Prostate-specific Klf6 Inactivation Impairs Anterior Prostate Branching Morphogenesis through Increased Activation of the Shh Pathway

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Krüppel-like factor 6 (Klf6) belongs to a family of zinc finger transcription factors known to play a role in development and tumor suppression. Although Klf6 is highly mutated in prostate cancer, its function in prostate development is unknown. We have generated a prostate-specific Klf6-deficient mouse model and report here a novel role for Klf6 in the regulation of prostate branching morphogenesis. Importantly, our study reveals a novel relationship between Klf6 and the Shh pathway. Klf6-deficiency leads to elevated levels of hedgehog pathway components (Shh, Ptc, and Gli) and loss of their localized expression, which in turn causes impaired lateral branching.

Klf6 belongs to the family of Krüppel-like zinc finger transcription factors that regulate cell proliferation and differentiation (1). All members of the Klf gene family contain a highly conserved zinc finger DNA binding domain at their C terminus and an activation domain at its N terminus, distinct to each Klf gene and accounting for their wide-ranging biological capabilities (2–4). Similar to other members of the Klf family (5, 6), Klf6 is reported to act as a tumor suppressor (7–10).

Materials and Methods

Generation, Genotyping, and Reverse Transcription-PCR of Klf6 Prostate-specific Mutant Mice—We generated prostate-specific deletion of Klf6 exons 2 and 3 by crossing Klf6f/f mice (C57BL/6, 129Sv) with Nkx3.1Cre/+ mice, which expressed Cre recombinase from the Nkx3.1 locus (5). The Nkx3.1Cre/+ knock-in mice used in the crosses were phenotypically identical to the published Nkx3.1 mutant mice (25) and, thus, exhibited normal prostate development. PIN lesions were detected in Nkx3.1Cre/+ mice only after they have been aged beyond 1 year (6).

Acknowledgments

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

The abbreviations used are: Hh, hedgehog; BMP, bone morphogenic protein; FGF, fibroblastic growth factor; Shh, sonic hedgehog; Micro-CT, micro-computed tomography; SMA, smooth muscle actin; H&E, hematoxylin and eosin; TCRD, T-cell receptor delta chain.

References

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4. The work was supported, in part, by National Institutes of Health Grant CA115985 (to M. M. S.).
5. The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.
6. M. Shen, unpublished data.
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erations (data not shown), and subsequent generations were genotyped by PCR analysis using the forward primer, 5′-GTC TCT TGA CAC CTT GAC TAT CTC TCC-3′, and the reverse primer, 5′-CTA CAG GAT TCG TCC CTC TGC-3′, from genomic DNA obtained from tail biopsies. We prepared RNA from TRIzol (Invitrogen) extractions from various prostate lobes, brain, testes, and kidney tissues. The RNA was then used for reverse transcription–PCR to confirm Klf6 deletion through detection of a 695-bp PCR product using forward primer 5′-GAA TAC TCT TGG AGT GCT AGG and reverse primer 5′-CTG CTC TTT CAG AGG TGC. RNA loading control was performed by employing a housekeeping gene called T-cell receptor delta chain (TCRD). TCRD was employed to ensure that equal RNA was loaded into each lane and was detected using forward primer 5′-CAA ATG TTG CTT GTC TGG TG and reverse primer 5′-GTC AGT CGA GTG CAC AGT TT. In addition, the Ptc-lacZ reporter mice, also known as Ptch1D11 mice, were then imaged with the Scanco APO microscope was used to acquire images of the micro-dissected prostates. Tissues were fixed in 4% paraformaldehyde. High resolution ultrasound imaging was employed to evaluate the interior of anterior prostate. The fixed tissue samples were placed in a bath of phosphate-buffered saline to provide acoustic coupling for ex vivo three-dimensional ultrasound micro-imaging. Ultrasound imaging was performed with a high resolution dedicated small animal ultrasound imaging system (Vevo 770, VisualSonics, Toronto, Canada). Images were acquired with a single-element (mechanical sweep) ultrasound transducer operating at a center frequency of 55 MHz and a 4.5 mm focal length, resulting in an axial resolution of 30 μm and a lateral resolution of 50 μm. Three-dimensional acquisition was performed by the linear translation of the transducer that was controlled by a motorized drive mechanism, where images were acquired every 50 μm to provide a three-dimensional field-of-view of 7×7×10 mm. Images were processed with image analysis software provide with the Vevo 770 imaging.

Micro-CT was used to provide a quantitative analysis of the number of ductal tips and branch points. Microdissected tissues were soaked in 5% Isovue saline, and ex vivo three-dimensional ultrasound imaging. Samples were then imaged with the Scanco μCT40 (Bassendorf, Switzerland) micro-CT system at an isotropic voxel size of 12 μm. Images were acquired with the x-ray tube operating at an energy level of 45 kV and a current of 177 microamperes with a mechanical sweep. High resolution ultrasound imaging was employed to provide acoustic coupling for in vivo micro-CT imaging. In addition, the Ptc-lacZ reporter mice, also known as Ptch1D11 mice, were then imaged with the Scanco APO microscope was used to acquire images of the micro-dissected prostates. Tissues were fixed in 4% paraformaldehyde prior to Declere antigen retrieval (Cell Marque) using a pressure cooker for 10 min and then blocked in 10% donkey serum/phosphate-buffered saline. Primary antibodies used include rabbit anti-Klf6 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Ki67 (LabVision), rabbit anti-β-galactosidase (1:10,000, MP Biomedical, without antigen retrieval) (19), rabbit anti-androgen receptor (Upstate), rabbit anti-smooth muscle actin (LabVision), rabbit anti-desmin (EuroDiagnostica), rabbit anti-cdk4 (Covance), mouse anti-ck8 (Novus), rabbit anti-α-smooth muscle actin (Labvision), mouse anti-α-smooth muscle actin (BD Pharmingen). Secondary antibodies used to detect respective primary antibodies were anti-rabbit Alexa 488 (Invitrogen) and anti-mouse Alexa 594 (Invitrogen). Respective IgG controls were used on serial sections placed on each slide. Sections were mounted using Fluoromount containing 4',6-diamidino-2-phenylindole stain (Dako).

Prostate tissues for non-radioactive in situ hybridization were harvested and fixed in 4% paraformaldehyde prior to embedding in an OCT compound and freezing on dry ice. Pre-treatment of frozen tissue sections included proteinase K digestion (40 μg/ml, 6 min, and 25 °C) and acetylation (0.1 M triethanolamine, pH 8.0, 0.25% acetic anhydride, 10 min, 25 °C). Sections were hybridized with Riboprobe probes against Shh, Ptc, and Gli1 at 60 °C overnight, washed sequentially with 2× SSC, 0.5× SSC, and blocked with 1× Digoxygenin-Block (Roche Applied Science) for 1 h before hybridization with alkaline phosphatase conjugated anti-Dig antibody (Roche Applied Science) for 1 h at room temperature.

Radioactive in situ hybridization was performed as previously described (27). PCR primers were designed to amplify a 415-bp fragment of mouse BMP4 spanning from nucleotides 948 to 1362 of mouse BMP4 reference sequence NM_007554.2 (upper, 5′-CAGGGCTTCCTCACGGTATAAAC-3′; lower, 5′-AATGGCGACCCGAGTT-3′). Primers included extensions encoding 27-nucleotide T7 or T3 RNA polymerase initiation sites to allow in vitro transcription of sense and antisense probes, respectively, from the amplified products. Formalin-fixed, paraffin-embedded sections 5-μm thick were deparaffinized, deproteinized in 10 μg/ml Proteinase K for 30 min at 37 °C, and further processed for in situ hybridization as previously described (Jubb et al. (27)). [33P]UTP-labeled sense and antisense probes were hybridized to the sections at 55 °C overnight. Unhybridized probe was removed by incubation in 20 μg/ml RNase A for 30 min at 37 °C, followed by a high stringency wash at 55 °C in 0.1× SSC for 2 h and dehydration through graded ethanol. The slides were dipped in NTB nuclear track emulsion (Eastman Kodak), exposed in sealed plastic slide boxes containing desiccant for 4 weeks at 4 °C, developed, and counterstained with H&E. Images were analyzed in Metamorph (version 7.0r4, MDS Analytical, Sunnyvale, CA). Briefly, regions of interest (epithelial ductal units)
RESULTS

Generation of Mice with Prostate-specific Deletion of Klf6—To understand the developmental role of Klf6 in the prostate, we first defined the spatial and temporal expression pattern of Klf6. Anti-Klf6 immunofluorescent staining revealed that Klf6 expression was highly expressed in the nuclei of prostate epithelium as early as postnatal day 1 (P1) (data not shown), P4 (arrow, Fig. 1A), and mature prostate (arrow, Fig. 1B). The specificity of the anti-Klf6 antibody was confirmed by the lack of immunofluorescent staining in the nuclei of cells in which the Klf6 were deleted (see below, Fig. 2, N and P).

To generate a prostate-specific deletion of exons 2 and 3 that encompass all three zinc fingers, which inactivates Klf6 transcription factor activity (Fig. 1C), we crossed Klf6 conditional mutant mice containing loxP sites flanking exons 2 and 3 (Klf6f/f) with Nkx3.1 Cre/+/H11001 knock-in mice in which the Nkx3.1 promoter directs expression of Cre-recombinase to the prostate epithelium.5 To demonstrate prostate-specific deletion of exons 2 and 3 from Klf6, we performed reverse transcription-PCR analysis on mRNA from various genotypes and tissues. A 695-bp PCR product, amplified when exons 2 and 3 were deleted, could only be detected in Klf6f/fNkx3.1 Cre/+/H11001 prostate but not in brain, testes, and kidney or Klf6f/fNkx3.1 Cre/+/H11001 prostate. The highest level of Cre-mediated deletion of Klf6 was detected in the anterior prostate (Fig. 1E).

Branching and Canalization Defects in Klf6-deficient Prostate—Progression of prostate branching morphogenesis varies from lobe to lobe. Main ducts are present in all lobes at birth, but lateral branching in the ventral prostates begins at birth (P1), the ante-

were created manually to restrict analysis to the lumen. A threshold value was applied for each set of images (~95% of maximum pixel value) to identify positively stained areas. The total area of each region and the positive stained area for each region were outputted directly to Excel (Microsoft, Redmond, WA). A value of 10.3 pixels per silver grain was used to calculate the total number of silver grains associated with the positively stained area.
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FIGURE 2. Abnormality in ductal branching in the Klf6-deficient prostate epithelium. Morphological ductal aberrations in Klf6-deficient anterior prostates were first observed at P5 and retained in adult mice. Light microscopy images (A, B, E, and F) and H&E staining (C and D) of Klf6f/fNkx3.1Cre+/ (A, C, and E) and Klf6f/fNkx3.1Cre+/ (B, D, and F) from P5 and 1-year-old anterior prostates that were microdissected prior to imaging. Only one side of the symmetrical anterior lobe was imaged, and three mice were imaged from each stage. Branching was visible in both microdissected (arrow, A) and H&E-stained (arrow, C) P5 sections from Klf6f/fNkx3.1Cre+/ but not in Klf6f/fNkx3.1Cre+/ (B and D). Ex vivo ultrasound imaging allowed visualization of the interior of anterior prostate of Klf6f/fNkx3.1Cre+/ (G) that appears dense and white due to extensive epithelial infolding in the ducts, and the single layer of white epithelial lumen that outlines the main ducts of Klf6f/fNkx3.1Cre+/ mice (H). Arrowhead (in H) points to the significantly enhanced luminal space present within the main duct of Klf6f/fNkx3.1Cre+/ anterior prostate. H&E staining of prostates obtained from 6-month-old (J and K) Klf6f/fNkx3.1Cre+/ and (L and M) Klf6f/fNkx3.1Cre+/ mice. Note that the widest region at the base of Klf6f/fNkx3.1Cre+/ prostate (K) fits into the field of view, whereas the narrowed region of Klf6f/fNkx3.1Cre+/ prostate (L) does not fit into the field of view. Epithelial infolding that spans the lumen was more extensive in Klf6f/fNkx3.1Cre+/ prostates (arrow, L) than in Klf6f/fNkx3.1Cre+/ prostates (arrow, J). Insets represent higher magnification of the regions marked by the dotted square in K and L, respectively, that revealed nuclei with normal characteristics. Immunofluorescent staining of Klf6 expression in prostate tissues obtained from 8-week-old Klf6f/fNkx3.1Cre+/ (M and O) and Klf6f/fNkx3.1Cre+/ (N and P) mice. Klf6, which is a transcription factor, was highly localized to the nuclei of Klf6f/fNkx3.1Cre+/ prostate epithelium (arrows, O), and depletion of Klf6 in the Klf6f/fNkx3.1Cre+/ prostate was confirmed by the absence of green signal in the nuclei (arrowhead, P). I, J, M, and N, low magnification and high magnification (K, L, O, and P) of respective images. Bar in I, 500 μm for A and B; 1000 μm for E and F, and 750 μm for G and H. Bar in C, 50 μm for C and D. Bar in K, 200 μm for I and J and 100 μm for K–N. Bar in O, 50 μm for O and P.

Anterior prostate at P5, and the dorsolateral prostate by P10 (11, 28). Gross examination of all three lobes of Klf6f/fNkx3.1Cre+/ prostates revealed a prominent branching phenotype primarily in the anterior prostate, which was consistent with the highest level of Klf6 depletion. The branching defect in the Klf6f/fNkx3.1Cre+/ anterior prostates was evident as early as postnatal day 5 (P5) (Fig. 2, B and D). Impaired branching was 100% penetrant in the Klf6f/fNkx3.1Cre+/ mice, whereas all Klf6f/fNkx3.1Cre+/, Klf6f/+ Nkx3.1Cre+/, and Klf6f/+ Nkx3.1Cre+/ prostates developed normally. To rule out a possibility of a delay in secondary branching, we examined Klf6f/f Nkx3.1Cre+/ prostate from 6-month-old (data not shown) and 1-year-old (Fig. 2F) mice and found that those mature prostates were still without secondary branches as first noted at P5. This was in contrast to the extensive secondary and tertiary branching observed in 1-year-old Klf6f/fNkx3.1Cre+/ mice (Fig. 2E). Distinguishing branching features in the dorsal and ventral prostates were not as apparent between Klf6f/fNkx3.1Cre+ and Klf6f/fNkx3.1Cre+/ except for larger luminal spaces in the ducts (supplemental Fig. S1, A–D). This milder phenotype could be due to less efficient Klf6 deletion in those lobes, because the greatest level of Klf6 deletion was primarily in the anterior lobe, the remainder of our analyses focused therefore on detailed characterization of the anterior prostate.

Canalization of the anterior prostate begins around P2 and progresses in a proximal to distal manner with elaboration of epithelial infolding or tufting within the luminal space (28, 29). A novel ex vivo application of three-dimensional, high resolution ultrasound micro-imaging technology was employed to visualize the luminal space of intricately microdissected prostates. Extensive epithelial infolding in the smaller luminal space of 1-year-old Klf6f/fNkx3.1Cre+ anterior prostates resulted in a dense, white, hyperechoic regions within the ultrasound image (Fig. 2G), which were also visible by H&E staining (Fig. 2, I and K). The absence of secondary branching from the two main ducts was evident in the Klf6f/fNkx3.1Cre+ anterior prostates (Fig. 2H). Ex vivo ultrasound imaging revealed a dark, hypoechoic luminal space in the main ducts, suggesting that canalization was unaffected (arrowhead, Fig. 2H). The epithelial infolding in the Klf6f/fNkx3.1Cre+ anterior prostate was significantly reduced resulting in a defined layer of epithelial cells outlining the main ducts (Fig. 2H). H&E-stained sections also revealed that the epithelial infolding rarely spanned across the lumen, and the eosinophilic lumen suggests a functional epithelium, capable of secretion (Fig. 2, J and L). Further histopathological analysis of the H&E-stained Klf6f/fNkx3.1Cre+ sections did not reveal any signs of hyperproliferation, nuclear anomalies, or mitotic figures (insets, Fig. 2, J and L). Given the normal appearance of the luminal epithelium, we performed anti-Klf6 immunofluorescent staining to confirm that Klf6 protein was indeed depleted in the prostate epithelium. In Klf6f/fNkx3.1Cre+ mice, nuclear Klf6 expression was detected in the prostate epithelium (arrow, Fig. 2O), whereas positive nuclear staining was absent from Klf6f/fNkx3.1Cre+ epithelium (arrowhead, Fig. 2P).

To quantify the number of ductal tips and branch points we used ex vivo micro-CT to image the anterior prostate. It was necessary to microdissect away connective tissues encapsulating the prostate to reveal adequately separated ducts ideal for micro-CT imaging. We found significantly (p < 0.001) more ductal tips (red dot) and branch points (red Y) in the anterior prostate of Klf6f/fNkx3.1Cre+ mice (Fig. 3, A, C, and E) than Klf6f/fNkx3.1Cre+ mice (Fig. 3, B, D, and F). It should be pointed out that, although Nkx3.1 null mice show a defect in prostatic branching morphogenesis (25, 30), the ductal epithelium in Nkx3.1+/ mice is essentially indistinguishable from wild type at 2 months of age and only shows much milder phenotype than that in Nkx3.1−/− mice at an age of 10–12 months.
or older (Fig. 4E of (30)). The branching defect due to conditional Klf6 deletion reported in the present study is far more severe than that found even in Nkx3.1 homozygous knockouts (Figs. 2F and 3 of the present study, as compared with Fig. 4I and J of Ref. 25). These findings together argue strongly that Klf6 inactivation makes important contribution to the defect in prostate branching morphogenesis.

Characterization of Gene Expression in Abnormal Prostate Resulting from Loss of Klf6—Murine prostate development begins in late gestation, in a process involving reciprocal interactions between the prostate epithelium and mesenchyme (31, 32). During development, the mesenchyme condenses around the ducts in a proximal to distal fashion to encase the ducts with smooth muscle actin (SMA), the earliest smooth muscle marker expressed, and desmin, a later marker (33). To determine the state of smooth muscle differentiation, we examined the expression of SMA and desmin at P5, which corresponds to the initiation of secondary branching in the anterior prostate. We found SMA (Fig. 4A, C, E, and G) and desmin (Fig. 4B, D, F, and H) expressed proximally but not at the distal tips (arrow) of newly branched lateral ducts of Klf6f/fNkx3.1Cre/+H11001 prostates. In contrast, SMA (Fig. 4B, D, F, and H) and desmin (Fig. 4F and H) were expressed continuously along the entire length of each main duct of Klf6f/fNkx3.1Cre/+H11001, but not at the distal tip (arrowhead). Interestingly, this is consistent with previous findings where the relative thickness of the smooth muscle layer is inversely proportional to epithelial ductal growth (34). This suggests that, although there was diminished smooth muscle expression around
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emerging lateral ducts of Klf6<sup>f/f</sup>Nkx3.1<sup>1+/+</sup> prostates, lateral epithelial branching in Klf6<sup>f/f</sup>Nkx3.1<sup>Cre/+</sup> was prevented as a result of a continuous layer of smooth muscle along the main ducts. On the other hand, it is also possible that the continuous layer of smooth muscle formed around the main ducts as a result of the absence of branching. To further understand this branching defect, we examined expression of androgen receptor by immunofluorescent staining, because several investigators had demonstrated that postnatal prostate branching morphogenesis and differentiation of the stroma are highly regulated by androgen (32, 33, 35). Androgen receptor was expressed continuously around the primary and secondary ducts of Klf6<sup>f/f</sup>Nkx3.1<sup>1+/+</sup> (supplemental Fig. S2, A and C) and the main ducts of Klf6<sup>f/f</sup>Nkx3.1<sup>Cre/+</sup> prostates (supplemental Fig. S2, B and D). There were no apparent differences in proliferation by anti-Ki67 staining (supplemental Fig. S3, A and B), cell death by anti-active caspase-3 (data not shown) and epithelial differentiation by anti-CK8, anti-CK14 and anti-E-cadherin staining (supplemental Figs. S2 (E–H) and S3 (C and D)). Therefore, the lack of lateral branching could not be attributed to androgen receptor expression, proliferation, cell death, or epithelial differentiation in the mutant mice.

Factors in the epithelium and mesenchyme essential for proper ductal branching include Nkx3.1, Hh, BMP, FGF, Notch, and Wnt pathway components (12–19, 25, 36). Our proper ductal branching include Nkx3.1, Hh, BMP, FGF, thelial differentiation in the mutant mice.

To confirm the change in expression pattern of Ptc, we crossed Klf6<sup>f/f</sup>Nkx3.1<sup>Cre/+</sup> with Ptc-lacZ reporter mice (26) in which lacZ is driven under the Pct promoter, to obtain Klf6<sup>f/f</sup>Nkx3.1<sup>Cre/+</sup>; Ptc-lacZ mice. This Ptc-lacZ reporter line is a sensitive indicator of endogenous Ptc expression and Hh signaling activity (26). Consistent with the in situ hybridization results, we found that there was increased β-galactosidase staining in the peripherich epithelial stoma of Klf6<sup>f/f</sup>Nkx3.1<sup>Cre/+</sup>; Ptc-lacZ mice compared with the staining observed in Klf6<sup>f/f</sup>Nkx3.1<sup>1+/+</sup>; Ptc-lacZ mice (Fig. 6). There were multiple cell layers of β-galactosidase-positive cells surrounding the epithelium in the Klf6<sup>f/f</sup>Nkx3.1<sup>Cre/+</sup>; Ptc-lacZ mice (Fig. 6, A and C). In sharp contrast, only one to two layers of β-galactosidase-positive cells were seen in the Klf6<sup>f/f</sup>Nkx3.1<sup>1+/+</sup>; Ptc-lacZ control littermates (Fig. 6, B and D). Together these findings indicate that Klf6 expression leads to broader expression of Hh signaling around the developing prostate ducts.

In an attempt to further understand how epithelial branching morphogenesis is inhibited in the Klf6<sup>f/f</sup>Nkx3.1<sup>Cre/+</sup> mice, we also performed radioactive in situ hybridization using a probe specific for BMP4, which was previously shown to be expressed in the prostatic stroma and can inhibit prostatic epithelial duc-
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Consistent with previous reports from our group and others on the role of Shh signaling in prostate branching (12–15, 37), both Ptc-lacZ reporter mice crossed to Klf6fl/flNkx3.1 Cre/+ mice and in situ hybridization data in the present study indicate that impaired lateral branching in the Klf6-deficient prostate correlate with an up-regulation and loss of spatial localization of Shh, Ptc, and Gli1. Further support for the involvement of focal Hh signaling in prostate epithelial branching comes from a study by Bushman and co-workers (15), showing a close association between prostate ductal bud formation and localized expression of Shh at the growing tips of elongating prostate ducts. In addition, Shh regulates prostate branching morphogenesis in concert with FGF10 and BMP4 (37). Shh secreted from the epithelial ductal tips can down-regulate FGF10 that stimulates epithelial cell growth (16) and/or up-regulate BMP4 that inhibits epithelial cell growth (17) in the mesenchyme, which results in epithelial ductal branching (37). In the present study, we show that loss of Klf6 leads to dispersed, rather than focal, Shh expression throughout the epithelial ducts and corresponding dispersed Ptc and Gli1 expression in the adjacent mesenchyme (Fig. 5). This broader domain of Hh pathway activity leads to an up-regulation of BMP4 expres-
sion, resulting in reduced ductal branching. As proposed by Pu and coworkers, the interplay and localized expression pattern of Shh and BMP4 are key to conveying critical branching information (37).

Klf6 is reported to be a tumor suppressor that is inactivated in a large subset of prostate cancer (47). However, our detailed histological characterization of Klf6f/fNkx3.1Cre/+ mice as old as 2 years failed to reveal any prostate tumors.7 The lack of tumor phenotype in the Klf6 mutant mice could be due to compensations/redundancy with other Klf family members. It is also possible that loss of Klf6 might not be sufficient to initiate prostate tumorigenesis in the mouse and that loss/mutation of additional tumor suppressors might be required.

Acknowledgments—We thank Leisa Johnson, Mallika Singh, Hua Tian, and Zhenyu Gu for critical discussions. We also thank Margaret Fuentes, Christine Olsson, and Luz Orellana for assistance with the animal colony, Allison Bruce with Graphics, Jeffrey Eastham-Anderson for Metamorph analysis of BMP4 in situ hybridization signal, and Howard Stern for his assistance with some histopathological review.

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