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DEVELOPMENT AND ANALYSIS OF A STROMAL MICROENVIRONMENT EXPERIMENTAL MODEL CONTAINING PROTO-MYOFIBROBLASTS: A NEW TOOL TO STUDY THE CROSSTALK BETWEEN STROMAL AND MELANOMA CELLS

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ABSTRACT

Solid tumours are characterized by tumour microenvironment, composed of both neoplastic cells and a variety of tissue resident and recruited non-cancerous stromal cells, secreted factors, extracellular matrix (ECM) proteins, blood and lymphatic tumour vessels. The stromal compartment of tumour microenvironment, consisting also of fibroblasts, can favour or impede tumour growth and thus it has been recognized as an active and essential component of all solid tumours. Melanoma is one of the most aggressive solid tumours, composed of not only cancer cells but also the supporting stroma, which includes fibroblasts, fibroblast aggregates and cancer associated fibroblasts (CAFs). These fibroblast compartments affect differently melanoma growth during its distinct stages. Therefore, in this work, we have *in vitro* developed an experimental system resembling a part of the stromal microenvironment, represented by inactivated fibroblasts, proto-myofibroblasts, myofibroblasts and aggregates of inactivated myofibroblasts, namely spheroids. We have generated from human primary cutaneous myofibroblasts, isolated from normal skin, proto-myofibroblasts, which represent the intermediate cell type during fibroblast to myofibroblast differentiation. In particular, we have obtained proto-myofibroblasts from myofibroblast spheroids transferred to cell culture dishes and reverted to adhesion growth after 216 h of 3D culture. Likewise cells found *in vivo*, the proto-myofibroblasts of our experimental system are characterized by very low levels of α-smooth muscle actin (α-SMA) and cyclooxygenase-2 (COX-2) proteins, a cytoskeleton structure with less developed stress fibers, and increased proliferative and migratory capabilities. The analysis of the crosstalk between A375 or A2058 melanoma cell lines and fibroblasts in different stages of activation is the remarkable novelty of this work. In particular, for the first time, our study showed the anti-tumour role exerted by proto-myofibroblasts *in vitro*. Furthermore, the secretome analysis of proto-myofibroblast- and melanoma cell-conditioned media suggests that the down-

regulation of some cytokines and growth factors in the conditioned medium of proto-myofibroblasts could be associated with their anti-tumour activity. This study has also showed that the viability, outgrowth and migration of proto-myofibroblasts from spheroids are not influenced by melanoma cell-conditioned media. Furthermore, the conditioned medium of proto-myofibroblasts does not affect the viability of both BJ-5ta cells and myofibroblasts. In the light of this evidence, it is possible to assume that proto-myofibroblasts could be useful in the study of new therapeutic strategies targeting melanoma.

Chapter 1

INTRODUCTION

1.1 Fibroblasts

1.1.1 Fibroblast origin and heterogeneity

Human fibroblasts represent a very heterogeneous population of mesenchymal cells, characterized by elongated spindle or stellate shape with a multitude of cytoplasmic projections [1]. Fibroblasts are defined as non-muscle [2], non-vascular and non-epithelial cells of the connective tissue and represent its principal cellular component [3]. Fibroblasts are ubiquitous and multifunctional cells, involved in many cellular processes [4], found in different tissues, healthy or diseased, and at different stages of development. Furthermore, fibroblasts in different anatomical sites have different embryonic origins. In particular, head and neck cutaneous fibroblasts originate from the neural crest (ectoderm), while cutaneous fibroblasts from other body regions are mostly considered mesodermal cells [5,6]. Adult fibroblasts retain features of the embryonic pattern of HOX code both *in vitro*, when they are placed in culture, and *in vivo*, when they are transplanted to a new location. This spatial pattern of HOX gene expression delineates the "positional memory and identity" and the heterogeneity of fibroblasts [7,8]. Indeed, fibroblasts from different sites have distinct and characteristic gene expression patterns [7], different properties in their proliferative and migratory capability, in the extracellular matrix (ECM) synthesis and degradation [9], in matrix metalloproteinase (MMP) expression under basal or inflammatory conditions and in immunomodulatory function [10,11]. Even within a single tissue, fibroblasts represent a very complex and heterogeneous group of cells [12]. The dermis and the human mammary gland provide two examples of how

fibroblasts show remarkable morphological, molecular and functional differences in the same body site and in a single development stage [5,13]. Particularly, in the papillary (upper) layer of dermis, there are more fibroblasts with high enzymatic activity compared with these located in the reticular (lower) layer. Papillary fibroblasts are spindle-shaped cells involved in the formation of the basement membrane, in the growth of keratinocytes [14] and in the regulation of immune response in the skin. In fact, papillary fibroblasts express high levels of genes related to the complement activation pathway [15]. Conversely, reticular fibroblasts have a stellate shape [16], and overexpress genes involved in cell motility, cytoskeletal organization, smooth muscle contraction and neuronal development [15]. On the other hand, the human mammary gland contains two distinct types of fibroblasts: intralobular and interlobular fibroblasts [13]. Intralobular fibroblasts, which are involved in the branching of mammary epithelial cells, exhibit a myofibroblast-related signature including expression of transforming growth factor beta 1 (TGF-β1), tenascin-C, and α-smooth muscle actin (α-SMA). Conversely, interlobular fibroblasts show an immune-related signature, that is characterized by the expression of interleukin 1 receptor like 1 (IL1R1), interleukin 33 (IL-33), and membrane transporter SCL39A8 [17]. Interestingly, all these molecular, morphological, and functional features are maintained by fibroblasts *in vitro*, even after prolonged culture, reflecting their specific identity and positional memory [17,18].

Fibroblasts can be easily isolated from tissues and cultured *in vitro*. They can be identified based on their fusiform morphology and expression of the fibroblast specific protein-1 (FSP-1), desmin, vimentin and procollagen Iα2 chain. However, these markers are not specific for fibroblasts [9,19] and it has been demonstrated that these markers are not expressed by all fibroblast types. Additionally, *in vitro* studies, carried out on primary fibroblasts, are likely to be representative only of activated fibroblasts, named myofibroblasts. Indeed, primary fibroblasts retain fibroblast markers only in early passage cultures, and in the second passage they begin to differentiate into

myofibroblasts, characterized by expression of α-SMA, paxillin, vinculin, tensin [20] and cyclooxygenase-2 (COX-2) [21].

1.1.2 Fibroblast functions

Fibroblasts are the primary source of ECM components, such as structural proteins (e.g., fibrous collagen and elastin), adhesive proteins (e.g., laminin and fibronectin), and ground substance (e.g., glycosaminoglycans) [22]. Hence, they control the production, the maintenance and renovation of the ECM, which, in addition to providing a scaffold for cells, plays a very important role in determining cell phenotype and function. Besides their role in the regulation of normal tissue homeostasis, fibroblasts participate in wound healing and senescence [23]. These cells also act as accessory cells in many immune and inflammatory processes. Fibroblasts can produce or respond to a wide range of cytokines, which allow fibroblasts and leukocytes to cooperate during wound healing [24].

Furthermore, many diseases are associated with dysregulation of the injury repair response and fibroblast function, leading to increased or decreased deposition of ECM proteins, changes in tissue architecture and function, and sometimes, significant morbidity and mortality [25]. Fibroblasts are implicated in diseases characterized by excessive fibrosis and scarring such as scleroderma, keloids, graft-versus-host disease, pulmonary fibrosis [26] and tumours [27]. Wound healing and cancer share striking similarities, such as changes in ECM deposition, fibroblast recruitment and activation, infiltration of immune cells and neovascularization. However, during wound healing, fibroblasts are activated transiently into myofibroblasts, and at the end of this process, they undergo apoptosis or a transition back to a resting state [28]. Conversely, in tumours, that are described as wounds that never heal, myofibroblasts are resistant to apoptotic stimuli and are constitutively activated [28].

1.1.3 Fibroblast to myofibroblast differentiation

The differentiation of fibroblasts into myofibroblasts, i.e. activated fibroblasts, is a step by step process, regulated by several mechanical and molecular factors [29] (Figure 1). Indeed, fibroblast activation occurs in response to changes in the composition, organization, and mechanical property of the ECM [30], and to cytokines and growth factors released by inflammatory and resident cells [31]. In healthy tissues, fibroblasts remain in a quiescent state until stimuli activate protein synthesis and contractile mechanisms. During wound healing and cancer, they become activated and differentiate into myofibroblasts, which exhibit different morphological and functional features compared to quiescent fibroblasts [32]. Therefore, in intact and normal tissues, fibroblasts are generally stress-shielded by the cross-linked ECM, organize actin filaments into their cortical meshwork, without forming stress fibers or adhesion complexes with the surrounding ECM [29,33]. Under mechanical tension, fibroblasts differentiate into proto-myofibroblasts, which represent an intermediate step between fibroblasts and myofibroblasts [34]**.** Proto-myofibroblasts acquire contractile bundles, i.e. stress fibers that contain β- and γ-cytoplasmic actins [35] and generate small traction forces [36]. Proto-myofibroblasts predominantly express N-cadherins type adherens juntions that are frequently associated with increased cell migration [37]. Stress fibers of these migratory fibroblasts are connected to the ECM by using a specialized adhesion complex, named focal adhesions (FAs) *in vitro* [38] and fibronexus *in vivo* [39]. The fibronexus uses transmembrane integrins to link intracellular actin with extracellular fibronectin fibrils. Proto-myofibroblasts also express and organize cellular fibronectin, including the ED-A splice variant at the cell surface [29]. The presence of proto-myofibroblasts in some adult tissues suggests that they can persist and work as an independent and stable cell type without differentiating into myofibroblasts [29].

During wound healing, in many fibro-contractive diseases and even in developing and specialized normal tissues, mechanical stress and specific factors

[29,40] induce *de novo* expression of α-SMA and the differentiation of proto-myofibroblasts into myofibroblasts. Myofibroblast differentiation from proto-myofibroblast is essentially due to the combined action of the splice variant ED-A of cellular fibronectin and TGF-β. In particular, TGF-β1, that is first produced by phagocytic cells and then by proto-myofibroblasts themselves, increases the expression of ED-A fibronectin, initiating a vicious autocrine loop that together with mechanical tension, leads to the formation of myofibroblasts. TGF-β induces the synthesis of α-SMA and stimulates the production of collagen type I in myofibroblasts [41]. Other cytokines, including platelet-derived growth factor (PDGF), interleukin 4 (IL-4) and insulin-like growth factor II (IGFII) are involved in fibroblast differentiation into myofibroblast [42]. *In vitro* fibroblasts differentiate into myofibroblasts when cultured on high stiffness substrate, such as cell culture plates, in the presence of TGF-β [29]. Moreover, it is known that fetal bovine serum (FBS), which is the most widely used growth supplement for cell culture media, induces COX-2 expression in fibroblasts and activates a transcriptional program related to physiology of wound repair and associated with myofibroblasts differentiation [43]. Myofibroblasts represent activated fibroblasts, characterized by a cytoskeleton with extensively developed stress fibers, containing α-SMA, their most commonly used molecular marker [44], and increased expression of OB-cadherin either exclusively or in conjunction with N-cadherin in adhesion junctions [45]. The change from N- to OB-cadherin and the incorporation of α-SMA in stress fibers are associated with the switch from migratory phenotype of proto-myofibroblasts to the contractile one, characteristic of myofibroblasts [45]. In culture, the presence of α-SMA in stress fibers confers to myofibroblasts at least 2-fold stronger contractile activity compared with proto-myofibroblasts [44]. Myofibroblasts have the prominent rough endoplasmic reticulum of fibroblasts and the contractile myofilaments of smooth muscle cells, although these myofilaments in myofibroblasts are few and located under the membrane with respect to those found in smooth muscle cells [46]. Furthermore, similarly to smooth muscle cells, myofibroblasts are also connected directly to each

other by specific cell-cell contacts, including adherens and gap junctions [33,47] and expresses α and β integrins that allow myofibroblasts adhesion to matrix proteins [29].

Besides their role in wound contraction and closure, and ECM synthesis, myofibroblasts can acquire an immunomodulatory phenotype [48]. In fact**,** fibroblasts express a variety of Toll-like receptors (TLRs), and the subsequent ligand activation of those receptors can directly activate fibroblasts and induce their activation and differentiation into myofibroblasts [49,50]. Fibroblast activation is also mediated by direct cell-cell communication and contacts with leukocytes through adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 (VCAM-1) [51]. Fibroblasts are also activated by reactive oxygen species (ROS), complement factor C1 and impaired ECM [52]. Consequently, myofibroblasts produce large amounts of COX-2, pro-inflammatory cytokines [6,53] and participate in the regulation and maintenance of the inflammatory response**.** Activated fibroblasts not only regulate the recruitment of immune cells, but also control their behavior, retention, and survival in damaged tissues. Myofibroblasts are responsible for the switch from acute to chronic persistent inflammation [54].

It is noteworthy that fibrosis as well as tumours can develop as a complication of an adverse chronic inflammatory process. *In vivo*, myofibroblasts can be found both in normal tissues, where mechanical forces are required, and in pathological tissues, such as hypertrophic scarring, fibromatoses and fibrocontractive diseases as well as tumours [55]. Therefore, the elimination of an inflammatory insult and the resolution of the inflammation may be important for the prevention of an aberrant activation of fibroblasts in fibrotic diseases and tumour development [54].

1.2 Fibroblast clusters *in vivo* and *in vitro*

In vivo fibroblasts form clusters, under diverse physiological and pathological conditions. For example, in the hair follicles, cluster of specialized fibroblasts, i.e. the dermal papilla, furnishes the inductive signals that force skin epidermal cells to

differentiate into hair follicle cells [56]. Furthermore, nodules of highly contractile, aggregated fibroblasts are found in fibrotic tissues, where clusters of myofibroblasts are known as fibroblastic foci [56]. *In vitro* studies revealed that fibroblasts derived from neonatal foreskin, when forced to cluster, undergo a new pathway of cell activation, named nemosis, associated with a massive pro-inflammatory, proteolytic and growth factor response. This process starts about two days after clustering but simultaneously fibroblasts in spheroids undergo a programmed necrosis-like death, associated with spheroids decomposition [53,57]. Nemosis is induced early in neonatal foreskin fibroblasts grown as spheroids and is characterized by a massive production of COX-2 and prostaglandins. COX-2 is expressed throughout the spheroid and not only in the centre where nutrient deprivation and hypoxia are presumably the highest. This result demonstrated that hypoxia is not the cause of COX-2 induction in spheroids formed by neonatal foreskin fibroblasts [57]. On the contrary, a recent work demonstrated that primary cutaneous myofibroblasts isolated from normal neck skin, when forced to cluster, do not undergo apoptosis or nemosis. Fibroblast clustering leads to neither COX-2 protein induction nor spheroids decomposition, typical features of nemosis. Hence, during spheroids formation, myofibroblast aggregation is not associated with an inflammatory response. Furthermore, the significant decrease of α-SMA protein levels in fibroblasts within spheroids also indicates that three-dimensional (3D) aggregation of myofibroblasts can induce a deactivation process and could represent a reservoir of fibroblasts. For many years, it was supposed that myofibroblasts were terminally differentiated cells, which could not revert to inactivated fibroblasts [21]. Recent studies support the concept that myofibroblasts can revert to a non-activated phenotype [21,58,59]. Furthermore, the study of fibroblasts grown as spheroids can be useful to mimic the *in vivo* conditions, during which fibroblasts lose their contact to connective tissue. In particular, fibroblasts can lose their contacts to ECM during inflammation, wound healing and cancer. Indeed, in these conditions several proteases can liberate fibroblasts from ECM and trigger fibroblast aggregation [21,32].

Nodular growth is common in malignant, fast-growing cancers and can also arise from the skin as a result of the hyperproliferation of dermal fibroblasts, as in dermal fibrosarcomas [60]. Furthermore, fibroblast aggregates are also found in the dermis at early stage of melanoma development, before the metastatic dissemination. Particularly, in melanoma in situ, a distal paracrine interaction between melanoma cells, located in the epidermis, and dermal fibroblasts leads to the dermal tumour niche formation, by inducing the differentiation of normal fibroblasts into cancer associated fibroblasts (CAFs) [61]. Fibroblasts are dangerous travel companions, because they form heterotypic aggregates with disseminating cancer cells, thereby determining the success of the metastatic colonization and growth. In fact, circulating cancer cells hardly establish secondary growth. This lack of success is likely due to anoikis (literally meaning "without home") that occurs when cancer cells leave the stromal microenvironment of the primary tumour mass [62]. CAFs, or CAF aggregates, by moving from the primary tumour towards a secondary site, could provide a provisional and congenial stroma and establish a metastatic niche that facilitates the formation and the growth of metastasis [63,64]. Heterotypic aggregates forming by cancer cells and by an inner core of stromal cells, comprising of fibroblasts, are responsible for the metastatic success of high-grade serous ovarian cancer (HGSOC), which represents one of the most lethal female malignancy [62]. Additionally, CAFs, together with circulating cancer cells, and CAF aggregates are also found in the peripheral blood of breast cancer patients with metastatic disease [64]. Hence, circulating CAFs are promising biomarkers for metastatic cancers, because they can be detected in peripheral blood and quantified following a minimally invasive liquid biopsy [64].

1.3 Towards the understanding of tumours

1.3.1 The biology and pathophysiology of wound healing

Wound healing represents a response to an injury, leading to a rapid repair of damaged tissues, where fibroblast activation plays a key role in the restoration of tissue integrity [65,66]. Wound healing is a dynamic process, which requires strict control of degradative and regenerative phases involving the integrated actions of numerous cell types, of ECM and cytokines [67] (Figure 2). Immediately after injury, platelets adhere to damaged blood vessels to stop excessive bleeding, by initiating the clotting cascade. The so formed blood clot, constituted by cross-linked fibrin and ECM proteins, provides a provisional protection for the wounded area. Thereafter, inflammatory cells, such as leukocytes, neutrophils, and macrophages, respond to platelet-released factors and arrive in the wound bed. Leukocytes clear the wound area of foreign bodies and bacteria by producing and releasing ROS [68]. Additionally, inflammatory cells produce proteinases and growth factors that induce collagen production [31], furnishing a provisional matrix that is necessary for the recruitment of epidermal and dermal cells. The proliferative phase of wound repair is characterized by increased levels of keratinocyte and fibroblast proliferation, migration, and ECM synthesis. In particular, keratinocytes support wound re-epithelialization and restoration of tissue continuity. Fibroblasts, differentiating into proto-myofibroblasts, migrate and proliferate into the wound and produce large amounts of ECM proteins by forming the granulation tissue [68]. The granulation tissue is so called for the granular appearance of the numerous capillaries and new blood vessels led by massive angiogenesis [69]. In response to TGF-β, tissue tension, and certain matrix proteins (such as ED-A fibronectin and tenascin-C) present in the granulation tissue, proto-myofibroblasts acquire a contractile phenotype with α-SMA containing stress fibers and differentiate into myofibroblasts. Myofibroblasts play a pivotal role in the contraction and maturation of the granulation tissue. In fact, they synthesize and deposit ECM components (such as collagen I) that replace gradually

the provisional matrix [68,70], mainly composed by collagen III, hyaluronic acid, fibrin, and fibronectin [65]. Elastin, an ECM protein absent in the granulation tissue and involved in skin elasticity, reappears [70]. Additionally, fibronexus junctions of myofibroblasts permit them to strongly attach, contract and remodel the ECM [70]. In physiological conditions, during the resolution phase of healing, myofibroblasts disappear, either by reverting to a quiescent state or by apoptotic cell death. The result of all these processes is the reconstitution of tissue integrity and the formation of a permanent connective scar [71].

Therefore, temporal and spatial control of myofibroblast activities is crucial for the normal and controlled healing process. In fact, in pathological conditions, myofibroblasts fail to undergo apoptosis, persist, and participate in the uncontrolled deposition of ECM, resulting in excessive scar tissue deposition, and thus fibrosis [70,72]. Fibrosis appears when the synthesis of collagen by myofibroblasts overcomes the rate of its degradation [73] and can occur in a broad range of prevalent chronic diseases, such as diabetes, hypertension, heart failure and cardiomyopathy, idiopathic pulmonary disease, scleroderma, and also in tumours [74]. Recently, fibrosis has been shown to precede or follow cancer development, growth and progression [75]. In fact, several *in vivo* studies support the etiologic role for fibrosis in pancreatic cancer progression [76,77]. Furthermore, progressive lung scarring associated with idiopathic pulmonary fibrosis represents high risk factor for the development of lung cancer [78]. On the other hands, a dense fibrotic tissue or desmoplastic tissue, which is characterized by excessive ECM production and stromal cell proliferation, can occur in response to the malignant transformation. In fact, fibrosis and tumours can arise from a persistent and exacerbated healing response [79]. This pathological wound healing response can generate a vicious cycle between fibrosis and cancer, which both reciprocally support each another by establishing a bidirectional cause-effect relationship [79].

Tumours are described as “never-healing wounds” [80]. Similar to wound healing processes, solid tumours promote fibroblast recruitment and activation, ECM deposition, infiltration of immune cells, neovascularization, cell heterogeneity and plasticity [28]. However, all the mechanisms regulating this uncontrolled and persistent wound healing process can generate an abnormal and lethal microenvironment, supporting transformation, growth and dissemination of malignant cells [81].

1.3.2 The microenvironment of solid tumours

The tumour microenvironment is a dynamic, interactive and constantly changing mass, composed of both cancerous and non-cancerous components (Figure 2 D-F) [82]. Tumour microenvironment consists of ECM components, such as laminin and collagen, growth factors, nutrients, such as glucose, varying concentrations of oxygen [83,84]. Additionally, apart from cancer cells, the tumour microenvironment also comprises non-malignant cells of the immune system, blood and lymphatic vessels, as well as fibroblasts, named CAFs [85]. In particular, the dense, fibrotic nature of most solid tumours is considered a direct consequence of fibroblast infiltration and the subsequent deposition of ECM proteins [86]. Non-cancerous cells present within the tumour mass constitute the tumour stroma, whose importance in cancer has been increasingly recognized. Indeed, it is known that tumour cannot develop without the parallel expansion of the tumour stroma [87]. This results in the co-evolution of cancer cells and their microenvironment, leading to a continuous crosstalk between tumour and the surrounding stromal cells in order to support cancer growth, progression and metastasis [86]. In particular, once tumour has occurred, cancer cells promote a wound healing response that is unable to resolve, leading to a persistent and uncontrolled inflammation and to a constitutively activation of the stroma (Figure 2 D-F) [88]. Cancer cells recruit host tissue cells, such as immune cells and fibroblasts that in turn participate actively in tumour progression and

dissemination, by producing growth factors and inflammatory cytokines [89]. The inflammatory response in turn triggers an over-production of ROS that not only perpetuate the inflammatory cascade and thus stroma activation [27] but also cause high rate of mitochondrial DNA mutation [84]. Variations in mitochondrial DNA copy number or mitochondrial genome mutations result in abnormal mitochondrial functions, characterized by oxidative phosphorylation system (OXPHOS) alterations, increased ROS production and enhanced genomic instability [84]. Besides oxidative stress, other conditions within the tumour microenvironment, such as hypoxia, nutrient deprivation, and low pH contribute to genetic instability in cancer cells. Therefore, the mutator phenotype of cancer cells in turn allows both stroma activation and the creation and selection of more aggressive cancer cell clones, and thus tumour progression [90]. Thereby, the surrounding tumor stroma can be considered to be equally important as tumour cells [91].

In 1980, Hart *et al.* first demonstrated that metastasis and development of secondary tumours are not arbitrary or random events, but they are strictly linked to the microenvironment of the metastatic site [92]. Upon intravenous injection into mice, B16 melanoma cells form tumours preferentially in the lung and in grafts of pulmonary or ovarian tissue implanted either subcutaneously or intramuscularly, but not in renal tissue [92]. The 'seed and soil' hypothesis is the result of favorable interactions between cancer cells (the "seed") and their specific and compatible "soil" microenvironment, i.e. the pre-metastatic niche [93]. Factors secreted by primary cancer cells can activate and reprogram resident fibroblasts at a pre-metastatic site, resulting in the generation of a fibrotic microenvironment capable of supporting the growth of secondary tumours [94]. The old concept of 'seed and soil' is still valid, but it has been integrated with the new concept of receptor–ligand interactions. For example, the small intestine, where there is abundant expression of the CCL25 ligand, can provide an ideal microenvironment for the colonization by a specific subset of melanoma cells. The epithelium of the small intestine by producing high levels of CCL25, attracts CCR9-positive melanoma cells and provide a more permissive

microenvironment for melanoma metastasis [95]. Furthermore, lung, bones, lymph nodes and brain, all of which are CXCL12-rich organs, provide a preferential microenvironment for breast cancer metastasis [96,97]. In particular, breast CAFs (BCAFs) secrete CXCL12 ligand, which in turn binds to CXCR4 receptor expressed on cancer cells and induces actin polymerization and pseudopodia formation. Thus, the CXCL12-CXCR4 binding promotes the migration of CXCR4-positive breast cancer cells towards CXCL12-rich secondary metastatic sites [97]. Taken together all these data confirm the concept that cancer cells are able to metastasize specific and preferential tissues depending on their own properties and on the type of microenvironment available in the ectopic organ site [92].

1.3.3 Cancer Associated fibroblasts origin, features and functions

CAFs are one of the most abundant non-cancerous cell types within the reactive stroma of many solid tumours [98]. In breast and pancreatic carcinomas, CAFs represent up to 80% of the tumour mass, as a result of a widespread desmoplasia that activates fibroblasts by generating mechanical forces [23]. Desmoplasia is a histological hallmark of cancer tissue and consists of an abundant tumour stroma formed by ECM and myofibroblasts, inflammatory cells, blood and lymphatic vessels. Desmoplasia wrecks the organ's normal architecture and creates an optimal niche for cancer cells. In particular, desmoplasia produces and maintains an oxidative stress condition in cancer tissue and generates mechanical forces that are responsible of myofibroblast formation from fibroblasts and other precursors. Furthermore, desmoplasia establishes a hypoxic and acidic microenvironment that compromises chemotherapeutic treatment [23].

The main source of CAFs is represented by pre-existing resident fibroblasts. CAFs can also originate from other precursor cells, such as bone marrow derived mesenchymal stem cells, epithelial cells, carcinoma cells, pericytes, smooth muscle cells, adipocytes, fibrocytes, stellate cells in pancreas and liver, myoepithelial cells in

breast, and pericryptal myofibroblasts of the gastrointestinal tract [99]. In melanoma and pancreatic cancer models, the endothelial cells can undergo endothelial-to-mesenchymal transition (EndMT) mediated by autocrine and paracrine TGF-β signalling, that induces them to transdifferentiate into CAFs [100].

Genetic changes occurring in tumour cells remain an important driver of cancer. At early phase of cancer development, in association with emerging epithelial molecular lesions, normal fibroblasts are reprogrammed into CAFs [101]. However, this conversion may precede the genetic mutations of cancer cells and lead to CAFs the task to trigger malignant transformation [102]. Indeed, recent works have demonstrated that CAFs mediate field cancerization, transform primary epithelial cells, and enhance cancer aggressiveness [103]. De Filippi *et al.* demonstrated that fibroblasts derived from cancer-free breast tissues obtained from healthy women with high mammographic density exhibit desmoplastic/protumorigenic phenotype respect to fibroblasts isolated from normal breast tissues obtained from women with low mammographic density. This fibroblast phenotype is associated with high risk for developing cancer, especially in high mammographic density tissues [104].

CAFs are described as a heterogeneous fibroblastic cell population in a permanently activated state, sharing similarities with normal activated fibroblasts, found during inflammation and wound healing [102]. CAFs are similar in morphology to myofibroblasts, which are large spindle-shaped cells with well-developed stress fibers [105]. However, compared with normal fibroblasts, CAFs have a larger number of lamellipodia, the thin, sheet-like membrane protrusions normally found at the leading edge of moving cells. As a consequence, CAFs exhibit a more migratory phenotype compared to normal fibroblasts. Additionally, normal fibroblasts and CAFs have different stress fibers orientation. Particularly, while stress fibers in normal fibroblasts are usually randomly organized, in CAFs they are oriented towards one direction [106]. Unexpectedly, the proliferation rate of CAFs is slower than normal fibroblasts, because of their different metabolic profiles [107,108]. In particular, CAFs,

which acquire a catabolic phenotype, produce high-energy metabolites, specially lactate, pyruvate, and ketone bodies. These energy-rich fuels are not used by CAFs for their own biosynthesis but are necessary for the growth of adjacent anabolic cancer cells [109].

CAFs, as well as myofibroblasts, express high levels of α-SMA protein, the typical marker of fibroblast activation and CAF differentiation [99]. However, not all CAFs express α-SMA. Indeed, based on different levels of α-SMA, Ohlund *et al.* identified two subtypes of CAFs: myofibroblast CAFs (myoCAFs) characterized by high α-SMA expression levels and inflammatory CAFs (iCAFs) with high expression of tumour-promoting cytokines and chemokines [110]. Additionally, fibroblast-activating protein (FAP), FSP-1, also known as S100A4, desmin, osteonectin, platelet derived growth factor receptor (PDGFR), periostin (POSTN), podoplanin (PDPN), neuron-glial antigen-2 (NG2), tenascin-C and the mesenchymal protein vimentin are highly expressed by CAFs and can be used as CAF markers [99,102,111]. However, all these CAF markers are not specific for this cell type and can be also expressed by other cell types of the tumour mass. For example, PDPN is a marker of lymphatic vessels and can be also expressed by cancer cells, and PDGFR is also expressed by pericytes [98]. Furthermore, CAF markers are not synchronously expressed, due to the existence of different subpopulations of CAFs [98]. For example, circulating CAFs detected in the peripheral blood of patients with metastatic breast cancer were characterized as $FAP^+/\alpha\text{-}SMA^+/Cytokeratin^-/CD45^-$ [102].

Comparative gene expressing profiling of normal fibroblasts and CAFs demonstrated that CAFs produce factors that are lower or not expressed by the normal counterpart [98]. In particular, CAFs upregulate genes associated with angiogenesis, inflammation, epithelial-mesenchymal transition (EMT), cell adhesion and cell interactions [112,113]. CAFs secrete a variety of growth factors, such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), connective tissue growth factor (CTGF), insulin-like growth factor (IGF), vascular

endothelial-derived growth factor (VEGF), TGF-β, and cytokines, chemokine including CXCL12 and interleukin 6 (IL-6). CAFs also secrete high levels of ECM remodelling enzymes and ECM proteins, such as fibronectin, type I and type II collagen, and express oncofetal isoforms of fibronectin [114]. CAFs alter the architecture and physical properties of tumour ECM, influencing cell migration, invasion, and growth. In particular, CAFs organize the fibronectin matrix into aligned fibers generating tracks that cancer cells efficiently and directionally follow. Conversely, the healthy matrix produced by normal fibroblasts resembles a random meshwork of fibers that do not promote directional migration of cancer cells [114]. CAF-secreted factors are involved in paracrine signalling or activate CAFs in autocrine loops, thus contributing to tumour formation, development, invasion, metastasis and resistance to treatment [98,102,115]. The ECM remodeling by CAFs can generate a chemoprotective environment and thus hinder the effective delivery of chemotherapeutic drugs to cancer cells [116].

Several studies have reported that not only genetic mutations but also epigenetic reprogramming plays a crucial role in the induction and maintenance of the permanent activated state in CAFs [117–119]. For example, hypermethylation of the *RASAL1* promoter regulates its transcriptional suppression, and promotes Ras-GTP activity and a persistent fibroblast activation, which often occurs in renal fibrosis [118]. Furthermore, a global hypomethylation of CAF genes was also reported by Jiang *et al.* [120]**.** The positional memory exhibited by normal fibroblasts, is also maintained in CAFs and contributes to differences among CAFs of different anatomical sites [98].

1.3.4 CAF differentiation

CAF formation represents a step by step activation program, characterized by a lot of molecular and functional changes. Normal fibroblasts become activated during the initial phase of oncogenesis and initiate the remodelling of the tumour microenvironment by exerting an initial anti-tumour function [121]. However, as the

tumour grows, cancer cells force normal fibroblasts to increase the expression of TGF-β, which in turn can induce a reversible "primed state" in normal fibroblasts. These primed fibroblasts can promote tumour initiation and progression even though they do not exhibit all the phenotypic and molecular characteristics of the constitutively activated CAFs [122]. Comito *et al.* speculated that hypoxia can be considered as the synergizing factor needed to achieve a complete fibroblasts activation, by inducing oxidative stress and hypoxia inducible factor-1 (HIF-1) stabilization. In fact, ROS scavenging with N-acetyl cysteine (NAC) dramatically abrogates activation of fibroblasts as well as their effects on cancer cells [123].

CAFs adopt a large number of self-stimulating and cross-communicating pathways in order to maintain myofibroblast phenotype, and establish CAF property and their tumour promoting functions, independently of the presence of tumour cells [102].

1.4 Fibroblasts and cancer associated fibroblasts in melanoma

1.4.1 Dual role of fibroblasts in melanoma growth

Melanoma is the most severe and fatal form of skin cancer, that arises from the malignant transformation of skin melanocytes [124]. Nowadays, its incidence rate is still rising dramatically [125].

Normal skin structure consists of two layers: the outer, the thinner epidermis, and the inner, thicker dermis. In particular, normal melanocytes reside in the basal layer of the epidermis, where form an 'Epidermal Melanin Unit' that contains one melanocyte and up to thirty-six keratinocytes (Figure 3A). Moreover, normal melanocytes are in contact with keratinocytes trough the expression of the cell-cell adhesion molecule, E-cadherin. However, during oncogenic insults, malignant melanocytes proliferate, penetrate the basal layer, and invade the underlying dermis

[126]. Furthermore, melanoma progression is linked to a switch in cell-cell communication: in this case, melanoma cells lose their preferential interactions with epidermal keratinocytes, and adhere and interact with host fibroblasts [127]. In fact, during the process of melanoma invasion and metastasis, malignant melanocytes lose E-cadherin expression, produce N-cadherin, that allows melanoma cells to bind to N-cadherin-expressing fibroblasts [126] (Figure 3A). This switch enables melanoma cells to escape from the epidermal microenvironment and to communicate with stromal cells important for their metastatic spread [128,129].

However, it is noteworthy that fibroblasts can favour or impede melanoma development (Figure 3B). In particular, at early stages of tumour development, normal dermal fibroblasts can suppress growth and progression of pre-malignant lesions [130]. Furthermore, normal dermal fibroblasts can also block melanoma cell migration and invasion through the surrounding tissues by strictly controlling MMP1, MMP2, MMP9, and MMP13, and membrane-type matrix metalloproteinases (MT-MMPs) expression, thereby impeding the degradation of basement membrane [126]. How normal dermal fibroblasts are converted into CAFs, entering a continuous state of irreversible activation in melanoma, remains poorly understood [131]. However, experiments of co-culture demonstrated that melanoma cells and fibroblasts influence each other, effecting their gene expression profiles, with dramatic alterations in fibroblasts compared with a more modest effect on cancer cells. Upon physical and humoral contact with melanoma cells, fibroblasts are activated and start to up-regulate a large number of genes associated with the matrix proteolysis, cellular proliferation and pro-inflammatory pathways, including cytokines and chemokines, such as IL-1α, CXCL1/GROα, and CXCL8/IL-8 [132]. Furthermore, it is known that melanoma cells secrete specific growth factors, including TGF-β1, PDGF, FGF-2. These growth factors activate fibroblasts, which subsequently support melanoma progression [133,134]. TGF-β has an important and pleiotropic role in melanoma progression and induces normal fibroblast conversion into CAF [126,135,136]. Yin *et al.* found that blocking TGF-β signalling with a TGF-β receptor-I/ALK5 inhibitor or TGF-β–neutralizing

antibodies effectively inhibits the activation of fibroblasts and their ability to promote the invasive growth of melanoma [137]. Furthermore, Berking *et al.* showed that TGF-β1 produced by melanoma cells remodel the surrounding stroma by increasing fibroblasts deposition of ECM proteins, such as collagen, fibronectin, tenascin, and α2 integrin, within and around the tumour mass. These proteins produced by melanoma associated fibroblasts (MAFs) provide a physical and biological support for melanoma cells, which increase their survival and metastasis formation [135]. Furthermore, Flach *et al.* demonstrated that melanoma cells enhance matrix production in fibroblasts and stimulate CAF motility. In particular, melanoma cells induce fibroblasts to migrate toward, surround, and then infiltrate the tumour mass [131]. These results are consistent with the observations made by pathologists, showing that melanoma samples often show signs of fibroplasia, and may possess desmoplastic characteristics, for the presence of fibroblasts, fibrocytes and clearly visible fibers of the ECM [138]. Furthermore, Whipple *et al.* demonstrated that $BRAF^{V600E}$ melanoma cells, by expressing high levels of IL-1β, IL-6, and IL-8 cytokines and MMP-1, trigger a constitutive activation of dermal fibroblasts, which differentiate into CAFs [139].

In addition, it has been reported that hypoxia promotes tumour microvescicles (MVs) secretion [140], which could exert a wide range of biological functions in melanoma, including tumour-stroma communication and induction of a tumour-associated phenotype in normal dermal fibroblasts. In particular, Zhao *et al.* demonstrated that fibroblasts, after melanoma-derived MVs incubation, increase IL-6, FAP and EGF expression simultaneously. Furthermore, MV-educated fibroblasts enhance VCAM-1 expression, in an ERK1/ 2-activation-dependent manner, favouring in this way the attachment of highly metastatic melanoma cells to the stroma in a pre-metastatic niche[141]. Izar *et al.* demonstrated that melanoma invasiveness can depend on physical interactions with CAFs. In fact, MAF-derived conditioned medium modestly enhances melanoma invasiveness in only one of three patient samples examined. Instead, co-culture experiments of MAFs and melanoma cells reveal that

MAFs only after a direct physical interaction with cancer cells, are able to strongly enhance melanoma invasiveness in all the experiments [142].

Chapter 2

AIM OF WORK

Fibroblasts represent a heterogeneous population of mesenchymal cells, involved in many physiological and pathological processes, such as ECM turnover, tissue homeostasis, wound healing, senescence and tumour [27]. In physiological conditions, fibroblasts can display a quiescent phenotype. However, upon various stimuli, the quiescent fibroblasts differentiate before into proto-myofibroblasts, acquiring a proliferative and migratory phenotype, and then into myofibroblasts, acquiring an activated phenotype, characterized by protein synthetic activity and contractile functions [143]. Once myofibroblasts have fulfilled their function, they have to disappear, either by reverting to a quiescent state or by apoptotic cell death [71]. Conversely, during tumorigenesis, upon interaction with cancer cells, normal fibroblasts can acquire a constitutive activated state, fail to undergo apoptosis, differentiate into CAFs and thus promote cancer initiation and progression [144]. However, before CAF differentiation, fibroblasts, which can be present in the tumour stroma as activated fibroblasts, non-activated fibroblasts and aggregates [61,145], can suppress cancer initiation and progression via direct cell-cell contact, paracrine signalling by soluble factors, and by influencing ECM structure [143].

The aim of the work was to generate and analyse an *in vitro* experimental system, mimicking successfully the heterogeneity, the complexity, and the dynamicity of a part of the stromal microenvironment observed *in vivo*, before CAF differentiation. In particular, BJ-5ta fibroblasts and primary myofibroblasts from normal skin of neck have been chosen to better recall the *in vivo* functions and behaviors of inactivated fibroblasts and activated fibroblasts, respectively. Furthermore, spheroids from

human primary myofibroblasts have been generated to reproduce the *in vivo* functions of fibroblast aggregates and their interaction with cancer cells [56,61,145,146].

The first goal of this study was to demonstrate that fibroblasts of spheroids reverted to adhesion and monolayer growth, namely reverted-fibs, maintain the de-activated phenotype acquired during spheroids formation.

Since in the tumour stroma inactivated fibroblasts and activated fibroblasts can affect differently tumour progression, it has been considered important to analyze the paracrine interaction between all the fibroblast populations and melanoma cells. In particular, we have evaluated the viability and migratory capability of melanoma cells in response to signals from the different fibroblast populations of our experimental system. Furthermore, we have evaluated the effect of melanoma cell signals on all the fibroblast types. Next, we have compared cytokines and growth factors secretion profile of proto-myofibroblasts respect to that one of melanoma cells.

By providing the answers to the above questions and understanding the effects of the crosstalk between melanoma and stromal cells, the present work should improve our knowledge on fibroblast contribution in cancer tissue regulation. In particular, this study could represent an experimental system to study fibroblast deactivation process, with a possible application in the modulation of tumour progression.

Chapter 3

RESULTS

3.1 Characterization of fibroblast populations

3.1.1 Morphological analysis of human primary fibroblasts

We isolated primary human fibroblasts from normal neck skin, as reported in Materials and Methods. We first examined human primary fibroblast morphology under an inverted light microscopy. Primary fibroblasts appear as elongated spindle-shaped cells as showed in figure 4A. Additionally, we analysed the cytoskeleton organization of primary fibroblasts by evaluating the presence of intermediate filaments and stress fibers by vimentin immunofluorescence and phalloidin staining, respectively (Figure 4B). Confocal immunofluorescence analysis showed the presence of vimentin intermediate filaments, cytoskeletal markers of mesenchymal cells and well-developed stress fibers. Therefore, the cytoskeleton organization of primary fibroblasts resembles that of myofibroblasts [29].

3.1.2 Evaluation of inflammatory and activation markers in primary and immortalized fibroblasts

To evaluate the levels of activation and inflammation markers specific of fibroblasts, the levels of α-SMA and COX-2 proteins were analysed in extracts of human primary fibroblasts from neck skin and BJ-5ta cells by western blotting (Figure 5A,B). This analysis showed that human primary fibroblasts are myofibroblasts because they express high levels of both α-SMA and COX-2 proteins. In fact, it is known that α-SMA and COX-2 are markers of fibroblast to myofibroblast differentiation [23,29]. The confocal analysis confirmed the western blotting results.

Conversely, the immortalized foreskin fibroblast cell line, BJ-5ta, expressed neither COX-2 nor α-SMA proteins. The absence of these two proteins in BJ-5ta cells indicated that these cells represent inactivated fibroblasts.

3.1.3 Analysis of fibroblast three-dimensional cultures

In our previous work, we have demonstrated that spheroids, collected after 72 h and 216 h of 3D culture, show an impressive and comparable reduction of α-SMA levels respect to myofibroblasts grown as monolayer [21]. Hence, in the present work, we have generated and compare spheroids from human primary cutaneous myofibroblasts [21] up to 72 h and 216 h of 3D culture. In particular, to evaluate the presence of necrosis areas based on the typically increased eosinophilia of necrotic areas, we have performed haematoxylin and eosin staining on spheroids collected after 72 h and 216 h of 3D culture (Figure 5C). This analysis did not detect any evident necrotic area in spheroids collected at different timepoints and excluded the presence of inflammatory process [57]. Furthermore, immunohistochemical analysis of paraffin-embedded sections of spheroids showed positivity for vimentin in all spheroids (Figure 5D). These results demonstrated that spheroids collected at both 72 h and 216 h are formed by cells preserving their mesenchymal origin (Figure 5D). The little decrease of the volume observed in the spheroids collected at 216 h is due to a compaction process, as previously described [21]. Taken together all these data suggested that spheroids collected at 72 h and 216 h are comparable in their activation, inflammation and mesenchymal states. Hence, they can equally be used as fibroblast aggregates in our experimental system.

3.1.4 Reversion of spheroids to adhesion and monolayer growth

In our previous work, we have demonstrated that the myofibroblast deactivation within spheroids collected at 216 h, then transferred to plastic culture plates and maintained in bidimensional (2D) culture for 12 days, could be an

irreversible process. In particular, fibroblasts derived from these spheroids present stress fibers less developed and express lower α-SMA levels, with respect to the cells from spheroids collected at 96 h and reverted to adhesion growth for 12 days [21]. Therefore, to deepen the deactivation process of fibroblasts from spheroids collected after 216 h of 3D culture on agar, we have generated the so-called reverted-fibs, i.e. fibroblasts outgrowing from myofibroblast spheroids collected after 216 h of 3D culture and then transferred to standard culture dishes, to allow cell adhesion and spreading. Reverted-fibs were expanded and maintained in culture as 2D monolayers for about 30 days in order to allow the achievement of a stable phenotype.

3.1.5 Evaluation of inflammation, activation and mesenchymal markers

To analyse the state of activation and inflammation of our cultured fibroblasts, we have evaluated, by western blotting, the levels of α-SMA and COX-2 in protein extracts of cutaneous myofibroblasts, spheroids collected at 72 h and reverted-fibs (Figure 6). The densitometric analysis showed a dramatic and significant decrease of both α-SMA and COX-2 levels in reverted-fibs and spheroids compared with myofibroblasts, whereas it did not show any difference between reverted-fibs and spheroids. These results have indicated that the deactivation state of fibroblasts within spheroids was stably maintained in reverted-fibs.

The remarkable standard error in the densitometric analysis of α-SMA and COX-2 levels in reverted-fibs (Figure 6B,C) is due to the presence of specimens that do not express both the proteins. Hence, the significant differences in α-SMA and COX-2 levels indicated that myofibroblasts, cells of spheroids and reverted-fibs represent distinct stages of fibroblast differentiation.

Furthermore, we have analysed by western blotting the protein levels of the mesenchymal marker vimentin. The densitometric analysis did not show any significant differences of vimentin levels in all samples (Figure 6D). Hence, all the analysed fibroblast populations preserve their mesenchymal origin.

3.1.6 Evaluation of fibroblast viability

The total amount of ATP produced by cells can be used as an indicator of the cell proliferation rate, because ATP levels are directly proportional to the number of metabolically active cells that are present in culture [147]. Therefore, to analyse and compare the proliferative capability of BJ-5ta cells, reverted-fibs and myofibroblasts, we have performed the ATP cell viability assay on these three fibroblast populations incubated with cell culture standard medium (Figure 7). This analysis has showed that cell viability of reverted-fibs is significantly greater than that of both BJ-5ta and myofibroblasts. Hence, reverted-fibs have acquired a proliferative phenotype.

3.1.7 Evaluation of fibroblast migratory capability

We have analysed the migratory capability of BJ-5ta cells, reverted-fibs and myofibroblasts by wound healing assays (Figure 8). This analysis showed a greater wound healing capability of both BJ-5ta cells and reverted-fibs with respect to myofibroblasts. In particular, at 24 h after wounding, the quantitative analysis (Figure 8B) showed that in both BJ-5ta and reverted-fib cultures the scratch area was almost totally closed. On the other hand, at the same time point, in myofibroblast culture the percentage of open surface area was still about 50 %.

It is known that fibroblasts belonging to distinct stages of differentiation, display different migratory capability [29]. Furthermore, α-SMA protein levels and fibroblast migration are inversely correlated [148]. Therefore, it is possible to hypothesize that the significant greater migratory capability of both BJ-5ta cells and reverted-fibs may be due to their lower levels of α-SMA protein. Furthermore, the observed differences in the migratory capability between BJ-5ta, reverted-fibs and myofibroblasts, also support the hypothesis that the three fibroblast populations belong to different stages of differentiation [29,149].

3.1.8 Cytoskeleton organization analysis of reverted-fibs and myofibroblasts

Confocal fluorescence and immunofluorescence analysis showed that both reverted-fibs and myofibroblasts contained stress fibers and vimentin intermediate filaments (Figure 9). However, the analysis of stress fibers by phalloidin staining showed fewer and less developed stress fibers in reverted-fibs compared with myofibroblasts (Figure 9). In particular, the cytoskeleton organization of reverted-fibs resembled this one of proto-myofibroblasts, which represent the intermediate step during fibroblast to myofibroblast differentiation [29].

Furthermore, it is known that most non-motile cell types contain thick fibers, whereas most motile cell types contain very few and thin stress fibers [150]. This is consistent with our results regarding the different migratory capability of myofibroblasts and reverted-fibs. Indeed, myofibroblasts displayed slower motility and more developed stress fibers respect to reverted-fibs, which are highly motile cells and have thin stress fibers [29].

3.1.9 *In vitro* model of fibroblasts belonging to different stages of differentiation

Therefore, we have developed an *in vitro* experimental model, resembling a part of the stromal microenvironment that is composed of inactivated fibroblasts, proto-myofibroblasts, myofibroblasts and fibroblast aggregates. BJ-5ta cells are inactivated fibroblasts, because these cells do not express α-SMA and COX-2, display lower proliferation and greater motility compared with myofibroblasts. Furthermore, we have identified reverted-fibs as proto-myofibroblast-like cells. Indeed, likewise proto-myofibroblats, reverted-fibs are characterized by a dramatic decrease of α-SMA and COX-2 protein levels, few and less organized stress fibers, and a proliferative and migratory phenotype [29,151]. Therefore, the *in vitro* generation of proto-

myofibroblasts from human primary cutaneous myofibroblasts, support the concept that myofibroblasts are not terminally differentiated cells. Hereafter, we refer to reverted-fibs as proto-myofibroblasts.

3.2 Influence of stromal cell signals on melanoma cells

3.2.1 Evaluation of melanoma cell viability

Several studies have documented that the interaction of melanoma cells with their surrounding microenvironment promotes and sustains metabolic reprogramming, melanoma growth and progression [61,84,126,134,142,152]. In particular, during melanoma development, the crosstalk between skin fibroblasts and malignant melanocytes triggers the differentiation of fibroblasts into CAFs, which are known to promote and sustain cancer [84]. On the other hand, the role of normal stromal fibroblasts in solid tumours such as melanoma, before CAF differentiation, is largely unexplored and poorly understood. Therefore, in order to study the crosstalk between normal fibroblasts and melanoma cells, we have analysed the effect of the conditioned media of BJ-5ta cells, proto-myofibroblasts, myofibroblasts and spheroids on the viability, migration and invasion of A375 and A2058 melanoma cell lines. Hence, to evaluate the viability of melanoma cells we have performed ATP cell viability assay on A375 and A2058 cells treated with the conditioned media of fibroblasts of our experimental system: BJ-5ta cells, proto-myofibroblasts, myofibroblasts and fibroblast spheroids (Figure 10). The controls were represented by A375 and A2058 cells treated with their own conditioned media.

This analysis showed that the viability of both cell lines treated with the conditioned media of proto-myofibroblasts, myofibroblasts and spheroids was significantly lower when compared with cells treated with standard culture medium and their own conditioned medium, used as control (Figure 10). Particularly, compared with control, A375 cells treated with proto-myofibroblast-, myofibroblast- and spheroid-derived conditioned media showed a reduction in cell viability of about

80 %, 50 % and 70 %, respectively (Figure 10A). The conditioned media of proto-myofibroblasts, myofibroblasts and spheroids reduced A2058 cell viability of about 90 %, 75 % and 90 %, respectively (Figure 10B). Interestingly, the conditioned medium of proto-myofibroblasts triggered a higher reduction of cell viability compared with myofibroblast-derived conditioned medium. Conversely, BJ-5ta-derived conditioned medium did not affect he viability of both A375 and A2058 cells. Furthermore, it is noteworthy that both A375 and A2058 cells incubated with standard culture medium, containing 10 % fresh serum, did not display any difference in cell viability compared with their respective controls. This experimental evidence indicates that diverse amounts of serum contained in melanoma cell-derived conditioned media and standard culture medium affect melanoma cell viability to the same extent. To further evaluate if different amounts of serum in conditioned media could affect cell viability of both melanoma cell lines, we have analysed cell viability of A375 (Figure 11A) and A2058 (Figure 11B) cells exposed to their own conditioned medium, with or without addition of 10 % fresh serum, or to proto-myofibroblast-derived conditioned medium, with or without addition of 10 % fresh serum. No differences were observed in the viability of both A375 and A2058 cells treated with either proto-myofibroblast-derived conditioned medium or the same conditioned medium supplemented with fresh serum. Similarly, there were no differences in the viability of both melanoma cell lines incubated with their own conditioned medium (Control) and the cells treated with the same conditioned medium supplemented with fresh serum. On the contrary, a significant decrease in cell viability was observed in both melanoma cell lines treated with proto-myofibroblast-derived conditioned media, supplemented or not with fresh serum, compared with melanoma cells incubated with their own conditioned media with or without addition of fresh serum (Figure 11). These results confirm the anti-proliferative effect exerted by proto-myofibroblasts on both A375 and A2058 cells, and exclude that diverse serum amounts in the conditioned media affect melanoma cell viability.

3.2.2 Evaluation of melanoma cell death

To investigate if the reduction in melanoma cell viability was associated with cell death, we have evaluated the effect of fibroblast culture-derived conditioned media on the number of nuclei with a sub-diploid content through cytofluorimetric analysis. The controls were represented by A375 and A2058 cells treated with their own conditioned media. This analysis showed a significant increase of cell death in A375 cells treated with the conditioned media of proto-myofibroblasts, myofibroblasts or spheroids, when compared with cells treated with BJ-5ta-derived conditioned medium, standard culture medium or control (Figure 12A). It is important to note that the trend of cell viability and cell death is comparable. Additionally, proto-myofibroblast- and spheroid-derived conditioned media induced an impressive and significant cell death increase in A2058 cells (Figure 12B). In particular, A2058 cells treated with the conditioned media of proto-myofibroblasts and spheroids have displayed 6-fold and 3-fold increase of cell death, respectively, when compared with control and cells treated with BJ-5ta-derived conditioned medium. No differences in cell death were observed between A2058 cells treated with standard culture medium, their own conditioned medium, BJ-5ta- or myofibroblast-derived conditioned media. It is important to highlight that melanoma cell-derived conditioned media and standard culture medium influence the cell death of both melanoma cell lines to the same extent. Therefore, these results exclude that diverse serum amounts in the conditioned media affect melanoma cell death.

3.2.3 Evaluation of melanoma cell migration and invasion

Wound Healing Assay was used to test the migratory capability of melanoma cells under the influence of conditioned media derived from BJ-5ta, proto-myofibroblasts, myofibroblasts and spheroids. Controls were represented by A375 and A2058 cells treated with their own conditioned media. As shown in figure 13, the conditioned media of both proto-myofibroblasts and spheroids reduced significantly

the migration of the low metastatic A375 cells [153], respect to control and cells treated with BJ-5ta-derived conditioned medium. Conversely, the conditioned medium of myofibroblasts did not significantly influence the migratory capability of A375 cells compared with control. In particular, at 48 h after wounding, the quantitative analysis indicated that in A375 cell culture containing proto-myofibroblast- or spheroids-derived conditioned media the wound was almost open. Conversely, the percentage of open surface area in the control and in A375 cells cultured with BJ-5ta-conditioned medium was about 55 % and 40 %, respectively (Figure 13B). Additionally, also the wound healing capability of the high metastatic A2058 cells [153] treated with the conditioned media of BJ-5ta, proto-myofibroblasts, myofibroblasts and spheroids was significantly decreased compared with control (Figure 14). In particular, at 48 h after wounding, the percentage of open surface area was about 20 % in A2058 cells containing their own conditioned medium (Control). Conversely, in A2058 cells treated for 48 h with BJ-5ta-, proto-myofibroblast-, myofibroblast- or spheroid-derived conditioned media the percentages of open wound areas were about 60 %, 90 %, 50 % and 75 %, respectively (Figure 14B).

Additionally, to further evaluate if different amounts of serum present in conditioned media could affect the migratory capability of both A375 and A2058 cells, we have performed wound healing assays on both melanoma cell lines treated with their own conditioned medium, with or without addition of 10 % fresh serum, or with proto-myofibroblast-derived conditioned medium, with or without addition of 10 % fresh serum (Figure 15). This analysis has showed no differences in the migratory capability between A375 cells treated with proto-myofibroblast-derived conditioned medium and melanoma cells exposed to the same conditioned medium refreshed with 10 % fresh serum. Similarly, there were no differences between A375 cells cultured with their own conditioned medium (Control) and cells incubated with the same conditioned medium refreshed with 10 % fresh serum. On the contrary, the conditioned media of proto-myofibroblasts, supplemented or not with fresh serum,

decreased significantly the migration of A375 cells compared with the same cells treated with their own conditioned media with or without addition of fresh serum (Figure 15A,C). Similarly, our results revealed no differences in cell migration between A2058 cells treated with proto-myofibroblast-derived conditioned medium and melanoma cells incubated with the same medium refreshed with fresh serum (Figure 15B,D). Instead, it is important to note that proto-myofibroblast-derived conditioned media, with or without addition of fresh serum, significantly decreased the migratory capability of A2058 cells respect to melanoma cell-derived conditioned media, with or without addition of fresh serum. Finally, taken together all these data demonstrated that the different amounts of serum present in conditioned media of melanoma cells and proto-myofibroblasts did not modify the anti-migratory effect exerted by proto-myofibroblasts on both melanoma cell lines.

Invasion assays performed on A375 and A2058 cells treated with the conditioned media of the different fibroblast populations, did not show any difference in the invasiveness capability of both cell lines (data not shown).

3.3 Influence of melanoma cell signals on fibroblast populations

3.3.1 Evaluation of fibroblast viability

During melanoma progression, the continuous interaction with malignant melanocytes triggers skin fibroblasts to differentiate into CAFs [84], which are known to play a crucial role in melanoma progression and dissemination [61,154]. Upon interaction with cancer cells, CAFs acquire a catabolic phenotype and exhibit a slower proliferation rate and an increased migratory capability compared with normal fibroblasts [27,155,156].

Therefore, to analyse the influence of melanoma cell signals on the viability of fibroblasts at different stages of differentiation, we have performed ATP cell viability

assay on BJ-5ta cells, proto-myofibroblasts and myofibroblasts incubated for 48 h with standard culture medium or with the conditioned media of A375 or A2058 cells. Controls were represented by fibroblast cells incubated with their own conditioned medium. This analysis showed that the viability of BJ-5ta cells treated with A375- or A2058-derived conditioned media was significantly lower compared with the same cells treated with either standard culture medium or their own conditioned medium (Control) (Figure 16A). Conversely, the different serum amounts present in standard culture medium and in conditioned media of BJ-5ta cells influenced the cell viability equally.

Interestingly, the conditioned media of both melanoma cell lines did not exert any effect on the viability of both proto-myofibroblasts and myofibroblasts. Indeed, the viability of proto-myofibroblasts treated for 48 h with melanoma cell-derived conditioned media was comparable with that observed in the same cells incubated with their own conditioned medium (Control) (Figure 16B). Similarly, myofibroblasts incubated with A375- or A2058-derived conditioned medium did not show differences in cell viability compared with myofibroblasts treated with either standard culture medium or their own conditioned medium (Control) (Figure 16C). Furthermore, the different serum amounts present in standard culture medium and in the conditioned media of both proto-myofibroblasts and myofibroblasts affected the cell viability to the same extent (Figure 16).

3.3.2 Evaluation of fibroblast migration under 3D culture conditions

To evaluate the influence of melanoma cell signals on the 3D migration of fibroblasts at different stages of differentiation, we have performed a spheroid migration assay. To this aim, we have cultured BJ-5ta cells, proto-myofibroblasts and myofibroblasts as spheroids. We have placed spheroids formed by the different types of fibroblasts on top of a conventional cell culture dishes in presence of either standard culture medium or A375- or A2058-derived conditioned media. Therefore, we have

analysed the outgrowth and migration of fibroblasts for 24 h (Figure 17). This analysis showed that the conditioned media of both melanoma cell lines did not affect the outgrowth and the migration of proto-myofibroblasts from the spheroids (Figure 17B,E). Conversely, melanoma cell-derived conditioned media significantly increased the outgrowth and migration of both BJ-5ta cells (Figure 17A,D) and myofibroblasts (Figure 17C,F) compared with the same fibroblast spheroids incubated with standard culture medium. In particular, BJ-5ta spheroids incubated for 24 h with standard culture medium, conditioned media of A375 or A2058 cells, showed 1.5-, 2.8-, 2.9-fold increase in their migration areas, respectively, compared with spheroids at 0 h (Figure 17D). Additionally, myofibroblast spheroids treated with standard culture medium or A375- or A2058-derived conditioned media, showed 1.9-, 2.6-fold and 3.6-fold increase in their migration areas, respectively (Figure 17F). Interestingly, the conditioned medium of the high aggressive A2058 cells significantly increased the outgrowth and migration of myofibroblasts from spheroids compared with myofibroblast spheroids incubated with either standard culture medium or A375-conditioned medium (Figure 17F).

To the best of our knowledge, aggregates of fibroblasts are formed in dermis at early stage of melanoma development. A distal paracrine interaction between melanoma cells and fibroblast aggregates leads to fibroblast reprogramming associated with increased migration, a typical feature of CAFs [61]. In fact, it is well-known that CAFs have an increased migratory capability than normal fibroblasts [61,106]. Therefore, our results indicated that melanoma cells could trigger a CAF-like migratory phenotype in BJ-5ta cells and myofibroblasts, whereas they had no effect on proto-myofibroblast. In fact, the conditioned media of both melanoma cell lines did not alter the 3D migratory capability of proto-myofibroblasts.

3.4 Influence of stromal cell signals on fibroblast populations

We have evaluated if potential paracrine signals from the stromal microenvironment of our *in vitro* experimental system, could affect the metabolic activity and the viability of fibroblast cell types grown as monolayers. For this purpose, we have performed ATP cell viability assay on BJ-5ta cells (Figure 18A), proto-myofibroblasts (Figure 18B) and myofibroblasts (Figure 18C) incubated for 48 h with either standard culture medium or with the conditioned media collected from BJ-5ta, proto-myofibroblasts and myofibroblasts, grown as monolayers, or from spheroids formed by myofibroblasts. This analysis showed that the viability of BJ-5ta, proto-myofibroblasts and myofibroblasts was not affected by the conditioned media derived from the different fibroblast populations of our experimental system. Furthermore, no differences in cell viability were observed between fibroblasts incubated with standard culture medium and fibroblasts exposed to stroma-derived conditioned media. Therefore, once again, this experimental evidence suggests that diverse quantity of serum present in fibroblastic conditioned media and standard culture medium influences fibroblast viability equally. Additionally, these data also suggest that the cytotoxic activity of proto-myofibroblasts may be specific for melanoma cells, because the conditioned medium of proto-myofibroblasts did not affect the viability of BJ-5ta cells that represent ubiquitous non-activated fibroblasts, present in all body tissues.

3.5 Secretome profiles of melanoma cells and proto-myofibroblasts

It is known that tumour-stroma interactions are influenced by cytokines and growth factors [126]. Therefore, we have analysed the secretome profile of A375 cells, A2058 cells and proto-myofibroblasts. To this aim, the conditioned media of melanoma cells and proto-myofibroblasts were hybridized on cytokine array membranes, which detect 42 different cytokines. The cytokines/chemokines production levels in the

conditioned medium of proto-myofibroblasts were compared with the levels of the factors secreted in A375- and A2058-derived conditioned media (Figure 19). Densitometric analysis assessed by ImageJ software indicated a significant decrease of some cytokines and growth factors secretion in the conditioned medium of proto-myofibroblasts compared with the conditioned media of melanoma cells. Of these 42 cytokines, 6 (TGF-β1, GM-CSF, RANTES, IL-13, IL-8 and GRO-α) were significantly downregulated in the conditioned medium of proto-myofibroblasts. In particular, in proto-myofibroblast-derived conditioned medium, TGF-β1, GM-CSF, RANTES, IL-13, IL-8 and GRO-α showed 0.31-, 0.50-, 0.54-, 0.59-, 0.75- and 0.81- fold change respectively in comparison with the same secreted factors detected in A375-conditioned medium (Figure 19A). Furthermore, in proto-myofibroblast-derived conditioned medium TGF-β1, GM-CSF, RANTES, IL-13, IL-8 and GRO-α showed 0.28-, 0.57-, 0.64-, 0.69-, 0.74- and 0.76- fold change, respectively, compared with the same factors secreted in A2058-derived conditioned medium (Figure 19B). Additionally, this analysis also revealed a significant downregulation of CCL2 in proto-myofibroblast-conditioned medium (0.72- fold change) compared with CCL2 secreted in A2058-conditioned medium (Figure 19B). Taken together all these results indicate that the conditioned medium of proto-myofibroblasts could represent an unfavorable microenvironment for melanoma cell viability and migration.

Chapter 4

DISCUSSION

Solid tumours are abnormal masses of tissue that consist of cancer cells and non-malignant components, which together represent the tumour microenvironment [157]. In particular, the microenvironment of solid tumors is extremely complex and consists of non-cellular components, such as the ECM, and non-malignant cells such as fibroblasts, endothelial cells, pericytes, immune and inflammatory cells, bone marrow derived cells, which establish a complex crosstalk with cancer cells [158].

Among solid tumours, melanoma is one of the most aggressive, metastasizing and drug resistant malignancies in human and is responsible for approximately 60% of lethal skin tumours [84,159]. The evolution, structure and activities of the cells in the tumour microenvironment show many parallels with the processes occurring during wound healing and inflammation [85]. In fact, melanoma development is associated with recruitment and activation of stromal cells, such as immune and inflammatory cells and fibroblasts [88] and is deeply influenced by the crosstalk between melanoma cells and the tumour microenvironment [84]. The primary stage of melanoma is represented by an uncontrolled proliferation of melanocytes from the basal epidermis toward the upper epidermis (melanoma in situ). Later, during the vertical growth phase, melanoma cells invade the dermis and interact actively and extensively with mesenchymal elements, mainly fibroblasts, and blood vessels, allowing metastasis [160,161]. Therefore, the dynamic interplay between melanoma cells and fibroblasts forces fibroblasts to enter a continuous state of constitutive activation and to differentiate into CAFs [61,126,162]. Zhou *et al.* demonstrated that, before CAF differentiation, dermal fibroblasts block the onset of melanoma by inducing cell cycle arrest and by preventing EMT [152]. Conversely, when fibroblasts

differentiate irreversibly into CAFs, they furnish the building blocks for macromolecules biosynthesis and ATP production, which are both required for the rapid proliferation and growth of cancer cells, and for the progression of metastatic melanoma [142,163]. In addition to normal fibroblasts and CAFs, the stromal microenvironment of melanoma consists also of fibroblast aggregates. In fact, Dror *et al.* showed that fibroblasts form aggregates in the dermis at early stages of melanoma development and that a paracrine interaction with melanoma cells leads to CAF differentiation and formation of a dermal tumour niche [61]. Therefore, in this work, we have developed an *in vitro* experimental system mimicking part of the stromal microenvironment consisting of fibroblasts in different stages of activation, such as inactivated fibroblasts, proto-myofibroblasts and myofibroblasts, and aggregates of inactivated myofibroblasts, namely spheroids. In particular, we have generated proto-myofibroblasts from primary myofibroblasts forced to grow as spheroids for 216 h and then transferred to cell culture dishes to allow the adhesion growth. Therefore, we have provided evidence for myofibroblast de-activation *in vitro*, during spheroids formation. This de-activation is maintained in fibroblast spheroids transferred to plastic culture plates, expanded, and then maintained in culture as monolayers for about 30 days to allow the achievement of a stable phenotype. Thus we have supported the concept that myofibroblasts are not terminally differentiated cells [21,58,59]**.** However, further studies are necessary to identify the mechanisms that regulate the de-differentiation of myofibroblasts into proto-myofibroblasts during *in vitro* 3D cultures.

Proto-myofibroblasts represent the intermediate cell type during the activation process of fibroblasts into myofibroblasts and are characterized by α-SMA-negative stress fibers. They appear in early granulation tissue of open wound during tissue repair [29]. Furthermore, proto-myofibroblasts are present as a stable cell phenotype in specialized normal connective tissue, such as normal alveolar septum. During lung injury, the repair process is associated with the neoformation of differentiated myofibroblasts [44]. Furthermore, proto-myofibroblasts have been described as cells

with a proliferative and migratory phenotype, whereas myofibroblasts are cells involved in the contraction and synthesis of ECM and characterized by α-SMA-positive stress fibers [29,149]. Additionally, it is known that fibroblasts cultured *in vitro* on high stiffness substrate, such as plastic dishes, differentiate into myofibroblasts and express COX-2 in the presence of culture medium supplemented with FBS [21]. In this work we have showed that the proto-myofibroblasts of our stromal system, as well as proto-myofibroblasts found *in vivo*, are characterized by a dramatic decrease of α-SMA and COX-2 protein levels and an increase of migratory capability and cell viability when compared with myofibroblasts. Additionally, when compared with inactivated fibroblasts, represented by BJ-5ta cells, proto-myofibroblasts showed a greater proliferative rate. Furthermore, the confocal analysis of cytoskeleton showed that proto-myofibroblasts have fewer and less developed stress fibers compared with myofibroblasts.

To the best of our knowledge, this is the first study that combines the development of an *in vitro* stromal microenvironment containing primary proto-myofibroblasts and its crosstalk with melanoma cells. In this work, we have demonstrated that the conditioned medium of proto-myofibroblasts triggers a decrease of cell viability and an increase of cell death in both A375 and A2058 cells, even if these effects were more evident in A2058 cells. A significant reduction of cell viability was also detected in both melanoma cell lines treated with the conditioned medium of either myofibroblasts or spheroids, even if a stronger decrease of cell viability was detected in melanoma cells incubated with proto-myofibroblast-derived conditioned medium. Furthermore, we have demonstrated that the anti-proliferative and cytotoxic role of proto-myofibroblast-derived conditioned medium is specific for melanoma cells because it does not affect the viability of BJ-5ta fibroblasts, which could represent ubiquitous mesenchymal cells localized in almost all adult tissues.

Metastasis is a crucial step for tumour progression, and it also represents the biggest threat to survival for patients with solid tumours [164]. The migratory and invasiveness capabilities are needed for the dissemination of cancer cells from the

primary tumour towards a secondary site, leading to a more aggressive, invasive and lethal phenotype [165]. In this scenario, stromal cells, especially fibroblasts, play a very important role in the regulation of tumour progression and dissemination [62–64]. On this basis, we decided to investigate how fibroblasts in different stages of activation influence the migratory capability of the low metastatic A375 and high metastatic A2058 cells [153]. In particular, we have demonstrated that the conditioned medium of proto-myofibroblasts inhibits dramatically the migratory capability of both melanoma cell lines. Although myofibroblast- and spheroid-derived conditioned media reduce significantly the migration of A2058 cells with respect to control, a stronger anti-migratory response was observed in melanoma cells incubated with the conditioned medium of proto-myofibroblasts. Furthermore, it is noteworthy that the addition of fresh serum in the conditioned media of melanoma cell lines and proto-myofibroblasts did not influence the paracrine interactions between these cell populations. Our data support other studies showing the crucial role of fibroblasts on melanoma growth and progression also before fibroblast to CAF transition. Conversely, we did not observe any effect on the invasiveness capability of both melanoma cell lines treated with the conditioned media of the fibroblasts of our experimental system. Of note, melanoma invasiveness can depend on physical interactions with fibroblasts. In fact, Izar *et al.* reported that only after a direct physical interaction with cancer cells, CAFs strongly enhance melanoma invasiveness, whereas CAF-derived conditioned medium fails to affect melanoma invasiveness [142]. Hence, we supposed that also in our experimental system fibroblasts could need cancer cell proximity to affect the invasion capability of melanoma cells.

Subsequently, in order to identify chemokines, cytokines and/or growth factors associated with the cytotoxic and anti-migratory effects of proto-myofibroblast-derived conditioned medium, we have analysed the secretome profile of the conditioned media of melanoma cells and proto-myofibroblasts. This analysis showed that in the conditioned medium of proto-myofibroblasts there are lower levels of some cytokines and growth factors such as TGF-β1, GM-CSF, RANTES, IL-13, IL-8 and

GRO-α compared with the levels of the same factors secreted in the conditioned media of both A375 and A2058 cells. Additionally, the levels of CCL2 is significantly downregulated in proto-myofibroblast-derived conditioned medium with respect to A2058-conditioned medium. Therefore, we supposed that the cytokine environment created by proto-myofibroblasts could counteract melanoma proliferation and migration. In fact, the cytokines and growth factors that are downregulated in proto-myofibroblast-derived conditioned medium, are known to be increased and involved in melanoma progression. In particular, it is known that TGF-β signalling promotes melanoma development, aggressiveness and metastasis [166,167]. Furthermore, TGF-β pathway drives immune evasion and influences the efficacy of anti-CTLA-4 immunotherapy [166,168]. It was reported that the granulocyte–macrophage colony-stimulating factor (GM-CSF) promotes tumour growth and angiogenesis *in vivo* [169]. Furthermore, some preclinical data indicated that GM-CSF may sometimes promote tumour growth, whereas other studies reported its anti-tumour role [170]. The C-C chemokine receptor type 5 (CCR5) and its ligand named regulated upon activation, normal T-cell expressed, and secreted (RANTES) are involved in the paracrine interaction between cancer cells and fibroblasts. In particular, cancer cells secrete RANTES or induce fibroblasts to secrete RANTES which in turn binds to its receptor CCR5 expressed by tumour cells. The activation of RANTES/CCR5 axis promotes melanoma progression by sustaining tumour cell proliferation and dissemination, recruitment of immunosuppressive cells (T-reg cells, monocyte), osteoclast activation, bone metastasis and neoangiogenesis [171]. Additionally, in melanoma, higher levels of RANTES secretion are associated with higher malignancy and increased tumour formation *in vivo* [171,172]. Interleukin-13 (IL-13) and its receptor interleukin (IL)-13Rα2 promote migration and metastasis in solid tumours [173]. Furthermore, interleukin-8 (IL-8) and its receptors, C-X-C chemokine receptor 1 and (CXCR1 and CXCR2), sustain the initiation, immunosuppression, angiogenesis and metastasis in many solid tumours, including melanoma. In particular**,** the metastatic potential of IL-8 is linked to its ability to support vascularization, MMP-2 activation, and anoikis

resistance [174]. Additionally, growth-regulated oncogene α (GRO-α), also called chemokine (C-X-C motif) ligand 1 (CXCL1), is overexpressed in melanoma and contributes to the establishment and the maintenance of the tumour potential of melanoma. In particular, GRO-α supports melanoma growth, survival, angiogenesis and metastasis [175]. C-C motif chemokine ligand 2 (CCL2) together with its receptor CCR2 play key roles in cancer metastasis by promoting cancer cell proliferation and survival, migration and invasion, and by inducing inflammation and angiogenesis [176].

As well as fibroblasts play an important role in the modulation of cancer cell proliferation, viability and migration, also tumour cells can affect the viability, the proliferation and migration of fibroblasts. The dynamic interactions with cancer cells lead to CAF differentiation, which is associated with changes in the proliferation rate, metabolic activity and migratory capability of fibroblasts [27]. In particular, it has been reported that in solid tumours, CAFs acquire a catabolic phenotype, which is associated with a decrease of cell proliferation [108]**.** CAFs are enslaved by tumour and sustain the massive and uncontrolled proliferation of cancer cells by providing high energy metabolites, especially lactate, pyruvate and ketone bodies, which are used by cancer cells [27,177]. Furthermore, at early stages of melanoma, the crosstalk between cancer cells and fibroblast aggregates leads to fibroblast reprogramming associated with increased migration that is a typical feature of CAFs [61]. In this study, we have demonstrated that the conditioned media of both melanoma cell lines reduced only the viability of BJ-5ta cells, without affecting the viability of proto-myofibroblasts and myofibroblasts. Furthermore, we have demonstrated that melanoma cell-derived conditioned media increased the outgrowth and migration of BJ-5ta cells and myofibroblasts from spheroids. Interestingly, melanoma cell-derived conditioned media did not modify the outgrowth and the migratory capability of proto-myofibroblasts grown as spheroids. Therefore, these data indicate that the interactions between melanoma cells and proto-myofibroblasts, mediated by melanoma cell-derived conditioned media, did not trigger the reprogramming of proto-

myofibroblasts towards a CAF-like phenotype. Although further investigations are necessary to identify the mechanisms responsible for the potential anti-tumour activity exerted by proto-myofibroblasts, the current study shows that proto-myofibroblasts could represent a cell type useful to study new therapeutic strategies targeting melanoma. In this perspective, *in vivo* studies should be employed in order to evaluate the anti-tumour activity of proto-myofibroblasts.

Chapter 5

CONCLUSIONS

We have developed an *in vitro* experimental system that is able to reproduce the phenotypic and functional heterogeneity of fibroblast populations observed *in vivo* during wound healing process. In particular, this *in vitro* experimental system consists of inactivated fibroblasts, proto-myofibroblasts, myofibroblasts and fibroblast aggregates. The generation of proto-myofibroblast-like cells from primary cutaneous myofibroblasts support the concept that myofibroblasts are not terminally differentiated cells and can return to a less activated state. It is well known that fibroblasts, proto-myofibroblasts and myofibroblasts play an active and important role during wound healing process. Furthermore, fibroblast aggregates have been found in both wound healing process and cancer, which is often described as wound that never heals. The tumour microenvironment, as well as the wound microenvironment, consists of inactivated fibroblasts, activated fibroblasts and fibroblast aggregates. Therefore, we demonstrated that fibroblasts in different stages of activation influence differently melanoma cell proliferation and migration. In particular, for the first time, this work documented the inhibitory role of proto-myofibroblasts in melanoma cell proliferation and migration. In fact, to the best of our knowledge, there is no experimental evidence describing the *in vitro* and *in vivo* interaction between proto-myofibroblasts and melanoma cells. We showed a greater *in vitro* anti-tumour activity exerted by the proto-myofibroblast-conditioned medium on melanoma cells with respect to myofibroblast-derived conditioned medium. Hence, this study also indicates that the processes leading to the de-differentiation of myofibroblasts into proto-myofibroblasts could be pivotal to enhance the anti-tumour activity of normal fibroblasts. However, further studies are needed to elucidate the mechanisms that

regulate myofibroblast to proto-myofibroblast de-differrentiation and the anti-tumour activity of proto-myofibroblasts.

Chapter 6

MATERIALS AND METHODS

6.1. Cutaneous tissues

Normal skin specimens were obtained from donors (n = 11 females; mean age 46.7 ± 5.6) undergoing neck surgery for benign pathology. Patients with metabolic and connective tissue diseases were excluded. The investigation was in agreement with the principles outlined in the Declaration of Helsinki [178] and informed consent was obtained from all the patients. The study reported in the manuscript has received the approval from the Ethic Committee of the University of Naples Federico II (Comitato Etico Università Federico II). The assigned protocol number of the study is 228/18.

6.2 Cell cultures, spheroids generation and preparation of conditioned medium

Human cutaneous fibroblasts were obtained by using an explant technique and cultured as previously described [21]. Reverted-fibs were obtained from spheroids grown for 216 h on agar and then transferred to plastic culture plates, expanded and maintained in culture as 2D monolayers for about 30 days to allow the achievement of a stable phenotype. All the experiments were performed only with cells from early passage, within the ninth passages.

The human melanoma (A375 and A2058) and foreskin fibroblast (BJ-5ta) cell lines were kindly provided by CEINGE (Naples, Italy). Cells were cultured in Dulbecco's minimal essential medium (DMEM) (Sigma-Aldrich, Saint Louis, Missouri, USA), supplemented with 10 % FBS (GIBCO, Grand Island, New York, USA), antibiotics (100 mg/ml penicillin, 100 mg/ml streptomycin) (all purchased by Sigma-

Aldrich) (standard culture medium). The plates were maintained at 37°C with 5% CO_2 and 95 % humidity in a HeraCel (Heracus) incubator, with fresh culture medium changed every 3 days. Cell treatments were carried out after 24 h from plating. Cells were observed with a phase contrast microscope Olympus CKX41 model and the images were acquired with a camera connected to its by means Cell-A software. For the conditioned media preparation, melanoma cell lines and all the fibroblastic cell types (1 x 10^4 cells/well) were seeded in 96-well plate and cultured in standard culture medium. After 48 h, 50 µl of fresh standard culture medium were added in each well. Then, conditioned media were collected after 72 h and stored at -20 °C or -80 °C until use. To exclude that the observed effects on melanoma cell viability and migration were due to the amounts of serum in the conditioned media, we have refreshed proto-myofibroblast- and melanoma cell- conditioned media by adding the same amount (10 %) of fresh serum, before performing ATP and wound healing assays. For spheroid conditioned medium preparation, myofibroblasts were grown as spheroids and the medium was collected after 72 h of 3D culture. Alternatively, for the spheroid outgrowth experiments, 1 x 10^6 A375 and A2058 melanoma cells were seeded in 10-cm cell culture dishes, incubated in standard culture medium and the media was collected after 24 h.

All chemicals were of analytical grade and were purchased from Sigma-Aldrich.

6.3 Immunofluorescence analysis of fibroblasts

Morphological analysis of myofibroblasts and reverted-fibs was performed by plating the cells on glass coverslips (Bio-Optica, Milan, Italy). Once adherent cells were more than 75 % confluent, they were washed in 1 X PBS, fixed in 4% paraformaldehyde (Merk Millipore, Billerica, Massachusetts, USA) for 20 minutes at room temperature and washed again, according to standard techniques. Then cells were permeabilized with 0.1 % Triton X-100 (VWR International Srl, Milan, Italy) and blocked in donkey serum (Millipore, Billerica, MA, USA), diluted 1 : 10 in 1× PBS, for 30 min at room temperature. Glass coverslips were incubated with a mouse monoclonal anti-vimentin primary

antibody (Sigma-Aldrich), diluted 1 : 50, for 1 h at 37 °C. After three washes in 1× PBS, glass coverslips were subsequently incubated with a FITC donkey anti-mouse secondary antibody (Jackson ImmunoResearch, Suffolk, UK) diluted 1 : 50 and phalloidin-TRITC (Sigma-Aldrich) diluted 1 : 100, for 1 h at 37 °C. The cell nuclei were counterstained with DAPI (Vector Laboratories, Inc, Burlingame, CA, USA). Glass coverslips were mounted in Vectashield (Vector Labs, Peterborough, UK). The immunofluorescence and fluorescence analysis of both myofibroblasts and reverted-fibs were carried out on an inverted and motorized microscope (Axio Observer Z.1) equipped with a 63×/1.4 Plan-Apochromat objective. The attached laser-scanning unit (LSM 700 4× pigtailed laser 405-488-555-639; Zeiss, Jena, Germany) enabled confocal imaging. For excitation, 405, 488, and 555 nm lasers were used. Fluorescence emission was revealed by Main Dichroic Beam Splitter and Variable Secondary Dichroic Beam Splitter. Triple staining fluorescence images were acquired separately using ZEN Black 2012 software in the red, green and blue channels at a resolution of 512×512 pixels, with the confocal pinhole set to one Airy unit and then saved in TIFF format.

All other chemicals were of analytical grade and were purchased from Sigma-Aldrich.

6.4 Protein extraction and Western blotting analysis

Total protein extracts were prepared from fibroblast monolayer harvested by tripsin/EDTA, and from fibroblast spheroids collected at 72h. Samples were incubated on ice-cold for 30 min in a RIPA lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 % Nonidet P-40, 0.25 % sodium deoxycholate, 1 mM NaF and 1 mM Na3VO4, supplemented with proteases inhibitors cocktails (1 mM DTT, 2 mM PMSF, 2 μg/ml aprotinin and 10 μg/ml leupeptin) (Roche Diagnostics Corporation, Mannheim, Germany). Lysates were centrifuged at 12000 g for 30 min at 4 °C and protein concentration in the supernatants was determined by the Bradford method (Bio-Rad Laboratories, Hercules, California, USA) [179]. Albumin from bovine serum (BSA,

Sigma Aldrich) was used to construct the standard curve. The absorbances were read by Cary Eclipse spectrophotometer (Varian, Santa Clara, California, USA) at 595 nm. Western blotting was performed with equal amounts of total protein extracts (20 μg). Briefly, protein samples were dissolved in SDS-sample buffer, size-fractionated by electrophoresis on 10 % SDS-polyacrylamide gel, under reducing and denaturing conditions at 120 V and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Merck Millipore) at 360 mA for 90 minutes. Molecular weight marker was loaded onto gel as a weight indicator. The membranes were blocked and then incubated for 1 hour with one of the following antibodies: COX-2 (Abcam, Cambridge, UK and Elabscience Biotechnology Inc. USA) α-SMA (Abcam, Cambridge, UK and and Elabscience Biotechnology Inc. USA), Vimentin (Cell Signal Technology Inc., Danvers, MA, USA) and GAPDH (Cell Signal Technology) and then with horseradish peroxidase-linked specific secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h. The membranes were analysed by an enhanced chemiluminescence reaction, using Super Signal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific) according to the manufacturer's instructions. The signals were visualized by autoradiography. The images were acquired by Epson Perfection 2480 Photo (Epson, Suwa, Japan) and densitometric analysis was performed using the Image J 1.48i version (Wayne Rasband National Institutes of Health, USA).

6.5 Immunohistochemistry

Spheroids collected at 72 h and 216 h were fixed in 10 % neutral buffered formalin (Bio-Optica), embedded in paraffin (Bio-Optica), then sliced into serial 4 μm-thick sections and place d on poly-l-lysine coated glass slides (Menzel-Glaser, Brunswick, Germany). Slides were deparaffinized twice in xylene and rehydrated in graded series of ethanol and immersed in 10 mM citric acid (Sigma-Aldrich), pH 6, in a microwave oven (VWR) for three cycles of 5 minutes at 650 Watt, to exclude epitope masking owing to fixation. Spheroid sections were haematoxylin and eosin stained (Bio-

Optica), according to the manufacturer's protocol, for necrosis analysis. Other sections were immunostained with primary antibodies against Vimentin (Ventana Medical Systems, Tucson, AZ, USA) detected by ULTRA View UNIVERSAL DAB DETECTION KIT (Ventana Medical Systems), according to the manufacturer's protocol. Glass coverslips were mounted with BioMount (Bio-Optica). Therefore, these microscopic slides were digitized into high-resolution files through NanoZoomer-2.0RS digital scanner (Hamamatsu, Tokyo, Japan, Asia).

6.6 ATP cell viability assay

To evaluate the influence of the conditioned media collected from BJ-5ta cells, proto-myofibroblasts, myofibroblasts, spheroids and melanoma cell lines on cell viability, fibroblasts or melanoma cells (1×10^4 cells/well) were seeded in 96-well plates and let to grow for 24 h. Then, the culture medium was removed and replaced with 100 µl of conditioned medium. After 48 h of incubation ATP assay was performed by CellTiter-Glo® Luminescent Cell Viability Assay (Promega), according to the manufacturer's protocol.

6.7 Cytofluorimetric analysis of cell death

To evaluate cell death, 1×10^4 melanoma cells/well were seeded into 96-well/plates and let to grow for 24 h. Then, the culture medium was removed and replaced with 100 µl of conditioned medium. After 48 h of incubation, cell suspensions were centrifuged and the pellets were resuspended in a hypotonic lysis solution containing 50 mg/mL propidium iodide (PI). After incubation at 4 °C for 30 min, cells were analysed by flow cytometry to evaluate the number of nuclei with a DNA content lower than the diploid [180].

All chemicals of analytical grade and were purchased from Sigma-Aldrich.

6.8. Wound healing and cell invasion assays

Wound healing assay was performed by seeding 2.5 x 10^5 cells into 35 mm tissue culture plates and growing them to confluence. Then, the confluent monolayer of cells was carefully scratched with a sterilized pipette tip. After wounding, the plates were rinsed twice with 1 X PBS to remove cell debris. Cells were incubated with the conditioned media of all types of fibroblasts and melanoma cells. Photographs of the same fields were taken at 0 h and 48 h with a camera connected to a phase contrast microscope Olympus CKX41 model and the images were acquired using Cell-A software. Quantitative analysis of open wound was performed by measuring the gap area at different time points using Image J Software (National Institute of Health, USA). The ratio between the relative open surface area at indicated time points and at baseline was calculated.

Cell invasion assay was carried out in 24-well matrigel invasion chambers (Corning BioCoat Matrigel Invasion Chamber, Bedford, MA, USA). In brief, A375 and A2058 cells were seeded in invasion chambers (upper chambers, 2×10^4 cells per 24-well invasion chamber) in serum-free medium. The lower chambers were filled with BJ-5ta-, proto-myofibroblast-, myofibroblast- or spheroid-conditioned media and A375- or A2058-conditioned media, used as controls. Following culturing at 37°C and 5% CO_2 for 24 h, the cells that did not migrate through the matrigel membrane were subsequently removed with a cotton swab and the cells at the bottom of the membrane were stained with Diff-Quik Stain Set (Medion Diagnostics, Miami, FL, USA). Then, randomly chosen fields were photographed (X 10) and the number of invaded cells was calculated as a percentage of invasion. The images were taken in bright field with a digital camera (Leica DC200; Leica Microsystems) connected to the microscope (Leica DMLB; Leica Microsystems).

All chemicals were of analytical grade and were purchased from Sigma-Aldrich.

6.9 Spheroid migration assay

We have generated homotypic spheroids from BJ-5ta, proto-myofibroblasts and myofibroblasts by using hanging-drops and coated plates methods, as previously described [21]. Each fibroblast spheroid was transferred to an individual well of a 24-well plate and incubated with standard culture medium or melanoma cell-conditioned media. The area covered by fibroblasts migrating out from the spheroids and spreading on a plastic surface was used as an index of cell migration [181]. The images of the three different types of spheroids were captured at 0 h and 24 h and acquired with a camera connected to a phase contrast microscope Olympus CKX41 model, using Cell-A software. The migration area was determined by calculating the difference between the final area (24 h) and the initial area (0 h) using Image J analysis program.

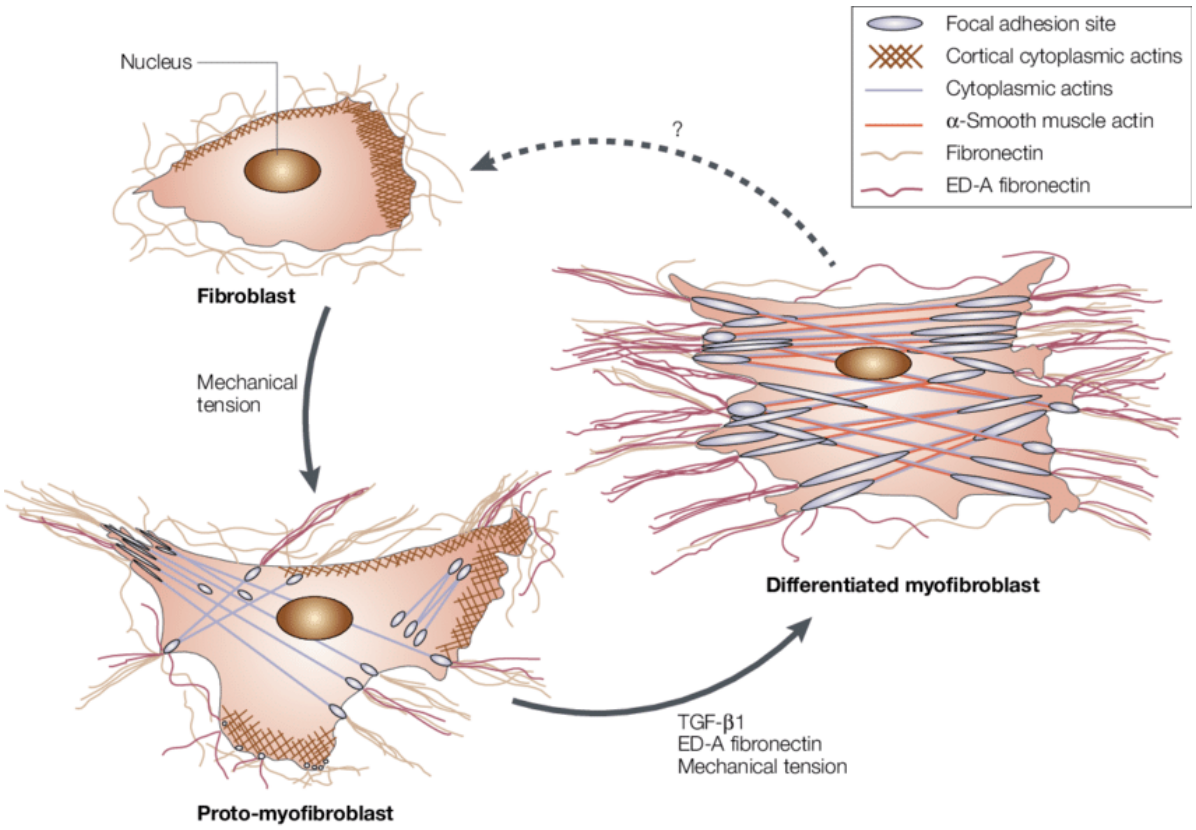
6.10 Statistical analysis

All numerical data were reported in Kaleida Grafh 4.0 and analysed by Student's t-test; one-way ANOVA, with Bonferroni corrections, was used for multiple comparisons.

Chapter 7

ICONOGRAPHY

Figure 1



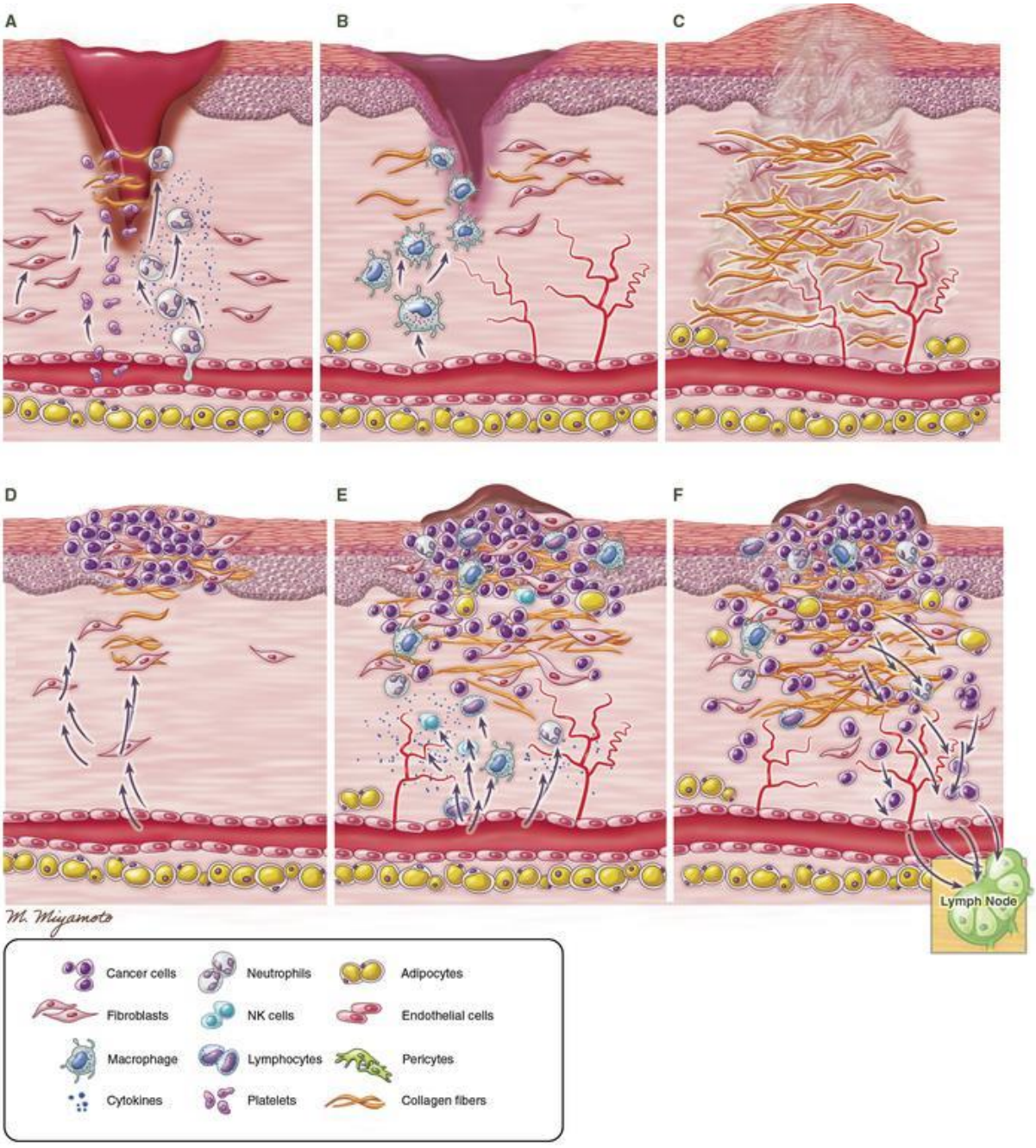
Tomasek *et al.*, Nature Reviews Molecular Cell Biology. 3, 349–363 (2002).

Figure 1

Fibroblast differentation. *In vivo*, quiescent fibroblasts contain actin in their cortical meshwork, and show neither stress fibers nor adhesion complexes with the surrounding ECM. Under mechanical stress, fibroblasts differentiate into proto-myofibroblasts, which represent the intermediate step between fibroblast to myofibroblast transition. Proto-myofibroblasts acquire a proliferative and migratory phenotype, contain contractile bundles, i.e. stress fibers that include β- and γ-cytoplasmic actin and generate small traction forces. Furthermore, proto-myofibroblasts express fibronectin-including the ED-A splice variant, whose expression is further enhanced by TGF-β1, produced by proto-myofibroblasts. Both ED-A fibronectin and TGF-β1 in the presence of mechanical tension, promote the

differentiation of proto-myofibroblasts into myofibroblasts. Differentiated myofibroblasts have more extensively developed stress fibers, that contain α -SMA and show large fibronexus adhesion complexes (*in vivo*) or supermature focal adhesions (*in vitro*). Myofibroblasts are also characterized by a great protein synthetic activity and contractile functions.

Figure 2



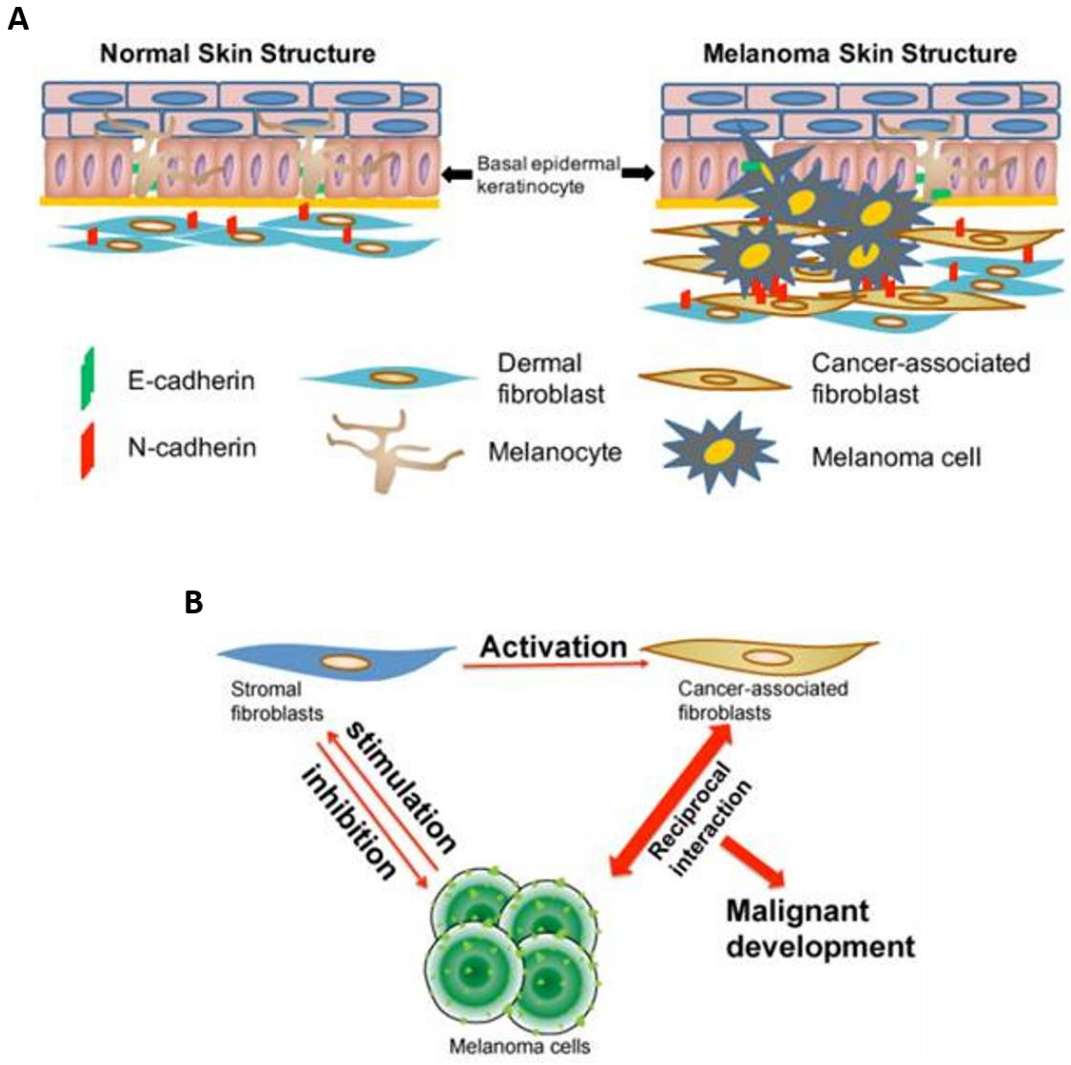
Foster *et al.*, JCI Insight. 3(18), e99911 (2018).

Figure 2

Comparative model of wound healing and reactive tumor microenvironment. (**A**) In normal tissue, fibroblasts are inactivated and do not present developed contractile apparatus. Immediately after injury, the full-tickness dermal wound is

filled by a fibrin clot, neutrophils are recruited to the wound, cytokines and growth factors are released and together stimulate fibroblasts from the adjacent intact dermis to migrate to the wound site. (**B**) During the proliferation phase of wound healing macrophages clear dead tissue and debris, keratinocytes support wound re-epithelialization and restoration of tissue continuity. Additionally, migrating fibroblasts differentiate before into proto-myofibroblasts, and then into myofibroblasts, which are involved in wound contraction, ECM production and remodeling, and finally wound closure. (**C**) When a healing wound closes, myofibroblasts disappear by apoptosis or by reverting to a quiescent state. (**D-F**) When tumor occurs, cancer cells promote a wound healing response that is unable to resolve, leading to a persistent and uncontrolled inflammation and to a constitutively activation of the stroma. In particular, fibroblasts are recruited to the tumor site, are constitutively activated and reprogrammed into CAFs. CAFs support tumor progression by providing a suitable molecular and physical scaffold for tumor proliferation. Additionally, apart from the abnormal ECM, cancer cells and CAFs, the tumor microenvironment also comprises inflammatory and immune cells, adipose cells, endothelial cells, blood and lymphatic tumor vessels, which all together participate in cancer progression and dissemination.

Figure 3



Zhou *et al.*, J. Cancer. 6(8), 717-726, (2015).

Figure 3

Fibroblasts in normal skin and melanoma skin tissues. (**A**) Comparative model of normal skin and melanoma skin structure. In normal skin tissue, normal melanocytes reside in the basal layer of the epidermis, where interact with keratinocytes and thus form the "Epidermal Melanin Unit". In melanoma skin tissue, malignant melanocytes proliferate, penetrate the basement membrane (thick yellow line), invade the underlying dermis and interact with N-cadherin-expressing fibroblasts (indicated by red bars). (**B**) Schematic representation of the dual role of

fibroblasts in melanoma. At early stage of melanoma, fibroblasts create a barrier and produce chemical factors, which altogether inhibit melanoma cell growth. However, the continuous crosstalk between melanoma cells and fibroblasts induce epigenetic, cellular and biochemical changes in fibroblasts that consequently become CAFs. CAFs subsequently generate an optimal microenvironment for melanoma growth, progression and dissemination.

Figure 4

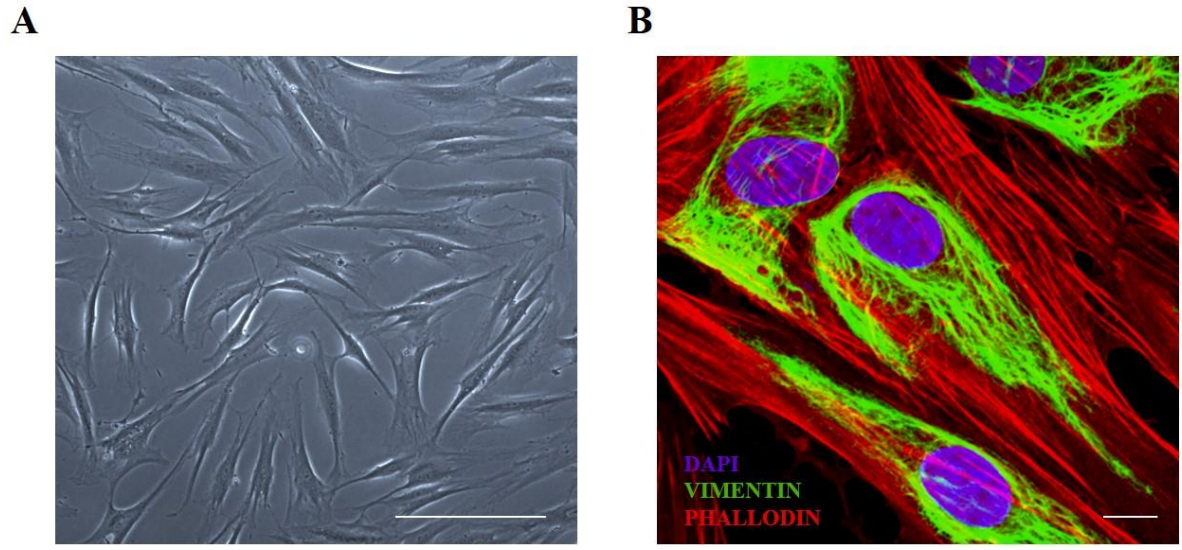


Figure 4

Primary human cutaneous fibroblasts. (**A**) Representative image of fibroblasts obtained by outgrowth from tissues of normal neck skin. Scale bar 200 μm. Magnification X 10. (**B**). Cytoskeleton analysis of primary human cutaneous fibroblasts, isolated from normal neck skin, though confocal fluorescence evaluation of vimentin immunostaining (green channel) and phalloidin staining (red channel). DAPI (blue channel) was used to locate nuclei. Scale bar 10 μm. Magnification X 100.

Figure 5

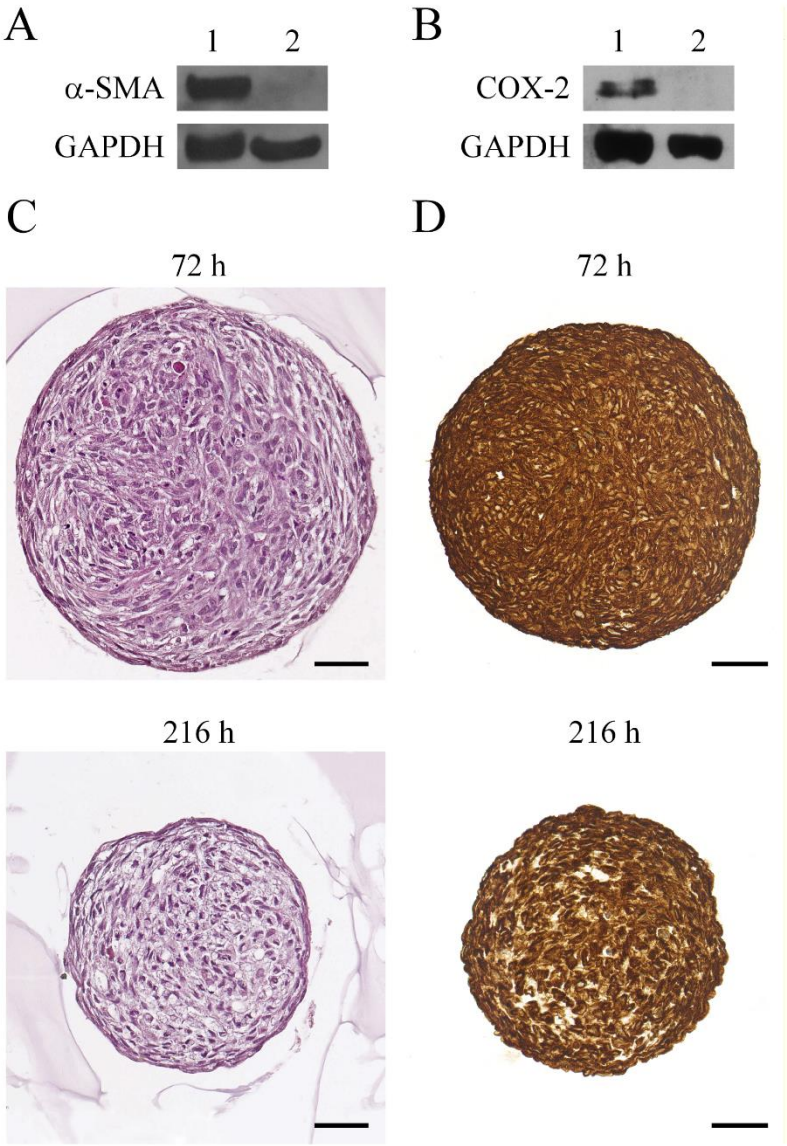


Figure 5

Evaluation of fibroblast activation markers in human primary fibroblasts and Bj-5ta cells, and analysis of myofibroblast spheroids collected at 72 h and 216 h. Western blotting analysis of α-SMA (**A**) and COX-2 (**B**) in protein extracts of cutaneous fibroblasts (1) and BJ-5ta cells (2). GAPDH was used as loading control. Representative image of three independent experiments is shown. (**C**) Haematoxylin and eosin staining and (**D**) vimentin immunohistochemical analysis of paraffin-embedded sections of spheroids collected at 72 h and 216 h. Scale bar

50 μm. Magnification X 40. All images are representative of three independent experiments.

Figure 6

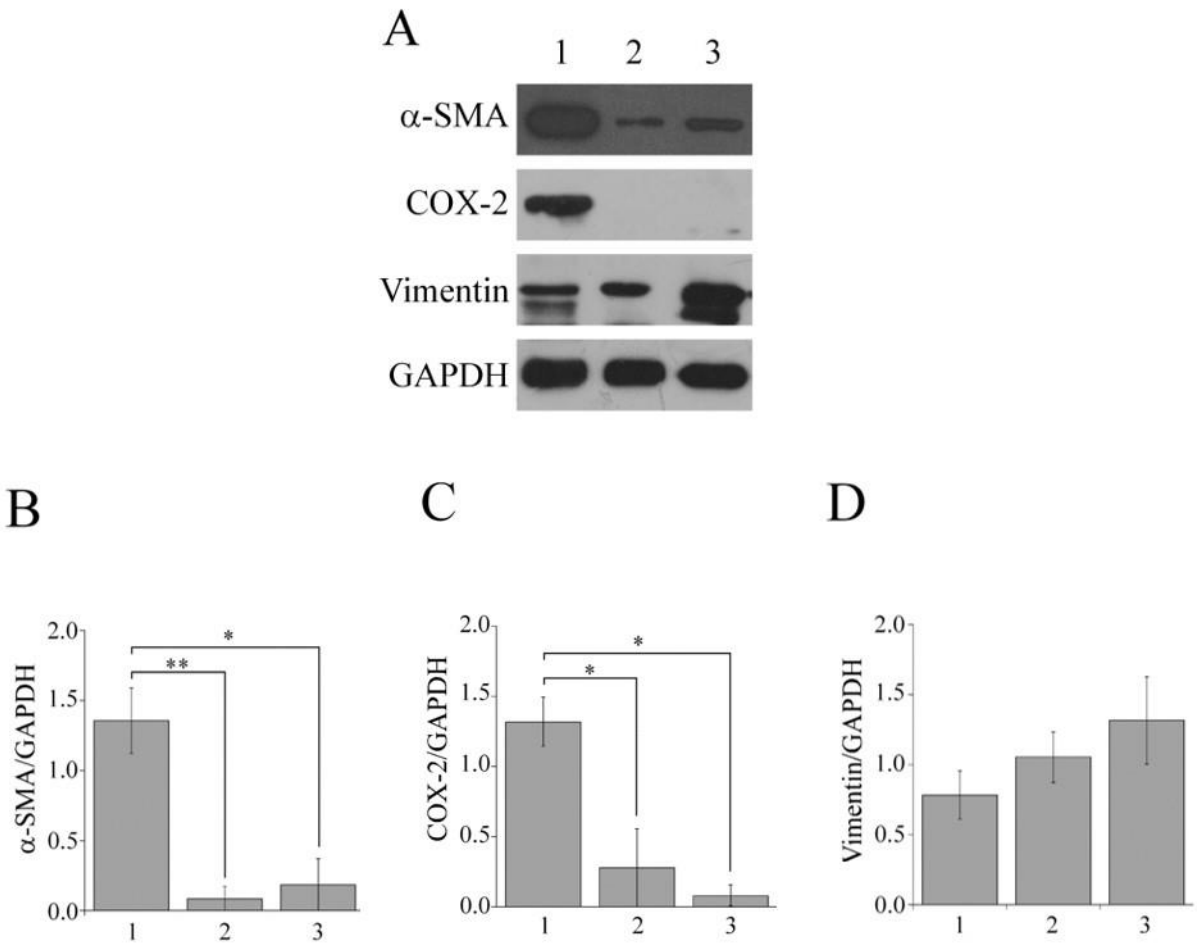


Figure 6

Evaluation of activation, inflammation and mesenchymal markers. (**A**) Western blotting analysis of α-SMA, COX-2 and vimentin. Protein extracts of myofibroblasts (1), spheroids collected at 72 h (2) and reverted-fibs (3). GAPDH was used as loading control. Representative image of three independent experiments is shown. (**B-D**) Densitometric analysis of α-SMA, COX-2 and vimentin protein levels. Data are reported as means of three independent experiments ± S.E. *P<0.05, **P<0.01.

Figure 7

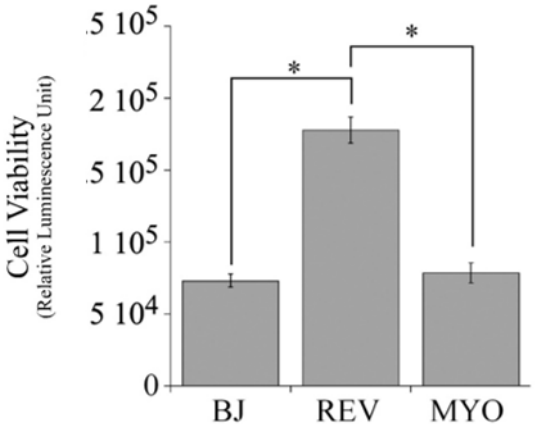


Figure 7

Evaluation of fibroblast cell viability. ATP cell viability assay of BJ-5ta cells (BJ), reverted-fibs (REV) and myofibroblasts (MYO) cultured for 48 h with standard culture medium. Data are means of at least three independent experiments ± S.E. *P<0.0001.

Figure 8

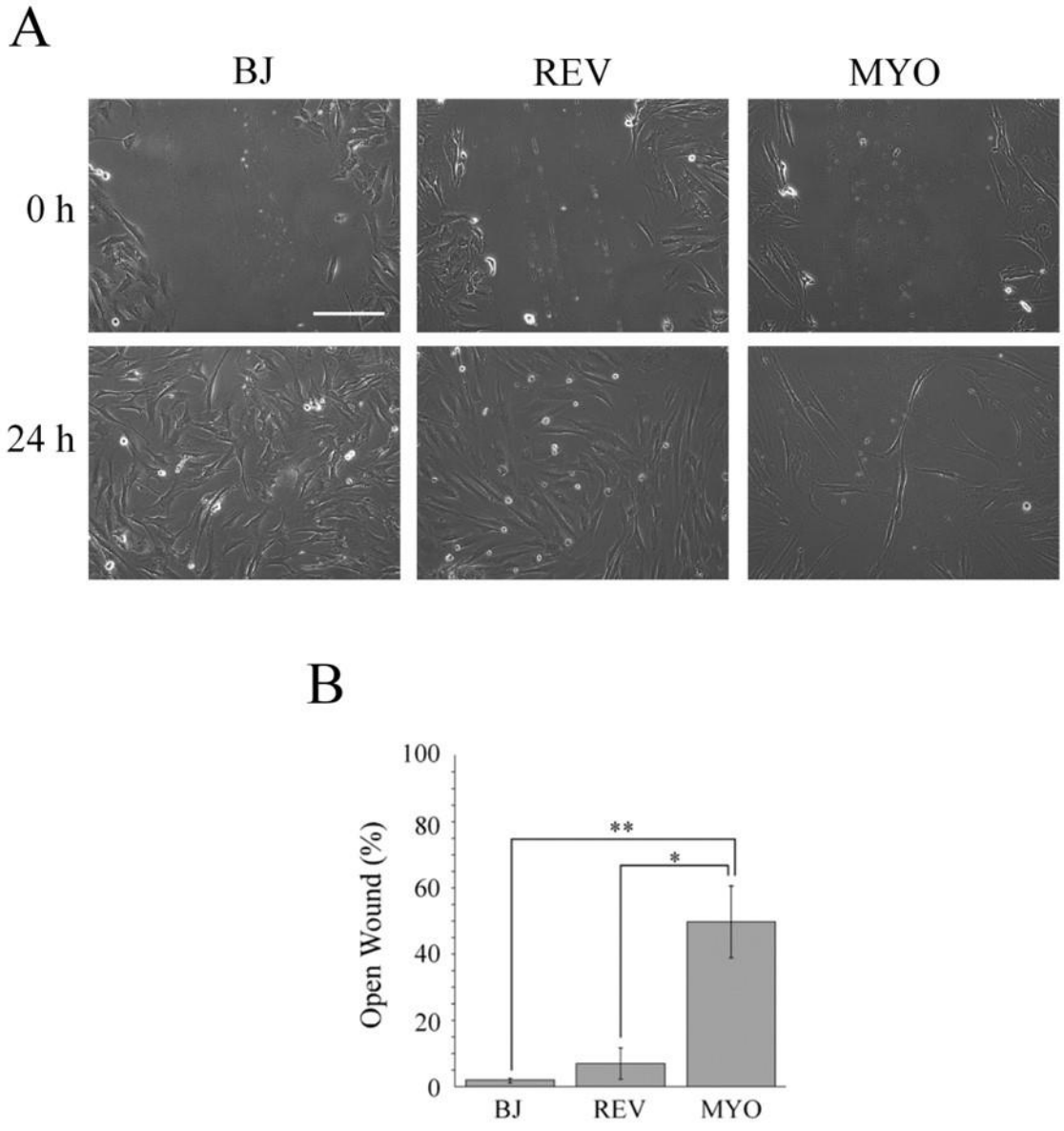


Figure 8

Evaluation of migratory capability of fibroblast populations. (**A**) Wound healing assay of BJ-5ta cells (BJ), reverted-fibs (REV) and myofibroblasts (MYO). The representative images show the same field at 0 h and 24 h after wounding. (**B**) Data are expressed as percentage of fold-decrease of the open wound area compared with

control (0 h) set as 100 % and are reported as mean of three independent experiments ± S.E. *P<0.05, **P<0.01. Scale bar 200 μm. Magnification X 10.

Figure 9

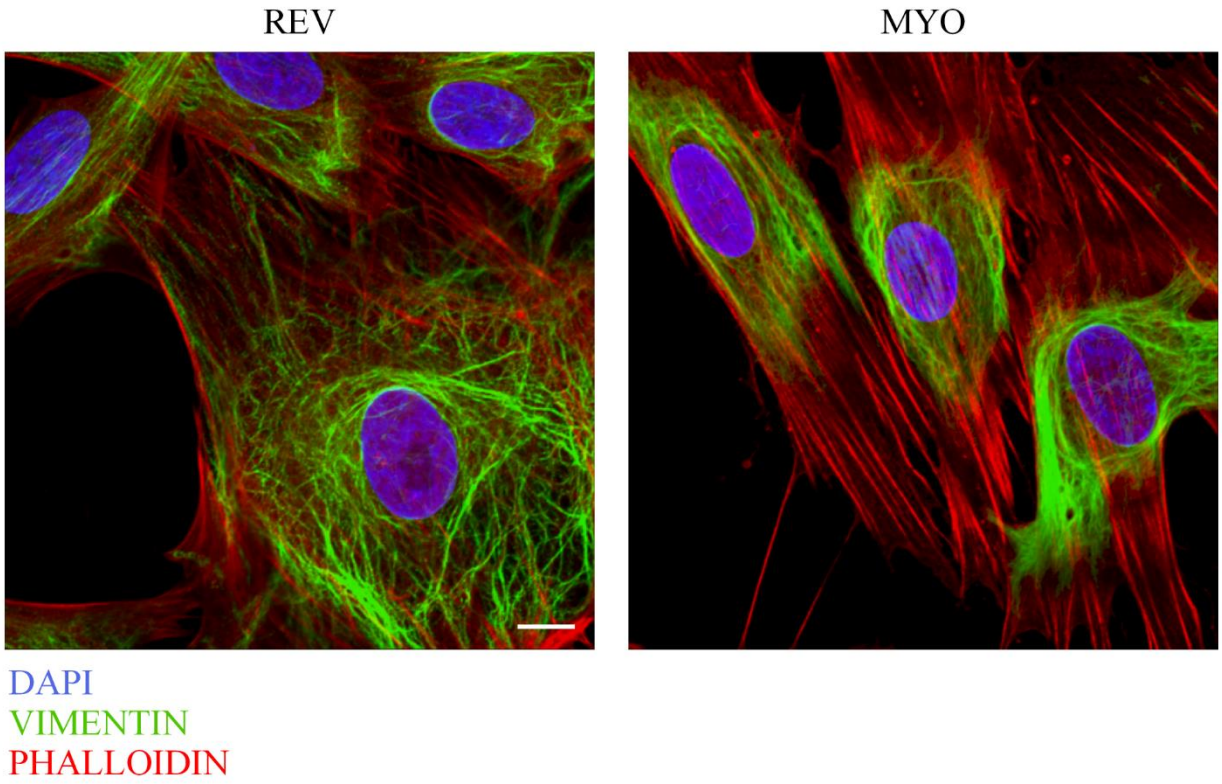
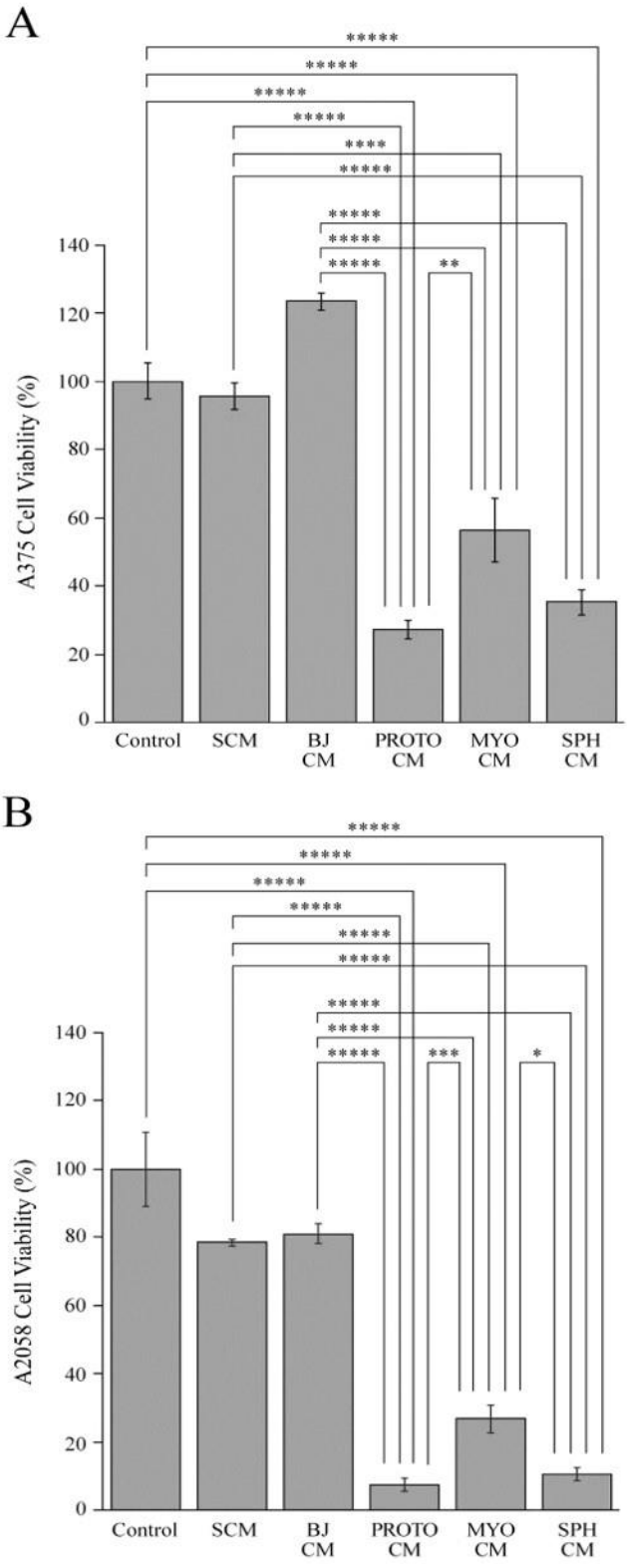


Figure 9.

Morphological analysis of the cytoskeleton organization in reverted-fibs and myofibroblasts. Confocal fluorescence analysis of vimentin immunostaining (green channel) and phalloidin staining (red channel) of reverted-fibs (REV), and myofibroblasts (MYO). DAPI (blue channel) was used to locate the nuclei. Images correspond to the 3D reconstruction of Z-planes acquired from the top to the bottom of the cell. Scale bar 10 μm. The images are representative of three independent experiments.

Figure 10


A
A375 Cell Viability (%)
0
20
40
60
80
100
120
140
Control
SCM
BJ CM
PROTO CM
MYO CM
SPH CM
*****
*****
*****
*****
****
*****
*****
*****
*****
**
B
A2058 Cell Viability (%)
0
20
40
60
80
100
120
140
Control
SCM
BJ CM
PROTO CM
MYO CM
SPH CM
*****
*****
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*

Figure 10

Effect of fibroblast-derived conditioned media on melanoma cell viability. (**A**) A375 and (**B**) A2058 melanoma cells were grown for 48 h with the conditioned media of BJ-5ta (BJ CM), proto-myofibroblasts (PROTO CM), myofibroblasts (MYO CM) and spheroids (SPH CM), their conditioned media (Control) or with standard culture medium (SCM). Cell viability was evaluated by ATP assay. Data are means of at least three independent experiments ± S.E. *P<0.05, **P<0.01, ***P<0.005, ****P<0.0005, *****P<0.0001.

Figure 11

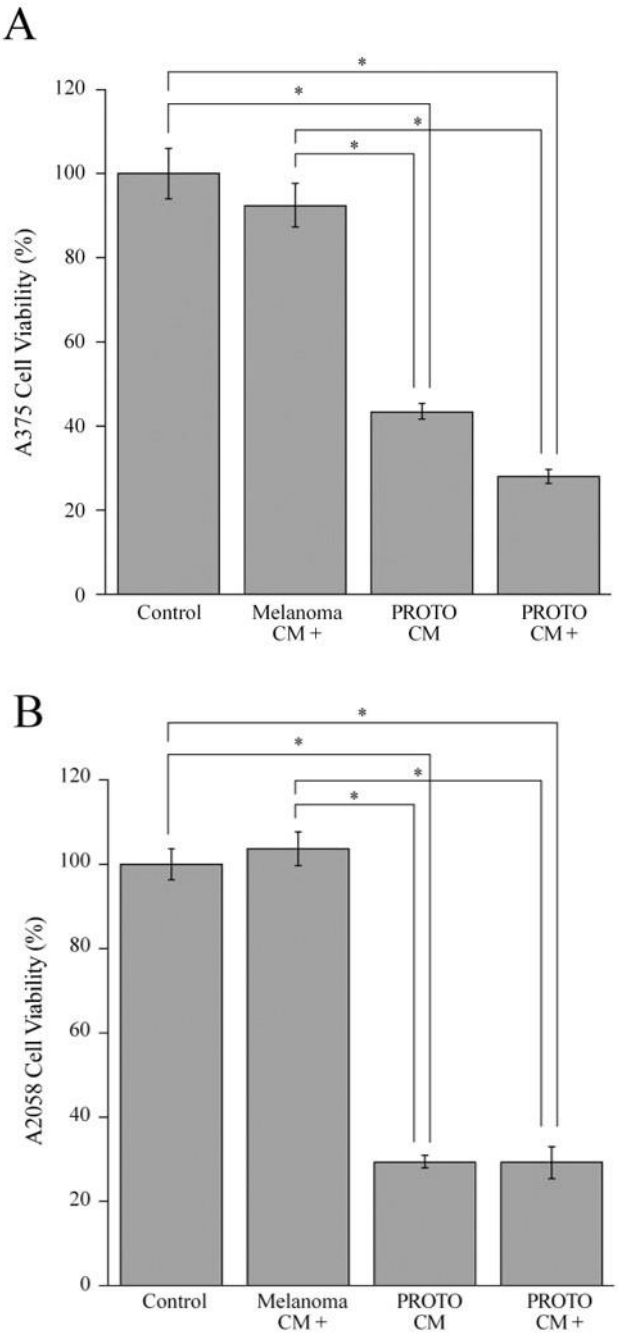

Figure 11

Effect of different serum amounts, present in the conditioned media, on melanoma cell viability. (**A**) A375 and (**B**) A2058 cells were grown for 48 h with their own conditioned medium, without or with addition of 10% fresh serum (Control and melanoma CM +, respectively) or with the conditioned medium of proto-

myofibroblasts, without or with addition of 10 % fresh serum (PROTO CM and PROTO CM +, respectively). Cell viability was evaluated by ATP assay. Data are means of three independent experiments ± S.E . *P<0.0001.

Figure 12

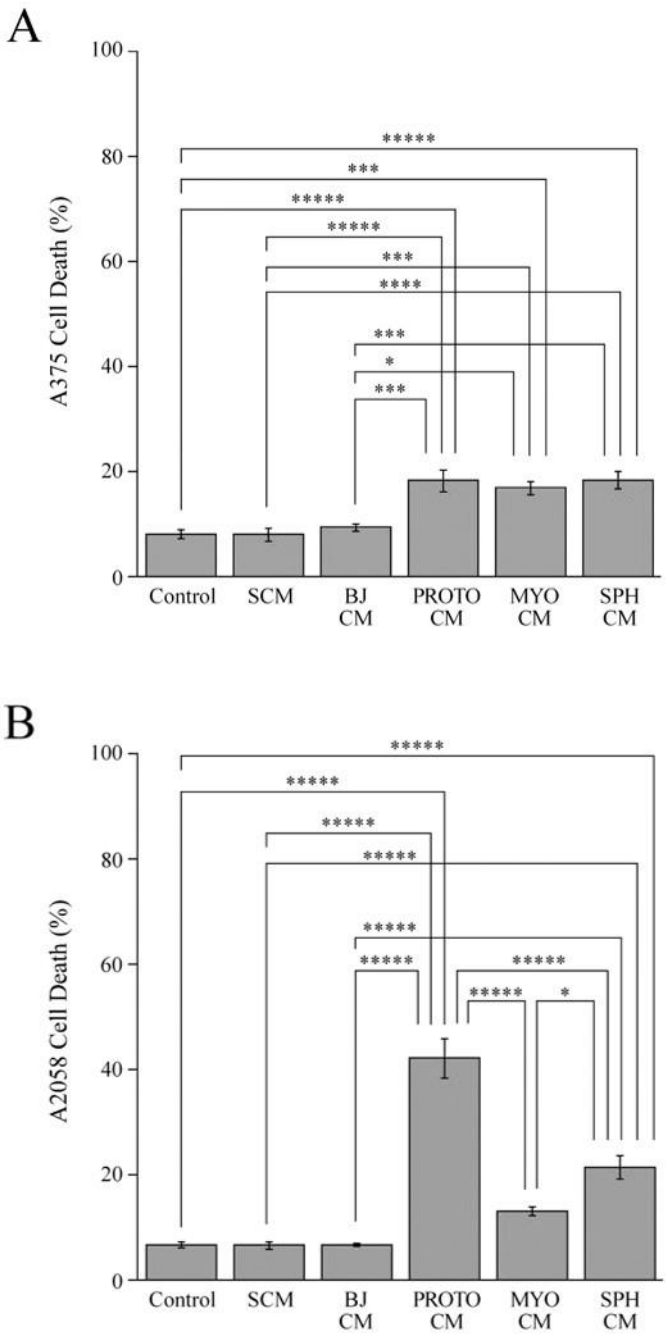

Figure 12

Effect of fibroblast-derived conditioned media on melanoma cell death. (**A**) A375 and (**B**) A2058 melanoma cells were grown for 48 h with the conditioned media of BJ-5ta (BJ CM), proto-myofibroblasts (PROTO CM), myofibroblasts (MYO CM), spheroids (SPH CM), their conditioned media (Control) or with standard culture

medium (SCM). Cytotoxic effect was evaluated by cytofluorimetric analysis of the number of nuclei with a subdiploid DNA content. Data are means of at least three independent experiments ± S.E. *P<0.05, **P<0.01, ***P<0.005, ****P<0.0005, *****P<0.0001.

Figure 13

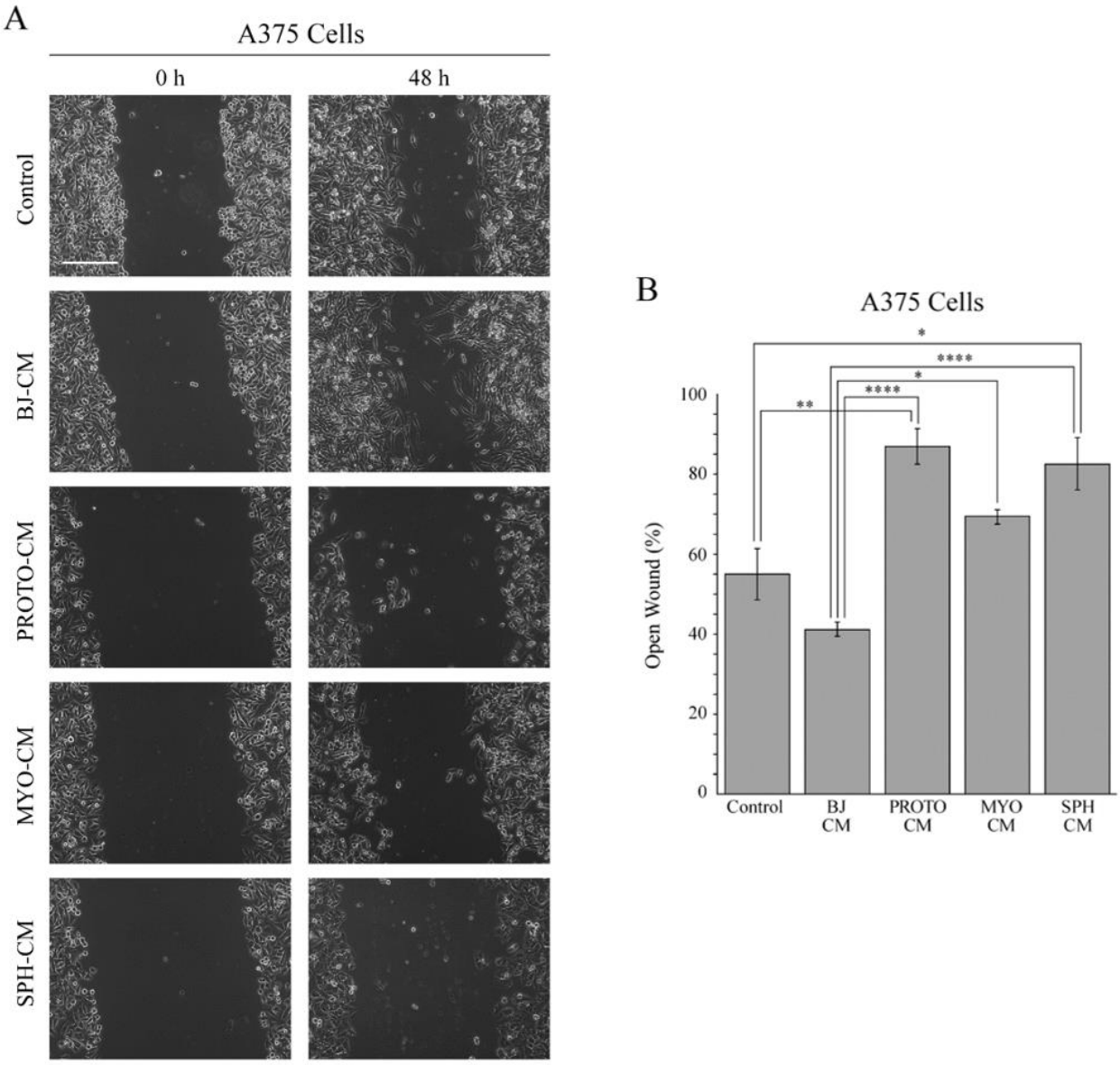

Figure 13

Effect of fibroblast-derived conditioned media on A375 melanoma cell migration. (**A**) Wound healing assay of A375 cells incubated for 48 h with the conditioned media of A375 cells (Control), BJ-5ta (BJ CM), proto-myofibroblasts (PROTO CM), myofibroblasts (MYO CM), or spheroids (SPH CM). The representative images of three independent experiments show the same fields with scratching at 0 h and 48 h after wounding. Scale bar 200 μm. Magnification X 10. (**B**) Quantification of

the migratory capability of A375 cells. Data are expressed as percentage of fold-decrease of open wound area compared with control (0 h) set as 100 % and are reported as mean of three independent experiments ± S.E. *P<0.05, **P< 0.005, ***P<0.001, ****P<0.0005.

Figure 14

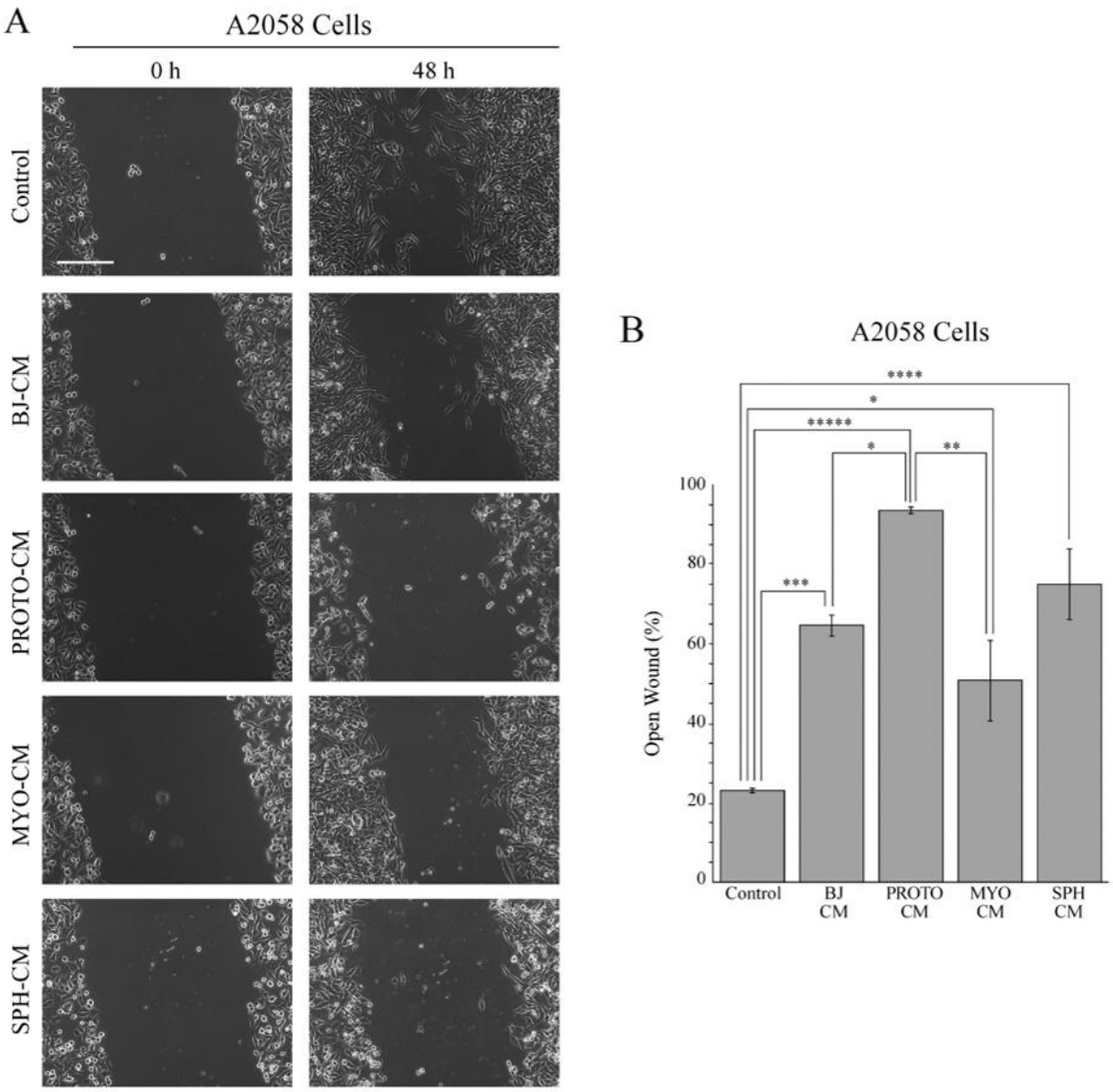

Figure 14

Effect of fibroblast-derived conditioned media on A2058 melanoma cell migration. (**A**) Wound healing assay of A2058 cells incubated for 48 h with the conditioned media of A2058 cells (Control), BJ-5ta (BJ CM), proto-myofibroblasts (PROTO CM), myofibroblasts (MYO CM), or spheroids (SPH CM). The representative images of three independent experiments show the same fields with scratching at 0 h and 48 h after wounding. Scale bar 200 μm. Magnification X 10. (**B**) Quantification of

the migratory capability of A2058 cells. Data are expressed as percentage of fold-decrease of open wound area compared with control (0 h) set as 100 % and are reported as mean of three independent experiments ± S.E. *P<0.05, **P< 0.005, ***P<0.001, ****P<0.0005, *****P<0.0001.

Figure 15

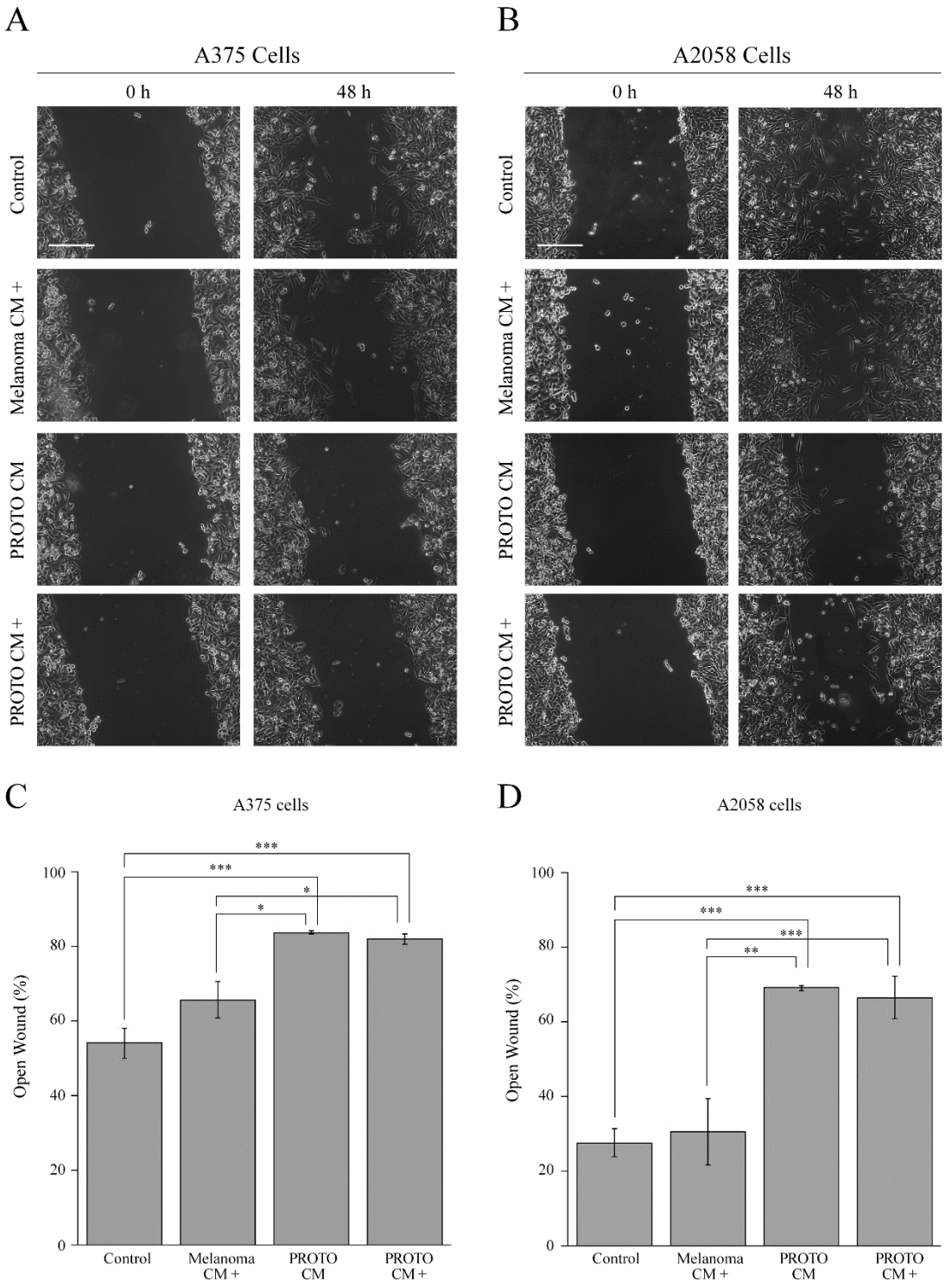

Figure 15

Effect of different serum amounts, present in the conditioned media, on melanoma cell migration. Wound healing assay of A375 (**A**) and A2058 (**B**) cells

incubated for 48 h with their own conditioned medium (Control), melanoma cell-conditioned medium supplemented with 10% fresh serum (Melanoma CM +), conditioned medium of proto-myofibroblasts (PROTO CM) and proto-myofibroblast-conditioned medium supplemented with 10 % fresh serum (PROTO CM +). The representative images of three independent experiments show the same fields with scratching at 0 h, and 48 h after wounding. Scale bar 200 μm. Magnification X 10. Migratory capability quantification of A375 (**C**) and A2058 (**D**) melanoma cells. Wound widths were measured at 0 h and 48 h after wounding. Data are expressed as percentage of fold-decrease of open wound area compared to control (0 h) set as 100 % and are reported as mean of three independent experiments ± S.E. *P<0.05, **P< 0.01, ***P<0.005.

Figure 16

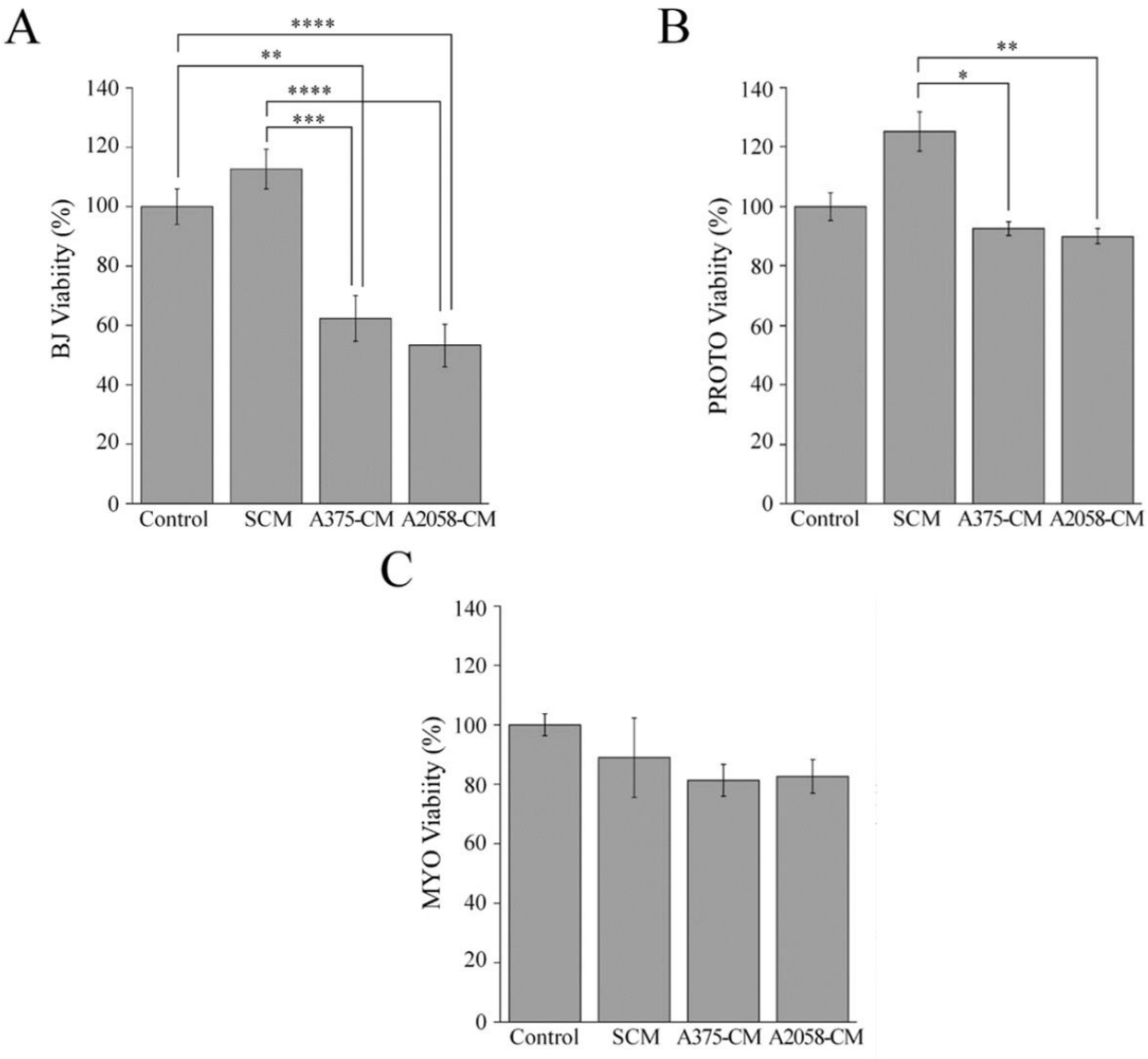

Figure 16

Effect of melanoma cell-derived conditioned media on fibroblast viability. (**A**) BJ-5ta cells (BJ), (**B**) proto-myofibroblasts (PROTO) and (**C**) myofibroblasts (MYO) were grown for 48 h with their conditioned media (Control), standard culture medium (SCM), conditioned media of A375 (A375 CM) and A2058 (A2058 CM) cells. Cell viability was evaluated by ATP assay. Data are means of at least three independent experiments ± S.E. *P< 0.01, **P<0.005, ***P<0.0005, ****P<0.0001.

Figure 17

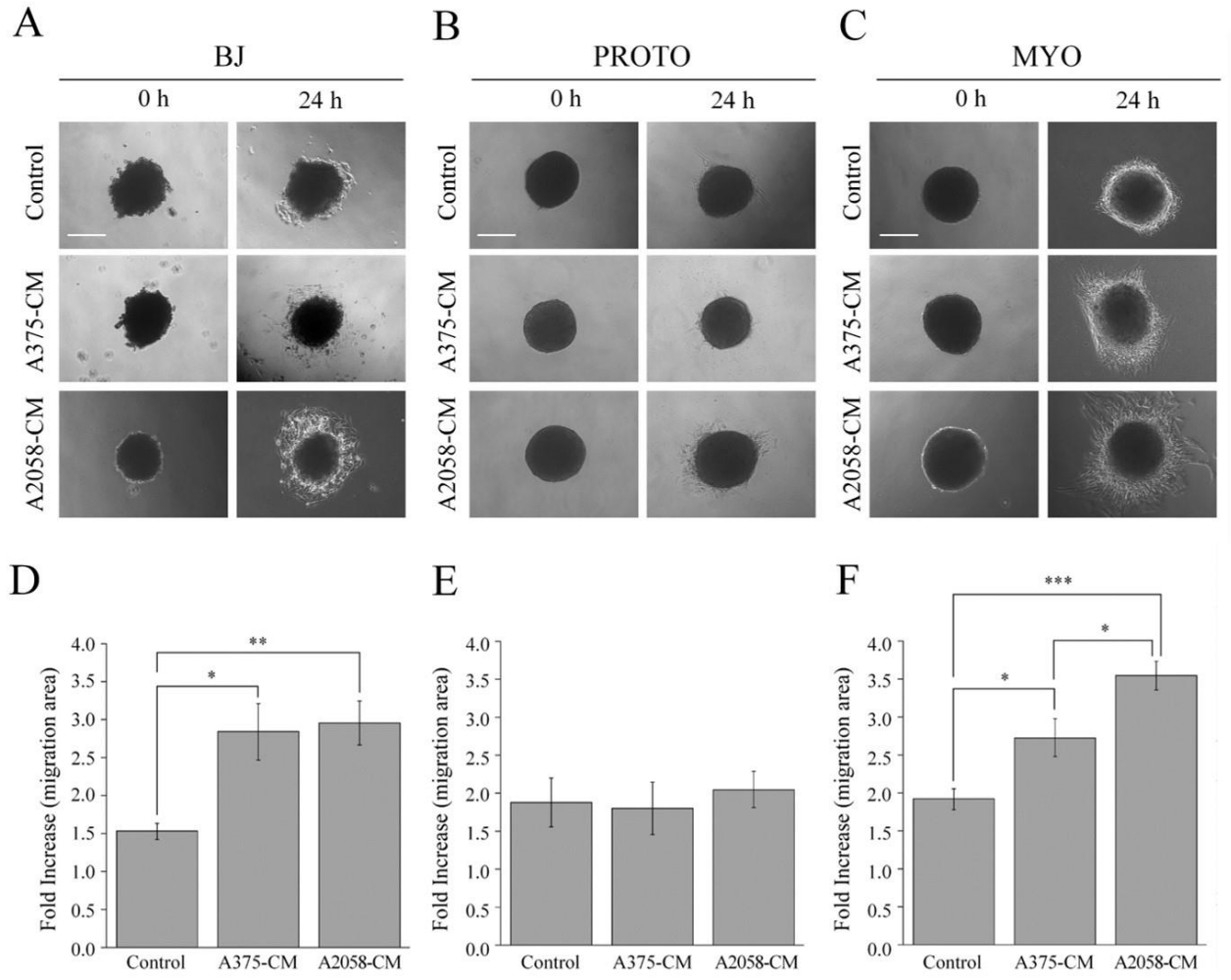

Figure 17

Effect of melanoma cell-derived conditioned media on fibroblast migration. Evaluation of cell outgrowth and migration from spheroids derived from (**A**) BJ-5ta (BJ), (**B**) proto-myofibroblasts (PROTO) and (**C**) myofibroblasts (MYO) exposed to the conditioned media of A375 (A375 CM) and A2058 (A2058 CM) cells for 24 h. Quantification analysis of the area covered by BJ-5ta cells (**D**), proto-myofibroblasts (**E**) and myofibroblasts (**F**) migrating out from the spheroids and spreading on a plastic surface. Data are means of at least three independent experiments ± S.E. *P<0.05, **P<0.005, ***P<0.0001.

Figure 18

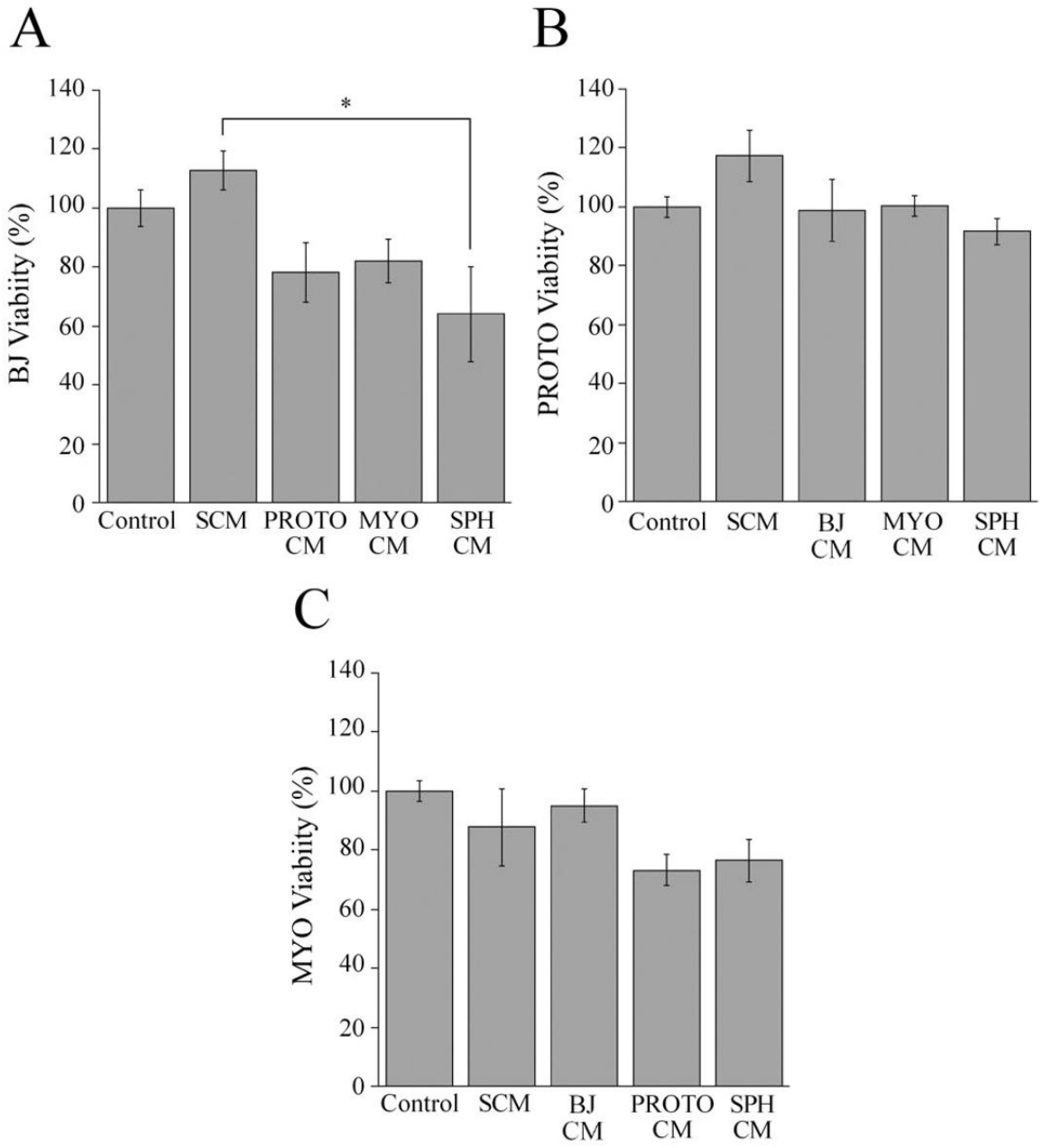

Figure 18

Influence of fibroblast-derived conditioned media on fibroblast monolayer viability. (**A**) BJ-5ta (BJ), (**B**) proto-myofibroblasts (PROTO), (**C**) myofibroblasts (MYO) were grown for 48 h with standard culture medium (SCM), or with the conditioned media of BJ-5ta cells (BJ CM), proto-myofibroblasts (PROTO CM), myofibroblasts (MYO CM) and spheroids (SPH CM). Controls are represented by BJ-

5ta cells, proto-myofibroblasts or myofibroblasts incubated with their own conditioned medium. Cell viability was evaluated by ATP assay. Data are means of at least three independent experiments ± S.E. *P<0.05.

Figure 19

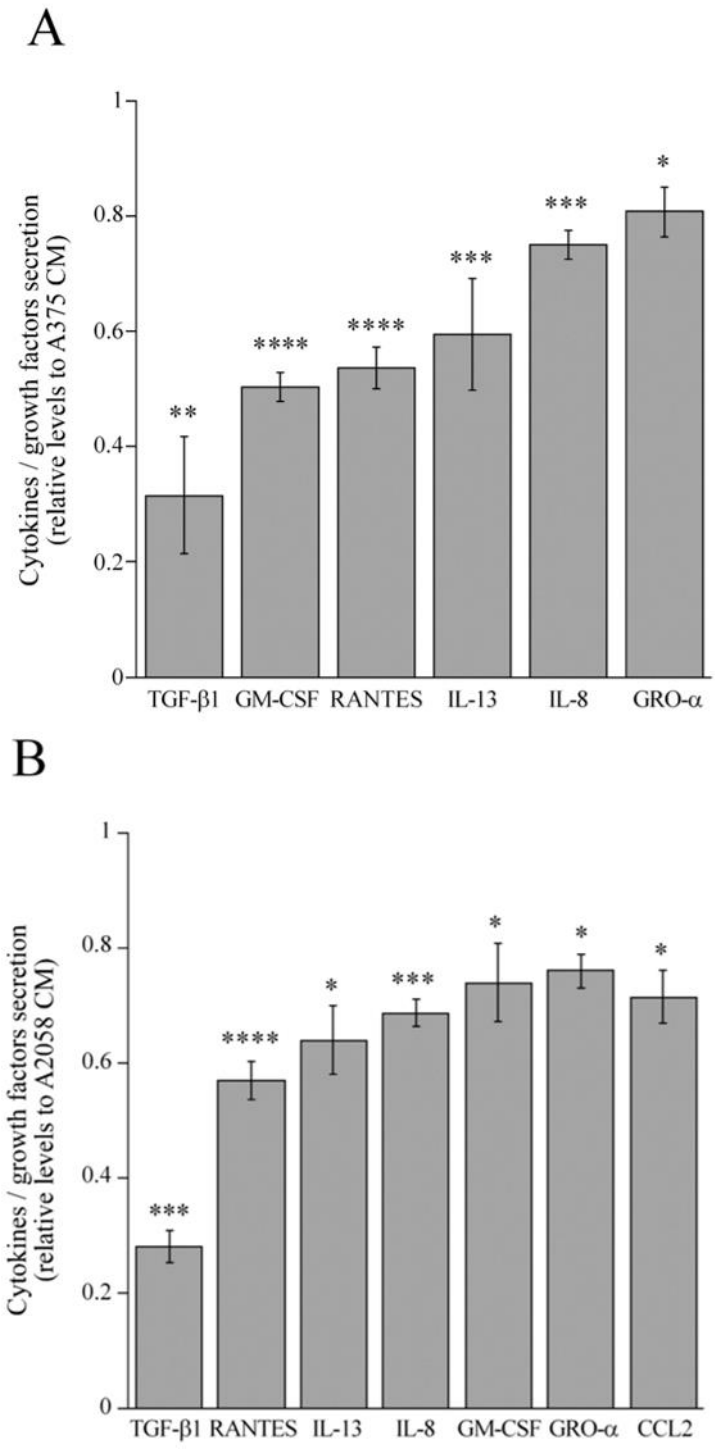

Figure 19

Evaluation of the secretome profiles of proto-myofibroblasts and melanoma cells. The cytokines/chemokines levels in proto-myofibroblast-, A375- and A2058-

derived conditioned media were compared. Densitometric analysis of cytokines and growth factors of proto-myofibroblast-conditioned medium is shown. Control values, set as 1, are represented by cytokines/growth factors levels of A375- (**A**) and A2058- (**B**) conditioned media. Values are represented by mean± SE (n= 3) compared with the signal intensity of A375- (A375 CM) and A2058- (A2058 CM) conditioned media, set as 1. The asterisks show a statistically significant difference between melanoma cell- and proto-myofibroblast-derived-conditioned media, according Student's t test (*P< 0.05, **P<0.01, ***P<0.005, ****P<0.0001).

Chapter 8

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