



THÈSE DE DOCTORAT

Etude du role de la rigidité matricielle dans la résistance des cellules de carcinomes squameux aux thérapies anti-cancéreuses

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Summary

Role of matrix stiffness in the resistance of squamous cell carcinoma cells to anti-cancer therapies

Resistance to epidermal growth factor receptor (EGFR) targeted therapy triggered by the tumor niche in head and neck squamous cell carcinoma (HNSCC) represents a challenge in research and in clinics. Despite the fact that over 15% of HNSCC overexpress EGFR, HNSCC are refractory to EGFR Tyrosine Kinase Inhibitors (TKIs) targeted therapy and yet **the molecular and cellular mechanisms of EGFR-TKIs resistance in HNSCC are unknown**. The tumor niche plays an important role in conventional chemotherapeutic resistance. Cancer associated fibroblasts (CAFs), the most prominent stromal cell in tumor niche, participate in this process. Notably, CAFs are responsible for tumor tissue fibrosis an excessive extracellular matrix (ECM) remodeling that increases matrix stiffness. In carcinoma cells, adhesion to stiff substrate triggers mechano-dependent intracellular signaling pathways that favor tumor resistance to conventional chemotherapies.

My work demonstrates that ECM stiffening is responsible for a significant increase of squamous cell carcinoma (SCC) survival upon the treatment with EGFR TKIs, conventional chemotherapies and combination of both. Over 60% more cells survive treatment with the gefitinib EGFR TKI compared to cells plated on soft matrix. Same effect was observed on matrix derived from CAFs that is known to be stiffer compared to the one derived from fibroblasts isolated from normal skin. Further analysis revealed an induction of partial epidermal-to-mesenchymal transition (EMT) in cells plated on rigid matrices. EMT is know to play a role in resistance of cancer cells to treatments, and I have demonstrated that downregulation of know transcriptional factors involved in EMT leads to an increase of cell susceptibility to EGFR TKI when plated on stiff matrix. To understand in more detail what drives the resistance of SCC cells when plated on stiff we conduced an RNA sequencing.

RNA sequencing of SCC12 cells plated on soft and stiff matrix revealed AXL as main driver of EGFR TKI resistance in HNSCC. I was able to demonstrated that inhibiting AXL in SCC cells, lying on stiff matrices, reverts the EGFR TKI resistance triggered by the tumor niche. Moreover, I show in 3D cell culture the importance of combining AXL and EGFR TKI in treatment of SCCs. Our overall goal was to identify novel therapeutic targets with reduced resistance opportunity.

Finally, research presented in this manuscript carries potential in establishing a prediction biomarker to the response of HNSCCs and other cancers to EGFR TKIs.

Résumé

Etude du rôle de la rigidité matricielle dans la résistance des cellules de carcinomes squameux aux thérapies anti-cancéreuses

Dans notre modèle de carcinome épidermoïde humain de la tête et du cou (HNSCC), si l'on observe une surexpression du récepteur au facteur de croissance épidermique (EGFR) dans plus de 15 % des cas, les cellules cancéreuses (SCC) sont réfractaires à un traitement par des inhibiteurs de l'activité Tyrosine kinase de l'EGFR (EGFR TKI) tels que le Gécitinib et l'AG1478. A ce jour, les mécanismes moléculaires et cellulaires impliqués dans la résistance des HNSCC aux EGFR TKI sont encore inconnus. On sait aujourd'hui que les niches tumorales jouent un rôle crucial dans la résistance des cellules cancéreuses aux traitements chimiothérapeutiques conventionnels et que les fibroblastes associés au cancer (CAF) présents dans ces niches, participent très certainement à ce processus. Les CAF sont notamment responsables de la fibrose du tissu tumoral et d'un remodelage excessif de la matrice extracellulaire (MEC) aboutissant à une augmentation de sa rigidité. Dans les cellules de carcinome, l'adhésion sur un substrat rigide déclenche des voies de signalisation intracellulaires mécano-dépendantes qui favorisent la résistance des tumeurs aux chimiothérapies conventionnelles.

Mes travaux démontrent que la rigidification de la MEC est responsable d'une augmentation significative de la survie des cellules de carcinome épidermoïde (SCC) en réponse aux traitements par des ITK de l'EGFR, aux chimiothérapies conventionnelles ou à une combinaison des deux. Comparé à des cellulesensemencées sur matrice molle, des cellules cultivées sur matrice rigide survivent mieux (+60%) au traitement avec le Gécitinib (EGFR TKI). Le même effet a été observé sur une matrice dérivée de CAF dont on sait qu'elle est plus rigide que celle dérivée de fibroblastes isolés d'une peau normale. Une analyse plus poussée a révélé une induction partielle de la transition épidermo-mésenchymateuse (EMT) dans des cellules SCC étalées sur des matrices rigides. L'EMT joue un rôle dans la résistance des cellules cancéreuses aux traitements, j'ai ainsi démontré que la régulation négative des facteurs de transcription connus pour être impliqués dans l'EMT conduit, dans le cas de cellulesensemencées sur une matrice rigide, à une augmentation de la sensibilité cellulaire aux EGFR TKI.

Pour comprendre plus en détails ce qui induit la résistance des cellules SCC lorsqu'elles sont étalées sur une plaque rigide, nous avons effectué un séquençage d'ARN. Le séquençage de l'ARN des cellules SCC12 étalées sur une matrice souple et rigide a révélé que la protéine AXL pouvait contribuer à la résistance au Gécitinib des HNSCC. J'ai pu démontrer que l'inhibition de l'expression d'AXL dans les cellules SCCensemencées sur des matrices rigides, réduit totalement la résistance à l'EGFR TKI induite par la niche tumorale. De plus, je montre, en culture cellulaire 3D, l'importance de combiner AXL et EGFR TKI dans le traitement des SCC.

Notre objectif global était d'identifier de nouvelles cibles thérapeutiques présentant une opportunité de résistance réduite. Les recherches présentées dans ce manuscrit ont le potentiel d'établir un biomarqueur de prédiction de la réponse des HNSCC et d'autres types de cancers aux EGFR TKI.

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Hvala svima!

*To my Daddy (1957-2016),
and to all of those who lost their battle against cancer*

FAGUS SYLVATICA

*Never wish I be
free of pain for you!*

*Sneaking through my day
Following my steps
Hidden in the back,
back of every story
That you never told*

*Waiting for my night
Sleeping next to me
Present in my dreams,
dreams I have you back
Just to keep you safe*

*Taking all myself
Living just for you
Kept forever in the heart,
heart that worries much
Memories of truth*

*Pain does not belong to me
I belong to pain
Sneaking through my day
Turning into love,
Love beyond a life*

*I am not alone,
pain will keep me safe!*

List of abbreviations

2D: Two Dimensional	ERK: Extracellular signal–Regulated Kinase
2DRT: Two-Dimensional Radiation Therapy	FAK: Focal Adhesion Kinase
3D: Three Dimensional	FAP: Fibroblast Activation Protein
3DCRT: Three-Dimensional Conformal Radiation Therapy	FDA: Food and Drug Administration
ABL: Abelson Murine Leukemia	FGF: Fibroblast growth factor
ADAMs: A Disintegrin and Metalloproteinases	FGF-BP: Fibroblast growth factor Binding Protein
ADCC: Antibody-Dependent Cellular Cytotoxicity	FRET: Fluorescence Resonance Energy Ttransfer
AJCC: American Joint Committee on Cancer	FSP1: Fibroblast-Specific Protein 1
AML: Acute Myeloid Leukemia	G3BP2: Ras GTPase-activating protein-Binding Protein 2
ATP: Adenosine TriPhosphate	GAGs: GlycosAmino Glycans
AR: Amphiregulin	Gas6: Growth arrest-specific gene 6
AXL: Anexelekt0	Gla: Gamma-carboxyglutamic acid
BCL-2: B-cell lymphoma 2	GPCR: G-Protein Coupled Receptors
BTC: β-cellulin	HER: Human Epidermal growth factor Receptor
CAFs: Cancer Associated Fibroblasts	HGF: Hepatocyte Growth Factor
CAM-DR: Cell Adhesion-Mediated Drug Resistance	HNSCCs: Head and Neck Squamous Cell Carcinomas
CCND1: Cyclin D1	HPV: Human Papilloma Virus
CDKN2A: Cyclin-Dependent Kinase Inhibitor 2A	ICAM1: Intercellular Adhesion Molecule 1
CTCA: Cancer Treatment Centres of America	IL-1: InterLeukin-1
DAPI: 4',6-DiAmidino-2-PhenylIndole	IL-6: InterLeukin-6
DDR: Discoidin Domain Receptors	ILK: Integrin-Linked Kinase
DNA: DeoxyriboNucleic Acid	IMRT: Intensity-Modulated Radiation Therapy
ECM: ExtraCellular Matrix	IRF: Interferon Regulatory Factor
ECS: ExtraCapsular Spread	JAK: Janus Kinase
EGF: Epidermal Growth Factor	JNK: c-Jun N-terminal kinase
EGFR: Epidermal Growth Factor Receptor	KRAS: Kirsten rat sarcoma 2 viral oncogene homolog
EMT: Epithelial-to-Mesenchymal Transition	
EPG: Epigen	
EPR: Epiregulin	

LA-SCCHN: Locally Advanced Squamous Cell Carcinoma of the Head and Neck

LIF: Leukemia Inhibitory Factor

LOX2: Lysil Oxidase-like 2 protein

MIS: Minimally Invasive Surgery

MKK6: Mitogen-activated protein Kinase Kinase 6

MMP: Metalloproteinase

MRI: Magnetic Resonance Imaging

MRTF: Myocardin and Related Transcription Factors

MSCs: Mesenchymal Stem Cells

NF- κ B: Nuclear Factor-kappa B

NRG: Neuregulin

NSCLC: Non-Small Cell Lung Carcinoma

NURD: Nucleosome Remodeling Deacetylase

PBRT: Proton-Beam Radiation Therapy

PDAC: Pancreatic Cancer

PDGF: Platelet-Derived Growth Factor

PGs: ProteoGlycans

PI3KCA: Phosphatidylinositol-4,5-bisphosphate 3-Kinase Catalytic subunit Alpha

PKB/ AKT: Protein Kinase B

PLC γ : PhosphoLipase C- γ

pRb: Retinoblastoma protein

PTB: Phosphotyrosine binding domain

PTEN: Phosphatase and Tensin homolog

Raf: Rapidly Accelerated Fibrosarcoma

RNA: RiboNucleic Acid

ROCK: Rho-associated protein kinase

RTKs: Receptor Tyrosine Kinases

SCC: Squamous Cell Carcinoma

SDF-1: Stromal cell-derived factor 1

sE-cad: soluble E-cadherin

SHBG: Sex Hormone-Binding Globulin

siRNA: Small Interfering RNA

SPARC: Secreted Protein Acidic and Rich in Cysteine

SRC: Sarcoma

SRF: Serum Response Factor

STAT: Signal Transducer and Activator of Transcription

TAMs: Tumor-Associated Macrophages

TAZ: Transcriptional co-activator with PDZ-binding motif

TFs: Transcription Factors

TGF α : Transforming Growth Factor alpha

TGF β : Transforming Growth Factor beta

TKIs: Tyrosine Kinase Inhibitors

TLM: Transoral Laser Microsurgery

TME: Tumor microenvironment

TNF α : Tumor necrosis factor alpha

TNFAIP3: Tumor Necrosis Factor α -Induced Protein

TNM: tumors tumor-node-metastasis

TORS: TransOral Robotic Surgery

TRAIL: Tumor Necrosis Factor-related Apoptosis-Inducing Ligand

VEGF: Vascular Endothelial Growth Factor

WHO: World Health Organisation

YAP: Yes-Associated Protein

α SMA: alpha Smooth Muscle Actin

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Chapter 1:

INTRODUCTION

1.1. HEAD and NECK SQUAMOUS CELL CARCINOMAS

Head and Neck Squamous Cell Carcinomas (HNSCCs) are a heterogenic group of cancers. Not only do they affect the whole head and neck region, driving the heterogeneity of their form (Vokes et al., 1993), but their molecular signature reveals a plethora of genetic changes not yet fully disclosed (Chung et al., 2004).

According to the statistics gathered by the World Health Organization (WHO) in 2014, HNSCCs are the 7th most frequent cancer and 9th most frequent cause of death from cancer, killing approximately 400 000 people worldwide (World Health Organization, World Cancer Report 2014). Common cause of HNSCCs are tobacco and alcohol that have a synergistic effect, but other risk factors are reported like human-papilloma virus (HPV) and Epstein-Barr virus (Argiris et al., 2008). As in many other cancers, HNSCC is oftentimes curable if it is diagnosed early, however if it is diagnosed later outcomes can be unfavorable due to the disease reoccurrence and development of metastasis. In developed countries, 5-year survival in average is between 40 to 60% (**Figure 1**). It is very common that therapies designed for HNSCCs (chemotherapy, radiotherapy, targeted therapy and their combinations) fail in time, and patients become unresponsive to them (Beyzadeoglu et al., 2014, National Cancer Institute, Cancer Stat Facts: Oral Cavity and Pharynx Cancer). In this chapter I will review generalities and the molecular background of HNSCCs, whilst treatments designed for these cancers will be detailed in an individual chapter.

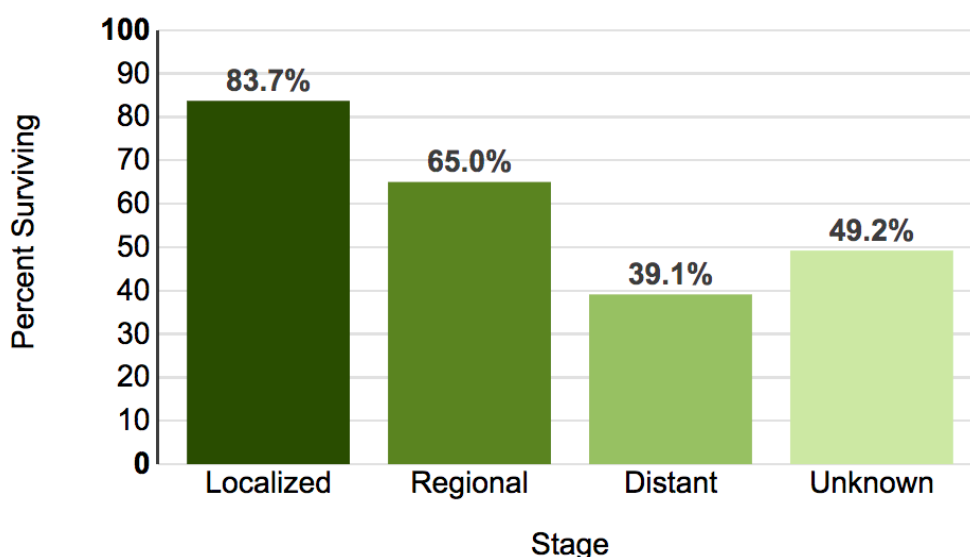


Figure 1. 5-year Relative Survival of Oral Cavity and Pharynx Cancers depending on the stage

If the cancer is found only in one part of the human body it is considered to be localised, and those tumors have the best 5-year prognosis, unfortunately if the cancer has spread regionally or distantly this prognosis is much worse. (Adapted from National Cancer Institute).

1.1.1. CHARACTERISTICS of HNSCCs

Majority of cancers (up to more than 90%) found in head and neck region are of squamous cell origin (Sanderson and Ironside, 2002), hence the name Head and Neck Squamous Cell Carcinomas (HNSCCs). Squamous cells are found in the outer layer of the skin and in the mucous membranes which are linings of ectodermic origin, covered in epithelium, and are involved in absorption and secretion. Mucous membranes line body cavities such as the airways and intestine (Fry and Vahabi, 2016). Squamous cell carcinoma (SCC) is characterized by squamous differentiation and invasive growth with a high occurrence of metastasis. Invasion into the surrounding tissue is followed by an increase in fibrosis and infiltration of inflammatory cells, notably lymphocytes and plasma cells (Organization and Cancer, 2005). Therefore, all cancers of squamous cell origin found in the upper aero-digestive tract are labelled as HNSCCs. Due to the multiple locations, HNSCCs can be classified according to four different regions it encompasses, 1) the nasal cavity and paranasal sinuses, 2) the nasopharynx, 3) the hypopharynx, larynx, and trachea, and 4) the oral cavity and orophary (Figure 2).

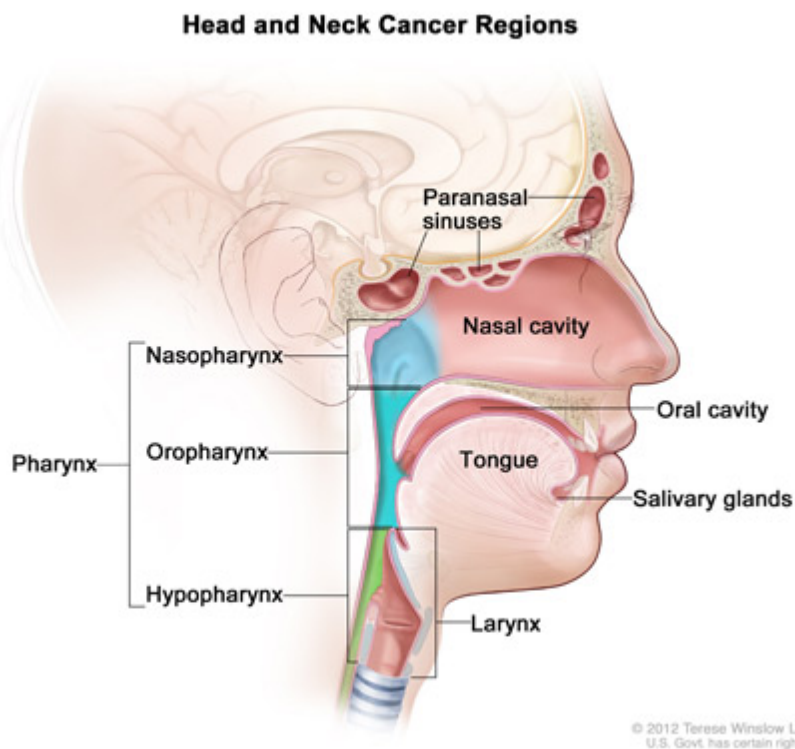


Figure 2. Head and neck regions affected by the squamous cell carcinomas

This schematic representation describes all possible location of squamous cell carcinoma of head and neck; paranasal sinuses, nasal cavity, oral cavity, tongue, salivary glands, larynx, and pharynx (including the nasopharynx, oropharynx, and hypopharynx). Adapted from National Cancer Institute report on Head and Neck Cancers.

A) HNSCCs epidemiology

In general, HNSCCs are more common in men than in women. A disproportion between two sexes is attributed to the higher exposure to risk factors in men, such as tobacco, alcohol and UV light. In terms of age, HNSCCs are usually diagnosed in patients over the age of 50 (Warnakulasuriya, 2009). Nevertheless, 6% of cancers occur in people younger than 45 years and in counties with a higher incidents disease occurs before the patients turn 40 years old. Worldwide, HNSCCs affects approximately 600 000 people yearly and only 40-50% of patients will survive more than 5 years (Leemans et al., 2011). In European Union there is 67 000 new cases registered each year with highest male incident rates in France and Hungary. Indeed, in France, in 2010, the number of new cases was estimated to be 14 000, the majority of which (75%) were men. Low survival rate can be attributed to the fact that two third of patients are diagnosed in the advanced stage. Stage of the disease is determined by the extent of the tumor, presence of lymph-nodes metastases and distant metastases.

B) Risk factors involved in the development of HNSCCs

Tobacco and alcohol consumption are the two main causes of HNSCCs, and they appear to have a synergistic effect (Blot et al., 1988). Due to the campaigns against smoking in western world, occurrence of HNSCCs in the past decade has been slowly declining. On the other hand, subgroup of HNSCCs (in particular those of oropharynx) caused by the infection with HPV type16 and 18 are becoming more frequent, which could be correlated with in increase of HPV infections. Nowadays, 25% of HNSCCs are positive for the genomic DNA of HPV that exhibit their carcinogenic effects through viral proteins E6 and E7, that inactivate P53 and pRb (retinoblastoma protein) tumor repressor genes (D'Souza et al., 2007; Münger and Howley, 2002). HPV-negative and HPV-positive are usually distinguished by different clinicopathological and molecular characteristics. Interestingly HPV-positive tumors area associated with a favourable prognosis for the patients because of the positive response to the treatment (radiotherapy and chemotherapy). Besides these external factors, some genetic diseases such as Fanconi anemia provide intrinsic susceptibility to HNSCCs (Cloos et al., 1996; Kutler et al., 2003).

C) *Molecular classification of HNSCCs*

As I mentioned in the very beginning, 90% of head and neck cancers are squamous cells that led researchers to believe it is a rather homogenous disease (Pai and Westra, 2009). However, recent observations have revealed that HNSCCs are quite heterogeneous, making it hard to establish accurate prognostics, plan the treatment, and finally identify the genes responsible for cancer development.

In 2004 by taking advantage of expression profiling, Chung *et al.* highlighted the heterogeneity of HNSCCs and sought to identify novel markers for survival predictions. They analysed gene expression patterns of 60 HNSCCs tumors and identified 4 different subtypes with different clinical outcomes. The worst outcome was associated with **Group 1** tumors subtype that were characterized by the high expression of Transforming Growth Factor Beta (TGF β) that is known to correlate with poor clinical outcomes (Endo *et al.*, 2000). Most HNSCCs express Epidermal Growth Factor Receptor (EGFR), but there is an additional evidence that Group 1 cancers have an activation of EGFR pathway. Besides the upregulated genes from EGFR signaling pathway such as Transforming growth factor alpha (TGF α), an EGFR ligand, a kinase downstream of EGFR – Mitogen-activated protein Kinase Kinase 6 (MKK6) and angiogenic switch molecule induced by Epidermal Growth Factor (EGF) - Fibroblast growth factor binding protein (FGF-BP); 15 out of 19 tumors in Group 1 tested by IHC were positive for activated, Tyr-1173 phosphorylated, EGFR. Data suggests that patients in Group 1 should be evaluated for the benefit of EGFR inhibitor treatments. The **Group 2** tumors expressed strong mesenchymal cell signature due to the presence of fibroblasts and lack of epithelial characteristics, or cells that may have undergone epithelial to mesenchymal transition (EMT). Tumors organized under **Group 3** are characterized by Cytokeratin 14 and 15 expression patterns that are similar to those found in normal tonsil epithelium samples suggesting that there are very few differences between premalignant and malignant state. These tumors showed fewer relapses and therefore best outcomes. The **Group 4** tumors had an expression pattern very similar to the gene pattern expression induced by cigarette smoke. Since majority of the cohort were heavy smokers, data obtained suggest that there could be variations in the response of smokers to cigarette smoke (Chung *et al.*, 2004). Two years later, same authors went a step further and determined a high-risk signature for HNSCCs using formalin fixed and frozen tumor samples. They showed that genes involved in EMT and nuclear factor- κ B (NF- κ B) signalling are the most relevant molecular signature of high-risk tumors (Chung *et al.*, 2006). EMT has been associated with late stage tumor progression and metastasis (Boyer *et al.*, 2000) and, in HNSCCs has been previously associated with resistance to EGFR tyrosine kinase inhibitors such as erlotinib (Thomson *et al.*, 2005; Yauch *et al.*, 2005). It is worth to mention that they came up with a 75-gene list of potential prognostic markers some of which are metalloproteinase (MMP) 2, keratin 14, lysyl oxidase-like 2 protein (LOX2), stratifin and galectin 1. Interestingly for the research I have done, MMP2, LOX2 and galectin 1 are all proteins involved in tumor microenvironment regulation which could potentially suggest that the HNSCCs tumors with strongly deregulated tumor stroma are the ones classified as high-risk. Nevertheless, data

acquired should be considered preliminary and more efforts are necessary to completely elucidate the HNSCCs subgroups since no clear connection has been established between the genetically identified subgroups and molecular classification. However, it is widely accepted that HPV-positive HNSCCs are a specific subgroup since the tumors are distinctive on a molecular level and have favourable clinical outcomes (Ragin and Taioli, 2007).

1.1.2. ONCOGENIC DRIVERS in HNSCCs

In HNSCCs the most common mutations are in the Transformation related Protein 53 (*TP53*) gene - 53%, deletions and mutations in the tumor suppressor gene Cyclin Dependent Kinase Inhibitor 2A (*CDKN2A*) - 58%, amplification of Cyclin D1 (*CCND1*) - 31%, Phosphatidylinositol 3-kinase (*PI3KCA*) - 34%, and lastly, *EGFR* genes - 15% (Argiris et al., 2008), leading to gene instability (**Figure 3**). Interestingly, EGFR is expressed in 90% of HNSCCs but gene amplification is observed in only 15%, and studies have shown that overexpression of EGFR is associated with poor prognosis (Ang et al.; Grandis and Tweardy, 1993; Rubin Grandis et al., 1998). However, no link has been established between EGFR expression and the effectiveness of treatments, and no other biomarker other than HPV is used for prognosis or treatment establishment.

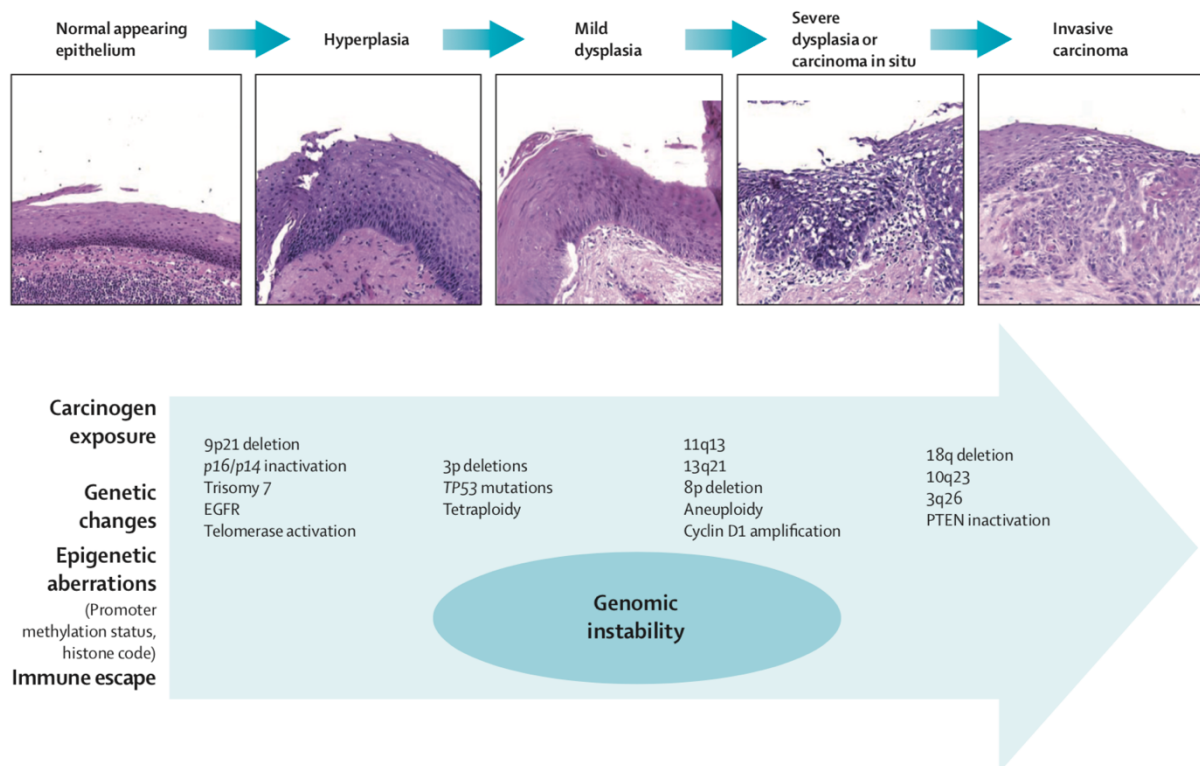


Figure 3. Accumulation of molecular changes in cancerogenesis of head and neck cancers and phenotypical progression

Heamatoxylin and eosin staining of histological changes in HNSCCs shows the evloution of the tumor in parallel with the accumulation of genetic and epigenetic alterations. (Adaoted from (Argiris et al., 2008).

A) *TP53*

Malformations of p53 are the most common events in all cancers and HNSCCs are no exception. *TP53* tumor suppressor gene is often referred to as a gatekeeper gene due to its role in regulation of the key events in cell, such as cell cycle, metabolism of cancer cells, DNA-repair, apoptosis and senescence. It is located in chromosome 17p13 and can be induced by a plethora of stress-like events like DNA-damage and inflammation (Bensaad and Vousden, 2007; Hussain and Harris, 2006). In HNSCCs point mutations of *TP53* are often seen and disruptive mutations have been associated with short survival rate *post* surgical removal. According to International Agency for Research on Cancer's (IARC) *TP53* mutation database, codons 238-245 are a hotspot region for mutations in HNSCCs. Somatic mutations are found in 50-80% of HNSCCs

Mutations of *TP53* are associated with the use of tobacco (Soussi et al., 2005), although the relationship is not always significant and varies from study to study. Moreover, in the analysis of 420 patients *TP53* mutations were more frequent in patients with advanced-stage HNSCCs and showed positive correlation with the poor survival of patients (Poeta et al., 2007). As for HPV-positive HNSCCs in which viral E6 protein binds and inactivates *TP53*, low frequency of mutations is observed. Regardless of the data, prognostic value of p53 is not yet evident.

B) *CDKN2A*

CDKN2A is a tumor suppressor gene located on chromosome 9p2 and associated with the cell cycle progression. *CDKN2A* codes for 16^{INK4a} protein that plays an important role in cell cycle and senescent through the regulation of cyclin D and cyclin-dependant kinase (CDK) 4/6 (Zhao et al., 2016). In HNSCCs, *CDKN2A* is often inactivated by mutations or methylations, chromosome loss or homozygous deletion (Reed et al., 1996). In cell cycle, an interaction between cyclins, CDKs and their inhibitors regulates the restriction points, and a stimulation from growth factor is necessary to break G1 cell cycle restriction point in order for cells to enter S phase.

Same as in the case of *TP53*, tobacco and alcohol consumption correlate with the apparent loss of p16 protein. Methylation in the promoter region, however, did not show any significant correlation with tobacco exposure in HNSCCs like it did in lung cancer (Ai et al., 2003; Kim et al., 2001). Relationship between genetic and epigenetic changes within p16 have been studied by a variety of groups, but consensus has not been reached (Bazan et al., 2002; Bova et al., 1999; Danahey et al., 1999; Kwong et al., 2002). Half of the studies showed a significant positive correlation between p16 expression and poor patient survival, and half did not.

C) *CCND1*

CCND1, which encodes for cyclin D1, is located on chromosome 11q13, and is amplified or overexpressed in more than 30% of HNSCCs cases and even more frequently in HPV-negative tumors (D'Souza et al., 2007; Rothenberg and Ellisen, 2012). Some research shows, that cyclin D is overexpressed in 80% of HNSCCs (Kutler et al., 2003). Main function of cyclin D is a cell cycle regulator, but it can act as a cofactor by binding to transcriptional factors and DNA repair proteins (Poeta et al., 2007). For example, Cyclin D and CDK2 form a complex that phosphorylates Rb important for the S phase progression (Day et al., 2009), therefore these two mutations are not necessarily mutually exclusive explaining the high prevalence of these mutations. Overexpression of cyclin D is a good predictor of patient death caused by tongue cancer and overexpression of cyclin D and loss of P16 expression, together, are an indicator of a worse 5-year survival (Napier and Speight, 2008). Together with the abrogation of p53, these changes cause cellular immortalization (Smeets et al., 2011).

D) *PI3KCA*

PI3K gene is located on chromosome 3q26 and represents complex family of lipid kinases that regulate pivotal cellular processes such as survival, growth, proliferation, motility, cell adhesion, differentiation, morphology, cytoskeletal rearrangement and apoptosis (Murugan et al., 2008). PI3K family is divided into three classes (I, II and III) and different roles have been associated with each three, as well as with different isoforms within each group (Jean and Kiger, 2014) For example, class I consists of two subgroups IA and IB. Interestingly, IA is often recruited and activated at the cell membrane by growth factor receptor tyrosine kinase as EGFR and insulin receptor (Cantley, 2002; Vanhaesebroeck et al., 2001)

In a variety of cancers, notably breast cancer, melanoma and lung squamous cell carcinoma, *PIK3CA* codes for p110, catalytic subunit of PI3-kinase that is somatically mutated. In HNSCCs, study has been done on 151 tumors sample and around 30% of HNSCCs had a mutation in the PI3K pathway, making it the most mutated oncogenic pathway (Lui et al., 2013). Same was previously observed by others on tumor samples, but as well in HNSCCs cell lines (Murugan et al., 2008). Frequent mutations include E542K, E545K and H1047R hotspots, but others are reported as well. However, mutation responsible for the gain of function of the *PIK3CA* is still unknown.

What has been established is the correlation with mutations of multiple PI3K pathways and advanced stage of the disease (Lui et al., 2013). It is suggested that *PIK3C* can be used as a predictive biomarker and a potential target for further treatment development, although it has not yet been used for these purposes.

E) *EGFR*

When it comes to EGFR things are more complicated than they normally are. In HNSCCs it is believed that *EGFR* is overexpressed in 90% of the patients, but this data is still not corroborated and different ways of *EGFR* activation exists.

Besides the “standard” treatment for HNSCCs that includes surgery, radiotherapy, chemotherapy and often combinations of the three (that will be reviewed in more detail later on), EGFR is the only molecular target that has been exploited for treatment of patients. However, the success of the targeted therapy (EGFR-specific antibody) is debatable and more understanding is necessary to unveil the role of EGFR in HNSCCs.

Since the subject of my research is indeed EGFR and its inhibitors, following part is dedicated to it.

1.1.3. EGFR and CANCERS

EGFR is the most studied, and one of the first characterized cellular oncogenes and the very first identified member of the receptor tyrosine kinase (RTK) family (Burgess et al., 2003; Cohen, 1962). EGFR belongs in the family of EGF receptor or ErbB family that includes EGFR itself, ErbB2 (HER2/Neu), ErbB3 (HER3) and ErbB4. ErbB receptors participate in a variety of cellular functions such as growth, differentiation, proliferation and motility (Ceresa and Peterson, 2014). Consequently, the role of each and every one of them is well established during cancerogenesis, for example in 1987 overexpression of HER2 was associated with poor prognosis in breast cancer (Slamon et al., 1987). EGFR is expressed in the majority of carcinomas. For example, besides in HNSCCs, it is found in 50 to 70% of lung, breast, prostate, kidney, ovarian and colon carcinomas (Normanno et al., 2003; Prenzel et al., 1999; Salomon et al., 1995).

Soon enough, EGFR became an important therapeutic target for many cancer types including HNSCCs. Thus, the concept of targeted therapy, intended to affect only tumor cells to reduce the side effects of conventional treatments, appeared in 1998 with the commercialization of the blocking antibody trastuzumab (Herceptin®) targeting HER2 (Gschwind et al., 2004). Since then, many anti-EGFR therapies have emerged, yet they only benefit a minority of patients because of innate or adaptive resistance. Therefore, understanding the molecular mechanisms implicating EGFR in tumor progression is important for the development of new therapeutic strategies.

A) *Activation of ErbB receptors*

1) *Activation by ligand*

ErbB receptors, including EGFR, exist in an inactive monomeric form. Most common way of activating EGFR is by numerous ligands, of which EGF is the most common and studied one (Cohen, 1962; Cohen and Carpenter, 1975). These ligands can be secreted in an autocrine (produced by the cells expressing the receptor), paracrine (secreted by other cells) or juxtacrine manner (Singh and Harris, 2005). Juxtacrine manner is explained as “where the molecule that induces the functional changes in the target cell remains associated with the plasma membrane of the signaling cell, rather than acting in the fluid phase” (Anklesaria et al., 1990).

The binding of a ligand causes a conformational change of the receptor, releasing the domain II responsible for the formation of homo or heterodimers.

Besides EGF, other ErbB receptors ligands can be classified in three groups according to their selectivity for the receptor: EGF, TGF α , amphiregulin (AR) and epigen (EPG) preferentially bind to EGFR; β -cellulin (BTC), heparin binding EGF (HB-EGF) and epiregulin (EPR) specifically activate EGFR and HER4; finally, neuregulins (NRG1 to 4) can bind to HER3 and / or HER4 (Carraway et al., 1997; Chang et al., 1997; Harari et al., 1999; Jura et al., 2009).

Upon activation, ErbB receptors are autophosphorylated on tyrosine residues present in the intracellular domain. Such phosphorylated residues allow anchoring of proteins with SH2 (Src homology) or phosphotyrosine binding (PTB) domains, that build adapter proteins as Shc, Crk, Grb2, Grb7 and Gab1, the Src, Chk and PI3K kinases, and the phosphatases SHP1 and SHP2. Adapter proteins transmit signals by activating various signaling pathways. Besides adapter proteins, ErbB receptors can activate transcription factors, such as proto-oncogenes c-fos, c-jun and c-myc (Yaffe, 2002). Diversity among activated signaling pathways is a consequence of the complexity of the dimers, their ligands and different phosphorylation sites of the receptor. All ErbB receptors and their ligands activate the RAS / RAF / MEK / Erk signaling pathway (Bazley and Gullick, 2005; Carpenter, 2003; Citri et al., 2003) and PI3K pathway, however, the degree of activation and kinetics vary among receptors (Elenius et al., 1999; Fedi et al., 1994; Prigent and Gullick, 1994). Conversely, the activation of the phospholipase PLC γ , Esp15 and Cbl is specific for the EGFR receptor (Chattopadhyay et al., 1999; Levkowitz et al., 1999).

II) Transactivation

ErbB receptors can be activated in the absence of ligand by mechanism that are known as transactivation. Janus Kinase 2 (JAK2) can activate (phosphorylate) ErbB receptors via induction by growth factors like cytokines and hormones (prolactin) (Yamauchi et al., 1997). Similarly, Src kinase can activate EGFR by phosphorylating several tyrosine residues (Biscardi et al., 1999). On the other hand, G-protein coupled receptors (GPCRs) can also transactivate ErbB receptors. GPCRs are transmembrane proteins coupled to heterotrimeric G proteins composed of three subunits: G α , G β and G γ . The binding of a ligand to the GPCR results in the dissociation of the subunits of the G protein into G α -GTP and G $\beta\gamma$. G α -GTP and G $\beta\gamma$ subunits control the activity of several enzymes including kinases, phospholipase C and adenylate cyclase. Daub *et al* were the first to demonstrate in 1996 the transactivation of EGFR and HER2 by GPCR ligands; lysophosphatidic acid, thrombin and endothelin (Daub et al., 1996). Upon transactivation, proteases from the family of A Disintegrin And Metalloproteinases (ADAMs) are activated and allow the cleavage of EGF family, such as HB-EGF, at the cell surface (Carpenter, 2003; Gschwind et al., 2003; Prenzel et al., 1999). Similarly, another study showed that the activation of the estrogen receptor resulted in the release of HB-EGF dependent on MMP2 and MMP9 proteases (Razandi et al., 2003). In conclusion, ErbB receptors, notably EGFR, allow the regulation of many signaling pathways, which influences key biological phenotypes, such as cell proliferation, differentiation, motility, and survival.

Importantly, EGFR along with other ErbB receptors participates in all stages of carcinogenesis and historically plays a very important role in tumor development, from initiation to progression.

B) *EGFR mutations often found in HNSCCs*

In 1986, *EGFR* was postulated as overexpressed in many cases of HNSCCs (Yamauchi et al., 1997), and later confirmed by different studies (Grandis and Tweardy, 1993; Hama et al., 2009). However, these observations are based on the immuno-stainings of tumor samples that are not always consistent due to the different protocols used and lack of negative controls (normal healthy tissue). Therefore, it is fair to say that *EGFR* is expressed in majority (90%) of HNSCCs rather than overexpressed, and only 15% of patients have *EGFR* amplified. Amplification was first reported by Ishitoya *et al* in 1989 and data about the percentage of tumors being oncogenically activated varies as well (Ishitoya et al., 1989). High-resolution single-nucleotide polymorphism arrays described 31% of cancers carrying the amplification at 7p11. This study was later confirmed on a higher number of samples using *in situ* hybridisation and immuno-staining (Sheu et al., 2009).

Besides amplification, there are other *EGFR* activating mutations found in HNSCCs such as point mutations, but also specific mutant forms of *EGFR* such as *EGFRvIII* that are found more frequently (Ekstrand et al., 1992). *EGFRvIII* corresponds to the deletion of exons 2 and 7 of the extracellular part of the receptor and codes for a constitutively active receptor. It has a particular effect on the intracellular signaling pathways causing increase in proliferation, and it has been show to decrease the effect of treatment (Sok et al., 2006).

In the matter of altered signalling, it is interesting to mention that signaling function of *EGFR* might differ from tumor to tumor. In some cases, *EGFR* activates Protein Kinase B (PKB) also known as Akt pathway, in others Ras-MAPK pathway, or it can induce *CCND1* expression. This kind of multi-faced role can significantly alter the role of *EGFR* in HNSCCs and influence the outcome of the anti-*EGFR* treatment. Again, it is not so clear weather the overexpression of *EGFR* correlates with bad prognosis. For the moment 60% of studies show a correlation and 40% does not (Leemans et al., 2011). Although, for example, early study done by Maurizi *et al.* showed that the 5-year survival of patients with non-*EGFR*-expressing tumors was 81% versus 25% in positive patients (Maurizi et al., 1996).

1.1.4. CONCLUSION

HNSCCs are more complicated than it has been described at the early stage of research (Vokes et al., 1993). Nowadays, with the usage of new technologies it has become apparent that the story behind HNSCCs is far more complicated and, despite enormous efforts (Chung et al., 2004, 2006), it is still very difficult to pin-point and identify a specific molecular signature of HNSCCs. It would be of great value to identify prognostic markers based on a molecular signature of the tumor or even its histological transformation (Uramoto et al., 2010) in order to place patients into certain categories and predict the best treatment and, therefore, the outcome.

At the same time, tumor development is not limited to the intrinsic properties of cancer cells only and changes within them (many of which are described in this chapter). It is now widely accepted that microenvironment surrounding cancer cells undergoes changes as well, becoming tumor microenvironment and influencing all stages of cancerogenesis (Hanahan and Coussens, 2012). To fully understand HNSCCs we must look beyond the tumor cells themselves and expand our efforts to the environment they belong to. Several researchers have already taken this approach and explored the role of fibroblasts and tumor microenvironment in HNSCCs describing a dynamic relationship (Liu et al., 2011; Sweeny et al., 2012; Tong et al., 2012a).

1.2. TUMOR MICROENVIRONMENT

Tumor development and progression is not solely dependant on the malignancy of cancer cells (Kalluri and Zeisberg, 2006). Cancer epithelial cells are supported by a complex microenvironment referred to as “Tumor MicroEnvironment” (TME) that provides a fertile soil for all stages of tumor advancement (Kuzet and Gaggioli, 2016a). Therefore, it is not surprising that the “seed and soil” hypothesis (Paget, 1889) is still as relevant as it was when it was first published in 1889 and numerous studies have endeavoured in further deciphering the role of TME in such a context. A collaborative nature of cancer cells and the supporting stroma results in chronically proliferative and disseminating organ-like structures characteristic for most human cancers, resulting in characteristic morphological cellular changes (large nucleus, irregular size and shape (Baba and Côté, 2007)), invasion and metastases (Hanahan and Coussens, 2012).

Therefore, the role of TME in tumor progression cannot be emphasised enough. It is implicated in all stages of cancer development, from growth, proliferation, invasion, metastasis to chemoresistance (Junttila and de Sauvage, 2013). In this chapter I will further elucidate its composition and role during cancerogenesis.

1.2.1. COMPOSITION of TME– GENERAL OVERVIEW

In the literature there is more than one classification of TME according to the cell origin, cell type, location, immune response-associated gene expression and gene expression.

When it comes to the origin of cells found in TME, we distinguish three mayor parts; cells of hematopoietic origin, cells of mesenchymal origin and non-cellular components. The proportion of these three components vary according to the tumor type and its progression (Egeblad et al., 2010; Quail and Joyce, 2013).

Cells of hematopoietic origin derived from red bone marrow are divided in two groups: (1) cells from lymphoid lineage (T cells, B cells and natural killer cells) and (2) cells from myeloid lineage (macrophages, neutrophils). These are immune cells found infiltrated within TME as a response to strong induction of inflammation at the tumor site. Besides being an immune response, these cells are implicated in the angiogenesis, tumor growth, migration and metastatic progression (Condeelis and Pollard, 2006; Murdoch et al., 2008).

Cells of mesenchymal origin include fibroblasts, mesenchymal stem cells (MSCs), adipocytes and endothelial cells. These cells participate in the angiogenesis, lymphogenesis, remodelling of extracellular matrix, tumor growth and, as well, in invasion of cancer cells. Bone marrow-derived fibroblasts and MSCs directly support cancer cells creating a favourable environment-specific niche and facilitating tumor progression (Quante et al., 2011). Until recently, adipocytes were considered merely an energy storage cells; however recent studies have revealed the importance of adipocyte-secreted factors (e.g. hepatocyte growth factor, HGF) in the progression of breast cancers (Dirat et al., 2011; Huang et al., 2017). Endothelial cells and pericytes, which form the walls of blood vessels, play a major role in vascular function and angiogenesis, as well as in the regulation of cancer cell diffusion (Butler et al., 2010).

Principal **non-cellular component** of TME is extracellular matrix (ECM) secreted by the cells of mesenchymal origin. Unlike physiological conditions, tumor ECM is strongly altered. Altered on the level of fibre deposition but as well fibre alignment and increased number of pro-tumorigenic factors. Therefore, tumor ECM influences tumor progression on architectural and biochemical manner (Bissell et al., 1982; Dvorak, 1986)

Further on, the classification of TME on the functional and organisational level is more diverse due to the high complexity of structure and the multiple roles each cell type can play within TME. Some of the authors describe TME as a structure composed of tumor cells and complex surrounding stroma (Ramamonjisoa and Ackerstaff, 2017). Others, in turn, classify it as non-cancerous cells and their stroma (Whiteside, 2008). One of the most common ways of describing TME of developing tumors is that it is an organ-like entity composed of numerous cell types including proliferating tumor cells, the tumor stroma, blood vessels, infiltrating inflammatory cells and variety of associated tissue cells (Whiteside, 2008) (**Figure 4**). It is important to note that, despite having pro-cancerogenic function, non of cells

within stroma are malignant themselves, but rather are explained as “non-cancerous cells of the tumor organ”. Indeed, many authors compare TME, due to its complexity, to the function of an organ (Egeblad et al., 2010).

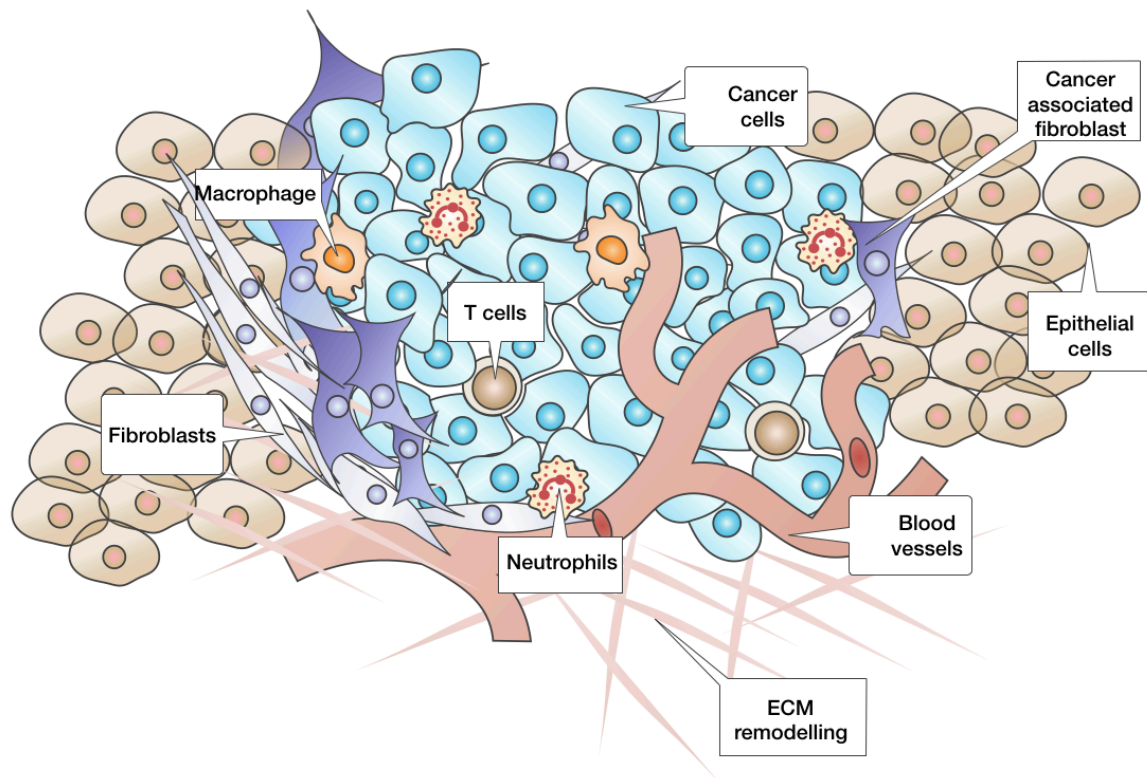


Figure 4. Composition of tumor microenvironment

Tumor microenvironment TME is an entity comprised of various cell types including cancer cells, fibroblasts and cancer associated fibroblasts, immune cells such as macrophages, T-cells, neutrophils and blood vessels that “feed” TME. Tumor tissue is embedded in extracellular matrix (ECM) that in tumor tissue is severely remodelled and stiffer.. Adapted from (Junttila and de Sauvage, 2013)).

To follow the context of the research delivered in this manuscript, TME will be explained as a complex tumor stroma. General overview of two stromal mayor components will be presented in the following chapters, extracellular matrix and stromal cells. Higher emphasis will be put on the role of the remodelled ECM and cancer associated fibroblasts (CAFs) that secret it. Nonetheless, due to the extremely collaborative nature of all cell types in TME, each cell type will be mentioned and their role explained to certain extent.

1.2.2. EXTRACELLULAR MATRIX FOUND in TUMORS

The tumour stroma is responsible for tumor initiation, progression and metastasis (Hanahan and Weinberg, 2011). Tumor stroma can be classified as following: the non-cellular connective ECM composed of proteoglycans, hyaluronic acid, fibrous proteins (e.g. fibronectin, collagen and laminin) and cellular components - stromal cells. The stromal cells include mesenchymal supporting cells such as adipocytes, fibroblasts, and CAFs, mesenchymal stromal cells, fibrocytes (Raffaghello and Dazzi, 2015).

Besides this, tumor stroma is abundant in immune and inflammatory cells that will be mentioned briefly in this manuscript but not detailed since they are not in the scope of the research presented.

ECM is an assembly of macromolecules of protein and carbohydrate nature that bind homologous and heterogeneous cells together and organize them into tissues. ECM surrounds cells that synthesise it and, in turn, helps to determine the phenotype by providing not only physical support, but also the biochemical and biomechanical signals necessary for morphogenesis, cell differentiation and tissue homeostasis (Frantz et al., 2010). The macromolecules of the extracellular matrix are grouped into the four categories:

1. Collagens – family of 27 distinct members, grouped into two large subfamily fibrillar collagens and non-fibrillar collagens (Mylyharju and Kivirikko, 2004)
2. ProteoGlycans (PGs) – superfamily containing more than 30 large protein chains often linked to one or more GlycosAminoGlycans (GAGs) chains, such as heparin sulphate (Casu et al., 2010; Iozzo, 1998).
3. Elastin fibres – essential ECM macromolecules comprising an elastin core surrounded by a mantle of fibrillin-rich microfibrils and fibulin (structural glycoproteins) (Kielty et al., 2002)
4. Glycoproteins – proteins which contain short chains of carbohydrates covalently attached to amino acid side chains (Chothia and Jones, 1997). Some of the members of glycoprotein family are fibronectin, laminin, tenascin-C, integrin, etc.

Microenvironment includes endothelial cells that line the blood and lymphatic vessels, and pericytes that surround these vessels; a wide variety of bone marrow derived cells such as mast cells, macrophages, lymphocytes, neutrophils and immunosuppressive myeloid cells (MICs); MSCs as well as many fibroblasts (Chen et al., 2018a). All these cells are surrounded by a pro-tumor extracellular matrix having, in particular, the characteristic of being more rigid than in healthy tissues (Frantz et al., 2010).

The ECM is not only a network in which cells are integrated, but it also establishes cell-matrix interactions that take place through specialized receptors (adhesion receptors) and allows the communication between the cells and their environment. Among these receptors are integrins, discoidin domain receptors (DDR) and syndecans (Harburger and Calderwood, 2009; Leitinger and Hohenester,

2007; Xian et al., 2010). Integrins, principal transmitters of signals from ECM to the cells are a family of 24 transmembrane heterodimers composed of alpha and beta subunit. Upon their activation and binding to the ECM (through their extracellular domain) (Harburger and Calderwood, 2009) adhesome is recruited to the cytoplasmic tail of an integrin. Adhesome is a protein complex composed of proteins involved in signalling, scaffolding and cytoskeleton (Horton et al., 2015, 2016). These protein complexes, in one hand allow anchoring of the cell cytoskeleton to ECM, and on the other hand support bidirectional transmission of signals between the cell and its environment (signaling controlling migration, survival, proliferation and differentiation) (Ridley et al., 2003). Generally, outside-inside integrin mediated signalling involves the recruitment of the focal adhesion kinase (FAK) and its downstream target Src (Humphries et al., 2006). It is important to stress that, integrins are crucial for mechanical sensing, stiffening and remodelling of ECM, and all fundamental steps in tumor progression including invasion, metastases and drug resistance, which is the main topic of the research presented in this manuscript (Hamidi and Ivaska, 2018).

Besides integrins, various enzymatic and non-enzymatic processes have the power to influence ECM behaviour in terms of structure, remodelling and soluble factors found within. At the same time, molecular components undergo a very large number of post-translational modifications. Under physiological conditions all these processes are tightly regulated and homeostasis is maintained. If homeostasis is disrupted, no control is established over any type of cellular processes including ECM deposition and remodelling. ECM formed under this dis-balance acquires pathological properties that results in fibrosis and, ultimately tumor development (Eckes et al., 1999). ECM found in tumors represents an architectural structure profoundly different from the one of “normal” healthy tissue (Butcher et al., 2009). During tumor progression, there are multiple changes in the production and assembly of ECM molecules secreted and arranged by the different cell populations of the TME (Jinka et al., 2012). Excessive deposition and remodelling of ECM by CAFs is responsible for increased fibrosis that in turn promotes tumor development. (Lu et al., 2012; Samuel et al., 2011). Matrix remodelling is one of the key events during tumor development, and it mostly coordinated by the activity of MMPs and their inhibitors. In cancers, MMPs can be secreted by tumor cells themselves, CAFs and tumor-associated macrophages (TAMs) and they facilitate the invasion and migration of cancer cells by collagen degradation (Rosenblum et al., 2007). Their activity is regulated by *Timp* gene family of inhibitors that control not only a wide range of MMPs but also ADAMs by post-translational inhibition (Khokha et al., 2013). In parallel, collagen and elastin fibres of the tumor stroma are organized, re-oriented and assembled by LOX enzymes leading to the increase in width and, importantly, stiffness of these fibres (Butcher et al., 2009; Levental et al., 2009; Paszek et al., 2005). Increased deposition of ECM proteins such as collagens (in particular collagen type I, III, and IV), fibronectin, hyaluronic acid and laminin is observed in many cancers (Brichkina et al., 2016; Fullár et al., 2015; Provenzano et al., 2006). On top of these proteins, ECM found in tumors is packed with secreted protein acidic and rich in

cysteine (SPARC) protein, periostin, Tenasin C, osteopontin and thrombospondin (Albo et al., 1997; Nong et al., 2015; Wang and Ouyang, 2012)

Tumors, besides being characterized by ECM remodelling, have know to be stiffer than normal tissue as discussed later on.

Simultaneously, various chemokines (CXCL12, CXCL10, CCL21), growth factors (insulin growth factor (IGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), angiogenic factors such as vascular endothelial growth factor (VEGF) and TGF β are released in ECM and bound to it (Gocheva et al., 2006; Kessenbrock et al., 2010; Lerner et al., 2011). Often, growth factors bind to glycosaminoglycan chains attached to ECM and membrane proteins, but recent evidence suggest a specific biding of growth factors to ECM proteins themselves. As an example fibronectin binds HGF and VEGF directly (Rahman et al., 2005; Wijelath et al., 2006).

Given the evidence , the importance of ECM in variety of cellular process (both in physiological and pathological conditions), and its role in the behaviour of epithelial cells is obvious (Pickup et al., 2014; Werb, 1997) (**Figure 5**) and remains an important aspect to investigate during cancerogenesis, and finally in chemoresistance which is the main subject of my research.

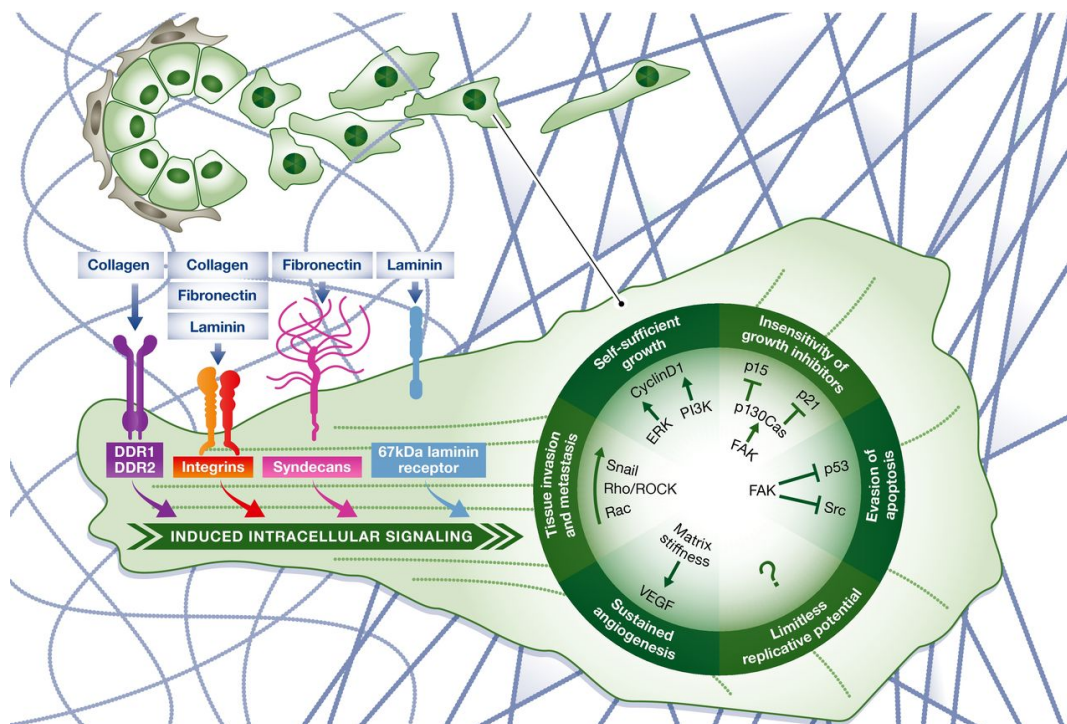


Figure 5. Molecular influence of extracellular matrix on tumor cells

From the initiation of a tumor to the formation of metastases, ECM supports and sustains all hallmarks of cancers. ECM molecules bind to cell surface receptors, activating intracellular signaling pathways. Adherence of cells to ECM induces activation of Erk and PI3K kinases, which promotes autonomic growth of tumor cells. Activation of the FAK kinase inhibits p15 and p21 growth suppressors, and induces apoptosis resistance by p53 inhibition. The molecular components and biophysical properties of ECM promote the induction of epithelio-mesenchymal transition, and increase tumor cell migration, particularly via TGF β signaling, and RhoA / Rac GTPases. Matrix rigidity also promotes angiogenesis by increasing VEGF signaling in endothelial cells. Adapted from (Pickup et al., 2014).

1.2.3. RIGIDITY and REMODELLING of the ECM

I would like to dedicate this part to elucidating the role of ECM in tumor progression, specifically to the role of ECM remodelling and stiffness. Earlier on, I have mention that composition of tumor ECM is modified compared to normal ECM, and that various players participate in its remodelling leaving architecture of tumor ECM crooked. For example, ECM secreted by CAFs is organised in a more liner manner with increased levels of fibronectin and collagen, notably cross-linked collagen. ECM plays multiple, equally relevant, roles in TME such as facilitating migration and invasion, nevertheless I would like to focus on the fact that ECM found in tumors is significantly stiffer that the one in normal tissue (Butcher et al., 2009) and the fact that stiffness can influence the faith of cancer cells in a process called mechanotransduction.

A) What is stiffness of the ECM?

Cells behaviour does not rely only on biochemical pathways but also on forces applied on them. Cells, including cancer cell, are subjected to isometric nanoscale forces or tensions generated by cell-cell contact or cell-ECM contact. Upon the application of force, cell functions are modified by actomyosin contractility and actin dynamic in collaboration with biomechanical cues ultimately modulating cell behaviour. Process described is mechanotransduction and will be further discussed. Generally, mechanical stress is quantified in Pascals (Pa) and measured as force per unit (N per m²).

As I have established now, in cancers, homeostasis is lost, thus it is not surprising that the level of stiffness is as well disturbed. Indeed, cancerous tissue can be up to 10 times more rigid than healthy tissue (**Figure 6**), which is correlated with tumor cell survival and increased proliferation (Jalouk and Lammerding, 2009; Levental et al., 2009). In addition, it is associated with a higher risk of metastasis, and is a factor of poor prognosis (Schwartz, 2010).

In diagnostic, stiffness of tumors can be exploited for a palpation diagnosis of certain tumors such as breast cancer, magnetic resonance imaging (MRI) or ultrasound that detect cancerous tissue (Poterucha et al., 2015; Zaleska-Dorobisz et al., 2014).

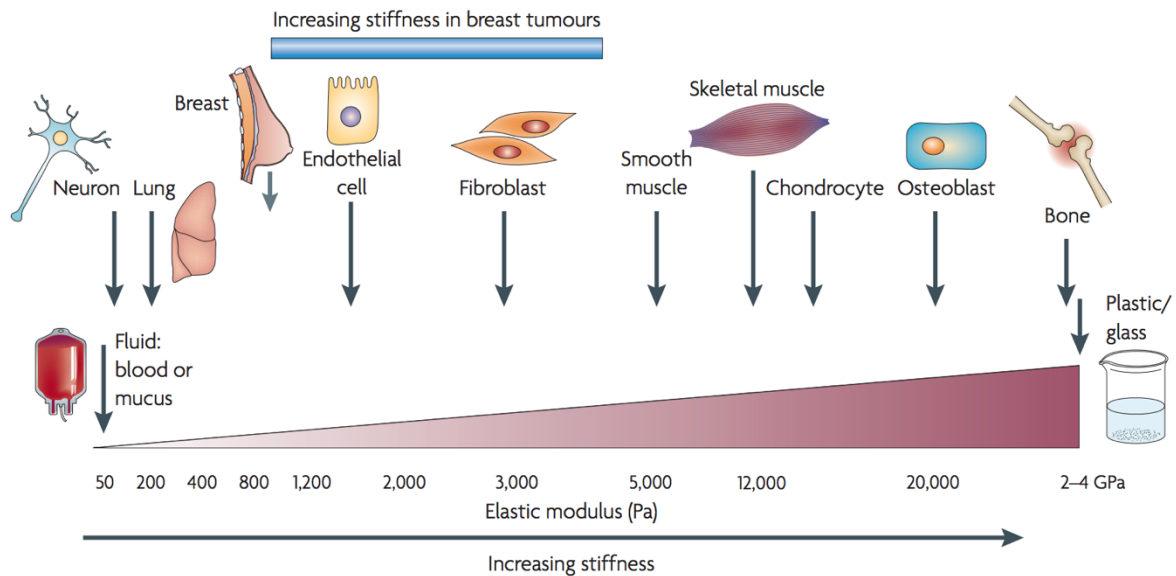


Figure 6. Difference in rigidity of various tissues measured in pascals, including tumor tissue

All cells are exposed to tension forces generated by cell-cell interactions, or importantly cell-ECM interactions. Well-being of cells corresponds to their homeostatic stiffness, for example brain tissue is a very soft one, therefore an optimal lifecycle of neural cells is favoured by soft matrix. Quite similar is with epithelial cells that are adjusted to a soft matrix. In case of a tumor formation, ECM becomes more stiff and epithelial cells become hyper-responsive to the change alter their function into a malignant one. Adapted from Butcher et al.

B) Role of stiffness in cancer progression

In recent years, the importance of tumor matrix stiffness has emerged and accumulating number of publications are dedicated to exploring its role in tumor development.

Matrix rigidity increases the proliferation of tumor cells. In fact, in contact with a rigid matrix, the cells increase the phosphorylation of the FAK, Erk and PI3K kinases, which accelerates the cell cycle progression by increasing the expression of cyclin D1 (Bae et al., 2014; Levental et al., 2009; Paszek et al., 2005; Provenzano et al., 2009). Moreover, by modulating the expression and activity of growth factor receptors such as EGFR, stiffness increases proliferation in response to growth factors (Song et al., 2015). During cancerogenesis mutations occur in tumor suppressor genes leading to the loss-of-function and non-responsiveness to growth inhibitory signals from these tumor suppressors (Collins et al., 1997). Besides mutations, binding of cells to a tumor matrix inhibits the expression of some of these tumor suppressors such as BRCA1 (O'Connell and Martin, 2000) or phosphatase and tensin homolog (PTEN) (Mouw et al., 2014). Cell-ECM interaction leads to the adhesion of the tumor cells to the ECM and bypasses several cell growth suppression pathways in order to promote malignant transformation. For example, the formation of metastases in lung cancer is increased by the alteration of the ECM at the metastatic site. In turn, tumor growth is stimulated by the increase of fibronectin and matrix stiffness through inhibition of tumor suppressors (Erler et al., 2009). In addition, the mechanical activation of Yes-Associated Protein (YAP) allows tumor cells to be insensitive to the suppression of cell growth by

contact inhibition, and thus to proliferate uncontrollably (Dupont et al., 2011; Zhao et al., 2007). Matrix stiffness also allows tumor cells to resist apoptosis. Indeed, the activity of integrins influences apoptosis by inactivating pro-apoptotic molecules such as Bax (BCL2 associated X protein), or over-expressing anti-apoptotic molecules such as BCL2 (B-cell CLL / Lymphoma 2) (Frisch et al., 1996; Gilmore et al., 2000; Ruoslahti and Reed, 1994). In addition, several teams demonstrated that activation of FAK and PI3K kinases inhibited apoptosis (Golubovskaya and Cance, 2011; Lim et al., 2008). Induction of proliferation by stiffness and anti-apoptotic stability indicate to a correlation between ECM stiffness and chemoresistance (Hayashi et al., 2012; Schrader et al., 2011). This notion is the foundation of my PhD research, and the role of matrix stiffness in chemoresistance will be reported in following chapters. Further more, tumor development is followed by neo-angiogenesis allowing the transport of oxygen and nutrients necessary for the growth and survival of cancer cells. Angiogenesis is stimulated by growth factors such as VEGF and FGF, which induce proliferation and migration of endothelial cells (Bergers and Benjamin, 2003). Stiffness promotes angiogenesis by regulating the expression of VEGF receptor (Lederle et al., 2010; Mammoto et al., 2009). Moreover, matrix stiffness contributes to the formation of invadopodia and complex focal adhesions leading to the invasion of tumor cells (Menon and Beningo, 2011; Parekh et al., 2011). Invasion of cancer cells is supported by the activation of the Rho and Rac GTPases that regulate cellular contractility and tumor migration (Parri and Chiarugi, 2010; Sanz-Moreno et al., 2008). In addition, , the rigidity of the ECM has recently been implicated in tumor metabolism. Research published in 2014 show that in pancreatic cancer, activation of FAK kinase by stiffness induces glucose consumption by tumor cells and their dependence on glutamine (Zhang et al., 2014). Finally, I would like to underline that recently a connection between ECM rigidity and EMT has been established and it is know that stiffness promotes EMT (Leight et al., 2012; Rice et al., 2017a; Wei et al., 2015). In the light of the data I obtained, relationship between stiffness and EMT will be further developed. In conclusion, remodelled matrix and therefore stiff matrix plays an important role in all stages of tumor development (**Figure 7**). Since all these process would not be possible without the act of mechanotransduction, next part of this chapter will be dedicated to understanding mechanotransduction in this context.

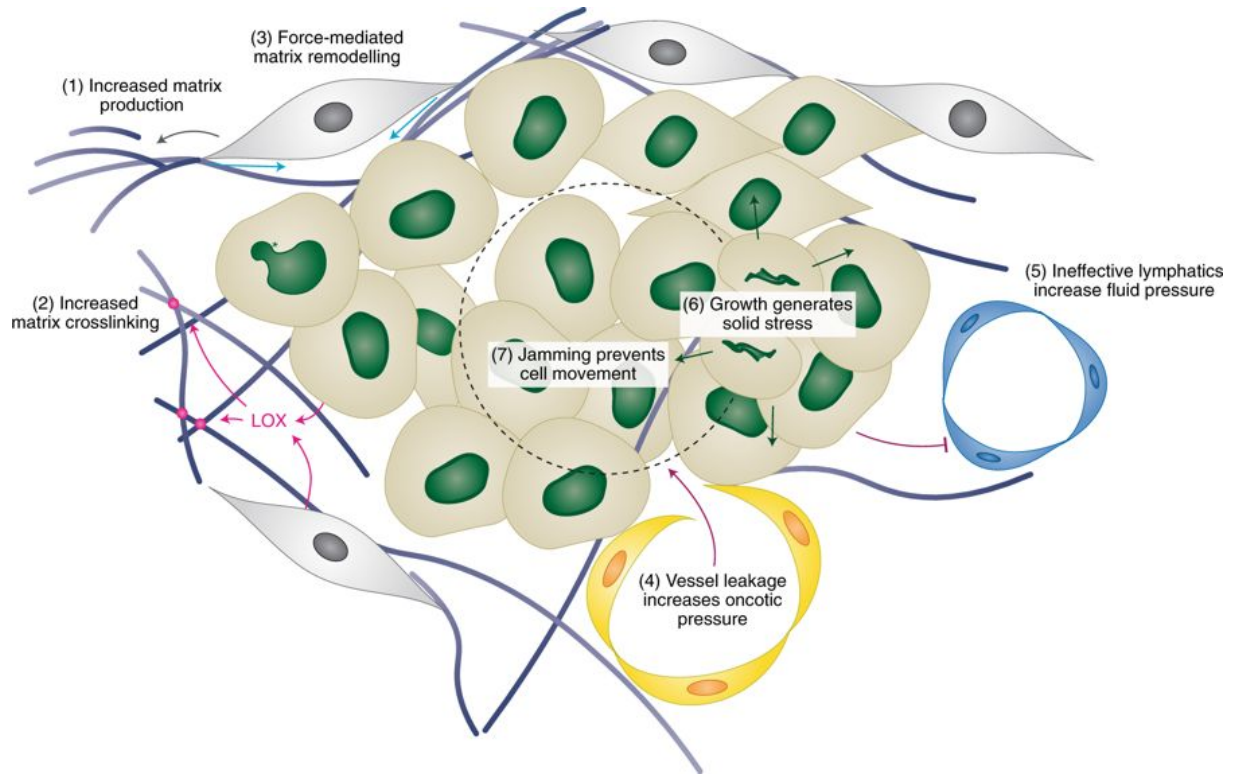


Figure 7. Matrix remodelling impacts cancer cells behaviour on multiple levels

Tumor cells are labelled in green, stromal fibroblasts in grey, endothelial cells in yellow for blood vessels and green for lymphatic. Lastly, ECM is dark blue. Changes in tissue stiffness leads to (1) increased matrix deposition, (2) crosslinking, (3) contractile forces controlling ECM stretching, (4) blood vessel leakage, (5) fluid pressure increase due to ineffective lymphatics, (6) cell growth, (7) high cell density – increase of proliferation. Adapted from (Mohammadi and Sahai, 2018).

C) Mechanotransduction

Mechanotransduction is a cellular process in which mechanical stimuli are translated into biochemical cues (pathways), allowing cells to adapt to their environment.

Mechanism of mechanotransduction involves the detection of external forces, or biomechanical properties of the ECM, and the translation of this information into specific intracellular signals. Mechanotransduction influences cellular behaviours, including growth, differentiation and malignant development.

Many cellular actors have been described to participate in this mechanism, but until this day all processed have not been fully understood (Moore and Sheetz, 2011). In particular, the actin cytoskeleton plays a crucial role in mechanotransduction by linking the different cellular compartments to the physical signal detection apparatus: integrins. Cells actively detect the rigidity of their environment by exerting traction forces on the ECM *via* integrins (Hynes, 2002). Matrix stiffness influences the binding time of integrins with ECM, stimulating their activation (Friedl et al.; Litvinov et al., 2011). Activation of integrins leads to their clustering and the formation of focal adhesion complexes containing adapter proteins talin and vinculin, kinases such as Src and FAK, and the actin cytoskeleton (**Figure 8**). The

main adapter proteins sensitive to matrix stiffness are talin (del Rio et al., 2009), vinculin (Grashoff et al., 2010), and P130Cas (Sawada et al., 2006). These proteins undergo a conformational change depending on the tensile forces and matrix stiffness. For example, the contraction of actomyosin stretches the talin, which frees its cryptic binding sites and recreates vinculin at the focal adhesion complexes (del Rio et al., 2009). The vinculin then allows the attachment of the P130Cas protein. Stretching the P130Cas core domain releases phosphorylation sites for Src kinases (Hotta et al., 2014; Janoštiak et al., 2014; Sawada et al., 2006) which regulates the formation of focal adhesion complexes and the actin cytoskeleton (Meenderink et al., 2010). Mechanotransduction induced by integrins also modifies cellular behaviours by activating various signaling pathways regulating gene expression (Janmey et al., 2013). I will review several important pathways involved in mechanotransduction.

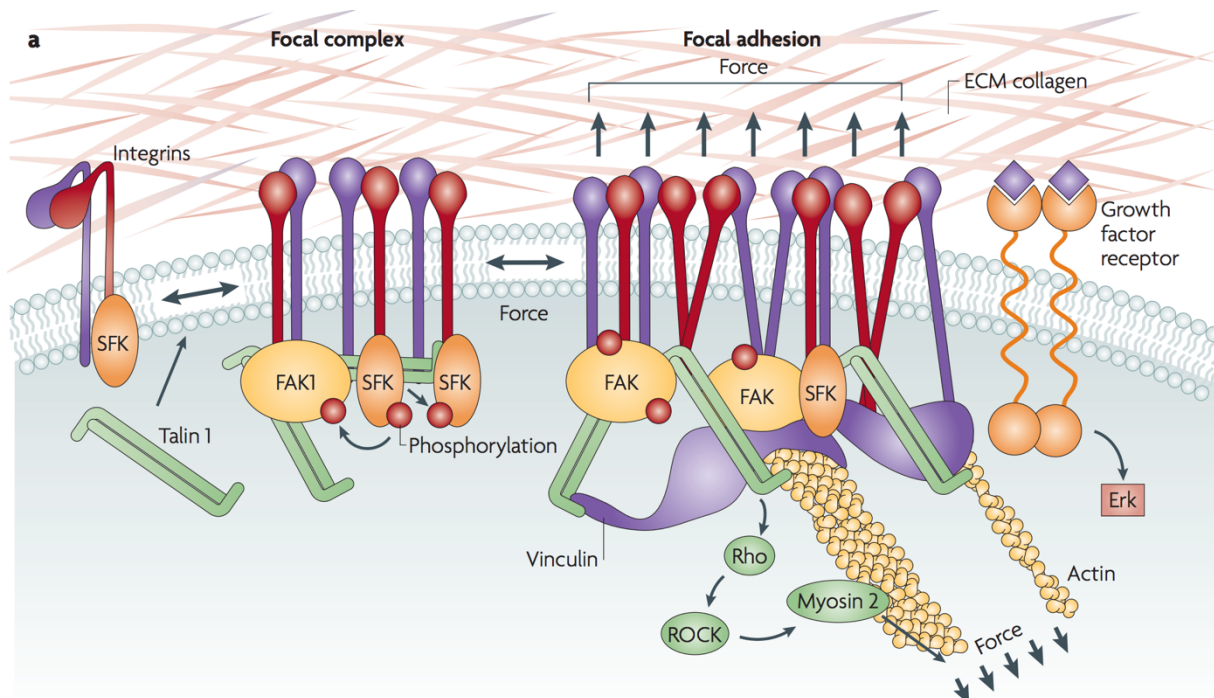


Figure 8. Schematic representation of mechanotransduction carried out by integrin activation

Majority of integrins found on the membrane are in an in-active state. Upon matrix remodelling and ECM stiffening, integrins are activated and initiate the formation of focal adhesion complex. Effector proteins such as talin, FAK and Src are recruited and stiffness dependant conformational changes occur resulting in “mechanosensing” or precisely in activation of FAK and Src kinase activation and Rho / ROCK-dependent actomyosin contractility. Adapted from (Butcher et al., 2009).

I) FAK-SRC pathway and its connection to mechanotransduction

Focal adhesion plate formation and cellular contractility leads to phosphorylation of the FAK kinase (Paszek et al., 2005), a non-receptor tyrosine kinase (Peng et al., 2008). The importance of FAK in mechanotransduction has been demonstrated by Wang *et al.* (2001), research done by this group shows that cells that do not express FAK lost their ability to migrate in a stiffness-dependent manner, but by phenomenon of durotaxis (Lo et al., 2000). Src and FAK kinases are responsible for the phosphorylation of proteins within focal adhesion complexes. Inhibition of their kinase activity has no effect on the formation of these complexes and the detection of matrix stiffness. However, it blocks the cellular response induced by changes in matrix stiffness (Bae et al., 2014; Horton et al., 2016). In addition, growth factor receptors interact with integrins, particularly during cell migration (Lee and Juliano, 2004). Interestingly, in the presence of a rigid matrix, Src kinases activates EGF receptors independently of their ligand, therefore EGF receptors participate in mechanotransduction and rigidity-induced cell phenotype (Saxena et al., 2017).

II) YAP-TAZ co-transcriptional factors and stiffness

Another link between stiffness dependent integrin activation and gene transcription is Hippo pathway. Stiff substrate stimulates integrin and F-actin dependant activation of YAP and TAZ (transcriptional co-activator with PDZ-binding motif), two transcriptional regulators and key players of the Hippo pathway. Generally, Hippo pathway is involved in the control of organ size and its kinase cascade phosphorylates and inhibits YAP/TAZ transcriptional co-activators (YAP/TAZ are one of few transcriptional factors that are inactive when phosphorylated) (Zhao et al., 2007). In the set up of stiffness, YAP/TAZ can become activated and therefore translocated to the nucleus upon integrin activation and cellular contractility through Rho-ROCK signaling pathway (Dupont et al., 2011). Consequently, YAP and TAZ modulate the expression of target genes (Halder and Johnson, 2011; Zhao et al., 2007). Similar mode of activation is associated with another family of transcriptional co-activators known as MLK family transcriptional regulators or myocardin and related transcription factors (MRTF) that regulate expression of serum response factor (SRF) (**Figure 9**) (Minami et al., 2012). The rigidity-dependent signaling mediated by MRTF-A involves its direct binding to the actin cytoskeleton. Indeed, by binding to the actin monomers, MRTF-A inhibits their polymerization. However, in the presence of a rigid matrix, the contraction of actomyosin induces the actin polymerization thereby releasing MRTF-A, which can then move into the nucleus and induce the expression of SRF (Miralles et al., 2003).

When in nucleus, YAP/TAZ modulate the expression of target genes (Halder and Johnson, 2011; Zhao et al., 2007). YAP/TAZ are responsible of transcribing genes involved in proliferation, survival and migration (Mohammadi and Sahai, 2018). The activity of these factors is associated with poor prognosis in many cancers such as lung cancer (Noguchi et al., 2017; Zanconato et al., 2016), breast (Cordenosi et al., 2011) and colon (Yuen et al., 2013).

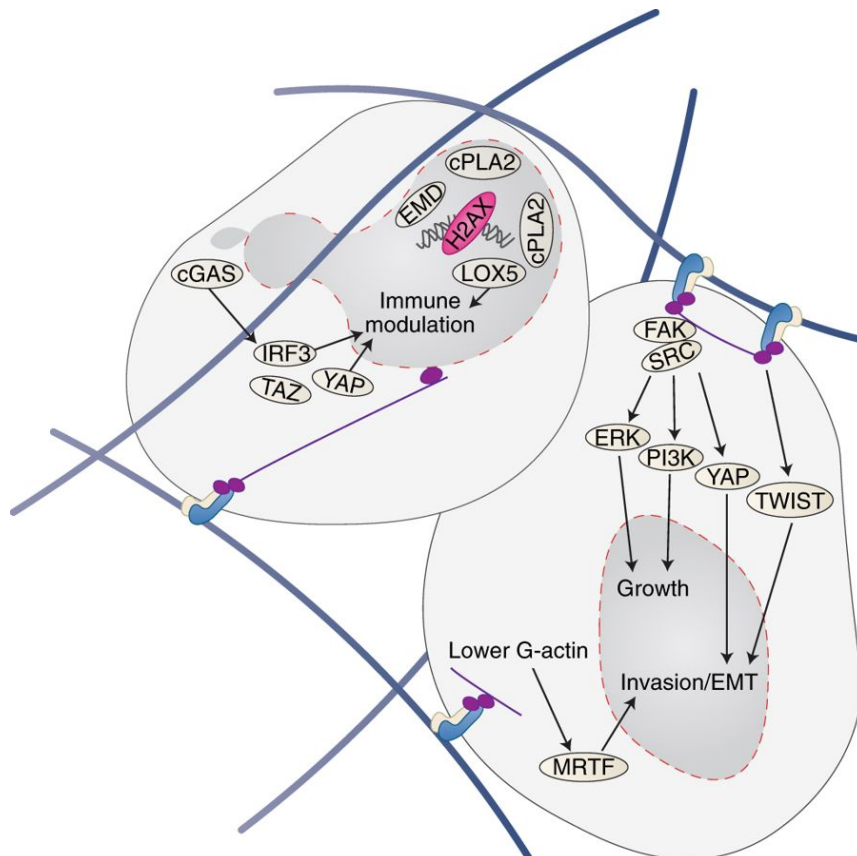


Figure 9. Different signaling pathways that are modified as a result of ECM stiffening

One of the most important factors in mechanotransduction are FAK/SRC, YAP/TAZ and MRTF transcriptional factors. For instance, YAP is translocated into the nucleus and able to transcribe the genes responsible for tumor growth. At the same time, TWIST (an EMT transcriptional factor) is also allowing the cell to invade and change phenotype into a mesenchymal one. Adapted from (Mohammadi and Sahai, 2018).

III) Rho-ROCK signaling cascade in response to rigid matrix

Following mechanical stress, focal adhesion complex forms, allowing the activation of the small Rho, Ras, and Rac GTPases (Clark et al., 1998; Ridley et al., 2003) which reinforces cellular contractility and actin cytoskeleton. It is established that matrix stiffness promotes tumor development, notably via the regulation of cellular contractility by Rho GTPase and its Rho-associated protein kinase (ROCK) (Paszek et al., 2005). Furthermore, Rho-ROCK signalling is, as mentioned before, a big wheel in matrix remodelling stimulated by cancer cells (Provenzano et al., 2008) and CAFs (Gaggioli et al., 2007) remodelling, that results in ECM stiffening that strengthens the Rho-ROCK signaling pathway by positive feedback loop.

Lastly, one of the most studied pathways involves the family of MAPKs including Erk, p38 and JNK kinases. Activated by tyrosine kinase receptors such as EGFR, MAPKs can induce the phosphorylation of nuclear substrates and modulate the expression of certain genes. They have been described as a key regulator of response to mechanical stimulation of osteoblasts (Hamamura et al., 2012; Khatiwala et al., 2006), endothelial cells, smooth muscle cells (Zebda et al., 2012) and breast cancer cells (Levental et al., 2009; Paszek et al., 2005) (**Figure 9**).

Besides the described molecular process many others participate in modulating the cell response to mechanical stimulations. Nevertheless, a big chunk of it is yet to be identified and studied, specially in the context of cancer. Mechanotransduction is a complex and crucial process that determines cell faith, in cancers cell faith becomes viscous upon crooked mechanical stimulations. The stiffer the matrix the worse the outcome (Gkretsi and Stylianopoulos, 2018).

1.2.4. EPITHELIAL TO MESENCHYMAL TRANSITION and its RELATIONSHIP with STIFF MATRIX

A) General information about EMT

EMT was first described in the 1980s by Elizabeth Hay during chick embryogenesis as a transformation that gives to epithelial cells high plasticity crucial for morphogenesis (Hay, 1995).

In the context of cancerogenesis, EMT is considered as a first major step towards tumor development, and a second one, and every step after in the advanced stages of cancer progression, mostly by favoring metastasis and resistance to therapy (Brabletz et al., 2018). It is a dynamic process during which cells lose epithelial “E” characteristics and gain mesenchymal “M” properties by complex functional and architectural modifications. In the Brabletz *et al.* review, I quite enjoyed, Dr. Angela Nieto comments on the “T” in the EMT in a very interesting fashion. Indeed, as she says, “T” suggests the existence of a transitional stage, a stage that is not yet fully mesenchymal and not fully epithelial anymore. In literature, this stage is described as a partial EMT (Kalluri and Weinberg, 2009). In cancers, partial EMT is established when cells are exhibiting both epithelial and mesenchymal markers at the same time. Epithelial markers notably include E-cadherin, occludin family, cytokeratins, while mesenchymal markers are N-cadherin, vimentin, fibronectin (Mani et al., 2008).

Behind these functional and morphological changes lies a panel of transcriptomic regulators of EMT: the transcription factors (EMT-TFs). Three major families of EMT-activating transcription factors have been the focus of cancer research: SNAIL (containing Snai1 and Snai2/slug), ZEB (with two factors Zeb1 and Zeb2) and basic Helix Loop Helix or TWIST (with Twist1 and Twist2) families (Polyak and Weinberg, 2009; Puisieux et al., 2014; Yang and Weinberg, 2008). EMT-TFs can modulate the expression of their target genes by directly binding to DNA or by cooperating with other transcriptional regulators. While Twist1 induces the expression of N-cadherin by binding to its promoter, downregulation of E-cadherin requires the interaction of TWIST1 with nucleosome remodeling deacetylase (NURD) complex (Alexander et al., 2006; Cheng et al., 2007). SNAIL1, SNAIL2, TWIST1 and ZEB1 are each independently responsible for an induction of partial EMT programme in tumors (Barrallo-Gimeno and Nieto, 2005; Jolly et al., 2015; Yang et al., 2004).

EMT-TFs control more than 20 signaling pathways that have been described to induce EMT in various cell types during physiological and pathological processes (Thiery et al., 2009). In literature, all these signaling pathways are grouped under five “stresses” associated with cancerogenesis. These five stresses are physical constraints, hypoxia, inflammation and oncogenic or metabolic stress (Puisieux et al., 2014). Of course, physical constraints include hydrostatic pressure, shear stress and tension forces (Desprat et al., 2008) or, as I like to call it, stiffness

B) Relationship between stiffness and EMT

Study published in Nature Cell Biology in 2015, provides compelling evidence that matrix stiffness drives EMT (Wei et al., 2015). Authors report that, in breast tumors, TWIST1 is a mechano-mediator that promotes EMT in response to increase in ECM stiffness. Matrix stiffness induces integrin-dependent phosphorylation events that translocate TWIST1 from cytoplasm to nucleus. Upon increase of stiffness, TWIST1 is released from its anchoring partner G3BP2 (Ras GTPase-activating protein-binding protein2) to nucleus. Constitutively located in nucleus, it drives the transcription of genes responsible for proliferation, invasion and metastasis. Interestingly, this mechano-regulation is independent of YAP/TAZ signaling implying a different regulatory system, not yet fully understood.

Shortly after, another research has been published demonstrating that matrix stiffness induces EMT in pancreatic cancer (PDAC) and therefore promotes chemoresistance (Rice et al., 2017a). Induction of EMT correlates with the induction to stiffness and resistance to Paclitaxel (chemotherapy used for treatment of various cancers, including pancreatic). Highly epithelial cell lines have greater sensitivity to the treatment compared to the intermediate cell lines and cells grown on *in vitro* stiff conditions that exhibit mesenchymal properties (**Figure 10**) (Rice et al., 2017a).

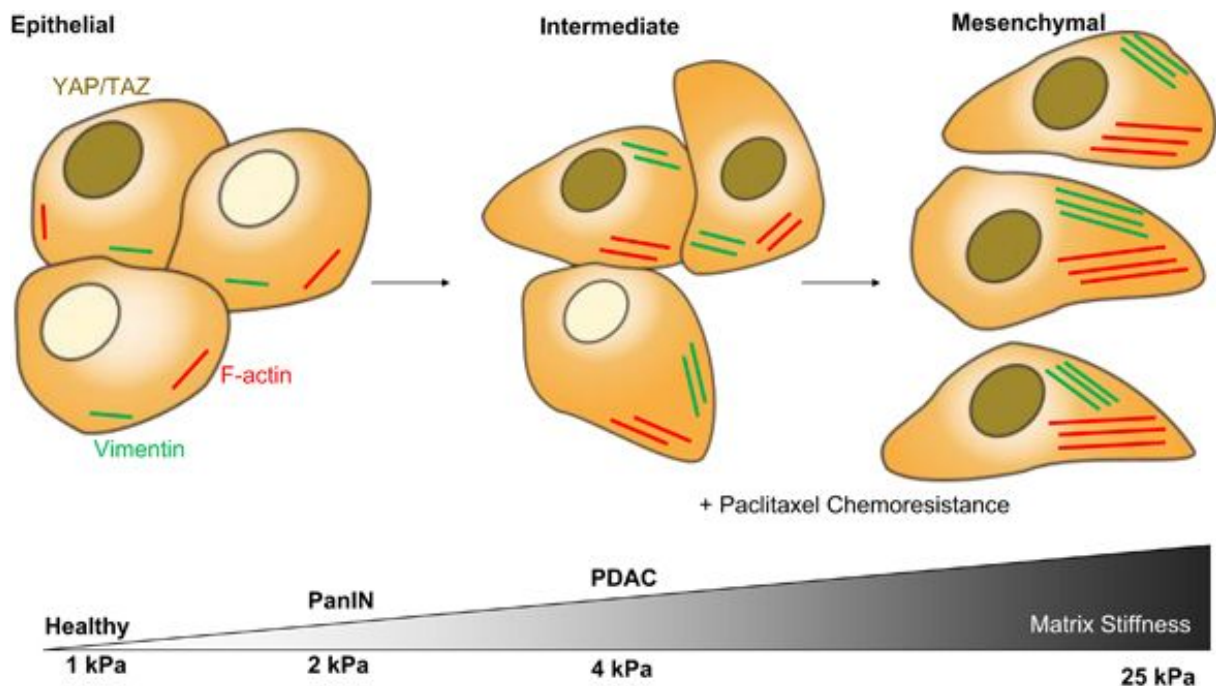


Figure 10. Relationship between stiffness and progression of EMT

In the event of an increase ECM stiffness, cells undergo an EMT that tightly correlates with a degree of stiffness. Even at intermediate state, in pancreatic cancer, treatment is no longer effective and cancer cells become resistant to Paclitaxel. Adapted from Rice et al.

Another research done on endometriosis, a gynaecological disorder responsible for infertility and pelvic pain showed that endometrial epithelial cells grown on stiff surface (plastic) underwent EMT-like process and acquired partial mesenchymal phenotype (Matsuzaki et al., 2017).

It is obvious that there is a strong link between stiffness, EMT and ultimately chemoresistance, but as you may understand from the data reviewed, mechanistically we are still on the surface of such an elaborated process. More research and more efforts are needed to fully understand what is beneath the surface.

1.2.5. STIFF ECM AS A THERAPEUTIC TARGET

Given the importance of ECM remodelling and stiffness in tumor progression, ability to prevent this occurrence or reverse its function provides an interesting direction to pursue in future drug development. However, due to its complexity it is difficult to predict will targeting a single protein involved in stiffening yield a favourable result.

A very exhaustive review published in Nature Cell Biology earlier in 2018 gives an overview of drugs in pre-clinical and clinical trials that are designed to target tumor mechanobiology (**Table 1**). Another fact, that draws the attention immediately, is the low number of drugs being currently tested suggesting a need for further efforts on this front.

Table 1 Overview of drugs designed to target tumor mechanobiology and their current developing status

Target	Name	Biological agent	Mechanism	Current status
Integrin $\alpha_v\beta_3$	Cilengitide	Ligand mimetic	Integrin binding	Clinical trials stopped due to lack of efficacy
Integrin $\alpha_v\beta_6$	GSK2634673F and BG00011	Ligand mimetic	Integrin binding	Preclinical and fibrosis trials
FAK	Defactinib (VS-6063, PF-04554878)	Small molecule	Downstream of integrin signalling	Clinical trials ongoing
Abl and Src kinases	Dasatinib	Small molecule	Downstream of integrin signalling	Clinical trials ongoing, some reported lack of efficacy
Hedgehog	IPI-926 (saridegib) and vismodegib	Small molecule	Reduces CAF activation	Clinical trials ongoing, some reported lack of efficacy
ROCK	AT13148	Small molecule	Contractility	Phase I clinical trial completed
LOXL2	Simtuzumab (GS 6624)	Blocking antibody	Anti-crosslinking	Preclinical and fibrosis trials
CTGF	FG-3019	Blocking antibody	Blocks receptor binding ^a	Early phase clinical trials ongoing
Hyaluronidase	PEGPH20	PEGylated enzyme	ECM degradation	Clinical trials ongoing

Matrix crosslinking is driven by LOX enzymes, and they are a rational target for overcoming matrix stiffening. Indeed, in breast cancer model inhibition of these enzymes by β -aminopropionitrile (BAPN) or blocking antibodies resulted in reduce tumor size and metastasis (Levental et al., 2009). Similar results were obtained in *in vivo* studies of breast, pancreatic and gastric tumors (Barry-Hamilton et al., 2010; Li et al., 2015; Miller et al., 2015). Unfortunately, clinical trials testing LOX2 inhibitors so far were not a great success, potentially due to an inability to reach doses high enough to reduce the stiffening and the fact that crosslinking is only one part of the story with actomyosin contractility on the other side (Mohammadi and Sahai, 2018). Contractility can be reversed by using ROCK inhibitors, that have been approved for vasospasm (Shimokawa et al., 2002) and have been show in pre-clinical trials to control (to some extent) pancreatic cancer (Vennin et al., 2017). Again, unfortunately, ROCK inhibitors did not find their way in oncology.

Since the first to respond to mechanical stress are integrins, targeting them has been examined. Both by usage of ligand mimetic peptides and blocking antibodies. Most studied integrins for drug development are $\alpha_v\beta_3$ and $\alpha_v\beta_6$ heterodimers. No apparent efficacy in phase III clinical trials has been observed for a peptide targeting α_v integrins (Stupp et al., 2014) and antibodies against $\alpha_v\beta_6$ are in a very beginning of development (Mas-Moruno et al., 2010).

Besides integrins, integrin regulated signaling pathways are being investigated as well, such as FAK-Src kinase complex and MAPKKK cascade (Golubovskaya and Cance, 2011). However, since the implication of FAK-Src in numerous signaling streams it is difficult to understand is the compound targeting mechanosignaling or growth-factor.

Certainly, it remains a great challenge to exploit ECM stiffness as a therapeutical strategy specially as a single regulatory event. Tissue mechanics have not been well characterised as biomarkers such as proliferation and other biochemical processes are. It is important to put technology to use and evaluate the tissue mechanics *in vivo* and therefore evaluate the potential drug response. Possibility of patient stratification according to the high levels of fibrillar ECM is another avenue that should be considered in future (Vennin et al., 2017).

1.2.6. STROMAL CELLS in TME

Through out this part of the introduction, I will discuss two types of stromal cells; fibroblasts and cancer associated fibroblasts. In normal tissue only fibroblasts are found while in TME presence of both cell type is reported.

Besides these cells, role of adipocytes as stromal cells is described, but since their role was not the scope of the research we conducted, just like in the case of immune cells, they will not be discussed in more detail.

A) Fibroblasts

Fibroblasts, first described in 19th century (Virchow, 1859) are the most abundant cell type of connective tissue, and are derived from heterogeneous cells of mesenchymal lineage origin (Baum and Duffy, 2011). These non-vascular, non-epithelial, non-inflammatory cells are large (100uM) spindle-shaped or star-shaped cells with a large cytoplasmic extension. In a physiological contexts, fibroblast are considered to be quiescent with a low rate of proliferation (Kalluri, 2016). Fibroblast are usually distinguished on the basic of their morphology by presence of certain markers such as vimentin intermediate filament, integrin $\alpha 1 \beta 1$ or fibroblast-specific protein 1(FSP1). Importantly, non of these markers are fibroblast-specific, for example FSP1 can be found in macrophages, other immune cells and, sometimes, in cancer cells (Österreicher et al., 2011). Since there is no precise criteria for fibroblast differentiation nor specific markers the isolate them, a common classification has not been clearly established. The physiological role of normal fibroblasts includes several processes such as: (1) secretion and assembly of the major components of ECM; collagen (notably type I, III and V), fibronectin, elastin, fibrillin, glycoproteins, proteoglycans, etc.(Bremnes et al., 2011; Rodemann and Müller, 1991; Tomasek et al., 2002), (2) regulation of tissues homeostasis, (3) inflammation and (4) differentiation of surrounding cells through secretion of cytokines, growth factors (Wiseman and Werb, 2002), enzymes, previously mentioned MMPs and protease inhibitors that maintain a homeostatic balance between ECM deposition and degradation (Chang et al., 2002). Fibroblast are sensitive to changes in their environment and any kind of alteration has a great effect on their function. One of the main characteristics of fibroblast is their ability be activated (and therefore become contractile) following various stimuli. Once activated, their properties change and the ECM they secrete is of altered architecture. Besides that they recruit immune cells and secrete different chemokines and cytokines (Hinz et al., 2001; Kalluri and Zeisberg, 2006) that disrupt previously established homeostasis. Activated fibroblasts – myofibroblasts are introduced next.

B) Myofibroblasts

In case of a wound healing process, under stress, resting fibroblasts become activated and acquire different physiological and mechanical properties (Gabbiani et al., 1971). These activated fibroblasts are named myofibroblasts by their fibroblastic nature and the presence of a large network of micro-filaments composed of alpha-smooth muscle actin (α SMA) (Darby et al., 1990). Morphologically, myofibroblasts are identified by their ability to contract due to the significant activity of actomyosin. Unlike myofibroblasts, normal fibroblasts have cortical organisation of actin. The differentiation of fibroblasts into myofibroblasts is separated in two phases (1) mechanical stress of damaged tissue (and sometimes platelet-derived growth factor (PDGF)) induces the formation of proto-myofibroblasts that through stress fibres generate contractile forces on the ECM, but do not express α SMA (Tomasek et al., 2002). (2) Only after the induction of α SMA expression by TGF β , ECM proteins such as fibronectin and mechanical stress, are myofibroblasts differentiated (Hinz, 2007). As mentioned in the beginning, differentiation of fibroblasts is particularly prominent during wound healing when myofibroblasts synthesise ECM and generate traction forces necessary for wound closure (Estes et al., 1994). When the healing process is over, myofibroblasts disappear in a cascade of events. It has been shown that myofibroblasts undergo apoptosis and the tissue is later re-populated by “new” normal fibroblasts (Darby et al., 2014; Desmoulière et al., 1995). However, in a prolonged wound-like state, such as fibrosis or scarring (as well as in tumors) myofibroblasts avoid cell death, persist and contribute to the ongoing pathological state, notably cancerogenesis (Darby et al., 2014). We must remind ourselves that cancer has been accurately described as a “wound that never heals” (Dvorak, 1986) therefore, the role of “activated fibroblasts” is self-explanatory. Although the role of myofibroblasts in wound healing is well understood, their role in cancer progression remains to be elucidated. What is known is that myofibroblasts can play a dual role - tumor promoting and tumor suppressing. There is a very thin, well controlled line between tumor promoting CAFs and myofibroblasts. CAFs, undoubtedly, act in the same manner as (myo)fibroblasts during wound healing, express similar markers and acquire contractility.

I) FIBROSIS

I have already mentioned that prolonged wound state such as fibrosis leads to the constitutive activation of myofibroblasts and eventually development of CAFs. At the same time, one of the main roles of CAFs is production and remodelling of ECM that leads to fibrosis creating a positive feedback loop. Chronic fibrosis in many organs eventually leads to the development of tumors (Li et al., 2014; Rybinski et al., 2014). Fibrosis is a consequence of a chronic inflammation process induced by, for example, persistent infections, autoimmune reactions, allergic responses, exposure to chemical agents and tissue injury (Wynn, 2008). It is associated with excessive deposition of ECM, notably collagen and its characteristics are overgrowth, stiffening and scarring of tissues (Friedman, 2004). Although fibrosis is strongly connected with inflammation and treatments for fibrotic diseases (such as pulmonary fibrosis, liver cirrhosis, systemic sclerosis, progressive kidney disease, and cardiovascular fibrosis) mostly target the inflammatory response, recent evidence suggest that the mechanism behind fibrosis is somewhat different than the one behind inflammation. In fact, myofibroblasts are a key cellular advocate of fibrosis and are becoming an important target for antifibrotic drugs. In most cases, fibrosis is sustained by production of growth factors (TGF β , VEGF, FGF2 and IGF) (Chaudhary et al., 2007), proteolytic enzymes, angiogenic factors and fibrogenic cytokines (IL-4 and IL-13) (Borthwick et al., 2013) and chemokines, all of which stimulate ECM deposition and tissue remodelling. I have mention all of these factors previously in association with tumor microenvironment, development of CAFs and tumor formation, therefore role of myofibroblasts in fibrosis is far more than just significant. In fact, TGF β that is upregulated in response to injury and causes fibrosis associated with EMT and synthesis of ECM, has been explored in therapeutic purposes through development of TGF β signaling modifiers (Wynn, 2008). Another explored avenue of treatment for fibrosis is possibility to target tropomyosin 1.6/7 isoform in order to reduce α SMA expression in myofibroblasts with the consequent reduction of their remodelling activity and therefore fibrosis (Gunning et al., 2015; Hinz et al., 2002; Prunotto et al., 2015). Lastly, it has been shown that the ROCK inhibitor Y-27632 decreases myofibroblasts remodelling activity and tissue contraction leading to decrease in fibrosis (Tomasek et al., 2006).

C) *Cancer associated fibroblast*

CAFs are, besides cancer cell, the greatest villain of TME and the collaboration between the two is extremely fruitful. Through this chapter I will fully disclose this relationship, role of CAFs and potential of exploiting them as a therapeutic target.

1) *General overview*

Term CAFs defines activated fibroblasts displaying similar phenotype to myofibroblasts during wound healing, only contrast is they do not undergo apoptosis and “disappear” (Mueller and Fusenig, 2004). Similar to fibroblasts, CAFs are involved in the same physiological process from ECM deposition and remodelling to cytokine and growth factor secretion, with one mayor difference – they are all altered and support tumor development. CAFs are involved in every step of cancerogenesis from initiation to metastases, and produce many different tumor components. In brief, CAFs are responsible for architectural modification of ECM including linear re-organisation of collagen and fibronectin fibres that in turn facilitate migration and invasion of cancer cells (Conklin et al., 2011; Provenzano et al., 2006).

At the same time, collagen crosslinking increases cellular proliferation and metastatic spread with niche formation (Cox et al., 2013). Importantly, these modification lead to the stiffening of the ECM, an important characteristic of tumors. In fact, tumor tissue can be up to 10 times more stiff compared to healthy tissue. ECM stiffening is positively correlated with tumor cell survival, increased proliferation and chemoresistance (Jalouk and Lammerding, 2009; Levental et al., 2009; Lu et al., 2012).

When it comes to cytokines and growth factor receptors, CAFs have a tremendous impact as well. Through secretion of growth factors such as SDF-1, FGF2, VEGF, TGF β and HGF, various cytokines, proteases such as MMPs and extracellular proteins such as Tenasin-C CAFs directly and indirectly influence the tumorigenic behaviour of other cell types of TME including cancer cells, adipocytes, inflammatory and immune cells. Paracrine signals derived from these cells contribute largely to the tumor growth. CAFs are a heterogenic group of cells that exhibit different markers according to the tumor type, location and function. Until this day it remains a challenge to identify plethora of CAF populations and sub-populations in order to further understand their ole.

II) Markers used to identify CAFs population

Although there are no specific CAFs markers, variety of them are used, often in combination, to identify and distinguish CAFs population. The most frequently used marker is α SMA (Kalluri and Zeisberg, 2006) despite that, research from our group identified an α SMA negative population of CAFs (Albregues et al., 2014). Similar to myofibroblasts, CAFs express fibroblast activation protein (FAP), vimentin, desmin, tenascin C (De Wever et al., 2004), but as well SPARC, chondroitin sulphate proteoglycan (Sugimoto et al., 2006), PDGF (Pietras et al., 2003), prolyl 4-hydroxylase (Kojima et al., 2010), integrin alpha 11 (Zeltz and Gullberg, 2016) and FSP1 (Strutz et al., 1995). Markers named here are just some of many that CAFs express. Generally speaking, CAF markers are expressed differentially, not only between different tumors, but also between cells within the same tumor which is making it extremely difficult to identify and potentially target. In the field, it is believed that more than one sub-population exists within the tumor and exert distinct function. There is more and more research dedicated to describing different subpopulations of CAFs in tumors and pathological conditions. In ovarian cancer, presence of four distinct CAFs subtypes is reported and a specific role of CAF-S1 (identified by the accumulation of beta isoform of the CXCL12 chemokine) subtype in resistance to immunotherapies is described (Givel et al., 2018)

Single-cell RNA sequencing approaches are shedding a new light to the divergence of CAFs, for example, Xie *et al.* have identified previously not reported myofibroblasts subtypes markers in pulmonary fibrosis (Xie et al., 2018). On top of that, by the end of 2018 same technique was used to describe different subclasses of CAFs in breast cancer. In more detail, the group has identified three spatially and functionally distinct subsets of CAFs with a distinctive gene signature that can be used as a prediction for metastasis in breast cancer (Bartoschek et al., 2018).

Each day, specially with the emerging of new technologies we are one step closer to fully understanding the convolution of TME.

III) Activation of fibroblasts

Fibroblasts can become activated by numerous mechanisms involving cancer and immune cells. Epithelial cancer cells have the ability to secrete growth factors into their surroundings and these growth factors stimulate and recruit fibroblasts. One of the most famous growth factors, often used *in vitro* for fibroblasts activation is TGF β (Roberts et al., 1992). Importantly, TGF β is implicated in ECM synthesis and degradation by upregulating the expression of fibronectin, collagen (Igotz and Massagué, 1986) and MMPs (Sonnylal et al., 2010). As an example of the bi-directional communication between cancer cells and CAFs, TGF β /SMAD2/3 and CXCL12/CXCR4 ((C-X-C) chemokine receptor) pathways are crucial for, in one hand, activation of CAFs that, in the other hand, release chemokines that trigger tumor invasion and metastases (Mishra et al., 2011). Equally important as TGF β is hepatocyte growth factor (HGF) expressed by fibroblasts and its receptor Met present in the cancer cells (Bhowmick et al., 2004). HGF is responsible for fibroblast-derived invasion (Kawaida et al., 1994) and stimulation of angiogenesis *in vitro* and *in vivo* (Bussolino et al., 1992). In response to HGF/Met signalling cancer cells undergo EMT, colony dispersion and gain cell motility (Birchmeier et al., 2003; Thiery, 2002). In gastric cancer, crosstalk between the cancer cells and CAFs contributes to further tumor growth and fibroblasts to CAFs metamorphosis (Wu et al., 2013). Another two growth factors known to activate fibroblasts secreted by cancer cells are FGF2 and PDGF (Elenbaas and Weinberg, 2001). PDGF is well described as pro-angiogenic, pro-proliferating factor. In most cases, PDGF is synthesised and secreted from cancer cells and it exerts its function in a paracrine manner on stromal cells (Bronzert et al., 1987). Dissimilar to TGF β , PDGF does not promote the phenotype switch but rather recruits fibroblasts to tumor site and induces its proliferation (Shao et al., 2000). FGF2 belongs to a family of angiogenic endothelial mitogens that function in autocrine, intracrine and paracrine manner (Seghezzi et al.). Upregulation of FGF2 is connected to fibrogenesis and with fibroblast phenotype switch leading to their activation (Strutz et al., 2000). Interestingly, FGF2 interacts in a synergistic manner with one of the isoforms of PDGF to promote tumor angiogenesis and metastasis (Nissen et al., 2007). Besides growth factors, signalling pathways such as the Sonic Hedgehog (Shh) - Smoothened (Smo) are involved in the transition of stromal cells to “CAF” like phenotype. Hedgehog ligands (Hh) secreted by the tumor cells allow activation of Smo *via* binding to the Patched1 receptor, which is only expressed by stromal cells (Ng and Curran, 2011; Tian et al., 2009). The role of Hh-Smo pathway has not been fully disclosed, while the genetic depletion of Shh in pancreatic tumors leads to decreased desmoplastic response and tumor fibrosis (Bailey et al., 2009). These tumors become much more aggressive, undifferentiated, and vascularized (Lee et al., 2014; Rhim et al., 2014). The controversial role of Hh-Smo signaling pathway and fibroblasts in pancreatic cancer is an example of an heterogeneity of CAFs, not only on a molecular level, but also in a disease-dependant context (Gore and Korc, 2014). Recent studies revealed a cooperation between TGF β and Wnt in breast cancer. Mechanism of action is independent of canonical Wnt pathway and acts as an enhancer of TGF β signalling loop. Wnt7a is secreted by cancer cells and

induces fibroblast activation, matrix remodelling and invasion (Avgustinova et al., 2016). In our lab, we have described a novel pro-invasive fibroblast activator and tumor promoter - LIF (Leukemia Inhibitory Factor), a member of IL-6 proinflammatory cytokine family (Albregues et al., 2014). Increased production of LIF by tumor cells is known to correlate with their invasiveness (García-Tuñón et al., 2008) and fibroblast activation through autocrine and paracrine mechanisms. Unlike autocrine manner that relies on TGF β , paracrine is independent of α SMA expression as discussed in previous part. Principal of LIF fibroblast activation lies on the crosstalk between JAK1/STAT3 and Rho/ROCK/MLC2 signalling pathways. LIF constitutively activates JAK/STAT3 signaling in fibroblasts as a consequence of epigenetic modifications and in turn activates Rho/ROCK/MLC2 responsible for contractility and activation (Albregues et al., 2015). Lastly, accumulation of reactive oxygen species (ROS) drives the activation of fibroblasts by modulating the crosstalk between the cancer cells and fibroblast by stimulating the expression of growth factors like TGF β and PDGF that eventually leads to the secretion of chemokine CXL12 (Costa et al., 2014). ROS are usually released by various inflammatory cells found in TME. Inflammation has been established as one of the hallmarks in cancer and the role of inflammation in cancer development is crucial (Hanahan and Weinberg, 2011). During inflammation, TME becomes infiltrated with immune cells including macrophages, neutrophils, basophils, myeloid-derived suppressor cells, dendritic cells, natural killer cells and lymphocytes (de Visser et al., 2006). All these cells secrete various growth factors, cytokines and chemokines that drive the communication with cancer cells and TME. Some of the growth factors are TGF β , FGF, EGF, TNF, VEGF, as well as members of the Interleukin family IL-1 and IL-6 (interleukin 1 and 6), (Calvo and Sahai, 2011). In general, immune cells are able to secrete variety of factors involved in angiogenesis, motility, invasion and finally metastasis (**Figure 11**).

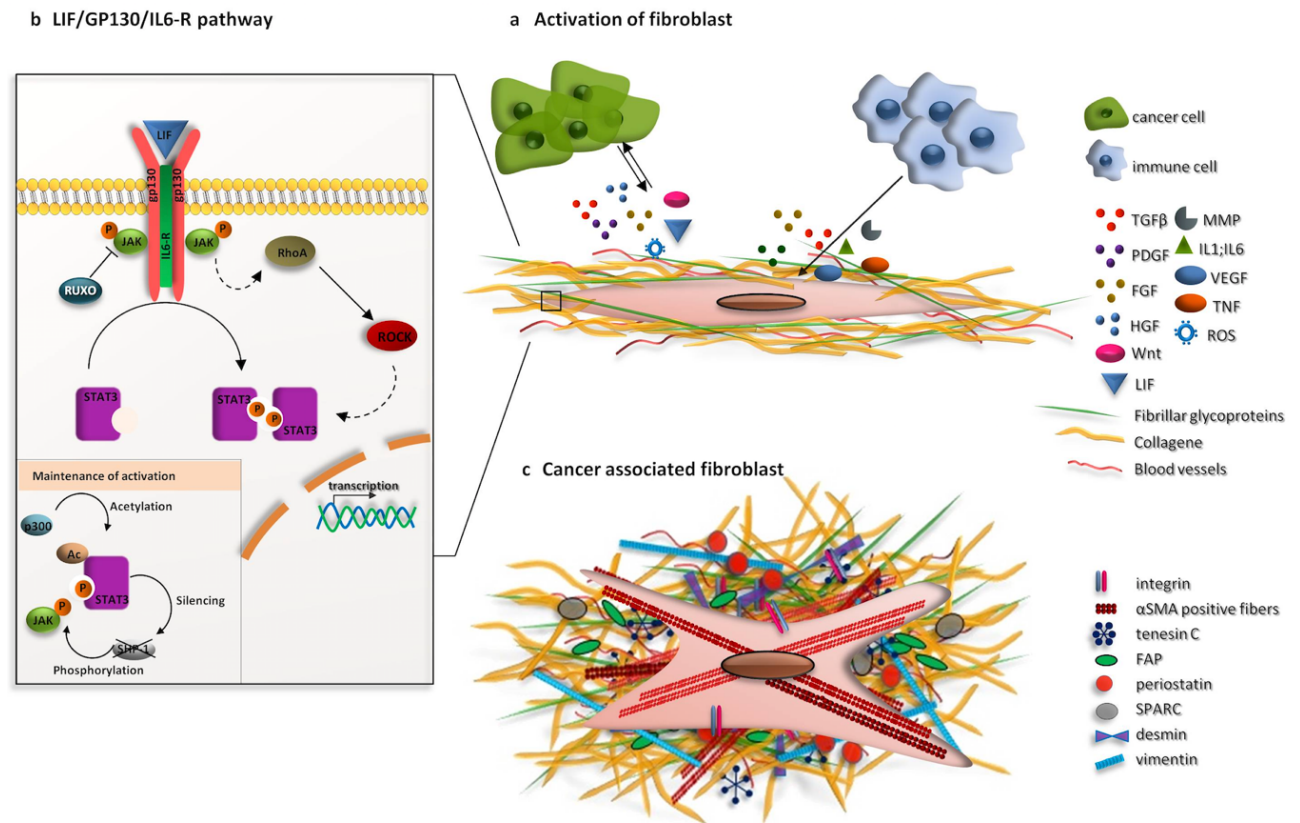


Figure 11. Various mechanisms of fibroblast activation

. *a* Normal fibroblasts, found in TME can be activated by various processes. Often by growth factors secreted from either cancer or immune cells found in TME. Besides common growth factors such as TGFβ, PDGF, HGF and FGF, interleukins, metalloproteinases and reactive oxygen species contribute to fibroblast activation. *b* research from our group identified a novel mechanism of fibroblast activation, independent of αSMA. Upon activation of LIF, JAK-STAT pathways is mobilized and translocated into the nucleus where it promotes the transcription of genes responsible for cell growth, differentiation, proliferation and apoptosis. *c* Activated fibroblasts or cancer associated fibroblasts (if constitutively active) undergo a phenotype switch and express various markers such as α-SMA, FSP1, vimentin and periostatin.

IV) Role of CAFs in cancer progression

CAFs participate in all stages of cancerogenesis, from tumor initiation, development of primary tumor, invasion and formation of metastatic niche (Attieh and Vignjevic, 2016). In order to do so, CAFs act in different ways by emitting oncogenic signals through secretion of many factors, direct interaction with cancer cell and remodelling of ECM.

During tumor initiation, CAFs are a key player. Experimental studies shown *in vitro* and *in vivo* that CAFs induce the formation of tumors. For example, when immortalised (but not malignant) prostatic epithelial cells were co-injected with CAFs and normal fibroblasts, however only when CAFs were co-injected tumor formation was induced in nude mice (Olumi et al., 1999). Another example is a research done by Trimboli *et al* that demonstrated that inactivation of tumor suppressor *Pten* in mouse mammary fibroblasts leads to the malignant transformation of epithelial cells (Trimboli et al., 2009).

CAFs strongly influence the proliferation of tumor cells, particularly through the secretion of many cytokines, hormones and growth factors. Among these, we find mainly HGF, EGF, FGF, SDF-1, CCL5 (CC motif chemokine ligand 5) and IL-6, all of which have mitogenic effects on tumor cells (Cirri and Chiarugi, 2012). In addition, CAFs influence the metabolism of tumor cells, which is necessary for their growth. Indeed, cancer cells undergo a major metabolic change called the "Warburg effect" which involves the use of glycolysis as the primary source of biomass in place of oxidative phosphorylation, used in healthy cells (Vander Heiden et al., 2009). Altered metabolism of cancer cells can influence the behaviour of CAFs and *vice versa*, for example cancer cells have the ability to maximize glutamine synthesis in stroma, reprogramming CAFs directly influencing tumor size, nodules formation and metastasis (Yang et al., 2016b). Recently our group published a paper on that exact topic, we have shown that ECM stiffening mechano-activates metabolic crosstalk between CAFs and cancer cells allowing them to acquire more aggressive phenotype (Bertero et al., 2018).

CAFs as well participate in angiogenesis and recruitment of endothelial cells and the formation of new vessels used by cancer cells for intravasation (Fukumura and Jain., 2007; Orimo et al., 2005). As I discussed in previous chapter, various pro-inflammatory molecules are secreted in TME, often by CAFs including cytokines, chemokines and interleukins. In general, CAFs create a protective ecosystem that "protects and serves" cancer cells every step of the way.

During metastatic invasion, CAFs again secret growth factors and remodel ECM that enables migration and, finally, invasion of cancer cells into surrounding tissue (De Wever et al., 2014). In prostate cancer, secretion of chemokine Stromal cell-derived factor 1 (SDF-1), in addition to promoting angiogenesis, induces invasion and migration of tumor cells by inducing an EMT (Jung et al., 2013). Same is true for the invasion of mammary and pancreatic carcinoma cells (Matsuo et al., 2009; Orimo et al., 2005). CAFs have the ability to turn non-invasive tumor cells into invasive ones (Dimanche-Boitrel et al., 1994). To do so, CAFs remodel the ECM that serves as a barrier for cell invasion (Wolf et al., 2013) and create "tracks" within the matrix that tumor cells use as an escape route (Gaggioli et al., 2007). It has been

shown that CAFs drive invasion through fibronectin assembly and integrin- $\alpha\beta 3$ signaling (Attieh et al., 2017). On top of that, same research shows that CAFs induce the collective migration of tumor cells as "leader" cells, guiding the invasive cohort.

I have previously mentioned the process of matrix remodelling, nevertheless it is worth to stress out once more that, in on hand it is dependant on degradation by MMPs and on the other hand on the tension forces that reorganise fibres of ECM. Tension forces rely on the coordinated binding of integrins to ECM and the generation of contractility via actomyosin cytoskeleton. In CAFs these forces are generated by RhoA GTPase signaling pathway and its ROCK effector kinase that induces the phosphorylation of MLC-II (Albregues et al., 2014). In addition, the autophosphorylation of JAK1 phosphorylates STAT3 transcription factor which regulates the expression of many genes involved in the generation of contractile forces. *In vitro*, this matrix remodelling is mimicked by the contraction of gels rich in collagen I and laminin (Gaggioli et al., 2007). These results, obtained *in vitro*, were confirmed *in vivo* by Goetz *et al.* who showed that activation of small GTPase Rho by caveolin-1 resulted in the rigidity and matrix organization required for tumor cell invasion (Goetz et al., 2011). Recent study shows that CAFs exert physical forces on the cohort of tumor cells by binding of N-cadherine (expressed on CAFs surface) to E-cadherine (expressed on the membrane of tumor cells). Inhibition of this binding blocks the ability of CAFs to guide the collective migration of tumor cells and therefore blocks their invasion (Labernadie et al., 2017).

In addition, to actively participate in the development of the primary tumor, CAFs also promote the formation of metastases by developing metastatic niche. If we go back to the very beginning of this chapter, I have introduced Paget's "seed and soil" hypothesis that could be explained through the role of TME. Several studies have demonstrated the importance of the CAFs in developing these metastatic niches, including the activation of fibroblasts in the secondary organ, and the recruitment of bone marrow-derived cells that are a source of CAFs. CAFs allow the preparation of "soil" at the metastatic site by the secretion and remodelling of ECM. Deposition of tenascin C, fibronectin and periostin allows tumor cells to implant and develop (Malanchi et al., 2011; Oskarsson et al., 2011). For example, Kaplan *et al.* observed an increase in fibronectin produced by CAFs 72h before the appearance of tumor cells in the bronchioles of the lungs in mouse models of melanoma and lung carcinoma (Kaplan et al., 2005). Another example published by Costa-Silva *et al.* is the activation of liver cells (fibroblast-like cells) that allow the production of fibronectin and a fibrotic microenvironment following the secretion of exosomes by pancreatic adenocarcinoma cells (Costa-Silva et al., 2015).

V) CAFs and therapies

Besides many roles, CAFs are involved in the response of tumor to therapies, whether it is chemotherapies, targeted therapies or immunotherapies. It would be naive to assume that the only enemy are cancer cells when the connection between them and their environment is so profound. Therefore, research has been directed in deciphering the role of TME, notably CAFs as therapeutic targets.

For obvious reasons, CAFs are a reasonable target for chemotherapy. Nowadays single-drug treatments are considered outdated and more and more emphasis is being put in combinational therapy. Combinational therapy might include two agents targeting cancer cells but modern science has focused its efforts into targeting both cancer cells and surrounding stroma (Joyce, 2005).

Despite their heterogeneity, CAFs are genetically stable cells, which limits the emergence of resistance by acquisition of mutations, as observed in tumor cells (Cirri and Chiarugi, 2012). In the past years, different approaches have been established in order to (1) target CAFs directly and eliminate them, (2) inhibit secretion of certain molecules from CAFs, (3) block the remodelling of ECM and contact of CAFs to it (Togo et al., 2013).

For instance, blocking antibodies or inhibitors of the HGF / c-Met signaling pathways, SDF-1 / CXCR4, Shh / Smo (sonic hedgehog / smoothed, frizzled class receptor) or PDGF / PDGFR are used to reduce the tumor growth and overcome chemoresistance in murine models (Crawford and Ferrara, 2009; Olive et al., 2009; Orimo et al., 2005; Pietras et al., 2008). Some multi-target tyrosine kinase inhibitors (TKIs) such as Imatinib, Sorafenib and Sunitinib have been tested in the context of inhibiting the PDGF kinase activity and are currently in clinical trials (Faivre et al., 2006). Another interesting approach is the possibility of targeting FAP positive CAFs. Suppression of FAP expression inhibits the migration, invasion and formation of ovarian carcinoma metastases (Wang et al., 2014). Unfortunately, clinical trials using FAP blocking antibodies showed no significant benefit for the patients (Hofheinz et al., 2003). Despite that, pre-clinical testings are underway to evaluate the efficacy of modified T cells for targeting FAP-expressing cells (Schuberth et al., 2013).

Indeed, inhibition of matrix remodelling blocks tumor cell invasion *in vitro* and *in vivo* in mouse models of breast carcinoma. This led Albregues *et al.* to explore the role of Ruxolitinib, a pharmacological inhibitor of JAK kinases, used clinically for other conditions, to reverse the remodelling of matrix. Besides that, new strategies are being developed to re-program CAFs activity, rather than eliminate them. One reason is definitely the controversial role of CAFs in TME, one research on pancreatic cancer suggests an anti-tumor role for CAFs in pancreatic cancer and elimination of α SMA induced the formation of more invasive and undifferentiated tumors (Özdemir et al., 2014). Nevertheless, it is important to remember that not all CAFs express α SMA and depletion of α SMA might not have any impact on CAFs population.

1.2.7. CONCLUSION

As I said in the beginning of this chapter, tumor microenvironment participates in all stages of cancerogenesis, from its very beginning to the very end. From the initiation of cancer to its fight against the chemotherapy, TME is an ally and accomplice. Understanding cancer is impossible without the understanding of the tumor stroma. Throughout this chapter I tried to paint a very complex picture of each and every member of TME and how well organized they are in order to expand the reach of cancer. One of the most studied members of TME are CAFs, despite that their role is still not fully understood, and the role of ECM they secrete is known even less. Matrix found in tumors is up to 10 times more stiff than of one in normal tissue (Rice et al., 2017a). Mechanosensing has become an important aspect of tumor development and more and more research is emerging trying to understand how can we reverse its actions. Unfortunately, frontiers of the greatest battle on Earth, one against cancer, are many and it is nearly impossible to tackle them at the same time. Therefore, during my PhD, I focused exclusively on the role of matrix stiffness in cancer's ability to evade treatment. Although chemoresistance is far more complicated than we can imagine, understanding a critical response to changes in the environment is crucial and not studied enough. A clear mechanism underlying stiffness dependent drug resistance is not yet established and we hope to provide a novel direction to follow.

1.3. TREATMENT of HNSCCs

As in other tumors, the treatment of HNSCCs carcinomas depends on many factors such as the site of the primary tumor, the presence or absence of distant or local metastases, the degree of differentiation of tumor cells and recurrence. Due to the complexity of the diseases (variety of anatomy sites and diverse clinical outcomes) it has been a challenge to generate a consistent staging system for tumors arising in the head and neck region. Two main methods are used to classify HNSCCs; tumors tumor-node-metastasis (TNM) staging system and American Joint Committee on Cancer (AJCC) classification. TNM describes the anatomic expansion of primary tumors and their metastatic reach (Sobin, 2003). It has been adapted for HNSCCs classification based on experts' opinion and literature and it is continuously updated. AJCC classifies tumors according to three parameters: the primary tumor (size, local invasion), local metastases in the lymph nodes, and distant metastases. AJCC has been widely used it is accepted by medical staff in characterisation and treatment choice in patients (Patel and Shah, 2005). Treatments used for HNSCCs are surgical removal, radiotherapy in combination or not with chemotherapy and targeted therapy.

1.3.1. SURGICAL REMOVAL of HNSCCs

When it comes to the surgical removal of cancers in the head and neck region, although being the first-line treatment option, it is quite limiting due to the degree of organ corruption or/and the importance of preserving the functionality of the affected organ, and can the morbidity of surgery be justified (Homer, 2016). Often, clinicians turn to chemotherapy or radiotherapy treatments to preserve the organ and maintain life-quality of the patients. For example, removal of tumor from larynx can significantly impair the overall life-quality of the patients in terms of diminishing the ability to speak, swallow and even breath. One of the surgeries that has been very well described is radical neck surgery performed in patients with lymph node metastasis and it includes the complete removal of lymph nodes from one side of the neck (Cognetti et al., 2008)(Figure 12)

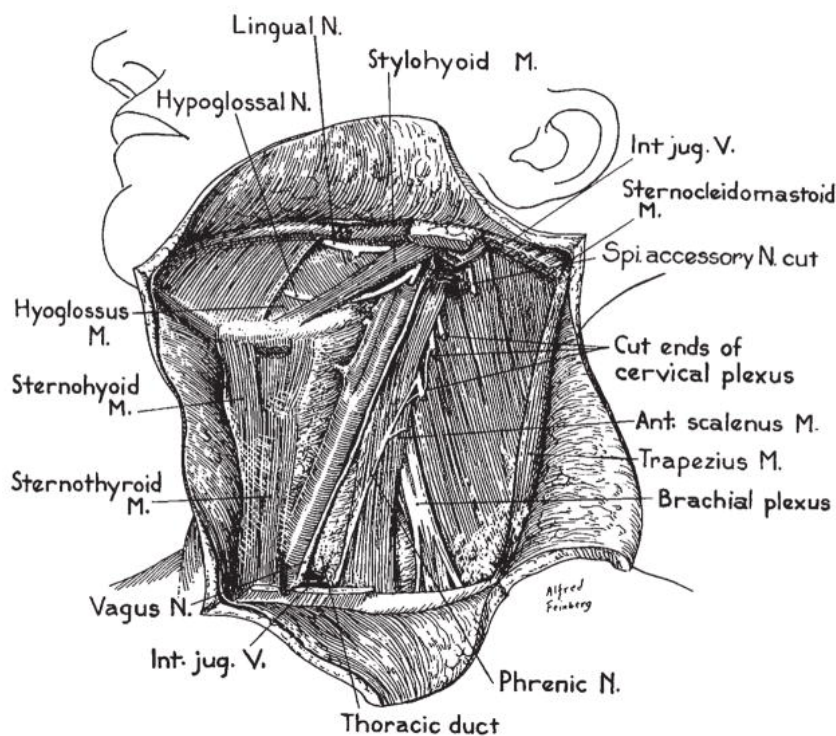


Figure 12. Radical neck dissection in patients suffering from HNSCCs

Illustration of the radical neck dissection by Hayes Martin, MD in 1951.. N indicates nerve; M muscle; V vein; Int.jug.V., internal jugular vein, Ant., anterior.(Martin et al., 1951)

Underlying foundation of head and neck surgery is a complete, microscopic removal of the tumor with the proper margin according to the type, site and stage of cancer. Commonly, debulking surgeries serve no purpose in improving the chance of chemoradiation success, but may be necessary for airway preservation and symptom palliation (Cooper et al., 2004; Homer, 2016). Excision margins are a decisive parameter in treatment choice and prognosis of patient survival (Hinni et al., 2013).

Nowadays, with a significant progress made in technology, novel minimally invasive surgical (MIS) methodologies have emerged. Some endoscopic methods include, transoral laser microsurgery (TLM) and transoral robotic surgery (TORS), where TORS is a technological improvement of TLM,

particularly beneficial for tongue base and supraglottic surgeries. Advantages of minimal access surgery compared to open ones is an obvious reduction in morbidity. Of course, as always, there are certain limitations to these methods (proof of surgical margins) that require a strong collaboration between the pathologist and the surgeon (Smits et al., 2016). If an open, more radical, surgery is needed in advanced disease, there are few key points to consider such as (1) can a complete tumor removal be achieved, if not morbidity of a surgery is not rationalized (2) even if the complete removal is achievable are the risk of the surgery, morbidity of it and a chance of survival justified enough and (3) if radical surgery is to be done, it should be done thoroughly with no compromise in the incision area. As an illustration, pharyngolaryngectomy will be done instead of laryngectomy (Homer, 2016).

However, in the case of an extensive surgery, reconstructive microsurgeries can be performed on a patient to improve post-operational life-quality. It is possible to reconstruct throat and other parts of head and neck region by utilizing muscle and skin from nearby areas. After a total laryngectomy, a tracheotomy can be done to create an alternative pathway for breathing (Cancer Treatment Centres of America (CTCA)).

Recent improvements in surgical methodology and frequent long-term and short-term consequences of non-surgical treatments of HNSCCs (radiotherapy and chemotherapy) have led to a resurgence in surgery, specially in light of minimal invasion approaches such as TORS (Yeh et al., 2015). Nevertheless, various protocols have been deployed including radio- and chemotherapy together with surgical removal to optimize patient response and to preserve organ function. In 1991, three hundred thirty-two patients were randomly assigned to either a combination of chemotherapy (cisplatin and fluorouracil) and radiotherapy or surgery followed by radiotherapy. Survival rates were not significantly distinctive but latter group of patients retained their larynx (Department of Veterans Affairs Laryngeal Cancer Study Group et al., 1991). Same was confirmed in a later study (Lefebvre et al., 1996) and is still somewhat true today, therefore the use of radiotherapy and/or chemotherapy is widely used.

1.3.2. RADIOTHERAPY in TREATMENT of HNSCCs

Radiotherapy utilises the high-energy rays to induce DNA-damage in form of single- or double-stranded breaks in cancer cells with little damage to surrounding tissue. The intensity of radiation is measured in Gray (Gy) and when radiotherapy is applied alone it usually (but not always) scheduled as 2 Gy in single fraction per day, five days a week for seven weeks. Radiation does not have an immediate effect; it may take days and weeks of radiotherapy for cancer cells to start dying, but they will continue to do so for weeks and months after the treatment. Radiotherapy can stop or slow down the tumor progression and shrink the tumor size reducing the symptoms and relieving the pain (John M. Eisenberg Center for Clinical Decisions and Communications Science, 2005)

In HNSCCs it is often used (1) after surgery, alone or in combination with chemotherapy, (2) in combination with chemotherapy (chemoradiation) without the surgery or (3) in combination with targeted therapy.

In the very beginning of radiotherapy, before 1948, there were plentiful limitations to it mostly due to the usage of orthovoltage generators. Orthovoltage radiation was characterised by low depth beam delivery with no efficacy against tumors localised in deep tissue and severe side-effects on skin. During this time, poor response in patients to radiotherapy led to steady trend towards surgery (Mead, 1963). However, after the deployment of linear accelerators and telecobalt units that achieved supervoltage radiations (Langlands, 1982), treatment was greatly improved (improvement was reflected in the ability of radiation to penetrate in deeper tissue in order to eliminate tumor cells and preserve skin at the same time) and became an important aspect of HNSCCs treatment. Although radiotherapy was used throughout the whole first half of 20th century (Maccomb and Fletcher, 1957), in 1980s research done by Kramer *et al* established that postoperative treatment with radiotherapy shows significant improvement compared to preoperational, approximately 20% better response to be precise (Kramer et al., 1987).

Generally, there are four different external beam radiation systems used by clinicians, three of which have proved to be successful in HNSCCs (John M. Eisenberg Center for Clinical Decisions and Communications, Science, 2005).

Two-Dimensional Radiation Therapy (2DRT), the oldest system that is nowadays considered outdated and has been replaced by more sophisticated protocols. It functions on the principal of targeting tumors using two-dimensional image and the beams utilised cannot be aimed at tumor but at a few angles and do not increase strength while being administered.

Three-Dimensional Conformal Radiation Therapy (3DCRT), a newer system that uses three-dimensional images, as its name suggests, allowing doctors to take a better “hit” on the tumor. The beams can be aimed at more angles compared to 2DRT, therefore limiting the damage to surrounding tissue.

The most recent system, **Intensity-Modulated Radiation Therapy (IMRT)** is quite similar to 3DCRT with a higher degree of freedom in optimising the level of radiation of each beam. This upgrade is highly beneficial in preserving healthy areas of the tissue.

Proton-Beam Radiation Therapy (PBRT) utilises a different kind of energy source - protons (sub-atomic particles) are accelerated through “particle accelerator” and delivered directly to cancer cells. Once the proton beam reaches cancer cells, it stops causing very few damage to tissue. However, method is quite controversial and very few data is available on its efficacy and it is still not a conventional approach for HNSCCs.

Besides this classification based on merely technical specifications of energy source and its delivery, a more “disease-specific” classification was described by Cognetti *et al.* (Cognetti *et al.*, 2008)

Definitive radiation therapy. In a subset of HNSCCs, radiation therapy is used as primary and single-modality treatment, specially in early-stage cancers and its success has been compared to the one of surgery with lower morbidity rate (Fletcher and Jessee, 1977; Mendenhall *et al.*, 2006; Montana *et al.*, 1969; Nakamura *et al.*, 2006).

Preoperative radiation therapy. Although preoperative radiotherapy showed promising results, as mention few paragraphs earlier, it has been replaced by postoperative treatment after a direct comparison in clinical trials (Fu *et al.*, 1996; Kramer *et al.*, 1987).

Adjuvant or postoperational radiotherapy. Patients that undergo adjuvant radiotherapy after being placed under operation have a higher 5-year survival rate. This is particularly relevant in advanced disease patients with extracapsular spread (ECS) and lymph node-positive HNSCCs, where 10% increase in 5-year survival is observed (Lavaf *et al.*, 2008). In recent times, chemotherapy has been added to radiotherapy to increase the control of the disease and disease-free survival (Bernier *et al.*, 2004; Cooper *et al.*, 2004). Despite having a 10% increase in response and disease-free survival, these two clinical studies noted adverse effects of combinational treatments with a higher mortality rate cause directly by the treatment itself.

Salvage and palliative radiation therapy. Unfortunately, HNSCC is a recurrent disease making it difficult to handle with an unfavourable prognosis. Often, surgery is used for salvage after all other treatments have fails (if it is possible to operate). Radiation (with or without chemotherapy) can be used in patients that have not received radiotherapy before. If a patients received irradiation previously it represents a greater challenge in choosing the right strategy. Nevertheless, some studies show that irradiation is possible in some cases, specially after operation for recurrent primary HNSCCs (Kasperts *et al.*, 2006) or can be administered with chemotherapy (Spencer *et al.*, 2008). In case of distant metastases, curing the patients is nearly impossible and palliative care is a rational option, often in form of chemotherapy (Choong and Vokes, 2008).

Altered fractionation radiation therapy. In the need of improvement of patients’ survival, radiotherapy has been altered to deliver the best result. Hyperfractionation and accelerated fractionation are one of those enhancements. Hyperfractionation delivers the total dose of radiotherapy in smaller

doses per fraction (during one day) without changing the duration of treatment. This approach increases the activity of radiation on tumor and not normal tissue. Accelerated fractionation, in contrast, delivers the total dose of treatment in shorter time period to decrease tumor growth during treatment. Several studies showed benefits for the patients with an acceptable tolerance (Million et al., 1985; Wang et al., 1985).

I would like to mention the importance of combining radiotherapy with traditional chemotherapy and targeted therapy.

As it was mentioned briefly in the beginning, radiotherapy is often use postoperational in combination with chemotherapeutical agents, in particular cisplatin and flourouracil. It is believed that cisplatin in combination with ionizing radiation enhances the formation of platinum intermediates due to the presence of radiation-induced free radials and in turn radiotherapy is linked to the increase in platinum uptake creating a positive feedback loop (Creagan et al., 1981; Fu et al., 1987; Tannock, 1989; Vokes et al., 1991). However, the toxicity level of the combination is drastically increased and represents a big challenge. Patients receive intravenous rehydration, feeding tubes and strong pain-killers for severe pain and often, chemoradiation is not adequate for patients (Bernier and Cooper, 2005).

In the Head and Neck Squamous Cell Carcinoma chapter of this manuscript, role of EGFR in HNSCCs is emphasised and during this chapter I will address developed therapies targeting the receptor. Nevertheless, since targeted therapies, in the case of HNSCCs cetuximab, are used in combination with radiotherapy I will present the ratio behind it in following lines. It is now known that radiation increases already (over)expressed EGFR and blocking EGFR drives cancer susceptibility to radiation (Bonner et al., 1994; Liang et al., 2003), therefore conjunction of cetuximab with radiotherapy shows beneficial results. Clinical study done in 2006, combining cetuximab and radiotherapy shows clear benefits for the patients with 10% of absolute survival and very low in-field toxic effects compared to radiotherapy alone and chemotherapy as well (Bonner et al., 2006a). This protocol is now standardly used worldwide. Since the radiotherapy has a prolonged way of acting, side-effects following the treatment can be present for a long time as well. Common side-effects include tiredness, mouth sores, dry mouth (xerostomia), pain while swallowing, weightless, hair loss, skin damage, etc. Some side-effects may be present for days, weeks, month and some can be permanent such as xerostomia (Head and neck radiotherapy side effects, Cancer Research UK).

1.3.3. CHEMOTHERAPEUTICAL APPROACH in HNSCCs

I have tackled the use of chemotherapy in HNSCCs before, and here I will devote the attention to the chemotherapeutic protocols used in patients.

First chemotherapy was developed in 1940s and it has been through renaissance of its own since then. Chemotherapies utilise the most basic property of cancer cells – fast proliferation - to eliminate them. There are different types of chemotherapies based on their properties, some include alkylating agents (cell-cycle non-specific) that encompass platinum based drugs as cisplatin, plant alkaloids (made from certain types of plants, cell-cycle specific) that include docetaxel, antimetabolites (mechanism of action involves incorporation of foreign substances into the cellular metabolism preventing the division) like fluorouracil and topoisomerase inhibitors (interfere with the action of topoisomerase I and II). After chemotherapies gained popularity, specially for the treatment of leukaemia under the leadership of Sidney Farber, known as the father of modern chemotherapy, various protocols have been used. These protocols regularly include combination of more than one agent, pushing the limits of their toxicity for the patients (Mukherjee, 2010). Chemotherapy is by far the most used form of treatment for all types of cancers, and therefore is a standard protocol for HNSCCs as well.

Before being administered regularly for HNSCCs managing, chemotherapy did not receive as much attention during first half of 20th century as surgery and radiotherapy did. It was often used as a palliative treatment where previous treatments failed (Watne, 1984). Things started to change with an introduction of cisplatin, an antineoplastic agent, in 1970s when a shift from palliative use of chemotherapy to adjuvant occurred. Soon after, chemotherapy was used independently or in combination with radiotherapy. Today, chemotherapeutic drugs commonly used for HNSCCs are cisplatin, fluorouracil, methotrexate, carboplatin, paclitaxel and docetaxel (Drugs approved for head and neck cancer, NCI, 2011). Adding chemotherapy into the standard treatment protocols (surgery and radiotherapy) of HNSCCs patients has two goals in mind; increase the patient's survival and decrease the organ morbidity (minimizing the need for radical surgery, particularly important for laryngeal cancer) (Clark and Frei, 1989; Vokes et al., 1993). Besides laryngeal cancer where chemotherapy is often used to avoid laryngectomy, chemotherapy is commonly used for patients with advanced, recurrent and metastatic disease, stage III and IV. As a comparison, stage I and II patients receive standard therapy including surgery and radiation in order to cure the disease with a success rate between 60-80% (Lippman et al., 1994). Survival rates for stage III and IV are drastically lower (30%) (Tupchong et al., 1991) and great efforts have been put in developing strategies to increase overall survival with few side-effects, including the addition of chemotherapy to surgery or radiation.

There are three common approaches of chemotherapy inclusion in the treatment and are discussed as following.

Induction (neoadjuvant) chemotherapy. Neoadjuvant type of therapy is carried out before the definitive therapy in order to maximise its success. Response to neoadjuvant therapy can be used to select the definitive treatment and to predict the prognosis for patient. In HNSCCs, early studies showed tumor regression after administering 5-fluorouracil prior to radiotherapy compared to radiotherapy alone, however it had no impact on patient survival (Gollin and Johnson, 1971). Later, a combination of cisplatin with other chemotherapeutic agents showed improvement in a neoadjuvant setting (Ensley et al., 1986). Similar studies were done, showing low toxicity and tumor shrinkage but had no impact on overall survival. Regardless of the absence of survival improvement, preliminary studies identified a valuable role for neoadjuvant chemotherapy. Response to initial chemotherapy can be used as a prediction tool for further treatment response of patients and therefore impact indirectly the survival (Forastiere et al., 1992). For example, chemotherapy followed by radiotherapy has been traditionally compared with surgery followed by radiotherapy as a method of organ preservation. One study in particular compared two cycles of chemotherapy (cisplatin and fluorouracil) and assessed the response of patients suffering from advanced laryngeal cancer. Responders were placed under radiotherapy and completed an organ-preservation protocol while non-responders underwent a surgical removal of the tumor (Department of Veterans Affairs Laryngeal Cancer Study Group et al., 1991). Later on similar study was done on patients with hypopharyngeal cancer, showing same results; induction chemotherapy followed by radiotherapy showed similar results as drastic surgery and radiotherapy (Lefebvre et al., 1996).

However, no trial demonstrated a direct impact on the patients survival, or reduced number of distant metastases and laryngeal preservation (Forastiere et al., 1992). Most common combination of chemotherapies used in neoadjuvant approach are cisplatin and fluorouracil, but more and more combinations are merging strongly impacting the degree of side-effects, often lethal for the patient (Haddad et al., 2018). Despite decades of research, the use of induction chemotherapy is still under investigation.

Adjuvant chemotherapy is used in order to completely eliminate the microscopic lesions remaining after surgical removal of the tumor, radiation or both (Vokes et al., 1993). In 1996, Johnson and colleagues did a trial on 371 patients using three different approaches; patients were either just operated, or operated and later treated with radiotherapy or radiotherapy and chemotherapy for 6 months. Results obtained from the non-randomized study indicated that chemotherapy in combination with radiotherapy after surgery may improve survival rate in patients (Johnson et al., 1996). However, a randomised, more controlled, trial resulted in no apparent impact on survival of patients while using adjuvant chemotherapy in addition to radiotherapy postoperational (Laramore et al., 1992). More randomized studies followed displaying the same feature, no real, significant impact on the survival of patients but did impact the rate of proliferation of the tumor (Laramore et al., 1992). At the same time, some studies did not report overall survival increase and improvement in the rate of distant metastasis, but did note

2-year disease-free survival increase for almost 30% (Tishler et al., 1999). It is obvious that results obtained from various sources are not consistent and that the role of chemotherapy as an adjuvant agent is not completely clear, however majority of publications will agree that there is no impact on patients' survival. Similarly to induction chemotherapy, benefits are still under investigation.

Concomitant chemotherapy is a very interesting concept corroborated with multiple research. Concomitant chemotherapy is way of administering chemotherapy together with radiotherapy with a goal to eliminate microscopic lesions of tumor while increasing the cytotoxicity of the radiation on those exact remainings (Vokes and Weichselbaum, 1990). Among different chemotherapeutical agents, paclitaxel has been described as a radiation sensitizer with greatest success. A preliminary research done on 14 patients previously treated or chemotherapy-naïve with paclitaxel showed good response to radiotherapy in paclitaxel treated condition (Tishler et al., 1999). Concomitant chemoradiotherapy has been show to improve overall survival of patients in more elaborated trials. Different approaches have been used to assess the very best approach to concomitant treatment, using single and multiple drugs combination. In single drug settings carboplatin and fluorouracil were used for treatment of oropharynx carcinoma and yielded in statistically significant improvement in survival (Calais et al., 1999). Same result was obtained for a single drug use of cisplatin followed by radiation (Brizel et al., 1998), other showed the same in Locally Advanced Squamous Cell Carcinoma of the Head and Neck (LA-SCCHN) using platinum-based chemoradiations (Langer et al., 2008). Some groups engaged in a combination of more than one chemotherapy, Taylor *et al* used a simultaneous treatment of fluorouracil and cisplatin followed by radiation and observed a better result than using only single drug approach (Taylor et al., 1989). Similar approach has been used later on, utilising fluorouracil and cisplatin led to promising results with somewhat increase in toxicity and was later used a basis of phase III clinical trials (Adelstein et al., 1993). Today, the protocol is established ad further tested (Zampino et al., 2011; Zhang et al., 2015).

As we can see, benefits of chemotherapy in HNSCCs are not yet completely elucidated. If we keep in mind that this group of cancers are highly heterogenic in terms of location and molecular signature, it does not come as a surprise. Still, scientists are putting enormous efforts to gather all the pieces of information to generate a better understanding of the disease and how to properly manage it.

In 2000, a very comprehensive study was done using data available on all published and un-published randomized trails done between 1965 and 1993 on patients with carcinoma in oral cavity, larynx, oropharynx and hypopharynx. Authors wanted to evaluate the effects of chemotherapy on non-metastatic HNSCCs, and to do so they used three meta-analyses of the impact chemotherapy has on overall survival added to locoregional treatment (Pignon et al., 2000). Analysis took in consideration age and sex of the patient, tumor site, TNM classification, histology, treatment given, but also type of chemotherapy (platinum based, with or without fluorouracil, multi-agent chemotherapy without platin, and single-agent chemotherapies) and their setting (neoadjuvant, adjuvant and concomitant). Data

acquired is exhaustive and detailed, however authors conclude that there is no significant benefit of neoadjuvant and adjuvant chemotherapy but with concomitant strategies (8%). Overall, they observe a small statistical benefit of using chemotherapy, mostly due to concomitant chemotherapy, therefore routine of using chemotherapy is still nowadays debatable and under investigation (Tsukahara et al., 2015; Weiss et al., 2018).

1.3.4. TARGETED THERAPY in HNSCCs

Today, we have a better understanding of molecular mechanisms involved in HNSCCs than ever. I have already described in detail the role EGFR has in these cancers, and how it has become a rational target for more advanced therapeutic strategies. In this paragraph, I will display the role of cetuximab, the only EGFR targeted therapy approved for HNSCCs, but also other know single-selective and multiple-selective tyrosine kinase inhibitors that could potentially be beneficial in future (**Figure 13**).

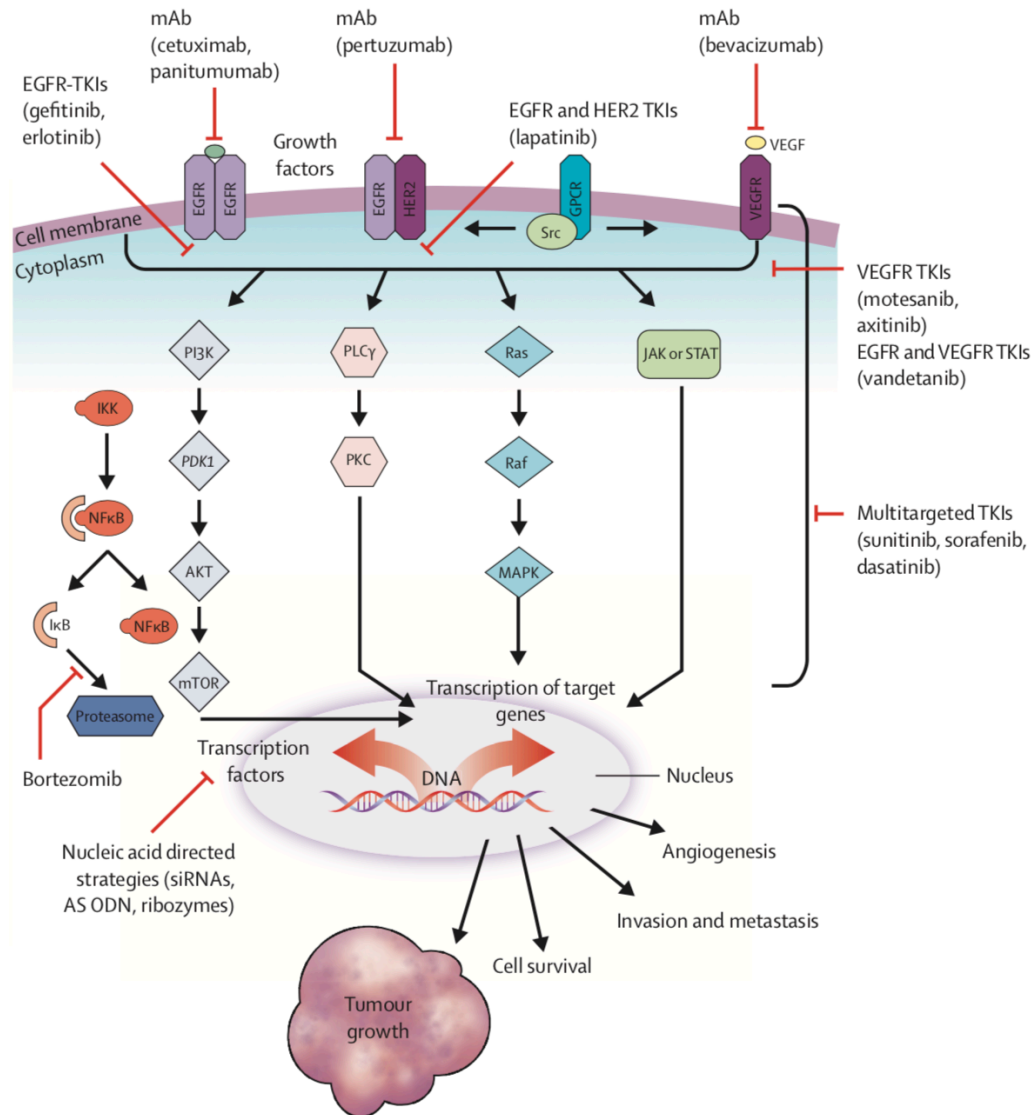


Figure 13. Targeted therapies developed against common ErbB receptors for the treatment of HNSCCs and corresponding molecular pathways

Considering the fact that Erb receptors, notably EGFR in HNSCCs, are on the top of a molecular cascade of multiple signaling pathways, their crosstalk is inevitable. Diverse strategies have been developed to target and inhibit these pathways, strategies such mAb, single- and multiple- selective tyrosine kinase inhibitors and nucleic acid-directed gene silencing molecules (eg. AS ODN and RNA interference—siRNA and ribozymes). Some of them are represented in the figure. Adapted from (Argiris et al., 2008).

A) *ErbB receptor blocking antibodies - Cetuximab*

Blocking antibodies developed against ErbB receptors are monoclonal antibodies designed to target extracellular part of the the EGFR or HER2 receptors. Mechanism of action includes inhibiting receptors activity by competing with endogenous ligands with a higher affinity. By ligand-binding inhibition, blocking antibodies prevent the autophosphorylation of the receptors and activation of downstream signaling pathways (Albanell et al., 2003; Yarden, 2001). Besides that, blocking antibodies can induce internalization and degradation of the receptors and thus decrease their expression (Sunada et al., 1986). Another described mechanism of a specific antibody, cetuximab, is proposed. It is described as antibody-dependent cellular cytotoxicity (ADCC) and it represents a situation when the antibody is bound to the receptor on the cancer cell and is recognized by the Fc receptor of NK immune cells or monocytes. Upon recognition, immune cells release toxic granules and induce the lysis of cancer cells (Kimura et al., 2007; Wang et al., 2015).

Cetuximab, also known as IMC-C225 or Erbitux®, is a monoclonal antibody that specifically binds to domain II of EGFR (Baselga, 2001; Mendelsohn and Baselga, 2000). In more detail, it is a recombinant, human/mouse chimeric monoclonal antibody that binds to the extracellular domain of EGFR (Goldstein et al., 1995). It has the same mechanism of action as other blocking antibodies, it competes with the ligand (for example EGF), blocks the phosphorylation of EGFR and activation of receptor kinases and downstream signaling (**Figure 14**).

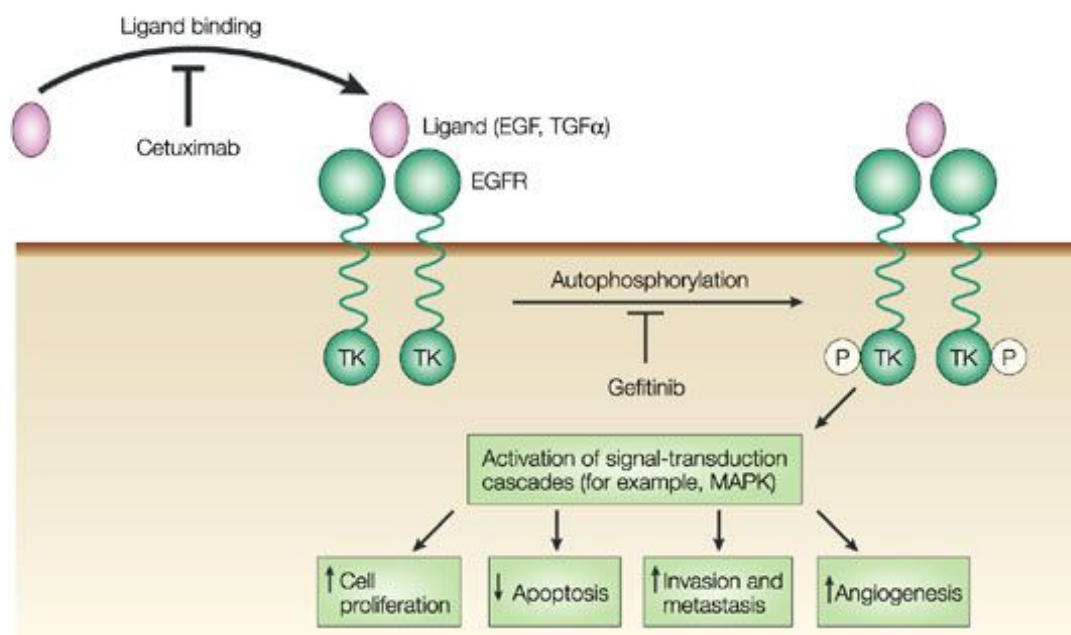


Figure 14. Cetuximab blocks the binding of EGF to its receptor and prevents its activation

Extracellular domain of EGFR binds various ligands (EGF, TGF- α) which causes its dimerization and tyrosine kinase activation. Activated (and phosphorylated) receptor activates downstream signals involved in proliferation, apoptosis, invasion, metastasis and angiogenesis. Cetuximab blocks the binding of ligand to the receptor and therefore its dimerization and activation. Agents as gefitinib inhibit the tyrosine kinase activity of EGFR. Adapted from (Kirkpatrick et al., 2004)

Cetuximab was first approved in Switzerland in 2003 and next year in the rest of Europe.

In 2004 it was FDA approved for the treatment of metastatic colon cancer in patients expressing EGFR, with non-mutated RAS and not responding to traditional chemotherapy as oxaliplatin or irinotecan. If RAS is mutated it causes a constitutive activation of the MAPK pathway, independently of the EGFR and therefore its inhibition has no effect. Cetuximab, in colorectal cancer, is administered alone or in combination with irinotecan. In clinical trials, combination yielded in an positive response rate of 22.9% and cetuximab alone 10.8% (Kirkpatrick et al., 2004). Importantly, Cetuximab is the only FDA approved therapy targeting EGFR in HNSCCs. In patients with metastatic HNSCCs, protocols involving cetuximab in combination with chemotherapy and radiotherapy are applied. In combination with radiotherapy it has a positive effect on the survival of patients by approximately 10% (Bonner et al., 2006a). In the beginning cetuximab and radiotherapy were considered a promising alternative to chemoradiotherapy, specially in cases where tolerance to chemotherapy is an issue. Clinical trials comparing the two came quite late, and several studies done since 2011 showed no relevant data to support the use of either modality treatment. Same group published two retrospective studies showing that cetuximab and radiotherapy give worse outcomes than previously established concurrent chemotherapy (cisplatin, carboplatin, fluorouracil) and radiotherapy (Koutcher et al., 2011; Shapiro et

al., 2014). Other retrospective studies confirmed the same, nevertheless, all these studies have great limitations (patients in the cetuximab arms were older and had poorer performance status) and a clear conclusion cannot be drawn (Ley et al., 2013; Ye et al., 2013). A more controlled phase II clinical trial was done, and a conclusion is that there is no real benefit of using cetuximab in these settings and it can even be considered unsafe for the patients (Magrini et al., 2015). Indeed, cetuximab is associated with hypersensitivity infusion, systemic toxicity, severe complications and death (Acevedo-Henao et al., 2012; Hopps et al., 2013). It is hypothesised that some patients are more prone to have severe complications like organ failure, septic shock and heart arrest (Numico et al., 2013). Compellingly, recent evidence in non-small cell lung carcinoma and colorectal cancer indicates that skin rash, that frequently occurs during cetuximab (and other anti-EGFR antibodies) intake, is a positive predictive marker of how successful the treatment will be (Liu et al., 2013; Petrelli et al., 2013).

Cetuximab combined with chemoradiotherapy was tested in clinical trials as well, but again no clear benefit was observed in numerous, often conflicting research (Ang et al., 2014; Argiris et al., 2011, 2016; Kao et al., 2011; Tong et al., 2012b). In colorectal cancer cetuximab has been shown to be inefficient in most of the cases and a need for a novel biomarker led researchers to identify downstream signaling mediator FOX3. Indeed, expression of FOXO3 and its activator p38 is associated with better response to cetuximab in patients with colorectal cancer patients (Marzi et al., 2016). In non-small lung carcinoma, despite promising results, cetuximab showed no significant clinical benefit (Gridelli et al., 2014). However, by the end of 2004, in US one month dose of cetuximab cost 16 000 dollars making it one of the most expensive cancer drugs ever (Kirkpatrick et al., 2004).

Other blocking antibodies

Trastuzumab (Herceptin®) is a monoclonal antibody against the IV domain of the HER2 receptor. Its binding, in addition to inhibiting receptor activation, blocks proteolytic cleavage of its extracellular domain, an activation mechanism observed only for HER2. It is used in early and metastatic breast cancer, in patients that expressing HER2, in combination or not with chemotherapeutic agents. Trastuzumab can also be used in combination with chemotherapy for the treatment of HER2-positive metastatic gastric cancer. However, in this case, its use has a modest advantage in terms of median overall survival (gain of 4.2 months) without benefit on the quality of life (Tan et al., 2018).

Another HER2 monoclonal antibody is **Pertuzumab** (Perjeta®) designed to target domain II. Usually it is used in combination with trastuzumab and docetaxel or paclitaxel in patients with metastatic or locally recurrent, HER2-positive breast cancer.

First monoclonal antibody against EGFR used for treatment of metastatic colon cancer is **Panitumumab** (ABX-EGF or Vectibix®). Nowadays, it is used as the first line of defence for patients without mutation of RAS genes, in combination with oxaliplatin and 5-fluorouracil, or in second-line patients who do not respond to fluoropyrimidine-based chemotherapy (Hurwitz and Kabbinavar, 2005). It can also be used alone in patients who do not respond to any conventional chemotherapy

B) Receptor tyrosine kinase inhibitors

Receptor tyrosine kinase inhibitors (TKIs) are small molecule inhibitors of ErbB receptor kinase activity that bind reversibly or irreversibly. TKIs are adenosine triphosphate (ATP) analogues that inhibit receptor phosphorylation by competition with ATP in the catalytic domain of the active kinase (Ciardiello et al., 2000). Irreversible inhibitors bind covalently to the kinase, and due to their molecular function have a prolonged effect, but are usually less specific and less tolerated by the patients (Slichenmyer and Fry, 2001). TKIs can be taken orally, facilitating the management of the disease.

Most well known TKIs available commercially and FDA approved are gefitinib (Iressa®) and erlotinib (Tarceva®), reversible inhibitors of EGFR used in the treatment of patients with non-small cell lung cancers carrying a mutation in the catalytic domain of the EGFR. In addition, erlotinib is also used in combination with chemotherapy in pancreatic cancer (Kelley and Ko, 2008). Another available TKIs is Lapatinib (Tyverb®), a reversible inhibitor of both EGFR and HER2. It is currently used in the treatment of EGFR, HER2 and estrogen-receptor positive breast cancer, and metastatic breast cancer positive for HER2 (Schroeder et al., 2014)

From irreversible inhibitors, only Afatinib (Giotrif®) is commercially available. Afatinib can bind to EGFR, HER2 or HER4 and therefore also inhibits HER3 by blocking the activity of all its interactions. It is currently approved for the treatment of locally advanced or metastatic non-small cell lung cancer positive for EGFR activating mutation (High Authority of Health) (Wu et al., 2014).

Many other inhibitors have been developed but did not show sufficient benefits in clinical trials. For example, pelitinib (EKB 569) and canertinib (CI 1033), irreversible inhibitors of EGFR and HER2, seemed promising because of their many effects in preclinical models, but failed in later trials. Another quite specific inhibitor of EGFR, AG1478 was tested in preclinical models, but showed severe side-effects such as hypomagnesaemia due to therapy-induced magnesium wasting (Weglicki et al., 2012). All targeted anti-ErbB therapies have side effects such as skin rash and hypomagnesaemia that can vary in its severity. Interestingly, rashes have been associated with better patient response and are a consequence of many roles of ErbB signaling pathways.

1.3.5. CONCLUSION

HNSCCs is complex disease with a variety of options when it comes to treatment. Choosing the right one is not always easy and we have seen in this chapter that it can depend on multiple factors such as stage of the disease, location of the tumor, histology, initial response to chemotherapy, age, tolerability to chemotherapies, and so on. In severe cases such as LA-SCCHN standard treatment paradigm in developed and its schematic representation gives a nice example of crucial factors to consider while choosing between options and just how complicated it can get (**Figure 15**). Options are many and their combinations numerous. However, treatments like cetuximab show no real impact on the survival of patients, yet they are standardly used without a biomarker decision driven approach. Need for a better understanding of how an individual patient will respond is emerging and personalised approaches are slowly taking over (Malone and Siu, 2018) specially in the light of novel therapies such as cetuximab.

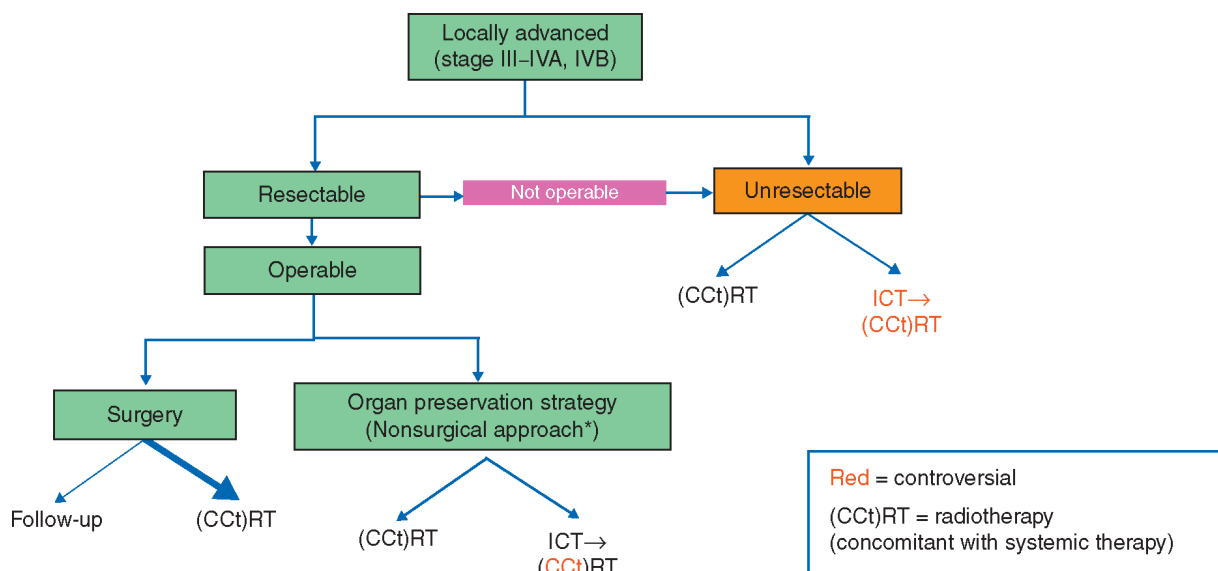


Figure 15. Treatment decision tree for locally advanced squamous cell carcinoma of the head and neck (LA SCCHN)

Induction chemotherapy followed by radiotherapy (ICT→RT) is an adequate for larynx preservation in locoregionally advanced larynx and hypopharynx cancer. Adapted from (Haddad et al., 2018).

At the same time, despite different therapies, approximately 50% of patients diagnosed with advanced HNSCCs develop resistance to given treatments and re-grow local or distant recurrences. In this case, no effective treatment is applied and only palliative care is used. That is why more understanding of chemoresistance mechanism is crucial to overcome the phenomena and act in time, appropriately.

1.4. CANCER RESISTANCE

Cancer resistance to therapeutic treatments (chemoresistance, radiotherapy and targeted therapy resistance) is a complex and highly situation-specific phenomena. Specific in a way that it varies greatly between cancer types, patients, treatments and origin. It is a consequence of an exhaustive interplay of mentioned factors. There is not a single way to describe or approach chemoresistance.

I focused on means of cancer resistance significant for the research I deliver here. I have decided to split this chapter into several paragraphs; role of CAFs in chemoresistance and ECM they secrete, resistance to EGFR targeted therapy and finally, AXL and A20 and their take on the subject.

Each paragraph is there to bring all elements of the research done in this manuscript closer together. My subject deals with the role of stiffness in resistance to EGFR targeted therapy in HNSCCs. Firstly, resistance to EGFR targeted therapy can occur in many different ways I describe in this chapter.

Secondly, without CAFs there would be no stiff matrix to promote the resistance and I find it important to look at both in context of resistance to have a clearer image of just how complex the system is. Lastly, AXL comes as a connector of the specific model I have worked with and is the molecular target I focused my research on. Its role in different types of resistance has been described, but the full mechanism is not yet characterized.

1.4.1. CAFs and THEIR ROLE in RESISTANCE of CANCER CELLS to THERAPIES

Signals from stroma, notably CAFs, provide necessary survival skills to cancer cells in order to adapt to and/or evade chemotherapies (Paraiso and Smalley, 2013). CAFs can interfere with the distribution of drugs within the tumor and provide innate or adaptive resistance to cancer cell. First, the secretion of growth factors and cytokines by CAFs are involved in many types of resistances. In particular, the secretion of HGF by CAFs in lung cancer induces resistance to EGF receptor inhibitors (erlotinib and gefitinib) by enabling EGFR to interact with several proteins: CDCP1, EphA2 and Axl (Gusenbauer et al., 2013). Inhibition of HGF in combination with EGFR inhibitors restores the sensitivity of tumor cells (Wang et al., 2009). In addition, HGF secretion by CAFs has also been described in resistance to RAF (proto-oncogene serine / threonine-protein kinase) inhibitors in B-RAF mutant melanoma cells. HGF induces MAPK activation (mitogen-activated protein kinase) and PI3K / PKB pathway (Protein Kinase B), independently of RAF (Straussman et al., 2012; Wilson et al., 2012). Another example is PDGF-C secretion by CAFs (**Figure 16**) that induces angiogenesis but also resistance to VEGF inhibitors (anti-angiogenic treatment) (Crawford and Ferrara, 2009). In addition, CAFs induce tumor hypoxia through the production of growth factors that support tumor proliferation such as TGF β and HGF. The creation of this hypoxic environment leads to a decrease in drug uptake and efficacy (Gerweck and Seetharaman, 1996; Harrison and Blackwell, 2004; Vukovic and Tannock, 1997) and a reduction in the sensitivity of tumor cells to the expression of MDRs, channels involved in the export of molecules outside cells (Wartenberg et al., 2003). Surprisingly, some evidence show that the DNA damaged induced by chemotherapies results in the secretion of WNT16B by the TME. WNT16B is a cytokine associated with the expression of NF- κ B, which in turn activates Wnt signalling pathway in cancer cells and therefore promotes survival (Östman, 2012). It is important to mention that NF- κ B is a transcriptional factor described as “playing the first violin” of the inflammation response (Ben-Neriah and Karin, 2011). Multiple other mechanisms of resistance rely on the ECM remodelling by CAFs (Netti et al., 2000) and they will be discussed in the “ECM and stiffness”. Finally, the secretion of MMPs by the CAFs also plays an important role in therapeutic treatments. Indeed, secretion of MMPs induce the resistance of head and neck squamous cell carcinoma (HNSCC) to cetuximab in a dose-dependent manner even though the mechanism is still unclear (Johansson et al., 2012).

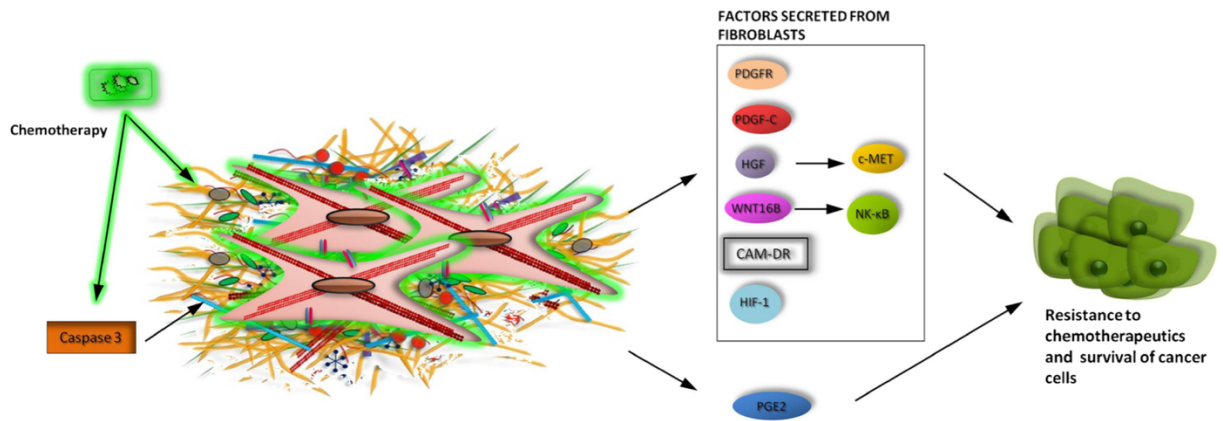


Figure 16. CAFs play an important role in chemoresistance through various mechanisms

CAFs are able to emit survival signals to the cancer cells around them. Signals emitted depend greatly on the chemotherapy in use, cancer type and the established relationship between CAFs and cancer cells, and often are growth factor receptors, transcription factors and adhesion molecules.

1.4.2. ROLE of ECM in CANCER RESISTANCE

Truth to be told, not a lot is known about the emerging role of stiffness in chemoresistance, and all potential therapies designed to override the role of stiff ECM have not yet reached patients as discussed previously.

Matrix remodelling and therefore stiffness influences the response of cancer cells to chemotherapies in various ways. Remodelled ECM perturbs the access of chemotherapeutics to tumor, for example a research published in 2012 shows that hyaluronic acid - an ECM component, creates a physical barrier that blocks the access of drugs to the tumor. Another example would be the cell-adhesion-mediated drug resistance (CAM-DR), which modulates the adhesion of integrins found on cancer cells to CAFs or ECM (in particular, its components fibronectin, collagen, etc.) (Meads et al., 2009). Recently, an interesting research from Hirata et al. (2015) demonstrated *in vitro* and *in vivo* that matrix remodelling, including a rigid and fibronectin-rich environment, induced tolerance to BRAF inhibitor PLX4720 of melanoma cells through the integrin signaling pathway - $\beta 1$ / FAK / Src / Erk (extracellular signal-regulated kinase). Moreover, BRAF inhibitors can even activate stromal fibroblasts and promote ECM remodelling. This leads to decreased drug efficacy by promoting integrin-dependant pro-survival and growth signals leaving patients receiving the treatment with negative consequences (Hirata et al., 2015). Matrix stiffness has been demonstrated to play a role in tumor proliferation and dissemination and it has been connected with reduced chemotherapy-induced apoptosis in hepatocellular carcinoma (Schrader et al., 2011). A very recent study shows that progressive stiffening of ECM has a positive impact on breast cancer response to chemotherapeutic treatment, doxorubicin to be more precise, leaving cells *in vitro* resistant to it (Joyce et al., 2018). The study demonstrates a correlation between stiffness, localisation of YAP in the nucleus, increase of EMT markers and ultimately chemoresistance.

On a similar note, ECM remodelling can be used as a prediction tool for patient's response to therapy, indeed one study shows a model system that predicts the drug response in prostatic cancer (PCa) in the context of TME. According to the research, TME is an "innovative measure" that adds new insights into tumor complexity. In more detail, pFAK and active conformation of $\alpha_5\beta_1$ -integrin are a signature of tumor-permissive stroma that helps cells fight the drug treatment (Shafi et al., 2018). As a reminder, FAK and integrins are crucial in mechanosensing and cancer cells response to stiffness.

Finally, I would like to mention two studies identifying that stiff ECM promotes EMT (Wang et al., 2016b) that consecutively promotes chemoresistance (Rice et al., 2017a; Wei et al., 2015).

Role of stiffness in chemoresistance is undeniable, nevertheless, mechanism of its action is not yet fully understood and very few studies give a comprehensive understanding. Hence we sought to explore the complexity of mechanotransduction in resistance to cancer treatment and provide new knowledge to the scientific community.

1.4.3. RESISTANCE of TUMORS to EGFR TARGETED THERAPIES

Despite the huge progress made in terms of developing novel therapeutically strategies and initial beneficial response of patients to EGFR targeted therapies, all patients eventually develop resistance. Generally, EGFR targeted therapy resistance falls into two categories (1) intrinsic (or primary) resistance and (2) acquired resistance. Intrinsic resistance is defined as the failure of tumor response to therapeutic inhibitors or antibodies. It must be distinguished from the inability to respond due to insufficient drug exposure (too low concentrations). Acquired resistance occurs in patients who have been treated and responded to treatment or in patients who have been treated for more than six months without tumor progression (Jackman et al., 2010). In contrast to resistance mechanisms to chemotherapeutic agents, mostly mediated by a change in the import or export of molecules, resistance to targeted therapies of EGFR can occur through multiple mechanisms; secondary EGFR mutations, activation of other signaling pathways (Wheeler et al., 2010), inhibition of apoptosis induced by anti-EGFR therapies (Ng et al., 2012a) and resistance by histological transformations (Chong and Jänne, 2013) (**Figure 17**)

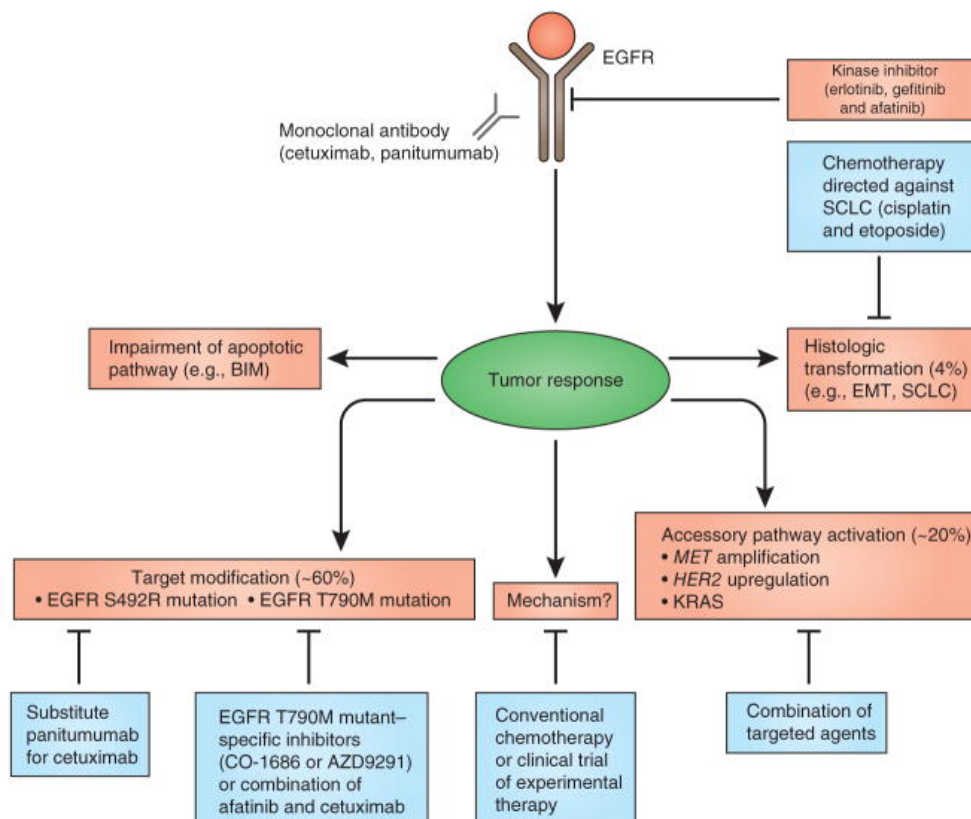


Figure 17. Mechanism of resistance to EGFR inhibitors

Tumor responds to treatment with EGFR targeted therapy in various ways. Cancer cells develop a mutation that confers resistance ((T790M or S492R) or are driven to an oncogenic shift (activation, upregulation or amplification of a bypass pathway), apoptosis is inhibited and lastly, histological transformation occurs. Adapted from (Chong and Jänne, 2013).

A) Secondary mutations leading to the resistance of cancer to EGFR inhibitors

In lung cancer, more than 60% of patients with EGFR mutation acquire T790M mutation that drives the resistance to RTKi. This single amino acid mutation increases the kinase affinity for ATP by approximately 5-fold, which decreases its sensitivity to competitive ATP inhibitors such as gefitinib or erlotinib (Yun et al., 2007). Interestingly, T790M mutation is also found in patients that have not been treated with RTKi (Inukai et al., 2006; Oxnard et al., 2012). In colorectal cancers, acquired S492R mutation in the extracellular domain of EGFR blocks the binding of cetuximab, leading to resistance in patients. Despite the mutation and acquired resistance, patients are still sensitive to panitumumab that binds to another EGFR binding site (Montagut et al., 2012). In HNSCCs, Sok et al. (2006) reported an EGFRvIII mutation as an origin to cetuximab resistance in 46% of patients (Sok et al., 2006), however genetic analysis of greater sample number only found this mutation in 1 out of 279 patients (The Cancer Genome Atlas Network, 2015). Until today, relevance of EGFRvIII mutation is not completely clear.

B) Activation of other signaling pathways contribute to the resistance of tumors to EGFR inhibitors

Upon blocking of EGFR, it is very common for downstream signaling pathways to become activated and carry on the transcription of unfavourable genes leading to resistance to apoptosis and the antiproliferative effect normally caused by the inhibition of EGFR. Mechanism behind involves the overexpression of genes such as the increase of the proto-oncogene MET (coding for the HGF receptor tyrosine kinase) which allows the maintenance of the activation of the PI3K/Akt pathway (Engelman et al., 2007). The increase in HER2 receptor and its activity has also been described to induce resistance (Takezawa et al., 2012; Yonesaka et al., 2011). Other mechanisms have been described as well such as the decrease in PTEN (Laurent-Puig et al., 2009; Sartore-Bianchi et al., 2009), the activation of KRAS (Diaz et al., 2012; Misale et al., 2012), PI3KCA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (Sartore-Bianchi et al., 2009) and / or BRAF (Ohashi et al., 2012). Mechanisms of resistance by activation of other signaling pathways can be both intrinsic or acquired. For example, in the treatment of colorectal cancer, only patients with no intrinsic KRAS mutations are treated with EGFR inhibitors (Knickerbein and Zhang, 2014).

C) *Inhibition of apoptosis induced by anti-EGFR therapies*

Patients resistant to EGFR-TKIs often carry a common polymorphism, an intronic deletion in the gene coding for BIM, a member of the pro-apoptotic factor family BCL2 (Ng et al., 2012a). Upon translation, BIM lacks a BH3 domain, essential for its pro-apoptotic effects. Indeed, the increase in BIM expression has already been described as essential for inducing TKI-mediated apoptosis (Costa et al., 2007; Cragg et al., 2007; Gong et al., 2007). Therefore, resistance conferred by the polymorphism in the *BIM* gene is an intrinsic one through the resistance to apoptosis.

D) *Resistance of cancers to EGFR therapies by histological transformation.*

I have already discussed in the second chapter, paragraph 1.4, how relevant EMT is in tumor progression and the unambiguous relationship it has with stiffness. In the same chapter I have highlighted that EMT participates in the resistance of cancers to chemotherapies including Paclitaxel in pancreatic cancer (Rice et al., 2017b), but also that the exact mechanism is not completely understood. EMT has also been described in resistance to TKIs in lung adenocarcinoma (Uramoto et al., 2010). Resistance in non-small cell lung cancer (NSCLC) can be mediated by a rather rare mechanism (3% of tumors) involving activation of AXL kinase and maintenance of Akt kinase activity. Zhang *et al* describe an activation of AXL and evidence of an EMT in various *in vivo* and *in vitro* models with erlotinib acquired resistance. Indeed, the use of AXL inhibitor restores the sensitivity of tumor cells to erlotinib and decreases the markers of EMT (Zhang et al., 2012). Other factors including Notch-1, Skp2 and TGF β have also been implicated in tumor EMT, conferring resistance to TKIs targeting EGFR in breast and lung cancer (Xie et al., 2012; Yang et al., 2014; Yao et al.). Interestingly, it has been shown that EGF/EGFR signaling axis drives EMT itself in cholangiocarcinoma facilitating cancer cell invasion. However, these processes were abrogated with the action of gefitinib (Clap eron et al., 2014). Recently, one research describes TWIST1, a transcriptional factor involved in EMT, as a driver of resistance to EGFR TKIs in non-small lung carcinoma (Yochum et al., 2019).

1.4.4. ROLE of AXL in RESISTANCE to EGFR TARGETED THERAPY

A) AXL- a general introduction

AXL is a 140kDa tyrosine kinase membrane receptor and an established member of the TAM (TYRO3-AXL-MER) family of RTKs. It is coded by an *AXL* gene (named after a Greek word *anexelekto* meaning uncontrolled) that was first cloned and characterized from primary human chronic myelogenous leukemia (CML) cells in 1991 (O'Bryan et al., 1991). AXL protein is transmembrane protein composed of two immunoglobulin-like domains (Ig) and two fibronectin III domains (FNIII) in the extracellular region, a single-pass trans-membrane domain, and an intracellular protein-tyrosine kinase domain that has a unique KWIAIES sequence conserved between the members of the TAM family (O'Bryan et al., 1991). Indeed, similar extracellular structure containing two Ig and fibronectin III like domains is found in other two receptors, TYRO3 protein tyrosine kinase and c-MER proto-oncogene tyrosine kinase (MERTK or MER) (Graham et al., 1994; Lai et al., 1994)(Figure 18). Interestingly, the Ig domains are frequent in the FGF, VEGF, PDGF receptor families, and the FNIII is common in the ephrin and insulin families.

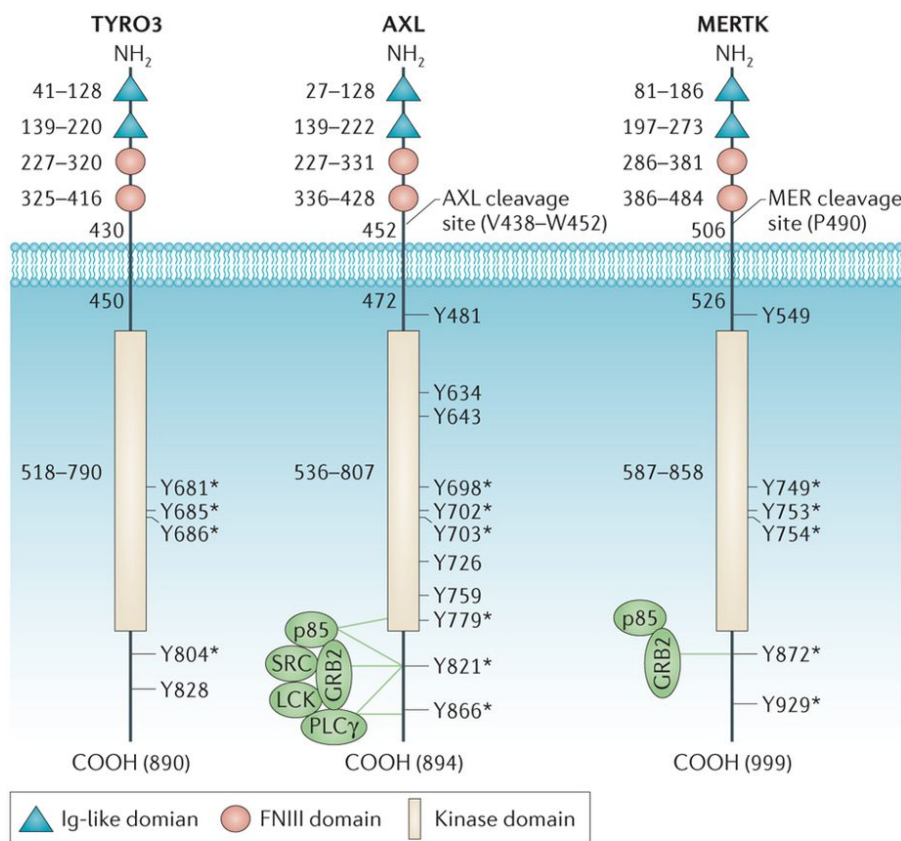


Figure 18. Structural differences between three members (TYRO3, AXL and MERTK) of the TAM family receptors

All three receptors are built with two extracellular fibronectin type III (FNIII) and two immunoglobulin (Ig)-like domains, as well as a conserved kinase domain featuring the unusual KWIAIES sequence that is unique to this family of receptor tyrosine kinases (RTKs). All phosphorylated tyrosine residues are shown on the kinase domain. Autophosphorylation is labelled with asterisks. GRB2, growth factor receptor-bound protein 2; PLC γ , phospholipase C γ . Adapted from .(Graham et al., 2014)

AXL receptor activation can occur in a ligand-dependent or ligand-independent manner and it results in the autophosphorylation of the intracellular tyrosine kinase domain and receptor dimerization. Only known ligand that binds to the extracellular domain of AXL is growth arrest-specific gene 6 (Gas6) (Nagata et al., 1996). Gas6 contains sex hormone-binding globulin (SHBG) and gamma-carboxyglutamic acid (Gla) domains that drive its activity. The SHBG domain mediates receptor dimerization and activation via Ig domain binding (Varnum et al., 1995). Ligand-independent activation is achieved by homodimerization or heterodimerization with other RTKs such as EGFR (Goyette et al., 2018; Huang et al., 2007; Meyer et al., 2013) and results in fast phosphorylation of the AXL and activation of downstream signaling pathways.

Generally, TAM receptors function as homeostatic regulators influencing immune, reproductive, hematopoietic, vascular and nervous system. Their role is crucial in phagocytosis of apoptotic cells in tissues and in innate inflammatory response to pathogens (Lemke, 2013). As opposed to the majority of RTKs, deletion of the TAM receptors does not result in embryonic lethality. *Tyro3-Axl-Mertk* triple knockout mice are viable and fertile, however, with age, these mice develop degenerative diseases correlated with the inability to clear apoptotic cells (Lu et al., 1999).

As for AXL in specific, it is expressed in a variety of normal tissue such as heart, brain, liver, kidney, endothelial cell, skeletal muscle and bone marrow (Lemke and Rothlin, 2008). However, its overexpression is common for a majority of cancers including colon, oesophageal, thyroid, breast, lung, liver, and astrocytoma–glioblastoma (Graham et al., 2014; Verma et al., 2011). Increase in expression of AXL is associated with proliferation, EMT, invasion, and chemoresistance (Antony et al., 2016; Goruppi et al., 1996; Hafizi and Dahlbäck, 2006; Sainaghi et al., 2005). Besides that, using immunohistochemical analysis of primary tumors it has been established that increased expression of AXL correlates with metastasis appearance and/or poor patients survival in lung adenocarcinoma, glioblastoma, pancreatic, renal cell carcinoma, esophageal adenocarcinoma, oral squamous carcinoma, pleural mesothelioma, ovarian adenocarcinoma, colon cancer, urothelial carcinoma, esophageal cell carcinoma, hepatocellular carcinoma and, importantly, HNSCCs (Brand et al., 2015; Chen et al., 2013; Dunne et al., 2014; Gjerdrum et al., 2010; Gustafsson et al., 2009; Han et al., 2013; Hattori et al., 2016; Hector et al., 2010; Hsieh et al., 2016; Hutterer et al., 2008; Koorstra et al., 2009; Lee et al., 2012; Liu et al., 2016; Pinato et al., 2013; Rea et al., 2015; Shieh et al., 2005).

B) *AXL signaling pathways in cancer*

In order to exert its function, downstream signals of AXL regulate core cellular processes including proliferation, survival, and migration (Graham et al., 2014) as well as in motility and invasiveness of cancer cells (Lay et al., 2007; Zhang et al., 2008). It is important to mention that very few activating mutations within the AXL kinase domain are found in cancers (The Cancer Genome Atlas, TCGA) and, as mentioned before, most of the AXL signaling arise through Gas6 binding (ligand-dependent manner). Indeed, in clinics Gas6 expression in tumors is used as a prognostic marker in urothelial, ovarian, lung adenocarcinoma, gastric cancer, and glioblastoma (Buehler et al., 2013; Hattori et al., 2016; Hutterer et al., 2008; Sawabu et al., 2007; Whitman et al., 2014; Zhang et al., 2012). Moreover, elevated serum level of GAS6 is a prognostic biomarker in patients with oral squamous cell carcinoma (Jiang et al., 2015).

Some of the signaling networks mobilized by the AXL signaling are Ras/Raf/MEK/ERK cascade, PI3K/Akt signaling pathways and protein kinase C (PKC) signal transduction cascade (Scaltriti et al., 2016). Ras/Raf/MEK/ERK cascade is commonly involved in proliferation, PI3K signaling is responsible for cell survival through the Akt/ribosomal s6 kinase (S6K) axis (Goruppi et al., 1997) and PCK signal transduction is involved in proliferation and anti-apoptotic signals (Garg et al., 2014). AXL causes an increase of variety of anti-apoptotic proteins like B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-xL) (Hasanbasic et al., 2004). As well it is responsible for the destabilisation of the the I κ B α -NF- κ B complex mediated by the Akt that results in a nuclear translocation of NF- κ B leading to the transcription of anti-apoptotic proteins such as cyclin D1, survivin, and focal adhesion kinase (Ammoun et al., 2014; Pancez et al., 2013). It, as well, inhibits pro-apoptotic proteins like caspase-3 to promote survival (Cavet et al., 2008).

AXL enables migration of cancer cell by regulating the PI3K/Ras/Rac signaling axis, responsible for the reorganisation of actin (Allen et al., 2002; Nielsen-Preiss et al., 2007). As it was briefly mention before, AXL signaling in cancers has been strongly associated with the EMT (Asiedu et al., 2014; Linger et al., 2008; Verma et al., 2011) during which cells lose their apical/basal polarity and gain motility and invasiveness (Thiery, 2002). Indeed, in a research that analysed 643 human cancer cell lines, AXL was strongly correlated with a mesenchymal phenotype particularly in NSCLC and triple-negative breast cancer (Wilson et al., 2014). Same relationship is described in ovarian cancers, where EMT is shown to regulate AXL signaling. Upon AXL inhibition in ovarian cancers, invasion and MMPs activity are drastically reduced (Rankin et al., 2010). In pancreatic cancer, *AXL* knockdown resulted in reduction of EMT transcriptional factors SNAI1, SNAI2, and TWIST leading to the decrease in invasion, migration and metastases (Koorstra et al., 2009). In mesenchymal set up, AXL associates and activates EGFR, HER2 and protein kinase Met (cMET) resulting in phosphorylation of ERK, motility and invasion (Antony et al., 2016)(**Figure 19**). EMT effects on PI3K pathway have also been reported, where

epithelial system relies on the HER3 signaling to activate PI3K and mesenchymal on growth factor stimulation, p110 α , and upregulated PI3K (Salt et al., 2014).

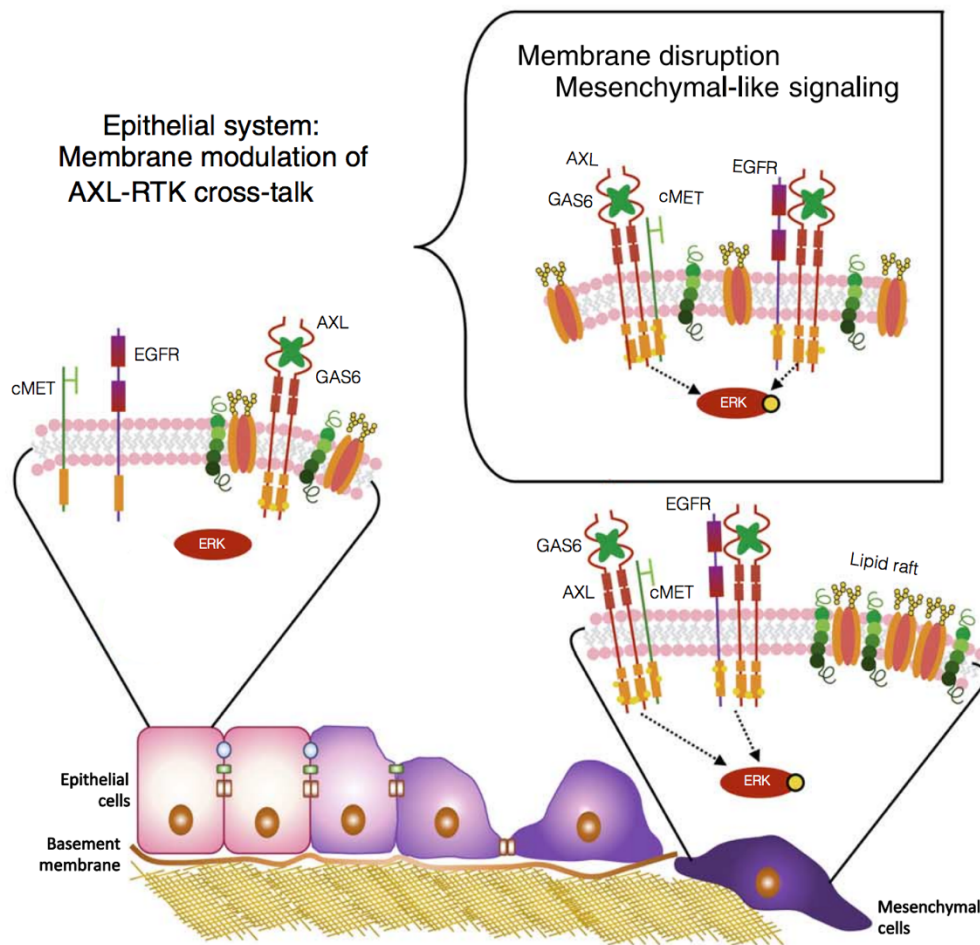


Figure 19. AXL drives the changes in cellular signaling pathways during EMT

Normally, epithelial cells are under modulation of RTK networks and phosphorylation of ERK response. and loss of both these regulatory mechanisms will allow a signaling phenotype that is mesenchymal like. As cells turn from epithelial to mesenchymal state, AXL associates with other RTK such as EGFR and phosphorylates ERK. Adapted from (Antony et al., 2016).

Besides EMT, AXL has been directly associated with the contractility of cells, by phosphorylating tropomyosin 2.1. This very interesting research, describes how AXL controls cells mechanics (contractions) responsible for rigidity sensing, in the absence of ligands. Mechanically, phosphorylated AXL localizes to myosine filaments that leads to the tropomyosin 2.1 activation, known to be critical for adhesion, formation directly controlling mechanosensory contractions. Downregulation of AXL alters rigidity sensing and increases the magnitude or duration of local contraction events (Yang et al., 2016a).

AXL's communication with the integrins is mediated by the adapter protein Nck2. In detail, AXL-Nck2 interaction enables AXL-modulation of the integrin-linked kinase (ILK), a major element of focal adhesion responsible for cytoskeleton dynamics (Verma et al., 2011). Lastly, autophosphorylation of the cytoplasmic residues of AXL docks the phospholipase C- γ (PLC γ), PI3K, and growth factor

receptor-bound protein 2 (Grb2) (Braunger et al., 1997; Fridell et al., 1996). Interestingly PLC γ is involved in calcium signaling (Hokin, 1966) and a recent publication from our group describes a cooperation between EGFR and matrix stiffness in collective invasion of cancer cells through calcium signaling (Grasset et al., 2018). Some of the AXL/Gas6 signaling pathways are schematically represented in the **Figure 20**.

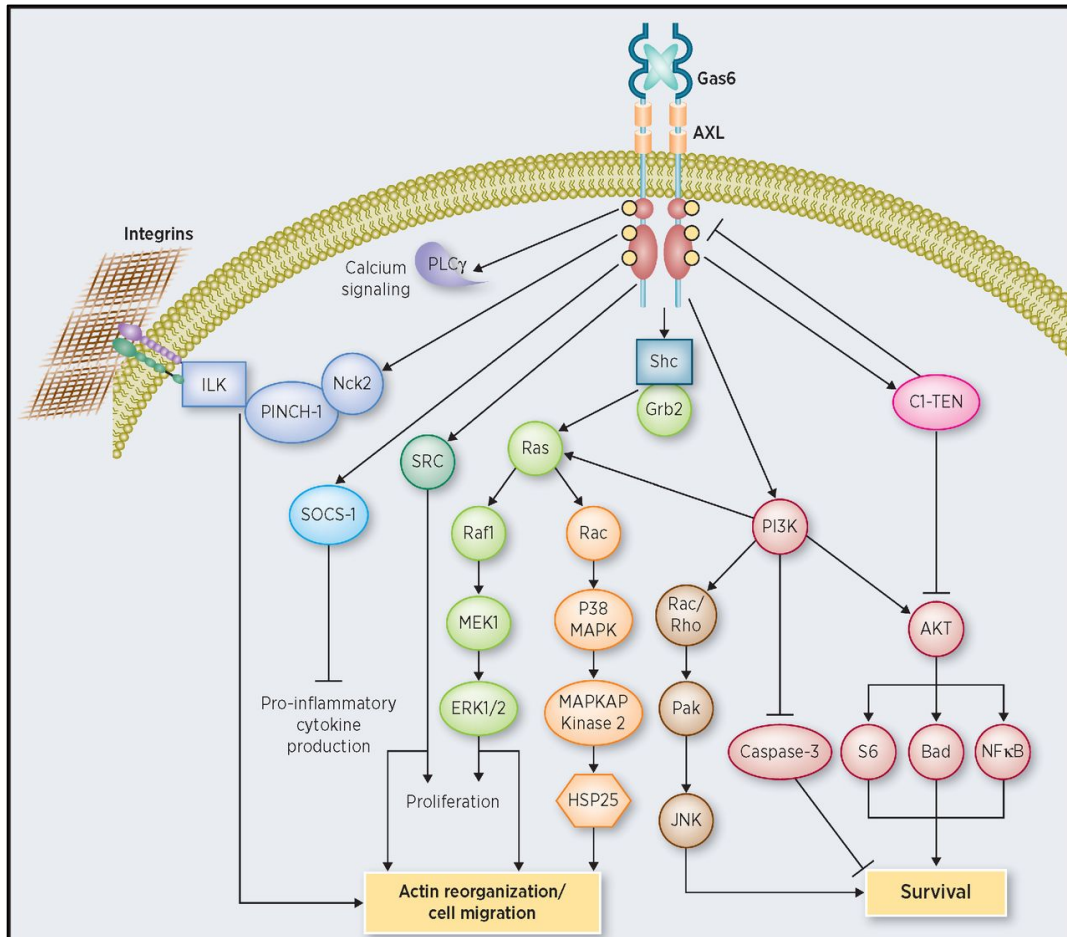


Figure 20. Gas6/AXL signaling schema

AXL activation can occur through ligand-dependant (activation by ligand Gas6 in a 2:2 stoichiometry) or ligand independent manner. Either way AXL is phosphorylated and active. It exerts its activity by influencing various signaling pathways and through them deciding upon cell faith. Ras/Raf/MEK/ERK pathway as well as Src converges on proliferation and migration. PI3K kinase axis involving S6, Akt, or JNK results in cell survival and protection from apoptosis. AXL signaling affect actin reorganization through integrins. It also modulates calcium signaling through PCL γ and proinflammatory cytokine production through SOCS-1. Adapted from (Antony and Huang, 2017).

Regulation of AXL activity is not yet completely described, but several evidence have linked the phosphatase C1-TEN (Hafizi et al., 2002) (know to prevent signaling downstream of Akt (Hafizi et al., 2005)) to AXL as well as the E3-ubiquitin ligase Cbl-b that counteracts AXL signaling by ubiquitination and degradation of AXL (Gioia et al., 2015; Paolino et al., 2014).

The reach of AXL signalling is extraordinarily far, as it is common for the majority of RTKs, and it influences main cellular processes. Increasing knowledge of AXL regulating many aspects of cancer

progression and response to therapy has justified a growing interest in the role of AXL in resistance to cancer therapies and development of AXL inhibitors.

C) *Role of AXL in resistance of cancer cells to therapeutic strategies*

Role of AXL in resistance to multiple anti-cancer strategies including chemotherapies, radiotherapy and targeted therapy has been documented in literature in variety of cancers such as AML (Acute Myeloid Leukemia), NSCLC, triple negative breast cancer, oesophageal and ovarian cancer, pancreatic cancer, glioblastoma and HNSCCs among others (Brand et al., 2015; Hong et al., 2008, 2013; Meyer et al., 2013; Wilson et al., 2014; Zhang et al., 2012). For example, AXL is overexpressed in cisplatin chemoresistant oesophageal cancer where it promotes survival through the activation of Akt and ERK1/2 and block apoptosis by modulating c-ABL (Hong et al., 2013). In breast cancer, AXL confers resistance to doxorubicin through the Akt/GSK-3 β / β -catenin leading to the upregulation of ZEB1 that regulates DNA damage repair and EMT (Wang et al., 2016a).

Despite a significant role AXL plays in resistance to common chemotherapies and radiotherapy, I will be mostly focused on its role in resistance to targeted therapies.

In NSCLCs where upregulation of AXL is strongly associated with EMT. Indeed, EMT-associated transcriptional program, mostly by upregulation of vimentin, drives the *AXL* overexpression in *EGFR* mutant lung cancer cells with acquired EGFR TKI resistance to erlotinib. Preclinical data connects *AXL* upregulation with multiple pathways such as MAPK, Akt and NF- κ B that may govern erlotinib resistance, but no clear connection has been established yet (Zhang et al., 2012). Brand *et al* described that overexpression of AXL is sufficient to drive cetuximab resistance in NSCLC and HNSCCs. AXL activation stimulates cell proliferation, EGFR activation and MAPK signaling in cetuximab resistant cells. Research also demonstrates a direct regulation of AXL mRNA expression by EGFR through MAPK signaling and the transcription factor c-Jun in cetuximab resistant cells in NSCLC creating a positive feedback loop that maintains EGFR activation by AXL (Brand et al., 2014) (**Figure 21**)

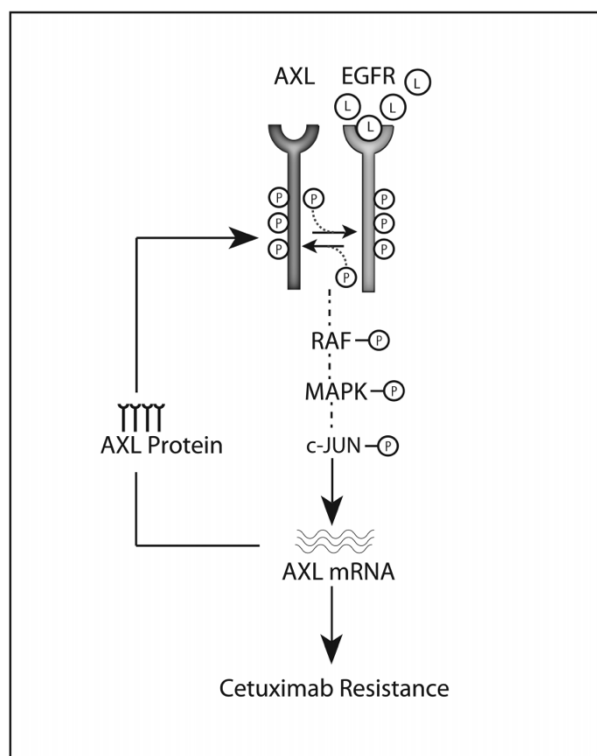


Figure 21. AXL and EGFR cooperate in cetuximab resistance

In cetuximab resistant cells, increase of EGFR ligand (L) leads to an AXL and EGFR association and transactivation. Consequently, MAPK and c-Jun signaling is activated leading to the increase in AXL transcription. In turn, elevated levels of AXL protein result in maintenance of EGFR activation and signaling creating a positive feedback loop that results in constitutive activation of AXL and EGFR. Adapted from (Meyer et al., 2013).

Indeed, a possibility that AXL may drive resistance to EGFR inhibitors by associating with EGFR and other ErbB receptor family members, MET and PDGFR was shown previously in triple-negative breast cancer. Data suggests that AXL interacts with other RTK, activating them (and *vice versa*) limiting the ligand-blocking therapies such as cetuximab (Meyer et al., 2013). Furthermore, authors demonstrated via small-molecule inhibitors screen that AXL inhibition is synergistic with antimitotic pharmaceuticals in cells that exhibited an EMT phenotype and resistance to RTKi (Wilson et al., 2014). However, authors were not able to overcome acquired resistance to EGFR inhibitors by AXL inhibition like it was previously reported by Zhang *et al* two years earlier, but they do describe a sensitizing effect. Importantly, in triple-negative breast cancer, AXL is transactivated by EGFR rather than by ligand-binding and it leads to the expansion of EGFR signaling and ultimately to resistance to EGFR inhibitors, in this particular case - erlotinib (Meyer et al., 2013). Furthermore, AXL dimerization and phosphorylation of EGFR leads to the activation of phospholipase C gamma and PKC that activate mTOR signaling to resistance to PI3K inhibition in HNSCCs and oesophageal squamous cell carcinomas (Elkabets et al., 2015). AXL has been implicated in the resistance of breast cancer to HER2-targeted therapy through EMT-mediated mechanism (Creedon et al., 2016).

Most importantly, inhibition of AXL restores the sensitivity of cancer cells to EGFR inhibitors (Brand et al., 2014; Zhang et al., 2012). Besides directly targeting AXL to overcome the resistance, inhibition of upstream AXL regulator YAP has been shown to impair lung cancer resistance to EGFR targeted therapy (Ghiso et al., 2017; Lee et al., 2016).

These data imply that the AXL signaling is an important player in cancer development, growth, metastasis and chemoresistance. In recent years, efforts have been put in deciphering the molecular mechanism behind this tumor promoting signaling pathway that has led to the development of variety of AXL inhibitors that are being tested in preclinical and clinical studies.

D) Anti-cancer therapies developed to target AXL

As mentioned above, more and more data is confirming the role of AXL in drug resistance, patient mortality and a predictor of poor patient outcome, highlighting AXL as an appealing target for therapeutic development (Han et al., 2013; Holland et al., 2010; Hutterer et al., 2008).

Wide range of small-molecule kinase inhibitors that target AXL are described in several studies, including Foretinib, Cabozantinib, Merestinib, Bosutinib, Gilteritinib, Crizotinib, Amuvatinib, Sunitinib, MGCD265, ASLAN002, NPS-1034, LDC1267, SGI-7079, TP-0903, UNC2025 and BGB324 (Graham et al., 2014). These inhibitors are a broad spectrum RTKs inhibitors and its primarily target was not AXL but rather an “off-target” resulting from the similarities between kinase domains of RTKs such as MET and Bcr-Abl. Since these inhibitors are not AXL-specific, they exhibit less potency for AXL than their main target. However, research done with BGB324 or R428 has shown that it is an AXL-selective inhibitor and it has advanced into clinical trials for that purpose (Sheridan, 2013). Interestingly, a company that has enter clinical trials with BGB324 is co-founded by professor James B. Lorens that has been studying role of AXL in cancers for decade as a group leader at University of Bergen, Norway. His research has demonstrated that EMT (in particular vimentin) is required for induction of AXL expression in breast cancer that is necessary for metastasis formation and cancer progression (Vuoriluoto et al., 2011).

BGB324 inhibits AXL activity and blocks AXL-dependent events, including Akt phosphorylation, cancer cell invasion, and proinflammatory cytokine production. Pharmacological research has demonstrated favourable effects after oral administration of BGB324 followed by a reduction in expression of cytokine granulocyte macrophage colony-stimulating factor and Snail, an EMT transcriptional factor, in a dose-dependant manner. Besides that, BGB324 reduced the rate of metastasis and extended the survival of orthotopic mouse models of breast cancer metastasis (Wu et al., 2018). It also has a synergistic effect with cisplatin in suppression of liver micrometastases (Holland et al., 2010). BGB324 is shown to block AXL in glioblastoma multiforme (GBM), AML and Ewing sarcoma, indicating the possibility of using BGB324 in variety of cancers (Fleuren et al., 2014; Janning et al., 2015; Vouri et al., 2015).

BGB324 is currently in clinical development (alone or in combination with other drugs used for that specific cancer) and more than one trial is registered at ClinicalTrials.gov for this AXL inhibitor in various clinical centres for NSCLC, AML and metastatic melanoma (trial identifiers: NCT02922777, NCT02488408, NCT02424617 and NCT02872259). Besides kinase inhibitors, several monoclonal antibodies targeting AXL are described including 12A11 (Li et al., 2009), Mab173 (Li et al., 2009), YW327.6S2 (Ye et al., 2010) and, D9 and E8 (Leconet et al., 2014).

Lastly, studies using shRNA against AXL have reported to be effective in inhibiting AXL signaling, for example in xenograft model of breast carcinoma, shRNA knockdown of AXL significantly reduces the tumor size (Gjerdrum et al., 2010).

Taken together, these data provide confidence that soon enough a commercial therapy against AXL will be available for patients suffering from various cancers.

1.4.5. ROLE of TNFAIP3 in CANCER RESISTANCE

A) *General introduction of TNFAIP3*

Tumor necrosis factor α -induced protein (TNFAIP)3 or A20, named after its cDNA clone number, is a zinc finger protein and ubiquitin-editing enzyme and it was first described in endothelial cells as a primary response to TNF (Dixit et al., 1990). Originally it was characterized as a protein protecting cells from TNF-induced cytotoxicity (Opipari et al., 1992) and today is known for its role in negative regulation of inflammation and immunity. (Catrysse et al., 2014). Further research demonstrated that it is also a negative regulator of NF- κ B activation in response to IL-1, CD40, and of signaling through pattern recognition receptors (PRRs), and T cell and B cell antigen receptor activation (Beyaert et al., 2000). Due to the role of A20 as anti-inflammatory protein, A20-deficient mice are hypersensitive to TNF and die prematurely due to severe multi-organ inflammation and cachexia (Lee et al., 2000). Besides its well characterized role in inflammation and regulation of NF- κ B signalling, A20 function in a cytoprotective manner (Won et al., 2010). It has been shown that the overexpression of A20 protects different cell types from TNF-mediated apoptosis and necrosis. For example, A20 was shown to inhibit caspase 8 dependent and independent cell death pathway in macrophages stimulated with TNF (Tran et al., 2009). However its role in cell death remains controversial since it highly depends on cell type and context (Lee et al., 2000; Verstrepen et al., 2010). The underlying mechanism by which A20 inhibits apoptosis is still under investigation. Next to its role in restricting inflammation and cell death, A20 has been described to regulate various signaling pathways such as Wnt, autophagic response and interferon regulatory factor (IRF) pathway (Lin et al., 2006; Saitoh et al., 2005; Shao et al., 2013; Shi and Kehrl, 2010). Activation and expression of A20 is a tightly regulated process. Under basal conditions most of the cells express very low level of the protein, nevertheless upon NF- κ B activation A20 is actively expressed (Düwel et al., 2009; Verstrepen et al., 2010). It is also regulated by phosphorylation. Stimulation with TNF leads to the A20 phosphorylation by inhibitor of nuclear factor kappa-B kinase (IKK- β) that increases its ability to inhibit NF- κ B signaling by an unknown mechanism (Hutti et al., 2007). Function of A20 has been described as cell and disease-specific (Vereecke et al., 2014) and it plays an important role in auto-immune and auto-inflammatory diseases (Zhou et al., 2016).

In cancers it has been described to play both tumor-suppressor and oncogenic role. A20 is highly expressed in inflammatory breast cancer (Vendrell et al., 2007), glioma stem cells (Hjelmeland et al., 2010), HNSCCs and undifferentiated nasopharyngeal carcinoma (Codd et al., 1999). Recent research describes a role of A20 in metastasis of aggressive breast cancers and a marker of poor prognosis. Researchers report that A20 is able to monoubiquitylate Snail1 at three lysine residues facilitating TGF- β 1-induced EMT of breast cancer and metastasis (Lee et al., 2017). However in multiple B cell lymphomas, Hodgkin's lymphoma, and follicular lymphoma it acts as a tumor suppressor (Kato et al., 2009; Novak et al., 2009; Schmitz et al., 2009). Together, these studies suggest that depending on the cell type and tumor stage, A20 may act as in a pro- or anti-cancerogenic manner.

B) Role of A20 in resistance of tumors to therapies

Recent finding report that A20 is overexpressed in a number of solid tumors, and therefore contributes to carcinogenesis and response to anti-cancer therapies (da Silva et al., 2014). However, these correlations are not fully studied and few research dealt with the role of A20 in resistance of cancer to therapies.

For example, in hepatocellular carcinoma it has been associated with resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis (Dong et al., 2012). Same was reported in glioblastoma that revealed a potential therapeutic property of A20 since its inhibition can overcome TRAIL resistance (Bellail et al., 2012). In acute lymphoblastic leukemia, upregulation of A20 leads to an increase of proliferation and chemotherapy resistance to daunorubicin (Chen et al., 2015). In breast cancer, where its role has been somewhat described, A20 has been shown to drive the resistance to tamoxifen (Vendrell et al., 2007). A very recent study discussed how A20 is a key factor mediating cancer cell resistance to DNA-damaging therapy (Yang et al., 2018).

Due to its dual role in cancer, A20 has been described in resistance context but with a different role. Some studies focused on deciphering molecular targets that will drive the expression of A20 and therefore modulate the resistance of colorectal cancer (Liu et al., 2018).

Until this day there is no commercially available inhibitor of A20, however a patent exists on the list of A20 inhibitors (a molecule selected from the list comprising of a ribozyme with a specificity for the A20 gene, a siRNA with a specificity for the A20 gene, an artificial microRNA with a specificity for the A20 gene, an antisense nucleic acid construct with a specificity for the A20 gene, a peptide with a specificity for the A20 protein or an antibody with a specificity for the A20 protein) proposed to use for treatment of respiratory viral infections. No clinical study has been done with any of the proposed methods of A20 inhibition.

Due to its nature, A20 is a complex molecular target to be considered in future of cancer research, protocols must consider the cell-specific function of A20 and focus on expressing or attenuating its function on a tissue-specific level.

1.4.6. CONCLUSION

Cancerogenesis is a dynamic process and so is acquiring resistance to carefully designed drugs. Many scientist tried to explain cancer through evolutionary and ecological processes (Casás-Selves and DeGregori, 2011; Merlo et al., 2006; Vineis, 2003) and I would agree. Cancer development has an uncanny resemblance to an evolution strategy taken by so many species, including humans. Same as species evolve by mutations and selection of favourable individuals, tumors evolve by mutations and selection of (un)favourable cells. Same as the environment has a tremendous impact on the destiny of the species, it can either nurture it or starve it, tumor microenvironment plays a crucial role in cancer development. Since the evolution and adaption of cancers is so fast, it can easily evolve resistance to any given therapy. Therefore, we must act fast in understanding the mechanisms behind these resistances in order to develop new therapeutic strategies, aimed at new targets or based on the combination of several therapeutic agents to prevent or overcome these resistances in patients, or ideally gain enough knowledge to be able to predict each patient's outcome.

I would like to conclude by quoting Siddhartha Mukherjee and his marvelous book "*The Emperor of all Maladies: A Biography of Cancer*" where he described resistance of cancer.

"If the vigilance was dropped, even for a moment, then the weight of the battle would shift. In Lewis Carroll's *Through the Looking-Glass*, the red Queen tells Alice that the world keeps shifting so quickly under her feet that she has to keep running just to keep her position. This is our predicament with cancer: we are forced to keep running merely to keep still."

Chapter 2: *OBJECTIVES*

Cancers develop therapeutic resistance to treatments such as as radiotherapy, chemotherapy and targeted therapy through various mechanisms. These mechanisms can be genetic and epigenetic changes in cancer cells or/and in the tumor microenvironment. The underlying mutations are followed or, often, generated by the changes in biochemical and biophysical features of the surrounding tissue (Singh et al., 2018). Extra Cellular Matrix (ECM) found in tumor tissue is significantly more stiff than the one in normal tissue (Rice et al., 2017a). Mechanical stimuli exerted on cancer cells by stiff ECM are transduced to chemical cues via process of mechanotransduction (Jaalouk and Lammerding, 2009). Stiffness and mechanotransduction influence the faith of cancer cells and promote proliferation, angiogenesis, invasion, EMT and survival (Bertero et al., 2018; Lim et al., 2008; Singh et al., 2018). Therefore, role of stiffness in cancer resistance to therapeutics has been investigated (Hirata et al., 2015; Rice et al., 2017a). However, its clinical relevance remains elusive.

In Head and Neck Squamous Cell Carcinomas (HNSCCs), many different therapeutic approaches have been taken in order to eliminate cancer and its metastatic spreading. Epidermal Growth Factor Receptor (EGFR) is found to be expressed, overexpressed or mutated in these cancers and a marker of poor prognosis (Ang et al.). Due to its role in HNSCCs, EGFR found its way to clinics as one of the rational targets in treatment strategies. (Baselga, 2001; Rubin Grandis et al., 1998). However, only a small fraction of patients benefits from these therapies and the majority of them develop resistance. These failures can be explained by the limitations of the *in vitro* study models used, taking into account only the tumor cells alone.

Therefore, studying the resistance in the context of matrix stiffness is a potential approach in understanding the failure of therapies in HNSCCs as well as paving the path for new ones.

Very first objective of my thesis was to characterize the role of stiff ECM in survival of SCC cell lines upon various treatments such as conventional chemotherapies, targeted therapies and a combination of both, treatments that are deployed in treating HNSCCs patients. To do so, we have utilised 2.5D cell cultures *in vitro* where cells were plated on top of a tick layer of ECM (soft and deformable collagen I rich matrix) or on a thin coating of collagen I rich stiff matrix. This approach allowed us to understand the response of cancer cells to anti-cancer treatment upon their interaction with the environment.

Second objective was to identify a specific molecular target that would override stiffness mediated resistance. Identification of such a target would further decipher the mechanism of resistance and allow exploration of novel therapeutic agents. To do so we have preformed RNA sequencing of SCC cell line plated on stiff and soft matrix. This method allowed us to classify genes differentially regulated in cancer cells in contact with rigid environment. Our goal was to describe a new inhibitor that would reverse stiffness driven resistance in cancer cells and stimulate cell death both in 2.5D culture and 3D structures, spheroids embedded in ECM scaffolds.

Furthermore, we aim to provide a novel biomarker of drug efficacy prediction. In many EGFR-dependant cancers such as pancreatic cancer, breast cancer, NSCLC and HNSCCs, EGFR-TKIs failure is common and not yet explained. We believe that stiffness plays a major role in the response of cancer

cells to TKIs and we wish to allow clinicians to treat their patient with an appropriate agent. If stiffness is indeed as important as we hypothesize, our data should pave the path to more personalized and adequate approach to treating these cancers.

Chapter 3: RESULTS

I. Summary of the results

Tumor microenvironment and matrix stiffness are considered key players in cancer development and resistance to anti-cancer agents. In HNSCCs, EGFR-targeted therapies often show no significant benefit for the patients and their failure is considered a major problem in clinics. Despite significant efforts focused on understanding the mechanism behind this occurrence, it is yet to be completely elucidated. We hypothesised that stiffness could be the reason behind EGFR-targeted therapy failure.

In order to study the relationship between matrix rigidity and resistance of HNSCCs to EGFR-targeted therapies, but also to traditional chemotherapies that are frequently used for the treatment of patients, we have used various *in vitro* approaches.

More specifically, we have shown that EGFR TK inhibitors – gefitinib and AG1478 are not efficient when the human SCC cell line SCC12 is plated on stiff matrix.

When SCC12 cells are plated on a soft and deformable collagen I rich matrix or on a collagen I rich stiff matrix for 48h, to get accustomed to their environment, their sensitivity to gefitinib changes. On soft substrate not more than 20% of cells are able to survive treatment 5 μ M gefitinib, while on stiff 78% survives the same. Additionally, when we measured the induction of apoptosis by gefitinib, SCC12 cells were positive for cleaved caspase-3 only when plated on soft matrix. Following these results, we tested cetuximab, monoclonal antibody against EGFR, in the same fashion. When SCC12 cells were plated on stiff substrate they were significantly more protected from cetuximab treatment than when plated on soft substrate. In addition, an interference RNA approach has demonstrated that the EGFR knock out reduces SCC12 cell number only when plated on soft matrices, that is counteracted by the rigidity of the ECM when cells are plated on stiff matrix. We tested our hypothesis on more than one HN-SCC cancer cell lines, CAL27, CAL166 and SCC25 cell lines isolated from patients' biopsies at the "Centre Antoine Lacassagne" anti-cancer centre in Nice, France. Indeed, our hypothesis proved to be correct in a variety of HNSCCs – stiffness provided massive protection to treatment with both gefitinib and AG1478. To understand will fine-tuning of matrix rigidity change the level of protection to SCC12 cells we tested the ability of gefitinib to kill cells in two more pathophysiological conditions. First, we plated SCC12 cells on substrate of known stiffness values to mimic normal (1kPa) and tumor (12 and 50kPa) tissue. It was shown that even at more relevant values of rigidity, stiffness was able to protect SCC12 cells from gefitinib treatment. Second, we aimed to produce ECM that can be found in normal or cancerous tissue respectively. Therefore, we produced the matrix using fibroblast and activated (CAF-like) fibroblasts *in vitro*, plated SCC12 cells on top of it and treated them with gefitinib. Not only were the fibres more aligned when produced from activated fibroblasts, but it also allowed 39% increase of SCC12 cells survival of 48h treatment with gefitinib.

Since in HNSCCs, traditional chemotherapy is often used alone or in combination with EGFR-targeted therapy, we wondered will the difference in matrix rigidity maintain the same type of protection to

SCC12 cells. To test our notion, we placed SCC12 cells on either soft or stiff matrix and treated them with (1) cisplatin, docetaxel, 5-fluorouracil alone or in combination with either (2) gefitinib or (3) cetuximab. In all three situations, stiffness of the matrix was able to protect SCC12 cells despite the different mechanism of action of these therapies.

[These data suggest an important role of ECM rigidity in SCC12 cell survival and protection from various treatments, EGFR-targeted therapy, chemotherapy or both.](#)

To verify our hypothesis that protection is indeed stiffness induced, we knocked out co-transcriptional factors YAP and TAZ that mediate mechanical stress induced by matrix rigidity. Our conclusion proved to be correct as SCC12 cells were more susceptible to gefitinib treatment in the absence of YAP/TAZ, even of stiff matrix.

Having established our proof of concept hypothesis, we sought to understand why is stiffness so crucial in resistance of HNSCCs.

We noticed that, when plated on stiff matrices, SCC12 cells become more elongated therefore, adopt a more mesenchymal phenotype. We hypothesised that stiffness induced an EMT. Indeed, N-cadherin and fibronectin were found to be overexpressed in SCC12 cells plated on stiff.

[However, epithelial marker E-cadherin was also expressed in this condition suggesting a partial EMT.](#) Since EMT was reported in some models to drive the resistance of cancers, we assumed that the downregulation of EMT transcriptional factors will have an effect on cells susceptibility to gefitinib treatment. Accordingly, knock out of Zeb1, Zeb2, Snail1, Snail2 and Twist in SCC12 cells using siRNA showed that cells were more susceptible to gefitinib treatment when plated on stiff matrix when one of the EMT transcriptional factors was attenuated compared to control (siLuc). However, more than 40% of cells were still surviving the treatment. Our goal was to identify a novel target that will lead to a complete responsiveness to EGFR targeted treatments.

In order to get a full grasp on the changes that occur in SCC12 cells in the response to increased matrix rigidity we performed RNA sequencing of SCC12 cells plated on soft and stiff. RNAseq revealed numerous genes to be differentially regulated in cells plated on stiff (50kPa) compared to soft (1kPa) substrate. Among the plentiful genes that were upregulated, notably 72 RTK were differentially regulated in SCC12 cells plated on stiff compared to soft. Besides that, various inflammatory regulators were found to be upregulated in SCC12 plated on stiff, *NFKB1*, *NFKB2*, *RELA* and *RELB*, *TNAFAIP3* and *ICAMI*. Lastly, significant amount of genes coding for ECM proteins was upregulated in SCC12 cells plated on stiff compared to soft as well, such as fibronectin, tenascin-C, vimentin and collagen IV. Interestingly, fibronectin, tenascin-C and vimentin are involved in EMT that further confirm our findings. One RTK was particularly interesting, AXL kinase, due to its role in resistance of various cancers and a direct link to EMT, and it became the focus of our further research. We confirmed the overexpression of AXL in SCC12 cells plated on stiff substrate on a protein level and established a direct link of AXL

and stiffness. YAP/TAZ knock out led to a decreased level of AXL in SCC12 cells. On top of that, inhibition of AXL in combination with EGFR inhibitor led to a complete overturn of stiffness mediated protection of SCC12 cells – upon combinational therapy with R428, and AXL inhibitor, and gefitinib only 8% of cells plated on stiff survived the treatment. We have as well showed using 3D cell structures (spheroids) that combinational approach of targeting both kinases AXL and EGFR induced a significant increase of cell death. Finally, we demonstrate that combining traditional chemotherapeutic agents with R428 is far more effective in killing SCC12 cells plated on stiff then combining them with EGFR inhibitors.

In conclusion, our data demonstrates that AXL is overexpressed upon an increase of stiffness and could be exploited as a potent target in HNSCCs patients.

II. Results

Matrix rigidity promotes resistance of HNSCCs to conventional chemotherapeutic compounds and to EGFR-targeted therapy.

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Key words: Extracellular matrix, stiffness, EGFR, head and neck squamous cell carcinoma, resistance

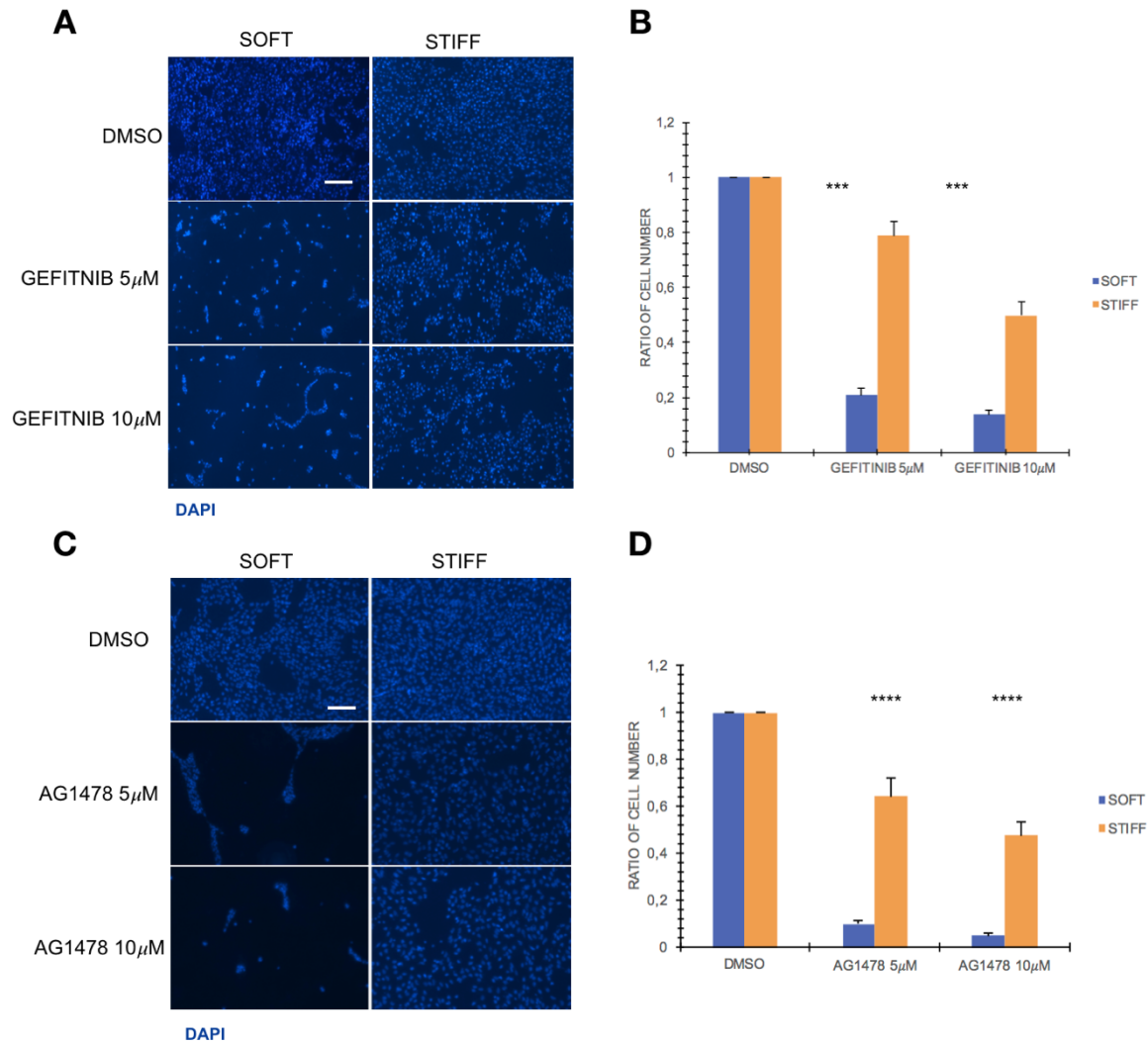


Figure 22. SCC12 cells plated on the stiff matrix are more resistant to treatment with EGFR-TKIs

A) Representative DAPI staining pictures of SCC12 cultivated on a stiff and soft matrix for 48h, treated with 5 and 10 μ M of gefitinib for another 48h. Scale bar 200 μ m. B) Quantification of DAPI staining from experiment shown in A. (n=4, mean + SEM) C) Representative DAPI staining pictures of SCC12 cultivated on a stiff and soft matrix for 48h, treated with 5 and 10 μ M of AG1478 for another 48h. D) Quantification of DAPI staining from experiment shown in C. (n=3, mean + SEM). Paired samples were compared by 2-tailed Student's t test (NS: not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

3.1. PROOF of CONCEPT – STIFFNESS PROMOTES RESISTANCE in HNSCCs

Resistance to therapies in cancers represents a growing problem and many researchers have sought the find the best approach to resolve this Gordian knot. In HNSCCs resistance is a particular challenge due to the various combinations of therapies, often given simultaneously. A recent publication from our group investigated mechanism of EGFR-downstream signalling in response to ECM stiffness in the context of cancer cell invasion in SCCs (Grasset et al., 2018; Rosenthal, 2017). Data obtained led us to hypothesized that stiffness itself promotes resistance to common EGFR-targeted therapies, we thus further investigated the potential role of the extracellular matrix rigidity in HNSCC cells resistance to anti-cancerous therapeutic compounds.

To test this hypothesis, we conducted a proof of concept experiment using the human SCC cell line SCC12, plated either on a soft and deformable collagen I rich matrix or on a collagen I rich stiff matrix. SCC12 cells were plated on the matrices for 48h to get adjusted to their environment and then, treated with two TKIs targeting EGFR; the gefitinib and AG1478 compounds at indicated final concentration for a period of 48h In **Figure 22** (A and C), we show that SCC12 cells plated on the stiff matrix were significantly more resistant to EGFR inhibitors treatment-induced cells death compared to SCC12 cells plated on the soft matrix as demonstrated in **Figure 22**. We found that 78% of SCC12 cells plated on stiff matrix are still alive and viable after 48h treatment of gefitinib at 5 μ M concentration and that 49% of SCC12 cells are viable after 48h treatment of gefitinib at 10 μ M final concentration. However, treatment of SCC12 cells plated on the soft matrix resulted in only 20% of cells viability at 5 μ M and 13% at 10 μ M concentration of gefitinib (**Figure 22B**). Same was true when SCC12 cell plated on soft and stiff for 48h were treated with 5 μ M and 10 μ M of AG1478 (**Figure 22C**). 64% of SCC12 cells plated on stiff matrix survived the treatment with 5 μ M final concentration of AG1478 and only 10% when SCC12 cells were plated on soft matrix. When cells plated on stiff were treated with and 10 μ M final concentration of AG1478, 47% of them were viable post-treatment and only 5% when cells were plated on soft substrate. This proof of concept experiment indicates that indeed, the rigidity of collagen I rich matrix substrate promotes a significant resistance to anti-EGFR treatment-induced cell death in SCC12 human cancer cell line.

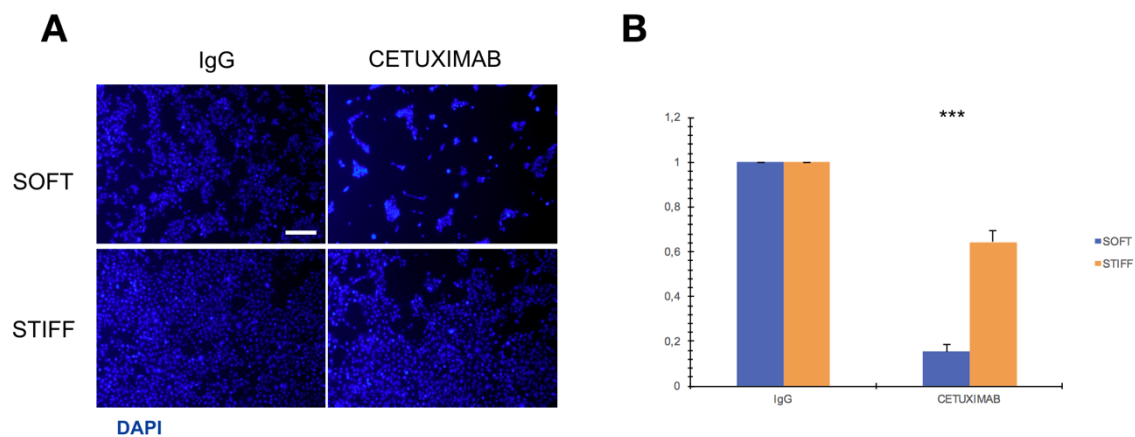


Figure 23. *SSC12 cells plated on the stiff matrix are more resistant to treatment with EGFR-targeted therapy, monoclonal antibody cetuximab*

A) Representative DAPI staining pictures of SCC12 cultivated on a stiff and soft matrix for 48h, treated with 10 μ M of cetuximab for another 48h. Scale bar 200 μ m. B) Quantification of DAPI staining from experiment shown in A. (n=3, mean + SEM). Paired samples were compared by 2-tailed Student's t test (NS: not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

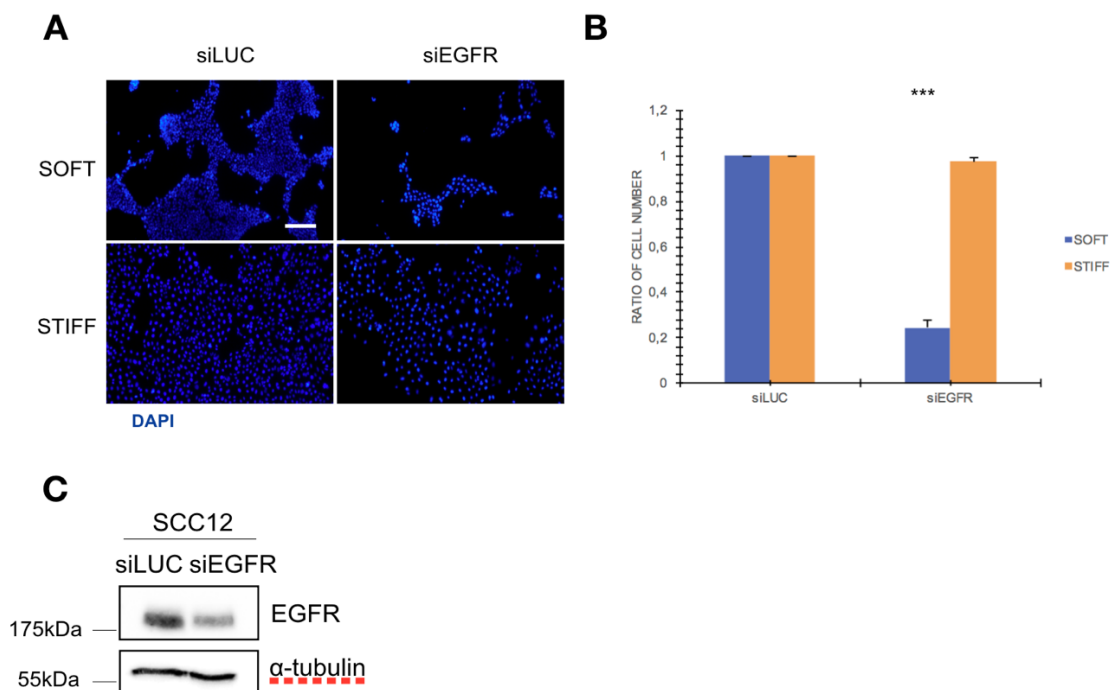


Figure 24. *SCC12 cells transfected with siRNA against EGFR lose their ability to proliferate on soft but not on stiff matrix*

A) Representative DAPI staining pictures of SCC12 transfected either by siLUC as a control or siEGFR and cultivated on a stiff and soft matrix. Scale bar 200 μ m. B) Quantification of DAPI staining from experiment shown in A. (n=3, mean + SEM). C) Immunoblot of total EGFR in SCC12 cells corresponding to the assay presented in A. Tubulin used as control. Paired samples were compared by 2-tailed Student's t test (NS: not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

We further investigated the role of EGF/EGFR signaling inhibition in SCC12 cells viability and the role of matrix rigidity. To do so, we performed the similar experimentation as in **Figure 1** and studied the use of cetuximab, a selective monoclonal antibody against EGFR, in SCC12 cells viability and survival. SCC12 cells were cultured on either a soft or stiff matrix rich in collagen I for 48h, then, the cetuximab monoclonal antibody was added at the final concentration of 10 μ M. We show that addition of cetuximab induced a strong reduction of cell number, quantified by DAPI staining and counting of cell number after 48h, compared to the control condition, in which a IgG peptide was added (**Figure 23A**). Cetuximab induced a 85% reduction in cell number when cells were plated on the soft matrix, however, when the SCC12 cells were plated on a stiff matrix, we noticed a reduction of cell number of only 36% (**Figure 23B**). Thus, we conclude that inhibition of EGFR signaling in SCC12 cells, either using RTKIs or monoclonal antibody, promotes SCC12 cells death *in vitro* but only when plated on soft matrix. To provide more specificity in the effect of EGFR inhibition-dependent SCC12 cells death relative to the substrate rigidity, we performed an *in vitro* cells knock out of EGFR using a small interference RNA oligonucleotide sequence that specifically target the RNA messenger of the EGFR gene (siEGFR) (**Figure 24C**). SCC12 cells were transfected with an siEGFR oligonucleotide sequence and plated on soft or stiff matrix for a period of 48h. At the end of this period, SCC12 cells were fixed, stained with DAPI and the number of cells was quantified by DAPI staining (**Figure 24A**). We showed that knock out of EGFR expression led to a strong and significant decrease in cell viability on soft matrix (76%), however, the reduction of cell number, when cells were plated on the stiff matrix, is limited to 3%, when compared to control transfected cells with a siRNA targeting the Luciferase gene (siLuc) (**Figure 24B**). All together, our data showed that inhibition of EGFR activity or expression leads to a strong decrease of SCC12 cells viability. This loss of cell viability is counteracted by the rigidity of the ECM. Our finding could potentially bring the idea, that in tumor microenvironment context, in which a strong tissue fibrosis and increased rigidity occur through the action of the carcinoma associated fibroblasts, SCC cancer cells are protected to cell death induced by anti-EGFR targeted therapy.

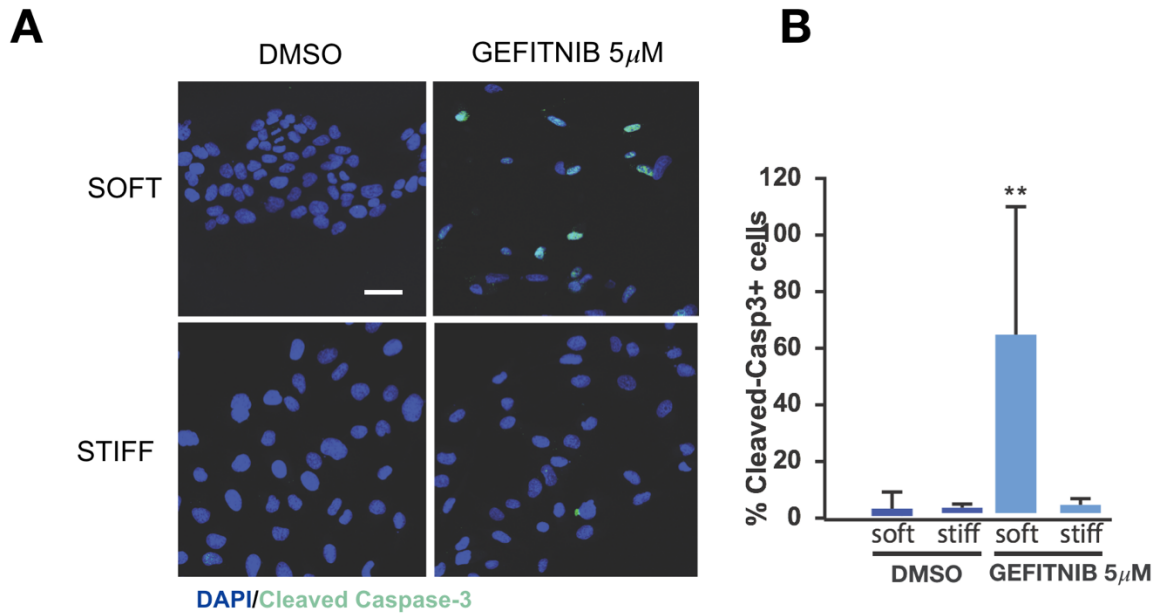


Figure 25. Stiff matrix protects SCC12 cells from cell death

*A) Representative confocal merged images of DAPI and cleaved caspase-3 staining in SCC12 cells plated 2 days on soft or stiff condition and then treated with 5 μ M of gefitinib for 48h. Scale bar, 50 μ m. B) Quantification of cleaved caspase-3 positive cells shown in A. (n=3, mean + SEM). Paired samples were compared by 2-tailed Student's t test (NS: not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).*

Intracellular signalling pathways are critical regulator of cell survival and thus, addition of RTKi, including gefitinib, are known to induce cell death in tumors (Cragg et al., 2007). To have a better insight into how SCC12 cells die in response to EGFR inhibition, we measured apoptosis activity in SCC12 cells following gefitinib treatment (**Figure 25A**). Number of cells undergoing apoptosis were assessed by the number of cells positive for cleaved caspase-3 staining. Caspase-3 is one of the key players in executing apoptosis and its activation is fully achieved once cleaved (Crowley and Waterhouse, 2016). As above, SCC12 cells were treated using gefitinib at 5 μ M final concentration, with substrate rigidity variation. After 48h of gefitinib treatment, SCC12 cells were fixed and subjected to a staining for the active form of the caspase 3 pro-apoptotic molecule. Quantification of caspase-3 positive cells revealed, in one hand, that stiff matrix provides an absolute protection to SCC12 cells from apoptosis and in the other hand, SCC12 cells plated on soft matrix were significantly (63%) more susceptible to cell death (**Figure 25B**). These data suggest an important role of stiffness in protecting cells from targeted therapy.

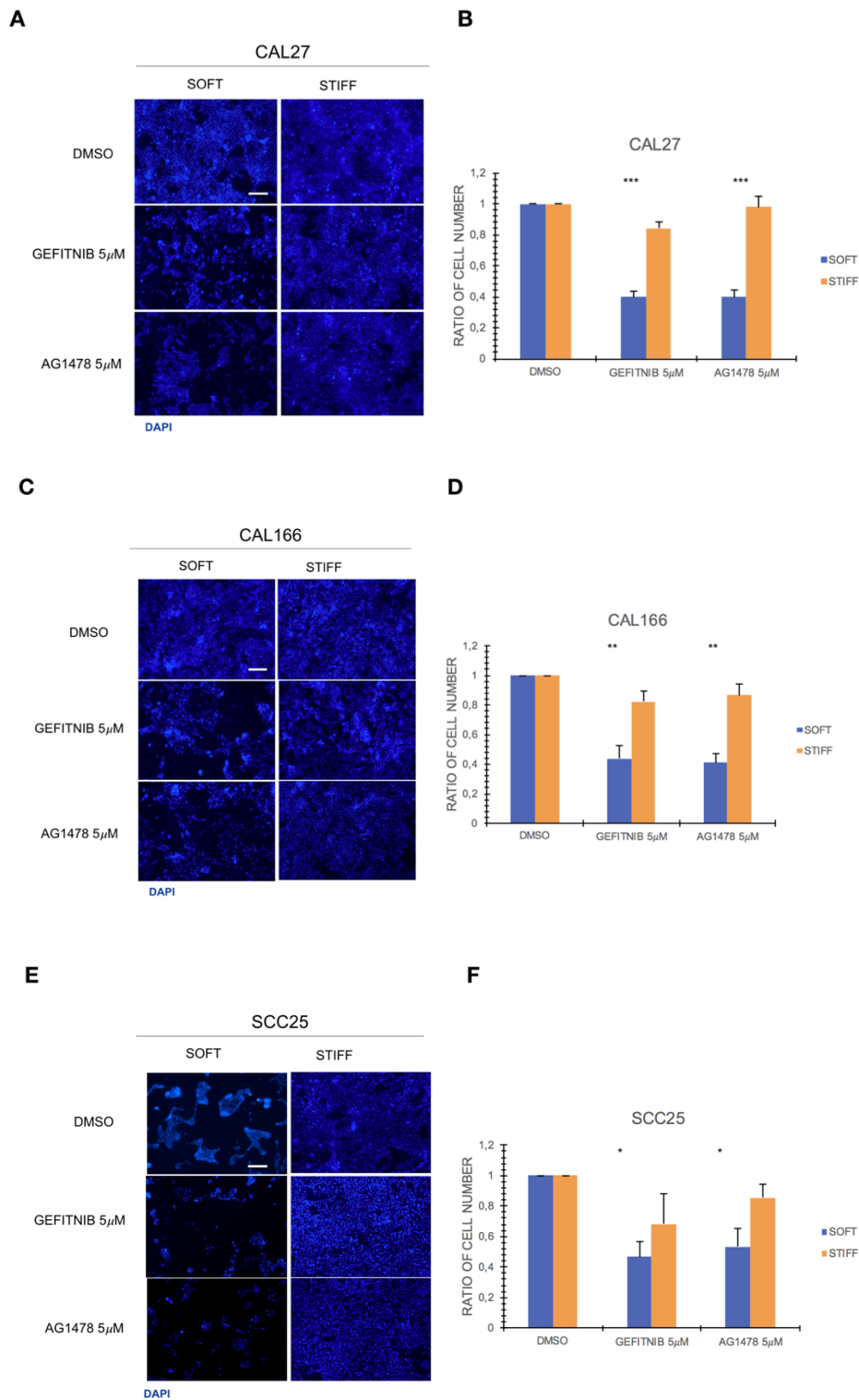


Figure 26. Stiffness mediates protection against TKIs gefitinib and AG1478 in CAL27, CAL166 and SCC25 cell lines

A) Representative DAPI staining pictures of CAL27 cultivated on a stiff and soft matrix for 48h, treated with 5 μ M of gefitinib and AG1478 for another 48h. Scale bar 200 μ m. B) Quantification of DAPI staining from experiment shown in A. (n=3, mean + SEM). C) Representative DAPI staining pictures of CAL166 cultivated on a stiff and soft matrix for 48h, treated with 5 μ M of gefitinib and AG1478 for another 48h. Scale bar 200 μ m. D) Quantification of DAPI staining from experiment shown in A. (n=3, mean + SEM). E) Representative DAPI staining pictures of SCC25 cultivated on a stiff and soft matrix for 48h, treated with 5 μ M of gefitinib and AG1478 for another 48h. Scale bar 200 μ m. F) Quantification of DAPI staining from experiment shown in A. (n=3, mean + SEM). Paired samples were compared by 2-tailed Student's t test (NS: not significant, * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001).

We next wondered if gefitinib also triggers cell death and if the protective effect of the matrix substrate rigidity is a general mechanism in human head and neck SCC cell lines. To answer this question, we performed a similar experimental design on a variety of HN-SCC cell lines isolated from patients' biopsies at the "Centre Antoine Laccasgne", anti-cancer centre in Nice, France. We tested our hypothesis using CAL27, CAL166 and SCC25 cell lines isolated from tongue. CAL27 (**Figure 26 A-B**), CAL166 (**Figure 26 C-D**), and SCC25 (**Figure 26 E-F**), were plated on soft and stiff matrix for 48h before we started the treatment with gefitinib and AG1478. As it is shown in **Figure 26**, all three cell lines were more resistant to treatment-induced cell toxicity when plated on the stiff substrate by 22 to 58% more compared to those plated on the soft matrix (**Figure 26 B, D and F**). Interestingly, SCC25 cells failed to reach the same growth rate when plated on soft compared to stiff. It has been shown that stiffness promotes proliferation and that when cells are plated on stiff substrate can reach up to 12.2-fold higher rate of proliferation, usually through regulation of β 1-integrin and FAK (Schrader et al., 2011). Therefore, SCC25 cells are possibly more dependant on stiffness than other cell lines are.

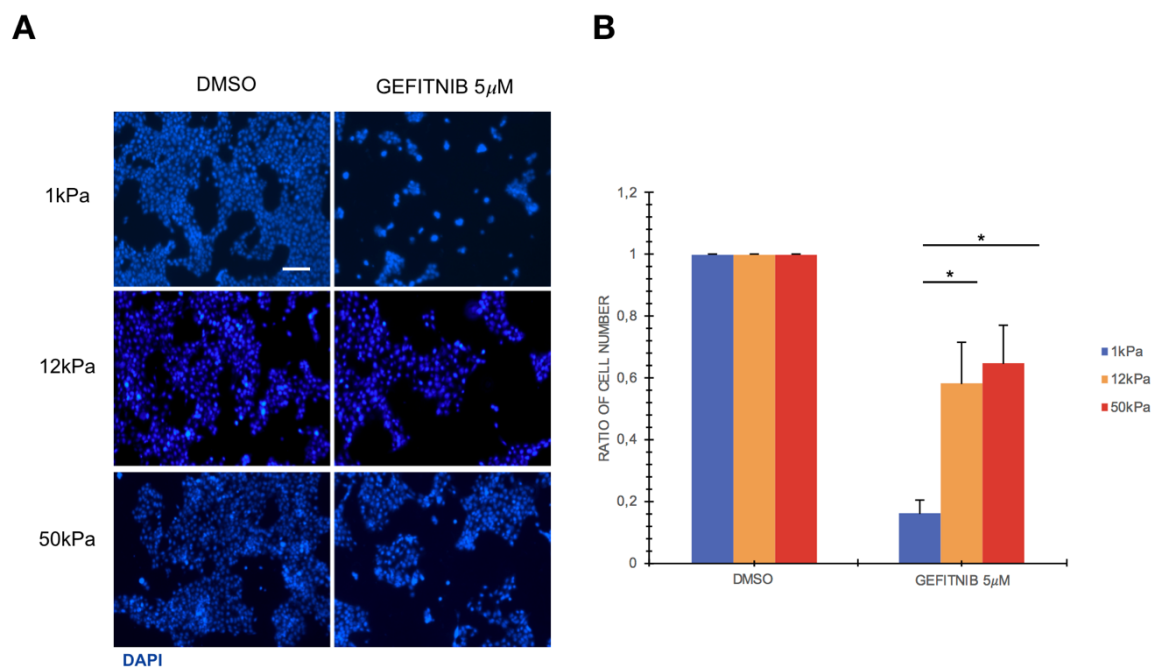


Figure 27. SSC12 cells plated on the 12 and 50kPa are more resistant to treatment with gefitinib

*A) Representative DAPI staining pictures of SSC12 cells cultivated on a 1kPa (soft) and 12 and 50kPa (stiff) collagen-I coated Matrigen Petrisoft dishes for 48h, treated with 5 μ M of gefitinib for another 48h. Scale bar 200 μ m. B) Quantification of DAPI staining from experiment shown in A. (n=3, mean + SEM). Paired samples were compared by 2-tailed Student's t test (NS: not significant, *p<0.05, **p<0.01, ***p<0.001, **** p<0.0001).*

In pancreatic cancer, tumor tissue is known to be 25 to 50 times more stiff than normal tissue (Rice et al., 2017a). To be precise, normal tissue can be about 1kPa of stiffness where cancer tissue can be more than 50kPa (Rice et al., 2017a). In our set up of in-house made soft and stiff matrix, difference in stiffness can be in GPa, so we decided to design experiments in more relevant pathophysiological conditions, similar to the one found in clinics. To do so, we have used Matrigen Petrisoft culture dishes of know rigidity values, 1kPa, 12kPa and 50kPa to mimic these conditions, previously coted with collagen-I. 1kPa represents value associated with normal tissue and 12kpa and 50kPa represent the rigidity found in tumors. Once again, we have plated SCC12 cells on soft (1kPa) and stiff (12kPa and 50kPa) to evaluate their response to gefitinib (**Figure 27A**). Cells plated on 1kPa Petrisoft dish were far more, up to 48%, susceptible to gefitinib-induced cell death than when plated on 12 or 50kPa(**Figure 27B**), indicating the importance of substrate rigidity in cell response to gefitinib. Here, we propose that tissue stiffness specifically plays a major role of the efficacy of anti-EGFR compounds, either TKIs or blocking antibody, to induce tumor cell toxicity.

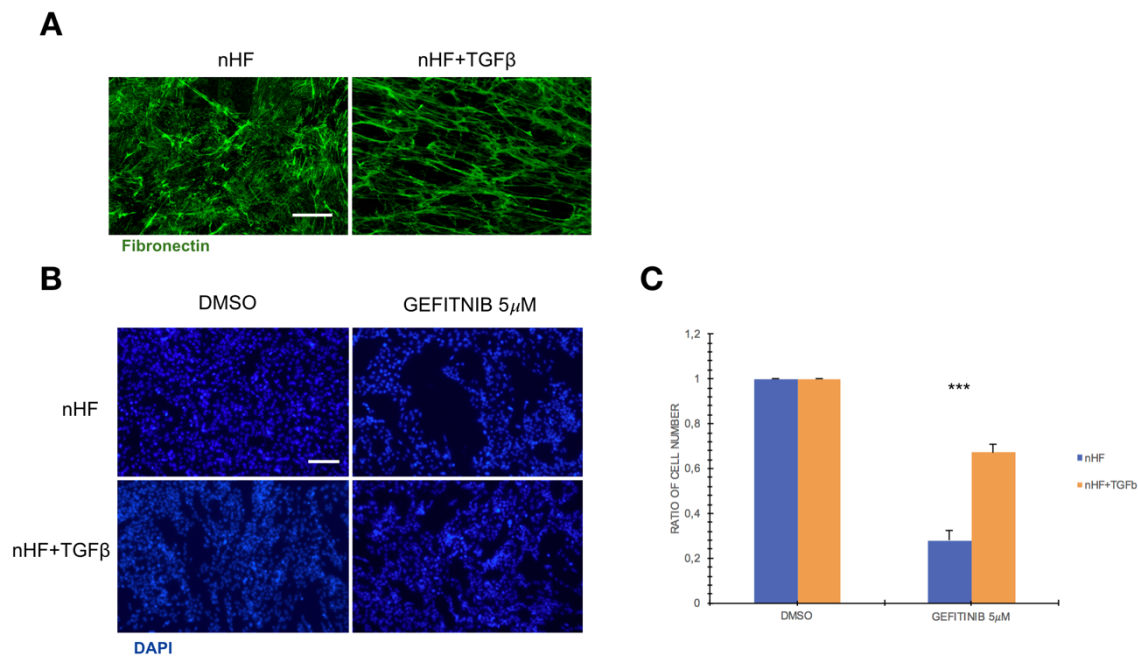


Figure 28. SCC12 cells plated on matrix produced by nHF+TGFβ are more resistant to treatment with gefitinib

*A) Confocal images of fibronectin staining of NHF or nHF+ TGFβ derived matrices. Scale bar 60 μm. B) Representative DAPI staining pictures of SCC12 cultivated on a stiff and soft matrix for 48h, treated with 5μM of gefitinib for another 48h. Scale bar 200μm. C) Quantification of DAPI staining from experiment shown in B. (n=5, mean + SEM). Paired samples were compared by 2-tailed Student's t test (NS: not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).*

We went one step further in a pursuit of an even more pathophysiological conditions, therefore we decided to investigate the role of fibroblasts-derived extracellular matrix in the protection of cell death triggered by EGFR-targeting TKI. Thus, we produced *in vitro* matrix from human fibroblasts (nHF) and compared it to the matrix produced by human fibroblasts activated by TGF β (nHF+ TGF β), in order to mimic a cancerous ECM. Matrix produced by fibroblasts represents the actual matrix that would be found *in vivo*. It has been established that upon *in vitro* stimulation of nHF with TGF β their phenotype becomes CAF-like (Bonan et al., 2016). Matrix produced by CAFs is know to be stiffer when compared to the one produced by fibroblasts (Grasset et al., 2018; Kharraishvili et al., 2014). Staining of fibronectin fibres within matrix produced by nHF are not organised similarly to those in matrix deposited by nHF + TGF β (**Figure 28A**) that are well describe to be aligned and dense (Goetz et al., 2011; Grasset et al., 2018). Our data demonstrates that SCC12 cells plated on a derived matrix made by *in vitro* TGF β activated fibroblasts are 39% more resistant to gefitinib-induced cell death, when compared to SCC12 cells plated on derived matrix made by control fibroblasts (**Figure 28B-C**).

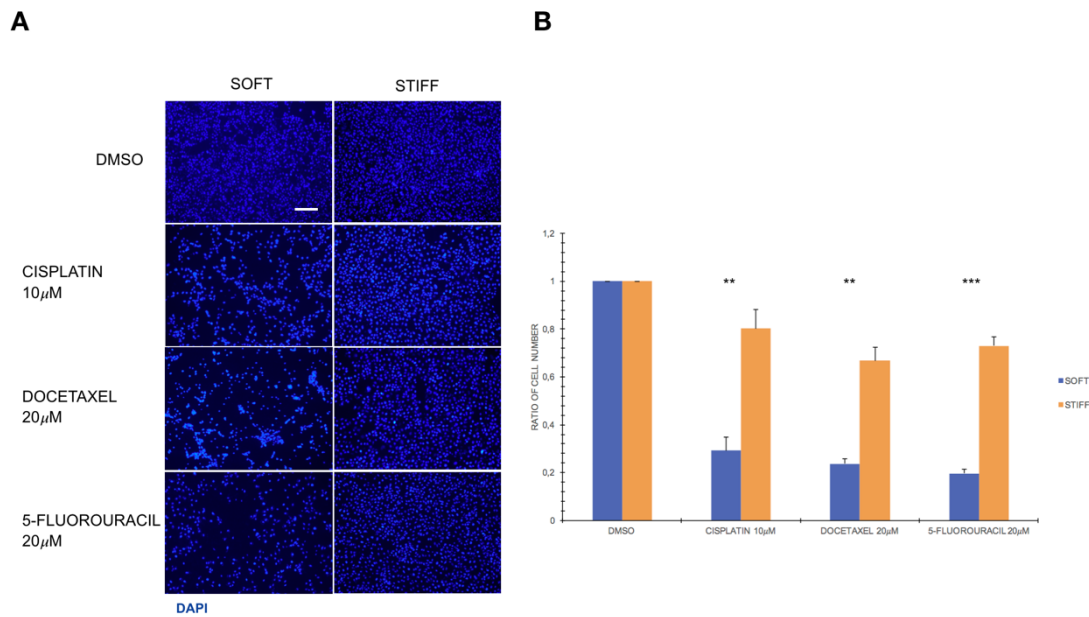


Figure 29. *SSC12 cells plated on the stiff matrix are more resistant to treatment with traditional chemotherapeutic agents, cisplatin, docetaxel and 5-fluorouracil*

*A) Representative DAPI staining pictures of SCC12 cultivated on a stiff and soft matrix for 48h, treated with 10 μ M of cisplatin, 20 μ M of docetaxel and 20 μ M 5-fluorouracil for 48h. Scale bar 200 μ m. B) Quantification of DAPI staining from experiment shown in A. (n=3, mean + SEM). Paired samples were compared by 2-tailed Student's t test (NS: not significant, * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001).*

In HNSCCs, common treatment of patients includes traditional chemotherapeutics such as cisplatin, docetaxel and 5-fluorouracil, however despite the initial progress patients often develop resistance and are submitted to a combination of two or more of these agents leading to an increase in toxicity. We hypothesised that, the reason behind the resistance of HNSCCs tumors to traditional chemotherapy is an increase in tumor rigidity as well. To test that hypothesis, we cultured SCC12 cells on soft and stiff matrix for 48h and then treated them with cisplatin (final concentration of 10 μ M), docetaxel (final concentration of 20 μ M) and 5-fluorouracil (final concentration of 20 μ M). We show that all three chemotherapeutic agents cause a decrease in SCC12 cell number when plated on soft matrix, while SCC12 cells plated on stiff matrix resist the treatment (**Figure 29A**). Cell number was quantified by cell count of positive DAPI staining using Image J. Cisplatin induced 71% reduction of cell number when plated on soft compared to only 20% reduction when plated on stiff. Docetaxel reduced SCC12 cell number by 77% when plated on soft and by 34% when plated on stiff. Lastly, 5-fluorouracil similarly to docetaxel decreased SCC12 cell number by 81% when they were plated on soft substrate, and only a 27% decrease of cell number was observed in SCC12 cells plated on stiff (**Figure 29B**).

Therefore, we conclude that matrix rigidity protects SCC12 cells from treatment with cisplatin, docetaxel and 5-fluorouracil.

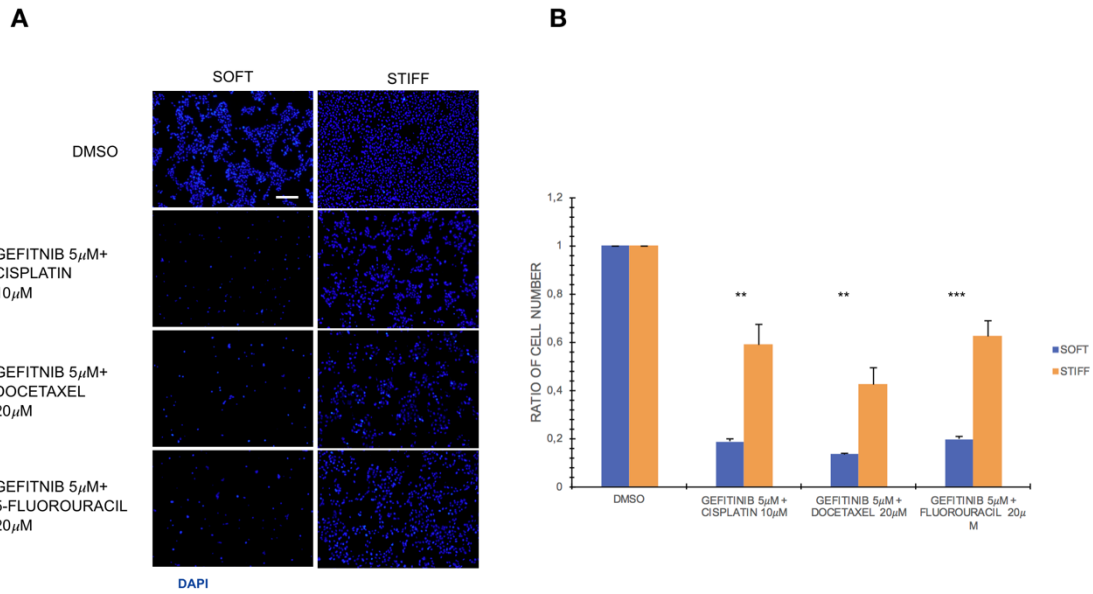


Figure 30. SCC12 cells plated on the stiff matrix are more resistant to treatment with traditional chemotherapeutic agents, cisplatin, docetaxel and 5-fluorouracil in combination with gefitinib

A) Representative DAPI staining pictures of SCC12 cultivated on a stiff and soft matrix for 48h, treated with 10 μ M of cisplatin, 20 μ M of docetaxel and 20 μ M 5-fluorouracil in combination with 5 μ M of gefitinib for 48h. Scale bar 200 μ m. B) Quantification of DAPI staining from experiment shown in A. (n=3, mean + SEM). Paired samples were compared by 2-tailed Student's t test (NS: not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

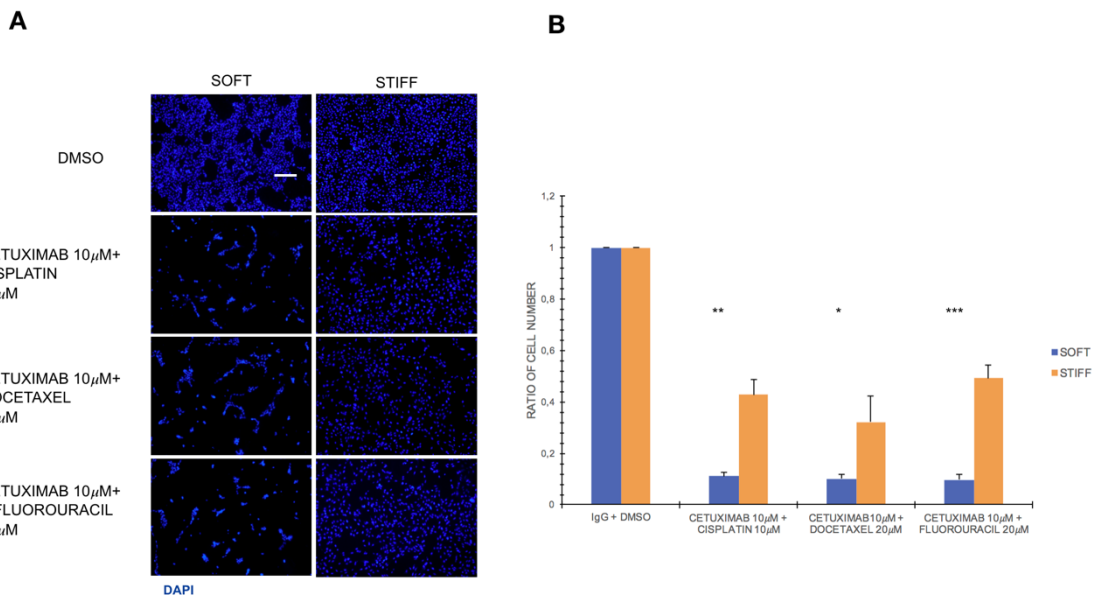


Figure 31. SCC12 cells plated on the stiff matrix are more resistant to treatment with traditional chemotherapeutic agents, cisplatin, docetaxel and 5-fluorouracil in combination with cetuximab

A) Representative DAPI staining pictures of SCC12 cultivated on a stiff and soft matrix for 48h, treated with 10 μ M of cisplatin, 20 μ M of docetaxel and 20 μ M 5-fluorouracil in combination with 10 μ M of cetuximab for 48h. Scale bar 200 μ m. B) Quantification of DAPI staining from experiment shown in A. (n=3, mean + SEM). Paired samples were compared by 2-tailed Student's t test (NS: not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Next step in our approach was to test, using same experiment, will matrix rigidity promote resistance to combinational therapy of EGFR-targeted agents gefitinib and cetuximab with traditional agents, cisplatin, docetaxel and 5-flourouracil. This approach in treating HNSCCs has been adapted but very little efficacy was shown in patient, therefore we argue that the treatment failure is strongly associated with the tumor stiffness. To test our belief, we first plated SCC12 cells on soft and deformable collagen I rich matrix or on a collagen I rich stiff matrix for 48h followed by a 48h treatment of gefitinib in pair with either cisplatin, docetaxel and 5-flourouracil. DAPI staining demonstrates that SCC12 cells plated on soft substrate, when treated with gefitinib in combination with any of the three traditional chemotherapies, are sensitive to the treatment and do not survive it. However, SCC12 cells plated on stiff substrate resist the combinational treatment (**Figure 30.**) Quantification of positive DAPI staining gave us a precise percentage of cells that survived the treatment on stiff matrix compared to cells plated on soft matrix. For a combination of gefitinib and cisplatin treatment, 58% cells plated on stiff survived the treatment while only 18% of cells plated on soft survived. Gefitinib and docetaxel treatment allowed 42% of cells plated on stiff matrix to survive the treatment and only 14% of cells plated on soft matrix survived. Lastly, combination of gefitinib and 5-flourouracil led to a survival of 63% SCC12 cells plated on stiff and merely 19% of SCC12 cells survived the treatment when plated on soft (**Figure 30.**)

Next, we submitted SCC12 cells, that were plated on soft and stiff for 48h, to a treatment with monoclonal antibody cetuximab in combination with cisplatin, docetaxel and 5-flourouracil. After 48h of combinational treatment experiment was analysed and positive DAPI staining was quantified. As expected, SCC12 cells plated on stiff matrix were once again more resistant to the treatment (**Figure 31A**). In more details, when SCC12 cells were treated with cetuximab in combination with cisplatin, 31% cells more survived the treatment when plated on stiff matrix. Survival of SCC12 cells upon cetuximab treatment in combination with docetaxel increased for 22% when cells were plated on stiff substrate. Combination of cetuximab and 5-flourouracil led to the 49% more cell surviving the treatment when plated on stiff compared to soft (**Figure 31B**).

Taken together, these experiments demonstrate that regardless of the mechanism of action of various therapies used in HNSCCs and their combinations, rigidity of the substrate significantly influences the overall SCC12 cell survival.

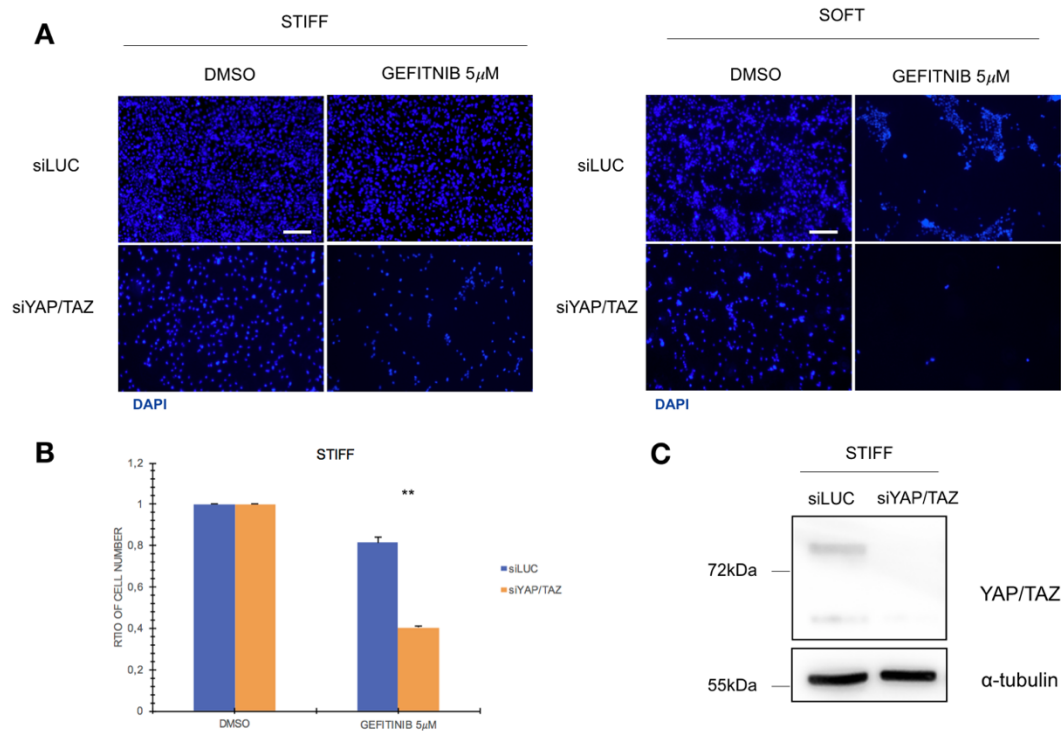


Figure 32. YAP/TAZ knock down in SCC12 cells leads to an increase in sensitivity to gefitinib when plated on stiff matrix

A) Representative DAPI staining pictures of SCC12 transfected either by siLUC as a control or siYAP/TAZ and cultivated on a stiff and soft matrix for 48h before treatment with 5 μ M gefitinib. Scale bar 200 μ m B) Quantification of DAPI staining from experiment shown in A. (n=3, mean + SEM). C) Immunoblot of YAP and TAZ in SCC12 cells corresponding to the assay presented in A. Tubulin used as control. Paired samples were compared by 2-tailed Student's t test (NS: not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Having established that matrix stiffness promotes resistance to common EGFR inhibitors-induced cell death, we further investigated the role of the stiffness-dependent intracellular signalling pathway, regulated by the co-transcription factors YAP and TAZ in SCC12 cells. To do so, we knocked out a know mediators of mechanical stressed induced by microenvironment; YAP/TAZ. Our hypothesis was that, if the protection is stiffness meditated, downregulation of YAP/TAZ transcriptional factors should override the protection and make cells plated on stiff susceptible to EGFR inhibition-induced cell toxicity. we transfected SCC12 cells with siRNA against YAP and TAZ to knock out their expression and plated the SCC12 cells (Figure 32C) on either a soft and a stiff matrix for 48h. Next, cells were treated using the gefitinib compound and cell survival was assessed 48h later by quantification of DAPI positive cells (Figure 32A). we show that cells in which YAP and TAZ were knock out, cells were 41% more sensitive to gefitinib treatment compared to those transfected with an siRNA control (siLuc) (Figure 32B).

All together, my data show that the efficacy of EGFR targeted therapies to induce cell death is subjected to the rigidity of the cell substrata and suggest that the efficacy for patient treatment may vary depending on the fibrotic condition found on the tumor.

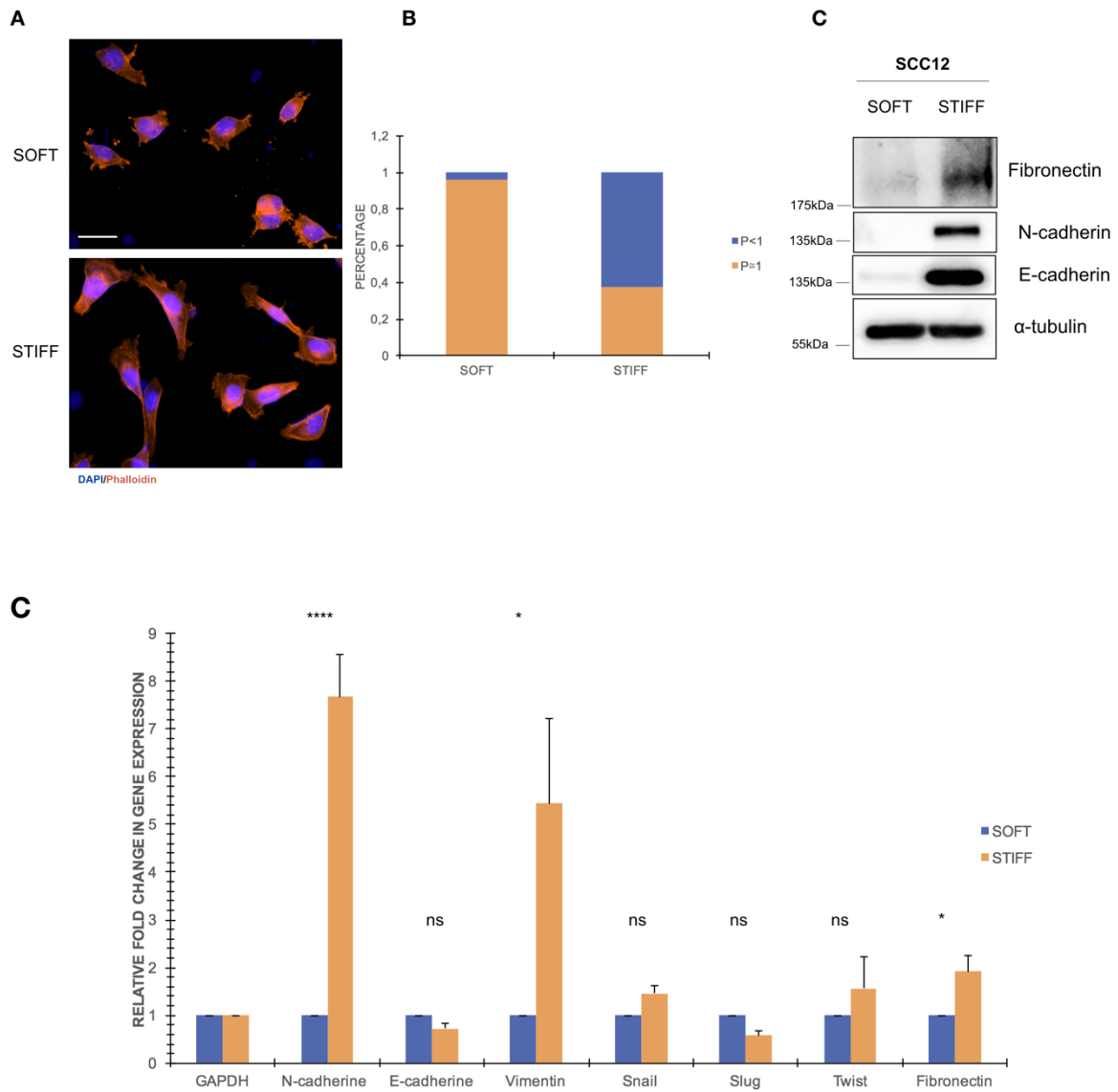


Figure 33. SCC12 cells in stiff condition display partial EMT

A) Confocal images of DAPI and Phalloidin staining of SCC12 cells plated on soft and stiff matrix. Scale bar 40 μ M. B) Quantification of cell elongation from experiment shown in A. Elongation of cells was quantified using ImageJ. Cells with a value of 1 or close to 1 are considered round ($P \approx 1$), while if the value is smaller than 1 cells are considered elongated ($P < 1$). ($n = 30$ cells at least, representative experiment of 3 independent experiments). C) RT-qPCR analysis of SCC12 cell. Gene expression was normalized relative to the expression of GAPDH and cells plated on soft matrix. Data are represented as mean \pm SEM ($n = 6$). Paired samples were compared by 2-tailed Student's *t* test (NS: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

3.2. WHAT MAKES CELLS on STIFF MATRIX RESISTANT?

Having established that stiffness promotes resistance to EGFR targeted therapy in SCC12 cells. We next aimed at understanding the molecular mechanisms of it. Identification of the mechanism behind stiffness driven resistance in HNSCCs patients would provide valuable insights that could potentially lead to implementation of novel therapeutic strategies.

During cells handling and experiments we have noticed that SCC12 cells plated on stiff substrate are becoming more elongated in shape after 48h in comparison to cells plated on soft. This observation led us to hypothesis that SCC12 cells, in response to increased matrix rigidity are undergoing an EMT and that this process may be responsible for the resistance of cells to anti-EGFR targeted therapy. Recently, EMT has been strongly associated with resistance of cancer cell to anti-cancer therapies (Rice et al., 2017a; Wang et al., 2016b).

To verify that the observation of the changes in cells morphology are significant and are indeed an indicator of EMT, we first quantified the elongation of cells. To do so, we phalloidin stained SCC12 cells, to reveal their actin cytoskeleton architecture, that were plated on soft and stiff matrix for 48h (**Figure 33A**). Using the perimeter of each cell we calculated the surface of a perfect circle that perimeter would make, if the ration of the actual surfaces measured by ImageJ and an ideal surface was one or close to one, that cell was considered round. However, if the ratio was lower than one, we considered cell elongated. As seen in **Figure 33B**, we show that SCC12 cells plated on stiff became elongated. Indeed, 96% of cells plated on a soft substrate had a score of one or close to one demonstrating their roundness shape and only 4% were considered elongated, while cells plated on a stiff matrix were round in 38% of the cases, and elongated in 62%. Taking this quantification in consideration, we propose that SCC12 cells undergo EMT when plated on a stiff substrate rich in collagen I. To further characterise the possible induction of EMT by the matrix rigidity in SCC12 cells, we used Western blot to detect the expression of known biomarkers of EMT such as fibronectin and N-cadherin in SCC12 cells that were plated either on soft or stiff matrix. Blot shows that both fibronectin and N-cadherin are drastically overexpressed in SCC12 cells plated on a stiff matrix, when compared to SCC12 cells plated on a soft matrix. Besides fibronectin and N-cadherin, we immunoblotted for E-cadherin as well, an adhesion molecule expressed in epithelial cells and describe to be decreased in expression during EMT. We expected to have a decrease of epithelial marker in cells plated on stiff matrix due to the EMT, however this was not the case. Interestingly, E-cadherin is also upregulated in cells plated on stiff condition (**Figure 33C**). Together, acquisition of mesenchymal markers, without lost of the epithelial marker E-cadherin in SCC12 cells, suggest that SCC12 cells undergo a partial EMT in response to matrix rigidity. Following Western blot, we measured the expression of genes at the mRNA level, therefore we

performed RT-qPCR using primers for N-cadherine, E-cadherine, vimentin, Snail, Slug, Twist and fibronectin. Again, EMT markers N-cadherine, fibronectin and vimentin were significantly upregulated in cells plated on stiff compared to soft substrates. Change in N-cadherine expression was more than 7 fold, change in vimentin expression increased 5 folds and fibronectins' 2 folds. We also measured the mRNA expression of Snail, Slug and Twist, EMT transcriptional factors. Their mRNA expression did not significantly change, but we do observe a slight, 1.5. fold change increase of Snail and Twist. Lastly, mRNA levels of E-cadherin did not change (**Figure 33D**), despite an overexpression at a protein level suggesting a different mechanism of activation or posttranslational modifications. Overall, these analyses suggest that SCC12 cells plated on a collagen with stiff matrix undergo a partial EMT, while when plated on soft substrate maintain their epithelial shape and phenotype.

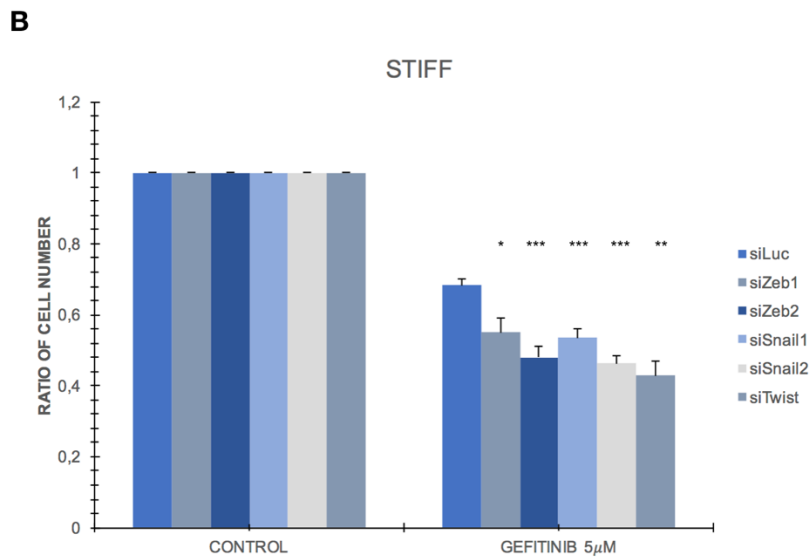
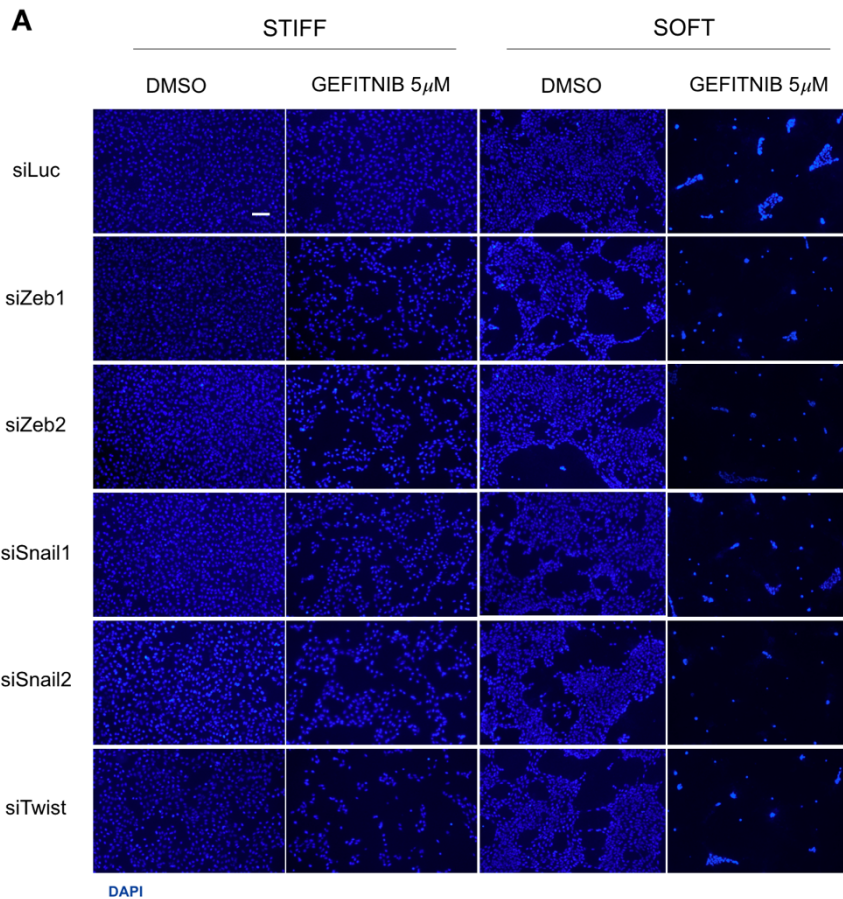


Figure 34. Knock out of Zeb1, Zeb2, Snai1, Snai2 and Twist in SCC12 cells leads to an increase in sensitivity to gefitinib when plated on stiff matrix

A) Representative DAPI staining pictures of SCC12 transfected either by siLuc as a control or siZeb1, siZeb2, siSnail1, siSnail2, siTwist and cultivated on a stiff and soft matrix followed by a 48h treatment with 5 μ M gefitinib. Scale bar 200 μ m B) Quantification of DAPI staining from experiment shown in A. (n=4, mean + SEM). Data are represented as mean + SEM (n=6). Paired samples were compared by 2-tailed Student's t test (NS: not significant, * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001).

Our data suggest that a partial EMT is ongoing in SCC12 cells plated on a stiff matrix, therefore, we wanted to study if the downregulation of the partial EMT would promote a SCC12 susceptible to EGFR inhibition-induced cell death, using the gefitinib compound, even in presence of a stiff matrix. We speculated that the knock out of master regulators of EMT, the transcriptional factors Zeb1, Zeb2, Snail1, Snail2 and Twist, would lead to the blockade of this EMT induction (Zhang et al., 2018b). To do so, SCC12 cells were transfected with siRNA against Zeb1, Zeb2, Snai1, Snai2 and Twist to knock out their expression and plated them on soft and stiff matrices for 48h. After cells got accustomed to the environment, we treated them with the gefitinib compound for another 48h and quantified DAPI positive cells to evaluate cell survival. In **Figure 34A** we show that cells plated on a stiff matrix in which Zeb1, Zeb2, Snai1, Snai2 and Twist are knocked out are more responsive to gefitinib treatment compared to those transfected with an siRNA control (siLuc). In particular, knock out of these transcriptional factors led to 15 to 30% decrease in cell number compared to control in stiff condition. In control (siLuc), 70% of cells plated on stiff substrate survive a 48h treatment of gefitinib, while when Zeb1, Zeb2, Snail1, Snail 2 and Twist are knocked out, 55%, 47%, 53%, 46% and 42% of cells survive the treatment, respectively. Greatest impact on overall cell survival was observed in SCC12 cells when Twist was knocked out (**Figure 34B**). These results suggest that EMT plays a role in resistance of SCC12 to EGFR-targeted therapies and that signaling mechanism behind EMT could at least, partially promote the resistance. Although 28% decrease in cell survival is a relevant result, 40% of cells are still surviving the treatment which is sufficient for cancer to progress. We rationalised that transfecting SCC12 cells with a combination of two or more siRNAs against Zeb1, Zeb2, Snail1, Snail 2 and Twist will have a higher impact on survival of SCC12 cells plated on stiff matrix. However, even the combination of all siRNAs did not yield a more relevant decrease of cell numbers upon 48h of gefitinib treatment compared to cells transfected with individual siRNAs (data not shown).

Overall, these data led us to speculate that EMT is a consequence of an upstream changes in molecular mechanisms behind EGFR-targeted therapies resistance in HNSCCs.

3.3. RNA sequencing

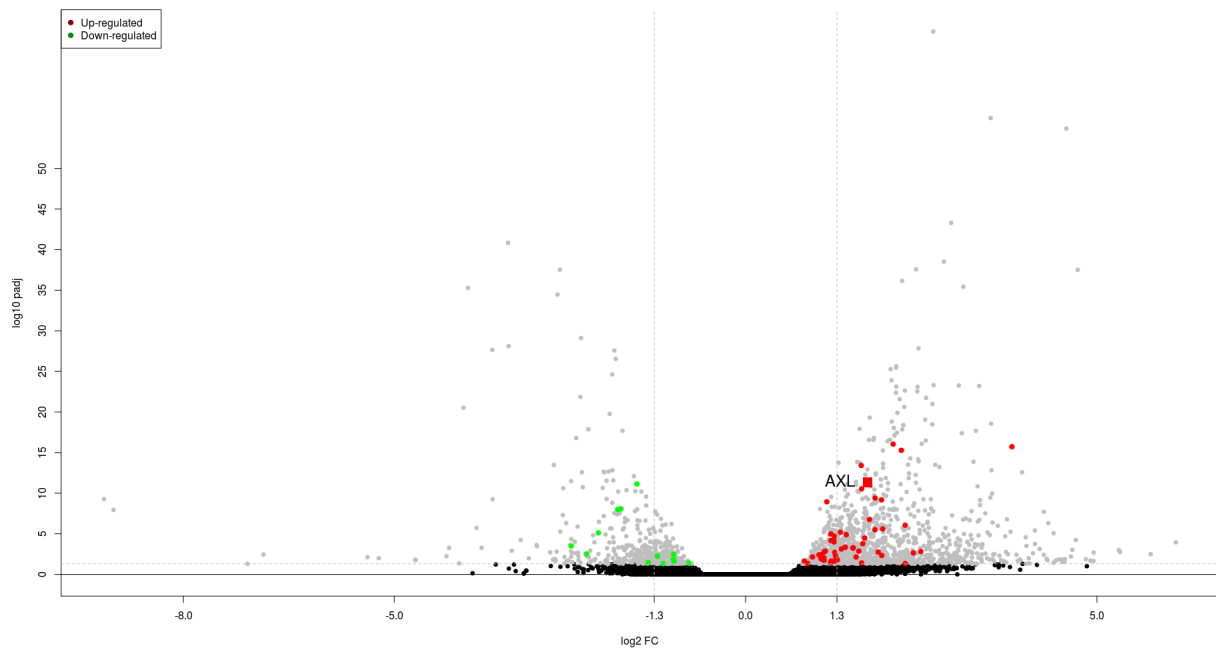


Figure 35. RNA sequencing revealed an upregulation of RTK *AXL*.

Volcano plot showing downregulated and upregulated RTKs in SCC12 cells plated on 50kPa stiff matrix compared to SCC12 plated on 1kPa stiff matrix. Downregulated RTKs are shown in green, and upregulated ones in red. ($n=4$)

To better understand why SCC12 cells plated on stiff matrix display such a strong resistance to EGFR-targeted therapy as well as traditional chemotherapy compared to SCC12 cells plated on soft matrix, we performed RNA sequencing of SCC12 cells plated on both substrates. SCC12 cells were grown in four different experiments on 1kPa (soft) and 50kPa (stiff) Petrisoft dishes to mimic normal and tumor tissue respectively. After 48h, total RNA were isolated as described in materials and methods, RNA transcriptome was enriched by ribosomal RNA depletion and quadruplicates were sent for sequencing at UCAGenomiX - IPMC platform (Nice).

After the raw data was processed using bioinformatics methods by the same platform, a strong upregulation of numerous RTK was observed in SCC12 cells plated on 50kPa. Among differently regulated 72 RTK either by downregulation or upregulation, vast majority of them was upregulated as seen in **Figure 35**. One of the most interesting RTKs that was upregulated in SCC12 cells plated on 50kPa was *AXL*. Reason why we found it interesting is that, in literature, *AXL* was reported to be an important player in resistance of various types of cancers to targeted therapy (Antony and Huang, 2017; Antony et al., 2016; Brand et al., 2015; Zhang et al., 2012). Besides that, *AXL* has been known to regulate EMT in cancer progression (Antony and Huang, 2017; Asiedu et al., 2014). Taking all that together, we though there are reasons to assume that *AXL* is a key regulator of matrix rigidity driven resistance to EGFR-targeted therapy.

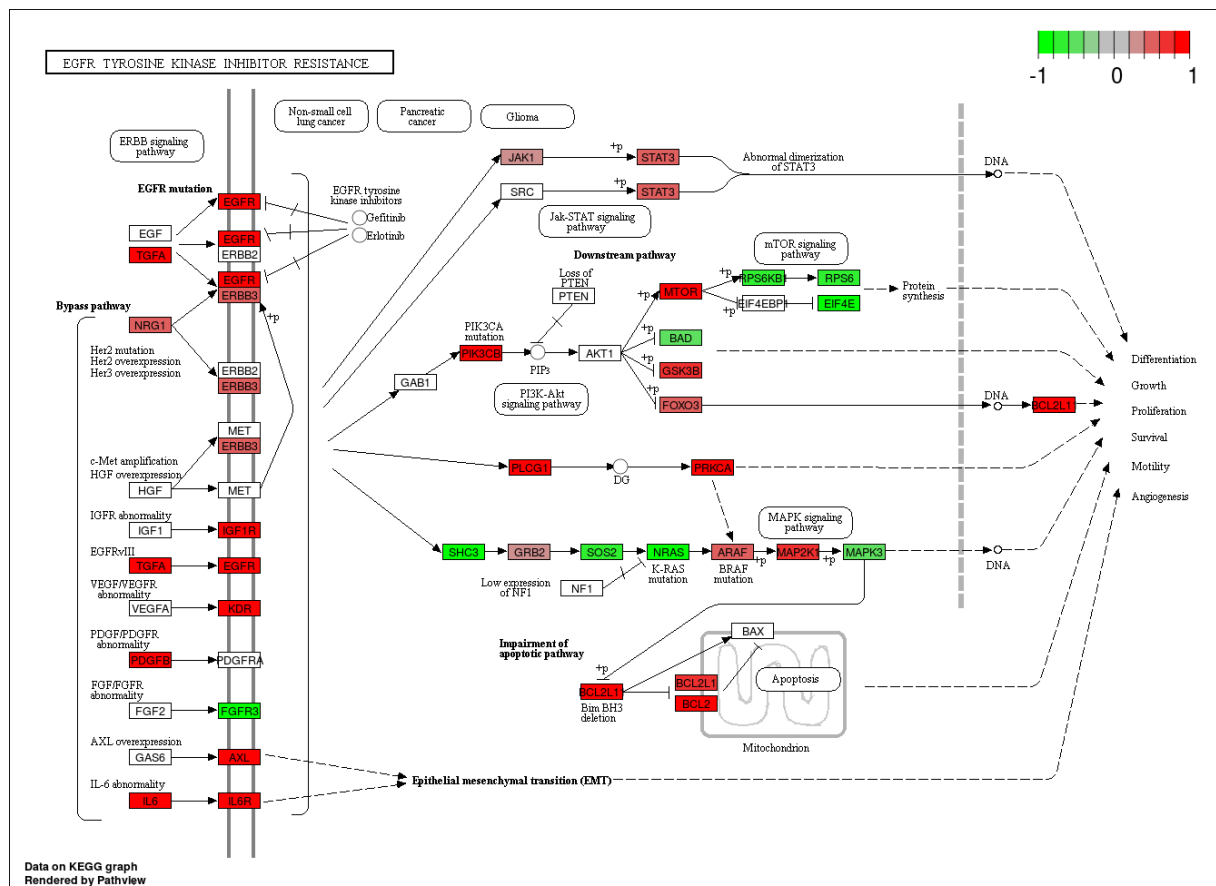


Figure 36. EGFR Tyrosine Kinase inhibitor resistance pathway

Pathview tool representation of EGFR inhibitors gefitinib and erlotinib resistance pathways and genes that are upregulated (red) and downregulated (green) RTKs in SCC12 cells plated on 50kPa Petrisoft dishes compared to SCC12 plated on 1kPa Petrisoft dishes. Grade of downregulation or Upregulation of genes is defined by a -1 to 1 scale.

Further analysis of the RNA sequencing data using Pathview tool, a tool set that allows mapping of a wide range of data on relevant pathways graphs, placed *AXL* on the top of a signaling pathway responsible for EMT that ultimately leads to motility, differentiation, growth, proliferation, angiogenesis and survival (Figure 36). Interestingly, *Gas6*, an *AXL* ligand, was not shown to be overexpressed in the RNA sequencing analysis. However, other known downstream signaling targets of *AXL* such as *PIK3C*, *MAP2K*, *BCL2* and *STAT* are overexpressed in SCC12 cells plated on 50kPa suggesting an activation of *AXL* kinase and its downstream targets. Besides that, RTKs *EGFR* and its ligand *TGFA*, and *IGF1R* are also overexpressed in SCC12 cells plated on stiff substrate. Graph shown in Figure 36, highlights all the genes known to be implicated in the EGFR-TKI resistance that are upregulated and downregulated in the RNA sequencing of SCC12 cells plated on 50kPa compared to SCC12 cells plated on 1kPa substrate.

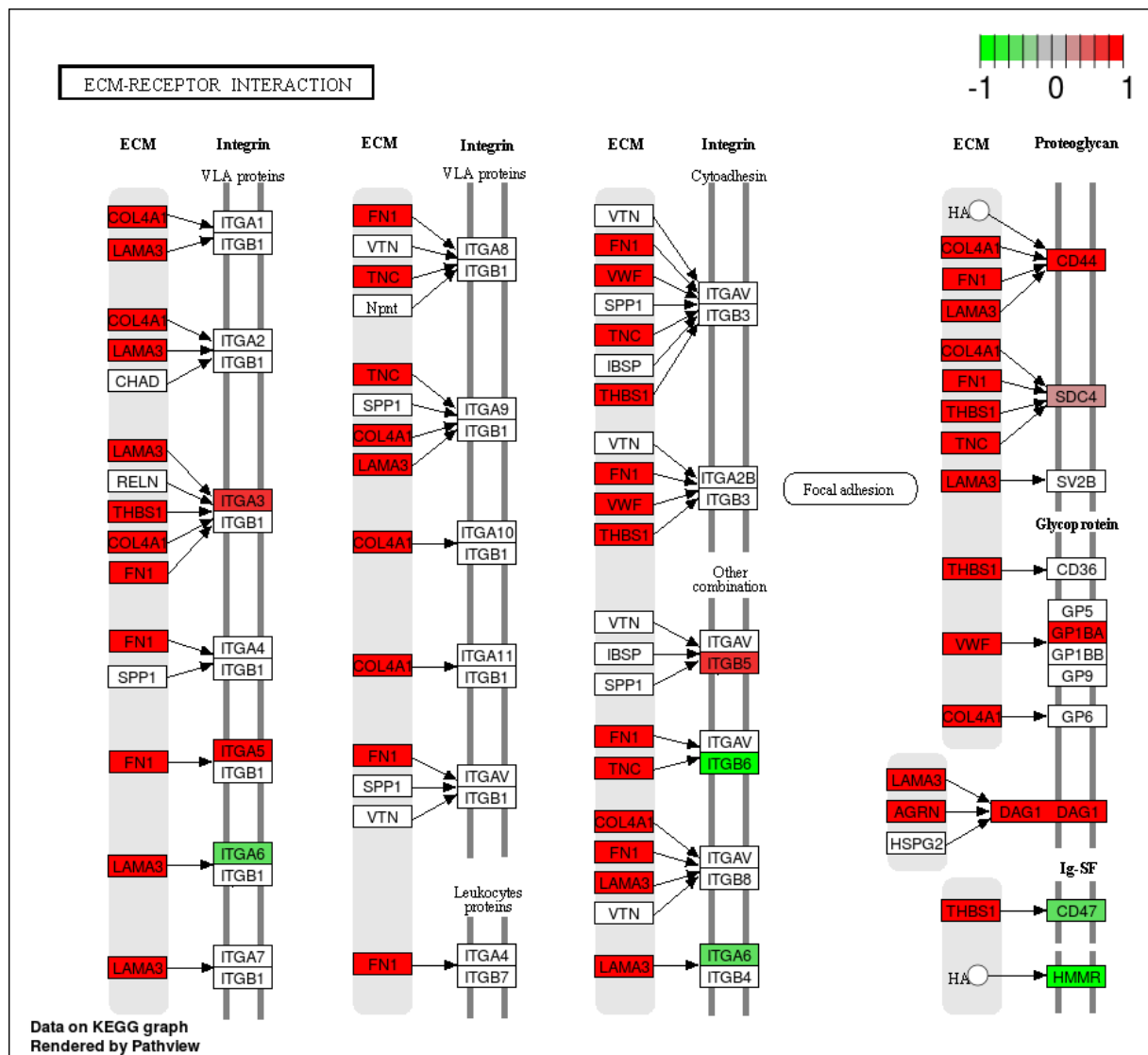


Figure 37. ECM-receptor interactions

Pathview tool representation of ECM proteins and their interaction with integrin receptors. Genes that are upregulated (red) and downregulated (green) in SCC12 cells plated on 50kPa Petrisoft dishes compared to SCC12 plated on 1kPa Petrisoft dishes are labelled in the graph. Grade of downregulation or Upregulation of genes is defined by a -1 to 1 scale.

Besides various RTK, what was interesting is that the majority of genes coding for ECM proteins were upregulated, notably fibronectin, tenascin-C, vimentin and collagen IV (**Figure 37**). Collagen IV is a founding member of the basement membrane, while fibronectin, tenascin-C and vimentin are all implicated in the transition of epithelial cells to mesenchymal one. Therefore, RNA sequencing confirmed the data we have obtained previously that describes a mesenchymal phenotypical switch that SCC12 cells go while plated on stiff matrices (**Figure 33**),

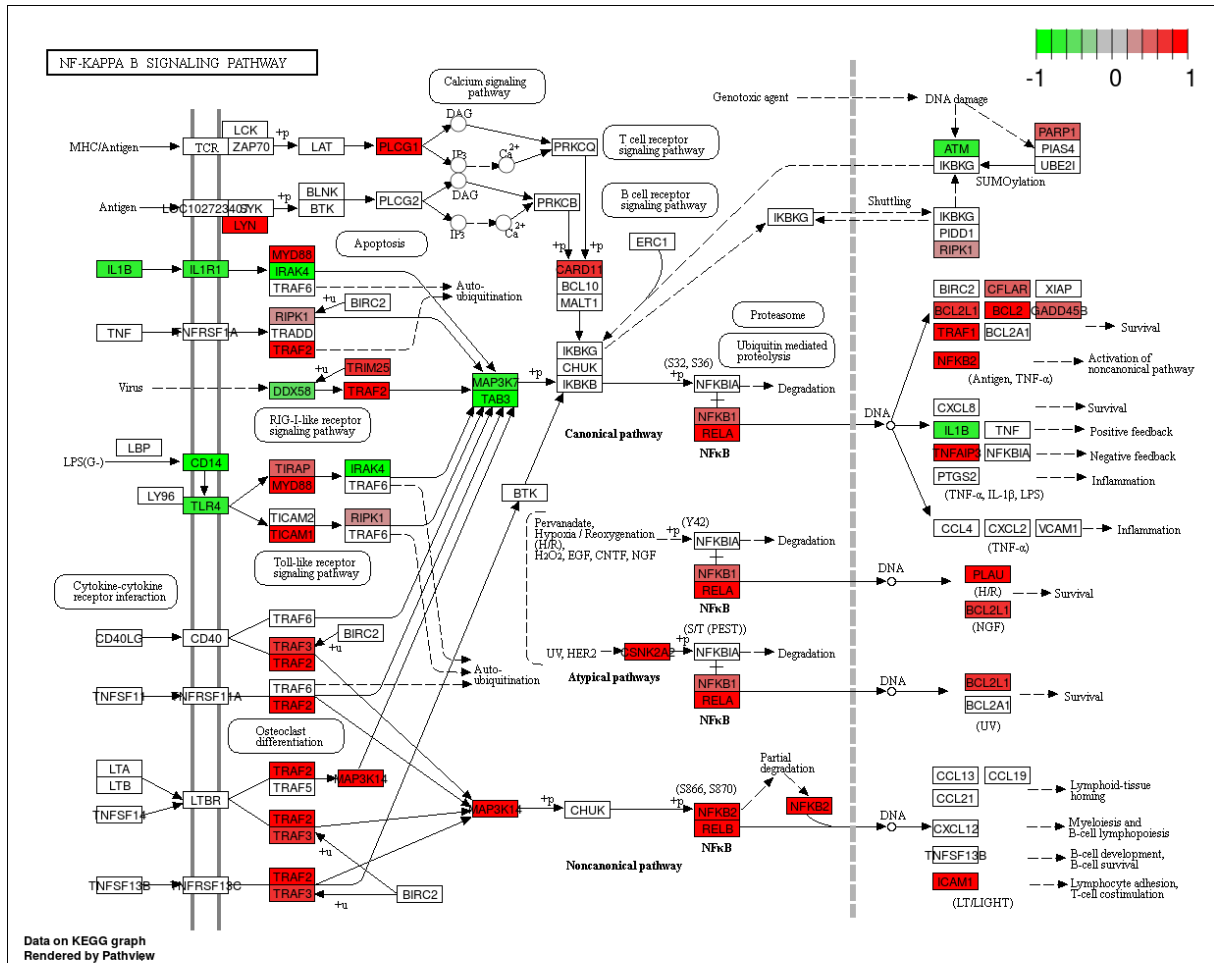


Figure 38. NF-KAPPA B signaling pathway

Pathview tool representation of NFκB signaling pathway responsible for the inflammation reaction in cells. All members of the pathway are labelled in the graphic representation, while genes that were found upregulated by the sequencing of SCC12 cells plated on stiff matrix compared to cells plated on soft matrix are portrayed in red, and downregulated genes in green. Their interactions are indicated with arrows, as well as the outcomes of the interactions. Grade of downregulation or upregulation of genes is defined by a -1 to 1 scale.

Moreover, RNA sequencing revealed an upregulation of genes coding for members of NF-κB transcriptional factors family *sig* in SCC12 cells plated on stiff matrix in comparison to soft (**Figure 38**). As mentioned in the introduction, NF-κB signaling pathway has been described to play a massive role in inflammation, favouring cancer progression and survival. It is as well important to emphasise that AXL is known regulator of NF-κB (Paccez et al., 2015). Besides NF-κB family members, *TNFAIP3* gene coding for A20 protein is found to be upregulated in SCC12 cells plated on 50kPa stiff matrix. A20 is as well known to inhibit and regulate canonical NF-κB activation and also itself is a NF-κB dependant gene (Coornaert et al., 2009). Importantly, it has been shown to monoubiquitylate Snail1 and facilitate TGFβ1-induced EMT (Lee et al., 2017) and it has been involved in anti- and pro-apoptotic functions making it an interesting target in overcoming chemoresistance (Bredel et al., 2006; Chen et al., 2015; da Silva et al., 2014). *ICAM1*, gene coding for Intracellular adhesion molecule (ICAM)1, a

transmembrane glycoprotein of the immunoglobulin (Ig)-like superfamily is as well upregulated in SCC12 cells when plated on stiff substrate compared to SCC12 cells plated on soft substrate. Generally, ICAM-1 is induced upon inflammation by cytokines Interleukin (IL)-1 and TNF- α and it is involved in immune response that requires intercellular contact and collaboration (Benedicto et al., 2017). It is used in research as a marker of inflammation (Raguz et al. 2016). These RNA sequencing results indicate that a very strong induction of inflammation is occurring in SCC12 cells in response to stiff substrate. Indeed, it has been shown that ECM rigidity promotes inflammation and activation of inflammatory pathways, notably NF- κ B (Johnson et al., 2013; Li et al., 2010).

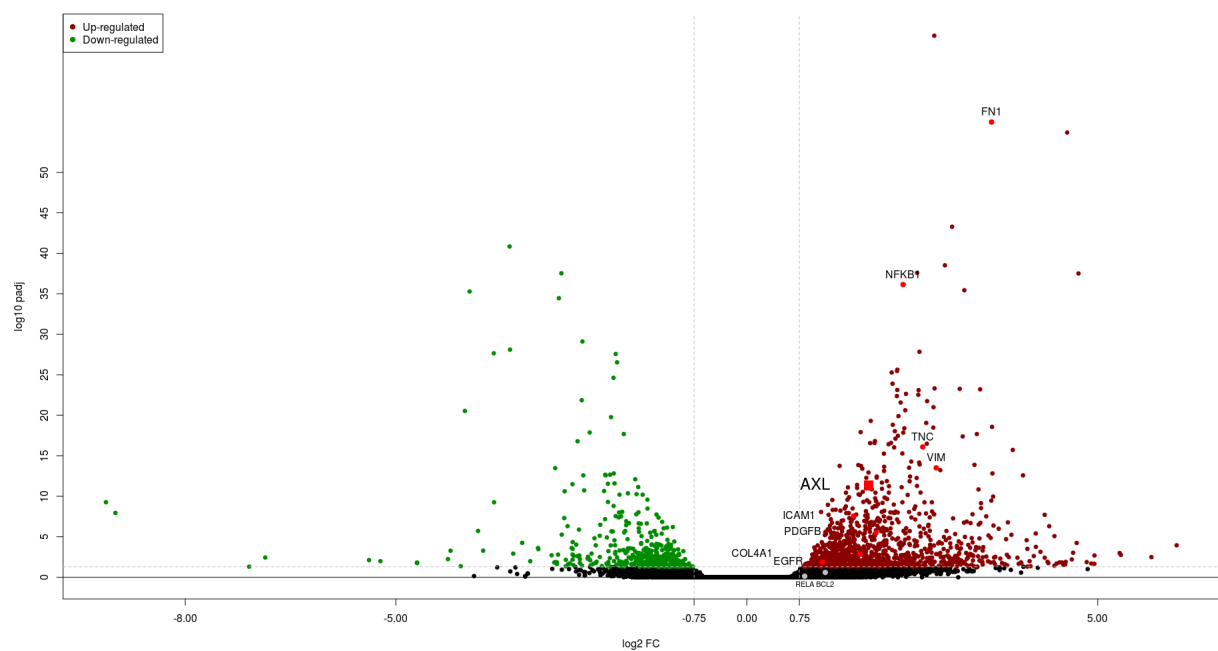


Figure 39. RNA sequencing revealed an upregulation of *AXL*, *EGFR*, *COL4A1*, *VIM*, *TNC*, *FN1*, *ICAM1*, *NFKB1*, *RELA* and *BCL2*

Volcano plot showing relevant downregulated and upregulated genes in SCC12 cells plated on 50kPa stiff matrix compared to SCC12 plated on 1kPa stiff matrix. Downregulated genes shown in green, and upregulated ones in red. (n=4)

Finally, **Figure 39** shows a volcano plot with all the upregulated and downregulated genes in SCC12 cell plated on 50kPa in contrast to SCC12 cells plated on 1kPa. We have labelled in the plot most relevant genes that are upregulated in “stiff condition”. These genes are RTK coding genes *AXL* and *EGFR*, growth factor coding gene *PDGFB*, genes coding for ECM proteins *FN1*, *VIM*, *TNC*, *COL4A1* and inflammatory molecules *NFKB*, *RELA* and *ICAM1*, and finally gene coding for signaling protein involved in apoptosis *BCL2*.

3.4. MECHANISM BEHIND SCCs RESISTANCE to EGFR INHIBITORS

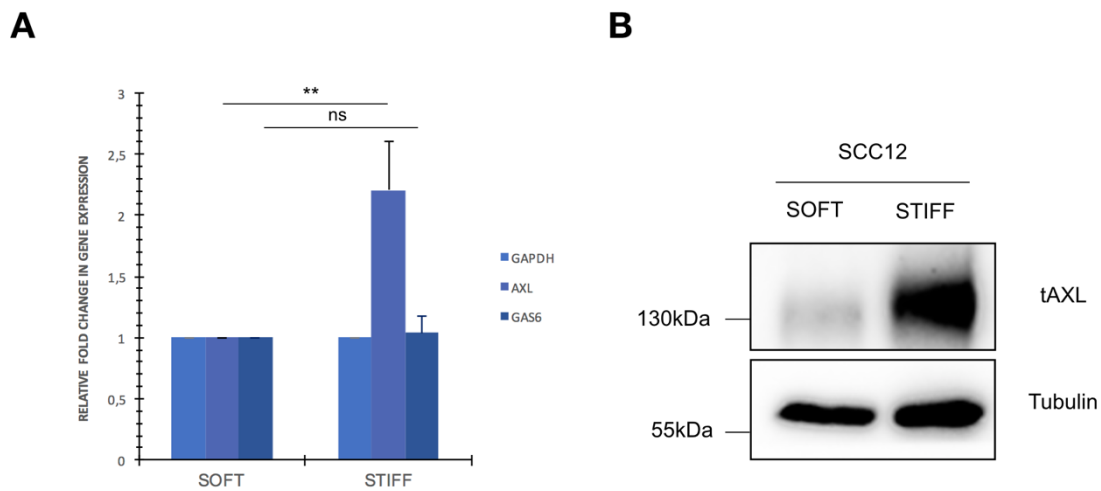


Figure 40. AXL is overexpressed in SCC12 cells plated on stiff matrix

A) RT-qPCR analysis of SCC12 cells plated on soft or stiff matrices. Gene expression was normalized relative to the expression of GAPDH and cells plated on soft matrices. Data are represented as mean + SEM (n=3). B) Representative immunoblot of total AXL in SCC12 cells plated on soft or stiff matrices. Tubulin used as control. Paired samples were compared by 2-tailed Student's t test (NS: not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

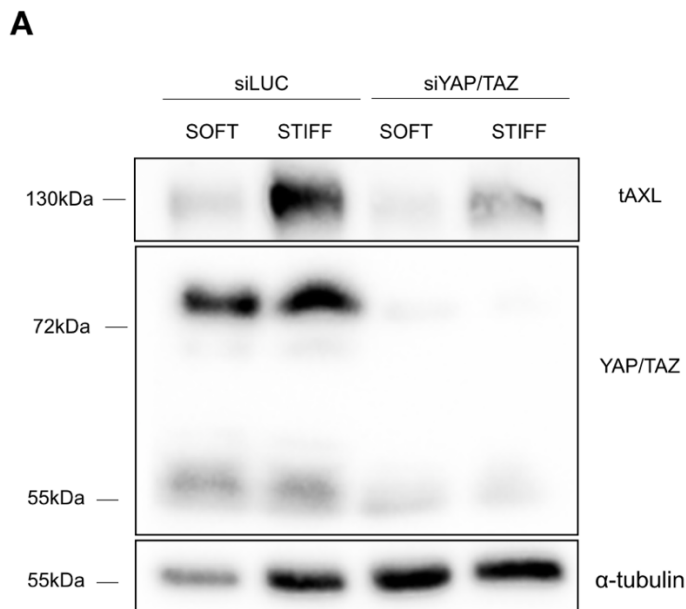


Figure 41. Expression of AXL is under the control of YAP/TAZ co-transcriptional factors.

A) Representative immunoblot of total AXL and YAP/TAZ in SCC12 cells transfected with siLuc as control and siRNA against YAP and TAZ plated on soft or stiff matrices for 48h. Tubulin used as control.

Among the plethora of genes that were upregulated in SCC12 cells plated on 50kPa compared to those plated on 1kPa, we hypothesised that AXL could be the one responsible for the resistance we observed. Role of AXL in resistance to EGFR-targeted therapy in NSCLC has already been described in literature (Zhang et al., 2012), and more over it is know to induce EMT in various settings (Antony and Huang, 2017; Asiedu et al., 2014).

First step we took was to confirm the RNA upregulation of AXL in SCC12 cells plated on stiff that was identified by RNA sequencing. Therefore, we plated SCC12 cells on soft collagen I rich matrix and collagen I rich stiff matrix prepared in house for 48h and measured the expression of AXL and its ligand Gas6 on a gene level using RT-qPCR. We have observed a 2.2-fold increase of AXL on a mRNA level in SCC12 cells plated on stiff matrix. At the same time, mRNA levels of Gas6 did not increase (**Figure 40A**). Results obtained with RT-qPCR confirm the data form RNA-sequencing, AXL is upregulated on a gene level in SCC12 cells plated on stiff compared to soft, while its only ligand Gas6 is not. To further get a better understanding, we analysed protein expression of total AXL using Western blot. Immunoblot revealed a strong overexpression of the total AXL in SCC12 cells plated on stiff matrix compared to soft (**Figure 40B**). Very low level of total AXL was present in cells plated on soft suggesting that AXL is overexpressed due to mechanical stress induced by the increased rigidity of stiff matrix.

To confirm the notion that stiffness induces overexpression of AXL and establish the connection between stiffness and AXL, we decided to knock out the co-transcription factors YAP and TAZ in SCC12 cells. As mentioned earlier, YAP and TAZ serve as a mechanotransducers of matrix rigidity and are responsible for activation of many signaling pathways upon stiffness increase. We hypothesised that, if the AXL overexpression is stiffness induced, knock out of YAP and TAZ will lead to the downregulation of AXL. We plated SCC12 cells that were transfected with siRNA against YAP and TAZ on soft and stiff matrix for 48h and afterwards analysed by Western blot the expression of AXL. In accordance with our hypothesis, upon the knock out of YAP/TAZ in SCC12 cells plated on stiff, level of AXL protein expression was comparable to the level observed in cells plated on soft matrix transfected with siLuc as control (**Figure 41A**). Therefore, we confirm that knock out of YAP/TAZ in SCC12 cells abrogates the overexpression of AXL on stiff matrix.

These findings establish a strong correlation of AXL overexpression and increase in matrix rigidity. Based on the role of AXL on NSCLC resistance to treatments, we hypothesis that the downregulation of AXL in SCC12 cells plated on a stiff matrix could lead to increase the cell sensitivity to gefitinib treatment.

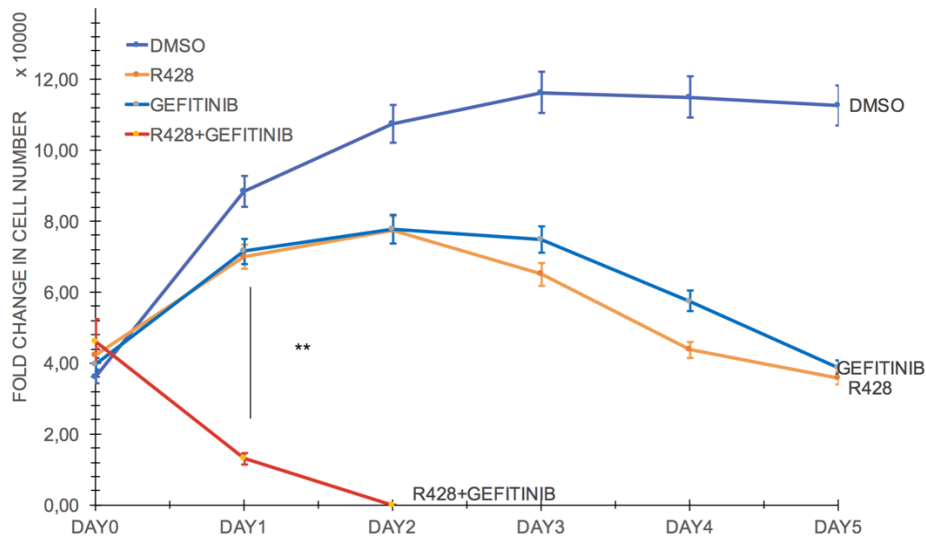
A

Figure 42. Treatment of R428 and gefitinib overrides stiffness mediated resistance in SCC12 cells

A) Fold change of SCC12 cells number plated on stiff matrices for 5 days with treatment of R428 (10 μ M), gefitinib (5 μ M) and combination of both (R428 10 μ M and gefitinib 5 μ M) ($n=3$, mean \pm SD One-way ANOVA was used for group comparisons (NS: not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$))

In order to test this hypothesis, we plated cell on a stiff matrix for 48h prior to the treatment with R428, a small molecule inhibitor of AXL kinase, gefitinib and a combination of both. We counted the cell number in each condition every day during a course of 5 days to get a full understanding of cell survival. 24h after the treatment with the combination of AXL and EGFR inhibitors, SCC12 cells survival was significantly lower compared to gefitinib treatment only. Difference in fold change of cell number was more than six after 24h, and after 48h of treatment with the combination of inhibitors cells died completely (**Figure 42A**). Gefitinib treatment of SCC12 cells resulted in, as shown earlier, 30% decrease in cell number after 48h of treatment, same was true for the cells treated with only R428. It is important to mention that SCC12 cells were kept in 0,5% serum media and continued to proliferate upon treatment with gefitinib and R428 until the end of the second day of treatment, afterwards the cell number started to decrease. However, SCC12 cells in control (DMSO) condition proliferated until the end of the third day and their number remained the same until the end of the experiment.

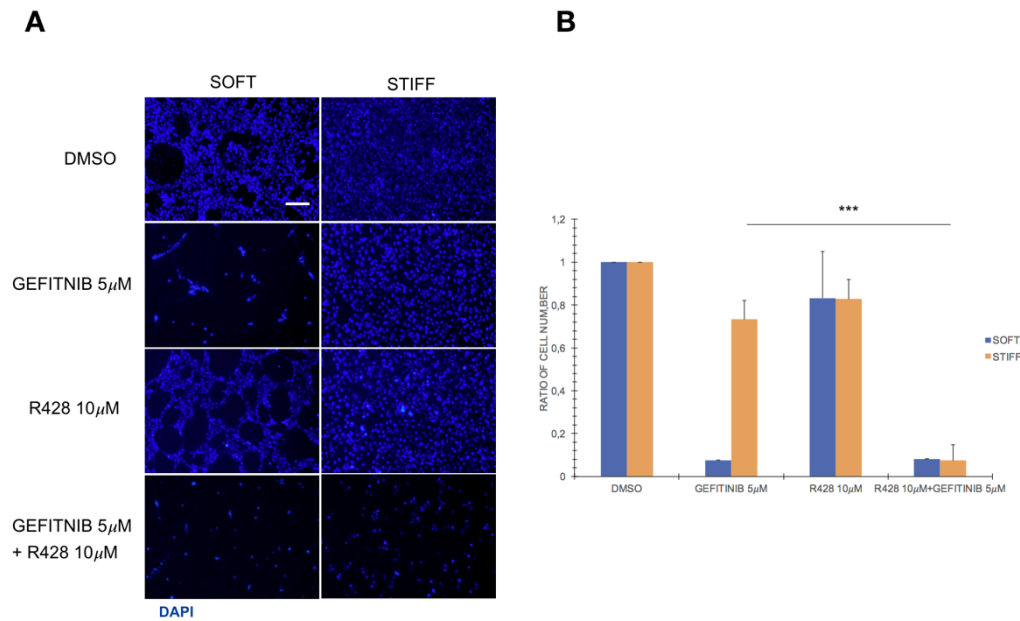


Figure 43. SCC12 cells plated on the stiff matrix are not resistant to treatment with AXL inhibitor and EGFR-TKI gefitinib

A) Representative DAPI staining pictures of SCC12 cultivated on a stiff and soft matrix for 48h, treated with 5µM of gefitinib, 10µM of R428 and a combination of both (R428 10µM and gefitinib 5µM) for another 48h. Scale bar 200µm. B) Quantification of DAPI staining from experiment shown in A. (n=3, mean + SEM) One-way ANOVA was used for group comparisons (NS: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Next, to confirm that combination of R428 and gefitinib kills SCC12 cells plated on stiff matrix, we plated cells either on a soft or a stiff substrate for 48h and then treated them with gefitinib, R428 or combination of both for 48 additional hours. As seen in **Figure 43A**, upon the combination of the two inhibitors, SCC12 cells plated on stiff matrix are almost completely gone. Quantification of DAPI positive cells shows 65% decrease of cell number when cells were plated on a stiff matrix and treated with R428 and gefitinib together compared to gefitinib treatment alone (**Figure 43B**). Only a small percentage (8%) of cells plated on stiff survived the treatment with both inhibitors, while treatment with only AXL inhibitor, R428, has few effects on cell number both in soft and stiff condition.

Taken together, these data suggest an important role of AXL in the resistance of SCC12 cells to targeted therapy. AXL inhibition leads to the increase of the efficiency of EGFR targeted therapy in inducing cell death in SCC12 cells plated on stiff matrix. In other words, inhibition of AXL kinase overrides rigidity mediated resistance of SCC12 cells to EGFR targeted therapy.

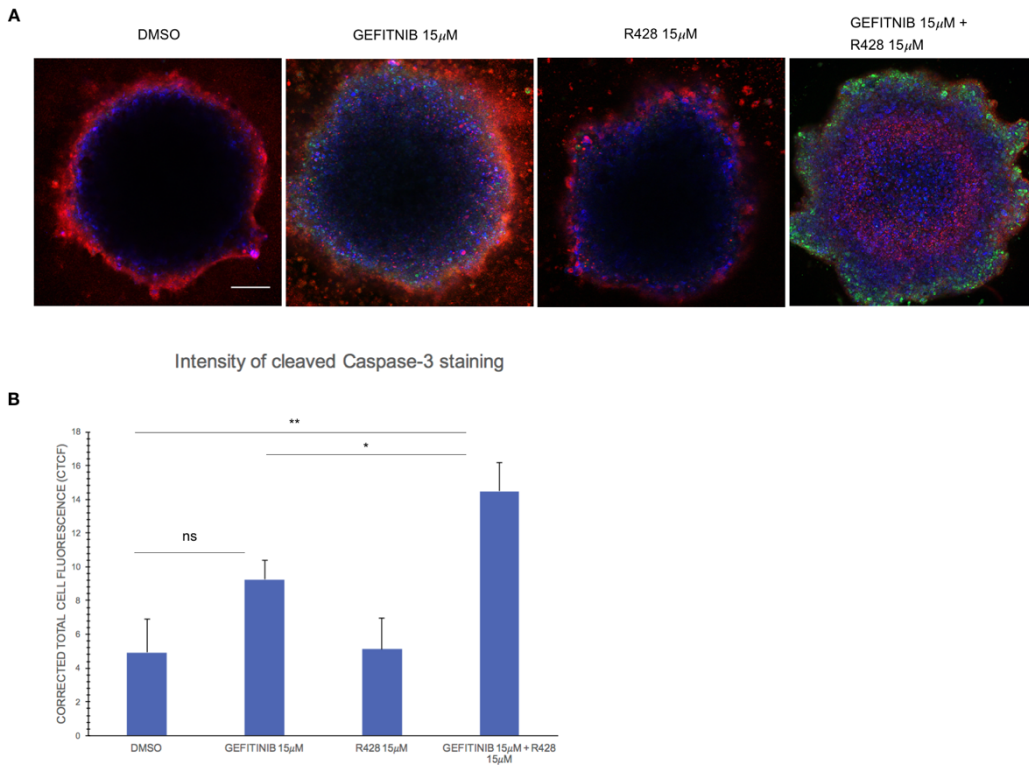


Figure 44. R428 and gefitinib induce an increase of cell death in SCC12 spheroids

*A) Representative confocal merged images of phalloidin, cleaved caspase-3 and DAPI staining in SCC12 spheroids embedded in collagen-I rich matrix for 5 days in presence of 5 μ M of gefitinib, 10 μ M of R428 and of both (R428 10 μ M and gefitinib 5 μ M). Scale bar, 100 μ m B) Quantification of cleaved caspase-3 fluorescence intensity represented in A. (mean + SD). One-way ANOVA was used for group comparisons (NS: not significant, * p <0.05, ** p <0.01, *** p <0.001).*

So far, use of two-dimensional (2D) cell monolayer has proven to be an effective method in research. However, with new advances, it has been slowly replaced with three-dimensional (3D) cultures or spheroids. Spheroids overcome the limitation of 2D cell culture (Pampaloni et al., 2007) and partially mimic the function of living tissue. 3D cell structures establish more relevant cell-cell and cell-ECM interactions. Due to their properties, spheroids have been proven to be an effective approach to cancer drug validation and response (Chatzinikolaidou, 2016).

Our hypothesis was that, if we culture SCC12 cells in a 3D spheroids and treat them with gefitinib, R428 and combination of both, we will observe a more physiologically relevant cell response to the drugs and have a better understanding of how efficient they are. In particular, we expected to have a higher level of cell death in spheroids treated with the combination of both agents then with single-agent approach. We first formed three SCC12 cells spheroids for each condition in droplets for 48h and after included them in collagen-I rich matrix to imitate their interaction with the environment. Once spheroids were included in matrix, we treated them for 5 days with 15 μ M of gefitinib, 15 μ M of R428 and a combination of both compounds. We have increased the concentrations compared to those used in 2D cell culture due to the spheroids anatomy that makes drug diffusion more difficult. After the treatment, we have stained the spheroids for DAPI, phalloidin and cleaved-caspase 3 to quantify the occurrence of cell death

in each condition. As seen in representative confocal images (**Figure 44A**) there is more positive cleaved-caspase 3 staining (green) in spheroids that were treated with both gefitinib and R428 than in spheroids treated with each compound individually. Quantification of the cleaved caspase-3 staining confirmed our hypothesis that combination of treatment leads to more cell death of SCC12 cells. Intensity of cell fluorescence was 37% higher in spheroids treated with both drugs, compared to gefitinib alone (**Figure 44B**), while intensity difference between control and gefitinib failed to be significant, once again emphasising the inefficiency of EGFR-targeted therapy in SCC cells.

Overall, our data suggests that inhibition of AXL kinase activity along with EGFR activity leads to a significant increase of cell death and decrease of cell survival. Moreover, we propose that AXL activity is stiffness dependant and that response of HNSCCs patients to AXL inhibition can be predicted on the fibrotic and mesenchymal signature of the tumor.

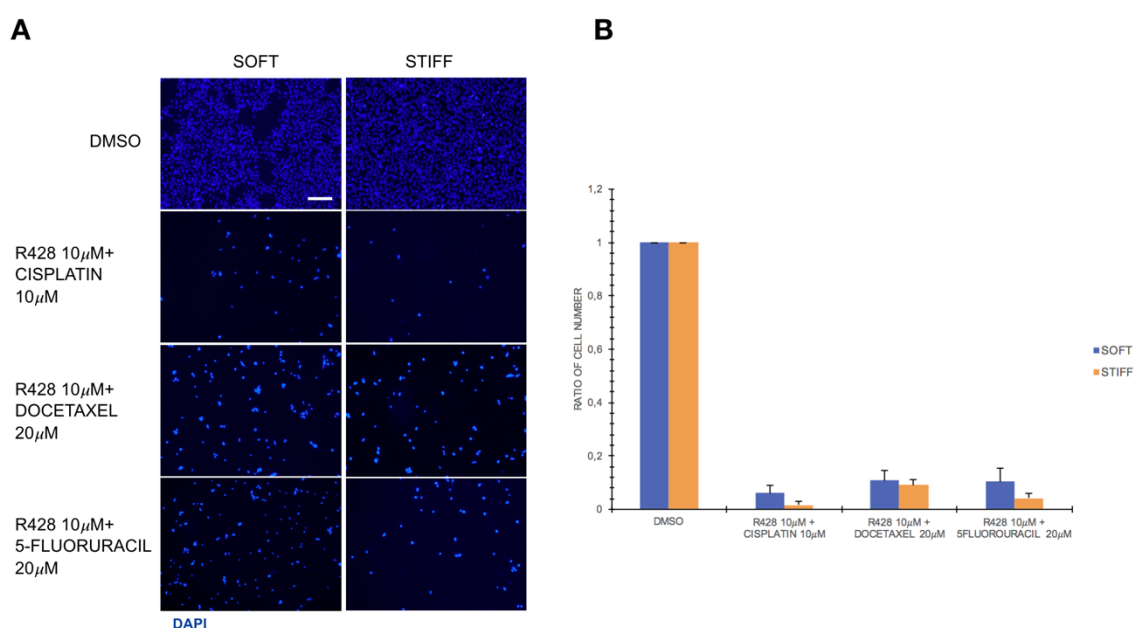


Figure 45. *SSC12 cells plated on the stiff matrix are not resistant to treatment with traditional chemotherapeutic agents, cisplatin, docetaxel and 5-fluorouracil in combination with R428*

*A) Representative DAPI staining pictures of SCC12 cultivated on a stiff and soft matrix for 48h, treated with 10 μ M of cisplatin, 20 μ M of docetaxel and 20 μ M 5-fluorouracil in combination with 10 μ M of R428 for 48h. Scale bar 200 μ m. B) Quantification of DAPI staining from experiment shown in A. (n=3, mean + SEM). Paired samples were compared by 2-tailed Student's t test (NS: not significant, * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001).*

As mentioned earlier, in HNSCCs one of the approaches to treatment is a combination of chemotherapy and targeted therapy, despite the fact that it has been shown not to be completely efficient (Yamano et al., 2010). Here we aim at demonstrating that EGFR targeted therapy can be accompanied with AXL inhibitor to overcome the matrix rigidity-induced resistance of SCC12 cells we have observed (**Figure**

29). Once again, we have plated SCC12 cells on either a soft or a stiff matrix for 48h and treated them with the combination of R428 and cisplatin, docetaxel or 5-fluorouracil. Positive DAPI staining of the cells after 48h of treatment revealed that very few cells remain after the treatment of combination of cisplatin, docetaxel or 5- fluorouracil with AXL inhibitor (**Figure 45**. As expected, quantification of the positive DAPI staining confirmed that when AXL inhibitor is added to the traditional chemotherapy agents, SCC12 cells do not survive the treatment and are no longer resistant. In fact, only 5% of SCC12 cells are left on stiff matrix after the combinational treatment, level that is more than comparable with the SCC12 cells plated on soft and sensitive to treatment (**Figure 45**.

Taken together, we demonstrate that AXL is a rational target to take in consideration when approaching the treatment of HNSCCs. We have as well suggest that EMT is part of the rigidity induced resistance process, and having in mind that AXL is very well described in literature to induce EMT, we believe that stiffness induced EMT can be used as a predictive marker for the failure of EGFR-targeted therapy as well as a predictive marker of the successful use of AXL inhibitors.



Figure 46. Expression of AXL is overturned by a combination of EGFR and AXL TKIs

A) Representative immunoblot of total AXL in SCC12 cells plated on soft or stiff matrices for 48h and treated with 5μM of gefitinib/R428 and combination . Tubulin used as control.

It has been shown in literature that one of the ligand-independent manners of activating AXL is by homodimerization or heterodimerization with other RTK, notably EGFR, and that this association may drive the resistance to EGFR-targeted therapies (Meyer et al., 2013). It has been shown that stiffness induces the activation of EGFR expression and kinase activity independently of EGF ligand binding (Grasset et al., 2018; Umesh et al., 2014; Yarwood and Woodgett, 2001).

We hypothesise that stiffness stimulates the activation of both EGFR and AXL, and in turn the relationship of EGFR and AXL becomes reciprocal and maintains their activation. Therefore, inhibition of EGFR by gefitinib should impact the expression of EGFR. We have plated SCC12 cells on stiff substrate and treated them for 24h with 5 μ M of R428, gefitinib or the combination of both. Protein expression of AXL was analysed with Western blot, and as seen in **Figure 46A**, AXL was only partially downregulated with R428 and more importantly, gefitinib was able to downregulate the expression of AXL as well. Only a combination of treatment abolished completely the expression of AXL (**Figure 46A**).

This immunoblot confirms that AXL activation is partially maintained by EGFR, and downregulation of both is necessary to overturn stiffness induced expression of AXL.

3.5. SUPPLEMENTAL DATA

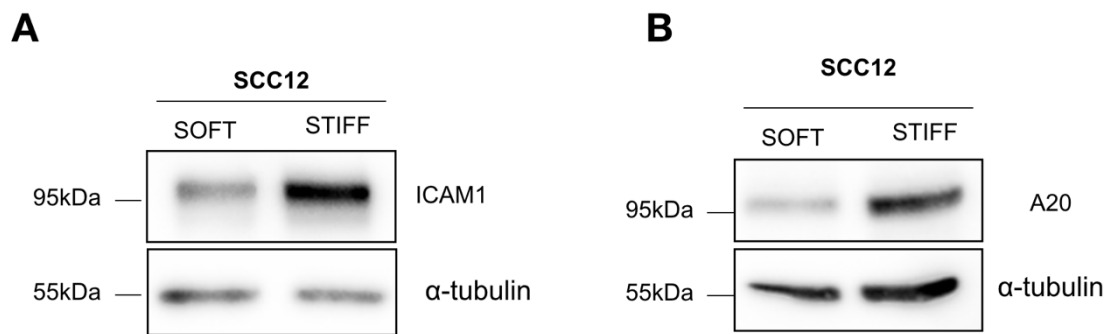


Figure 47. ICAM1 and A20 are overexpressed in SCC12 cells plated on stiff matrix

A) Representative immunoblot of ICAM1 in SCC12 cells plated on soft or stiff matrices for 48h. Tubulin used as control. B) Representative immunoblot of A20 in SCC12 cells plated on soft or stiff matrices for 48h. Tubulin used as control.

As a very last part of results we show here, we wanted to verify the data obtained with RNA sequencing that suggests a strong inflammation in SCC12 cells in contact with stiff matrices (**Figure 38**).

Since RNA sequencing demonstrated an upregulation of *ICAM1*, inflammatory marker, we hypothesised that ICAM-1 will be overexpressed on protein level also.

To verify our hypothesis, we plated SCC12 cells for 48h on soft and stiff matrix and analysed the expression of ICAM1 protein using western blot. Indeed, ICAM1 is found to be overexpressed in SCC12 cells plated on stiff compared to SCC12 cells plated on soft (**Figure 47A**). Next, we aimed to confirm that A20 is upregulated in SCC12 cells in contact with increased rigidity matrix. Upon 48h adjustment to either soft or stiff the environment, SCC12 cells were subjected to western blot analysis. Here we report that A20 is overexpressed in SCC12 cells plated on stiff matrices (**Figure 47B**).

These immunoblots confirm and induction of inflammation as a response to a rigid matrix in SCC12 cells, particularly ICAM1 and A20.

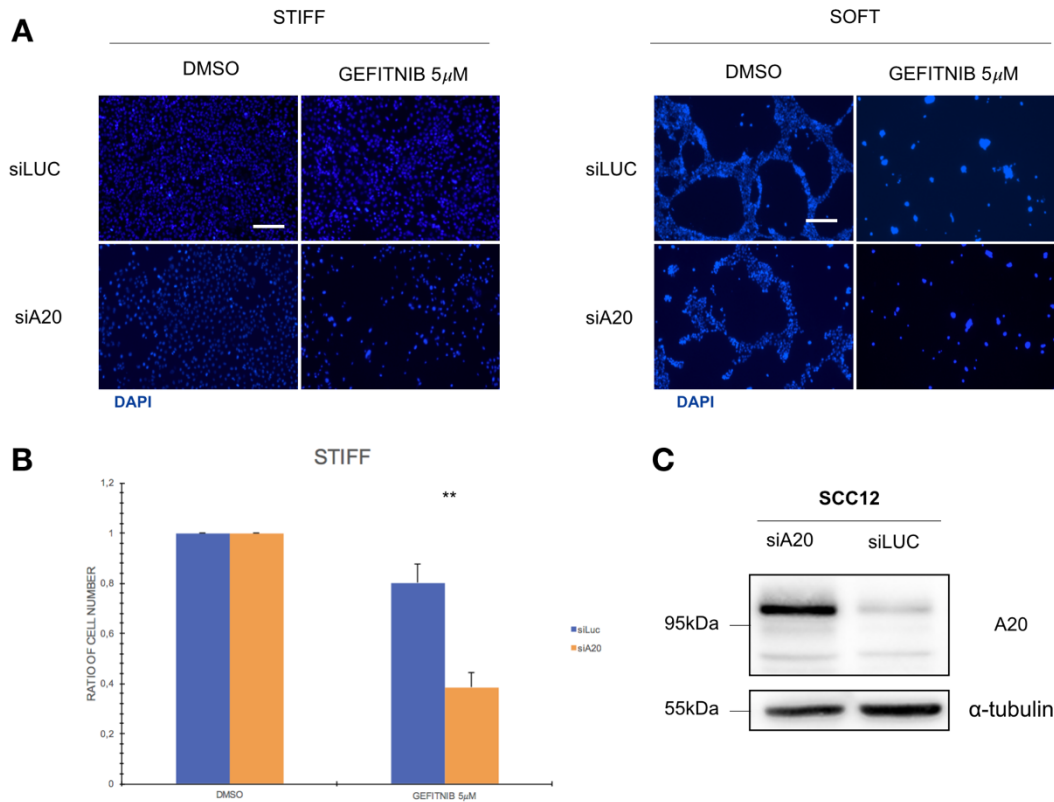


Figure 48. Knock out of A20 in SCC12 cells leads to an increase in sensitivity to gefitinib when plated on stiff matrix

A) Representative DAPI staining pictures of SCC12 transfected either by siLUC as a control or siA20 and cultivated on a stiff and soft matrix. Scale bar 200 μ m B) Quantification of DAPI staining from experiment shown in A. (n=3, mean + SEM). C) Immunoblot of A20 in SCC12 cells corresponding to the assay presented in A. Tubulin used as control. Paired samples were compared by 2-tailed Student's t test (NS: not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Since A20 is known to be involved in anti- and pro-apoptotic signals (Storz et al., 2005), and is overexpressed in SCC12 cells plated on stiff substrate (Figure 47B) we assumed that knock out of A20 will sensitise cells plated on stiff matrix to EGFR inhibitor-induced cell death and override matrix rigidity induced protection. To verify our notion, we knocked out A20 in SCC12 cells using siRNA against A20 (Figure 48C) and plated them on soft and stiff matrices for 48h followed by a 48h treatment of 5 μ M gefitinib. We have showed that cells in which A20 was knocked out, were more sensitive to gefitinib treatment compared to those transfected with an siRNA control (siLuc) (Figure 48A). Quantification of DAPI positive staining showed that when cells were transfected with siA20 and plated on stiff were 42% more responsive to gefitinib treatment (Figure 48B).

These data suggest that inhibiting A20 in SCC12 cells can have a positive impact on the EGFR inhibitor-induced cell death and that resistance of the cells on stiff substrate is partially driven by the overexpression of A20.

Chapter 4: *MATERIALS*
and METHODS

Cell Culture

SCC12 and SCC25 cells were cultured in FAD media (21765, Gibco), supplemented with 10 % FCS (fetal calf serum), 2mM glutamine, 5 µg ml⁻¹ insulin-transferrin-selenium (41400-045; Invitrogen) and 0.5µg/mL hydrocortisone (H-0135; Sigma), 10 ng ml⁻¹ EGF (E9644, Sigma) and 1% Pen/Strep. CAL27 and CAL166 were maintained in in DMEM supplemented with 10 % FCS (fetal calf serum), 2 mM glutamine and 1% Pen/Strep. Primary normal human fibroblasts (nHF) were cultured in DMEM supplemented with 10 % FCS and 2 mM glutamine. SCC12 cells were a kind gift from E. Sahai lab, SCC25, CAL27 and CAL166 are cell lines isolated from HN-SCC patients' biopsies at the "Centre Antoine Lacassagne, Nice. All cells were grown at 37°C in a humidified 5 % CO₂ atmosphere. Experiments were performed at passages 3-12.

Soft matrix refers to cells plated on surface of 100 µL of matrix gel for 6 well plates. Briefly, matrix gel is composed of 4 mg/mL collagen (354249, Corning), 2 mg/mL matrigel (354234, Corning), 5x DMEM, HEPES (15630-056, Gibco) and 0.5 % FCS DMEM. Stiff conditions correspond to cells cultivated on collagen (40 µg/mL)/matrigel (20 µg/mL) coated plates. Hydrogels were purchased from Matrigen and coated with collagen (50 µg/mL, 354249, Corning).

Cytokines and inhibitors.

TGFβ₁ was purchased from Peprotech (#100-21, Peprotech, Rocky Hill, NJ) and was used at 2 ng/ml. Inhibitors and chemotherapeutics were used between 5µM and 20µM in this research and are listed in the **Table 2**.

Table 2. TKIs and chemotherapeutic agents used

Name	Supplier	Catalogue number
Gefitinib (ZD1839)	Selleckchem	S1025
AG1478 hydrochloride	TOCRIS bioscience	1276
R428 (BGB324)	Selleckchem	S2841
Cetuximab	Gift from A.Bozec	/
Cisplatin	Sigma Aldrich	C2210000
Docetaxel	Sigma Aldrich	01885-5MG-F
5-Fluorouracil	Gift from A.Bozec	/

Transfection

Cells were plated at 70 % confluence and subjected to siRNA (25 nM) transfection the following day using Lipofectamine RNAiMax (Invitrogen #13778-150) according to the manufacturers' instructions. siRNA Smartpool On-target plus were purchased from Dharmacon and control siRNA (siLuc) from Eurogentec All are listed in **Table 3**

Table 3. List of siRNA used

Target	Type	Supplier	Reference code/sequence
YAP	Smartpool On-target plus siRNA	Dharmacon	J-012200-07
TAZ	Smartpool On-target plus siRNA	Dharmacon	J-016083-05
SNAI1	Smartpool On-target plus siRNA	Dharmacon	J-010847-09
SNAI2	Smartpool On-target plus siRNA	Dharmacon	J-017386-05
ZEB1	Smartpool On-target plus siRNA	Dharmacon	J-006564-10
ZEB2	Smartpool On-target plus siRNA	Dharmacon	J-006914-21
TWIST1	Smartpool On-target plus siRNA	Dharmacon	J-006434-08
TNFAIP3	Smartpool On-target plus siRNA	Dharmacon	J-009919-05
EGFR	Smartpool On-target plus siRNA	Dharmacon	J-003114-10
Luciferase	Control siRNA	Eurogentec	CGUACGCGGAAUACUUCGA

Messenger RNA extraction and Quantitative RT-PCR

Total RNA was extracted using Trizol reagent (15596018; Lifetechnologies) according to the manufacturer's instructions. Total RNA concentration was determined using ND-1000 micro-spectrophotometer (NanoDrop Technologies). 1 µg of total RNA was used for cDNA synthesis with random hexamers. Messenger RNAs were reverse transcribed using the Multiscript RT kit (18064-014; Life Technologies) to generate cDNA. Real time PCR was performed using Fast SYBR Green Master Mix (18064-014; Applied Biosystems) and executed on a Step One Plus Real-Time PCR system (Applied Biosystems). Fold-change of RNA species was calculated using the formula $(2^{-\Delta Ct})$, normalized to RPLP0 expression. Primers sequences are listed in **Table 4**

Table 4. RT-qPCR primers

Gene Name	Forward (5' → 3')	Reverse (5' → 3')
GAPDH	ACCCAGAAGACTGTGGATGG	TCTAGACGGCAGGTCAGGTC
N-cadherin	ATCCTACTGGACGGTTTCG	TTG GCTAATGGCACTTGA
E-cadherin	TTCCCAACTCCTCTCCTG	AAACCTTGCCTTCTTTGTC
Vimentin	GAACGCCAGATGCGTGAAATG	CCAGAGGGAGTGAATCCAGATTA
Slug	AAGCATTTCAACGCCTCCAAA	GGATCTCTGGTTGTGGTATGACA
Snail	TCGGAAGCCTAACTACAGCGA	AGATGAGCATTGGCAGCGAG
Twist	CCGGAGACCTAGATGTCATTG	CCACGCCCTGTTTCTTTG
Fibronectin	CCATCGCAAACCGCTGCCAT	AACACTTCTCAGCTATGGGCTT
AXL	AGACATCGCCAGTGGCATG	AGGCGATTTCTGCTTCAGG
Gas6	CATCAACAAGTATGGGTCTCCGT	GTTCTCCTGGCTGCATTTCGTTGA

Matrix production

Cell-derived matrices were generated as previously described (Beacham et al., 2007). Briefly, nHF or nHF+TGFβ1 (nHF were treated with TGFβ1 at least for 7 days prior to the matrix production and were maintained in TGFβ1 during the matrix production) were seeded at a density of 3.10^5 cells per well in a 6-well plates coated with gelatin (G1890, Sigma Aldrich) and cultured for 10 days, with medium being changed every 48 hours to 0.5 % FCS medium supplemented with 50 mg/mL ascorbic acid (A4403; Sigma-Aldrich). Mature matrices were then denuded of cells using lysis buffer (PBS containing 20 mM NH₄OH and 0.5 % (vol/vol) Triton X-100). Following PBS washes, matrices were stored in PBS at 4°C before use.

Cell counting assay

SCC12 cells were plated in triplicate in 24 well plates at 2.10^4 cells per well. After overnight incubation for cells to adhere, 24 wells were counted to determine initial count at time of treatments (gefitinib or/and R428). Between day 1 and day 5 the entire contents of the well was trypsinized and counted and proliferation rate was calculated.

Immunoblotting and antibodies

Cells were washed with PBS on ice and immediately lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM, NaCl, 5 mM EDTA, pH 8.0, 30 mM NaF, 1 mM Na₃VO₄, 40 mM β-glycerophosphate, 0.1 mM PMSF, protease inhibitors, 10% glycerol and 1% Nonidet-P40). Equal amounts of protein from each sample were resolved by SDS-PAGE and transferred onto a PVDF membrane (Millipore). Membranes were blocked 1 hour at room temperature in 5 % BSA (A7030, Sigma Aldrich) in TN buffer - 0.1 % Tween (10mM Tris-HCl (pH7,5) , 500 mM NaCl, 0,1 % Tween 20 (Sigma # P1379)) and incubated in the presence of the primary antibody at 4°C overnight. After washing in TN buffer containing 0.1 % tween, membranes were incubated for 1 hour at room temperature with secondary antibody. After washing in TN buffer - 0.1 % Tween, immunodetection was performed using chemiluminescent HRP substrate (WBKLS0500; Millipore). Appropriate secondary antibodies (anti-rabbit P0448, anti-mouse P0447 and anti-goat P0449, Dako) coupled to HRP were used. Primary antibodies are listed in **Table 5**.

Immunofluorescence

After the different treatments cells or matrices were fixed with PBS/PFA 3.7 % for 20 min and incubated with blocking buffer (3 % BSA Triton 100X 0.01 %) for 1 hour at room temperature. Then, cells or matrices were incubated with primary antibody

Table 5) or phalloidin (P1951; 2 μ g/mL; Sigma Aldrich) diluted in blocking buffer overnight at 4°C. Secondary antibodies coupled with Alexa-594, Alexa-488 or Alexa 647 (A21209, A21206, A31571, Thermo Scientific) were used at 1:400. Nuclei were counterstained with DAPI (1050-A, Euromedex) and mounted in Montant, Permafluor (TA-030-FM, Thermo Scientific). Cell number and intensity of staining was measured using ImageJ software. All images acquisition was performed using an Olympus Bx51 microscope or ZEISS LSM Exciter confocal microscope.

Table 5. Primary antibodies used

Name	Supplier	Species	Dilution WB	Dilution IF
α-tubulin	Sigma Aldrich	Mouse	1:10000	/
Total AXL	R&D systems	Goat	1:1000	/
A20	Santa Cruz	Mouse	1:1000	/
ICAM1	Cell Signaling Technology	Rabbit	1:1000	/
YAP and TAZ	Cell Signaling Technology	Rabbit	1:1000	/
N-cadherin	Santa Cruz	Rabbit	1:750	/
E-cadherin	Cell Signaling Technology	Rabbit	1:750	/
Cleaved caspase-3	Cell Signaling Technology	Rabbit	/	1:400
Fibronectin	Sigma Aldrich	Mouse	1:1000	1:400

Cell elongation quantification

Morphology of SCC12 was visualised using phalloidin (P1951; 2 μ g/mL; Sigma Aldrich) and cells elongation was quantified with ImageJ software. In brief, radius (r) of a hypothetical perfect circle each cell would form was measured with ImageJ and it was used to calculate the “perfect circle” surface using the $A=\pi r^2$ formula. Following that, the actual surface of the cell was measured using ImageJ. If the ratio between the actual surface and ideal one calculated using the cell radius was equal to 1 or close to 1, cell was labelled as round. If the ratio was equal less than 1, cell was considered elongated. Not less than 30 cells were quantified per condition in 3 independent experiments.

Three-dimensional cell culture

Spheroid assay was performed as we described before (Albregues et al., 2013). Briefly, cancer cells (SCC12) were removed from the cell culture dishes with trypsin and re-suspended in FAD media 10% FCS, no EGF. Fifty-microliter droplets containing 50 000 cells were plated onto the underside of a 10 cm culture dish and allowed to form spheroids in a 37°C incubator for 48 hours. The spheroids were then embedded in a collagen I/Matrigel gel mix at a concentration of approximately 4 mg/mL collagen I and 2 mg ml⁻¹ Matrigel (BD Bioscience) in 35mm glass-bottomed cell culture plates (MatTek). The gel was incubated for at least 45 min at 37°C with 5% CO₂. The gel was covered with FAD media with corresponding inhibitors. Forty-eight hours later, the spheroids were imaged with an inverted microscope at a magnification of 10x and 20x.

RNA sequencing

RNA was extracted from SCC12 cells plated on 1kPa and 50kPa hydrogels for 48h as described earlier. Experiment was performed independently 4 times, RNA transcriptome was enriched by ribosomal RNA depletion and quadruplicates were sent for sequencing at UCA GenomiX - IPMC platform (Nice).

Data was processed by Bioinformatics platform at IRCAN, Nice as following.

Reads from multiple lanes per sample were grouped together and basic QC performed using FastQC; low quality bases and adapter sequences were trimmed with Trimmomatic. Reads were aligned with STAR (Dobin et al. 2013), to the reference genome. GRCh38.primary_assembly. genome.fa, downloaded from ENSEMBL. To improve spliced alignments quality, annotation file gencode_hg38_GRCh38.gtf from GENCODE was provided. RSEM (Li, B. & Dewey (2011)) was used to perform read summarization at gene level. Differential expression was assessed by the Bioconductor package DESeq2 (Love MI, Huber W, Anders S (2014)), with a cut-off on adjusted p-value (< 0.05) and fold change (> 1). However, second sample of SCC12 cells plated on 50kPa hydrogel was removed from the analysis due to the differentially expressed genes visualised on the heatmap. S2 has slightly different expression signature compared to other samples, and although it did not change significantly the data, we removed it from the analysis. Therefore, three samples of SCC12 cells plated on 50kPa were compared to four samples of SCC12 cells plated on 1kPa hydrogel.

Gene set enrichment for pathway analysis, then pathway visualisation was done by Bioconductor package Gage (Luo, Weijun, Friedman, Michael, Shedden, Kerby, Hankenson, Kurt, Woolf, Peter (2009) and pathview Luo, Weijun, Brouwer, Cory (2013)).

Statistics

Cell culture experiments were performed at least three times independently. Numerical quantifications for *in vitro* experiments using cultured cells represent mean \pm standard error of the mean (SEM). Immunoblot images are representative of experiments that have been repeated at least three times. Paired samples were compared by a 2-tailed Student's t test for normally distributed data. For comparisons among groups, one-way ANOVA and post-hoc Tukey testing was performed. P-value less than 0.05 was considered significant. All statistical analyses have been performed with Microsoft Excel.

Chapter 5: *DISCUSSION*
and PERSPECTIVES

Treatment of patients with advanced, non-operable, recurrent HNSCCs represents a major challenge in clinics. In effort to preserve organ functions, multidisciplinary therapies are administered involving multiple chemotherapies and radiotherapy. With the advancement of research, it was suggested in 1986 that EGFR is overexpressed in many cases of HNSCCs (Ozanne et al., 1986). More studies followed confirming these results (Hama et al., 2009; Rubin Grandis et al., 1998) and finally it led to a first clinical trial 20 years later, that showed efficacy of combining radiotherapy and EGFR-specific antibody, cetuximab. However, clinical studies involving anti-EGFR targeted therapies in HNSCCs are not consistent, while some show significant benefits others mark no improvement (Ang et al., 2014; Bonner et al., 2006b, 2010; Burtneess et al., 2005; Vermorken et al., 2008). These deviations in patient response could be due to the fact that reports of EGFR overexpression are established by immunostaining studies that vary greatly in antibodies and protocols used, and often lack controls. Furthermore, it has been shown that small percentage of HNSCCs that have EGFR overexpression also have receptor cross-phosphorylation, suggesting an active autocrine loop (Chung et al., 2004; Hama et al., 2009). Moreover, there are at least 13 tyrosine phosphorylation sites in EGFR that mediate interactions with different partner proteins, causing different downstream effects (Morandell et al., 2006). In reality, there is no biomarker that predicts the efficiency of EGFR-targeted treatments or any other therapy and patients still do develop distant metastases and second primary tumors, or with one word - resistance. Interestingly, in colorectal cancer KRAS mutation is a well-established biomarker of cetuximab resistance (Lièvre et al., 2006), however it is rarely present in HNSCCs and is proven to not be useful (Wang et al., 2012). Thus, development of the predictive strategy could lead to an improvement of cetuximab efficacy.

Patients not only suffer from resistance to EGFR-targeted therapies but also to chemotherapy, radiotherapy and combination of all three (López-Verdín et al., 2018). One of the reasons for the failure of these therapies can be explained by the fact that the majority of the tests carried out only take into account the tumor cells, without considering the microenvironment. However, the latter influences all the tumor characteristics (Hanahan and Weinberg, 2011), including resistance to therapies (Hanahan and Coussens, 2012; Quail and Joyce, 2013). One of the major components of TME is ECM, a complex network of macromolecules with distinctive physical, biochemical, and biomechanical properties. ECM found in tumors is remodelled, fibres are aligned (Levental et al., 2009) and significantly more rigid (Butcher et al., 2009; Cox and Epler, 2011; Grasset et al., 2018; Paszek et al., 2005). Recent evidence suggest a cooperation between rigidity of the ECM and EGFR expression (Grasset et al., 2018; Okawa et al., 2007; Rosenthal, 2017; Thomas et al., 2018) and studying resistance of HNSCCs to EGFR-targeted therapies in the context of stiffness could help identify potential therapeutic strategies and biomarkers of EGFR-targeted therapy resistance.

5.1. EGFR and STIFFNESS

Relationship between stiffness and EGFR expression has been first proposed by Levental *et al* in 2005. They demonstrated that matrix stiffness increased PI3K activity downstream of EGFR in mammary carcinoma cells. Subsequently, it was shown that the amount of EGF required for epithelial cell proliferation was much lower when the cells were in contact with a rigid matrix compared to a flexible matrix, allowing the authors to conclude that matrix stiffness sensitizes cells to EGFR signaling (Kim and Asthagiri, 2011). It was soon after validated that EGFR expression is controlled by the transcription factor YAP, which is activated by matrix stiffness, and that this regulation contributes to the chemoresistance of oesophageal cancers (Song *et al.*, 2015). Interestingly, Xia *et al* have recently demonstrated that EGFR signaling promotes YAP activation in hepatocellular carcinoma (Xia *et al.*, 2018). YAP, a core effector of Hippo pathway is as well one of the most famous mechanotransducers responsible for direct translation of mechanical stimuli into chemical reaction and is known to be activated upon stiffness (Dupont *et al.*, 2011; Nukuda *et al.*, 2015). Even more peculiar are two studies demonstrating that YAP expression is associated with cetuximab resistance in colon cancer and HNSCCs (Jerhammar *et al.*, 2014; Lee *et al.*, 2015).

Another publication displayed that both EGFR and HER2 activity increases in contact with a rigid matrix by the Src kinase, regardless of the presence of ligand (Saxena *et al.*, 2017). Finally, our group described a relationship between stiffness, EGFR overexpression, calcium channels and cancer cell invasion (Grasset *et al.*, 2018). On the other hand, analyzes of EGFR expression in solid tumors revealed that a number of patients overexpress this receptor without amplification of the gene (Salomon *et al.*, 1995). For example, 90% of HNSCCs carcinomas express EGFR, and only 15% of patients have genetic amplification (The Cancer Genome Atlas Network, 2015).

Taken together, these data led us to believe that the reason behind EGFR-targeted therapy failure in HNSCCs is extracellular matrix rigidity. By combining various *in vitro* approaches, such as 2.5D cultures that mimic difference in rigidity of normal *versus* tumor tissue we have demonstrated a strong protection against EGFR-targeted therapy, chemotherapy and combination of both dependent cancer cell death induced by an increase of stiffness. The use of polyacrylamide hydrogels further confirmed this observation on a more relevant pathophysiological matrix rigidity scale. Indeed, cells resist to the treatment-dependent cell death with gefitinib when plated on 12kPa hydrogels compared to cells plated on 1kPa. Furthermore, analyzes of the biophysical properties of ECM by atomic force microscopy (AFM) have shown that the tumor matrix is more rigid than healthy tissue in the majority of solid tumors (Grasset *et al.*, 2018). Therefore, we have shown that the cells plated on a stiffer matrix derived from activated fibroblasts are more resistant to gefitinib induced cell death, compared to when plated on a softer matrix derived from fibroblast isolated from dermis. Since we have shown that matrices derived from activated fibroblasts are more stiff, we argue that the stiffness is responsible for observed protection. However, we cannot exclude the potential effect of soluble factors within matrix such as

cytokines, chemokines and growth factors. Indeed, the role of soluble factors mentioned has been shown by other groups (Pontiggia et al., 2012). In perspective, we could analyze the matrix derived from nHF and CAF-like using mass spectrometry to identify differentially expressed proteins between the two. Potentially, it could lead to an identification of a novel target within matrix that, once downregulated, would sensitize the cells to treatment with EGFR-TKIs. Besides that, we could understand will the downregulation of the desired target in CAFs lead to production of the matrix that is significantly more soft, comparable to the one produced by nHF. With this approach, we could propose a double targeting, with TME in mind, that would have tremendous benefits for the patients. It is important to mention, that a need for a drug targeting stiff matrix is emerging. Very few agents enter pre-clinical and clinical trials and non has been approved yet (Mohammadi and Sahai, 2018). Therefore, identifying a new target that would abrogate matrix stiffness would have huge clinical significance.

Furthermore, we sought to provide a strong connection between stiffness and cell survival upon anti-cancer treatments. To do so, we exploited the fact that mechanotransduction is carried out by two co-transcriptional factors YAP and TAZ (Dupont et al., 2011). We, and others, have shown that upon an increase of rigidity YAP is activated and translocated into the nucleus where it is able to transcribe genes involved in carcinogenesis (Bertero et al., 2018; Rice et al., 2017a). Upon silencing of *YAP* and *TAZ*, cells plated on stiff substrate are no long resistant to gefitinib treatment confirming our hypothesis that stiffness is responsible for promoting cancer cells survival upon treatment with EGFR-targeted therapy.

Another approach we could have take, is to combine EGFR-targeted therapy or chemotherapy with inhibitors against FAK and Src kinase that are known to mediate mechanical signals (Bae et al., 2014). We assume that upon FAK and Src inhibition, stiffness mediated signals would be abolished and cells plated on stiff would be more sensitive to treatment with gefitinib, similarly to the silencing of *YAP* and *TAZ*.

5.2. EMT and STIFFNESS

Among many different processes known to be stimulated in cancer cells by stiffness, EMT has been described recently. Many studies connecting matrix rigidity with EMT are emerging in variety of cancers, including oral squamous cell carcinoma (Engler et al., 2016; Matte et al., 2018; Rice et al., 2017a; Wei et al., 2015). At the same time, it has been established that EMT promotes resistance to anti-cancer therapies in NSCLC, HNSCCs, pancreatic and breast cancers (Rice et al., 2017a; Sarkar et al., 2009; Singh and Settleman, 2010; Wang et al., 2016b). In our settings, we have observed a strong elongation of cancer cells plated on stiff compared to the morphology of cancer cells plated on soft. Indeed, after quantification we have established that 62% of cells plated on stiff obtain an elongated phenotype while 38% of the cells showed an epithelial morphology. Cells plated on soft maintain their morphology with only 4% exhibiting an elongated shape. These results led us to hypothesize that an EMT is occurring. Major hallmark of EMT is the loss of E-cadherin that is balanced by an increase of mesenchymal markers N-cadherin, vimentin, fibronectin and α SMA (Wheelock et al., 2008; Yilmaz and Christofori, 2009). We show an overexpression of two mesenchymal markers N-cadherin and fibronectin on both protein and gene level in cells plated on rigid substrate. Interestingly, we also show a strong overexpression of E-cadherin at the protein level, however we did not confirmed this overexpression at the mRNA level by qRT-PCR. Recent study demonstrated what they call “epithelial paradox” or the fact that patients with breast cancer tumors expressing vimentin and E-cadherin had the worst disease-free and overall survival among all tumors (Yamashita et al., 2018). Despite its well established role as a tumor suppressor, E-cadherin has recently been a subject of many debates. One study shows that Twist1-induced metastatic spreading requires E-cadherin expression, and on top of that authors observe differential regulation of E-cadherin on a RNA and protein level (Shamir et al., 2014). These results go in line with our data and suggest posttranslational regulation of adherent junction components such as E-cadherin. More *in vitro* studies showed that overexpression of E-cadherin did not inhibit motility and invasion of cancer cells as it would be expected (Nieman et al., 1999; Rajasekaran et al., 2001). Additionally, tumors isolated from patients with breast, colorectal and salivary gland cancer that were E-cadherin positive displayed an increase of invasion into surrounding stroma (Christiansen and Rajasekaran, 2006). In prostate cancer, E-cadherin expression is associates with an aggressive form of tumor (Putzke et al., 2011), and in glioblastoma with growth, invasion and poor outcome for patients (Lewis-Tuffin et al., 2010). E-cadherin is also highly expressed in cancers undergoing severe inflammation such as breast cancer, and its blocking inhibits cancer cell invasion (Dong et al., 2007). Interestingly, E-cadherin in ovarian cancer enhances proliferation and survival by activating EGFR in an ligand-independent manner (Reddy et al., 2005). Role of E-cadherin in cell signaling is well known (Klezovitch and Vasioukhin, 2015). Clustering of E-cadherin molecules in *cis* following intercellular homophilic interaction can promote ligand-independent dimerization and activation of EGFR (Pece and Gutkind, 2000; Pece et al., 1999; Reddy et al., 2005). Extracellular domain of E-cadherin interacts with

EGFR that leads to the cleavage and shedding of this domain into cytosol forming 80 kDa soluble fragment of E-cadherin (sE-cad) that serves as a paracrine/autocrine signaling molecule (Hazan and Norton, 1998). Elevated levels of sE-cad in cancer patients' sera are commonly associated with invasiveness of cancer and poor prognosis (De Wever et al., 2007). What is interesting in the context of our research is the fact that signaling by sE-cad can also inhibit apoptosis by activating EGFR (Inge et al., 2011), however its role in the context of resistance to cancer therapies is not yet explored. Another research reported the activation of EGFR by sE-cad, but the difference between these two studies is in the requirement of full-length E-cadherin expression. While one publication claims sE-cad signaling occurs in the absence of full-length E-cadherin, other finds interaction between sE-cad and full-length E-cadherin essential to mediate survival signaling (Inge et al., 2011; Najy et al., 2008). Relationship between sE-cad and EGFR is reciprocal since EGFR promotes MMP- and ADAM-dependent generation of sE-cad (Grabowska et al., 2012; Zuo et al., 2011). Zuo *et al* suggest that EGFR activation in HNSCCs promotes cell migration and invasion by inducing EMT-like phenotype characterised with MMP-9 cleavage of E-cadherin into sE-cad. Therefore, a positive feedback loop is generated where EGFR activation produces sE-cad that in turn activates EGFR signaling to promote oncogenic proliferation and invasion (**Figure 49**). Taking all these data into account, we can hypothesize that stiffness promotes EGFR expression which could mediate production of sE-cad that is able to protect cells from cell death induced by therapies in the presence of full-length E-cadherin. To verify this hypothesis, further studies are necessary and would include, measuring the levels of sE-cad in the conditioned media of cells cultivated on soft and stiff substrate, as well as degradation of sE-cad to sensitise cells plated on stiff to cell death.

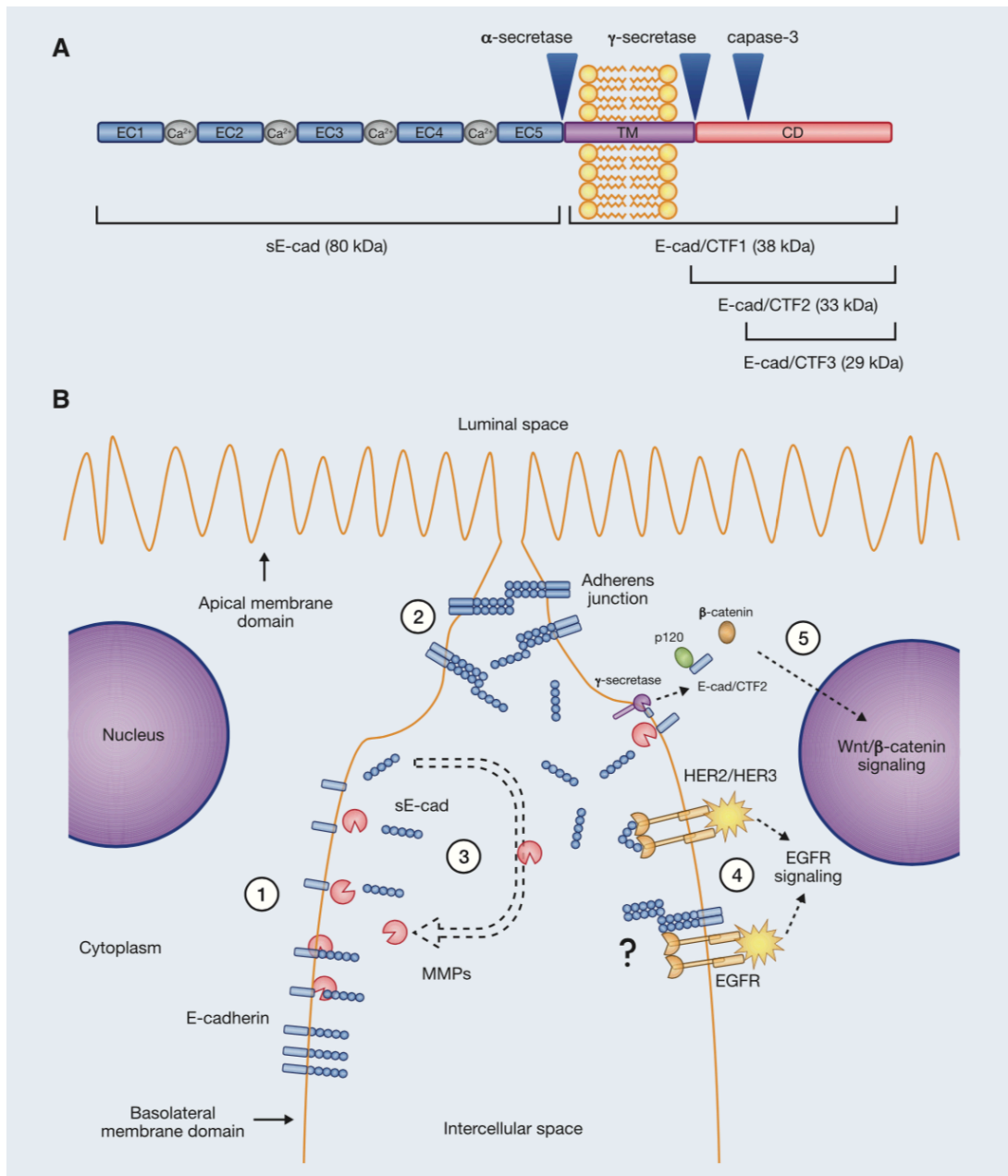


Figure 49. Oncogenic functions of soluble fragment of E-cadherin

sEcad is generated by cleavage of full length E-cadherin. *sEcad* activates the expression of metalloproteinases (MMPs) to enhance its own shedding and it activates EGFR signaling pathway by different mechanisms. Cleavage of E-cadherin activates Wnt/ β -catenin signaling pathway. Wnt/ β -catenin pathway may serve to support rapid tumor cell proliferation and also augment tumor cell survival. Adapted from (David and Rajasekaran, 2012).

Another conclusion we can draw from the fact that E-cadherin is overexpressed in cancer cells plated on stiff substrate is that process we observe is not a full, but rather partial, EMT. This can be further corroborated by the RNA levels of EMT transcriptional factors Snail, Slug and Twist. Gene expression of Slug did not change, while we observe a small, but not significant increase of Snail and notably Twist. EMT is a very dynamic and reversible, multi-state process that includes all intermediate phenotypes between purely epithelial and purely mesenchymal cell type (Jordan et al., 2011). These intermediate phenotypes are in literature described as “intermediate EMT”, “incomplete EMT”, “EMT-like” and my favourite, “partial EMT” (Futterman et al., 2011). Partial EMT is not yet completely understood, and

several theories of what it really is are proposed: (1) a mixed cell population composed of epithelial and mesenchymal cells; (2) a pure cell population composed of hybrid epithelial/mesenchymal cells (E/M); or (3) a mixed cell population composed of epithelial, mesenchymal, and hybrid epithelial/mesenchymal cells (Grigore et al., 2016). Indeed, a subpopulation of cells that co-express epithelial and mesenchymal markers has been identified (Grosse-Wilde et al., 2015; Hong et al., 2015).

According to the quantification we performed, as I stated before, 62% of cells found on stiff substrate are elongated while the other persist with an epithelial morphology, suggesting that we have a mixed population of epithelial and mesenchymal cells. However, we cannot exclude the possibility that hybrid epithelial/mesenchymal cells are present in our set up. To further understand the partial EMT process ongoing in cells plated on rigid substrate, we would need to perform additional analysis of EMT markers and their co(localisation). Nevertheless, we hypothesize that in our case mixed cells population composed of epithelial, mesenchymal, and hybrid epithelial/mesenchymal cells exists. We know that 60% or more cells plated on stiff survive the treatment with anti-cancer agents, same percentage that corresponds to the elongated cells. We could argue that it is exactly these mesenchymal or hybrid epithelial/mesenchymal, low proliferative cells, that survive the treatment, and would still explain the increase in proliferation of cells plated on stiff (data not shown) attributed to the rest of the epithelial-like cells. However, to confirm this notion we would need additional analysis. In particular, it would be interesting to perform immunostaining of mesenchymal markers such as N-cadherin and epithelial markers such as cytokeratin 14 on soft and stiff matrices, with or without treatment. Immunostaining as such would allow us to understand are the mesenchymal cells the one that resist the treatment, and are the cells with epithelial phenotype the one that do not survive. In addition, to get a complete understanding of the type of cells present on stiff matrix in terms of EMT stage, we could perform a single cell RNA sequencing. Same approach was taken by Pastushenko *et al* to identify tumor transition states during EMT. They show using single cell RNA sequencing that different intermediate states exhibit different invasive, metastatic and differentiation characteristics, but also are localized in different microenvironments and in contact with different stromal cells (Pastushenko et al., 2018). We can propose the idea that the stage of EMT found in cancer cells, and therefore their resistance, is dependant on the degree of stiffness found in TME and inflammation that further drives fibrosis and tissue stiffening (Georges et al., 2007). On the other hand, analysing the intermediate states of cells that resist the conventional and targeted treatment would help us develop an appropriate strategy for their elimination. Nowadays, strategies for pharmacological approaches to target EMT are being explored, with a focus on micro-RNAs regulating EMT and, interestingly, TME (Elaskalani et al., 2017). Indeed, role of miR-200 family of micro-RNAs has been reported in EMT. In more detail, it acts as a tumor suppressor and drives Mesenchymal-to-Epithelial Transition (MET) (Mongroo and Rustgi, 2010). In perspective, we could restore the miR-200 expression in SCC12 cells plated on stiff substrate to sensitise them to anti-cancer treatments. Finally, we could carry out a rescue experiment where we would induce EMT in SCC12 cells plated on soft matrix using TGF β or TNF α , that are known to promote EMT *in*

vitro, and monitor their resistance to treatment. We expect to observe a significant increase of cells that survive the treatment due to their mesenchymal properties, confirming that resistance we observe in cells plated on stiff matrix is EMT driven.

It is reassuring that the knock down of known transcriptional factors such as Zeb1, Zeb2, Snail1, Snail2 and Twist involved in EMT leads to the increased sensitivity of cancer cells plated on stiff matrices to EGFR-targeted therapy. Besides that, greatest effect was observed upon Twist knock out, same transcriptional factor that we observed slightly upregulated in SCC12 cells plated on a stiff matrix. Interestingly, NF- κ B is known to be implicated in EMT through regulation of Twist expression (Kang and Massagué, 2004) and we showed using RNA sequencing that NF- κ B is upregulated in SCC12 cells plated on stiff suggesting a potential connection between the two.

Taken together, we provide sufficient evidence of the EMT, or in this case partial EMT, in stiffness mediated resistance of HNSCCs to targeted therapy.

5.3. AXL SIGNALING in RESISTANCE of HNSCCs to EGFR INHIBITORS and CONVENTIONAL CHEMOTHERAPY

RNA sequencing, we performed on SCC12 cells plated on 50kPa (stiff) and 1kPa (soft) hydrogels, revealed a strong upregulation of gene coding for AXL RTK in cells plated on stiff matrices. We rationalised that it is a good target to explore in HNSCCs since its role in various other cancers is well documented. Moreover, role of AXL was prominent in cancers that were EGFR-dependant such as NSCLC, triple-negative breast cancer, colorectal cancer and finally HNSCCs (Brand et al., 2014; Meyer et al., 2013; Spano et al., 2008; Zhang et al., 2012). Interestingly, AXL was found to be important in resistance of melanoma to BRAF inhibitors (Seip et al., 2016). This study reports that stromal fibroblasts reduce melanoma sensitivity to BRAF inhibition by inducing a mesenchymal switch in melanoma cells through upregulation of AXL and fibronectin. Indeed, role of AXL in EMT-mediated resistance has been well described in NSCLS, breast, pancreatic, ovarian and oesophageal cancer (Antony and Huang, 2017; Byers et al., 2013; Gjerdrum et al., 2010; Zhang et al., 2018a). If we look back on the previous paragraph of discussion it is easy to connect EMT, AXL, EGFR and finally stiffness. Before proposing an explanation for this correlation it is important to mention that several mechanism of acquired resistance to EGFR-therapies have been described, such as EGFR mutations, MET amplification, PIK3CA mutations, but in more than third of cases mechanism remains unknown (Postel-Vinay and Ashworth, 2012). We can assume, although we do not confirm, that in these 30% of cases patients potentially develop resistance through EMT or/and AXL signaling. Therefore, our hypothesis is that stiffness could be responsible for such a course of events. We have demonstrated stiffness dependant activation of EGFR, partial EMT and upregulation of AXL. On top of that, we have showed that blocking AXL in combination with gefitinib has a tremendous effect on the survival of SCC12 cells plated on stiff matrix. Even more so, a combination of AXL and conventional chemotherapy led to a complete eradication of cells plated on stiff substrate, while both gefitinib and cetuximab in combination with chemotherapeutic agents failed to accomplish the same. Combination of EGFR-targeted therapy with chemotherapy is a standard procedure in treating HNSCCs patients, however, as mentioned before, no real and consistent clinical benefit has been observed. In pancreatic cancers situation is more or less the same. EGFR expression is found in 82% of colorectal carcinomas (Goldstein and Armin, 2001), but no clear association between tumor EGFR expression and response to EGFR-targeted therapy was evidenced (Van Cutsem, 2006). Pre-clinical and clinical trials showed a similar response of patients with high EGFR expression and low or negative EGFR expression to cetuximab, panitimumab and gefitinib plus chemotherapy (Berlin et al., 2006; Ogino et al., 2005; Saltz et al., 2004), emphasising the need for a new biomarker that will predict the success or failure of EGFR targeted therapy.

Despite having shown the upregulation of AXL on a protein and gene level, unfortunately we were not able to demonstrate its activation in SCC12 cells due to the technical issues. Despite using two different antibodies against phosphorylated AXL by different companies developed for Western blot use, we

were not able to get a meaningful result, nor positive nor negative. We continue our efforts to generate data on the activity of AXL.

To understand how AXL is activated more data is necessary. For example, we have shown that levels of Gas6, only known AXL ligand (Nagata et al., 1996), did not change upon stiffness on a gene level. Despite not verifying the level of Gas6 on a protein level, we argue that AXL expression is ligand-independent and stiffness-driven. It is known that AXL can be activated in two manners (1) ligand dependant by Gas6 and (2) ligand independent by homodimerization or heterodimerization with other RTKi such as EGFR (Meyer et al., 2013; Varnum et al., 1995). Knock down of *YAP* and *TAZ* lead to a strong downregulation of AXL suggest that AXL expression could potentially be stiffness mediated. More studies have confirmed a relationship between YAP and AXL where YAP drives the expression of AXL (Ghiso et al., 2017; Hong and Guan, 2012; Xu et al., 2011). Ghiso *et al* have, in particular, describe YAP-dependant overexpression of AXL that mediates resistance to EGFR inhibitors in NSCLC. We can propose that this transcriptional regulation of AXL by YAP is potentiated through stiffness activation of YAP as it has been previously described (Bertero et al., 2018). In future, we could impair other members of mechanotransduction cascade by pharmacologic inhibition of FAK and ROCK and monitor the effect on the expression of AXL.

Nevertheless, it has been shown that AXL can be activated by forming heterodimers with EGFR (Goyette et al., 2018; Huang et al., 2007; Meyer et al., 2013). To understand are AXL and EGFR activations mediated by a crosstalk between the receptors, we could measure the level of ERK activity upon AXL/EGFR inhibitors and both. Indeed, quantification of activated ERK would also allow us to understand the cooperation of EGFR and AXL. We hypothesise that AXL and EGFR inhibitors will partially inhibit activation of ERK, and combination of both inhibitors would lead to a complete blocking of ERK activity. If so is true, we can argue that stiffness includes the activation of both EGFR and AXL that later maintain and reinforce each others activation. Furthermore, we could perform co-immunoprecipitation, a pull-down assay or crosslinking experiments to verify protein-protein interactions between AXL and EGFR, and characterize their nature. Benefit of doing crosslinking experiment is the fact that we would be able to determine exactly which AXL and EGFR domains are interacting, that would provide crucial knowledge in further development of targeting strategies. Another approach would be to fluorescently label both kinases and monitor their co-localisation using confocal microscope. Ideally, depending on the intensity of interaction between AXL and EGFR, we can utilise Fluorescence Resonance Energy Transfer (FRET) technique for further confirmation of the relationship between two kinases. In parallel, regardless of evidence of stiffness mediated upregulation of AXL is ligand independent, more studies are necessary to completely exclude Gas6 activation of AXL. We have performed RT-qPCR that showed no upregulation of Gas6, however we did not measure its protein level, therefore we should consider doing ELISA or Western blot in future to confirm our data. In addition, to eliminate the possibility of ligand-dependant activation of AXL, we could mutate extracellular domain of AXL to alter its conformation and prevent its activation by ligand. Recently,

Moody *et al* have developed two blocking antibodies, GMAB1 and GMAB2, that led to a 90% inhibition of Gas6-induced phosphorylation of Akt, a downstream target of AXL (Moody et al., 2016). Utilising described blocking antibodies would allow us to exclude any possibility of ligand-dependent AXL activation.

Overall, our conclusion is that AXL upregulation upon stiffness drives the resistance of cancer cells and that AXL is an excellent target to consider in future of HNSCCs treatment as well as other cancers. To further confirm the role of AXL in resistance of SCC12 cells plated on stiff, it would be beneficial to quantify the resistance of cells plated on soft to EGFR-TKIs upon AXL overexpression. For example, we expect to have an increase of cell survival despite the EGFR inhibitor when cells are plated on soft substrate and AXL is forcefully overexpressed.

Furthermore, in RNA sequencing analysis we reported an upregulation of A20, a context-specific anti-inflammatory protein. As mentioned in the introduction, its role in resistance of tumors has been reported but not studied enough. We have demonstrated that its knock down leads to an increase of cell susceptibility to gefitinib when plated on stiff substrate. Besides that, A20 is a known negative regulator of NF- κ B (Beyaert et al., 2000) that is also shown to be upregulated by RNA sequencing in SCC12 cells on stiff substrate. Relationship between NF- κ B and A20 is more complex than purely one-sided since upon NF- κ B activation, A20 is actively expressed (Düwel et al., 2009; Verstrepen et al., 2010). Interestingly enough, a study established a connection between A20 and Snail1 in promoting metastasis of aggressive breast cancer (Lee et al., 2017) suggesting a role of A20 in EMT.

It is now worth to mention again that NF- κ B is a known and often described as sufficient player in inducing EMT (Huber et al., 2004; Pires et al., 2017). Besides that, in oesophageal squamous cell carcinoma NF- κ B pathway is activated in an AXL dependant manner (Paccez et al., 2015). Taken all together, it can be proposed that upon stiffness AXL is upregulated and in turn drives the activation of NF- κ B signaling and A20 resulting in EMT that allows SCC12 cells to survive the treatment. Since NF- κ B is a pivotal mediator of inflammation, and we have observed an upregulation of ICAM1, an inflammatory marker, in SCC12 cells plated on stiff. Here, we speculate that AXL drives this inflammation process, specially if we take in consideration high expression of E-cadherin we report that has been previously associated with severe inflammation occurring in invasive breast cancer (Dong et al., 2007). More data is necessary to validate such a complex interaction of key players in signaling of cancer cells in contact with stiff environment.

In **Figure 50**, we display a schematic summary of molecular changes we report occur in SCC12 cells upon increase of matrix rigidity and that lead to their resistance to treatment.

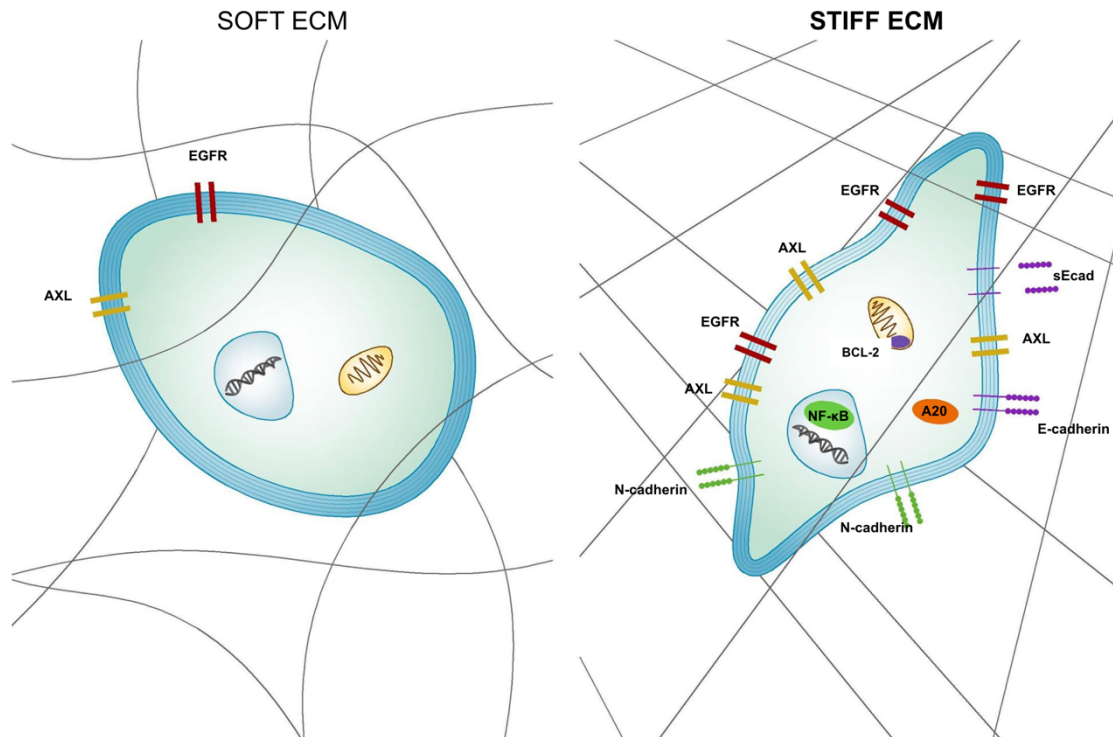


Figure 50. Stiffness regulates resistance of SCC12 cells to anti-cancer therapies by inducing overexpression of various proteins involved in EMT, inflammation and cell survival

Upon interaction with stiff substrate, SCC12 cells gain mesenchymal phenotype and express elevated levels of N-cadherin. However, we report an upregulation of E-cadherin that is potentially a consequence of a strong inflammation we observe in these cells. Indeed, RNA sequencing of SCC12 cells revealed an upregulation of NF- κ B. On the other hand, E-cadherin could be cleaved by EGFR to its soluble form sE-cad and further reinforce EGFR expression, and induce protection from apoptosis. Another anti-apoptotic molecule, BCL2 was shown to be upregulated in SCC12 cells plated on stiff substrate. Lastly, we have previously shown that stiffness induces EGFR activation, and here we demonstrate an upregulation of AXL kinase that has the potential to interact with EGFR but also induce observed partial EMT through regulation of NF- κ B and A20. All these molecular changes eventually lead stiffness induced protection of SCC12 from anti-cancer treatments.

Lastly, I would like to stress out that we have identified 72 differentially regulated RTKs between SCC12 cells plated on soft and stiff substrate. Among these, majority was upregulated including the Abelson (ABL) tyrosine kinases, ABL1 and ABL2. ABL kinases are oncogenes first described in development of leukemias (Greuber et al., 2013), and their role in solid tumors is emerging. It has been reported that increased expression of ABL changes cell polarity, and stimulates invasion and growth of breast carcinoma, ovarian cancers, lung adenocarcinoma and lung squamous cell carcinoma (Cerami et al., 2012; Simpson et al., 2005; Sos et al., 2009). Subsequent studies demonstrated that ABL kinases are tyrosine phosphorylated and activated in breast, lung, colorectal, gastric, and prostate cancer cells as well as in melanoma (Drake et al., 2012; Furlan et al., 2011; Rikova et al., 2007; Srinivasan and Plattner, 2006; Yang et al., 2006). Majority of these cancers rely on EGFR signaling, therefore it is not surprising that ABL is activated downstream of EGFR and in response to epidermal growth factor (EGF) stimulation (Panjarian et al., 2013; Stuhlmiller et al., 2015). Besides that, Hong *et al* established a relationship between

AXL and ABL in oesophageal cancer. In more detail they show that AXL activation of ABL is responsible for cisplatin resistance in these cancers (Hong et al., 2013).

Having that in mind, it would be interesting to analyse their relationship in our context and provide a more refined mechanism of resistance. At the same time, a screen of TKIs against RTKs that were upregulated in SCC12 cell plated on stiff would possibly allow us to identify a novel, not yet reported, kinase that plays a role in stiffness mediated resistance.

5.4. CLINICAL RELEVANCE

This work suggests that increase matrix stiffness is responsible for resistance of HNSCCs to EGFR-targeted therapy and chemotherapy. In order to confirm these data in more relevant preclinical models we can take several approaches.

We have already shown in 3D culture using SCC12 cells that the combinational treatment of AXL and EGFR inhibitor leads to an increase of cell death. Next step would be to form human patient derived HNSCC multicellular spheroid embedded in matrices to test the efficiency of the combination of the agents. By using patients' cells extracted from fresh tumor biopsies we would be able to understand exactly how relevant is the use of AXL inhibitor in treating patients. Furthermore, characterization of these biopsies in terms of presence of mesenchymal cells and stromal cells would allow us to establish a correlation between EGFR-targeted therapy failure and tumor signature, but also allow us to predicts good response to AXL inhibition. It has been shown that expression of strong mesenchymal cell signature due to the presence of fibroblasts and lack of epithelial characteristics, or presence of cells that have undergone EMT is a molecular signature of HNSCCs high-risk tumors (Chung et al., 2004). In the light of our data, this seems to be true for the resistance of HNSCCs to therapies as well. We hypothesis that tumors that have a strong relationship with their stiff stroma are characterised by mesenchymal signature that leads to the failure of EGFR-targeted therapy and that this resistance can be overcome with the synergistic use of EGFR and AXL inhibitors. Targeted therapy in HNSCCs, but also other tumors such as colorectal cancer, breast cancer, NSCLC and pancreatic cancer is in high demand for more predictive markers of therapy response. One could argue that AXL positive staining is a good marker to identify whether or not EGFR-targeted therapy would be beneficial for the patient. However, we propose a different approach. In our system, AXL is a consequence of an increase of fibrosis and matrix stiffening of the tumor, a profound stroma that mediates cancer resistance. At the same time, we have seen how non-consistent EGFR staining is in these tumors possibly due to the difference in antibodies and protocols used – same can be true for AXL. Therefore, we propose to identify EGFR-targeted therapy resistant tumors by analysing the “stiffness” of the stroma. To do so, we can stain collagen fibres using Picrosirius Red and monitor their crosslinking under polarized light. Crosslinking of collagen fibres has been associated with an increase of matrix stiffness (Koshy et al., 2003; Levental et al., 2009; Lin and Gu, 2015; Pankova et al., 2016). Assessing collagen crosslinking is an effective, fast and accurate method that would allow clinicians to identify tumors that are not sensitive to EGFR-targeted therapy.

Although β -aminopropionitrile - BAPN (irreversible inhibitor of LOX) can block matrix remodelling and verteporfin (YAP/TAZ inhibitor) can inhibit signaling induced by the rigidity of tumor ECM, these treatments are mainly effective if given in the beginning of the tumor development (Bondareva et al., 2009; Dasari et al., 2017). However, when tumors are diagnosed in a patient, the ECM is already impaired limiting the inhibition of remodelling. Therefore, targeting AXL, as a stiffness induced kinase,

may help in the development of new therapeutic strategies. To fathom the idea of stiffness induced expression of AXL, we could as well correlate the collagen crosslinking in patient samples with positive AXL staining, that could give us an idea is AXL the right treatment for a specific patient.

Another approach would be to do a retrospective study in collaboration with Centre Antoine Lacassagne, a research based anti-cancer centre in Nice. Retrospective study would allow us to connect the unresponsive tumors with their histological characteristics to get a better comprehension of the reason behind treatment failure. Utilising these approaches could help patients receive the right therapy for the tumor and prevent side-effects of therapies that will in the end show very little to no benefit.

Next step would be to generate patient-derived xenografts, by injecting human HNSCC primary cancer cells derived from tumor biopsies into the flank of nude mice. Treatment of mice with EGFR TKI or AXL TKI either alone or in combination would allow us to validate combination of inhibitors in HNSCC expansion *in vivo*. Other *in vivo* approaches can be used to further elucidate the role of AXL inhibitor in treatment of, not only HNSCCs but also, NSCLC, breast and pancreatic cancer. Since both *EGFR* and *AXL* knock out mice are viable, we can think about using transgenic mice to further elucidate the role of AXL inhibitor in treatment of these cancers.

Resistance of various types of cancers to EGFR targeted therapies continues to puzzle clinicians and researchers. Despite promising results, it is impossible to predict will EGFR-TKIs show any benefit to the patient or will the patient ultimately develop resistance. During my PhD we were able to describe a strong relationship between stiffness and resistance of cancer cells to EGFR-inhibitors and convention therapy. We suggest that ECM remodelling and increase in rigidity is an unenviable factor in the treatment outcome. RNA sequencing allowed us to explore various targetes in abrogating stiffness mediated resistance, notably, AXL. Interestingly, AXL is known to have a strong relationship with EMT and we have demonstrated sufficient evidence that cells plated on stiff matrix undergo a partial EMT. In literature, relationship between AXL, EMT and cancer resistance has been documented, and we propose the idea of stiffness mediated AXL overexpression that acts upon numerous molecular targets, including ones responsible for EMT.

In conclusion, our data provides a strong understanding of a common mechanism of cancer resistance to a variety of treatments that can be extrapolated to a plethora of cancers, specially those that show EGFR expression. We have seen that multiple cancers exhibit a similar signature when it comes to their resistance, that is often accompanied with strong inflammation and EMT. Data presented herein has a potential to generate a common biomarker of tumors responsiveness to common therapies, and on top of that enable the use of a more appropriate strategy. As I mentioned in the very beginning, understanding cancer is impossible without the understanding of the tumor stroma and cancer therapies must not neglect its role.

Chapter 6: REFERENCES

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Chapter 7: APPENDIX

Targeting elevated STAT3 activity in dystrophic epidermolysis bullosa skin has limited effect on fibrosis but confers dual prevention for skin cancer progression. Venugopal Rao Mittapalli, Tobias Kühl¹, [Sanya Eduarda Kuzet²](#), Christine Gretzmeier, Dimitra Kiritsi, Cedric Gaggioli, Leena Bruckner-Tuderman, Alexander Nyström . *In revision*

Membrane-bound ICAM-1 contributes to the onset of proinvasive tumor stroma by controlling acto-myosin contractility in carcinoma-associated fibroblasts. Bonan S, Albregues J, Grasset E, [Kuzet SE](#), Nottet N, Bourget I, Bertero T, Mari B, Meneguzzi G, Gaggioli C. *Oncotarget*. 2017 Jan 3;8(1):1304-1320. doi: 10.18632/oncotarget.13610.

Fibroblast activation in cancer: when seed fertilizes soil. [Kuzet SE](#) and Gaggioli C, *Cell Tissue Res*, 2016

In revision: Targeting elevated STAT3 activity in dystrophic epidermolysis bullosa skin has limited effect on fibrosis but confers dual prevention for skin cancer progression

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Abstract

Dystrophic epidermolysis bullosa (DEB) is a genetic skin blistering disease. Secondary to skin blistering, DEB manifests as paradigmatic injury- and inflammation-driven fibrosis. Chronically injured and stiffened areas are also the breeding grounds for early-onset aggressive squamous cell carcinomas. From a therapeutic point of view, interfering with processes relating to inflammation and fibrosis may offer substantial symptom-relief for DEB. The janus kinase (JAK)1/2 – signal transducers and activators of transcription 3 (STAT3) axis conveys injury- and inflammation-induced cues to epidermal and dermal activation, and is thus a potentially attractive DEB therapy target. Here, we repurposed the JAK1/2 inhibitor ruxolitinib for treatment of DEB in a preclinical study. In vitro, ruxolitinib reduced the contractile activity of dermal fibroblasts, reduced invasion of tumor cells in 3D skin models and lowered gene expression associated with tumor progression in keratinocytes. In DEB mice, it offered protection against development of mechanically induced fibrosis of back skin, but not against injury-induced progressive fibrosis of forepaws in vivo. Taken together, our study illustrates the need of careful preclinical assessment in relevant models of potential symptom-relief drugs but nevertheless reveals a potential dual benefit in the context of cancer of targeting the JAK1/2-STAT3 axis in DEB.

Introduction

Dystrophic epidermolysis bullosa (DEB) is a skin blistering disease caused by mutations in *COL7A1* encoding the primary constituent of anchoring fibrils – collagen VII (Intong and Murrell, 2012). Secondary to skin fragility, DEB manifests as chronic wounds, healing with scarring and progressive soft tissue fibrosis. As a consequence of a chronically injured and stiffened dermal microenvironment people with severe DEB are prone to develop metastatic cutaneous squamous cell carcinomas (cSCC) with early onset (Cho et al., 2018; Fine et al., 2009; Kim and Murrell, 2015; Mittapalli et al., 2016).

The progressive dermal fibrosis in DEB is paradigmatic of injury and inflammation-driven activation of fibrogenic processes (Nyström and Bruckner-Tuderman, 2018). Such activation underlies most fibrotic conditions and DEB, being a monogenic disease with early onset of fibrosis, may thus be used as a model to increase the understanding of pathomechanisms at play in fibrosis. Current knowledge points to that fibrosis in DEB is initiated by repeated wounding of the papillary dermis that places the skin in a state of constant healing. Increased tissue stiffness from vascular leakage, exposure to serum, and altered bioavailability of growth factors and cytokines from destruction of the dermal extracellular matrix (ECM) and inflammation activate dermal fibroblasts (Nyström and Bruckner-Tuderman, 2018). The activated fibroblasts are the main producers of the fibrotic ECM, which is deposited in an unorganized manner and stiffened through crosslinking by lysyl oxidases and transglutaminase 2 (Küttner et al., 2014; Mittapalli et al., 2016). In this cascade, the transforming growth factors betas (TGFβs) are important mediators of fibroblast activation (Ng et al., 2012b; Nyström et al., 2015). We have previously shown that limiting inflammation and TGFβ activity by angiotensin II type 1 receptor antagonism through the small molecule losartan effectively halts fibrotic disease progression in the collagen VII hypomorphic mouse model (Nyström et al., 2015).

The collagen VII hypomorphic mouse was engineered to globally express significantly diminished levels of wild-type collagen VII – ~ 10% of levels in wild-type littermates – which are insufficient for firm epidermal-dermal cohesion but allow for survival into adulthood (Fritsch et al., 2008). The mouse

faithfully replicates most signs of severe human DEB, including progressive formation of mutilating deformities, and is here referred to as the DEB mouse.

The DEB mouse has been successfully employed to increase the understanding of DEB and for preclinical therapy studies (Chen et al., 2018b; Fritsch et al., 2008; Kopecki et al., 2011; Kühl et al., 2015; Nyström et al., 2013, 2015, 2018; Vanden Oever et al., 2016). Fur stabilizes its skin over the trunk and protects it from frictional damage. In accordance with injury-driven fibrosis in DEB, back skin in this mouse model is less fibrotic and represents an earlier disease stage than skin from forepaws, which is not protected by fur, is exposed to constant shear forces and injury and becomes heavily fibrotic (Nyström et al., 2015). Using this DEB mouse model, systemic overexpression of decorin – a small leucine-rich proteoglycan with a large interactome, including TGF β ligand-sequestering properties – was also shown to increase survival and reduce fibrosis (Cianfarani et al., XXX). The study was initiated based on observations from monozygotic twins disparately affected with DEB (Odorisio et al., 2014), in whom increased decorin abundance was associated with milder disease. Intriguingly, however, the more mildly affected twin also displayed reduced levels of interleukin-6 (IL6) (Odorisio et al., 2014). This is in line with the observation that losartan reduced elevated IL6 levels in DEB mice (Nyström et al., 2015).

In a fibrotic disease progression cascade, enhanced TGF β s and IL6 in skin could be placed as parallel, co-dependent events occurring in response to tissue damage by tissue-resident cells and also, subsequently, by inflammatory cells. A prominent downstream mediator of IL6 signaling is signal transducers and activators of transcription 3 (STAT3) (Huynh et al., 2017). The janus kinase (JAK)1/2-STAT3 signaling pathway is one linker of inflammation and fibrosis, as its activity contextually intersects with TGF β signaling (Sarközi et al., 2011; Zehender et al., 2018). In dermal fibroblasts it promotes profibrogenic activities, including synthesis of fibrillar collagens (Kuzet and Gaggioli, 2016b; Lim et al., 2006; Zehender et al., 2018). Targeting JAK1/2 and STAT activity using e.g. ruxolitinib/Jakavi is effective against myelofibrosis (Verstovsek et al., 2010). Paradoxically, selective

STAT-3 activation in macrophages may protect against fibrosis (Do et al., 2018). Thus, the pro- or antifibrotic benefits of STAT3 targeting are context-dependent and cannot be generalized for all conditions and settings.

Here we first showed that the epidermis and the dermis display elevated levels of phospho-STAT3 (pSTAT3) in DEB mouse skin. Targeting STAT3 activity in vitro with the clinically approved JAK1/2 inhibitor ruxolitinib reduced pro-fibrotic and cancer-promoting activities in human dermal RDEB fibroblasts and keratinocytes, respectively. Next, we proceeded to evaluate ruxolitinib for treatment of DEB mice at preclinical level. Topical application of ruxolitinib onto wounds, in accordance with the proven importance of STAT3 activity for skin regeneration (Macias et al., 2013; Sano et al., 1999), delayed macroscopic wound closure and re-epithelialization. Importantly, also dermal healing was reduced, suggesting antifibrotic potential of ruxolitinib in vivo. In a short-term and well-controlled setting, topical application of ruxolitinib reduced fibrosis in repeatedly injured back skin of DEB mice. However, long-term treatment with ruxolitinib of forepaws disclosed no discernable antifibrotic activity on naturally progressing fibrosis. Taken together, our study suggests that for DEB the antifibrotic activities of ruxolitinib are weak. Nevertheless, given the multiple benefits of targeting JAK1/2-STAT3 activities in the setting of cancer, safer and more directed compounds could be considered as stage-dependent, symptom-relief therapies for DEB.

Results

STAT3 activity is increased in DEB mouse skin

Our previous studies had shown elevated pSTAT3 in keratinocytes in human and murine DEB wounds (Nyström et al., 2013). Although large skin wounds and erosions are rarely observed in DEB mice, subclinical blistering is prominent by histological analysis. The blistering becomes more restricted after growth of fur and, thus, blistering-evoked activation of signaling pathways could be expected to subside in fur-covered back skin. We analyzed pSTAT3 in back skin from nude newborn and fur-covered 2-week-old wild-type and DEB mouse littermates. Western blots showed elevated pSTAT3 levels in DEB mice of both ages (Figure 1a). However, there was a tendency to more enhanced pSTAT3 in the less stable back skin from newborn DEB mice. To obtain information of the subtissue localization of pSTAT3 in areas undergoing active fibrosis we analyzed forepaws from 10-week-old mice (Nyström et al., 2015) by immunofluorescence staining. The staining showed that pSTAT3 was enhanced in both the epidermis and the dermis (Figure 1b).

In vitro studies on fibroblasts and keratinocytes reveal potential dual benefit for DEB of STAT3 targeting via ruxolitinib

Dermal STAT3 activity was interesting as it posited it to be involved in profibrotic fibroblast activation and potentially a target to limit fibrosis in DEB. Consequently, next we assessed the effect of interfering with STAT3 in dermal fibroblasts. For direct translational relevance we chose to target STAT3 activity with the clinically-approved JAK1/2 inhibitor ruxolitinib/Jakavi. Activity of the signal transducer JAK1/2 is essential for phosphorylation of STAT3, leading to its nuclear localization and activation of transcription. One-day treatment of human and murine dermal DEB fibroblasts with ruxolitinib led to potent and dose-dependent reduction in pSTAT3 (Supplemental Figure S1a and b). Functionally, ruxolitinib significantly impaired contraction of free-floating collagen lattices populated with human DEB dermal fibroblasts, pointing to its potential in reducing dermal fibrosis (Supplemental Figure S1c).

We next turned to evaluating JAK1/2 targeting via ruxolitinib in the setting of cSCC, which is the main cause of death in the severe forms of DEB (Fine et al., 2009). Previous work from others and us has

shown that progression of the extremely aggressive cSCCs in DEB is to a large extent driven by injury- and inflammation-induced fibrotic remodeling of the dermal ECM (Föll et al., 2018; Guerra et al., 2017; Mittapalli et al., 2016; Ng et al., 2012b). Increased JAK1/2-STAT3 activity is a common feature of solid tumors (Huynh et al., 2017), including skin carcinomas (Macias et al., 2013). Elevated STAT3 activity may confer several tumor progression-promoting activities; in tumor cells it promotes survival and proliferation, whilst in the tumor microenvironment it activates stromal cells to evoke tumor invasion-supporting ECM remodeling and to quench antitumor immunity (Huynh et al., 2017). Thus, targeting elevated STAT3 activity could on multiple levels delay the progression of DEB cSCCs.

To evaluate the ability of ruxolitinib to counteract invasion-promoting changes in the dermal microenvironment we employed 3D organotypic co-cultures composed of DEB cSCC keratinocytes and DEB fibroblasts (Mittapalli et al., 2016). In this system, the invasion of cancer keratinocytes into the dermal stroma is driven by fibroblast-mediated ECM remodeling (Mittapalli et al., 2016). As expected from its fibroblast deactivating properties (Supplemental Figure S1), ruxolitinib strongly and significantly reduced DEB cancer keratinocyte invasion (Figure 2a and b).

Next, we studied the effect of ruxolitinib on keratinocytes. While JAK1/2-STAT3 axis activity promotes carcinoma progression (Huynh et al., 2017), its activity can also be tumor initiating (Bromberg et al., 1999). We focused our studies on non-tumor keratinocytes – representing early events of tumor initiation. A recent study implicated mutagenic events from activity of the cytidine deaminases apolipoprotein B mRNA editing enzyme catalytic polypeptide-like (APOBEC) protein family in the initiation of DEB cSCCs (Cho et al., 2018). The APOBECs constitute an important cell-intrinsic defense to viral challenges (Stavrou and Ross, 2015). Although the exact triggers of APOBEC upregulation in DEB keratinocytes are unknown, inflammatory processes are likely involved and the transcription factor Nuclear Factor kappaB (NF- κ B) has been shown to promote expression of various APOBECs (Tilborghs et al., 2017). STAT3 supports NF- κ B-promoted gene expression (Lee et al., 2009). To investigate a possible link between JAK1/2-STAT3 activity and APOBEC expression we treated E6/E7 immortalized keratinocytes from donors with DEB with 0.5 μ M ruxolitinib for 24h and analyzed the

expression of *APOBEC3A*, which was reported to be the most prominently increased APOBEC in DEB cSCCs (Cho et al., 2018). Notably, ruxolitinib led to a strong downregulation of *APOBEC3A* expression (Figure 3a).

JAK1/2-STAT3 axis activity also promotes expression of immune checkpoint inhibitor proteins, including programmed death ligand 1 (PD-L1/CD274) (Prestipino et al., 2018), to create an immune-evasive, tumor-accommodating microenvironment. PD-L1 is one effective target of immunotherapies for SCCs (Seiwert et al., 2016). DEB keratinocytes grown for 24h in the presence of 0.5 μ M ruxolitinib significantly reduced gene expression of *PD-L1* (Figure 3b). Importantly, this resulted in decreased abundance of PD-L1 protein on DEB keratinocytes (Figure 3c and d). To conclude, in the context of DEB cSCC initiation and progression, targeting the JAK1/2-STAT3 axis may have multiple synergistic benefits, since it could counteract cell self-intrinsic tumor-initiating mutational activities and the creation of an immune-evasive and biomechanically tumor-permissive microenvironment.

Ruxolitinib downmodulates healing activities of acute wounds in the DEB mouse

To assess the in vivo aptitude of ruxolitinib as an antifibrotic, symptom-relief therapy for DEB, we started with investigating its effect on cutaneous wound healing. DEB is associated with a very high wound burden, and a potential therapy should not majorly obstruct wound healing. For ruxolitinib this was a particular concern since epidermal JAK1/2-STAT3 activity is essential for re-epithelialization (Sano et al., 1999). One case report has also suggested development of foot ulcers to be attributed to ruxolitinib treatment (Del Rosario et al., 2018). On the other hand, ruxolitinib protects hair follicle stem cells from destructive immunity occurring in the setting of graft versus host disease (Takahashi et al., 2018), and it was also reported to promote graft take and healing of a highly-inflamed chronic leg ulcer (Shanmugam et al., 2013). Thus, the effect of ruxolitinib on wound closure may be context dependent and governed by factors including inflammation and the preservation status of the skin stem cell compartments. Healing of excision wounds in DEB mice replicates many features of naturally-occurring DEB wounds (Nyström et al., 2013) and therefore we assessed the outcome of ruxolitinib treatment on healing of excision wounds in DEB mice.

Directly after wounding and every day until the end of observation (day 15) the wounds received topical treatment with either 15 mg/ml ruxolitinib in DMSO or DMSO alone. Ruxolitinib delayed macroscopic wound healing (Figure 4a and b). Importantly, however, although delayed all, ruxolitinib-treated wounds healed. The timing of the delay implicated reduced re-epithelialization speed after ruxolitinib exposure. Indeed, histological analyses showed significantly shorter length of epithelial wound tongues in early and later stages of re-epithelialization (Figure 4c and d). These findings are consistent with that mice with epidermal deficiency of Stat3 show severely protracted wound re-epithelialization (Sano et al., 1999). Re-epithelialized wounds at day 14 displayed thicker epidermis after ruxolitinib, which was an additional evidence of delayed re-epithelialization and subsequent epidermal maturation after wounding (Figure 4e). The histological analyses disclosed that ruxolitinib alleviated the enhanced inflammation of DEB wounds (Figure 4e) (Kühl et al., 2015; Nyström et al., 2013). Importantly, staining for α -smooth muscle actin revealed that ruxolitinib delayed the occurrence and subsequent loss of myofibroblasts in the granulation tissue (Figure 4f). The ability to reduce the speed of dermal healing pointed to antifibrotic activities of ruxolitinib in vivo in DEB skin.

Ruxolitinib is protective against progression of expedited, experimentally-induced dermal fibrosis in DEB mice

To obtain direct support of the antifibrotic potential of ruxolitinib we then tested the effect of ruxolitinib on injury-induced dermal remodeling in a controlled setting. The back skin of DEB mice usually meets few frictional challenges, is stabilized by fur and is consequently protected against development of dermal fibrosis (Nyström et al., 2015). In order to evoke fibrotic remodeling of back skin we applied standardized frictional challenges by inducing blisters through rubbing with a rubber eraser (Figure 5a). After blister induction, 15 mg/ml ruxolitinib in DMSO or DMSO alone was applied topically daily at the site of the blister. Three days after blister induction the skin was re-challenged to induce blisters and the cycle repeated nine times (Figure 5a). In total the mice were treated with ruxolitinib for 30 days.

The mice tolerated the treatment well. Ocular inspection revealed less prominent scarring of healed blisters treated with ruxolitinib (Figure 5b). Histological analysis showed blistering in both treatment groups and unexpectedly inflammatory infiltrates were still highly present in ruxolitinib-treated skin (Figure 5b). Antibody staining confirmed significant reduction of pSTAT3 after ruxolitinib exposure (Figure 5c). Importantly, picrosirius red stained skin sections visualized under polarizing light, revealed significant reduction of the number of parallelly aligned and/or thickened collagen fibrils in blistered skin receiving ruxolitinib (Figure 5d). Thus, in the setting of induced and expedited fibrosis, a relatively short treatment with ruxolitinib showed protection against fibrotic remodeling of the dermal microenvironment.

Ruxolitinib is minimally protective against naturally-progressing DEB fibrosis

After having obtained support of targeting JAK1/2-STAT3 to limit fibrosis in DEB in vitro and in vivo, we proceeded to assess its effect on development of mutilating deformities of forepaws, which is driven by fibrosis (Nyström et al., 2015). We decided to apply ruxolitinib in a DMSO solution topically onto forepaws, as the highly fragile oral mucosa of DEB mice made systemic delivery via oral gavage unfeasible. 25 μ l ruxolitinib in DMSO at a concentration of 15 mg/ml was applied topically daily on forepaws for 80 days, starting from when the mice were 1 month old. Per mouse one paw received ruxolitinib and one paw control treatment with DMSO only.

Unfortunately, the mice did not tolerate this treatment well. The mice showed reduced activity and deterioration in the general health. Five out of six mice had to be sacrificed before the planned end of the treatment. In none of the mice we observed clear protection of ruxolitinib against formation of mutilating deformities (as an example the forepaws from the mouse that had undergone treatment for the full 80 days is shown, Figure 6a). Importantly, the lack of efficacy did not appear to be connected to insufficient exposure to ruxolitinib, as pSTAT3 was significantly reduced in ruxolitinib-treated compared to DMSO-treated paws (Figure 6b). Histological analyses revealed no apparent reduction in inflammation or changes in the arrangement of collagen fibrils, as revealed by H&E staining and picrosirius red staining visualized under polarizing light, respectively (Figure 6c and d).

Discussion

Our study illustrates the trial of developing therapies for genetic skin diseases, and the necessity for careful, multi-contextual examination of their benefits. Specifically for DEB, the opposing needs of promoting wound healing and skin regeneration, whilst reducing scarring and fibrosis and not causing enhanced keratinocyte activation to risk epidermal malignancy, pose a substantial challenge. Ruxolitinib reduced scarring in a controlled setting but also delayed wound closure. However, its ability to quench epidermal activities appeared favorable in the context of tumor initiation and progression. Thus, systemic application of ruxolitinib or other safer compounds targeting the JAK1/2-STAT3 axis are not candidates for general management of symptoms in DEB. Nevertheless, they could be used for targeting selected events during disease progression – especially those linked to carcinoma progression.

There are however obvious challenges in targeting STAT3 for the treatment of, or the prevention of the development of solid tumors. On the one hand, limiting tumor-promoting ECM remodeling in the microenvironment and reducing tumor-promoting inflammation appear beneficial. Indeed, there are indications that ruxolitinib treatment improves the response to capecitabine in advanced pancreatic adenocarcinoma in a subset of patients with evidence of high levels of systemic inflammation (Hurwitz et al., 2015). An additional benefit would be the increased recognition by the immune system of tumor cells through downregulation of PD-L1 after STAT3 targeting. On the other hand, the immunosuppressive effects of interfering with the JAK1/2-STAT3 axis are potential drawbacks. It is known that general immunosuppressive therapy increases the risk of developing skin cancers (DePry et al., 2011). Ruxolitinib has been reported to interfere with natural killer cell function (Schönberg et al., 2015) and thus hamper antitumor immunity and making patients more susceptible to infections. The latter is a particular concern for DEB, as loss of lymphoid collagen VII leads to impairment of antibacterial immunity (Nyström et al., 2018). In line with this, shotgun proteomics on human cSCCs suggested a positive correlation of bacterial challenges with the conversion to high-risk cSCCs (Föll et al., 2018).

As for improving treatment of DEB, a better understanding of the molecular processes involved in the specific aspects of disease progression is needed. At the moment these processes are rather broadly defined – we know that injury, inflammation and stiffening are involved (Guerra et al., 2017; Nyström and Bruckner-Tuderman, 2018), but detailed knowledge of these events in time and space are missing. In addition, the extracutaneous functions of collagen VII need to be considered. Together this could allow for disease stage-specific treatments, and combinations of small molecule drugs or small molecule drugs with a causal therapy approach to optimize the benefit of therapies.

In summary, targeting elevated JAK1/2-STAT3-axis activity in DEB skin could be effective against specific events during fibrosis and SCC progression. However, the observed delay in skin wound healing, and reported immunosuppressive properties caution the clinical testing of such drugs at the moment, and call for a better understanding of mechanisms involved in progressing DEB.

Materials and Methods

More information about the materials and methods used in the study can be found in the supplemental information.

Ethics statement

All animal experiments were approved by the regional ethics review board (Regierungspräsidium Freiburg) approval numbers: G14/90, G14/93 and G16/17. The collagen VII hypomorphic mouse model has been described before (Fritsch et al., 2008). Mice were housed in a special-pathogen-free facility with 12:12 h light-dark cycle, and food, supportive nutrition and water *ad libitum*.

Antibodies and reagents

Antibodies used were: Phospho-Stat3 (Tyr705) (D3A7) rabbit monoclonal antibody #9145, Stat3 (124H6) mouse monoclonal antibody #9139, PDL1 (E1L3N) rabbit monoclonal antibody # 13684 from Cell Signaling, β -tubulin rabbit polyclonal antibody (ab6046) from Abcam, α -Smooth Muscle mouse monoclonal clone 1A4 and β -actin mouse monoclonal antibody (A5441) from Sigma-Aldrich. Ruxolitinib/Jakavi was purchased from Novartis.

Cells

Fibroblasts from newborn collagen VII hypomorphic (DEB mice) was isolated as previously described (Nyström et al., 2013) and cultured in DMEM:F12 supplemented with 10% FCS and antibiotics (Thermo Fischer Scientific). Human dermal fibroblasts (2 donors) and keratinocytes (4 donors) were from donors with molecularly confirmed complete collagen VII deficient recessive DEB. Generation of HPV16 E6/E7 immortalized keratinocytes have previously been described (Thriene et al., 2018). Human fibroblasts were cultured in DMEM + 10% FCS and keratinocytes in KGM + supplements (Thermo Fischer Scientific).

3D organotypic co-cultures

The RDEB cancer keratinocytes used for the 3D organotypic cultures have previously been described (Mittapalli et al., 2016) and the co-cultures were assembled, executed and invasion index analyzed in accordance with the same publication. Ruxolitinib in a concentration of 1 μ M dissolved in DMSO was given fresh, daily to the cultures; control cultures received an equal volume of DMSO.

PCR

mRNA was isolated using RNeasy Mini Kit (Qiagen) and transcribed to cDNA using First Strand cDNA Synthesis Kit (Thermo Scientific). Real-time quantitative PCR was performed using SYBR-green detection (Bio Rad) on a C100 Thermal Cycler-CFX96 Real-Time System (Bio Rad). The primers used were: *GAPDH* forward GGCCTCCAAGGAGTAAGACC; *GAPDH* reverse AGGGTCTACATGGCAACTG; *PD-L1 (CD274)* forward TGGCATTGCTGAACGCATTT; *PD-L1 (CD274)* reverse TGCAGCCAGGTCTAATTGTTTT; *APOBEC3A* forward TGGCATTGGAAGGCATAAGAC; *APOBEC3A* reverse TTAGCCTGGTTGTGTAGAAAGC.

Statistics

Statistical analysis was performed using the GraphPad Prism 5.03 software. The data were tested for normal distribution and equal variance, and analyzed using Student's unpaired or paired *t*-test.

Conflict of interests

The authors report no conflict of interest.

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Figure legends

Figure 1 STAT3 is activated in DEB mouse skin epidermis and dermis. (a) Western blotting of whole-skin lysates from newborn and 2-week-old wild-type and DEB mouse littermates. The blot was probed for pSTAT3, total STAT3 and β -tubulin to ensure equal loading. (b) Staining of sections of forepaws from 10-week-old wild-type and DEB mice for pSTAT3 (green) and DAPI (blue) to visualize nuclei. Top shows staining in the epidermis and bottom shows staining in the dermis. Scale bar = 50 μ m.

Figure 2 Reduced invasion of DEB cancer keratinocytes in 3D organotypic skin after treatment with ruxolitinib. (a) Hematoxylin (H&E) and keratin 14 (green) staining of sections of 3D organotypic skin co-cultures composed of DEB cancer keratinocytes and DEB fibroblasts treated with control (DMSO) or ruxolitinib (1 μ M) for three weeks. (b) Quantification of the invasion index as described in (Mittapalli et al. 2016). Scale bar = 100 μ m, *** $P < 0.001$; values represent mean \pm S.E.M.

Figure 3 Targeting JAK1/2-STAT3 axis with ruxolitinib reduces expression of cancer-promoting associated genes and proteins in keratinocytes. (a,b) RT-PCR analysis for *APOBEC3A* and *PD-L1* in immortalized DEB keratinocytes \pm 0.5 μ M ruxolitinib for 24h. (c) Western blot of DEB keratinocytes treated as in a for pSTAT3, STAT3, PD-L1 and β -actin to ensure equal loading. (d) Quantification of PD-L1 abundance from blots as in c. N = 4, * $P < 0.05$; values represent mean \pm S.E.M.

Figure 4 Ruxolitinib delays healing of excision wounds in DEB mice. (a) Photographs of DMSO or ruxolitinib-treated wounds directly after injury and at indicated timepoints to day 12. The wound edges are marked with a white dotted line. (b) Quantification of the macroscopic wound healing; six wounds per group were quantified. (c) Hematoxylin and eosin (H&E) staining of sections of wounds three days after wounding. The black arrows indicate start and end of epithelial tongue and the epidermal tongue is underlined with a red dotted line. (d) Quantification of epithelial tongues as shown in c for the indicated timepoints; 6 epithelial tongues per timepoint were quantified. (e) Hematoxylin and eosin

(H&E) staining of sections from wounds fourteen days after wounding. The black double-headed arrows indicate, thicker epidermis in wounds treated with ruxolitinib. Note the notably lower inflammation in wounds that had received ruxolitinib. (f) Staining of α -smooth muscle actin (α SMA, red) positive fibroblasts in 5, 9 and 12 days after wounding in wounds treated with DMSO or ruxolitinib. Scale bars = 200, 100 or 50 μ m as indicated, $*P < 0.05$; $*P < 0.01$, values represent mean \pm S.E.M.

Figure 5 Ruxolitinib treatment does not majorly delay formation of fibrotic mutilating deformities in DEB mice. (a) Photographs of ruxolitinib- and DMSO-treated forepaws at the start and after treatment for 80 consecutive days. (b) Left, staining for pSTAT3 in sections of forepaws after treatment with ruxolitinib or DMSO for 80 days. Right, quantification of staining of four ruxolitinib-treated and four DMSO-treated paws. (c) Hematoxylin and eosin (H&E) staining of paws in b. (d) Left, picrosirius red (PSR) staining visualized under polarizing light of sections of forepaws after treatment with ruxolitinib or DMSO for 80 days. Right, quantification of the stained area after application of a digital threshold, of five ruxolitinib-treated and five DMSO-treated paws. Scale bars = 100 or 50 μ m as indicated, $*P < 0.05$, values represent mean \pm S.E.M.

Figure 6 Ruxolitinib treatment delays experimentally-induced dermal fibrosis in DEB mice. (a) Schematic of the treatment protocol. (b) Top, photographs of ruxolitinib- and DMSO-treated back skin after treatment for 30 consecutive days. Bottom, H&E staining of skin sections from skin as presented in the top panel. Asterisks indicate signs of inflammation in the ruxolitinib-treated blisters. Note that ruxolitinib does not protect against skin blistering (c) Left, staining for pSTAT3 of sections of skin after treatment with ruxolitinib or DMSO for 30 days. Right, quantification of staining of four ruxolitinib-treated and four DMSO-treated back skins. (d) Left, picrosirius red (PSR) staining visualized under polarizing light of sections from skin treated with ruxolitinib or DMSO for 30 days. Right, quantification of the stained area after application of a digital threshold, of three ruxolitinib-treated and three DMSO-treated paws. Scale bars = 200 or 100 μ m as indicated, $*P < 0.05$, values represent mean \pm S.E.M.

Supplemental information

Supplemental figure S1 Ruxolitinib reduces STAT3 activity and gel contractile

Figure legends

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Supplemental information

Supplemental figure S1 Ruxolitinib reduces STAT3 activity and gel contractile activities in DEB

fibroblasts. (a) Western blots for pSTAT3 and β -tubulin on protein lysates from fibroblasts isolated from the back skin of a newborn DEB mouse and treated with two concentration of ruxolitinib for 24h as indicated. (b) Western blots as in a, on protein lysates from dermal fibroblasts from a human donor with DEB. The cells were treated \pm 0.5 μM ruxolitinib for 24h. (c) Top, photos human DEB fibroblast-populated free-floating collagen lattices \pm 0.5 μM ruxolitinib for 24h. Bottom, the quantification of the area of the collagen lattices treated as above. Values were normalized to the area of vehicle-treated lattices and represent mean \pm S.E.M.

Supplemental Materials and Methods

Treatment of mice

Collagen VII hypomorphic mice (DEB mice) were on a mixed C57BL/6N:129SV background (Nyström et al., 2015). For the experiments equal number of male and female mice was used, at the start of the experiments the mice were on average 5 weeks old. For treatment of forepaws, the mice were restrained, paws photographed. Per mouse, to one forepaw 25 μl 15 mg/ml ruxolitinib dissolved in DMSO was applied and to the other 25 μl DMSO. The DMSO was allowed to be absorbed before release of the mice. The application was repeated daily, for a planned time of 80 days, the paws were photographed every two weeks. Due to the adverse reactions to ruxolitinib, 6 mice received treatment before the decision was made to stop the study due to ethical concerns.

Blisters were induced on shaved back skin at the mid-back of the mice. The mice were sedated with isoflurane and the skin was rubbed five times with a rubber eraser until dermal-epidermal separation was macroscopically visible. The mice were divided into two groups, one group received 25 μ l 15 mg/ml ruxolitinib dissolved in DMSO onto the blister, the other group received 25 μ l DMSO. Ruxolitinib or DMSO was applied daily, the blisters were photographed daily. Re-blistering was performed every third day for a total of nine times before killing. Eight mice received ruxolitinib treatment and eight mice were treated with DMSO only.

Wounding was performed on the shaved back skin of isoflurane-sedated mice by marking the edges of a 6 mm punch biopsy tool and then cutting out the marked area with surgical scissors. Photographs were made immediately after wounding and daily thereafter. The macroscopic wound healing was calculated using Image J (NIH) as previously described (Nyström et al., 2013). Directly after wounding, the wounded mice were divided into two groups and the wounds in one group received 25 μ l 15 mg/ml ruxolitinib dissolved in DMSO daily topically onto the wound, the other group received 25 μ l DMSO daily, onto the wound. In total eight mice received ruxolitinib treatment and eight mice were treated with DMSO only.

After sacrificed the tissues were fixed in 10% formalin and processed for immunostaining and histological analyses.

Western blotting

Proteins from cultured cells were extracted with RIPA buffer and protein from whole skin after crushing of snap-frozen skin and boiling with Laemmli loading buffer containing 4M urea (Nyström et al., 2015). Western blotting was performed as previously described (Nyström et al., 2015).

Immunostaining and histological analyses

Picrosirius red staining was performed and analyzed as previously described (Mittapalli et al., 2016). For pSTAT3 staining, heat and citrate buffer-mediated antigen retrieval of paraffin sections was performed. Quantification of picrosirius red staining was performed using Image J as previously described (Nyström et al., 2015).

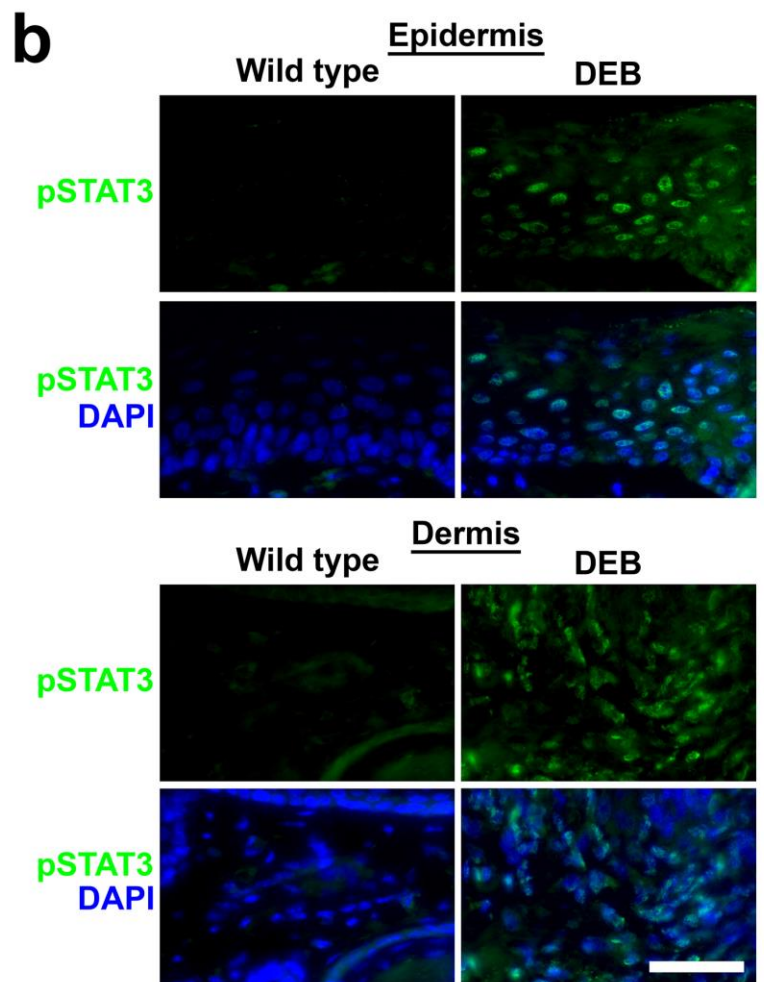
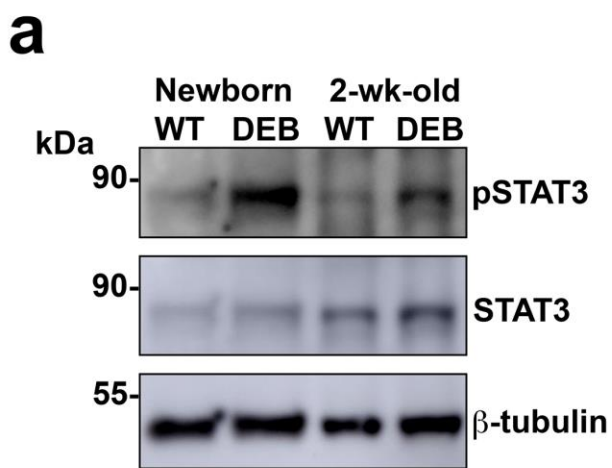
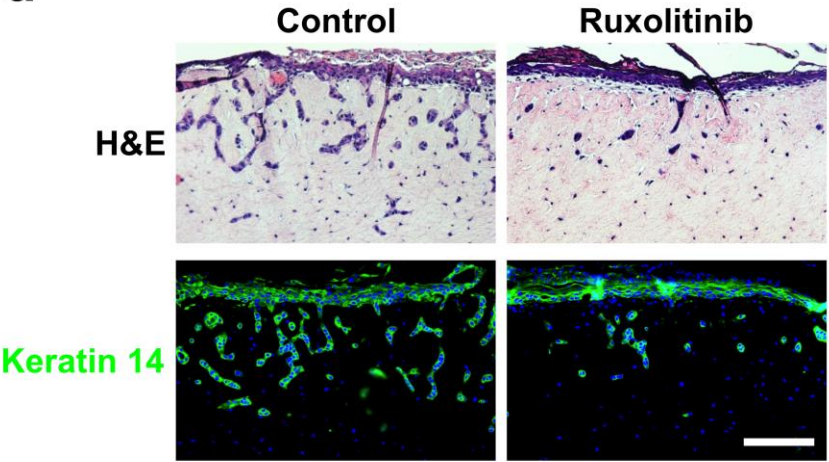


Figure 1

a



b

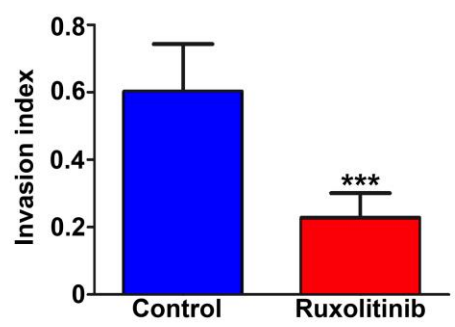


Figure 2

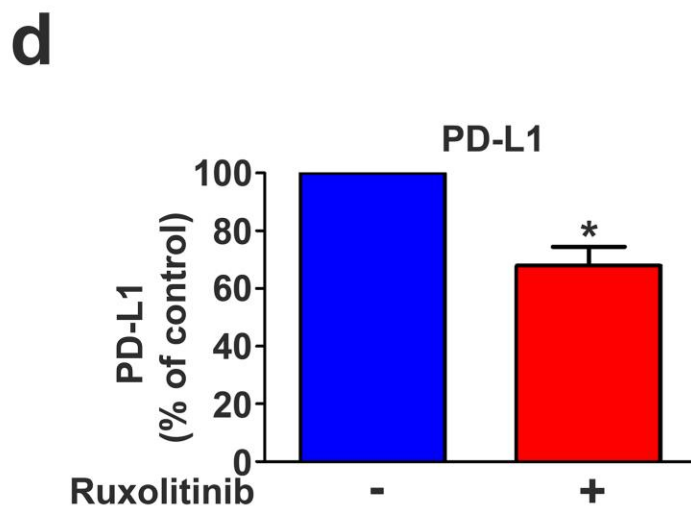
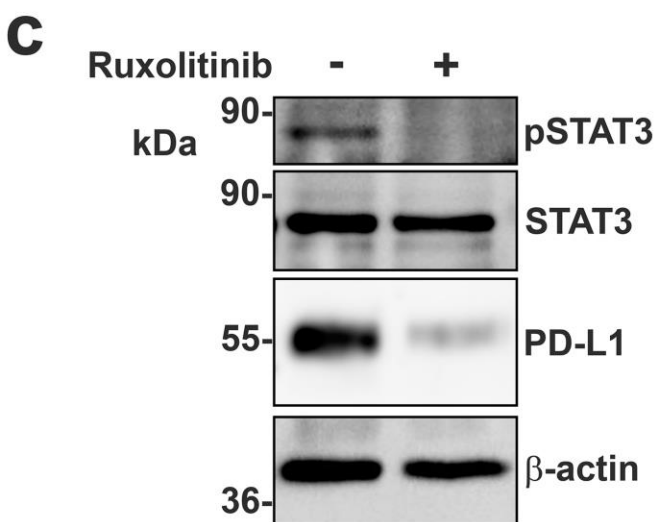
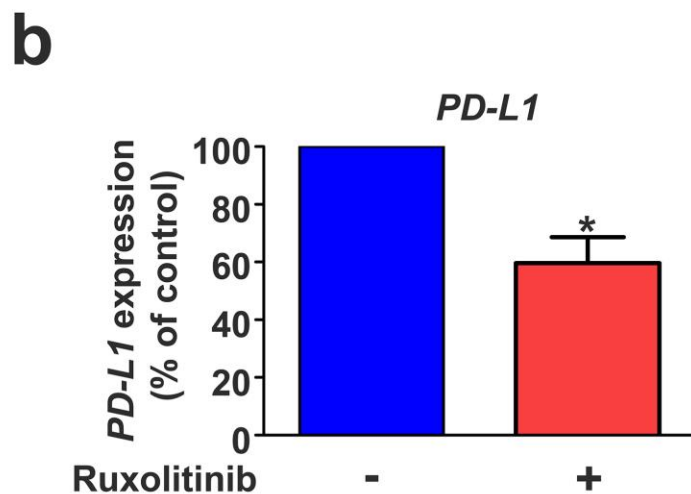
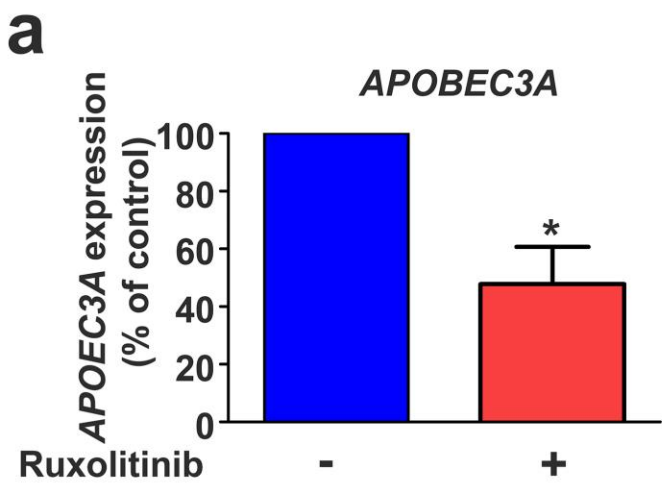
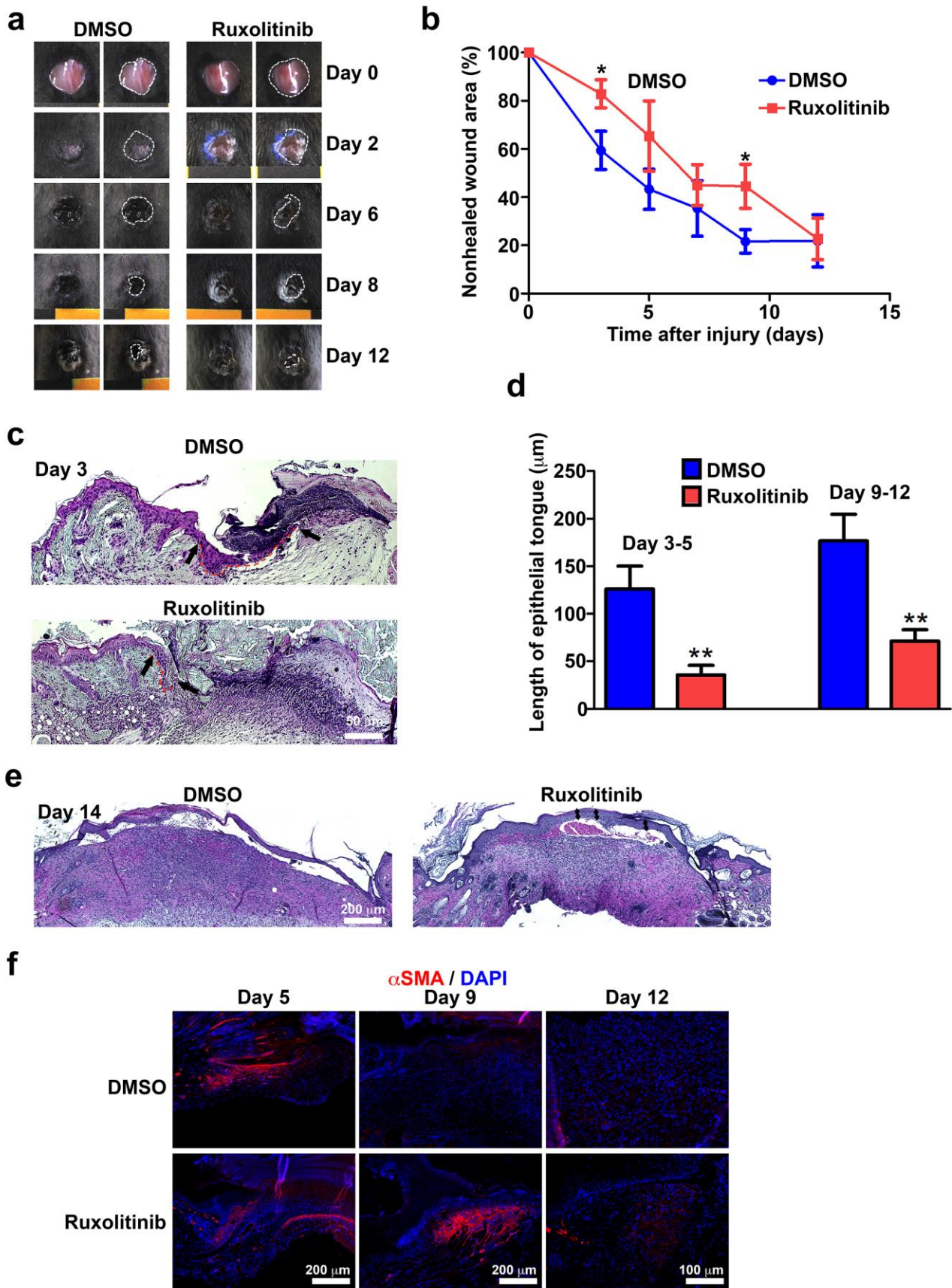


Figure 3



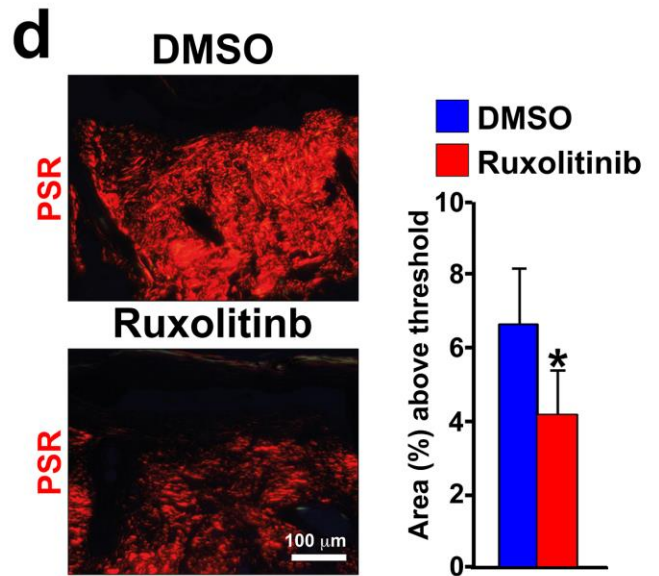
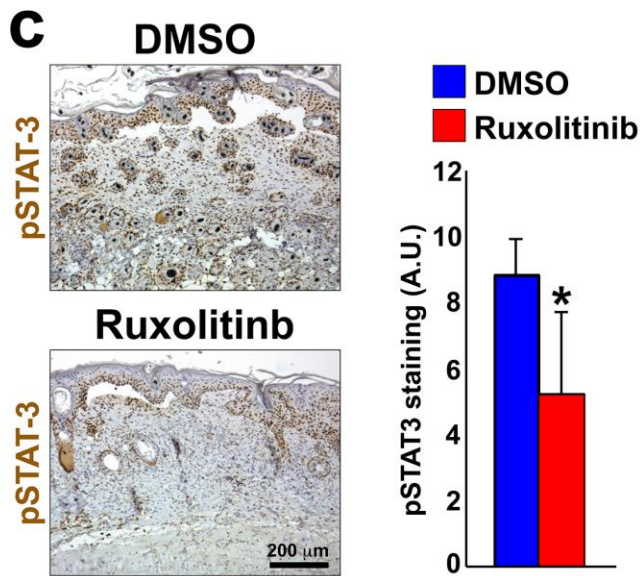
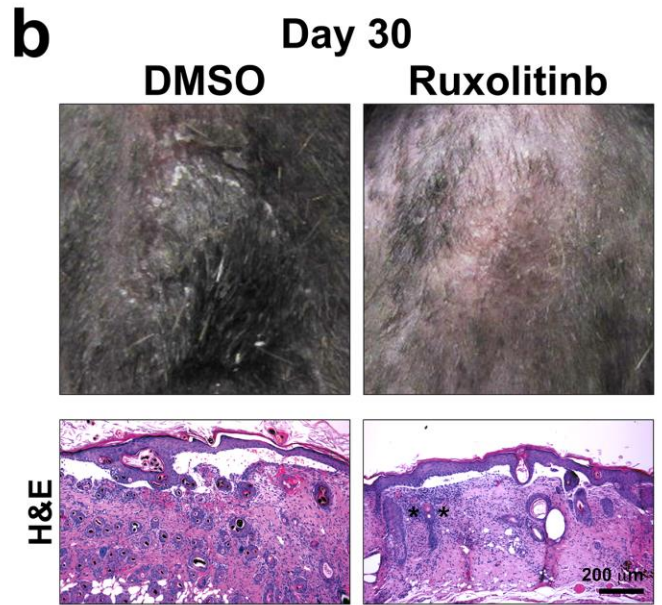
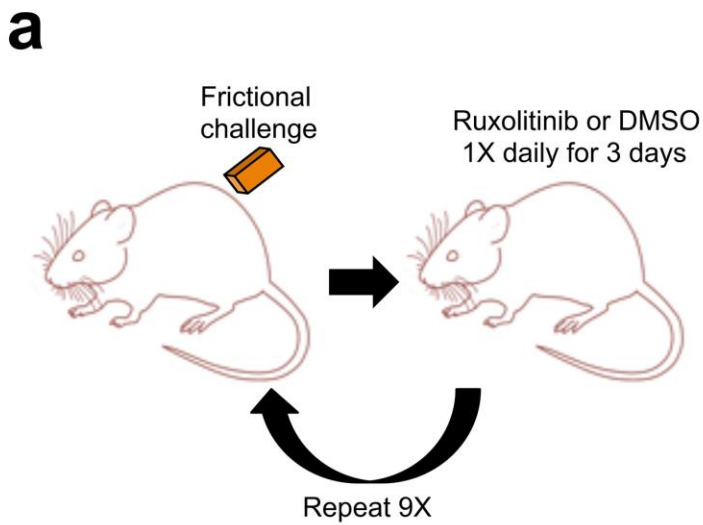


Figure 5

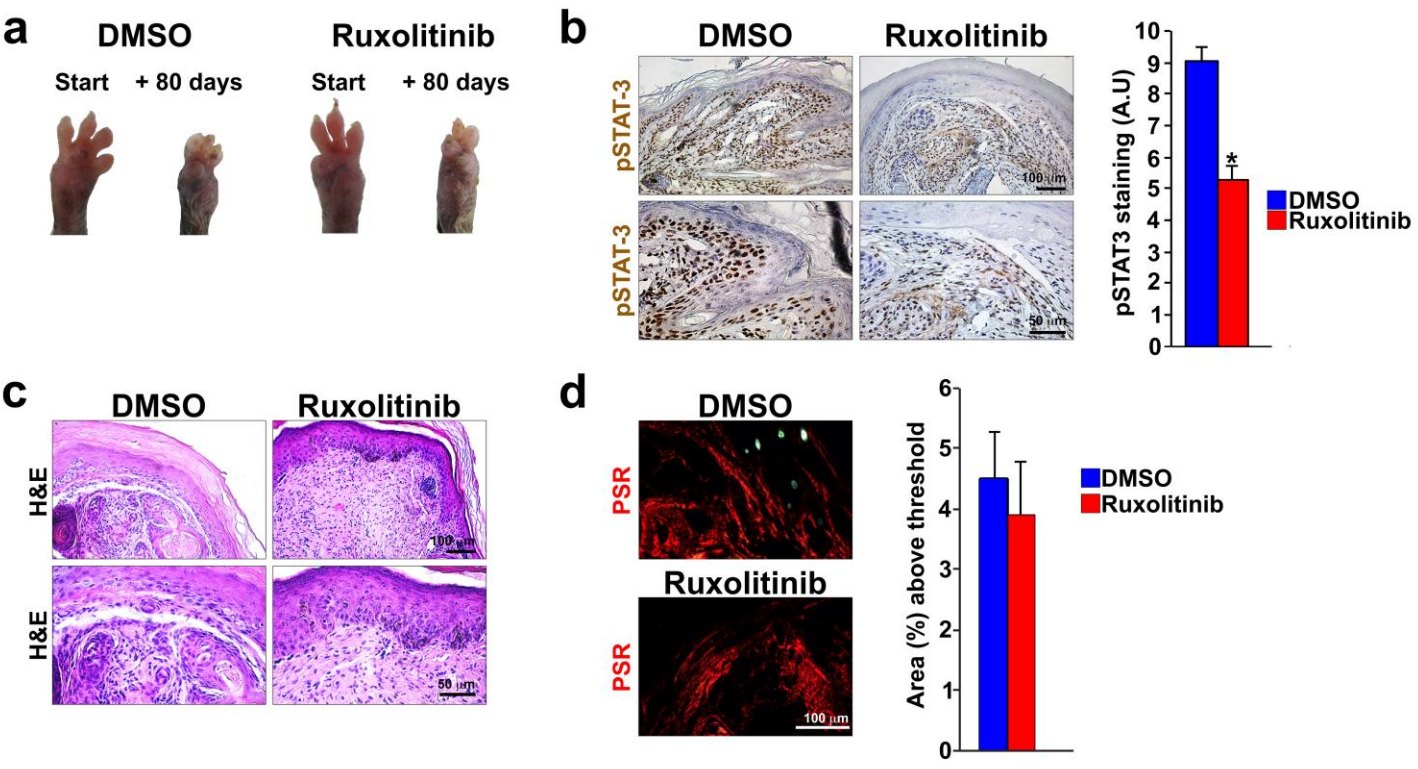


Figure 6

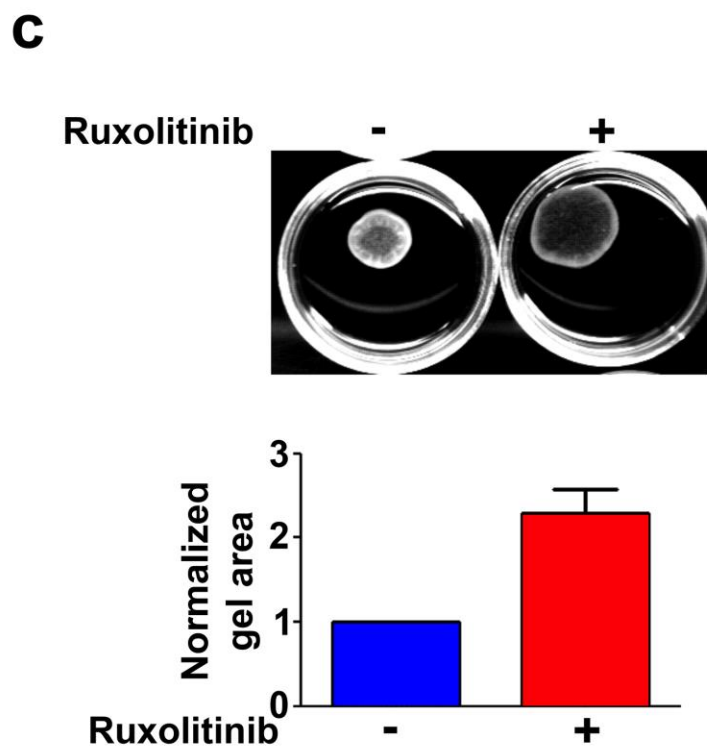
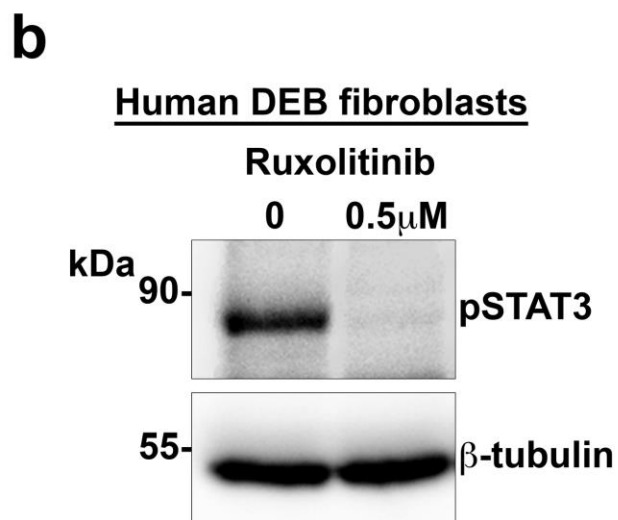
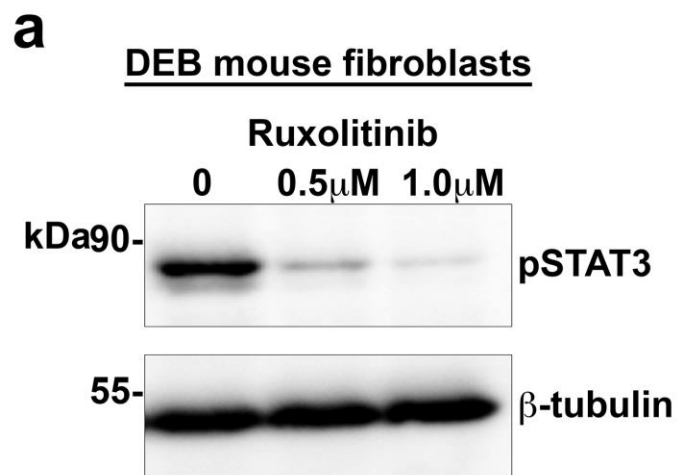


Figure S1

Membrane-bound ICAM-1 contributes to the onset of proinvasive tumor stroma by controlling acto-myosin contractility in carcinoma-associated fibroblasts

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ABSTRACT

Acto-myosin contractility in carcinoma-associated fibroblasts leads to assembly of the tumor extracellular matrix. The pro-inflammatory cytokine LIF governs fibroblast activation in cancer by regulating the myosin light chain 2 activity. So far, however, how LIF mediates cytoskeleton contractility remains unknown. Using phenotypic screening assays based on knock-down of LIF-dependent genes in fibroblasts, we identified the glycoprotein ICAM-1 as a crucial regulator of stroma fibroblast proinvasive matrix remodeling. We demonstrate that the membrane-bound ICAM-1 isoform is necessary and sufficient to promote inflammation-dependent extracellular matrix contraction, which favors cancer cell invasion. Indeed, ICAM-1 mediates generation of acto-myosin contractility downstream of the Src kinases in stromal fibroblasts. Moreover, acto-myosin contractility regulates ICAM-1 expression by establishing a positive feedback signaling. Thus, targeting stromal ICAM-1 might constitute a possible therapeutic mean to counteract tumor cell invasion and dissemination.

INTRODUCTION

Carcinoma-associated fibroblasts (CAF) are the most representative non-cancerous cell population of the tumor stroma. In several instances, presence of CAF dictates the tumor outcome [1–4]. CAF participate to all steps of carcinomagenesis, from tumor initiation to metastatic spreading in secondary organs, essentially by generating a proinvasive tumoral stroma that favors tumor cell propagation and dissemination from the primary tumor [5–8]. Indeed, secretion of inflammatory molecules, including chemokines of the IL6 family, triggers a proinvasive fibroblast activation. In such context, we have demonstrated the crucial role that Leukemia Inhibitory Factor (LIF) plays in the proinvasive ECM remodeling by inducing acto-myosin contractility in fibroblasts [9]. In fibroblasts, LIF activates the GP130/JAK1/STAT3 signaling pathway, which initiates tensile force generation through regulation of the RhoA/ROCK/MLC2 signaling

pathway. Moreover, in activated fibroblasts and CAF, constitutive activation and crosstalk of these two signaling pathways lead to the generation of fibrotic and tumorigenic cancer-associated ECM [10]. However, the LIF-dependent genes that mediate the crosstalk between inflammation and acto-myosin contractility in fibroblast remain to be identified.

Intercellular Adhesion Molecule 1 (ICAM-1), a member of the immunoglobulin superfamily, is a cell surface glycoprotein receptor for LFA-1 (Lymphocyte Function-associated Antigen 1) and MAC-1 (Macrophages Adhesion Ligand 1) integrins, but also for ECM proteins [11–16]. ICAM-1 is considered to be an inflammatory responsive gene, whose expression is highly induced in injured tissues [17, 18]. The membrane-bound ICAM-1 isoform is expressed at the cell surface of a variety of cell types including endothelial, epithelial, immune cells and fibroblasts [19–21], but it can also be found as a soluble secreted form (sICAM-1) [22–24]. ICAM-1 is mainly

responsible for intercellular adhesion and trafficking of inflammatory cells. Membrane-bound ICAM-1 engagement at the cell surface results in an out-side-in signaling triggered by the activation of specific tyrosine kinases, which, depending on the cellular contexts, leads to transcription factors activation, inflammation, production of reactive oxygen species and cell proliferation [19]. Membrane-bound ICAM-1 expression by colorectal cancer cells has been associated with reduced tumor cell dissemination and metastatic potential [25, 26]; whereas ICAM-1 expression by stromal fibroblasts suggests a tumor promoting effect potentially through increased monocytic cell recruitment to the tumor mass [27]. Yet, the mechanisms by which CAF-associated ICAM-1 acts as a tumor promoter remain unclear.

Using organotypic cell cultures submitted to three-dimensional collagen-rich contraction assays after RNAi-mediated knock down of LIF-responsive genes, we show that membrane-bound ICAM-1 triggers tumorigenic ECM remodeling in CAF and in fibroblasts undergoing activation by tumor cells. ICAM-1 is thus identified as a crucial regulator of the inflammation-dependent ECM remodeling in cancer. We demonstrate that ICAM-1 promotes proinvasive ECM remodeling through regulation of acto-myosin contractility. Moreover, inhibition of acto-myosin contractility in CAF induces a decrease of ICAM-1 expression, which suggests that a positive feedback signaling mediates excessive ECM deposition and fibrotic tissue formation in cancers. We also show that CAF are heterogeneous for the membrane-bound ICAM-1 expression at the cell surface, and that such expression is specific to sub-sets of contractile and proinvasive CAF populations. Finally, we demonstrate that ICAM-1 is expressed in the tumor stroma of human head and neck cancers in correlation with the presence of clusters of invasive cancer cells.

RESULTS

Long-term TGF β -activated fibroblasts display a LIF-dependent gene signature

To unveil the genes controlling acto-myosin cytoskeleton contractility in stromal fibroblasts and thus involved in generation of a proinvasive matrix, we first conducted a pan-genomic transcriptome analysis of human primary dermal fibroblasts (hDF) following a short- (48 hours) or long-term (15 days) *in vitro* stimulation by TGF β or LIF (Figure 1A). A short-term stimulation led to distinct transcriptomic changes with only 33 genes significantly modulated by LIF, while TGF β induced a wide transcriptomic response involving several thousand genes (Figure 1B and 1C). In fibroblasts stimulated by TGF β , a LIF blocking antibody (α LIF) failed to alter response to TGF β (Figure 1B and 1C). Conversely, in the case of the long-term stimulation, LIF or TGF β induced a

comparable signature (Figure 1D) with more than 1,000 genes significantly regulated by both factors (Figure 1E). However, addition of the LIF-blocking antibody completely inhibited the TGF β effect (Figures 1B, right panel and Figure 1C), which unveiled a transcriptomic switch from an early TGF β -specific to a long-term LIF-dependent gene signature. The pivotal role of LIF in the gene modulation associated with maintenance of the proinvasive phenotype acquired by the long-term TGF β -activated fibroblasts was thus demonstrated. We next assessed whether such a LIF-dependent gene signature is also shared by the CAF isolated from tissue biopsies of patients with head and neck, lung or breast carcinomas. A subset of 10 genes (eight up-regulated and two down-regulated) was selected, firstly to confirm the microarray data by qRT-PCR analysis in both TGF β and LIF activated fibroblasts (Supplementary Figure S1A) and secondly for comparative mRNA quantification analysis with CAF and the hDF control (Supplementary Figure S1B). qRT-PCR analysis confirmed that both LIF and TGF β regulate expression of all the 10 genes that, importantly, are similarly regulated in CAF. These findings demonstrate that the genes regulated by LIF in *in vitro* TGF β -activated fibroblasts are similarly regulated in CAF.

Membrane-bound ICAM-1 governs the onset of proinvasive ECM

Having demonstrated that LIF induces and sustains a contractile and proinvasive phenotype in activated fibroblasts [9, 28], we speculated that the genes essential for fibroblast acto-myosin cytoskeleton contractility, and thus for CAF-dependent proinvasive matrix remodeling, could be transcriptionally regulated by LIF. Accordingly, the LIF-blocking antibody is expected to inhibit contractility of *in vitro* long-term TGF β -stimulated fibroblasts. A three-dimensional RNAi-based phenotypic screening was thus set up to identify the genes governing onset of CAF-dependent proinvasive ECM remodeling that strongly correlates with matrix contraction [29]. In light of our results on the LIF-dependent up-regulation of fibroblasts and the inhibitory effect of the LIF-blocking antibody on TGF β -stimulated hDF, 50 genes were selected on the basis of their known or putative biological functions described in the literature on ECM remodeling, cytoskeleton organization, cell contractility, metabolism and transcription (Figure 1E in red and Supplementary Table S1A). Using hDF and CAF, three independent screenings were implemented to identify genes that specifically regulate the initiation and the maintenance phase of the cell contractility activation by LIF (Figures 2A-2C and Supplementary Table S1B-S1E). Thus, hDF were transfected with smart pools of RNAi targeting the 50 selected genes (Supplementary Table S2). Non-targeting RNAi were negative controls, while RNAi targeting the JAK1 kinase were positive controls. The next day, hDF

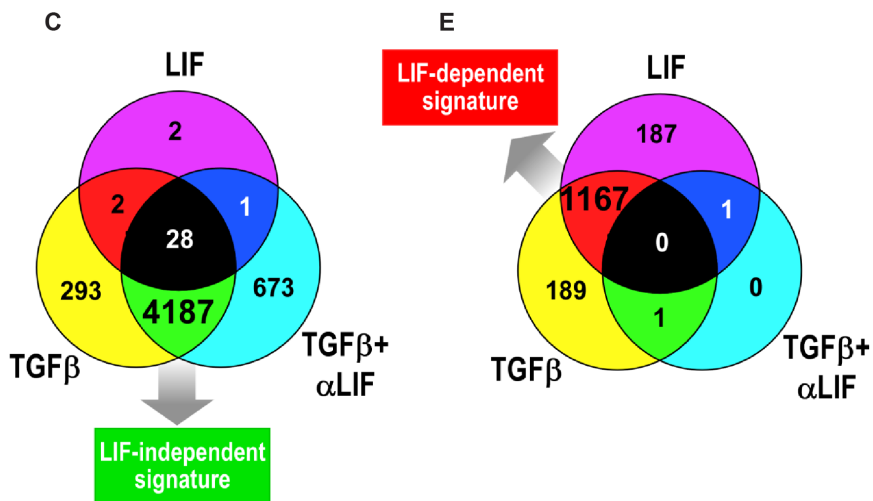
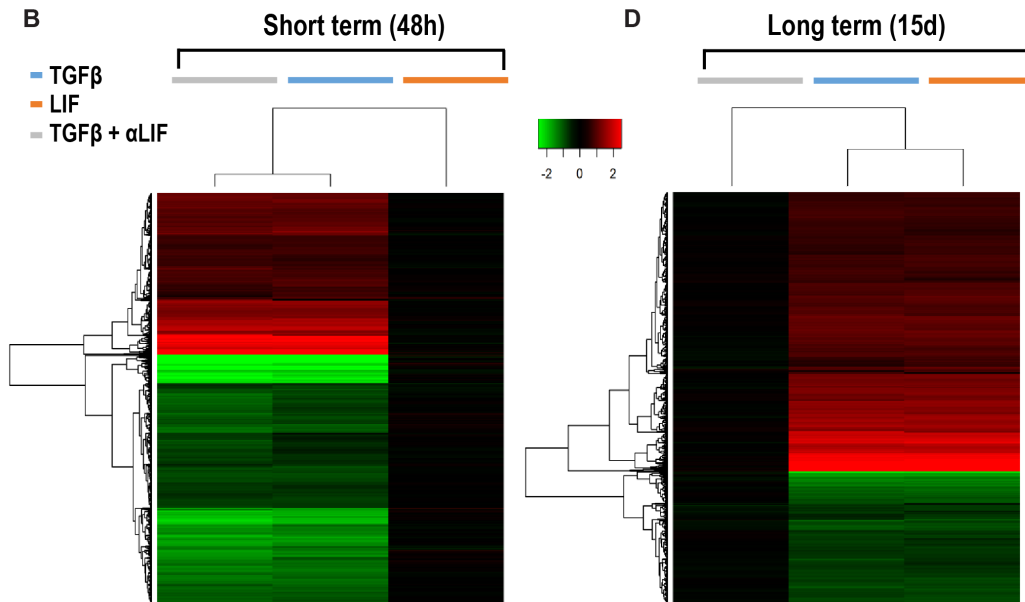
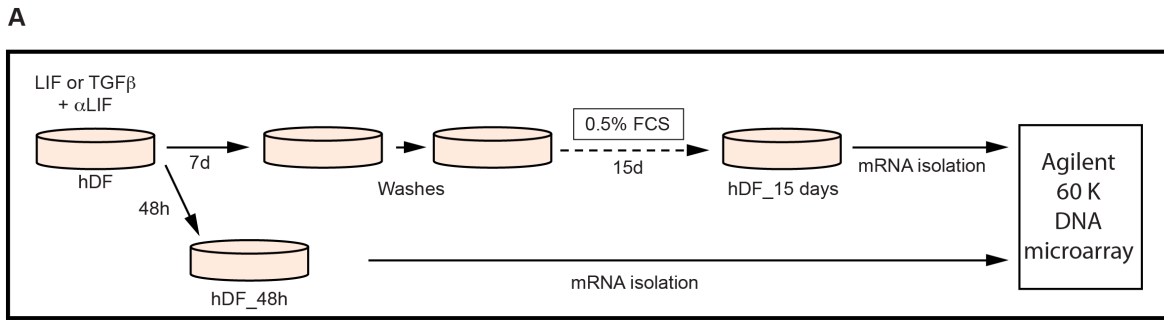


Figure 1: LIF supports long-term TGFβ-activated fibroblasts transcriptomic signature. **A.** Schematic representation of the experimental design of the short- or long-term hDF stimulation by LIF or TGFβ in presence or absence of LIF blocking antibody (αLIF). Short-term cytokines stimulation: **B.** Heatmaps comparing the normalized log₂ ratio between stimulated hDF versus control cells at short-term. **C.** Venn diagrams showing the overlapping set of genes regulated (both up-regulated and down-regulated) by the three experimental conditions at short-term. **D.** Heatmaps comparing the normalized log₂ ratio between stimulated hDF versus control cells at long-term. **E.** Venn diagrams showing the overlapping set of genes regulated (both up-regulated and down-regulated) by the three experimental conditions at long-term.

were embedded in a three-dimension collagen lattices, then low serum media supplemented with LIF was added. Six days later, gel contraction was quantified, which revealed four genes (HRH1, DBC1, BCL3 and ICAM-1) essential for initiation of LIF-dependent contractility in fibroblasts (Figure 2A, Supplementary Tables S1B and S1E). Next, the genes sustaining the contractile activity in long-term LIF-activated hDF were investigated. HDF were activated *in vitro* for 15-days, then transfected using the RNAi smart pools. Collagen lattice contraction and quantification were then assessed as above. Six genes (HRH1, DBC1, BCL3, ICAM-1, GGT5 and ANGPTL4) appeared to be crucial for the maintenance of the LIF-dependent contractility of hDF (Supplementary Figure S2A, Supplementary Tables S1C and S1E). To confirm the LIF-dependent gene signature and, more specifically, the role of the identified genes in CAF contractility, CAF isolated from human head and neck carcinoma were transfected using the 50 RNAi bank and embedded in collagen lattices 24 hours later. Six genes (HRH1, DBC1, BCL3, ICAM-1, ANGPTL4 and BCL2L14) resulted to be crucial for CAF-dependent collagen lattice contraction (Figure 2B, Supplementary Tables S1D and S1E). Interestingly, most of the genes found to be crucial in LIF-activated hDF also support CAF contractility. RNAi to genes HRH1, DBC1, BCL3, ICAM-1 consistently blocked the activated fibroblast matrix contraction with no significant impact on CAF viability (data not shown).

Because the membrane-bound adhesion molecule ICAM-1 may serve as a preferential target for immune-cancer therapies, its potential role in the CAF-dependent onset of a proinvasive ECM remodeling was further analyzed. We first confirmed that ICAM-1 is induced by both LIF and TGF β (Figure 2C) and showed that ICAM-1 expression in hDF is stimulated by tumor cells conditioned media (Supplementary Figure S2B). Interestingly, ICAM-1 appeared overexpressed in CAF isolated from head and neck, lung and breast carcinomas when compared to hDF (Figure 2D). The role of membrane-bound ICAM-1 in CAF-dependent SCC cell collective invasion was then assessed using organotypic three-dimensional invasion assays. Inhibition of ICAM-1 expression in CAF by specific knock-down expression by four independent RNAi oligonucleotides confirmed the involvement of ICAM-1 in matrix contraction (Figure 2E and Supplementary Figure S2C) and also revealed the crucial role for ICAM-1 in the onset of a proinvasive ECM remodeling (Figure 2F and Supplementary Figure S2C). Additionally, ICAM-1 was found to support LIF-dependent contractile and proinvasive fibroblast activation (Figures 2G-2H and Supplementary Figure S2D). Interestingly, interfering in both CAF and LIF-activated fibroblasts using a specific anti-ICAM-1 blocking antibody (α ICAM-1) dramatically reduced both collagen gel contraction and collective invasion of SCC12 cells (Supplementary Figures S2E-S2G). Taken together, these data identify membrane-

bound ICAM-1 as a crucial regulator of contractile and proinvasive CAF activities, and highlight a novel potential therapeutic target for the procarcinogenic activity of CAF in cancer development.

Membrane-bound ICAM-1 triggers inflammation-dependent cancerous ECM

ICAM-1 has been identified as an inflammatory responsive gene displaying increased expression in pathological tissues [21]. Therefore, we hypothesized that inflammation may induce development of a cancerous and proinvasive ECM *in vitro*. To verify this idea, hDF cells were grown in low serum media supplemented with pro-inflammatory cytokines known to play major roles in cancer development. All the tested pro-inflammatory cytokines, including TGF β , LIF, TNF α , GCSF and IL6, induced a strong ICAM-1 expression in hDF (Figure 3A) and proinvasive activation of normal fibroblasts, which resulted in invasion of SCC12 cells in organotypic invasion assays (Figure 3B). Moreover, RNAi-mediated silencing of ICAM-1 blocked the proinvasive activity of inflammation-activated fibroblasts (Figures 3B and Supplementary Figure S3A). This result demonstrates that membrane-bound ICAM-1 expression in fibroblasts supports the inflammation-dependent extracellular matrix remodeling and may drive inflammation-dependent fibrosis leading to organ failure in multiple pathologies [30].

To investigate whether ICAM-1 is sufficient to support fibroblast-dependent matrix remodeling and proinvasive activities, hDF constitutively expressing high level of membrane-bound ICAM-1 were generated (Figure 3C). Their contractile and proinvasive capacities *in vitro* were assessed using two independent hDF cell lines (hDF-ICAM-1-GFP#A and #B). ICAM-1 expression resulted sufficient to induce both matrix contraction (Figure 3D) and proinvasive activities (Figure 3E). Addition of an ICAM-1 blocking antibody during matrix contraction strongly decreased the contractile capacity of the ICAM-1 overexpressing fibroblasts to a level comparable to that of the control parental cell (Figure 3D). It is well established that CAF consist of highly heterogeneous subpopulations within the tumor, a heterogeneity conserved during culture *in vitro* [31]. Accordingly, heterogeneity of CAF for membrane-bound ICAM-1 expression was confirmed by FACS cell sorting (Supplementary Figure S3B), which allowed to investigate the possible correlation between the contractile and proinvasive capacities of CAF subpopulations with the levels of ICAM-1 expression at the cell surface (Supplementary Figure S3C). It could also be demonstrated that the ability of collagen contraction by CAF depends on the level of ICAM-1 expression (Supplementary Figure S3D) and that ICAM-1 expressing cells acquire proinvasive capacities compared

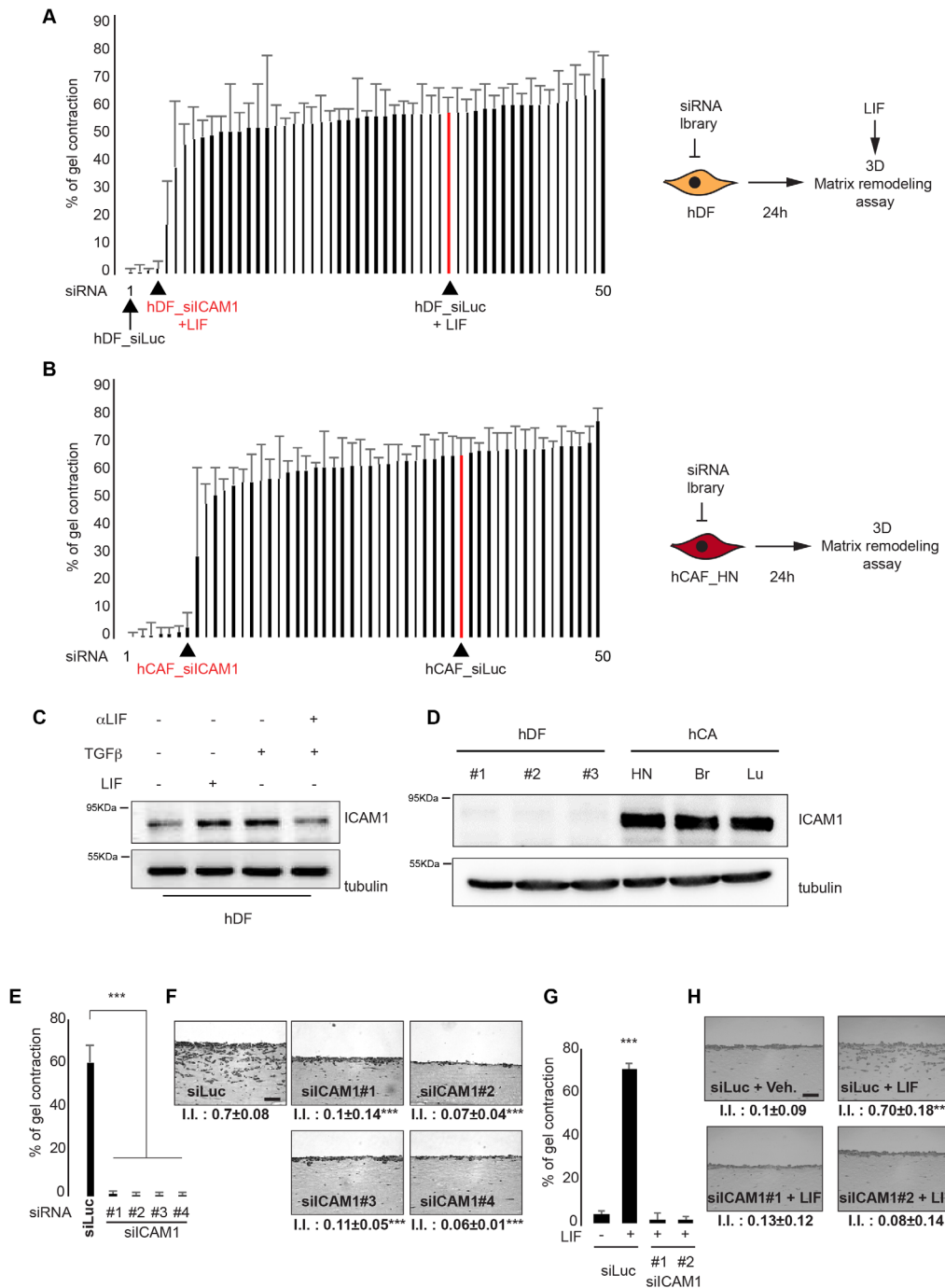


Figure 2: ICAM-1 controls proinvasive ECM remodeling. **A.** Percentage of gel contraction by hDF six days after RNAi transfection and subsequent LIF stimulation (left panel, n=2 in triplicates). Schematic representation of the experimental conditions (right panel). **B.** Percentage of gel contraction by CAF 6 days after RNAi transfection (left panel, n=2 in triplicates). Schematic representation of the experimental conditions (right panel). **C.** Immunoblot of ICAM-1 in hDF following LIF or TGFβ1 stimulation, in the presence (48h) or absence of the LIF blocking antibody. Immunoblot of tubulin was the internal control. **D.** Immunoblot of ICAM-1 in three hDF #1, #2 and #3) and in three CAF (Head and Neck, Breast and Lung). Immunoblot as control. **E.** Percentage of gel contraction by CAF transfected with control RNAi (siLuc) or RNAi targeting ICAM-1 (siCAM-1#1, #2, #3 and #4) (n=3 in triplicates, mean + s.d., ***p<0.001). **F.** Representative images of H&E coloration of paraffin-embedded sections of SCC12 3D-cultures in response to CAF transfected with control (siLuc) or targeting ICAM-1 (siCAM-1#1, #2, #3 and #4) siRNA (n=3, I.I., invasion index, mean ± s.d., ***p<0.001). Scale bar 100µm. **G.** Percentage of gel contraction by LIF-stimulated or not (Veh.) hDF subsequently transfected with control (siLuc) or ICAM-1-targeting ICAM-1 (siCAM-1#1 and #2) RNAi (n=3 in triplicates, mean + s.d., ***p<0.001). **H.** Representative images of H&E staining of paraffin-embedded sections of SCC12 in response to control (veh) or LIF-activated hDF subsequently transfected with control (siLuc) or ICAM-1-targeting siCAM-1#1 and #2) RNAi (n=3, I.I., invasion index, mean ± s.d., ***p<0.001). Scale bar 100µm.

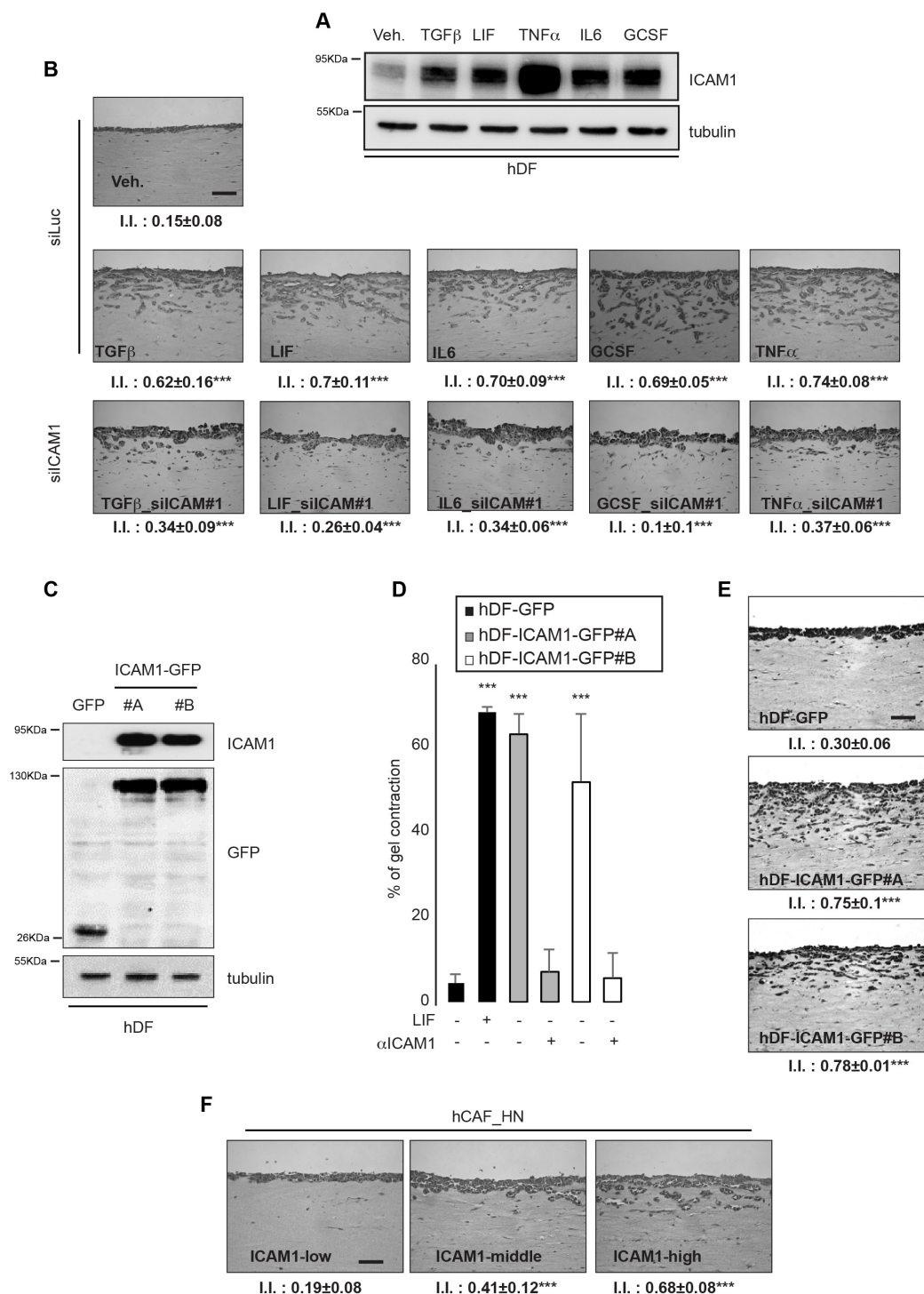


Figure 3: ICAM-1 drives inflammation-dependent proinvasive ECM remodeling. **A.** Immunoblot of ICAM-1 in hDF following cytokines stimulation for 24 hours. Immunoblot of tubulin shown as control. **B.** Representative images of H&E staining of paraffin-embedded sections of SCC12 in response to control hDF (Veh.; siLuc) or hDF stimulated by inflammatory cytokines and subsequently transfected using RNAi targeting ICAM-1 (siICAM-1#1) (n=3, I.I., invasion index, mean ± s.d., ***P<0.001). Scale bar 100µm. **C.** Immunoblot of ICAM-1 in hDF transfected with an empty vector (GFP) or ICAM-1 (ICAM-1-GFP). Immunoblot of GFP and tubulin as internal controls. **D.** Percentage of gel contraction by control (hDF-GFP) or hDF stimulated by LIF or overexpressing ICAM-1 (hDF-ICAM-1-GFP#A and #B) in presence or absence of a specific ICAM-1 blocking antibody (αICAM-1) (n=3 in triplicates, mean + s.d., ***p<0.001). **E.** Representative images of H&E staining of paraffin-embedded sections of SCC12 in response to control (hDF-GFP) or ICAM-1 overexpressing hDF (hDF-ICAM-1-GFP#A and #B) (n=33, I.I., invasion index, mean ± s.d., ***P<0.001). Scale bar 100µm. **F.** Representative images of H&E staining of paraffin-embedded sections of SCC12 in response to CAF sorted for ICAM-1 expression (n=3, I.I., invasion index, mean ± s.d., ***P<0.001). Scale bar 100µm.

to low-ICAM-1 expressing CAF (Figure 3F). Membrane-bound ICAM-1 expression and association with CAF-marker expression in CAF was further investigated (Supplementary Figure S3E), which showed that CAF expressing high level of membrane-bound ICAM-1 also express high level of the CAF markers FAP1, α SMA and fibronectin. These data demonstrate that membrane-bound ICAM-1 is necessary and sufficient to promote proinvasive ECM remodeling by CAF, and that membrane-bound ICAM-1 may serve as a marker to identify the proinvasive stromal fibroblasts.

Src kinases mediate ICAM-1-dependent regulation of CAF actomyosin contractility

Actomyosin contractility is a key driver for CAF-dependent proinvasive ECM remodeling and its regulation by pro-inflammatory cues, such as IL6 family cytokines, requires cooperation between the JAK1 and ROCK kinases [10]. Thus, we speculated that membrane-bound ICAM-1 might drive proinvasive ECM remodeling by CAF via regulation of actomyosin contractility and subsequently through MLC2 phosphorylation on Serine 19, which reflects MLC2 activation. Accordingly to this hypothesis, RNAi-mediated ablation of ICAM-1 expression was found to lead to a strong decrease of MLC2 phosphorylation (Figure 4A) subsequent to a reduced activity of the RhoA small GTPase upon ICAM-1 depletion (Supplementary Figure S4A). A strong correlation between ICAM-1 expression and the endogenous level of activated MLC2 was also established (Supplementary Figure S4B). In light of these data we confirmed that membrane-bound ICAM-1 regulates the onset of a proinvasive tumor microenvironment potential in CAF by regulation of the actomyosin cytoskeleton contractility.

The molecular mechanisms underlying the ICAM-1-dependent MLC2 regulation in hCAF were then deciphered. Src family kinases have been linked to ICAM-1-dependent cell signaling [32] and to MLC2 regulation [33]. The potential role of the Src family kinases in CAF-dependent ECM remodeling remains, however, poorly studied. Whether the Src family kinases could play a role in CAF-dependent ECM remodeling and SCC12 collective invasion was thus investigated *in vitro* using a three-dimensional organotypic invasion assay. In our hands, addition of SU6656, a Src kinase family inhibitor, strongly impaired both gel contraction and SCC12 cell collective invasion (Figure 4B and 4C). Moreover, CAF presented high levels of endogenous Src-activated phosphotyrosin 416. However, RNAi-mediated ablation of ICAM-1 expression, or addition of SU6656, induced a strong decrease of 416 tyrosin residue phosphorylation (Figure 4D and Supplementary Figure S4C). Accordingly, the crucial role for Src kinases during proinvasive fibroblast activation was demonstrated. Indeed, LIF-

mediated Src phosphorylation at Y416 (Supplementary Figure S4D) was mandatory for LIF-induced ECM remodeling and also SCC12 cell invasion (Supplementary Figures S4E and S4F). Co-immunoprecipitation assays demonstrated that LIF stimulation induces binding of Src kinases to ICAM-1 in hDF, (Figure 4E and 4F), which further reinforces the hypothesis that ICAM-1 mediates fibroblast actomyosin contractility through a Src-dependent regulation of MLC2 activity. Finally, we confirmed that ICAM-1 overexpression is sufficient to promote activation of the RhoA/ROCK/MLC2 signaling pathway activation. Indeed, forced expression of ICAM-1 in hDF triggered an increased RhoA-GTP bound state together with an increased activity and expression of MLC2 protein (Figure 4G). Accordingly, the ICAM-1-overexpressing hDF displayed an increased contractility capacity that was abolished in presence of SU6656 (Figure 4H). On the other hand, inhibition of Src activity in hCAF resulted in decreased ICAM-1 expression and reduction of both MLC2 expression and activity (Supplementary Figure S4G). These data therefore demonstrate that membrane-bound ICAM-1 governs the onset of a proinvasive ECM remodeling through a Src/RhoA/ROCK/MLC2 signaling pathway. Interestingly, in CAF, inhibition of either activity or expression of JAK, a kinase family regulated by the IL6 family cytokines such as LIF, impaired ICAM-1 expression (Supplementary Figures S4H and S4I). Having demonstrated that JAK and ROCK signaling pathways cooperate to control actomyosin contractility [10], we investigated whether cytoskeleton contractility in CAF could regulate ICAM-1 expression. Figure 4I and 4J show that forced expression of an active form of ROCK (ROCK-ER) [34] following 4-hydroxytamoxifen (4OHT) treatment is sufficient to increase *ICAM-1* expression at mRNA and protein levels, respectively. Moreover, inhibition of the mechano-responsive signaling pathway, which includes ROCK/MLCK and the YAP-TEAD interaction, using the Y27632, beblistatin and verteporfin chemical compounds, respectively, reduced the ICAM-1 protein content in CAF (Figure 4K). Taken together, these data demonstrate that membrane-bound ICAM-1 regulates, and is regulated, by actomyosin contractility, which attributes a central role to ICAM-1 in regulation of actin cytoskeleton contractility in the stroma fibroblasts.

ICAM-1 expression in tumor stroma correlates with the presence of invasive cohorts of tumor cells in human head and neck carcinoma

Overexpression of ICAM-1 in cancer tissues, both in tumor cells and stroma, has previously been reported. Indeed, ICAM-1 is up-regulated in CAF associated with colorectal cancer [27], but no information was so far available on possible functional consequences. Because our *in vitro* results suggest a novel role for ICAM-1 in tumor ECM remodeling and onset of proinvasive tumor

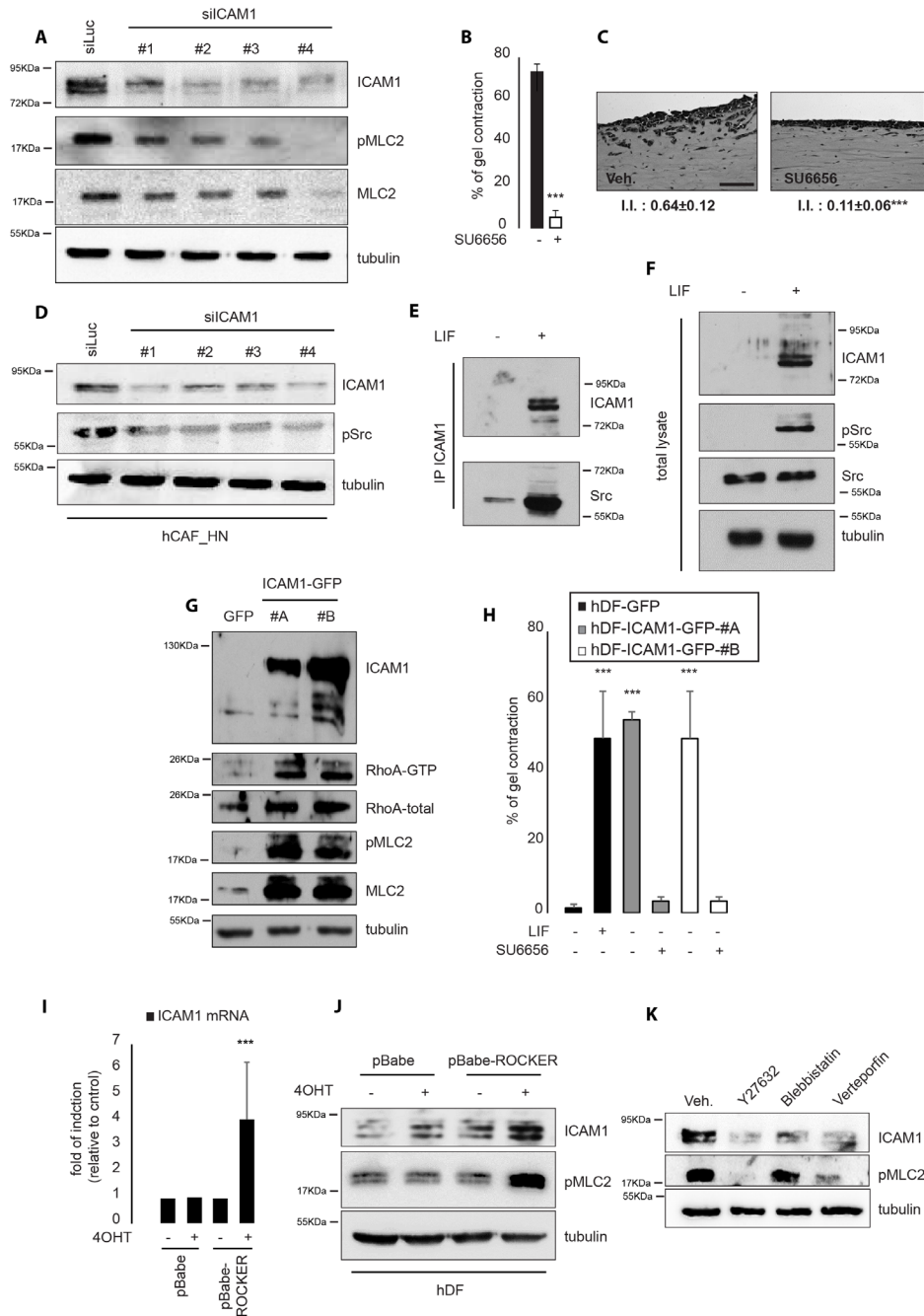


Figure 4: ICAM-1 regulates and is regulated by acto-myosin contractility. **A.** Immunoblot of ICAM-1, pMLC2 and MLC2 in CAF following transfection of RNAi targeting ICAM-1 (siCAM-1#1, #2, #3 and #4). Tubulin as internal control. **B.** Percentage of gel contraction by CAF in presence or absence of SU6656. (n=3 in triplicates, mean + s.d., ***p<0.001). **C.** Representative images of H&E staining of paraffin-embedded sections of SCC12 in response to CAF in absence or presence of SU6656 (n=3, I.I., invasion index, mean ± s.d., ***P<0.001). Scale bar 100µm. **D.** Immunoblot of ICAM-1, pSrc in CAF following transfection of RNAi targeting ICAM-1 (siCAM-1#1, #2, #3 and #4). Tubulin shown as control. **E.** Immunoblot of ICAM-1 and Src in hDF stimulated by LIF following ICAM-1-specific immunoprecipitation. **F.** Immunoblot of total cell lysate from experiment shown in E. Immunoblot of ICAM-1 and pSrc in hDF stimulated by LIF. Tubulin and Src shown as control. **G.** Immunoblot of ICAM-1 and RhoA following RhoA-GTP pull down assay in hDF cell transfected with an empty (GFP) or ICAM-1-expressing vector (ICAM-1-GFP#A and #B). Immunoblot of pMLC2, MLC2 and total RhoA shown as controls. **H.** Percentage of gel contraction after 6 days by control (hDF-GFP) or ICAM-1-overexpressing hDF (hDF-ICAM-1-GFP#A and #B) in presence or absence of SU6656 (n=3 in triplicates, mean + s.d., ***p<0.001). **I.** Quantification of ICAM-1 mRNA levels in control or HDF overexpressing an active form of ROCK following 4OHT treatment (n=3 in triplicates, mean + s.d., ***p<0.001). **J.** Immunoblot of ICAM-1 in control or HDF overexpressing an active form of ROCK following 4OHT treatment. Immunoblot of pMLC2 and tubulin shown as controls. **K.** Immunoblot of ICAM-1 in hDF cell control (veh.) or treated with Y27632, blebbistatin or verteporfin. Immunoblot of pMLC2 and tubulin shown as controls.

stroma, expression of ICAM-1 was investigated in invasive tumor cell clusters in human head and neck carcinomas. Interestingly, ICAM-1 was found in the tumoral stroma and co-localized with vimentin, a fibroblast marker (Figure 5A). Analysis of ICAM-1 expression level in 48 human head and neck carcinomas, using the quick score method,

was consistent with the quick score for the presence of the invasive cohorts of tumor cells observed in the tumor samples (Figures 5B and 5C and Supplementary Figure S5A). In conclusion, association of high levels of ICAM-1 with presence of invasive cohorts of tumor cells in human carcinomas validates our *in vitro* observations, and

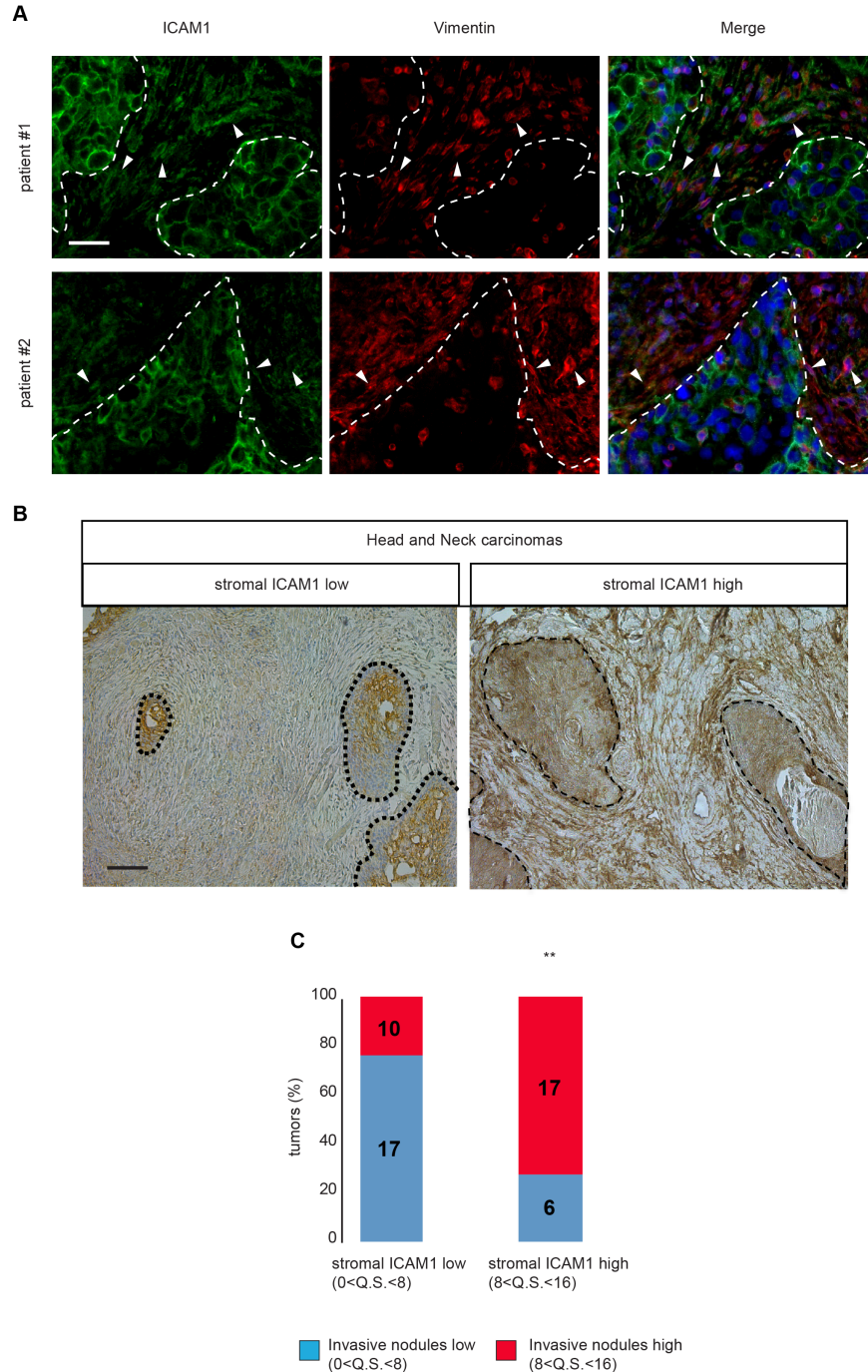


Figure 5: ICAM-1 is overexpressed in head and neck cancer stroma. **A.** Immunofluorescence of ICAM-1 (green) and vimentin (red) in representative cancer biopsies from two distinct patients with head and neck carcinoma. **B.** ICAM-1 immunohistological staining in human head and neck (n=50) carcinomas. Left panel shows low ICAM-1 detection and right panel shows high ICAM-1 detection. Scale bar, 100 μ m. **C.** Plot of mean quick score quantification for ICAM-1 specific decoration in tumor stroma from 48 head and neck carcinoma samples relative to the mean quick score for presence of invasive carcinoma cell nodules.

further indicates that ICAM-1 detection in tumor stroma may serve as a diagnostic tool to define the proinvasive potential of the tumor microenvironment.

DISCUSSION

We have identified a novel role for *ICAM-1*, an inflammatory responsive gene, in the establishment of a protumorigenic tumor stroma. In reaction to inflammatory signaling cues, fibroblasts populating the tumor stroma are activated, which induces ICAM-1 expression at the cell surface. As a consequence, activated fibroblasts promote the onset of proinvasive ECM remodeling leading to tumor cell invasion (Figure 6).

The pro-inflammatory cytokine LIF induces [9] and sustains [28] the proinvasive capacity of stromal fibroblasts through the constitutive activation of the JAK1/STAT3 signaling pathway [10]. In this study, we demonstrate that LIF supports the transcriptomic signature of fibroblasts long-term activated by TGF β , a well-known CAF activator both *in vitro* and *in vivo* [7]. In addition, we show that LIF-responsive genes *in vitro* are those regulated in CAF, which validates the essential role of LIF in CAF activation during tumor progression.

Using three-dimensional phenotypic contraction assays, following RNAi-mediated knock-down expression of 50 LIF-responsive genes, we identified four genes (ICAM-1, DBC1, HRH1 and BCL3) consistently crucial for activated-fibroblast contractility. While DBC1, also

called BRINP1 (Bone Morphogenic Protein/Retinoic Acid Inducible Neural-Specific 1), a cell proliferation and cell death regulator, has never been linked to cell contractility, which deserves investigation, HRH1 (Histamine Receptor 1) was shown to promote human lung fibroblast collagen lattice contraction *in vitro* [35]. Interestingly, RNAi-mediated ablation of BCL3 (B-cell CLL/Lymphoma 3) expression in activated fibroblasts, leads to a strong decrease of ICAM-1 expression both in LIF-activated fibroblasts and in CAF (data not shown). BCL3 is a transcriptional co-activator of NF- κ B transcription factor that mediates TNF α -dependent ICAM-1 expression [21]. Because we consider that the *in vitro* three-dimensional collagen lattices contraction assay is a powerful tool to unveil new genes or signaling pathways that regulate cell contractility [10, 28, 29], such a screen was used to disclose a novel role for ICAM-1 during tumor progression.

Pro-inflammatory cytokines production is a key characteristic of tumor microenvironments [36–38] and cytokine signaling contributes to the establishment of a proinvasive ECM [9, 10]. The central role of the membrane-bound ICAM-1 that we have unveiled in the cytokine-dependent regulation of RhoA/ROCK/MLC2 acto-myosin cytoskeleton contractility in CAF is reinforced by the function that this protein plays in endothelial cells during leukocytes transendothelial migration [39, 40]. Also the fact that membrane-bound ICAM-1 regulates the Src kinases activity, which controls the RhoA/ROCK/MLC2 signaling pathway [32], is in accordance

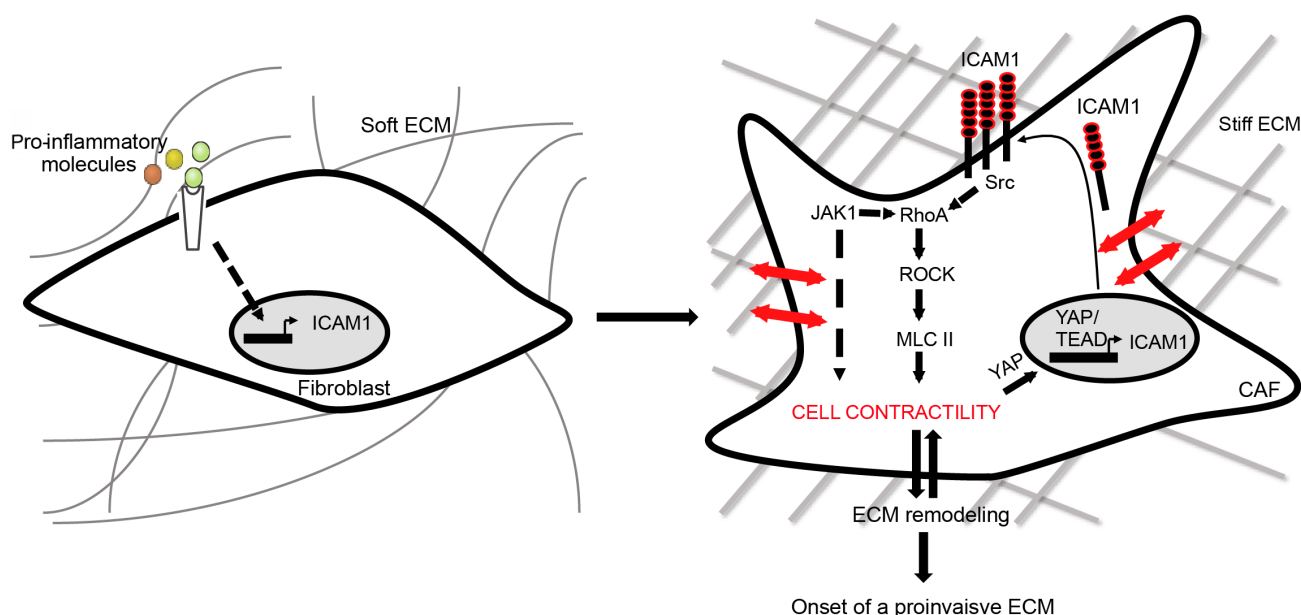


Figure 6: ICAM-1 mediates inflammation-dependent onset of a proinvasive ECM remodeling. Pro-inflammatory cues from the tumor microenvironment trigger ICAM-1 expression in fibroblasts, which leads to proinvasive ECM remodeling. In activated fibroblasts, membrane-bound ICAM-1 expression controls cellular contractility through regulation of a Src/RhoA/ROCK/MLC2-dependent signaling. In return, acto-myosin contractility in CAF regulates expression of membrane-bound ICAM-1 at the cell surface. In CAF, ICAM-1 acts as a crucial hub to sustain acto-myosin contractility and matrix remodeling of tumor stroma.

with previous data suggesting a role for Src in fibroblast contractility [41] and during kidney and lung fibrosis [42, 43]. Multiple cytokines signaling converge to ICAM-1 to promote and sustain proinvasive matrix remodeling, which makes membrane-bound ICAM-1 a potential target for therapeutic protocols for patients suffering from aggressive carcinoma. Indeed we demonstrate that membrane-bound ICAM-1 is overexpressed in CAF isolated from head and neck, lung and breast cancers, which strongly suggests that in such cells ICAM-1 plays a role consistent with the functions we unveiled *in vitro*. Because the need for specific CAF sub-population identification is compelling [7, 31], detection of ICAM-1 in the tumor stroma, coupled with a specific fibroblast marker might constitute an interesting biomarker for clinical evaluation of tumor stroma proinvasiveness. ICAM-1 belongs to a family of five members (ICAM-1 to ICAM-5) that share some, but not all, structural similarities and disclose diverse patterns of expression and downstream effector behaviors [44]. For instance, ICAM-1 blocking antibodies do not interfere with ICAM-2-dependent leukocyte adhesion to endothelial monolayers [45]. Moreover, ICAM-2 is not regulated by inflammatory cues [46]. ICAM-3 shows a different binding property to LFA-1 [47, 48]. All together, these data suggest that ICAM-1 present unique features and biological activities, such as matrix remodeling in CAF. Accordingly, our pan-genomic data show that ICAM-1 is the only family member to be induced by LIF and TGF β in hDF cells, and specific knock down expression of ICAM-1 drastically blocks CAF contractility, which indicates that in the context of proinvasive matrix remodeling by CAF no compensation mechanisms are provided by the other ICAM family members. In contrast, little is known about the source of sICAM-1 that is found to correlate with tumor stage and metastasis development in sera of patients with carcinoma [49–54]. It is proposed that sICAM-1 acts as a de-adhesive molecule that triggers cancer cell migration: investigating the potential secretion of sICAM-1 by CAF would therefore be of general interest in cancer biology.

Interestingly, our results demonstrate that ICAM-1 regulates fibroblast contractility. In CAF, inhibition of ROCK, MLCK and the YAP-TEAD complex drastically downregulates ICAM-1 expression. Accordingly, forced expression of an active form of ROCK is sufficient to trigger ICAM-1 expression in fibroblasts. These observations are consistent with the fact that, in endothelial cells, application of mechanical forces to ICAM-1 clusters induces a Rho GEF 12-dependent RhoA activation [55], and ICAM-1 clustering at the cell surface is sufficient to promote traction forces [56]. Moreover, it has been suggested that a positive feedback signaling between actomyosin contractility and matrix stiffness may sustain CAF contractility in the tumor stroma [41]. Based on our results, one can speculate that ICAM-1 acts as a central hub that coordinates a signaling loop between actomyosin contractility and matrix stiffening that sustains the onset of a proinvasive tumor

microenvironment (Figure 6). However, how ICAM-1 could sense matrix stiffness remains to be elucidated. Integrins are considered the main mechanoreceptors able to transduce out-side-in signaling in response to matrix stiffness [57]. In CAF, α 3 β 1 and α 5 β 1 integrins are involved in matrix remodeling and proinvasive behavior through the regulation of cell contractility and matrix remodeling [58–60]. We can thus speculate that membrane-bound ICAM-1, similarly to its role of tyrosine kinase co-receptor [61], may interact with integrins to transduce the out-side-in signaling. On the other hand, ICAM-1 interacts within the first immunoglobulin domain of fibrinogen to support the Src-dependent mitogenic activity in B cells [15, 62]. In light of this data, it is tempting to speculate that ICAM-1 may bind to extracellular fibrinogen to promote actomyosin contractility in CAF.

In conclusion, we identify membrane-bound ICAM-1 as a major regulator of proinvasive CAF activity in head and neck carcinoma, but a similar role could also be played in lung and breast carcinomas. We demonstrate that membrane-bound ICAM-1 promotes inflammation-dependent extracellular matrix remodeling, which leads to tumor cell dissemination. Membrane-bound ICAM-1 is overexpressed in CAF and acts as a crucial hub to sustain actomyosin contractility and matrix remodeling in tumor stroma. Therefore our data suggest that inhibition of membrane-bound ICAM-1 function using ICAM-1 specific blocking antibodies might constitute an interesting possibility to counteract tumor cell invasion and dissemination.

MATERIALS AND METHODS

Cell culture

Human primary Dermal Fibroblasts (hDF) and human HEK293 Phoenix cells were maintained in DMEM supplemented with 10% FCS (fetal calf serum). Human Carcinoma-Associated Fibroblasts (CAF) isolated from patients with head and neck, lung and breast cancers were cultured in DMEM supplemented with 10% FCS and insulin-transferrin-selenium (#41400-045; Invitrogen, Carlsbad, CA). SCC12 cells were cultured in FAD media, as described in Gaggioli et al [59].

Long-term LIF and TGF β 1-activated fibroblasts (hDF_LIF and hDF_TGF β 1) have been performed in DMEM supplemented with 0.5% FCS containing 2ng/ml final concentration of human recombinant proteins for seven days. Next, activated-hDF was cultured for 7 days in 0.5% FCS media prior to experiments.

Cytokines and neutralizing antibodies and inhibitors

TGF β 1 was purchased from Peptrotech (#100-21, Peptrotech, Rocky Hill, NJ) and was used at 2 ng/ml; recombinant human GCSF (#300-23) and IL-6 (#200-

06) were purchased from Peprtech and were used at 10ng/mL, recombinant human LIF was purchased from Millipore (#LIF1005, Millipore, Billerica, MA), and was used at a concentration of 2 ng/ml. Recombinant TNF alpha was produced in E Coli and purified under native conditions using an N-terminal 6-his tag. ICAM-1 neutralizing antibody (#BBA3, R&D, Minneapolis, MN) was used at 10 µg/ml. The following inhibitors were used in this study: Ruxolitinib (#1598, Axon medchem, Groningen, The netherlands) at 10µM, CYT387 (#S2219, Selleckchem, Huston, TX) at 10µM Y27632 (#1254, Tocris bioscience, Ellisville, MO) at 10µM, Blebbistatin (#B0560, Sigma, Saint Louis, MO) at 10µM, SU6656 (#572635, CalbioChem, Los Angeles, CA) at 10µM and Verteporfin (#SML0534, Sigma, Saint Louis, MO) at 4µg/mL.

RNAi transfections

Cells were plated at 60% confluence and subjected to transfection the following day using Dharmafect 3 (#T-2002-02; Dharmacon, inc., Lafayette, CO) at 20nM final concentration of RNAi. RNAi sequences are listed in Supplementary Table S2.

Organotypic invasion assays and matrix remodeling assay

In organotypic invasion assays, $5 \cdot 10^5$ fibroblasts were embedded in 1ml of matrix gel, made of collagen I and Matrigel, yielding a final collagen concentration of $4.6 \text{ mg} \cdot \text{ml}^{-1}$ and a final Matrigel concentration of approximately $2.2 \text{ mg} \cdot \text{ml}^{-1}$. After 1h at 37°C, matrix gel were overlaid with $5 \cdot 10^5$ SCC12 cells and lifted at the cells-air interface 24h later. After 5 days, organotypic cultures were fixed, embedded in paraffin block, sectioned and stained for invasion index quantification using ImageJ [63]. For gel contraction assay, $25 \cdot 10^3$ cells were embedded in 100µl of matrix gel [29] and seeded in triplicate into 96 wells plate. After 1h at 37°C, matrix gels were overlaid with 100µl of 0.5% FCS medium (with indicated cytokines or inhibitors) and changed every two days. At day 6 the relative diameter of the well and the gel were measured using ImageJ. The percentage of gel contraction was calculated using the formula $100 \times (\text{well diameter} - \text{gel diameter}) / \text{well diameter}$.

Neutralizing antibody method

Neutralizing antibody against ICAM-1 (10µg/mL) was incubated one hour with trypsinized fibroblasts at 37°C before being used for matrix remodeling assay or organotypic cultures. Neutralizing antibody against LIF (10µg/mL) was incubated one hour with media before fibroblasts stimulation.

Antibodies

Antibodies against STAT3 (#9139; 1/1000), pY705-STAT3 (#9145; 1/1000), JAK1 (#3332; 1/500), pY1022/1023-JAK1 (#3331; 1/200), MLC2 (#3672; 1/500), pThr18/19-MLC2 (#3674; 1/500) Src (#2109; 1/2000), pTyr416-Src (#2101; 1/500), vimentin (#5741) were purchased from Cell Signaling (Cell Signaling Technology, Beverly MA), α -tubulin from sigma (T4026, Sigma, Saint Louis, MO; 1/5000) ICAM-1 (#sc-8439; 1/1000), RhoA (#sc-418; 1/500) from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA).

Western blot and coimmunoprecipitation analysis

Western blot analysis was performed as previously described [9]. For coimmunoprecipitation analysis, cells were lysed on ice in modified RIPA buffer (50mM Tris pH 7.4 150 mM NaCl, 1%NP-40, 0.1% SDS, 0.5% SD Dextroxycholate, 5mM NaF, 2.5 mM Nappi, and protease inhibitor (#04693159001, Roche) for 30 minutes and isolated by centrifugation (15 min, 10000g, 4°C). Supernatants were precleared during 1 hour at 4°C with Magna CHIP protein G bead (#16-662, Millipore) and normal mouse IgG (#sc-2025, Santa Cruz technology) and the cleared lysate incubated with primary antibody or IgG overnight at 4°C. Immune complexes were captured by adding 35µl of protein G magnetic beads, rotated for 1h at 4°C and washed three times with lysis buffer (without SDS, Sodium Dextroxycholate and protease inhibitors). Immunoprecipitation products were separated by SDS-PAGE.

Microarrays analysis

Total RNA of hDFs stimulated by LIF, TGF β or TGF β + α Lif mAb was extracted using the RNeasy kit (Qiagen, Hilden, Germany). The integrity of the RNA was assessed using an Agilent BioAnalyzer 2100 (Agilent Technologies). RNA samples were then labeled and hybridized on 8×60K high density SurePrint G3 gene expression human Agilent microarrays following the manufacturer's instructions. Two biological replicates were performed for each experimental condition. The microarray experimental data were deposited in the NCBI GEO under the serial record number GSE81996 (short term response) and GSE81997 (long term response).

The data were quantile normalized using the Bioconductor package limma [64]. Means of ratios from all comparisons were calculated and the moderated t-statistic of the limma package provided the per gene *P* values. The Benjamini-Hochberg procedure was used to control the experiment-wise false discovery rate (FDR) from multiple testing procedures. Differentially expressed genes were selected based on an adjusted p-value below 0.05 and an absolute log₂ (fold change) >0.7.

RT-qPCR analysis

RNA isolation was performed using RNeasy Mini kit (#217004, Qiagen, Turnberry Ln, Valencia, CA) according to the manufacturer's instructions. Reverse Transcription of 500ng RNA by Superscript II reverse transcriptase (#18064-014, Invitrogen, Carlsbad, CA) was followed by Real time PCR using Fast SYBR Green Master Mix (#18064-014; Applied Biosystems, Foster City, CA) and performed on a Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, CA).

Primers sequences are listed in Supplementary Table 2 :

Relative expression of the respective gene was determined after normalization to GAPDH and calculated with the following formula: relative expression level = $2^{-\Delta\Delta CT}$

Statistical analysis

Student's t test was performed for statistical analysis of invasion assay, gel contraction assay, and qPCR results. *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$. Error bars are + standard deviation (+ s.d.). Pearson's correlation coefficient was used to assess the relationship between ICAM-1 and invasion quick score within human samples.

Immunohistochemical staining and quantification methods

Fifty head and neck tumor biopsies were fixed (3.7% formaldehyde in PBS) for 4 h and transferred to 70% ethanol (24 h), embedded in paraffin wax and sectioned at 7 μ m. After deparaffination, microwave antigen retrieval was performed in Na-citrate buffer (10mM, pH6; 5min at 900W, 10min at 150W and 30 min at room temperature). Sections were washed three times in PBS (5min per wash) before endogenous peroxidase activity was blocked in 1% H₂O₂ for 10 min and washed 3 times in PBS. After incubation in blocking buffer for two hours (10% serum (S-5000, S-1000; Vector, Burlingame, CA); 0.3% Triton X100 in PBS), sections were incubated with primary antibody diluted 1:50 in blocking buffer overnight at 4°C. After three washes in PBS, sections were incubated with biotinylated secondary antibody (#BA-1000 Vector, Burlingame, CA) diluted 1:400 in PBS for 30 min and washed 3 times in PBS. Samples were then processed using Vectastain ABC kit (#PK4001, Vector, Burlingame, CA) and DAB peroxidase substrate kit (#SK4100, Vector, Burlingame, CA) according to manufacturer's instructions. Sections were next counterstained with hematoxylin for 5 sec, rinsed in water, blued 10 sec in 0,08% ammonia water, dehydrated, cleared, and mounted with cover clips. Two authors, blinded to each other's assessment, scored the slides using the Quick Score method to determine ICAM-1 status within the tumor bulk and stroma.

For immunofluorescence staining, after deparaffination and microwave antigen retrieval, sections were washed three times in PBS (5min per wash) before incubation in blocking buffer (3% BSA #A7030, Sigma; 0.2% Triton X100 in PBS) for 1 hour. Sections were incubated with primary antibodies diluted 1:50 in blocking buffer overnight at 4°C. After three washes in PBS, sections were incubated with secondary antibody conjugated to Alexa 488 (#A21202, Life Technology) or Alexa 594 (#A21207, Life Technology) diluted 1:400 in PBS for 1 hour and washed 2 times in PBS, stained with DAPI (2 μ g/mL) for 5min, rinsed in water and coverslips mounted onto glass slides using mounting media (#TA-030-FM, Thermo).

Plasmids constructs

ICAM-1 was cloned in pEGFPN1 vector between BamHI and AgeI sites after PCR amplification using following primers: forward : CGCGGGGATCCGCCACCATGGCTCCCAGCAGCCCCCG reverse : CGCGGGA CCGGTGTGGGAGGCGTGGCTTGTGTG; The ICAM-1-GFP insert was retrieved by cutting with BamHI and HpaI restriction enzymes and subcloned in the pBABE puro vector between the BAMHI/SnaBI sites, generating the pBABE-ICAM-1-GFP Puro construct. The same operation was realized with the GFP gene alone to make the pBABE-GFP Puro control vector.

Production of recombinant retroviruses

Phoenix cells were transiently transfected with 1 μ g of the previously described vectors using calcium phosphate mediated transfection using classical procedures. Six hours after transfection, cells were washed with PBS and complete media was added. The day after, media was replaced by a heat-inactivated serum medium and cells were moved to 32°C for 24 hours. Forty eight hours post transfection clarified supernatants (retroviral particles) were collected and used to infect either hDF. Retrovirus infection was performed in the presence of 5 μ g/ml polybrene. Stably transduced cells were selected with 5 μ g/ml puromycin.

Flow cytometry

For ICAM-1 labelling, fibroblasts were trypsinized, resuspended in DMEM 10% FCS and washed in PBS. 1×10^6 cells/100 μ L were blocked in PBS containing 2% FCS and 0,5mM EDTA (FACS buffer) for 1 hour at 4°C. PE-conjugated antibody for ICAM-1 (#353105, BioLegend) or normal mouse IgG (#400113, BioLegend) were add at 1/100 dilution in blocking buffer during 1 hour at 4°C. Cells were washed three times in PBS and resuspended in FACS buffer at 1×10^6 cells/100 μ L. Analysis was performed on a FacsCanto flow cytometer (Becton Dickinson) with BD diva software. Cell sorting was performed on FacsAria 3 (Becton Dickinson). Cells collected were cultured for 7 days in 0.5% FCS containing 1% of Penicilin-Streptomycin (#15140-122,

Gibco) and 0,5% of Fongizone (#15290-018, Gibco) prior to experiments.

Conditioned media preparation

Cancer cells were grown to confluence, washed twice with PBS and then incubated in serum-free medium at 37°C. After 48 hours, conditioned media (CM) were collected, centrifuged at 5000g for 5 min to remove cell debris and the supernatant stored at -80°C.

Pulldown RhoA activity

Fibroblasts were grown to 80% of confluence and lysed on ice in lysis buffer (50mM Tris pH 7.5, 500mM NaCl, 0.1% SDS, 1% triton, 0.5mM MgCl₂ and protease inhibitor (#04693159001, Roche)) for 5 minutes and isolated by centrifugation (5 min, 10000g, 4°C). Equal concentration and volume of each sample were used and 30µg of GST-RBD beads was added. Samples were incubated at 4°C with rotation for 30 minutes. Beads were washed 4 times with lysis buffer (150mM NaCl, without SDS) and resuspended in Laemli buffer. Samples were loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

Author contributions

S.B, J.A. and C.G. designed and performed most of the experiments and analyzed data. E.G. and I.B. performed experiments provided in Figure 3. S.E.K. and T.B. performed experiments provided in Figure 4. N.N. and B.M. performed pan-genomic and bioinformatics analysis provided in Figure 1. CG supervised the whole work and wrote the manuscript with contribution of G.M.

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Fibroblast activation in cancer: when seed fertilizes soil

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Abstract In solid cancers, activated fibroblasts acquire the capacity to provide fertile soil for tumor progression. Specifically, cancer-associated fibroblasts (CAFs) establish a strong relationship with cancer cells. This provides advantages to both cell types: whereas cancer cells initiate and sustain CAF activation, CAFs support cancer cell growth, motility and invasion. This results in tumor progression, metastasis and chemoresistance. Numerous studies have detailed the mechanisms involved in fibroblast activation and cancer progression, some of which are reviewed in this article. Cancer cells and CAFs are “partners in crime”, and their interaction is supported by inflammation. An understanding of the enemy, the cancer cell population and its “allies” should provide novel opportunities for targeted-drug development.

Keywords Carcinoma-associated fibroblasts · Tumor microenvironment · Inflammation · Cancer

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Introduction

Tumor development and progression is not only dependent on the malignancy of cancer cells (Kalluri 2003). Like their normal counterparts, cancer epithelial cells are supported by a complex microenvironment that is mainly composed of extracellular matrix (ECM), immune cells, blood vessels, cytokines, growth factors and importantly, non-epithelial cells such as fibroblasts (Liotta and Kohn 2001). Fibroblasts constitute the most abundant population within the family of connective-tissue cells. The physiological role of fibroblasts includes the synthesis of ECM components, notably collagens, the regulation of tissue homeostasis and inflammation and the differentiation of the surrounding cells (Tomasek et al. 2002). Furthermore, fibroblasts are responsible for the production of the matrix metalloproteinases (MMPs; Simian et al. 2001) that are involved in matrix remodeling, cell proliferation, motility and death, further underscoring the role of fibroblasts in the maintenance of ECM homeostasis. The ability of fibroblasts to secrete diverse growth factors is another essential function that facilitates interactions between mesenchymal and epithelial cells (Wiseman and Werb 2002). Proper communication between cells is essential for the physiological growth and differentiation of mesenchymal tissue and relies not only on cell-to-cell interactions but also on cell-ECM interactions. In addition, fibroblasts are star players in the wound healing processes (Shaw and Martin 2009). They are involved in all the key steps of injury repair, from the initiation of the inflammatory response to cell proliferation, migration and contraction of granular tissue (Park et al. 1999). Fibroblasts endowed with contraction properties are designated as myofibroblasts that represent a subpopulation of cells entering an activated state (Desmoulière 1995). Myofibroblasts that exhibit properties characteristic of fibroblasts and smooth muscle cells can be identified by their expression of specific markers, such as the intermediate filament

proteins vimentin (Mørk et al. 1990) and desmin (Lazarides and Balzer 1978), the mesenchymal marker α -smooth muscle actin (α -SMA; Tomasek et al. 2002) and the fibroblast activation protein (FAP; Rettig et al. 1993). Activated fibroblasts are found in tissue undergoing regeneration, inflammation and matrix remodeling and are abundant in fibrosis. Thirty years ago, cancer was defined as a “wound that never heals” (Dvorak 1986); since then, the critical role of fibroblasts and of fibroblasts activation in cancer progression has emerged.

The changes occurring within cells during carcinogenesis are not restricted to epithelial cells. When the epithelium undergoes modifications, the stroma follows. Under the control of a variety of stroma-modulating factors, the cancer cells themselves generate a permissive microenvironment favoring further tumor development and invasion (Bhowmick et al. 2004). The microenvironment modifies its own morphological features leading not only to an increase in the number of immune and inflammatory cells such as macrophages but also and more importantly, to the development of CAFs. This term defines activated fibroblasts displaying a phenotype similar to that acquired by myofibroblasts during the wound healing process (Mueller and Fusenig 2004). Similar to myofibroblasts, CAFs express α -SMA, FAP, vimentin and desmin but also tenascin C (TNC; De Wever et al. 2004), periostin (Malanchi et al. 2012), secreted protein acidic and rich in cysteine (SPARC), chondroitin sulfate proteoglycan (Sugimoto et al. 2006), platelet-derived growth factor (PDGF) receptor (PDGFR; Pietras et al. 2003a), prolyl 4-hydroxylase (Kojima et al. 2010), integrin α 11 (Zeltz and Gullberg 2016) and fibroblast-specific protein 1 (FSP1; Strutz et al. 1995; Fig. 1c) The expression levels of these markers can vary from one cell to another thereby attesting to the heterogeneity of CAF populations, a factor that contributes to the overall difficulties encountered in the treatment of cancer. One particular piece of research tackled this subject in detail. Wide screening of the most relevant and commonly used fibroblast/mesenchymal cell markers was performed by using two well-studied mouse models of human cancer (Sugimoto et al. 2006). Among the various markers, FSP1 displayed the most limited localization and selectively identified a unique sub-population of CAFs. Other markers, such as α -SMA and PDGFR β , showed a large but not completely overlapping, labeling pattern; the non-overlapping staining provides evidence for a significant proportion of diverse CAF populations (Sugimoto et al. 2006). Moreover, FSP1 appeared to be a unique marker of resting and activated fibroblasts that shared minimal overlap with other markers, thereby indicating that CAFs consist of heterogeneous cell populations possibly sharing distinct roles in cancer progression. The presence of CAFs in tumor stroma is associated with poor prognosis outcome for patients suffering from cancer (Shimoda et al. 2010). Indeed, CAFs are well established to be able to stimulate tumor initiation (Trimboli et al. 2009), progression and growth (Taddei

et al. 2013), angiogenesis (Montesano et al. 1986), cancer cell proliferation (Olumi et al. 1999) and invasion (Gaggioli et al. 2007) and to favor metastatic spreading by inducing epithelial to mesenchymal cell transition (EMT; Thiery et al. 2009). Recently, several studies have shown that CAFs co-migrate with cancer cells in the bloodstream (Haugsten et al. 2010), supporting the idea that the activation of fibroblasts is essential for the formation of pre-metastatic niches (Kaplan et al. 2005); this is consistent with the “seed and soil” hypothesis postulated in the 19th century (Paget 1889).

Growth factors and cytokines trigger fibroblast activation

To acquire invasive and tumor-promoting phenotypes, normal fibroblasts (NFs) undergo activation via diverse mechanisms. Epithelial cancer cells secrete growth factors into the surrounding microenvironment; these factors stimulate the recruitment and activation of fibroblasts. Among these factors, transforming growth factor beta (TGF- β) is the most prominent, because of the wide range of effects that it exerts on ECM (Roberts et al. 1992; Fig. 1a). Moreover, TGF- β plays a pivotal role in ECM synthesis and degradation by increasing both expression of fibronectin and collagens (Ignatz and Massagué 1986) and that of the MMPs involved in matrix remodeling (Leask and Abraham 2004). Orimo et al. (2005) showed that resident fibroblasts can be activated by the cooperative signaling of autocrine TGF- β /SMAD2/3 and CXCL12/CXCR4 ([C-X-C] chemokine receptor) pathways. These pathways are connected in a positive feedback loop enabling bidirectional communication between tumor cells and CAFs. Such crosstalk is carried out by the release of TGF- β into the tumor stroma; TGF- β acts upon CAFs and enhances their potential to secrete tumor-promoting chemokines. Furthermore, chemokines act back on cancer cells and promote their invasive properties (Mishra et al. 2011). Intracellular signaling mechanisms that mediate a fibroblast response to TGF- β stimulation are achieved by the binding of TGF- β to the ubiquitous type II serine/threonine kinase receptor (TGF- β RII), binding that enables heterodimerization with TGF- β RI and its activation. Upon activation of TGF- β RI, the signal is transduced downstream by a family of the intercellular transcription co-factors, namely SMAD (Derynck and Zhang 2003), which play a central role as the effector of TGF- β -dependent fibroblast activation and chemokine secretion. Chemokines, such as CXCL12, CXCL10, CCL21 and CCL25 and CXCR4, CXCR3 and CCR9, have previously been documented to induce tumor cell invasion and motility, respectively. Lately, major emphasis has been placed on the role of chemokines in the occurrence of organ-specific metastases (Balkwill 2004). In addition, compelling evidence has shown that CAFs also release

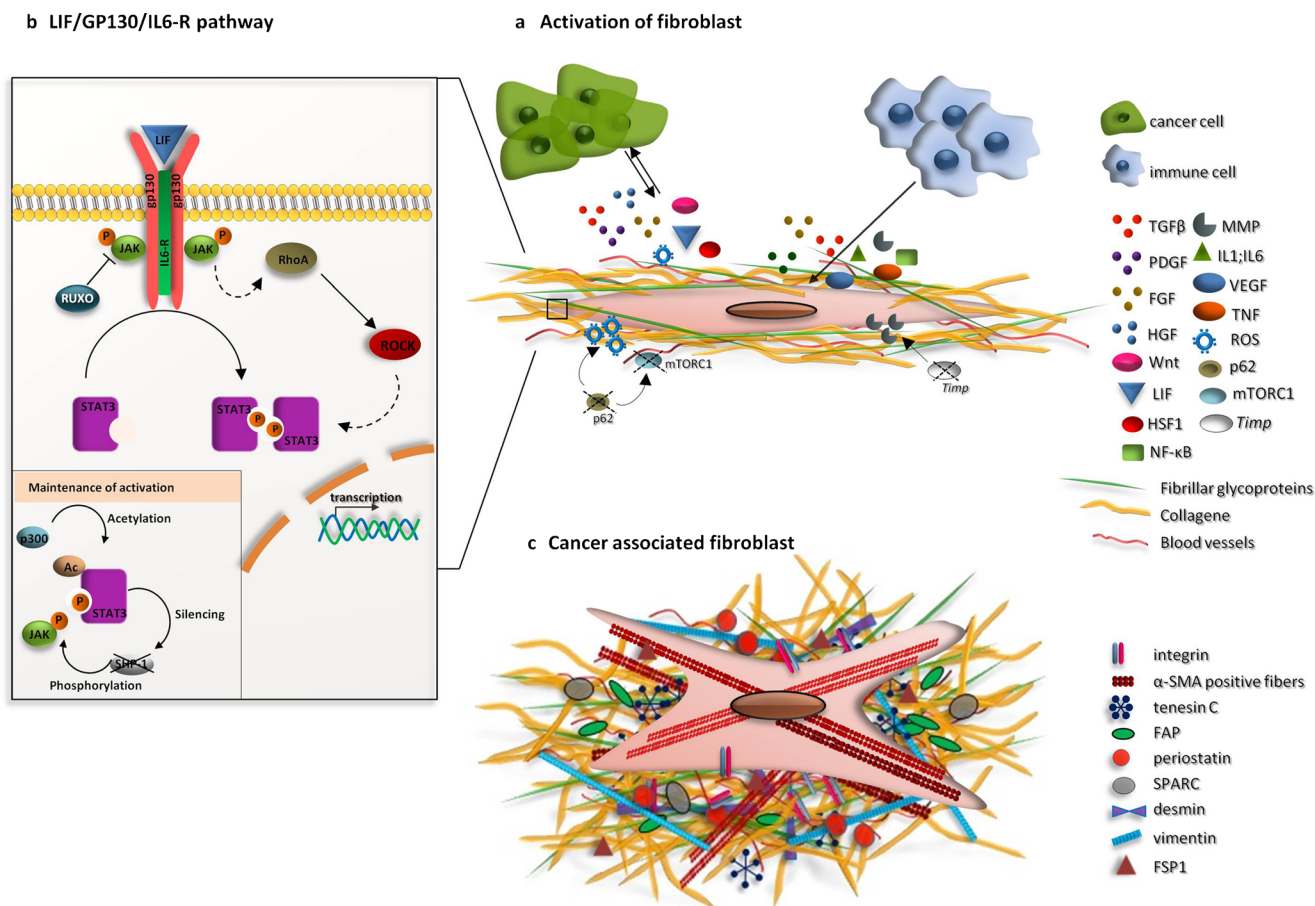


Fig. 1 Molecular mechanism of fibroblast activation. **a** Normal fibroblasts are the most common cell type in the extracellular matrix and are responsible for the synthesis of collagens and fibrillar proteins. Under normal conditions, fibroblasts maintain tissue homeostasis and contribute to proper cell communication and function. Fibroblasts can be activated by a diverse set of factors secreted from cancer or immune cells. Not only growth factors such as TGF-β, PDGF, HGF and FGF but also interleukins, metalloproteinases and reactive oxygen species can promote activation. Likewise, transcriptional factors such as NF-κB and HSF-1 play an important role, as do the gene family of metalloproteinase inhibitors, namely *Timp* and NF-κB subunit, p62. Interestingly, fibroblasts themselves can stimulate cancer cells to support activation further. **b** Once activated, fibroblasts undergo a phenotype switch and

become cancer-associated fibroblasts (CAFs) expressing various markers such as α-SMA, FSP1, vimentin and periostatin. **c** Recently, the LIF/GP130/IL6-R pathway was identified as the signaling cascade involved in fibroblast activation. Upon LIF stimulation, JAK is phosphorylated and further activates STAT3, a transcriptional factor, which is then translocated into the nucleus where it promotes the transcription of genes responsible for cell growth, differentiation, proliferation and apoptosis. Ruxolitinib can inhibit JAK and prevent STAT3 activation. Further on, the maintenance of JAK activation is supported by epigenetical changes and posttranslational modifications. Once pSTAT3 is acetylated by histon acetyltransferase, p300, it leads to the loss of the expression of SHP-1 that is a negative regulator of the JAK/STAT pathway. Silencing of SHP-1 steers the constitutive activation of JAK and STAT3

chemokines, such as CXCL12 and that they promote the malignancy of cancer cells by inducing cell proliferation, migration and angiogenesis (Balkwill 2004). Moreover, TGF-β/SMAD signaling is responsible for the exosome secretion of CXCL12-CXCR4 chemokines in CAFs (Webber et al. 2015).

Despite its undeniable importance, TGF-β shares the spotlight with the hepatocyte growth factor (HGF) (Fig. 1a) and its receptor, the tyrosine kinase Met (Bhowmick et al. 2004). The Met receptor is expressed by epithelial cells but, more importantly, fibroblasts can express HGF (Nakamura et al. 1997). HGF is a mesenchymal- or stromal-derived factor that induces mitogenic, motogenic and morphogenic activities on various cell types, predominantly in a paracrine fashion. HGF

promotes fibroblast-derived invasion (Matsumoto et al. 1994) and is known to stimulate angiogenesis, both in vivo and in vitro (Bussolino et al. 1992). Epithelial cells in response to HGF/Met signaling undergo several processes such as colony dispersion, EMT and increased cell motility (Birchmeier et al. 2003; Thiery 2002). These dissociated epithelial cells are able to invade the ECM and migrate, properties that attest to their metastatic ability (Maina and Klein 1999). Nonetheless, although CAFs never become malignant themselves and display features different from those of the malignant epithelial cells, CAFs and epithelial cancer cells are codependent. Although cancer cells influence the behavior of the surrounding stroma, the latter helps cancer cells to acquire a fully transformed

potential. For instance, the HGF-induced crosstalk between gastric cancer cells and their adjunct CAFs contributes to tumor growth and the further transformation of NFs to CAFs. Indeed, experiments *in vitro* and *in vivo* identified HGF as a significant factor in fibroblast activation (Wu et al. 2013).

PDGF and the fibroblast growth factor 2 (FGF-2) are two other pro-fibrotic growth factors that are secreted by cancer cells and that induce fibroblast activation (Elenbaas and Weinberg 2001) (Fig. 1a). PDGF was one of the first growth factors to be described (Paul et al. 1971) and its involvement in various cell processes, such as proliferation, growth and angiogenesis, has been well documented (Hannink and Donoghue 1989). All five PDGF isoforms (PDGF A to D) function as secreted homodimers but only PDGF-A and -B can form functional PDGF-AB heterodimers: their signaling network includes PDGFR α and PDGFR β receptors. PDGF is a potent mitogen and chemoattractant for mesenchymal cells, including fibroblasts, strongly correlating with cancer progression. Interestingly, most cancer cells do not express PDGFR themselves but, nevertheless, display an up-regulated level of PDGF suggesting that PDGF exerts its function in a strictly paracrine manner on stromal cells, especially endothelial cells and fibroblasts *in vivo* (Bronzert et al. 1987). This notion is confirmed by the observation that the secretion of PDGF-AA by tumor cells fails to stimulate the synthesis of VEGF (vascular endothelial growth factor) but leads to the recruitment of fibroblasts that participate in tumor growth and neo-angiogenesis (Heldin and Westermark 1999). Importantly, unlike TGF- β , which promotes fibroblast activation and phenotypic switch, the PDGF primary functions are the recruitment and the proliferation of fibroblasts with no influence on their differentiation into myofibroblasts (Shao et al. 2000). Consistent with all these findings, PDGF has been proposed to act indirectly through macrophages that secrete TGF- β upon recruitment at the tumor site (Shimokado et al. 1985; Elenbaas and Weinberg 2001). PDGF, therefore, represents an important factor in tumor vascularization and in the establishment of pro-tumorigenic stroma, which consequently enhances tumor growth.

Notably, the accumulation of reactive oxygen species (ROS; Fig. 1a) also drives the switch of resident fibroblasts into CAFs. ROS modulates the crosstalk between the cancer cells and fibroblasts via increased expression of growth factors such as PDGF and TGF- β , which eventually leads to the secretion of chemokines such as CXCL12 (Costa et al. 2014).

Angiogenic endothelial cell mitogens, also known as basic FGF (Fig. 1a), are pleiotropic molecules that function in an intracrine, autocrine and paracrine manner on both epithelial and mesenchymal cells (Strutz et al. 2000). This family of growth factors comprises nine different members, including FGF-2, which has been exhaustively described as a potent angiogenesis inducer (Folkman et al. 1988). FGF-2 acts through low- and high-affinity receptors (heparan

sulfateproteoglycans [HSPG] and tyrosine kinase receptors) with a wide range of effects on various cell types (Haugsten et al. 2010). Cell growth stimulation and differentiation represent the most important functions of this growth factor and the ability of FGF-2 to induce the proliferation of epithelial cells provides a strong indication of the way that it is involved in cancer development and progression. Moreover, the upregulation of FGF-2 has been linked to fibrogenesis and to the changes in the fibroblast phenotype leading to cell activation (Strutz et al. 2000). FGF-2 is a strong promoter of angiogenesis and fibrogenesis and also has an active role in the metastatic properties of cancer cells. Previous studies have unveiled the interplay between FGF-2 and the pro-angiogenic factor PDGF-BB and their synergistic action on tumor angiogenesis and metastasis (Nissen et al. 2007).

Together, the intertwining sets of signaling molecules, such as growth factors and cytokines, play a lead role in the establishment of the CAF-like phenotype; however, inflammation and metabolic stress further provide an impulse for the development of the tumor microenvironment. Notably, increased levels of interleukin-6 (IL-6) are also linked to metabolic reprogramming through c-Myc inactivation accomplished by the inactivation of mTORC1 in p62-deficient stromal fibroblasts in the model of prostate cancer (PCa). Downregulation of p62 leads not only to IL-6 activation but also to impaired metabolic detoxification and the release of ROS (Fig. 1a). Despite the opposite role that p62 plays in epithelial cells, p62 regulation of mTORC1 in the tumor microenvironment and in stromal fibroblasts arises as an anti-inflammatory tumor suppressor (Valencia et al. 2014). Interestingly, p62 is a subunit of nuclear factor kappa B (NF- κ B), a factor that will be further discussed below.

A recent study revealed an interesting cooperation between TGF- β and Wnt7a in breast cancer (Avgustinova et al. 2016). Wnt7a is a factor secreted by aggressive tumor cells and is able to induce fibroblast recruitment and activation both *in vitro* and *in vivo* (Fig. 1a). The mechanism of Wnt7a action is independent from the classic Wnt signaling pathway and instead enhances TGF- β signaling. Experimental evidence suggests that Wnt7a acts as a modulator of TGF- β receptor activity without interfering with downstream signaling elements. In breast cancer cell lines, isolated from *in vivo* orthotopic tumors, no modulation of TGF- β expression is detected. Nevertheless, because tumors display major differences in stromal composition, Wnt7a has been concluded to control the complexity of TGF- β signaling in a temporal and spatial context. Thus, similar to LIF (leukemia inhibitory factor), Wnt is hypothesized to sustain the establishment of an autocrine TGF- β signaling loop that promotes and maintains the CAF phenotype. Functionally, the upregulation of Wnt7a induces a desmoplastic response that is characterized by an activated fibroblast phenotype, matrix remodeling and pro-invasive properties of the tumor cells.

Moreover, LIF, a member of the IL-6 proinflammatory cytokine family, is identified as a novel pro-invasive fibroblast activator and a tumor promoter (Albregues et al. 2014). LIF is known to be overexpressed in a variety of solid tumors and its enhanced production by tumor cells correlates with their invasive potential (García-Tuñón et al. 2008; Wysoczynski et al. 2007). LIF supports the pro-invasive activation of fibroblasts by both autocrine and paracrine mechanisms; whereas the autocrine route relies on TGF- β signaling, the paracrine action is independent of α -SMA expression. We suggest that, through autocrine secretion, LIF mediates STAT3 phosphorylation upon TGF- β stimulation and contributes to the TGF- β -dependent pro-invasive activation in fibroblasts by actomyosin contractility and pro-invasive ECM remodeling. On the other hand, LIF paracrine activities promote and sustain the pro-invasive conversion of fibroblasts independently of α -SMA expression. The underlying molecular mechanism is based on crosstalk between the JAK1/STAT3 and RhoA/ROCK/MLC2 signaling pathways. Starting from the idea that the term CAFs defines a highly heterogeneous cell population expressing a variety of cell markers, including α -SMA, which is expressed by only a set of CAFs (Sugimoto et al. 2006), we detected increased levels of PDGFR α in fibroblasts upon LIF stimulation. Indeed, we established that LIF stimulation *in vitro* leads to the generation of a CAF subpopulation able to promote invasive tumor cell evolution independently of α -SMA expression. The novelty is the demonstration that fibroblast exposure to LIF is sufficient to promote a microenvironment permissive for tumor cell invasion. Because the synergistic action of TGF- β and JAK relies on the ability of JAK to stimulate the profibrotic effect of TGF- β signaling (Dees et al. 2012), we have shown that the JAK1/2 inhibitor Ruxolitinib can counteract the TGF- β - and LIF-mediated fibroblast-dependent carcinoma cell invasion. Mechanistically, the LIF-induced constitutive activation of JAK1/STAT3 signaling in fibroblasts is a consequence of epigenetic modifications leading to the loss of expression of *PTPN6*, which encodes for SHP-1 (Src homology region 2 domain-containing phosphatase-1) (Albregues et al. 2015). Moreover, we deciphered the course of action that drives the epigenetic silencing of SHP-1 tyrosine phosphatase, a negative regulator of the JAK/STAT pathway, by acetylated STAT3 (Fig. 1b). Indeed, acetylated STAT3 activates DNMT3b DNA methyltransferase, which, in turn, methylates CpG sites in the SHP-1 promoter, silencing its expression and leading to the constitutive activity of the JAK1/STAT3 signaling pathway (Albregues et al. 2015).

HSF1 (Heat shock factor protein 1) is another transcriptional factor that has been implicated in the transcriptional reprogramming of stromal cells leading to a switch from a tumor-repressive phenotype to a tumor-promoting one (Fig. 1a). HSF1 is a ubiquitously expressed transcriptional factor known for its tumor suppressor role in a wide range of

human cancer cell lines and also for its obliteration in mice providing great resistance to tumorigenesis (Mendillo et al. 2012). A similar role of HSF1 in tumor stroma has been elucidated. In cancer cells, the HSF1-dependent program is stress-activated and in the tumor microenvironment, the stromal-HSF1 program drives the activation of two central signaling pathways: TGF- β and SDF1 (Scherz-Shouval et al. 2014).

In addition to the action of soluble factors, the modification of the tumor microenvironment via signaling pathways can be achieved by changes in the physical properties and the mechanical forces of the surrounding ECM. Disruption of the matrix stiffness and cell shape leads to the unusual cell behavior characteristic of malignant cells, notably the enhancement of metastatic and invasive properties. Indeed, cells can translate mechanical forces into biochemical signals mediated by members of known signaling pathways. In particular, a stiff matrix, a characteristic of several solid cancers, promotes the YAP/TAZ (Yes-associated protein/Tafazzin) transcriptional activity that is responsible for the delivery of mechanical messages through Rho GTPase activity and actomyosin cytoskeleton tension but independent of the Hippo pathway (Dupont et al. 2011). Increased levels of YAP and TAZ are known to promote tumorigenesis, cell proliferation, EMT and migration. As CAFs are the main cell type found in the tumor microenvironment and as they are essential for matrix remodeling, the role of YAP in the establishment and maintenance of CAFs has therefore been investigated. YAP is involved in a positive feedback loop orchestrated by two interdependent events. In response to stimulation by soluble factors, such as TGF- β , the matrix becomes stiffer, which leads to the enhanced expression of YAP, which in turn regulates the transcription of genes involved in matrix stiffening and pro-carcinogenesis. Matrix remodeling and YAP activation are both essential for the sustained activation and phenotype maintenance of CAFs (Calvo et al. 2013). Interestingly, CAFs activities can be modified, promoted and sustained not only through biochemical interactions within the tissue but also by physical modifications of tissue. The role of integrin $\alpha 11 \beta 1$, which is expressed by CAFs in collagen cross-linking and stiffness, has recently been found to correlate with the growth and metastasis of non-small cell lung carcinoma, a finding that supports the notion that mechanical stress strongly impacts tumor progression (Navab et al. 2015). Matrix remodeling is essentially regulated by the coordinated expression of a variety of MMPs and their inhibitors, which, under physiological conditions, orchestrate ECM organization and homeostasis. In cancer, homeostasis is disrupted and MMPs facilitate the invasion and migration of cancer cells by degrading collagen (Wolf et al. 2007). The *Timp* gene family of MMP inhibitors controls a wide range of MMPs and ADAMs (a disintegrin and MMPs) by post-translational inhibition (Khokha et al. 2013). The role of TIMPs in generating a tumor-permissive stroma has been inferred from the observation that the complete loss of *Timp* is

sufficient for fibroblasts to acquire a CAF-like state. Moreover, exosomes secreted from TIMP-less fibroblasts are enriched in ECM proteins and the MMP ADAM10 inducing cancer cell motility and stemness (Shimoda et al. 2014).

Clearly, well-coordinated mechanisms activating fibroblasts support the complex cascade of events resulting in cancer development that, like planned sabotage, overwhelm any possible defense. Indeed, cancer cells display a wide range of actions that manage to “tame” the surrounding cells and, even worse, turn them into “preachers of malignancy”.

Inflammation drives fibroblast activation

In addition to the established hallmarks of cancer (Hanahan and Weinberg 2000), inflammation has been defined as a fundamental process in tumor development. Despite its main function of “serving and protecting”, the immune system plays a dual role in cancer development. The activation of adaptive immune cells results in the destruction of malignant cells but in response to the chronic activation of the innate immune system, the contrary occurs: the immune system provides all the ingredients required to bypass all the steps of carcinogenesis (DeNardo et al. 2010). Indeed, inflammation is a response to tissue injury justifying the idea that cancer is a “wound that never heals”. Keeping in mind that the activation of fibroblasts is crucial in wound healing, the role of chronic inflammation in carcinogenesis becomes evident. Inflammatory responses are involved in various stages of tumor development, initiation, promotion, malignant progression, invasion and metastasis. Upon inflammation, the tumor microenvironment becomes infiltrated with immune cells, including innate immune cells (macrophages subtypes, neutrophils, basophils, mast cells, myeloid-derived suppressor cells, dendritic cells and natural killer cells) and adaptive immune cells (lymphocytes) (de Visser et al. 2006). All these various immune cells engage themselves in dynamic communication with the cancer cells, either by direct contacts or by the autocrine or paracrine production of chemokines and cytokines that promote and induce tumor growth and progression. Specifically, the tumor microenvironment is rich in immune-cell-derived growth factors, such as the previously mentioned TGF, FGF and EGF and also tumor necrosis factor, VEGF and members of the interleukin family, namely IL-1 and IL-6. Paracrine secretion of these variable molecules by cancer cells has been demonstrated to promote tumorigenesis (Calvo and Sahai 2011). In this context, we proposed that the induction of pro-invasive activity in human dermal fibroblasts requires transient stimulation by TGF- β , which is then sustained by JAK1/STAT3-dependent signaling. Indeed, JAK1 activity induces contraction in stromal fibroblasts leading to ECM remodeling and the creation of migration tracks for squamous carcinoma cells (Sanz-Moreno et al. 2011). In addition, we

should mention the production of proangiogenic and/or pro-invasive matrix-degrading enzymes, including MMP-9 and other MMPs, cysteine cathepsin proteases and heparanase (Grivennikov et al. 2010). Expression of all these different factors triggers the induction of multiple signaling pathways acting on both cancer cells and stroma. Moreover, inflammatory cells can release chemicals, particularly ROS, that are highly mutagenic for cancer cells and that accelerate their deleterious evolution (Hanahan and Weinberg 2011). Signaling routes directed by JAK/STAT, TGF- β and the transcriptional factor NF- κ B are the most prominent actors in the inflammatory response. NF- κ B has been designated by Ben-Neriah and Karin (2011) as “playing the first violin, if not the “conductor” of the inflammatory response”. Several studies have focused on various roles that NF- κ B can have in the context of the tumor microenvironment. Previously described chemokines such as *CXCL1*, *CXCL2* and *CXCL5* and cytokines *IL-6* and *IL-1 β* , which are upregulated in CAFs, are also known targets of NF- κ B transcription factor. Therefore, not only is the upregulation of NF- κ B is inflammation-linked in various cancers (Karin and Greten 2005; Pikarsky et al. 2004), but it also establishes a proinflammatory gene signature responsible for the tumor-promoting behavior of CAFs in models of pancreatic and mammary skin cancer (Erez et al. 2010). Functionally, the activation of NF- κ B is dependent on I κ B kinase β (IKK β), which is a catalytic component of the IKK complex. Intriguingly, two independent and rather similar studies have disclosed quite opposite functions of IKK β . In the model of colitis-associated cancer (CAC), IKK β is described to play a tumor-suppressive role in Col1a2-expressing fibroblasts, but a tumor-promoting role in intestinal mesenchymal cells (IMCs). Whereas the Col1a2 fibroblast-restricted deletion of *Ikk β* leads to increased cell proliferation, cell death evasion, accumulation of T cells angiogenesis and ultimately promotes colonic tumor growth (Pallangyo et al. 2015), IMC-specific *Ikk β* deletion in vivo leads to a decreased tumor proportion (Koliaraki et al. 2015). Moreover, IKK β has been shown to exert its protumorigenic function through the upregulation of proinflammatory cytokines and chemokines, including IL-6, which is responsible for STAT3 activation. Such contradictory results can be explained by the difference in the subpopulation of mesenchymal cells that, in each case, leads to distinct phenotypes and mechanisms responsible for IKK β function.

As mentioned above, JAK/STAT and TGF- β are necessary for fibroblast activation. Inflammation is an extremely complex and chaotic multistep process that influences a wide pattern of responses from immune cells recruited by the tumor microenvironment. The recruited cells then drive the production of a variety of factors that promote angiogenesis, motility, invasion and metastasis. Just by focusing on fibroblasts, we can see that inflammation plays an essential role in the production of activating molecules, spanning from growth factors

to interleukins. Therefore, inflammation can be suggested as a driving force of fibroblast activation.

Chemoresistance

Today, despite the remarkable progresses made in the development of anti-cancer treatments, most of the protocols are ultimately unsuccessful over time. The reasons behind such a failure remain obscure but large amounts of evidence suggest that signals from the stroma cells, notably CAFs, provide the survival skills necessary for a small population of cancer cells to gain the capability to adapt to treatments and evade chemotherapies (Paraiso and Smalley 2013). Apparently, CAFs can interfere with the distribution of drugs within the tumor and provide innate and adaptive resistance to cancer treatments. Single-drug treatments are progressively replaced by protocols combining two or more antitumor agents. Although mechanisms of chemoresistance are obviously complex and rely on a variety of cross-linked signaling pathways, the targeting of both cancer cells and the surrounding stroma provides an interesting perspective for the development of novel therapeutic strategies (Hirata et al. 2015).

Among the various mechanisms of chemoresistance, Crawford et al. (2009) reported that tumor cells show resistance to anti-VEGF therapy and stimulate fibroblast activation in the stroma (Fig. 2). Upon activation, CAFs secrete PDGF-C, an important mediator of CAF-induced angiogenesis and carcinogenesis. Nearly all patients treated with one of the anti-angiogenesis drugs, such as Bevacizumab, Sorafenib, or

Sunitinib, become unresponsive after an initial promising response (Kerbel 2008). Additionally, tumors with intrinsic resistance to anti-VEGF drugs have been observed, even when the specific drug targets are highly expressed by the cells. Nowadays, combinational therapy with two or more chemical compounds is employed to achieve a more efficient anti-tumor response. However, even combinations of drugs fail to arrest the tumor growth completely indicating the existence of multiple complex mechanisms of chemoresistance. Another example of a chemoresistance mechanism is the secretion of HGF by fibroblasts isolated from lung cancer patients in whom the activation of c-MET is stimulated in tumor cells (Fig. 2). Consequently, c-MET raises tumor cell resistance to conventional treatments, such as the potent EGFR inhibitor Gefitinib (Wang et al. 2009). Accordingly, the inhibition of the HGF/Met signaling pathway makes cancer cells more susceptible to chemotherapeutics. Moreover, the secretion of HGF by CAFs is also involved in the resistance to treatments with the RAF inhibitors, Vemurafenib and Dabrafenib, which are used in the case of melanoma patients carrying a mutated *B-RAF* and which act on the regulation of MAPK and PI3K/PKB (Straussman et al. 2012; Wilson et al. 2012). B-RAF, which belongs to a family of serine/threonine kinases, is a member of the MAPK/ERK pathway that controls numerous cell functions whose deregulation is associated with a broad range of tumors (Maurer et al. 2011). In detail, B-RAF inhibitors for *BRAF* V600E mutant melanoma at first show high efficiency in “melting the tumor” but, shortly after the initial shrinkage, tumor resistance occurs and the life expectancy of patients decreases (Long et al. 2012; Sosman et al. 2012). The

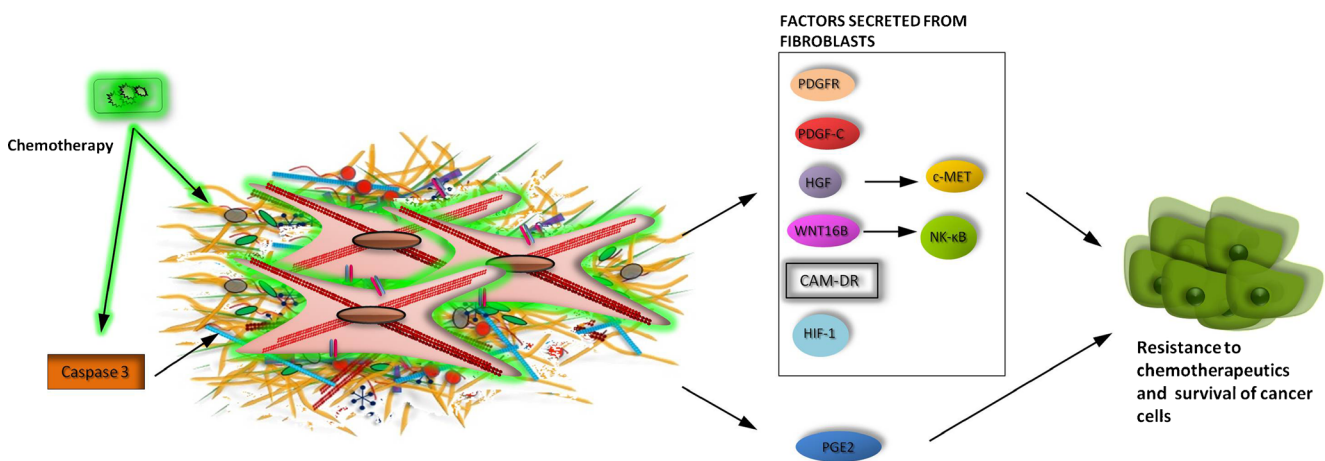


Fig. 2 Cancer-associated fibroblasts mediate chemoresistance. During chemotherapeutic treatments, a significant number of cancer cells develop pro-survival properties. CAFs play an important role in this mechanism by expressing signals that promote cancer resistance. CAF signals vary depending on the chemotherapy used and cancer type. Signals include several growth factors, growth factor receptors, transcription factors and adhesion molecules. Treatment of patients with EGFR and B-RAF inhibitors leads to CAF secretion of HGF, which stimulates the expression of c-MET providing cancer resistance. PDGF and its

receptor PDGFR also contribute to cancer resistance. The DNA damage that is induced after chemotherapeutic treatment activates WNT16B cytokine, which regulates NF-κB transcriptional factor. Chemotherapeutics can also induce apoptotic escape, cell adhesion-mediated drug resistance (CAM-DR) and hypoxia. The expression of caspase 3 stimulates CAFs to produce prostaglandin E2 (PGE2), whereas in an hypoxic environment, hypoxia-inducible factor 1 (HIF1) is secreted from CAFs. Through distinct mechanisms and cooperative behaviour, CAFs facilitate the adaptation of cancer cells to treatment (*NF-κB* nuclear factor kappa B)

mechanism of resistance relies on diverse possibilities, such as an increase in RTK signaling, the emergence of B-RAF splice mutants and the acquisition of secondary mutations in *NRAS* (Fedorenko et al. 2011; Poulikakos et al. 2011). Another approach has disclosed the dual mechanism of B-RAF mutant melanoma cell resistance to treatment with Vemurafenib. Cell survival upon treatment is stroma- and adhesion-signal-dependent. Initially, the tumor stroma is activated by B-RAF inhibition but this is then followed by B-RAF independent ERK/MAP kinase activity (Hirata et al. 2015). Interestingly, even though resistance follows different routes, the recovery of the MAPK signaling pathway upon treatment is common to all mechanisms. This observation has led to a double-target treatment strategy, targeting both B-RAF and MEK (Flaherty et al. 2012). Despite the positive results in early phase II trials, resistance still arises suggesting the complexity of melanoma is greater than expected. Similarly to B-RAF inhibition, the inhibition of EGFR with small molecules leads to the generation of genetically resistant cells within the tumor. As it is now known, the mechanism behind this phenomenon relies either on acquired mutations within the EGFR kinase domain or on MET amplification but the evolution of resistance is not fully understood. Tumor heterogeneity contributes to the distinct compliance of tumor cells and it influences the outcome of targeted therapies. Tumor cells have been shown to be able to follow different paths for developing chemoresistance prior to or post treatment depending on the intrinsic novel properties that they have acquired and on external stimuli from the microenvironment (Hata et al. 2016).

Intriguingly, one study unveiled that DNA damage induced by chemotherapeutic treatment causes secretion of WNT16B by the tumor environment (Fig. 2). Expression of this cytokine is associated with the regulation of the transcriptional factor NF- κ B, which activates the Wnt signaling pathway in cancer cells and promotes resistance to treatment (Ostman 2012). The impact that epigenetic modifications of tumor cells have on the surrounding stroma cells that then become involved in cancer adaptive resistance is intriguing.

Several other systems show that matrix remodeling by CAFs themselves perturbs the access of chemotherapeutics to the tumor by providing a chemoprotective niche and thus contributes to resistance to treatments, apoptotic escape and cell-adhesion-mediated drug resistance (CAM-DR; Meads et al. 2009; Fig. 2). CAM-DR modulates the adhesion of cancer cells to the ECM; this was first described in multiple myeloma in which the adhesion of myeloma cells to fibronectin provides resistance to Melphalan (Hazlehurst and Dalton 2001). A number of cell adhesion receptors are present on the cell membrane of CAFs, including integrins, non-integrin collagen receptors and cell surface proteoglycans (Multhaupt et al. 2016; Zeltz and Gullberg 2016). All these molecules are potentially involved in cell adhesion mechanisms contributing to chemoresistance. Some of these cell-adhesion-related mechanisms have recently

been reviewed (Eke and Cordes 2015). As an example, the presence of hyaluronic acid, an ECM component abundant in the tumor stroma, is associated with a poor prognosis in human cancer, specifically in pancreatic cancer. This product of matrix degradation plays an important role in the innate immune response by interacting with the cellular receptor CD44 (Iijima et al. 2011). Recently, hyaluronic acid has been shown to form a physical barrier that blocks the access of drugs to the tumor (Provenzano et al. 2012). Furthermore, increasing evidence demonstrates that enzymatic depletion of hyaluronic acid in the ECM before treatment with Gemcitabine (a pancreatic cancer treatment) augments the effect of the drug in a mouse model of adenocarcinomas and pancreatic cancer. Because hyaluronic acid helps to maintain pressure between cells, its degradation causes a drop of intercellular pressure. This leads to an increase of microvascularization that results in higher levels of Gemcitabine cytotoxicity, which then blocks tumor growth and metastatic spread (Provenzano et al. 2012). This mechanism is restored through Hh-Smo (Hedgehog-Smoothed) signaling; indeed, the inhibition of the Hh-Smo pathway diminishes the desmoplastic response and fibrosis, which facilitates the intra-tumoral distribution of Gemcitabine (Olive et al. 2009; Yauch et al. 2008). In pancreatic cancers, provocative data suggest that CAFs also play a tumor-suppressive role (Özdemir et al. 2014; Rhim et al. 2014).

CAF's influence the transport of drugs into the bloodstream via mechanisms involving the PDGFRs and regulation of the intracellular pressure in blood vessels (Heldin et al. 2004). Indeed, the inhibition of the PDGFR signaling by the kinase inhibitors STI571 or PDGF-B antagonists reduces vascular intercellular pressure and facilitates drug transport into the tumors (Pietras et al. 2003b).

Among other roles, CAFs contribute to the development of hypoxia by producing a variety of growth factors that increase cell proliferation, such as TGF- β , HGF and pro-angiogenic factors VEGF, PDGF and SDF1. Hypoxia can modify the efficiency of chemotherapeutic treatments, whose action is frequently affected by the oxygenation of the tumor microenvironment (Fig. 2). As shown in vivo and in vitro, the cytotoxic and cyclophosphamide effects of Doxorubicin are oxygen-dependent and, therefore, diminished in hypoxic environments (Harrison and Blackwell 2004). Moreover, hypoxia generates an acidic environment through the anaerobic respiration that causes the production of lactic acid within the ECM, while maintaining intact the intracellular pH (Harrison and Blackwell 2004), thereby preventing the assimilation of chemotherapeutic agents by the cancer cells (Gerweck and Seetharaman 1996; Vukovic and Tannock 1997). Cell sensitivity to cytotoxic drugs is dramatically reduced by hypoxic environments. Indeed, specific drugs are designed to target dividing cells that are in a state of hypoxia attributable to the upregulation of the transcription hypoxia-inducible factor 1 (HIF-1). These drugs have a positive effect that results in cell

cycle arrest and that consequently limits the adverse effect of hypoxia on conventional treatment (Vukovic and Tannock 1997). In addition, in response to the hypoxic environment, HIF-1 induces the expression of proteins encoded by the family of drug resistance genes (multi-drug resistance; Wartenberg et al. 2003). Such proteins belong to a family of ABC transporters that allow cancer cells to eliminate anti-tumor drugs (Gottesman et al. 2002). In a brief summary, the hypoxic environment created by CAFs causes a decrease in chemotherapeutic assimilation and efficiency and, also, higher resistance of cancer cells to treatment, thereby allowing their survival. On the contrary and rather surprisingly, Erler and coworkers provided evidence that prolonged hypoxia can eventually lead to the deactivation of CAFs and reduced ECM remodeling (Madsen et al. 2015). Paradoxically, hypoxia is known to be correlated with tumor invasiveness, metastasis, poor prognosis and chemoresistance (Wilson and Hay 2011). Nonetheless, Madsen et al. (2015) suggested that chronic hypoxia deactivates CAFs, finally abrogating cancer cell invasion and metastasis.

Unexpectedly, evidence has been provided that signals from necrotic cancer cells during cytotoxic cancer therapy can deliver survival signals favoring a therapeutic escape (Huang et al. 2011). Such signals are linked to the cellular processes responsible for cell death and the regulation of the growth-promoting properties of the surrounding cells. Caspase 3, the “master executioner”, is found to drive such an unusual effect that associates cell death with tumor repopulation (Fig. 2). The underlying mechanism depends on the ability of caspase 3 to promote the synthesis of prostaglandin E2 (PGE2) in the microenvironment (Huang et al. 2011). PGE2 is a key regulator of tumor growth and promotes the survival of cancer cells (Castellone et al. 2005). During this “survival” process, a few cells emit signals leading to the reconstruction of the damaged tumor population by inducing high rates of proliferation of the surviving cells. This phenomenon is poorly understood but further investigation will certainly uncover relevant roles for this mechanism, which may be harnessed in the “new age” radio- and chemotherapy.

Perspectives

Despite the great efforts devoted to the understanding of the molecular mechanisms lying behind fibroblast pro-tumoral activation and their roles in cancer progression, several relevant questions lack answers. Various methodological approaches have unveiled the complexity of novel interlaced pathways, such as the LIF and Wnt fibroblast activation mechanisms that drive the phenotype switch in fibroblasts. Intriguingly, several mechanisms rely on TGF- β signaling and/or on bi-directional communication between the fibroblasts and the cancer cells, which makes the general overview of the signaling pathways

extremely complex. The complexity of the phenomenon not only relies on the two-way communication between CAFs and cancer cells but is also further fueled by the immune responses. On the other hand, novel studies have focused increasingly on the role of mechanotransduction and epigenetic alterations that lead to CAFs activation and tumor promotion; this, we believe, is an important direction to follow in the future. Another feature that requires greater attention is the presence of diverse subpopulations of CAFs in the tumor stroma; these populations can be distinguished from each other by the expression of one or more specific markers. Hence, CAFs might constitute a constellation of cells of diverse origins and locations playing distinct roles in tumorigenesis. In general, CAFs seem to exert the common function of altering the tumor microenvironment but not only by promoting tumorigenesis. Some studies have reported the anti-tumorigenic effects of CAFs (Öhlund et al. 2014), specifically in pancreatic cancer in which the depletion of CAFs induces immunosuppression and promotes the development of cancer per se (Özdemir et al. 2014). Therefore, future work must take into consideration alternative approaches aimed at unraveling all possible functions that CAFs may have and, accordingly, at designing personalized treatments. Indeed, one of the most interesting aspects of studies of the role of fibroblasts in cancer is the possible implementation of therapeutic strategies that would target both the cancer cells and their microenvironment. The ability of CAFs to regulate the responses of cancer cells to targeted curative treatments and chemotherapeutics constitutes an attractive area of investigation that is focused on the identification of specific protocols aimed at the well-identified “deleterious” activities of the fibroblasts. Of course, the multifaceted nature of CAFs constitutes a difficult challenge for the identification of specific drugs presenting a real potential for the development of therapeutic approaches able to counteract the harmful action of CAFs. When all the data are taken together, the future of cancer research appears to be promising. However, it will nevertheless remain problematic because a long succession of lost and won battles are expected in which fibroblasts represent the front line that has to be broken in order for novel generations of anti-tumoral drug treatments to gain access to the cancerous cells.

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