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Role of fibroblasts in wound healing and cancer

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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 extracellular matrix (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

Fibroblasts are the main cells that form the connective tissue. They are flattened, elongated or spindle shape cells and have 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. Fibroblasts do not form flat monolayers and are not polarized cells (Duffy, 2011; Kalluri, 2016).

Fibroblasts' main function is the production and secretion of a complex variety of molecules with structural and biological roles: the ECM. The ECM provides a scaffold for other cells to adhere and is involved in tissue and organ morphogenesis and function (Bonnans et al., 2014).

Fibroblasts are very dynamic and heterogeneous cells. The phenotypic and functional characteristics of fibroblasts depend on the anatomic site of their origin, the pathologic status and the specific roles they are to play (Chang et al., 2002; Szabo et al., 2013).

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).

Fibroblast 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 markers most commonly used nowadays are Vimentin, an intermediate filament, FSP-1, an intracellular protein, TE-7, a membrane protein, HSP-47, a collagen-specific molecular chaperone and CD-13 an ectopeptidase (Cheng et al., 2016; Goodpaster et al., 2008; Kundrotas, 2012; Kuroda and Tajima, 2004; Ogawa et al., 2007; Strutz et al., 1995).

Fibroblasts are present in the adult body in a quiescent state, however, during tissue remodeling situations such as injury and embryonic development, quiescent fibroblasts become active. 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 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).

Active fibroblasts can differentiate into myofibroblasts, cells specialized in contractile function. Myofibroblasts contain stress fibers, frequently made of α-SMA bundles (Tomasek et al., 2002). The main stimulation factor involved in the myofibroblast differentiation is TGF-β1 (Klingberg et al., 2018). TGF-β1 signaling cascade 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). Chronic presence and continued activity of myofibroblasts is involved in pathological processes like fibrosis and neoplastic proliferation (Bugyik et al., 2018; Darby et al., 2016).

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 (Egeblad et al., 2010). ECM provides physical support and tissue integrity and elasticity. Besides, components of the ECM interact with the embedded cells through adhesion receptors providing contextual information and transmitting signals that regulate cell behavior. ECM can work as a biological reservoir of signaling molecules (Hynes, 2009).

Moreover, 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).

Furthermore, fibroblasts have an important role in immunoregulation. Fibroblasts can express toll-like receptors (TLR)s, antimicrobial peptides, proinflammatory cytokines, chemokines, and growth factors, which are clue participants of the innate immunity (Bautista-hernández et al., 2017). Fibroblasts can secrete proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interferon (INF)-γ, IL-6, IL-12, and IL-10 are some of the most important inflammatory agents in the acute phase (Barnes et al., 2011). 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 indoleamine2,3-dioxygenase (IDO), prostaglandin E2 (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 tumor associated macrophages, reduce the infiltration of cytotoxic T cells and inhibit natural killer cell (NKC) cytotoxicity (Liu et al., 2019).

2. WOUND HEALING AND THE ROLE OF FIBROBLASTS

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. First, active thrombocytes induce the release of clotting factors that leads to the clot formation (Velnar et al., 2009). The next phase is known as the inflammatory phase: the cytokines and growth factors released by the thrombocytes attract neutrophils, monocytes (Hantash et al., 2008). In the next stage, the proliferative phase, fibroblasts migrate to the inflammation site, degrade the fibrin clot and replace it with a provisional ECM that acts as a support and signal for angiogenesis and re-epithelialization (Desmoulière et al., 2005). 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 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). 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).

3. CANCER STROMA

A tumor or neoplasm is an uncoordinated growth of tissue. They are considered malignant if they grow and invade the surrounding tissue, often reducing the function of the affected organ. 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).

Malignant cells also need a microenvironment to support their growth. Cancer cells produce factors that activate and recruit mesenchymal cells to create an environment that meets the tumor development needs (Nwani et al., 2016). 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 (Lacina et al., 2018).

Fibroblasts present in the tumor stroma are called CAFs. CAFs express markers like α-SMA, FAP. The presence of FAP in CAFs play an important role in tumor progression, influencing invasiveness, proliferation, ECM remodeling, vascularization, and immunoresistance (Wen et al., 2017).

CAFs are a complex and heterogeneous population. Such heterogeneity may be partially explained by its multiple cellular precursors. The most common source of CAFs are local quiescent fibroblasts that are recruited and activated by malignant cells. Bone marrow-derived MSC can be source of CAFs. Endothelial cells can transform into CAFs through endothelial to mesenchymal transition (Piera-Velazquez et al., 2016). Epithelial cells can be a source of CAFs through epithelial to mesenchymal transition (EMT). EMT occurs during embryonic development, wound healing and also metastasis (Antony et al., 2019).

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 (Butcher et al., 2009).

CAFs also communicate with the cells in their environment via secretion of growth factors, chemokines and cytokines (Yamamura et al., 2015). The communication established by CAFs is often aberrant (Zhang and Liu, 2013) and leads to uncontrolled proliferation, invasion, neoangiogenesis, apoptosis, cancer stemness, metabolism, inflammation, immunosuppression, drug resistance, and metastasis (Yamamura et al., 2015).

Traditional therapies are not fully efficient for most forms of cancer and malignant cells often developed resistance 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.

FAP is being exploited to target CAFs since early 1990 for its restricted expression pattern and its unique post proline dipeptidyl peptidase activity (Busek et al., 2018). One approach consists in the use of low molecular inhibitors that inactivate its enzymatic activity. Another approach exploits FAP unique enzymatic activity to design prodrugs that would be activated solely by FAP. 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).

4. CANCER STROMA

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). 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). 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.

HYPOTHESIS AND OBJECTIVES

Scar formation and pathological wound healing represents a major medical challenge and causes physiological and psychological problems in patients. In contrast to adults, young newborns heal in an almost scarless manner. Fibroblasts are key during the course of wound healing. Thus, the study of the fibroblasts in the wound environment in adults and scarless young newborns could be relevant to develop new therapies to prevent pathological healing.

Wound healing and tumor progression have many features in common, tumors exploit the wound healing response to generate the stroma necessary to sustain tumor growing. CAFs are predominant cells in the tumor stroma. Hence understanding wound healing could also be beneficial to treat tumors by preventing the generation of tumor stroma.

Thus, the hypotheses of this work are:

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

RESULTS AND DISCUSSION

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.

We analyzed fibroblasts' plasticity, assaying their capacity to differentiate into other mesenchymal cell types. We observed that early newborn fibroblasts could differentiate into adipocytes and chondrocytes *in vitro*. This suggests that newborn fibroblasts retain partially the low differentiated phenotype observed in embryonic dermal fibroblasts. 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, they failed differentiating into any of these two mesenchymal cell types in our experiments.

We also analyzed the expression of nestin, an intermediate filament expressed in the germ layers, during development and in pathological conditions (Yusuf et al., 2013). As expected, most of newborn fibroblasts (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. The positivity increased in coculture with keratinocytes in both newborn and adult

fibroblasts. These results are consistent with previous works (Krejčí et al., 2015). Several studies suggested that nestin could be a marker of multilineage progenitor cells (Sellheyer and Krahl, 2010). This suggests that NFs possess characteristics of multi-potentiality in opposition to adult fibroblasts (AFs), which lack nestin expression and cannot differentiate into any other mesenchymal subtype cells.

We observed by immunostaining that the fibroblasts that expressed nestin also expressed nuclear markers 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).

We observed in our experiments that NFs expressed frequently α-SMA, while AFs did not, in agreement with the literature (Hinz, 2016). 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). 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).

We also studied functional assays relevant to wound healing such as migration of keratinocytes and fibroblasts. Newborn keratinocytes were able to completely cover a biopsy punch-made round defect in a confluent monolayer faster than adult keratinocytes. Moreover, newborn keratinocytes closing the defect were all very small and expressed K8, while adult keratinocytes 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). 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). Altogether our results suggest that newborn keratinocytes are more often undifferentiated than the adult ones.

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. 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 the ability.

A work that compares migration in fibroblasts from fetal origin, 2 days old newborn, 8 month old child, and adult found out that fetal fibroblasts migration abilities are driven by a different mechanism than adult fibroblasts. 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 PDGR receptor (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.

Previous studies have shown that fetal keratinocytes are able to increase fibroblasts migration (Wang et al., 2015). 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 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 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). 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; Lacina et al., 2007), showing similarities between wound healing and tumor progression.

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, chemokine (CXC motif) ligand (CXCL)-1, CXCL-6, CXCL-14, CXCL-16, TGFB2, Vascular endothelial growth factor (VEGF)-A and -B. 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 enzyme-linked immunoabsorbent assay (ELISA).

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.

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.

Moreover 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, older child cleft lip fibroblasts (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. As we expected, the gene expression patterns of newborn and older children's fibroblasts were very similar.

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. The levels of TGF-β3 were upregulated in our newborn and child samples, this is possibly affecting the better wound outcome in these groups. Our results are supported by other works that demonstrated the anti-fibrotic effects of TGF-β3 (Gilbert et al., 2016; Lichtman et al., 2016). Moreover it is known that higher protein expression of TGF-β3 combined with lower 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). TGF-β3 causes a reduction in fibronectin and collagen I and III deposition and stimulates keratinocyte migration, macrophage recruitment and attenuates cell proliferation, promoting scarless healing

(Mahmoudi Rad et al., 2015; Moore et al., 2018). 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).

The duration of expression of these three members of the TGF-β family is also clue to determine the outcome of a wound healing. 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 (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). 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).

We detected a growth inhibition in OCCFs and AFs' when an inhibitor of TGF-βR2 was applied in the medium. On the contrary, the inhibitor did not affect newborn fibroblasts' growth. 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 (Walraven et al., 2014).

Scientists have tried to explain the reason why adults cannot repair wounds in a regenerative or scarless way from an evolutionary point of view. They 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, leading to a scar formation (Kathju et al., 2012).

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. 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). However, we did not detect changes in BLM invasion after culture with conditioned media from human healthy fibroblasts, in accordance with other works (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).

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. Other authors reached the same conclusions while working with low invasive melanoma cell lines such as WM793 (Yin et al., 2012).

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., 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).

It is known in the literature that melanoma cells stimulate CAFs to secrete cytokines and growth factors such as VEGF, CXCL12, HGF, matrix metalloproteinase (MMP)-2, monocyte chemotactic protein (MCP)-1, IL-6 and IL-8 to promote melanoma invasion. 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). 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, we observed that the production of IL-6 in monoculture was lower in melanoma cell lines, BLM and A2058, than in fibroblasts and CAFs. However, melanoma cell lines increased the

production of IL-6 when cocultivated with fibroblasts or CAFs. 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.

We further analyzed the levels of IL-6 and IL-8 in our 3D model, which would reflect their concentration throughout the experiment more accurately than in monoculture. 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. 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.

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.

In the paper ‘Cancer-associated fibroblasts are not formed from cancer cells by epithelial-to-mesenchymal transition in nu/nu mice‘ we address the origin of CAFs in a xenografted mice model.

Previous works showed that breast cancer can generate CAFs through EMT (Petersen et al., 2003), and that EMT is occurring during the process of metastasis (López-Nouoa and Nieto, 2009).

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.

Inflammation could have a role initiating EMT (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. 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, epithelial growth factor (EGF), PDGF and FGF-2 (Liu et al., 2019).

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.

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