Two succeeding fibroblastic lineages drive dermal development and the transition from regeneration to scarring

Dongsheng Jiang[1,ξ], Donovan Correa-Gallegos[1,ξ], Simon Christ[1,ξ], Anna Stefanska[1], Juan Liu[1], Pushkar Ramesh[1], Vijayanand Rajendran[1], Darcy Wagner[1], Yuval Rinkevich[1,2*]

[1] Institute of Lung Biology and Disease, Helmholtz Zentrum München, Munich, Germany

[2] Member of the German Center for Lung Research (DZL), Munich, Germany

[ξ] These authors have contributed equally

* To whom correspondence should be addressed:
Yuval Rinkevich
Phone: +49 (89) 3187 4685
Fax: +49 (89) 3187 4661
yuval.rinkevich@helmholtz-muenchen.de

Abstract

Dermal tissues are an organized lattice of fibroblasts and extracellular matrix protein fibers. Upon skin injury this lattice is replaced by scars, which diminish the skin's tensile strength, growth and functions. Early fetuses respond to injuries by simply regenerating, and the scarring response only arrives later in embryonic development. Understanding this irreversible transition is key to new clinical avenues for regenerative medicine and for developing anti-scar therapies.

Here we characterize dermal morphogenesis by following two distinct embryonic fibroblast lineages, which either do or do not have a history of expression of the Engrailed1 gene. We use single cell fate mapping, live confocal 3D imaging and *in silico* analysis coupled with immuno-labeling to reveal unanticipated structural and regional complexity and dynamics within the dermis. We show that dermal development is driven by Engrailed1-history-naive fibroblasts. Dermal lattice regenerates during fetal life exclusively from these cells, whose cell numbers subsequently decline. We show that the lineage of fibroblasts with a history of Engrailed1 expression has scarring abilities at this early stage and their expansion later on in embryogenesis drives scar emergence.

We demonstrate that the transition can be reversed, locally, by transplanting Engrailed1-naive cells. Fibroblastic lineage replacement thus couples the decline of regeneration with the emergence of scarring, and creates potential new clinical avenues to reduce scar damage.

Introduction

Skin develops in the fetus when dermal fibroblasts establish a porous, ‘basket-weave’ scaffold. This porous scaffold is accessible to blood and lymph infiltration, as well as peripheral nerve innervations. The fibroblastic network has the tensile strength and extensibility needed for the skin’s structural integrity, and for its protective and sensing functions. Injuries to the skin commonly lead to loss of these frameworks and replacement by scar tissue which has greatly limited functions. Scar tissues have lower tensile strength, extensibility and growth and excessive scarring can even lead to end-stage organ failure.

‘Skin scarring’ groups a range of pathologies that occur in response to dermatologic injuries, syndromes and diseases. Clinically speaking, skin scars cover a wide spectrum of phenotypes from thin-line scars to abnormal widespread, atrophic, hypertrophic, and keloid scars and scar contractures. For example, atrophic scars commonly arise after acne or chickenpox, while stretch marks (abdominal striae) that develop after pregnancy or weight gain are both versions of dermal scars in which the epidermis is unbreached. Overall then, scars are an enormous biomedical, clinical and cosmetic problem.

Each year in the developed world 100 million patients are diagnosed with scars that result from surgical procedures alone. There are an estimated 11 million keloid scars and four million burn scars, 70% of which occur in children. These figures include only clinical cases, so the overall global incidence of scarring is doubtless much higher. People with abnormal skin scarring may face physical, aesthetic, psychological, and social consequences that may be associated with substantial emotional and financial costs.

Scar tissue is rarely observed in lower vertebrates where the normal response to injury is a complete regeneration of the original dermal structure. Scarring is however frequent in mammals which have evolved to heal with scar tissue[1-2]. Mammals undergo a regeneration-to-scar phenotypic transition during fetal life. This transition has been documented in the back-skin of all mammalian embryos studied to date, including mice, rats, marsupials, rabbits, pigs, non-human primates, and in human fetuses that had undergone corrective spinal surgery for *Spina bifida*[3-7]. In the first two trimesters of fetal life (gestational stage E16.5 in mice), injuries regenerate without scarring, as the wounded dermis regenerates the 'basket-weave' architecture of intact dermis. From the third trimester (gestational stage E18.5 in mice) on and throughout adulthood, humans and most mammals patch wounds with scars, which are tightly packed parallel collagen bundles and very unlike the original reticular structure.

We know surprisingly little about how dermal structure develops, how it becomes regionally specified and less about why this structure is replaced by scars in adults at the sites of skin injuries. Current treatment options to eradicate or prevent scarring in humans and domestic animals are unreliable and there are no prescription drugs for the prevention or treatment of dermal scarring. The fetal transition from automatic regeneration to the ability to form scars provides a unique assay to understand loss of regeneration in mammals, and explore potential anti-scar therapies.

Previous studies into the fetal commencement of scarring ability have focused on a multitude of environmental differences between early and late fetal dermal stages, including inflammatory responses to injury, expressions of morphogenetic proteins, growth factors and extracellular matrix components such as hyaluronic acid[8]. In the early

90s, Longaker and colleagues addressed the influence of the environment on the commencement of scarring by performing fetal/adult heterochronic transplantations of back-skin tissues in sheep. They found that donor back-skin tissues respond to injury (scar/regenerate) independent of host microenvironment or developmental stage. These authors thus concluded that the determining factor/s of the commencement of scarring are likely intrinsic to the transplanted back-skin graft, most likely its fibroblasts, which are the tissue's primary secretors of extracellular matrix[9-10].

More recently, we have determined that fibroblasts include functionally diverse cell types[11]. We used genetic lineage tracing to show distinct but coexisting embryonic lineages in the mouse back-skin and oral cavity. We discovered that embryonic cells that have expressed Engrailed-1 (En1), termed EPFs (for En1-lineage-Past fibroblasts), have many of the phenotypic attributes of pathologic scars. EPFs are the primary contributors to scarring in various injury models, including postnatal cutaneous wounding, irradiation-induced fibrosis, as well as to a melanoma tumor stroma, which is a model that develops scar tissue.

EPFs are intermixed with En1-lineage-Naive fibroblasts (ENFs) that have never expressed Engrailed. ENFs do not participate in scar tissue formation. By transplanting adult ENFs & EPFs, separately, in different anatomical locations, we determined that the difference in the capacity of EPFs & ENFs to form a scar is cell-intrinsic, and permanent, and that these are *in vivo* behaviors of two distinct fibroblastic cell types[11].

Here, we followed the fates of EPF & ENF progenitors. We used genetic fate mapping approaches at single cell and lineage levels, live confocal 3D imaging of lineage-specific cellular migrations and immuno-labeling coupled with *in silico* approaches to determine

how the dermal structure in the back-skin develops. We find that ENFs form the sculptures of the dermal lattice and that their cell lineage declines during development, concurrent with a surge in EPF numbers that predisposes back-skin to scarring.

ENFs are replaced by EPFs during back skin development

The engrailed gene is expressed in a small subset of early embryonic cells and switched off permanently later in embryogenesis[11]. To distinguish engrailed-expressing cells and follow their fibroblastic descendants in the developing back-skin, we used a transgenic mouse system where Engrailed1 gene expression drives genetic rearrangements (*En1*^{Cre}). Crossing *En1*^{Cre} with a reporter mouse system (*R26*^{mTmG}), generated offspring where the genetic rearrangement replaced membrane bound tomato red protein (RFP) expression with membrane bound green fluorescence protein (GFP) expression[12] (Fig. 1a). The replacement of RFP with GFP is permanent, and includes all sibling cells. This system allowed purification schemes of fibroblastic lineages based of GFP^{+}RFP^{-}Lin^{-} and GFP^{-} RFP^{+}Lin^{-} fluorescence combinations, respectively (see Methods).

We thus performed flow cytometry analysis of EPFs (Engrailed-Past) Fibroblasts and Engrailed-history-Naive Fibroblasts (ENFs) within the total fibroblast population (Lin^{-}, see Methods) in early fetal back-skin dermis. We found dermal fibroblasts are mostly engrailed Naive early on. Thereafter, ENFs initially decline slowly during subsequent development, followed by a steep decline in ENF percentage between E14.5-E18.5 (~90% down to ~20%, Fig. 1b-c). The decline in ENFs was proportional to total dermal cells (including hematopoietic, endothelial, lymphatic) and to dermal fibroblasts alone (Lin-), indicating a clonal disadvantage to ENFs as compared to all other dermal

progenitors. Conversely, EPFs increase from ~2% of dermal cells at E14.5 (where EPF/ENF ratio stood at 1 to 33) to ~72% at P0 (where EPF/ENF ratio stood at ~4 to 1, Fig. 1b-c) and in proportion to all other dermal progenitors (Fig. 1c). We also looked at markers that have been reported in literatures to identify subpopulations of dermal mesenchymal cells (Extened Data Fig. 1). About 3% Lin^- cells were fibro-adipogenic progenitors (FAPs[13-15], Lin^-integrin $\alpha7^-Sca1^+PDGFR\alpha^+$), which were enriched but not only in EPF lineage (Extended Data Fig. 1c, f, g); while Lin^-CD146^+ pericytes[16] comprised 5% of Lin- cells were enriched but not only in ENF lineage (Extended Data Fig. 1d, f, g). As expected, the surface makers such as $Lin^-CD29^+CD105^+$ were not able to discriminate mesenchymal stem cells[17] from fibroblasts, and they were abundant in both ENF and EPF lineages (Extended Data Fig. 1e-g). Complementing the flow cytometryic data, immunostainings on $En1^{Cre};R26^{VT2/GK3}$ neonates sections for adipocytes (FABP4) or pre-adipocytes[18] (Dlk1 and, Sca1) showed a more prominent but not restricted expression on EPFs (Extended Data Fig 2a-c). Similarly, the smooth muscle marker (αSMA) expression was enriched in EPFs compared to ENFs (Extended Data Fig 2d) while the endothelial marker (CD31) is expressed mostly in ENFs (Extended Data Fig 2e). Staining for reticular and papillary dermis markers (Dlk1, TNC, CD26) showed that EPFs and ENFs don't allocate preferentially on either sub-location, and furthermore, the expression of these markers is not restricted in either of these lineages (Extended Data Fig 2b, f, g). We thus consider that the classical discrimination of papillary and reticular dermis does not account for the functional specification of the embryonic lineages.

To determine if the decline in ENFs numbers is due to programed cell death or a decrease in proliferation, we performed immuno-labeling of cleaved Caspase 3 (Cas3) and the

proliferation marker Ki67 in histological sections of E16.5 and E18.5 *En1*^{Cre}; *R26*^{mTmG} embryos. ENFs underwent more apoptosis than EPFs at E18.5 (Fig 1e-f). Conversely, the number of proliferating EPFs significantly increased at E18.5 while ENF proliferation remained unchanged (Fig 1d, f). Together, our results show that the decay in ENF population is primarily due to a clonal disadvantage.

To study the clonal dynamics of ENF-to-EPF replacements *in situ*, and at single cell levels, we used a transgenic 'Rainbow' reporter system[19]. Using this new strategy we genetically marked individual EPF progenitors from the moment of Engrailed1 expression (*En1*^{Cre};*R26*$^{VT2/GK3}$), with one out of three alternate fluorescent colors (YFP, RFP, CFP), while in this strategy ENFs express GFP only. EPF progenitors were absent in dermis at E9.5 and earlier and first appeared at E10.5 (Fig 1g-j). We cut coronal sections at E10.5 and observed single EPFs and mono-clones develop in close association with ectoderm and then extend from the most anterior regions (neck level) down to half of the trunk (Fig 1h-i). EPFs remained absent in most posterior regions (including hindlimbs, data not shown) at that stage of development. At E11.5 the EPFs form two parallel mid-lateral lines that cover the entire back (Fig 1k). To image the EPF arrangements throughout the back-skin, we used solvent-based clearing methods to generate transparent E12.5 embryos where EPF's fluorescence could be visualized within the entire embryo, and in 3D (see Methods). The EPFs arrange themselves in an arc across the back-skin, with migration 'protrusions' at anterior sites (Fig 1l and Video 1).

We next performed immuno-labeling of extracellular matrix (ECM) protein fibers on histological sections of *En1*^{Cre}; *R26*$^{VT2/GK3}$ early developing dermis. We expected ECM deposition to associate with ENFs and EPFs, equally. Surprisingly, we found that ENFs

deposit Fibronectin protein fibers, but not Collagen I & Collagen III (Fig 1m). This indicates that ENFs just have a provisional matrix. At anterior sites, Collagen I & Collagen III fibers were visible within the dermal matrix in association with EPF clones. This indicates EPFs form a more 'mature' dermis (Fig 1n-o). Indeed, at later stages, Collagen I expression was also strongly around EPFs (Extended Data Fig 1). To view the dynamics of ENF-to-EPF replacement, we performed live 3D confocal imaging, of the developing back-skin of *En1*^{Cre}; *R26*^{mTmG} embryos (E12.5), at the anterior margin of the EPF arc (Extended Data Fig 2). We found that the ENF cytological structure actively displaced to create open gaps into which EPFs migrated (Fig 1p, Extended Data Fig 2, and Video 2). The EPFs colonized the provisional dermis, through both dorsal and lateral trajectories (Fig 1p arrows). This local dynamic displacement of ENF-to-EPF cytological structures was completed within 24 hours.

We then analyzed the migration behaviors of single cells in three dimensions by automatically tracking the migration paths of EPFs at 15min intervals. Using computational software, we obtained 144 high-quality tracks. Fibroblasts were tracked across an area of ~170 microns^2 and ~100 microns deep (Fig 2a). From a coronal plane of view, we observed characteristic reticular patterns wherein the EPFs follow seemingly collective behaviors (Fig 2b). The EPFs migrated in vertical columns along the dorsal-ventral axis (Fig 2c-d). Anterior tracks had more dispersed points along the dorsal-ventral axis than posterior tracks, which indicate that anterior EPFs migrated faster than posterior EPFs (Fig 2e-f).

We expected fibroblasts to migrate uniformly. Surprisingly, the EPFs migrated in three distinctive ways: (1) "converging" migration to a fixed space (Fig 2b), (2) "localized"

migration where EPFs move in a limited space without directly contacting each other (Fig 2g), or (3) "diverging" migration of several EPFs from a space unit (Fig 2h). These three migration types occurred in clusters of 3-6 fibroblasts that shared common behaviors. Sequences of several units with varied migration behaviors could be detected even along a relative short distance of 170 microns. This indicates that EPF movements in situ are directed locally, by micro-environmental cues. These observations are in contrast to observations from fibroblast migration assays in two-dimension, and demonstrates that EPFs exhibit intricate migration repertoires *in vivo*.

Dermal lattice development follows Engrailed1 fibroblast replacement

Having documented the change in cellular composition from Engrailed-Naive fibroblasts to cells with a history of Engrailed1 expression during dermal development, we went on to describe dermal lattice development. Since dermal structure is too complex to be analyzed with simple Euclidean geometry parameters such as length, area, and angles, we turned to fractal analysis to measure the complexity of arrangements of cells and of matrix fibers. Fractal dimensions (FD) and lacunarity (L) are two values derived from the fractal analysis that have been used to assess and quantify morphologically complex objects like vessels and tumors[20-22]. Complex cellular arrangements, like blood vessels, score higher FD values, while simpler arrangements (e.g. geometrical shapes) score lower FD values. On the other hand, L values reflect the "gappiness" or empty spaces between shapes (porosity). Porous structures (eg. sponges) score higher L values than smooth surfaces (eg. scales). We used these two complementary values, together, to describe the general organization of dermal structures from two-dimensional images. For instance,

high FD and L values imply complex, porous shapes (e.g. arborized patterns like blood vessels, plant roots, and tree branches), while simple, smooth patterns (e.g. floor tiles) have lower FD and L values (Extended Data Fig. 3).

We first used the fractal analysis to resolve the cellular organization during dermal morphogenesis using histologic sections of *En1*^{Cre}; *R26*$^{VT2/GK3}$ embryos. Early (E11.5-12.5), Engrailed1-Naive regions were smoother (lower L) and had more complex shapes (higher FD), and at later stages (E14.5-E16.5) they became simpler and more porous (low FD and high L (Extended Data Fig. 4). This indicates that there is a drift from a compact arrangement (characteristic of a mesenchymal tissue) to a more interspaced cellular organization (characteristic of a mature dermis). We could not detect differences between anterior and posterior regions at any stages analyzed apart from E12.5, where anterior engrailed-naive cell arrangements were more complex and smoother (high FD and low L) than posterior cells (Extended Data Fig. 4b-c). This indicates that, at E12.5, anterior and posterior regions have different cellular organizations.

We next analyzed EPF cyto-architecture and cell morphology. EPF arrangements were more complex (high FD) at early stages (E10.5-11.5) and then simpler (low FD) at E12.5 followed by a progressive increase at later stages (Fig. 3a). Simultaneously, EPF arrangements increase constantly in porosity (L) throughout development (Fig. 3b). This suggests that EPFs undergo two major cellular rearrangements during development: 1) From E10.5-12.5 EPFs cluster tightly in complex patterns but with individual cells having smooth shapes, and after E12.5 these clusters split into individual cells (simpler shapes), but with each cell showing more complex arrangements. Interestingly, at E12.5 only anterior EPFs decreased in FD value, while posterior EPFs retained similar

complexity to their earlier stage. To view these cell morphology differences, we generated high magnification images, and generated 3D rendered pictures of single EPFs from E12.5 embryos. Posterior EPFs were simpler in shape then anterior EPFs, which had more complex morphologies (Fig. 3c). Remarkably, the sudden increase in complexity (FD) of anterior EPFs in E12.5 embryos occurs in the same time and place as the migration of EPFs (Fig. 1l, p, Fig. 2, Extended Data Fig. 2, and Video 1-2). This suggests that changes in EPF morphology cause or are caused by migrations. Together, our fractal analysis was able not only to identify different spatiotemporal patterns in two embryonic populations but also to predict complex cellular behaviors reflected on the morphology patterns, such as migration.

To determine if dermal lattice organization is changed following EPF's expansion, we analyzed ~~Fibronectin~~ matrix fiber alignment at different stages in dermal regions that do or do not contain EPFs. Since Collagen can be used only from a later stage of development and precludes the earlier stages of development where EPFs initially seed the back-skin, we primarily used Fibronectin to study the patterns of dermal lattice development. The distance between fibers increased progressively from E11.5 to E16.5 (Fig. 3d and Extended Data Fig. 5), concurrent with EPF development. At the same time there was a progressive decrease in complexity (FD) and an increase in porosity (L, Fig. 3e-f). This demonstrates that a gradual transformation of the dermal Fibronectin matrix occurs, from a relaxed and disorganized framework towards a more stretched conformation (Fig. 3g). Our fractal analysis from EPF cellular arrangements stresses that E12.5 is a pivotal time-point when anterior regions are actively changing while posterior regions are still dormant. The co-occurrence of Fibronectin framework transformation

and EPF morphological changes links extra-cellular matrix arrangements with distinct cell migration behaviors during development. To directly prove this link, we used an inducible-Cre labeling system (see Methods) to follow single fibroblastic cells (either ENFs or EPFs) and their sibling cells during back-skin development, and compared behaviors between anterior and posterior sites.

Fibroblastic clone size increased and clone expansion extended across the dorsal-ventral axis in the most anterior dermal locations. At the same time, singly labeled cells and small clones remained close to the ectoderm in posterior regions (Fig. 3h-i). These experiments show that the dynamics of structure changes across the anterior-posterior dermal axis influence both the cell division rate of fibroblastic precursors and the migration trajectories of their clonal colonies.

Collectively, these results document general cellular and ECM matrix arrangements changes in dermal morphogenesis across developmental time. Next we aimed to detect in more detail discrete cellular arrangements at later stages, when fibroblast clones are too widespread and intermixed. We generated a tissue-level description of dermal cellular organization by calculating local FD and L values using high (63x) and low (10x) power images from E16.5 $En1^{Cre}$; $R26^{VT2/GK3}$ embryos. Our low-power images included epidermis, dermis, hypodermis, two distinctive muscle layers, rib cartilage and three internal RFP-expressing layers (Extended Data Fig. 6a). As expected, more compact tissues such as epidermis, hypodermis, muscle and cartilage had higher FD values (more complex arrangements) than the dermis (Extended Data Fig. 6b). Interestingly, the upper muscle layer was more complex (high FD) and smoother (low L) than the muscle layer

beneath (Extended Data Fig. 6b), suggesting these are morphologically and perhaps functionally distinct muscle compartments.

By observing cellular arrangements on high power images we could identify five distinct dermal layers (DL-1-5) organized in parallel sheets across the back-skin (Fig. 3j left panel). The first dermis layer (DL-1), which is in direct contact with the epidermis, was composed of mostly EPFs (~85%) and cells were more densely packed compared to the other layers, except DL-5. The layer below (DL-2) was also composed of mostly EPFs (~75%) and was much thicker but had a lower cell density than DL-1. In contrast, the third dermal layer (DL-3) was composed mainly of elongated ENFs (~75%). Similar to DL-2, the fourth layer (DL-4) was thicker, was less dense than DL-1 and was primarily composed of EPFs (~90%). The fifth (DL-5) layer was similar to DL-1, composed mostly of densely packed EPFs (~75%) that were in direct contact with the underlying hypodermis (Fig. 3j-l).

DL-2 and -4 have simpler (low FD) and more porous (high L) cellular organizations than the other layers. Conversely, DL-1 and -5 were more complex (high FD) and smoother (low L). DL-3 was both complex and porous (high FD and L, Fig. 3m-n). The cellular organization of the dermis at E16.5 therefore has a mirrored pattern. The outer flanking dermal layers that are in direct contact with the epidermis and hypodermis have similarly compact fibroblastic cell arrangements, while the intermediate dermal layers are more porous and simply organized. The symmetrical ‘sandwich’ is separated by a more complex and porous middle layer that is devoid of EPFs. Overall our analyses reveal that the dermis at E16.5 is composed of 5 distinct sub-structures across the dorsal-to-ventral axes. Each layer has its own unique cytological arrangements and structural complexity

values. Critically, within each internal layer there are micro-domains with distinct complexity values across the anterior-posterior axes. Tissue structure complexity and porosity does not vary linearly across these microdomains. In fact, in several places the deep dermis is abruptly interspersed with distinct cellular arrangements (Fig. 3j right panels), which might result from local migration patterns at earlier stages of development (Fig. 2).

Scarring transition follows an anterior-to-posterior sequence

We showed above that EPF's gradual increase within the dermal back-skin follows an anterior to posterior sequence. We previously showed that adult EPFs produce scars[11]. We therefore speculated that the fetal regeneration-to-scar transition is linked to engrailed lineage replacement and could be due to the gradual increase in EPF abundance during dermal development. If this is so, we would expect that wounds inflicted at early fetal stages would regenerate from ENFs. If lineage replacement is correct, then during the regeneration-to-scar transition there should be also an anterior to posterior sequence in the extent of scar phenotype.

To test our 1st hypothesis, we generated wounds in the back-skin of *En1*^{Cre}; *R26*^{mTmG} E12.5 embryos and analyzed both lineage compositions and ECM depositions (Fig. 4a). At 48 hours post-wounding, ENFs had migrated into wound beds, where a provisional Fibronectin-rich matrix had been generated. There were very few EPFs near the wound margins at these time points (Fig. 4b arrows and dotted line). We detected little to no Collagen I fibers at the injury sites (Fig. 4c). Moreover, the fractal analysis showed that the arrangement (both FD and L values) of the newly deposited fibronectin at the injury

site was not different from that of the adjacent fetal skin (Fig. 4d-f). Thus, during fetal skin regeneration, ENFs sculpt the wound bed matrix without the intervention of EPFs.

To test our 2nd hypothesis, we cultured skin biopsies from different anterior-posterior regions of E19.5 WT embryos (Fig. 4d). Skin biopsies from anterior regions deposited significantly more Collagen-rich scars (Fig. 4e right panel arrow). More posterior sites had a much less pronounced matrix deposition (Fig. 4e left panel arrow). We performed fractal analysis on the ECM arrangements and found that posterior matrix depositions were significantly simpler (lower FD) than anterior ones, although there were no changes on L values (Fig. 4f), suggesting that at this stage anterior fibroblasts are more fitted to create a scar than posterior fibroblasts. Collectively, these experiments demonstrate that the fetal regeneration-to-scar transition follows the increase in EPF abundance during dermal development.

We next asked whether EPFs from early stages possess scarring capacities or if, alternatively, they undergo a developmental maturation, whereby they acquire scar-producing abilities, over time. To test this idea, we used the same mice that label EPFs with GFP (*En1*^{Cre}; *R26*^{mTmG}). We purified EPFs from the back-skin of embryos/neonatal mice at regenerating (E16.5) or scarring (P1) stages. Because of the lower number of EPFs at regenerative stages compared to scarring stages, we purified and pooled EPFs from the back-skin of eleven E16.5 embryos. We generated 5 mm full-thickness splinted wounds on the back-skin of adult immuno-deficient mice and transplanted same numbers of EPFs from E16.5 and P1 around the wound edges. Skin wounds were harvested at 14 days post EPF transplantations and processed for histological analysis to assess the contribution of the EPFs from different ages to scar formation (Fig. 5a). Transplanted

EPFs from P1 that migrated into the wounds, deposited Collagen I extensively within host wound beds (Fig. 5b). Some EPFs did not migrate into wound beds, but instead deposited ectopic Collagen I at their original transplanted location (Fig. 5c). Strikingly, transplanted EPFs from E16.5 embryos had the same adult scarring capacities as new-born mouse (P1) EPFs. EPFs from E16.5 contributed to Collagen I deposition both within adult wounds and at ectopic un-injured dermal sites (Fig. 5d-e). EPFs from both E16.5 and P1 had pathologically active cell morphologies, with membrane protrusions that span across and intermingle with the Collagen I fibers (Fig. 5e').

We analyzed the Collagen I matrix architecture generated by the transplanted EPFs and compared them to non-injured adult or embryonic, or adult scar matrices (Fig. 5f). In non-injured adult and fetal dermis, Collagen I arrangements were simple and porous (low FD and high L, squares and circles). In adult scars (day 21 post-wounding) and in EPF-transplanted regions (day 14 post-wounding), Collagen I arrangements were more complex and smoother (high FD and low L, diamonds and triangles) than non-injured dermis. EPF-transplanted sites far away from the wound-bed showed similar patterns to wounded scars, indicating that the degree of Collagen I deposition and the complexity of collagen fiber arrangements in the dermis is directly related to the amount of EPFs. Our results also demonstrate that fetal EPFs are capable of producing and organizing a scar tissue, just as well as newborn EPFs, and that the absence of scar-formation ability in early fetal skin is due scarcity of EPFs, rather than their developmental immaturity. Taken together, we conclude that the mechanism behind the dynamic transition from regeneration to scarring in the fetus is Engrailed1-Past lineage replacement during back-skin development.

ENFs or native lattice reduce scarring in adult wounds

Since we found that ENF abundance in dermal tissues is associated with regenerative outcomes at early stages, and since we also show that ENFs sculpt a provisional lattice during development, we asked whether transplantations of ENFs alone would regenerate adult dermal wounds. In opposite to our earlier EPF transplantation experiments that aimed to prove the scarring abilities of EPFs, here we assess the regenerative potential of transplanted ENFs as a 'cell therapy' approach to adult dermal injuries. To test this hypothesis, we transplanted fetal (E16.5) ENFs into back-skin wounds of adult immuno-deficient mice and allowed wounds to heal for up to 14 days (Fig. 6a). As expected, sites of ENF transplantations had a more reticular ECM, compared to fibrotic ECM at the sites of EPF transplantations (Fig. 6b, c). The fractal analysis showed that ENF transplantation led to simpler and porous matrix pattern at the injury site compared to EPF or mock transplanted ones (Fig. 6d), together with the analysis showed in Fig. 5f suggesting less fibrosis. By immunostaining of the endothelial marker CD31, we found a significant higher infiltration of blood vessels into the wound beds at sites of ENF transplantation compared to EPF or mock transplanted wounds (Fig. 6e-i). Many of the transplanted ENFs were closely associated with blood vessels, which were interlaced with one another to generate vascular networks within the transplanted sites (Fig. 6g).

To test if the ENF-generated lattice itself would promote a regenerative outcome, we transplanted de-cellularized extra-cellular matrix from P5 WT mouse skin into splinted wounds on the back-skin of immune-deficient mice (Fig. 6e). 10 days later we isolated dermal tissues from the wound site. While fibroblasts in the control wounds and in the

borders between the wound and the transplanted matrix had a typical active morphology with membrane protrusions (Fig. 6f-g), resident cells within the transplanted matrix had a typical inactive morphology (round cytoplasm, Fig. 6h). We also used immuno-labeling against α-SMA to detect activated fibroblasts, which were present in the control wound beds and in the matrix-wound border (Fig. 6i-j), while the transplanted matrix was mostly devoid of activated fibroblasts (Fig. 6k). These experiments show that a 'healthy' dermal lattice can be imposed, locally, and in two ways. First, transplanting ENFs expands the existing pool of ENFs that are already there, and allows ENFs to sculpt a provisional matrix. Alternatively, transplantations of de-cellularized dermal lattice, in essence, mimic dermal development, and in the presence of which EPFs do not promote a pathologic scar. Both approaches, if they could be made industrially, provide novel therapeutic possibilities for a range of dermal pathologies, including patients suffering from large skin burns or wounds.

Discussion

The ENF and EPF lineages were demonstrated in our previous study[11] in adult settings, including wound healing, irradiation fibrosis and melanoma cancer growth. In the current study, by demonstrating that during the development EPFs and ENFs have vastly different migratory, matrix secretions, and proliferative capacities, we have further characterised how dermal development is established and why the phenotypic transition occurs during fetal life, from regeneration to scarring. Here, we outlined dermal morphogenesis by characterizing dermal cell and dermal lattice development. Changes in dermal lattice are driven by Engrailed1 cell lineage replacements, and these two are

interconnected. Moreover, our single cell tracking of EPFs shows distinct migration behaviors *in vivo* including localized, diverging or converging patterns. To our knowledge, these patterns have never been observed in 2D or 3D migration assays to date. Migration patterns are most likely instructed locally to generate local lattice structures with distinct complexity values. They must also be instructed across dorsal-to-ventral axes, to generate 5 distinct dermal layers. Mechanisms that may establish dermal segmentation from dorsal-to-ventral, could include a morphogenetic gradient or a cellular gradient. Morphogenetic gradients establish segmentation during the development of the larval cuticle in drosophila embryogenesis[23] from which distinct tissue structures develop. It is possible that fibroblasts across a morphogenetic gradient acquire unique proliferation and secretion behaviors that, in turn, establish regionally unique dermal structures.

Secondly, our findings explain the phenotypic transition from regeneration-to-scarring, and have the developmental, evolutionary and clinical implications outlined below.

Developmental implications: Recent findings from other groups implicated other distinct fibroblastic lineages of the back-skin in the differentiation of the skin's epidermis[18,24-25]. Fetal processes of epidermal development, hair follicle and sebaceous gland differentiation, may require specialized stromal signals and cells. The priority for adults is a quick fix of breached skin, which likely requires different stromal signals and cells to produce a scar. These two diverged requirements at different stages of fetal life are maintained through dynamic cellular shuffling of fibroblastic populations, allowing specialized types of fibroblasts to accommodate specific fetal and adult requirements.

Evolutionarily implications: We demonstrate here that regeneration and scarring are distinct phenotypic consequences of ENFs and EPFs respectively. These two separate phenotypes become linked during fetal life because of the fibroblastic successions between a lineage without a history of Engrailed1 expression to a lineage with past expression of that gene. This switch imposes a phenotypic transition in response to injury. In evolutionary terms this switch provides a model to explain how dermal regeneration was retained in some adult rodent species such as African spiny mice[26]. We suggest that species such as the African spiny mice have retained the fibroblastic cell compositions of fetal stage dermis (ENFs>EPFs), and have resisted the ENF-to-EPF lineage successions.

Clinical implications: We describe, and map in detail, here a mechanism for the general acquisition of scarring ability in the skin and we show that the temporal and spatial determinants of scarring depend on the dynamics of Engrailed1-past history fibroblasts. We believe both the temporal and spatial-dynamics of this Engrailed-past history fibroblast in the skin will be widely clinically applicable. Consideration of lineages with engrailed history will outline a favorable time window for surgeons to work in, when scarring outcomes will be predicted to be at a bare minimum, such as during corrective surgery for *Spina bifida*, or equally, it could be applicable to the removal of pediatric malignancies.

Our findings also carry implications for adult scarring. There are no definitive strategies to prevent scar formation, and current clinical practice is focused on scar tissue acceptance rather than its amelioration. We have demonstrated two separate strategies that reduce scar outcomes in adult injured skin. Both our ENF cell transplantation and de-

cellularized dermal transplantation assays provide a proof-of-concept for a therapeutic option that could be used to treat large wounds, scolds or burns.

Our findings also create new opportunities for the intense field of bio-engineered autologous dermo-epidermal human skin grafts that are currently being tested in Phase I and II clinical studies. Skin grafts currently include all fibroblastic lineages within dermis and are therefore likely to produce some scarring upon transplantation. We predict it would be advantageous to graft pure human ENFs or ENFs combined with dermo-epidermal skin. In essence, this dermal milieu would override the ENF-to-EPF population shift and favor regeneration over scarring.

Our current knowledge of fibroblast physiology is largely based upon monolayer culture studies. Here, by using single cell clonal analysis, 3D imaging, computational cell tracking and in silico analysis, we follow the behaviors of fibroblast lineages in vivo to document their dynamics of cellular proliferation, cellular migrations and cell secretion at both cellular and lattice levels, to demonstrate how such a fundamental process as dermal morphogenesis actually occurs. In sum, our finding of Engrailed-lineage replacement links dermal morphogenesis with the phenotypic shift in response to dermal injury. We believe our findings generate new clinical approaches and opportunities on which to ameliorate scar tissues in injured adult skin.

Methods

Mice strains and genotyping

All Mouse strains (C57BL/6 [WT], $En1^{Cre}$, $Actin^{CreER}$, $R26^{VT2/GK3}$, $R26^{mTmG}$, Fox Chase SCID) were either obtained from Jackson laboratories, Charles River, or generated at the

Stanford University Research Animal Facility as described previously[11]. They were housed at the Helmholtz center animal facility and all animal experiments were conducted under strict governmental and international guidelines. The rooms were maintained at constant temperature and humidity with a 12-h light cycle. Animals were allowed food and water *ad libitum*. Cre-positive embryos were identified by detection of relevant fluorescence into the dorsal dermis. Genotyping was performed to distinguish mouse lines containing 200 bp Cre fragment (Cre+/-) from the wildtypes (Cre-/-). The genomic DNA from the ear clips was extracted using QuickExtract DNA extraction solution (Epicenter, USA) following the manufacturers guidelines. 1µl of DNA extract was added to each 24µl PCR reaction. The reaction mixture was set up using Taq PCR core kit (Qiagen, USA) containing 1x coral buffer, 10mM dNTPs, 0.625 units Taq polymerase, 0.5µM forward primer "Cre_genotype_4F"- 5´ ATT GCT GTC ACT TGG TCG TGG C- 3´" (Sigma, Germany) and 0.5µM reverse primer "Cre_genotype_4R"- 5´ GGA AAA TGC TTC TGT CCG TTT GC- 3´ (Sigma, Germany). PCR reactions were performed with initial denaturation for 10 mins at 94°C, amplification for 30 cycles (denaturation for 30s at 94°C, hybridization for 30s at 56°C, elongation for 30s at 72°C) and final elongation for 8min at 72°C then cooled to 4°C. In every experiment, negative controls (non-template and extraction) and positive control were included. The reactions were carried out in an Eppendorf master cycler (Eppendorf, Germany). Reactions were analyzed by gel electrophoresis.

Cell harvesting and sorting

Dorsal skin of $En1^{Cre};R26^{mTmG}$ embryos, neonates or adult mice were harvested and washed with HBSS. Skin was minced into small pieces with surgical scissors and washed

again with HBSS. The cells was re-suspended in 2 ml of digestion solution containing 1 mg/ml of Collagenase I, 0.5 mg/ml of Hyaluronidase, and 25 U/ml of DNase I, and incubated in a 37°C water bath for 30 min with agitation. 10 ml of DMEM containing 10% FBS was added to stop the enzymatic reaction and the suspension was filtered through a 100 µm and then 40 µm cell strainer. After centrifugation at 300x g for 5 min, the cell pellet was re-suspended in 1 ml of FACS buffer (0.5% FBS in PBS) and incubated with 1 µg of APC-conjugated anti-mouse CD31 (PECAM-1), CD45, Ter119, Tie2 (CD202b) or EpCam (CD326) antibodies (BioLegend) and eFluor660 conjugated anti-mouse Lyve-1 antibody (Thermo Fisher) on ice for 30 min. A small aliquot of cells was incubated with the respective APC-conjugated isotype controls (BioLegend). After washing with 5 ml FACS buffer, the antibody-conjugated cell pellet and the isotype-conjugated cell pellet were resuspended in 2 ml or 0.5 ml of FACS buffer, respectively. The cells were sorted on a BD FACSAria III cell sorter with a 100 µm nozzle. The lineage-negative cells (Lin^-: $CD31^-$, $CD45^-$, $Ter119^-$, $Tie2^-$, $EpCam^-$, $Lyve\text{-}1^-$) were sorted into ENFs ($Lin^-TomatoRed^+GFP^-$) and EPFs ($Lin^-TomatoRed^-GFP^+$) based on TomatoRed and GFP fluorescence. For the flow cytometric analysis of surface markers, following antibodies were used: anti-mouse CD105-PE-Vio770, CD146-PerCP-Vio700, CD140a (pdgfra)-PE-Vio770, integrin α-7-APC (Miltenyi); and anti-mouse Sca-1-PerCP-Cy5.5, CD29-PerCP-eFluor710 (eBioscience).

Splinted wounds on adult mice

Splinting rings were prepared from 0.5 mm silicone sheet (Grace Bio-Labs, CWS-S-0.5) by cutting rings with outer diameter 12 mm and inner diameter 6 mm. After washing with

detergent, rinsing with water, the splints were sterilized with 70% ethanol for 30 min and air dried in cell culture hood and kept in a sterile bottle.

Mice were anesthetized with 100 µl of MMF (medetomidine, midazolam and fentanyl). Dorsal hair was removed by hair clipper (Aesculap Schermaschine Exacta), followed by hair removal cream for 3-5 min. Two full-thickness excisional wounds were created with a 5 mm diameter biopsy punch (Stiefel). One side of a splint was applied with silicone elastomer super glue (Kwik-Sil Adhesive, World Precision Instruments~~, KWIK-SIL~~) and placed around the wound. The splint was secured with 5 sutures of 6.0 nylon, and the wound was covered with Tegaderm transparent dressing (3M). Mice were recovered from anesthesia with MMF antagonist, and were supplied with Metamizol (500 mg Metamizol/ 250 ml drinking water) as postoperative analgesia.

Cell transplants

Sorted cells were washed with PBS and re-suspended in PBS at $4x10^6$ cells/ml and mixed with an equal volume of ice-cold Matrigel (growth factor reduced, phenol red free) (Corning, #356231) to obtain EPF and ENF suspensions in matrigel at a final concentration of $2x10^6$ cells/ml, and stored on ice until injection for transplantation. The Fox Chase SCID mice (Charles River) at 10-12 weeks of age were used as recipients of transplanted EPFs and ENFs. Two 5 mm diameter full-thickness excisional wounds were created on the back-skin of SCID mice as described above. Then two injections of 50 µl of prepared EPF- or ENF-matrigel solution at concentration of $2x10^6$ cells/ml were made intra-dermally around each wound, which resulted intradermal transplantation of $2x10^5$ EPFs or ENFs to each wound. Control wounds received two intradermal injections of 50 µl PBS. 15 min after injection, the silicone splints were applied and fixed around the

wounds as described above. Scar tissues were harvested 14 days post-wounding, and fixed with 2% PFA at 4°C overnight. After washing three times with PBS, the tissues were processed for histology as described below.

Transplantation of decellularized skin

The dorsal skin of C57BL/6J mice (age P5) were subjected to de-cellularization as described previously[27]. Briefly, the skin tissue was incubated sequentially with, 1) distilled water containing 0.1% Triton X-100, 5x penicillin/Streptomycin at 4°C for 24 h with stirring, then, 2) distilled water containing 2% sodium deoxycholate at 4°C for 24 h with stirring, 3) distilled water containing 1 M NaCl, 5x 5x penicillin/Streptomycin at room temperature for 1 h, and finally, 4) distilled water containing 30 µg/ml porcine pancreatic DNase, 1.3 mM MgSO4, 2 mM CaCl2, 5X penicillin/Streptomycin at room temperature for 1 h. The decellularized skin was stored in PBS at 4°C until transplantation. Two 5 mm diameter full-thickness excisional wounds were created on the dorsal back of each recipient SCID mouse. A 5 mm diameter biopsy of de-cellularized donor skin was transplanted to the recipient's wound with silicone elastomer super glue (Kwik-Sil Adhesive, World Precision Instruments). Wounds without transplant served as control. Subsequently, the wounds were covered with 12 mm diameter silicone sheet (Grace Bio-Labs), fixed with 5 sutures, and transparent dressing was applied (3M Tegaderm Film). The scar tissues were harvested 10 days post-wounding and processed for histology.

Ex vivo wounding

E12.5 embryos were harvested from *En1*^{Cre};*R26*^{mTmG} breeding. Cre-positive embryos were selected based on GFP-positivity by observing them under a fluorescence

stereomicroscope (Leica M205 FA). Small excisional wounds were created on the dorsal back of embryos by using fine microsurgical scissors (Vannas Spring Scissors, Fine Science Tools) under the stereomicroscope (Leica M50). The embryos were kept in Knockout DMEM (Thermo Fisher) supplemented with 10% KnockOut™ Serum Replacement (Thermo Fisher), 1% Penicilin/Streptavidin (Thermo Fisher), 1% GlutaMAX™ (Thermo Fisher) and 1% Non-Essential Amino Acids solution (Thermo Fisher) in a 37°C, 5% CO_2 incubator. Embryos were fixed with 2% PFA at 12 h, 24 h and 48 h after culture and processed for histology.

Ex vivo tamoxifen treatment

E12.5 embryos were harvested from *Actin^{CreER};R26$^{VT2/GK3}$* breeding. The embryos were kept in Knockout DMEM (Thermo Fisher) supplemented with 10% KnockOut™ Serum Replacement (Thermo Fisher), 1% Penicilin/Streptavidin (Thermo Fisher), 1% GlutaMAX™ (Thermo Fisher), 1% Non-Essential Amino Acids solution (Thermo Fisher) and 1nM 4-Hydroxytamoxifen (Sigma Aldrich) in a 37°C, 5% CO2 incubator. 24 h later, embryos were fixed overnight with 2% PFA at 4°C and processed for histology.

Histology

Tissue samples were fixed overnight with 2% PFA in PBS at 4 °C. Samples were rinsed three times with PBS, immersed in OCT (Sakura Finetek) embedding compound, and flash-frozen on dry ice. 6-8 micron sections were made in a Hyrax C50 Zeiss Cryostat.

Masson's trichrome staining was performed with a Sigma-Aldrich Trichrome stain kit, according to manufacturer instructions.

For immunostainings, sections were air-dried for 5 min followed by fixation with -20 °C-chilled Acetone for 20 min. Sections were rinsed thrice with PBS and blocked for 1h at

room temperature in PBS plus 5 % serum. Then, the sections were incubated with the primary antibody in PBS-serum overnight at 4°C or for 90 min at room temperature. Sections were then rinsed thrice with PBS, and incubated with the secondary antibody in PBS-serum for 60 min at room temperature. Finally, sections were rinsed thrice in PBS and mounted with fluorescent mounting media with DAPI.

Antibodies used: Ki67 (Abcam), Cas3 (Cell signal), Collagen I (Rockland), Fibronectin (Abcam), FSP1 (Abcam), Collagen III (Abcam), Collagen VI (Abcam), CD31 (Novus Biologicals), α-SMA (Abcam), CD26 (Abcam), TNC (Abcam), Dlk1 (Abcam), Sca1 (Biolegend), FABP4 (Abcam) and AF647 secondary antibody (Molecular Probes).

Live-Imaging

Pregnant mice were sacrificed 12.5 dpc by cervical dislocation and uteri were excised. Embryos were dissected in ice-cold medium and tested for Cre+-fluorescence. Excised Cre+ embryos were incubated with SiR-DNA (Spirochrome, Switzerland) for 1h (37°, 5% CO_2). The embryos were embedded in 35mm glass bottom dishes (Ibidi, Germany) and Matrigel (Corning, NY, USA) and the gel was allowed to solidify for 30 min. Imaging medium (DMEM/F-12; SiR-DNA 1:1000) was then added. 24h time-lapse imaging was performed using a Zeiss LSM 710, equipped with 488nm, 515nm and 633nm laser lines and a 20x objective. Temperature control was set to 37°C with 5% CO_2-supplemented air. Three channels (GFP, tdTomato, AF647) were recorded every 15 min as a z-stack of 188 µm thickness. The 4D data was processed with Imaris 8.4.1 (Bitplane, UK) and ImageJ. Contrast and brightness was adjusted for better visibility.

Cell-tracking

3D Drift correction was applied on the live-imaging 4D data using the ImageJ`s "Correct 3D Drift" in reference to the EPF channel. The resultant drift-corrected datasets were subjected to maximum intensity projection and visualized to verify compensation of the apparent drift in reference to the imaging plane. The corrected datasets were subjected to cell migration and cell tracking analysis using "Trackmate". Variables such as blob diameter, threshold, detector (LoG -Laplacian of Gaussian segmentation), and the tracker: LAP tracker- Linear Assignment Problem (LAP), were adjusted to suit the nature of the data and the samples as described in the plugin workflow[28]. Filters such as spots with good quality, mean intensity, and uniform color were applied and tracked using LAP Tracker. Individual track information and the coordinates of these data points were exported as an Excel file. Graphical representation and visualization of 4D Datasets were performed using a R-script employing the "scatterplot3D" package to represent the tracks on a 3D plane. For the fourth dimension of the tracks, color ramp was applied to the individual tracks as a function of time (Blue=first time point; Red= last time point of the track). To visualize the data points on a 3D interactive space (360° rotation and zoom function), the R - package "rgl: 3D visualization using openGL" was integrated into the script.

3D-Imaging

Samples were fixed in 2% paraformaldehyde at 4°C overnight and imaged in a 35 mm glass-bottom dish (Ibidi, Germany).

Cleared samples were processed using a modified 3Disco protocol. In short, samples were stained with anti-GFP (Abcam) and anti-RFP (SICGEN) for 4 days, followed by

672 incubation with AF488 and AF647 secondary antibodies (Molecular Probes) for 2 days.
673 Clearing was performed after dehydration in an ascending Tetrahydrofuran (Sigma)
674 series, with 30 min incubation in Dichloromethane (Sigma) and eventually with
675 immersion in Dibenzyl ether (DBE, Sigma). Imaging was performed in DBE with an
676 inverted confocal (Zeiss, LSM 710) using a 10x or 20x objectives. Stacks were recorded
677 in the green and far-red channels. 3D-visualization and recording of movies was
678 performed using Imaris 8.4.1 (Bitplane, UK).

679 **Image analysis**

680 Single 10X or 63X images were taken of the EPF-containing anterior and posterior fields,
681 in the same section, using a confocal LSM 710 (Zeiss).

682 Image processing and analysis were performed with ImageJ (ImageJ 1.47[29]). For
683 separating EPF and ENF fluorescent signals, only the red population of EPFs was
684 analyzed from sections from 'Rainbow' mouse, as it provided the strongest signal.
685 Channels from the raw images were split, background was subtracted (rolling ball radius
686 = 50 pixels), and contrast was enhanced (pixel saturation = 0.1, normalized). The
687 "Unsharp Mask" and "Median" filters were applied (radius = 10, mask = 0.60; and radius
688 = 1, respectively). Auto Threshold method "Yen" was used to make binary images except
689 for the immunostaining signal of Fibronectin and Collagen I, for which "Default" method
690 was used. To avoid the inclusion of background signal into the analysis, the pixels from
691 the ENF signal were subtracted from the EPF signal and vice versa using the image
692 calculator function. For segmentation of the blue color from trichrome stains, 10X bright-
693 field images were taken with a vertical Axio scope (Zeiss). Images were transformed into
694 CMYK and only cyan channel was used for further separation, background was

subtracted (rolling ball radius = 20 pixels), and contrast was enhanced (pixel saturation = 0.1, normalized). The "Unsharp Mask" and "Median" filters were applied (radius = 2, mask = 0.60; and radius = 1, respectively). Auto Threshold method "Moments" was used to make binary images and "Despeckle" function was used to avoid the inclusion of background signal into the analysis.

The fractal analysis was performed using the ImageJ plug in "FracLac"[30] (FracLac 2015Sep090313a9390. Fractal dimension (FD) was calculated using the box counting scan (20 slipping and tighten grids at default sampling sizes, minimum pixel density threshold = 0.40). Lacunarity (L) was calculated using the sliding box scan (slipping and tighten grid at default sampling sizes, minimum pixel density threshold = 0.40). Local FD and L were calculated using the subsample scan (1 grid at default sampling size, minimum pixel density threshold = 0.40, sub scan in rectangles). Individual values within discernible distinct dermal layers were manually pooled.

For the Collagen I intensity analysis, sections of *En1*^{Cre}; *R26*$^{VT2/GK3}$ embryos at different stages were immuno-stained for Collagen I. 63X images were segmented as before. Segmented EPF and ENF binary images were further processed (fill holes, close) and a ROI selection was created to measure the mean gray values (mgv) from the raw images. The mgv from the ROI were normalized with the general mgv of the image. Three images from every condition were analyzed.

For the Fibronectin fiber distance measurements, sections of *En1*^{Cre}; *R26*$^{VT2/GK3}$ embryos at different stages were immuno-stained for fibronectin. 63X images were segmented as before. We use the plot profile function in diagonal lines of fibronectin segmented images

and the distance between the fibers was calculated as the gaps between the signal peaks. Three images from every condition were analyzed.

Statistics

Statistical analyses were performed with GraphPad Prism 6 software. A minimal of 3 samples was analyzed at any time.

Data availability

Data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author Contributions

Y.R. outlined and supervised the research narrative, and designed all experiments. D.J. performed the wound healing studies, performed flow cytometric analysis and purified fetal and adult EPFs and ENFs, performed cell transplantations and transplanted the de-cellularized dermis. D.C.G. and A.S. performed and analyzed the clonal analysis studies and generated the confocal images. D.C.G. performed the fractal analysis. S.C. and P.R. performed live imaging and cell tracking analysis. J.L. and V.R. assisted with clonal analysis, confocal imaging and histological sectioning. D.W. generated the de-cellularized ~~fetal~~ dermis. Y.R. wrote the manuscript.

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Figure 1 EPFs replace ENFs during mouse back-skin development. a, Genetic labeling systems used in this study. **b**, Representative flow cytometry plots of ENFs/EPFs from *En1*^{Cre};*R26*^{mTmG} embryos at different developmental stages. All Cre^{+} embryos (ranging from 3-6 embryos) from an entire litter were pooled together for a single analysis. Two independent experiments were performed for the key development stages at E14.5, 16.5, E18.5 and P0.5. GFP intensity (X-axis) is plotted against RFP intensity (Y-axis). **c**, EPF/ENFs percentages of Lin^{-} (lines), or total live cells (dashed lines). **d**,**e**, Representative immunofluorescence images of Ki67 (**d**) or Cas3 (**e**) in E18.5 *En1*^{Cre};*R26*^{mTmG} embryos, arrows indicate Ki67 or Cas3 positive cells. **f**, EPFs/ENFs positive for Ki67 or Cas3. Data derived from cryosections of 3 Cre^{+} embryos at E16.5 and E18.5, respectively. *, $p<0.05$ by two tailed unpaired Student's *t*-test. **g-k**, Coronal sections of E9.5-E11.5 En1^{Cre}; R26$^{VT2/GK3}$ embryos. Arrows indicate single EPFs (**h**, **j**). Dashed line indicates ectoderm border (**h**).

Doted lines delimitate EPFs clones (**i-k**). **l**, E12.5 $En1^{Cre}$; $R26^{VT2/GK3}$ embryo showing EPFs arc across the back skin. **l'**,**l''**, high magnification from boxes in **l**. Arrows indicate migrating EPFs. Cross indicate embryonic axes. **m-o**, Immunofluorescences for Fibronectin (**m**), Collagen I (**n**), or Collagen III (**o**) in coronal section of E9.5 or E11.5 $En1^{Cre}$; $R26^{VT2/GK3}$ embryos. Doted lines delimitate EPFs. **p**, Orthogonal view from time-lapse images (0 h and 24 h) from the anterior region of E12.5 $En1^{Cre}$; $R26^{mTmG}$ embryo. Doted lines in X-Y frame delimitate the EPF-induced ENF gap. Green channel (EPFs) was omitted in X-Y frames for clarity. Doted lines from X-Z and Y-Z delimitate EPFs. Dashed lines mark the dorsal surface. Arrows indicate EPFs movement direction. Scales: d-e, g-j = 50 microns. k-l = 500 microns. l'-l''-m-o = 200 microns. p = 100 microns. ec = ectoderm, Ant = anterior, Pos = posterior, D = dorsal, V = ventral.

Figure 2 EPFs exhibit non-stochastic migrating behaviors. a, 4D scatter plot of individual tracks, the color ramp indicates time (Blue = first; Red = last time point). **b**, Top X-Y view showing the reticular patterns of migration. Dashed lines in zoomed view (right panel) delimitate "converging" track points. **c**, **d**, Lateral Y-Z views of posterior (**c**) or anterior (**d**) tracks split in dorsal (up) and ventral (down) regions. **e, f**, Frontal X-Z views of posterior (**e**) or anterior (**f**) tracks. **g**, **h**, Amplified view of single tracks showing "localized" (**g**) or "diverging" (**h**) migrations. Color ramps of tracks of no interest (**g**, **h**) were changed to gray-scale for clarity. Axes units = microns.

Figure 3 Dermal lattice is actively changing during development. a, **b**, Box and whiskers plots of FD (**a**) and L (**b**) from EPFs of different regions and developmental stages. RM ANOVA, alpha = 0.05, Newman-Keuls test, 10-90 percentiles. **c**, 3D reconstructions from high magnification images of posterior (up) or anterior (down) $En1^{Cre}$; $R26^{VT2/GK3}$ EPFs. **d**, Mean + SEM distances between fibronectin fibers in microns. Unpaired T-test, * $p < 0.05$. **e**, **f**, Box and whiskers plots of Fibronectin FD (**e**) and L (**f**) from different regions and developmental stages. RM ANOVA, alpha

= 0.05, Newman-Keuls test, 10-90 percentiles. **g**, Graphical representation of Fibronectin matrix changes from a relaxed framework to a rigid matrix. **h**, Immunofluorescence for Fsp1 in anterior (left) or posterior (right) regions of E12.5 Actin^{CreER}; R26$^{VT2/GK3}$ embryos after 24 h of 1 nM 4-Hydroxytamoxifen exposure. Doted lines delimitate single clones. **i**, Percentage of labeled cells in clones from two independent embryos. **j**, Local (subsampled) fractal analysis of high magnification images from E16.5 En1^{Cre}; R26$^{VT2/GK3}$ dermis. Dashed lines delimitate dermal layers. **k**, Percentages + SEM of EPFs/ENFs of each layer. Two-way ANOVA, alpha = 0.05, Tukey test. **l**, Mean + SEM number of cells per 1000 microns^2 of each layer. One-way ANOVA, alpha = 0.05, Tukey test. **m**, **n**, Box and whiskers plots of FD (**m**) and L (**n**) from different dermal layers. One-way ANOVA, alpha = 0.05, Tukey's test, 10-90 percentiles. Scales: c = 20 microns, h = 200 microns, j = 50 microns.

Figure 4 Regeneration-to-scar transition is coupled to EPFs development. a, Embryo back-skin wounding experiment. E12.5 En1^{Cre}; R26^{mTmG} embryos were collected, wounded on the back-skin, and kept in culture for 48 h. **b**, **c**, Immunofluorescence for Fibronectin (**b**) or Collagen I (**c**). Dotted lines delimitate lesion site. Arrows indicate EPFs. **d, e,** Representative immunofluorescence images of fibronectin staining at adjacent fetal skin (d) and the wounded site (e) and at 48 h post-wounding. **f,** Box and whiskers plots of FD (left) and L (right) from fibronectin staining at adjacent fetal skin and the wounded site at 24 h and 48 h after wounding. Unpaired T-test two-tailed, confidence interval 95%. **g,** Cultures biopsies experiment. 2 mm biopsies were taken from anterior, middle, and posterior regions of E19.5 WT embryos and cultured for 5 days. **h**, Brightfield images of Massons Trichrome Stained sections of posterior (left) or anterior (right) biopsies. Arrows indicate the scar-like deposition of ECM. **i**, Box and whiskers plots of FD (left) and L (right) from ECM cyan channel of stained biopsies. One-way ANOVA, alpha = 0.05, Tukey test, 10-90 percentiles. Scales: 200 microns.

Figure 5 EPFs are a mature scar-matrix-depositing population. a, EPFs transplantation experiment. E16.5 or P1 $En1^{Cre}$; $R26^{mTmG}$ EPFs were sorted and transplanted ($2x10^5$) into freshly made splinted wounds of SCID immune-deficient adult mice and the tissue was collected two weeks after. **b**-**e**, Immunofluorescence for Collagen I of scars (**b**, **d**) or ectopic un-injured sites (**c**, **e**) containing transplanted EPFs from P1 mice (**b**-**c**) or E16.5 embryos (**d**-**e**). **e'**, High magnification image of a Collagen I-attached EPF. Dotted line delimitate EPFs cell-body. **f**, Collagen I FD and L scatter plot of E12.5 (squares) and adult (circles) normal dermis, adult scars (triangles), and scars derived from E16.5 EPFs transplants (diamonds). Scales: b-e = 50 microns, e' = 20 microns.

Figure 6 ENFs and ECM matrix transplantations have a better outcome in wound healing. a, ENFs transplantation experiment. E16.5 $En1^{Cre}$; $R26^{mTmG}$ EPFs were sorted and transplanted ($2x10^5$) into freshly made splinted wounds of adult SCID ~~immune-deficient~~ mice and the tissue was collected two weeks after. **b, c,** Representative images of Masson's trichrome staining of EPFs-transplant scar (b) and ENF-transplant scar (c). **d**, Box and whiskers plots of FD (left) and L (right) from ECM cyan channel of stained sections. One-way ANOVA, alpha = 0.05, Tukey test, 10-90 percentiles. **e-g,** Representative immunofluorescence images of CD31 staining on mock-transplant scars (e), EPFs-transplant scars (f) or ENFs-transplant scars (g). **h-i,** Quantification of CD31 integrated fluorescence density (h) and $CD31^+$ cells per low power (20x) image (i) of mock-transplant, EPFs-transplant, and ENFs-transplant scars. One-way ANOVA, alpha = 0.05, Tukey test, 10-90 percentiles. AU, arbitrary unit. **j**, Decellularized matrix transplantation experiment. Back-skin explants from P5 WT mice were decellularized and transplanted into freshly made splinted wounds of SCID immune-deficient adult mice and the tissue was collected ten days after. **k-m,** Brightfield images of Trichrome stained wound regions of control wounds (**k**), the border between the transplanted matrix and the wound (**l**), or within the transplanted matrix

(**m**). **n-p,** Immunofluorescence for αSMA and Collagen I in control wounds (**n**), the border between the transplanted matrix and the wound (**o**), or within the transplanted matrix (**p**). Scales: 50 microns.

Extended Data Figure 1 Flow cytometric analysis of mesenchymal markers on ENFs and EPFs. **a,** single cell suspension was prepared from the pooled back-skin tissue from three E12.5, three E15.5 and one E19.5 *En1*^{Cre};*R26*^{mTmG} embryos, and subjected to flow cytometric analysis. **b**, Sytox blue was used to exclude dead cells, and APC-conjugated lineage markers (CD45, CD31, Ter119, EpCAM, Tie-2, Lyve-1) were used to exclude non-mesenchymal cells. **c-e**, representative analysis plots of fibro-adipogenic progenitors (FAPs) (Lin-ITGA 7-Sca1+PDGFRα+) (c), pericytes (Lin-CD146+) (d), and mesenchymal stem cells (Lin-CD29+CD105+) (e), and their distributions in ENFs and EPFs. **f-g**, the percentages of ENFs and EPFs (f) and the ratio of ENFs:EPFs (g) within Lin- cells (initial), FAPs, pericytes and MSCs.

Extended Data Figure 2 Expression of mesoderm markers on ENFs and EPFs by immunofluorescence staining. Cryosections were prepared from *En1*^{Cre};*R26*$^{VT2/GK3}$ neonates (P1), and stained with primary antibodies against mouse FABP4 (a), Dlk1 (b), Sca1 (c), αSMA (d), CD31 (e), TNC (f), and CD26 (g), and AF647-conjugated respective secondary antibodies. Depicted are representative high-power images of AF647 channel for antibodies (left), GFP-RFP channels for ENFs-EPFs (middle), and merged channels (right). Dotted lines indicate the border of epidermis and dermis, and the border of upper dermis (UD) and lower dermis (LD). Squares indicate the locations of enlarged inserts in merged images. Scales: 50 microns.

Extended Data Figure 3 EPFs associate with Collagen I fibers. a-d, Immunofluorescence for Collagen I of E11.5 (**a**), E12.5 (**b**), E14.5 (**c**), or E16.5 (**d**) En1^{Cre}; R26$^{VT2/GK3}$ embryos. **e-h**, EPF- or ENF-signal-derived ROI mean gray value (mgv) + SEM of the Collagen I signal from anterior or

posterior regions of E11.5 (**e**), E12.5 (**f**), E14.5 (**g**), or E16.5 (**h**) $En1^{Cre}$; $R26^{VT2/GK3}$ embryos. Unpaired T-test. * $p < 0.05$. Scale: 50 microns.

Extended Data Figure 4 EPFs actively displace provisional ENF dermis. 3D representations of 24 h time-lapse images of EPF-positive anterior region in E12.5 $En1^{Cre}$; $R26^{mTmG}$ embryo (up). Max projection of whole live embryo showing the area analyzed (down). Scales: 100 and 500 microns, respectively.

Extended Data Figure 5 Graphical representation of FD and L quantitatively describing patterns in terms of complexity and porosity. Complex patterns like tree branches (up, right) or Escher's tessellations (down, right) have a higher FD value than simpler patterns like honeycombs (up, left) or floor tiles (down, left). Complementarily, porous structures like tree branches and honeycombs have higher L values than smoother structures like tessellations and floor tiles.

Extended Data Figure 6 Fractal analysis of ENFs organizations during development. a, Representative confocal images of clumped posterior E11.5 EPFs (left), and stretched anterior E12.5 (middle) and posterior E16.5 EPFs (right) from $En1^{Cre}$; $R26^{VT2/GK3}$ embryos. **b**, **c**, Box and whiskers plots of FD (**b**) and L (**c**) from ENFs of different regions and developmental stages. RM ANOVA, alpha = 0.05, Newman-Keuls test, 10-90 percentiles. Scale: 50 microns.

Extended Data Figure 7 Fibronectin matrix stiffens concurrent with EPF expansion. a-d, Immunofluorescence for Fibronectin in E11.5 (**a**), E12.5 (**b**), E14.5 (**c**), or E16.5 (**d**) $En1^{Cre}$; $R26^{VT2/GK3}$ embryos. **e-h**, Representative linear profiles of diagonal lines on the Fibronectin signal from E11.5 (**e**), E12.5 (**f**), E14.5 (**g**), or E16.5 (**h**) images. Dashed lines above the plots represent the calculated distances between fibers. Scale: 50 microns.

Extended Data Figure 8 Tissue-level fractal analysis. a, Local (subsampled) fractal analysis (right) from a low magnification image from E16.5 $En1^{Cre}$; $R26^{VT2/GK3}$ embryo (left). Lines

delimitate tissue layers. **b**, Box and whiskers plots of FD (up) and L (down) from different tissues. One-way ANOVA, alpha = 0.05, Tukey's test, 10-90 percentiles. Scale: 200 microns.

Video 1: EPFs arch in the mouse developing embryo. 3D reconstruction of an $En1^{Cre}$; $R26^{VT2/GK3}$ E12.5 embryo showing the EPFs formations across the dorsum of the embryo. Note that the EPFs at the anterior regions of the embryo showed cells migrating from the dermomyotome. Green: Autofluorescence, Magenta: EPFs.

Video 2: EPFs actively displace ENFs. 3D reconstruction of time-lapse imaging of an $En1^{Cre}$; $R26^{mTmG}$ E12.5 embryo at the anterior region of the EPFs arch showing that EPFs migration actively displaces ENFs-provisional dermal cyto-architecture. Green: EPFs, Red: ENFs.

Video 3: EPFs actively displace ENFs (Only ENFs). 3D reconstruction of time-lapse imaging of an $En1^{Cre}$; $R26^{mTmG}$ E12.5 embryo at the anterior region of the EPFs arch showing that ENFs-provisional dermal cyto-architecture is displaced. Red: ENFs.