# To Serum or Not to Serum:

# Reduced-serum based methods for contact-based co-culture of human dermal fibroblasts (HDFa) and epidermal keratinocytes (HaCaT) for wound bed studies

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**Abstract:** Contact-based co-culture of human dermal fibroblasts and epidermal keratinocytes is important to study wound bed structure and functions. Co-culture of these two cell types in direct contact with each other has been a long-standing and challenging issue, owing to specific and different serum and growth factor requirements of the two cell types. Existing protocols employ high-serum concentrations (up to 10% FBS), complex cell feeder systems, and a range of supplemental factors. These approaches are not only technically demanding, labor- and material-intensive but also pose scientific and ethical limitations associated with high concentrations of animal serum. On the other hand, serum-free conditions often fail to support the attachment and proliferation of one or both cell types when cultured together. Given this, we have developed two reduced-serum based approaches (1-2% serum), using commonly-available media components, to support the contact-based co-culture of HDFa and HaCaT cells. Using these approaches, HDFa and HaCaT were co-cultured by layering keratinocytes over confluent fibroblasts or by co-seeding the two cell types simultaneously. Under these conditions, both cell types showed robust attachment and proliferation, and characteristic cellular morphology in co-culture. Further, these co-cultured platforms enabled the study of important wound bed functions such as cell migration and wound closure. We believe that these methods can be leveraged for a range of 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 wound 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 [3,4] **(Figure 1A).** Migration of keratinocytes and fibroblasts results in regions of direct contact between the two cell types [5,6]. Interactions between these two cell types critically impacts wound bed structure and functions, including cellular architecture, cell migration and wound closure [6–9]. Therefore, it is important to develop approaches that not only enable the co-culture of these two cell types in direct contact with each other but can also be leveraged to study important features and functions of the wound bed.
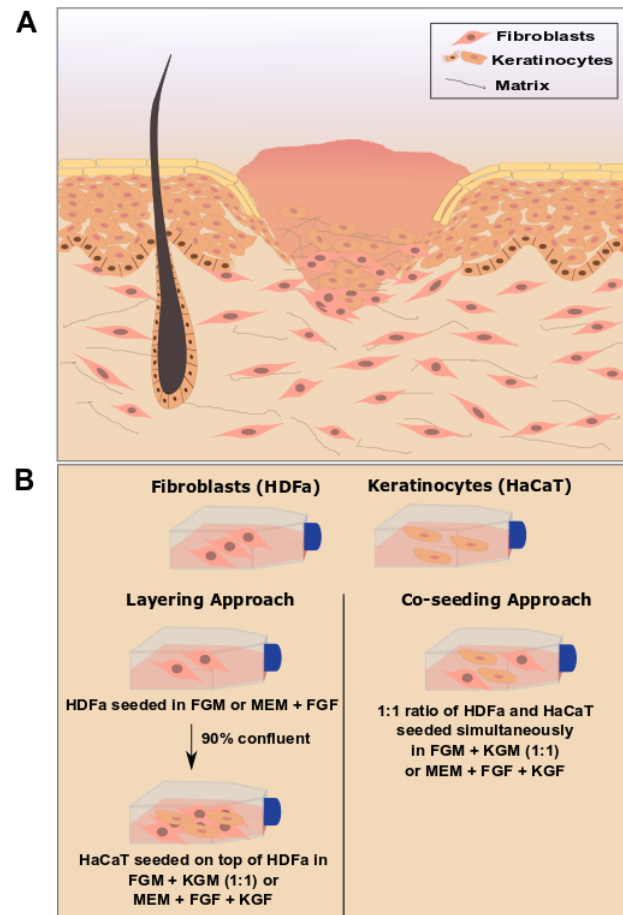
**Figure 1.** Contact-based co-culture of fibroblasts and keratinocytes is important to study the wound bed. **(A)** In the proliferative stage of wound healing, the wound bed consists of fibroblasts and keratinocytes, in close association with each other. Fibroblasts from the wound edges migrate into 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 (HDFa) and keratinocytes (HaCaT), based on a mixture of specialized cell media (FGM+KGM) or minimal media supplemented with growth factors (MEM+GF), using layering and co-seeding approaches.

Contact-based co-culture of human dermal fibroblasts (fibroblasts) and epidermal keratinocytes (keratinocytes) has been a long-standing and challenging issue in the field of wound and skin studies [10–19] (Table 1). Fibroblasts and keratinocytes (primary cells and cell lines), as well as cell lines, require a host of growth factors and supplements to support their *in vitro* proliferation [20–23], the concentrations and compositions of which vary across the two cell types. Animal serum (fetal bovine serum (FBS) or fetal calf serum (FCS)) contains a range of growth factors and is widely used as a supplement in cell culture media [10,12–14,17,24]. However, the use of serum poses a discrepancy in the context of fibroblast and keratinocyte co-culture. The presence of serum is required to support the proliferation of fibroblasts, which is in contrast to keratinocytes, which grow poorly or fail to attach and grow in the presence of serum, and are therefore cultivated in serum-free media [16,25–28]. To overcome this, a large number of protocols for contact-based co-culture of fibroblasts

and keratinocytes, start with growing confluent layers of fibroblasts in media supplemented with high concentrations of animal serum (up to 10%), after which keratinocytes are seeded on fibroblast layers in serum-free media [10,11,15,16,19]. In doing so, the fibroblasts serve as a cell feeder system (feeder fibroblasts may be irradiated to provide additional factors that aid keratinocyte attachment) for proliferating keratinocytes, enabling their expansion [14].

**Table 1.** Comparison of different approaches for contact-based co-culture of dermal fibroblasts and epidermal keratinocytes

| Co-culture Approaches | Serum Concentrations | Additional Growth Factors | Technical Ease | Ease of Media Formulation | Key Features | References |
|---|---|---|---|---|---|---|
| **Serum-based Approaches** | | | | | | |
| Keratinocytes layered on top of Fibroblasts (including cell feeder platforms) | ~5-10% FBS/FCS to grow fibroblast layers; certain protocols switch to serum-free media to layer keratinocytes | Present in commercial media or added individually as growth supplements | Utilizes standard cell culture techniques to layer cells; usually does not require irradiated cells or complex feeder systems | Media prepared from standard, commercially-available media components; supplements, if used, added individually | Supports co-culture of fibroblasts and keratinocytes (primary human cells and cell lines) using a layered approach | 5, 10, 16, 19, 24, 32, 33 |
| Fibroblasts co-seeded with Keratinocytes | ~10% FBS/FCS | Additional growth factors, if used, added individually | Utilizes standard cell culture techniques to co-seed cells | Standard, commercially-available media components | Variable attachment of keratinocytes depending on serum and growth factors in media used | 11, 12, 14, 17, 24 |

**Serum-free Approaches**

| | | | | | |
|---|---|---|---|---|---|
| Keratinocytes layered on top of Fibroblasts | 0% | Additional growth factors, if used, added individually | Certain protocols lethally irradiate feeder layer of fibroblasts, on which keratinocytes are seeded | Media prepared from standard media components; supplements, if used, added individually | Supports expansion of keratinocytes; if irradiated feeder cells are used, unsuitable for cell migration and wound closure studies | 24, 32 |
| Fibroblasts co-seeded with Keratinocytes | 0% | Additional growth factors, if used, added individually | Utilizes standard cell culture techniques to co-seed cells | Media prepared from standard media components; supplements, if used, added individually | Poor proliferation of HDFa cells and abnormal co-culture morphology without growth supplements | 12, 24 |

**Our Reduced-Serum Approaches**

| | | | | | | |
|---|---|---|---|---|---|---|
| Fibroblasts layered on top of Keratinocytes | 1-2% FBS to form a confluent layer of fibroblasts and to seed keratinocytes over the fibroblast layer | Present in composite media or added via mixture of growth factors | Utilizes standard cell culture techniques to layer cells; does not use irradiated cells or complex feeder systems | Composite media prepared from cell-specific media or minimal media supplemented with mixture of cell-specific growth factors; precludes addition of individual growth supplements | Supports robust attachment, proliferation and cell morphology of HDFa and HaCaT cells in co-culture; cell migration and wound closure studies possible | This study |
| Fibroblasts co-seeded with Keratinocytes | 1-2% FBS | Present in composite media or added via mixture of growth factors | Standard cell culture techniques; two cell types mixed and co-seeded | Composite media prepared from cell-specific culture media or minimal media supplemented with mixture of cell-specific growth factors; precludes addition of individual growth supplements | Supports robust attachment, proliferation and cell morphology of HDFa and HaCaT cells in co-culture; cell migration and wound closure studies possible | This study |

While they have been successful, these approaches have certain limitations (Table 1). Several protocols employ high concentrations of animal serum (up to 10% FBS or FCS). The usage of animal serum in cell culture studies is associated with a range of well-known scientific, technical and ethical limitations, [29–31], and there has been a push to develop serum-free or serum-alternative approaches [31]. Protocols that employ different media compositions for fibroblasts and

keratinocytes require the two cell types to be cultivated in a layered fashion, with keratinocytes seeded on top of fibroblasts. While this broadly resembles the structure of the wound bed, to recapitulate the entire range of cell-to-cell interactions, the simultaneous attachment, proliferation, and contact of the two cell types is desirable [4,11,12,17].

Along these lines, a recent study compared co-culture results for human dermal fibroblasts (HDFa) and immortalized human epidermal keratinocytes (HaCaT) in the presence of serum and under serum-free conditions [12]. HaCaT were either added soon after (~40 minutes) seeding HDFa (resembling co-seeding) or layered over confluent HDFa (after five days of HDFa growth). In both these approaches, the presence of serum was observed to variably influence the early attachment and proliferation of the two cell types. HaCaT added soon after seeding HDFa cells (~40 minutes), in the presence of 10% FBS (in Dulbecco's modified Eagle's medium; DMEM), displayed significantly impaired attachment to tissue culture plastic substrates; attachment of HaCaT cells was significantly higher under serum-free conditions. On the other hand, under serum-free conditions, HDFa displayed impaired proliferative capacity even after five days of culture [12]. Notably, both media conditions, with serum and serum-free, did not contain additional cell-specific growth factors.

Other serum-free approaches employ the use of cell feeder platforms and synthetic media formulations [24,32–35]. Cell feeder protocols typically lethally irradiate the feeder cells, usually fibroblasts, on which keratinocytes are seeded (Table 1). This results in growth-arrested fibroblasts, which are unsuitable for cell migration and wound closure studies. On the other hand, protocols using non-irradiated fibroblasts use media compositions that require a host of growth factors and supplements to be procured and added individually to the basal cell culture media [24].

Taken together, existing approaches for contact-based co-culture of dermal fibroblasts and epidermal keratinocytes are not only technically demanding and labor-intensive, but are also limited by differential effects of serum on the two cell types, need for complex cell feeder platforms, and material-intensive media formulations (**Table 1**).

Given this, it is necessary to develop methods that overcome these limitations, to successfully support the contact-based co-culture fibroblasts and keratinocytes, using approaches that can be leveraged to study cellular architecture, structure and functions of the wound bed.

We have formulated two reduced-serum based methods (1-2% FBS) to co-culture HDFa and HaCaT, in direct contact with each other. These approaches are simple, easy to formulate and use commercially-available media components. Using these reduced-serum approaches, fibroblasts and keratinocytes could be co-cultured by layering keratinocytes over confluent fibroblasts or by co-seeding the two cell types simultaneously. 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. These approaches can be used and adapted for a range of wound studies, as well as skin, tissue and cell engineering applications, potentially reducing concerns with high-serum formulations.

## 2. Materials and Methods

### 2.1. Cell culture and maintenance

Adult primary human dermal fibroblasts (HDFa) were obtained from PromoCell (Germany) and maintained in an all-in-one, ready-to-use Fibroblast Growth Media (FGM) (a proprietary formulation

from Cell Applications, 116-500) containing 2% 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. Imaging was performed with a 10X objective (total magnification 100X) 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 of 1X.

## 2.2. *Reduced-serum media formulations for co-culture*

To co-culture HDFa and HaCaT in the presence of reduced-serum and cell-specific growth factors, two media formulations were developed using commercially-available, cell-culture grade media components.

### 2.2.1 Reduced-serum media based on All-in-One Fibroblast and Keratinocyte Growth Media

For this formulation, 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). FGM and KGM are commercially-available, proprietary formulations, serving as an 'all-in-one' composition of required growth factors, supplements, and antibiotics, to support fibroblast and keratinocyte cell culture respectively. Notably, FGM contains 2% FBS, whereas KGM is serum-free. Media formulation was freshly-prepared prior to each use.

### 2.2.2 Reduced-serum formulation based on Minimal Essential Medium supplemented with Cell-Specific Growth Factors

For this formulation, Minimum Essential Medium (MEM) containing Earle's salts, 2 mM L-glutamine, 1mM sodium pyruvate, non-essential amino acids (NEAA) and 1.5 gms per liter 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 (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 (information from manufacturer). KGF contains bovine pituitary extract, recombinant human insulin-like growth factor-I, hydrocortisone, bovine transferrin and human epidermal growth factor, and is serum-free. Media formulation was freshly-prepared prior to each use.

## 2.3. *Co-culture of HDFa and HaCaT cells*

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

### 2.3.1 Layering approach to co-culture HDFa and HaCaT cells

For the layering approach in FGM+KGM media, HDFa cells were counted (~$10^5$ cells) and seeded in tissue-culture treated T25 flasks in FGM (2% serum), incubated at 37°C in a 5% $CO_2$ incubator 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 (~$10^5$ cells) and seeded on the confluent layer of fibroblasts. Flasks were imaged with a 10X objective (total magnification 100X) every 24 hours for five days.

For the layering technique in MEM+GF, HDFa cells were counted (~$10^5$ cells) and seeded in tissue-culture treated T25 flasks in MEM supplemented with 1X FGF, incubated at 37°C in a 5% $CO_2$ incubator and grown to 90% confluency. When confluent, the media was replaced with MEM containing both FGF and KGF (MEM+GF, 1X each, 2% serum). HaCaT cells were counted (~$10^5$ cells) and seeded on the confluent layer of fibroblasts. Flasks were imaged with a 10X objective (total magnification 100X) every 24 hours for five days. For the wound scratch assays, the layering approaches were similarly performed in 6-well plates (Figure S2).

2.3.2 Co-seeding approach to co-culture HDFa and HaCaT cells

For the co-seeding approach, HDFa and HaCaT cells were counted and ~$10^5$ cells of each type was mixed (resulting in a 1:1 ratio) in FGM+KGM or MEM+GF, and co-seeded into T25 flasks. Co-seeded cells were incubated at 37°C in a 5% $CO_2$ incubator. The T25 flasks were imaged with a 10X objective (total magnification 100X) every 24 hours for five days. For the wound scratch assays, the co-seeding approach was similarly performed in 6-well plates (Figure S2).

*2.4. Wound scratch assay on co-cultured platforms*

HDFa and HaCaT were seeded in 6-well tissue culture-treated plates under reduced-serum conditions, using the co-seeding or layering approaches, with culture and media conditions as previously described.

When the co-cultured cells reached 90% confluency, the media was replaced with fresh reduced-serum media (FGM+KGM or MEM+GF), and a scratch was made across each well using a sterile 200uL tip. Following this, the wells were imaged with a 10X objective (total magnification 100X) at 0, 4, 24 and 48 hours (three images were captured per scratch at different regions). The images were analyzed in ImageJ [36] by drawing a freehand outline of the scratch and using ImageJ tools to calculate the area within the outlined region **(Figure S2)**. 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 and Discussion**

*3.1 Reduced-serum conditions developed using commercial media components*

To co-culture HDFa and HaCaT cells, two reduced-serum media were formulated using commercially-available media components **(Figure 1B).** The first formulation consisted of specialized cell culture media for fibroblasts and keratinocytes ((Fibroblast Growth Medium (FGM) and Keratinocyte Growth Medium (KGM)) in a 1:1 ratio, referred to as FGM+KGM. FGM contains 2% FBS, resulting in a final 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 (information from manufacturer); KGF is serum-free.

Taken together, these formulations result in effective concentrations of 1% FBS in FGM+KGM, and 2% in MEM+GF, representing a 5-10 fold reduction in serum concentration as compared with previous studies (that use up to 10% serum) [10–12,14–16,19,37]. In addition, our formulations also contain cell-specific growth factors recommended for fibroblast and keratinocyte cells. In our experience, the reduced-serum formulations were simple to develop and use, using widely-available and easy to procure media components. Notably, our formulations have leveraged commercial, 'all-in-one' formulations (of media and growth factors) to develop reduced-serum concentrations and incorporate cell-specific growth factors. Previous approaches for fibroblast and keratinocyte co-culture often add serum, growth factors and supplements individually to the cell culture media, which is resource-, time-, and labor-intensive [10,11,15,16,19,24].

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

For the layering approach, HDFa cells (~$10^5$ cells) were seeded and grown to 90% confluency in tissue-culture treated T25 flasks, following which HaCaT cells were counted (~$10^5$ cells) and seeded on layers of confluent HDFa. HDFa cells were initially cultured in FGM (containing 2% FBS) or MEM+ 1X FGF (final concentration 2% FBS), where they demonstrated robust attachment, proliferation and formed confluent layers in 2-3 days, under reduced-serum conditions (**Figure 2**). This is notable, given that previous protocols employ media containing 10% FBS to grow these HDFa layers [10,11,15,16,19], and under serum-free conditions HDFa cells show poor proliferation [12]. After HDFa layers grew to confluency, HaCaT were introduced in FGM+KGM (1% FBS) and MEM+GF (2% FBS. Starting from Day 1, HaCaT cells were observed to attach on to HDFa layers under both reduced-serum conditions, FGM+KGM (1% FBS) and MEM+GF (2% FBS). As seen in **Figure 2A**, HaCaT cells proceeded to form distinct clusters on and between sheaths of HDFa cells from Day 1 (MEM+GF) or Day 2 (FGM+KGM), which continued to proliferate and increase in size. The morphology of HDFa and HaCaT co-cultured by layering under reduced-serum conditions **(Figure 2A)** is similar to that observed with previous layering approaches using 10% FBS containing media to grow HDFa layers, followed by layering keratinocytes in serum-free conditions [10,11,15,16,19]. In doing so, our formulations represent an advancement to previous protocols by enabling layered co-culture of HDFa and HaCaT cells using reduced-serum concentrations at all stages of the protocol.
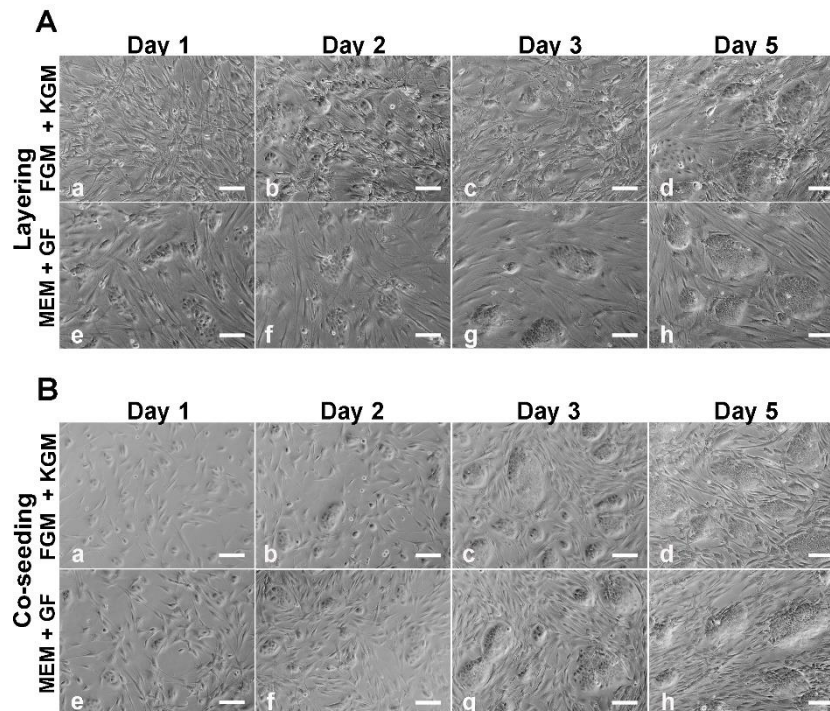
**Figure 2.** Co-culture of HDFa and HaCaT cells using layering and co-seeding (in 1:1 ratio) techniques under reduced-serum (1-2%) conditions. **(A)** When layered over HDFa, HaCaT cells form distinct clusters on HDFa cells starting from Day 1 (MEM+GF) or Day 2 (FGM+KGM), which continue to proliferate and increase in size. **(B)** When co-seeded together, under both reduced-serum conditions, HaCaT cells form distinct colonies by Day 2, which become more dense and tightly-packed over subsequent days, surrounded by sheaths of proliferating fibroblasts. Scale bars = 150 μm.

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

Given that the reduced-serum formulations supported the co-culture of HDFa and HaCaT using the layering approach, we proceeded to employ a co-seeding approach, where both cell types were seeded simultaneously.

For the co-seeding approach, HDFa and HaCaT were seeded in a 1:1 ratio (~$10^5$ cells of each cell type) in tissue-culture treated T25 flasks. 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 2B)**. 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 2B)**. This is in accordance with previous co-seeding protocols that demonstrated successful co-culture of normal human fibroblasts and keratinocytes when co-seeded at cell numbers ~$10^5$ cells of each cell type (1:1 ratio), albeit under different media conditions [11,24].

Few previous studies have reported successful co-seeding approaches to co-culture fibroblasts and keratinocytes [11,12,17]; reports have either employed media containing 10% FBS or serum-free conditions. However, when added soon after the introduction of HDFa cells (~40 min), in the presence of 10% serum, HaCaT cells displayed very poor (almost minimal) attachment [12], and under serum-free conditions, the proliferation and cell morphologies of the co-cultured cells were impaired [12].

Therefore, by supporting the co-seeding approach, our reduced-serum formulations provide a significant advancement to existing co-culture protocols.

*3.4 Reduced-serum conditions 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 were surrounded by characteristic sheaths of spindle-shaped cells HDFa cells **(Figure 2 and Figure S1A).** This characteristic co-culture arrangement was observed with both layering and co-seeding approaches and is similar to that seen in previous co-culture studies [12,17,24] (with 10% FBS or serum-free conditions with growth supplements). While we did not quantify the attachment or proliferation of each cell type serial images from Day 1 to Day 5 **(Figure 2)**, show both cell types displaying robust attachment and subsequent proliferation, in co-culture under reduced-serum conditions.

It is important to note that the reduced-serum conditions in this study include cell-specific growth factors (as components of the 'all-in-one' compositions of specialized cell media or as a mixture of growth factors added to minimal media), which, along with optimum serum concentrations, was observed to be critical to establish co-culture **(Figure S1B).** As seen in **Figure S1B**, when co-seeded 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. On the other hand, in the presence of 10% FBS alone (no growth factors), HDFa and HaCaT showed minimal proliferation, and HDFa did not form typical sheaths around HaCaT colonies. This underscores the need for optimal serum concentrations and cell-specific growth factors for successful co-culture of HDFa and HaCaT, both of which are included in the reduced-serum formulations in this study.

*3.5 Co-cultured platforms, under reduced-serum conditions, enable the study of cell migration and wound closure using wound scratch assays*

To enable the study of wound bed functions using the scratch assay, HDFa and HaCaT cells were co-cultured in tissue culture treated 6-well plates **(Figure S2A)** under reduced-serum conditions, FGM+KGM (1% serum) and in MEM+GF (2% serum). As seen in **Figure S2A**, using both layering and co-seeding approaches, HDFa and HaCaT cells showed characteristic co-culture morphology in tissue culture treated 6-well plates, which was similar to our previous results in tissue culture-treated T25 flasks (**Figure 2).**

To these co-cultured cells in 6-well plates, a sterile 200uL pipette tip was used to apply a scratch across the confluent layers. Following this, the wound area was imaged at 0, 4, 24 and 48 hours. From images for each time point, the wound area was measured (using ImageJ) and percent wound closure was calculated **(Figure S2B)**.

As seen in **Figure 3A**, in the layering approach under both reduced-serum conditions, HDFa and HaCaT cells started filling the wound gap by 4 hours, with near-complete closure of the gap by 24 hours **(Figure 3A)**. The wound gap was primarily filled by migrating HDFa cells, while HaCaT clusters were seen to increase in size and fill the wound area from the periphery. 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, mainly

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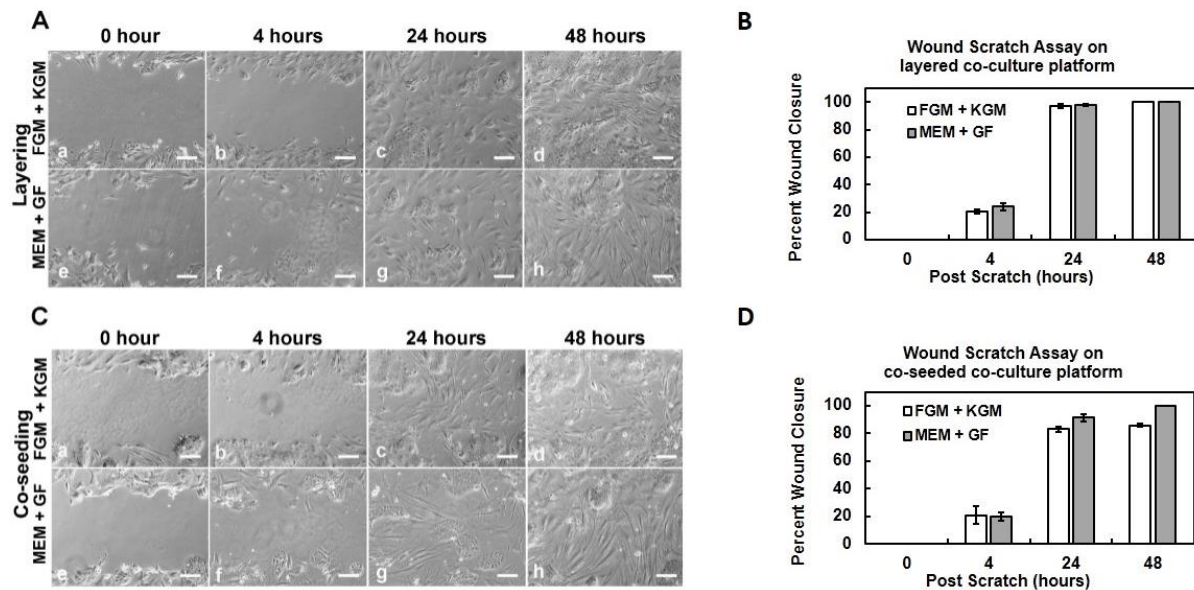
**Figure 3.** Wound scratch assay for cell migration and wound closure. Co-cultured HDFa and HaCaT cells under reduced-serum conditions (layering and co-seeding approaches), were scratched (to mimic wound injury) and wound closure was monitored. **(A and B)** In the layering approach, HDFa and HaCaT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

These results are similar to those seen in a previous study [11], where co-culture scratch assays were performed using L929 mouse fibroblasts and HaCaT keratinocytes. The two cell types were co-seeded in the presence of 10% serum, following which scratch assays were performed in 10% serum-supplemented or serum-free media. Under both conditions, L929 mouse fibroblasts migrated into the wound area prior to HaCaT keratinocytes, such that at complete closure of the wound (72 hours), the fibroblasts appeared to fill the wound area almost to the exclusion of HaCaT cells.

Taken together, HDFa and HaCaT co-cultured platforms under reduced-serum conditions support the study of wound bed functions, such as cell migration and wound closure.

## 4. Conclusion

We have developed two reduced-serum based approaches that support the co-culture of HDFa and HaCaT cells in direct contact with each other. These co-cultured systems support the robust attachment, proliferation and cell morphology of both cell types, using both layering and co-seeding techniques, and enable the study of wound bed functions such as cell migration and wound closure. These approaches are simple to formulate, easy to adopt and execute, and use commercial media components. In addition to be widely available and accessible, these media components

include necessary cell-specific growth factors, as part of their all-in-one formulation, precluding the need for procurement and addition of individual growth supplements. Finally, till serum-alternatives or synthetic cell culture media become part of routine use, these reduced-serum approaches will serve to reduce the scientific, technical and ethical limitations [29] associated with the usage of animal serum in cell culture.

To the best of our knowledge, this is the first study to report the successful direct contact-based co-culture of HDFa and HaCaT cells in the presence of reduced-serum conditions (1-2% serum). Taken together, these approaches lend themselves well for co-culture of these two cell types for wound and skin studies, tissue bioengineering and cell therapy applications.

## 5. Supplementary Figures
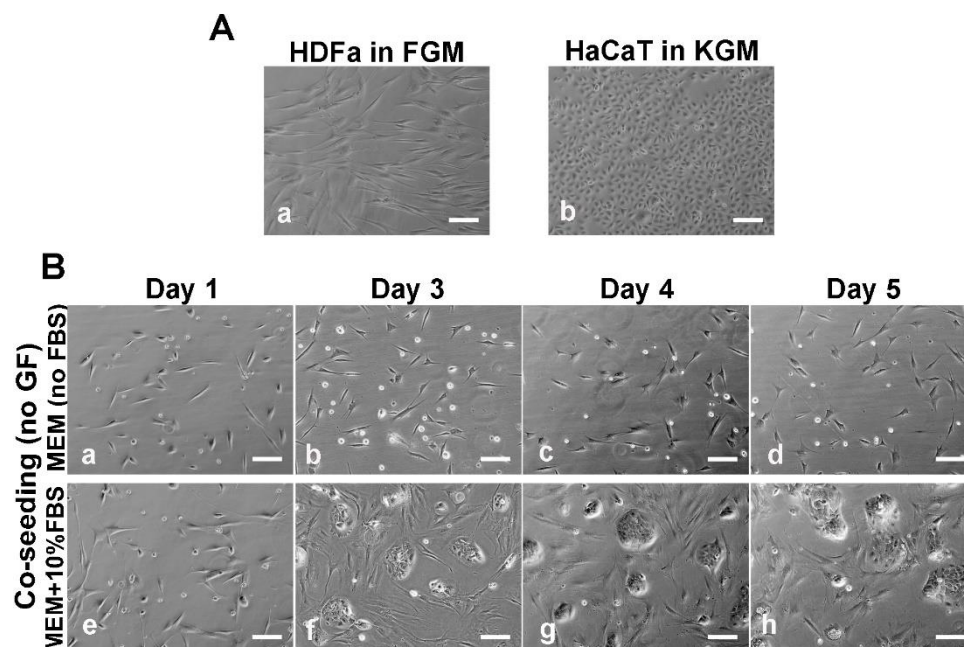
**Figure S1.** Morphology and proliferation of HDFa and HaCaT, mono-culture and co-culture, under different media conditions. **(A)** HDFa growing in Fibroblast Growth Medium (FGM) with characteristic spindle-shaped morphology and HaCaT (immortalized) growing in Keratinocyte Growth Medium (KGM) with 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 attach and proliferate. In the presence of 10% FBS (no growth factors, e-h), HDFa and HaCaT show minimal proliferation, but HDFa do not form typical sheaths around HaCaT colonies. Scale bars = 150 μm.
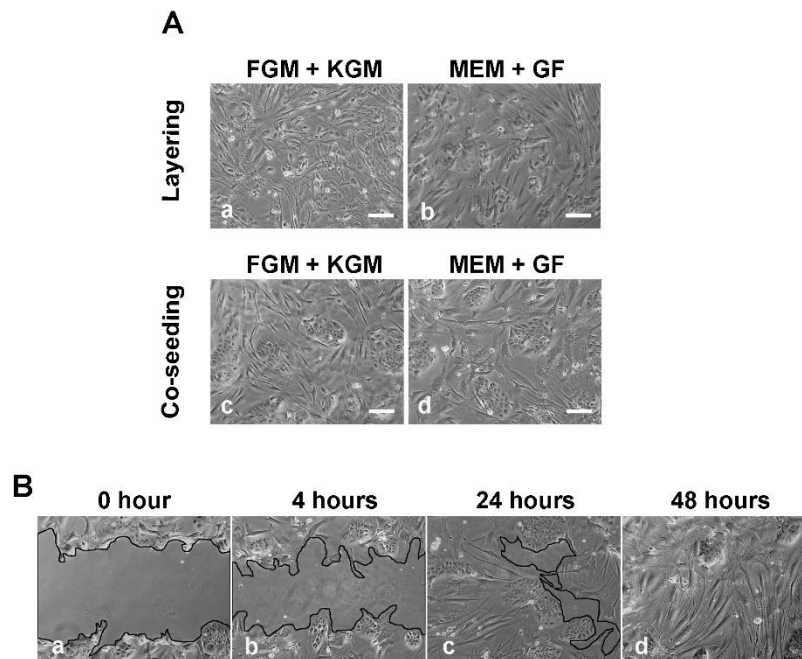
**Figure S2.** Co-culture wound scratch assay set up and measurements **(A)** HDFa and HaCaT were co-cultured under reduced-serum conditions on tissue culture-treated six well plates (for the wound scratch assay). **(B)** Representative images of the wound scratch assay for HDFa and HaCaT co-seeded co-culture 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: Morphology and proliferation of HDFa and HaCaT, mono-culture and co-culture, under different media conditions. Figure S2. Co-culture wound scratch assay set up and measurements

**Author Contributions:** SK and MV (equal contributions) designed and performed the experiments and analyzed the data. SK, MV and KSK wrote the manuscript.

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