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Ultrastructural investigation on fibroblast interaction with collagen scaffold

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Abstract: Collagen-based scaffolds are used as temporary or permanent coverings to help wound healing. Under natural conditions, wound healing is affected by such factors as cell types, growth factors and several components of the extracellular matrix. Due to the complexity of the cell-to-matrix interaction, many cell based mechanisms regulating wound healing *in vivo* are not yet properly understood. However, the whole process can be partially simulated *in vitro* to determine how cells interact with the collagen scaffold in relation to such features as physico-chemical properties, matrix architecture and fiber stability. Under these conditions, cell migration into the collagen matrix can be easily assessed and causally correlated with these features. In this study, we aimed at providing a structural analysis of how NIH3T3 fibroblasts migrate and proliferate *in vitro* when seeded on a native type-I collagen scaffold. To this end, samples were collected at regular time intervals and analyzed by light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Through this experimental approach we demonstrate that collagen is gradually frayed into progressively thinner fibrils as fibroblasts migrate into the matrix, embrace the collagen fibers with long filopodia and form large intracellular vacuoles. A key role in this process is also played by microvesicles shed from the fibroblast plasma membrane and spread over long distances inside the collagen matrix. These observations indicate that a native type-I equine collagen provides favorable conditions for simulating collagen processing *in vitro* and eventually for unraveling the mechanisms controlling cell uptake and intracellular degradation. © 2015 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2015.

Key Words: fibroblasts, collagen matrix, electron microscopy, *in vitro* culture, microvesicles

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INTRODUCTION

Wound healing is a physiological process naturally addressed to repairing skin injury. It consists of a complex cascade of molecular events, from removal of damaged tissues to restoration of normal morphogenesis.[1,2] To this end, the extracellular matrix provides a structural scaffold for cells to migrate through the wound area and differentiate into specific functional types.[3] As a major constituent of the extracellular matrix, the collagen plays a key role in the regenerative process of the wound by acting as an anchoring support for the migrating cells.[4,5] Because of its relative abundance in bodily proteins, low immunogenicity and high stability, the collagen is *par excellence* the best biomaterial employed as a prosthetic medical device to treat dermal injury.[6,7] However, to satisfy these requirements, the collagen scaffold should be endowed with such physico-chemical properties as mechanical integrity, structural uniformity and have a matrix porosity suitable for sustaining cell migration.[8] In addition, the matrix itself should be such to be retained on the wound bed for time intervals sufficient to complete tissue remodelling.[9]

Prosthetic collagen matrices differ in absorption and stability depending on the type and concentration of chemical substances added to modulate these capacities.[10–12] Therefore, a wide variety of collagen-based dressings differing in structural and biochemical properties have been developed with the intent to simulate the overall function of the extracellular matrix and improve the regenerative process of the injured area in normal or chronic situations.[13,14] Amongst the physiological variables that characterize the collagen matrix, the overall three-dimensional architecture and porosity are the most relevant since they condition such cell behaviours as proliferation, migration and, ultimately, signalling and differential gene expression.[15–17] Matrices with high porosity values provide healthier healing environments, especially in case of chronic wounds, due to their capacity to absorb more efficiently blood and lymphatic fluids from the wound area.[9,18]

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Wound healing entails proliferation of a variety of cell types including fibroblasts, endothelial cells and epithelial cells, besides restoration of the extracellular matrix. Since it is highly improbable that such a complexity could be mimicked in monolayer cell cultures, cell-to-matrix interactions have been explored in 3D collagen matrices in order to create a more realistic *in vivo*-like environment.[9,19] Under these conditions, the structural alterations of the collagen scaffold may be experimentally monitored with relative ease. Because of these unique structural features of 3D cultures,[20–22] we have recently examined how different collagen products become structurally modified upon *in vitro* exposure to migrating cells and how long they may persist invariant before being fully disaggregated. We found that the best structural conditions for cells to adhere and migrate inward are provided by native scaffolds characterized by a high matrix porosity, a maximally extended inner surface and thick collagen bundles.[14] For a scaffold to satisfy these requirements, the collagen matrix should be made of highly interlaced strands comprised of properly aligned and tightly packed native collagen fibers. In this study, we report how fibroblasts behave when cultured in this type of native collagen matrix and we envision their changing morphological features by transmission electron microscopy (TEM) and scanning microscopy (SEM). We focus on the structural modifications the native collagen matrix undergoes in relation to the migratory behaviour of the fibroblasts and to their ability to shed microvesicles in the surrounding milieu as a prelude to take up and process collagen fragments intracellularly. Since metalloproteinases, especially MMP-2 and MMP-9, are known to cleave collagen I and III in their native form,[23] we wonder whether the process of microvesicle shedding may be causally related with the release of the collagenolytic activity by the fibroblasts. By documenting the extent and the modalities by which fibroblasts and native collagen fibers interact *in vitro* we intend to verify this possibility and to describe the morphological counterpart of this functional activity.

MATERIALS AND METHODS

Collagen scaffold preparation

The collagen used in this study is type I collagen (Biopad) extracted under non-denaturing conditions using the procedure previously described[24] and more recently adapted for the equine Achilles tendon. Briefly, tendons were removed of all surrounding tissues, harvested and cleaned in deionized water. They were then treated with 10% sodium chloride at 4°C for 24 h under continuous magnetic stirring. The mixture was centrifuged and the precipitated collagen fibers repeatedly washed and eventually recovered with a 0.5% *w/v* solution of acetic acid. The resulting collagen slurry was freeze-dried and soon sterilized with a γ-ray dose of about 1 Mrad. Collagen titre was evaluated by determining the total nitrogen and hydroxyproline contents of the lyophilized product, followed by electrophoretic and electron microscopic analyses. By using this extraction procedure, the collagen scaffold contained no additional proteins from the extracellular matrix, at least within the detection limits of both western blotting and immunoelectrophoretic assay.[25]

Cell culturing

Stabilized murine fibroblasts (NIH 3T3) were used throughout this study. To collect enough cells for plating on collagen matrices, NIH 3T3 fibroblasts were first cultured in 75 cm^2 flasks containing DMEM (Dulbecco's Modified Eagle's Medium) culture medium, supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin/streptomycin, and maintained in an incubator at 37°C under continuous 5% CO_2 flow and controlled humidity. Upon reaching confluence, cells were passed into new culture vessels in a 1:5 ratio and the medium was changed every three days.

3D collagen scaffolds were cut with a sterile razor blade into blocks of approximately 1 cm^2 of surface area each and inserted into 24-well plates. Aliquots of 2×10^5 cells were seeded on the upper surface of every collagen block and incubated at 37°C in 1.5 mL of culture medium under a continuous flow of 5% CO_2 for time intervals ranging from 4 h to 14 days. Medium was then renewed every three days.

For 2D control experiments, NIH 3T3 fibroblasts were seeded on 24 well plates and incubated in 1 mL of culture medium at the same conditions as the 3D cell-cultures and assayed at regular time intervals for cell viability and microvesicle release.

Cell viability

Cell viability was monitored by measuring the activity of the mitochondrial enzyme succinate dehydrogenase by using the MTT test. Since reduction of MTT is correlated with cell metabolic activity, cells metabolically active are expected to yield high MTT values. At each incubation time, 1 mL aliquots of culture medium, containing both collagen scaffolds and cells, were withdrawn from each well. Subsequently 0.1 mL MTT were added to every aliquot and left to incubate for 4 h in the dark at 37°C under a 5% CO_2 flow. By the end of this incubation time, the resulting formazan crystals were completely dissolved in DMSO glycine buffer at pH 10.5 and the relative absorbance measured at a wavelength of 570 nm. Absorbance values for NIH 3T3 cells were measured at each time point of the entire culture period in both 2D plates and 3D matrix.

Cell retention

The initial number of cells trapped onto the collagen scaffolding at 4 h of incubation time (Ci) was considered equivalent to the number of cells present in the original cell suspension (Cs) subtracted of the cells adhering to the well (Ca). Cs was determined by manual cell count using a Neubauer chamber, while Ca was determined by counting cells stripped from each well following trypsin treatment.

Collagen matrix structure

The scaffold architecture of the collagen matrix was structurally characterized by scanning electron microscopy (SEM). To this end, collagen scaffolds were cut with a sterile razor blade into a number of small blocks and attached to aluminum stubs with a carbon tape. Blocks were oriented in such a way as to expose either the upper or the lower surfaces, while others were glued on their longitudinal cut

profile. They were sputter-coated with gold in a Balzers MED 010 unit and observed in a JEOL JSM 6010LA electron microscope. The native structure of collagen was also checked by TEM as specified below.

Fibroblasts and collagen matrix co-culturing

Collagen scaffolds inclusive of their cellular loads were cut into a number of small blocks with a sterile razor blade and fixed overnight at 4°C with 3% glutaraldehyde in phosphate buffer (PB) at pH 7.2. Following extensive rinsing with the same buffer at 4°C, they were immersed for 1 h in 0.5% tannic acid in PB at 4°C. They were then rinsed again four times in the same buffer for 15 min at 4°C, and eventually post-fixed for 1 h with 1% osmium tetroxide in PB at 4°C. Specimens were finally washed in distilled water, block-stained with 1% uranyl acetate in distilled water and then dehydrated in a graded series of ethanols. For SEM, samples were first dried by the critical point method in a Balzers Union CPD 020, sputter-coated with gold in a Balzers MED 010 unit and then observed in a JEOL JSM 6010LA electron microscope. For TEM samples were fixed and dehydrated as described above and then infiltrated for two days with graded mixtures of LRWhite resin/ethanol. By the end of this procedure, samples were embedded in fresh LRWhite resin and cut with Reichert Ultracut ultramicrotome equipped with a diamond knife. Ultrathin sections (60–80 nm thick) were collected on copper grids, stained with uranyl acetate and lead citrate, and observed with a JEOL 1200 EX II electron microscope. Micrographs were acquired by the Olympus SIS VELETA CCD camera equipped the iTEM software.

For light microscopy (LM), 1 μm thick sections were collected on glass slides, stained with toluidine blue and observed with an optical Zeiss Axioskop2 plus microscope. Images were collected with a video camera AxioCam MRC and elaborated with the software Axiovision4.

Matrix porosity

SEM images were subjected to thresholding to convert matrix pores and collagen fibrils into black and white images. This procedure allows to adjust pixel density and to eliminate collagen fibrils from the image background. The 2D black and white matrix representations that are so obtained are then evaluated as particle sizes and their distribution analyzed with an image program (Image J).

Statistical analysis

The data from eight independent experiments on cell migration in 3D collagen scaffolds were used and expressed as means ± SD. Statistical analysis was preformed using one-way ANOVA test with a Stat-Plus software (AnalystSoft ©2009) with the threshold of significance set at $p < 0.05$.

RESULTS

Scaffold characterization

To assess the overall stability of the collagen scaffold, 0.5 × 0.5 mm blocks were cultured *in vitro* for periods ranging from a minimum of 4 h for up to 14 days, either in the presence or absence of cells. Figure 1 shows how the

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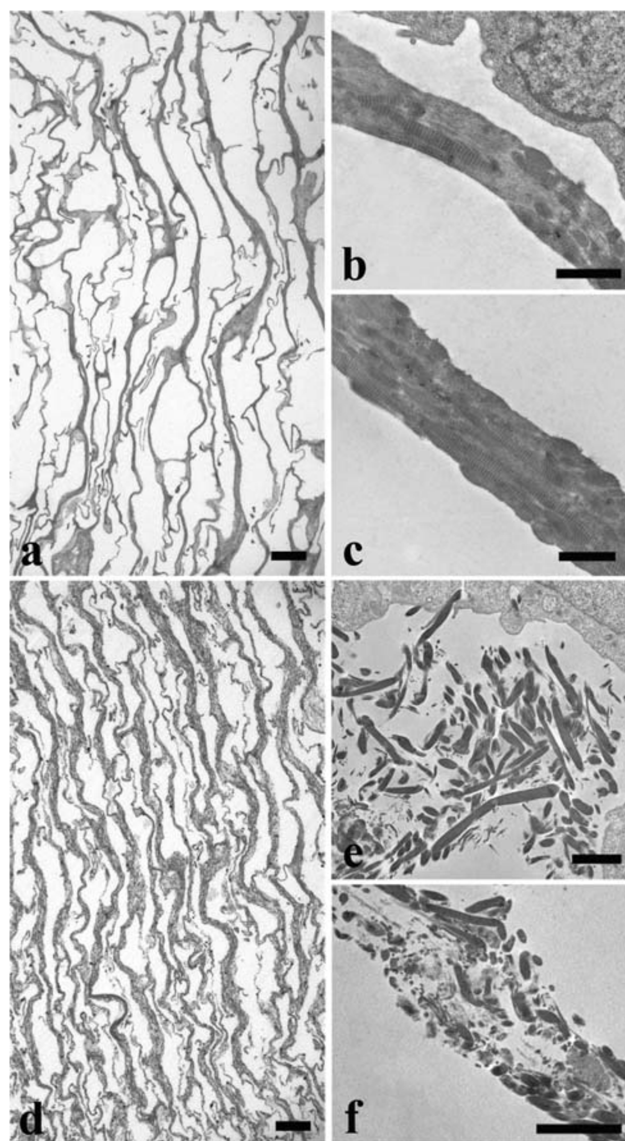

FIGURE 1. LM and TEM images showing laminas of the collagen scaffold following 4 h (a and b) and 14d (c and d) of *in vitro* culture in the presence of cells (a and c) or without cells (b and d); (a) and (d), Bars 50 μm; (b) and (c), Bars 1 μm; (e), Bar 2,5 μm. (f), Bar 3 μm.

collagen scaffold changes during this culturing period. From a compact structure with highly intertwined fibers (Fig. 1a–c), the scaffold is gradually transformed into a rather loose assembly of dispersed collagen fibers (Fig. 1d–f). These changes occur regardless of whether cells are present or not. However, they are somehow accelerated and collagen fibers become more dispersed if the scaffold is cultured in the presence of cells (compare Fig. 1e,f).

Cell/collagen matrix interaction

NIH 3T3 fibroblasts were cultured on collagen scaffold matrices as specified in Materials and Methods, and their distribution assessed at regular time intervals by LM and SEM. Figure 2 shows that, at 4 h after seeding, numerous fibroblasts were retained on the upper scaffold surface and

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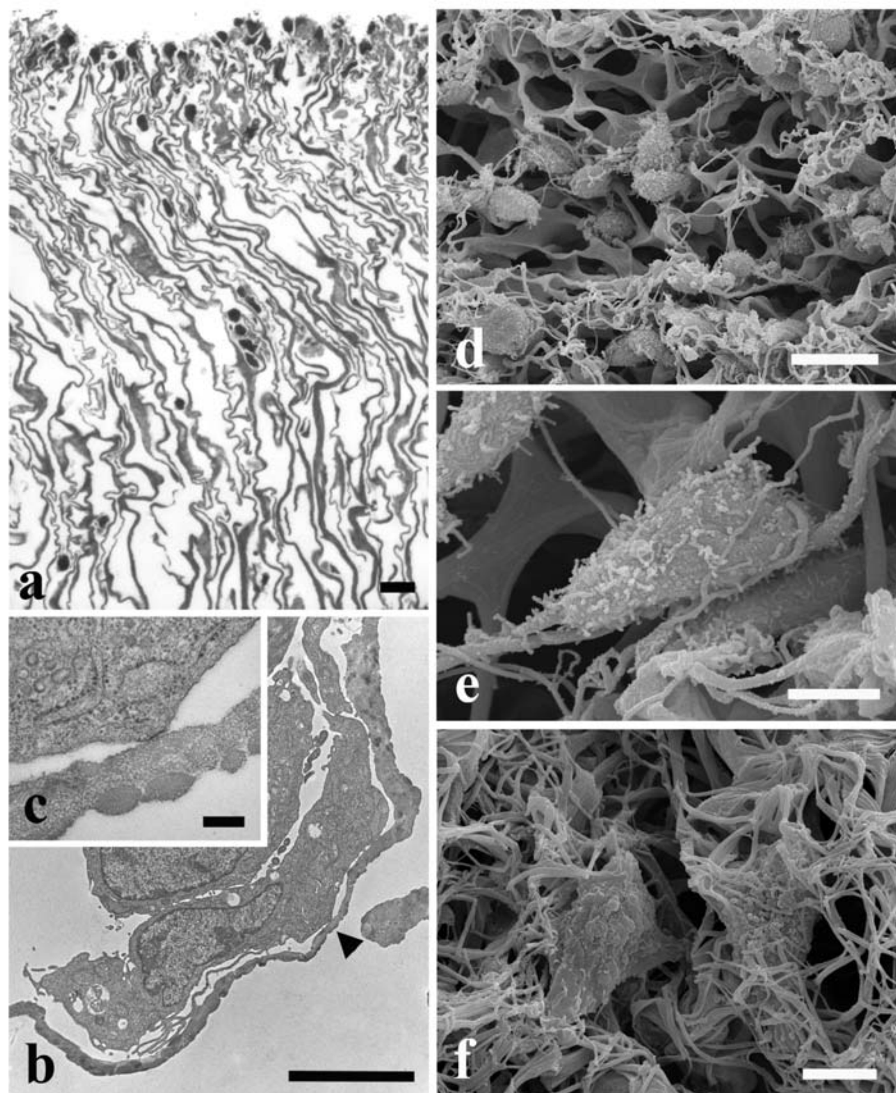

FIGURE 2. (a) LM image showing numerous fibroblasts on the upper surface of the collagen scaffold. Note that some cells have already migrated inward, Bar 20 μm. (b) TEM micrograph showing fibroblasts closely adhering to a collagen fiber (see arrowhead), Bar 5 μm. (c) enlargement of the adhesion point, Bar 200 nm. (d) SEM micrograph showing a number of fibroblasts on the top surface of the collagen scaffold, Bar 20 μm. (e) SEM micrograph showing a fibroblast amidst collagen fibers 4 h after seeding, Bar 5 μm. (f) SEM micrograph showing fibroblasts on the collagen scaffold 7 days after seeding, Bar 5 μm.

some cells had already migrated into deeper regions of the collagen matrix (Fig. 2a). On entering the matrix, fibroblasts became highly intertwined with the collagen fibers, having most of their cell surface in close contact with the collagen fibers and occasionally forming specific adhesion plaques (Fig. 2b,c). In the presence of the collagen scaffold, fibroblasts apparently assumed different shapes, ranging from very roundish cells (Fig. 2d) to highly polarized ones (Fig. 2e). As culturing time was prolonged to 14 days, fibroblasts became even more entangled with the collagen fibers, eventually forming thick bundles of weaved filaments (Fig. 2f).

Cell/collagen matrix interaction: incorporation

Following 1 day of culture, fibroblasts were already anchored onto the collagen laminas, but on average they maintained a low cell surface roughness with very few filopodia extending outwardly (Fig. 3a,b). However, with the continuation of the co-culture period for up to 14 days, the fibroblast plasma membrane weaved intimately with the collagen matrix by extending with an increasing number of filopodia (Fig. 3c). Figure 3d shows how some of these filopodia can actually embrace entire collagen fibers. A number of small vesicles appeared to be shed from the fibroblast cell surface in the proximity of the embraced fibril (Fig. 3e). Additional disaggregation of the collagen scaffold could be observed when fibers started to be gradually frayed into thinner fibrils (Fig. 3f). As these changes occurred, fibrils were seen gradually reduced in mean diameter, deprived of their staggered banding patterns and eventually taken up into large vacuoles by fibroblasts (Fig. 3g). Fibroblast filopodia are likely to play an active role

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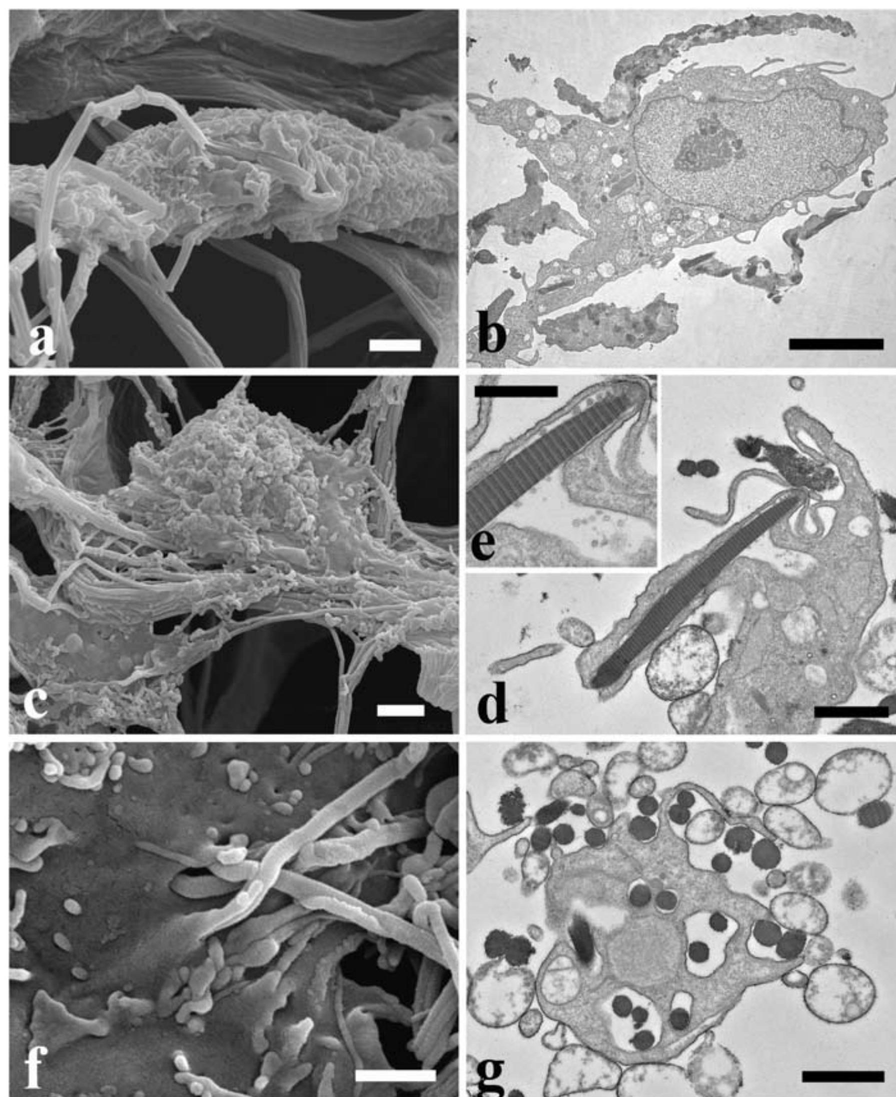

FIGURE 3. 3T3 fibroblasts cultured on collagen scaffold for up to 7 days. SEM (a) and TEM (b) micrographs showing fibroblast anchored onto collagen fibers after 1 day of culture. (a) Bar 2 μm. (b) Bar 5 μm. SEM (c) and TEM (d) micrographs showing a fibroblast highly intertwined with a collagen fiber after 4 days of culture. (c) and (d) Bars 2 μm. (e) enlargement of Figure 3d showing a number of microvesicles, Bar 500 nm. SEM (f) and TEM (g) micrographs showing an enlargement of a fibroblast surface with several collagen fibers entering the cell interior, as seen in cross sections. (f) and (g), Bars 1 μm.

in this engulfment process by first forming hairpin-like protrusions and then folding them back onto the cell surface to trap collagen fibers in a sort of vacuolar enclosure (Fig. 3d).

An extensive ultrastructural analysis indicated that filopodia are dynamic structures, highly variables both in mean diameter and overall length (Fig. 4a). Besides taking direct contact with the collagen fibers (Fig. 4b), filopodia are also involved in releasing a number of microvesicles onto the collagen matrix. In all likelihood, these microvesicles emerge from the fibroblast surface through an active process of budding from the filopodial tips. Instances of actual continuities of filopodia with emerging microvesicles have been recorded several times (Fig. 4c). As microvesicles bud off from the fibroblast cell surface they recruit inside part of the cortical cytoplasm, including a number of smaller intraluminal vesicles (Fig. 4d). From this cell site they may migrate at a certain distance from the cell surface and disperse into the collagen matrix (Fig. 4e). With the continuation of the co-culture for up to 14 days, microvesicles increase in number, appearing also at a far distance from the fibroblast cell surface and highly intermingled with frayed collagen fibrils (Fig. 4f).

F4

With the continuation of the cell culture, both fibroblasts and collagen matrix undergo additional changes. In particular, the fibroblast cytoplasm gradually filled with vacuoles containing material of varying consistency and density. Occasionally, some of the material in these vacuoles was seen in actual continuity with collagen fibrils (Fig. 5a). These vacuoles are likely to be formed by phagocytosis of collagen fibers through embracement and folding of the filopodia followed by invagination of the enclosed plasma membrane (Fig. 5b). However, these collagen vacuoles are complex organelles containing not only exogenous material

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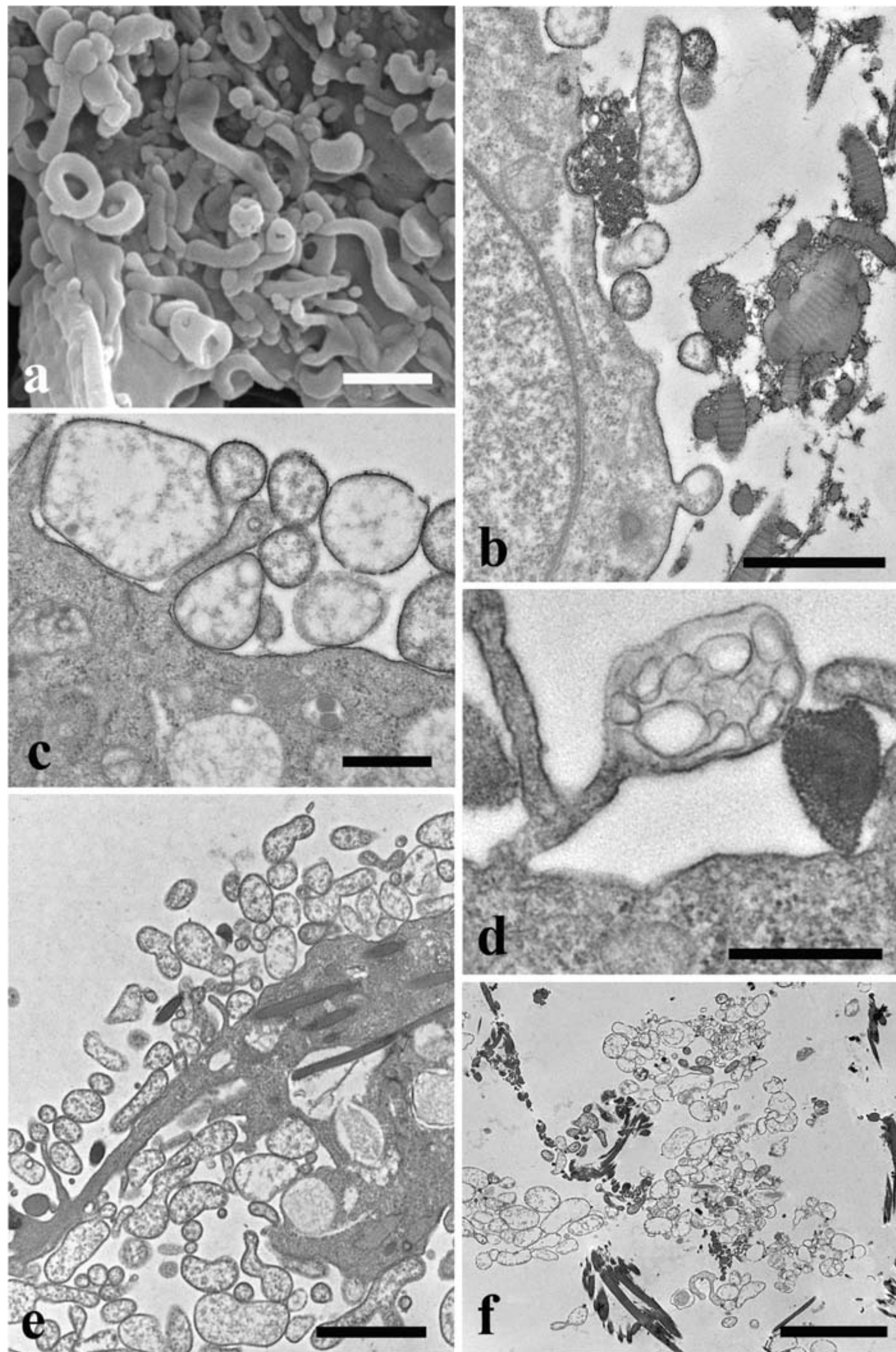

FIGURE 4. (a) SEM micrograph showing the rough surface of a fibroblast, Bar 1 μm. (b) TEM micrograph showing a fibroblast surface with numerous filopodia and microvesicles closely adjoined to collagen fibers, Bar 1 μm. (c) TEM micrograph of fibroblast showing numerous filopodia and microvesicles protruding from the cell surface, Bar 500 nm. (d) TEM micrograph showing a microvesicle budding from a filopodium tip, Bar 500 nm. (e) TEM micrograph of a 7 day fibroblast highly entangled with collagen fibers and microvesicles, Bar 2 μm. (f) TEM micrograph of collagen fibers and microvesicles in regions of the collagen matrix far way form the fibroblast cell body, Bar 5 μm.

but also material conveyed by other vesicles. These latter are likely to be Golgi-associated vesicles delivering their load of hydrolytic enzymes to the collagen containing vacuoles for additional processing (Fig. 5b,c).

In vitro cultured 3T3 fibroblasts

As a control of the cell-to-collagen interaction experiments, fibroblasts were also cultured *in vitro* and allowed to grow for up to 14 days. Under these conditions, fibroblasts grew and migrated as flat elongated cells, while remaining firmly attached onto the substrate (Fig. 6a,b). Although variable both in shape and extension in relation to such conditions as cell crowding and incubation time, the number of filopodia present on the fibroblast cell surface appeared to increase steadily during the entire culture period (Fig. 6c,d). Numerous microvesicles were also present along the cell

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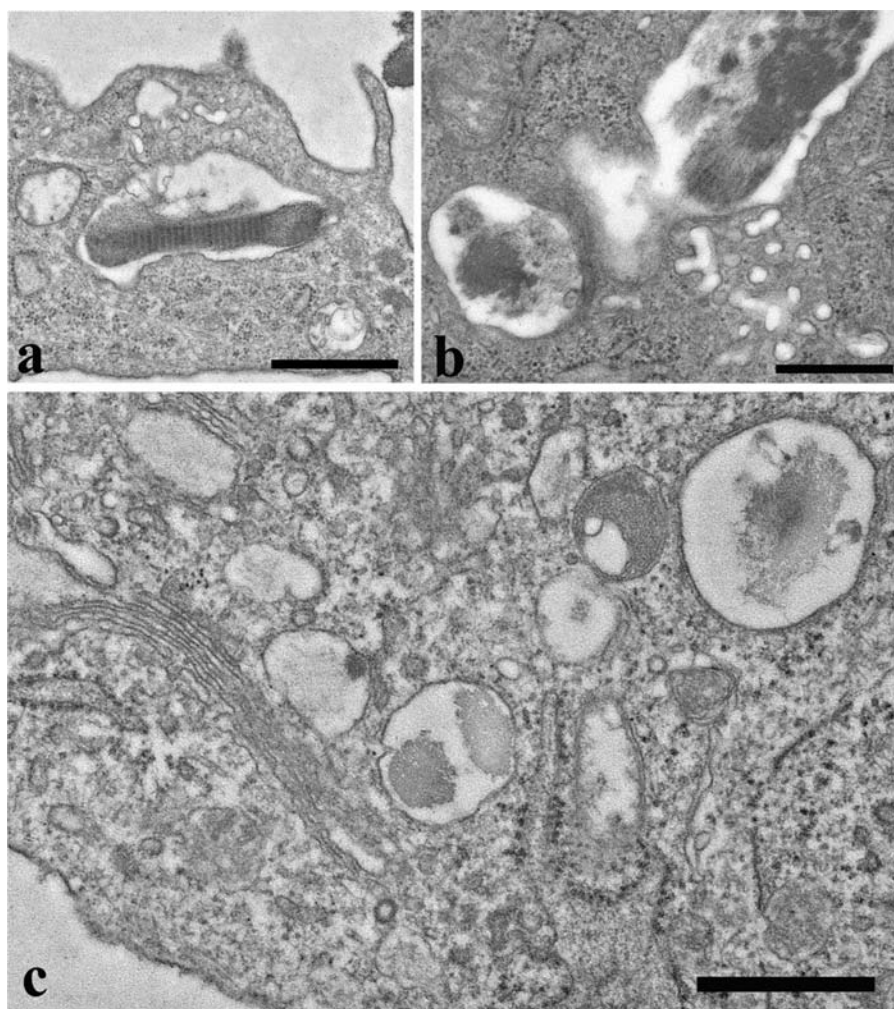


FIGURE 5. (a) TEM micrograph showing a large vacuole in the cortical cytoplasm of a fibroblast containing a collagen fiber, Bar 1 μm. (b) TEM micrograph showing some collagen-containing vacuoles merging with smaller vesicles deriving from the Golgi apparatus, Bar 500 nm. (c) TEM micrograph showing several vesicles with some electron dense material therein, close to a Golgi apparatus, Bar 500 nm.

contours of many fibroblasts, even though they could not be discerned in regions far from the cell surface, indicating that they had to remain in the cell proximity for lack of any additional anchoring site. Fibroblasts appeared to release microvesicles *in vitro* in much the same way as in the presence of collagen scaffolds, that is, as buds from the filopodial tip, the only difference being their number and position relative to the cell body (Fig. 6e,f) that appeared to be smaller than in the presence of the collagen scaffold.

Cell kinetics

A rough estimate of the number of fibroblasts migrating in collagen scaffolds as a function of culturing time indicated that a progressively higher number of fibroblasts could be found in matrix regions further away from the seeded side of the scaffold (data not shown).

However, taking the MTT test as a reliable tool to measure cell viability and cell proliferation, the fibroblast/collagen interaction seems to be more complex than simple cell counting could suggest. Table I indicates that cells increased their MTT activity quite rapidly within a day and then stayed constant for the rest of the incubation period. This is probably caused by fibroblasts starting to proliferate soon after entering the collagen matrix, and declining upon becoming involved in collagen breakdown and absorption. By comparison, cells cultured *in vitro* with no collagen scaffold grew according to a symmetrical kinetics inclusive of slow rise followed by an equally slow decline. This is probably due to the fact that confluence *in vitro* is attained after 3 days of cell culture. Afterward, cells may stop growing and presumably start to be removed by apoptosis (Table I).

T1

DISCUSSION

A variety of collagen scaffolds are available today to help wound healing and skin regeneration. To accomplish these goals, scaffolds ought to satisfy several requirements. First, the pore size has to be maintained within a restricted range for cells to adhere to the collagen matrix and migrate inside the scaffold. Second, the scaffold itself should be sufficiently

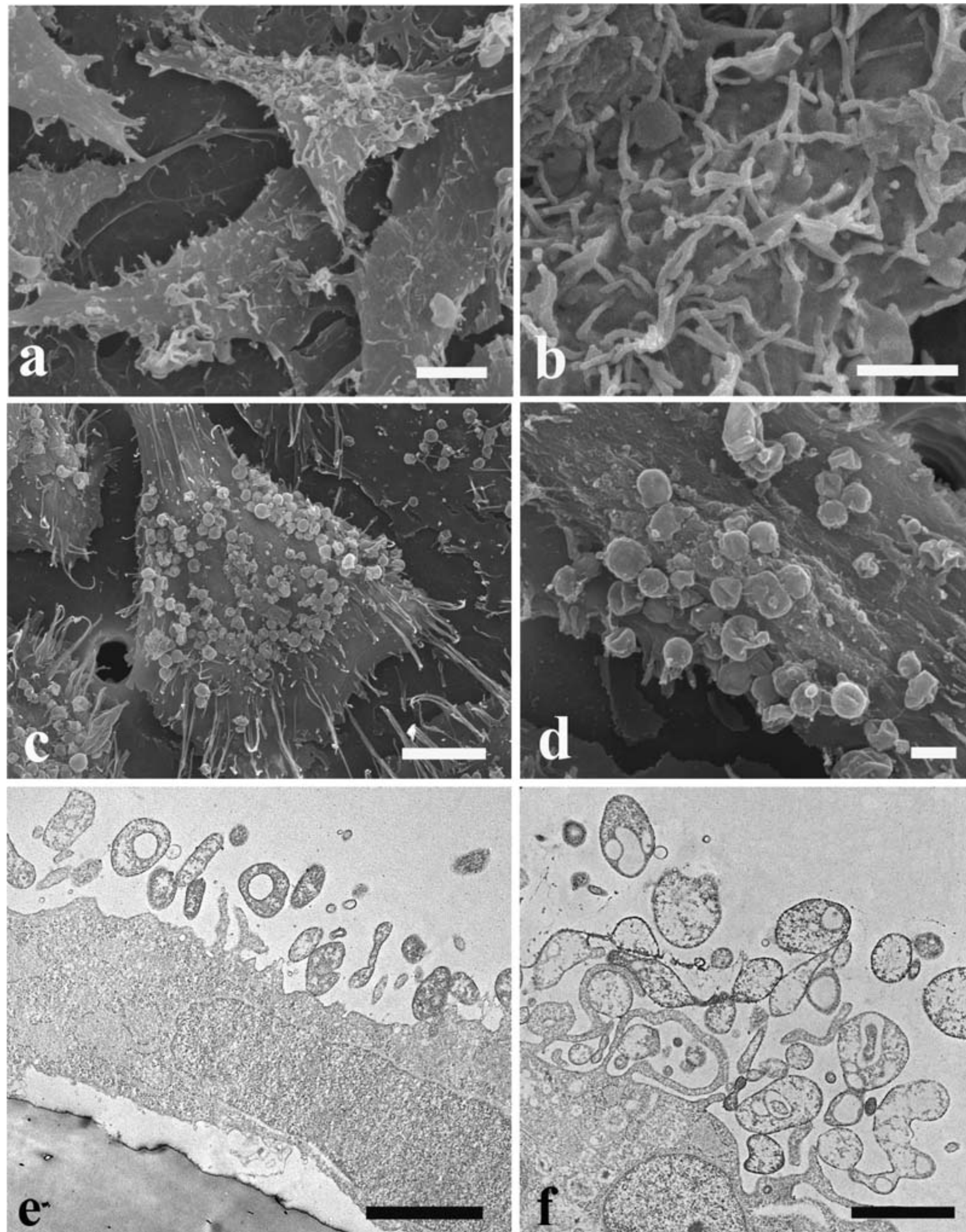
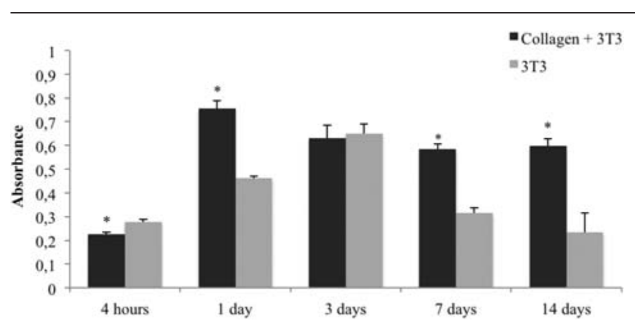


FIGURE 6. (a) SEM micrograph of 1 day *in vitro* cultured fibroblasts, Bar 5 μm. (b) SEM micrograph showing a number of filopodia on the fibroblast cell surface, Bar 2 μm. (c) SEM micrograph of 7 day *in vitro* cultured fibroblasts showing a number of cells highly enriched with filopodia and microvesicles, Bar 5 μm. (d) enlargement of the previous figure showing the extent of microvesicle budding from the cell surface, Bar 1 μm. (e) TEM micrograph of an *in vitro* cultured fibroblast showing a number of filopodia and microvesicles protruding from the cell surface, Bar 2 μm. (f) TEM micrograph showing several microvesicles amidst some tortuous filopodia, Bar 2 μm.

stable to allow new *granulation tissue* to mature, while the injured *tissue* is replaced by new collagen deposition. Third, the collagen matrix should not persist indefinitely, but be degradable within the time limits compatible with the healing process. All of these requirements were apparently verified in the current *in vitro* study where the use of a native collagen scaffold allowed fibroblasts to migrate and adhere to the collagen matrix much more efficiently than in other type of scaffolds tested in earlier investigations.[14] A uniform matrix architecture and a pore size range of about 100 ± 150 mm[8] are in fact among the best structural conditions that a prosthetic collagen scaffold should provide to sustain a cell migratory behaviour compatible with *in vivo* wound healing, as shown by a number of recent studies on scaffolds differing in either collagen compositions[14] or pore sizes.[26] In fact, the extent by which cells migrate and interact with the scaffold *in vitro* are important parameters to determine the overall quality of the 3D microenvironment[27]

TABLE I. Histogram Showing Cell Viability of 3T3 Fibroblasts Cultured for Over a Period of 14 Days (Grey Columns) or Co-cultured for the Same Length of Time in the Presence of a Biopad Collagen Scaffold (Black Columns). Asterisks (*) for $p < 0.05$.



and to predict its propensity to mimic the extracellular matrix on the wound bed.[28,29]

Wound healing is a rather complex phenomenon requiring the contribution of several cell types and the activation of a variety of growth factors and cytokines[30] for the injured tissues to be properly remodelled. Since fibroblasts appear to play a major role in remodelling the extracellular matrix,[31] in this study we have followed the fate of 3T3 fibroblasts as they migrate and proliferate in 3D collagen scaffolds for up to 14 days in culture. During this time period, cells were seen to adhere to the collagen matrix and change their overall morphology, while the collagen fibers themselves were gradually frayed, disaggregated and eventually taken up for intracellular digestion.

As well known, *in vivo* remodelling of the extracellular matrix entails *de-novo* collagen synthesis[32] and removal of the fragmented collagen fibers.[33,34] A balance between the two activities has to be maintained for the wound to be properly healed,[35] as otherwise excessive collagen accumulation may lead to fibrotic changes in the skin or to arterial stenosis in the cardiovascular system.[36] Likewise, enhancement or reduction of collagen degradation by fibroblast phagocytosis may result in the alteration of the extracellular matrix, eventually leading to the deregulation of cell proliferation and/or loss of cell differentiation.[37]

Even though collagen degradation by phagocytosis may appear to be the main pathway to remodel the extracellular matrix in wound healing, it may not suffice to account for the complexity of the morphological alterations exhibited by collagen prior to cellular uptake, as described in this study (see Figs. 3c–g and 5a–c). The process of collagen degradation is certainly comprised of an intracellular pathway involving phagocytosis, as reported by a number of earlier investigations,[38–40] but it includes also an additional pathway requiring the release of metalloproteinases for processing the collagen fibers extracellularly.[37] Fibroblasts are in fact known to express and secrete a number of diverse metalloproteinases (MMPs) as inactive pro-enzymes and activate them extracellularly in response to a variety of stimuli.[41,42] In spite of this knowledge, however, it is still uncertain how collagen fibrils are partially processed by MMPs and how eventually they are recruited by fibroblasts for intracellular digestion.[43] In recent years, it has become apparent that metalloproteinases are released directly from the fibroblast plasma membrane in the form of shedding microvesicles.[44,45] It has also been repeatedly shown that, by doing so, microvesicles affect both matrix invasiveness and cell migration by acting on the integrity of the collagen itself.[46]

We think the sequence of micrographs reported in this study on 3D scaffolding to be highly suggestive of the occurrence of a collagen processing brought about by proteolytically active microvesicles, followed by the endocytic uptake of the resulting frayed collagen fibrils. As clearly documented here, 3T3 fibroblasts cultured in the presence of a native equine collagen scaffold undergo dramatic changes both in cell shape and in cell surface extensions, becoming highly convoluted with numerous filopodia and microvesicles budding from the plasma membrane. In our experience, prosthetic dressings with lower collagen contents or denatured by cross-linking do not elicit similar cell responses when co-cultured with fibroblasts,[14] even though cross-linking may *per se* improve the collagen tensile properties and its implanted durability.[47] Since protrusion of filopodia are typical of cells capable of altering their actin cytoskeleton in response to variations in the substrate roughness,[48,49] it is conceivable that only adhesion to native collagen fibers may induce fibroblasts to express such a responding repertoire. By doing so they are likely to act as cellular tentacles pulling collagen fragments toward the fibroblast cell surface,[50] and merge into newly developed luminal compartments in the fibroblast cytoplasm.[51]

Although the role played by membrane shedding in relation to collagen processing remains to be experimentally proved, it is quite likely that microvesicles may serve as vectors transferring metalloproteinases onto the collagen matrix. Microvesicles are in fact known to be a universal type of organelle sustaining cell communication in a variety of metabolic and developmental functions.[52] Thus by proposing microvesicles as key actors in the extracellular processing of the collagen scaffold we are in line with the large body of evidence that attribute multifunctional roles to this new type of organelle in cell-to-matrix relationships.[45,53]

CONCLUSION

This study is an electron microscope investigation documenting how fibroblasts and collagen matrices are morphologically modified when co-cultured *in vitro* for up to 14 days. The extent by which cells and matrix interact depend on such factors as pore size range, mechanical stability and susceptibility to proteolytic degradation by metalloproteinase. We do believe that these requirements are more likely to be satisfied if the scaffold is comprised of 100% native collagen, as earlier reports have already demonstrated.[14] Under these conditions, cells are allowed to proliferate, migrate and adhere to collagen fibers through

extended filopodia and microvesicle shedding. We interpret the micrographs depicting this interaction as the morphological counterpart of a collagen proteolysis brought about by microvesicles transferring metalloproteinases to the collagen matrix as a prelude to its degradation by fibroblasts.

REFERENCES

1. Flanagan M. The physiology of wound healing. J Wound C 2000; 9:299–300.
2. Diegelmann RF, Evans MC. Wound healing: An overview of acute, fibrotic and delayed healing. Front Biosci 2004;9:283–289.
3. Clark RAF. Fibrin and wound healing. Ann NY Acad Sci 2001;936: 355–367.
4. Gattazzo F, Urciuolo U, Bonaldo P. Extracellular matrix: A dynamic microenvironment for stem cell niche. Biochim Biophy Acta 2014;1840:2506–2519.
5. Friedl P, Bröcker EB. The biology of cell locomotion within three-dimensional extracellular matrix. Cell Mol Life Sci 2000;57:41–64.
6. Murphy PS, Evans GR. Advances in wound healing: A review of current wound healing products. Plast Surg Int 2012;2012:190436
7. Chattopadhyay S, Raines RT. Collagen-based biomaterials for wound healing. Biopolymers 2014;101:821–833.
8. Angele P, Abke J, Kujat R, Faltermeier H, Schumann D, Nerlich M, Kinner B, Englert C, Ruszczak Z, Mehrl R, Mueller R. Influence of different collagen species on physico-chemical properties of crosslinked collagen matrices. Biomaterials 2004;25:2831–2841.
9. Lawrence BJ, Madihally SV. Cell colonization in degradable 3D porous matrices. Cell Adh Migr 2008;2:9–16.
10. Lin YK, Liu DC. Studies of novel hyaluronic acid-collagen sponge materials composed of two different species of type I collagen. J Biomater Appl 2007;21:265–281.
11. Brett DA. Review of collagen collagen-based wound dressings. Wounds 2008;20:1–12.
12. Miron-Mendoza M, Seemann J, Grinnell F. The differential regulation of cell motile activity through matrix stiffness and porosity in three dimensional collagen matrices. Biomaterials 2010;31:6425–6435.
13. Ghibaudo M, Trichet L, Le Digabel J, Richert A, Hersen P, Ladoux B. Substrate topography induces a crossover from 2D to 3D behavior in fibroblast migration. Biophys J 2009;97:357–368.
14. Karr JC, Taddei AR, Picchietti S, Gambellini G, Fausto AM, Giorgi F. A morphological and biochemical analysis comparative study of the collagen products Biopad, Promogram, Puracol, and Colactive. Adv Skin Wound Care 2011;24:208–216.
15. Astashkina A, Mann B, Grainger DW. A critical evaluation of in vitro cell culture models for high-throughput drug screening and toxicity. Pharmacol Ther 2012;134:82–106.
16. Iyer P, Walker KJ, Madihally SV. Increased matrix synthesis by fibroblasts with decreased proliferation on synthetic chitosan-gelatin porous structures. Biotechnol Bioeng 2012;109:1314–1325.
17. Lu H, Oh HH, Kawazoe N, Yamagishi K, Chen G. PLLA–collagen and PLLA–gelatin hybrid scaffolds with funnel-like porous structure for skin tissue engineering. Sci Technol Adv Mat 2012;13:1–9.
18. Wang HM, Chou YT, Wen ZH, Wang ZR, Chen CH, Ho ML. Novel biodegradable porous scaffold applied to skin regeneration. PLoS One 2013;8:e56330
19. Beningo KA, Dembo M, Wang Y. Responses of fibroblasts to anchorage of dorsal extracellular matrix receptors. Pnas 2004;101: 18024–18029.
20. Carlson MA, Longaker MT. The fibroblast-populated collagen matrix as a model of wound healing: A review of the evidence. Wound Rep Reg 2004;12:134–147.
21. Doillon CJ, Whyne CF, Brandwein S, Silver FH. Collagen-based wound dressings: Control of the pore structure and morphology. J Biomed Mater Res 1986;20:1219–1228.
22. Tuan TL, Song A, Chang S, Younai S, Nimni ME. In vitro fibroplasia: Matrix contraction, cell growth, and collagen production of fibroblasts cultured in fibrin gels. Exp Cell Res 1996;223:127–134.
23. Bigg HF, Rowan AD, Barker MD, Cawston TE. Activity of matrix metalloproteinase-9 against native collagen types I and III. Febs J 2007;274:1246–1255.
24. Beghé F, Menicagli C, Neggiani P, Zampieri A, Trallori L, Teta E, Rosini S. Lyophilized non-denatured type-I collagen (Condress) extracted from bovine Achilles' tendon and suitable for clinical use. Int J Tissue React 1992;14:11–19.
25. Ignacchiti V, Giorgi F, Menicagli C, Chiaverini L. Contaminating proteins in collagen type I from bovine Achilles' tendon. Chimica Oggi-Chemistry Today 1996;14:68–71.
26. O'Brien FJ, Harley BA, Yannas IV, Gibson LJ. The effect of pore size on cell adhesion in collagen-GAG scaffolds. Biomaterials 2005;26:433–441.
27. Burgstaller G, Oehrle B, Koch I, Lindner M, Eickelberg O. Multiplex profiling of cellular invasion in 3D cell culture models. PLoS One 2013;8:e63121
28. Beningo KA, Dembo M, Kaverina I, Small JV, Wang YL. Nascent focal adhesions are responsible for the generation of strong propulsive forces in migrating fibroblasts. J Cell Biol 2001;153: 881–888.
29. Abraham LC, Vorrasi J, Kaplan DL. Impact of collagen structure on matrix trafficking by human fibroblasts. J Biomed Mater Res 2004;70A:39–48.
30. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. Physiol Rev 2003;83:835–870.
31. Lamme EN, van Leeuwen RT, Jonker A, van Marle J, Middelkoop E. Living skin substitutes: Survival and function of fibroblasts seeded in a dermal substitute in experimental wounds. J Invest Dermatol 1998;111:989–995.
32. Zhou S, Salisbury J, Preedy VR, Emery PW. Increased Collagen Synthesis Rate during Wound Healing in Muscle. PLoS ONE 2013; 8:e58324
33. Madsen DH, Engelholm LH, Ingvarsen S, Hillig T, Wagenaar-Miller RA, Kjøller L, Gårdsvoll H, Høyer-Hansen G, Holmbeck K, Bugge TH, Behrendt N. Extracellular collagenases and the endocytic receptor, urokinase plasminogen activator receptor-associated protein/Endo180, cooperate in fibroblast-mediated collagen degradation. J Biol Chem 2007;282:27037–27045.
34. McCulloch CA, Knowles GC. Deficiencies in collagen phagocytosis by human fibroblasts *in vitro*: A mechanism for fibrosis? J Cell Physiol 1993;155:461–471.
35. Brett D. A review of collagen and collagen-based wound dressings. Wounds 2008;20:347–356.
36. Rekhter MD. Collagen synthesis in atherosclerosis: Too much and not enough. Cardiovasc Res 1999;41:376–384.
37. Lu P, Takai K, Weaver VM, Werb Z. Extracellular matrix degradation and remodelling in development and disease. Cold Spring Harb Perspect Biol 2011;3:a005058.
38. Svoboda EL, Melcher AH, Brunette DM. Stereological study of collagen phagocytosis by cultured periodontal ligament fibroblasts: Time course and effect of deficient culture medium. J Ultrastruct Res 1979;68:195–208.
39. Melcher AH, Chan J. Phagocytosis and digestion of collagen by gingival fibroblasts *in vivo*: A study of serial sections. J Ultrastruct Res 1981;77:1–36.
40. Knowles GC, McKeown M, Sodek J, McCulloch CA. Mechanism of collagen phagocytosis by human gingival fibroblasts: Importance of collagen structure in cell recognition and internalization. J Cell Sci 1991;98:551–558.
41. Kobayashi T, Hattori S, Shinkai H. Matrix metalloproteinases-2 and −9 are secreted from human fibroblasts. Acta Derm Venereol 2003;83:105–107.
42. Lindner D, Zietsch C, Becher PM, Schulze K, Schultheiss H-P, Tschope C, Westermann D. Differential expression of matrix metalloproteases in human fibroblasts with different origins. Biochem Res Int 2012;2012:875742
43. Arora PD, Wang Y, Bresnick A, Dawson J, Janmey PA, McCulloch CA. Collagen remodeling by phagocytosis is determined by collagen substrate topology and calcium-dependent interactions of gelsolin with nonmuscle myosin IIA in cell adhesions. Mol Biol Cell 2013;24:734–747.
44. Hakulinen J, Sankkila L, Sugiyama N, Lehti K, Keski-Oja J. Secretion of active membrane type 1 matrix metalloproteinase (MMP-14) into extracellular space in microvesicular exosomes. J Cell Biochem 2008;105:1211–1218.
45. Li C-J, Liu Y, Chen Y, Yu D, Williams KJ, Liu M-L. Novel proteolytic microvesicles released from human macrophages after exposure to tobacco smoke. Am J Pathol 2013;182:1552–1562.

46. Menck K, Scharf C, Bleckmann A, Dyck L, Rost U, Wenzel D, Dhople VM, Siam L, Pukrop T, Binder C, Klemm F. Tumor-derived microvesicles mediate human breast cancer invasion through differentially glycosylated EMMPRIN. J Mol Cell Biol 2015;7:143–153.
47. Tomihata K, Burczak K, Shiraki K, Ikada Y. Cross-linking and biodegradation of native and denatured collagen. In: Shalaby SW, Ikada Y, Langer R, Williams J, editors: Polymers of Biological and Biomedical Significance. American Chemical Society Symposium Series; 1993. p 275–286.
48. Dalby MJ, Riehle MO, Sutherland DS, Agheli H, Curtis AS. Changes in fibroblast morphology in response to nano-columns produced by colloidal lithography. Biomaterials 2004;25:5415–5422.
49. Albuschies J, Vogel V. The role of filopodia in the recognition of Nanotopographies. Sci Rep 2013;3:1658
50. Kress H, Stelzer EHK, Holzer D, Buss F, Griffiths G, Rohrbach A. Filopodia act as phagocytic tentacles and pull with discrete steps and a load-dependent velocity. PNAS 2007;104:11633–11638.
51. Bayless KJ, Davis GE. The Cdc42 and Rac1 GTPases are required for capillary lumen formation in three-dimensional extracellular matrices. J Cell Sci 2002;115:1123–1136.
52. Taverna S, Ghersi G, Ginestra A, Rigogliuso S, Pecorella S, Alaimo G, Saladino F, Dolo V, Dell'Era P, Pavan A, Pizzolanti G, Mignatti P, Presta M, Vittorelli ML. Shedding of membrane vesicles mediates fibroblast growth factor-2 release from cells. J Biol Chem 2003;278:51911–51919.
53. Muralidharan-Chari V, Clancy JW, Sedgwick A, D'Souza-Schorey C. Microvesicles: Mediators of extracellular communication during cancer progression. J Cell Sci 2010;123:1603–1611.