Requirement of a Myocardin-Related Transcription Factor for Development of Mammary Myoepithelial Cells

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The mammary gland consists of a branched ductal system comprised of milk-producing epithelial cells that form ductile tubules surrounded by a myoepithelial cell layer that provides contractility required for milk ejection. Myoepithelial cells bear a striking resemblance to smooth muscle cells, but they are derived from a different embryonic cell lineage, and little is known of the mechanisms that control their differentiation. Members of the myocardin family of transcriptional coactivators cooperate with serum response factor to activate smooth muscle gene expression. We show that female mice homozygous for a loss-of-function mutation of the myocardin-related transcription factor A (*MRTF-A*) gene are unable to effectively nurse their offspring due to a failure in maintenance of the differentiated state of mammary myoepithelial cells during lactation, resulting in apoptosis of this cell population, a consequent inability to release milk, and premature involution. The phenotype of *MRTF-A* mutant mice reveals a specific and essential role for MRTF-A in mammary myoepithelial cell differentiation and points to commonalities in the transcriptional mechanisms that control differentiation of smooth muscle and myoepithelial cells.

In contrast to most organs, the mammary gland develops primarily after birth in response to endocrine signals. During embryogenesis, the nascent mammary gland forms by budding of embryonic ectoderm and invasion of adjacent mesenchyme to give rise to a primitive ductal tree, which increases in size and branching pattern in response to hormonal signaling during puberty. During pregnancy, mammary ductal branching further increases and a secretory lobuloalveolar compartment forms at the termini of the ductal branches, allowing for production and secretion of milk. After weaning of the offspring, the mother's lobuloalveolar compartment returns to the virgin-like state. Thus, the mammary gland undergoes a cyclical process of hormone-dependent differentiation and dedifferentiation (15, 34, 40).

The mammary tree in adult females is composed of a luminal epithelial layer of milk-producing cells surrounded by a basal layer of myoepithelial cells that provides structural support and contractility required for milk release (18). Myoepithelial cells possess characteristics of both epithelial cells and smooth muscle (SM) cells (SMCs). They are true epithelial cells since they are derived from ectoderm, they express cytokeratins as the major component of the intermediate filament system, they form desmosomes, hemidesmosomes, and cadherin-mediated junctions, and they are permanently separated from surrounding stroma by a basement membrane. On the other hand, like SMCs, myoepithelial cells contain numerous fine filaments in their cytoplasm, express several smooth muscle structural proteins, and possess contractile ability (10, 57). Contraction of myoepithelial cells is triggered by oxytocin stimulation, resulting in the release of milk (9, 34, 36). Although

numerous studies have focused on the differentiation and functions of luminal epithelial cells, little is known of the mechanisms that control the development of myoepithelial cells, and no transcription factors that control their differentiation have yet been identified.

Differentiation of SMCs is dependent on serum response factor (SRF), a MADS (MCM1, agamous, *deficiens*, SRF) box transcription factor that binds a DNA sequence known as a CArG [CC(A/T)₆GG] box associated with smooth muscle structural genes such as the smooth muscle α -actin and myosin heavy chain genes (16, 20, 23, 27, 29, 30, 32). Members of the myocardin family of transcriptional coactivators interact with SRF and potentially enhance the expression of SRF-dependent genes (7, 11, 26, 52, 54–56, 58). Myocardin is expressed specifically in cardiac and smooth muscle cells, whereas the myocardin-related transcription factors (MRTFs) MRTF-A/MAL/MKL1 and MRTF-B/MKL-2 are expressed in a wide range of cell types (4, 5, 28, 31, 42, 53).

Deletion of the *Srf* gene in mice results in early embryonic lethality, precluding an analysis of possible functions of *Srf* after birth (2). Similarly, myocardin gene knockout mice die at embryonic day 10.5 (E10.5) from an apparent failure in differentiation of SMCs (26), and *MRTF-B* null mice die at about E13.5 with abnormalities in SMCs within the aortic arch arteries (38). In the skeletal muscle lineage, SRF and MRTFs are important for muscle fiber growth and maturation (25).

Here we describe the phenotype of *MRTF-A* mutant mice. In contrast to mice lacking *myocardin* or *MRTF-B* genes, mice homozygous for a null mutation in the *MRTF-A* gene are viable. However, postpartum *MRTF-A* mutant females are unable to productively nurse their offspring. Analysis of the molecular basis of this maternal abnormality reveals an essential role of MRTF-A in sustaining differentiation and function of mammary myoepithelial cells, which are required for ejection of milk from the mammary gland during lactation. Failure in

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maintaining myoepithelial cell differentiation results in apoptosis of this specific cell population. We conclude that MRTF-A is a highly specific regulator of myoepithelial cell development and survival in the mammary gland and that each member of the myocardin family is dedicated to the control of smooth muscle genes in specific subsets of cells during embryogenesis and adulthood, although they may play redundant roles in certain cell types.

MATERIALS AND METHODS

Generation of *MRTF-A* knockout mice. The gene structure of *MRTF-A* has been described previously (53). An *MRTF-A*-targeting vector was constructed to delete exons 9 and 10 by using a pN-Z-TK2 vector, which contains a nuclear *LacZ* cassette and a neomycin resistance gene under the control of the RNA polymerase II promoter and two herpes simplex virus thymidine kinase gene cassettes (a generous gift of R. Palmiter, University of Washington, Seattle). Genomic DNA flanking *MRTF-A* exons 9 and 10 was PCR amplified from a mouse 129SvEv genomic DNA library (Stratagene) and inserted into the targeting vector as short and long arms, respectively. The targeting vector was electroporated into 129 SvEv-derived embryonic stem (ES) cells, and selection was performed with G-418 and FIAU, respectively. Five hundred ES cell clones were isolated and analyzed by Southern blotting for homologous recombination. Three clones with a disrupted *MRTF-A* gene were injected into 3.5-day-old mouse C57BL/6 blastocysts, and the resulting chimeric male mice were bred to C57BL/6 females to achieve germ line transmission of the mutant allele.

RT-PCR. Total RNA was purified from tissues with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For reverse transcription (RT)-PCR, total RNA was used as a template for reverse transcriptase and random hexamer primers. Primer sequences are available on request.

Immunostaining and histology. As described previously (43), the fourth pair of mammary glands was surgically dissected, fixed with Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 1 h, washed with 70% ethanol and distilled water, and then stained with carmine alum staining solution (0.2% carmine, 0.5% aluminum potassium sulfate) overnight, washed again, and cleared with xylenes for visualization of the stained lobular-alveolar structure.

Histological sectioning and staining with hematoxylin/eosin were performed according to standard techniques. For immunostaining, sections were deparaffinized in xylenes, rehydrated through graded ethanol to phosphate-buffered saline (PBS), and permeabilized in 0.3% Triton X-100 in PBS. A standard heat antigen retrieval method was applied for antibodies against cytokeratin (CK) 14, CK18, cleaved caspase 3, and proliferating cell nuclear antigen (PCNA). Non-specific binding was blocked by 1.5% normal goat serum in PBS, and primary antibodies were applied at a 1:200 dilution in 0.1% bovine serum albumin in PBS overnight at 4°C. Sections were washed in PBS, and fluorescein- or Texas red-conjugated secondary antibody (Vector Laboratories) was applied at a 1:200 dilution in 1% normal goat serum for 1 h. Antibodies used were mouse SM α -actin antibody (clone 1A4; Sigma), rabbit cytokeratin 14 antibody (Zymed), mouse CD10 (anti-common acute lymphoblastic leukemia antigen [CALLA]) antibody (56C6; Labvision), rabbit cytokeratin 18 antibody (Santa Cruz), rabbit cleaved caspase 3 antibody (Cell Signaling), and mouse PCNA antibody (Santa Cruz).

TUNEL staining. A dead-end fluorometric terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) system was purchased from Promega (Madison, WI), and staining was performed according to the user's manual.

RNA in situ hybridization. In situ hybridization of paraffin sections was performed as described previously (52). Identical bright and dark field images were captured, and silver grains were pseudocolored red using Adobe Photoshop, after which images were superimposed.

Western blot analysis. Total protein was extracted from mammary gland tissue in radioimmunoprecipitation assay protein extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, pH 7.5) supplemented with protease and phosphatase inhibitors. The samples were homogenized and subsequently incubated on ice for 30 min and centrifuged at $9,500 \times g$ for 20 min at 4°C. Supernatants were transferred to a fresh tube, and protein concentration was measured with the bicinchoninic acid colorimetric assay (Bio-Rad). Samples (30 μ g/lane) were run on sodium dodecyl sulfate-polyacrylamide gels, blotted onto polyvinylidene difluoride membranes and incubated with blocking solution (5% milk in Tris-buffered saline with 0.1%

Tween 20) for 1 h. Membranes were incubated with primary antibodies (rabbit Stat3 antibody [Santa Cruz] and rabbit phospho-Stat3-Tyr705 antibody [Cell Signaling]) diluted in blocking solution overnight at 4°C, and specifically bound antibody was detected using horseradish peroxidase-conjugated secondary antibodies in conjunction with a chemiluminescent substrate (ECL; AP Biotech).

RESULTS

Generation of *MRTF-A* mutant mice. The mouse *MRTF-A* gene, located on chromosome 15, contains 14 exons distributed across ~37 kb of DNA. To introduce a loss-of-function mutation in the gene, we deleted a 1.7-kb region encompassing a portion of exon 9 and all of exon 10, which encode the basic, glutamine-rich, and SAP domains (Fig. 1A). The basic domain is required for the interaction of myocardin and MRTFs with SRF, and the SAP domain confers target gene specificity (33, 52, 54, 56). Deletion of these domains results in functional inactivation of MRTF-A. The deleted genomic region was replaced with a *lacZ* expression cassette fused in-frame with exon 9 and a neomycin resistance gene. The targeted *MRTF-A* locus was identified by Southern blot analysis of genomic DNA (Fig. 1B).

MRTF-A null offspring were produced at predicted Mendelian ratios from intercrosses of *MRTF-A* heterozygous mutant mice, indicating that *MRTF-A* is not required for embryonic or postnatal development. Heterozygous and homozygous *MRTF-A* mutant mice were viable and fertile, and intercrosses of null mice yielded normal-sized litters.

To confirm the gene-targeting event and determine whether the mutant allele might encode truncated *MRTF-A* transcripts, we performed RT-PCR with mRNA isolated from hearts of adult mice of the different genotypes, using primers representing exon sequences within and surrounding the deleted region of the gene (Fig. 1C and D). These assays confirmed that the targeted exons were deleted and also showed that exon 8 was spliced to exon 12, thereby deleting the *lacZ-neo* cassette (Fig. 1C). Sequencing of the RT-PCR product from the mutant allele showed that this aberrant splicing event caused an in-frame fusion such that the mutant transcript would create a truncated protein with residues 1 to 209 fused to residues 687 to 929 and lacking the SRF interaction domain. Without the SRF binding region, this truncated protein is nonfunctional and does not act as a dominant-negative mutant on SRF target genes (33, 52).

***MRTF-A* mutant females are unable to productively nurse their offspring.** Although *MRTF-A* null mice showed no obvious abnormalities, we noticed that the offspring of *MRTF-A* null females failed to thrive. The pups raised by *MRTF-A* null females showed growth retardation from postnatal day 4, and none survived beyond 20 days of age (Fig. 2A). The growth retardation of offspring of *MRTF-A* null females was independent of their genotype, suggesting an abnormality in the mutant mothers rather than the offspring. Indeed, wild-type pups fostered to *MRTF-A* null females also failed to thrive, whereas *MRTF-A* null pups fostered to wild-type mothers grew normally (Fig. 2B). *MRTF-A*-deficient females attended to their young and allowed them to suckle from the nipples, suggesting that they did not exhibit abnormal maternal nurturing behavior. These findings suggested that *MRTF-A* is required specifically for females to productively nurse their young. We observed the same phenotype with mutant mice in mixed

FIG. 1. Generation and analysis of *MRTF-A* knockout mice. (A) Gene targeting strategy. The mouse *MRTF-A* protein is schematized at the top. Amino acid positions are indicated, and functional domains are shown in color on the corresponding exons. The targeting vector, which contained a 2.4-kb 5' arm and a 5-kb 3' arm, replaced a 1.7-kb region of the gene with a *LacZ*-neo cassette. Positions of 5' and 3' probes are indicated. Positions of PCR primers used for genotyping are indicated at the bottom by horizontal arrows. WT, wild type; tk, thymidine kinase; NTD, N-terminal domain; Q, glutamine-rich domain; ++, basic region; LZ, leucine zipper; TAD, transactivation domain. (B) Southern analysis. Genomic DNA from ES cell clones was isolated from tail biopsies and analyzed by Southern blotting with 5' and 3' probes after digestion with *Hind*III. Mut, mutant. (C) Positions of primers used for RT-PCR. A schematic of the exons of the *MRTF-A* gene and positions of primers used for RT-PCR is shown. The expected mutation would contain the *LacZ*-neo cassette between exons 9 and 11. However, RT-PCR from mRNA isolated from heart tissue of mutant mice revealed that exon 8 was spliced to exon 12, as shown at the bottom. (D) RT-PCR was performed with RNA from heart tissue using primers shown in panel C. Genotypes of mice are shown at the top.

129SvEv/C57BL6 and isogenic 129SvEv backgrounds, indicating that genetic background did not affect the phenotype. **Abnormal mammary development in *MRTF-A* mutant mice.** Consistent with the notion that *MRTF-A* mutant females dis-

played a defect in nursing, the mammary glands dissected from mutant lactating females contained excessive milk and were pale compared to those of wild-type lactating females (Fig. 3A). To visualize the ductal and alveolar structures in the

FIG. 2. Growth retardation of neonates nursed by *MRTF-A*^{-/-} mothers. (A) Postnatal growth curves. Body weights of wild-type (WT) pups raised by wild-type ($n = 22$; 3 litters) and *MRTF-A* mutant ($n = 19$; 3 litters) mothers were determined on successive days after birth. Pups raised by *MRTF-A* mutant mothers failed to thrive. (B) Pups on day 5 (top) and day 10 (bottom) after birth are shown. The genotypes of the mothers and pups are shown. Wild-type and *MRTF-A* mutant pups thrive with wild-type mothers, whereas wild-type and *MRTF-A* mutant pups failed to thrive with *MRTF-A* mutant mothers.

mammary glands of females at different maternal stages, we performed whole-mount staining. In the mutant females, the large club-shaped terminal-end bud formed normally during puberty, and elongation and branching of the mammary tree showed no apparent abnormalities (Fig. 3B, a and b). During pregnancy, additional ductal branching occurred, and terminal alveoli formed in the mutants just as in the wild-type females (Fig. 3B, c and d). On day 1 after delivery, the mutant females

also completed ductal-alveolar development, and the mammary trees of wild-type and mutant females were indistinguishable (Fig. 3B, e to f). However, beginning at day 4 of lactation, the density of alveoli in mutant mammary glands became substantially reduced relative to that in the wild-type mammary gland (Fig. 3B, g to j). Large ducts, which were completely surrounded by extensive alveoli in the wild-type mammary gland, were often visible in the mutant (Fig. 3B, h). On day 12, the wild-type mammary gland was filled with alveoli, whereas there were spaces between the alveoli in the mutant mammary gland (Fig. 3B, j). Notably, at this stage, the alveoli of the mutant females appeared larger and less organized than those of the wild-type females (Fig. 3B, j). After weaning, the mammary gland undergoes a remodeling process, known as involution, which occurred in the same fashion in wild-type and mutant animals. Regression of the gland to the resting phase also occurred normally in the mutant (Fig. 3B, k to n).

Histological analysis confirmed normal ductal-alveolar development during the resting stage and pregnancy (Fig. 3C). In both wild-type and mutant 8-week-old virgins, the mammary gland was filled with fat tissue, and the ducts were lined by a single layer of epithelial cells surrounded by myoepithelial cells and dense stroma (Fig. 3C, a and b). During pregnancy, both ducts and alveoli were visible on the sections, and the epithelial cells began to secrete milk protein and lipid in the mutant and wild-type mammary glands (Fig. 3C, c and d). The overall appearance of ductal-alveolar structures of the mutant was normal on the first day after parturition (Fig. 3C, e and f). However, on lactation day 12, histological sections revealed that the mammary glands of wild-type lactating females were filled with alveoli and that their lumens were defined by highly organized thick alveolar walls. On the contrary, in the mutant lactating mammary glands, adipocytes were still present between alveoli, and the alveoli were dilated and had much thinner walls than in the wild-type glands (Fig. 3C, g and h). Milk was also trapped in the lumens of mutant mammary glands, indicated by purple protein staining and lipid droplets. After weaning of the pups, wild-type and mutant mammary glands undergo involution in a similar manner (Fig. 3C, i to l).

Myoepithelial cell defects in *MRTF-A* mutant mice. To pinpoint the cell type responsible for the nursing defects of *MRTF-A* mutant mothers, we examined markers of the different mammary cell types using RT-PCR analysis with RNA samples from mammary tissues of 8-week-old virgins and females at 14 days of pregnancy, 4 days of lactation, 12 days of lactation, and 4 days of involution. Transcripts encoding milk proteins (α -lactalbumin, β -casein, and whey acidic protein) (3, 41) were expressed normally at all stages in *MRTF-A* mutant mammary glands (Fig. 4A). The luminal epithelial cell-specific cytoskeletal protein cytokeratin 18 (50) was also expressed at a normal level in the mutants (Fig. 4A). Thus, luminal epithelial cell differentiation and function appeared unperturbed in the mutant mammary glands, and the nursing defect of *MRTF-A* mutant females is likely caused by defects of mammary myoepithelial cells and milk release.

Myoepithelial cells express both smooth muscle genes and certain epithelial genes. We first examined the expression of the myoepithelium-specific epithelial genes, *CK14* and *CALLA* (*CD10*), in the mammary gland (14, 50). The RT-PCR analysis of *CK14* and *CALLA* indicated that expression of these genes

FIG. 3. Mammary gland defects of *MRTF-A* mutant females. (A) Gross appearance of mammary glands at day 12 of lactation. The mammary gland from the mutant lactating female is pale compared to that of the wild-type lactating female. (B) Whole-mount staining of lobular-alveolar networks. (a to f) Normal mammary development in virgins, 14-day-pregnant, and 1-day-lactating females. (g to j) Lobular-alveolar structures of *MRTF-A* mutant females are underdeveloped during mid- and late lactation. Large tubular structures are still visible at day (d) 4 of lactation in the mutant (h, arrowhead). In the mutant mammary gland, at day 12 of lactation, the alveoli are enlarged compared to those of the wild type (j, arrow), and there is space among the alveoli (j, asterisk). (k to n) Normal appearance of *MRTF-A* mutant mammary gland undergoing involution. (C) Histological sections of mammary glands from different developmental stages. (a to f) Normal ductal-alveolar development of *MRTF-A* mutant females during resting and pregnancy and on day 1 of lactation. Black arrowheads, milk ducts; arrows, alveoli; blue arrowheads, milk droplets; asterisks, adipocytes. (g and h) During lactation, the alveolar lumen of *MRTF-A* mutant females are enlarged with thin walls (arrowheads), and milk protein and lipids are trapped in the alveoli (arrows). Fat tissue is present between alveoli (asterisk). (i to l) *MRTF-A* mutant mammary glands undergo normal involution after weaning of pups.

was reduced at the early lactating stage (L4) and almost abolished at the late lactating stage (L12).

Smooth muscle genes are up-regulated in the mammary glands of wild-type females during the pregnancy-lactation transition (19). There was a slight reduction in expression of smooth muscle genes in mammary tissue from *MRTF-A* mutant virgins and 14-day-pregnant mice compared to that from

wild-type females. However, we observed a pronounced loss of smooth muscle markers in the mammary myoepithelial cells of lactating *MRTF-A* mutant females (Fig. 4A). The dramatic down-regulation of smooth muscle gene expression in myoepithelial cells from mutant mammary gland was confirmed by in situ hybridization (Fig. 4B).

It is intriguing that the ablation of smooth muscle gene

FIG. 4. Analysis of transcripts for mammary gland cells. (A) RT-PCR analysis of mammary genes. MRTF-A transcript is absent in the mutant, while MRTF-B is constantly expressed during the mammary cycle in both wild-type and *MRTF-A* mutant females. Milk protein (α -lactalbumin, β -casein, and whey acidic protein [WAP]) genes are expressed at the same level in wild-type and mutant mammary glands during lactation. Expression of the secretory epithelium-specific CK18 gene is also unchanged in the mutant. Smooth muscle (SM α -actin, SM22, SM MHC, SM myosin light-chain kinase [MLCK], and SM caldesmon) genes are down-regulated specifically during lactation in the mutant. Other myoepithelium-specific (cytokeratin 14, CD10, and oxytocin receptor [OTR]) genes are down-regulated specifically during late lactation. The GAPDH gene is a loading control. wk, weeks; d, days. (B) Transcripts for the indicated smooth muscle genes were detected by in situ hybridization to sections of mammary gland from wild-type and *MRTF-A* mutant females at 12 days of lactation. Silver grains are pseudocolored red.

expression was restricted to the lactation phase, while the initial differentiation of mammary myoepithelial cells appeared normal in *MRTF-A* mutant virgins. *MRTF-A* and *MRTF-B* were expressed constantly during the mammary developmental cycle (Fig. 4A), while myocardin gene expression was not detected in mammary tissue. RT-PCR analysis with human mammary epithelial cell line Hs578T and myoepithelial cell line Hs578Bst showed that *MRTF-A* and *MRTF-B* are both expressed in these two cell lines (data not shown). Given the functional redundancy of these two transcription factors, it is possible that MRTF-B alone is able to initiate smooth muscle gene expression of mammary myoepithelial cells at the resting stage; however, the rapid proliferation and differentiation of myoepithelial cells during pregnancy and lactation require MRTF-A.

Abnormalities in myoepithelial cell differentiation in *MRTF-A* mutant mothers. To further examine the differentiation of myoepithelial cells, we performed immunohistochemistry using antibodies against myoepithelial protein SM α -actin and the epithelial protein cytokeratin 18 (Fig. 5A). CK18 antibody labels the inner layer of epithelial cells of the milk ducts and alveoli, and its expression is comparable between wild-type and *MRTF-A* mutant animals at all stages examined. SM α -actin-positive myoepithelial cells form a single layer around the ducts. SM α -actin was expressed at a comparable level in wild-type and *MRTF-A* mutant 8-week-old virgin females (Fig. 5A, a and b). However, during pregnancy, the wild-type myoepithelial cells around the mammary ducts showed a stronger and

thicker staining pattern with a stellate shape, while the mutant myoepithelial cells maintained a staining pattern similar to that of 8-week-old virgins (Fig. 5A, c and d). On day 1 of lactation, SM α -actin expression in the mutant appeared similar to that in the wild type (Fig. 5A, e and f). However, SM α -actin eventually decreased during the lactation process in the mutant (Fig. 5A, g to i), and strikingly, during late lactation, the mutant myoepithelial cells showed almost no SM α -actin expression, while the wild-type myoepithelial cells formed a discontinuous, basket-like single layer around the alveolar lumens (Fig. 5A, k and l). However, upon involution (4 days after weaning), the expression of SM α -actin in *MRTF-A* mutants returned to a level comparable to that of wild-type females (Fig. 5A, o and p). Immunohistochemistry with other smooth muscle proteins, such as SM calponin and SM-myosin heavy chain (MHC), showed similar expression patterns in *MRTF-A* mutant mammary glands (data not shown), which is consistent with the mRNA expression of these genes.

Lack of myoepithelial cells in *MRTF-A* mutant mammary gland at the late lactating stage. Immunostaining using antibody against CALLA (CD10) and CK14 confirmed that on day 12 of lactation, expression of CALLA and CK14 was abolished (Fig. 5, B and C). Double immunostaining with antibodies against SM α -actin and CK14 indicated that on day 4 of lactation, both of these proteins were still expressed in most of the myoepithelial cells of the *MRTF-A* mutant, although at a decreased level compared to that in the wild type. On day 10, the expression of these two genes were further reduced, while by

FIG. 5. Detection of smooth muscle markers in mammary glands of wild-type and *MRTF-A* mutant females. (A) SM α -actin (green) and cytokeratin 18 (red) were detected in histological sections of mammary glands from wild-type and *MRTF-A* mutant females at the indicated stages. Cytokeratin 18 is expressed normally at all stages in the mutant. (a and b) A similar pattern of SM α -actin-positive myoepithelial cells is seen in wild-type and *MRTF-A* mutant virgin females. (c and d) In pregnant wild-type females, smooth α -actin staining around milk ducts becomes thicker and stronger, while the mutant mammary glands maintain a thin, single-layered pattern (arrows). (e to l) SM α -actin expression eventually decreases during the process of lactation. While SM α -actin is expressed at a normal level on day 1, it is absent around the alveoli at day 12 in the mutant mammary gland (arrows). The vascular smooth muscle which strongly expresses SM α -actin is not affected (arrowheads). (m to p) SM α -actin expression recovers during involution in the mutant mammary gland, and it is similar to that of the wild type 4 days after weaning of the pups. Blue indicates 4',6'-diamidino-2-phenylindole (DAPI) staining of the nuclei. (B) Immunostaining with antibodies against SM α -actin and cytokeratin 14 during lactation. Myoepithelial cells eventually lose expression of these proteins. At day 12 of lactation, expression of both proteins is abolished, and cell numbers around alveoli are decreased in *MRTF-A* mutant mammary gland. During involution, the expression of these two proteins recovers. (C) Expression of CD10, which is also specifically expressed in myoepithelial cells, is diminished during the late lactating stage in the *MRTF-A* mutant. wk, weeks; d, days.

day 12, expression of SM α -actin and CK14 was no longer detectable (Fig. 5B). Noticeably, at day 4 of lactation, the cell number around the lumens of the alveoli in mutant mammary glands was similar to that of the wild type, and typically there were three layers of cells, comprising two layers of epithelial cells separated by one layer of myoepithelial cells. In contrast, at day 12 of lactation, the number of cells surrounding the lumens was greatly reduced in the mutant mammary tree, and the walls between adjacent alveolar lumens were composed of only two layers of cells, which resulted in a thin appearance of these walls (Fig. 5C).

All these results implied that the myoepithelial cells were

ablated during the late lactating stages in *MRTF-A* mutant females. TUNEL assays, which specifically label the DNA of apoptotic cells, showed no increase in apoptosis in the mutant mammary gland at the resting or pregnant stages or at days 2, 4, 7, and 12 of lactation, while massive apoptosis was detected in the mutant mammary gland at day 10 of lactation. The mutant mammary gland showed an apoptotic rate during involution that was similar to that of the wild type (Fig. 6A and B).

To determine which cell population died during late lactation in the mutant mammary tissue, we performed double immunofluorescence with antibodies against cleaved caspase 3,

FIG. 6. Myoepithelial cell death, premature involution during lactation, and increased myoepithelial proliferation during involution in *MRTF-A* mutant mammary gland. (A) Excessive apoptotic cell death in *MRTF-A* mutant mammary gland at lactating day 10, detected by TUNEL. Red, TUNEL labeling of apoptotic cells; blue, DAPI staining of nuclei. (B) Quantification of apoptosis in wild-type and *MRTF-A* mutant mammary glands during lactation and involution. (C) Double immunofluorescence with antibodies against SM α -actin (red) and cleaved caspase 3 (green). (a and b) SM α -actin and cleaved caspase 3 double-positive cells (arrows) account for over 50% of the apoptotic cells in *MRTF-A* mutant mammary glands at day 10 of lactation. (c and d) Most apoptotic cells are SM α -actin-negative epithelial cells in both wild-type and mutant involuting mammary

TABLE 1. Quantification of apoptotic and proliferative cells during lactation and involution

Mouse phenotype	% of SM α -actin-positive cells among apoptotic cells at:		Overall proliferation rate at day 4 of involution (%)	% of SM α -actin-positive cells among all proliferative cells at day 4 of involution
	Day 10 of lactation	Day 4 of involution		
<i>MRTF-A</i> ^{+/+}	ND ^a	8.9 $\pm$ 0.9	7.5 $\pm$ 1.3	3.1 $\pm$ 0.6
<i>MRTF-A</i> ^{-/-}	50.4 $\pm$ 1.7	9.8 $\pm$ 0.9	8.2 $\pm$ 1.6	12.7 $\pm$ 1.0

^a ND, not determined.

which labels apoptotic cells, and SM α -actin. About 50% of the cells that underwent apoptosis at day 10 of lactation in the mutant were SM α -actin positive, while in the involuting mammary glands of both wild-type and mutant animals, less than 10% of apoptotic cells were SM α -actin positive (Fig. 6C; Table 1). These data suggest that numerous myoepithelial cells die during late lactation and that due to severe milk accumulation, the mutant mammary gland undergoes involution to some extent and a number of epithelial cells also die. We speculate that the residual smooth muscle proteins present during early lactation were adequate to maintain a low level of milk ejection that allowed the survival of offspring of the *MRTF-A* mutant females. However, during the late phase of lactation, when the myoepithelial cells were almost completely ablated in the mutant mammary tree, milk could not be ejected and the milk stasis induced premature involution, resulting in starvation of the offspring.

Up-regulation of certain involution-related genes during lactation by milk stasis in *MRTF-A* mutant mammary gland. It is well known that milk stasis, together with the decline in lactogenic hormones, leads to mammary involution, which is characterized by cessation of milk synthesis, apoptosis of epithelial cells, collapse of alveoli, and eventual remodeling to a morphological state similar to that of the adult virgin. It has been demonstrated by teat-sealing experiments that local signals generated by accumulated milk are sufficient to induce apoptosis and down-regulation of milk synthesis; however, maintenance of systemic lactogenic hormones by suckling prevents such sealed mammary glands from undergoing complete regression (24). In lactating *MRTF-A* mutant mammary glands, milk could not be released efficiently and accumulated within the alveolar lumens due to the myoepithelial cell defects. Although milk accumulation, dilation of alveoli, excessive fat tissue, and a transient burst of apoptosis were observed during lactation in *MRTF-A* mutant mammary glands, the integrity of the lobuloalveolar architecture was preserved, and milk synthesis appeared normal throughout lactation as revealed by RT-PCR analysis of the major milk protein genes. It is unlikely

that premature involution was the primary defect of losing *MRTF-A* and caused reduction of myoepithelial gene expression, since myoepithelial genes are expressed normally during involution in wild-type mammary tissue (Fig. 5A, m and o).

Involution is also associated with characteristic changes in expression of a battery of genes, especially those related to cell death and immune responses, as shown recently by systematic microarray analysis (8, 47). To further examine whether involution-related genes were induced by milk stasis in *MRTF-A* mutant mammary glands, we analyzed the expression of a series of genes that have been shown to be involved in involution by semiquantitative RT-PCR (Fig. 6D). Signal transducer and activator of transcription 3 (STAT3) is a key regulator of mammary involution involved in both programmed cell death and the acute phase response (6, 17). STAT3 expression was comparable in wild-type and *MRTF-A* mutant mammary glands at both the mRNA and protein levels during different stages of the mammary cycle. It is known that STAT3 is highly phosphorylated upon involution. The phosphorylation status of Stat3 was examined by Western blotting using an antibody against phosphorylated STAT3, which showed that indeed Stat3 is hyper-phosphorylated in the mutant mammary gland during late lactation (Fig. 6E). The CEBP δ gene, a target of STAT3, is also up-regulated during lactation. Several other involution-related genes were up-regulated during lactation in the mutant mammary tissue. These genes include monocyte differentiation antigen CD14, serum amyloid A2, and the neutrophilic granulocyte marker LRG1 (leucine-rich alpha 2-glycoprotein 1), which are all involved in immune responses; IGFBP5 (insulin-like growth factor binding protein 5), which is important for the induction of apoptosis; and tyro3 (protein-tyrosine kinase 3), whose function in mammary involution has not been fully defined. Interestingly, the gene encoding lipopolysaccharide binding protein, which is involved in the acute phase response and also a target of STAT3 (47), was expressed at a normal level in *MRTF-A* mutants. These data indicate that milk stasis induces immune responses and up-regulation of certain involution genes in *MRTF-A* mutant mammary gland, while the hormonal level, which is sustained by continued suckling of the pups, controls milk secretion and the expression of other involution-related genes.

It is interesting that myoepithelial cell gene expression returned to a relatively normal level a few days after weaning of the pups. To examine whether more myoepithelial cells were generated by excessive proliferation, double immunostaining with antibodies against PCNA and SM α -actin was performed. Proliferation rates were not disturbed during pregnancy and lactation in the mutant mammary gland (data not shown). However, during involution, the overall proliferation rate was slightly higher in the mutant, and more importantly, there was

glands (arrows). Blue indicates DAPI staining of nuclei. (D) RT-PCR analysis of involution-related genes. GAPDH is the loading control. LBP, lipopolysaccharide binding protein. (E) Western blot analysis of STAT3 expression and phosphorylation. STAT3 is expressed at comparable levels in the wild type and the mutant. STAT3 is hyperphosphorylated (P-STAT3) in the mutant from midlactation, and its phosphorylation levels are comparable in the wild type and the mutant during involution. (F) Double immunofluorescence with antibodies against SM α -actin (red) and PCNA (green), a proliferative cell marker. (a and b) There are more proliferating myoepithelial cells (SM α -actin and PCNA double-positive cells) in *MRTF-A* mutant mammary tissue during involution (arrows). (c and d) DAPI staining of the corresponding sections of panels a and b. d, days; wk, weeks.

a significant number of proliferating cells that were SM α -actin positive in the mutant, suggesting that new myoepithelial cells may be generated from progenitor cells after weaning (Fig. 6F; Table 1).

DISCUSSION

The results of this study reveal an essential role for MRTF-A in the differentiation of mammary myoepithelial cells, a specialized smooth muscle-like cell type required for milk ejection from the mammary gland. As a consequence of the failure in myoepithelial cell differentiation, *MRTF-A* mutant mothers are unable to productively nurse their offspring. These findings reveal a commonality in the molecular mechanisms that control differentiation of smooth muscle and myoepithelial cells, both of which depend on activation of contractile protein genes by members of the myocardin family of SRF coactivators.

Myoepithelial development. Myoepithelial cells are found within the secretory and ductal portions of most glands. Their contractile function, which is controlled by hormonal and neural signals, is essential for ductal secretion. Myoepithelial cells also transport metabolites to secretory cells and provide structural integrity to glandular tissues through their association with the basement membrane. The ultrastructure, gene expression pattern, and contractile properties of myoepithelial cells are strikingly similar to those of SMCs. However, in contrast to SMCs, which are derived from mesodermal precursors and neural crest cells, myoepithelial cells of the mammary gland are derived from ectoderm. To our knowledge, the molecular mechanisms responsible for myoepithelial cell differentiation have not been previously defined.

The mammary ductal tree forms normally in *MRTF-A* mutant mice, but myoepithelial cells fail to differentiate upon lactation, as shown by the lack of expression of smooth muscle contractile protein genes such as those encoding SM α -actin, SM MHC, and SM caldesmon during lactation. As a result, mutant mothers are unable to release their milk. Oxytocin, a neurohypophyseal hormone essential for stimulating myoepithelial cell contraction and milk ejection, is expressed normally in *MRTF-A* mutants (data not shown). The oxytocin receptor is exclusively expressed in myoepithelial cells within the mammary gland (12, 13). Expression of oxytocin receptor transcripts is unchanged in the mutant mammary gland during early lactation (day 4), when the myoepithelium-specific smooth muscle proteins and cytokeratins are already down-regulated. At lactating day 12, oxytocin receptor expression is diminished since the myoepithelial cells are lost due to excessive apoptosis. Thus, it is unlikely that defective oxytocin circulation or signaling contributes to the nursing deficiency of *MRTF-A* mutant females.

It is curious that the phenotype of *MRTF-A* mutant mice is so restricted to myoepithelial cells of the mammary gland. Myoepithelial cells are also associated with salivary and lacrimal glands, but we did not detect abnormalities in these glandular tissues, raising the possibility that other members of the myocardin family may substitute for MRTF-A function in those tissues. In this regard, it is interesting to note that *MRTF-B* is expressed at normal levels in mammary glands from *MRTF-A* mutant females. *MRTF-A* and *-B* are also expressed in the mammary epithelial and myoepithelial cell lines

Hs578T and Hs578Bst, respectively. This could suggest that MRTF-A is uniquely required for differentiation of mammary myoepithelial cells or that the loss of MRTF-A reduces the level of myocardin family members below a critical threshold required for myoepithelial cell differentiation. It is also interesting that the myoepithelial cell defects of *MRTF-A* mutant mice are restricted to pregnancy and lactating stages, while smooth muscle gene expression is normal in the resting mammary gland. It is possible that during the resting stage, MRTF-B is able to take the place of MRTF-A and sustain the smooth muscle program, while during pregnancy and lactation, when extensive proliferation and differentiation of myoepithelial cells is necessary, MRTF-B cannot compensate for the loss of MRTF-A. It will also be interesting to examine whether transcriptional activities of MRTF-A and *-B* are differentially regulated by female hormones during mammary development.

MRTF-A is required for maintenance of differentiation and survival of mammary myoepithelial cells. In the lactating mammary glands of *MRTF-A* mutant females, not only the smooth muscle genes but also other myoepithelial cell-specific genes, such as the cytokeratin 14 and CALLA genes, were down-regulated. There are no conserved SRF binding sites within 40 kb upstream of the transcription initiation sites of these genes, and there have been no reports that expression of these genes might be under the control of SRF. Since smooth muscle structural proteins and cytokeratins together compose the cytoskeletal structure of these cells, we speculate that loss of smooth muscle proteins leads to the dispensability of cytokeratins and in turn to degradation of these proteins or repression of their expression, although we cannot exclude the possibility that MRTF-A has partners other than SRF that control the expression of these myoepithelium-specific genes. There was a dramatic increase of apoptotic cells specifically at lactating day 10; at day 12 the alveolar walls were much thinner and composed of fewer cells, although the overall alveolar structures were still maintained. Notably, the decrease in expression of smooth muscle and myoepithelium-specific genes preceded apoptotic cell death. Thus, the impaired function of myoepithelial cells resulting from a failure in expression of smooth muscle and myoepithelium-specific genes appears to result in programmed cell death of myoepithelial cells.

It has been previously reported that failure of milk ejection promotes mammary involution (39, 51). In mice in which milk removal is disrupted by teat sealing, milk synthesis declines, and programmed cell death can proceed without disruption of the alveolar structure, although gene expression profiling has not been performed with these mice (24). Milk trapped within the alveoli was observed in *MRTF-A* mutant mammary glands from early lactation. However, excessive apoptosis was not detected until day 10 of lactation, and unlike in normal involution, myoepithelial cells account for a large fraction of the apoptotic cells. Moreover, the lobular-alveolar structure of the mutant mammary gland maintained the lactating appearance throughout lactation, and milk protein expression was sustained at a normal level. The reason mutant epithelial cells still produced milk and maintained their integrity might be that trace amounts of smooth muscle contractile proteins were still able to release a small amount of milk, as suggested by the survival of pups until late lactation, and that the stress of milk accumulation was not as severe as that resulting from weaning

or sealing the teats. However, the stress was able to induce an immune response, and at the level of gene transcription, some, but not all, involution-related genes were up-regulated. We cannot exclude the possibility that MRTF-A has an anti-involution or anti-inflammatory effect; however, given the clear myoepithelial defects and milk accumulation we observed, it is highly likely that the up-regulation of the involution genes is secondary to milk stasis.

Regulation of cell migration and cytoskeletal development by the myocardin family and SRF. We showed previously that myocardin gene null mice die at E10.5 from an apparent lack of differentiated SMCs (26). However, the interpretation of this mutant phenotype was complicated by the fact that the *myocardin* gene is expressed in only a small subset of SMCs at this stage of development. In addition, *myocardin* gene mutant embryos displayed abnormalities in yolk sac development, making it difficult to distinguish whether the effects of myocardin gene deletion on embryonic vascular development are primary or secondary to yolk sac abnormalities.

A null mutation in the *MRTF-B* gene also results in embryonic lethality at ~E12.5 due to a spectrum of cardiovascular defects (38). Thus, each member of the myocardin gene family is required for the activation of smooth muscle gene expression, but each is uniquely required in a different cell type at a different developmental stage. It will eventually be interesting to generate mice with different combinations of mutations of the myocardin family genes in order to determine whether there are cell types in which they are functionally redundant and whether there might be alternative pathways leading to smooth muscle gene expression in a subset of cell types.

MRTF-A has been shown to mediate the effects of Rho signaling and changes in the actin cytoskeleton on SRF-dependent transcription (21, 33). Mice overexpressing a dominant-negative form of MRTF-A in skeletal muscle showed skeletal myopathy and hypoplasia (25). Similarly, a dominant-negative mutant of MRTF-B/MKL2 inhibits differentiation of skeletal muscle cells in vitro (44). Remarkably, however, *MRTF-A* mutant mice display no obvious abnormalities in skeletal, cardiac, or smooth muscle, presumably due to the redundancy between MRTF-A and MRTF-B.

Implications. In addition to their role in milk secretion, myoepithelial cells have been suggested to possess tumor suppression activities (1, 22, 48, 49). Myoepithelial cells produce anti-invasive protease inhibitors and antiangiogenic molecules, such as the protease nexin II, α 1-antitrypsin, tissue inhibitor of metalloproteinase 1, thrombospondin 1, and soluble basic fibroblast growth factor receptor (35, 45, 46). Thus, myoepithelial cells can induce growth arrest and apoptosis of breast carcinoma cells by interfering with the invasive behavior of tumor cells and inhibiting angiogenesis. Given the roles of SRF and MRTFs in controlling expression of growth responsive genes, such as *c-fos* and *egr-1* (5, 37), it will be of interest to determine whether MRTF-A plays a role in breast cancer.

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