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Mast Cells Are Dispensable for Normal and Activin-Promoted Wound Healing and Skin Carcinogenesis

Maria Antsiferova,* Caroline Martin,* Marcel Huber,† Thorsten B. Feyerabend,‡ Anja Förster,§ Karin Hartmann,§ Hans-Reimer Rodewald,‡,1 Daniel Hohl,†,1 and Sabine Werner*

The growth and differentiation factor activin A is a key regulator of tissue repair, inflammation, fibrosis, and tumorigenesis. However, the cellular targets, which mediate the different activin functions, are still largely unknown. In this study, we show that activin increases the number of mature mast cells in mouse skin in vivo. To determine the relevance of this finding for wound healing and skin carcinogenesis, we mated activin transgenic mice with CreMaster mice, which are characterized by Cre recombinase-mediated mast cell eradication. Using single- and double-mutant mice, we show that loss of mast cells neither affected the stimulatory effect of overexpressed activin on granulation tissue formation and reepithelialization of skin wounds nor its protumorigenic activity in a model of chemically induced skin carcinogenesis. Furthermore, mast cell deficiency did not alter wounding-induced inflammation and new tissue formation or chemically induced angiogenesis and tumorigenesis in mice with normal activin levels. These findings reveal that mast cells are not major targets of activin during wound healing and skin cancer development and also argue against nonredundant functions of mast cells in wound healing and skin carcinogenesis in general. ***The Journal of Immunology*****, 2013, 191: 000–000.**

Mast cells are tissue-resident cells and well known for their role in mediating IgE-driven allergic reactions. Over the past years, many other functions of mast cells have been described, including contributions to tissue remodeling, angiogenesis, and cancer development and progression (1). In addition, it was proposed that mast cells regulate all phases of wound healing from the initial inflammatory response to reepithelialization and collagen remodeling (2). Experiments with mast cell–deficient Kit^{W}/Kit^{Wv} mice, which have mutations in the gene encoding the receptor tyrosine kinase Kit (3), gave inconsistent results. One study showed reduced neutrophil infiltration after wounding in these mice, whereas new tissue formation, including reepithelialization, angiogenesis, and collagen synthesis, was not affected (4). Two other studies confirmed that wound closure was not affected by loss of mast cells (5, 6), whereas alterations in collagen remodeling were observed (5). A fourth study then confirmed the previously observed impairment in recruitment of neutrophils to the wounded areas of Kit^{W}/Kit^{Wv} mice and reported a delayed wound closure (7). However, these results may have been in part influenced by other abnormalities of Kit^{W}/Kit^{Wv} mice, which could affect the wound-healing response. Therefore, it is important to analyze mast cell function during wound healing using mast cell–deficient mice without *Kit* mutation, and ideally without a mutation in another gene. Such animals were recently generated by expression of Cre recombinase under control of the mast cell–specific carboxypeptidase A gene promoter (CreMaster mice) (8). This leads to efficient depletion of mast cells during their development due to the genotoxicity of Cre. Thus, CreMaster mice are constitutively devoid of mast cells, and these cells do not reappear during postnatal development. Interestingly, various previously predicted mast cell functions could not be confirmed in studies with CreMaster mice (8).

Activin, a pleiotropic growth and differentiation factor of the TGF-β superfamily, is strongly upregulated upon skin injury in mice and humans [(9) and M. Antsiferova and S. Werner, unpublished observations] as well as in human cutaneous squamous cell carcinomas (SCC) (10). Interestingly, it was shown to act on mast cell precursors by inducing their migration and maturation in vitro (11). However, it is as yet unclear whether this activity is important for the different in vivo functions of activin, including its remarkable capacity to enhance new tissue formation during wound healing and to promote skin carcinogenesis. Thus, we previously showed that transgenic mice overexpressing activin A in keratinocytes under control of the keratin 14 promoter (Act mice) are characterized by enhanced granulation tissue formation and accelerated reepithelialization after wounding, and also by enhanced skin carcinogenesis and malignant progression (10, 12). Interestingly, acceleration of wound repair by activin is at least in part mediated via stromal cells (13). The role of the stroma in the protumorigenic effect of activin was even more remarkable, because inhibition of activin signaling in keratinocytes did not reduce tumorigenesis. By contrast, the enhanced tumor formation

*Department of Biology, Institute of Molecular Health Sciences, Swiss Federal Institute of Technology Zurich, 8093 Zürich, Switzerland; †Department of Dermatology, University of Lausanne, 1011 Lausanne, Switzerland; ‡German Cancer Research Center, 69120 Heidelberg, Germany; and §Department of Dermatology, University of Cologne, 50937 Cologne, Germany

[1]H.-R.R. and D.H. contributed equally to this work.

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Address correspondence and reprint requests to Prof. Sabine Werner, Institute of Molecular Health Sciences, ETH Zurich, Schafmattstrasse 22, HPL F12, 8093 Zurich, Switzerland. E-mail address: sabine.werner@biol.ethz.ch

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Abbreviations used in this article: BMMC, bone marrow–derived mast cell; DMBA, 7,12-dimethylbenz(a)anthracene; HPV, human papilloma virus; MPO, myeloperoxidase; qRT-PCR, quantitative RT-PCR; SCC, squamous cell carcinoma; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; wt, wild-type.

and malignant progression resulted from the generation of a protumorigenic microenvironment by activin, which included depletion of epidermal γδ T cells and an increase in epidermal αβ T cells and Langerhans cells (10). However, a possible involvement of mast cells in the protumorigenic phenotype of activin or in its healing-promoting activity had previously not been considered. To test these possibilities, we first analyzed the effect of activin on mast cells in vivo. In addition, we crossed Act mice with CreMaster mice and performed wound-healing and skin carcinogenesis studies. These experiments also allowed us to address the general role of mast cells in wound repair and carcinogenesis by comparing these processes in wild-type (wt) and CreMaster mice.

Materials and Methods

Animals

Act mice in CD1 genetic background (14) were mated with CreMaster mice in C57BL/6 genetic background (8). All experiments were performed with the F_1 progeny. Genotyping of these mice was previously described (8, 10).

Mice were housed under optimal hygiene conditions and maintained according to Swiss animal protection guidelines. All procedures with mice were approved by the local veterinary authorities of Zurich or Lausanne, Switzerland.

In vivo activin injection

Mice were anesthetized, and a 2 × 2-cm area of the back skin was shaved. A quantity amounting to 1 μg recombinant human activin A (provided by Chiron, Emeryville, CA) in a volume of 100 μl 0.9% NaCl/0.5% BSA was injected intradermally into the center of the shaved area, and the site of injection was marked with a pen. Vehicle solution was injected in a group of control mice. Six, 12, or 24 h later, mice were sacrificed and the marked area of the back skin was excised and bisected. One half was fixed in 95% ethanol/1% acetic acid and processed for paraffin embedding; another half was snap frozen in liquid nitrogen for RNA isolation. Sections (7 μm) were analyzed for mast cells, as described below.

Mast cell staining

For visualization of mast cells, paraffin sections of 95% ethanol/1% acetic acid–fixed tissue samples were stained with 0.5% toluidine blue (Sigma-Aldrich, Munich, Germany) in 0.5 N HCl for 30 min. Alternatively, chloroacetate esterase histochemistry was performed to visualize chymase-like activity of mast cells (15).

Stained sections were photographed using an Imager.A1 microscope equipped with an Axiocam Mrm camera and enhanced-contrast Plan-Neofluor objectives (10×/0.3 NA, 20×/0.5 NA; all from Carl Zeiss, Oberkochen, Germany). Axiovision 4.6 software (Carl Zeiss) was used for data acquisition.

RNA isolation and quantitative RT-PCR

Total RNA was isolated using the RNeasy fibrous tissue mini kit (Qiagen, Hilden, Germany). Remaining DNA was removed by incubation with RQ1 DNase (Promega, Madison, WI). cDNA was synthesized using the iScript kit (Bio-Rad Laboratories, Hercules, CA). Relative gene expression was determined using the Roche LightCycler 480 SYBR Green system (Roche, Rotkreuz, Switzerland). Alternatively, semiquantitative RT-PCR was performed, and the amplification products were analyzed by agarose gel electrophoresis.

Primers used for quantitative and semiquantitative RT-PCR

Primers are as follows: *Scf* (5′-CATTTATCTTCAACTGCTCCTATTT-3′; 5′-GGTCATCCACTATTTTCCCAAG-3′); *Ngf* (5′-GTGCCTCAAGCCAGTGAAAT-3′; 5′-GCGGCCAGTATAGAAAGCTG-3′); *Ccl2* (5′-TTCTGGGCCTGCTGTTCAC-3′; 5′-GAGCCAACACGTGGATGCT-3′); *Ccl5* (5′-GCAGTCGTGTTTGTCACTCG-3′; 5′-ATTACTGAGTGGCATCCCCA-3′); *Acvr1b* (5′-CTCCAAAGACAAGACGCTCC-3′; 5′-AGCAGCAATAAAGCCAAGGA-3′); *Acvr1c* (5′-TATCACACTGCACCTTCCCA-3′; 5′-ACCAAGAGAGGCAGACCAGA-3′); *Acvr2a* (5′-CGTTCGCCGTCTTTCTTATC-3′; 5′-GCCCTCACAGCAACAAAAGT-3′); *Acvr2b* (5′-ACTACAACGCCAACTGGGAG-3′; 5′-TGGCTCGTACGTGACTTCTG-3′); *Cpa3* (5′-TGGTCATGGACACAGGATCG-3′; 5′-GTGGATGCTATTGGGCCGTA-3′); *Mcp4* (5′-AGAAAAGATCGGCATACAAGGG-3′; 5′-TCTCCGCGTCCATAAGATACA-3′); *Mcp6* (5′-CTGGCTAGTCTGGTGTACTCA-3′; 5′-CAGGGCCACCTACTCTCAGAA-3′); *Rps29* (5′-GGTCACCAGCAGCTCTACTG-3′; 5′-GTCCAACTTAATGAAGCCTATGTCC-3′); *Gapdh* (5′-TCGTGGATCTGCCGTGCCGCCTG-3′; 5′-CACCACCCTGTTGCTGTAGCCGTAT-3′).

Isolation of mast cells from mouse skin and culture of bone marrow–derived mast cell precursors

Skin tissue from mouse ears was pooled from 10 C57BL/6 mice, minced, and resuspended in IMDM containing 2 mg/ml collagenase type IV. After two rounds of digestion (30 min at 37°C each), liberated cells were stained using anti–Kit-allophycocyanin and anti–CD45-PeCy7 Abs. RNA was isolated from 20,000 cells with RNAzol (Sigma-Aldrich), according to the manufacturer's instructions, pretreated with heparinase (NEB, Beverly, MA) for 90 min, followed by reverse transcription (SuperScript first strand cDNA synthesis kit; Invitrogen, Carlsbad, CA) with oligodT priming.

Bone marrow–derived mast cells (BMMCs) were isolated from the femoral and tibial bone marrow of a C57BL/6 mouse and cultured in DMEM (high glucose; Invitrogen) supplemented with 20% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 30% WEHI-3B–conditioned medium (as a source of IL-3). RNA was isolated from ∼200,000 cells using the RNeasy Plus micro kit (Qiagen, Hilden, Germany). The complete RNA sample was used for cDNA synthesis using the iScript kit (Bio-Rad).

Semiquantitative RT-PCR was performed with 1/50 of the cDNA (1 μl) using Taq polymerase (Invitrogen).

Wounding and preparation of wound tissue

Mice (8–10 wk old) were anesthetized by i.p. injection of ketamine (75 mg/kg)/xylazine (5 mg/kg). Four full-thickness excisional wounds of 5 mm diameter were generated on the back of mice by excising the skin and the rodent-specific s.c. muscle *panniculus carnosus*, as described previously (16). Wounds were left uncovered and photographs were taken with a digital camera at different time points after injury. For macroscopic analysis of wound area, photographs were analyzed using ImageJ software. For histological analysis, wounds were excised, including 2–3 mm of the adjacent back skin, bisected, fixed in 95% ethanol/1% acetic acid, and embedded in paraffin. H&E staining of 7-μm sections from the middle of the wounds was performed. Images were acquired with Slide Scanner (3DHistech, Budapest, Hungary), and histomorphometrical measurements were performed using Fiji software (17). Only mice of the same age and gender were used for direct comparison.

Ly6G immunostaining

Three-day wounds were harvested, bisected, fixed with 95% ethanol/1% acetic acid, and embedded in paraffin. The 7-μm sections were stained with a Ly6G Ab (BD Biosciences, San Diego, CA), followed by an anti–rat-biotin secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA), and by ABC Vectastain solution and AEC Substrate kit. Images were acquired with Slide Scanner (3DHistech), and Ly6G-positive cells were quantified in the newly formed granulation tissue and in the adjacent dermis at the wound edges using Fiji software (17).

Masson trichrome staining

Masson's trichrome staining was performed with a Masson-Goldner Trichrome kit (Merck, Darmstadt, Germany), according to manufacturer's instructions.

Myeloperoxidase enzymatic activity assay

To estimate the tissue neutrophil content, myeloperoxidase (MPO) activity assay was performed according to (18) with some modifications. MPO was extracted by homogenizing frozen tissue samples in hexadecyltrimethylammonium bromide extraction buffer (0.5% hexadecyltrimethylammonium bromide, 50 mM potassium phosphate [pH 6.0]) using an Ultra Turrax homogenizer (Janke & Kunkel, Staufen, Germany), followed by sonication for 10 s in an ice bath. Specimens were freeze thawed three times, after which sonication was repeated. Suspensions were then centrifuged at 4000 rpm for 30 min at 4°C, and the supernatant was assayed. A total of 50 μl of the sample was mixed with 150 μl reaction buffer (0.229 mg/ml *o*-dianisidine dihydrochloride, 0.0005% H_2O_2, 50 mM potassium phosphate buffer), and the change in absorbance at 450 nm was immediately measured in kinetic mode at 25°C for 100 s. Results were analyzed using Prism software. Nonlinear regression, straight line fit was used to calculate the slope (OD/min). A total of 1 mU MPO activity was assigned to the amount of enzyme that gives an absorbance increment of 0.001 OD/min.

Chemical skin carcinogenesis experiment

A total of 25 μg 7,12-dimethylbenz(a)anthracene (DMBA; Sigma-Aldrich) dissolved in acetone was applied on the back skin of 8- to 10-wk-old female

mice 2 d after shaving. One week later, 7.5 μg 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma-Aldrich) in acetone was applied weekly for 20 wk. Tumor number and size were documented biweekly. Tumors were grouped into four categories, as follows: small papilloma (<2 mm diameter), medium papilloma (2–6 mm diameter), large papilloma (>6 mm diameter), and SCC (ulcerating/invasive tumor). Twenty or 32 wk after DMBA treatment, biopsies of nontumorigenic back skin and of tumors with at least 2 mm diameter were taken and used for histology, immunohistochemistry, or RNA isolation. All large/ulcerating tumors (classified as SCC macroscopically) were analyzed histologically for signs of invasiveness to confirm the malignancy.

Acute DMBA/TPA-induced inflammation and MECA-32 immunostaining

Female 13-wk-old mice were treated with 25 μg DMBA and 1 wk later with 7.5 μg TPA to induce acute inflammation. Twenty-four hours later, they were sacrificed, and the treated back skin was fixed with 95% ethanol/1% acetic acid and embedded in paraffin. Sections were stained with a *Pan* endothelial cell Ag Ab (clone MECA-32; BD Biosciences), followed by an anti–rat-Cy3 secondary Ab (Jackson ImmunoResearch Laboratories), and counterstained with Hoechst.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0a for Mac OS X (GraphPad Software, San Diego, CA). Mann–Whitney *U* test was used for comparing two groups of data; two-way ANOVA test was used for comparing mice of four different genotypes. For statistical analysis of tumor incidence, comparison of the curves showing the number of mice with tumors was performed using log-rank χ^2 test. Tumor multiplicity was analyzed using two-way repeated measures ANOVA. The *p* values were $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

Results

Activin increases mast cell numbers in vivo

Activin had previously been shown to act as a chemoattractant and maturation factor for cultured BMMCs (11). To test whether it can also affect mast cells in vivo, we injected activin A intradermally into the skin of wt mice. Six and 12 h after injection, there was a slight increase in the number of toluidine blue–positive mast cells at the site of activin injection (Fig. 1A), and the increase was statistically significant 24 h after injection (Fig. 1A, 1B). To test whether the effect of activin might be mediated via upregulation of known mast cell survival or chemotactic factors, we analyzed the mRNA levels of stem cell factor (*Scf*), nerve growth factor (*Ngf*), and the chemokines *Ccl2* and *Ccl5* in activin- or vehicle-injected skin using quantitative RT-PCR (qRT-PCR). *Ccl5* (RANTES), which had been shown to increase the number of mast cells in the skin 4 h after s.c. injection (19), was slightly and transiently upregulated 6 h after activin injection, whereas expression of the other factors was not altered or even reduced in the presence of activin (Supplemental Fig. 1A). It was recently reported that activin induced Th9 differentiation in a model of allergic airway disease, resulting in recruitment and activation of mast cells in the lung (20). However, we were not able to detect mRNA for IL-9, neither in normal skin of wt or Act mice, nor after activin injection (data not shown), probably due to a generally low number of T cells in the skin.

Overall, these results suggest that the effect of activin on mast cells could be mediated at least in part via Ccl5, but also through a direct attraction of mast cells from the bone marrow. Consistent with the latter assumption, we found that BMMC precursors that had been cultured with conditioned medium from WEHI3 cells, which is rich in IL-3, express all types of activin receptors (Supplemental Fig. 1B). However, none of the receptors was expressed in mast cells that had been isolated from ear skin by FACS (Supplemental Fig. 1B). Together, these findings suggest that mast cell progenitors can be attracted to the skin by activin, but downregulate activin receptors upon differentiation in this tissue.

We then analyzed mast cell numbers in the skin of Act mice under homeostatic conditions. Toluidine blue staining revealed a slight increase in mast cell number in the dermis of Act mice (Fig. 1C, 1D). Similar results were obtained with chloroacetate esterase histochemistry (data not shown). The generally higher number of mast cells in the dermis that we identified in this experiment (102 ± 26 cells/mm^2 in wt mice) compared with the experiment shown in Fig. 1A and 1B (15 ± 2 cells/mm^2 in vehicle-injected mice) most likely results from the use of a different mouse strain and the different gender (male B6D2F1 mice [Fig. 1A, 1B] versus female CD1 mice [Fig. 1C, 1D]). Thus, male mice were shown to have lower histamine levels in dorsal and ventral skin as compared with female mice (21), and C57BL/6 mice have a generally low mast cell content in the skin (22). Besides, male mice have a significantly thinner layer of hypodermis (s.c. fat), hardly distinguishable from the dermis on toluidine blue–stained sections. Therefore, mast cells were quantified and related to the total area of dermis and hypodermis in male mice, but only to the area of dermis in female mice.

As an independent approach, we analyzed the expression levels of mast cell–specific enzymes in the skin of wt and Act mice using qRT-PCR. Expression of mast cell proteases (*Mcpt*)-4 and -6, which are expressed by mature mast cells (23), and which are upregulated by activin in mast cell progenitors (*Mcpt6*) (24), was indeed higher in the skin of Act mice (Fig. 1E), suggesting an increase in mature mast cells. In summary, these results demonstrate that activin A increases the number of mature mast cells in the skin in vivo.

We next analyzed mast cell numbers during wound healing of control and Act mice. There was no significant increase in the number of mast cells in Act mice compared with wt controls within 2 mm from the edge of full-thickness excisional wounds at day 5 or 10 (Fig. 1F, 1G). Surprisingly, there were only very few mast cells in the granulation tissue of wt and Act mice at days 5, 10, and 13 after wounding, and their number was even lower in 10-d wounds of Act mice compared with wt controls (Fig. 1H, 1I, Supplemental Fig. 1C). Even 13 d after injury there were very few mast cells in the wound tissue (Supplemental Fig. 1C), and these were located at the periphery of the granulation tissue (data not shown). However, their number increased in the vicinity of the wounds at this stage, indicating that mast cell accumulation is a late event in wound healing. It seems likely that mast cells are not efficiently attracted to the granulation tissue of healing wounds, even in the presence of high levels of activin. This may be due to the lack of additional factors required for mast cell migration/survival in the newly formed granulation tissue or to the lack of expression of certain proteases that are required for invasion into the dense granulation tissue. These deficiencies can obviously not be overcome by activin overexpression.

Wound closure is not impaired in mast cell–deficient mice

To determine whether loss of mast cells affects wound healing in a wt background and/or the accelerated healing seen in Act mice, we crossed Act mice with mast cell–deficient CreMaster mice (8) and performed wound-healing experiments with the F_1 generation. Lack of expression of the mast cell–specific enzyme *Cpa3* in normal skin of CreMaster or Act/CreMaster mice strongly suggested the complete lack of mast cells (Supplemental Fig. 2A). This was confirmed by toluidine blue staining of untreated or wounded back skin of CreMaster mice, as well as of Act/CreMaster double-transgenic animals. Mast cells could not be detected in these mice at any stage of the healing process (Supplemental Fig. 2B–E).

Because it was previously suggested that the ability of mast cells to regulate vascular permeability and neutrophil infiltration is important for wound closure (7), we first measured levels of the neutrophil-specific enzyme MPO in early wounds of mice of all genotypes. However, neither activin overexpression nor mast cell

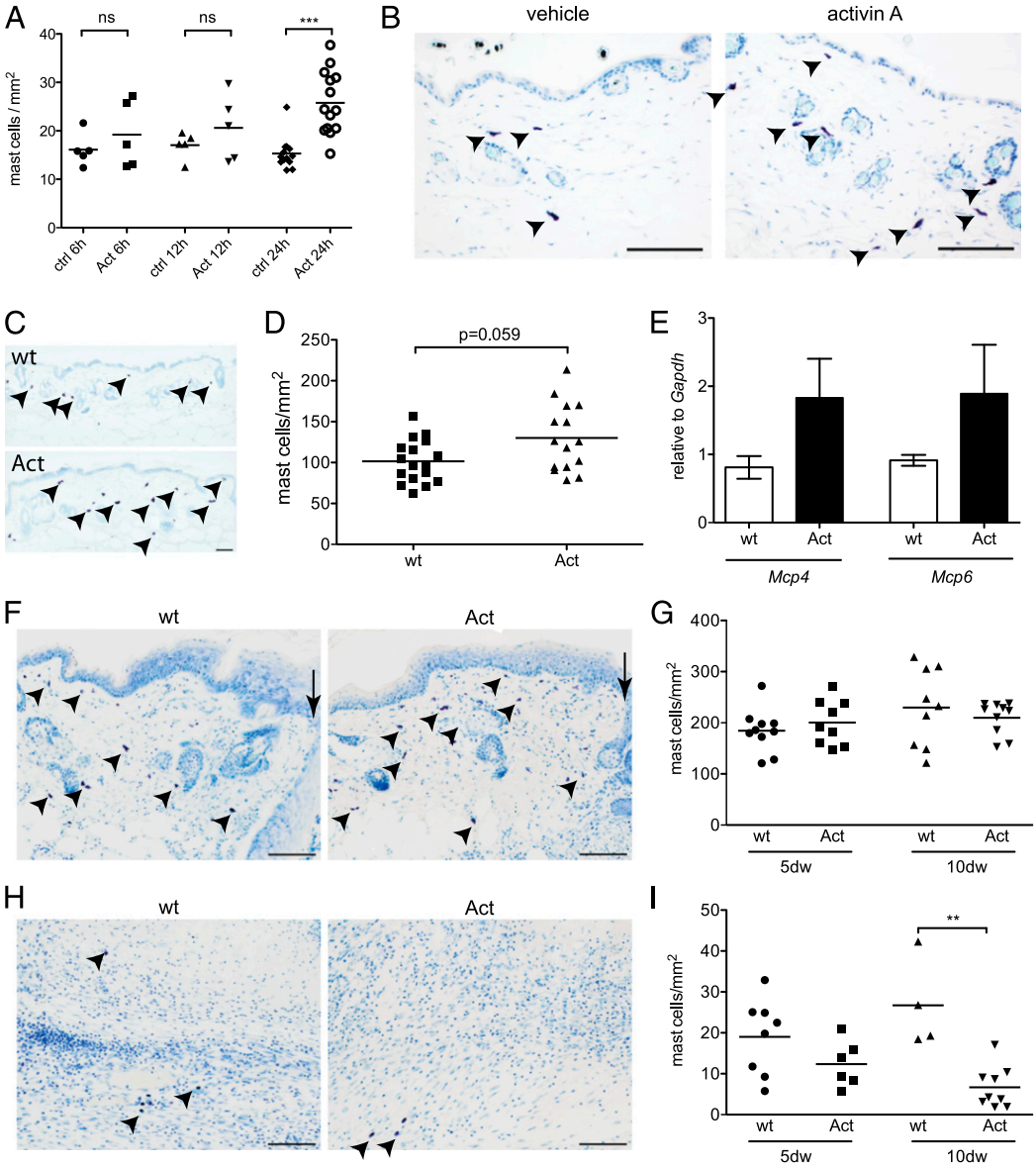


FIGURE 1. Activin A increases the number of mast cells in murine skin. (**A** and **B**) A total of 1 μg recombinant activin A or vehicle control was injected intradermally into the skin of male wt mice (B6D2F1 background). They were sacrificed 6, 12, or 24 h after injection, and mast cells were identified by toluidine blue staining of skin sections. Representative pictures from mouse skin 24 h after activin or vehicle injection are shown in (B); arrowheads point to mast cells. Scale bars, 100 μm. Quantification of the data (scatter plots and mean values) is shown in (A). The 24-h results are combined from three independent experiments. n = 5–14 per time point and treatment group, ***$p < 0.001$ according to one-way ANOVA with Bonferroni's multiple comparison test. (**C** and **D**) Mast cells in the dermis of female wt and Act mice (CD1 background) were identified by toluidine blue staining and quantified. Representative pictures are shown in (C). Scale bars, 100 μm. Quantification of the result is shown in (D) (scatter plot and mean values). Results were combined from four independent experiments. n = 17 wt and 15 Act mice, $p = 0.059$, according to Mann–Whitney U test. (**E**) RNA samples from the skin of wt and Act mice were analyzed for expression of *Mcp4* and *Mcp6* (relative to *Gapdh*) by qRT-PCR. Bars indicate mean ± SD. n = 3 wt and 3 Act mice. (**F**–**I**) Sections from the middle of 5-d or 10-d full-thickness excisional wounds of female Act mice and wt controls were stained with toluidine blue, and mast cell numbers were determined in the dermis adjacent to the wound (within ~2 mm from the wound edge, indicated by the arrow) (F, G) or in the granulation tissue (H, I). Representative pictures of the wound edge (F) or of the center of the granulation tissue (H) of 5-d wounds are shown. Scale bars, 100 μm. (G) Quantification of mast cells at the wound edge. (I) Quantification of mast cells in the granulation tissue. Scatter plots and mean values are shown. At least four wounds from four mice were analyzed. **$p = 0.0028$ according to Mann–Whitney test.

deficiency affected the MPO activity in the wound tissue at day 1 or 3 after injury (Fig. 2A and data not shown). As an alternative approach, we also quantified the number of Ly6G-positive cells on the sections of 3-d wounds and found no major difference between genotypes (Supplemental Fig. 3A).

We then macroscopically analyzed the wound surface area by taking photographs of the wounds and subsequent quantification of the wound area. In contrast to the results obtained with other mast cell–deficient mice (7), wound closure was not decreased in CreMaster mice (Fig. 2B). Rather, at the early stages of wound healing, these mice had slightly smaller wounds than corresponding controls.

Macroscopic analysis of the wound area mainly detects differences in wound contraction, a process that is particularly efficient in rodent wounds (25). To detect potential differences in granulation tissue formation or reepithelialization, we performed histomorphometrical analysis of sections from the middle of the wounds at day 5 after injury (Fig. 2C–G). This time point was chosen because it

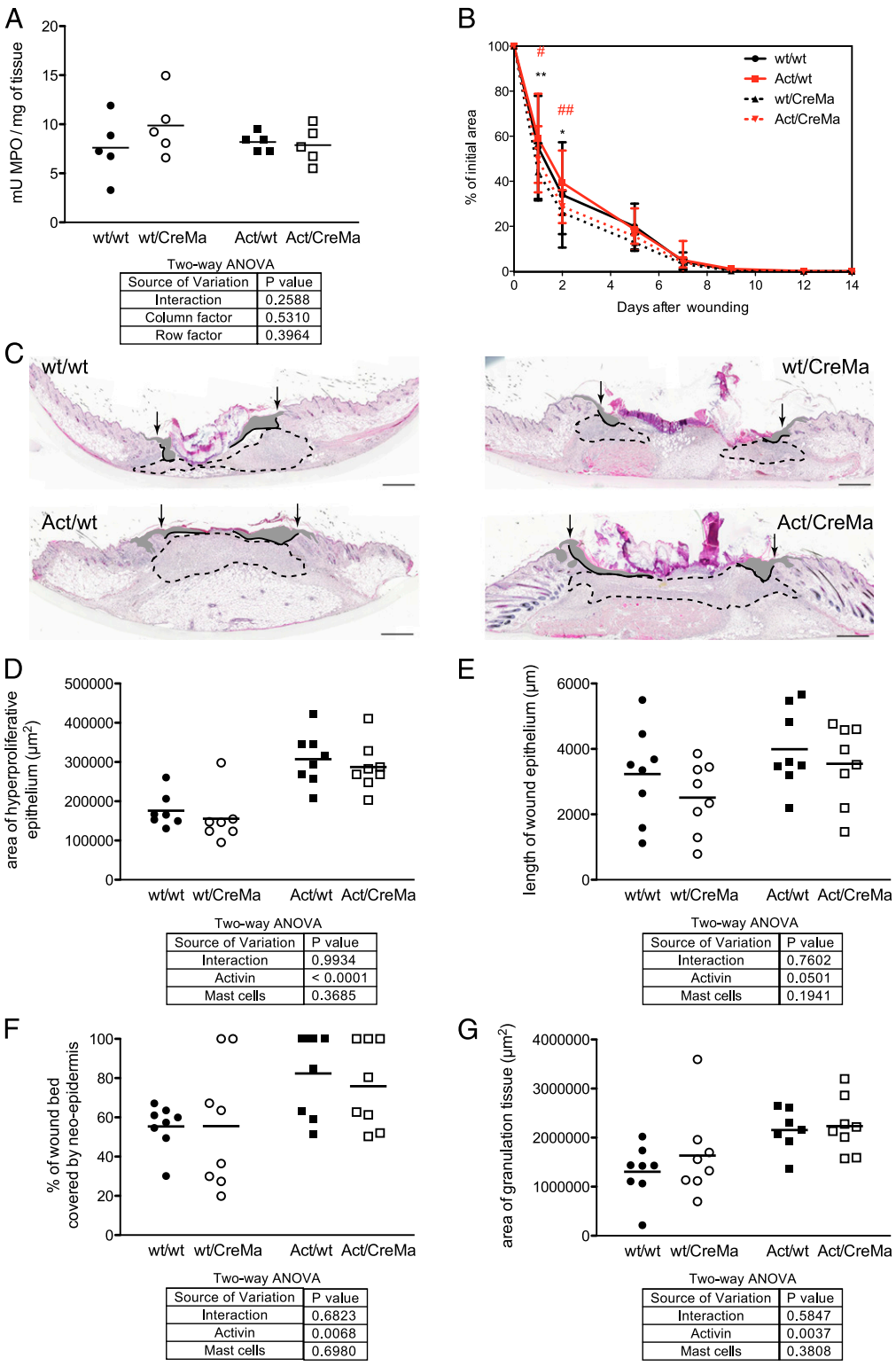


FIGURE 2. Loss of mast cells does not affect neutrophil recruitment to wounded skin and does not impair the wound-healing process in wt or activin-overexpressing mice. Full-thickness excisional wounds were generated in female Act and CreMaster single-transgenic mice, Act/CreMaster double-transgenic mice, and wt controls. (**A**) Lysates from 1-d wounds were analyzed for the activity of the neutrophil-specific enzyme MPO. $n = 5$ wounds from five mice. Dot plot and mean values are shown. (**B**) The wound area was determined on photographs taken at the indicated time points after wounding. Note the slightly enhanced wound closure in CreMaster mice at days 1 and 2 after injury. Twenty wounds from five animals were analyzed at each time point. Mean values ± SD are shown. $^{*}p < 0.05$, $^{**}p < 0.01$ for the comparison between wt/wt and wt/CreMaster mice; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ for the comparison between Act/wt and Act/CreMaster mice (two-way repeated measures ANOVA with Bonferroni posttests; first factor: genotype, second factor: time). (**C**) Representative microphotographs of sections from the middle of 5-d wounds from mice of all genotypes. The parameters analyzed by histomorphometrical measurements are schematically depicted: black line indicates the length of the wound epithelium (neo-epidermis), shadowed area indicates the area of hyperproliferative epithelium, arrows indicate wound edges, and dashed line demarcates the area of granulation tissue. Scale bars, 500 µm. (**D**–**G**) Histomorphometric analysis of the area of the hyperproliferative epithelium (D), the length of wound epithelium (E), the percentage (*Figure legend continues*)

represents the peak of reepithelialization and granulation tissue formation in the wound model that we used (26). Consistent with our previous nonquantified observations (12), Act mice had a significantly larger area of hyperproliferative epithelium and of granulation tissue compared with control mice (Fig. 2D, 2G). The increase in both parameters in the absence of a difference in wound contraction also provides an explanation for the unaltered wound closure rate of these animals that was detected by macroscopic wound analysis. Furthermore, the length of the wound epithelium (neo-epidermis), which reflects keratinocyte migration, was also slightly increased in Act mice (Fig. 2E). However, none of these parameters was affected by the absence of mast cells (Fig. 2D–G). Loss of these cells also did not impair wound reepithelialization or granulation tissue formation in mice with normal activin levels (Fig. 2D–G). Finally, loss of mast cells did not affect the area and density of the late granulation tissue/early scar tissue at day 13 after injury in mice of a wt background, neither did it reduce the hyperthickened neo-epidermis and enlarged scar area seen in Act mice at this time point (Supplemental Fig. 3B, 3C). In summary, these results strongly suggest that mast cells are dispensable for the healing of full-thickness excisional wounds in mice and also do not obviously affect the scarring response.

Mast cell deficiency does not affect the activin-induced acceleration of chemically induced skin carcinogenesis

Because there are remarkable parallels between wound healing and cancer (27) and because mast cells had been proposed, based on work in *Kit* mutants, to contribute to human papilloma virus (HPV) 16-induced skin carcinogenesis (28), we asked whether these cells also play a role in chemically induced skin carcinogenesis. This possibility was particularly intriguing, because we recently showed that Act mice are strongly susceptible to skin carcinogenesis induced by topical application of the mutagen DMBA followed by 20 applications of the tumor promoter TPA (10), and because elevated numbers of mast cells were seen in the skin of Act mice 24 h after treatment with DMBA, DMBA plus TPA, or vehicle acetone, which induces mild inflammation (Supplemental Fig. 4A). Most importantly, the number of mast cells in the dermis below the papillomas collected 20 wk after the last TPA application was significantly higher in Act mice compared with control mice (Supplemental Fig. 4B). To determine a potential role of mast cells in activin-induced promotion of tumorigenesis, we performed a DMBA/TPA-induced skin carcinogenesis study using the F_1 progeny of a breeding of Act mice with CreMaster mice (Act mice, CreMaster mice, Act/CreMaster double-transgenic mice, and wt controls). The absence of mast cells in the tumors of CreMaster and Act/CreMaster mice was confirmed by toluidine blue staining (Supplemental Fig. 4C). Tumor incidence and multiplicity were similar in wt/wt and wt/CreMaster mice, indicating that mast cells do not contribute to skin tumorigenesis in this model (Fig. 3A, 3B). Most of the tumors were classical papillomas, and there was no major difference in tumor size between mice of different genotypes (Fig. 3C). Histological analysis of large/invasive tumors collected 12 wk after the last TPA application revealed a similar incidence of SCC in wt and CreMaster mice (data not shown). Because the tumor multiplicity in wt and wt/CreMaster mice was low in this experiment (Fig. 3B), a possible effect of mast cell loss on tumor load could not be determined in mice with normal activin levels. However, in Act mice, the loss of mast cells did not reduce

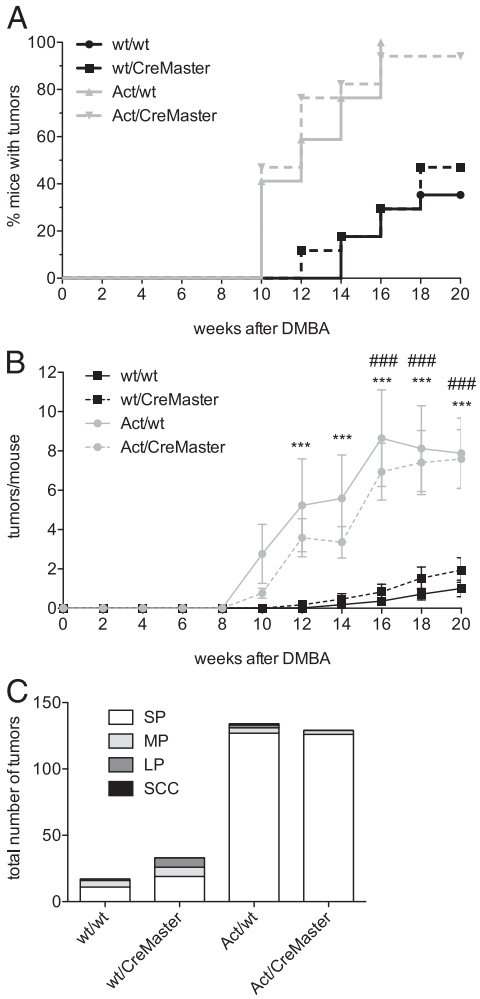


FIGURE 3. Mast cell deficiency does not significantly reduce the protumorigenic effect of activin in DMBA/TPA-induced skin carcinogenesis. Kinetics of tumor incidence (**A**) or multiplicity (**B**) in Act/wt and wt/CreMaster single-transgenic mice, Act/CreMaster double-transgenic mice, and wt/wt controls. $n = 17$ mice per genotype. Mean ± SEM are shown in (B). ***$p < 0.001$ for the comparison between wt/wt and Act/wt mice and between wt/CreMaster and Act/wt mice, ###$p < 0.001$ for the comparison between wt/wt and Act/CreMaster and wt/CreMaster and Act/CreMaster mice (two-way repeated measures ANOVA and Bonferroni posttest; first factor: genotype, second factor: time). (**C**) Distribution of tumors, collected after 20 TPA applications, according to tumor diameter and tumor type: SP, small papilloma (<2 mm); MP, medium papilloma (2–6 mm); LP, large papilloma (>6 mm).

tumor incidence (Fig. 3A) and only slightly, but nonsignificantly, reduced tumor multiplicity during the early stages of carcinogenesis (Fig. 3B). Similarly, the incidence of SCC did not differ significantly between Act and Act/CreMaster mice (data not shown). Thus, mast cells are obviously not major regulators of activin-induced skin carcinogenesis.

Tumorigenesis-associated vascularization is not impaired in CreMaster mice

Because mast cells had been invoked in HPV8-induced tumorigenesis via their effects on angiogenesis (28), we characterized the

of the wound surface covered by neo-epidermis (wound epithelium) (F), and the area of granulation tissue (G) using sections from the middle of 5-d wounds from mice of all genotypes. Dot plots and mean values are shown. At least eight wounds from four mice were analyzed. The results of two-way ANOVA are shown below each graph (first factor: presence of CreMaster transgene, second factor: presence of activin transgene).

vasculature during chemically induced skin carcinogenesis. MECA-32 staining revealed that, upon induction of acute inflammation by DMBA and TPA application (24 h after the first TPA application), vessel size or area covered by vessels was not affected by mast cell deficiency or activin overexpression (Fig. 4A–C). Vessel size in chemically induced tumors collected 20 wk after DMBA initiation was slightly reduced in activin-overexpressing mice, but was not influenced by the loss of mast cells (Fig. 4D). These data demonstrate that loss of mast cells does not obviously affect angiogenesis during chemically induced skin carcinogenesis.

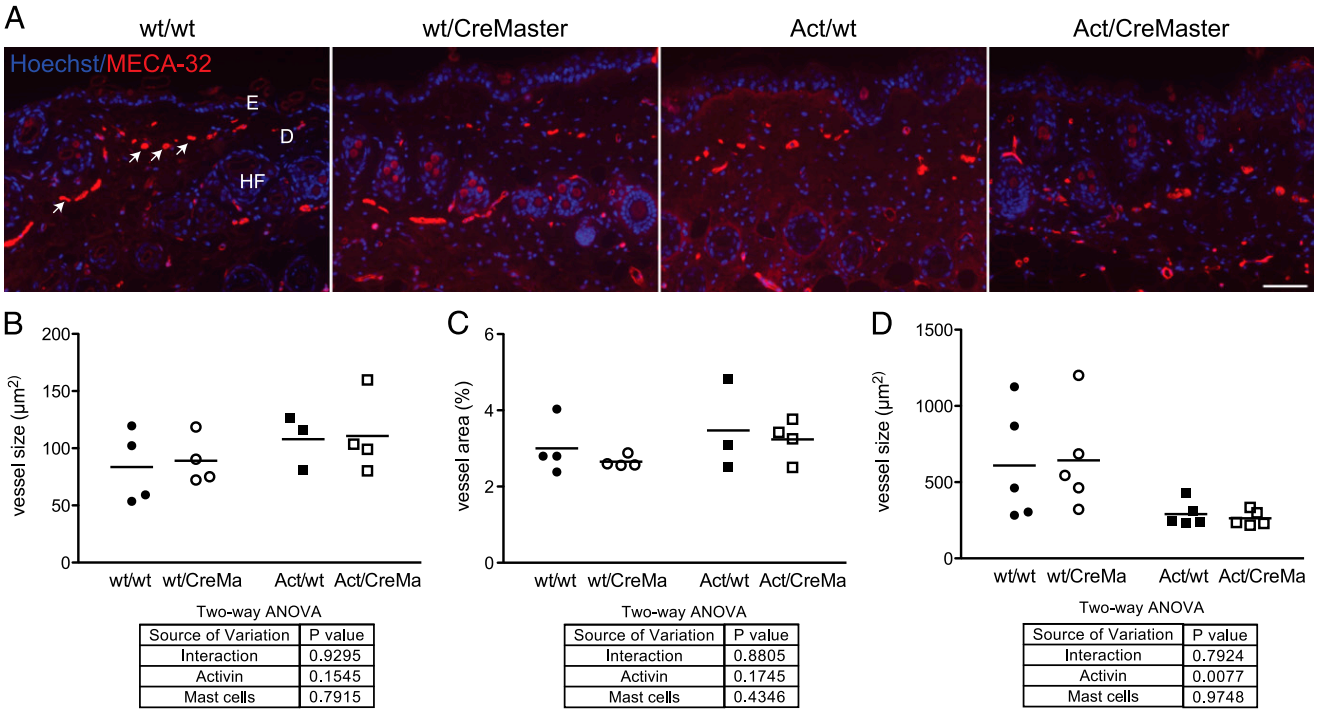
Discussion

In previous studies, we demonstrated that activin promotes wound healing and chemically induced skin carcinogenesis, in particular by activating various types of stromal cells (10, 12, 13, 29). Furthermore, other laboratories demonstrated that activin affects different types of immune cells in vitro and in vivo [reviewed by (29, 30)]. In particular, activin was shown to directly induce migration and differentiation of BMMC precursors via activation of activin receptors on these cells (11). Consistent with this finding, we detected expression of all types of activin receptors in BMMCs after in vitro differentiation in the presence of IL-3. These data suggest that activin directly increases the number of mature mast cells in vivo by inducing migration and/or differentiation of mast cell precursors. Because we could not detect expression of activin receptors in mast cells isolated from the skin, it seems likely that, once settled in the skin, mast cells downregulate activin receptors and are thus not further affected by activin. Therefore, activin-induced stimulation of mast cell proliferation in the skin is unlikely to contribute to the increase in cutaneous mast cells after activin injection. In addition to a direct effect of activin on mast cell precursors, the upregulation of *Ccl5* expression that we observed in response to activin injection may contribute to the effect of activin on mast cells in vivo.

Because mast cells were suggested previously to contribute to wound healing (2, 5, 7), we hypothesized that activin promotes wound healing at least in part via attraction and/or differentiation of mast cells. Surprisingly, however, the strong promotion of new tissue formation in healing wounds and the acceleration of skin carcinogenesis by overexpression of activin were not or only marginally reduced in the absence of mast cells. Thus, mast cells are obviously not the key mediators of these activities of activin.

Our previous data revealed an important role of different types of T cells in the protumorigenic effect of activin (10), but the major target cells of activin in healing wounds remain to be determined. Because activin strongly accelerated granulation tissue formation, a role of fibroblasts in activin-promoted wound healing seems likely. This hypothesis is supported by the potent effect of activin on matrix production by skin fibroblasts (31), by the enhanced scar formation that we observed in Act mice (Supplemental Fig. 3B, 3C, and M. Antsiferova and S. Werner, unpublished observations), and by the activin-induced promotion of fibrotic processes in other organs (32). Because carcinogenesis is also promoted by a fibrotic microenvironment (33), the effect of activin on matrix production by fibroblasts may also contribute to its protumorigenic activity in the skin.

To our surprise, we found that mast cells are dispensable for normal wound healing. This is contradictory to results from previous studies using the same wound model, which demonstrated that mast cells contribute to wounding-induced inflammation and new tissue formation (4, 7). However, these studies were performed with Kit^{W}/Kit^{Wv} mice, known to have additional abnormalities in the immune system (3, 34). In particular, they are neutropenic (35), which may explain the observed reduction in neutrophil infiltration



Two-way ANOVA

Source of Variation	P value
Interaction	0.9295
Activin	0.1545
Mast cells	0.7915

Two-way ANOVA

Source of Variation	P value
Interaction	0.8805
Activin	0.1745
Mast cells	0.4346

Two-way ANOVA

Source of Variation	P value
Interaction	0.7924
Activin	0.0077
Mast cells	0.9748

FIGURE 4. Neither activin overexpression nor mast cell deficiency affects blood vessel size and number in TPA-treated skin and in tumors. Sections from inflamed back skin collected 24 h after the first TPA application (**A**–**C**) were stained with a MECA-32 Ab to visualize blood vessels. Representative micrographs are shown in (A). Vessels are indicated with arrowheads. Scale bars, 100 µm. D, Dermis; E, epidermis; HF, hair follicle. Vessel size (B) and percentage of dermis area covered by vessels (C) were quantified on 11–19 micrographs per mouse. (**D**) Tumors, collected after 20 TPA applications, were stained with a MECA-32 Ab, and vessel size was quantified. Dot plot and mean values are shown in (B)–(D). Results of two-way ANOVA are shown below each graph (first factor: presence of CreMaster transgene, second factor: presence of activin transgene). n = 3–5 mice per genotype.

upon wounding (4, 7). Although the impaired healing of Kit^{W}/Kit^{Wv} mice was rescued by reconstitution of the mice with mast cells (7), this finding does not provide a final proof for a role of mast cells in wound healing. This conclusion is in agreement with several other in vivo experiments in which *Kit* mutant mice reconstituted with mast cells had a different behavior than mice with wt *Kit* alleles with and without mast cells, depending on the experimental setup (8). The normal wound closure in CreMaster mice that we observed at the macroscopic and histological level is consistent with recent data, which demonstrated that wound closure as assessed macroscopically was not affected in splinted excisional wounds of three different types of mast cell–deficient mice, including Kit^{W}/Kit^{Wv} mice (6).

Mast cells had previously been shown to contribute to collagen remodeling after completion of healing (5) and to scar formation during fetal wound healing (36). However, the analysis of 13-d wounds revealed no obvious difference in the area and density of the late granulation tissue/early scar tissue, although more subtle differences in collagen remodeling after completion of wounding cannot be excluded. In the future, it will therefore be interesting to perform a detailed analysis of the scar tissue and to study other fibrotic processes in CreMaster mice. This will reveal whether mast cells are indeed regulators of organ fibrosis in vivo.

Finally, the results presented in this study strongly suggest that mast cells are not key regulators of DMBA/TPA-induced skin carcinogenesis. This experiment should be repeated in the future with mice of another genetic background that are more susceptible to skin carcinogenesis than the CD1/C57BL/6 F_1 mice used in this study. Furthermore, a potential role of mast cells in the progression from papillomas to carcinomas remains to be determined. It was recently shown that expression of mast cell proteases correlates with angiogenesis during progression of chemically induced tumors (37), but a functional role of mast cells in angiogenesis and tumor formation had not been addressed in this model. The results obtained in our study, however, argue against a major role of mast cells in the control of blood vessels in TPA-treated skin and in skin tumors. Interestingly, mast cells had previously been shown to contribute to early neoplastic progression in a model of HPV16-induced carcinogenesis, in which transgenic mice expressing the HPV16 oncogenes in keratinocytes were bred onto the Kit^{W}/Kit^{Wv} background. Due to a high lethality of the double-mutant mice in FVB/N background, however, only one mast cell–deficient HPV16-transgenic animal was available for this analysis (28). Nevertheless, it may well be that the reduced neoplastic progression in HPV16/Kit^{W}/Kit^{Wv} mice is due to the Kit deficiency rather than to the loss of mast cells. Alternatively, mast cells may have different roles in chemically versus virus-induced carcinogenesis. This possibility is supported by a previous study showing that DMBA/TPA-induced skin carcinogenesis is not affected in *Kit* mutant mice (38). However, it needs to be considered that mast cells reappear in *Kit*-deficient mice upon induction of skin inflammation, including TPA treatment (8, 39, 40). Therefore, it was unclear whether the reappearing mast cells were sufficient to drive carcinogenesis. The use of the CreMaster mouse line that is completely deficient in mast cells even under inflammatory conditions therefore clarified this issue and revealed that DMBA/TPA-induced skin carcinogenesis is indeed not obviously affected by the loss of mast cells.

Taken together, our results revealed that mast cells are not the major targets of activin in wound healing and carcinogenesis and argue against the previously suggested nonredundant functions of mast cells in these processes in general.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Rodewald, H. R., and T. B. Feyerabend. 2012. Widespread immunological functions of mast cells: fact or fiction? *Immunity* 37: 13–24.
2. Noli, C., and A. Miolo. 2001. The mast cell in wound healing. *Vet. Dermatol.* 12: 303–313.
3. Nocka, K., J. C. Tan, E. Chiu, T. Y. Chu, P. Ray, P. Traktman, and P. Besmer. 1990. Molecular bases of dominant negative and loss of function mutations at the murine c-kit/white spotting locus: W37, Wv, W41 and W. *EMBO J.* 9: 1805–1813.
4. Egozi, E. I., A. M. Ferreira, A. L. Burns, R. L. Gamelli, and L. A. Dipietro. 2003. Mast cells modulate the inflammatory but not the proliferative response in healing wounds. *Wound Repair Regen.* 11: 46–54.
5. Iba, Y., A. Shibata, M. Kato, and T. Masukawa. 2004. Possible involvement of mast cells in collagen remodeling in the late phase of cutaneous wound healing in mice. *Int. Immunopharmacol.* 4: 1873–1880.
6. Nauta, A. C., M. Grova, D. T. Montoro, A. Zimmermann, M. Tsai, G. C. Gurtner, S. J. Galli, and M. T. Longaker. 2013. Evidence that mast cells are not required for healing of splinted cutaneous excisional wounds in mice. *PLoS One* 8: e59167.
7. Weller, K., K. Foitzik, R. Paus, W. Syska, and M. Maurer. 2006. Mast cells are required for normal healing of skin wounds in mice. *FASEB J.* 20: 2366–2368.
8. Feyerabend, T. B., A. Weiser, A. Tietz, M. Stassen, N. Harris, M. Kopf, P. Radermacher, P. Möller, C. Benoist, D. Mathis, et al. 2011. Cre-mediated cell ablation contests mast cell contribution in models of antibody- and T cell-mediated autoimmunity. *Immunity* 35: 832–844.
9. Hübner, G., Q. Hu, H. Smola, and S. Werner. 1996. Strong induction of activin expression after injury suggests an important role of activin in wound repair. *Dev. Biol.* 173: 490–498.
10. Antsiferova, M., M. Huber, M. Meyer, A. Piwko-Czuchra, T. Ramadan, A. S. MacLeod, W. L. Havran, R. Dummer, D. Hohl, and S. Werner. 2011. Activin enhances skin tumourigenesis and malignant progression by inducing a pro-tumourigenic immune cell response. *Nat. Commun.* 2: 576.
11. Funaba, M., T. Ikeda, K. Ogawa, M. Murakami, and M. Abe. 2003. Role of activin A in murine mast cells: modulation of cell growth, differentiation, and migration. *J. Leukoc. Biol.* 73: 793–801.
12. Munz, B., H. Smola, F. Engelhardt, K. Bleuel, M. Brauchle, I. Lein, L. W. Evans, D. Huylebroeck, R. Balling, and S. Werner. 1999. Overexpression of activin A in the skin of transgenic mice reveals new activities of activin in epidermal morphogenesis, dermal fibrosis and wound repair. *EMBO J.* 18: 5205–5215.
13. Bamberger, C., A. Schärer, M. Antsiferova, B. Tychsen, S. Pankow, M. Müller, T. Rülicke, R. Paus, and S. Werner. 2005. Activin controls skin morphogenesis and wound repair predominantly via stromal cells and in a concentration-dependent manner via keratinocytes. *Am. J. Pathol.* 167: 733–747.
14. Munz, B., G. Hübner, Y. Tretter, C. Alzheimer, and S. Werner. 1999. A novel role of activin in inflammation and repair. *J. Endocrinol.* 161: 187–193.
15. Junankar, S. R., A. Eichten, A. Kramer, K. E. de Visser, and L. M. Coussens. 2006. Analysis of immune cell infiltrates during squamous carcinoma development. *J. Investig. Dermatol. Symp. Proc.* 11: 36–43.
16. Werner, S., H. Smola, X. Liao, M. T. Longaker, T. Krieg, P. H. Hofschneider, and L. T. Williams. 1994. The function of KGF in morphogenesis of epithelium and reepithelialization of wounds. *Science* 266: 819–822.
17. Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, et al. 2012. Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9: 676–682.
18. Bradley, P. P., D. A. Priebat, R. D. Christensen, and G. Rothstein. 1982. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J. Invest. Dermatol.* 78: 206–209.
19. Conti, P., M. Reale, R. C. Barbacane, M. Felaco, A. Grilli, and T. C. Theoharides. 1998. Mast cell recruitment after subcutaneous injection of RANTES in the sole of the rat paw. *Br. J. Haematol.* 103: 798–803.
20. Jones, C. P., L. G. Gregory, B. Causton, G. A. Campbell, and C. M. Lloyd. 2012. Activin A and TGF-beta promote T(H)9 cell-mediated pulmonary allergic pathology. *J. Allergy Clin. Immunol.* 129: 1000-1010.e3.
21. Ponvert, C., L. Galoppin, P. Scheinmann, P. Canu, and C. Burtin. 1985. Tissue histamine levels in male and female mast cell deficient mice (W/Wv) and in their littermates (Wv/+, W/+ and +/+). *Agents Actions* 17: 1–4.
22. Koizumi, T., and J. Hayakawa. 1987. Single-locus control of the mast cell population in mouse skin. *Immunogenetics* 26: 36–39.
23. Miller, H. R., and A. D. Pemberton. 2002. Tissue-specific expression of mast cell granule serine proteinases and their role in inflammation in the lung and gut. *Immunology* 105: 375–390.
24. Funaba, M., T. Ikeda, M. Murakami, K. Ogawa, and M. Abe. 2005. Up-regulation of mouse mast cell protease-6 gene by transforming growth factor-beta and activin in mast cell progenitors. *Cell. Signal.* 17: 121–128.
25. Wang, X., J. Ge, E. E. Tredget, and Y. Wu. 2013. The mouse excisional wound splinting model, including applications for stem cell transplantation. *Nat. Protoc.* 8: 302–309.
26. Kümin, A., M. Schäfer, N. Epp, P. Bugnon, C. Born-Berclaz, A. Oxenius, A. Klippel, W. Bloch, and S. Werner. 2007. Peroxiredoxin 6 is required for blood vessel integrity in wounded skin. *J. Cell Biol.* 179: 747–760.
27. Schäfer, M., and S. Werner. 2008. Cancer as an overhealing wound: an old hypothesis revisited. *Nat. Rev. Mol. Cell Biol.* 9: 628–638.

28. Coussens, L. M., W. W. Raymond, G. Bergers, M. Laig-Webster, O. Behrendtsen, Z. Werb, G. H. Caughey, and D. Hanahan. 1999. Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. *Genes Dev.* 13: 1382–1397.
29. Antsiferova, M., and S. Werner. 2012. The bright and the dark sides of activin in wound healing and cancer. *J. Cell Sci.* 125: 3929–3937.
30. de Kretser, D. M., R. E. O'Hehir, C. L. Hardy, and M. P. Hedger. 2012. The roles of activin A and its binding protein, follistatin, in inflammation and tissue repair. *Mol. Cell. Endocrinol.* 359: 101–106.
31. Mukhopadhyay, A., S. Y. Chan, I. J. Lim, D. J. Phillips, and T. T. Phan. 2007. The role of the activin system in keloid pathogenesis. *Am. J. Physiol. Cell Physiol.* 292: C1331–C1338.
32. Werner, S., and C. Alzheimer. 2006. Roles of activin in tissue repair, fibrosis, and inflammatory disease. *Cytokine Growth Factor Rev.* 17: 157–171.
33. Otranto, M., V. Sarrazy, F. Bonté, B. Hinz, G. Gabbiani, and A. Desmoulière. 2012. The role of the myofibroblast in tumor stroma remodeling. *Cell Adhes. Migr.* 6: 203–219.
34. Bernstein, A., B. Chabot, P. Dubreuil, A. Reith, K. Nocka, S. Majumder, P. Ray, and P. Besmer. 1990. The mouse W/c-kit locus. *Ciba Found. Symp.* 148: 158–166, discussion 166–172.
35. Zhou, J. S., W. Xing, D. S. Friend, K. F. Austen, and H. R. Katz. 2007. Mast cell deficiency in Kit(W-sh) mice does not impair antibody-mediated arthritis. *J. Exp. Med.* 204: 2797–2802.
36. Wulff, B. C., A. E. Parent, M. A. Meleski, L. A. DiPietro, M. E. Schrementi, and T. A. Wilgus. 2012. Mast cells contribute to scar formation during fetal wound healing. *J. Invest. Dermatol.* 132: 458–465.
37. de Souza, D. A., Jr., V. D. Toso, M. R. Campos, V. S. Lara, C. Oliver, and M. C. Jamur. 2012. Expression of mast cell proteases correlates with mast cell maturation and angiogenesis during tumor progression. *PLoS One* 7: e40790.
38. Muto, S., M. Katsuki, and S. Horie. 2007. Decreased c-kit function inhibits enhanced skin carcinogenesis in c-Ha-ras protooncogene transgenic mice. *Cancer Sci.* 98: 1549–1556.
39. Gordon, J. R., and S. J. Galli. 1990. Phorbol 12-myristate 13-acetate-induced development of functionally active mast cells in W/Wv but not Sl/Sld genetically mast cell-deficient mice. *Blood* 75: 1637–1645.
40. Waskow, C., S. Bartels, S. M. Schlenner, C. Costa, and H. R. Rodewald. 2007. Kit is essential for PMA-inflammation-induced mast-cell accumulation in the skin. *Blood* 109: 5363–5370.