

Human Melanoma Progression in Skin Reconstructs

Biological Significance of bFGF

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Human skin reconstructs are three-dimensional *in vitro* models consisting of epidermal keratinocytes plated onto fibroblast-contracted collagen gels. Cells in skin reconstructs more closely recapitulate the *in situ* phenotype than do cells in monolayer culture. Normal melanocytes in skin reconstructs remained singly distributed at the basement membrane which separated the epidermis from the dermis. Cell lines derived from biologically early primary melanomas of the radial growth phase proliferated in the epidermis and the basement membrane was left intact. Growth and migration of the radial growth phase melanoma cells in the dermal reconstruct and tumorigenicity *in vivo* were only observed when cells were transduced with the basic fibroblast growth factor gene, a major autocrine growth stimulator for melanomas. Primary melanoma cell lines representing the more advanced stage vertical growth phase invaded the dermis in reconstructs and only an irregular basement membrane was formed. Metastatic melanoma cells rapidly proliferated and aggressively invaded deep into the dermis, with each cell line showing typical invasion and growth characteristics. Our results demonstrate that the growth patterns of melanoma cells in skin reconstructs closely correspond to those *in situ* and that basic fibroblast growth factor is critical for progression. (Am J Pathol 2000, 156:193–200)

Human skin reconstructs consist of artificial skin constructed from isolated cutaneous cell populations.¹ The reconstructs comprise a stratified, terminally differentiated epidermal compartment and a dermal compartment consisting of fibroblasts embedded in collagen.^{2–4} The

coculture of epidermal and dermal cells in a physiological context simulates human skin, and has been used as an alternative to animal testing for studies of skin barrier function,⁵ skin irritation,^{6,7} wound healing,⁸ and ultraviolet light-induced damage.^{9,10} Recently, skin reconstructs have been used for treatment of burns¹¹ and other wounds.^{12,13} Most studies with human skin reconstructs have focused on keratinocytes, whereas melanocytes have received little attention. In normal human skin, melanocytes are aligned at the basement membrane, separating the epidermis from dermis, and communicate through dendritic extensions with multiple keratinocytes to form an epidermal melanin unit.

Methods for the isolation and culture of human melanocytes^{14,15} are now routine and have been useful in studying melanocyte function. Such studies indicate the role of cell-cell and cell-matrix interactions in the regulation of melanocytic phenotype and function.^{10,16,17} In monolayer culture, human melanocytes proliferate, display a bi- or tripolar morphology, and express melanoma-associated antigens.¹⁸ However, in cocultures with keratinocytes, melanocytes more closely resemble the phenotype seen *in situ*, maintaining a constant ratio with keratinocytes, exhibiting a multidendritic morphology, and expressing no melanoma-associated antigens.^{19–22} Melanocytes in coculture are functional, and melanosomes from melanocytes have been successfully transferred to keratinocytes in a skin equivalent model.^{23,24} More recently, several skin reconstruction models have been used to investigate the biological properties of normal human melanocytes.^{9,10,25–27}

Tissue culture of melanoma cells also induces a different phenotype due to the two-dimensional growth conditions, and the biological properties of cultured melanoma cells only partially resemble those *in situ*.²⁸ Cultured cells from radial growth phase (RGP) melanoma have characteristics of both malignant and non-malignant cells: they are immortal but do not grow anchorage independently in soft agar nor are they tumorigenic in mice.²⁹ In addition, RGP cells require exogenous growth factors³⁰ for continuous growth in culture due to limited autoproduction of

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mitogens. In a typical RGP lesion, melanoma cells predominantly reside in the epidermis, with little invasion into the dermis. RGP primary melanomas are considered metastasis-incompetent, ie, they do not invade lymphatics and capillaries. Melanoma cells from the biologically advanced vertical growth phase (VGP) of primary lesions have an infinite lifespan³¹ they proliferate independently of exogenous growth factors, with the exception of insulin,³² because they produce a variety of growth factors.³³ Of these, basic fibroblast growth factor (bFGF) appears to be the most important, because inhibition of bFGF or its receptor in melanoma cells with antisense oligodeoxynucleotides inhibits their growth.³⁴⁻³⁷

In this study, we analyzed the growth patterns of melanoma cells representing different stages of tumor progression. As a model, we used human skin reconstructs with normal melanocytes, RGP and VGP primary and metastatic melanomas, which were all incorporated into the epidermis to simulate the physiological context. We found distinct growth patterns that mirrored the lesions from which the cells were originally derived, as well as a remarkable stability in the biological phenotype of melanoma cells over time in culture. With melanoma progression, there was an aberrant formation of the basement membrane and the ability of VGP and metastatic melanoma cells to invade the dermis and proliferate there. RGP melanomas survived in the dermal *in vitro* environment and were tumorigenic *in vivo* only when cells were transduced to overexpress the bFGF gene.

Materials and Methods

Tissue Culture

Keratinocytes, fibroblasts, and melanocytes were isolated from neonatal foreskins (obtained from Cooperative Human Tissue Network, Philadelphia, PA) after routine circumcision, and cultured as described.²¹ All reagents were obtained from Sigma Chemical Co. (St. Louis, MO) except for recombinant human epidermal growth factor (EGF) and bovine pituitary extract (BPE), which were obtained from Gibco-BRL (Grand Island, NY). Keratinocyte cultures were maintained in keratinocyte growth medium (KGM) composed of MCDB 153 medium supplemented with amino acids,³⁸ 0.1 mmol/L ethanolamine, 0.1 mmol/L O-phosphorylethanolamine, 5×10^{-7} mol/L hydrocortisone, 5 μ g/ml insulin, 5 ng/ml recombinant human EGF, and 100 μ g/ml BPE. Keratinocytes at passage 2-5 were used for skin reconstructs.

Human primary (RGP: WM35; VGP: WM793 and WM115) and metastatic melanoma (WM852) cells were isolated and cultured as described.²⁹⁻³¹ Sbc12 RGP-like cells were a gift of Dr. B. Giovanelli (Stehlin Foundation for Cancer Research, St. Joseph Hospital, Houston, TX). The highly aggressive melanoma cell lines 451Lu and 1205Lu were selected from lung metastatic lesions in mice after subcutaneous (s.c.) injection of WM164 and WM793 melanoma cells, respectively.^{39,40} Melanoma cells were maintained in melanocyte growth medium in the absence of EGF, phorbol ester, and BPE. All cell lines

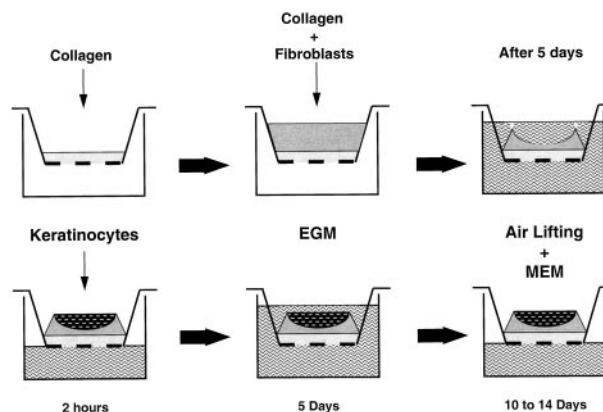


Figure 1. Schematic of *in vitro* reconstruction of human skin. Collagen is layered into inserts followed by a layer of collagen with fibroblasts. After 5 days, collagen is constricted by the fibroblasts. Keratinocytes/melanocytes/melanoma cells are added into the developing nest, grown submerged in epidermal growth medium (EGM) for 5 days, followed by air lifting and medium change to maintenance medium (MEM).

were used between 50 and 100 passages in culture except Sbc12 cells, which were at more than 200 passages. All cultures were tested twice a year for absence of mycoplasma contamination.

In Vitro Reconstruction of Human Skin and Melanoma

Reconstructs were generated using described techniques and media formulations^{3,30,41} with modifications. For dermal reconstruction, 1 ml of a cell-free buffered collagen solution consisting of rat tail collagen, type I (Collaborative Biomedical, Bedford, MA), at a final concentration of 1.35 mg/ml in DMEM with 10% FCS was added to tissue culture inserts (Transwell, Costar, Cambridge, MA) in 6-well plates (Figure 1). This precoated acellular layer was then overlaid with 3 ml of fibroblast-containing collagen (1.125×10^5 /ml). After a 5-day incubation at 37°C, fibroblasts had contracted the collagen gel, which formed a concave central area for subsequent seeding of epidermal cells. For epidermal reconstruction, the mature dermal reconstructs were rinsed and equilibrated with 2 ml of epidermal growth medium (EGM) as described.⁴¹ Minor modifications included the addition of 2% dialyzed FCS and the omission of linoleic acid. After 1 hour, EGM was removed, and the surface of the dermal reconstructs was allowed to dry. Neonatal foreskin keratinocytes (1.5×10^5 /reconstruct, in a total volume of 50 μ l) were seeded onto the concave center of the dermal reconstructs and incubated at 37°C for 2 hours to allow attachment of the seeded cells. Composite cultures were then submerged by adding 3 ml of EGM outside and 2 ml inside of the insert. Seeded keratinocytes were allowed to attach and proliferate. After 4 to 6 days of submerged culture, with regular feedings every 2 days, skin reconstructs were lifted to the air-liquid interface and medium was switched to maintenance medium⁴¹ with similar modifications described for EGM above. After 10 to 14 days of air exposure, skin reconstructs were harvested. Skin reconstructs were fixed overnight with 4% parafor-

maldehyde, dehydrated, and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E). For incorporation of melanocytes or melanomas, cells were seeded together with keratinocytes onto dermal reconstructs at a 1:5 ratio of melanocytic cells to keratinocytes. Culture conditions were the same as for reconstructs containing keratinocytes alone. Human melanoma cell lines derived from RGP (WM35, Sbc12) and VGP primary (WM793, WM115) and metastatic melanomas (451Lu, 1205Lu, WM852) were tested in the reconstructs. All experiments were performed in duplicate. For testing tumorigenicity of Sbc12 cells, 5×10^6 cells were injected s.c. into 5 SCID mice per group. Tumor growth was monitored for 4 weeks. Paraffin sections of reconstructs and tumor tissues were stained immunohistochemically with antibodies detecting type IV collagen, laminin, Ki67 proliferation marker, or S100 melanocytic markers all using standard methods. Apoptosis in paraffin sections was evaluated using an *in situ* apoptosis detection kit (ApoptTag, Oncor, Gaithersburg, MD).

Adenoviral Vectors

The adenoviral vector carrying the lacZ reporter gene (LacZ/Ad5) was obtained from the Vector Core, University of Pennsylvania (Philadelphia, PA). The adenoviral vector carrying the bFGF gene (bFGF/Ad5) has been described in detail elsewhere.⁴² bFGF/Ad5 induces in normal melanocytes, at 20 plaque forming units (PFU)/cell, at 48 hours, a biologically active 18-kd protein that is found predominantly in the cytoplasm but that can also be found in the culture supernatant. Through the use of adenoviral vectors expressing β galactosidase or green fluorescent protein we showed that, in preliminary experiments, 100% of melanoma cells were transduced after 48 hours when infected at 20 PFU/cell. Melanocytes and melanoma cells transduced with bFGF/Ad5 express the protein *in vitro* and *in vivo*.⁴²

Results

In Vitro Reconstruction of Human Skin

Human skin reconstructs resembled the architecture of skin *in situ* (Figure 2). The dermal equivalent contained extracellular matrix material (collagen) with interspersed fibroblasts. The fibroblasts were single, bipolar, and horizontally oriented, and migrated into the acellular collagen gel, forming a homogeneously structured dermis. The epidermal equivalent comprised proliferating basal cells and sequentially differentiated stratified cell layers (Figure 2A). The basal cells were vertically oriented, well-organized and cuboidal to columnar, containing oval-shaped nuclei. Numerous layers of progressively flattening keratinocytes with less prominent nuclei constituted the stratum spinosum. A stratum granulosum, approximately 3 layers thick, formed above the stratum spinosum. A stratum corneum consisting of flattened, anucleate corneocytes developed on top. Compared to human skin, skin reconstructs lacked rete ridges. When melano-

cytes were mixed with keratinocytes, they settled at the interface between dermis and epidermis (Figure 2, B and C), where they remained singly with a multidendritic morphology throughout the life span of the reconstructs of 28 to 35 days.

Reconstruction of Primary and Metastatic Melanomas

To study the biological properties of melanoma cells from different stages of progression, we incorporated seven human melanoma cell lines derived from RGP and VGP primary and metastatic melanomas into skin reconstructs. RGP melanoma cells Sbc12 formed nests within the epidermis and band-like tumor cell aggregates at the epidermal-dermal junction (Figure 2D). RGP primary melanoma cells, WM35 (Figure 2E), were disposed as individual cells and as small nests within the epidermis and at the epidermal-dermal junction. Staining for S-100 protein (Figure 2F) demonstrated the lack of competence for invasive growth into the dermis. VGP primary melanoma cells WM793 (Figure 2G) and WM115 (Figure 2H) formed nests and clusters at the epidermal-dermal junction, and exhibited invasive growth into the dermis as demonstrated by staining for S-100 protein (Figure 2I). Whereas WM793 VGP melanoma cells invaded the dermis in clusters with only a few individual cells preceding the cluster, WM115 cell invaded the dermis more individually.

When metastatic melanoma cells were incorporated into skin reconstructs, they displayed rapid proliferation and aggressive invasive growth deep into the dermis. As shown in Figure 2J, 451Lu metastatic melanoma cells formed tumor cell clusters at the epidermal-dermal junction, and exhibited growth of tumor cell nests in the dermis. Figure 2K reflects that 1205Lu metastatic cells were disposed as tumor cell nests at the epidermal-dermal junction and showed vertical invasive growth of tumor cell strands deep into the dermis. WM852 metastatic cells (Figure 2L) also displayed rapid vertical growth of individual tumor cells deep into the dermis.

Basement Membrane Development

Staining for type IV collagen (a component of the lamina densa) of normal human skin reconstructs revealed linear deposition along the epidermal-dermal junction (Figure 3A), indicating that in a physiological context, keratinocytes and fibroblasts synthesize and deposit basement membrane proteins *in vitro*. Laminin was also deposited in the basement membrane zone, with diffuse and weak staining in the epidermis (not shown). When RGP primary melanoma WM35 were seeded together with keratinocytes, they produced collagen type IV within the epidermal nests (Figure 3, B and C). At the same time, a seemingly intact basement membrane was formed. Seeding of VGP primary melanoma cells WM115 together with keratinocytes resulted in epidermal and dermal growth (Figure 3, D and E). The basement membrane was irregular and collagen type IV was scattered throughout the lesion. When metastatic melanomas were

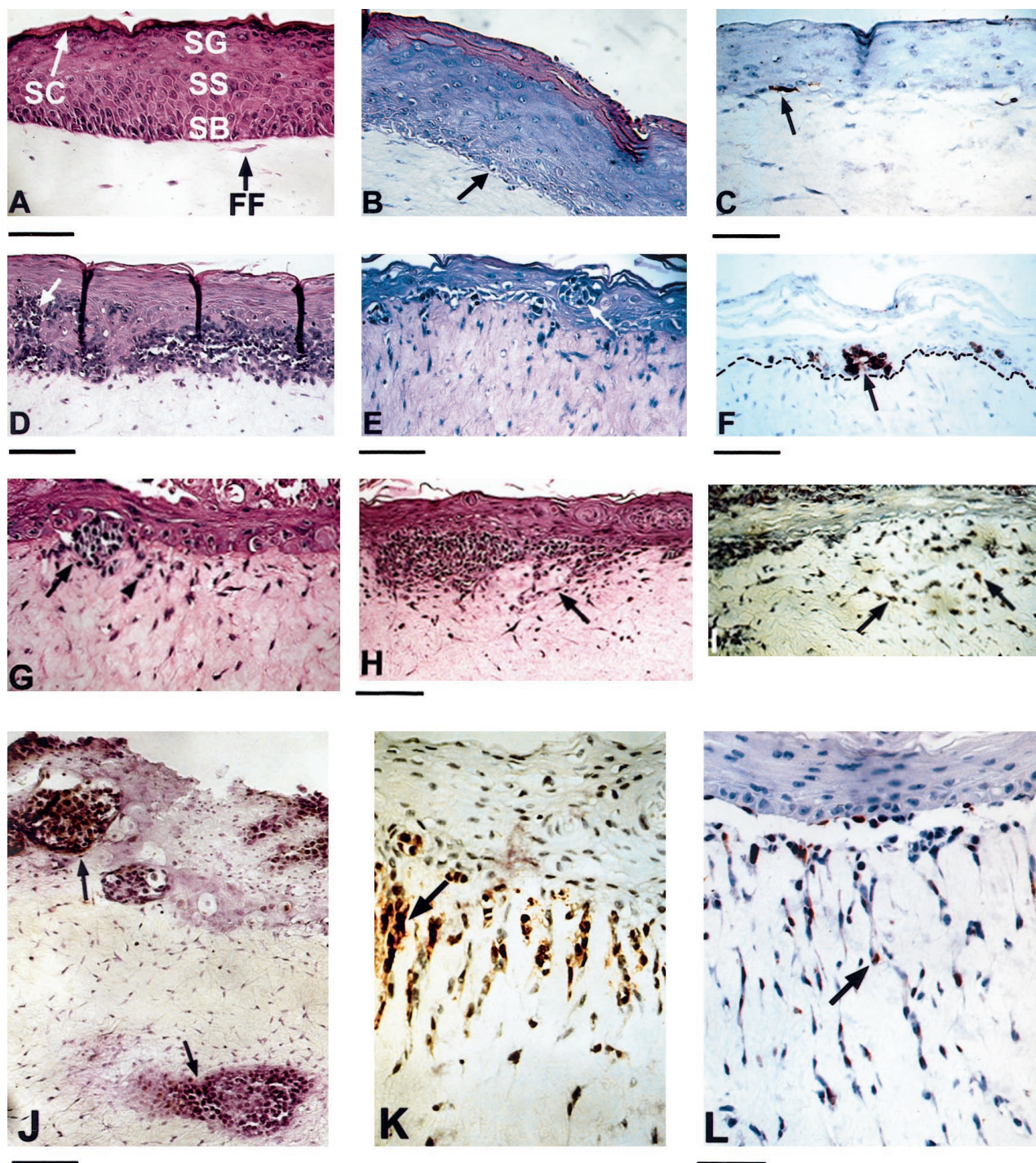


Figure 2. Characterization of human skin reconstructs. Reconstructs containing no melanocytic cells (**A**), normal melanocytes (**B** and **C**), or RGP (**D-F**), VGP (**G-I**), and metastatic (**J-L**) melanoma cells. **A:** H&E staining of reconstructs containing keratinocytes as the only cellular component in the epidermis. Keratinocytes form the typical epidermal structure, consisting of proliferating basal cells and sequentially differentiated stratified cell layers. SB, stratum basale; SS, stratum spinosum; SG, stratum granulosum; SC, stratum corneum; FF, fibroblast embedded in collagen. Bar, 17.2 μm . **B:** H&E staining of skin reconstruct with fibroblasts, keratinocytes, and melanocytes. Bar, 9.9 μm . Melanocytes (**arrow**) are singly located within the stratum basale and maintain a ratio of 1:5 to 1:10 with basal keratinocytes. **C:** Staining of melanocyte (**arrow**) for S-100 protein. Bar, 14.85 μm . **D:** H&E staining of melanoma reconstructs containing RGP melanoma cells Sbc12. Melanoma cell nests within the epidermis and band-like melanoma cell aggregates at the epidermal-dermal junction. **Arrowhead** indicates apoptotic bodies in melanoma cells. Bar, 23.7 μm . **E:** H&E staining of RGP melanoma cells WM35, which form nests in the epidermis (**arrow**). Bar, 16.95 μm . **F:** Staining of WM35 melanoma cells for S-100. The broken line highlights the basement membrane. **G:** H&E staining of VGP melanoma WM793 in skin reconstructs. **Arrow** indicates melanoma cell cluster at the epidermal-dermal junction. **Arrowhead** shows individual melanoma cells infiltrating the dermis. Bar, 34.4 μm . **H:** H&E staining of reconstruct with VGP melanoma WM115 cells. **Arrow** indicates a melanoma cells infiltrating the dermis. Bar, 12.25 μm . **I:** Staining of WM793 for S-100 expression. Numerous melanoma cells infiltrate the dermis (**arrows**). Bar, 17.1 μm . **J:** S-100 staining of reconstructs of metastatic melanoma 451Lu cells. Melanoma cells form clusters (**arrows**) within the epidermis, at the epidermal-dermal junction, and in the dermis. Bar, 11.25 μm . **K:** S-100 staining for metastatic 1205Lu cells. **Arrow** indicates vertical invasive growth of melanoma cell strands deep into the dermis. Bar, 11.25 μm . **L:** Staining of metastatic WM852 reconstructs. **Arrow** shows aggressive vertical invasive growth of individual melanoma cells deep into the dermis. Bar, 11.1 μm .

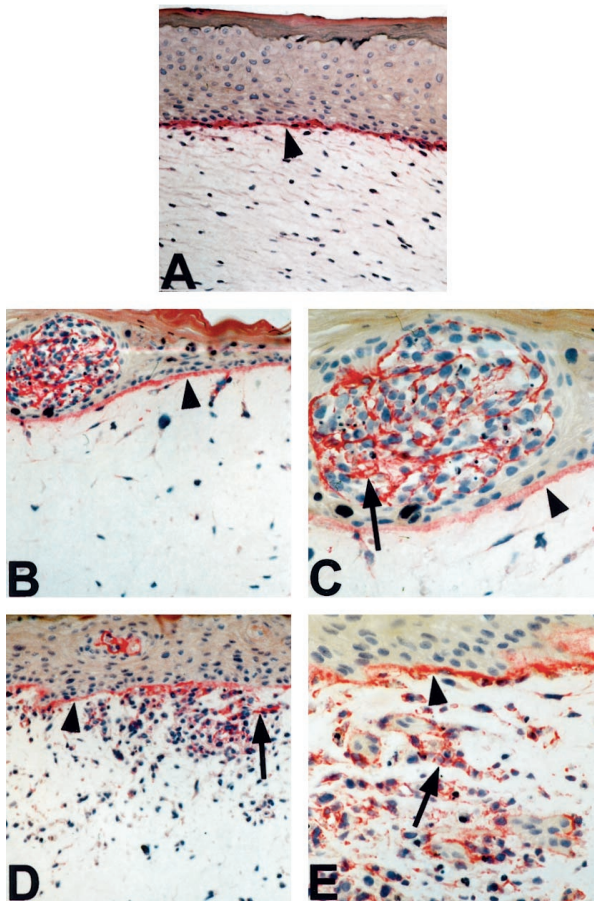


Figure 3. Presence of collagen type IV in reconstructions containing melanocytic cells at different stages of tumor progression. **A:** Normal skin reconstructions. Linear deposition of collagen type IV (arrowhead) in the basement membrane zone at the dermo-epidermal junction. Magnification, $\times 50$. **B:** Deposits of collagen type IV with an epidermal nest of RGP melanoma WM35. Arrowhead indicates the basement membrane. Magnification, $\times 50$. **C:** Same reconstruct as **B**. Magnification, $\times 100$. **D:** Collagen deposits surrounding VGP melanoma WM115 (arrow), which have invaded the basement membrane (arrowhead). Magnification, $\times 50$. **E:** Same reconstruct as **D**. Magnification, $\times 100$.

seeded together with keratinocytes, collagen type IV distribution was similar to VGP melanomas within and surrounding tumor cell nests.

bFGF Production Supports RGP Primary Melanomas in Dermis and in Vivo

RGP melanomas either cannot penetrate the basement membrane for migration into the dermis (see Figure 2, D-F), or on entering the dermis they undergo apoptosis.⁴ Because bFGF is a major growth and survival factor for melanocytes and melanomas, we determined whether bFGF could induce proliferation of RGP melanoma cells in the dermis in the absence of an overlying keratinocyte layer. Sbc12 transduced with the control vector LacZ/Ad5 and then embedded together with fibroblasts remained singly distributed in the dermal matrix (Figure 4A), and the cells did not proliferate (Figure 4C). Parental Sbc12 cells or cells transduced with control do not produce bFGF as determined by immunostaining and Western

blotting.⁴² On the other hand, when the melanoma cells were transduced with the bFGF gene before incorporation into reconstructions, they expressed bFGF and formed small nests within the matrix (Figure 4B). The bFGF-overexpressing Sbc12 cells also migrated outside of the dermis and adhered to its surface, where they proliferated and formed nests (Figure 4D). The Sbc12 cells transduced with bFGF were also tumorigenic when injected s.c. into SCID mice, whereas LacZ transduced control cells were not. The control Sbc12 cells all died within 4 days after injection and the injected cells were no longer detectable. On the other hand, bFGF-transduced cells developed into palpable lesions within 10 days. After 14 days a tumor nodule had formed (Figure 4E) with numerous cells expressing the proliferation marker Ki67 (Figure 4F). Due to the transient nature of adenovirus-induced gene expression, tumors regressed after 3 to 4 weeks.

Discussion

The clinical and histological features of melanoma development and progression have been well described,⁴³ and a sequence of steps has been proposed: common acquired nevi, dysplastic nevi, RGP and VGP primary melanomas, and metastatic melanomas. Due to the easy accessibility of the lesions and recent advances in culture techniques, cell lines from various stages of tumor progression are available in culture and have been extensively characterized.⁴⁴ The biological properties of cultured melanocytic cells resemble those *in vivo*, but typical characteristics of cultured cells such as growth factor requirements, anchorage-independent growth, growth factor production and infinite life span cannot be directly related to *in vivo* growth. The antigenic profile of VGP primary and metastatic melanoma cells is comparable between cultured cells and those *in situ* but the profile of melanocytes and RGP cells is very different.^{21,22,45} Cultured tumor cells also acquire chromosomal abnormalities such as mutations or deletions in the *p16* gene,⁴⁶ which could make it difficult to interpret results obtained with cell lines. Our model represents a new tool for the *in vitro* characterization of cell lines from different stages of progression that mimics biological properties *in vivo* better than previous attributes. In skin reconstructions consisting of artificial dermis and epidermis, melanocytic cells from different stages of progression show remarkable consistency in their growth and migration patterns, as would be expected from observations in normal skin and patients with melanoma. Normal melanocytes home to the basement membrane, where they are placed singly within the basal keratinocyte without apparent proliferation.^{23,24} The reconstructions confirm clinical observations that RGP primary melanomas proliferate predominantly in the epidermis, whereas VGP melanomas grow invasively into the dermis. Metastatic melanomas aggressively invade both epidermis and dermis. Within each group, cell lines show individual growth patterns, confirming the unique nature of each lesion and the derived cell line. The reconstruction of melanoma also confirms our previous *in vivo* studies, in which melanoma

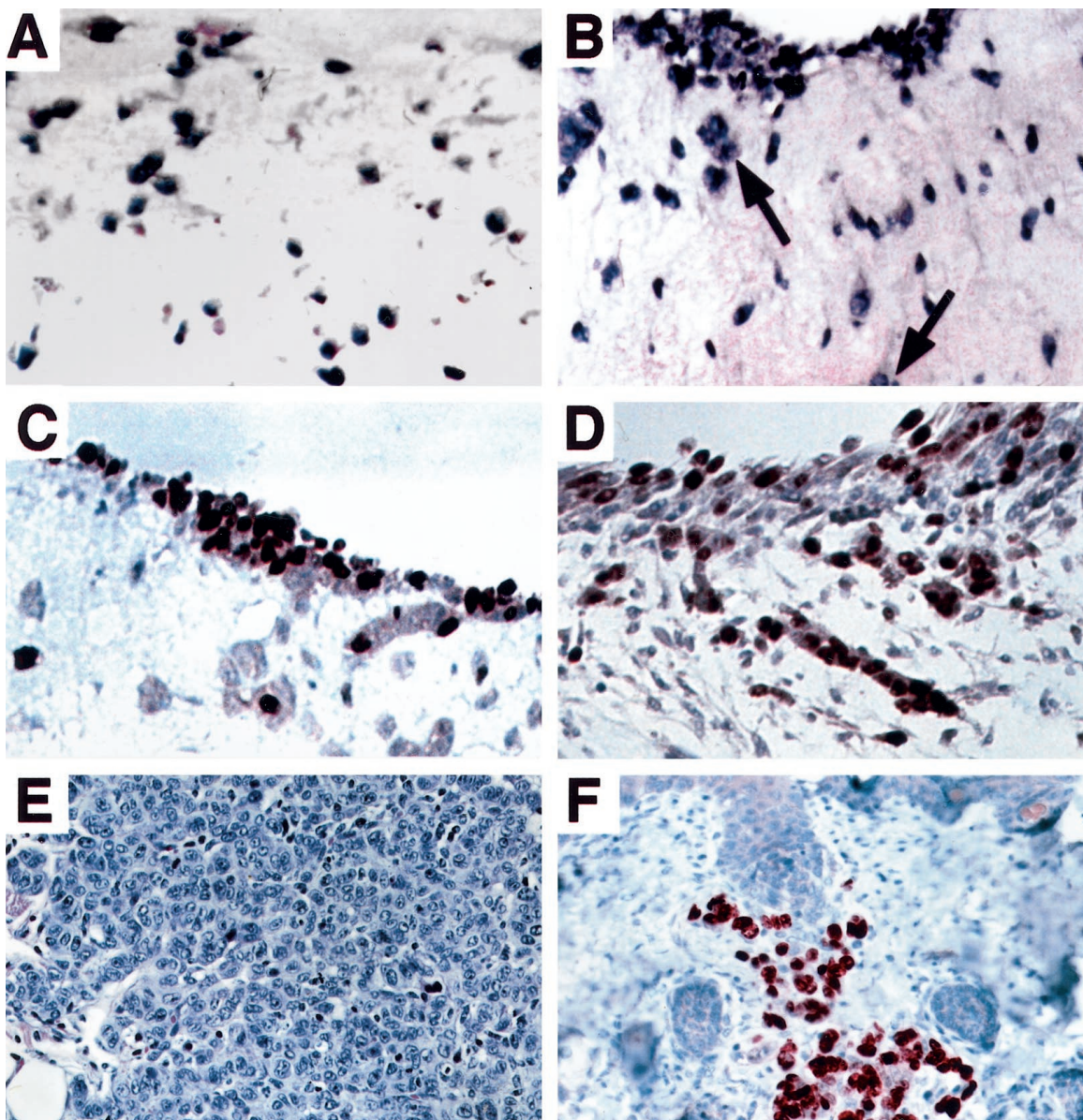


Figure 4. Survival, growth, and migration of RGP melanoma cells Sbc12 in dermal equivalent after transduction with bFGF gene. **A:** Sbc12 melanoma cells transduced with lacZ control gene and embedded together with fibroblasts in dermis were stained with hematoxylin and eosin. Magnification, $\times 50$. **B:** Sbc12 cells transduced with bFGF and mixed with fibroblasts in collagen form small clusters in the collagen and migrate out of the matrix for nest formation (**arrows**). Magnification, $\times 50$. **C:** Sbc12 cells transduced with LacZ control vector and stained for proliferation marker Ki67. Magnification, $\times 50$. **D:** Sbc12 cells transduced with bFGF, stained for Ki67. Section was counterstained with Mayer's hematoxylin. Magnification, $\times 50$. **E:** H & E stain of tumor from SCID mouse injected s.c. 4 days earlier with bFGF-transduced Sbc12 cells of section. Magnification, $\times 40$. **F:** Section of bFGF-transduced Sbc12 cells stained after 14 days for Ki67 proliferation marker. Magnification, $\times 40$.

cells proliferated orthotopically in human skin grafted to immunodeficient mice with characteristics similar to those in patients.⁴⁰ Thus, despite long-term culture of at least 50 subcultures melanoma cell lines remained stable in their biological phenotype when grown *in vitro* or *in vivo* in a cutaneous environment that is similar to that in patients.

Melanocytes adhere to keratinocytes through E-cadherin.⁴⁷ This close interaction allows keratinocytes to regulate expression of cell surface antigens on melanocytes and to control cell growth.^{21,22} Melanoma cells do not express E-cadherin⁴⁸ and thus grow independently of keratinocytes. Although RGP melanomas grow separated

from the basement membrane in the upper layers of the epidermis, they remain dependent on keratinocytes. When RGP melanoma cells are transduced with the $\beta 3$ subunit of the $\alpha v \beta 3$ vitronectin receptor, they grow invasively into the dermis⁴ and are tumorigenic in immunodeficient mice. It remains unknown which genes are activated to trigger the increased growth and invasiveness after overexpression of an adhesion receptor.

The present study suggests that RGP primary melanoma cells require activation of the gene bFGF for survival, proliferation, and migration into the dermis. bFGF is apparently the most important growth factor in melanoma.²⁸ bFGF has autocrine growth stimulatory functions

because cells cannot survive *in vitro* and *in vivo* without it.^{17,34-37} RGP melanomas do not express bFGF protein when cell extracts are tested by Western blotting, although RNA can be detected after 30 cycles by reverse transcriptase-polymerase chain reaction.⁴⁹ The low level production of bFGF by RGP melanomas is apparently insufficient for survival of those RGP melanoma cells that begin to enter the dermis from the epidermis. In the epidermis, keratinocytes provide the necessary bFGF for melanocytes¹⁷ and apparently also for RGP cells. bFGF appears to stimulate the expression of an enzyme(s) that degrades collagen I which is tightly constricted by the embedded fibroblasts. The up-regulation of metalloproteinases for collagens by bFGF has been reported.⁵⁰

Reconstructs displayed the basement membrane that separates the epidermis and dermis. Apparently both keratinocytes and fibroblasts contribute to its formation in normal skin reconstructs.⁵¹ In RGP melanoma reconstructs, fibroblasts and keratinocytes can still form an intact basement membrane, but the melanoma cells begin to synthesize their own collagen IV. The synthesis of collagen type IV and laminin by melanomas has also been demonstrated in patients' lesions.⁵² VGP melanomas traversing into the dermis no longer allow the formation of an intact basement membrane. Instead, collagen type IV appears to be randomly distributed throughout the lesion. It is not clear whether this reflects increased degradation or decreased production of the collagen type IV. However melanoma cells producing their own collagen type IV are unlikely to increase the production of degradative enzymes. It is possible that the malignant cells transmit signals to fibroblasts in the stroma to produce less collagen. Further studies are needed to determine the cross-talk between normal and malignant cells for the production of proteolytic enzymes and their activators. The human skin reconstruct model should be ideally suited to investigate the relative contribution of individual genes for invasion. Molecular engineering of each normal skin constituent and of the tumor cells will allow a better dissection of each step of invasion.

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