Role of p63 and basal cells in the prostate

Takeshi Kurita[1],*, Roanna T. Medina[1], Alea A. Mills[2] and Gerald R. Cunha[1]

[1]Department of Anatomy, University of California, San Francisco, CA 94143-0452, USA
[2]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA
*Author for correspondence (e-mail: kurita@itsa.ucsf.edu)

Summary

The prostate contains two major epithelial cell types – luminal and basal cells - both of which develop from urogenital sinus epithelium. The cell linage relationship between these two epithelial types is not clear. Here we demonstrate that luminal cells can develop independently of basal cells, but that basal cells are essential for maintaining ductal integrity and the proper differentiation of luminal cells. Urogenital sinus (UGS) isolated from $p63^{+/+}$ and $p63^{-/-}$ embryos developed into prostate when grafted into adult male nude mice. Prostatic tissue that developed in $p63^{-/-}$ UGS grafts contained neuroendocrine and luminal cells, but basal cells were absent. Therefore, p63 is essential for differentiation of basal cells, but p63 and thus basal cells are not required for differentiation of prostatic neuroendocrine and luminal epithelial cells. $p63^{-/-}$ prostatic grafts also contained atypical mucinous cells, which appeared to differentiate from luminal cells via activation of Src. In the response to castration, regression of $p63^{-/-}$ prostate was inordinately severe with almost complete loss of ducts, resulting in the formation of residual cystic structures devoid of epithelium. Therefore, basal cells play critical roles in maintaining ductal integrity and survival of luminal cells. However, regressed $p63^{-/-}$ prostate did regenerate in response to androgen administration, indicating that basal cells were not essential for prostatic regeneration.

Key words: Androgen, Mucin, Epithelial differentiation, Apoptosis, Urogenital sinus, Cell linage, MAPK, Src

Introduction

The prostate contains two major epithelial cell types: luminal and basal epithelial cells. Both luminal and basal epithelial cells develop from the urogenital sinus epithelium (UGE), which is stratified squamous (Wang et al., 2001). Columnar luminal secretory cells express prostate-specific markers such as Nkx3.1 (Bhatia-Gaur et al., 1999) and prostate-specific secretory proteins (Donjacour et al., 1990). Prostatic basal cells reside on the basement membrane and express markers in common with basal epithelial cells in other epithelia, e.g. cytokeratin 14 (K14) (Hayward et al., 1996) and p63 (Yang et al., 1998). Whereas luminal cells are the major functional component of prostate, the role of basal cells is unclear. Prostatic epithelium also contains rare neuroendocrine cells (Aumuller et al., 2001; Schalken and van Leenders, 2003). The developmental cell-linage relationships between luminal, basal and neuroendocrine cells have been debated without resolution for years.

Development and growth of the prostate depend upon androgen. The androgen signals regulating prostatic development (and maintenance) are mediated by androgen receptor (AR) in stromal cells (Cunha and Lung, 1978; Donjacour and Cunha, 1993; Kurita et al., 2001; Sugimura et al., 1986). Androgen receptors in luminal cells are required for secretory protein production (Donjacour and Cunha, 1993). The prostate regresses in response to androgen withdrawal, and restoration of systemic androgen elicits regeneration of the prostate. Androgen receptors in the stroma regulate these processes (Kurita et al., 2001). In male seasonal breeders such as the ram, the prostate undergoes this regression/regeneration cycle naturally in response to the seasonal changes in the systemic androgen levels. This ability of the prostate to go through multiple cycles of regression/regeneration implies the existence of stem cells in prostatic epithelium. Although it has not been definitively demonstrated, basal cells are widely believed to contain prostatic stem cells, which can differentiate into basal, luminal and neuroendocrine cells (Collins et al., 2001; Foster et al., 2002; Hudson et al., 2000; Uzgare et al., 2004; van Leenders and Schalken, 2001; Wang et al., 2001). In contrast, a recent study has suggested that stem cells may also reside in luminal cell layer as a slow proliferating/self-reserve population in the proximal part of prostatic ducts (Tsujimura et al., 2002). The presence and nature of prostatic stem cells continue to be debated because stem cells may be the target of carcinogenesis.

p63 (also named KET, p51A, p51B, p40 or p73L) is a homologue of the p53 tumor suppressor gene (Yang et al., 1998). p63 is essential for development of squamous epithelia. $p63^{-/-}$ mice have severe defects in stratified epithelia, which causes newborn lethality, and lack organs arising from epidermis such as mammary and salivary glands (Mills et al., 1999; Yang et al., 1999). In the female reproductive tract, p63 is an identity switch for cell-fate determination, and loss of p63 causes transformation of cervical/vaginal epithelial cells into uterine epithelial cells (Kurita et al., 2004). In the prostate, p63 is expressed in the basal cells (Yang et al., 1998). Signoretti et al. proposed that p63 is the stem cell factor for prostate because

the prostate is not formed in embryonic/newborn $p63^{-/-}$ mice (Signoretti et al., 2000). However, we have generated $p63^{-/-}$ prostate through rescue of $p63^{-/-}$ urogenital sinus (UGS) by transplantation. $p63^{-/-}$ prostate contains luminal and neuroendocrine but not basal cells. In this study, we elucidate the role of p63 and basal cells in development and maintenance of the prostate.

Materials and methods

Animals and grafting

Mice were maintained in accordance with the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals, and the UCSF Laboratory Animal Resource Center committee approved all procedures described here. Adult nude mice were purchased from Charles River (Wilmington, MA, USA).

$p63^{-/-}$ embryos were produced by heterozygous intercrosses of $p63^{Brdm2}$ mice (Mills et al., 1999). Pregnant females were sacrificed at 16-18 days postcoitum, and embryos were harvested. UGSs from $p63^{-/-}$ and $p63^{+/+}$ embryos were used. $p63^{-/-}$ mice were identified visually by the limbless phenotype. The genotypes of embryos were confirmed by PCR. The phenotypes of $p63^{+/+}$ and $p63^{+/-}$ prostates were essentially identical in the intact host. Therefore, analysis was concentrated on $p63^{+/+}$ and $p63^{-/-}$ UGE. Urogenital sinuses were grafted under the kidney capsule of athymic male nude mice. The results presented here are based upon analysis of 80 $p63^{-/-}$ and 61 $p63^{+/+}$ UGS grafts.

Some hosts were castrated at the time of grafting or one month after grafting. Compressed pellets of testosterone propionate (Sigma) (25 mg T-pellet) were implanted into castrated host mice one month after the castration.

Histochemistry

Alcian blue staining (pH 2.8) was used for detection of acid mucopolysaccharides (Putt, 1971). Methods for immunohistochemical detection in paraffin-embedded tissues have been described (Kurita et al., 1998). Mouse monoclonal antibodies were used at the following concentrations: anti-p63 4A4 (1:100) (1:20, Santa Cruz Biotechnology), anti-Ki67 (1:100, Novacastra Laboratories, Burlingame, CA, USA), anti-K8 LE41 (1:2) and anti-K14 LE001 (1:2, gift from E.B. Lane, University of Dundee, Dundee, UK). Mouse monoclonal antibody against the active form of Src (clone 28, 1:500) was kindly provided by Hisaaki Kawakatsu, Lung Biology Center, UCSF (San Francisco, CA, USA) (Kawakatsu et al., 1996). Rabbit polyclonal antibodies were used at the following concentrations: anti-AR (1:100, Affinity BioReagents, Golden, CO, USA), anti-phospho-histone H3 rabbit (1:100), anti-phospho-ERK1/2 (1:100), anti-phospho-MEK1/2 (1:100) and anti-phospho-p65 NF-kB (1:100, Cell Signaling Technology, Beverly MA, USA), anti-Cdk4 (1:100), anti-maspin (1:200, Santa Cruz Biotechnology), anti-pan-Ras (1:500, LabVision, Fremont, CA, USA), anti-ERβ rabbit (1:30, BioGenx, San Ramon, CA, USA) and anti-involucrin (1:2000, Covance, Princeton, NJ, USA). Rabbit antiserum for Nkx3.1 (1:100) was a gift from C. Abate-Shen (University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School) and uroplakin (1:2000) was a gift from T. T. Sun (New York University School of Medicine, New York, USA) (Wu et al., 1994). Anti-serum for secretory proteins from mouse dorsolateral prostate (mDLP), mouse ventral prostate (mVP), mouse bulburethral gland (mBUG) and mouse seminal vesicle (mSV) were used at 1:5000 dilution (Donjacour et al., 1990). Anti-K19 rabbit monoclonal antibody (1:1) was obtained from Robert Pytela (UCSF). Positive signals were visualized as brown precipitates utilizing 3,3′-diaminobenzidine tetra-hydrochloride (Sigma). Some immunohistochemistry (IHC)-stained slides were also stained with Alcian Blue. Hematoxylin was used for counter staining. Apoptotic cells were detected by 3′-hydroxyl termini of DNA in situ (TUNEL assay) using ApoTag kit (Oncor, Gaithersburg, MD, USA).

Preparation of ultra-thin JB4 sections

The tissues were fixed with 1.8% paraformaldehyde, 2.5% glutaraldehyde, ~0.1% picric acid in cacodylate buffer (pH 7.2) by perfusion of host mice. The tissue samples were embedded using a JB4 plus embedding kit (Polysciences, Warrington, PA, USA) following the manufacturer's instructions. Thin sections (0.8 μm) were cut with a glass-knife, and stained with Hematoxylin and Eosin (H&E).

Image analysis

For image analysis, images were captured with CCD camera interfaced with Macintosh G3 computer (Apple, Cupertino, CA, USA) and at least four specimens for each group were analyzed. For labeling index of phospho-histone H3 (pH3), the epithelial cells positive and negative for pH3 were counted on the screen in at least two frames for each specimen (more than 600 total epithelial cells). To assess the complexity of ducts, area of the grafts and length of basement membrane lined with epithelial cells were measured on the screen. The complexity of the ductal structure was expressed as the total length of epithelium (basement membrane) (μm) divided by area (μm^2) of prostatic tissue in the graft.

RT-PCR

Total RNA was extracted from three $p63^{+/+}$ and two $p63^{-/-}$ UGS grafts four weeks after grafting with TRI reagent (Sigma). The cDNA was synthesized from 2 μg total RNA by using Super Script II (Invitrogen, Carlsbad, CA, USA) with random primer (Invitrogen). All PCR primers were synthesized at the UCSF Biomolecular Resource Center. For detection of the keratin 14, forward primer 5′-GCTCTTGTGGT-ATCGGTGGT and reverse primer 5′-TTGCTCTTCAGGTCCTC-GAT were used (496 bp product). For the detection of the β-actin, forward primer 5′-AGCCATGTACGTAGCCATCC and reverse primer 5′-CTCTCAGCTGTGGTGGTGAA were used (392 bp product). RT reaction was performed in 20 μl reaction mixture and 1 μl of RT-product was used as template for PCR-reaction. For all primer sets, PCR was performed with the following protocol: denaturing, 94°C, 15 seconds; annealing, 58°C, 15 seconds; and extension, 72°C, 1 minute; for 40 cycles, with Advan Taq Platinum Taq polymerase (Clontech Laboratory, Palo Alto, CA, USA).

Results

Ontogeny of p63 in UGS and phenotypes of $p63^{-/-}$ UGS

At E11 only the epithelium lining the ventral portion of the cloaca was positive for p63 (Fig. 1A). On E12 the entire epithelium of the cloaca expressed p63 (Fig. 1B). As the cloaca differentiated into the UGS and hindgut, p63 expression gradually disappeared from hindgut epithelium, whereas its expression was maintained in UGE (Fig. 1B,D). By E16, colorectal epithelium was negative for p63 except for the caudal end connected to skin (anus) (Fig. 1D). UGE was p63-positive at E14 and E16 (Fig. 1D,E).

The differentiation markers of $p63^{+/+}$ and $p63^{-/-}$ UGSs were studied in E17.5 male embryos (Fig. 1E-N). The caudal part of the $p63^{-/-}$ UGS was thinner than that of the $p63^{+/+}$ UGS (not shown), but the area in which the prostate develops did not appear to be affected by loss of p63 (Fig. 1I-L). UGSs of $p63^{-/-}$ embryos showed typical sexual dimorphism; the bladder neck of male $p63^{-/-}$ embryos was thicker and was more angulated than the female $p63^{-/-}$ UGS [compare Fig. 1H,L (male) versus Fig. 1O (female)]. The presence of mesonephric (Wolffian)
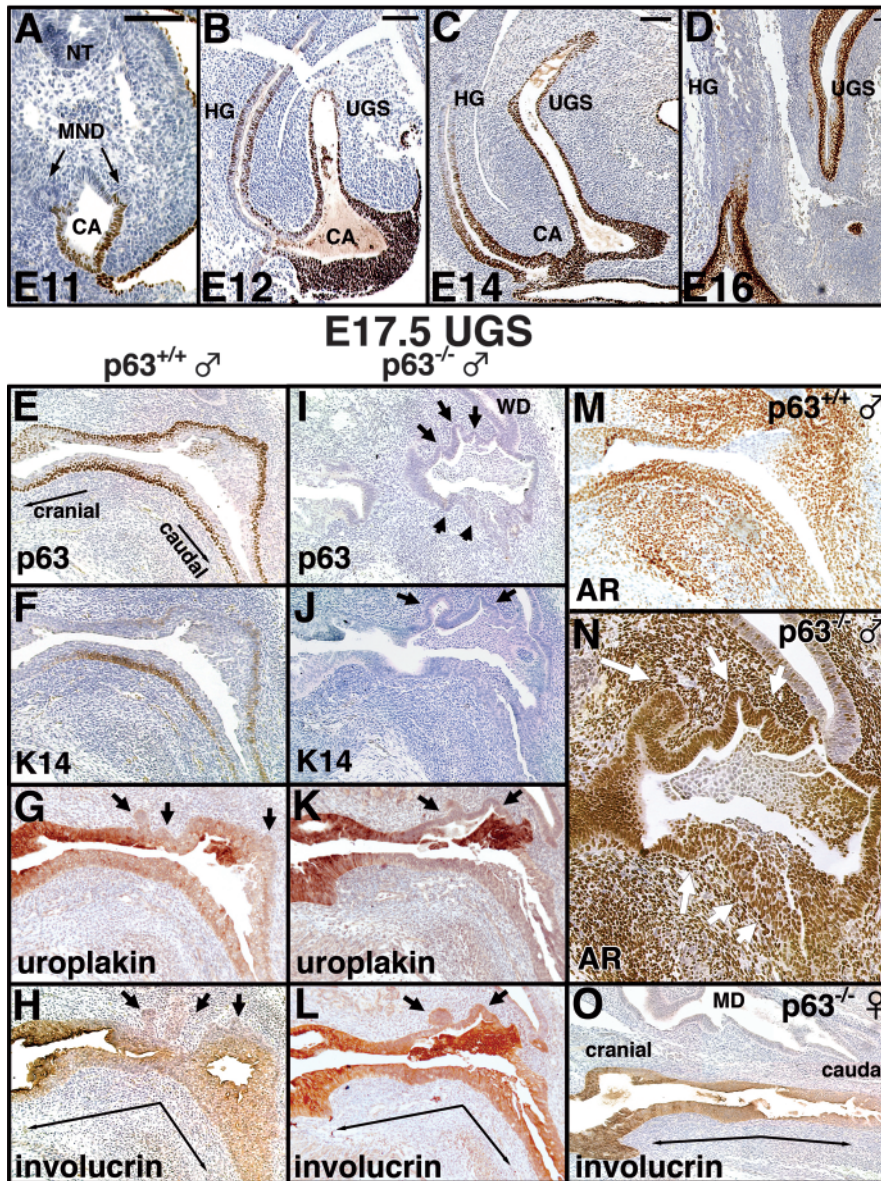
Fig. 1. Ontogeny of p63 in urogenital sinus (UGS) and phenotype of *p63⁻/⁻* UGS. (A-D) Ontogeny of p63 in embryonic UGS. CA, cloaca; HG, hind gut; MND, mesonephric duct; NT, neural tube. Cross section (A) and saggital section (B-D) of mouse embryos. Scale bar: 50 μm. (E-O) Marker expression was examined in male *p63⁺/⁺* UGS (E-H,M) and male (I-L,N) and female (O) *p63⁻/⁻* UGSs at E17.5. MD, Müllerian duct; WD, Wolffian duct. Gross morphology of the *p63⁻/⁻* UGS was normal and showed normal sexual dimorphism at E17.5 (compare O [female] with I-L [male]). *p63⁻/⁻* urogenital sinus epithelium (UGE) expressed differentiation markers identical to that of *p63⁺/⁺* UGE [uroplakin (G,K), involucrin (H,L) and androgen receptor (AR) (M,N)] with the exception that p63 (E,I) and K14 (F,J) were absent in *p63⁻/⁻* UGE. In both male *p63⁺/⁺* and *p63⁻/⁻* UGSs, notice epithelial outgrowths into stroma in the area where prostates normally form (black and white arrows).

duct derivatives indicated sufficient androgen levels for sex differentiation in *p63⁻/⁻* male embryos (Fig. 1I). The *p63⁺/⁺* and *p63⁻/⁻* UGE showed essentially identical expression patterns of differentiation markers for UGE such as uroplakin (Wu et al., 1994) (Fig. 1G,K), involucrin (Walts et al., 1985) (Fig. 1H,L), K19 (Wang et al., 2001) (not shown) and AR (Fig. 1M,N) (Cooke et al., 1991). Therefore, p63 is dispensable for expression of several normal differentiation markers for UGE. However, *p63⁻/⁻* UGE was columnar (Fig. 1N) and negative for K14 (Fig. 1J) indicating that p63 was essential for squamous differentiation, as suggested earlier (Mills et al., 1999; Yang et al., 1999).

Androgen induces development of prostate, and androgen action is mediated by AR in urogenital sinus mesenchyme (Cunha and Lung, 1979). AR was highly expressed in mesenchyme of *p63⁻/⁻* UGS (Fig. 1N). The columnar *p63⁻/⁻* UGE appeared to be responding to androgen-dependent mesenchymal signal to induce prostatic development as

indicated by formation of epithelial evaginations in the regions where prostate normally forms (Fig. 1I-L,N, arrows). As expected, similar epithelial evaginations were absent in females (Fig. 1O). Whereas normal prostatic buds are solid cords of stratified epithelium, the epithelial cells in the in-folds were simple columnar and had definitive lumen.

Phenotype of *p63⁻/⁻* prostate

p63⁻/⁻ and *p63⁺/⁺* UGSs were dissected from E16-18 embryos, transplanted and grown under renal capsule of adult male nude mice. *p63⁻/⁻* UGS developed ductal structures resembling normal prostate (compare Fig. 2A and 2B). Development of *p63⁻/⁻* prostate was androgen-dependent, because *p63⁻/⁻* UGS grafted into castrated male (Fig. 2C) or female (not shown) hosts formed large cysts and never developed ducts. In general, ducts of *p63⁻/⁻* prostate were less dilated than *p63⁺/⁺* prostate, but otherwise, the ductal morphology of *p63⁻/⁻* prostate appeared normal. Ducts of *p63⁻/⁻* prostate were surrounded by
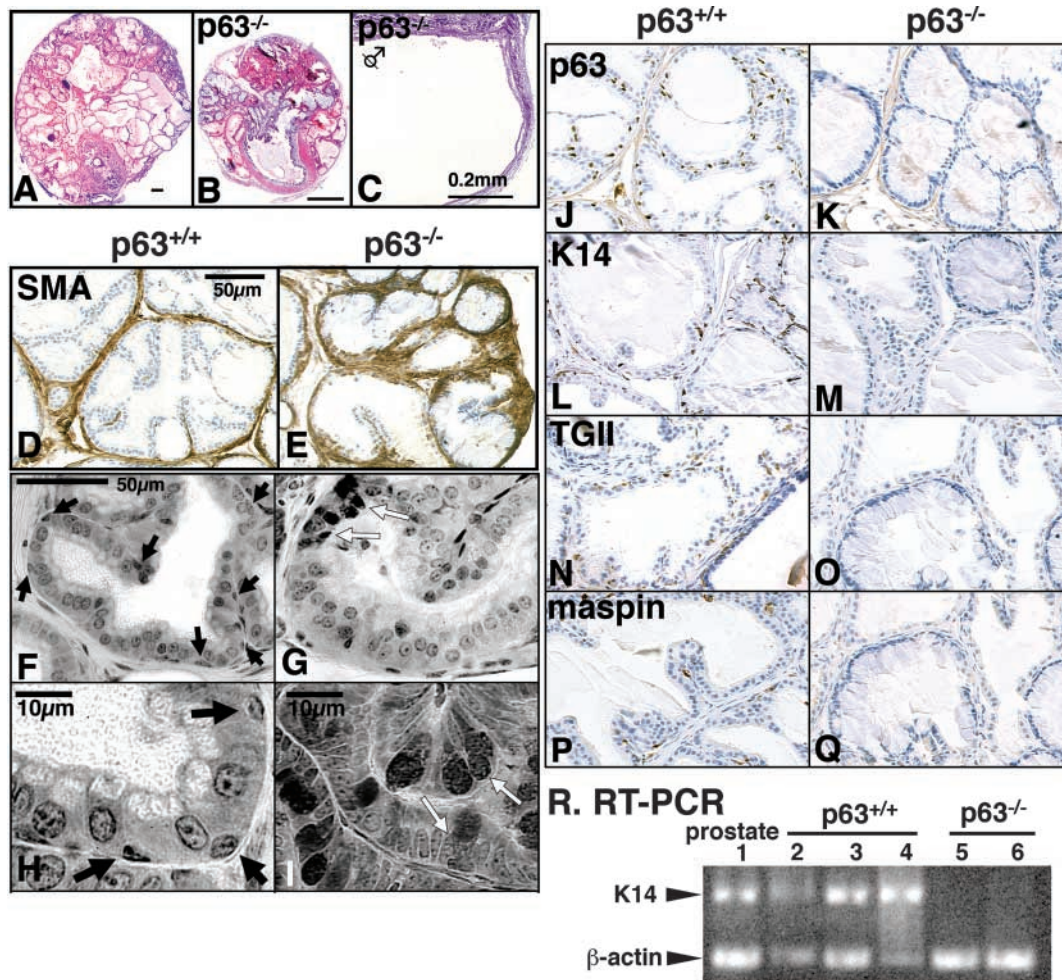
Fig. 2. *p63⁻/⁻* prostate lacks basal cells. Both *p63⁺/⁺* (A) and *p63⁻/⁻* (B) urogenital sinus (UGS) formed prostate when grafted into intact male hosts. When *p63⁻/⁻* UGS was grafted into castrated male hosts, the UGS developed into a large cyst without ducts (C). The prostatic ducts were surrounded by smooth muscle cells expressing smooth muscle actin (SMA) (D,E). Morphologically definable basal cells (black arrow in F,H) were not observed in *p63⁻/⁻* prostate (G,I). *p63⁻/⁻* prostate contained goblet cells (G,I, white arrows) (see also Fig. 3). Elongated/flat nuclei of stromal cells should not be confused with basal cells. Expression of basal cell markers was examined in *p63⁺/⁺* (J,L,N,P) and *p63⁻/⁻* (K,M,O,Q) prostates. p63 (J,K), K14 (L,M), transglutaminase II (TGII,N,O) and maspin (P,Q) were detected only in *p63⁺/⁺* prostate. All basal cell markers were absent in *p63⁻/⁻* prostate (K,M,O,Q). RT-PCR for K14 (R) confirmed results of IHC. K14 mRNA was detected in host prostate (coagulating gland + ventral prostate + dorsolateral prostate, lane 1) and three *p63⁺/⁺* prostatic grafts (lanes 2-4) but not in *p63⁻/⁻* prostates (lanes 5 and 6). β-actin was used for control.

smooth muscle cells as in normal prostate (Fig. 2D,E). Although its gross morphology was similar to *p63⁺/⁺* prostate, *p63⁻/⁻* prostate lacked morphologically definable basal epithelial cells (Fig. 2F-I). Instead, *p63⁻/⁻* prostate contained goblet cell-like cells (Fig. 1G,I, white arrows). Basal cell markers [p63 (Fig. 2J,K), K14 (Fig. 2L,M), K19 (not shown), transglutaminase II (Friedrichs et al., 1995) (Fig. 2N,O)] were undetectable in *p63⁻/⁻* prostate epithelium. Maspin, which is expressed in the basal cells of normal prostate (Pierson et al., 2002) (Fig. 2P), was also absent in *p63⁻/⁻* prostatic epithelium (Fig. 1Q). Therefore, p63 is essential for development of prostatic basal cells. The result of IHC was confirmed by RT-PCR for K14 (Fig. 2R). K14 mRNA was detected in host prostate and *p63⁺/⁺* prostate, but not in *p63⁻/⁻* prostate (Fig. 2R).

Ducts of *p63⁻/⁻* prostate were lined by columnar luminal and atypical mucin-producing cells (Fig. 3A-C). These mucinous cells stained for PAS (not shown) and Alcian blue (pH 2.8)

(Fig. 3C). In time-course experiments the mucinous (Alcian blue-positive) cells were undetectable in *p63⁻/⁻* UGS seven days after grafting (an early stage of prostatic development) (Fig. 3D). Ten to 14 days after grafting, a small number of Alcian blue-positive cells were detected in the prostatic ductal epithelium of *p63⁻/⁻* UGS grafts (Fig. 3E). This observation suggests that differentiation of mucinous cells is secondary to the development of prostatic ducts.

Expression of luminal cell markers was examined in *p63⁺/⁺* (Fig. 3F,H,J,L) and *p63⁻/⁻* (Fig. 3G,I,K,M) prostates. AR (Fig. 3F,G), Nkx3.1 (Fig. 3H,I), and the secretory proteins mDLP (Fig. 3J,K) and mVP (Fig. 3L,M) were expressed in the luminal cells in *p63⁺/⁺* and *p63⁻/⁻* prostates. AR and Nkx3.1 were expressed in some mucinous cells of *p63⁻/⁻* prostate. mDLP-positive (Fig. 3K) and mVP-positive (Fig. 3M) cells were found among Alcian blue-positive cells. In some mucinous cells, mDLP was co-expressed with mucin (Fig. 3K, black
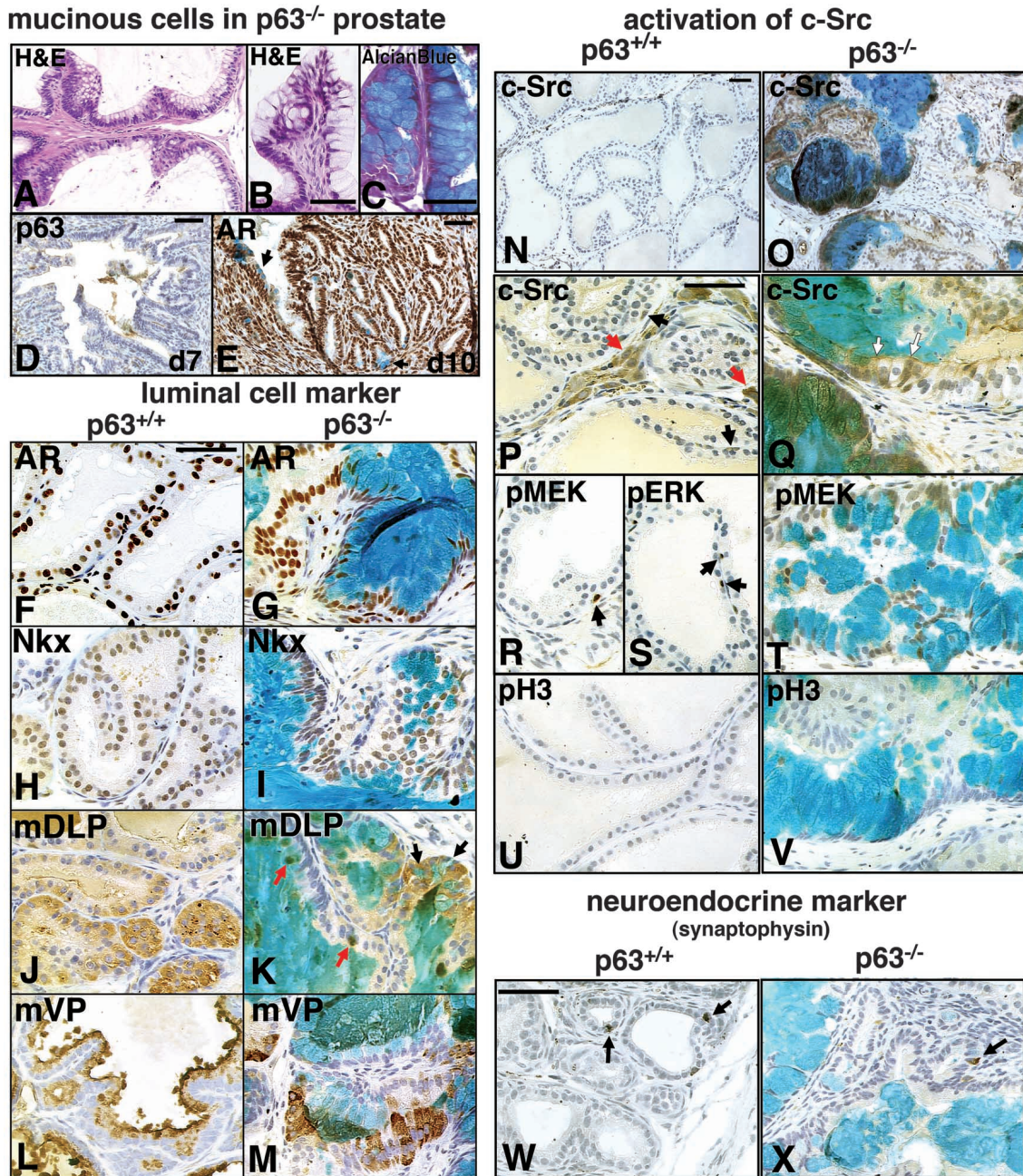
Fig. 3. Epithelial differentiation in *p63*−/− prostate. Scale bars: 50 μm. Goblet mucinous cells were detected in only *p63*−/− prostate (A,C). (C) The mucinous secretion stained for Alcian blue (pH 2.8). (D) Double staining for p63 and Alcian blue; one week after grafting the mucinous (Alcian blue-positive) cells were undetectable in *p63*−/− urogenital sinus (UGS). Double staining for androgen receptor (AR) and Alcian blue (E) in *p63*−/− UGS grafts; a small number of Alcian blue-positive cells was detected in epithelium of proximal ducts at 10 days after grafting (E, arrows). Expression of luminal cell markers was examined in *p63*+/+ (F,H,J,L) and *p63*−/− (G,I,K,M) prostates. AR (F,G), Nkx3.1 (Nkx, H,I), and secretory proteins, mouse dorsolateral prostate (mDLP) (J,K) and mouse ventral prostate (mVP) (L,M) were expressed in the luminal cells in *p63*+/+ and *p63*−/− prostates. mDLP-positive and mVP-positive cells were found among Alcian blue-positive cells (K,M). mDLP was co-expressed with mucin (K, red and black arrows). Secretory granules contained both mucin and mDLP in some mucinous cells (K, red arrows). Activity of Src was examined in *p63*+/+ and *p63*−/− prostates (N-Q). In the *p63*+/+ prostate, activation of Src was detected in neurons (P, red arrows) and a subset of basal cells (P, black arrows) but not in luminal cells (N,P). In *p63*−/− prostate, Src activity was detected mainly in Alcian blue-positive cells, but some non-mucinous luminal cells were also strongly positive for active Src (Q, white arrows). Downstream effecters in the Src-Ras-MAP kinase signal transduction pathway (MEK1/2 and ERK1/2) were also activated (phosphorylated) in neurons (not shown) and basal cells (R,S, arrows) in *p63*+/+ prostate and in (mucinous and non-mucinous) luminal cells in *p63*−/− prostate (T). Even though MAP kinase signaling was active, *p63*−/− prostate epithelium was proliferation-quiescent one month after grafting. Phosphorylation of histone H3 (pH3, U,V) was not detected in areas where phosphorylation of MEK was detected in *p63*+/+ (U) or *p63*−/− (V). Both *p63*+/+ and *p63*−/− contained rare neuroendocrine cells as assessed by expression of synaptophysin (W,X).

arrows), and the large secretory granules contained both mucin and mDLP (Fig. 3K, red arrows). The ontogeny of mucinous cells and the luminal cell marker expression pattern strongly suggested a luminal cell origin of the mucinous cells. Secretory markers of mBUG and mSV were undetectable in *p63*^{+/+} and *p63*^{–/–} UGS grafts as expected (not shown).

Activation of Ras-MAPK pathway by Src has been demonstrated to play key roles in overproduction of mucin in respiratory tract epithelium (Li et al., 1998). Activation of Ras and ERK1/2 has been implicated in the mucin production in intestinal epithelium (Lee et al., 2002). Recently, a transgenic mouse model (*Pb-RAS* mouse) demonstrated that expression of constitutively active mutant H-Ras (H-Ras[V12]) in prostatic epithelium transforms luminal cells into mucinous cells (Scherl et al., 2004). Therefore, we examined activation of Src (Fig. 3N-Q) and MEK1/2 (Fig. 3R,T). In the *p63*^{+/+} prostate, activated Src was detected in neurons (Fig. 3P, red arrows) and in a subset of basal cells (Fig. 3P, back arrows) but not in luminal cells (Fig. 3N,P) after one month of growth in intact male hosts. In contrast, in *p63*^{–/–} prostate, Src activity was detected in luminal epithelium (Fig. 3O,Q). Active Src was detected mainly in Alcian blue-positive cells, but some non-mucinous luminal cells were also strongly positive for active Src (Fig. 3Q, white arrows). In *p63*^{–/–} prostate, focal upregulation of Ras was also detected in the luminal cells which appeared to be in the process of trans-differentiation into mucinous cells (not shown). In the *p63*^{+/+} prostate, downstream effecters in the Src-Ras-MAPK signal transduction (MEK1/2 and ERK1/2) were activated (phosphorylated) in the same cell types as Src activation; in neurons (not shown) and a subset of basal cells (Fig. 3R,S). In *p63*^{–/–} prostate, MEK1/2 was phosphorylated and translocated into nucleus in both mucinous and non-mucinous luminal cells (Fig. 3T). Even though MAP kinase signaling was active, *p63*^{–/–} prostate was quiescent in regard to proliferation one month after grafting. Phosphorylation of histone H3 was equally low in *p63*^{+/+} and *p63*^{–/–} prostate in intact male hosts (Fig. 3U,V).

Neuroendocrine cells developed independent of basal cells

Both *p63*^{+/+} and *p63*^{–/–} prostates contained rare neuroendocrine cells as assessed by expression of synaptophysin (Fig. 3W,X). There was no distinctive difference between *p63*^{+/+} and *p63*^{–/–} prostates in the distribution and concentration of synaptophysin-positive cells.

Effect of castration and testosterone-treatment

One month after the grafting, *p63*^{+/+} and *p63*^{–/–} UGSs developed prostate with complex ductal structure (Fig. 4A,B). The *p63*^{+/+} prostate was negative for Alcian blue (Fig. 4A), whereas ducts of *p63*^{–/–} prostate were filled with Alcian blue-positive mucin (Fig. 4B). The wet weight of prostate is determined mostly by the water content. Therefore, *p63*^{+/+} prostate, which had more dilated ducts, was significantly heavier than *p63*^{–/–} prostatic gland (Fig. 4G). In the intact hosts, apoptotic cells were almost undetectable in both *p63*^{+/+} and *p63*^{–/–} prostate (Fig. 4I,J). In response to androgen deprivation epithelial apoptosis was detected in both the *p63*^{+/+} and *p63*^{–/–} prostates three days after the castration of the host (Fig. 4J,K). One month after the castration, *p63*^{+/+} prostatic regression was complete with marked reduction in luminal cells, reduction in the content of the ducts, and reduced ductal size (Fig. 4C). As a result, the entire *p63*^{+/+} prostatic graft was reduced in size with approximately 60% reduction in the original weight (Fig. 4G). Even though castration reduced its size, ductal structure remained and retained its complexity in *p63*^{+/+} prostate (Fig. 4C,H). In the shrunken ducts, p63-positive basal cells were enriched (Fig. 4M). In *p63*^{–/–} prostatic grafts, castration elicited a much more severe reduction in the number of ducts, and in all cases the entire graft became cystic (compare Fig. 4B with 4D, arrow indicates large cystic ducts). Although the wet weight was maintained following castration, the complexity of duct reduced dramatically one month after castration (Fig. 4H). In some areas of *p63*^{–/–} prostate, there was a complete loss of luminal cells leaving pools of mucinous material in the stroma (Fig. 4D,N,*). The mucinous pools and cysts filled with Alcian blue-positive material were observed in all *p63*^{–/–} prostate grafts subjected to androgen depletion by castration (18/18). The mucinous pools in the stroma were connected to residual ducts or cysts lined at least in part by epithelial cells (not shown). Dead cells were also observed in the mucinous pools (Fig. 4N, black arrows), suggesting that the mucin was originally circumscribed by epithelium. In the surviving ducts of *p63*^{–/–} prostate, both luminal and atypical mucinous cells were present. Mucinous cells were stained for Alcian blue in the castrated host (Fig. 4D,N).

One month after castration, a 25 mg testosterone (T)-pellet was implanted into some of the hosts. After one month of continuous T-treatment, both *p63*^{+/+} and *p63*^{–/–} prostate regenerated (Fig. 4E,F). In *p63*^{+/+} prostate, ducts were filled with secretions (Fig. 4E) and the wet weight significantly (*P*=0.05) exceeded the original weight (Fig. 4G). Ducts of *p63*^{–/–} prostate also regenerated and exhibited increased complexity of ductal structure (Fig. 4F,H), but the wet weight remained the same (Fig. 4G).

The expression of proliferation makers clearly demonstrated the ability of *p63*^{+/+} and *p63*^{–/–} prostate to regenerate. In the castrated hosts, pH3, phospho-MEK1/2 (pMEK) and Cdk4 were very low to undetectable in both *p63*^{+/+} (Fig. 5A) and *p63*^{–/–} (Fig. 5B) prostates. Therefore, phosphorylation of MEK in the epithelium of *p63*^{–/–} prostate in the intact hosts (Fig. 3T) is androgen-dependent. Five days after 25 mg T-pellet implantation, all three proliferation markers were upregulated in both *p63*^{+/+} (Fig. 5C) and *p63*^{–/–} (Fig. 5D) prostates. In *p63*^{+/+} prostate, the lumen of existing ducts became enlarged, and the luminal epithelial cells increased in height as expected (compare Fig. 5A with Fig. 5C). In the *p63*^{–/–} prostate, most ducts disappeared after the castration. Therefore, regeneration of *p63*^{–/–} prostate started as outgrowths of tightly packed ducts from the large cysts, similar to the early prostatic development (Fig. 5D). Epithelial labeling indices for pH3 clearly showed regeneration of prostatic tissue in both *p63*^{+/+} and *p63*^{–/–} prostates. Epithelial pH3 labeling index was low (<1%) in the intact and castrated host in both *p63*^{+/+} and *p63*^{–/–} prostates. T-pellet treatment significantly increased pH3 labeling index in both *p63*^{+/+} and *p63*^{–/–} prostates (Fig. 5E,*). One month after T-pellet treatment to the castrated hosts, *p63*^{+/+} and *p63*^{–/–} prostates were fully regenerated and the pH3 labeling index returned to the basal level (Fig. 5E, +T one month).

Basal cells seem to play a minimum role in the regulation of luminal epithelial proliferation, because the labeling index for pH3 was identical between *p63*^{+/+} and *p63*^{–/–} prostate in the

all-hormone treatment groups. The regenerated ducts of *p63⁻/⁻* prostates contained luminal cells positive for mucin, AR (Fig. 5F) and mDLP (Fig. 5G) or mVP (Fig. 5H), but not for mSV or mBUG (not shown), indicating prostatic differentiation.

Discussion

Studies with *p63⁻/⁻* mice have shown that p63 is essential for differentiation of epidermis (Mills et al., 1999; Yang et al., 1999). In the Müllerian (paramesonephric) duct, loss of p63 transformed stratified squamous cervicovaginal epithelium into simple columnar uterine epithelium (Kurita et al., 2004). Unlike the case of Müllerian duct, *p63⁻/⁻* UGE expressed proper differentiation markers for normal embryonic UGE (uroplakin, involucrin, K19 and AR) even though it failed to undergo squamous differentiation. Development of UGE into prostatic epithelia is regulated by androgens through AR in stromal cells (Cunha and Lung, 1978). In response to stromal signal(s), wild-type UGE proliferates, develops ducts and differentiates into luminal and basal prostatic epithelial cells. Our data clearly demonstrated that p63 is essential for development of prostatic basal cells. The unique phenotype of

Fig. 4. Effect of castration and androgen treatment. Double staining for androgen receptor (AR) and Alcian blue on *p63⁺/⁺* (A,C,E) and *p63⁻/⁻* (B,D,F) prostates. After one month of growth in intact male hosts, *p63⁺/⁺* and *p63⁻/⁻* urogenital sinus (UGS) developed prostate having a complex ductal structure (intact, A,B). The ducts of *p63⁺/⁺* prostates (A) were more dilated and had larger lumens than that of *p63⁻/⁻* prostate (B). In grafts grown in intact hosts for one month followed by one month of castration, the lumen of ducts in *p63⁺/⁺* prostate became smaller and the entire graft was reduced in size (C). In *p63⁻/⁻* prostate, castration reduced the number of ducts, and the entire graft became cystic (D, arrows; large cystic ducts). Pools of secretion devoid of epithelium were always observed (D,N,*). One month after the T-pellet implantation to castrated hosts, ducts in *p63⁺/⁺* prostate became dilated and filled with secretion (E). Ducts of *p63⁻/⁻* prostate also regenerated and exhibited increased complexity of ductal structure (F). (G) Wet weight of prostatic ducts. Data was analyzed with Fisher's PSLD and ANOVA factorial tests. Data are expressed as mean+s.d. Lowercase letters indicate groups statistically distinguished. The data was significantly higher in the following order, c>a>b (*P*<0.05). (H) Ductal complexity. Total length of epithelial basement membrane (epithelial length, μm) in prostatic tissue was measured in sections of UGS grafts. The epithelial length was divided by the area of prostatic tissue (μm²). Data was analyzed with Fisher's PSLD and ANOVA factorial tests. Data are expressed as mean+s.d. Lowercase letters indicate groups statistically distinguished. There is no significant difference between a versus b, or a versus c. c is significantly higher than b. a, b and c are significantly higher than d (*P*<0.05). Detection of apoptosis in *p63⁺/⁺* (I,K) and *p63⁻/⁻* (J,L) prostate. In the intact hosts, apoptotic cells were rarely detected (I,J). Three days after the castration of the host, apoptotic epithelial cells were detected in both *p63⁺/⁺* and *p63⁻/⁻* prostates (K,L, white arrows). Double staining for p63 and Alcian blue (M,N). The concentration of p63-positive basal cells increased in the shrunken duct of *p63⁺/⁺* prostate (M). Pools of Alcian blue-positive material devoid of intact epithelium but containing dying/dead cells (N, black arrow) were consistently observed in *p63⁻/⁻* prostate one month following castration (N, *).
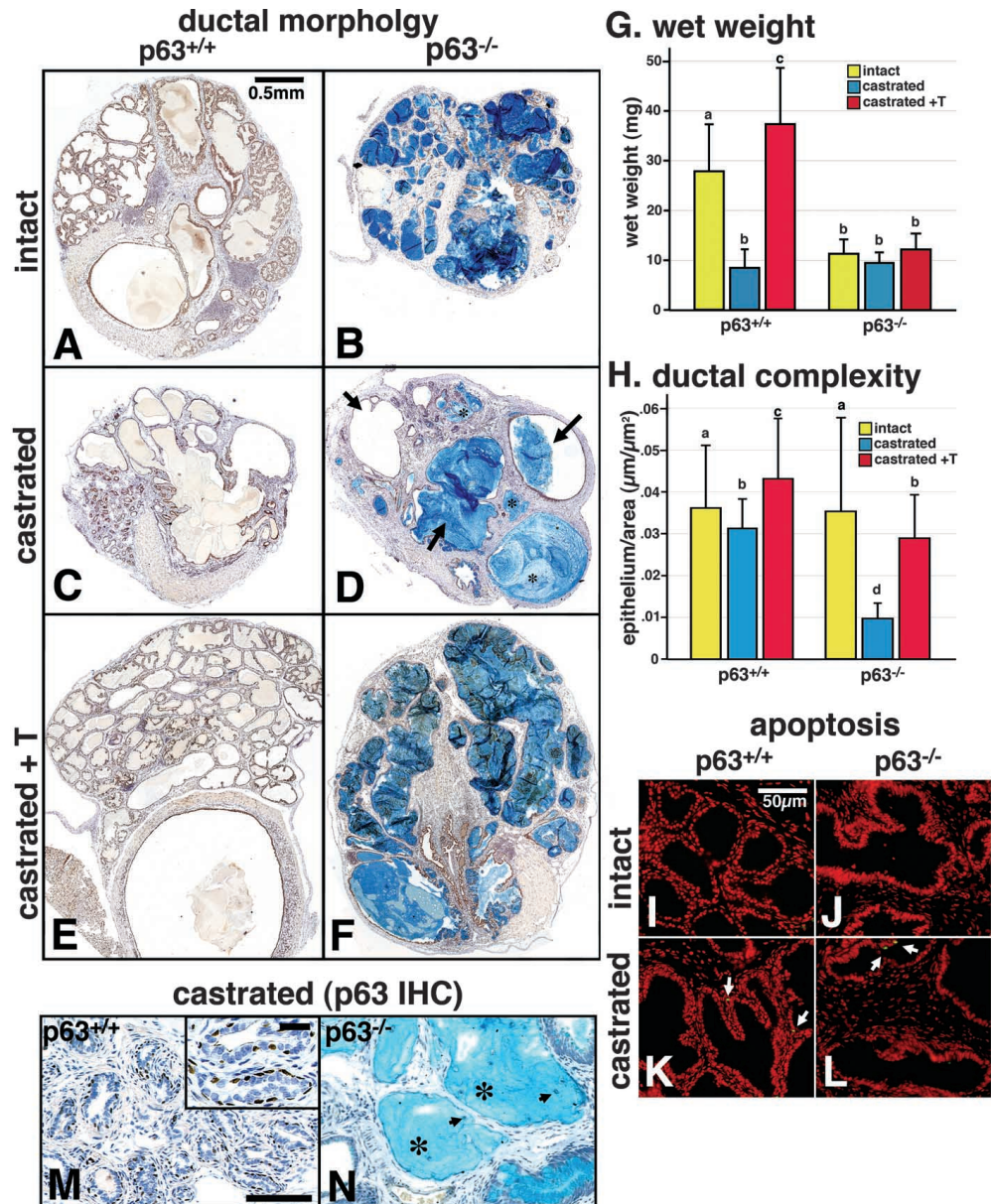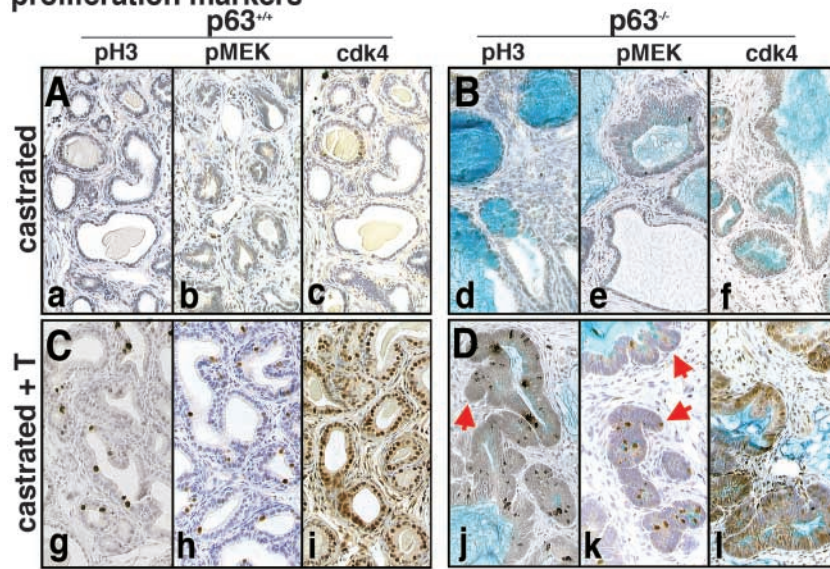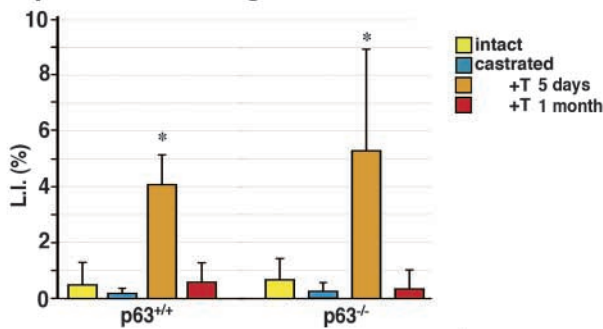
Fig. 5. Effect of testosterone. One month after castration (castrated A and B), proliferation markers, phospho-histone H3 (pH3; a,d,g,j), phospho-MEK (pMEK; b,e,h,k) and Cdk4 (c,f,i,l) were very low to undetectable in both *p63*^{+/+} (A) and *p63*^{−/−} (B) prostates. Five days after implantation of a 25 mg T-pellet (castrated+T; C,D), all three proliferation markers were upregulated in both *p63*^{+/+} (C) and *p63*^{−/−} (D) prostates. In the *p63*^{−/−} prostatic grafts, small bud-like outgrowths were visible (D, red arrows). (E) Labeling index for pH3. Data was analyzed with Fisher's PSLD and ANOVA factorial test. Data was indicated as mean+s.d. The bars with asterisk are significantly higher than others ($P<0.05$). There's no significant difference among bars without asterisk. Both *p63*^{+/+} and *p63*^{−/−} prostates were proliferation-quiescent in the intact and castrated hosts. T-treatment of castrated hosts increased pH3-positive cells equally in luminal cells of *p63*^{+/+} and *p63*^{−/−} prostates (+T 5 days). However, luminal cells in both *p63*^{+/+} and *p63*^{−/−} prostates became proliferation-quiescent one month after T-pellet implantation (+T one month). One month after T-pellet implantation, regenerated *p63*^{−/−} prostate expressed markers specific for prostatic luminal epithelium [androgen receptor (AR) (F), and secretory proteins, mouse dorsolateral prostate (mDLP) (G) and mouse ventral prostate (mVP) (H)].

p63^{−/−} prostate lacking basal cells provides insights into the function of basal cells in prostate in vivo. It is evident that development of prostatic ducts and differentiation of luminal cells do not require p63-positive basal cells. Likewise, p63-positive basal cells are not required for differentiation of neuroendocrine cells. Moreover, basal cells are not required for smooth muscle differentiation of UGS mesenchyme, which is regulated by prostatic epithelium (Cunha et al., 1996).

Tsujimura et al. have suggested that luminal epithelium contains a self-renewing population of putative stem cells that are presumably required for prostatic regeneration (Tsujimura et al., 2002). Our results also demonstrated regenerative capacity of prostate lacking basal cells. Therefore, stem cell function (at least regenerative capacity) is not necessarily associated with 'basal cells'. However, our results do not exclude possible trans-differentiation of basal to luminal cells in the normal adult prostate as suggested previously (Collins et al., 2001; Foster et al., 2002; Hudson et al., 2000; Uzgare et

al., 2004; van Leenders and Schalken, 2001; Wang et al., 2001). Moreover, our results do not exclude the presence of prostatic stem cells in the basal cells of normal wild-type prostate.

Although basal cells are not required for the differentiation of luminal cells, basal cells appear to be essential to maintain normal luminal cell differentiation. Luminal cells of *p63*^{−/−} prostate transformed into mucinous cells. Activation of Src, Ras and MAPK signaling can induce mucinous differentiation or overproduction of mucin in epithelial tissues (Lee et al., 2002; Li et al., 1998; Meerzaman et al., 2001; Perrais et al., 2002), and in the *p63*^{−/−} prostate Src and MEK were activated in the luminal cells. The activation of Src and MAPK signaling almost certainly plays a key role in mucinous transformation of luminal cells in *p63*^{−/−} prostate. Src can be activated by signals via adhesion molecules, cytokines or growth factors (Abram and Courtneidge, 2000; Thomas and Brugge, 1997). It is notable that in human prostate laminin 5 ($\alpha3\beta3\gamma2$) is produced exclusively by basal cells (Calaluce et al., 2001) and

downregulation of laminin 5 affects membrane stability of integrin α6β4 and gene expression in the prostatic luminal cells (Hao et al., 2001). These observations clearly demonstrate that loss of basal cells and/or change in cell adhesion molecule indeed affect gene expression and phenotype of luminal cells in the prostate. Moreover, in the adult prostate, epithelial growth and functional differentiation are regulated by androgen via stromal paracrine factors. In the normal prostate, p63-positive basal cells reside on the basement membrane, and may mediate or modulate interactions between stromal cells and luminal cells. Therefore, it is likely that loss of basal cells disturbs the interaction between stromal cells and epithelial cells, and causes activation of Src in luminal cells. In the normal prostate, Src was active in a subset of basal cells in the proliferation-quiescent adult state, indicating the existence of Src-activating signals in the normal prostatic epithelium. The factors activating Src in luminal cells of $p63^{-/-}$ prostate are yet to be identified, even though a wide spectrum of autocrine and paracrine growth factors have been described in the prostate (Cunha et al., 1998).

$p63^{-/-}$ prostate expressed proper secretory proteins specific for mouse prostate. Therefore, expression of these androgen-regulated genes in luminal cells does not require basal cells. However, ducts in 63$^{-/-}$ prostate were less dilated than ducts in the $p63^{+/+}$ prostate, and the ducts in $p63^{-/-}$ prostate were unable to reduce luminal secretory products in response to castration. This phenotype may be secondary to the mucinous differentiation of luminal cells, otherwise, basal cells may play a role in regulating secretory activity of luminal cells.

Basal cells also play key roles in maintaining ductal structure of the prostate in the absence of androgen. In response to castration, normal prostatic ducts lose luminal secretion content, and a substantial portion of luminal cells die via apoptosis. The entire process is highly coordinated, and therefore, normal prostatic ducts are able to shrink while maintaining their morphologic integrity. In contrast, in $p63^{-/-}$ prostate complete loss of ducts occurred leaving only residual cystic structures or pools of mucinous secretion in the stroma following castration. In normal prostate, a substantial portion of luminal cells dies in response to castration, but always a subset of luminal cells survive to maintain ductal structure and to permit regeneration when androgens are restored. The mechanism controlling death or survival of luminal cells following castration is not known. Because castration selectively eliminates luminal (versus basal) cells, the ratio of basal cells to luminal cells increases (Rouleau et al., 1990), and after castration most surviving luminal cells are in direct contact with basal cells. Taken together, the phenotypes of the $p63^{-/-}$ prostate strongly suggest that basal cells play an important role in the control of luminal cell differentiation and survival/apoptosis.

Adult prostate is a proliferation-quiescent organ in the mouse. Although androgen is a potent mitogen for developing and regenerating prostatic epithelia, androgen maintains prostatic tissue but does not stimulate epithelial proliferation in adult prostate. Luminal epithelial cells in developing or regenerating $p63^{+/+}$ and $p63^{-/-}$ prostatic grafts became proliferation-quiescent once the grafts reached a certain size. Therefore, the mode switch for androgen function, growth to maintenance, appears to be intact in $p63^{-/-}$ prostate. Moreover, the labeling index for pH3 was identical between $p63^{+/+}$ and

$p63^{-/-}$ prostates in response to hormonal manipulation, suggesting that p63 and basal cells are not essential for regulation of luminal epithelial proliferation.

Mucinous metaplasia of prostate has been suggested to be a pre-neoplastic condition (Scherl et al., 2004). Loss of basal cell is also a common characteristic in prostatic carcinogenesis. These observations suggest that changes caused by loss of basal cells may have an impact on carcinogenesis of the prostate. The effect of the loss of basal cells in carcinogenesis of the prostate is currently under investigation utilizing $p63^{-/-}$ UGS and the hormonal carcinogenesis model with T+E$_2$-treatment (Wang et al., 2000).

In conclusion, p63 is essential for differentiation of prostatic basal cells, and basal cells are essential in maintaining normal differentiation of luminal cells and integrity of prostatic ducts. However, basal cells (therefore p63) are not required for development and regeneration of prostate. Further experimentation is required to define the role of p63 in basal cell differentiation. p63 isoforms are functionally distinct in regard to cell fate commitment, particularly in epidermal differentiation (Koster et al., 2004; McKeon, 2004). The differentiation of epidermis appears to be regulated by the balance between isoforms containing and lacking the transactivation domain. To understand the function of p63 in basal cell differentiation in prostate may require detailed analysis of isoform expression in the developing UGS and the adult prostate.

References

Abram, C. L. and Courtneidge, S. A. (2000). Src family tyrosine kinases and growth factor signaling. *Exp. Cell Res.* **254**, 1-13.

Aumuller, G., Leonhardt, M., Renneberg, H., von Rahden, B., Bjartell, A. and Abrahamsson, P. A. (2001). Semiquantitative morphology of human prostatic development and regional distribution of prostatic neuroendocrine cells. *Prostate* **46**, 108-115.

Bhatia-Gaur, R., Donjacour, A. A., Sciavolino, P. J., Kim, M., Desai, N., Young, P., Norton, C. R., Gridley, T., Cardiff, R. D., Cunha, G. R. et al. (1999). Roles for Nkx3.1 in prostate development and cancer. *Genes Dev.* **13**, 966-977.

Calaluce, R., Kunkel, M. W., Watts, G. S., Schmelz, M., Hao, J., Barrera, J., Gleason-Guzman, M., Isett, R., Fitchmun, M., Bowden, G. T. et al. (2001). Laminin-5-mediated gene expression in human prostate carcinoma cells. *Mol. Carcinog.* **30**, 119-129.

Collins, A. T., Habib, F. K., Maitland, N. J. and Neal, D. E. (2001). Identification and isolation of human prostate epithelial stem cells on alpha(2)beta(1)-integrin expression. *J. Cell Sci.* **114**, 3865-3872.

Cooke, P. S., Young, P. and Cunha, G. R. (1991). Androgen receptor expression in developing male reproductive organs. *Endocrinology* **128**, 2867-2873.

Cunha, G. R. and Lung, B. (1978). The possible influences of temporal factors in androgenic responsiveness of urogenital tissue recombinants from wild-type and androgen-insensitive (Tfm) mice. *J. Exp. Zool.* **205**, 181-194.

Cunha, G. R. and Lung, B. (1979). The importance of stroma in morphogenesis and function of urogenital epithelium. *In Vitro* **15**, 50-71.

Cunha, G. R., Hayward, S. W., Dahiya, R. and Foster, B. A. (1996). Smooth muscle-epithelial interactions in normal and neoplastic prostatic development. *Acta Anat.* **155**, 63-72.

Cunha, G. R., Hayward, S. W., Hom, Y. K., Donjacour, A. A., Kurita, T., Cooke, P. S. and Lubahn, D. B. (1998). Growth factors as mediators of stromal-epithelial interactions in steroid hormone target organs. In

Hormones and Growth Factors in Development and Neoplasia (ed. R. B. Dickson and D. S. Salomon), pp. 207-228. New York: Wiley-Liss.

Donjacour, A. A. and Cunha, G. R. (1993). Assessment of prostatic protein secretion in tissue recombinants made of urogenital sinus mesenchyme and urothelium from normal or androgen-insensitive mice. *Endocrinology* **131**, 2342-2350.

Donjacour, A. A., Rosales, A., Higgins, S. J. and Cunha, G. R. (1990). Characterization of antibodies to androgen-dependent secretory proteins of the mouse dorsolateral prostate. *Endocrinology* **126**, 1343-1354.

Foster, C. S., Dodson, A., Karavana, V., Smith, P. H. and Ke, Y. (2002). Prostatic stem cells. *J. Pathol.* **197**, 551-565.

Friedrichs, B., Riedmiller, H., Goebel, H. W., Rausch, U. and Aumuller, G. (1995). Immunological characterization and activity of transglutaminases in human normal and malignant prostate and in prostate cancer cell lines. *Urol. Res.* **23**, 301-310.

Hao, J., Jackson, L., Calaluce, R., McDaniel, K., Dalkin, B. L. and Nagle, R. B. (2001). Investigation into the mechanism of the loss of laminin 5 (alpha3beta3gamma2) expression in prostate cancer. *Am. J. Pathol.* **158**, 1129-1135.

Hayward, S. W., Brody, J. R. and Cunha, G. R. (1996). An edgewise look at basal epithelial cells: three-dimensional views of the rat prostate, mammary gland and salivary gland. *Differentiation* **60**, 219-227.

Hudson, D. L., O'Hare, M., Watt, F. M. and Masters, J. R. (2000). Proliferative heterogeneity in the human prostate: evidence for epithelial stem cells. *Lab. Invest.* **80**, 1243-1250.

Kawakatsu, H., Sakai, T., Takagaki, Y., Shinoda, Y., Saito, M., Owada, M. K. and Yano, J. (1996). A new monoclonal antibody which selectively recognizes the active form of Src tyrosine kinase. *J. Biol. Chem.* **271**, 5680-5685.

Koster, M. I., Kim, S., Mills, A. A., DeMayo, F. J. and Roop, D. R. (2004). p63 is the molecular switch for initiation of an epithelial stratification program. *Genes Dev.* **18**, 126-131.

Kurita, T., Mills, A. A. and Cunha, G. R. (2004). Roles of p63 in the diethylstilbestrol-induced cervicovaginal adenosis. *Development* **131**, 1639-1649.

Kurita, T., Wang, Y. Z., Donjacour, A. A., Zhao, C., Lydon, J. P., O'Malley, B. W., Isaacs, J. T., Dahiya, R. and Cunha, G. R. (2001). Paracrine regulation of apoptosis by steroid hormones in the male and female reproductive system. *Cell Death Differ.* **8**, 192-200.

Kurita, T., Young, P., Brody, J. R., Lydon, J. P., O'Malley, B. W. and Cunha, G. R. (1998). Stromal progesterone receptors mediate the inhibitory effects of progesterone on estrogen-induced uterine epithelial cell deoxyribonucleic acid synthesis. *Endocrinology* **139**, 4708-4713.

Lee, H. W., Ahn, D. H., Crawley, S. C., Li, J. D., Gum, J. R., Jr, Basbaum, C. B., Fan, N. Q., Szymkowski, D. E., Han, S. Y., Lee, B. H. et al. (2002). Phorbol 12-myristate 13-acetate up-regulates the transcription of MUC2 intestinal mucin via Ras, ERK, and NF-kappa B. *J. Biol. Chem.* **277**, 32624-32631.

Li, J. D., Feng, W., Gallup, M., Kim, J. H., Gum, J., Kim, Y. and Basbaum, C. (1998). Activation of NF-kappaB via a Src-dependent Ras-MAPK-pp90rsk pathway is required for *Pseudomonas aeruginosa*-induced mucin overproduction in epithelial cells. *Proc. Natl. Acad. Sci. USA* **95**, 5718-5723.

McKeon, F. (2004). p63 and the epithelial stem cell: more than status quo? *Genes Dev.* **18**, 465-469.

Meerzaman, D., Shapiro, P. S. and Kim, K. C. (2001). Involvement of the MAP kinase ERK2 in MUC1 mucin signaling. *Am. J. Physiol. Lung Cell Mol. Physiol.* **281**, L86-91.

Mills, A. A., Zheng, B., Wang, X. J., Vogel, H., Roop, D. R. and Bradley, A. (1999). p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* **398**, 708-713.

Perrais, M., Pigny, P., Copin, M. C., Aubert, J. P. and Van Seuningen, I. (2002). Induction of MUC2 and MUC5AC mucins by factors of the epidermal growth factor (EGF) family is mediated by EGF receptor/Ras/Raf/extracellular signal-regulated kinase cascade and Sp1. *J. Biol. Chem.* **277**, 32258-32267.

Pierson, C. R., McGowen, R., Grignon, D., Sakr, W., Dey, J. and Sheng, S. (2002). Maspin is up-regulated in premalignant prostate epithelia. *Prostate* **53**, 255-262.

Putt, F. A. (1971). Alcian dyes in calcium chloride: a routine selective method to demonstrate mucins. *Yale J. Biol. Med.* **43**, 279-282.

Rouleau, M., Leger, J. and Tenniswood, M. (1990). Ductal heterogeneity of cytokeratins, gene expression, and cell death in the rat ventral prostate. *Mol. Endocrinol.* **4**, 2003-2013.

Schalken, J. A. and van Leenders, G. (2003). Cellular and molecular biology of the prostate: stem cell biology. *Urology* **62**, 11-20.

Scherl, A., Li, J. F., Cardiff, R. D. and Schreiber-Agus, N. (2004). Prostatic intraepithelial neoplasia and intestinal metaplasia in prostates of probasin-RAS transgenic mice. *Prostate* **59**, 448-459.

Signoretti, S., Waltregny, D., Dilks, J., Isaac, B., Lin, D., Garraway, L., Yang, A., Montironi, R., McKeon, F. and Loda, M. (2000). p63 is a prostate basal cell marker and is required for prostate development. *Am. J. Pathol.* **157**, 1769-1775.

Sugimura, Y., Cunha, G. R. and Bigsby, R. M. (1986). Androgenic induction of deoxyribonucleic acid synthesis in prostatic glands induced in the urothelium of testicular feminized (Tfm/y) mice. *Prostate* **9**, 217-225.

Thomas, S. M. and Brugge, J. S. (1997). Cellular functions regulated by Src family kinases. *Annu. Rev. Cell Dev. Biol.* **13**, 513-609.

Tsujimura, A., Koikawa, Y., Salm, S., Takao, T., Coetzee, S., Moscatelli, D., Shapiro, E., Lepor, H., Sun, T. T. and Wilson, E. L. (2002). Proximal location of mouse prostate epithelial stem cells: a model of prostatic homeostasis. *J. Cell Biol.* **157**, 1257-1265.

Uzgare, A. R., Xu, Y. and Isaacs, J. T. (2004). In vitro culturing and characteristics of transit amplifying epithelial cells from human prostate tissue. *J. Cell. Biochem.* **91**, 196-205.

van Leenders, G. J. and Schalken, J. A. (2001). Stem cell differentiation within the human prostate epithelium: implications for prostate carcinogenesis. *BJU Int.* **88 Suppl. 2**, 35-42; discussion 49-50.

Walts, A. E., Said, J. W., Siegel, M. B. and Banks-Schlegel, S. (1985). Involucrin, a marker of squamous and urothelial differentiation. An immunohistochemical study on its distribution in normal and neoplastic tissues. *J. Pathol.* **145**, 329-340.

Wang, Y., Hayward, S., Cao, M., Thayer, K. and Cunha, G. (2001). Cell differentiation lineage in the prostate. *Differentiation* **68**, 270-279.

Wang, Y., Hayward, S. W., Donjacour, A. A., Young, P., Jacks, T., Sage, J., Dahiya, R., Cardiff, R. D., Day, M. L. and Cunha, G. R. (2000). Sex hormone-induced carcinogenesis in Rb-deficient prostate tissue. *Cancer Res.* **60**, 6008-6017.

Wu, X. R., Lin, J. H., Walz, T., Haner, M., Yu, J., Aebi, U. and Sun, T. T. (1994). Mammalian uroplakins. A group of highly conserved urothelial differentiation-related membrane proteins. *J. Biol. Chem.* **269**, 13716-13724.

Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M. D., Dotsch, V., Andrews, N. C., Caput, D. and McKeon, F. (1998). p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol. Cell.* **2**, 305-316.

Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R. T., Tabin, C., Sharpe, A., Caput, D., Crum, C. et al. (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* **398**, 714-718.