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Developmental Biology 267 (2004) 418–429

DEVELOPMENTAL BIOLOGY

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Loss of connexin 26 in mammary epithelium during early but not during late pregnancy results in unscheduled apoptosis and impaired development

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Received for publication 5 May 2003, revised form 7 November 2003, accepted 24 November 2003

Abstract

Gap junctions are intercellular channels that are formed by the protein family of connexins (Cxs). In mammary tissue, Cx26 and Cx32 are present in the secretory epithelium and Cx43 is localized in the myoepithelium. The expression of Cx26 and Cx32 is induced during pregnancy and lactation, respectively, thus suggesting unique roles for them in the functional development of the gland. The requirement for these connexins was explored using several strains of genetically altered mice: mice with an inactivated *Cx32* gene, mice in which the *Cx43* gene had been replaced with the *Cx32* gene (Cx43KI32 mice) and mice in which the *Cx26* gene was specifically ablated in mammary epithelium at different stages of development using Cre-loxP-based recombination. Normal mammary development was obtained in Cx32-null mice and in Cx43KI32 mammary tissue. In contrast, loss of Cx26 in mammary epithelium before puberty resulted in abrogated lobulo-alveolar development and increased cell death during pregnancy, which was accompanied by impaired lactation. Loss of Cx26 in mammary epithelium during the later part of pregnancy did not adversely interfere with functional mammary development. These results demonstrate that the presence of Cx26 is critical during early stages but not during the end of pregnancy when the tissue has completed functional differentiation. Cx26 is considered a tumor suppressor gene and Cx26-null mammary tissue was evaluated after five pregnancies. No hyperproliferation or hyperplasia was observed, suggesting that Cx26 does not function as a tumor suppressor.
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Keywords: Connexins; Gap junction; Mammary gland; Apoptosis; Alveolar development; Differentiation

Introduction

Cells within the context of an organ communicate directly with each other and thereby coordinate their activities. Such communication is facilitated by gap junctions, which are unique membrane structures composed of clusters of channels connecting neighboring cells. The channels are built by hexamers of connexins (Cxs), which are integral membrane proteins. Molecules smaller than 1 kDa, such as calcium and cyclic AMP, can travel through these channels and convey rapid communications between cells. Nineteen genes encoding connexins have been identified in the mouse genome and many of them are expressed in a rather cell-restricted pattern (Willecke et al., 2002). Connexins 26, 32 and 43 are highly expressed in mammary tissue and several lines of investigation suggest that they might be important for its development during pregnancy and function during lactation (Pozzi et al., 1995). Most notably, connexins 26 and 32 have been co-localized in gap junctions of mouse mammary epithelium during lactation and both homomeric and heteromeric connexins have been identified (Locke et al., 2000). The unique expression patterns of connexins 26 and 32 suggest distinct and overlapping roles in mammary development and function. Cx26 mRNA and protein have been detected in mouse mammary tissue as early as day 4 of pregnancy, which is followed by a steady increase and maximal expression during lactation (Locke et al., 2000; Monaghan

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0012-1606/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.ydbio.2003.11.022

et al., 1994). In contrast, a significant induction of Cx32 expression occurs only within a few hours after parturition (Locke et al., 2000). A similar expression profile has been established in the rat (Yamanaka et al., 2001). In contrast to Cx26 and Cx32, Cx43 is not detected in the secretory epithelium but rather the myoepithelium, and its expression increases sharply after parturition (Monaghan et al., 1996; Yamanaka et al., 1997). A role in the coordination of the contraction of the myoepithelium was hypothesized.

The finding that Cx26 is the dominant gap junction protein in acinar cells during pregnancy suggests that these channels may be critical for normal development, such as alveolar expansion and differentiation. Conversely, the induction of Cx32 after parturition points to a role in the establishment and/or maintenance of secretory functions. Experimental mouse genetics provides a powerful tool to define the function of a protein, but it has its limitations in that the redundancy of proteins within a family may obscure their function. For example, the loss of Cx32 does lead to a rather restricted set of lesions (for review, see Willecke et al., 2002) and the presence of Cx26 is essential for placental function (Gabriel et al., 1998). The expression pattern of Cx26 during pregnancy (Locke et al., 2000) mimics that of some milk proteins, including WDNM1 and β-casein (Robinson et al., 1995) suggesting that the gene is subject to similar hormonal control mechanism, including the Jak2/Stat5 pathway. In support of this, a Stat5 binding site has been identified in the promoter region of the human and mouse *Cx26* gene (Kiang et al., 1997).

To explore the unique contributions of Cx26 to alveologenesis and epithelial differentiation and function, we inactivated the gene specifically in mammary epithelium using Cre-loxP-based recombination. Two specific Cre-expressing mouse lines were used that exhibit distinct expression patterns. WAP-Cre (WC) mice display Cre expression in the later part of pregnancy when the alveoli have already formed, and differentiation has been initiated (Wagner et al., 1997, 2001). In contrast, the MMTV-Cre (MC) mice express Cre recombinase already in mammary epithelium at the time of birth (Wagner et al., 1997, 2001), and the *Cx26* gene will be inactivated at all stages of mammary development. Furthermore, the role of connexin 32 was established during the differentiation and lactation phase of mammary tissue in Cx32-null mice. Lastly, the contribution of Cx43 was evaluated in mice in which the *Cx43* genes had been replaced with *Cx32* alleles.

Materials and methods

Mice

Mice in which the *Cx32* gene had been inactivated (Nelles et al., 1996) and the *Cx43* gene had been replaced with the *Cx32* gene (Plum et al., 2000) have been described. The *Cx26* gene was inactivated in mammary epithelium using MMTV-Cre and WAP-Cre transgenes (Wagner et al., 1997, 2001) that were introduced into mice where exon 2 of Cx26, the coding region of the gene, is flanked by loxP site (Cohen-Salmon et al., 2002) (Fig. 4I). To distinguish between wild-type and floxed alleles, primers were used that flank a loxP site. Primer 1 (5′-ACA GAA ATG TGT TGG TGA TGG-3′) is located 204 bp before exon 2 and primer 2 (5′-CTT TCC AAT GCT GGT GGA GTG-3′) is located 65 bp after the beginning of exon 2. A fragment of 289 bp was amplified from the wild-type allele and a 390-bp fragment from the floxed allele. The MMTV-Cre transgene was identified with two primers (5′-CGT TCT GAT CTG AGC TCT GAG TG-3′ and 5′-CAT CAC TCG TTG CAT CGA CCG G-3′) that generated a 280-bp fragment. The WAP-Cre transgene was identified with two primers (5′-TAG AGC TGT GCC AGC CTC TTC C-3′and 5′CAT CAC TCG TTG CAT CGA CCG G 3′) that generated a 240-bp PCR fragment. Mice carrying two floxed Cx26 alleles and the WAP-Cre or MMTV-Cre transgene are referred to throughout the text as Cx26 fl/fl; WC and Cx26 fl/fl; MC mice, respectively.

Mammary epithelial transplantation

The technique has been described elsewhere (Robinson and Hennighausen, 1997). Mammary tissue from virgin Cx43KI32 mice was transplanted into cleared fat pads of 3-week-old female athymic nude mice (nu/nu). A total of 17 transplanted mice was used for this analysis. Eight weeks after transplantation, 8 transplanted mice were mated. Transplanted mammary tissue was harvested from virgin mice 11 weeks after transplantation and at day 1 of lactation.

Whole-mount analysis

After anesthesia with proper anesthetic, number 4 glands were harvested from all the different transgenic mice at 6 weeks of age and at lactation day 1. Virgin and lactating mammary tissues were fixed 4 h in Carnoy's fixative (6 parts 100% ethanol, 3 parts $CHCl_3$, 1 part glacial acetic acid), washed in ethanol 70%, dehydrated by passing through decreasing ethanol concentration, stained overnight in carmine alumin, rehydrated through increasing ethanol concentrations, cleared in xylene and mounted with Permount.

H&E analysis

For histological analyses, we harvested number 4 glands of 6-week-old virgin and lactation day 1 mice from all the transgenic mice. Tissues were fixed in 4% paraformaldehyde (PFA) for 4 h at room temperature, dehydrated and embedded in paraffin. Tissue blocks were sectioned at 5 μm and stained with hematoxylin and eosin by standard methods.

Southern blot analysis

Number 4 glands of Cx26 fl/fl; WC and Cx26 fl/fl; MC mice were harvested at day 1 of lactation and genomic DNA was isolated by digesting the tissue in a solution consisting of proteinase K (20 ng/ml) in 1% SDS, 50 mM Tris–HCl at pH 8.0, 20 mM NaCl and 1 mM EDTA. Upon extraction with phenol–chloroform, the DNA was precipitated in ethanol, air-dried and resuspended in TE. Thirty micrograms of genomic DNA was digested O/N at 37°C with *Spe*I and *Bam*HI and separated in a 0.8% TAE gel. The DNA was subsequently blotted onto a nylon membrane and hybridized with a ^{32}P-labeled probe (described below) O/N at 65°C with QuickHyb (Stratagene) and sheared salmon sperm DNA. Membranes were washed with 2× SSC, 0.1% SDS for 20 min at 65°C and twice with 2× SSC, 0.01% SDS for 20 min at 65°C, and then exposed on autoradiography film.

Template DNA to be used as a probe in the Southern analysis was amplified from C57BL/6 mouse genomic DNA using the following primers: 5′-CCAGCTCTGTATTTTGGGTCAGG-3′and 5′-ACCAGTTGCTGGTCCAAGGAGC-3′. This probe is part of the intron upstream of Cx26 exon 2 (Fig. 4I). The PCR product was separated in a 1% TAE gel and purified using a gel extraction kit (Qiagen).

Immunohistochemical analysis

Tissues specimens were harvested from 6-week-old virgin and lactation day 1 mice from all genotypes described previously. The tissues were fixed in PFA for 4 h at room temperature and embedded in paraffin. Sections were boiled in an antigen unmasking solution (Vector Laboratories, Inc.) for 2 min followed by 10 min cooling down. Primary antibodies were applied (NKCC1, 1:1000; Npt2b, 1:100; E-cadherin, 1:100; β-catenin, 1:100; K5, 1:200; SMA, 1:1000; H-3, 1:100; Stat 5a, 1:100; Stat 3, 1:100; Cx26, 1:100) and the sections were incubated for 1 h at 37°C or O/N at 4°C for Stat 5, Stat 3 and Cx26 antibodies. Subsequently, sections were incubated with fluorescent-conjugated secondary antibodies (Molecular Probes, Inc.) for 1 h at room temperature in the dark. Mounting medium (Vectashield, Vector Laboratories, Inc.) was applied, and the sections were analyzed under a Zeiss Axioskop (Carl Zeiss, Inc.) equipped with filters for fluorescein isothiocyanate (FITC), tetramethyl rhodamine isothiocyanate (TRITC) and FITC:TRITC and DAPI.

TUNEL assay

The TUNEL assay was performed according to the manufacturer's protocol (Apoptag kit, Intergen) on samples from Cx26 fl/f; MC and wild-type control mice at pregnancy day 19. A total of three mice of each genotype was used. Cells from 10 fields at 40× magnification were counted from each sample. The number of apoptotic cells is expressed as a percentage of the total number of DAPI-stained cells.

Cell proliferation

To mimic the pregnancy and thus study the proliferation function of the glands, 8-week-old virgin mice (wild type and Cx26 fl/fl; MC) were treated during 48 h with 1 μg β-estradiol and 1 mg progesterone in 100 μl sesame oil via interscapular subcutaneous injection (one injection each 24 h). Number 4 glands were harvested and fixed in 4% PFA during 4 h at room temperature. Immunostaining was performed as described above using anti-phosphorylated Histone-3 (H-3) antibodies. This antibody is specific for mitotic cells. Six glands per genotype were used for each experiment. Cells from five fields at 40× magnification were counted from each sample. The number of H-3-positive cells in a given field was expressed as a percentage of the total number of DAPI-stained cells.

Results

Expression of connexins in mouse mammary tissue

Although it had been shown that the genes encoding connexins 26, 32 and 43 are expressed in mammary tissue, their relative expression levels were not known. Unigene clusters were established from the entire set of EST databases as a means to determine the relative expression levels of Cx26, Cx32 and Cx43. More than 300,000 EST clones had been sequenced from a variety of mouse mammary cDNA libraries (Dupont et al., 2002; Miyoshi et al., 2002). Because most of these libraries were not normalized, it was possible to directly compare the expression levels of individual genes and estimate the relative expression in different physiological states of the gland. While 27% of the Cx26 ESTs were derived from mammary libraries, 6% of the Cx32 and 5% of the Cx43 EST clones were from mammary libraries (Table 1). The Cx26 unigene cluster contained 210 EST clones, with 43 from a cDNA library prepared from hyperplastic mammary tissue during lactation (WAP-TGFα transgenic mice), and 3 were found in libraries prepared from virgin (NbMMG) and lactating (NMLMG) tissues. In contrast, 189 EST clones corresponded to Cx32 mRNA, 4 of which came from the RIKEN lactating library and 6 from the WAP-TGFα library. The highest expression level with 932 matches was determined for Cx43, with 46 hits in mammary tissue. Twenty-five of these were found in the cDNA library from transgenic mice that expressed the SV40TAg. From the three connexins, Cx26 exhibited the highest expression levels in developing and functional mammary tissue, suggesting a role in the differentiation process.

Table 1
Relative expression of Cx26, Cx32 and Cx43 as measured by their abundance in EST libraries from mammary and non-mammary origin

	EST total	EST in mammary libraries	EST LactL/ EST Mam L	NbMMG	**NMLMG**	**Riken lactation**	Mam1	Mam2	Mam3	Mam4	**Mam5**	Mam6
Cx26	210	27%	83%	1	2	3	6	3	0	0	43	0
Cx32	189	6%	92%	1	1	4	0	0	0	0	6	0
Cx43	932	5%	13%	4	0	2	1	9	0	1	4	25

Column 2: Percentage of clones in mammary libraries compared to the entire set of EST clones in GenBank databases. Column 3: Percentage of EST present in lactating mammary libraries compared to the total of ESTs expressed in mammary libraries. The following libraries of mouse mammary origin have been searched. NbMMG, from virgin mice (39,638 clones); NMLMG, from lactating mice (422,777 clones); RIKEN, day 10 lactation (9921 clones); Mam_1, derived from a mammary tumor dissected from a virgin mouse carrying a human TGFα transgene under control of a metallothionein promoter (Smith et al., 1995b); Mam_2, derived from MMTV-LTR-Int3/Notch4 transgenic mouse mammary tumor (Smith et al., 1995a); Mam_3, from mice in which the BRCA1 gene had been inactivated (Xu et al., 1999); Mam_4, from mice which express the SV40Tag under control of the WAP gene promoter (Li et al., 1996); Mam_5, from lactating mice which express TGFα under control of the WAP gene promoter (Humphreys and Hennighausen, 2000; Sandgren et al., 1995); Mam_6; from mice which express the SV40Tag under control of the C3(1) promoter (Shibata et al., 1999). Bold, lactating mammary library.

Connexin 26, but not 32 and 43 is required for alveologenesis

To explore whether functional development of mammary epithelium during puberty and pregnancy was uniquely dependent on specific connexins, mice were analyzed in which the genes encoding the individual connexins had been inactivated. Three types of mice were used: mice that carried an inactive *Cx32* gene (Cx32-null), mice in which the *Cx43* gene had been replaced with the *Cx32* gene (Cx43KI32) and mice in which the *Cx26* gene was inactivated specifically in mammary epithelium. Development of mammary tissue was evaluated during puberty (Fig. 1). The mammary anlage is established during the fetal stage but ductal and alveolar development is confined to puberty and pregnancy, respectively. The elongation and branching of mammary ducts occurs during puberty and is activated by ovarian steroid hormones (Figs. 1A–D). Puberty-mediated ductal elongation and branching was normal in Cx32-null and Cx43KI32 females (Figs. 1M–T). To inactivate the *Cx26* gene specifically in mammary epithelium, two distinct Cre transgenes were introduced into Cx26 fl/fl mice, which resulted in the loss of the protein coding exon. While Cre expression under control of the WAP gene promoter (WC mice) is confined to differentiating mammary epithelium after mid-pregnancy, Cre expression under control of the MMTV-LTR (MC mice) occurs already in ductal epithelium before puberty and throughout subsequent stages (Wagner et al., 1997, 2001). Puberty-mediated ductal elongation and branching was normal in Cx26 fl/fl; and WC Cx26 fl/fl; MC mice (Figs. 1E–L). This demonstrates that Cx26 is not required for the formation of ductal epithelium.

Development of mammary epithelium was further evaluated during pregnancy and lactation (Fig. 2). Normal mammary epithelial cells proliferate and differentiate during pregnancy (Figs. 2A–D), a process that is completely dependent on a functional prolactin receptor, Jak2 and Stat5. At the end of pregnancy, the fat pad is filled with alveolar units that display a secretory behavior. Fat droplets and other milk components are being secreted into the lumen of alveoli. Cx32-null dams were able to nurture their pups and proliferation and differentiation of mutant mammary tissue during pregnancy was indistinguishable from that of wild-type epithelium, as judged by histology (Figs. 2M–P). This demonstrates that Cx32 is not required for functional mammary development. Because Cx43KI32 mice are infertile, it was necessary to transplant their mammary epithelium into wild-type hosts and evaluate its development after one pregnancy (Figs. 2Q–T). On the basis of histological criteria, Cx43KI32 mammary epithelium had undergone functional differentiation, lumina were filled with secretion and displayed lipid droplets (Fig. 2T). WAP-Cre-mediated inactivation of the Cx26 gene did not impair functional mammary development during pregnancy and the mice could lactate and nurse their pups. In contrast, lactation of Cx26 fl/fl; MC mice was severely impaired. Thirteen litters with a total of 82 pups were obtained and only 46 pups survived to weaning age. Two dams were incapable to nurse and their pups were dead within 24 h after parturition. While the histological features of Cx32-null, Cx43KI32 and Cx26 fl/fl; WC mammary tissue at parturition were identical to those observed in wild-type mice (Figs. 2A–H and M–T), mammary tissue in Cx26 fl/fl; MC mice was severely underdeveloped (Figs. 2I–K). Notably, there was a paucity of alveolar epithelium (Fig. 2K). However, some alveoli developed during pregnancy and they displayed features of differentiation with the secretion of lipid droplets (Fig. 2L). The lack of sufficient mammary epithelium suggests that Cx26 is required for the proliferation and/or survival of epithelial cells (see below).

To further define the time window when Cx26 was critical for alveolar development, mammary tissue from Cx26 fl/fl; MC was analyzed at days 8.5, 9.5, 13.5, 15.5 and 19 of pregnancy. Alveolar development, as judged by epithelial density within the fat pad, appeared to be normal at days 8.5 and 9.5 of pregnancy (data not shown). However, a clear paucity of alveolar epithelium was observed in Cx26 fl/fl; MC mice at day 15.5 of pregnancy (Fig. 3C). Wild-type tissue had twice as many alveolar units as mutant tissue (710 alveoli in panel A versus 280 alveoli in panel C).

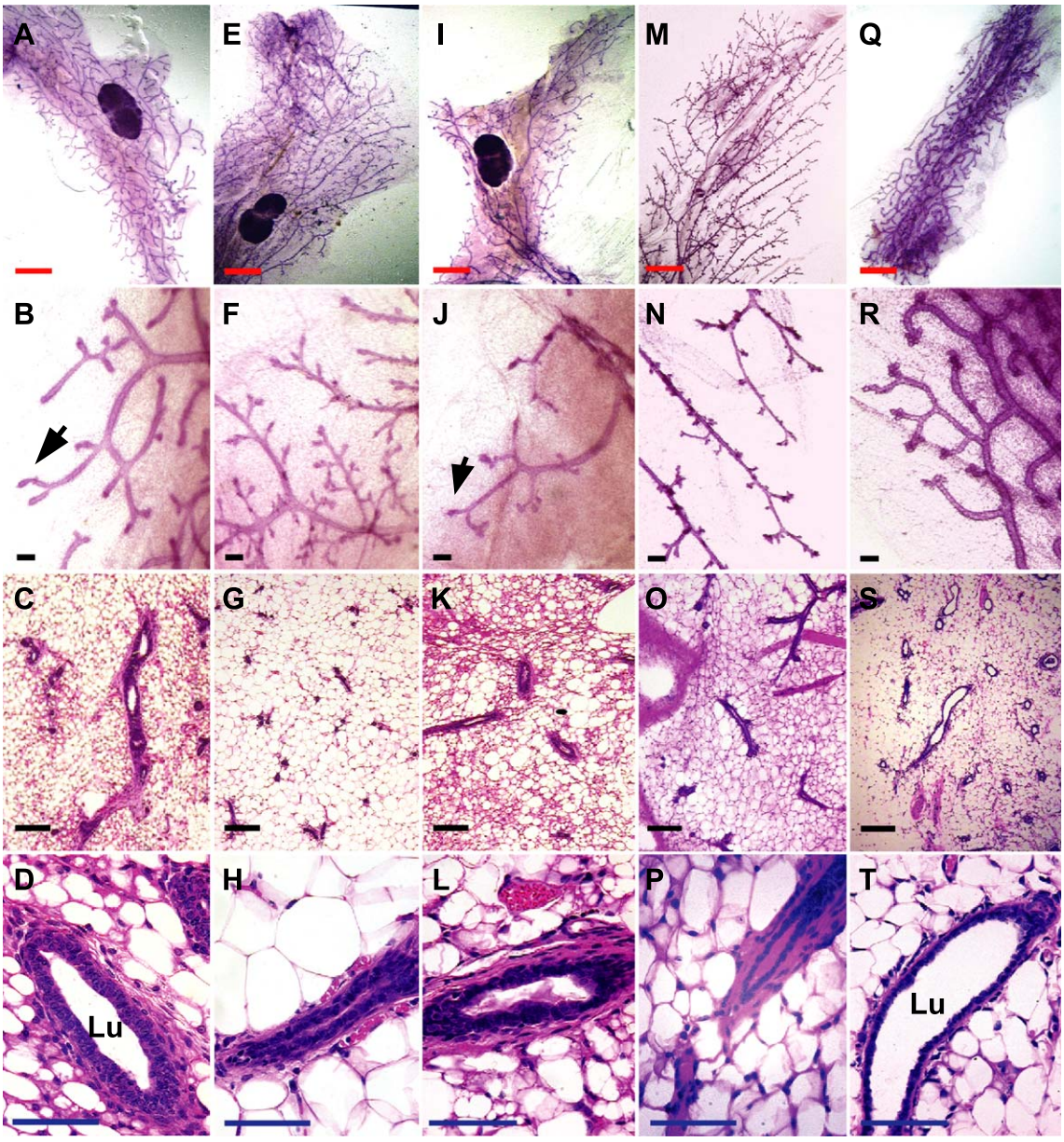

Fig. 1. Histological analyses of mammary tissue from wild-type control and mutant virgin mice (6 weeks old). (A–D) Wild type. (E–H) Cx26 fl/fl; WC. (I–L) Cx26 fl/fl; MC. (M–P) Cx32KO. (Q–T) Cx43KI32. (A, E, I, M, Q, B, F, J, N, R) Whole-mount analyses. (C, G, K, O, S, D, H, L, P, T) H&E analyses of sectioned tissue. Puberty-mediated ductal elongation and branching in mutant tissues was indistinguishable from wild-type controls. Arrows, terminal end buds; Lu, lumen; red scale bar, 1 mm; black scale bar, 100 μm; blue scale bar, 50 μm.

Moreover, wild-type tissue displayed more extensive differentiation than mutant tissue as evidenced by its secretory activity (Figs. 3B and 3D). No discrepancies in the differentiation status were observed at parturition.

A critical aspect in using the Cre-loxP recombination system is its overall efficiency, which depends on the Cre-expressing mouse strain and possibly the target gene. WAP-Cre and MMTV-Cre induced recombination and inactivation of the *Cx26* gene at day 1 of lactation was demonstrated by Southern blot analysis (Fig. 4). Restriction of genomic DNA from wild-type mice with *Bam*HI and *Spe*I, followed by separation in agarose gels and probing with an internal probe, resulted in the detection of a 4-kbp fragment (Fig. 4 I). In contrast, the floxed and recombined alleles provided 6.1 and 2.1 kbp fragments, respectively. To avoid problems with selective pressure and the potential loss of Cx26-null cells, DNA from fl/+; MC mice was initially tested. Because the MC transgene is active in B-cells in the spleen (Wagner et al., 2000), this tissue was used as a positive control. The presence of the 2.1-kbp recombined fragment demonstrated the activity of the MC transgene (Fig. 4, IIA). Analysis of mammary tissue from a day 15 pregnant fl/+; MC mouse demonstrated that the floxed allele had undergone Cre-mediated recombination (Fig. 4, IIB). Because the MC

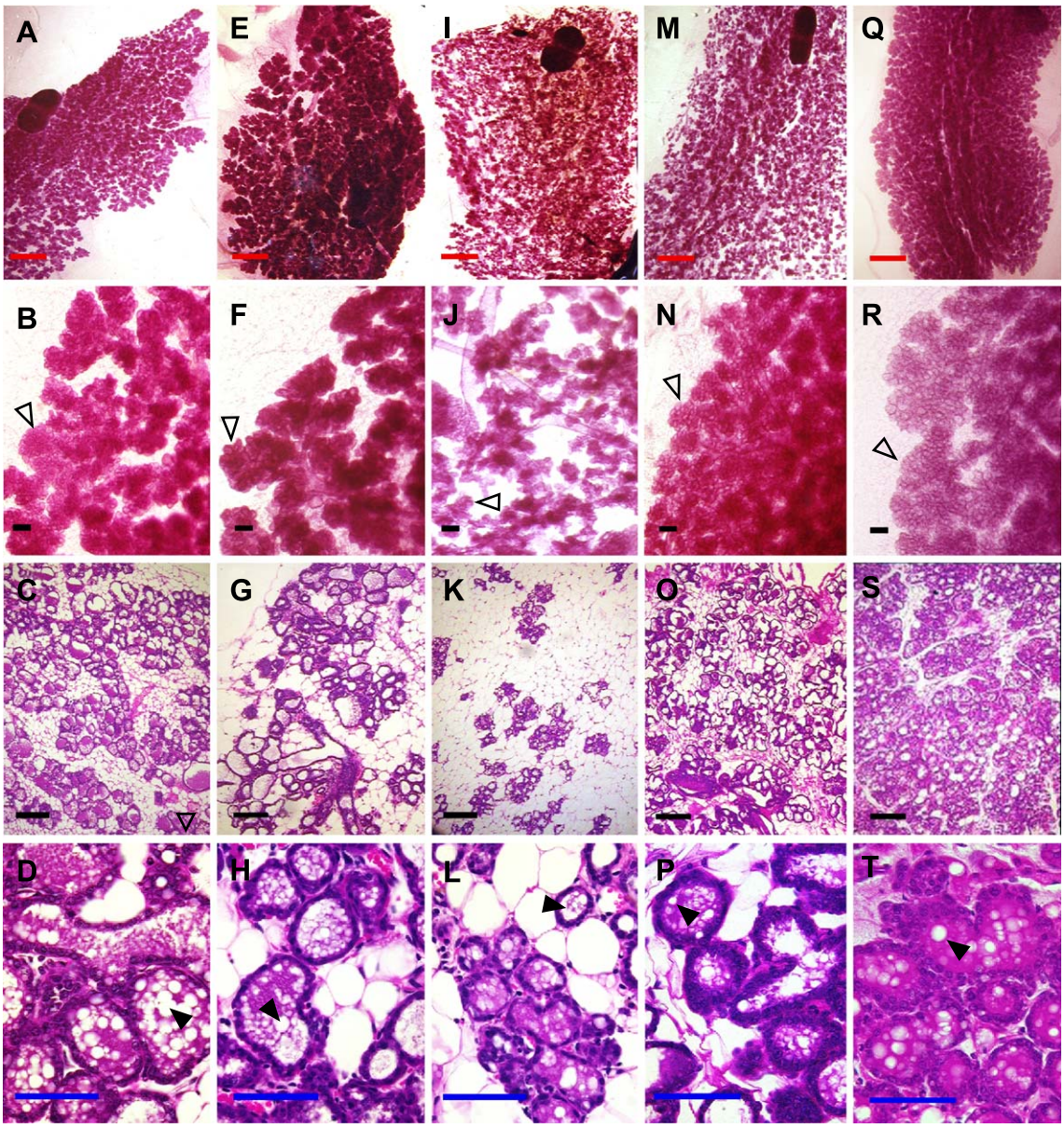

Fig. 2. Histological analyses of mammary tissue from wild-type control and mutant lactating mice (lactation day 1). (A–D) Wild type. (E–H) Cx26 fl/fl; WC. (I–L) Cx26 fl/fl; MC. (M–P) Cx32KO. (Q–T) Cx43KI32. (A, E, I, M, Q, B, F, J, N, R) Whole-mount analyses. (C, G, K, O, S, D, H, L, P, T) H&E analyses of sectioned tissue. Cx26 fl/fl; MC tissue mammary was underdeveloped (I–K): we observed less alveoli (J) and the fat pad was not fully filled (K) but the resident alveoli appeared functional as they contained lipid droplets (K and L). Open arrowheads, alveoli; solid arrowheads, lipid droplets; red scale bar, 1 mm; black scale bar, 100 μm; blue scale bar, 50 μm.

transgene is not active in the stromal part of mammary tissue, it is likely that the remaining signal for the 6.1-kbp floxed allele was derived from the stromal compartment. Analysis of mammary tissue from Cx26 fl/fl; MC and Cx26 fl/fl; WC dam at parturition revealed that the majority of the floxed alleles had undergone recombination (Fig. 4, IIC and IID).

To further establish the loss of Cx26 protein from mammary epithelium an immunostaining approach was used (Fig. 4, III). While Cx26 was observed between adjacent epithelial cells in tissue from wild-type mice (Fig. 4, IIIA), no Cx26 plaques were detected in the epithelium from Cx26 fl/fl; MC mice (Fig. 4, IIIB).

Analysis of epithelial cell differentiation in the absence of Cx26, 32 and 43

To further determine the extent of differentiation of mammary epithelium in the absence of Cx26, the temporal expression pattern of proteins that characterize either ductal or secretory alveolar epithelium was established. In lactating wild-type mice, β-catenin is preferentially located in the lateral membranes of the epithelium (Fig. 5A, in green), whereas Keratin 5 (K5) is expressed in the basally located myoepithelial cells (Fig. 5A, in red). Smooth muscle actin (SMA) characterizes myoepithelial cells

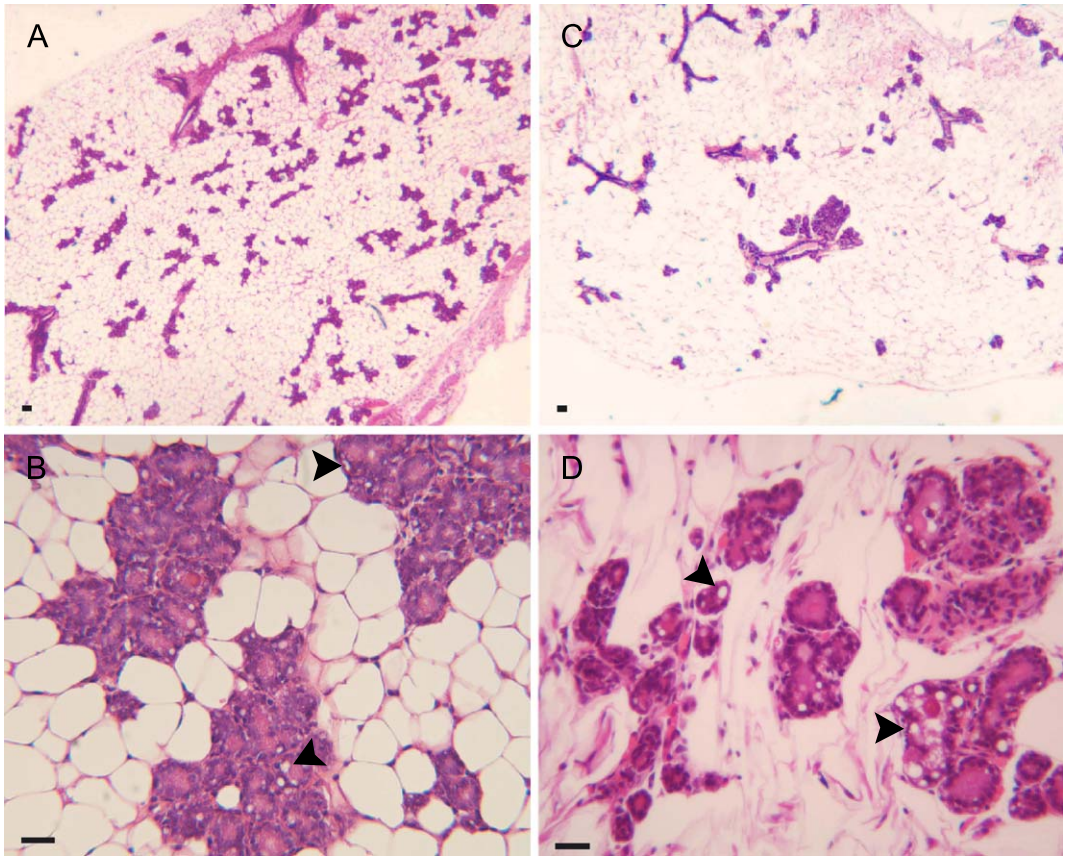

Fig. 3. Histological analysis of mammary tissue at pregnancy day 15.5. (A, B) H&E staining of WT gland. (C, D) H&E staining of Cx26 fl/fl; MC gland. At early stages of pregnancy, mammary development is already severely impaired in the transgenic model. Arrow, lipid droplets. Scale bar: 50 μm.

and is expressed throughout development (Fig. 5B, in green) (Shillingford et al., 2002). In contrast, the Na–K–Cl-cotransporter NKCC1 is expressed at high levels in ductal epithelial cells of virgin mice (data not shown) and is undetectable in secretory epithelium after parturition (Fig. 5B) (Shillingford et al., 2002, 2003). In secretory epithelial cells, E-cadherin is preferentially located on the lateral membranes of the epithelium (Fig. 5C, in green), whereas the Na–Pi cotransporter Npt2b is located on the apical membranes and thus serves as a marker of differentiation during lactation (Fig. 5C, in red) (Shillingford et al., 2002). In secretory epithelial cells, Stat5a is found in the nucleus (Fig. 5D, in red). As lactation was impaired in Cx26 fl/fl; MC mice, we addressed the question if milk ejection could be involved and then evaluated the expression of K5 and SMA. Expression of β-catenin/K5 (Figs. 5E and 5I) and SMA/NKCC1 (Figs. 5F and 5J) in both Cx26 fl/fl; WC and Cx26 fl/fl; MC mice at parturition was indistinguishable from control tissue. The expression of Npt2b was normal in Cx26 fl/fl; WC (Fig. 5G) and Cx26 fl/fl; MC (Fig. 5K) mice. Although Cx26 fl/fl; MC mammary tissue did not expand during pregnancy, expression of Npt2b was apparent on the apical membrane of alveolar-like structures (Fig. 5K) and Stat 5a had accumulated in the nucleus (Fig. 5L), suggesting that these cells had acquired a differentiation program that leads to secretion.

Although Cx43 is highly expressed in the myoepithelium, its loss did not alter the expression of the specific markers SMA and K5 (data not shown), suggesting that myoepithelial cells could develop in the absence of Cx43. The presence and localization of all the markers demonstrate that Cx26 is not involved in the process of differentiation of the epithelium during pregnancy (data not shown) and lactation. In addition, the known markers of contractile function in mammary glands are present, indicating that the survival problems of pups from Cx26 fl/fl; MC mice are not likely due to a failure of milk ejection problem but to an underdevelopment of the epithelium (Fig. 2K).

Loss of Cx26 does not adversely affect mammary epithelial cell proliferation but results in increased apoptosis

The lack of full expansion of mammary epithelium upon deletion of the Cx26 gene could be the result of impaired cell proliferation or increased apoptosis. Extensive proliferation occurs during the first few days of pregnancy. To mimic early pregnancy and study cell proliferation, acute hormone stimulation was performed for 48 h. Cell proliferation in three wild type and three

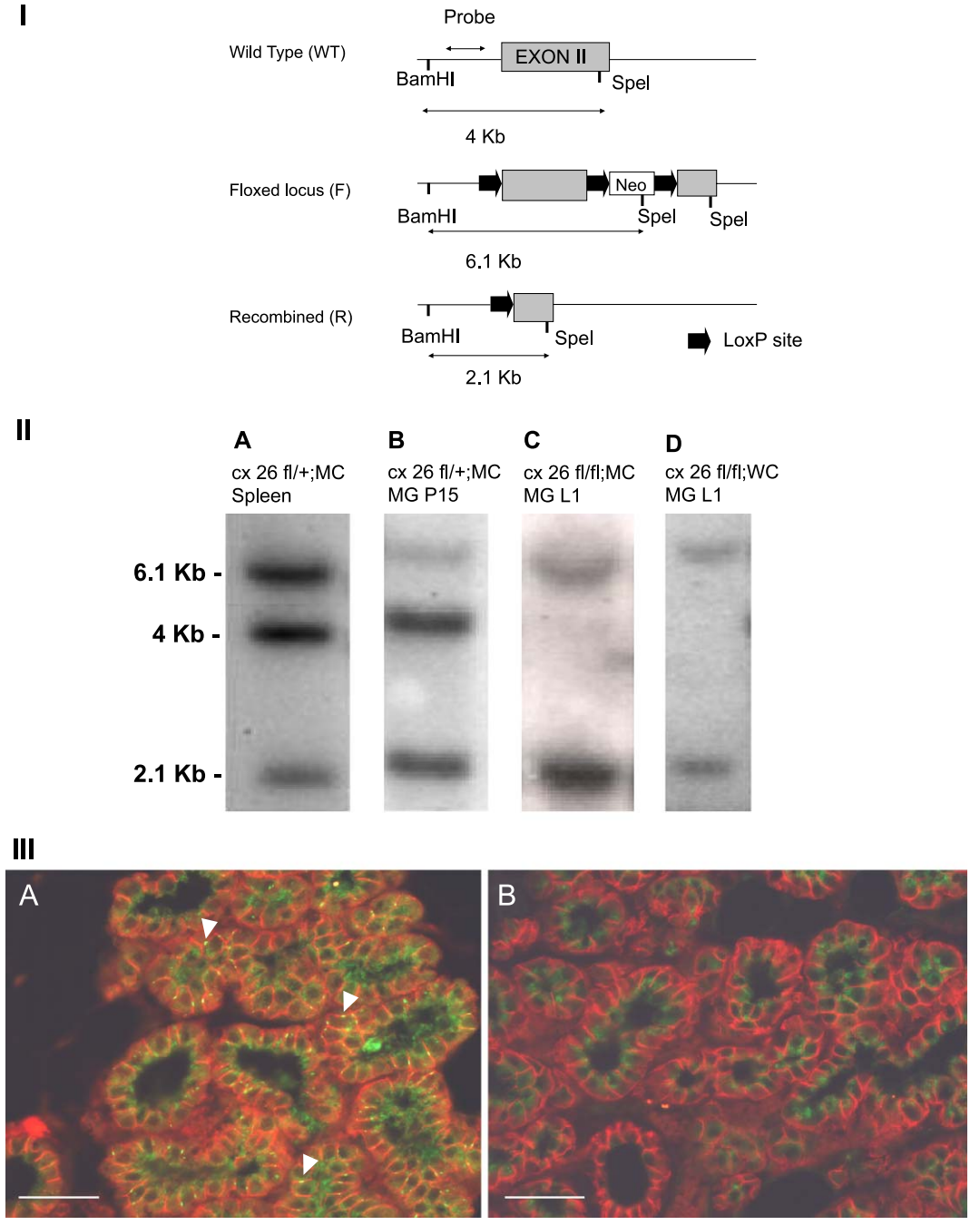

Fig. 4. Southern blot analyses of the targeted *Cx26* gene. (I) Structures of the wild-type, floxed and recombined Cx26 alleles. A neomycin cassette flanked by two loxP sites was inserted into exon 2 and another loxP site was inserted 5′ of exon 2 (Cohen-Salmon et al., 2002). Restriction of genomic DNA with *Spe*I and *Bam*HI resulted in the following diagnostic fragments. Wild-type allele: 4 kbp; floxed allele: 6.1 kbp; Cre-mediated recombined allele: 2.1 kbp. (II) Southern blot analyses. (A) Spleen DNA from a Cx26 fl/+; MC male. Note: The presence of 2.1-kbp fragments demonstrates the recombination event. (B) DNA from mammary tissue from a Cx26 fl/+; MC female at day 15 of pregnancy. (C) DNA from mammary tissue from a Cx26 fl/fl; MC female at day 1 of lactation. Note: The majority of the floxed alleles have undergone recombination. The remaining signal is likely due to the stromal compartment, in which neither the MMTV-Cre nor the WAP-Cre transgenes are active. (D) DNA of mammary tissue from a Cx26 fl/fl; WC female at day 1 of lactation. (III) Analyses of Cx26 expression in parturing Wt and Cx26fl/fl; MC mice by IFC. Green, Cx26; red, E-cadherin. (A) Wild type, observe in yellow (white arrow) the expression of Cx26 protein between two adjacent epithelial cells. (B) Cx26fl/fl; MC, Cx26 is absent from epithelium in this transgene at lactation day 1. Scale bar: 50 μm.

Cx26 fl/fl; MC mice was evaluated in 8-week-old virgin females after injection of progesterone and estrogen. Proliferating cells were counted after immunostaining with anti-phosphorylated Histone-3 antibodies. No significant difference was observed between wild-type and Cx26 fl/fl; MC mammary tissue, indicating that the early loss of Cx26 in mammary epithelium does not affect the ability of the gland to proliferate (data not shown). Furthermore,

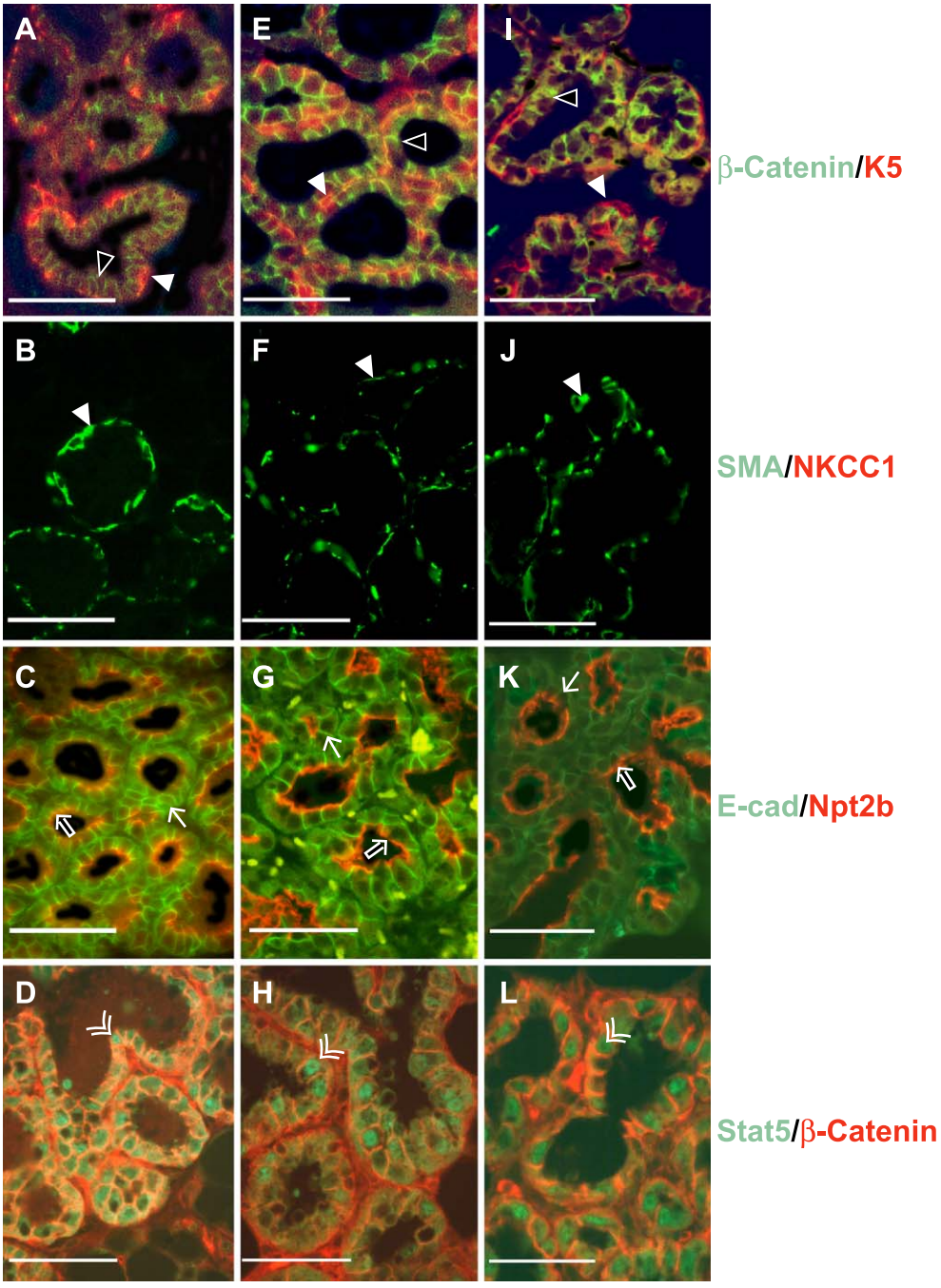

Fig. 5. Immunohistochemical analyses of lactating mammary tissue. (A–D) Wild type. (E–H) Cx26 fl/fl; WC. (I–L) Cx26 fl/fl; MC. (A, E, I) Staining with anti β-catenin and K5 antibodies. (B, F, J) Staining with anti SMA and NKCC1 antibodies. (C, G, K) Staining with anti E-cadherin and Npt2b antibodies. (D, H, L) Staining with Stat 5a and β-catenin. The expression of the different proteins in mutant and wild-type tissues was indistinguishable. Arrowheads, myoepithelial cells; open arrowheads, luminal epithelial cells; arrows, baso-lateral epithelial cells; open arrows, apical epithelial cells; double arrows, intranuclear localization of Stat5a. Scale bar: 50 μm.

proliferation was not affected in the mutant at P8.5, P9.5, P13.5 and P15 (data not shown).

The extent of cell death in wild-type and Cx26 fl/fl; MC mice was evaluated using TUNEL assays. In case the impaired lactation would be caused by precocious apoptosis, we studied the glands at pregnancy day 19. While in wild-type tissue 1.2 ± 0.7% of the cells were apoptotic, 5.6 ± 2.1% were TUNEL positive in the Cx26 fl/fl; MC mice (Fig. 6). Absence of Stat 3 nuclear protein staining in the same samples confirmed that observed apoptosis was not related to aberrant involution of the gland (data not shown). This demonstrated that Cx26 is required for the survival of mammary epithelium. Data were significant with $P < 0.005$.

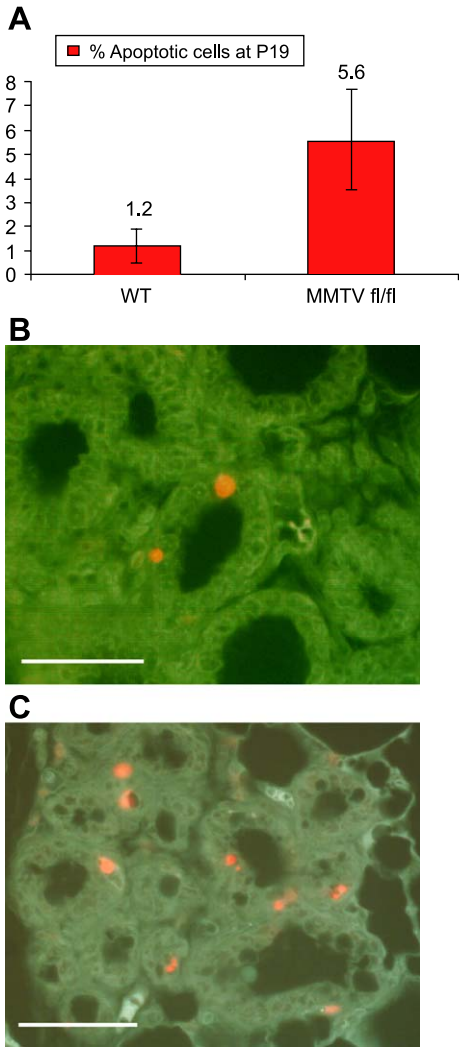

Fig. 6. Increased apoptosis in Cx26 fl/fl; MC mammary tissue. (A) The apoptotic rate in Cx26 fl/fl; MC tissue at pregnancy day 19 was 5.6 ± 2.1% versus 1.2 ± 0.7% in the wild-type tissue. (B) TUNEL assay of wild-type mammary tissue at parturition. (C) TUNEL assay of Cx26 fl/fl; MC mammary tissue at parturition. Apoptotic cells are shown in red. The values comparing Wt and Cx26 fl/fl; MC glands at parturition were significantly different ($P < 0.005$). Scale bar: 50 μm.

Lack of Cx26 does not result in the appearance of mammary tumors

It has been observed that connexins are frequently lost in breast cancer (Carystinos et al., 2001), suggesting that *Cx26* is a tumor suppressor (Hirschi et al., 1996; Muramatsu et al., 2002). We tested this hypothesis and explored whether loss of Cx26 in the mammary tissue would lead to the appearance of tumors. Mammary tissue from five Cx26 fl/fl; WC and five Cx26 fl/fl; MC was analyzed after five and three pregnancies, respectively. No signs of hyperplasia and tumors were detected, suggesting that the lack of Cx26 by itself does not predispose the mouse to mammary tumors (data not shown).

Discussion

Through the use of four genetically altered mouse strains, we were able to decipher the importance of Cx26 in mammary gland development. While the loss of Cx32 and the replacement of Cx43 with Cx32 did not adversely affect mammary development and differentiation, ablation of the *Cx26* gene from mammary epithelium before puberty compromised alveolar development during pregnancy and function during lactation. In contrast, ablation of Cx26 during the later part of pregnancy in the secretory epithelial compartment did not adversely affect alveolar development and function. Several conclusions can be drawn from these findings. First, functional development of mammary alveolar epithelium depends on the presence of Cx26 only during the early phase of pregnancy. Loss of Cx26 in secretory alveolar cells in the later part of pregnancy remains without consequences. Second, Cx32 itself is either not required at all or its absence can be compensated for by other connexins. Third, myoepithelial cells form in the absence of Cx43.

The distinct expression patterns of connexins 26, 32 and 43 in mammary tissue during development (Locke et al., 2000; Monaghan et al., 1994; Yamanaka et al., 2001) had suggested defined functions in mammary physiology. Inactivation of the *Cx26* gene before puberty using the MMTV-Cre mice demonstrated the need for this connexin in the pregnancy-mediated alveolar development. Thus, Cx26 appears to have a unique and non-redundant role during the early stages of pregnancy that are characterized by alveolar cell proliferation. This function coincides with its expression pattern during pregnancy (Locke et al., 2000). Loss of Cx26 in the alveolar epithelium resulted in a sharp increase of apoptosis without a major impairment of cell proliferation. Controlling epithelial cell survival emerges as a key function of Cx26. Similarly, inactivation of the *Cx26* gene in the epithelial network of the inner ear does not impair development per se but results in cell death within the organ of Corti (Cohen-Salmon et al., 2002). At this point, it is not clear which pathway controls cell survival and death in mammary epithelium during pregnancy. Although the Bcl-2 family of proteins controls the balance of cell survival and death in several settings, loss of the most prominent member in mammary epithelium, Bcl-x, does not influence mammary epithelial cell survival during pregnancy (Walton et al., 2001). While Cx26 is required for epithelial cell survival, it appears to be dispensable for cell function, both in the inner ear (Cohen-Salmon et al., 2002) and the mammary gland as shown in this study. Loss of Cx26-containing epithelial gap junctions does not affect the functioning of the vestibular apparatus or mammary secretory epithelium. In Cx26-deficient mammary epithelium, proteins linked to differentiation are expressed similar to wild-type tissue and the failure of these mice to lactate properly is probably due to the lack of sufficient amounts of epithelium. Because mammary epithelial differentiation occurs around parturition, the time when Cx32 is detected, it

can be speculated that the loss of Cx26 is compensated by Cx32. In support of this, Cx26 and Cx32 are co-expressed in acinar cells (Yamanaka et al., 2001) and organized as homomeric and heteromeric connexins (Locke et al., 2000).

Loss of Stat5a (Liu et al., 1995) results in impaired mammary development and function, which displays similarities to that seen in Cx26-null mammary epithelium. In particular, Stat5a is required for the survival of mammary epithelium after parturition (Humphreys and Hennighausen, 1999). This suggests that Cx26 is a direct or indirect downstream mediator of Stat5a. In support of this, a Stat5 binding site has been identified in the promoter of the *Cx26* gene (Kiang et al., 1997). Moreover, the expression pattern of the *Cx26* gene during pregnancy is reminiscent of some milk protein genes (Robinson and Hennighausen, 1997), suggesting that it is under similar hormonal control.

Inactivation of the *Cx26* gene in mammary secretory epithelium during the late stage of pregnancy using WAP-Cre transgenic mice did not lead to impaired development and the tissue was functional as demonstrated by the lactational performance of the dams. Because *Cx32* is expressed around parturition, it is likely that its presence will compensate for the lack of Cx26. Similarly, loss of Cx32 did not result in impaired alveolar function and lactation and can be explained by the presence of Cx26 after parturition (Locke et al., 2000). Normal pregnancy-mediated epithelial proliferation and development in the absence of Cx32 might have been anticipated because strong induction of Cx32 occurs only around parturition. While in the mammary gland the lack of Cx32 has no apparent impact on the lactational competence, its presence in lacrimal glands is necessary for optimal fluid secretion (Brink et al., 2002). The requirement for Cx26 and Cx32 in the physiology of other secretory organs remains to be determined.

It has been suggested that connexins function as potent tumor suppressors (Hirschi et al., 1996; Muramatsu et al., 2002) and retroviral-mediated overexpression of Cx26 in breast cancer cell lines resulted in a suppression of tumor growth in nude mice (Qin et al., 2002). Similarly, introduction of Cx26 into malignant human hepatoma cells reduced their malignant potential (Muramatsu et al., 2002). The in vivo significance of Cx26 as a tumor suppressor was evaluated in mammary epithelium from mice that had gone through several pregnancies. No hyperproliferation or hyperplasia were observed, suggesting that Cx26 is not a tumor suppressor in the absence of additional genetic. To investigate further the potential importance of Cx26 in mammary tumor development, it would be necessary to introduce activated oncogenes.

In the mammary gland, the presence of Cx43 is confined to the myoepithelium (Pozzi et al., 1995) which is located at the basal side of the secretory and ductal epithelium. Ordered myoepithelium that expressed the characteristic smooth muscle actin was present in mice in which the Cx43 gene had been replaced with Cx32 alleles. This suggests that either the loss of Cx43 can be compensated by the presence of Cx32 or that ordered gap junctions between the myoepithelium and the secretory epithelium are not required for the establishment and presence of myoepithelial cells.

In conclusion, of the connexins investigated (Cx26, Cx32 and Cx43), only Cx26 was shown to have a unique and non-redundant role in mammary development. Deletion of the *Cx26* gene in mammary epithelium before pregnancy resulted in increased apoptosis and a reduced alveolar compartment. However, deletion of the *Cx26* gene during the later part of pregnancy did not abrogate mammary development, which could be the result of compensation by Cx32 that is expressed during this time window.

References

Brink, P.R., Valiunas, V., Moore, L., Birzgalis, A., Walcott, B., 2002. The role of gap junctions in lacrimal acinar cells: the formation of tears. Adv. Exp. Med. Biol. 506, 109–113.

Carystinos, G.D., Bier, A., Batist, G., 2001. The role of connexin-mediated cell–cell communication in breast cancer metastasis. J. Mammary Gland Biol. Neoplasia 6, 431–440.

Cohen-Salmon, M., Ott, T., Michel, V., Hardelin, J.P., Perfettini, I., Eybalin, M., Wu, T., Marcus, D.C., Wangemann, P., Willecke, K., Petit, C., 2002. Targeted ablation of connexin26 in the inner ear epithelial gap junction network causes hearing impairment and cell death. Curr. Biol. 12, 1106–1111.

Dupont, J., Renou, J.P., Shani, M., Hennighausen, L., LeRoith, D., 2002. PTEN overexpression suppresses proliferation and differentiation and enhances apoptosis of the mouse mammary epithelium. J. Clin. Invest. 110, 815–825.

Gabriel, H.D., Jung, D., Butzler, C., Temme, A., Traub, O., Winterhager, E., Willecke, K., 1998. Transplacental uptake of glucose is decreased in embryonic lethal connexin26-deficient mice. J. Cell Biol. 140, 1453–1461.

Hirschi, K.K., Xu, C.E., Tsukamoto, T., Sager, R., 1996. Gap junction genes Cx26 and Cx43 individually suppress the cancer phenotype of human mammary carcinoma cells and restore differentiation potential. Cell Growth Differ. 7, 861–870.

Humphreys, R.C., Hennighausen, L., 1999. Signal transducer and activator of transcription 5a influences mammary epithelial cell survival and tumorigenesis. Cell Growth Differ. 10, 685–694.

Humphreys, R.C., Hennighausen, L., 2000. Transforming growth factor alpha and mouse models of human breast cancer. Oncogene 19, 1085–1091.

Kiang, D.T., Jin, N., Tu, Z.J., Lin, H.H., 1997. Upstream genomic sequence of the human connexin26 gene. Gene 199, 165–171.

Li, M., Hu, J., Heermeier, K., Hennighausen, L., Furth, P.A., 1996. Apoptosis and remodeling of mammary gland tissue during involution proceeds through p53-independent pathways. Cell Growth Differ. 7, 13–20.

Liu, X., Robinson, G.W., Gouilleux, F., Groner, B., Hennighausen, L., 1995. Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue. Proc. Natl. Acad. Sci. U. S. A. 92, 8831–8835.

Locke, D., Perusinghe, N., Newman, T., Jayatilake, H., Evans, W.H., Monaghan, P., 2000. Developmental expression and assembly of connexins into homomeric and heteromeric gap junction hemichannels in the mouse mammary gland. J. Cell. Physiol. 183, 228–237.

Miyoshi, K., Meyer, B., Gruss, P., Cui, Y., Renou, J.P., Morgan, F.V.

Smith, G.H., Reichenstein, M., Shani, M., Hennighausen, L., Robinson, G.W., 2002. Mammary epithelial cells are not able to undergo pregnancy-dependent differentiation in the absence of the helix–loop–helix inhibitor Id2. Mol. Endocrinol. 16, 2892–2901.

Monaghan, P., Perusinghe, N., Carlile, G., Evans, W.H., 1994. Rapid modulation of gap junction expression in mouse mammary gland during pregnancy, lactation, and involution. J. Histochem. Cytochem. 42, 931–938.

Monaghan, P., Clarke, C., Perusinghe, N.P., Moss, D.W., Chen, X.Y., Evans, W.H., 1996. Gap junction distribution and connexin expression in human breast. Exp. Cell Res. 223, 29–38.

Muramatsu, A., Iwai, M., Morikawa, T., Tanaka, S., Mori, T., Harada, Y., Okanoue, T., 2002. Influence of transfection with connexin 26 gene on malignant potential of human hepatoma cells. Carcinogenesis 23, 351–358.

Nelles, E., Butzler, C., Jung, D., Temme, A., Gabriel, H.D., Dahl, U., Traub, O., Stumpel, F., Jungermann, K., Zielasek, J., Toyka, K.V., Dermietzel, R., Willecke, K., 1996. Defective propagation of signals generated by sympathetic nerve stimulation in the liver of connexin32-deficient mice. Proc. Natl. Acad. Sci. U. S. A. 93, 9565–9570.

Plum, A., Hallas, G., Magin, T., Dombrowski, F., Hagendorff, A., Schumacher, B., Wolpert, C., Kim, J., Lamers, W.H., Evert, M., Meda, P., Traub, O., Willecke, K., 2000. Unique and shared functions of different connexins in mice. Curr. Biol. 10, 1083–1091.

Pozzi, A., Risek, B., Kiang, D.T., Gilula, N.B., Kumar, N.M., 1995. Analysis of multiple gap junction gene products in the rodent and human mammary gland. Exp. Cell Res. 220, 212–219.

Qin, H., Shao, Q., Curtis, H., Galipeau, J., Belliveau, D.J., Wang, T., Alaoui-Jamali, M.A., Laird, D.W., 2002. Retroviral delivery of connexin genes to human breast tumor cells inhibits in vivo tumor growth by a mechanism that is independent of significant gap junctional intercellular communication. J. Biol. Chem. 277, 29132–29138.

Robinson, G.W., Hennighausen, L., 1997. Inhibins and activins regulate mammary epithelial cell differentiation through mesenchymal–epithelial interactions. Development 124, 2701–2708.

Robinson, G.W., McKnight, R.A., Smith, G.H., Hennighausen, L., 1995. Mammary epithelial cells undergo secretory differentiation in cycling virgins but require pregnancy for the establishment of terminal differentiation. Development 121, 2079–2090.

Sandgren, E.P., Schroeder, J.A., Qui, T.H., Palmiter, R.D., Brinster, R.L., Lee, D.C., 1995. Inhibition of mammary gland involution is associated with transforming growth factor alpha but not c-*myc*-induced tumorigenesis in transgenic mice. Cancer Res. 55, 3915–3927.

Shibata, M.A., Liu, M.L., Knudson, M.C., Shibata, E., Yoshidome, K., Bandey, T., Korsmeyer, S.J., Green, J.E., 1999. Haploid loss of bax leads to accelerated mammary tumor development in C3(1)/SV40-TAg transgenic mice: reduction in protective apoptotic response at the preneoplastic stage. EMBO J. 18, 2692–2701.

Shillingford, J.M., Miyoshi, K., Flagella, M., Shull, G.E., Hennighausen, L., 2002. Mouse mammary epithelial cells express the Na–K–Cl cotransporter, NKCC1: characterization, localization, and involvement in ductal development and morphogenesis. Mol. Endocrinol. 16, 1309–1321.

Shillingford, J.M., Miyoshi, K., Robinson, G.W., Bierie, B., Cao, Y., Karin, M., Hennighausen, L., 2003. Proteotyping of mammary tissue from transgenic and gene knockout mice with immunohistochemical markers. A tool to define developmental lesions. J. Histochem. Cytochem. 51, 555–565.

Smith, G.H., Gallahan, D., Diella, F., Jhappan, C., Merlino, G., Callahan, R., 1995a. Constitutive expression of a truncated INT3 gene in mouse mammary epithelium impairs differentiation and functional development. Cell Growth Differ. 6, 563–577.

Smith, G.H., Sharp, R., Kordon, E.C., Jhappan, C., Merlino, G., 1995b. Transforming growth factor-alpha promotes mammary tumorigenesis through selective survival and growth of secretory epithelial cells. Am. J. Pathol. 147, 1081–1096.

Wagner, K.U., Wall, R.J., St-Onge, L., Gruss, P., Wynshaw-Boris, A., Garrett, L., Li, M., Furth, P.A., Hennighausen, L., 1997. Cre-mediated gene deletion in the mammary gland. Nucleic Acids Res. 25, 4323–4330.

Wagner, K.U., Claudio, E., Rucker III, E.B., Riedlinger, G., Broussard, C., Schwartzberg, P.L., Siebenlist, U., Hennighausen, L. 2000. Conditional deletion of the Bcl-x gene from erythroid cells results in hemolytic anemia and profound splenomegaly. Development 127, 4949–4958.

Wagner, K.U., McAllister, K., Ward, T., Davis, B., Wiseman, R., Hennighausen, L., 2001. Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. Transgenic Res. 10, 545–553.

Walton, K.D., Wagner, K.U., Rucker III, E.B., Shillingford, J.M., Miyoshi, K., Hennighausen, L. 2001. Conditional deletion of the bcl-x gene from mouse mammary epithelium results in accelerated apoptosis during involution but does not compromise cell function during lactation. Mech. Dev. 109, 281–293.

Willecke, K., Eiberger, J., Degen, J., Eckardt, D., Romualdi, A., Guldenagel, M., Deutsch, U., Sohl, G., 2002. Structural and functional diversity of connexin genes in the mouse and human genome. Biol. Chem. 383, 725–737.

Xu, X., Wagner, K.U., Larson, D., Weaver, Z., Li, C., Ried, T., Hennighausen, L., Wynshaw-Boris, A., Deng, C.X., 1999. Conditional mutation of Brca1 in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. Nat. Genet. 22, 37–43.

Yamanaka, I., Kuraoka, A., Inai, T., Ishibashi, T., Shibata, Y., 1997. Changes in the phosphorylation states of connexin43 in myoepithelial cells of lactating rat mammary glands. Eur. J. Cell Biol. 72, 166–173.

Yamanaka, I., Kuraoka, A., Inai, T., Ishibashi, T., Shibata, Y., 2001. Differential expression of major gap junction proteins, connexins 26 and 32, in rat mammary glands during pregnancy and lactation. Histochem. Cell Biol. 115, 277–284.