Cellular forces and matrix assembly coordinate fibrous tissue repair

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Planar in vitro models have been invaluable tools to identify the mechanical basis of wound closure. Although these models may recapitulate closure dynamics of epithelial cell sheets, they fail to capture how a wounded fibrous tissue rebuilds its 3D architecture. Here we develop a 3D biomimetic model for soft tissue repair and demonstrate that fibroblasts ensconced in a collagen matrix rapidly close microsurgically induced defects within 24 h. Traction force microscopy and time-lapse imaging reveal that closure of gaps begins with contractility-mediated whole-tissue deformations. Subsequently, tangentially migrating fibroblasts along the wound edge tow and assemble a progressively thickening fibronectin template inside the gap that provides the substrate for cells to complete closure. Unlike previously reported mechanisms based on lamellipodial protrusions and purse-string contraction, our data reveal a mode of stromal closure in which coordination of tissue-scale deformations, matrix assembly and cell migration act together to restore 3D tissue architecture.
Closure of open gaps within a tissue, a morphogenetic process involving rearrangement of cells and assembly of extracellular matrix (ECM), is fundamental for normal development and repair of damaged tissues and organs. During embryogenesis, many tissue structures such as the neural tube, eyelid, and dorsal epidermis require closure as a key step in forming a contiguous anatomical structure. In adults, tissue closure is again invoked during the wound-healing response after injury, to restore mechanical integrity and function.

The mechanical basis of wound healing has been studied using in vitro models that measure cellular forces during epithelial gap closure through collective cell migration on planar substrates. These studies revealed one class of closure that involves covering a bare surface in which leading cells in an advancing epithelial monolayer migrate across the surface in an adhesion-dependent manner. Across non-adhesive gaps, epithelial cells employ a different mechanism by generating traction forces parallel to the wound margin through the contraction of a multicellular actin purse string to close the gap. Although these mechanisms explain many aspects of repair of fibrous tissues wherein mesenchymal cells ensconced in a fibrillar matrix restore the three-dimensional (3D) architecture of the tissue.

Here we introduce a 3D bioengineered culture system to study the cellular and biophysical processes during stromal gap closure.

We generated arrays of 3D microscale tissues (microtissues) consisting of NIH-3T3 fibroblasts embedded in a type I collagen matrix. The microtissues were suspended between flexible cantilevers that simultaneously constrain the microtissues and report microtissue tension in real time. After formation, microtissues were damaged using a microsurgical knife mounted on a microrobotic manipulation platform and closure was monitored using time-lapse confocal microscopy. We demonstrate that, in contrast to mechanisms previously described by planar in vitro models, fibroblasts close the open gap through the coordinated action of force-dependent contraction of the whole tissue, circumferential cell migration around the wound edge and assembly of fibronectin scaffolding that allows cells to repair the gap and restore tissue integrity.

Results

Fibroblasts repair gaps in 3D collagen microtissues. To generate microtissues, we seeded NIH-3T3 fibroblasts in a suspension of collagen type I into microfabricated substrates with arrays of wells containing vertical cantilevers (Fig. 1a and Supplementary Fig. 1). Cells contracted the collagen to form a dense fibrocellular microtissue around the caps of the engineered cantilevers in each well. To examine the response of these microtissues to damage, we wounded them in the centre of the tissue with a microdissection knife mounted on a teleoperated micromanipulator and then observed how they evolved (Fig. 1b).
Within minutes after the full-thickness incision was made, the gap further widened (Fig. 1c). As the area of the gap stabilized over the following several hours, the rough wound edge smoothed to form an ellipse (Fig. 1c), a process associated with alignment and elongation of the cells along the circumferential boundary of the wound edge (Fig. 1d). Over the course of the next 24 h, the gap progressively closed, while maintaining its elliptical shape and keeping the centroid position of the wound stationary (Fig. 1e and Supplementary Movie 1). Temporal analysis of the gap area (Fig. 1f) showed a constant rate of closure (1.344 ± 0.150 μm² h⁻¹) throughout the process independent of the initial gap size (Fig. 1g), until the gap closed.

**Closure is driven by contractility rather than proliferation.** During wound healing, cell proliferation is necessary to populate the wound with ample matrix-producing fibroblasts. To test whether cell proliferation and resulting tissue expansion could contribute to the filling of the gap, we administered the nucleotide analogue 5-ethyl-2'-deoxyuridine (EdU) to label fibroblasts undergoing proliferation during closure and treated microtissues with Aphidicolin, a mitogenic inhibitor. EdU uptake was substantial over the 24 h of wound closure; however, whereas aphidicolin nearly eliminated EdU uptake by the cells, undergoing proliferation during closure and treated microtissues

**Fibroblasts tow fibronectin into the gap area.** The non-correlated movements of the fibroblasts suggested that cells primarily coordinate with the ECM rather than via cell–cell adhesions, to mediate the gap closure response. Indeed, knockdown of N-cadherin in 3T3 fibroblasts did not impede gap closure (Supplementary Fig. 5). This finding led us to investigate how the ECM evolved during repair. The microtissue matrix consists mainly of type I collagen; thus, we asked whether closure was primarily driven by the contraction of the existing collagen matrix, or the process involved assembly of new matrix inside the gap. Time-lapse microscopy of gap closure in microtissues containing fluorescently tagged collagen type I matrix showed that reduction of gap area for the first 10 h was primarily associated with contraction of the hole within the existing collagen matrix (Supplementary Movie 5). Interestingly, a later stage of closure followed in which the cells continued to enter and fill the gap area without any collagen matrix (Fig. 4a and Supplementary Movie 5).

The lack of type I collagen within the gap suggested that cells employed a second, more provisional matrix for later stages of wound closure. Indeed, fluorescently labelled fibronectin spiked into the medium showed that fibronectin was being deposited into the gap throughout the closure (Fig. 4a,b). High-resolution time-lapse microscopy revealed that fibroblasts first migrated circumferentially around the wound edge, while towing, remodelling and extending existing fibronectin fibres into the gap (Fig. 4c and Supplementary Movie 6). Furthermore, fibroblasts also deposited cellular fibronectin during this remodelling process (Fig. 4d). The newly formed fibrillar fibronectin template then served as a substrate for cells to migrate further into the gap area (Fig. 4c). During the final phases of healing, a single layer of cells then fully closed the gap with this fibronectin, and then this layer thickened and reinforced as additional cells entered the region, ultimately resulting in a multilayered, matrix-rich tissue.

**Cell-matrix adhesion governs provisional matrix assembly.** Remodelling of ECM is regulated by the binding of matrix proteins to cells through integrin receptors. Once bound to ligands, integrins cluster and activate focal adhesion kinase (FAK), leading to assembly of focal adhesions and modulation of Rho GTPases. In our model, inhibition of FAK kinase activity using the small compound PF-228 abolished closure (Fig. 5a). Interestingly, despite FAK is activated by most integrins, PF-228 primarily hindered closure 8–10 h after injury, which coincided with the onset of fibronectin scaffolding. To further elucidate the role of integrins and cell–matrix adhesion in stromal wound closure, we treated tissues with blocking antibodies that affect interactions with fibronectin. Using α5- or β3-integrin

**Contractility controls migration along the wound edge.** The contractility requirement for closure and the alignment of cells along the wound edge suggested the possibility that the fibroblasts adopted an actomyosin purse string, as has been described as a key element to gap closure by the epithelium. However, immunofluorescence imaging of phosphorylated myosin light chain was characterized by discontinuous, punctate distributions inconsistent with purse strings (Supplementary Fig. 4). Furthermore, time-lapse movies of cells (Supplementary Movie 4) showed that neighbouring fibroblasts followed non-correlated trajectories, especially around the wound edge (Fig. 3a), which are distinct from the collective motions described for epithelial sheets. Cells at the wound edge migrated tangentially in both directions along the circumference of the wound, whereas cells located distal from the wound edge generally moved radially towards the centre of the gap (Fig. 3b,c).

As cell-generated forces drive gap closure (Fig. 2), we hypothesized that this could be mediated via cell migration. Indeed, treatment of microtissues with Y27632 or Blebbistatin reduced more the maximum migration speeds of cells (Fig. 3d) located at the wound edge than those distal from the wound edge.
antibodies targeting α5β1 and αVβ3, we observed a small decrease in closure rate, during the collagen contraction phase of gap closure, and a more pronounced inhibition of the fibronectin-dependent phase of closure (Fig. 5b). These findings are consistent with a more critical role for fibronectin in the later stages of gap closure.
of new scaffolding was further characterized by tangentially moving fibroblasts at the wound edge that assembled fibronectin into fibrillar networks within the gap, thus providing a provisional substrate for cell entry into the gap area. Once the opposing gap edges were close to each other, fibroblasts were able to span the gap and reinforce the region by thickening the tissue in the vertical plane (Fig. 5d). Interestingly, a similar closure mechanism involving ECM remodelling has been observed during eyelid closure in embryonic development. In this process, epidermal cells at the eyelid border pull on their surrounding ECM and intercalate perpendicularly to the closure axis, a process dependent on α5β1-integrin/fibronectin interactions. In agreement with these findings, we observed a similar dependence of gap closure on fibronectin-binding integrins and FAK signalling, although here intercalation did not occur. The fact that our model appeared to capture a distinct wound contraction phase and a deposition of provisional new matrix, to complete healing, further highlights the role of distinct cell–ECM processes to control this morphogenetic process.

Actomyosin contractility provides a central driving force for mediating many of the major structural reorganizations during morphogenesis. Here, by establishing an approach to measure such forces during the repair of gaps, we demonstrated a staged process comprising relaxation after damage, followed by tissue contraction and a steady-state sub-baseline tension stage. Whereas rapid collagen contraction has been described in fibroblast-populated collagen lattices, the demonstration of a relaxation phase and matrix deposition stage on either end of this process may provide a more complete picture of wound healing. Although such tension profiles have not yet been investigated in vivo after injury, the presence of such dynamics could regulate the wound-healing process at multiple levels. For example, transient loss of contractility has been described to stimulate increased motility, and increased myosin activity is not only critical for ECM and tissue contraction, but also important for the assembly of fibronectin matrix. Thus, there are several links between cytoskeletal forces and the many ensuing cellular remodelling events engaged during wound repair, and additional approaches to investigate and deconvolve these links are needed.

In adult skin, the tissue movements of wound repair involve re-epithelialization and fibrous tissue contraction. Hence, bringing the wound margins together is a collective effort of epithelial cells and fibroblasts. Experiments with animal and 3D organotypic models revealed two distinct mechanisms for the repair of the dermis. In a first mechanism, myofibroblasts contract the central granulation tissue in the wound to bring the wound margins closer. Alternatively, fibroblasts residing at the wound edge can pull the intact dermis inwards by directional mass migration towards the centre of the wound. In this latter mechanism, granulation tissue is not required for closure. Similarly, in our in vitro model, gaps spontaneously closed in the absence of a granulation tissue. The mechanical conditions and cellular origin that require the formation of a granulation tissue for the closure of full thickness wounds is unknown. A bioengineered model, such as ours, which allows one to controllably re-introduce the complexity of a wound environment, could provide a critical new strategy to help parse out the role of different cell types, matrix components, contractility and tissue remodelling in dermal tissue repair.

In summary, this study provides a new model to examine how cells are able to fill a void in free space, and demonstrates that stromal cells can close a tissue gap through the coordinated action of matrix contraction, cell migration and ECM remodelling. Importantly, morphogenetic events such as described herein involve spatial reorganization and deformation of ECM that...
cannot be captured by planar substrates. Hence, this bioengineered 3D model of wound closure may become a valuable tool to investigate the underlying mechanics of gap closure and ECM remodeling.

**Methods**

**Device fabrication.** Devices were fabricated as described previously. Briefly, layers of SU-8 photoresist (Microchem) were patterned onto silicon wafers by successive spin coat, alignment, ultraviolet exposure and baking steps. All masters were developed in a single step in propylene glycol methyl ether acetate (Sigma-Aldrich) and the PDMS templates were sterilized in 70% ethanol followed by ultraviolet sterilization for 15 min before treatment with 0.02% pluronic-F127 (Sigma-Aldrich) solution for 10 min at room temperature.

**Cell culture.** NIH 3T3 cells (American Type Cell Culture) were expanded in high-glucose (4.5 g/l) DMEM containing (GIBCO) 10% bovine serum and 100 U/mL Penicillin and 100 mg/mL Streptomycin. Passage 4 to Passage 18 cells were used in our experiments.

**Microtissue formation and wound repair model.** One million 3T3 cells were suspended in 2 mg/ml liquid neutralized collagen type I from rat tail (BD Biosciences) and seeded in the device. The entire assembly was centrifuged to drive cells into the chambers. Excess solution was removed, leaving only within the chambers, and the remaining constructs were centrifuged once again in an inverted configuration to resuspend the cells into the collagen matrix before polymerization. A few hours after polymerization, we observed the spontaneous contraction of the collagen matrix by the cells. Cantilevers incorporated within each chamber spatially restricted the contraction of the collagen matrix, whereby the contraction gels slide up the cantilevers and are then caught by the larger end caps, resulting in a large array of microtissues anchored to the tips of the cantilevers (Supplementary Fig. 1). To visualize the collagen and fibronectin matrix, 4% (w/w) Alexa-568-labeled collagen (Alexa Fluor 568 labelling dye from ThermoFisher, A-20000) was added to the medium during polymerization. A few hours after polymerization, the collagen matrix was fixed in a single step in propylene glycol methyl ether acetate (Sigma-Aldrich), followed by hard bake. Polydimethylsiloxane (PDMS, Sylgard 184, Dow-Corning) were developed in a single step in propylene glycol methyl ether acetate (Sigma-Aldrich), followed by hard bake. Polydimethylsiloxane (PDMS, Sylgard 184, Dow-Corning) were patterned onto silicon wafers by successive spin coat, alignment, ultraviolet exposure and baking steps. All masters were developed in a single step in propylene glycol methyl ether acetate (Sigma-Aldrich) and the PDMS templates were sterilized in 70% ethanol followed by ultraviolet sterilization for 15 min before treatment with 0.02% pluronic-F127 (Sigma-Aldrich) solution for 10 min at room temperature.

Inhibitor experiments. Medium containing pharmacological inhibitors was added just before damaging the microtissues. In this study we used RhoActivator II (10 ng/ml, Cytoskeleton), Rhoinhibitor (100 ng/ml, Cytoskeleton), Y27632 (25 μM, Tocris), Blebbistatin (20 μM, Sigma), CytoD (4 μM, Sigma) and PF573.228 (5 μM, Tocris). For the antibody-blocking experiments, we added rat anti-mouse...
Two-dimensional elliptical scratch assay. PDMS stencils with four 500-µm-tall cylindrical pillars were microfabricated, treated with 0.2% F127 for 10 min and placed in a 12-well plate before seeding with 3T3 fibroblasts in growth medium. After the cells formed confluent monolayers, the pillars were removed and closure was evaluated in the presence of cytotoxic drugs using time-lapse microscopy.

Time-lapse microscopy. For wide-field imaging, microtissues were labelled with Hoechst 33342 (Sigma), to visualize the nuclei of the cells. Phase-contrast and fluorescent images were acquired every 30 min and 20 min, respectively, with a Polaview Evolve 16-bit-multiplying charge-coupled device camera (Photometrics) and an A-Plan 10 objective mounted on a Nikon Ti Eclipse (Nikon Instruments, Inc.) microscope equipped with a live cell incubator. To visualize fibronectin dynamics, Alexa-488-conjugated fibronectin (from human plasma, 8 µg ml⁻¹) was added to the medium during microtissue formation. The next day, medium was replaced before damaging. Twelve hours after wounding, samples were imaged with an LD-C apochromat 63 × 1.15 numerical aperture, water-immersion objective mounted to a Zeiss Axiovert 200M inverted microscope (Carl Zeiss) equipped with a CSU10 spinning disk confocal scan head (Yokogawa Corp.) and a white light illuminator. Time-lapse data were acquired every 20 min and 3-µm spacing in the axial plane. To reduce phototoxicity, Oxyfuor (Fisher) was added to the medium (1:100). After image acquisition, the hyperstack was processed in imageJ using noise reduction filters, log transformation of the histogram, maximal z-projection and contrast/brightness enhancement. To make contrast of the fibronectin fibres in Fig. 5c, the histogram of maximal z-projections of image stacks taken at three different time points was inverted and recoloured using Photoshop CS4 (Adobe).

Image processing and contractility measurements. Algorithms to measure the wound area and the size of the tissues from time-lapse videos were implemented in Matlab (Mathworks, MA). The programme accepts an input video and based on a brightness threshold generates regions that fill empty spaces inside and around the microtissues. These regions are analysed to calculate tissue width, gap shape and tissue shape. TrackMate, a plugin for ImageJ, was used to automatically track individual nuclei and characterize their trajectories from time-lapse videos. The tracks were then imported into Matlab and analysed for direction of motion and velocity. Windrose graphs were generated from histograms of several data points at different locations within the microtissues. Contractility measurements were performed as described in our previous work.39,40 Fluorescent microbeads (Fluoresbrite 17147; Polysciences, Inc.), embedded in the caps of the cantilevers, were used for computerized deflection tracking using the Spot Tracker plug-in in Fiji.38 Briefly, the position of fluorescent beads located at the top surface of the cantilevers was measured during the course of the experiment. After recording the time-lapse data, tissues were disintegrated with collagenase, to determine the baseline position of the same beads. Cantilever displacements were measured by subtracting the baseline position of fluorescent beads from the position at a given time point. Total contractility was calculated by multiplying the sum of cantilever displacement with the spring constant (k = 2.67 ± 0.31 μN m⁻¹) of the cantilevers at a PDMS-curing agents ratio of 1:10. The spring constant of cantilevers was measured using capacitive force sensors (FT-S100, FemtoTools GmbH) mounted on a microrobotic manipulation platform.39

Immunohistochemistry. Microtissues were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS, blocked in 10% Goat serum for 1 h at room temperature, followed by incubation with antibodies against phospho-myosin light chain 2 (Thr19/Ser19) (Cell Signaling Technologies, #3674L, 1:100), fibronectin (Abcam, ab82143, 1:100), cellular fibronectin (Sigma, clone 5H10–27, Abcam) and ArH anti-mouse (Abcam, ab18430) was used as isotype control.

Statistical analysis. Results are presented as mean ± S.E.M. Statistical analysis was performed using JMP Pro 11 (SAS Institute). Differences between experimental conditions were compared by analysis of variance followed by post-hoc Dunnett’s test. For the migration data, a Kruskal–Wallis test followed by a Dunn Method For Joint Ranking post-hoc analysis was used. Significance values are indicated as *P<0.05, **P<0.01 and ***P<0.001.

Code availability. Please see Supplementary Data 1 and 2 to access the computer code generated for processing the time-lapse images of microtissues and quantifying tissue morphology.

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Author contributions

M.S.S., J.E. and C.S.C. designed the study. M.S.S. and J.E. performed experiments and analyzed data. M.S.S., J.E., R.P. and D.E. contributed new reagents and analytical tools. M.S.S., J.E., B.J.N. and C.S.C. wrote the paper.

Additional information

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