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Smooth muscle α actin is specifically required for the maintenance of lactation

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ABSTRACT

Smooth muscle α-actin (*Acta2*) is one of six highly conserved mammalian actin isoforms that appear to exhibit functional redundancy. Nonetheless, we have postulated a specific functional role for the smooth muscle specific isoform. Here, we show that *Acta2* deficient mice have a remarkable mammary phenotype such that dams lacking *Acta2* are unable to nurse their offspring effectively. The phenotype was rescued in cross fostering experiments with wild type mice, excluding a developmental defect in *Acta2* null pups. The mechanism for the underlying phenotype is due to myoepithelial dysfunction postpartum resulting in precocious involution. Further, we demonstrate a specific defect in myoepithelial cell contractility in *Acta2* null mammary glands, despite normal expression of cytoplasmic actins. We conclude that *Acta2* specifically mediates myoepithelial cell contraction during lactation and that this actin isoform therefore exhibits functional specificity.

Introduction

Actin is a highly abundant structural protein of the cytoskeleton found in eukaryotic cells, which serves critical roles in cell motility and muscle contraction. At least six actin isoforms have been identified in eukaryotic cells (Vandekerckhove and Weber, 1978), which are products of six different genes (Herman, 1993; Miwa et al., 1991; Rubenstein, 1990). Although the isoactins are expressed in spatial, temporal, and tissue specific patterns, they exhibit remarkable amino acid identity. Two "nonmuscle" actins, cytoplasmic β and γ-actins, are thought to be present in all cells. The other four actin isoforms, which include skeletal, cardiac, vascular, and enteric actins, are typically found in specific adult muscle types, although they are also present in specialized cell types. The muscle specific actins differ from the nonmuscle cytoplasmic actins at less than 10% of the total 375 amino acid residues that make up the primary sequence, while the muscle specific isoforms differ from each by only a few residues (McHugh et al., 1991; Miwa et al., 1991).

The remarkable conservation of actin isoforms suggests that actin isoforms are functionally similar. Several cell-based experiments support this conclusion (Gunning et al., 1984; Rubenstein and Spudich, 1977; von Arx et al., 1995). For example, when the cytoplasmic β-actin isoform was over expressed in C2 myoblasts, it caused an increase in cellular surface area while over expression of γ-isoform caused cells to roundup (Lloyd et al., 1992). Myogenic cells have been shown to regulate the expression of specific actin isoforms in a manner that alters the expression of cytoplasmic actins to muscle isoforms. Quiescent aortic smooth muscle cells express a significant amount of *Acta2*. However, when these cultured cells begin to proliferate and migrate, they increase the expression of cytoplasmic actins, which suggests a level of functional specificity (Barja et al., 1986). Considerable debate exists about the conclusiveness of such cell culture-based studies and raises questions about the function of actin isoforms (Bulinski, 2006).

Phenotypic analysis of actin isoform deficient mice provides some evidence supporting the concept that actin isoforms mediate specific functions. Mice deficient in skeletal muscle α-actin (*Acta1*) died early in neonatal development due to defects in skeletal muscle growth and/or function (Crawford et al., 2002). Deletion of cardiac muscle α-actin (*Actc1*) led to defects in myocardial contractility, sarcomeric organization, and an apparent compensatory increase in *Acta1* mRNA as well as *Acta2* protein levels in the hearts of null mice (Kumar et al., 1997). A hypomorphic allele in cytoplasmic β-actin (*Actb*) proved to be embryonic lethal prior to the beginning of muscle development (Shawlot et al., 1998). A conditional allele for cytoplasmic γ-actin (*Actg1*) in which this actin was deleted only from muscle tissue was characterized by a progressive myopathy (Sonnemann et al., 2006) without an apparent change in isoactin expression. *Thus, all actin isoform mouse knockouts, except smooth muscle γ-actin (Actg2), have been reported and have yielded data suggesting some element of isoform specificity, as well as overlapping cellular functions* (Perrin and Ervasti, 2010).

Here, we have hypothesized *Acta2*, while exhibiting a high degree of identity to the other actin isoforms, exhibits functional specificity. We further postulated that such functional specificity was likely to be observed in a tissue specific fashion and/or under stress conditions. Given *Acta2*'s implied role in myofibroblast contraction (Rockey et al., 1993) and the predominance of this isoform in

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myoepithelial cells (Skalli et al., 1986), we propose that *Acta2* serves an essential function in myoepithelial contraction and the maintenance of lactation. We discovered that *Acta2* deficient (−/−) dams failed to nurse their pups effectively and that the mechanism underlying this phenotype is linked to hypocontractility of myoepithelial cells due to a lack of *Acta2*.

Materials and methods

Antibodies

Primary antibodies used were as follows: anti-Acta2 Cy3 (1A4; Part# C6198; Sigma, St Louis, MO), anti-keratin 14 (Part# PRB-155P; Covance, Berkeley, CA), anti-troma1 or anti-keratin 8 (Part# TROMA-1; University of Iowa Hybridoma Bank), anti-vimentin (Part# AB 5733; Chemicon, Temecula, CA), anti-GFAP (6F2; Part# MO761; Dako, Carpinteria, CA), anti-SM22α (Polyclonal antibody # 1387-4; a gift from Dr. M. Parmacek), anti-total actin (C4; Part# MAB1501; Chemicon), anti-β-actin ((*Actb*); AC-15; Part# A1978; Sigma), anti- smooth muscle α-actin ((*Acta2*); 1A4; Part# A5228; Sigma), anti-smooth muscle γ-actin ((*Actg2*); B4;(Lessard, 1988)), anti-cy*topla*smic γ-actin ((*Actg1*); monoclonal- (Dugina et al., 2009); polyclonal- (Hanft et al., 2006)), anti-cardiac and skeletal α-actin ((*Actc1 and Acta1*); 5C5; Part# A2172; Sigma), anti-muscle actins (HUC 1-1; Part# 691391; MP Biomedicals, Solon, OH), anti-myosin sarcomere (MF20; Part# MF20; University of Iowa Hybridoma Bank), anti-myosin cytoplasmic non-muscle (CMII 23; Part# CMII 23; University of Iowa Hybridoma Bank), anti-myosin smooth muscle (Part# BT-562; Biomedical Technologies, Stoughton, MA) and anti-GAPDH (1D4; Part# MMS-580S; Covance, Princeton, NJ). Secondary antibodies used were as follows: Donkey anti-mouse Cy2, Donkey anti rat Cy5, Donkey anti mouse Cy2 (Molecular Probes, Carlsbad, CA), and Donkey anti mouse IgG HRP (Jackson Immunoresearch; West Grove, PA). Milk antibodies: anti-rat β casein antibody (a gift from Dr. C. Kaetzel) and anti-mouse specific milk proteins (anti α-casein, β-casein and WAP; Part# YNRMTM; Accurate Chemical, Westbury, NY). Nuclear stains included BOPRO (Molecular Probes) and DAPI (Vector Labs, Burlingame, CA).

Mice husbandry, maternal care, and genotyping

The targeted disruption of the *Acta2* gene by homologous recombination has been previously described (Schildmeyer et al., 2000). Using a PCR screen, the genotype of litters from heterozygous matings revealed normal Mendelian ratios, as previously reported (Schildmeyer et al., 2000).

In rodents, maternal behavior involves a complex set of activities, which include nest building and repair, sniffing of pups, pup retrieval, licking, grooming as well as blanket nursing. Indeed, several genes have been shown to be essential for maternal behavior. We carefully monitored *Acta2*−/− dams for a full range of maternal behavior as previously described (Yeo and Keverne, 1986) and found that these mice exhibited entirely normal mother–pup interactions.

Acta2−/− mice were maintained on an out bred (BalbC/129/SvJ) genetic background. *Acta2* mice were backcrossed to a BalbC strain. All animal care and experiments were carried out in accordance with NIH guidelines and approved by the local Animal Care and Use Committee. All mice were housed in cages with filter top cages and subjected to 12L:12D cycles. For the purposes of breeding and time pregnancies, 10–14 week old females were placed with stud males, checked daily for copulation plugs, and weighted during gestation in order to determine the timing of pregnancy. If a copulation plug was observed, the female was housed individually and given nesting materials. The presence of a copulation plug was designated as day 0.5 of pregnancy. *Acta2*+/+ females (BalbC/129/SvJ and BalbC) birthed between 20 and 21 days after the observation of the copulation plug. However, 10% of *Acta2*−/− pregnant females (BalbC/129/SvJ and BalbC) exhibited a failure or delay of parturition (by about 5 days). Thus, monitoring the presence of copulation plugs and recording weights of pregnant females were important to determine the correct timing of mammary gland development.

Cross fostering and administration of oxytocin

Acta2+/+ and −/− dams that delivered a litter within 24 h of each other were used in cross-fostering experiments. *Acta2*+/+ dams were mated with *Acta2*+/+ males and *Acta2*−/− dams were mated with *Acta2*−/− males. The litters born to *Acta2*−/− and +/+ dams were swapped as described (Chen and Capecchi, 1999). In some experiments, synthetic oxytocin (Sigma, St. Louis, MO) was injected into the appropriate mice twice daily 24 h postpartum until weaning or an experimental endpoint. Administration of daily oxytocin rescued several milk letdown phenotypes such as that observed in *mf3* null mice (Labosky et al., 1997) and oxytocin (*oxt*) null mice (Nishimori et al., 1996).

Serum total protein and albumin measurements

Blood chemistry measurements were done as described (Kwon et al., 2001) with several modifications. Blood from pups (n = 10–12) of three litters for each indicated measurement were collected and placed in heparinized coated tubes. Serum fraction was isolated from the clotted blood by centrifugation at 4 °C and frozen at −80 °C. The levels of total protein and albumin were determined by University of Texas Southwestern Mouse Metabolic Phenotyping Core using a Vitros 950 Chemistry Analyzer (Johnson and Johnson, Rochester, NY).

Histology and immunohistochemistry

Mammary glands were dissected and embedded in paraffin as described (Seagroves et al., 1998). Immunohistochemistry was performed on formalin fixed specimens, subjected to an antigen retrieval step as previously described (Katoh et al., 1997). Sections were incubated with primary antibody for 3 h at room temperature, washed in PBS for 20 min, and incubated with fluorescent labeled secondary antibody diluted in PBS for 1 h. Sections were washed and mounted (Gel/Mount™ BiØmedia, Fisher Scientific, Ashville, NC). Myoepithelial cells were fixed in a 50:50 mixture of acetone and methanol, incubated with antibodies, and immunofluorescent signals were detected using a confocal microscope (LSM 510; Carl Zeiss, Inc, Thornwood, NY). Images were imported and arranged in Adobe Illustrator CS2 (Adobe, San Jose, CA).

Analysis of alveolar diameters

Whole-mount preparation was done as previously described (Robinson et al., 1998). The diameter of the duct lumen was measured using a calibrated eyepiece reticule as described (Van Nguyen and Pollard, 2002) in sections from lactation time points. Twenty blinded measurements were taken for each time point in 5 different mammary glands.

TUNEL assay

Mammary glands were fixed and sectioned at specific time points and analyzed for apoptosis by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) as described (Tepera et al., 2003). The TUNEL terminal transferase was purchased from (Roche, Mannheim, Germany) and utilized per instructions. For each time point, 2000–4000 total cells were counted by identification of DAPI stained nuclei. At least three mammary glands, including