

Fibroblast growth factor-2 but not Mel-CAM and/or $\beta 3$ integrin promotes progression of melanocytes to melanoma

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Abstract: A variety of melanoma-associated antigens have been identified that mediate adhesion, growth, proteolysis, and modulation of immune response. However, the mechanisms by which human normal melanocytes become malignant are not clearly understood. Among the most consistent observations is the up-regulation of fibroblast growth factor-2 (FGF-2) and of the adhesion molecules $\beta 3$ integrin and Mel-CAM during melanoma progression. To evaluate the potential role of FGF-2, $\beta 3$ integrin and Mel-CAM in melanoma development we overexpressed FGF-2, $\beta 3$ integrin and Mel-CAM in normal human melanocytes using replication-deficient adenoviruses as a gene delivery vehicle. Fibroblast growth factor-2 overexpressing melanocytes in monolayer culture displayed cytological atypia. Furthermore, in human skin reconstructs where the physiological milieu is recreated *in vitro*, FGF-2-overexpressing melanocytes exhibited marked proliferation, upwards migration, cluster formation and type IV collagen expression within the epidermal compartment, simulating early radial growth phase melanoma. In contrast, overexpression of $\beta 3$ integrin and/or Mel-CAM in melanocytes did not affect their biological behaviour in human skin reconstructs. The described results of the current and previous studies emphasise the key role of FGF-2 in melanoma development and progression, underscoring the promise of FGF-2 as a target for therapy.

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Introduction

Based on clinical and histopathological features, a model of melanoma development and progression has been proposed (1). The model suggests that melanoma may develop and progress in a sequence of steps: melanocyte → nevus → dysplastic nevus → radial growth phase primary melanoma without competence for metastasis → vertical growth phase (VGP) primary melanoma with competence for metastasis → metastatic melanoma. The mechanisms by which melanocytes become malignant are still largely unknown. A variety of melanoma-associated antigens have been identified that mediate cell-cell or cell-substrate adhesion, growth regulation, proteolysis, and modulation of immune response (2). During melanoma progression, up-regulation of fibroblast growth factor-2 (FGF-2)

or basic fibroblast growth factor and adhesion molecules such as $\beta 3$ integrin and Mel-CAM have been consistently observed.

The adhesion molecule Mel-CAM is rarely detected in benign melanocytic lesions and is strongly expressed in melanomas (3). Mel-CAM expression in melanoma cells correlates with tumour thickness (3) and metastatic potential (4). Mel-CAM appears to be involved in melanoma–endothelial cell interactions, suggesting a role for Mel-CAM in tumour extravasation and angiogenesis (5). In addition, Mel-CAM-transfected melanoma cells exhibit increased activity of matrix metalloproteinase-2 (MMP-2) facilitating tumour cell migration and invasion (6).

The adhesion molecule $\beta 3$ integrin is exclusively expressed in VGP and metastatic melanomas and correlates with proliferation (7), invasion (8), re-

currence and mortality (9,10). *In vitro* studies provide evidence that the survival and proliferation of melanoma cells in the dermal extracellular matrix is regulated by collagen degradation and $\beta 3$ integrin interaction with denatured collagen (11,12). *In vitro* and *in vivo* findings suggest that $\beta 3$ integrin-mediated signalling affects the expression, processing and activation of membrane-type matrix metalloproteinase-1 and MMP-2, thereby inducing matrix degradation and facilitating cellular invasion (13). Previous studies have demonstrated an important role of $\beta 3$ integrin in melanoma angiogenesis (14,15).

Whereas normal melanocytes do not express the growth factor FGF-2, nearly all melanomas produce FGF-2 with increasing levels during tumour progression (16–18). Unlike FGF-2, high affinity membrane receptors for FGF-2, predominantly fibroblast growth factor receptor-1 (FGFR-1), are expressed in both normal melanocytes and melanoma cells. An autocrine growth stimulatory role of FGF-2 in melanoma progression has been demonstrated (19,20). Furthermore, paracrine effects of FGF-2 in angiogenesis and stroma formation have been postulated (21).

To identify some of the early events during melanoma development, we overexpressed FGF-2, Mel-CAM and $\beta 3$ integrin in human melanocytes using replication-deficient adenoviruses as a gene delivery vehicle. The biological effects of Mel-CAM, $\beta 3$ integrin and FGF-2 on normal melanocytes were analysed in three-dimensional human skin reconstructions. Earlier studies from ours and other laboratories have demonstrated close resemblance of artificial skin reconstructions to *in vivo* situations. Skin reconstructions comprise a stratified, terminally differentiated epidermal compartment and a dermal compartment consisting of fibroblasts embedded in collagen. Cells in skin reconstructions where the physiological milieu is recreated *in vitro* more closely recapitulate the *in situ* phenotype than cells in monolayer culture (22). When FGF-2-, Mel-CAM- and $\beta 3$ integrin-transduced melanocytes were incorporated into human skin reconstructions, FGF-2-transduced but not Mel-CAM- and $\beta 3$ integrin-transduced melanocytes exhibited a phenotype resembling early radial growth phase melanoma. These results emphasise the central role of FGF-2 in the transformation of melanocytes to melanoma.

Materials and methods

Construction of adenoviral vectors

Control adenoviral vector, LacZ/Ad5, encoding β -galactosidase enzyme from *Escherichia coli* was obtained from the Vector Core (Institute of Human Gene Therapy, University of Pennsylvania, Philadelphia, PA). In preliminary experiments we have

shown high levels of expression in 99% of human melanocytes infected with 10–20 plaque-forming units (pfu)/cell of adenoviral vector expressing β -galactosidase after 24–48 h. High expression levels were maintained for a long period of time in human melanocytes after 40 days in culture (23).

The replication defective recombinant adenovirus expressing Mel-CAM (Mel-CAM/Ad5) was constructed according to methods described by He et al. (24). In brief, full-length human Mel-CAM cDNA was cloned into the multiple cloning site of pShuttle-CMV and recombined with the adenovirus genome (Ad5Easy 1) in bacteria. The linearized viral genome was transfected into 293 cells using calcium phosphate precipitation. All the viruses were propagated and determined for infectivity as described earlier (25). The construction and characterisation of adenoviral vectors carrying the human integrin $\beta 3$ subunit and FGF-2 genes ($\beta 3$ /Ad5, FGF-2/Ad5) have been described elsewhere (21,26).

Infection of human melanocytes by adenoviral vectors

Subconfluent human melanocytes were infected with 20 pfu/cell of replication-deficient adenoviruses (LacZ/Ad5, Mel-CAM/Ad5, $\beta 3$ /Ad5, FGF-2/Ad5) for 2 h at 37°C in serum-free Dulbecco's modified Eagle's medium (Gibco, Eggenstein, Germany). After 2 h of incubation, the viral suspension was removed, and regular medium was added. Infected cells were allowed to recover for 24–48 h before use.

Double infection with Mel-CAM/Ad5 + $\beta 3$ /Ad5, FGF-2/Ad5 + Mel-CAM/Ad5, FGF-2/Ad5 + $\beta 3$ /Ad5 and LacZ/Ad5 + LacZ/Ad5 was performed as described. Before second infection, cells were allowed to recover for 24 h.

Cell culture

After obtaining informed consent, keratinocytes, fibroblasts, and melanocytes were isolated from human foreskin and cultured as described (22,27,28). Melanocyte cultures were maintained in defined melanocyte growth medium M2 (Promocell, Heidelberg, Germany). Conditioned medium was prepared by incubating monolayer cultures of FGF-2/Ad5-infected melanocytes and control melanocytes in defined culture medium M2 for 24 h. Conditioned medium was cleared of cell debris by passing through 0.2- μ m filters. Keratinocytes were used at passage 2–3, melanocytes at passage 4–6, and fibroblasts at passage 5–7.

Cell culture on slides

Infected and control melanocytes from subconfluent cultures were trypsinised, washed, resuspended in melanocyte growth medium M2 and plated in duplicates at 1.5×10^5 cells/well in 4-well chamber slides (FALCON® and BIOCOAT™ CultureSlides, Becton Dickinson Labware, Franklin Lakes, NJ). After 2 days of culture, culture slides were harvested, fixed, and subjected to immunohistochemistry.

In vitro reconstruction of human skin and invasion assays

Human skin reconstructions were generated using described techniques and media formulations with modifications (22).

Dermal reconstruction.

A cell-free buffered collagen solution was prepared consisting of rat tail collagen type I (Collaborative Biomedical Products, Bedford, MA) at a final concentration of 1.35 mg/ml in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Gibco, Eggenstein, Germany). One ml of the cell-free collagen solution was added to the culture plate inserts (MILLICELL-PC, Millipore, Eschborn, Germany) placed in the six-well tissue culture plates. While the acellular collagen layer was solidifying, a second collagen solution was prepared similar to the first but with the addition of human fibroblasts (11.25×10^4 /ml). Three ml of the fibroblast-containing collagen solution was placed over the solidified acellular collagen layer. After 5 days of incubation at 37°C, the fibroblast contraction force caused the collagen gel to contract and form a concaved central area. This structure represented the dermal reconstruct.

Epidermal reconstruction with incorporation of melanocytes.

The dermal reconstruct was rinsed with 2 ml of epidermal growth medium (EGM) (22). After 1 h, the EGM was removed, and the surface of the dermal reconstruct was allowed to dry. Human keratinocytes together with human melanocytes were seeded into the concaved centre of the dermal reconstruct at a density of 3.0×10^6 /ml and a 2.5: 1 keratinocyte-melanocyte ratio. The skin reconstructs were incubated at 37°C for 2 h to allow attachment of the seeded cells. For submerged culture conditions, 3 ml of EGM were added beneath the insert and 2 ml inside the insert to allow proliferation of the seeded cells. The EGM was changed every 2 days. After 5 days of submerged culture, the skin reconstructs were lifted to the air-liquid interface to induce differentiation of the epidermis. For the air-exposed culture period, the medium was switched to maintenance medium (22). After 10–14 days of air-exposed culture, the skin reconstructs were harvested, fixed, and evaluated using light microscopy, including immunohistochemistry and immunofluorescence staining. All experiments were carried out as duplicates, most of which were repeated twice or more.

Invasion assays were performed, as described earlier, in the absence of keratinocytes.

Light microscopy, immunohistochemistry and immunofluorescence staining

Cell cultures on slides were fixed in 4% formaldehyde and subsequently submitted to immunohistochemistry. Culture slides were incubated with monoclonal antibodies for Mel-CAM (1: 5 dilution) (6), $\beta 3$ integrin subunit (1: 4 dilution) (8), FGF-2 (1: 100 dilution) (21), and type IV collagen (1: 100 dilution) (Dako, Glostrup, Denmark), using the APAAP technique as previously described (29). Final staining was developed with FAST Red TR (Sigma, Deisenhofen, Germany). Hemalaun was used for counterstaining.

Skin reconstructs and invasion assays were fixed with 4% paraformaldehyde overnight, dehydrated, and embedded in paraffin. Sections were stained with hematoxylin and eosin for routine light microscopy. For immunohistochemistry, paraffin sections were stained with HMB45 as melanocytic marker (1: 100 dilution) (Dako, Carpinteria, CA), Ki-67 as proliferation marker (1: 50 dilution) (Dianova, Hamburg, Germany), anti-type IV collagen (1: 100 dilution), and anti- $\beta 3$ integrin subunit (1: 4 dilution). The sections were processed and visualised as described earlier. For immunofluorescence staining of Mel-CAM, 15- μ m cryo-sections of Mel-CAM/Ad5-infected melanocytes seeded on dermal reconstructs were fixed in acetone at 4°C for 90 s. For FGF-2 and FGFR-1 immunofluorescence staining, 5- μ m paraffin sections of FGF-2/Ad5-infected mel-

anocytes seeded on dermal reconstructs were dehydrated and pretreated with microwave. All sections were blocked with normal donkey serum, and incubated with monoclonal mouse anti-Mel-CAM antibody (1: 5 dilution) (Chemicon, Hofheim, Germany), rabbit anti-FGF-2 antibody (1: 50 dilution) (Santa Cruz, Heidelberg, Germany) or rabbit anti-FGFR-1 antibody (1: 20 dilution) (Santa-Cruz, Heidelberg, Germany) for 1 h. Sections were subsequently stained with donkey antimouse and donkey antirabbit antibodies, respectively, labelled with Cy3 (Molecular Probes, Leiden, the Netherlands). Finally, all nuclei were stained with YOPRO (Molecular Probes). For double immunofluorescence staining of type IV collagen and laminin, 15- μ m vertical cryo-sections of skin reconstructs and invasion assays were fixed in acetone at 4°C for 90 s, blocked with normal donkey serum, and incubated with a 1: 20 dilution of goat anti-type IV collagen (Southern Biotechnologies Associates, Birmingham, AL) and a 1: 20 dilution of monoclonal antilaminin 5 (Harlan Sera Laboratory, Loughborough, UK) antibodies for 1 h. Sections were subsequently stained with donkey anti-goat antibodies labelled with FITC (Dianova, Hamburg, Germany) and with donkey antimouse antibodies labelled with Cy3 (Molecular Probes). Nuclei were stained with TOPRO (Molecular Probes). All sections were analysed using a confocal laser scanning microscope (Leica TCS SP, Leica Microsystems, Bensheim, Germany).

Results*Expression of Mel-CAM and/or $\beta 3$ integrin does not affect the biological behaviour of normal human melanocytes*

Melanocyte cultures infected with Mel-CAM/Ad5 and/or $\beta 3$ /Ad5 and control melanocytes (melanocyte cultures infected with LacZ/Ad5, non-infected melanocyte cultures) expressed Mel-CAM and $\beta 3$ integrin by immunohistochemistry (Figs 1.a–d). Others and we have demonstrated that human normal melanocytes acquire expression of melanoma-associated antigens, such as Mel-CAM and $\beta 3$ integrin, after isolation and subsequent monolayer culture (22). Increased expression of Mel-CAM and/or $\beta 3$ integrin was evident in Mel-CAM/Ad5- and/or $\beta 3$ /Ad5- infected melanocytes exhibiting strong staining (Figs 1a,b). Monolayer cultures of $\beta 3$ /Ad5-infected melanocytes showed cytologically distinctive cells (Fig. 1c) compared with control melanocytes (Fig. 1d); the individual cells were large and epitheloid with abundant cytoplasm and large nuclei (Fig. 1c). When Mel-CAM/Ad5- and/or $\beta 3$ /Ad5-infected and control melanocytes were seeded on dermal reconstructs and harvested after 3 weeks of three-dimensional culture, more than 50% of Mel-CAM/Ad5- and/or $\beta 3$ /Ad5-infected melanocytes still expressed Mel-CAM and/or $\beta 3$ integrin (Figs 1e,g) whereas control melanocytes were completely negative (Figs 1f,h).

When incorporated into the epidermis of human skin reconstructs melanocytes expressing Mel-CAM (Fig. 2a), $\beta 3$ integrin (data not shown) or both (Fig. 2c) resembled phenotypical control melanocytes (LacZ/Ad5-infected melanocytes, LacZ/

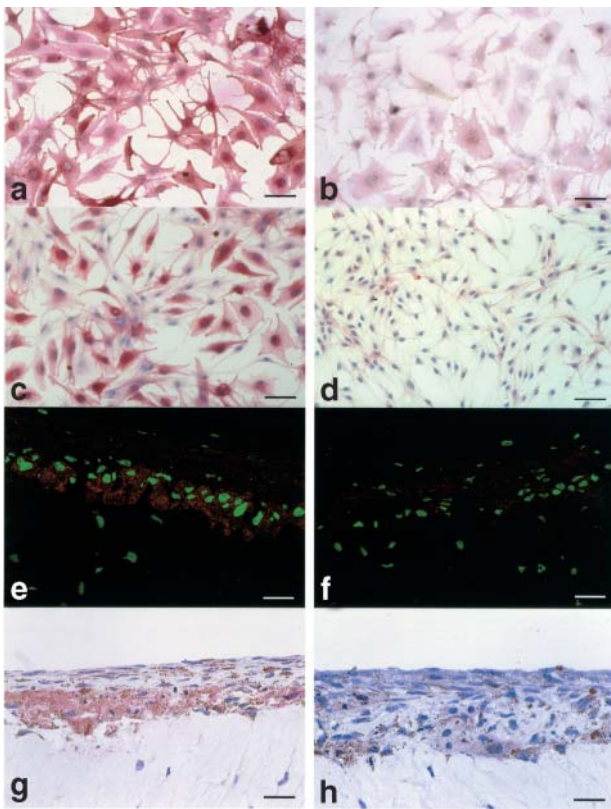


Figure 1. Expression of Mel-CAM and $\beta 3$ integrin after infection of human melanocytes with Mel-CAM/Ad5 and $\beta 3$ /Ad5. Both melanocytes infected with Mel-CAM/Ad5 (a) and control melanocytes (b) express Mel-CAM (red) in monolayer culture 4 days after infection with stronger labelling of Mel-CAM/Ad5-infected melanocytes. Ninety per cent of $\beta 3$ /Ad5-infected melanocytes (c) exhibit good labelling of $\beta 3$ integrin (red) 4 days after infection whereas control melanocytes (d) show weak labelling. Note the distinctive cytology of $\beta 3$ /Ad5-infected melanocytes in monolayer culture with large epitheloid cells. Immunofluorescence staining of melanocytes seeded on dermal reconstructs for Mel-CAM (red) 3 weeks after infection (e,f). Nuclei are stained with YOPRO (green). More than 50% of Mel-CAM/Ad5-infected melanocytes (e) express Mel-CAM whereas control melanocytes are negative for the same (f). Staining of melanocytes seeded on dermal reconstructs for $\beta 3$ integrin (red) 3 weeks after infection (g,h). More than 50% of $\beta 3$ /Ad5-infected melanocytes show expression of $\beta 3$ integrin (g) whereas control melanocytes do not express this integrin (h). Scale bars (a–d): 50 μm ; (e–h): 25 μm .

Ad5 + LacZ/Ad5 – infected melanocytes, non-infected melanocytes) (Figs 2b,d). Mel-CAM- and/or $\beta 3$ integrin-expressing melanocytes and control melanocytes singly located within the stratum basale of the epidermis, exhibited a multidendritic morphology and maintained a constant ratio of 1 : 5–1 : 10 with basal keratinocytes.

When invasion assays were performed neither Mel-CAM- and/or $\beta 3$ integrin-expressing melanocytes nor control melanocytes exhibited an invasive phenotype (Figs 2e,f), suggesting that expression of

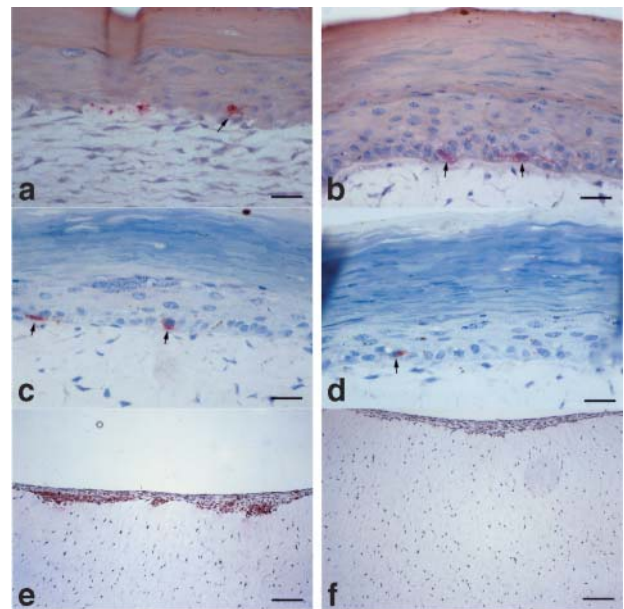


Figure 2. Expression of Mel-CAM and/or $\beta 3$ integrin does not affect the biological behaviour of human melanocytes. Staining of human skin reconstructs with HMB45 melanocytic marker (red) (a–d). Both Mel-CAM-overexpressing melanocytes (a) and not infected control melanocytes (b) exhibit the biological behaviour of normal melanocytes *in situ*. Melanocytes (\rightarrow) settle within the stratum basale of the epidermis where they remain singly and maintain a constant ratio with keratinocytes. Melanocytes double infected with Mel-CAM/Ad5 + $\beta 3$ /Ad5 (c) as well as control melanocytes double infected with LacZ/Ad5 + LacZ/Ad5 (d) recapitulate the *in situ* phenotype of normal melanocytes (\rightarrow). $\beta 3$ integrin staining (red) of invasion assays (e,f). Neither $\beta 3$ integrin-expressing melanocytes (e) nor $\beta 3$ integrin-negative melanocytes (f) are able to invade the dermis. Scale bars (a–d): 25 μm ; (e–f): 100 μm .

Mel-CAM and/or $\beta 3$ integrin does not initiate invasive growth in normal melanocytes.

Expression of FGF-2 induces an altered phenotype in normal human melanocytes resembling early radial growth phase melanoma

More than 50% of the FGF-2/Ad5-infected melanocytes showed expression of FGF-2 (Fig. 3a), whereas the control cells were negative (Fig. 3b). Cytologically, FGF-2/Ad5-infected melanocytes were atypical. Unlike control melanocytes (Fig. 3b), FGF-2-transduced melanocytes were not uniform and did not exhibit contact-inhibited growth in monolayer culture. Cytoplasm and nuclei varied in size, and multilayers were formed (Fig. 3a). Conditioned media from FGF-2-overexpressing melanocytes were collected after 24h and tested for the effect on normal melanocytes. However, no alterations in cell morphology and growth were observed when compared with normal melanocytes incubated with control media (data not

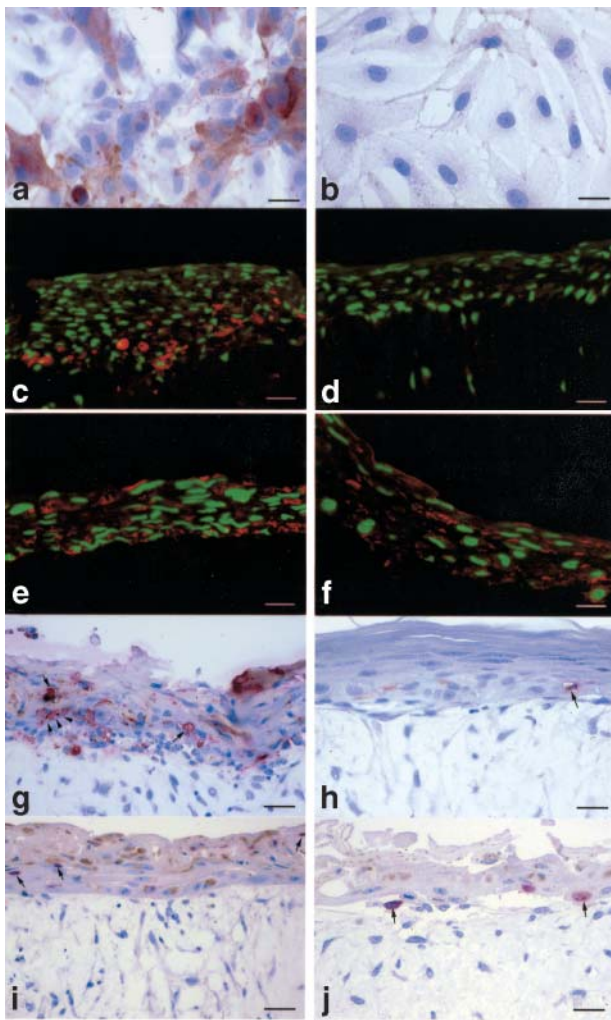


Figure 3. Expression of fibroblast growth factor-2 (FGF-2) induces an altered phenotype in human melanocytes, resembling early radial growth phase melanoma. More than 50% of FGF-2/Ad5-infected melanocytes (a) exhibit expression of FGF-2 (red) 4 days after infection, whereas control melanocytes (b) do not express FGF-2 in monolayer culture. Note the marked cytological atypia of FGF-2-expressing melanocytes. Immunofluorescence staining of melanocytes seeded on dermal reconstructs for FGF-2 (a,b, red) and fibroblast growth factor receptor-1 (FGFR-1) (c,d, red) 3 weeks after infection (c-f). Nuclei are stained with YOPRO (green). Up to 40% of FGF-2/Ad5-infected melanocytes (c) express FGF-2, whereas control melanocytes (d) remain negative for the same. Both melanocytes infected with FGF-2/Ad5 (e) and control melanocytes (f) express FGFR-1 without detectable differences in expression levels. Fibroblast growth factor-2-expressing and control melanocytes were incorporated into skin reconstructs and stained with HMB45 melanocytic marker (g,h, red) and Ki-67 proliferation marker (i,j, red). Fibroblast growth factor-2-expressing melanocytes (g) increase in cell number, move upwards (→) and form clusters (arrowheads) within the epidermal compartment, simulating early radial growth phase melanoma. In contrast, control melanocytes (h) exhibit the biological properties of normal melanocytes *in situ* singly locating within the stratum basale of the epidermis (→). Skin reconstructs with FGF-2-expressing melanocytes (i) display marked proliferation of upwards migrating melanocytes with numerous Ki-67 positive cells (→) at all levels of the epidermis, whereas skin reconstructs with control melanocytes (j) only show staining of proliferating basal keratinocytes (→). Scale bars (a-d): 25 μm; (e,f): 16 μm; (g-j): 25 μm.

shown). High expression levels of FGF-2 were maintained for at least 3 weeks after infection. Three weeks after infection, up to 40% of FGF-2/Ad5-infected melanocytes seeded onto dermal reconstructs expressing FGF-2 (Fig. 3c), whereas control melanocytes remained negative for the same (Fig. 3d). No obvious differences in expression levels of FGF-2 receptor FGFR-1 between FGF-2/Ad5-infected and control melanocytes were detectable (Figs 3e,f).

When FGF-2-overexpressing melanocytes were incorporated into the epidermis of human skin reconstructs, increased pigmentation and disarray of the epidermis were observed (Fig. 3g). Fibroblast growth factor-2-overexpressing melanocytes increased in cell number, moved upwards, and formed clusters within the epidermal compartment of skin reconstructs resembling melanoma *in situ*, i.e. early radial growth phase melanoma (Fig. 3g) whereas control melanocytes recapitulated the *in situ* phenotype of normal melanocytes (Fig. 3h). Skin reconstructs with FGF-2-overexpressing mel-

anocytes showed marked proliferation in upwards migrating melanocytes with numerous cells expressing the proliferation marker Ki-67 at all levels of the epidermis (Fig. 3i), whereas skin reconstructs with control melanocytes only exhibited staining of proliferating basal keratinocytes (Fig. 3j). Type IV collagen staining of skin reconstructs with FGF-2-overexpressing melanocytes and control skin reconstructs revealed linear deposition of collagen type IV along the epidermal-dermal junction (Figs 4a,b). Furthermore, double immunofluorescence staining of collagen type IV and laminin displayed linear deposition of both basement membrane proteins, indicating the presence of a seemingly intact basement membrane (Fig. 4c). Interestingly, in skin reconstructs with FGF-2-overexpressing melanocytes, single cells at all levels of the epidermis appeared to express type IV collagen (Fig. 4a), whereas the epidermis of control skin reconstructs was completely negative (Fig. 4b).

To evaluate invasive competence of FGF-2-overexpressing melanocytes in the absence of keratinocyte-mediated control and to determine the source of type IV collagen found in the epidermis of skin reconstructs, invasion assays were performed. Fibroblast growth factor-2-overexpressing melanocytes and control melanocytes were plated on top of dermal reconstructs without keratinocytes. Similar to control melanocytes, FGF-2-over-

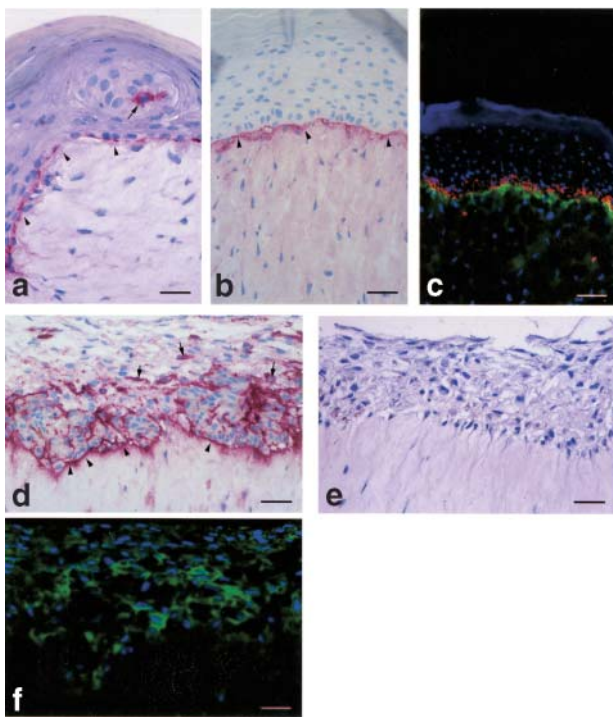


Figure 4. Expression of fibroblast growth factor-2 (FGF-2) in human melanocytes promotes synthesis and deposition of collagen type IV. Type IV collagen staining (red) of skin reconstructs with FGF-2/Ad5-transduced melanocytes (a) and control melanocytes (b), which both display linear deposition of this basement membrane protein along the epidermal-dermal junction (arrowheads). Unlike skin reconstructs with control melanocytes, skin reconstructs with FGF-2-overexpressing melanocytes (a) display suprabasal type IV collagen expressing cells (→). Double immunofluorescence staining of control skin reconstructs for type IV collagen (green) and laminin (red) (c). Nuclei are stained with TOPRO (blue). Linear deposition of both basement membrane proteins along the epidermal-dermal junction is seen. Type IV collagen staining (red) of invasion assays with FGF-2-overexpressing melanocytes (d) and control melanocytes (e). Both FGF-2-overexpressing and control melanocytes lack competence for invasive growth. In contrast to control melanocytes, FGF-2-overexpressing melanocytes (d) display intense intracellular (→) and extracellular (arrowheads) type IV collagen staining. Double immunofluorescence staining of invasion assays with FGF-2-overexpressing melanocytes for collagen type IV (green) and laminin (red) (f). Nuclei are stained with TOPRO (blue). Fibroblast growth factor-2-overexpressing melanocytes synthesise and deposit abundant type IV collagen but not laminin. Scale bars (a–f): 25µm.

expressing melanocytes lacked competence for invasive growth into the dermis (Figs 4d,e). In contrast to control melanocytes (Fig. 4e), intense type IV collagen staining was observed within and around FGF-2-overexpressing melanocytes (Fig. 4d). Double immunofluorescence staining of collagen type IV and laminin revealed that FGF-2-overexpressing melanocytes produce abundant type IV collagen but not laminin (Fig. 4f).

We also examined whether conditioned media of

FGF-2-overexpressing melanocytes induce type IV collagen synthesis in normal melanocytes. Interestingly, when monolayer cultures of FGF-2-overexpressing melanocytes and control melanocytes were stained with anti-type IV collagen, both the groups were positive with stronger labelling in FGF-2-overexpressing melanocytes (data not shown). These observations may reflect the variable biological behaviour of cells with the use of organotypic vs. monolayer culture. When control melanocytes were incubated in the presence or absence of conditioned media collected from FGF-2-overexpressing melanocytes, there were no detectable differences in expression levels of collagen type IV between them (data not shown).

Co-expression of FGF-2 and Mel-CAM or FGF-2 and β3 integrin in normal human melanocytes does not elicit greater biological effects than FGF-2 alone

Fibroblast growth factor-2-overexpressing melanocytes simulate the biological behaviour of early radial growth phase melanoma cells. However, these cells do not initiate invasive growth. Alternatively, a combination of growth factor with the tumour-associated adhesion molecule might provide an aggressive phenotype. To test this, human skin reconstructs were separately incorporated with double-infected melanocytes (FGF-2/Ad5 + Mel-CAM/Ad5-infected melanocytes, FGF-2/Ad5 + β3/Ad5-infected melanocytes) and control melanocytes (LacZ/Ad5 + LacZ/Ad5-infected melanocytes, non-infected melanocytes). A comparative histological and immunohistochemical analysis of paraffin sections from skin reconstructs showed that a combination of FGF-2 with Mel-CAM or FGF-2 with β3 integrin does not result in additive biological effects (data not shown).

We next assessed the invasiveness of melanocytes coexpressing FGF-2 and Mel-CAM or FGF-2 and β3 integrin in invasion assays. Comparative histology and immunohistochemistry revealed that coexpression of FGF-2 and Mel-CAM or FGF-2 and β3 integrin is not sufficient to confer an invasive phenotype in melanocytes (data not shown).

Discussion

The mechanisms by which normal human melanocytes become malignant are largely unknown. Our previous studies showed that introduction of the adhesion molecules Mel-CAM (30) or β3 integrin (26) into radial growth phase melanoma cell lines induces conversion from radial to vertical growth phase. So far it is not clear whether Mel-CAM and β3 integrin are involved in the transformation of

normal melanocytes to malignant melanoma. Melanoma cells express multiple growth factors. Apparently, FGF-2/FGFR-1 is the only growth factor/growth factor receptor combination that is expressed in all primary and metastatic melanoma specimens and cell lines analysed to date (19). Furthermore, melanoma growth can be arrested by interfering with the production or biological activity of FGF-2 alone (31–33). Of all the growth factors known, FGF-2 appears to be a prime candidate for a key role in the transformation of normal human melanocytes to malignant melanoma (32,34). To further investigate the potential role of Mel-CAM, β 3 integrin and FGF-2 in melanoma development, we overexpressed Mel-CAM, β 3 integrin and FGF-2 in cultured melanocytes using replication-deficient adenoviruses as a gene delivery vehicle. The biological effects of Mel-CAM, β 3 integrin and FGF-2 on normal human melanocytes were analysed in three-dimensional human skin reconstructs where the physiological context is recreated *in vitro*.

The present study demonstrates that overexpression of the adhesion molecules Mel-CAM and/or β 3 integrin in normal human melanocytes did not affect the biological behaviour of melanocytes in three-dimensional skin reconstructs despite the distinctive cytology of β 3 integrin-transduced melanocytes in monolayer culture. The discrepancies might reflect variability in the biological behaviour of cells with the use of monolayer vs. organotypic culture. Despite the profound effect of Mel-CAM and β 3 integrin expression on the biological properties of radial growth phase melanoma cells, normal human melanocytes appear to be less susceptible to alterations induced by Mel-CAM and/or β 3 integrin overexpression. This observation suggests that Mel-CAM and β 3 integrin play a decisive role in the progression of radial growth phase to vertical growth phase melanoma but not in the progression of normal melanocytes to malignant melanoma. Previously, decreased metastatic potential of a human β 3-negative metastatic melanoma cell line was reported upon β 3 transduction (35). Thus, the biological functions of the adhesion molecules Mel-CAM and β 3 integrin in melanoma may depend on the cellular background of a given stage of melanoma development and progression.

Unlike the adhesion molecules Mel-CAM and β 3 integrin, up-regulation of the growth factor FGF-2 in normal human melanocytes appears to have profound effects. In monolayer culture, FGF-2-overexpressing melanocytes displayed marked cytological atypia. These cytological changes could not be induced by incubating control melanocytes with conditioned media collected from FGF-2-overexpressing melanocytes, suggesting these ef-

fects were from endogenous expression of FGF-2. No differences in expression levels of FGF-2 receptor FGFR-1 between FGF-2-transduced and control melanocytes were detectable, suggesting that FGF-2 up-regulation does not modulate FGFR-1 expression. When FGF-2-overexpressing melanocytes were incorporated into the epidermis of skin reconstructs, increased pigmentation of the epidermis was noted. This observation is in agreement with previous experiments with human FGF-2-transduced melanocytes (21,36); however, this is in contrast to murine melanocytes, which lost pigmentation after FGF-2 transduction (37). The disparity between these observations might be the result of species differences. Fibroblast growth factor-2-overexpressing melanocytes displayed marked proliferation, upwards migration and cluster formation within the epidermis of human skin reconstructs resembling melanoma *in situ*, i.e. early radial growth phase melanoma. A previous study also demonstrated a transformed phenotype in FGF-2-transduced human melanocytes (21). Here, FGF-2-transduced melanocytes in monolayer culture were not dependent on exogenous FGF-2, insulin/insulin-like growth factor 1, and cyclic AMP enhancers, requiring only phorbol ester as mitogen. Furthermore, FGF-2-transduced melanocytes grew anchorage independently in soft agar-like primary melanoma cells. When FGF-2-transduced cells were injected into the dermis of human skin grafted to mice, they proliferated in the dermal compartment, whereas control melanocytes showed poor survival in the dermal environment. Consistent with this observation, FGF-2 has been reported to act as a survival factor for melanocytic cells in the dermal environment (38). Nevus cells expressing FGF-2 survived in three-dimensional collagen gels, while normal melanocytes underwent apoptosis. In the presence of exogenous FGF-2, melanocytes also survived in collagen gels. Taken together, the described results of the current and previous studies suggest that FGF-2 provides a growth advantage to normal melanocytes in the epidermal and dermal compartment of human skin, emphasising a decisive role of FGF-2 in the progression of melanocytes to melanoma. To support this conclusion, antagonistic studies for the FGF-2/FGFR-1 system are desirable. However, these studies are not feasible because it has been consistently demonstrated that melanocytes or melanoma cells cannot survive if FGF-2 or FGFR-1 are targeted (19,33). Conflicting results have been yielded by other experimental approaches using retroviral vectors for FGF-2 overexpression in normal human melanocytes (39). Here, FGF-2-overexpressing melanocytes still required exogenous FGF-2 for growth in monolayer

culture, and did not form colonies in soft agar. The authors conclude that expression of FGF-2 alone is not enough to cause aberrant growth of normal human melanocytes. However, the discrepancies might reflect variability in the degree of FGF-2 expression induced in cells with the use of retroviral vs. adenoviral vectors. In our study FGF-2-overexpressing melanocytes migrated upward in human skin reconstructs. Expression of FGF-2 has been reported in nevi (40). Nevus cells, however, rarely display upward migration in contrast to melanoma cells. These discrepancies may indicate that epidermal melanocytes and nevus cells represent different melanocytic cell subpopulations with different localisation and growth characteristics. Fibroblast growth factor-2 may induce different biological effects in epidermal melanocytes and nevus cells because of intrinsic and/or environmental differences.

Skin reconstructs with incorporated FGF-2-overexpressing melanocytes displayed type IV collagen-expressing cells at all levels of the epidermis. Fibroblast growth factor-2-overexpressing melanocytes seeded onto three-dimensional dermal reconstructs with the omission of keratinocytes displayed abundant intracellular and extracellular type IV collagen but not laminin, while control melanocytes did not express these basement membrane proteins. In monolayer culture both FGF-2-overexpressing melanocytes and control melanocytes expressed type IV collagen with stronger labelling of FGF-2-overexpressing melanocytes. These observations presumably reflect the variable biological behaviour of cells with the use of organotypic vs. monolayer culture, and are consistent with the results of a previous study by Yaar et al. (41). When control melanocytes were incubated with conditioned media collected from FGF-2-overexpressing melanocytes there were no detectable differences in expression levels of collagen type IV between melanocytes cultured in conditioned media and those cultured in control media suggesting that the stronger labelling of FGF-2-overexpressing melanocytes is the result of endogenous expression of FGF-2. Taken together, the presented data suggest that overexpression of FGF-2 in normal melanocytes induces synthesis and deposition of basement membrane-like material containing type IV collagen. Indeed, production of collagen type IV and/or laminin by melanomas has been found in patients' lesions as well as in three-dimensional skin reconstructs (22,42). A recent immunohistochemical and electron microscopic study including immunoelectron microscopy has demonstrated that nevus and melanoma cells synthesise basement membrane (BM) and basement membrane-like products (BM-like material) con-

taining type IV collagen and/or laminin (43). The production of BM or BM-like material is probably advantageous for melanocytic growth, as it can act as a scaffold for growth factors (44). Vukicevic et al. identified various endogenous growth factors in reconstituted basement membrane, suggesting that extracellular matrix components provide a reservoir for growth factors (45). Furthermore, BM material may serve as a protective wall against undesirable effects from the stroma. On the other hand, FGF-2 up-regulates and activates extracellular degenerative enzymes assuming invasive capacity for FGF-2-expressing cells (46). Matrix metalloproteinase-2, a 72-kDa type IV collagenase, could be located in melanoma cells and adjacent fibroblasts by immunoelectron microscopy (43). Expression of MMP-2 has been shown to be associated with invasion and metastasis in melanoma cell lines and melanoma lesions (47,48). Matrix metalloproteinase-2 located in melanoma cells and neighbouring fibroblasts is likely to degrade portions of BM material in order to promote migration, invasion and dissemination of melanoma cells. Apparently, BM material is immediately resynthesized by melanoma cells and/or fibroblasts. Interactions between melanocytic cells and extracellular matrix of which the BM is a part, can be traced back to the migration of melanocytes from the neural crest to the epidermis (49,50). Further studies are needed to elucidate the complex cross talk between melanocytic cells, surrounding cells and extracellular matrix, including basement membrane and its modulation with the stage of melanocytic tumour progression. Although type IV collagen and laminin represent the major constituents of basement membranes, other basement membrane proteins may also play an important role in regulating cell-cell and cell-matrix interactions.

Fibroblast growth factor-2-overexpressing melanocytes appeared to induce disarray of the epidermal compartment of human skin reconstructs. This phenomenon might be as a result of the aberrant biological behaviour of FGF-2-overexpressing melanocytes with proliferation, upward migration, cluster formation and type IV collagen production. Alternatively, FGF-2-overexpressing melanocytes might secrete FGF-2 or even other factors that affect the keratinocytes. However, when we incubated melanocytes with conditioned media collected from FGF-2-overexpressing melanocytes, no differences in the cellular behaviour was noted, suggesting that the observed biological effects such as alterations of cell morphology and growth and collagen type IV expression were caused by endogenous expression rather than release of FGF-2. It is known that FGF-2 is not readily secreted because of the lack of a signal pep-

tide sequence (51). Nesbit et al. showed that FGF-2/Ad5-infected melanocytes secrete only a small fraction of the total FGF-2 into the culture supernatant (21). It remains unclear whether these low levels of secreted FGF-2 are sufficient to affect the biological behaviour of keratinocytes.

It will be important to determine how the described FGF-2-induced alterations of cell morphology and growth are characterised on a molecular level, e.g. whether they are associated with changes of intracellular signalling. Nesbit et al. observed that FGF-2-overexpressing melanocytes grew independently of FGF-2, insulin/IGF-1 and α -MSH, suggesting that overexpression of FGF-2 is sufficient to activate signalling pathways induced by insulin/IGF-1 and α -MSH (21). Fibroblast growth factor-2 also appears to activate the protein kinase A pathway and enhances cAMP levels which human melanocytes require for proliferation in monolayer culture (52). Furthermore, FGF-2 can enhance intracellular protein kinase C activity by interacting with the FGFR-1 receptor (52). Interestingly, a recent study suggests that Src-family kinases(s) are a major downstream target for activated FGFR-1 in normal and malignant human melanocytic cells (20).

In our study, FGF-2-overexpressing melanocytes were not able to invade the dermis. This finding suggests that expression of FGF-2 provides a significant growth advantage but is not sufficient to confer an invasive phenotype in melanocytes. Apparently, additional factors are required to promote competence for invasive growth. Combined expression of FGF-2 and Mel-CAM or β 3 integrin did not elicit pronounced biological effects, i.e. coexpression of FGF-2 and Mel-CAM or β 3 integrin in melanocytes was not sufficient to trigger invasive growth. Recently, experimental induction of melanoma in human skin grafted to immunodeficient mice by overexpression of FGF-2 in the dermis and concomitant UVB irradiation was reported (36). However, melanocyte transformation resembling lentiginous melanoma was only observed in one of four skin grafts, suggesting that other major factors for development of invasive melanoma are missing. Therefore, further studies will be required to identify additional factors, which potentiate melanocytic competence for invasive growth.

Taken together, these findings suggest that the growth factor FGF-2 but not the adhesion molecules Mel-CAM and β 3 integrin plays an essential role early in melanoma development. The described results of the current and previous studies provide the rational basis for targeting FGF-2/FGFR-1-mediated signalling in order to develop effective treatment strategies for melanoma patients with poor prognosis.

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