

Effect of Serum and Insulin Modulation on the Organization and Morphology of Matrix Synthesized by Bovine Corneal Stromal Cells

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The *in vitro* production of highly organized collagen fibrils by corneal keratocytes in a three-dimensional scaffold-free culture system presents a unique opportunity for the direct observation of organized matrix formation. The objective of this investigation was to develop such a culture system in a glass substrate (for optical accessibility) and to directly examine the effect of reducing serum and/or increasing insulin on the stratification and secretion of aligned matrix by fourth- to fifth-passage bovine corneal stromal keratocytes. Medium concentrations of 0%, 1%, or 10% fetal bovine serum and 0% or 1% insulin–transferrin–selenium were investigated. High-resolution differential interference contrast microscopy, quick-freeze/deep-etch, and conventional transmission electron microscopy were used to monitor the evolution, morphology, and ultrastructure of the cell–matrix constructs. In a medium containing 1% each of serum and insulin–transferrin–selenium, stromal cells stratified and secreted abundant and locally aligned matrix, generating the thickest cell–matrix constructs (allowing handling with forceps). The results of this study have the potential to significantly advance the field of developmental functional engineering of load-bearing tissues by (i) elucidating cues that modulate *in vitro* cell secretion of organized matrix and (ii) establishing an optically accessible cell culture system for investigating the mechanism of cell secretion of aligned collagen fibrils.

Introduction

KERATOCYTES ARE THE PRINCIPAL cellular component of the adult corneal stroma. They express aldehyde dehydrogenase and keratocan,^{1,2} exhibit a dendritic/stellate morphology, interconnect using multiple long cellular processes,^{2,3} and help maintain the extracellular matrix (ECM) by synthesizing its components.^{4–8} After injury to the corneal stroma, keratocytes transform via a wound healing response into the more active phenotype of fibroblasts,⁹ and from there sometimes into myofibroblasts.^{10,11} These repair cells migrate to the wound area and secrete a repair ECM (collagen and proteoglycans)² that is often fibrotic, fails to restore normal function, and may cause scarring and contraction of the cornea.¹² Repair fibrotic tissue contains collagen types I and III and fibronectin,^{13,14} and altered proportions of proteoglycans.^{15,16} Stromal fibroblasts are spindle shaped, exhibit long cellular processes,¹⁷ actively secrete matrix metalloproteinases (MMPs), fibronectin, and $\alpha 5$ integrin,^{18–20} and are associated with corneal ulceration,²¹ low contractility, and

lack of α -smooth muscle actin (α -SMA) expression or organized focal adhesions (reviewed in Refs.^{14,22}). On the other hand, myofibroblasts actively express α -SMA and $\alpha 5 \beta 1$ integrin, exhibit pronounced stress fibers and focal adhesions,^{11,17,18,22} synthesize collagen types I and III and fibronectin,^{23,24} exhibit strong biomechanical activity,²² and when compared with fibroblasts show reduced expression of MMPs^{25,26} and are larger during *in vitro* culture.²⁷

Precisely how the exquisite two-dimensional plus organization of the corneal stroma arises during development is not well understood.²⁸ Two competing theories address the deposition of organized collagen. The first suggests that collagen is directly placed (via fibripositors) by the invading neural crest–derived mesenchymal cells,²⁹ while the second suggests that collagen fibrils condense from concentrated solutions of collagen monomer.³⁰ Determining the method by which fibroblastic cells produce organized collagen arrays is not only an important basic science question, but can also inform efforts to engineer load-bearing tissue in general.²⁸ In a recent investigation by our laboratory, dedifferentiated

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human stromal keratocytes stratify and secrete a corneal-like organization of type I/V collagen lamellae *in vitro* in a scaffold-free tissue engineering system.³¹ To our knowledge, this is one of the first culture system capable of reproducing alternating arrays of collagen with organization similar to the corneal stroma. The principal purpose of this investigation is to robustly transfer the scaffold-free system onto a glass surface that will allow multiple differential interference contrast (DIC) investigations of the same culture at a high-temporal resolution and over long periods. We also are interested in reducing the dependence of culture on serum, which represents an uncontrolled variable. Due to the quiescent nature of stromal keratocytes, transformation into a more active phenotype (such as fibroblast or myofibroblast) must occur for sufficient ECM secretion. One way to guide *in vitro* stromal keratocyte transformation toward cell stratification and secretion of organized collagen is through the formulation of the culture medium.^{10,22,27,32,33} For example, keratocytes transform into myofibroblasts *in vitro* in the presence of transforming growth factor β (TGF- β). However, myofibroblasts produced via TGF- β -induced transformation of corneal stromal keratocytes exhibit contractile activity *in vitro*, which occasionally leads to a pulling^{23,34} of the construct into highly cellular clumps. This pulling is detrimental to our ultimate goal of generating organized arrays of collagen similar to those in the corneal stroma, and therefore we avoid the addition of exogenous TGF- β to the culture medium in the present study.

Stromal keratocytes undergo fibroblastic transformation *in vitro* in the presence of serum,^{23,35} while myofibroblastic transformation occurs in the presence of TGF- β 1, regardless of serum concentration.^{23,35} Myofibroblastic transformation can be suppressed by the addition of basic fibroblast growth factor (bFGF)²³ or entirely prevented by cultivation of primary keratocytes in the serum-free medium.^{23,36} However, for corneal stromal keratocytes, serum stimulates proliferation by 5- to 10-fold.²³ It is important to minimize the concentration of serum because the exact composition of serum is both undefined and variable and because low-serum and serum-free culture media reduce the risk of contamination by pathogenic agents such as viruses and mycoplasma. Insulin, transferrin, and selenium have been shown to be critical components of serum-free media.^{37,38} Insulin promotes the uptake of glucose and amino acids and the synthesis of proteins and nucleic acids³⁹; transferrin acts as an iron carrier (reviewed in Ref.⁴⁰), and selenium is a powerful anti-oxidant (reviewed in Ref.⁴¹). Insulin-transferrin-selenium (ITS) is a precisely defined medium supplement that enhances the proliferation and differentiation of several cell types,⁴²⁻⁴⁸ increases the contraction of collagen gels by rat cardiac fibroblasts,⁴⁹ and the synthesis of collagen by avian tendon cells.⁴⁴ However, elimination of serum by replacement with insulin or ITS is often detrimental to cell survival, differentiation, and/or proliferation.^{42,50-52} Although the mechanisms of ITS action on *in vitro* cell proliferation and ECM synthesis are not entirely understood, its main component, insulin, increases type I collagen synthesis,^{53,54} and maintains keratocyte proliferation and morphology in the serum-free medium.³² It has been postulated that ascorbic acid, a cofactor in the production of collagen (for review, see Refs.⁵⁵⁻⁵⁶), and glucose share structural similarities that cause their metabolisms to interact during

membrane transport and cellular processes. Thus, high glucose concentrations may inhibit the stimulatory action of ascorbic acid in the secretion of collagen, an effect that can be abolished by insulin.⁵⁷

In this study, the effect of culture medium component modulation (serum 1-10%; ITS 0-1%) on cell stratification and secretion of aligned matrix by bovine corneal stromal fibroblasts cultured on glass was examined. It was hypothesized that the low-serum medium supplemented with ITS/bFGF could maintain organized collagen synthesis while converting the cells to a nonfibrotic phenotype.

Materials and Methods

Materials

Unless otherwise indicated, all materials and reagents were from Fisher Bioreagents (Fair Lawn, NJ). Sigma Aldrich (St. Louis, MO) was the source of antibiotic-antimycotic, bovine serum albumin (BSA), goat serum, and trypsin-ethylenediaminetetraacetic acid (EDTA). L-Ascorbic acid phosphate (a stable form of vitamin C) was from Wako Pure Chemical Industries (Osaka, Japan), bovine brain-derived bFGF and anti-human α -SMA phycoerythrin monoclonal antibody were from R&D Systems (Minneapolis, MN), ITS-A and rhodamine phalloidin were supplied by Invitrogen (Carlsbad, CA), and rabbit anti-bovine type I collagen polyclonal antibody and fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG were from Chemicon International (Temecula, CA).

Cell culture

Bovine eyes were delivered within 8 h of slaughter from a local abattoir. The epithelium was scraped, and a square incision was made to remove the cornea. The endothelium and remaining limbus were then gently scraped. Corneas were rinsed several times with phosphate-buffered saline (PBS) with 1% antibiotic/antimycotic and minced into approximately 10 equally sized pieces. Excess liquid was removed, and corneal segments were allowed to attach to the bottom of six-well plastic culture dishes for 10 min. A small volume (0.04 mL/cm² surface area) of the culture medium (Dulbecco's modified Eagle's medium with 1% antibiotic/antimycotic and 10% fetal bovine serum [FBS]) was added to the wells, and the corneas were cultivated overnight at standard incubator conditions (37°C, 5% CO₂). Two milliliters of the culture medium was added to each well on the next day, and the corneas were cultivated in this manner for approximately 2 weeks, exchanging the medium every 5 days. After this period of time, the corneas were discarded and the wells were rinsed with PBS and treated with trypsin-EDTA (2.5 g trypsin/L and 2 g EDTA/L) for 5 min to release the cells that had migrated out of the corneas onto the tissue culture plastic. Cells were centrifuged, resuspended in the culture medium, and cultivated in standard tissue culture flasks. Fourth or fifth passage bovine corneal fibroblasts thus obtained were seeded on 1-cm-diameter glass-bottom cell culture dishes (MatTek, Ashland, MA) at 3×10⁵ cells/cm². Cultures were fed with 2 mL of basal culture medium (Dulbecco's modified Eagle's medium, 50 mg/L vitamin C, 10 ng/mL bFGF, and 1% antibiotic/antimycotic) supplemented with one of five different formulations described in Table 1.

TABLE 1. FORMULATIONS OF CULTURE MEDIA

Group	% FBS in medium	% ITS in medium
A	10	0
B	1	0
C	0	0
D	1	1
E	0	1

ITS, insulin–transferrin–selenium; FBS, fetal bovine serum.

Cells were thus cultivated for 3 weeks with 100% medium exchanges every 3–4 days (i.e., twice a week).

DIC microscopy

Cells were monitored using DIC microscopy every 3–4 days. DIC microscopy has excellent out-of-plane noise rejection (clearly resolved focal depths of ~ 200 nm) and is

capable of detecting organized fibrillar structures with x–y resolution on the order of 1/10th the wavelength of light. DIC allows for a clear observation of the cell morphology and reveals the organization of the aligned fibrils surrounding each individual cell.⁵⁸ The procedure has been described elsewhere.^{58–60} Axial scans and snapshots were acquired using a Nikon TE2000U inverted microscope fitted with a 60 \times 1.45 NA oil-immersion objective (Nikon, Melville, NY), a Coolsnap EZ camera (Photometrics, Tucson, AZ), and a Dell desktop computer. The thickness of the cell layer was determined from the axial scans and the microscope calibration. The number of cell layers was quantified by manual examination of at least six axial scans per time point and medium formulation.

Transmission electron microscopy

Samples for transmission electron microscopy (TEM) were prepared in two different ways: traditional preparation and

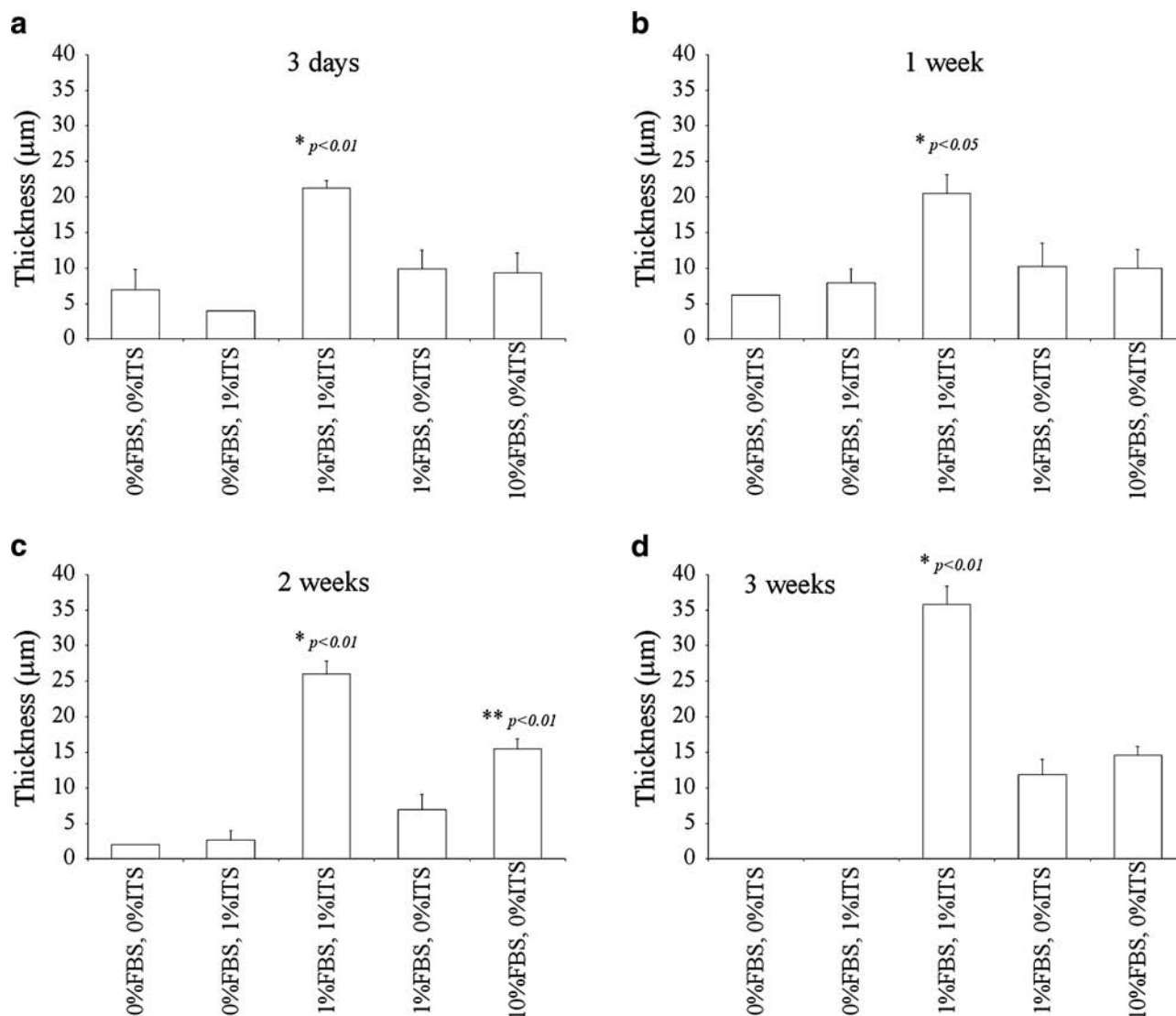


FIG. 1. Thickness of constructs for the culture medium formulations described in Table 1, measured through differential interference contrast (DIC) microscopy. Results are average \pm standard error of a minimum of four and a maximum of six independent experiments. *Significantly different to all other groups and **significantly different to serum-free groups. (a) 3 days, (b) 1 week, (c) 2 weeks, and (d) 3 weeks.

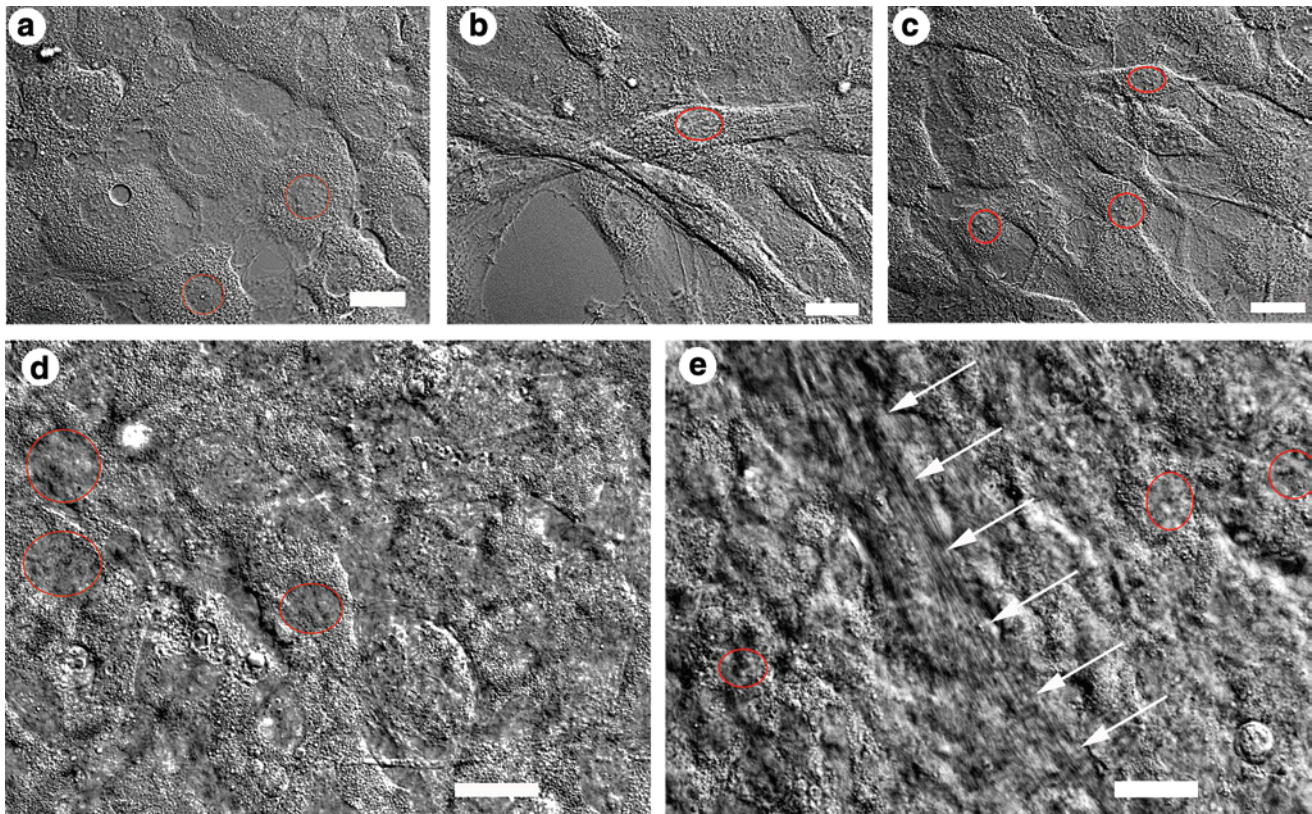


FIG. 2. DIC microscopy images illustrating the differences in the morphology of bovine corneal fibroblasts cultivated for 1 week with the basic culture medium supplemented with (a) 0% fetal bovine serum (FBS) and 0% insulin–transferrin–selenium (ITS), (b) 0% FBS and 1% ITS, (c) 1% FBS and 0% ITS, (d) 10% FBS and 0% ITS, and (e) 1% FBS and 1% ITS; white arrows indicate streak of aligned matrix (most likely deposited collagen). Scale bars are 20 μm . Drawn circles or ellipses indicate the contour of some cell nuclei and are used to illustrate shape and size. Color images available online at www.liebertonline.com/ten.

quick-freeze/deep-etch (QFDE). Each of these types of sample preparation allowed for distinctive qualities of detail preservation and observation. In the traditional sample preparation, samples were immersed in $\frac{1}{2}$ strength Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1M cacodylate buffer, pH 7.4) for 1 h. Postfixation in 2% osmium tetroxide and serial dehydration in graded ethanol followed. After dehydration, constructs were embedded in Epon-Araldite and thin-sectioned *en face* or in cross section. After staining with uranyl acetate and lead citrate in methanol, construct sections were viewed and photographed on a JEOL JEM-1000 TEM (JEOL, Tokyo, Japan).

For QFDE sample preparation, after 3 weeks of cultivation the glass bottom of culture dishes was carefully removed from its plastic frame, and the cell sheets peeled off and cut into small pieces that were then preserved by slam freezing onto a copper block cooled to -194°C using a portable cryogun (Delaware Diamond Knives, Dover, DE). Frozen cell sheets were transferred under liquid nitrogen into a modified CFE-40 Freeze fracture/freeze etch apparatus (Cressington Scientific, Watford, United Kingdom). The cell sheets were superficially fractured at -150°C and etched for 15 min at -95°C . They were then rotary-shadowed at a 20° angle with Pt/C followed by a carbon coating applied at a 90° angle. After removal from the CFE-40, the sheets were digested in household bleach for 24 h, picked up on 600-mesh grids,

and viewed at an acceleration of 70 kV on a JEOL JEM-1000 TEM.

In situ immunofluorescence

Cell constructs were stained *in situ* to investigate actin filament organization, detect α -SMA, and verify the secretion and large-scale organization of type I collagen. Compared to fibroblasts, myofibroblasts exhibit stronger stain for organized α -SMA and f-actin filaments. Cells for immunofluorescence were rinsed in PBS, fixed in 3% formalin for 10 min, and rinsed three times in PBS. For intracellular staining, fixed cells were permeabilized with 0.5% Triton X-100 and thoroughly rinsed with PBS. Cells were then treated according to one of the following protocols: (i) incubation with 1:20 phycoerythrin-conjugated antibody for α -SMA (1 h, room temperature), (ii) incubation with 1:40 rabbit anti-bovine collagen type I polyclonal antibody (1 h room temperature) followed by blocking with 1% goat serum (30 min, room temperature), wash and exposure to 33 $\mu\text{g}/\text{mL}$ fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG (1 h, room temperature), or (iii) blocking with 1% BSA (10 min, room temperature) followed by wash and incubation with 100 μL of rhodamine phalloidin in BSA (1:40) (20 min, room temperature). After three 5-min washes in PBS, all stained cells were observed on a Nikon

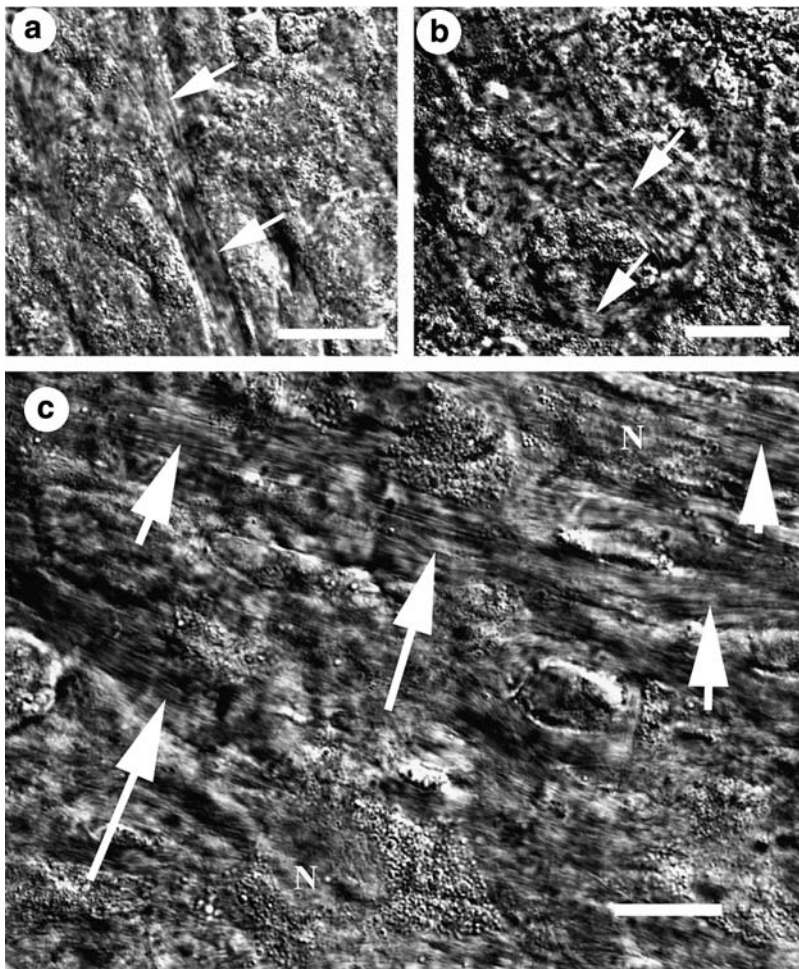


FIG. 3. DIC microscopy image of (a) 3-day cultures in 1% FBS + 1% ITS, (b) 10-day cultures in 10% FBS, and (c) 3-week cultures in 1% FBS + 1% ITS. Arrows point to what we believe are aligned collagen fibrillar structures in the extracellular matrix. N, cell nucleus. Scale bars are 20 μm .

TE2000E inverted microscope fitted with an epifluorescence attachment.

Statistics

An independent experiment was defined as the use of cells from one bovine specimen to seed a set of culture dishes at a minimum of one dish per culture medium formulation. The results are presented as average \pm standard error. Student's *t*-test assuming equal variances was used to determine significant differences across groups. Significance was defined as $p < 0.05$.

Results

Cell proliferation and survival

In all cultures where serum was added to the medium (groups A, B, and D in Table 1), cells reached confluence within 1 week of cultivation. In contrast, cells cultured in the serum-free medium (groups C and E in Table 1) did not show appreciable proliferation, and failed to survive after approximately 1.5 weeks.

Culture microscopic morphology (DIC imaging)

Stratification and thickness. Multiple layers of cells were observed as early as the third day in cultures supplemented

with 1% FBS + 1% ITS (group D in Table 1) and in cultures supplemented with either 1% or 10% FBS but no ITS (groups A and B in Table 1) (results not shown). The thickness of the cell sheets was significantly higher in cultures supplemented with 1% FBS + 1% ITS than in all other cultures throughout the culture period (Fig. 1). Cell sheets cultivated with 1% FBS + 1% ITS were $21.3 \pm 1.0 \mu\text{m}$ thick on day 3 (Fig. 1a), and continued to thicken with time, reaching $35.8 \pm 0.5 \mu\text{m}$ thickness at the end of the third week (Fig. 1d). This corresponds to a 1.7-fold increase in cell sheet thickness within an 18-day period. In comparison, cells cultivated with 10% FBS (group A in Table 1) formed sheets that were $9.3 \pm 2.8 \mu\text{m}$ thick on day 3 (Fig. 1a) and had grown to $14.6 \pm 1.2 \mu\text{m}$ at 3 weeks (Fig. 1d), corresponding to a 1.6-fold increase in thickness. Finally, 1% FBS cultures (group B in Table 1) exhibited less stratification, with an increase in thickness from $9.9 \pm 2.7 \mu\text{m}$ to $11.9 \pm 2.1 \mu\text{m}$ between the 3rd day (Fig. 1a) and 21st day (Fig. 1d) of cultivation (1.2-fold increase in thickness within the time frame). Three-week ITS-free cultures supplemented with 1% FBS or 10% FBS (groups A and B in Table 1) were only 33.1 ± 8.3 or $40.6 \pm 6.2\%$ (respectively) as thick as cultures supplemented with 1% FBS + 1% ITS at the same time point (Fig. 1d). The same comparison (thickness with respect to 1% FBS + 1% ITS culture) at 2 weeks yielded $26.7 \pm 10.2\%$ and $59.4 \pm 9.6\%$ for groups A and B in Table 1, respectively (Fig. 1c), and at 1 week it was $50.0 \pm 22.5\%$ and $48.8 \pm 19.2\%$ (Fig. 1b).

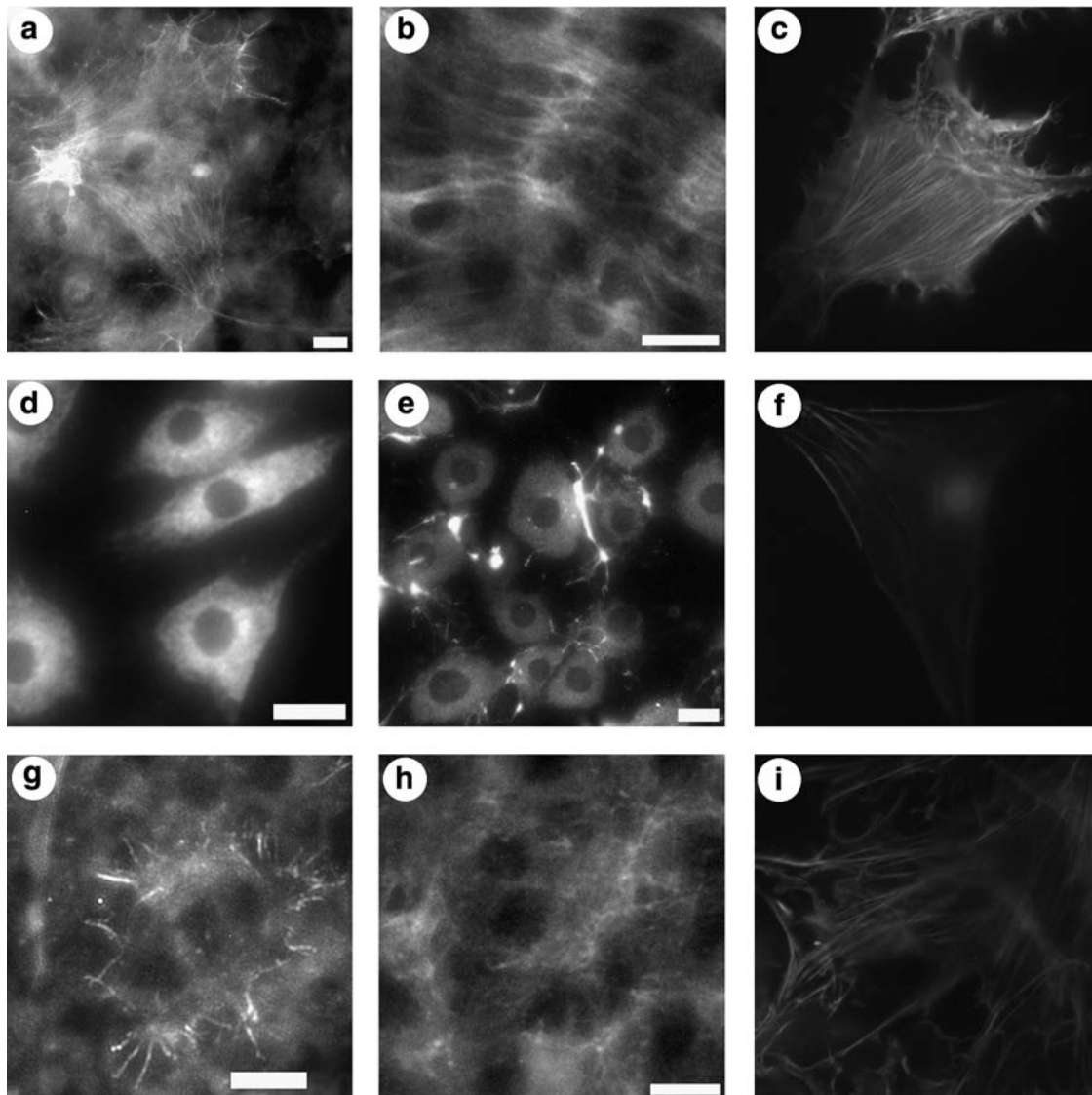


FIG. 4. Fluorescent images of 4-week cell sheets cultivated in 1% FBS + 1% ITS (a–c), 1% FBS (d–f), or 10% FBS (g–i) and stained with a phycoerythrin-conjugated antibody for α -smooth muscle actin (a, d, g), a collagen type I polyclonal antibody and a fluorescein isothiocyanate-conjugated secondary antibody (b, e, h), or phalloidin-rhodamine (c, f, i). All images were acquired using a 20 \times objective; however, some images were cropped to remove artifacts. Scale bars are 20 μ m. Asterisks in bindicate cell processes or filipodia.

Cultures supplemented with 10% FBS were also significantly thicker than serum-free ones (groups C and E in Table 1) and thicker than those supplemented with 1% FBS but no ITS (group B in Table 1) on the second week of culture (Fig. 1c). At that particular time point, serum-free, ITS-free cultures (group C in Table 1) were only $12.9 \pm 1.2\%$ as thick as 10% FBS cultures, whereas for serum-free, 1% ITS cultures (group E in Table 1) and ITS-free, 1% FBS cultures (group B in Table 1) the same comparison yielded $17.2 \pm 10.2\%$ and $44.9 \pm 18.0\%$. Interestingly, past the second week of cultivation, the thickness of cell sheets cultivated with 1% or 10% FBS but no ITS (groups A and B in Table 1) was statistically similar.

Cell morphology. Cells cultivated without FBS and ITS appeared round with round nuclei during the first week of cultivation (Fig. 2a). On the second week of cultivation, these cells became more dendritic and in some cases assumed a

“T” shape. An appreciable decrease in cell number was observed, such that by the third week of cultivation there were virtually no cells left on the culture surface (not shown). Cells cultivated with 1% ITS and no FBS (group E in Table 1) were confluent during the first week of cultivation (Fig. 2b). They were elongated with elliptical nuclei. During the second week of cultivation, the cell number started to decrease, such that there were virtually no cells left in the culture area on the third week of cultivation (not shown).

In contrast, cells cultivated with 1% FBS and 0% ITS (group B in Table 1) proliferated actively, and the cell sheet was confluent by the end of the first week. Initially, these cells exhibited round nuclei and elongated cell bodies with long processes (Fig. 2c). A few ellipsoid cells were also observed (not shown). By the second week of cultivation, about one half of the cells were round, while the other half were ellipsoidal.

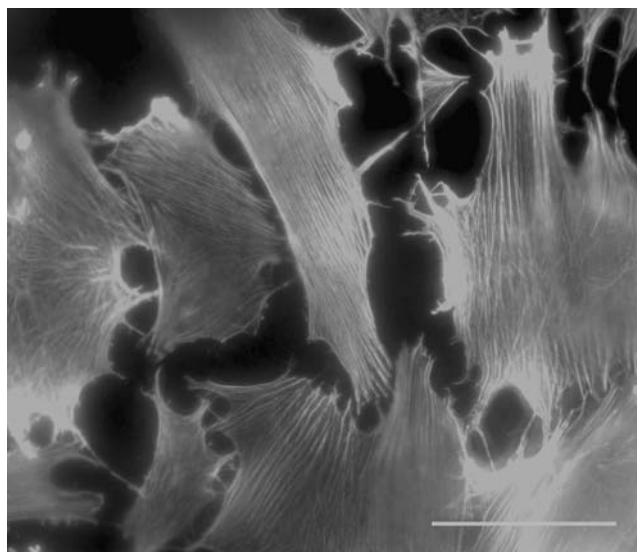


FIG. 5. Rhodamine phalloidin staining of 4-week cell sheet cultivated in 1% FBS + 1% ITS. Scale bar is 100 μ m.

Cells cultivated in the presence of 10% FBS and 0% ITS (group A in Table 1) were confluent throughout the cultivation period (Fig. 2d). Due to a dense cell and ECM content, cellular features are not easily appreciable on the DIC micrographs in these cultures (Fig. 2d). However, it was observed that the nuclei of these cells were round, with cell bodies that were elongated with numerous processes during the first week of cultivation and that became more spread out by the end of the second week of cultivation (not shown).

Finally, cells cultivated simultaneously with 1% each of FBS and ITS (group D in Table 1) were confluent throughout the cultivation period, and similarly to the case of group A, dense cellular and ECM content obscured their cellular features in DIC images (Fig. 2e). Nonetheless, cells in the 1%

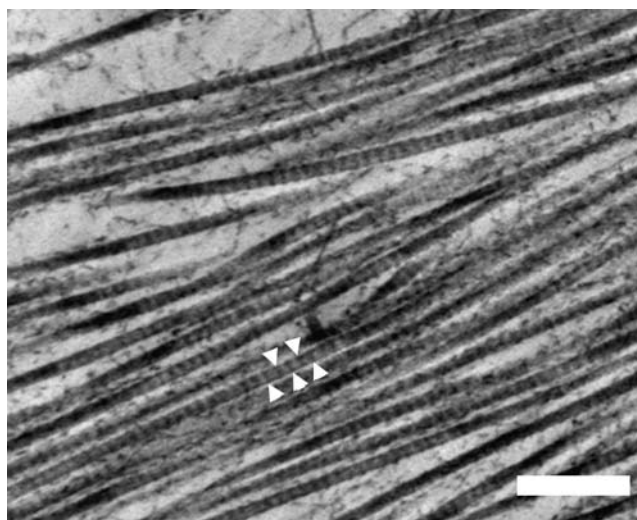


FIG. 6. Transmission electron microscopy (TEM) of bovine corneal stroma fibroblast cultures showing extracellular matrix fibrils with diameter (\sim 60 nm) and banding (arrowheads) characteristic of collagen. Scale bar is 500 nm.

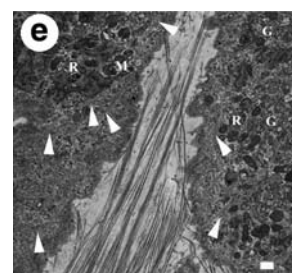
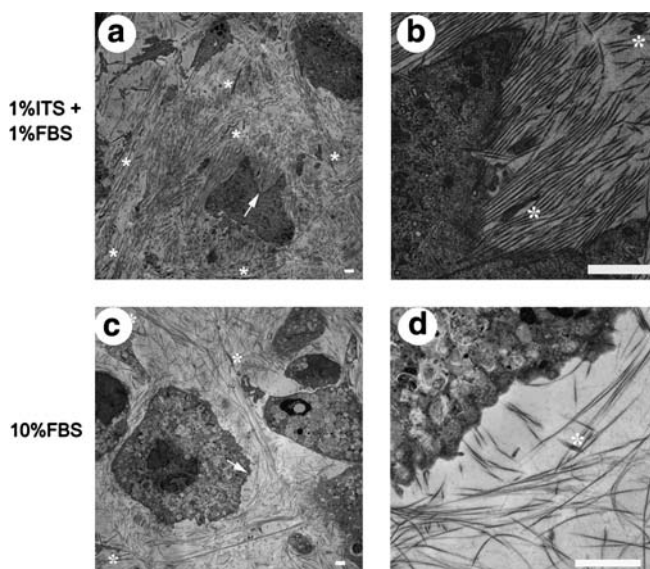


FIG. 7. TEM images of *en face* sections from 3-week bovine corneal stroma fibroblasts cultivated in the culture medium supplemented with 1% FBS + 1% ITS (a, b, e) and 10% FBS (c, d). Panels (b) and (d) were taken at higher magnification at the same spot indicated by arrows in (a) and (c), respectively. Asterisks are used to indicate the presence of filopodia aligned in the same direction as surrounding collagen fibrils. Mitochondria (M), Golgi apparatus (G), residual bodies (R), and intracellular fibril bundles that may be actin or pro-collagen (arrowheads) are indicated in (e). Scale bars are 2 μ m in (a–d) and 500 nm in (e).

FBS + 1% ITS group appeared less spread out than in all other groups and displayed round or elliptical nuclei. Cultures appeared to comprise more cell layers (i.e., increased cell stratification) than those in the other experimental groups, as assessed by qualitative examination of the library of DIC z-scans such as those in Supplemental Movies 1 and 2 (available online at www.liebertonline.com/ten). Although the previous statement does not convey statistical significance, it is supported by the observation of significantly increased construct thickness (Fig. 1). Cells in the 1% FBS + 1% ITS group did not exhibit numerous processes as did those cultivated in 10% FBS, nor did they possess a dendritic appearance upon visual inspection.

Extracellular fibrillar structures. Aligned ECM (presumably bundles of collagen fibrils) was detected by DIC microscopy, which has very high spatial resolution in the z-plane, as early as the third day in cultivation for cultures

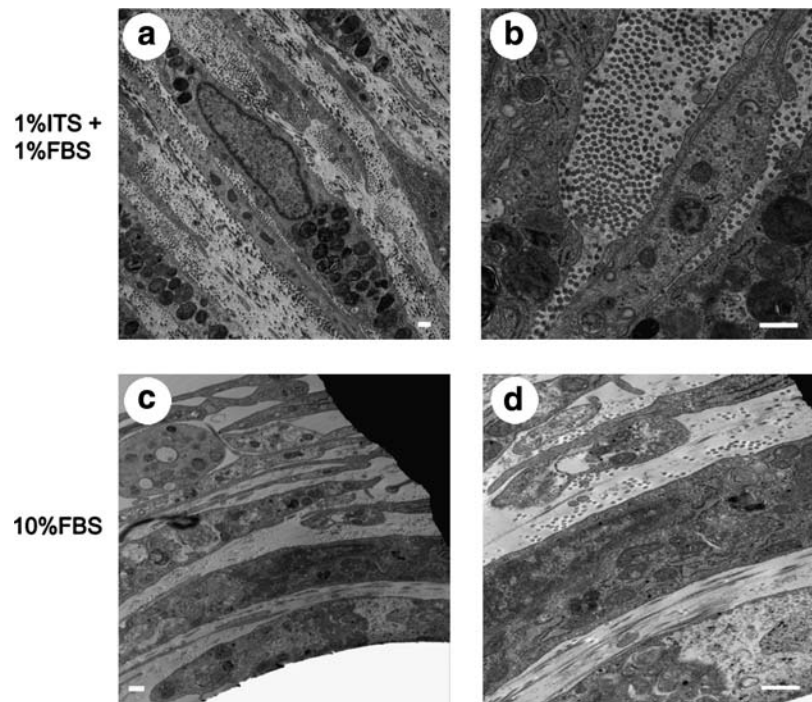


FIG. 8. TEM images of cross sections from 3-week bovine corneal stroma fibroblasts cultivated in the culture medium supplemented with 1% FBS + 1% ITS (**a, b**) and 10% FBS (**c, d**). Scale bars are 500 nm.

supplemented with 1% FBS + 1% ITS (arrows in Fig. 3a). Matrix fibrils were detected after 7–10 days in those supplemented with either 1 or 10% FBS (arrows in Fig. 3b–10% FBS shown). Of these early fibrillar structures, only those in 1% FBS + 1% ITS cultures were visibly aligned (Fig. 3a). After 3 weeks, fibrillar ECM in 1% FBS + 1% ITS cultures was densely packed and demonstrated uniaxial alignment over reasonably long lateral length scales ($\sim 100\ \mu\text{m}$; Fig. 3c), whereas in 10% FBS cultures the synthesized ECM remained sparse and disorganized (not shown).

Spatial organization of constructs. Representative z-scan DIC microscopy of culture systems that demonstrated appreciable stratification is provided in two Supplemental Movies (available online at www.liebertonline.com/ten). The z-scans begin at the glass substrate surface and move toward the culture medium–air interface. For the 10% FBS cultures, z-scans reveal a mostly cellular stratified construct interspersed with a loose fibrillar matrix. The 1% ITS + 1% FBS culture system typically comprised a dense matrix–cellular construct in which highly aligned fibrillar matrix occupied the center part of the constructs. Matrix fibrils were observed to align typically only in one direction throughout the axial scans of these cultures, suggesting that alternating orthogonal arrays of collagen similar to those found in the cornea were either not present or not discernible under DIC microscopy.

Cell contractility and pulling-off of the cell layer into highly cellular clumps was observed in $n=2$ cultures that used 1% FBS + 1% ITS supplementation, in $n=5$ cultures supplemented with 1% FBS but no ITS, and in $n=1$ cultures supplemented with 10% FBS (not shown).

Biochemistry/immunostaining

To detect differences in the density and/or organization of type I collagen and to investigate myofibroblastic transfor-

mation, 4-week cultures of dedifferentiated bovine corneal stromal keratocytes were stained for α -SMA, f-actin, and type I collagen. Cells in 1% FBS + 1% ITS showed prominent organization of actin filaments and intracellular α -SMA, suggestive of stress-fiber formation and myofibroblastic transformation. These cells also showed organized extracellular type I collagen (Figs. 4a–c and 5). 1% FBS + 0% ITS cultures did not stain robustly for f-actin, α -SMA, or type I collagen (Fig. 4d–f). Cells cultivated with 10% FBS + 0% ITS stained only peripherally for f-actin or α -SMA, and showed a diffuse type I collagen network (Fig. 4g–i).

Cell and ECM ultrastructure

While DIC microscopy was used to observe large scale cell–matrix interaction and organization, standard preparation TEM (sTEM) micrographs provided a more detailed local account of the cell–matrix interaction, matrix organization, and fibrillar structure (e.g., collagen banding and periodicity). In addition to sTEM, we also used QFDE imaging to examine the fibroblasts and their synthesized ECM with higher fidelity and with fewer fixation and processing artifacts (although QFDE is not artifact free).

sTEM images from sections obtained at a distance of approximately $10\ \mu\text{m}$ from the glass culture substrate corroborated the observations made using DIC microscopy. Measurements of $n=40$ individual fibrils pooled from >10 TEM images served to positively identify ECM fibrils as collagenous due to their size and to the presence of characteristic banding (see Fig. 6 for representative high magnification TEM of cell-derived collagen). Though it is known that sTEM affects fibril diameter due to the dehydration processing, we felt that it was acceptable to determine fibril diameters directly from micrographs for direct comparison. Measurements of the smallest dimension of collagen fibrillar cross sections from sTEM images revealed a fibril diameter of $61.2 \pm 0.9\ \text{nm}$ for 1% FBS + 1% ITS cultures and $32.2 \pm 0.7\ \text{nm}$ for 10% FBS

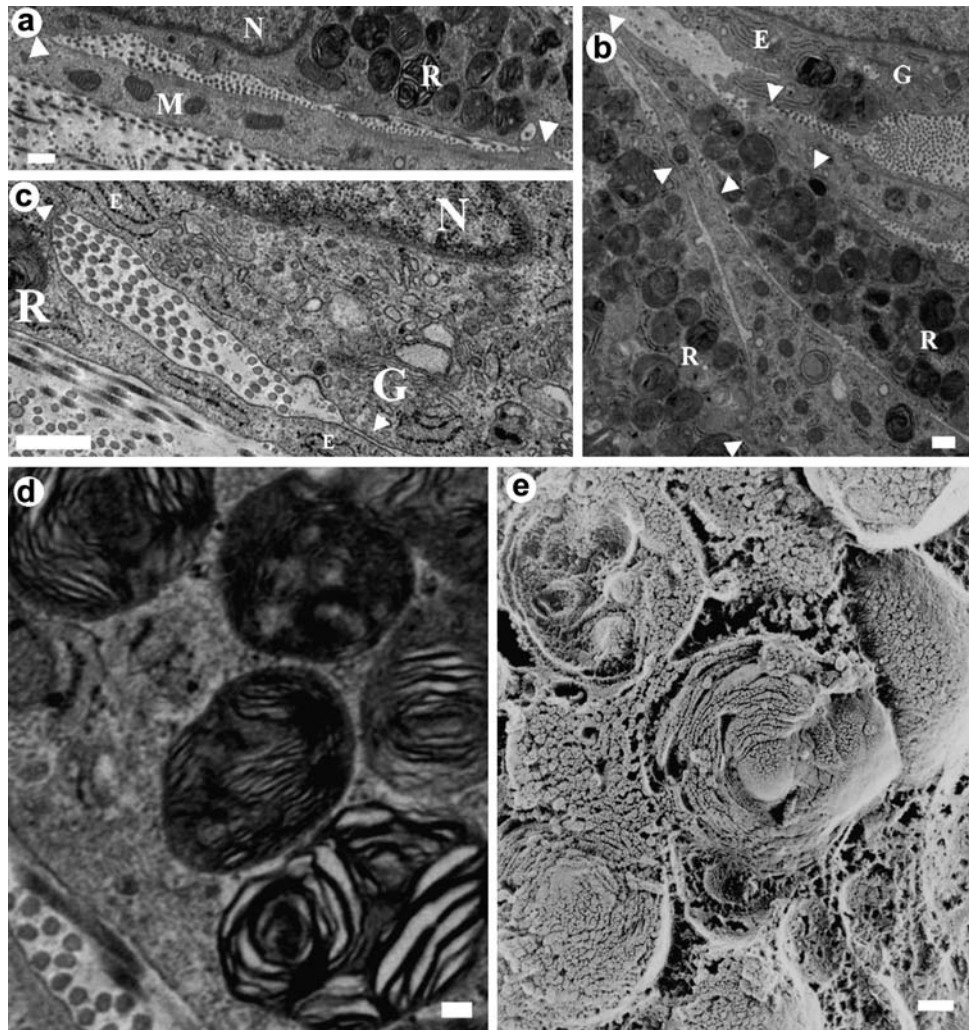


FIG. 9. Images from 3-week bovine corneal stroma fibroblasts cultivated in the culture medium supplemented with 1% FBS + 1% ITS: (a–d) cross-sectional traditional TEM and (e) quick-freeze/deep-etch (QFDE)/TEM. Arrowheads point to cell–cell junctions. Note the presence of matrix fibrils in between contacting cells. M, mitochondria; G, Golgi apparatus; E, rough endoplasmic reticulum; R, residual bodies; N, cell nucleus. (d, e) Close-up details of intracellular residual bodies with a concentric-ring shape observed in a traditional cross section. Scale bars are 500 nm in (a–c) and 100 nm in (d, e).

cultures, and this difference was significant ($p < 1 \times 10^{-10}$). Banding periodicity was 68.7 ± 2.2 nm and 60.3 ± 0.7 nm for 1% FBS + 1% ITS and 10% FBS cultures, respectively, and this difference was significant as well ($p < 0.05$). In 1% FBS + 1% ITS cultures, a higher density of collagen fibrils was observed, and these fibrils were generally oriented unidirectionally over length scales of 10–50 μ m (Figs. 7a, b and 8a, b). In contrast, collagen fibrils were less dense in 10% FBS cultures, and did not show unidirectional alignment over the same length scale (Figs. 7c, d and 8c, d). However, occasional orthogonality in the direction of collagen layers separated by one cell layer was observed in these cultures (Fig. 8d).

Interesting cellular features observed via traditional TEM included collagen fibrils appearing to emerge through the cell's membrane (Fig. 7b). In addition, extensive mitochondria, lysosomes, and residual bodies and what is believed to be either intracellular actin or collagen fibril bundles (Fig. 7e) were observed. Numerous cell–cell interactions and cell junctions between lamellar layers were discerned, in some cases the junctions produced a confined space between two

cells where aligned collagen fibrils were present (Fig. 9a, c). In the intracellular space, there could be observed an abundance of residual bodies (Fig. 9b, d, and e). Further, abundant filopodia were observed within the extracellular space, and they appeared to orient in the same direction as surrounding collagen fibrils (Fig. 10).

The TEM images obtained from samples prepared via QFDE (Figs. 11 and 12) corroborated the observations made on traditionally embedded TEM samples (Figs. 6–10) and DIC microscopy (Figs. 2 and 3; Supplemental Movies 1 and 2, available online at www.liebertonline.com/ten), as well as provided further insight into microstructural differences in the matrix secreted by cells cultivated with 10% FBS-supplemented or 1% FBS + 1% ITS-supplemented medium. Increased density of collagen fibrils was observed in 1% FBS + 1% ITS cultures (Fig. 11a vs. b, c vs. d). An increased number of cell processes (Fig. 11b) were observed in 10% FBS cultures. In both culture systems, collagen fibrils appeared in close relationship or attached to a secondary matrix (Fig. 11c, d), which may be material left behind after cell fracture by

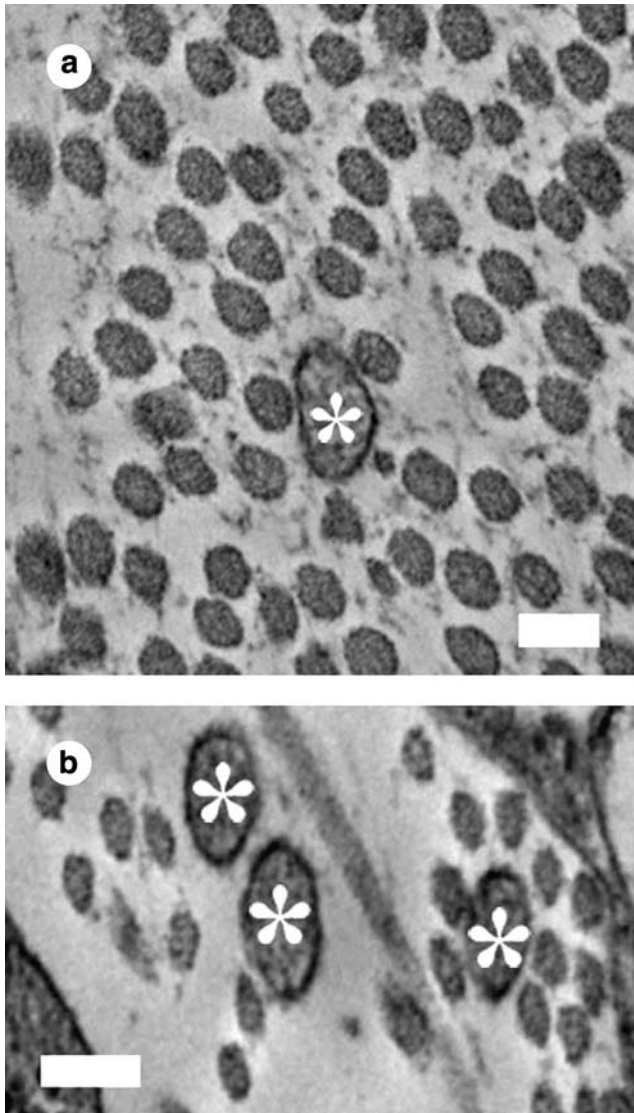


FIG. 10. High-magnification TEM images of cross sections from 3-week bovine corneal stroma fibroblasts cultivated in the culture medium supplemented with 1% FBS + 1% ITS (a) or 10% FBS (b). Asterisks indicate filopodia in alignment with surrounding collagen fibrils. Scale bars are 100 nm.

the knife during QFDE sample processing. In addition, a fibril-positor-like structure was observed in a cell membrane. What appears to be a bundle of collagen fibrils is observed exiting the fibril-positor-like structure (Fig. 12). In addition, the differences in collagen fibril diameter reported for traditionally prepared TEM images (Figs. 6–10) persisted in the QFDE specimens (Figs. 11 and 12), with 61.3 ± 1.1 nm and 42.1 ± 7.7 nm for 1% FBS + 1% ITS and 10% FBS cultures, respectively ($p < 1 \times 10^{-10}$). Banding of collagen fibrils in the QFDE specimens (such as those in Figs. 11 and 12) had a periodicity of 66.4 ± 1.8 nm and 63.6 ± 1.6 nm for 1% FBS + 1% ITS and 10% FBS cultures, respectively ($p > 0.05$). Fibril diameters measured using traditionally prepared versus QFDE-prepared TEM samples were statistically similar for 1% FBS + 1% ITS cultures ($p = 0.09$), but significantly dif-

ferent for 10% FBS cultures ($p = 6 \times 10^{-7}$), with fibrils in traditionally prepared TEM samples showing approximately 77% of the diameter of those in QFDE-prepared TEM samples.

Discussion

During the *in vivo* development of the mammalian corneal stroma, cell stratification and organization occur before organized ECM production. Similarly, using an *in vitro* developmental corneal tissue engineering model, we recently reported that the stratification and organization of human corneal fibroblastic cells was an important factor preceding the production of organized collagenous lamellae.³¹ In the current investigation, both cell stratification and synthesis of aligned bundles of collagen by dedifferentiated bovine corneal stromal keratocytes cultivated on glass substrates were appreciably enhanced by using the culture medium supplemented with 1% each serum and ITS. However, this medium formulation induced unexpected phenotypic changes consistent with myofibroblastic transformation, and the resulting cell–matrix constructs were less mimetic to the ultrastructure of the cornea with respect to those in our previous study³¹ because they were not as thick and did not contain prolific alternating arrays of organized collagen lamella. The morphology of the collagen fibrils and the myofibroblastic transformation of the cells suggested that matrix production was more similar to scar formation than to developmental histogenesis. Cultures supplemented with 10% FBS only (no ITS) stratified and produced fine collagen that had individual fibril morphology similar to that found in the adult cornea. However, this collagen was not as prolific or locally aligned as that found in the combined serum/ITS cultures. The relative success in producing stratified cultures containing collagenous matrix on glass substrates in a scaffold-free system provides a unique opportunity to further observe matrix synthesis *in vitro*, even though it remains unclear how the observed myofibroblastic transformation in cell phenotype relates to what takes place during wound healing or developmental histogenesis *in vivo*.

Serum-free versus serum-supplemented medium

The inherent variability of serum composition and its potential for pathogenic agent contamination make its elimination from the culture medium highly desirable. A beneficial effect of serum is that it mediates the transformation of corneal stromal keratocytes into repair fibroblasts.²³ However, the TGF- β found in serum⁶¹ has the potential to facilitate their further, undesirable transformation into myofibroblasts.^{23,35,62} The presence of bFGF in media can suppress fibroblast to myofibroblast transition.²³ In an attempt to preclude the formation of scar-like tissue containing significant numbers of contractile myofibroblasts, a basal medium containing bFGF and no TGF- β was employed. A 10-fold reduction in the serum concentration was achieved (compared to our previous model³¹) while maintaining cell stratification and substantial aligned collagen secretion (if the medium contained 1% ITS). Interestingly, the use of 10% FBS culture medium (which mimicked that used in our scaffold-free corneal developmental model³¹) resulted in less fibrotic cultures (small, cornea-like collagen fibrils and dendritic cells, weak f-actin, and α -SMA staining) than the reduced

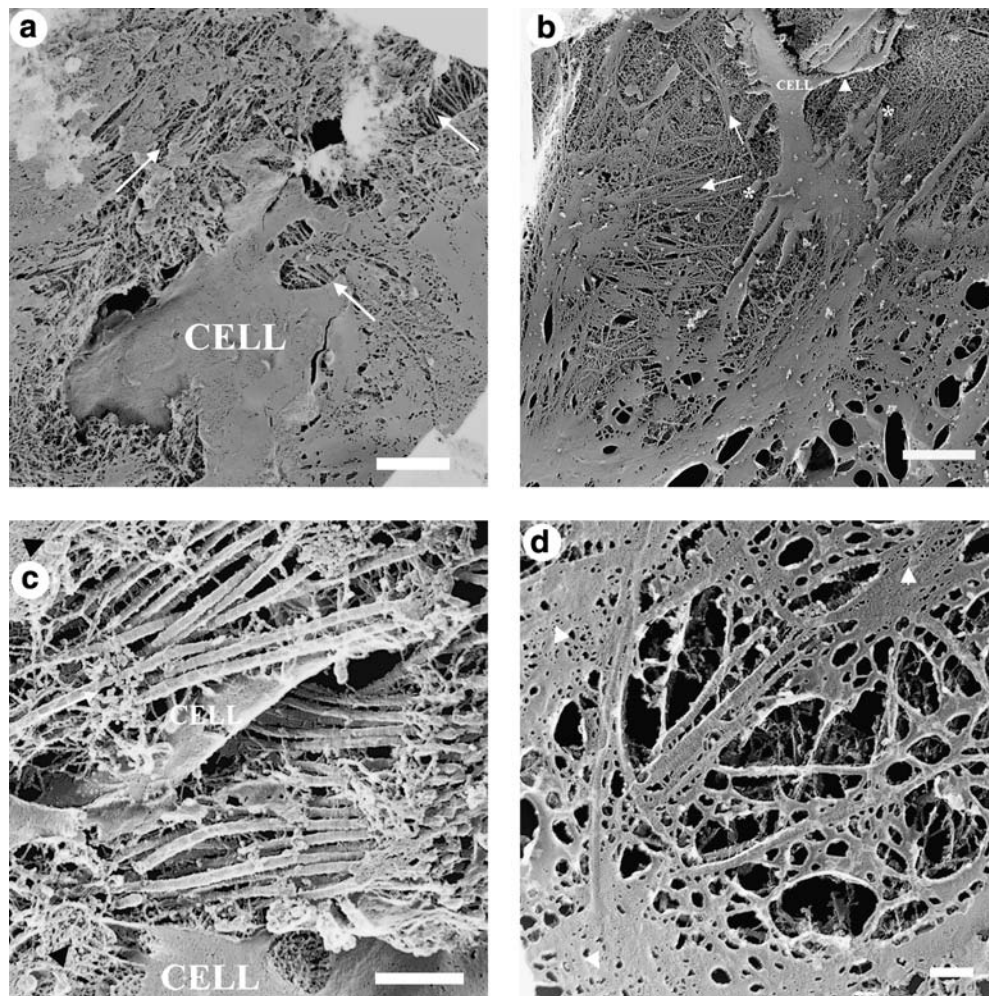


FIG. 11. TEM images showing fine cellular and matrix structures in culture samples that were prepared via QFDE. Panels (a) and (c) correspond to 1% FBS + 1% ITS cultures, (b) and (d) correspond to 10% FBS cultures. Remaining portions of the cell membrane in the fractured replicas are indicated by the word "CELL"; collagen fibrils are indicated by long arrows drawn parallel to the apparent direction of fibril orientation, and arrowheads point to what appears to be secondary matrix associated with the collagen fibrils. Scale bars are 2 μm in (a, b), and 500 nm in (c, d). Asterisks in b indicate cell processes or filipodia.

serum culture system with ITS, which exhibited scar-like behavior (large fibrils and strong phalloidin and α -SMA staining consistent with the formation of stress fibers and myofibroblastic transformation). This result was unexpected and counterintuitive, given what is known about the role of TGF- β in mediating myofibroblastic transformation both *in vivo* and *in vitro*. Although no exogenous TGF- β was purposely added to any of the cultures, serum contains unspecified amounts of this growth factor, and thus higher serum concentrations should have resulted in increased myofibroblastic transformation.

Complete deletion of serum from our medium was detrimental to cell viability in the longer-term cultures (1.5–3 weeks). Others have maintained viability (and consistently reported quiescence) of bovine corneal stromal cells in serum-free cultivation for up to 11 days;^{1,36,63–65} however, they have used mostly plastic culture substrates (in some cases treated to enhance cell attachment), they did not stimulate the culture with ascorbic acid and they used primary cells, compared with our uncoated glass substrate and fourth- to fifth-passage cells.

ITS stimulated organized collagenous matrix secretion and organization

ITS in the reduced-serum culture medium significantly improved cell stratification and organized ECM secretion relative to the other medium formulations. This is consistent with reports of *in vitro* cell stratification in the insulin-supplemented culture medium.^{66–68} ECM fibrils were collagenous showing typical crossbanding in TEM images. ITS in low-serum cultures induced increased staining of organized actin filament bundles and α -SMA as well as organized extracellular type I collagen (Fig. 4), suggesting that these cells underwent myofibroblastic transformation.^{11,17,18} Although double labeling was not carried out in the present study, we expect that actin filaments and α -SMA would colocalize as they did in a study that reported myofibroblastic transformation in cultures of human corneal stromal keratocytes in the serum-free medium containing TGF- β .⁶⁹ Compared with fibroblasts, myofibroblasts are more active in the production of α -SMA, collagen, and fibronectin, and in the formation of organized intracellular actin filaments and focal adhesions,^{11,17,18,23} and

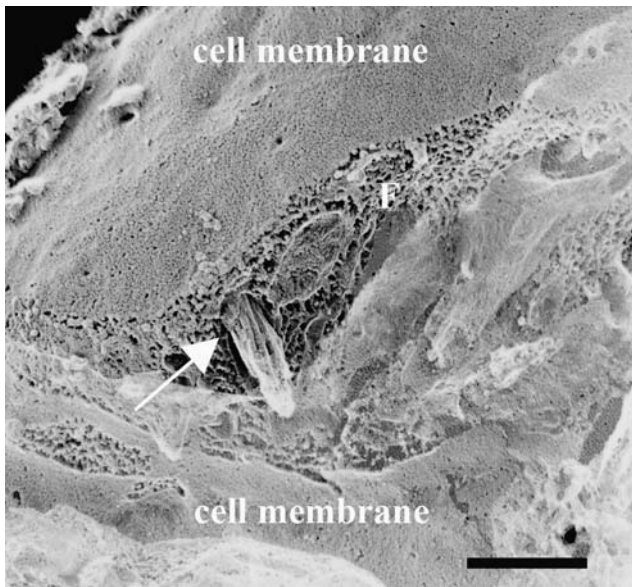


FIG. 12. TEM image showing a fibripositor-like structure in a 1% FBS + 1% ITS culture sample that was prepared via QFDE. Remaining portions of the cell membrane in the fractured replica are indicated; "F" indicates the plane of fracture across the cell membrane. A fibripositor-like pore of approximately 530-nm-diameter is indicated by the arrow, with a tight bundle of fibrils extruding through the pore. Each fibril is approximately 30–40 nm in diameter. The bundle appears to be covered in a ground substance. Scale bar is 500 nm.

less active in the secretion of matrix-digesting MMPs.^{25,26} It is thus possible that cells in 1% FBS + 1% ITS medium retained more ECM than did their 10% FBS or 1% FBS counterparts, although MMP expression was not quantified.

Most available literature reports stimulation of collagen synthesis *in vitro* by ITS's main component, insulin,^{32,53,70,71} with few exceptions.⁵⁷ It is possible that cellular uptake of ascorbic acid (~0.07–1 mM) may be inhibited by glucose (~1–25 mM), preventing its stimulatory effect on collagen synthesis; this inhibition may be reduced or abolished by insulin (~10⁻⁹ to 10⁻⁶ mM).^{57,72,73} Ascorbic acid concentration accumulates in the intracellular space to a level 100-fold that in the medium, and this uptake directly correlates to intracellular hydroxyproline synthesis (reviewed in Ref.⁷⁴). During wound healing, ascorbic acid autooxidates into dehydroascorbate,^{75,76} which is cytotoxic⁷³ but can be reduced to ascorbate inside the cell. Insulin stimulates the uptake of dehydroascorbic acid by the cells under high glucose conditions.⁷³ Thus, it is likely that in the present study (i) ascorbic acid stimulated collagen synthesis, (ii) glucose may have partially inhibited ascorbic acid cellular uptake, and (iii) insulin (1% ITS) abolished this effect.

Intracellular structures

Intracellular 8–12-nm-diameter fibrils (Fig. 7e) were similar to procollagen filaments or thin collagen fibrils reported elsewhere.^{77,78} Intracellular collagen fibrillogenesis can occur in activated fibroblasts when collagen production exceeds the cell's excretion capabilities.^{77,78} Thus, Figure 7e may suggest overstimulation of collagen synthesis in 1% FBS + 1% ITS cultures. Some of the smaller-diameter (~7 nm) intracellular

fibrils may correspond to actin microfilaments consistent with myofibroblastic transformation. Concentric-ring residual bodies (Fig. 9) may correspond to packed lipid membranes of incompletely digested aged organelles,⁷⁹ and their abundance also suggests elevated synthetic activity.

ECM structures

Our observations agree with recent literature,⁸⁰ where stem cells from human corneal stroma cultured in the serum-free medium secreted fibrillar collagen only if cultured in three-dimensional pellets (i.e., forced stratification). Collagen fibrils produced in the stratified 1% ITS + 1% FBS culture appear denser and more aligned than those of Du *et al.*'s pellet cultures.⁸⁰ This difference may be due to our culture substratum (glass instead of pellet), medium formulation (we employed more glucose and ascorbic acid than Du *et al.*, plus insulin and serum), or cell type.

The diameter of the collagen synthesized by the 1% FBS + 1% ITS system was larger than that produced in the normal bovine cornea, while the collagen produced by the 10% FBS system had nearly the correct diameter. Corneal fibroblasts and myofibroblasts express type III and type V collagen *in vitro*,^{2,81,82} both of which influence fibril diameter. Collagen types I and III and types I and V form heterotypic fibrils, with an inverse correlation between the concentrations of types III/I and V/I and the fibril diameter.^{83–91} Although the diameter of type I/V collagen fibrils in the bovine corneal stroma *in vivo* is 38.2 nm,⁹² *in vitro* cell-secreted collagen fibrils may be larger and/or more broadly distributed.⁹³ Cells and fibrils appeared embedded in a matrix that we tentatively categorized as fibronectin-like (Fig. 11), based on its similarity to QFDE images of Zea-Aragon *et al.*,⁹⁴ who identified similar structures via immunogold. Qualitatively, our QFDE image library showed that more of this fibronectin-like matrix was present in the 1% ITS + 1% FBS culture (not shown).

Figures 7b and 12 are consistent with the fibripositor mechanism of aligned collagen secretion.^{95,96} Fibripositors are invaginations in the plasma membrane that align parallel to the axis of tendons and vectorially discharge collagen fibrils into the ECM. To our knowledge, Figure 12 constitutes the first three-dimensional observation of a cell-membrane fibripositor extruding a bundle of collagen fibrils (although the fibrils in this structure were not positively identified). We hold the opinion that fibripositors are more likely utilized by cells in wound healing mode because they permit tight control of the fibril forming environment. We found much more evidence of fibripositors in the 1% ITS + 1% FBS system that we did in the 10% FBS system. The observation of filopodia in close relation to and aligned in the same direction as collagen fibrils in Figures 7 and 10 is similar to that made in developing chick corneas.⁹⁷

Concluding remarks

Few corneal tissue engineering studies have focused on discerning the details of organized matrix production by fibroblasts. Because matrix production is a temporally varying event, it is critical to develop a highly controlled system that produces organized collagen while permitting maximum microscopic accessibility. In this study, bovine corneal fibroblasts were grown and stimulated to produce collagen in various culture medium formulations on glass substrates.

The most successful of these (1% FBS + 1% ITS) produced highly aligned collagen but also promoted a myofibroblastic-like transformation in the cells. The other successful culture medium (10% FBS) retained a nonfibrotic cell phenotype but produced fewer, less regular (but more cornea-like) collagen fibrils. Although the 1% ITS + 1% FBS culture system did not result in the *in vitro* utter replication of the ultrastructure of the cornea, it constituted an important advance toward the developing of highly controlled cell culture systems that permit the direct observation of the production of organized collagen secretion with high-spatial and temporal resolution.

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Disclosure Statement

No competing financial interests exist.

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