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Expression of Snail in Epidermal Keratinocytes Promotes Cutaneous Inflammation and Hyperplasia Conducive to Tumor Formation

Fei Du¹, Yoshikazu Nakamura¹, Tuan-Lin Tan¹, Pedro Lee¹, Robert Lee², Benjamin Yu², and Colin Jamora¹,²

Abstract

Although metastasis is the most lethal consequence of tumor progression, comparatively little is known regarding the molecular machinery governing this process. In many carcinomas, there is a robust correlation between the expression of the transcription factor Snail and a poor prognosis, but the contribution of this protein to the metastatic process remains unresolved. Interestingly, the prolonged expression of Snail in epidermal keratinocytes is sufficient to recapitulate early features of metastasis. However, it does so without inducing a complete epithelial-mesenchymal transition (EMT), a developmental phenomenon mediated by Snail that is extensively invoked as the mechanism fueling tumorigenesis. Instead, we found that the local invasiveness of keratinocytes is the consequence of the recruitment and activity of macrophages. Moreover, keratinocyte proliferation is the product of an IL-17/IL-6/Stat3 signaling module initiated by activated resident γδT cells in the transgenic skin. Together, these phenotypes prime the transgenic skin for the formation and metastasis of tumors in response to chemically induced carcinogenesis. Thus, the contribution of Snail to the progression of carcinomas is largely through the creation of a hyperproliferative and inflammatory niche that facilitates tumor development and dissemination.

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Introduction

A decisive factor in the multistage process of metastasis is the early step of local invasion of carcinoma cells (1). However, the mechanisms coordinating the increased motility of proliferating cancer cells remain elusive. Members of the Snail family of transcription factors have garnered widespread interest in this context, as they are expressed in a variety of carcinomas (2, 3) and are associated with recurring or metastasizing tumors (4). Snail proteins have a well-established role in embryogenesis during which they mediate a process known as an epithelial-mesenchymal transition (EMT) to facilitate tissue formation (5). The Snail-mediated EMT causes cells to lose their epithelial characteristics such as E-cadherin–mediated adhesion and polarity while adopting phenotypes of mesenchymal cells such as an increased migratory capacity (6). Given the similarities to its effects in development, it is widely extrapolated that Snail functions similarly in the metastasis of somatic cells during tumorigenesis (7). The ability of Snail to induce an EMT in multiple cancer cell lines supports this notion, but the absence of in vivo evidence that this likewise occurs in cancerous cells of the body (8) suggests that additional mechanisms are operational in this disease.

We previously found that Snail is expressed during budding morphogenesis of the hair follicle, wherein proliferating keratinocytes in the epidermis invade into the underlying dermal compartment of the skin (9). Moreover, transgenic expression of Snail in the epidermis of young mice leads to features commonly seen in cutaneous cancers (10). This suggests that additional mechanisms are operational in this disease.

Generation of Snail transgenic mice

Mice engineered to express the Snail transgene in the epidermis was previously described (9). All animal work was approved and adhered to the guidelines of IACUC.
Transwell cell migration and invasion assays
For the cell migration assay, 5 x 10^5 Raw264.7 cells (ATCC) were seeded in Transwell inserts with an 8.0-μm pore (Corning) with DMEM + 10% heat-inactivated FBS. CM from the epidermis of P7 mice was added to the bottom chamber at a 1:2 dilution in medium with or without 1.6 μg/mL of M-CSF neutralizing antibody (R&D Systems) or goat IgG control antibody. After 8-hour incubation, cells were stained with 0.1% Crystal Violet. The membrane inserts were removed and mounted on a slide. For the keratinocyte invasion assay, the Transwell inserts were coated with Collagen I (Sigma) and 1 x 10^5 HaCat cells (ATCC) were used. CM from thioglycollate-elicited peritoneal macrophages stimulated with (TAM CM) or without (Mac CM) 20 ng/mL of IL-4 (eBioscience) was collected and added to the bottom chamber and incubated for 14 hours.

Proliferation assays
After overnight starvation, primary mouse keratinocytes were trypsinized and resuspended in mouse keratinocytes medium with 2% FBS. A total of 1,500 cells were inoculated in 96-well dishes with or without 10 ng/mL of IL-6 (eBioscience), 25 ng/mL of IL-17 (R&D Systems), 2 μg/mL of IL-6 neutralizing antibody (R&D Systems), or 50 μmol/L of STAT3 inhibitor peptide (Calbiochem). Cell numbers were measured by Cell Titer 96 Aqueous One Solution (Promega) as suggested by the manufacturer.

Dexamethasone and indomethacin treatment
Mice were injected subcutaneously with a mixture of 0.05 mg/kg of dexamethasone (Sigma-Aldrich) and 0.05 mg/kg of indomethacin (Fluka) starting from newborn mice for 5 consecutive days. Control mice were injected with vehicle (0.4% ethanol in PBS).

Histology, in situ hybridization, and immunohistochemistry
Mouse skin or lymph nodes from WT and Snail transgenic animals were either frozen in OCT (Tissue-Tek) or embedded in paraffin depending on the application. Paraffin sections were prepared for histology and counterstained with hematoxylin and eosin-Y (H&E). Antibodies used were anti-phospho-Akt, c-Jun, CD31, and phospho-STAT3 (Tyr 705) all from Cell Signaling, CD44, fibronectin, phospho-NFκB (Ser276; Cell Signaling), CD3 (BD Biosciences), MAC-1 (BD Biosciences), and CD206. For nuclear staining, Hoechst 33342 (Calbiochem) was added in a final concentration of 1 mg/mL to the secondary antibody dilution. Immunofluorescence (IF) was detected using rhodamine-X or FITC-conjugated secondary antibodies (Jackson Immunoresearch) or expression was developed using the Vectastain ABC kit (Vector Labs) according to the manufacturer’s instructions. Images were acquired on an Olympus BX51 microscope with an Olympus DP70 camera. A 40 x 1.3 UPPlan FL N objective (Olympus) was used for acquisition.

Quantitative real-time PCR
Total RNA was extracted from whole skin of WT (n = 5) and Snail transgenic (Tg; n = 5) mice at postnatal day 7 (P7) using Trizol reagent (Invitrogen) according to manufacturer’s instructions. Similarly, epidermis from P7 WT (n = 5) and Tg (n = 5) mice was isolated with dispase treatment and total RNA was isolated using the Trizol protocol. cDNA was synthesized by reverse transcription using oligo-dT as primers (Superscript III kit; Invitrogen). Real-time PCR analysis was carried out with previously described primers (12). Experiments were carried out in triplicate from cDNA isolated from 5 different animals.

Zymography
Presence of active MMP-9 and MMP-2 was detected using a previously described protocol (13).

Two-stage chemically induced skin carcinogenesis
Seven- to 8-week-old mice were subjected to the 2-stage skin chemical carcinogenesis protocol as previously described (14), using 400 nmol of DMBA as the initiating agent and 10 nmol of TPA as the promoting agent.

Results
The extensive attention paid to the role of Snail in tumorigenesis is partly due to its expression in numerous carcinomas (3) including those of the skin (Supplementary Fig. S1). Interestingly, we found that a transgenic mouse engineered to overexpress Snail in epidermal keratinocytes shares some features with these carcinomas including an elevated proliferative index and local invasiveness (9). We, therefore, investigated the extent to which the Snail transgenic skin recapitulates the biochemical features of the metastatic program. We found that several factors known to promote tumor dissemination are upregulated in the Snail transgenic skin of neonatal mice (Fig. 1A). Among these are the activated Akt kinase, which is necessary for many events of the metastatic pathway (15), and c-Jun/AP-1, which has been linked to invasive properties of aggressive breast cancer cells (16).

The growth of tumors within somatic tissues imposes an increased metabolic burden that is met by an increased supply of nutrients delivered by the targeted growth of new blood vessels to the cancer cells. Moreover, these vessels provide a route for metastatic dissemination by providing a site for entry into the circulation (17). The level of CD31 staining that denotes endothelial vessels is markedly increased in the transgenic dermis, indicating augmented vascularization (Fig. 1A). This phenotype is assisted by an increase in the level of the proangiogenic factor, vascular endothelial growth factor (VEGF), and matrix metalloproteinase-9 (MMP-9), which facilitates remodeling of the local extracellular matrix and directional growth of the blood vessel (Fig. 1B; ref. 18). This restriction of MMP-9 to the dermal compartment of the skin is unexpected, as it has previously been shown that introduction of Snail into an epithelial cell line is sufficient to induce expression of MMP-9 (19). In addition, MMP-2, which is also known to facilitate angiogenesis, has relatively low activity in both the wild-type (WT) and transgenic dermis (Fig. 1B).

Increasing evidence shows that the transcription factor Stat3 plays an indispensable role in various aspects of onco
Figure 1. Presence of metastasis-associated markers in the Snail transgenic skin. A, WT (left) and Snail transgenic (Snail Tg; right) skin sections were subjected to IF with antibodies recognizing keratin 5 (K5) in red and phospho-Akt, c-Jun, and the blood vessel marker CD31 in green. Dotted lines denote the basement membrane, which separates the epidermis (epi) and hair follicle (hf) from the dermis (der). B, reverse transcription PCR of VEGF (left) from RNA extracted from 2 WT and 3 transgenic skin samples, with GAPDH as a loading control, and zymography of MMP-9 and MMP-2 activity (right column). C, immunohistochemistry of phosphorylated Stat3 (pStat3). D, IF of CD44 expression. Bars, 30 μm.
macrophages (TAM). Evidence of this is seen in the Snail transgenic skin by the coexpression of the lectin CD206 on a subset of macrophages (Fig. 2C) and the presence of other markers of TAMs (Supplementary Fig. S3B and C). Interestingly, it has been reported that TAMs are a source of MMP-9, which is found exclusively in the transgenic dermis (Fig. 1B), and this enzyme contributes to their role in the metastatic cascade (27). The importance of these various immune cells in manifesting the changes in the Snail transgenic skin was shown by the ability of an immunosuppressive cocktail to significantly reduce the epidermal involution and hyperplasia (Fig. 2D) and cutaneous inflammation (Supplementary Fig. S4) in the mutant mouse.

Among these infiltrating immune cells, macrophages have garnered extensive attention for their remarkable ability to promote tumor proliferation and metastasis (28). Thus, the mechanism by which Snail expressing keratinocytes induces their recruitment into the skin becomes an important problem to resolve. We observed that the transgenic epidermis had an elevated level of monocyte colony-stimulating factor-1 (CSF-1), which is a well-known chemoattractant for macrophages (Fig. 3A; ref. 29). The induction of CSF-1 seems to be cell autonomous, as transfection of Snail into primary keratinocytes is sufficient to elicit CSF-1 expression (Fig. 3A).

Importantly, we found, using a Transwell assay, that conditioned medium (CM) from epidermal explants of transgenic mice is capable of recruiting macrophages (Fig. 3B). An inhibitory antibody against this cytokine shows that CSF-1 is a required component of the CM to stimulate macrophage mobilization. Because the epidermal explants used to condition the media are a heterogeneous population of cells, we tested whether Snail expression in keratinocytes is directly involved in the recruitment of macrophages by reconstituting this process completely in vitro. Primary keratinocytes transfected with Snail are capable of synthesizing and secreting CSF-1 to promote macrophage recruitment (Fig. 3C). These macrophages can, in turn, potently stimulate invasion of primary keratinocytes through an extracellular matrix (Fig. 3D). Moreover, the cytokine milieu present in the transgenic skin favors the polarization of the macrophages into TAMs (Fig. 2B and C; Supplementary Fig. 3B and C), which have been localized to areas of metastasis and shown to promote tumor cell invasion (30, 31). Consistent with this scenario, we found that TAMs can stimulate keratinocyte invasion at even higher levels than the classically activated macrophages (Fig. 3D).

Given TAMs ability to stimulate proliferation of breast carcinoma cells (32), we hypothesized that they may also...
be responsible for the elevated keratinocyte proliferation found in the Snail transgenic skin (Supplementary Fig. S5; ref. 9). Surprisingly, we found that both M1 macrophages and M2/TAMs have no effect on the growth rate of keratinocytes (Supplementary Fig. S6). To decipher the mechanism of increased keratinocyte proliferation in the transgenic skin, we focused on the activation of Stat3 (Fig. 1C), which is a prerequisite for keratinocyte proliferation during carcinogenesis (20). Profiling of the cytokines released from the transgenic epidermis revealed an increased level in the amount of secreted IL-6, which can stimulate the phosphorylation and nuclear translocation of Stat3 (Fig. 4A). Moreover, IL-6 can increase the rate of keratinocyte growth in a Stat3-dependent fashion (Fig. 4A). IL-6 is not normally expressed in the WT epidermis, and transfection of Snail into keratinocytes is incapable of inducing its expression (Fig. 4B). However, it is found in inflammatory skin diseases such as psoriasis (33), suggesting that the stimulus may be derived from an activated leukocyte. A clue into this mechanism came from reports that IL-17 can induce IL-6 expression in both autoimmune (34) and tumor settings (35). While profiling cytokine expression, we observed that IL-17 was induced in the transgenic skin and that the activated γδT cells in the transgenic dermis are a source of this cytokine (Fig. 4B). The analysis of the biological activity of this cytokine on epidermal keratinocytes revealed that IL-17 can promote keratinocyte proliferation via IL-6 signaling (Fig 4B), and this cascade is competent to activate Stat3 (Fig. 4C). As noted earlier, c-myc is a target of Stat3 and the increased expression of this proto-oncogene upon treatment of keratinocytes with IL-17 verified that the IL-17/IL-6/Stat3 signaling cascade was operational (Fig. 4C).

In light of the effect of Snail expression in the skin, we investigated whether this transgene renders mice more susceptible to inflammation-driven skin cancer. To test this hypothesis, we subjected both WT and transgenic mice to the 2-stage chemical carcinogenesis protocol using 7,12-dimethylbenz(a)anthracene (DMBA) as the mutagen and 12-O-tetradecanoylphorbol-13 acetate (TPA) as the promoter (14). This protocol was facilitated by the fact that the neonatal phenotypes decrease as the mice reach adulthood (Fig. 5A) and Snail protein levels diminish (data not shown), thus allowing us to test whether Snail primes the epithelial cells
of the skin for tumor formation. Transgenic mice have a higher frequency and incidence of tumor formation than WT litter-mate controls (Table 1; Supplementary Fig. S7). The transgenic skin responded with a significant epidermal hyperplasia and involution of the tissue reminiscent of migrating cells (Fig. 5A). Moreover, all of the adult transgenic mice displayed a hyperplastic sebaceous gland, an appendage of the epidermis, and treatment with DMBA + TPA led to the development of sebaceous carcinomas. The dermis of the DMBA + TPA-treated transgenic mouse verified a substantial increase in the number of lobular acini relative to the WT skin as marked by Oil Red O staining (Fig. 5B). Histologic analysis shows that these sebaceous carcinomas invade the blood vessels (Fig. 5B), induce epidermal ulceration, and invasion into both the adipose and stromal tissues of the skin (Fig. 5C). These sebaceous carcinomas were indeed metastatic, as 15 of 16 transgenic mice (Table 1) had sebocytes in their lymph nodes (Fig. 5D) that were positive for keratin 5 expression (Fig. 5E).

### Discussion

A model summarizing the epithelial-leukocytic cross talk stimulated by the expression of Snail in epidermal keratino-cytes to promote an early metastatic phenotype is presented in Figure 6. In the aggregate, these findings highlight the non–cell autonomous role that Snail has in promoting oncogenesis in vivo. We found no evidence of a complete or permanent EMT but can attribute many of the phenotypes to the recruit-ment and activity of immune cells recruited to the skin of the Snail transgenic mouse. This is consonant with the lack of reports of definitive evidence of an EMT occurring in any carcinoma cells in vivo (8). The intermediate phenotype of the Snail transgenic mouse may therefore be more appropriately referred to as ‘EMT like’ (36). Our data suggest that the cell autonomous function of Snail during carcinogenesis in vivo may be to maintain the undifferentiated state of a metastasiz-ing cell (23, 37) as it disseminates to new tissues, thereby
Figure 5. Tumor development and metastasis in the Snail transgenic mouse. Seven- to 8-week-old mice were treated with DMBA + TPA unless otherwise noted. A, histologic staining with H&E of WT and Snail transgenic (Tg) and DMBA and TPA. B, Oil Red O staining to detect sebaceous glands in WT (left) and transgenic (middle) skin. Right, transgenic skin section stained with H&E—arrowheads point to blood vessel. C, H&E staining of Snail transgenic skin showing an epidermal ulcer (left), invasion into adipose tissue, and stromal tissue (asterisk). D, top, H&E staining of lymph nodes from WT (left) and transgenic (middle, 10×; right, 40×) mice treated with DMBA and TPA. Bottom, IF of keratin 5 (red) in lymph nodes. Bars, 30 μm.
contributing to the maintenance of the "cancer stem cell" pool. On the other hand, the driving force for the local invasion of these cancer cells is the reciprocal interactions between the carcinoma cells and leukocytes. These findings concur with recent reports implicating Snail in mediating inflammation (38, 39). Interestingly, the epidermal hyperplasia and cutaneous inflammation that is prominent in neonatal mice significantly dissipate in the adult mice (Fig. 5). This is likely due to the inherent instability of Snail expression (6) and reduction in protein levels despite the continual transcription driven by the keratin-14 promoter in epidermal keratinocytes. This reversibility of the phenotype implies that continual intercellular signaling stimulated by Snail is required to preserve the changes we documented in the transgenic skin.

At first glance, the activity of resident γδT cells in the Snail transgenic skin seems to contradict their anticancer capability (40). However, γδT cells are activated and play a critical role in wound healing (41), which shares many processes in common with tumorigenesis such as inflammation/cytokine signaling and cell proliferation. In fact, tumor promotion and development are often found at sites of wounds/tissue damage (42). The activation and function of γδT cells in the transgenic skin likely occur along the lines of a wound-healing program to elicit a protumor response from these cells. Another important subset of T cells in this metastatic cascade seems to be TH2 cells that contribute to the inflammatory microenvironment and polarizes the macrophages into M2/TAMs. These TH2 cells are probably recruited to the skin by the chemokines CCL18, 22, and 27, which are upregulated in the epidermis of the transgenic skin (data not shown).

We postulate that the sebaceous gland carcinoma induced via chemical carcinogenesis is likely the response to the fact that the alteration in the sebocytes is the strongest remaining phenotype in adult transgenic mice. Moreover, these

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**Table 1. Incidence and frequency of tumorigenesis**

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<th>Adult mouse + treatment</th>
<th>No. of animals</th>
<th>Incidence of epidermal hyperplasia, %</th>
<th>Incidence of sebaceous hyperplasia, %</th>
<th>Incidence of sebaceous carcinoma, %</th>
<th>No. of sebaceous carcinoma per mouse</th>
<th>Incidence of lymph node metastasis, %</th>
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<tr>
<td>WT + DMBA/TPA</td>
<td>10</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Snail Tg + DMBA/TPA</td>
<td>16</td>
<td>100</td>
<td>100</td>
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**NOTE:** Quantitation of phenotypes in WT and transgenic mice with or without chemical carcinogenesis. Data are compiled from mice that are 8 weeks after the promotion phase of the 2-step chemical carcinogenesis protocol.

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Figure 6. Signaling in the Snail transgenic skin. Expression of Snail in epidermal keratinocytes leads to a program that maintains the undifferentiated state of the transgenic cells. Snail also transcriptionally upregulates CSF-1 to cause homing of macrophages (MΦ) into the skin. A subset of these macrophages is polarized along the M2/TAM lineage. Together, these macrophages then stimulate the local invasion of the keratinocytes into the underlying dermis. Snail expressing keratinocytes also leads to the wound-like activation of resident γδT cells that migrate into the dermis and begin secreting IL-17. Epidermal keratinocytes respond to IL-17 by inducing expression of the cytokine IL-6, which works in a paracrine fashion to activate the transcription factor Stat3. Stat3 activation contributes to tumorigenesis by augmenting proliferation, cell survival, and angiogenesis.
hyperplastic cells seem to be positive for keratin 15 (data not shown), which is another marker for sebaceous neoplasms as well as the sebaceous carcinoma of Murre–Torre syndrome (43), which is a subtype of hereditary nonpolyposis colorectal cancer, and sebaceous differentiation (Fig. 5B and C) seems to be a strong phenotypic marker of this disease. Other genes associated with hair follicle morphogenesis that were expressed in the basal layer of the epidermis, such as a mutated Lef1, also generated sebaceous skin tumors (44). Altogether, a clearer picture is emerging regarding the mechanism by which genes associated with budding morphogenesis of the hair follicle can be usurped by carcinomas to serve similar, albeit unregulated, roles in tumor development and metastasis.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


