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Original Research

Comparative functional aspects of 3T3 fibroblasts in 2D and 3D cell-culture environment

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Abstract: Tissue engineering is a versatile and valuable approach that has been widely used for constructing tissue architecture and evaluating drug efficacy, among other applications. It particularly focuses on techniques aimed at restoring or replacing parts of or entire tissues and organs. The development of in-vitro bioengineered skin models holds a significant potential for clinical applications. Most existing methods for skin bioengineering primarily rely on two-dimensional (2D) cell cultures, which present notable limitations, particularly in assessing the safety and effectiveness of topical pharmaceutical agents due to the absence of dermal-epidermal interactions. In contrast, three-dimensional (3D) skin bioengineering models enable the culture of epidermal keratinocytes in combination with fibroblasts embedded within 3D matrices, better mimicking natural skin structure and function. In this study, we designed a comprehensive experimental approach to investigate the role of Swiss 3T3 fibroblast cells, and their variants pre-exposed to a single pulse of varying doses of Mitomycin C, in supporting the growth of keratinocytes when embedded in collagen. We examined the proliferation and viability of 3T3 cells to assess their ability to promote the growth of epidermal cells within this embedded niche. Additionally, we analysed the level of paracrine secretion of Keratinocyte Growth Factor (KGF) by fibroblasts in both conditions of 2D and 3D cell culture. Furthermore, we conducted a histological comparison of the epidermal layers and evaluated the expression of various epidermal markers to elucidate their structural and functional differences in 2D and 3D culture conditions. This study demonstrates the crucial role of both 2D and 3D skin bioengineering models and their potential applications in dermatological research and pharmaceutical development.

Keywords: 2D culture, 3D culture, 3T3 feeder cells, Epidermal culture

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1. Introduction

Tissue engineering is a field dedicated to the regeneration of damaged or non-regenerative tissues and organs. Its scope is vast, encompassing a wide range of applications and employing various techniques to prepare tissues, ranging from partial replacements to entire organs [1]. Skin bioengineering has predominantly relied on 2D cell culture techniques for the purposes of skin grafting [2]. However, 2D cultures lack the essential components of the natural environment, prompting a shift towards 3D cell culture systems, which are gaining popularity due to their ability to provide a more physiologically relevant environment compared to conventional 2D cultures [3, 4]. In vitro skin culture relies on a substratum of growth-arrested 3T3 feeder cells. These fibroblasts, originally derived from mouse embryonic tissue, are widely used as feeder layers after growth arrest, providing extracellular matrix components and soluble factors that support keratinocyte attachment, survival, and proliferation.

Recent advances in cytotechnology within tissue engineering have been greatly influenced by the adoption of 3D cell culture systems [5, 6]. Cells cultured in 3D environments exhibit distinct functional behaviours concerning cell morphology, metabolism, migration, and cell division when compared to their counterparts in 2D cultures [7, 8]. While these functional differences are well-documented, the underlying mechanisms responsible for these alterations are not yet fully understood. This shift towards 3D cell culture systems has opened new avenues for enhancing our understanding of cell behaviour and tissue development, offering promising prospects for the field of tissue engineering.

However, the cell proliferation rates in 3D matrices have been observed to be slower than in 2D cultures, although the 3D environment provides a more physiologically relevant in-vivo environment. This property is attributed to the limited availability of nutrients to the cells that are minimally distributed in the 3D matrices [9]. Given these similar nutrient restrictions, it might be expected that toxic agents would have a more pronounced impact on well-dispersed cells in 2D cultures compared to those cells in 3D cultures. Various reports have indicated lower toxicities of chemical agents in 3D cultures [6, 10, 11]. Studies have shown that cell viability within 3D cultures have predominantly focused on assessing the efficacy of chemotherapeutic agents on cancer cells. These studies have consistently demonstrated that 3D cultures yield results closer to in vivo situations, in contrast to the lethal outcomes observed in 2D cultures [9]. However, the key factor driving these differential outcomes between 2D and 3D culture environments remains unclear. Interestingly, this ambiguity is the methodological concern regarding cell viability assessments in 3D cultures. Reports on 3D in vitro toxicological assays rely on colorimetric dye-based assays, which may introduce potential unreliability's due to variations in the diffusion rates of the dye through 3D matrices [12, 13]. The intricate interplay between culture dimensionality, nutrient availability, and the response to toxic agents in 2D and 3D cellular systems remains an area of active research.

In addition to considering cell viability, it is important to recognize that dermal fibroblasts are a complex cell population known to exhibit physiological variations [14, 15]. These variations arise from the presence of morphologically and physiologically heterogeneous subpopulations within the fibroblast population [16-18]. Investigating these variant subpopulations of a specific fibroblast cell type in both their original and altered physiological states, when cultured in 3D environments as compared to 2D environments, holds the potential to shed light on the regulatory aspects of culture environments [19]. By exploring the diverse facets of fibroblast physiology and subpopulation dynamics in both 2D and 3D cultures, it can provide the understanding of how culture conditions impact cell behaviour and function.

In view of these observations, this study was designed to investigate the effects of different culture conditions on Swiss 3T3 cells and their variants. In particular, we wanted to understand how these cells would behave in their original state and after exposure to different toxicants such as X-irradiation or varying doses of mitomycin C when cultured within a 3D collagen matrix. To achieve this, we analysed the cell proliferation and the decline in cell number by directly counting the viable cells after releasing them from the collagen matrix through a digestion process. This allowed us to quantify and compare the growth and survival of the cells under different experimental conditions.

Furthermore, the physiological status of fibroblasts within both 2D and 3D environments was assessed in part by measuring the levels of Keratinocyte Growth Factor (KGF), a key signaling molecule involved in interactions between fibroblasts and keratinocytes, providing an indication of growth factor support under different culture conditions. In addition to these cellular assessments, we conducted a histological comparison of the epidermal layers that were constructed under 2D and 3D conditions. The histological analysis allowed us to examine the structural and compositional differences between the epidermal tissues cultivated in these two distinct environments.

2. Materials & methods

2.1 3T3 fibroblast culture

The Swiss 3T3 fibroblast cells used in this study were procured at the 115th passage from the American Type Culture Collection (CCL-92, ATCC; www.atcc.org) and expanded for a total of 6 passages before cryopreservation by adopting a published 2-tiered banking protocol [18]. Upon retrieval from cryopreservation, the frozen working

bank vials were quickly thawed, and the cells were cultured in 3T3 medium. This culture medium contains Dulbecco's modified eagle medium (Gibco-Invitrogen) with 10% donor calf serum (HyClone) with the addition of 1.5 grams of sodium bicarbonate. The cell cultures were maintained under standard conditions involving a controlled temperature of 37°C and a humidity-controlled atmosphere with a 5% CO_2 concentration. The experimental cells were established in either T25 or T75 flasks by subculturing the working bank cells once, as mentioned in our previously published report [18]. The expanded cells were tested for confirming the absence of Mitomycin C (MC) resistance and their capacity for anchorage-independent growth prior to their use in the experiments in accordance with the detailed procedure [18]. All cultures were confirmed to be free of Mycoplasma contamination [20].

2.2 Generation of transformed clones of 3T3 cells

The generation of transformed clones was a crucial step in this study to discern the differential effects of MC-treated feeder cells prepared with transformed clones compared to normal 3T3 cells. The transformed clones of 3T3 cells were derived from a spontaneously induced transformation focus on a confluent culture (Figure 1) that was established by incubating 3000 cells per cm^2 in 3T3-FBS medium containing 10% fetal bovine serum [18]. The formation of transformation foci was subsequently expanded through subculturing of a prominent confluent focus, leading to an increase in the number of foci. This was accomplished by carefully trypsinizing and transferring the cells from the scraped foci to initiate an expanded culture. The expanded culture, derived from these cells was subjected to trypsinization and approximately 1000 cells were cultured in methylcellulose for 12 to 14 days. This culture system facilitated the growth and formation of spheroids from transformed cell. The discrete spheres formed by the transformed variants were subjected to the single sphere cloning in 24-well plates (Figure 2). Three such clone cultures, each derived from a single sphere, were further expanded by two passages before experiment in this study.

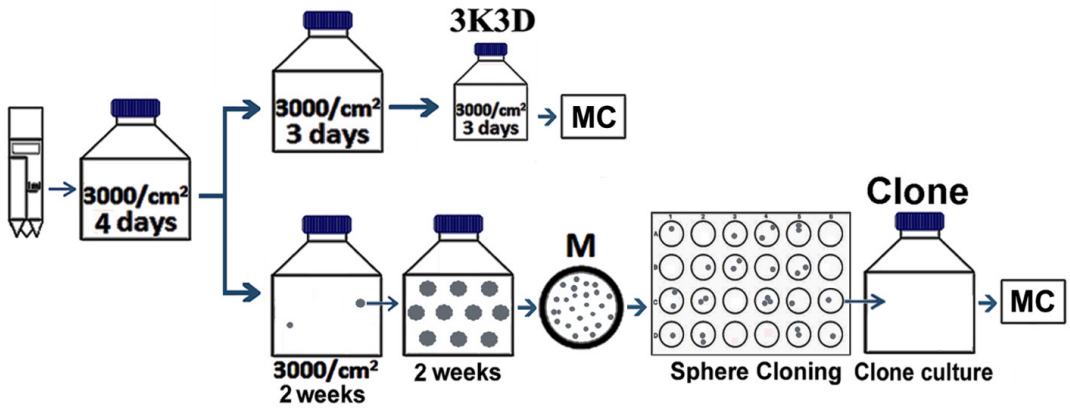

Figure 1. Swiss 3T3 clone flow chart

Notes: This flowchart outlines the experimental procedure employed to culture Swiss 3T3 cells, as well as to create transformed variants and to assess their responsiveness to Mitomycin C (MC). M: Methylcellulose, MC: Mitomycin C.

2.3 Preparation of dermal equivalent

The preparation of dermal equivalents involved mixing acid-soluble collagen (3.5 mg/mL; Millipore, catalogue No. 08-115), 10× DMEM, and reconstitution buffer (2.2 g $NaHCO_3$ and 4.77 g HEPES in 100 mL of 0.05 M NaOH) at a ratio of 8:1:1, respectively, as previously described [21]. Fibroblasts were then added to this mixture at a density of 1×10^5 cells per well for a 48-well plate or 3×10^5 cells per well for a 24-well plate. The gel was immediately polymerized at 37°C in a humidified CO_2 incubator. The dermal equivalents were prepared for the construction of the composite skin model in culture inserts of a 24-well format, which allow the composite skin model to be constructed in a structured and organized manner. While, for the periodic evaluation of cell viability after treatment with MC, the dermal equivalents were directly prepared within 48-well plate format (Figure 3a).

2.4 Induction of cell death with mitomycin C or irradiation

The experimental cells were exposed to a two-hour pulse of 3, 4, 5 and 10 g/ml of MC. In order to obtain different toxic outcomes within a single concentration, a concentration of 4 g per ml was further sub-divided

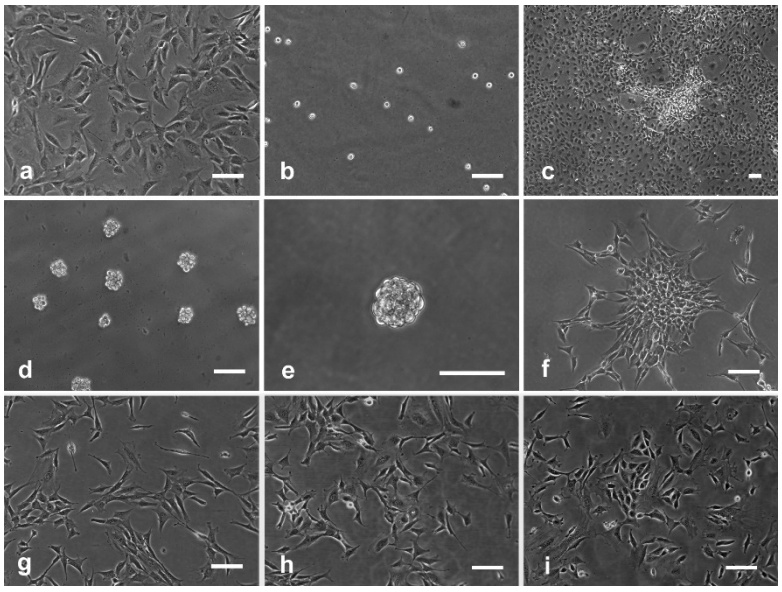

Figure 2. Establishment of control and spontaneously transformed clones.
(a). Normal Swiss 3T3 fibroblasts cells in culture; (b). no spheroids yielded by cells from normal Swiss 3T3 fibroblasts when plated in methylcellulose; (c). normal Swiss 3T3 fibroblasts cultured with 10% fetal bovine serum showing transformation foci characterized by discrete clusters of small cells with compact cell bodies and a high nucleus-to-cytoplasm ratio; (d). cells from transformed foci re-plated in methylcellulose, resulting in the formation of discrete spheres; (e). representation of single sphere; (f). single sphere cloning anchored overnight and expanded to form small colonies within three days and densely populated colonies over ten days; (g, h, i). three selected clones for the study due to their varied responses to Mitomycin C (MC) and distinct morphological differences from the control cells as shown in (a).

Note: Scale bar: 100 μm.

into doses of 15, 150, and 450 g/cell that corresponded to different volumes of MC which are specific to the previously tested permutations of concentration per se and dose per cell [22]. Each concentration of 3, 5, 10 g/ml was tested at single permutation that corresponded to doses of 10, 15 and 30 pg/cell, respectively and served as low, median, and high concentration controls for comparison. Each permutation is depicted with the concentration and the dose shown on the left and right side of a hyphen, respectively. The cells in T75 flasks were treated with a 2 hour-pulse of MC (Sigma-Aldrich Cat. No. M4287), which was prepared by proportionately diluting MC stock solution of 200 g per ml of HEPES Buffered Earl's Salt (HBES) with 3T3 culture medium to yield the desired dose permutation. A medium volume of culture medium containing HBES was used as a vehicle control. At the end of MC exposure, the cells were washed three times with PBS to remove the traces of MC and cells were trypsinized using a solution of 0.25% trypsin and 0.03% EDTA, and the viable cells were counted using the gold standard trypan blue exclusion method. For the comparative assessment of viability between irradiated (Irr) feeder cells and those treated with MC, Irr-3T3 cells (48-X, ATCC), originating from a Swiss 3T3 (CCL-92, ATCC) source culture, were employed in the study. Which allowed for the systematic evaluation of cellular responses to varying MC concentrations and doses, as well as comparisons with Irr. feeder cells.

2.5 Post-exposure 3T3 viability

2D cultures, cells exposed to MC or X-Irr were subsequently replated in triplicate wells of 24 well-plates. These cells were seeded at a density of 15,000 cells per cm2 and allowed to incubate. At specified intervals ranging from 3 days to 12 days, the cells were trypsinized for viable cells and were quantified using the Trypan Blue exclusion method.

For 3D cultures, the 3T3 cells exposed to MC were seeded in triplicate into dermal equivalents. These dermal equivalents were prepared in 48 well-plates, with each well containing cells at a density of 1×10^5 cells per ml. The cultures were then incubated, and at intervals of 3 to 12 days, the cells were detached from the collagen gel by digesting collagenase at a concentration of 2.5 mg/ml. Subsequently, the viable cells were quantified using the Trypan Blue exclusion method.

2.6 Keratinocyte culture

Primary human epidermal keratinocyte cells (Cat. No. PH10205A), frozen at the end of primary culture under

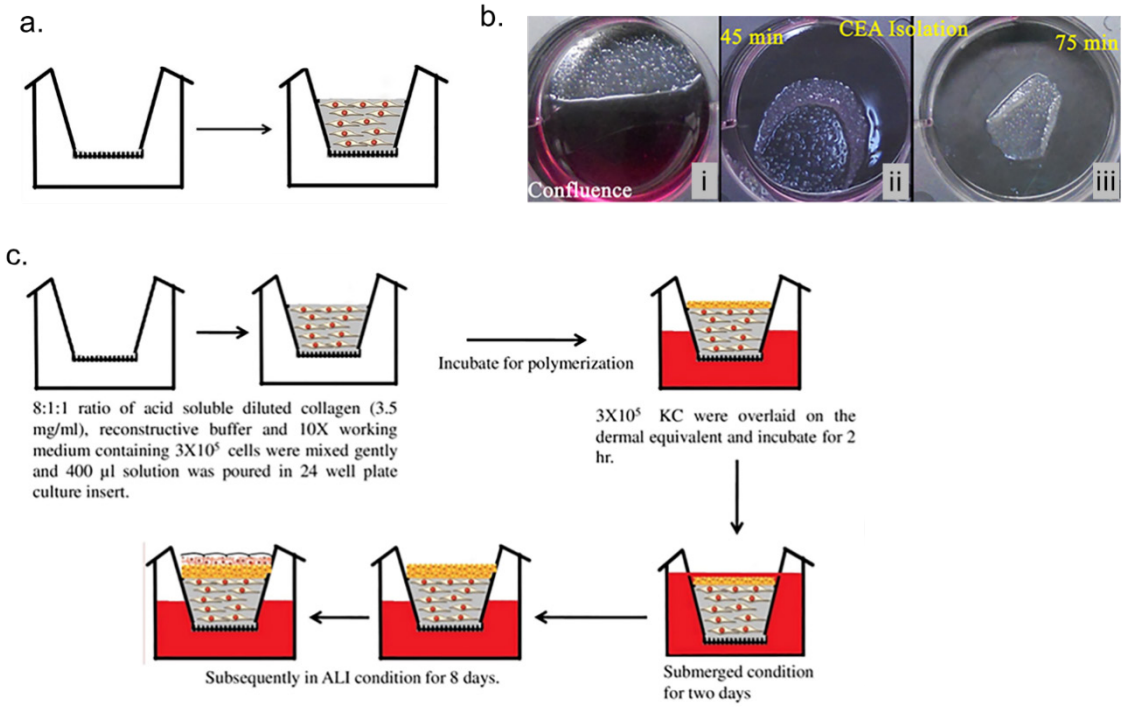

Figure 3. Representation of 2D and 3D culture system.
(a). Dermal equivalent showing MC treated 3T3 feeder cells embedded in collagen matrix;
(b). representation of Isolation of 2D culture epithelial sheet (Cultured epithelial autograft: CEA);
(c). schematic representation of generation of stratified epithelium in 3D culture.

feeder-free and serum-free conditions, were obtained from Genlantis, USA. The keratinocyte cells were co-cultured with 3T3 feeders using the Rheinwald-Green technique [23]. The culture medium for keratinocytes consisted of a mixture of DMEM and Ham's F12 in a volume ration of 1:3. This medium, supplemented with several essential components, including 10% fetal calf serum, adenine at a concentration of 1.8×10^{-4}M, transferrin at 5 µg/ml, tri-iodo-L-thyronine at 2×10^{-9}M, dexamethasone at 1 µg/ml, hydrocortisone at 0.4 µg/ml, insulin at 5 µg/ml, Cholera Toxin at 1×10^{-10}M, L-serine at 20 µg/ml, and L-glutamine at 100 µg/ml. Furthermore, EGF was added to the culture at a concentration of 10 ng/ml, on the second day after the initiation of the culture.

2.7 Generation of stratified epidermis in 2D culture

The epidermal cultures were initiated in 6-well plates by seeding 800 viable first passage keratinocyte cells per cm^2 in the presence of Irr or MC treated feeder cells at a seeding density of 15,000 feeders per cm^2. The cultures were subsequently placed in a CO_2 incubator and allowed to grow for a period of 10 days, with the culture medium being changed every other day. The stratified epithelium from confluent keratinocyte cultures was obtained by incubating in a serum-free keratinocyte culture medium containing dispase solution at a concentration of 2 mg/ml and incubated at 37°C for 50-70 minutes. The dispase solution allowed controlled detachment and collection of the stratified epitheliums, as shown in Figure 3b.

2.8 Generation of stratified epidermis in 3D culture

The composite skin model was constructed by transferring 400 µl of dermal equivalent collagen solution containing 3×10^5 fibroblast cells into culture inserts of 24-well format. After polymerization of the gel, 3×10^5 human epidermal keratinocytes were overlaid on the dermal equivalent and incubated for 2 hours. The insert was then submerged in keratinocyte culture medium for 2 days and subsequently incubated at the air-liquid interface for 8 days to allow the formation of a stratified epithelium (Figure 3c).

2.9 Histology and immunohistochemistry

The stratified cultured epidermis from 2D cultures and the composite skin from 3D cultures were fixed in 4% paraformaldehyde and embedded in paraffin. For histological analysis, 5 µm thick serial sections were stained with haematoxylin and eosin. For immunohistochemistry, serial sections were treated

in a microwave oven for 30 minutes in sodium citrate buffer (pH 6.0) at 90°C for antigen retrieval for filaggrin, cytokeratin-10, cytokeratin-14. The slides containing these sections were then allowed to cool at room temperature for 30 minutes. For involucrin staining, the sections were incubated in freshly prepared 0.1% trypsin and 0.1% $CaCl_2$ in TBS for 7 minutes at room temperature, followed by blocking with 2% normal goat serum in PBS-T for 1 hour at 37°C. The sections were then incubated overnight at 4°C in a humidified chamber with the specific primary antibody diluted in PBS-T as follows: Filaggrin (1:100; Santa Cruz Biotechnology, Inc., sc25896), cytokeratin-10 (1:100; Santa Cruz Biotechnology, Inc., sc51581), cytokeratin-14 (1:100; Santa Cruz Biotechnology, Inc., sc58724), Involucrin (1:150; Santa Cruz Biotechnology, Inc., sc21748). Negative controls were performed with their respective IgG control in PBS-T. After washing, the sections were incubated with a FITC linked secondary antibody for 1 hour at room temperature. Finally, the sections were visualized under a fluorescence microscope to assess the expression of the specific markers.

2.10 Keratinocyte growth factor assay

Keratinocyte growth factor (KGF) plays a crucial role in promoting the proliferation and survival of keratinocytes, which are the predominant cell type in the epidermis, the outermost layer of the skin. KGF is primarily produced by fibroblast cells within the skin's dermal layer. The paracrine secretion of KGF by fibroblast feeders in both the culture system was measured by ELISA. The spent culture media from both the 2D keratinocyte-feeder culture and the 3D composite skin culture were collected at 2, 4 and 6 days. These collected spent media were centrifuged at 10,000 rpm for 15 minutes to remove all any cell debris, and the resulting supernatants were carefully collected into separate tubes. Subsequently, aliquots of these samples were stored at -20°C until the ELISA test was performed.

The mouse KGF ELISA kit (Cusabio, Cat no. CSB-E13046m) was used to detect KGF in the spent medium as per the manufacturer's instruction. Briefly, 100 µl serial standard dilutions of KGF and experimental conditioned medium from both 2D and 3D cultures were added to a 96-well plate coated with anti-KGF. The plate was sealed and incubated at 37°C for 2 hours. After the incubation period, the samples were then aspirated and 100 µl of the biotin conjugated antibody was added to each well and incubated for 1 hour at 37°C. Subsequently, the wells were washed three times with wash buffer and 100 µl of a secondary polyclonal Streptavidin-HRP antibody was added and allowed to incubate for 1 hour at 37°C. Following three more washes with wash buffer, 100 µl of TMB (3,3',5,5'-Tetramethylbenzidine) was added to all the wells and incubated for 20 minutes in dark at 37°C. The reaction was halted by adding 50 µl of a stop solution, and the absorbance of each well was measured at 450 nm using a microplate reader.

2.11 Statistics

The periodic cell counts after MC treatment were converted into fold changes in the number of viable cells with reference to the number of seeded cells. Data from 2D and 3D culture systems for each cell type and treatment group are presented as line diagrams with fold change on the y-axis against post-treatment time points on the x-axis. The difference in cell viability at each time point between the two culture systems was individually tested for significance by using the Mann-Whitney Utest. This test provided a rigorous assessment of whether the observed variations in cell viability between the 2D and 3D culture systems were statistically meaningful and not the result of random fluctuations.

3. Results

3.1 Fold change in cell number in 2D and 3D culture

3.11 In control cells, post Mitomycin treated and irradiated cells

The assessment of cell number fold change in control 3T3 cells cultured in both 2D and 3D culture environments, at initial cell densities of 15,000 cells/cm² and 50,000 cells/cm² respectively, showed noteworthy variations across all assessed time points, including day 3, day 6, and day 9 ($p < 0.05$), as shown in Figure 4a. The fold change in 3T3 cell number after MC exposure with concentrations of 3, 4-15, 4-150, 4-450, 5 and 10µg/ml showed a similar overall decline in cell number in both culture groups. The cell number on day 3 in 2D cultures was significantly higher in comparison to 3D cultures in all MC concentrations except the 4-150 group, while on the subsequent day 6 and 9, there was a significant decrease in cell number in all MC concentrations except day 6 at a concentration of 4-150 and day 9 at a concentration of 5 and 10 µg/ml (Figure 4b-g).

These results highlight the significant influence of culture dimension and initial cell density on the proliferation dynamics of control 3T3 cells. These observations provide valuable insight into how MMC or irradiation responses differ in physiologically relevant 3D environments compared to conventional 2D monolayers. This has implications for interpreting preclinical cytotoxicity data, as reliance solely on 2D cultures may underestimate cell survival potential and treatment resistance in more tissue-like 3D cell environment.

Furthermore, we conducted a comparative analysis of the fold change in cell number between Irr cells in 2D and 3D cultures. Irr cells are often used as an alternative to

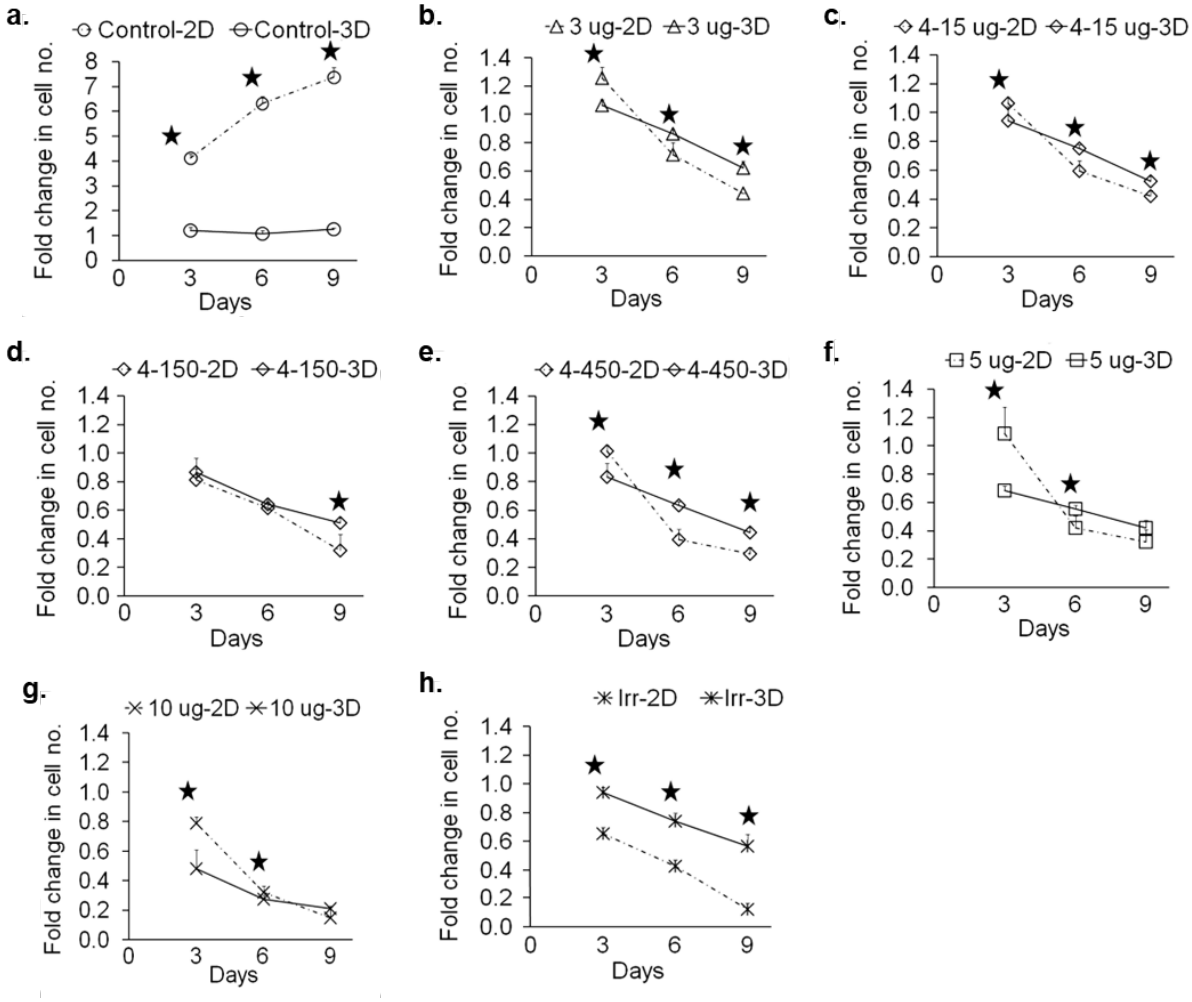

Figure 4. Fold change in cell number in control cells, MC treated cells and gamma irradiated cells.
(a). The assessment of cell number fold change in control 3T3 cells cultured in both 2D and 3D culture demonstrated significant variations on day 3, day 6, and day 9; (b-g). The fold change in 3T3 cell number after Mitomycin C exposure at the concentrations of 3, 4-15, 4-150, 4-450, 5, and 10 µg/ml displayed a consistent decrease in cell number in both 2D and 3D cultures; (h). Comparative analysis of cell number fold change between irradiated (Irr) cells in 2D and 3D cultures.

Note: Data presented as the mean ± SD. (Significant level, *: $p<0.05$).

MC-treated feeder cells in various experimental setups. Our findings revealed a significant reduction ($p < 0.05$) in cell number of Irr cells at all assessed time points. Interestingly, this reduction occurred in a parallel manner in both 2D and 3D culture environments (Figure 4h). These findings highlight the consistent impact of both Irr and MC treatment on cell proliferation, suggesting that both methods effectively inhibit cell growth. Moreover, the parallel response observed in both 2D and 3D cultures suggests that the choice of culture dimension does not significantly alter the inhibitory effect of these treatments on cell number. This comparative analysis provides valuable insights for considering the use of MC treated cells as an alternative to standard Irr-treated cells in various experimental setups.

3.12 In clone cells and post MC clone cells

In this study with clone cells and clone cells after treatment with post-Mitomycin C (MC), we identified nine different clones by cloning single cell from spontaneously transformed foci. All these identified clones exhibited notable variations in mean cell size, saturation density, and doubling time compared to control 3T3 cells. Subsequently, we subjected these clones to different doses of MC treatment and cultured them with keratinocytes to assess the resumption of feeder cell growth (data not shown). Three clones out of nine clones (clone 1, 3, and 5) displaying higher proliferation rates were selected for inclusion in this study. When examining the fold change in cell number, we found that cell numbers in 2D cultures increased rapidly over time in all three clones. In contrast, in 3D cultures, all three clones exhibited a slower rate of cell proliferation (Figure 5a-c).

Upon treatment with an MC concentration of 4 µg/ml, significant differences ($P < 0.05$) were noted in both

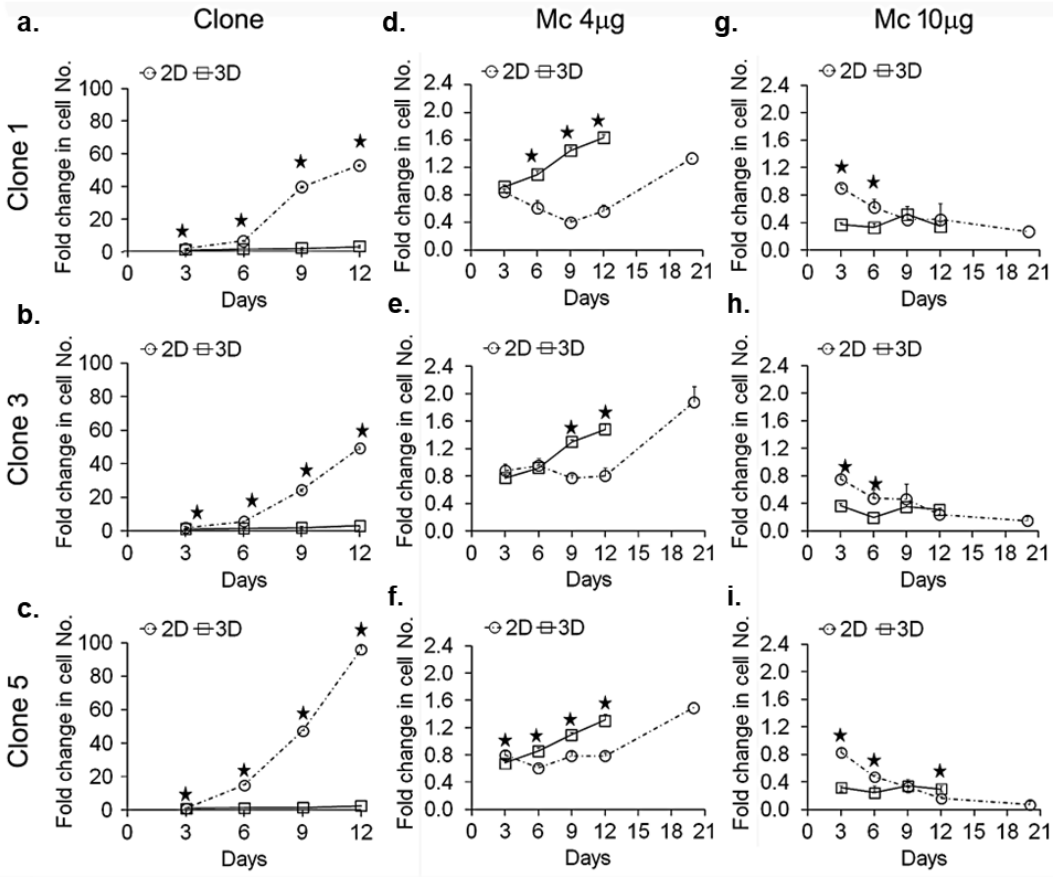

Figure 5. Proliferation pattern of selected clones in response to mitomycin C treatment in 2D and 3D cultures. (a-c). Proliferation of clones: Three selected clones (clone 1, clone 3, and clone 5) were assessed for cell number fold change in response to MC treatment; (d-f). Proliferation of clones after treatment with 4 µg/ml MC concentration showed significant differences in both 2D and 3D cultures; (g-i). Proliferation of clones after treatment with 10 µg/ml MC concentration resulted in significant differences on different days.

Notes: In 2D cultures, increasing time led to rapid cell proliferation in all three clones.
However, in 3D cultures, a slower rate of cell proliferation was observed.
Data presented as the mean ± SD. (Significant level, *: $p<0.05$).

2D and 3D cultures. In 3D cultures, the cell numbers continued to increase in all three clones, while in 2D cultures, the cell numbers initially decreased in the case of clone 1 until day 9, followed by an increase in subsequent days. For clones 3 and 5, the cell numbers remained relatively constant until day 12 and then increased rapidly (Figure 5d-f). This suggests that an MC concentration of 4 µg/ml may not be sufficient to completely arrest mitotic activity in all clones tested in this study. Furthermore, treatment with a 10 µg/ml MC concentration resulted in significant differences ($P < 0.05$) on day 3 and 6 for clones 1 and 3. In the case of clone 5, significant differences were observed on day 3, 6, and 12 only. In 2D cultures, the cell numbers steadily declined from day 3 until day 21, while 3D cultures maintained a more stable cell number (Figure 5g-i).

These findings highlight the complex and clone-specific responses to MC treatment in both 2D and 3D culture environments. The observed differences in proliferation dynamics highlight the significance of the chosen treatment concentration and the influence of the culture dimensionality on cell behaviour.

In accordance with our findings, a boxplot was generated by combining all the data points from all clones in both 2D and 3D culture conditions separately. This analysis revealed a statistically significant increase in the total fold change in cell number in 2D cultures compared to 3D cultures (Figure 6a). Interestingly, when these clones were exposed to treatment with a concentration of 4 µg/ml of MC, 3D cultures provided a homeostatic environment for MC-treated cells. This environment effectively slowed the rate of decline in cell number, resulting in a significantly higher fold change in cell number from day 6 onwards (Figure 6b). On the other hand, when a higher MC concentration (10 µg/ml) was used to treat the cells, significant differences were observed on days

3 and 6, but no significant differences were noted from day 9 onwards (Figure 6c). These findings suggest that in instances where cellular homeostatic control is disrupted during transformation, the surrounding environment can provide a degree of homeostasis. However, it is essential to understand that if this dysregulation is not subsequently controlled, it may lead to the development of a neoplastic phenotype. These findings underscore the critical role of the microenvironment in influencing the behaviour of transformed cells and offer insights into the dynamics of cell responses to varying concentrations of MC, which shed light on potential implications of MC for cancer research and therapeutic approaches.

3.2 Histology analysis of 2D and 3D epidermal culture

Histological examinations of cultured epidermis revealed the distinct structural differences between the 2D and 3D culture systems. In the 2D culture system, it showed a well-organized basal cell layer with minimal stratification (Figure 7a, b). However, in the 3D culture system, a more complex multi-cell layered structure developed, comprising basal, granular, and stratified layers. These stratified layers of the cultured skin developed in 10 days of the experimental period (Figure 7c-d). These histological findings emphasize the profound influence of culture dimension on the development and organization of cell layers. The 3D culture system appeared to better recapitulate the multi-layered structure seen in native tissue, which is of considerable relevance when considering tissue engineering and modelling approaches. These results highlight the importance of selecting an appropriate culture system to mimic in-vivo tissue characteristics and provide valuable insights into the morphological changes that occur in different culture environments over time.

3.3 Immunohistochemistry of 2D and 3D epidermal culture

The immunohistochemistry analysis of the cultured epidermis in both 2D and 3D culture showed that cytokeratin-10, a structural component found predominantly in the suprabasal layer of keratinocyte, exhibited positive staining in the 3D epidermal culture, while no such positive reaction was detected in the 2D epidermal culture (Figure 7b). Interestingly, cytokeratin-14, a marker indicative of the basal layer, showed positive staining in both the 2D and 3D culture systems, highlighting the structural similarity of the basal layer in both culture conditions (Figure 7b). The involucrin, which is a marker, associated with epidermal differentiation, exhibited positive staining particularly in the 3D cultured epidermis, whereas it was conspicuously absent in the 2D cultured epidermis (Figure 7b). While Filaggrin, a marker for epithelial keratinization, showed no reactivity in either 2D or 3D cultured epidermis (Figure 7b). The nuclei were visualized using DAPI staining, and FITC-tagged secondary antibodies were used that bind to their respective primary antibodies (Figure 7b). Notably, the expression of specific markers provided valuable insights into the structural and functional prosperities of the cultured epidermal tissues.

These findings collectively suggest that the 3D epidermal culture model, in contrast to the 2D culture system, provides a more comprehensive and realistic characterization of the epithelial markers, which encompass both the basal and suprabasal layers as well as the markers of differentiation. This heightened fidelity to normal human skin properties makes the 3D epidermal culture a superior option for many applications. However, it is important to note that practical considerations such as cost and time constraints may sometimes make the 2D epidermal culture a more practical choice, particularly in the context of applications such as burn research.

3.4 Keratinocyte growth factor secretion estimation

The expression of KGF, a paracrine growth factor that is crucial for promoting epithelial cell growth, exhibited significant differences between the spent medium of 2D and 3D cultures. Specifically, KGF expression levels were notably higher in the spent medium of 2D cultures in comparison to 3D cultures. In the 2D culture system, KGF secretion levels were measured at 12.8 pg/ml, 17.4 pg/ml, and 6.13 pg/ml on days 2, 4, and 6, respectively. In contrast, the 3D culture system displayed a significantly lower KGF secretion levels, 0.9 pg/ml, 7.7 pg/ml, and 0.65 pg/ml on days 2, 4, and 6, respectively (Figure 8).

This finding that a particularly elevated KGF secretion was observed on day 4 in 2D cultures has a very important functional implications, as it plays a role in inhibiting the process of epidermal differentiation. Elevated KGF levels during this phase can slow down the differentiation process. In contrast, the lower levels of KGF secretion in the 3D culture system, especially in comparison to the air-liquid interface contribute to a more favourable environment for epidermal differentiation. All these observations highlight the complex regulatory role of KGF in the growth and differentiation of epithelial cell. This might be an important and valuable finding for understanding the factors that influence epidermal development and differentiation in different culture settings.

4. Discussion

Several published studies have suggested that the relatively low proliferation of cells within 3D collagen

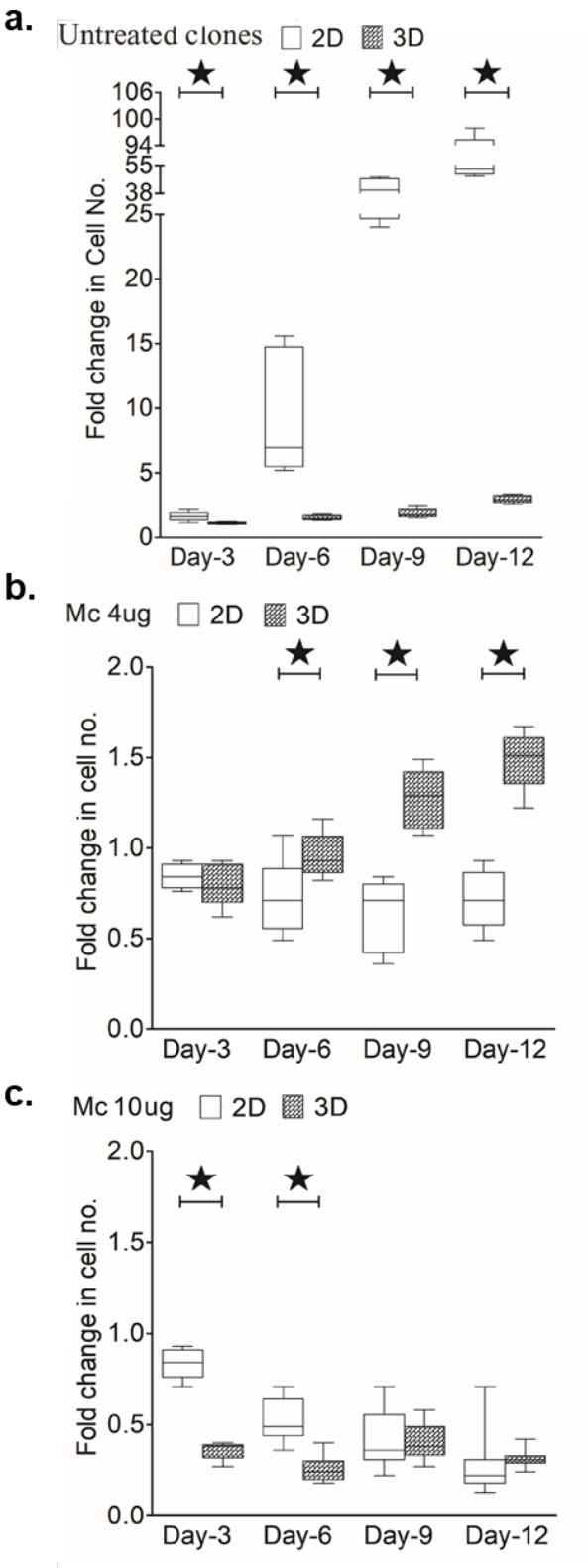

Figure 6. Comparative analysis of cell proliferation in 2D and 3D cultures in response to Mitomycin C treatment. (a). Boxplot shows a statistically significant increase in the total fold change in cell number in 2D cultures compared to 3D cultures; (b). Exposure to a concentration of 4 μg/ml MC in 3D cultures resulted in a slowing the rate of decline in cell number and leading to a significantly higher fold change in cell number from day 6 onwards; (c). Higher MC concentration (10 μg/ml), significant differences were observed on day 3 and 6, but no significant differences were noted from day 9 onwards.

Notes: To compare the effect of MC treatment on cell proliferation between 2D and 3D cultures, data from all clones were combined separately for each culture dimension.Data presented as the mean ± SD. (Significant level, *: $p<0.05$).

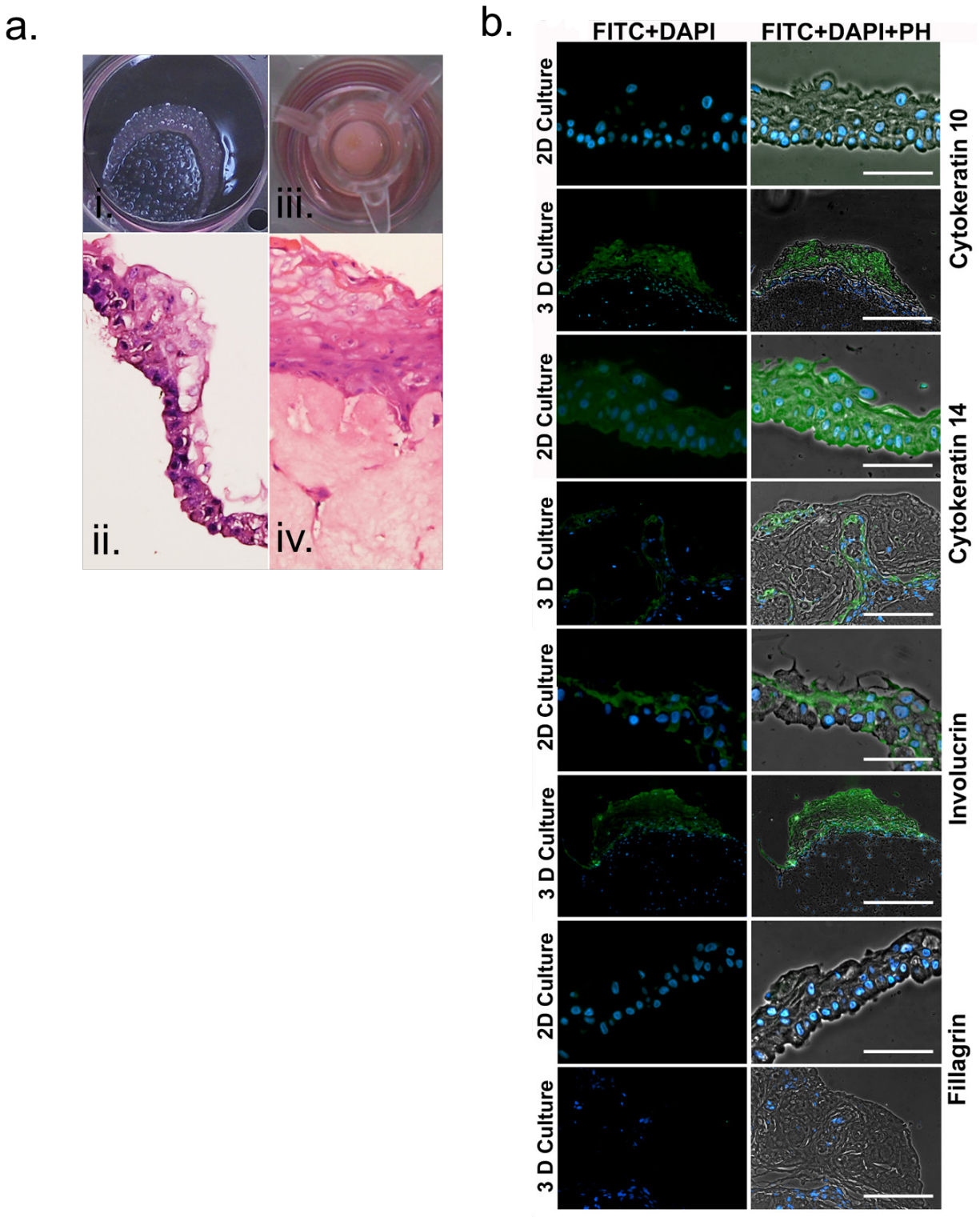

Figure 7. Histological comparison of cultured epidermis in 2D and 3D culture systems.
(a, i-iv). Cultured epidermis grown in 2D and 3D cell-culture environments with histological examination;
(b). Immunohistochemistry of cultured epidermis from 2D and 3D culture systems revealed distinct staining patterns for keratinocyte suprabasal layer marker cytokeratin-10, cytokeratin-14, involucrin, and filaggrin marker expression.

Notes: Nuclei were visualized using DAPI staining. Scale bar: 100μm.

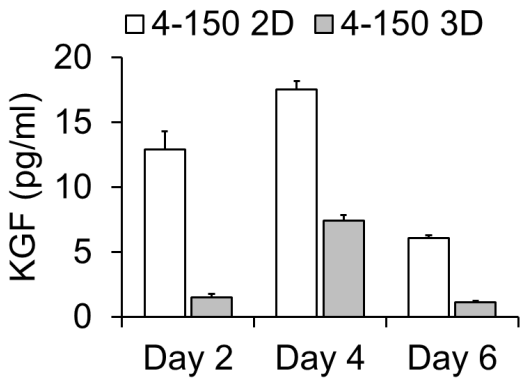

Figure 8. Expression of KGF in 2D and 3D Cultures on day 2, 4, 6.

Notes: The expression of Keratinocyte Growth Factor (KGF), a paracrine growth factor crucial for promoting epithelial cell growth, showed significant variations between the spent medium of 2D and 3D cultures. Data presented as the mean ± SD.

gels could be attributed to potential limitations in nutrient availability, reduced gaseous exchange, and the hindered removal of metabolic end products [24-26]. However, our observations challenge this assumption. Specifically, we anticipated that the extent of the decline in cell proliferation would vary between the fast-replicating clones and the slower 3K3D cells if these factors were the main causes. Surprisingly, we found that the degree of reduction in cell proliferation was comparable among the untreated cell types. Additionally, it is worth noting that the diffusion rates of various essential nutrients, dissolved gases, and metabolic end products within a collagen gel are unlikely to be significantly affected. This is primarily due to the relatively loose packing of collagen fibres within the gelated matrix, as corroborated by previous research [27]. These findings challenge the conventional assumptions regarding the factors influencing cell proliferation in 3D collagen gels. While the availability of nutrient and the removal of metabolic waste are undoubtedly important factors, our observations suggest that additional complex factors may be at play. Further research is needed to elucidate the precise mechanisms governing cell behaviour in 3D culture environments, which will contribute to a more comprehensive understanding of cell biology in such settings.

The viability of 3T3 cells within collagen I matrices has been demonstrated to exhibit a direct correlation with the concentration of collagen I itself. Notably, this relationship inversely affects the degree of gel contraction [28]. The reduction in collagen gel concentration within the matrix and the subsequent decrease in gel contraction have been identified as key factors that negatively regulate cell viability. This effect is mediated through the transmission of a mechanical stimulus to β1 integrin, a receptor protein on the cell surface. β1 integrin, in turn, plays a pivotal role in regulating Akt activity through a phosphatidylinositol-3-kinase-dependent mechanism [28, 29]. These findings emphasize the intricate interplay between collagen concentration, gel contraction, and the signalling pathways that govern cell viability. Understanding these regulatory mechanisms provides valuable insights into the dynamic interactions between cells and their extracellular microenvironment, which have significant implications for various biomedical and tissue engineering applications.

In this study, we conducted a comparative analysis of the fold change in cell number between 2D and 3D culture systems and observed significant differences in cell number dynamics between these systems. Initially, in the early days of culture (up to day 3), the overall cell number in 2D cultures was significantly higher than that in 3D cultures. However, as the experiment progressed on day 6 and day 9, we noted a substantial decline in cell numbers in the 2D culture system when compared to the 3D cultures. These observations are of particular significance in the context of 3T3 cells, as they possess the inherent potential for spontaneous transformation that may not be readily detectable in routinely passaged cultures. This property of losing contact inhibition, which serves as an indicator of cellular transformation, may not manifest prominently when transformed mutants, whether or not they exhibit distinct phenotypic characteristics or not, are present in low numbers during routine subculturing, as previously mentioned in various reports [30-32]. These findings highlight the dynamic nature of cell behaviour and transformation potential of 3T3 cells and emphasize the importance of considering both culture dimensionality and time in cellular research and experimentation. This is critical for understanding the restraints of cellular transformation and behaviour in vitro conditions.

Considering the widespread use of 3T3 cells worldwide, owing to their widespread popularity as feeder cells in cell culture applications [33-35] and their versatile utility in fundamental biological research [36], it becomes important to investigate the potential occurrence of spontaneous

transformation in these cells. The inherent possibility of unintended and frequent spontaneous transformation in 3T3 cells cannot be disregarded. Moreover, this transformation may take place surreptitiously, eluding routine detection methods. In line with this concern, there is the potential for such transformed variants to develop resistance to MC, a commonly used agent to inhibit cell proliferation. This resistance, if it is established, could lead to the contamination of the target cell cultures not only by viable but also by actively proliferating feeder cells. Therefore, it is important to conduct a vigilant monitoring and to implement quality control in cell culture procedures involving 3T3 cells. The potential for spontaneous transformation and drug resistance highlights the need for stringent protocols and ongoing surveillance to ensure the integrity and reliability of cell-based experiments and applications. Awareness of these factors is crucial for maintaining the reliability of research outcomes and the reproducibility of experimental results in tissue engineering fields.

In this study, we generated 3T3 cells transformed clones from a transformed focus derived from a confluent culture. The transformed focus was characterized by densely packed and rapidly proliferating cells. This induction was accomplished by maintaining a 7th passage of 3K3D culture at confluence for a duration of 4 weeks. The culture medium used to generate transformed focus was 3T3-FCS, supplemented with 10% fetal calf serum [31]. This approach was undertaken deliberately to derive transformed clones with specific characteristics and it is a critical component of our study.

A total of nine distinctly transformed clones were successfully isolated during our experiments. Out of nine clones, we specifically selected three clones for in this study, namely clone-1, clone-3 and clone-5. These clones were chosen based on their demonstrated resistance to mitomycin C (10 µg/ml) and their higher proliferation rate in culture. The fold change in cell number for these selected clones (clone-1, clone-3, and clone-5) showed notable differences between the 2D and 3D cultures. In 2D cultures, these clones exhibited a rapid increase in cell numbers compared to their counterparts in 3D cultures. However, upon treatment with a concentration of 4 µg/ml MC, a distinct trend emerged. In the 3D culture environment, the cell number continued to rise, in contrast to 2D cultures where the cell number increased at a slower rate. Furthermore, when these clones were exposed to a higher concentration of MC (10 µg/ml), striking differences were observed. In 2D cultures, the cell number experienced a continuous decline. In stark contrast, the 3D culture system maintained a stable cell number over time. These findings strongly suggest that 3D cultures, which more closely mimic the natural cellular environment, appear to offer a form of homeostasis for MC-treated cells. This homeostatic effect is characterized by a slower rate of decline in cell number, which is particularly evident when cells are exposed to a higher concentration of Mitomycin C. This demonstrates the significance of the 3D culture model and its potential to provide a more supportive and dynamic environment for treated cells, with implications for both research and therapeutic applications.

In addition, we conducted epidermal cultures in 2D and 3D culture systems utilizing Swiss 3T3 fibroblast cells as feeder cells, which are renowned for their ability to produce a range of growth factors that are pivotal for promoting keratinocyte proliferation [22, 37]. In the 2D culture system, keratinocytes were co-cultured together with post-mitotic 3T3 feeder layers, fostering an environment conducive to keratinocyte proliferation. In contrast, 3D culture system introduced a more intricate approach. In which, keratinocytes were seeded on top of collagen gel infused with feeder cells, simulating a three-dimensional microenvironment. This 3D culture system aimed to emulate a full-thickness skin equivalent, closely mimicking the complexities of natural skin tissue. The assessment of various key epidermal markers, such as cytokeratin 10, cytokeratin 14, and involucrin, within the 3D culture system provided compelling evidence of its heightened functionality in comparison to the 2D culture system. This observation shows that the 3D culture system is able to better reproduce the properties and functionality of natural skin tissue. This observation shows the significance of the 3D culture model as a valuable tool for studies involving skin equivalents and emphasize its potential for advancing our understanding of skin biology, as well as its applications in dermatological research, drug development, and tissue engineering.

KGF, also known as FGF7, is a crucial paracrine signaling molecule produced by viable fibroblasts. Its primary role is to facilitate the migration and proliferation of keratinocytes, a fundamental process in skin biology [38]. In this study, we estimated the secretion of KGF by analysing the spent medium collected at different time intervals. Notably, our results revealed a significant difference in KGF secretion between 2D and 3D culture systems. Interestingly, KGF secretion was significantly lower in the three-dimensional culture system compared to the two-dimensional counterpart. A plausible explanation for this divergence is the presence of the collagen matrix in the 3D culture. It is conceivable that the collagen matrix may act as a barrier and hinder the release of KGF into the spent medium. These findings are important for the dynamic interplay between extracellular matrix components such as collagen and the secretion of key paracrine factors such as KGF. Understanding these interactions is critical for unravelling the complexities of cell behaviour within various culture systems and highlights the importance of selecting the most appropriate cell culture model for a specific research.

5. Conclusion

This study highlights the importance of the dimensionality

in cell culture systems by comparing the behavior of 3T3 cells and keratinocytes in both 2D and 3D cell culture environments. Our results demonstrate that 3D collagen-based cultures not only provide a more physiologically relevant microenvironment, but also exhibit distinct differences in proliferation dynamics, resistance to Mitomycin C, and paracrine signalling compared to conventional 2D cultures. Importantly, 3D cell culture systems appear to buffer cellular responses and maintain homeostasis under stressful conditions such as drug treatment. Additionally, the enhanced expression of epidermal markers in 3D co-cultures with keratinocytes highlights the value of this model for developing skin equivalents.

Taken together, these findings emphasize that while 2D cultures remain useful for basic observations, 3D culture systems offer a superior and more biologically relevant platform for studying cell transformation, drug resistance, and tissue regeneration. This has significant implications for tissue engineering, regenerative medicine, and translational applications, where accurate modelling of the in vivo environment is critical.

Limitation of the study

While the findings of this study provide new insights into the comparative behavior of 3T3 cells and keratinocyte co-cultures in 2D and 3D collagen-based systems, several limitations must be acknowledged. One limitation of this study is the use of trypan blue exclusion after trypsinization, which may underestimate the viability of cells treated with MMC or irradiation. Although all groups were processed identically to minimize bias, future studies should incorporate other assays such as MTT, WST-1, or Alamar Blue. This study is limited by the use of in-vitro 2D and collagen I-based 3D models, which may not fully capture the complexity of the native tissue microenvironments. Furthermore, the reduced KGF secretion observed in 3D cultures could result from collagen-mediated sequestration or altered fibroblast signaling, mechanisms that were not directly investigated. Future studies incorporating multi-component extra-cellular matrices systems and in vivo validation will be essential to strengthen these findings. The integration of molecular and functional assays will be crucial to understand the mechanisms underlying spontaneous transformation, drug resistance, and paracrine signaling. In vivo validation will further enhance the translational relevance of these findings.

Authors' contributions

Conceived and designed the experiments: LKY, RMC. Performed the experiments: RMC, MC. Analysed the data: LKY RMC MC. Wrote the paper: LKY, RMC, MC. Edited the manuscript: RMC. All the authors read and approve the final version of the manuscript.

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Competing interest statement

All the authors declare that there is no competing interest related to this manuscript.

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