Charles University
First Faculty of Medicine

Role of fibroblasts in wound healing and cancer

Rosana Mateu Sanz

PhD Thesis in Cell Biology and Pathology

Supervisor: Professor Karel Smetana, MD, DSc

Institute of Anatomy

Prague 2021

I hereby, declare that this thesis is my own work and that, to the best of my knowledge and belief, it has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications. And I also declare that I have acknowledged all material and sources used in its preparation.

Rosana Mateu Sanz

ACKNOWLEDGEMENTS

First and foremost I am extremely grateful to my supervisor Prof. MUDr. Karel Smetana ml., DrSc. for his invaluable supervision, bright ideas and tutelage during the course of my PhD degree and to RNDr. Barbora Dvořánková, Ph.D. for her support inside and sometimes outside of the laboratory.

I would like to thank prof. MUDr. Aleksi Šedo DrSc without whom I would not have been able to complete this research and doc. MUDr. Petr Bušek, Ph.D. for his advice, indispensable help and patience.

I would also like to thank my colleagues from the Institute of Anatomy and Institute of biochemistry and experimental oncology for the fruitful work and the time spent together.

I per últim, també m'agradaria donar les gràcies als meus amics, especialment Ondra, Sandra i Bastien per la seva ajuda, i la meva familia per estar sempre al meu cosat.

LIST OF ABREVIATIONS

3D	Tridimensional
ADAM	Disintegrin and metalloproteinase
ADAMTS	ADAMs with thrombospondin motifs
AF	Adult fibroblast
AGE	Advanced glycation end products
AGE-RAGE	Advanced glycation end products-receptor for advanced glycation end products
AK	Adult keratinocyte
ANG-1	Angiopoietin-1
Bcl-2	B-cell lymphoma 2
CAF	Cancer-associated fibroblasts
CAR	Chimeric antigen receptor
CTGF	Connective tissue growth factor
CXCL	Chemokine (C-X-C motif) ligand
DAPI	4',6-diamidino- 2-phenylindole
DDR2	Collagen-specific receptor tyrosine kinase
DKK1	Dickkopf 1
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPPIV	Dipeptidyl peptidase IV or CD26
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunoabsorbent assay
EMT	Epithelial to mesenchymal transition
EndMT	Endothelial to mesenchymal transition
FABP4	Fatty acid binding protein
FACITs	Fibril-associated collagens with interrupted triple helices
FaDu	Human squamous cell carcinoma isolated from pharynx; HTB-43
FAP	Fibroblast Activation Protein
FBS	Fetal bovine serum
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein IsoTioCyanate
FSP-1	Fibroblast-specific protein-1
GDF	Growth differentiation factor
GM-CSF	Granulocyte-macrophage-colony stimulating factor
GPER	G-protein-coupled estrogen receptor
HDGF	Hepatoma-derived growth factor
HFP3	Human adult fibroblasts
HGF	Hepatocyte growth factor
HIAR	Hypoxia-induced angiogenesis regulator
HIF	Hypoxia-inducible factor
HSP-47	Heat shock protein 47
HT-29	Human colorectal adenocarcinoma; HTB-38
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
INHBB	Inhibin subunit beta B
JAK	Janus kinases

JNK	c-Jun N-terminal kinase
K	Keratin
KAZALD1	Kazal type serine peptidase inhibitor domain 1
LIF	Leukemia inhibitor factor
MACITs	Membrane-associated collagens with interrupted triple helices
MCP	Monocyte chemotactic protein
MDSC	Myeloid-derived suppressor cells
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cells
NCF	Neonatal cleft lip fibroblasts
NF	Newborn fibroblasts
NF-κB	Nuclear factor-Kb
NGF	Nerve growth factor
NK	Newborn keratinocytes
NKC	Natural killer cells
OCCF	Older child cleft lip fibroblasts
Oct4	Octamer-binding transcription factor 4 or POU5F1
PBS	Phosphate- buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PEDF	Pigment epithelium derived factor
PET	Polyethylene terephthalate
PCOLCE	Procollagen C-Endopeptidase Enhancer
PTEN	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase
qPCR	Real-time polymerase chain reaction
RER	Rough endoplasmic reticulum
RhoA	Ras Homolog Family Member A
ROCK	Rho associated protein kinase
ROS	Reactive oxygen species
SCA-1	Stem cells antigen-1
SDF	Stromal cell-derived factor
Shh	Sonic hedgehog
α-SMA	Smooth muscle actin alpha
SMC	Smooth muscle cells
Smo	Smoothened
SPARC	secreted protein acidic and rich in cysteine
Src	Steroid receptor coactivator
STAT	Signal transducer and activator of transcription
Sw620	Human colorectal adenocarcinoma; CCL-227
TGF- α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
TGF-βR	Transforming growth factor receptor
TIMP	Tissue inhibitors of metalloproteinases
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	T regulatory lymphocytes
TRITC	Tetramethylrhodamine
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WB	Western blot
YAP	Yes-associated protein

TABLE OF CONTENT

SUMMARY

Fibroblasts are stromal cells ubiquitously present in the human body. They often appear in a quiescent state and can become activated in response to tissue remodeling signals. Activated fibroblasts acquire biosynthetic, pro-inflammatory and contractile properties, key functions for wound healing. In addition, the presence of permanently activated fibroblasts is one of the hallmarks of cancer. The purpose of this work is to investigate the differences between newborn and adult fibroblasts and keratinocytes in their implication in scarless wound healing, the origin of cancer associated fibroblasts (CAF)s and the influence of fibroblasts in melanoma invasion.

Evidence suggests that wounds heal almost without scar in newborns. To understand the mechanisms that contribute to scarless wound healing we focused on the differences between newborn and adult fibroblasts and keratinocytes, which are cells present in human skin and participating in wound healing process. A comparison of the expression profile between newborn and adult fibroblasts showed differentially regulated genes related to the acute phase of the inflammatory response and ECM organization, traits involved in wound healing. We also found that newborn fibroblast showed higher differentiation potential, exhibited markers of pluripotency and poor differentiation and expressed smooth muscle actin α (α-SMA) more frequently.

α-SMA expressing fibroblasts are called myofibroblasts and they are the main producers of ECM, and are key players in wound healing. Transforming growth factor beta (TGF-β) signaling pathway triggers the expression of α-SMA in fibroblasts. We noticed that newborn fibroblasts showed an upregulation of transcripts for TGF-β2 and TGF-β3, and downregulation of the transforming growth factor receptor II (TGF-R2) compared to adult fibroblasts.

In addition, the expression of α-SMA in both adult and newborn fibroblasts can be increased in coculture with newborn keratinocytes, indicating the importance of the crosstalk of fibroblasts with epithelial cells. The newborn keratinocytes showed expression of keratins (K)- 8, -14 and -19, markers of poor differentiation. This reminds the keratin expression profile of malignant cells.

In the second part of this work we examined the role of fibroblasts in melanoma invasion. We studied how the secretome from human fibroblasts and CAFs affected human melanoma cell line invasiveness *in vitro*. Melanoma cells appeared more invasive when cultivated in conditioned media from CAFs. Cocultivation of CAFs with melanoma cells induced secretion of interleukin 6 (IL)-6 in

fibroblasts and IL-8 in melanoma cells. High levels of IL-6 and IL-8 have been observed in melanoma patient serum. Moreover simultaneous blocking of IL-6 and IL-8 reversed fibroblasts induced melanoma cell invasiveness.

Since the source of CAFs is unclear we investigated the possibility that they can originate from cancer cells through epithelial-to-mesenchymal transition. For this purpose human cancer cells were grafted to nu/nu mice. Tumors were formed, they contained a well structured stroma containing typical smooth muscle actin cancer-associated fibroblasts. We observed that these cells did not originate from the xenografted cells, instead they were from the host origin.

Findings summarized in this thesis suggest that fibroblasts are a dynamic heterogeneous cells population and are key players in both wound healing and cancer.

SOUHRN

Fibroblasty jsou stromální buňky, které jsou rozšířené v celém lidském těle. Často se vyskytují v neaktivním stavu a k jejich aktivaci dochází až při remodelaci tkáně. Aktivované fibroblasty produkují extracelulární matrix, mají prozánětlivé a kontraktilní vlastnosti, což jsou klíčové momenty v hojení tkání. Na druhou stranu je přítomnost aktivovaných fibroblastů jedním z typických znaků nádorového bujení. Cílem této práce je porovnání rozdílů mezi novorozeneckými a dospělými fibroblasty a keratinocyty ve vztahu k „bezjizevnatému“ hojení, k původu nádorově asociovaných fibroblastů a vlivu fibroblastů na invazivitu melanomu.

Klinické zkušenosti ukazují, že se u novorozenců hojí rány téměř bez jizvení. Abychom lépe porozuměli mechanizmům, které k tomuto hojení přispívají, zaměřili jsme se na rozdíly mezi novorozeneckými a dospělými fibroblasty a keratinocyty, kožními buňkami, které jsou zásadní pro hojivý proces. Srovnání expresního profilu novorozeneckých a dospělých fibroblastů ukázalo, že jsou rozdílně regulovány geny, které se vztahují k akutní fázi zánětlivé odpovědi a organizaci extracelulární matrix, což úzce souvisí s procesem hojení. Také jsme zjistili, že novorozenecké fibroblasty vykazují vyšší diferenciační potenciál, exprimují znaky nízké diferenciace a pluripotence a také častěji produkují hladký svalový aktin a (α-SMA).

Fibroblasty, které produkují α-SMA, jsou nazývány myofibroblasty a jsou hlavními producenty extracelulární matrix a klíčovými hráči v hojení ran. Signalizační dráha transformujícího růstového faktoru beta (TGF-β) spouští expresi α-SMA ve fibroblastech. Zjistili jsme, že novorozenecké

fibroblasty vykazují ve srovnání s dospělými zvýšenou expresi transkriptu TGF-β2 and TGF-β3 a naopak sníženou expresi receptoru II transformujícího růstového faktoru (TGF-R2).

Navíc může být exprese α-SMA zvýšena jak u dospělých, tak i novorozeneckých fibroblastů jejich kokultivací s novorozeneckými keratinocyty. To ukazuje na (nebo podtrhuje) význam vzájemné komunikace mezi fibroblasty a epitelovými buňkami. Novorozenecké keratinocyty exprimují (produkují) keratin-8, -14 a -19, které jsou charakteristické pro nízce diferencované buňky. Tento expresní profil keratinů připomíná profil nádorových epitelových buněk.

Ve druhé části této práce jsme zkoumali roli fibroblastů v invazivitě melanomů. Sledovali jsme, jak kondiciovaná média z lidských fibroblastů a nádorově-asociovaných fibroblastů (CAFs) ovlivňují in vitro invazivitu buněk lidské melanomové linie. Melanomové buňky vykazovaly vyšší invazivitu, jestliže byly kultivovány v médiu kondiciovaném nádorově asociovanými fibroblasty. Kokultivace nádorově asociovaných fibroblastů s melanomovými buňkami vyvolávala zvýšenou sekreci IL-6 u fibroblastů a IL-8 u melanomových buněk, avšak současná blokace IL-6 a IL-8 dokázala zvrátit zvýšenou invazivitu melanocytů, vyvolanou přítomností fibroblastů. Vysoké hladiny IL-6 a IL-8 byly také stanoveny v sérech pacientů s melanomem.

Vzhledem k tomu, že původ nádorově asociovaných fibroblastů je nejasný, soustředili jsme se na možnost, že mohou vznikat z buněk karcinomu epitelo-mezenchymální tranzicí. Jestliže byly lidské karcinomové buňky inokulovány do nu/nu myší, vytvořily nádory, které obsahovaly dobře strukturované stroma s nádorově asociovanými fibroblasty produkujícími hladký svalový aktin. Zjistili jsme, že tyto buňky nepocházely z xenotransplantátu, ale že byly myšího, tedy hostitelského původu.

Získané výsledky uvedené v této dizertační práci potvrzují, že fibroblasty jsou dynamickou, heterogenní buněčnou populací, která hraje klíčovou roli jak v hojení ran, tak i při tvorbě nádoru.

INTRODUCTION

1. FIBROBLASTS

1.1. FIBROBLAST DEFINITION

Fibroblasts are the main cells that form the connective tissue. These mesenchymal cells can acquire different morphologies depending on their location but generally they can be recognized by flattened, elongated or spindle shape and branched cytoplasm in culture, yet they acquire more complex morphologies in tissues. They can contain one or two flat, elliptical nuclei and a well-developed rough endoplasmic reticulum and Golgi apparatus. They can adhere and migrate on tissue culture substrates. Fibroblasts do not form flat monolayers and are not polarized cells. Fibroblasts make up approximately 30% of the tissue mass (Duffy, 2011; Kalluri, 2016).

Fibroblasts' main function is the production and secretion of a complex variety of molecules with structural and biological roles called extracellular matrix (ECM). The ECM provides a scaffold for other cells to adhere and is involved in tissue and organ morphogenesis and function. Fibroblasts synthesize and reorganize the ECM in many organs such as skin, lung, heart, kidney, liver, eye, etc. (Bonnans et al., 2014).

However, it is difficult to find a more specific and detailed definition, because these cells are very dynamic and heterogeneous. The phenotypic and functional characteristics of fibroblasts depend on the anatomic site of their origin, the pathologic status and the specific roles they play (Chang et al., 2002; Szabo et al., 2013). Presumably these differences reflect particular requirements of each tissue. Furthermore, in the same tissues, we can find different population of fibroblasts with specific functions and characteristics (Lynch and Watt, 2018; Nolte et al., 2008; Sorrell and Caplan, 2004). Specific fibroblast populations have different morphology, proliferation rate, contractibility, as well as collagen and matrix metalloproteinase (MMP) production (Lindner et al., 2012; Sorrell et al., 2007). Genomic work using cDNA microarray technology performed by Brown's group made possible to characterize the expression pattern of fibroblasts from different anatomical regions of the body. They were able to demonstrate a site-specific patterning with differences on fibroblasts expression from the anterior-posterior part, proximal-distal and dermal vs non-dermal fibroblasts. In addition, it was also demonstrated that genes involved in ECM synthesis, cell migration, growth and

differentiation are also expressed differentially in different parts of the body (Chang et al., 2002; Parsonage et al., 2003; Rinn et al., 2006).

Fibroblasts build a structural framework for tissues and organs and are key players in the support and homeostasis of nearly every tissue in the body. Even though in the adult body they appear in a quiescent state, fibroblasts are very dynamic cells and can respond to many types of stimuli. In case of tissue remodeling conditions such as embryonic development or tissue injury, the neighboring fibroblasts get activated, proliferate, migrate and secrete ECM components and cytokines and growth factors (Amadeu et al., 2003). Fibroblast malfunction is known to be involved in pathological processes such as hypertrophic scars, keloids, fibrosis and even tumors (Dick; et al., 2020).

We will discuss the function of fibroblasts in following chapters, mainly their principal role producing and remodeling ECM, and also their role in angiogenesis and as immunoregulator. We will consider fibroblasts in healthy tissues and also in pathologic conditions, specifically in wound healing and tumor development.

1.2. ONTOGENY

Most of the fibroblasts in the human body arise developmentally from the dermomyotome (Scaal and Christ, 2004). However, fibroblasts in the scalp and facial skin, arise from a completely different origin: the neural crest (Noden and Trainor, 2005). Fibroblasts from the head and legs express different Hox genes. Hox genes are a set of genes that specify regions of the body of an embryo along the anteroposterior axis of animals. This differential expression illustrates and confirms the different ontogenic origin of fibroblasts from the head and fibroblasts from the rest of the body (Chang et al., 2002; Rinn et al., 2006).

In the following sections we will see how the fibroblasts from the body and the head arise during the development of animals.

1.2.1. MESENCHYME

Mesenchyme is an animal tissue constituted by loose cells embedded in a net of proteins and fluid. And gives rise to the connective tissues in the body. Fibroblasts, like other cells in the connective

tissue, are derived from mesenchyme. Mesenchymal cells originate from the mesoderm. The mesoderm is one of the three layers that originate during gastrulation (Thiery et al., 2009) (Fig 1).

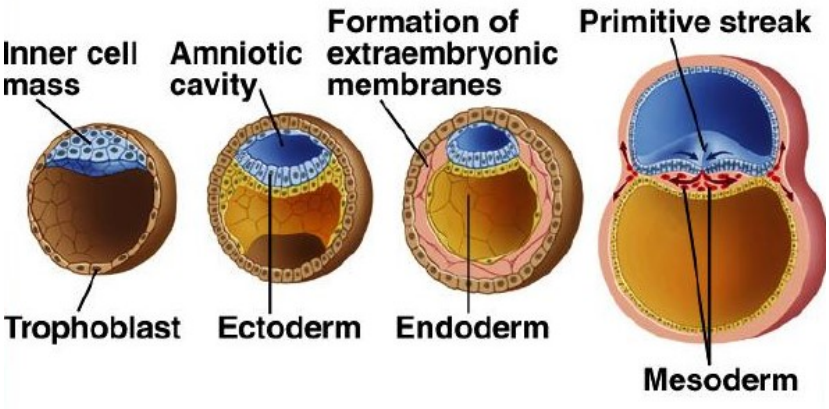

Figure 1: Overview of gastrulation. College of Atlantic's website. Bar Harbor. Maine. As a zygote divides, two layers develop: the trophoblast and the inner cell mass. The inner cell mass differentiate later into the hypoblast and the epiblast, forming a flat disc (Hassoun et al., 2009). Next, in the middle of the epiblast the primitive node is formed, from which the primitive streak extends caudally. Epiblast cells undergo an epithelial to mesenchymal transition (EMT) and migrate ventrally through the primitive streak and locate between the epiblast and the hypoblast (Williams et al., 2012). The first cells to invaginate form the endoderm, the intermediate layer constitute the mesoderm and the cells that remained in the epiblast form the ectoderm. This process is known as gastrulation. (Thiery et al., 2009) (Fig 1).

The mesenchyme is composed mainly of extracellular substance with embebbed cells. Mesenchymal cells exhibit self-renewal and multipotent differentiation capacity, and these cells give rise to bones, cartilage, lymphatic, cardiovascular systems and connective tissues.

Since no specific mesenchymal markers have been established, mesenchymal cells are identified by a combination of presence and absence of various markers. They generally express fibroblast-specific protein (Fsp1), stem cells antigen-1 (SCA-1), collagen-specific receptor tyrosine kinase (DDR2), CD44, CD71, CD73, CD90, and CD105 (Dominici et al., 2006; Lv et al., 2014). They also show high expression of heat shock protein 47 (HSP-47), collagen α1, collagen α2, vimentin and S100A4 (Zeisberg and Neilson, 2009). On the other hand there is an absence of hematopoietic and endothelial markers: CD45, CD34, CD19, CD11b, CD11c, CD79a, and CD31 (Ferrell et al., 2014). Additionally to the previous markers, we can also observe the expression of ECM proteins such as fibronectin in mesenchymal cells (Assis-Ribas et al., 2018).

Most of the mesenchyme is derived during the embryo development from the mesoderm except for a small part which is derived from the ectoderm and it is called ectomesenchyme.

1.2.2. ECTOMESENCHYME

During gastrulation, some cells migrate from the primitive node and establish the notochord, which will later form the vertebral column. The notochord induces the formation of a transient structure called neural crest (Weston et al., 2004) (Fig. 2). The neural crest will form a multipotent population of migratory cells that is exclusive of vertebrate embryos (Zhang et al., 2014). Once the cells from the neural crest migrate to its ultimate location, they interact with other embryonic structures and differentiate into divers cell types such as neurons, and glial cells of the peripheral nervous system, pigment cells of the skin, mesodermal lineages like cartilage, bone, connective tissue of the face, and mesenchyme and smooth muscle cells (SMC) in the cardiovascular system (Stuhlmiller and Garcıía-Castro, 2012). The wide range of cellular types originated from the neural crest migrating cells prompted to stablish the term „ectomesenchyme“, which is also known as the „fourth germ layer“ (Hall, 2000).

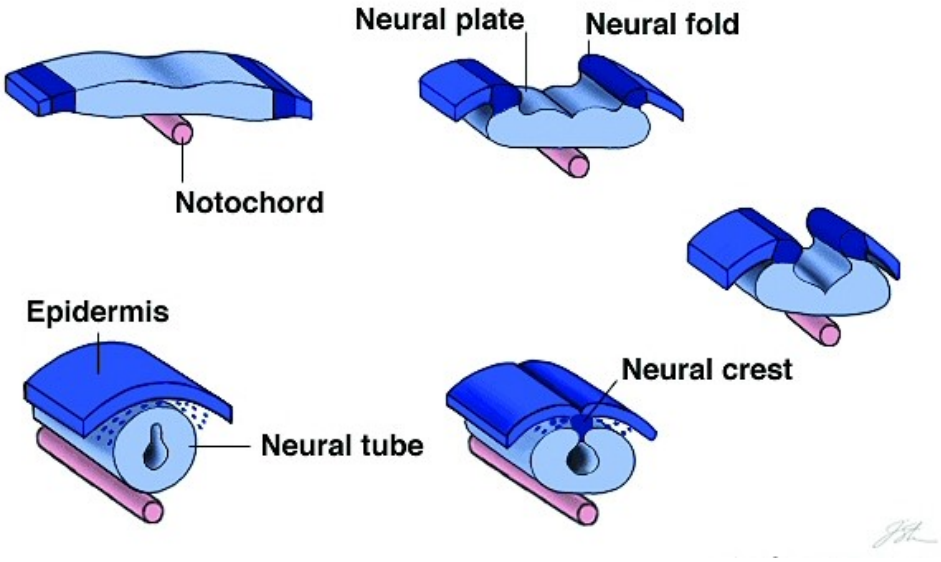

Figure 2: Formation of the neural tube. Professor Patricia E. Phelps UCLA. The National Science Foundation, the UCLA Office of Instructional Development, and the Norton Simon Research Foundation supported the development of these multimedia teaching tools.

1.3. FIBROBLAST MARKERS

As mentioned above, fibroblasts are present in almost every organ and tissue in the body performing different functions. Their ubiquitousness and heterogeneousness make difficult to find a comprehensive definition and the identification of these cells (Blankesteijn, 2015; Nolte et al., 2008; Sorrell and Caplan, 2004). The identification of fibroblasts using surface markers is problematic, because the markers used currently are often not exclusively expressed by fibroblasts, but often shared by other mesenchymal cells (Kahounov et al., 2017). The lack of specific markers has stalled the study of these cells considerably. The markers used to identify fibroblasts currently are summarized in the table 1.

Table 1. Fibroblast markers.

Marker	Description	Reference
Vimentin	It is a type III intermediate filament. One of the most reliable markers for fibroblasts	(Cheng et al., 2016)
FSP-1 or S100A4	A member of the S100 superfamily of intracellular proteins	(Strutz et al., 1995)
TE-7	A membrane protein. Reacts with fibroblasts in tissue as well as cultured fibroblasts.	(Goodpaster et al., 2008)
HSP-47	It is a collagen-specific molecular chaperone localized in the endoplasmic reticulum present in fibroblasts.	(Kuroda and Tajima, 2004; Ogawa et al., 2007)
CD-13	This ectopeptidase is highly expressed at sites of epithelial/mesenchymal interactions in human skin and in developing human breast tissue. However, all subpopulations express this antigen when human dermal fibroblasts are placed into culture.	(Kundrotas, 2012; Lysy et al., 2007)

None of these markers alone can identify fibroblasts, therefore, several of these markers are used, combined with the absence of markers specific of other mesenchymal cell types.

1.4. FIBROBLAST ACTIVATION

Fibroblasts are present in all the tissues in the body, often in quiescent state. Quiescent fibroblasts are inert and appear as fusiform or spindle-shaped single cells in the interstitial space embedded in the ECM. These cells undergo reversible exit from the cell cycle and they do not have contractile properties. However, during tissue injury and other tissue remodeling situations such as embryonic development, quiescent fibroblasts become active (Foster et al., 2018).

Fibroblasts activation is initiated by changes in the ECM and signaling molecules produced by epithelial and immune cells. The most relevant signaling molecules stimulating fibroblast activation are cytokines and growth factors such as TGF-β signaling, platelet derived growth factor (PDGF), fibroblast growth factor (FGF)-2, hepatocyte growth factor (HGF), insulin-like growth factor (IGF) and connective tissue growth factor (CTGF). Wnt signaling pathway, integrin expression and cell-cell interactions are also factors involved in the fibroblast activation (Foster et al., 2018).

In response, fibroblasts acquire stellate shape, shift to a migratory phenotype and change the expression pattern of molecular markers (Fig.3) with respect to quiescent fibroblasts. Active fibroblasts start to proliferate and increase ECM production, secretion and remodeling. These changes in the fibroblasts facilitate the process of wound healing but interestingly also promotes tumor progression (Kalluri, 2016).

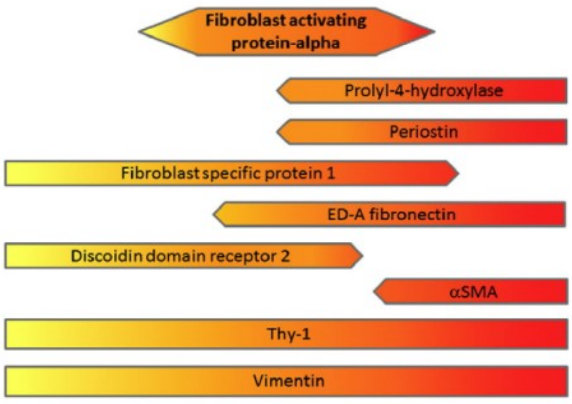

Figure 3: Markers of fibroblast activation. Simplified representation of the changes in the marker expression as fibroblasts acquire their activated phenotype (Blankesteijn, 2015). There is a significant heterogeneity in markers of fibroblast activation. Generally, activated fibroblasts progressively lower the expression of FSP-1, discoidin domain receptor 2 and α1β1 integrin, which are markers associated with quiescent fibroblasts. Additionally, activated fibroblasts show progressively higher expression of α-SMA, DPP4, fibroblast activation protein (FAP) and periostin (Blankesteijn, 2015; Foster et al., 2018).

1.4.1. MYOFIBROBLASTS

Myofibroblasts are active fibroblasts specialized in contractile function, morphologically and functionally different from fibroblasts. They were discovered by Gabbiani and coworkers more than 50 years ago in wound granulation tissue (Gabbiani et al., 1971). Myofibroblasts have some features in common with smooth muscle cells and fibroblasts (Gabbiani, 2003). Myofibroblasts have these three essential features:

- They contain stress fibers, bundles of microfilaments, normally parallel to the long axis of the cytoplasm.

- The rough endoplasmic reticulum (RER) and the Golgi apparatus are very active and occupy a considerable amount of the cytoplasm in myofibroblasts, because these cells are the most active producers of ECM.

- Myofibroblasts are well connected to other cells and to the ECM through well-developed GAP junctions and specially fibronexus, which is an organelle characteristic of myofibroblasts. Fibronexus is the area on the cells surface of fibroblasts in which the intracellular myofilaments attach indirectly to the fibronectin in the ECM establishing a strong adhesion to the stroma to allow contractile force.

The basic component of the mature myofibroblasts' contractile apparatus is α-SMA (Tomasek et al., 2002). α-SMA is one of the main groups of actin isoforms. It is fundamental part of the contractile apparatus thus present almost exclusively in muscle tissues and in myofibroblasts. Actin monomers form microfilaments that polymerize in bundles. These bundles of actin appear associated with other

contractile proteins such as non-muscle myosin (Hinz et al., 2002, 2001a, 2001b). α-SMA is frequently used as molecular marker to detect myofibroblasts.

Fibroblasts differentiate into myofibroblasts through a two steps differentiation process. In the first step, activated fibroblast acquire stress fibers and increase substantially the secretion of collagen and fibronectin rich ECM. These cells are called protomyofibroblasts. Mechanical tension of the ECM is necessary to maintain the protomyofibroblast phenotype (Hinz et al., 2001b; Tomasek et al., 2002). Protomyofibroblasts produce a class of fibronectin that resembles that found during early embryogenesis and in the ECM of tumors. This protein contains two splice segments called ED-A and ED-B which are absent in normal fibronectin. ED-A is important for further steps of differentiation of protomyofibroblasts to myofibroblasts (Klingberg et al., 2018).

ECM remodeling triggers an increasing mechanical stress, necessary to maintain the protomyofibroblast phenotype. The mechanical stress together with ED-A fragments collaborate in the maturation of protomyofibroblasts. Nevertheless, the main stimulation factor involved in the myofibroblast differentiation is TGF-β1 (Klingberg et al., 2018). TGFβ1 binds to TGF-βRII and triggers a signaling cascade that ultimately leads to an upregulation of α-SMA expression and other components of the myofibroblast contractile apparatus as well as an increase in the production and secretion of ECM components (Amadeu et al., 2003; Carthy, 2018; Malmstrom et al., 2004). TGF-β1 by itself can stimulate fibroblasts to transform into mature myofibroblasts *in vitro* (Dvoránková et al., 2011; Hecker et al., 20011), however *in vivo* myofibroblasts still require to reach specific mechanical stress on the ECM to form α-SMA fibers (Tomasek et al. 2002).

Other cytokines and growth factors such as PDGF, granulocyte-macrophage-colony stimulating factor (GM-CSF), IL-1β and tumor necrosis factor alpha (TNF)-α and FGF are believed to stimulate myofibroblast differentiation too (Amadeu et al., 2003; Werner and Grose, 2003). However this is still under discussion (Gailit et al., 2001; Mia et al., 2014).

Myofibroblasts activity is beneficial in response to tissue injury and wound closure. In an injured tissue, platelets, macrophages and epithelial cells can produce TGF-β1 to stimulate myofibroblast differentiation (Delavary et al., 2011; Guo and DiPietro, 2010; Wan et al., 2008). Another source of TGF-β1 can be autocrine production by fibroblasts, this is essential to keep the phenotype of the myofibroblasts once the inflammation is over (Wipff and Hinz, 2008).

1.5. FUNCTIONS OF FIBROBLASTS

1.5.1. STRUCTURAL

Fibroblasts function is to maintain the structural integrity of the connective tissue. To accomplish that, fibroblasts synthesize, secrete and remodel the components of the ECM. The molecules that form the ECM are synthesized intracellularly and secreted into the interstitial matrix that surrounds and supports cells. These ECM components form a three-dimensional (3D) structure that is present in all tissues and is essential for cell survival, growth and interaction (Egeblad et al., 2010; Engler et al., 2009). In fact, mutations or deletions in genes that encode elements of ECM cause severe defects or even lethality in embryos, highlighting the importance of the ECM (Rozario and DeSimone, 2010). The ECM has a dynamic and complex organization and can trigger multiple biological activities that are essential for a normal organ development and tissue homeostasis:

- The ECM provides physical support and tissue integrity and elasticity, acting as a cushion for compression when tissues are subjected to deforming stresses and defining the characteristic shape and dimensions of organs and complex tissues (Hynes, 2009).

- The components of the ECM interact with the embedded cells through adhesion receptors such as integrins, providing contextual information and transmitting signals that regulate adhesion, polarity, migration, proliferation, apoptosis, survival or differentiation. Recently it has been suggested that physical characteristics of the ECM such as stiffness or deformability also provide information and modulate the behavior of the embedded cells (Butcher et al., 2009; Hynes, 2009; Paszek and Weaver, 2004).

- The ECM can work as a biological reservoir of signaling molecules. The components of the matrix can sequester a wide range of growth factors and cytokines, and other signaling molecules. Consequently, changes in the ECM integrity (e.g., after tissue injury) would provide rapid release of signaling molecules without *de novo* synthesis (Simpson et al., 2010; Wells and Discher, 2008).

The biomechanical features of the ECM are defined by its composition, the architecture or spatial distribution of the molecules that compose it and also by post-translational modifications, such as glycosylation, transglutamination and cross-linking. These characteristics are crucial for the homeostasis of the tissues and organs and must be accurately regulated (Butcher et al., 2009; Erler

and Weaver, 2009). For example, irregularities in the collagen fibrils assembly in the cornea may cause defects in the transparency or refraction which would led to pathologies in the cornea (Chen et al., 2015).

The properties of the ECM differ according to the location and specific functions of given organs and tissues: notable differences are evident between the ECM found in the cornea and cartilage, for instance (Chen et al., 2015; Gentili and Cancedda, 2009). Populations of fibroblasts from different tissues or locations within a tissue can produce specific ECM in composition and architecture in order to adapt to the needs of the tissue or organ (Ghetti et al., 2018). We can find in the dermis an example that illustrates how different populations of fibroblasts can produce morphologically, functionally and compositionally different ECMs(Ghetti et al., 2018)(Ghetti et al., 2018)(Ghetti et al., 2018)(Ghetti et al., 2018): papillary fibroblasts produce an ECM with thin not well organized collagen fibrils, while reticular fibroblasts produce a well organized collagen bundles (Ghetti et al., 2018).

ECM is not a static structure, on the contrary, it is subjected to changes during development, growth, repair, pathogenesis and in reaction to an external mechanical pressure (Butcher et al., 2009). Even in homeostasis, the microscopic structure of the ECM is under permanent remodeling or turnover, as part of healthy tissue maintenance, where old proteins are degraded and new proteins formed (Sand et al., 2015).

1.5.1.1. ECM COMPOSITION AND DEGRADATION

The ECM is composed of two main classes of macromolecules: proteoglycans and fibrillar proteins. Among the fibrillar components, the most abundant are collagens, elastins, fibronectins and laminins.

Collagens are a big family of ECM proteins present in the ECM of all tissues. They are the main structural protein and the most abundant component of the ECM. Collagens build fibers, networks and filaments. Collagen are made of amino acids linked together to form a triple helix. Collagens can be classified in fibrillary and non-fibrillar.

- Fibrillar collagens: include collagen types I, II, III, V, XI, XXIV, and XXVII. They are the major components of collagen fibrils in the body (Harris and Hulmes, 2017). Collagen I represents more than 90% of the total collagen content in a human adult body.
- Non-fibrillar collagens: Network forming collagens (Theocharis et al., 2019), fibril-associated collagens with interrupted triple helices (FACITs) (Harris and Hulmes, 2017), membrane-

associated collagens with interrupted triple helices (MACITs) (Theocharis et al., 2019) and other collagens.

Elastin is an abundant component of the ECM in tissues exposed to stretch or contraction. Elastin allows a restoration of the tissue original shape in the ECM of skin, ligaments, blood vessels, and bladder among others. Elastin is synthesized by the assembly of several molecules of tropoelastin (Theocharis et al., 2019).

Laminins are especially abundant in the basement membranes, a type of ECM. Laminins are large cross-shaped heterotrimeric proteins that contain an α, β, and γ-chain. They interact with collagen IV and with epithelial cells through cell surface receptors influencing cell differentiation, migration, and adhesion (Theocharis et al., 2019).

Fibronectin is secreted as a fibrillar glycoprotein dimer, and then assembled into an insoluble matrix in a complex process at the cell surface. It is involved in cell adhesion interacting with glycosaminoglycans, integrins, and other components of the ECM (Theocharis et al., 2019).

Proteoglycans fill the spaces between collagen molecules. These are proteins with a variable number of glycosaminoglycan chains attached, which makes proteoglycans highly diverse molecules. They interact with other ECM components, growth factors, cytokines and cell receptors and can appear intra or extracellularly (Theocharis et al., 2019).

Matricellular proteins are non-structural proteins present in the ECM. These proteins have a very dynamic turnover and are relevant for their regulatory roles and are key for cell–matrix communication. Some examples are thrombospondins, tenascins (TNs), osteopontin and periostin (Theocharis et al., 2019).

ECM homeostasis, rely on tightly regulated ECM synthesis and degradation. Dysregulation of this balance can cause abnormal deposition and stiffness or excessive degradation. On one hand, excessive ECM degradation is linked to osteoarthritis (Song et al., 2017; Wang et al., 2017). On the other hand excessive deposition is associated with diseases such as fibrosis and tumor development (Butcher et al., 2009).

MMPs are the main group of ECM-degrading enzymes. MMP is an extensive family of proteins with endopeptidase activity. The activity of MMP degradating the ECM not only facilitates cell movement but also releases growth factors and chemokines that are contained within the ECM (Egeblad et al., 2010).

Disintegrin and metalloproteinase (ADAM) and ADAMs with thrombospondin motifs (ADAMTS) are another two important families of ECM-degrading enzymes. They can cleave transmembrane protein ectodomains, thus releasing the complete ectodomain of cytokines, growth factors, receptors and adhesion molecules. The activity of ADAM, ADAMTS is low in normal conditions but increased during repair or remodeling processes and in diseased or inflamed tissue (Giebeler and Zigrino, 2016; Itoh, 2017; Yang et al., 2017; Zhong et al., 2018).

1.5.2. OTHER ROLES OF FIBROBLASTS

The role of fibroblasts goes beyond the synthesis of ECM components. Fibroblasts are also able to regulate the behavior of other cell populations. Fibroblasts interact with other cells through the secretion of cytokines, chemokines and growth factors. Fibroblasts have thus a role in many other biological processes, among them angiogenesis and immunoregulation (Wiseman and Werb, 2002). This is of particular interest for their relevance in wound healing and tumor development.

1.5.2.1. ANGIOGENESIS

Angiogenesis is a process that occurs during embryonic development, tissue growth or remodeling. In these circumstances, endothelial cells receive signals from the environment, via cell-cell signaling, cell-ECM signaling or by soluble factors, to proliferate, migrate and differentiate in order to form new blood vessels. Angiogenesis is a tightly regulated process, and a lack of control in angiogenesis can cause uncontrolled proliferation and tumorigenesis (Pollina et al., 2008).

Fibroblasts are thought to play a role in angiogenesis regulation. Activated fibroblasts secrete proangiogenic factors in tissues under growth or remodeling, and quiescent fibroblasts secrete angiogenesis inhibitors in quiescent tissues (Pollina et al., 2008).

Several studies demonstrated that endothelial cells sprouting and lumen formation are enhanced in the presence of fibroblasts in culture. Fibroblasts promote endothelial cell sprouting and lumen formation by secreting angiogenic factors such as vascular endothelial growth factor (VEGF), TGF-β1, PDGF, basic FGF (Newman et al., 2011). These factors promote vessel formation through stimulation of matrix protease production, endothelial cell mobility and reduction in endothelial cell apoptosis (Pollina et al., 2008).

The presence of fibroblasts is essential for the correct lumen formation even when the endothelial cells are exposed to a combination of angiogenic regulators. Newman et al. observed that endothelial cells exposed to angiopoietin-1 (ANG-1), angiogenin, HGF, transforming growth factor-α (TGF-α), and tumor necrosis factor (TNF) were able to form endothelial cell sprouting, but failed to form lumens. When fibroblasts were present, the lumens acquired the correct structure. Collagen 1, Procollagen C-Endopeptidase Enhancer (PCOLCE), secreted protein acidic and rich in cysteine (SPARC), insulin-like growth factor binding protein (IGFBP) 7, and βig-h3 were identified as the additional proteins secreted by fibroblasts and necessary for a correct lumen formation. These proteins are all components or modifiers of the ECM (Newman et al., 2011).

On the other hand, quiescent fibroblasts secrete higher levels of anti-angiogenenic factors such as pigment epithelium derived factor (PEDF) and thromobospondin-2 when compared to active fibroblasts (Pollina et al., 2008). These factors would prevent the generation of new vessel formation in quiescent tissues, and by doing so, may prevent uncontrolled tissue growth or neoplasm formation.

1.5.2.2. IMMUNOREGULATION

Fibroblasts have an important role in immunoregulation, both in innate and adaptive immunity. The mechanism through which fibroblasts regulate the immune system is very complex. Fibroblasts can interact with tissue-resident lymphocytes and also with immune cells in secondary lymphoid organs (Buechler and Turley, 2018).

Fibroblasts can express toll-like receptors (TLR)s, antimicrobial peptides, proinflammatory cytokines, chemokines, and growth factors, which are clue participants of the innate immunity. Moreover they can synthesize antimicrobial peptides such as defensins hBD-1, and hBD-2 (Bautista-hernández et al., 2017).

Importantly, proinflammatory cytokines secreted by fibroblasts such as TNF-α, INFγ, IL-6, IL-12p70, and IL-10 are some of the most important inflammatory agents in the acute phase. They participate in vasodilatation, differentiation of lymphocytes and their infiltration. IL-6 is crucial during acute inflammation and fibroblasts can rapidly upregulate IL-6 to amplify the immune reaction and promoting plasma cell differentiation and antibody production, neutrophil and macrophage infiltration, collagen release, cell proliferation (Barnes et al., 2011). IL-6 secreted by fibroblasts is also critical during wound healing (Foster et al., 2018).

Moreover, fibroblasts synthesize and secrete other chemokines, such as CCL1, CCL2, CCL5, chemokine (C-X-C motif) ligand (CXCL)1, CXCL8, CXCL10, CXCL13 and CX3CL1 involved in the cellular immune response (Bautista-hernández et al., 2017), e.g. fibroblasts promote lymphocytes B1 accumulation and organization through secretion of high levels of CXCL13 (Buechler and Turley, 2018).

Fibroblasts not only activate the immune system reaction, but they can also suppress it through the production and secretion of immunosuppressive molecules such as TGF-β1 or HGF, the tryptophan-catabolizing enzyme IDO, PGE_2, and coregulatory molecules such as the programmed death-1 (PD-1)–binding molecules B7-H1 (PD-L1) and B7-DC (PD-L2), as a consequence they can induce monocyte recruitment and differentiation into TAMs, reduce the infiltration of cytotoxic T cells and inhibit natural killer cell (NKC) cytotoxicity (Tongyan Liu et al., 2019).

It was demonstrated that IFN-γ secreted by T lymphocytes induced IDO expression in dermal fibroblasts and, in response, fibroblasts suppressed T cell proliferation through monocyte interaction (Haniffa et al., 2007). However a similar experiment demonstrated that the presence of the fibroblasts was able to increase the secretion of IFN-γ and IL-17A by T lymphocytes, to which fibroblasts responded with an increase of IL-6. This in turn activated T lymphocytes via CD3/CD28. This work showed an interaction between lymphocytes and fibroblasts (Barnas et al., 2010). However we can see that fibroblasts reacted to IFN-γ secreted by T lymphocytes in very different manner: the outcome of one experiment is the inactivation of T lymphocytes, while in the other is inactivation. This illustrates that the role of fibroblasts in immunoregulation is very complex and context-dependent and need to be further examined.

1.6. AGEING DERMIS

During the development of the embryo, the dermis appears as a cellular network lacking fibrous ECM (Coolen et al., 2010). The main cellular component present in the dermis are fibroblasts, these cells appear surrounded by hyaluronic acid, which retains high water content, and thin collagen fibrils. Later in the development, the collagen fibrils associate into fibrillar bundles and two distinct regions appear in the dermis: papillary dermis, the upper layer and reticular dermis, the deeper layer.

After birth, the dermis organization eventually becomes similar to that of an adult, but during the first days, the newborn dermis possesses transient characteristics between fetal and adult (Haydont et al., 2019).

In the adulthood, the skin starts to present some signs of aging, the epidermis reduces its epithelial turnover speed and the dermis becomes thinner and experiences a progressive loss in elasticity, vascularity, thermoregulation capabilities, reduction in mechanical protection, immune responsiveness, sensory perception, sweat and sebum production, vitamin D synthesis, vascular reactivity and a decrease in the number of cells. These changes result in histological and physiological deterioration (Strnadova et al., 2019). Genetic factors and changes in the body can cause aging, that is called intrinsic aging, but dermis is also exposed to extrinsic aging, caused by the influence of the environment (Rinnerthaler et al., 2015).

Fibroblasts are the main cells responsible of dermal aging. It is still not known whether it is due to the progressive reduction in the number of fibroblasts in the dermis, or to the alterations in the remaining ones, or perhaps a combination of both. As dermis ages, dermal fibroblasts change their phenotype, lose their characteristic spindle shape, they experience a reduction in proliferation, migratory capacity and change their gene expression pattern. They upregulate genes promoting cytoskeletal extensions, genes involved in inflammation, and genes related to the lipid metabolism (adipogenesis, lipid metabolism, and fat cell differentiation) (Salzer et al., 2018). In addition, fibroblasts in aged dermis reduce the production and secretion of ECM components and increase the production of MMP while downregulate MMP inhibitors, namely tissue inhibitors of metalloproteinases (TIMP)-1 and -3 (Rinnerthaler et al., 2015; Shin et al., 2019).

As a consequence, there is a reduction in hyaluronic acid, glycosaminoglycans, elastin and collagen (Haydont et al., 2019; Salzer et al., 2018). Collagen, as the most abundant protein in the ECM, is an important contributor of the reduced structural integrity typical of aged skin (Marcos-Garcés et al., 2014). As dermis ages, the thickness of the collagen bundles decreases, appear fragmented and coarsely distributed. The lack of collagen bundles prevents fibroblasts attachment to the ECM (Shin et al., 2019). In this condition fibroblasts produce lower levels of collagen and high levels of collagen degrading enzymes. This lack of balance acts as a positive feedback contributing to the progress of the ageing process (Haydont et al., 2019; Varani et al., 2006).

Moreover, collagen is a protein with a long turnover. In its 15 to 18 years of half-life collagen accumulates non-enzymatic modifications. For example, collagen exposure to sugars for a long period can lead to the formation of advanced glycation end products (AGEs). Over the years AGEs accumulation in collagen leads to stiffer tissues and decreased elasticity (Jeanmaire et al., 2001).

Cells have receptors for AGEs, activation of these receptors activated the transcription of the nuclear factor--κB (NF-κB), which in turn increases the transcription of RAGE, acting as a positive feedback (Rinnerthaler et al., 2015).

The dermis is exposed to ultraviolet (UV)-irradiation, which is probably the most significant extrinsic factor affecting aging. UVA can result in damage in the DNA and the non-enzymatic production of reactive oxygen species (ROS), main contributors in dermal aging. ROS activates a signaling pathway that reinforces the expression of MMPs and NF-κB, an important element to keep the balance between apoptosis and proliferation (Rinnerthaler et al., 2015).

Age-related changes in the skin also affect the wound healing process. It has been observed that newborns heal rapidly and almost scarless, probably due to lack of acute inflammatory activity and absence of granulation tissue formation accompanied by a faster infiltration of macrophages and fibroblasts(Hu et al., 2018). Along with the previous affirmation, early newborns that underwent lip cleft surgery presented almost scar-less healing (Borsky et al., 2012; Valentova and Malina, 2018). On the contrary, as humans age, the skin decreases the re-epithelization rate after injury and loses its capacity to repair wounds (Akamatsu et al., 2016; Aunin et al., 2017). Therefore, knowledge of the fetal skin composition and characteristics may help to understand fetal wound healing aiming to help aged skin to heal faster and more efficiently after being damaged or after an operation.

2. WOUND HEALING AND THE ROLE OF FIBROBLASTS

2.1. PHASES OF WOUND HEALING

Skin acts as a protection for the body against mechanical forces and infections, fluid imbalance, and thermal dysregulation. The skin loses mechanical integrity when it is injured, thus restoration of the mechanical stability is crucial to recover its homeostasis and functions. After an injury, the cells in the damaged area acquire an active phenotype in order to repair the damage. There are two ways to do it: one is regeneration, which replace the damaged tissue exactly as it was before the injury. The other is replacement, it repairs the damage with connective tissue, resulting in a scar formation. The latter represents the main form of healing in adult skin (Sorg et al., 2017).

After a wound occurs, the skin goes through a series of phases to recover its integrity. The first step is the coagulation or hemostasis phase. The main goal of this process is to avoid the loss of blood, prevent microorganisms' entrance and to provide a matrix for invading cells that will migrate to the wounded area in subsequent phases. After an injury, damaged vessels leak blood into the site of injury. As a consequence, thrombocytes present in the blood come into contact with collagen, Von Willebrand factor and thrombin, and this induce thrombocyte activation. Active thrombocytes induce the release of clotting factors that leads to the clot formation. The blood clot is formed by fibronectin, fibrin, vitronectin and thrombospondin (Velnar et al., 2009).

Thrombocytes have different types of granules that can be exocytose during the hemostasis phase and contribute to wound healing. α-granules are filled with growth factors and cytokines, such as PDGF, TGF-β, epidermal growth factor (EGF), FGF and IGF that will activate and attract other cells to the wound (Velnar et al., 2009).

The next phase is known as the inflammatory phase. The cytokines and growth factors released by the thrombocytes attract neutrophils (Hantash et al., 2008). Neutrophils kill local bacteria and help to degrade necrotic tissue. Next, monocytes arrive to the injury site, and participate in the phagocytosis and synthesize growth factors such as TGF-β, TGF-α, FGF, PDGF and VEGF, which promote cell proliferation (Werner and Grose, 2003). TGF-β1 is one of the most important cytokines in wound healing. It causes fibroblast chemotaxis and activation (Behm et al., 2012; Martin and Leibovich, 2005).

In the next stage, the proliferative phase, fibroblasts from the dermis in adjacent skin and other sources migrate to the inflammation site (Desmoulière et al., 2005). Fibroblasts infiltrate and degrade the fibrin clot by producing various MMP, replacing it with a provisional tissue consisting in ECM components, such as collagen I–IV, XVIII, glycoproteins, proteoglycans, laminin, thrombospondin, glycosaminoglycans, hyaluronic acid and heparan sulphate called granulation tissue (Li and Wang, 2011). This provisional tissue is a complex matrix that supports and regulates the migration and activity of the fibroblasts, as well as acting as a support and signal for angiogenesis and re-epithelialization from the wound edges (Rozario and DeSimone, 2010). Angiogenesis is crucial for wound healing because the processes of repair are highly energy-consuming and require high amounts of oxygen (Behm et al., 2012). In this phase, macrophages switch their proinflammatory functions and release proangiogenic factors such as VEGF and molecules that stimulate collagen synthesis and fibroblast proliferation (Reinke and Sorg, 2012).

IL-6 produced by fibroblasts, macrophages, endothelial cells and keratinocytes also has important functions in the proliferative phase. IL-6 binds to its receptor IL-6Rα, this is followed by the activation of the Janus kinases (JAK)s- signal transducer and activator of transcription proteins (STAT) signaling pathway. The effect of IL-6 downstream pathway induces neutrophil and macrophage infiltration, angiogenesis, collagen release, cell proliferation through the induction of TGF-β1, IL-1 and VEGF production. Mice deficient in IL-6 have impaired wound healing due to defects in the granulation tissue formation, re-epithelialization, angiogenesis, macrophage and neutrophil infiltration and matrix remodeling. On the other hand, high concentrations of IL-6 were detected in chronic wounds. This proves that wound healing is tightly controlled process regulated by the balanced secretion of cytokines, chemokines and growth factors (Behm et al., 2012).

The final phase of wound healing is the tissue remodeling, a process in which the granulation tissue will be replaced by the permanent tissue eventually. This can take weeks or even years. This process is characterized by apoptosis of the high cellular component, the substitution of collagen III by the stronger collagen I fibers and the contraction of the wound (Behm et al., 2012; Werner and Grose, 2003). Myofibroblasts cause wound contractions by attaching to the collagen in the ECM and contracting its strength fibers in order to decrease the surface of the wound (Reinke and Sorg, 2012).

An appropriate balance between degradation and synthesis of ECM is essential for tissue remodeling, and it is achieved by growth factors, chemokines and cytokines which control the synthesis of MMP and its inhibitors (Behm et al., 2012).

Once the wound is closed the cytokine release ceases due to a negative feedback loop. The angiogenic formation decreases as well as the blood flow and the metabolic activity diminishes

(Behm et al., 2012). As a consequence, most of the inflammatory cells that migrated and proliferated during the inflammation phase dissipate. Myofibroblasts enter into apoptosis due to the lack of growth factors and also because in the remodeling phase the ECM regains its original mechanical properties, the mechanical stress release in the ECM leads to instant loss of internal tension in the myofibroblast, a circumstance that prompt it to enter apoptosis (Desmoulière et al., 2005).

The final appearance of the skin after an injury in adults will not appear exactly as it was before the injury, and it will contain fibrotic tissue and a scar. This phenomenon was observed in humans and other vertebrate species and it is age-dependent (Colwell et al., 2007). Scars are different from healthy tissue: stiffer, lack epidermal appendages like hair follicles and sebaceous glands, moreover the collagen pattern is different from the normal skin. In scared tissue, new collagen fibers are densely packed to fill the wound site while in normal skin it has a reticular structure (Behm et al., 2012; Hinz and Gabbiani, 2010). In early stages of development, embryos can heal without scar formation. This ability is maintained in young newborns (1 to 8 days after birth), who can heal with minimal scar formation (Borsky et al., 2012).

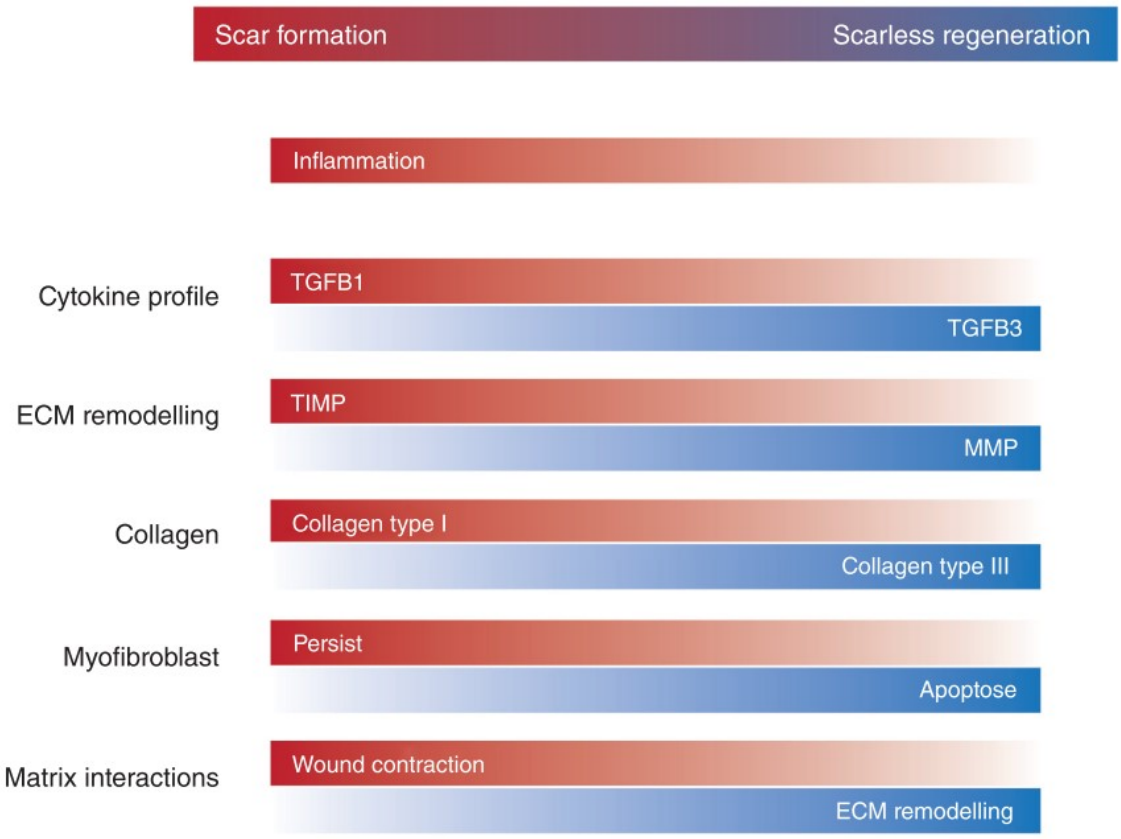

Figure 4: Schematic representation of the characteristics of the scar and regenerative wound healing (Leavitt et al., 2016).

Many comparative studies tried to explain the different outcomes in adult and fetus wound healing. During gestation, low levels of transforming growth factor alpha (TGF-α)1 and high levels of TGF-α3 are expressed. Furthermore, in fetal wound experiments, a high TGF-β3/TGF-β1 ratio was associated with scarless healing, suggesting that the relative proportion of each subtype may be crucial for the wound outcome (Fig.4) (Bullard et al., 2003; Delavary et al., 2011). Fibroblasts from newborn secrete less TGF-β1, higher levels of collagen and IGFBP-3. Moreover, in scarless wounds, neutrophils, macrophages, and mast cells are immature and smaller (Kathju et al., 2012; Wulff et al., 2012). Fetal wounds also show lower levels of the pro-inflammatory cytokines IL-6 and IL-8, as well as higher levels of the anti-inflammatory cytokine IL-10 as compared to adult ones (Pratsinis et al., 2019).

2.2. CHRONIC WOUNDS

As we have just seen, in a healthy situation a wound undergoes four phases during wound healing: inflammation, proliferation, epithelization and remodeling. These steps must be accurately regulated by cytokines, chemokines and growth factors. The disequilibrium of local and systemic signaling may impair wound closure and lead to the formation of a chronic wounds. Chronic wounds seem to be arrested in the inflammatory phase of wound healing (Zhao et al., 2016).

Acute wounds resolve normally within three weeks maximum, while chronic wounds persist for minimally three months (Berberich et al., 2020). Chronic wounds represent a serious cause of morbidity and mortality that is increasing due to the increasing number of elders, malnutrition and some diseases such as obesity and diabetes that are linked with problems in healing (Berberich et al., 2020).

A characteristic of the chronic wounds is the excessive or persistent neutrophil infiltration, while in healthy wounds neutrophils disappear after 72 hours normally. Several possible causes for the persistence of activated neutrophils have been suggested: infection in the wounded area, reperfusion injury, aged neutrophils with diminished ability to phagocytose bacteria, hypoxia, etc. (Menke et al., 2007). Neutrophils release proteases such as elastase and other MMPs that degrade growth factors such as PDGF and TGF-β and components of the ECM. But most importantly, neutrophils also secrete proinflammatory cytokines such as IL-1α, IL-1β, IL-6 and TNF-α that increase MMP production and lower the production of inhibitors of MMPs, causing tissue degradation that further recruits more neutrophils in a self sustained cycle (Menke et al., 2007). Neutrophils also increase the formation of ROS that cause damage in the ECM and cell membranes as well as

promoting the secretion of inflammatory cytokines (Zhao et al., 2016). The result of the persistence of neutrophils in the wound is a continued inflammation, degradation of ECM and reduced concentration of factors that promote proliferation and matrix deposition.

As consequence, fibroblasts cannot build ECM necessary for the wound closure, because matrix degradation occurs faster than its synthesis. Besides, the excessive ECM degradation reduces fibroblasts proliferation, migration and prevents matrix deposition (Menke et al., 2007). Moreover, the excessive inflammatory environment, together with hypoxia present normally in chronic wounds, impairs fibroblasts differentiation into myofibroblasts causing a delay in the wound contraction (Hinz, 2016; Martin and Nunan, 2015).

Fibroblasts are also responsible of the development of chronic wounds. Fibroblasts in chronic wounds show premature senescence, lower migration capacity and reduced amount of growth factor receptors, so they cannot respond effectively to the environmental signals (Demidova-Rice et al., 2012). Some authors hypothesize that the interaction between neutrophils and fibroblasts during an acute inflammation phase is what sustained neutrophil survival and persistence of the inflammation (Buckley, 2012). While previous therapies to improve chronic wounds were focused on promoting the reepithelization, novel therapies aim is to eliminate the causes of the persistent inflammation.

2.3. FIBROSIS

As we have seen, chronic wounds are a pathological condition in which a wound do not heal and cannot progress to closure. Fibrosis would be on the other end of the expectrum. Fibrosis describes the pathological wounds in which there is excessive connective tissue formation, to the point where normal tissue is replaced by scars leading to loss of function in the tissue/organ (Johnson et al., 2020).

Contrary to chronic wounds, fibrosis presents increased growth factor activity, decreased protease activity and excessive matrix formation, continued fibroblast activation and excessive inflammation response (Elliott and Hamilton, 2011).

In fibrosis, the inflammation persists after the wound closure and it is characterized by a high macrophage infiltration. Macrophages secrete profibrotic growth factors PDGF and TGFβ. The excess of TGF-β1 increase excessively fibroblast survival, migration, activation and differentiation into myofibroblasts. Myofibroblasts secretes TGF-β1, acting as a positive feedback loop that perpetuates

this pathological response (Elliott and Hamilton, 2011). Moreover, TGF-β1 also upregulates the expression of VEGF, that promotes angiogenesis. New vasculature provides oxygen and nutrient supply to allow the fibrosis to perpetuate (Johnson et al., 2020).

Other possible factors affecting fibrosis are the lack of growth factors released by platelets during the first stage of wound healing. This fact seem to prevent collagen reorganization in the wound matrix and this promotes myofibroblasts persistence (Elliott and Hamilton, 2011). Some studies demonstrate that changes in the tension of the dermis during wound healing promote vascular permeability, and this leads to a sustained inflammation in which proinflammatory cytokines are released to the medium. Among them, IL-6, a cytokine that promotes myofibroblast differentiation and resistance to apoptosis (Johnson et al., 2020).

3. CANCER STROMA

3.1. TUMOR

A tumor or neoplasm is an uncoordinated growth of tissue. Tumors can be delimited in a specific area, the so-called benign tumors, but in some cases they can persistently grow and invade the surrounding tissue, often reducing the function of the affected organ becoming malignant tumors.

Accumulation of various genetic alterations in normal cells may cause the development of malignant cells that may eventually lead to the formation of a tumor (Hanahan and Weinberg, 2011). Tumor initiation and progression can be studied from an evolutionary point of view. The hypothesis proposes that cells are exposed to selection pressure and the accumulation of somatic mutations or epigenetic changes that control cell division occasionally gives a selective growth advantage. As the cell grows and divides, the population diverges further from the original cell population and is likely to accumulate more mutations. Eventually this can lead to uncontrolled proliferation and malignancy (Tianyi Liu et al., 2019). An alternative hypothesis proposes that there is an initial mutation in genes that maintain genetic stability in normal cells, which can generate a cascade of mutations throughout the genome. Some of the resulting mutations will confer a selective advantage, allowing the mutation carrier cells to expand and achieve clonal dominance (Attolini and Michor, 2009).

The acquired characteristics that give advantage for a cell are: rapid division, evasion of tumor suppression mechanisms, inhibition of programmed cell death, the ability to create a microenvironment containing blood vessels, stromal and immune cells and the acquisition of invasive and metastatic potential. These characteristics that give advantage to a cell population and tumor growth are detrimental to the body (Davis et al., 2017).

As any other cells in our body, malignant cells also need a microenvironment to support their growth. All solid tumors, regardless of their site of origin, require a stroma if they are to grow beyond a minimal size of 1 to 2 mm (Connolly et al., 2003). Tumors are composed not only of malignant cells, but they are complex ecosystems that include many different types of cells and non-cellular components. Cancer cells produce factors that activate and recruit mesenchymal cells to create an environment that meets the tumor development needs (Nwani et al., 2016).

Some works show that stromal cells can also present genetic or epigenetic alterations (Moinfar et al., 2000). Somatic mutations in TP53 and phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN) were frequently observed in stromal cells of invasive breast carcinoma (Fukino et al., 2004; Kurose et al., 2002). Aneuploid karyotypes were observed in stromal cells from melanoma and prostate cancer and it was suggested to be caused by inactivation of p53 (Du and Che, 2017).

Epigenetic changes, such as methylation, can be also found in tumor stromal cells of breast, cervical, gastric and esophageal squamous cancer (Du and Che, 2017; Fiegl et al., 2006; Hu et al., 2005). However the genetic instability of stromal cells and whether genetic alteration is necessary to transform healthy fibroblasts towards a tumor stromal phenotype is still under discussion. Many other works have not find genetic alterations in stromal cells (Du and Che, 2017).

3.2. TUMOR STROMA

Tumor stroma is a complex microenvironment essential for tumor cell growth and progression. It is mechanically and biologically active, and indeed, it is continuously being remodeled and adapting to the dynamic needs of the tumor. Tumor stroma used to be considered for its role sustaining malignant cells. However the perspective has changed, and nowadays we know that tumor stoma has an active role in the development of tumors.

Tumor stroma is composed of ECM, cytokines, growth factors and a cellular component: endothelial cells, pericytes, bone marrow mesenchymal stem cells (MSC)s, adipocytes, macrophages, immune cells, and the most abundant: fibroblasts. Thus a tumor is composed of a very heterogeneous population of cells (Fig. 5) (Lacina et al., 2018; Plzák et al., 2019). The composition of the tumor stroma varies in different tumors: in some tumors stroma take up an important part of the tumor, while in others, like glioma, stroma represents just a small fraction of the tumor. Due to the relevance of the stroma, nowadays tumors are studied not like isolated and autonomous neoplastic cells, but in physiological context together with their stroma(Werb et al., 2016).

The cellular component of the stroma initially has initially tumor-suppressing activities, but this changes during the progression of tumor (Bissell and Hines, 2011). Malignant cells can recruit and manipulate their microenvironment through production of various growth factors, chemokines, and cytokines in order to build a more permissive environment for tumor survival (Mishra et al., 2011; Radisky et al., 2001). Some of these modifications include the recruitment of supporting cells that promote ECM remodeling, cellular migration, neoangiogenesis, invasion, cancer cell proliferation, drug resistance, immunosuppression and metastasis (Erdogan and Webb, 2017). But this communication is bidirectional, and stromal cells also contribute to tumorigenicity by communicating with the malignant cells via a large variety of soluble factors (Littlepage et al., 2005; Mishra et al., 2011). Malignant cells and stromal cells co-evolve to further promote tumor progression.

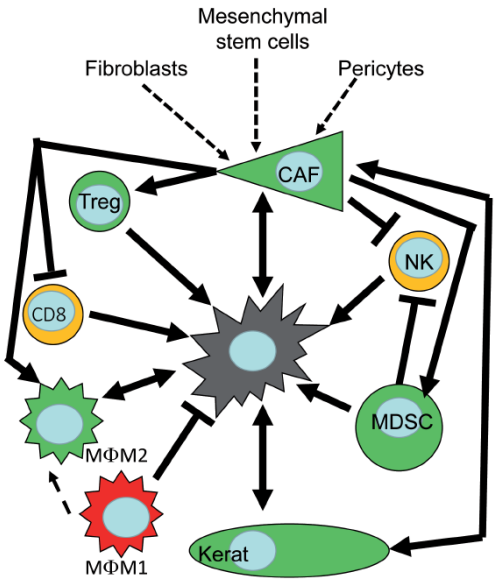

Figure 5: Schematic representation of a melanoma cell and the cells in its stroma. CAF, NKC, myeloid-derived suppressor cells (MDSC), keratinocytes, M1 polarized macrophages, M2 polarized macrophages, CD8 cytotoxic T lymphocytes (CD8) and T regulatory lymphocytes (Treg) participate in formation of the melanoma cell niche. Cells that stimulate melanoma growth are marked in green and cells with an inhibitory effect are red. Yellow colored cells indicate cells with initially inhibiting activities and pro-tumorigenic properties thereafter (Lacina et al., 2018).

Several experiments demonstrated that cells with malignant genotype became phenotypically normal in an antitumorigenic environment, suggesting that it might be possible to diminish aggressiveness in malignant cells by manipulating their environment towards a less pro-tumorigenic one (Radisky et al., 2001). There is a series of experiments in which tumorigenic agents were injected in embryos and showed that the embryonic environment was not permissive for a tumor formation (Bissell and Hines, 2011). In other experiments, melanoma cells were exposed to human embryonic stem cells matrices and in this microenvironment melanoma cells started to show a melanocyte-like phenotype (Bissell and Hines, 2011).

On the other hand, it has been hypothesized that abnormal microenvironment signaling can directly drag epithelial cells toward neoplasia. For instance, mice genetically engineered to not express TGF-β receptors in fibroblasts developed tumors in the prostate and stomach (Bissell and Hines, 2011). Another study showed that genetically modified fibroblasts to overexpress HGF or/and TGF-β1 could prompt an abnormal epithelial growth. The posterior analysis of the tumors formed revealed genetic alterations in malignant cells (Kuperwasser et al., 2004). It is still uncertain whether genetically

abnormal stroma is enough to initiate tumor formation in healthy epithelial cells or genetic alterations in epithelial cells are required too.

Tumor environment also influences the secondary tumor growth or metastasis. Already in 1889 Steven Paget published his “seed and soil” work, in which he hypothesized that for a tumor to spread in secondary sites it is necessary the cooperation between cancer cells ‘seed’ and the host tissue ’soil’ (Paget, 1889). A suitable microenvironment must be created for tumor cells to grow in distant sites. Primary tumors can release secretory factors to the circulation to induce the creation of an optimal environment in a distant tissue, to allow a metastatic spread of the tumor (Psaila and Lyden, 2009). Further, some studies have shown that CAF can 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).

3.3. CANCER ASSOCIATED FIBROBLASTS

Cancer-associated fibroblasts (CAFs) are one of the most abundant and critical components of the tumor stroma. CAFs are different from normal, healthy fibroblasts and have different properties. CAFs are characterized by a more rapid proliferation rate, production and degradation of components of the ECM and secretion of cytokines and growth factors that promote proliferation, migration and invasion of malignant cells, providing physical support for tumor cells and modulating tumorigenesis (Raffaghello and Dazzi, 2015).

CAFs Moreover CAFs can also alter other stromal cells in the microenvironment promoting angiogenesis, proliferation and infiltration of subpopulation of immune cells with pro-tumorigenic activities (Mishra et al., 2011).

CAFs are thought to originate from local quiescent fibroblasts. These fibroblasts respond to changes in the environment derived from a growing neoplasm. This activation is similar to the activation that occurs in response to an injury during wound healing (LeBleu and Kalluri, 2018).

Most of the surface markers present in CAFs are the same markers we find in normal active fibroblasts. However some markers such as platelet-derived growth factor receptor A (PDGFR)α, VEGF and FAP, are highly expressed in CAFs while absent in normal fibroblasts. These markers are frequently used to identify CAF populations and also as therapeutic target (Busek et al., 2018; Ferrara, 2005; Papadopoulos and Lennartsson, 2018). The disadvantage of these markers is their low

specificity and that they are not present in all subpopulations of CAFs (Nurmik et al., 2019). But a combination of various markers may help identify CAFs. However, negative selection is crucial in order to exclude other cell types that can be typically found in tumor tissue samples and are not CAFs. These are the most frequently used markers to identify CAFs (Table 2).

Table 2: Positive and negative markers for CAFs definition according to different authors (*used in our studies)

Positive markers	Negative markers
Vimentin	Keratin*
α-SMA*	CD31*
FAP	CD34*
FSP-1 or S100A4	CD45*
Periostin	HMB-45*
NG2	MELANA*
PDFGRb	
Tenascin-C*	
Desmin	
FGF-2	
VEGF*	
Ang-1	
MFAP5	
Col XI	

The study of the CAFs' surface markers is interesting not only to recognize CAFs, but also for their value as prognostic markers. For example the level of expression of FSP1 in breast cancer negatively correlates with patient survival and FAP expression correlates with bad prognosis in colon cancer patients (Foster et al., 2018). There is an overlap between several of the typical cell markers of activated fibroblasts and CAFs, the reason is that some of their functions are very similar, and it is still nowadays difficult to discriminate them.

α-SMA is one of the most widely used markers to detect activated fibroblasts during tissue repair and CAFs. As it was said before, this protein is present in myofibroblasts. The disadvantage of α-SMA is that not all the activated fibroblasts or CAFs exhibit a myofibroblast-like phenotype. On the other

hand, we can find other cells like smooth muscle cells and pericytes to express significant levels of α-SMA (Latif et al., 2015). This limits its use as a possible therapeutic target.

FAP is a serine protease with post-proline dipeptidyl peptidase and endopeptidase enzymatic activity. It is upregulated in fetal mesenchymal tissues and during embryogenesis. In adults it is mainly expressed in activated fibroblasts during tissue repair, fibrosis and ECM degradation. FAP is also upregulated in CAFs in squamous cell carcinoma, melanoma, colorectal cancer and breast tumor to mention some examples. FAP is absent in most healthy adult tissues. Nevertheless, there is FAP expression in the alpha cells of Langerhans islets and there are traces of FAP expression in multipotent bone marrow stromal cells, in the cervix and in the uterine stroma, placenta and in the surroundings of hair follicles (Busek et al., 2018).

The presence of FAP in activated fibroblasts contributes to their migratory potential in tissue remodeling and its presence in CAFs play an important role in tumor progression, influencing invasiveness, proliferation, ECM remodeling, vascularization, and immunoresistance (Wen et al., 2017). FAP is one of the most strongly expressed genes in the tumor stroma and is upregulated in over 90% of epithelial carcinomas (Nurmik et al., 2019) and it is associated with poor prognosis in some malignancies (Busek et al., 2018). FAP constitutes a potential therapeutic target.

Summarizing, so far there are no universal reliable markers to identify all CAF populations. Indeed, considering the multiple roles of CAFs in the tumor environment: from tumor-promoting to even tumor-suppressive roles it becomes questionable whether such a universal marker exists.

During this work we focused on α-SMA because α-SMA expressing fibroblasts, also known as myofibroblasts, are a common element and clue cells in the development of both processes of interest in this work: wound healing and tumor progression. FAP is also a protein of interest in this work since we consider that it is nowadays one of the most promising therapeutic targets for tumors.

3.3.1. FIBROBLAST CONVERSION INTO CAFs

Resting fibroblasts become activated and recruited to form part of the tumor stroma, the process of activation is similar to what happens during wound healing and it is mainly coordinated by the soluble factors released by malignant cells and to a lesser degree, infiltrating immune cells. Cancer cells secrete growth factors like VEGF, PDGF, basic-FGF, Hepatoma-derived growth factor (HDGF), IGF, TGF-β and chemokines and cytokines such as CCL2, CXCL1, CXCL2, CXCL12, IL-6 and IL-8.

Fibroblasts have receptors for these growth factors and cytokines, and once the ligand binds to its correspondent receptor, fibroblasts migrate towards the tumor or acquire an active phenotype. After fibroblasts undergo activation, they acquire invasive and tumor-promoting phenotypes (Raffaghello and Dazzi, 2015).

TGF-β1 is one of the main growth factors because it causes a wide range of pro-tumorigenic changes: recruits fibroblasts, activates them and causes in them an increase in the expression of fibronectin and collagens (Coleman et al., 2016). Besides TGF-β1, there are other soluble signaling molecules that activate stromal fibroblasts. Another important growth factor is PDGF, it is secreted by cancer cells, and upon binding, fibroblast begin to secrete FGF-2, a potent pro-angiogenic molecule, that stimulates tumor progression (Jain et al., 2008). It was seen that gastric cancer cells cocultured with healthy fibroblasts triggered a change in the fibroblasts towards a CAF phenotype, including the newly expression of FAP, α-SMA, and stromal cell-derived factor (SDF)-1 mRNA expression. They observed that this effect was absent when a HGF neutralizing antibody was added to the medium, so they hypothesized that HGF secreted by cancer cells was acting in a paracrine way on CAFs (Wu et al., 2013).

Interleukins appear overexpressed frequently in cancer tissues. Interleukins down-regulate important tumor suppressor genes like p16, p21, and p53 in breast stromal fibroblasts. The suppression of these genes stimulates fibroblasts activation towards a pro-tumorigenic phenotype (Hendrayani et al., 2014). Leukemia inhibitory factor (LIF) is an IL-6 class cytokine secreted by tumor cells which promotes stromal fibroblasts activation. LIF upregulation has been observed in different types of carcinomas, and it correlated with stiffer ECM and dense collagen fibers, tumor invasion and negative outcome (Albrengues et al., 2014).

Soluble growth factors and cytokines are not the only signal to induce fibroblasts conversion into CAFs. Hypoxia is a common feature of many tumors and in hypoxic conditions malignant cells produce high concentration of ROS. The accumulation of ROS reinforces the shift of the resident fibroblasts towards a CAFs phenotype through the increase in the expression of PDGF and TGF-β. Moreover, hypoxia-inducible factor (HIF)-1 induces the transcription of pro-angiogenic and inflammatory mediators in fibroblasts (Kuzet and Gaggioli, 2016).

Changes in the ECM composition or architecture contribute to fibroblasts conversion into CAFs. Changes in the rigidity or stiffness of the ECM induce changes in the cytoskeleton tension in fibroblasts. This is perceived by the cells and mechanotransduction systems can translate them into biochemical signals leading to activation of the yes-associated protein (YAP)/ PDZ-binding motif (TAZ) pathway, focal adhesion kinase, steroid receptor coactivator (Src) family kinases and Ras Homolog

Family Member A (RhoA) (Calvo et al., 2013; Dupont et al., 2011). The activation of these signaling pathways leads to an activated fibroblast phenotype. Activated fibroblasts increase ECM deposition which increases the tension in the ECM leading to further YAP activation in a positive feedback loop (Calvo et al., 2013).

Some authors have demonstrated that CAF activation is dependent on the environment and can be reversed. In fact, some works demonstrated that hypoxic conditions inhibit prolyl hydroxylase domain protein 2 that leads to stabilization of HIF, in these conditions CAFs reduced their α-SMA and periostin expression, collagen matrix contraction ability and the invasion of squamous cell carcinoma cells *in vitro* was reduced as well as the spontaneous metastases to lungs and liver (Madsen et al., 2015). Dauer et al. proved that CAFs treated with triptolide presented a downregulated autocrine TGF-β signaling pathway in the CAFs. This induced CAF inactivation, as it was observed by a decrease in the secretion of fibronectin, periostin, collagen, hyaluronic acid, MMP2 and MMP9. Addition of TGF-β to the culture system reverted the decreased ECM production caused by triptolide (Dauer et al., 2018). Approaches to understand the inactivation of CAFs may represent a good strategy to treat tumors.

3.3.2. CAFs PRECURSORS

CAFs are a complex and heterogeneous population. Such heterogeneity may be partially explained by its multiple cellular precursors (Fig. 6). The most common source of CAFs are local quiescent fibroblasts that are recruited and activated by malignant cells (Chen and Song, 2019).

Bone marrow-derived MSC can be source of CAFs. Osteopontin is highly expressed in tumor tissue and seems to be involved in the conversion of MSC into CAFs via the osteopontin-myeloid zinc finger 1 (MZF1)-TGF-β1 pathway. In a mouse model of inflammation-induced gastric cancer, Shiga et al demonstrated that more than 20% of the CAFs present in the stroma were derived from MSC (Quante et al., 2011). In addition to the local sources, fibrocytes recruited from bone marrow and can be converted into CAFs. Fibrocytes contribute to the myofibroblast population during wound healing and to the CAF population in breast cancer (Chen and Song, 2019).

Even non-mesenchymal cells, such as endothelial cells can transform into CAFs through endothelial to mesenchymal transition (EndMT). EndMT occurs during development in the formation of the heart and, several studies found similar process occurring in the tumor stroma. Mouse lung endothelial cells were able to acquire fibroblast characteristics and surface markers such as FSP-1, α-SMA when exposed to TGF-β1 (Piera-Velazquez et al., 2016; Shiga et al., 2015; Zeisberg and Neilson, 2009).

Epithelial cells can be a source of CAFs through EMT. EMT occurs during embryonic development, wound healing and also metastasis. During EMT epithelial cells have to change their phenotype towards a more motile mesenchymal one. Epithelial cells have specialized membrane structures such as the tight junctions, adherens junctions, desmosomes and gap junctions. These structures keep the epithelial cells immobilized and interconnected close together organized in a layer with apical–basolateral organization. During the EMT these connections are lost, as well as the apico-basal polarity, and the cells become motile, acquiring mesenchymal characteristics. Experiments in alveolar epithelial type 2 cell line RLE-6TN showed that upon treatment with TGF-β1, the epithelial cells were converted into myofibroblasts through Ras-ERK pathway. Recently EMT has gained more attention for its implication in tumor dissemination and propagation. Interestingly it has been hypothesized that some of fibroblasts present in the tumor stroma could be cancer cells that have undergone EMT, but this hypothesis needs further research (Antony et al., 2019; Drake and Macleod, 2014).

Other sources of CAFs less studied and probably less common are adipocytes, pericytes and smooth muscle cells (Osman et al., 2020).

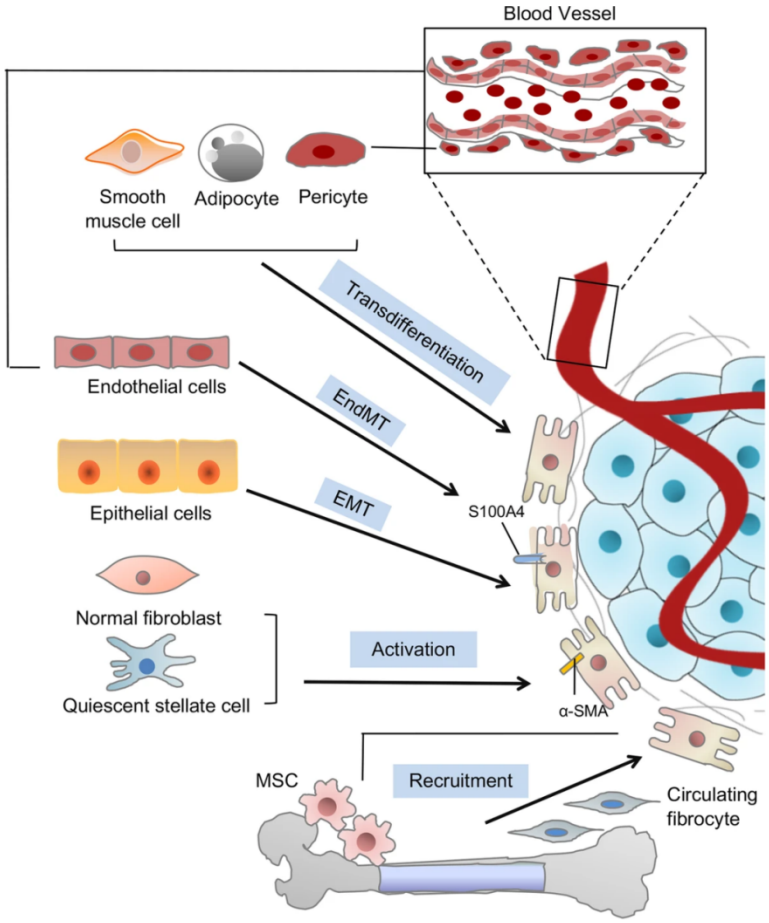

Figure 6: Representation of the possible cellular sources of CAFs (Tongyan Liu et al., 2019).

3.4. CAF FUNCTIONS

In most cases, CAFs arise as the host's response to a neoplasm growing (LeBleu and Kalluri, 2018). Some authors hypothesize that, during the initial stages of tumor development fibroblasts role may be to organize tissue repair and keep homeostasis. Later on, as tumor grows, the crosstalk between tumor cells and CAFs might turn CAFs gradually into pro-tumorigenic cells (LeBleu and Kalluri, 2018).

CAFs main function is to synthesize and remodel ECM, like healthy fibroblasts do. However, tumor-derived ECM differs in its composition and characteristics from ECM of healthy tissue. ECM in tumors is typically stiffer than ECM in normal stroma (~400 Pa compared with 150 Pa, respectively) (Butcher et al., 2009). The ECM secreted by CAFs contains higher amounts of collagen and increased collagen crosslinking than the ECM built by healthy fibroblasts. Higher amounts of collagen cause an increase in stiffness, which is frequently observed in tumor ECM. Moreover, CAFs contract the collagen fibrils to align them to their long axis leading to a different ECM architecture. This condensed architecture negatively correlated with the patients' survival (Hanley et al., 2015). It is speculated that this fibril organization, with collagen fibers paralelly align creates 'roads' for the malignant cells to diffuse easily. The ration of collagen I/collagen III is higher in tumor ECM than in healthy tissue ECM, since CAFs have a decrease in the collagen III production. It has been demonstrated that high collagen I/collagen III ration promotes tumorigenesis. It has been hypothesized that collagen III suppresses protumorigenic microenvironment by breaking collagen fibers alignment (Theocharis et al., 2019).

Tumor ECM contains higher amount of fibronectin than healthy ECM and, specifically two splice variants: fibronectin-EDA and fibronectin-EDB, which are present only during fetal development and in tumors. Moreover it has been demonstrated that fibronectin-EDB is involved in angiogenesis (Theocharis et al., 2019).

The content in proteoglycans in tumor ECM is slightly different than the proteoglycan composition in healthy ECM. For instance, a large proteoglycan secreted by CAFs, versican, is known to accumulate in tumor stroma. It promotes cancer invasion in a TGF-β-dependent manner and induces the expression of proinflammatory molecules such as IL-1β, IL-6, IL- 12, and chemokine (C-C motif) ligand 2, as well as the conversion of fibroblasts into myofibroblasts. Versican is also overexpressed in hypertrophic scars and fibrosis (Theocharis et al., 2019). Other proteoglycans such as brevican, neurocan, tenascin-C and versican are implicated in astrocytoma progression (Varga et al., 2012).

CAFs synthesize matricellular proteins, such as tenascin-C, osteopontin or periostin, that initiate signaling pathways that lead to cell proliferation, invasion and matrix remodeling. Tenascin-C

increases Notch signaling in breast cancer and it is involved in lung metastasis among other functions. This might be due to the fact that tenascin-C attenuate the binding of malignant cells to fibronectin (De Wever et al., 2004).

In tumors, abnormal production of the ECM simultaneously coexists with abnormal matrix degradation. ECM-degrading enzymes are often overexpressed in CAFs, the presence of ECM-degrading enzymes coupled with increased ECM synthesis leads to the progressive destruction of normal ECM and its replacement by tumor-derived ECM, a more permissive niche for tumor development (Cox and Erler, 2011; Lu et al., 2011).

Tumor cells and CAFs secrete a variety of ECM-degrading enzymes to dismantle the basement membranes and change the ECM architecture to allow tumor cells to invade. Moreover, ECM-degrading enzymes also free growth factors and cytokines stored in the ECM to enhance tumor cell growth, invasion and neovascularization (Theocharis et al., 2019).

For example overexpression of MMP2 has been shown in mammary cancer cells, whereas MMP3, MMP11, MMP12 and MMP13 overexpression has been observed in tumor stroma in breast cancer (Butcher et al., 2009). Polymorphisms in the human MMP3 promoter that increase its expression have been clinically associated with an increased tumor incidence, highlighting the important nature of MMPs in cancer (Biondi et al., 2000). Therefore MMPs and their inhibitors are tested in several studies for their potential as anticancer therapy (Jabłońska-Trypuć et al., 2016).

Healthy fibroblasts maintain tissue homeostasis through an accurately regulated crosstalk with the cells in their environment, similarly CAFs communicate with the cells in their environment via secretion of growth factors, chemokines and cytokines (Yamamura et al., 2015). However the communication established by CAFs is often aberrant (Zhang and Liu, 2013). In fact some studies demonstrated that the fibroblast isolated from pathologic conditions presented a cytokine secretion pattern that differed from the cytokine pattern from fibroblasts isolated from a healthy tissue (Brouty-Boyé et al., 2000).

The growth factors and cytokines secreted by CAFs causes changes in the transcription, secretion and phenotype of malignant, endothelial and immune cells that results in detrimental effects such as uncontrolled proliferation, invasion, neoangiogenesis, apoptosis, cancer stemness, metabolism, inflammation, immunosuppression, drug resistance, and metastasis (Yamamura et al., 2015).

An important contribution of CAFs to the tumor progression is the secretion of growth factors that increase the proliferation of malignant cells as well as other cells in the tumor microenvironment. TGF-β secretion is increased in CAFs and, it stimulated proliferation, EMT, migration and invasion.

TGF-β produced by fibroblasts increased the migration and invasion of tumor cells through the activation of Smad, p38 and c-Jun N-terminal kinase (JNK) signaling pathways *in vitro* (Zhang and Liu, 2013). CAFs are also the main producers of HGF in the tumor microenvironment. This factor regulates proliferation, differentiation and migration in epithelial cells that express the cell surface receptor c-Met. E.g. CAFs promoted invasion of esophageal squamous cell carcinoma cells mediated by the secretion of HGF. This CAF mediated increase in invasion could be inhibited when specific HGF siRNAs were used. CAF derived HGF also stimulates cell invasion. In response to HGF/c-Met signaling pathway, malignant cells increase cell motility, EMT and colony dispersion (Suárez-Causado et al., 2015).

CAFs can modulate angiogenesis by stimulate cancer cells to secrete VEGF (factor required for neo-angiogenesis), or by secreting pro-angiogenic factors themselves such as VEGF and other growth factors like FGF, PDGF, IGF, TGF-β, angiopoietin and angiomodulin (AGM) (Wang et al., 2019). Tenascin C present in the ECM in the tumor microenvironment also induces angiogenesis and blood vessel permeability in a pancreatic β-cell tumor model, by downregulating Dickkopf 1 (DKK1) and increasing WNT signaling (Saupe et al., 2013). In hypoxic conditions, frequently seen in tumors, activation of G-protein-coupled estrogen receptor (GPER), HIF-1α, formation of reactive oxygen species (ROS) and secretion of IL-6 promote CAF up-regulation of VEGF (Saupe et al., 2013).

CAF also facilitate tumor acquired resistance to drugs. E.g. CAFs were able to stimulate drug resistance in non-small cell lung carcinoma through IGF2 pathway. IGF2 induce the expression of Sox2, and this activates the expression of P-glycoprotein 1, also known as multidrug resistance protein. They also observed that the IGF2 expression correlates with chemoresistance in non-small cell lung cancer tumor samples (Zhang et al., 2018). CAFs are also an important source of CXCL12. CXCL12 upon binding to its receptor, stimulates the expression of anti-apoptotic proteins in tumor cells that contribute to its chemoresistance (Li et al., 2015).

The immune system can recognize and eliminate malignant cells. However CAFs can help tumors scape the immune control by modifying the tumor environment towards a more immunosuppressive status. CAFs increase the release of immunosuppressive cytokines and chemokines in order to model the function of immune cells towards a tumor permissive behavior, recruit immune cells that promote tumor growth, and interfere in the ability of lymphocytes to kill malignant cells (Monteran and Erez, 2019). Moreover some studies observed that CAFs are able to maintain an inflammatory environment that promotes tumor growth by the secretion of inflammatory mediators such as IL-6, IL-11, CXCL1, CXCL2 (Monteran and Erez, 2019).

Although CAFs mainly promote tumor growth, tumor suppressor functions of some CAF subsets have been reported. A mice model confirmed that depleting the tumor microenvironment of α-SMA expressing fibroblasts accelerated tumor growth. The lack of tumor suppressive fibroblasts caused a reduction in immune surveillance and increased infiltration of Treg cells (Gieniec et al., 2019).

3.5. POSSIBLE THERAPEUTIC TARGETS

Nowadays one in six death worldwide is caused by cancer (WHO, 2020). Traditionally tumor therapies were directed against cancer cells, oncogenes, dysregulated signaling pathways and epigenetic modifications. Traditional therapies are not fully efficient for most forms of cancer and malignant cells often developed resistance to chemotherapy or radiotherapy due to their genomic instability and the resistance provided by the stroma that surrounds them. The close link between tumor cells and their environment makes stromal cells promising therapeutic targets.

1- Direct targeting of CAFs to eliminate them or return them to a quiescent state:

CAFs may seem a very interesting target for the treatment of tumors, however the lack of specific cell surface markers and its heterogeneity makes it difficult to target all CAFs population without damaging other cell populations. Despite this, the number of therapies targeting CAFs is increasing (Chen and Song, 2019).

Some proteins are specifically expressed in CAFs, and therefore perfect targets for anti-tumor therapy. Some examples are: FAP, PDGFR, Sonic hedgehog (Shh)-Smoothened (Smo), CXCR4, NF-kB and Ets (Chen and Song, 2019).

Recently PDGFR blocking antibodies, such as Olaratumab (IMC-3G3, Lartruvo™) have been used in antitumor therapy. Olaratumab specifically binds to PDGFRa and reduces the growth of several tumor models *in vivo*, and in combination with doxorubicin it increases the survival in patients. However this approach was inefficient in some tumors (Tap et al., 2016). Another approach to block PDGFR signaling is through the use of low molecular weight inhibitors of its enzymatic activity. The problem of this methodology is the difficult to design specific inhibitor, due to the highly conserved structure among other families of tyrosine kinases. Some examples are dasatinib, nilotinib, bosutinib, and ponatinib. These inhibitors increase drug delivery efficiency, thereby increasing the efficacy of chemotherapy and attenuating tumor growth (Papadopoulos and Lennartsson, 2018).

Also FAP is being exploited to target CAFs since early 1990 for its restricted expression pattern and its unique post proline dipeptidyl peptidase activity. In physiological conditions is only expressed in alpha cells of Langerhans islets and bone marrow stromal cells in humans, however we can see FAP expression in pathological conditions such as tissue remodeling, liver cirrhosis, fibrosis, osteoarthritis and rheumatoid arthritis and tumors, in malignant cells as well as in cells of the stroma as we describe in our review (Busek et al., 2018).

One approach consists in the use of low molecular inhibitors that inactivate its enzymatic activity. Several compounds have been tested, showing a reduction in tumor growth in animal models that seemed to be mediated by the immune system. Only Talabostat made it to the clinical trials but failed in showing significant clinical effect in patients (Busek et al., 2018). Another approach exploits FAP unique enzymatic activity. A prodrug was designed by attaching Z-Gly- Pro dipeptide to desacetyl-vinblastine monohydrazide. FAP enzymatic activity released the active toxin viniblastine showing tumor regression in mouse xenografts (Chen et al., 2017). Other techniques use anti-FAP antibodies to detect tumors. These antibodies did not show anti-tumor effects but could be used to deliver toxins to the tumor (Busek et al., 2018).

The F19, the antibody was used to identify FAP in the original studies, in its humanized version was tested as a possible antitumor drug. In a phase I clinical trial in metastatic cancer patients demonstrated accumulation in the tumor stroma, unfortunately it did not show therapeutic improvements for the patients (Busek et al., 2018). CD8+ human T cells were transduced with FAP-chimeric antigen receptor (CAR) based on the antibody F19. These CAR-T cells demonstrated their specifity by releasing INF-γ in contact with FAP-positive mesothelioma cells *in vitro*. Other studies tried FAP-targeting CAR T cells, however none of the attempts made it to the clinical trial due to severe secondary effects (Busek et al., 2018).

Some drugs attempt to reverse the CAFs activation, returning them to a quiescent or anti-tumorigenic state. Minnelide, which is a drug currently tested in clinical trial (NCT03117920) that targeted TGF-β signaling pathway in CAF, showed a switch from their activated state back into a quiescent state (Dauer et al., 2018). Calcipotriol, a vitamin D analogue, decreased CAF proliferation, migration and the release of pro-tumorigenic factors in a 3D pancreatic tumor model (Gorchs et al., 2020).

2- Targeting the CAF-immune cell interactions:

CAFs are involved in the modulation of the immune response in the tumor microenvironment by the production and release of chemokines such as IL1-b, IL-6, CXCL1, CXCL2, CXCL12, CXCL14, CCL2, CCL5 and growth factors such as TGF-β1. CAFs induce inflammation, immune evasion and poor responses to cancer immunotherapy. Thus targeting CAF-derived cytokines and chemokines may be a good therapeutic target to diminish inflammation in the tumor site and control its progression (Togo et al., 2013).

CAFs are known to be big producers of IL-6. Upregulation of IL-6 causes constitutive activation of STAT3 which has effects in the tumor immune evasion and it is correlated with poor prognosis. Inhbitors for the cytokine IL-6 or its signaling pathway intermediates like rho associated protein kinase (ROCK) and signal transducer and activator of transcription (STAT)3 have been developed (Johnson et al., 2018). An example is Siltuximab (CNTO 328), an IL-6 neutralizing antibody (Song et al., 2014) which show efficacy in the treatment of ovarian, prostate and lung cancer in preclinical studies.

A research group has identified a trihydroxyphenolic compound that inhibits TGFβRI kinase in a fibroblast-selective manner. This results in a decrease in pathologic collagen deposition, and facilitated T cell penetration, since TGF-β1 is known to contribute to T cell exclusion into the tumor microenvironment (Wei et al., 2017). Inhibition of TGF-β1 release by using tranilast improved antitumor immune responses in a xenograft mouse model (Ohshio et al., 2015).

3- Targeting the CAF-tumor cell interactions:

CAFs and cancer cells interact with each other through the secretion of cytokines and growth factors, mainly: CXCL12, TGF-β, HGF, PDGFs, Shh, Cox-2 and CCL2 and transcription factors such as NF-kB (Togo et al., 2013). Interrupting the communication between CAFs and tumor cells hampers tumor growth. For example Cox-2 is often overexpressed in various cancers and it is thought to increase tumor cell invasiveness and cancer cell resistance to chemo- and radiotherapy. COX-2 inhibitors, such as celecoxib or refecoxib, reverse the COX-2-mediated expression of multidrug resistance proteins in cancer cells. COX-2 inhibition by NASIDs is known to reduce cancer recurrence and increase of patient life expectancy (Cirri and Chiarugi, 2012; Goradel et al., 2018).

4- Targeting the CAF-endothelial cell interactions:

Some of the molecules released by CAFs are involved in the interaction with endothelial cells. Some examples are VEGF, CTGF, FGFs, CXCL12, PDGFs or TGF-β, which have been involved in neoangiogenesis and vessel stability. For example CTGF expression levels are elevated in a various cancers where it is produced by several tumor cell types, among them CAFs. And exogenous addition of CTGF to the medium increases pancreatic cancer cells invasion and proliferation *in vitro*. A specific antibody against CTGF, FG-3019, is being tested in a pancreatic cancer model where a decrease in angiogenesis as well as tumor growth was observed (Hofmeister et al., 2008). A recent study shows that hypoxic CAFs upregulate a series of proteins, among them, the most upregulated was hypoxia-induced angiogenesis regulator (HIAR). Silencing of HIAR decreased CAF mediated pro-angiogenic effect by decreasing secretion of VEGFA (Kugeratski et al., 2019).

5- Targeting the CAF-ECM interactions:

Some of the efforts to target CAFs are focused in the proteins of the ECM synthesize by these cells. For example Halofuginone, an inhibitor of collagen I gene expression, exhibits anti-tumor activities in mouse models of prostate, pancreatic and lung cancer (Valkenburg et al., 2017). HU177, a cryptic integrin binding site within collagen, is often present within structurally altered forms of collagen. D93/TRC093 is a humanized monoclonal antibody that specifically binds to HU177 and disrupts ECM signaling and, as a consequence, reduces angiogenesis and α-SMA expressing stromal cells in B16F10 melanomas (Caron et al., 2018). L19 is a fully human monoclonal antibody, specific to ED-B domain of fibronectin, specifically expressed in tumors. An anti-angiogenesis drug was attached to L19 and applied into several animal models and demonstrated anti-tumor effects (Tianyi Liu et al., 2019). L-19 conjugated with IL-2 or TNF (L19-IL-2 and L19-TNF), applied in combination, are in a phase III clinical trial in patients with advanced melanoma (ClinicalTrials.gov Identifier: NCT03567889).Tenascin C, a protein expressed mainly by CAFs, was also used to target tumor stroma. A B-cell lymphoma 2 (Bcl-2) inhibitor, Navitoclax, that induces apoptosis in myofibroblasts, was enclosed in nanoliposomes which bind specifically to tenascin C (Chen et al., 2016). These nanoliposomes, in combination with Trametinib and Navitoclax, are being evaluated in clinical trial II in patients with advanced or metastatic tumors (ClinicalTrials.gov Identifier: NCT02079740).

In addition, ECM remodeling enzymes have been reviewed as possible therapeutic targets. CAFs are responsible for the aberrant composition of the ECM and the enzymes that degrade it. Various MMPs promote tumor growth, and several inhibitors have been assayed in preclinical or clinical

trials. Unfortunately, none presented antitumor effect. Further efforts must be done in the development of more specific inhibitors (Hofmeister et al., 2008).

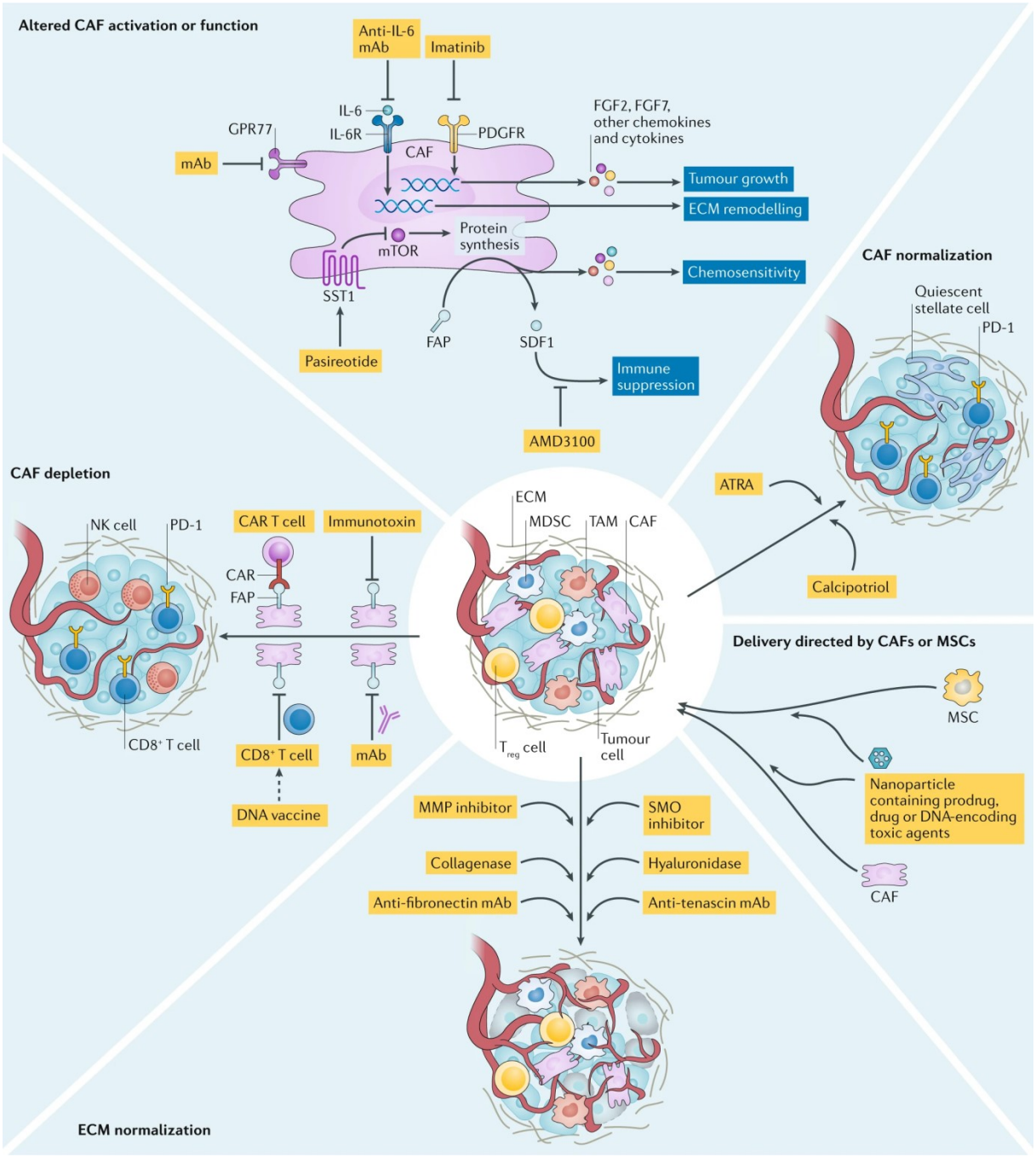

Figure 7. Schematic representation of the different CAF-directed therapeutic approaches to treat tumors. Targeting CAFs or its activation: by the use of DNA vaccines, CAF-directed CAR Tells or drugs like calcipotriol. Targeting soluble signals and effectors secreted by CAFs to inhibit the CAF mediated promotion of angiogenesis, pro-tumorigenic immunomodulation or tumor cell proliferation or invasion. Targeting CAF-derived ECM proteins by the use of drugs such as collagenase inhibitors (Chen and Song, 2019).

3.6. TUMORS: WOUNDS THAT DO NOT HEAL

Dvořak described more than 30 years ago the parallelism between wound healing and tumor formation, suggesting a model in which tumors would use an abnormal activation of the wound healing response in order to induce the stroma they require for their maintenance and growth (Dvorak, 1988). The hypothesis was extended not only to tumors, but also to other chronic inflammatory diseases that have characteristics of aberrant wound healing responses such as: rheumatoid arthritis and psoriasis (Wong and Whited, 2020). In fact, the crosstalk between epithelial cells and fibroblasts during wound healing and the signaling between cancer cells and CAFs have many similarities (Lacina et al., 2015).

As we have seen in the previous chapter, wound healing process starts with the activation of thrombocytes. As thrombocytes become active they secrete cytokines and growth factors, including VEGF and form a blood clot by deposition of fibrin, vitronectin and fibronectin. The cytokines and growth factors secreted attract neutrophils and macrophages to the site of injury. These two cell populations initiate the inflammation stage of wound healing. Subsequently fibroblasts become active and are recruited to the site of injury to synthesize a temporary ECM characterized by high vasculature and high number of cells called, granulation tissue. This temporary ECM is crucial for other cells to migrate, including keratinocytes, and favoring re-epithelization. Finally, fibroblasts remodel the ECM to its final state and facilitate wound contraction (Foster et al., 2018).

Tumor stroma formation occurs in a very similar process, and can be organized into equivalent phases. VEGF expression is upregulated in keratinocytes during a growing neoplasm. VEGF initiates a sequence of events very similar to the events happening during wound healing: including blood extravasation, formation of a clot, immune cells recruitment and inflammation. Fibroblasts are thereafter recruited to the site of neoplasm growth and activated. Fibroblasts begin with synthesis and deposition of components of the ECM generating a scaffold that allows tumor cells to continue proliferating and growing. The ECM is stiffer and its composition differs from an ECM in healthy tissues. This abnormal ECM promotes angiogenesis and tumor progression. Newly formed blood vessels tend to be dysfunctional, thus causing hypoxia in the tumor microenvironment. Hypoxic environment in a tumor, as well as in a wound, induces the overexpression of VEGF, acting as a positive feedback and reinforcing the inflammation and progression of tumor growth (Foster et al., 2018).

In a wound, once and the lesion is closed, the stimuli cease and fibroblasts' activity fade, as well as inflammation: it is a temporal event. However, in tumors, the activation of the host 'repair program' persists and becomes chronic due to the continuous tumor cell growth, the presence of hormones, cytokines and growth factors in the stroma (Dvorak, 2016). Exploring the similarities between wound healing and tumor might lead as to a discovery of possible stromal-based therapeutic targets.

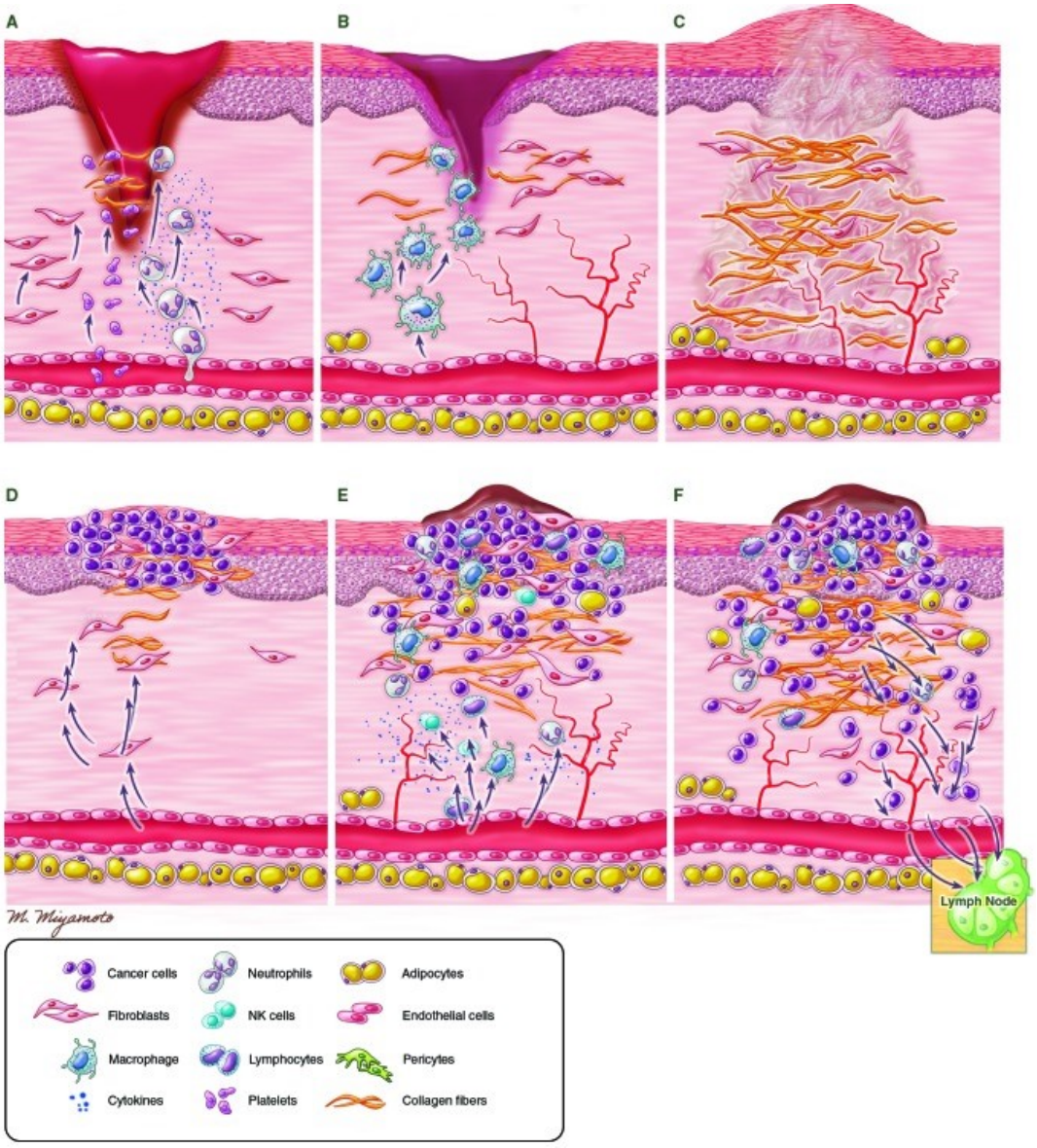

Figure 8. Comparative scheme of wound healing (top) and tumor (bottom). (A) The first phase of is coagulation or hemostasis phase: thrombocytes in the blood form a clot at the site of injury. The blood clot is formed by fibronectin, fibrin, vitronectin and thrombospondin. Neutrophils migrate to the wound and cytokines are released defining the beginning of the inflammatory phase. Fibroblasts are recruited and synthesize ECM. (B) During the proliferation phase, macrophages clear dead tissue and debris. En reepithelization begins. (C) During the remodeling phase the temporary ECM is remodeled and there is scar formation. (D) Fibroblasts are recruited to the tumor microenvironment and transformed into CAFs, CAFs then

start synthesizing an abnormal ECM in which tumor cells can keep growing. (E) Inflammatory cells are recruited to the site of neoplasm and they release cytokines. (F) The inflammation keeps the fibroblasts active and ECM formation continues as well as tumor cells growth. The leaky vasculature frequently seen in tumors can allow tumor cells extravasation and metastasis. Illustrated by Mao Miyamoto (Foster et al., 2018).

HYPOTHESIS AND OBJECTIVES

Hypothesis:

- Differences between newborn and adult fibroblasts contribute to the different outcomes of wound healing.

- Fibroblasts crosstalk with melanoma cells increase melanoma cell invasion.

- Cancer cells are a potential source of CAF formation.

Objectives:

- Evaluate functional and morphological differences between adult and newborn fibroblasts and their crosstalk with keratinocytes in functional assays relevant for wound healing.

- Determine the presence of myofibroblast-like α-SMA positive cells in newborn, older children and adult fibroblast and the signaling pathway involved in the conversion of fibroblasts to myofibroblasts.

- Analyze the effects of fibroblasts and cancer associated fibroblasts in the invasiveness of melanoma cells and the molecular mechanism responsible of it.

- Asses the ability of xenografted human cancer cells to create tumors containing CAFs derived from cancer cells through EMT in a mouse model.

MATERIAL AND METHODS

Material and methods are explained only briefly, their detailed description can be found in attached studies.

Cell lines and culture media

-FaDu (human squamous cell carcinoma isolated from pharynx; HTB-43), propagated in E-MEM with 2 mM L-glutamine, 1.0 mM sodium pyruvate, 10 mM HEPES , 1.5 mg/mL sodium bicarbonate, 100 U/mL penicillin, 100 µg/ mL streptomycin (Sigma-Aldrich Co., St. Louis, MO, USA) and heat-inactivated 10 % fetal calf serum (FCS) (GIBCO, Invitrogen, Life Technologies, Carlsbad, CA, USA) added.

-Sw620 (human colorectal adenocarcinoma; CCL-227) and HT-29 (human colorectal adenocarcinoma; HTB-38) in McCoy's cultivation medium (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10 % FCS, 2 mM l-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin.

-BLM, A2058, WM3629 and WM3670 melanoma cells, human adult fibroblasts derived cells, cancer-associated fibroblasts (Mel Fib) and mouse 3T3 were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % fetal bovine serum (FBS), 1 % antibiotic - antimitotic solution (Sigma-Aldrich Co., St. Louis, MO, USA) and 50 µg/ml gentamicin, at 37 °C with 5 % CO2.

All the cell lines and primary cultures were grown in cultivation flasks at 37 °C with 5 % CO2 in the recommended medium.

Animal models

All the *in vivo* experiments were performed with 8-week-old female nu/nu mice (AnLab Ltd., Prague, Czech Republic). Food and water were given *ad libitum*.

Isolation of cells

Samples were obtained at the Ear, Nose and Throat Department of the Second Faculty of Medicine and the Department of Plastic Surgery of the Third Faculty of Medicine, at Charles University in Prague, department of the University hospital in Motol and from the Department of Plastic surgery, University Hospital Královské Vinohrady (Prague, Czech Republic), department of dermatovenerology, General University Hospital (Prague, Czech Republic).

The isolation of cell populations was performed as described by Krejčí and colleagues (Krejčí et al., 2015) and fibroblasts and cancer cells sprouting from the tissues were collected and cultivated in vitro in DMEM (Biochrom, Berlin, Germany) containing 10 % fetal calf serum, 2 mM l-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (all Biochrom, Berlin, Germany) at 37 °C and 5 % CO_2 as described (L. Lacina et al., 2007). Harvested keratinocytes were cultured on mitomycin C-treated (Sigma-Aldrich, Prague, Czech Republic) 3T3 feeder cells in keratinocyte medium DMEM + F12 3:1 with 10% FBS, supplemented with 0.4 µg/ml hydrocortisone, 10^{-10}M cholera toxin, 10 ng/ml EGF (all from Sigma-Aldrich Co., St. Louis, MO, USA) and 0.12 U/ml insulin Actrapid (Novo Nordisk, Bagsværd, Denmark).

All samples were collected under Local Ethics Committee approval in accordance with the ethical standards of the Institutional and National research Committee, and according to the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The informed consent was obtained from all individual participants or their legal representatives in the case of minors.

Immunocytochemistry/ Immunohistochemistry/hematoxylin-eosin staining

Coverslips were processed and stained with hematoxylin and eosin and/or immunofluorescence method to evaluate the morphology of tumors and the expression pattern of the cells.

- Immunocytochemistry. The cells grown on coverslips were fixed with 2% (w/v) paraformaldehyde in phosphate- buffered saline (PBS) for 10 min, then washed with PBS and permeabilized by 0.05% Triton X-100 (Sigma-Aldrich Co., St. Louis, MO, USA) in PBS. The non-specific binding of immunoglobulins via Fc receptors was blocked by 3.3% non-immune porcine serum (DAKO, Glostrup, Denmark).

- Immunohistochemistry. Samples were cryoprotected using Tissue-Tek (Sakura Finetek Europe b.V., Zoeterwoude, The Netherlands) and frozen in liquid nitrogen. The 10 µm sections were prepared using CryoCut (Reichert-Jung, Vienna, Austria). Formaldehyde-fixed paraffin-embedded sections were routinely rehydrated, and heat-induced epitope retrieval in citrate buffer (pH 6, DAKO, Glostrup, Denmark) was consequently performed. Endogenous peroxidase activity was blocked by 1 % hydrogen peroxide (20 min), and non-specific interaction of immunoglobulins was blocked by incubation in diluted 10 % non-immune goat serum (20 min).

Antibodies were used following the specifications from the manufacturer:

- α-SMA mouse monoclonal antibody (DAKO, Glostrup, Denmark)

- Fibronectin rabbit polyclonal antibody (DAKO, Glostrup, Denmark)

- Anti-CD31 rabbit polyclonal antibody (Abcam, Cambridge, UK)

- Desmin rabbit polyclonal antibody (DAKO, Glostrup, Denmark)

- Vimentin rabbit polyclonal (Sigma-Aldrich, St. Louis, MO, USA)

- Hu-Vimentin mouse monoclonal (DAKO Glostrup, Denmark)

- Wide spectrum cytokeratin rabbit polyclonal anti- (Abcam, Cambridge, UK)

- IL-6 mouse monoclonal (Abcam, Cambridge, UK)

- IL-8 mouse monoclonal (Abcam, Cambridge, UK)

Secondary antibodies:

- Fluorescein IsoTioCyanate (FITC)-labeled swine anti-rabbit immunoglobulins (DAKO, Glostrup, Denmark)
- Tetramethylrhodamine (TRITC)-labeled goat anti-mouse immunoglobulins (Sigma-Aldrich Co., St. Louis, MO, USA)

All the samples were counterstained with 4',6-diamidino- 2-phenylindole (DAPI); (Sigma-Aldrich, Co., St. Louis, MO, USA) and mounted in Vectashield (Vector Laboratories, Peterborough, UK). Visualized with Eclipse 90i fluorescence microscope (Nikon, Prague, Czech Republic) equipped with a Cooled-1300Q CCD camera (Vosskühler, Osnabrück, Germany) and LUCIA 5.1 computer-assisted image analysis system (Laboratory Imaging, Prague, Czech Republic).

Conditioned media:

Cells were seeded at confluency 10000 cells/cm^2 with recommended medium for 48 hours. Then the medium was replaced and let for 24 hours. After these 24 hours the medium was harvested and filtrated with 0,22μm pore diameter filter.

Spheroid invasion assay:

Cells were grown in Micro-tissues® 3D Petri Dish® in the shape of spheres and later embedded in 2 mg/ml Collagen R (Serva Electrophoresis, Heidelberg, Germany) solution, which contained DMEM, 5 % NaHCO3 and 10 % FBS. DMEM or conditioned media were added to the wells. Images were taken

at time zero and 48 hours using a Nikon-Eclipse TE2000-S objectives 4x/0,13 PHL and 10x/0,13 MHC and NIS-Elements software (Nikon, Prague, Czech Republic).

Wound healing assay:

BLM or A2058 cells were grown to confluency and a P200 pipette was used to create uniform gaps. Cells were cultivated in the proper media and images were taken at 0, 6 and 12 h using Nikon-Eclipse TE2000-S 10x/0,13 MHC, and NIS-Elements software (Nikon, Prague, Czech Republic).

Proliferation assay:

A total of 6×10^3 BLM or A2058 melanoma cells were plated in a 96-well plate overnight, media was then removed, cells were washed with PBS, and DMEM with 10 % FBS or conditioned media was added to the cells. Proliferation was analyzed using AlamarBlue® (GIBCO, Invitrogen, Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. Absorbance was measured using TECAN infinite® m200 PRO and i-control™ software (Tecan Group, Männedorf, Switzerland).

Enzyme-linked immunosorbent assay (ELISA):

IL-6 and IL-8/CXCL8 levels in conditioned media, and from spheroids in collagen, were measured using sandwich ELISA according to the manufacturer's protocol (R&D Systems, Prague, Czech Republic). Human IL-8, IL-6 and TGF-β1 kits (cat. nos. el1008-1, el1006-1 and eT3102-1; BioVendor Laboratory Medicine Inc.) and analyzed at a wavelength of 450 nm using a universal Microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Multipotency assay:

The adipogenic, chondrogenic and osteogenic potential of newborn and adult fibroblasts was tested using a commercial Human Mesenchymal Stem Cell Functional Identification kit (R&D Systems, Minneapolis, MN, USA).

Migration of fibroblasts:

The cells were inoculated in seven 10ul drops containing 5000 cells each in 6-well plates (Corning, Rochester, NY, USA). 6 h after inoculation, 2 ml of DMEM + 10% FBS were added and the cells were then cultured for 7 days. The culture medium was changed on day 3 and 5. MTT assay was used to evaluate the diameter reached by the cells using ImageJ software. Later blue formazan was dissolved in 2 ml of dimethyl sulfoxide (DMSO; Sigma-Aldrich Co., St. Louis, MO, USA) and the absorbance of

200 µl of blue colored solution was measured at 570 nm using the microplate reader EL800 (Bio-Tek, Winooski, VT, USA).

Migration of keratinocytes:

Newborn and adult keratinocytes were inoculated on the coverslips placed in an 8-well dish Nunc (Thermo Fisher Scientific, Rochester, NY, USA) on a mitomycin C-treated feeder layer (3T3 cells, 30,000 cells/cm^2) till confluency. Uniform circular defects were made using the 8 mm biopsy punch (Kai Medical, Seki City, Japan). Pictures were taken after 1, 2 and 4 days. The size of the defect and the phenotype of the keratinocytes was analyzed by immunocytochemistry.

Coculture of fibroblasts and keratinocytes:

Newborn or adult fibroblasts were inoculated in the same manner as mentioned above on 6 well plate or coverslips. After their adhesion, collagen inserts (Corning, New York, NC, USA) were placed into the wells and newborn and adult keratinocytes were inoculated at a density of 40,000 cells/cm2. The growth of fibroblasts in 6 well plate was evaluated by MTT assay after 5 days. The coverslips were used to study the phenotype of fibroblasts by immunocytochemistry after 7 days.

In the same manner newborn or adult keratinocytes were seeded on coverslips, 24 hours later, Falcon polyethylene terephthalate (PET) inserts (Corning, New York, NC, USA) were put into the wells and newborn or adult fibroblasts were inoculated at a density of 5,000 cells/ insert. The cells were cocultured under the same conditions as mentioned above. The phenotype of keratinocytes was detected by immunocytochemistry.

Real-time polymerase chain reaction (qPCR) analysis:

RNA from subconfluent cultures of newborn and adult fibroblasts was isolated using an RNeasy micro kit (Qiagen, Gaithersburg, MD, USA). DNase I (Qiagen, Gaithersburg, MD, USA) was applied in RNA solution to properly remove genomic DNA, and the purification procedure was repeated. Reverse transcription was performed with 1 µg of total RNA and an AccuScript High Fidelity First Strand cDNA Synthesis kit (Stratagene, San Diego, CA, USA) according to the manufacturer's instructions. For negative control, the same reverse transcription reaction without reverse transcriptase was performed. The polymerase chain reaction **(**PCR) reaction was performed with REDTaq ReadyMix (Sigma-Aldrich Co., St. Louis, MO, USA). All primers are listed in Table II.

Transcriptome analysis:

Total RNA was isolated using an RNeasy micro kit (Qiagen, Gaithersburg, MD, USA) according to the manufacturer's instructions and its concentration measured with NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The RNA integrity was analyzed with an Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). Illumina HumanHT-12 v4 Expression BeadChips (Illumina, San Diego, CA, USA) were used for the microarray analysis following the standard protocol. GenomeStudio software (version 1.9.0.24624; Illumina) was used to analyze the data.

Western blot analysis:

The cells of each fibroblast population were seeded at a density 1,000 cells/cm^2 into 10 cm diameter Petri dishes (Corning, Inc., Corning, NY, USA) and cultured for 7 days (95-100% confluence). The culture medium was refreshed every two days. The cell lysates were harvested according to the standard protocol in NP-40 cell lysis buffer (Thermo Fisher scientific, Inc.). The lysates were centrifuged and the supernatant was collected into a fresh microtube containing Protease Inhibitor Cocktail (Sigma-Aldrich Co., St. Louis, MO, USA; Merck kGaa). The total protein concentration was detected using the Bradford method for protein quantitation (28). Samples were resolved by 1-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (sds-PaGe) gels according to standard techniques. Equal quantities of total protein (10 µg) were subjected to 12% sds-PaGe (electrophoresis for 60 min, 100 V, in cold room at 4˚C) and transferred onto polyvinylidene difluoride membranes. The membranes were subsequently blocked with 5% goat serum (Sigma-Aldrich Co., St. Louis, MO, USA) for 1 h and probed with specific α-SMA primary antibody (dilution, 1:1,000; overnight at 4˚C), followed by the appropriate horseradish peroxidase-conjugated secondary antibody (dilution, 1:5,000; 60 min at room temperature). Proteins were detected using the kPL Trueblue blotting detection reagents (BioVendor Laboratory Medicine, Inc., Brno, Czech Republic).

Cell proliferation assay and TGF-β treatment/inhibition:

Fibroblasts from different donors were seeded at a density of 750 cells/well in 96-well plates overnight in DMEM with 10% FBS. Inhibitor sb-431542 (Sigma-Aldrich Co., St. Louis, MO, USA) was added to the media to block TGF-β signaling (10 µmol/l). Recombinant human TGF-β1, TGF-β2 and TGF-β3 (Sigma-Aldrich Co., St. Louis, MO, USA) were dissolved in 4 mM HCl with 0.1% of bovine serum albumin to a final concentration of 10 ng/ml in DMEM. Cell proliferation was continuously monitored using an IncuCyte ZooM kinetic live cell imaging system (Essen bioscience, Ann arbor, MI, USA).

Statistical analysis:

Statistical analysis was performed using Paleontological statistics (version 3.14; university of Oslo, Oslo, Norway) and the nonparametric Kruskal-Wallis test (by ranks) was performed. $p<0.05$ was considered to indicate statistical significance. ANOVA followed by Tukey's honest significant difference test was also used. Statistical analysis was performed using PAST (version 3.12) free scientific analysis software kindly provided by Dr Ø. Hammer, University of Oslo, Norway. Normality of distribution was assessed using Shapiro-Wilk test, Levene's test was used for homoscedasticity and t-test was performed to compare the size of newborn and adult fibroblasts covered areas.

RESULTS

Result I:

Functional differences between neonatal and adult fibroblasts and keratinocytes: Donor age affects epithelial-mesenchymal crosstalk *in vitro*

Rosana Mateu, Veronika Živicová, Eliška Drobná Krejčí, Miloš Grim, Hynek Strnad, Čestmír Vlček, Michal Kolář, Lukáš Lacina, Peter Gál, Jiří Borský, Karel Smetana jr and Barbora Dvořánková

Int. J. Mol. Med. 38: 1063-1074, 2016, IF: 2,242, **Number of citations: 9** (auto citations are excluded)

Recent data suggests that newborns, during the first days after birth, are able to heal without scar, almost in a regenerative way as it is observed during fetal development in human and other mammals (Borsky et al., 2012). Newborn dermis might be the clue for these excellent results during wound healing. Thus, the objective of this research was to characterize the phenotic differences

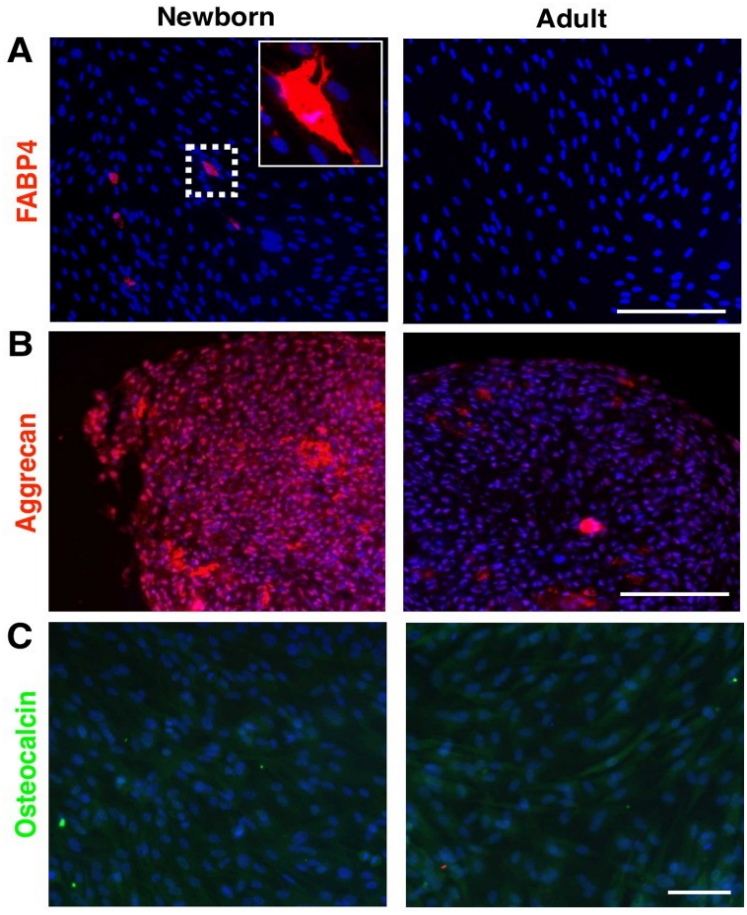

Figure 9: Plasticity of fibroblasts. NFs can be transformed to (A) adipocytes and (B) chondroblasts but not to (C) osteoblasts. Fatty acid binding protein (FABP4)(red signal), aggrecan (red signal) and osteocalcin (green signal) were detected by immunofluorescence. Scale bar is 100 µm.

between newborn and adult fibroblasts. In individual cultures, and also in a coculture system, in order to study their reciprocal influence and their correlation with wound healing process. For this purpose we isolated three newborn and three adult fibroblasts (NF and AF, respectively) and keratinocytes (NK and AK, respectively) from cleft lip and aesthetic surgery residual tissue respectively.

We analyzed fibroblasts' plasticity, assaying their capacity to differentiate into other mesenchymal cell types. Fibroblasts were cultured during three weeks in a medium providing signals for adipocyte, chondrocyte or osteoblasts differentiation (see material and methods). We performed immunohistochemical detection using the adipocyte marker fatty-acid-binding proteins (FABP4) and we could observe FABP4 expressing cells in the newborn fibroblast culture. In addition, we observed lipid droplets content in the cells, which is a typical feature in adipocytes, confirming that NFs were able to differentiate into adipocytes. Immunohistochemical detection using the chondrocyte markers aggrecan showed aggrecan positive cells, confirming that NFs were able to differentiate into chondrocytes too. However, NFs failed to differentiate into osteoblasts. On the contrary, AFs were not able to differentiate into adipocytes, chondrocytes or osteoblasts. These results suggested that the plasticity of NFs disappears during the course life and thus AFs lack this differentiating capacity (Fig. 9).

Next, we assayed the migratory properties of fibroblasts, since their migration to the wound site is necessary in order to reconstitute the dermis after an injury. We did it by performing an inoculum spreading assay in which 10 µl-drops containing 5000 NFs or AFs were cultured. After 7 days the inoculum spreading assay was visualized using the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as colorimetric method to observe and quantify the area covered by the fibroblasts as they migrated from the initial 10 µl-drops. We observed that AFs were able to cover a bigger area than NFs (Fig. 10). The MTT test is a colorimetric assay for assessing cell

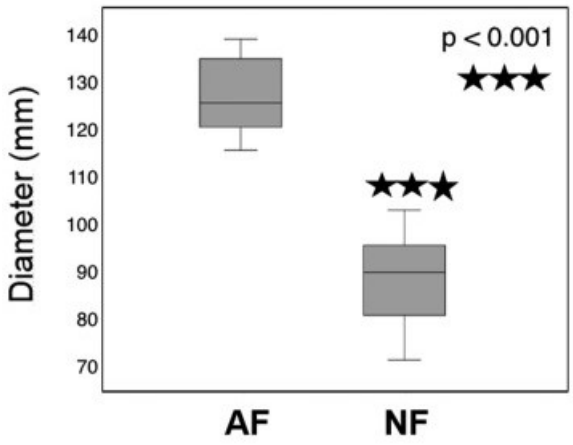

Figure 10: Migration of fibroblasts. Migration of both cell types from microdrops significantly differed at day 7. Twenty clusters of each cell type (from 3 biological replicates) were used for the statistical evaluation. A statistically significant difference between AFs and NFs was found (P<0.001).

metabolic activity that indirectly reflects the cell number of viable cells present so we could measure the densities of NFs and AFs. The cell densities of NFs and AFs were similar, demonstrating that the AFs were able to cover a bigger area due to higher migration rate, not due to an increase in the cell division rate.

In wound healing conditions *in vivo*, keratinocytes and fibroblasts coexist and interact and influence each other's behaviors. We wanted to study the influence of keratinocytes in fibroblast migration. To evaluate this, we used a scratch assay. We cultured fibroblasts in monolayer till confluency was reached, and then we scraped the cell layer in a homogeneous straight line to create an empty space. We cultured NFs and AFs alone and in coculture system with keratinocytes. In the coculture system NFs and AFs were cultured in the lower chamber and NKs or AKs in collagen inserts in the upper compartment, allowing the crosstalk, but avoiding direct contact between the cell populations. AF closure rate was not affected by coculture with keratinocytes (no differences regardless the use of NK or AK), while strikingly NF increased its migratory potential in coculture. These results demonstrated that NF, upon crosstalk with keratinocyte population, can change their phenotype.

The migratory properties of the keratinocytes were also assayed. Keratinocytes were cultured till confluency and then using a biopsy punch we made a gap in the keratinocyte layer. NKs were able to regrow the defect completely in 96 hours, while AKs could not. These results indicate that NKs would perform a faster closure of a wound. Moreover by immunocytochemistry we observed a higher percentage of keratin 8 (K8) expressing keratinocytes in the migrating front of NKs compared to AKs. K8 is a marker of simple epithelia, thus indicating the low differentiation status of the keratinocytes.

Contraction of the wound is a crucial step in wound healing. Myofibroblasts, α-SMA expressing fibroblasts, are responsible of contraction. We wanted to evaluate the presence of α-SMA fibrils in NFs and AFs. Fibroblasts were cultured in coverslips during 7 days and then α-SMA was detected using immunocytochemistry. α-SMA positive fibroblasts were abundant in NFs but rare in AFs cultures (Fig.11). Interestingly, coculture with keratinocytes increased the conversion of fibroblasts to α-SMA positive both newborn and adult fibroblasts although the effect was more prominent in NKs. These observations could partially explain why newborns are able to heal faster and almost without scar.

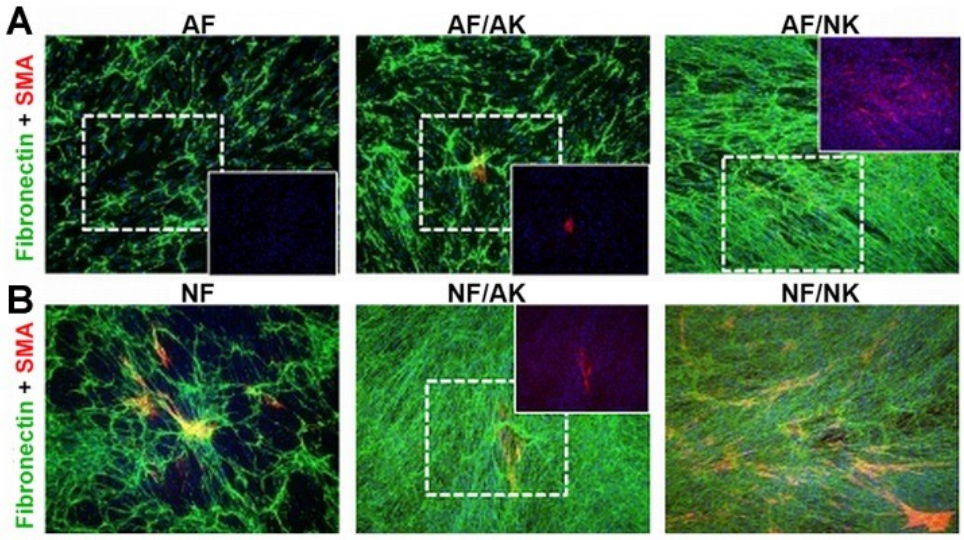

Figure 11: Presence of α-SMA in fibroblasts. AF culture was negative for the presence of myofibroblasts exhibiting α-SMA while we can observe the presence of α-SMA in NFs. The coculture of fibroblasts with either NKs or AKs enhanced the incidence of α-SMA expressing fibroblasts in both NFs and AFs. Both fibroblast cultures expressed a fibronectin rich ECM. Fibronectin (green) α-SMA (red).

ECM is a crucial element during wound healing, since a rich in protein ECM lay a support for other cells, such as keratinocytes, to migrate to the wound site. So we selected an ECM protein, fibronectin, to check its presence in fibroblast cultures. Fibronectin is an adhesive molecule that is important in wound healing for its interaction with other cell types, cytokines and other components of the ECM, the organization of the granulation tissue and basement membrane. Fibroblasts were cultured on coverslips and after adhesion collagen inserts with the keratinocytes were placed. Both NFs and AFs produced a rich in fibronectin matrix, as it was observed by fibronectin immunocytochemistry staining. Fibronectin matrix was increased when fibroblasts where cocultured with keratinocytes, corroborating a crosstalk communication between fibroblasts and keratinocytes. Surprisingly, AKs could only stimulate fibronectin production on NFs, highlighting once more the differences between adult and newborn cells (Fig.11).

Using the same set up, nestin expression was analyzed. Nestin is a cytoskeletal protein broadly expressed during development and in pathological conditions. The percentage of nestin positive fibroblasts in NFs was noteworthy, while they were very rare in AFs. The positivity increased in coculture with keratinocytes in both AF and NF cultures. Nestin is also found in fetal fibroblasts, suggesting that NFs share more similarities with fetal fibroblasts that the AFs do.

Next, we focused on changes in the keratin expression pattern and morphology of the colonies of keratinocytes alone and after coculture with fibroblasts. Keratins are expressed abundantly in

keratinocytes and, the expression of the different keratins change during keratinocyte differentiation. We were specifically interested in keratins 8, 19 and 14, which are normally found in early stage of development and in malignant tumors, indicating poor differentiation. Only NFs (but not AFs) were able to induce the presence of small rounded cells on the periphery of the keratinocyte colonies which lacked intercellular contacts and were positive for keratin 14, K8 and K19 (Fig. 12). These data indicates that NKs present a poor differentiation state when compared to AKs.

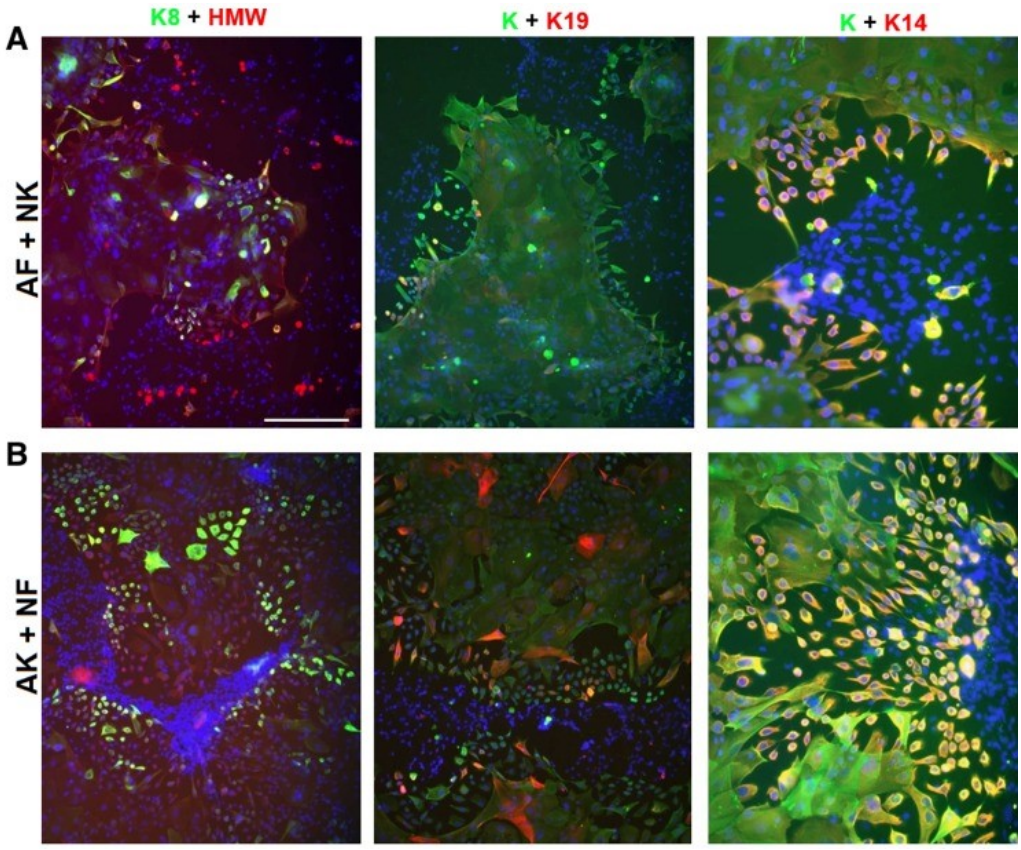

Figure 12: Influence of the age of fibroblasts' donor on the phenotype of keratinocytes. AFs had a negative effect on the incidence of small NKs located on peripheries of compact colonies positive for keratin 8 (K8; green signal), keratin 19 (K19; red signal) and keratin 14 (K14; red signal). HMW represents high molecular weight cytokeratin and K wide spectrum cytokeratin. The nuclei of the cells were visualized with DAPI (blue color). (B) NF had a strong stimulatory effect on the occurrence of small AK positive for the above-mentioned keratins. Scale bar is 100 µm.

Finally we analyzed NFs and AFs expression profile using microarray technology. We found 1,509 differentially-regulated genes. From those, we specifically focused in genes related to the expression of growth factors, interleukins and other signaling molecules because they are relevant to explain the crosstalk communication between fibroblasts and keratinocytes and also relevant for cell chemotaxis and the acute phase of inflammatory response during the process of wound healing.

Among the upregulated genes in NFs we found genes involved in the cell division regulation (FGF-5, TGFB2, MDK, TGFB3, IL1B, VEGFB, FGF-1, PGF and VEGFA), cell growth (TGFB2, IGFBP5, IGFBP4, IGFBP2, IGFBP7, nerve growth factor (NGF), kazal type serine peptidase domain 1 (KAZALD1), CTGF, CXCL16, VEGFA, growth differentiation factor (GDF)10, IL-6, TGFB3, inhibin subunit beta B (INHBB), FGF-1 and bone morphogenic protein 6, proteins of the ECM, and interleukins and other cell signaling molecules, involved in the acute inflammatory response like IL1B, IL-6, CXCL1, CXCL6, CXCL14, CXCL16, TGFβ2, VEGFA and VEGFB.

The differentially expression genes between NFs and AFs could partially explain the phenotypical differences observed in these experiments and more importantly, the better wound healing outcome observed in newborns.

In conclusion, the results obtained in this work showed differences between NFs and AFs which are relevant for wound healing. We observed differences in plasticity, migration, expression of nestin and α-SMA, as well as differences in the expression pattern in genes involved in cell division, growth, acute inflammatory response, and chemotaxis, which could have a role in the better wound healing outcome. We observed that NFs also influenced keratinocytes towards a less differentiated phenotype. This data could help to better understand the process of wound healing and improve and minimize scar formation. Furthermore, it also could help to understand the similarities between wound healing and tumor progression, specifically the role of fibroblasts and CAFs, respectively.

For further details, please see annex 1

Result II:

Analysis of dermal fibroblasts isolated from neonatal and child cleft lip and adult skin: Developmental implications in reconstructive surgery

Veronika Živicová, Lukáš Lacina, Rosana Mateu, Karel Smetana Jr, Radana Kavková, Eliška Drobná Krejčí, Miloš Grim, Alena Kvasilová, Jiří Borský, Hynek Strnad, Miluše Hradilová, Jana Šáchová, Michal Kolář and Barbora Dvořánková

Int. J. Mol. Med. 40:1323-1334, 2017, IF: 2,712, **Number of citations: 5** (auto citations are excluded)

In the previous paper we observed that NFs and AFs presented some phenotypical differences relevant for the wound healing. In this work we wanted to expand the characterization of neonatal cleft lip fibroblasts (NCF) (24 samples) and older children cleft lip fibroblasts (OCCF) (8 samples) and their correlation with cleft lip outcome. NCF and OCCF were also further analyzed in order to compare the expression of pluripotency markers. In addition, we compared those results with the expression profiles from normal adult skin fibroblasts isolated from face (5 samples) and breast (3 samples) and, fibroblasts from scar (3 samples).

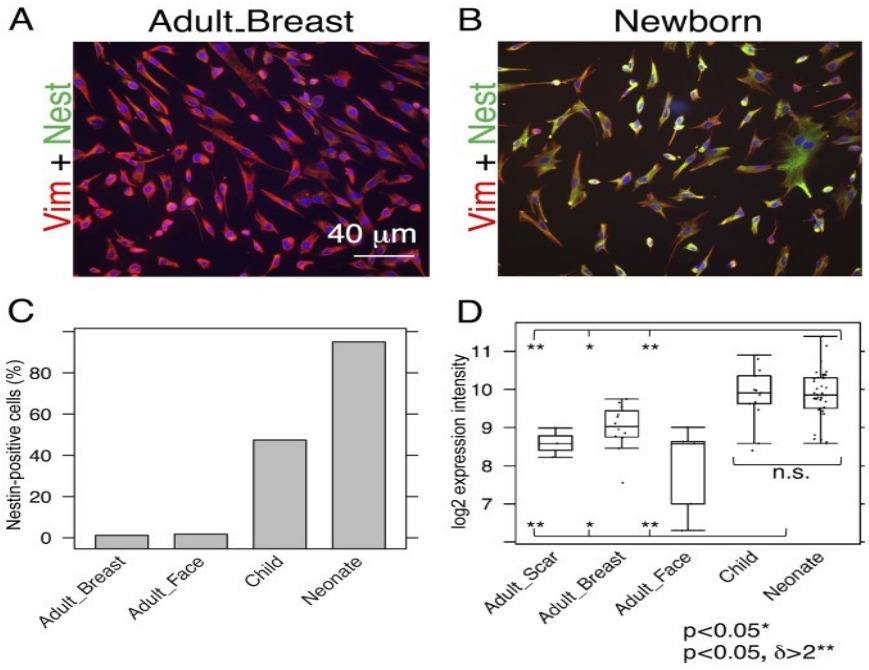

Figure 13. Expression of nestin in newborn, older children and adult facial, scar and breast fibroblasts. Vimentin and nestin immunocytochemistry staining in (A) adult breast and (B) newborn derived fibroblasts. Percentage of Nestin-positive cells (C) and transcriptomics data of Nestin expression (D).

First, we studied the differentiation status of the fibroblasts. We focused on the expression of nestin, a marker of immature cells. NCF were mostly nestin positive, its expression in OCCF was reduced and negligible in adult fibroblasts from breast and facial origin (Fig. 13 A, B and C). These results were confirmed by transcriptomics (Fig. 13 D) and by immunohistochemistry in sections (See Fig. 2 in the annex II).

Next we asked whether the nestin expressing fibroblasts expressed, in addition, pluripotency markers nanog and octamer-binding transcription factor 4 (oct4). Nanog and oct4 are transcription factors active in undifferentiated embryonic stem cells. To answer that, we performed an immunostaining for these markers. In NCF cultures, nestin positive cells also expressed the nuclear markers oct4 and nanog. However, at the expression level, we did not find significative differences on the expression levels of oct4 and nanog among the different groups NCF, OCCF and adult fibroblasts from breast, face and scar (Fig. 3 in the annex II).

During tissue remodeling, fibroblasts often express α-SMA and become myofibroblasts, key cells during wound closure and predominant producers of ECM. We observed α-SMA positive fibroblasts in NCFs and OCCFs in roughly 50% of the tested cultures from each group. Almost no positivity was found in any of the adult fibroblast cultures. We also analyzed the α-SMA content in the total protein lysates of the different cultures using western blot. Adult fibroblast lysates did not show α-SMA detectable levels, while NCF lysates showed α-SMA presence in most of the cultures, even those that lacked α-SMA typical fibrillary pattern in immunocytochemistry (Fig. 14). We analyzed the expression of the actin α2 gene, which encodes α-SMA. actin α2 was highly expressed in NCFs and OCCFs when compared to adult fibroblasts from breast, face or scar. Besides actin α2, humans have other isoforms of actin such as actin γ2. It is also a component of the cytoskeleton, and unlike actin α2, it is expressed in all cells. We further analyzed the expression level of actin γ2, which appeared higher in NCFs, OCCFs and adult facial fibroblasts as compared to adult fibroblast from breast and scar (Fig. in the annex II).

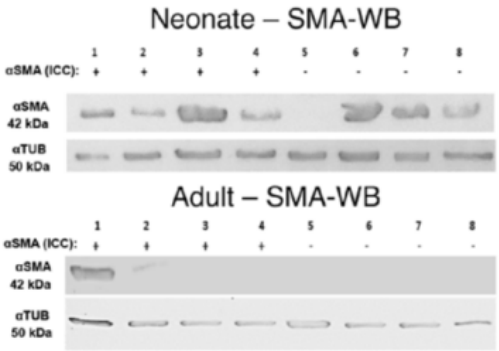

Figure 14. Expression of α-SMA. Western blot (WB) analysis of representative NF and AF.

The frequency of α-SMA -positive fibroblasts is considerably higher in NCFs and OCCFs than in adult fibroblast cultures. It is known that TGF-β1 stimulates the conversion of fibroblasts into α-SMA expressing myofibroblasts. Thus, we wanted to evaluate the TGF-β1 production and secretion to the medium in fibroblast cultures. We measured the concentration of TGF-β1 in DMEM medium conditioned for 24 hours in fibroblasts using ELISA. We did not observe differences in the concentration of TGF-β1 secreted by the different groups of fibroblasts analyzed.

Next we analyzed the production of cytokines relevant for wound healing, such as IL-8 and IL-6, in the different fibroblast groups. IL-6 and IL-8 are important cytokines that stimulate keratinocytes low differentiation state, migration and invasiveness. On the other hand, ELISA test of the conditioned media showed that the production of IL-6 was higher in NCFs compared to adult fibroblasts. The transcription of IL-6 was upregulated in NCF and especially in OCCF samples compared to adult fibroblast samples (Fig. 15 A and C). On the other hand, concentrations of IL-8 were similar in all fibroblast samples, and interestingly the transcription levels of IL-8 were higher in the samples from adult groups when compared to the transcription levels in NCF and OCCF samples (Fig.15 B and D).

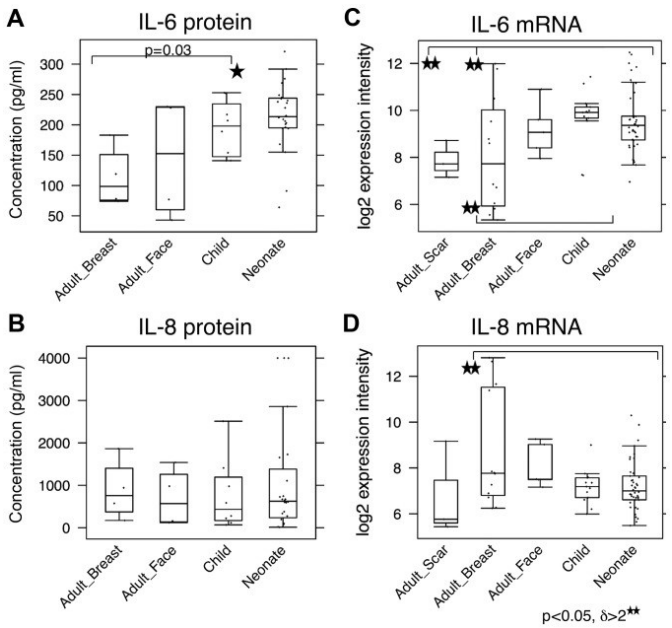

Figure 15. Production and expression levels of IL-6 and IL-8 in fibroblast cultures. ELISA analsis of the production of (A) IL-6 and (B) IL-8 to cultivation media compared with detection of (C) IL-6 and (D) IL-8 gene expression. Statistical differences between the different fibroblast types was marked by a line; *p<0.05 and **p<0.05 indicates a statistically significant difference and a minimum of a 2-fold change.

Considering the observed differences between the studied samples we wanted to further study the possible differences in the gene expression of the different groups (Fig. 16). The microarray genomic analysis showed hundreds of genes differentially expressed in NCF and OCCF with respect to the adult fibroblasts. These expression differences were in genes involved in cell signaling and metabolic pathways confirming that the phenotypical differences between newborn and adult fibroblasts reproduce differences at the gene expression level.

We were also interested in the differences between adult fibroblasts from breast and face. We observed some genes differentially expressed, among them some belonging to the homeobox family (important genes for the formation of several body structures during early embryonic development). This result reflects the different embryonic origin of these fibroblasts, since it is known that facial fibroblasts originate from the neural crest while fibroblasts from the rest of the body originate from the mesoderm (Noden and Trainor, 2005).

Differentially regulated pathways between NCF and OCCF samples compared to adult ones were identified. Among them the advanced glycation endproducts-receptor for advanced glycation endproducts (AGE-RAGE), TNF and TGF-β signaling pathways. We observed that changes in these pathways have an effect in the TGF-β, IL-6, MMP-2 and VEGFA expression.

The gene set analysis on the dysregulated transcripts between adult breast fibroblasts and NCFs and OCCFs resulted in differences of genes connected to organ morphogenesis and neural crest development (as expected by their distinct ontological origin), and strong differences in several genes involved in chemotactic pathways, among them TGF-β pathway.

We found substantial differences between NCF and adult fibroblasts from scar. The differentially expressed genes were virtually the same we found dysregulated between OCCF and adult fibroblasts from scar. The same genes we found differentially expressed between NCF and OCCF with the exception of the p53 signaling pathway and apoptosis.

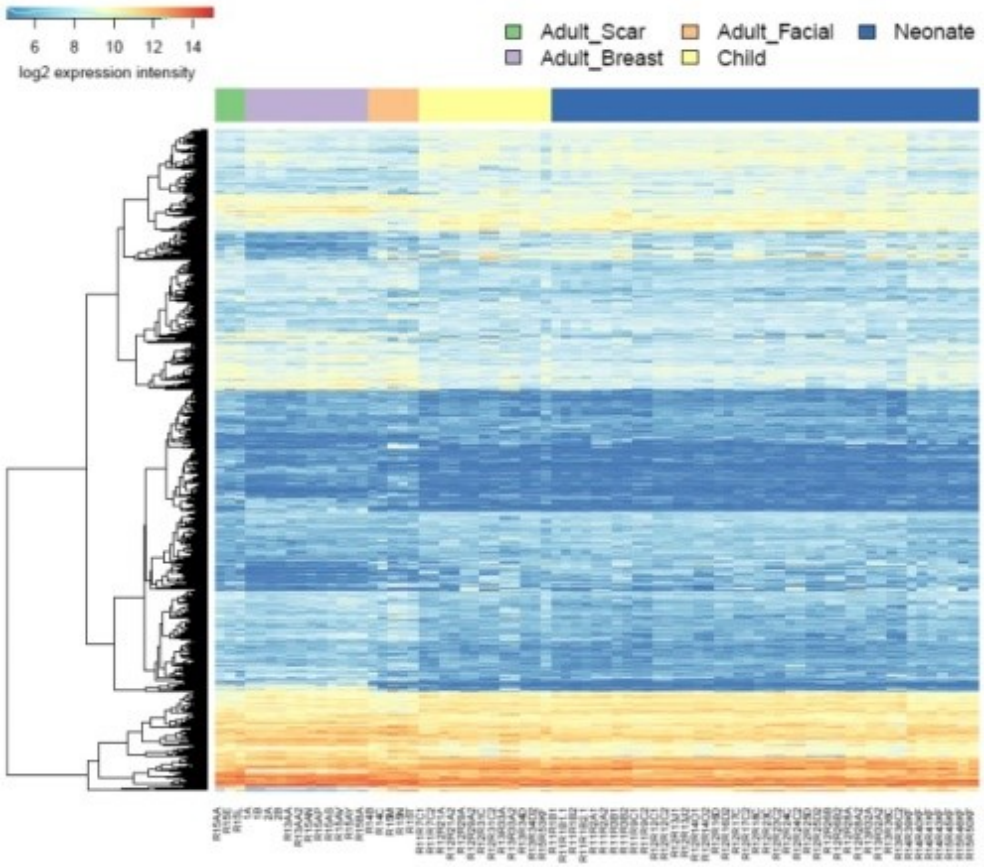

Figure 16. Heat map of the expression profiles of NCF, OCCF, adult fibroblasts from face, breast and scar. The differences between NCF and OCCF are minimal. But they differ from the adult fibroblasts in similar genes.

Only one protein-coding gene was differentially expressed between NCF and OCCF: the gene that encodes the Structural Maintenance of Chromosomes 6 protein.

Microarray genomic analysis exposed TGF-β signaling as one of the most dysregulated between adult and NCFs-OCCFs, which also correlated with higher presence of myofibroblasts in NCFs and OCCFs. Thus we decided to study TGF-β thoroughly. No significant differences between the expression levels of TGF-β1 in NCF, OCCF and adult fibroblasts were found. The expression of other members of the TGF family was also studied. We found upregulation of TGF-β3 on NCFs and OCCFs, and downregulation of transforming growth factor receptor (TGF-βR)2 when compared to their adult counterparts (Fig.18). Besides, the activity of the smad3 gene, a product of which is downstream of the TGF-β signaling cascade, was also upregulated on NCF and OCCF compared to the adult fibroblasts (Fig.16). Additionally, we observed changes in in the transcriptional activity of the genes that possibly regulate TGF-β signaling like CD2AP, CD34, HIF-1α, CD24, PTGS2 and MET.

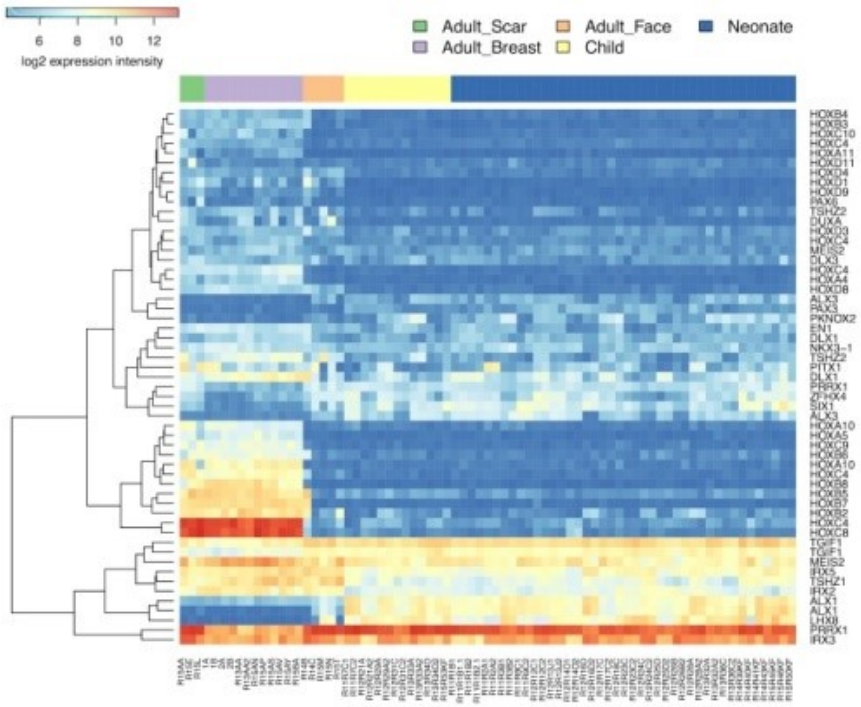

Figure 17. Heat map of the expression profiles of HOX genes NCF, OCCF, adult fibroblasts from face, breast and scar. We can observe two blocks or groups of expression pattern: fibroblasts originated from the neural crest and fibroblasts originated from mesoderm.

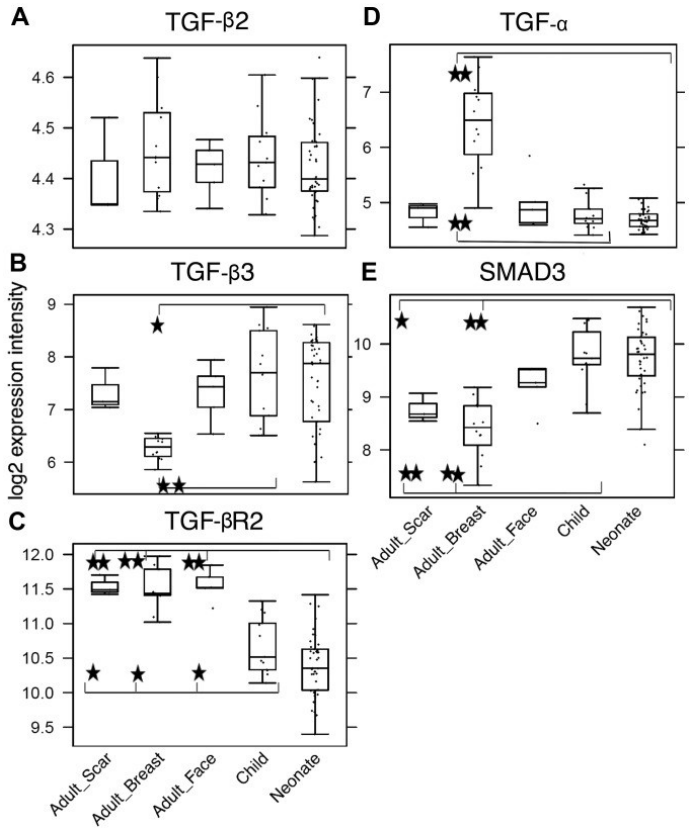

Figure 18. Detection of (A) TGF-β2, (b) TGF-β3, (C) TGF-βR2 receptor, (d) TGF-α and (E) smad3 gene expression in all of the tested types of fibroblast. Statistical differences between the different fibroblast types was marked by a line; *p<0.05 and **p<0.05 indicates a statistically significant difference and a minimum of a 2-fold (δ>2) change.

We further wanted to explore if these upregulation in the expression levels of TGF-β3 and downregulation in TGF-βR2 on NCFs and OCCFs when compared to their adult counterparts had implications in the cell growth. Addition of TGF-β3 to the culture media had no effect in any of the samples studied: newborn, child and adult fibroblasts. On the contrary OCCFs and adult fibroblasts were sensitive to TGF-βR inhibition while newborn fibroblasts were not (Fig.19).

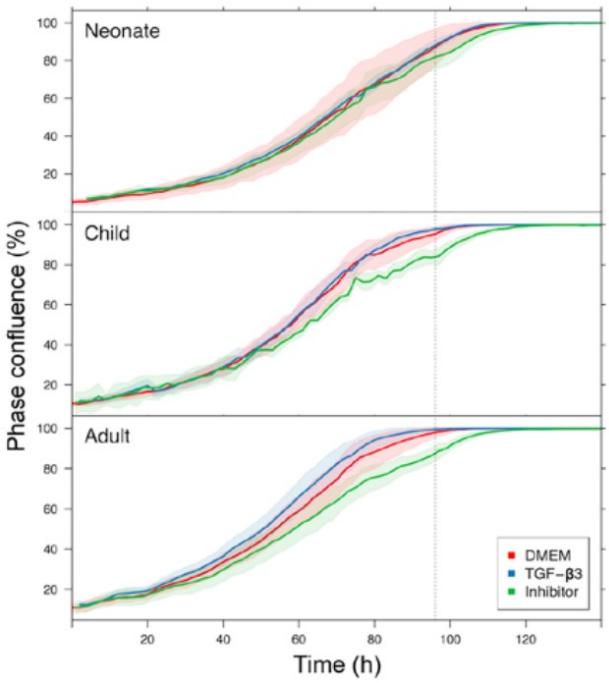

Figure 19. Growth curves of neonate, child, and adult fibroblasts cultured with DMEM, DMEM with TGF-β3 or DMEM with TGF-β inhibitor measured using IncuCyte. The graphs show the percentage of confluency during the time in hours. The shadows represent the standard deviation of the technical replicates.

Altogether these data provide information about the differences between NCFs and OCCFs respect adult fibroblasts. We found differences in the α-SMA positive fibroblasts (also referred as myofibroblasts) content, as well differences in the transcription activity of several genes, especially in members of the TGF-β pathway, which is involved in the conversion of fibroblasts to myofibroblasts and other roles in wound healing. This may partially explain why the outcome of wound healing in newborns is better that in adults. Moreover, we found similar differences between the gene expression pattern of fibroblasts originated from the mesoderm: adult fibroblasts from breast and scar as compared to the fibroblasts originated from the neural crest: newborn, older children and adult facial fibroblasts. These differences lay especially in hox genes, the genes that direct the regions of the body plan during the development of an embryo, demonstrating the differences between fibroblasts from different ontological origins.

Contribution: Derivation of fibroblast cultures from residual skin during cleft lip reconstructive surgery from newborns (1-16 days old) and older children (from 3 to 6 months old), breast, face and scar in adults. Immunocytochemistry staining for nestin, oct4, nanog and α-SMA. Preparation of conditions media and subsequent ELISA analysis of TGF-β, IL-6 and IL-8. Preparation of lysates for microarray analysis.

For further details, please see annex 2

Results III:

Simultaneous blocking of IL-6 and IL-8 is sufficient to fully inhibit CAF-induced human melanoma cell invasiveness

Njainday Pulo Jobe, Daniel Rösel, Barbora Dvořánková, Ondřej Kodet, Lukáš Lacina, Rosana Mateu, Karel Smetana, Jr., Jan Brábek

Histochem. Cell Biol. 146: 205-217 2016, IF:1,934, **Number of citations: 21** (auto citations are excluded)

Fibroblasts in the stroma of tumors have shown to promote tumor initiation and progression producing specific stimuli that influences malignant cells as well as changes the environment towards more pro-tumorigenic conditions. In this work, we examined the influence of fibroblasts on the migration of the melanoma cells. To mimic the migration conditions of tumor cells, we prepared collagen-embedded spheroids of melanoma cells lines (highly invasive BLM cells and the less invasive A2058) that resemble the tridimensional tumor environment *in vivo*. The spheroids were exposed to conditioned media from human adult fibroblasts (HFP3) and CAFs (Mel fib).

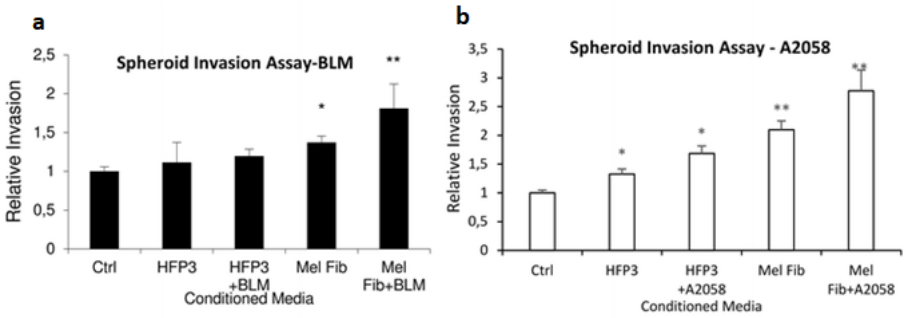

Figure 20. Graphs show relative invasion of BLM (a) and A2058 (b) cells. The spheroids were measured at time 0 and time 48 cultivated in DMEM or conditioned media from HFP3 or Mel Fib alone or previously cultivated with the respective melanoma cell line. Each graph is representative of one experiment. Ten spheroids were observed per condition, and the experiment was repeated at least three times. *$p < 0.05$; **$p < 0.01$ compared to the control.

The spheroids were formed with melanoma cells, then embedded in collagen matrix and afterwards DMEM or conditioned media was added. We measured the invasion of the spheroid forming cells towards the surrounding collagen matrix after 48 hours. The spheroids cultivated with conditioned media from CAFs were more invasive than those cultivated in conditioned media from healthy fibroblasts. Greater invasion was observed if the conditioned media was prepared with CAFs

previously ‚activated' by coculture with melanoma cells (Fig. 20). Similar results were obtained in 2D-wound healing closure assay too (See Fig. 2 a-b in the annex III). The increase in invasion was not related to an increased number of cells, because the conditioned media had no effect on the proliferation of melanoma cells as it was analyzed using the AlamarBlue® assay (See Fig. 1 e-f in the annex III).

We then analyzed the migration of melanoma cells in a 2D scratch assay in which cells were grown till confluency and then an empty linear space was created, and the wells observed at 0, 6 and 12 hours. The results showed that for BLM cells, conditioned media from CAFs and CAFs cocultured with BLM cells significantly increased the migration rate. However conditioned media from normal fibroblasts or normal fibroblasts cocultured with BLM had no effect on BLM migration compared to the control. A2058 showed a lower rate of basal migration compared to BLM, and all the conditioned media assayed produced an increase in migration rate (Fig. in the annex III).

IL-6 and IL-8 play an important role in the pathological behavior of the cancer cells in tumors. Specifically IL-6 promotes melanoma invasion *in vitro*, and CAFs stimulate cancer metastasis through IL-6. IL-8 also promotes melanoma cell migration and metastasis. Hereby, we speculated that the increase in invasion and migration in melanoma cells when cultivated with conditioned media could be due to the presence of IL-6 and IL-8 secreted by fibroblasts. In consequence, we wanted to explore the production of these interleukins in conditioned media from melanoma cells, fibroblasts and fibroblasts cocultivated with melanoma cells in a ratio 9:1 in a 2D culture. Using ELISA test we detected that the melanoma cells produces high amounts of IL-8 and, on the contrary, it was very low in fibroblasts. However, when fibroblasts were in coculture with melanoma cells the production of IL-8 increased, suggesting that the crosstalk with melanoma cells enhanced the production and secretion of IL-8 by fibroblasts (Fig. 21 a and b). The basal production of IL-6 by melanoma cells, especially A2058 cells, was much lower than the amount of IL-6 produced by fibroblasts in monocultures. Conditioned media from coculture of healthy fibroblasts were able to stimulate a higher production of IL-6 only when cocultured with the melanoma cell line BLM, while conditioned media from coculture of CAFs increased the production and release of IL-6 in both melanoma cell lines (Fig. 16 c and d). These results show that in 2D culture melanoma cells are the main producers of IL-8 and fibroblasts are the main producers of IL-6, whereas, coculture of melanoma cells with fibroblasts increases the secretion of IL-8 in fibroblasts.

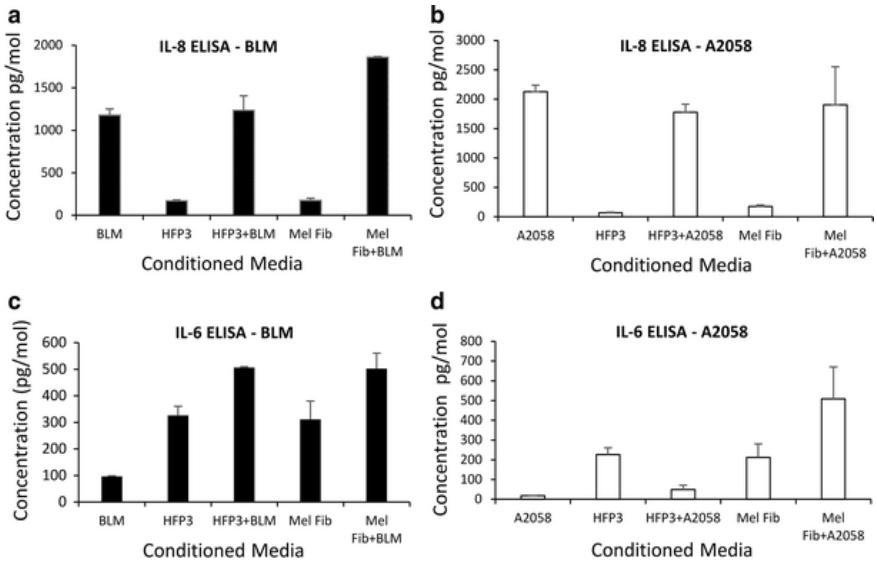

Figure 21. IL-6 and IL-8 concentration in the conditioned media. IL-8 (a and b) and IL-6 (c and d) concentrations in conditioned media of monocultures of melanoma cells, fibroblasts and coculture of fibroblasts with melanoma cells in a ratio 9:1 were measured using ELISA.

We next wanted to confirm that the increase in migration and invasion in melanoma cell lines was due to the presence of IL-6 and IL-8 in the conditioned media. Therefore, we used blocking antibodies in our collagen-embedded spheroid model. Invasion of melanoma cells with conditioned media supplemented with IL-6 blocking antibody was similar to the levels of invasion of the cells cultivated in control media (See Fig. 4 a-d in the annex III). Likewise, invasion of melanoma cells with conditioned media supplemented with IL-8 blocking antibody was reverted to the levels of invasion of the cells in control media (See Fig. 5 a-d in the annex III).

Additionally we tested the effect of the blocked IL-8 in IL-6 using neutralizing antibodies simultaneously. When both IL-8 and IL-6 antibodies were blocked, there was a decrease in the *in vitro* invasiveness to the level of control cells (without conditioned media), in both melanoma cell lines: A2058 and BLM cells (Fig. 22).

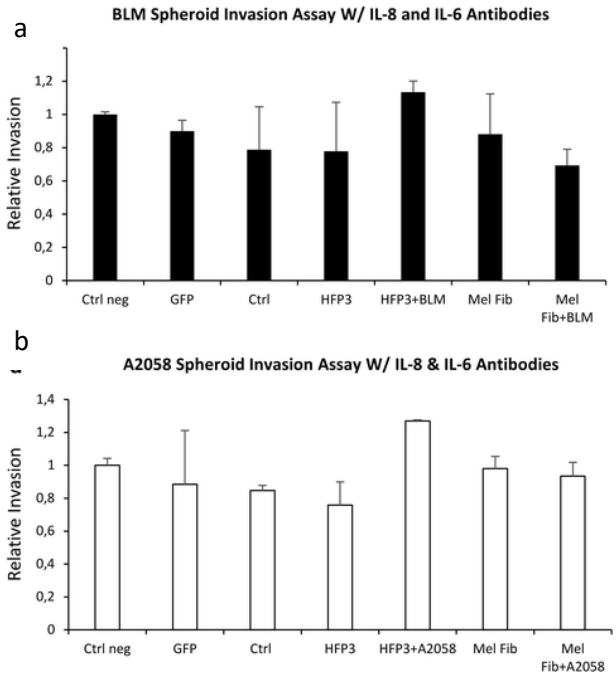

Figure 22. Blocking IL-6 and IL-8 in conditioned media fully inhibits CAF-induced invasion. The graphs show relative invasion in BLM (a) and A2058 (b) after 48 h. These results are representative of three independent experiments.

Our results showed that the medium conditioned by CAFs, particularly by CAFs and melanoma cells together, increased invasion of melanoma cells and the addition of IL-6 and IL-8 blocking antibodies to the medium was able to reverse this increase in invasiveness.

Nevertheless, the measured concentration of IL-6 and IL-8 in the system did not reflect accurately the changes in the induction of invasiveness. In the measurements the amount of IL-6 and IL-8 which were considered included only the levels of interleukins in the conditioned media in the moment it was added to the spheres, at the beginning of the experiment. However we did not take into consideration the levels of the cytokines present throughout the experiment. Thus, we measured the concentration of these two interleukins present in the media and collagen gel in which the sphere is embedded at the end of the experiment. The results show that BLM are big producers of both IL-6 and IL-8, and the addition of conditioned media did not significantly change the final concentration of these interleukins. Whereas, A2058 produced low concentrations of IL-8 and undetectable levels of IL-6. Addition of conditioned media to A2058 culture considerably increases the final concentration of both IL-6 and IL-8 (Fig. 23). These results disagree with the results obtained previously (Fig. 21), showing a disagreement between interleukin production in 2D and 3D cultures.

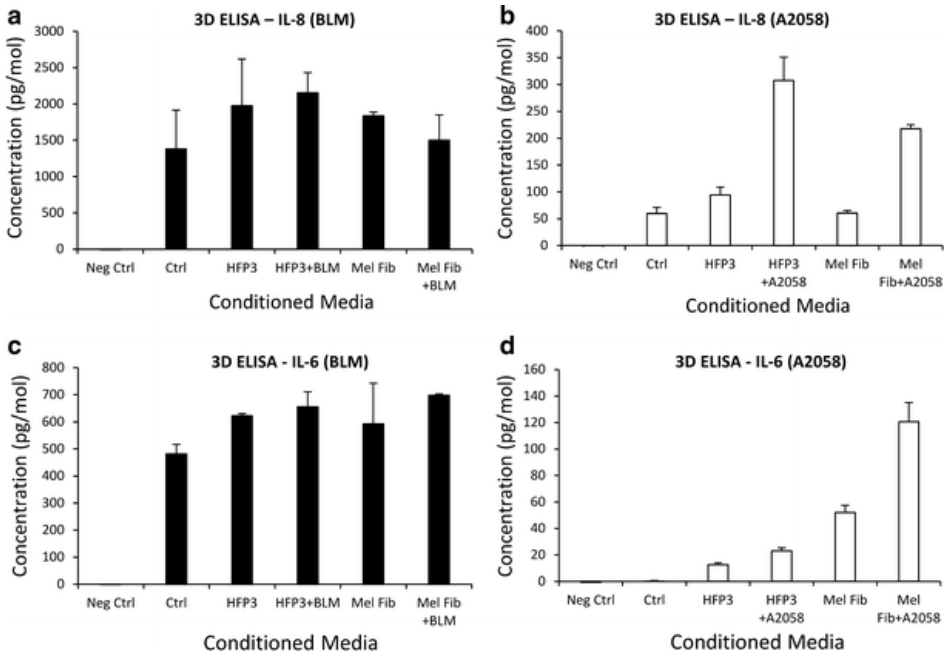

Figure 23. IL-6 and IL-8 concentration at the end of the experiment. Media and collagen from the spheroid culture were collected after 48 hours and concentration of IL-8 (a, b) and IL-8 (c, d) were analyzed using ELISA.

We wanted to evaluate which of the cultures, 2D or 3D, was closer to the *in vivo* conditions. Therefore, we analyzed the expression of IL-6 and IL-8 by immunohistochemistry in sections of human melanoma samples. As we could observe, the 3D model reflects better the expression of IL-8 and IL-6 in human melanoma specimens, where we could see expression of both interleukins associated with melanoma cells (See fig. 8 a-d in the annex III).

The results obtained in this work indicate that IL-6 and IL-8 are possible target to block migration induced by fibroblasts and CAF in melanoma cells. Targeting the supportive function of the tumor microenvironment in combination with current therapies targeting melanoma cells could represent novel and promising treatment of this disease.

Contribution: I contributed to the preparation of the different conditioned media HFP3, HFP3 and BLM or A2058, Mel Fib, Mel Fib and BLM or A2058.

For further details, please see annex 3

Result IV:

Cancer-associated fibroblasts are not formed from cancer cells by epithelial-to-mesenchymal transition in nu/nu mice

Barbora Dvořánková, Karel Smetana Jr., Blanka Říhová, Jan Kucera, Rosana Mateu, Pavol Szabo

Histochemistry and Cell Biology 143:463–469 2015, IF: 2,199, **Number of citations: 4** (auto citations are excluded)

CAF in the tumor stroma are a very heterogeneous population which origin is still under debate. The aim of this paper was to elucidate the origin of CAFs in the stroma of nu/nu mice xenografted with human cancer cell lines.

For this purpose, 8 week-old female nu/nu mice were subcutaneously implanted with three different human cancer cell lines. The first group was xenografted with FaDu, a human squamous cell carcinoma cell line, the second with Sw620 which is a human colorectal adenocarcinoma cell line and the last group with HT-29, a human colorectal adenocarcinoma cell line.

The resulting tumors exhibited a distinctive structure consisting of cancer cells and a well-formed stroma (Fig. 24). The sections showed a stroma rich in fibronectin and α-SMA positive CAFs. We could observe the formation of capillaries with cells expressing the endothelial specific marker CD31 surrounded by desmin positive cells, presumably SMC (See fig. 3 A-D annex IV).

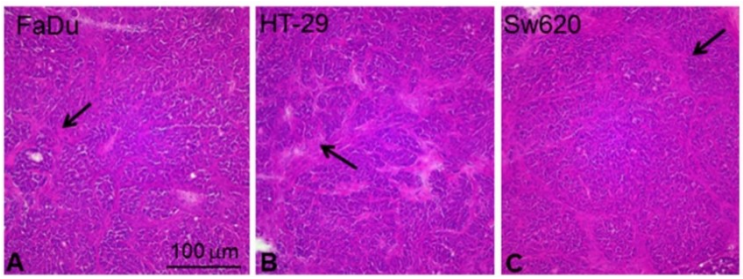

Figure 24. Subcutaneous tumors. Sections of the subcutaneous tumors formed by grafting FaDu cells (a), HT-29 (b) and Sw 620 (c) cells clearly exhibited well-formed stroma (arrows). Hematoxylin eosin staining, bar is 100 µm.

To corroborate the origin of the CAFs present in the stroma of the developed tumors, we used a specific antibody that discriminates between human and mouse vimentin. A Human specific antibody was used for detection of human vimentin and a monoclonal antibody was used for human and mouse vimentin detection (Fig. 25). The selectivity of both anti-vimentin antibodies was tested

employing mouse 3T3 and human dermal fibroblasts. The antibody specific for human was not able to react with vimentin in 3T3 mouse cell culture.

Sections from the harvested tumors were stained to detect human and mouse vimentin. The sections showed vimentin expressing cells present among the tumors and the in connective tissue surrounding the tumors. When the immunodetection was performed using anti human vimentin antibody we did not observe any positive cells in the sections. This indicates that the fibroblast-like cells in the stroma of the tumors were not from human origin.

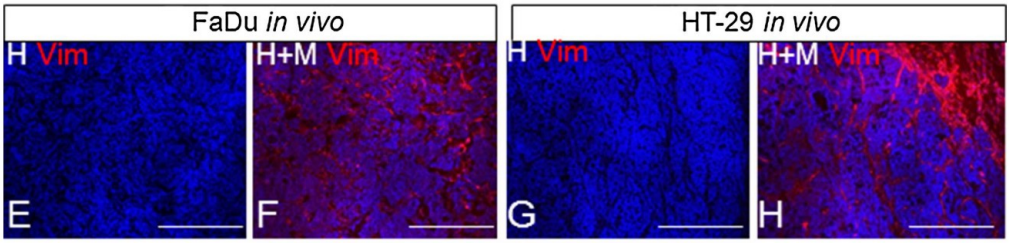

Figure 25. Human vimentin is absent in the stroma of mice tumors. Stromal fibroblasts in FaDu tumors (e) and HT-29 (g) exhibited no fibroblasts recognized by antibody against human vimentin (H Vim, red signal). Stromal fibroblasts in both these tumors (f, h) were well recognized by antibody against mouse and human vimentin (M + HVim, red signal). Nuclei were counterstained with DAPI, bar is 100 μm.

The cells sprouting from the tumor tissues treated with trypsin were tested in an immunocytochemistry assay. The cancer cells, appearing as small round cells, were stained with anti-keratin. We could not see fibroblasts exhibiting human vimentin in contrast to the presence of fibroblasts reacting to anti mouse and human vimentin (Fig. 26).

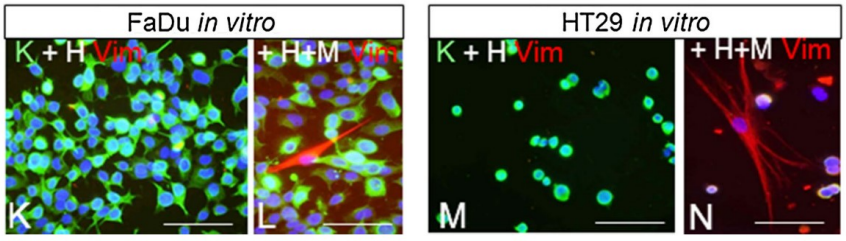

Figure 26. Immunocytochemistry detection of vimentin in fibroblasts obtained from our tumor samples. No fibroblasts exhibiting human vimentin were observed in the cells emigrated from FaDu (k) and HT-29 (m) tumors in contrast to detection of fibroblasts positive for mouse and human vimentin (l, n). Cancer cells were visualized with anti-keratin (k) antibody (K–N, green signal). Nuclei were counterstained with DAPI, bar is 100 μm.

This study shows that human cancer cells subcutaneously grafted in immunodeficient mice induce tumor stroma formation, containing typical CAF with a fibronectin rich matrix and containing smooth

muscle actin positive cells. More importantly, these cells were from the host origin because they were not recognized by an antibody specific for human vimentin in immunohistochemistry/sections, as well as in immunocytochemistry.

This suggests that the cancer cells from human origin are able to attract cells from the host origin and stimulate the formation of functional stroma through a cell crosstalk.

Contribution: I contributed to the derivation of cells from the harvested tumors. Immunocytochemistry and immunohistochemistry.

For further details, please see annex 4

Result V:

Targeting fibroblast activation protein in cancer – Prospects and caveats

Petr Busek, Rosana Mateu, Michal Zubal, Lenka Kotackova, Aleksi Sedo

Frontiers In Bioscience, Landmark 23, 1933-1968, IF: 2,25, **Number of citations:** 24 (autocitations excluded)

This review focuses on the protein FAP. FAP is a serine protease with post-proline dipeptidyl peptidase and endopeptidase enzymatic activity. It is a transmembrane protein, with a short cytoplasmic N-terminal and a long extracellular domain. It can be found in blood plasma as a soluble form in humans. FAP is a promising target for therapeutic research due to these two characteristics:

- Its singular enzymatic activity: It can cleave protein bonds adjacent to a proline.
- Its restricted distribution in adult tissues: it is almost absent in normal adult healthy tissues, however, it is present in various cell populations in the microenvironment of human malignancies, including malignant cells and CAFs.

In adult humans, we can find FAP protein expression in the alpha cells of Langerhans islets, multipotent bone marrow stromal cells, cervix, uterine stroma and placenta and occasionally in dermal fibroblasts surrounding hair follicles. However, the physiological role of FAP in these tissues has not yet been clarified.

FAP is a relevant molecule because it is expressed in activated fibroblasts during wound healing, where it seems to be involved in the migration of bone marrow mesenchymal stem cells. FAP protein expression can also be found in CAFs in the tumor stroma. Some experiments demonstrated that the ECM produced by FAP-positive CAFs was able to promote motility to a higher extent than the ECM produced by FAP negative CAFs, probably due to FAP collagenase enzymatic activity. FAP positive cells in the tumor stromal are associated with an increase in the expression of the proangiogenic factors VEGF or ANG-1 and a decrease in the expression of PEDF. Moreover, in a tumor model depleted of FAP expressing cells there was a decrease in vascular density. FAP+ stromal cells also play an immunoregulatory effect secreting immunosuppressive factors such as heme oxygenase 1, prostaglandin E synthase, CXCL12, and CCL2 as well as promoting a pro-inflammatory environment and infiltration of macrophages. According to these data, FAP and FAP-expressing cells present in the tumor microenvironment have a pro-tumorigenic role, promoting invasiveness, proliferation, tumor vascularization, immunosuppression and ECM remodeling through mechanisms involving FAP

enzymatic activity and other functions of FAP. However there are other examples, in which FAP positive cells showed tumor suppressor functions.

FAP is an enzyme expressed during situations of tissue remodeling. Thus, FAP is a common element in the two processes of interest in this work: wound healing and tumor development. The study of the role of FAP could be interesting to understand better the similarities and differences between these two processes and FAP could also represent an attractive target for both tumor therapies and wound healing improvement.

For further details, please see annex 5

DISCUSSION

Fibroblasts are one of the main cells involved in wound healing and tumor progression. Fibroblasts maintain skin homeostasis and become active during tissue repair. Activated fibroblasts establish an interaction with their microenvironment that results in wound closure and can influence the final outcome of wound healing. Likewise, fibroblasts become active in response to tissue damage caused by cancer cells and remain associated with tumors during all stages of the disease progression. Fibroblasts' interaction with the tumor microenvironment is similar to that occurring during the process of wound healing, leading to the idea that a tumor behaves as a wound that does not heal (Dvorak, 2016).

In the paper 'Functional differences between neonatal and adult fibroblasts and keratinocytes: Donor age affects epithelial-mesenchymal crosstalk *in vitro*' and 'Analysis of dermal fibroblasts isolated from neonatal and child cleft lip and adult skin: Developmental implications in reconstructive surgery' we intended to bring new data about the phenotypic characteristics of newborn and adult fibroblasts that could be relevant to explain the differences between neonatal and adult wound healing outcome. This knowledge may be of great use for the design new strategies to treat wound healing in the clinic.

Human fetuses are able to undergo regenerative healing during the first and second trimester of development, they can generate an exact copy of the damaged or lost tissue. This ability is diminished during the third trimester and lost in the postnatal period, in which the damaged tissue is repaired with the formation of fibrotic tissue or scar (Larson et al., 2010; Lo et al., 2012). However some recent studies showed that early newborns still retain partially this improved, almost scarless healing (Borsky et al., 2012; Valentova and Malina, 2018). This scarless outcome could be partly attributed to the properties of some of the cells involved in this process: keratinocytes and fibroblasts. In agreement with this, we observed that newborn and adult fibroblasts and keratinocytes have structural and functional differences that could partially explain the successful wound healing outcomes in young newborns as compared to adults.

We demonstrated that early newborn fibroblasts retain partially the low differentiated phenotype observed in embryonic dermal fibroblasts by differentiating human newborn fibroblasts into adipocytes and chondrocytes *in vitro*. Multipotency has been observed by others in human and mouse embryonic fibroblasts (Chen et al., 2007; Lee et al., 2016; Yusuf et al., 2013). They demonstrated that, contrary to adult fibroblasts, their embryonic counterparts are multipotent cells

than can differentiate in osteocytes, adipocytes and chondrocytes. Yusuf et al. proved that mouse fetal fibroblasts were similar to mesenchymal stem cells in their morphology and expression of surface markers like CD90, CD73, CD105 and vimentin (Yusuf et al., 2013). This multipotency seems to be lost in adult fibroblasts, since they failed differentiating into any of these two mesenchymal cell types in our experiments.

However, we did not succeed in the differentiation of newborn human fibroblasts into osteocytes, in opposition to what it was reported by other groups that used embryonic human fibroblasts (Chen et al., 2007). This leads us to the concept that newborn fibroblasts retain only partially the multipotency abilities of embryonic fibroblasts.

Based on previous data that shows that nestin is an intermediate filament expressed in the germ layers (Yusuf et al., 2013), we hypothesized that our sample from NF would express nestin. As expected, most of NFs were positive for nestin, while in fibroblasts from older children the number of cells expressing nestin was vastly reduced, and nestin expressing cells were almost absent in adult samples. These results are consistent with our previous work (Krejčí et al., 2015).

Several studies suggested that nestin could be a marker of multilineage progenitor cells (Sellheyer and Krahl, 2010) which agrees with our previous experiments showing the plasticity of NFs. This suggests that NFs possess characteristics of multi-potentiality in opposition to AFs, which lack nestin expression and cannot differentiate into any other mesenchymal subtype cells.

The cells that express nestin were also positive for the nuclear marker of pluripotency, oct4 and nanog. These markers have been associated with embryonic stem cells because their function as transcription regulators is key to maintain the self-renewal and pluripotency abilities of embryonic stem cells (Boiani and Schöler, 2005). This suggests that fibroblasts from dermis of young donor, specially very young newborns, have some traits of pluripotency and, in fact, this is being explored for its potential application in wound healing treatment (Esteban-Vives et al., 2019).

Interestingly oct4 and nanog are also present in malignant cells, and can regulate tumor cancer progression (Rasti et al., 2018; You et al., 2018). Malignant cells expressing these stem cell markers are present specially in the tumor invasive front suggesting that these cells are important for tumor infiltration and metastasis (Luo et al., 2013).

Fibroblasts produce ECM, which is a key component of the dermis and is a very active structure during wound healing. The composition of the ECM has been hypothesized as one of the factors contributing to a scarless healing. Human oral mucosa is a tissue that, similarly to fetal tissues, heals without scar. It has been observed that, prior to wounding, both fetal ECM and the ECM of the oral

mucosa exhibits differences in their composition as compared to the ECM of normal adult skin. Among these differences they have increased content of chondroitin sulfate, fibronectin and its splice variant ED-A (Leavitt et al., 2016).

Differences in the ECM production by NFs and AFs could be in part responsible for the different outcomes of wound healing in newborns and adults. However we did not observe any differences in the content of fibronectin between newborn and adult fibroblasts.

Nevertheless, we observed differences in the expression of α-SMA between newborn and adult fibroblasts, in agreement with previous findings in the literature (Hinz, 2016). NFs expressed frequently α-SMA, while AFs did not. α-SMA expression increases fibroblast contractile activity and it is a hallmark of mature myofibroblasts, which are important cells involved in the proper wound closure (Hinz, 2016), so the high incidence of α-SMA expressing fibroblasts or myofibroblasts in NF cultures could partially explain the good wound healing outcome in newborns. Other authors demonstrated that the lack of α-SMA causes a delay in healing, specifically a delay in wound contraction and immature organization of cutaneous wounds: α-SMA knockout mice showed lower cellularity, less collagen deposition and immature collagen organization in wounds (Ibrahim et al., 2015). Moreover different studies observed high levels of α-SMA in fetal fibroblasts, a period in the development known to heal wounds in an scarless manner (Sarrazy et al., 2011).

On one hand, higher incidence of myofibroblasts is associated with scarless wound healing, but on the other hand, an excess of myofibroblasts or its persistence has been associated with scars and fibrosis (Hinz et al., 2007). A possible explanation for this contradictory data lies probably in the timing: in healthy wounds, myofibroblasts are abundant during the wound healing but, disappear from granulation tissue after the wound is closed (Sarrazy et al., 2011). Myofibroblasts are also responsible of the wound closure by exerting tension in the collagen fibrils of the ECM. An experiment designed to study the mechanical tension caused by myofibroblasts in collagen fibers demonstrated that fetal fibroblasts were able to contract collagen gels more than AFs when the collagen gels were floating in a cell culture well. In the same experiment, AFs performed better than fetal fibroblasts when the collagen gels were anchored to the cell culture well and the tension was higher (Parekh and Hebda, 2018). These results suggest that AFs may need a specific tension in their microenvironment to become active, while NFs do not.

Another cellular process that may be relevant for wound healing is cell migration. Fibroblasts from surrounding tissues migrate to the wound at the end of the inflammation phase, followed by

keratinocytes. Keratinocytes migrate in a sheet across the damaged area and proliferate from the edges of the injured site until the wound is completely closed (Werner et al., 2007).

We also studied functional assays relevant to wound healing such as migration of keratinocytes and fibroblasts. NKs were able to completely cover a biopsy punch-made round defect in a confluent monolayer faster than AKs. Moreover, NKs closing the defect were all very small and expressed K8, while AKs covered the defect in organized layers in which small round cells appeared only at the front border. These observations agree with other studies that reported undifferentiated keratinocytes to be smaller and more motile than differentiated keratinocytes (Wong et al., 2019). It also agrees with our previous work, in which the keratinocytes from newborn origin showed expression of K8, K19 and vimentin, markers of low differentiation (Krejčí et al., 2015). Altogether our results suggest that NKs are more often undifferentiated than AKs.

The expression of K8 in NKs has been previously reported by other authors. This keratin is present in simple epithelial cells (Moll et al., 2008), and appears normally co-expressed together with K18. Both keratins are absent in differentiating keratinocytes (Moll et al., 2008). Moreover this pair of keratins are also expressed in in most carcinomas (Moll et al., 2008), cells that are also in a low differentiated state. Other works observed that K8 could play a role in epithelial cell migration, although this information is still uncertain, since other research pointed that epithelial cells not expressing K8 performed a faster wound closure *in vitro* (Magin et al., 2007).

We also compared the migration of adult and newborn fibroblasts. According to our observations, in an inoculum spreading test, AFs migrated to a greater extent in the same time period than NFs. It is known that fetal fibroblasts migrate faster than adult fibroblasts (Nguyen et al., 2009; Parekh and Hebda, 2018), however our observations suggested that postnatal fibroblasts do not retain this ability.

A study that compared cell migration in fibroblasts from fetal origin, 2 days old newborn, 8 month old child, and adult fibroblasts, found out that fetal fibroblasts migration abilities are driven by a different mechanism than adult fibroblasts migration. Fetal fibroblast migration is stimulated by the FGF and type I collagen produced by themselves while the migration in adult fibroblast was dependent on exogenous factors present in the FCS added in the cultivation medium, such as PDGFR. They determine that this transition occurred before birth (Kondo and Yonezawa, 1995). This could explain why we do not observe faster migration in our newborn isolated fibroblasts.

Moreover, a study performed with fibroblasts from young (22 to 30 years) and aged (older than 81) donors determined that human skin fibroblast migration capabilities decline during ageing (Reed et al., 2001). Possibly contributing to impaired wound healing in elders.

One of the limitations in the process of wound healing is the poor recruitment of fibroblasts and keratinocytes from neighboring healthy regions. Migration of fibroblasts and keratinocytes during wound healing is induced by microenvironmental stimuli such as hypoxia and oxidative stress, and mainly by cytokines and growth factors. After injury, keratinocytes secrete chemokines to the microenvironment that recruit fibroblasts to the wound site and activate them (Menon et al., 2012; Qiang et al., 2020). The keratinocytes are stimulated to grow and migrate in response to growth factors and cytokines produced by fibroblasts (Childs and Murthy, 2017). Thus, understanding the reciprocal interaction between these two populations and the molecules involved in this communication is becoming increasingly important in order to find out the functioning of wound healing and try to improve its outcome.

Previous studies have shown that fetal keratinocytes are able to increase fibroblasts migration. In a recent study it has been observed that upon coculture with fetal keratinocytes, fibroblasts upregulated MMP-2 and MMP-9 in fibroblasts (Wang et al., 2015). The enzymatic activity of MMPs degrading the ECM may be the cause of enhanced fibroblast migration.

We studied whether adult and newborn keratinocytes could influence fibroblasts' migration in order to understand the differences between wound healing process in newborns and adults. In our work, we observed that NFs increased their migratory potential in coculture with keratinocytes, regardless of the donor age of the keratinocytes and were able to close a wound healing assay faster than their adult counterparts. On the other hand, AFs did not increase their migration upon cocultivation with either NKs or AKs.

Our findings evidence that, besides fetal keratinocytes, also adult and newborn keratinocytes are able to increase fibroblast migratory potential in NFs, but not in AFs regardless of the age of the keratinocytes employed in the coculture experiment. The experiments performed by Wang et al. suggest that the molecular basis behind this enhancement in migration might be related to a keratinocyte mediated increase in the expression of enzymes that degrade ECM in fibroblasts, however this has to be further studied (Wang et al., 2015). AFs showed again a more differentiated phenotype compared to NFs, as they do not change their migration rate in coculture or in monoculture.

The interaction between keratinocytes and fibroblasts is reciprocal. It is known that in the context of wound healing, fibroblasts regulate keratinocytes‘ migration, proliferation and even their final differentiation once the reepithelization is completed. Fibroblasts release growth factors to enhance keratinocytes division as well as migration from the edges of the injury, and facilitates its migration by building an ECM in which cells can migrate (Childs and Murthy, 2017; Schumacher et al., 2014; Wang et al., 2012).

In our experiments, we observed that coculture of keratinocytes with fibroblasts extensively influenced keratinocyte‘s behavior. NFs, but not AFs stimulated the appearance of small, round keratinocytes in the borders of the colonies of AKs. These small keratinocytes lacked intercellular contacts and expressed keratins 8, 19 and 14. These small keratinocytes have been observed also in neonatal human epidermis (Krejčí et al., 2015) and fetal porcine epidermis (Klíma et al., 2007). K8 and K19 are keratins typical for simple epithelia, and are absent in differentiating keratinocytes (Moll et al., 2008). Overexpression of K8 has been associated with loss of cell-cell adhesion in epidermal keratinocytes (Zhang et al., 2012) and increased migration rate (Bordeleau et al., 2010). Interestingly K8 together with K18 are frequently present in malignant tumors, associated with invasiveness and poor prognosis and are often used as diagnostic tool in immunohistochemical stainings (Moll et al., 2008). These results suggest that NFs, but not AFs are able to create an optimal environment to support and induce a low differentiated status in keratinocytes. Remarkably, CAFs are able to induce the appearance of these small round keratinocytes expressing low differentiation keratins (Cirillo et al., 2017; Lukas Lacina et al., 2007), showing similarities between wound healing and tumor progression.

We observed functional and phenotypical differences between newborn and adult fibroblasts such as the ability to differentiate into adipocytes and chondrocytes, expression of nestin and nuclear pluripotency markers oct4 and nanog, α-SMA presence and migration rate. These differences were further confirmed by their gene expression pattern.

We analyzed the gene expression of adult and newborn fibroblasts by microarray direct hybridization and we observed genes differentially expressed between these two groups. Some of the upregulated genes in NFs were involved in cell division, proliferation, chemotaxis and inflammation such as IL-6, IL1B, CXCL1, CXCL6, CXCL14, CXCL16, TGFB2, VEGFA and VEGFB. These soluble signaling molecules are involved in the acute phase of wound healing (Behm et al., 2012). The observed higher level of IL-6 gene expression in newborn and older children’s fibroblasts was consistent with IL-6 protein level, as it was confirmed by ELISA.

One of the hallmarks of fetal and oral mucosa scarless wound healing is the low concentration of IL-6, together with an also low concentration of IL-8 and high concentration of the anti-inflammatory cytokine IL-10 (Zgheib et al., 2014). Moreover, experimental exogenous application of IL-6 has been proved to worsen wound healing and increase scar formation. On the contrary, adult healing, associated with scar formation, is characterized by excessive inflammation (Hedayatyanfard et al., 2020).

Young neonates heal in an almost scarless fashion, as it was observed in neonatal cleft lip reconstructive surgery performed in very young newborns (Borsky et al., 2012). However in our samples, we observed that IL-6 was more extensively expressed, produced and secreted to the medium in NFs than in their adult counterparts. This data seems to be in contradiction to the general belief that low production of IL-6 is associated with scarless wound healing (Bermudez et al., 2011; Hedayatyanfard et al., 2020; Kathju et al., 2012). A possible explanation for the higher levels of IL-6 in NFs when compared to AFs could reflect the status and the origin of the sample, since NFs were obtained from a sample of cleft lift during repair surgery.

IL-6 is crucial during the first stages of healing and its total absence can cause incorrect wound healing process and delay in wound closure. Moreover, topically applied IL-6 improved the barrier repair in a concentration-dependent manner (Wang et al., 2004). IL-6 induces the release of proinflammatory cytokines and chemotaxis of leukocytes, later, as the healing process progresses, IL-6 promotes a switch to a reparative environment (Johnson et al., 2020). Besides, despite being traditionally regarded as a pro-inflammatory cytokine, IL-6 has also anti-inflammatory activity such as regulation of the immune system, regenerative processes, metabolism, bone homeostasis, cardiovascular protection and neural function (Schett, 2018). However, high concentrations of IL-6 can result in scar formation, and even fibrosis in some cases (Johnson et al., 2020). Thus, we can conclude that IL-6 is necessary for a healthy wound healing and it needs to be tightly regulated, since excess or lack of it would turn out in pathological healing.

The expression of IL-8 at the transcriptomic level appeared lower in newborn and older children fibroblasts when compared with adult breast fibroblasts. However we did not find any striking differences in the concentration of IL-8 released in the medium between NFs and AFs. IL-8 is present in human skin wounds in higher concentration than in healthy skin (Takada et al., 2017). It is a strong chemoattractant for neutrophils, and during the reepithelization phase it has a role promoting migration and proliferation of keratinocytes (Barrientos et al., 2008; Takada et al., 2017). During fetal development, cells express low levels of IL-8, probably this contributes to the scarless wounds and the diminished inflammation response during prenatal wound healing (Barrientos et al., 2008).

The gene expression profile of the fibroblasts showed differences between some of the age groups studied. We found abundant differences in the gene expression of newborn and older children fibroblasts as compared to adult breast fibroblasts. The differences were mostly in the expression of genes involved in signaling pathways. However, the differences between newborn and older children fibroblasts as compared to adult facial fibroblasts were focalized mostly in the expression of genes involved in metabolic pathways. Probably, this can be explained by the common ontological origin of newborn, older children and adult facial fibroblasts. While newborn, children and adult breast fibroblasts differ not only on the age of the donor but also in their origin.

In fact, when we compared all the fibroblasts from facial origin (including NFs, OCCFs and also facial adult fibroblasts), these differed from fibroblasts from the breast in 364 genes including some of the homeobox gene family, genes whose products direct the structure of the body during early embryonic development (Robertis et al., 1990). This illustrates once more their different developmental origin.

The most dysregulated genes between fibroblasts from newborn and older children versus adult fibroblasts were in the AGE-RAGE, TNF and TGF-β signaling. Binding of advanced glycation end products (AGE) and its most frequent receptor, RAGE, leads to downstream activation of several pathways including TGF-β and inflammatory pathways (Li et al., 2004).

AGE accumulates in the tissues and organs as humans age, and are partially responsible of the typical features of aged skin, such as wrinkles, loss in elasticity and water content. AGE has a role in most of the age-related diseases including cancer. Interestingly, the accumulation of these macromolecules could also negatively influence the wound healing process, causing the appearance of aberrant collagen fibers that delay the process of wound closure. Moreover, data suggest that as AGEs accumulates, scars appear more rigid and contractile with unremitting redness. Activation of the AGE/RAGE pathway has deleterious effects for the cells (Van Putte et al., 2016).

As we expected, the gene expression patterns of newborn and older children's fibroblasts were very similar, and only two genes were differentially expressed, exhibiting the similarities between these two populations.

Overall, the gene expression analysis showed us that TGF-β pathway was the most dysregulated pathway between adult fibroblasts (regardless of the origin) and newborn and older children's fibroblasts. In a deeper look in the TGF-β pathway we observed that the levels of TGF-β1 and TGF-β2 were similar in adult and newborn fibroblasts. However the levels of TGF-β3 were upregulated in our newborn and child samples, this is possibly affecting the better wound outcome in these groups. This

observation is consistent with other works stating that TGF-β3 has anti-fibrotic effects in different tissues (Gilbert et al., 2016; Lichtman et al., 2016). Higher protein expression of TGF-β3 together with lower protein expression of TGF-β1 and TGF-β2 are a hallmark of fetal tissues, both healthy and in wounded fetal skin in mouse, rabbit and human models (Gilbert et al., 2016; Lichtman et al., 2016; Walraven et al., 2014). High expression of this TGF-β3 during fetal wound healing causes a reduction in fibronectin and collagen I and III deposition and, it stimulates keratinocyte migration, macrophage recruitment and attenuates cell proliferation, promoting scarless healing (Mahmoudi Rad et al., 2015; Moore et al., 2018). According to the existing literature, selective expression of TGF-β3, faster migration, proliferation and collagen synthesis and specific proteolytic enzyme expression in fetal fibroblasts are key factors for a regenerative wound healing (Nguyen et al., 2009). In fact, TGF-β3 has been used as an antiscarring therapeutic, to accelerate the wound closure and to potentiate a better scarring (Ferguson et al., 2009; So et al., 2011).

Additionally to the proportion between the TGF-β isotypes, the duration of expression of these three members of the TGF-β family is also clue to determine the outcome of a wound healing. In general, long-lasting TGF-β signaling is one of the aspects causing scars and fibrosis, and therefore the brief response, seen in fetal skin, might contribute to the scarless healing. Some studies in rat model showed that TGF-β1 expression in adult skin wounds remain higher during longer time than in fetal wounds before going back to baseline levels (Walraven et al., 2014). This could be due to the fact that TGF-β1 promoter activation in adults has a positive autoregulatory feedback activation while fetal fibroblasts do not have an autocrine loop in response to TGF-β1 (Walraven et al., 2014).

Furthermore, we observed that the levels of TGF-βR2 were significantly lower in newborn and older children's fibroblasts when compared to adult fibroblasts. The levels of TGF-βR2 are known to be downregulated in during fetal development in humans and also in tumors (Shah et al., 2018). This might represent a relevant molecular pathway involved in tumor progression as well as early embryonic development. On the other hand, total absence of TGF-βR2 has deleterious consequences in the development of the neural crest derivatives, including palate cleft and other skull defects (Ito et al., 2003).

Our experiments also revealed that SMAD3, a protein downstream in the TGFβ signaling pathway, appeared upregulated in NCFs and oCCFs when compared to adult fibroblasts.

Differences in the gene expression of SMAD, TGF-β3 and TGF-βR2 makes TGF-β pathway the most prominent in the differential expression profile between newborn and adult fibroblasts, which interestingly, is also one of the major signaling pathway involved in wound healing.

The next approach was to check whether the exogenous application of TGF-β3 was affecting fibroblasts growth. We observed that TGF-β3 does not affect the growth of newborn, older children and adult fibroblasts. OCCFs and AFs' growth was diminished when an inhibitor of TGF-βR2 was applied in the medium. On the contrary, the inhibitor did not affect newborn fibroblasts' growth.

Probably this is due to the lack of proliferative effect of TGF-β on newborn fibroblasts. This might be a characteristic shared with fetal fibroblasts, as it is known that contrary to adult fibroblasts, fetal fibroblasts seem to not respond to this mitogenic effect of TGF-β, in some cases, TGF-β even decreases proliferation. Induction of proliferation in fetal fibroblasts occurs via PKA-dependent mechanism, while adult cells regulate proliferation via FGF-2 and the MEK–ERK pathway (Walraven et al., 2014). Since newborn fibroblast proliferation might be triggered by other signals than TGF-β, thus we do not see any diminished proliferation when an inhibitor of TGF-βR2 is applied.

This information could be useful to design treatments to avoid excessive scaring and fibrosis. However, total arrest of the expression of TGF-β isotypes could impair healing, since these growth factors seem to be necessary during the first stage of wound healing. Treatment with antibodies blocking TGFβ-1, -2 and -3 during the first stage of healing caused a delay in the wound closure without reducing the resulting scar. However these same treatment applied later in the healing process caused a reduction in the scar formation (Lu et al., 2005). This suggests that TGFβ-1, -2 and -3 are key chemotactic and proliferative stimuli necessary for the progress of wound healing. This has to be taken into account for the design of strategies to decrease hypertrophic scars if we want to avoid a delayed wound closure.

Although fibroblasts and keratinocytes are clue in wound healing, we should take into account that the process of repair is much more complex than the model we studied, and there are other players that could contribute to the differences between newborn and adult wound healing outcome, such as infiltrated immune cells, endothelial cells, etc.

Scientists have tried to explain the reason why adults cannot repair wounds in a regenerative or scarless way from an evolutionary point of view. The integrity of the skin is important for the survival of the organism. Intact skin prevents infections, fluid loss and many other vital functions. Scientists have hypothesized that, in a non-sterile environment, it is more advantageous to give priority to a quick closure of the wound. This evolved in a rapid deposition of ECM and a strong pro-inflammatory response to prevent infections (Kathju et al., 2012). This implies the formation of a scar as opposed to a perfect regeneration of the skin (Penn et al., 2012). The goal of modern medicine is to understand regenerative repair. The study of the characteristics of newborn fibroblasts and keratinocytes can contribute to broaden the knowledge on scarless wound healing and can be useful

for the development of novel treatments to improve scarring, hypertrophic scars, keloids and fibrosis.

In our other work 'Simultaneous blocking of IL-6 and IL-8 is sufficient to fully inhibit CAF-induced human melanoma cell invasiveness' we address the role of CAFs in melanoma progression. The interaction between malignant cells and its microenvironment is crucial for tumor progression. CAFs are the major component of the tumor microenvironment and have demonstrated to play a critical role in the regulation of processes such as tumor migration and invasion, the hallmarks of metastasis (Zhou et al., 2015).

We hypothesized that the invasiveness of melanoma cells could partly depend on the interaction with CAFs present in the tumor microenvironment. Thus we studied the effect of conditioned media from fibroblasts and CAFs in invasion of melanoma cell lines in a 3D collagen model.

We observed that the conditioned media from CAFs increased invasion of the melanoma cell line BLM. The conditioned media from CAFs cocultured with BLM further increased BLM invasion. Other authors also observed that fibroblast-derived increase in melanoma invasion was enhanced if the fibroblasts were pre-stimulated by previous cultivation with melanoma cells (Pessotti et al., 2020). This suggests that the growth factors and cytokines produced by melanoma cells modulate the fibroblasts in the microenvironment to favor melanoma invasion. Li et al. demonstrated that conditioned media from melanoma cell lines was able to modify the expression profile of fibroblasts (Li et al., 2009). In their experiments they used conditioned media from 5 different melanoma cell lines and concluded that the changes in the fibroblasts' expression were dependent on the characteristics of the melanoma cell lines (Li et al., 2009). Interestingly, Pessoti et al. observed that the proteome of melanoma cells stimulated with conditioned media from fibroblasts showed 28 exclusive proteins not present in the proteome of melanoma cultivated without fibroblast conditioned media. Melanoma cells stimulated with conditioned media from preactivated fibroblasts (meaning fibroblasts cultured with conditioned media from melanoma cells) showed 51 exclusive proteins (Pessotti et al., 2020).

However, we did not detect changes in BLM invasion after culture with conditioned media from human healthy fibroblasts. A recent work shows similar results in oral squamous cell carcinoma, in which conditioned media from dermal fibroblasts fail to strongly stimulate cancer cell invasion while conditioned media from CAFs increase cancer cell invasion more than twice (Zhang and Hwang, 2019). Our results would fit in the belief that normal fibroblasts are inhibitors of melanoma progression in the first stages of the disease, by preventing EMT, thus avoiding invasion and metastasis and inducing G1/S cell cycle arrest in melanoma cells. As tumor progresses, and

melanoma cells secret signaling molecules, fibroblasts become activated, acquire tumor promoting properties and physiological characteristics of myofibroblasts (Shelton et al., 2020). In our model, fibroblasts cultured with melanoma conditioned media could be considered as activated fibroblasts, thus making them more pro-tumorigenic, for instance, enhancing their ability to promote melanoma invasion.

We performed the same assay with a less invasive melanoma cell line, A2058. This resulted in increased invasion under conditioned media from CAFs and melanoma-stimulated CAFs as well human fibroblasts and melanoma-stimulated fibroblasts. Conditioned media from human fibroblasts was thus ineffective in BLM but it increased the invasion of A2058. This apparent contradiction can be explained by the nature of the melanoma cell line, since BLM is highly invasive, while A2058 is not. Other authors reached the same conclusions while working with low invasive melanoma cell lines such as WM793 (Yin et al., 2012). This might be due to the fact that highly invasive melanoma cell lines often overexpress cathepsin B and MM-1 and -9 (Klose et al., 2006; Yin et al., 2012) and this may enhance their invasion without need of any paracrine cue.

The fibroblast or CAF-mediated increase in melanoma invasion was not due to a raise in proliferation. We did not observed significant changes in the proliferation rate of melanoma cell lines cultured with conditioned media. Our observations differ from other contemporary published works. Izar et al. showed that melanoma cells increase their proliferation when cultured with conditioned media from human fibroblasts or CAFs to a comparable extent (Izar et al., 2016). Other authors explained this controversy and claimed that at initial stages of melanoma progression, conditioned media from fibroblasts or CAFs can influence melanoma proliferation, while late primary and metastatic melanoma do not respond to paracrine growth signals because they have a constitutive activation of MAP kinase (Bai et al., 2017; Kim et al., 2017; Li et al., 2003). This could be the reason why we do not observe a proliferation effect in our melanoma cell lines cultivated with fibroblast‘s conditioned media.

It is known in the literature that melanoma cells stimulate CAFs to secrete cytokines and growth factors such as VEGF, CXCL12, HGF, MMP-2, monocyte chemotactic protein (MCP)-1, IL-6 and IL-8 to promote melanoma invasion. Moreover, fibroblasts cultured with conditioned media from highly invasive cell lines: BLM and MV3, resulted in a higher production of IL-8, IL-1B and CCL2, when compared to the effect of conditioned media from less invasive melanoma cell line WM164 (Li et al., 2009). Among those factors, IL-6 and IL-8 are some of the most relevant (Grimm et al., 2015; Li et al., 2009; Zhou et al., 2015). IL-6 has been identified as key player in tumor invasiveness and metastasis through IL-6/Stat3 signaling pathway (Kumari et al., 2016; Sun et al., 2014). And IL-8 has been

associated to increase in invasion in melanoma cell line in several experiments (Wu et al., 2012). Moreover, melanoma patients frequently showed increased levels of IL-6 and IL-8 and it correlated with worse prognosis (Hoejberg et al., 2012; Kucera et al., 2015).

In our experience, the production of IL-6 in monoculture was veryy low in melanoma cell lines, BLM and A2058, as compared to the IL-6 production in fibroblasts and CAFs. It is well-know that CAFs are an important source of IL-6 in the tumor microenvironment (Shiga et al., 2015), and even healthy fibroblasts express this interleukin according to other authors that used dermal fibroblasts as controls in their experiments (Antonelli et al., 2012; Zalewska et al., 2006). Coculturing fibroblasts or CAFs with melanoma cell lines caused an increase in the IL-6 production.

With regard to IL-8, we observed that both melanoma cell lines used in our experiments secreted high amounts of this interleukin when cultivated in monolayer. However, fibroblasts and CAFs secrete insignificant amount of IL-8. This data is in agreement with the literature (Wu et al., 2012). However, when fibroblasts and CAFs were cocultutivated with melanoma cells, the levels of IL-8 secreted by fibroblasts increased drastically, suggesting that melanoma cell lines are able to modify the expression pattern of fibroblasts and CAFs.

To determine the role of IL-8 and IL-6 in melanoma invasion, we blocked these interleukins using neutralizing antibodies and we observed that the conditioned media mediated increase in melanoma invasion is suppressed with IL-6 and IL-8 neutralizing antibodies. This pointed out that IL-6 and IL-8 play an important role in melanoma invasion.

The levels of IL-6 and IL-8 previously measured in our CM by ELISA reflected the levels of this cytokines added to the system initially, but does not consider the production of cytokines by the spheres of melanoma cells in our 3D system. Thus, we further studied the secretion of IL-6 and -8 in our 3D model, we observed that the levels of both IL-6 and -8 in BLM were high in our 3D model. Therefore, the addition of conditioned media did not affect the final concentration of these interleukins. Hoejberg et al. also demonstrated that metastatic melanoma cells have high expressions of both IL-6 and IL-8 (Hoejberg et al., 2012). On the contrary, the melanoma cell line A2058 expressed low levels of IL-8 and IL-6 level was undetectable, therefore the addition of conditioned media significantly increases the concentration of both interleukins.

The analysis of IL-8 and IL-6 expression in human melanoma samples showed that melanoma cells produced both IL-6 and IL-8.This proves that our 3D model better reflects the conditions *in vivo* than a culture in monolayer. *In vitro* models will always have some flaws and will not reflect entirely an *in*

vivo system, however they may substitute animal models in some particular experiments or purposes.

BLM and A2058 have different metastatic potential and, as we observed, they differed in the expression of IL-6 and IL-8. Other comparative studies showed abundant differentially expressed genes between high and low metastatic melanoma cell lines. Li et al. found approximately 700 genes downregulated genes in high metastatic melanoma cell lines (BLM and MV3) as compared to low metastatic melanoma cell line (WM164). And around other 700 genes were upregulated, some of which were involved in the ECM and cell communication (Li et al., 2009).

Fibroblasts can induce changes in melanoma cell lines to stimulate melanoma cells to modify their invasive potential and secretory phenotype (Pessotti et al., 2020). Likewise, melanoma cell lines can secrete signaling molecules to induce proteome changes in fibroblasts and CAFs, which ultimately creates a protumorigenic microenvironment necessary for tumor progression (Pessotti et al., 2020). This suggests that tumor cells actively create a microenvironment to allow their progression and development.

This data demonstrates the importance of the CAFs in the tumor microenvironment, but the origin of these cells is still under debate. In the paper 'Cancer-associated fibroblasts are not formed from cancer cells by epithelial-to-mesenchymal transition in nu/nu mice' we address this question.

Some studies have claimed that quiescent fibroblasts located in the surrounding host tissues become active in response of the injury generated by a neoplasm (Kalluri, 2016; Osman et al., 2020). Other hypothesis suggest that CAFs could originate from adipocytes, pericytes, endothelial, bone marrow-derived cells, cancer stem cells and even epithelial cells (Chen and Song, 2019; Kurashige et al., 2018; LeBleu and Kalluri, 2018; Osman et al., 2020).

Previous works showed that breast cancer can generate CAFs through EMT (Petersen et al., 2003). Kopantzev et al. observed that a percentage of fibroblast cells present in the stroma of some tumor and healthy tissues expressed epithelial markers (Kopantzev et al., 2010) suggesting these cells evolved from epithelial cells. Other works claim an EMT occurring during metastasis at the invasive front (López-Nouoa and Nieto, 2009), however other authors could not corroborate this (Fischer et al., 2015).

Our work showed that the human cancer cells xenografted in mice were able to form subcutaneous tumor with well defined stroma in immunodeficient mice. The analysis of these tumors demonstrated that the fibroblasts present in the tumor stroma in our model were from host origin revealing that these fibroblasts were not originated from cancer cells through EMT.

According to the literature, inflammation could have a role initiating the EMT. Rhim et al. proved that in a mice model of pancreatic tumor formation, the creation of pancreatitis enhanced the EMT and the number of circulating pancreatic cells, whereas the use of dexamethasone, an anti-inflammatory drug, reduced considerably the number of circulating pancreatic cells (Rhim et al., 2012).

In our experiments, we used an immunosuppressed mice model, thus the lack of inflammation could be one of the reasons we did not observe EMT. On the other hand, local fibroblasts can be activated or recruited without inflammation by other signals present in the microenvironment such as hypoxia, oxidative stress, and the growth factors released from the nearby tumor cells such as TGF-β, EGF, PDGF and FGF-2 (Tongyan Liu et al., 2019).

In a tumor *in vivo*, most probably the population of CAFs is a mixed population arising from diverse origins, which could explain its heterogeneity and functional diversity (Madar et al., 2013). Regardless of the new techniques that could allow genetic lineage tracing, clarifying the origin of CAFs is problematic due to the lack of specific markers for fibroblasts (LeBleu and Kalluri, 2018). Despite this, finding the origin of fibroblasts could help scientists to understand which signals lead to their activation, how it occurs and to help in the search for possible therapeutic targets.

CAFs are a dynamic part of the tumor microenvironment and strongly influence tumor progression. Understanding the complexity of their role and crosstalk with malignant cells as well as other cells in the microenvironment could offer an advantageous understanding into the regulation of tumor progression and could represent an opportunity to control cancer disease.

Understanding fibroblasts and CAFs and how they interact with their environment represents a challenge due to the complexity of their functions but also an approach to understand pathological and physiological processes such as wound healing, fibrosis, ageing or tumor development. Likewise fibroblasts are an excellent candidate to explore possible therapeutic targets to treat wound healing and tumors.

CONCLUSIONS

All questions were successfully solved. Briefly:

- Newborn fibroblasts have a higher differentiation potential and greater nestin and α-SMA expression, hence myofibroblasts incidence. These characteristics can be a possible explanation for a better wound healing outcome in newborns.

- Newborn fibroblasts can regulate the phenotype of keratinocytes towards a less differentiated status in cocultivation, demonstrating the importance of epithelial-mesenchymal interactions.

- Newborn fibroblasts exhibit higher secretion and gene expression of some chemotactic and pro-inflammatory cytokines.

- Newborn fibroblasts express markers of pluripotency such as oct4 and nanog.

- Newborn and older children fibroblast cultures frequently express fibrillar α-SMA, while α-SMA is absent in most of the adult fibroblast samples tested.

- Newborn and older children secret higher amounts of IL-6 compared to adult fibroblasts.

- Newborn and older children fibroblast expression patters differ from those in adult fibroblasts in the TGF-β pathway, specifically an upregulation in the expression of TGF-β3 and downregulation of TGF-βR2.

- Transcriptomic comparison of fibroblast from facial origin and fibroblasts from breast revealed a differential expression in homeobox genes, due to their different ontological origin.

- Cancer associated fibroblasts have no effect on melanoma cell proliferation but they stimulate their invasiveness in a 3D model.

- Fibroblasts and cancer associated fibroblasts increase their secretion of IL-6 and IL-8 in cocultivation with melanoma cell lines.

- Blocking IL-6 and IL-8 in a 3D melanoma model reverse the fibroblast-induced melanoma cell invasiveness.

- Cancer associated fibroblasts are not formed by EMT from human cancer cells xenografted in nu/nu mice. Xenografted cells are able to recruit stromal cells of host origin.

BIBLIOGRAPHY

Akamatsu, H., Hasegawa, S., Yamada, T., Mizutani, H., Nakata, S., Yagami, A., Matsunaga, K., 2016. Age-related decrease in CD271+ cells in human skin. J. Dermatol. 43, 311–313. https://doi.org/10.1111/1346-8138.13048

Albrengues, J., Bourget, I., Pons, C., Butet, V., Hofman, P., Tartare-Deckert, S., Feral, C.C., Meneguzzi, G., Gaggioli, C., 2014. LIF mediates proinvasive activation of stromal fibroblasts in cancer. Cell Rep. 7, 1664–1678. https://doi.org/10.1016/j.celrep.2014.04.036

Amadeu, T.P., Coulomb, B., Desmouliere, A., Costa, A.M.A., 2003. Cutaneous Wound Healing: Myofibroblastic Differentiation and in Vitro Models. Int. J. Low. Extrem. Wounds 2, 60–68. https://doi.org/10.1177/1534734603256155

Antonelli, A., Fallahi, P., Ferrari, S.M., Giuggioli, D., Colaci, M., Di Domenicantonio, A., Ferri, C., 2012. Systemic sclerosis fibroblasts show specific alterations of interferon-γ and tumor necrosis factor-α-induced modulation of interleukin 6 and chemokine ligand 2. J. Rheumatol. 39, 979–985. https://doi.org/10.3899/jrheum.111132

Antony, J., Thiery, J.P., Huang, R.Y.-J., 2019. Epithelial-to-mesenchymal transition: Lessons from development, insights into cancer and the potential of EMT-subtype based therapeutic intervention. Soc. biofisicos Latinoam.

Assis-Ribas, T., Forni, M.F., Winnischofer, S.M.B., Sogayar, M.C., Trombetta-Lima, M., 2018. Extracellular matrix dynamics during mesenchymal stem cells differentiation. Dev. Biol. 437, 63–74. https://doi.org/10.1016/j.ydbio.2018.03.002

Attolini, C.S.O., Michor, F., 2009. Evolutionary theory of cancer. Ann. N. Y. Acad. Sci. 1168, 23–51. https://doi.org/10.1111/j.1749-6632.2009.04880.x

Aunin, E., Broadley, D., Ahmed, M.I., Mardaryev, A.N., Botchkareva, N. V., 2017. Exploring a Role for Regulatory miRNAs in Wound Healing during Ageing:Involvement of miR-200c in wound repair. Sci. Rep. 7, 1–10. https://doi.org/10.1038/s41598-017-03331-6

Bai, X., Kong, Y., Chi, Z., Sheng, X., Cui, C., Wang, X., Mao, L., Tang, B., Li, S., Lian, B., Yan, X., Zhou, L., Dai, J., Guo, J., Si, L., 2017. MAPK pathway and TERT promoter gene mutation pattern and its prognostic value in melanoma patients: A retrospective study of 2,793 cases. Clin. Cancer Res. 23, 6120–6128. https://doi.org/10.1158/1078-0432.CCR-17-0980

Barnas, J.L., Simpson-abelson, M.R., Brooks, P., Kelleher, R.J., Bankert, R.B., Bankert, R.B., 2010. Reciprocal Functional Modulation of the Activation of T Lymphocytes and Fibroblasts Derived from Human Solid Tumors. J Immunol 185, 2681–2692. https://doi.org/10.4049/jimmunol.1000896

Barnes, T.C., Anderson, M.E., Moots, R.J., 2011. The Many Faces of Interleukin-6 : The Role of IL-6 in Inflammation , Vasculopathy , and Fibrosis in Systemic Sclerosis. Int. J. ofRheumatology 2011. https://doi.org/10.1155/2011/721608

Barrientos, S., Stojadinovic, O., Golinko, M.S., Brem, H., Tomic-Canic, M., 2008. Growth factors and cytokines in wound healing. Wound Repair Regen. 16, 585–601. https://doi.org/10.1111/j.1524-475X.2008.00410.x

Bautista-hernández, L.A., Gómez-olivares, J.L., Buentello-volante, B., Lucio, V.M.B., 2017. Fibroblasts: the unknown sentinels eliciting immune responses against microorganisms. Eur. J. Microbiol.

Immunol. 7, 151–157. https://doi.org/10.1556/1886.2017.00009

Behm, B., Babilas, P., Landthaler, M., Schreml, S., 2012. Cytokines, chemokines and growth factors in wound healing. J. Eur. Acad. Dermatology Venereol. 26, 812–820. https://doi.org/10.1111/j.1468-3083.2011.04415.x

Berberich, B., Thriene, K., Gretzmeier, C., Kühl, T., Bayer, H., Athanasiou, I., Rafei-Shamsabadi, D.A., Bruckner-Tuderman, L., Nyström, A., Kiritsi, D., Dengjel, J., 2020. Proteomic Profiling of Fibroblasts Isolated from Chronic Wounds Identifies Disease-Relevant Signaling Pathways. J. Invest. Dermatol. 140, 2280-2290.e4. https://doi.org/10.1016/j.jid.2020.02.040

Bermudez, D.M., Canning, D.A., Liechty, K.W., 2011. Age and pro-inflammatory cytokine production: Wound-healing implications for scar-formation and the timing of genital surgery in boys. J. Pediatr. Urol. 7, 324–331. https://doi.org/10.1016/j.jpurol.2011.02.013

Biondi, M.L., Turri, O., Leviti, S., Seminati, R., Cecchini, F., Bernini, M., Ghilardi, G., Guagnellini, E., 2000. MMP1 and MMP3 polymorphisms in promoter regions and cancer. Clin. Chem. 46, 2023–2024.

Bissell, M.J., Hines, W.C., 2011. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. Nat. Med. 17, 320–329. https://doi.org/10.1038/nm.2328

Blankesteijn, M.W., 2015. Has the search for a marker of activated fibroblasts finally come to an end? J. Mol. Cell. Cardiol. 88, 120–123. https://doi.org/10.1016/j.yjmcc.2015.10.005

Boiani, M., Schöler, H.R., 2005. Regulatory networks in embryo-derived pluripotent stem cells. Nat. Rev. Mol. Cell Biol. https://doi.org/10.1038/nrm1744

Bonnans, C., Chou, J., Werb, Z., 2014. Remodelling the extracellular matrix in development and disease. Nat rev mol cell biol 15, 786–801. https://doi.org/10.1038/nrm3904.Remodelling

Bordeleau, F., Galarneau, L., Gilbert, S., Loranger, A., Marceau, N., 2010. Keratin 8/18 Modulation of Protein Kinase C-mediated Integrin-dependent Adhesion and Migration of Liver Epithelial Cells. Mol. Biol. Cell 20, 4524–4530. https://doi.org/10.1091/mbc.E09

Borsky, J., Veleminska, J., Jurovčík, M., Jiri, K., Hechtova, D., Tvrdek, M., Cerny, M., Kabelka, Z., Fajstavr, J., Janota, J., Zach, J., Peterkova, R., Peterka, M., 2012. Successful early neonatal repair of cleft lip within first 8 days of life. Int. J. Pediatr. Otorhinolaryngol. 76, 1616–1626. https://doi.org/10.1016/j.ijporl.2012.07.031

Brouty-Boyé, D., Pottin-Clémenceau, C., Doucet, C., Jasmin, C., Azzarone, B., 2000. Chemokines and CD40 expression in human fibroblasts. Eur. J. Immunol. 30, 914–919. https://doi.org/10.1002/1521-4141(200003)30:3<914::AID-IMMU914>3.0.CO;2-D

Buckley, C.D., 2012. Why does chronic inflammation persist: An unexpected role for fibroblasts. Immunity 138, 12–14. https://doi.org/10.1016/j.imlet.2011.02.010.Why

Buechler, M.B., Turley, S.J., 2018. A short field guide to fibroblast function in immunity. Semin. Immunol. 35, 48–58. https://doi.org/10.1016/j.smim.2017.11.001

Bullard, K.M., Longaker, M.T., Lorenz, H.P., 2003. Fetal wound healing: Current biology. World J. Surg. 27, 54–61. https://doi.org/10.1007/s00268-002-6737-2

Busek, P., Mateu, R., Zubal, M., Kotackova, L., Sedo, A., 2018. Targeting fibroblast activation protein

in cancer – Prospects and caveats. Front biosci 23, 1933–1968.

Butcher, D.T., Alliston, T., Weaver, V.M., 2009. Forcing tumor Progresion. Nat Rev Cancer 9, 1–31. https://doi.org/10.1038/nrc2544.A

Calvo, F., Ege, N., Grande-Garcia, A., Hooper, S., Jenkins, R.P., Chaudhry, S.I., Harrington, K., Williamson, P., Moeendarbary, E., Charras, G., Sahai, E., 2013. Mechanotransduction and YAP-dependent matrix remodelling is required for the generation and maintenance of cancer-associated fibroblasts. Nat. Cell Biol. 15, 637–646. https://doi.org/10.1038/ncb2756

Caron, J.M., Han, X.H., Contois, L., Vary, C.P.H., Brooks, P.C., 2018. The HU177 Collagen Epitope Controls Melanoma Cell Migration and Experimental Metastasis by a CDK5/YAP-Dependent Mechanism. Am. J. Pathol. 188, 2356–2368. https://doi.org/10.1016/j.ajpath.2018.06.017

Carthy, J.M., 2018. TGFβ signaling and the control of myofibroblast differentiation: Implications for chronic inflammatory disorders. J. Cell. Physiol. 233, 98–106. https://doi.org/10.1002/jcp.25879

Chang, H.Y., Chi, J.-T., Dudoit, S., Bondre, C., van de Rijn, M., Botstein, D., Brown, P.O., 2002. Diversity, topographic differentiation, and positional memory in human fibroblasts. Proc. Natl. Acad. Sci. 99, 12877–12882. https://doi.org/10.1073/pnas.162488599

Chen, B., Wang, Z., Sun, J., Song, Q., He, B., Zhang, H., Wang, X., Dai, W., Zhang, Q., 2016. A tenascin C targeted nanoliposome with navitoclax for specifically eradicating of cancer-associated fibroblasts. Nanomedicine Nanotechnology, Biol. Med. 12, 131–141. https://doi.org/10.1016/j.nano.2015.10.001

Chen, F.G., Zhang, W.J., Bi, D., Liu, W., Wei, X., Chen, F.F., Zhu, L., Cui, L., Cao, Y., 2007. Clonal analysis of nestin- vimentin+ multipotent fibroblasts isolated from human dermis. J. Cell Sci. 120, 2875–2883. https://doi.org/10.1242/jcs.03478

Chen, M., Lei, X., Shi, C., Huang, M., Li, X., Wu, B., Li, Z., Han, W., Du, B., Hu, J., Nie, Q., Mai, W., Ma, N., Xu, N., Zhang, X., Fan, C., Hong, A., Xia, M., Luo, L., Ma, A., Li, H., Yu, Q., Chen, H., Zhang, D., Ye, W., 2017. Pericyte-targeting prodrug overcomes tumor resistance to vascular disrupting agents. J. Clin. Invest. 127, 3689–3701. https://doi.org/10.1172/JCI94258

Chen, S., Mienaltowski, M.J., Birk, D.E., 2015. Regulation of corneal stroma extracellular matrix assembly. Exp. Eye Res. 133, 69–80. https://doi.org/10.1016/j.exer.2014.08.001

Chen, X., Song, E., 2019. Turning foes to friends: targeting cancer-associated fibroblasts. Nat. Rev. Drug Discov. 18, 99–115. https://doi.org/10.1038/s41573-018-0004-1

Cheng, F., Shen, Y., Mohanasundaram, P., Lindström, M., Ivaska, J., Ny, T., Erikss, J.E., 2016. Vimentin coordinates fibroblast proliferation and keratinocyte differentiation in wound healing via TGF-β-Slug signaling. Proc. Natl. Acad. Sci. U. S. A. 113, E4320–E4327. https://doi.org/10.1073/pnas.1519197113

Childs, D.R., Murthy, A.S., 2017. Overview of Wound Healing and Management. Surg. Clin. North Am. 97, 189–207. https://doi.org/10.1016/j.suc.2016.08.013

Cirillo, N., Hassona, Y., Celentano, A., Lim, K.P., Manchella, S., Parkinson, E.K., Prime, S.S., 2017. Cancer-associated fibroblasts regulate keratinocyte cell-cell adhesion via TGF-β-dependent pathways in genotype-specific oral cancer. Carcinogenesis 38, 76–85. https://doi.org/10.1093/carcin/bgw113

Cirri, P., Chiarugi, P., 2012. Cancer-associated-fibroblasts and tumour cells: A diabolic liaison driving

cancer progression. Cancer Metastasis Rev. 31, 195–208. https://doi.org/10.1007/s10555-011-9340-x

Coleman, D.T., Gray, A.L., Stephens, C.A., Scott, M.L., Cardelli, J.A., 2016. Repurposed drug screen identifies cardiac glycosides as inhibitors of TGF-β-induced cancer-associated fibroblast differentiation. Oncotarget 1–10. https://doi.org/10.18632/oncotarget.8609

Colwell, A.S., Yun, R., Krummel, T.M., Longaker, M.T., Lorenz, H.P., 2007. Keratinocytes modulate fetal and postnatal fibroblast transforming growth factor-β and Smad expression in co-culture. Plast. Reconstr. Surg. 119, 1440–1445. https://doi.org/10.1097/01.prs.0000256049.53562.39

Connolly, J.L., Schnitt, S.J., Wang, H.H., Longtine, J.A., Dvorak, A., Dvorak, H.F., 2003. Tumor Structure and Tumor Stroma Generation, in: Holland-Frei Cancer Medicine. 6th Edition.

Coolen, N.A., Schouten, K.C.W.M., Middelkoop, E., Ulrich, M.M.W., 2010. Comparison between human fetal and adult skin. Arch. Dermatol. Res. 302, 47–55. https://doi.org/10.1007/s00403-009-0989-8

Cox, T.R., Erler, J.T., 2011. Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer. Dis. Model. Mech. 4, 165–178. https://doi.org/10.1242/dmm.004077

Dauer, P., Zhao, X., Gupta, V.K., Sharma, N., Kesh, K., Gnamlin, P., Dudeja, V., Vickers, S.M., Banerjee, S., Saluja, A., 2018. Inactivation of cancer-associated-fibroblasts disrupts oncogenic signaling in pancreatic cancer cells and promotes its regression. Cancer Res. 78, 1321–1333. https://doi.org/10.1158/0008-5472.CAN-17-2320

Davis, A., Gao, R., Navin, N., 2017. Tumor evolution: Linear, branching, neutral or punctuated? Biochim. Biophys. Acta - Rev. Cancer 1867, 151–161. https://doi.org/10.1016/j.bbcan.2017.01.003

De Wever, O., Nguyen, Q.-D., Van Hoorde, L., Bracke, M., Bruyneel, E., Gespach, C., Mareel, M., 2004. Tenascin-C and SF/HGF produced by myofibroblasts in vitro provide convergent pro-invasive signals to human colon cancer cells through RhoA and Rac. FASEB J. 18, 1016–1018. https://doi.org/10.1096/fj.03-1110fje

Delavary, B.M., van der Veer, W.M., van Egmond, M., Niessen, F.B., Beelen, R.H.J., 2011. Macrophages in skin injury and repair. Immunobiology 216, 753–762. https://doi.org/10.1016/j.imbio.2011.01.001

Demidova-Rice, T.N., Hamblin, M.R., Herman, I.M., 2012. Acute and impaired wound healing: Pathophysiology and current methods for drug delivery, part 1: Normal and chronic wounds: Biology, causes, and approaches to care. Adv. Ski. Wound Care 25, 304–314. https://doi.org/10.1097/01.ASW.0000416006.55218.d0

Desmoulière, A., Chaponnier, C., Gabbiani, G., 2005. Tissue repair, contraction, and the myofibroblast. Wound Repair Regen. 13, 7–12. https://doi.org/10.1111/j.1067-1927.2005.130102.x

Dick;, M.K., Miao, J.H., Limaiem, F., 2020. Histology, Fibroblast.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F.C., Krause, D.S., Deans, R.J., Keating, A., Prockop, D.J., Horwitz, E.M., 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8, 315–317. https://doi.org/10.1080/14653240600855905

Drake, L.E., Macleod, K.F., 2014. Tumour suppressor gene function in carcinoma-associated fibroblasts: From tumour cells via EMT and back again? J. Pathol. 232, 283–288. https://doi.org/10.1002/path.4298

Du, H., Che, G., 2017. Genetic alterations and epigenetic alterations of cancer-associated fibroblasts (Review). Oncol. Lett. 13, 3–12. https://doi.org/10.3892/ol.2016.5451

Duffy, H.S., 2011. Fibroblasts, myofibroblasts, and fibrosis: Fact, fiction, and the future. J. Cardiovasc. Pharmacol. 57, 373–375. https://doi.org/10.1097/FJC.0b013e3182155a38

Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M., Zanconato, F., Le Digabel, J., Forcato, M., Bicciato, S., Elvassore, N., Piccolo, S., 2011. Role of YAP/TAZ in mechanotransduction. Nature 474, 179–184. https://doi.org/10.1038/nature10137

Dvorak, H.F., 2016. Tumors : Wounds that do not heal--Redux 3, 1–11. https://doi.org/10.1158/2326-6066.CIR-14-0209.Tumors

Dvorak, H.F., 1988. Tumors: wounds that do not heal. Society 114, 187–219.

Dvoránková, B., Szabo, P., Lacina, L., Gal, P., Uhrova, J., Zima, T., Kaltner, H., André, S., Gabius, H.J., Sykova, E., Smetana, K., 2011. Human galectins induce conversion of dermal fibroblasts into myofibroblasts and production of extracellular matrix: Potential application in tissue engineering and wound repair. Cells Tissues Organs 194, 469–480. https://doi.org/10.1159/000324864

Egeblad, M., Rasch, M.G., Weaver, V.M., 2010. Dynamic interplay between the collagen scaffold and tumor evolution. Curr. Opin. Cell Biol. 22, 697–706. https://doi.org/10.1016/j.ceb.2010.08.015

Elliott, C.G., Hamilton, D.W., 2011. Deconstructing fibrosis research: Do pro-fibrotic signals point the way for chronic dermal wound regeneration? J. Cell Commun. Signal. 5, 301–315. https://doi.org/10.1007/s12079-011-0131-5

Engler, A.J., Humbert, P.O., Wehrle-Haller, B., Weaver, V.M., 2009. Multiscale modeling of form and function. Science (80-.). 324, 208–212. https://doi.org/10.1126/science.1170107

Erdogan, B., Webb, D.J., 2017. Cancer-associated fibroblasts modulate growth factor signaling and extracellular matrix remodeling to regulate tumor metastasis. Biochem. Soc. Trans. 45, 229–236. https://doi.org/10.1042/BST20160387

Erler, J.T., Weaver, V.M., 2009. Three-dimensional context regulation of metastasis. Clin. Exp. Metastasis 26, 35–49. https://doi.org/10.1007/s10585-008-9209-8

Esteban-Vives, R., Ziembicki, J., Sun Choi, M., Thompson, R.L., Schmelzer, E., Gerlach, J.C., 2019. Isolation and Characterization of a Human Fetal Mesenchymal Stem Cell Population: Exploring the Potential for Cell Banking in Wound Healing Therapies. Cell Transplant. 28, 1404–1419. https://doi.org/10.1177/0963689718817524

Ferguson, M.W., Duncan, J., Bond, J., Bush, J., Durani, P., So, K., Taylor, L., Chantrey, J., Mason, T., James, G., Laverty, H., Occleston, N.L., Sattar, A., Ludlow, A., O'Kane, S., 2009. Prophylactic administration of avotermin for improvement of skin scarring: three double-blind, placebo-controlled, phase I/II studies. Lancet 373, 1264–1274. https://doi.org/10.1016/S0140-6736(09)60322-6

Ferrara, N., 2005. VEGF as a therapeutic target in cancer. Oncology 69, 11–16. https://doi.org/10.1159/000088479

Ferrell, P.I., Hexum, M.K., Kopher, R.A., Lepley, M.A., Gussiaas, A., Kaufman, D.S., 2014. Functional assessment of hematopoietic niche cells derived from human embryonic stem cells. Stem Cells Dev. 23, 1355–1363. https://doi.org/10.1089/scd.2013.0497

Fiegl, H., Millinger, S., Goebel, G., Müller-Holzner, E., Marth, C., Laird, P.W., Widschwendter, M., 2006. Breast cancer DNA methylation profiles in cancer cells and tumor stroma: Association with HER-2/neu status in primary breast cancer. Cancer Res. 66, 29–33. https://doi.org/10.1158/0008-5472.CAN-05-2508

Fischer, K.R., Durrans, A., Lee, S., Sheng, J., Li, F., Wong, S.T.C., Choi, H., El Rayes, T., Ryu, S., Troeger, J., Schwabe, R.F., Vahdat, L.T., Altorki, N.K., Mittal, V., Gao, D., 2015. Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance. Nature 527, 472–476. https://doi.org/10.1038/nature15748

Foster, D.S., Jones, R.E., Ransom, R.C., Longaker, M.T., Norton, J.A., 2018. The evolving relationship of wound healing and tumor stroma. JCI insight. https://doi.org/10.1172/jci.insight.99911

Fukino, K., Shen, L., Matsumoto, S., Morrison, C.D., Mutter, G.L., Eng, C., 2004. Combined total genome loss of heterozygosity scan of breast cancer stroma and epithelium reveals multiplicity of stromal targets. Cancer Res. 64, 7231–7236. https://doi.org/10.1158/0008-5472.CAN-04-2866

Gabbiani, G., 2003. The myofibroblast in wound healing and fibrocontractive diseases. J. Pathol. 200, 500–503. https://doi.org/10.1002/path.1427

Gabbiani, G., Ryan, G.B., Majno, G., 1971. Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. Experientia 27, 549–550. https://doi.org/10.1007/BF02147594

Gailit, J., Marchese, M.J., Kew, R.R., Gruber, B.L., 2001. The differentiation and function of myofibroblasts is regulated by mast cell mediators. J. Invest. Dermatol. 117, 1113–1119. https://doi.org/10.1046/j.1523-1747.2001.15211.x

Gentili, C., Cancedda, R., 2009. Cartilage and Bone Extracellular Matrix. Curr. Pharm. Des. 15, 1334–1348. https://doi.org/10.2174/138161209787846739

Ghetti, M., Topouzi, H., Theocharidis, G., Papa, V., Williams, G., Bondioli, E., Cenacchi, G., Connelly, J.T., Higgins, C.A., 2018. Subpopulations of dermal skin fibroblasts secrete distinct extracellular matrix: implications for using skin substitutes in the clinic. Br. J. Dermatol. 179, 381–393. https://doi.org/10.1111/bjd.16255

Giebeler, N., Zigrino, P., 2016. A disintegrin and metalloprotease (ADAM): Historical overview of their functions. Toxins (Basel). 8. https://doi.org/10.3390/toxins8040122

Gieniec, K.A., Butler, L.M., Worthley, D.L., Woods, S.L., 2019. Cancer-associated fibroblasts—heroes or villains? Br. J. Cancer 121, 293–302. https://doi.org/10.1038/s41416-019-0509-3

Gilbert, R.W.D., Vickaryous, M.K., Viloria-Petit, A.M., 2016. Signalling by transforming growth factor beta isoforms in wound healing and tissue regeneration. J. Dev. Biol. 4. https://doi.org/10.3390/jdb4020021

Goodpaster, T., Legesse-Miller, A., Hameed, M.R., Aisner, S.C., Randolph-Habecker, J., Coller, H.A., 2008. An immunohistochemical method for identifying fibroblasts in formalin-fixed, paraffin-embedded tissue. J. Histochem. Cytochem. 56, 347–358. https://doi.org/10.1369/jhc.7A7287.2007

Goradel, N.H., Najafi, M., Salehi, E., Farhood, B., Mortezaee, K., 2018. Cyclooxygenase - 2 in cancer : A review. Cell. Physiol. 1, 1–17. https://doi.org/10.1002/jcp.27411

Gorchs, L., Ahmed, S., Mayer, C., Knauf, A., Fernández Moro, C., Svensson, M., Heuchel, R., Rangelova, E., Bergman, P., Kaipe, H., 2020. The vitamin D analogue calcipotriol promotes an anti-tumorigenic phenotype of human pancreatic CAFs but reduces T cell mediated immunity. Sci. Rep. 10, 1–15. https://doi.org/10.1038/s41598-020-74368-3

Grimm, S., Jennek, S., Singh, R., Enkelmann, A., Junker, K., Rippaus, N., Berndt, A., Friedrich, K., 2015. Malignancy of bladder cancer cells is enhanced by tumor-associated fibroblasts through a multifaceted cytokine-chemokine loop. Exp. Cell Res. 335, 1–11. https://doi.org/10.1016/j.yexcr.2015.04.001

Guo, S., DiPietro, L.A., 2010. Critical review in oral biology & medicine: Factors affecting wound healing. J. Dent. Res. 89, 219–229. https://doi.org/10.1177/0022034509359125

Hall, B.K., 2000. The neural crest as a fourth germ layer and vertebrates as quadroblastic not triploblastic 5, 3–5.

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: The next generation. Cell 144, 646–674. https://doi.org/10.1016/j.cell.2011.02.013

Haniffa, M.A., Wang, X., Holtick, U., 2007. Adult Human Fibroblasts Are Potent Immunoregulatory Cells and Functionally Equivalent to Mesenchymal Stem Cells. J. Immunol. 179, 1595–1604. https://doi.org/10.4049/jimmunol.179.3.1595

Hanley, C.J., Noble, F., Ward, M., Bullock, M., Drifka, C., Mellone, M., Manousopoulou, A., Johnston, H.E., Thirdborough, S., Liu, Y., Smith, D.M., Mellows, T., John, W., Garbis, S.D., Mirnezami, A., Underwood, T.J., Eliceiri, K.W., Thomas, G.J., 2015. A subset of myofibroblastic cancer-associated fibroblasts regulate collagen fiber elongation , which is prognostic in multiple cancers. Oncotarget 7, 6159–6174.

Hantash, B.M., Zhao, L., Knowles, J.A., Lorenz, H.P., 2008. Adult and fetal wound healing Basil. Front. Biosci. 51–61.

Harris, J.R., Hulmes, D.J.S., 2017. Fibrous Proteins: Structures and Mechanisms, Subcellular Biochemistry. https://doi.org/10.1007/978-3-319-49674-0_14

Hassoun, R., Schwartz, P., Feistel, K., Blum, M., Viebahn, C., 2009. Axial differentiation and early gastrulation stages of the pig embryo. Differentiation 78, 301–311. https://doi.org/10.1016/j.diff.2009.07.006

Haydont, V., Bernard, B.A., Fortunel, N.O., 2019. Age-related evolutions of the dermis: Clinical signs, fibroblast and extracellular matrix dynamics. Mech. Ageing Dev. 177, 150–156. https://doi.org/10.1016/j.mad.2018.03.006

Hecker, L., Jagirdar, R., Jin, T., Thannickal, V.J., 20011. Reversible Differentiation of Myofibroblasts by MyoD. NIH 23, 1–7. https://doi.org/10.1038/jid.2014.371

Hedayatyanfard, K., Haddadi, N.S., Ziai, S.A., Karim, H., Niazi, F., Steckelings, U.M., Habibi, B., Modarressi, A., Dehpour, A.R., 2020. The renin-angiotensin system in cutaneous hypertrophic scar and keloid formation. Exp. Dermatol. https://doi.org/10.1111/exd.14154

Hendrayani, S.F., Al-Khalaf, H.H., Aboussekhra, A., 2014. The cytokine il-6 reactivates breast stromal fibroblasts through transcription factor STAT3-dependent up-regulation of the RNA-binding

protein AUF1. J. Biol. Chem. 289, 30962–30976. https://doi.org/10.1074/jbc.M114.594044

Hinz, B., 2016. The role of myofibroblasts in wound healing. Curr. Res. Transl. Med. 64, 171–177. https://doi.org/10.1016/j.retram.2016.09.003

Hinz, B., Celetta, G., Tomasek, J.J., Gabbiani, G., Chaponnier, C., 2001a. Alpha-Smooth Muscle Actin Expression Upregulates Fibroblast Contractile Activity. Mol. Biol. Cell 12, 2730–2741. https://doi.org/10.1091/mbc.12.9.2730

Hinz, B., Gabbiani, G., 2010. Fibrosis: Recent advances in myofibroblast biology and new therapeutic perspectives. F1000 Biol. Rep. 2, 1–5. https://doi.org/10.3410/B2-78

Hinz, B., Gabbiani, G., Chaponnier, C., 2002. The NH2-terminal peptide of α-smooth muscle actin inhibits force generation by the myofibroblast in vitro and in vivo. J. Cell Biol. 157, 657–663. https://doi.org/10.1083/jcb.200201049

Hinz, B., Mastrangelo, D., Iselin, C.E., Chaponnier, C., Gabbiani, G., 2001b. Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation. Am. J. Pathol. 159, 1009–1020. https://doi.org/10.1016/S0002-9440(10)61776-2

Hinz, B., Phan, S.H., Thannickal, V.J., Galli, A., Bochaton-Piallat, M.L., Gabbiani, G., 2007. The myofibroblast: One function, multiple origins. Am. J. Pathol. 170, 1807–1816. https://doi.org/10.2353/ajpath.2007.070112

Hoejberg, L., Bastholt, L., Schmidt, H., 2012. Interleukin-6 and melanoma. Melanoma Res. 22, 327–333. https://doi.org/10.1097/CMR.0b013e3283543d72

Hofmeister, V., Schrama, D., Becker, J.C., 2008. Anti-cancer therapies targeting the tumor stroma. Cancer Immunol. Immunother. 57, 1–17. https://doi.org/10.1007/s00262-007-0365-5

Hu, M., Yao, J., Cai, L., Bachman, K.E., Van Den Brûle, F., Velculescu, V., Polyak, K., 2005. Distinct epigenetic changes in the stromal cells of breast cancers. Nat. Genet. 37, 899–905. https://doi.org/10.1038/ng1596

Hu, M.S., Borrelli, M.R., Hong, W.X., Malhotra, S., Cheung, A.T.M., Ransom, R.C., Rennert, R.C., Morrison, S.D., Lorenz, H.P., Longaker, M.T., 2018. Embryonic skin development and repair. Organogenesis 14, 46–63. https://doi.org/10.1080/15476278.2017.1421882

Hynes, R.O., 2009. Extracellular matrix: not just pretty fibrils. Science (80-.). 326, 1216–1219. https://doi.org/10.1126/science.1176009.Extracellular

Ibrahim, M.M., Chen, L., Bond, J.E., Medina, M.A., Ren, L., Kokosis, G., Selim, A.M., Levinson, H., 2015. Myofibroblasts contribute to but are not necessary for wound contraction. Lab. Investig. 95, 1429–1438. https://doi.org/10.1038/labinvest.2015.116

Ito, Y., Yeo, J.Y., Chytil, A., Han, J., Bringas, P., Nakajima, A., Shuler, C.F., Moses, H.L., Chai, Y., 2003. Conditional inactivation of Tgfbr2 in cranial neural crest causes cleft palate and calvaria defects. https://doi.org/10.1242/dev.00708

Itoh, Y., 2017. Metalloproteinases in Rheumatoid Arthritis: Potential Therapeutic Targets to Improve Current Therapies, 1st ed, Progress in Molecular Biology and Translational Science. Elsevier Inc. https://doi.org/10.1016/bs.pmbts.2017.03.002

Izar, B., Joyce, C.E., Goff, S., Cho, N.L., Shah, P.M., Sharma, G., Li, J., Ibrahim, N., Gold, J., Hodi, F.S., Garraway, L.A., Novina, C.D., Bertagnolli, M.M., Yoon, C.H., 2016. Bidirectional cross talk

between patient-derived melanoma and cancer-associated fibroblasts promotes invasion and proliferation. Pigment Cell Melanoma Res. 29, 656–668. https://doi.org/10.1111/pcmr.12513

Jabłońska-Trypuć, A., Matejczyk, M., Rosochacki, S., 2016. Matrix metalloproteinases (MMPs), the main extracellular matrix (ECM) enzymes in collagen degradation, as a target for anticancer drugs. J. Enzyme Inhib. Med. Chem. 31, 177–183. https://doi.org/10.3109/14756366.2016.1161620

Jain, R.K., Lahdenranta, J., Fukumura, D., 2008. Targeting PDGF Signaling in Carcinoma- Associated Fibroblasts Controls Cervical Cancer in Mouse Model. PLoS Med. 5, 0123–0138. https://doi.org/10.1371/journal.pmed.0050019

Jeanmaire, C., Danoux, L., Pauly, G., 2001. Glycation during human dermal intrinsic and actinic ageing: An in vivo and in vitro model study. Br. J. Dermatol. 145, 10–18. https://doi.org/10.1046/j.1365-2133.2001.04275.x

Johnson, B.Z., Stevenson, A.W., Prêle, C.M., Fear, M.W., Wood, F.M., 2020. The role of IL-6 in skin fibrosis and cutaneous wound healing. Biomedicines 8, 1–18. https://doi.org/10.3390/BIOMEDICINES8050101

Johnson, D.E., O'Keefe, R.A., Grandis, J.R., 2018. Targeting the IL-6/JAK/STAT3 signalling axis. Nat Rev Clin Oncol. 15, 234–248. https://doi.org/10.1016/j.physbeh.2017.03.040

Kahounov, Z., Kurf, D., Bouchal, J., Kharaishvili, G., Kozub, A., 2017. The Fibroblast Surface Markers FAP , anti-Fibroblast , and FSP are Expressed by Cells of Epithelial Origin and may be Altered During Epithelial-to-Mesenchymal Transition. https://doi.org/10.1002/cyto.a.23101

Kalluri, R., 2016. The biology and function of fibroblasts in cancer. Nat. Publ. Gr. https://doi.org/10.1038/nrc.2016.73

Kathju, S., Gallo, P.H., Satish, L., 2012. Scarless integumentary wound healing in the mammalian fetus: Molecular basis and therapeutic implications. Birth Defects Res. Part C - Embryo Today Rev. 96, 223–236. https://doi.org/10.1002/bdrc.21015

Kim, H.S., Jung, M., Kang, H.N., Kim, H., Park, C.W., Kim, S.M., Shin, S.J., Kim, S.H., Kim, S.G., Kim, E.K., Yun, M.R., Zheng, Z., Chung, K.Y., Greenbowe, J., Ali, S.M., Kim, T.M., Cho, B.C., 2017. Oncogenic BRAF fusions in mucosal melanomas activate the MAPK pathway and are sensitive to MEK/PI3K inhibition or MEK/CDK4/6 inhibition. Oncogene 36, 3334–3345. https://doi.org/10.1038/onc.2016.486

Klíma, J., Motlík, J., Gabius, H.J., Smetana, K., 2007. Phenotypic characterization of porcine interfollicular keratinocytes separated by elutriation: A technical note. Folia Biol. (Praha). 53, 33–36. https://doi.org/FB2007A0006 [pii]

Klingberg, F., Chau, G., Walraven, M., Boo, S., Koehler, A., Chow, M.L., Olsen, A.L., Im, M., Lodyga, M., Wells, R.G., White, E.S., Hinz, B., 2018. The fibronectin ED-A domain enhances recruitment of latent TGF-β-binding protein-1 to the fibroblast matrix. J. Cell Sci. 131, 1–12. https://doi.org/10.1242/jcs.201293

Klose, A., Wilbrand-Heinnes, A., Zigrino, P., Weber, E., Krieg, T., Mauch, C., Hunzelmann, N., 2006. Contact of high-invasive, but not low-invasive, melanoma cells to native collagen I induces the release of mature cathepsin B. Int. J. Cancer 118, 2735–2743. https://doi.org/10.1002/ijc.21700

Kondo, H., Yonezawa, Y., 1995. Fetal-adult phenotype transition, in terms of the serum dependency and growth factor requirements, of human skin fibroblast migration. Exp. Cell Res.

https://doi.org/10.1006/excr.1995.1342

Kopantzev, E.P., Vayshlya, N.A., Kopantseva, M.R., Egorov, V.I., Pikunov, M., Zinovyeva, M. V., Vinogradova, T. V., Zborovskaya, I.B., Sverdlov, E.D., 2010. Cellular and molecular phenotypes of proliferating stromal cells from human carcinomas. Br. J. Cancer 102, 1533–1540. https://doi.org/10.1038/sj.bjc.6605652

Krejčí, E., Kodet, O., Szabo, P., Borskỳ, J., Smetana, K., Grim, M., Dvořánková, B., 2015. In vitro differences of neonatal and later postnatal keratinocytes and dermal fibroblasts. Physiol. Res. 64, 561–569.

Kucera, R., Topolcan, O., Treskova, I., Kinkorova, J., Windrichova, J., Fuchsova, R., Svobodova, S., Treska, V., Babuska, V., Novak, J., Smejkal, J., 2015. Evaluation of IL-2, IL-6, IL-8 and IL-10 in Malignant Melanoma Diagnostics. Anticancer Res. 35, 3537–41.

Kugeratski, F.G., Atkinson, S.J., Neilson, L.J., Lilla, S., Knight, J.R.P., Serneels, J., Juin, A., Ismail, S., Bryant, D.M., Markert, E.K., Machesky, L.M., Mazzone, M., Sansom, O.J., Zanivan, S., 2019. Hypoxic cancer-associated fibroblasts increase NCBP2-AS2/HIAR to promote endothelial sprouting through enhanced VEGF signaling. Sci. Signal. 12, 1–18. https://doi.org/10.1126/scisignal.aan8247

Kumari, N., Dwarakanath, B.S., Das, A., Bhatt, A.N., 2016. Role of interleukin-6 in cancer progression and therapeutic resistance. Tumor Biol. 37, 11553–11572. https://doi.org/10.1007/s13277-016-5098-7

Kundrotas, G., 2012. Surface markers distinguishing mesenchymal stem cells from fibroblasts. Acta medica Litu. 19, 75–79. https://doi.org/10.6001/actamedica.v19i2.2313

Kuperwasser, C., Chavarria, T., Wu, M., Magrane, G., Gray, J.W., Carey, L., Richardson, A., Weinberg, R.A., 2004. From The Cover: Reconstruction of functionally normal and malignant human breast tissues in mice. Proc. Natl. Acad. Sci. 101, 4966–4971. https://doi.org/10.3109/00365548.2010.515606

Kurashige, M., Kohara, M., Ohshima, K., Tahara, S., Hori, Y., Nojima, S., Wada, N., Ikeda, J. ichiro, Miyamura, K., Ito, M., Morii, E., 2018. Origin of cancer-associated fibroblasts and tumor-associated macrophages in humans after sex-mismatched bone marrow transplantation. Commun. Biol. 1, 1–13. https://doi.org/10.1038/s42003-018-0137-0

Kuroda, K., Tajima, S., 2004. HSP47 is a useful marker for skin fibroblasts in formalin-fixed, paraffin-embedded tissue specimens. J. Cutan. Pathol. 31, 241–246. https://doi.org/10.1111/j.0303-6987.2003.00166.x

Kurose, K., Gilley, K., Matsumoto, S., Watson, P.H., Zhou, X.P., Eng, C., 2002. Frequent somatic mutations in PTEN and TP53 are mutually exclusive in the stroma of breast carcinomas. Nat. Genet. 32, 355–357. https://doi.org/10.1038/ng1013

Kuzet, S.E., Gaggioli, C., 2016. Fibroblast activation in cancer: when seed fertilizes soil. Cell Tissue Res. 365, 607–619. https://doi.org/10.1007/s00441-016-2467-x

Lacina, Lukas, Dvoránkova, B., Smetana, K., Chovanec, M., Plzák, J., Tachezy, R., Kideryová, L., Kucerová, L., Cada, Z., Boucek, J., Kodet, R., André, S., Gabius, H.-J., 2007. Marker profiling of normal keratinocytes identifies the stroma from squamous cell carcinoma of the oral cavity as a modulatory microenvironment in co-culture. Int. J. Radiat. Biol. 83, 837–48. https://doi.org/10.1080/09553000701694343

Lacina, L., Kodet, O., Dvořánková, B., Szabo, P., Smetana, K., 2018. Ecology of melanoma cell. Histol. Histopathol. https://doi.org/10.14670/HH-11-926

Lacina, L., Plzak, J., Kodet, O., Szabo, P., Chovanec, M., Dvorankova, B., Smetana, K., 2015. Cancer microenvironment: What can we learn from the stem cell niche. Int. J. Mol. Sci. 16, 24094–24110. https://doi.org/10.3390/ijms161024094

Lacina, L., Smetana, K., Dvořánková, B., Pytlík, R., Kideryová, L., Kučerová, L., Plzáková, Z., Štork, J., Gabius, H.J., André, S., 2007. Stromal fibroblasts from basal cell carcinoma affect phenotype of normal keratinocytes. Br. J. Dermatol. 156, 819–829. https://doi.org/10.1111/j.1365-2133.2006.07728.x

Larson, B.J., Longaker, M.T., Lorenz H., P., 2010. Scarless Fetal Wound Healing: A Basic Science Review Barrett. Plast. Reconstr. Surg. 126, 1172–1180. https://doi.org/10.1097/PRS.0b013e3181eae781.Scarless

Latif, N., Sarathchandra, P., Chester, A.H., Yacoub, M.H., 2015. Expression of smoothmuscle cellmarkers and co-activators in calcified aortic valves. Eur. Heart J. 36, 1335–1345. https://doi.org/10.1093/eurheartj/eht547

Leavitt, T., Hu, M.S., Marshall, C.D., Barnes, L.A., Lorenz, H.P., Longaker, M.T., 2016. Scarless wound healing: finding the right cells and signals. Cell Tissue Res. 365, 483–493. https://doi.org/10.1007/s00441-016-2424-8

LeBleu, V.S., Kalluri, R., 2018. A peek into cancer-associated fibroblasts: Origins, functions and translational impact. DMM Dis. Model. Mech. 11, 1–9. https://doi.org/10.1242/dmm.029447

Lee, S.B., Shim, S., Kim, M.J., Shin, H.Y., Jang, W.S., Lee, S.J., Jin, Y.W., Lee, S.S., Park, S., 2016. Identification of a distinct subpopulation of fibroblasts from murine dermis: CD73−CD105+ as potential marker of dermal fibroblasts subset with multipotency. Cell Biol. Int. 40, 1008–1016. https://doi.org/10.1002/cbin.10623

Li, B., Wang, J.H.C., 2011. Fibroblasts and myofibroblasts in wound healing: Force generation and measurement. J. Tissue Viability 20, 108–120. https://doi.org/10.1016/j.jtv.2009.11.004

Li, G., Satyamoorthy, K., Meier, F., Berking, C., Bogenrieder, T., Herlyn, M., 2003. Function and regulation of melanoma-stromal fibroblast interactions: When seeds meet soil. Oncogene 22, 3162–3171. https://doi.org/10.1038/sj.onc.1206455

Li, J.H., Huang, X.R., Zhu, H.J., Oldfield, M., Cooper, M., Truong, L.D., Johnson, R.J., Lan, H.Y., 2004. Advanced glycation end products activate Smad signaling via TGF-beta-dependent and independent mechanisms: implications for diabetic renal and vascular disease. FASEB J. 18, 176–178. https://doi.org/10.1096/fj.02-1117fje

Li, L., Dragulev, B., Zigrino, P., Mauch, C., Fox, J.W., 2009. The invasive potential of human melanoma cell lines correlates with their ability to alter fibroblast gene expression in vitro and the stromal microenvironment in vivo. Int. J. Cancer 125, 1796–1804. https://doi.org/10.1002/ijc.24463

Li, X.Y., Hu, S.Q., Xiao, L., 2015. The cancer-associated fibroblasts and drug resistance. Eur. Rev. Med. Pharmacol. Sci. 19, 2112–2119.

Lichtman, M.K., Otero-Vinas, M., Falanga, V., 2016. Transforming growth factor beta (TGF-β) isoforms in wound healing and fibrosis. Wound Repair Regen. 24, 215–222. https://doi.org/10.1111/wrr.12398

Lindner, D., Zietsch, C., Becher, P.M., Schulze, K., Schultheiss, H.P., Tschöpe, C., Westermann, D., 2012. Differential expression of matrix metalloproteases in human fibroblasts with different origins. Biochem. Res. Int. 2012. https://doi.org/10.1155/2012/875742

Littlepage, L.E., Egeblad, M., Werb, Z., 2005. Coevolution of cancer and stromal cellular responses. Cancer Cell 7, 499–500. https://doi.org/10.1016/j.ccr.2005.05.019

Liu, Tongyan, Han, C., Wang, S., Fang, P., Ma, Z., Xu, L., Yin, R., 2019. Cancer-associated fibroblasts: An emerging target of anti-cancer immunotherapy. J. Hematol. Oncol. 12, 1–15. https://doi.org/10.1186/s13045-019-0770-1

Liu, Tianyi, Zhou, L., Li, D., Andl, T., Zhang, Y., 2019. Cancer-associated fibroblasts build and secure the tumor microenvironment. Front. Cell Dev. Biol. 7, 1–14. https://doi.org/10.3389/fcell.2019.00060

Lo, D.D., Zimmermann, A.S., Nauta, A., Longaker, M.T., Lorenz, H.P., 2012. Scarless fetal skin wound healing update. Birth Defects Res. Part C - Embryo Today Rev. 96, 237–247. https://doi.org/10.1002/bdrc.21018

López-Nouoa, J.M., Nieto, M.A., 2009. Inflammation and EMT: An alliance towards organ fibrosis and cancer progression. EMBO Mol. Med. 1, 303–314. https://doi.org/10.1002/emmm.200900043

Lu, L., Saulis, A.S., Liu, W.R., Roy, N.K., Chao, J.D., Ledbetter, S., Mustoe, T.A., 2005. The temporal effects of anti-TGF-β1, 2, and 3 monoclonal antibody on wound healing and hypertrophic scar formation. J. Am. Coll. Surg. 201, 391–397. https://doi.org/10.1016/j.jamcollsurg.2005.03.032

Lu, P., Takai, K., Weaver, V.M., Werb, Z., 2011. Extracellular Matrix degradation and remodeling in development and disease. Cold Spring Harb. Perspect. Biol. 3, 1–24. https://doi.org/10.1101/cshperspect.a005058

Luo, W., Li, S., Peng, B., Ye, Y., Deng, X., Yao, K., 2013. Embryonic Stem Cells Markers SOX2, OCT4 and Nanog Expression and Their Correlations with Epithelial-Mesenchymal Transition in Nasopharyngeal Carcinoma. PLoS One 8. https://doi.org/10.1371/journal.pone.0056324

Lv, F.-J., Tuan, R.S., Cheung, K.M.C., Leung, V.Y.L., 2014. Concise Review: The Surface Markers and Identity of Human Mesenchymal Stem Cells. Stem Cells 32, 1408–1419. https://doi.org/10.1002/stem.1681

Lynch, M.D., Watt, F.M., 2018. Fibroblast heterogeneity: implications for human disease. J. Clin. Invest. 128, 26–35. https://doi.org/10.1172/JCI93555

Lysy, P.A., Smets, F., Sibille, C., Najimi, M., Sokal, E.M., 2007. Human skin fibroblasts: From mesodermal to hepatocyte-like differentiation. Hepatology 46, 1574–1585. https://doi.org/10.1002/hep.21839

Madsen, C.D., Pedersen, J.T., Venning, F.A., Singh, L.B., Moeendarbary, E., Charras, G., Cox, T.R., Sahai, E., Erler, J.T., 2015. Hypoxia and loss of PHD 2 inactivate stromal fibroblasts to decrease tumour stiffness and metastasis . EMBO Rep. 16, 1394–1408. https://doi.org/10.15252/embr.201540107

Magin, T.M., Vijayaraj, P., Leube, R.E., 2007. Structural and regulatory functions of keratins. Exp. Cell Res. 313, 2021–2032. https://doi.org/10.1016/j.yexcr.2007.03.005

Mahmoudi Rad, M., Mahmoudi Rad, N., Mirdamadi, Y., 2015. Expression of TGF-β3 in isolated fibroblasts from foreskin. Reports Biochem. Mol. Biol. 3, 76–81.

Malmstrom, J., Linberg, H., Lindberg, C., Bratt, C., Wieslander, E., Delander, E.L., Särnstrand, B., Burns, J.S., Mose-Larsen, P., Fey, S., Marko-Varga, G., 2004. Transforming growth factor-β1 specificically induce proteins involved in the myofibroblast contractile apparatus. Mol. Cell. Proteomics 3, 466–477. https://doi.org/10.1074/mcp.M300108-MCP200

Marcos-Garcés, V., Molina Aguilar, P., Bea Serrano, C., García Bustos, V., Benavent Seguí, J., Ferrández Izquierdo, A., Ruiz-Saurí, A., 2014. Age-related dermal collagen changes during development, maturation and ageing - a morphometric and comparative study. J. Anat. 225, 98–108. https://doi.org/10.1111/joa.12186

Martin, P., Leibovich, S.J., 2005. Inflammatory cells during wound repair: The good, the bad and the ugly. Trends Cell Biol. 15, 599–607. https://doi.org/10.1016/j.tcb.2005.09.002

Martin, P., Nunan, R., 2015. Cellular and molecular mechanisms of repair in acute and chronic wound healing. Br. J. Dermatol. 173, 370–378. https://doi.org/10.1111/bjd.13954

Menke, N.B., Ward, K.R., Witten, T.M., Bonchev, D.G., Diegelmann, R.F., 2007. Impaired wound healing. Clin. Dermatol. 25, 19–25. https://doi.org/10.1016/j.clindermatol.2006.12.005

Menon, S.N., Flegg, J.A., Mccue, S.W., Schugart, R.C., Dawson, R.A., Sean McElwain, D.L., 2012. Modelling the interaction of keratinocytes and fibroblasts during normal and abnormal wound healing processes. Proc. R. Soc. B Biol. Sci. 279, 3329–3338. https://doi.org/10.1098/rspb.2012.0319

Mia, M.M., Boersema, M., Bank, R.A., 2014. Interleukin-1β attenuates myofibroblast formation and extracellular matrix production in dermal and lung fibroblasts exposed to transforming growth factor-β1. PLoS One 9. https://doi.org/10.1371/journal.pone.0091559

Mishra, P., Banerjee, D., Ben-Baruch, A., 2011. Chemokines at the crossroads of tumor-fibroblast interactions that promote malignancy. J. Leukoc. Biol. 89, 31–39. https://doi.org/10.1189/jlb.0310182

Moinfar, F., Man, Y.G., Arnould, L., Bratthauer, G.L., Ratschek, M., Tavassoli, F.A., 2000. Concurrent and independent genetic alterations in the stromal and epithelial cells of mammary carcinoma: Implications for tumorigenesis. Cancer Res. 60, 2562–2566. https://doi.org/10.1158/0008-5472.can-08-4253

Moll, R., Divo, M., Langbein, L., 2008. The human keratins: Biology and pathology. Histochem. Cell Biol. 129, 705–733. https://doi.org/10.1007/s00418-008-0435-6

Monteran, L., Erez, N., 2019. The dark side of fibroblasts: Cancer-associated fibroblasts as mediators of immunosuppression in the tumor microenvironment. Front. Immunol. 10, 1–15. https://doi.org/10.3389/fimmu.2019.01835

Moore, A.L., Marshall, C.D., Barnes, L.A., Murphy, M.P., Ransom, R.C., Longaker, M.T., 2018. Scarless wound healing: Transitioning from fetal research to regenerative healing. Wiley Interdiscip. Rev. Dev. Biol. 7, 1–19. https://doi.org/10.1002/wdev.309

Newman, A.C., Nakatsu, M.N., Chou, W., Gershon, P.D., Hughes, C.C.W., 2011. The requirement for fibroblasts in angiogenesis: Fibroblast-derived matrix proteins are essential for endothelial cell lumen formation. Mol. Biol. Cell 22, 3791–3800. https://doi.org/10.1091/mbc.E11-05-0393

Nguyen, D.T., Orgill, D.P., Murphy, G.F., 2009. The pathophysiologic basis for wound healing and cutaneous regeneration. Biomater. Treat. Ski. Loss A Vol. Woodhead Publ. Ser. Biomater. 25–57. https://doi.org/10.1533/9781845695545.1.25

Noden, D.M., Trainor, P.A., 2005. Relations and interactions between cranial mesoderm and neural crest populations. J. Anat. 207, 575–601. https://doi.org/10.1111/j.1469-7580.2005.00473.x

Nolte, V.S., Xu, W., Rennekampff, H., 2008. Diversity of Fibroblasts – A Review on 165–176. https://doi.org/10.1159/000111805

Nurmik, M., Ullmann, P., Rodriguez, F., Haan, S., Letellier, E., 2019. In search of definitions: Cancer-associated fibroblasts and their markers. Int. J. Cancer. https://doi.org/10.1002/ijc.32193

Nwani, N.G., Deguiz, M.L., Jimenez, B., Vinokour, E., Dubrovskyi, O., Ugolkov, A., Mazar, A.P., Volpert, O. V., 2016. Melanoma cells block PEDF production in fibroblasts to induce the tumor-promoting phenotype of cancer-associated fibroblasts. Cancer Res. 76, 2265–2276. https://doi.org/10.1158/0008-5472.CAN-15-2468

Ogawa, Y., Razzaque, M.S., Kameyama, K., Hasegawa, G., Shimmura, S., Kawai, M., Okamoto, S., Ikeda, Y., Tsubota, K., Kawakami, Y., Kuwana, M., 2007. Role of heat shock protein 47, a collagen-binding chaperone, in lacrimal gland pathology in patients with cGVHD. Investig. Ophthalmol. Vis. Sci. 48, 1079–1086. https://doi.org/10.1167/iovs.06-0601

Ohshio, Y., Teramoto, K., Hanaoka, J., Tezuka, N., Itoh, Y., Asai, T., Daigo, Y., Ogasawara, K., 2015. Cancer-associated fibroblast-targeted strategy enhances antitumor immune responses in dendritic cell-based vaccine. Cancer Sci. 106, 134–142. https://doi.org/10.1111/cas.12584

Osman, A., Afify, S.M., Hassan, G., Fu, X., Seno, A., Seno, M., 2020. Revisiting cancer stem cells as the origin of cancer-associated cells in the tumor microenvironment: A hypothetical view from the potential of iPSCs. Cancers (Basel). https://doi.org/10.3390/cancers12040879

Paget, S., 1889. The Distribution of Secondary Growths in Cancer of the Breast. Lancet 133, 571–573. https://doi.org/10.1017/CBO9781107415324.004

Papadopoulos, N., Lennartsson, J., 2018. The PDGF/PDGFR pathway as a drug target. Mol. Aspects Med. 62, 75–88. https://doi.org/10.1016/j.mam.2017.11.007

Parekh, A., Hebda, P.A., 2018. The Contractile Phenotype of Dermal Fetal Fibroblasts in Scarless Wound Healing. curr pathobiol rep 5, 271–277. https://doi.org/10.1016/j.physbeh.2017.03.040

Parsonage, G., Falciani, F., Burman, A., Filer, A., Ross, E., Bofill, M., Martin, S., Salmon, M., Buckley, C.D., 2003. Global gene expression profiles in fibroblasts from synovial, skin and lymphoid tissue levels distinct cytokine and chemokine expression patterns. Thromb. Haemost. 90, 688–697. https://doi.org/10.1160/th03-04-0208

Paszek, M.J., Weaver, V.M., 2004. The tension mounts: Mechanics meets morphogenesis and malignancy. J. Mammary Gland Biol. Neoplasia 9, 325–342. https://doi.org/10.1007/s10911-004-1404-x

Penn, J.W., Grobbelaar, A.O., Rolfe, K.J., 2012. The role of the TGF-β family in wound healing, burns and scarring: a review. Int. J. Burns Trauma 2, 18–28.

Pessotti, D.S., Andrade-Silva, D., Serrano, S.M.T., Zelanis, A., 2020. Heterotypic signaling between dermal fibroblasts and melanoma cells induces phenotypic plasticity and proteome rearrangement in malignant cells. Biochim. Biophys. Acta - Proteins Proteomics 1868, 140525. https://doi.org/10.1016/j.bbapap.2020.140525

Petersen, O.W., Nielsen, H.L., Gudjonsson, T., Villadsen, R., Rank, F., Niebuhr, E., Bissell, M.J., Rønnov-Jessen, L., 2003. Epithelial to mesenchymal transition in human breast cancer can

provide a nonmalignant stroma. Am. J. Pathol. 162, 391–402. https://doi.org/10.1016/S0002-9440(10)63834-5

Piera-Velazquez, S., Mendoza, F., Jimenez, S., 2016. Endothelial to Mesenchymal Transition (EndoMT) in the Pathogenesis of Human Fibrotic Diseases. J. Clin. Med. 5, 45. https://doi.org/10.3390/jcm5040045

Plzák, J., Bouček, J., Bandúrová, V., Kolář, M., Hradilová, M., Szabo, P., Lacina, L., Chovanec, M., Smetana, K., 2019. The head and neck squamous cell carcinoma microenvironment as a potential target for cancer therapy. Cancers (Basel). 11. https://doi.org/10.3390/cancers11040440

Pollina, E.A., Legesse-Miller, A., Haley, E.M., Goodpaster, T., Randolph-Habecker, J., Coller, H.A., 2008. Regulating the angiogenic balance in tissues: A potential role for the proliferative state of fibroblasts. Cell Cycle 7, 2056–2070. https://doi.org/10.4161/cc.7.13.6240

Pratsinis, H., Mavrogonatou, E., Kletsas, D., 2019. Scarless wound healing: From development to senescence. Adv. Drug Deliv. Rev. 146, 325–343. https://doi.org/10.1016/j.addr.2018.04.011

Psaila, B., Lyden, D., 2009. The Metastatic Niche: Adapting the Foreign Soil. nat Rev cancer 9, 285–293. https://doi.org/10.1177/070674378402900106

Qiang, L., Yang, S., Cui, Y.H., He, Y.Y., 2020. Keratinocyte autophagy enables the activation of keratinocytes and fibroblasts and facilitates wound healing. Autophagy 0. https://doi.org/10.1080/15548627.2020.1816342

Quante, M., Tu, S.P., Tomita, H., Gonda, T., Wang, S.S.W., Takashi, S., Baik, G.H., Shibata, W., DiPrete, B., Betz, K.S., Friedman, R., Varro, A., Tycko, B., Wang, T.C., 2011. Bone Marrow-Derived Myofibroblasts Contribute to the Mesenchymal Stem Cell Niche and Promote Tumor Growth. Cancer Cell 19, 257–272. https://doi.org/10.1016/j.ccr.2011.01.020

Radisky, D., Hagios, C., Bissell, M.J., 2001. Tumors are unique organs defined by abnormal signaling and context. Semin. Cancer Biol. 11, 87–95. https://doi.org/10.1006/scbi.2000.0360

Raffaghello, L., Dazzi, F., 2015. Classification and biology of tumour associated stromal cells. Immunol. Lett. 168, 175–182. https://doi.org/10.1016/j.imlet.2015.06.016

Rasti, A., Mehrazma, M., Madjd, Z., Abolhasani, M., Saeednejad Zanjani, L., Asgari, M., 2018. Co-expression of Cancer Stem Cell Markers OCT4 and NANOG Predicts Poor Prognosis in Renal Cell Carcinomas. Sci. Rep. 8, 1–11. https://doi.org/10.1038/s41598-018-30168-4

Reed, M.J., Ferara, N.S., Vernon, R.B., 2001. Impaired migration, integrin function, and actin cytoskeletal organization in dermal fibroblasts from a subset of aged human donors. Mech. Ageing Dev. 122, 1203–1220. https://doi.org/10.1016/S0047-6374(01)00260-3

Reinke, J.M., Sorg, H., 2012. Wound repair and regeneration. Eur. Surg. Res. 49, 35–43. https://doi.org/10.1159/000339613

Rhim, A.D., Mirek, E.T., Aiello, N.M., Maitra, A., Bailey, J.M., McAllister, F., Reichert, M., Beatty, G.L., Rustgi, A.K., Vonderheide, R.H., Leach, S.D., Stanger, B.Z., 2012. EMT and dissemination precede pancreatic tumor formation. Cell 148, 349–361. https://doi.org/10.1016/j.cell.2011.11.025

Rinn, J.L., Bondre, C., Gladstone, H.B., Brown, P.O., Chang, H.Y., 2006. Anatomic demarcation by positional variation in fibroblast gene expression programs. PLoS Genet. 2, 1084–1096. https://doi.org/10.1371/journal.pgen.0020119

Rinnerthaler, M., Bischof, J., Streubel, M.K., Trost, A., Richter, K., 2015. Oxidative stress in aging human skin. Biomolecules 5, 545–589. https://doi.org/10.3390/biom5020545

Robertis, E.M. De, Oliver, G., Wright, C.V.E., 1990. Homeobox Genes and the Vertebrate Body Plan of cells that eventually become limbs and other structures. Sci. Am.

Rozario, T., DeSimone, D.W., 2010. The extracellular matrix in development and morphogenesis: A dynamic view. Dev. Biol. 341, 126–140. https://doi.org/10.1016/j.ydbio.2009.10.026

Salzer, M.C., Lafzi, A., Berenguer-Llergo, A., Youssif, C., Castellanos, A., Solanas, G., Peixoto, F.O., Stephan-Otto Attolini, C., Prats, N., Aguilera, M., Martín-Caballero, J., Heyn, H., Benitah, S.A., 2018. Identity Noise and Adipogenic Traits Characterize Dermal Fibroblast Aging. Cell 175, 1575-1590.e22. https://doi.org/10.1016/j.cell.2018.10.012

Sand, J.M.B., Knox, A.J., Lange, P., Sun, S., Kristensen, J.H., Leeming, D.J., Karsdal, M.A., Bolton, C.E., Johnson, S.R., 2015. Accelerated extracellular matrix turnover during exacerbations of COPD. Respir. Res. 16, 1–8. https://doi.org/10.1186/s12931-015-0225-3

Sarrazy, V., Billet, F., Micallef, L., Coulomb, B., Desmoulière, A., 2011. Mechanisms of pathological scarring: Role of myofibroblasts and current developments. Wound Repair Regen. 19, s10–s15. https://doi.org/10.1111/j.1524-475X.2011.00708.x

Saupe, F., Schwenzer, A., Jia, Y., Gasser, I., Spenlé, C., Langlois, B., Kammerer, M., Lefebvre, O., Hlushchuk, R., Rupp, T., Marko, M., vanderHeyden, M., Cremel, G., Arnold, C., Klein, A., Simon-Assmann, P., Djonov, V., Neuville-Méchine, A., Esposito, I., Slotta-Huspenina, J., Janssen, K.P., deWever, O., Christofori, G., Hussenet, T., Orend, G., 2013. Tenascin-C Downregulates Wnt Inhibitor Dickkopf-1, Promoting Tumorigenesis in a Neuroendocrine Tumor Model. Cell Rep. 5, 482–492. https://doi.org/10.1016/j.celrep.2013.09.014

Scaal, M., Christ, B., 2004. Formation and differentiation of the avian dermomyotome. Anat. Embryol. (Berl). 208, 411–424. https://doi.org/10.1007/s00429-004-0417-y

Schett, G., 2018. Physiological effects of modulating the interleukin-6 axis. Rheumatol. (United Kingdom) 57, ii43–ii50. https://doi.org/10.1093/rheumatology/kex513

Schumacher, M., Schuster, C., Rogon, Z.M., Bauer, T., Caushaj, N., Baars, S., Szabowski, S., Bauer, C., Schorpp-Kistner, M., Hess, J., Holland-Cunz, S., Wagner, E.F., Eils, R., Angel, P., Hartenstein, B., 2014. Efficient keratinocyte differentiation strictly depends on JNK-induced soluble factors in fibroblasts. J. Invest. Dermatol. 134, 1332–1341. https://doi.org/10.1038/jid.2013.535

Sellheyer, K., Krahl, D., 2010. Spatiotemporal expression pattern of neuroepithelial stem cell marker nestin suggests a role in dermal homeostasis, neovasculogenesis, and tumor stroma development: A study on embryonic and adult human skin. J. Am. Acad. Dermatol. 63, 93–113. https://doi.org/10.1016/j.jaad.2009.07.013

Shah, K., Patel, S., Mirza, S., Rawal, R.M., 2018. Unravelling the link between embryogenesis and cancer metastasis. Gene 642, 447–452. https://doi.org/10.1016/j.gene.2017.11.056

Shelton, M.., Anene, C.A.., Nsengimana, J.., Roberts, W.., J.5, N.-B., Boyne, J.R., 2020. The Role of CAF derived Exosomal MicroRNAs in the Tumour Microenvironment of Melanoma. Mater. Des. 108947. https://doi.org/10.1016/j.bbcan.2020.188456

Shiga, K., Hara, M., Nagasaki, T., Sato, T., Takahashi, H., Takeyama, H., 2015. Cancer-associated fibroblasts: Their characteristics and their roles in tumor growth. Cancers (Basel). 7, 2443–2458. https://doi.org/10.3390/cancers7040902

Shin, J.W., Kwon, S.H., Choi, J.Y., Na, J.I., Huh, C.H., Choi, H.R., Park, K.C., 2019. Molecular mechanisms of dermal aging and antiaging approaches. Int. J. Mol. Sci. 20. https://doi.org/10.3390/ijms20092126

Simpson, R.M.L., Wells, A., Thomas, D., Stephens, P., Steadman, R., Phillips, A., 2010. Aging fibroblasts resist phenotypic maturation because of impaired hyaluronan-dependent CD44/epidermal growth factor receptor signaling. Am. J. Pathol. 176, 1215–1228. https://doi.org/10.2353/ajpath.2010.090802

So, K., McGrouther, D.A., Bush, J.A., Durani, P., Taylor, L., Skotny, G., Mason, T., Metcalfe, A., Okane, S., Ferguson, M.W.J., 2011. Avotermin for scar improvement following scar revision surgery: A randomized, double-blind, within-patient, placebo-controlled, phase II clinical trial. Plast. Reconstr. Surg. 128, 163–172. https://doi.org/10.1097/PRS.0b013e318217429b

Song, L., Smith, M.A., Doshi, P., Sasser, K., Fulp, W., Altiok, S., Haura, E.B., 2014. Antitumor efficacy of the anti-interleukin-6 (IL-6) antibody siltuximab in mouse xenograft models of lung cancer. J. Thorac. Oncol. 9, 974–982. https://doi.org/10.1097/JTO.0000000000000193

Song, Y., Zheng, S., Wang, J., Long, H., Fang, L., Wang, G., Li, Z., Que, T., Liu, Y., Li, Y., Zhang, X., Fang, W., Qi, S., 2017. Hypoxia-induced PLOD2 promotes proliferation, migration and invasion via PI3K/Akt signaling in glioma. Oncotarget 8, 41947–41962. https://doi.org/10.18632/oncotarget.16710

Sorg, H., Tilkorn, D.J., Hager, S., Hauser, J., Mirastschijski, U., 2017. Skin Wound Healing: An Update on the Current Knowledge and Concepts. Eur. Surg. Res. 58, 81–94. https://doi.org/10.1159/000454919

Sorrell, J.M., Baber, M.A., Caplan, A.I., 2007. Clonal characterization of fibroblasts in the superficial layer of the adult human dermis. Cell Tissue Res. 327, 499–510. https://doi.org/10.1007/s00441-006-0317-y

Sorrell, J.M., Caplan, A.I., 2004. Fibroblast heterogeneity: More than skin deep. J. Cell Sci. 117, 667–675. https://doi.org/10.1242/jcs.01005

Strnadova, K., Sandera, V., Dvorankova, B., Kodet, O., Duskova, M., Smetana, K., Lacina, L., 2019. Skin aging: the dermal perspective. Clin. Dermatol. 37, 326–335. https://doi.org/10.1016/j.clindermatol.2019.04.005

Strutz, F., Okada, H., Lo, C.W., Danoff, T., Carone, R.L., Tomaszewski, J.E., Neilson, E.G., 1995. Identification and characterization of a fibroblast marker: FSP1. J. Cell Biol. 130, 393–405. https://doi.org/10.1083/jcb.130.2.393

Stuhlmiller, T.J., Garcıía-Castro, M.I., 2012. Current perspectives of the signaling pathways directing neural crest induction. Cell. Mol. Life Sci. 3715–3737. https://doi.org/10.1007/s00018-012-0991-8

Suárez-Causado, A., Caballero-Díaz, D., Bertrán, E., Roncero, C., Addante, A., García-Álvaro, M., Fernández, M., Herrera, B., Porras, A., Fabregat, I., Sánchez, A., 2015. HGF/c-Met signaling promotes liver progenitor cell migration and invasion by an epithelial-mesenchymal transition-independent, phosphatidyl inositol-3 kinase-dependent pathway in an in vitro model. Biochim. Biophys. Acta - Mol. Cell Res. 1853, 2453–2463. https://doi.org/10.1016/j.bbamcr.2015.05.017

Sun, W., Liu, D.B., Li, W.W., Zhang, L.L., Long, G.X., Wang, J.F., Mei, Q., Hu, G.Q., 2014. Interleukin-6 promotes the migration and invasion of nasopharyngeal carcinoma cell lines and upregulates the expression of MMP-2 and MMP-9. Int. J. Oncol. 44, 1551–1560.

https://doi.org/10.3892/ijo.2014.2323

Szabo, P., Valach, J., Smetana, K., 2013. Short Communication Comparative Analysis of IL-8 and CXCL-1 Production by Normal and Cancer Stromal Fibroblasts 137, 134–137.

Takada, K., Komine-Aizawa, S., Hirohata, N., Trinh, Q.D., Nishina, A., Kimura, H., Hayakawa, S., 2017. Poly I: C induces collective migration of HaCaT keratinocytes via IL-8. BMC Immunol. 18, 1–10. https://doi.org/10.1186/s12865-017-0202-3

Tap, W.D., Jones, R.L., Van Tine, B.A., Chmielowski, B., Elias, A.D., Adkins, D., Agulnik, M., Cooney, M.M., Livingston, M.B., Pennock, G., Hameed, M.R., Shah, G.D., Qin, A., Shahir, A., Cronier, D.M., Ilaria, R., Conti, I., Cosaert, J., Schwartz, G.K., 2016. Olaratumab and doxorubicin versus doxorubicin alone for treatment of soft-tissue sarcoma: an open-label phase 1b and randomised phase 2 trial. Lancet 388, 488–497. https://doi.org/10.1016/S0140-6736(16)30587-6

Theocharis, A.D., Manou, D., Karamanos, N.K., 2019. The extracellular matrix as a multitasking player in disease. FEBS J. https://doi.org/10.1111/febs.14818

Thiery, J.P., Acloque, H., Huang, R.Y.J., Nieto, M.A., 2009. Review Epithelial-Mesenchymal Transitions in Development and Disease 871–890. https://doi.org/10.1016/j.cell.2009.11.007

Togo, S., Polanska, U.M., Horimoto, Y., Orimo, A., 2013. Carcinoma-associated fibroblasts are a promising therapeutic target. Cancers (Basel). 5, 149–169. https://doi.org/10.3390/cancers5010149

Tomasek, J.J., Gabbiani, G., Hinz, B., Chaponnier, C., Brown, R.A., 2002. Myofibroblasts and mechano: Regulation of connective tissue remodelling. Nat. Rev. Mol. Cell Biol. 3, 349–363. https://doi.org/10.1038/nrm809

Valentova, S.S., Malina, R., 2018. Primary Repair of Cleft Lip and Nose in the Neonatal Period. Acta Medica Martiniana 18, 42–48. https://doi.org/10.2478/acm-2018-0006

Valkenburg, K., Groot, A.E. de, Pienta, K.C., 2017. Targeting the tumour stroma to improve cancer therapy. Nat Rev Clin Oncol. 15, 366–381. https://doi.org/10.1186/s40945-017-0033-9.Using

Van Putte, L., De Schrijver, S., Moortgat, P., 2016. The effects of advanced glycation end products (AGEs) on dermal wound healing and scar formation: a systematic review. Scars, Burn. Heal. 2, 205951311667682. https://doi.org/10.1177/2059513116676828

Varani, J., Dame, M.K., Rittie, L., Fligiel, S.E.G., Kang, S., Fisher, G.J., Voorhees, J.J., 2006. Decreased collagen production in chronologically aged skin: Roles of age-dependent alteration in fibroblast function and defective mechanical stimulation. Am. J. Pathol. 168, 1861–1868. https://doi.org/10.2353/ajpath.2006.051302

Varga, I., Hutóczki, G., Szemcsák, C.D., Zahuczky, G., Tóth, J., 2012. Brevican , Neurocan , Tenascin-C and Versican are Mainly Responsible for the Invasiveness of Low-Grade Astrocytoma. Pathol. Oncol. Res. 18, 413–420. https://doi.org/10.1007/s12253-011-9461-0

Velnar, T., Bailey, T., Smrkolj, V., 2009. The wound healing process: An overview of the cellular and molecular mechanisms. J. Int. Med. Res. 37, 1528–1542. https://doi.org/10.1177/147323000903700531

Walraven, M., Gouverneur, M., Middelkoop, E., Beelen, R.H.J., Ulrich, M.M.W., 2014. Altered TGF-β signaling in fetal fibroblasts: What is known about the underlying mechanisms? Wound Repair

Regen. 22, 3–13. https://doi.org/10.1111/wrr.12098

Wan, M., Li, C., Zhen, G., Jiao, K., He, W., Jia, X., Wang, W., Shi, C., Xing, Q., Chen, Y.-F., Beur, S.J. De, Yu, B., Cao, X., 2008. Injury-Activated TGFβ Controls Mobilization of MSCs for Tissue Remodeling. Bone 23, 1–7. https://doi.org/10.1038/jid.2014.371

Wang, F.T., Sun, W.E.I., Zhang, J.T., Fan, Y.Z., 2019. Cancer-associated fibroblast regulation of tumor neo-angiogenesis as a therapeutic target in cancer (Review). Oncol. Lett. 17, 3055–3065. https://doi.org/10.3892/ol.2019.9973

Wang, X.P., Schunck, M., Kallen, K.J., Neumann, C., Trautwein, C., Rose-John, S., Proksch, E., 2004. The interleukin-6 cytokine system regulates epidermal permeability barrier homeostasis. J. Invest. Dermatol. 123, 124–131. https://doi.org/10.1111/j.0022-202X.2004.22736.x

Wang, Y., Yu, D., Liu, Z., Zhou, F., Dai, J., Wu, B., Zhou, J., Heng, B.C., Zou, X.H., Ouyang, H., Liu, H., 2017. Exosomes from embryonic mesenchymal stem cells alleviate osteoarthritis through balancing synthesis and degradation of cartilage extracellular matrix. Stem Cell Res. Ther. 8, 1–13. https://doi.org/10.1186/s13287-017-0632-0

Wang, Z., Liu, X., Zhang, D., Wang, X., Zhao, F., Shi, P., Pang, X., 2015. Coculture with human fetal epidermal keratinocytes promotes proliferation and migration of human fetal and adult dermal fibroblasts. Mol. Med. Rep. 11, 1105–1110. https://doi.org/10.3892/mmr.2014.2798

Wang, Z., Wang, Y., Farhangfar, F., Zimmer, M., Zhang, Y., 2012. Enhanced keratinocyte proliferation and migration in co-culture with fibroblasts. PLoS One 7, 1–12. https://doi.org/10.1371/journal.pone.0040951

Wei, Y., Kim, T.J., Peng, D.H., Duan, D., Gibbons, D.L., Yamauchi, M., Jackson, J.R., Le Saux, C.J., Calhoun, C., Peters, J., Derynck, R., Backes, B.J., Chapman, H.A., 2017. Fibroblast-specific inhibition of TGF-β1 signaling attenuates lung and tumor fibrosis. J. Clin. Invest. 127, 3675–3688. https://doi.org/10.1172/JCI94624

Wells, R.G., Discher, D.E., 2008. Matrix elasticity, cytoskeletal tension, and TGF-β : The insoluble and soluble meet. Sci. Signal. 1, 1–6. https://doi.org/10.1126/stke.110pe13

Wen, X., He, X., Jiao, F., Wang, C., Sun, Y., Ren, X., Li, Q., 2017. Fibroblast Activation Protein-alpha-Positive Fibroblasts Promote Gastric Cancer Progression and Resistance to Immune Checkpoint Blockade. Oncol. Res. 25, 629–640. https://doi.org/10.3727/096504016X14768383625385

Werb, Z., Lu, P., Francisco, S., 2016. The Role of Stroma in Tumor Development 21, 250–253. https://doi.org/10.1097/PPO.0000000000000127.The

Werner, S., Grose, R., 2003. Regulation of Wound Healing by Growth Factors and Cytokines 835–870.

Werner, S., Krieg, T., Smola, H., 2007. Keratinocyte-fibroblast interactions in wound healing. J. Invest. Dermatol. 127, 998–1008. https://doi.org/10.1038/sj.jid.5700786

Weston, J.A., Yoshida, H., Robinson, V., Nishikawa, S., Fraser, S.T., 2004. Neural Crest and the Origin of Ectomesenchyme : Neural Fold Heterogeneity Suggests an Alternative Hypothesis 118–130. https://doi.org/10.1002/dvdy.10478

WHO, 2020. Who report on cancer: setting priorities, investing wisely and providing care for all, World Health Organization.

Williams, M., Burdsal, C., Periasamy, A., Lewandoski, M., Sutherland, A., 2012. Mouse primitive

streak forms in situ by initiation of epithelial to mesenchymal transition without migration of a cell population. Dev. Dyn. 241, 270–283. https://doi.org/10.1002/dvdy.23711

Wipff, P.J., Hinz, B., 2008. Integrins and the activation of latent transforming growth factor beta 1 - An intimate relationship. Eur. J. Cell Biol. 87, 601–615. https://doi.org/10.1016/j.ejcb.2008.01.012

Wiseman, B.S., Werb, Z., 2002. Development: Stromal effects on mammary gland development and breast cancer. Science (80-.). 296, 1046–1049. https://doi.org/10.1126/science.1067431

Wong, A.Y., Whited, J.L., 2020. Parallels between wound healing, epimorphic regeneration and solid tumors. Development. https://doi.org/10.1242/dev.181636

Wong, C.W., LeGrand, C.F., Kinnear, B.F., Sobota, R.M., Ramalingam, R., Dye, D.E., Raghunath, M., Lane, E.B., Coombe, D.R., 2019. In Vitro Expansion of Keratinocytes on Human Dermal Fibroblast-Derived Matrix Retains Their Stem-Like Characteristics. Sci. Rep. 9, 1–17. https://doi.org/10.1038/s41598-019-54793-9

Wu, S., Singh, S., Varney, M.L., Kindle, S., Singh, R.K., 2012. Modulation of CXCL-8 expression in human melanoma cells regulates tumor growth, angiogenesis, invasion, and metastasis. Cancer Med. 1, 306–317. https://doi.org/10.1002/cam4.28

Wulff, B.C., Parent, A.E., Meleski, M.A., Dipietro, L.A., Schrementi, M.E., Wilgus, T.A., 2012. Mast cells contribute to scar formation during fetal wound healing. J. Invest. Dermatol. 132, 458–465. https://doi.org/10.1038/jid.2011.324

Yamamura, Y., Asai, N., Enomoto, A., Kato, T., Mii, S., Kondo, Y., Ushida, K., Niimi, K., Tsunoda, N., Nagino, M., Ichihara, S., Furukawa, K., Maeda, K., Murohara, T., Takahashi, M., 2015. Microenvironment and Immunology Akt-Girdin Signaling in Cancer-Associated Fibroblasts Contributes to Tumor Progression. Cancer Res. 75, 813–823. https://doi.org/10.1158/0008-5472.CAN-14-1317

Yang, C.Y., Chanalaris, A., Troeberg, L., 2017. ADAMTS and ADAM metalloproteinases in osteoarthritis – looking beyond the 'usual suspects.' Osteoarthr. Cartil. 25, 1000–1009. https://doi.org/10.1016/j.joca.2017.02.791

Yin, M., Soikkeli, J., Jahkola, T., Virolainen, S., Saksela, O., Hölttä, E., 2012. TGF-β Signaling, activated stromal fibroblasts, and cysteine cathepsins B and L drive the invasive growth of human melanoma cells. Am. J. Pathol. 181, 2202–2216. https://doi.org/10.1016/j.ajpath.2012.08.027

You, L., Guo, X., Huang, Y., 2018. Correlation of cancer stem-cell markers OCT4, SOX2, and NANOG with clinicopathological features and prognosis in operative patients with rectal cancer. Yonsei Med. J. 59, 35–42. https://doi.org/10.3349/ymj.2018.59.1.35

Yusuf, B., Gopurappilly, R., Dadheech, N., Gupta, S., Bhonde, R., Pal, R., 2013. Embryonic fibroblasts represent a connecting link between mesenchymal and embryonic stem cells. Dev. Growth Differ. 55, 330–340. https://doi.org/10.1111/dgd.12043

Zalewska, A., Głowacka, E., Wyczółkowska, J., Tchórzewski, H., Narbutt, J., Sysa-Jędrzejowska, A., 2006. Interleukin 6 and 8 levels in plasma and fibroblast cultures in psoriasis. Mediators Inflamm. 2006, 1–6. https://doi.org/10.1155/MI/2006/81767

Zeisberg, M., Neilson, E.G., 2009. Review series personal perspective Biomarkers for epithelial-mesenchymal transitions 119, 1429–1437. https://doi.org/10.1172/JCI36183.protected

Zgheib, C., Xu, J., Liechty, K.W., 2014. Targeting Inflammatory Cytokines and Extracellular Matrix Composition to Promote Wound Regeneration. Adv. Wound Care 3, 344–355. https://doi.org/10.1089/wound.2013.0456

Zhang, D., Ighaniyan, S., Stathopoulos, L., Rollo, B., Landman, K., Hutson, J., Newgreen, D., 2014. The neural crest: A versatile organ system. Birth Defects Res. Part C - Embryo Today Rev. 102, 275–298. https://doi.org/10.1002/bdrc.21081

Zhang, J., Liu, J., 2013. Tumor stroma as targets for cancer therapy. Pharmacol. Ther. 137, 200–215. https://doi.org/10.1016/j.pharmthera.2012.10.003

Zhang, L.J., Bhattacharya, S., Leid, M., Ganguli-Indra, G., Indra, A.K., 2012. Ctip2 is a dynamic regulator of epidermal proliferation: And differentiation by integrating EGFR and Notch signaling. J. Cell Sci. 125, 5733–5744. https://doi.org/10.1242/jcs.108969

Zhang, Q., Yang, J., Bai, J., Ren, J., 2018. Reverse of non-small cell lung cancer drug resistance induced by cancer-associated fibroblasts via a paracrine pathway. Cancer Sci. 109, 944–955. https://doi.org/10.1111/cas.13520

Zhang, X., Hwang, Y.S., 2019. Cancer-associated fibroblast stimulates cancer cell invasion in an interleukin-1 receptor (il-1r)-dependent manner. Oncol. Lett. 18, 4645–4650. https://doi.org/10.3892/ol.2019.10784

Zhao, R., Liang, H., Clarke, E., Jackson, C., Xue, M., 2016. Inflammation in chronic wounds. Int. J. Mol. Sci. 17, 1–14. https://doi.org/10.3390/ijms17122085

Zhong, Y., Lu, Y.T., Sun, Y., Shi, Z.H., Li, N.G., Tang, Y.P., Duan, J.A., 2018. Recent opportunities in matrix metalloproteinase inhibitor drug design for cancer. Expert Opin. Drug Discov. 13, 75–87. https://doi.org/10.1080/17460441.2018.1398732

Zhou, L., Yang, K., Andl, T., Randall Wickett, R., Zhang, Y., 2015. Perspective of targeting cancer-associated fibroblasts in melanoma. J. Cancer. https://doi.org/10.7150/jca.10865