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Development of a mini 3D cell culture system using well defined nickel grids for the investigation of cell scaffold interactions

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Abstract A bioreactor system was developed using a series of fine mesh nickel grids as free standing scaffolds to investigate the behaviours of fibroblasts and keratinocytes in tissue culture. It was found that the mesh size of the suspended grids, but not of the grids that attached to tissue culture surface, had significant influences on cell behaviour and there was a maximum size for fibroblast to span within the defined culture period. Time lapse video microscopy demonstrated fibroblasts cultured on these grids initially migrated onto the struts but then worked together to fill in the voids between struts with a membranous sheet of tissue. In contrast keratinocytes barely migrated from the initial site of cell deposition and when they moved (to a modest extent) it was as an integrated sheet of cells. Similar results were observed when both types of cells were co-cultured in the system.

1 Introduction

Extracellular matrix (ECM) plays a major role in controlling cell behaviour in vivo and one of the major research efforts in tissue engineering is to mimic the natural three-dimensional (3D) ECM by generating scaffolds with well-defined architectures—controlled pore size, porosity and topography—which favour cell and tissue growth [1, 2]. Not surprisingly there is a wealth of research into the nature of the scaffold materials and considerable debate on whether macro-length scale features alone will prove adequate information or whether nano-features on scaffolds are also required to induce cells to form reasonably biomimetic tissues for clinical transplantation.

In order to optimise the integration of skin cells into 3D scaffolds for skin tissue engineering, we previously investigated the influences of fibre diameter and interfibre spaces of different scaffolds on the behaviour of human dermal fibroblasts and of a keratinocyte cell line (HaCat) [3, 4]. This yielded valuable data on the ability of fibroblasts and HaCats to migrate along fibres of varying width and to span gaps between fibres. We also observed clustering of fibroblasts around fibres and particularly around intersections of fibres—a behaviour that is not observed in cells grown in two-dimensional (2D) cultures on tissue culture plastic.

The aim of this research was to examine the organisational behaviour of skin cells in 3D culture using simple nickel grids of varying strut spacing. These allowed an examination of the ability of cells to span and fill in open spaces in these grids. Mini cell culture systems were designed and fabricated using commercially available nickel specimen supporters with well-defined mesh sizes (or distances between struts) of 37–420 μm. These grids were either attached directly to the surface of normal tissue culture plates for 2D cell culture or mounted in pre-fabricated silicone sheets for 3D cell culture. The influence of grid architecture on cell behaviour was investigated in both 2D and 3D cell culture for human dermal fibroblasts and

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epidermal keratinocytes examined as single cultures and in co-culture.

Our research indicated that the nickel grids in 2D culture had negligible effects on cell culture compared to the suspended nickel grids. The mesh size of the suspended grids had significant influences on cell behaviour and there was a maximum size for fibroblast to span within the defined culture period. Time lapse video microscopy demonstrated fibroblasts cultured on these grids initially migrated onto the struts but then worked together to fill in the voids between struts with a membranous sheet of tissue. In contrast keratinocytes barely migrated from the initial site of cell deposition and when they moved (to a modest extent) it was as an integrated sheet of cells. Similar results were observed when both types of cells were co-cultured in the system.

2 Materials and methods

2.1 Cell culture

Normal human fibroblasts and keratinocytes were isolated from split thickness skin (STS) harvested from theatre specimens removed following routine plastic surgical abdominoplasties and breast reductions skin under a licence (Licensing Number 12179) granted under Section 16(2)e(ii) of the Human Tissue Act 2004 by the Human Tissue Authority (UK). Fully informed written consent was obtained from each patient prior to operation with explicit permission that removed tissue could be used for research purposes. The methodologies of cell isolation, media preparation and cell culture were as described previously [5, 6]. For keratinocyte culture Green's medium was used. This consisted of DMEM and Ham's F12 medium in a 3:1 (v/v) ratio supplemented with 10% (v/v) foetal calf serum (FCS), 10 ng/ml EGF, 0.4 μg/ml hydrocortisone, 10^{-10} mol/l cholera toxin, 1.8×10^{-4} mol/l adenine, 5 μg/ml insulin, 5 μg/ml transferrin, 2×10^{-3} mol/l glutamine, 2×10^{-7} mol/l triiodothyrionine, 0.625 μg/ml amphotericin B, 100 IU/ml penicillin and 100 μg/ml streptomycin. For the co-culture of fibroblasts and keratinocytes both Green's media with or without serum were used as described previously [5, 6]. Fibroblasts were cultured in DMEM medium supplemented with 10% (v/v) FCS, 2×10^{-3} mol/l glutamine, 0.625 μg/ml amphotericin B, 100 IU/ml penicillin and 100 μg/ml streptomycin.

2.2 Nickel grids used for cell culture

Nickel specimen supporters (3.05 mm in diameter, thickness: 10–20 μm, bar width: 25–90 μm) are commercially available products for supporting tissue samples for transmission electron microscopy (TEM) and were purchased from TAAB Laboratories Equipment Ltd (Berk, UK). Nickel specimen supporters with both square and rectangular meshes were used for the development of mini 2D and 3D cell culture systems in this study. The nickel grids with square meshes were G-400 (mesh size 37 μm), HF-35 (mesh size 60 μm), G-175 (mesh size 108 μm), G-100 (mesh size 205 μm), G-75 (mesh size 285 μm) and G-50 (mesh size 420 μm). The nickel grids with rectangular meshes were G-400P (gap size 37 μm), G-200P (gap size 90 μm), HF-46 (gap size 103 μm), G-100P (gap size 205 μm), G-75P (gap size 285 μm) and G-50P (gap size 420 μm).

2.3 Development of mini 3D and 2D cell culture systems

Two types of very similar mini 3D cell culture systems were developed. In the module-I 3D cell culture system (as illustrated in Figs. 1a, b, 2a), two silicone sheets were fabricated to hold a piece of nickel specimen supporter (TAAB, UK, 3.05 mm in diameter of 10–20 μm thickness and bar widths of 25–90 μm). In the upper silicone sheet, two separate holes with diameters of approximately 2.5–2.9 mm were created and connected by a channel (0.4–0.6 mm wide, 1.0–1.5 mm long), while only one hole (2.5–2.9 mm in diameter) was created in the lower silicone sheet. A piece of nickel grid was carefully mounted between two holes of the upper and lower sheets so that only the edge of the nickel grid was covered by the silicone while most of it was exposed on both sides. As shown in Figs. 1a and 2a-the suspended nickel grid and the two holes on either side made up a cell culture chamber, while the other hole on the upper silicone sheet and the surface of the lower sheet made up a cell-seeding chamber. The cell seeding chamber and the cell culture chamber were connected by the cell migration chamber made up by the channel on the upper sheet and the surface of the lower sheet. In this system, cells were seeded specifically in the cell seeding chamber as illustrated by the dashed circle in Fig. 1a.

A similar approach was used to develop the module-II mini 3D culture system (shown in Fig. 1c, d). However, the two holes (e.g. the cell-seeding chamber and the cell culture chamber) on the upper silicone were created such that they partly overlapped. This was designed for cells (e.g. keratinocytes) with relatively low motility, and these cells were seeded directly onto the edge of the grid as illustrated by the dashed circle in Fig. 1c. For both modules, the outsides of both the upper and lower silicone sheets were tailored so that they were easily situated in wells of either 24 well (15 mm in diameter) or 6-well (35 mm in diameter) tissue culture plates. Multiple mini cell culture systems were set up in 24 wells or 6 wells for parallel experiments.

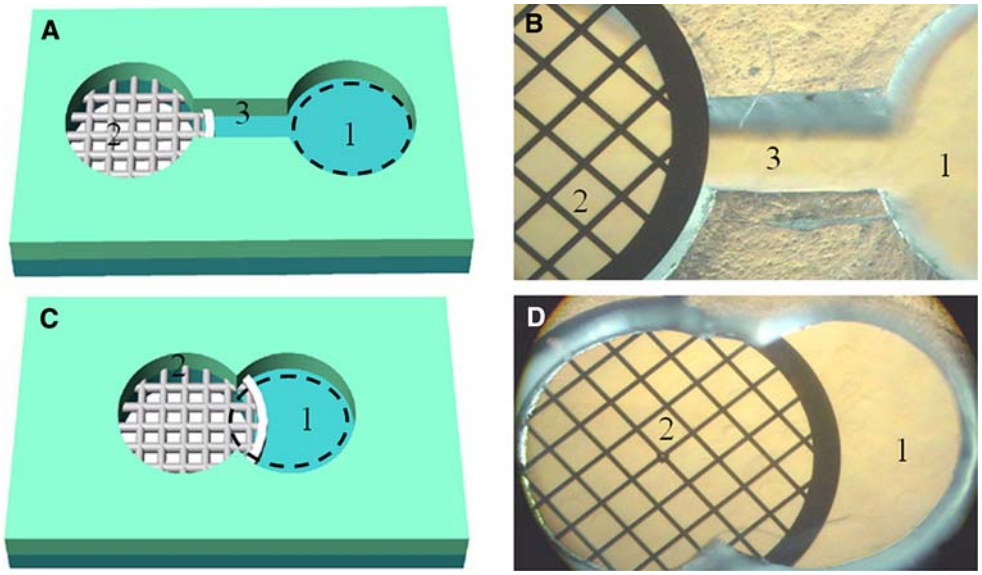

Fig. 1 **a** Schematic diagram of the Module-I mini 3D cell culture system: two silicone sheets were used to hold a piece of nickel grid (1) the cell seeding chamber and (2) the cell culture chamber were connected by (3) a cell migration channel. **b** Appearances of the upper silicone sheet and a piece of nickel grid used in Module-I mini 3D cell culture system. **c** Schematic diagram of the Module-II mini 3D cell culture system: (1) the cell seeding chamber and (2) the cell culture chamber were partly overlapped so cells could be seeded directly onto part of the scaffold. **d** Appearances of the upper silicone sheet and a piece of nickel grid used in the Module-II mini 3D cell culture system. *Dashed circles* indicate the cell seeding areas

As a control for the mini 3D cell culture systems, the nickel grids were also attached directly onto the surface of either 24 well or 6 well tissue culture plates so that only one side of the nickel grid was exposed to the cell culture media. In each of these 2D cell culture systems, the nickel grid was fixed in its position by a piece of silicone sheet as used in the 3D cell culture systems (shown in Fig. 2b). Thus the nickel grid and one hole of the silicone sheet made up the cell culture chamber, while the other hole, the channel of silicone sheet and the tissue culture surface made up a cell-seeding chamber and a cell migration chamber.

All of the above 2D and 3D cell culture systems were sterilised with 70% ethanol for 24 h, washed thoroughly with PBS (×3), sterilized water (×3) and dried before seeding cells. The subsequent operations such as cell seeding, cell culture, cell staining and microscopic study were all performed consecutively in the tissue culture wells.

2.4 Cell seeding and cell culture in 2D and 3D culture systems

A fibrin clot was used to seed cells at specific sites in the 2D and 3D cell culture systems. Briefly, a cell suspension was prepared by thoroughly mixing fibrinogen (3.5 mg/ml), thrombin (10 units/ml) and cells together in

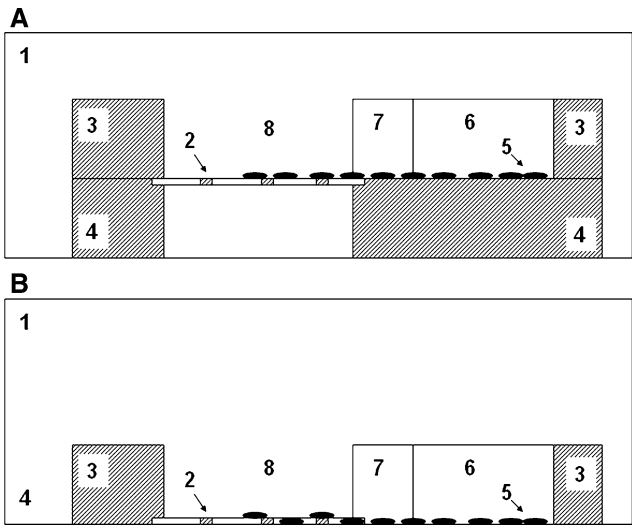

Fig. 2 **a** Schematic diagram of the cross section of the Module-I mini 3D cell culture system. The system was situated in (1) a culture well of a tissue culture plate, (2) a piece of nickel grid was suspended by (3) the upper silicone sheet and (4) the lower silicone sheet. (5) Cells seeded in (6) the cell seeding chamber migrated through (7) the cell migration channel onto the nickel grid in (8) the cell culture chamber. **b** Schematic diagram of the cross section of the mini 2D cell culture system. The system was situated in (1) a culture well of a tissue culture plate, (2) a piece of nickel grid was fixed by (3) the upper silicone sheet directly onto (4) the surface of a cell culture well. (5) Cells were seeded in (6) the cell seeding chamber migrated through (7) the cell migration channel onto the nickel grid in (8) the cell culture chamber

DMEM media without FCS. For single culture of fibroblasts or keratinocytes the cell density in the fibrin clot was 5×10^5 cells/ml. For fibroblast and keratinocyte co-culture, equal numbers of both cells were mixed and the total cell density was 1×10^6 cells/ml. Using a pipette, tiny drops (10–20 μm) of the cell suspension were quickly delivered into the cell seeding chambers of either 2D or 3D cell culture systems. The tissue culture plates were then kept in a tissue culture incubator (37°C and 5% CO_2) for 20–40 min for fibrinogen polymerisation. Cell culture medium was then gently added into each well to cover the fibrin gel containing cells and the grids. During cell culture, phase contrast and or live cell fluorescent microscopy were conducted using a microscope (Leica, Germany) to monitor the performance of the cells in these culture systems. After culture, the grids with cells were washed with PBS and the cells were immunostained for epifluorescent microscopy.

2.5 Phase contrast and time-lapse video microscopy

The morphologies and behaviours of live cells cultured in the 2D or 3D mini cell culture systems were studied using an inverted phase contrast light microscope (Leica, Germany). Cell orientation and outgrowth were then determined from the micrographs using image analysis software (Openlab 4.0.2 and Volocity 3.0.2, Improvision, UK). The same microscope was used for time-lapse experiments as it was situated in a specifically designed cell culture chamber (37°C and 5% CO_2). At certain times in the cell culture, the mini 3D cell culture system was transferred into the specifically designed cell culture chamber for time lapse experiment for 18–20 h and with frames taken every 20 min.

2.6 Cell labelling and detection using Cell Tracker™

After single culture in the Module-I 3D cell culture systems for certain periods of time, fibroblasts on the nickel grids were labeled with Cell Tracker™ Red CMTPX (C34552) (Invitrogen Ltd, UK) for visualisation using fluorescent microscopy. The cells on grids in the mini 3D cell culture systems were washed gently using pre-warmed (37°C) serum free DMEM medium in the tissue culture plate, incubated in fluorescent probe Cell Tracker™ reagent (0.5–25 μM, in serum free DMEM medium) for 15–45 min at 37°C and subsequently in normal DMEM medium (with serum) for 30 min at 37°C. Cell culture was then continued and the fluorescently labelled cells were observed under fluorescence microscope (Leica, Germany). When the cell culture was finished, the cells were also fixed in 4% (w/v) paraformaldehyde in PBS and stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI, 300 μM, 15 min, Vector Laboratories Inc., Ca, USA) for further epifluorescence microscopy using ImageXpress™ (AXON, USA).

2.7 Immunofluorescence microscopy

Expression of pancytokeratin was used to specifically identify keratinocytes when the epidermal cells were single cultured or co-cultured with fibroblasts. After cell culture, the cells cultured in the mini 3D cell culture systems were washed gently with PBS, fixed in 4% (w/v) paraformaldehyde in PBS for 30 min at 37°C and permeabilised with 0.2% (w/v) Triton X-100 in PBS for 5 min. After blocking with 1% (w/v) BSA in PBS for 45 min, cells were incubated in primary antibody solution (mouse anti-cytokeratin, M3515, Dako, 1:500 dilution in PBS) and subsequently with secondary antibody solution (FITC conjugated rabbit anti-mouse, F0232, Dako, 1:1000 dilution in PBS), each for 1 h with individual wash (×3 PBS). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI, 300 μM, 15 min, Vector Laboratories Inc., Ca, USA). After a final wash (×3 PBS), the cells were ready for immunofluorescent microscopy.

The epifluorescence images of Cell Tracker™ labelled or immunostained cells were taken with either a fluorescence microscope (Leica, Germany) or an ImageXpress™ (AXON, USA) at $\lambda_{ex} = 495$ nm, $\lambda_{em} = 515$ nm (for FITC/pancytokeratin visualization), $\lambda_{ex} = 580$ nm, $\lambda_{em} = 650$ nm (for TRITC/Cell Tracker™ visualization) and $\lambda_{ex} = 358$ nm, $\lambda_{em} = 461$ nm (for DAPI/nuclei visualization).

2.8 Evaluation of the ability of cells cultured on the grids to bridge gaps

After a defined length of culture (2 weeks) the ability of cells to span the gaps between struts in the various nickel grids in either 2D or 3D cell culture systems was simply assessed as either "bridged" or "not bridged".

3 Results

3.1 Culture of fibroblast on nickel grids placed in tissue culture wells

Nickel grids were placed directly on tissue culture plastic in culture wells and fibroblasts were seeded in the cell seeding chamber using a fibrin clot as detailed in the methods. After cultured for 3–4 days, cells proliferated inside the fibrin clot then migrated out of it along the migration channel toward the nickel scaffold. They appeared to migrate and proliferate on the nickel grids as well as on the tissue culture plastic surface. Cells growing on the tissue culture plastic (Fig. 3c) or nickel grids with small gaps between the struts (Fig. 3d, e) spread in all directions without any obvious orientation. Cells were

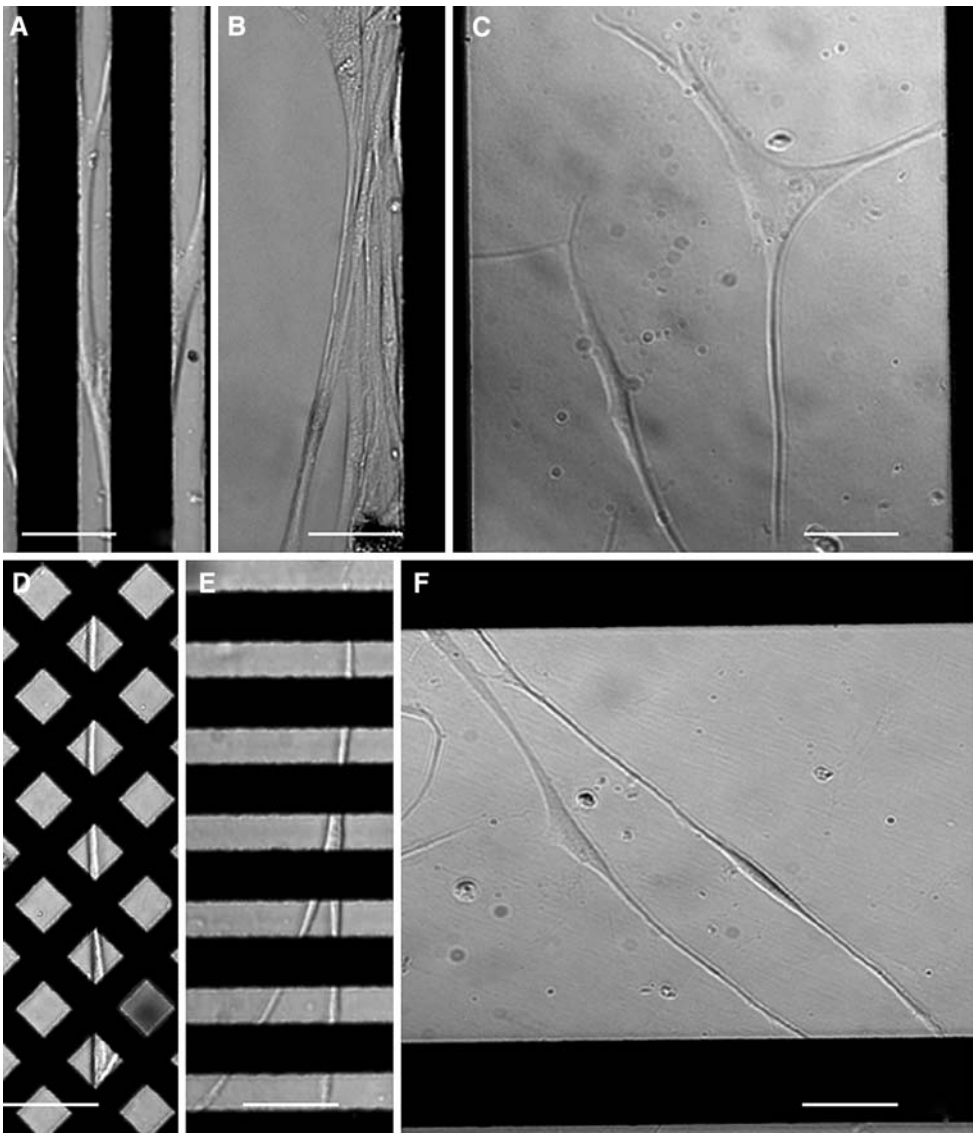

Fig. 3 Phase contrast normal light micrographs of fibroblasts cultured in the mini 2D cell culture systems in DMEM medium for 5 days. The nickel grids used were: G-400P with bar gaps of 37 μm for (**a**, **e**), G-50P with bar gaps of 420 μm for (**b**, **c**, **f**), G-400 with mesh sizes of 37 μm for (**d**). Scale bars are 100 μm

found to span gaps of 37–420 μm in these grids within 2 weeks. There was no overall influence of these nickel grids on the pattern of fibroblast organisation with the exception of fibroblasts seen close to nickel struts where these cells were observed to elongate parallel to these metal struts (as shown in Fig. 3a, b)

3.2 Culture of fibroblast on suspended nickel grids

Fibroblasts were then seeded onto nickel grids held suspended in media (see Figs. 1a, 2a Module-I) and cultured for 2 weeks. Cells were observed to migrate from the cell seeding chamber towards the culture chamber through the migration channel as illustrated in Fig. 4a, b. Cells which reached the grids initially attached and migrated on the struts (Fig. 4c). However as the cultures continued, cells started to aggregate and stratify and began to fill in the gaps between the struts in the suspended grids as shown in Fig. 4d.

At the corners of the grids where struts intersected cells changed their shape and aggregated (Fig. 4e, f), suggesting that the intersections of scaffolds may play key roles in influencing cell behaviour in 3D cell culture. The ability of fibroblasts to span the gaps in these suspended grids was then examined and as shown in Figs. 5a–l and 6a, b, fibroblasts were able to fill in gaps as big as 200 μm within 2 weeks.

3.3 Time-lapse video microscopy of fibroblasts cultured on suspended nickel grids

Time lapse studies were then conducted to investigate how fibroblasts actually span and fill in gaps in the suspended nickel grids. Cells initially migrated along the nickel bars

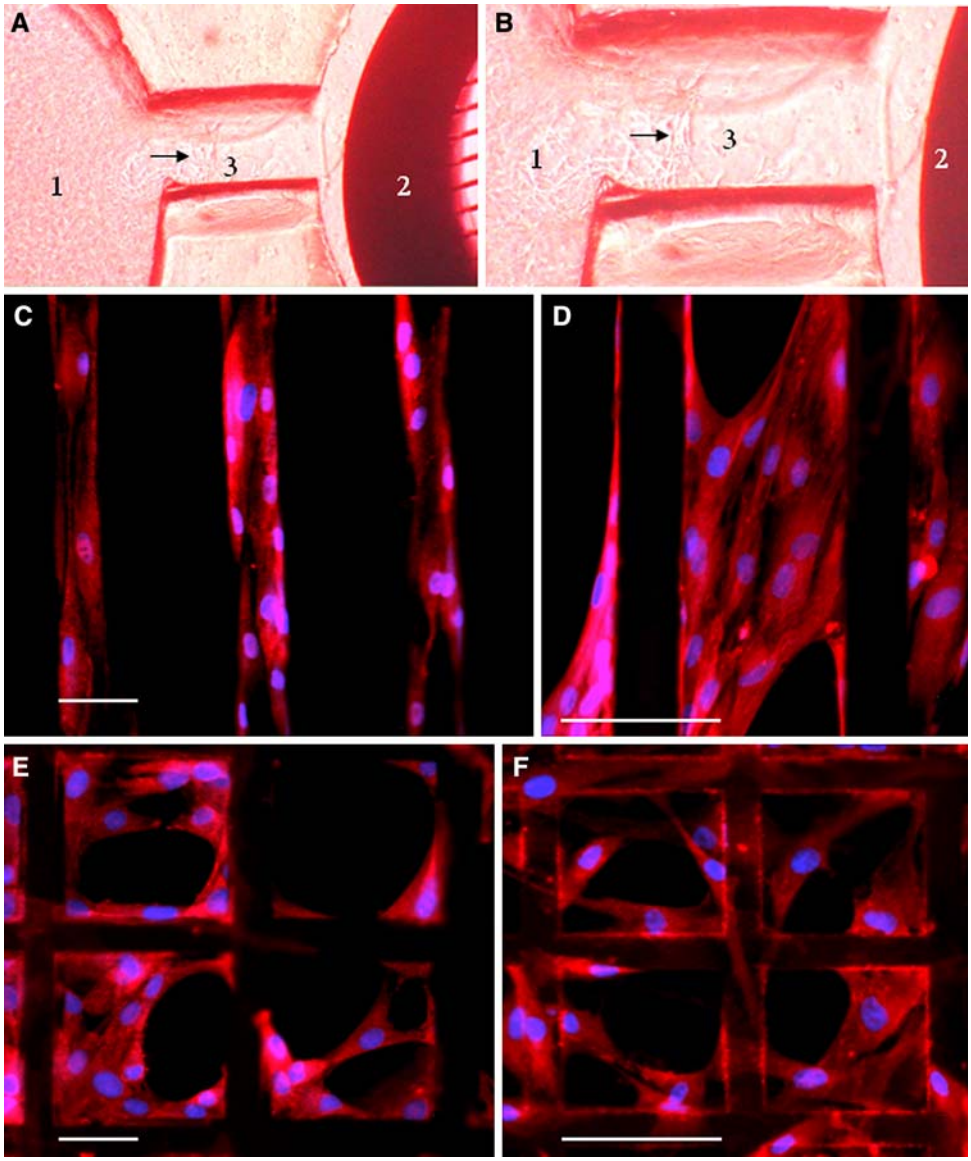

Fig. 4 **a, b** Appearances of fibroblasts migrated from (1) the cell-seeding chamber toward (2) the cell culture chamber through (3) the migration channel in Module-I mini 3D cell culture system. Epifluorescent micrographs of DAPI (*blue*) and Cell Tracker™ Red CMTPX (*red*) stained fibroblasts in Module-I mini 3D cell culture system. The nickel grids used were: G-100P with bar gaps of 205 μm for (**c**), HF-46 with bar gaps of 103 μm for (**d**), G-100 with mesh sizes of 205 μm for (**e**), G-175 with a mesh size of 108 μm for (**f**). Scale bars are 100 μm

without any obvious aggregation or stratification for 3–4 days culture. Then around 5–7 days, a few fibroblasts started to slide backwards and forwards by elongating their cell bodies while maintaining attachments at both ends of their cell bodies to these intersecting nickel struts. This initially occurred within a very small area close to the intersections of the grids. Then as cultures continued, more and more cells joined in this active movement, sliding backwards and forwards now as coherent cell sheets rather than as single cells as shown in Fig. 7a–c. Videos illustrate close interactions between these cells. Gradually, the sliding sheets of fibroblasts at the corners increased in size and formed a continuous cell sheet which appeared to be a few cell layers thick. This eventually completely filled in the voids as shown in Fig. 7d–i.

It was obvious from the videos that that the cell sheets in these voids were complex dynamic structures with cells aligned in structures which continued to move as though seeking to pull the struts of the nickel grids together. Cells showed a considerable amount of motility in forming these sheets and many of the cells underwent cell division. Time lapse experiments of the fibroblasts cultured on the grids with larger mesh sizes indicated that voids as big as 420 μm could be filled by fibroblasts if the cultures were continued for approximately 1 month.

3.4 Culture of keratinocytes on nickel grids

Keratinocytes were then seeded in the cell seeding chamber of the Module-I 3D culture system using a fibrin clot and cultured for 2 weeks in Green's medium. However, keratinocytes did not migrate along the migration channel toward the suspended nickel grids during this period. Accordingly, a second Module-II 3D cell culture system

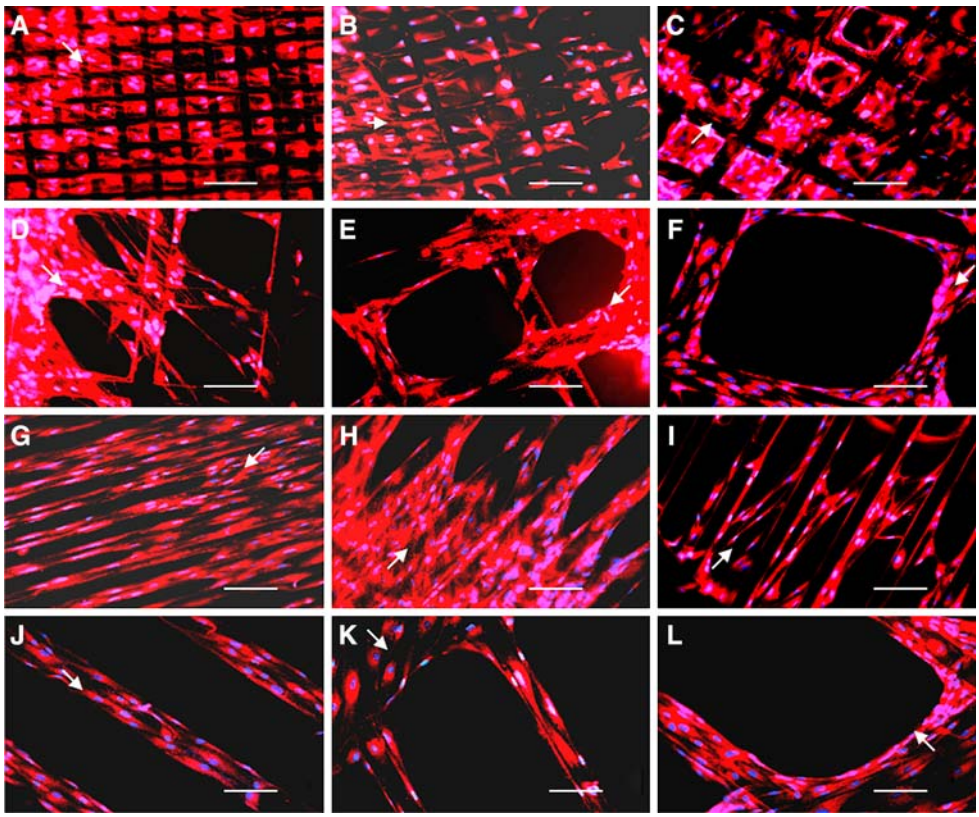

Fig. 5 Fluorescent micrographs of DAPI (*blue*) and Cell Tracker™ Red CMTPX (*red*) stained fibroblasts on different nickel grids mounted in the Module-I mini 3D cell culture system in DMEM medium for 2 weeks. The nickel grids used in this experiment were: **a** G-400 with a mesh size of 37 μm, **b** HF-35 with a mesh size of 60 μm, **c** G-175, with a mesh size of 108 μm, **d** G-100 with a mesh size of 205 μm, **e** G-75 with a mesh size of 285 μm, **f** G-50 with a mesh size of 420 μm, **g** G-400P with bar gaps of 37 μm, **h** G-200P with bar gaps of 90 μm, **i** HF-46 with bar gaps of 103 μm, **j** G-100P with bar gaps of 205 μm, **k** G-75P with bar gaps of 285 μm, **l** G-50P with bar gaps of 420 μm. *Arrows* point to the cell migration direction. Scale bars are 100 μm

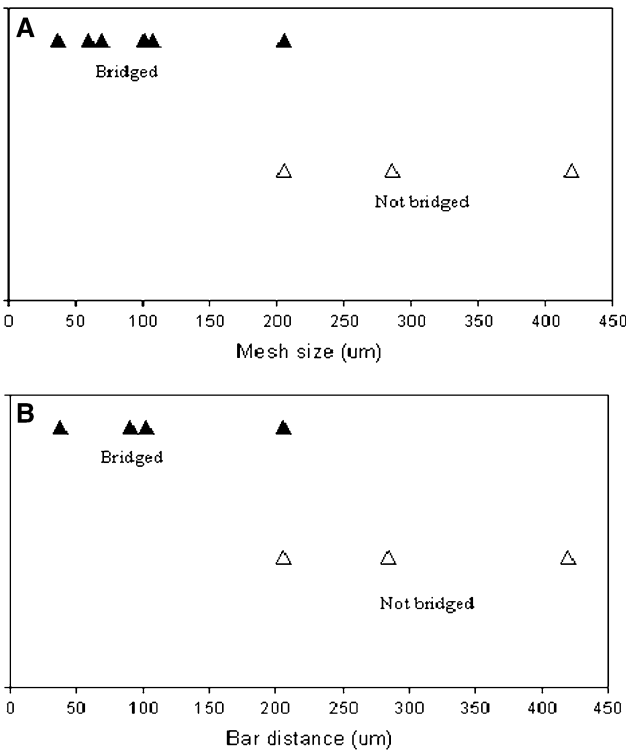

Fig. 6 Effect of **a** mesh size and **b** bar gaps of nickel grids on the ability of fibroblasts cultured in Module-I mini 3D cell culture system for 2 weeks to bridge gaps

was developed specifically for the culture of keratinocytes. In this module the cell seeding chamber was deliberately overlapped with the nickel grids so that keratinocytes could be seeded directly onto the edge of the grids using a fibrin clot and then followed microscopically.

Cells were cultured for 2 weeks and keratinocytes were observed to proliferate and migrate as a coherent cell sheet mainly around the cell seeding area. The migration of keratinocytes was very different to that of the fibroblasts—cells remained together as a sheet and the movement of the whole sheet was very slow (confined in the cell seeding area within 2 weeks culture) compared to that of the individual fibroblasts. Keratinocytes did not migrate appreciably outside of the cell seeding area (as shown in Fig. 8a–d) in these experiments. Similar results were obtained when keratinocytes were cultured in a 2D cell culture system (data not shown).

3.5 Co-culture of fibroblasts and keratinocytes on nickel grids

In these experiments fibroblasts and keratinocytes were mixed together and seeded directly onto the nickel scaffolds in the Module-II 3D cell culture system. However, the only cells which migrated during 2 weeks were those with a fibroblast-like morphology (spindle shaped and

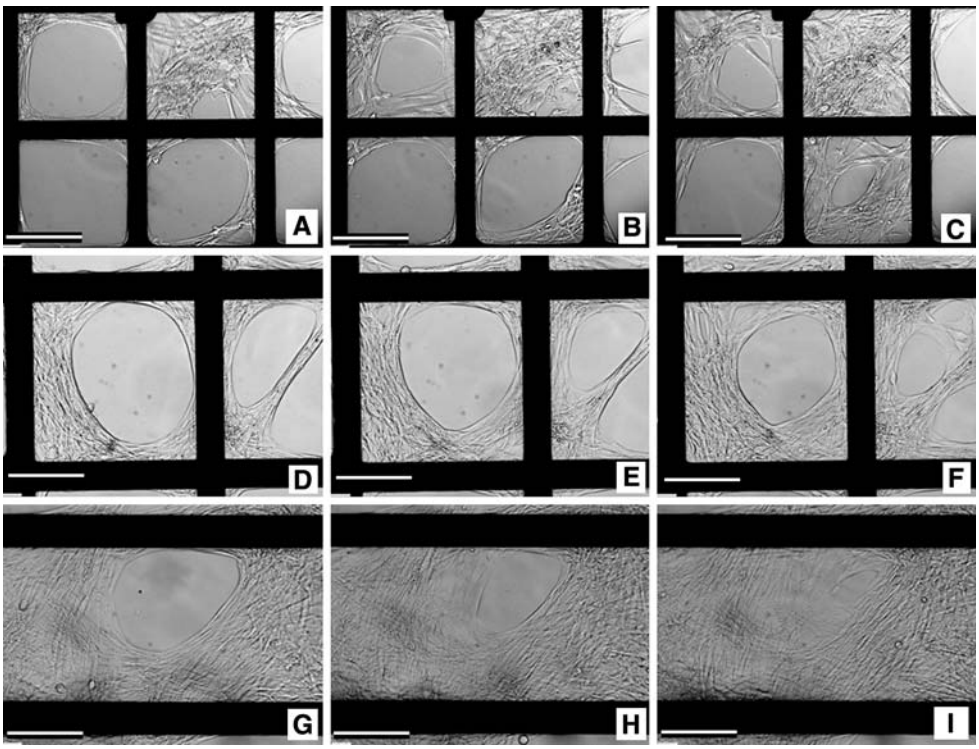

Fig. 7 Micrographs from time-lapse videos of fibroblasts cultured on three different nickel grids suspended in Module-I mini 3D cell culture systems in DMEM medium at various time points. **a–c** show cells cultured on G-175 at 16th, 24th h of the 6th day of culture, and at 9th h of the 7 day of culture, respectively, **d–f** are the cells cultured on G-100 at 16th and 24th h of the 10th day of culture, and at 9th h of the 11th day of culture, respectively, **g–i** are the cells cultured on G-100P at 16th and 24th h of the 13th day of culture, and at 9th h of the 14th day of culture, respectively. Scale bars are 100 μm

elongated) and these spanned the gaps between the nickel grids beyond the initial cell seeding area. In contrast cells with the morphology of keratinocytes remained around the initial cell seeding area.

Immunostaining for pancytokeratin while identifying all cell nuclei by staining with DAPI confirmed that the cells with the rounded shapes which remained in the cell seeding chamber were keratinocytes and the cells which migrated onto the grids were fibroblasts as shown in Fig. 8e–h. Co-culture of fibroblasts and keratinocytes was compared in Green's media both with and without serum and similar results were obtained—fibroblasts migrating and forming cell sheets but keratinocytes tending to stay at the site of seeding.

4 Discussion

It is well accepted that ECM encodes a wealth of information within it to form a multidimensional map in which the cells have been shown to be sensitive to features of this matrix at all length scales from the macro down to the nano scale [7]. In wound healing there is a loss of tissue and of mature matrix and the cells in the wound, fibroblasts, keratinocytes and endothelial cells, produce a temporary transitional matrix which is eventually matured to restore the normal matrix function [8–10]. Current tissue engineered scaffolds consist of 3D open pore networks in which multiple layers of cells and ECM span and fill the void spaces between the structural members of the scaffolds [11]. The majority of matrices used in tissue engineering are based on natural materials (of which bovine collagen is the most predominant) or biodegradable synthetic materials such as PLA and PGA mixtures [12, 13]. In all cases these scaffolds are designed to be temporary—to provide structure and organization to these tissues prior to transplantation but then to be replaced by the patients own new matrix post implantation. However if one starts off by adding cells to these pre-existing scaffolds then it is difficult to observe in vitro how the cells remodel the scaffolds except by using conventional destructive histology at fixed time intervals.

In this project we have deliberately used very thin grids of nickel (used as TEM specimen supports) composed of struts at right angles to each other in which to examine several aspects of how skin cells migrate, proliferate and become organized into early stage tissues. These allow us to see the behaviour of cells growing between the struts of the grids.

Grids were assembled either to lie flat on tissue culture surfaces where, as their height was so small (10–20 μm), it turned out they had relatively little influence on the growth of fibroblasts or keratinocytes migrating over them or, more informatively, grids were suspended in media and cells introduced to them so that the cells had the opportunity to migrate over the grids and fill in the spaces between them. This revealed very different behaviours of keratinocytes and fibroblasts. Firstly, keratinocytes in standard

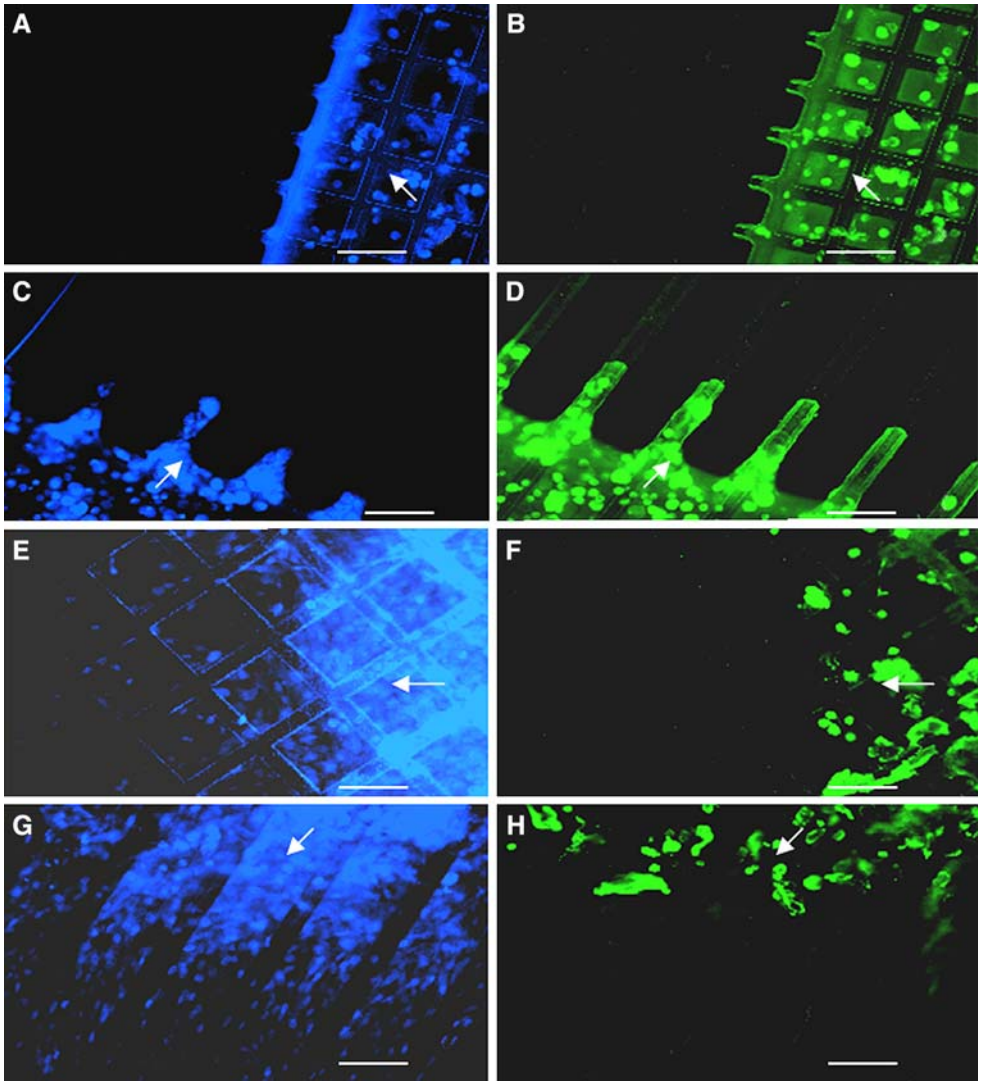

Fig. 8 Epifluorescent micrographs of keratinocytes cultured on two different nickel grids mounted in the Module-II mini 3D scaffold in Green's medium supplemented with 10% FCS for 2 weeks and stained with **a**, **c** DAPI (*blue*) and **b**, **d** pan-cytokeratin antibodies (*green*). Fluorescent micrographs of keratinocytes and fibroblasts co-cultured on two different nickel grids mounted in the Module-II mini 3D scaffold in Green's medium without FCS for 2 weeks and stained with **e**, **g** DAPI (*blue*) and **f**, **h** pancytokeratin antibodies (*green*). The nickel grids investigated were: HF-35 with a mesh size of 60 μm for (**a**, **b**); HF-46 with bar gaps of 103 μm for (**c**, **d**, **g**, **h**); G-175 with a mesh size of 108 μm for (**e**, **f**). *Arrows* point to the cell migration direction. Scale bars are 100 μm

media containing physiological levels of calcium were strongly adherent to each other (as has been extensively described previously [14–16] and showed relatively little ability to migrate as single entities. They migrated as an integrated sheet of cells in 2D cell culture.

In contrast fibroblasts were much more motile and migrated apparently singly or in small groups of cells without any apparent strong bonds between them until they completely occupied the culture surfaces available to them. At this point the fibroblast behaviour changed markedly in 3D cell culture. Fibroblasts started to fill in the voids between the grids by interacting with each other. Video movies showed that they filled in these voids by hanging on to each other. They appeared to form new "tissue" by initially attaching to the grids across the intersections and then moving outwards into the void while apparently pulling a contiguous sheet of fibroblasts behind them. Fibroblast divisions were obvious in this sheet and the cell sheet appeared to be 2–3 layers thick.

To the best of our knowledge this has not been reported previously and this offers a very simple low cost method for observing cell–cell behaviour of fibroblasts forming early stage tissue structures. Our observation clearly confirmed that this was a highly coordinated multi-cellular behaviour as suggested by Engelmayr et al. [11], as opposed to the phenomenon of contact guidance on structured surfaces which affects the behaviour of individual cells [17].

The maximum distance which fibroblasts spanned within 2 weeks was approximately 200 μm, (confirming our previous research in this area [3, 4]. Time lapse experiments of fibroblasts cultured on the grids with larger mesh sizes indicated that the voids as big as 420 μm were filled by the cells if the cultures were continued for approximately 1 month.

As previously noted by many [18–22] keratinocyte behaviour is tightly regulated—cells exhibiting strong cell–cell adhesion immediately from the point of seeding in

fibrin clots. In these experiments, relatively small numbers of keratinocytes were seeded in a confined cell seeding area and it appears that the colonies created by the seeded cells were too small to expand beyond the cell seeding chamber. We have recently reported that the sizes of keratinocyte colonies are tightly controlled by autoregulation mechanisms [23]. These have been described by a number of authors [18–22] and can actually be modelled mathematically [23]. Other literature also shows that epidermal cells when they do migrate do so as a cohesive cell sheet rather than as single entities in vivo and in vitro in Green's media [14–16]. Accordingly it would not be expected that individual cells would readily migrate out of a cohesive keratinocyte sheet to form separate colonies. The biological differences in migratory behaviour of fibroblasts and keratinocytes undoubtedly contribute to the way that keratinocytes and fibroblasts become organised with keratinocytes forming integrated cell layers above more widely distributed fibroblasts in 3D as observed in this and our previous studies [6].

The mini 3D cell culture systems we describe based on nickel grids have many advantages for investigation of cell biology and tissue organization. These are low cost commercially available items. Multiple mini cell culture systems can be set up in tissue culture plates for parallel experiments. The sterilization, cell seeding, cell culturing, cell staining and time-lapse video microscopy studies can be carried out consecutively in the same tissue plates. Also the use of fibrin clots to seed cells specifically in cell seeding chambers helps locate cells so that they can be observed readily with time. Thus the adverse effects of conventional random cell seeding methods on cellular behavior [24–26] were avoided. Cells growing out from the grids can be readily imaged over at least 2 weeks by light and fluorescent microscopy as well as video imaging.

The usefulness of this model for studying cells was demonstrated in the observation of fibroblasts interacting together to form new tissue in the gaps between the struts on the nickel grids. One could hypothesize that the leading edge fibroblasts were "seeking out" new space and pulling the fibroblasts behind them as a consequence of this. Alternatively, one could hypothesize that fibroblasts at the rear of the tissue were expanding and pushing the fibroblasts at the leading edge into empty space. Clearly these 3D models will allow us to further investigate these hypotheses, which are relevant not just to tissue engineering but may also help our understanding of how skin grafts undergo extreme contraction in many patients where it is known that cells acquire a myofibroblast phenotype [27–29].

In summary, we have developed mini 2D and 3D cell culture systems using nickel grids with fine open pore structures for the investigation of cell–scaffold interactions. These systems can now be used for a range of future investigations such as examining the cell/cell adhesions between cells and the organisation of the cytoskeleton during tissue formation.

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References

1. J.A. Hubbell, Curr. Opin. Biotechnol. **14**, 551 (2003). doi:10.1016/j.copbio.2003.09.004
2. X.H. Zong, C.Y. Chung, L.H. Yin, D.F. Fang, B.S. Hsiao, B. Chu et al., Biomaterials **26**, 5330 (2005). doi:10.1016/j.biomaterials.2005.01.052
3. T. Sun, D. Norton, R.J. McKean, J.W. Haycock, A.J. Ryan, S. MacNeil, Biotechnol. Bioeng. **97**, 1318 (2007). doi:10.1002/bit.21309
4. T. Sun, D. Norton, R.J. McKean, A.J. Ryan, S. MacNeil, J.W. Haycock, J. Mater. Sci.: MIM **18**, 321 (2007)
5. T. Sun, M. Higham, C. Layton, J. Haycock, R. Short, S. MacNeil, Wound Repair Regen. **12**, 626 (2004). doi:10.1111/j.1067-1927.2004.12609.x
6. T. Sun, S.M. Mai, J. Haycock, A. Ryan, S. MacNeil, Tissue Eng. **11**, 1023 (2005). doi:10.1089/ten.2005.11.1023
7. M.S. Molly, H.G. Julian, Science **310**, 1135 (2005). doi:10.1126/science.1106587
8. S. Werner, R. Grose, Physiol. Rev. **83**, 835 (2003)
9. M.A. Carlson, M.T. Longaker, Wound Repair Reg. **12**, 134 (2004). doi:10.1111/j.1067-1927.2004.012208.x
10. R.A.F. Clark, K. Ghosh, M.G. Tonnesen, J. Invest. Dermatol. **127**, 1018 (2007). doi:10.1038/sj.jid.5700715
11. G.C. Engelmayr, G.D. Papworth, S.C. Watkins, J.E. Mayer, M.S. Sacks, J. Biomech. **39**, 1819 (2006). doi:10.1016/j.jbiomech.2005.05.020
12. K. Ceonzo, A. Gaynor, L. Shaffer, K. Kojima, C.A. Vacanti, G.L. Stahl, Tissue Eng. **12**, 301 (2006). doi:10.1089/ten.2006.12.301
13. Blackwood K, McKean R, Canton I, Freeman C, Franklin K, Cole D, et al. (2008). Biomaterials (in press)
14. G. Fenteany, P.A. Janmey, T.P. Stossel, Curr. Biol. **10**, 831 (2000). doi:10.1016/S0960-9822(00)00579-0
15. K. Woolley, P. Martin, Bioessays **22**, 911 (2000). doi:10.1002/1521-1878(200010)22:10<911::AID-BIES6>3.0.CO;2-V
16. R. Farooqui, G. Fenteany, J. Cell Sci. **118**, 51 (2005). doi:10.1242/jcs.01577
17. X.F. Walboomers, J.A. Jansen, Odontology **89**, 2 (2001). doi:10.1007/s10266-001-8178-z
18. U.B. Jensen, S. Lowell, F.M. Watt, Development **126**, 2409 (1999)
19. A.E. Aplin, A.K. Howe, R.L. Juliano, Curr. Opin. Cell Biol. **11**, 737 (1999). doi:10.1016/S0955-0674(99)00045-9
20. A.J. Zhu, I. Haase, F.M. Watt, Proc. Natl. Acad. Sci. USA **96**, 6728 (1999). doi:10.1073/pnas.96.12.6728
21. S. Lowell, P. Jones, I.L. Roux, J. Dune, F.M. Watt, Curr. Biol. **10**, 491 (2000). doi:10.1016/S0960-9822(00)00451-6
22. N.J. Savill, J.A. Sherratt, Dev. Biol. **258**, 141 (2003). doi:10.1016/S0012-1606(03)00107-6
23. T. Sun, P. McMinn, S. Coakley, M. Holcombe, R. Smallwood, S. MacNeil, J. R. Soc. Interface **4**, 1077 (2007). doi:10.1098/rsif.2007.0227
24. A. Chevallay, N. Abdul-Malak, D. Herbage, J. Biomed. Mater. Res. **49**, 448 (2000). doi:10.1002/(SICI)1097-4636(20000315)49:4<448::AID-JBM3>3.0.CO;2-L

25. T.A. Desai, Med. Eng. Phys. **22**, 595 (2000). doi:10.1016/S1350-4533(00)00087-4
26. V.F. Sechriest, Y.J. Miao, C. Niyibizi, L.A. Westerhausen, H.W. Matthew, C.H. Evans et al., J. Biomed. Mater. Res. **49**, 534 (2000). doi:10.1002/(SICI)1097-4636(20000315)49:4<534::AID-JBM12>3.0.CO;2-#
27. J. Gailit, R.A.F. Clark, Curr. Opin. Cell Biol. **6**, 717 (1994). doi:10.1016/0955-0674(94)90099-X
28. A.J. Bogaerdt, A.E. Ghalbzouri, P.J. Hensbergen, L. Reijnen, M. Verkerk, Biochem. Biophys. Res. Commun. **315**, 428 (2004). doi:10.1016/j.bbrc.2004.01.069
29. U. Mirastschijski, C.J. Haaksma, J.J. Tomasek, M.S. Agren, Exp. Cell Res. **299**, 465 (2004). doi:10.1016/j.yexcr.2004.06.007