J. Dairy Sci. 99:1–9
http://dx.doi.org/10.3168/jds.2016-11550

Impaired mammary development in tamoxifen-treated prepubertal heifers is associated with altered development and morphology of myoepithelial cells[1]

H. L. M. Tucker,* K. L. Beaudry,* C. L. M. Parsons,* S. E. Ellis,† and R. M. Akers*[2]
*Department of Dairy Sciences, Virginia Polytechnic Institute and State University, Blacksburg 24061
†Biological Sciences Directorate, National Science Foundation, 4201 Wilson Boulevard, Arlington, VA 22230

ABSTRACT

Prepubertal mammary development involves elongation and branching of ducts and stromal tissue remodeling. This process is closely linked with ovarian and pituitary hormones, growth factors, and local regulators. Accumulating evidence suggests that the myoepithelial cells also play a role in ductal development in addition to their well-recognized importance in the milk ejection reflex. Following reports that myoepithelial cells changed in correspondence with decreased mammary growth after ovariectomy of prepubertal heifers, we evaluated myoepithelial cells in mammary tissue collected from prepubertal heifers treated with the antiestrogen tamoxifen. Briefly, heifers were given placebo (n = 7) or tamoxifen (n = 8; 0.3 mg/kg per day) beginning on d 28 of life until the animals were euthanized on d 120. Tissues were collected from each of 3 zones (near the gland cistern, midway between the gland cistern and mammary fat pad, and at the interface of the parenchyma and mammary fat pad). Samples were processed to measure expression of transformation-related protein 63 (p63), smooth muscle actin, and common acute lymphoblastic leukemia antigen. We found that smooth muscle actin and common acute lymphoblastic leukemia antigen were expressed in the cytoplasm and p63 in the nuclei of myoepithelial cells. In concert with a 50% impairment in mammary growth after tamoxifen, we found that the number of myoepithelial cells around developing mammary ducts was reduced. But the average intensity of p63 expression per nucleus was not affected. We used the very distinct and exclusive staining of p63 in myoepithelial cell nuclei to capture hundreds of nuclear images for subsequent analysis using CellProfiler software. From this image analysis, we found that the area of myoepithelial cell nuclei and perimeter distances were reduced by tamoxifen. When nuclei were classified based on nuclear shape (eccentricity), we found differences in area, perimeter, and patterns of p63 expression based on Zernike number evaluations as well as treatment differences within each shape classification. These data provide support to the concept that myoepithelial cells are also the involved in mammary development in the prepubertal bovine mammary gland and that use of multispectral imaging combined with image analysis software can provide quantitative data to better understand the complex cellular interactions that ultimately regulate mammary morphogenesis in the bovine.

Key words: CellProfiler, bovine mammary, myoepithelial, tamoxifen

INTRODUCTION

Most prior studies of early mammary development have focused on ductal development, and specifically on the mammary epithelial cells. However, myoepithelial cells are also present in the developing mammary gland. Whereas several studies have evaluated the ontogeny of myoepithelial cells in the developing murine mammary gland (Deugnier et al., 1995; Gudjonsson et al., 2001, 2006) and suggested that these cells play an important role in ductal growth and morphogenesis (Sopel, 2010), data specific to dairy ruminants are rare and frequently it is assumed that the basal-oriented cells of the epithelium are primarily myoepithelial cells (Ballagh et al., 2008; Safayi et al., 2012). A more recent rodent study (Forster et al., 2014) provides compelling direct evidence to support the significance of myoepithelial cells in mammary development. Those authors used mice in which transformation-related protein 63 (**p63**) expression was knocked out in mammary tissue and demonstrated a complete failure to develop the secretory epithelium. This is relevant because, as we demonstrate in the current report, p63 is strongly and exclusively expressed only in the nuclei of the myoepi-

Received June 2, 2016.
Accepted August 18, 2016.
[1]The views expressed in this article do not necessarily reflect those of the National Science Foundation or the United States Government.
[2]Corresponding author: rma@vt.edu

thelial or basal cells in the developing bovine mammary gland. We (Ballagh et al., 2008; Safayi et al., 2012) previously used quantitative multispectral imaging and showed that early ovariectomy of prepubertal heifers (also known to suppress mammary growth) altered the expression of both smooth muscle actin (**SMA**) and common acute lymphoblastic leukemia antigen (**CD10**) in the basal or presumptive myoepithelial cells. Indeed, CD10 and SMA are excellent cytoplasmic markers for myoepithelial cells. In these studies, we noted that mammary ductal cell stratification and morphogenesis was also altered by ovariectomy. In ovariectomized heifers, myoepithelial cells appeared smaller, irregularly shaped, and generally flattened. In ovariectomized heifers, $CD10^+$ myoepithelial cells were also observed adjacent to the ductal lumen (rare in ovary intact animals). We have subsequently noted that p63 is co-expressed in the nuclei of bovine myoepithelial cells expressing CD10 and SMA. Indeed, nuclear expression of p63 provides an especially good marker for identification of myoepithelial cells.

We have recently discovered that heifers treated with antiestrogen tamoxifen have significantly reduced (50%) mammary development and experienced dramatic alterations in the expression of estrogen and progesterone receptors by the mammary epithelial cells (Tucker et al., 2016). These responses are similar but not identical to those occurring after ovariectomy of prepubertal heifers. For example, Velayudhan et al. (2015) reported the complete loss of progesterone receptor expression after ovariectomy. But this was not true after treatment with tamoxifen (Tucker et al., 2016). Marked alterations in the development of the reproductive tract tissues also occurred in these heifers (Al Naib et al., 2016), further indicating the efficacy of tamoxifen as an antiestrogen in the prepubertal heifer. Because of the increasing evidence that the myoepithelial cells are important players in control of mammary ductal elongation and mammogenesis, we used tissues collected from tamoxifen-treated heifers to determine if the differentials in mammary growth attributed to tamoxifen treatment were associated with changes in the number and distribution of myoepithelial cells in developing prepubertal bovine mammary gland. We also used the distinct, focused expression of p63 in myoepithelial cell nuclei to quantitatively evaluate the morphology of myoepithelial epithelial cell nuclei using images collected via multispectral imaging and the powerful software available as part of the CellProfiler analysis system (Carpenter et al., 2006; Kamentsky, et al., 2011; Bray et al., 2016). Our data support the idea that the organization and development of myoepithelial cells are altered in correspondence with mammary ductal development in heifers and that the myoepithelial cells likely play a role in the regulation of ductal development in the bovine.

MATERIALS AND METHODS

Animals

All experimental procedures were conducted under the review and approval of the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (11–208 DASC). Sixteen female heifer Holsteins were housed individually in calf hutches and randomly assigned to 1 of 2 groups: control (**CON**; n = **7**) or tamoxifen (**TAM**; n = 8). All calves were fed milk replacer twice daily and were weaned at 8 wk of age. One CON calf died during the trial. The data from this animal was omitted before statistical analysis. Calves were managed and reared following standard practices at the Virginia Tech Dairy Center.

Injection Management

Heifers were enrolled at 28 d of age. Daily subcutaneous injections were given over the scapula until 120 d of age. Tamoxifen-treated heifers were given 0.3 mg/kg of tamoxifen. Heifers in the control group were injected with an equivalent volume of the carrier. Injection volumes were adjusted every 14 d. The carrier for the tamoxifen injections contained 30% ethanol, 30% benzyl benzoate, and 40% corn oil. A 10 mg/mL stock solution of tamoxifen citrate was prepared by dissolving the tamoxifen citrate in a solution of 50% ethanol and 50% benzyl benzoate. The stock solution was sterilized by passing through a 0.45-μm filter into sterile bottles. Corn oil was sterilized by autoclave and injectable solutions were aseptically prepared and stored in sterile vials with septum closures. Because no information exists in the literature to suggest an optimal dose of tamoxifen to use to suppress estrogen receptor (ESR1) action in the bovine mammary gland, we chose an amount equivalent to that administered to human breast cancer patients on tamoxifen therapy (Jordan, 2008). Heifers were euthanized using Euthasol (Virbac Animal Health, Fort Worth, TX) and exsanguination at 121 ± 1 d of age.

Mammary Tissue Sampling

At slaughter, udders were immediately removed following exsanguination. The udder was bisected medially into left and right hemispheres. The right hemisphere was used to determine gross weight and immediately immersed in liquid nitrogen. The left rear quarter was trimmed of excess mammary fat pad tissue, butterflied,