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Hoxb13 is required for normal differentiation and secretory function of the ventral prostate

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SUMMARY

The murine prostate is a structure that is made up of four distinct lobes; the dorsal and lateral prostates (often grouped together as the dorsolateral prostate), the anterior (coagulating gland) and the ventral prostate. Previous work has implicated *Hox* genes in the development of these structures, but how each lobe acquires unique identities for specific functions has not been addressed. In this study, the ventral prostate-specific function of Hoxb13 is described. Mice lacking *Hoxb13* function show normal numbers of duct tips, but mice mutant for both *Hoxb13* and *Hoxd13* exhibit severe hypoplasia of the duct tips, revealing a role for *Hoxb13* in ventral prostate morphogenesis. Additionally, a ventral lobe-specific defect was identified in *Hoxb13* mutants wherein the epithelium is composed of simple cuboidal cells rather than of tall columnar cells. Ventral prostate ducts appear devoid of contents and do not express the ventral prostate-specific secretory proteins p12, a kazal-type protease inhibitor and p25, a spermine binding protein. These defects are not due to reduction of *Nkx3.1* expression or to a global effect on androgen receptor signaling. These results suggest a specific role for *Hoxb13* in a differentiation pathway that gives the ventral prostate epithelium a unique identity, as well as a more general role in ventral prostate morphogenesis that is redundant with other *Hox13* paralogs.

Key words: Prostate, *Hox* genes, Secretory proteins, *Hoxb13*, Mouse

INTRODUCTION

Vertebrate *Hox* genes are known to play an important role in assigning positional identity to various organs along the anterior-posterior axis of the developing embryo (Chisaka et al., 1992; Krumlauf, 1994; Davis et al., 1995; Duboule, 1995; Podlasek et al., 1997). Developmental defects in these organs are readily distinguishable by the absence of structures, reduction of tissue, and/or morphological changes (Patterson et al., 2001; Wellik et al., 2002). *Hox* genes are also known to play roles in the development of secondary sex characteristics such as the mammary glands (Chen and Capecchi, 1999; Garcia-Gasca and Spyropoulos, 2000), and male accessory sexual organs (Dolle et al., 1993; Podlasek et al., 1997; Podlasek et al., 1999). The *Hox13* paralogs are especially important to the development of the prostate. For example, *Hoxa13* and *Hoxd13* both have functions in the outgrowth and patterning of the lobes of murine prostate glands, as indicated by reduced branching in prostate ducts of *Hoxa13* and *Hoxd13* loss-of-function mutants (Kondo et al., 1997; Podlasek et al., 1997; Warot et al., 1997; Podlasek et al., 1999). Nkx3.1, a non-Hox homeodomain protein, is also required for the proper development of the prostate and displays an androgen-dependent expression profile (Bieberich et al., 1996; Sciavolino et al., 1997). Similar to *Hox13* mutants, *Nkx3.1* mutants also show reductions in duct tips and impaired secretory function. In addition, *Nkx3.1* mutants display a high incidence of prostatic intraepithelial neoplasias (PINs) (Bhatia-Gaur et al., 1999; Abdulkadir et al., 2002), which are more frequent and progress to carcinogenesis when the mice are also heterozygous for mutations in PTEN, a tumor suppressor gene (Kim et al., 2002). *Hoxb13*, the last identified vertebrate *Hox* gene (Zeltser et al., 1996), has been shown to be expressed highly and in an androgen-independent manner in the prostate (Sreenath et al., 1999).

The murine prostate is divided into four distinct lobes, the ventral prostate, the dorsal prostate, the lateral prostate and the anterior prostate (coagulating gland) (Abate-Shen and Shen, 2000). These lobes function independently to supply proteins to the seminal fluid (Takeda et al., 1990). The anterior, dorsal and lateral prostates secrete many of the same proteins while the ventral prostate has a distinct secretory protein profile (Donjacour et al., 1990). The major proteins that are secreted by the ventral prostate are p12 (Mills et al., 1987a), a 6 kDa kazal-type serine protease inhibitor (Chen et al., 1998; Mirosevich et al., 2001), and p25, a 25 kDa spermine-binding protein that is N-linked glycosylated (Mills et al., 1987b). An additional feature that distinguishes the ventral prostate from all other lobes is the relative absence of PINs in *Nkx3.1* mutants (Bhatia-Gaur et al., 1999; Abdulkadir et al., 2002) and the absence of carcinogenesis in *Nkx3.1/PTEN* compound mutants (Kim et al., 2002).

To study the role of *Hoxb13* in the developing and adult prostate, we generated loss-of-function alleles of *Hoxb13* by disrupting the homeodomain. Mice homozygous for mutant *Hoxb13* alleles show, with 100% penetrance, complete absence of the secretory proteins p12 and p25 in the ventral prostates. Moreover, the luminal cells of the ventral prostate epithelium are simple cuboidal in appearance in contrast to the tall columnar morphology of these cells in wild-type or heterozygote animals. We show that the defective secretory function of the ventral prostate is unique to *Hoxb13* mutants by comparing and crossing *Hoxb13* mutants to a previously described mutant, *Hoxd13* (Davis and Capecchi, 1994). We also determined whether *Nkx3.1* expression is affected in *Hoxb13* mutants and propose that in *Hoxb13* homozygous mutants, the ventral prostate is partially transformed into anterior prostate.

MATERIALS AND METHODS

Generation of *Hoxb13* mutant mice

To generate a targeting vector, a fragment containing *lacZ* was cloned into the *Bgl*II site of the second exon, interrupting the homeodomain at the 33rd amino acid residue and in frame with the aid of a custom linker (GATCCGAGATCTCG). A *Bgl*II fragment containing a loxP-flanked MC1 promoter driven Neo^R cassette was cloned into the *Bgl*II site downstream of the *lacZ* reporter. The *Bgl*II *NeoR* cassette was cloned directly into the genomic *Hoxb13 Bgl*II site to create loss-of-function mice without a *lacZ* reporter. Both lines were crossed to the Cre-deletor mouse line (Schwenk et al., 1995) to remove the *NeoR* cassette. $Hoxd13^{neo}$ mutant mice were as previously described (Davis and Capecchi, 1994).

X-gal staining of prostates

The bladder and the male accessory organs (seminal vesicles, ductii deferens, anterior, dorsal, dorsolateral and ventral prostates) were dissected from adult male mice. Organs were stained for *lacZ* activity with X-gal as described previously (Mansour et al., 1993), but stained for only 1.5 hours at 37°C to avoid background staining typical of adult prostatic ducts (M. Reynon and M. Shen, personal communication).

Prostate microdissections

Ventral prostate and attached urethra were placed in phosphate-buffered saline (PBS) containing 0.2-0.4% collagenase. Stromal cells were gently teased away until only prostate ducts remained. Prostate ducts were teased apart and photographed under bright-field and dark-field optics. Duct tips were counted directly.

Immunofluorescence of tissue sections

Ventral prostates were removed in cold PBS, washed, and immediately frozen in OCT. Prostates were sectioned (10 μm) and mounted on VWR Superfrost Plus slides. Sections were fixed in fresh cold 4% paraformaldehyde/PBS for 5 minutes, and rinsed three times for 5 minutes in cold PBS. Sections were then blocked in PBS/3% goat serum/0.5% Triton X-100, for 30 minutes at room temperature and incubated overnight with block and primary antibodies at the following dilutions: 1:1000 anti-β-gal (Rockland cat. 100-4136), 1:250 anti-AR (ABR-Affinity Bio Reagents) 1:50 CD44 (Supernatant-Developmental Hybridoma). Sections were washed three times for 5 minutes in PBS and blocked again in PBS/3% goat serum/0.5% Triton X-100. Sections were incubated in anti-mouse or anti-rabbit FITC or Texas Red (1:500, Molecular Probes), washed again, mounted with Vectashield (Vector Labs) and examined by confocal microscopy.

Examination of ventral prostate secretory proteins

Secretory proteins were isolated by modification of the method of Donjacour et. al. (Donjacour et al., 1990). Briefly, prostates were washed in PBS and 3-5 mm sections were cut with a razor into 500 μl PBS containing a protease inhibitor cocktail (Roche Complete mini-EDTA free). Cut prostates in protease inhibitor solution were transferred to 1.5 ml Eppendorf tubes and spun at 12,000 ***g*** for 2 minutes at 4°C. Supernatants were removed to new tubes, sodium dodecyl sulphate (SDS) was added to 1% and samples were boiled for 10 minutes. Proteins from pellets were also extracted by sonication in extraction buffer (20 mM Hepes pH 7.5, 450 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol). Microsequencing of protein bands was performed by transfer of proteins to polyvinylidene fluoride (PVDF) and Edmund degradation followed by HPLC.

RT-PCR

Total RNA was extracted from prostates by dounce homogenization in Trizol (Gibco-BRL). First strand synthesis was performed using reverse-transcriptase with poly(dT) primers (Fermentas). RT-PCR primers for p12 were 5′GCACCCTGTATAGTTCTTCTGG3′ (sense) and 5′AAGTGTTCATGAAGCGATTTATTCAA3′ (antisense), for p25 were 5′TCCTGGCCAGTCCCACATGCA3′ (sense) and 5′CGCCCCTTGTTTGTAGTGAAGG (antisense) and were designed across introns. Primers for GAPDH were 5′ACCACAGTCC-ATGCCATCAC3′ (sense) and 5′TCCACCACCCTGTTGCTGTA3′ (antisense). The conditions for the RT-PCR were 94°C for 5 minutes (94°C 30 seconds, 60°C 30 seconds, 72°C 60 seconds) 25 cycles.

Western blots

Total proteins or cellular-only proteins (from secretory protein isolation pellets) were electrophoresed on Novex 4-12% acrylamide gels in MES-SDS (Invitrogen). 20 μg of each sample was loaded. Gels were stained with Coomassie Blue and volumes were readjusted to a reference band (murine serum albumin). Proteins were electrophoresed and electroblotted to nitrocellulose. Equal loading and transfer were confirmed by staining with PonceuS Red. Nitrocellulose membranes were blocked in 3% milk/Tris-buffered saline with 0.1% Tween (TBST) for 1 hour and incubated O/N with primary antibodies Nkx3.1 (1:6000) or anti-VP (1:20,000) (kind gifts from C. Abate-Shen and M. Kim).

RESULTS

Generation of mutant mice

Embryo-derived stem (ES) cell lines containing an inframe fusion of the bacterial *lacZ* gene plus a floxed neo^r gene in the second exon of *Hoxb13*, or simply a disruption of the second exon with a floxed neo^r, gene were generated by gene targeting as described in Materials and Methods. Two ES cell lines containing each *Hoxb13* mutant allele were used to generate male mouse chimeras capable of transmitting the mutant allele to their progeny. The floxed neo^r gene was removed from the genome of both *Hoxb13* mutant mouse strains (i.e., $Hoxb13^{lacZneo}$, $Hoxb13^{neo}$) by breeding to a Cre deleter strain to generate the $Hoxb13^{lacZlxp}$, $Hoxb13^{lxp}$ mouse strains. The phenotypes resulting from all four mutant alleles were indistinguishable with respect to the formation and function of the ventral prostrate, as well as all other mutant phenotypes associated with the *Hoxb13* loss-of-function mutation. The *lxp* mutant allele was generated to control for the potential artefact due to the silencing of the target locus and nearby genes by the methylation of the *lacZ* bacterial gene as has been previously reported (Thorey et al., 1993; Cohen-Tannoudji et al., 2000;

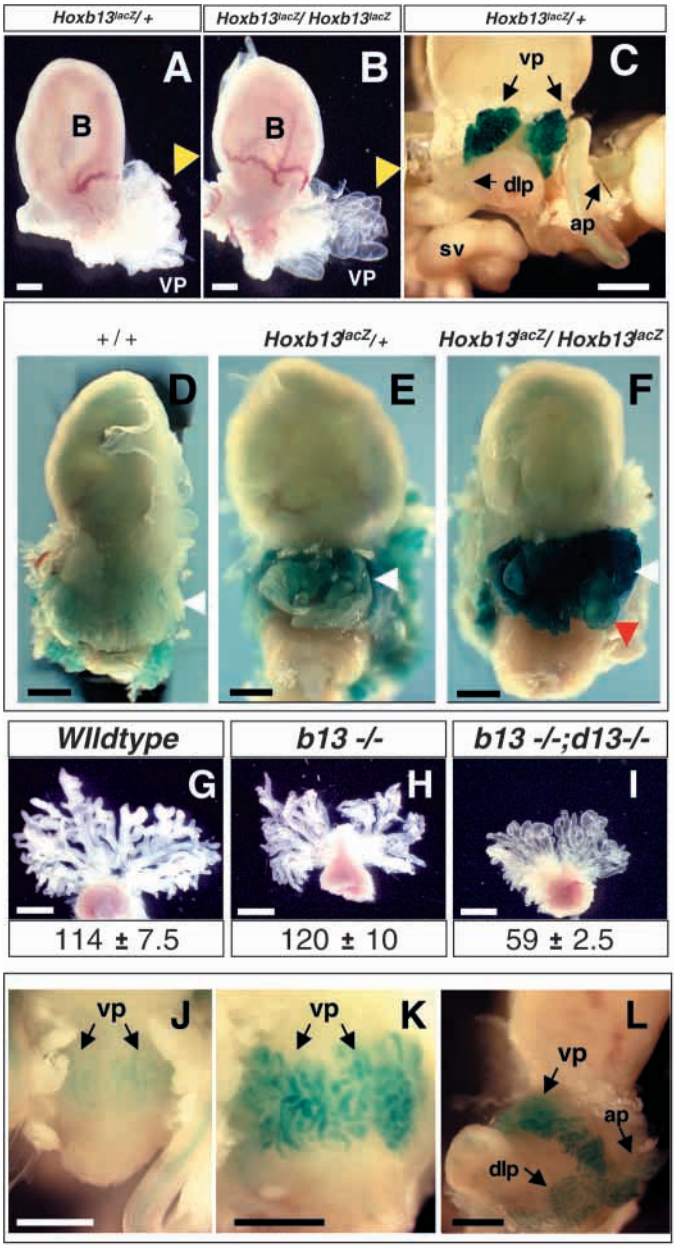

Fig. 1. Gross morphology of mutant ventral prostates. Ventral prostate showing the opaque appearance of control ducts (A) and the transparent appearance of *Hoxb13* homozygous mutant ducts (B) (yellow arrows). (C) X-gal staining of 5-week-old *Hoxb13*^{lacZ} heterozygote reproductive organs show preferential expression of Hoxb13 within the ventral prostate; *Hoxb13* continues to be strongly expressed in the ventral prostate of 1-year-old animals. (D-F) There is a dose-dependent X-gal staining intensity between wild-type (D), *Hoxb13* heterozygote (E) and *Hoxb13* homozygous mutant (F) ventral prostates (white arrowheads). Homozygous mutant prostates frequently exhibit swelling in individual ducts (E; red arrowhead). (G-I) Reductions in ventral prostate duct tips are observed in *Hoxb13/Hoxd13* double homozygous mutants. Dark-field micrographs of ventral prostate microdissections of wild type (G), *Hoxb13* homozygous mutants (H) and *Hoxb13/Hoxd13* double homozygous mutants (I). Significant reductions in number of duct tips and outgrowth of branches are only seen in double *Hoxb13/Hoxd13* mutants (averages and standard deviations for number of duct tips from 3 prostates for each genotype are shown below representative photo). (J-L) Postnatal X-gal staining of developing prostates showing Hoxb13 expression during the time of extensive ductile morphogenesis. At P10, X-gal staining is barely detectable (J), but is very strong by P12 in the ventral prostate (K). (L) During development, all prostatic lobes show strong X-gal staining (P16 shown). B, bladder; VP,ventral prostate; AP, anterior prostate; dlp, dorsolateral prostate; SV, seminal vesicle. Scale bars (A-I) 2 mm, (J-L) 1 mm.

Greer and Capecchi, 2002). The experiments described in this study made use of mice with the *Hoxb13*^{lacZlxp} or *Hoxb13*^{lxp} mutant alleles.

Hoxb13 mutant phenotypes

Females and males homozygous for both *Hoxb13* mutant alleles are viable and fertile. *Hoxd13* mutants, in contrast, have severe limb malformations and the males are infertile (Dolle et al., 1993). Mice homozygous for *Hoxb13* loss-of-function mutations show overgrowth in all major structures derived from the tail bud, including the developing secondary neural tube, the caudal spinal ganglia and the caudal vertebrae (Economides et al., 2003). Because mice carrying mutations in both *Hoxd13* and *Hoxa13* have defects in the development of male accessory sex organs (Podlasek et al., 1997; Warot et al., 1997; Podlasek et al., 1999) and *Hoxb13* is expressed in the developing and adult prostate (Sreenath et al., 1999; Prinsac et al., 2001), analyses of *Hoxb13* mutants for defects in the formation and/or function of the prostate were carried out.

Gross analysis of male secondary sexual organs

Seminal vesicles, dorsolateral prostates, dorsal prostates and anterior prostates from *Hoxb13* homozygous mutants appeared normal. The ventral prostates from *Hoxb13* homozygous mutants were normal in size and morphology (i.e., number of duct tips) but the ducts had a transparent appearance (Fig. 1B). X-gal staining of reproductive organs of adult *Hoxb13*^{lacZ} heterozygotes revealed preferential expression in the ventral prostate of adult males (Fig. 1C). Ventral prostates from heterozygous and homozygous mutants strongly expressed the reporter gene for β-galactosidase in a dosage-dependent manner (Fig. 1D-F). In addition, ducts from prostates of older homozygous mutant males (>1 year old) frequently were swollen (4/7) (Fig. 1F).

Hoxb13 and *Hoxd13* cooperate in the development of the ventral prostate

To determine if *Hoxb13* and *Hoxd13* cooperate in the development of the ventral prostate, animals with both mutations were bred to generate mice harboring compound mutations. Prostate lobes were microdissected and duct tips counted (Sugimura et al., 1986). Mutants homozygous for either *Hoxb13* or *Hoxd13* alone did not show significant reductions in duct tips (Fig. 1H and data not shown). Double mutants for *Hoxb13* and *Hoxd13*, however, showed a 50% reduction in the number of duct tips. Reduced outgrowth of prostate ducts was also apparent in the double mutants (Fig. 1I). *Hoxa13* mutants have also been reported to exhibit reduced ductile branching in addition to absence of the dorsolateral lobes of the prostate (Podlasek et al., 1999). Mutations in

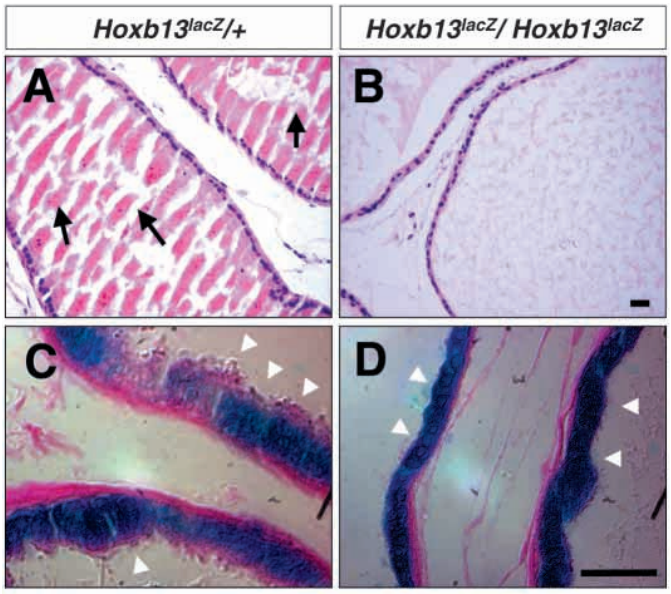

Fig. 2. Sections of *Hoxb13* mutant ventral prostates**.** (A,B) Hematoxylin and Eosin stained sections, and (C,D) high magnification X-gal and eosin stained sections of heterozygous (A,C) and mutant (B,D) ventral prostates. There is a lack of secretory proteins within the ducts of the homozygous mutant ventral prostates (B) when compared to heterozygotes (A, black arrows). X-gal staining reveals strong expression of the *Hoxb13lacZ* reporter in the luminal epithelium (C,D) Note the tall columnar epithelium in heterozygotes compared with the simple cuboidal epithelium in homozygous mutants (compare C and D, white arrowheads). Scale bars: 25 μm (in B for A,B; in D, for C,D).

another homeobox gene known to play a role in prostate development and morphogenesis, *Nkx3.1*, also result in significant reduction of duct tips in the ventral prostate, as well as in the anterior and dorsolateral prostate (Bhatia-Gaur et al., 1999). The double mutant phenotypes provide evidence for redundant functions of *Hoxb13* and *Hoxd13* in the development of the ventral prostate. To further determine the expression of *Hoxb13*, an expression analysis using the β-galactosidase reporter in *Hoxb13*^{lacZ} heterozygotes was carried out (i.e., post-natal days (P) P2, P5, P10, P12, P14, P16 and P20). X-gal staining was not observed in developing male structures until P10. At P10, X-gal staining was evident in the ventral prostate (Fig. 1J), as well as anterior and dorsolateral prostates (data not shown). Expression in all lobes persisted until P20 (days 10, 12 and 16 shown, VP only, Fig. 1J-L). Female reproductive organs from *Hoxb13*^{lacZ} heterozygotes ranging from P2 until adult stages were also stained with X-gal and never showed positive staining (data not shown). The onset of expression in the prostate at P10 after birth is consistent with the requirement of Hoxb13 for branching morphology.

Defects in the morphology of epithelial cells

Ventral prostate sections were examined for changes in morphology and overall appearance through analysis by Hematoxylin and Eosin staining (Fig. 2A,B) and for *Hoxb13* reporter expression by X-gal staining (Fig. 2C,D). Histology revealed reduced protein content within the prostatic ducts (Fig. 2A, black arrows, compare with 2B), indicating a reduction in secretory function of the ventral prostate. The reporter allele was expressed strongly within the luminal cells of prostate ducts. Mutant epithelial cells appeared simple cuboidal in contrast to the tall columnar epithelia seen in normal prostates (Fig. 2C,D; white arrowheads). The morphology of mutant prostate epithelia is similar to that seen in the prostatic epithelium of castrated mice (Mirosevich et al., 1999). Degeneration of the ventral prostate, which is a characteristic of castrated mice, however, was not evident in *Hoxb13* homozygous mutants. Tissue sections of *Hoxb13* mutant mice did not reveal any evidence of PINs similar to those described in *Nkx3.1* mutants in any prostate lobe.

Hoxb13 mutants do not produce major secretory proteins

To further characterize the apparent loss of ventral prostate secretory function in *Hoxb13* homozygous mutants, secretory proteins were isolated from wild-type, heterozygous, and mutant homozygous mice (Donjacour et al., 1990). SDS-PAGE followed by Coomassie Blue staining revealed that the major secretory proteins, p12 and p25, are not secreted into the ducts of *Hoxb13* homozygous mutants (Fig. 3A; lane 3). The identity of these proteins in wild-type and heterozygous animals was confirmed by microsequencing. p12 (Mills et al., 1987c) is a kazal-type protease inhibitor (Chen et al., 1998) while p25, which migrates as a broad band because of the N-linked glycosylation, is a spermine binding protein (Mills et al., 1987b). The ventral prostate is the only prostate lobe that secretes these proteins, although p12 is also supplied by the seminal vesicle. *Hoxb13* heterozygotes in a *Hoxd13* homozygous mutant background did not appear to have a secretory defect and showed normal amounts of secretory proteins (Fig. 3A, lane 4). By contrast, *Nkx3.1* animals show a dosage sensitive response, with heterozygotes producing less secretory proteins than wild types and homozygous mutants producing less than heterozygotes, however, some secretory function remains intact in the *Nkx3.1* mutant homozygotes (Bhatia-Gaur et al., 1999). To determine if other prostatic lobes are affected, we compared the secretory protein profiles of anterior prostate and ventral prostates. Anterior prostates appear normal in *Hoxb13* homozygous mutants while ventral prostates not only lack the major secretory proteins, but also have some novel protein bands within their profiles (Fig. 3B). Two of these bands have been identified by microsequencing, one is polymeric immunoglobulin receptor (pIgR) while another is androgen binding protein beta subunit (ABPβ).

To rule out the possibility that secretory proteins are made, but not secreted, or that they are rapidly degraded, we analyzed p12 and p25 mRNA levels by RT-PCR. Both transcripts were undetectable or greatly reduced (Fig. 3C). While p12 transcripts were detected in both *Hoxb13* mutant and wild-type seminal vesicles, we did not detect this transcript in dorsolateral prostates or anterior prostates (data not shown), contradicting an earlier characterization of the expression pattern of this gene (Chen et al., 1998).

Luminal epithelial markers are present in *Hoxb13* mutants

One possibility for the absence of secretory proteins in the ventral prostate of *Hoxb13* homozygous mutants is that the

luminal cells fail to differentiate properly. Expression patterns of luminal cell markers were examined in homozygous mutant and heterozygous ventral prostates. Antibodies to β-galactosidase were used to determine localization of the Hoxb13-β-gal fusion protein within the luminal cells and anti-androgen receptor (anti-AR) antibodies were used to determine if this luminal epithelial-specific marker was present. In both homozygous mutants and heterozygotes, AR and the Hoxb13-β-gal fusion protein appear intact and properly localized to the nucleus (Fig. 4). Using CD44 as a marker for the basal cell layer, however, yielded a surprising result. In *Hoxb13* mutant ventral prostates CD44 was localized to both the apical and basal surfaces of luminal cells. This result suggests that although the markers for luminal epithelium are expressed, mutant epithelial cells appear to lack polarity.

Nkx3.1 levels are not reduced in the VP

Mice mutant for *Nkx3.1* show reductions in the levels of both p12 and p25 in the ventral prostate (Bhatia-Gaur et al., 1999). Since, *Hoxb13* mice have similar, although more severe, secretory function defects in the ventral prostate compared to *Nkx3.1* mutant mice, the possibility that *Hoxb13* may regulate *Nkx3.1* expression was explored. Western immunoblots were performed using an antibody to Nkx3.1 (Kim et al., 2002) on protein extracts from ducts that had the majority of their secretory proteins removed. Ducts were separated from secretory proteins for two reasons. (1) The major band SBP (25-40 kDa) overlaps with Nkx3.1 (39 kDa); the large amount of secretory protein that gets transferred to the membrane might cause attenuation of signal because the proteins exceed the binding capacity of the membranes. (2) Removal of the bulk of secretory proteins allows for a more equal quantitation of cellular proteins between mutants and controls. Even with secretory proteins removed, a significant amount of total protein from heterozygotes and wild-type mice are secretory proteins (Fig. 5A). Thus, 20 μg of protein per lane was used as a first approximation between mutants and non-mutants, and then normalized to common bands, such as murine serum albumin. Surprisingly, Nkx3.1 levels were not reduced in *Hoxb13* mutant homozygotes, but were comparable to wild-type and heterozygous mice (Fig. 5A). When we compared relative levels of Nkx3.1 between heterozygotes and homozygous mutants on western immunoblots of total protein samples versus samples where the secretory proteins were first removed, attenuation of the signal by excess secretory proteins was not observed (compare Fig. 5B with 5A). A strong Nkx3.1 signal in homozygous mutants was observed by both methods. An antibody to ventral prostate secretory proteins (kind gift from C. Abate-Shen and M. Kim) was used to determine relative amounts of secretory proteins in the cellular-only samples. Note that p12 was detected in mutant and heterozygote seminal vesicles in the total protein immunoblots (Fig. 5B), but was barely detectable in the cellular-only immunoblots (Fig. 5A). The anti-VP secretory protein-specific antibody confirmed that ventral-specific secretory function was completely absent in homozygous mutant ventral prostates, whereas homozygous mutant seminal vesicles were not unaffected in their ability to produce p12, which supported our results from the RT-PCR experiments described earlier.

Transformation of ventral prostate

Gels loaded with total protein from mutant and heterozygote urogenital sinus-derived organs revealed that samples from *Hoxb13* mutant ventral prostates shared similar protein profiles with anterior prostates, while certain prominent bands had more in common with the ventral prostates from normal mice (Fig. 5C). While one of the common bands with the anterior

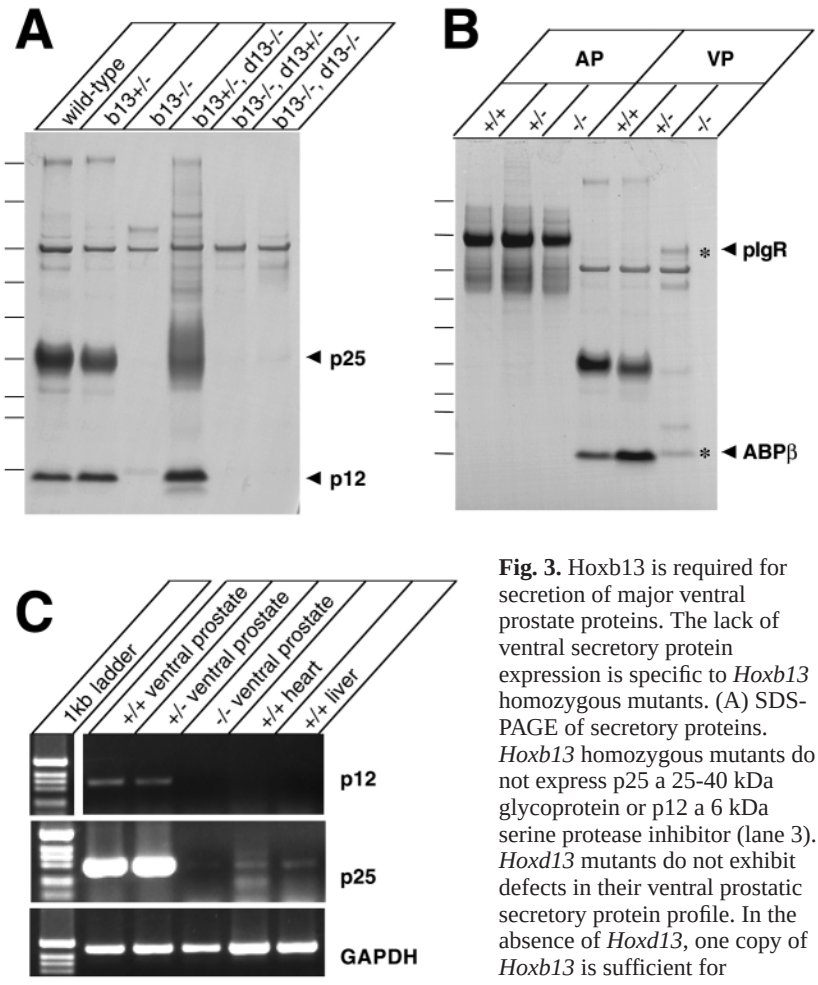

Fig. 3. Hoxb13 is required for secretion of major ventral prostate proteins. The lack of ventral secretory protein expression is specific to *Hoxb13* homozygous mutants. (A) SDS-PAGE of secretory proteins. *Hoxb13* homozygous mutants do not express p25 a 25-40 kDa glycoprotein or p12 a 6 kDa serine protease inhibitor (lane 3). *Hoxd13* mutants do not exhibit defects in their ventral prostatic secretory protein profile. In the absence of *Hoxd13*, one copy of *Hoxb13* is sufficient for expression of both p12 and p25 (lane 4). (B) The *Hoxb13* mutation appears to affect secretory function of only the ventral prostate. SDS-PAGE of secretory proteins from anterior prostates (AP) ventral prostates (VP). (C) *Hoxb13* homozygous mutant ventral prostates do not produce secretory protein mRNA. Semi-quantitative RT-PCR using primers for the two major ventral secretory proteins p12 and p25, reveals absence of these mRNAs in ventral prostates from *Hoxb13* homozygous mutants. Heart and liver RNAs are used as negative controls, GAPDH is an internal loading control. Molecular mass markers indicated by bars on the left of A and B are 188, 98, 62, 49, 38, 28, 17, 14, and 6 kDa.

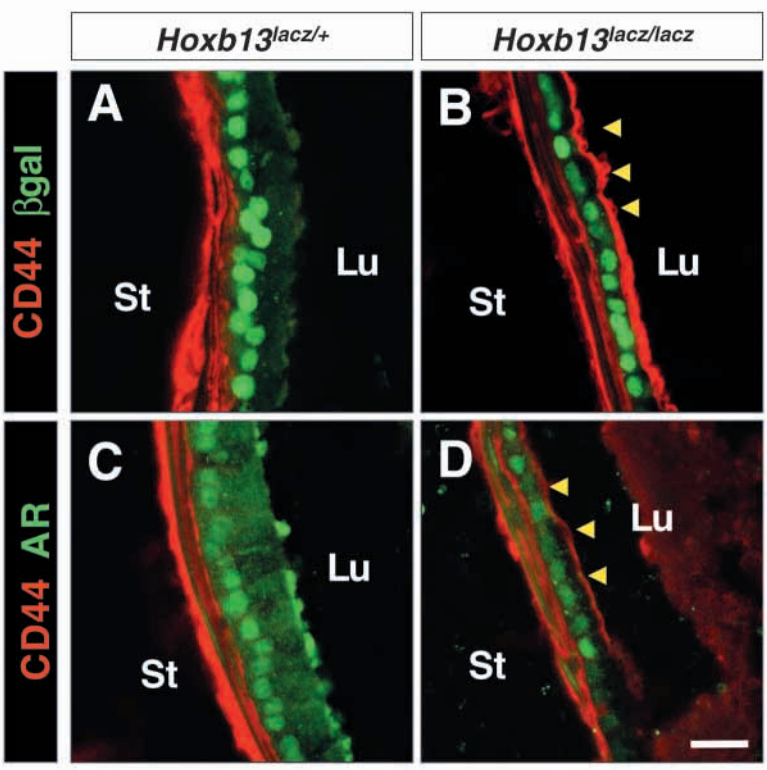

Fig. 4. Immunofluorescence of basal and epithelial markers. *Hoxb13* homozygous mutant luminal cells display loss of polarity. (A,B) *Hoxb13* expression and localization was determined by using an antibody to the β-galactosidase reporter. (C,D) Androgen Receptor (AR) expression is intact in homozygous mutant epithelium. Basal cell marker CD44 is mis-expressed on apical surface of mutant epithelium (B,D yellow arrowheads). The tall-columnar morphology of heterozygous luminal cells (A,C) is evident when compared to the simple-cuboidal morphology of homozygous mutant luminal cells (B,D). St, stroma; Lu, lumen. Scale bar: 10 μm.

prostate appeared to be a secretory protein, the secretory protein profiles of mutant ventral prostates did not appear to be similar to that of anterior prostates (Fig. 3B). The possibility exists that some anterior-specific secretory proteins are made in homozygous mutant ventral prostates, but the luminal cells are defective in secretory function and do not release them into the lumen. The fact that some bands are still common to both homozygous mutant and heterozygote ventral prostates indicates that mutant cells appear to have lost their identity and that transformation to different cell types such as those of the anterior prostate is not complete, since a complete transformation to anterior prostate should maintain anterior-specific secretory function.

DISCUSSION

Mice with a disruption in the homeobox gene *Hoxb13* exhibited an absence of ventral prostate-specific secretory proteins and defects in the cellular morphology of the ventral prostate epithelium. The secretory defects appeared to be limited to the ventral prostate, indicating that *Hoxb13* is specifically required for the proper differentiation of the ventral prostate epithelium in the adult.

Hox genes in general play a role in providing identity to developing organs along the anterior-posterior axis of an embryo. *Hoxd13* and *Hoxa13* have roles in the development of accessory sex organs in the male (Kondo et al., 1997; Podlasek et al., 1997; Warot et al., 1997; Podlasek et al., 1999). *Hoxd13* and *Hoxa13* appear to be involved in the outgrowth and branching morphology of the prostate by controlling local cell proliferation rates within the developing ducts. Our study suggests that *Hoxb13* shares a redundant function with *Hoxd13* in the developing prostate based on the severe truncations and branching defects observed in *Hoxb13/Hoxd13* double mutants. The expression pattern of *Hoxb13* in the prostates of young mice coincides with a period of extensive branching morphogenesis. Defects in branching morphogenesis, however, do not correlate with impaired secretory function. While *Hoxd13* homozygous mutants show developmental defects in the ventral prostate, secretory function is intact. Additionally, *Hoxd13* shows a dramatic decrease in its expression during the progression from birth to adult (Podlasek et al., 1997), whereas *Hoxb13* continues to be expressed at high levels in adult tissues (our observations) (Sreenath et al., 1999).

It is clear from the absence of ventral secretory protein expression that the luminal epithelium is not appropriately specified. Ventral prostate luminal cells are present and branching morphogenesis appears normal in *Hoxb13* homozygous mutants. The epithelial cells express luminal cell-specific markers such as AR, Nkx3.1, and are positive for the *Hoxb13^{lacZ}* reporter. However, they misexpress a basal cell marker, CD44, on their apical surface, indicating loss of cellular polarity. The simple cuboidal epithelium evident in the *Hoxb13* homozygous mutant luminal cells, in contrast to the tall columnar epithelium found in normal cells, may be secondary to the absence of secretory function in these mutant cells. This hypothesis is supported by the observation that castrated mice develop a very similar cellular morphology in the prostate epithelium and that these changes in cellular morphology can be reverted to a tall columnar morphology by treatment of the castrated mice with testosterone (Mirosevich et al., 1999). The abnormal balloon-like ducts evident in older males may be explained by the altered cellular morphology and swelling due to osmotic changes within the duct.

Another homeobox gene involved in prostate development and ventral prostate function is *Nkx3.1*. Mice homozygous for mutations in *Nkx3.1* show reduced branching in all lobes and reduced ventral prostate secretion, but not complete absence of the major secretory proteins as observed in *Hoxb13* mutants. Frequently, *Nkx3.1* homozygous mutants display balloon-like swelling of individual ducts. *Nkx3.1* mutants also develop prostatic intraepithelial neoplasias (PINs), which are an early step in prostate tumor progression. Interestingly, PINs are not detected in the ventral prostate of *Nkx3.1* mutant homozygotes (Bhatia-Gaur et al., 1999; Abdulkadir et al., 2002). The expression of *Nkx3.1* is more robust in anterior prostate lobes when compared to ventral prostates, which suggests that

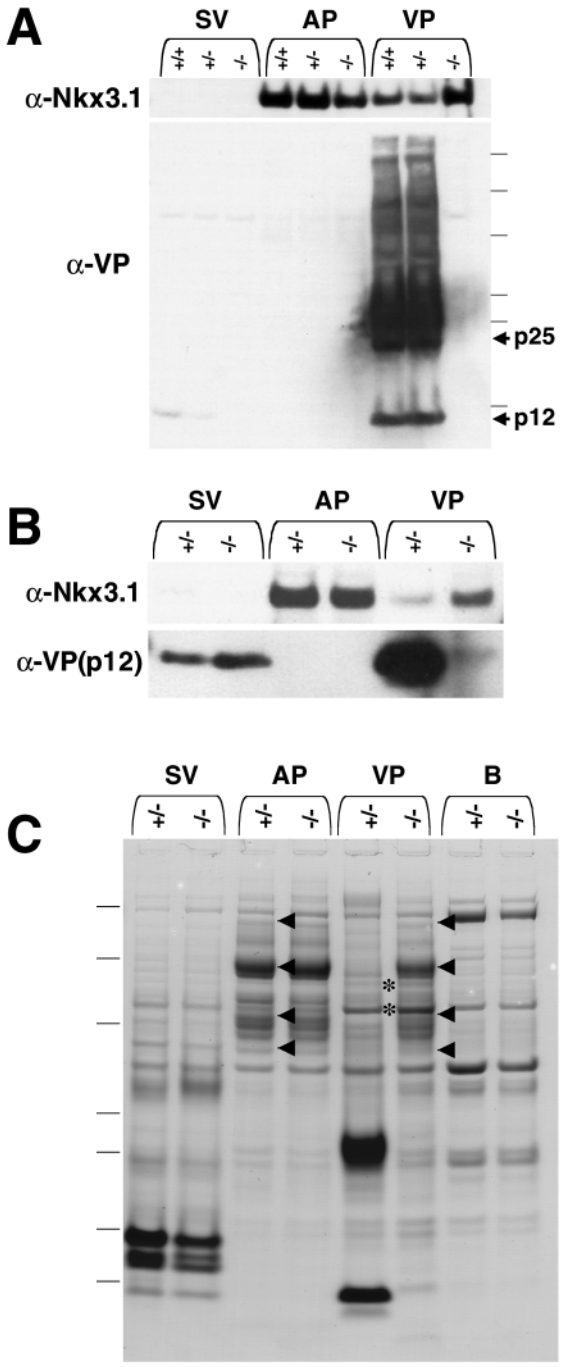

Fig. 5. Nkx3.1 expression is not reduced in *Hoxb13* homozygous mutants. (A) Western blots using an anti-Nkx3.1 antibody and an anti-ventral prostate protein antibody were performed on samples of protein extracts from ventral duct cells where secreted proteins were first removed. Nkx3.1 levels are not reduced in mutant ventral prostates, while antibodies to the ventral prostate proteins show complete absence of ventral prostate-specific proteins in the mutant VP. (B) Western blots using an anti-Nkx3.1 antibody and an anti-ventral prostate protein antibody on total protein samples show that Seminal vesicles but not anterior prostates secrete p12. Furthermore, expression of p12 in the seminal vesicle is not affected in *Hoxb13* homozygous mutants. (C) Coomassie Blue stained gel of total proteins shows that homozygous mutant ventral prostates share many common bands with control anterior prostates (black arrowheads), but also display some prominent ventral-specific bands (asterisks). SV, seminal vesicle; AP, anterior prostate; VP, ventral prostate, B, bladder.

Nkx3.1 may have a more extended role in anterior prostate development than in ventral prostate development. In contrast, the expression of *Hoxb13* is more robust in the ventral prostate than in the anterior prostate, indicating a more extended role for *Hoxb13* in the development of the ventral prostate.

The similarity in the ventral prostate phenotypes observed in *Nkx3.1* and *Hoxb13* homozygous mutants suggests a common pathway for secretory protein production. It is important to note, however, ventral prostate proteins p12 and p25 are androgen dependent, as is expression of Nkx3.1, while Hoxb13 expression has been reported to be androgen independent (Sreenath et al., 1999). A potential model would be that Hoxb13 functions in a parallel pathway with androgen receptor signaling, directly affecting Nkx3.1 expression which in turn affects the expression of secretory protein genes. This clearly is not the case since expression of Nkx3.1 is not decreased in *Hoxb13* mutants. The result that Nkx3.1 is still expressed in the absence of functional Hoxb13 leads to three important conclusions. (1) The ventral prostate secretory defect is not due to loss of Nkx3.1 expression. (2) The presence of Nkx3.1, the earliest known marker for prostate epithelium, confirms that the epithelium in *Hoxb13* homozygous mutants is in fact prostatic. (3) Finally, and most importantly, the presence of Nkx3.1 in *Hoxb13* homozygous mutant prostates implies that the androgen signaling pathways are still intact in these prostates since the expression of Nkx3.1 is androgen dependent.

The simplest explanation for our observations is that *Hoxb13* acts in a hierarchy of homeobox genes to assign ventral prostate fates to cells in the ventral luminal epithelium. *Nkx3.1*, *Hoxa13*, *Hoxd13*, and *Hoxb13* all cooperate to form the prostate, but only *Hoxb13* is necessary to mediate the final steps in ventral prostate differentiation. One possibility is that anterior and dorsolateral prostate fates are ‘default’ pathways and that elevated levels of Hoxb13 direct a ventral-specific differentiation. Consistent with this hypothesis is the observation that the total protein profiles of mutant ventral prostates resemble those of anterior prostates. However, this cannot be entirely explained by a simple transformation to another type of lobe, because cells of the homozygous mutant ventral prostates also have a secretory defect. Ventral prostates in *Hoxb13* mutants do not secrete anterior- or dorsolateral-specific secretory proteins, although they do secrete novel proteins such as polymeric immunoglobulin receptor (pIgR) and androgen binding protein beta subunit (ABPβ). pIgR is a receptor molecule that is involved in binding dimeric IgA and transcytosis across epithelial cell layers. pIgR expression has been observed in many mucosal epithelia including uterine, mammary and lung epithelia, and has been implicated in viral infection (Kaetzel et al., 1991; Mostov, 1994; Lamm et al., 1995; de Groot et al., 1999; Kaetzel, 2001). Although pIgR has been shown to have important functions in female reproductive organs (Kaushic et al., 1995; Richardson et al., 1995; Kaushic et al., 1997), no known function of pIgR has been described in the prostate. We do not detect pIgR in the ventral secretions of wild-type and heterozygous ventral prostates. One possibility

is that the proposed immunosuppressive function (Maccioni et al., 2001) of the ventral secretions is compromised in *Hoxb13* homozygous mutants and pIgR is trancytosed from the basal surface in response.

The other misexpressed protein, the beta subunit of androgen binding protein (ABPβ) (Karn and Russell, 1993) is one of three known subunits of ABP (Dlouhy et al., 1987) which has been shown to form heterodimeric complexes with ABPα or ABPγ and is expressed in both male and female salivary glands in response to testosterone treatment (Dlouhy and Karn, 1984). The question this finding poses is: why is the mutant ventral prostate secreting one subunit of a salivary androgen-binding protein? One possibility is that ABPβ expression represents a default state for mucosal epithelia and is expressed in these mutant cells because of the absence of specification to a ventral prostatic fate by *Hoxb13*.

A question of particular interest is: what is the function of the ventral prostate? Clearly, in a controlled environment, a properly functioning ventral prostate is not required to confer fertility to males as no decreases in litter size or mating frequency were observed. The ability to form copulatory plugs was also unaffected (data not shown), although the major component of these plugs is from secretory proteins of the seminal vesicle (Bradshaw and Wolfe, 1977) which appears to be unaffected by the *Hoxb13* mutation. The major proteins that the ventral prostate supplies to the seminal fluid are the protease inhibitor, p12, and the spermine binding protein, p25. The absence of p12 may be compensated for by the contribution of this protein from the seminal vesicle, but the other major secretory protein, p25, is produced only by the ventral prostate. In an environment with controlled breeding and no competition from other males, *Hoxb13* homozygous mutants lacking p25 appear not to be compromised in their ability to generate offspring. This may not be the case in the wild.

In this study, the function of *Hoxb13* in the mouse ventral prostate was examined. *Hoxb13* is expressed strongly and localized to the nuclei of ventral prostate luminal cells in which it directs differentiation of prostate epithelium into a ventral prostate-specific tissue. In the absence of *Hoxb13*, genes encoding ventral-specific secretory proteins are not expressed, while genes not normally expressed in this tissue such as pIgR and ABPβ are misexpressed. Secretory function in general is also affected and the luminal cells are transformed from a tall columnar morphology to a simple cuboidal morphology. The expression of CD44, a basal cell marker, on the apical surface, suggests loss of polarity in these cells. Although total protein profiles of the mutant ventral prostates show some similarity to other prostatic lobes, secretory profiles for the mutant ventral prostate are unique. Given these data, it is likely that there is a loss of identity and impaired secretory function of cells in the luminal epithelium rather than a complete transformation to a different prostatic lobe. The misexpression of CD44 on the apical surface in luminal cells in the *Hoxb13* homozygous mutant ventral prostate is consistent with preneoplastic lesions in many tissue types (Sugar et al., 1997; Lagorce-Pages et al., 1998; Wimmel et al., 2001) and in prostate tumors (Paradis et al., 1998). Interestingly, loss of heterozygosity of the 17q21 locus, which spans the *Hoxb* cluster, in humans is linked to early events of prostate carcinogenesis such as preneoplastic lesions (Brothman et al., 1995; Brothman, 1997; Deubler et al., 1997). A database search of the Serial Analysis of Gene Expression (SAGE) at the Cancer Genome Anatomy Project (CGAP) at the NIH reveals that Hoxb13 is expressed in virtually all human prostate cancers as well as normal prostates listed within the SAGE database (http://cgap.nci.nih.gov/). It is interesting to note that *Nkx3.1* mutations do not cause PINs in the VP. The commitment of prostate tissues to a ventral prostate fate by *Hoxb13* may provide protection to this tissue from neoplasia. Future work will address how *Hoxb13* interacts with other homeobox genes such as *Nkx3.1* in the formation and function of the prostatic lobes and the potential role of these genes in the early events of prostate carcinogenesis.

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