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Epidermal Notch1 Loss Promotes Skin Tumorigenesis by Impacting the Stromal Microenvironment

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SUMMARY

Notch1 is a proto-oncogene in several organs. In the skin, however, *Notch1* deletion leads to tumor formation, suggesting that Notch1 is a "tumor suppressor" within this context. Here we demonstrate that, unlike classical tumor suppressors, *Notch1* loss in epidermal keratinocytes promotes tumorigenesis non-cell autonomously by impairing skin-barrier integrity and creating a wound-like microenvironment in the skin. Using mice with a chimeric pattern of *Notch1* deletion, we determined that *Notch1*-expressing keratinocytes in this microenvironment readily formed papillomas, showing that Notch1 was insufficient to suppress this tumor-promoting effect. Accordingly, loss of other Notch paralogues that impaired the skin barrier also predisposed *Notch1*-expressing skin to tumorigenesis, demonstrating that the tumor-promoting effect of *Notch1* loss involves a crosstalk between barrier-defective epidermis and its stroma.

INTRODUCTION

Notch proteins are transmembrane receptors activated upon binding of transmembrane ligands and are implicated in many developmental and cellular processes, including carcinogenesis. Notch activation involves two sequential proteolytic cleavages releasing the Notch intracellular domain, a transcription regulator, into the nucleus (Lubman et al., 2004). Based on its role in most cancers involving Notch signaling (e.g., T-acute lymphoblastic leukemia), Notch1 is a proto-oncogene, driving carcinogenesis cell autonomously when hyperactivated or hyperstabilized (Radtke et al., 2006; Weng et al., 2004). In contrast, an opposite role for Notch1 is observed in skin keratinocytes, where it acts as a "tumor suppressor" (Koch and Radtke, 2007; Nicolas et al., 2003). The mechanism enabling Notch1 to deliver this unique function in skin remains controversial.

The vertebrate skin is a barrier-forming organ in which keratinocytes form a highly organized, stratified epithelium protecting the internal milieu from the outside environment. To achieve this, proliferating keratinocyte progenitors residing within the innermost (basal) layer of the epidermis constantly divide and replenish the upper barrier-forming layers (Clayton et al., 2007). Cells exiting the basal layer gradually commit to terminal differentiation in the spinous layer and, under normal conditions, complete their differentiation program, giving rise to granular and cornified layers (Fuchs and Raghavan, 2002; Jamora and Fuchs, 2002). Three Notch paralogues (*Notch1*, *-2*, and *-3*) are expressed in the epidermis and their activation is evident in suprabasal keratinocytes. Importantly, reduction in Notch signaling within keratinocytes impairs their ability to execute the terminal differentiation program, resulting in formation of a defective skin-barrier and death if the areas involved are sufficiently large (Blanpain et al., 2006; Demehri et al., 2008).

One of the major adverse consequences of Notch loss in epidermis is the development of skin tumors (Nicolas et al., 2003; Proweller et al., 2006), evoking the use of the term "tumor suppressor" to specifically describe the role of Notch1 in the epidermis (Nicolas et al., 2003). However, the exact mechanism underlying Notch1 tumor suppressor function, and why specific consequences of *Notch1* loss cannot be compensated for by

SIGNIFICANCE

In contrast to the current dogma, we demonstrate unequivocally that the non-cell autonomous consequences of defective barrier formation are responsible for the tumor-promoting effects of *Notch1* loss in mouse skin. Thus, individuals with subacute skin-barrier defects may also be prone to carcinogenesis upon exposure to initiating carcinogens like UV rays. As *Notch1* deletion in skin tumors enhanced their progression to invasive carcinomas, patients with benign hyperplasic skin lesions receiving γ-secretase inhibitor therapy may, therefore, be at additional risk. More broadly, given that chronic injury causatively effects the development of several human carcinomas, *Notch1*-deficient mice with mild skin-barrier defects can serve as an experimental model in which to study the tumor-promoting elements of chronic injury/wound and develop relevant therapies.

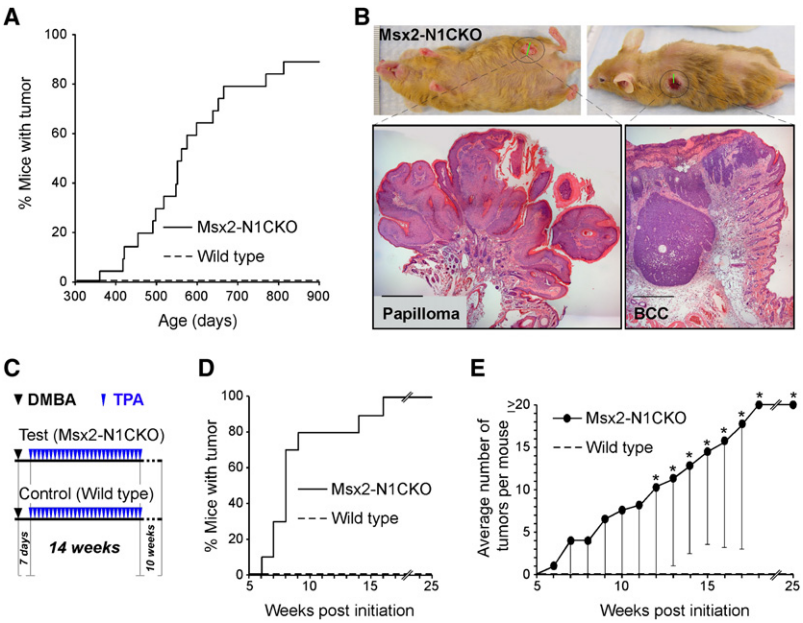

Figure 1. Msx2-N1CKO Mice Develop Skin Tumors Spontaneously and in Response to Chemical Carcinogens

(A) Spontaneous skin tumorigenesis of Msx2-N1CKO mice and their wild-type littermates (n = 20 for each group; $p < 0.0001$, log rank test).

(B) A representative Msx2-N1CKO mouse with two papillomas (left) and one BCC (right) at P593. Scale bars, 500 μm.

(C) Schematic diagram outlining the standard DMBA/TPA treatment protocol used for skin carcinogenesis studies. Two days prior to treatment with DMBA (black arrowhead), the back skin of the animals are shaved to ensure the hair follicles are in telogen (rest) phase.

(D) DMBA/TPA-induced skin tumorigenesis of Msx2-N1CKO and their wild-type littermates (n = 10 for each group; $p < 0.0001$, log rank test).

(E) The average number of papillomas per each tumor-bearing mutant mouse observed over a 25 week follow-up period. Beginning at 20 weeks after initiation, >50% of Msx2-N1CKO mice have more than 20 papillomas (*$p < 0.05$, Student's t test). These findings are confirmed in additional independent experiments. All error bars represent ± SD.

other Notch paralogues present in the skin (i.e., Notch2 and -3), are not fully understood. It has been proposed that the tumor suppressor activity of Notch1 reflects its unique ability to antagonize keratinocyte proliferation through one or more cell autonomous signaling mechanisms. Most of these findings were established in precancerous hyperplastic epidermis of Notch-deficient animals (Nicolas et al., 2003; Proweller et al., 2006) and did not examine the early changes following Notch loss. Other conclusions were based on in vitro studies with isolated keratinocytes (Devgan et al., 2005; Nguyen et al., 2006; Nicolas et al., 2003), failing to take into account the complexity of the skin microenvironment, including the contribution of other skin components to carcinogenesis. Notably, in vivo studies have revealed that loss of Notch signaling during embryogenesis induces epidermal hypoplasia and low proliferative capacity in the keratinocytes, proposing that reactive, i.e., secondary, hyperplasia accounts for the late epidermal hyperproliferation detected in adult Notch-deficient skin (Blanpain et al., 2006; Demehri et al., 2008). This also implies that the molecular changes outlined above may reflect secondary events following epidermal hyperplasia in Notch-deficient mice.

In this study, we investigate the mechanism underlying tumor development in *Notch1*-deficient skin in vivo using the multistage skin chemical carcinogenesis model (Zoumpourlis et al., 2003). In this well-established carcinogenesis model, treating the skin with an initiating carcinogen, 7,12-dimethylbenz[a] anthracene (DMBA), results in an activating mutation in the H-ras gene and creation of "initiated cells." Thereafter, the continual exposure of the skin to a tumor-promoting agent, tetradecanoylphorbol acetate (TPA), leads to expansion of the initiated cells and eventually to tumor development. In addition, we took advantage of two Cre-expressing transgenes. *K14CreERT* (Vasioukhin et al., 1999) allows us to generate mice in which we can control the timing of epidermal *Notch1* deletion relative to DMBA treatment. *Msx2-Cre* is ectopically expressed at embryonic day (E) 9.5 in clusters of ectodermal cells prior to the onset of skin morphogenesis, but its expression is never again detected in the epidermis after E13. This chimeric pattern of Cre expression allows us to generate mice with a chimeric pattern of *Notch1* deletion in skin keratinocytes with three types of epidermal territories: (1) clones of epidermal cells on dorsal and ventral midline with complete deletion of *Notch1*-floxed alleles showing alopecia; (2) territories with functional proteins expressed from undeleted floxed alleles exhibiting normal epidermal and hair growth; and (3) border regions between the two in which cells of both genotypes mingle (Demehri et al., 2008; Pan et al., 2004) (see Figure S1 available online). This chimeric animal model permits a direct comparison of cells with identical genetic backgrounds, other than the deleted alleles, in the same microenvironment.

RESULTS

Animals with Chimeric Loss of *Notch1* in the Epidermis Develop Skin Tumors

As reported in animals with 4-hydroxytamoxifen (4-OHT)-induced deletion of *Notch1* in the epidermis (Nicolas et al., 2003), most 20-month-old *Msx2-Cre; Notch1*$^{flox/flox}$ (Msx2-N1CKO) mice spontaneously developed one to four skin tumors each (Figure 1A). These lesions were predominantly composed of benign papillomas; however, 4 out of 41 spontaneous tumors characterized progressed to basal cell carcinoma (BCC; Figure 1B). To study the mechanism linking *Notch1* loss to skin carcinogenesis in an in vivo setting that permitted analysis of mutant and wild-type cells in the same environment, we examined the response of Msx2-N1CKO animals to the multistage chemical skin carcinogenesis model (Figure 1C). Treating 6- to 10-week-old Msx2-N1CKO and wild-type (*Notch1*$^{flox/flox}$) littermates with a single initiating dose of DMBA, followed by a twice weekly dose of TPA for 14 weeks (Nicolas et al., 2003), resulted in development of more than 20 papillomas/mouse in all DMBA/TPA-treated Msx2-N1CKO animals, whereas none of the control

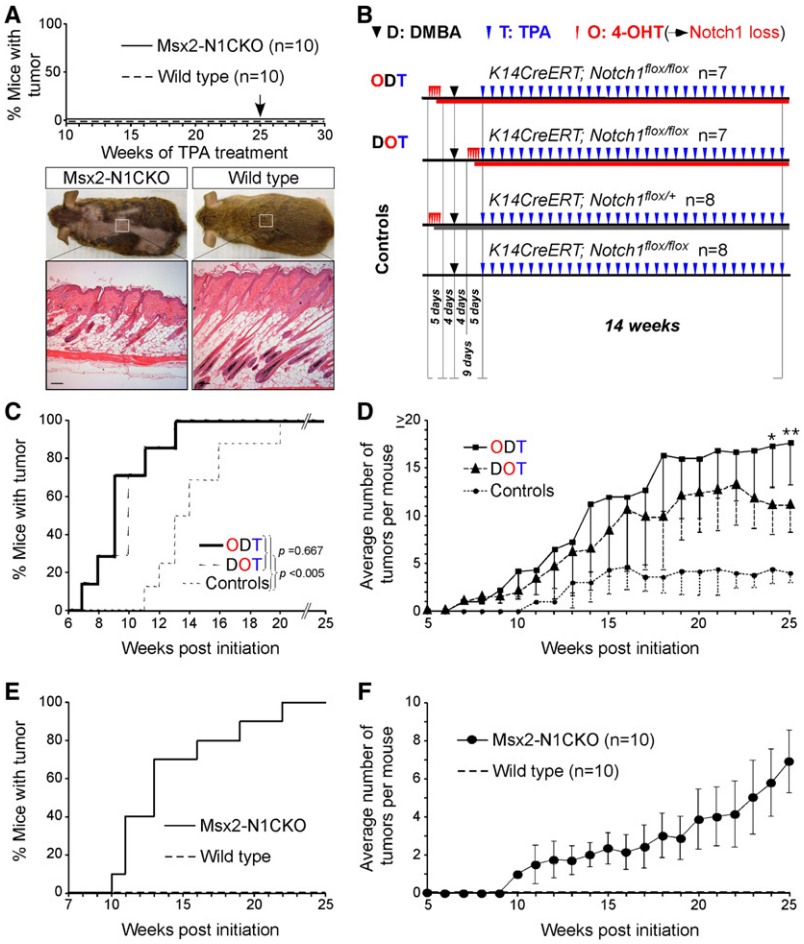

Figure 2. Epidermal Deletion of *Notch1* Leads Primarily to Skin Tumor Promotion

(A) Treating Msx2-N1CKO and wild-type skin with TPA (18 μg/mouse) twice weekly for 25 weeks (arrow) does not result in tumor growth in the mutant mice. An example of Msx2-N1CKO and wild-type skins with high doses of TPA (18 μg per mouse; arrow) twice weekly for 25 weeks. Scale bars, 100 μm.

(B) Schematic diagram detailing K14ERT-N1CKO treatment protocol. Six- to ten-week-old K14ERT-N1CKO mice are treated with 4-OHT for 5 days at the indicated points (red arrowheads) to induce *Notch1* deletion.

(C and D) *Notch1* deletion was induced by 4-OHT injections beginning 4 days after DMBA-mediated tumor initiation (DOT) induced skin carcinogenesis to a similar level as did *Notch1* deletion 9 days prior to DMBA treatment (ODT). Both *Notch1*-deficient cohorts develop skin tumors earlier (C) and in significantly higher numbers than controls ($p < 0.05$ for all tumor counts after week 9, Student's t test) (D). The ODT group has significantly more tumors than the DOT group at the last two time points (*p = 0.017; **p = 0.014, Student's t test).

(E and F) Msx2-N1CKO mice treated topically with one dose of DMBA (150 μg per mouse) develop skin tumors in the absence of TPA (E) starting at 10 weeks after DMBA treatment and gain a few papillomas per each tumor-bearing mouse (F). No papilloma is formed in DMBA-treated, wild-type littermates (n = 10 for each group; $p < 0.0001$, log rank test). These data are confirmed in additional independent experiments. All error bars represent ± SD.

animals developed any papillomas after 25 weeks of follow-up (Figures 1D–1E and Tables S1 and S2). These results reproduce the tumor phenotype reported (Nicolas et al., 2003) and establish that *Notch1* loss, even in a fraction of epidermal cells, is sufficient to sensitize the animals to chemical carcinogenesis.

Notch1 Loss Primarily Acts as a Tumor Promoter in the Epidermis

We next tested the impact of *Notch1* loss on distinct stages of skin tumor development (Zoumpourlis et al., 2003). The appearance of spontaneous tumors on Msx2-N1CKO skin could reflect a role for Notch1 in initiation, in which case tumors would be expected to develop earlier in Msx2-N1CKO mice exposed to TPA alone. However, 6- to 10-week-old Msx2-N1CKO animals treated only with a high dose of TPA twice weekly for 25 weeks developed hyperplasia but did not develop any tumors (n = 10; Figure 2A), indicating that deletion of *Notch1* did not act as a tumor initiator nor did its loss lead to the activation of one. Accordingly, we were unable to detect an elevated *Gli2* expression or elevated Wnt/β-catenin signaling (seen at 6 weeks of age or older) (Nicolas et al., 2003; Proweller et al., 2006) in 2-week-old Notch-deficient skin (Figure S2). However, given that the direct Notch1 targets in skin keratinocytes, Hes1 and Hey1, are repressors of HDM2 (Huang et al., 2004), *Notch1* loss might have led to elevated MDM2 and destabilization of p53, thereby facilitating H-ras-mediated transformation of DMBA-treated keratinocytes (Zhao et al., 2006). In addition, *Notch1* loss could contribute to tumor initiation by lowering $p21^{WAF1/Cip1}$ expression in keratinocytes (Nicolas et al., 2003; Rangarajan et al., 2001). Therefore, we examined the possibility that *Notch1* loss contributed to the fixation rate of DMBA-induced DNA mutation in basal keratinocytes. We treated 6- to 10-week-old *K14CreERT; Notch1*$^{flox/flox}$ (K14ERT-N1CKO) animals with 4-OHT to remove *Notch1* either before (OHT→ DMBA→ TPA, or ODT) or after (DMBA→ OHT→ TPA, or DOT) DMBA exposure (Figure 2B). As judged by time to tumor onset, both cohorts displayed a similarly elevated susceptibility to carcinogenesis over the 4-OHT-treated controls (*K14CreERT; Notch1*$^{flox/+}$), indicating that *Notch1* loss did not contribute to a $p21^{WAF1/Cip1}$- or p53-controlled checkpoint (Figure 2C and Table S1). Although the ODT cohort developed more tumors in the final 2 weeks of the follow-up period, both cohorts had comparable tumor counts during the first 23 weeks that were significantly higher than the wild-type tumor count (Figure 2D and Table S1). Although our animals were kept in an outbred background with variable strain susceptibility, nested ANOVA analysis showed that the differences reported in this study were solely conferred by the presence or absence of *Notch1* (Tables S3). Collectively, these data suggest that the main consequence of *Notch1* loss is in tumor promotion. Indeed, 100% of Msx2-N1CKO mice treated only once with DMBA (n = 10) developed tumors, whereas none of the wild-type controls (*Notch1*$^{flox/flox}$; n = 10) did ($p < 0.0001$; Figures 2E and 2F and Tables S1 and S4).

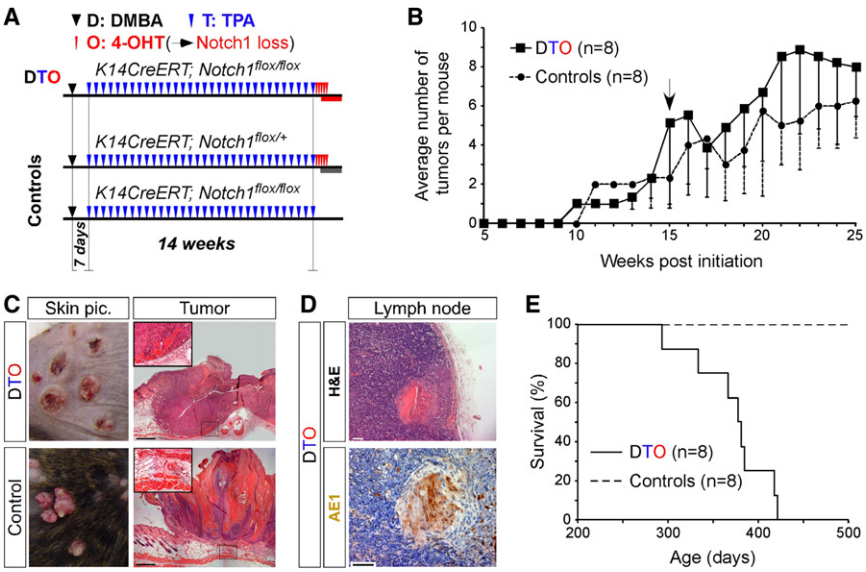

Figure 3. *Notch1* Loss in the Epidermis Causes Malignant Progression of Skin Tumors

(A) Schematic diagram outlining the method used to address the role of *Notch1* in tumor progression. *Notch1* deletion was induced after papilloma formation in *K14CreERT* background.

(B) Treatment of mice with 4-OHT starting after the last TPA application (arrow) does not impact the total number of tumors formed in the *Notch1*-deleted (DTO) or control animals (n = 8 for each group).

(C) A significant fraction (~50%) of tumors in DTO cohort progress to SCC, distinguishable on H&E-stained histological section. In contrast, papillomas on *Notch1*-expressing control skin show no sign of malignant progression. Note that global loss of Notch1 results in hair loss phenotype and that SCC cells in *Notch1*-deficient skin invade through the subcutaneous muscle layer (insets). Scale bars, 500 μm.

(D) Malignant skin cancer in DTO mice has metastatic features shown by the presence of $AE1^+$ epidermal keratinocytes in a draining lymph node of a representative DTO animal. Scale bars, 50 μm.

(E) Due to their severe skin cancers, DTO mice become moribund and demise prematurely ($p < 0.0001$, log rank test). All error bars represent ± SD.

As expected (Nicolas et al., 2003), subsets of papillomas from the ODT and DOT groups progressed to invasive squamous cell carcinoma (SCC) (Figure S3). Therefore, we examined the impact of *Notch1* loss on malignant progression. Intraperitoneal injection of 4-OHT into K14ERT-N1CKO and control mice after completion of the DMBA/TPA protocol (DTO) did not alter tumor numbers (Figures 3A and 3B). Interestingly, malignant conversion of papillomas to metastatic SCC was seen only in DTO animals (Figures 3C and 3D). On average, 50% of *Notch1*-deficient tumors progressed to SCC, which metastasized and necessitated euthanasia of the moribund mice (Figure 3E). Together, these in vivo cancer studies indicate that the primary role of Notch1 in suppressing skin tumors is in blocking tumor promotion and progression; the latter could be associated with reduction in p53 (Huang et al., 2004) or with elevated activity of Wnt and HH signaling in hyperplastic, *Notch1*-deficient cells (Nicolas et al., 2003).

Notch1-Deficient Epidermis Creates a Microenvironment Promoting Carcinogenesis

The tumor-promoting effect of *Notch1* deletion can be mediated by cell autonomous changes in the initiated cells that acquired H-ras mutations, by non-cell autonomous signals emanating from the surrounding environment that is responding to signals produced by *Notch1*-deficient keratinocytes (Lee et al., 2007), or by a combination of both. To determine which of these possibilities best describes the contribution of *Notch1* loss to skin tumorigenesis, we reanalyzed Msx2-N1CKO mice exposed to DMBA/TPA (Figure 1) and performed an intracomparison of adjacent wild-type and *Notch1*-deficient territories within each animal after DMBA/TPA treatment (Figure 4A). If Notch1 acted like a classical tumor suppressor, initiated cells expressing *Notch1* would not be able to form tumors. In contrast to this prediction, DMBA/TPA-treated Msx2-N1CKO; Rosa26R mice had a comparable number of Cre-negative (white) papillomas adjacent to the Cre-positive (blue) territory (Figure 4B). This is consistent with *Notch1*-expressing tumors developing in Msx2-N1CKO; Rosa26R animals. To confirm that tumors can arise from initiated cells that did not experience *Notch1* deletion, we performed direct amplification of the *Notch1* locus in tumor DNA randomly collected from DMBA/TPA-treated Msx2-N1CKO animals to estimate how many cells underwent *Notch1* deletion (Figure 4C and Figure S4). *Notch1* deletion was undetectable in 33 out of 91 benign exophytic papillomas analyzed by PCR with conditions sufficient to detect deletion in 1% of genome equivalents. Since a significant subset of papillomas contained more than 99% of genome equivalents contributed by wild-type cells, we concluded that these tumors arose from initiated cells that did not experience *Notch1* deletion. Because none of the wild-type littermates used in this study (n = 10) developed any tumors, this finding demonstrates that loss of *Notch1* provides a promoting, non-cell autonomous signal that can support papilloma formation from nearby initiated cells independent of their *Notch1* status. Given the absence of SCC among DMBA/TPA-treated Msx2-N1CKO animals and the global nature of Cre activation in K14ERT-N1CKO mice (Vasioukhin et al., 1999), we could not examine if tumor progression to SCC was a strict cell autonomous consequence of *Notch1* loss.

Epidermal Differentiation/Barrier Formation Defects Can Explain Tumor Promotion upon *Notch1* Loss

Loss of Notch signaling in the skin causes impaired epidermal differentiation, which in turn results in defective skin-barrier formation (Figure S5) (Blanpain et al., 2006; Demehri et al., 2008). After birth, the reactive epidermal hyperplasia masks the physical consequences of skin-barrier impairments (i.e.,

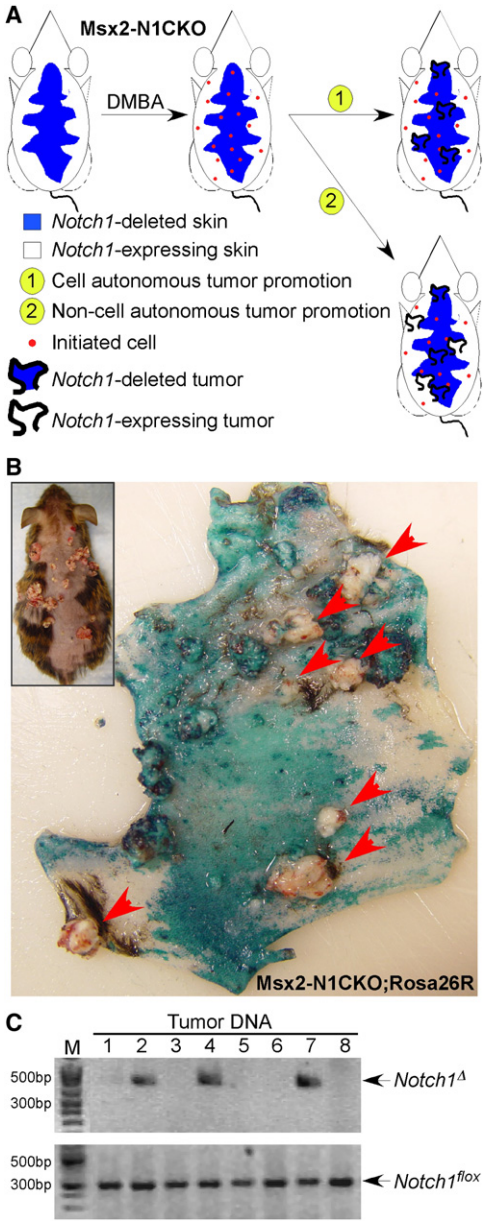

Figure 4. *Notch1* Deletion in Epidermal Keratinocytes Acts as a Non-Cell Autonomous Tumor Promoter

(A) The chimeric pattern of *Notch1* deletion in Msx2-N1CKO animals enables us to determine if the tumor-promoting effect of *Notch1* loss in the skin is the consequence of cell autonomous or non-cell autonomous changes. *Notch1*-expressing skin patches (white) in Msx2-N1CKO animals serve as an internal control to determine if *Notch1*-expressing initiated cells can form skin tumors in an environment modified by neighboring, *Notch1*-deficient cells. Considering that in this genetic background none of the DMBA/TPA-treated wild-type littermates developed tumors (Figure 1), tumor growth in white territory of Msx2-N1CKO skin would point to a non-cell autonomous tumor-promoting signal from the environment conditioned by *Notch1*-deleted (blue) cells.

(B) X-gal staining of representative Msx2-N1CKO;Rosa26R mouse treated with DMBA/TPA shows the presence of a significant number of completely white tumors (arrows) adjacent to *Notch1*-deleted (blue) skin territory. Note the comparable size and number of white and blue tumors, consistent with a dominant non-cell autonomous effect downstream of *Notch1* loss that results in tumor promotion in wild-type cells.

dye penetration or transepidermal water loss) to ensure survival in terrestrial life (Kuramoto et al., 2002). Nonetheless, thymic stromal lymphopoietin (TSLP) and antimicrobial peptide overexpression can serve as reliable biomarkers for postnatal barrier impairments (Aberg et al., 2008; Demehri et al., 2008; Kuramoto et al., 2002). Based on these criteria, a mild barrier defect is also detectable in Msx2-N1CKO skin at postnatal day (P) 9 (Figure S6A) (Demehri et al., 2008). The persistence of this barrier defect was confirmed by documenting the overexpression of antimicrobial peptides mRNA in the epidermis (Figure S6B) and upregulation of serum TSLP levels in adult Msx2-N1CKO mice (Figure S7).

Notch pathway-deficient skin present initially with an epidermal hypoplasia that resembles a chronic wound, thus recruiting an array of cellular responders to repair the site of the breach and resulting in the development of a reactive epidermal hyperplasia over time (Blanpain et al., 2006; Segre, 2006). Accordingly, Msx2-N1CKO epidermis was mildly hypoplastic at birth, but exhibited a significant stromal hyperplasia as early as P9 (Figure 5A). Following the proliferative changes in the dermis, significant epidermal hyperplasia developed in adult mutants (Figure 5A and Figure S8; Lee et al., 2007). Importantly, epidermal hyperplasia extended beyond *Notch1*-deleted territories and into adjacent *Notch1*-expressing epidermal regions (Figure 5B), indicative of a non-cell autonomous proliferative mechanism.

The major stromal responses to a breach in the skin barrier include infiltration of immune cells, activation of fibroblasts, and angiogenesis, all of which provide proliferative signals to keratinocytes as part of an integrated wound healing/barrier repair response (Segre, 2006; Werner et al., 2007). To determine the contribution of immune cells to reactive epidermal hyperplasia and tumor promotion in Msx2-N1CKO skin (Proweller et al., 2006), we examined the number and composition of dermal leukocytes in Msx2-N1CKO skin. An increase in $CD45^+$ leukocytes was evident in Msx2-N1CKO skin by P9 (Figure 5C), which was due predominantly to accumulation of $CD4^+$ T cells in the dermis of Msx2-N1CKO mice (Figure 5D and Figure S9). Prominent increase in number of $CD4^+$ T cells in dermis and peripheral blood of adult Msx2-N1CKO mice together with serum IgE elevation (Figure S10) is reminiscent of a mild atopic dermatitis (AD)-like allergic inflammation, which represents a typical immune response to skin-barrier defects (Segre, 2006). In addition, dermal mast cells, another component of AD-like inflammation (Navi et al., 2007), were significantly increased in Msx2-N1CKO skin (Figure S11). Mast cell activation can enhance skin carcinogenesis (de Visser et al., 2005). To examine if this was critical for tumor promotion, we impaired mast cells and neutrophils function by generating Msx2-N1CKO mice deficient for cathepsin C (dipeptidyl peptidase I [DPPI] [Pham, 2006]). DMBA/TPA treatment of *Msx2-Cre; Notch1$^{flox/flox}$; DPPI$^{-/-}$* (Msx2-N1CKO;DPPI$^{-/-}$), Msx2-N1CKO, DPPI$^{+/-}$, and wild-type littermates demonstrated that mast cells did not play a dominant role in tumor promotion since the inhibition of mast

(C) PCR analysis of DNA isolated from eight randomly selected Msx2-N1CKO tumors from one individual confirms that *Notch1* locus is intact (i.e., *Notch1* is not deleted) in >99% of tumor-forming cells in a substantial number of tumors (Δ, deleted allele; M, molecular marker), demonstrating that these tumors have arisen from *Notch1*-expressing initiated cells (see Figure S4 for the sensitivity of the method).

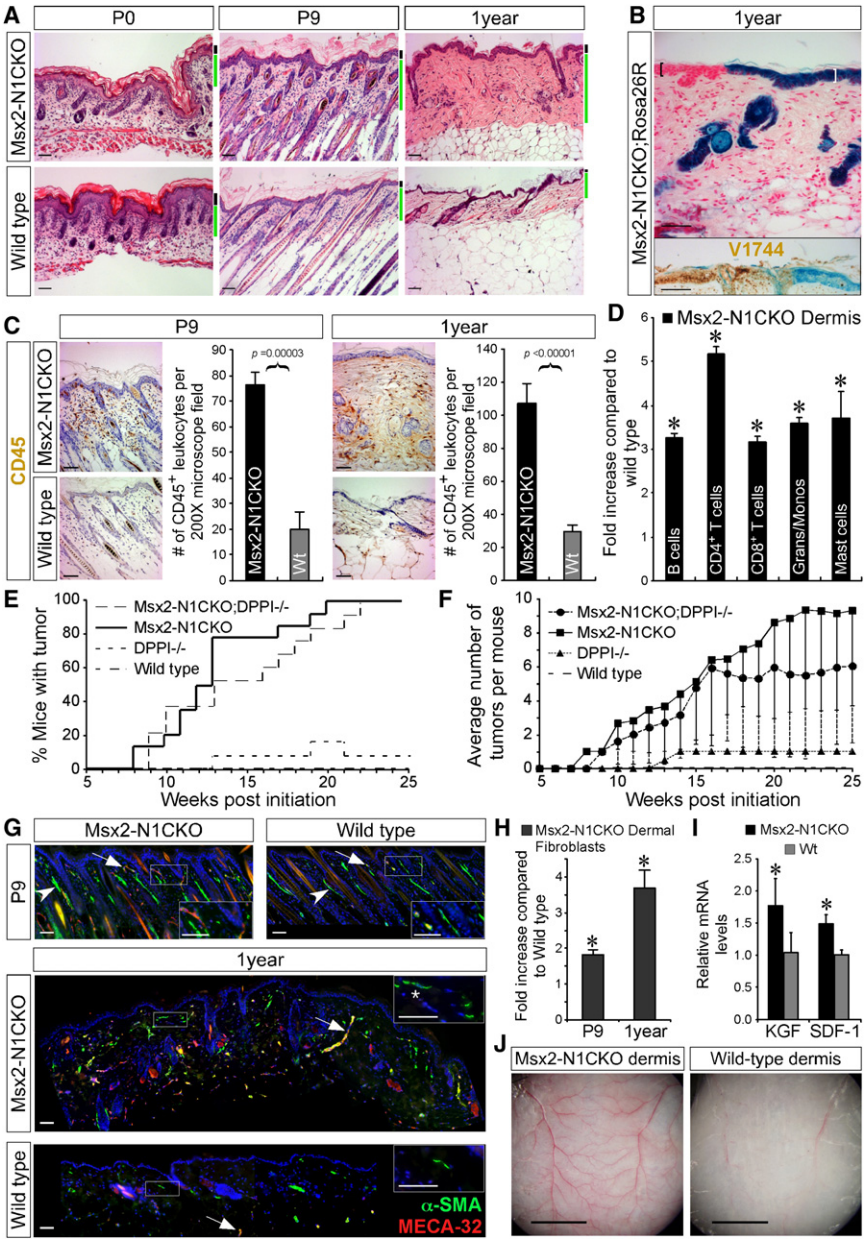

Figure 5. *Notch1*-Deficient Epidermis Creates a Wound-like Microenvironment in the Skin

(A) H&E staining of Msx2-N1CKO and wild-type skin at birth (P0), P9, and 1 year of age demonstrates the progression from relatively hypoplastic epidermis in newborn Msx2-N1CKO animals to distinct epidermal hyperplasia at 1 year. The dermal hyperplasia, however, is evident as early as P9. The bars on the left side of each picture show the average thickness of epidermis (black) and dermis (green) across three 100× microscope fields. Scale bars, 50 μm.

(B) X-gal staining of 1-year-old Msx2-N1CKO skin shows similar degree of hyperplasia (brackets) in *Notch1*-expressing epidermis (red) and in the *Notch1*-deleted territory (blue), consistent with the presence of a dominant proliferative microenvironment in Msx2-N1CKO skin. Immunohistochemical staining for the V1744 epitope (detecting activated Notch1) confirms the presence of Notch1 activity in hyperplastic epidermis adjacent to the *Notch1*-deficient epidermis. Scale bars, 50 μm.

(C) Leukocyte infiltration into the dermis of Msx2-N1CKO is detectable as early as P9 using CD45 pan-leukocyte marker (average number of $CD45^+$ leukocytes in six randomly selected 200× microscope fields is tabulated). Scale bars, 50 μm.

(D) FC and cell counting methods are used (Figure S9) to calculate the fold increase of dermal immune cells in 6- to 8-month-old Msx2-N1CKO animals relative to their wild-type controls (*$p < 0.0001$, Student's t test).

(E and F) Treating Msx2-N1CKO;$DPPI^{-/-}$ (n = 13), Msx2-N1CKO (n = 14), DPPI−/− (n = 12), and wild-type (*Notch1*$^{flox/flox}$; *DPPI*$^{+/-}$; n = 12) littermates with DMBA/TPA excludes a dominant tumor-promoting role for mast cells in *Notch1*-deficient skin. *Notch1*-deficient mice are significantly more prone to DMBA/TPA-induced tumor growth than their *Notch1*-expressing counterparts ($p < 0.0001$, log rank test), and no major difference was detected between Msx2-N1CKO;$DPPI^{-/-}$ and Msx2-N1CKO animals with regard to time to tumor onset (E) ($p = 0.31$, log rank test) or average number of tumors per each tumor-bearing mouse (F) ($p = 0.12$ at week 25 after initiation, Student's t test).

(G) Double staining of skin with α-SMA and an endothelial marker (MECA-32) demonstrates the abundance of myofibroblasts in adult *Notch1*-deficient skin (asterisk). Note the expansion of microvasculature in *Notch1*-deficient skin as early as P9 (arrows). The arrowheads point to α-SMA-expressing arrector pili muscles. Scale bars, 50 μm.

(H) The number of dermal fibroblasts is significantly increased in *Notch1*-deficient skin. Dermal fibroblasts are counted in six random 200× microscope fields, and the average ratio of Msx2-N1CKO to wild-type fibroblast counts at P9 and 1 year of age is presented (*$p < 0.001$ compared to wild-type, Student's t test).

(I) Dermal fibroblasts in *Notch1*-deficient skin overexpress two major keratinocyte proliferative signals, SDF-1 and KGF, which are detected by qRT-PCR on total skin mRNA samples (but not in epidermal mRNA [data not shown]) from P9 Msx2-N1CKO and wild-type mice (*$p < 0.001$ compared to wild-type, Student's t test).

(J) Dermal view of dorsal skin shows expanded vascular network in 1-year-old Msx2-N1CKO skin compared to a wild-type littermate. Scale bars, 1 cm. All error bars represent ± SD.

cell/neutrophil function did not completely ameliorate the tumor-promoting effect of the stroma in *Notch1*-deficient background (Figures 5E and 5F). When nested ANOVA was applied to account for the contribution of gender-based differences, a trend toward delayed tumor onset and a significant reduction in tumor counts at 20 weeks after DMBA treatment were detected in Msx2-N1CKO;$DPPI^{-/-}$ relative to Msx2-N1CKO mice (Table S5). Thus, mast cells contribute to tumor promotion, but loss of this contribution can be largely overcome by other tumor-promoting factors.

Msx2-N1CKO epidermis overproduces TGF-β1 and TGF-β2 (Lee et al., 2007), the major diffusible keratinocyte factors that recruit Gr-$1^+$$CD11b^+$ myeloid suppressor cells (Yang et al., 2008) and activate dermal fibroblasts (Werner et al., 2007).

Gr-1$^+$CD11b$^+$ myeloid suppressor cells, which promote carcinogenesis by suppressing immunosurveillance apparatus, including cytotoxic CD8$^+$ T cells (Bronte et al., 2000), were increased in spleen of adult Msx2-N1CKO mice (Figure S12). Activated fibroblasts, known to play an important role in tumor promotion (Orimo and Weinberg, 2006), could also secrete matrix-modifying proteins and mitogens leading to epidermal hyperproliferation, establishing a "vicious cycle" (Lee et al., 2007). In agreement with a vicious cycle involving TGF-β signaling (Werner et al., 2007), Msx2-N1CKO dermal fibroblasts were increased relative to wild-type at P9 and 1 year of age, acquiring a myofibroblast phenotype as indicated by the expression of α-smooth muscle actin (α-SMA) in dermis of adult *Notch1*-deficient mice (Figures 5G and 5H and Figure S13). Accordingly, the expression levels of two fibroblast-derived epidermal mitogens, keratinocyte growth factor (KGF or FGF-7) and stromal cell-derived factor 1 (SDF-1 or CXCL-12) (Szabowski et al., 2000; Werner et al., 2007), were modestly but significantly upregulated in *Notch1*-deficient skin at P9 (1.6-fold, $p < 0.001$; Figure 5I). SDF-1 mRNA remained elevated in 6- to 8-month-old Msx2-N1CKO skin relative to wild-type littermates, confirming the persistent overexpression of this factor over time (Figure S14). Of note, the dermal vasculature also showed increased branching and dilation in P9 and adult Msx2-N1CKO skin, underscoring the gestalt of non-cell autonomous events contributing to the tumor phenotype (Figures 5G and 5J). Taken together, the consequences of persistent skin-barrier defects in Msx2-N1CKO mice create a wound-like, proliferative microenvironment capable of driving epidermal hyperplasia and carcinogenesis over time (Alberts et al., 2002; Eming et al., 2007; Parkinson, 1985).

All Notch Paralogues Involved in Barrier Formation also Participate in Suppressing Skin Carcinogenesis

Loss of either *Notch2* (*Msx2-Cre; Notch2$^{flox/flox}$* or Msx2-N2CKO) or *Notch3* (*Notch3$^{-/-}$* or N3KO) in the skin had no phenotypic consequences and, accordingly, no spontaneous epidermal tumors appeared over the entire life span of these animals ($n > 10$ for each genotype; Figure S15A). Furthermore, the response of *Notch2*- or *Notch3*-deficient mice to DMBA/TPA carcinogens was indistinguishable from the wild-type littermates, reflecting their respective strain's baseline susceptibility (Figures S15B and S15C). This result could reflect a unique contribution of Notch1 to tumor suppression, perhaps by maintaining *p21$^{WAF1/Cip1}$* expression (Devgan et al., 2005; Nguyen et al., 2006; Nicolas et al., 2003; Okuyama et al., 2004). Alternatively, if tumor promotion is mainly a consequence of an impaired epidermal differentiation/barrier formation (the "defective barrier" hypothesis), the lack of tumor susceptibility upon the deletion of *Notch2* or *Notch3* could be because their loss, unlike *Notch1*, did not compromise the skin barrier.

To differentiate between these possibilities, we first assessed the contribution of Notch2 and Notch3 to the Notch1 "tumor suppressor" function by analyzing an allelic series in which *Notch2* and *Notch3* alleles were progressively removed on the Msx2-N1CKO background. Removal of *Notch2* and *Notch3* in *Notch1*-deficient skin exacerbates the barrier defects (Demehri et al., 2008). In agreement with the defective barrier hypothesis, this stepwise removal of *Notch2* and *Notch3* alleles in Msx2-N1CKO animals resulted in progressive enhancement of epidermal hyperplasia at P9 (Figures 6A and 6B). We also noticed a tight correlation between time to spontaneous tumor onset and global Notch dose in keratinocytes (Figure 6C). This demonstrates the existence of an additive function for Notch paralogues in suppressing skin tumors as long as the overall Notch dosage is reduced below a threshold; however, it does not determine whether Notch1 makes a unique contribution to tumor suppression in addition to its shared effect on proper skin-barrier formation.

To examine if Notch1 has any unique tumor-suppressing activity sufficient to prevent tumor formation in barrier-impaired skin, we generated animals lacking *Notch2* and *Notch3* while retaining the expression of *Notch1* (*Msx2-Cre; Notch2$^{flox/flox}$; Notch3$^{-/-}$* or Msx2-N2N3CKO). P9 Msx2-N2N3CKO epidermis overexpressed TSLP and antimicrobial peptides, demonstrating that keratinocytes lacking Notch2 and Notch3 formed a defective skin barrier (Figures 7A and Figure S6). TSLP overexpression persisted in adult Msx2-N2N3CKO mice and was accompanied by serum IgE elevation that signified the development of a subacute AD-like inflammation in response to persistent barrier defects in these animals, similar to Msx2-N1CKO mice (Figures 7B and 7C and Figures S6 and S10B). Importantly, Msx2-N2N3CKO epidermis retained normal expression of *p21$^{wAF1/Cip1}$*, confirming the context-specific role of Notch1 in regulating *p21$^{wAF1/Cip1}$* expression (Figure 7A) (Nicolas et al., 2003). Nonetheless, deletion of *Notch2* in the skin of *Notch3* null animals resulted in skin hyperplasia (Figure 7D) and spontaneous tumors in the skin of adult *Notch1*-expressing Msx2-N2N3CKO animals (Figures 7E and 7F). As predicted from the defective barrier hypothesis, exposure of Msx2-N2N3CKO mice to DMBA/TPA resulted in a significantly higher tumor burden than that seen in control littermates (Figures 7G and 7H), with many tumors progressing to SCC (Figure 7I). Thus, blocking tumor progression is a *p21$^{wAF1/Cip1}$*-independent, shared function of the three Notch receptors. Taken together, these findings indicate that the presence of Notch1 and p21$^{wAF1/Cip1}$ is not sufficient to protect barrier-defective skin from chemical carcinogens and instead demonstrates that the tumor phenotype mirrors the progressive defects in barrier formation and keratinocyte differentiation.

DISCUSSION

The fundamental observation that *Notch1* deletion in epidermal keratinocytes causes skin carcinogenesis is a clear deviation from Notch1's role as a proto-oncogene in several other organs (Koch and Radtke, 2007). We examined the mechanism underlying the tumor-prone behavior of *Notch1*-deficient skin in mice with a global or chimeric deletion pattern in their epidermis. We established that Notch1 resembled most tumor suppressors in that its loss was not involved in the initiating event of multistage skin carcinogenesis (Zoumpourlis et al., 2003) by deleting *Notch1* either before or after DMBA treatment in the *K14CreERT* system. However, *Notch1* loss could effectively substitute for TPA in the chemical carcinogenesis paradigm, establishing unequivocally that its loss acts as a tumor-promoting event. Delaying *Notch1* deletion in K14CreERT mice until after the tumor-promotion stage of carcinogenesis demonstrated that late deletion of *Notch1* contributed to malignant progression of

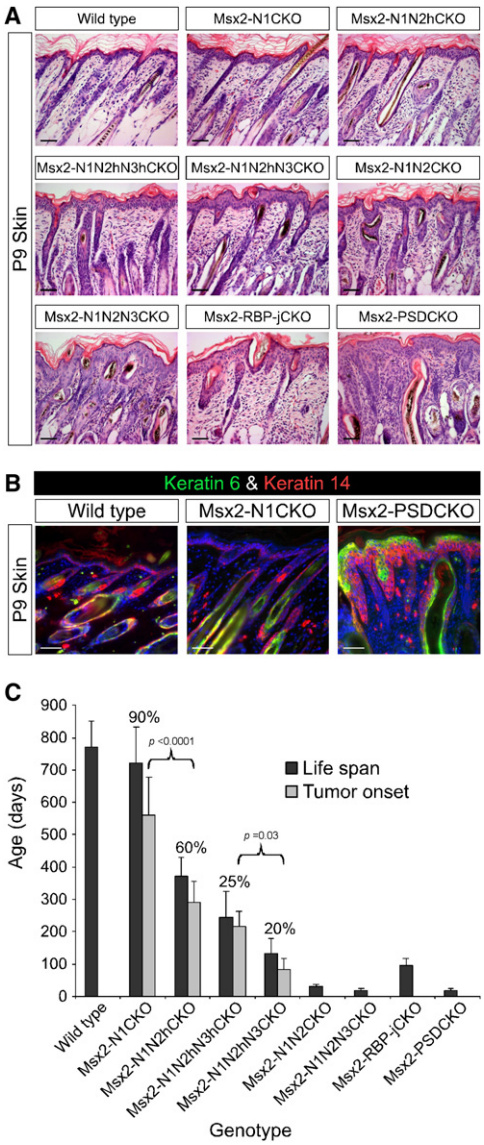

Figure 6. Stepwise Removal of Notch Paralogues in Msx2-N1CKO Animals Leads to Progressively Worse Epidermal Hyperplasia that Accelerates the Onset of Spontaneous Skin Carcinogenesis

(A) Skin histology of compound Notch-deficient mice at P9 demonstrates progressive epidermal hyperplasia, hyperkeratosis, and dermal hyperplasia as more *Notch* alleles are deleted. Note the disorganized epidermal cells with atypia in Notch pathway-deficient skin (i.e., Msx2-N1N2N3CKO and Msx2-PSDCKO). Scale bars, 50 μm.

(B) Although Msx2-N1CKO epidermis appears relatively normal at P9, Msx2-PSDCKO skin that lacks all Notch signaling shows severely hyperplastic epidermis, which overexpresses keratin 14 in suprabasal keratinocytes (marking epidermal hyperplasia) and expresses keratin 6 (marking epidermal dysplasia). Note that Msx2-PSDCKO skin is severely hypoplastic at birth (Figure S5) (Demehri et al., 2008); Msx2-PSDCKO skin has thus undergone an acute reactive hyperplasia in 9 days to repair its severe skin-barrier defect. Scale bars, 50 μm.

(C) Progressive reduction in Notch signal dosage reduces the life span (Demehri et al., 2008) and time to spontaneous tumor onset. Lifelong monitoring of 10 to 20 mice for each genotype reveals that severity of skin pathology (shown in A) correlates inversely with lifespan, time to tumor onset, and tumor penetrance (i.e., the percentage of animals with a given genotype that developed tumors) in compound Notch-deficient mice. Significant differences in average time to tumor onset between adjacent genotypes are marked by brackets. Although the shorting in life span for most genotypes is due directly to their intrinsic skin phenotype including exfoliation, bleeding, and infection, Msx2-N1N2CKO, Msx2-N1N2N3CKO, and Msx2-PSDCKO animals die shortly after birth due to a lethal blood disorder (Demehri et al., 2008). Note that Msx2-N2CKO and *Notch3*$^{-/-}$ (N3KO) mice have normal skin and behave like wild-type. Abbreviations used are as follows: *Msx2-Cre/+; Notch1*$^{flox/flox}$ (Msx2-N1CKO), *Msx2-Cre/+; Notch1*$^{flox/flox}$*; Notch2*$^{flox/+}$ (Msx2-N1N2hCKO), *Msx2-Cre/+; Notch1*$^{flox/flox}$*; Notch2*$^{flox/+}$*; Notch3*$^{+/-}$ (Msx2-N1N2hN3hCKO), *Msx2-Cre/+; Notch1*$^{flox/flox}$*; Notch2*$^{flox/+}$*; Notch3*$^{-/-}$ (Msx2-N1N2hN3CKO), *Msx2-Cre/+; Notch1*$^{flox/flox}$*; Notch2*$^{flox/flox}$ (Msx2-N1N2CKO), *Msx2-Cre/+; Notch1*$^{flox/flox}$*; Notch2*$^{flox/flox}$*; Notch3*$^{-/-}$ (Msx2-N1N2N3CKO), *Msx2-Cre/+; RBP-j*$^{flox/flox}$ (Msx2-RBP-jCKO), and *Msx2-Cre/+; PS1*$^{flox/flox}$*; PS2*$^{-/-}$ (Msx2-PSDCKO). All error bars represent ± SD.

benign papillomas, a phenotype that is observed upon loss of p53 but not loss of p21$^{WAF1/Cip1}$ (Weinberg et al., 1999), a specific Notch1 target in the skin (Rangarajan et al., 2001). Taken together, we have conclusively determined that the main effect of *Notch1* loss is to provide the initiated cells with a proliferative signal to form tumors and proceed to invasive carcinoma.

The proliferative signal that lies downstream of *Notch1* loss could be originated from within the initiated cells, substantiating Notch1's role as a classical tumor suppressor in skin keratinocytes (Nicolas et al., 2003). Alternatively, this signal could be delivered by the skin microenvironment reacting to *Notch1* loss in the epidermis (Lee et al., 2007; Orimo and Weinberg, 2006; Vauclair et al., 2007; Watt et al., 2008). The system we studied allowed us to distinguish between these two possibilities; the chimeric pattern of *Notch1* deletion by *Msx2-Cre* created neighboring territories of *Notch1*-expressing and *Notch1*-deficient keratinocytes coexisting in the same microenvironment. Examining a large number of tumors isolated from DMBA/TPA-treated Msx2-N1CKO mice clearly demonstrated that tumors comprised mostly (>99%) of *Notch1*-expressing cells were as likely to form as tumors comprised predominantly of *Notch1*-deleted cells in the same environment. Thus, *Notch1* loss in the epidermis generates a non-cell autonomous signal, promoting tumorigenesis from any initiated cell exposed to the microenvironment conditioned by *Notch1*-deficient keratinocytes. This finding emphasizes the importance of the environment as an active contributor to tumor development (Bissell and Radisky, 2001) by showing that it can be the primary source of proliferative signals to initiated cells.

To determine the identity of the tumor-promoting microenvironment formed as a consequence of *Notch1* deletion in keratinocytes, we reexamined the earliest effects of *Notch1* loss on the skin. As previously shown, loss of Notch signaling leads to impaired keratinocyte proliferation/differentiation culminating in epidermal cell loss and defective skin-barrier function at birth (Blanpain et al., 2006; Demehri et al., 2008; Rangarajan et al., 2001). Therefore, we examined the hypothesis that *Notch1*-deficient skin encompassed a chronic wound-like microenvironment developing in response to barrier defects, which were the direct consequence of *Notch1* deletion in the epidermis (Blanpain et al., 2006; Demehri et al., 2008). Indeed, the dermis of *Notch1*-deficient skin contained the critical components of an activated stroma responding to the breach in the skin barrier including inflammatory cell infiltrate, activated fibroblasts, and expanded vasculature (Mueller and Fusenig, 2004). To further

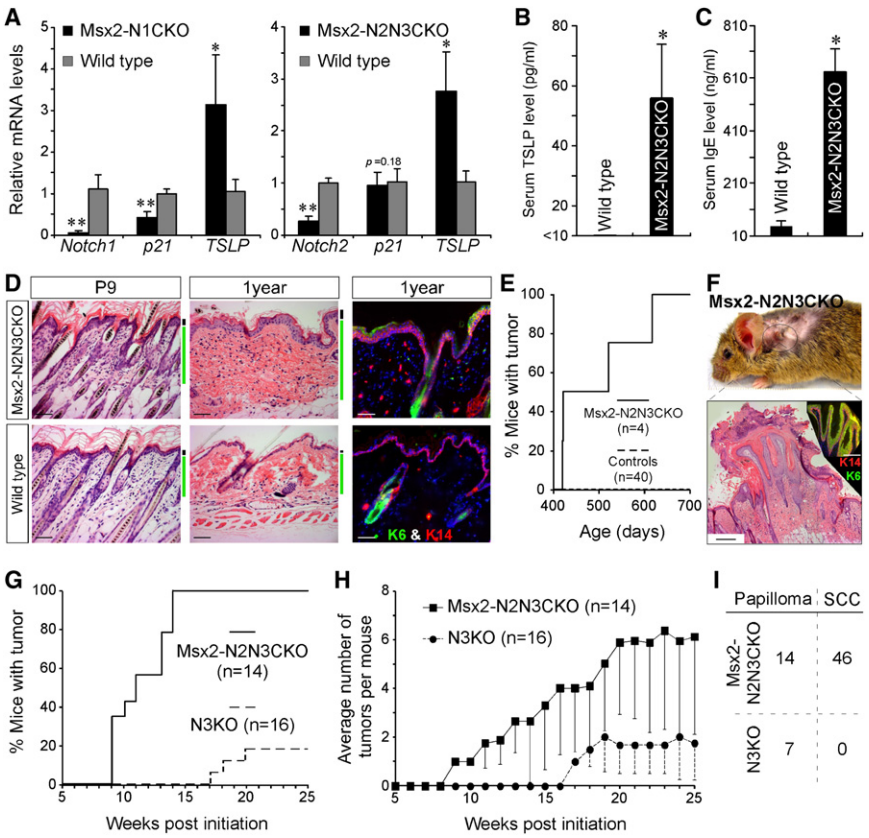

Figure 7. Mice Lacking Notch2 and Notch3 in the Skin Develop Skin-Barrier Defect, Epidermal Hyperplasia, and Skin Tumors

(A) TSLP overexpression (a biomarker for postnatal skin-barrier defects [Demehri et al., 2008]) is seen in both *Notch1*-deficient and *Notch2/3*-deficient epidermis. mRNA for qRT-PCR analysis is isolated from epidermis of P9 Msx2-N1CKO, Msx2-N2N3CKO, and their wild-type littermates. $p21^{wAF1/Cip1}$ expression (a direct target of Notch1), which is reduced in *Notch1*-deficient epidermis and thought to be essential for tumor formation in *Notch1*-deficient skin (Nicolas et al., 2003), is not altered in Msx2-N2N3CKO epidermis. However, *TSLP* mRNA level is highly elevated in both Msx2-N1CKO and Msx2-N2N3CKO relative to their wild-type controls (*p < 0.001; **p < 0.0001, Student's t test).

(B) Serum TSLP levels remain elevated in adult (6 to 8 months old) Msx2-N2N3CKO animals implying the persistence of defective skin-barrier in these mice (*p < 0.01, Student's t test).

(C) The presence of a mild allergic inflammation responding to skin-barrier defects in adult Msx2-N2N3CKO mice is evident based on their elevated serum IgE levels (n = 4 for each group; *p < 0.01, Student's t test).

(D) H&E staining of *Msx2-Cre; Notch2*$^{flox/flox}$*; Notch3*$^{-/-}$ (Msx2-N2N3CKO) and wild-type skin at P0, P9, and 1 year of age shows a Msx2-N1CKO-like pattern of dermal hyperproliferation starting at P9 and subsequent epidermal hyperplasia in 1-year-old mutant skin. The bars on the left side of each picture show the average thickness of epidermis (black) and dermis (green) across three 100× microscope fields. Keratin 6 (K6) and keratin 14 (K14) immunofluorescence staining shows distinct epidermal hyperplasia with dysplastic changes in 1-year-old Msx2-N2N3CKO skin. Scale bars, 50 μm.

(E) While none of *Notch2*$^{flox/flox}$*; Notch3*$^{-/-}$ (N3KO) or wild-type controls develop any skin tumor, Msx2-N2N3CKO mice develop spontaneous skin tumors over time (n = 4; p < 0.0001, log rank test).

(F) A representative Msx2-N2N3CKO mouse (P418) is shown to demonstrate that the spontaneous tumors in Msx2-N2N3CKO skin are benign papillomas; H&E-stained Msx2-N2N3CKO papilloma is also examined using K6 and K14 immunostaining (bottom). Scale bar, 200 μm.

(G) Treating Msx2-N2N3CKO and N3KO littermates with DMBA/TPA results in tumor formation in all Msx2-N2N3CKO mice (n = 14). In contrast, only 3 out 16 DMBA/TPA-treated N3KO animals develop tumors (p < 0.0001, log rank test).

(H) The average number of tumors per each tumor-bearing Msx2-N2N3CKO mouse is significantly higher than that among N3KO controls over the 25 week experimental period (p < 0.05 for all tumor counts after week 8, Student's t test).

(I) Analysis of all tumors present at week 25 after initiation show that N3KO control mice have only developed papillomas. In contrast, the majority of skin tumors in DMBA/TPA-treated Msx2-N2N3CKO skin progress to SCC as confirmed by histological examination. All error bars represent ± SD.

demonstrate that tumor promotion was the consequence of an activated stroma responding to a general breach in the skin barrier, we showed that mice lacking Notch2 and Notch3 in their epidermis also developed skin tumors. This is in contrast to a mechanistic model proposing that cell autonomous oncogenic changes, specific to *Notch1* loss (Rangarajan et al., 2001), are the initial events mediating tumorigenesis in *Notch1*-deficient skin. We find that the tumors in *Notch1*-deficient skin are the end product of a complex interaction between a barrier-defective epidermis and its underlying stroma, which creates a tumor-promoting feed-forward loop. This Notch1-independent, barrier-dependent phenotype distinguishes Notch1 from classical tumor suppressors. Accordingly, we predict that any mouse model with mild chronic skin-barrier defects will also be prone to skin tumorigenesis.

Fibroplasia, angiogenesis, and inflammation are stromal elements intimately linked to wound repair (Martin, 1997). These cellular changes are also closely associated with neoplastic transformation (Bissell and Radisky, 2001; Mueller and Fusenig, 2004). It is proposed that the microenvironment of the non-healing wound/defective skin barrier could be a risk factor for carcinogenesis (Eming et al., 2007). This association is supported by chemical carcinogenesis studies showing that tumors grow at the edges of skin wounds (Parkinson, 1985). In addition, it is suggested that chronic injury can predispose various organs to cancer (Bissell and Radisky, 2001) and there is clinical evidence linking chronic skin wounds to BCC and SCC (Nguyen and Ho, 2002). For instance, leg ulcers significantly increase the risk of SCC in patients (Baldursson et al., 1995). Nonetheless, experimental evidence establishing that stromal changes in chronic wound microenvironment can drive skin carcinogenesis is lacking (Eming et al., 2007). Therefore, Msx2-N1CKO and Msx2-N2N3CKO skin present a model demonstrating that a lengthened stromal attempt to repair a non-healing wound or a persistent skin-barrier defect predisposes the skin to carcinogenesis. We speculate that the plurality of the cellular effectors (i.e., fibroplasia,

angiogenesis, and inflammation) responding to breaches, in skin-barrier collectively contribute to tumor promotion in this model.

We have previously identified several of these factors. Matrix metallopeptidases (MMP8 and MMP9) are elevated in *Notch1*-deficient skin at P9 as is osteopontin (Demehri et al., 2008). All these stromal-derived factors are potential tumor promoters (Pazolli et al., 2009; van Deventer et al., 2008). In addition, dermal fibroblasts in *Notch1*-deficient skin are overproducing SDF-1 and KGF that directly stimulate keratinocyte proliferation; this is reminiscent of carcinoma-associated fibroblasts known to promote tumor development from a non-tumorigenic cell population (Bhowmick et al., 2004; Orimo and Weinberg, 2006). Furthermore, accumulation of immune cells and development of a subacute inflammation in Notch1-deficient skin, triggered by cytokines/chemokines released from barrier-defective epidermis (Demehri et al., 2008; Segre, 2006; Yoo et al., 2005), have been shown to promote skin carcinogenesis (Johansson et al., 2008). From this large pool of tumor-promoting stromal cells/factors present in *Notch1*-deficient skin, we examined the contribution of a single component (Mast cells), which has been previously deemed critical in skin carcinogenesis (Coussens et al., 1999; de Visser et al., 2005; Tlsty and Coussens, 2006), and asked if the components listed above act redundantly or are all required individually. Our results are consistent with the possibility that removal of any single stromal component would not significantly alter the tumor-promoting effect of the wound microenvironment. Therefore, we propose that the tumor promotion in *Notch1*-deficient skin results from the additive contributions of fibroplasia, angiogenesis, and inflammation. The cumulative effect of these factors on skin carcinogenesis in the presence of severe barrier defects that cause a full-blown inflammatory disease (i.e., AD) remains a topic for future investigation. Taken together, *Notch1*- and *Notch2/3*-deficient mice demonstrate that stroma of a chronic skin wound is analogous to tumor stroma and can be used to determine the specific contribution of each stromal component to tumor development, a worthy question that falls outside the scope of the current study.

In conclusion, the persistent barrier defects in Notch-deficient skin, which resemble chronic wounds, recruit several mesenchymal components necessary to repair the barrier. In turn, the vascularized and growth factor-rich stroma provides initiated cells with nutrients and proliferative signals that can directly promote tumor formation. Thus, Notch1 is not a classical tumor suppressor that solely exerts its effects cell autonomously (e.g., promotes cell death or cell-cycle arrest). Notch-deficient mice provide instead a suitable system in which to dissect out the molecular mediators and the cellular interactions that are responsible for oncogenic effect of chronic wound/tumor stroma. Based on such an analysis, new therapeutic targets can be identified in the tumor microenvironment that will be useful in developing molecular therapies for cancers of skin and perhaps other organs (Albini and Sporn, 2007).

EXPERIMENTAL PROCEDURES

Generation of Mutant Mice

All the mice were maintained in the Washington University animal facility according to animal care regulations, and the Animal Studies Committee of Washington University approved the experimental protocols.

The mutant strains of mice analyzed in the current study were generated following the protocol described previously (Pan et al., 2004). All the animals were maintained in mixed C57BL/6 and CD1 genetic backgrounds, which were overall resistant to DMBA/TPA skin carcinogenesis. In some experiments, remnant contributions from 129sv and FVB strains might have also been present. In all cancer experiments, age-matched littermates were compared and nested ANOVA was used to confirm that strain-based differences did not confound our analysis. In studies related to spontaneous carcinogenesis and longevity, mice were monitored regularly for onset, number, and size of tumors and any sign of failure to thrive. Moribund mice are euthanized and skin, tumors, and lymph nodes are harvested.

Chemical Skin Carcinogenesis Studies

For DMBA/TPA experiments in *Msx2-Cre* background, mutant mice and their age-matched littermate controls were treated with standard protocols for skin chemical carcinogenesis models as previously described (Nicolas et al., 2003). Further details are presented in Supplemental Experimental Procedures.

Histology, Immunohistochemistry, and Flow Cytometry (FC)

For hematoxylin and eosin (H&E), toluidine blue, and immunostaining using paraffin-embedded tissue sections, skin, tumor, and lymph node samples from various mutant and wild-type animals were fixed in 4% paraformaldehyde in PBS, dehydrated with ethanol, and embedded in paraffin, which were then sectioned at 5 μm. X-gal staining was done on the skin prior to fixation with 4% paraformaldehyde as previously described (Pan et al., 2004). Antibodies used for immunohistochemistry are listed in the Supplemental Experimental Procedures. For FC analysis, single-cell suspensions from dermis, peripheral blood, and spleen were prepared as described previously (Demehri et al., 2008). Dermal cells were isolated using a Brinkmann Tissue Chopper and crude collagenase (Sigma) digestion for 90 min at 37°C. Single-cell suspensions were stained with antibodies listed in the supplemental information.

ELISA and Immunoblotting

Serum TSLP concentrations were measured using Quantikine mouse TSLP kit (R&D Systems). Serum IgE was measured using Mouse IgE ELISA kit (Immunology Consultants Laboratory Inc.). Epidermal samples were collected in NP40 lysis buffer as previously shown (Lee et al., 2007; Nicolas et al., 2003). Protein lysates were run on SDS-PAGE gels after adjusting for protein concentration and analyzed using anti-active β-catenin antibody (Upstate Biotechnology) and total β-catenin (BD Biosciences).

Dye Penetration Assay

To detect defects in skin-barrier function (Hardman et al., 1998), intact E18.5 embryos were stained in X-gal (pH 4.5) for 12 hr at 37°C. After X-gal staining, the embryos were washed in PBS three times and photographed with a digital camera.

PCR and Quantitative RT-PCR

Conventional PCR for *Notch1* allele was performed on genomic DNA isolated from skin tumors of DMBA/TPA-treated Msx2-N1CKO mice using KlenTaq10 (DNA Polymerase Technology) supplemented with 1.3 M final concentration of betaine (amplification cycles = 32). Quantitative RT-PCR (qRT-PCR) was performed on mRNA isolated from skin and epidermis of Msx2-N1CKO, Msx2-N2N3CKO mice and their wild-type littermates as previously described (Lee et al., 2007). The primers used are listed in Table S7.

Statistical Analysis

The studies in this report were conducted in outbred cohorts of mice resembling human population. To minimize the effect of susceptibility differences due to genetic background on tumor phenotype observed in each study, age-matched littermates were used as controls. We used power analysis to estimate the number of mice needed in each group to reach statistical significance (Table S8). In addition, to confirm that the differential tumor parameters we measured were conferred by status of gene (e.g., *Notch1*) deletion and not by heritable factors (strain) or gender, we used nested ANOVA (SPSS; Tables S2–S6). Further details are presented in Supplemental Experimental Procedures. "Time to tumor onset" and "survival" data were analyzed using log

rank test to determine significant differences. Tumor counts and other quantitative measurements were assessed using Student's t test. These quantitative data are presented as mean ± SD for each measured parameter.

SUPPLEMENTAL DATA

Supplemental Data contain Supplemental Experimental Procedures, 15 figures, and eight tables and can be found with this article online at http://www.cell.com/cancer-cell/supplemental/S1535-6108(09)00178-0.

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