Carcinogen-Induced Skin Tumor Development Requires Leukocytic Expression of the Transcription Factor Runx3

Omri Bauer, Shay Hantisteanu, Joseph Lotem, and Yoram Groner

Abstract
Carcinogen-induced skin tumorigenesis depends heavily on proinflammatory tumor-promoting processes. Here, we show that leukocytic Runx3 expression is central to the two-stage DMBA/TPA-induced skin tumorigenesis. Runx3-null mice were highly resistant to this process and concomitant ablation of Runx3 in dendritic and T cells fully recapitulated this resistance. Mechanistically, this resistance was associated with a shift in the skin cytokine milieu toward a tumor nonpermissive microenvironment. Specifically, leukocytic Runx3 loss substantially increased the antitumorigenic cytokine thymic stromal lymphopoietin (TSLP) and profoundly decreased two protumorigenic cytokines, interleukin-17a and osteopontin. Therefore, inflammation-mediated tumor promotion requires leukocytic Runx3 expression, as its loss creates a unique cytokine composition that polarizes the tumor microenvironment to a potent antitumorigenic state. Cancer Prev Res; 7(9); 913–26. ©2014 AACR.

Introduction
The two-stage (DMBA/TPA) skin tumorigenesis assay has been instrumental to our understanding of carcinogenesis as a multistep process (1, 2). Like in other epithelial cancer models, tumors induced by this assay evolve through primary genetic and epigenetic changes that are propagated through interactions with the tumor microenvironment (3). Tumor-promoting mechanisms are only partly defined, involving chronic recruitment of inflammatory cells, fibroblasts, and endothelial cells that reside within the tumor stroma and contribute to its survival and proliferation (4, 5). Although inflammatory cells in the tumor microenvironment can eliminate initiated cells and suppress tumor growth (6), there is increasing recognition of their tumor-promoting roles (3, 7).

The tumor microenvironment cytokine/chemokine milieu is a major determinant of cancer-related inflammation. It coordinates communication between transformed cells and supporting stroma, thereby affecting several aspects of tumor promotion and progression, including immune cell recruitment and function, tumor cell senescence, proliferation and survival, matrix remodeling, angiogenesis, and tumor invasion and metastasis (8, 9). Thus, tumor bed cytokines can polarize host immunity to either inflammatory-promotive or -protective responses, leading to one of two opposite outcomes: tumor progression or regression (10, 11). Therefore, there is a great clinical promise in modulating the tumor microenvironment cytokine repertoire so that immune response is shifted toward a tumor-protective mode (8, 12).

The runt-related transcription factor Runx3 is highly expressed in murine skin leukocytes and mesenchyme, including hair follicle dermal papillae, but not in epithelia (13, 14). In lymphocytes, Runx3 is involved in Cd4 repression in differentiating CD4+ /CD8+ thymocytes (15, 16), in regulation of cytokine gene expression in CD4+ Th1 lymphocytes (17), and in growth/terminal differentiation of mature CD8+ T cells (15). Runx3 is also mandatory for the perinatal homing of dendritic epidermal T cells (DETC) to the epidermis, as reflected by their complete loss in Runx3-null mice (18). In dendritic cells (DC), Runx3 is part of the tumor growth factor β (TGFβ) signaling cascade (19, 20).

When lost, the development of TGFB-dependent epidermal Langerhans cells (LC) is abrogated, whereas dermal DCs (dDC) seem unaffected (18, 19). Runx3 germine inactivation enhanced lung DC maturation, resulting in hyper-mature/-active alveolar DCs (19, 20) and reprogrammed gene expression of CD11b +Esam + splenic DCs (21). Despite these profound immune anomalies, in vivo studies of wound closure, contact hypersensitivity, and epidermal apoptosis were unaltered in skins of Runx3-null mice (18). Runx3 is also highly expressed in respiratory and gastrointestinal tract leukocytes, but is absent from their epithelia (13, 22, 23). Significantly, Runx3-null mice spontaneously develop airway and colon inflammation (19, 20, 22), attesting to Runx3 involvement in leukocyte homeostasis in these tissues. These earlier findings prompted us to investigate the potential role of Runx3 in inflammation-mediated tumor promotion in the skin.
Materials and Methods

Mice
The mice (2–6 months old) used in the study include the previously described strains: Runx3-null (ICR background; ref. 24); lomP-flanked Runx3 (Runx3\textsuperscript{lomPfl}) and Runx3\textsuperscript{fl/fl}/Pgy-cre (25), and Runx3\textsuperscript{K14-loxP/EGFP} (23) and Runx3\textsuperscript{K14-loxP/CD11c-cre} (21). Transgenic Lck- and K14-cre mice were obtained from The Jackson Laboratory. Transgenic hemizygous TG.AC mice (FVB background) were purchased from Taconic and crossed with Runx3-null and wild-type (WT) mice to yield Runx3-null/TG.AC and WT/TG.AC control mice (using FVB/ICR F1 only). Control mice, that is, WT mice to yield Runx3-null/TG.AC and WT/TG.AC control mice, obtained from The Jackson Laboratory. Transgenic hemizygous TG.AC mice (FVB background) were purchased from Taconic and crossed with Runx3-null and wild-type (WT) mice to yield Runx3-null/TG.AC and WT/TG.AC control mice (using FVB/ICR F1 only). Control mice, that is, WT for Runx3-null and Runx3\textsuperscript{+/−} for all other transgenic mice (unless stated otherwise), were gender-matching litters from heterozygote breeding. Specific ablation of Runx3 in dDCs (D\textsuperscript{Runx3−/−} mice) or in basal keratinocytes (Runx3\textsuperscript{fl/fl}/K14-cre mice) was verified by PCR on DNA from CD11c\textsuperscript{high} FACS-sorted dermal or DETC/LC–depleted epidermal cell suspensions, respectively, isolated using the Arcturus Picopure DNA Extraction Kit (Applied Biosystems). Cell-specific Runx3 ablation in sorted splenic CD8\textsuperscript{+} thymocytes from Runx3\textsuperscript{fl/fl}/Lck-cre mice was validated by western blotting, using an in-house polyclonal anti-Runx3 antibody (13). Mice husbandry details are given in Supplementary Materials and Methods.

Immunohistochemistry and histology
Runx3 immunohistochemistry (IHC) was performed on formalin-fixed paraffin-embedded sections, as described previously (16), using an in-house polyclonal anti-Runx3 antibody (1:1,000; ref. 13). Further information about assessments of epidermal hyperplasia, keratinocyte proliferation, and apoptosis is included in Supplementary Materials and Methods.

Flow cytometry
Epidermal or dermal single-cell suspensions were generated from cohorts of mice (n ≥ 3), as described previously (26). Further details are included in Supplementary Materials and Methods.

Chemical carcinogenesis assay
The DMBA/TPA skin tumorigenesis assay was conducted as described previously (27), including several modifications, as detailed in Supplementary Materials and Methods.

Epidermal sheet preparation, immunostaining, and LC quantification
Epidermal sheets were prepared from adult mouse ears as described previously (19), including modifications described in Supplementary Materials and Methods.

Cytokine analysis
Naïve and DMBA/TPA–treated (single/6 biweekly applications, respectively) whole-skin biopsies of Runx3-null and control WT mice (pooled; n = 3 mice/genotype) were weighed, snap-frozen, and stored (−80°C) for further processing. Tissue protein lysate (R&D Systems/ARY006) and RNA (Qiagen/RNeasy Microarray Tissue Kit) were obtained according to the manufacturer’s instructions and analyzed using the Proteome Profiler/Mouse Cytokine Array Dot-Blot Kit (R&D Systems), the RT\textsuperscript{2} Profiler PCR Array System/Mouse Cytokines and Chemokines (SABiosciences/PAMM-150Z) and qRT-PCR, as detailed in Supplementary Materials and Methods.

Statistical analysis
Data were presented as mean ± standard deviation (SD). Datasets were compared using the Student two-tailed t test. Statistical significance was considered at P < 0.05.

Results

Runx3 expression in mouse skin
To evaluate Runx3 expression in mouse skin, we performed IHC on normal and neoplastic skin sections. In line with previous studies (13, 14), we found that Runx3 expression was confined to large scattered cells of the interfollicular epidermis and to distinct hair follicle cells (Fig. 1A). However, Runx3 was not detected in either embryonic or postnatal (normal or neoplastic) epidermal keratinocytes (Fig. 1A). We noted intense staining of dispersed morphologically heterogeneous cells in normal and pathologic dermis of adult mice (Fig. 1A), and therefore performed flow cytometry on single-cell suspensions from dermis and epidermis of Runx3/GFP knockin mice (23). Analysis revealed intense expression of Runx3/GFP in resident T cells and CD11b\textsuperscript{+} DCs (Fig. 1B), two well-established Runx3-expressing cell lineages (16, 19, 21, 23). Similar epidermal analysis identified Runx3 expression in subsets of steady-state LCs and DETCs (Fig. 1C), corresponding with their complete absence in Runx3-null mice (18, 19). Together, these findings demonstrated Runx3 expression in skin leukocytes and lack of its expression in normal or neoplastic epidermal keratinocytes.

Significant reduction in CD11b\textsuperscript{+} DCs in naïve Runx3-null dermis
Steady-state murine dermis contains two distinct DC subtypes—classical dermal (d) (CD11b\textsuperscript{+}) and langerin\textsuperscript{+} (CD11b\textsuperscript{low/−} and Langerin\textsuperscript{+}) DCs—that share the CD11c pan-DC marker (28). The eE-β7 integrin CD103, found on DC subsets in various organs, including lung (29) and colon (30), further specifies langerin\textsuperscript{+} dDCs. To evaluate changes in the proportion of dDC subsets in Runx3-null mice, we performed flow cytometry analysis on ear skin-isolated cells. Naïve Runx3-null skin displayed a significant decrease in CD11b\textsuperscript{+} dDCs (30.3% ± 11.6%), compared with WT controls (62.6% ± 10.2%; P < 0.01; Fig. 1D, left). Furthermore, while the proportion of CD11b\textsuperscript{low/−}CD103\textsuperscript{−} dDCs did not change significantly, approximately five times increase in a unique population of CD11b\textsuperscript{+}CD103\textsuperscript{−} dDCs was recorded in Runx3-null mice (27.3% ± 1.6% vs. 4.9% ± 4.2% in WT controls; P < 0.01; Fig. 1D, left and middle). DC maturation/activation state analyses indicated comparable MHCII and T-cell costimulatory molecule CD86 levels in Runx3-null...
Figure 1. Runx3 expression in naïve and papillomatotic skin leukocytes and changes in immunocytes in Runx3-null skin. A, top, IHC showing intense Runx3 expression in scattered nonkeratinocytic cells of naïve WT skin. Note stained nuclei in large ovoid cells in basal epidermis (red arrows), in distinct hair follicle cells and in dispersed heterogeneous dermal cells (black arrows), but not in keratinocytes. Dorsal skin, 2-month-old WT mouse. ×200 (left); enlarged micrograph of boxed area, ×400 (right); E, epidermis; D, dermis; SC, subcutis (hypodermis); HF, hair follicle. Bottom, a section through a cauliflower-shaped benign papilloma showing outwardly proliferating keratinocytes. H&E, ×10 (left); Runx3 IHC on a papilloma showing marked infiltration of Runx3-expressing leukocytes into the tumor stroma (black arrows) in ×50 and ×100 micrographs of boxed area. Note lack of Runx3 in neoplastic keratinocytes (red arrows); pK, papillomatotic keratinocytes. Runx3 expression in CD3εhigh T cells and CD11b+ DCs from naïve WT dermis (B) and in LCs and DETCs (C) from epidermis. Shown are flow cytometry analyses of cells from Runx3pro-amino-acid knockin mice, depicting GFP-expressing CD3εhigh, CD103+CD11c+ T cells, and the CD11c+/CD11b+ dDC subsets (B) and CD11c+/MHCII+ LCs and CD3ε+γδ TCR+ DETCs (C). D, flow cytometry analysis of WT and Runx3-null dDC subsets in naïve dermis. Note marked reduction in CD11b+ dDCs and CD11b+/CD103+ dDC emergence (∼5× more prevalent) in Runx3-null mice (values are mean ± SD; n = 4 mice/genotype; P < 0.01). Right, comparative MHCII and CD86 levels in dDCs. Results in B–D represent one of three experiments with similar findings.
and WT dDCs (Fig. 1D, right), suggesting that in contrast to lung and splenic Runx3-null DCs (19), naïve Runx3-null dDCs are neither hypermature nor hyperactivated.

**Runx3 loss markedly attenuates DMBA/TPA-induced papilloma outgrowth**

Despite the above-mentioned anomalies in major components of Runx3-null cutaneous immunity, no quantifiable skin deficit was noted in a series of *in vivo* experiments addressing wound closure, contact hypersensitivity, and epidermal apoptosis (18). To investigate Runx3 role in skin tumorigenesis, we compared tumor development in Runx3-null and WT mice, using the two-stage tumorigenesis protocol (1). Our results revealed a striking resistance of Runx3-null mice to DMBA/TPA tumorigenesis (Fig. 2A and B), reflected by (i) an order-of-magnitude lower mean papilloma yield (multiplicity) in Runx3-null vs. WT control mice (peak of 1.1 ± 1.8 vs. 12.1 ± 6.9 papillomas/mouse at 17 and 20 weeks after DMBA initiation, respectively; *P < 0.01*); (ii) a doubled period to first papilloma appearance (92 vs. 46 days from DMBA initiation, respectively); and (iii) a decreased rate of papilloma appearance in Runx3-null mice (0% vs. 90% of mice bearing ≥1 papilloma at 8 weeks after DMBA initiation, respectively). Furthermore, tumors that did form grew significantly slower in the absence of Runx3, indicating that Runx3 is involved in papilloma formation and growth. Similar results were obtained when Runx3fl/fl/Pgk-cre mice, Runx3-null functional analogs, were subjected to DMBA/TPA treatment (Fig. 2C). Compared with WT, these mice yielded reduced multiplicity (peak of 0.6 ± 0.8 vs. 12.6 ± 8.3 papillomas/mouse at 20 weeks after DMBA initiation; *P < 0.01*), a significantly lower papilloma rate (0% vs. 100% and 43% vs. 100% of mice bearing ≥1 papilloma at 10 and 20 weeks after DMBA initiation, respectively) and an increased latency period (99 compared with 39 days). Collectively, these data showed that Runx3-null mice exhibit a profound resistance to DMBA/TPA-induced tumorigenesis.

Although our analyses failed to detect Runx3 in murine keratinocytes, we repeatedly observed its presence in hair follicle cells. Given that keratinocyte stem cells, residing in the hair follicle bulge and basal interfollicular epidermis, are the primary cellular targets of the DMBA-induced initiation phase of carcinogenesis (2), we addressed whether lack of Runx3 in these cells contributed to the observed DMBA/TPA resistance. Using Runx3fl/fl/K14-cre mice that lack Runx3 in the hair follicle and epidermal basal layer cells (Supplementary Fig. S1), we found similar papilloma yield, rate, and kinetics in these mice and their WT littermates (Fig. 2D), indicating that lack of Runx3 in epidermal keratinocytes was not involved in the observed tumor resistance of Runx3-null mice.

**Runx3 loss mildly increases 3'-methylcholanthrene–induced carcinogenesis**

To evaluate Runx3 function in other neoplastic settings, we assessed fibrosarcoma induction by 3'-methylcholanthrene (3'-MCA), a complete carcinogen that mediates its action solitarily (31). Although 3'-MCA carcinogenicity does not involve a substantial inflammatory component (32–34), adaptive immunity plays a role in preventing late-induced sarcomas in this model (35). Mice were injected subcutaneously with 3'-MCA (50 or 100 μg) and inspected twice-weekly for tumor development (Fig. 2E). In contrast to their resistance to DMBA/TPA, Runx3-null mice displayed a mildly increased susceptibility, compared with WT littermates (Fig. 2F). This was reflected by a higher finite tumors/injected site in Runx3-null vs WT (22.7% vs. 14.3% and 50.0% vs. 22.7%, in the low- and high-dose groups, respectively) and an earlier appearance of first tumor (106 vs. 141 days and 89 vs. 105 days in the low- and high-dose groups, respectively). Once established, however, sarcoma growth behavior was similar between Runx3-null and WT mice (Fig. 2F). Thus, Runx3 loss results in a mildly increased susceptibility to 3'-MCA–induced tumorigenicity, conceivably due to Runx3-null impaired immunosurveillance-mediated immune response against the malignant sarcoma cells (6, 35). Taken together, these 3'-MCA results, the opposed DMBA/TPA findings, and Runx3 skin expression pattern indicated that while leukocytic Runx3 is required for DMBA/TPA inflammation-dependent tumorigenesis, it is nonessential for inflammation-independent chemical carcinogenesis.

**Altered TPA-mediated tumor promotion in Runx3-null mice**

To address whether papillomagenesis suppression in Runx3-null mice stemmed from a failure in DMBA-mediated initiation or in TPA-induced tumor promotion, we crossed Runx3-null mice onto the TG.AC strain, which constitutively expresses an activated Ha-ras gene (36). TG.AC mice are considered "pre-initiated," because they develop papillomas following topical TPA application, without prior DMBA initiation (36). Using a biweekly TPA promotion schedule, WT/TG.AC mice developed large papillomas, as of 4 weeks after the first TPA application (Fig. 3A), consistent with their increased sensitivity (36). In contrast, Runx3-null/TG.AC mice only occasionally developed small papillomas that often regressed spontaneously (Fig. 3A). Thus, even in "pre-initiated" mice with a mutant/activated Ha-ras, Runx3 loss suppressed TPA-induced papillomagenesis, demonstrating that tumor promotion was affected by Runx3 loss.

**Runx3 ablation decreases TPA-induced epidermal proliferation**

To determine whether Runx3 absence affects keratinocyte proliferation, we assessed changes in TPA-induced epidermal hyperplasia. The number of nucleated cell layers and thickness of untreated Runx3-null and WT epidermis were not significantly different (Fig. 3B, top left). Although TPA application induced epidermal hyperplasia in both genotypes, the number of nucleated cell layers in Runx3-null mice was lower as compared with WT mice (Fig. 3B, top left). Specifically, following 2 or 10 TPA applications, Runx3-null epidermal thickness has increased approximately 1.5-fold less than in WT mice (Fig. 3B, bottom left). These data
Figure 2. Runx3-null mice are resistant to DMBA/TPA tumorigenesis but susceptible to 3-MCA-induced skin tumors. A, representative mice showing multiple papillomas on a WT versus a single papilloma on a Runx3-null mouse at 20 weeks after DMBA initiation. B and C, delayed onset, reduced yield (multiplicity), and decreased rate of papillomas in DMBA/TPA-treated Runx3-null (B) and Runx3fl/fl/Pgk-cre mice (n = 7, each), compared with WT (n = 10) and control (n = 9; bearing ≥1 WT allele) littermate mice, respectively. D, similar papilloma yield, rate, and kinetics in DMBA/TPA-treated Runx3fl/fl/K14-cre mice (n = 10) and WT littermate mice (n = 16). E, macro appearance of an autochthonous fibrosarcoma developing in a WT mouse lower back (left, circled). A section through this tumor (right) shows its subcutaneous localization (H&E, ×63) and the proliferating cancerous fibroblasts (inset, H&E ×400; bar, 50 μm); E, epidermis; D, dermis; M, hypodermal muscle; FS, fibrosarcoma. F, tumor formation kinetics and frequency following 3-MCA injection. Shown are cumulative tumor numbers/injection sites (%) over time. Left, low dose (50 μg; 11 Runx3-null and 14 WT mice). Right, high dose (100 μg; 10 Runx3-null and 11 WT mice). Values in B–D are mean ± SD.
indicated that while Runx3 loss had no effect on naïve epidermis, its ablation significantly attenuated TPA-induced epidermal hyperplasia.

We then used bromodeoxyuridine (BrdUrd) IHC to evaluate TPA-induced changes in keratinocyte proliferation. Whereas naïve Runx3-null mice exhibited BrdUrd labeling that was similar to WT mice (1%–2% of stratum basale cells), TPA-treated skins of Runx3-null mice showed a significantly lower percentage of BrdUrd+ cells (11.4% ± 8.2%) as compared with WT littermate mice (29.0% ± 4.5%); P < 0.01 (Fig 3B, middle). These findings suggested that Runx3 loss abrogated TPA-induced keratinocyte proliferation. To determine whether enhanced keratinocyte apoptosis also contributed to Runx3-null mice resistance to DMBA/TPA-induced papillomagenesis, we used the terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling (TUNEL) assay to measure in vivo apoptosis. Analysis showed that TPA-treated skins from WT and Runx3-null mice had comparable numbers of epidermal TUNEL-positive nuclei (Fig 3B, right top and bottom), indicating that Runx3-null mice lower papilloma incidence largely stemmed from diminished TPA-induced keratinocyte proliferation.

Runx3-null mice chronically inflamed skin shows altered leukocyte recruitment

To probe into the cellular microenvironment that opposes tumor promotion in Runx3-null mice, we quantified the cellular infiltrate in DMBA-initiated skin persistently treated with TPA. As indicated, Runx3-null mice had a lower percentage of inflammatory cells in epidermis and dermis when compared with WT littermate mice (Fig 3B, bottom). These findings suggested that Runx3-null mice have a diminished cellular response to TPA treatment, which may contribute to their resistance to TPA-induced papillomagenesis.
with TPA (8–10 biweekly applications) to establish chronic dermatitis. Flow cytometry revealed comparable proportions of several leukocyte populations, including DC subsets, neutrophils, macrophages, and myeloid-derived suppressor cells (MDSC) in Runx3-null and WT chronically inflamed dermis (Supplementary Fig. S2A). However, a significant difference was found in the dermal T-cell compartment (Fig. 4A). Despite similar overall numbers of CD3ε⁺ T cells
in these two genotypes, we noted a 2-fold decrease in the γδ⁺ T-cell subset (26.0% ± 5.1% in WT vs. 12.0% ± 2.5% in Runx3-null mice; P < 0.01) accompanied by a mild increase in the proportion of Runx3-null γδ⁺ T cells (69.9% ± 4.9% in Runx3-null vs. 60.4% ± 2.8% in WT mice; P = 0.02; Fig. 4A). Furthermore, a CD3e⁺γδ⁺ T-cell subset, which comprised 1% to 4% of inflamed WT dermal T cells, was absent from Runx3-null dermis (Fig. 4A). Similar analyses of the DMBA/TPA-treated epidermal compartment corresponded with these dermal findings (Supplementary Fig. S2B). In the epidermis, the DC proportion decreased 4-fold in Runx3-null mice (1.4% ± 0.4% in Runx3-null vs. 6.0% ± 3.0% in WT mice; P = 0.03; Fig. 4B). This decrease was probably due to a decline in the CD11b⁺ DC subset (1.2% ± 0.6% in Runx3-null vs. 3.5% ± 2.3% in control mice; P = 0.07). Collectively, these data demonstrate that Runx3 loss affected recruitment of T and DC lineage cell subsets to the TPA-treated skin, attesting to the crucial role of Runx3 in shaping the inflamed skin microenvironment.

**DC- or T cell–specific Runx3 ablation partially phenocopy Runx3-null resistance to DMBA/TPA skin tumorigenesis**

Because Runx3 expression was confined to skin leukocytes, we addressed whether specific elimination of Runx3 in DCs would endow resistance to DMBA/TPA-mediated tumorigenesis. Upon DMBA/TPA treatment, Runx3fl/fl/CD11c-cre (DCRunx3-/-/) mice (Fig. 5A, left) developed fewer papillomas/mouse than Runx3+/- littermates (11.9 ± 11.4 vs. 29.5 ± 11.6 peak papillomas/mouse at 20 weeks after DMBA initiation, respectively; P < 0.01; Fig. 5A, middle). However, the percentage of DCRunx3-/- mice with >1 papilloma did not differ significantly from controls (63% vs. 81% and 88% vs. 100% of mice at 9 and 20 weeks after DMBA initiation, respectively; P < 0.05). Furthermore, tumor latency period was 45 days in both DCRunx3-/- and Runx3+/- groups (Fig. 5A). These results suggested that although Runx3-expressing DCs were essential, they were not sufficient to furnish Runx3-null full tumor resistance.

Modi and colleagues (37) showed that absence of epidermal LCs confers complete resistance to DMBA/TPA–induced tumorigenesis. Therefore, we evaluated the involvement of LCs in Runx3-null tumor resistance by immunostaining of epidermal sheets from naïve DCRunx3-/- ear skin and flow cytometry (Fig. 5B). Although morphologically sound MHCIIs were present in naïve DCRunx3-/- epidermis (Fig. 5B and Supplementary Fig. S3), their numbers were significantly lower, compared with Runx3+/- mice (Fig. 5B, right top and bottom). This finding indicates that while DCRunx3-/- mice tumor resistance occurred en face low numbers of epidermal LCs, additional dermal components contributed to Runx3-null mice resistance.

We then evaluated the involvement of T cells in Runx3-null mice tumor resistance. Treatment of Runx3fl/fl/Lck-cre (TRunx3-/-) mice (Fig. 6A, left) with DMBA/TPA revealed a papilloma yield that was lower, compared with littermate Runx3+/- mice (peak of 4.4 ± 5.5 vs. 9.5 ± 7.8 papillomas/mouse at 20 weeks after DMBA initiation; P = 0.03; Fig. 6A, middle). Papilloma rate in TRunx3-/- mice was also lower, compared with Runx3-/- control mice (17% vs. 50% and 83% vs. 96% of mice bearing ≥1 papilloma at 10 and 20 weeks after DMBA initiation, respectively; Fig. 6A, right). However, like in DCRunx3-/- mice, a similar time span to first papilloma was observed in TRunx3-/- and littermate Runx3+/- mice (43 days from DMBA initiation). These results show that Runx3 ablation in T cells conferred partial resistance to DMBA/TPA–mediated skin tumorigenesis.

Given that skin of germline-inactivated Runx3-null mice lacks DETCs (18), which are known to protect against DMBA/TPA–induced neoplasms (27), skins of TRunx3-/- mice were examined. Using whole-mount epidermal sheet immunostaining, we readily detected morphologically mature DETCs in naïve epidermis of adult TRunx3-/- mice (Fig. 6B and Supplementary Fig. S3). This finding indicated that Lck-cre–mediated Runx3 deletion in early CD4⁺/CD8⁻ T cells did not affect the generation/survival of epidermal DETCs. This result further implied that TRunx3-/- mice partial resistance to DMBA/TPA–mediated tumorigenicity was mediated by other T-cell species.

Together, the above results show that specific Runx3 ablation in either the DC or T-cell compartments confers resistance to DMBA/TPA–induced skin tumorigenesis onto these mice. However, the extent of resistance in both genotypes, based on the relative finite tumor yield, rate, and latency period, was not as robust as in the two germline Runx3-deficient mouse strains, Runx3-null and Runx3fl/fl/Pgk-cre. These findings implied that a cooperative activity between Runx3-deficient DCs and T cells is required to fully recapitulate observed resistance in Runx3-null mice.

**Combined Runx3 deletion in DCs and T cells fully recapitulated Runx3-null mice resistance to skin tumorigenesis**

Mice concurrently lacking Runx3 in DCs and T cells were generated by cross-breeding Runx3fl/fl/CD11c-cre and Runx3fl/fl/Lck-cre mice (DCRunx3-/-/TRunx3-/-). These mice and their littermate controls were subjected to the DMBA/TPA protocol. Significantly, DCRunx3-/-/TRunx3-/- mice conferred an extended resistance, similar to that noted in the two Runx3-deficient mouse strains (Runx3-null and Runx3fl/fl/Pgk-cre). Accordingly, by 20 weeks of promotion, mean tumor multiplicity was 8.3-fold lower in DCRunx3-/-/TRunx3-/- mice, compared with control group (peak of 2.5 ± 1.9 vs. 20.1 ± 15.5 papillomas/mouse, respectively; P = 0.015; Fig. 6C). Furthermore, the delay period to first papilloma was doubled in DCRunx3-/-/TRunx3-/- mice, compared with controls (77 vs. 39 days from DMBA initiation; Fig. 6C), and papilloma rate was significantly lower (0% vs. 40% and 25% vs. 93% of mice bearing ≥1 papilloma at 10 and 15 weeks after DMBA initiation, respectively; Fig. 6C). Collectively, these findings indicated that Runx3 cell-autonomous function in DCs and thymocytes is required for DMBA/TPA–mediated tumorigenesis, and thus Runx3 loss in both these cell types causes the
pronounced tumor resistance. But what is the underlying mechanism?

Cytokine composition in Runx3-null naïve and TPA-treated skin generates a robust antitumor microenvironment

The fact that communication between tumor cells and their supporting stroma is largely regulated by inflammatory cytokine/chemokine networks raised the possibility that an altered cytokine/chemokine milieu in Runx3-null skin participates in conferring the observed tumor resistance. To address this possibility, we used qRT-PCR and proteomic dot-blot assays to comparatively measure the expression levels of multiple cytokines and chemokines. Using manual qRT-PCR and the RT2 Profiler PCR Array, which simultaneously monitors the expression of 84
cytokines/chemokines (Supplementary Tables S1 and S2), we analyzed WT or Runx3-null skin before and after DMBA/TPA treatment. A 15-fold increase in Tslp (thymic stromal lymphopoietin) expression was noted in naïve Runx3-null skin (Fig. 7A). Tslp is a master cytokine that confers a dominant and lasting CD4⁺/CD8⁺ T cell–mediated protection against skin tumorigenesis (26, 38). In addition, we also observed intense upregulation of Cd70 and Cxcl5, indicating the existence of immunologically active leukocytes (39) in naïve Runx3-null skin. On the other hand, expression of Spp1, encoding the protumorigenic cytokine osteopontin (4), was 6-fold lower in naïve Runx3-null skin, compared with WT skin (Fig. 7A). We then used a Proteome Profiler Array that concurrently detects the levels of 40 cytokines/chemokines (Supplementary Table S3) to assay protein extracts from DMBA/TPA-treated WT or Runx3-null skins (Fig. 7B). This analysis revealed a ≥2.5-fold increase in several chemokines, including Ccl17, Ccl1, and Ccl12, as well as cytokines such as colony-stimulating factor (Csf) 2 and 3, tumor necrosis factor α (Tnfα), and interleukin-6 (Il6) in Runx3-null skin. These proteomic results were further validated by qRT-PCR (Fig. 7B). Notably, a striking decrease in Il17a was also evident in DMBA/TPA-treated Runx3-null skin (Fig. 7C). Il17a is a signature Th17
cytokine, the deletion of which has been correlated with marked resistance to the DMBA/TPA protocol (40). Similar to naïve skin, Spp1 levels in DMBA/TPA–treated Runx3-null skin were lower, compared with WT (Fig. 7B). Collectively, these expression experiments show that leukocytic Runx3 loss generates a unique assembly of cytokines that is associated with a tumor-refractory microenvironment in the skin.

Discussion

We have used mouse genetic models to demonstrate the pivotal role of Runx3-expressing skin leukocytes in shaping the tumor microenvironment, thereby promoting the DMBA-initiated keratinocyte to a mature tumor. Specifically, we show that Runx3 cell-autonomous function in the innate (DC) and adaptive (T-cell) immune compartments hubs the induction of a protumorigenic cytokine microenvironment in the DMBA/TPA model of skin tumorigenesis. Accordingly, subjecting mice lacking Runx3 in DCs and T cells to DMBA/TPA generates an inflammatory tumor nonpermissive stromal environment that strongly resists papillomagenesis. Our analysis also revealed approximately 2-fold decrease in the proportion of “classical” CD11b+ DCs and a concomitant approximately 5-fold increase in CD11b+CD103+ dDCs in Runx3-null mice naïve dermis. Although an increase in CD11b+/CD103+ DCs was noted in inflamed murine gut lamina propria (30), as well as in steady-state mesenteric lymph nodes (28) and lungs (41), their in vivo functions remain largely obscure. Given that Runx3 was correlated with both positive and negative regulation of Cdl03, during CD8+ T-cell differentiation (18, 42), it is tempting to speculate that its loss in “classical” CD11b+ dDCs leads to Cdl03 de-repression and the subsequent emergence of CD11b+/CD103+ DCs, observed in Runx3-null mice. The finding that Runx3 skin expression was restricted to immunocytes and was not detected in naïve or DMBA/TPA–treated keratinocytes excludes the possibility of a cell-autonomous oncogenic Runx3 function in the murine DMBA/TPA tumor target cell itself. This conclusion is further supported by the Runx3fl/fl/K14-cre mice

Figure 7. Runx3-null mice skin cytokine analysis. A, qRT-PCR analysis of Runx3-null mice naïve skin RNA using the RT2 Profiler PCR Array and/or manual qRT-PCR. Results show four highly significant differentially expressed cytokines of 84 analyzed genes (Supplementary Tables S1 and S2). Primers used in Tslp analysis are depicted in Supplementary Materials and Methods. B, semiquantitative proteomic dot-blot panel (left), densitometry and RT2 Profiler qRT-PCR (right) analyses of differentially expressed cytokines in DMBA/TPA–treated skins of Runx3-null and WT littermate mice (Supplementary Table S3). Ccl17 and IL6 signals were undetected in Runx3-null mice. C, semiquantitative proteomic dot-blot panel (left), densitometry (middle), and qRT-PCR (right) analyses of differentially expressed cytokines in DMBA/TPA–treated skins of 3 mice (each genotype) and pooled. Dot-blot reference (Ref) signals in B and C are membrane positive controls. Data in A–C represent three and five independent experiments with similar findings, respectively.
findings, demonstrating that epidermal keratinocytes were not involved in Runx3-null mice tumor resistance.

Other mouse models displaying resistance to DMBA/TPA have been described, including mice deficient in Tnfα, cyclooxygenase-2, and Il12 (43–45). Although the magnitude of tumor resistance in these models was probably affected by genetic background and promotion regimen differences, none has—as far as we know—resulted in resistance as dramatic as that reported here for Runx3-deficient mice. Moreover, although DMBA/TPA-induced papillomas persisted >100 days after TPA cessation, with approximately 7% progressing to malignant carcinoma in WT mice, the low overall incidence of these benign papillomas in Runx3-null mice resulted in null carcinomas in this genotype. Therefore, our study did not address the possibility that lack of leukocytic Runx3 could also inhibit papilloma-carcinoma transition.

In contrast to Runx3-null mice resistance to DMBA/TPA tumorigenicity, these mice were slightly more susceptible than control mice to complete 3'-MCA-induced carcinogenesis. Several possible explanations were considered in previously observed differential susceptibility to DMBA/TPA vs. 3'-MCA treatments (33, 34): (i) the obvious differences in tumor characteristics (proliferating keratinocytes/DMBA vs. malignant fibroblasts/3'-MCA); (ii) the tumor location (epidermal vs. subcutaneous, respectively); and (iii) the stromal microenvironment [persistent inflammation (46) vs. inflammation-independent "foreign-body" granuloma formation, respectively (33, 34)]. Hence, the observation that Runx3-null mice are refractory to DMBA/TPA-induced tumorigenesis, but are susceptible to inflammation-independent 3'-MCA carcinogenesis, underlines Runx3 involvement in inflammation-dependent skin tumorigenesis.

LC-deficient mice are almost completely resistant to DMBA/TPA tumorigenesis (47). When LCs are absent, metabolic activation of chemical carcinogens (e.g., DMBA) that induce tumor initiation was impaired (37). Moreover, although DETC-depleted mice manifest higher susceptibility to DMBA/TPA tumorigenesis by themselves (27), combined depletion of DETCs and LCs retained the robust LC-deficient tumor-resistant phenotype (47). Significant differences exist between our observation that Runx3 loss confers marked resistance to DMBA/TPA tumorigenesis and the tumor-resistant phenotype of LC-deficient mice. The T<sup>Runx3<sup>−/−</sup></sup> mice, which have epidermal LCs, are approximately three times more resistant than WT mice to the DMBA/TPA assay. More convincingly, Runx3-null/Tg.AC mice are refractory to DMBA/TPA papillomagenesis, a fact that provides strong evidence that Runx3-null resistance is exerted at the post-initiation phase. Thus, although we cannot rule out the possibility that LC decrease or absence contributes to the DMBA/TPA resistance, by yet another noninitiation mechanism, we conclude that the unique inflammatory milieu drives Runx3-null mice robust tumor resistance.

Studies of cytokine function in tumorigenesis are complicated by their pleiotropic effects and redundancy and by the ways in which the overall cytokine environment modulates individual cytokine impact (48). Despite these difficulties, loss- and gain-of-function experiments have yielded important insights into the complex cytokines–tumor associations. Remarkably, each of the highly dysregulated cytokines in naïve and DMBA/TPA–treated Runx3-null skin—Tslp, Il17a, and osteopontin—has been implicated in decreased susceptibility to DMBA/TPA-induced tumorigenicity (4, 38, 40). Interestingly, ChlP-seq analysis in CD8<sup>+</sup> T and natural killer (NK) cells revealed Runx3 binding to both Il17a and Spp1 genes (49), suggesting that Runx3 might be involved in regulating these genes. In summary, loss of Runx3 in DCs and T cells affects tumor and/or inflammatory cell cytokine production, thereby generating a unique antitumor microenvironment that resists skin tumorigenesis.

Tumor biology can no longer be understood without considering the tumor microenvironment contributions to tumorigenicity (3). Various observations, from human epidemiology to gene-modified mice, have highlighted inflammation as a predisposing cause of cancer and it is now considered an enabling characteristic of cancer (3). Current estimates imply that a quarter of cancers are associated with chronic inflammation (of infective or sterile origin; ref. 5). Moreover, inflammatory cells and mediators are present within the microenvironment of most tumors, including those that are not epidemiologically related to inflammation (e.g., breast cancer; ref. 50). Our findings indicate that Runx3-null mice provide a model for dissecting the critical events affecting clonal progression of initiated cells to a mature neoplasm. Under the two-stage DMBA/TPA regimen, loss of leukocytic Runx3 induces a nonpermissive microenvironment that potently opposes the growth of initiated cells. Whether the DMBA/TPA protocol faithfully mimics human skin tumorigenesis is uncertain. Yet, whether targeting Runx3 in leukocytes blocks the expansion of initiated epithelial cells in other malignant settings is an important issue to address in the future, implying a significant preventive impact on cancer occurrence in the human clinical arena.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: O. Bauer, Y. Groner
Development of methodology: O. Bauer, Y. Groner
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): O. Bauer, S. Hantisteanu, J. Lotem
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): O. Bauer, J. Lotem, Y. Groner
Writing, review, and/or revision of the manuscript: O. Bauer, J. Lotem, Y. Groner
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): O. Bauer
Study supervision: Y. Groner

Acknowledgments
The authors thank Avraham Keter, Zamir Kokhavi, Rafi Saka, Arie Gumberol, and Ofir Higfa for help in animal husbandry; Ori Brenner for histopathologic analyses and helpful advice; Steffen Jung and Uri Gat for...
helpful discussions; and Edna Schechtman and Yisrael Parmet for assistance in statistical analysis.

Grant Support
This study was supported by a grant from Israel Science Foundation (ISF) individual grants to Y. Groner

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 21, 2014; revised June 10, 2014; accepted June 12, 2014; published OnlineFirst June 24, 2014.

References

27. Demehri S, Turkoz A, Manivasagam S, Yockey LJ, Turkoz M, Kopan R. Elevated epidermal thymic stromal lymphopoietin levels

www.aacajournals.org

Cancer Prev Res; 7(9) September 2014

925

Downloaded from cancerpreventionresearch.aacajournals.org on May 4, 2021. © 2014 American Association for Cancer Research.


Cancer Prevention Research

Carcinogen-Induced Skin Tumor Development Requires Leukocytic Expression of the Transcription Factor Runx3

Omri Bauer, Shay Hantisteanu, Joseph Lotem, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1940-6207.CAPR-14-0098-T

Supplementary Material
Access the most recent supplemental material at:
http://cancerpreventionresearch.aacrjournals.org/content/suppl/2014/06/28/1940-6207.CAPR-14-0098-T.DC1

Cited articles
This article cites 50 articles, 18 of which you can access for free at:
http://cancerpreventionresearch.aacrjournals.org/content/7/9/913.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://cancerpreventionresearch.aacrjournals.org/content/7/9/913.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerpreventionresearch.aacrjournals.org/content/7/9/913.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.