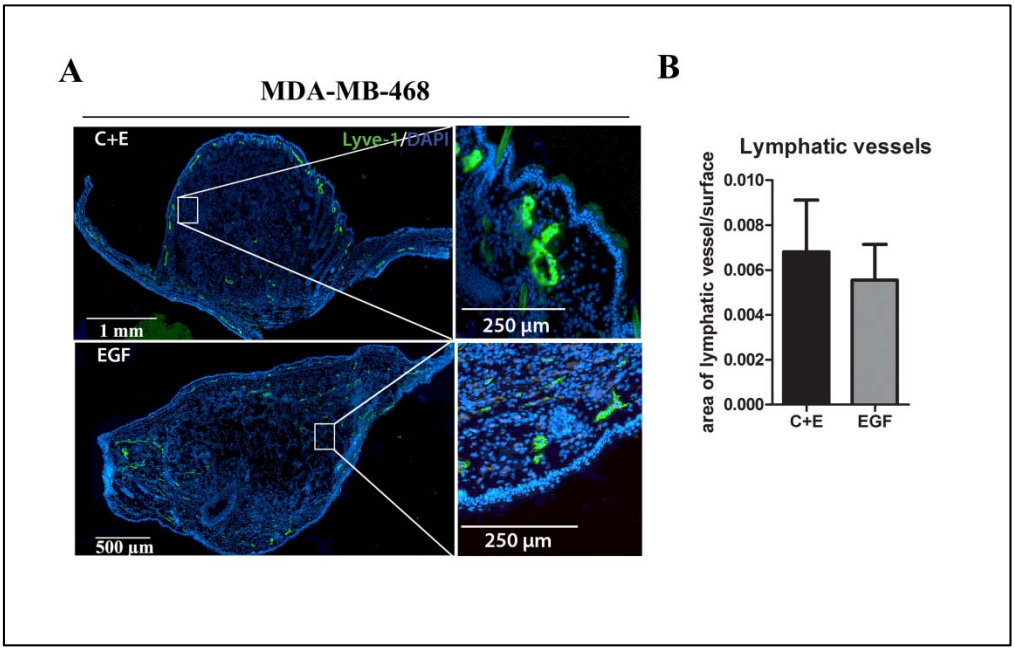


Figure 21: Effect of conditioned medium from EMT-derived cells on lymphangiogenesis. (A) Microscopy images illustrating LYVE-1 staining (in green, labelling lymphatic vessels) on ear sections containing 3 week-sponges soaked in conditioned medium from untreated (C+E) or EGF-treated (EGF) MDA-MB-468 cells. Nuclei are stained in blue with DAPI. EGF was added to the control condition to exclude any EGF-related effects. (B) Results of the computer-assisted quantification of the LYVE-1 staining as shown in (A). Results of one experiment with n=5 (C+E) and n=6 (EGF) are shown.

3.3 Discussion

We here demonstrate that conditioned medium from EMT-positive cells (EGF-treated MDA-MB-468 and TGF-β-treated A549) significantly increased angiogenesis *in vivo* in the sponge assay. Additionally, conditioned medium from EMT-positive MDA-MB-468 stimulated vessel sprouting from rat aortic rings *ex vivo* and enhanced HUVEC cell migration *in vitro* in a PAI-1 and IL-6-dependent way. Surprisingly, IL-8 and sICAM-1 inhibition with blocking antibodies did not affect EMT-induced HUVEC cell chemo-attraction, despite their well

described pro-angiogenic action (Gho et al., 1999; Heidemann et al., 2003; Koch et al., 1992). We do not preclude any effect of IL-8 and ICAM-1 in chemo-attracting endothelial cells but we hypothesize that any effect of IL-8 and ICAM-1 depletion on HUVEC cell recruitment could be masked by the high levels of IL-6 and PAI-1 present in the conditioned medium from EGF-treated MDA-MB-468 in our model. Our results thus rather involve PAI-1 and IL-6 as EMT-induced mediators of HUVEC cell migration and are supported by several lines of evidence implicating IL-6 in angiogenesis. Indeed, Nilsson and colleagues have shown that recombinant IL-6 stimulates angiogenesis in the sponge assay (Nilsson et al., 2005). Furthermore, recombinant IL-6 has been shown to stimulate HUVEC cell proliferation, tubule formation and vessel sprouting in aortic ring assays (Hernandez-Rodriguez et al., 2003). Finally, fibroblast-derived IL-6 has recently been shown to promote angiogenesis in colorectal tumor xenografts (Nagasaki et al., 2014). In this latest study, IL-6 is shown to increase angiogenesis in colorectal carcinoma in mice by stimulating VEGF production by fibroblasts. Our results using IL-6 blocking antibody in the HUVEC migration assay suggest that IL-6 is additionally able to directly chemo-attract endothelial cells. PAI-1 has a much less well defined effect on angiogenesis. Data, including those of our laboratory, suggest that PAI-1 contextually either stimulates or inhibits angiogenesis, as described in the introduction of this manuscript (Bajou et al., 2014; Bajou et al., 2004; Bajou et al., 1998).

Taken together, our results demonstrate that conditioned medium from EMT-positive cells is angiogenic and identify IL-6 and PAI-1 as major mediators of EMT-associated angiogenesis.

4 Conditioned medium from EMT-positive cells recruits myeloid cells

4.1 Introduction

We took advantage of the above-detailed sponge model which, if sponges are collected at earlier time points, is well suited to characterize the recruitment of inflammatory cells.

Several pieces of evidence prompted us to first analyze macrophages. Macrophages are indeed the most represented inflammatory cell type infiltrating breast tumors and play a

pleiotropic and decisive role in metastasis. They indeed promote angiogenesis, tumor cell survival, migration and invasion and elaborate an immunosuppressive microenvironment (Pollard, 2004; Qian and Pollard, 2010). More specifically suggesting a link between tumor-associated macrophages (TAMs) and EMT, data have shown that TAMs accumulate in hypoxic regions where they can promote cancer cell invasion. TAMs secrete EMT-inducing factors such as TGF-β and IL-6 (Wynn et al., 2013). Accordingly, in non-small cell lung carcinoma samples from patients, EMT features are associated with TGF-β and tumor infiltration by macrophages (Bonde et al., 2012). *In vitro*, prolonged culture of murine epithelial cell lines with conditioned medium from macrophages led to EMT-like changes, as evaluated by decrease in E-cadherin, activation of β-catenin pathway and increased invasive properties. TGF-β was further identified as the key EMT-inducer in this conditioned medium (Bonde et al., 2012).

Increasing evidence also link several EMT-regulated soluble factors to another cell type well known for its accumulation in cancer called MDSCs. IL-6 and GM-CSF are indeed used for *in vitro* generation of MDSCs from hematopoietic stem cells isolated from murine bone marrow. Also, clinical trials have shown that high levels of tumor-derived GM-CSF are associated with an infiltration of the tumor by MDSCs (Morales et al., 2010; Serafini et al., 2004). Finally, in mice expressing hIL-8 under the control of its human promoter, inflammatory and neoplastic sites are infiltrated by a higher number of MDSCs (Asfaha et al., 2013).

4.2 Results

A preliminary experiment of sponge dissociation followed by FACS analysis was performed on sponges soaked in the conditioned medium of either untreated (control) or EGF-treated MDA-MB-468 cells. EGF was extemporaneously added to the control conditioned medium to exclude any EGF-mediated effect. This enabled us to roughly characterize the infiltration of the sponge by macrophages and myeloid cells after three days. F4/80+ macrophages infiltrating the sponge were relatively scarce (13.7%) (Figure 22A). However, FACS staining revealed that the most abundant cell population in the sponge was a myeloid $CD11b^{+}GR1^{high}$ (91%) (Figure 22B). Ly6G and Ly6C stainings revealed that the

most abundant population infiltrating the sponge was the granulocytic $CD11b^{+}Ly6G^{high}Ly6C^{low}$ cells (76%) rather than the monocytic $CD11b^{+}Ly6G^{-}Ly6C^{high}$ cells (5.9%) (Figure 22B).

Because the dissociation technique of the sponges did not reach an acceptable level of reproducibility to perform quantitative analyzes, we performed immunofluorescent stainings on frozen section realized in the sponges to compare further the infiltration of this myeloid population in EMT-negative and EMT-positive groups. Supporting the FACS results, these immunostaining results showed a high infiltration by $CD11b^{+}GR1^{+}$ myeloid cells around the sponge (Figure 22C). Moreover, sponges soaked into conditioned medium from EGF-treated MDA-MB-468 cells contained a higher relative $CD11b^{+}Gr1^{+}$ hotspot surface compared to control condition (Figure 22D). Suspecting a recruitment of these cells from the bone marrow, we correspondingly showed by FACS that mice bearing EMT-positive sponges contained a significantly decreased proportion of $CD11b^{+}Ly6G^{high}Ly6C^{low}$ cells in their bone marrow compared to the control mice (Figure 22F). Proportions of $CD11bLy6C^{high}Ly6G^{-}$ were unchanged in the bone marrow (Figure 22F).

4.3 Discussion

Using the sponge assay model, we were able to show that $CD11b^{+}Gr1^{+}$ myeloid cells represent an important population of cells attracted by conditioned medium from EMT-derived cells. Immunofluorescent staining indeed showed that sponges soaked in conditioned medium from EMT-positive cells were invaded by more $CD11b^{+}GR1^{+}$ cells compared to control sponges. More specifically, FACS analysis revealed that EMT-positive sponges are infiltrated by an elevated ratio of granulocytic/monocytic MDSC. Accordingly, FACS analysis of bone marrows of sponge-bearing mice showed that proportions of $CD11b^{+}Ly6G^{hi}$ cells were decreased in the EMT-positive group, whereas $CD11b^{+}Ly6C^{hi}$ cell proportion was unchanged. These data suggest that conditioned medium from EMT-positive cells is able to mobilize granulocytic MDSCs from the bone marrow.

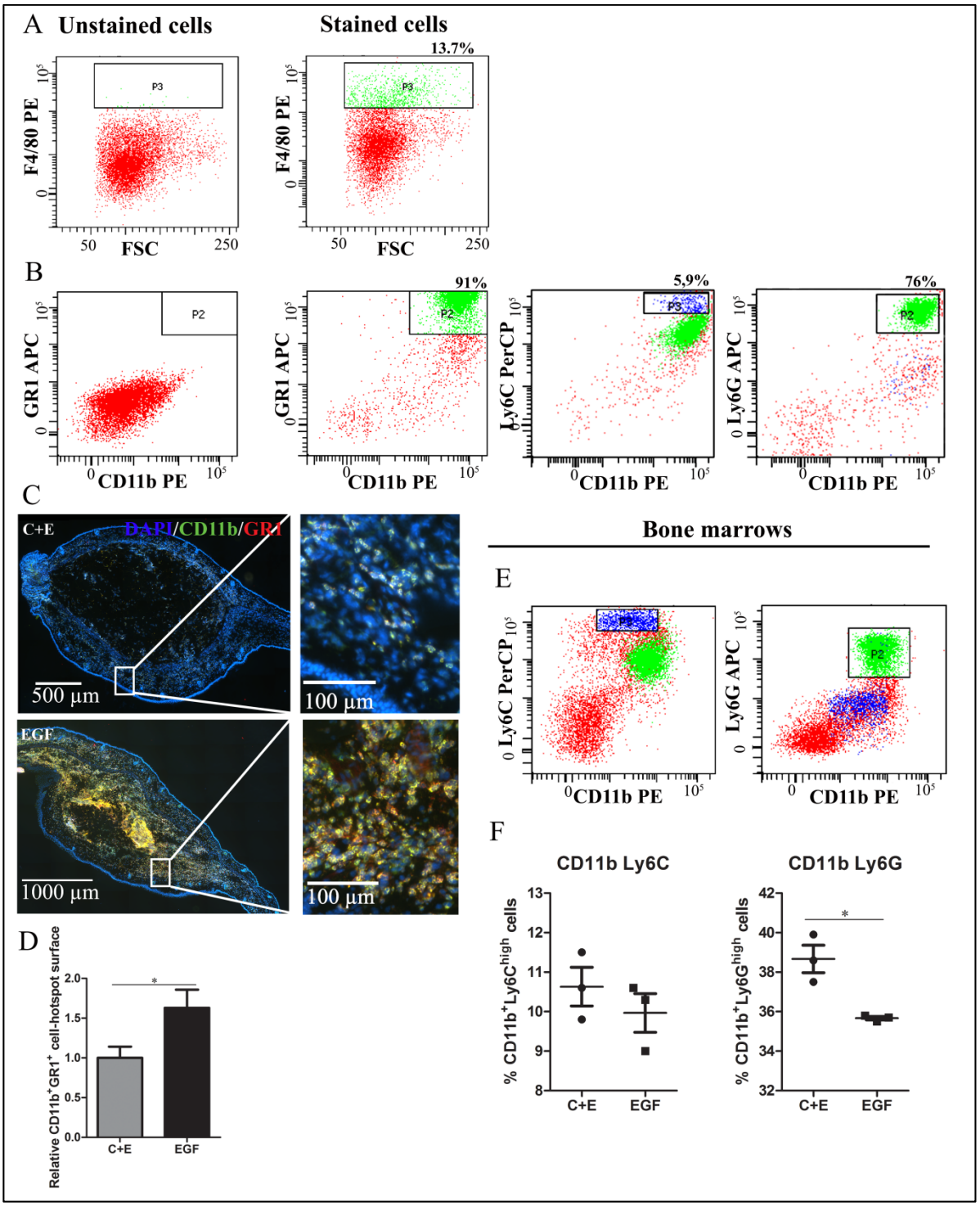


Figure 22: Effect of conditioned medium of EMT-derived cells on inflammatory cell recruitment. (A-B) FACS analyzes on immunolabelling for (A) F4/80, (B) CD11b, GR1, Ly6G or Ly6C on cells collected from 3 day-sponges soaked in conditioned medium from EGF-treated MDA-MB-468 cells (EGF). (C) Microscopy images illustrating staining for CD11b and GR1 on ear sections containing 3 day-sponges soaked in conditioned medium from non-treated (C+E) or EGF-treated MDA-MB-468 cells (EGF). EGF was added to the control conditioned medium to exclude any EGF-related effects. Nuclei are stained in blue with DAPI. (D) Results of the quantification of the relative $CD11b^{+}GR1^{+}$ cell hotspot surface on the staining shown in (C). A pool of two independent experiments leading to n=11 (C+E) and n=13 (EGF), quantified by two independent observers in double-blind settings is

shown. (E) FACS analyzes of CD11b, Ly6G and Ly6C staining on bone marrows collected from mice bearing 3 day-sponges as described in (A-B). (F) Quantifications by FACS of CD11b, Ly6C and Ly6G staining on cells collected from bone marrow of sponge-bearing mice as shown in (E). Graphs illustrate the proportions of $CD11b^{+}Ly6C^{hi}$ (left) and $CD11b^{+}Ly6G^{hi}$ (right) cells in the bone marrow. Each dot represents the pooled analysis of two mice.

Our results are supported by evidence linking some of the identified EMT-regulated soluble factors, namely IL-6, GM-CSF and IL-8 to MDSC biology as explained in the introduction of this section (Asfaha et al., 2013; Morales et al., 2010). Furthermore, two independent studies suggest that MDSCs can be recruited to tumors in a CXCR2-dependent mechanism, in a murine model of chronic inflammation-induced colorectal cancer (Katoh et al., 2013) and in a transgenic murine model of melanoma (Toh et al., 2011).

There is however no evidence attributing a direct causative role to EMT for the recruitment of MDSC to our knowledge but conversely, several works suggest that induction of EMT might be triggered by MDSCs. For example, Toh and colleagues have shown a functional role for MDSCs in EMT induction in a murine model of metastatic melanoma (the RETAAD mice, in which the RET oncogene is specifically expressed in skin and eye melanocytes, that develop uveal melanomas with skin metastases) (Toh et al., 2011). In that model, MDSCs preferentially infiltrate primary tumors rather than metastases. The depletion of granulocytic MDSCs by treatment with an anti-Ly6G antibody reduces expression of two EMT-markers in the primary tumor: FSP1 (Fibroblast-specific protein 1) and vimentin. Supportively, the co-culture of either dissociated primary tumors or NTBII (a cancer cell line inducible for EMT) with MDSCs triggered EMT-like changes in the cancer cells, as evaluated by morphological changes, decreased E-cadherin expression and increased migration (Toh et al., 2011). Recently, another study addressed the effect of MDSCs on canine mammary cancer cells (Mucha et al., 2014). Co-culture experiments between mammary cancer cells and MDSCs triggered EMT-like changes in cancer cells, such as decreased cytokeratin and increased vimentin expression, and increased invasive properties. Because IL-28/IL-28RA are increased in supernatants from the co-culture, along with phosphorylated STAT3 in cancer cells, and because IL-28 knockdown in MDSCs dampens the EMT-inducing effect of MDSCs on cancer cells, the authors suggest IL-28/IL-28RA/STAT3 signaling is responsible for MDSC-mediated EMT-induction in that model. A third study addressed the potential pro-EMT effect of MDSCs in pancreatic cancer (Panni et al., 2014). After co-culture of human pancreatic cell

lines with monocytic MDSCs, tumor cells exhibited EMT features such as decreased E-cadherin, increased vimentin and enhanced invasion in Matrigel, while co-culture of cancer cells with granulocytic MDSCs had no effect. Cancer cells co-cultured with Mo-MDSCs and subsequently injected subcutaneously in mice displayed enhanced tumor initiated capacity and increased mRNA expression of EMT-TFs (Slug, Twist, ZEEB1) and CSC markers (Nanog and Oct4).

Taken together and accordingly to the increasing evidence suggesting a functional role for MDSCs in EMT induction *in vivo* as detailed above, our results suggest that EMT-regulated soluble factors recruit MDSCs *in vivo*, thereby initiating a double-regulatory loop in which MDSC infiltration to the tumor and the presence of EMT features in cancer cells are mutually sustained.

5 The presence of EMT features relates to increased angiogenesis and tumor infiltration by myeloid cells in human triple-negative breast cancers

5.1 Introduction

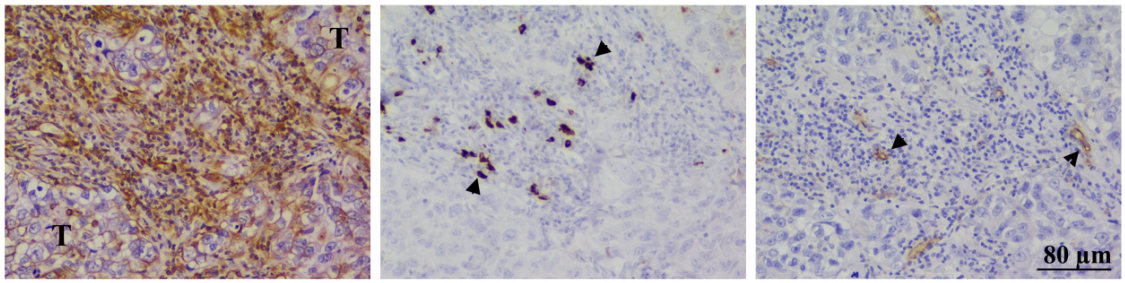
Based on our results obtained with the mouse ear sponge assay, we concluded that conditioned medium from EMT-derived cells promotes angiogenesis and myeloid cell recruitment *in vivo*. To address the clinical relevance of our observations, we examined the potential association between EMT features, angiogenesis and myeloid cell infiltration in a collection of 40 TNBCs in collaboration with Professors Myriam Polette and Philippe Birembaut from Laboratory Paul Bouin (Reims, France). The triple-negative histological subtype was chosen because of the well-described enrichment in EMT features in these cancers compared to the other breast cancer types (Sethi et al., 2011).

5.2 Results

Forty specimens of TNBC were stained on serial sections for vimentin (for detection of EMT-derived cancer cells), CD33 (for myeloid cells) and CD105 (for angiogenesis) (Figure 23A). CD33 is a marker expressed at the surface of all cells from the myeloid lineage. CD105 (endoglin), a TGF-β receptor, is expressed at the surface of "activated" endothelial cells as illustrated by its reactivity for vessels inside tumors, around skin lesions or in infarcted areas after a stroke and is a widely accepted marker for active angiogenesis (Kumar et al., 1996).

The results of immunohistochemical detection were scored independently by two pathologists. When a discrepancy was found between the two investigators, a consensus was reached via a simultaneous examination using a double-headed microscope. For vimentin, staining extent was graded into 5 categories as follows: 0 = no detection, 1 = detection in <10% of tumor cells, 2 = detection in 10-25% of tumor cells, 3 = detection in 26-50% of tumor cells, 4 = detection in 51-75% of tumor cells, 5 = detection > in 76% of tumor cells. For CD33 and CD105, staining extent was graded into 4 categories as follows: 0 = no expression, 1 = low expression, 2 = moderate expression and 3 = high expression. Vimentin expression was divided to 0-1 or more and CD33 and CD105 were managed as either 0-1 (low expression) or 2-3-4 (high expression) in analyzes.

A



B

Variables	Vim ⩽ 10 % n=20	Vim > 10% n=20	*p*
CD33 expression			
Low	16 (64%)	9 (36%)	**0.0484**
High	4 (27%)	11 (73%)	
CD105 expression			
Low	17 (71%)	7 (29%)	**0.0031**
High	3 (19%)	13 (81%)	

Figure 23: Expression of EMT features, myeloid and angiogenesis markers in a bank of TNBC. (A) Illustrative microscopy images of vimentin, CD105 and CD33 immunostainings on serial sections in a bank of TNBC. T = tumor cluster. Arrows point to myeloid cells (CD33 staining) or blood vessels (CD105 staining). (B) Contingency table illustrating the association between EMT features, tumor infiltration by myeloid cells and tumor angiogenesis in a bank of 40 TNBC. Statistical test: Fisher's exact test.

Accordingly to the documented enrichment of EMT features in triple-negative breast cancers, 50% of the samples (20/40) contained EMT features, as evaluated by expression of vimentin by at least 10% of tumor cells in the sample (Figure 23B). Most interestingly, the presence of EMT markers was associated with increased density of CD105-positive blood vessels, compared to tumors containing less than 10% of vimentin-positive cancer cells (Figure 23B). Likewise and corroborating our findings suggesting an EMT-mediated recruitment of MDSCs, the presence of EMT markers in TNBCs was associated with increased tumor infiltration by CD33-positive myeloid cells (Figure 23B).

5.3 Discussion

Emphasizing the clinical relevance of our observations made *in vitro* and in animal models suggesting a functional effect of conditioned mediums from EMT-derived cancer cells on angiogenesis and myeloid cell recruitment, we here establish the association

between the presence of EMT features in a bank of 40 TNBCs, enhanced angiogenesis and myeloid cell infiltration.

Accordingly, several clinical observations associate the triple-negative subtype of breast cancer with a pro-angiogenic signature (Bernardi and Gianni, 2014). TNBCs express higher levels of pro-angiogenic factors such as VEGF, Angptl4 (Angiopoietin-like 4) or semaphorins (Bender and Mac Gabhann, 2013; Linderholm et al., 2009; Yotsumoto et al., 2013) and are characterized by a higher microvessel density (Mohammed et al., 2011) compared to non-TNBCs.

Our results are most pertinently supported by two independent studies linking EMT and angiogenesis in TNBCs. On one hand, CD146, an inducer of EMT and angiogenesis, was recently found associated to TNBC (Zeng et al., 2012). On the other hand, aiming at subdividing TNBCs into subtypes based on genomic analysis, Lehmann and colleagues have recently identified a subtype called mesenchymal stem like, in which expression of EMT-related genes is high. The mesenchymal stem like subtype is also particularly enriched in genes involved in angiogenesis (Lehmann et al., 2011).

Also supporting our results showing infiltration of myeloid cells in TNBCs, a recent study particularly showed a link between high HIF-1α in TNBCs and the recruitment of monocytic MDSC (Chaturvedi et al., 2014). However, there is no data linking MDSC recruitment to EMT features in TNBCs to our knowledge, even though MDSCs are well known to accumulate in late stage breast cancer (Markowitz et al., 2013). There is nevertheless a documented association between MDSC infiltration of tumors and EMT induction in animal models, as described in the previous section (paragraph 4.3) (Toh et al., 2011).

Our data thus suggest the existence of a sub-group of TNBC displaying EMT features with enhanced angiogenesis and myeloid cell infiltration.

Discussion and perspectives

Many studies in the past two decades have addressed the question of the "microenvironmental regulation of metastasis" (Joyce and Pollard, 2009). The tumor microenvironment is composed of a heterogeneous population of cancer cells and of a host compartment collectively called the tumor stroma and including vascular cells, fibroblasts, leukocytes and the extracellular matrix. Cancer cells are able to secrete high amounts of soluble factors such as cytokines, chemokines and growth factors, which act locally and systemically to recruit stromal cells. These become a second source of soluble factors, fuelling the described 'cytokine storm' observed in cancer (Joyce and Pollard, 2009). The interplay between the varieties of cells in the tumor microenvironment enables the acquisition by some cancer cells of pro-metastatic properties such as EMT, the sustained activation of angiogenesis or the evasion of the host immune system. Accordingly, overexpression of cytokines, chemokines, growth factors and their respective receptors by cancer cells have been described and initiate a crosstalk between cancer cells and stromal cells that might play a key role in promoting metastatic dissemination (Kudo-Saito et al., 2013; Lazennec and Richmond, 2010; Mestdagt et al., 2006; Singh et al., 2007; Soria and Ben-Baruch, 2008; Su et al., 2014). Nevertheless, the mechanisms of such an overexpression of soluble factors by cancer cells are not well understood.

While it is now clearly established that tumor cells are heterogeneous, the impact of such heterogeneity on the tumor stroma is unclear. During this work, we examined the possibility that EMT-derived pro-invasive subpopulations of cells might differently affect the tumor stroma and establish a specific microenvironment that could favor metastatic spread. We more particularly hypothesized that EMT might affect the secretion profile of cancer cells and thereby impact the tumor-stroma crosstalk.

Confirming our hypothesis, we have established that a panel of soluble factors, i.e. IL-8, IL-6, PAI-1, sICAM-1 and GM-CSF, are consistently upregulated in cell lines either induced for EMT following growth factor treatment or displaying endogenous EMT features. These factors are in part regulated by EMT-TF Snail and some of them (IL-8, IL-6 and PAI-1) are

enriched in EMT-positive areas of tumors formed in mice with human cancer cells. Our results, pertinently supported by several published works that were discussed in the specific discussions in the "results" section (Bates et al., 2004; Freytag et al., 2010; Lyons et al., 2008; Palena et al., 2012), validate these soluble factors as target genes of EMT.

We further demonstrate a functional consequence of EMT-induced soluble factor expression on the tumor stroma. Indeed, conditioned medium from EMT-derived cells promotes angiogenesis *in vivo* (in the mouse sponge assay) and *ex vivo* (in the rat aortic ring assay), and furthermore stimulates the chemotactic migration of primary endothelial cells in an IL-6 and PAI-1-dependent mechanism. Our data are in accordance with published works suggesting pro-angiogenic effects of EMT (Fantozzi et al., 2014) and specifically of IL-6 (Middleton et al., 2014) and PAI-1 (Bajou et al., 2004). Moreover, the association between EMT and tumor angiogenesis that we observed in a series of forty TNBCs uniquely demonstrates the clinical relevance of our observations.

We also collected some data suggesting a potential impact of EMT on myeloid cell recruitment. In the bank of forty TNBCs mentioned above, we found an association between the presence of EMT features and tumor infiltration by CD33-positive myeloid cells. Although CD33 is not a specific marker for MDSCs but stains all cells from the myeloid lineage, including neutrophils, we suspected an association between EMT features and MDSC recruitment. Accordingly, in our mouse ear sponge model, conditioned medium from EMT-derived cells stimulates recruitment of myeloid cells with markers of MDSCs, and more specifically of granulocytic MDSCs ($CD11b^{+}$ $Ly6G^{high}$), even though a better characterization needs to be performed (see the perspectives of the work below).

The impact of EMT on MDSC recruitment is an unexploited promising field. Indeed, levels of MDSCs in the blood stream are correlated positively with tumor stage, numbers of CTCs and metastatic burden, and negatively with overall survival in breast cancer patients (Cole et al., 2010; Diaz-Montero et al., 2009). The association between MDSCs and metastatic progression would essentially be caused by MDSC immunosuppressive activity (Gabrilovich and Nagaraj, 2009). However, while MDSC accumulation in primary tumors in cancer patients and in mice has raised much interest during the past few years, the mechanisms of MDSC recruitment to tumor sites are still unclear (Cole et al., 2010; Diaz-

Montero et al., 2009; Katoh et al., 2013; Maenhout et al., 2014; Solito et al., 2014). Some preclinical studies in mice suggest that MDSC recruitment to the tumor might involve CXCR2 expression by MDSCs and expression of its ligands by cancer cells (Katoh et al., 2013; Toh et al., 2011).

Interestingly, a growing number of works have suggested a link between MDSCs and angiogenesis. For instance, when co-injected with tumor cells in mice, MDSCs promote vascularization, in a TGF-β-dependent way (Gabrilovich and Nagaraj, 2009). In another murine model, MDSCs promote angiogenesis through the release of the pro-angiogenic metalloproteinase MMP9 (Yang et al., 2004b). Furthermore, MDSCs have been shown to be able to incorporate vascular wall in a process called "vascular mimicry" (Coussens and Pollard, 2011; Yang et al., 2008). Finally, immunotherapy combined with low normalizing doses anti-VEGFR2 monoclonal antibody decreases tumor infiltration by MDSCs and increases survival in breast cancer-bearing mice (Huang et al., 2012). Conversely, tumor-related aberrant vasculature has been described to promote MDSC accumulation (see introduction paragraph 3.1.4 on anti-angiogenic treatments).

In light of our results, it is tempting to speculate that EMT-regulated soluble factors might, through the promotion of both angiogenesis and MDSC recruitment, elaborate a permissive milieu for cancer spread, even though further research is required.

Therefore, our immediate line of research will aim at confirming the preliminary results concerning myeloid cells obtained in mouse models. The lack of specific markers for MDSCs indeed creates a need for functional characterization of any cell type presenting markers of MDSCs. In order to better characterize $CD11b^{+}Ly6G^{hi}$ cells infiltrating the sponge, sponge dissociation, sorting of $CD11b^{+}Ly6G^{hi}$ cells has been tried but any subsequent cell culture is precluded, because of the relatively low amount of collected cells in a sponge. However, staining for functional markers of MDSC immunosuppressive activity, such as arginase, which is highly expressed by granulocytic MDSCs (Gabrilovich and Nagaraj, 2009), would be feasible. We also plan to isolate *bona fide* MDSCs from mouse bone marrow using MACS (magnetic activated cell sorting) and test for their capacity to inhibit T cell proliferation and/or activity. Culture of these MDSCs with conditioned medium from EMT-positive or

negative cells should allow us to know whether EMT-derived soluble factors are able to activate MDSC immunosuppressive function. The question of chemotactic activity of EMT-soluble factors on MDSCs could also be addressed by chemotaxis assay on MDSCs isolated from bone marrows.

Moreover, the potential impact of EMT-regulated soluble factors on metastatic spread needs to be examined. For this, we would like to exploit murine models in which human tumor cells are subcutaneously injected. We have very recently received in the laboratory the MCF7 cell line stably expressing Snail under a Doxycycline-inducible promoter. We have observed an robust increase in soluble factors upon Snail induction and we would like to inject these cells in SCID mice, in order to observe on one hand the angiogenic activity and MDSC recruitment to the tumor, and on the other hand the effect of Snail-induced soluble factor expression on metastatic spread, as evaluated by CTC detection and lung metastatic burden.

In parallel, the PyMT mouse model will be exploited to study the expression of Snail and soluble factors by cancer cells. PyMT mice are genetically engineered so as to express the oncogenic Middle T antigen of the Polyoma virus under the control of the prolactin promoter and they consequently develop mammary adenocarcinomas. In these mice, EMT-like changes characterized by expression of vimentin by cancer cells have been observed, intensify with time and correlate with the metastatic load (unpublished observations). In these tumors, we plan to microdissect the epithelial compartment to measure soluble factor expression and stain for by blood vessels and myeloid cells, in order to know whether they all increase in time during metastatic spread.

In a second line of *in vivo* experiments, it would be most interesting to block angiogenesis and to deplete mice bearing MCF7/Snail cell-formed tumors in MDSCs (as can be done by treating them with a blocking anti-Ly6G antibody) in order to evaluate whether the impact of Snail on metastatic spread can be reverted. Similarly, blocking angiogenesis and MDSC recruitment to the tumor could also be tested on the PyMT model.

Finally, our results on the bank of TNBCs, though requiring further confirmation, suggest that there exists a subgroup consisting of approximatively 50% of TNBCs that displays EMT features, increased angiogenesis and abundant myeloid infiltration of the tumor and might

therefore be more aggressive. This identified subgroup of TNBCs might correspond to or overlap with the mesenchymal stem like subtype of breast cancer, categorized based on genetic analysis and in which expression of EMT and angiogenesis-related genes is high (Lehmann et al., 2011). This group could also overlap with the claudin-low subtype of TNBCs, which, similarly to the mesenchymal stem like, contains a substantial proportion of EMT-positive cancer cells and elevated angiogenic markers (Prat et al., 2010).

Therefore, in perspective to our present results, it would be mostly interesting to better characterize the subgroup of TNBCs that we identified, in order to establish whether the features we observed in the primary tumors are translated in clinics by a more aggressive subgroup, characterized by a higher grade or stage, a specific metastasis burden, or worse patient outcome. We also would like to correlate the presence of EMT features in primary tumors with the levels of circulating MDSCs, as accurately defined by FACS analysis as a $CD11b^{+}CD33^{+}HLA\text{-}DR^{-}$ $lineage^{-}$ (i.e. $CD3^{-}$, $CD19^{-}$, $CD14^{-}$ and $CD57^{-}$) population (Diaz-Montero et al., 2009), and, since pro-metastatic activities of MDSCs are linked to their immunosuppressive function, we wish to evaluate the antitumor immune response in our samples, with the study of immune cell (T lymphocytes and natural killer) infiltration and activity in the primary tumors.

Taken together, our research works are part of a more global research line aiming at better deciphering breast cancer heterogeneity in order to come up with new treatment strategies. Teasing out specific subgroups and characterizing them seems especially important to select the most appropriate treatment for each patient. Moreover, identifying subgroup-specific targetable factors – such as soluble factors, which are targetable with small molecule inhibitors or blocking antibodies – related to features of aggressiveness such as angiogenesis or immunosuppression appears to us important to develop treatment options for cases that are resistant to currently available drugs.

Linked publication

Epithelial-to-mesenchymal transition (EMT)-regulated soluble factors mediate tumor angiogenesis and myeloid cell recruitment

Journal of Pathology (under revision)

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Short running title: EMT-regulated angiogenesis and myeloid cell recruitment.

The authors have no conflict of interest to declare.

Word count for main text: 3999 words.

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Abstract

Epithelial-to-mesenchymal transition (EMT) programs provide cancer cells with invasive and survival capacities that might favor metastatic dissemination. Whilst signaling cascades triggering EMT have been extensively studied, the impact of EMT on the crosstalk between tumor cells and the tumor microenvironment remains elusive. We here aimed at identifying EMT-regulated soluble factors that could facilitate the recruitment of host cells in the tumor. Our findings indicate that EMT phenotypes relate to the induction of a panel of secreted mediators, namely IL-8, IL-6, sICAM-1, PAI-1 and GM-CSF and implicate the EMT-transcription factor Snail as a regulator of this process. We further show that EMT-derived soluble factors are pro-angiogenic *in vivo* (in the mouse ear sponge assay), *ex vivo* (in the rat aortic ring assay) and *in vitro* (in a chemotaxis assay). Additionally, conditioned medium from EMT-positive cells stimulates the recruitment of myeloid cells. Most interestingly, in a bank of 40 triple-negative breast cancers, tumors presenting figures of EMT were significantly more angiogenic and infiltrated by a higher quantity of myeloid cells compared to tumors with little or no presence of EMT markers. Taken together, our results show that EMT programs trigger the expression of soluble mediators in breast and lung cancer cells that stimulate angiogenesis and recruit myeloid cells *in vivo*, which might in turn favor metastatic dissemination.

Keywords: epithelial-to-mesenchymal transition, cancer, angiogenesis, myeloid cells

Introduction

Known as developmental programs transforming epithelial cells into mesenchymal ones [1, 2], epithelial-to-mesenchymal transitions (EMT) trigger a morphological switch from a cobblestone shape to an elongated form, alongside with a decreased expression of epithelial markers (i.e. E-cadherin) and a gain of mesenchymal markers (i.e. vimentin) [3, 4]. A variety of EMT-inducing extracellular signals and signaling pathways converge to the expression of EMT-transcription factors (EMT-TF) [5, 6] including those of the Snail family (comprising snail and slug), the ZEB family (comprising ZEB1 and ZEB2), Twist, E47, Brachyury and others. Although a major and well-described regulation of EMT-TF is E-cadherin repression [6-8], EMT-TF can activate or repress a variety of target genes of EMT [9-11] (see [12] for a review). In the most studied Snail family, the two human homologs Snail and Slug contain a Zinc finger cluster enabling interaction with specific DNA sequences called E-boxes, first discovered on the chd1/E-cadherin promoter [7, 13]. Snail expression is connected to disease progression. For instance, its expression correlates with lymph node metastasis in breast cancer [14] and with poor overall survival in ovarian cancer [15]. In mouse models, Snail has been shown to trigger inflammation and hyperplasia followed by tumor formation [16], to accelerate metastasis through enhanced invasion and immunosuppression [17] and to increase blood vessel density [18]. Snail has also been associated to cancer stemness in colorectal cancer cells [19].

Partial, reversible EMT has been suggested to occur at different steps of cancer progression [3, 20-22] and recent findings indicate that EMT is particularly involved in tumor cell escape from the primary tumor and in the release of circulating tumor cells (CTCs) [23, 24]. CTCs from breast cancer patients have accordingly been shown to express EMT markers [20, 25, 26] and EMT-derived phenotypes are observed in about 15% of invasive ductal carcinomas and relate to a higher histological grade, the aggressive triple-negative histological type [14, 27-29], a higher rate of loco-regional recurrence [8], chemoresistance [30] and presence of lymph node metastases [14].

Although the cellular pathways leading to EMT and their contribution to intrinsically enhanced invasive properties of tumor cells have been extensively studied, the impact of EMT programs on the crosstalk between tumor cells and the tumor microenvironment remains elusive. Yet, metastatic dissemination largely relies on tumor-stroma interactions and cytokines represent major soluble mediators implicated in tumor-stroma crosstalk [31-33]. Cytokine overexpression by cancer cells has been reported in many cancers including breast and lung cancer [34-36], although the mechanisms of such a regulation are still largely uncovered.

We hypothesized here that EMT programs might modulate the cytokinic microenvironment thereby stimulating the recruitment of a variety of host cells in the tumor and elaborating a permissive milieu for tumor progression.

Materials and methods

Immunohistochemical study on human samples

Human breast tissues were obtained from 40 biopsies of ductal invasive triple-negative breast carcinomas from Reims University Hospital Biological Resource Collection n° DC-2008-374 and staged according the 2009 WHO classification. This study was approved by the Institutional Review Board of Reims University Hospital.

Stainings were performed for vimentin, CD105 and CD33 on serial sections as previously described [21] to determine the extent of EMT, angiogenesis and myeloid cell recruitment in the samples. A list of antibodies is provided in supplemental Table S1. Semi-quantitative scorings are described in Supplemental materials and methods.

Cell culture and reagents

Cancer cell lines were from ATCC (American Type Culture Collection, Manassas, VA). The A549 and the MDA-MB-468 cells used are luciferase-expressing clones respectively purchased from Caliper Life Sciences (Hopkinton, MA) and generated as previously described [21]. Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Verviers, Belgium). A description of culture reagents is provided in Supplemental Material and methods.

Growth factor induction of EMT

For EMT induction, MDA-MB-468 and A549 were seeded ($2x10^5$ and $1.5x10^5$ cells respectively) in 6-well plates and immediately treated with 20 ng/ml recombinant EGF (Sigma, St-Louis, MO) or 5 ng/ml recombinant TGF-β (R&D Systems, Minneapolis, MN) respectively. Medium was replaced by serum-free medium 24h later and cells were treated again and left 24 supplemental hours before conditioned media were collected and RNA extracted.

Plasmid and siRNA transfections

Cells were seeded as described above and transfected 48 hours later. The protocol for siRNA transfections has been described previously [37]. Sequences of siRNA are provided in supplemental material and methods. Plasmids were transfected using the ExtremeGENE 9 reagent (Roche, Basel, Switzerland) in serum-free medium according to the manufacturer's instructions and RNA was extracted 48 hours later. The plasmids used were pCMV-Tag2B Snail-6SA (plasmid 16221, Addgene, Cambridge, MA, USA) and pCMV-Tag2B as an empty control vector.

Recombinant proteins, antibodies, cytokine array and ELISA

Recombinant IL-8 was from R&D and recombinant ICAM-1 was from Peprotech (Hamburg, Germany). ELISAs were performed with Duoset kits for IL-8, GM-CSF, PAI-1, sICAM-1, EGF and TGF-β (R&D) according to the manufacturer's instructions. IL-6 ELISA kit was a Cytoset kit (Invitrogen). For cytokine screening,

Human Cytokine Array Kit, Panel A (R&D) was used.

Reverse Transcription and realtime PCR

After RNA extraction (High Pure RNA Isolation Kit, Roche) and reverse transcription (First Strand cDNA Synthesis kit, Roche), real time PCR was performed on the LightCycler480 (Roche) with the LightCycler480 Probes Master and the Universal Probe Library system. Primer sequences are provided in supplemental Table S2. Gene expression was normalized to GAPDH expression or, in the case of microdissection, to TBP expression.

Sponge Assay and immunofluorescence

Sponge assays were performed as previously described [38]. Briefly, gelfoam sponges (Pfizer, New-York, NY) fragments were soaked into Amicon-(Millipore, Darmstadt, Germany) concentrated conditioned medium from control or EGF-treated MDA-MB-468 or from control or TGF-β-treated A549. Remaining levels of EGF and TGF-β in the treated conditions were measured by ELISA and the equivalent amounts were added into corresponding control conditions in order to exclude EGF- of TGF-β-related effects. Sponges were subcutaneously inserted in mice ears and left for 3 days or 3 weeks. Groups of 6 C57BL/6 mice (Charles River, Chatillon-sur-Chalarone, France) were used. A detailed description of ear processing, immunofluorescent staining and computer-assisted quantification of angiogenesis (CD31 staining) and myeloid cell distribution (GR1/CD11b staining) is provided in Supplemental materials and methods and Table S1.

FACS analysis of sponges and bone marrows

Sponges were collected and gently dissociated using PBS-EDTA for 10 minutes at 37°C. Sponges isolates and flushed bone marrows were centrifuged and suspended in PBS- containing 3% FBS, filtered and processed for staining. Antibodies are listed in supplemental Table S1. Analysis was performed with the FACS CantoII (GIGA-Imagery platform).

Aortic ring assays

The protocol for aortic ring assays has been described [39]. Briefly, rat aortic rings were cultured in presence of conditioned medium from EGF-treated or control MDA-MB-468. EGF was extemporaneously added to untreated MDA-MB-468 conditioned medium to exclude EGF-related effects. After 9 days of culture, pictures were taken and analyzed with a semi-automatic detection software.

Transwell migration assay

HUVEC cells (10^5 cells) were suspended in 200 µl of serum-free medium containing 0.1% BSA and placed on the upper compartment of a Transwell (Corning, Tewksbury, MA). The lower compartment was filled with conditioned medium from control or EGF-treated MDA-MB-468 complemented with 1% BSA and, for inhibitory experiments, with blocking antibodies (see supplemental Table S1) or Corresponding negative control antibodies (Dako). After 24h of migration through a 8 µm-pore gelatin-coated filter, cells were methanol fixed and stained with Giemsa. The upper side of the membranes was

scraped to remove non migrating cells. Conditions were run in triplicates. Image processing and computerized analysis is described in supplemental material and methods.

Laser-Capture Microdissection on MDA-MB-468 xenografts

MDA-MB-468 cell tumors were grown following subcutaneous injection in SCID mice as previously described [21]. Mice were sacrificed after 100 days and tumors were preserved in OCT. Laser-capture microdissection of vimentin-positive and -negative areas was performed based on vimentin staining on a seriated reference slide as previously described [21] using the Leica LMD7000 (GIGA-Imaging platform) and followed by RNA extraction (TriZOL, Invitrogen) and purification (RNeasy mini kit, Qiagen).

Statistical analyzes

Statistical analysis was performed with Prism software (Graphpad Software, San Diego, CA, USA). For the immunohistochemistry study on human samples, associations between vimentin and CD33 or CD105 were studied using Chi-square or Fisher exact tests. For other experiments, results were analyzed with the non-parametric Mann Whitney test with one-tailed p-value. A p-value <0.05 was considered statistically significant. In histograms, mean ± SEM is represented.

Results

EMT phenotypes associate with the expression of a panel of soluble factors in vitro and in vivo

To examine whether EMT programs are involved in the regulation of potential soluble modulators of angiogenesis and inflammation, two models of inducible EMT were used: the MDA-MB-468 human mammary adenocarcinoma cell line and the A549 human lung carcinoma cell line, in which EMT was induced following EGF or TGF-β treatment respectively. EMT induction was confirmed by observation of morphological changes (Figure 1A) and by RT-qPCR analysis showing a decrease in E-cadherin and an increase in vimentin mRNA expression (Figure 1B). A cytokine array was performed on conditioned medium from control (EMT-negative) and EGF-treated (EMT-positive) MDA-MB-468 and showed an increase in many secreted factors (data not shown). The five most overexpressed molecules were Interleukin-8 (IL-8), Interleukin-6 (IL-6), soluble form of InterCellular Adhesion Molecule-1 (sICAM-1), Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF) and Plasminogen Activator Inhibitor-1 (PAI-1). We confirmed by RT-qPCR (Figure 1C) and ELISA (Figure 1D) that these 5 factors were significantly upregulated upon EMT induction in MDA-MB-468. It is worth mentioning that, if IL-8, Il-6, GM-CSF and PAI-1 are secreted molecules, ICAM-1 can be either transmembrane or soluble, due to both alternative splicing and protein shedding [40]. Primers recognizing all forms of ICAM-1 were used, while only the soluble form (sICAM-1) was detected by

ELISA. Similar regulations were obtained with A549 cells, although sICAM-1 secretion could not be detected (Figure S1).

To extend these observations, the expression of the five factors of interest was assessed in well-known EMT-negative (vimentin-negative, E-cadherin-positive) and EMT-positive (vimentin-positive, E-cadherin-negative) breast cancer cell lines by RT-PCR. Supporting our results on the inducible models, higher expression levels of IL-8, IL-6, sICAM-1, PAI-1 and GM-CSF were detected in well-known EMT-positive human breast cancer cell lines (MDA-MB-231, Hs578T, BT-549) compared to epithelial-like cell lines (MCF7 and T47D) (Figure 1E).

To further investigate the association between EMT processes and secreted factor expression *in vivo*, MDA-MB-468 cells were used to induce tumors in SCID mice. As previously described, these cells undergo EMT *in vivo* with large tumors arising after subcutaneous injection [21]. These tumors indeed contained vimentin-positive and vimentin-negative zones (Figure 2A) which were microdissected (Figure 2B). IL-6 and PAI-1 mRNA expression was significantly higher in vimentin-positive zones compared to vimentin-negative ones (Figure 2C). IL-8 mRNA expression tended to be higher in the vimentin-positive compartment, although this did not reach statistical significance. No change in ICAM-1 mRNA expression was observed. GM-CSF was not detectable in the *in vivo* situation, in accordance with its already low expression level *in vitro*.

To establish a functional contribution of EMT pathways in soluble factor regulation, we investigated the potential implication of well described EMT-associated transcription factors Slug and Snail in the process. Indeed, their expression was increased upon EMT induction in the MDA-MB-468 (Figure 3A). As we observed that Snail siRNA knockdown affected Slug expression and vice versa (data not shown), we knocked both genes down. Combined siRNA repression of Slug and Snail strongly inhibited mRNA expression of IL-8, IL-6 and moderately that of ICAM-1 both in control and in EGF-treated conditions in MDA-MB-468 (Figure 3A). PAI-1 and GM-CSF expression remained unaffected. Inversely, we used a degradation-resistant form of Snail, Snail-6SA [41] to show that its ectopic expression in MDA-MB-468 cells increases mRNA expression of all five factors of interest, reaching significance for IL-6 and PAI-1 (Figure 3B). Altogether, these results suggest a functional contribution of EMT pathways, and particularly of Snail, in the specific regulation of the five factors of interest, even though other transcription factors are most likely involved.

EMT-positive cell-conditioned medium is angiogenic

The above-exposed results clearly showed that EMT is associated with the secretion of specific pro-angiogenic and pro-inflammatory mediators. This prompted us to investigate the functional consequences of these EMT-induced factors on host cell recruitment *in vivo* using a sponge assay. This assay is indeed particularly well suited to address the effect of conditioned media

on sequential inflammatory cell infiltration and angiogenesis by observing stromal cell infiltration at different time points. Subcutaneously inserted sponges, previously soaked in conditioned medium of EMT-positive cells or EMT-negative cells, were thus collected after 3 and 21 days to analyze inflammation and angiogenesis respectively. After 21 days, blood vessels were detected by CD31 immunostaining (Figure 4A) and blood vessel density was determined by computer-assisted quantification. Sponges soaked in conditioned medium from EGF-treated MDA-MB-468 were infiltrated by a higher number of blood vessels compared to sponges soaked into control conditioned medium (i.e. conditioned medium from untreated MDA-468 supplemented extemporaneously with EGF to exclude a direct effect of EGF in the sponge assay) (Figure 4B). Similar results were obtained with A549 cells (Figure S2). Confirming the enhanced pro-angiogenic activity observed in the sponge assay, conditioned medium from EGF-treated MDA-MB-468 significantly increased vessel sprouting in a rat aortic ring assay (Figure 4C-E). EMT-induced secreted factors thus clearly triggered an enhanced angiogenic response.

To further characterize this EMT-associated pro-angiogenic activity, conditioned medium from EGF-treated MDA-MB-468 cells was tested for its chemotactic activity on HUVEC cells in a chemotaxis assay and significantly increased HUVEC cell migration compared to control cell-derived conditioned medium (Figure 4F). Any direct EGF-related effects were excluded by the extemporaneous addition of EGF into the control conditioned medium, which did not affect HUVEC cell migration. The inhibition of soluble factors in conditioned medium from EMT-positive cells with specific blocking antibodies showed that IL-6 and PAI-1 are necessary for HUVEC cell migration (Figure 4G). Surprisingly, the inhibition of ICAM-1 or IL-8, which are well-known angiogenic compounds [42-45], did not affect HUVEC cell migration, though the efficiency of blocking antibodies was validated using recombinant IL-8 and ICAM-1 proteins (Figure S3). Taken together, these results suggest that IL-6 and PAI-1 are functional secreted mediators of the pro-angiogenic activity associated with EMT in our models.

EMT-positive cell conditioned medium recruits myeloid derived suppressor cells (MDSC)

Seeking to investigate the impact of EMT-induced cytokines on inflammatory cell recruitment, we took advantage of the 3 day-sponge model to screen infiltrating cell types. Sponge dissociation followed by FACS analysis enabled us to roughly characterize the infiltration of the sponge by macrophages and myeloid cells. A first F4/80 staining revealed that macrophages are not importantly recruited in the sponges (data not shown). We thus examined more in details the potential recruitment of cells of the myeloid lineage. CD11b, Ly6G and Ly6C stainings revealed that the most abundant cell population in the sponge was a myeloid $CD11b^{+}Ly6G^{high}Ly6C^{low}$ followed by a $CD11b^{+}Ly6G^{-}Ly6C^{high}$ population (Figure 5A). Because the dissociation technique of the sponges did not reach an acceptable

level of reproducibility to perform quantitative analyzes, we performed immunofluorescent stainings on frozen sections realized in the sponges to compare further the infiltration of this myeloid population in EMT-negative and EMT-positive groups (Figure 5B). Supporting the FACS results, these immunostaining results showed a high infiltration by $CD11b^{+}GR1^{+}$ myeloid cells around the sponge (Figure 5B). Moreover, sponges soaked into conditioned medium from EGF-treated MDA-MB-468 cells contained a higher relative $CD11b^{+}Gr1^{+}$ hotspot surface compared to control condition (Figure 5C). Suspecting a recruitment of these cells from the bone marrow, we correspondingly showed by FACS that mice bearing EMT-positive sponges contained a significantly decreased proportion of $CD11b^{+}Ly6G^{high}Ly6C^{low}$ cells in their bone marrow compared to the control mice. Proportions of $CD11bLy6C^{high}Ly6G^{-}$ were unchanged in the bone marrow (Figure 5D).

The presence of EMT figures relates to increased angiogenesis and tumor infiltration by myeloid cells in triple-negative breast cancers

Based on our aforementioned results obtained *in vitro* and in animal models, we examined the potential relationship between EMT, angiogenesis and myeloid cell recruitment in human cancers. We conducted a study on 40 triple-negative breast cancers and performed vimentin, CD105 (marker of angiogenesis) and CD33 (marker of myeloid cells) staining on serial sections (Figure 6A). The triple-negative subtype was selected because of the well-described enrichment of EMT markers in this subtype compared to all breast cancer types [29]. Indeed, 20 of the 40 samples displayed at least 10% of vimentin-positive cancer cells (score = 2) (Figure 6B). Most interestingly, presence of EMT markers (as defined by vimentin expression by at least 10% of cancer cells, equaling to a score of 2) was associated with increased density of CD105-positive blood vessels, compared to tumors containing less than 10% of vimentin-positive cancer cells. Likewise and corroborating our findings suggesting an EMT-mediated recruitment of MDSC, the presence of EMT markers in triple-negative breast cancers was associated to increased tumor infiltration by CD33-positive myeloid cells (Table 1).

Discussion

Although sound evidence has accumulated linking EMT to cancer progression in breast cancer patients [25-27, 29], corroborated by *in vitro* models [28, 46] and animal experiments [21, 24], mechanisms relating EMT to cancer stroma interaction and cancer progression remain elusive. We here show that EMT phenotypes relate to increased expression of a panel of pro-angiogenic and pro-inflammatory soluble mediators (IL-8, IL-6, sICAM-1, PAI-1 and GM-CSF) in several cellular systems. These factors are consistently increased in two cell lines (MDA-MB-468 and A549) following growth factor treatment (EGF and TGF-β respectively) and strongly expressed in cell lines displaying endogenous EMT traits. Our results are pertinently supported by a few data linking EMT-signaling and specific cytokine

secretion. The association between EMT features and IL-8 has already been reported by us and others in breast cancer cell lines [37] and in colorectal carcinoma cell lines [47]. Clinically, EMT features were found enriched in IL-8-positive hepatocellular carcinoma tissues [48]. Regarding the regulation of other cytokines through EMT, data are scarce. PAI-1 expression is nevertheless induced during EMT in Ras-transformed keratinocytes (HaCaT II-4) [49]. Concerning GM-CSF, a very recent study revealed that mesenchymal-like breast cancer cells secrete high amounts of GM-CSF [50]. Although some data suggest an implication of the membrane bound ICAM-1 in accelerating TGF-β-induced EMT in renal cells in presence of its ligand (LFA-1) expressed at the surface of monocytes [51, 52], no data has clearly linked sICAM-1 expression to EMT. Conversely, certain chemokines have also been shown to act as EMT inducers. Independent studies have shown that IL-8 [53] and IL-6 [54] act as key mediators of EMT induction and maintenance in several cancer cell lines (from breast, lung and pancreatic cancer) [53]. Also, a functional role of PAI-1 in the modulation of EMT programs has been reported in the Ras-induced EMT model of HaCAT II cells [49] and in a model of EMT-induced lung fibrosis [55]. In the latter, PAI-1 repression by siRNA inhibited TGF-β-triggered EMT in murine epithelial cells. Taken together, these data suggest that induction of IL-8, IL-6 and PAI-1 might create a positive regulatory loop to maintain EMT.

Our findings also indicate that EMT-associated cytokines are regulated by EMT-transcription factor Snail, even though other transcription factors are most likely involved. Supporting our results, ectopic expression of Snail in the canine renal MDCK cell line accentuates IL-6 and IL-8 increase upon IL-1β stimulation [56] and Snail expression in head and neck carcinoma cells enhances IL-6 and IL-8 expression [57]. Functional inhibition of Snail by ectopic expression of a dominant negative form in breast cancer cells decreases PAI-1 expression [58]. This somehow contrasts with our data showing no PAI-1 regulation after siRNA snail inhibition though this study was performed using other cell systems. The particular molecular EMT pathways involved in the modulation of a specific cytokine expression are indeed likely various and dependent on the cellular context. Other EMT-transcription factors such as Brachury [53] or Twist [59] have also been shown to promote IL-8 expression. Previous data of our laboratory have also shown an increased expression of IL-8 in breast cancer cell lines depending on EMT-induced ZO-1 delocalization [37]. Taken together, our data support the involvement of various EMT pathways in regulating the expression of specific soluble mediators by cancer cells. Our results, consistently identifying the 5 factors of interest, modulated through a major EMT transcription factor such as Snail, in different cellular systems, support their relevance as target genes of EMT.

In agreement with the known properties of these soluble factors on angiogenesis and inflammatory response, our results further show that EMT in our cell models create a pro-angiogenic and pro-inflammatory

context. Regarding angiogenesis, our original data obtained on the triple negative breast cancers evidenced a clear correlation between the presence of EMT areas in these cancers and increased angiogenesis. Corroborating these observations and further suggesting a functional role of EMT-derived soluble factors, conditioned medium from EMT-positive cells significantly increases angiogenesis *in vivo* in the sponge assay, stimulates vessel sprouting from rat aortic rings *ex vivo* and enhances HUVEC cell migration *in vitro* in PAI-1 and IL-6-dependent ways. Accordingly, reports have implicated IL-6 in angiogenesis. Nilsson and colleagues [60] have shown that IL-6 stimulates angiogenesis in the sponge assay. Furthermore, IL-6 stimulates HUVEC cell proliferation, tubule formation, and vessel sprouting in aortic ring assays [61]. At last, fibroblast-derived IL-6 was recently shown to promote angiogenesis in colorectal tumors [62]. In this latest study, IL-6 increases angiogenesis in colorectal carcinoma by stimulating VEGF production by fibroblasts. In contrast, our results using IL-6 blocking antibody in the HUVEC migration assay suggest that IL-6 is able to directly chemoattract endothelial cells. PAI-1 exerts dual effects on angiogenesis. Data, including those of our laboratory, have indeed shown that PAI-1 contextually either stimulates or inhibits angiogenesis [63-65]. Taken together, our results demonstrate on one hand that EMT relates to increased angiogenesis in triple-negative breast cancer, and, on the other hand, that conditioned medium from EMT-positive cells is angiogenic. We further pinpoint IL-6 and PAI-1 as major mediators of EMT-associated angiogenesis.

In addition to our observations linking EMT and angiogenesis, examining the bank of triple-negative breast cancers further allowed us to correlate the presence of EMT markers to increased tumor infiltration by CD33-positive myeloid cells. Accordingly, in the sponge assay model, we showed that sponges containing conditioned medium from EMT-positive cells are invaded by more $CD11b^{+}GR1^{+}$ myeloid cells compared to control sponges. $CD11b^{+}GR1^{+}$ cells, so-called MDSC (myeloid-derived suppressor cells), represent a heterogeneous group of immature myeloid cells of granulocytic ($CD11b^{+}Ly6G^{high}$) or monocytic ($CD11b^{+}Ly6C^{high}$) morphology which share a common immunosuppressive activity. We further show by FACS analysis that EMT-positive sponges are infiltrated by an elevated ratio of granulocytic/monocytic MDSC. Accordingly, FACS analysis of bone marrows of sponge bearing mice showed that proportions of $CD11b^{+}Ly6G^{hi}$ cells were decreased in the EMT-positive group, whereas $CD11b^{+}Ly6C^{hi}$ cell proportion was unchanged. Over the last years, clinical importance of MDSC has been revealed by trials showing their circulating levels are not only predictive of survival in metastatic breast cancer patients [66] but also correlated to the tumor burden and the number of CTCs [67]. Accordingly, it is interesting to note that MDSC have been suggested to promote not only EMT and cancer cell dissemination [68] but also angiogenesis [69]. Most interestingly, immunotherapy combined with low-dose

anti-VEGFR2 monoclonal antibody decreases tumor infiltration by MDSC and increases survival in breast cancer-bearing mice [70].

Our original results suggest that conditioned medium from EMT-positive cells is able to attract MDSC. Supportively, pieces of evidence link several cytokines that we found induced by EMT to MDSC biology. First, IL-6 and GM-CSF are used for *in vitro* generation of MDSC from hematopoietic stem cells. Second, clinical trials have shown that high levels of tumor-derived GM-CSF are associated with an infiltration of the tumor by MDSC [71, 72]. Third, in mice expressing hIL-8 under the control of its human promoter, inflammatory and neoplastic sites are infiltrated by a higher number of MDSC [73].

Taken together, our results thus reveal that EMT programs, by regulating a consistent panel of several soluble mediators, functionally promote both angiogenesis and MDSC recruitment, both of which may independently or synergistically promote metastatic dissemination. Emphasizing the clinical relevance of our observations, we further established the correlation between the presence of EMT features in triple-negative breast cancers, enhanced angiogenesis and myeloid cells infiltration.

Acknowledgment

This work was supported by grants from the Fonds de la Recherche Scientifique Médicale, the Fonds de la Recherche Scientifique - FNRS (F.R.S.-FNRS, Belgium), the Foundation against Cancer (foundation of public interest, Belgium), the C.G.R.I.-F.N.R.S.-INSERM Coopération, the Fonds spéciaux de la Recherche (University of Liège), the Centre Anticancéreux près l'Université de Liège, the Fonds Léon Fredericq (University of Liège), the Direction Générale Opérationnelle de l'Economie, de l'Emploi et de la Recherche from the S.P.W. (Région Wallonne, Belgium). CG is a Senior Research Associates at the F.R.S-FNRS, MSC is a Research Fellow at the F.R.S.-FNRS. We thank Emilie Fereysen, Isabelle Dasoul, Marie Dehuy, Guy Roland, Laure Volders and Nathalie Lefin for technical assistance. We thank the GIGA Bioinformatics Platform for providing access to computing servers and to the Cytomine software, and the GIGA-Imaging and Microdissection platform for providing access to FACSCanto II (BD) and to LMD7000 microdissector (Leica).

Statement of author contributions

MSC and CG conceived and carried out experiments. MB, PH and LS carried out experiments. JL, MP and PB provided and analyzed the stainings on human samples. SB analyzed images. CE helped with the development of sponge assays. All authors, including PD, JMF and AN were involved in writing the paper and had final approval of the submitted and published versions.

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Figure 1

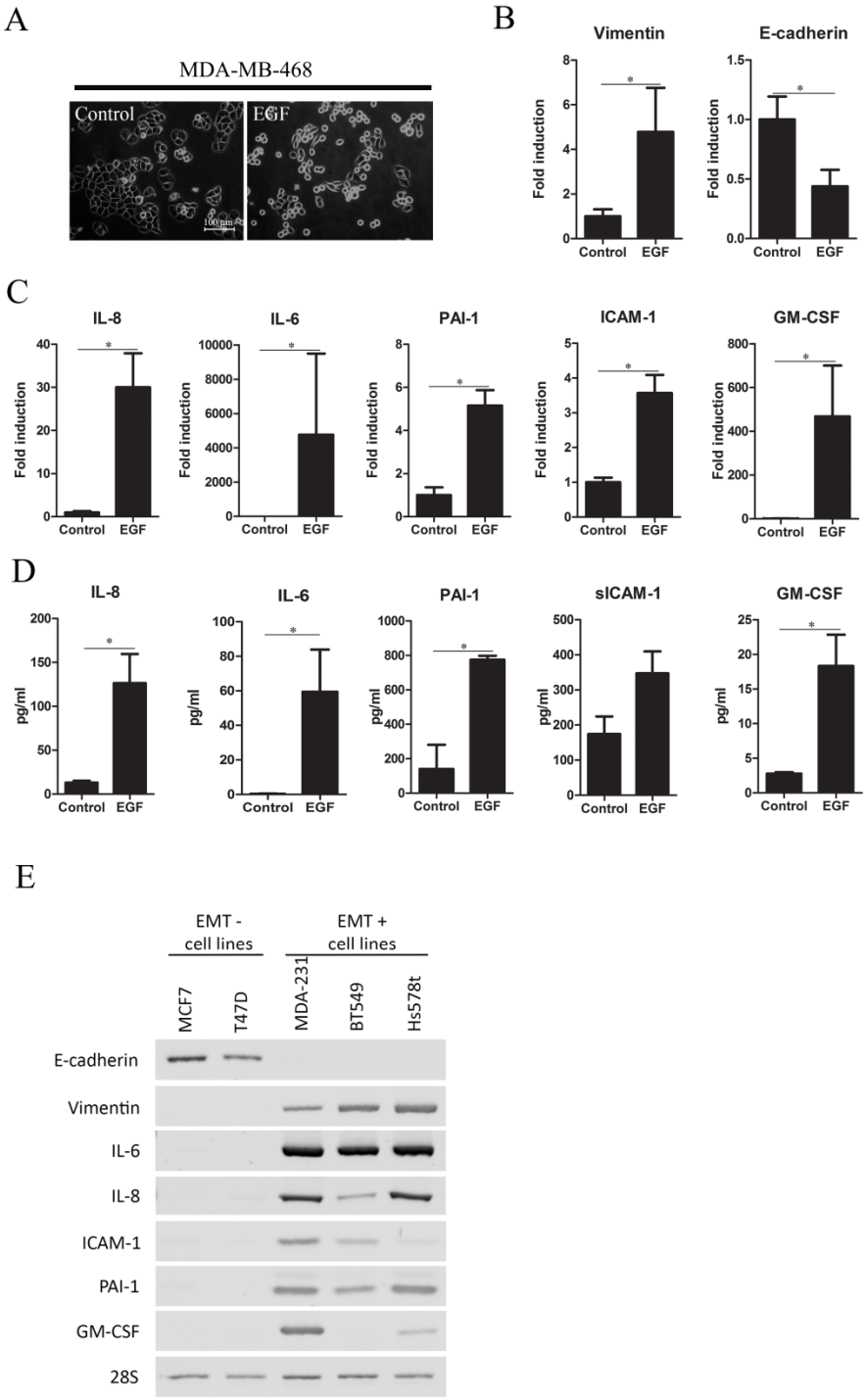


Figure 1. Cytokine expression upon EMT induction. (**A**) Microscopy images showing morphological changes in MDA-MB-468 cell line upon EGF treatment. (**B**) RT-qPCR analyzes of vimentin and E-cadherin in control or EGF-treated MDA-MB-468 cells. Data are expressed as fold induction in treated cells relative to the controls. (**C-D**) Analyzes of IL-8, IL-6, PAI-1, ICAM-1 and GM-CSF in control or EGF-treated MDA-468 by RT-qPCR (C) or ELISA (D). Analyzes by endpoint RT-PCR of E-cadherin, vimentin, IL-8, IL-6, ICAM-1, PAI-1 and GM-CSF in a panel of well-known epithelial-like and EMT-derived human breast cancer cell lines. *: $p<0.05$.

Figure 2

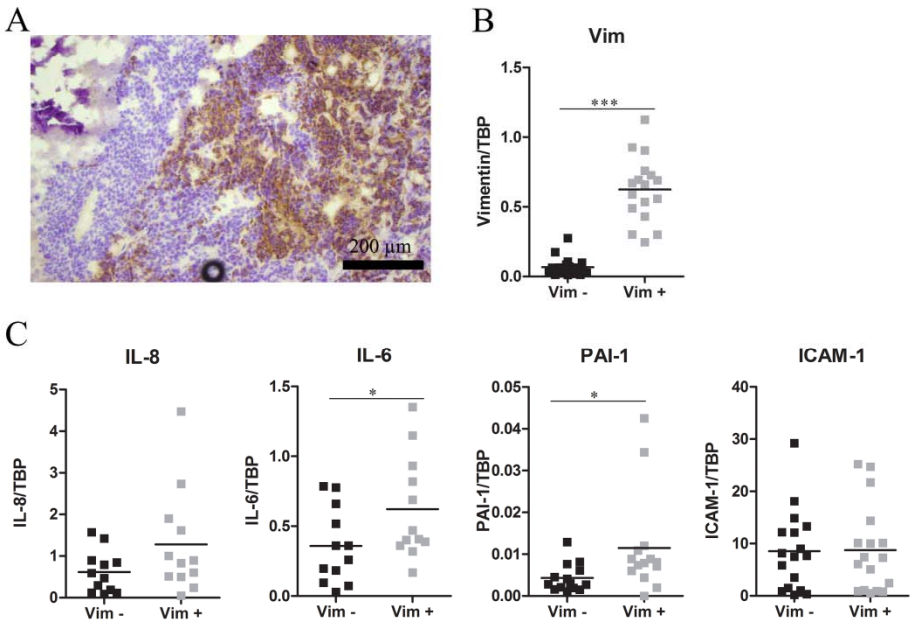


Figure 2. Expression of EMT-related soluble factors in microdissected vimentin-positive and vimentin-negative areas in MDA-MB-468-formed tumors in SCID mice. (**A**) Microscopy image of human vimentin specific immunostaining (brown) performed on an section of a MDA-MB-468-tumor formed in SCID mice. (**B**) RT-qPCR analysis of human vimentin on RNA extracted from microdissected vimentin-negative (Vim-) and vimentin-positive (Vim+) tumor areas. (**C**) RT-qPCR analyses of human IL-8, IL-6, PAI-1 and ICAM-1 on RNA extracted from microdissected areas. *: $p< 0.05$ and ***: $p< 0.001$.

Figure 3

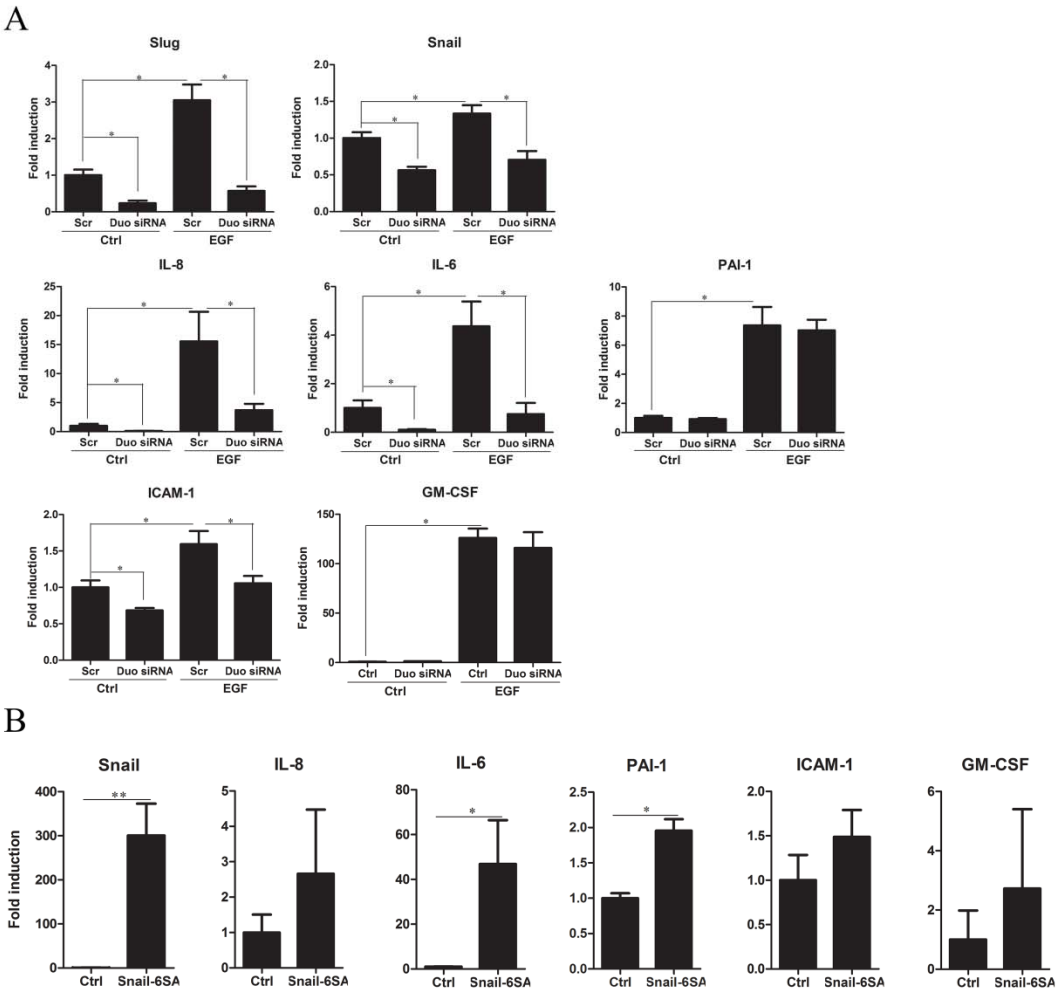


Figure 3. Regulation of soluble factors by combined repression of Slug and Snail. (**A**) RT-qPCR analyzes of Slug, Snail, IL-8, IL-6, ICAM-1, PAI1 and GM-CSF in MDA-MB-468 transfected with a combination of Snail and Slug siRNA or a control siRNA. Data are expressed as fold induction relative to the non-treated control siRNA condition. (**B**) RT-qPCR analyzes of Snail, IL-8, IL-6, ICAM-1, PAI1 and GM-CSF in MDA-MB-468 cells transfected with cDNA encoding a degradation-resistant form of Snail (Snail-6SA) or with the corresponding empty vector (Ctrl). *:p<0.05, **:p<0.01.

Figure 4

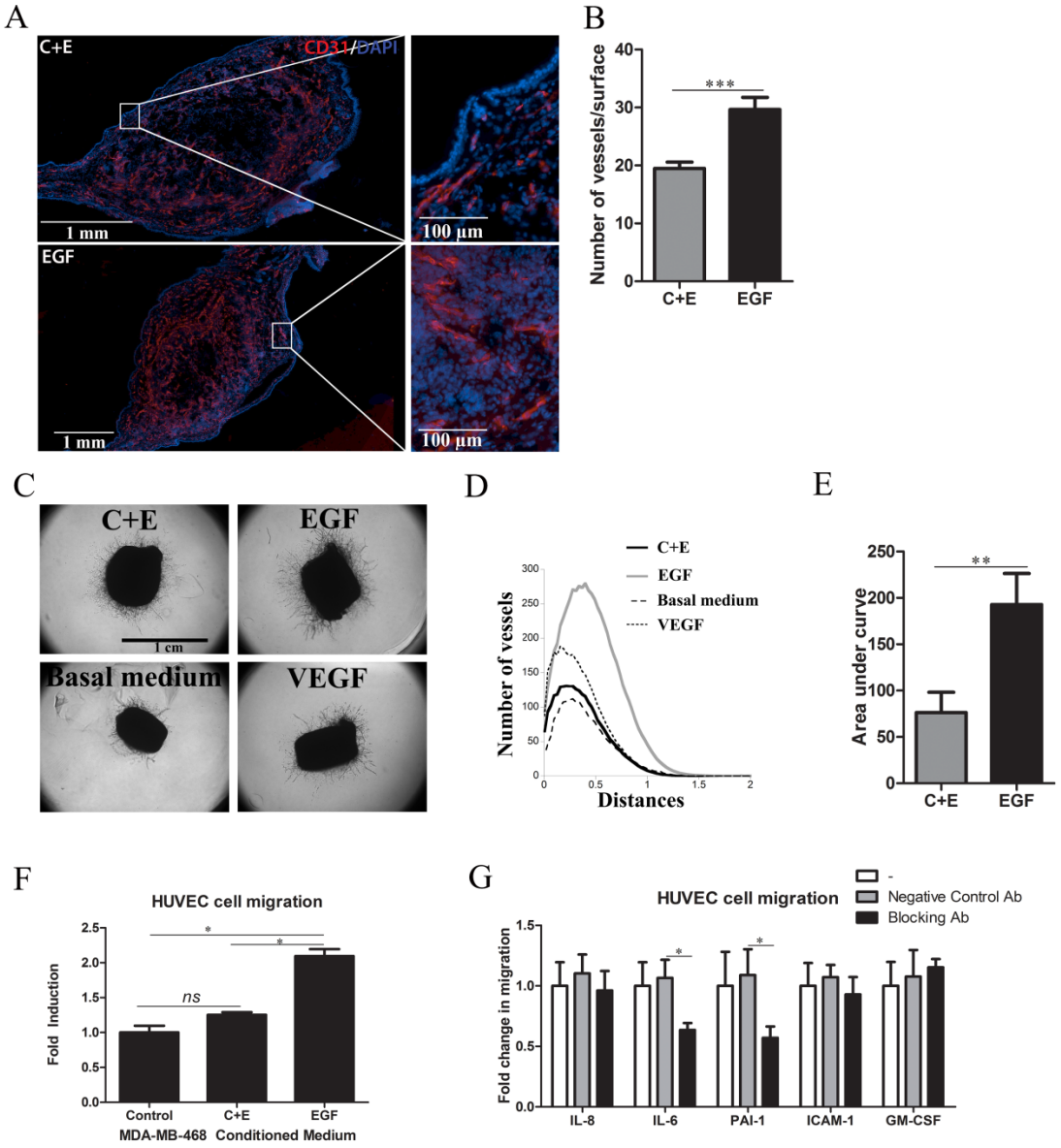


Figure 4. Effect of conditioned medium from EMT-derived cells on angiogenesis. (**A**) Immunofluorescent staining for CD31 (in red, labeling blood vessels) on ear sections containing 3 week-sponges soaked in conditioned medium of non-treated (C+E) or EGF-treated (EGF) MDA-MB-468. EGF was added to the control conditioned medium to exclude EGF-related effects (C+E). DAPI (in blue) is used to label nuclei. (**B**) Results of the computer-assisted quantification of the CD31 staining as shown in A representing blood vessel density. Pooled results of three independent mice experiments leading to n = 14 (C+E) and n= 15 (EGF) are shown. (**C**) Microscopy images illustrating 9-day-aortic rings accompanied by (**D**) results of the computer-assisted quantification of images shown in C. (**E**) Area under curve quantification graphs corresponding to the analyzes shown in D. One representative experiment out of 3 is shown. (**F**) Primary endothelial cell (HUVEC) chemotaxis assay using conditioned medium from EGF-treated (EGF) or untreated (Control) MDA-MB-468. A condition using control medium of untreated MDA-MB-468 in which EGF was added extemporaneously (C+E) was performed to exclude any direct effect of EGF on HUVEC chemotaxis. (**G**) HUVEC chemotaxis assay using conditioned medium of EGF-treated MDA-MB-468 containing blocking antibodies for the factors of interest (IL-8, IL-6, sICAM-1, PAI-1 or GM-CSF) or the corresponding control antibodies (Negative control).

Figure 5

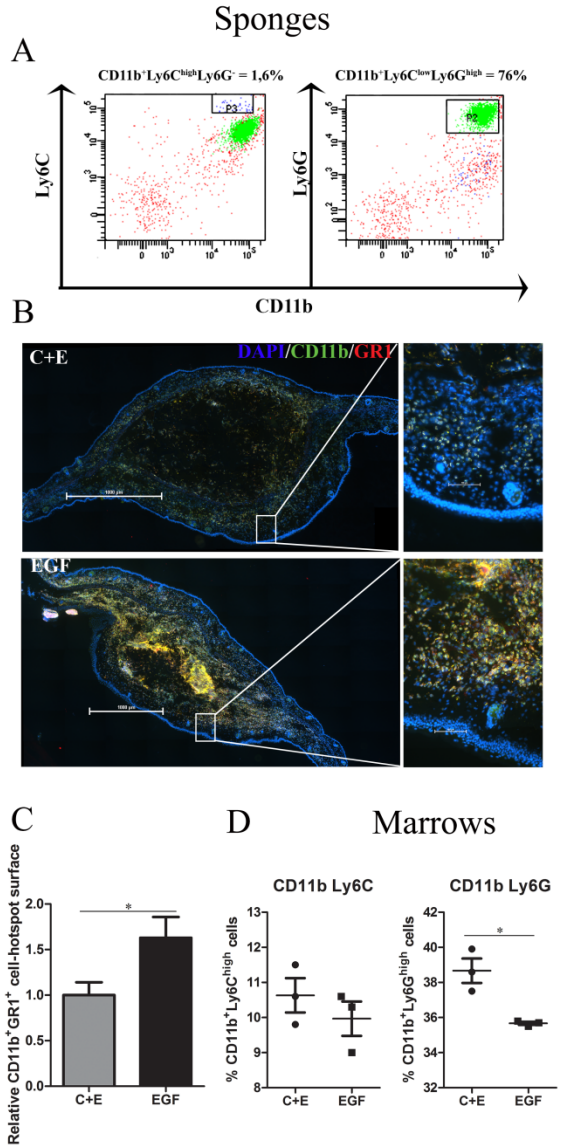
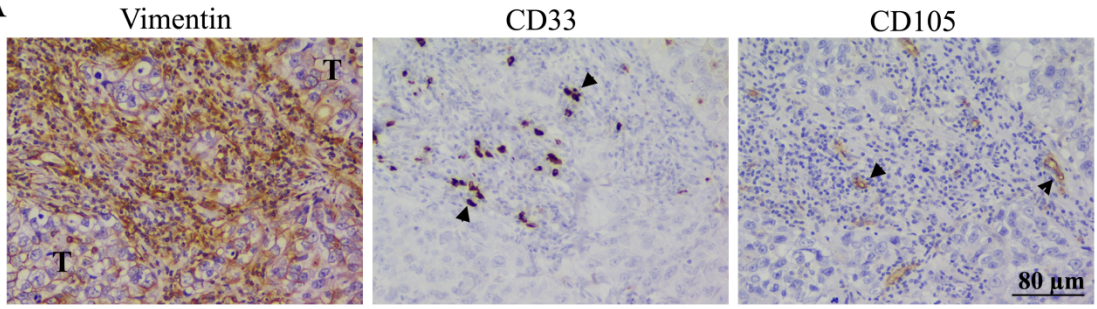


Figure 5. Effect of conditioned medium from EMT-derived cells on myeloid cell recruitment *in vivo*. (**A**) FACS analyzes of immunolabelling by CD11b, Ly6G and Ly6C antibodies on cells collected from 3 day-sponges soaked in conditioned medium of EGF-treated MDA-MB-468. The $CD11b^{+}Ly6G^{hi}$ and $CD11b^{+}Ly6C^{hi}$ populations are delineated by boxes (P2 and P3 respectively) and $CD11b^{+}Ly6G^{hi}$ cells are represented in green. (**B**) Microscopy images illustrating CD11b/GR1 fluorescent double staining on ear sections containing 3-day-sponges soaked beforehand in conditioned medium of non-treated (C+E) or EGF-treated (EGF) MDA-MB-468. EGF was added to the control conditioned medium to exclude EGF-related effects. DAPI (in blue) is used to label nuclei. (**C**) The graph reports the quantification of the relative $CD11b^{+}GR1^{+}$ hotspot surface on the staining shown in B. A pool of two independent experiments leading to n=11 (C+E) and n=13 (EGF), quantified by two independent observers in double-blind settings is shown. (**D**) Quantifications by FACS of CD11b, Ly6G and Ly6C immunolabellings on cells dissociated from bone marrow of sponge-bearing mice. Graphs illustrate the proportions of $CD11b^{+}Ly6C^{hi}$ (left) and $CD11b^{+}Ly6G^{hi}$ cells (right) in the marrow. Each dot represents the pooled analysis of two mice.

Figure 6

A



B

Variables	Vim ⩽ 10 % n=20	Vim > 10% n=20	*p*
CD33 expression			
Low	16 (64%)	9 (36%)	**0.0484**
High	4 (27%)	11 (73%)	
CD105 expression			
Low	17 (71%)	7 (29%)	**0.0031**
High	3 (19%)	13 (81%)	

Figure 6. (**A**) Illustrative microscopy images of vimentin, CD105 and CD33 stainings on serial sections in a triple negative breast cancer. T = tumor cluster. Arrows point to myeloid cells (CD33 labelling) or blood vessels (CD105 labelling). (**B**) Correlation between vimentin and CD105 or CD33 in triple negative breast cancers.

Figure S1

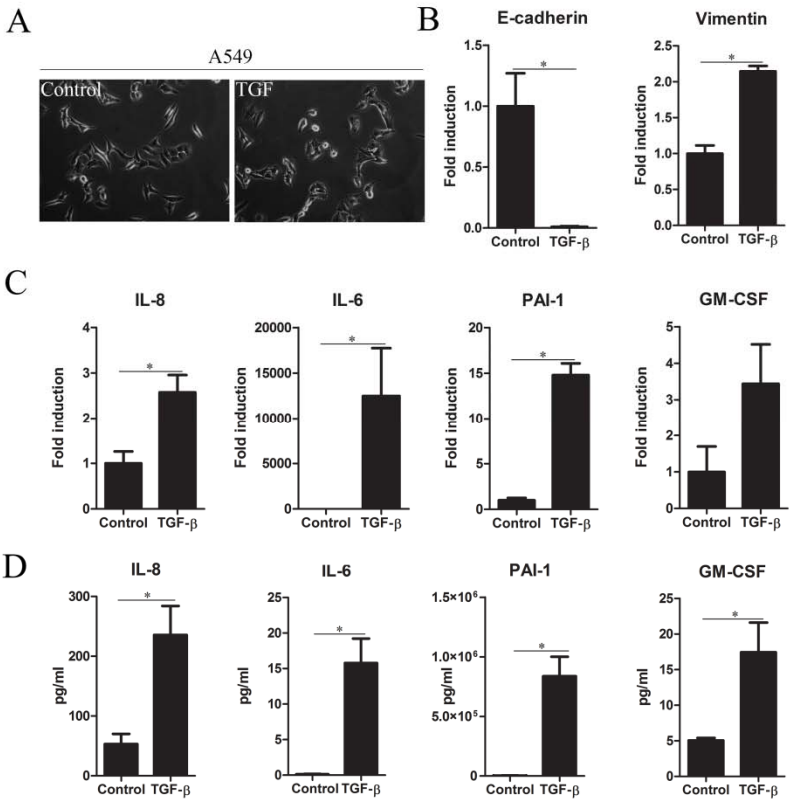


Figure S1. EMT induction and soluble factors expression in A549 cell line. (**A**) Microscopy images illustrating morphological changes upon TGF-β treatment in A549 (left) and RT-qPCR analyzes of E-cadherin and vimentin in control or TGF-β-treated A549. (**B**) RT-qPCR and (**C**) ELISA analyzes for IL-8, IL-6, PAI-1 and GM-CSF in control or TGF-β-treated A549.

Figure S2

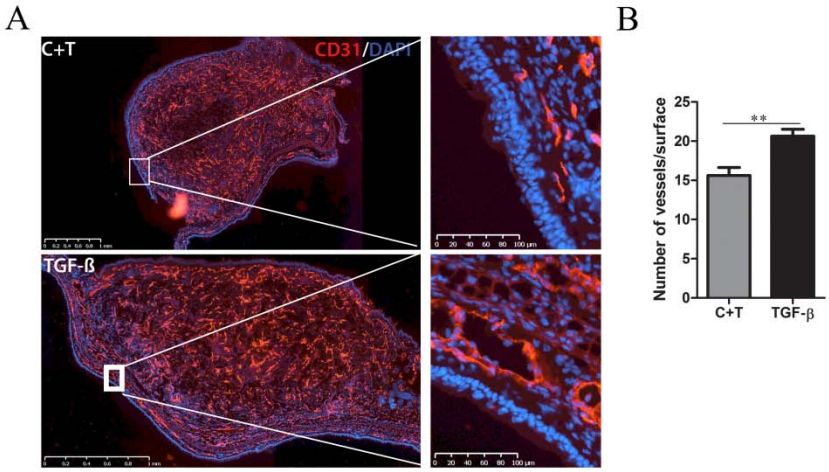


Figure S2. Effect of conditioned medium from EMT-derived cells on angiogenesis. (**A**) Immunofluorescent staining for CD31 (in red, labeling blood vessels) on ear sections containing 3 week-sponges soaked beforehand in conditioned medium of non-treated (C+T) or TGF-β-treated (TGF-β) A549 cells. TGF-β was added to the control conditioned medium to exclude TGF-β-related effects (C+T). DAPI (in blue) is used to label nuclei (**B**) Results of the computer-assisted quantification of the CD31 staining as shown in A representing blood vessel density. Pooled results of two independent mice experiments leading to n = 10 (C+T) and n = 11 (TGF-β) are shown. **: $p<0.01$.

Figure S3

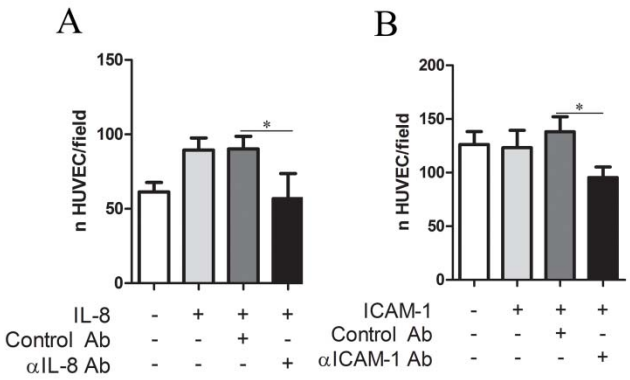


Figure S3. Validation of anti-IL-8 and anti-ICAM-1 blocking antibodies. HUVEC cell chemotaxis assay using conditioned medium from untreated MDA-MB-468 with addition of (**A**) recombinant IL-8 (IL-8) with or without anti-IL-8 antibody (αIL-8 Ab) or control antibody (Control Ab) and (**B**) recombinant ICAM-1 (ICAM-1) with or without anti-ICAM-1 antibody (αICAM-1 Ab) or control antibody (Control Ab).

Supplemental material and methods

Immunohistochemical study on human samples: quantifications

The results for immunohistochemical detection were scored independently by two pathologists who had no knowledge of the clinical data. When a discrepancy was found between the two investigators, a consensus was reached via simultaneous examination using a double-headed microscope. For vimentin, staining extend was graded into 5 categories as follows: 0 = no detection, 1 = detection in <10% of tumor cells, 2 = detection in 10-25% of tumor cells, 3 = detection in 26-50% of tumor cells, 4 = detection in 51-75% of tumor cells, 5 = detection > in 76% of tumor cells. For CD33 and CD105, staining extent was graded into 4 categories as follows: 0 = no expression, 1 = low expression, 2 = moderate expression and 3 = high expression. Vimentin expression was divided to 0-1 or more and CD33 and CD105 were managed as either 0-1 (low expression) or 3-4 (high expression) in analyses.

Cell culture

Cancer cell lines (MCF7, T47D, MDA-MB-231, BT549 and Hs578t) were cultured in DMEM medium (Gibco-Invitrogen, Carlsbad, CA) complemented with 10% fetal bovine serum (Gibco), 1% glutamin (Gibco) and 1% penicillin-streptomycin (Gibco). Human umbilical vein endothelial cells (HUVEC) were cultured on 0.2% gelatin-coated dishes in EBM medium supplemented with EGM Singlequot reagents (Lonza), 2% serum (Lonza) and 1% penicillin-streptomycin (Gibco).

Plasmid and siRNA transfections

For siRNA transfections (19N) TT (N, any nucleotide) duplexes were generated as previously described. One duplex which does not recognize any sequence in the human genome was used as control siRNA (5'-GUCUAGUCUAGACGAUACU-3'). The sequences were: 5'-GCUACCCAAUGGCCUCUCU-3' (Slug siRNA) and 5'-GCGAGCUGCAGGACUCUAA-3' (Snail siRNA) from Eurogentec (Liège, Belgium) and were transfected at a concentration of 20 nM by the calcium phosphate technique. Cells were washed 16 hours after transfection, serum-starved and treated again with EGF or TGF-β. RNA and proteins were extracted 24 hours later.

Sponge Assay

After sacrificing the mice, ears were collected and preserved in Optimal Cutting Temperature (OCT) compound. For immunofluorescent staining, ten 10 micron-thick sections were realized across the sponge, fixed in acetone and permeabilized with methanol before immunostaining (for antibodies, see supplemental Table S1). Nuclei were counter-stained and slides were coverslipped with Vectashield Hardset mounting medium with DAPI (Vector, Peterborough, UK). For blood vessel density analysis, slides labeled with CD31 antibody were scanned using the Nanozoomer (Hamamatsu Photonics, Mont-Saint-Guibert, Belgium) at a 20x magnification. Blood vessel stainings were automatically detected and blood vessel density was calculated as previously described [37]. For CD11b-Gr1 staining analysis, the whole slides were scanned using the Eclipse microscope (Nikon

Instruments, Melville, NY, USA), at the 20x magnification. Quantification of CD11b-GR1 immunostainings was performed with the Cytomine software from the GIGA-Bioinformatics Platform [38]. Areas occupied by CD11b+Gr1+ cells were manually delimited by two independent observers in double blind settings. The total of positive surfaces (hotspots) was normalized by the total sample area.

All mice experiments were performed with approval of the ethics committee from the University of Liège.

Transwell migration assay

For Transwell result analysis, 12 images were taken per membrane and measurements were performed using the toolbox of image analysis of MATLAB 7.9 (R2009b) software (Mathworks, Inc.). Briefly, stained cell images were extracted from the enhanced red component using automatic entropy thresholding (Kapur et al., 1985). Morphological filters were used to eliminate small artifacts (Soille, 2003). From the resulting binary images, cells were counted automatically. Random images were selected for confirmation by manual counting.

1. Kapur J, Sahoo PK, Wong AK. A new method for gray-level picture thresholding using the entropy of the histogram. *Computer vision, graphics, and image processing* 1985; **29**: 273-285.
2. Soille P. Morphological image analysis: principles and applications. Springer-Verlag New York, Inc. 2003.

Supplemental table 1

List of antibodies used for stainings and neutralization experiments.

Application	Antibody	Clone	Source
Immunofluorescent staining	Anti-mouse CD31	MEC 13.3	BD, San Diego, CA,
	Anti-mouse CD11b	M1/70	BD
	Anti-mouse GR1	RB6-8C5	eBioscience, Vienna, Austria
FACS staining	Anti-mouse PE-F4/80	BM8	eBioscience
	Anti-mouse PE-CD11b	M1/70	BD
	Anti-mouse APC-Ly6G	1A8	BD
	Anti-mouse APC-GR1	RB6-8C5	eBioscience
	Anti-mouse PerCP-Ly6C	HK1.4	BioLegend
Immunohisto-chemistry on human samples	Anti-human vimentin	V9	Dako
	Anti-human CD105	-	Thermoscientific
	Anti-human CD33	6C5/2	Abcam
Neutralization experiments in Transwell assays	Anti-human IL-8	6217	R&D
	Anti-human PAI-1	3785	American Diagnostica, Pfungstadt, Germany
	Anti-human ICAM-1	G-5	Santa Cruz Biotech, Snta Cruz, CA
	Anti-human IL-6	Polyclonal	Pierce, Rockford, IL
	Anti-human GM-CSF	Polyclonal	Pierce

Supplemental table 2

List of primers used for RT-qPCR

Gene	Primer	Sequence
hGAPDH[1]	Forward	5'-ACCAGGTGGTCTCCTCTGAC-3'
	Reverse	5'-TGCTGTAGCCAAATTCGTTG-3'
hTBP[2]	Forward	5'-GACTCCCATGACCCCCAT-3'
	Reverse	5'-CAACCAAGATTCACTGTGGATAC-3'
hVimentin	Forward	5'-GCGTGACGTACGTCAGCAATATGA-3'
	Reverse	5'-GTTCCAGGGACTCATTGGTTCCTT-3'
hE-cadherin	Forward	5'- GACACATTTATGGAACAGAAAATAACA -3
	Reverse	5'-AGTGGAAATGGCACCAGTGT-3'
hIL-8	Forward	5'-AGACAGCAGAGCACACAAGC-3'
	Reverse	5'-ATGGTTCCTTCCGGTGGT-3'
hIL-6	Forward	5'- CAGGAGCCCAGCTATGAACT-3'
	Reverse	5'- GAAGGCAGCAGGCAACAC-3
hICAM-1	Forward	5'-AACCTCAGCCTCGCTATGG-3'
	Reverse	5'-GATGACTTTTGAGGGGGACA-3'
hGM-CSF	Forward	5'-TCTCAGAAATGTTTGACCTCCA-3'
	Reverse	5'-CCATCATGGTCAAGGGGC-3'
hPAI-1	Forward	5'-CTCCTGGTTCTGCCCAAGT-3'
	Reverse	5'-CAGGTTCTCTAGGGGCTTCC-3'
hSnail	Forward	5'-GCTGCAGGACTCTAATCCAGA-3'
	Reverse	5'-ATCTCCGGAGGTGGGATG-3'
hSlug	Forward	5'-ACAGCGAACTGGACACACAT-3'
	Reverse	5'-GATGGGGCTGTATGCTCCT-3'

[1]Glycéraldéhyde-3-phosphate déshydrogénase, [2]TATA box binding protein

Part II – DeltaNp63 isoform-mediated β-defensin family upregulation is associated with (lymph-) angiogenesis and poor outcome in patients with squamous cell carcinoma

1 Introduction

Member of the p53 family of transcription factors, p63 is required during the development of pluristratified epithelia (Yang et al., 1999), especially for stratification (Koster et al., 2004), differentiation (Truong et al., 2006) and maintenance of a pool of proliferative stem cells (Pellegrini et al., 2001; Senoo et al. 2007). However, in contrast to p53, the role of p63 in cancer is unclear because p63 can be either overexpressed or lost in some squamous cell carcinomas (SCC) (Flores, 2007; Urist et al., 2002; Di Como et al., 2002). The loss of p63 is for instance associated with bladder cancer progression (Urist et al., 2002) but p63 expression promotes pediatric neuroblastoma and osteosarcoma progression (Bid et al., 2014).

The existence of two isoforms, TA- and ΔNp63, generated by alternative transcription initiation sites and comprising or not a transactivation domain respectively, and the existence of three main isotypes (p63α, β and γ) due to alternative splicing, give rise to six possible forms of p63 with potentially different roles (Yang et al., 2002). Accordingly, several tumor suppressive functions have been described specifically for TAp63. TAp63 is for instance required for p53-mediated apoptosis in response to DNA damage (Flores et al., 2002) and TAp63α is induced by chemotherapeutic drugs and is able to trigger apoptosis both by upregulating the expression of death receptors and through a mitochondrial pathway (Gressner et al., 2005). The implication of ΔNp63 in cancer, on the other hand, is still controversial. DeltaNp63 is for instance able to upregulate the expression of oncogenic factors such as the β-catenin/c-Myc pathway in esophageal cancer cells, resulting in increased invasion and metastasis (Lee et al., 2014), but inhibits EMT in human bladder cancer cells (Tran et al., 2013). In breast cancer, ΔNp63α confers stem cell properties to cancer cells (DeCastro et al., 2014).

Small conserved cationic peptides, defensins share a potent antimicrobial activity against viruses, bacteria and parasites and are categorized into α- and β-defensins according to the organization of their disulfide bridges. While α-defensins are essentially expressed by leukocytes (HNP1-4) or Paneth cells (HD5-6), β-defensins (or hβDs, comprising hβD1, 2, 3 and 4) are expressed by epithelial cells of the skin and mucosae (Klotman and Chang, 2006).

Beside their role in innate immunity, defensins have been suspected to participate to other biological processes such as adaptive immune system response, wound healing, male fertility and cancer, as we recently reviewed (see linked publication).

The specific implication of hβDs in cancer seems to be context and defensin-dependent. The expression of hβD1, for example, can be decreased or even lost in cancers such as oral SCC, renal and prostate carcinoma (Donald et al., 2003). The expression of hβD2 and hβD3, on the other hand, seems to be either increased or decreased depending on tumor location or differentiation (Kesting et al., 2009; Shi et al., 2014; Shuyi et al., 2011). The potential effect of hβDs in cancer are unclear but include alteration of cancer cells themselves through the regulation of cell proliferation (Gerashchenko et al., 2013; Shi et al., 2014), invasive properties (Han et al., 2014; Shi et al., 2014), and effects on the tumor microenvironment, i.e. inflammation and immunity (Hubert et al., 2007; Li et al., 2014).

The purpose of this study was to investigate the effect of each form of p63 on the expression of hβDs in cervical and in head and neck SCC, as well as the functional repercussions of hβD expression in cancer spread.

2 Results

Based on a published microarray performed on SCC cells transfected with a siRNA against p63 in which p63 knockdown results in decreased hβD1 and hβD4 expression (Barbieri et al., 2006), we hypothesized that one p63 isoform might be a transcriptional regulator of β-defensins. Accordingly, in a set of uterine cervix SCC cell lines and in head and neck SCC (HNSCC) cell lines, we observed a positive association between ΔNp63 expression and the mRNA expression of hβD1, hβD2 and hβD4 but not hβD3. In HaCaT cells and in HT-3 cervical SCC cells, ectopic expression of ΔNp63α, ΔNp63β or ΔNp63γ increased the mRNA expression of hβD1, hβD2 and hβD4 while ectopic expression of TAp63α, TAp63β or TAp63γ had no effect. Conversely, the knockdown of ΔNp63 (all isotypes) decreased mRNA and protein expression of hβD1, hβD2 and hβD4. Taken together, these results suggest that ΔNp63 transcriptionally regulates the expression of hβD1, hβD2 and hβD4 in normal and cancer epithelial cells.

In vivo, ΔNp63 immunoreactivity was associated with hβD1, hβD2 and hβD4 expression in a series of cervical SCC and in HNSCC. Indeed, specimens with a high expression of ΔNp63 had a stronger staining for hβD1, hβD2 and hβD4 compared to specimens with weak or absent expression of ΔNp63. Most interestingly and in agreement with some published data showing an association between ΔNp63 expression and a poor outcome in patients with HNSCC (Lo Muzio et al., 2005) or melanoma (Matin et al., 2013), we demonstrated that ΔNp63 strong immunoreactivity was associated with poorer overall survival in a cohort of 39 patients with HNSCC.

Because of the aforementioned results demonstrating the regulation of hβD1, hβD2 and hβD4 by ΔNp63 and the association between ΔNp63 to a poor clinical outcome in patients with HNSCC, we addressed the effects of hβD1, hβD2 and hβD4 on the tumor microenvironment. Staining for newly-formed blood vessels (with CD105) revealed that tumors with high ΔNp63 expression were infiltrated by a higher density of blood vessels compared with tumors with weak or absent ΔNp63 staining. Suspecting a role for ΔNp63-regulated defensins in tumor angiogenesis, we tested hβD1, hβD2 and hβD4 for angiogenic effects and showed that each defensin stimulates primary endothelial cell chemotactic migration in a CCR6-dependent mechanism *in vitro*, and angiogenesis in a chicken chorioallantoic membrane assay (CAM assay) *in vivo*. HβD1 further promotes endothelial cell viability and proliferation.

With the same rationale, we addressed the effect of defensins on lymphangiogenesis and similarly, we observed that tumors with strong expression of ΔNp63 are invaded by a higher quantity of lymphatic vessels. hβD1, hβD2 and hβD4 promote lymphatic endothelial cell chemotactic migration in a CCR6-dependent mechanism *in vitro* and lymphangiogenesis in the CAM assay *in vivo*, but have no effect on lymphatic endothelial cell viability or proliferation.

3 Discussion

Our findings indicate that hβD1, hβD2 and hβD4 are expressed in cancer cells under the transcriptional regulation of transcription factor ΔNp63. Most interestingly, ΔNp63 immunoreactivity is associated to the expression of hβD1, hβD2 and hβD4 both in uterine cervix SCC and in HNSCC. In the latter, high tumor ΔNp63 immunoreactivity also relates to a poor outcome, in agreement with previous studies performed in oral SCC (Lo Muzio et al., 2005; Moergel et al., 2010). However, the mechanisms of ΔNp63-favored cancer spread are unclear.

Even though β-defensins were initially discovered as antimicrobial peptides, a growing body of evidence has characterized their pleiotropic chemotactic activity. Indeed, defensins have been implicated in the recruitment of immature dendritic cells (Yang et al. 1999; Hubert et al., 2007), macrophages, mast cells and lymphocytes (Grigat et al., 2007). We here demonstrate that hβD1, hβD2 and hβD4 are associated with high blood and lymphatic vessel densities in both uterine cervical SCC and head and neck SCC. Functionally, hβDs stimulate chemotactic migration of primary blood and lymphatic endothelial cells in a CCR6-dependent way.

Cancer-related angiogenesis is a metastasis-driving process (Carmeliet, 2003). Lymph-angiogenesis, although less extensively studied, also plays a key role in early metastatic dissemination in cancer and has been considered a potential therapeutic target as well (Duong et al., 2012). However, molecular actors involved in (lymph-) angiogenesis are numerous and not all are characterized. In this context, we establish the potent pro-(lymph-) angiogenic function of hβDs, which might be considered as potential new targetable compounds in cancer. Moreover, characterizing the implication of ΔNp63 in hβD transcriptional regulation, we provide a mechanistic explanation to the observed poor outcome linked to ΔNp63 overexpression in cervix and head and neck SCC.

Linked publications

ΔNp63 isoform-mediated β-defensin family up-regulation is associated with (lymph)angiogenesis and poor prognosis in patients with squamous cell carcinoma

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Keywords: p63, defensins, (lymph)angiogenesis, prognosis, squamous cell carcinoma

Received: *January 17, 2014* ***Accepted***: *March 19, 2014* ***Published***: *March 21, 2014*

ABSTRACT:

Beside a role in normal development/differentiation, high p63 immunoreactivity is also frequently observed in squamous cell carcinoma (SCC). Due to the complexity of the gene, the role of each p63 isotype in tumorigenesis is still confusing. Constitutively produced or induced in inflammatory conditions, human beta-defensins (HβDs) are cationic peptides involved in host defenses against bacteria, viruses and fungi. Here, we investigated both the role of p63 proteins in the regulation of HβDs and the implication of these antimicrobial peptides in tumor (lymph)angiogenesis. Thus, in contrast to TAp63 isotypes, we observed that ΔNp63 proteins (α, β, γ) induce HβD1, 2 and 4 expression. Similar results were observed in cancer tissues and cell lines. We next demonstrated that ΔNp63-overexpressing SCC are associated with both a poor prognosis and a high tumor vascularisation and lymphangiogenesis. Moreover, we showed that HβDs exert a chemotactic activity for (lymphatic) endothelial cells in a CCR6-dependent manner. The ability of HβDs to enhance (lymph)angiogenesis in vivo was also evaluated. We observed that HβDs increase the vessel number and induce a significant increase in relative vascular area compared to negative control. Taken together, the results of this study suggest that ΔNp63-regulated HβD could promote tumor (lymph)angiogenesis in SCC microenvironment.

INTRODUCTION

Member of the p53 family, *TP63* gene gives rise to transcripts that encode either full-length isoforms containing an amino-transactivation (TA) domain (TAp63) or truncated isoforms that lacks this TA domain (ΔNp63). Both TA and ΔN transcripts undergo C-terminal alternative splicing to yield six further carboxyl-terminal isotypes (α, β, γ) [1]. In the last decade, the implication of p63 proteins in epithelial stratification [2], differentiation [3] and in the maintenance of the proliferative potential of epithelial stems cells [4] has been well established. In addition to their role in normal development and homeostasis, the large majority of squamous malignancies display p63 immunoreactivity suggesting that, similar to p53, p63 is also acting during tumorigenesis [5]. However, due to both the complexity of the gene and the lack of reliable antibodies for each individual isotype, the role of p63 in cancer is still controversial and subject to debate [6, 7]. Recent data suggested that *p63* could play a dual function. Indeed, several studies have highlighted the oncogenic potential of ΔNp63α [8-11]. In contrast, other data show

that the *p63* gene, especially TAp63 isoforms, could act as a tumor suppressor [12-14], although *p63* is rarely mutated in human cancer in contrast to classic tumor suppressor genes.

Defensins are a family of small (2–6 kDa) cationic, antimicrobial peptides either constitutively secreted or induced in inflammatory conditions. Based on their amino acid sequence and pattern of disulfide bonding, mammalian defensins are classified into two main subfamilies: α and β defensins. Abundantly expressed by polynuclear neutrophils, α defensins were also isolated from subpopulations of macrophages and Paneth cells of the small intestine. To date, six human beta defensins (HβD1 to 6) have been discovered and cloned. Whereas HβD5 and HβD6 are specifically produced in the human epididymis, HβD1-4 are expressed by epithelial cells lining numerous organs (oral, nasal and epidermal mucosa, lungs, gastrointestinal and urogenital tracts) [15-17]. Through their direct antimicrobial activities, HβDs have emerged as important effectors of innate immunity [17]. Moreover, HβDs induce T cell and immature dendritic cell chemotaxis through chemokine receptor CCR6 and, therefore, might also link innate and adaptive immune responses [18-20]. Besides their role in the host defense, recent reports suggest that HβD expression could enhance tumor progression through unclear mechanisms [21]. By inducing dendritic cell and tumor-associated macrophage chemoattraction into cancerous lesions, it was proposed that HβDs could stimulate the production of tumor-promoting cytokines [22]. Moreover, *in vitro* data support that HβD2 could have some pro-angiogenic abilities [23].

The purpose of this study was to examine the regulation of HβD expression by p63 isoforms, as suggested in published microarray analyses [3, 24], and the implication of these small antimicrobial peptides in tumor vascularization and lymphangiogenesis. We showed that ΔNp63 proteins (α, β, γ) induce HβD1, 2 and 4 up-regulation whereas TAp63 isotypes do not modify HβD expression. These *in vitro* data were congruent with results obtained in cancer tissues [squamous cell carcinoma (SCC)]. Through a series of *in vitro* and *in vivo* experiments, we also demonstrated that ΔNp63-regulated HβDs are associated with tumor angiogenesis and lymphangiogenesis.

RESULTS

Positive association between ΔNp63 expression and HβD1, 2 and 4 levels in human keratinocytes and SCC cell lines.

To determine the possible relationship between p63 isoforms and HβD family, we first analyzed their expression (Western blot and/or RT-PCR) in human normal keratinocytes (HaCaT) as well as in two head and neck (Detroit 562, RPMI 2650) and three genital (A431, HT-3, SiHa) SCC cell lines. As shown in Figure 1A-B, all these cells expressed TAp63. Regarding ΔNp63 expression, normal keratinocytes and several SCC cell lines (A431, HT-3, Detroit 562) exhibited extremely high protein and mRNA levels of ΔNp63 whereas SiHa and RPMI 2650 cells did not express this p63 isoform. Interestingly, in contrast to other cell lines, no or a weak mRNA expression of HβD1, 2 and 4 was observed in SiHa and RPMI 2650. No difference in HβD3 expression was detected between ΔNp63-positive and negative cells. Therefore, our data suggest that ΔNp63 could be involved in the regulation of HβD1, 2 and 4 expression.

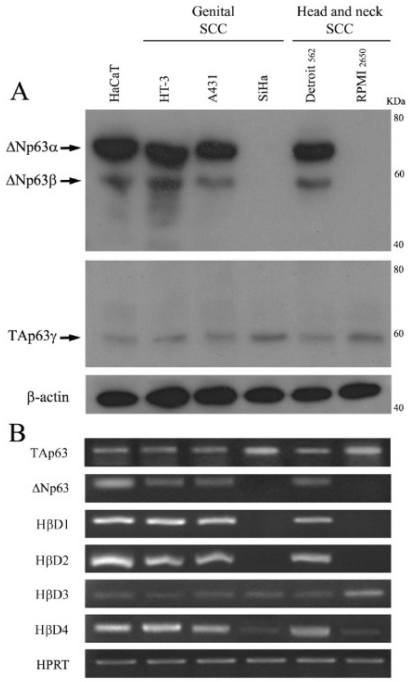

Figure 1: Expression of the p63 isoforms and HβDs in human normal keratinocytes and SCC cell lines. A: Both ΔNp63 and TAp63 isotypes were detected by Western blot using anti-p40 (ΔN) and anti-TAp63 antibodies. B: RT PCR analysis of HβD1, HβD2, HβD3, HβD4, ΔNp63 and TAp63 isoform expression was performed on mRNA isolated from normal keratinocytes (HaCaT cells) and five human SCC cell lines. HPRT was used as controls for RNA loading. 10^5 cells (from every analyzed cell line) were plated in six-well plates. When 60–70% confluence was reached, cells were lysed and subsequent Western Blot or RT PCR analyses were performed. The experimental procedures are extensively described in the "Materials and Methods" section. A representative experiment is shown of three independent experiments performed.

ΔNp63 isoforms regulate HβD1, 2 and 4 expression.

To examine whether p63 proteins could regulate HβD expression, we transfected different p63 isoform cDNA sequences in normal keratinocytes and HT-3 SCC cell lines. p63 isoforms were upregulated at 24h (data not shown) and remained overexpressed until 48h (Figure 2A). HβD expression was assessed by real-time RT-PCR. Each experiment was normalized to the amount of HPRT mRNA from the same sample. As a control, cells were transfected with the corresponding empty vector. We showed that transient transfection of ΔNp63 isoform cDNAs (Figure 2B-E) for 48h significantly induce HβD1, 2 and 4 expression in HaCaT cells. No statistical difference in HβD3 expression was observed (Figure 2D). In contrast to ΔNp63 isoforms, TAp63α, β and γ did not alter the pattern of HβD expression (Figure 2B-E). No synergistic effect was detected when cells were simultaneously transfected with all ΔNp63 isoform cDNAs (data not shown). Similar results were observed with the cervical HT-3 SCC cell line (data not shown). In order to determine whether ΔNp63 isoforms influence HβD mRNA stability, we evaluated the rate of mRNA degradation. The RNA transcription activity was inhibited by actinomycin D and the mRNA level of HβD 1, 2 and 4 relative to GAPDH mRNA was then determined by quantitative real-time PCR. HaCaT cells were transiently transfected with ΔNp63α cDNA and, 24h after transfection, the cells were treated with 5μg/ml actinomycin D. The levels of HβD 1, 2 and 4 mRNA transcripts were determined at 1, 2, 4 and 6h following actinomycin D addition. Compared to control cells (empty vector), no significant difference of the rate of HβD 1, 2 and 4 decay was observed in ΔNp63α-transfected cells (Figure 2F). Similar results were obtained with other ΔNp63 isotypes (data not shown). These data suggest that ΔNp63 do not modulate HβD transcript levels via an increased mRNA stability.

Inversely, we investigated the effect of an inhibition of ΔNp63 on HβD1, 2 and 4 expression using RNA interference strategy both in normal keratinocytes and cancer cells. siRNA transfection efficiency was assessed by flow cytometry (Figure 3A). ΔNp63 silencing efficiency (24, 48 and 72h after transfection) was analyzed by real-time RT-PCR (Figure 3B), Western blot (Figure 3C) and immunohistochemistry (Figure 3G). As a

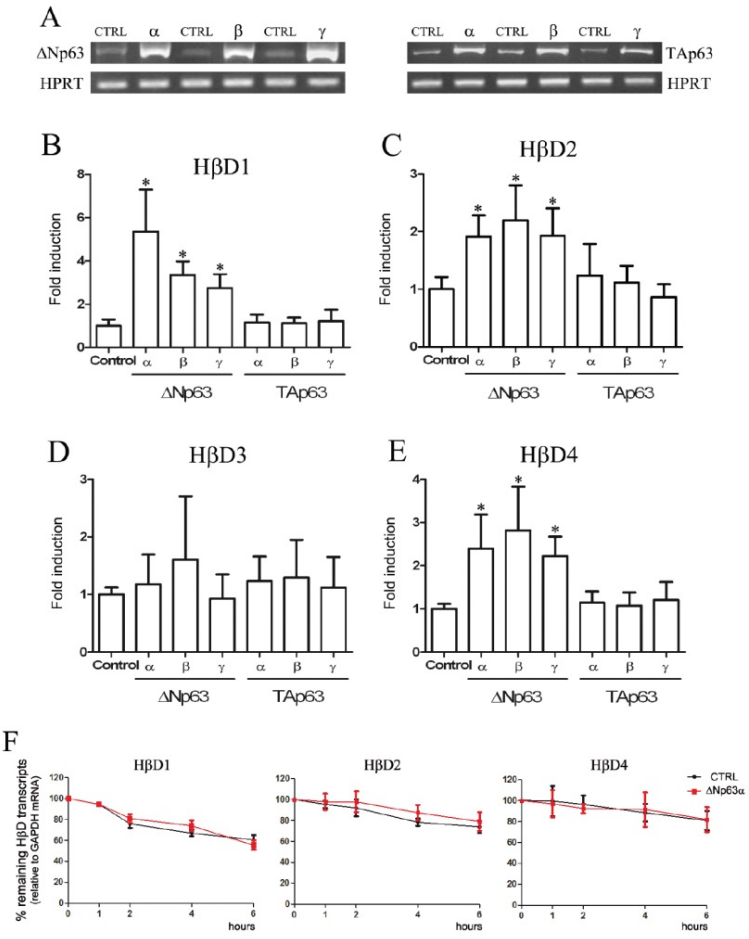

Figure 2: HβD1, HβD2 and HβD4 expression is up-regulated by ΔNp63 transfection. A: cDNA corresponding to different p63 isotypes were transfected in HaCaT cells. Forty-eight hours after transfection, mRNAs were extracted and RT-PCR analyses were performed using primers specific for each p63 isoform. B-E: Real-time RT-PCR analysis of HβD1, HβD2, HβD3 and HβD4 expression were performed on mRNA isolated from HaCaT cells transiently transfected with the different p63 isoform cDNAs. At 48h after transfection, cells were collected for transcriptional analysis. The experimental procedures are extensively described in the "Materials and Methods" section. F: ΔNp63-transfected cells were treated with 5μg/ml actinomycin D 24h after transfection. mRNAs were isolated at the indicated times after actinomycin D application. Each real-time RT-PCR experiment was normalized to the amount of GAPDH mRNA from the same sample. Results are the means ± SD of four independent transfection experiments performed in duplicate. Asterisks indicate statistically significant differences (*$P < 0.05$).

control, cells were transfected with a siRNA which does not match to any sequence in the human genome. Results indicated that ΔNp63 silencing significantly reduce HβD1, 2 and 4 expression in normal and cancer cells (at least 30% decrease compared to siRNA control-transfected cells) (Figure 3D-F). This downregulation of HβD1, 2 and 4 in ΔNp63-silenced cells was also observed at the protein level (Figure 3G).

ΔNp63 immunoreactivity is associated with high levels of HβD1, 2 and 4 expression in cervical and head and neck SCC

By immunohistochemistry, the expression of HβD1, 2, 4 and ΔNp63 was then investigated in 18 cervical and 39 head and neck SCC specimens (Figure 4). Positive staining for ΔNp63 was observed in 54 tissue samples (94.7%). However, variable degrees of nuclear ΔNp63 expression were detected (Figure 4A). High expression of ΔNp63 (score >3) was observed in 9 (50%) cases of cervical SCC and in 21 (53.8%) cases of head and neck SCC. Furthermore, we analyzed HβD1, 2 and 4 expression in all these tissue specimens. These peptides were distributed in the cytoplasm of neoplastic cells. A nuclear HβD1 staining was also observed in 16 cases (28.1%) (Supplemental Figure 1). We showed that tumors with a highly positive ΔNp63 immunoreactivity were significantly associated with a global up-regulation of HβD1, 2 and 4 (Figure 4B). Indeed, a co-expression of ΔNp63 and hβDs was observed in numerous serial sections of SCC (Supplemental Figure 2). A similar association between hβD immunoreactivity and ΔNp63 expression was observed in normal squamous epithelia (Supplemental Figure 3). A Spearman correlation between ΔNp63 and HβD scores was also observed both in patients with cervical and head and neck SCC (Supplemental Figure 4). These results support the involvement of ΔNp63

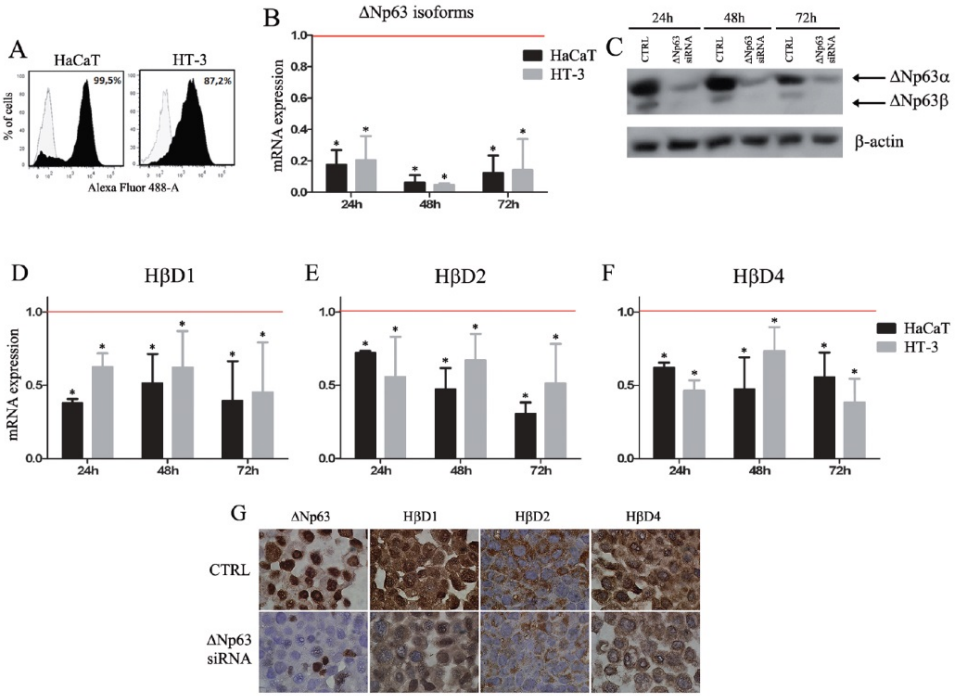

Figure 3:ΔNp63 silencing reduces HβD1, HβD2 and HβD4 expression in both normal keratinocytes and cancer cells. A: First, siRNA transfection efficiency was assessed by flow cytometry using a Alexa Fluor 488-labeled control siRNA. B: Real-time RT-PCR analyses of ΔNp63 isoform expression were performed on mRNA isolated from ΔNp63 siRNA-transfected HaCaT and HT-3 cells. As a control, these cell lines were transfected with a siRNA which does not match to any sequence in the human genome. Each experiment was normalized to the amount of HPRT mRNA from the same sample. Results are the means ± SD of four independent transfection experiments performed in duplicate. C: ΔNp63 protein levels were also determined by Western blot in the ΔNp63-silenced HaCaT cells compared with the control cells. A representative experiment is shown of three independent experiments performed. D-F: Real-time RT-PCR analyses of HβD1, HβD2 and HβD4 expression were performed on mRNA isolated from ΔNp63-silenced HaCaT and HT-3 cells. Each real-time RT-PCR experiment was normalized to the amount of HPRT mRNA from the same sample. Results are the means ± SD of four independent transfection experiments performed in duplicate. Red bars represent corresponding controls for each condition. Asterisks indicate statistically significant differences (*$P < 0.05$). G: The HβD protein level was also evaluated on HaCaT cells transfected or not with ΔNp63 siRNA. A reduced HβD1, 2 and 4 immunoreactivity was observed in ΔNp63-silenced cells.

in the regulation of HβD1, 2 and 4 in cancer tissues.

Prognostic value of ΔNp63 expression in head and neck SCC samples.

Due to the very few number of clinical data available [6 (33%) out of 18 patients], the prognostic value of cervical SCC samples was not analyzed. The follow-up data of the head and neck SCC patients for up to 138 months were used to evaluate the impact of ΔNp63 expression on overall survival. The series of head and neck SCC specimens presented in this study was composed of men (n=33) and women (n=6) from 40 to 79 years of age. Eighteen (46.2%) of these cancers were infected by high-risk HPV. Importantly, 27 (69.2%) and 12 (30.7%) of these patients were respectively active smokers or drinkers. None of these clinicopathological features were correlated with a high ΔNp63 expression (Table 1). During the follow-up period, 11 of 21 (52.3%) patients in the "ΔNp63++" group and 4 of 18 (22.2%) patients in the "ΔNp63+/-" group died. Based on Kaplan-Meier survival analysis, overall survival for patients with ΔNp63-overexpressing SCC was significantly decreased compared to that for individuals with cancers displaying a weakly positive ΔNp63 immunoreactivity (Figure 4C).

HβD1, 2 and 4 enhance tumor angiogenesis in ΔNp63-positive SCC through promoting endothelial cells migration.

We next analyzed the blood vessel density in high and low ΔNp63-expressing cervical and head and neck SCC using anti-CD105 antibody. Also called endoglin, this accessory protein of the transforming growth factor

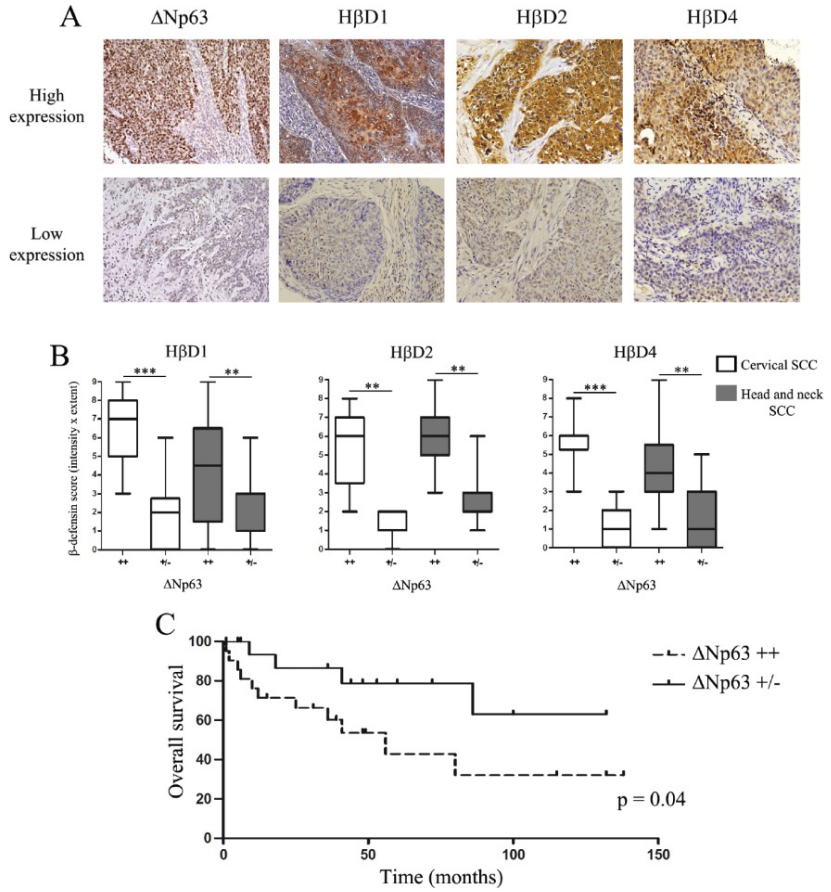

Figure 4: High HβD1, 2 and 4 immunoreactivity is observed in human SCC overexpressing ΔNp63. A: HβD1, HβD2, HβD4 and ΔNp63 expression in paraffin-embedded sections of human SCC specimens was assessed by immunohistochemistry. We observed variable degrees of HβD1, HβD2, HβD4 and ΔNp63 expression. B: Semiquantitative evaluation of HβD1, HβD2 and HβD4 expression in 18 cervical and 39 head and neck SCC specimens. The tissue samples were classified into two groups according to ΔNp63 immunoreactivity (high: ++, low: +/-). Asterisks indicate statistically significant differences (**$P < 0.01$, ***$P < 0.001$). Original magnifications: X200. C: Kaplan-Meier curve (overall survival) for patients with head and neck SCC expressing low (n= 18) or high (n=21) levels of ΔNp63.

Table 1: Variable analysis for ΔNp63 expression in head and neck SCC			
	High ΔNp63	Low ΔNp63	
			P
Clinical factors			
Age (y), median (SD)	58.1 (8.4)	57.6 (10.1)	
Sex			0,6674
Male	17	16	
Female	4	2	
Smoker			1
yes	15	12	
no	6	6	
Drinker			1
yes	6	6	
no	15	12	
Primary site			0,5279
Oral cavity	8	9	
Oropharynx	13	9	
T stage			0,7424
T1-T2	14	13	
T3-T4	7	5	
HPV DNA			0,738
positive	9	9	
negative	10	7	

beta receptor system is highly expressed on vascular endothelial cells [25]. Through CD105 immunolabeling on tumor sections, the average number of neoformed blood vessels per mm^2 was evaluated by image analysis. As shown in Figure 5A-B, we observed that ΔNp63-overexpressing SCC are associated with a significantly higher tumor vascularisation compared to cancers displaying a weakly positive ΔNp63 immunoreactivity.

In order to explore the ΔNp63-regulated HβD impact on the endothelial cell recruitment, Boyden Chamber migration assays were performed. As expected, VEGF-A elicited a considerable HUVEC migration, rising to 340% of the control (Figure 5C). A significant chemotactic activity of HβD1, 2 and 4 was observed for endothelial cells at a concentration as low as 0.25 µg/ml. When employed at a 0.5 µg/ml concentration, these three HβDs elicited a chemotactic effect similar to that exerted by VEGF-A, the positive control (Figure 5C). Furthermore, the HβD1, 2 and 4-dependent chemotaxis was similar when 0.5 µg/ml or higher concentrations were used (Figure 5C). We next investigated the involvement of chemokine receptor CCR6 on the capacity of vascular endothelial cells to migrate toward HβDs. Interestingly, pretreatment of the endothelial cells with a CCR6-blocking antibody partially abrogated the migration induced by HβDs (Figure 5D). We also observed that, similar to VEGF-A, HβD1 promoted endothelial cell proliferation/viability (Figure 5E-F). In contrast, cell growth was not significantly modified when HβD2 and 4 were used. The angiogenic activity of HβDs *in vivo* was then investigated using the CAM assay (Figure 5G). On day 10, there was a high density of blood vessels within and around the methylcellulose sponge in CAMs exposed to VEGF-A and HβDs. Each CAM section was immunostained for alpha SMA, a marker whose expression is relatively restricted to vascular smooth muscle cells. We observed that both VEGF-A and HβDs induce a significant increase in relative vascular area compared to negative control (PBS) (Figure 5H). This higher blood vessel area observed in the presence of VEGF-A and HβDs relied on an increased vessel number (Figure 5I). Confirming the implication of HβDs in angiogenesis, an association between HβD expression level (mainly HβD1 and 2) and the density of blood vessels in both cervical and head and neck tumor specimens was observed (Supplemental Figure 5). Although we fail to reach a statistical significance in SCC tissues with HβD4, our data suggest that these peptides play a relevant role in tumor angiogenesis.

ΔNp63-regulated HβDs senhance lymphangiogenesis by inducing lymphatic endothelial cell chemotaxis

The lymphatic vasculature was finally investigated in cervical and head and neck SCC, by evaluating the average number of podoplanin$^+$ vessels per mm^2. Significantly increased density of lymphatic vessels was detected in high ΔNp63-expressing SCC when compared to tumors with a weakly positive ΔNp63 immunoreactivity (Figure 6A-B).

The influence of ΔNp63-regulated HβDs on chemotactic migration of lymphatic endothelial cells was then assessed using a Boyden chamber assay. As shown in Figure 6C, a significant increased migration of immortalized (hTERT-LEC) lymphatic endothelial cells was observed in the presence of both VEGF-C (positive control) and HβD1, 2, 4 compared to negative control. Moreover, the chemotactic activity of HβDs on lymphatic endothelial cells was similar when we used 0.5 µg/ml or higher concentrations (Figure 6C). Similar results were obtained with normal cells (HMVEC) (Supplemental Figure 6). HβD-dependent enhanced migration was totally abrogated by addition of a CCR6-blocking antibody (Figure 6D). Regarding cell growth and viability, no difference was observed when lymphatic endothelial cells were treated with HβDs 1, 2 or 4 (Figure 6E-F). The lymphangiogenic activity of HβDs *in vivo* was then investigated using the CAM assay. Numerous vascular

structures, lined by alpha SMA-negative endothelial cells, were detectable on CAM sections. Immunohistochemical staining for the lymphatic biomarker Prox-1 confirmed the lymphatic nature of these vessels (Figure 6G). Image analysis was used to quantify CAM lymphangiogenic response from optical microscopy observation (Prox-1 staining). We demonstrated that both serum (positive control) and HβDs induce a significant increase in relative lymphatic vascular area compared to PBS (Figure 6H). This increased lymphatic vessel area was relied on a higher vessel number (Figure 6I). Similar to angiogenesis, HβD-overexpressing tumor specimens were associated with a higher tumor lymphangiogenesis compared to cancers displaying a weakly positive HβD immunoreactivity (Supplemental Figure 7). Collectively, our results support the implication of HβDs in lymphangiogenesis.

DISCUSSION

Whatever the tumor location (cervix, head and neck, esophagus…), the overall survival rate for SCC is low, largely due to the capacity of cancer cells to disseminate via blood and/or lymphatic circulations. The formation of new blood vessels is well-known to play a key role during the tumor growth and metastasis processes [26]. Similarly, accumulating evidence suggests that lymphangiogenesis also promotes the tumor progression and regional lymph node involvement is both early sign of metastasis and one of the strongest poor prognostic indicators. Understanding the interplay between cancer cells and (lymph)angiogenesis could define new molecular targets that might prevent the very initial stage of tumor spreading from the primary site and, therefore, increase

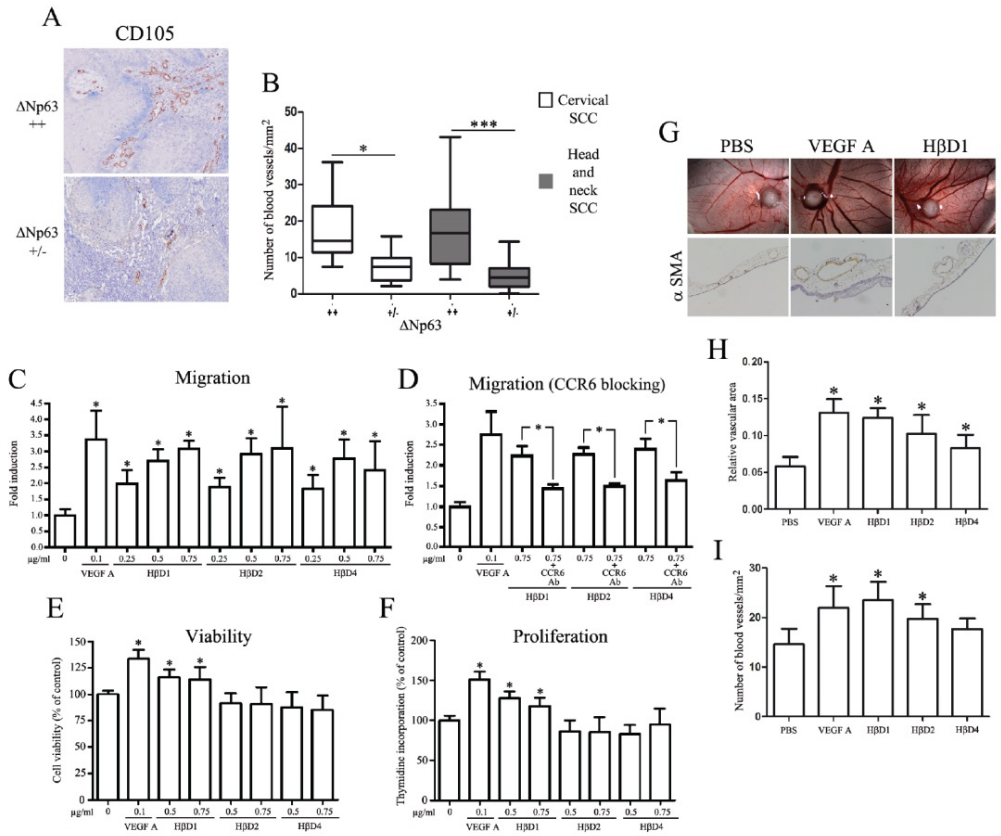

Figure 5: ΔNp63-regulated HβDs enhance endothelial cell migration. A: CD105 immunostaining showing the neoformed blood vessel density in high and low ΔNp63-expressing SCC. Increased counts of $CD105^+$ blood vessels were detected in high ΔNp63-expressing cervical and head and neck SCC when compared to tumors with a weakly positive ΔNp63 immunoreactivity (B). The average number of blood vessels per mm^2 was measured as described in the "Materials and Methods" section. Influence of HβDs on endothelial cell migration in a Boyden Chamber assay (C), viability (E) and proliferation (F). The HβDs-related migratory ability of blood vessel cells was also measured with a CCR6-blocking antibody (D). PBS and VEGF-A were used as negative and positive control, respectively. Data are presented as the means ± standard deviation of four different experiments. G: Enhancement of angiogenesis by HβDs in the CAM assay. Representative images of alpha-SMA positive-blood vessels are shown. Quantification of blood vessels has been performed by computerized image analysis. (H) Relative vascular area and (I) number of blood vessels per mm^2 were determined. Asterisks indicate statistically significant differences (*$P < 0.05$).

patient survival [27]. However, the molecular and cellular basis of blood and lymphatic abnormalities associated with cancers remains unclear and the subject of numerous investigations.

HβDs are antimicrobial peptides produced primarily by epithelial cells. Recently, several studies have showed the implication of HβDs in immune cell chemotaxis, activation as well as in wound healing suggesting that the collective effects of these small peptides extend well beyond their antiviral/bacterial activities [21]. Moreover, reports have analyzed the expression of HβDs in normal and (pre)neoplastic tissues at mRNA and protein levels and showed that HβD-1 and 2 were particularly expressed in well differentiated oral SCC [28]. Although pro-inflammatory cytokines, bacterial products and TGF-β have been shown to induce HβD upregulation, transcription factors and associated signaling pathways that regulate the expression of HβDs still remain unknown. Furthermore, these data suggest that HβD expression could be regulated by multiple factors and be cell or tissue-type dependent [21].

In the current study, upon examination of several human SCC cell lines, we found a clear association between ΔNp63 and HβD-1, 2 and 4 expression. No

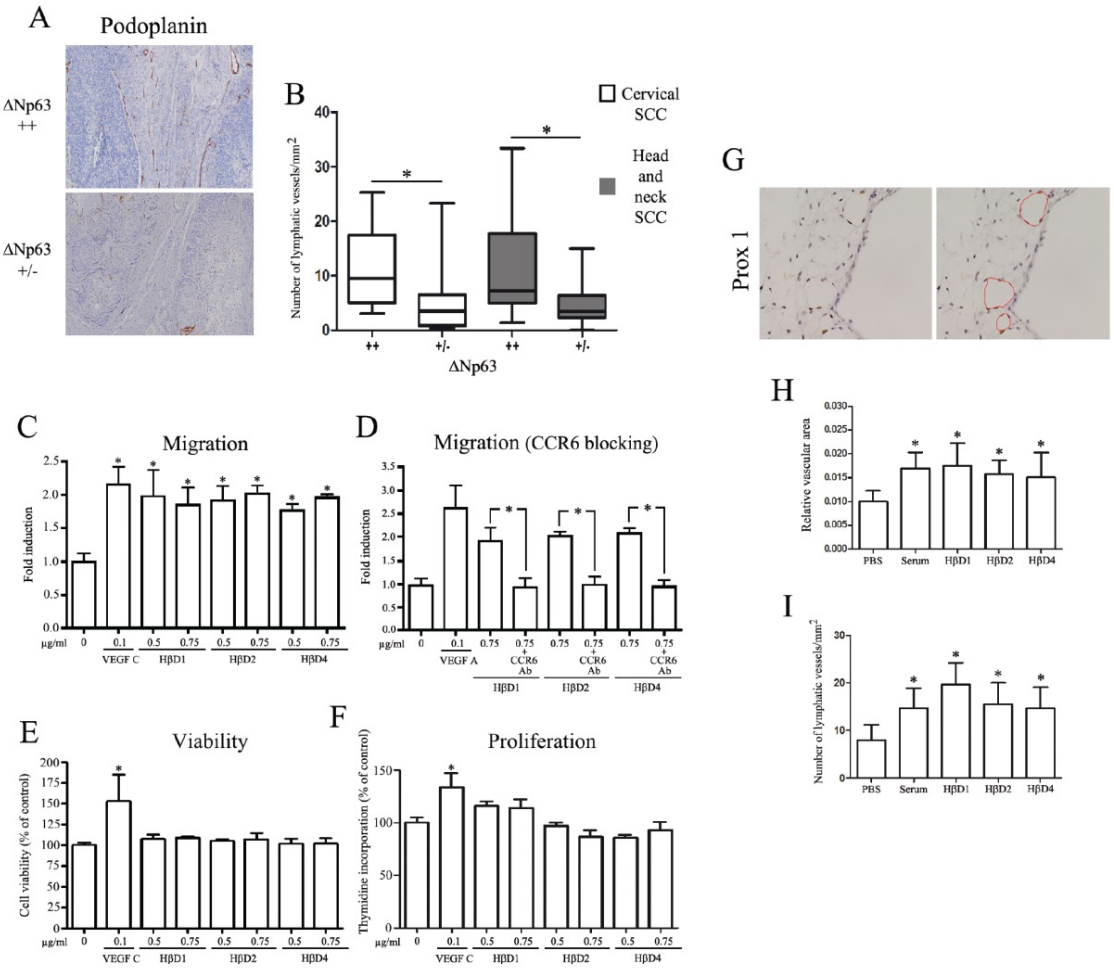

Figure6:ΔNp63-regulated HβDs promote lymphangiogenesis. A: Anti-Podoplanin antibody was used to determine the lymphatic vessel density in high and low ΔNp63-expressing SCC. Increased counts of podoplanin$^+$ lymphatic vessels were observed in high ΔNp63-expressing cervical and head and neck SCC when compared to neoplastic lesions with a weakly positive ΔNp63 immunoreactivity (B). The average number of lymphatic vessels per mm^2 was determined. Influence of HβDs on lymphatic endothelial cell migration (C), viability (E) and proliferation (F) was analyzed as described in the Materials and Methods section. Boyden chamber assay of lymphatic endothelial cells with CCR6-blocking antibody was also performed (D). PBS and VEGF-C were used as negative and positive control, respectively. Data are presented as the means ± standard deviation of four different experiments. G: Enhancement of lymphangiogenesis by HβDs in the CAM assay. Representative images of Prox1 positive-blood vessels are shown. Quantification of lymphatic vessels has been performed by computerized image analysis. (H) Relative vascular area and (I) number of lymphatic vessels per mm^2 were determined. Asterisks indicate statistically significant differences (*P < 0.05).

difference in terms of HβD3 expression was observed between ΔNp63-positive and negative cells. In order to demonstrate this relationship between p63 and HβDs, we transfected p63 isotype cDNAs in normal and SCC cells and demonstrated that, in contrast to TAp63 isoform, ΔNp63α, β and γ increase HβD expression at the mRNA levels. This HβD upregulation was not related to an increased HβD mRNA stability. Inversely, a decrease of HβD-1, 2 and 4 expression following ΔNp63 silencing was shown in HaCaT and HT-3 cells. By immunohistochemistry, we demonstrated that this downregulation of HβD1, 2 and 4 in ΔNp63-silenced cells was also observed at the protein level. We tried to confirm all these results by Western blot analysis. Numerous protocols were used. However, similarly to other studies and probably because of the small size of these peptides (2–6 kDa), it was impossible to detect HβDs, excepted when 0.5 or 1μg of recombinant proteins (positive control) was used. In agreement with our results and according to published microarray analyses, HβD1 and 4 were two genes highly down-regulated (3 to 10 fold) after disruption of p63 expression in several cancer cell lines [24]. These data were obtained using a siRNA targeting all p63 transcripts.

To evaluate the association between HβD-1, 2 and 4 and ΔNp63 isoform expression in human SCC specimens, immunohistochemical analyses were performed. A significant increase in HβD1, 2 and 4 immunoreactivity was observed when ΔNp63 was overexpressed. In addition, ΔNp63 and HβD scores were correlated both in cervical and head and neck SCC confirming our *in vitro* results. Previous data from our laboratory and others showed variable degrees of nuclear p63 expression in SCC tissues [29, 30]. Furthermore, marked overexpression of ΔNp63 has been recently associated with increased proliferation, radiation resistance and unfavorable outcome in the context of several cancers [9, 31-33]. Although ΔNp63 may reduce apoptosis and promote cell proliferation, the implication of ΔNp63 isoforms in soluble factor expression and cancer microenvironment has not been extensively explored. We demonstrated that ΔNp63-regulated HβDs stimulate the migration of (lymphatic) endothelial cells in a CCR6-dependent manner which may explain partially the increased density of blood and lymphatic vessels observed in high ΔNp63-expressing SCC. Beside HβD upregulation, *in vitro* data showed that ΔNp63 overexpression increases secretion of Interleukin 6, 8 and VEGF-A which could also promote angiogenesis in SCC microenvironment [34-35]. Regarding CCR6, the requirement of this chemokine receptor in HβD-dependent enhanced migration was, however, higher in lymphatic cells suggesting that these peptides could promote blood vascular cell chemotaxis via an alternative receptor. The *in vitro* results showing an implication of HβDs in (lymph) angiogenesis were confirmed using the *in vivo* CAM assay. In contrast to chemoattraction, HβDs display a limited effect on viability and proliferation of endothelial cells. In agreement with our results, Röhrl and collaborators recently showed an increased vascularization in mouse β defensin 14 overexpressing tumors. These authors also found that this enhanced angiogenesis is reduced in CCR6 knockout mice, indicating the requirement of CCR6 expression on mouse endothelial cells [36]. This chemokine receptor is also involved in HβDs-mediated human macrophage and mast cell chemoattraction.

Finally, the impact of ΔNp63 expression on overall survival was evaluated. Overall survival was significantly higher for patients with low ΔNp63 expression compared with those displaying a high expression for this p63 isoform. In agreement with our results, data reported that the overexpression of p63 in oral SCC was associated with poor radiation response and shorter survival [31, 32]. However, in contrast to our data, these studies did not reveal a potential mechanism and used a pan-antibody targeting all p63 transcripts; thus different p63 isoforms were not analyzed separately.

In conclusion, we demonstrated that ΔNp63 isoforms affect HβD expression in human SCC leading to increased blood and lymphatic vessel density in the tumor microenvironment and indirectly to a poor prognosis. Therefore, a treatment strategy aimed at reducing the adverse effects of HβDs may be effective at reducing metastasis dissemination and/or recurrence after surgery.

MATERIALS AND METHODS

Patients and tissue samples

Fifty-seven paraffin-embedded specimens of SCC [18 cervical SCC (mean age: 47 ± 9 years) and 39 head and neck SCC (33 men and 6 women; mean age: 58 ± 9 years)] were obtained from Pathology archives (University Hospital Center of Liege or Mons in the period between 2002 and 2010). Ten cases of normal ectocervical squamous epithelium were also retrieved. All cases were re-examined by a pathologist to confirm the diagnosis. Clinicopathological features were available for head and neck SCC patients (Table 1). These tissue samples were collected at the Tissue Bank of the University of Liege. The protocol was approved by the Ethics Committee of the University Hospital of Liege.

Primary cells, cell lines and cell culture media

Normal human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells (HMVEC) were purchased from Lonza (Verviers, Belgium) and were grown in MCDB 131 medium (Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 2mM glutamine, endothelial cell

growth supplement (12 ng/ml; BD Biosciences, Bedford, MA) and 2.5 mg/ml heparin. Immortalized lymphatic endothelial cells (hTERT-LEC) were cultured in EGM2-MV (Lonza) supplemented with 5% fetal bovine serum and glutamine. Immortalized human keratinocytes (HaCaT cells) and three genital SCC cell lines (A431, HT-3, SiHa) were grown in a 3:1 mixture of DMEM (Gibco) and Ham's F12 (Gibco) containing 10% fetal calf serum (FCS) and supplied with 1% non-essential amino acid (Gibco), 1% sodium pyruvate (Gibco) and 0.5% penicillin-streptamycin (Gibco). Two head and neck SCC cell lines (Detroit 562, RPMI 2650) were maintained in MEM (Gibco) containing 10% FCS and supplemented with 1% L-glutamine (Gibco). All the cell lines were incubated until a 60–70% confluence was reached.

Immunohistochemistry

Immunohistochemical analysis of paraffin-embedded specimens was performed as previously described [29, 37, 38]. Antibodies anti-ΔNp63 (anti-p40; Calbiochem, Gibbstown, NJ, USA), anti-HβD1 (Biologo, Kronshagen, Germany), anti-HβD2 (Abcam, Cambridge, MA, USA), anti-HβD4 (Abcam), anti-CD105 (Thermo Scientific, Waltham, MA, USA), anti-Podoplanin (Clone D2-40, Dako, Glostrup, Denmark), anti-alpha Smooth muscle actin (SMA) (Abcam) and anti-Prox1 (ReliaTech GmbH, Wolfenbuettel, Germany) were used for the primary reaction. Immunoperoxidase staining was performed using the Envision kit (Dako, Glostrup, Denmark) or the BrightVision Plus kit (Immunologic, Duiven, Netherlands) according to the supplier's recommendations. Positive cells were visualized using a 3, 3'-diaminobenzidine (DAB) substrate and the sections were counterstained with hematoxylin. A control IgG was used as negative control (Santa Cruz Biotechnology, Santa Cruz, CA, USA). To test the specificity of the HβD staining, the different anti-HβD antibodies were neutralized by the incubation with an excess of peptide. No immunoreactivity was observed in this condition.

Immunostaining assessment

According to a protocol previously described [29, 37], two independent histopathologists evaluated the immunolabeled tissues by using a semi-quantitative score of the intensity and extent of the staining according to an arbitrary scale. For staining intensity, 0 represented samples in which the immunoreactivity was undetectable whereas 1, 2 and 3 denoted samples with, respectively, a low, moderate and strong staining. For staining extent, 0, 1, 2 and 3 represented samples in which the immunoreactivity was detectable, respectively, in <5%, 6–33%, 34–66% and >67% of the tumor cells. In order to provide a global score for each case, the results obtained with the two scales were multiplied, yielding a single scale of 0, +1, +2, +3, +4, +6 or +9. This scoring system was validated using a computerized image analysis system (CAS; Becton Dickinson, Erembodegen, Belgium). The biopsies were classified into two groups: high (score >3) or low (score ≤3) ΔNp63 expression. Moreover, both the relative vascular area and the density of blood ($CD105^+$) and lymphatic ($Podoplanin^+$ or $Prox\text{-}1^+$) vessels in tumor microenvironment was quantified by computerized count (FSX 100 computerized image analysis system, Olympus, Hamburg, Germany), verified by manual counting and supervised by a histopathologist. The number of blood and lymphatic vessels was reported to the area around tumor cells yielding a count expressed as number of vessels/mm^2. A similar computerized quantification method was used to quantify the number and the vessel density in lymph/angiogenic CAM images.

Chicken chorioallantoic membrane (CAM) angiogenesis assay

On embryonic day 3, a window was open into the shell of fertilized chicken eggs. Four days later, methylcellulose 3 mm sponges saturated with 5 µg HβDs were laid onto the egg chorionallantoic membrane. VEGF-A (1.5 µg) (R and D systems, Minneapolis, MN, USA) or serum and PBS were used as positive and negative controls, respectively. Blood vessel density was evaluated and photographed on day 10. CAMs were then fixed (formalin) for 24 h, remove from the eggs and paraffin embedded using standard protocol. To identify blood and lymphatic vessels, anti-alpha SMA and anti-Prox1 were used, respectively. Thirteen CAMs were analyzed in each test group.

RT-PCR and quantitative real-time PCR analysis

Total RNA was extracted (RNeasy mini kit, Qiagen, Valencia, CA, USA) and cDNA was generated by reverse transcription as previously described [39]. For quantitative real-time PCR experiments, 15 ng of cDNA were amplified in 25 µl of 1× SYBR-Green I qPCR master mix plus (Eurogentec, Seraing, Belgium), containing 200 or 300 nmol/L of each primer. Primer sequences were as follows: TAp63 forward, 5'-TGTATCCGCATGCAGGACT-3'; TAp63 reverse, 5'-CTGTGTTATAGGGACTGGTGGAC-3'; ΔNp63 forward, 5'-GAAAACAATGCCCAGACTCAA-3'; ΔNp63 reverse, 5'-TGCGCGTGGTCTGTGTTA-3'; HβD1 forward, 5'-GTCGCCATGAGAACTCCCTACC-3'; HβD1 reverse, 5'-CATTGCCCTCCACTGCTGAC-3'; HβD2 forward, 5'-CCAGTTCCTGAAATCCTGAG-3'; HβD2 reverse, 5'-CTCTGTAACAGGTGCCTTGA-3'; HβD3 forward, 5'-AGTGACCAAGCACACCTTTTCA-3'; HβD3

reverse, 5'-CCAAAAACAGGAAGAGCAAAGC-3'; HβD4 forward, 5'-CCTGTTACCTGCCTTAAGAGTG-3'; HβD4 reverse, 5'-GAATCCGCATCAGCCACAG-3'; HPRT reverse, 5'-GGTCCTTTTCACCAGCAAGCT-3'; HPRT forward, 5'-TGACACTGGCAAAACAATGCA-3'; GAPDH reverse, 5'- ACCAGGTGGTCTCCTCTGAC-3'; GAPDH forward, 5'- TGCTGTAGCCAAATTCGTTG-3'. Thermal cycling conditions were: 50 °C for 2 min, 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60 °C for 1 min. All the experiments were performed in triplicate, using the ABI-Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and negative controls (master mix without any cDNA or RNA) were added in each run. Each quantitative real-time PCR experiment was normalized to the amount of HPRT or GAPDH mRNA from the same sample. The acquired data were analyzed by Sequence Detector software, Version 1.9 (Applied Biosystems). As an additional control, PCR products were run on 1.8% agarose gels containing ethidium bromide and visualized with an UV transilluminator.

Western blotting analysis

Cells were lysed in a buffer containing 50 mM TRIS pH 7,5, 300 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 (Igepal CA-630) (Sigma, Saint Louis, USA), 1 mM PMSF (Sigma) and protease/phosphatase inhibitors (Roche, Bale, Switzerland). After quantification (BCA protein assay; Pierce, Rockford, USA), western blot analysis was performed as previously described [37]. Anti-β-actin (Sigma), anti-ΔNp63 (anti-p40, Calbiochem) and anti-TAp63 (Biolegend, San Diego, CA, USA) were used as primary antibodies.

siRNA transfection and gene silencing

Small interfering RNA (siRNA) targeting specifically human ΔNp63 (5'-UGCCCAGACUCAAUUUAGU-3') was designed previously [29] and purchased from Eurogentec. The day before transfection, 10^5 cells per well of a six-well plate were seeded in 2 ml of appropriate growth medium. For each experiment, 50 ng of siRNA duplexes were transfected with the TransFectin lipid reagent (Bio-Rad, Hercules, CA, USA) according to the supplier's recommendations. A siRNA which does not match to any sequence in the human genome (Eurogentec) was used as control. The transfection of an Alexa Fluor 488-labeled control siRNA (Eurogentec) was performed in order to analyze the percentage of cells with siRNA uptake.

Transient transfections of p63 isoform cDNAs

pcDNA3 expression vectors (Invitrogen) encoding each p63 isoform were kindly provided by Dr. Caron de Fromentel (INSERM U590, Lyon, France). 1.25 x 10^5 cells were plated in six-well plates. Twenty-four hours after plating, cells were transiently transfected with ExGene reagent (Fermentas, Burlington, Canada) (2 µg plasmid DNA, 9 µl ExGene in 200 µl of 150 mmol/L NaCl). As a control, cells were transfected with the corresponding empty vector. A control transfection condition using a plasmid encoding GFP (pEGFP-IRESpuro, Clontech, CA, USA) was performed in parallel to determine the transfection efficiency. All experiments were set up to obtain at least 60% of transfected cells.

Boyden chamber migration assay

Chemotactic migration of HUVEC, HMVEC and hTERT-LEC were assessed using the Boyden chamber assay [18]. 3 x 10^4 cells were suspended in serum-free medium supplemented with 0.1% BSA and placed in the upper compartment of a 48-well Boyden microchamber (Neuroprobe, Cabin John, MD, USA). For inhibitory experiments, blocking anti-CCR6 antibody (Clone 53103, R and D systems) was added 20 min at 37 °C before treatments were started. The lower compartment was filled with a VEGF-A (R and D systems) (0.1 µg/ml), VEGF-C (R and D systems) (0.1 µg/ml), HβD1 (PeproTech, Rocky Hill, NJ, USA) (0.25 to 0.75 µg/ml), HβD2 (PeproTech) (0.25 to 0.75 µg/ml) or HβD4(PeproTech) (0.25 to 0.75 µg/ml) solution, containing 0.1% BSA. After 24 h of incubation at 37 °C, the cells that had migrated to the underside of the 8µm gelatin-coated filter (Poretics Corp., Livermore, CA, USA) were fixed and stained with Diff Quick Stain set (Baxter Diagnostics AG, Düdingen, Switzerland). The upper side of the filter was scraped to remove residual non migrating cells. One random field was counted per well using an eyepiece with a calibrated grid to evaluate the number of fully migrated cells. Experiments were performed four times in sixplicate.

Cell viability/proliferation

Cell viability and proliferation were determined by using the Alamar blue colorimetric-based assay (AbD Serotec, Dusseldorf, Germany) and the 3H-thymidine incorporation assay, respectively. Twenty four hours before stimulation with HβDs, 50,000 cells per well of a six well plate (viability) or 5,000 cells per well of a ninety-six well plate (proliferation) were seeded in appropriate growth medium. Both cell viability and 3H-thymidine uptake were measured after 4 days of culture. For the proliferation assay, the incorporated 3H-thymidine was

normalized to the total number of cells.

Statistical analysis

Statistical analysis was performed with Instat 3 software (Graph-Pad Software, San Diego, CA, USA). The statistical significance of the results was calculated by using a Student's *t* test (immunostaining) or a Mann-Whitney test (cell proliferation, migration, viability, quantitative real-time PCR). The correlations among the staining intensity of ΔNp63, HβD1, HβD2 and HβD4 were assessed using Spearman's correlation analysis. Categorical data from independent groups were compared using the Fisher's Exact Test. Overall survival was defined as the time from the date of registration to the date of death. Standard survival time analyses were performed using Kaplan–Meier curves. Differences were considered as statistically significant when *P* values were less than 0.05.

ACKNOWLEDGEMENT AND GRANT SUPPORT

This study was supported by the Belgian Fund for Medical Scientific Research, by the Centre Anti-Cancereux près l'Université de Liège, by the Faculty of Medicine of the University of Liège and by the Fonds Léon Frédéricq. The authors acknowledge the GIGA-imaging and Flow Cytometry facility. M.H. and M.S.C. are Postdoctoral Researcher and Research fellow of the Belgian National Fund for Scientific Research, respectively.

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CGFR-860; No. of Pages 10
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Cytokine & Growth Factor Reviews xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Cytokine & Growth Factor Reviews

journal homepage: www.elsevier.com/locate/cytogfr

Mini review

Defensins: "Simple" antimicrobial peptides or broad-spectrum molecules?

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ARTICLE INFO

Article history:
Available online xxx

Keywords:
Defensin
Immune system
Carcinogenesis
Biomarker

ABSTRACT

Small cationic peptides highly conserved in vertebrates, both α- and β-defensins were primarily identified as anti-microbial compounds involved in innate immunity. While human α-defensins are mostly expressed by neutrophils, β-defensins are secreted by epithelial cells of the skin and mucosae. Besides their anti-microbial activity, accumulating data emerged in the past decade indicating that defensins have extended functions in human physio(patho)logy. Indeed, defensins appeared as modulators of the adaptive immune system and angiogenesis, key mediators of wound healing and determinant players in male fertility. Furthermore, the impact of defensin expression in cancer and the potential use of these small peptides as biomarkers or even therapeutic tools should not be ignored. In the present review, we describe recent research works regarding the diversified functions of defensins, by mainly focusing on human models.

1. Introduction

Members of the antimicrobial peptide superfamily, defensins are small (~4–6 kDa) cationic peptides secreted in various species including humans and other mammals but also fishes, birds, insects, filamentous fungi, plants, etc. This wide distribution suggests that the production of such peptides is an ancient and well-conserved mechanism of host defense [1]. In humans, defensins are classified into two subgroups (α and β), based on both unique amino acid sequences and disulfide connectivities (Table 1). Primarily detected in neutrophils, α-defensins 1–4, known also as human neutrophil peptides (HNP), are also expressed in NK cells, monocytes and some T lymphocytes subsets. Human α-defensins (HD) 5 and 6 were initially detected in Paneth cells of the small intestine suggesting a role of protecting the intestinal stem cells within the crypt [2]. Moreover, the expression of these peptides was also reported in both respiratory and *gynecological tracts* [3–5]. Although genomic analyses reported at least 26 transcriptionally active human β-defensin (HβD) genes [6], to date, only a few have been cloned, isolated and fully characterized at the protein level. Whereas HβD1-4 are secreted by a large variety of mucosal epithelia including those lining urogenital, gastrointestinal and respiratory tracts [7], HβD5-6 genes seem to be specifically expressed in the epididymis [8]. Other human epididymal β-defensins (HβD14, HβD18, HβD23 and HβD26 encoded by DEFB114, DEFB118, DEFB123 and DEFB126, respectively) were also identified [9–11]. These latter peptides are, however, still largely unknown.

Originally discovered due to their antimicrobial activity, accumulating data suggest that the collective functions of defensins extend well beyond their activities in innate immunity. The present review is mainly based on recent studies on both α- and β-defensins of human origin and highlights the recent advances in the involvement of these arginine-rich peptides in the immune system, injury and carcinogenesis. The potential diagnostic/clinical relevance of their broad effects is also addressed.

2. Defensins: synthesis, processing, conformation and regulation

Despite the lack of similarity at the genetic level, all human defensins are synthesized *in vivo* as large prepro-peptide (up to ~110 amino acid residues) consisting of an N-terminal extension followed by the pro-peptide. This latter is composed of the pro-domain and the C-terminal mature defensin sequence (~35 residues) [12]. Typically very short and devoid of any inhibitory function in HβD pro-peptides, the pro-domain in α-defensin precursors contains ~40 amino acids and is essential to maintain the biologically active peptide in an inactive state. Through the

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http://dx.doi.org/10.1016/j.cytogfr.2014.12.005
1359-6101/

Table 1
Expression patterns of main human alpha- and beta-defensins.

	Defensin	Gene (location)	Main cell/tissue sources	Synthesis and stimuli
α-Defensins	HNP 1	DEFA1 (8p23.1)	Neutrophils, monocytes, macrophages, NK cells	Constitutive
	HNP 2	DEFA2 (8p23.1)		
	HNP 3	DEFA3 (8p23.1)		
	HNP 4	DEFA4 (8p23)		
	HD-5	DEFA5 (8p23.1)	Paneth cells, cervico-vaginal epithelial cells	Constitutive (but upregulated in the case of bacterial infection)
	HD-6	DEFA6 (8p23.1)		
β-Defensins	HβD 1	DEFB1 (8p23.1)	Epithelial cells (lining numerous organs)	Constitutive
	HβD 2	DEFB4A (8p23.1)	Epithelial cells (lining numerous organs)	Inducible by bacterial, fungal and viral products or proinflammatory cytokines
	HβD 3	DEFB103 (8p23.1)		
	HβD 4	DEFB104 (8p23.1)		
	HβD 5	DEFB105 (8p23.1)	Epididymis	Unknown
	HβD 6	DEFB106 (8p23.1)		
	HβD 14	DEFB114 (6p12.3)		
	HβD 18	DEFB118 (20q11.21)		
	HβD 23	DEFB123 (20q11.1)		
	HβD 26	DEFB126 (20p13)		

interaction with the mature peptide, the pro-segment acts as an efficient intramolecular inhibitor avoiding autocytotoxicity [13,14]. During neutrophil differentiation, HNP1-4 precursors would be excised by neutrophil elastase and proteinase 3 [15]. Resulting mature α-defensins are then stored in azurophilic granules which are released into phagocytic vacuoles containing ingested pathogens. Although neutrophils express various enzymes (*e.g.* elastase, proteases) and other bactericidal polypeptides (*e.g.* lactoferrin), HNPs constitute >40% of the total protein content within the azurophilic granules [16,17]. Almost undetectable in the extracellular fluids in normal healthy individuals, the plasma level of HNPs may reach concentrations up to 100 mg/ml in the setting of sepsis or bacterial meningitis [18]. This release of a large amount of HNPs is a consequence of neutrophil degranulation (exocytosis), lysis and/or leakage during phagosome formation [19]. In contrast to HNPs, processing of HD-5 in the intestinal crypt occurs extracellularly and is mediated by a trypsin isoform [20]. Interestingly, multiple processing intermediates of HD-5 and HD-6 were observed *in vivo*, especially in the female reproductive tract [3]. However, the proteases involved in the generation of these naturally occurring forms remain unknown. Regarding HβDs, a mixture of forms containing variable numbers of amino acids (between 36 and 47 residues in length) was detected in urine, blood plasma and cervicovaginal lavage, reflecting variable N-terminal proteolytic processing [21]. Despite the relatively minor changes in the peptide sequence, HβD intermediates display marked differences in their functional activity. Similar to HNPs, the enzyme(s) involved in HβD propeptide processing is still subject to investigations. Both HNP1 and HβD1 were recently identified as targets for matrix metalloproteinase 7. However, its apparent absence of expression in both squamous epithelia and neutrophils suggests that this protease is not involved in the intracellular processing of pro-defensins [22].

Both α and β defensins adopt a relatively compact molecular conformation with rigid β-sheet structures that are stabilized by three intramolecular disulfide bonds. However, as mentioned above, the disulfide-pairing pattern is specific for each defensin subgroup. Indeed, α-defensins are linked by cysteines 1–6, 2–4, and 3–5, whereas the six cysteine residues in HβDs are connected 1–5, 2–4 and 3–6 [7,23]. Although these disulfide connectivities are essential for the CCR2/CCR6-mediated chemotactic activities of defensins [24], unexpectedly, several studies demonstrated that they are not required for their antimicrobial activities. For example, linear unstructured HD-5 and HβD3 analogs exhibited similar anti-HIV and antimicrobial (against *Escherichia coli*) activities, respectively [24,25]. However, one exception was recently reported. In reducing environment, an increased antimicrobial activity of HβD1 against commensal bacteria and pathogens was demonstrated suggesting that redox-regulation could modulate the structure of HβD1 and, therefore, its function in mucosal surfaces [26].

Whereas HNPs are abundantly and constitutively expressed, in particular, in neutrophils, HD-5 and HD-6 basal expression levels are modulated in response to sexually transmitted infections (urethral secretions) [27] or to a down-regulation of the Wnt-signaling pathway (Tcf4 transcription factor in Crohn's disease) [28]. A recent study also identified IFN-γ as a trigger for HD-5 release in the intestinal epithelium [29]. Regarding HβDs, a ubiquitous expression of HβD1 was observed in all epithelia irrespective of their differentiation [2,7]. In mucosal surfaces lined by a squamous epithelium, ΔNp63 isoforms would be involved in the constitutive HβD1 mRNA expression, as recently demonstrated in squamous cell carcinoma [30]. However, this "master regulator" of squamous differentiation cannot explain the HβD1 secretion observed in both columnar and ciliated epithelia [5]. In contrast to HβD1, HβD2 and HβD3 are induced in inflamed/infected mucosa by bacterial, fungal and viral products or proinflammatory cytokines [*e.g.* interleukin (IL) 1β and tumor necrosis factor (TNF) α] [7,31]. These two latter soluble factors do not seem to affect HβD4 expression [32]. Although epidermal growth factor receptor (EGFR) signaling pathway could also participate in HβD up-regulation [33,34], the HβD synthesis is predominantly mediated by NF-κB and *Mitogen-activated protein kinase* (*MAPK*) pathways leading to AP-1 transcriptional activation [35]. Indeed, consensus NF-κB and AP-1 binding sites are present in the gene promoters of all inducible HβDs. Through the inhibition of AP-1 activity, retinoic acid has been shown to completely abolish HβD expression in keratinocytes. These results suggest that the balance between active metabolites of vitamin A and proinflammatory cytokines could be critical for HβD regulation in mucosal surfaces [36].

3. Defensins as antimicrobial peptides

All human defensins have demonstrated the ability to exert antimicrobial activities (killing and/or inactivation) over a wide variety of bacteria, viruses, fungi and protozoa. Although further investigations are still needed, the direct microbicidal mechanisms of these cationic effectors of innate immunity are presumably initiated by an interaction with the negatively charged membranes of pathogens [37,38]. The variability in the phospholipid composition of the bacterial membranes or viral envelopes might explain the differential antimicrobial effects of defensins which have been highlighted in numerous studies. Similarly, human lipid bilayer

membranes would be electrically neutral, therefore protecting efficiently host cells against defensin-induced cytotoxicity [37,38]. After the initial electrostatic interaction, defensins accumulate into the membrane of microbes and cause depolarization which would ultimately induce altered metabolism and death. In addition, defensins use their capacity to aggregate in order to form channels/pores stimulating membrane permeabilization. Mainly described in the HIV setting, defensins can also inactivate pathogens or prevent their cellular internalization by interacting with membrane/envelope glycoproteins [39]. Besides this direct interaction with microbes, interesting data revealed that HNP1-3 neutralize several secreted bacterial enzymes such as anthrax lethal toxin and, therefore, could protect the host against fatal consequences [40]. Some defensins extend their antiviral activity by acting on infected target cells. Through the interaction with cell surface receptors, HNPs could block intracellular signaling cascades resulting in the inhibition of viral replication/transcription [7,41]. The exact mechanism involved in this latter indirect antiviral activity is, however, still poorly understood. Moreover, both HNPs and HD-5 can inhibit virion escape from late endocytic vesicles as demonstrated *in vitro* with influenza virus and human papillomavirus (HPV) [7,42]. In contrast to α-defensins, and, for unclear reasons, HβDs failed to alter viral transduction [42]. Another aspect of antiviral activity of defensins is indirectly mediated by the induction of soluble factor expression by epithelial/immune cells. A well-documented example is the up-regulation of C–C chemokines by HNPs which could partially inhibit HIV infection through competition for host receptors [43]. Although numerous data support the involvement of both α- and β-defensins in antimicrobial immunity, in some cases, these peptides could enhance a viral infection. By increasing viral particle attachment to the target cells, HD-5 was demonstrated to promote HIV and adenovirus infection [44–46]. However, it is still unknown if this enhancement takes place *in vivo*. Furthermore, contradictory data were recently reported [47].

4. Defensins, inflammation and adaptive immune system

The idea that defensins could promote inflammation and stimulate adaptive immune response arose fifteen years ago with the works of Yang and colleagues demonstrating HβD2-induced CCR6-dependent chemotactic migration of immature myeloid dendritic cells (mDCs) and T cells [48]. Accumulating evidence has then suggested that defensins act as modulators of inflammation and activators of adaptive immune system. Indeed, defensins efficiently link innate and adaptive immunity mostly by directly stimulating immune cell migration, promoting the release of pro-inflammatory cytokines and by recruiting antigen-presenting cells and/or activating them to induce a Th1-skewed immune response. The receptors involved in immune cell chemoattraction/activation are listed in Table 2.

Besides CCR6-mediated mDC chemotaxis, HβDs also promote monocyte/macrophage migration. In contrast to data collected with mDCs, HβD2- and HβD3-dependent chemotactic migration of human monocytes was shown to be mediated by CCR2 receptor [49]. Similar to HβDs, α-defensins are also considered as pleiotropic chemotactic factors. Indeed, an increased macrophage (naïve, activated, memory) T cell and immature mast cell migration was observed *in vitro* in the presence of HNP1 HNP3 and HD-5 [50]. Although this chemoattraction is speculated to be mediated by a G protein-coupled receptor, this latter is still subject to investigations.

In addition to their chemotactic activity for professional antigen presenting cells, HβDs also induce their activation as assessed by the up-regulation of HLA complexes and surface costimulatory molecules such as CD80 and CD86. HβD3 activates mDCs and monocytes independently of CCR6 or CCR2 but *via* TLR1/2-MyD88 signaling pathway [51]. Demonstrated in mouse and still not confirmed in humans, TLR4 could also be involved in β-defensin-mediated mDC activation [52]. Furthermore, HβD2 and HβD3 are able to activate mDCs *via* an indirect mechanism. These two defensins attach to host or foreign DNA and support CpG nucleotide uptake by plasmacytoid DCs (pDCs) through endosomal TLR9 which in turn stimulates their activation. Activated pDCs consecutively release IFN-α, which activates mDCs to present antigens and initiate a T-cell immune response [53]. More recently, HβD3 was also shown to induce maturation of both Langerhans cell-like DCs (LC-DCs) and primary Langerhans cells (LC) from skin explants. HβD3-activated LC-DCs expressed CCR7 and migrated toward lymph node homing CCR7 ligands CCL19 and CCL21. Consistently with their activity of activated antigen-presenting cells, they induced proliferation of naïve T cells. Moreover, HβD3-activated LCs induced a strong production of IFN-γ by T lymphocytes, suggesting that HβD3 promotes Th1-skewing activity of LCs [54]. Interestingly, the addition of pertussis toxin, an inhibitor of Gi-coupled protein receptors, did not inhibit HβD3-induced LC maturation indicating that this effect is not mediated by CCR2, CCR6 or CXCR4. In contrast to previous works using mDCs [51], the involvement of TLR1-2-induced MyD88 signaling pathway was also precluded. The discrepancy between these results could reflect the phenotypic differences between mDCs and LCs.

In vitro and *in vivo* data suggest that both α- and β-defensins can act as proinflammatory compounds. Along with the recruitment of DCs and macrophages, HβDs induced cytokine secretion by inflammatory cells. As shown by protein array, HβD1, HβD2

Table 2
Cell type- and receptor-dependent effects of defensins.

Proposed receptor	Cell type	Defensin	Effect	Refs.
CCR6	Immature DCs and memory T cells	HβD2	Chemotactic migration	[48]
	Enterocytes	HβD3		[74]
	Vascular and lymphatic endothelial cells	HβD1, HβD2 and HβD4		[30]
	B220+ B lymphocytes	Mouse βD14		[64]
	DC precursors	Mouse βD29		[63]
	Sperm cells	HβD1	Ca^{2+} flux, mobilization	[82]
CCR2	Monocytes	HβD2-3	Chemotactic migration	[49]
	Monocytes and macrophages	HβD3		[61]
TLR4	DCs	Mouse βD2	Activation and Th1 skewing activity	[52]
TLR1/2	DCs and monocytes	HβD3	Activation	[51]
Endosomal TLR9	Plasmacytoid DCs	HβD2/CpG HβD3/CPG	Activation	[53]
Unknown GPCR	Macrophages, naïve and memory T cells and immature mast cells	HNP1, HNP3 and HD5	Chemotactic migration	[50]
Unknown	LCs	HβD3	Activation and Th1 skewing activity	[54]

and HβD3 stimulate massive cytokine production by peripheral blood mononuclear cells. In those cells, HβD2 was the most active peptide and mainly induced IL-6, IL-8 and CCL2 release [55]. In response to HD-5, intestinal epithelial cells were shown to secrete numerous pro-inflammatory soluble factors such as IL-2, IL-8, CCL20 and TNF-α [56]. Low doses of this α-defensin also induced IL-2, IL-8 and IFN-γ release by primary $CD4^+$ T cells suggesting a role for HD-5 in both promoting inflammation and participating in cell-mediated immune response [56]. In a mouse model, HNP-1 injection aggravated dextran sulfate sodium-induced colitis. Colon from HNP-1 treated mice was infiltrated by higher numbers of $F4/80^+$ macrophages and lymphocytes. Moreover, in colon culture supernatants from these mice, increased levels of IFN-γ, TNF-α and IL-1β were detected. These results are in accordance with a previous study showing that HNP-1 induces, at very low concentrations (10^{-9} M), the secretion of IL-1β and TNF-α from activated monocytes [57]. Altogether, these results further support the implication of defensins in promoting inflammation [58]. Conversely, some less characterized β-defensins only expressed in the epididymis might display an anti-inflammatory activity. As an example, recombinant HβD14 and HβD23 were shown to prevent LPS-induced release of TNF-α by macrophages and to protect mice from LPS-induced sepsis [59]. However, the mechanism of action of these epididymal defensins (LPS neutralization and/or repression of its proinflammatory action) is still unknown.

5. Defensin and (lymph-)angiogenesis

Angiogenesis is a key process in wound healing but can also be excessive in pathological conditions such as cancers or retinopathies. For all these reasons, modulators of angiogenesis have raised a lot of interest. Alpha- and β-defensins appear to have opposite consequences in neovascularization. While an inhibitory effect has been documented for α-defensins, β-defensins could promote (lymph-)angiogenesis by several mechanisms.

Recent evidence support that HβDs are able to directly affect blood and lymphatic endothelial cells. In a study investigating the effects of HβDs on the tumor microenvironment, we demonstrated that HβD1, 2 and 4 stimulate both vascular and lymphatic endothelial cell recruitment in a CCR6-dependent mechanism *in vitro* and promote vasculogenesis *in vivo* [30]. Similarly, HβD2 was found to induce primary endothelial cells chemotaxis *via* αvβ3 vitronectin receptor and to enhance tubule formation in a VEGF-independent way [60]. Whereas HβDs did not affect metabolic activity or cell proliferation at physiological concentrations, high doses (5–10 μg/ml) of HβD2 increased blood endothelial cell proliferation [60]. This unclear result could be related to factors such as serum or salt which are well-known to alter defensin functions in *in vitro* models. Beta-defensins might also induce angiogenesis *via* an indirect mechanism. For instance, intratumoral HβD3 increases the expression of the pro-angiogenic factor IL-8 by macrophages [61] and induces secretion of VEGF from both normal monocytes and macrophages [62]. These results attributing a pro-angiogenic role to HβDs are further supported by interesting data collected with β-defensins from mouse origin. CCR6-positive DC precursors, recruited in the tumor microenvironment by mouse β-defensin 29, were found to commit into endothelial-like cells in response to VEGF-A. In human ovarian carcinoma, such DC precursors expressing both DC and endothelial cell markers were detected suggesting that this cooperative effect of defensins and VEGF-A may occur in human as well [63]. More recently, another murine defensin (mouse β-defensin 14, an ortholog of HβD3) was also shown to be indirectly involved in tumor angiogenesis. Indeed, the expression of this defensin by fibrosarcoma cells injected in mice enhanced blood vascularization through chemoattraction of $CCR6^+$ lymphocytes which both interacted with cancer cells and stimulated pro-angiogenic CXCL2 secretion by these latter [64].

In contrast, inhibitory effects on angiogenesis have been described for α-defensins. In a model of hypoxia-induced retinal neovascularization, treatment of mice with a mixture of HNP1-3 significantly impaired angiogenesis [65]. HNPs also inhibited neoangiogenesis in the chicken chorioallantoic membrane assay [66]. *In vitro* results confirmed that α-defensins mainly inhibit α5β1-integrin-dependent migration of endothelial cells toward fibronectin, adhesion and reduce VEGF-induced vessel permeability [65,66]. HNPs also abrogated VEGF-induced endothelial cell proliferation and triggered endothelial cell apoptosis [66]. Regarding HD-5, high concentrations (5–10 μg/ml) were recently found to alter endothelial cell viability and growth and to prevent the sprouting of new blood vessels from rat aortic rings [67].

6. Defensins and wound healing

Wound healing is a multistep process requiring inflammatory response to injury, re-epithelialization and angiogenesis. In the past decade, a growing body of evidence has pointed out defensins as cornerstones to this process. Beta-defensins were shown to be expressed at wound sites and were shown to promote expression of pro-inflammatory cytokines by keratinocytes, cell migration and angiogenesis. For instance, HβD2 protein expression is induced in human skin wounds [68]. However, in patients with diabetes mellitus suffering from non-healing skin ulcers, HβD2 expression was detected but did not increase compared to normal skin [69]. Wound chronicity and/or disease context could partially explain this insufficient or absent induction of HβD2 [69]. In agreement with this hypothesis, high glucose levels were found to inhibit HβD2 expression in human keratinocytes [70]. In an extensive study, Niyonsaba and colleagues demonstrated that HβD2, 3 and 4, but not HβD1, stimulate mRNA expression and protein secretion of a large variety of pro-inflammatory cytokines in primary human keratinocytes [71]. They also observed that these three defensins further trigger chemotactic migration of keratinocytes and wound closure *in vitro*, along with cell proliferation. These effects were dependent on EGF receptor phosphorylation and activation of STAT1 and STAT3 signaling pathways. Similarly, the topic administration of HβD3 to infected wounds in Yorkshire pigs significantly accelerated wound closure [72]. In recent studies, HβD2 and HβD3 were shown to promote intestinal wound healing *in vitro* and in a neonatal rat model, respectively [73,74]. In contrast to keratinocytes, these defensins induced intestinal cell migration but not proliferation in a CCR6-dependent manner, suggesting that effects of β-defensins on epithelial cells are cell type and context-dependent.

7. Defensins and fertility

The urogenital tract and particularly the male reproductive tract is a major source of most β-defensins, whose expression changes with age and peaks during sexual maturation, suggesting a role for them in male reproductive function [75]. HβDs are expressed in testis and in diverse parts of the epididymis, where sperm cells maturate to acquire mobility and fertilization ability. They are secreted into the lumen and can bind to *spermatozoa* plasma membrane [76]. Emerging data from the literature suggest that β-defensins could participate in sperm cell maturation, motility and fertilization efficiency alongside with protecting *spermatozoa* from infections.

In a micro-array analysis, an altered expression of several genes encoding β-defensins was found in the epididymis of infertile men compared to fertile counterparts. Among these genes, two defensins were particularly down-regulated in

epididymis of infertile men: DEFB106 (encoding HβD6) and DEFB126 (encoding HβD26) [77]. In contrast to HβD6, HβD26 seems to be a major component in the sperm glycocalyx, a thick protective coating of sperm cells consisting of glycoproteins and polysaccharides. Unlike other β-defensins, HβD26 has a long C-terminal domain of 52 amino acids very rich in glycosylation sites. A deletion of 2 nucleotides generating a reading frame shift and a "non-stop mRNA" has recently been observed in men and associated with decreased O-linked glycosylation of the peptide, reduced gel-penetration ability of the *spermatozoa* and diminished male fertility [9]. Accordingly, several studies mostly conducted in non-human primates such as macaques had previously shown that HβD26 is a chief component of the sperm plasma membrane glycocalyx, mediates attachment of sperm cells to oviducts, favors penetration through cervical mucus and protects sperm cells against binding of anti-sperm antibodies [78–81]. HβD1 mRNA expression was also decreased in a transcriptomic analysis led by Dube and colleagues, albeit to a lesser extent. Suggesting an implication for HβD1 in male fertility, levels of HβD1 appeared lower in sperm from infertile men because of two specific diseases (leukocytospermia and asthenozoospermia, affecting bactericidal activity and sperm motility respectively) compared to the sperm of fertile men. Most interestingly, the authors showed that recombinant HβD1 restores sperm quality in infertile men. Mechanistically, HβD1 binds to CCR6 expressed in sperm cells and triggers Ca^{2+} mobilization which enhances cell mobility [82].

Along with glycocalyx integrity and sperm cell mobility, *spermatozoa* maturation in the epididymis involves a gain of fertilization ability and may be regulated by β-defensins as well. A recent genetic study performed in mice demonstrated that deletion of a 9 defensin gene cluster is sufficient to induce sterility in male mice. Sperm cells in mutant mice displayed alterations in their intracellular calcium contents, early acrosome reaction and decreased ability to bind oocytes [76].

HβDs are primarily anti-microbial peptides and, accordingly, also protect sperm cells against pathogens in the male and female reproductive tracts. HβD18 (encoded by DEFB118) for instance, an androgen-regulated sperm-binding defensin, displays antibacterial activities against bacteria such as *Escherichia coli* [11]. Defensins are nonetheless only part of a variety of sperm-bound or secreted antimicrobial peptides which comprise other defensin-like molecules such as rat 2D6 glycopeptide [83] or defensin-like Bin1B [84]. As mentioned above and in contrast to HβD2-4, some epididymal β-defensins (especially HβD14 and HβD23) could surprisingly mediate an anti-inflammatory activity and thereby preserve sperm function [10].

8. Defensin in carcinogenesis

8.1. Genetic variations

Human β-defensin genes are grouped in five genomic regions called defensin clusters [6]. In the 200 kb long "DEF cluster b", genes are extremely variable in copy numbers. Two to twelve copies per diploid genome were reported. In the past few years, copy number variation appeared as potentially linked to the susceptibility of developing cancer. In a cohort study involving patients with pancreatic adenocarcinoma and an age-matched control group of healthy individuals, striking differences were observed between groups in terms of gene copy numbers within DEF cluster b. In cancer patients, the presence of 5–6 copies was rarely encountered whereas occurrence of 3 or fewer copies was more frequent compared to the control group [85]. Similarly, a low copy number of DEFB4, the gene encoding HβD2, was found to increase the risk of cervical cancer development [86]. Although such genomic analyses should be extended to other cancers, these data suggest a potential protective effect of a high β-defensin gene copy number.

Another genetic source of variation in the expression of defensins is named multi-site variation and involves the presence of several single nucleotide polymorphisms (SNPs) in a gene. For instance, among the four haplotypes detected around exon 1 of DEFB104, two were under-represented among patients with prostate cancer (GGGC and CAAT) whereas two other ones (GAAT and GAAC) were over-represented in those patients, compared to the control group [87]. Likewise, two SNPs in DEFA6 (encoding HD-6) and DEFB1 (encoding HβD1) were linked to an increased susceptibility to develop gastric cancer [88]. One haplotype of DEFB1 determined by four SNPs was also overrepresented in oral cancer population [89]. More recently, a SNP in DEFB126 was found to create a haplotype over-represented in follicular lymphoma [90].

8.2. Altered defensin expression in cancer

Various studies have reported altered expression of both α- and β-defensins in neoplastic tissues suggesting their potential involvement in cancer initiation/progression. However, as shown in Table 3, these variations in defensin expression seem to be dependent on the cell differentiation as well as the origin of the tumors. Data from the literature consistently support a tumor suppressor function of HβD1 based on its cancer-specific loss or decreased expression. Indeed, a lower mRNA expression pattern of HβD1 in oral squamous cell carcinoma cell lines compared to normal epithelial cells [49] and a decrease in HβD1 protein expression from oral preneoplastic lesions to cancer was documented [51]. A cancer-specific down-regulation of HβD1 was also observed in renal and prostate carcinoma [91]. In contrast to HβD1, HβD2 and HβD3 expression is either increased or diminished in cancer depending on cancer type. An overexpression of HβD2 was recently reported in patients with esophageal carcinoma [92]. An increased expression of HβD3 in head and neck carcinoma was also shown at the mRNA and protein levels when compared to paired healthy tissues [34,93]. In disagreement with these works, Joly and colleagues showed a decrease in both HβD2 and HβD3 inductibility in oral cancer cells. However, this study was only performed on cell lines and addressed mRNA expression exclusively [89].

Data linking expression of α-defensins and cancer is scarce. Nonetheless, in patients with bladder cancer, plasma levels of HNP1-3 are elevated and reflect clinical stage [94]. Similar results were observed in colorectal cancer patients [95,96]. These data suggest a pro-tumoral activity of HNP1-3. However, since α-defensins are primarily expressed by neutrophils, which are more abundantly found in blood with cancer progression, these data should therefore be interpreted with caution and require further investigation.

8.3. Potential effects of defensins on both cancer cells and tumor microenvironment

Because of the aforementioned data regarding altered expression of defensins in several cancer types, one could hypothesize that defensins might play a role in cancer progression. Accordingly, accumulating evidence showed that these peptides may modify both cancer cell phenotypic properties and tumor microenvironment through various mechanisms (Fig. 1).

First, defensins can alter cancer cells in a cell autonomous way, modifying their capacity to proliferate or survive, migrate and invade adjacent tissues. In a human esophageal cell line, HβD2 gene silencing abrogated cell proliferation [92]. In contrast, ectopic expression of HβD1 had no effect on oral cancer cell proliferation [97]. Although the effect of HβD4 in cancer is poorly characterized, this defensin seems to have dose-dependent effects on cancer cell

Table 3
Expression of defensins in cancer patients.

Tumor	Defensin	Expression	RNA or protein	Location	Refs.
Oral (head and neck) cancer	HβD1	Down	mRNA	Cells, cancer tissue	[89,128]
	HβD1	Down	Protein	Cancer tissue	[97]
	HβD2	Down	mRNA	Cell lines	[89]
	HβD3	Up	mRNA and protein	Cancer tissue	[34,93]
Renal cancer	HβD1	Down	Protein	Cancer tissue	[91]
Prostate cancer	HβD1	Down	mRNA and protein	Cancer tissue	[91]
Esophageal cancer	HβD2	Up	mRNA and protein	Cancer tissue	[92]
Bladder cancer	HNP1-3	Up	Protein	Plasma	[94]
Cervical cancer	HD5	Down	Protein	Cancer tissue	[67]
Colorectal cancer	HD6	Up	mRNA	Cancer tissue	[129]
	HNP1-3	Up	Protein	Cancer tissue, serum	[95,96]
Lung cancer	HβD1-2, 4	Up	mRNA	Cancer tissue	[130]
	HβD1	Up	Protein	Serum	[122]
Skin (basal cell) carcinoma	HβD1	Down	mRNA	Cancer tissue	[131]
	HβD2	Up	mRNA	Cancer tissue	[131]
	HβD3	Unchanged	mRNA	Cancer tissue	[131]

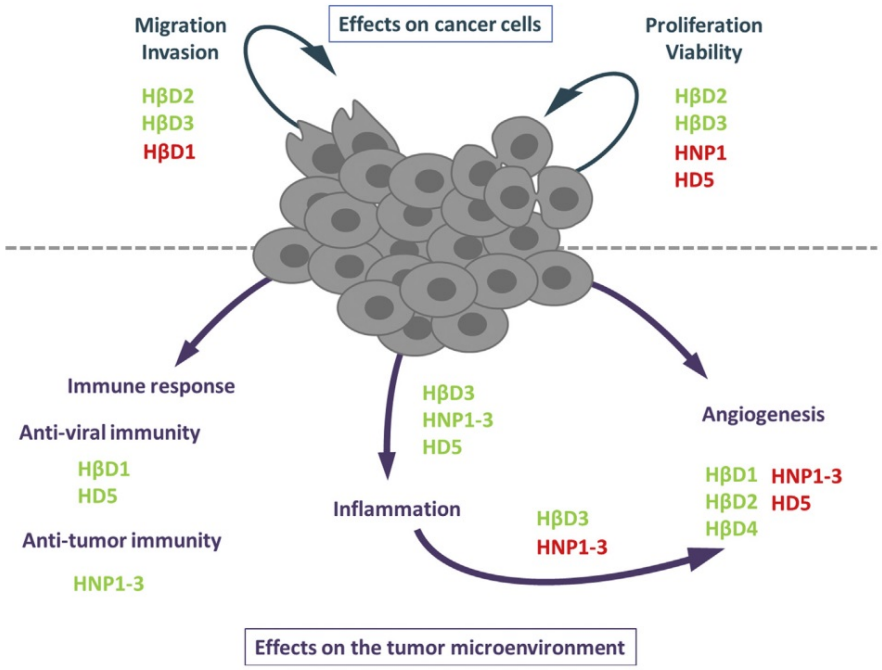

Fig. 1. Schematic effects of defensins on tumor cells and microenvironment. Defensins directly affect cancer cells by modulating their proliferation, survival, migration and invasive properties. Defensins also alter the tumor microenvironment by chemoattracting and/or activating host cells such as endothelial cells and immune/inflammatory cells. In green: promoted; in red: repressed.

line proliferation. Indeed, HβD4 promotes cell cycle progression and proliferation at low doses (<100 nM) but completely blocks cell cycle at higher concentrations (>500 nM) [98]. To the best of our knowledge, no effect on cancer cell proliferation was reported with α-defensins. However, several articles showed that, at high doses, HNPs and HD-5 impair cancer cell viability [67,99–101]. Conversely, HβD3 expression in head and neck cancer cells triggers anti-apoptotic signaling [102].

Cancer cell migration and invasion could also be affected by defensins. Migration and invasion of oral squamous cell carcinoma cells are inhibited by HβD1 *in vitro* [97], which is in accordance with its presumed tumor suppressor role based on its cancer-specific loss or decreased expression. On the contrary, HβD2 promotes neoplastic cell migration and invasion in esophageal cancer [92]. Concerning HβD3, interesting results indicate that HβD3 increases head and neck cancer cell migration and expression of CCR7 [102], a lymph node homing receptor, which may corroborate the association between HβD3 expression and oral cancer positive lymph node status [34]. Similarly to its effect on cancer cell proliferation, HβD4 induces cell migration *in vitro* at low concentrations [98].

Alongside with affecting tumor cell properties, defensins also impact the tumor microenvironment and particularly (lymph-)angiogenesis, inflammation and anti-tumor immunity. Aberrantly activated angiogenesis is a hallmark of cancer and promotes metastatic dissemination [103]. Indeed, because of the massive production of pro-angiogenic compounds by the tumor, accelerated angiogenesis gives rise to leaky and distorted blood vessels, which, besides providing the tumor with nutrients, favor metastatic dissemination and generate hypoxic areas, which are

well-known to promote both resistance to therapy and the emergence of more malignant clones [104]. As detailed in a previous section, defensins are able to modulate cancer-related angiogenesis. Beta-defensins mostly promote angiogenesis while α-defensins could be anti-angiogenic.

Tumor progression and metastatic dissemination also require tumor cell evasion from the immune system. For example, a better prognosis is generally observed in patients whose tumors are infiltrated by a higher quantity of effector CD8 + T lymphocytes, which mediate cellular anti-tumor immune response [105]. Suggesting an effect of defensins on anti-tumor immunity, genetic therapy with mouse β-defensin 2 significantly inhibits tumor growth in mice models of lung cancer and sarcoma by promoting anti-tumor immune response [106]. This contrasts with data obtained in rat esophageal cancer showing that HβD2 expression rather predicts resistance to treatment [92]. Of course this apparent discrepancy could be accounted for by the various animal models used and the different cancer cell types tested [99,100]. Additionally, results from our laboratory and others showed that HNPs are expressed in human tumors such as cervical (pre)neoplastic lesions [107] where they recruit immature mDCs. In a mouse model, HNP1 expression in the tumor environment was triggered by intratumoral injection of liposomes containing a plasmid encoding HNP1. HNP1 release in the tumor was associated with the recruitment and activation of immature DCs, enhanced CD8+ T cell infiltration and specific cellular response, increased antibody levels in sera of mice and tumor growth inhibition [108]. The effects of HNPs on DCs and lymphocytes are consistent with their well-documented chemotactic capacity for these cell types [109]. Similarly, high doses of HD-5 stimulated DC migration and proliferation in a mouse xenograft model of cervical cancer and induced activated lymphocyte chemoattraction *in vitro* [67]. However, it should be kept in mind that, although the well-described defensin-mediated immature mDC chemoattraction to the tumor theoretically facilitates antigen capture, maturation of DCs and subsequent antigen presentation to effector T cells along with Th1-skewing of the immune response, tumors are a source of numerous soluble immunosuppressive compounds such as IL-10 or TGF-β [110]. The possibility that this immunosuppressive microenvironment partially prevents mDCs maturation and induces tolerogenic cells and thereby prevents appropriate immune response should not be excluded. Besides antigen-presenting cells and lymphocytes, tumor-associated macrophages are also chief cellular compounds of the tumor stroma and their pro-metastatic effects are well documented (for a review, see [111]). In oral squamous carcinoma, HβD3 expression was shown to be related to a massive $CD68^+$ $CCR2^+$ macrophage infiltration and to subsequently trigger the formation of larger tumors in nude mice compared to control animals [61].

Finally, since defensins are primarily known for their anti-microbial activity, their downregulation could favor oncogenic virus infection in mucosal surfaces. Supporting this hypothesis, HβD1 expression has been shown to be lost or decreased in head and neck squamous cell carcinoma which was described as a HPV-related cancer. In addition, we recently noticed that HD-5 expression is reduced in cervical (pre)neoplastic lesions. Furthermore, HD-5 was shown to be particularly efficient at preventing HPV entry in keratinocytes, through the inhibition of virion escape from endocytic vesicles [42]. Taken together, these data suggest that impaired expression of defensins might promote HPV infection and related carcinogenesis.

9. Defensins: biomarkers and potential therapeutic agents

As mentioned above, the expression of human α- and β-defensins was extensively reported in inflamed and (pre) neoplastic tissues as well as in associated biological fluids. In the last years, accumulating data revealed that some defensins may have some utility in cancer diagnosis and screening. The most detailed studies were performed in colon adenocarcinoma patients. By mass spectrometry and/or ELISA assay, increased serum levels of HD-6 and HNP1-3 were associated with cancer progression and metastatic dissemination [95,112–114]. In intestinal-type gastric cancer, HNP1 expression was also shown to be associated with a poor prognosis independent of major clinical covariates [115]. However, the interpretation of these results and, overall, the use of HNPs as cancer biomarkers need some degree of caution. Although a few anecdotic and controversial results showed that cancer cells produce HNPs by themselves [116], we may assume that the high HNP levels in cancer patient serum is related to the tumor infiltrating immune cells. Therefore, an elevation of circulating HNP levels may not directly be linked to cancer development but rather related to the inflammatory microenvironment. This is supported by the up-regulation of HNP peptides in several chronic inflammatory and auto-immune processes such as Crohn's disease [117], ulcerative colitis [118], Wegener's granulomatosis [119] or lupus nephritis [120]. In addition to monitor cancer behavior, the variation of HNP secretion could help to predict the resistance to treatment as shown in chronic myeloid patients treated with Imatinib [121]. However, these results need to be confirmed with a larger cohort of patients. Regarding β-defensin expression, the survival rate of head and neck cancer patients with an intense HβD1 immunoreactivity was significantly higher compared to individuals displaying a weak/negative staining [97]. Compared to healthy subjects, serum HβD2 level was higher in patients with lung cancer suggesting that this inducible defensin could be used as a diagnostic tool and/or indicator of respiratory tract tumors [122]. A study recently published also demonstrated that HβD2 is overexpressed in the esophagus of both N-nitrosomethylbenzylamine (NMBA)-treated rats and in patients with esophageal cancer [92]. Interestingly, rats with lower HβD2 expression responded better to treatment, suggesting that HβD2 might serve as a biomarker of resistance to therapy. Besides the cancer setting, HβD2 was also shown to be a helpful serum biomarker for both disease activity in psoriasis [123] and poor outcome prediction in the acute phase of stroke [124].

Due to their broad spectrum activity in human physio(patho)l-ogy, defensins could be considered as promising pharmacological molecules. Given their well-documented antimicrobial function, defensins could be directly applied on infected/inflamed lesions, in order to both promote wound healing and to act as local antibiotics [125]. The ability of defensins to block several sexually transmitted viral pathogens such as HIV, HPV and herpes simplex virus suggests that HNPs could be interesting antiviral candidates as well [42,67,126].

Through the inhibition of blood vessel formation, α-defensins might be interesting potential therapeutic agents in neovascularization-related diseases, such as hypoxia-induced retinal angiogenesis which is observed in diabetes patients and represents a major cause of adult blindness. This defensin-induced anti-angiogenic effect could also be valuable in cancer therapy.

In the cancer microenvironment, these peptides could also act as immune modulators. By inducing DC, LC and T lymphocyte chemotaxis, defensins could partially restore antitumor immunity which is altered in cancer microenvironment. In order to overcome tumor-related immunosuppression/immunotolerance, an approach in which defensins would be used in combination with an immune system activator such as Imiquimod could provide even greater anti-cancer efficiency.

In a mouse model of immunization, HβD3 addition increased the serum levels of both anti-ovalbumin antibodies and IFN-γ suggesting that HβD3 may therefore be considered as an

interesting adjuvant for vaccination [53]. In another murine model, vaccination with a fusion protein composed of mouse β-defensin 2 and ovalbumin enhanced antigen presentation, TLR4-dependent expansion of adoptively transferred ovalbumin-specific CD8+ T cells and longer survival when mice were challenged with lethal doses of B16 cancer cells expressing ovalbumin, once again emphasizing the potent role of defensins as vaccine adjuvant [127].

Since treatment with recombinant HβD1 or HβD26 restored sperm quality in infertile men, some defensins might also be part of a potential therapeutic strategy for male infertility.

10. Conclusion

Although many questions still exist regarding defensin expression, processing and function, solid evidence suggests that these cationic peptides are much more than "simple" antimicrobial peptides. We support the notion that an altered defensin expression is involved in cancer progression, male infertility and in the pathogenesis of several inflammatory disorders. A better characterization of the diversified activities of these small peptides would be relevant for both clinical and translational studies aimed to elucidate whether defensins could be efficiently used as biomarkers and/or as novel pharmacological agents.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments

This work was supported by the Belgian Fund for Medical Scientific Research (FNRS), by the Centre Anti-Cancereux près l'Université de Liège, by the Faculty of Medicine of the University of Liège and by the Fonds Léon Frédéricq.

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