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Cellular immune regulation in skin wound healing

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vorgelegt von
Ruoxuan Dai

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Erstes Gutachten: Prof. Dr. Jürgen Behr

Zweites Gutachten: Dr. Yuval Rinkevich

Drittes Gutachten: Prof. Dr. Markus Reinholz

Viertes Gutachten: Prof. Dr. Rudolf A. Rupec

Dekan: Prof. Dr. med. Thomas Gudermann

Tag der mündlichen Prüfung: 07.12.2023

Affidavit

Affidavit

Dai, Ruoxuan

_____
Surname, first name

Max-Lebsche-Platz 31

_____
Street

81377, Munich, Germany

_____
Zip code, town, country

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Dai, Ruoxuan

_____
Surname, first name

Max-Lebsche-Platz 31

_____
Street

81377, Munich, Germany

_____
Zip code, town, country

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List of abbreviations

ECM	Extracellular matrix
PDGF	Platelet derived growth factor
ROS	Reactive oxygen species
H_2O_2	Hydrogen peroxide
DAMPs	Damage associated molecular patterns
NETs	Neutrophil extracellular traps
IL	Interleukin
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
EGF	Epidermal growth factor
IGF	Insulin-like growth factor
NGF	Nerve growth factor
FGF	Fibroblast growth factor
NO	Nitric oxide
SOD	Superoxide dismutase
TGF	Transforming growth factor
NLRP3	NLR family pyrin domain containing 3
MCP-1	Monocyte chemoattractant protein-1
PPAR-γ	Peroxisome proliferator activated receptor-γ
NETs	Neutrophils release extracellular traps
PAD4	Peptidyl deaminase 4
HTS	Hypertrophic scars
Treg cell	Regulatory T cell
MMP	Matrix metalloproteinases
MCS	Mesenchymal stem cell
En1	Engrailed-1
EPFs	En1-past fibroblasts
GFP	Green fluorescence protein
ENFs	En1-naïve fibroblasts
RFP	Red fluorescence protein
ePTFE	Polytetrafluoroethylene

PC	Panniculus carnosus
DT	Diphtheria toxin
YAP	Yes-associated protein
sh	Short hairpin
dWAT	Dermal white adipose tissue
sWAT	Subcutaneous white adipose tissue
Plin1	Perilipin-1

List of publications

1. Rajendran V, Ramesh P, **Dai R**, Kalgudde Gopal S, Ye H, Machens HG, Adler H, Jiang D, Rinkevich Y. Therapeutic Silencing of p120 in Fascia Fibroblasts Ameliorates Tissue Repair. J Invest Dermatol. 2022 Nov 26:S0022-202X(22)02821-4. doi: 10.1016/j.jid.2022.10.018. Epub ahead of print. PMID: 36442618.

2. Shruthi Kalgudde Gopal*, **Ruoxuan Dai***, Ania Maria Stefanska, Meshal Ansari, Jiakuan Zhao, Pushkar Ramesh, Johannes W. Bagnoli, Donovan Correa-Gallegos, Yue Lin, Simon Christ, Ilias Angelidis, Valerio Lupperger, Carsten Marr, Lindsay C. Davies, Wolfgang Enard, Hans-Günther Machens, Herbert B. Schiller, Dongsheng Jiang, Yuval Rinkevich. Wound infiltrating adipocytes are not myofibroblasts. *Nature Communications*. ***First author with equal contribution** (Accepted by editor)

Publications

1.1   Publication I

Rajendran V, Ramesh P, **Dai R**, Kalgudde Gopal S, Ye H, Machens HG, Adler H, Jiang D, Rinkevich Y. Therapeutic Silencing of p120 in Fascia Fibroblasts Ameliorates Tissue Repair. J Invest Dermatol. 2022 Nov 26:S0022-202X(22)02821-4. doi: 10.1016/j.jid.2022.10.018. Epub ahead of print. PMID: 36442618.

Publisher's version is available online in: https://pubmed.ncbi.nlm.nih.gov/36442618/

In this work, my contributions include: a) Immunostaining of samples of virous markers. b) Statistical analysis of different markers expression.

In particular:

(1) Figure 2c, 2d: Immunostaining GFP$^+$ wound resident fibroblasts from 7dpw expressing myofibroblast marker(αSMA) and fibroblast markers (CD26; ERTR7)
(2) Figure S1: Immunostaining of different cell type (epidermal marker CK14, endothelial marker CD31, immune cell marker CD45, pan fibroblast cell marker PDGFR, fascia fibroblast marker N-cadherin and Sca1) of AAV8-GFP transduced cells.
(3) Figure S2: Immunostaining of p120 in *En1^{Cre};R26^{mTmG}* mice and different cell markers (N-cadherin, CD45, Caspase-3) in AAV8 control shRNA and AAV8 p120 shRNA injected wounds.

1.2 Publication II

Shruthi Kalgudde Gopal*, **Ruoxuan Dai***, Ania Maria Stefanska, Meshal Ansari, Jiakuan Zhao, Pushkar Ramesh, Johannes W. Bagnoli, Donovan Correa-Gallegos, Yue Lin, Simon Christ, Ilias Angelidis, Valerio Lupperger, Carsten Marr, Lindsay C. Davies, Wolfgang Enard, Hans-Günther Machens, Herbert B. Schiller, Dongsheng Jiang, Yuval Rinkevich. Wound infiltrating adipocytes are not myofibroblasts. *Nature Communications.* ***First author with equal contribution** (Accepted by editor)

The above study is a result of my contribution as a "shared-first author" or "first-author-with equal contribution". My major contribution include a) Completed full-thickness excisional wounding experiments with $En1^{Cre};R26^{mTmG}$ mice and $Adipoq^{Cre};R26^{mTmG}$ mice at day 0, day 10 and day 21. b) Fascia tissue isolation and digestion for FACS sorting of adipocytes and fibroblasts with negative depletion strategy and live/dead staining, mRNA extraction, cDNA conversion and expansion and bead purification, quality control, sample delivery for bulk sequencing. c) Cryosections for the samples and various immunostainings of Perilipin, αSMA, Vimentin, Cathelicidin of $En1^{Cre};R26^{mTmG}$ mice and $Adipoq^{Cre};R26^{mTmG}$ mice. d) Completed full-thickness excisional wounding experiments with $Procr^{CreER};Ai14$ mice and $PDPN^{CreER};Ai14$ mice at day 7. e) Immunostainings of Perilipin, PDGFRα, and ER-TR7 were performed with cryosections of healthy skin and day 7 wounds of $Procr^{CreER};Ai14$ mice and $PDPN^{CreER};Ai14$ mice. f) Modified and draw the flowchart. g) Revision of the manuscript and data statistical analysis.

In particular:

(1) Figure 1a: Modified and draw the scheme.
(2) Figure 5a, 5b, 5c, 5d, 5e, 5h: Implementation of in vivo experiment and immunostainings of adipocytes/fibroblasts markers to prove adipocytes are non fibrogenic in wounds.
(3) Figure S5a, S5b, S5c: Heatmap analyze the core signature gene of FACS sorting GFP⁺ cells and immunostainings of Ki67 in wounds.
(4) Figure S6 (delete according to Reviewer 3): Implementation of in vivo experiment and immunostainings of adipocytes cell marker to proof adipocytes in adult wounds do not overlap with wound fibroblasts.

2.   Introductory summary

2.1   Physiological structure and function of the skin

With a surface area of around 1.8 square meters, the human skin serves as the body's primary physical barrier and aids in defending the body against external diseases and environmental toxins [1]. The most outer epidermis layer, the inner dermis layer, and the deepest layer of subcutaneous or hypodermis tissue make up the three primary layers of normal human skin.

The epidermis, the skin's outermost layer, can be subdivided into five sublayers [2, 3]. Stratum corneum, stratum lucidum and stratum granulosum are the outermost layers of the epidermis of the skin, which is made up of dead cells that can shield the body from friction and moisture loss. Keratinocytes, which may create keratin and make up the fundamental structure of skin, are abundant in the stratum spinosum layer. The deepest layer of epidermis is stratum basale, it comprises constantly dividing basal cells that push older cells upwards. It also contains melanocytes, which control skin pigmentation.

The next inner dermis layer predominantly consists of extracellular matrix, blood vessels, lymphatics, nerves, sweat glands and pilosebaceous units. This layer is divided into two sublayers: the upper papillary and the lower reticular dermis layers. The papillary dermis layer has tiny collagen fibers that are tightly packed and is just 0.3 – 0.4 mm thick. The reticular dermis layer, in contrast, develops interwoven bundles of denser fibers [4].

The dermis and skeletal muscles are wedged between the subcutaneous or hypodermis tissue, the skin's deepest layer. Proteoglycans and glycosaminoglycans are particularly abundant in the hypodermis tissue, which draws fluid into the tissue and gives it mucous-like qualities [5]. Fibroblasts, adipose cells, and macrophages are the types of cells found in the hypodermis, and they play a specific function in adipocyte homoeostasis in obesity, likely connected to tissue remodeling and could promote fat thermogenesis when exercising and being exposed to cold temperatures [6]. The hypodermis of pig and human skin was discovered that the structure was made up of a lattice network of fibrous tissue that was mostly vertically oriented and was structured into geometric forms. This loose connective tissue structure was later defined as fascia. Increasingly, its mechanisms and functions are being studied.

2.2   The repair process of skin wound healing

Both the rate and the integrity of skin wound healing differ under various physiological circumstances. For instance, wounds can last a long period in diabetic and immunocompromised patients. After healing, the lesions in certain people with scarring sometimes develop into keloids. As a result, the following healing states will be used to explain how skin wounds heal.

2.2.1   Normal wound healing of the skin

Acute wounds are typically caused by tissue injuries that can usually heal completely within the expected timeframe of 8-12 weeks, with minimal scarring [7]. The normal acute wound healing sequence is: hemostasis, inflammation, proliferation and remodeling [8].

1. Hemostasis

The hemostatic process, the first line of defense against skin wounds, is triggered when the blood vessels of the skin are damaged. This results in the production of a quick hemagglutination response in the wound. This process involves vasoconstriction and platelet aggregation [9].

When an injury occurs, the body's response includes rapid vasoconstriction. This is achieved through a reflex contraction of the vascular smooth muscle, which is triggered by the release of vasoconstrictors like endothelin from injured endothelial cells. These vasoconstrictors cause the blood vessels to narrow, reducing blood flow to the injured area [10]. Additionally, platelets generate platelet-derived growth factor (PDGF), which has the ability to contract smooth muscle in the artery wall by activating it [11-13]. However, the first reflex contraction only stops bleeding for a brief period. Passive muscle relaxation is caused by increased acidity and hypoxia in the wound, which in turn promotes the recovery of bleeding. Following activation of the coagulation cascade requires long-term relief of bleeding issue as well as further regulation of vasoconstriction by mediators of bradykinin, fibrinopeptide, 5-hydroxytryptamine, and thromboxane A2 [14, 15].

Primary hemostasis and secondary hemostasis occur simultaneously and are mechanistically interrelated [16]. Primary hemostasis involves platelet aggregation and the formation of platelet plug. Platelets bind to the matrix via G protein-coupled receptors on their surface and trigger an inside-out signaling cascade that activates integrins and enhances platelet adherence to the surrounding extracellular matrix (ECM) and other platelets to plug the injury [17]. When the coagulation cascade is activated, soluble fibrinogen is transformed into insoluble fibrin chains that form a fibrin network, reinforcing and solidifying the clot. This process is known as secondary hemostasis. Platelets serve a hemostatic role by adhering to the fibrin network to form a thrombus, and their release of complement and growth factors creates a temporary scaffold for invading cells that is essential for wound healing [18].

2. Inflammation

The inflammatory phase is characterized by a large number of immune cells infiltrating the wound, such as neutrophils, monocytes, and lymphocytes [19]. This phase of wound healing lasts 48 hours following injury [20]. Pro-inflammatory mediators are abundant at this stage, flooding the area around the wound and recruiting more immune cells from the blood vessels. During the inflammatory phase, phagocytes play a crucial role in removing necrotic material from the wound. The primary objective of this phase is to provide the host with sufficient time to protect itself from any infections that might penetrate the wound site.

Large numbers of chemokines, lipid mediators, hydrogen peroxide (H_2O_2), damage-associated molecular patterns (DAMPs) released by damaged cells, signaling the recruitment of inflammatory cells, particularly neutrophils. Neutrophils play a crucial role in fighting pathogens by deploying different mechanisms. Firstly, they release proteases from their intracellular granules to break down invading microbes. Additionally, neutrophils can generate neutrophil extracellular traps (NETs) through a process known as NETosis. During NETosis, neutrophils extrude chromatin filaments coated with proteases outside their cell membrane to ensnare and neutralize pathogens, effectively eliminating them from the host [21]. It was discovered that inhibiting the formation of NETs or removing NETs may improve wound healing [22]. Also, phagocytosis by neutrophils plays an important role in the early stages of wounding. Surface receptors and integrins on neutrophils help them recognize antigens. The antigen is then engulfed by a phagocytic cup formed

by the neutrophil granules, where proteases break it down [23]. Proteases are the main component of toxic particles. When active, proteases can digest surrounding tissue to let neutrophils exit the vasculature and reach the site of damage. Proteases are crucial for antibacterial action, for dissolving fibrillary ECM, and for breaking down basement membranes [24]. Clearing neutrophils in a timely manner is essential for resolving tissue inflammation. Neutrophils can either be phagocytosed by macrophages, or they can re-enter the vasculature in a distinct process and leave sites of tissue damage referred to as neutrophil reverse transendothelial migration.

Throughout the first 24 to 48 hours after a wound is formed, macrophages then gather at the site of the lesion [25]. The release of monocyte chemoattractant protein-1 (MCP-1) plays a crucial role in the recruitment of monocytes from the bone marrow to the site of inflammation, which promotes monocytes rapidly differentiate into macrophages and actively participate in the immune response. To combat wound infection, macrophages then produce copious amounts of pro-inflammatory cytokines including interleukin (IL)-6, tumor necrosis factor (TNF)-α, and IL-1 to active fibroblasts [26]. Meanwhile, macrophages engulf pathogens and cellular debris through their phagocytosis. Phagocytosis of apoptotic neutrophils by macrophages signals the end of the inflammatory phase of wound healing [27].

3.   Proliferation

During the proliferative phase of the wound (after approximately 3-10 days), the main goals of the wound healing process are to cover the wound surface, restore the vascular network, and form granulation tissue.

In the initial proliferation phase of wound healing, keratinocytes migrate upward from the injured dermis, which is defined as re-epithelialization. During this process, localized keratinocytes from the margins of the lesion and stem/progenitor cells from sweat glands or interfollicular epidermis progressing from the margins to the wound center [28-30]. The signaling pathways of epithelium and non-epithelial cells along the wound edge, which produce a wide range of cytokines and growth factors including epidermal growth factor (EGF), insulin-like growth factor (IGF)-1, and nerve growth factor (NGF), promote this process [31].

During the next proliferative phase, new blood vessels are formed, and capillaries sprout and link to fibroblasts and macrophages. Granulation tissue then works to remove the fibrin matrix, paving the way for the creation of a new matrix. This new matrix serves as a foundation on which keratinocytes can regenerate the damaged epidermis, restoring the skin's integrity [32]. Growth factors, including VEGF, PDGF, fibroblast growth factors (FGF), and serine protease thrombin, bind to receptors on existing vascular endothelial cells, thereby activating intracellular signaling cascades. Proteolytic enzymes are secreted by the activated endothelial cells, which lyse the basal lamina. Endothelial cells can proliferate and move into the wound as a result, then the freshly constructed endothelial sprouts create tiny tubular canals that join to one another to form a vessel loop [33].

The proliferative phase culminates in the formation of acute granulation tissue, which is characterized by a dense network of capillaries and loosely arranged collagen fibers. This process also involves the recruitment of numerous granulocytes, macrophages, and fibroblasts. Collagen and other ECM components (e.g., fibronectin, glycosaminoglycan, proteoglycan, and hyaluronic acid) can be produced by fibroblasts [4, 34]. The temporary wound matrix, on which the relevant cell migration and organization takes occur, is therefore created by fibroblasts. The number of mature fibroblasts is reduced by myofibroblast differentiation and terminated by subsequent apoptosis at the conclusion of this phase.

4. Remodeling

Remodeling, which usually starts 2 to 3 weeks after damage and lasts for a year or longer, is the last stage of wound healing. All post-injury activation processes are gradually decreased and terminated during this period. The majority of endothelial, macrophage, and myofibroblast cells either suffer apoptosis (programmed cell death) or wither away from the wound, leaving a mass made of collagen and other extracellular matrix components and a few cells. In addition, the cell-free matrix undergoes an active remodeling process within 6–12 months, changing from a matrix with a largely type III collagen foundation to one with a predominantly type I collagen [35] . This form of collagen differs from the basket-like collagen present in healthy dermis as it is organized in short parallel bundles. And through their many collagen-attachments, myofibroblasts produce wound contraction and aid in reducing the surface of growing scars.

2.2.2 Chronic wound healing of the skin

Wounds that are challenging to cure and remain longer than 6 weeks or regularly reopen are known as chronic wounds [36]. The most prevalent chronic wound disease in the U.S. is diabetic ulcers [37]. With a mortality rate greater than most malignancies, more than half of patients who have limbs amputated due to ulcers will die within 5 years [38]. Peripheral neuropathy can cause patients to be unaware of small wounds, leading to undetected ulcer development and progression. Additionally, diabetes can damage the larger blood vessels in the legs, resulting in peripheral arterial disease and impaired circulation. This vascular issue can significantly impede ulcer healing. The long-lasting and recurring ulcers that result from diabetes place a substantial burden on the healthcare system [39].

1. Platelets

A crucial element of hemostasis, platelets are in charge of some of the early reactions to damage. The thrombus can play a temporary scaffold role in facilitating the proliferation and migration of cells that are involved in the process of wound healing. However, for complete healing to occur, the thrombus must eventually be removed or broken down to allow for the regeneration of healthy tissue. It has been shown that under diabetes circumstances, platelets are less susceptible to the nitric oxide (NO) that the vascular endothelium releases, which ordinarily lessens vessel wall aggregation [40]. This is further worsened by poor insulin signaling, which causes the vascular endothelium to produce less NO and endothelial nitric oxide synthase and superoxide dismutase (SOD) to function less efficiently [41]. Consequently, as has been shown in diabetic patients, platelet dysfunction plays a role in the development of microvascular illness.

2. Neutrophils

Neutrophils play a crucial role in the inflammatory stage of wound healing and are responsible for removing germs. Nevertheless, chronic, non-healing wounds are linked to ongoing neutrophil recruitment and activation. Increased neutrophil-derived protease levels (e.g. elastase and matrix metalloproteinases (MMP)-8 and -9) are hypothesized to have a role in chronic inflammation and delayed wound healing [42]. In addition to proteases, neutrophil release of extracellular traps (NETs) is increased in diabetes and affects the resolution of inflammation during wound healing [43]. It has been demonstrated that peptidyl deaminase 4 (PAD4), the protein that causes NETs, is higher in neutrophils from both diabetic mice and patients, making them more prone to NETosis and potentially causing persistent inflammation and tissue damage [22].

3. Monocyte/macrophages

Early in the inflammatory phase, monocytes are attracted to the wound site where they undergo differentiation into macrophages and dendritic cells. The establishment of the initial inflammatory phase and the promotion of the change from a pro-inflammatory to an anti-inflammatory environment depend critically on the infiltration of monocytes and macrophages [44, 45].

During the normal healing process, there is a shift from inflammatory to proliferative activities which is reflected by a change in the ratio of M1 to M2 macrophage phenotypes. To encourage pathogen-killing activity and early wound debridement, classically activated M1 macrophages release high quantities of pro-inflammatory cytokines (e.g. IL-1, IL-6, IL-12, TNF-α) and oxidative metabolites (e.g. NO, superoxide) [46, 47]. Inflammation is induced and aids in its resolution when M2 macrophages are activated, especially through IL-4 and IL-13 signaling. IL-10, VEGF, transforming growth factor (TGF)-β1, and PDGF, which encourage the formation of granulation tissue, angiogenesis, and cell proliferation, are among the cytokines and growth factors secreted by classical M2 macrophages that are upregulated to indicate the phenotypic switch.

In diabetic wounds, macrophages remain in a persistent M1 inflammatory state, wherein macrophages promote the destruction of surrounding tissues directly or indirectly by recruiting other pro-inflammatory immune cells. Matrix metalloproteinase 1 (MMP1) and the pro-inflammatory cytokine TNF-α are both downregulated by M1 macrophages, which inhibits keratinocyte migration and slow wound healing [48]. Through the NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome and Peroxisome proliferator-activated receptor-γ (PPAR-γ) signaling, excess glucose and adipose tissue increase the expression of inflammatory markers (e.g. TNF-α, IL-1, IL-6) and this also increases the expression of pro-inflammatory genes, which further activates M1 macrophage polarization [49-51]. This vicious cycle results in chronic inflammation and wounds that are unable to heal. Moreover, hyperglycemia causes an increase in histone acetylation and methylation, which supports the M1 macrophage phenotype.

4. Fibroblasts

Fibroblast function changes also lead to defective epithelialization and delayed wound healing in diabetics. These mainly involve decreased proliferation, increased apoptosis, and impaired migration to the wound site [52, 53]. In patients with diabetic foot ulcers (DFUs), specific populations of fibroblasts were found to be overexpressing *MMP1, MMP3, MMP11, HIF1A, CHI3L1, and TNFAIP6*, which helped in promoting the polarization of M1 macrophages. Furthermore, through spatial transcriptomic analysis of patients, it was found that fibroblasts associated with wound healing showed a preference towards the wound bed rather than the wound edge or healthy skin [38]. Hyperglycemia can impair cytokine signaling pathways that affect fibroblast differentiation (e.g. TGF-β pathway), alter fibroblast apoptosis, dysregulate dermal lipolysis, and increase hypoxic injury. As a result, myofibroblast formation is impaired, the extracellular matrix is improperly regulated, and wound contractility is decreased [54].

2.2.3 Excessive wound healing

Excessive healing is characterized by the overproduction of extracellular matrix, increased local vascularity, and excessive cell proliferation. These changes may lead to the formation of hypertrophic scars or keloids, which can be unsightly and uncomfortable. This hypertrophic scarring may be due to a severe injury (e.g., burn) or may manifest after an acute injury in a keloid with a

genetic basis. Large disfiguring scars can physically deform surface structures (e.g., nose, eyelids) [55].

1. Macrophages

In keloid tissues, macrophages are primarily of the M2-phenotype, which are strongly linked to tissue healing and remodeling [56]. Keloid-derived macrophages exhibit a hyperactivated state with greater M2 polarization and increased overall expression of M1 and M2 activators as compared to normal skin macrophages. By secreting PDGF-CC and TGF-β, which both favor collagen deposition and scar formation, these M2 macrophages can promote the conversion of fibroblasts into myofibroblasts, indicating a potential role for M2 macrophages in fibrosis [57, 58]. In patients with hypertrophic scars (HTS), CD163-positive M2 macrophages may be seen 4-6 weeks after injury, but only after 2 weeks in patients with normal scar formation [59]. Moreover, as HTS formation advances, IL-10 and IL-1RN concentrations continue to fall and return to normal levels after 6 months [60]. 24 months after injury, the expression of Arg-1 and CD206 declines, and the quantity of M2 macrophages (CD68$^+$ and CMAF$^+$) reaches the pre-injury level.

Researchers have created a nude mouse model of human hypertrophic scar (HTS) by transplanting scar tissue from HTS patients into the mice. This model exhibits increased thickness, higher collagen levels, and infiltration of myofibroblasts. M2 macrophages occurred in considerable numbers late 3–4 weeks after transplantation, which was concurrent with the development of fibrosis in human skin tissue. In allografted skin, M1 macrophages were observed primarily 1-2 weeks after grafting [61].

2. T Lymphocytes

Due in part to the variety of T cell subsets, T cells contribute to the development of scars through multifaceted functions. In addition to the distinction between CD8$^+$ cytotoxic T cells and CD4$^+$ helper T cells, Th1, Th2, Th17, Tfh, and regulatory T (Treg) subpopulations of CD4$^+$ T cells can be distinguished based on transcriptome and effector cytokines. By secreting IFN-γ and MMPs, which prevents fibroblast proliferation and suppresses the expression of type I and III collagen, Th1 cells can decrease tissue fibrosis [62]. In contrast, Th2 cells' released IL-4, IL-5, IL-13 and TIMPs can stimulate the production and metabolism of collagen in a fibrosis model, resulting in the accumulation of reticular fibrin protein [63]. The growth of IL-10-producing Treg cells may also be assisted by TGF-1β-producing Treg cells [64]. In fibroblasts, IL-10 directly reduces the production of collagen [65]. In addition to regulating fibroblasts directly, Treg cells also work with other T helper cells to influence the deposition of collagen and subsequent scar formation [66].

3. Fibroblasts

Myofibroblasts, the primary contractile and scar-depositing cell type in charge of ECM buildup, can be produced from resting resident skin fibroblasts either by trans-differentiation or from multilineage-differentiating, self-renewing fibroblast progenitors. In keloids, certain fibroblasts may be seen embedded in the collagen matrix (e.g., types I, III, V, and VI collagen), frequently displaying myofibroblastic characteristics and prominently expressing marker proteins, including α-SMA and vimentin. In keloid scars, fibroblasts have a variety of characteristics compared to normal wound healing.

Studies show that a reduction in apoptosis leads to a cumulative rise in the number of keloid fibroblasts [67-69]. By the elevation of anti-apoptotic capability, telomere dysfunction, and senes-

cence abnormalities, apoptosis is also decreased in keloid fibroblasts. The activation of telomerase and subsequent telomere lengthening seen in keloid fibroblasts supports the life-extending impact of telomere dysfunction for this skin disease [70]. In 3D invasion experiments, keloid fibroblasts also exhibit greater migratory and invasion potential [71].

According to the heterogeneity of keloid fibroblasts, they can be divided into 4 subpopulations: secretory-papillary, secretory-reticular, mesenchymal, and pro-inflammatory [55]. As compared to normal scars, keloid scars have a much higher number of fibroblast subpopulations, which are essential for the exuberant collagen deposition seen in keloids. It's interesting to note that various scar forms may have varying levels of cellular heterogeneity. For instance, keloid fibroblasts' propensity to develop into adipocytes in response to BMP4 or when co-cultured with human scalp hair follicle cells [72]. According to studies, keloid fibroblasts have a great ability for self-renewal and are resistant to drugs, which allows them to sustain themselves through asymmetric cell division [73]. The typical tumor-like keloid development is thus set in motion by the ongoing production of new aberrant fibroblast cells, which also helps to explain their high post-treatment recurrence rates. The pathological keloid microenvironment may also be the primary cause of keloid cell production, with overexpression of growth factors within its microenvironment in turn leading to an enhanced and persistent inflammatory response.

2.3   Skin fibroblast subpopulations in mobilized skin fascial tissue promote wound healing

Deep skin wounds may be assisted by muscle contraction, fibroblast differentiation, and extracellular matrix deposition, according to earlier studies. Our research revealed that during deep wound healing, the fascial layer of the skin, a loose layer of connective tissue containing a large number of fibroblasts, ECM, blood vessels, and nerves, migrate toward the center of the wound and contracts, thus promoting wound healing [74].

The fascia is a viscoelastic tissue that is filled with fluid, and it serves to create a smooth and slidable interface between organs and tissues throughout the body [75]. The cutaneous ligaments provide a three-dimensional network between the fat lobules by connecting the deep fascia and superficial fascia to the epidermis [76]. In humans, the fascial layer is composed of sensory neurons, fibroblasts, lymphatic arteries, adipose tissue, and a neurovascular network [77]. Skin fibroblasts are one of them, making up a diverse cell population with a variety of as-yet-undefined traits. We found that the main cause of numerous types of pathological scars was attributed to En1-past fibroblasts (EPFs), which were characterized by transient expression of the engrailed-1 (En1) transcription factor during the embryonic stage. On the contrary, En1-lineage-naïve fibroblasts (ENFs) were not involved in the formation of scars. Both EPFs and ENFs co-inhabit similar skin dermal compartments. To monitor EPFs, we crossed *En1^{Cre}* mice with *R26^{mTmG}* mice which express green fluorescence protein (GFP) in vivo, and ENFs, which retain the original red fluorescence protein (RFP) [78]. We found that ENFs were responsible for dermal development and regeneration once injured at early fetal stages of development [79]. Whereas EPFs gradually increased in population and outnumbered ENFs at newborn stages. EPFs primary role was during adult wound healing, and their ablation reduced connective tissue buildup and scarring around the wound.

To curb fascial tissue from moving toward the wound bed, we grafted polytetrafluoroethylene (ePTFE) membranes between fascial tissue and the panniculus carnosus (PC) muscle in a full thickness skin wound healing mouse model [74]. We discovered that the wounds in the ePTFE group of mice remained completely open, whereas the wounds in the control group healed completely within 21 days. Two months later, the EPFs pulled out from the borders of the incision and from the fascia underneath the membrane without leaving scars. As a result, when fascial mobility was absent, the dermal EPFs or other resident dermal fibroblasts were incapable of effectively healing wounds. These findings serve a clear indication that scar tissue is predominantly of fascial origin. The fascia of *En1^{cre};R26^{iDTR}* animals and the dermis of wild-type mice were then combined to form chimeric grafts. Diphtheria toxin (DT) was used to genetically deplete facial EPFs from the chimeric tissues, and the matrix was fluorescently tagged before grafting. The study's results showed that the tagged matrix persisted in the fascial layer underneath the incision, but when the fascial EPFs were ablated, it prevented the matrix from entering the wound. Thus, the resident EPFs that originate from the fascia play an active role in guiding the fascial matrix to close the open wound.

Shamik Mascharak et al. identified a subset of dermal ENFs that create EPFs by inducing the expression of *En1* during adult wound healing using cell transplantation and transgenic mice models [80]. They discovered that reticular dermal ENFs activate through the normal *En1* mechano-transduction signal by observing how ENFs react to matrix mechanics. According to mouse wound models, suppressing Yes-associated protein (YAP) reduces *En1* activation, increases wound regeneration of ENFs, and restores skin appendages, ultrastructure, and mechanical strength through limiting mechano-transduction signaling.

Hence, research into EPFs may open new therapy options for diseases that cause skin scarring. The p120 catenin protein was initially discovered through a screening for substrates of the Src tyrosine kinase. It is a 120kDa protein, and its phosphorylation on tyrosine residues has been found to correlate with cellular transformation [81]. It has been demonstrated that p120 catenin controls intercellular adhesion through its interaction with the cytoplasmic association membrane domain of cadherins.

We discovered that AAV8 had the highest transactivation efficacy in fascial fibroblasts in vivo after screening for AAV pseudotypes in **Publication I**. Using AAV8 as a gene expression modification tool, we next examined the efficiency of AAV8 transfection of fascia in vivo and found that most of it transfected into fibroblasts of the fascial layer (Pdgfrα$^+$). Moreover, we discovered that p120 expression dramatically increased at day 5 after injury and co-localized with EPFs in the fascia, before declining at day 21 after injury. p120 knockdown lowered N-cadherin and α-SMA expression in wounds and decreased collagen in ECM, allowing scarless wound healing outcomes to resurface. This was accomplished via AAV8-mediated silencing of p120 by short hairpin (sh) RNA.

Previous research had shown that fascial resident EPFs fibroblasts mobilized an influx of ECM from the fascial layer into the wound [74, 82, 83]. By fluorescently labeling the skin fascia's ECM with NHS ester dye, we tested the hypothesis that inhibiting p120 in EPFs would stop fascial ECM from moving in vivo. As compared to the AAV8 control shRNA group, the matrix from the wound bed in the fascial layer was dramatically reduced in AAV8 p120 shRNA group.

In conclusion, our studies demonstrated that therapeutic manipulation of p120 in fascial fibroblasts improved wound healing. AAV8 p120 shRNA administered subcutaneously efficiently in-

hibited fascial fibroblasts from migrating in a group, promoting wound healing, and greatly reducing scarring. The ability of the AAV8 vector to target fascial mobilization and the translational application of this technology in altering the endogenous healing response could be used to restore the function of injured tissue without scarring.

2.4 Migrating adipocytes cannot be converted to myofibroblasts in skin wound healing

Adipocytes are being recognized as key participants in maintaining tissue homeostasis and modulating organ regeneration in various organ systems beyond adipose tissue. They can be found in two types of white adipose tissue in the skin: dermal white adipose tissue (dWAT) and subcutaneous white adipose tissue (sWAT). Recent studies in mice have revealed that dWAT differs from sWAT in terms of its development, morphology, and function. Specifically, dWAT plays a critical role in regulating body temperature, regulating hair growth, and facilitating wound healing [84].

Myofibroblasts, on the other hand, have traditionally been thought of being terminally differentiated cells. A recent study has demonstrated that myofibroblasts, which are considered to be non-adipogenic cells, may be converted to adipocytes during wound healing, demonstrating the trans-differentiating capability of these cells [72]. Fatty acids and hormones that control inflammation and glucose metabolism can be produced by adipocytes [85, 86]. The role of adipocytes has led to the hypothesis that they may play a crucial role in wound healing through growth factor secretion, angiogenesis, and the deposition of type VI collagen.

Shook et al. investigated the impact of dermal adipocytes on in vivo wound healing [87]. Tamoxifen-induced dermal adipocyte ablation was achieved by using *Adipoq^{CreER};iDTR* transgenic mice, although other adipose depots were mostly unaffected. Skin wound healing was significantly delayed as a result of the loss of dermal adipocytes, and the recruitment of inflammatory macrophages was also compromised. Dermal adipocytes completed a dedifferentiation process to express Pdgfrα/Pdgfrβ resembling fibroblasts. *Adipoq^{CreER};R26$^{mT/mG}$* mice lineage-tracing strategies had revealed that adipocytes located near the edge of a wound undergo a series of transformations. These cells broke down their lipid droplets, which led to the downregulation of adipocyte-specific genes such as PPAR-γ and Perilipin-1 (Plin1). As a result, the adipocytes acquired a fibroblast-like appearance before migrating into the wounded tissue.

It is widely believed that communication between fibroblasts and adipocytes involves the exchange of cell markers. For instance, fibroblasts may express the adipocyte marker Plin1, while adipocytes may express the fibroblast marker αSMA. However, these markers alone are insufficient to determine cell identity. In addition to marker expression, cellular plasticity is also dependent on morphological changes. For instance, adipocytes can transition from a rounded shape to a flattened, bipolar shape characteristic of fibroblasts. Nonetheless, relying solely on phenotype can be misleading, as mature adipocytes can lose their lipids under certain metabolic conditions, making them harder to distinguish from fibroblasts. Therefore, a more comprehensive study of the molecular and cytoarchitectural changes involved in the injury response is necessary to fully understand the cellular plasticity between fibroblasts and adipocytes.

In **Publication II**, we investigated the stepwise transcriptional and functional responses of mature adipocytes and fibroblasts following skin injury using a combination of genetic lineage tracking

strategies, single-cell transcriptomes, live cell imaging and computational tracking, transplantation assays, and in vivo injury models. Morphological regulation of wounded adipocytes in injured tissues was observed, but there was no evidence of an adipogenic origin of fibrotic cells during injury, nor was there evidence of transformation or cell fusion from adipocytes to fibroblasts by assessing transcriptomic, behavioral, or functional criteria.

We analyzed the gene expression of GFP$^+$ cells in the skin of $En1^{Cre};R26^{mTmG}$ mice and $Adipoq^{Cre};R26^{mTmG}$ mice by ex vivo culturing at different days with single-cell RNA sequencing, and found no overlap and no confluence in these 12 clusters of single-cell sequencing data, which remained independent. By performing live imaging of the in vitro experiments, we found that from day 0 to day 2 the adipocytes had a gradual reduction in round cells and an increase in the oval, spindle, and elongated morphology. Between day 3 and 5, this phenotype reversed and returned to a strictly round morphology. In vivo wound experiments had shown that adipocyte lineage cells undergo a transition in morphology following injury. By day 7, these cells acquired a fibroblast-like appearance and migrate from their original location in the dWAT to the wound bed. By day 21, elongated adipocyte lineage cells could be observed clustering near the scar area, while nearby adipocytes in non-wounded skin retained their characteristic rounded morphology. These findings suggest that adipocytes are capable of undergoing a transitional change in morphology and actively migrating while maintaining their identity as adipocytes.

$En1^{Cre};R26^{mTmG}$ mice and $Adipoq^{Cre};R26^{mTmG}$ mice were used to perform full-thickness excisional wounds on the dorsal back skin, and on day 10 and 21, FACS was used to sort GFP$^+$ cells (adipocytes from Adipoq-lineage mice, and fibroblasts from En1-lineage mice) for bulk sequencing. Wound fibroblasts were shown to be enriched for matrix deposition and matrix regulation, and cell-matrix adhesion by using gene ontology enrichment analysis. Adipocytes from wounds on day 10 were, in contrast, more highly enriched in sensory perception, immunological regulation, and antimicrobial responses. Migrating adipocyte-lineage cells, which were distinct from fibrogenic cells, lacked the fibroblast markers αSMA and vimentin as well as the proliferation marker Ki67.

In summary, we demonstrate an irreversible cell fate of mature adipocytes during skin injury, despite their significant morphological changes and migratory behavior. Wounded adipocytes remain linearly restricted in their response to tissue injury and do not differentiate and do not fuse with or convert to myofibroblasts. Adipocytes respond to traumatic stimuli with a shift in motility and function and play an active but distinct role in the endogenous tissue healing response.
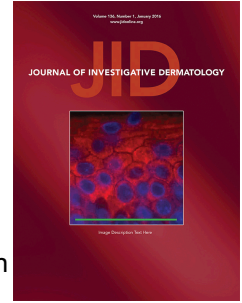
3. Paper I

Therapeutic silencing of p120 in fascia fibroblasts ameliorate tissue repair

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Haifeng Ye, Hans-Günther Machens, Heiko Adler, Dongsheng Jiang, Yuval Rinkevich

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Therapeutic silencing of p120 in fascia fibroblasts ameliorate tissue repair

Vijayanand Rajendran[1], Pushkar Ramesh[1], Ruoxuan Dai[1], Shruthi Kalgudde Gopal[1], Haifeng Ye[1], Hans-Günther Machens[2], Heiko Adler[3], Dongsheng Jiang[1], Yuval Rinkevich[1,*]

[1]Institute of Regenerative Biology and Medicine, Helmholtz Zentrum München, Munich, Germany

[2]Department of Plastic and Hand Surgery, Klinikum rechts der Isar, School of Medicine, Technical University of Munich, Munich, Germany

[3]Institute of Asthma and Allergy Prevention, Helmholtz Zentrum Muenchen, Munich, Germany, and German Center for Lung Research (DZL), Giessen, Germany

*Correspondence:

Dr. Yuval Rinkevich

Address: Max-Lebsche-Platz 31, 81377 Munich, Germany

Telephone: +49 (89) 3187 4685

Fax: +49 (89) 3187 4661

Email: yuval.rinkevich@helmholtz-muenchen.de

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ORCID:

Vijayanand Rajendran	0000-0002-6583-4044
Pushkar Ramesh	0000-0002-6815-4626
Ruoxuan Dai	0000-0003-4669-1022
Shruthi Kalgudde Gopal	0000-0001-7053-919X

Haifeng Ye	0000-0001-6762-7260
Hans-Günther Machens	0000-0002-3852-6433
Heiko Adler	0000-0002-6481-6709
Dongsheng Jiang	0000-0003-0961-2671
Yuval Rinkevich	0000-0003-3658-0257

ABSTRACT

Deep skin wounds rapidly heal by mobilizing extracellular matrix and cells from the fascia, deep beneath the dermal layer of the skin, to form scars. Despite wounds being an extensively studied area and an unmet clinical need, the biochemistry driving this patch-like repair remains obscure. Lacking also are efficacious therapeutic means to modulate scar formation in vivo. Here, we identify a central role for p120 in mediating fascia mobilization and wound repair. Injury triggers p120 expression, largely within engrailed-1 lineage positive fibroblasts (EPFs) of the fascia that exhibit a supra-cellular organization. Using adeno-associated virus (AAV) mediated gene silencing, we show that p120 establishes the supracellular organization of fascia EPFs, without which fascia mobilization is impaired. Gene silencing of p120 in fascia fibroblasts disentangles their supracellular organization, reducing the transfer of fascial cells and extracellular matrix into wounds, and augments wound healing. Our findings place p120 as essential for fascia mobilization, opening a new therapeutic avenue for targeted intervention in the treatment of a variety of skin scar conditions.

INTRODUCTION

Tissue scarring is a pivotal step in mammalian wound repair following injury. Scars replace the healthy reticular connective tissue foundation with irregular dense meshwork of connective tissue matrix that severely restrict the tissue's biomechanical, physiological and functional performance. Scarring is ubiquitous after surgery or acute trauma, and excessive scarring is a crucial stage of many chronic diseases that leads to organ dysfunction, failure, and death (Sen, 2019). Up to 45 percent of all deaths in the world are attributed to the progression of scar disorders (Eming et al., 2014, Gurtner et al., 2008, Marshall et al., 2018). Several key limitations hinder anti-scar therapies from becoming widespread and utilized across diverse clinical indications.

The first limitation arises from our current incomplete knowledge of how scars are formed in animals and the precise pathomechanism underpinning this process for effective, targeted treatments to be developed. Scar formation has traditionally been viewed as a pathology of *de novo* matrix synthesis and deposition by fibroblasts on site (Eming et al., 2014; Gurtner et al., 2008; Sen, 2019). This has provided the foundation for most anti-scar and anti-fibrotic approaches under clinical development, focusing on reducing fibroblast activation and collagen synthesis (such as transforming growth factor-beta [TGF-β] signalling). Success in translating such drug candidates into effective treatments in clinical trials is slow and hampered by the fact that this mechanism is difficult to precisely target, resulting in many adverse effects systemically.

In sharp contrast to the view of scarring as a pathology of excessive and *de novo* matrix deposition, we recently demonstrated that scars in skin originate from pre-existing extracellular matrix (ECM) positioned underneath the dermis. In response to injury, a viscoelastic membranous sheet of loose connective tissue termed superficial fascia, which creates a thin gelatinous and frictionless interface between the skin and the body's interior rigid structures,

is dragged upwards and mobilized into wounds, resulting in scar formation (Correa-Gallegos et al., 2019). These findings highlighted fascia mobilization as an untapped avenue to identify anti-scarring treatment mechanisms independent of *de novo* collagen synthesis (Jiang & Rinkevich, 2021). Our follow-up studies have demonstrated that fascia mobilization during wound repair is driven by a swarming-like collective migration of fascia fibroblasts from the wound periphery towards the wound center (Jiang et al., 2020). This directional collective migration of fascia fibroblasts forms a myofibroblast cell aggregate at sites of wounds that requires elevated intercellular communication and adhesion via N-cadherin-based adherens junctions and Connexin 43-based gap junctions (Jiang et al., 2020; Wan et al., 2021). The upstream signal that instructs upregulation of N-cadherin and Connexin 43 in fascia fibroblasts upon injury has not been identified.

The second limitation is the lack of a delivery vehicle that can effectively target dermal and fascia fibroblasts. The most attractive vectors of choice are Adeno-Associated Viruses (AAVs). These are small (26 nm in diameter) non-enveloped parvoviruses (Wang et al., 2019) that provide persistent high-level transgene expression and minimal post-infection immunogenicity and pathogenicity. AAV vectors have been evaluated in 149 clinical trials worldwide (Kuzmin et al., 2021), providing evidential support for the safety and feasibility of AAV-mediated gene delivery in therapeutic development. In preclinical models, AAV-mediated gene transfer has demonstrated in vivo efficacy with proven tropism to epidermal muscle cells, hepatocytes, pancreatic cells, and heart muscle (Galeano et al., 2003; Jazwa et al., 2010; Keswani et al., 2012). AAV pseudotypes that show efficient transduction to stromal cells in vivo have been not optimal (Kumar et al., 2004, Mcmahon et al., 2006, Stender et al., 2007). Thus, identifying gene transfer applications that can target and transduce dermal and fascia cells at high efficiency in vivo would theoretically enable their widespread applicability in treating wounds across diverse medical conditions.

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Here, we undertake a screen of AAV pseudotypes and determine AAV8 as having the highest transduction efficacy in fascia fibroblasts *in vivo*. Using AAV8 as a gene-expression modification tool, we explore the potential role of p120-catenin (p120) in fascia mobilization, wound healing, and scar formation. p120 catenin has been demonstrated to regulate cell-cell adhesion through its interaction with the cytoplasmic juxta-membrane domain of cadherins (Yanagisawa and Anastasiadis, 2006), and it is essential for intercellular adhesion (Pettitt et al., 2003; Myster et al., 2003). In this study, we demonstrate in animals that AAV8 mediated short hairpin (sh) RNA silencing of p120, predominantly in fascia fibroblasts, reduces ECM mobilization and enables re-emergence of scarless wound repair outcomes. Our findings demonstrate the potential for targeting mechanisms of fascia mobilization with AAV8 vectors and the translational applications of this technology in modulating endogenous repair responses to restore the function of injured tissues in a scarless fashion.

RESULTS

AAV Capsid Screening for efficient transduction of fascial fibroblasts.

Our initial experiments were designed to evaluate the transduction capability of different AAV capsids and to identify the most suitable serotype for transducing fibroblasts in an established murine whole skin-fascia explants (Jiang et al., 2020). Several AAV capsids (AAV2, AAV6, AAV7, AAV8, AAV9 and AAV-DJ) were used for packaging the AAV reporter genome expressing green fluorescent protein (GFP) under the control of the human cytomegalovirus (CMV) promoter. All AAVs were incubated with murine skin-fascia explants and GFP expression in transduced cells was assessed by immunohistochemical staining using an anti-GFP antibody on cryosections of transduced explants with epidermis facing upwards and fascia bellow, three days post-infection (**Figure 1a**). AAV8 displayed a significantly higher percentage of transduced GFP-expressing cells among the serotypes tested (**Figure 1a and b**). Further, to test AAV8's ability to target the fascia fibroblasts in vivo, AAV8-GFP was injected

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subcutaneously into C57BL/6 mice at postnatal day 3 (P3), and GFP expression was assessed three days post-injection. CMV-driven GFP was detected in the fascia layer and within fibrogenic lineage cells that express fibroblast specific protein 1 (Fsp1), demonstrating that subcutaneous delivery routes of AAV8 effectively transduce fascia fibroblasts (**Figure 1c**). Furthermore, we subcutaneously injected AAV8-GFP surrounding the wounds on the back of P3 mice, and examined the cell types that were transduced in wounded region by immunostaining for various cell type specific markers. Within total transduced GFP-positive cells, 4.6% were CK14$^+$ epidermal cells (Supplementary Figure 1a and g), 6.5% were CD31$^+$ endothelial cells (Supplementary Figure 1b and g), 10.1% were CD45$^+$ immune cells (Supplementary Figure 1c and g), and the majority of transduced cells in wounds were PDGFRα^+ fibroblasts (Supplementary Figure 1d and g). Within the fibroblast population, 58.3% and 79.3% of GFP$^+$PDGFRα^+ fibroblasts expressed the fascia fibroblast markers N-cadherin (Supplementary Figure 1e and g) and Sca1 (Supplementary Figure 1f and g), respectively. This data indicates that fascia fibroblasts are the most prominent cell population transduced by AAV8 in wounds.

AAV8 transduced fascia fibroblasts efficiently establish a wound scar

We have previously shown that fascia fibroblasts respond to skin injury by steering the fascia's ECM and cells into open wounds, thereby establishing a provisional wound scar. We reasoned that subcutaneous injection of AAV viral particles into the fascia surroundings the wound could yield an efficiently transduced population of wound myofibroblasts. To evaluate whether AAV8 transduced fascia fibroblasts are steered into wounds in vivo, we injected the AAV8-GFP virus into the fascia surrounding wounds on the dorsal back skin. At 7 days post-wounding (dpw), 71.1% of wound resident fibroblasts were GFP positive, in which majority of them were derived from the transduced fascia (**Figure 2a and b**). The transduced GFP-positive fibroblasts

in wound bed expressed myofibroblast markers alpha Smooth Muscle Actin (αSMA), Dipeptidyl peptidase-4 (also known as Cluster of Differentiation 26; CD26) and ER-TR7 (**Figure 2c and d**). Taken together, our findings indicate that subcutaneous delivery of AAV8 particles represents a valuable method for targeted manipulation of fascia-sourced wound myofibroblasts.

We next examined the efficacy of AAV8 to transfect the fascia in vivo in an independent genetic system. AAV8 capsids were used for packaging a Cre plasmid before subcutaneous injection into the fascia of R26$^{mT/mG}$ reporter mice. These reporter mice harbor a loxP-flanked STOP cassette that prevents transcription of the downstream GFP in the absence of Cre recombinase (**Figure 2e**). Healthy non-injured skin tissue at sites of AAV8 injection were excised at day 7 post-injection, and placed with the fascia-side facing up for imaging. Robust GFP signals at sites of AAV8-Cre injection were observed, indicating achievement of transduction and recombination in fascia cells (**Figure 2f**). Subsequently, AAV8-Cre was injected subcutaneously followed by generating full thickness wounds. Transverse cross-section of wounds at day 7 post-wounding showed accumulation of transduced GFP positive fascia cells in wound bed that expressed the myofibroblast marker αSMA (**Figure 2g**).

p120 is upregulated in fascia EPFs upon injury

Having established a translational system for modulating gene expression of fascia fibroblast in vivo (as our predominant target), we examined the gene modulatory prowess of p120-catenin (p120) to steer wounds into scarless repair. Excisional wounds generated on the dorsal backs of wild-type (WT) mice showed upregulation of p120 in wound fibroblasts in vivo compared to negligible expression levels in unwounded skin (**Figure 3a and b**). p120 protein was specifically upregulated in the pro-fibrotic lineage En1-lineage positive fibroblasts (EPFs) when full-thickness excisional wounds were generated on the back-skin of En1^{Cre};R26^{mTmG}

double transgenic mice. At 5 days post-wounding, p120 was robustly expressed and colocalized with fascia EPFs within the wound bed (**Figure 3c**), and P120 expression returned to lower level at 21 days post-wounding (Supplementary Figure 2a and b). These results collectively demonstrated that p120 is highly expressed in the scar-forming fibroblast lineage throughout the wound repair process. p120 has been reported to bind directly to N-cadherin thereby stabilizing intercellular adherent junctions (Yanagisawa and Anastasiadis, 2006), and our previous study showed a crucial role of N-cadherin in fibroblast collective migration (Jiang et al., 2020). We, therefore, went on to target p120 in fascia fibroblasts in order to investigate whether p120 plays functional role in collective migration, fascia mobilization and scar severity. To effectively knock down the p120 expression in vivo, we generated an AAV8 virus expressing either a p120 shRNA or a non-targeted shRNA (control shRNA) coupled to GFP under the human cytomegalovirus (CMV) promoter. To demonstrate the effectiveness of the p120 shRNA system, primary fascia fibroblasts were transduced with an AAV8 p120 shRNA and AAV8 Control shRNA virus (**Figure 3d**). Western blot results showed that transduction with AAV-P120 ShRNA significantly reduced p120 protein expression (**Figure 3e**). In addition, qRT-PCR of RNA extracts from primary fibroblasts was performed to verify the knockdown specificity. AAV8-p120 shRNA transduction significantly reduced p120 mRNA relative to AAV Control shRNA (**Figure 3f**), validating our AAV8 p120 ShRNA construct system to reduce p120 expression in the fascia effectively.

p120 promotes fascia mobilization into wounds

To directly examine the relevance of injury-induced upregulation of p120 to fascia mobilization, scar formation and wound repair in vivo, we turned to our AAV8 system in order to ablate the expression of p120 in fascia fibroblasts in the context of full thickness wounds on the backs of mice. Quantitative immunofluorescence measurements of wounds revealed a

significant reduction in p120 and αSMA intensity in wounds from mice injected with AAV8 p120 shRNA compared to control shRNA (**Figure 4a-d**). Knocking down p120 significantly reduced N-cadherin expression levels in wounds (Supplementary Figure 2c and d). Whereas immune cell infiltration and cell viability in wounds were not affected by the treatment of AAV8 p120 shRNA, as demonstrated by immunostaining of CD45 (Supplementary Figure 2e and f) and active Caspase 3 (Supplementary Figure 2g and h), respectively. At day 7 post-injection, we observed significantly lower collagen expression within the wound area in our AAV8 p120 shRNA injected group (**Figure 4e and f**). In addition, scar size from AAV8 p120 shRNA transduction experiments was significantly smaller than those from controls (**Figure 4g and h**).

To further study how p120 silencing reduces wound myofibroblasts, we established an *ex vivo* live imaging setup. Following skin injury, WT mice received a subcutaneous injection of AAV8 p120 shRNA or AAV8 control shRNA. Wounds were excised on day 5 post-injection, and kept in culture media in a 37°C chamber with 5% CO_2. Live imaging was then performed with fascia-side face-up for 24 h in order to directly visualize the transduced GFP$^+$ cells inside the wound bed. Transduced fascia cells in AAV8 control shRNA treated wounds exhibited polarized morphologies with extensive cellular protrusions and a collective migration phenotype reminiscent of wounds in untransfected mice. Conversely, p120-silenced fascia cells were poorly polarized with a significant reduction in fibroblast protrusion length compared to control fibroblasts (**Figure 5a and b**). Moreover, transduced fascia cells in control wounds clustered together and formed multicellular clusters. By contrast, in AAV8 p120 shRNA treated wounds, intercellular junctions were completely lost (**Figure 5c**), and fibroblasts showed significant reduction of cell membrane branches (**Figure 5d**). To directly analyze changes in fibroblast dynamics in response to p120 silencing, we performed particle image velocimetry (PIV) analysis on live image videos tracking single fascia fibroblasts. Transduced

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fascia cells in control wounds exhibit directed (peripheral-to-central) movement with collective migration towards the wound region, whereas p120 silenced fascia cells had substantially reduced migration distances and lacked collective migration behaviors (**Figure 5e and f**). These results indicate a crucial role for p120 in stabilizing the supracellular organization of fascia fibroblasts needed for effective mobilization of the fascia tissue into wounds.

Recently we showed that fascia resident EPF fibroblasts physically mobilize the fascia ECM into the wound (Correa-Gallegos et al., 2019; Jiang et al., 2020; Wan et al., 2021). To further investigate whether p120 depletion in fibroblasts blocks mobilization of fascial ECM in vivo, we fluorescently 'tagged' the fascia ECM with NHS ester dye by directly injecting NHS-fluorescein into the subcutaneous space of the back skin prior to creating full-thickness excisional wounds (**Figure 5g**). After the injury, AAV8 control shRNA or AAV8 p120 shRNA was subcutaneously injected into the fascia surrounding the wound edge. Wounds receiving AAV8 control shRNA showed accumulation of ECM within wounds with 64.8% of the wound bed matrix labelled with NHS ester and therefore mobilized from fascia adjacent to the wound. In contrast, wounds receiving AAV8 p120 shRNA had drastically reduced ECM, with only 28.9% of wound bed matrix originating from the fascia (**Figure 5h and i**).

Collectively, our experiments demonstrate enhanced wound repair by therapeutic modulation of p120 in fascia fibroblasts. Functionally, we show that subcutaneous delivery of AAV8 p120 shRNA effectively blocks fascia fibroblast collective migration needed for fascia mobilization into wounds, thereby producing wound healing with significantly reduced scars.

DISCUSSION

Clinical practice of dermal scars relies primarily on scar tissue management rather than its therapeutic amelioration. For example, topical management options normally include invasive procedures such as surgical scar corrections, intralesional application of corticosteroids,

physical laser- or radiotherapy, and radiotherapy directly after surgical scar correction, either separately or in combination. Additional non-invasive treatment options exist, including the physical application of pressure forces, often in combination with applied silicone sheets and gels, static/dynamic splints, massage therapy or the use of dermatologic ointments, oils, lotions, creams (Monstrey et al., 2014), all of which show limited clinical efficacy in reducing scars during wound management.

To date, there has been no efficient demonstration of therapeutic improvement of wound repair by targeted delivery methods into the fascia. This is primarily due to a lack of mechanistic understanding of the central role of the fascia in wound repair but also due to a lack of efficacious approaches for targeted modulation of fascia tissue in vivo. Here, we overcome these milestone gaps by (a) identifying p120 as a targetable molecule for scar prevention that is highly expressed in fascia EPFs in response to injury and (b) discovering the fundamental role of p120 in steering fascia cells and ECM into wounds, and (c) demonstrating translational efficacy of AAV8 p120 shRNA in improving wound repair outcomes in animals.

p120 is an adhesion junction protein known to function as a link between intercellular adhesion and signal transduction in various cells. p120 in glioma cells has been shown to play a critical role in cancer cell invasion, migration, and axonal growth (Gritsenko et al., 2020) as well as in motility of cultured stromal cells (Grosheva et al., 2001). Our previous studies identified N-cadherin as an essential intercellular adhesion molecule needed for fascia fibroblast collective cell migration into wounds (Jiang et al., 2020). p120 is essential for the formation of N-cadherin-based junctional complexes and cell polarity (Ozaki et al., 2010, Cell Struct Funct), and both N-cadherin and p120 are essential for directional collective migration of fibroblasts. N-cadherin has been shown to promote tumor cell invasion (Thoreson et al., 2000), and we speculate that p120 is involved in cellular invasion by either 1) upregulating and stabilizing N-cadherin through direct binding, or 2) directly remodeling the cytoskeleton via modulating

Rho-GTPase activity (Yanagisawa and Anastasiadis, 2006; Anastasiadis et al., 2000). Under the conceptual frame that tumors are wounds that do not heal (Dvorak, 1986; Dvora, 2015), we speculate that p120 may have similar physiological functions during wound repair.

Our study confirms that p120 is an essential regulator of the supracellular organization needed to mobilize fascia cells and matrix into wounds. Silencing of p120 modulates the extent of connective tissue matrix and cells being steered into wounds, thereby reducing wound scar severity in animals. Multiple doses of AAV8-mediated p120 knockdown are required for a longer period of p120 silencing. The development of p120 genetically modified animal models or p120-specific chemical inhibitors may also enable therapeutic modulation of wound repair. Our findings open a wide range of possibilities for exploring the therapeutic efficacy of gene delivery into the dermis and fascia with broad clinical implications and pharmaceutical value.

MATERIALS AND METHODS

Detailed materials and methods are described in Supplementary Materials and Methods.

Animals

For in vivo studies, C57BL/6J, and En1^{Cre} mice were purchased from Jackson Laboratories and ROSA26^{mTmG} (R26^{mTmG}) reporter mice were obtained from Stanford University. The mouse lines were kept at the Helmholtz animal facility in compliance with the German Law for Welfare of Laboratory Animals. All experiments were approved by the Government of Upper Bavaria.

Antibodies

The following antibodies were used: anti-goat FSP1 (1:200, ab58597), anti-mousep120 catenin (1:250, BD610133), anti-rabbit αSMA (1:250, ab5694), anti-rabbit Collagen type I (1:250, Rockland 600-401-103-0.1), anti-rabbit Collagen type III (1:200,ab7778), anti-goat CD26 (1:200, SAB2500328), anti-rabbit collagen type VI (1:200,ab6588,1:150), anti-rat ERTR7 (1:500,ab51824), anti-rabbit GFP (1:250,ab290).Tissue sections were over layed with relevant secondary antibodies (1:500) for 1 hour at Room Temperature (RT).

Excisional wound model and AAV injection

Two-week wildtype mice were anaesthetized with MMF (Medetomidine at 0.5 mg.kg^{-1}, midazolam at 5 mg.kg^{-1}, and fentanyl at 0.05 mg.kg^{-1}). Under analgesia, two 2mm full-thickness deep wounds (i.e., epidermis, dermis, and fascia) were generated on the back skin. The mouse received an injection of 10 μl of AAV8-GFP, AAV8 p120 ShRNA, and AAV8 Control ShRNA virus at a titre of 2x10^{12} GC /ml in the area between the two wounds. After 7 days post-injury, the scar tissue was cryo-sectioned for histological and immunofluorescent analysis. Similarly, two 2-mm full-thickness excisional wounds were created on the back of

R26mTmG mice, and 20 µl of AAV8-Cre virus at a viral titer of 1×10^{12} GC/ml was injected at the area between the wounds. The control mice received the injection of PBS. Scar tissues were harvested on day 7 post-wounding. Subsequently, the scar tissue was processed for cryo-sections for histology and immunofluorescence.

Ex vivo **live imaging**

Live imaging of transduced wound tissues was performed as described previously (Ramesh et.al, 2022). AAV8 p120 ShRNA or AAV8 Control ShRNA virus injected wound tissue were embedded in 4% agarose in a 35 mm-dish (Corning) and submerged in imaging medium phenol-red free DMEM/F-12 (Thermo Fisher Scientific) containing 10% KnockOut Serum Replacement, 1x GlutaMAX, 1x Penicillin/streptomycin, and 1x MEM non-essential amino acids. Time-lapse imaging was performed under a Leica SP8 MP (Leica, Germany), equipped with a modified incubation system with heating and gas control (ibidi) was used during imaging (37 °C, 5% CO_2). 250 z-stacks and 65 time-frames were recorded for each imaged sample in approximately 24 h, which gives z-plane resolution of 2.4 – 2.6 µm, and time interval of 22 – 29 min for each frame. Images were visualized and analyzed with the Imaris software package (v9.3.0, Bitplane, UK).

Particle Image Velocimetry (PIV) analysis of images

Particle image velocimetry (PIV) has been used to describe collective cell migration. It compares the localization of signals in 2 images from 12-hour videos and computes the pixel flow trajectories between them. PIV analysis was performed using Fiji Plugin Iterative PIV from the 12 hrs live imaging video stacks of AAV8 p120 ShRNA, AAV8 Control ShRNA virus injected wound tissue. Stable pictures from the timelapse devoid of tissue drifts from early and final time points were converted into a 2-picture stack to derive these trajectories. Images were converted to an 8-bit stack, and the "iterative PIV" function was applied. Default values with

"Vector spacing" of 128, 64 and 32 pixels sub windows were applied. Calculated magnitude vectors were then used to measure direction and velocity.

Cell tracking and displacement analysis

Drift correction was performed on live imaging dataset using ImageJ "Correct 3D drift" in reference to the non-cellular tissue autofluorescence. The Initial and final time point z stacks of drift corrected dataset were extracted and converted to 2D stacks using maximum intensity projection. The resulting x, y, t stacks were converted to binary images and further processed using the analysis pipeline of ImageJ plugin "Trackmate" (Jiang et al., 2018). Cell tracks and (x, y) spot coordinates were exported as .xml to excel and spot displacement values were computed using formula $d = \sqrt{((x2 - x1)2 + (y2 - y1)2)}$.

Matrix labelling and AAV injection into the wound

2-week-old C57BL6/J mice were injected with 5µl of NHS-fluorescein dye at 10mg/ml in PBS with 0.1 M sodium bicarbonate pH 9 (46,409, Life technologies) one day before wounding. 2 mm full-thickness excisional wounds were created in the labeled area and 10µl of AAV8 p120 ShRNA or AAV8 Control ShRNA virus at a $2x10^{12}$ GC/ml titre range was injected around the wounds. Wounds were harvested on day 7 post-wounding and fixed in 2% PFA at 4 °C overnight. After washing with PBS, tissue was embedded in OCT and cryo-sectioned.

Quantification and statistical analysis

All statistical analyses were performed with GraphPad Prism 9.2 software. Data are presented as the means ± standard error of the mean (SEM) or mean ± standard deviation (SD). A two-tailed t-test (Student´s t-test) was carried out for the statistical analysis to compare the two groups. $P < 0.05$ was considered statistically significant. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; and $****P < 0.0001$.

DATA AVAILABILITY STATEMENT

All the data are available with the corresponding authors upon reasonable request. No large datasets were generated in the current study.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists

ACKNOWLEDGMENTS

AUTHOR CONTRIBUTIONS

Conceptualization: YR, DJ; Data curation: VR, PR, RD, SKG, HY, HA, H-GM, DJ; Writing: VR, DJ, YR.

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FIGURE LEGENDS

Figure 1. AAV8 transduces fascia fibroblasts. (a) Representative confocal images show the comparison of several AAV serotypes with GFP transgene expression in skin fascia explants of C57BL/6 mice. Green: GFP Blue: DAPI. Scale bar, 100 µm. (b) Quantification of the percentage of GFP$^+$ cells as a fraction of total cells in each group corresponding to a. Data is shown as mean ± SD. The unpaired two-tailed t-tests were performed between AAV8 and other AAV serotypes, p-value **p < 0.01, ***p < 0.001, n = 3. (c) C57BL/6J mice were transduced with the AAV8-GFP virus into the fascia. Mice were sacrificed at 3 days post-viral injection. Immunofluorescence images of GFP-positive cells expressing mesenchymal/fibroblast marker (Fsp1). pc, panniculus carnosus. The lower panel shows the high magnification images of the white boxes in the upper panel. Scale bars, 100 µm.
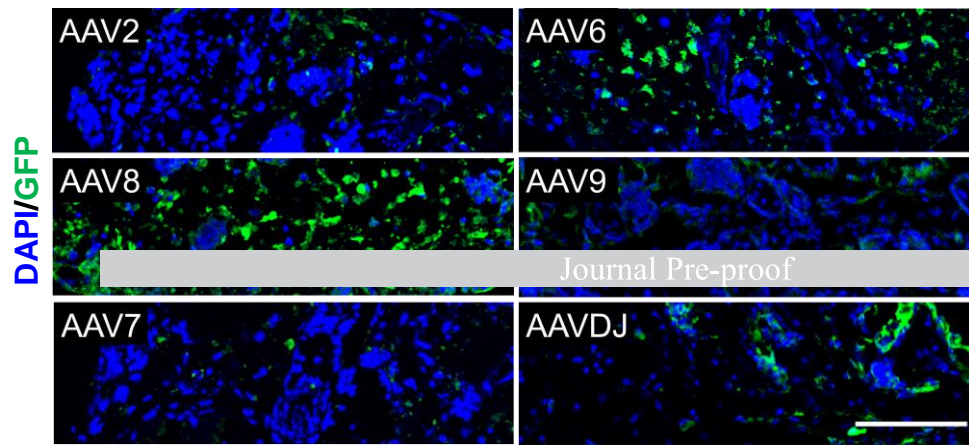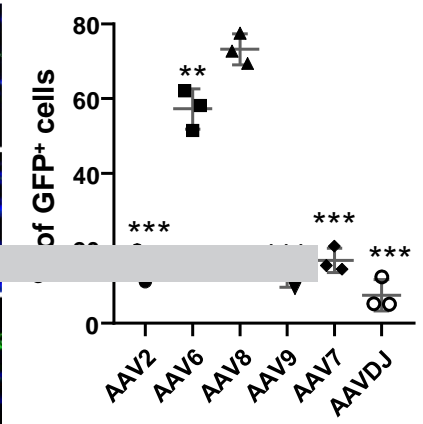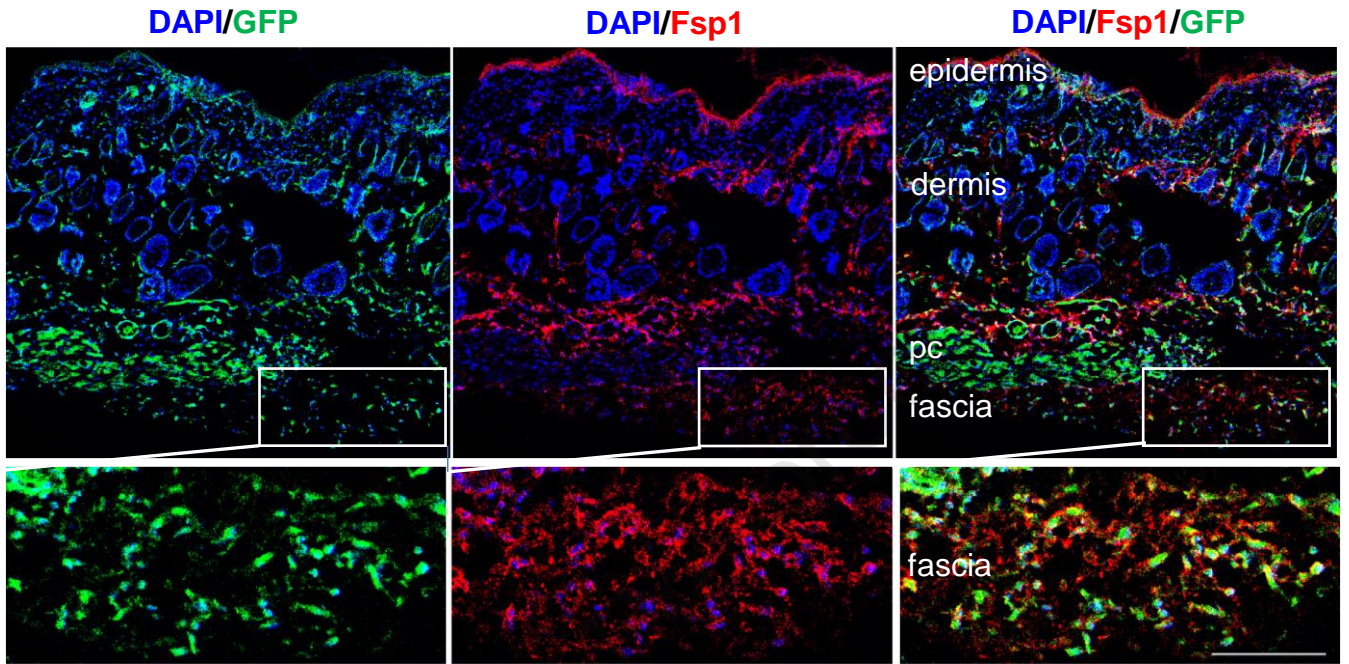
Figure 2. AAV8 gene delivery into the fascia effectively targets the wound myofibroblasts. (a) Histology shows GFP-expressing cells in uninjured skin (left) and 7dpw (right) and (b) percentage of GFP-expressing cells per high magnification filed. Data are mean ± SD, n = 6 images analysed from 3 biological replicates. Arrow heads indicate the border of wounds. wb, wound bed. (c) Immunolabelling of GFP-positive wound resident fibroblasts from 7dpw expressing myofibroblast marker(αSMA) and fibroblast markers (CD26; ERTR7). (d) Percentage of GFP cells corresponding to c. Data are mean ± SD, n = 5. (e) Experimental scheme and schematic depicting the AAV8 vector mediated Cre/Loxp recombination in the R26$^{mT/mG}$ mice allele, and the ex vivo imaging setup showing the imaging plane of wound bed and fascia. (f) Representative images of skin tissue from mT/mG mice treated with saline and AAV8-Cre virus. (g) Representative images of cross-section of wounds at 7dpw showing AAV8-Cre transduced cells in wound bed express myofibroblast marker αSMA. wb, wound bed; pc, panniculus carnosus. Scale bars, 100 µm.

Figure 3. p120 expression is elevated in physiological wounds. (a) Representative confocal images of p120 expression in the uninjured fascia, 3dpw and 7dpw of C57BL/6J mice. Arrow heads indicate the border of wounds. The lower panel shows the high magnification images of the white boxes in the upper panel. (b) Quantification of p120 fluorescence intensity corresponding to a. Data is mean $\pm$ SEM. The student's t-test calculates the p-Value. ****$p < 0.0001$. n=5. (c) Immunolabelling of p120 expression at 7dpw from En1Cre;R26mTmG mice. pc, panniculus carnosus; wb, wound bed. Arrow heads indicate the border of wounds. The right panel shows the high magnification of the white box. (d) Experimental scheme of in vitro transduction and schematic representation of AAV Control ShRNA and AAV p120 ShRNA constructs. (e) Western blot image for validation of p120 knockdown after AAV8 mediated p120 ShRNA transduction in primary fibroblasts of fascia. (f) qRT-PCR analysis of p120 expression in primary fibroblasts with AAV8 expressing control ShRNA or p120 ShRNA. Data is mean $\pm$ SD; n = 3 independent experiments. The student's t-test calculates the p-Value. **$p < 0.01$.
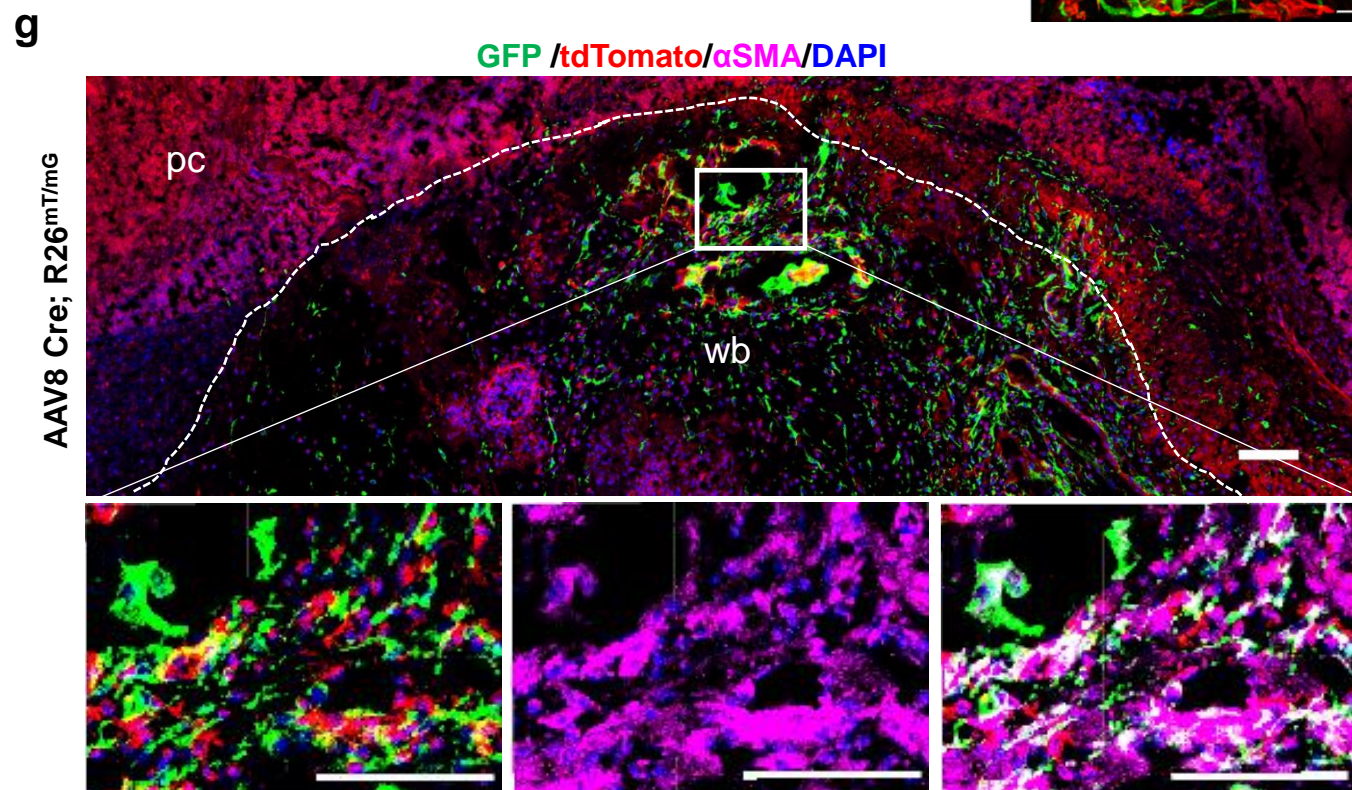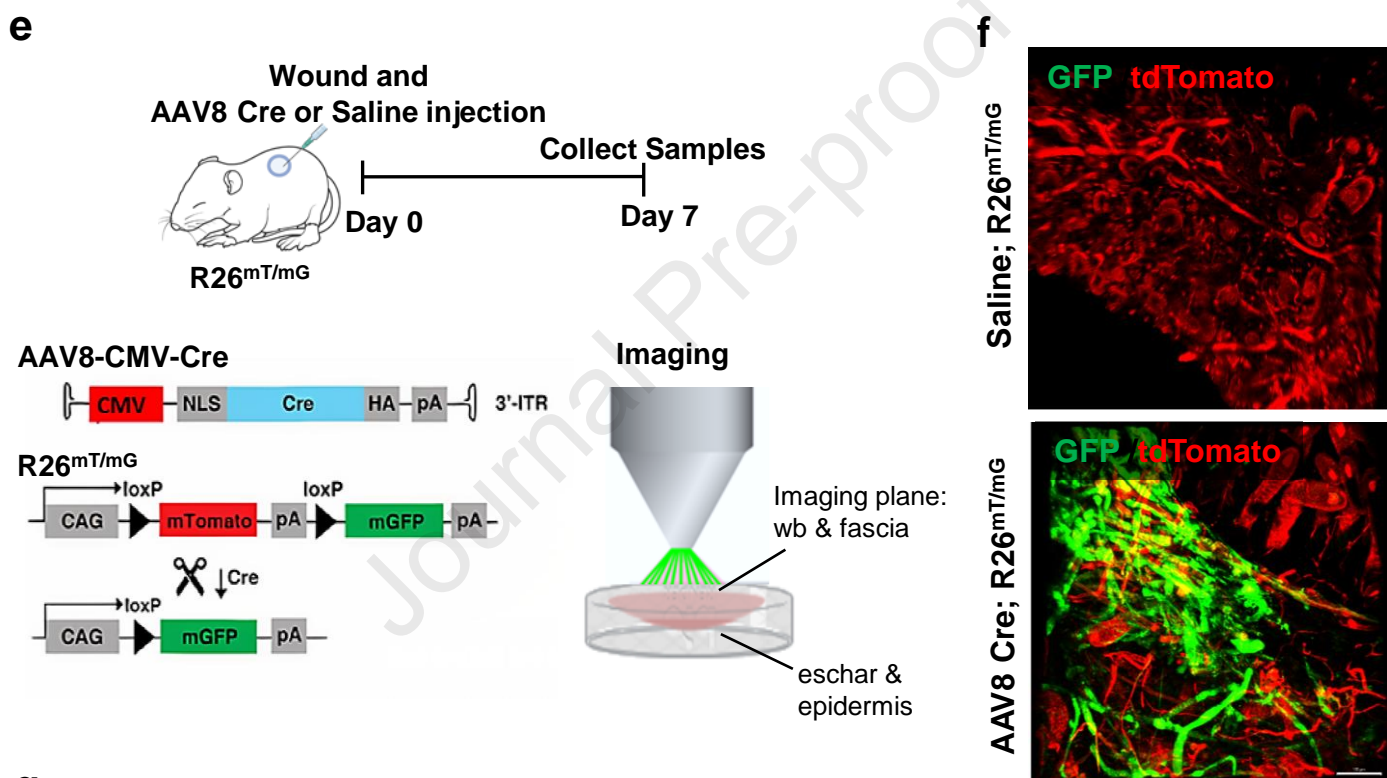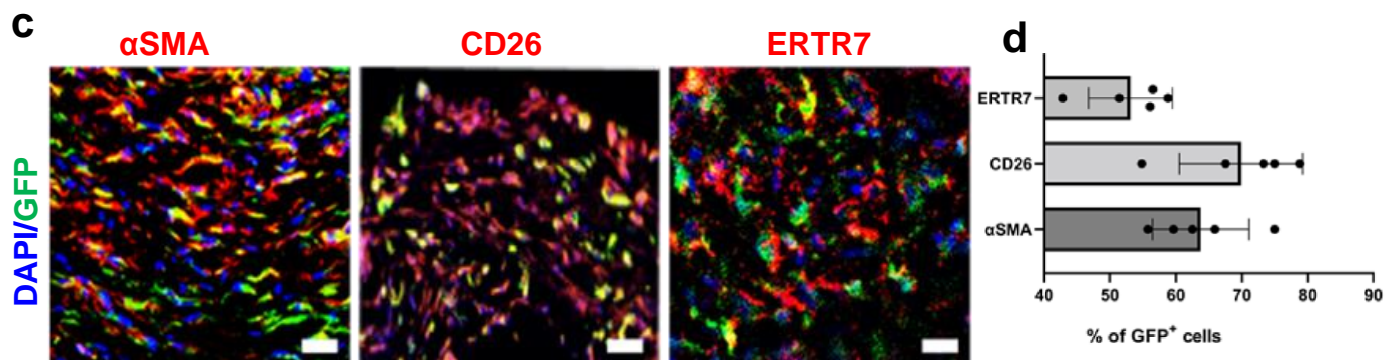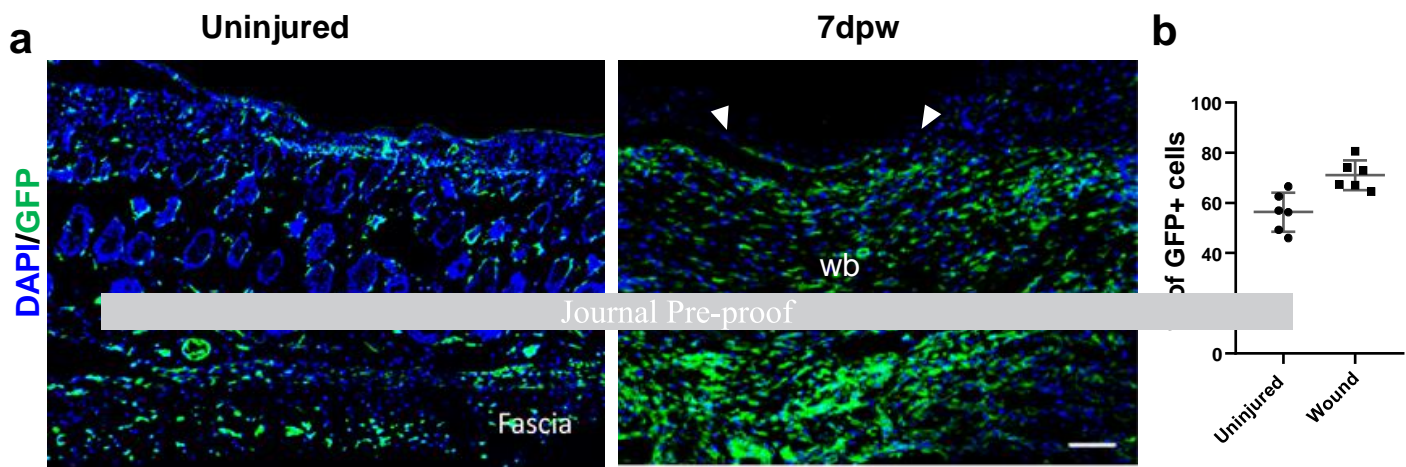
Figure 4. AAV8-mediated shRNA silencing of p120 reduces the scarring. (a) Representative confocal images of p120 expression in 7dpw from AAV8 Control ShRNA or AAV8 p120 ShRNA injected C57BL/6 mice. Arrow heads indicate the border of wounds. The right panel shows the high magnification of the white boxes. (b) Quantification of p120 intensity corresponding to a. Data is mean $\pm$ SEM. The student's t-test calculates the p-Value. ****$p < 0.0001$. n = 5. (c) Representative images of myofibroblast marker αSMA staining in the 7dpw of AAV8 Control ShRNA or AAV8 p120 shRNA injected C57BL/6J mice. The right panel shows the high magnification of the white boxes. (d) Quantification of αSMA intensity corresponding to c. Data is mean $\pm$ SEM. The student's t-test calculates the p-Value. ***$p < 0.001$. n = 5. (e) Representative images of collagen I+III+IV staining in the 7dpw of
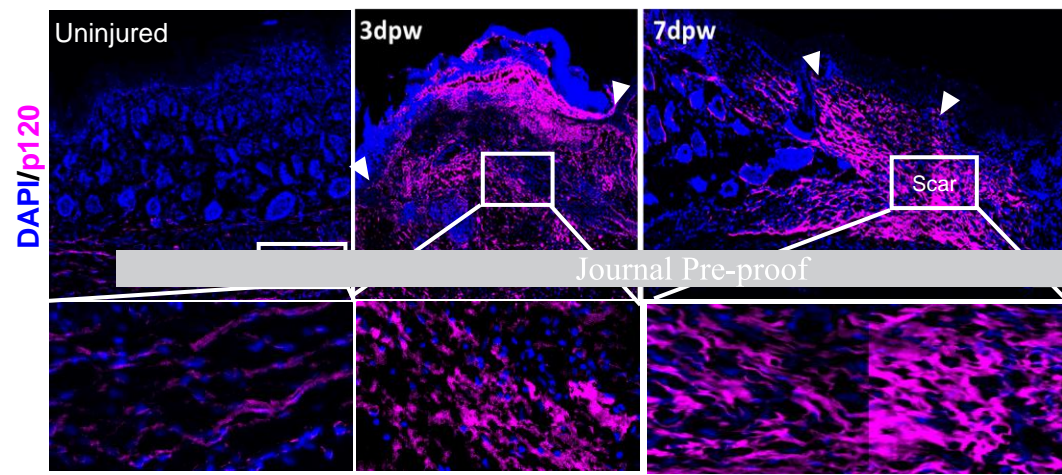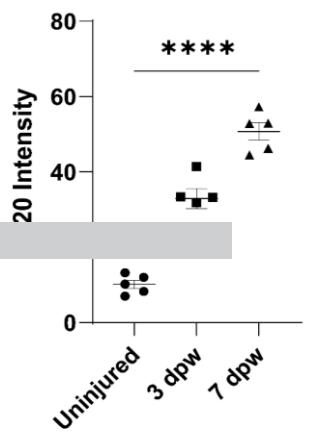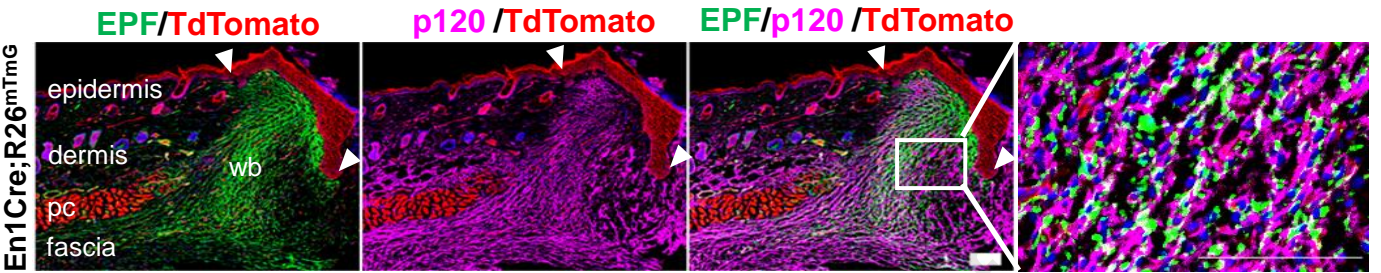
AAV8 Control ShRNA or AAV8 P120 ShRNA injected C57BL/6J mice. The right panel shows the high magnification of the white boxes. (f) Quantification of collagen I +III+VI intensity corresponding to e. Data is mean ± SEM. The student's t-test calculates the p-Value. ****$p < 0.0001$. n = 5. (g) Masson's trichrome stained 7dpw of AAV8 Control ShRNA or AAV8 p120 ShRNA injected C57BL/6J mice. dotted lines indicate the scar area. (h) Quantification analysis of scar area in g. Data is mean ± SEM. The student's t-test calculates the p-Value. ***$p < 0.001$. n = 5.

Figure 5. AAV mediated silencing of p120 prevents ECM mobilization. (a) live imaging representing the morphology of wound resident fibroblasts in response to AAV8 control shRNA, and AAV8 p120 shRNA transduction are visualized with GFP. (b) Quantification of protrusion length of the transduced cells. Error bars show the SEM. (c) Schematic representation of transduced cell clusters. (d) Quantification of the number of the secondary branches of intercellular junctions. (e) Particle image velocimetry (PIV) analysis of the movement pattern of transduced cells of the last imaging frame. The colored scale bar indicates migratory speeds from slow (blue) to fast (red). Scale bar unit: pixel. (f) Quantification of the displacement of all transduced cells in the last frame compared to the first frame of the live imaging videos. (g) Experimental scheme of matrix labelling with AAV Control ShRNA or AAV p120 ShRNA injection after injury in C57BL/6 mice. (h) Images show NHS ester labelled matrix in 7dpw. Labeled fascia matrix in green and autofluorescence in red. The lower panel shows the high magnification images of the white boxes in the upper panel. (i) Percentage of labelled fascia matrix coverage in g. Data is Mean ± SEM, n = 6 sections from 3 independent experiments. unpaired two-tail Student's t-test, ****$p<0.001$.

a

AAV2 | AAV6
AAV8 | AAV9

Journal Pre-proof

AAV7 | AAVDJ

b

DAPI/GFP

of GFP+ cells

80

40

0

**

***

***

***

AAV2 AAV6 AAV8 AAV9 AAV7 AAVDJ

c

DAPI/GFP | DAPI/Fsp1 | DAPI/Fsp1/GFP

epidermis

dermis

pc

fascia

fascia

Journal Pre-proof

a Uninjured — 7dpw

DAPI/GFP

wb

~~Journal Pre-proof~~

Fascia

b of GFP+ cells

Uninjured Wound

c αSMA CD26 ERTR7

DAPI/GFP

d

ERTR7

CD26

αSMA

% of GFP+ cells

e Wound and
AAV8 Cre or Saline injection

Collect Samples

Day 0 Day 7

R26^mT/mG

AAV8-CMV-Cre

CMV — NLS — Cre — HA — pA 3'-ITR

R26^mT/mG

CAG ▶ loxP — mTomato — pA ▶ loxP — mGFP — pA

↓ Cre

CAG ▶ loxP — mGFP — pA

Imaging

Imaging plane: wb & fascia

eschar & epidermis

f GFP tdTomato

Saline; R26^mT/mG

GFP tdTomato

AAV8 Cre; R26^mT/mG

g GFP /tdTomato/αSMA/DAPI

AAV8 Cre; R26^mT/mG

pc

wb

a

Uninjured | 3dpw | 7dpw

DAPI/p120

Scar

Journal Pre-proof

b

p120 Intensity

****

Uninjured   3 dpw   7 dpw

c

En1Cre;R26^mTmG

EPF/TdTomato   p120 /TdTomato   EPF/p120 /TdTomato

epidermis
dermis
pc
fascia

wb

d

Primary fascia fibroblasts isolation

AAV8 p120 shRNA
or AAV8 Control
shRNA transduction

5´ITR — U6 — ShRNA — CMV — EGFP — 3´ITR

Control shRNA
Or p120 shRNA

e

AAV8 Control shRNA   AAV8 p120 shRNA

p120

β-Actin

f

p120 relative expression

**

AAV8 Control ShRNA   AAV8 p120 ShRNA

a DAPI/p120/GFP

AAV8 Control shRNA

AAV8 p120 shRNA

Journal Pre-proof

b

p120 Intensity

AAV8 Control ShRNA   AAV8 P120 ShRNA

****

c DAPI/αSMA

AAV8 Control shRNA

AAV8 p120 shRNA

d

αSMA Intensity

***

AAV8 Control ShRNA   AAV8 p120 ShRNA

e DAPI/Collagen I+III+IV

AAV8 Control ShRNA

AAV8 p120 ShRNA

f

Collagen I+III+VI Intensity

****

AAV8 Control ShRNA   AAV8 p120 ShRNA

g

AAV8 Control shRNA

AAV8 p120 shRNA

h

Scar area (mm^2)

***

AAV8 Control ShRNA   AAV8 p120 ShRNA

a AAV8 Control shRNA AAV8 p120 shRNA

GFP GFP

Journal Pre-proof

b

Protrusion length (µm)

***

AAV8 ctrl shRNA AAV8 p120 shRNA

c

d

Secondary branches

***

AAV8 ctrl shRNA AAV8 p120 shRNA

e AAV8 Control shRNA AAV8 p120 shRNA

88
71
53
35
18
0

f

Displacement (µM)

***

AAV8 ctrl shRNA AAV8 p120 shRNA

g Label with NHS-ester

Injury and
AAV8 Control shRNA or
AAV8 p120 shRNA injection

Collect Samples

Day -1 Day 0 Day 7

h AAV8 Control shRNA AAV8 p120 shRNA

NHS-matrix Autofluorescence

i

% Fascia matrix

****

AAV8 Control ShRNA AAV8 p120 ShRNA

SUPPLEMENTARY

Supplementary Figure Legends

Supplementary Figure 1. Cell type of AAV8 transduced cells. Cryo-sections from AAV8-GFP transduced wounds 7 days post-wounding were immunostained with epidermal marker CK14 (a), endothelial marker CD31 (b), immune cell marker CD45 (c), pan fibroblast cell marker PDGFRα (d), and fascia fibroblast marker N-cadherin (e) and Sca1 (f), respectively. In the left panel, the dotted lines indicate the border of wounds, and white boxes indicate the regions showed in higher magnification in the right panel. Scale bars: left panel = 200 µm; right panel = 50 µm. (g) Quantification the percentage of each cell type that was transduced by AAV8-GFP in total transduced cells in wound bed and wound margin.

Supplementary Figure 2. Expression of p120 and effects of p120 knockdown in wounds. (a) immunofluorescence staining of p120 in wounds of 7 dpw and 21 dpw from En1^{Cre};R26^{mTmG} mice. (b) quantification of p120 expression in 7 dpw and 21 dpw as fluorescence intensity unit. (c) immunofluorescence staining of N-cadherin in AAV8 control shRNA and AAV8 p120 shRNA injected wounds. (d) quantification of N-cadherin expression as fluorescence intensity unit. (e) immunofluorescence staining of CD45 in AAV8 control shRNA and AAV8 p120 shRNA injected wounds. (f) quantification of percentage of wound infiltrating CD45$^+$ cells in total cells. (g) immunofluorescence staining of active Caspase 3 in AAV8 control shRNA and AAV8 p120 shRNA injected wounds. (f) quantification of percentage of Caspase 3$^+$ cells in total cells in wounds. Arrow heads indicate the border of wounds. dpw, days post-wounding; FIU, fluorescence intensity unit. Data are mean ± SD. *** p<0.001, with unpaired two-tailed t-test. Scale bar = 200 µm.

Supplementary Materials and Methods

Tissue fixation and cryosections

The skin and wound tissue was fixed overnight with 2% paraformaldehyde (PFA) at 4 °C. Following the removal of PFA, samples were rinsed in PBS three times for a total of 5 minutes each, and immersed in 30% sucrose overnight. The fixed wound tissue was cut into two halves in the middle, and embedded in Tissue-Tek® O.C.T medium for snap frozen on dry ice. The cryosections at the thickness of 10 µm were generated from the middle of the wounds. Each 10 µm cut was followed 30 µm trim. Five continuous sections were collected, and the average was calculated.

Immunofluorescence staining

Cryosections were fixed with ice-cold acetone for 5 minutes and gently washed the slides 3X in PBS. Overlay the tissue section with a blocking solution. Incubate the slides in a humidified chamber at RT for 1 hr. After that, slices were overlayed with a primary antibody with recommended dilution in blocking solution overnight at 4°C. The next day, gently wash the slides 3X in PBS (5 min/wash). Overlay the tissue sections with secondary antibody diluted in blocking solution. Incubate the slides in a humidified chamber for 1 hr. at room temperature. Gently wash the slides 3X in PBS (5 min/wash) and stain with DAPI solution (1:5000 dilution in PBS) for 5 min. Gently blot the slide with absorbent paper to remove excess liquid. FluoromountG™ Mounting Medium (Invitrogen) was used for Mounting the slides. The mounted slides were imaged using a confocal laser scanning microscope (Zeiss LSM710).

Masson's trichrome staining

Fix the slides by immersion in precooled cold acetone (-20°C) for 5 min. Incubate the tissue slides with Bouin's solution (Sigma-Aldrich HT10132) at RT overnight. After washing the slides for 2 minutes with cold tap water, they are stained for 10 minutes with iron Hematoxylin solution (Sigma-Aldrich HT1079). Discard the Hematoxylin solution. Wash the slides with

running tap water for 10 minutes. Following that, rinse the slides with distilled water for 2 minutes. Incubate the Slides for 5 minutes with Biebrich Scarlet-Acid solution. Wash the slides with 3X distilled water (1min/wash). Incubate the slides with Phosphotungstic /Phosphomolybdic Acid Solution for 10 minutes in the glass chamber and directly incubate the slides with Aniline Blue solution for 10 minutes. Wash the slides with 3X distilled water for 1 minute each. Further, incubate the slides with Glacial acetic acid (1%) for 2 minutes and wash the slides with 2X distilled water for 1 minute each. Post staining, the tissue slides were dehydrated with 70% ethanol for 3 minutes - 90% ethanol for 3 minutes and 100% ethanol for 3 minutes. Finally, keep the slides in Xylol for 5 minutes. After dehydration, the slides were mounted with 2-3 drops of ROTI®Mount media. Images were acquired using the Zeiss Axio imager with 20X magnification. The stained structure in the slides represents the following, Nuclei- Black, Cytoplasm-Red and Collagen in Blue.

ShRNA design and plasmid construction

AAV U6-p120-ShRNA and AAV U6-Control ShRNA plasmids were constructed and purchased from Vector Biolabs (Malvern, PA). To knock down the p120 expression, a distinct ShRNA sequence targeting mouse (5'CGAGGCTATGAACTCTTATTTCTCGAGAAAT AAGAGTTCATAGCCTCG3') and a scrambled ShRNA sequence (5'CCTAAGGTTAAGTC GCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG3') was designed using Vector Biolabs (Malvern, PA). U6 promoter was used to drive p120 ShRNA or scrambled ShRNA expression followed by green fluorescent protein (GFP) separately driven by a CMV promoter.

Adeno-associated virus (AAV) production

Replication-incompetent AAV was packaged via triple transfections of the AAV transgene plasmid (AAV p120 ShRNA-GFP, AAV Control ShRNA-GFP, AAV Cre, AAV-GFP), pRC8

encoding Rep and cap proteins and helper plasmids into AAVpro 293 T cell line (Takara Bio, cat. no. 632273). The cells should reach the confluence of 75%-80% on the day of transfection. Prepare a 1:1:1 molar ratio of triple plasmids (pRC6, helper plasmids and AAV transgene plasmids). The total DNA is calculated based on 0.5µg of DNA/cm^2 of cell culture surface area. The volume of Polyethyleneimine (PEI) was used based on 4:1 ratio of PEI (ug): total DNA (ug). Four-day post-transfection, AAV viruses were extracted according to the AAVpro® Purification Kit (Takara. Cat. #6232) procedures. AAVpro® Titration Kit was utilized to measure the genomic titer of AAV stock.

Ex vivo skin tissue culture

Back-skin was collected from newborn (postnatal day 0-1) C57BL/6J and washed twice with cold DMEM/F-12 (Thermo Fisher Scientific 11320074) medium to remove contaminating blood, and then washed once with Hank's Balanced Salt Solution (HBSS, Thermo Fisher Scientific 14175095). After careful removal of ventral non-skin tissue with a surgical scalpel, round skin pieces were cut out with a disposable Ø 2 mm biopsy punch (Stiefel 270130) down to below the panniculus carnosus muscle and cultured in 200 µl of DMEM/F-12 medium containing 10% FBS, 1x GlutaMAX (Thermo 338 Fisher Scientific 35050038), 1x Penicillin/streptomycin (Thermo Fisher Scientific 339 15140122), and 1x MEM non-essential amino acids (Thermo Fisher Scientific 11140035) in 96-well plates, in a humidified 37°C, 5% CO2 incubator. (Note: the 2 mm skin pieces were cultured submerged in medium with fascia side face up). After 2 hrs of culture, the skin explants were exposed to 5×10^{10} GC AAV for 24 h, and a fresh medium was supplied every other day. The skin tissues were harvested after 72 hrs. The tissue samples were fixed in 2% PFA overnight at 4°C. After washing in PBS, the tissues were embedded and frozen in an optimal cutting temperature compound, and 6 µm cryo-sections were prepared with a cryostat.

In vitro transduction assay for p120 knockdown

Primary fibroblasts from fascia were obtained from neonatal (P0-P4) back skin tissue. Fascia layers were minced with scalpels in a 10cm dish. The tissue fragments were transferred into a centrifuge tube containing collagenase (1000U/mL) final concentration. Incubate the tube at 37°C for 30 minutes and swirl the tubes every 5 minutes centrifuge at 200 x g for 10 min. Remove the supernatant and resuspend the pellet with 2 ml of HBSS medium. Centrifuge the cell suspension again at $200 \times g$ for 5 minutes and 0.5ml of 0.05 % trypsin, swirl the tube vigorously and place the tube for 30 min at 37°C. Then centrifuge the cell suspension at $200 \times g$ for 5 minutes and discard the resuspend. Resuspend the cell pellet with 2 ml of fibroblast culture medium. The cell suspension was filtered with a 70µm cell strainer. Transfer the cells to a 10 cm cell culture dish and 10 ml of culture medium. Incubate the cells at 37°C, CO2 at 5%, and 95 % relative humidity in an incubator. Approximately 1×10^5 primary fibroblasts were used for transductions with AAV8 Control ShRNA or AAV8 p120 ShRNA virus in 12-well plates and harvested after 4 days for the western blot and qPCR analysis.

Western blot analysis

Cells were directly lysed using RIPA buffer (Sigma-Aldrich, UK) supplemented with a phosphatase inhibitor cocktail (Protease Inhibitor Cocktail Set III, EDTA-Free, Calbiochem, UK (1:200 dilution). With the BSA protein assay kit, the protein concentration was determined. All assays were performed using a total of 30µg of protein. Proteins were separated on Invitrogen™ NuPAGE™ 4 to 2% Mini gels using an electrophoresis technique described by the manufacturer (running conditions 200V and 135 mA). Proteins were blotted onto polyvinylidene difluoride membranes (PVDF) from polyacrylamide gels. The membranes were

kept with a specific anti-p120 antibody (and developed using an ECL™ detection kit. p120 protein bands were visualized using a chemiluminescent imaging system such as a ChemiDoc in a dark room. β-actin was used as a loading control.

Quantitative RT-PCR (qPCR) analysis

Qiagen RNeasy Mini-Kit to extract the RNA from AAV transduced primary fibroblasts and Verso Reverse Transcriptase (Invitrogen) was utilised for cDNA synthesis. Quantitative RT-PCR procedures were carried out using a LightCycler® 480 II platform (Roche Diagnostics). Relative mRNA expression was then calculated using comparative methods ($2-\Delta\Delta C_T$) using RPL-13A as a reference gene. Following p120 primer sequences were used, forward primer (5´CTGTGATGGTGTTCCTGCTCTG3´) and reverse primer (5´TGGGATGAGAGATTC CA CAGGG 3´)

4.   Paper II

Wound infiltrating adipocytes are not myofibroblasts

Shruthi Kalgudde Gopal[1,2,#], Ruoxuan Dai[1,#], Ania Maria Stefanska[1], Meshal Ansari[2,3], Jiakuan Zhao[1], Pushkar Ramesh[1], Johannes W. Bagnoli[4], Donovan Correa-Gallegos[1], Yue Lin[1], Simon Christ[1], Ilias Angelidis[2], Valerio Lupperger[3], Carsten Marr[3], Lindsay C. Davies[5], Wolfgang Enard[4], Hans-Günther Machens[6], Herbert B. Schiller[2,*], Dongsheng Jiang[1,*], and Yuval Rinkevich[1,*]

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Yuval Rinkevich[1,*]

[1] Institute of Regenerative Biology and Medicine, Helmholtz Center Munich, Munich, Germany

[2] Institute of Lung Health and Immunity, Helmholtz Center Munich, Munich, Germany

[3] Institute of AI for Health, Helmholtz Center Munich, Munich, Germany

[4] Anthropology and Human Genomics, Faculty of Biology, Ludwig-Maximilian University Munich, Germany.

[5] Department of Microbiology, Tumour and Cell Biology (MTC), Karolinska Institute, Stockholm, Sweden

[6] Department of Plastic and Hand Surgery, Klinikum rechts der Isar, School of Medicine, Technical University of Munich, Munich, Germany

[#] These authors contributed equally

* Correspondence to: herbert.schiller@helmholtz-munich.de (H.B.S.),

dongsheng.jiang@helmholtz-munich.de (D.J.) & yuval.rinkevich@helmholtz-munich.de

(Y.R.)

Abstract

The origins of wound myofibroblasts and scar tissue remains unclear, but it is assumed to involve conversion of adipocytes into myofibroblasts. Here, we directly explore the potential plasticity of adipocytes and fibroblasts after skin injury. Using genetic lineage tracing and live imaging in explants and in wounded animals, we observe that injury induces a transient migratory state in adipocytes with vastly distinct cell migration patterns and behaviours from fibroblasts. Furthermore, migratory adipocytes, do not contribute to scar formation and remain non-fibrogenic *in vitro*, *in vivo* and upon transplantation into wounds in animals. Using single-cell and bulk transcriptomics we confirm that wound adipocytes do not convert into fibrogenic myofibroblasts. In summary, the injury-induced migratory adipocytes remain lineage-restricted and do not converge or reprogram into a fibrosing phenotype. These findings broadly impact basic and translational strategies in the regenerative medicine field, including clinical interventions for wound repair, diabetes, and fibrotic pathologies.

Introduction

Cells undergo a gradual stepwise restrictive specification during embryonic development, acquiring lineage-specific differentiation fates to become specialized adult cell types. This gradual segregation of cellular potential during development is thought to be restrictive and maintained into, and throughout, adulthood[1]. However, many studies have challenged this notion, and called lineage-restriction into question, by proposing that fully committed cells can respond to tissue challenges, such as injury, disease or aging, by acquiring new fates. This phenotypic adaptability is termed plasticity.

The prototypical example of plasticity is the terminally differentiated stromal mesenchymal cell[2]. Mesenchyme includes mature adipocytes and fibroblasts, which have been proposed to cross-convert between lineages in response to tissue challenges, in skin and in internal organs of both mice and humans[3-11]. The impact of lineage interplay between adipocytes and fibroblasts is evident clinically in numerous disorders including diabetes, where complications associated with the disease include chronic wound healing and fibrotic pathologies such as renal disease and diabetic retinopathy[12].

Lineage interplay between adipocytes and fibroblasts is also evident in fibrotic induction, irrespective of organ. Loss of dermal adipose tissue and adipogenicity in favour of expanding stromal fibroblasts responsible for ECM deposition are consistent features of fibrosis in both mice and men. This is exemplified in skin biopsies from patients with systemic sclerosis[13]. Similar imbalances in the adipocyte : fibroblast stromal environment are seen in obese or diabetic patients undergoing fibro-inflammation in their adipose tissue, where adipocyte numbers decrease in favour of fibroblasts, a transition that is associated with fibrosis and scar formation within the connective tissues[14, 15].

The relevance of changing cellular ratios and phenotypes of fibroblasts and adipocytes within organ systems extends far beyond diabetes and fibrotic disease however. Increased frailty and deterioration in organ function have been directly linked to changes in adipose and fibroblast

66 communities with age[16, 17]. There is a clear need for further delineation of each cell lineage's

67 role in tissue homeostasis and repair.

68 Three main hypotheses have been proposed as to how cells may transition between lineages:

69 de-differentiation (and re-differentiation), (direct) trans-differentiation and heterotypic cell

70 fusion[18]. (i) De-differentiation is where a cell loses its lineage-specific differentiation state and

71 reverses into a more immature cellular state, sometimes associated with a multi-lineage

72 differentiation potential, as seen in adult stem/progenitor cells. (ii) Trans-differentiation is the

73 transition from one lineage to another either directly or indirectly i.e. de-differentiation

74 followed by subsequent re-differentiation. (iii) The third conversion mechanism, heterotopic

75 cell fusion, involves merging of two terminally differentiated and functionally distinct cell

76 types. This form of fusion results in the formation of a hybrid cell with the combined functions

77 of both precursor cells, and has been shown to occur in low frequency during injury[19].

78 Most reported observations of interchangeability between fibroblasts and adipocytes have been

79 based on one cell type acquiring markers associated with the other, e.g. fibroblasts expressing

80 Perilipin-1 (*Plin1*), or adipocytes expressing alpha smooth muscle actin (*Acta2*). However,

81 these markers are insufficient to establish the identity categorically[20, 21]. Designating plasticity

82 has equally relied on morphological changes, for example between round and lipidated

83 adipocytes to flat bipolar fibroblasts. However, phenotype can also be deceptive, as mature

84 adult adipocytes can shed their lipid content under certain metabolic conditions making them

85 even harder to distinguish from fibroblasts. Such fluidity in the homeostatic properties of

86 adipocytes means that a more in-depth characterization of the molecular and cytostructural

87 changes involved in response to injury is needed to definitively demonstrate cellular plasticity.

88 Cellular identity is not just a construct of markers and morphology. Formal proof of

89 physiological plasticity requires the observation of a defined mature cell losing its cellular

90 identity and functions, and metamorphosing into a distinctly different cellular identity with new

91 functions. Such changes are, in the main, inherently achieved through transcriptional regulation,

4

92   characterized by the loss of mature differentiation markers (as for de-differentiation), the

93   expression of immature cell markers and acquisition of a new transcriptional landscape (as for

94   trans-differentiation), supporting the attainment of these new functional properties[22].

95   Here, we directly explore the plasticity of fibroblasts and white adipocytes in skin by closely

96   analysing various transcriptomic, phenotypic, and functional criteria across these two cellular

97   lineages. Using a combination of genetic lineage-tracing strategies, single-cell transcriptomics,

98   live-cell imaging and tracking, transplantation assays and *in vivo* injury models, we study

99   stepwise transcriptional and functional responses in mature adipocytes and fibroblasts after skin

100   injury. We conclude that adipocytes remain lineage-restricted in response to tissue injury, and

101   do not dedifferentiate, fuse with, or convert to myofibroblasts. Adipocytes respond to wounding

102   stimuli, experience shifts in their motility and functionality, and play active but distinct roles

103   from fibroblasts in the endogenous tissue healing response.

104
105
106   **Results**

107
108   **Adipocytes retain their cell lineage identities in a skin explant model**

109   To address a potential phenotypic convergence of adipocyte and fibroblast lineages we

110   performed single-cell RNA-seq (scRNA-seq) experiments. To track fibroblasts in the back-

111   skin, we employed an En1[Cre] reporter that labels dermal fibroblasts, and to a small extent

112   adipocytes. The vast majority of En1-lineage cells termed Engrailed-lineage positive fibroblasts

113   (EPFs) have been demonstrated to be the fibrogenic cell lineage responsible for scar formation

114   in the back-skin[23-25]. Mature adipocytes were selectively tracked using an adiponectin lineage-

115   specific reporter (Adipoq[Cre]), a hormone involved in regulating glucose levels and fatty acid

116   breakdown, and a key biomolecule for anti-diabetic clinical interventions[26]. The two lineage-

117   specific transgenic lines were individually crossed to a double-colour fluorescent reporter

118   (R26[mTmG]), thereby marking mature adipocytes and fibrogenic cells with membrane-bound

119   green fluorescent protein (GFP) in two separate transgenic reporter mice (Supplementary Fig.

5

120   1a). Skin explants were harvested from both adipocyte (Adipoq^{Cre};R26^{mTmG}) and fibroblast

121   (En1^{Cre};R26^{mTmG}) double-transgenic reporter mice, cultured for up to 5 days, followed by

122   purification of adipocytes and fibroblasts for scRNA-seq (Fig. 1a)[27-29].

123   In two-dimensional uniform manifold approximation and projection (UMAP) embeddings of

124   the single-cell transcriptomes, the two lineages largely separated but with partial overlap (Fig.

125   1b). The overlap was mostly observed on day 4 after culture (Fig. 1c). To characterize the

126   heterogeneity of cell states within the two lineage-labelled populations of single cells we used

127   Louvain clustering analysis. Engrailed marks a very early embryonic population of cells in the

128   dermomyotome, primarily of fibroblasts but also a subset of adipocyte progenitors (APs).

129   Indeed, two clusters of APs (cluster 1 and 9) were identified in the En1-lineage population

130   (Supplementary Fig. 1b, c) in neonatal skin of the explant model. They were excluded in the

131   analyses to enable mature adipocytes alone to be studied for potential cellular conversion into

132   fibroblasts. We observed six distinct sub-clusters of cells in each of the Adipoq^{Cre} and the En1^{Cre}

133   lineages (Fig. 1, Supplementary Fig. 1d, e). All twelve cluster identities have highly distinct

134   marker genes and distinct enriched gene categories (Supplementary Fig. 1h, Supplementary

135   Table 1). Next, we used Pearson's correlation co-efficiency and measured the magnitude of

136   relationships between the twelve clusters (Fig. 1d). This analysis indicated that the twelve

137   clusters did not overlap, or converge, and remained separate. Despite signature variability

138   within lineages, the two lineages stayed distinct and clustered apart under all experimental

139   conditions.

140   These data indicated that adipocyte and fibroblast lineage signatures are not lost in the explants

141   that mimic wounding and scarring. We identified a core set of 88 definitive adipocyte genes

142   and 198 definitive fibroblast genes that remained specific to both lineages in the explants (Fig.

143   1e, Supplementary Fig. 1i, Supplementary Table 2). Gene ontology (GO) gene set enrichment

144   analysis confirmed that the core set of adipocyte genes was significantly enriched for terms

6

145   such as "fat cell differentiation" and "lipid metabolism" (Fig. 1f), while the fibroblast core gene

146   set was significantly enriched for extracellular matrix genes (Fig. 1g). To further address this

147   lineage restriction at the pathway level, we scored transcriptomic signatures of both adipocytes

148   and fibroblasts against a selected set of pathway gene lists taken from the RT^2 profiler PCR

149   array (n=84). We found that fibroblast signatures were enriched for fibrogenically active

150   pathways like ECM and adhesion molecules, fibrosis, wound healing, focal adhesions, etc.

151   Whereas, adipocytes were enriched for adipogenic pathways such as fatty acid metabolism,

152   Lipoprotein signalling and cholesterol metabolism (*Lscm*), Peroxisome proliferator-activated

153   receptor gamma (*Pparγ*) targets etc.

154   The adipocyte core signature contained genes such as Pyruvate dehydrogenase lipoamide

155   kinase isozyme 4 (*Pdk4*), a gene involved in reactive oxygen species (ROS) production, which

156   has a vital role acting as a second messenger recruiting immune cells, as well as defensive

157   against invading bacteria at the site of injury[30, 31]. Core adipocyte markers also included

158   Orosomucoid 1 (*Orm1*) known to be immunomodulatory, Stearoyl Coa desaturase 1 (*Scd1*)

159   which is involved in fatty acid biogenesis and Serum amyloid A 3 (*Saa3*), which is responsible

160   for acute phase response and is induced by pro-inflammatory stimuli[32].

161   Fibroblast core signature genes included Bone morphogenetic protein 7 (*Bmp7*), which is likely

162   involved in ECM degradation, Insulin like growth factor binding protein 5 (*Igfbp5*) involved in

163   ECM production promoting fibrosis[33], and Nerve growth factor (*Ngf*), known to promote

164   myofibroblast differentiation[34, 35]. This indicated to us that definitive adipogenic and fibrogenic

165   lineage markers are not lost in explants during culture (as assumed in de-differentiation), nor

166   are they re-acquired in alternative lineages (as assumed in trans-differentiation).

167   Next, we focused on the individually clustering differentiation trajectories of adipocytes and

168   fibroblasts in explants. Mature adipocytes (cluster A2) were characterized by high metabolic

169   activity, and lipid biogenesis i.e. by expressing the Complement factor d (*Cfd*), Adiponectin

7

170   (*Adipoq*), Perilipin1 (*Plin1*), and Resistin (*Retn*) genes (Supplementary Fig. 1h). Interestingly,

171   adipocyte cluster A1 was enriched for a gene signature consistent with a migratory phenotype,

172   embellished with active cytoskeletal remodelling, including increased expression of Was/Wasl

173   interacting protein family member 1 (*Wipf1*), Tripartite motif containing 44 (*Trim44*), Opticin

174   (*Optc*), and NLR family apoptosis inhibitory protein 6 (*Naip6*). It was this adipogenic cluster

175   that appeared transcriptionally closest to fibroblasts (Fig. 1h). Still, migratory adipocytes

176   (cluster A1) could be consistently distinguished from fibroblast subpopulations based on their

177   expression of adipogenic markers such as *Cfd* (Supplementary Fig. 1f), an adipokine involved

178   in cell signalling and insulin secretion, and *Cidec* (Supplementary Fig. 1g), a gene encoding

179   cell death activator CIDE-3 and involved in lipid droplet enlargement. Together, these data

180   indicated that mature adipocytes might undergo rearrangement and active remodelling of

181   cytoskeletal actin filaments in explants, reminiscent of a migratory mesenchymal cell, while

182   still retaining the definitive adipogenic-lineage markers and not acquiring the definitive

183   fibrogenic-cell markers.

184   Analysis of cell states of En1-lineage positive cells identified enrichment for several different

185   biological processes (Fig. 1i). Fibroblast cluster F1 was enriched for cell migration associated

186   genes such as Vav guanine nucleotide exchange factor 2 (*Vav2*), Slingshot protein phosphatase

187   2 (*Ssh2*), Myosin light chain kinase (*Mylk*), Unc-51 like kinase 4 (*Ulk4*), IQ motif containing

188   GTPase activating protein 1 (*Iqgap1*), and was transcriptionally the closest fibroblast cluster to

189   the migratory adipocyte cluster. We identified a state consistent with fibrogenic gene expression

190   (cluster F2), including Collagen type 1 alpha 1 chain (*Col1a1*), Collagen type 3 alpha 1 chain

191   (*Col3a1*), Fibronectin (*Fn1*), Periostin (*Postn*), Decorin (*Dcn*), Microfibril associated protein 5

192   (*Mfap5*). Cluster F3 demonstrated a genotypic profile central to endoplasmic reticulum (ER)

193   stress processes with expression of Mesemcephalic astrocyte derived neurotrophic factor

194   (*Manf*), Cysteine rich with EGF like domains 2 (*Creld2*), Heat shock protein 90 beta family

8

195   member 1 (*Hsp90b1*). Cluster F4 however, was enriched for ECM remodelling genes and

196   processes, expressing Matrix metallopeptidases 9 and 13 (*Mmp9, Mmp13*), Cathepsin h (*Ctsh*),

197   Tenascin C (*Tnc*), Lumican (*Lum*), Argininosuccinate synthase 1 (*Ass1*).

198   The pathway analysis of the top DEGs highlighted anti-microbial (anti-bacterial, anti-fungi,

199   anti-virus) pathways in Adipoq-lineage positive cells in wounds; and highlighted fibrosis,

200   wound healing, and ECM deposition pathways in En1-lineage positive wound fibroblasts

201   (Supplementary Fig. 1j). The genes with high expression level in each pathway were plotted as

202   a heat map. Those anti-microbial genes were drastically upregulated in day 1 explants and the

203   expression returned to low levels in day 4 explants (Fig. 1k).

204   In conclusion, our data provide tantalizing evidence that fibroblasts and adipocytes refrain from

205   cross-converting between lineages or de-differentiating in whole-skin explant models, and

206   remain genetically bound to their original fibroblast or adipocyte identity.

207

208   **Terminally differentiated adipocytes mobilize and reposition during injury**

209   To study the behaviour of mature, terminally differentiated adipocytes and fibroblasts in

210   response to injury, we employed whole-skin explants, allowing visualization and tracking of

211   resident cells, in unprecedented detail over five days in culture, thereby overcoming restrictions

212   associated with short imaging durations *in vivo*[36]. *Ex vivo* models, such as this, allow delineation

213   of the phenotypic and behavioural responses of stromal cells after tissue damage. Briefly,

214   whole-skin explants were isolated from the back-skin of both lineage reporter mouse strains

215   and grown in a specialized incubation chamber that maintains constant O_2/CO_2, humidity and

216   temperature. We also modified the incubation chamber with specialized silicone rings, to allow

217   multi-photon microscopy objectives to record GFP$^+$ adipocytes and fibroblasts within the

218   explants, in high resolution, without compromising environmental conditions. Using this

219   system, explants remained viable and underwent contraction, scar formation and re-

220   epithelialization[36].

221   With this set-up we obtained time-lapse videos, directly after explant culture, of clusters of

222   mature and round adipocyte bundles, in both the subcutaneous fascia and in the papillary dermis

223   where the bundles wrapped around individual hair follicles (Fig. 2a, Supplementary Movie 1).

224   Adipocyte-lineage cells remained stationary initially, with the first signs of morphologic change

225   visible at around twenty minutes, including the appearance of small filaments or protrusions

226   extending from the cell membrane (Fig. 2b, c). At twenty-four hours, explants had adipocyte-

227   lineage clusters that continued the morphologic transformation, with multiple round adipocytes

228   acquiring oval and spindle-shaped morphologies (n=110 cells, n=3 explants). Two days after

229   wounding, 36% of all adipocytes in papillary and fascia deposits gained filopodial extensions

230   (Fig. 2b) that elongated to >100µm (length of individual cell). Supplementary Movies 2 and 3

231   illustrate two stages of this morphologic change. Supplementary Movie 2 (and Fig. 2c) shows

232   oval adipocyte-lineage cells extending small membrane filaments with membrane ruffling,

233   indicative of active cytoskeletal reorganization seen in motile cells. Supplementary Movie 3

234   shows a back-and-forth change of round-to-elongated morphology during movement. Explants

235   on day 2 and day 3 illustrate further filopodia-like extensions, with cells acquiring sleek and

236   slender cell bodies, resembling motile cells. Morphologically transformed adipocyte-lineage

237   cells were interspersed with individual adipocytes adopting a fibroblast-like bipolar

238   morphology, stretching to about 50-200 µm, whereas rounded adipocytes were much smaller,

239   between 10-30 µm. As suggested by our transcriptomics data, adipocytes acquired mobility,

240   moving away from their original fat deposit towards the formative scar region. For example, a

241   single illustrative cell repositioned 380 µm away from its original location (Supplementary

242   Movie 4). Furthermore, elongated adipocytes originated from multiple niches, including fat

243   deposits within the papillary dermis surrounding hair follicles and from within the subcutaneous

fascia. On day 4, motile adipocytes, had reached the centre of the scar region but still retained their elongated morphology (Fig. 2a). Supplementary Movie 5 (and Fig. 2c) shows an adipocyte acquiring a bipolar elongated morphology with extending filopodia (11.11 hrs), with a gradually protruding cell body (14.23 hrs).

A succession of snap-shot images of whole-skin explants indicated that adipocyte-lineage cells constantly revert back-and-forth between stationary and migratory states, with accompanying dynamic remodelling of cellular morphology between rounded and elongated/spindle-shapes (Fig.2b, Supplementary Movie 3). Those migrating adipocyte-lineage cells that had relocated at day 5 moved into the centre of the explants where they reverted to their original, lipid-filled, round morphology (Fig. 2a, Supplementary Movie 6, Supplementary Fig. 2). Quantification of morphologies of adipocyte-lineage cells revealed a gradual decrease of rounded cells from day 0 to day 2 and an increase of oval, spindle shaped and elongated morphologies. A reversal of this phenotype was seen back to a strictly rounded morphology between days 3 and 5 (Fig. 2b). All forms of adipocyte-lineage cells including migratory ones retained Plin1 expression (Fig. 2d).

Next, we investigated if mature adipocytes migrated and repositioned in tissues, in the same way as in the *ex vivo* explant model, in *in vivo* splinted wounds on the dorsal backs of Adipoq[Cre]; R26[mTmG] adult mice. Confocal imaging of wounds at day 7 revealed adipocyte-lineage cells clearly acquired fibroblast-like morphologies and translocate from their initial location within dermal white adipose tissue into the wound bed. On day 21, multiple elongated adipocyte-lineage cells aggregated near the scar region, whereas adjacent non-wounded skin adipocytes retained the classical round morphology associated with dermal white adipose tissue (Fig. 2f). These findings confirm that transcriptional changes associated with motility and phenotypic shifts are functionally relevant, with adipocytes transitionally switching morphology and actively migrating, while retaining adipocyte-committed identity.

269

Absence of cell fusion events between mature adipocytes and fibroblasts

Having found mature adipocytes switching morphology and migrating both *in vitro* and *in vivo,* we wanted to formally exclude all possibility of lineage crossover, therefore we examined the possibility of cell fusion between adipocytes and fibroblasts. Cell fusion includes cytoplasmic intermixing, a phenomenon that can be observed and quantified using red and green lineage-specific fluorescence reporters. Cytoplasmic intermixing would be evidenced as co-labelling of green and red (yellow fluorescence) within migratory adipocytes and fibroblasts. To do this, we analysed and quantified single cells from Adipoq^{Cre};R26^{mTmG} (n=122) and En1^{Cre};R26^{mTmG} lineages (n=135) in both tdTomato$^+$ and GFP$^+$ background at various stages (days) of the explant assay. Lack of co-localization between green and red labels demonstrated that both cell populations, and wound myofibroblasts, shared no history of cell fusion (Supplementary Fig. 3). Collectively, our findings prove that during skin injury adipocytes and fibroblasts remain transcriptionally, behaviourally, and functionally distinct cells, with no inter-conversion or cell fusion between these two stromal lineages.

Distinct migratory behaviours in adipocytes and fibroblasts

Live imaging data indicated that migratory adipocytes share morphological features with fibroblasts despite there being no cross-lineage plasticity or fusion. To visualize and analyse these distinct motility features, and determine whether adipocytes and fibroblasts share migratory behaviours, we crossed both adipocyte and fibroblast mouse lines with a nuclear mCherry reporter (R26$^{LSL-H2B-mCherry}$), allowing tracking of individual cellular nuclei across whole skin. Furthermore, this technique allowed us to compare migratory patterns and features such as velocity, distance travelled, directionality, and collectivity between migrating fibroblasts and adipocytes. Back-skin explants were harvested from Adipoq^{Cre};R26$^{LSL-H2B-}$

294   mCherry and En1Cre;R26LSL-H2B-mCherry double-transgenic mice and cell migration videos recorded

295   in 3D.

296   Adipocyte-lineage cells initiate their migration from their fat deposits within hours after culture

297   (Fig. 3a, Supplementary Movie 7). On day 1, manual tracking of cells in the scar region revealed

298   that 53% of all evaluated adipocytes move away from their original position, yet only 20%

299   directionally moved towards the formative scar region (defined by a minimal movement of 5

300   µm) at day 1 (Fig. 3b, c). Fibroblasts, by comparison, rapidly relocating in explants. At day 1

301   after culture, around 70% of fibroblasts had moved towards the formative scar region, with only

302   22% of the fibrogenic cell lineage moving in other directions (Fig. 3a, b and Supplementary

303   Movie 8).

304   On day 4, both adipocytes and fibroblasts remained motile but with widely different patterns of

305   migration. Migration of adipocyte-lineage cells was stochastic at a population level, with

306   individual cells generating migration tracks that were non-coordinated (Fig. 3d, and

307   Supplementary Movie 9). In contrast, groups of hundreds of fibroblasts migrated collectively,

308   in a uniform and coordinated manner, towards the formative scar region (Fig. 3d,

309   Supplementary Movie 10). This indicates that most of the fibrogenic lineage cells respond by

310   directional cell migrating into wounds, whereas adipocyte-lineage cells appear to respond and

311   migrate less purposefully after culture.

312   Migration dynamics were further quantified using automated cell tracking in the whole explant.

313   The average velocity of adipocyte migration differed significantly from fibroblasts, particularly

314   at day 4. The velocities for individual adipocyte-lineage cells had greater variability throughout

315   all time points, whereas fibroblast velocities remained consistent (Fig. 3e, f). Furthermore,

316   movement similarity analysis confirmed that adipocyte-lineage cells move randomly, in

317   contrast to fibroblasts (Fig. 3g). This suggests that fibroblasts act collectively, whereas

318   adipocytes behave individually. Indeed, directional analysis of adipocyte-lineage cells and

13

319   fibroblasts further confirmed the collective migration path of fibroblasts, in contrast to

320   adipocyte-lineage cells (Fig. 3h). These findings indicate that migratory adipocytes and

321   fibroblasts respond in a vastly different manner in terms of velocity, directionality, and

322   collectivity. It further indicates that collective cell migration is a defining characteristic of

323   migratory fibroblasts, but not adipocytes.

324   **Adipocytes do not contribute to scar formation**

325   Accretion of extracellular connective tissue matrix is another defining characteristic of

326   fibrogenic cells[37]. We previously demonstrated that the fibroblasts that go on to have the ability

327   to form scars (by depositing ECM and accumulating dense fibrous tissue) express the *Engrailed*

328   gene temporarily during embryogenesis[25]. We thus compared, the scar-competent 'Engrailed

329   Past Fibroblasts' (EPFs) with adipocytes, for their ability to express and secrete ECM proteins

330   in skin explants; mature adipocytes were coloured green with GFP from Adipoq[Cre];R26[mTmG]

331   mice, and the scar-forming fibroblasts, EPFs, were coloured red with tdTomato.

332   Expression of the myofibroblast marker αSMA[38, 39] was assessed by immunohistochemical

333   localization at day 0. Here expression patterns correlated with a perivascular niche environment,

334   in the absence of established scar tissue in explants. At day 4 parallel fibrils of αSMA were

335   evident within the scar region, and the fibroblasts started to retract from the developing fibrotic

336   tissue. At this time the adipocyte-lineage cells were distant from the scar developing region,

337   suggesting a less active role in deposition. Furthermore, in day 4 explants the ECM protein

338   Fibronectin was deposited by GFP[-] tdTomato[+] fibroblasts and not by GFP[+] adipocyte-lineage

339   cells (Fig. 4a). Migratory adipocytes did not express fibroblast proteins Fibroblast Specific

340   Protein 1 (FSP1), Transcription Factor 21 (TCF21), or Col3a1 (Supplementary Fig. 4a-e).

341   Myofibroblast and fibrogenic markers, such as *Ddr2*, *Lrrc17*[33, 34], and ECM markers *Col1a1*

342   and *Col3a1*, were upregulated across all fibroblast subsets, but not within adipocytes (Fig. 4b).

343   To evaluate directly whether adipocyte-lineage cells produce and deposit ECM proteins, we

344   performed 3D immuno-labelling of deposited ECM proteins (Fig. 4c). Adipocyte-lineage cells

345   showed minimal contribution of Fibronectin or Type-I Collagen, irrespective of their

346   morphology or motility status (Fig. 4d). As a control, baseline matrix production was compared

347   to that induced by recombinant transforming growth factor beta 1 (TGFβ1) stimulation, a

348   known pro-fibrotic cytokine upregulated during the early stages of the wound healing

349   response[40]. Fibroblasts deposited significant amounts of Fibronectin and Type-I Collagen,

350   whereas adipocyte showed minimal contribution in ECM production (Fig. 4d-f). Stimulation

351   with TGFβ1 significantly increased Fibronectin and Type-I Collagen in En1-lineage fibroblasts,

352   but not in adipocyte-lineage cells (Fig. 4d-f). Interestingly, TGFβ1 altered cellular contour to

353   the same fibroblast-like morphology seen in migratory adipocyte-lineage cells *in vivo*

354   (Supplementary Fig. 4f). Despite their morphologic conversion then, we have seen that

355   adipocyte-lineage cells are not fibrogenic, and they do not contribute to the ECM, which is

356   exclusively deposited by fibroblasts.

357   We then went on to investigate mature adipocytes in wounds *in vivo*. Full-thickness excisional

358   wounds were generated on the backskin of adult Adipoq^{Cre};R26^{mTmG} and En1^{Cre};R26^{mTmG} mice,

359   in which the Adipoq-lineage adipocytes and En1-lineage fibroblasts express GFP, respectively.

360   At day 10 and day 21, wounds were harvested and GFP^{+} cells were sorted by FACS. The

361   transcriptomic profiles of the sorted wound adipocyte-lineage cells and fibroblasts were

362   analysed by mRNA-sequencing, with three independent biological repeats for each cell type at

363   each time point. Pearson correlation analysis revealed that adipocytes from day 10 and day 21

364   wounds were clustered together with adipocytes from adjacent uninjured skin; whereas

365   fibroblasts from day 10, day 21 wounds and healthy skin were clustered together. The

366   transcriptome of the adipocyte cluster was clearly distinct from the fibroblast cluster across all

367   wound time points (Fig. 5a). Gene ontology (GO) enrichment of differentially expressed genes

368 (DEG) in adipocytes and fibroblasts in day 10 wounds indicated that wound fibroblasts and

369 adipocytes performed distinct biological processes. Wound fibroblasts were enriched for cell-

370 matrix adhesion, matrix deposition and regulation. By contrast, wound adipocytes in day 10

371 wounds were enriched for sensory perception, immune regulation, and anti-microbial responses

372 (Fig. 5b). Comparison across time points showed that the transcriptome of adipocytes from day

373 21 wounds was closer to adipocytes from the adjacent healthy skin; whereas adipocytes from

374 day 10 wounds upregulated gene features consistent with a migratory mesenchymal cell, which

375 are also highly expressed in fibroblast groups; however, the expression levels of those genes

376 were much lower than En1-lineage fibroblasts (Supplementary Fig. 5a). Still, DEG analysis

377 within adipocytes at different time points showed they retained biological processes of anti-

378 microbial responses, inflammation, and immune regulation (Supplementary Fig. 5b).

379 Immunofluorescence staining of wound sections showed that the migratory adipocyte-lineage

380 cells were at higher number in day 7 wounds compared to day 21 scars (Fig. 5c). Those

381 migratory adipocyte-lineage cells were negative for fibroblastic markers αSMA (Fig. 5d) and

382 vimentin (Fig. 5e), and proliferation marker Ki67 (Supplementary Fig. 5c), and therefore

383 separated from fibrogenic cells. To evaluate the relevance of these observations further *in vivo*,

384 we transplanted 2.5×10^5 individual cells of each lineage into full-thickness dorsal wounds of

385 immuno-deficient Rag2$^{-/-}$ mice. Wounds were harvested 7 days post-transplantation. ECM

386 within the transplanted regions and adjacent non-transplanted areas were quantified as merged

387 immunofluorescence intensities of type-I collagen and fibronectin (Fig. 5f). Transplanted

388 fibrogenic lineage cells generated ectopic scar ECM architectures, whereas transplanted

389 purified adipocyte-lineage cells had negligible effects on scar formation and connective tissue

390 architecture (Fig. 5f, g).

391 Gene ontology analyses of the adipocyte lineage from both skin explants (Fig. 1j, k) and from

392 in vivo wounds (Fig. 5a, b; Supplementary Fig. 5a, b) indicated they retained anti-microbial

16

responses, immune regulation, and sensory perception, while acquiring a mesenchymal migratory phenotype. To explore the physiological role of adipocytes in wounds, we performed immunostaining of mouse cathelicidin-related antimicrobial peptide (CRAMP), an ortholog of human cathelicidin (the active form is known as LL-37), on day 7 wound section from Adipoq[Cre];R26[mTmG] mice. 60% of migratory adipocytes in day 7 wounds showed clear expression of CRAMP, while the expression markedly reduced in adipocytes in day 21 wounds (Fig. 5h). This data confirms that migratory adipocytes retain anti-microbial peptide functions in wounds.

Discussion

In this paper we demonstrate irreversible cell fate of mature adipocytes during skin injury, despite their remarkable morphological changes and migratory behaviours. Wound adipocytes do not convert their cellular identity into fibrogenic cells, as had been previously assumed[9, 10]. Morphological modulation of wound adipocytes in injured tissues were observed, but there was no evidence for adipogenic origins of fibrosing cells during injury or evidence of conversion or cell fusion from adipocytes to fibroblasts by assessment of transcriptomic, behavioural, or functional criteria.

Our findings that differentiated adipocytes remain committed to their lineages during the healing process, has direct implications to our understanding of homeostasis and repair of stromal tissues; impacting both basic and clinical paradigms for wound repair, regeneration, and various cell transplantation strategies for regenerative medicine. Clinically, today's standard of care for skin grafts and scaffold constructs provide closure of a wound area, but leave patients with disfiguring and debilitating scars. Current pre-clinical and clinical studies employ tissue equivalents composed of epidermal and stromal fibroblast populations embedded in various matrices. The incorporation of a dermal component into skin equivalents provides mechanical stability and supports a functional repair tissue by prevention of stricture and scar

418   formation. Clinically approved grafts, such as Apligraf®, have already received Food and Drug

419   Administration (FDA) approval for usage in venous leg ulcers and diabetic foot ulcers. Clear

420   delineation of stromal subsets, and understanding of their tissue specific roles and

421   responsiveness to the environmental milieu, is crucial in the development of novel skin

422   substitutes that enable superior 'scarless' regenerative outcomes in the treatment of severe skin

423   defects, burns, accidents, congenital diseases, tumours, or chronic wounds.

424   Fat grafting, or lipofilling, is another clinical example where lineage conversions from

425   adipocytes to fibroblasts have been proposed to enhance tissue repair processes not only by

426   providing tissue volume, but also by reducing scar formation and fibrosis, thereby improving

427   the functionality of the repair tissue[41]. Convincing clinical evidence for the effectiveness of fat

428   grafting remains elusive. Data from our study highlights that transplanted mature adipocytes

429   will not morphos into stromal fibroblasts, contributing to repair tissue; rather this remains the

430   feat of the resident stromal fibroblasts. However, key reparative signals and a supportive milieu

431   provided by the adipocytes may promote the function and response of the injured stroma. This

432   remains an area warranting further investigation and clinical refinement as our knowledge

433   deepens.

434   Previous studies from Horsley's group suggested a conversion of wound adipocytes to

435   myofibroblasts by using lineage tracing coupled with scRNA-seq[9, 10]. However, the cell

436   numbers of tracked GFP expressing adipocytes in wounds included in the analysis was

437   extremely low, and in the background noise of large numbers of fibroblasts and contaminated

438   leukocytes and endothelial cells[9]. Conversion was reported based on scRNAseq data from 13

439   wound adipocytes, whereas our analysis was conducted on a much larger population (200 -

440   2000 wound adipocytes per sample). Nonetheless, even if the conversion happened in such few

441   cells, it remains unclear whether they play physiologic roles in scar formation, where the

442   predominant numbers of myofibroblasts originate from $En1^+$ fibroblast lineages. In our mRNA-

443   seq analysis of in vivo wound fibroblasts and adipocytes, we used bulk RNA-seq with FACS

444   enriched En1-lineage positive fibroblasts and Adipoq-lineage positive adipocytes, ranging from

445   200 to 2000 cells per sample, which largely increased the cell numbers of input adipocytes, and

446   therefore remarkably reduced the noise. There are a couple of limitations to this study. The

447   scRNA-seq data is derived from skin explants, which lack immune cells, blood supply and

448   innervation, all of which could influence the transcriptomics data and therefore we have

449   confirmed functional separation of fibroblasts and adipocytes in vivo in wounded animals.

450   Another limitation relates to the preparation procedure of purifying adipocytes that is

451   considered to be technically challenging and therefore relatively low numbers of adipocytes are

452   analysed, as compared to fibroblasts. Nevertheless, we believe the sequencing analyses

453   presented in this study are closer to the genuine physiologic roles of wound adipocytes.

454   The scRNA-seq analysis of adipocyte in the whole-skin explant model, and the mRNA-seq data

455   of adipocytes in excisional wounds have suggested that the migratory mature adipocytes are

456   involved in anti-microbial responses, immune regulation, and sensory perception. The

457   adipocytes in day 7 wounds express high level of cathelicidin-related antimicrobial peptide,

458   confirming their anti-microbial functions in vivo. In line with these findings, skin adipocytes

459   have been shown to execute anti-microbial functions during injury repair or skin infection, by

460   releasing anti-microbial peptide cathelicidin[42, 43]. Such behaviour is believed to be regulated by

461   toll-like receptors and retinoic acid[44]. Furthermore, the antimicrobial function of wound

462   adipocytes is evolutionary conserved. Fat body cells, the equivalent of vertebrate adipocytes in

463   *Drosophila* have been demonstrated by live imaging that they participate in wound healing, by

464   actively migrating to wounds and physically sealing wounds. The fat body cells also help to

465   fight against the infection by locally releasing antimicrobial peptides such as Attacin[45]. The

466   other predicted physiological functions of wound adipocytes such as immune regulation and

467   sensory perception require further investigation.

468   This study delineates multi-modality in lineage-restricted adipocytes in their response to tissue

469   injury. Understanding the limitations of lineage interplay between stromal cells provides key

470   knowledge to tissue repair responses central to multiple organ systems, as well as supporting

471   translational knowledge for the development of novel tissue engineering and stromal cell-based

472   therapeutics for impaired healing and fibrotic disorders.

473

Methods

475 **Transgenic & reporter mouse lines**

476 This study complies with all relevantethical regulations. All animal experiments were reviewed

477 and approved by the Government of Upper Bavaria and registered under the projects under

478 projects ROB-55.2-2532.Vet_02-16-61, ROB-55.2-2532.Vet_02-19-23, and ROB-55.2-

479 2532.Vet_02-21-153, and conducted under strict governmental and international guidelines.

480 C57BL/6J wild type mice were purchased from Charles River (strain code 632). En1^{Cre} (JAX

481 stock No. 007916), ROSA26LSL-H2B-mCherry (R26$^{LSL-H2BmCherry}$) (JAX stock No. 023139),

482 Rag2$^{-/-}$ mice and ROSA26mTmG (R26^{mTmG}) reporter mice were from Stanford University[25].

483 Adipoq^{Cre} (Jax stock No.028020) mice were from Helmholtz Center. En1^{Cre} or Adipoq^{Cre}

484 transgenic mice were crossed with R26^{mTmG} or R26$^{LSL-H2BmCherry}$ reporter mice for use in the

485 described experiments. Animals were housed at the Helmholtz Center animal facility rooms

486 that were maintained at constant temperature and humidity with a 12-h light cycle. Animals

487 were given food and water ad libitum. No sex selection was performed in this study.

488 **Mouse genotyping**

489 Cre^{+} animals from double-transgenic reporter mice were classified based on appropriate

490 fluorescence present in the dorsal back of the mice. A fluorescence microscope was used for

491 identification of Cre^{+} neonatal mice. For adult mice, genotyping was performed to detect a 200

492 base pair Cre band (fragment). Filter tips were used to prevent cross contamination. Genomic

493 DNA was extracted normally from ear clip tissue. Quick Extract (QE) DNA extraction solution

494 (Biozym, 101094) was used. Extracted Genomic DNA solution 1 μl, was added to each PCR

495 reaction mix containing 24 μl. The PCR reaction mixture was prepared using Taq PCR core kit

496 (Qiagen, 201205) containing Coral buffer (1X concentration final), dNTPs (10mM each), Taq

497 polymerase enzyme (0.625 units per reaction), 0.5 μM of each forward primer (FP) 5′ ATT

498 GCT GTC ACT TGG TCG TGG C-3' and reverse primer (RP) 5′ GGA AAA TGC TTC TGT

499 CCG TTT GC-3′. PCR cycling temperature was set to 10 minutes at 94 °C for initial

500   denaturation. Followed by, amplification of 30 cycles: 30 seconds denaturation at 94 °C, 30

501   seconds, annealing at 56 °C, 30 seconds, elongation at 72 °C. The final elongation at 72 °C was

502   for 8 minutes, and an infinite temperature of 10 ° C was maintained. Negative controls with no

503   template and positive controls were included. The Eppendorf master cycler instrument was

504   used, and samples were analyzed by agarose gel electrophoresis

505   *Ex vivo* **explant culture**

506   The skin-fascia explant assay was performed by following our established protocol[36, 46]. Briefly,

507   back-skin was collected from new-born (postnatal day 0-1) two-colour membrane reporter

508   En1[Cre];R26[mTmG] or Adipoq[Cre];R26[mTmG] reporter or En1[Cre];R26[LSL-H2B-mCherry] mice or

509   Adipoq[Cre];R26[LSL-H2B-mCherry] nuclear reporter mice, and washed twice with DMEM/F-12

510   (Thermo Fisher Scientific, 11320074) medium to remove contaminating blood, and then

511   washed once with Hank's Balanced Salt Solution (HBSS, Thermo Fisher Scientific 14175095).

512   Dorsal back skin was cut out and explants were made using a disposable Ø 2 mm biopsy punch

513   (Stiefel, 270130) down to below the panniculus carnosus muscle, and cultured in 2 ml of

514   DMEM/F-12 medium containing 10% FBS, 1x GlutaMAX (Thermo Fisher Scientific,

515   35050038), 1x Penicillin/streptomycin (Thermo Fisher Scientific, 15140122), and 1x MEM

516   non-essential amino acids (Thermo Fisher Scientific, 11140035) in 6 well plates, in a

517   humidified 37°C, 5% CO_2 incubator. Fresh medium was supplied every other day and the skin

518   tissues were harvested at the indicated time points (day 1 to day 5 after culture), with the fresh

519   tissues serving as day 0 control. Post harvesting on designated days, explants were fixed in 2%,

520   paraformaldehyde (PFA), overnight at 4ºC for whole mount imaging and immuno-labelling, or

521   directly used for live imaging by multiphoton microscopy or mcSCRB-seq experiments.

522   **Sorting of adipocytes and fibroblasts**

523   Transgenic mouse lines (En1[Cre];R26[mTmG] or Adipoq[Cre];R26[mTmG]) at P0 or P1 stage were used

524   for sorting. Multiple skin explants for each day was pooled to maximize yield. Tissue was

525   minced and digested using 0.5mg/ml Collagenase A (Sigma Aldrich, 10103586001) and 25

526   U/ml of DNase 1 (Sigma, 10104159001) for 1hr at 37˚C with shaking. Cells were washed with

527   complete media and filtered through cell strainers. Centrifugation was carried out for 5 min at

528   200g and the cell pellet was resuspended in 1 ml of FACS buffer containing 2% FBS in PBS.

529   Adipocyte and fibroblast sorting were performed based on GFP$^+$ fluorescence. En1cre$^+$

530   fibroblasts were incubated with the following antibodies for lineage-negative gating. 1 μg of

531   APC-conjugated anti-mouse CD45 (BioLegend, 103112, 1:200), EpCAM (CD326)

532   (BioLegend, 118214, 1:200), PECAM1(CD31) (BioLegend, 102410, 1:200), Ter119

533   (BioLegend, 116212, 1:200), Tie2 (CD202b) (BioLegend, 124008, 1:200) and eFluor660-

534   conjugated anti- LYVE1 (eBioscience, 50-0443-82, 1:200) in FACS buffer (2% [v/v] FBS in

535   phosphate buffered saline [PBS]), on ice for 30 min. The antibody-conjugated cell pellet was

536   washed in 5ml FACS buffer then re-suspended in 1 ml of FACS buffer (PBS +2% FBS). The

537   cells were sorted using the a FACS Aria III sorter with a 120 μm nozzle. Fibroblasts were sorted

538   based on the following gating scheme: Lin-TomatoRed$^-$GFP$^+$ from En1^{Cre};R26^{mTmG}.

539   Adipocytes were sorted based on the following gating scheme: TomatoRed$^-$GFP$^+$ from

540   Adipoq^{Cre};R26^{mTmG}.

541   **Cell sorting for mcSCRB-seq**

542   Dorsal back skin was taken from En1^{Cre};R26^{mTmG} or Adipoq^{Cre};R26^{mTmG}, postnatal day 0 or day

543   1 mice and washed three times with HBSS. The tissue was minced and incubated with 5ml of

544   digestion mix for preparation of single cell suspension. mcSRCB-seq was performed with 1000

545   adipocytes or fibroblasts sorted from day 1 and day 4 explants. The indexing parameter in Aria

546   III was used and cells were sorted directly into 96-well DNA LoBind plates (Eppendorf). Each

547   well was aliquoted with 5 μl lysis buffer prior to sorting. Lysis buffer consisted of Guanidine

548   Hydrochloride (5M concentration; Sigma Aldrich), β-Mercaptoethanol (1%, Sigma Aldrich),

549   and Phusion High Fidelity (HF) buffer (1:500 dilution, New England Biolabs: M0531L).

550   Immediately post sorting, plates were spun down, placed on dry ice, and later stored at -80° C.

551   **cDNA synthesis step of mcSCRB-seq (pre amplification step)**

552   A full step by step protocol for mcSCRB-seq is deposited in the protocols.io repository[27].

553   Before the preparation of libraries, SPRI beads were used to clean up each well. Beads were

554   resuspended in 4 µl double distilled water along with a reverse transcription master mix (5 µl)

555   containing Maxima H-RT enzyme (20U, Thermo Fisher), Maxima H buffer 2x (Thermo

556   Fisher), dNTPs at concentration 2 mM each (Thermo Fisher), template switching oligo (from

557   IDT) 4 µM and Poly ethylene gycol (PEG) 8000 15% (Sigma-Aldrich). After addition of 1µl

558   of 2µM barcoded oligo-dT primer (E3V6NEXT, Integrated DNA technologies), cDNA was

559   prepared according to the mcSCRBseq protocol. Template-switching and cDNA synthesis was

560   performed at 42 ° C for 90 minutes. Barcoded cDNA was later pooled in 2 ml DNA LoBind

561   tubes followed by clean-up using SPRI bead buffer. Purified cDNA was eluted in 17 µl and

562   residual primers digested with enzyme Exonuclease I (from Thermo Fisher) at 37 ° C for 20

563   min. After heat-inactivation at 80 ° C for 10 min, 30 µl PCR master mix containing Terra direct

564   polymerase, 1.25 Units (Clonetech), Terra direct buffer 1.66 X, and SINGV6 primer (IDT) 0.33

565   µM, was added. PCR was cycling was performed as follows: 98 ° C, 3 min for initial

566   denaturation, 19 cycles of 15 seconds at 98 ° C, 65 ° C for 30 seconds and at 68 ° C for 4

567   minutes. Lastly, final elongation was performed at 72 ° C for 10 minutes.

568   **scRNA-seq library preparation**

569   All samples were purified with SPRI beads (ratio 1: 0.8) after pre-amplification. Final elution

570   was in 10 µL of nuclease-free water (Invitrogen). Later cDNA was quantified using the Quant-

571   iT PicoGreen double stranded DNA Assay Kit (Thermo Fisher). A High Sensitivity DNA chip

572   (Agilent Bioanalyzer), was used to verify size distributions. Samples that passed the quality and

573   quantity control parameter, were used for constructing Nextera XT libraries from 0.8 ng of pre-

574   amplified cDNA. Later, 3 'end sequences were enriched with a custom made P5 primer

575   (P5NEXTPT5, IDT), during PCR of the libraries. Following this, Libraries were pooled and

576   selected based on their size using 2% E-Gel Agarose EX Gels (Life Technologies); a size range

577   from 300bp to 800 bp was cut out and extracted using the MinElute kit (Qiagen). All Procedures

578   were in accordance with the manufacturer's recommendations.

Sequencing of scRNA-seq libraries

580   An Illumina HiSeq 1500 instrument was used to sequence libraries. Paired-end sequencing of

581   libraries was performed on high output flow cells. To generate molecular and cellular barcodes,

582   16 bases were sequenced with the 1st read, 50 bps sequenced in the 2nd read into the cDNA

583   fragment, and 8 bases were read to obtain the i7 barcode.

Full-thickness excisional wound model

585   Adult (8-16 weeks old) Adipoq^{Cre};R26^{mTmG} or En1^{cre};R26^{mTmG} mice were anesthetized with

586   MMF (medetomidine at 500 μg/kg, midazolam at 5mg/kg and fentanyl at 50 μg/kg body

587   weight). Dorsal back hair was shaved with a hair clipper, and further removed with depilatory

588   cream. Two full-thickness excisional wounds were created with a 5 mm diameter biopsy punch

589   (Stiefel). Mice were supplied with analgesic. Wounds were harvested at days 7, 10, or 21 post-

590   wounding depending on the subsequent analyses.

Bulk RNA-sequencing of adipocytes and fibroblasts from in vivo wounds

592   Full-thickness excisional wounds were made on the back of Adipoq^{Cre};R26^{mTmG} mice and

593   En1^{Cre};R26^{mTmG} mice. The digested samples were filtered through 100 μm cell strainers to

594   allow collection of adipocytes with bigger size, and centrifuged at 300 g for 5 minutes, and

595   floating adipocytes were isolated using a pipette. Adipocytes and fibroblasts were FACS sorted

596   from day 10 and day 21 wounds of respective transgenic lines as GFP$^+$ cells. 200 – 2000 FACS

597   sorted cells were lysed and cDNA synthesis and the subsequent expansion with 8 PCR cycles

598   were performed using the Smart-Seq® v4 Ultra® Low Input RNA Kit for Sequencing (Takara

599   #634889), according to manufacturer's instructions. The amplified cDNA was purified with

600   NucleoMag NGS Clean-up and Size Select beads (Takara 7449705). The library preparation

601   and sequencing were performed by Biomarker Technologies (BMKGene) GmbH (Münster,

602   Germany) with Illumina Novaseq 6000. Standardized RNAseq pipelines written in Nextflow,

603   nf-core/rnaseq were adapted for trimming, alignment to mm10 by STAR and counts calling by

604   salmon as default setting[47]. The raw counts were used for a differential gene expression analysis

605   (DEG) using DEseq2 (version 1.34.0 with R version 4.1.2)[48]. Normalized counts were scaled

606   for Pearson's correlation matrices and plotting feature genes expression in heat map. Gene

607   ontology (GO) enrichment of DEGs was done using clusterProfiler (version 4.2.2) and viewed

608   by ggplot2 (Version 3.3.6)[49].

609   **Primary data processing of RNA-seq data**

610   Processing of all raw FASTQ data was done using zUMIs pipeline along with STAR to obtain

611   expression profiles for barcoded UMI data[28, 29]. Mapping was done to the reference genome

612   (mm10 / 09) concatenated with the ERCC and GFP reference. Gene annotations were obtained

613   from Ensembl (GRCm38 / mm10 - GRCh38.84).

614   **Processing of mcSCRB-seq single cell data set**

615   The raw count matrices output by the zUMIs pipeline were analyzed using Scanpy[50] (v.1.6.0).

616   For barcode filtering, we excluded barcodes with less than 1000 detected genes. We assessed

617   the number of unique molecular identifiers (UMIs) for each sample using violin plots, and

618   retained cells with a number of UMIs below 250000. Genes were only considered if they were

619   expressed in at least 3 cells in the data set.

620   Normalization was performed based on scran's approach[51], in which size factors are calculated

621   and used to scale the counts in each cell. Log transformation was used via Scanpy's pp.log1p.

622   Top variable genes were established with scanpy's pp.highly_variable_genes and flavor set to

623   "cell_ranger". Highly variable genes were the basis for the principal component analysis (PCA)

624   and neighborhood graph construction via pp.pca and pp.neighbours (adipocytes: n_pcs = 15,

625   n_neighbors = 5; fibroblasts: (n_pcs = 20, n_neighbors = 10). For clustering the louvain

626   algorithm was employed at resolution 0.5, resulting in 6 clusters within the adipocyte and

627   fibroblast populations, respectively. For both subsets the UMAPs and diffusion maps were

628   generated using Scanpy's functions tl.umap and tl.diffmap.

629   As we anticipate a small fraction of contamination with adipocyte cells in the fibroblast labelled

630   population, we cleared away such adipocyte cells. For that we assessed the similarity to

631   adipocytes for each cell in the fibroblast subset with scanpy's tl.score_genes function, using an

632   adipocyte reference signature (adipocyte cell type from Mouse Cell Atlas, link:

633   http://bis.zju.edu.cn/MCA/gallery.html?tissue=Neonatal-Skin,cluster3). Unsupervised louvain

634   clustering at resolution 1 revealed one cluster with particularly high scoring cells, which was

635   then removed from the analysis.

636   For the comparison of both lineages, the two refined subsets were re-combined into one object

637   and the list of variable genes set to the union of the list established on the two subsets. The PCA

638   was re-calculated and the neighbourhood graph established for the concatenated object (n_pcs

639   = 15, n_neighbors = 20). Cell type marker genes for the 12 subclusters were established with

640   tl.rank_genes_groups and method = "wilcoxon".

641   **Differential Gene Expression across the two lineages**

642   We performed differential expression analysis with diffxpy (v.0.7.4). In a first analysis we

643   compared the differences between adipocyte and fibroblast lineage using the Wald test. To

644   circumvent the problem that certain genes are highly upregulated in only one of the 6

645   subclusters per lineage, we constrained the model to each subcluster by adding constraint_loc

646   = [subcluster: lineage] to the model. Genes are labelled as differentially expressed if their

647   Benjamini-Hochberg corrected p-value was less than 0.5, have a log2foldchange of greater 1

648   and are expressed in at least 10% of cells in the relevant lineage. This resulted in a list of 88

649   genes for adipocytes and 198 genes for fibroblast.

650   Gene Set Enrichment Analysis was performed using these differentially regulated genes as

651   input for the python package GOATOOLS[52].

652   **Whole mount 3D imaging by multi-photon microscopy**

653   Post fixation, explants were thoroughly washed in PBS 3X times, 1 hour each, followed by,

654   embedding in NuSieve GTG agarose (2%, Lonza, cat.no. 859081) in a 35mm dish (Falcon,

655 cat.no. 351008). Imaging was performed under a Leica SP8 Multiphoton microscope (Leica,

656 Germany). Tiles were merged with a LAS X (v4.8, Leica) with smooth overlap blending, and

657 data was finally visualized with Imaris image analysis software (v9.1.0 and v9.2, Bitplane, UK)

658 using contrast and brightness adjustments.

659 **Live 3D multi-photon microscopy**

660 Live samples were embedded as above, and live imaging was performed using multi-photon

661 microscopy. Imaging medium included DMEM/F-12. Time-lapse imaging was performed over

662 15 hours under the multi photon microscope. A modified incubation system, with heating and

663 gas control (ibidi 10915 & 11922), was used to guarantee physiologic and stable conditions

664 during imaging. Temperature control was set to 37 °C with 5% CO_2-supplemented air. 3D data

665 was processed with Imaris 9.1.0 (Bitplane, UK) and ImageJ (1.52i). Contrast and brightness

666 were adjusted for better visibility.

667 **Manual cell tracking**

668 Manual cell tracking was performed on explants from nuclear reporter lines (En1^{Cre}; R26^{LSL-}

669 $^{H2B-mCherry}$ or Adipoq^{Cre};R26$^{LSL-H2B-mCherry}$). ImageJ software with "Manual Tracking" plugin

670 (version 2.1.1) was used. In brief, an area of 700µm X 700µm in the scar region of the explant

671 was cropped from 3D-Time lapse datasets. Nuclear spots were identified after subjecting to

672 maximum-intensity projection. Migration of individual cells was tracked over time.

673 Trajectories and individual track information with coordinates were exported as TIFF and excel

674 file respectively. Graphical visualization and analysis of these trajectories were performed using

675 "R". Color-ramps were generated for each track as a function of time (Blue; first time point;

676 Red; last time point). Cell movement patterns were quantified based on whether a cell moved

677 away (towards to epidermis) or towards the scar region (center of the explant).

678 **Automated cell tracking**

679 Automated cell tracking was performed using 3D-Time lapse datasets of whole explants made

680 from nuclear reporter lines using the Imaris software package (v9.2.1, Bitplane, UK). Live

681   videos were generated with a 15 min interval from explant stages of day 1 and day 4. Tracks

682   were generated from 3D data using mCherry fluorescence and an intensity-based spot detection

683   tool. Tracks were visualized in time-coded color representation, ranging from purple to red. For

684   snapshot images, full tracks representing the last 10 time points were shown for better visibility

685   and to prevent overcrowding. Dragon tail representation was used in live videos to better

686   visualize directed collective and non-directed migration patterns.

687   **Quantification of cell migration**

688   Employing the tracked cell trajectories, the displacement in 3D for every cell between

689   consecutive time frames was calculated. To compare experiments, mean cell displacements

690   were added up and plotted over time. To analyse the movement similarity of neighbouring cells,

691   the neighbourhood was determined via Delaunay triangulation. Neighbours were defined as

692   cells that are direct neighbours in the resulting neighbourhood graph. Next, the 3D movement

693   vectors for a cell and its neighbours for consecutive time points were calculated. The 3D

694   movement similarity for cell '*u*' and neighbour '*v*' can be assessed by calculating the

695   intermediate angle between the respective movement vectors:

696    $$\alpha_{u,v} = \cos^{-1}\left(\frac{u_x v_x + u_y v_y + u_z v_z}{\sqrt{u_x^2 + u_y^2 + u_z^2}\sqrt{v_x^2 + v_y^2 + v_z^2}}\right)$$

697
698   Finally, all angles were averaged to produce one movement similarity score. For display

699   reasons the angles were inverted and the movement similarity ranges from 90° (random

700   movement) to 180° (coordinated movement).

701   **Whole mount and conventional immunostaining**

702   Whole-mount samples were pre-incubated in Dulbecco's Phosphate-Buffered Saline (DPBS,

703   Thermo Fisher Scientific 14190169) containing 0.2% gelatin (Sigma G1393), 0.5% Triton-

704   X100 (Sigma X100) and 0.01% Thimerosal (Sigma T8784) (PBS-GT), for 24h at room

705   temperature. They were then incubated with primary antibodies. Primary antibody incubation

706   was carried out at room temperature for 72 hours, followed by washing with PBSGT, 3X times,

707   1 hour each. Fluorophore-conjugated secondary antibodies were purchased from Thermo Fisher

708   Scientific. The samples were incubated, rotating, with the labelled antibodies in PBSGT

709   (1:1000) for 24h at room temperature. Samples were then washed with PBSGT 3X times, 1

710   hour each and stored in fresh PBSGT at 4ºC in the dark until imaging. The following primary

711   antibodies were used: Collagen I (1:200 Rockland, cat.no. 600-401-103-0.1), Collagen III

712   (1:200, Abcam ab7778), Fibronectin 1 (1:250, Abcam ab23750), a-SMA (1:200, Abcam

713   ab5694), Perilipin1 (1:200, Abcam, ab3526), CRAMP (1:200, Novus Biologicals, NB100-

714   98689), FSP1 (1:200, Abcam, ab58597), PDGFRα (1:200, R&D Systems AF1062), Ki67

715   (1:200, Abcam, ab15580), TCF21 (1:250, Abcam, ab32981), Vimentin (1:200, Abcam,

716   ab8978). AlexaFluor488-, AlexaFluor568-, or AlexaFluor647-conjugated secondary antibodies

717   against suitable species were used for fluorescence labelling, for example: AF488-conjugated

718   goat anti-rabbit IgG (Life Technologies, A11008, 1:500), AF568-conjugated donkey anti-rabbit

719   IgG (Life Technologies, A10042, 1:500), AF647-conjugated donkey anti-rabbit IgG (Life

720   Technologies, A31573, 1:500). The images were taken with a Thunder Imaging Systems

721   (Leica) acquired by Leica Application Suite v4.8, or an AxioImager (Carl Zeiss) acquired by

722   Zen v3.0 blue edition.

723   **Lipid tox staining**

724   All procedures followed were in accordance with manufacturer's instructions. HCS Lipid TOX

725   Deep Red (Thermo Scientific, H34477) was used at 1:200 dilution on PFA fixed whole mount

726   explants and incubated at 37° C for 2 h. Whole mount imaging used a Multiphoton microscope.

727   **Extracellular matrix deposition assay**

728   In vitro culture of FACS-sorted cells were obtained from transgenic mouse lines (En1^{Cre};

729   R26^{mTmG} or Adipoq^{Cre};R26^{mTmG}) at the P0 or P1 stage. Explants from Cre+ mice were collected

730   on day 1 and day 4. Tissue was minced and digested using 0.5mg/ml Collagenase A and 25u/ml

731   of DNase 1 for 1hr at 37˚C with shaking. Cells were washed with complete media and filtered

732   through cell strainers. En1^{cre+} samples were incubated with the following antibodies for lineage-

733   negative gating: APC- anti-mCD31, mCD45, EpCam (CD326), PECAM-1, mTie2(CD202b),

734   mTer119, anti-mLyve-1 on ice for thirty minutes. The antibody-conjugated cell pellet was then

735   washed with, 5 ml FACS buffer and re-suspended in 1 ml of FACS buffer (PBS +2% FBS).

736   The cells were sorted by a FACS Aria III sorter with a 120 μm nozzle. EPFs were sorted based

737   on Lin⁻TomatoRed⁻GFP⁺ fluorescence. For Adipoq[Cre] positive samples, digestion procedures

738   were similar to those mentioned above but excluded lineage markers. En1+ fibroblasts and

739   Adipocytes were later plated in 384 well glass bottom plates coated with 1% Porcine Gelatin.

740   3000 cells were plated per well and cultured in complete medium with or without 5ng/ml

741   recombinant TGFβ1 (rTGFβ1). Adipocytes were cultured in complete medium with or without

742   5ng/ml rTGFβ1 and 5ng/ml bFGF (basal FGF). Both cultures were maintained in 37ºC

743   incubator for 72 hours and media was replenished once.

744   **Decellularization and extracellular matrix immunostaining**

745   Adipocytes and fibroblasts in 384-well plates were cultured up to 3 days. Confluent fibroblasts

746   or adipocytes were de-cellularized using an established protocol[53, 54]. Briefly, confluent culture

747   dishes were incubated at 4°C for 45 mins with very gentle stirring, sequentially, first in DDW

748   (double distilled water) with Triton X 100 (0.1%) at 4°C, then in freshly prepared 2% sodium

749   deoxycholate. This was followed by two 30-minute incubations at room temperature, first in

750   DDW containing 1 M NaCl and second in DDW containing pancreatic DNase (30 μg/ml, source

751   porcine), MgSO₄ (1.3 mM) and CaCl₂ (2 mM). Plates were later rinsed very gently with distilled

752   water, and 4% PFA was used to fix the deposited extracellular matrix for 15 mins at RT. After

753   washing twice gently with PBS, permeabilization was performed in PBS containing BSA (1%)

754   and Triton X (0.1%). Blocking was carried out for 1hr at RT in PBS containing 1% BSA and

755   10% serum of the species in which secondary antibody was raised. Next followed a primary

756   antibody incubation at 4˚C overnight, gentle rinsing with PBS and later secondary antibody was

757   applied for 1hr at RT. Finally, nuclei were stained with DAPI for 10mins at RT. Plates were

758   washed 3X times with 1X PBS and stored in PBS, and images were taken with a confocal

759   microscope (LSM 710, Zeiss). Percent fluorescence was measure after converting the images

760   to binary format in Image J and calculating the area of the fluorescence signal.

761   **Cell transplantation**

762   FACS sorted cells were first thoroughly and gently washed with PBS. Cells were later

763   resuspended in PBS at 5×10^5 cells per ml. An equal volume of ice-cold Matrigel was mixed

764   with the sell suspension (Phenol red free, Corning, 356231). Cell suspensions in Matrigel were

765   made to a final concentration of 2.5×10^5 cells per ml and stored in an ice bucket until

766   intradermal injections. Rag2$^{-/-}$ mice, at age 10–12 weeks, received adipocyte lineage and

767   fibroblast lineage cell - Matrigel transplants. 5 mm diameter excisional wounds (full-thickness,

768   2 wounds) were made on the dorsal back skin of mice, as described above. Two 50 µl injections

769   of adipocyte lineage and fibroblast lineage cell-Matrigel suspensions were made near the wound

770   site. Two similar intradermal injections of 50 µl PBS alone were made for control wounds.

771   Cell-Matrigel suspensions were left for 10 minutes, followed by placing silicone splints around

772   the wound. Gentle press was applied around the wound as mentioned above in the splinted

773   wound method. Whole skin including scar tissue was harvested 7 days post wounding. Lastly,

774   harvested tissue was fixed at 4 ° C, overnight using 2% PFA. After 3 PBS washes, the tissues

775   were prepared for histological analysis.

776   **Statistics and Reproducibility**

777   GraphPad Prism 8 was used for all statistical analyses except for sequencing data. Unless

778   otherwise indicated, mean ± SD values are reported in the graphs. The exact statistical analyses

779   used to quantify data, the exact values of n, and the exact p values are stated in the respective

780   figure legends. For simplicity, p values below 0.0001 were stated as equal to 0.0001. All

781   experiments were performed at least three times independently with similar results.

782   No statistical method was used to predetermine sample size. Required experimental sample

783   sizes were estimated based on previous established protocols in the field. The sample sizes were

784   adequate as the differences between experimental groups were reproducible. No data were

785 excluded from the analyses. The experiments were not randomized. The Investigators were not

786 blinded to allocation during experiments and outcome assessment.

787

788 **Data availability**

789 The generated scRNA-seq data has been deposited in the Gene Expression Omnibus under the

790 accession number GSE175650

791 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE175650]. The generated mRNA-

792 seq data has been deposited in the Gene Expression Omnibus under the accession number

793 GSE215912 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE122159]. The raw

794 sequence data has been deposited in the Sequence Read Archive (SRA) with the BioProject ID

795 PRJNA889718 [https://www.ncbi.nlm.nih.gov/bioproject/PRJNA889718/]. Source data for

796 Figure 2, 4, 5 has been provided in the Supplementary Table 3.

797

798 **Code availability**

799 scRNA-seq data analysis was performed with scanpy (v1.6.0) and diffxpy (v0.7.4). Gene set

800 enrichment analysis was performed with goatools (v0.18). Custom R and Python code to

801 reproduce the analyses and figures based on scRNA-seq data generated in this manuscript has

802 been deposited in GitHub [https://github.com/schillerlab/2021_Stromal_Fate_Restriction].

803
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Author contributions

Conceived and designed the analysis: Y.R., D.J., H.B.S.; Collect the data: S.K.G., R.D., A.M.S., P.R., J.W.B., Y.L., S.C., D.J.; Contributed data or analysis tools: D.C.-G., L.C.D., H.-G.M.; Performed the analysis: S.K.G., R.D., A.M.S., M.A., J.Z., P.R., D.C.-G., Y.L., I.A., V.L., C.M., W.E., H.-G.M. H.B.S., D.J., Y.R.; Wrote the paper: S.K.G., L.C.D., H.B.S., D.J., Y.R.

Competing interests

The authors declare no competing interests.

Figure Legends

Figure 1. Adipocyte and fibroblast lineages retain their identities in skin explant model.

a, Schematic workflow of *ex vivo* whole-skin explant assay and molecular crowding single cell

RNA barcoding and sequencing (mcSCRBseq). The skin explants from neonatal

Adipoq^{Cre};R26^{mTmG} or En1^{Cre};R26^{mTmG} skin were cultured in 96-well plate with fascia side face

up. The GFP$^+$ cells were FACS sorted from explants that were collected 1 day or 4 days after

culture for single cell sequencing. F, fascia; D, dermis; E, epidermis. **b**, Dimension-reduced

single cell transcriptomic data is visualized through Uniform Manifold Approximation and

Projection (UMAP), coloured by Louvain cluster and **c**, time point of extraction. **d**, Similarities

of marker gene signatures for the 12 cell states (6 states per lineage) along with relative

frequency of each cell state per time point. Colour indicates Pearson correlation coefficients for

each pairwise comparison across transcriptional cell states in adipocyte and fibroblast lineages.

e, The heatmap shows relative expression of the indicated genes across cell states and lineages.

f, Gene set enrichment results in an adipocyte core signature gene list (88 genes). **g**, Gene set

enrichment results in a fibroblast core signature gene list (198 genes). **h**, Diffusion maps show

adipocyte cell states and the gene expression levels of the indicated genes. **i**, Diffusion maps

show fibroblast cell states and gene expression levels of the indicated genes. **j**, Pathway focused

gene expression analysis of adipocytes and fibroblasts at day 1 and day 4. **k**, Expression of

feature genes of listed pathway in adipocytes and fibroblasts at day 1 and day 4. Z score of

individual gene was normalization read counts across samples.

Figure 2. Spatio-temporal characterization of migratory adipocytes.

a, Morphological changes of adiponectin-lineage positive cells (GFP) in skin explants from

Adipoq^{Cre};R26^{mTmG} neonates in culture from Day 0 to Day 5 at low (upper panel, scale bar 200

µm) and high (lower panel, scale bar 50 µm) magnification. **b**, Quantification of adipocyte

morphologies throughout 5-day explant assay, n = 3 explants per timepoint, mean ± SD. **c**,

973   Morphology dynamics of adipocytes. Snapshots of single representative cells from Day 0 to

974   Day 5 showing transition from characteristic mature round to migratory states of adipocytes.

975   Time format-hour.min. Scale bar: 20 microns. **d**, Three migratory morphologies of adipocytes

976   are positive for Perilipin1 by immuno-labelling. Scale bar: Classic round 20 µm, Oval spliky:10

977   µm and Elongated fibroblast-like morphologies: upper panel 20 µm, lower panel 30 µm. This

978   experiment was repeated three times independently with similar results. **e**, Quantification of

979   Perilipin1-positive and -negative cells in explants, n = 3 biological repeats, mean ± SD. **f**,

980   Adipocytes superficially resemble fibroblasts after wounding in live mice: Control back skin of

981   Adipoq^{Cre};R26^{mTmG} mice, GFP$^+$ adipocytes are round, located around hair follicles. Following

982   the wound healing experiment at day 7, Adipoq-lineage (GFP) cells seen at the wound periphery

983   have a fibroblast-like elongated morphology. At day 21 after injury, activated adipocytes still

984   have a fibroblastic shape as the skin tissue is undergoing remodelling. Arrowheads indicate

985   round adipocytes, and arrows indicate elongated, activated fibroblast-like cells. Scale bars: 50

986   µm. This experiment was repeated three times independently with similar results.

987

988   **Figure 3. Distinct adipocytes and fibroblast migrations.**

989   3D whole mount time-lapse imaging snapshots of single-cell tracks skin explants generated

990   from Adipoq^{cre} or En1^{Cre} crossed to R26$^{LSL-H2B-mCherry}$ reporter mice. **a**, Snapshots of adipocyte-

991   and fibroblast-migration tracks on day 1. **b**, Adipocyte and fibroblast tracks on day 4, generated

992   by automated cell tracking using Imaris version 9.2. (Bitplane). **c**, Manual tracks of adipocytes

993   and fibroblasts in the scar region of explants at day 1 and day 4; the plot shows the difference

994   in migration distance and type of movement in the scar region of both adipocytes and

995   fibroblasts. N=2 videos per time point. Scar regions were cropped (700µm X 700µm) from

996   whole explant and cells manually tracked. Blue indicates starting time and red is the end-point.

997   **d**, 3 main types of movement quantified using manually annotated single cell tracks present in

998   c, n = 3 biological repeats, mean ± SD. **e**, Velocity of migratory adipocytes and fibroblasts is

999   calculated using time-lapse videos and automated single cell tracks. Velocity variation and

1000   amplitude difference from time point 4 -9 hours across all samples are shown in higher

1001   magnification (lower panel). The red crosses (+) indicate the mean velocities of the indicated

1002   time points. **f**, Spline graph of day 4 showing differences of mean velocity between adipocytes

1003   and fibroblasts. **g**, Neighbour similarity analysis of day 4 explants using automated single-cell

1004   tracks generated from 3D time lapse videos. The colour bar represents the movement angles 0°

1005   (red, coordinated movement) to 90° (blue, random movement). Fibroblast migrations are

1006   coordinated and collective, whereas adipocyte migrations are random and individual. **h**,

1007   Directed and non-directed movement of fibroblasts and adipocytes respectively at day 4. Scale

1008   bars:100 µm.

1009

1010   **Figure 4. Adipocytes are non fibrogenic in *ex vivo* models.**

1011   **a**, Immunostainings of Adipoq^{Cre};R26^{mTmG} explants at day 0 and day 4. Adiponectin-lineage

1012   cells in green, fibroblasts in red, and marker gene αSMA (top) and Fibronectin 1 (bottom)

1013   expression in magenta. Merged channel image of the whole explant (left), magnified area of

1014   individual channels (right). Scale bars: 100 µm in low magnification images, 20 µm in high

1015   magnification images. This experiment was repeated three times independently with similar

1016   results. **b**, Feature plots generated from combined analysis of mcSCRBseq showing adipocyte,

1017   myofibroblast, and extracellular matrix-specific enrichment in cell type-specific clusters. **c-f**,

1018   Adipocyte-lineage cells deposit marginal matrix proteins than fibroblasts under scarring

1019   conditions. **c**, Schematic of *in vitro* matrix deposition assay and quantification using Image J.

1020   **d**, FACS-sorted adipocytes, and fibroblasts were cultured *in vitro*, with and without rTGFβ1

1021   stimulation for 72hrs, followed by decellularization and immunolabelling of deposited matrix

1022   Collagen 1 and Fibronectin 1. Scale bars: 50 µm. **e,f**, Quantification of percent fluorescence of

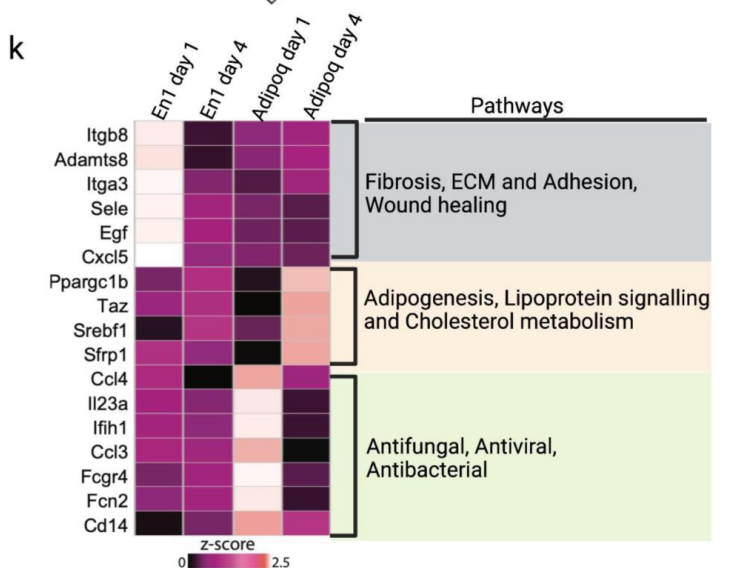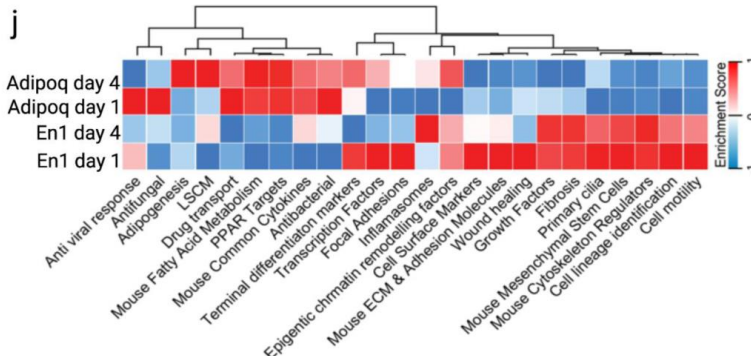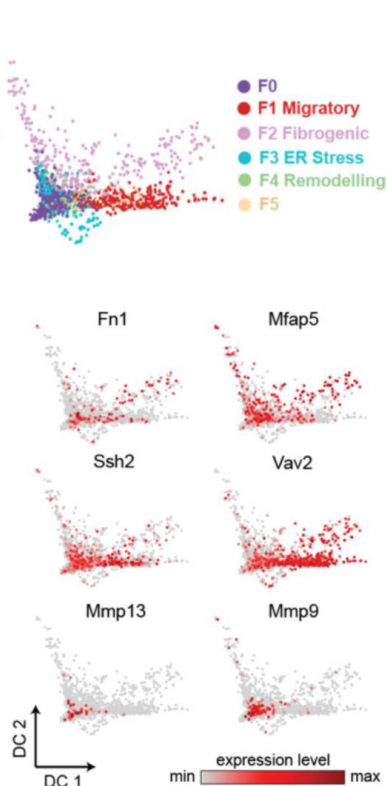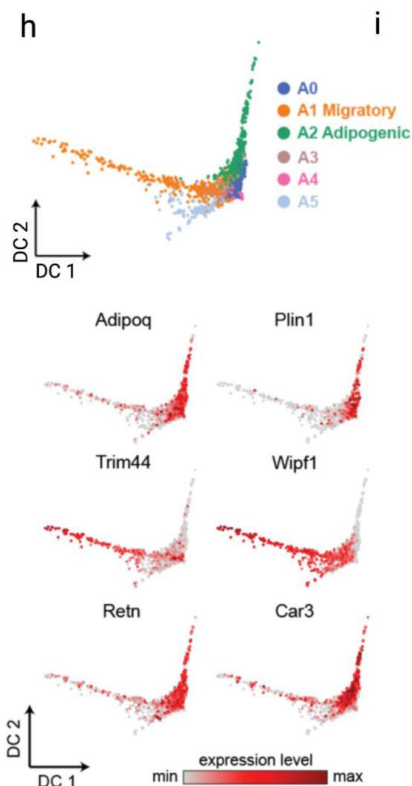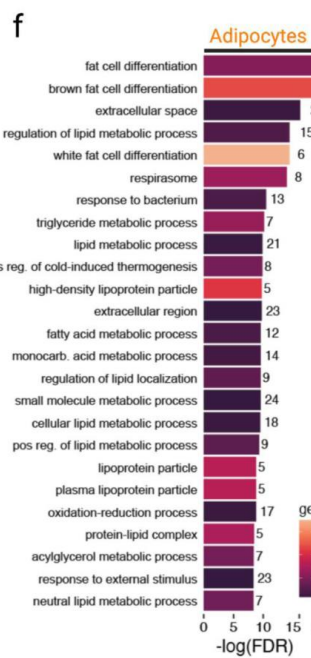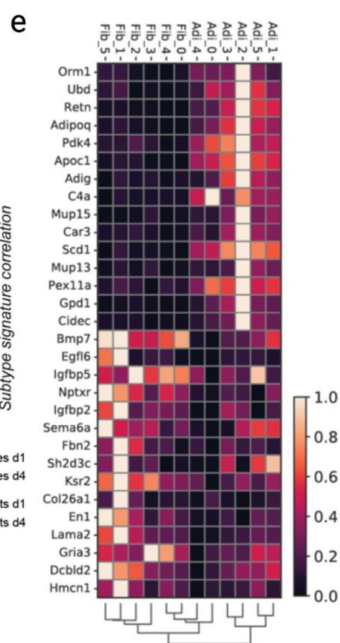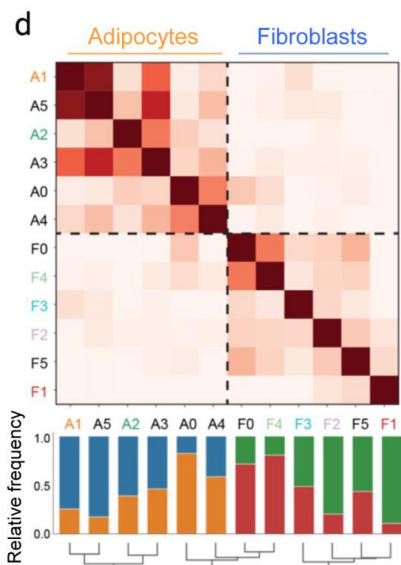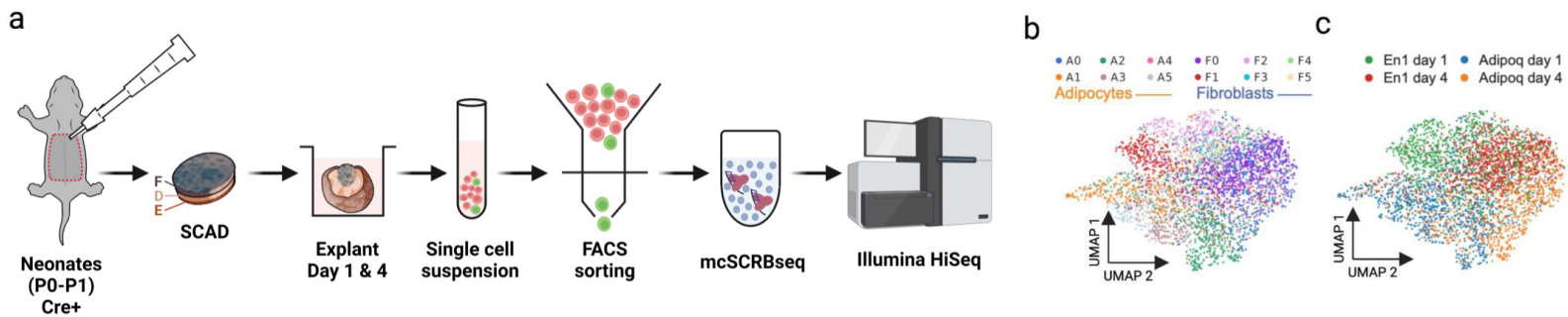1023   deposited matrix showing higher percentage of deposited matrix when compared to adipocytes,

1024   n = 3 biological replicates and 4 images of each replicate, Mean ± SD, Two-way ANOVA with

1025   95% CI.

1026

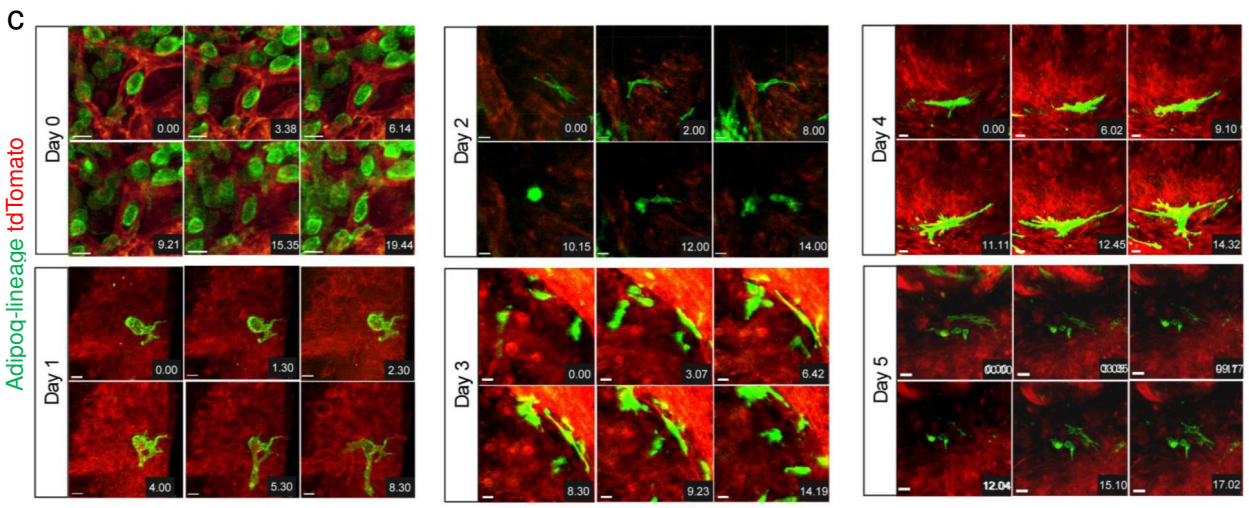1027   **Fig. 5. Adipocytes are non fibrogenic in wounds.**

1028   mRNA-seq was performed with FACS sorted adipocytes and fibroblasts from day 7 and 21

1029   wounds and adjacent skin of Adipoq^{Cre};R26^{mTmG} and En1^{Cre};R26^{mTmG} mice, respectively. Each

1030   cell type at each time point includes three independent biological replicates. **a**. Pearson

1031   correlation analysis of all 18 samples. Colour in each cells represented Pearson correlation

1032   coefficients for every pairwise comparison. **b**. GO term enrichment based on DEGs of

1033   adipocytes and fibroblasts from day 10 wounds. Filled colour represented number of genes

1034   enriched relative to all DEGs. Cryosections of day 7 and day 21 wounds from

1035   Adipoq^{Cre};R26^{mTmG} mice were subjected for immunofluorescence staining. **c**, Representative

1036   images and quantification of Perilipin (magenta) in GFP positive cells. Data are numbers of

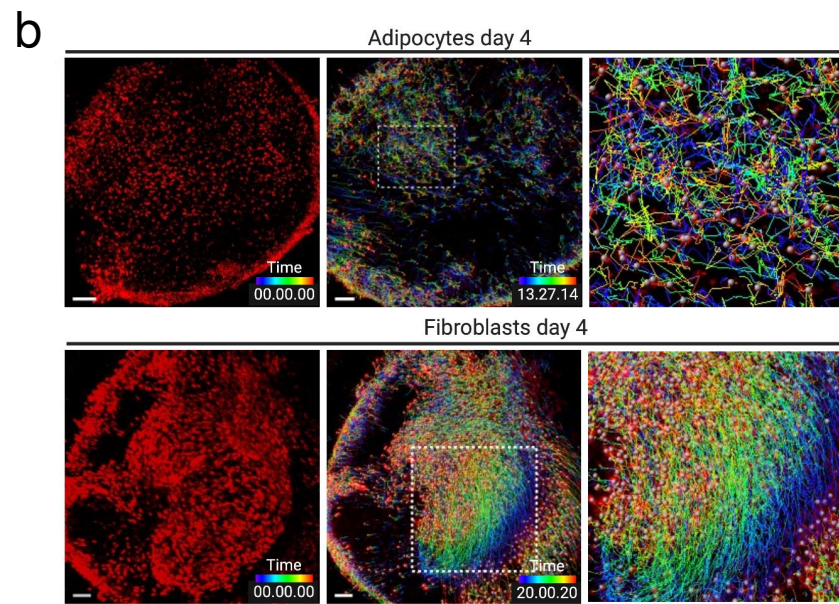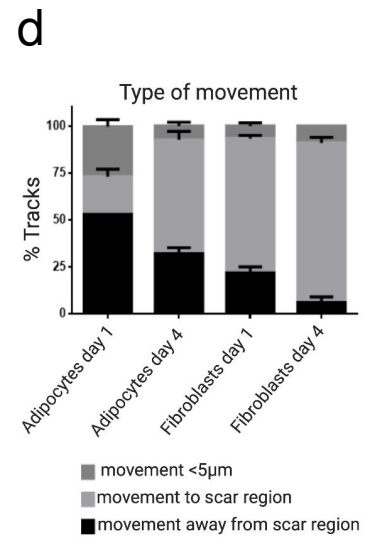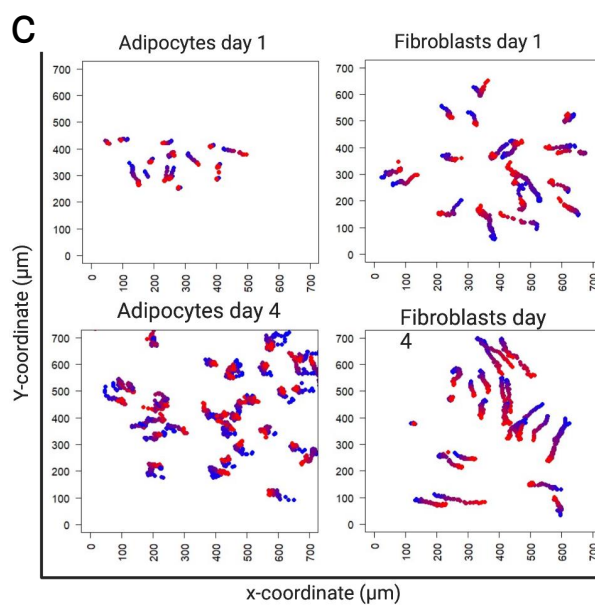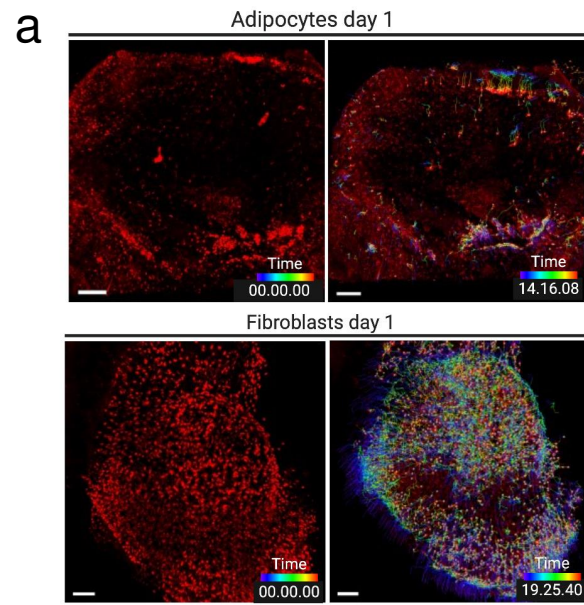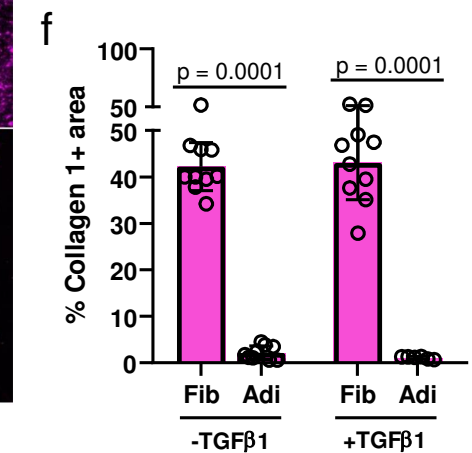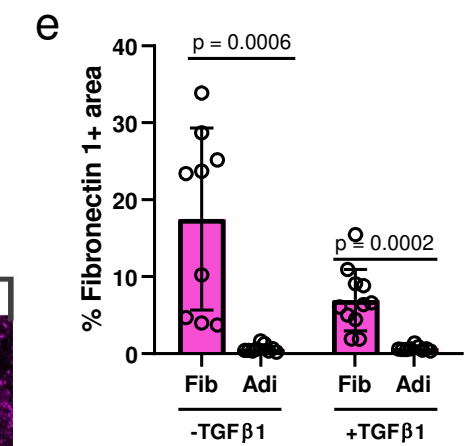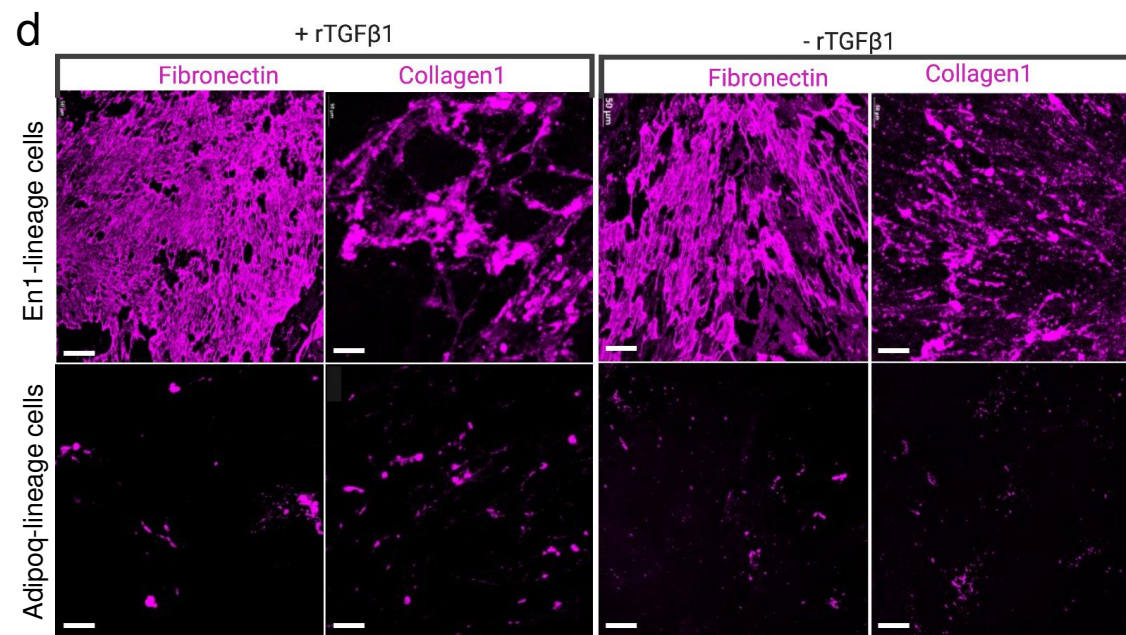1037   GFP^{+}Perilipin^{+} cells per high magnification field, n = 6 independent samples, mean ± SD,

1038   unpaired two-tailed t-test. **d**, Representative images and quantification of αSMA (magenta) in

1039   GFP positive cells. The migratory elongated and rounded adipocytes are negative for αSMA.

1040   At day 7 there is widespread αSMA staining in the centre of the wound, whereas only

1041   physiological αSMA is found in the hair follicle dermal sheath at day 21. Data are percentage

1042   of αSMA^{+}GFP^{+} cells and αSMA^{-}GFP^{+} cells in total GFP^{+} cells, n = 6 independent samples,

1043   mean ± SD. **e**, Representative images and quantification of vimentin (magenta) in GFP positive

1044   cells. Data are percentage of Vimentin^{+}GFP^{+} cells and Vimentin^{-}GFP^{+} cells in total GFP^{+} cells,

1045   n = 6 independent samples, mean ± SD. **f**, Transplantation of FACS-sorted adipocytes or

1046   fibroblasts from P1 new born mice into adult Rag2$^{-/-}$ immunodeficient mouse back skin into an

1047   excisional wound model. Immunolabelling with anti-Collagen1 or anti-Fibronectin 1 and

1048   quantification of associated extracellular matrix in the transplanted regions. **g**, Quantification

1049   of adipocyte- and fibroblast-associated ECM in the transplanted regions. n = 3 independent

1050   adipocyte samples, n = 6 independent fibroblast or control samples, mean ± SD, unpaired two-

1051   tailed t-test. **h**, Representative images and quantification of cathelicidin-related antimicrobial

1052   peptide (CRAMP) in GFP positive cells. Data are percentage of CRAMP$^+$GFP$^+$ cells in total

1053   GFP$^+$ cells, n = 6 independent samples, mean ± SD, unpaired two-tailed t-test. Arrow heads

1054   indicate the wound borders, the stars indicate the examples of double positive staining. Scale

1055   bars: c-f, h = 100 μm.

1056

a

Neonates (P0-P1) Cre+ → SCAD → Explant Day 1 & 4 → Single cell suspension → FACS sorting → mcSCRBseq → Illumina HiSeq

b

A0 · A1 · A2 · A3 · A4 · A5 | F0 · F1 · F2 · F3 · F4 · F5
Adipocytes — Fibroblasts

c

En1 day 1 · En1 day 4 · Adipoq day 1 · Adipoq day 4

d

Adipocytes | Fibroblasts

e

f Adipocytes

fat cell differentiation — 13
brown fat cell differentiation — 9
extracellular space — 26
regulation of lipid metabolic process — 15
white fat cell differentiation — 6
respirasome — 8
response to bacterium — 13
triglyceride metabolic process — 7
lipid metabolic process — 21
pos reg. of cold-induced thermogenesis — 8
high-density lipoprotein particle — 5
extracellular region — 23
fatty acid metabolic process — 12
monocarb. acid metabolic process — 14
regulation of lipid localization — 9
small molecule metabolic process — 24
cellular lipid metabolic process — 18
pos reg of lipid metabolic process — 9
lipoprotein particle — 5
plasma lipoprotein particle — 5
oxidation-reduction process — 17
protein-lipid complex — 5
acylglycerol metabolic process — 7
response to external stimulus — 23
neutral lipid metabolic process — 7

-log(FDR)

g Fibroblasts

extracellular matrix — 22
microfibril — 5
extracellular region — 37
collagen-containing extracellular matrix — 17
reg. of multicellular organismal process — 64
extracellular matrix structural constituent — 11
extracellular space — 29
regulation of developmental process — 54
protein binding — 123
regulation of cell migration — 26
regulation of biological process — 134
regulation of cell motility — 26
reg. of multicellular organismal development — 44
regulation of locomotion — 26
cellular component organization or biogenesis — 75
cellular component organization — 74
plasma membrane — 63
cell adhesion — 21
regulation of cellular component organization — 47
cell morphogenesis involved in differentiation — 9
biological adhesion — 21
regulation of cellular component movement — 26
pos. reg. of multicellular organismal process — 44
biological regulation — 136
regulation of cellular process — 126

-log(FDR)

h

A0 · A1 Migratory · A2 Adipogenic · A3 · A4 · A5

Adipoq · Plin1 · Trim44 · Wipf1 · Retn · Car3

expression level min — max

i

F0 · F1 Migratory · F2 Fibrogenic · F3 ER Stress · F4 Remodelling · F5

Fn1 · Mfap5 · Ssh2 · Vav2 · Mmp13 · Mmp9

expression level min — max

j

Pathways

k

Pathways: Fibrosis, ECM and Adhesion, Wound healing | Adipogenesis, Lipoprotein signalling and Cholesterol metabolism | Antifungal, Antiviral, Antibacterial

a

Day 0   Day 1   Day 2   Day 3   Day 4   Day 5

Adipoq-lineage tdTomato

b

Classic-Round   Oval-Spiky
Elongated-Fibroblast like

%Adipocytes

D0   D1   D2   D3   D4   D5

Explant Assay Days

c

Adipoq-lineage tdTomato

Day 0   Day 2   Day 4

0.00 3.38 6.14   0.00 2.00 8.00   0.00 6.02 9.10
9.21 15.35 19.44   10.15 12.00 14.00   11.11 12.45 14.32

Day 1   Day 3   Day 5

0.00 1.30 2.30   0.00 3.07 6.42   00.00 03.05 09.17
4.00 5.30 8.30   8.30 9.23 14.19   12.04 15.10 17.02

e

Perilipin1⁻
Perilipin1⁺

Adipocyte Number

Classic round   Oval spiky   Elongated Fibroblast-like

d

Adipoq-lineage   Perilipin   Merge

Classic Round and Oval

Oval Spiky

Elongated Fibroblast like

f

Control   Day 7   Day 21

wound   wound

a

Adipocytes day 1

Time 00.00.00 — Time 14.16.08

Fibroblasts day 1

Time 00.00.00 — Time 19.25.40

b

Adipocytes day 4

Time 00.00.00 — Time 13.27.14

Fibroblasts day 4

Time 00.00.00 — Time 20.00.20

c

Adipocytes day 1 — Fibroblasts day 1

Adipocytes day 4 — Fibroblasts day 4

Y-coordinate (µm)

x-coordinate (µm)

d

Type of movement

% Tracks

Adipocytes day 1 — Adipocytes day 4 — Fibroblasts day 1 — Fibroblasts day 4

movement <5µm
movement to scar region
movement away from scar region

e

Velocity (µm/min)

Adipocytes day 1 — Fibroblasts day 1

Adipocytes day 4 — Fibroblasts day 4

Time (h)

g

Adipocytes day 4 — Fibroblasts day 4

Y [µm]

X [µm]

Mean similarity [°]

h

Adipocytes day 4 — Fibroblasts day 4

f

Velocity (µm/min)

Adipocytes day 4
Fibroblasts day 4

Time (h)

a

Explant day 0 | Explant day 4

Adipoq-lineage tdTomato αSMA

Merged | tdTomato
Adiponectin-GFP | αSMA

Adipoq-lineage tdTomato Fibronectin

Merged | tdTomato
Adiponectin-GFP | Fibronectin

b

Adipocyte Marker: Cfd, Cidec

Myofibroblast Marker: Lrrc17, Ddr2

ECM Marker: Col3a1, Col1a1

c

Adipoq or En1 cre+ → Digestion Single cell prep → GFP+ sorting → +/- rTGFb1 +/- Decel → Image quantification

d

+ rTGFβ1 | - rTGFβ1

En1-lineage cells: Fibronectin | Collagen1 | Fibronectin | Collagen1

Adipoq-lineage cells: Fibronectin | Collagen1 | Fibronectin | Collagen1

e

% Fibronectin 1+ area

p = 0.0006
p = 0.0002

Fib Adi | Fib Adi
-TGFβ1 | +TGFβ1

f

% Collagen 1+ area

p = 0.0001 | p = 0.0001

Fib Adi | Fib Adi
-TGFβ1 | +TGFβ1

a — Correlation heatmap of Adipocytes (AD) and Fibroblasts (FB) samples. Corr scale 0.2–1.0.

b — Gene Ontology enrichment bar charts.

Fibroblasts:
- extracellular structure organization — 33
- extracellular matrix organization — 33
- external encapsulating structure organization — 33
- collagen metabolic process — 14
- cell–substrate adhesion — 26
- collagen fibril organization — 10
- extracellular matrix assembly — 9
- ossification — 26
- regulation of cell–substrate adhesion — 19
- regulation of cellular response to growth factor stimulus — 22
- protein maturation — 20
- retrograde vesicle–mediated transport, Golgi to endoplasmic reticulum — 8
- positive regulation of cell–substrate adhesion — 13
- membrane lipid catabolic process — 6
- bone mineralization — 11
- endoplasmic reticulum to Golgi vesicle–mediated transport — 12
- cell–matrix adhesion — 16
- regulation of BMP signaling pathway — 10
- positive regulation of canonical Wnt signaling pathway — 11
- collagen biosynthetic process — 7

Adipocytes:
- G protein–coupled receptor signaling pathway — 49
- sensory perception of chemical stimulus — 22
- sensory perception of smell — 20
- sensory perception — 35
- regulated exocytosis — 18
- chemical synaptic transmission — 32
- exocytosis — 20
- myeloid leukocyte activation — 10
- system process — 62
- regulation of trans–synaptic signaling — 23
- microtubule–based movement — 12
- regulation of interferon–gamma production — 7
- leukocyte mediated cytotoxicity — 7
- positive regulation of immune effector process — 11
- myeloid leukocyte mediated immunity — 6
- regulation of leukocyte mediated cytotoxicity — 6
- regulation of ion transport — 20
- positive regulation of cytosolic calcium ion concentration — 8
- response to molecule of bacterial origin — 8
- negative regulation of leukocyte activation — 10
- leukocyte activation — 30

Gene ratio 0.02–0.08. −log(FDR) axis.

c — Adipoq-lineage (GFP) Plin1, Day 7 and Day 21. No. of GFP+Plin1+ cells, Day7 vs Day21, p=0.0001.

d — Adipoq-lineage (GFP) αSMA, Day 7 and Day 21. % in total GFP+ cells; GFP+αSMA− and GFP+αSMA+.

e — Adipoq-lineage (GFP) Vimentin, Day 7 and Day 21. % in total GFP+ cells; GFP+Vimentin− and GFP+Vimentin+.

f — Collagen1/Fibronectin. ECM Control area, ECM EPF+ area, ECM Adipocytes+ area.

g — Marker area (%); Fibroblasts, Adipocytes, Control. p=0.0142, p=0.0033.

h — Adipoq-lineage (GFP) CRAMP, Day 7 and Day 21. % of CRAMP+GFP+ in total GFP+, Day7 vs Day21, p=0.0001.

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