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Hot Topic: Prepubertal Ovariectomy Alters the Development of Myoepithelial Cells in the Bovine Mammary Gland

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ABSTRACT

Prepubertal ovariectomy can dramatically inhibit mammary development, but the mechanism of inhibition is not well characterized. Holstein heifers were ovariectomized (OVX) or sham operated but left intact (INT) at d 40 and then sacrificed at d 55, 70, 85, 100, 130, or 160 to provide tissues for histologic analysis of cell proliferation. Our histologic analyses unexpectedly revealed a pronounced effect of ovariectomy on myoepithelial cell development. Myoepithelial cells were identified on the basis of location, morphology, and immunohistochemical staining for α-smooth muscle actin (SMA). Vascular smooth muscle staining served as an internal positive control for all immunohistochemical analyses. Mammary tissues from d 40 heifers had an abundance of SMA+ cells associated with the ductal parenchyma. In INT heifers, the frequency of SMA+ cells decreased as development progressed. Only a limited number of isolated SMA+ cells were observed in d 70 to d 160 INT heifers. In OVX heifers, SMA+ cells were abundant, had elongated morphology, and frequently stained more intensively than vascular smooth muscle cells. The intense SMA staining and altered morphology was most prominent in older heifers. Limited analysis of gene expression revealed that maspin, a protease inhibitor expressed by myoepithelial cells, was expressed in parenchyma from both INT and OVX heifers. Our hypothesis is that ovarian secretions stimulate epithelial proliferation, and block myoepithelial differentiation. Myoepithelial cells are known to limit parenchymal cell proliferation. Ovariectomy may thus remove an estrogenic growth stimulus and permit the emergence of inhibitory cell populations that further limit parenchymal expansion. Our observation has important implications for control mechanisms that regulate parenchymal development.

Key words: ovariectomy, myoepithelial, parenchymal development

Sinha and Tucker (1969) showed that allometric growth of mammary parenchyma begins well before puberty in heifers. Ovariectomy can inhibit allometric mammary development (e.g., Wallace, 1953; Purup et al., 1993), but the mechanisms of ovarian influence on prepubertal mammary development are poorly defined. Berry et al. (2003) reported that heifers ovariectomized earlier than ~2.5 mo of age had virtually no subsequent mammary parenchymal growth. Heifers ovariectomized between 2.5 and 3.0 mo of age had reduced, but not completely impaired, mammary growth. The age-dependent effects of ovariectomy suggest that prepubertal ovarian activity is especially important in determining the rate and extent of mammary development. During a recent trial to assess the effect of prepubertal ovariectomy on proliferative parenchymal cell populations, we noted an unexpected and dramatic effect on the appearance of myoepithelial cells after ovariectomy.

Thirty-nine Holstein heifers were purchased from regional suppliers between 7 and 21 d of age. Commercial milk replacer and starter ration were fed according to manufacturer's instructions. At d 40 of age, all heifers underwent flank laparotomy to perform either an ovariectomy (**OVX**; n = 16) or sham (**INT**; n = 21) operation. All procedures were approved and monitored by the Clemson University Institutional Animal Care and Use Committee. At 55, 70, 85, 100, 130, or 160 d of age, INT and OVX heifers were sacrificed to provide tissues for histologic analysis. Two INT heifers were sacrificed at d 40 to serve as a control for developmental divergence for both INT and OVX heifers. Parenchymal tissues were fixed in 4% paraformaldehyde in pH 7.2 PBS overnight, dehydrated through a graded series of ethanol solutions (50, 75, 95, 100, and 100%), infiltrated, and embedded in ImmunoBed resin according to the manufacturer's instructions (Polysciences Inc., Warrington, PA). To detect smooth muscle actin (**SMA**), sections (1.5

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μm) were stained with a prediluted primary antibody (anti-SMA IMMH2, Sigma, St. Louis, MO) and secondary gold-conjugated antibody (10 nm goat-anti-rabbit, 0.6 g/mL working concentration as a 1:50 dilution of the stock; BBInternational, Lianishen, UK). Silver enhancement (BBInternational) was performed according to the manufacturer's instructions, and sections were counterstained with sequential immersions in 1% Azure II in a 1% Na-borate buffer and 1% basic fuchsin in 2.5% ethanol.

Primer pairs for PCR were designed to analyze the expression of maspin, a protease inhibitor expressed by myoepithelial cells (Zou et al., 1994), in mammary tissue samples after reverse transcription of total cellular RNA using the Superscript III kit according to manufacturer's protocols (Invitrogen, Carlsbad, CA). The maspin primers (forward: 5′-ATGCCAAAGTCAAACTCTCCAT-3′ and reverse: 5′-GACATCCCAGAGAAATCAGAGG-3′) were used in a standard 35-cycle reverse transcription-PCR reaction with 30 s of denaturation at 94°C, 30 s of annealing at 60°C, followed by extension for 30 s at 72°C in each cycle. The PCR product was subcloned and sequenced to ensure that the amplified product was maspin. Results of the BLAST search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) showed several bovine expressed sequence tags with 100% identity and ≥90% homology for equine, human, chimpanzee, and orangutan maspin sequences (data not shown).

Parenchymal tissues in 15 or more randomly selected microscope fields from several nonadjacent sections were observed to count at least 1,000 cells per animal, resulting in a total of 42,245 cell observations. Basal position within the parenchymal epithelium, positive immunoreactivity for SMA, and cell morphology were used to identify and characterize myoepithelial cells (Deugnier et al., 1995). Parenchymal cells were assessed for SMA staining and scored as SMA– or SMA+. Cells in the SMA+ category had staining patterns that ranged from sparsely distributed punctate staining to heavy continuous staining. Cells with more intense staining tended to have the elongated cytoplasmic processes expected of differentiated myoepithelial cells. Intense staining was also found in some rounded cells in the basal strata, suggesting a series of progressive differentiation stages. The present experimental design provided no context to discern sequential stages of myoepithelial differentiation once they became SMA+, so the staining results are presented in a conservative manner with only 2 categories (SMA+ and SMA–). Statistical analyses were performed with Z-tests to compare the proportion of SMA+ cells at each age. The *P*-values were adjusted using the Bonferroni procedure and considered significant if the adjusted *P*-values were < 0.05.

Vascular smooth muscle staining served as an internal positive control for SMA+ staining. Vascular smooth muscle cells were identified on the basis of their fusiform shape, association with endothelial cells, and position within the vascular tunica media. There were no noticeable differences in smooth muscle morphology in samples from OVX or INT heifers. The SMA staining in vascular smooth muscle cells had a consistent and moderate intensity that served as a convenient reference during observation of histologic sections.

Figure 1 includes representative micrographs of parenchymal tissues from d 40 control, and d 100 INT and OVX heifers (panels A to C). Our interest in myoepithelial cells and SMA staining was sparked by the observation of strikingly altered basal cell morphology in samples from OVX heifers. During the processing of coded mammary tissue samples, it became very obvious from inspection of Azure II and basic-fuchsin'stained sections that 2 distinct morphologies were present in our samples (data not shown). One set of slides had the typical rounded basal cell morphology we have come to expect after numerous experiments with normal, intact heifers (e.g., Capuco et al., 2002). In the other set of slides, basal cells had extended cytoplasmic processes, rich cytoplasmic staining, and were associated with altered epithelial stratification patterns. When the sample codes were checked, all of the OVX heifers had the altered basal cell histomorphology. In OVX heifers most of the parenchymal epithelium was organized into a single layer of lumenal cells and an essentially continuous layer of basal myoepithelial cells. Our observations of altered gross tissue histology prompted us to stain for SMA as an indicator of myoepithelial differentiation.

As depicted in Figure 1 (panel D), our quantitative assessment of SMA labeling in histologic samples from OVX and INT heifers validated our subjective impressions of altered myoepithelial cell populations. In samples from heifers slaughtered at d 40 ($n = 2$), 80% of basal epithelial cells were SMA+. By d 55, only 27% of basal parenchymal cells in INT heifers were SMA+ ($P < 0.05$ relative to d 40), and SMA staining was not observed in the micrographs collected from the INT d 70 samples ($P < 0.05$ relative to d 40). After d 55, labeling for SMA remained greater ($P < 0.05$) in OVX (>47%) than in INT (<0.6%). At later ages, SMA+ cells in OVX heifers tended to have much more pronounced SMA staining and more elongated cell morphology. The few SMA+ cells observed in INT heifers after d 55 tended to have only a very light, diffuse SMA staining pattern (data not shown). The very low frequency of SMA+ cells observed in intact heifers (e.g., 0.6% at d 100) suggests that although ovarian secretions can suppress myoepithelial differentiation, there is no absolute lack of myoepithelial cells in intact heifers. It is therefore possible