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Scarless wound healing: From development to senescence

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ABSTRACT

An essential element of tissue homeostasis is the response to injuries, cutaneous wound healing being the most studied example. In the adults, wound healing aims at quickly restoring the barrier function of the skin, leading however to scar, a dysfunctional fibrotic tissue. On the other hand, in fetuses a scarless tissue regeneration takes place. During ageing, the wound healing capacity declines; however, in the absence of comorbidities a higher quality in tissue repair is observed. Senescent cells have been found to accumulate in chronic unhealed wounds, but more recent reports indicate that their transient presence may be beneficial for tissue repair. In this review data on skin wound healing and scarring are presented, covering the whole spectrum from early embryonic development to adulthood, and furthermore until ageing of the organism.

Contents

Abbreviations: AGE, advanced glycation end-product; α-SMA, α-smooth muscle actin; BLIMP, B lymphocyte-induced maturation protein; CTGF, connective tissue growth factor; CDK, cyclin-dependent kinase; DDR, DNA damage response; DPP-4, dipeptidyl peptidase-4; ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; FGF-R1, fibroblast growth factor receptor-1; GAG, glycosaminoglycan; HA, hyaluronic acid; HAS, hyaluronan synthase; HGF, hepatocyte growth factor; HIF-1, hypoxia inducible factor-1; IGF-I, insulin-like growth factor-I; IGFBP, insulin-like growth factor binding protein; IGF-IR, insulin-like growth factor-I receptor; IL, interleukin; LTBP-1, latent transforming growth factor-β binding protein-1; MCP-1, monocyte chemoattractant protein-1; MIF, migration inhibitory factor; MMP, matrix metalloproteinase; MSC, mesenchymal stem cell; MSF, migration stimulation factor; NF-κB, nuclear factor-κB; PDGF, platelet-derived growth factor; PG, proteoglycan; Sca1, stem cells antigen 1; SCID, severe combined immunodeficiency; SDF-1α, stromal cell-derived factor-1α; SA β-gal, senescence-associated β galactosidase; SASP, senescence-associated secretory phenotype; Shh, Sonic hedgehog; TGF-α, transforming growth factor-α; TGF-β, transforming growth factor-β; TGF-βR, transforming growth factor-β receptor; TRIP-1, transforming growth factor-β receptor interacting protein-1; TIMP, tissue inhibitor of matrix metalloproteinases; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

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1. Introduction

Life is dependent upon the preservation of homeostasis, which is manifested, among others, by the ability of the various tissues to heal and/or regenerate. In lower organisms, including non-amniote vertebrates such as zebrafish and salamander, injuries are healed by executing a process analogous to the embryonic development, recapitulating the original tissue in form and function [1,2]. In more evolved species, like mammals, after birth, a different process is followed, which is remarkably controlled and efficient, however it leads to a less functional tissue, the so-called scar, characterized by fibrosis [3]. Nevertheless, even in these species, the ability of scarless tissue regeneration exists throughout the largest part of the prenatal period [2].

Among the various tissues, skin wound healing serves as a paradigm. Human skin is the largest organ in our body and it serves the vital role of the barrier to a wide range of external insults, such as bacteria and other pathogens [4]. This may explain the repair strategy followed by adult organisms, since any skin damage must be rapidly and efficiently mended for the barrier function to be restored. Hence, a temporary repair is achieved in the form of a clot that plugs the defect, and then efforts begin for the restoration of the missing parts [5]. On the other hand, fetuses are protected from environmental insults, most importantly due to the sterile environment of the amniotic sac, and their wounds close following regenerative processes. Scar-related disfigurement and disability represent major challenges for medicine and even cosmetics, with the final goal for scientists to induce adult wound healing in a scarless mode [5,6].

Ageing can be defined as a progressive loss of the homeostasis of the organism [7], hence it is expected to be accompanied by a decline in the wound healing capacity [8]. Given the rapid increase of the elderly population ratios in our societies [9], the socioeconomic burden for chronic wound treatment will continue to grow, making necessary the research for novel and more efficient therapeutic interventions [10]. On the other hand, it seems that — in the absence of other comorbidities — the quality of wound healing in the elderly may be better than in young adults, notably with diminished scarring [11,12]. Furthermore, it has been hypothesized that senescent cells accumulating with ageing may affect the healing process, but recent data indicate that their temporary presence during the early phases of tissue damage may be beneficial for healing [13–15]. Accordingly, here we present data from the literature regarding mainly skin wound healing and its mechanisms, focusing on fibrotic responses and scarring from early embryonic development to adulthood, and furthermore to ageing.

2. Wound healing

Almost two thousand years ago, the Roman encyclopaedist Aulus Cornelius Celsus used the latin terms tumor, rubor, calor, and dolor (i.e. swelling, redness, heat, and pain) to describe the responses of human tissues to injuries [16]. Nowadays, we know that the wound healing process is a complex sequence of events, traditionally classified into three main phases: inflammation, new tissue formation, and remodeling [3,17]. These phases are not mutually exclusive but rather overlapping [17], while some authors may use alternative terms to describe them [18,19].

2.1. Inflammation

The initial event triggering the wound healing process is blood vessel injury, causing blood extravasation and coagulation, a cascade of events driven by a number of proteins called coagulation factors, as well as, by the activation and degranulation of platelets [20]. The final outcome is the blood clot (Fig. 1), a network of cross-linked fibrin fibers containing also extracellular matrix (ECM) proteins, such as fibronectin, vitronectin, and thrombospondin, functioning mainly as a seal against further blood loss (haemostasis) and as a barrier against invading microorganisms [21]. As mentioned above, haemostasis is considered a part of the inflammatory response, since the coagulation factors trigger also classical and alternative complement cascades and attract neutrophils and monocytes to the injury site [17]. At the same time, the blood clot serves as a reservoir for the cytokines and growth factors released from the granules of activated platelets [22]. These growth factors constitute the primary trigger that activates the subsequent processes of wound healing, such as recruitment of the circulating inflammatory cells, activation of fibroblasts and epithelial cells, angiogenesis, cell proliferation, and ECM biosynthesis, to name a few.

The very first type of inflammatory cells attracted to the wound are the polymorphonuclear leukocytes or neutrophils [23], which begin the debridement of devitalized tissue and phagocytosis of infectious agents releasing reactive oxygen species, cationic peptides, eicosanoids and a variety of proteases [24]. Normally, neutrophils disappear quickly from the wound environment being phagocytosed by tissue macrophages, unless there is substantial wound contamination with bacteria or other foreign objects [17]. Actually, a sterile wound can heal in the absence of neutrophils [25], and, moreover, neutropenia may accelerate the repair process [26]. Monocytes are also recruited from the bloodstream, and upon their extravasation they are activated into tissue macrophages, whose principal function is the removal and degradation of injured tissue debris [27]. Resident tissue macrophages replicating in situ may contribute to this process, as well as, in the subsequent phases of wound healing due to the broad spectrum of cytokines, growth factors and enzymes they secrete [23]. Interestingly, in early healing phases macrophages exhibit an M1 profile, i.e. enhanced microbicidal capacity and secretion of high pro-inflammatory cytokines' levels, in contrast to later phases, where they adopt an M2 profile characterized by secretion of anti-inflammatory and ECM molecules [28,29]. As shown long ago, absence of monocytes and local tissue macrophages not only inhibits tissue debridement, but also causes a marked delay in fibroblast proliferation and subsequent wound fibrosis [30]. Beyond neutrophils and monocyte/macrophages, resident mast cells of the skin tissue [31], as well as, T lymphocyte subsets, such as the dendritic epidermal T cells play significant roles in later phases of the healing process [32,33].

2.2. New tissue formation

While inflammation is still in progress, the second phase of wound healing begins, characterized by migration and proliferation of a variety of cell types, such as, keratinocytes, fibroblasts, and endothelial cells [3]. The so-called re-epithelialization of the wound, i.e. the re-establishment of the epidermal layer, starts with a lag of several hours, corresponding to the time necessary for the keratinocytes to perform a drastic modification in their gene expression pattern [34,35]. By changing the

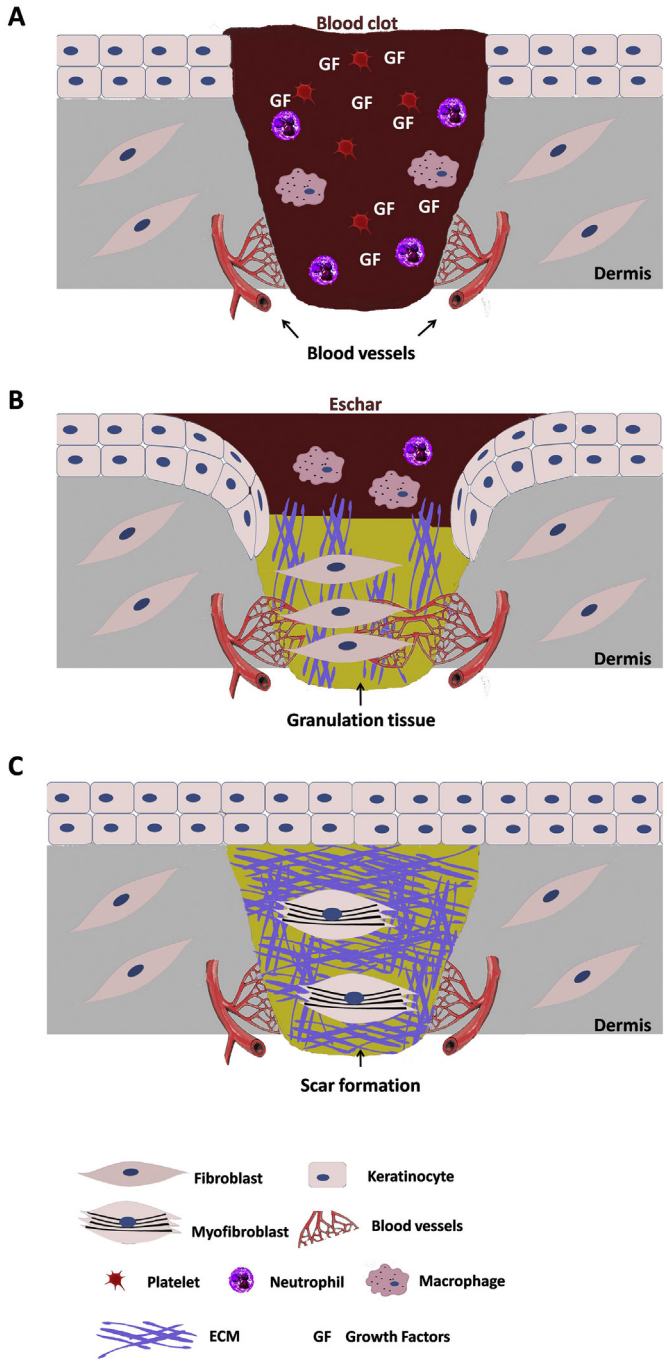

Fig. 1. Simplified illustration of wound healing phases. (A) A snapshot of the inflammatory phase with the clot, the activated platelets releasing growth factors, the neutrophils and the macrophages (approx. first day post-wounding); (B) New tissue formation, i.e. re-epithelialization, fibroblast migration, ECM accumulation and neovascularization (approx. first week post-wounding); (C) Remodeling below the complete neoepidermis, involving decrease in granulation tissue cellularity, vascular involution, and contraction by the α-SMA expressing myofibroblasts (approx. first month post-wounding).

repertoire of a family of heterodimeric cell surface receptors called integrins on their surface [36], and contracting their intracellular actinomyosin filaments, keratinocytes are able to start a locomotion across the damaged area [5]. Later on, keratinocytes at the wound margin start also to proliferate ensuring an adequate supply of cells to encase the wound [34]. These events are induced by a variety of growth factors and cytokines, which are either of platelet origin or secreted by the various immunocytes of the wound or by dermal fibroblasts or even by the keratinocytes themselves in an autocrine fashion, such as epidermal growth factor receptor (EGFR) ligands, members of the fibroblast growth factor (FGF) superfamily, transforming growth factor-β (TGF-β), interleukin (IL)-1 and -6, and tumor necrosis factor-α (TNF-α) [37]. Furthermore, the activated epidermal keratinocytes secrete also proteolytic enzymes, such as members of the zinc-dependent endopeptidases' family called matrix metalloproteinases (MMPs), and in particular MMP-1, -2, -3, -10, -14, -19, and -28, in order to facilitate their movement through the ECM of the wound [38], but also produce ECM components forming a fresh provisional matrix [17,39].

The second phase of wound healing involves also the formation of the granulation tissue at the dermal layer level, consisting of macrophages, fibroblasts, a loose connective tissue, and new blood vessels, that produce its characteristic pink granular appearance [17,40]. All these granulation tissue constituents function in an interdependent manner to gradually replace the initial fibrin clot, and they are usually studied as two parallel processes, called fibroplasia and neovascularization [41].

Fibroplasia refers to dermal fibroblast migration, proliferation, and ECM production, the latter consisting mostly of fibronectin and collagen [42]. Migration of skin fibroblasts is a response to the chemotactic factors released from platelets and inflammatory cells, the most potent being platelet-derived growth factor (PDGF) and TGF-β [43]. Fibroblast proliferation is a response to the above growth factors, as well as, to factors produced by fibroblasts themselves in an autocrine manner [44], such as insulin-like growth factor-I (IGF-I), basic FGF (bFGF), epidermal growth factor (EGF), and TGF-β [45]. Furthermore, fibroblasts are triggered by these growth factors, and especially by TGF-β, to produce collagen - in loosely organized bundles - fibronectin, glycosaminoglycans (GAGs), proteoglycans (PGs) and hyaluronic acid (HA) [46,47]. These ECM molecules serve multiple functions, e.g. they can provide scaffolds for contact guidance (fibronectin and collagen), and low impedance for cell mobility (HA), they may act as reservoirs for growth factors [17], and as ligands for cytokine receptors through their so-called matrikine domains [48,49] or they can modulate growth factor actions [50]. Hence, through the course of fibroplasia, fibroblasts, growth factors and ECM molecules are mutually regulated in a dynamic reciprocity [17].

Neovascularization is based on microvascular endothelial cells, that become activated, migrate into the injury area, start proliferating and form tubular structures, which upon stabilization by β1 integrins generate new vessels [51]. Various growth factors, such as bFGF, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), TGF-β, connective tissue growth factor (CTGF), and angiopoietins, as well as, hypoxia, and high lactic acid levels are all considered to contribute to endothelial cell activation [41], though the final common pathway for all these stimuli appears to be the production of VEGF and bFGF [52,53]. ECM molecules play also important role in stimulating the migration of endothelial cells and affecting their phenotype [54], most notably fibrin [55] and osteopontin [56]. Moreover, the stiffness of the ECM, as well as, the mechanical forces transmitted by it play important role in angiogenesis, regulating self-assembly of endothelial cells into capillary tubes [57].

After the establishment of granulation tissue - at least, in humans and tight-skinned mammals - a differentiation of fibroblasts into myofibroblasts occurs, the latter being the driving force behind wound contraction [58]. Myofibroblasts, discovered more than forty years ago [59], feature an increased expression of α-smooth muscle actin (α-SMA), a contractile cytoskeletal protein enabling them to bring together the edges of the wound, but they do not express other smooth muscle cell markers [60]. It appears that the main factors triggering the conversion of fibroblasts to myofibroblasts are the increased stiffness of the wound compared to normal dermis [61], in combination with the action of TGF-β [62,63] in the presence of the ED-A fibronectin splice variant [64]. Interestingly, in the course of various pathologies, myofibroblasts may originate also from other cell types beyond fibroblasts, such as smooth muscle cells in coronary atherosclerosis [65], pericytes in various fibrotic tissues [66], local or circulating mesenchymal stem cells (MSCs) in tumors or wounds [67,68], circulating fibrocytes in wounds

[69] and chronic inflammation sites [70], and even epithelial cells through their transition to mesenchymal ones in kidney, liver and lung fibrosis, as well as, in the tumor microenvironment [71–74].

2.3. Remodeling

Contraction is considered also as part of the third phase of the healing process, i.e. tissue remodeling. The main characteristic of this phase is that the cellularity of the wound gradually decreases, i.e. many of the cell types involved in the previous phases, such as macrophages, endothelial cells, and myofibroblasts undergo apoptosis, thus leading to resolution of inflammation, vascular involution, and cessation of contraction, respectively [75,76]. Moreover, the wound ECM undergoes significant alterations, the most important being the replacement of collagen type-III by the more robust collagen type-I, as well as, the decrease of hyaluronan content and the transition from GAGs into the more resilient and stiff molecules of the PG family [77]. These remodeling events include ECM synthesis and deposition in parallel with ECM degradation, which is effected through a variety of MMPs secreted by the diverse cell types present in the wound area, as well as, the corresponding tissue inhibitors of MMPs (TIMPs) [78]. The final outcome of the remodeling phase is a new tissue, in the case of adult organisms the scar, which in terms of its composition is similar to the normal skin, however with altered organization [79]. For example, while the collagen fibers in normal dermis are arranged in a basket weave pattern, in scar the thicker collagen fibers are arranged parallel to the skin surface [79]. Moreover, in contrast to the recoil and resistance imparted by elastin in normal skin, after scar formation only a disorganized elastic fiber network is present, contributing to the reduced elasticity and resilience of the mature scar [80]. Hence, scar is less extensible than dermis and fractures at lower tensile stress. Scar lacks the complex rete ridge configuration of normal skin, it does not contain hair follicles or sebaceous or sweat glands, and it is characterized by 16% higher dermatan sulfate content but 35% lower HA content compared to normal skin [81]. Although scarring to some extent is a normal consequence of wound healing, there are also cases of excessive scars (hypertrophic scars and keloids), which are considered to be pathologic [6]. In particular, hypertrophic (as opposed to normotrophic) scars are overgrowths of scar tissue that remain within the boundaries of a wound, whereas keloids extend beyond the boundaries of the original lesion [82,83]. While the term scar refers primarily to cutaneous wound healing, it may be used also to describe fibrosis of various parenchymal organs, such as liver, kidney, heart, and lung, i.e. a detrimental pathology sharing common histomorphological characteristics in all these organs, most notably the excessive deposition of ECM [84,85]. However, there are also fundamental differences between excessive scarring and parenchymal organ fibrosis, such as diverse temporal sequences of events [84], variable involvement of myofibroblasts in skin hypertrophic scars and keloids [86] as opposed to a consistent presence in all parenchymal organ fibrotic pathologies [29], as well as, various organ-specific mechanisms underlying fibrosis [85].

3. Scarless wound healing

3.1. Fetal vs. adult wound repair

As already mentioned, at variance with adult wound healing, in fetuses a perfect tissue regeneration is observed, characterized by the absence of a scar [43]. The transition from scar-free healing to wound repair exhibiting an adult-like scar formation is taking place in all mammals at a point before the end of gestation, e.g. in rats and mice between days 16.5 and 18.5 of gestation with the term being at day 21.5 [87,88], in sheep between days 100 and 120 (term at day 145) [89], in rhesus monkeys between days 85 and 100 (term at 165 days) [90]. In humans, this transition takes place at the beginning of the third trimester of pregnancy [91]. A major difference between fetuses and adult organisms regarding tissue repair lies in the setting where the latter takes place — i.e. the sterile environment of the amniotic fluid and the concomitant diminished inflammatory response (Table 1) — hence this was thought to be responsible for fetal scarless healing, especially since the presence of bacteria in fetal wounds induces neovascularization and fibroplasia, classic features of adult-like healing [92]. Other characteristics of the fetal environment that could contribute to scar-free healing are the lower oxygen tension [53,93,94], the existence of a wet milieu [95], and the growth factor and protease-inhibitor profile of the amniotic fluid [45,96,97]. On the other hand, several studies support the significance of intrinsic differences between the fetal and the adult tissue. In particular, wounds inflicted on fetal tissue grafted subcutaneously in adult animals can heal without a scar [91], while, vice versa, wounds implemented on full-thickness adult skin transplanted in early gestation fetuses lead to scar formation [98]. Finally, the embryos of the marsupial opossum *Monodelphis domesticus* exhibit typical features of fetal wound healing, such as fast re-epithelialization, marginal inflammation and lack of fibrosis, although they are exposed to an adult external environment at an early developmental stage, roughly corresponding to a human fetus at the sixth gestational week [99].

Table 1
Key parameters in fetal vs. adult cutaneous wound repair.

Parameter	Fetal	Adult	Refs.
Morphology/environment			
Scar	Absent	Present	[83]
Oxygen tension	Low	High	[51,79,80]
Wet milieu	Present	Absent	[81]
Sterile milieu	Present	Absent	[83]
Inflammation			
Immunocytes' numbers	Low	High	[163–166]
IL-6, -8 levels	Low	High	[167,168]
IL-10 levels	High	Low	[168]
Re-epithelialization			
Keratinocyte levels of TGF-β1, -β2, -βRs, Smad4	Low	High	[161]
Fibroplasia			
Fibroblast CTGF levels	Low	High	[86]
Fibroblast TGF-β3 levels	High	Low	[87]
Fibroblast proliferation: response to TGF-βs	Inhibition	Stimulation	[94,103–105]
Fibroblast proliferation: response to IGFs	Weak stimulation	Intense stimulation	[95]
Fibroblast migration	Enhanced	Weak	[107]
Levels of HA, HA receptors	High	Low	[108–110,124]
Fibroblast migration: response to TGF-β	Inhibition	Stimulation	[115]
Collagen synthesis rate	Fast	Slow	[116]
Collagen type III/I ratio	High	Low	[92,117]
TGF-β stimulation of procollagen-I	Weak	Intense	[118]
Contraction			
Wound contraction	Absent	Present	[135]
Fibroblast populated collagen gel contraction	Moderate	Intense	[135,149,151,152]
TGF-β effect on fibroblast populated collagen gel contraction	Inhibition	Stimulation	[136]
Remodeling			
MMP-1, -2, -9, -14	Low	High	[142,143]
MMP/TIMP ratio	High	Low	[143]

3.1.1. Fibroblasts in fetal vs. adult wound repair

All together the above indicate that scarless healing is in large part an intrinsic characteristic of the fetal tissue, possibly resulting from certain distinctive properties of fetal cells. Since scar is practically a fibrotic tissue, a comparison of fetal skin fibroblasts to ones of adult origin could reveal the causes of scarless healing. Among the properties that could differentiate fetal from adult fibroblasts is the expression of growth

factors and/or their receptors. In this direction, fetal mouse skin fibroblasts were reported to express lower basal CTGF mRNA levels compared to adult cells [100]. Also, TGF-β3 was found to have higher expression levels in fetal than adult mouse fibroblasts, while concerning the expression of TGF-β1, TGF-β2, TGF-βRI and TGF-βRII no differences have been observed [101]. These observations, in combination with the previous finding that exogenous addition of anti-TGF-β1 and -β2 neutralizing antibodies or of the TGF-β3 isoform to cutaneous rat full-thickness incisional wounds reduces scarring at day 70 post-wounding [102], could provide an explanation of how fetal fibroblasts contribute to scar-free healing. However, during similar comparisons of growth factor expression in fetal vs. adult human skin fibroblasts, contradictory results have been obtained. For example, whereas gene expression levels of TGF-β1, FGF-1 and FGF-2 in adult cells have been reported to exceed these in fetal cells [103], the opposite has been observed regarding the expression of these three growth factors at the protein level [104]. Especially regarding TGF-β1 levels, it seems to be secreted by human fetal fibroblasts in their conditioned medium at higher levels than by adult ones [105], however fetal fibroblasts express significantly lower levels of both latent TGF-β binding protein-1 (LTBP-1), which may make TGF-β available to its receptor, and IGF binding protein-3 (IGFBP-3), acting as TGF-β mediator, than adult ones [106]. A more recent study has not revealed any difference between fetal and adult human skin fibroblasts in the expression levels of all three TGF-β isoform genes [107]. More importantly, although fetal fibroblasts express higher TGF-βRI and lower TGF-βRII protein levels compared to adult ones, this difference is not followed by any variation in the downstream phosphorylation of Smad-2 and -3 [108]. Hence, it seems that the expression of growth factors and/or their receptors has to be examined in correlation with functional properties of the cells.

3.1.1.1. Fibroblast proliferation. Accordingly, many other studies have focused on the proliferative response of fibroblasts from various developmental stages to the above growth factors. For instance, human fetal skin fibroblasts have been reported to respond to IGFs less intensely than postnatal ones, although they express equivalent receptor levels, a fact correlated also with differences in the tyrosine phosphorylation pattern of certain signal transduction substrates [109]. Moreover, the role of TGF-β on fibroblast proliferation has been extensively studied — as expected due to its well-known multifunctionality [110,111] — and conflicting results have been reported. Originally, TGF-β was described as a negative regulator of human skin fibroblast proliferation, based on the fact that it inhibited DNA synthesis stimulation by PDGF depending on the cell density [112]. Other data support a weak stimulation of the proliferation of both fetal and postnatal cells by TGF-β [113]. Most studies concur that TGF-β is mitogenic for newborn and adult skin fibroblasts mainly by inducing another growth factor acting in autocrine fashion [114–116], without providing information regarding fetal skin fibroblasts. Notably, through a methodical evaluation of a panel of cell strains cultured simultaneously under the same conditions, we have shown a definite change in the response of fetal and adult human skin fibroblasts to TGF-β, i.e., it inhibits the proliferation of the former, whereas it stimulates that of the latter [117]. This differential response of fetal vs. adult fibroblasts is independent of the TGF-β isoform, while there is no difference between the responses of neonatal and adult fibroblasts [117]. Hence, this alteration in fibroblast proliferative response to TGF-β is analogous to the transition from fetal-like to adult-like healing, which is also observed at the third trimester of gestation. Furthermore, we have reported that the inhibitory effect of TGF-β on the proliferation of fetal cells requires PKA phosphorylation and the ensuing overexpression of the cyclin-dependent kinase inhibitors $p21^{WAF1}$ and $p15^{INK4B}$ [118]. On the other hand, in fibroblasts from adult donors, instead of phosphorylating PKA, TGF-β up-regulates extracellular bFGF levels, and, through the receptor of the latter and the downstream MEK/ERK pathway, stimulates cell proliferation in an autocrine manner [118]. In both fetal and adult human skin fibroblasts, both Smad2 and Smad3 are phosphorylated in response to TGF-β [113,118,119]. Smad2/3 phosphorylation after TGF-β treatment was shown to persist longer in adult human skin fibroblasts than in fetal ones [113], however the Smad pathway seems to be necessary for the regulation of proliferation by TGF-β – both inhibition in fetal fibroblasts and stimulation in adult ones – as shown by Smad4 silencing using siRNA [108]. Interestingly, most of the above mentioned in vitro studies of fibroblast proliferation have been performed using traditional two-dimensional culture systems and no additional ECM supplements. We have assessed the mitogenic effect of TGF-β in the presence of fibronectin or collagen, roughly simulating the provisional wound matrix and the granulation tissue, respectively, and observed that environments rich in these two ECM components, in two- or three-dimensional (3-D) culture settings, do not alter the inhibitory or stimulatory effects of TGF-β on human skin fibroblasts [108]. However, beyond IGFs and TGF-β, three other important mitogens for skin fibroblasts, i.e. PDGF, EGF, and bFGF, are exhibiting their proliferative effects regardless of the developmental stage of the donor [43,120].

3.1.1.2. Fibroblast migration. A further functional property studied in fetal vs. adult fibroblasts, in an effort to identify possible mechanisms underlying scarless healing, is their migratory capacity. Generally fetal skin fibroblasts exhibit an enhanced tendency to migrate compared to adult ones. In particular, fetal fibroblasts migrate more efficiently in 3-D collagen lattices compared to adult ones, and they are able to migrate regardless of the culture density, in contrast to adult fibroblasts, the migration of which is facilitated at lower densities [121]. These differences are most probably correlated with the increased HA quantity, since cell migration is enhanced by high HA levels, while it is annulled by hyaluronidase pretreatment [122,123]. Fetal fibroblasts have been reported to express four-fold more CD44 receptors for HA than their counterparts from adults [124]. Furthermore, fetal skin fibroblasts secrete migration stimulation factor (MSF), which has been reported to induce migration by promoting HA production by the cells [123]. Therefore, fetal fibroblasts differ from adult ones in terms of both HA production and the response to it, which leads to distinctive migratory behavior. Moreover, fetal skin fibroblasts can migrate under serum-free conditions, in contrast to adult fibroblasts [125]. This was associated with autocrine secretion of bFGF by fetal fibroblasts, since an anti-bFGF-antibody was found to block their migration [126]. Furthermore, the migratory response of fetal and adult fibroblasts to growth factors is also affected by the presence of ECM components and the cell culture density [127]. In particular, when adult fibroblasts are cultured on polymerized collagen, their migration is induced by PDGF and EGF, while this does not happen in the case of fetal fibroblasts [128]. Important role in the effects of another growth factor, TGF-β, on fibroblast migration play the isoform type and the culture density, i.e. the β1 and β2 isoforms inhibit the migration of fetal fibroblasts only in confluent cultures, and that of adult ones only in subconfluent cultures, while the β3 isoform inhibits migration of fetal fibroblasts irrespective of cell density and of adult ones in subconfluent cultures, although it stimulates adult cell-migration in confluent cultures [129]. In summary, regarding cell migration, fetal and adult skin fibroblasts display a divergent behavior due to intrinsic differences, which furthermore affect their interaction with growth factors, ECM components, and neighboring cells.

3.1.1.3. Fibroblast-mediated ECM synthesis. As mentioned above, a major difference between scar and normal skin lies in collagen organization. Hence, it is reasonable to assume that there are differences in collagen synthesis and remodeling between fetal and adult skin fibroblasts. In this vein, it was reported that the overall collagen synthesis by fetal skin fibroblasts is increased compared with adult ones due to higher prolyl hydroxylase activity (a rate-limiting enzyme crucial for collagen synthesis) in the former [130]. Furthermore, in normal fetal skin the ratio of collagen type III to type I is higher than in the adult tissue, a fact that can be attributed to the higher collagen-III production by

fetal fibroblasts [106,131]. Beyond basal levels, mid-gestational mouse fibroblasts have been shown to express less type I procollagen [132] after TGF-β induction (an important regulator of collagen production in tissue repair [133]), while in fibroblasts from later developmental stages procollagen-I is up-regulated and procollagen-III down-regulated [132]. IGFs have also different effects on collagen synthesis in fetal vs. adult fibroblasts, i.e. IGF-II acts as an inducer in fetal fibroblasts while IGF-I has no effect, and vice versa only IGF-I acts as an inducer in adult fibroblasts [109], a fact correlated with these growth factors' circulating levels, since IGF-II is predominant in embryonic life while IGF-I increases gradually up to the onset of puberty [134]. Notably, in fetuses collagen synthesis is an immediate response to injury, in contrast to its delayed onset in the adult [135].

Another key difference between fetal and adult skin lies in the higher HA content of the former [136,137]. This has not only structural but also functional consequences, since the high HA production has been correlated with the increased migratory capacity of fetal fibroblasts [138]. Furthermore, as mentioned above, fetal fibroblasts express higher levels of the main HA receptor CD44 than adult fibroblasts [124]. Moreover, fetal and adult fibroblasts exhibit differential responses to growth factors towards HA synthesis. TGF-β1 induces HA synthesis in confluent cultures of cells from both developmental stages, however in subconfluent cultures it acts as an HA-synthesis inhibitor only for adult but not for fetal fibroblasts [139]. In murine skin from different developmental stages, TGF-β1, as well as, Wnt (Wnt is not an abbreviation but represents a gene belonging to the int1/Wingless family) have been shown to regulate differentially the gene expression of the three enzymes synthesizing HA [140], i.e. hyaluronan synthase-1 (HAS-1), -2, and -3, known to differ in their catalytic activities (HAS-1 being less active than -2, which is less active than -3) and in the HA chains they produce (HAS-3 produces shorter chains) [141]. Both TGF-β1 and Wnt induced HAS-2 and HAS-3 gene expression in embryonic fibroblasts, while in postnatal fibroblasts HAS-1 was induced along with hyaluronidase, the enzyme responsible for HA catabolism [140]. Beyond growth factors, pro-inflammatory cytokines, such as TNF-α and IL-1β, exert diverse regulatory effects on the three HAS isoenzymes; in particular, in fetal fibroblasts both TNF-α and IL-1β stimulate HAS-1 mRNA levels, whilst in adult fibroblasts TNF-α stimulates HAS-3 gene expression, the final effect being higher levels of HA in fetal cells [142]. In general, increased amounts of HA and for longer periods have been observed in the fetal wound environment in various species, and this fact has been proposed to create a permissive milieu promoting fetal fibroblast movement and proliferation while inhibiting differentiation, directing healing to regeneration rather than scarring [143,144]. In this vein, in keloids (which represent extreme cases of scarring, see above) HA, as well as, HAS expression are even less than in normal skin and normotrophic scars [145].

Communication of cells with the ECM is mediated in large part through integrins [146], hence the latter play pivotal role during tissue repair [147]. Interestingly, integrin signals may interfere or co-operate with growth factor-receptor signaling [50], as typically observed in the fibrotic processes induced by TGF-β [148]. Accordingly, a role of integrins in scarless healing has been proposed, based on the fact that human fetal skin fibroblasts express lower α1 and α3 integrin subunit levels and higher α2 subunit ones compared with adult fibroblasts [149]. Moreover, TGF-β inhibits α1, α2, and β1 integrin expression without affecting α3 integrin in fibroblasts from fetuses, in contrast to adult donor cells, where TGF-β stimulates α3 and β1 integrin subunits without affecting α1 and α2 levels [150]. Data from mice where α1 or α2 or both integrin subunits were genetically ablated suggest their importance for wound angiogenesis and tensile strain, although some other wound repair parameters, e.g. re-epithelialization, are not affected by integrin knockout [151,152].

3.1.1.4. Fibroblast-mediated ECM remodeling. In all phases of tissue repair, and especially in remodeling, ECM degrading enzymes play an important role, most prominent among them being the MMPs, a family of endopeptidases produced by the cells as inactive precursors that become active after proteolytic cleavage [78,153]. Negative regulation of their activity is effected by TIMPs [78,154], while the extracellular milieu significantly affects MMPs, e.g. human skin fibroblasts embedded into a 3-D collagen lattice produce more MMP-activity compared to the same cells cultured under traditional 2-D conditions, as a positive feedback response [155].

The expression of many MMPs and TIMPs in non-wounded skin of animals and humans has been shown to increase during the embryonic development and becomes higher in adults [88,156]. Especially a lower TIMP to MMP ratio has been proposed to contribute to scarless healing, by promoting ECM remodeling and assisting the migratory movements of the cells [88,156]. On the other hand, studies with human fibroblasts have shown higher levels of activated MMP-9 to be secreted by fetal than by neonatal cells, while there was no substantial difference in MMP-2 levels [157]. Growth factor regulation of MMPs was also found to be different between fetal and neonatal fibroblasts, since TGF-β1 is inducing both MMPs only in the former, and PDGF-AB is inhibiting MMP-2 in the former while inducing MMP-9 in all the above cell types [157]. Proteomic analysis has revealed differential expression of various other proteins in fetal vs. adult skin fibroblasts, such as HSP-71, tubulin, actin, cofilin-1, peroxiredoxin-1, galectin-1, profilin-1 etc., however their functional significance remains to be elucidated [158].

3.1.1.5. Fibroblast contractility. As mentioned above, wound contraction is a vital step of the wound healing process, that is effected by myofibroblasts, cells with fibroblastic appearance but also with enhanced α-SMA expression [159]. Fetal skin, on the other hand, heals in the absence of contraction. A model system used by numerous research teams, in order to identify whether this is an intrinsic feature of fetal cells, is the contraction of fibroblast-populated 3-D collagen gels [160]. Since the ability of fibroblasts to contract these gels is affected by multiple parameters, a wide range of results has been reported in the literature. Such a parameter pertains to relaxed vs. stressed collagen lattices, the former being more intensely contracted by human fetal skin fibroblasts than by adult ones [161], which vice versa exert higher contractile forces in stressed collagen gels [162]. Notably, in the stressed collagen model the fibroblasts experience mechanical strain equivalent to the tractional force they generate, corresponding to contracting wounds [163]. Another parameter pertains to the prolonged in vitro culture time, since human fetal skin fibroblasts tested immediately after their isolation from the biopsy seem to possess inferior contractility than the corresponding adult cultures [149]. This agrees with reports using fibroblasts of animal origin (e.g. murine and ovine), indicating that early fetal fibroblasts exhibit a diminished contractile capacity vs. adult ones [164,165].

The most important growth factor that regulates contraction seems to be TGF-β, due to its ability to induce α-SMA and fibroblast-differentiation into myofibroblasts [58]. Early fetal murine skin fibroblasts have been reported to underperform in comparison with late fetal and adult fibroblasts regarding collagen gel-contraction, as well as, latent TGF-β secretion and its activation [164]. Concerning human lung fibroblasts, cultures of fetal origin exhibit also inferior collagen contraction than the ones from adults, a fact that may be explained by the higher levels of TGF-β receptor interacting protein 1 (TRIP-1) in fetal fibroblasts [166], a protein acting as repressor of many TGF-β-induced genes [167]. In human skin fibroblasts as well, data support the inability of TGF-β to induce α-SMA expression by fetal cells, thus inhibiting their contractile capacity, while it stimulates contraction in adult-donor cells [150]. Furthermore, TGF-β induces collagen gel contraction at an increasing degree as the gestational age of the donor increases [165]. Another growth factor from platelets, PDGF, acts in an analogous manner to TGF-β, strongly inducing adult skin fibroblast-contractility [168,169]. Fetal human skin fibroblast contraction seems also to be enhanced by PDGF at an extent similar to fibroblasts from

adult donors (our unpublished observations). In general, scarring is associated with increased and persistent application of mechanical forces on fibroblasts. For example, it has been proposed that stretching of the ECM around various cell types can activate the latent form of TGF-β that is deposited in the ECM, a fact that in the case of wound healing may boost the profibrotic program of fibroblasts [63]. Furthermore, mechanical signals can be transformed to biochemical responses through the so-called mechanotransduction pathways, such as the integrin-focal adhesion kinase axis, which in turn leads to stimulation of inflammation and collagen production [170]. These observations are being exploited in various interventions, either against chronic wounds, such as the application of negative pressure [171], or against scarring, such as a specially designed device to offload tension [172].

3.1.2. Other cell types in fetal vs. adult wound repair

Although the lion's share of the articles regarding fetal vs. adult wound repair are studies on skin fibroblasts, there are also other cell types that have attracted considerable attention, among them the keratinocytes, the central players of re-epithelialization. The TGF-β system (TGF-β1, TGF-β2, TGF-βRs, and Smad4) and collagen-I exhibit increased expression with increasing gestational age in rat keratinocytes, suggesting an amplified profibrotic response during skin differentiation at the gestational ages associated with scarring [173]. Interestingly, murine newborn keratinocytes have an overall anti-fibrotic influence on both fetal and postnatal fibroblasts in co-culture conditions, supporting the importance of intrinsic differences between fibroblasts from these two developmental stages [101]. Regarding human skin keratinocytes from fetuses at mid- and late-gestation, i.e. before and after the transition from scar-free to scar-bearing healing, mid-gestational keratinocytes have been found to secrete more heparin-binding EGF and IL-1α than late-gestational ones, and to possess an improved ability to modulate in a paracrine manner the expression of MMPs and TIMPs by skin fibroblasts from adult donors towards ECM remodeling [174].

Immunocytes have been also studied, since fetal wounds are characterized by the presence of very few inflammatory cells compared to adult ones [175,176]. More specifically, studies in rodents have shown that fetal wounds contain lower numbers of macrophages than adult ones [177,178]. Moreover, fewer B and T lymphocytes are attracted in the wound area in fetuses. The lower numbers of inflammatory cells are in accordance with the lower levels of the pro-inflammatory cytokines IL-6 and IL-8, as well as, the higher levels of the anti-inflammatory cytokine IL-10 observed in fetal wounds, compared to adult ones [179,180]. Both the sterile environment of the amniotic fluid and the diminished secretion of Th1-polarizing cytokines, as a means to prevent an alloimmune reaction between mother and fetus may explain these differences [119].

Since fetal scarless wound healing involves regeneration rather than repair of the injured tissue injury, stem cells may also represent key players in this process [181]. There are many kinds of stem cells in the skin, and especially in the epidermis: sweat gland-specific progenitors [182], multipotent hair follicle stem cells [183], as well as, interfollicular ones [184]. Their contribution in general epidermal homeostasis is contradictory [185,186], however it has been proved that following wounding, they can undergo reprogramming to become repopulating epidermal progenitors [187]. MSCs may also contribute to scarless healing, especially since they can be found in abundance in tissues and fluids surrounding the fetus, such as, the placenta, the umbilical cord, the amniotic membrane and the amniotic fluid [181]. So far, MSCs from umbilical cord blood and Wharton's jelly directly injected into full-thickness skin defect sites in nude mice have failed to improve wound healing quality [188]. On the other hand, combination of Wharton's jelly-MSCs with a decellularized amniotic membrane scaffold in a severe combined immunodeficiency (SCID) mouse model leads to considerably improved wound healing, diminished scar and ameliorated biomechanical properties of the regenerated skin as opposed to the use of MSCs solely [189]. Also a group of cells isolated from murine blood (both fetal and adult) expressing markers of germ, embryonic stem and adult stem cells, as well as, E-cadherin, upon transplantation to adult mice contributes to skin wound repair characterized by decreased α-SMA and collagen expression, and, hence, by limited scar tissue [190,191].

Apart from stem cells, other cell types, such as skin fibroblasts, that were traditionally considered quite uniform, have recently been shown to possess significant heterogeneity, as well as, plasticity. In particular, in murine fetuses skin fibroblasts arise from two distinct lineages, one expressing CD26/dipeptidyl peptidase-4 (DPP-4) and B lymphocyte-induced maturation protein (BLIMP) but not stem cells antigen 1 (Sca1) and forming the upper dermis and the other expressing Sca1 but not CD26/DPP4 or BLIMP and being responsible for lower dermis formation [192,193]. During wound healing, the initial wave of dermal repair is mediated by the second lineage and the first one is recruited only during re-epithelialization [192]. Interestingly, fibroblasts respond to paracrine signaling from epidermis through Wnt/β-catenin activation and subsequent Sonic hedgehog (Shh) and TGF-β induction in a lineage-specific manner: Shh stimulates proliferation of the papillary fibroblasts lineage and ECM remodeling by them, whereas TGF-β regulates proliferation, differentiation and ECM synthesis by reticular fibroblasts [194]. In the same vein, it has been shown that in murine dorsal skin at least two fibroblast lineages are present, but only one of them featuring the surface marker CD26/DPP4 accounts for the majority of the connective tissue produced during cutaneous wound healing, while blocking the CD26/DPP4 enzymatic activity during the wound repair process, by means of small molecule inhibitors, leads to decreased short-term cutaneous scarring [195].

3.2. Oral mucosa: an adult tissue healing in a fetal-like manner

Although the various stages of wound healing do not differ between skin and oral mucosa, it was observed long ago that in the latter the whole process is accomplished much faster [196], and shares some similarities with fetal wound healing, the most important being the absence of scar formation [197]. This difference has been attributed to evolutionary reasons, since scarring in the oral cavity would have hampered food intake by ancient humans [198]. Nevertheless, variations exist also inside the oral environment, since incisions in the buccal mucosa lead to scarring, in contrast to incisions in the tongue or gingiva [198,199]. Hence, human gingival fibroblasts have been studied and found to resemble more closer fetal skin fibroblasts than adult ones, regarding various phenotypic parameters in vitro, such as saturation cell density, migratory capacity, and HA synthesis [200]. Furthermore, gingival fibroblast proliferation is inhibited by TGF-β in contrast to the stimulatory activity of this growth factor on skin fibroblast proliferation [201], in accordance with the similar differential response of fetal vs. adult skin fibroblasts (see above and [117]). This inhibitory effect of TGF-β on gingival fibroblasts is correlated with their lower HA synthesis and can be reversed to a stimulatory one by forced overexpression of HAS-2 [201,202]. Regarding ECM reorganization, oral mucosal fibroblasts are able to contract a fibroblast-populated collagen lattice more rapidly than their dermal counterparts [203]. In experiments employing 3-D cultures simulating the cells' natural ECM environment, gingival fibroblasts have been shown to express higher levels of inflammatory regulators and ECM remodeling molecules (MMPs) in contrast to skin fibroblasts expressing higher levels of ECM molecules, of TGF-β signaling-mediators, as well as, of myofibroblast and cell contractility-related genes [204]. Moreover, gingival fibroblasts are expressing lower levels of the profibrotic transmembrane glycoprotein CD26/DPP-4 (see above) than their skin counterparts [205]. Collectively, these data indicate the distinctive intrinsic characteristics of gingival fibroblasts which may account for the fetal-like wound healing of oral mucosa, possibly reflecting their diverse ontogenetic origin (neural crest) from skin fibroblasts (mesoderm) [206].

Table 2
Changes in the aged skin.

	Refs.
Cells	
Keratinocytes present variability in cell morphology, differences in cytokine production and reduced proliferation and migration potential	[218–220]
Melanocytes are fewer	[221–223]
Langerhans cell density remains unaltered/Langerhans cells are fewer and show impaired migration, dendritic function and antigen presenting role	[224]/[220,225–228]
Fibroblasts are fewer, have a lower proliferation capacity and have been shown to become dysfunctional in terms of responsiveness to growth factors, migration rate, integrin function, actin cytoskeletal organization and differentiation to myofibroblasts	[229–234]
Leukocytes, macrophages and mast cells increase in number. Macrophages are characterized by a declined function, a lower phagocytic activity and an altered profile of produced cytokines/mast cell populations are decreased	[205,209,220,229, 235–237,239]/[238]
B lymphocytes present a limited antibody range	[220]
T lymphocytes possess a lower cytolytic activity, a diminished repertoire of T cell antigen receptors, a decreased mitogenic potential and an altered profile of secreted cytokines when activated and move from the naïve towards the memory phenotype	[220]
Skin-derived progenitor cells are less and show a reduced differentiation potential	[240]
Epidermal stem cells retain a constant density throughout lifespan	[241–243]
Vascular endothelial cells are larger in size, have a lower proliferation and migration rate and overexpress fibronectin. They present a better adherence to leukocytes, an increased IL-1 production and a higher response to TNF-α	[211,244,245]
A reduced subcutaneous adipose layer exists. Adipocytes show diminished ability to accumulate lipids. Differentiated adipocytes are susceptible to senescence and may be among the main cell types secreting inflammatory cytokines during ageing	[246–249]
ECM	
Collagen is reduced and fragmented, resulting in disruption of dermis' structural integrity and thus hindering fibroblasts' attachment and mechanical stimulation	[250–255]
Tensile strength of collagen fibers is increased due to the presence of advanced glycation end-products and enhanced crosslinking	[256,257]
Elastase-type protease activity is increased correlated to AGEs	[258,259]
Elastotic material replaces normal elastin leading to an increase of skin rigidity and wrinkle formation caused by a fibrobrast-derived elastase activity	[260–262]
PGs are aberrantly distributed in the elastotic material. Specific types of GAGs appear in different ages and in a skin layer-dependent manner. Lumican decreases. A catabolic fragment of decorin with reduced ability to bind collagen I accumulates	[263–266]
Fibronectin expression and deposition changes	[267–269]
The dermal-epidermal junction is flattened permitting higher susceptibility to mechanical forces	[270,271]
Reduced vascularity of the dermis hampers thermoregulation and adaptability to injury	[272,273]
Defects in the ability of the skin to remove fluid in response to gravitational stress favor the development of intradermal oedema	[274]
MMP expression increases, while TIMP-1 is down-regulated	[275–279]
Activation of the TGF-β/Smad/CTGF axis is impaired, ultimately leading to collagen loss in the aged skin	[280]

Aside from fibroblasts, gingiva-derived MSCs when compared to skin-derived ones exhibit greater proliferation and migration capacity, and less matrix contraction in full thickness tissue equivalents, thus contributing to the superior oral wound healing [207]. In general, the better quality of oral healing has been associated with a more transient and diminished inflammatory response, due to lower levels of pro-inflammatory cytokines, such as IL-6 and IL-8, leading to less macrophage, neutrophil, and T-cell infiltration [199,208], with a richer vasculature, as well as, with enhanced proliferation of oral keratinocytes as opposed to the higher differentiation of skin ones [209]. Furthermore, a prolonged expression of the integrin αvβ6 along with TGF-β3 in the gingival wound epithelium has been proposed to protect gingiva from scar formation [210]. Finally, in an analogy to the amniotic fluid in the case of fetal wound healing, the presence of saliva in the oral environment is considered a factor contributing to healing quality, by ensuring a wet milieu, as well as, due to its distinctive growth factor content [211,212]. The above positive characteristics of oral mucosa cells, and particularly gingival fibroblasts, in combination with their easy collection have rendered them into attractive sources for regenerative medicine applications [213].

4. Wound healing in ageing

Since the first publication of the military surgeon DuNuoy reporting a delayed cutaneous wound healing in older soldiers during World War I [214], numerous studies have focused on the existence of putative age-related differences in the quality and/or rate of wound healing in humans [8,11,215–219] and animals [220–226] with contradicting results. Based on the existing literature describing an impaired wound healing process in the aged and taking into account that the elderly population is nowadays growing fast, investigation of tissue repair with increasing age becomes of great importance. The financial burden for prevention and treatment of non-healing wounds worldwide is enormous, reaching – according to the latest published data – an annual cost of US$20 billion in the USA [227] and more than 3% of the healthcare expenditure in the UK [10,228]. Given that advanced chronological age is characterized by changes in skin morphology, but is also usually accompanied by comorbidities, healing in the aged becomes a multifactorial and multiparametric process.

4.1. The skin in the elderly

Among the key factors affecting wound healing in old individuals is ageing skin, which by default presents several differences compared to young skin (Table 2). First, changes in the behavior and physiology have been observed in all types of cells embedded in the epidermis and the dermis, including the remodeling of the skin immune system, being part of the general organismal inflammageing [229]. In the epidermis, keratinocytes present variability in cell morphology, differences in cytokine production and reduced proliferation and migration potential with advancing age [230–232], while melanocytes are fewer in aged individuals [233–235]. Even though it has initially been reported that Langerhans cell density remains unaltered with age in normal skin and mucosal tissues [236], more recent studies refer to ageing as a negative regulator of number, migration, dendritic function and antigen presenting role of Langerhans cells [232,237–240]. In the dermis, a high negative correlation between age and fibroblasts' density, as well as proliferation capacity has been reported [241–243]. Aged fibroblasts have also been shown to become dysfunctional in terms of responsiveness to growth factors, migration rate, integrin function, actin cytoskeletal organization and differentiation to myofibroblasts [244–246]. A reverse relationship has been described for leukocytes, macrophages and mast cells, which have been found to increase in number with advancing age, suggesting an enhanced inflammatory status [241,247–249]. However, decreased mast cell populations in the aged human skin have also been reported [250]. Macrophages in older individuals are also characterized by a declined function, a lower phagocytic activity and an altered profile of produced cytokines [217,221,232,251]. B lymphocytes present a limited antibody range, while T lymphocytes possess a lower cytolytic activity, a diminished repertoire of T cell

antigen receptors, a decreased mitogenic potential and an altered profile of secreted cytokines when activated and move from the naïve towards the memory phenotype [232]. Although in the work of Gunin et al. all bone marrow-derived cells were studied reciprocally and demonstrated an age-related change [241], conflicting evidence exists regarding skin stem cells' behavior with age progression: skin-derived progenitor cells have been reported to be less and to show a reduced differentiation potential in aged subjects [252], while epidermal stem cells have been documented to retain a constant density throughout lifespan [253–255]. Vascular endothelial cells from older donors are larger in size, have a lower proliferation and migration rate and overexpress fibronectin [256]. They have also been shown to present a better adherence to leukocytes, an increased IL-1 production and a higher response to TNF-α [223,257]. Furthermore, an age-related reduced subcutaneous adipose layer [258,259] and diminished ability of adipocytes to accumulate lipids have been documented [260], at the same time that differentiated adipocytes have been shown to be susceptible to senescence [261] and may be among the main cell types secreting inflammatory cytokines during ageing [260].

In addition to the alterations observed in the cellular constituents of the skin, ECM composition and structure shows differences in aged subjects compared to young ones. Collagen is reduced and fragmented in older individuals, resulting in disruption of dermis' structural integrity and thus hindering fibroblasts' attachment and mechanical stimulation [262–267]. Tensile strength of collagen fibers is increased with age due to the presence of advanced glycation end-products (AGEs) and enhanced crosslinking [268,269]. An increase in elastase-type protease activity has been shown in mouse skin [270] that was correlated to AGEs [271]. Non-functional, degraded and stiffened elastic fibers with a disordered orientation (elastotic material) replace normal elastin with advancing age [272,273] leading to an increase of skin rigidity and wrinkle formation caused by a fibrobrast-derived elastase activity [274]. PGs are aberrantly distributed in the elastotic material [275], while specific types of GAGs have been shown to appear in different ages and in a skin layer-dependent manner [276]. Additionally, lumican has been reported to decrease in the aged skin [277] and a catabolic fragment of decorin with reduced ability to bind collagen-I has been described to accumulate with age in adult human skin with putative implications in collagen fibrillogenesis [278]. Fibronectin expression and deposition has been found to change in aged individuals, but not following the same pattern among published studies [279–281]. Notably, the most universal change met in the aged skin is the flattening of the dermal-epidermal junction [282] permitting higher susceptibility to mechanical forces [283]. Finally, reduced vascularity of the dermis [284,285] hampers thermoregulation and adaptability to injury, whereas defects in the ability of the skin to remove fluid in response to gravitational stress favor the development of intradermal oedema [286]. Given that skin structure homeostasis is regulated by a firm equilibrium between ECM components' biosynthesis and degradation, changes in expression and activity of degrading enzymes in the aged skin are of outmost importance. Indeed, MMP expression has been shown to increase with both chronological and photo-ageing [287–290], while TIMP-1 is down-regulated in the aged skin [291].

Finally, cutaneous ageing is characterized by alterations in the expression patterns of growth factors and their receptors, along with the changes in the response of indigenous cells to these growth factors also mentioned above. An impaired activation of the TGF-β/Smad/CTGF axis attributed to CTGF/CCN2 underexpression has been shown in human dermal fibroblasts of aged donors, ultimately leading to collagen loss in the aged skin [292]. On the other hand, the lower mitogenic potential described for aged fibroblasts and keratinocytes early on may be due to a decrease in the expression of specific growth factor receptors, a possible dysfunction in the signal transduction machinery following ligand binding, a reduced secretion of growth factors and/or an inadequate responsiveness of the cells to the particular molecules [293].

4.2. Changes in wound healing with ageing

Taking into consideration the aforementioned changes in the aged skin, it could be easily assumed that wound healing would also present age-related alterations. In fact, it has been demonstrated that all phases of wound healing are modified with advancing age [217]. Overall an altered inflammatory response, a decreased cellular proliferation and migration capacity, a reduced cytokine production at the same time with a lower response to growth factors and a changed endothelial cell adhesion molecule profile have been described, all of them resulting in insufficient ECM components' deposition, defective angiogenesis and delayed tissue repair [11,217,221,294]. According to some older reports, inflammatory response is initially enhanced with age due to increased platelets' aggregation and to better adherence of endothelial cells to monocytes [257,295]. Other authors describe a delayed inflammatory response in terms of macrophage/monocyte, B and T lymphocyte infiltration (but with a final elevated T lymphocyte population) in older subjects and a lower fibronectin deposition hindering keratinocytes', fibroblasts' and inflammatory cells' adherence [11,221,294]. In addition, an impaired function of macrophages during wound repair with age has been documented [251].

An age-associated decline in growth factors that are important for wound healing has been reported [296]. PDGF and EGF and their receptors, as well as, bFGF and TGF-β3 have been found to show a delayed expression in the wounds of aged individuals [297]. Wound macrophages from aged animals have been shown to possess reduced phagocytic activity and diminished production of FGF-2 and VEGF, both negatively affecting ultimate tissue repair [220,221].

Consequently, there is a delayed cytokine secretion which, in combination with the altered responsiveness of keratinocytes to cytokines and the hypoxic conditions generally met in the wound, lags proliferation and migration of keratinocytes in the aged thus retarding re-epithelialization, while contraction is also affected in older individuals [223,298–303]. Indeed, it has been shown that wound healing in an aged rabbit wound ischemia model is compromised due to defective granulation tissue formation and re-epithelialization. As this model was characterized by lower expression of TGF-β receptors and suppressed activation of TGF-β-induced intracellular pathways, the phenotype could not be rescued by TGF-β1 treatment [304,305].

Even though platelet release and aggregation and granulocyte adherence have been shown to increase with ageing in vitro [295,306,307], defective angiogenesis has been reported for aged animals, which may also contribute to the final outcome of impaired wound healing. This has been most probably attributed to lower levels of FGF, VEGF, IGF-I and TGF-β and to a hypoxia inducible factor-1α (HIF-1α)-dependent stromal cell-derived factor 1α (SDF-1α) deficiency [220,308–313]. In addition, altered ECM remodeling during ageing has been ascribed to the simultaneous overexpression of MMPs (mainly MMP-2 and MMP-9) and underexpression of TIMP-1 and -2 [314–317], to the up-regulation of elastase [318] and to the reduction of TGF-β1 levels [217] in the wound. AGE-modified proteins accumulating in older subjects in their turn stimulate monocytes to produce IL-1, TNF, PDGF and IGF-I and hence they may affect tissue remodeling [319].

4.3. Quality of wound healing in the elderly

Even though age-related changes have been described for all phases of wound healing [217], there is a consensus that the main effect of ageing on repair in the absence of comorbidities is rather a delay than an actual malfunction [11,220–223,225], followed by a shorter maturation time [320]. However, additional factors appear in older individuals that could act cooperatively with advanced ageing in compromising wound healing and include microbial infections, psychological stress, obesity, malnutrition, smoking, alcohol consumption, pathological conditions, such as diabetes and medication [321]. Tissue repair in the elderly may under suboptimal conditions (repeated ischemia-

reperfusion injury, diabetes, inveterate inflammation due to bacterial contamination, etc.) endure so long that wounds remain practically unhealed and become chronic. Chronic wounds mostly involve venous ulcers, diabetic ulcers and pressure sores and have been shown to occur more often in aged individuals [322,323] imposing a great annual economic burden for the healthcare systems [296,324], as stated above.

If aforementioned additional factors affecting wound healing are excluded, it is thus far well established that the quality of scarring in healthy aged individuals at the microscopic and macroscopic levels is superior compared to young ones, in terms of ECM components' deposition and organization, as well as of inflammatory responses and ultimately resembling normal uninjured skin [11,12,217,325–327]. Furthermore, in contrast to young individuals from the early adolescence to the early adulthood that often exhibit exuberant scarring after injury [328], aged rarely develop keloids or hypertrophic scars [231]. This could be the combined outcome of (i) increased proteolytic activity and decreased collagen deposition due to MMPs up-regulation and TIMPs down-regulation in aged individuals [217], (ii) an impaired HIF-1 signaling observed under the persistent hypoxic conditions prevailing in the wound of the aged that may lead to lower expression of collagen and α-SMA [53,321,329], (iii) changes in the distribution of elastin and fibrillin [326], (iv) a reduced proliferation rate during healing [12], (v) a dysregulating immune system [11] and (vi) an altered TGF-β isoform profile [297], in comparison to young subjects.

Besides chronological ageing per se, altered wound healing in older individuals has been connected with changes in reproductive hormones' levels [330], since fibroblasts, macrophages, epithelial and endothelial cells, all have been shown to express estrogen receptors [331–333], while fibroblasts, keratinocytes and inflammatory cells are known to express androgen receptors [334]. In fact, according to a recent microarray analysis, 83% and 80% of genes found to be down-regulated and up-regulated, respectively in aged compared to young human wounds were estrogen-regulated [335]. Sex hormones have an active role in cutaneous wound healing by regulating inflammatory response, cytokine production and release, ECM deposition and degradation, re-epithelialization and contraction and angiogenesis [330,336–338]. Estrogens in particular modulate inflammatory response by influencing leukocytes' populations and function and by down-regulating macrophage migration inhibitory factor (MIF), monocyte chemoattractant protein-1 (MCP-1) and IL-1 [339–343]. Furthermore, they promote re-epithelialization through the increase of keratinocytes' proliferation and migration rates and the decrease of their oxidative stress-induced apoptosis [344–348]. They enhance PDGF expression in macrophages and monocytes [349] and TGF-β1 expression in fibroblasts [325,350]. Finally, estrogens regulate ECM components' secretion and deposition [325,350,351] and have a positive effect on endothelial cells' migration to induce angiogenesis [352]. Ashcroft et al. have demonstrated that delayed but improved in terms of quality cutaneous wound healing in aged (postmenopausal) females is attributed to estrogen deficiency and to the consequent lower TGF-β1 levels released in the wound environment by skin fibroblasts [325], in agreement with Shah et al. showing that TGF-β1 neutralization has an anti-scarring effect [102]. Delayed tissue repair with ageing has also been reported to occur during wound closure in mucosal tissues, especially in females [353]. Moreover, it has been demonstrated that oophorectomized rats presented delayed wound contraction [354]. In addition, it has been shown that topical estrogen application on wounds of healthy aged individuals leads to a decrease in elastase levels and in fibronectin degradation, as well as to an altered inflammatory response resulting from the diminished chemotaxis and the different adhesion properties of neutrophils [351]. In the same direction, it has been reported that estrogen increases collagen production and decreases MMP degrading activity during cutaneous wound repair in ovariectomized rats [355] and that estrogen receptor modulators can lead to wound healing acceleration in ovariectomized mice [356]. On the other hand, topical use of the known anti-estrogenic drug tamoxifen in keloids can ameliorate scar formation by hindering fibroblasts' proliferation rates and collagen production via a diminished TGF-β expression [357–360]. Taking all the above into consideration, it becomes evident that in general wound healing is faster in the presence of estrogens in the young, but is of better quality in the absence of estrogens in the aged.

In contrast to estrogens, increasing testosterone levels have been associated with delayed tissue repair in aged males due to an enhanced inflammatory response [334,361,362] and to an inhibition of re-epithelialization assisted by β-catenin [363], thus rendering males more susceptible to delayed cutaneous wound healing with advancing age [330,364]. In this context, castration or androgen receptor blockade in rodents has been shown to attenuate increased IL-6 expression following trauma-hemorrhage, to down-regulate TNF-α and nuclear factor-κB (NF-κB), to increase TGF-β1 levels and to improve wound repair [334,361,365–367]. On the contrary, androgens have been reported to exert anti-fibrotic effects on the heart by down-regulating TGF-β signaling, inhibiting angiotensin II – known to support fibrosis in various tissues [368] – and playing a protective role against oxidative stress [369,370]. Even though andropause is not analogous to female menopause, a gradual decline – but not a complete withdrawal – of testosterone levels with increasing age is also observed for elderly males [365,371,372]. However, the slower rate of tissue repair in older male individuals compared to young ones, as well as the decreased response to exogenous estrogen supply compared to elderly females provides evidence that other factors besides androgen remainders are also implicated in the inhibition of wound healing in old male subjects [334,371].

Summarizing this part, we can conclude that the wound healing process during ageing is altered due to inherent changes in skin structure (i.e. alterations in containing cells' responses and ECM components' biosynthesis and degradation rates) and in all phases of tissue repair. However, increased age cannot unfortunately be easily disconnected from accompanying hormonal changes and comorbidities, which complicate monitoring and elucidating the final result. We can infer that pathological conditions in the aged could lead to impaired wound healing and to the occurrence of chronic wounds, while healthy older individuals under optimal conditions are characterized by a delayed but of higher quality, in terms of scar formation, tissue repair.

5. The multiple roles of cellular senescence in tissue repair

Normal cells – in contrast to immortalized cancer cells – cannot proliferate indefinitely in vitro, even under the appropriate culture conditions including a potent growth stimulation. After a certain number of cell doublings they are unable to multiply further, although they remain metabolically active. This phenomenon, called “replicative senescence”, was first reported by L. Hayflick in the early 1960's in human embryonic lung fibroblasts [373] and was then reproduced for fibroblasts from several other tissues (and species) and for various cell types, e.g. epithelial, endothelial, smooth muscle cells, lymphocytes, as well as for adult stem cells ([374] and references therein). It was later proven that replicative senescence is caused by a constant shortening of the chromosomal telomeres after each cell doubling [375]. This shortening can lead to a DNA damage response (DDR), encompassing stimulation of the ATM-Chk2-p53 axis, leading to up-regulation of the CDK inhibitor $p21^{WAF1}$, and hence to hypophosphorylation of pRB and cell cycle arrest; subsequently, the increased expression of $p16^{INK4a}$ (another CDK inhibitor) stabilizes permanent growth arrest and the senescent phenotype [376].

Interestingly, beyond consecutive subculturing, cells can become senescent when serially exposed to subcytotoxic doses of various genotoxic exogenous stresses (Fig. 2), such as ionizing radiation, oxidative stress, chemotherapeutic drugs, inflammatory cytokines, even the activation of certain oncogenes [377–380]; this process is termed “stress-induced premature senescence” (SIPS). It is worth mentioning that, apart from their identification in vitro, senescent cells have also been detected in the tissues in vivo by using several markers, such as

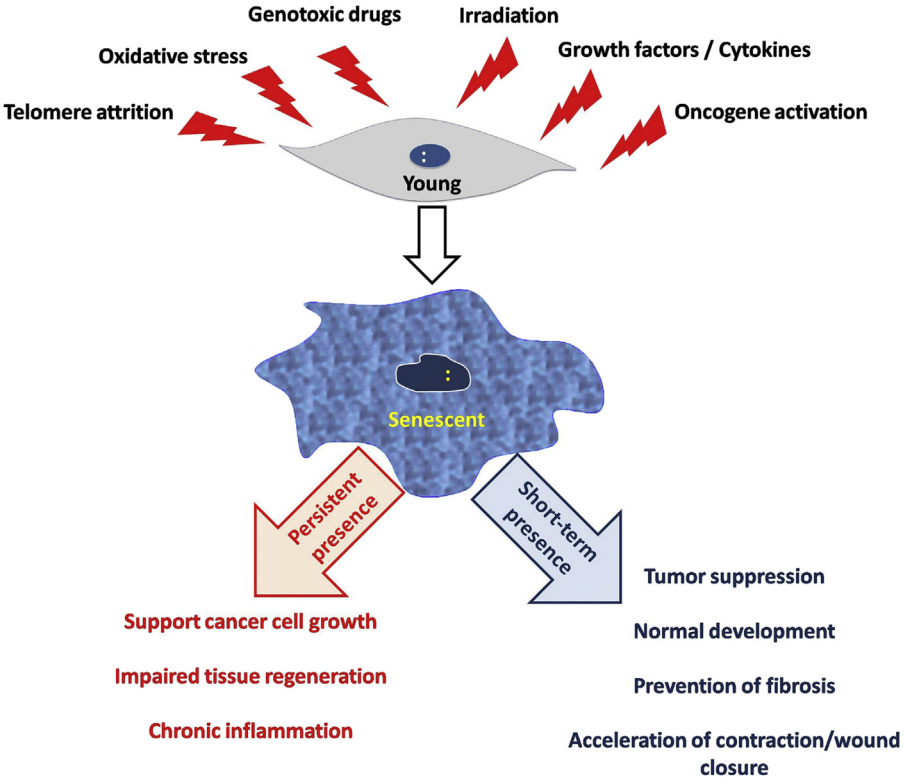

Fig. 2. Causes and physiological consequences of premature senescence.

the senescence-associated β galactosidase (SA β-gal) staining [381], the increased expression of the CDK inhibitor $p16^{INK4a}$ [28], and more recently a specific Sudan Black B staining [382,383].

At the functional level, based on the inability of senescent cells to proliferate, it is now widely accepted that cellular senescence represents a major obstacle against cancer development, thus preserving tissue homeostasis [7,377,384,385]. However, senescent cells exhibit a specific catabolic and inflammatory phenotype, featuring an increased secretion of a battery of products, like growth factors, ILs, chemokines and other proinflammatory molecules, MMPs and ECM components [386–390]. These factors – collectively called "senescence-associated secretory phenotype" (SASP) factors – seem to function in a paracrine manner affecting tissue homeostasis, while in addition, they reinforce senescence in an autocrine manner [386], leading to a vicious cycle of a local catabolism and inflammation. In this vein, senescent cells have been identified in sites of various age-related degenerative diseases, such as venous ulcers [391], atherosclerosis [388,392], chronic hepatitis [393], intervertebral disc degeneration [394], among others. Recent studies have shed light on the importance of the presence of senescent cells in age-related degenerative diseases. A number of research groups have shown that the removal of senescent ($p16^{INK4a}$-positive) cells from mice delayed the appearance of age-related deficits in a number of tissues [395–397].

Several studies have addressed the role of cellular senescence in the wound healing process. Initially, a role for senescent fibroblasts' accumulation in impaired or chronic wound healing was proposed, as an increased proportion of fibroblasts from venous ulcers was found to exhibit senescence characteristics [398]. It has been proposed that this can be due to the increased concentrations in the wound fluid of inflammatory cytokines (e.g. TNF-α), known to provoke premature senescence [399] and to induce skin catabolism [400]. Likewise, cultured pressure ulcer fibroblasts display signs of premature replicative senescence, overexpressing senescence-related molecules, like plasmin, plasmin activator inhibitor-1 and TGF-β1 [401,402]. In addition, chronic venous leg ulcer-derived fibroblasts morphologically resembled in vitro senescent cells and their proliferative response to PDGF-BB was poor [403]. In contrast, data from other groups suggest that no signs of senescence can be identified in such fibroblasts from chronic venous leg ulcers [404]. Nevertheless, human dermal fibroblast cultures, that have reached either replicative or stress-induced senescence, do not differ from cultures at low population doubling levels in terms of their in vitro response to paracrine factors suppressing α-SMA and collagen synthesis [405].

Impaired wound healing is a frequent comorbidity for patients suffering from diabetes mellitus. Diabetic patient-fibroblasts carry an excessive load of oxidants, leading them to SIPS and suppressing their capacity to proliferate and migrate [406]. Uncontrollable activation of macrophages can also affect wound healing quality. In this line it has been shown that macrophages with an unrestrained proinflammatory M1 activation state and incomplete switch to M2 secrete high TNF-α and hydroxyl radical levels, leading to $p16^{INK4a}$ upregulation and thus senescence of local fibroblasts, hence contributing to the inability of chronic venous ulcers to heal [28]. Finally, wound infections may also induce SIPS of skin fibroblasts. Pyocyanin, a virulence factor produced by certain *Pseudomonas* strains (notorious for their association with hospital-acquired infections) has been reported to induce oxidative stress leading to p38 MAPK activation, and, hence, to SIPS of dermal fibroblasts, in patients with infected burns [407].

Although the above mentioned studies indicate an involvement of senescent cells in impaired wound healing, more recent studies suggest a beneficial role of senescence in normal wound repair. Tissue damage in the liver stimulates the activation of hepatic stellate cells, which secrete ECM components leading to fibrosis. However, the increased proliferation of stellate cells provokes cell senescence and these cells via the secretion of SASP factors can limit fibrosis. In mice lacking the mechanisms leading to hepatic stellate cells' senescence, ECM is accumulated. The above indicate that senescence can prevent fibrosis during liver repair [408,409]. In an analogous manner, cardiac fibroblasts that became senescent following infarct-induced hypoxia limit local collagen production and heart fibrosis [410]. Similarly, at sites of cutaneous wound

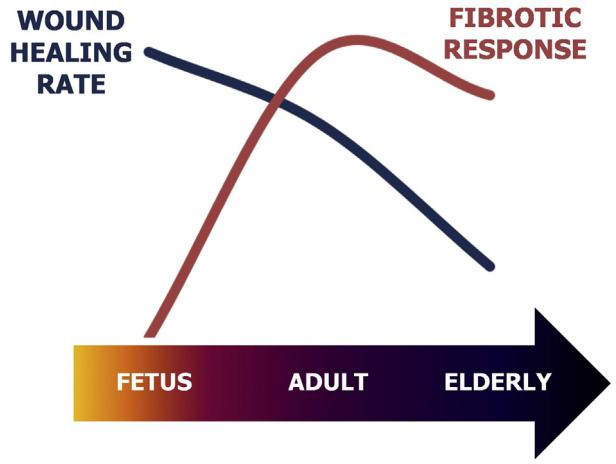

Fig. 3. Schematic representation of the wound healing rate and the presence of scar at the various periods of an organisms' life.

healing, the matricellular protein CCN1 can induce oxidative stress, which up-regulates $p16^{INK4a}$, thus provoking SIPS and indirectly stimulating an anti-fibrotic response, while in a knockin mouse model, where CCN1 is inactivated and cellular senescence is blocked, fibrosis is exacerbated [411]. In this vein, keloid fibroblasts have been found to exhibit a higher rate of senescence than normal ones, leading to the hypothesis that senescence may be a mechanism to keep keloid in a benign state [412].

In the aged skin senescent cells accumulate in the epidermis and the dermis, as found by various specific senescent markers [413,414] and due to their functional characteristics (e.g. decreased proliferation and increased SASP factors' secretion) can impair tissue homeostasis and wound repair. However, in young mice senescent cells appear transiently (between 3 and 12 days) at the wound site. These cells, most probably due to increased MMP secretion, limit fibrosis and, via the secretion of PDGF-AA, stimulate myofibroblast differentiation and thus contraction and accelerated wound closure [14,395,415]. In conclusion, cells can become senescent via different mechanisms and their transient or persistent presence can influence positively or negatively tissue repair and local tissue homeostasis (Fig. 2).

Finally, as mentioned above, fetal wounds are characterized by a scar-free phenotype. Interestingly, a senescent-like state in the murine fetus was recently discovered, which was proposed to contribute to tissue remodeling processes during embryonic development [416,417]. A major difference of this type of senescence from the classical one in the mature organism is the lack of a typical DDR, while it appears to be controlled mainly by developmental pathways. Accordingly, the complete characterization of cells exhibiting this developmentally programmed senescence and their presence and role in scarless fetal repair needs to be investigated.

6. Conclusion

In conclusion, the wound healing process follows diverse strategies at the various periods of an organisms' life. Especially, the speed of the whole process declines as the organism passes from the fetal stage to adulthood, and then ages. One of the main issues in wound healing in the adults is the formation of a scar, as an inevitable consequence of the main goal of repair at this stage, which is a fast healing to avoid fluid loss and contamination. In addition, in advanced chronological age suboptimal conditions (including malnutrition or various diseases) could permanently impair the process of healing and lead to chronic, non-healing wounds. However, under normal conditions and in the absence of these aggravating factors, although there is a decline in the speed of healing, the quality of the new tissue is highly improved with minimal scar formation. In this direction, the presence of senescent cells may be beneficial when they appear transiently in the wound area, but detrimental in chronic wounds. So, it seems that the two edges of life (embryonic life and advanced age) are characterized by a better quality of tissue repair, and specifically by an absence or a diminished fibrotic response, respectively (Fig. 3). Further understanding of the mechanisms of tissue repair in all these stages may lead to novel interventions leading to a more effective healing in the adults.

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This article is dedicated to the memory of our mentor Dr. Dimitri Stathakos.

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