# GENE EXPRESSION PROFILE IN PREPUBERTAL BOVINE MAMMARY PARENCHYMA AND EPITHELIAL CELLS IN RESPONSE TO OVARIECTOMY

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*Received* - 18. 06.13
*Accepted* - 20. 08.13

**Abstract**

*This study was carried out to compare the expression profile of steroid receptors and selected genes associated with cell signaling and proliferation in Mammary parenchyma (PAR) homogenates compared with mammary epithelial cells (MEC) in response to ovariectomy. Prepubertal dairy heifers were randomly assigned to one of two treatments, ovariectomized (n = 7) or sham operated (n = 12) and tissues were harvested 30 d after surgery. Parenchymal samples were snap frozen in liquid nitrogen for total RNA isolation. Samples of MEC were prepared from cryosections of parenchymal tissues using laser assisted microdissection and capture. Coupling two precise technologies like laser capture microdissection and quantitative real-time PCR enabled measurement of transcript abundance in MEC even at a very low level. Data were analyzed using Mixed procedure of SAS and significance was declared at P d" 0.05. The data strongly indicate a disparity in the response to ovariectomy between MEC and PAR tissue and suggest that ovariectomy very strongly impacted the non-epithelial cells in the PAR rather than the MEC themselves.*

**Keywords:** *mammary parenchyma, mammary epithelial cells, laser capture microdissection.*

Mammary parenchyma (PAR) is a complex tissue consisting of mammary epithelial cells (MEC) surrounded by intra lobular stroma composed of fibroblasts, adipocytes and blood vessels. It is well known that the ovary is an integral regulator of prepubertal mammary development and that many of the effects of estrogen are mediated through stromal cells (Haslam, 1988; Hovey *et al*., 1998). This is supported by the finding that isolated mammary epithelial cells in culture did not respond to estrogen (Woodward *et al*., 1994). We have previously reported that ovariectomy impacted transcript abundance of proliferation markers as well as selected estrogen responsive genes (Velayudhan *et al*., 2012). However, the magnitude of gene expression responses to estrogen and ovarian status were shown to be greater in mammary fat pad compared with parenchyma (Meyer *et al*., 2006). Moreover, the area occupied by epithelium per unit area of parenchyma was only 13-15 %, indicating that tissue homogenates prepared from parenchymal samples contain a mixture of cells other than epithelial cells. Therefore, we hypothesized that the response to ovariectomy in the transcript abundance in mammary parenchyma (**PAR**) is mainly from the intra lobular stromal cells, rather than the mammary epithelial cells (**MEC**). Advent of laser assisted microdissection (**LMD**) of histological preparations in 1990s has enabled precise

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separation of even single cells from tissues consisting of complex cell types. A combination of LMD along with quantitative real time polymerase chain reaction (**qRT-PCR**) is widely used to determine transcript abundance in single cell types. The objective of the study was to compare the gene expression profile between the PAR homogenate and MEC in response to ovariectomy employing the techniques of LMD and qRT-PCR.

## Materials and Methods

### *Animals and Treatments*

All animal care and use protocols used in the study were approved by the Clemson University Institutional Animal Care and Use Committee. Briefly, 24 Holstein heifers were fed with commercial milk replacers and calf starter diets according to the manufacturer's instructions prior to weaning and fed grains and hay thereafter. After a week's adaptation period at the facility, heifers were randomly assigned to either ovariectomy (**OVX**; n = 12) or sham operation (**INT**; n = 12) either at 2, 3 or 4 months of age. Animals of different age groups were acquired in batches and hence surgery was performed at different time points. In OVX heifers ovaries were removed surgically while in INT heifers surgery was performed, but ovaries were kept intact. Mammary tissues were harvested 30 d after surgery by humanely sacrificing the heifers using captive bolt pistol stunning and exsanguination. Only 19 animals were used in this study (INT, n = 12 and OVX, n = 7) for comparative gene expression analysis because of inadequate amount of tissue available from some animals.

### *Sample Collection and Analyses*

Mammary parenchymal samples were collected consistently from the inner parenchymal region of the left hind quarter for PAR homogenate preparation and from the left front quarter for MEC samples. For preparing tissue homogenate, PAR samples were immediately frozen by dipping tissue pieces into liquid nitrogen. For preparing MEC samples, parenchymal tissue pieces were first placed in plastic moulds containing a tissue embedding compound that ensures optimal cutting temperature (**OCT**) (Sakura Finetek U.S.A. Inc.; Torrence, CA) and then the tissues were also frozen in liquid nitrogen. Both samples were stored at -80°C until further processing. Isolation of total RNA from PAR tissue homogenate was performed as described (Velayudhan, *et al.*, 2012).

Cryo-preserved samples in OCT blocks were prepared for LMD following protocols of (Becker *et al.,* 1997; Saal *et al.,* 2003). Briefly, seven micrometer thick cryosections were made from OCT blocks and placed on polyethylene naphthalate membrane coated glass slides (Molecular Devices, Sunnyvale, CA). Slides were previously irradiated in a UV cross linker (FB UVXL-1000, Fisher Scientific, Pittsburgh, PA) for 30 min at maximum power to ensure cross linking of RNA onto the membrane. Slides were kept on dry ice while sectioning and stored at -80°C until LMD. Cryosections were thawed for less than 30 s and subjected to a quick staining procedure. Tissue sections were first rehydrated in 70 % ethanol followed by a rinse

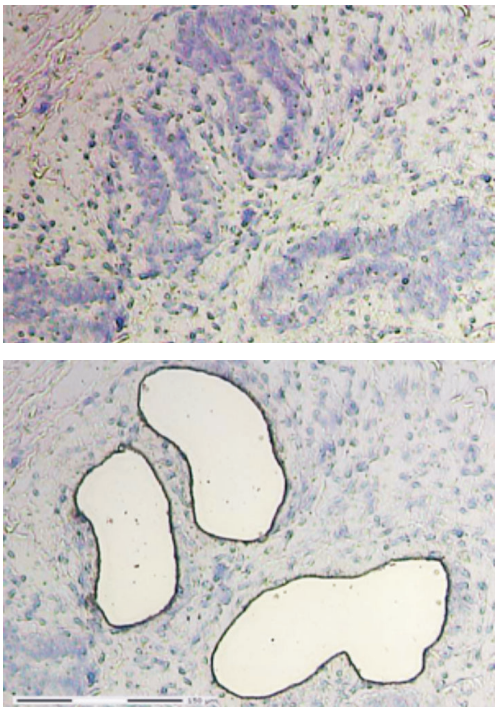
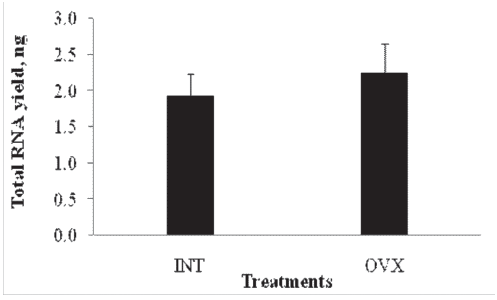


**Figure 1**. Representative figures of mammary parenchymal tissues before and after microdissection and catapulting. Mammary PAR tissue sections stained with toluidine blue before (A) and after (B) laser assisted microdissection and capture by catapulting. Thick arrows show the epithelial structures that were dissected and collected for RNA isolation for MEC samples and thin double headed arrows show the intralobular stroma.

in RNAse-free water for 30 s each. Sections were then treated with 1 % Toluidine blue stain (Fisher Scientific) containing 0.1 U/ ìL of RNAse-inhibitor (Qiagen Inc., Valencia, CA ) for 30 s followed by washing in RNAse-free water and dehydration in 80, 90 and100 % ethanol solutions, 30 s each. Tissue sections were made completely moisture free by air drying. Mammary epithelial cells were identified and the selected areas were precisely excised (Fig 1) using a UV-laser which was coupled through the illumination path of the microscope (PALM micro beam system, Carl Zeiss Micro Imaging, Inc.; Thornwood, NY). The excised cell zones were then catapulted against gravity into collecting tube caps containing 40 ìL of lysis buffer (RNeasy micro kit; Qiagen Inc.). The cells were then lysed immediately by vortexing in 350 ìL lysis buffer. On an average 85 to 90 epithelial zones depending on size were collected per heifer. Total RNA from MEC was isolated and purified using RNeasy Micro Kit (Qiagen Inc.). Quantity and quality of RNA were determined in a 2100 series Bioanalyzer from Agilent (Quantum Analytics, Inc, Foster City, CA) using pico-chips. Total yield of RNA from microdissected samples ranged from 0.84 – 4.04 ng and were not different between INT and OVX (*P* = 0.549; Fig 2).



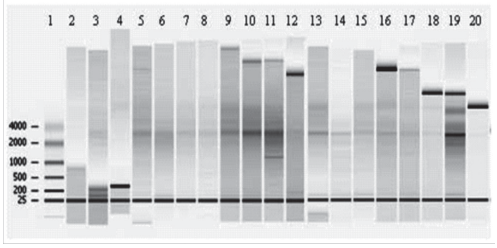




**Figure 2**. Quantity and quality of total RNA isolated from microdissected mammary epithelial cells. (A) Total yield of RNA isolated from microdissected mammary epithelial cells from sham operated (INT; n = 12) and ovariectomized (OVX; n = 7) Holstein heifers. Total yield of RNA was not different between INT and OVX (*P* = 0.549). Data are presented as LSM and bars represent SEM. (B) Gel image showing the quality of RNA isolated from OVX and INT heifers. Lane 1 represents the ladder and lanes 2-20 represent RNA samples from the isolated mammary epithelial cells.

### *qRT-PCR*

From each sample a total of 0.4 ng RNA was used to make single stranded cDNA in a 20 ìL reaction volume using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). A reaction tube containing no reverse transcriptase enzyme was used for each sample and this was later used in qRT-PCR as controls. A test run of qRT- PCR for different genes was performed with different concentrations of cDNA (stock and diluted) and 10 fold dilution of cDNA stock was used for qRT-PCR. A total of 4 pg cDNA was used in each reaction, along with 12.5 ml of SYBR Green dye (Applied Biosystems), 9.5 ml of sterile distilled water, 0.5 ml of 10 mM each of forward and reverse primers. The PCR conditions were: 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min. The reaction was set for 40 cycles in 7300 Series Real-Time System (Applied Biosystems).

Relative mRNA abundances of growth hormone receptor (**GHR**), insulin-like growth factor-1 ( IGF-1), IGF-I receptor (IGF-1R), IGF binding protein (**BP**)-3, proliferating cell nuclear antigen (**PCNA**), signal transducers and activators of transcription (**Stat**)-5b, and suppressors of cytokine signaling (**Socs**)-2 were determined in both PAR and MEC for OVX relative to INT heifers by comparative Ct ($2^{-\hat{A}\hat{A}Ct}$) method after normalizing the Ct values to the geometric mean of three endogenous control genes (Piantoni *et al.*, 2008). Primer sequences of target and endogenous reference genes are given in Table 1.

## Statistical Analysis

Relative abundance of mRNA in PAR homogenates was not different between different age groups and there was no interaction between treatment and age (P > 0.05). Additionally, the number of samples available for the 2 mo surgery group was very limited for MEC samples due to limited tissue resources. Therefore, for statistical analysis of comparative gene expression between the two types of sample preparations (PAR and

MEC) data were pooled within treatments and only treatment effect was tested. All data were analyzed using Mixed procedure of SAS (SAS 9.2; Cary, NC). Random effect error was heifer within treatment. Data distribution was analyzed using the "Proc Mixed Boxplot" statement (Appendix A.6). Significance was declared at P d" 0.05 for all analyses.

The model used in all analyses was

$Y_{ij} = ì + T_i + e_{(i)j}$ where

$Y_{ij}$ = variable being tested

ì = overall mean

$T_i$ = fixed effect of treatment (INT or OVX) (i = 1, 2)

$e_{(i)j}$ = residual error

Gene expression data for PAR and MEC were analyzed separately. The ÄCt data were used for statistical analyses and significance was determined based on the P values for ÄCt data. However, data was presented as fold change in gene expression for OVX relative to INT control using the comparative Ct ($2^{-ÄÄCt}$) method.

## Results and Discussion

There was a moderate degree of disintegration in the RNA isolated from MEC samples for both OVX and INT samples. However, we succeeded in obtaining a sufficient amplification for all the mRNA species evaluated and no-RT template controls had undetectable amounts in all the samples tested. Dissociation curves for each target gene had the same single peak in PAR and MEC samples indicating amplification of the same single product in both sample types. Additionally, there was no difference in the yield of total RNA between treatments (Fig 2). Therefore, we made the assumption that the low integrity of RNA did not hamper our ability to evaluate the relative gene expression between treatments using the highly sensitive qRT-PCR methodology. Quantitative determination of transcript abundance even in tissue extracts containing partially fragmented RNA is possible by qRT-PCR because this technique enables amplification of targets of very small size (Gibson *et al.,* 1996; Heid *et al.,* 1996) indicating that even though RNA undergo degradation, the resulting fragments are still large enough to be detected by qRT-PCR.

Relative abundance of individual mRNA species present in MEC samples were very low compared with that of PAR homogenate as indicated by greater Ct values for MEC samples in the qRT-PCR reactions (Fig 3). This could be due to the difference in the amount of total RNA used in the reverse transcription reactions. Because of the limited amount of MEC available for RNA extraction we could only use 0.4 ng total RNA to make cDNA whereas, 4 ug RNA was reverse transcribed for each PAR sample. We measured mRNA expression of IGF-1, IGF-IR, IGFBP-3, PCNA, GHR, Stat5b and Socs2 in PAR as well as MEC from OVX and INT heifers. Relative mRNA abundance of IGF-1 and PCNA was down regulated and IGF-1R mRNA was up regulated in PAR homogenate due to ovariectomy (P < 0.05; Fig 5). We and

**Table 1.** Primer pair sequences used in real-time PCR assays

| Gene | Forward (5'-3') | Reverse (5'-3') | Target/Endo[1] |
|---|---|---|---|
| GHR | CGTCTCTGCTGGTGAAAACA | AACGGGTGGATCTGGTTGTA | Target |
| IGF-1 | GTTGGTGGATGCTCTCCAGT | CTCCAGCCTCCTCAGATCAC | Target |
| IGF-1R | TCAAGGACGGAGTCTTCACC | GCTCAAACAGCATGTCAGGA | Target |
| IGFBP-3 | CAGAGCACAGACACCCAGAA | TGCCCCGTACTTATCCACACA | Target |
| PCNA | TCGTCTCAGGCGTTCATAGTC | AACATGGTGGCGGAGTCG | Target |
| Stat5b | TTTACCCGGACGGAATTACA | TAACTCAGGTCTCCCAAGCG | Target |
| Socs2 | TCGCATCGAATACCAAGATG | GTCCGCTTATCCTTGCACAT | Target |
| PPP1R11 | CCATCAAACTTCGGAAACGG | ACAGCAGCATTTTGATGAGCG | Endo |
| RPS15A | GAATGGTGCGCATGAATGTC | GACTTTGGAGCACGGCCTAA | Endo |
| MTG1 | CTTGGAATCCGAGGAGCCA | CCTGGGATCACCAGAGCTGT | Endo |

[1]*Whether the gene is treated as a target gene or endogenous control gene*

others (Akers *et al.,* 2000; Berry *et al.,* 2003) observed a decrease of IGF-1 mRNA in PAR due to ovariectomy. Transcript abundance of the proliferation marker gene PCNA was reduced in PAR ($P < 0.05$) but there was no difference in PCNA mRNA in MEC between OVX and INT. In support we did not see a difference in epithelial cell proliferation between intact and ovariectomized heifers (Velayudhan, *et al.,* 2012). Therefore, it seems likely that the reduction in PCNA mRNA in PAR as a response to ovariectomy was from the non-epithelial components of PAR.

Insulin-like growth factor-1 mRNA was not expressed in isolated primary MEC

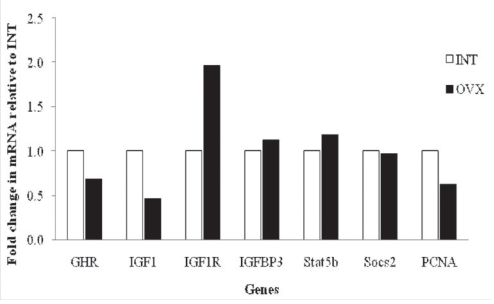



**Figure 3**. Effect of ovariectomy on transcript abundance in mammary parenchyma. Relative mRNA expression ($2^{-\ddot{A}\ddot{A}Ct}$) in PAR tissue homogenate samples collected from sham operated (INT; n = 12) and ovariectomized (OVX; n = 7) heifers. There was a reduction in transcript abundance for IGF-1 and PCNA while IGF-1R mRNA increased in OVX relative to INT.

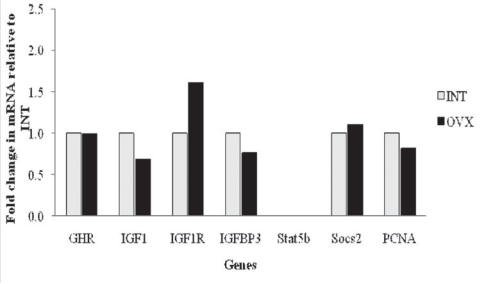



**Figure 4**. Effect of ovariectomy on transcript abundance in mammary epithelial cells. Relative mRNA expression ($2^{-\ddot{A}\ddot{A}Ct}$) in microdissected MEC collected from sham operated (INT; n = 12) and ovariectomized (OVX; n = 7) heifers. There was no detectable expression of Stat5b in mammary epithelial cells. Transcript abundance in mammary epithelial cells was not affected by ovariectomy.

(Berry, *et al.,* 2003). Contrary to this report we measured IGF-1 mRNA in MEC. However, IGF-1 mRNA expression in MEC was not affected by ovariectomy. Although there was numerical decreases in IGF-1 (46 %) mRNA in MEC by ovariectomy, the decrease was not significant ($P = 0.295$) which could be due to the low number of samples used in the current study. Similarly, transcript abundance of IGF-1R in MEC was also increased numerically (38 %), but was not statistically significant ($P = 0.207$). It could be due to the high animal to animal variation and comparatively less number of samples used in the assay. Bovine mammary epithelial cells express IGFBP-3 both in vivo and in cell culture systems (Cohick and Turner, 1998; Weber et al., 2000). Like previous reports we did not find difference in IGFBP-3 mRNA abundance between OVX and INT in PAR or MEC (Berry et al., 2001). Even though GHR is expressed in stromal and epithelial cells (Jiang et al., 1999; Plath-Gabler et al., 2001) ligand binding assays failed to demonstrate direct binding of GH in mammary parenchyma (Akers and Keys, 1984). However, GH increases transcription of milk protein genes in MAC-T cells expressing GHR, mediated through Stat5 (Zhou et al., 2008). Therefore, the activity of GHR in MEC may differ under in vivo and in vitro conditions as well as under different endocrine states. In the current study there was no detectable expression of Stat5b mRNA in MEC. However, Socs2 mRNA was detected in MEC although transcript abundance was not affected by ovariectomy. There was no detectable expression of Stat5b in mammary epithelial cells. Transcript abundance in mammary epithelial cells was not affected by ovariectomy.

In summary, coupling the technique of laser assisted microdissection and pressure catapulting with the precise and sensitive method of qRT-PCR enabled measurement of transcript abundance in MEC and PAR discretely from prepubertal mammary gland in ovariectomized and intact heifers. Our results strongly indicate a disparity in the response to ovariectomy between MEC and PAR tissue homogenate and suggest that response to ovariectomy in the gene expression profile in PAR is markedly impacted by the presence of the stromal cells adjacent to epithelium.

## Acknowledgements

The authors extend their gratitude to

Dr. Anthony Capuco (Bovine Functional Genomics, USDA, Beltsville, MD) for technical as well as intellectual support. The authors also thank Cathy Parsons (Virginia Tech) for technical support. In addition, we would like to acknowledge the facility center at Fralin Lifescience Institute, Virginia Tech, Blacksburg for letting us use their laser capture microdissection microscope. This project was supported by National Research Initiative Competitive Grant no. 2006-35206-16699,'Ovarian Regulation of Stem Cells and IGF-I Axis Molecules in Prepubertal Heifer Mammary Gland' to R. M. Akers and S. E. Ellis from the USDA National Institute of Food and Agriculture.

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