A Novel Fully Humanized 3D Skin Equivalent to Model Early Melanoma Invasion

David S. Hill1, Neil D.P. Robinson2, Matthew P. Caley3, Mei Chen4, Edel A. O’Toole3, Jane L. Armstrong1,5, Stefan Przyborski2, and Penny E. Lovat1

Abstract

Metastatic melanoma remains incurable, emphasizing the acute need for improved research models to investigate the underlying biologic mechanisms mediating tumor invasion and metastasis, and to develop more effective targeted therapies to improve clinical outcome. Available animal models of melanoma do not accurately reflect human disease and current in vitro human skin equivalent models incorporating melanoma cells are not fully representative of the human skin microenvironment. We have developed a robust and reproducible, fully humanized three-dimensional (3D) skin equivalent comprising a stratified, terminally differentiated epidermis and a dermal compartment consisting of fibroblast-generated extracellular matrix. Melanoma cells incorporated into the epidermis were able to invade through the basement membrane and into the dermis, mirroring early tumor invasion in vivo. Comparison of our novel 3D melanoma skin equivalent with melanoma in situ and metastatic melanoma indicates that this model accurately recreates features of disease pathology, making it a physiologically representative model of early radial and vertical growth-phase melanoma invasion. Mol Cancer Ther; 14(11): 2665–73. ©2015 AACR.

Introduction

Cutaneous metastatic melanoma remains one of the most deadly forms of cancer, with a rapidly increasing incidence, mortality, and public health burden. Although early-stage melanoma is largely curable through surgical resection, continued 5-year survival rates of only 5% to 19% for advanced disease (1) reflect the lack of consistently beneficial treatments for metastatic melanoma. Improved research models are therefore urgently needed to investigate the underlying biologic mechanisms mediating tumor invasion and subsequent metastasis, and to facilitate the development of more effective targeted therapies to improve clinical outcome.

Human skin comprises an upper epidermal layer containing mainly keratinocytes in close association with melanocytes, and a lower dermal layer containing multiple cell types, including fibroblasts that synthesize extracellular matrix (ECM) components to support cellular growth (2). Keratinocytes form a proliferative basal layer and differentiate as they move toward the surface of the skin, whereas melanocytes, the precursor cells of melanoma, proliferate less frequently and remain at the epidermal–dermal junction where they interact with basal layer keratinocytes to regulate tanning of the skin in response to UV radiation (3). A basement membrane, composed of matrix molecules, including laminin isoforms and type IV, VII, and XVII collagens, separate melanocytes and keratinocytes from the papillary dermis (4). However, when melanocytes become transformed, hyperproliferative and migratory melanoma cells invade through the basement membrane into the dermis. Therefore, models that aim to investigate early melanoma development must recreate the microenvironment of this distinct cellular niche (5).

Although mouse xenograft models of melanoma in immunocompromised mice are commonly used to investigate tumor development, progression, and therapeutic response, they do not accurately recreate the microenvironment of human melanoma at either the primary or distant site. As such, these models cannot recapitulate the initial events leading to early invasion through the basement membrane or dissemination of melanoma cells throughout the skin and to subsequent metastatic sites. Furthermore, although spontaneous mouse melanoma models (6–8) are useful for investigating the early stages of mouse melanoma development, significant differences between the architecture of human and rodent skin (9), as well as differences observed in the histopathologic features of human and murine melanoma subtypes (10) make it difficult to extrapolate results from these studies into a clinically relevant context.

To more accurately investigate early-stage human melanoma, full-thickness in vitro skin equivalent models incorporating melanoma cells have been developed, which allow investigation of melanoma migration and invasion from the epidermis into the dermis (11–14). However, such equivalents comprise a dermal component created from fibroblasts embedded in bovine or rat-tail collagen, which as well as contracting over time leading to distortion and disruption of the equivalent, are not
representative of the normal human skin microenvironment as they include nonhuman ECM components. Alternatively, although decellularized human skin models offer a human skin microenvironment, variability between donors results in inconsistent melanoma migration, which affects the reproducibility of these assays (15).

This study describes a novel in vitro model for the investigation of early melanoma invasion, such as that which occurs in radial and vertical growth phase melanoma, within a fully humanized cutaneous microenvironment. We have developed a unique full-thickness three-dimensional (3D) skin equivalent (organotypic skin culture) through the incorporation of an inert porous scaffold (16) with appropriate pore sizes to support the 3D growth and cell–cell contact of primary human dermal fibroblasts. Fibroblasts are stimulated to produce their own ECM constituents (17, 18), forming a stable dermal component that is physiologically representative of normal human skin. Following addition of primary human keratinocytes, cross-talk between fibroblasts and keratinocytes facilitates the development of a permissive microenvironment conducive to long-term culture (19). This is consistent with previous studies showing the stratum corneum of skin environment permissive for melanoma cells in their original microenvironmental niche without contact with the media but the upper surface remained exposed to the air and incubation continued at 37°C (20). Following detachment with trypsin/EDTA, keratinocytes were subsequently incubated with an equal volume of soybean trypsin inhibitor (Sigma-Aldrich) and centrifuged at 300 × g for 5 minutes prior to resuspension in fresh culture media and subsequent culture. The human metastatic melanoma cell line SK-mel-28 (LGC Standards; ATCC-HTB-72) and the primary human melanoma cell line WM35 (Coriell Cell Repositories) were obtained in 2011 and are tested every 6 months for Melan-A expression by immunofluorescence, with BRAF mutational status confirmed by real-time PCR (26), and cultured in Media A as previously described (27). All cells were cultured at 37°C in a humidified atmosphere with 5% CO2 in air.

Human skin equivalent preparation

Twelve-well format Alvetex scaffolds (Reinnervate Ltd., Reprocell Group) were pretreated with 70% ethanol in a 6-well plate according to the manufacturer’s instructions. A total of 2.0 × 106 primary human neonatal foreskin fibroblasts were seeded onto Alvetex in 100 mL Media A and incubated at 37°C in a humidified atmosphere of 5% CO2 in air for 1.5 hours. Nine milliliters of Media A + 100 mg/mL ascorbic acid (Sigma-Aldrich) were subsequently applied to the bottom of each well to gently flood the insert prior to incubation for a further 18 days, changing media every 3.5 days, to allow the formation of a dermal equivalent. Dermal equivalents were subsequently washed with 10 mL phosphate-buffered saline (PBS, Sigma-Aldrich) prior to the addition of 4 mL Media B to the outer side of the insert such that the bottom of each dermal equivalent was in contact with the media. To establish a melanoma 3D equivalent, 2.0 × 106 melanoma cells were added to the dermal equivalent in 100 mL Media B and incubation at 37°C continued for a further 3 hours. In the meantime, primary human keratinocytes were harvested by differential trypsinization, discarding the 3T3 feeder cells, and 2.0 × 106 keratinocytes seeded onto dermal equivalents (with or without melanoma cells) in 100 mL Media B and incubation continued for a further 3 hours. Five milliliters of Media B was then applied to the outer side of each well to gently flood the inside of the insert prior to further incubation at 37°C for 3 days, changing the media every day. On day 21, the insert was removed from the 6-well plate and placed into a well insert holder in a deep petri dish (Reinnervate Ltd., Reprocell Group) on the middle rung of the stand. Thirty milliliters of Media C was then added to the dish, such that the bottom of the equivalent was in contact with the media but the upper surface remained exposed to the air and incubation continued at 37°C in 5% CO2 for 14 days, changing the media every 3.5 days, to allow the formation of a full-thickness skin equivalent.

Scanning electron microscopy

Skin equivalent or primary tissue samples of normal human skin were fixed in a 1:1 mix of DMEM media and double strength fixation buffer (16% PFA, Sigma-Aldrich; 25% gluteraldehyde, Agar Scientific; 0.2 mol/L sodium cacodylate, Agar Scientific) for 5 to 10 minutes at room temperature. Samples were then transferred to a new tube and incubated in single-strength fixation buffer (8% PFA, 12.5% glutaraldehyde, 0.1 mol/L sodium cacodylate) at 4°C for 1 hour prior to washing in PBS 3 times for 5 minutes each. Samples were subsequently cut into 2- to 3-mm² squares and immersed in postfixation buffer (1% osmium tetroxide (Agar Scientific)) in 0.1 mol/L sodium cacodylate at 4°C for 60 minutes before washing in 0.1 mol/L sodium cacodylate buffer twice for 10 minutes each. Following dehydration through a series of ethanol washes (30%, 50%, 70%, 80%, 90%, 95%, and 100%)
Cutaneous Melanoma Invasion in Human 3D Skin Equivalents

Table 1. Media components for the production of full-thickness melanoma skin equivalents

<table>
<thead>
<tr>
<th>Media</th>
<th>Component (stock conc.)</th>
<th>Volume or weight</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Dulbecco’s Modified Eagle Medium</td>
<td>500 mL</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>— Fetal calf serum</td>
<td>50 mL</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>— Penicillin/streptomycin/amphotericin</td>
<td>5 mL</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>Dulbecco’s Modified Eagle Medium</td>
<td>375 mL</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>— Ham’s F12 nutrient mixture</td>
<td>125 mL</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>— Chelex-treated fetal calf serum</td>
<td>25 mL</td>
<td>—</td>
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<tr>
<td></td>
<td>— Cholera toxin (0.85 mg/mL)</td>
<td>5 µL</td>
<td>5.0 mg/mL</td>
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<tr>
<td></td>
<td>— Hydrocortisone (0.5 mg/mL)</td>
<td>400 µL</td>
<td>0.4 µg/mL</td>
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<tr>
<td></td>
<td>— Insulin (10 mg/mL)</td>
<td>250 µL</td>
<td>5 µg/mL</td>
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<tr>
<td></td>
<td>— Adenine (6 mg/mL)</td>
<td>2 mL</td>
<td>24 µg/mL</td>
</tr>
<tr>
<td></td>
<td>— Recombinant human epidermal growth factor (0.2 mg/mL)</td>
<td>50 µL</td>
<td>10 ng/mL</td>
</tr>
<tr>
<td></td>
<td>— Penicillin/streptomycin/amphotericin</td>
<td>5 mL</td>
<td>—</td>
</tr>
<tr>
<td>C</td>
<td>Dulbecco’s Modified Eagle Medium</td>
<td>375 mL</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>— Ham’s F12 nutrient mixture</td>
<td>125 mL</td>
<td>—</td>
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<tr>
<td></td>
<td>— Fetal calf serum</td>
<td>50 mL</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>— Cholera toxin (0.85 mg/mL)</td>
<td>5 µL</td>
<td>5.0 mg/mL</td>
</tr>
<tr>
<td></td>
<td>— Hydrocortisone (0.5 mg/mL)</td>
<td>400 µL</td>
<td>0.4 µg/mL</td>
</tr>
<tr>
<td></td>
<td>— Recombinant human epidermal growth factor (0.2 mg/mL)</td>
<td>50 µL</td>
<td>10 ng/mL</td>
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<tr>
<td></td>
<td>— Transferrin (00 mg/mL)</td>
<td>250 µL</td>
<td>5 µg/mL</td>
</tr>
<tr>
<td></td>
<td>— Penicillin/streptomycin/amphotericin</td>
<td>5 mL</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>— Ascorbic acid (10 mg/mL) added fresh</td>
<td>100 µL per 10 mL media</td>
<td>100 µg/mL</td>
</tr>
<tr>
<td>D</td>
<td>Dulbecco’s Modified Eagle Medium</td>
<td>500 mL</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>— Iron-fortified newborn calf serum</td>
<td>25 mL</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>— Penicillin/streptomycin/amphotericin</td>
<td>5 mL</td>
<td>—</td>
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<tr>
<td>E</td>
<td>— MCD1535 with γ-glutamine, 28 mmol/L HEPES</td>
<td>500 mL</td>
<td>—</td>
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<tr>
<td></td>
<td>— Histidine</td>
<td>18.65 mg</td>
<td>240 µmol/L</td>
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<tr>
<td></td>
<td>— Isoleucine</td>
<td>49.2 mg</td>
<td>750 µmol/L</td>
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<td></td>
<td>— Methionine</td>
<td>6.7 mg</td>
<td>90 µmol/L</td>
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<td></td>
<td>— Phenylalanine</td>
<td>7.45 mg</td>
<td>90 µmol/L</td>
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<tr>
<td></td>
<td>— Tyrosine</td>
<td>4.6 mg</td>
<td>45 µmol/L</td>
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<tr>
<td></td>
<td>— Ethanolamine (98% w/v)</td>
<td>9.775 mg</td>
<td>100 µmol/L</td>
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<tr>
<td></td>
<td>— Phosphorylethanolamine</td>
<td>3.05 mg</td>
<td>100 µmol/L</td>
</tr>
<tr>
<td></td>
<td>— Calcium chloride (1 mol/L)</td>
<td>7.05 mg</td>
<td>100 µmol/L</td>
</tr>
<tr>
<td></td>
<td>— Sodium bicarbonate</td>
<td>20 µL</td>
<td>40 µmol/L</td>
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<tr>
<td></td>
<td>— Human keratinocyte growth supplement</td>
<td>588 mg</td>
<td>15 mmol/L</td>
</tr>
<tr>
<td></td>
<td>— Penicillin/streptomycin/amphotericin</td>
<td>5 mL</td>
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NOTE: Volume or weight and final concentration of each component in Media A–E required for producing full-thickness melanoma skin equivalents (see Materials and Methods for use of each media). The suppliers of each component are indicated by superscripts: •, Scientific Laboratory Supplies Ltd.; ■, Sigma-Aldrich Company Ltd.; ●, Life Technologies Ltd.; ○, Bio-Rad Laboratories Ltd.

each for 15 minutes, samples were then dried using a critical point dryer (Balter CPD303; Pfeiffer Z1), coated in 5 nm of platinum using a Cressington Coating System 328 (Cressington Scientific Instruments) and visualized using a Leica S5200 scanning electron microscope (Leica Microsystems).

Immunofluorescent analysis of skin biomarkers

Formalin-fixed, paraffin-embedded primary human tissue samples derived from an in situ melanoma or an AJCC stage IV metastatic melanoma were used as a comparative to 3D human melanoma skin equivalents. All samples were processed for hematoxylin and eosin staining or immunohistochemistry, as previously described (28, 29). Sections (5 µm) were incubated with 1:1,000 mouse anti-human type III collagen (kindly supplied by Dr. Rachel Watson, Manchester University, Manchester, UK; Abcam; ab23445), 1:1,000 mouse anti-type IV collagen (Abcam; ab6586), 1:400 rabbit anti-type VII collagen (kindly supplied by Dr. Mei Chen, Norris Comprehensive Cancer Centre, University of Southern California, Los Angeles, CA; ref. 30), 1:1,000 rabbit anti-cytokeratin 1 (Abcam; ab93652), 1:1,000 mouse anti-cytokeratin 14 (Abcam; ab7800), 1:1,000 mouse anti-involutrin (Abcam; ab68), or 1:250 mouse anti-Melan-A (Abcam; ab731) primary antibodies diluted in PBS + 5% BSA overnight at 4°C. Primary antibody binding was detected with secondary Alexa Fluor 488 goat anti-mouse (Life Technologies) or Alexa Fluor 488 goat anti-rabbit antibodies (Life Technologies) and cell nuclei counter stained with DAPI (1 µg/mL). Sections were mounted under glass coverslips in Vectorshield mounting media (Vector Laboratories) and visualized using either a Leica DMI3000B (Leica Microsystems) or an Axioimager Z2 (Carl Zeiss Ltd.).

Results

Generation of a full-thickness human skin equivalent

Alvetex porous polymer scaffolds were used to create a full-thickness human skin equivalent in the absence of any animal matrix components (Fig. 1). Pretreatment of Alvetex by immersion in 70% ethanol rendered it hydrophilic allowing media and
cells to enter the 3D matrix. Alvetex scaffolds were subsequently washed with culture media to remove the ethanol and seeded with primary human neonatal foreskin fibroblasts, prior to culture for 18 days in Media A (Table 1) supplemented with ascorbic acid to promote synthesis of collagen polypeptides through the processing of procollagens to collagen α-chains (17). Primary human keratinocytes isolated from the epidermis of normal human skin were then seeded onto the upper surface and cultured for 3 days in Media B. The upper surface was subsequently exposed at the air-liquid interface for 14 days to induce keratinocyte differentiation, whereas the lower surface remained in contact with Media C, resulting in the formation of a full-thickness human skin equivalent.

Cell numbers, media components, and time intervals for each step of the protocol were optimized to allow full scaffold colonization by dermal fibroblasts (Fig. 2A) and the establishment of an intact, fully stratified epidermis with key morphologic features of a stratum basale, stratum spinosum, and stratum corneum (Fig. 2B, 20× magnification; Fig. 2C, 10× magnification). Electron micrographs indicate the structure and porosity of the Alvetex scaffold membrane (Fig. 2D), supporting fibroblast growth in three dimensions and facilitating the establishment of a full-thickness human skin equivalent (Fig. 2E) with clear morphologic similarities to normal human skin (Fig. 2F).

Primary human keratinocytes and fibroblasts in organotypic culture form a humanized skin microenvironment

Normal human skin comprises a dermal layer and a multilayered epidermis, each layer of which displays a distinct protein expression profile (Fig. 3A). The dermis contains extracellular matrix components, including type I and III collagens, whereas the epidermis is characterized by the expression of various cytokeratins that are differentially regulated within different layers of the epidermis, reflecting the progressive stages of normal human keratinocyte differentiation. Histologic analysis of our established 3D skin equivalent (Fig. 3B) demonstrated morphologic similarities to that of normal human skin (Fig. 3C), and a comparative commercially available model (Mattek EpidermFT; Fig. 3D); in particular, the presence of a fully developed stratum corneum was evident, indicating keratinocyte differentiation and barrier formation.

Immunofluorescent staining also revealed the expression of human type III, IV, and VII collagens, cytokeratin 1 and 14 as well as involucrin (Fig. 3), to varying degrees in the 3D skin equivalent, normal human skin, and the Mattek EpidermFT. Dermal fibroblasts contained within the 3D skin equivalent for 35 days clearly expressed type III collagen (Fig. 3T), which, albeit not as abundant as expression observed in normal human skin (Fig. 3U), nevertheless indicated the production of human extracellular matrix, critical to the long-term maintenance of the skin equivalent. In contrast, however, less human type III collagen expression was observed in the Mattek EpidermFT (Fig. 3V), likely due to their construction mainly being based on the use of bovine type I collagen that may suppress further ECM production by the dermal fibroblasts. The 3D skin equivalent model also demonstrated production of human type I collagen (data not shown).

The basement membrane components type IV and VII collagen were clearly expressed at the epidermal–dermal junction of both the 3D skin equivalent (Fig. 3N and Q) and normal human skin (Fig. 3O and I), indicating an interaction between fibroblasts and keratinocytes and synthesis of a de novo basement membrane. However, although expression of type IV collagen was partially observed between the epidermal and dermal layers within the Mattek EpidermFT model, there was no evidence for the organized expression of type VII collagen (Fig. 3P and S). It is possible, however, that the Mattek EpidermFT model may not have been cultured for sufficient time to enable type VII collagen organization and the formation of a basement membrane comparable to normal skin (31).

Expression of cytokeratin 14 by keratinocytes within the 3D skin equivalent also indicated the formation of a stratum basale (Fig. 3K), resembling that of normal human skin (Fig. 3L). Keratinocytes within the 3D skin equivalent appeared to undergo normal differentiation as demonstrated by the expression of cytokeratin 1 and involucrin in suprabasal and terminal layer keratinocytes, indicative of stratum spinosum and stratum granulosum formation, respectively (Fig. 3H and E), and again indicative of the pattern of epidermal differentiation observed in normal human skin (Fig. 3I and F). Furthermore, although expression of cytokeratin 14 (Fig. 3M) was observed in Mattek
EpidermFT, cytokeratin 1 (Fig. 3J), and involucrin (Fig. 3G) expression was less well defined, indicating formation of a stratum basale but ineffective keratinocyte differentiation in this model. The establishment of an organotypic skin equivalent on Alvetex scaffolds therefore accurately recreates the microenvironment of normal human skin. This was subsequently used to investigate melanoma cell behavior in vitro.

Melanoma cell invasion through the basement membrane of fully humanized 3D skin equivalents recreates the progressive histopathologic features of melanoma invasion in human skin.

The potential for human melanoma cell lines derived from either primary or metastatic tumors to invade the pore structure of Alvetex scaffolds was verified in the absence of primary fibroblasts (data not shown). To model melanoma invasion, metastatic melanoma cells were applied to preestablished fibroblast-containing Alvetex dermal equivalents prior to the incorporation of keratinocytes at a slightly lower ratio (100:1) to the physiologic ratio of keratinocytes-to-melanocytes in normal human skin (36:1; ref. 32), in order to prevent tumor cell overgrowth within the epidermis prior to the observation of dermal invasion. Histologic staining of a 3D skin equivalent 2 weeks after incorporation with metastatic SK-mel-28 melanoma cells demonstrated the development of melanoma nests at the epidermal–dermal junction (Fig. 4A), verified by the expression of the melanocyte lineage-specific marker Melan-A (Fig. 4E). Immunofluorescent staining for the human basement membrane components type IV collagen (Fig. 4I) and type VII collagen (Fig. 5A) 2 weeks after the incorporation of melanoma cells into the skin equivalent revealed intact expression of both markers and clear localization of melanoma cells above both type IV (Fig. 4M and Q) and type VII collagens (Fig. 5E and I). However, culture for a further 2 weeks resulted in the invasion of Melan-A–positive melanoma cells into the dermal component (Fig. 4B and F), accompanied by disruption of type IV collagen (Fig. 4J) and loss of type VII collagen (Fig. 5B). Furthermore, co-inoculating for Melan-A and either type IV collagen (Fig. 4N and R) or type VII collagen (Fig. 5F and J) demonstrated disruption of these basement membrane components coincided with melanoma invasion, indicating SK-mel-28 melanoma cells actively invade from the epidermis into the dermis of the skin equivalent through the basement membrane. Similar results were also obtained with skin equivalents incorporating Melan-A–positive primary WM35 melanoma cells, where again tumor invasion through the basement membrane, albeit less than metastatic SK-mel-28, was observed with a concurrent disruption of type IV collagen at 4 weeks (Supplementary Fig. S1).

To validate whether invasion of melanoma cells within a 3D skin equivalent accurately reflects the progressive stages of clinical disease, the effect of melanoma cells on type IV and VII collagens was investigated in a formalin-fixed paraffin-embedded in situ melanoma or in a primary tumor derived from a patient with metastatic disease. Histologic staining and Melan-A immunostaining of the melanoma in situ (Fig. 4C and G) confirmed a minimally invasive tumor accompanied by continuous and intact expression of both type IV (Fig. 4K) and type VII collagens (Fig. 5C) at the epidermal–dermal junction. Co-immunostaining demonstrated that in preinvasive melanomas, cells are located above type IV (Fig. 4O and S) and type VII collagens (Fig. 5G and K), indicating an intact basement membrane that reflects the histopathologic features observed in 3D skin equivalents 2 weeks.
after postincorporation with melanoma cells. Conversely, histology and immunostaining for the expression of Melan-A in the metastatic melanoma (Fig. 4D and H) revealed highly invasive tumor cells with disrupted type IV (Fig. 4L) and VII collagens (Fig. 5D) expression. Active invasion of this advanced metastatic melanoma, resulting in loss or disruption of type IV (Fig. 4P and
T) and type VII collagens (Fig. 5H and I), similarly reflected the histopathologic features observed in 3D skin equivalents 4 weeks after incorporation of SK-mel-28 metastatic melanoma cells. Representative photomicrographs showing hematoxylin and eosin (H&E) stained full-thickness melanoma skin equivalents (MSE) at 2 weeks (A) or 4 weeks (B) after inoculation with melanoma cells, highlighting clusters/nests of melanoma cells at the dermal/epidermal junction at week 2, which subsequently invade through the basement membrane at week 4 (black arrowheads); and H&E-stained sections of a melanoma in situ (C) or a primary superficial spreading malignant melanoma (D; invasive melanoma; black dotted lines illustrate the tumor boundary). Representative fluorescent photomicrographs for the expression of Melan-A (red; E–H) or type IV collagen (green; I–L) in 2-week (E and I) or 4-week (F and J) MSEs, melanoma in situ (G and K), or an invasive melanoma (H and L; red arrows illustrate intact type IV collagen whereas white arrows illustrate where type IV collagen is lost). M–P, overlay fluorescent photomicrographs showing relative expression of Melan-A and type IV collagen in 2-week (M) and 4-week (N) MSEs, melanoma in situ (O), and an invasive melanoma (P; note melanoma cells have invaded from right to left) with white boxes highlighting area magnified in Q–T (blue, DAPI). Q–T, 63x magnification of Melan-A and type IV collagen in 2-week (Q) and 4-week (R) MSEs, melanoma in situ (S), and an invasive melanoma (T). A–P, scale bars, 100 μm; Q–T, scale bars, 25 μm.

**Discussion**

This study demonstrates the generation of a novel full-thickness human skin equivalent containing morphologic and structural similarity to normal human skin within 35 days. We have optimized and validated a protocol for the construction of an organotypic skin model from primary human fibroblasts and keratinocytes that accurately recreates the microenvironment of normal human skin, as demonstrated by the production of human extracellular matrix component type III collagen, as well as the distinct expression profile of basement membrane proteins type IV and VII collagens, and epidermal differentiation markers cytokeratin 14, and involucrin. Incorporation of melanoma cells into their original environmental niche at the epidermal–dermal junction demonstrates that tumor cells retain their proliferative and invasive potential, forming melanoma clusters before invading through the basement membrane into the dermis.

Comparative histopathologic features observed in primary melanomas, derived from differing American Joint Committee on Cancer (AJCC) disease stages (33), confirm that the 3D skin equivalent model is physiologically representative of clinical disease. Conversely, although Mattek EpidermFT expressed type IV collagen, the lack of human type III and VII collagen expression suggests that the reduced longevity of this model will limit its use for the investigation of less-invasive melanoma cells.
Interestingly, our data demonstrate that, although invasion of both SK-mel-28 and WM35 melanoma cells through the basement membrane of the 3D skin equivalent resulted in the breakdown and disruption of type IV collagen, there appeared to be an increase in type IV collagen surrounding invading tumor cells, consistent with previous observations showing that increased type IV collagen expression parallels melanoma progression (34, 35) and which is directly required for melanoma metastasis (36). However, increased type IV collagen in this context is likely independent of its function as a basement membrane component as it does not form a continuous membrane structure. Collectively, these data support the validity of the 3D skin equivalent as a representative model of early melanoma invasion in vivo. Furthermore, because chemokines and growth factors, including IGF-1 (37), are known to drive melanoma invasion, the present model may also offer a means through which to study the effect of modulating such factors within melanoma cells on early tumor invasion.

In addition to confirming the presence of distinct skin layers within the skin equivalent, our data demonstrate the presence of regular compacted areas within the epidermis (Fig. 2B), which may represent important microenvironmental niche areas of the skin where skin stem cells may reside (38–41). Importantly, these data confer the additional potential utility of our 3D skin equivalent model for the investigation of dermal stem cell and hair follicle biology.

Furthermore, although the model presented is an allogeneic skin equivalent specifically developed for the investigation of melanoma invasion, it may be readily adapted into an autologous setting for the investigation of immunologic pathologies, or adapted through the addition of endothelial cells to the lower surface for studies of angiogenesis within the skin or development of tumor neovasculature. Grafting the 3D skin equivalent onto immunocompromised mice, in line with studies in alternative skin equivalent models (42), may also represent a useful means through which to investigate tumor cell dissemination from the skin to secondary sites.

In summary, the 3D skin equivalent model presented represents a robust and reproducible assay that is widely applicable to dermatological research, mimicking the morphology and microenvironment of normal human skin more accurately than previous assays. The demonstration of the applicability of this model for the investigation of the early stages of human melanoma invasion therefore renders it a valuable tool for defining and evaluating urgently required novel drug targets and personalized therapies.

**Disclosure of Potential Conflicts of Interest**

Stefan Przyborski is a consultant/advisory board member for and is former Director of Reinnervate. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: J.L. Armstrong, S. Przyborski, P.E. Lovat
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.S. Hill, N.D.P. Robinson, M.P. Caley, S. Przyborski, P.E. Lovat
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.S. Hill, N.D.P. Robinson, M.P. Caley, E.A. O'Toole, S. Przyborski, P.E. Lovat
Writing, review, and/or revision of the manuscript: D.S. Hill, N.D.P. Robinson, E.A. O'Toole, J.L. Armstrong, S. Przyborski, P.E. Lovat
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.S. Hill, M. Chen, S. Przyborski, P.E. Lovat
Study supervision: S. Przyborski, P.E. Lovat

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Cutaneous Melanoma Invasion in Human 3D Skin Equivalents

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