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REGULAR ARTICLE

Xeno-free expansion of adult keratinocytes for clinical application: the use of human-derived feeder cells and serum

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Abstract

Cultured epithelial autograft (CEA) was the birth of skin tissue engineering and encompassed methodologies for the isolation and expansion of autologous basal keratinocytes for burn treatment that are still practiced at some specialised units around the world. One of the limitations of CEA, however, is the reliance on animal-derived material during the manufacturing process and despite all efforts to date, no xeno-free alternative with proven efficacy has been reported. Here, we investigate whether human-derived fibroblast feeder cells and human serum can sufficiently and effectively provide a suitable microenvironment for adult keratinocyte isolation and expansion. Human dermal fibroblasts and epidermal keratinocytes were isolated from discarded skin during abdominoplasty and breast reduction procedures and cultured in xeno-free conditions. We report that these xeno-free adult keratinocytes form similar numbers of colony-forming units as those cultured using the Green's methods; however, xeno-free keratinocytes express lower levels of α6 integrin (CD49f; a progenitor and stem cell marker). We identified IL-8 as a potential growth factor secreted by adult human fibroblasts that may enhance keratinocyte colony formation in human serum. Finally, we propose a step-by-step xeno-free isolation and cultivation methodology for adult keratinocytes that can be tested further in serial cultivation for clinical application.

Keywords Adult keratinocytes · Skin tissue engineering · CEA · Burns · IL-8 (CXCL8) · CD49f (α6 integrin)

Introduction

Definitive wound closure of major burns in a timely fashion faces many challenges, and despite advancements in critical care, split skin grafting has remained the gold standard for large-wound closure. As an adjunctive treatment to split skin grafting, Rheinwald and Green introduced a long-term cultivation method for adult basal keratinocytes in their landmark publication in 1975 (Rheinwald and Green 1975b), which relies on mitotically inactive 3T3-J2 (a sub-clone of the mouse embryonic 3T3 fibroblast cell line, derived and maintained in bovine serum) as keratinocyte feeder cells. The resultant epithelial sheets, or cultured epithelial autograft (CEA), have been used in specialist burn units around the world since the 1980s, with variable success rates (Wood et al. 2006).

Despite reasonable CEA take rates (30–90%), a number of limitations are associated with this technique, in particular the presence of xeno-derived materials, specifically the murine embryo fibroblasts and bovine serum. According to the Good Manufacturing Practice (GMP) principles, such xeno-derived materials are a safety concern due to the possibility of introducing zoonoses (such as bovine spongiform encephalopathy) to the recipients. The focus of this study is to eliminate both the murine-derived feeder layer and bovine serum from the Green's adult basal keratinocyte culture method, which may improve the safety and quality of CEA by reducing the risk of transmitting animal-derived

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pathogens and antigens, which may in turn reduce graft rejection.

Feeder-free and serum-free alternatives have been reported for adult basal keratinocyte expansion using either commercial keratinocyte media or recombinant proteins. However, these cultures are typically slow to grow and/or incapable of serial cultivation and none have been translated to the clinic (Bisson et al. 2013; Lamb and Ambler 2013; Mujaj et al. 2010). Human-derived (neonatal or adult) fibroblasts have been considered effective alternatives to 3T3-J2 feeder cells for expansion of human adult keratinocytes (HAK) in culture; however, in these studies, the cultures were supplemented with either bovine serum or bovine pituitary extract and were not xeno-free (Bisson et al. 2013; Li et al. 2009). There has been some interest in the use of human platelet lysate as an alternative to bovine serum for cell therapies, including mesenchymal stem cells (Astori et al. 2016). Culture medium supplemented with platelet lysate at 2.5 and 5% has been used to replace bovine serum for HAK with short-term viability (3 days) but the doubling time was significantly increased (Witzeneder et al. 2013).

Despite all efforts, a xeno-free alternative to the Green's method of HAK expansion for clinical application has proven difficult to establish. This method thrives on mimicking the mesenchymal–epidermal interaction observed in vivo. In adults, dermal fibroblasts not only deposit extracellular matrix but also influence keratinocyte behaviour by modulating cell polarity, proliferation, migration, differentiation and homeostasis via secreted growth factors such as keratinocyte growth factors (KGF)-1 and 2, epidermal growth factor (EGF), granulocyte–macrophages colony-stimulating factor (GM-CSF), parathyroid hormone-related protein (PTHrP) and interleukin-6 (Borradori and Sonnenberg 1996; Sorrell et al. 2004; Sorrell and Caplan 2004; Szabowski et al. 2000). Simultaneously, fibroblast proliferation and function are regulated by factors secreted by keratinocytes, including oncostatin-M (OSM) (Canady et al. 2013).

This study aims to investigate human adult fibroblasts (HAF) and human serum (HS) as a complete xeno-free solution for isolation and cultivation of HAK that can be adapted to a clinical setting. Short-term culture of HAF in HS has already been found to be effective (Witzeneder et al. 2013). Here, we demonstrate that HS is able to maintain long-term HAF proliferation capacity and that HAF, in combination with HS-AB type (to ensure immune compatibility), is effective for HAK isolation and expansion and should be considered for further analysis as an alternative to the conventional Green's method in the clinical setting. Interleukin-8 (IL-8) may play a significant role in xeno-free HAK attachment and expansion in culture. The xeno-free condition supported similar HAK clonogenicity but with lower expression of α6 integrin marker, compared to the Green's method.

Methods

Access to human skin and serum

Skin tissue discarded during elective breast reduction or abdominoplasty surgery was obtained after informed consent, following approval by the Monash University, Monash Health and Alfred Health Research Ethics Committees, Melbourne, Australia. Human serum (HS) was obtained through the Australian Red Cross under ethical approval.

Primary HAF isolation in HS

Skin pieces were digested in Dispase II (4 mg/ml, Roche) over the first night of skin retrieval at 4 °C to separate the dermis from the epidermis. Adult fibroblasts were isolated from the dermis based on the protocol described by Paul et al. (2015) with some modification. Briefly, equal-sized dermal skin samples were minced and digested in an enzyme cocktail (20 ml per gramme tissue) composed of 3 mg/ml Collagenase (Gibco), 4 mg/ml Dispase (Gibco) and 10% calcium chloride (Sigma) in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen), supplemented with either 2% HS (Red Cross) or 5% Foetal Bovine Serum (FBS, Thermo Fisher Scientific) for 2 h at 37 °C under agitation. The enzyme reaction was halted by adding an equal volume of DMEM with FBS or HS as appropriate. After straining the quenched suspension through a 22-μm cell strainer, cell recovery and viability were determined using the trypan blue exclusion method.

HAF longevity in HS

Freshly isolated HAF from three donors were grown at 26,667 cells/cm^2 in DMEM containing 4 mM L-glutamine (Gibco), 50 mg/ml gentamicin (Life Technologies) and either 10.0% BCS (Thermo Fisher Scientific) or 5.0, 7.5, 10.0, or 12.5% HS at 37 °C, 5% CO_2. HS was pooled from three donors to reduce batch variation. Media were changed every 2–3 days. Cell banks were frozen down in their growth media plus 10% DMSO (Sigma) for further experiments. To determine the longevity of HAF isolated and cultured in HS, HAF from three separate donors were maintained in their corresponding serum for up to 20 passages in flasks at a density of 6400 cells/cm^2 in triplicate. The flasks were monitored daily for proliferation, morphology and confluency and were passaged once 80% confluency was observed to avoid contact-dependent inhibition (Gradl et al. 1995).

HAF proliferation rate in HS using MTT assay

The proliferation rate of HAF cultured in 5.0, 7.5, 10.0 and 12.5% HS was compared to the proliferation rate of HAF cultured in 10.0% BCS as described previously with

minor changes (Cleland et al. 2014). Briefly, HAF were seeded in triplicate at a density of 6400 cells/cm^2 at 37 °C, 5% CO_2 with media changed every 2 days. On days 2, 3, 4, 5, 6 and 7, media were aspirated and 1 ml of fresh culture media with 100 μl of MTT reagent (Sigma, 20 mg/ml in PBS) was added to each well. Plates were mixed for 30 s before incubation at 37 °C for 4 h. Media containing MTT was then removed and 500 μl of 2-methoxyethanol (Sigma) was added to each well and incubated at room temperature for 2 h. Plates were read on a FLUOstar Omega Microplate Reader (BMG Labtech) with absorbance set to 590 nm and the reference filter set to 660 nm.

HAF irradiation regime

HAF expanded in 12.5% HS or 10% BCS were gamma-irradiated (Gammacell 1000 Elite) at 60, 80 or 150 Gy and seeded. As a control, murine (3T3-J2) fibroblasts (expanded in DMEM containing 4 mM L-glutamine, 50 mg/ml gentamicin and 10.0% BCS) were irradiated at 60 Gy (Rheinwald and Green 1975a). Irradiated cells were seeded at 6400 cells/cm^2 and maintained with media changes every 2 days. On days 3, 5 and 10 post-irradiation, fibroblasts were detached and counted using the trypan blue exclusion method.

Growth factor expression in xeno-free HAF using real-time PCR

RNA from three HAF donors was isolated 24 h post-irradiation (80 Gy), using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. cDNA was prepared using a First-Strand cDNA Synthesis Kit for RT-PCR (AMV; Roche), and quantitative real-time PCR was performed in a LightCycler® 480 Multiwell Plate 384 (Roche) using LightCycler® 480 SYBR Green I Master (Roche) according to the manufacturer's instructions. KGF, fibroblast growth factor-2 (FGF-2), IL-6 and IL-8 were analysed as the target genes (Table 1). Three house-keeping genes, GAPDH, HPRT and TBP, were also amplified as reference genes. CT values were recorded, and the expression ratios between the irradiated and non-irradiated samples were calculated using the following formula:

$$\text{Ratio} = \left(E_{\text{target}}\right)^{\Delta \text{CT, target (non treated} - \text{irradiated)}} / \left(E_{\text{ref}}\right)^{\Delta \text{CT, target (non treated} - \text{irradiated)}}$$

A two-way ANOVA was used to determine statistical differences in expression levels, with P values less than 0.05 considered significant and the results were analysed using GraphPad Prism.

IL-8 detection by ELISA and immunofluorescence

HAFs (expanded in 12.5% HS or 10% BCS) and 3T3-J2 murine fibroblasts were gamma-irradiated at 80 and 60 Gy, respectively. After 24 h (day 1), the feeders were seeded with passage 2 HAK in their respective serum. Supernatants were collected on days 0, 1, 3, 5 and 7 post-irradiation, with media replenished for the remaining day(s). Cell supernatants were stored at − 80 °C for further analysis using a human IL-8 ELISA kit (BD Biosciences Pharmingen, San Diego, CA).

For immunofluorescence microscopy, non-irradiated and irradiated HAF were fixed and permeabilised in 0.2% Triton-X for 10 min, blocked in 10% BSA/goat serum for 30 min and stained with an IL-8 specific antibody (1:100, R&D Systems) overnight at 4 °C. Slides were washed and stained with Alexa Fluor 568 (1:400) and DAPI (1:500) for 45 min–1 h. Images were collected on an Olympus Bx 61 (Tokyo, Japan) equipped with an Olympus DP80 Camera (Tokyo, Japan) and analysed using ImageJ software.

Isolation of HAK in xeno-free conditions

Skin pieces were digested in Dispase II (4 mg/ml, Roche) over the first night of skin retrieval at 4 °C to separate the dermis from the epidermis. Epidermal sheets were minced using a scalpel blade, followed by enzymatic digestion in 5× TrypLE Select (Xeno-Free; Gibco) at either 37 °C or room temperature for 7 min. As a control, minced epidermis was digested in 0.25% Trypsin (animal-derived; Life Technologies) for 7 min at 37 °C. Digests were quenched in keratinocyte (FAD) media (Rheinwald and Green 1975b) containing DMEM and Ham's F12 (Life Technologies, 3:1 ratio), 4 mM L-glutamine, 0.18 mM adenine (Calbiochem), 0.4 μg/ml hydrocortisone (Calbiochem), 5 μg/ml insulin (Novo Nordisk), 2×10^9 M 3,3′,5-triiodo-L-thyronine sodium salt (Sigma), 5 μg/ml transferrin (Sigma), 50 μg/ml gentamicin, 0.2 μg/ml isoproterenol (Hospira) and supplemented with either 10.0% FCS (Green's method) or 5% HS-AB type (xeno-free).

HAK colony formation assay in xeno-free conditions

HAK freshly isolated from six donors according to either the Green's method (seeded on 60 Gy-irradiated 3T3-J2 feeder cells at 28,000 cells/cm^2) or our xeno-free method (seeded on 80 Gy-irradiated HAF feeder cells at 21,333 cells/cm^2) were seeded in 6-well plates in duplicate (5000 or 1000 HAK per well) in either FAD supplemented with 10.0% FCS or 5, 7.5, 10 and 12.5% HS-AB type. Colonies were maintained for 15 days and stained with

Table 1 List of specific primers used in real-time PCR

Protein of interest	Primers	NCBI mRNA reference number	Expected product size (bp)
KGF/FGF-7	5′-TTGTGGCAATCAAAGGGGTG-3′ 5′-CCTCCGTTGTGTGTCCATTTAGC-3′	NM_002009	159
FGF-2/bFGF	5′-GAAGAGCGACCCTCACATCAAGCTA-3′ 5′-CAGTTCGTTTCAGTGCCACATACC-3′	NM_002006	229
IL-6	5′-GGTACATCCTCGACGGCATCT-3′ 5′-GT GCCTCTTTGCTGCTTTCAC-3′	NM_000600.4	140
IL-8	5′-ACTGAGAGTGATTGAGAGTGGAC-3′ 5′-AACCCTCTGCACCCAGTTTTC-3′	BC013615.1	111
GAPDH	5′-CTCTGCTCCTCCTGTTCGAC-3′ 5′-AAATGAGCCCCAGCCTTCTC-3′	NM_002046.5	408
HPRT	5′-ATTGGTAATGACCAGTACCAGTCAACAG-3′ 5′-GCATTGTTTTGCCAGTGTCAA-3′	NM_000194.2	117
TBP	5′-CACGAACCACGGCACTGATT-3′ 5′-TTTTCTTGCTGCCAGTCTGGAC-3′	NM_003194	89

1% Rhodamine Blue. HAK colonies were analysed using GelCount™ (Oxford Optronix) according to the manufacturer's instructions. Individual colonies were imaged using an Olympus CKX41 (inverted) microscope equipped with a DP21 camera.

HAK marker expression analysis using flow cytometry and immunofluorescence

Cultured HAK 8–15 days post-isolation were blocked in 2% bovine serum albumin (MP Biomedicals New Zealand Limited)/2% FCS for 15 min at 4 °C and stained with either a FITC-conjugated α6-integrin (CD49f) antibody or isotype control (BD Biosciences) for 1 h at 4 °C under agitation. Samples were washed in staining buffer (SB, 2% FCS) and incubated in SB plus 1 μg/ml 7AAD (Sigma-Aldrich) prior to analysis. For cytoplasmic markers, HAK were fixed in 0.01% formaldehyde (Amber Scientific) and permeabilised in 0.2% Triton-X (Sigma-Aldrich)/SB prior to incubation in primary antibodies: mouse anti-human keratin 10 (Dako) or IgG1 isotype control (R&D Systems) at 1 in 50 dilution. Cells were washed in permeabilisation buffer and stained with anti-mouse Alexa Fluor 488 (1:400) for 45–60 min at 4 °C, shaking. HAK were washed and analysed using FACS Calibur (BD Biosciences) and plotted using Flow Logic software (Inivai Technologies). For confocal microscopy, HAK isolated and expanded according to the Green's method for 12–15 days on chamber slides were fixed, permeabilised and blocked as described previously. Slides were incubated in FITC-CD49f (1:5) and mouse anti-human keratin 10 antibodies (1:1000) or the IgG isotype control overnight at 4 °C. Optical sections of the sample were acquired using a Nikon A1R microscope (Nikon, Tokyo, Japan) equipped with Plan Fluor 20×/0.75 MImm lens used with glycerol immersion fluid and the appropriate collar setting.

Results

HS can effectively replace bovine serum for the isolation and expansion of HAF

Traditionally, FCS is used during isolation and expansion of HAF from the dermal tissue (Bisson et al. 2013; Bullock et al. 2006; Jubin et al. 2011). To determine the optimal concentration of HS supplement to replace FCS, skin pieces were digested in no serum, 2% HS or 5% HS (data not shown). There was some improvement in cell recovery/viability when 2% HS was included in the digestion cocktail and although not significant, this lends support to earlier work showing that serum-free extraction and culture of HAF leads to poor cell yield and growth rate (Bullock et al. 2006). Therefore, 2% HS was used in all subsequent HAF isolations. Xeno-free isolated HAF were cultured in media supplemented with either 5.0, 7.5, 10.0 or 12.5% HS, compared to those isolated and cultured in bovine serum (FCS and BCS). Short-term proliferation (7 days) was assessed using the MTT assay over early passages (passages 4–7; Fig. 1a). The MTT assay showed that HAF grown in HS have a comparative growth rate to HAF grown in 10.0% BCS for most of their growth period, until confluency is reached at day 6 or 7 ($P < 0.5$).

HAF have similar longevity in HS compared to BCS

HAF isolated and expanded in HS were maintained in culture and passaged over a period of 60 days to assess

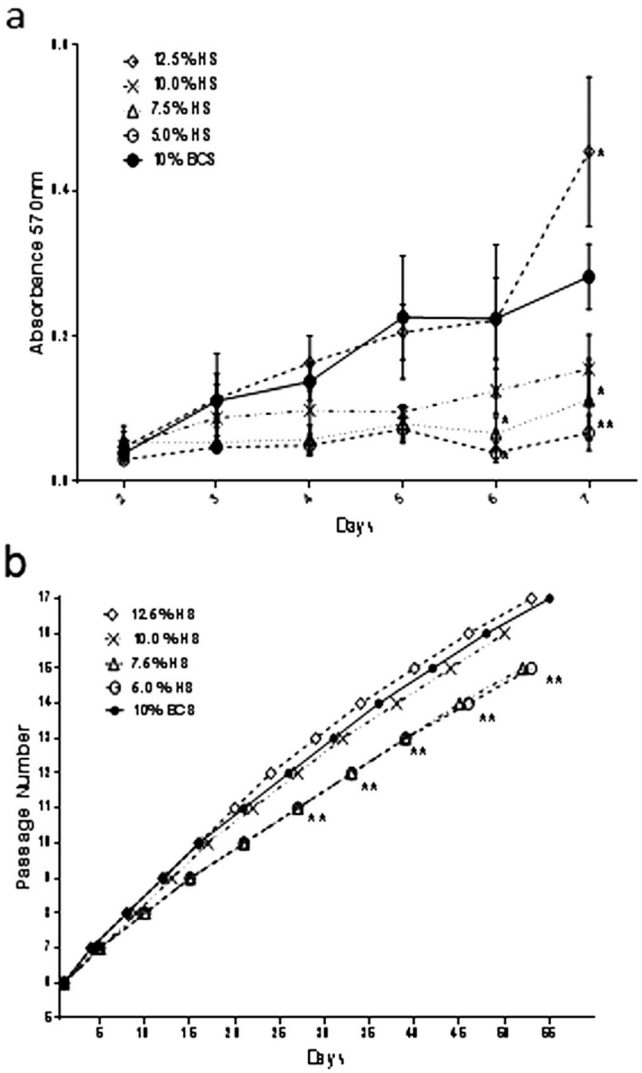


Fig. 1 HAF proliferation and longevity in HS. **a** Dermal HAF (passages 4–7) were grown in triplicate in 24-well plates in media supplemented with 5.0, 7.5, 10.0 or 12.5% HS or 10.0% BCS as a control for 7 days to assess their short-term proliferation using an MTT assay. Cells cultured with 5.0 and 7.5.0% HS have significantly reduced proliferation capacity compared with the standard 10.0% BCS that becomes significant on days 6 and 7. Cells cultured in 12.5% HS were significantly more proliferative, compared to the control on day 7. **b** HAF were maintained in an increasing concentration of HS, compared to control in culture flasks in triplicate to determine long-term longevity (over at least 19 passages in a 60-day period). Flasks were split and counted once they reached at least 80% confluency. HAF maintained in 5.0 and 7.5% HS lagged behind the control 10.0% BCS. However, cells grown in 10.0 and 12.5% HS had a comparable growth rate compared to the control. Data are representative of 3 independent experiments. Values represent mean ± SEM. Repeated-measure two-way ANOVA was performed. $*P < 0.05$; $**P < 0.01$

their longevity (Fig. 1b). HAF cultured in all media conditions reached 80% confluency after a maximum of 3–4 days for the first 8 passages (Fig. 1b). At this point, the growth rate of cells cultured in 5 and 7.5% HS began to slow, with HAF taking 5–6 days to reach 80% confluency. This lag in cell growth compared to 10.0% BCS reached statistical significance by passage 11 ($P < 0.01$, Fig. 1b). On the basis of these experiments, 12.5% HS was established as the optimal serum replacement for BCS for propagating HAF.

HAF require higher dosages of irradiation compared to 3T3-J2 cells to form an effective feeder layer for HAK culture

To determine the dosage of gamma irradiation required to arrest HAF growth, HAF grown in either HS or BCS were irradiated at 60, 80 or 150 Gy. 3T3-J2 murine fibroblasts cultured in bovine serum (Greens' method) were subjected to the same irradiation regimens for comparison. After irradiation, the fibroblasts were re-seeded and counted over a period of 10 days to determine if and when cell growth had been arrested. Figure 2 demonstrates that while 60 Gy was sufficient to arrest the growth of murine fibroblasts, HAF continued to proliferate. HAF growth arrest was achieved at 80 Gy, which was used as the standard irradiation dose for further experiments. HAF seeding density (21,333 cells/cm^2) for HAK co-culture was also determined using HAK colony formation assay (data not shown).

Human AB-type serum supports HAK clonogenicity

Freshly isolated HAK (next-day post-skin retrieval) in media containing HS-AB type (5.0, 7.5, 10.0 and 12.5%) or 10% FCS (Greens' method) were seeded on irradiated HAF or 3T3-J2 feeder cells, respectively, in a colony formation assay (Fig. 3a–h). There was a significant increase in colony numbers when HAK were grown in 5% HS-AB type, compared to 10%FCS in the presence of HAF feeder cells ($P < 0.05$). HS-AB type at 5, 7.5, 10 and 12.5% was able to support HAK clonogenicity. However, HS-AB type at 12.5% resulted in fewer HAK colonies, suggesting a possible inhibitory effect of HS at high concentrations, although this was not a statistically significant effect.

Interestingly, when HAF were utilised as feeder cells, regardless of the serum source used, HAK did not form round colonies, as they did when cultured according to the Green's method (Fig. 3i–n). HAK colonies always appeared to be elliptical or triangular and demonstrated polarity, with some HAK seemingly leading the colony expansion. This observation is similar to that seen for human embryonic colonies grown on either human- or mouse-derived fibroblast feeders (Dravid et al. 2006). Similar-shaped mouse embryonic colonies originating from early or late blastocysts are also observed when cultured on murine embryonic fibroblasts 10 or 12 days post-seeding (Lee 2013).

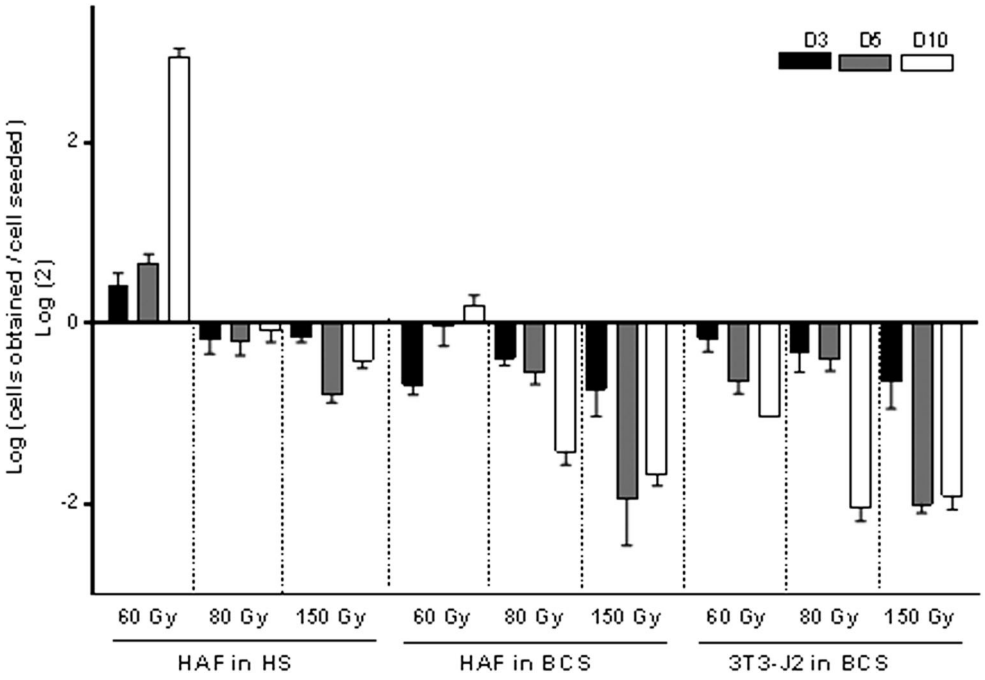


Fig. 2 Determining the optimal irradiation dose to growth arrest HAF. HAF (passages 4–7) were gamma-irradiated at 60, 80 and 150 Gy and reseeded. Murine 3T3-J2s were irradiated at 60 Gy according to the Green's method as a control. Cells were detached and counted on days 3, 5 and 10 post-irradiation using trypan blue exclusion method. The log of the number of cells counted/number of cells seeded is plotted. While 60 Gy was sufficient to arrest the growth of 3T3-J2 cells, HAFs required a dose of at least 80 Gy to prevent further proliferation. Negative readings were observed at higher irradiation dosage due to loss of loosely attached irradiated HAF over time. Data were pooled from 3 independent experiments. Values represent mean ± SEM

HAK expanded in xeno-free conditions show reduced expression of α6 integrin

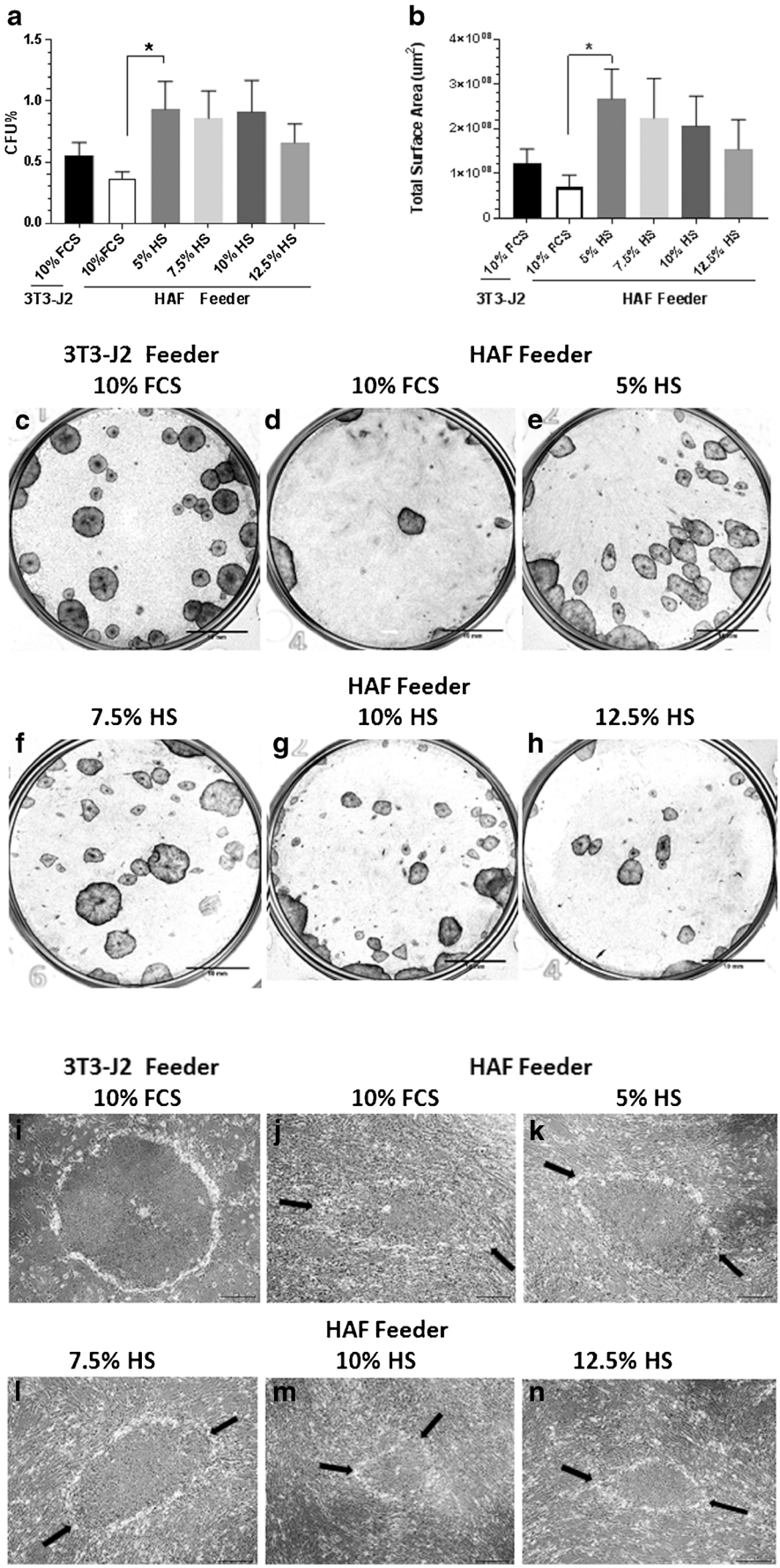
Expression of the cell surface marker α6 integrin, which is expressed on adult interfollicular stem and progenitor cells in native skin (Webb et al. 2004), was detected on expanded HAK using confocal microscopy (Fig. 4a, b). Differentiating HAK, marked with keratin 10, was detected above α6 integrin-expressing cells (a volume view of this image is provided in Supplementary Data 1). We employed flow cytometry to quantify the proportion of HAK that expressed α6 integrin grown in different conditions. The majority (75%) of the HAK expanded according to the Green's method expressed α6 integrin (Fig. 4c, f), whereas HAK grown on HAF feeders in either bovine (42%, $P < 0.0001$) or human serum (44%, $P < 0.0001$) showed significantly reduced α6 integrin-positive populations. This suggests that the fibroblast feeder/HAK interaction is more critical than serum nutrition in selecting for and maintaining α6 integrin-positive HAK in culture. The significance of reduced α6 integrin expression in xeno-free conditions is yet to be determined in HAK expanded for further consecutive passages.

The early differentiation marker, keratin 10, was expressed in a small proportion of HAK. Keratin 10 expression was not increased in HAK grown in the xeno-free condition (18%) compared to HAK grown according to the Green's method (15%), suggesting that the xeno-free culture condition does not induce differentiation in HAK. The gating strategy and an example of the HAK α6 integrin and keratin 10 expression profile are presented in Fig. 4(d–g).

IL-8 secreted by HAF may play a role in in vitro keratinocyte attachment/proliferation

In order to identify growth factors secreted by HAF that play a significant role in maintaining the proliferative capacity of isolated HAK in culture, real-time PCR was employed. The expression of candidate growth factors KGF, FGF-2, IL-6 and IL-8 was measured in three independent batches of HAF isolated and expanded in either bovine or human serum (passages 4–7, Fig. 5a). RNA was prepared from both irradiated (80 Gy) and non-irradiated HAF and the growth factor's mRNA levels

Fig. 3 HAK clonogenicity in xeno-free conditions. **a** Freshly isolated HAK were seeded on irradiated feeders in various serum conditions in a CFU assay. HAK colonies were fixed and stained with Rhodamine B on day 15. The bars represent mean CFU% in each condition. Unpaired *t* test was performed comparing each condition to the control (i.e., HAK CFU% in the Green's method). Differences did not reach significant value of $P < 0.05$ but there is a trend of HAK CFU inhibition at 12.5% HS compared to lower concentration of HS tested. HAK grown on HAF feeders in 5% HS-AB type formed significantly more CFU% compared to those grown on HAF feeder in 10%FCS (*$P < 0.05$). Data are representative of seven independent experiments, showing mean ± SEM. **b** HAK total surface area coverage (μm^2) in each culture condition, reflecting colony number, were measured. The bars represent mean value in five experiments ± SEM. HAK grown on HAF feeders in 5% HS-AB type (xeno-free condition) grew significantly more compared to those grown on HAF feeder in 10% FCS (*$P < 0.05$). **c**–**h** Representative Rhodamine Blue stain of HAK colonies (scale bar 10 mm) and **i**–**n** unstained colonies imaged on day 13. In contrast to HAK colonies cultured according to the Green's method, HAK colonies cultured on HAF do not form round colonies. The black arrows point at some of the leading cells (scale bar 500 μm)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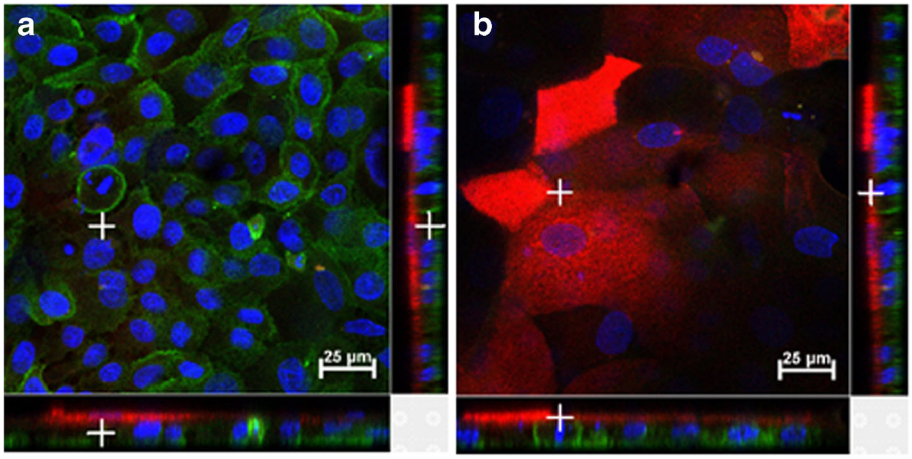
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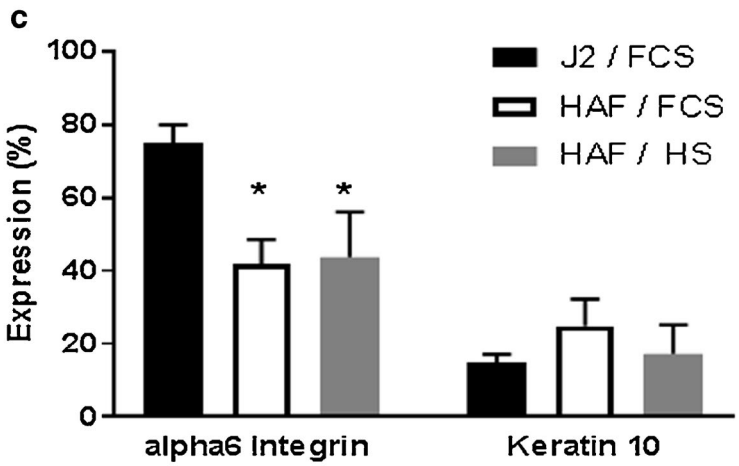
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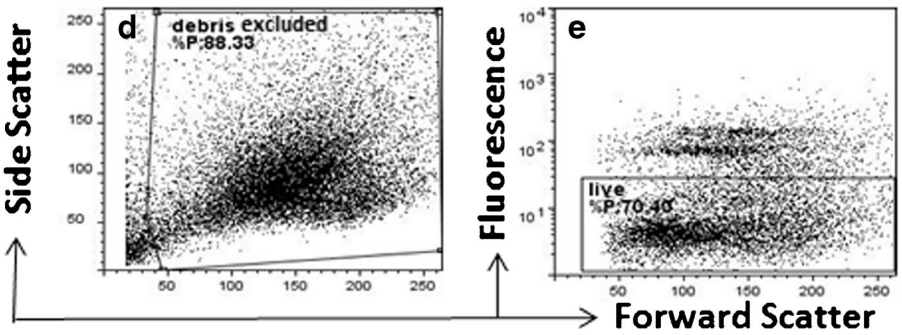
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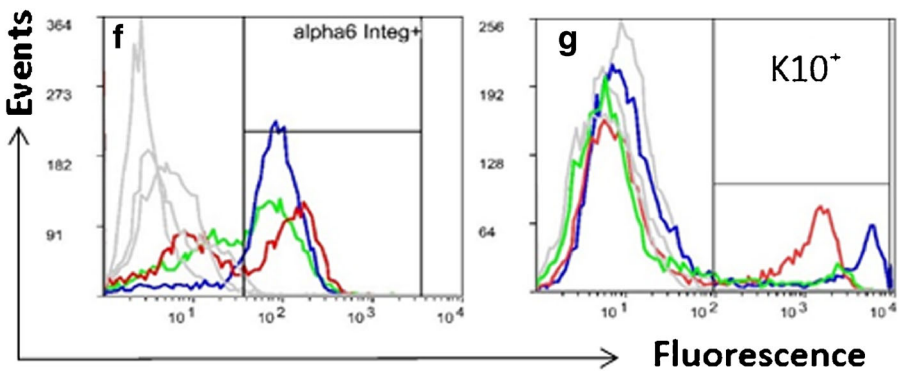
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K10+
Fluorescence

◀ **Fig. 4** Expression of proliferation and differentiation markers in xeno-free HAK. **a**, **b** Expression pattern of α6 integrin and keratin 10 in HAK expanded according to the Green's method was detected using confocal microscopy. Most cultured HAK expressed α6 integrin (green) on the cell membrane. Keratin 10 (red) was expressed in a few larger, flat cells that have moved to the top of the tissue, mimicking normal skin development. The bottom slice (**a**) and top slice (**b**) of a Z-section are presented here with the side scale bars in each image representing the volume view where the specimen is marked with a cross. The images are representatives of two independent experiments (scale bar 25 μm). **c** The α6 integrin and keratin 10 expression were quantified in HAK grown on HAF (in HS or BS), compared to the Green's method using flow cytometry. Values represent mean of four independent experiments ± SEM. Repeated-measure two-way ANOVA was performed for statistical analysis. $*P < 0.0001$. **d**–**g** Representative fluorescence intensity for α6 integrin and keratin 10 (K10) bright cells. Debris (**d**) and dead cells (**e**) were gated out and the proportion of HAK that express α6 integrin (**f**) or keratin 10 (**g**) was determined. Feeder: 3T3-J2/FCS (Green's method, blue line); HAF/FCS feeders (red line); HAF/HS-AB-type feeders (green line)

were quantified by amplification using specific primers. Data analysis revealed that KGF, FGF-2 and IL-6 levels were not altered by irradiation or source of serum in HAF. In contrast, IL-8 expression was substantially elevated in HAF isolated and grown in HS ($P < 0.0001$). When irradiated HAF in HS were compared to irradiated HAF in BCS, it was revealed that the HAF produced on average 100-fold more IL-8 mRNA when grown in HS. This increased IL-8 expression in HAF in HS was confirmed at the protein level by immunofluorescence (Fig. 5b–e) and may contribute to the increased HAK clonogenicity in human serum reported in Fig. 3. IL-8 secretion to the media was confirmed using ELISA (Fig. 5f). Extended analysis of IL-8 levels over the co-culture period of seven days revealed that IL-8 is also expressed by irradiated HAF in BCS but its expression is delayed compared to irradiated HAF in HS. Moreover, a marginal amount of IL-8 was secreted from HAK co-cultured according to the Green's method. IL-8 overexpression was also detectable at mRNA levels over the co-culture period (Supplementary Data 2).

Discussion

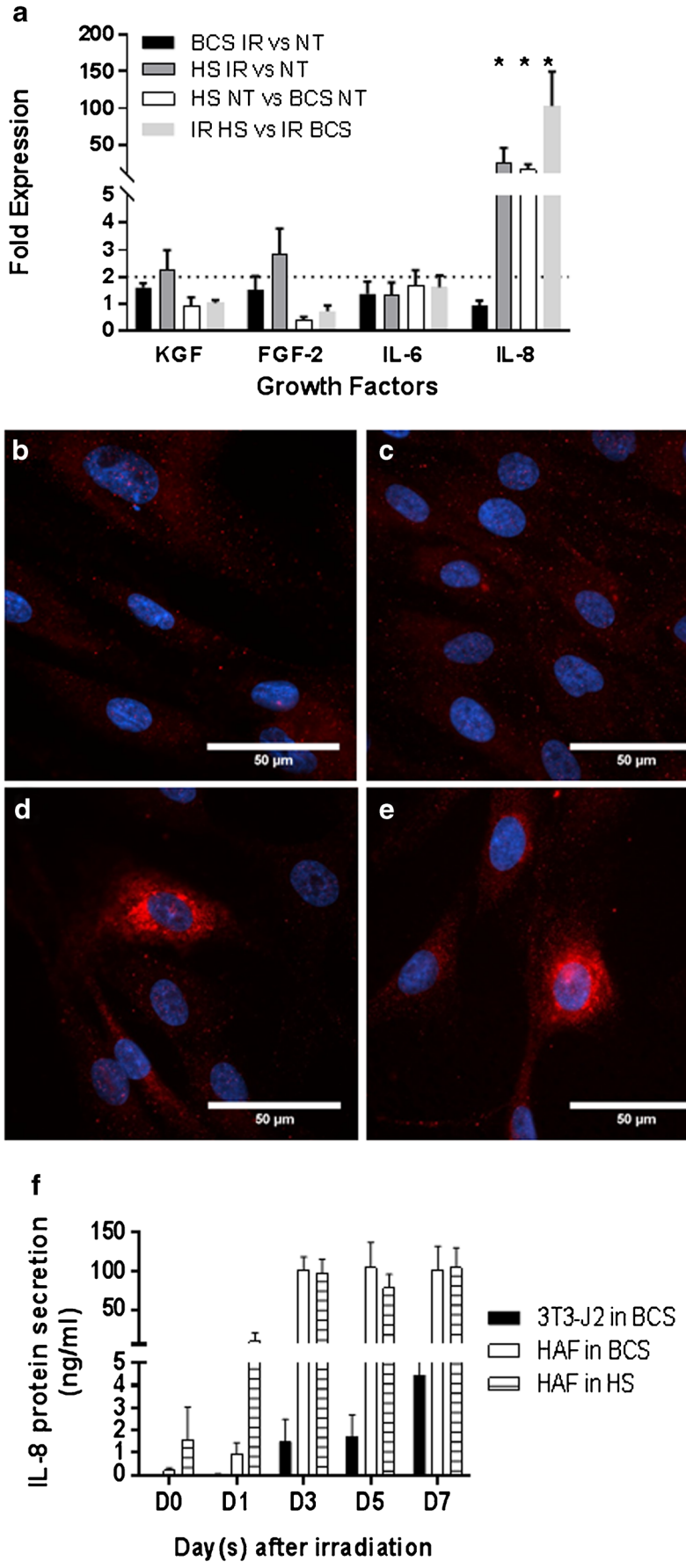
This study aimed to explore a clinically relevant alternative method to the classic Green's method for HAK expansion. One of the bottlenecks in adopting tissue-engineered skin in clinics is the ability to produce a product (such as CEA) that complies with the manufacturing standards (such as GMP) required by regulators. Here, we presented evidence that allogenic fibroblasts and serum should be considered as a novel, xeno-free, GMP-compliant alternative to the classic Green's method for keratinocyte expansion in the clinical setting. These findings will facilitate development of a truly autologous CEA in which keratinocytes are expanded with a patient's own fibroblasts and serum.

Specifically, HS was able to support both short- and long-term HAF isolation and proliferation, comparable to BCS (Fig. 1). However, unlike 3T3-J2 murine feeder cells, HAF proliferation began to slow after 8 passages in culture and HAF are therefore only suitable as feeders during these early passages. A higher irradiation regime (i.e., 80 Gy) was required to induce senescence in HAF compared to 3T3-J2 cells (Fig. 2). Nonetheless, HAF feeder cells in HS were able to support HAK attachment and proliferation in a similar manner to the Green's method (Fig. 3).

HS-AB type (at 5%), combined with HAF feeders, provided sufficient attachment factors and nutrition for HAK colony growth, whereas a trend towards reduced HAK clonogenicity was observed in 12.5% HS-AB type. This may be due to the presence of allogenic inhibitory factors in human serum that can inhibit the growth of HAK at higher serum concentrations. An inhibitory effect of high concentrations of blood-derived products on HAK growth has also been observed in platelet lysate applications (Altran et al. 2013; Stessuk et al. 2016). The choice of HS-AB type has practical implications for the potential use of autologous or allogenic serum during CEA manufacturing. One study has detected A, B and O human blood group antigens on cultured neonatal epidermal cells in vitro (Thompson et al. 1986). Expansion of epidermal cells in AB-type serum ensures that no AB type antibody is present in serum and therefore will not interfere with cell growth from other blood type donors. Similarly, HS-AB type (at 10%) has also been demonstrated to be an effective alternative to FCS for clinical expansion of mesenchymal stem cells (Aldahmash et al. 2011; Kocaoemer et al. 2007).

In this study, we used α6 integrin expression as a marker of proliferation to characterise xeno-free HAK. α6 integrin regulates interfollicular stem and progenitor cell attachment and has been used as a marker of these cells in other studies (Nanba et al. 2015; Webb et al. 2004). Our results confirmed earlier reports that most HAK expanded according to the Green's method are stem and progenitor cells and express α6 integrin (Lorenz et al. 2009). We found that although freshly isolated HAK grown in xeno-free conditions had similar clonogenicity to those expanded by the Green's method, a smaller proportion of the clones expressed α6 integrin. This was independent of the type of serum they were exposed to and could only be as a result of fibroblast feeder/HAK interaction. The effect of reduced expression of α6 integrin marker on HAK proliferation in subsequent expansion is yet to be determined but the fact that xeno-free conditions did not induce HAK differentiation (as assessed by keratin 10 expression) is encouraging.

IL-8 overexpression in HAF grown in HS compared to BCS in the immediate days post-irradiation was observed at both the RNA and protein levels, which may contribute to the improved HAK colony formation observed when HAK were grown in HS compared to bovine serum. No difference in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Fold Expression
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KGF
FGF-2
IL-6
IL-8
Growth Factors
b
c
d
e
50 µm
f
IL-8 protein secretion (ng/ml)
150
100
50
5
4
3
2
1
0
3T3-J2 in BCS
HAF in BCS
HAF in HS
D0
D1
D3
D5
D7
Day(s) after irradiation

Fig. 5 Growth factor expression pattern of HAF grown in BCS or HS. **a** Three independent batches of HAF (passages 4–7) isolated and cultured in either BCS or HS were γ irradiated. The expression pattern of KGF, FGF-2, IL-6 and IL-8 was compared in irradiated (IR) vs non-treated (NT) HAF, 24 h post-irradiation using specific primers in RT-PCR. An average copy numbers of three housekeeping genes (GAPDH, HPRT, TBP) were also analysed as reference genes to calculate $\Delta\Delta C_t$. (Pfaffl method). Values were statistically analysed using 2-way ANOVA analysis in GraphPad Prism. $^*P < 0.0001$. Error bars represent SEM. Irradiated (IR) and non-treated (NT) HAF in BCS (**b**, **c**, respectively), and IR and NT HAF in HS (**d**, **e**, respectively) were fixed and stained for IL-8 expression using Il-8 specific antibody. There were detectable levels of IL-8 in HAF cultured in HS pre- and post-irradiation. (**f**) IL-8 protein secretion was measured by ELISA at days 0, 1, 3, 5 and 7 after irradiation. Statistical analysis was performed using 2-way ANOVA in GraphPad Prism. Data are mean ± SEM values from $n = 4$ independent experiments

KGF, FGF-2 or IL-6 expression was observed in HAF post-irradiation. IL-8 (CXCL8) is a pro-inflammatory chemokine secreted by keratinocytes that signals through CXCR1 and CXCR2 members of the G protein coupled receptor superfamily to attract leukocytes. Dermal fibroblasts are not known to produce IL-8 under normal conditions. However, IL-8 expression by fibroblasts is observed during tissue injury and in inflammatory-related diseases, such as psoriasis and cystic fibrosis. Indeed, it is believed that in psoriasis, high levels of IL-8 are partly responsible for the hyper-proliferation of keratinocytes (Tuschil et al. 1992). Exposure of fibroblasts to burn exudates has also been reported to stimulate IL-8 expression by dermal fibroblasts (van den Broek et al. 2014). IL-8 is also upregulated in lung fibroblasts in fibrotic lung disease (Ludwicka-Bradley et al. 2000) and in rheumatoid synovial fibroblasts during chronic inflammation (Cho et al. 2007).

Grafting IL-8-stimulated keratinocytes may have important clinical implications. It is possible that IL-8-stimulated keratinocytes have a greater ability to close a wound once grafted onto a patient, by means of faster re-epidermisation and wound repair, compared to those expanded according to the Green's method. Although keratinocytes are a rich source of IL-8, addition of exogenous IL-8 has been shown to stimulate cell migration in a keratinocyte (HaCaT) cell line (Jiang et al. 2012). Other pathways activated downstream from IL-8 are not fully understood, although the angiogenic effect of IL-8 on human intestinal microvascular endothelial cells has been reported as one example (Heidemann et al. 2003).

Together, we provided data that human AB-type serum in combination with human-derived fibroblast feeder cells is capable of supporting human adult keratinocyte clonogenicity. These data are in agreement with studies showing that human fibroblast feeder cells in human serum can provide a complete xeno-free condition for expansion of human embryonic stem cells (Meng et al. 2008; Richards et al. 2002). We have shown that IL-8 may play a significant role in the effective expansion of xeno-free HAK, although the HAK may be somewhat less proliferative. Future work will determine whether serial cultivation, a requirement for clinical application, is feasible in such xeno-free conditions.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committees and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

References

Aldahmash A, Haack-Sorensen M, Al-Nbaheen M, Harkness L, Abdallah BM, Kassem M (2011) Human serum is as efficient as fetal bovine serum in supporting proliferation and differentiation of human multipotent stromal (mesenchymal) stem cells in vitro and in vivo. Stem Cell Rev 7:860–868

Altran SC, Yoshito D, Isaac C, Sufi BS, Herson MR, Conceicao RO, Ferreira MC, Mathor MB (2013) Human platelet lysate as a substitute for fetal bovine serum in human keratinocyte cell cultures conforming epithelia for transplantation. J Cell Tissue Res 13: 3603–3609

Astori G, Amati E, Bambi F, Bernardi M, Chieregato K, Schafer R, Sella S, Rodeghiero F (2016) Platelet lysate as a substitute for animal serum for the ex-vivo expansion of mesenchymal stem/stromal cells: present and future. Stem Cell Res Ther 7:93

Bisson F, Rochefort E, Lavoie A, Larouche D, Zaniolo K, Simard-Bisson C, Damour O, Auger FA, Guerin SL, Germain L (2013) Irradiated human dermal fibroblasts are as efficient as mouse fibroblasts as a feeder layer to improve human epidermal cell culture lifespan. Int J Mol Sci 14:4684–4704

Borradori L, Sonnenberg A (1996) Hemidesmosomes: roles in adhesion, signaling and human diseases. Curr Opin Cell Biol 8:647–656

Bullock AJ, Higham MC, MacNeil S (2006) Use of human fibroblasts in the development of a xenobiotic-free culture and delivery system for human keratinocytes. Tissue Eng 12:245–255

Canady J, Arndt S, Karrer S, Bosserhoff K (2013) Increased KGF expression promotes fibroblast activation in a double paracrine manner resulting cutaneous fibrosis. J Invest Dermatol 133:647–657

Cho ML, Ju JH, Kim HR, Oh HJ, Kang CM, Jhun JY, Lee SY, Park MK, Min JK, Park SH, Lee SH, Kim HY (2007) Toll-like receptor 2 ligand mediates the upregulation of angiogenic factor, vascular endothelial growth factor and interleukin-8/CXCL8 in human rheumatoid synovial fibroblasts. Immunol Lett 108:121–128

Cleland H, Wasiak J, Dobson H, Paul M, Pratt G, Paul E, Herson M, Akbarzadeh S (2014) Clinical application and viability of cryopreserved cadaveric skin allografts in severe burn: a retrospective analysis. Burns 40:61–66

Dravid G, Hammond H, Cheng L (2006) Culture of human embryonic stem cells on human and mouse feeder cells. Methods Mol Biol 331: 91–104

Gradl G, Faust D, Oesch F, Wieser RJ (1995) Density-dependent regulation of cell growth by contactinhibin and the contactinhibin receptor. Curr Biol 5:526–535

Heidemann J, Ogawa H, Dwinell MB, Rafiee P, Maaser C, Gockel HR, Otterson MF, Ota DM, Lugering N, Domschke W, Binion DG (2003) Angiogenic effects of interleukin 8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2. J Biol Chem 278:8508–8515

Jiang WG, Sanders AJ, Ruge F, Harding KG (2012) Influence of interleukin-8 (IL-8) and IL-8 receptors on the migration of human keratinocytes, the role of PLC-gamma and potential clinical implications. Exp Ther Med 3:231–236

Jubin K, Martin Y, Lawrence-Watt DJ, Sharpe JR (2011) A fully autologous co-culture system utilising non-irradiated autologous fibroblasts to support the expansion of human keratinocytes for clinical use. Cytotechnology 63:655–662

Kocaoemer A, Kern S, Kluter H, Bieback K (2007) Human AB serum and thrombin-activated platelet-rich plasma are suitable alternatives to fetal calf serum for the expansion of mesenchymal stem cells from adipose tissue. Stem Cells 25:1270–1278

Lamb R, Ambler CA (2013) Keratinocytes propagated in serum-free, feeder-free culture conditions fail to form stratified epidermis in a reconstituted skin model. PLoS One 8:e52494

Lee KH (2013) Conditions and techniques for mouse embryonic stem cell derivation and culture. In: Bhartiya G, Lenka N (eds) Pluripotent stem cells, vol 1. Intech, Rijeka, pp 85–116

Li A, Dearman BL, Crompton KE, Moore TG, Greenwood JE (2009) Evaluation of a novel biodegradable polymer for the generation of a dermal matrix. J Burn Care Res 30:717–728

Lorenz K, Rupf T, Salvetter J, Bader A (2009) Enrichment of human beta 1 bri/alpha 6 bri/CD71 dim keratinocytes after culture in defined media. Cells Tissues Organs 189:382–390

Ludwicka-Bradley A, Tourkina E, Suzuki S, Tyson E, Bonner M, Fenton JW 2nd, Hoffman S, Silver RM (2000) Thrombin upregulates interleukin-8 in lung fibroblasts via cleavage of proteolytically activated receptor-I and protein kinase C-gamma activation. Am J Respir Cell Mol Biol 22:235–243

Meng G, Liu S, Krawetz R, Chan M, Chernos J, Rancourt DE (2008) A novel method for generating xeno-free human feeder cells for human embryonic stem cell culture. Stem Cells Dev 17:413–422

Mujaj S, Manton K, Upton Z, Richards S (2010) Serum-free primary human fibroblast and keratinocyte coculture. Tissue Eng A 16: 1407–1420

Nanba D, Toki F, Tate S, Imai M, Matsushita N, Shiraishi K, Sayama K, Toki H, Higashiyama S, Barrandon Y (2015) Cell motion predicts human epidermal stemness. J Cell Biol 209:305–315

Paul M, Kaur P, Herson M, Cheshire P, Cleland H, Akbarzadeh S (2015) Use of clotted human plasma and aprotinin in skin tissue engineering: a novel approach to engineering composite skin on a porous scaffold. Tissue Eng Part C Methods 21:1098–1104

Rheinwald JG, Green H (1975a) Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma. Cell 6:317–330

Rheinwald JG, Green H (1975b) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 6:331–343

Richards M, Fong CY, Chan WK, Wong PC, Bongso A (2002) Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat Biotechnol 20:933–936

Sorrell JM, Caplan AI (2004) Fibroblast heterogeneity: more than skin deep. J Cell Sci 117:667–675

Sorrell JM, Baber MA, Caplan AI (2004) Site-matched papillary and reticular human dermal fibroblasts differ in their release of specific growth factors/cytokines and in their interaction with keratinocytes. J Cell Physiol 200:134–145

Stessuk T, Puzzi MB, Chaim EA, Alves PC, de Paula EV, Forte A, Izumizawa JM, Oliveira CC, Frei F, Ribeiro-Paes JT (2016) Platelet-rich plasma (PRP) and adipose-derived mesenchymal stem cells: stimulatory effects on proliferation and migration of fibroblasts and keratinocytes in vitro. Arch Dermatol Res 308:511–520

Szabowski A, Maas-Szabowski N, Andrecht S, Kolbus A, Schorpp-Kistner M, Fusenig NE, Angel P (2000) c-Jun and JunB antagonistically control cytokine-regulated mesenchymal-epidermal interaction in skin. Cell 103:745–755

Thompson C, Rosa B, Fletcher A, Harbour C, Cossart Y (1986) Expression of blood group antigens by cultured human epidermal cells. J Invest Dermatol 86:394–398

Tuschil A, Lam C, Haslberger A, Lindley I (1992) Interleukin-8 stimulates calcium transients and promotes epidermal cell proliferation. J Invest Dermatol 99:294–298

van den Broek LJ, Kroeze KL, Waaijman T, Breetveld M, Sampat-Sardjoepersad SC, Niessen FB, Middelkoop E, Scheper RJ, Gibbs S (2014) Differential response of human adipose tissue-derived mesenchymal stem cells, dermal fibroblasts, and keratinocytes to burn wound exudates: potential role of skin-specific chemokine CCL27. Tissue Eng A 20:197–209

Webb A, Li A, Kaur P (2004) Location and phenotype of human adult keratinocyte stem cells of the skin. Differentiation 72:387–395

Witzeneder K, Lindenmair A, Gabriel C, Holler K, Theiss D, Redl H, Hennerbichler S (2013) Human-derived alternatives to fetal bovine serum in cell culture. Transfus Med Hemother 40:417–423

Wood FM, Kolybaba ML, Allen P (2006) The use of cultured epithelial autograft in the treatment of major burn wounds: eleven years of clinical experience. Burns 32:538–544