Title: **Corneal keratocyte transition to mesenchymal stem cell phenotype and reversal using serum-free medium supplemented with FGF-2, TGF-ß3 and retinoic acid**

Short Title: Keratocyte to cMSC: transition and reversal

Authors: Laura E. Sidney, Andrew Hopkinson

Institution: Academic Ophthalmology, Division of Clinical Neuroscience, Queen's Medical Centre Campus, University of Nottingham, UK

Corresponding Author: Dr Laura Sidney, Academic Ophthalmology, B Floor, Eye and ENT Building, Queen's Medical Campus, University of Nottingham, NG7 2UH, UK.

Tel: +44 (0)115 924 9924 Ext:62025 **Email:** laura.sidney@nottingham.ac.uk

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Abstract

Keratocytes of the corneal limbal stroma can derive populations of mesenchymal stem cells (MSC) when expanded in vitro. However, once a corneal MSC (cMSC) phenotype is achieved, regaining the keratocyte phenotype can be challenging, and there is no standardised differentiation medium. Here, we investigated the transition of keratocytes to cMSC and compared different supplements in their ability to return cMSC to a keratocyte phenotype. Immunofluorescence and RT-qPCR demonstrated *in vivo* keratocyte expression of ALDH3A1, CD34 and keratocan, but not any of the typical MSC markers (CD73, CD90, CD105). As the keratocytes were expanded *in vitro*, the phenotypic profile reversed and the cells expressed MSC markers but not keratocyte markers. Differentiating the cMSC back to a keratocyte phenotype using non-supplemented, serum-free medium restored keratocyte markers but did not maintain cell viability or support corneal extracellular matrix (ECM) production. Supplementing the differentiation medium with combinations of fibroblast growth factor-2 (FGF-2), transforming growth factor-β3 (TGF-β3) and retinoic acid (RA) maintained viability, restored expression of CD34, ALDH3A1 and keratocan, and facilitated production of abundant ECM as shown by immunofluorescent staining for collagen-I and lumican, alongside quantitative assays for collagen and glycosaminoglycan production. However, no differentiation medium was able to downregulate the expression of MSC markers in the 21-day culture period. This study shows that the keratocyte to MSC transition can be partially reversed using serum-free media and supplementation with RA, FGF-2 and TGF-β3 can enhance this effect. This is relevant for development of corneal regenerative strategies that require the production of a keratocyte phenotype.

1. Introduction

The development of regenerative medicine therapies that include the use of stem cells to replace injured and diseased cells is one option for the treatment of corneal injuries (Patel *et al.* 2013). Effective vision is reliant on maintaining the structure and organisation of the extracellular matrix (ECM) of the corneal stroma (Jester *et al.* 1999, Funderburgh *et al.* 2003, Hassell and Birk 2010), which is produced and remodelled by the resident keratocytes (Poole *et al.* 1993, West-Mays and Dwivedi 2006). Keratocytes are mesenchymal cells with dendritic morphology, which when healthy remain quiescent. They replenish ECM proteins including collagen-I, and proteoglycans such as keratocan and lumican (Marshall *et al.* 1991, Michelacci 2003, Hassell and Birk 2010). Keratocytes also produce crystallins such as aldehyde dehydrogenase (ALDH) and transketolase (Jester *et al.* 1999, Sax *et al.* 2000, Jester *et al.* 2012) and express cell surface markers CD34 and CD133 (Joseph *et al.* 2003, Du *et al.* 2005, Perrella *et al.* 2007).

Trauma to the cornea causes keratocytes adjacent to the wound to take on a fibroblastic phenotype, in a process known as activation (West-Mays and Dwivedi 2006). Activation is associated with stromal tissue remodelling, scar formation and corneal opacity (Funderburgh *et al.* 2003). Activated cells begin to express markers, such as CD90 (Pei *et al.* 2004) and can go on to develop a contractile myofibroblast phenotype, expressing α-smooth muscle actin (α-SMA) (Jester *et al.* 1995, Helary *et al.* 2006, West-Mays and Dwivedi 2006).

Activation also occurs *in vitro*, stimulated when keratocytes are extracted and cultured on tissue culture plastic, particularly in serum-containing medium (Branch *et al.* 2012, Hashmani *et al.* 2013). We have previously demonstrated that keratocytes (often referred

to as corneal stromal cells (CSC) when cultured *in vitro*) that have been extracted from the peripheral and limbal stroma and cultured to passage 4, have a phenotype that conforms to the current criteria for multipotent mesenchymal stem cells (MSC) set by the International Society for Cellular Therapy (ISCT) (Branch *et al.* 2012, Hashmani *et al.* 2013). This criteria states that to be considered a population of MSC, over 95% of cells should express CD73, CD90 and CD105; under 2% of cells should express CD11b, CD14, CD19, CD34, CD45 and HLA-DR; and the cells should possess the ability to undergo adipogenesis, osteogenesis and chondrogenesis in vitro (Dominici *et al.* 2006). The resultant corneal MSC (cMSC), has a very different phenotype to an *in vivo* keratocyte.

There is little evidence to show that the keratocyte phenotype can be maintained from extraction *in vitro*, particularly in human cells. However, there is some evidence that the keratocyte phenotype can be regained once lost, using various techniques and culture medium supplementation. Several studies have shown bovine and rabbit CSC can be partially restored to keratocyte phenotype simply using serum-free medium with no other supplementation (Berryhill *et al.* 2002, He and Bazan 2008, Chen *et al.* 2009). However, there is considerable variation in keratocyte properties between species, and results in animals cannot always be translated to humans (Jester *et al.* 2005). For human CSC, there is limited evidence that serum-free medium alone can support differentiation, but in many cases, supplementation in the form of insulin, transferrin, selenium and ascorbate is required to maintain cell viability (Musselmann *et al.* 2005, Du *et al.* 2010, Foster *et al.* 2015). Additional supplements that have also been suggested include fibroblast growth factor-2 (FGF-2) (Du *et al.* 2010, Lakshman and Petroll 2012, Wu *et al.* 2012), transforming growth factor-β3 (TGF- β3)(Wu *et al.* 2013, Wu *et al.* 2014) and retinoic acid (RA) (Gouveia

and Connon 2013). However, the potency of these media supplements to differentiate cultured CSC or cMSC back to the keratocyte phenotype has never been compared.
It is critical that a reliable method to return CSC or cMSC to a keratocyte phenotype is developed, that results in cells that express keratocyte markers, do not express fibroblast markers and produce relevant stromal ECM. If it is achievable to obtain a keratocyte phenotype from fibroblast and myofibroblast populations, these populations could be grown to large numbers and then dedifferentiated back to the keratocyte, to be used clinically in cell therapies and tissue-engineered strategies. This would decrease the number of keratocytes, and therefore corneas, that are required to manufacture such therapies. Herein, we investigate the transition of keratocytes to the cMSC phenotype and compare various supplements in their ability to return cMSC to a keratocyte phenotype.

2. Methods

2.1 Materials

All reagents were purchased from Sigma-Aldrich, Poole, UK, unless otherwise stated.

2.2 Tissue

Human donor tissue was used with approval by the local ethics research committee and in accordance with the tenets of the Declaration of Helsinki, following consent obtained from the donors and/or their relatives.

2.3 Extraction, Culture and Differentiation of cMSC

Human keratocytes were isolated from corneoscleral rims, as previously described (Branch *et al.* 2012). Briefly, stromal tissue pieces were digested in 1 mg/mL collagenase type IA for 7 hours at 37°C. Digests were filtered through a 40 µm cell strainer, pelleted and cMSC were cultured in M199 supplemented with 20% (v/v) foetal bovine serum (FBS), 2 mM L-Glutamine and 20 ng/mL gentamicin and 0.5 ng/mL amphotericin B (antibiotics, Life Technologies, Paisley, UK). Cultured keratocytes were maintained at 37°C, 5% (v/v) CO_2, with medium changes every 2-3 days. Cells were passaged using treatment with TrypLE™ Express (Life Technologies).

For keratocyte differentiation, cMSC at P3 were seeded at 10,500 cells/cm^2, to be P4 in the culture plate. After a proliferation phase of 3 days, media was changed to one of six different keratocyte differentiation media (see table 1). Cells were maintained in differentiation media for up to 21 days before analysis. Controls in the differentiation experiments were performed in M199 containing 20% (v/v) FBS, 2 mM L-Glutamine and 20 ng/mL gentamicin and 0.5 ng/mL amphotericin B.

2.4 Immunofluorescent staining of cornea sections and fixed cells

Whole human corneas were snap frozen in optimal cutting temperature compound (VWR, West Sussex, UK). Specimens were cut into 8 µm sections using a cryostat-microtome (Leica Microsystems, UK) and fixed in ice-cold 100% acetone for 30 seconds.

Cellular samples were seeded at 10,500 cell/cm^2 in cultured in glass chamber slides, allowed to adhere overnight and were subsequently fixed in 4% buffered paraformaldehyde for 10

minutes. After washing in PBS, cells were permeabilised, where appropriate, in 0.1% (v/v) Triton X-100 for 5 minutes with subsequent PBS washing.

From this point, the immunofluorescent staining protocol for both sections and cell samples was identical. Samples were blocked in 1% (v/v) bovine serum albumin (BSA), 0.3 M glycine and 3% (v/v) donkey serum at room temperature for 1 hour. Incubation with primary antibodies (see table 2) took place at 4°C overnight. Samples washed, then incubated with secondary antibodies (table 2) for one hour at room temperature. Actin staining was performed with Alexa Fluor-488 conjugated phalloidin (Life Technologies). Counterstaining with 0.5 µg/mL DAPI was performed and slides were mounted in fluorescence mounting medium (Dako, Cambridgeshire, UK). Staining was viewed using an upright fluorescence microscope (BX51, Olympus) and images captured with a black and white camera (XM-10, Olympus) and Cell^F software (Olympus).

2.5 Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Epithelium and endothelium were stripped from whole human corneas and the stromal tissue snap frozen in liquid nitrogen and ground in a mortar and pestle. Ground tissue was then lysed in buffer RLT and homogenised in QIAshredder columns (Qiagen, Manchester, UK). Alternatively, cell monolayers were lysed directly in RLT buffer and homogenized using QIAshredder columns. Total RNA was extracted using an RNeasy mini kit (Qiagen) according to manufacturer's instructions. RNA was quantified using the L-Vis plate on a CLARIOstar plate reader (BMG LABTECH, Buckinghamshire, UK). 250 ng of RNA was transcribed into cDNA using Superscript III reverse transcriptase with random hexamer primers, according to

manufacturer's instructions (Life Technologies). For PCR reactions, 1 µL of cDNA was used with inventoried Taqman assays (table 3). Amplification was performed on an Mx3005P PCR system (Stratagene, Stockport, UK). Reactions were analysed using the Real Time PCR Miner algorithm (Zhao and Fernald 2005). All experimental values were normalised to endogenous reference gene *GAPDH*.

2.6 Cell proliferation and viability

Cell viability was assessed by PrestoBlue™ Cell Viability Reagent (Life Technologies), on days 3, 6, 10, 14, 18 and 21. Cells were washed in PBS and 10% (v/v) PrestoBlue reagent in medium was added to each well and incubated for 20 minutes at 37°C. Aliquots of 100 µL were transferred to black 96-well plates and fluorescence readings were taken at excitation 560 nm/emission 590 nm using a CLARIOstar plate reader.

2.7 Quantitative assay for sulphated glycosaminoglycans

Cell monolayers were digested in a 0.1 mg/mL papain solution in 0.2 M sodium phosphate buffer containing 8 mg/mL sodium acetate, 4 mg/mL ethylenediaminetetraacetic acid, and 0.8 mg/mL L-cysteine hydrochloride at 65°C overnight. The Blyscan™ 1,9 dimethyl methylene blue (DMMB) assay (Biocolor Ltd., Belfast, UK) assay was performed on samples according to manufacturer's instructions. Briefly, 200 µL of medium sample or papain digest was added to 1 mL DMMB dye solution and agitated for 30 minutes, before centrifugation at 10,000 x g for 10 minutes. The pellet was dissolved in 0.5 mL dissociation reagent and 200 µL transferred to each well of a 96-well plate. Absorbance was measured at 656 nm. sGAG

concentration was determined using a standard curve. sGAG readings were corrected for DNA content of cells present, determined by fluorescent Hoechst 33258 readings, at excitation wavelength 360 nm and emission wavelength 460 nm.

2.7 Hydroxyproline Assay

Hydroxyproline assays were performed as described previously (Edwards and O'Brien 1980) to estimate the concentration of collagen released into the culture medium and laid down in the cell monolayer. Briefly, acid hydrolysis of media samples and papain digested cell monolayers was achieved by heating samples with concentrated hydrochloric acid to 120 °C for 5 hours. Subsequently, samples were dried at 80 °C until only residue remained, which was dissolved in 0.2 M sodium phosphate buffer. Samples were transferred in triplicate to a 96-well plate, an equal volume of 70 mM chloramine T solution was added and incubated at room temperature for 20 minutes. Subsequently, an equal volume of 1.16 M dimethylaminobenzaldehyde solution was added and samples incubated at 60 °C for 30 minutes. Colour change was assessed by absorbance at 540 nm. Hydroxyproline concentration was calculated using a standard curve. Collagen concentration was estimated using a conversion factor of 7.6. Collagen readings were corrected for DNA content of cells present, determined by fluorescent Hoechst 33258 readings.

2.9 Statistical Analysis

Significances were analysed using GraphPad Prism version 6.02. Multiple groups were compared using one-way ANOVA with post-hoc Dunnett’s test.

3. Results

3.1 Keratocytes transition to a cMSC phenotype upon extraction and passage

Sections of human cornea peripheral and central stroma, extracted and cultured keratocytes at passage 1 (P1) and cultured keratocytes at P4 (cMSC) were stained for markers associated with keratocytes (ALDH3A1, CD34, keratocan, vimentin), MSC (CD73, CD90, CD105) and myofibroblasts (α-SMA (figure 1). The corneal stroma (both central and peripheral) showed positive staining for keratocyte markers ALDH3A1 (fig. 1 Ai), CD34 (fig. 1 Aii), keratocan (fig. 1 Aiii) and vimentin (fig. 1 Aiv). Expression of keratocyte markers decreased upon extraction and passage. All cells in the P1 population showed ALDH3A1 (fig. 1 Bi) and vimentin (fig. 1 Biv) staining. Some cells of the P1 population also stained for CD34 (fig. 1 Bii) and keratocan (fig. 1 Biii). In the P4 cMSC population, ALDH3A1 (fig. 1 Ci) and vimentin (fig. 1 Civ) were maintained but there was no longer any staining for CD34 (fig. 1 Cii) or keratocan (fig. 1 Ciii). The reverse trend occurred with MSC markers; markers were not present in the corneal stroma and appeared with extraction and passage. There was no expression of CD73 (fig. 1 Av), CD90 (fig. 1 Avi) or CD105 (fig. 1 Avii) in the corneal stroma. At P1, there was a low amount of staining for CD73 (fig. 1 Bv), CD90 (fig. 1 Bvi) and CD105 (fig. 1 Bvii). At P4, all cMSC stained positive for CD73 (fig. 1 Cv), CD90 (fig. 1 Cvi) and CD105 (fig. 1 Cvii). Myofibroblast marker α-SMA was present in individual cells at P1 (fig. 1 Bviii) and P4 (fig. 1 Cviii) but was not present in the healthy corneal stroma (fig. 1Aviii).

RT-qPCR was performed to explore differences in mRNA levels of keratocytes within the corneal stroma, at P1 and at P4 (figure 2). Expression of *ALDH3A1* (fig. 2A), *CD34* (fig. 2B) *and KERA* (fig. 2C) mRNA levels were significantly decreased in P1 and P4 cells compared to the cornea, but there were no significant differences between P1 and P4. *VIM* (fig. 2D) and

NT5E (fig. 2E) were significantly upregulated in extracted cells at both P1 and P4, but there was no significant difference between P1 and P4. *THY1* (fig. 2F) and *ENG* (fig. 2G) were significantly upregulated in P1 and P4 compared to cornea and there was also significant difference between P1 and P4. In contrast to the immunostaining, *ACTA2* (fig. 2H) mRNA levels were significantly lower in extracted cells than the cornea.

3.2 Supplemented keratocyte differentiation media support cell viability

Proliferation of cMSC (at P4) was monitored in six differentiation media and a non-differentiated control (figure 3). In the control (fig. 3A), cMSC proliferated rapidly up to day 10, but as cells became confluent, proliferation decreased and cell number was maintained. In serum-free medium (fig. 3B), viability did not increase significantly above that at day 3. By day 21, there were significantly less viable cells than there had been at day 3, indicating a small amount of cell death. cMSC in all other keratocyte media showed some level of proliferation, with significantly higher cell viability at day 14 than at day 3. However, metabolic activity decreased again at day 21, with the exception of cells differentiated in a combination of FGF-2, TGF-β3 and RA (fig. 3G) which had significantly increased cell viability on day 21.

3.3 Keratocyte differentiation media results in different cell morphologies

Morphology of cMSC in differentiation media was assessed by phalloidin staining of actin filaments at day 21 of differentiation. cMSC in control media (fig. 4A) were typically fibroblastic, long and spindle shaped. cMSC in serum-free medium (fig. 4B) were much more

sparse than in any other media, and were a mix of small cells with a dendritic morphology and larger fibroblastic cells. Both cMSC in FGF-2 (fig. 4C) and FGF-2 with TGF-β3 (fig. 4F) had different morphology to the other media. The cells were small and rounded with processes extending out from the cell body. cMSC in RA (fig. 4D) were mostly fibroblastic in morphology, but there were a few cells within the population of a dendritic morphology. cMSC in TGF-β3 (fig. 4E) and FGF-2, TGF-β3 and RA (fig. 4G) had similar morphology to the control but were more sparse, with large fibroblastic cells.

3.4 Keratocyte differentiation media stimulates expression of keratocyte markers but does not reduce MSC markers

Immunocytochemistry was performed on cMSC in keratocyte differentiation medium and a non-differentiated control at day 21 of culture (figure 5). In control medium, there were background levels of ALDH3A1 (fig. 5 Ai) but no staining for CD34 (fig. 5 Aii) or keratocan (fig. 5 Aiii). In the differentiation media, (fig 5 B-G) all cMSC stained for the keratocyte markers ALDH3A1, CD34 and keratocan, regardless of supplementation. Vimentin (fig. 5 iv), CD73 (fig. 5 v), CD90 (fig. 5 vi) and CD105 (fig. 5 vii) were present in all cells in all media, indicating that differentiation does not lead to a reduction in MSC markers over a 21 day time period. Myofibroblast marker α-SMA stained most prominently in control media (fig. 5 Aviii), with the majority of cell displaying fibres. α-SMA fibres were also seen in serum-free medium (fig. 5 Bviii), TGF-β3 supplemented (fig. 5 Dviii), RA supplemented (fig. 5 Eviii), and cells supplemented with FGF-2, TGF-β3 and RA combined (fig. 5 Gviii).

RT-qPCR was performed to compare mRNA levels due to differentiation (figure 6). Significances are shown compared to control on the figure, statistical comparisons of all other media can be found in supplementary table 1. There was significant upregulation of *ALDH3A1* (fig. 6A), *CD34* (fig. 6B), *KERA* (fig. 6C), and *VIM* (fig. 6D) in all differentiation media, compared to control. For *ALDH3A1* and *VIM*, there was also significantly higher expression in the serum-free medium compared to all other media. This was also true for *CD34*, with the exception of RA. There was also significant upregulation of *THY1* (fig. 6F), particularly in RA medium, and *ENG* (fig. 6G) in the differentiated samples. *NT5E* (fig. 6E) mRNA levels were not significantly different in serum-free and TGF-β3 containing media compared to control. In media containing FGF-2 and FGF-2 + TGF-β3, there was significant upregulation of *NT5E*, however, in media containing RA and FGF-2 + TGFβ3 + RA, there was significant downregulation compared to all other media. *ACTA2* was significantly upregulated in TGF-β3, RA and FGF-2+TGF-β3+RA media.

3.5 Inclusion of FGF-2 and TGF-β3 in the differentiation media increases production of ECM

Immunocytochemistry for collagen-I (fig. 7A) and lumican (fig. 7B) was performed at day 21 of differentiation to assess ECM deposition. There were low levels of staining for both in the non-differentiated controls (fig. 7 Ai and Bi, respectively). Staining for collagen-I and lumican was increased in serum-free media, but was intracellular and not deposited by the cells (fig. 7 Aii and Bii, respectively). There was subjectively increased staining for collagen-I in FGF-2 (fig. 7 Aiii), TGF-β3 (fig. 7 Aiv), FGF-2 + TGF-β3 (fig. 7 Avi) and FGF-2+TGF-β3+RA (fig. 7 Avii). Although there was deposition of collagen-I in RA containing media (fig. 7 Av), staining did

not appear as dense. Lumican stained strongly in all FGF-2 containing media (fig. 7 Biii; v and vii), but did not appear to be as brightly stained in TGF-β3 and RA differentiation media. RT-qPCR for *COL1A1* (fig. 7C) and *LUM* (fig. 7D) indicated that both genes were significantly upregulated in all differentiation media, compared to control. Significances are shown compared to control on the figure, statistical comparisons of all other media can be found in supplementary table 1. Serum-free media showed significantly higher upregulation of *COL1A1* than all other media, and significantly higher *LUM* than all meida except TGF-β3.

Collagen was released into the culture media during differentiation (fig. 7 Ei) and deposited in the cell monolayer (fig. 7 Eii). Significances are shown compared to control on the figure, statistical comparisons of all other media can be found in supplementary table 2. Combining media release and deposition (Fig. 7Eiii) revealed that collagen was produced at significantly higher rates compared to the control in all media containing either FGF-2 and/or TGF-β3, and the addition of RA had little effect on collagen production. Media that contained all three supplements was the only media to lead to significantly higher production of total collagen than all others.

Quantification of sGAG released into the culture medium (fig. 7 Fi) and deposited in the cell monolayer (fig. 7 Fii) was performed by DMMB assay. As sGAG are the polysaccharide units that are present in proteoglycans such as keratocan or lumican, this measurement estimates quantities of corneal ECM being produced. Significances are shown compared to control on the figure, statistical comparisons of all other media can be found in supplementary table 3. Release of sGAG into the culture medium was significantly higher than the control in all media except for serum-free. However, deposition of sGAG in the cell monolayer was only significantly higher in FGF-2 and TGF-β3 media. When media release and deposition of sGAG

were combined (fig. 7 Fiii), all media other than serum-free had significantly higher production than the control, however, media containing TGF-β3 had the highest sGAG production. Statistical differences in the total production of sGAG between media other than control and serum-free were all found to be non-significant.

4. Discussion

As the potential for corneal regenerative strategies is recognised, and research gathers momentum, it is essential for a greater understanding of stem cell differentiation to the keratocyte phenotype. Keratocytes are integral in producing and maintaining the complex ECM of the corneal stroma and essential in restoring and maintaining transparency. As it is difficult to maintain keratocyte phenotype whilst achieving proliferation in vitro, it would require a large amount of corneal tissue to extract enough keratocytes to use directly in the production of a cell therapy or tissue-engineered corneal stroma replacement. However, if keratocytes could be derived from cMSC populations that contain fibroblasts and myofibroblasts, this population could be cultured to much higher numbers and then the keratocyte phenotype restored during production of the cell therapy or tissue-engineered product. This study has addressed the feasibility of this process, identifying some of the issues in restoring a keratocyte phenotype for clinical use, through a comprehensive investigation of the phenotypic changes that occur to keratocytes upon *in vitro* culture, and comparison of media supplements for the reversion of this phenotypic change.

It has been well-documented that CSC extracted from the limbal region of the cornea can derive a cMSC phenotype (Choong *et al.* 2007, Polisetty *et al.* 2008, Branch *et al.* 2012), that conforms to ISCT criteria (Dominici *et al.* 2006) and have a multipotent potential, differentiating into lineages including adipogenic, osteogenic, chondrogenic and neurogenic

(Polisetty *et al.* 2008, Branch *et al.* 2012, Li *et al.* 2012, Greene *et al.* 2014). However, it has never been conclusively shown whether this MSC phenotype is present within the *in vivo* cornea, or is a product of *in vitro* culture. This study shows that, under these conditions, the extracted keratocytes become cMSC after several passages, but there is no evidence that the *in vivo* limbal stroma contains cells with the MSC phenotype. This is due primarily to a lack of visible expression of CD73, CD90 and CD105, and high expression of CD34. The absence of CD90 was to be expected, as it is rarely present in healthy corneas as it is a marker of activated keratocytes (Pei *et al.* 2004). Despite its use as a marker of keratocytes, CD34 is more commonly associated with haematopoietic cells, and it is for this reason it is considered to be an indicator that a cell is not an MSC, despite increasing evidence that CD34 is associated with a number of progenitor cell types, including bone marrow MSC (Sidney *et al.* 2014). In this study, we clearly showed that as the MSC phenotype begins to appear in vitro; the keratocyte phenotype proportionally disappears, with expression of the proteins and genes for ALDH3A1, CD34 and keratocan decreasing with culture, and CD73, CD90 and CD105 increasing.

Another well-documented phenomenon of *in vitro* cultured keratocytes is the appearance of a contractile myofibroblast phenotype (Masur *et al.* 1996, Pei *et al.* 2004), predominantly due to the serum concentration of the culture medium and more specifically the presence of TGF-β1 (Jester *et al.* 1996). In this study, distinctive α-SMA fibres were seen in cultured cMSC from P1. However, mRNA levels for *ACTA2*, the gene for α-SMA, were downregulated after culture, indicating some discrepancies between protein and gene expression.

Various media supplements have been used to differentiate cultured CSC back to a keratocyte phenotype. In most cases, the base media for differentiation is serum-free, to

induce the quiescence representative of a keratocyte population. In studies involving animal keratocytes, serum-free media alone can be sufficient in restoring the keratocyte phenotype (Berryhill *et al.* 2002, He and Bazan 2008, Chen *et al.* 2009). In this study, serum-free media (with ITS and ascorbate added) did restore keratocyte markers, and gene expression of these markers was actually higher in serum-free, than any of the supplemented media. However, serum-free media without supplementation did not support cell viability, with decreased cell numbers by day 21 of differentiation and was also not effective for the production of corneal ECM as the supplemented media. Addition of supplements FGF-2, TGF-β3 and RA, particularly in combination allowed some proliferation of the cMSC to occur and cell numbers were increased by the 21-day endpoint.

When looking at protein and gene expression of typical keratocyte (CD34, ALDH3A1, keratocan) and MSC (CD73, CD90, CD105, vimentin) markers of the differentiated cells, there was little difference between the different supplementations and serum-free media. All media showed significant upregulation of *ALDH3A1*, *CD34* and *KERA* compared to the non-differentiated control, with serum-free media showing the highest expression. In contrast to expectations, all differentiation media also showed significant upregulation of *VIM*, *THY1* and *ENG.* This was also reflected in the protein staining where ALDH3A1, CD34 and keratocan were clearly stained in the differentiated cells but not in the control. No differentiation media led to a decrease in the levels of staining of vimentin, CD73, CD90 or CD105. This suggests that there was only partial differentiation of the cells to the keratocyte phenotype, in all media. In many instances, *in vitro* keratocyte differentiation has only been demonstrated by increased expression of keratocyte markers, but has not been confirmed by a simultaneous decrease in fibroblast markers such as CD90 and vimentin (Berryhill *et al.*

2002, Park *et al.* 2012, Gouveia and Connon 2013, Byun *et al.* 2014, Foster *et al.* 2015). The only differences seen between the differentiation media in this study were a downregulation of *NT5E* in media containing RA and differences in α-SMA expression; α-SMA fibre staining was not seen in cultures supplemented with FGF-2. To achieve full keratocyte differentiation it may be that cells require longer in the differentiation media containing the three supplements, or require additional three-dimensional culture substrate such as a hydrogel or electrospun scaffold (Kim *et al.* 2010, Karamichos *et al.* 2014).

More differences were seen between the differentiation media when assessing production of ECM. Levels of *COL1A1* and *LUM* were significantly upregulated in all differentiation media, but there were no significant differences between them. However, immunofluorescent staining of collagen-I and lumican appeared to show decreased levels in serum-free and RA, compared to the other differentiation media. This was further corroborated by quantitative assessment of collagen and sGAG production. Production of ECM was significantly increased in media that contained FGF-2 and/or TGF-β3, particularly in combination. Literature has stated that RA promotes expression of ECM proteins and proteoglycans, by reducing the expression of MMPs (Gouveia and Connon 2013). However, our study found that RA on its own was not sufficient to stimulate ECM proteins and in combination with FGF-2 and TGF-β3 did not provide a more synergistic effect. For this reason, we would state that RA does not appear to be a necessary supplement when the other growth factors are present. FGF-2 and TGF-β3 have been reported to act synergistically in other studies to allow corneal stromal stem cells to produce an abundant organized, collagenous matrix on aligned synthetic substrates (Wu *et al.* 2013), to stimulate

collagen production by bone marrow-derived MSC (Perrier *et al.* 2011) and to support proliferation of CSC (Kay *et al.* 1998).

From a cell therapy viewpoint, it may be interesting as to whether a differentiation medium supplemented with FGF-2 and TGF-β3 , such as in this study could be used to derive keratocytes from MSC populations extracted from bone marrow or adipose tissue, or whether this is a property only cMSC possess. There is some evidence that MSCs extracted from non-corneal tissues can differentiate into keratocytes, as described in this review (Harkin *et al.* 2015); both in vivo (Arnalich-Montiel *et al.* 2008, Liu *et al.* 2010, Liu *et al.* 2012) and in vitro (Du *et al.* 2010, Zhang *et al.* 2013). As these MSC types are currently more readily available than MSC from the cornea, there would be advantages in using them to derive large numbers of keratocytes for clinical purposes.

In conclusion, the differentiation of stromal cells back to a keratocyte phenotype is more complicated than culturing in serum-free media, even with supplementation. Although, expression of keratocyte markers ALDH, CD34, collagen-I and specific proteoglycans may be shown to increase, a visible reduction of fibroblast, MSC and myofibroblast markers is also required, and was not shown in this study. However, with increased differentiation time and alternative substrates, a serum-free media containing FGF-2 and TGF-β3 shows more promise than serum-free media as a method of differentiating cultured cMSC back to a keratocyte phenotype.

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Conflict of Interest

The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

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Figure Legends

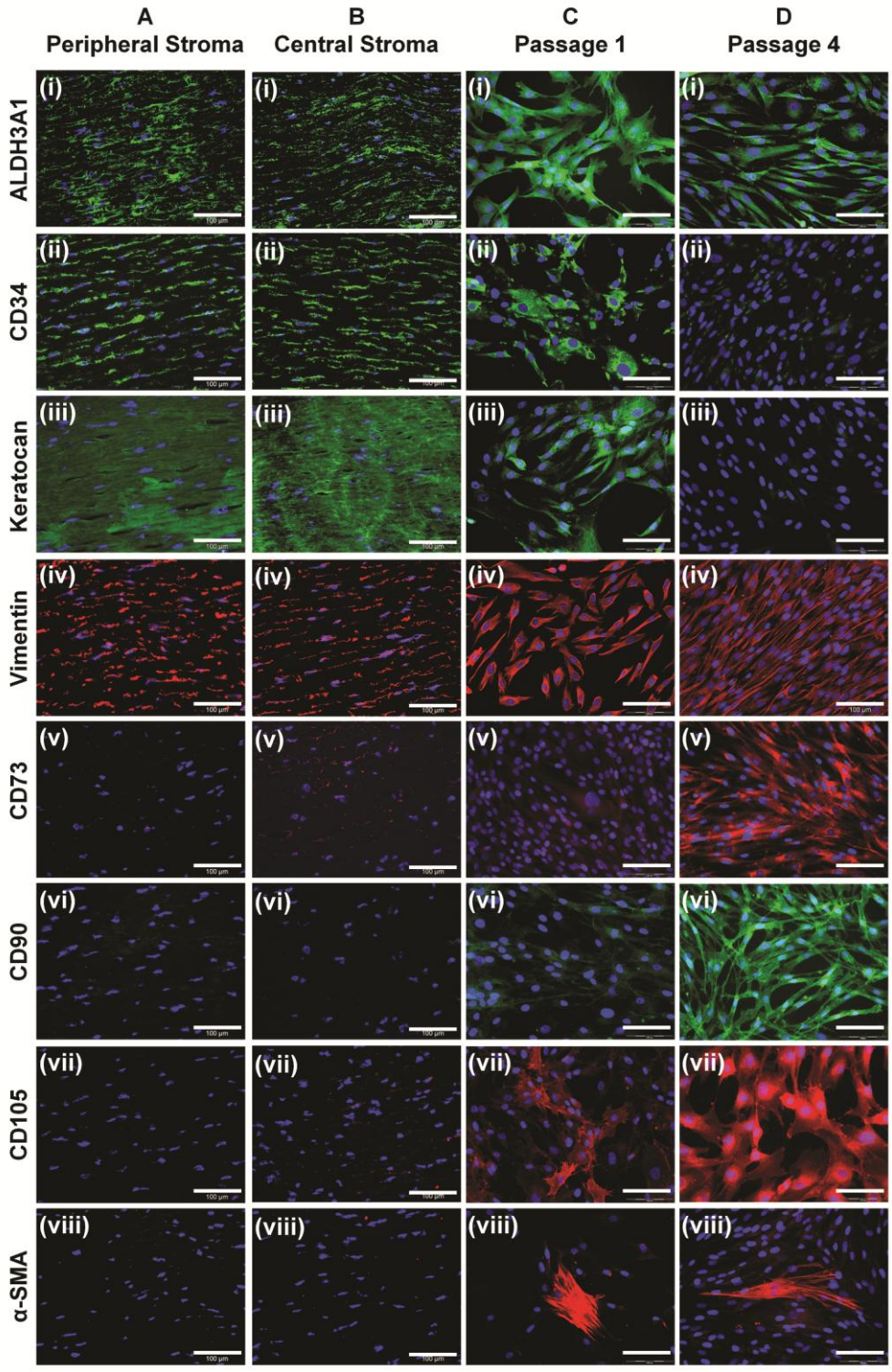

Figure 1. Change in protein expression upon transition of cells from keratocyte to MSC phenotype. Fluorescent immunostaining was performed on (A) peripheral corneal stroma

sections, (B) central corneal stroma sections, (C) passage 1 cMSC and (D) passage 4 cMSC for (i) ALDH3A1, (ii) CD34, (iii) Keratocan, (iv) Vimentin, (v) CD73, (vi) CD90, (vii) CD105 and (viii) α-SMA. Extraction and passage caused a reduction in expression of keratocyte markers and increased expression of MSC markers. Representative images shown. Scale bar = 100 μm.

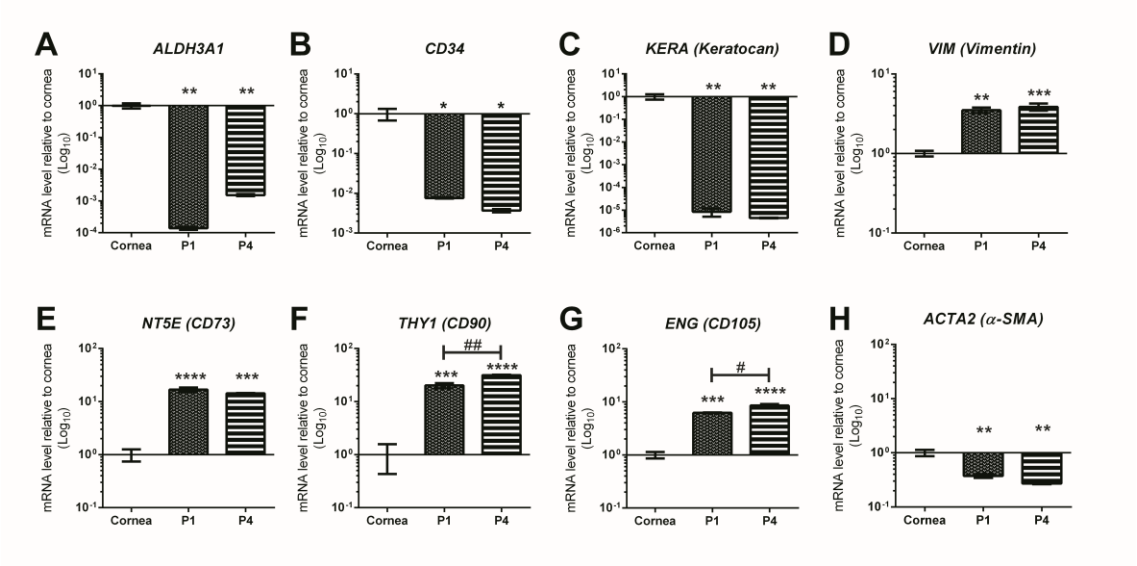

Figure 2. Change in mRNA levels upon transition of cells from keratocyte to MSC phenotype. RT-qPCR was performed on cornea, passage 1 (P1) cMSC and passage 4 (P4) cMSC for genes (A)*ALDH3A1*, (B) *CD34*, (C) *KERA*, (iv) *VIM*, (v) *NT5E*, (vi) *THY1*, (vii) *ENG* and (viii) *ACTA2*. Expression of each target gene was normalised to *GAPDH* and represented relative to mRNA levels in cornea. Data shown on $\log_{10}$ scale and represented by mean±SEM of three experiments (n=3) each with 2 replicates. Statistical significance vs. cornea shown by *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001. Statistical significance of P1 vs P4 shown by # p≤0.05, ##p≤0.01.

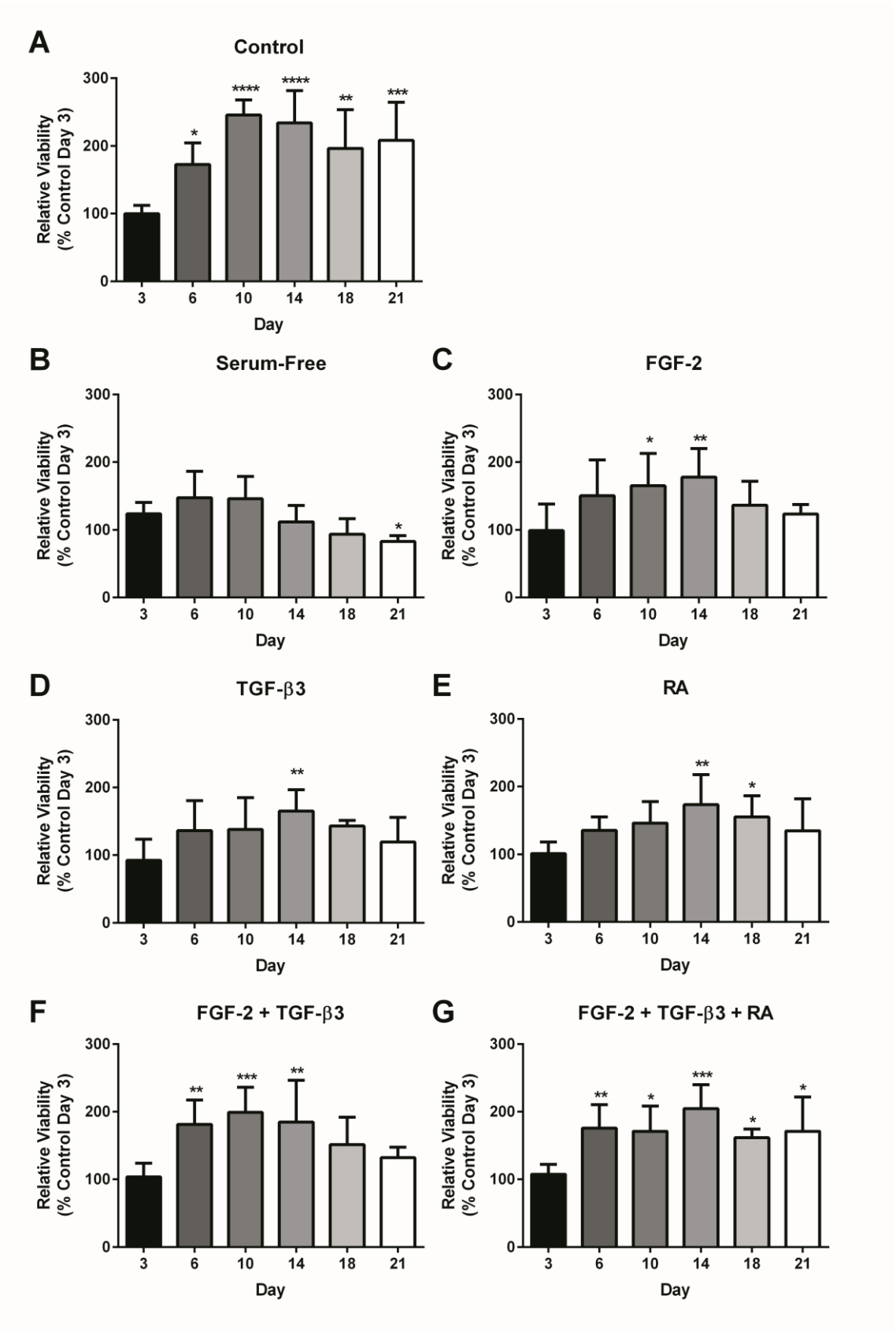

Figure 3. Effect of keratocyte differentiation media on cMSC proliferation and viability. Presto Blue proliferation assay was performed on day 3, 6, 10, 14, 18 and 21 of differentiation for cells in (A) non-differentiated control medium; (B) serum-free differentiation medium; (C) medium containing 10 ng/mL FGF-2; (D) medium containing 0.1 ng/mL TGF-β3; (E) medium containing 10 μM retinoic acid (RA); (F) medium containing 10 ng/mL FGF-2 and 0.1 ng/mL TGF-β3; and (G) medium containing 10 ng/mL FGF-2, 0.1 ng/mL TGF-β3 and 10 μM RA. Each timepoint is represented relative to the reading at day 3 for the control media. Data represented by mean±SEM of 5 experiments (n=5), each with 3 replicates. Statistical significance vs day 3 of same medium represented by *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

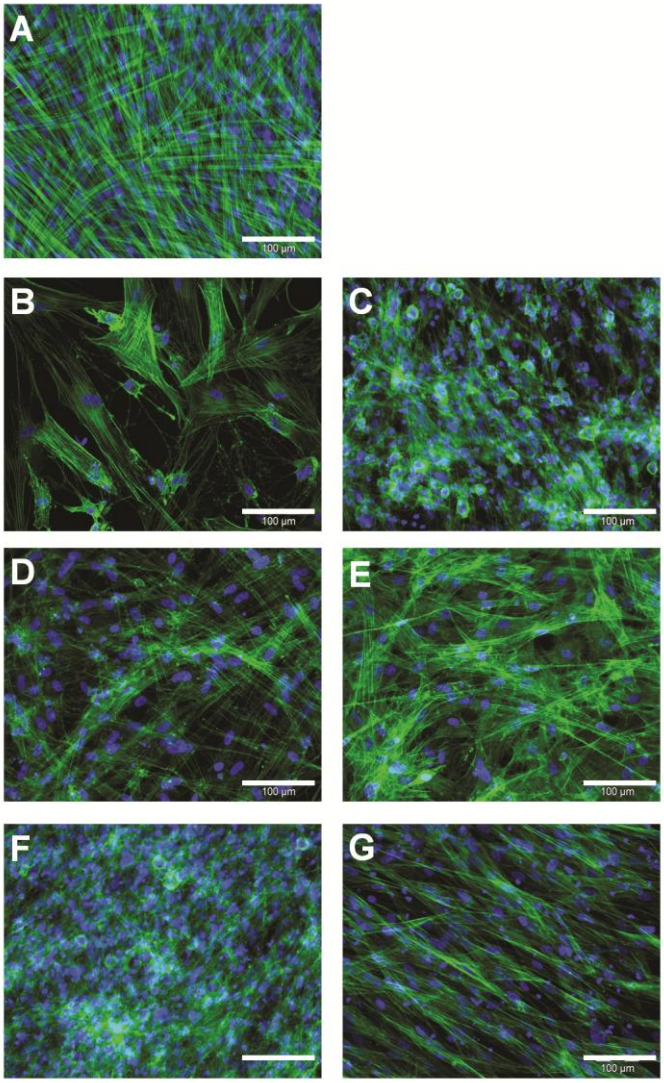

Figure 4. Changes in actin cytoskeleton due to keratocyte differentiation medium. Phalloidin was used to stain the actin filaments of cells differentiated in (A) non-differentiated control medium; (B) serum-free differentiation medium; (C) medium containing 10 ng/mL FGF-2; (D) medium containing 0.1 ng/mL TGF-β3; (E) medium containing 10 µM retinoic acid (RA); (F) medium containing 10 ng/mL FGF-2 and 0.1 ng/mL TGF-β3; and (G) medium containing 10 ng/mL FGF-2, 0.1 ng/mL TGF-β3 and 10 µM RA. Representative images shown. Scale bar = 100 µm.

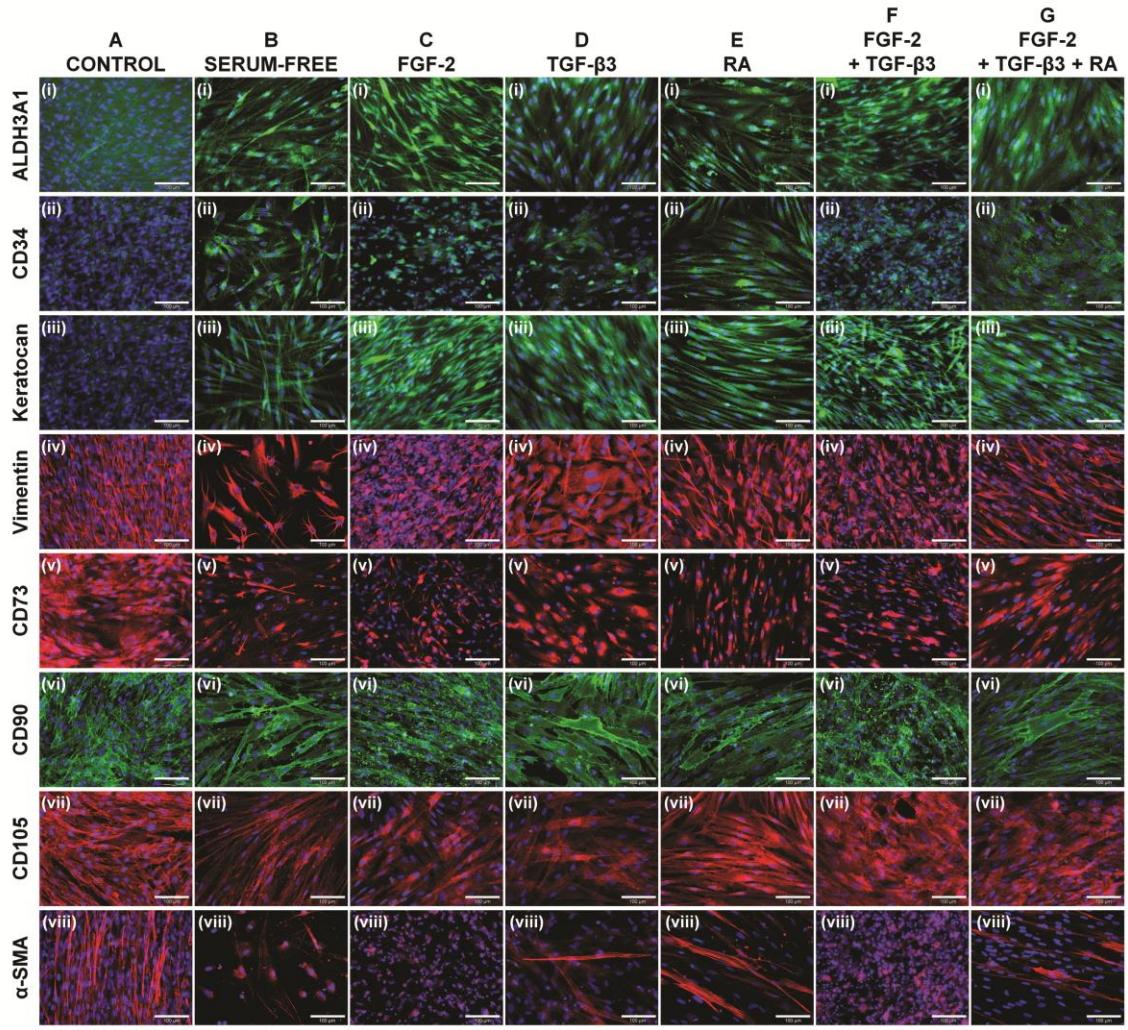

Figure 5. Effect of keratocyte differentiation media on cMSC morphology and protein expression. Fluorescent immunostaining was performed on cMSC samples after 21 days in (A) non-differentiated control medium; (B) serum-free differentiation medium; (C) medium containing 10 ng/mL FGF-2; (D) medium containing 0.1 ng/mL TGF-β3; (E) medium containing 10 µM retinoic acid (RA); (F) medium containing 10 ng/mL FGF-2 and 0.1 ng/mL TGF-β3; and (G) medium containing 10 ng/mL FGF-2, 0.1 ng/mL TGF-β3 and 10 µM RA. Staining was performed for for (i) ALDH3A1, (ii) CD34, (iii) Keratocan, (iv) Vimentin, (v) CD73, (vi) CD90, (vii) CD105 and (viii) α-SMA. Representative images shown. Scale bar = 100 µm.

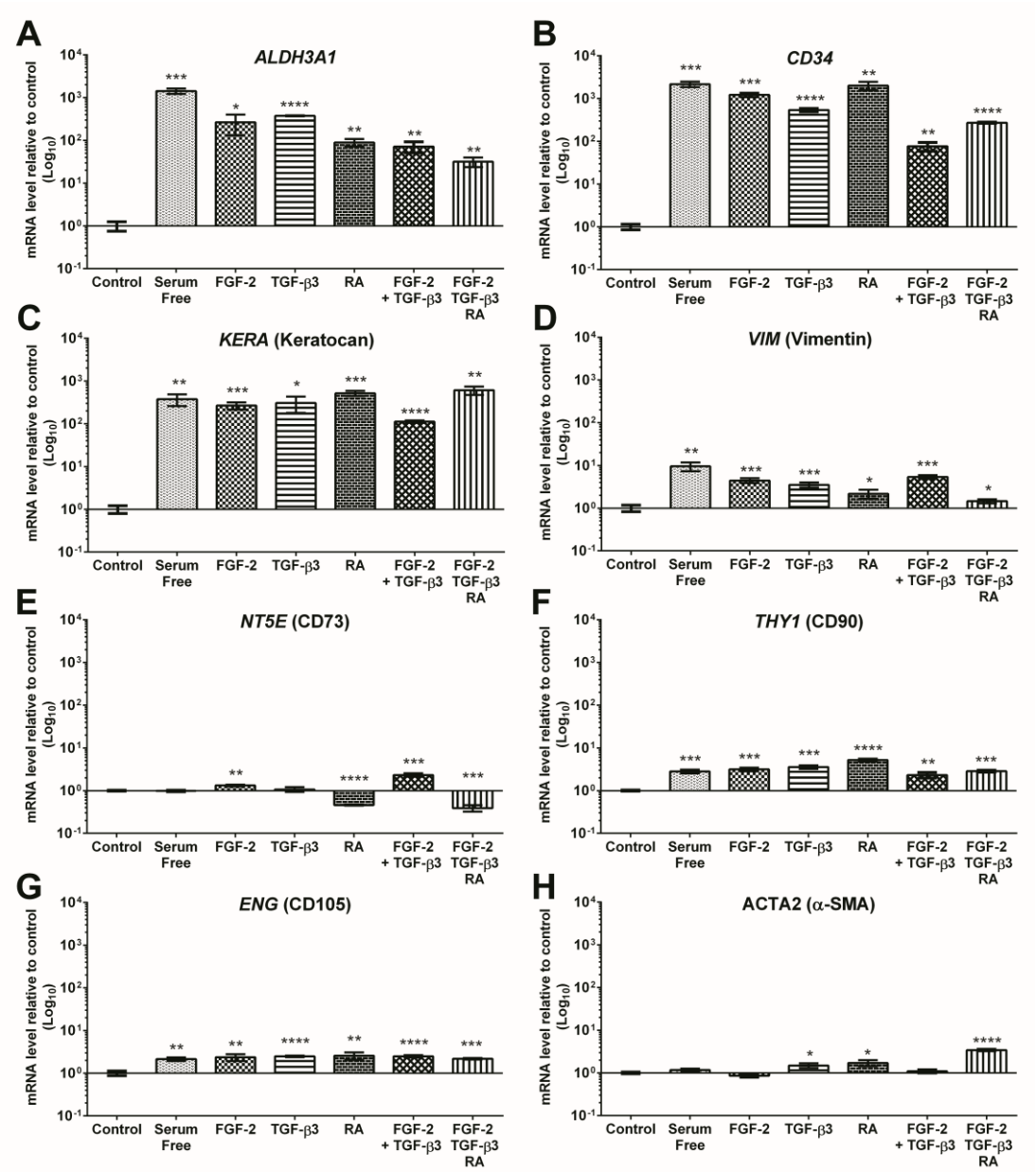

Figure 6. Change in mRNA levels upon culture in keratocyte differentiation medium. RT-qPCR was performed on cMSC cultured in control or differentiation media for 21 days. Genes tested were (A)*ALDH3A1*, (B) *CD34*, (C) *KERA*, (iv) *VIM*, (v) *NT5E*, (vi) *THY1*, (vii) *ENG* and (viii) *ACTA2*. Expression of each target gene was normalised to *GAPDH* and represented relative to mRNA levels in non-differentiated control. Data shown on $\log_{10}$ scale and represented by mean±SEM of three experiments (n=3) each with 2 replicates. Statistical significance vs. control shown by *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

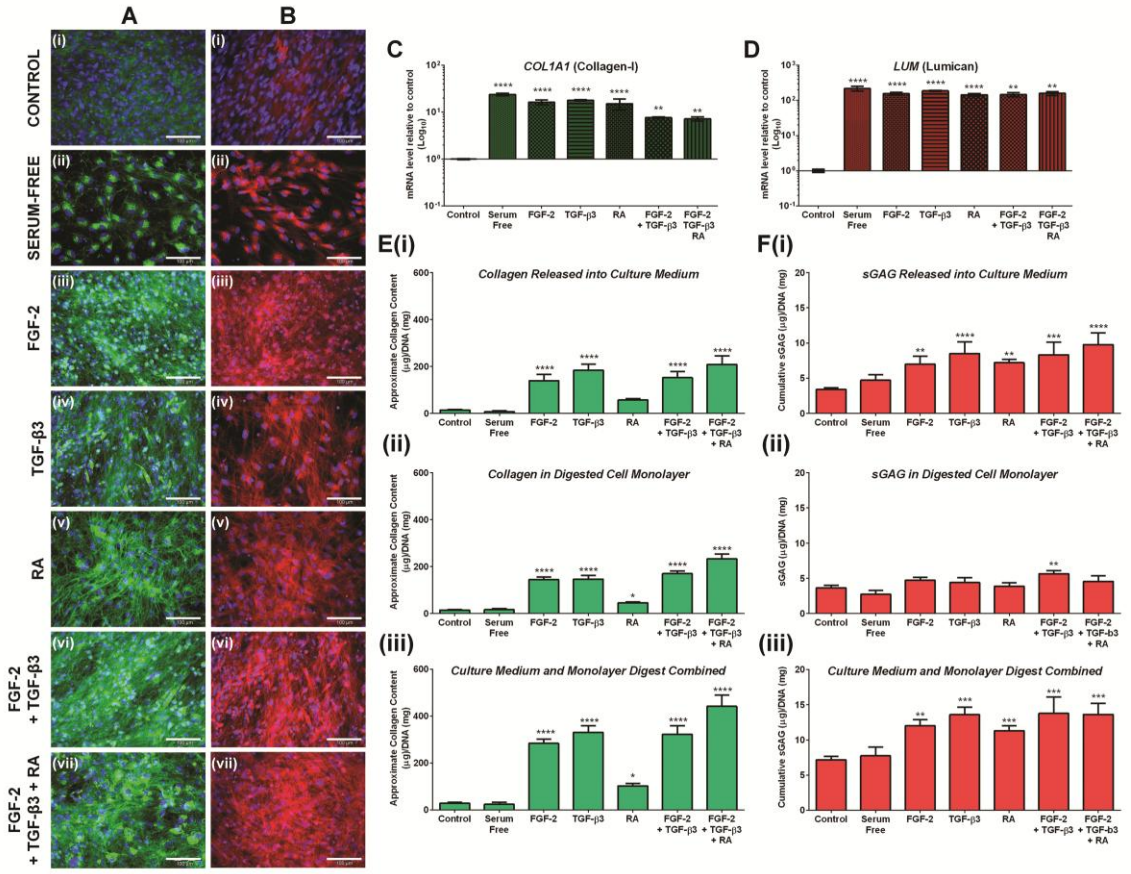

Figure 7. Production of ECM in different keratocyte differentiation media. Immunocytochemistry was performed for deposition of (A) Collagen-I and (B) Lumican in samples cultured for 21 days in (i) non-differentiated control medium; (ii) serum-free differentiation medium; (iii) medium containing 10 ng/mL FGF-2; (iv) medium containing 0.1 ng/mL TGF-β3; (v) medium containing 10 μM retinoic acid (RA); (vi) medium containing 10 ng/mL FGF-2 and 0.1 ng/mL TGF-β3; and (vii) medium containing 10 ng/mL FGF-2, 0.1 ng/mL TGF-β3 and 10 μM RA. Representative images shown. Scale bar = 100 μm. RT-qPCR was performed to assess mRNA levels of (C) *COL1A1* and (D) *LUM.* Expression of each target gene was normalised to *GAPDH* and represented relative to mRNA levels in non-differentiated control. Data shown on $\log_{10}$ scale and represented by mean±SEM of three experiments (n=3) each with 2 replicates. Statistical significance vs. control shown by **p≤0.01, ****p≤0.0001. (E) Hydroxyproline assays were performed to assess collagen

production: (i) collagen released into the culture medium, (ii) collagen deposited in the cell monolayer, (iii) collagen in media and monolayer combined. Data represented as mean±SD of 3 independent samples (n=3). Statistical significance vs. control shown by ****p≤0.0001. (F) DMMB assay was performed to assess sGAG production: (i) sGAG released into the culture medium, (ii) sGAG deposited in the cell monolayer, (iii) sGAG in media and monolayer combined. Data represented as mean±SD of 3 independent samples (n=3). Statistical significance vs. control shown by **p≤0.01, ***p≤0.001, ****p≤0.0001.

Tables

Table 1. Components of the various keratocyte differentiation media

Media	Components
Serum-Free	DMEM/F12 with 50 µg/mL ascorbate 2-phosphate; 10 µg/mL human insulin, 5.5 µg/mL human transferrin, 6.7 ng/mL sodium selenite (Life Technologies, ITS); 1% non-essential amino acids (Life Technologies); antibiotics
FGF-2	Serum-free with 10 ng/mL FGF-2 (Life Technologies)
TGF-β3	Serum-free with 0.1 ng/mL TGF-β3 (Life Technologies)
RA	Serum-free with 10 µM retinoic acid
FGF-2+TGF-β3	Serum-free with 10 ng/mL FGF-2 and 0.1 ng/mL TGF-β3
FGF-2+TGF-β3+RA	Serum-free with 10 ng/mL FGF-2, 0.1 ng/mL TGF-β3 and 10 µM retinoic acid.

Table 2. Antibody information

Antigen	Clone	Source (Catalogue #)	Host	Conjugate
ALDH3A1	Polyclonal	Abcam (ab76976)	Rabbit	-
CD34	QBEND10	Abcam (Ab8536)	Mouse	-
Keratocan	Polyclonal	Santa Cruz Biotechnology (sc-33243)	Goat	-
Vimentin	V9	Vector Labs (VPV684)	Mouse	-
CD73	Polyclonal	Thermo Scientific Pierce (PA5-11871)	Rabbit	-
CD90	F15-42-1	Thermo Scientific Pierce (MA5-16671)	Mouse	-
CD105	Polyclonal	R&D Systems (AF1097)	Goat	-
α-SMA	1A4	Abcam (ab7817)	Mouse	-
Collagen-I	Polyclonal	Abcam (ab34710)	Mouse	-
Lumican	Polyclonal	R&D Systems (AF2846)	Goat	-
Rabbit IgG	Polyclonal	Life Technologies (A-21206)	Donkey	Alexa Fluor-488
Mouse IgG	Polyclonal	Life Technologies (A-21202)	Donkey	Alexa Fluor-488
Goat IgG	Polyclonal	Life Technologies (A-11055)	Donkey	Alexa Fluor-488
Mouse IgG	Polyclonal	Life Technologies (A-21203)	Donkey	Alexa Fluor-594
Rabbit IgG	Polyclonal	Life Technologies (A-11056)	Donkey	Alexa Fluor-546
Goat IgG	Polyclonal	Life Technologies (A-10040)	Donkey	Alexa Fluor-546

Table 3. Taqman® Probe information

Gene Name	Protein	Assay ID
ALDH3A1	ALDH3A1	Hs00964880_m1
CD34	CD34	Hs00990732_m1
KERA	Keratocan	Hs00559942_m1
VIM	Vimentin	Hs00185584_m1
NT5E	CD73	Hs01573922_m1
THY1	CD90	Hs00174816_m1
ENG	CD105	Hs00923996_m1
COL1A1	Collagen-I	Hs00164004_m1
LUM	Lumican	Hs00158940_m1
GAPDH	GAPDH	Hs99999905_m1