Tumorigenesis and Neoplastic Progression

Genetic Ablation of αv Integrins in Epithelial Cells of the Eyelid Skin and Conjunctiva Leads to Squamous Cell Carcinoma

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Integrin-mediated cell adhesion and signaling events are essential for the proper development and homeostasis of most epithelial tissues. Dysregulation of integrin expression and function can cause abnormal epithelial cell proliferation and/or differentiation, contributing to the pathogenesis of malignant epithelial cancers. Here we report on the use of a conditional knockout strategy exploiting the Cre/Lox technology to study the in vivo functions of αv integrins during epithelial cell proliferation and differentiation. We show that genetic ablation of αv integrin expression in basal epithelial cells of the eyelid skin and conjunctiva causes the formation of tumors that are strikingly similar to the malignant epithelial cancer, squamous cell carcinoma. These data suggest a mechanism whereby αv integrins normally suppress epithelial cell proliferation, likely via adhesion to ECM ligands, as well as by the modulation of intracellular signaling cascades. We propose that defects in integrin expression and function can cause abnormal cell adhesion. Loss of α3 integrin expression leads to skin blistering phenotypes related to defective assembly of epidermal basement membranes. Selective ablation of the murine β1 integrin gene in the skin leads to severe defects in epidermal development and homeostasis, and activating mutations in the human β1 integrin gene are found in rare cases of SCC.

The αv integrin subfamily consists of αvβ1, αvβ3, αvβ5, αvβ6, and αvβ8, and various genetic ablation studies have implicated these integrins in the pathogenesis of squamous cell carcinoma, including defects in epidermal development and homeostasis. Integrins are a family of transmembrane receptors that mediate cell-cell and cell-matrix interactions. They are composed of α and β subunits, and the combination of distinct α and β subunits results in a diverse array of integrins with specific functions.

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ies in mice have shown that these integrins play important roles in multiple physiological and pathological contexts.11-16 However, in vivo genetic models to study αv integrin-mediated regulation of epithelial cell growth have not been reported. In this study we use Cre/lox technology to analyze the in vivo growth regulatory functions for the αv integrin subunit. We show that genetic ablation of αv expression selectively in basal cells of the eyelid skin and conjunctiva leads to development of epithelial tumors with pathological similarities to SCC. These data are direct molecular genetic evidence that αv integrins provide critical growth regulatory functions during epithelial proliferation and homeostasis. To our knowledge, these findings represent the first experimental data showing that genetic ablation of αv integrin expression and function in epithelial cells leads to dysregulation of normal cell growth and homeostasis, and suggest a physiological tumor suppressor-like function for αv integrins. Furthermore, this study provides a novel mouse genetic model to study the pathogenesis of SCC in the eyelid skin and conjunctiva.

Materials and Methods

Mouse Strains and Genotyping

This murine glial fibrillary acidic protein (mGFAP)-Cre transgene consists of a genomic fragment encompassing the minimal murine GFAP promoter, as well as regulatory intronic sequences flanking the Cre cDNA.17 Generation and characterization of mGFAP-Cre transgenic mice have been described elsewhere.18 The αv-flox mouse strain has been previously described.14 The Rosa26-LoxSTOPlox-LacZ reporter strain was purchased from The Jackson Laboratories.19 All mouse genotypes were confirmed by standard PCR-based genotyping of genomic DNA isolated from tail snips. The following primer sequences were used for PCR genotyping: Cre, 5'-ACCAGCCAGCTATCAACTC-3', and 5'-TATACCGGTGCTAGCAGATCTCCTCATTTCCAGCAG-3'. The Cre primers yield a single PCR product of ~200 bp. αv-flox primers, F1: 5'-GGTTGAATGCTCATTGCACGTTCA-3', F2: 5'-TTCAAGACGACGACAAAGACGTTG-3', and R: 5'-CACAAATCAAGATGACAAACTGAG-3'. The F1-R primer pair generates a PCR product of approximately 350 bp. The F2-R primer pair generates an 850 bp band representing the non-recombined αv-flox allele, or a 250bp band representing the recombined αv-flox allele. The αv<sup>flox<sup>-/- mutant animals, or eyelid tissue from control animals were fresh-frozen in Tissue Tek OCT (Miles). Sections (7 μm) were immunostained with rabbit IgG (10 μg/ml), or anti-αv antibody. A secondary antibody conjugated to horseradish peroxidase (Vector Laboratories) in combination with diaminobenzidine chromagenic substrate was used for immunohistochemistry. Alternatively, an Alexa488-conjugated goat anti-rabbit secondary antibody (Molecular Probes) was used for immunofluorescence. For histopathology studies, eyelids or eye tumors were excised and fixed overnight at 4°C with 4% paraformaldehyde in phosphate buffered saline. Tissue was subsequently dehydrated and processed for standard paraffin embedding and H&E staining. Alternatively, to visualize mucin-expressing goblet-like cells, paraffin sections from eye tumors were counterstained with periodic acid-Schiff and diastase, or Alcian Blue. Oil Red O staining was performed on sections prepared from unfixed, fresh-frozen ocular tumors.

Results

A Murine GFAP-Cre Transgene Is Expressed in Epithelial Cells of the Developing Eyelid and Conjunctiva

Previously, we used Cre/lox technology to selectively ablate the murine αv integrin gene in central nervous system (CNS) neural cells.14 These efforts involved analyzing the temporal and spatial expression patterns of various Cre transgenes reportedly expressed in specific cell types in the CNS. One such transgene, consisting of a fragment of the mGFAP promoter inserted 5’ to the cDNA encoding Cre recombinase (mGFAP-Cre), was reported to be expressed specifically in postnatal CNS glia and neurons.21 Indeed, using the ROSA26-loxSTOPlox-lacZ reporter mouse,19 we confirmed Cre activity in some neuronal and glial cells of the developing neural tube, as well as some cells in the subventricular zone of the brain (data not shown). However, we also detected transgene expression outside of the CNS. As shown in Figure 1, B and D, embryos (E13.5) harboring the mGFAP-Cre and ROSA26-loxSTOPlox-lacZ transgenes expressed robust levels of Cre in epithelial cells of the developing lens and conjunctiva. Additionally, analysis of neonatal (P7) transgenic mice revealed Cre expression in epithelial cells of the conjunctiva and cornea (Figure 1F), eyelid (Figure 1H), as well as occasional hair follicles in the eyelid epidermis (Figure 1J). We did not detect lacZ activity in embryos or neonates lacking the GFAP-Cre transgene (Figure 1, A, C, E, G, and I). The pattern of Cre expression was primarily localized to epithelial cells of the eyelid skin; we did not detect Cre activity in epithelia of other neonatal organs, including the intestine and lung (data not shown). It is likely that the epithelial expression pattern of the GFAP-Cre transgene is an aberrant consequence of the transgene insertion site. For example, the transgene may have inserted into a genomic region that is regulated by enhancer elements that activate gene expression in specific epithelial cells of the developing
Targeted Deletion of the αv Integrin Gene Using the mGFAP-Cre Transgene

We used an anti-αv integrin antibody\textsuperscript{14,20} to analyze the spatial expression pattern of αv integrin protein in the postnatal murine eyelid. αv integrin protein expression was detected in the basal epithelium of the normal eyelid, as well as by basal epithelial cells in sebaceous glands and hair follicles (Figure 2, A and B). The expression pattern of αv integrin protein was very similar to the pattern detected for β-catenin (Figure 2C), a protein commonly expressed in basal cells of stratified epithelial tissues.\textsuperscript{23} We selectively ablated the αv integrin gene by generating mice harboring a conditional αv allele (αv\textsuperscript{flox/flox}) in combination with the mGFAP-Cre transgene (Figure 3A). First, mGFAP-Cre hemizygotes were bred with αv\textsuperscript{+/–} mice to generate GFAP-Cre\textsuperscript{+}: αv\textsuperscript{+/–} progeny, which were subsequently bred with αv\textsuperscript{flox/flox} mice.\textsuperscript{13,14} The resulting mutant progeny are hemizygous for the mGFAP-Cre transgene, and carry one αv-flox allele and one αv-null allele. Littermate controls were hemizygous for the mGFAP-Cre transgene, and carry one αv-flox allele and one αv wild-type allele.

We used genomic PCR to test for mGFAP-Cre-mediated deletion of the αv integrin gene. We isolated genomic DNA from eyelid tissue, and analyzed αv-flox deletion using PCR to monitor a 350 bp PCR band representing the αv-flox allele (Figure 3B). Analysis of ear, eyelid, or eye tumor genomic DNA samples from control and mutant mice revealed recombination of the αv-flox allele selectively in the eye or eye tumor samples. We monitored deletion of the conditional αv allele using a second primer pair designed to amplify an 850 bp band representing the non-recombined αv-flox allele (Figure 3C). The same primer pair amplified a 250 bp band representing the recombined αv\textsuperscript{flox/flox} allele. Amplification of the complementary allele, which lacked loxP sites and is either wild-type or null for αv, yielded a 550 bp PCR product. Analysis of ear, eyelid, or eye tumor genomic DNA samples from control and mutant mice revealed recombination of the αv-flox allele selectively in the eye or eye tumor samples. This correlated with reduced intensity of the intact 850 bp αv-flox cassette, as well as an increase in the recombinated 250 bp PCR product (Figure 3C).

Genetic Ablation of αv Integrin Causes the Formation of Eyelid Skin Tumors Displaying Pathological Characteristics of Squamous Cell Carcinoma

mGFAP-Cre\textsuperscript{+}, αv\textsuperscript{flox/+} and mGFAP-Cre\textsuperscript{+}, αv\textsuperscript{flox/–} mutants were born in expected ratios, and displayed no grossly obvious developmental or behavioral abnormalities (data not shown). However, beginning as early as nine postnatal months, mGFAP-Cre\textsuperscript{+}; αv\textsuperscript{flox/–} mutant animals developed tumors surrounding one or both eyes (Figure 3, D–F). mGFAP-Cre\textsuperscript{+}; αv\textsuperscript{flox/–} control littermates (17/17 analyzed thus far) did not develop eye tumors like

Figure 1. A murine GFAP-Cre transgene is expressed in epithelial cells of the embryonic and postnatal eye. Embryos (E13.5) expressing the ROSA26-loxSTOPlox-LacZ reporter transgene in the absence (A) or presence (B) of the mGFAP-Cre transgene were dissected and whole-mounts were stained to determine the spatial pattern of β-galactosidase activity. β-galactosidase is minimally expressed in embryos lacking the mGFAP-Cre transgene (A); however, embryos harboring the mGFAP-Cre transgene display Cre-mediated expression of β-galactosidase (B). C, D: Embryonic heads were sectioned horizontally and the pattern of β-galactosidase activity was analyzed microscopically. Note the β-galactosidase activity in epithelial cells of the lens (arrow in D) and conjunctiva (arrowheads in D). Sagittal histological sections through the center of the neonatal eye (P7) from ROSA26-loxSTOPlox-LacZ transgenics in the absence (E, G, I) or presence (F, H, J) of the mGFAP-Cre transgene. Cre-mediated β-galactosidase activity is present in epithelial cells in the developing conjunctiva (arrows in F), cornea (arrowheads in F), eyelid (arrows in H), and hair follicles (arrows in J).
those observed in the mutant animals. Unilateral or bilateral eye tumors have developed in 12/12 mutant animals analyzed thus far, with most tumors being grossly obvious by 12 to 18 months of age. One mutant with an apparent unilateral tumor also displayed metastatic lesions in the cervical lymph nodes (data not shown). In most cases (7/12 mice analyzed thus far), postmortem analyses of mutant animals with one grossly obvious tumor also revealed a smaller, microscopic ocular tumor. Additionally, postmortem analysis of two adult mutants lacking grossly obvious tumors in either eye revealed microscopic tumors in at least one eye (data not shown). Many mutants developed tumors that were ulcerated (Figure 3D), and tumor growth often led to complete closure of one or both eyes (Figure 3, E and F). Postmortem analyses of tumor size revealed late-stage tumors as large as 1 cm³ (Figure 3F).

All tumors arose in the periorbital region subjacent to the palpebral and bulbar conjunctiva. Given the periorbital location of the tumors subjacent to conjunctiva and their recapitulation of the biphasic pattern of normal conjunctival epithelium, these tumors are best regarded as deriving from conjunctiva. Tumors exhibited malignant behavior, as evidenced by compression of the globe of the eye (Figure 4A), and invasion into periorbital tissues, including invasion into skeletal muscle in several cases (Figure 4F), and in one case invasion of the globe (Figure 4A). By microscopic analysis, all tumors showed similar morphological findings: an invasive squamous proliferation, many with admixed goblet-like cells displaying pale, homogeneous cytoplasms and eccentric nuclei (Figure 4B). These goblet-like cells were positive for mucin by staining with Alcian Blue (Figure 4G) and periodic acid-Schiff with diastase (data not shown), and negative for fat by Oil Red O stain performed on unfixed, frozen tumor sections. These morphological and histochemical features are most consistent with the interpretation that the goblet-like cells are mucin-secreting epithelial cells, and that they are not sebaceous in origin, nor are they macrophages recruited to the tumor for clearance of apoptotic cell debris.

In all tumors the squamous component predominated and was mostly nonkeratinizing (Figure 4C), though keratinization was present in some regions in some tumors (Figure 4D). All tumors had a distinctive tubulo-cystic pattern of growth, with desquamated cells and inflammatory cells present centrally located within the tubulo-cystic structures (Figure 4E). Since the potential glandular component is not clearly malignant, it is uncertain whether these tumors meet strict criteria for adenosquamous carcinoma. Characteristic features of mucoepidermoid carcinoma are not clearly identified. These tumors represent invasive carcinomas, most in keeping with invasive squamous cell carcinoma with scattered goblet-like cells. The latter feature is of unclear morphological significance, given that similar tumors of murine or human conjunctiva have not been well characterized or described.

**Discussion**

Here we have exploited Cre/Lox technology to analyze the functions of αv integrins in the eyelid skin and conjunctiva. A central result of this work is that genetic ab-
Large bilateral eye tumors (this 850 bp band is significantly reduced. Instead, a 250 bp band representing the recombined cell line lacks endogenous and colleagues have shown that a human SCC-derived wild-type (αv) allele and mutant mice harbor an αvfloxed allele and one αv wild-type (αv) allele and mutant mice harbor an αvfloxed allele and one αv null (−) allele via deletion of exon one. The primer pair F1 and R amplifies the 350-bp genomic region spanning exon 4 and the 3′ loxP site. The primer pair F2 and R amplifies the region (850 bp) containing both the 5′ and 3′ loxP sites. PCR-based amplification of genomic DNA isolated from ear or eyelid tissues from control (GFAP-Creαvfloxed) mice. Ear and eyelid tumor samples from mutant (GFAP-CreαvloxPloxP) animals were also analyzed. The primer pair F1 and R, amplifies a 350 bp band, containing the 3′ loxP sequence. The intensity of this band is reduced in eyelid and eye tumor samples, due to cre-mediated recombination of the αv-flox allele. Identical genomic samples described in (B) were used with the F2 and R primer pair, which amplify an 850 bp band containing 5′ and 3′ loxP sites. In samples from control eye and mutant tumor, amplification of this 850 bp band is significantly reduced. Instead, a 250 bp band representing the recombined αv-flox allele (lower panel in A) is detected. D: A twelve month-old GFAP-CreαvloxPloxP, αvloxPloxP mutant mouse. Note the macroscopic tumor displaying obvious ulceration (arrow). E: An 18 month-old GFAP-CreαvloxPloxP, αvloxPloxP mutant mouse displaying large bilateral eye tumors (arrows). F: The mutant mouse in (E) with the skin removed to expose the skull and tumor masses encompassing both eyes (arrows).

αv Integrins and Epithelial Tumorigenesis

The αv integrin subunit heterodimerizes with five different β subunits, and at least three of these integrins, αvβ1, αvβ5, and αvβ6, are expressed at varying levels in normal and malignant epithelial cells of the skin.2,3,24 Watt and colleagues have shown that a human SCC-derived cell line lacks endogenous αv integrin expression, which most likely contributes to enhanced in vitro proliferation and survival properties.3,25 These data are consistent with our in vivo gene deletion results, and support our model that αv integrin negatively regulates normal epithelial cell proliferation, and that loss of αv integrin expression or function causes aberrant cell growth. Other reports show that increased expression of αv integrins, particularly αvβ6, in SCCs correlate with advanced tumor progression and poor patient prognosis.26 Furthermore, inhibition of αvβ6 integrin expression and function leads to reduced tumorigenesis and invasiveness.27 It is possible that αv integrin expression levels regulate distinct phases of tumor onset and progression. For example, reduced αv integrin expression or function, via epigenetic or post-translational modifications, may promote tumor initiation, whereas subsequent increased αv and β6 integrin gene expression may drive tumor growth and malignancy. Our mouse genetic data support a role for reduced integrin expression and function being necessary for tumor initiation. Tumor progression in the mouse model described in this paper may occur via integrin-independent pathways, or integrin-dependent pathways unrelated to αvβ6 integrin overexpression. Indeed, there is an extended latency period from the time of Cre-mediated gene deletion (embryogenesis, Figure 1) to the formation of grossly obvious eyelid tumors (12 to 18 postnatal months, Figure 3). Thus, αv integrin probably influences other critical growth regulatory cascades that...
are progressively altered following gene deletion; together, these events contribute to the onset and progression of eyelid SCC. We are currently investigating the molecular alterations that occur as a result of \( \alpha v \) gene deletion, for example, whether tumor suppressor or oncogene signaling pathways are dysregulated, and how these events collectively lead to SCC. All five murine \( \alpha v \) subunit genes that pair with \( \beta 1 \) have been ablated individually or in various combinations, yet none of the published studies reveals a phenotype that relates to SCC. Thus, it is likely that the combined loss of two or more \( \alpha v \)-containing integrins, eg, \( \alpha v \beta 6 \), and \( \alpha v \beta 8 \), may contribute to SCC. We are currently generating mice that lack multiple \( \beta \) integrin genes to address this possibility.

**Figure 4.** Genetic ablation of \( \alpha v \) integrin in the murine eye epithelium leads to tumors with histological characteristics of squamous cell carcinoma. H&E staining of eye tumor sections from GFAP-Cre\(^{-}\), \( \alpha v \)\(^{flox} \) mutant animals. A: Tumor compressing and invading globe of the eye (arrows). B: Tumor showing admixed squamous cells (arrowheads) and goblet-like cells (arrows). C: Tumor with predominantly squamous differentiation (arrows) and intraluminal apoptosis (asterisks). D: Tumor showing keratinization (arrow). E: Tumor tubulo-cystic structures with marked lumenal accumulation of desquamated tumor cells (arrows). F: Tumor invading skeletal muscle of the eye (arrows). G: Tumor showing biphasic squamous and goblet-like cell differentiation. The Alcian Blue mucin stain highlights goblet-like cells (arrows). H, I: H&E stained sections from the ocular region of an \( \alpha v \)\(^{flox} \) mouse that did not harbor the GFAP-Cre transgene. Note the normal cytoarchitecture of the conjunctiva (arrows in H) and eyelid skin epidermis (arrows in I).

**Figure 5.** A model for \( \alpha v \) integrin-mediated regulation of epithelial proliferation and homeostasis. \( \alpha v \) integrins expressed in basal epithelial cells of the conjunctiva and eyelid skin bind to the latent forms of TGF\( \beta 1 \) and \( \beta 3 \) (latent associated peptide-TGF\( \beta 1/3 \)) in the epidermal basement membrane. Integrin binding leads to activation of TGF\( \beta \) signaling and suppression of epithelial cell proliferation, likely via an autocrine loop. Genetic ablation of \( \alpha v \) integrin expression in basal epithelia causes dysregulation of TGF\( \beta \) growth inhibition, leading to epithelial cell hyperplasia and tumor progression.

**\( \alpha v \) Integrins and Functional Links with Transforming Growth Factor \( \beta \) Signaling in SCC**

\( \alpha v \) integrins bind to RGD tripeptide motifs within the latent associated peptides of transforming growth factor (TGF) \( \beta 1 \) and TGF\( \beta 3 \). Latent associated peptides non-covalently associate with TGF\( \beta 1/3 \) in the ECM and maintain
TGFβ in an inactive form. Both αvβ6 and αvβ8 integrins mediate the physical dissociation of latent associated peptides, leading to release of bioactive TGFβ1 and TGFβ3 from the ECM.29–32 TGFβ’s and their receptors have been shown to negatively regulate epithelial cell growth.33 A recent report reveals that selective ablation of TGFβ-receptor I signaling leads to development of admixed squamous cell carcinomas and mucocutaneous or mucosal carcinomas in the periorbital and perianal regions.34 Fuchs and colleagues more recently published a study showing that genetic ablation of the TGFβ receptor II gene in basal cells of the mouse skin epithelium (via the Keratin5-Cre transgene) results in SCC development in perinatal and perivaginal areas, and these results correlate with reduced expression of TGFβ receptor II in human SCC samples.35 The TGFβ receptor I/II knockout results are consistent with a previous report showing that mice lacking Smad4, an intracellular signaling protein regulated by TGFβ receptors, develop SCC of the skin.36 Interestingly, genetic deletion of αv integrins in mouse dendritic cells leads to colitis and colon tumor formation, and these events are mostly due to defective αvβ8 integrin-mediated TGFβ activation.13,16 These various data strongly support our model that αv integrins, via activation of TGFβ signaling events, normally serve to suppress epithelial cell growth (Figure 5). Genetic ablation of αv integrins or TGFβ signaling components dysregulate this balance, leading to epithelial cell hyperplasia and tumor progression.

In conclusion, our molecular genetic strategies reveal important functions for αv integrins in regulating epithelial cell proliferation and homeostasis in the eyelid skin and conjunctiva. To our knowledge, these are the first direct genetic data supporting a tumor suppressor-like function for αv integrins in epithelial cells. Since the expression of the GFAP-Cre transgene has not been detected in epithelial cells outside of the eyelid skin and conjunctiva, or other stratified epithelial organs, we cannot yet conclude that αv integrins play more general roles in suppressing basal epithelial cell growth. We are currently deleting αv integrin expression using other Cre transgenes that are expressed in a broader range of epithelial organs to test this possibility. These various integrin knockout models will likely be useful translational tools to study SCC onset and progression, as well as to test and develop novel therapeutic compounds to treat or prevent SCC of the skin.

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