Impaired Notch Signaling Promotes De novo Squamous Cell Carcinoma Formation

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Abstract

Signaling through Notch receptors in the skin has been implicated in the differentiation, proliferation, and survival of keratinocytes, as well as in the pathogenesis of basal cell carcinoma (BCC). To determine the composite function of Notch receptor-mediated signaling in the skin and overcome potential redundancies between receptors, conditional transgenic mice were generated that express the pan-Notch inhibitor, dominant-negative Mastermind Like 1 (DNMAML1), to repress all canonical [CBF-1/Suppressor of hairless/LAG-1 (CSL)-dependent] Notch signaling exclusively in the epidermis. Here, we report that DNMAML1 mice display hyperplastic epidermis and spontaneously develop cutaneous squamous cell carcinoma (SCC) as well as dysplastic precursor lesions, actinic keratoses. Mice expressing epidermal DNMAML1 display enhanced accumulation of nuclear β-catenin and cyclin D1 in suprabasilar keratinocytes and in lesional cells from SCCs, which was also observed in human cutaneous SCC. These results suggest a model wherein CSL-dependent Notch signaling confers protection against cutaneous SCC. The demonstration that inhibition of canonical Notch signaling in mice leads to spontaneous formation of SCC and recapitulates the disease in humans yields fundamental insights into the pathogenesis of SCC and provides a unique in vivo animal model to examine the pathobiology of cutaneous SCC and for evaluating novel therapies. (Cancer Res 2006; 66(15): 7438-44)

Introduction

Notch signaling regulates multiple cellular processes, including differentiation, proliferation, and cell fate decisions, required for organogenesis and tissue homeostasis (for review, see refs. 1, 2). Notch receptor-mediated signaling is cell type dependent and developmentally regulated, underscoring the importance of other molecular pathways that modulate activity of this signaling pathway (3, 4). In mammals, there are four transmembrane Notch receptors (Notch1–Notch4) that are activated via binding ligands of the Delta and Jagged families. In the canonical Notch signaling pathway, Notch receptor stimulation results in cleavage of the Notch intracellular domain, which translocates to the nucleus and forms a ternary complex with the transcriptional coactivator, mastermind-like (MAML) protein, as well as the DNA-binding protein, CSL, which directs DNA-binding specificity and ultimately target gene expression (5). To date, only a limited number of Notch target genes have been identified and characterized, including basic-helix-loop-helix proteins of the hairy and enhancer of split (Hes) and Hes-related transcription factor (Hrt) families, which generally act as transcriptional repressors (6).

Notch receptors are expressed in the skin, although their precise function(s) remain uncertain (for review, see ref. 7). In keratinocytes, activated Notch1 induces p21 expression in a CSL-dependent manner, leading to cell cycle withdrawal and terminal differentiation (8). In addition, Notch1 directly activates caspase 3, which is required for terminal differentiation of embryonic keratinocytes (9). Consistent with its role in promoting differentiation of keratinocytes, mice with an induced epidermal deletion of the Notch1 gene exhibit extensive epidermal hyperplasia and spontaneously develop cutaneous basal cell carcinoma (BCC; ref. 10). This finding led investigators to propose that, in the skin, Notch1 acts as a tumor suppressor (10, 11). Moreover, in mice with conditional epidermal inactivation of Notch1, chemical injury induced the formation of cutaneous lesions resembling both BCC and squamous cell carcinoma (SCC), in addition to inducing numerous papillomas (10).

Neoplasms originating from cutaneous epithelial cells, including BCC and SCC, are the most common cancers in the United States with annual incidences of nearly 1 million and 250,000 cases, respectively (reviewed in refs. 12–14). Unlike BCC, SCCs are biologically aggressive, arise from precursor lesions (actinic keratoses), and display metastatic potential with frequencies approaching 12.5%. Mutations in components of the sonic hedgehog (SHH) signaling pathway have been implicated in the etiology of both human and mouse BCC (15–18). By contrast, genetic mutations uniquely causing human SCC have not been identified (17). Understanding the role of Notch signaling in BCC and chemically induced SCC is fundamentally important, given the dramatic differences in the pathobiology and clinical manifestations of these common cutaneous malignancies.

To better understand the role of Notch1-4 receptor signaling in the skin, we generated mice expressing a dominant negative MAML1 (DNMAML1) protein to inhibit CSL-dependent Notch signaling in the epidermis. DNMAML1 mice exhibited multiple skin defects including diffuse alopecia, epidermal hyperplasia, and hyperkeratinization. Surprisingly, these mice developed spontaneous lesions resembling human SCC and actinic keratoses, but did not develop BCC. In contrast to normal epidermis, keratinocytes and lesional cells from DNMAML1 mutant mice expressed nuclear β-catenin and cyclin D1 in a pattern similar to that observed in human cutaneous SCC, suggesting a conserved role for these molecules in SCC. Taken together, these data strongly suggest that functional interactions between Notch signaling and β-catenin and cyclin D1 play critical roles in the pathogenesis of cutaneous SCC.

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Materials and Methods

Generation and characterization of SM22-Cre\(^+\)/DNMAML1\(^+\) conditional transgenic mice. SM22-Cre transgenic mice, expressing Cre recombinase under the transcriptional control of the 2.8-kb mouse SM22a promoter, have previously been described (19). DNMAML1\(^{+\text{f/f}}\) mice, heretofoe designated as DNMAML1\(^{+\text{a}}\) conditional transgenic mice, expressing the DNMAML1-enhanced green fluorescent protein (EGFP) fusion protein, were previously described (20). DNMAML1\(^{+\text{a}}\) mice in the FVB/N background were intercrossed with SM22-Cre transgenic mice in the CD1 background, generating mice expressing DNMAML1 under the transcriptional control of the SM22a promoter, designated SM22-Cre\(^+\)/DNMAML1\(^{+\text{a}}\), and control littermates lacking Cre recombinase, designated SM22-Cre\(^-\)/DNMAML1\(^{+\text{a}}\), in a mixed FVB/N/CD1 genetic background. For the cell fate mapping studies, SM22-Cre transgenic mice were intercrossed to the R26R indicator strain (21) and β-galactosidase activity was assessed in the informative offspring as previously described (19). To assess the endogenous pattern of SM22a gene expression during mouse embryonic development, SM22a-LacZ mice, in which the LacZ gene was knocked into the endogenous SM22a allele (22), were harvested during embryonic development and stained for β-galactosidase activity as previously reported (19). All mouse experimentation was done under approved protocols from the University of Pennsylvania Animal Care and Use Committee and NIH guidelines.

Human tissue. Formalin-fixed, paraffin-embedded tissue samples of invasive SCC from skin were collected from the dermatopathology archives of the University of Pennsylvania Department of Dermatology with Internal Review Board approval under protocol 704450.

Immunohistochemistry and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining. Mouse skin and tumor specimens fixed in 2% paraformaldehyde were subjected to either standard H&E staining or immunohistochemical staining with Texas red–conjugated secondary antibody and Hoechst counterstain. Primary antibodies used in the study include anti-GFP (1:100, Molecular Probes, Eugene, OR), anti-β-catenin (1:100, Sigma, St. Louis, MO), and anti–cyclin D1 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining of embryonic and adult tissue was done as described (19) with light eosin counterstain. Histology protocols may be assessed on the University of Pennsylvania Molecular Cardiology website at http://www.uphs.upenn.edu/mcrc/histology/histologyhome.html.

Results

Generation of conditional transgenic mice with epidermal-restricted inhibition of Notch signaling. To determine the function of CSL-dependent Notch signaling in skin, SM22-Cre transgenic mice were intercrossed with genetically engineered DNMAML1\(^{+\text{a}}\) mice, in which the cDNA encoding DNMAML1 fused to GFP was knocked into the ROSA locus (20, 23). Our group and others have reported that during postnatal development, SM22a promoter activity is restricted to vascular and visceral smooth muscle cells, including the smooth muscle–containing arrector pili muscle within adult skin (Fig. 1A, arrows; refs. 24, 25). Surprisingly, we also observed transient activity of the SM22a promoter in the epidermis of the embryonic mouse at midgestation. At embryonic day (E)12.5, in SM22a-LacZ embryos, in which the LacZ gene was “knocked in” to the endogenous SM22a allele (22), β-galactosidase activity (blue stain) is clearly shown in the epidermis (Fig. 1C and E) but staining was not observed in control littermates (Fig. 1B and D). Consistent with these findings, intercrossing SM22-Cre transgenic mice with R26R-LacZ indicator mice, which provides a fate map of cells in which the SM22a promoter was ever active, showed intense blue staining indicative of β-galactosidase activity in the epidermis of adult SM22-Cre\(^-\)/R26R-LacZ\(^+\) progeny (Fig. 1G), which was not observed in littermate controls (Fig. 1F). Importantly, long-term LacZ expression was observed in both interfollicular (basal and suprabasal layers) and follicular (outer and inner root sheath) epithelium (Fig. 1G, arrows). Because stem cells within the basal epidermis and the bulge region associated with the hair follicle outer root sheath (Fig. 1F, arrows) are required for normal keratinocyte renewal and hair follicle cycling (26), these data show that prenatal Cre activation must occur in at least a subset of the proliferating epithelial cells destined to contribute to the adult epidermal stem cell population.

Characterization of SM22-Cre\(^+\)/DNMAML1\(^{+\text{a}}\) mutant mice. When SM22-Cre transgenic mice were intercrossed with DNMAML1 conditional transgenic mice, viable SM22-Cre\(^-\)/DNMAML1\(^{+\text{a}}\) progeny were born in the anticipated Mendelian ratio. Beginning 2 to 3 weeks after birth, these mice exhibited diffuse loss of body hair and whiskers, and with further aging, hyperkeratinization of the tail was observed [Fig. 2A (top) and data not shown]. Alopecia progressed to near completion by 10 months of age (data not shown). Generalized growth retardation was also noted in

![Figure 1](http://www.aacrjournals.org/)

**Figure 1.** SM22a promoter activity in embryonic skin directs Cre recombinase expression in keratinocytes required for normal adult skin homeostasis. A, X-gal staining of backskin from an adult SM22a\(^{+\text{a}}\)/R26R mouse (arrows, arrector pili). B to E, X-gal staining of cranial sections from day (E)12.5 SM22a\(^{+\text{a}}\) (B) and SM22a\(^{+\text{a}}\)/R26R (C) littermate embryos. Arrowheads, surface epithelium. V4, V3, and L, fourth, third, and lateral ventricular spaces, respectively. C, arrow, basilar artery. Boxed regions in (B and C) are shown enlarged in (D and E), respectively, with brackets encompassing the surface epithelium. F and G, X-gal staining of backskin from control SM22-Cre\(^-\) × R26R (F) and SM22-Cre\(^-\)/R26R (G) mice in telogen phase. Staining of the interfollicular and follicular epidermis (arrows) and the bulge region (bracket) is shown. Arrowheads, arrector pili.
SM22-Cre+/DNMAML1+ mice. By contrast, SM22-Cre−/DNMAML1− control littermates exhibited normal body hair and size (Fig. 2A, bottom). Immunohistochemical staining for GFP, indicative of expression of the DNMAML1-GFP fusion protein, showed GFP expression (red color) in the follicular and interfollicular epidermis in SM22-Cre+/DNMAML1+ mice (Fig. 2E-G) but not in corresponding SM22-Cre−/DNMAML1− skin (Fig. 2B-D). Of note, the keratinized surface of the skin generates a nonspecific fluorescent signal in both the control and mutant mice. These observations, together with the Cre recombinase fate mapping data described above (Fig. 1G), confirmed that SM22α promoter–directed Cre recombinase expression results in epidermally restricted expression of DNMAML1 that begins during midgestation and persists throughout postnatal development.

To examine cellular alterations in the skin of SM22-Cre+/DNMAML1+ mice, the backskin of mutant and control mice was biopsied and subjected to histologic analyses. Multiple aberrant features were observed in the skin of SM22-Cre+/DNMAML1+ mice including (i) hypoplastic dermis with increased cellularity, (ii) epidermal hyperplasia, (iii) keratin cyst formation, and (iv) aberrant hair follicle cycling (Fig. 3C and D). By contrast, none of these abnormalities was observed in the skin of SM22-Cre−/DNMAML1− control littermates (Fig. 3A and B). Similar histologic findings have been reported in other mouse models of skin-deficient Notch signaling (10, 27–29), further substantiating the conclusion that DNMAML1 represses Notch signaling in skin.

**SM22-Cre+/DNMAML1** mutant mice develop de novo SCC. As early as 6 months of age, most SM22-Cre+/DNMAML1+ mutant mice began to develop hyperkeratotic cutaneous nodules, with all SM22-Cre+/DNMAML1+ mice exhibiting multiple lesions by 10 months of age. Figure 4A shows a representative 8-month-old SM22-Cre+/DNMAML1+ mouse exhibiting exophytic hyperkeratotic lesions along the dorsal midline [Fig. 4A (lesion I) and B], near the proximal right upper limb [Fig. 4A (lesion II) and F] and near the left foot [Fig. 4A (lesion III) and J]. Histologic examination of these keratinized masses revealed hyperproliferative keratinocytic lesions exhibiting cellular atypia and an invasive growth pattern diagnostic of SCC (Fig. 4C-E and G-I). In addition, microscopic examination of the foot lesion revealed intraepidermal keratinocyte dysplasia and parakeratosis, consistent with a precursor lesion resembling an actinic keratosis (Fig. 4J and K). To date, SCC lesions have been observed in 14 of 16 SM22-Cre+/DNMAML1+ mice with an average of two SCC lesions per mouse. Histologic analyses of additional, although infrequent, cutaneous masses in these mice revealed papillomas, inflamed infundibular cysts, and one cystic teratoma.
Nuclear β-catenin accumulates in the epidermis of SM22-Cre\(^+/\)DNMAML1\(^+\) mice and in human SCC. The development of cutaneous SCC in the SM22-Cre\(^+/\)DNMAML1\(^+\) mice raised the mechanistic question of what alterations in downstream signaling pathways occurred in response to pan-Notch inhibition in the epidermis. Disruptions in Wnt/β-catenin signaling have been implicated in the pathogenesis of cutaneous SCC (30–32). In addition, mice harboring an epidermal-restricted null mutation in Dnm1, recapitulating observations made in human cutaneous SCC.

Cyclin D1 is up-regulated in the skin of SM22-Cre\(^+/\)DNMAML1\(^+\) mice. Cyclin D1 is up-regulated by β-catenin signaling and has been shown to regulate entry into the cell cycle (33, 34). Given the increased β-catenin activation in cutaneous SCCs from both SM22-Cre\(^+/\)DNMAML1\(^+\) mice and human tumors, we examined cyclin D1 gene expression in the skin of SM22-Cre\(^+/\)DNMAML1\(^+\) mice and human biopsy samples of cutaneous SCC. In control mice, in which the epidermis typically lacks multiple suprabasilar layers, expression of cyclin D1 protein (red staining) was observed in the nuclei of basal epithelial cells, consistent with the proliferative properties of this undifferentiated cell layer (Fig. 5G, I, arrowhead). In the skin of SM22-Cre\(^+/\)DNMAML1\(^+\) mice, nuclear basal cell expression of cyclin D1 was also observed. However, in the skin of mutant mice, cyclin D1 was enriched in the nuclei of suprabasilar epidermal cells (Fig. 5F-L, arrowheads). This observation is consistent with a model in which suprabasilar keratinocytes that normally differentiate fail to exit the cell cycle and continue to proliferate, thereby constituting an aberrant cell population.

Given these findings, we next examined whether cyclin D1 expression was altered in SCC lesions that arose spontaneously in the SM22-Cre\(^+/\)DNMAML1\(^+\) mice. As shown in Fig. 6C, cyclin D1 was expressed in strands of lesional cells, strongly suggesting that there is an enhanced replicative pool of undifferentiated cells supporting tumor growth. Remarkably, this pattern of cyclin D1 accumulation was recapitulated in human SCC (Fig. 6D), suggesting a conserved
functional role for cyclin D1 in the pathogenesis of SCC. Taken together, enhanced levels of nuclear β-catenin and cyclin D1 accumulation in SM22-Cre+/DNMAML1+ epidermis suggests a molecular model in which loss of CSL-dependent Notch activity promotes aberrant suprabasilar keratinocyte proliferation facilitating SCC formation. Furthermore, the presence of nuclear β-catenin and cyclin D1 expression in SCC may be important for sustained tumor growth.

**Discussion**

In this study, we determined that selective inhibition of Notch signaling in the epidermis results in the spontaneous formation of cutaneous SCC as well as its precursor lesion, actinic keratosis. To our knowledge, this is the first report linking inhibition of CSL-dependent Notch signaling with the de novo development of SCC. Moreover, in SM22-Cre+/DNMAML1+ mice, accumulation of nuclear β-catenin and up-regulation of cyclin D1 gene expression...
were observed, findings recapitulated in biopsies of patients diagnosed with cutaneous SCC. These observations extend the proposed function of the Notch1 receptor as a tumor suppressor of BCC (10) and reveal that pan-inhibition of canonical Notch signaling promotes spontaneous SCC. Taken together, these observations strongly suggest that Notch signaling may play a critical role in the pathogenesis of both BCC and SCC, disease processes originating from epidermal cells that exhibit profoundly different clinical manifestations (12, 14).

Previous studies revealed that activation of Notch signaling blocks formation of human cervical, prostate, liver, and lung malignancies (for review, see ref. 11). By contrast, activation of Notch signaling leads to oncogenic transformation in human T-cell acute lymphoblastic leukemia and murine mammary tumors (35–37). It has also been recently reported that activation of Notch signaling promotes formation of human melanoma (38), a cutaneous malignancy with significant metastatic potential and a grave prognosis if not diagnosed early in its course (39). These apparent contradictory functions of Notch signaling show that the ultimate effect of Notch receptor activation and signaling is dependent on its precise cellular context. Notch signaling has been shown to influence cellular differentiation, proliferation, and survival in both physiologic and oncogenic contexts. Which of these variables are affected by loss of Notch signaling in SCC remains to be determined. Ultimately, elucidating the precise transcriptional targets of Notch in SCC will provide important insights into the ability of this pathway to protect against both SCC and precursor lesion development.

Insights into the molecular pathogenesis of cutaneous SCC are slowly emerging (17, 40, 41). Previous studies have shown that loss of nuclear factor-kB (NF-kB) activity, through enhanced I-kB expression, or depletion of SMAD4 in murine skin confers susceptibility to SCC, in part, through promotion of unbalanced keratinocyte proliferation (41–46). The demonstration that inhibition of canonical Notch signaling also promotes de novo SCC suggests that combinatorial signaling pathways targeting the keratinocyte underlie the pathogenesis of cutaneous SCC. In this regard, it is noteworthy that hyperproliferative epidermis was observed in response to both disrupted NF-kB and Notch signaling, suggesting that hyperproliferation of keratinocytes may play an obligatory role in the pathogenesis of cutaneous SCC or in some way predispose to malignant transformation of keratinocytes. Moreover, as in humans, actinic keratoses were observed in SM22-Cre+/DNMAML1+ mice, showing that inhibition of Notch signaling may underlie the pathogenesis of the premalignant lesion, as well as cutaneous SCC. As such, at least in this mouse model, Notch signaling seems to repress the premalignant lesion that gives rise to the malignant SCC lesion.

The observed accumulation of nuclear β-catenin in epidermal cells and its detection in SCCs observed in the SM22-Cre+/DNMAML1+ mutant mice supports previous studies showing that β-catenin signaling is regulated (i.e., repressed) directly or indirectly by Notch signaling (47, 48). Consistent with these findings, the demonstration that signaling-active β-catenin inhibits keratinocyte differentiation (49) and that enhanced levels of β-catenin are observed in SCC lesions (ref. 30 and references therein) supports a model wherein β-catenin-mediated Wnt signaling plays a critical role in malignant transformation of keratinocytes.

Understanding how inhibition of Notch1 in the skin gives rise to BCC while pan-inhibition of canonical Notch signaling in the skin gives rise to de novo development of SCC is a fundamentally important question that should provide important insights into the molecular mechanisms that govern the behavior of these two common skin malignancies. Despite intense investigation in this area, CSL-dependent Notch target genes have not been clearly identified in adult epidermis in vivo (8). However, the finding that expression of the CSL-dependent Notch target genes, HRT1, HRT2, and HRT3, is down-regulated, and not induced, in vascular smooth muscle cells harvested from SM22-Cre+/DNMAML1+ mice (which express DNMAML1 protein) exposed to Jagged1 ligand supports the conclusion that de novo formation of SCC resulted from perturbations in CSL-dependent Notch signaling (data not shown). In this regard, it is noteworthy that in the conditional Notch1-deficient mice, activated β-catenin, as well as increased levels of Gli2, a target of activated sonic hedgehog (SHH), was observed (10). In addition, decreased p21 levels were observed. Thus, it is tempting to speculate that the magnitude of β-catenin activation in response to Notch inhibition may differentially lead to BCC versus SCC. Indeed, the level of β-catenin signaling activity, in part, has been proposed to account for differences in tumor types derived from follicular keratinocytes (31, 50). Alternatively, inhibition of Notch1 function may activate and/or repress only a subset of the genes regulated directly or indirectly by the canonical Notch signaling pathway in keratinocytes, suggesting that specific Notch receptors expressed on the surface of the keratinocyte may mediate distinct and critical functions in restricting malignant potential.

To our knowledge, this is the first Notch-deficient murine model that develops de novo cutaneous SCC. It is noteworthy that this mouse model recapitulates multiple features of human SCC including (i) its association with hyperproliferative epidermis, (ii) association with precursor lesions (actinic keratoses), (iii) spontaneous development associated with increasing age, (iv) cellular histology and pathology, and (v) accumulation of nuclear β-catenin. Consistent with these findings, a previous study reporting the phenotype of mice harboring a conditional null mutation of the CSL gene selectively in the epidermis showed progressive loss of hair follicles and the generation of dermal keratin cysts (27). However, in contrast to SM22-Cre+/DNMAML1+ mice, spontaneous formation of SCC was not described in these mice (27). As inhibition of CSL and expression of DNMAML1 protein should in theory both inhibit canonical Notch signaling, it remains unclear why SCC was not observed in these CSL conditional mutant mice. This difference could be related to differences in specificity of the promoter driving cell lineage–restricted Cre-mediated recombination (Nestin-Cre’ versus SM22-Cre’), timing of Cre-mediated excision, and/or efficiency of Cre excision. Nevertheless, because they recapitulate multiple aspects of human SCC, SM22-Cre’/DNMAML1+ mice should serve as a valuable animal model to elucidate the molecular basis of SCC and to evaluate novel therapeutic strategies.

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