 doi:10.20944/preprints202002.0430.v1

Article

To Serum or Not to Serum: Reduced-serum based approaches for contact-based co-culture of fibroblasts and keratinocytes for wound bed studies

Snehal Kadam[1,†], M Vandana[1,†] and Karishma S Kaushik[1,*]

[1] Institute of Bioinformatics and Biotechnology, Savitribai Phule Pune University, Pune, INDIA
† these authors contributed equally;
* Correspondence: karishmaskaushik@gmail.com;

Abstract: Contact-based co-culture of fibroblasts and keratinocytes is important to study the structure and functions of the wound bed. Co-culture of these two cell types in direct contact with each other has been challenging, requiring high serum concentrations (up to 10%), feeder systems and a range of supplemental factors. These approaches are not only technically demanding, but also present scientific, cost and ethical limitations associated with high-serum concentrations. We have developed two reduced-serum approaches (1-2%) to support contact-based co-culture of human dermal fibroblasts (HDFa) and human epidermal keratinocytes (HaCaT). The two approaches include (1) Specialized cell culture media for each cell type mixed in a 1:1 ratio (KGM+FGM), and (2) Minimal media supplemented with cell-specific growth factors (MEM+GF). Co-culture could be successfully achieved by co-seeding (two cell types were introduced simultaneously), or in a layered fashion (keratinocytes seeded on top of confluent fibroblasts). With wound scratch assays, the co-cultured platforms could demonstrate cell proliferation, migration and wound closure. The reduced-serum conditions developed are simple, easy to formulate and adopt, and based on commonly-available media components. These contact-based co-culture approaches can be leveraged for wound and skin studies, and tissue bioengineering applications, potentially reducing concerns with high-serum formulations.

Keywords: Co-culture, Reduced-serum, Wound bed, Fibroblasts, Keratinocytes

1. Introduction

In the proliferative phase of healing, the wound bed is progressively filled with granulation tissue [1], followed by re-epithelialization to restore surface integrity [2]. Granulation tissue consists of, among other components, fibroblasts from the dermis, which proliferate and migrate into the wound bed [3]. Fibroblasts secrete signaling factors that recruit adjacent epidermal keratinocytes from the wound edge and epithelial appendages [3] **(Figure 1A).** Migration of keratinocytes and fibroblasts results in regions of direct contact and communication between the two cell types [4,5]. Interactions between these two cell types critically impact wound bed structure and functions, including cellular architecture, cell migration and wound closure [5,6].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 February 2020 doi:10.20944/preprints202002.0430.v1

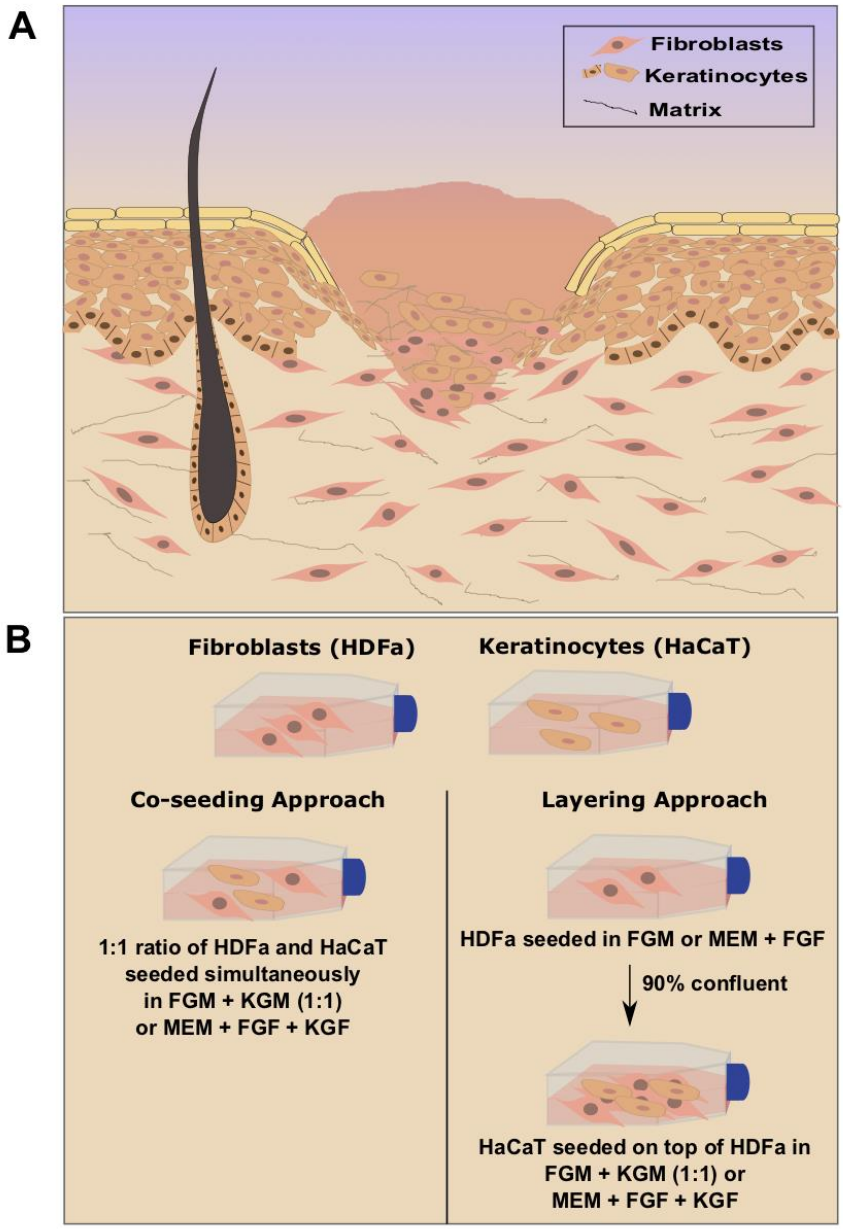

Figure 1: Contact-based co-culture of fibroblasts and keratinocytes is important to study the wound bed. **(A)** In the proliferative stage of wound healing, the wound bed consists of fibroblasts and keratinocytes, in close association with each other. Fibroblasts from the wound edges migrate into the wound bed, establishing signaling networks that recruit adjacent keratinocytes. Keratinocytes migrate from the wound edge and from around epithelial appendages, resulting in distinct regions of fibroblast-keratinocyte interactions. **(B)** Schematic of two reduced-serum conditions developed to co-culture fibroblasts and keratinocytes, based on a mixture of specialized cell media (FGM+KGM) or minimal media supplemented with growth factors (MEM+GF), using co-seeding and layering approaches.

Contact-based co-culture of human fibroblasts and keratinocytes has been challenging, with previous protocols employing high concentrations of animal serum (up to 10%), cell feeder systems and a range of cell-specific growth factors [7–10]. These approaches are not only tedious and labor-intensive, but the usage of high-serum concentrations poses a range of well-known technical and

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 February 2020 doi:10.20944/preprints202002.0430.v1

scientific limitations [11–13], including variable effects based on different cell types. For example, in the context of keratinocytes and fibroblasts, the presence of serum is observed to influence the attachment and proliferation [7] of the two cell types when cultured together. In one study [7], keratinocytes (HaCaT) co-cultured with fibroblasts (HDFa) in the presence of 10% fetal bovine serum, were seen to attach poorly to tissue culture plastic substrates; attachment was significantly higher under serum-free conditions. On the other hand, alternatives such as serum-free media or synthetic formulations either do not support the robust co-culture of fibroblasts and keratinocytes; fibroblasts have been shown to display impaired proliferative capacity under serum-free conditions [7], or require substantial optimization and are cost-intensive [11,13].

We have formulated two reduced-serum based approaches (1-2%) to co-culture fibroblasts and keratinocytes, in direct contact with each other. These approaches are simple, easy to formulate and use commonly-available media components. Using these reduced-serum approaches, fibroblasts and keratinocytes were co-cultured, by co-seeding the two cell types or by layering keratinocytes over confluent fibroblasts. Both cell types demonstrated robust attachment, proliferation, and characteristic cell morphology when cultured together. Further, the co-cultured platforms under reduced-serum conditions could be used to study wound bed features, such as cell migration and wound closure.

2. Materials and Methods

Cell culture and maintenance:

Adult primary human dermal fibroblasts (HDFa) were obtained from PromoCell (Germany) and maintained in a of all-in-one ready-to-use Fibroblast Growth Media (FGM) (a proprietary formulation from Cell Applications, 116-500) containing 2% serum (FBS). Immortalized adult human keratinocytes (HaCaT) were a gift from Dr. Madhur Motwani (Linq Labs, Jehangir Clinical Development Centre, Pune, India) and were maintained in an all-in-one, ready-to-use Keratinocyte Serum-free Growth Media (KGM) (a proprietary formulation from Sigma, 131-500A). Cells were maintained at 37°C and in a 5% CO_2 humidified incubator. Co-culture was done in T25 flasks (Tarsons) and 6-well tissue culture treated plates (Thermo Scientific), with total media volumes of 5mL and 2mL respectively. Cell counting was done with a hemocytometer. Cells were seeded at a density of ~10^5 cells/mL, unless stated otherwise. Imaging was performed at 10X magnification with a Magnus INVI microscope equipped with a Magcam DC5 camera. For all experiments in MEM, Penicillin-Streptomycin (Gibco) was added to media (with or without serum) in a final concentration 1X.

Reduced-serum media formulations for co-culture:

To co-culture HDFa and HaCaT in the presence of reduced-serum, two media formulations were developed. In one of them, equal parts of FGM (containing 2% serum) and KGM were mixed (in a 1:1 ratio), resulting in composite media with an effective concentration of 1% serum (FGM+KGM). In the second formulation, Minimum Essential Media (MEM) containing Earle's salts, 2mM L-Glutamine, 1mM Sodium pyruvate, Non-Essential Amino Acids (NEAA) and 1.5gms per litre sodium bicarbonate (MEM, Himedia, AL047S), was supplemented with fibroblast growth factor (FGF) (Gibco, S00310) and keratinocyte growth factor (KGF) (Gibco, S0015) at concentrations of 1X each

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 February 2020 doi:10.20944/preprints202002.0430.v1

(MEM+GF). FGF contains fetal bovine serum, hydrocortisone, human epidermal growth factor, basic fibroblast growth factor and heparin. When FGF is added to the media at a concentration of 1X, the resulting media contains 2% FBS. KGF contains bovine pituitary extract, recombinant human insulin-like growth factor-I, hydrocortisone, bovine transferrin and human epidermal growth factor, and is serum-free.

Co-culture using co-seeding and layering techniques:

HDFa and HaCaT cells were co-cultured in reduced-serum conditions, using two approaches using (1) a co-seeding technique, in which the two cell types were introduced simultaneously and (2) in a layered fashion, with keratinocytes seeded on top of a confluent layer of fibroblasts.

For the co-seeding approach, HDFa and HaCaT cells were counted and seeded in 1:1 ratio into both T25 flasks and 6-well plates. Both reduced-serum formulations, FGM+KGM and MEM+GF, were employed. The T25 flasks were imaged at 10X every 24 hours for 5 days. The 6-well plates were imaged at 10X every 24 hours intervals till 70-80% confluent.

For the layering approach in FGM+KGM media, HDFa cells were counted and seeded in T25 flasks in FGM (2% serum), incubated at 37°C and grown to 90% confluency. When confluent, the FGM was removed and replaced with FGM+KGM (in a 1:1 ratio; 1% serum). HaCaT cells were counted and seeded on the confluent layer of fibroblasts. Flasks were imaged at 10X every 24 hours for 5 days.

For the layering technique in MEM+GF, HDFa cells were counted and seeded in T25 flasks in MEM supplemented with 1X FGF, incubated at 37°C and grown to 90% confluency. When confluent, the media was replaced with MEM containing both FGF and KGF (1X each; 2% serum). HaCaT cells were counted and seeded on the confluent layer of fibroblasts. Flasks were imaged at 10X every 24 hours for 5 days. Similarly, the layering approach is also performed in 6-well plates for both the media conditions (**Figure S2A**).

Wound scratch assay on co-cultured platforms:

HDFa and HaCaT were counted and seeded in 6-well tissue culture treated plates in reduced-serum conditions, with MEM + GF, using the co-seeding or layering approach. When the co-cultured cells reached 90% confluency, the media was replaced with fresh media, and a scratch was made across each well using a sterile 200uL tip. Following this, the wells were imaged at 10X at 0, 4, 24, 48 and 72 hours (three images were captured per scratch at different regions). The images were analyzed in ImageJ [14] by drawing a freehand outline of the scratch and using ImageJ tools to calculate the area within the outlined region (Suppl. Fig. 2). This was done for three images per well for three replicates for each condition, and an average wound area was calculated.

Percentage of wound closure percentage was calculated as

% Wound closure = $((W_0 - W_x)/ W_0) \times 100$, where, W_0 = wound area at 0 hours and W_x = wound area at 'x' hours (calculated at 0, 4, 24 and 48 hours).

3. Results

3.1. Reduced-serum conditions developed using commonly-available media components

Two reduced-serum approaches were formulated using commonly-available media components **(Figure 1B).** The first formulation consisted of specialized cell culture media for fibroblasts and

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 February 2020 doi:10.20944/preprints202002.0430.v1

keratinocytes ((Fibroblast Growth Medium (FGM) and Keratinocyte Growth Medium (KGM)) in a 1:1 ratio, referred to as FGM+KGM. FGM contains 2% fetal bovine serum (FBS; information obtained from the manufacturer), resulting in an effective concentration of 1% serum in FGM+KGM. The second formulation consisted of minimal essential medium (MEM) supplemented with growth factors; Fibroblast Growth Factor (FGF) and Keratinocyte Growth Factor (KGF) were added in 1X concentration each, referred to as MEM+GF. When FGF is added to MEM at a concentration of 1X, the resulting media contains 2% FBS; KGF is serum-free. These approaches result in effective concentrations of 1-2% FBS, representing a 5-10-fold reduction as compared with previous protocols [7,8,10] (using up to 10% FBS).

3.2. *Reduced-serum conditions support the co-culture of HDFa and HaCaT cells using the co-seeding technique*

For the co-seeding approach, human dermal fibroblasts (HDFa) and adult human keratinocytes (immortalized, HaCaT) were seeded in a 1:1 ratio (~10^5 cells/mL of each cell type) in a T25 flask. Co-seeding was done in FGM+KGM (1% serum) and in MEM+GF (2% serum). After 24 hours, HDFa and HaCaT showed robust attachment, proliferation and characteristic cell morphology, in both reduced-serum conditions **(Figure 2A)**. Starting from Day 2, HaCaT cells were observed to cluster in colonies, which became progressively dense and tightly-packed, surrounded by sheaths of HDFa cells. The co-culture platform became fully confluent by Day 5 **(Figure 2A)**.

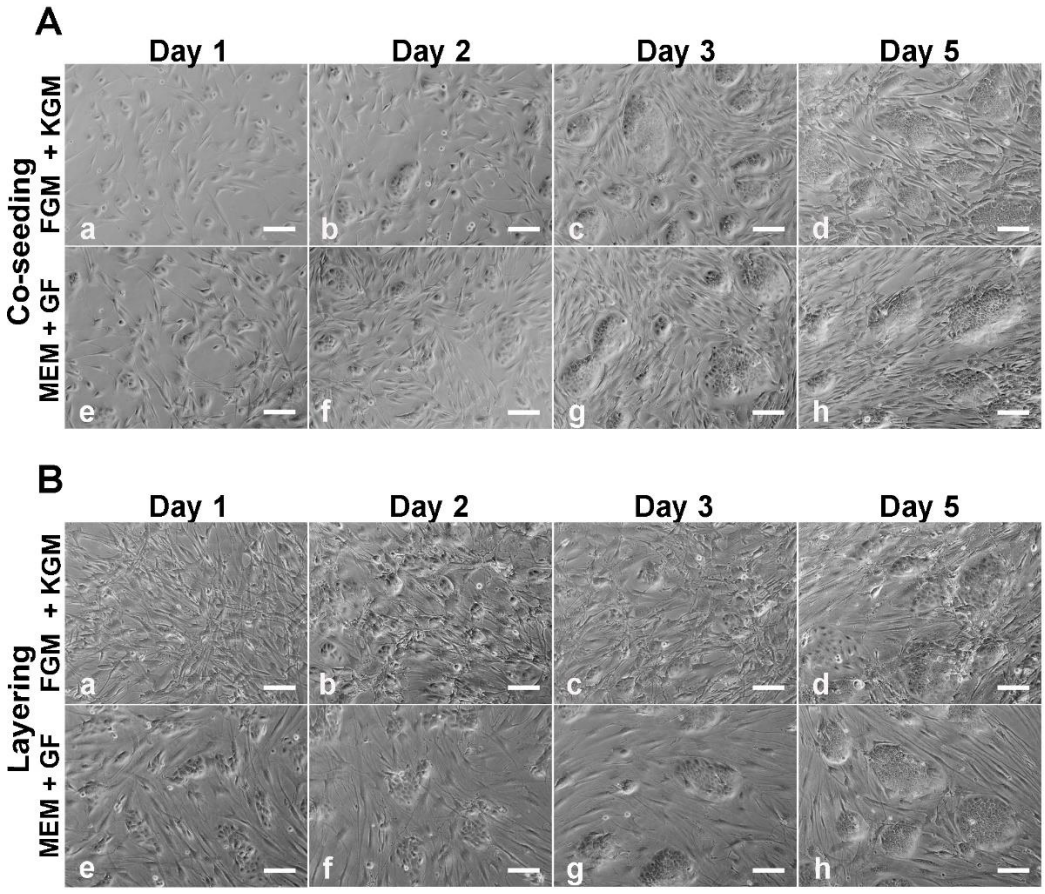

Figure 2: Co-culture of HDFa and HaCaT cells using co-seeding (in 1:1 ratio) and layering techniques under reduced-serum (1-2%) conditions. **(A)** When co-seeded together, under both reduced-serum

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 February 2020 doi:10.20944/preprints202002.0430.v1

conditions, HaCaT cells form distinct colonies by Day 2, which continue to become more dense and tightly-packed, surrounded by sheaths of proliferating fibroblasts. **(B)** When layered over HDFa, HaCaT cells form distinct clusters on HDFa cells starting from Day 1 (MEM+GF) or Day 2 (FGM+KGM), which continue to proliferate and increase in size. Scale bars = 150 μm.

3.3. *Reduced-serum conditions support the co-culture of HDFa and HaCaT cells using the layering technique*

For the layering approach, HDFa cells were grown to 90% confluency, following which HaCaT cells were counted (~10^5 cells/mL) and seeded on layers of confluent HDFa. Starting from Day 1, HaCaT cells were observed to attach on the HDFa layers under both reduced-serum conditions. As in the co-seeding approach, HaCaT cells proceeded to form distinct clusters on and between sheaths of HDFa cells starting from Day 1 (MEM+GF) or Day 2 (FGM+KGM), which continued to proliferate and increase in size **(Figure 2B)**.

3.4. *Reduced-serum approaches developed support characteristic HDFa and HaCaT morphologies under co-culture conditions*

In co-culture under reduced-serum conditions, HaCaT cells displayed typical polygonal cell clusters (colonies), with mosaic, cobblestone cell morphology, and HDFa cells were observed to form characteristic sheaths of spindle-shaped cells **(Figure 2 and Figure S1A).** Notably, the reduced-serum conditions developed included cell-specific growth factors (as components of specialized cell media or added into basal media), which, along with optimum serum concentrations, was observed to be critical to establish co-culture and characteristic cell morphology **(Figure S1B).** As seen in Suppl. Fig. 1B, in the absence of serum and growth factors, HDFa cells show poor proliferation even after five days, and HaCaT cells fail to attach and proliferate (likely due to lack of growth factors). On the other hand, in the presence of 10% FBS (no growth factors), HDFa and HaCaT showed minimal proliferation, but HDFa did not form typical sheaths around HaCaT colonies. This underscores the need for optimal serum concentrations and requisite growth factors for successful co-culture of fibroblasts and keratinocytes, both of which are included in the reduced-serum formulations.

3.5. *The co-cultured platform, under reduced-serum conditions, enables the study of cell migration and wound closure*

HDFa and HaCaT cells were co-cultured in tissue culture treated 6-well plates **(Figure S2A)** under reduced-serum conditions (with co-seeding and layering approaches), following which a scratch was applied across the co-cultured cells. As seen in Figure 3A, in the co-seeding approach under both reduced-serum conditions, HDFa and HaCaT proliferation and migration started filling the wound gap by 4 hours. The gap continued to be filled between 24-48 hours, primarily by migrating fibroblasts, with percent wound area showing complete closure (100%) with MEM+GF and 80% closure for FGM+KGM after 48 hours **(Figure 3A)**. In the layering approach, HDFa and HaCaT cells started filling the wound gap by 4 hours, with near-complete closure of the gap by 24 hours **(Figure 3B)**. Similar to that observed with the co-seeding approach, fibroblasts migrated into the wound gap, while keratinocyte clusters were seen to increase in size and fill the wound area from the periphery.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 February 2020 doi:10.20944/preprints202002.0430.v1

The wound area was determined by drawing a freehand outline of the scratch and using ImageJ tools to calculate the area within the outlined region **(Figure S2B)**. This was done for three images per well for three replicates for each condition, and an average wound area was calculated.

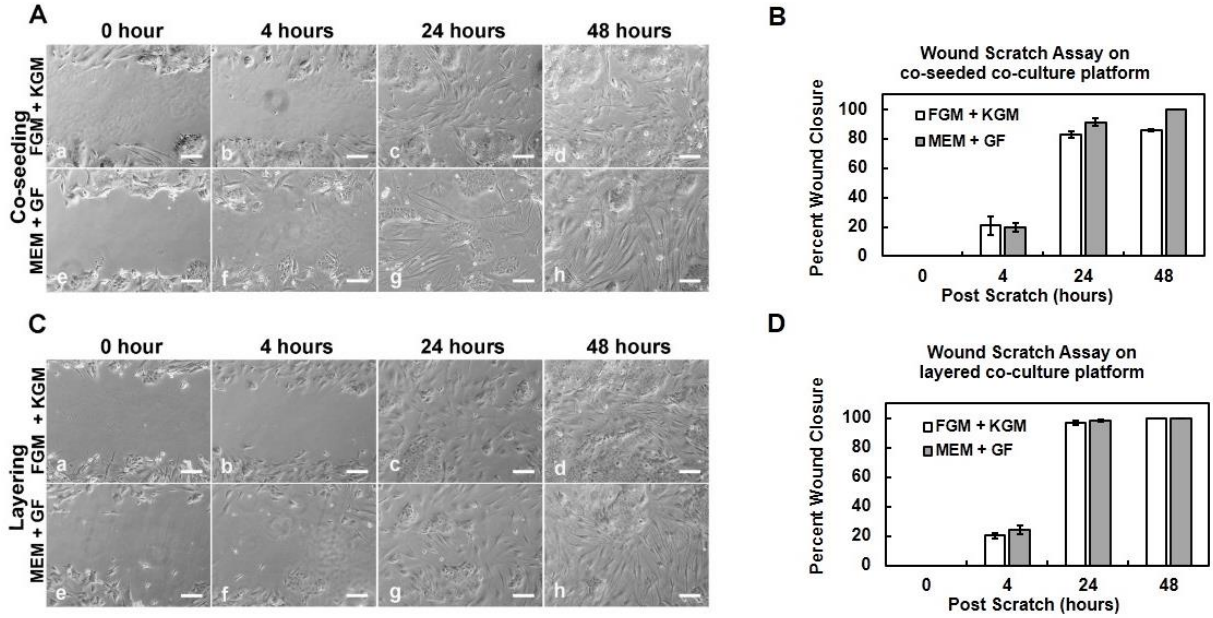

Figure 3: Wound scratch assay for cell migration and wound closure. Co-cultured HDFa and HaCaT cells under reduced-serum conditions (co-seeding and layering approaches), were scratched (to mimic wound injury) and wound closure was monitored. **(A and B)** In the co-seeding approach (a-h), HDFa and HaCaT cells start migrating into the wound gap by 4 hours, and progressively fill the wound area with 80-100% closure at 48 hours. **(C and D)** In the layering approach, HDFa and HaCaT cells start migrating into the wound gap by 4 hours, with near closure of the wound gap by 24 hours. Error bars represent SEM; at least 3 replicates. Scale bars = 150 μm.

4. Discussion

Contact-based co-culture of human dermal fibroblasts and epidermal keratinocytes is important for a range of wound and skin studies. Previous approaches to establish co-culture of fibroblasts and keratinocytes in direct contact with each other are often tedious and labor-intensive, and notably employ high-serum concentrations (often up to 10% FBS). Animal serum, for example fetal bovine serum (FBS), is widely-employed in cell culture studies, and possesses a range of active substances, known to support the proliferation and morphology of a variety of cell types. However, its usage in high concentrations is associated with several limitations [11–13], which are particularly relevant in the context of co-culturing fibroblasts and keratinocytes. The attachment, proliferation and cell morphology of fibroblasts and keratinocytes varies depending on the concentration of serum in the media and presence of specific growth factors. For example, keratinocytes (HaCaT) co-cultured with fibroblasts (HDFa) in the presence of 10% fetal bovine serum, displayed poor attachment to tissue culture plastic substrates; whereas when co-cultured in the complete absence of serum, HDFa cells demonstrated impaired proliferative capacity [7,9,15]. Further, in serum-free media, both cell types failed to form characteristic cellular arrangements. Given this, there is the need to develop

 doi:10.20944/preprints202002.0430.v1

approaches that successfully support the co-culture of fibroblasts and keratinocytes at concentrations of serum and growth factors, that not only overcome the differential effects of serum on the two cell types, but are also easy to formulate and adopt in experimental practice.

The reduced-serum approaches developed in this study, support the co-culture of fibroblasts and keratinocytes, using both co-seeding and layering approaches. These co-cultured systems support the proliferation and cell morphology of both cell types, and enable the study of wound bed functions such as cell migration and wound closure. The formulations developed result in effective concentrations of 1% FBS in KGM+FGM, and 2% in MEM+GF, representing a 5-10 fold reduction in serum concentration as compared with previous studies, using up to 10% serum [7,9,15]. Further, these approaches are easy to formulate and use commonly-available media components, making them relatively simple to adopt and use. Finally, reduced-serum approaches align with the push to reduce the scientific, technical and ethical limitations [11] associated with the use of animal serum in cell culture. These limitations include substantial batch-to-batch variation, presence of uncharacterized factors, and potential risk of microbial contamination, likely to influence experimental outcome and reproductivity. Further, procedures to harvest serum from animal sources are poorly regulated and standardized, posing major ethical and moral concerns. These limitations can be potentially alleviated with the development and application of reduced-serum formulations.

To the best of our knowledge, there is no previous work reporting the contact-based co-culture of fibroblasts and keratinocytes under reduced-serum conditions (1-2%) with cell-specific growth factors, as reported in this study. These approaches can be leveraged and adapted for a range of wound studies, as well as skin, tissue and cell engineering applications, potentially reducing concerns with high-serum formulations.

5. Conclusions

We have developed two reduced-serum based approaches that support the co-culture of fibroblast and keratinocytes in direct contact with each other. These co-cultured systems support the robust attachment, proliferation and cell morphology of both cell types, using co-seeding and layering techniques, and enable the study of wound bed functions such as cell migration and wound closure. Further, these approaches are simple to formulate, easy to adopt, use widely-available media components, and potentially reduce concerns associated with high concentrations of animal serum. Taken together, these protocols lend themselves well for co-culture of these two cell types for wound and skin studies, tissue bioengineering and cell therapy applications.

6. Supplementary Figures

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 February 2020 doi:10.20944/preprints202002.0430.v1

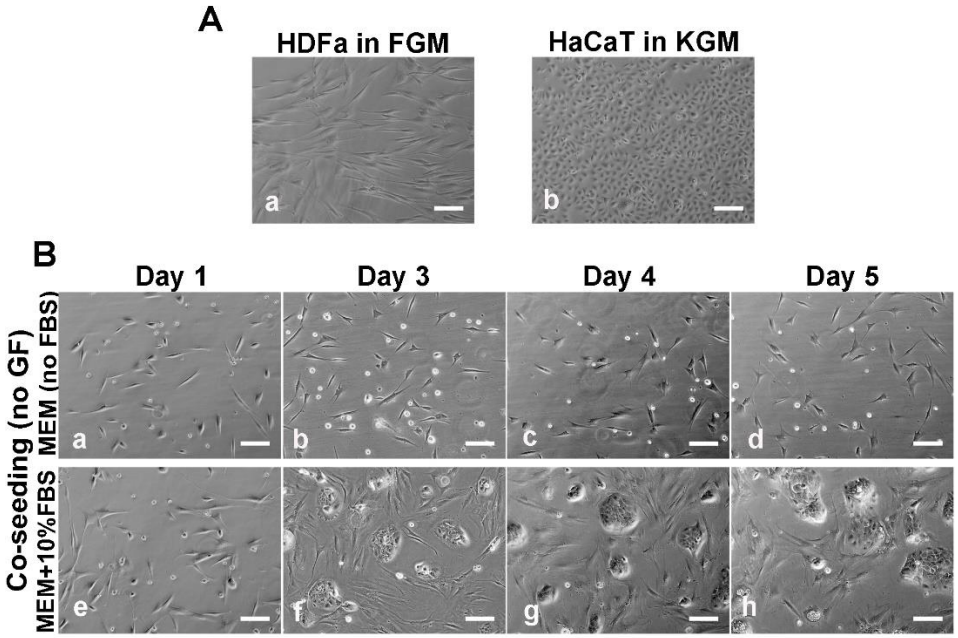

Figure S1: HDFa and HaCaT proliferation and growth in their optimum media individually, and co-cultured in varying FBS conditions. **(A)** Morphology and proliferation of HDFa and HaCaT under different conditions. (a) HDFa growing in Fibroblast Growth Medium (FGM) with characteristic spindle-shaped morphology and HaCaT (immortalized) growing in Keratinocyte Growth Medium (KGM) with typical mosaic, cobblestone morphology. **(B)** Co-seeding of HDFa and HaCaT (in a 1:1 ratio) in MEM alone (with no growth factors or FBS; a-d) and MEM with added 10% FBS (no growth factors) (e-h). In the absence of serum and growth factors (a-d), HDFa show poor proliferation, and HaCaT fail to proliferate. In the presence of 10% FBS (no growth factors, e-h), HDFa and HaCaT proliferate, but HDFa does not form typical sheaths around HaCaT colonies. Scale bars = 150 μm.

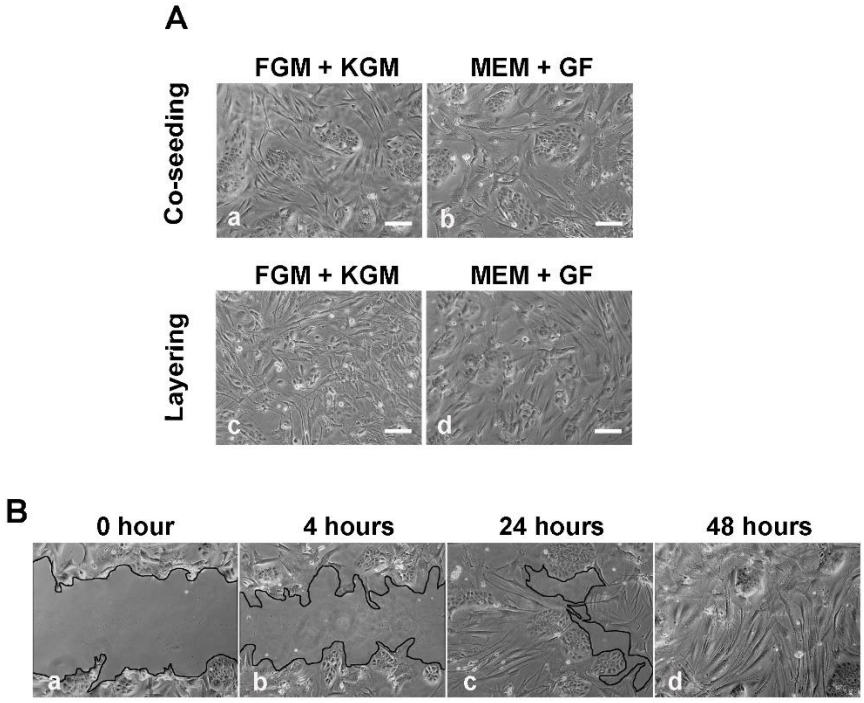

Figure S2: Analysis of Wound Scratch Assays in co-culture platforms **(A)** Co-culture of HDFa and HaCaT under reduced-serum conditions on tissue culture-treated six well plates (for the wound scratch assay). **(B)** Representative images of the wound scratch assay for HDFa and HaCaT co-

cultured platforms (in six well plates) from 0 hours to 48 hours showing the use of a freehand outline tool to calculate wound closure over time (ImageJ). This analysis was done for 3 images per well for three replicates for each condition, and an average of the wound area was calculated.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: HDFa and HaCaT proliferation and growth in their optimum media individually, and co-cultured in varying FBS conditions., Figure S2: Analysis of Wound Scratch Assays in co-culture platforms.

Author Contributions: SK and MV (equal contributions) designed and performed the experiments and analyzed the data. SK, MV and KSK wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the Ramalingaswami Re-entry Fellowship, Department of Biotechnology, Government of India (to Karishma S Kaushik).

Acknowledgments: We thank Dr. Madhur Motwani, Linq Labs, Jehangir Clinical Development Centre, Pune, India for the HaCaT cell line. We thank Abhishek Teli, Sukanya Gayan and Jay Dave (Institute of Bioinformatics and Biotechnology, Savitribai Phule Pune University) for technical inputs on microscopy.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. *Nature* (2008) **453**:314–321. doi:10.1038/nature07039

2. Landén NX, Li D, Ståhle M. Transition from inflammation to proliferation: a critical step during wound healing. *Cell Mol Life Sci* (2016) doi:10.1007/s00018-016-2268-0

3. Li J, Kirsner RS. "Wound Healing," in *Surgery of the Skin* (Elsevier Inc.), 97–115. doi:10.1016/B978-0-323-02752-6.50012-2

4. Wojtowicz AM, Oliveira S, Carlson MW, Zawadzka A, Rousseau CF, Baksh D. The importance of both fibroblasts and keratinocytes in a bilayered living cellular construct used in wound healing. *Wound Repair Regen* (2014) doi:10.1111/wrr.12154

5. Menon SN, Flegg JA, Mccue SW, Schugart RC, Dawson RA, Sean McElwain DL. Modelling the interaction of keratinocytes and fibroblasts during normal and abnormal wound healing processes. *Proc R Soc B Biol Sci* (2012) doi:10.1098/rspb.2012.0319

6. Ghahary A, Ghaffari A. Role of keratinocyte-fibroblast cross-talk in development of hypertrophic scar. *Wound Repair Regen* (2007) doi:10.1111/j.1524-475X.2007.00225.x

7. Gabbott CM, Sun T. Comparison of human dermal fibroblasts and hacat cells cultured in medium with or without serum via a generic tissue engineering research platform. *Int J Mol Sci* (2018) doi:10.3390/ijms19020388

8. Maas-Szabowski N, Stärker A, Fusenig NE. Epidermal tissue regeneration and stromal interaction in HaCaT cells is initiated by TGF-α. *J Cell Sci* (2003) doi:10.1242/jcs.00474

9. Jubin K, Martin Y, Lawrence-Watt DJ, Sharpe JR. A fully autologous co-culture system utilising non-irradiated autologous fibroblasts to support the expansion of human keratinocytes for clinical use. *Cytotechnology* (2011) doi:10.1007/s10616-011-9382-5

10. Wang Z, Wang Y, Farhangfar F, Zimmer M, Zhang Y. Enhanced keratinocyte proliferation and migration in co-culture with fibroblasts. *PLoS One* (2012) doi:10.1371/journal.pone.0040951

11. Yao T, Asayama Y. Animal-cell culture media: History, characteristics, and current issues. *Reprod Med Biol* (2017) doi:10.1002/rmb2.12024

12. Jochems CEA, Van der Valk JBF, Stafleu FR, Baumans V. The use of fetal bovine serum: Ethical or scientific problem? *ATLA Altern to Lab Anim* (2002) doi:10.1177/026119290203000208

13. Tekkatte C, Gunasingh GP, Cherian KM, Sankaranarayanan K. "Humanized" stem cell culture techniques: The animal serum controversy. *Stem Cells Int* (2011) doi:10.4061/2011/504723

14. Abràmoff MD, Magalhães PJ, Ram SJ. Image processing with imageJ. *Biophotonics Int* (2004) doi:10.1201/9781420005615.ax4

15. Mujaj S, Manton K, Upton Z, Richards S. Serum-free primary human fibroblast and keratinocyte coculture. *Tissue Eng - Part A* (2010) doi:10.1089/ten.tea.2009.0251