DETERMINATION OF EXPRESSION OF FLIZ1 DURING INVOLUTION OF THE MOUSE MAMMARY GLAND

A Thesis presented to
the faculty of the Department of Biology
Villanova University

By

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Abstract

Remodeling of the mouse mammary gland is a highly coordinated process that occurs after the removal of suckling pups from the mother. Involution, or shrinking of the mammary gland, after removal of the pups has been linked to apoptotic events within the mouse mammary tissue during forced weaning. Several transcription factors are hypothesized to be involved in this process. A transcription factor known as GATA-3, which was first identified in the thymus, is also important for maintenance of various tissue types within the mouse mammary gland; its loss leads to epithelial cell detachment and eventual death. Another transcription factor known as fetal zinc liver finger protein 1, or Fliz1, has been found to regulate GATA-3 in T-cells. This interaction had not been elucidated during involution in mouse mammary tissue. I hypothesized that Fliz1 is expressed at heightened levels during mouse mammary gland involution following forced weaning of pups, and that this expression correlates with a decrease in GATA-3 levels, with increased expression of the pro-apoptotic protein BAD. Using qRT-PCR, immunoblotting and immunohistochemistry I have shown that Fliz1 is indeed expressed in involuting mouse mammary gland tissue as well as several other tissue types. However, levels of Fliz1 remain fairly constant during involution. The findings also show that Cathepsin L, a known apoptotic marker for mammary gland involution, is substantially up-regulated during the process of mammary gland involution in the mouse. The study also revealed that GATA-3 levels as hypothesized decrease substantially during the process of mouse mammary gland involution, indicating that GATA-3 is required for maintenance of the mouse mammary gland.

Background

Development of the Mammary Gland

The mammary gland is a plastic organ that undergoes dramatic cycles of growth and regression. The embryonic development of the mammary gland is modulated largely by systemic maternal hormones, most notably parathyroid related hormone, which is responsible in large part for the stimulation of mammary bud epithelial sprouting (Sternlicht, 2006). All branched organ systems, whether the kidney, lungs or mammary gland, follow a similar developmental pathway involving: development and specification of an organ precursor, invagination, outgrowth of branches, and reiterative branching and tissue-specific formation of terminal structures (Sternlicht, 2006). Mammary buds act as the precursors to glandular development (Horseman, 1999). The buds form along the embryonic mammary ridge, which later generates the lactiferous ducts through which milk travels before draining into the teat. Parathyroid Related Hormone or PTHrP is functionally similar to that of Parathyroid hormone, and loss of PTHrP has been shown to result in lack of bud sprouting (Foley et al., 1998). In the event of proper PTHrP expression, the sprouting is continuous until the basal layer of the fat pad of the mammary tissue is reached, at which point, morphogenesis and continued reiterative branching of the epithelial ductal tissue begins. (Hens and Wysolmerski, 2005). Equally important as PTHrP, is the WNT signaling pathway which has been implicated in initiation of mammary morphogenesis (VanHouten et al., 2004). As with PTHrP loss, low levels of WNT expression prevents mammary morphogenesis initiation and results in abrogation of mammary gland development, indicating that mammary gland development is controlled by hormone and transcription factor related pathways and that

controlled temporal expression of each is necessary for proper initiation of mammary bud formation and ultimately gland development, Figure1 (Chu et al., 2004).

Mouse Terminal End Bud

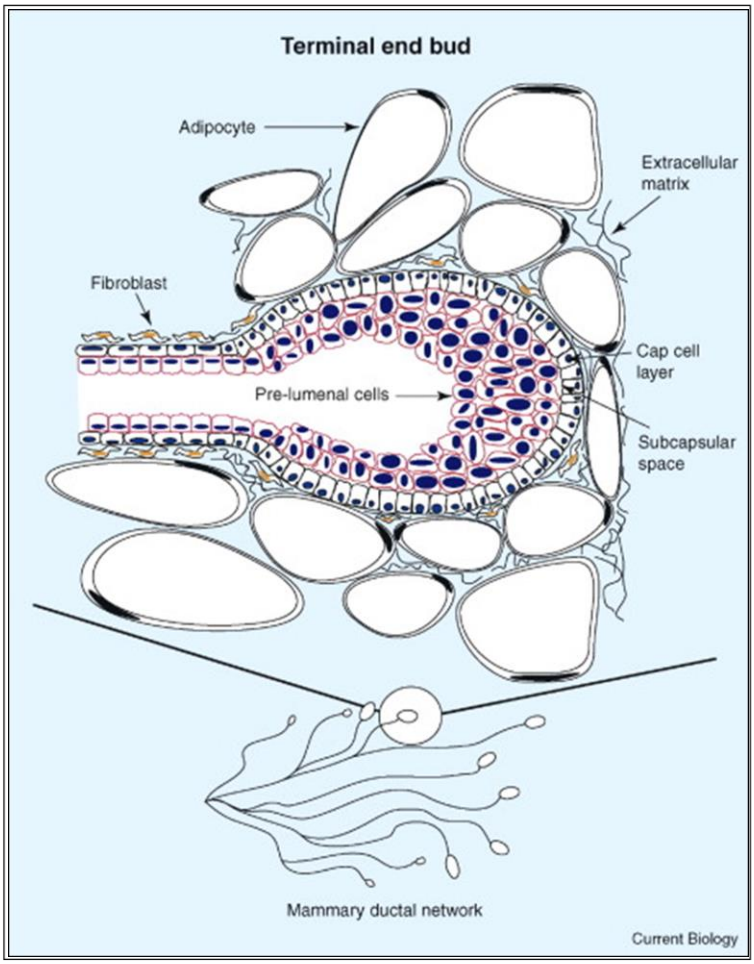

Figure 1. Terminal end bud showing luminal epithelial cells lining the interior of the lumen. Figure also shows adipocytes and milk secreting cuboidal cells (Slorach and Werb, 2003) .

Fetal lactiferous duct development is evident during gestation but is subsequently continued during puberty and adulthood by increased production of the hormone estrogen (Richert et al., 2000). Estrogen results in greater branching and differentiation of not only lactiferous ductal

tissue but also simultaneously gives rise to greater vascularization and increased fat content within the mature breast as is evident in Figure 2. Figure 2E also displays the profound physical changes that are evident after only three days of weaning and indicate massive remodelling of the mammary gland at a variety of different tissue levels.

Histological Whole Mounts

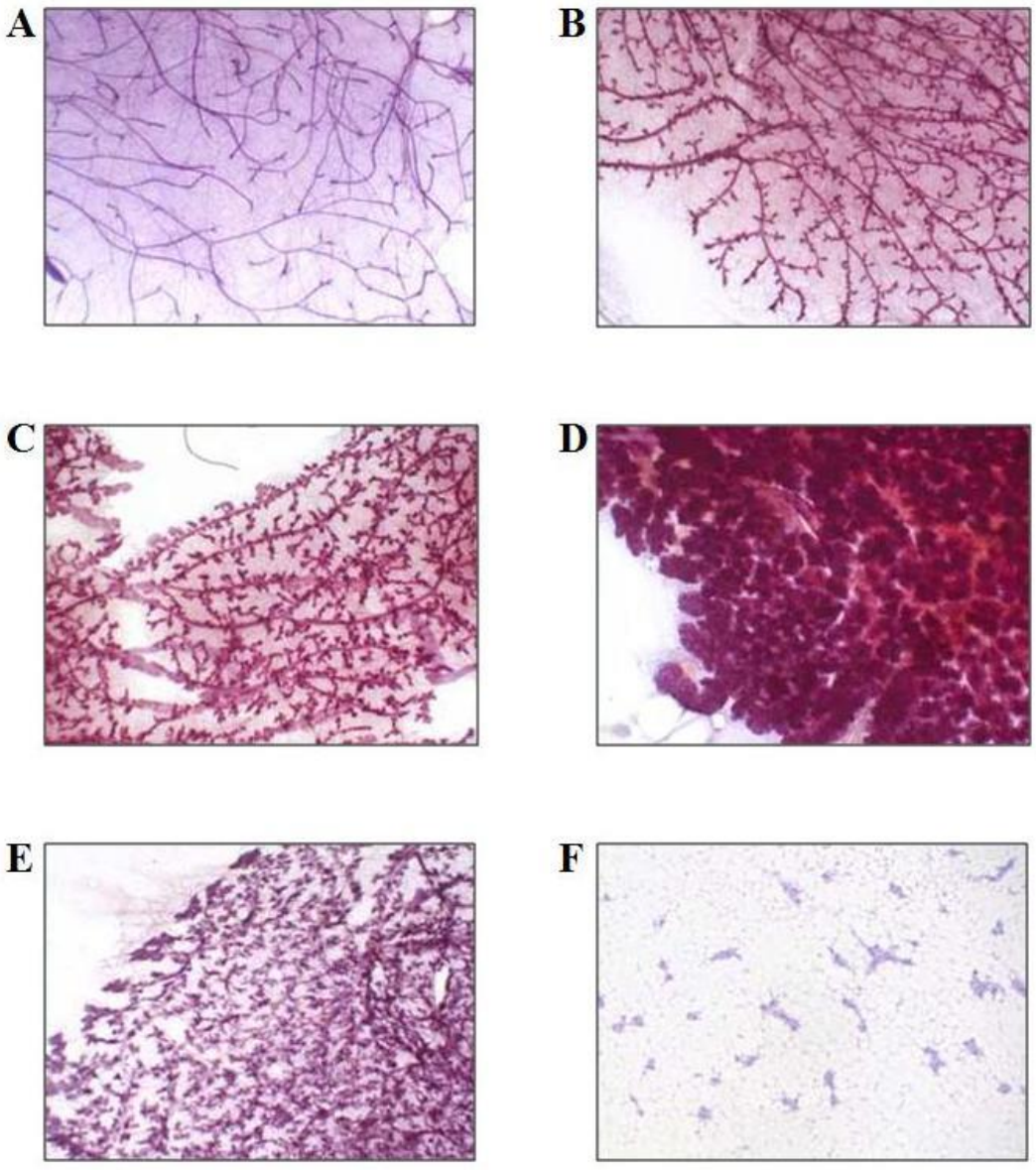

Figure 2. A. Stained histological whole mount of virgin mouse mammary gland. Note the lack of

convolution and the simple tree-like structure. **B.** Stained histological whole mount of the mammary gland in a slightly older virgin mouse. Convolution is greater and acini are evident. **C.** Stained histological whole mount of prelactating mammary gland in female five days following conception. Acini are more numberous as well as branching and overall convolution. **D.** Stained histological whole mount of lactating mammary gland one day post-partum. Alveoli form large clusters, filling the fat pad. **E.** Stained histological whole mount of mammary gland three days after weaning of the pups. Note that the alveoli are collapsing and the number of adipocytes in the fat pad is readily apparent. **F.** Histological cross section of mouse mammary gland several weeks after weaning. Note the lack of alveoli and the numerous adipocytes in the fat pad. (http://ccm.ucdavis.edu/bcancercd/22/development.html)

The mature breast is composed of a variety of tissue types ranging from mature milk-secreting cuboidal epithelial cells to adipocytes and fibroblasts which provide support and nutrients for the breast tissue (Horseman, 1999). During pregnancy, the breast undergoes continued remodeling and heightened levels of the hormone prolactin give rise to the production and extension of secretory alveoli as well as increased fat production and further levels of vascularization (Richert et al., 2000). Lactation follows with a decrease in levels of the hormone progesterone and rising levels of estrogen and prolactin, both of which aid in milk production via cuboidal cells (Horseman, 1999). The hormone oxytocin also plays a role not only in stimulating increased milk production, but also in efficient discharge of the milk from the nipple during suckling (Horseman, 1999). The cuboidal cells, which are the actual milk secreting elements of the breast line the alveoli and secrete into the luminal space. These numerous alveoli merge to

form lobules from which lactiferous ducts, the primary ducts through which milk travels, empty to the nipple (Richert et al., 2000).

Involution and Apoptosis

Upon cessation of lactation and weaning of the young, continued remodeling of breast tissue once again becomes apparent. Since pregnancy results in a massive proliferation and extension of mammary alveolar structures, and continued maintenance of such structures during lactation, cessation of lactation and weaning of the young results in substantial physiological changes to the mammary gland and supporting mammary tissue. Ultimately, involution results in regression to a virgin type model with the first histological signs of involution being the loss of lobuloaveolar units and cell junctions (Strange et al., 1992). However, the cellular mechanisms behind mammary gland tissue involution have yet to be fully elucidated. Involution or the general shrinking of the organ or tissue in question has been linked to controlled apoptosis within the mammary tissue (Bar-Dayan et al., 1999). Apoptosis serves a variety of developmental and physiological functions. A notable case of apoptosis occurs during embryonic development that involves the ordered death of cells comprising the webbing between the fingers and toes of the developing fetus (Crocoll et al., 2002). This controlled killing of the cells gives rise to fully separated digits. Apoptosis can also be organ specific. The thymus gland undergoes apoptosis and involution after puberty as the result of increased circulation of sex hormones. This results in a dramatic reduction in the overall activity of the thymus. The involution of the organ is also accompanied by a dramatic rise in fat content within the organ. Aside from having the capacity to efficiently remove supernumerary cells or cells that the body finds undesirable,

apoptosis has the capacity to kill cells without evoking an inflammatory response, which is normally induced by necrotic cell death. Physiologically, apoptosis also serves a variety of other roles from immune self-tolerance and killing to that of cell viability via hormonal regulation. Clearly the importance of apoptosis cannot be overstated given the fact that apoptosis and involution are causally linked in that apoptosis gives rise to a general loss of tissue and shrinking of the organ, or involution.

Involution of the mammary gland is a two stage process that initiates rapidly after pup removal. The first stage is characterized by limited loss of luminal epithelial cells and is reversible upon initiation of suckling by the pups (Furth et al., 1997). However, the second irreversible stage is characterized by more pronounced cell death and the destruction of lobuloaveolar epithelium (Lund et al., 1996). The second irreversible state is characterized by proteolytic degradation of the extracellular matrix and rapid destruction of the secretory epithelial cells by apoptosis. The transition from a reversible state to that of an irreversible state is characterized by increased levels of a lysosomal cysteine protease known as Cathepsin L (Burke et al., 2003). Cathepsin L is a major excreted protein that is highly expressed in mouse fibroblasts (Burke et al., 2003). This protein was found via immunoblot detection assay to be up-regulated during the temporal transition from the reversible stages of mouse mammary gland involution to the irreversible stage, clearly indicating that Cathepsin L not only plays an important role in mammary gland involution, but also that involution of the mammary gland is a highly fragile and time-dependent process involving multiple hormonal as well as enzymatic factors. Both involution and apoptosis are extremely important physiological responses to varying environmental conditions. For instance, maintenance of mammary gland tissue could be considered to be unnecessarily wasteful if lactation and feeding of offspring is no longer

necessary. If pups are removed from the mother, apoptosis and involution quickly become evident (Burke et al., 2003).

GATA-3

The transcription factor GATA-3, belongs to a family of zinc finger transcription factors and has been implicated in cell growth and differentiation as well as tumorigenesis (Merika and Orkin, 1993). The GATA family of DNA binding proteins is unique in that the transcription factors recognize a distinct site conforming to the consensus sequence, WGATAR (Merika and Orkin, 1993). The transcription factor GATA-3 in particular, which is expressed in in T-cells, has been shown to be involved in T-cell maintenance and cell fate determination (Ting et al., 1996). GATA-3, like other members of the GATA family, contains several conserved motifs, which are involved in both the functionality of the transcription factor and its level of specificity (Takemoto et al., 2002). For instance, the GATA-3 gene specifies a protein that contains two transactivation domains known as TA1 and TA2, as well as two zinc fingers known as Zn1 and Zn2. The transactivation domains are sites on the transcription factor where co-regulator factors can bind, thereby providing another level of transcriptional regulation. The zinc fingers are structural motifs on the translated protein that can act in a DNA binding role. These two motifs are highly conserved regulatory elements in the GATA family of transcription factors (Takemoto et al., 2002). Both the N- and C- zinc finger motifs are functionally unique. The C-terminal end has been shown to be essential for binding of the GATA sequence, whereas the N-terminus although seemingly superficial, provides for a greater level of binding specificity and is likely important in the overall stability of the binding domain (Takemoto et al., 2002). Heightened

levels of GATA-3 have been shown to maintain T-cell levels within the thymus and are likely involved in homeostasis of established T-cells. Ectopic expression of GATA-3 can induce differentiation of type II thymus cells (Pai et al., 2004). Equally fascinating is the ability of GATA-3 to stabilize the commitment of these cells lines via a feedback pathway (Takemoto et al., 2002). Conversely, loss of GATA-3 expression has been shown to drastically reduce the number of thymocytes as well as limit T-cell proliferation entirely (Pai et al., 2004). Reduction in the number of T-cells would indicate that GATA-3 expression is clearly involved in maintenance of mature T-cell lines in the thymus.

Experimentation has also extended the role of GATA-3 to regulation and maintenance of mammary gland tissue (Chou et al., 2010). This tissue undergoes rapid regression and involution upon weaning. Histological cross sections reveal decreased branching of ductal epithelial tissue and nearly complete loss of adipocytes within the mammary gland during low levels of GATA-3 expression, which was achieved through the use of conditional knockouts. Physiological expression of GATA-3 during involution of mouse mammary tissue has yet to be investigated. However, the use of conditional knockouts for GATA-3 indicates that GATA-3 expression is required for maintenance of the ductal epithelial network of the mammary gland and that loss of GATA-3 expression could lead to involution of the mouse mammary gland under normal physiological conditions. The importance of GATA-3 in mammary gland development becomes clearly evident in conditional deletion experiments of GATA-3 in adult mice (Kouros-Mehr et al., 2006). GATA-3 null mice display embryonic lethality at day 11; consequently, it is necessary to develop a conditional recombinant using the Cre/loxP system. Conditional Cre/loxP mice displayed not only a lack of ductal branching, but also defects in ductal invasion (Kouros-Mehr et al., 2006). Further experimentation using doxycycline inducible Cre/loxP recombinant

mice indicated that luminal epithelial cell populations are maintained in the presence of GATA-3 (Kouros-Mehr et al., 2006). Conditional deletion experiments of GATA-3 in mice results in loss of basement membrane attachment and undifferentiated luminal cell expansion, which ultimately leads to caspase mediated cell death (Kouros-Mehr et al., 2006).This lends credence to the idea that GATA-3 expression is necessary for the development and maintenance of healthy mammary gland tissue. If GATA-3 expression is necessary for maintenance of mammary gland tissue the question arises as to what specifically silences or down regulates the GATA-3 transcription factor and is this action integral to the process of involution in mammary gland tissue.

Fliz1

The fetal liver zinc finger protein 1, or Fliz1, is a zinc finger protein that is expressed both embryonically, and in adult organs (Dahm et al., 2001). Fliz1 is unique in that it contains three zinc finger C3H domains while lacking YKTEL peptides. Most C3H zinc finger proteins contain conserved YKTEL peptide sequences which are involved in RNA binding. The lack of conserved YKTEL peptides suggest a unique function of Fliz1 relative to other C3H zinc finger proteins (Hwang and Ho, 2002). Fliz1 protein expression is evident in several adult organs most notably the thymus, but is also expressed differentially in liver hematopoietic progenitors cells during embryonic development (Hwang and Ho, 2002). The researchers wished to determine the *in vivo* function of Fliz1 in the regulation and maintenance of thymocyte homeostasis. This was accomplished through use of an inducible Tet-On system that allowed for overexpression of human Fliz1 in transgenic mice (Hwang and Ho, 2002). This overexpression of Fliz1 resulted in lower levels of thymocytes within mature thymus tissue (Hwang and Ho, 2002). This could

possibly be due to the induction of Bad. Bad is an important pro-apoptotic factor that has a number of important developmental roles. Overexpression of Fliz1 in mouse thymus tissue has been shown to result in pronounced induction and expression of Bad (Hwang and Ho, 2002). Coincidentally, when Fliz1 expression is high, GATA-3 expression appears at a basal level, whereas when GATA-3 expression is high, Fliz1 expression is low. GATA-3 expression levels in T-cells are regulated by Fliz1 binding to the intronic regulatory region of GATA-3 (Hwang et al., 2002). This binding leads to a dramatic decrease in GATA-3 expression. The correlation between high levels of Fliz1 expression and a decrease in the total number of thymocytes is likely the result of greater levels of apoptosis within the thymus tissue. Experimental studies utilizing T-cell inducible transgenic mice overexpressing human Fliz1 have shown that Fliz1 is indeed necessary for thymocyte homeostasis (Hwang and Ho, 2002). Studies conducted by Hwang and Ho employed a tetracycline inducible system to generate transgenic mice that are capable of overexpressing human Fliz1, in a highly inducible fashion. In the studies, transgenic mice carrying either a full length myc-tagged hFliz1 or a truncated hFliz1-ΔN were fed doxycycline to induce Fliz1 expression. The hFliz1- ΔN lacked the N-terminus, thereby eliminating the serine-rich domain. Expression was found in all lines fed with Dox, while the sucrose control group did not express Fliz1 in either the full length hFliz1 or the hFliz1-ΔN. A lack of Fliz1 expression in the absence of Dox indicates that Fliz1 can be induced by the Tet-on system. The N-terminus of hFliz-1, which contains both acidic and serine rich domains appears necessary for Fliz1 action. Over expression of hFliz1 in Dox treated mice also resulted in pronounced thymic hypocellularity as well as decreased GATA-3 expression levels (Hwang and Ho, 2002). Continued research by Ho and Hwang also used mice from double transgenic founder lines to determine how reductions in total thymocyte number related to heightened levels of Fliz1

expression. Massive thymic hypocellularity would potentially indicate both apoptosis and induction of pro-apoptotic factors such as Bad.

BAD

The Bad gene is a pro-apoptotic gene that is involved in the initiation of apoptosis. The Bad protein interacts with Bcl-2 family members through a conserved BH3 domain (Adachi and Imai, 2002). Apoptosis is a highly complex cellular process involves multiple pathways and can be elicited by a variety of stimuli. During initiation of apoptosis, Bad functions to suppress the anti-apoptotic effects of Bcl-2 and Bcl-X_L, a family of conserved anti-apoptotic genes, that when suppressed lead to apoptosis and cell death, as depicted in Figure 3.

Intrinsic Apoptosis Pathway

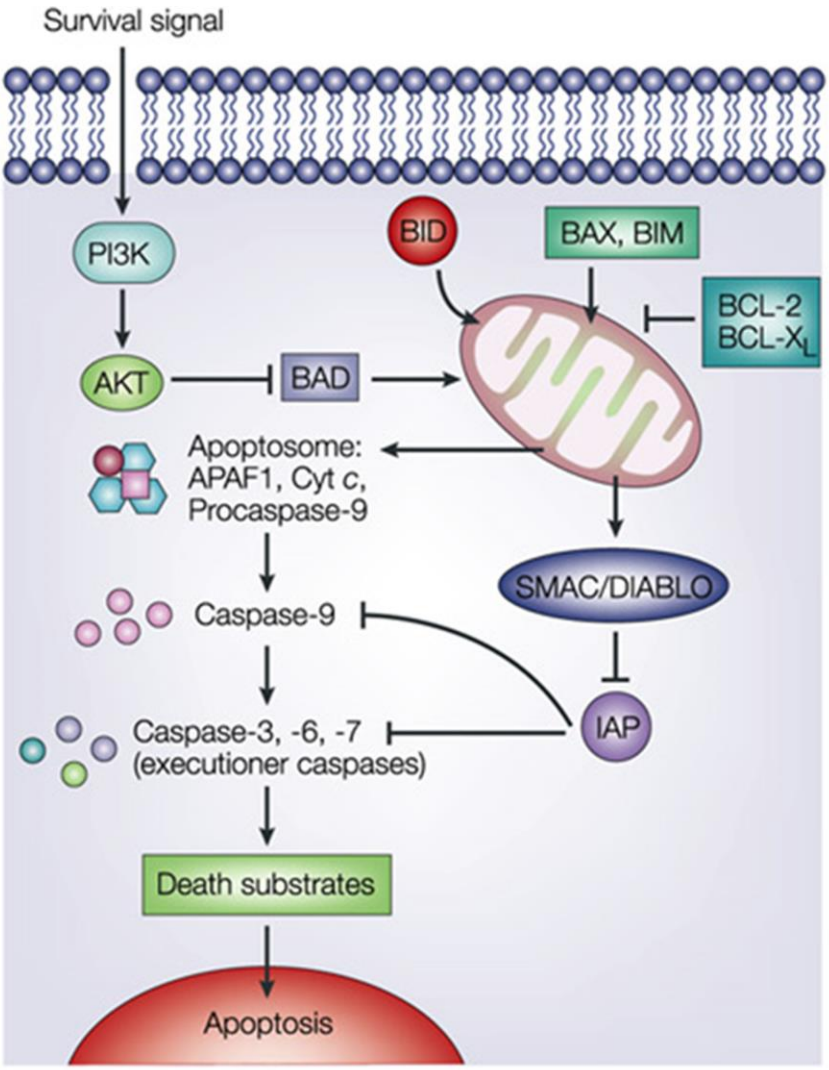

Figure 3. Intrinsic or mitochondria based apoptosis pathway showing relevant BCL_2 family members such as BAX, BIM and BAD. Figure also outlines the numerous caspases associated with the intrinsic pathway of apoptosis (Igney and Krammer, 2002).

Apoptosis involves several distinct morphological changes. Cells undergoing apoptosis display loss of cell-to-cell junctions as well as chromatin condensation and nuclear fragmentation. During involution or shrinking of the mouse mammary gland, apoptotic events mediate the controlled death of cells within mature breast tissue (Furth, 1999).

Hypothesis and Specific Aims

Based on these findings, it is possible that Fliz1 expression is regulated during involution of mammary gland tissue. Although mammary epithelial tissue differs from that of thymus tissue, both tissues have been shown to express GATA-3, which plays a central role in both differentiation and maintenance of thymus and breast tissue. My preliminary results showed that Fliz1 was clearly expressed in mouse mammary tissue. It is possible that binding of Fliz1 to the intronic regulatory region of GATA-3 prevents maintenance of mammary gland tissue and in turn results in increased levels of apoptosis followed by involution of the tissue. **I hypothesize that Fliz1 is expressed at heightened levels during mouse mammary gland involution following forced weaning of pups, and that this expression correlates with a decrease in GATA-3 levels**. The specific aims of the research were:

1. To determine if expression of Fliz1 is transcriptionally regulated during mammary gland involution, mRNA levels of Fliz1, GATA-3, and Cathepsin L were determined by quantitative RT-PCR and immunoblot. Expression was normalized to GAPDH.

Methods

Tissue Harvesting

Mammary tissue was harvested from BALB/c mice during lactation and at 24, 48, 72, and 96 hours post-lactation. Mammary gland tissue was also harvested at 12 days post-wean. Animals were sacrificed after forced weaning following humane and ethical guidelines consistent with Villanova University animal handling policies. Mice were sacrificed by CO_2 asphyxia and mammary tissue was promptly harvested, and flash frozen using liquid nitrogen to preserve RNA integrity, and then stored at -80 ° C for later RNA isolation. Mammary gland samples were harvested in triplicate from each time point, representing three individual mice for each time point indicated. All five mammary glands from each individual mouse were harvested and pooled together as individual samples from each mouse.

RNA Extraction

RNA isolation was performed with the use of TRI reagent (MRC). A total of 1mL of TRI reagent was used per 50-100mg of tissue sample. The tissues were first homogenized through manual grinding with the use of mortar and pestle. The homogenate was then allowed to sit for 5 minutes at room temperature. Phase separation was accomplished with addition of 0.1 mL bromochloropropane (BCP) reagent. The sample was then stored for 2-15 minutes at room temperature before centrifugation at 12,000g for 15 minutes at 4 °C. The aqueous layer was then transferred to an RNAase free 1.7 mL Eppendorf tube and 0.5 mL of cold isopropanol added. The sample was then vortexed and allowed to sit at room temperature for 10 minutes. The sample was then centrifuged a second time at 12,000 x g for 8 minutes, completing the

precipitation phase of the RNA extraction. Pellet formation was observed after removal of the sample from the centrifuge. The RNA pellet was then washed with 1 mL of 75% ethanol in DEPC-water and centrifuged a third time at 7,500 x g for 5 minutes at 4 °C. The supernatant was then removed and the sample allowed to air dry for 10 minutes. The pellet was then dissolved in DEPC-water. After incubation, Nanodrop spectrophotometer analysis was performed to assess the purity of RNA and to determine nucleic acid content. All isolated RNA samples were then stored at -80 °C for later cDNA synthesis.

cDNA Synthesis

Following RNA isolation the SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies) was used to convert isolated RNA into cDNA for later qRT-PCR amplification. The SuperScript® VILO™ cDNA Synthesis Kit is designed to maintain RNA linearity across a wide range of RNA inputs and is ideal for qRT-PCR analysis. A master mix consisting of 5X VILO™ Reaction Mix and 10X SuperScript® Enzyme Mix was combined with template RNA (1 µg) and DEPC-treated water to generate a 20 µL reaction. The reaction mixtures were gently mixed and the tube contents incubated at 25°C for 10 minutes in a thermocycler. Next, the tubes were incubated at 42°C for 60 minutes and the reaction terminated at 85°C for 5 minutes. Newly synthesized cDNA was then stored at –20°C until use.

PCR

Polymerase chain reaction was performed using EconoTaq Plus 2X Master Mix (Lucigen) on lactating mouse mammary gland cDNA, as well as 24, 48, 72 and 96 hour involuting mouse tissues. EconoTaq Plus 2X Master Mix utilized 25 µL reaction volumes and contained agarose

gel loading buffer. Primers used are shown in Table 1. Reactions performed on the thermocycler consisted of an initial 2 minute denaturation step at 94 °C, followed by 35 cycles of denaturation, annealing and extension. Denaturation was set at 94 °C for 30 seconds followed by annealing at 63°C for 30 seconds followed by extension at 72 °C for 30 seconds. Final extension was set at 72 °C for 5 minutes. Each reaction was set at 35 cycles.

qRT-PCR

Quantitative reverse transcriptase - polymerase chain reaction (qRT-PCR) was used to measure Fliz1, GATA-3 and Cathepsin L expression in lactating and involuting mouse mammary tissue. Mouse Fliz1, GATA-3 and Cathepsin L primers were used in conjunction with GAPDH as a control. A 1:32 cDNA dilution was used for all qRT-PCR runs performed in triplicate for each individual primer set: Fliz1, GATA-3 and Cathepsin L. Protocol for qRT-PCR amplification consisted of initial denaturation at 95 °C for 1 min followed by 39 cycles of denaturation, annealing and extension. Denaturation was set at 95 °C for 5 seconds followed by annealing. A gradient from 58 to 68 °C was used with annealing lasting for 30 seconds, followed by a plate read. The melting curve was set from 64 °C to 95 °C.

Primer	Sequence	Melting Temperature
Flizq1	Forward: 5'-TGAAGCGAAAGTGGCCTGGCACTGG-3' Reverse: 5'-TTGTGTTTCCGCAGTCAGCGGGGCG-3'	(T_m) 69.5 °C (T_m) 71.1 °C
Flizq2	Forward: 5'-CCGCCGACCCTGAGGAAAGAATTG-3' Reverse: 5'-GGAAGTAATGAGGGTTGAGCTGCGT-3'	(T_m) 68.0 °C (T_m) 66.2 °C
Cathepsin L	Forward: 5'-GCCATCCGTCTCTCCAGTTC-3' Reverse: 5'-CACTTCCCCAGCTGTTCTTGA-3'	(T_m) 64.5 °C (T_m) 62.6 °C
GAPDH	Forward: 5'-GACAACTTTGGCATTGTGG-3' Reverse: 5'-ATGCAGGGATGATGATCTTCTG-3'	(T_m) 58.0 °C (T_m) 58.0 °C
GATA3q1	Forward: 5'-GCTCAGCTTTCTCCTTTTGG-3' Reverse: 5'-GCTCAGAGACGGTTGCTCTT-3'	(T_m) 66.4 °C (T_m) 66.4 °C

Table 1. RT-PCR and qRT-PCR primer sets, sequences, and annealing temperatures.

qRT-PCR Quantification

In order to quantify changes in gene expression between lactating and involuting mouse mammary gland tissue, a relative quantification scheme was utilized. Relative quantification allows for the determination of changes in steady-state mRNA levels of a gene across multiple developmental samples. This expression is then normalized to that of an internal control such as GAPDH or Actin. Relative Quantification is a commonly employed quantification method when examining gene changes as part of a developmental profile. All samples were normalized to lactating mammary gland tissue. The ΔCT method uses calculations for the differences between the CT value of the reference gene and that of the target gene (Figure 4.).

$$2^{(CT(GAPDH)-CT(Fliz1))} = \text{Relative Expression}$$

Figure 4. Equation used to determine relative expression of Fliz1, GATA-3 and Cathepsin L in mouse mammary gland tissue samples. The CT value is the threshold value at which fluorescence rises above background.

Once the relative expression values have been determined for the control sample (lactating mammary gland) and each of the developmental samples (involuting tissues), the relative expression values are normalized to that of the lacating control sample to yield the expression relative to that of the control or lactating tissue (Figure 5).

Control(lactating)/Control(lactating) = Control Expression (1.0)

Sample(Involuting)/(Control(lactating)= Ratio of Expression to Control

Figure 5. Calculation scheme used to determine ratio of expression between lactating and involuting tissues. All samples were presented as a ratio to lactating control tissues.

Gel-Electrophoresis

Gel electrophoresis was performed after each qRT-PCR reaction to insure that a single amplicon was obtained at the correct molecular weight. The amplified product was run on a 1.2% Agarose gel in TAE for 60 minutes at 70 Volts. Gels were then imaged using an Alpha Innotech Alpha Imager (Cambridge Scientific).

SDS-PAGE and Western Blot

A rapid Bradford Assay was performed to determine protein levels in each sample. A total of 2 µL of protein extract was used and incubated for 5 minutes at room temperature. The spectrophotometer was then zeroed at 600 nm using a negative control consisting of 2 µL of lysis buffer. Each sample was then measured at A_{600}. The protein concentration was then calculated in µg/µL by multiplying the A_{600} by a factor of 10. After normalization of protein levels, samples were run on 4-12% Novex NuPage Bis-Tris mini gels (Life Technologies) for ninety minutes at 150 V. The transfer step was performed in an ice bath at 30 volts for eighty minutes on Immobilon P membrane (Millipore) using 1X NuPage transfer buffer (Life Technologies). The blot was then blocked in 10 mL of 5% milk solution. The milk solution was composed of 10 mL of TBST and 0.5 grams of powdered dry milk for 30 minutes at room temperature. The blot was then incubated overnight at 4° C with a rabbit polyclonal antibody ZC3H8 (Origene) at a 1:1,000 dilution in 5% TBST milk solution. The blot was then washed 3X with 10 mL TBST composed of Tris-buffered saline and Tween 20. Each wash lasted a total of 10 minutes. The blot was then

incubated with an anti-rabbit IgG Goat Polyclonal HRP conjugated secondary antibody (catalogue number AP187P) for 30 min at room temperature at a 1:20,000 dilution (Millipore). Next, the blot was washed 3X in 10 mL of TBST. After washing, the blot was incubated with 0.5 mL of Luminata Crescendo Western HRP substrate (Millipore) reagent for approximately five minutes and images were obtained on the Alpha Innotech Flurochem imager. Imaged blots were then subjected to densitometry analysis using ImageJ software. A rabbit polyclonal GATA-3 antibody (Santa Cruz) was also used. The same procedure was followed and a 1:1000 dilution for the primary antibody was also used. However, the secondary antibody dilution was at 1:10,000.

List of Antibodies

- **Fliz1**
- **Gene Name:** Rabbit polyclonal antibody ZC3H8 (zinc finger CCCH-type containing 8) from Origene **(TA309043)**
- **Gene Description:** *Homo sapiens* zinc finger CCCH-type containing 8 (ZC3H8)
- **Reactivity:** Human
- **Molecular Weight:** 43 kDa

- **Actin**
- **Gene Name**: ß-actin (13E5) Rabbit mAb monoclonal antibody from Cell Signaling (**4970S**)
- **Gene Description**: Detects endogenous levels of beta-actin protein
- **Reactivity:** Mouse
- **Molecular Weight**: 45 kDa

- **GATA-3**
- **Gene Name:** Rabbit polyclonal antibody GATA-3 raised against amino acids 167-214 of GATA-3 of human origin from Santa Cruz (**SC-9009**)
- **Gene Description:** Detects the zinc finger transcription factor GATA-3
- **Reactivity:** Reactive for human, mouse and rat
- **Molecular Weight:** 50 kDa

Table 2. Table showing description of antibodies with predicted molecular weights and species reactivity.

Statistical Analysis

Standard Error was performed using Sigma Plot graphing and analysis software. The Standard Error is defined as the measure of uncertainty based upon sampling, or as a measure of the variation of the sample points according to the equation:

$$SE = \frac{SD}{\sqrt{(n)}}$$

Results

In order to determine if Fliz1 is expressed in involuting mouse mammary tissue, it was first necessary to breed mice in order to generate pups. BALB/c mice were bred with prompt removal of males after-pregnancy was confirmed. Mammary glands of female mice were then harvested after forced weaning of pups. Tissue samples were extracted at 10 days lactation, and 24, 48, and 72, and 96 hours post wean, as well as 12 days post-lactation. Tissues were then used for isolation of RNA and protein and then fixed, embedded and processed for immunohistochemical analysis and apoptosis determination using Cathepsin L as a marker for involution.

Generalized Strategy of Molecular and Cellular Techniques Used

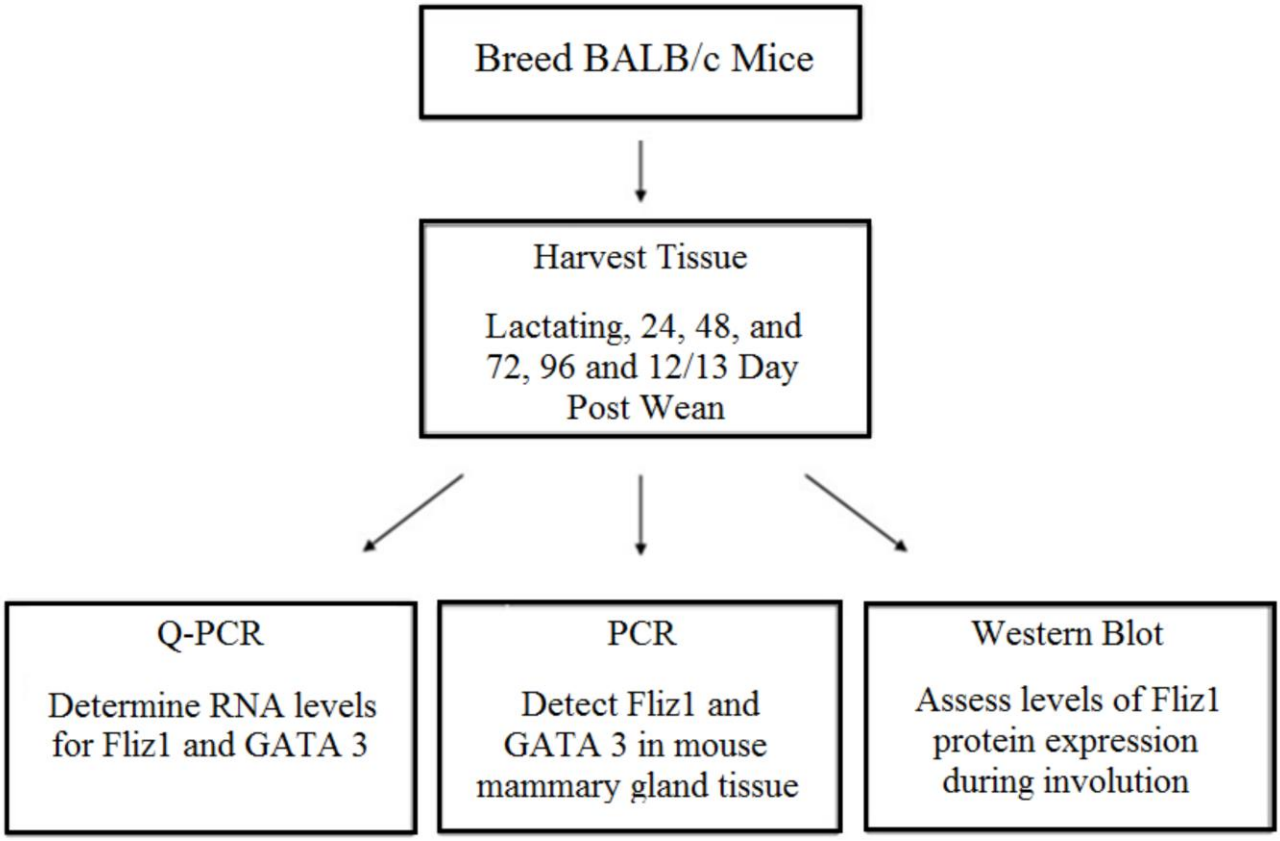

Figure 6. Generalized strategy outlining the molecular and cellular techniques utilized in the investigation of Fliz1 and GATA-3 expression in involuting mouse mammary gland.

In order to assess expression of Fliz1 and GATA-3 at the protein level I performed western blot analysis on isolated protein samples for lactating mouse mammary gland tissue as well as 24, 48, and 72 hour involuting tissue. Mouse fetal liver and extracts of HepG2 cells, a known human liver carcinoma cell line, were used as positive controls for Fliz1 expression. Fliz1 was detected in each of the tissue types probed with anti-Fliz1 antibody. Unfortunately, the signal remained weak in the lactating and involuting tissues, which required lengthy exposure times to allow for signal detection. Three blots, repeats of the same sample protein, did show increased Fliz1 expression in involuting mouse mammary gland tissue (Figure 7).

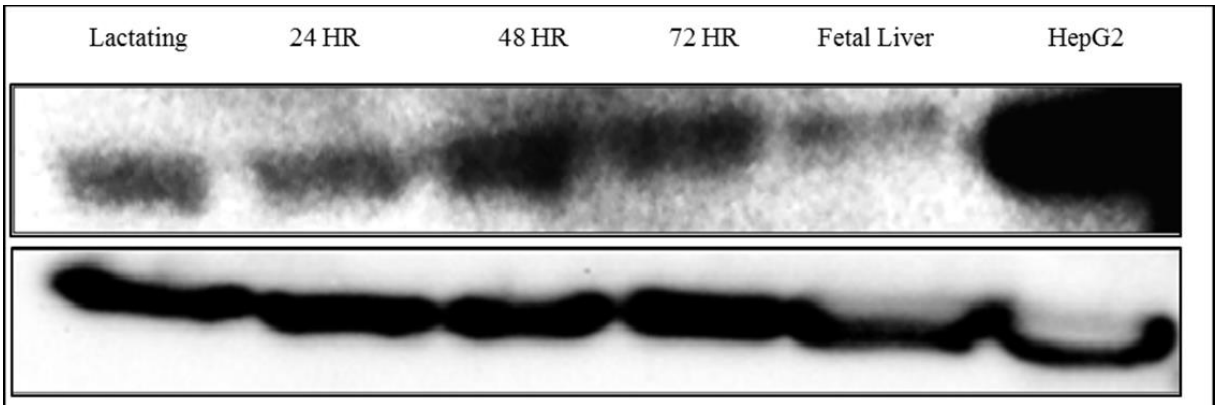

Figure 7. **Top Row:** Western Blot showing Fliz1 expression in lactating, 24, 48, 72 hour involuting mouse mammary gland tissue as well as in mouse fetal liver. Human hepatocellular carcinoma (HepG2) cells were used in conjunction with mouse fetal liver as a positive control. **Bottom Row:** Shows β-Actin expression in stripped and re-probed blot.

Fliz1 signal was detected not at the 34 kDa size predicted by the manufacturer but rather at 43 kDa. Fliz1 signal was also detected weakly in fetal liver, the murine positive control, and strongly in the HepG2 isolate. Quantified Fliz1 data is shown in Figure 8 and raw Fliz1, data in Table 3.

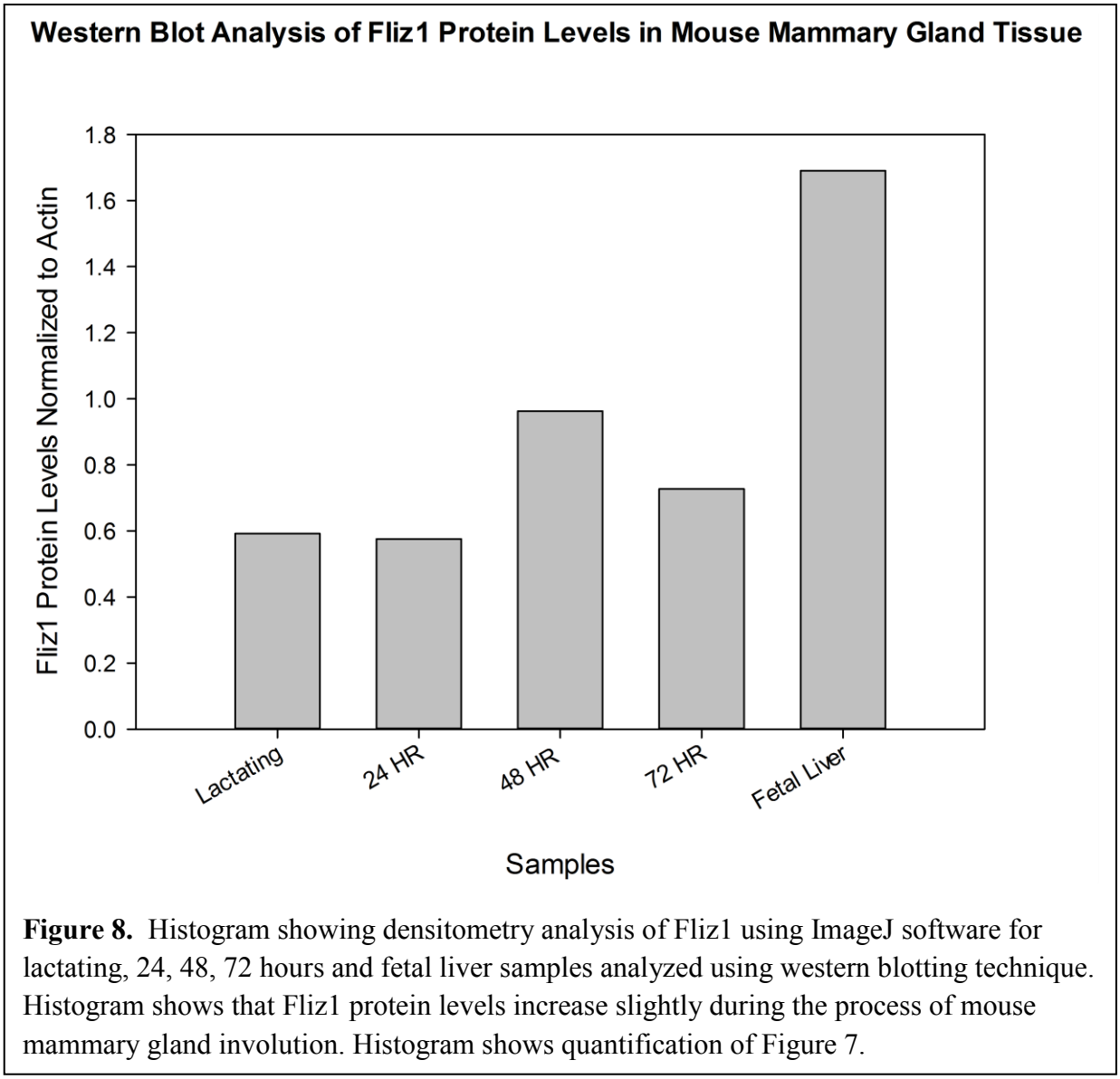

Figure 8. Histogram showing densitometry analysis of Fliz1 using ImageJ software for lactating, 24, 48, 72 hours and fetal liver samples analyzed using western blotting technique. Histogram shows that Fliz1 protein levels increase slightly during the process of mouse mammary gland involution. Histogram shows quantification of Figure 7.

Western Blot Data for Fliz1 Normalized to β-Actin

Sample	WB 1	WB 2	WB 3	Average	Standard Error
Lactating	1.052	0.905	0.5919	0.84963	0.234993837
24 HR	1.06	1.042	0.575	0.89233	0.274966059
48 HR	1.127	0.9186	0.963	1.00287	0.109770913
72 HR	0.8658	1.15	0.727	0.91427	0.215624705
Fetal Liver	0.903	*******	1.69	1.2965	0.556493037

Table 3. Western Blot data normalized to β-Actin using ImageJ software. Runs were completed in triplicate and then averaged and analyzed for standard error. The positive control fetal liver was only performed in duplicate as indicated by the asterisks.

GATA-3 expression at the protein level was also assessed using Western Blot analysis. Signal for GATA-3 was detected at 50 kDa, the size specified by the manufacturer in both lactating and involuting mouse mammary gland tissues. GATA-3 expression was also detected in murine fetal liver after probing with anti-GATA-3 antibody. The western blot finding demonstrates that GATA-3 is highest in lactating mammary gland tissue, but during the process of involution GATA-3 expression decreases (Figure 9).

Western Blot Analysis of GATA-3 and ß-Actin

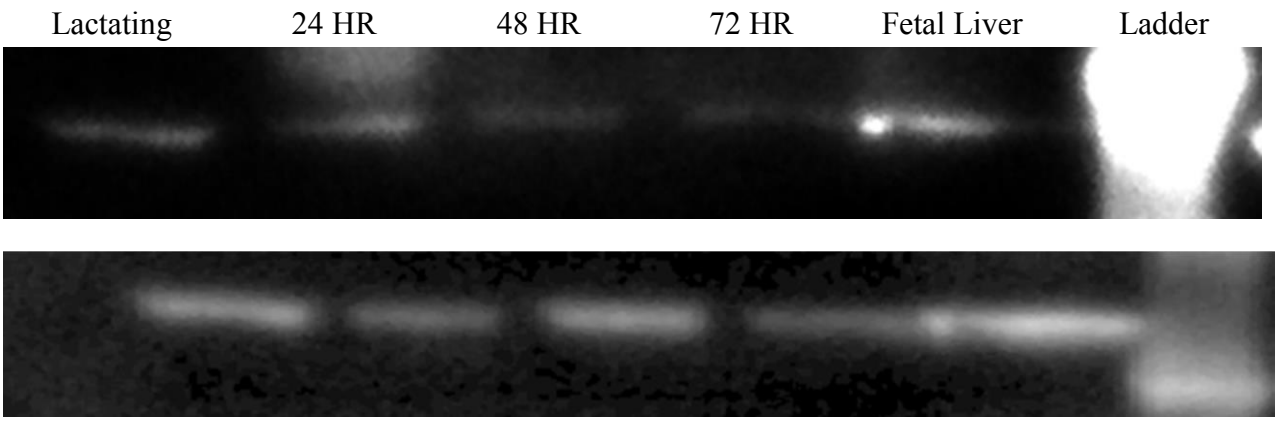

Figure 9. Top Row: Western Blot showing GATA-3 expression in lactating, 24, 48, 72 hours and fetal liver control. **Bottom Row:** Stripped and re-probed blot showing β-Actin in lactating 24, 48, 72 hour and fetal liver. Blot shows a decrease in GATA-3 protein levels in involuting mouse mammary gland tissue.

This finding is consistent with the qRT-PCR finding indicating decreased GATA-3 expression at the transcript level during involution and is also in support of my hypothesis.

The zinc finger transcription factor Fliz1, is known to be expressed during early embryonic development in the mouse and plays a known role in murine hematopoiesis. Fliz1 has also been shown to be expressed in the thymus at heightened levels during thymic involution in humans. In order to assess if Fliz1 is expressed in mouse mammary gland tissue, polymerase chain reaction was carried out using Fliz1 specific primers on cDNAs isolated from lactating mouse mammary gland as well as 24, 48, 72 and 96 hour involuting mouse mammary gland tissue. Stringent full length β- Actin primers were then used as a control during the reaction to assess cDNA quality. PCR analysis yielded a single Fliz1 amplicon at 303 base pairs consistent with predicted BLAST analysis. PCR analysis revealed that Fliz1 is expressed in both lactating and involuting mouse mammary gland tissues, as can be seen in Figure 10. However, the ability to resolve differences in Fliz1 expression between the tissue types at 35 cycles of amplification was not possible. Consequently, it was necessary to carry out qRT-PCR analysis on the developmental profile using qRT-PCR specific primers.

PCR Showing Fliz1 Expression in Mouse Mammary Gland Tissue

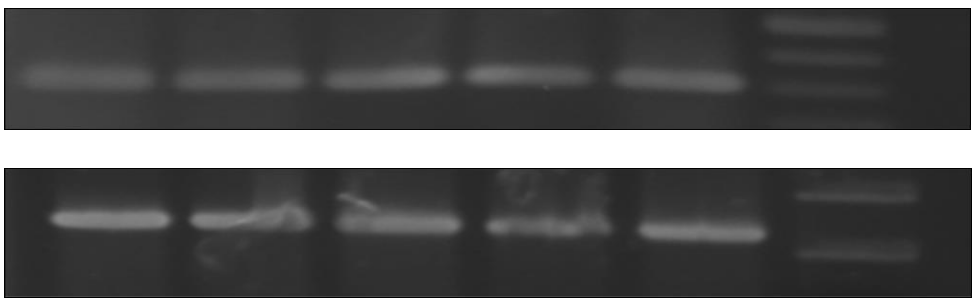

Figure 10. Top Row: Agarose gel showing Fliz1 (303 bp) PCR amplicon derived from lactating mouse mammary gland cDNA as well as 24, 48, 72 and 96 hour involuting mouse mammary gland. **Bottom Row**: Full length β- Actin (1.2 kb) amplicon for lactating, 24, 48, 72 and 96 hour mouse tissues.

In order to resolve differences in expression between Fliz1 and GATA-3 in normal lactating mouse mammary gland tissue and during involution, qRT-PCR analysis was carried out using qRT-PCR specific primer sets for Fliz1, GATA-3 and Cathepsin L. The cysteine protease Cathepsin L was used as a marker for involution and performed on each cDNA to ensure that tissue samples isolated from mice were indeed undergoing involution (Figure 11). Quantification of qRT-PCR results were also performed on Cathepsin L as can be seen in Figure 12. Quantification of qRT-PCR data for GATA-3 showed a decrease in GATA-3 transcript levels during involution up to 72 hours (Figure 13).

qRT-PCR Gel of Cathepsin L
A Known Marker of Mouse Mammary Gland Involution

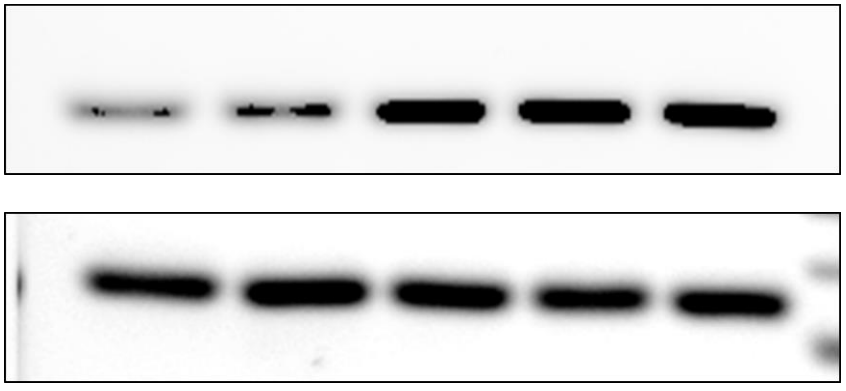

Figure 11. **Top Row**: qRT-PCR gel showing up-regulation of Cathepsin L (159 bp) in lactating, 24, 48, 72 and 96 hour mouse mammary gland tissue. **Bottom Row**: GAPDH control in lactating 24, 48 72 and 96 hour involuting mouse mammary gland tissue. Cathepsin L is a known marker of mouse mammary gland involution and was used as a control to ensure that tissues were indeed undergoing involuting.

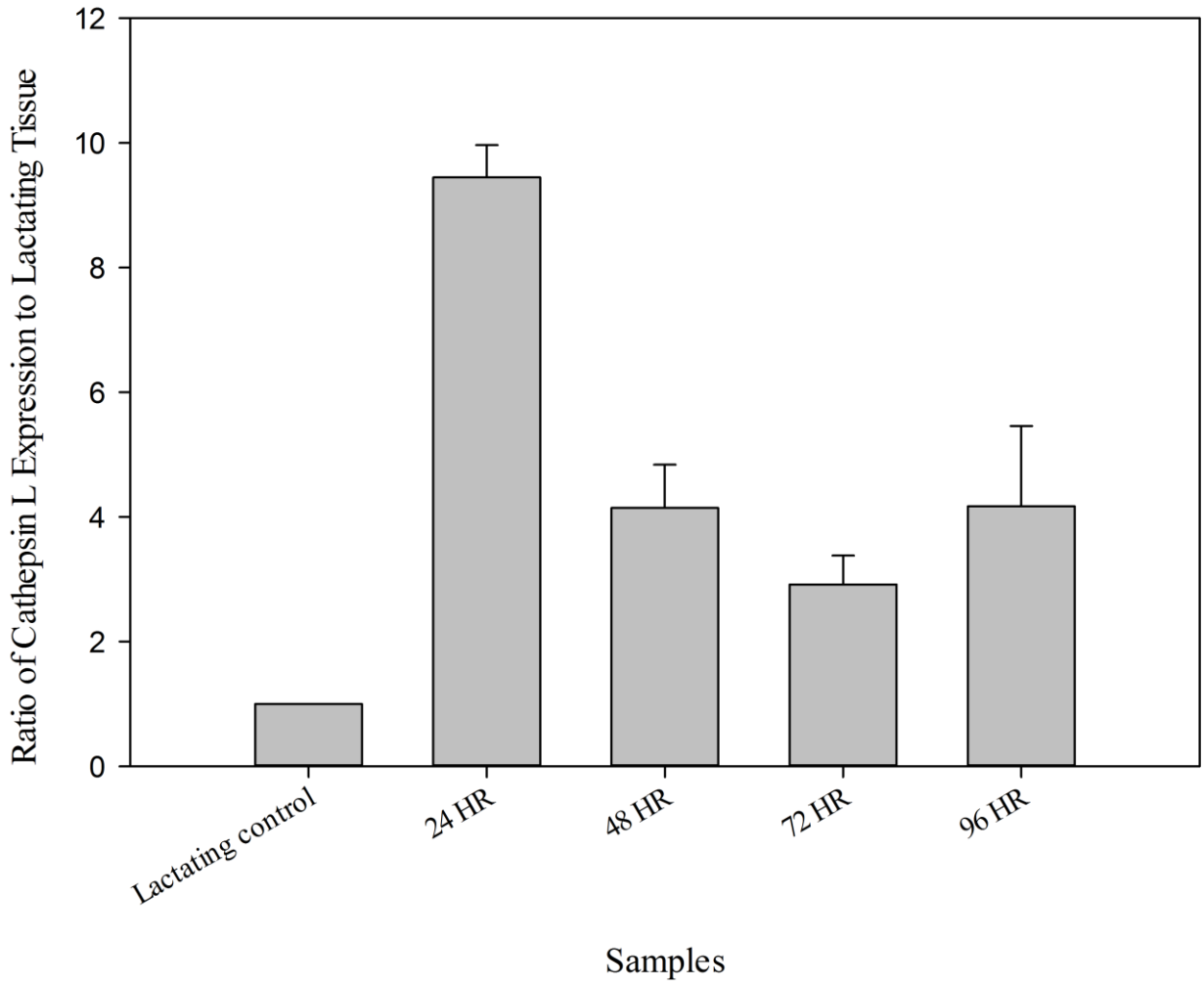

Figure 12. Histogram showing normalized qRT-PCR Cathepsin L data for lactating, 24, 48, 72 and 96 hour involuting mouse mammary gland samples. All involuting samples are shown relative to lactating mammary gland tissue. Histogram demonstrates that involuting tissues are indeed undergoing involution based on increased Cathepsin L expression relative to that of lactating tissue.

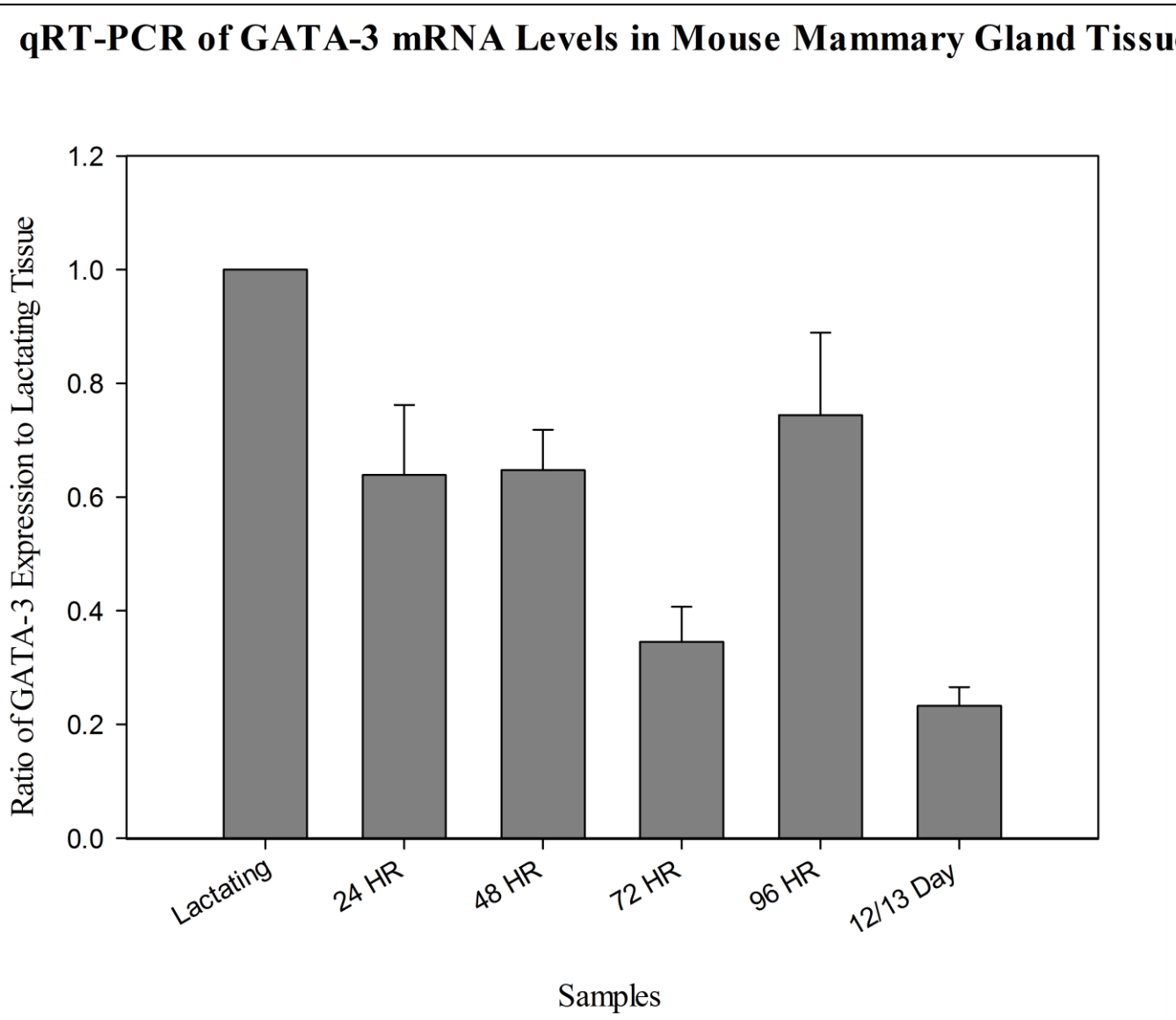

Figure 13. Histogram showing normalized qRT-PCR data for lactating, 24, 48, 72, 96 and 12/13 day involuting samples. All involuting samples are shown relative to lactating mammary gland tissue, which shows the highest levels of GATA-3 transcript. Histogram shows that GATA-3 mRNA levels decrease during the process of mouse mammary gland involution.

At 96 hours involution, the level of GATA-3 did increase, but never reached lactating levels. The 12 day involuting sample showed the lowest level of GATA-3 expression of any of the involuting samples harvested and analyzed through qRT-PCR analysis. The normalized qRT-PCR data for both Fliz1 and GATA-3 are shown in Table 4.

Table of Normalized Data for GATA-3, Fliz1 and Cathepsin L

	cDNA1			cDNA2			cDNA4					
GATA-3	**Run 1**	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**	**Average**	**Std Dev**	**Std Error**
lactating control	1	1	1	1	1	1	1	1	1	1	0	0
24 HR GATA	0.55	0.6028	0.437	0.593	0.5437	0.395	0.7572	1.1	0.77	0.638744	0.213431	0.123224
48 HR GATA	0.586	0.6363	0.425	0.7675	0.717	0.5632	0.65104	0.85	0.63	0.647338	0.123022	0.071027
72 HR GATA	0.369	0.239	0.236	0.554	0.4083	0.415	0.2708	0.363	0.25	0.345011	0.106733	0.061622
96 HR GATA	0.727	0.774	0.5944	1.33	0.901	0.6556	0.5416	0.675	0.5	0.744289	0.25117	0.145013
12 Day GATA	0.255	0.284	0.192	0.301	0.25	0.291	0.1407	0.2069	0.173	0.232622	0.056836	0.056836
	cDNA1			**cDNA2**			**cDNA4**					
FLIZ1	**Run 1**	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**	**Average**	**Std Dev**	**Std Error**
lactating control	1	1	1	1	1	1	1	1	1	1	0	0
24 HR Fliz	0.656	0.737	0.73	0.466	0.385	0.34	0.387	0.766	0.517	0.553778	0.16999	0.098144
48 HR Fliz	0.41158	0.66	0.418	0.459	0.322	0.363	0.812	0.598	0.517	0.506731	0.15776	0.091083
72 HR Fliz	1.777	1.21	0.717	0.44	0.37	0.364	0.737	1.326	1.32	0.917889	0.507454	0.292979
96 HR Fliz	1.198	1.243	0.327	0.5069	0.22	0.242	0.578	1.91	0.586	0.756767	0.572539	0.330556
12 Day Fliz	1.192	1.53	0.98	0.265	0.245	0.21	1.619	2.84	0.784	1.073889	0.852848	0.492392
	cDNA1			**cDNA2**			**cDNA4**					
Cathepsin L		**Run 1**			**Run 1**			**Run 1**		**Average**	**Std Dev**	**Std Error**
lactating control		1			1			1		1	0	0
24 HR CTL		9.98			9.9513			8.41		9.4471	0.89827	0.518616
48 HR CTL		5.46			3.85			3.12		4.143333	1.197261	0.691239
72 HR CTL		2.51			2.4			3.84		2.916667	0.801519	0.462757
96 HR CTL		6.6359			3.59			2.29		4.171967	2.230633	1.287857

Table 4. qRT-PCR data for GATA-3, Fliz1and Cathepsin L normalized to GAPDH. A total of three cDNAs were made for each time point then qRT-PCR runs performed in triplicate on each cDNA set with the exception of Cathepsin L. Data from each run and cDNA was averaged. Statistical significance was then assessed using standard deviation and standard error.

Expression of the fetal liver zinc finger transcription factor, Fliz1, did not show an increase above lactating mammary gland tissue during the first 48 hours of mammary gland involution (Figure 14). However, levels of Fliz1 transcript did return to those of lactating mammary gland in the 12 day involuting tissue sample. Comparison of Fliz1 and GATA-3 levels as a ratio to that of lactating tissue can be seen in the composite (Figure 15).

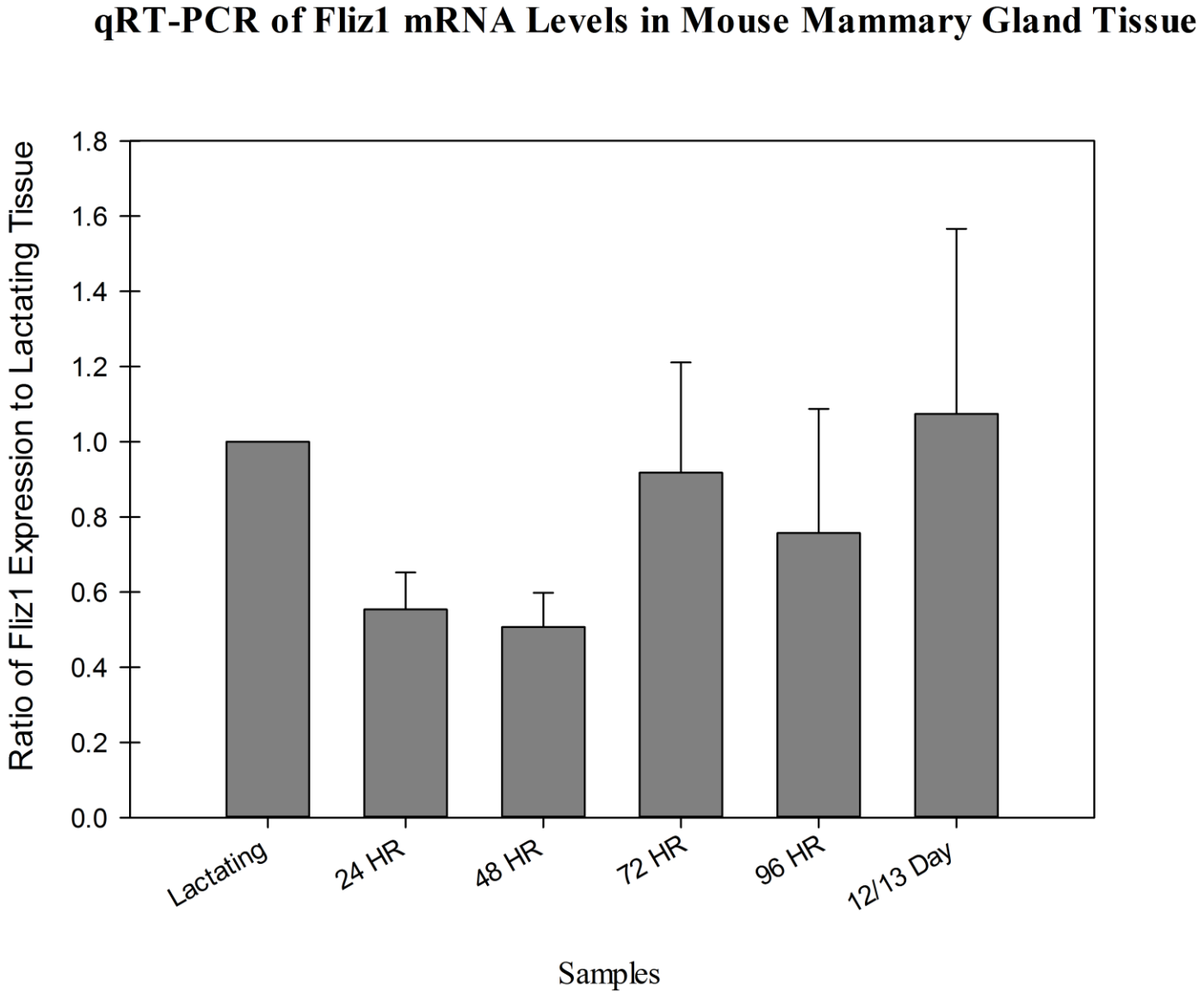

Figure 14. Histogram showing normalized qRT-PCR data for lactating 24, 48, 72, 96 hour and 12 day involuting tissues samples. All involuting samples are shown relative to lactating mammary gland tissue. Data indicates that Fliz1 expression does not noticeably increase during mouse mammary gland involution. Histogram shows that Fliz1 mRNA levels do not increase substantially during involution.

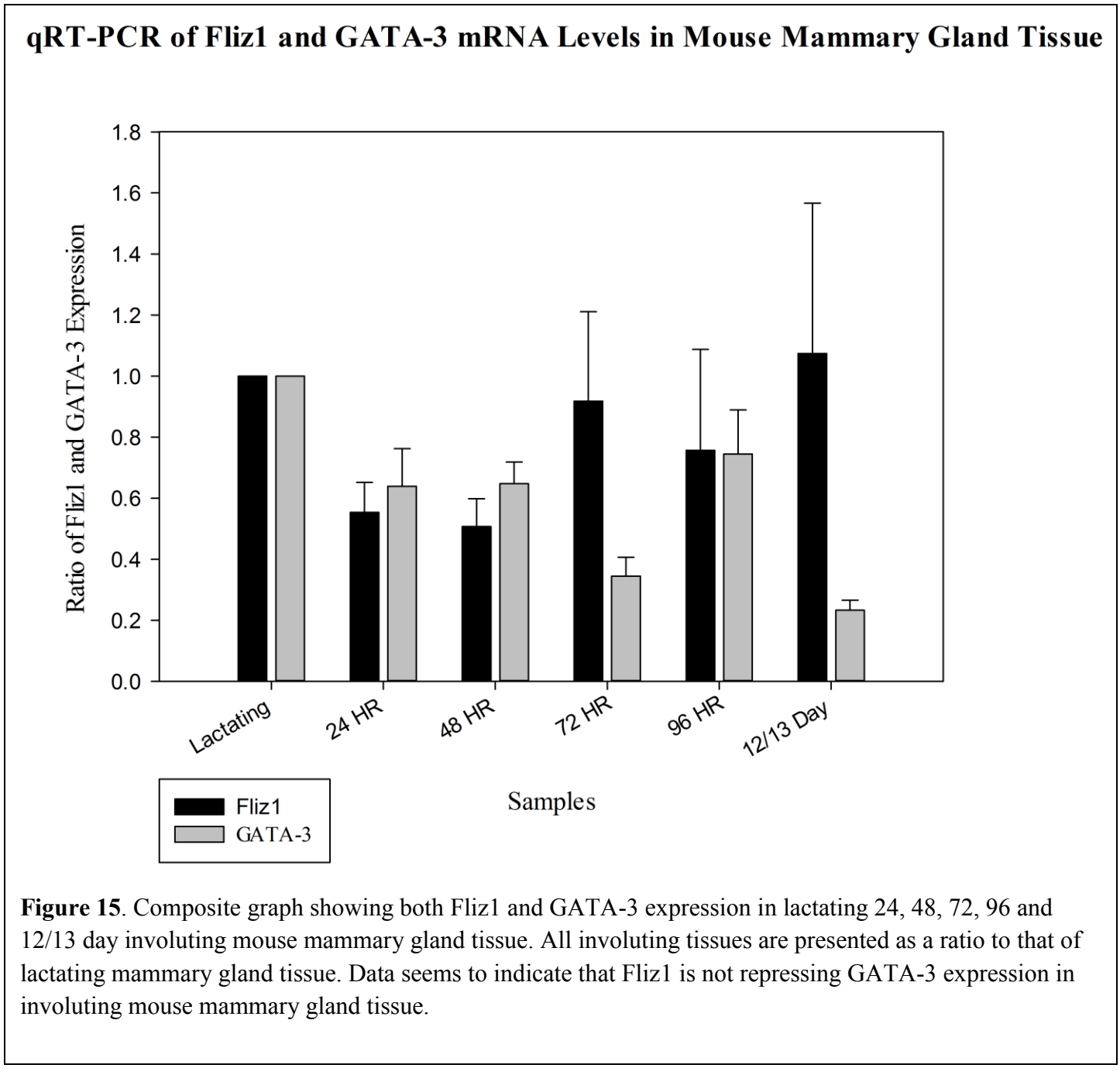

Figure 15. Composite graph showing both Fliz1 and GATA-3 expression in lactating 24, 48, 72, 96 and 12/13 day involuting mouse mammary gland tissue. All involuting tissues are presented as a ratio to that of lactating mammary gland tissue. Data seems to indicate that Fliz1 is not repressing GATA-3 expression in involuting mouse mammary gland tissue.

Discussion

The increase in Fliz1 expression at the protein level seemed promising, given that the rise in Fliz1 expression was coupled with a decrease in GATA-3 expression. This finding seemed to support the conclusions made by another lab exploring the role of Fliz1 in the thymus, which showed that increased Fliz1 expression led to thymocyte hypocellularity (Hwang and Ho, 2002). This increased Fliz1 expression was coupled with a concomitant loss of GATA-3 expression and thymic hypocellularity (Hwang and Ho, 2002). This finding also seemed to indicate that a potential link existed between increased Fliz1 expression and decreased GATA-3 expression leading to apoptotic events in the mouse mammary gland and subsequent involution. However, one of the major concerns regarding the western blot analysis involved the long exposure times required to detect Fliz1 signal. Long exposure times did result in background. This was fortunately not the case for the GATA-3 antibody. The Fliz1antibody, which is designed for detection of Fliz1 expression in human tissue did show cross-reactivity with mouse mammary gland tissue extracts, albeit at low levels. Nevertheless, signal was detected multiple times, and one of the analyzed blots did show an increase in Fliz1 expression, most noticeably at 48 and 72 hours of involution. The collated western blot data for Fliz1 (Table 3) did show a small increase in Fliz1 protein levels during involution from that of lactating tissue. Given these findings, I proceeded to perform RT-PCR using Fliz1 specific primers in order to determine if Fliz1 is expressed at the transcript level. RT-PCR analysis showed expression of Fliz1 in a variety of tissue types such as lactating mammary gland, as well as my involuting profile.

Detection of Fliz1 mRNA was also achieved in mouse embryo, pregnant mammary gland tissue, as well as in mouse tumor. The ability to detect Fliz1 using RT-PCR at 35 cycles of

amplification was encouraging. However, differences in expression between the lactating sample, and those of the involuting tissues could not be resolved visually using RT-PCR. The data generated during qRT-PCR analysis on lactating and involuting mouse mammary gland tissues did reveal a decrease in GATA-3 transcript levels during involution of the mouse mammary gland. The lowest levels of GATA-3 were detected at 72 hours of involution, but GATA-3 transcript levels begin to decline soon after the cessation of lactation. This is not surprising given that soon after the end of lactation and during the early stages of involution; there is a loss of the ductal glandular network in the mammary gland, which is coupled with apoptotic events (Kouros-Mehr et al., 2006). Given that GATA-3 expression is required for proper morphogenesis and maintenance of the mammary gland during both development and adulthood it is of little surprise that GATA-3 levels are low during the more pronounced periods of involution notably at 48 and 72 hours post-lactation. Interestingly, there did appear to be an increase in GATA-3 levels at 96 hours followed by a substantial decrease in the 12 day involuting sample. It is possible that GATA-3 levels cycle during the two stages of mammary gland involution.

The qRT-PCR analysis for Fliz1 did not reveal the increase in expression that was hypothesized. Transcript levels for Fliz1 remained highest in lactating mammary gland tissue, and showed a decrease during 24 hours post- lactation followed by an almost stepwise increase up to 72 hours. However, during the period of involution from 24 hours to 72 hours, Fliz1 transcript levels did not exceed those of lactating mammary gland samples. These data indicate that Fliz1 is not a major driver of mammary gland involution in the mouse. This finding also makes the link between Fliz1 negatively down-regulating GATA-3 during normal, physiological mammary gland involution unlikely. At this point, it seems that Fliz1 has yet to have a defined

function in mouse mammary gland tissue.

The observed increase in Fliz1 expression at the protein level is interesting given that the same trend is not seen at the transcript level for Fliz1. Fliz1 levels initially decrease after the end of lactation and slowly return to lactating levels by 72 hours post-wean. This inconsistency could potentially be due to limited sampling, in that the immunoblot analysis was performed only on one developmental series. Although the individual samples were probed three times, it will be necessary to reproduce the result with new a new developmental series taken from a new set of mice in order to assign any significance to the finding. Another potential source of error stems from the use of the Fliz1 antibody. The antibody is designed for detection of human Fliz1 protein and not mouse. Although the antibody does show cross reactivity, between species, the signal intensity remains weak despite prolonged exposure times. Hopefully, a commercial Fliz1 antibody designed for use on mouse tissue becomes available.

Analysis by qRT-PCR was performed in triplicate for each cDNA synthesized. A total of three cDNAs were isolated for each individual time-point and the qRT-PCR reactions for Fliz1, and GATA-3 was performed in triplicate as well. Performing each reaction in triplicate helped minimize instrument and technical error in the collated data, and allowed for comparison between samples using standard error. One cDNA series that was isolated did show abnormally low Fliz1 transcript levels. The Fliz1 data also showed variation between runs in a single cDNA sample. This variation could be due to pipetting error or potentially instrument error. This data series should be repeated in the future. However, GATA-3 qRT-PCR runs showed very consistent Ct fluorescent values above background. As a result, the standard error for the collated GATA-3 qRT-PCR data is lower than that of Fliz1.

Future Directions

Determination of the role of Fliz1 in normal development, and during mouse mammary gland involution might best be assessed using conditional knockout mice for Fliz1. The use of transgenic knockouts could provide invaluable insight regarding the function of Fliz1, not only in the mammary gland, but also potentially in hematopoiesis as well as in tumorigenesis. The latter being especially true given that Fliz1 is involved in lineage commitment during hematopoiesis. Consequently, there exists the potential that mice lacking Fliz1 expression might be more susceptible to a number of leukemias. Alternatively, the use of transgenic overexpressing mice for Fliz1 could reveal negative Fliz1 regulation of GATA-3. This method has been done before for other transcription factors such as FoxA1. Transgenic overexpressing FoxA1 have been shown to down regulate GATA-3 expression (Theodorou et al., 2013). The same could possibly hold true for Fliz1 and GATA-3. Although physiological expression of Fliz1 might not be enough to definitely down regulate GATA-3, transgenic overexpression might. Western blot analysis should be continued. Hopefully a commercial antibody, designed specifically for use on mouse tissue will be available for Fliz1 in the near future. Also, in order to assign any statistical relevance to the western blot findings it will be necessary to extend the study to multiple animals. Immunohistochemical analysis should also be continued. There is the possibility that Fliz1 expression becomes localized to areas undergoing apoptosis in the mammary gland. Perhaps a small sub-set of cells within the mammary gland begin to up-regulate Fliz1. These changes might not necessarily be detectable biochemically with standard assays such as PCR and western blot. It might be that despite there being no overall changes in the expression of Fliz1 at the transcript level; the physiological changes might be mediated by expression of Fliz1 amongst a small subset of epithelial cells in the ductal network of the mammary gland. Changes in

GATA-3 expression could relate to a loss of epithelial cells within the involuting mouse mammary gland. It is also a possibility that Fliz1 is regulating GATA-3 levels in the epithelial cells of the mammary gland. In order to determine, if Fliz1 is a regulator of GATA-3 in mouse mammary epithelial cells, it will be necessary to show co-localization of Fliz1 and GATA-3 in epithelial cells during involution of the mammary gland. Determination of Fliz1 as a negative regulator of GATA-3 could be accomplished through more extensive immunohistochemical analysis.

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