

Effects of varying energy intakes on mammary growth and development in prepubertal heifers

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Thesis submitted to the Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
in
Dairy Science

Committee

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May 9th, 2003
Blacksburg, VA

Key words: heifers, mammary, nutrition, estrogen receptor, extracellular matrix

ABSTRACT

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Rapid rearing of dairy heifers during late prepuberty has been linked to impairments in mammary development and reductions in milk yield. Our objective was to determine how varying energy intakes between 2 and 14 wk of age affect mammary parenchymal development. At 2 wk of age, Holstein calves were assigned to 1 of 4 treatments (HH, HL, LH, and LL) with 2 levels of energy intake (High or Low) and 2 periods of growth (2 to 8 and 8 to 14 wk of age). At 14 wk, parenchyma at the stromal interface, mid-gland, and above the cistern were collected, fixed, and embedded in paraffin. Digital images of stained sections were used to determine tissue composition (% epithelium, lumen, and stroma). Immunohistochemistry revealed estrogen receptor (ER) and Ki67 (nuclear proliferation antigen) positive cells, type IV collagen, fibronectin (FN), and laminin. Images representing 4 increasing grades were used to quantify ECM protein deposition. Luminal and stromal areas were $3.5 \pm 1.4\%$ higher ($p < 0.01$) and $4.0 \pm 1.7\%$ lower ($p < 0.01$), respectively, in HH and HL heifers. Ki67 labeling in terminal ductular units and subtending ducts was $2.1 \pm 0.8\%$ ($p < 0.01$) and $1.4 \pm 0.7\%$ ($p < 0.05$) lower for the same feeding level combination. FN deposition was also increased ($p < 0.05$) in HH and HL heifers. High rates of gain between 2 and 14 wk of age resulted in greater luminal area and reduced cell proliferation in mammary parenchyma at 14 wk of age. Changes in FN deposition could have mediated growth differences.

ACKNOWLEDGEMENTS

I would like to thank everyone who was involved with this thesis. Especially, I would like to thank Dr. Mike Akers, who has been a great advisor and a great scientist from whom to gain research experience from. I have genuinely enjoyed my time as Dr. Akers's graduate student. Also, thanks to the rest of my committee, Drs. Ron Pearson and Rick Howard, for all of their help and support pertaining to my studies and research.

The animal work for this thesis was carried out at Michigan State University, and I would like to thank everyone there that was involved. Special thanks to Erin Brown, Miriam Weber-Nielsen, and Mike Vandehaar. Erin's thesis was a product of the same animal study, and I obtained valuable information about our heifers from the work that she performed.

Thanks to all faculty and staff at the Virginia Tech Department of Dairy Science. The friendliness and helpfulness of everyone there has made my time as a graduate student very enjoyable. Pat Boyle, Karen Getzowitz, Chris Fletcher, and Kristy Daniels all helped out with work for this thesis, and their participation is greatly appreciated. To all the graduate students, thanks for all of the great times had outside of Litton-Reaves, it's been fun. Pat, thanks for the constant supply of fresh coffee and good times in the lab.

All of my friends and family have been extremely supportive during my years at Virginia Tech. Mom and Dad, your support, as well as occasional financial assistance, has been there from day one, and I could not have done it without you. Jessica, we have been side by side throughout our studies. Thank you so much for the love and support that always made the tough days seem a little less tough.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
ADG	average daily gain
BM	basement membrane
bST	bovine somatotropin
BW	body weight
Col I	type I collagen
Col IV	type IV collagen
CP	crude protein
d	day
DDR	discoidin domain receptor
DM	dry matter
ECM	extracellular matrix
EGF	epidermal growth factor
ER	estrogen receptor- α
ER ⁺	ER-positive
FGF	fibroblast growth factor
FN	fibronectin
GAG	glycosaminoglycan
GH	growth hormone
h	hour
H	high
HGF	hepatocyte growth factor
HSPG	heparan sulfate proteoglycan
IGFBP	insulin-like growth factor binding protein
IGF-I	insulin-like growth factor-I
kg	kilogram
KGF	keratinocyte growth factor
Ki67 ⁺	Ki67-positive
L	low
LM	laminin
MFP	mammary fat pad
min	minute
ml	milliliter
mm	millimeter
MMP	matrix metalloproteinase
OVX	ovariectomized
PBS	phosphate buffer saline
PR	progesterone receptor
PRL	prolactin
PTHrP	parathyroid hormone-related protein
s	second
Sdc1	syndecan-1
SUB	subtending duct
TDU	terminal ductular unit

TEB	terminal end bud
TGF β	transforming growth factor β
Timp	tissue inhibitor of matrix metalloproteinase
wk	week
WT	wild-type

LITERATURE REVIEW

Overview of Heifer Mammary Gland Development

The bovine mammary gland is an organ that undergoes dramatic cellular and morphogenic changes during its development. It is unique in that most of this development occurs after birth. Mammary epithelial cells (which will eventually form mature secretory alveoli) are derived from the embryonic ectoderm under the influence of the mammary mesenchyme, and by birth the beginning of the ductal system exists (Cunha 1994). At this point, there are a few rudimentary precursors for large mammary ducts next to the gland cistern, but neither terminal ducts nor alveoli are present (Sejrsen 1994), so the parenchymal tissue is rather underdeveloped. In contrast, the circulatory system and mammary stromal fat pad are almost fully developed at birth (Sejrsen 1994). These elements create a miniature version of the shape of the fully developed gland. Isometric growth of the gland continues after birth until approximately 3 months of age (Sinha and Tucker 1968), and during this period the non-epithelial tissue develops rapidly (Sejrsen 1994). Following this isometric growth phase, an allometric growth phase begins that is defined by rapid growth of the parenchymal tissue and ductal elongation (Sejrsen 1994). This phase of growth lasts throughout the first few estrous cycles, and then growth once again becomes isometric in nature. The next distinct phase of mammary development occurs following conception, and is marked by further ductal elongation and the formation of alveoli after mid-gestation (Tucker 1981). Epithelium has completely penetrated the fat pad at the end of gestation, and the secretory cells of the alveoli synthesize and secrete milk products (Tucker 1981). Following lactation, the

mammary gland enters a period of growth known as involution. This period is characterized by loss of secretory activity, decreased size of alveoli, and renewal of senescent epithelial cells (Capuco and Akers 1999).

As evidenced by the preceding description of mammary gland development, significantly more development and differentiation occurs in the mammary gland during gestation compared to the prepubertal and pubertal growth phases. However, there is a strong correlation between number of secretory cells and milk production (Tucker 1981), consequently, pre-pubertal mammary development must not be overlooked as an important factor in determining future milk yield. This assumption can be made due to the fact that prepubertal development is a period where precursors of mature secretory cells and ducts are actively growing. Discovery of means by which prepubertal mammary gland development can be optimized, such as ideal feeding level and rate of gain, are therefore relevant in determining the success of a mature dairy cow as it pertains to milk production.

Wiseman and Werb (2002) suggest that there are two mechanistically distinct processes involved in creation of branching ductal structures in the mammary gland. These processes have been outlined based on a mouse model, but may apply to bovine mammary development also. The first distinct process is terminal end bud (TEB) bifurcation. The TEB is the distal branching ductular structure present during development and is somewhat analogous to the bovine terminal ductular unit (TDU). TEB bifurcation involves the distal epithelial cells of the TEB that directly contact

mammary stroma through a sparse basement membrane (BM), and results in stromal extracellular matrix (ECM) being deposited to form a cleft at the site of bifurcation. The second process of mammary ductular branching involves sprouting of side branches from mature ducts. During this process, side branches extend through the myoepithelial cell layer, degrade the BM surrounding the mature epithelial ducts, and invade a periductal layer of fibrous stromal tissue. Interestingly, in the prepubertal bovine mammary gland, TDU are already highly branched and myoepithelial cells are not in evidence (Ellis and Capuco 2002). Regardless, there are likely important parallels in mechanisms of ductular elongation between rodents and ruminants.

Histology of Prepubertal Bovine Mammary Tissue

The growing mammary duct of the prepubertal calf is surrounded by stroma, which is a sheath of loose connective tissue (Capuco et al. 2001b). Few adipocytes are scattered throughout the densely packed stromal cells and stromal tissue. There is extensive ductal branching occurring along with elongation of the mammary duct (Capuco et al. 2001b), and cross-sectioned views of these radiating epithelial structures have a scalloped appearance, suggesting a complex tubular structure (Akers 2002). This type of duct development is in opposition to morphogenesis of prepubertal murine mammary tissue, where mammary ducts elongate without significant side branching (Capuco et al. 2001b). As the parenchymal tissue penetrates into the fat pad of the prepubertal mouse, TEB are present at the distal ends of growing ducts (Akers 2002). These end buds have a club-like appearance and their activity is necessary for the advancement of mammary ducts into the mammary fat pad (Akers 2002). During ductal elongation, there is considerable branching of the mammary ducts into a collagenous matrix of loose connective tissue that separates the epithelium from the adipocytes (Cardiff and Wellings 1999). In contrast, in the peripubertal ruminant mammary gland the developing ducts radiate from the gland cistern in a much more compact fashion. This pattern can be described as broccoli-like, with ductular elements closely aligned. Capuco et al. (2001b) have referred to these distal branching ductular structures as TDU. More mature supporting ducts are referred to as subtending ducts (SUB). These distinct morphological differences in mammary development between rodents and ruminants suggest that there are also likely important differences in mechanisms controlling development. Thus, despite extensive study of rodent mammary development, it is evident that results obtained in rodent models cannot

be uncritically applied to the bovine. This means that basic research must also be completed with ruminants. A photomicrograph of a histological section of the mammary gland of a prepubertal heifer is shown in Figure 1.1, and various structures within the developing mammary gland are illustrated.

Endocrine Control of Mammary Growth

Role of Estrogen in Mammary Gland Development

It has been clear for many years that normal mammary development in the heifer requires the ovary and its primary secretory product estrogen. This is evident from research reported by Wallace (1958). Jersey and Aberdeen Angus calves were ovariectomized (OVX) within a week of birth, and then slaughtered at 6 months of age to assess mammary development. The OVX calves demonstrated total failure in mammary gland development. This was characterized by an unextended ductal system that resembled that of bulls and steers (Wallace 1958). The deleterious effects seen with ovariectomized calves were prevented if the animals were treated regularly with estrogen (Wallace 1958). These treatments stimulated secretory development and ductal extension, resulting in developmental patterns similar to those exhibited by ovary intact heifers (Wallace 1958). These results indicate the obvious and important role of the ovary and estrogen in mammary development, however, the exact mechanism by which they support development is not known. It could be that estrogen promotes growth of the mammary gland directly through interactions with estrogen receptor- α (ER). This potential mechanism of interaction is supported by evidence that the ER is present in the epithelial, but not stromal, cell populations of the mammary gland (Capuco et al., 2000). If this is an accurate pathway of estrogen mediated mammary development, then prevalence of ER in the prepubertal heifer mammary gland may be a good predictor of future gland development and possibly future milk yield. There are also mechanisms through which estrogen might *indirectly* stimulate mammary development. Ruan et al. (1995) conducted a study to determine whether insulin like growth factor-I (IGF-I), a known

stimulator of mammary development, required estradiol (E_2) to act or whether it could stimulate growth without growth hormone (GH) and E_2 . Sexually immature, hypophysectomized, oophorectomized rats were utilized. The researchers found that without E_2 administration, IGF-I alone was not sufficient to promote normal mammary development, however E_2 supplementation significantly increased the effects from IGF-I on development. This suggests that estrogen induces its mitogenic effects through interactions with IGF-I. Ruan and colleagues also found that administering estrogen to these rats enhanced the effect of human GH on expression of IGF-I mRNA by 4 to 6 fold. This supports the idea that estrogen could act through both GH and IGF-I to induce mammary growth. Other researchers have also postulated that various growth factors such as epidermal growth factor (EGF) and IGF-I might activate the ER in the absence of its natural ligand (Woodward et al. 2000). Such a mechanism would also support the rationale that the number of ER present in the mammary epithelium would be proportional to the potential degree of mammary growth and development.

Role of Growth Hormone in Mammary Gland Development

The fact that GH plays a critical role in the development of the mammary gland has been known for many years. Studies dating as far back as the 1930's were successful in demonstrating the requirement for the pituitary gland (Reece et al. 1936, Gardner and White 1941, and Lewis et al. 1942). Following hypophysectomization and castration of rodents, estrogen replacement alone failed to stimulate development of the mammary gland. In contrast, estrogen supplementation following oophorectomy resulted in fully a developed mammary gland when the pituitary gland was left intact. Lyons et al. (1958)

and Nandi (1958) obtained similar results with hormone replacement studies. A hormone replacement combination consisting of prolactin (PRL), GH, estrogen, glucocorticoids, and progesterone resulted in full mammary development and induction of a secretory response in hypophysectomized, castrated, and adrenalectomized mice. Although it was not completely clear which pituitary hormone played the more crucial role in mammary gland development, some suggested that GH and PRL were primarily responsible for pubertal development and lactogenesis, respectively (Kleinberg et al. 2000 and Lyons 1993), an idea that would later be proven accurate. Kleinberg and colleagues selectively inhibited PRL in intact sexually immature monkeys by use of the dopamine agonist drug pergolide. This inhibition did not prevent E₂-induced mammary development, thus suggesting that PRL might not be a critical factor involved with mammary development. Kleinberg et al. (1990) also found that administration of GH and E₂ to hypophysectomized and ovariectomized rats significantly increased pubertal mammary development. In contrast, no effect on mammary development was observed upon treatment with E₂ alone or upon treatment with E₂ and PRL. These findings led to the hypothesis that GH acts through the GH receptor in the mammary stroma to produce IGF-I, which then stimulates mammary development via a paracrine manner (Kleinberg 1997). This hypothesis is supported by work from Feldman et al. (1993), who found significant increases in TEB of sexually immature, hypophysectomized, castrated, E₂ treated rats when given rGH, rhGH, rbGH, and one mutant rhGH. These four hormones also bound GH receptors with high affinity. Little development occurred when the rats were given hPRL, rPRL, rhPRL, oPRL, and mutant forms of rhPRL, rhPRL and rhGH, which was altered to reduce binding to GH and PRL receptors. Also, there was a 50-fold

decrease in GH receptor binding with these substances. Further support for a critical relationship between GH and mammary development was obtained by Ilkbahar et al. (1999). They found that the highest concentration of GH receptor in the mammary gland of mice was at 3 wk of age, which is a time when ducts and TEB are forming.

Local Control of Mammary Growth

The mammary gland is comprised of stromal and epithelial cells that communicate with each other through the ECM (Wiseman and Werb 2002). This communication, or “crosstalk”, is necessary for proper development and function of the mammary gland. The epithelial-stromal interaction may be particularly important during bovine mammary development because, compared to rodents, the mammary fat pad (MFP) of cows contains greater amounts of fibrous connective tissue in to which the glandular epithelium proliferates (Hovey et al. 2000). The mammary stroma contributes both instructive and permissive signals for the purposes of directing epithelial proliferation and expansion, and consists of many components: adipocytes, pre-adipocytes, fibroblasts, blood vessels, inflammatory cells, and ECM (Wiseman and Werb 2002). These components interact with the epithelial tissue via specific signaling pathways to either enhance or inhibit ductal expansion. The precise signals and interactions specifying development have not been determined, but many genes have been implicated in this process (Wiseman and Werb 2002). Components of the stroma, and their potential impact on mammary development, will now be examined.

Extracellular Matrix

The ECM is composed of a variety of versatile proteins and polysaccharides that are secreted locally and assembled into an organized meshwork that lies in close proximity to the surfaces of the cells that produce them (Alberts et al. 1994). Most of the ECM components in the mammary gland are secreted by fibroblasts. In the mouse mammary

gland, most if not all ECM proteins that make up the basement membrane are derived from stromal cells (Woodward et al., 2000). The first of the two most general classes of ECM macromolecules are glycosaminoglycans (GAG), which are usually linked to a protein to form a proteoglycan (Alberts et al. 1994). The second main class of ECM molecules are fibrous proteins that can be further divided into two functional types. The structural proteins in this class consist of proteins such as type IV collagen (Col IV), and the adhesive proteins in this class consist of proteins such as laminin (LM) and fibronectin (FN) (Alberts et al. 1994). Both of these classes of ECM molecules have been implicated as factors in the regulation of mammary growth and development.

Much of the attention in the research of mammary gland development is focused on hormone receptors, such as those for estrogen and progesterone. However, there are two types of receptors for the ECM that appear to play a critical role in ductular branching and morphogenesis (Wiseman and Werb 2002). These two types of receptors are discoidin domain receptor-1 (DDR1) and β 1 integrin. DDR1, along with DDR2, represent a subfamily of receptor tyrosine kinases and are known to be expressed in a variety of mouse and human tissues, including mammary tissue (Vogel 1999). Various types of collagen have been implicated as possible ligands and activators of DDR1 (Vogel et al. 2001). This is based on observations that type I through type VI collagens induce DDR1 autophosphorylation (Shrivastava et al. 1997 and Vogel et al. 1997). To examine the *in vivo* role of DDR1 on the growth and development of mice, Vogel and colleagues (2001) generated DDR1-null and DDR1-heterozygous mice. They found that homozygous mutant mice were born alive but remained smaller than their heterozygous

littermates. Mating between DDR1-null females and either mutant or wild type males was often unsuccessful. Implantation of a normal blastocyst into homozygous mutant females resulted in a 20% success rate, with the females giving birth to full litters. However, resulting pups appeared malnourished and only small amounts of milk were found in their stomachs. All pups that were left with their DDR1-null mothers died within days. To investigate the link between DDR1 and the reduced milk yield seen in mutant females, the researchers next examined the mammary glands of mutant females during pregnancy. At day 18.5 of gestation, mammary tissue from mutant mice was characterized by a more condensed alveolar structure, very few lipid droplets in the epithelium, and a fat pad almost completely filled with ducts. Tissue examined 1 day postpartum revealed a mammary gland largely composed of adipocytes and condensed alveoli that secreted very little milk. It is apparent that DDR1 has a significant impact on mammary gland development and functional capacity during gestation and lactation. To discover any defects that might occur earlier in the mammary gland development of DDR-null mice, ductal growth in the mammary fat pads of 3-week-old littermates was analyzed. Invasion of the fat pad by epithelial tissue was delayed in DDR1-null mice, with only the first quarter of the gland being filled with ducts at 3 weeks of age. Moreover, both TEB and mature epithelial ducts were notably larger in DDR1-null mice, which suggested a more proliferative epithelial cell population for mutant mice. This was the case, with cell proliferation analysis revealing a four to five fold greater number of Ki67 (nuclear proliferation antigen) positive cells in homozygous mice compared to the heterozygous controls. These results suggest that DDR1 is a key mediator of stromal-epithelial interaction during ductal morphogenesis of the mammary gland (Vogel et al.

2001), and that its normal physiological role might be inhibitory in nature. $\beta 1$ integrin is another type of receptor that appears to have a significant role in mammary development. Klinowska et al. (1999) attempted to assess the impact that $\beta 1$ integrin, along with the ECM protein LM and the $\alpha 6$ integrin receptor, has on mammary gland development. Pellets containing function-blocking anti- $\beta 1$ integrin, anti- $\alpha 6$ integrin, and anti-laminin antibodies were implanted into the mammary glands of pubertal mice. Blocking of $\beta 1$ integrins and laminin produced similar results, with both causing a reduction in the number of TEB and extent of mammary ductal network, relative to controls. This impaired development was TEB specific since the rest of the gland architecture remained intact and appeared normal. These findings were supported by *in vitro* studies showing that hepatocyte induced growth factor (HGF)–induced tubulogenesis was dependent on functional $\beta 1$ integrins, indicating a possible connection between HGF and $\beta 1$ integrins in ductal morphogenesis. This decrease in gland development upon reduction of $\beta 1$ integrin activity contrasts the hyperproliferative response seen with reduced DDR1 activity, and suggests a stimulatory role for $\beta 1$ integrin receptors under normal conditions. Results obtained for the ECM protein LM suggest a stimulatory role similar to $\beta 1$ integrin, but do not suggest a direct relationship between the two proteins.

One protein class that participates in the regulation of mammary development through direct interaction with ECM proteins such as LM is matrix metalloproteinases (MMP). MMP are locally derived enzymes that cleave ECM and other proteins in the cellular microenvironment (Wiseman and Werb 2002). Specifically, these proteins appear to be involved with cell migration and motility. Because cell motility is a determinant of

epithelial morphogenesis and regeneration (Thiery 1984), it can be considered especially critical to the ductal expansion that occurs during the prepubertal period of mammary growth. Addition of activated MMP2 to cultures of primary breast epithelial cells and the immortalized breast cell line MCF-10A resulted in cells becoming migratory on LM-5 (Giannelli et al. 1997), suggesting the direct involvement of the cleavage of LM-5. This appears to be accurate, because LM-5 γ 2 fragments, which correspond in size to those generated by MMP2 cleavage, are detectable in mammary glands that are remodeling, but not in those that are quiescent (Giannelli et al. 1999). To further investigate the nature of this proteolytic mechanism, Koshikawa et al. (2000) tested a panel of cell lines that constitutively migrated on LM-5, without exogenous addition of MMP. Western blot analysis revealed that this constitutive migration on LM-5 was correlated with the expression of MT1-MMP, a plasma membrane bound metalloproteinase. MT1-MMP activated MMP2 upon its addition to cell cultures, which allowed for greater cleavage of LM-5 and further migration of cells. These enzymes (MT1 through 5-MMP) have been proposed to be the “master switches” of ECM turnover based on this ability to activate other MMP (Ha et al. 2001). Constitutive migration of all cells on LM-5 was inhibited by tissue inhibitor of matrix metalloproteinase-1 (Timp-1) and Timp-2 in a dose-dependent manner. Timp are specific inhibitors of MMP that naturally occur in tissue (Koshikawa et al. 2000). Timp have also been implicated in the inhibition of three other MMP-mediated processes (Fata et al. 1999): the processing of cytokines (Gearing et al. 1995), the degradation of growth factor binding proteins (Thraillkill et al. 1995 and Fowlkes et al. 1994), and the release of ECM-bound growth factors (Whitelock et al. 1996). To assess the effect that Timp-1 has on mammary epithelial cell proliferation *in*

vivo, Fata and colleagues (1999) manipulated mammary Timp-1 levels genetically and biochemically. Down-regulation of epithelial-derived Timp-1 in transgenic mice resulted in augmented ductal expansion and an increased number of ducts, and there was an increase in luminal epithelial cell proliferation. BM integrity was disrupted in the parenchyma of these animals, thus supporting the concept that Timp-1 activity is mediated via MMP and degradation of LM. In contrast, biochemical addition of Timp-1 into the mammary gland decreased the level of ductal expansion and decreased luminal epithelial cell proliferation. The degradatory action of MMP on ECM proteins such as LM-5, along with the inhibitory actions of Timp, suggest that interactions between these stromal factors might be an important mechanism for TEB invasion *in vivo* (Wiseman and Werb 2002).

Proper side-branching of the developing ductal system is critical for normal mammary growth. Certain stromal factors also appear to be specifically involved in the regulation of ductular side-branching in the mammary gland. Ha et al. (2001) produced transgenic mice that over expressed MT1-MMP in the mammary gland. Histological abnormalities were exhibited in 82% of female mammary glands from these transgenic mice. These abnormalities included lymphocytic infiltration in the stroma, periductal fibrosis, epithelial hyperplasia in the mammary ducts, alveolar structure disruption in lactating glands, dysplastic change in the ductal epithelium, and adenocarcinoma. These morphologic changes resulted in very low levels of β -casein mRNA expression in transgenic mice, relative to control WT mice. In accordance with previous results implicating plasma bound MMP as master switches for ECM turnover, overexpression of

MT1-MMP resulted in significantly increased MMP-2 activation in the mammary gland. The fact that overexpression of MT1-MMP resulted in ECM remodeling, as well as tumor formation, in the mammary gland supports the involvement of MMP in normal mammary growth and development, but also implicates a possible link to breast cancer.

One growth/differentiation factor that appears to be involved in mammary ductular side-branching is Wnt-1 (Wiseman and Werb 2002). The Wnt family of secreted glycoproteins have been implicated in a variety of developmental processes such as cell differentiation, cell polarity, cell migration, and cell proliferation (Kuhl et al. 2000). This family of proteins has been divided into main functional groups based on biological activities in specific assays (Kuhl et al. 2000). Wnt-1, -3A, -8, and -8B belong to the group that induces a secondary axis in early *Xenopus* embryos (Du et al. 1995) and transforms C57mg mammary epithelial cells (Wong et al. 1994). The other functionally distinct group of Wnt, which consists of Wnt-4, -5A, and -11, does not induce a secondary axis or transform epithelial cells, but does alter cell movements and reduce cell adhesion when overexpressed in *Xenopus* embryos (Du et al. 1995, Moon et al. 1993, and Torres et al., 1996). To assess the effect that Wnt-1 has on mammary growth and development, Li et al. (2000) created mice overexpressing Wnt-1 in the mammary gland. The resultant mice exhibited extensive ductal hyperplasia early in life and mammary adenocarcinomas were present in 50% of mice at 6 months of age. These mitogenic effects did not require estrogen stimulation, with tumor formation occurring in ER-null mice. While overexpression of Wnt proteins caused an increase growth response and agonized ductular branching, underexpression of these proteins produces opposite results.

Brisken et al. (2000) harvested mammary buds from 14.5 d old Wnt-4-null and WT mice embryos and engrafted them into the cleared MFP of WT hosts. Both types of implants initially resulted in normal mammary development, however, at 12 d of pregnancy Wnt-4-null implants exhibited substantially less ductal branching relative to WT controls.

Specific pathways and signal transduction cascades that require the Wnt family of proteins are still unresolved, however, some relationships are known. There are likely many stimulatory and inhibitory factors acting upon these proteins. One of these factors is the progesterone receptor (PR). Expression of PR and Wnt-4 on sections of virgin and pregnant mouse mammary glands is restricted to the luminal epithelium (Brisken et al. 2000). This co-localization indicates that progesterone signaling activates Wnt-4 expression. To provide further support for this relationship, Wnt-4 expression was analyzed in the mammary glands of pregnant mice engrafted with PR^{+/+} epithelium in one fat pad and PR^{-/-} epithelium in the contralateral fat pad (Brisken et al. 2000). At 12 d of pregnancy, Wnt-4 mRNA expression was consistently 3-fold higher in glands with PR^{+/+} implants. PR and Wnt appear to act in concert to stimulate growth and development of the mammary gland via initiation of ductular side-branching, but exact mechanisms involved are not known. Work done by Brisken et al. (1998) suggests a paracrine mechanism. PR-null breasts were transplanted into WT mice. Those transplants lacking stromal expression of PR developed normally, whereas PR-null epithelial implants resulted in a cessation of development at a simple ductal system characteristic of young virgin mice. Interestingly, chimeric epithelia, which consisted of PR-null cells in close vicinity to PR-WT cells, grew normally and demonstrated complete alveolar

development. These results indicate that epithelial, not stromal, PR expression is crucial for normal mammary growth and development. It also indicates that normal growth and development of the mammary gland relies heavily on direct endocrinal cues, contradicting previous results regarding ER-null mice. However, only a fraction of PR^{+/+} epithelial cells appears to be sufficient, with apparent paracrine mechanisms stimulating the involvement of PR-null cells in the branching processes. This paracrine mechanism involving PR and Wnt is unknown, but one possibility is that Wnt, due to their association with the ECM, may mediate the paracrine signal from the PR through the ECM and syndecan-1 (Sdc1), which is a cell surface heparan sulfate proteoglycan (HSPG) expressed predominately by epithelial cells (Wiseman and Werb 2002). This possibility is based on work done by Alexander et al. (2000), who showed that generalized mammary hyperplasia caused by ectopic expression of Wnt-1 was reduced by 70% in Sdc1^{-/-} mice, compared to Sdc1^{+/+} counterparts. Also, Sdc1 ectodomain, which is a soluble version of Sdc1, stimulated activity of Wingless, a Wnt-1 homologue, in a tissue culture assay. These genetic and biochemical results indicate the critical nature of Sdc-1 in mediating the actions of Wnt proteins.

Stromal factors and processes that function to inhibit inappropriate growth, via repression of ductular branching, are also present in the cellular microenvironment of the mammary gland. Two of these factors are transforming growth factor β (TGF β) and P-cadherin (Wiseman and Werb 2002). TGF β represent a large family of polypeptide growth factors, some of which rapidly and reversibly inhibit mammary growth (Robinson et al.

1991). Pierce and colleagues (1993) created transgenic mice overexpressing TGF β 1, which resulted in significantly reduced side-branching, while alveoli developed normally. Also, overexpression of a function-ablating, dominant negative TGF β signaling receptor, resulting in low tissue levels of available TGF β , caused a dramatic increase in lateral branching that over-rode normal space keeping (Joseph et al. 1999). Expression of mutant receptors was concentrated in the periductal stroma, not the epithelium. These results suggest that TGF β has a definite role in the regulation of lateral branching. Signal receptor location indicates that the normal inhibitory effects of TGF β are mediated by interactions with the ECM, not epithelium. P-cadherin also appears to be an important factor involved in negative growth control of the mammary gland (Radice et al. 1997). Cadherins are a family of glycoproteins involved in Ca⁺⁺-dependant, homotypic cell-cell adhesion (Takeichi 1995 and Gumbiner 1996). Both E- and P-cadherin play important roles in maintaining the structural integrity of epithelial tissues (Radice et al. 1997). To investigate the role of the P-cadherin adhesion receptor in mouse mammary development, Radice et al. (1997) produced transgenic female mice carrying a mutated form of the P-cadherin gene. They found that P-cadherin expression was localized to the myoepithelial cell surrounding the luminal epithelial cells of the mammary gland. No differences between mutant and WT branching morphogenesis were observed at 5 wk of age, which is before the completion of puberty. However, at 10 wk of age, which is postpubertal, WT mice exhibited normal developmental patterns and P-cadherin-null mice demonstrated precocious alveolar development resembling an early pregnant gland. Transgenic mice were also predisposed to focal hyperplasia and dysplasia as well as leading to an increase in lymphoid cells in the mammary gland. These results suggest that

the inhibitory effects associated with P-cadherin are more important during growth associated with late puberty and just following the completion of puberty.

It is obvious that interactions between epithelium and ECM play a vital role in the development and function of the mammary gland. Multiple *in vitro* experiments demonstrate the highly favorable response of mammary epithelial cells when grown in the presence of ECM proteins. Liang Li et al. (1987) cultured mammary epithelial cells on a reconstituted BM derived from Engelbreth-Holm-Swarm tumor, and found that >90% of the cells cultured under these conditions produced high levels of β -casein, as determined by immunofluorescent staining procedures. In contrast, for cells cultured on type 1 collagen gels and plastic, the percentage of cells in culture producing high levels of β -casein were 30-40% and 2-10%, respectively. These measures were taken at d 6 of the experiment, which began at d 0 with 40% of cells in all cultures synthesizing high levels of β -casein. The cells grown on the reconstituted BM also resembled secretory alveoli morphologically. Individual ECM proteins were also examined for their ability to stimulate epithelial growth and function. Liang Li et al. (1987) found that Col IV and FN had little effect on cell morphology and β -casein expression levels, but that expression levels were increased by both LM and the proteoglycan, heparan sulfate.

Both *in vivo* and *in vitro* experiments demonstrate that interactions between the epithelium and stroma are necessary for mammary epithelial cells to respond to estrogen and progesterone (Woodward et al. 2000). However, the nature of the mechanisms that cause this response are not fully understood and likely occur via multiple pathways.

Epithelial/stromal interactions may be mediated through secretion of growth factors by the stromal cells, or stromal cells may alter the composition of the ECM proteins (Woodward et al. 2001). Although estrogen is necessary for normal postnatal growth of the mammary gland, current evidence suggests its effects are indirect. Possibly, estrogen stimulates the stromal cell population to produce paracrine factors such as growth factors/inhibitors and ECM proteins, which ultimately regulate growth of the mammary gland (Woodward et al. 2001). But, Capuco et al. (2001) showed in the bovine, that ER are expressed only in the epithelial cells. This supports the idea that estrogen acts on epithelial cells to produce paracrine growth factors. Woodward et al. (2000) conducted a study to determine the impact that ECM proteins would have on the proliferative responses of mouse mammary epithelial cells to EGF and IGF-I. The ECM proteins tested in this study were collagen I (Col I) and Col IV, LN, and FN. They found that all ECM tested promoted a highly synergistic proliferative response to both IGF-I and EGF (proliferative response measured as [³H] thymidine incorporation). EGF and IGF-I receptors were also increased by all ECM proteins, and there was a trend for decreased IGF-I binding proteins (IGFBP) when cells plated on ECM were treated with EGF. Furthermore, treatment with estrogen and the progestin R5020 decreased growth factor induced cell proliferation in an ECM dependent fashion (Woodward et al. 2000). EGF decreased IGFBP-2 and IGFBP-3 levels by greater than 50% in the presence of IGF-I on plastic and on collagen I. Woodward et al. (2000) speculated that these results could indicate an ECM-specific synergism that occurs in response to ECM up-regulation of IGF-I and EGF receptors and EGF down-regulation of IGFBP. These data show that the stroma is critical for the growth and development of mammary parenchyma, and that

stromal/epithelial interactions could mediate the responses to steroids, growth factors, and binding proteins, via various ECM proteins.

Proteoglycans have also been implicated as ECM factors involved in the chemical signaling between cells. They have been found to bind proteins such as growth factors, proteolytic enzymes, and protease inhibitors (Alberts et al. 1994). Binding of proteoglycans to fibroblast growth factor (FGF), which is a potent mitogen for cellular proliferation in many cell types, appears to be a necessary step for FGF in the binding of its cell surface receptor (Alberts et al. 1994). In contrast, binding of proteoglycans to the growth regulatory protein TGF β inhibits this proteins level of activity (Alberts et al. 1994).

Local Growth Factors Mediating Epithelial-Stromal Crosstalk

Several factors have been postulated to directly mediate the crosstalk between the stroma and epithelium during mammary gland development (Wiseman and Werb 2002). One of these factors is keratinocyte growth factor (KGF), which is a member of the FGF family. Rubin et al. (1989) first identified and purified this protein from medium containing human embryonic lung fibroblastic cells. KGF was a potent mitogen for epithelial, but not fibroblastic or endothelial, cells capable of stimulating DNA synthesis in keratinocytes by greater than 500-fold. The fact that KGF was secreted by fibroblasts and acted on epithelial cells suggested a role for KGF in the regulation of epithelial cell proliferation. Some evidence indicates that systemic endocrine regulation of mammary growth and development might be mediated via KGF. Koji et al. (1994) examined the

hormonal regulation and cellular localization of KGF mRNA expression in the rhesus monkey uterus. They found that treatment with progesterone or tissue taken during the luteal phase contained KGF mRNA levels that were 70-100-times greater than those observed following no treatment, E2, or tissue taken during the follicular phase. KGF mRNA was observed only in stromal and smooth muscle cells and was increased substantially after treatment with progesterone. Coinciding with these results, progesterone treatment caused an increase in cell proliferation in the glandular epithelium of the basal region. In rats, intravenous administration of KGF caused a dramatic increase in mammary epithelium proliferation, ductal neogenesis, and intraductal epithelial hyperplasia in nulliparous females (Ulich et al. 1994). To investigate whether or not these mitogenic properties of KGF apply in the bovine and ovine models, Hovey et al. (2001) manipulated MAC-T bovine mammary epithelial cells in culture.

Supplementation with 10 ng/ml human KGF caused a 390% increase in DNA synthesis, a phenomenon that was not observed when KGF was added to ovine mammary stromal cell cultures in a range of concentrations. In agreement with previous work, mammary stromal, but not epithelial cells, expressed abundant KGF mRNA. In the mammary tissue of nulliparous ewes, KGF expression was present in stromal cells of intra- and interlobular space and in fibroblasts and adipocytes located distally to the epithelial cells.

Another factor that might be involved in stromal/epithelial crosstalk is parathyroid hormone-related protein (PTHrP). During development, PTHrP is produced by many epithelial structures, and its respective receptor (type 1 PTH/PTHrP or PTH-1R) is often expressed on adjacent mesenchymal cells, thus suggesting a role for the hormone in

mediating crosstalk (Lee et al. 1995 and Wysolmerski and Stewart 1998). In PTHrP or PTH-1R knockout mice no mammary mesenchyme forms (Dunbar et al. 1999 and Foley et al. 2001). When mice constitutively express PTHrP in the fetal epidermis, contrasting results are obtained, with the entire ventral dermis being transformed into mammary mesenchyme (Wysolmerski et al. 1998 and Foley et al. 2001). These results indicate that PTHrP is critical for the formation of a mammary-specific mesenchyme. In addition to apparent actions by PTHrP on the mammary gland during embryonic development, PTHrP also appears to affect development during puberty. Wysolmerski et al. (1995) demonstrated that overexpression of the protein in mammary epithelial cells caused severe defects in ductal development during puberty and early pregnancy. These defects in development might be due to disruptions in the extracellular environment of the TEB, because PTHrP and PTH-1R are predominately expressed by the cap cells of the TEB and the stromal cells adjacent to TEB, respectively. To investigate the effect that PTHrP overexpression has at different points of mammary development, double transgenic mice were created whose expression levels could be manipulated by the presence/absence of tetracycline (Dunbar et al. 2001). Overexpression of PTHrP before birth and during puberty resulted in ductular branching defects during puberty and a decreased rate of ductal elongation, respectively. However, when PTHrP overexpression was induced after completion of ductal morphogenesis, lobulo-alveolar development was unaffected, thus suggesting a less critical role in periods of growth following cessation of puberty. Because the impairments in ductular elongation caused by pubertal overexpression of PTHrP were associated with an increase in epithelial cell apoptosis, and because

exogenous administration of estrogen and progesterone did not decrease apoptosis and increase TEB cell proliferation, PTHrP might play a role in mediating endocrine cues.

Another factor that has been recently implicated as a potential autocrine/paracrine signal in the mammary gland is leptin. Leptin is a product of the so-called “obesity gene” and is produced primarily by adipose tissue, however, leptin production also occurs in a variety of other tissues such as in mammary gland (Smith-Kirwin et al. 1998). In addition, leptin receptors, which are predominately found in the brain, have been identified in secretory epithelial cells of sheep mammary glands (Laud et al. 1999). Recently, leptin protein has been identified in bovine milk, and leptin mRNA has been identified in mammary tissue and cultured bovine mammary epithelial cells (Smith and Sheffield 2002). Real time RT-PCR techniques were utilized on the same cell culture to determine leptin responses to varying concentrations of insulin and IGF-I. There was a rapid increase in leptin mRNA in response to both hormones, with significant increases in response to as little as 1 ng/mL insulin and 10 ng/mL IGF-I, and maximum increases in response to 10 ng/mL insulin and 10 ng/mL IGF-I. Leptin production in mammary epithelial cells appears to be regulated by factors that are known to be involved in the growth and development of the mammary gland, thus implicating leptin as an important autocrine/paracrine signaling factor.

IGF-I and Mammary Gland Development

It has been demonstrated that IGF-I either fully or partially mediates the action of GH in pubertal mammary development (Kleinberg et al. 2000). Ruan and colleagues (1992)

implanted either native IGF-I or aminotermally-shortened des (1-3) IGF-I pellets into one lumbar mammary gland of sexually immature, hypophysectomized, and oophorectomized rats. A control pellet was implanted into the other lumbar mammary gland and E₂ was administered systemically. Quantification of TEB formation revealed that pubertal mammary development was occurring in the gland containing IGF-I, but not in the control gland. A similar study (Ruan et al. 1995) was carried out in which either bGH or des (1-3) IGF-I was implanted into a lumbar mammary gland, with some rats receiving E₂ and others not. E₂ administered with either hormone resulted in a highly significant increase in formation of TEB, while E₂ alone had no effect. These results indicate a clear connection between GH and IGF-I in mammary gland development. Further support for the direct involvement of IGF-I is demonstrated by the fact that IGF-I mRNA expression is at its highest in ewes during the prepubertal phase, which is a period characterized by rapid mammary growth (Hovey et al. 1998).

It has been proposed that the interaction between GH and IGF-I manifests itself via GH acting on the mammary stroma to induce production of IGF-I, which then directly stimulates mammary growth and ductal morphogenesis (Kleinberg et al. 2000). This relationship is supported by results indicating that GH-stimulated IGF-I mRNA expression was equally high in parenchymal and stromal areas of the mammary glands of hypophysectomized and castrated rats (Walden et al. 1998). Also, IGF-I mRNA is present in mammary stroma as well as within TEB, whereas IGF-1R is present only in TEB (Richert and Wood 1999). This suggests that the effects of IGF-I on mammary

growth arise from direct interactions with epithelial cells of the developing ductal structures.

A functional GH-IGF-I axis is a required phenomenon for normal mammary gland development, however, the extremely critical nature of IGF-I, along with its interactions with GH, has only recently been elucidated. Kleinberg and Ruan (1999) created IGF-I null female mice, and found that these mice exhibited grossly deficient mammary gland development compared to WT littermates. When these animals were treated with E₂ alone or in combination with hGH, no mammary development occurred. This indicates that GH alone is not sufficient in promoting normal mammary growth. However, administration of des (1-3) IGF-I alone or along with E₂ for a 5 day period caused a significant increase in the number of TEB, number of ductal structures, and percent parenchymal area. These results clearly demonstrate the crucial involvement of IGF-I in mammary development, and support the notion that IGF-I is the sole mediator of GH at puberty (Kleinberg and Ruan 1999).

Effects of Prepubertal Nutrition on Mammary Gland Development and Future Milk Yield

Whether or not rapid rearing of prepubertal heifers causes beneficial or detrimental effects on mammary gland development and future milk yield is controversial. Diets that promote a high rate of gain in the prepubertal period have the advantage of decreasing the age to sexual maturity, which allows calving and milk production to begin at a younger age (Akers et al. 2000). However, there is literature demonstrating that an increased feeding level, specifically an increased energy intake level, can adversely affect mammary gland development and future milk production (Akers et al. 2000). Lammers et al. (1999) conducted a study to assess the effects of accelerated growth rates and estrogen implants on mammary development and subsequent reproduction and milk yield in prepubertal heifers. Holstein heifers were randomly assigned to one of four treatments based on a 2x2 factorial design. The daily gains for the low and high feed treatments were 700g and 1000g, respectively. The treatments began at approximately 4.5 months of age and at a body weight of approximately 130kg, and were 20 weeks in duration. Following this 20-week treatment period, the heifers were group fed based on body weight and age so that these parameters would be similar at calving. Lammers and co-workers (1999) found that higher daily gains during prepuberty decreased age at puberty by 32 days, and decreased first lactation fat-corrected milk yield 7.1%.

Decreased milk yields following a rapid prepubertal growth regimen have been demonstrated in many studies, but there are also a number of experiments that fail to elicit these effects (Sejrsen and Purup 1997). In some cases, this lack of effect may be

contributed to factors such as short treatment periods, high pretreatment growth rates, small growth rate differences between treatment groups, and treatment periods outside the critical period (Sejrsen and Purup 1997). However, there are results where lack of effect due to feeding level can't be explained by factors such as these. Pirlo et al. (1997) fed 61 prepubertal Italian Friesian heifers at one of four feeding levels based on amounts of total digestible nutrients (TDN) and crude protein (CP). The four feeding levels were low energy/low protein, low energy/high protein, high energy/low protein, and high energy/high protein, and resulted in average daily gains of 608g, 659g, 794g, and 848g, respectively. These feeding levels were maintained until 300kg of BW, when all heifers were put on the same diet. Heifers were bred at roughly 370kg of BW. The two high energy feeding levels did not have an adverse effect on milk production through 36wks of the first lactation, with all four groups having similar milk yields (range of 20.2 kg/d to 22.7 kg/d). In another study that failed to show detrimental effects due to growth rate, Van Amburgh and co-workers (1998) fed 273 prepubertal Holstein heifers one of three dietary energy treatments that produced average daily gains of 680 g, 830 g, and 940 g. Heifers were bred at approximately 340 kg of BW. Initially, it appeared as though milk yields were significantly reduced for heifers grown at the highest daily gain. However, after taking into account posttreatment factors such as postcalving body weight, it was apparent that prepubertal rates of gain explained very little of the variation in milk yield. Therefore, prepubertal BW gains did not significantly affect milk yield during first lactation. Waldo et al. (1998) obtained similar results, finding that prepubertal heifers fed for a high rate of gain (994 g/d) did not exhibit altered milk production patterns compared

to heifers grown at lower rates of gain (785 g/d). Milk production was affected by age at calving and was strongly related to postcalving BW.

Results regarding high rates of growth and subsequent milk yield are mixed, with some studies indicating lowered milk production and others demonstrating no effects.

In cases where decreased milk yields are observed following high prepubertal growth rates, the negative results appear to be due to the impairment of mammary gland development. Sejrsen and colleagues (1982) conducted an experiment to determine the influence of plane of nutrition on mammary gland development in heifers. Prepubertal and postpubertal heifers were fed a ration 60:40 concentrate to forage in either restricted or ad libitum amounts. The heifers on restricted feeding had a dry matter intake that was 60% of that for heifers fed ad libitum. Average daily gains were 613g and 1218g for heifers on restricted feeding and ad libitum feeding, respectively. They found that, for prepubertal heifers, the increased daily gain lowered mammary secretory tissue weights and DNA content 23% and 32%, respectively, compared to restricted feeding. No differences between feeding treatments were observed with postpubertal heifers. These results indicate that higher average daily gains adversely affect mammary growth in prepubertal heifers and appear to have no effect on mammary growth during the postpubertal period, indicating the critical nature of development prior to puberty. The composition of mammary parenchyma was not affected by level of feeding/daily gain in both prepubertal and postpubertal groups. These results suggest that mammary gland development is compromised through decreased penetration of parenchymal tissue into the fat pad, and not through modifications of parenchyma composition. The link between

prepubertal rate of gain, mammary development, and resultant milk production is very significant. Although far more development and differentiation occurs in the mammary gland during gestation, it is apparent that the prepubertal period is critical to insure proper mammary development.

The exact mechanisms through which feeding level influence mammary growth and development are unknown. Many researchers have postulated that the impaired mammary growth seen with higher growth rates in prepubertal heifers is a result of decreased levels of circulating GH (Sejrsen and Purup 1997). Capuco et al. (1995) designed an experiment to determine whether prepubertal rate of gain had an impact on mammary gland development and subsequent milk production. Holstein heifers (N=116) were randomly assigned to 1 of 2 diets that promoted an average daily gain of 725 g/day or 950 g/day. They discovered that serum GH was reduced, and serum IGF-I was increased, in heifers fed for a high rate of gain on a corn silage diet. This same diet also resulted in lower total mammary parenchymal DNA and RNA, greater volume of parenchymal adipocytes, and lower volume of parenchymal epithelial cells compared to the heifers in other groups. Capuco et al. (1995) concluded that these results supported earlier research that rapid weight gain during the prepubertal period had a detrimental impact on mammary development when the gain in weight was accompanied by excess body fat deposition. Interestingly, the negative effect seen with mammary development did not cause a decline in future milk production. In a similar study, Sejrsen et al. (1983) found that feeding prepubertal heifers for daily gains of 1218 g caused a significant reduction in serum GH concentrations compared to heifers that averaged 613 g/d of gain.

The increased circulating GH found in heifers on the restricted diet was positively correlated with amount of mammary secretory tissue. These data (Capuco et al. 1995, Sejrsen et al. 1983) support the theory that GH has a direct effect on mammary gland development. However, this is likely not the case, because GH does not bind mammary gland parenchymal receptors (Akers 1985) and does not stimulate mammary cell growth *in vitro* (Purup 1995). It is possible that the effects of GH are mediated by IGF-I (Purup et al. 1999) based on the following observations: exogenous GH increases circulating IGF-I, IGF-I receptors are present in mammary tissue, mammary tissue binds IGF-I, and IGF-I stimulates mammary cell growth *in vitro* (Purup et al. 1993 and 1995, Purup et al. 1999). However, this hypothesis might not be accurate because serum IGF-I is actually increased in response to a high feeding level (Capuco et al. 1995, Vestergaard et al. 1995). Some studies have demonstrated impaired mammary growth after high growth rates, with neither GH nor IGF-I levels changing significantly (Mantysaari et al. 1995). It is obvious that the adverse effects associated with high prepubertal growth rates cannot be contributed solely to decreases in circulating GH or IGF-I. Because both GH and IGF-I bind to specific receptors, Purup et al. (1999) decided to investigate the influence of feeding level on the receptors for these two hormones, along with PRL, in mammary parenchyma and liver. Binding of GH and IGF-I to their respective receptors in mammary parenchyma and liver was unaffected by growth rate. However, a high level of feed increased specific binding of PRL to mammary tissue, suggesting the need for further investigation into feeding level and PRL response. Decreases in mammary development caused by high prepubertal growth rates apparently cannot be contributed to decreases in hormone receptors for GH and IGF-I.

Determining the specific physiological responses to a high prepubertal growth rate is necessary if there is to be an understanding of why mammary gland development is impaired. Discovery of these responses might also allow for the development of interventions that could alleviate mammary gland impairment, while still allowing rapid growth. This would allow calving and milk production to begin at a younger age, without the potential side effect of reduced milk yield. Recently, two groups of researchers attempted to prevent decreased milk yields following high rates of prepubertal growth with administration of bST. Radcliff and co-workers (2000) randomly assigned prepubertal Holstein heifers to one of three treatment groups. The average daily gains were designed to be 800g (standard), 1200g (high), and 1200g (high bST) for the three treatments, with one of the 1200g treatment groups receiving a daily injection of bovine somatotropin. The heifers were inseminated after reaching a body weight of 363kg, and diets and injections were terminated upon confirmation of pregnancy. Similar diets were administered to all pregnant heifers during gestation, parturition, and lactation. It is not surprising that the high and high-bST treatments resulted in greater prebreeding average body weight gains than those seen with the standard feeding level. However, standard heifers did have a greater average body weight gain during gestation than both high treatments. Heifers in the standard treatment produced 14% more milk than heifers in the high treatment, but did not produce more milk than heifers in the high-bST treatment. These results support other work that demonstrates decreased milk yields following high prepubertal gains, but also demonstrate that these adverse effects might be avoided. Daily injections of bST along with high gains during the prepubertal period of growth enabled heifers to be bred earlier

without decreasing milk production. Deleterious effects on milk production have also been observed in beef cattle following higher prepubertal rates of gain. Buskirk et al. (1996) conducted three similar trials, in which prepubertal crossbred heifers were assigned to a 2x2 factorial treatment design of moderate (MDE) or high dietary energy (HDE) and injections of vehicle (VEH) or injections of bST (bST) every 14 days. The average age of heifers upon initiation of treatments were 113 days, 123 days, and 134 days for trials 1, 2, and 3, respectively, and the treatment period was 112 days. They found that heifers receiving HDE in trials 2 and 3 had greater weight gains than those receiving MDE. In trial 2, HDE resulted in decreased milk production, calf weaning weight, and mammary dry fat free tissue and DNA. In contrast to work cited previously (Radcliff et al. 2000), the adverse affects on mammary development and milk yield associated with a high prepubertal rate of growth could not be offset with administration of bST.

Lowering a heifer's age to maturity and future milk production are certainly two issues that arise with increased feeding levels and subsequent increases in prepubertal weight. However, advantages and disadvantages of reducing the age to first calving can be considered from a broader perspective. Johnson (1988) cites advantages such as lower overhead costs, greater cumulative production per month of age, shorter generation interval and earlier sire evaluation. However, he also mentions disadvantages such as lower conception rates, increased dystocia, reduced milk yield, reduced longevity, and the cost of high energy rearing rations. It appears that feeding for a high rate of gain might be beneficial in some instances, but deleterious in others. Also, it is logical to

assume that there is a possible “cut off point” of feeding level, perhaps a level of prepubertal weight gain at which gains above that level are detrimental and gains below that level are beneficial (Akers et al. 2000).

Results regarding prepubertal growth rates and future milk production are inconsistent. Some studies demonstrate a negative effect associated with a high growth rate, while others fail to demonstrate any relationship. It is apparent that impaired mammary gland development is responsible for the decreased milk yields seen in some experiments. It is not clear what the cause of the impaired development is, but it is most likely mediated by changes in growth factor responses.

Rationale and Significance

Due to the inconclusive nature of the research that has been done regarding prepubertal weight gains in the heifer and mammary development/ future milk production, it is easy to see why further research is needed to investigate the relationship. There are about 9.3 million lactating dairy cows in the United States. Assuming each cow remains in the herd for an average of 3 years, a third of the cows must be replaced each year. With an average replacement cost of \$1400, these cows represent an annual investment of 4.3 billion dollars (Akers et al. 2000). Obviously, any means that could possibly increase or decrease milk production is of the utmost importance to the dairy industry.

At the cellular and molecular level, there little is known about the mechanisms and processes that regulate prepubertal mammary development. This prepubertal period of growth, as mentioned before, is quantitatively far less important than mammary growth after conception. However, important development is occurring in the prepubertal heifer that eventually will predict further development and thus resultant milk yield. The goal of this project is to elucidate these cellular and molecular pathways, to further understand prepubertal mammary gland development. Doing so will provide an answer as to what effects feeding for a high or low rate of gain has on the cellular and molecular level growth responses of mammary tissue. Once the complex processes governing prepubertal mammary growth have been specifically mapped, new management practices could be developed to maximize growth of mammary tissue in the prepubertal heifer, thus leading to greater future milk yield.

By measuring growth characteristics of the mammary gland, estrogen receptor content, etc., the samples in these studies will provide additional insight about high/low feeding levels and future milk production. As mentioned before, studies regarding this matter have been inconsistent, therefore the studies outlined in this proposal will provide greater insight into the effects of rate of gain on mammary gland development and future milk production, and possibly reasons as to why these effects occur.

Objectives

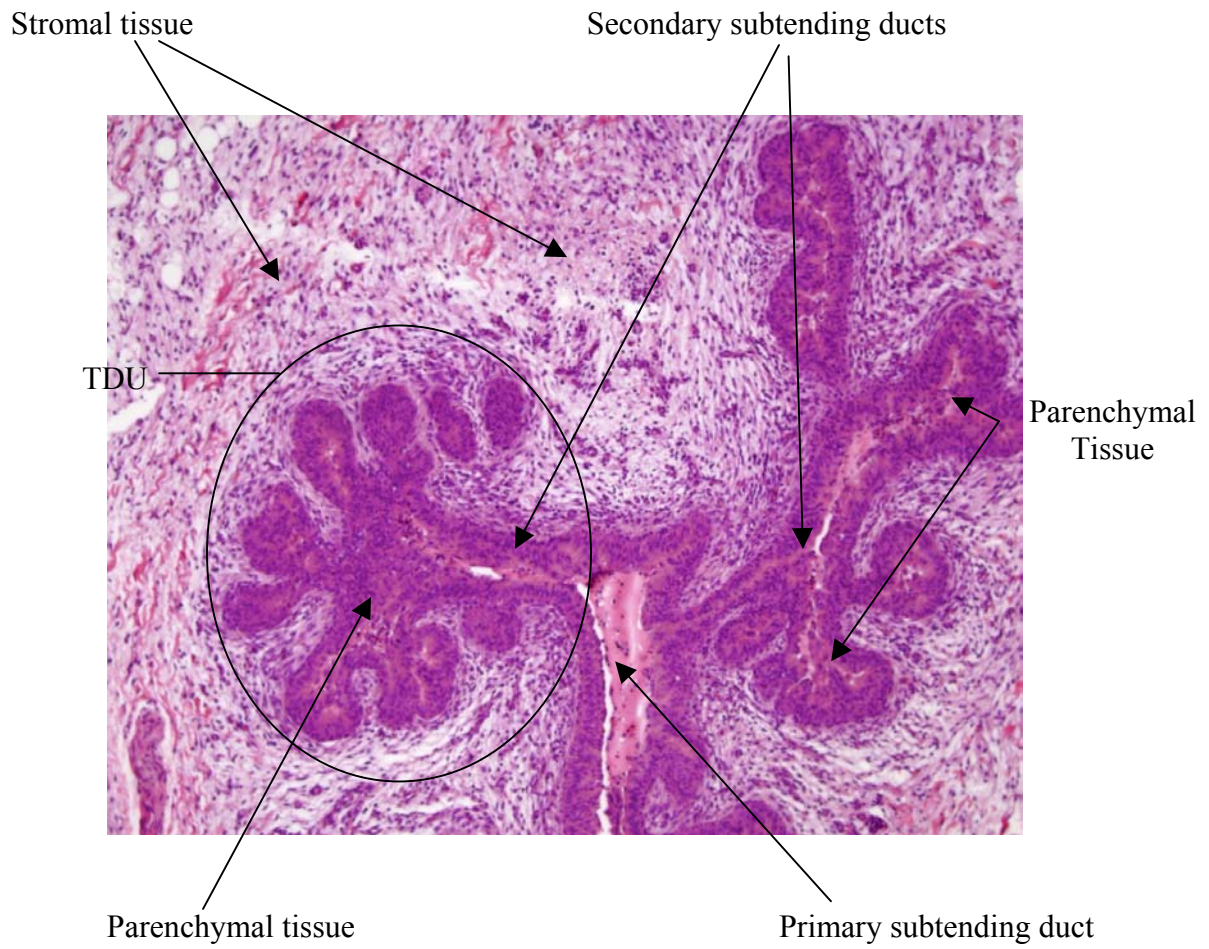
The overall objective of this study was to determine the effects of high and low rates of gain on mammary development of the prepubertal heifer.

Objective 1: To determine how a high or low rate of gain affects the role that the ovary has on pre-pubertal mammary development by measuring estrogen receptor content of mammary epithelial cells.

Objective 2: To determine how a high or low rate of gain affects cell proliferation and tissue development characteristics of the mammary gland.

Objective 3: To determine how a high or low rate of gain affects ECM protein deposition in the mammary parenchyma, and the impact that these ECM proteins have on growth and development of the mammary gland.

Figure 1.1: Histological section of the mammary gland of a prepubertal heifer.
H&E staining paraffin-embedded tissue (Capuco et al. 2000)



MATERIALS AND METHODS

Animals

The tissue samples utilized in this study were from animals that were purchased and treated as reported (Brown 2002) at Michigan State University. All experimental procedures were approved by the Michigan State University All University Committee on Animal Use and Care. Briefly, female Holstein calves (n=74, 43.8 ± 3 kg BW) were blocked by date of purchase, with 2 blocks each being purchased from sale barns in either Indiana (blocks 1 and 2, n=15 and n=20 calves, respectively) or Michigan (blocks 3 and 4, n=19 and n=20 calves, respectively). All calves at day of purchase were between 5 and 10 d of age, weighed over 45 kg, and were not freemartins. Each block arrived separately (roughly 1 m increments) at Michigan State University Dairy Cattle Teaching and Research Center, where they were weighed and housed in individual pens. The day following arrival, calves began a week-long adaptation period with feedings of 1.9 L milk replacer (21.3% CP, 21.3% fat on a DM basis reconstituted to 12.5% DM) at 7 a.m. and 5 p.m. Calves had water available at all times and 100 g/d calf starter (Gold Flake™ Calf Starter, Nutrena Feeds, Winnipeg, Manitoba) beginning on the third day after arrival. Some calves displayed health problems due to pneumonia, salmonella, cryptosporidiosis, mycoplasma ear infections, scours, or heart defects. This resulted in 6, 10, 4, and 1 calves from blocks 1,2,3, and 4, respectively, being eliminated from the study. One calf was later eliminated after being determined a freemartin. Final number of calves in the HH, HL, LH, and LL treatments for each block were 2, 2, 2, 1 for block 1; 2, 1, 2, 3 for block 2; 4, 3, 3, 3 for block 3; 3, 4, 3, 4, for block 4, respectively.

Feeding and Management of Calves

Feeding and management of calves was carried out as reported by Brown and colleagues (2002). Briefly, following the adaptation period, calves were stratified into highest and lowest BW and randomly assigned to 1 of 4 treatments (HH, HL, LH, and LL). Calves on the low diet (L) received standard milk replacer (21.3% CP, 21.3% fat on a DM basis) at 1.25% of BW (10% of BW after reconstituting to 12.5% solids), and were fed starter grain (20.5% CP, 3.4% fat on a DM basis) according to the Dairy NRC (2001) to gain 0.40 kg/d average daily gain (ADG) from 2 to 8 wk of age (period 1). Calves on the high diet (H) received a high-protein milk replacer (30.3% CP, 15.9% fat on a DM basis) at 2.25% of BW (15% of BW reconstituted to 15% DM) and calf starter (25.0% CP, 3.4% fat on a DM basis) for 1.2 kg/d ADG according to the Dairy NRC (2001) from 2 to 8 wk of age. Calves were weaned from milk replacer by 7 wk of age. From 8 to 14 wk of age (period 2), calves received only calf starter and corn grain. During wk 8, calves on the wk 8 to 14 L diet received standard calf starter to achieve an ADG of 0.40 kg/d. Calves on the wk 8 to 14 wk H diet were fed the high-protein calf starter ad libitum. At 9 wk of age, rolled corn was added to both diets, which resulted in new diets containing 70% of the respective calf starter and 30% rolled corn. Final CP percentages were 16.5% and 21.3% for the L and H diets, respectively. Fresh water was available to the calves at all times. A summary of the experimental design, along with actual ADG, is depicted in Figure 2.1.

Slaughter Procedure

Brown and colleagues carried out the slaughtering of the calves at Michigan State University as reported. Five L diet and 6 H diet calves were slaughtered at 8 wk of age. Following analytical procedures performed (Brown et al. 2002) on the mammary tissue from 8 wk calves, inadequate amounts of tissue were available for analyses in this study, therefore, no data from calves slaughtered at 8 wk was obtained. The remaining calves were slaughtered at 14 wk of age. All calves were slaughtered at the Michigan State University Meats Laboratory using captive bolt stunning followed by exsanguination. Reproductive tracts were examined to confirm that calves were not freemartins and had not reached puberty.

Mammary Tissue Collection

Collection of mammary tissue was carried out as reported (Brown et al. 2002).

Mammary glands were collected within 30 min of slaughter, then bisected into right and left hemi-glands. Three samples of tissue were taken from the right half, corresponding to the 3 “zones” of mammary gland that will be referred to hereafter. One sample was taken from the parenchymal tissue closest to the teat canal, or gland cistern (cistern zone or zone C). A second sample was taken from the parenchymal tissue midway between the teat canal and the exterior edge of the parenchymal tissue (medial zone or zone M). The last sample of parenchymal tissue was taken at the interface of the parenchymal tissue and mammary fat pad (interface zone or zone I). Thus, there are a total of 3 samples from each calf, one from each of the 3 zones. For example, sample 38-I refers to parenchymal tissue from the interface of the parenchyma and fat pad collected from animal 38. Following collection, samples were fixed for approximately 24 h in 10% neutral buffered formalin solution, processed through a graded series of ethanol washes, and sent to Virginia Tech.

Embedding of Tissue in Paraffin and Preparation of Slides

Once the samples arrived at Virginia Tech, they were cut to produce multiple tissue pieces from each sample. Replicate samples were then embedded in paraffin. This involved sequential dehydration with two incubations (60 min per incubation) each of: 70% ethanol, 80% ethanol, 95% ethanol, 100% ethanol, and xylene. The samples were then processed with two incubations (first incubation 60 min) of melted liquid paraffin. The samples incubated in the second liquid paraffin bath until they were transferred to blocks for embedding and were allowed to solidify. Sectioning of one replicate of paraffin-embedded tissue into 5 μm -thick sections was performed with a microtome (Model 2040, Reichert-Jung). Sections were then placed in a warm water bath (38° C) where they were “floated” onto microscope slides (Superfrost / Plus Microscope Slides, Fisher Scientific, Pittsburgh, PA). Generally, 4 to 5 tissue sections from a particular sample were placed on a slide, and 5 or 6 slides were made for each sample. Slides were allowed to dry at room temperature in an upright position. Once dry, slides were placed in slide boxes and stored until staining procedures were performed.

General Staining Procedures

Hydration of Tissue Sections

Prior to any staining procedure being performed, it was necessary that tissue sections be hydrated. Hydration of tissue sections began with a de-paraffinization step that involved washing slides twice in xylene at 5 min per wash. Slides were then transferred through a series of ethanol washes at 2 min per wash (100%, 95%, and 70% ethanol). The final step in hydration was a 5 min wash in distilled water.

Hematoxylin and Eosin Staining of Tissue Sections

Following de-paraffinization and hydration of sections, one slide representing each sample was placed in a hematoxylin and eosin solution (Sigma Chemical Co., St. Louis, MO) for 10 min. Sections were then de-stained by rinsing under running tap water for a period of 30 s to 1 min.

Staining of Tissue Sections using the Periodic acid-Schiff Reaction

Following de-paraffinization and hydration of sections, one slide from each sample was placed in a 0.5% periodic acid solution for 5 min. After 3 washes with distilled water at 2 min per wash, slides were placed in Schiff reagent (Protocol Schiff Reagent, Biochemical Sciences Inc., Swedesboro, NJ), which had been allowed to warm to room temperature, for 15 min. The sections were then washed in 2 solutions of 0.55% potassium *m*-bisulfite for 1 min each and distilled water for 10 min. Counterstaining was done for 30 sec using

a 48 mL hematoxylin + 2 mL acetic acid solution. Slides were then placed in the sink under running tap water for 10 min.

Dehydration of Tissue Sections and Coverslipping of Slides

Following completion of any staining procedure, tissue sections were dehydrated and slides were coverslipped. Dehydration began with a 5 min wash in distilled water. Slides were then put through a series of ethanol washes at 1 min per wash (70%, 95%, and 100% ethanol). The final step in dehydration involved 2 washes of xylene at 5 min per wash. Slides were removed from the second xylene wash, adhesive mounting medium (Permount, Fisher Chemicals, Fairlawn, NJ) was applied, coverslips were placed on the slides, and slides were allowed to dry overnight.

Immunochemical Staining Procedures for ER, Ki67, Col IV, FN, and LM

Identical staining procedures were utilized for the visualization of ER, Ki67, Col IV, FN, and LM. The only variation in procedure involved using different antibodies to identify each protein of interest. In contrast to the general staining procedures, which required an entire slide receiving the stain, multiple proteins were identified on the same microscope slide. ER and Ki67 staining was performed on serial sections on one slide from each sample, and Col IV, FN, and LM staining was performed on serial sections of a second slide.

Quenching of Sections in Hydrogen Peroxide

In order to remove any endogenous peroxidase activity, tissue sections were quenched in hydrogen peroxide. This was done following the previously described de-paraffinization and hydration steps, and involved placing the slides in a 3% H₂O₂ in PBS (Table 2.1 contains a listing of reagents used in the immunostaining procedures, along with their chemical compositions) solution for 10 min. The slides were then placed in three successive washes of PBS for 2 min each.

Microwave Antigen Retrieval and Blocking

To facilitate antigen retrieval, slides were placed in a citrate buffer solution (Table 2.1) and heated in a microwave on high power (650 W) for 2 periods of 5 min each. The two heating periods were each followed by a cooling period of 5 min and 30 min in length,

respectively. After the final cooling period, slides were once again washed in 3 solutions of PBS for 2 min each. Following removal of slides from the third PBS wash, individual tissue sections were circled with the Pap Pen (Sigma Chemical Co., St. Louis, MO), which served as a “barrier” around each section so that one slide could be used to stain for multiple proteins. The blocking step included a 30 min incubation with approximately 50 μ L of 5% goat serum (Table 2.1, Zymed Laboratories, Inc., San Francisco, CA) on each section of tissue.

Primary Antibody Incubation

Following removal of blocking agent, which was achieved by vigorously “flicking” the slide multiple times, approximately 50 μ L of primary antibody was added to each section. Specific antibodies, dilutions, etc. are described in Table 2.2. Two replicate slides from each sample were used for immunochemical staining procedures. On the first slide for each sample, anti-ER and anti-Ki67 antibodies were used in the incubation of one tissue section each. On the second slide for each sample, anti-Col IV, anti-FN, and anti-LM antibodies were used in the incubation of one tissue section each. Each slide also had a negative control section that received only 1% goat serum (Table 2.1), which served as the diluent for primary antibodies. This control section was subjected to the same conditions as the treatment sections during all other steps. Special care was taken to ensure that antibody from one section did not break its Pap Pen “barrier” to contaminate an adjacent section. Following addition of primary antibody, slides were placed in a humidifying chamber at 4°C overnight.

Secondary Antibody Incubation and Detection

After “flicking” off primary antibodies, slides were washed in 3 solutions of PBS for 5 min each. Two or 3 drops of biotinylated second antibody (Reagent 1B of Histostain Kit, Zymed Laboratories, Inc., San Francisco, CA) were then added to each section, and slides were placed back in the humidifying chamber for a period of 30 min. Slides were “flicked” to remove second antibody and washed 3 times in PBS for 2 min each. Two or 3 drops of streptavidin-peroxidase (Reagent 2 of Histostain Kit) were administered to each section and allowed to incubate for 10 min, after which came 3 more 5 min washes in PBS. Two or 3 drops of diaminobenzidine solution (Table 2.1, Liquid DAB Substrate Kit, Zymed Laboratories) was added to each section for approximately 5 min. A counterstaining period of 30 sec was performed with hematoxylin, and slides were dehydrated and coverslipped as previously described.

Determination of Mammary Parenchymal Tissue Composition

Digital Image Acquisition

Sections stained with hematoxylin and eosin were used to determine mammary parenchymal tissue composition. A small glass insert etched with a 10X10 grid was placed into the eyepiece of the microscope (Olympus BH-2, OPELCO, Dulles, VA), generating a 10X10 grid covering most of the field of view. Slides were first examined under 40X magnification, which generally allowed for complete grid coverage of the tissue section. Larger tissue sections that did not fit within the confines of the grid were simply centered. An “anchor point” was identified so that slides could be moved away from and returned to their original starting positions. At this point, the ocular grid was treated as a 5X5 grid, with the center of each of the 25 squares clearly defined by an intersection point. Intersection points were examined one at a time, and for each of the points that contacted parenchymal tissue, the slide was moved to a position where that particular point was centered in the field of view. The section of tissue was then focused under 400X magnification and a single image was captured with a top-mount digital microscope camera (Olympus DP10, OPELCO, Dulles, VA). Magnification was returned to 40X and the slide was moved back to its “anchor point” for examination of the next intersection point. Upon completion of each sample, images were transferred to a computer. The process of image collection resulted in image number variation between samples (approximately 6 images per sample), but allowed for a collection of images representative of parenchymal tissue.

Image Analysis for Percent Epithelium, Lumen, and Stroma

Digital images were examined using L-View Pro image analysis software (Mmedia Research Corporation, Hallandale, FL). Images were opened up at 50% zoom and an artificial 9X8 grid was superimposed over the image. Each of the 72 intersection points were determined to be in contact with either epithelium, lumen, or stroma, and a running total for each type of tissue was tallied. Intersection points not in contact with the tissue section were not counted. Once all images from a particular sample had been subjected to the grid and counted, tissue proportions were determined by dividing the number of tissue specific intersection points by the total number of points counted.

Developmental Categorization

The previously described procedure for obtaining tissue composition percentages was not considered sufficient in determining total amount of development, because only intersection points in contact with parenchymal tissue were used in acquiring the images utilized for analysis. We believed that this means of data collection might not reflect obvious differences between well-developed tissue sections that were completely filled with parenchymal tissue and those under-developed sections that possessed relatively small amounts of parenchymal tissue. Thus, three representative control images were obtained at 40X magnification and stored on the computer. The control images were assigned a developmental grade of 1 (least developed) to 3 (most developed) based on the amount of parenchymal tissue present (see Figure 2.3). Each LM stained tissue section was then viewed at 40X magnification, compared to control images opened with L View Pro, and given a developmental score of 1 to 3, resulting in one response for each sample.

Determination of Percent ER- and Ki67-positive Epithelial Cells

Digital Image Acquisition

Slides containing tissue sections stained with primary antibody for ER and Ki67 were examined under the microscope at 40X magnification. Examination under these low-power conditions allowed for epithelial structures to be distinguished as either SUB or TDU, but abundance of staining was not observable. Three areas of tissue containing SUB and 3 containing TDU were randomly selected from ER- and Ki67-stained sections. These areas were centered in the field of view and focused under 400X magnification. A single image was then captured with the digital microscope camera. Once all 12 images (3 for ER-SUB, ER-TDU, Ki67-SUB, and Ki67-TDU) were taken for each sample, they were transferred to a computer for storage and analysis. Some sections from underdeveloped tissue contained less than 3 areas with SUB or TDU. In these cases, as many pictures as possible were taken, and in a few cases (5, 2, 4, and 1 for ER-SUB, ER-TDU, Ki67-SUB, and Ki67-TDU, respectively) no pictures were taken due to the absence of either SUB or TDU in the tissue section.

Image Analysis for Percent ER- and Ki67-positive Epithelial Cells

Images were opened with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) at 50% zoom. Prior to any data being collected, a spatial calibration was performed on the first image opened, and this calibration was used for the remainder of the ER and Ki67 analyses. The spatial calibration window was obtained with the following

command sequence: Measure – Calibration – Spatial Calibration. The area of a representative epithelial cell was calculated using the image, and this specific area was defined in the unit box as a “cell area.”

To analyze the percentage of ER- and Ki67-positive epithelial cells in each image, the Measurements window was accessed (Measure – Measurements). Our particular measurement of interest (Area) was specified under the Select Measurements command. Using the mouse and cursor, each continuous epithelial structure on the image was outlined. An area measurement was generated for each outlined structure, which, due to the nature of our calibration, reflected the total number of epithelial cells within the boundaries of the structure. ER- and Ki67-positive epithelial cells were stained dark brown due to the horseradish peroxidase reaction, and thus could be distinguished and counted easily (Figure 2.2). The number of positive epithelial cells identified in one image was divided by the total number of epithelial cells present to obtain the proportion of either ER- or Ki67-positive cells. An average of images for each variable within a sample (ER-SUB, etc.) was recorded. Approximately 1200 cells were counted for each variable within a sample.

ECM Protein Staining Intensity

Control Images

Multiple tissue sections stained with primary antibodies for Col IV, FN, and LM were examined to establish the extremes of staining intensities, and to obtain four representative staining intensity pictures for each protein. The four pictures for each protein were assigned an intensity grade of 1 (lowest staining intensity) to 4 (highest staining intensity) based on the intensity of brown color produced by the horseradish peroxidase reaction. These control pictures were taken at 400X magnification with the digital microscope camera and saved on a computer. Figures 2.4, 2.5, and 2.6 show the staining intensity control pictures used for Col IV, FN, and LM analyses, respectively.

Col IV Analysis

The four Col IV control staining intensity pictures were opened up with L View Pro image analysis software and were retained on the screen during analysis for reference. Since the majority of Col IV staining activity was localized in the BM of epithelial structures (see figure 2.4), only BM staining was considered in the analysis for this protein. A Col IV stained tissue section was initially viewed at 40X magnification so that epithelial structures, but not amount of staining, could be identified. At this magnification, 5 random areas of tissue containing SUB and 5 random areas containing TDU were centered in the field of view, moved to 400X magnification, and brought into focus. Each of the 10 structures examined for each sample was analyzed for presence of

a Col IV stained BM (yes or no) and, when present, the staining intensity of the Col IV in the BM (1 to 4 based on control images). From this data, 4 response variables were obtained for each sample: Percentage of SUB containing a Col IV stained BM; Intensity of Col IV staining in the BM of SUB; Percentage of TDU containing a Col IV stained BM; and Intensity of Col IV staining in the BM of TDU. Structures that did not possess a Col IV stained BM were not given an intensity score. Also, some tissue sections lacked 5 independent SUB or 5 independent TDU structures. In these cases, available structures were analyzed.

FN and LM Analysis

Control staining intensity pictures for these two proteins were also opened using L View Pro. Tissue sections stained for FN and LM were viewed at 40X magnification. Five random areas of tissue adjacent to SUB, 5 adjacent to TDU, and 5 not adjacent to epithelium were centered in the field of view and then moved to 400X magnification and brought into focus. For well-developed tissue, distinct areas between lobules of epithelium (i.e. interlobular areas) were used for the 5 areas not adjacent to epithelium. However, in more under-developed tissue these interlobular areas were not apparent and thus the only criteria for this situation was that the 5 areas did not contact any epithelium at 400X magnification. Each area of tissue examined was given a staining intensity score (1 to 4 based on control pictures). In contrast to Col IV staining, FN and LM staining were generally present throughout the entire tissue section (see figures 2.5 and 2.6), thus intensity scores were assigned accordingly. Areas adjacent to SUB and TDU were also examined for the presence of a distinct FN or LM stained epithelial BM, resulting in each

sample being assigned a yes or no for presence of a distinctly stained BM. This resulted in 5 response variables for each sample: FN (LM) staining intensity adjacent to SUB; Presence of a distinct FN (LM) stained BM at a SUB; FN (LM) staining intensity adjacent to TDU; Presence of a distinct FN (LM) stained BM at a TDU; and FN (LM) staining intensity not adjacent to epithelium. Tissue sections lacking in any of the 5 distinct areas of tissue were analyzed based on available structures.

Control Experiments for Cross-Reactivity

Although our antibodies were purchased as being specific based on data from the suppliers, we also completed some additional cross-reactivity experiments. Batches of primary antibodies against Col IV and LM (see Table 2.2 for information) were prepared at dilutions of 1:10 and 1:50, respectively. Fifty μL of Col IV antibody was mixed with 50 μL (10 μg) Col IV protein (Catalog No. 1260-025, Southern Biotechnology Associates, Inc., Birmingham, AL), 50 μL (10 μg) Col I protein (Catalog No. 354236, BD Biosciences, San Jose, CA), and 50 μL diluent (1% goat serum). Fifty μL of LM antibody was mixed with 50 μL (10 μg) LM protein (Catalog No. 354232, BD Biosciences), 50 μL (10 μg) FN protein (Catalog No. 1390-01L, Southern Biotechnology Associates, Inc.) and 50 μL diluent (1% goat serum). These mixtures were incubated at 4°C overnight. The following day, 2 extra slides from sample 33-M were prepared for staining via de-paraffinization and hydration. The before-described procedure for immunochemical staining was performed on both slides, with 1 slide each being designated for those mixtures containing Col IV and LM antibodies. Sections were counterstained, dehydrated, and coverslipped. Digital images of sections were captured for the purposes of comparing staining intensities.

A similar procedure was performed with primary antibody for FN. However, some evidence of non-localized staining and lack of competition prompted us to perform a Western analysis. Fifty μg each of the four before-mentioned ECM proteins (Col I, Col IV, FN, and LM) were electrophoresed on a 7.5% SDS-PAGE gel, transferred to a

nitrocellulose membrane, and blocked in 5% BSA for 1 hr. The membrane was incubated with anti-FN antibody (see Table 2.2) at a 1:400 dilution for a period of 2 hr, then washed 3 times for 10-15 min in TBS-NP40. The membrane was incubated in second antibody (anti-Mouse IgG Peroxidase Conjugate, Sigma, St. Louis, MO) at a 1:1000 dilution for 1.5 hr and washed 3 more times in TBS-NP40. Detection was achieved with Super Signal West Chemi Substrate Kit (Pierce, Rockford, IL). In total, these data supported the use of the antibodies with bovine samples.

Statistical Analysis

Data were analyzed using the GLM procedure of SAS (SAS Institute, Inc., Cary, NC). The main effects in the model were feeding level, zone of parenchymal tissue, and the interaction of treatment with zone. The following main effects were used as blocking factors in the model: block (date of purchase), batch (slides stained under identical conditions), and observer (person collecting data). Batch was included as a blocking factor due to the inherent inability to produce an identical staining environment for each set of slides (20 slides per set). Observer was included as a blocking factor to rule out variation due to personal bias pertaining to data collection. Treatment contrasts included HH and HL vs. LH and LL, HH and LH vs. HL and LL, HH vs. LL heifers. Zone contrasts included I vs. M and C and I vs. C. For tissue composition (% epithelium, % lumen, and % stroma), ER, Ki67, and Col IV data, responses within SUB and TDU were grouped to determine differences between these two structures. For FN and LM data, responses at SUB, at TDU, and not adjacent to epithelium were grouped, and contrasts included NOT ADJ to EPI vs. SUB and TDU and SUB vs. TDU. Least squares means and standard errors were reported, significance was declared at $p < 0.05$, and trends were declared at $p < 0.10$. Analysis of the percentage of samples with a FN or LM stained BM in a SUB or TDU and developmental categorization also utilized the LOGISTIC procedure of SAS. All main effects were entered into the model and a forward selection procedure was used to determine the final model. Feeding treatments were compared against the LL treatment and zones I and M were each compared to zone C. Odds ratio estimates were reported for feeding level and zone when entered into the model, significance was declared at $p < 0.05$, and trends were declared at $p < 0.10$.

Figure 2.1: Summary of experimental design and actual ADG.

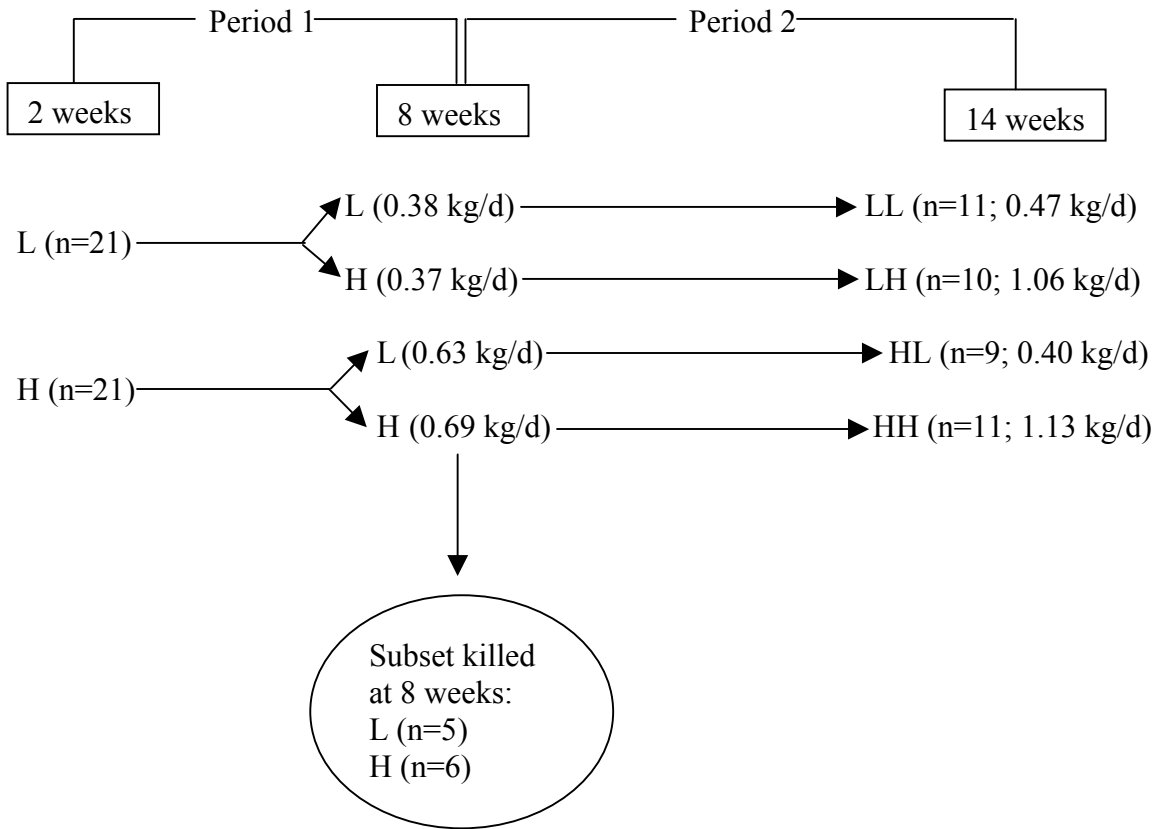


Figure 2.2: ER- and Ki67-positive epithelial cells within a SUB.

ER- (top) and Ki67-positive (bottom) cells are stained dark brown. Note the greater abundance of ER-positive cells within the epithelial cell layers (arrows). Also note the absence of ER expression in the stromal areas (asterisks).

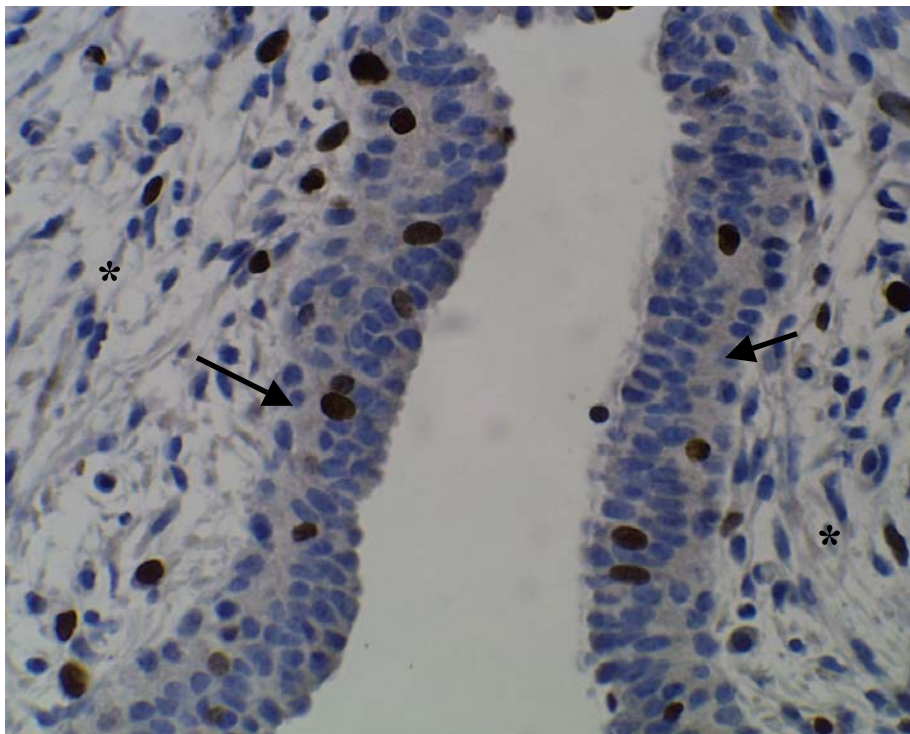
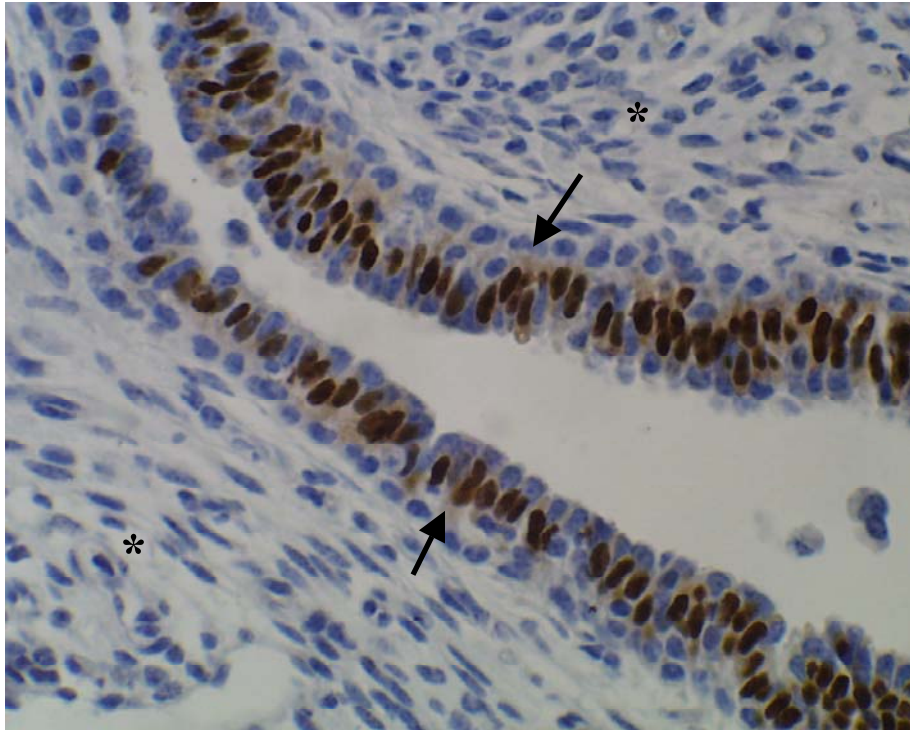


Figure 2.3: Control images used in assignment of developmental categorization.
Developmental grades 1 (least developed) to 3 (most developed, 40X).

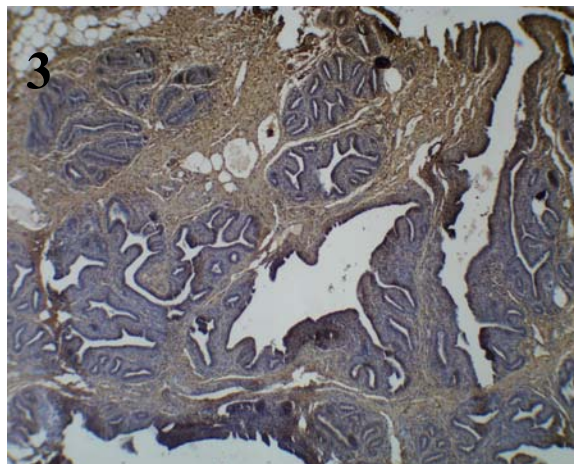
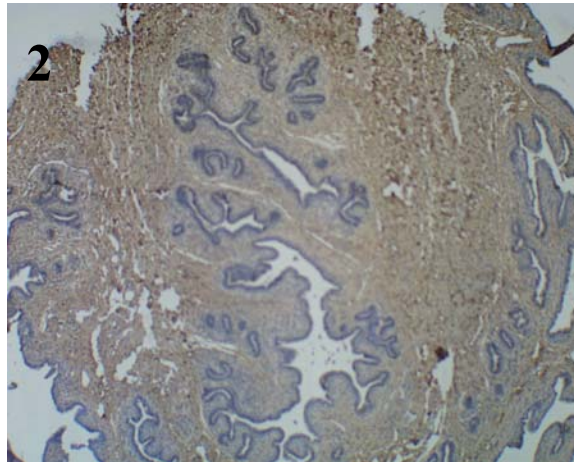


Figure 2.4: Control images used in assignment of Col IV staining intensity within BM.

Intensity grades 1 (lowest staining intensity) to 4 (highest staining intensity, 400X).

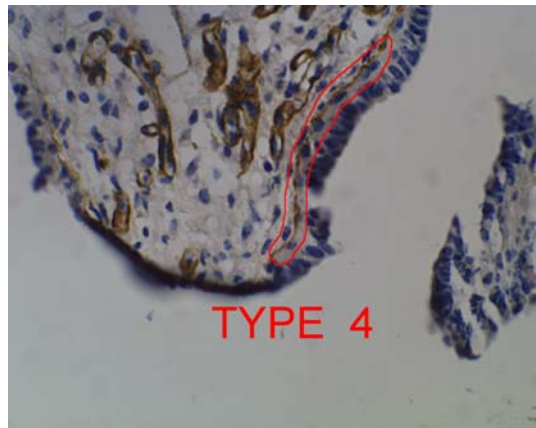
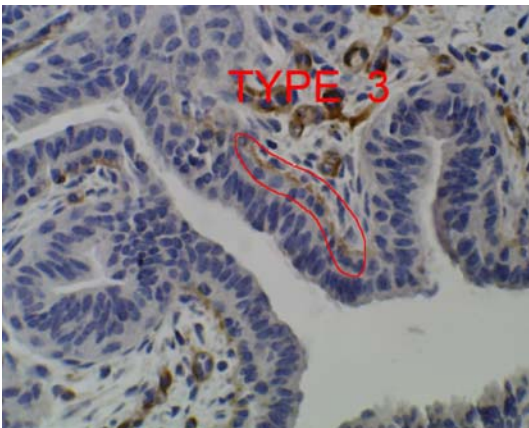
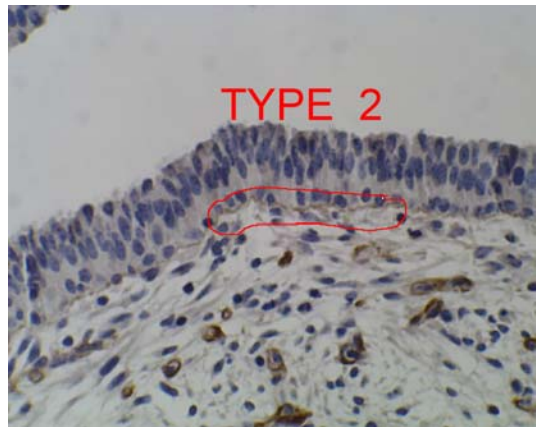
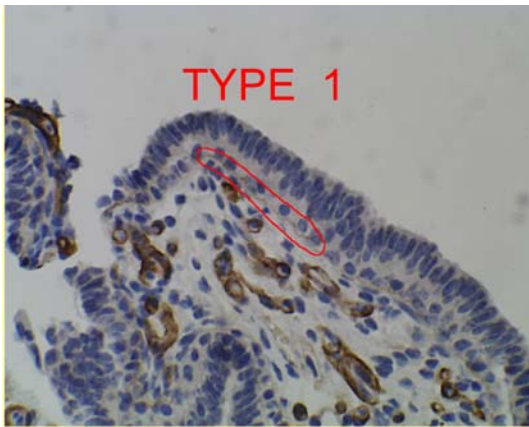


Figure 2.5: Control images used in assignment of FN staining intensity.
Intensity grades 1 (lowest staining intensity) to 4 (highest staining intensity, 400X).

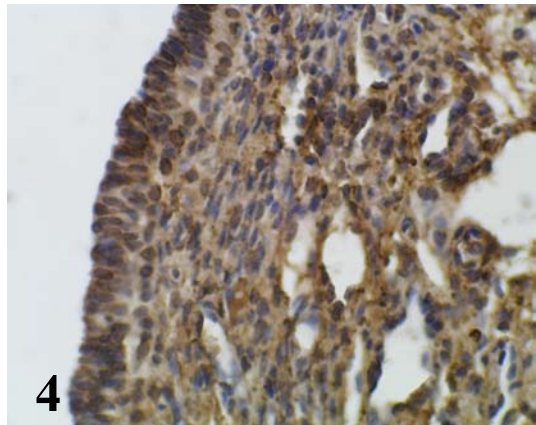
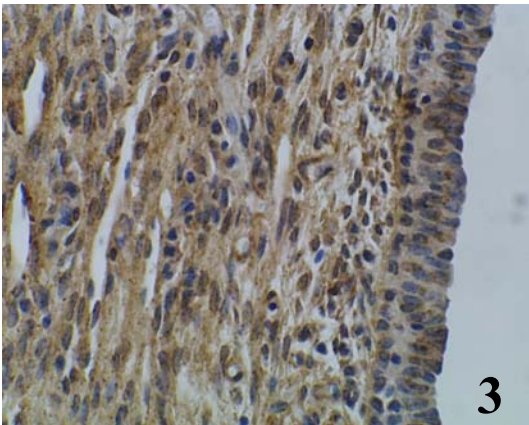
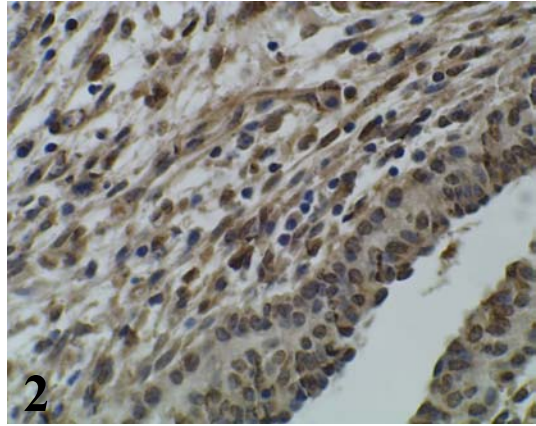
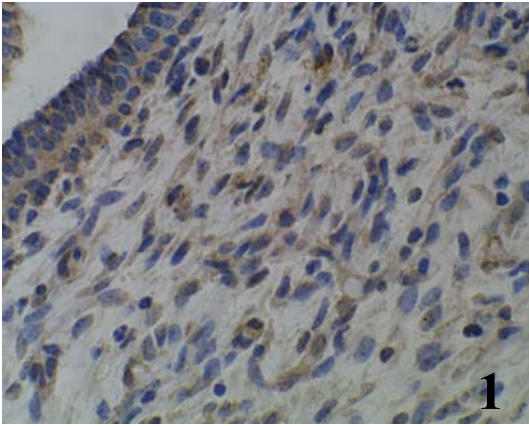


Figure 2.6: Control images used in assignment of LM staining intensity.
Intensity grades 1 (lowest staining intensity) to 4 (highest staining intensity, 400X).

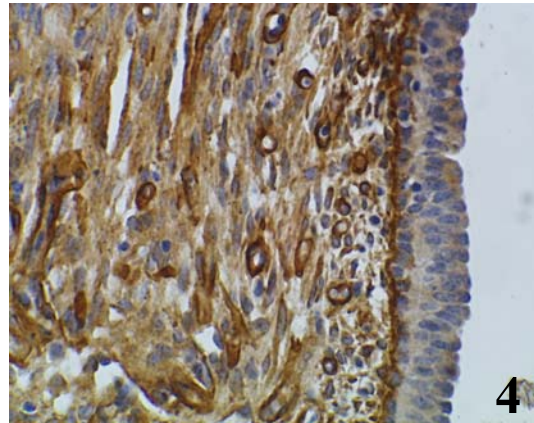
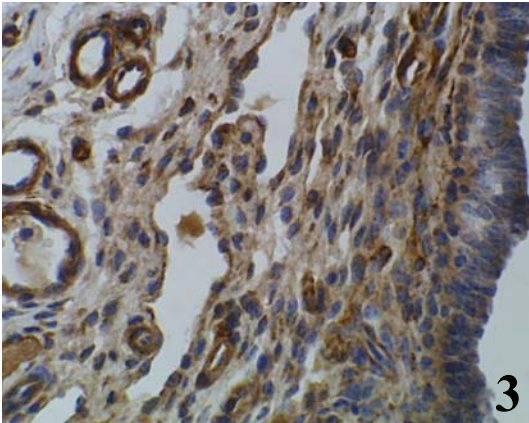
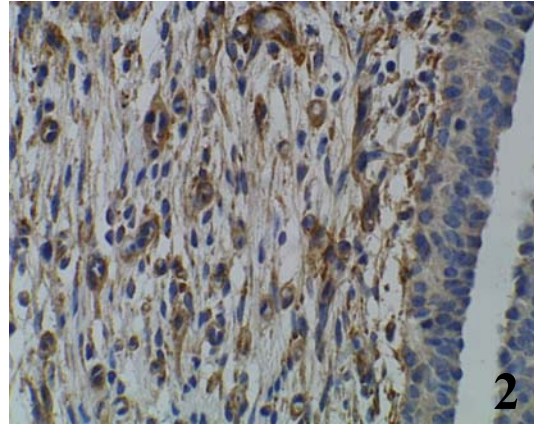
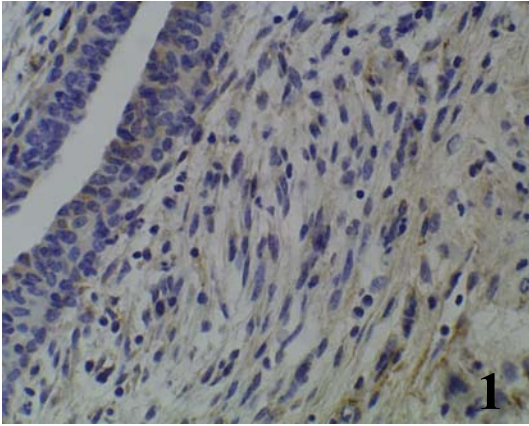


Table 2.1: Reagents used in immunochemical staining procedures.

Reagent	Composition
10X PBS (phosphate buffered saline)	21.4 g Na ₂ HPO ₄ + 5.76 g NaH ₂ PO ₄ + 87.8 g NaCl (adjust volume to 1L with distilled H ₂ O and pH to 7.4)
1X PBS	100 mL 10X PBS + 900 mL distilled H ₂ O
3% H ₂ O ₂ in PBS	30 mL 30% w/w H ₂ O ₂ + 270 mL PBS
10 mM citrate buffer	2.9 g sodium citrate (adjust volume to 1L with distilled H ₂ O and pH to 6.0)
5% goat serum	5 mL goat serum (info.) + 95 mL PBS
1% goat serum	1 mL goat serum + 99 mL PBS
diaminobenzidine (DAB) solution	5 drops Reagent A of DAB Kit + 5 mL distilled H ₂ O (mix) + 5 drops Reagent B + 5 drops Reagent C (mix)

Table 2.2: Primary antibody information.

Primary Antibody	Manufacturer	Catalog Number	Working Dilution*
Fibronectin Ab-11 (Clone FBN 11)	Lab Vision Corporation	MS-1351	1:100
Laminin Ab-1	Lab Vision Corporation	RB-082	1:50
Estrogen Receptor- α (C-311)	Santa Cruz Biotechnology	sc-787	2 μ g/mL
Collagen Type IV (Ab-2)	Oncogene Research Products	CP56	1:50
Ki67	Zymed Laboratories, Inc.	08-0156	pre-diluted

* Working solutions of 0.5 mL were prepared in 1% non-immune goat serum (Zymed Laboratories, Inc.).

RESULTS

Mammary Growth and Development

Tissue Composition

Feeding level did not significantly affect the percentage of epithelial tissue in mammary parenchyma (18.0 to 20.9%, Figure 3.14). However, percent lumen was 3.5% greater ($p=0.01$) in HH and HL vs. LH and LL heifers and 4.4% greater ($p=0.02$) in HH vs. LL heifers. Opposite treatment effects were demonstrated with respect to percent stroma, which was 4.0% lower ($p=0.02$) in HH and HL vs. LH and LL heifers and 5.7% lower ($p=0.01$) in HH vs. LL heifers.

Percentages of epithelium in mammary parenchymal tissue did not differ significantly between zones of the gland, averaging 19.6% (Figure 3.15). However, there was 3.9% less ($p=0.01$) luminal area in zone I vs. M and C, and 4.1% less ($p=0.02$) in zone I vs. C. The opposite effect was seen with percent stromal area, which was 5.3% greater ($p=0.004$) in zone I vs. M and C and 5.8% greater ($p=0.007$) in zone I vs. C.

Developmental Categorization

Mean developmental categorization scores for HH, HL, LH, and LL heifers were 2.30, 2.02, 2.31, and 1.60, respectively (Figure 3.16). Heifers receiving the HH and LH feeding treatments had a 0.50 unit higher ($p=0.0006$) developmental score than those heifers receiving the HL and LL diets. The developmental score for HH heifers was 0.71

higher ($p=0.0003$) than in LL heifers. Odds ratio analysis revealed significant differences between HH vs. LL and LH vs. LL heifers.

Differences between zones of the mammary gland were also seen with regard to level of development (Table 3.1). Zone I scored 0.40 and 0.31 units lower ($p=0.007$ and $p=0.07$, respectively) than zones M and C together and zone C alone.

Estrogen Receptor and Ki67

Feeding level did not significantly affect the percentage of ER-positive epithelial cells in SUB, averaging 46.6% across treatments (Figure 3.17). Diet also had no effect on the percentage of ER-positive epithelial cells in TDU, averaging 52.4% across treatments. However, Ki67 expression was affected by feeding level (Figure 3.18). In SUB, percent Ki67-positive epithelial cells was 1.4% lower ($p=0.05$) in HH and HL vs. LH and LL heifers, and 2.3% lower ($p=0.01$) in HH vs. LL heifers. Similar dietary effects were seen in TDU, with HH and HL heifers having 2.1% fewer ($p=0.01$) Ki67-positive cells than LH and LL heifers, and HH heifers having 2.4% fewer ($p=0.03$) Ki67-positive cells than LL heifers.

Neither ER nor Ki67 expression was affected by mammary zone (Table 3.2). The mean percentages of ER-positive epithelial cells in zones I, M, and C was 49.5%. The mean percentages of Ki67-positive epithelial cells in zones I, M, and C were 5.4, 3.9, and 5.0%.

Significant structure differences existed with respect to ER, but not Ki67, expression (Table 3.3). Percent ER-positive epithelial cells was 46.6 and 52.4% in SUB and TDU, respectively ($p < 0.0001$).

Extracellular Matrix

Type IV Collagen

Feeding level did not significantly affect Col IV deposition (Table 3.4). The percentages of SUB with a Col IV stained BM (Figure 3.1A) were 47.9, 44.4, 41.1, and 41.7% for HH, HL, LH, and LL heifers, respectively. Percentages of TDU with a Col IV stained BM were 9.8, 11.3, 7.5, and 8.3%, respectively. Although Col IV deposition was more likely to occur in the BM of more mature epithelial structures, staining prevalence generally was not consistent within sample or SUB (Figure 3.4A). Staining intensity scores for Col IV stained BM averaged 1.55 within SUB and 1.36 within TDU across dietary treatments. There was a trend ($p=0.09$) for HH and LH heifers to demonstrate less intense Col IV staining in the BM of SUB.

Col IV deposition did not differ by zone of mammary gland (Table 3.5), however, prevalence and intensity of Col IV deposition varied between structures (Table 3.6), with 43.3 and 9.7% of SUB and TDU ($p<0.0001$), respectively, containing a Col IV stained BM. Also, there was a tendency ($p=0.06$) for SUB to contain more intensely Col IV stained BM than TDU (1.55 vs. 1.36, respectively).

In well-developed tissue, Col IV deposition was greater in the interlobular than in the intralobular space (Figure 3.2A). This interlobular deposition was frequently arranged in a highly organized “rope-like” pattern, following the contour of the lobule. Both SUB and TDU were more likely to possess a Col IV stained BM when the structure was

adjacent to interlobular space. Less developed tissue was characterized by a general reduction in Col IV deposition that resulted in a more uniform, but less intense, staining pattern throughout the stroma (Figure 3.3A).

Fibronectin

Feeding level had a significant effect on FN deposition adjacent to both SUB and TDU (Figure 3.19). At SUB, FN staining intensity score was 0.27 greater ($p=0.05$) in HH and HL vs. LH and LL heifers, and 0.43 greater ($p=0.03$) in HH vs. LL heifers. Adjacent to TDU, FN intensity score was 0.35 higher ($p=0.008$) in HH and LH vs. HL and LL heifers, and 0.55 higher ($p=0.001$) in HH vs. LL heifers. Diet did not affect FN staining scores in areas away from epithelial structures, averaging 2.22 across treatments. The percentage of samples with a FN stained BM in a SUB (Figure 3.1B) was 18.5, 5.3, 17.4, and 11.5% for heifers that received the HH, HL, LH, and LL feeding treatments (Table 3.7). There were no FN stained BM in TDU.

FN deposition adjacent to SUB or areas away from epithelium did not vary significantly between the different zones of the mammary gland (1.64, 1.83, and 1.62 and 1.98, 2.12, and 2.25, respectively, for zones I, M, and C, Table 3.8). However, FN staining intensity at TDU was 1.41, 1.69, and 1.71 for zones I, M, and C, resulting in significant differences between zones I vs. M and C ($p=0.04$) and between zones I vs. C ($p=0.05$). The percentage of samples with a FN stained BM in a SUB was 9.4, 10.3, and 20.6% for zones I, M, and C.

FN deposition was significantly lower in areas of tissue away from epithelium compared to those areas adjacent to SUB and TDU (Table 3.9). FN staining intensity scores were 2.22, 1.80, and 1.68 for tissue not adjacent to epithelium, adjacent to SUB, and adjacent to TDU ($p < 0.0001$ for NO EPI vs. SUB and TDU). This resulted in greater interlobular FN deposition, compared to that present in the intralobular area (Figure 3.2B). This more intense staining pattern observed away from epithelium was associated with a decrease in stromal cell density (Figure 3.5A) relative to the greater cell concentration in stromal areas adjacent to SUB and TDU (Figure 3.5B). Higher relative interlobular FN deposition contrasts with LM staining, which was higher in areas adjacent to ductular structures. This difference was evidenced by the frequent non co-localization of FN and LM (Figure 3.7). FN staining in less-developed tissue was characterized by a more consistent, but less intense pattern throughout the entire section (Figure 3.3B). Because the majority of epithelial penetration into stroma occurs via expansion at TDU, these structures were typically observed in close proximity to unoccupied stromal areas. Frequently, a lack of FN staining was noted immediately adjacent to the apparent leading edge of an advancing TDU (Figure 3.5).

Laminin

Dietary treatment had no effect on LM deposition in the ECM (Table 3.10). The intensity of LM staining adjacent to SUB averaged 2.29 across treatments. At TDU, LM staining scores averaged 2.03 for the four feeding levels. Where there was no epithelium, LM staining intensity was similar. The percentage of samples with LM stained BM in a

SUB (Figure 3.1C) was 85.7, 55.0, 39.1, and 76.0% for heifers receiving diets HH, HL, LH, and LL ($p=0.05$ and $p=0.01$ for HH vs. LL and LH vs. LL, respectively). At TDU, prevalence of stained BM was reduced but the pattern of response was similar across treatments. As with Col IV, LM deposition was more likely to occur in SUB, but staining prevalence generally was not consistent within sample or SUB (Figure 3.4C).

LM staining intensity score was unaffected by zone of the mammary gland (Table 3.11). At SUB, 59.4, 64.5, and 72.7% of samples from zones I, M, and C possessed a LM stained BM. At TDU, 17.6, 28.1, and 17.7% of samples from each of the three zones had a LM stained BM.

LM staining score was 1.90, 2.29, and 2.03 at tissue not adjacent to epithelium, at SUB, and at TDU ($p=0.0002$ and $p=0.0009$ for NO EPI vs. SUB and TDU and SUB vs. TDU, respectively, Table 3.12). This resulted in less interlobular LM deposition, compared to that present in the intralobular space (Figure 3.2C). In contrast, LM staining in less-developed tissue was characterized by a more consistent, but less intense pattern throughout the entire section (Figure 3.3C).

PAS Reaction

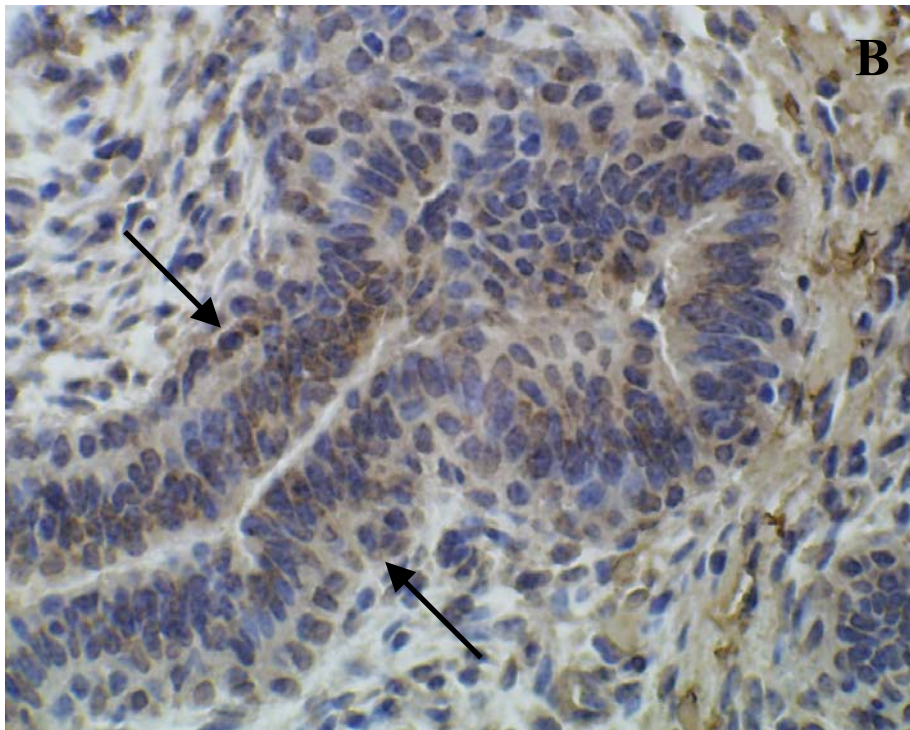
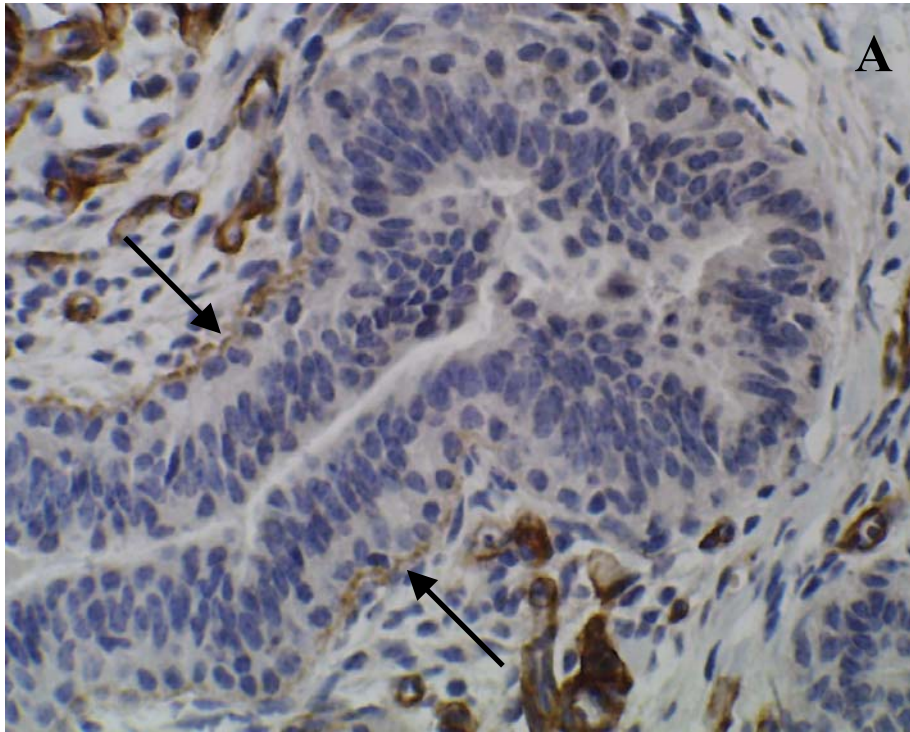
The PAS reaction visualizes carbohydrates, thus making it very useful for identification of BM and sugar-containing residues of the ECM (Sheehan and Hrapchak 1980). Very distinct PAS-positive BM were observed along SUB (Figure 3.8). BM were also

observed very frequently along TDU, although staining along these more immature structures was more diffuse compared to the more mature counterpart. BM at TDU appeared to lack the distinct “edge” that was apparent in BM of SUB. Also, BM staining was not confined to the aspect of ducts adjacent to the ECM, but was present on both sides of the basal epithelial cell layer (Figure 3.8). With respect to more developed tissue, PAS staining followed a pattern similar to that of LM, with staining in lobules having a greater intensity compared to the interlobular spaces (Figure 3.9). PAS staining between lobules also exhibited a more “sheet-like” appearance, which was characterized by a decreased intensity and increased consistency (Figure 3.10).

Cross-reactivity experiments

Antibodies used in this study were confirmed to be specific for their respective proteins of interest. Incubation of Col IV antibody with Col IV protein completely abolished the ability of the antibody to bind to Col IV sites within a section of tissue (Figure 3.11). Incubation of LM antibody with LM protein severely diminished the ability of the LM antibody to stain for LM in tissue (Figure 3.12). FN antibody incubation of a gel containing FN, LM, Col I, and Col IV proteins resulted in visualization of only FN protein (Figure 3.13).

Figure 3.1: Col IV, FN, and LM staining at a SUB and corresponding TDU. The images were taken at 400X magnification and represent serial sections from the same sample of mammary tissue. Note the presence of Col IV (A) and LM (C) stained BM (arrows) in the SUB, but not the TDU. Also, note the lack of distinct BM in the FN stained section (B).



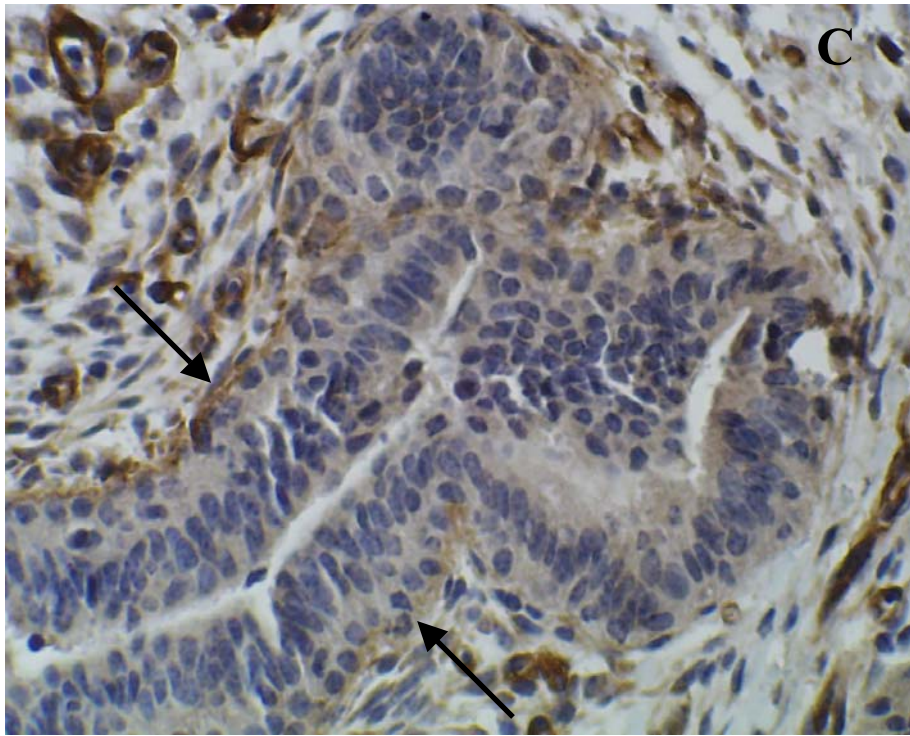
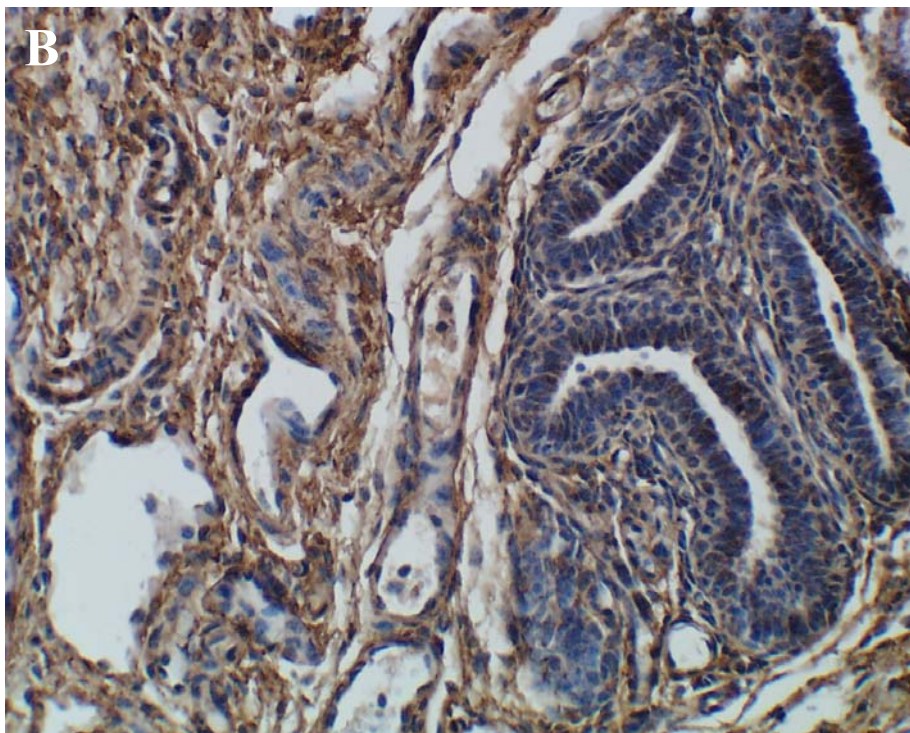
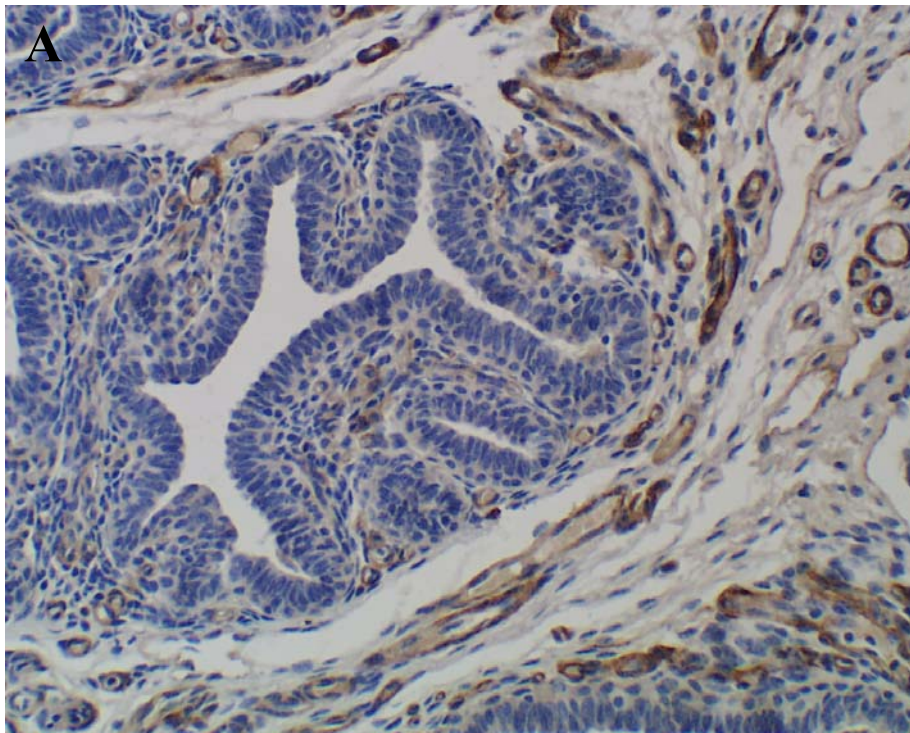


Figure 3.2: Col IV, FN, and LM deposition in well-developed tissue.
Note the greater interlobular deposition of Col IV (A) and FN (B), and the less intense interlobular deposition of LM (C). (400X, 400X, 200X)



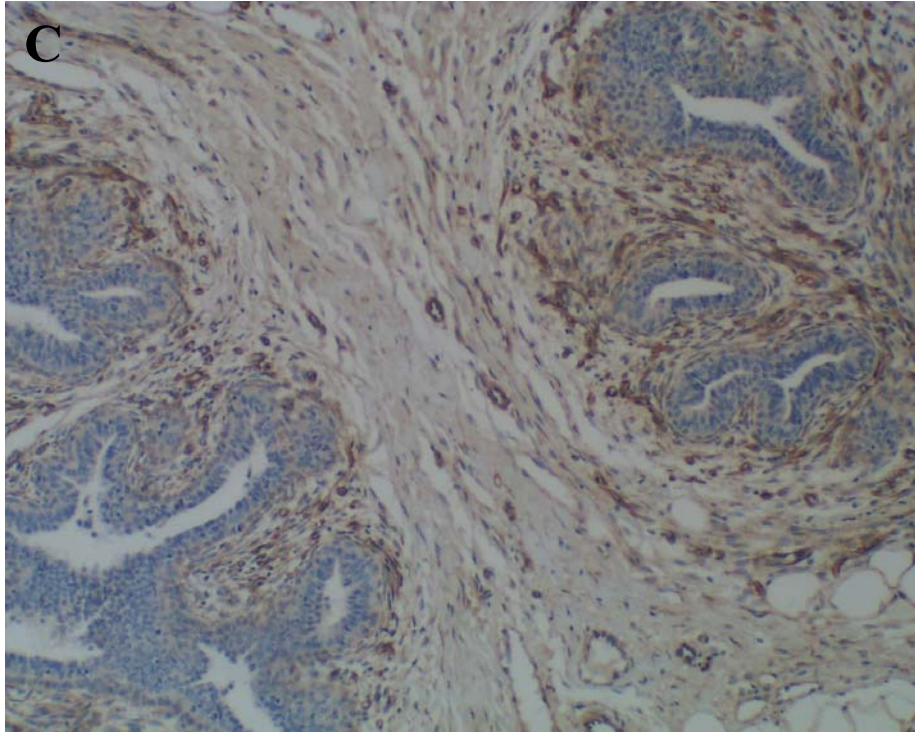
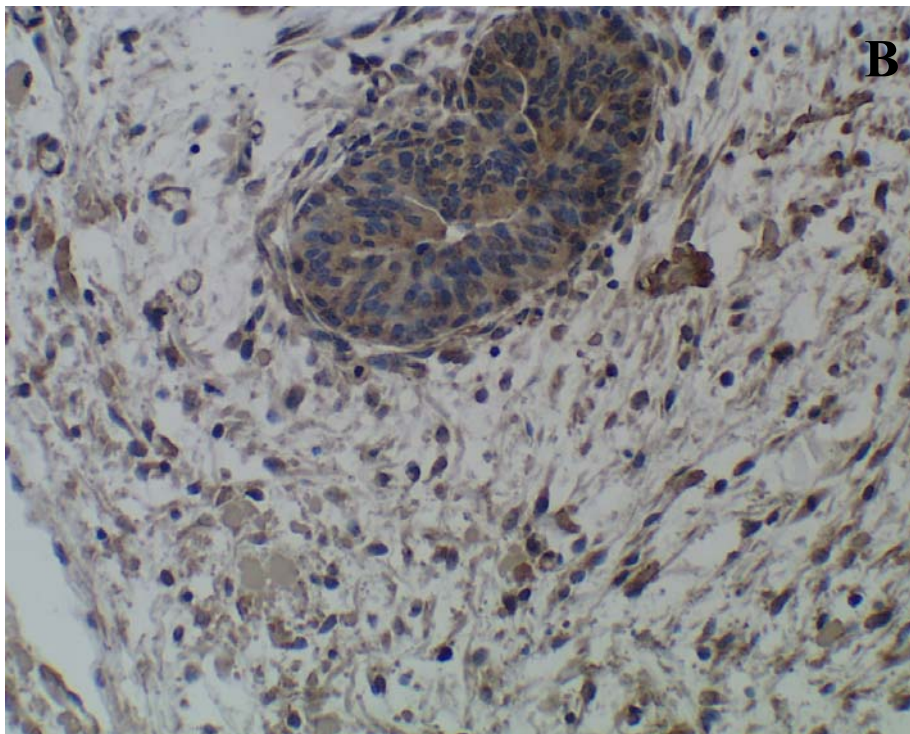
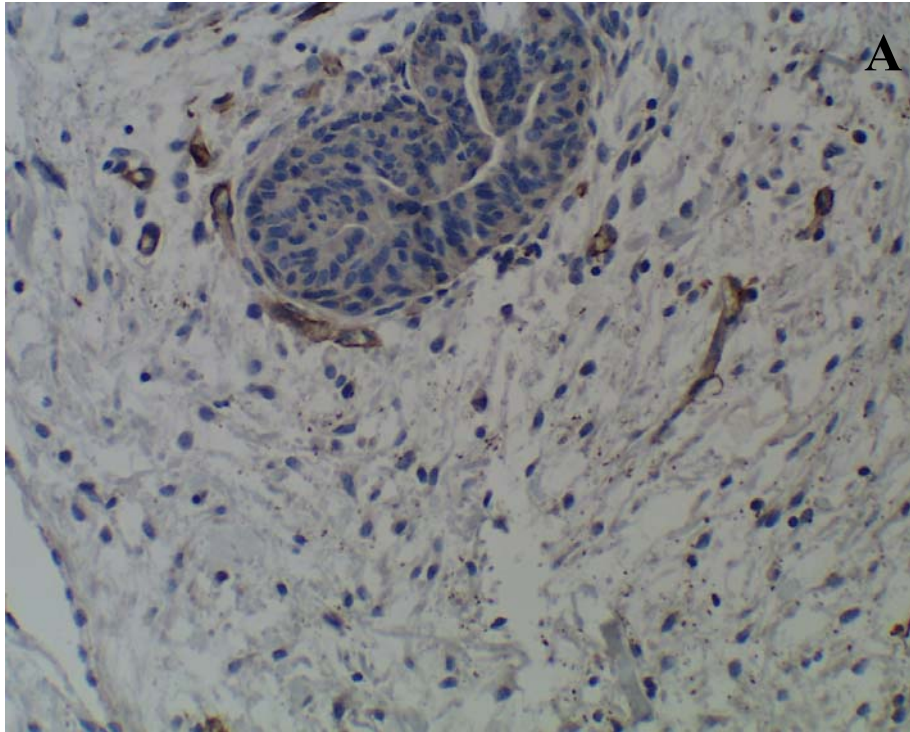


Figure 3.3: ECM protein deposition in less-developed tissue.

Images were taken at 200X magnification and represent serial sections of the same sample of mammary tissue. Note the more diffuse, less intense Col IV (A), FN (B), and LM (C) staining associated with the stroma.



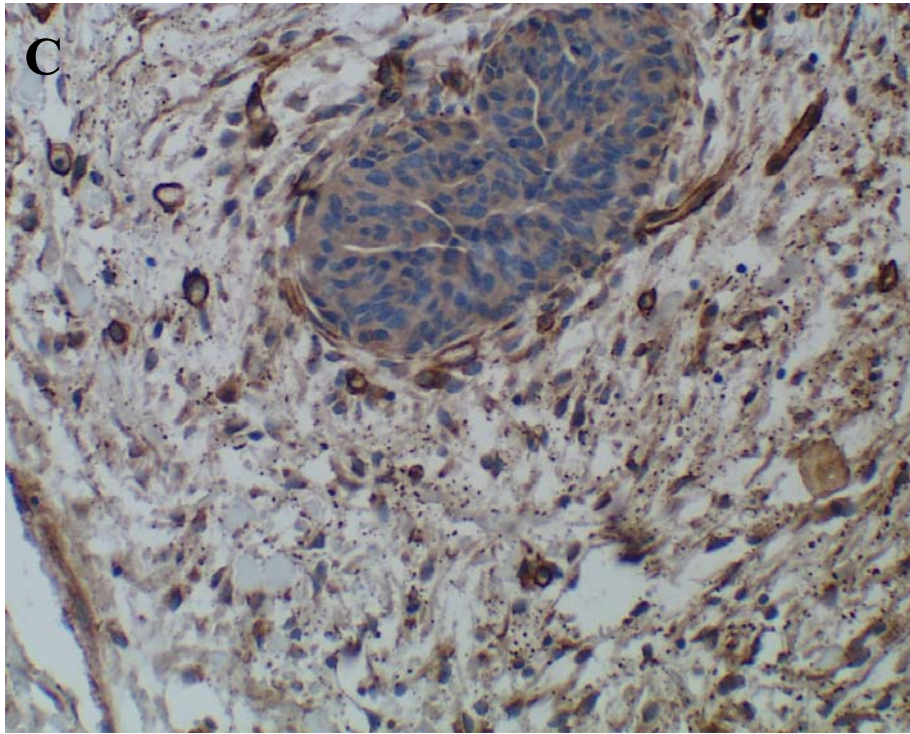
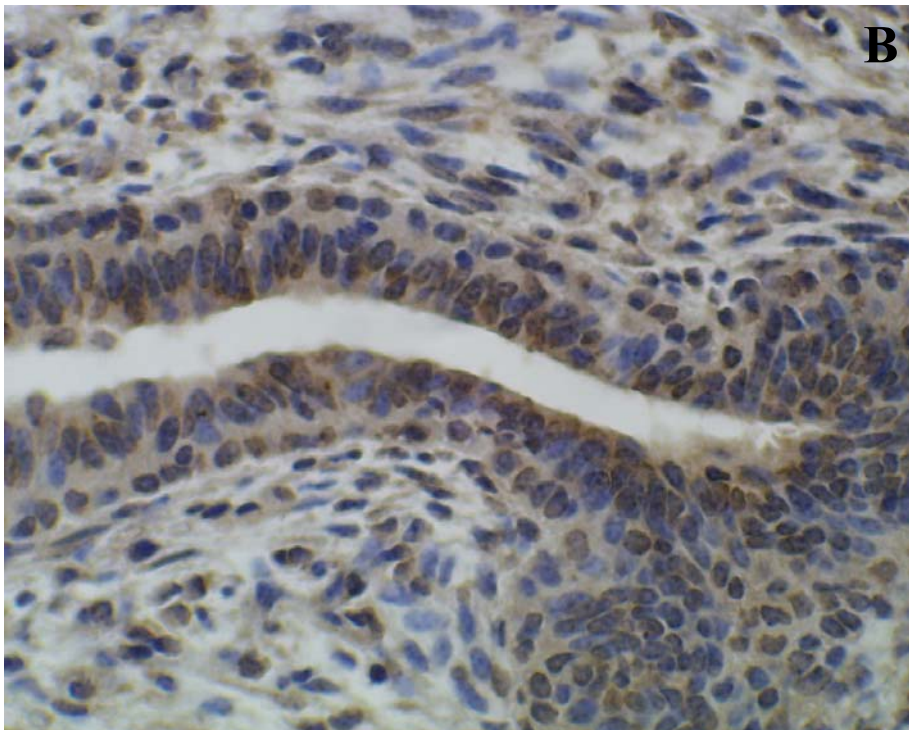
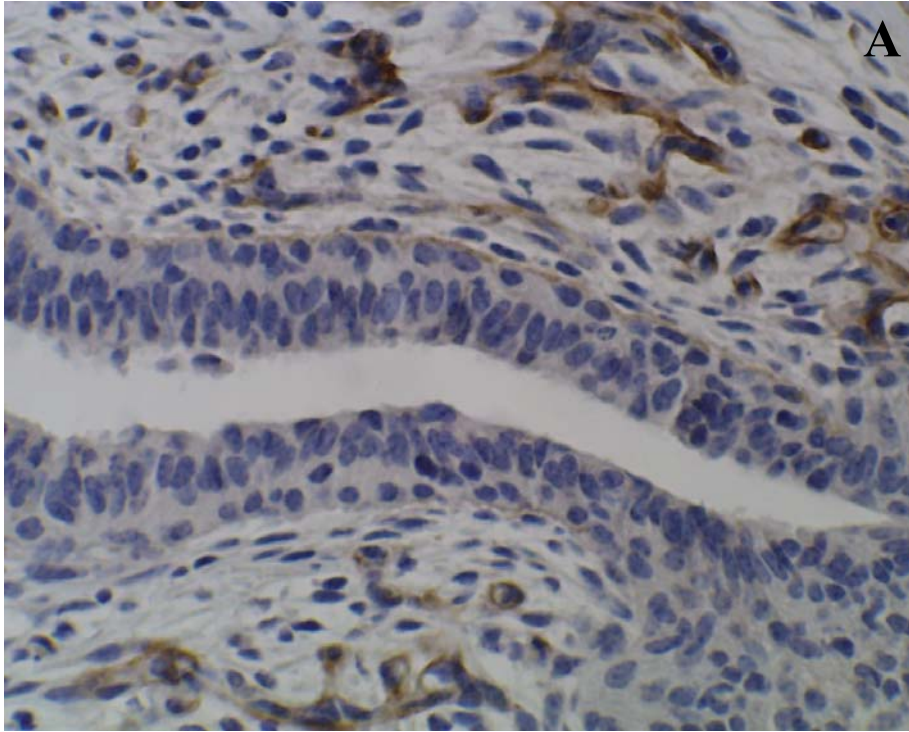


Figure 3.4: ECM protein deposition around a SUB.

Images were taken at 400X magnification from serial sections through the same sample. Note the more intense Col IV (A) and LM (C) BM staining along the upper epithelial structure, compared to the less intense staining associated with the opposing epithelial cell layer. There is no distinct FN (B) stained BM associated with either BM.



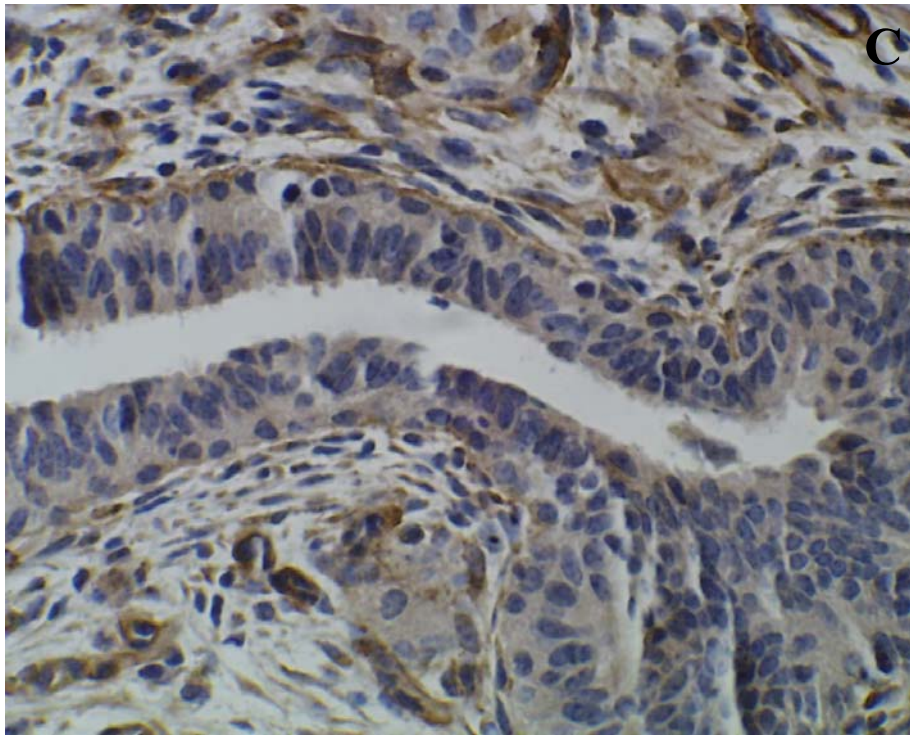


Figure 3.5: Epithelial penetration of stroma via growth at TDU.
Note the absence of FN staining (arrows) immediately adjacent to the leading edge of the epithelial cell layer (400X).

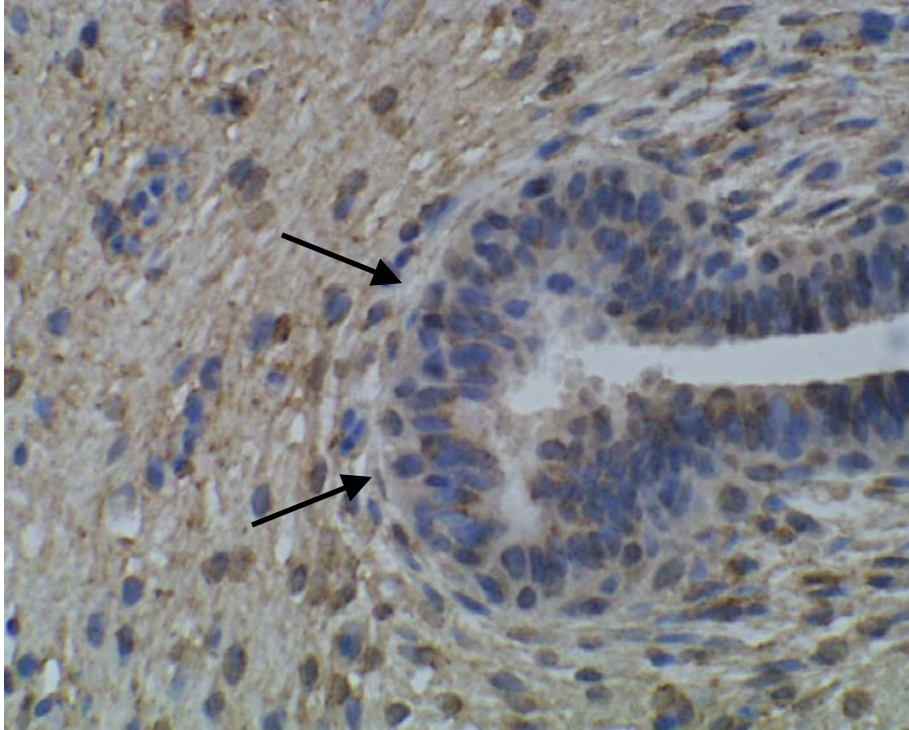


Figure 3.6: Fibroblast cell density and FN deposition adjacent to, and away from, epithelium.

Images are from adjacent portions of the same tissue section and were taken at 400X magnification. Note the greater FN staining and lower cell density observed in tissue away from epithelium (A), and the less intense staining and higher concentration of cells associated with the ductal structure (B).

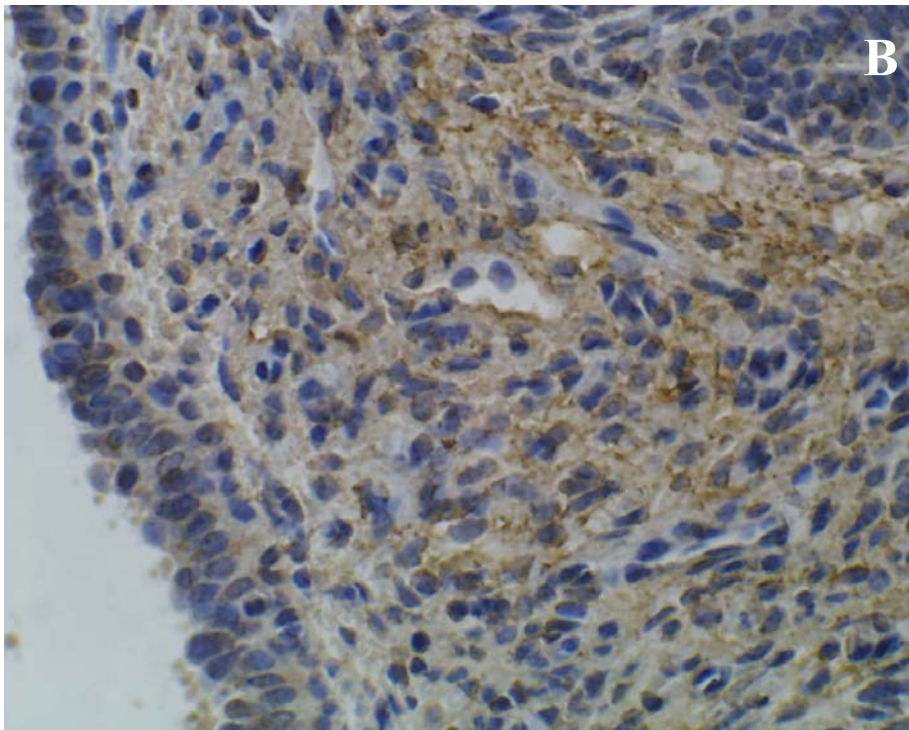
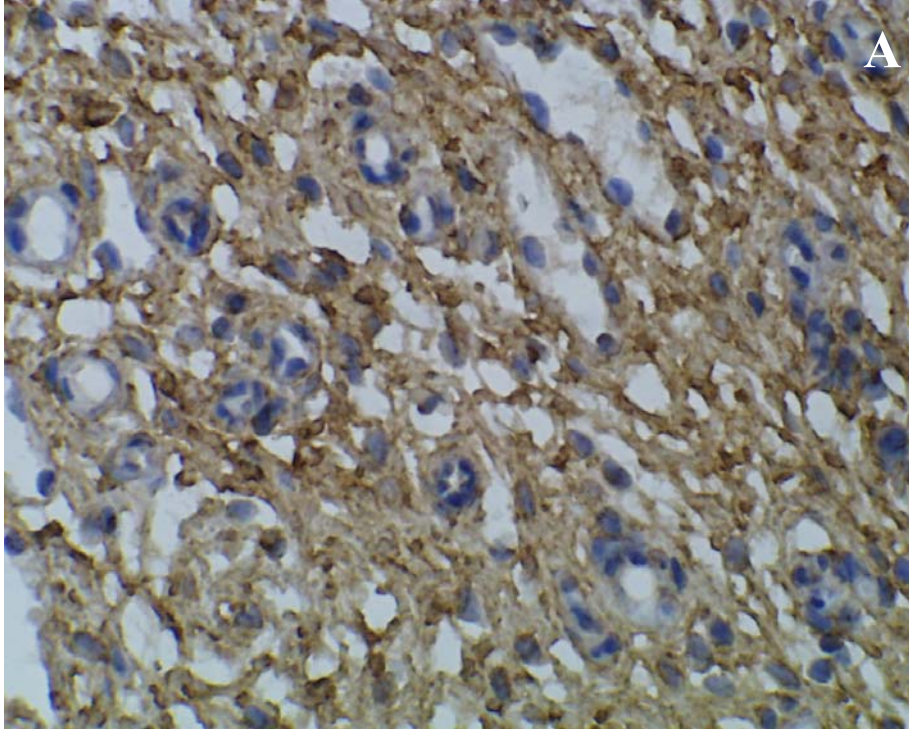


Figure 3.7: FN and LM staining at TDU.

Images were taken at 400X magnification from serial sections of tissue. FN (A) and LM (B) do not co-localize (arrows).

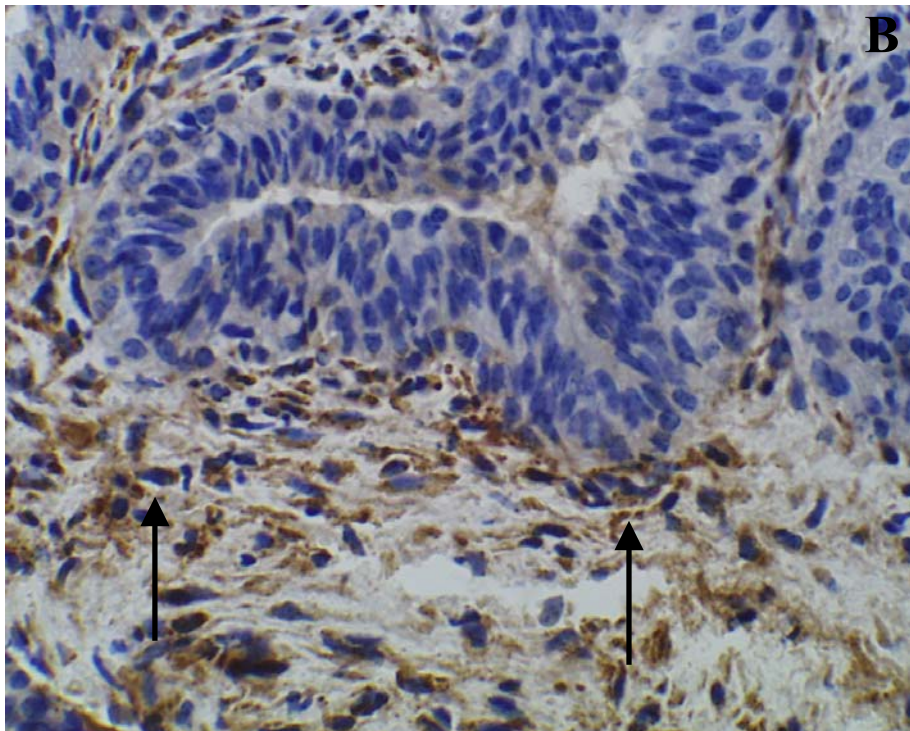
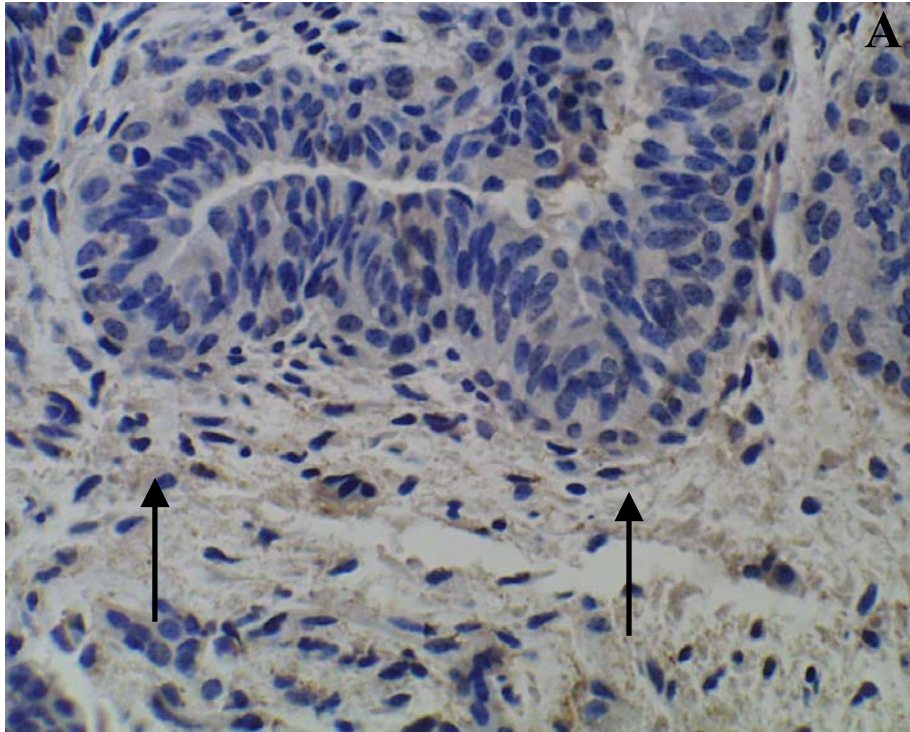


Figure 3.8: PAS-positive BM along SUB and TDU.

BM staining (arrows) is apparent on both sides of the basal epithelial cell layer, and is more distinct in BM of SUB (asterisks) compared to advancing TDU. Images **A** and **B** were captured at 400X magnification, and are from darker and lighter staining tissue, respectively.

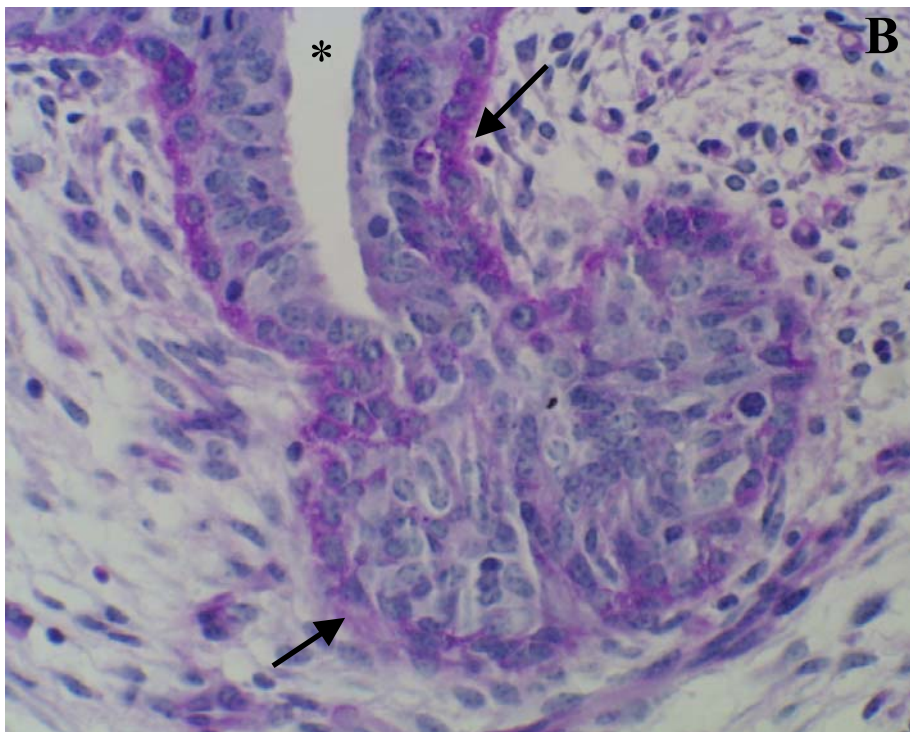
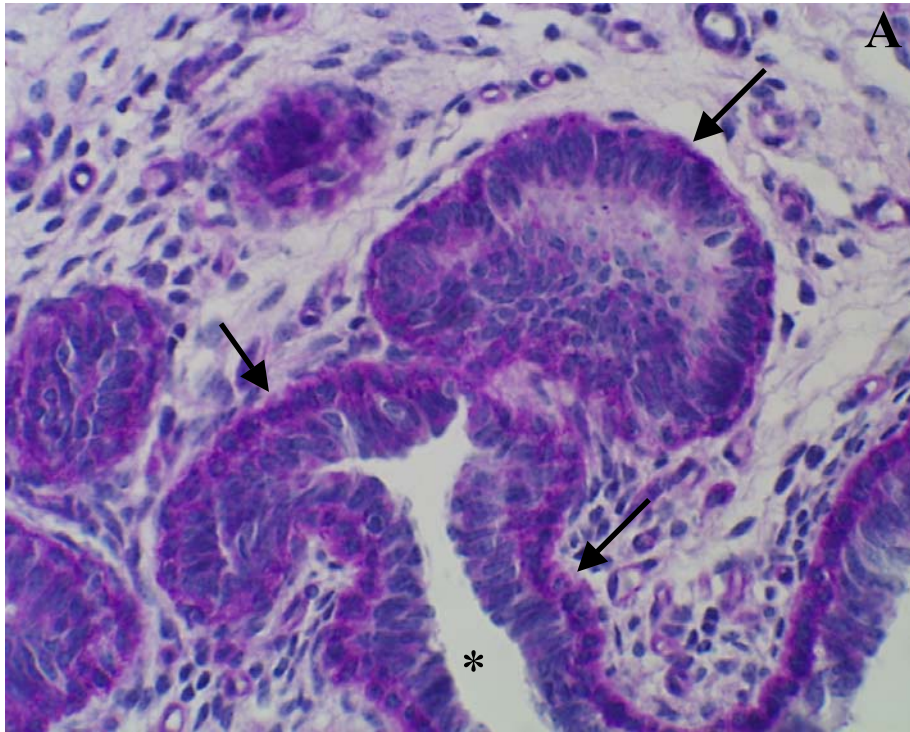


Figure 3.9: PAS staining in interlobular and intralobular areas.
Note the more intense ECM staining within intralobular areas, compared to the interlobular stroma.

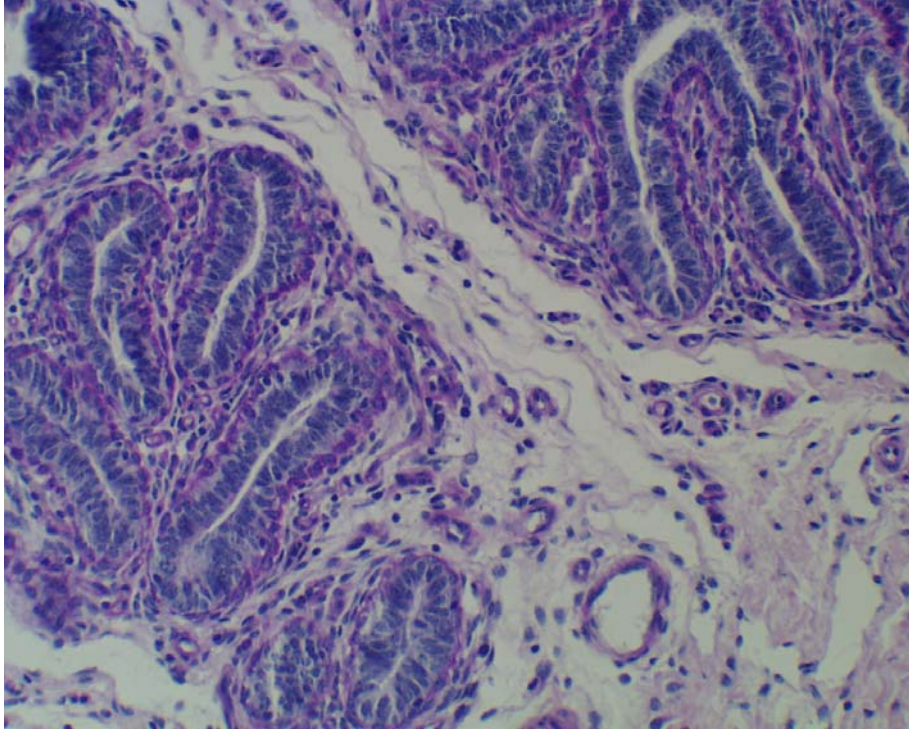


Figure 3.10: Interlobular PAS staining.
PAS staining in the interlobular ECM is less intense and has a “sheet-like” appearance.

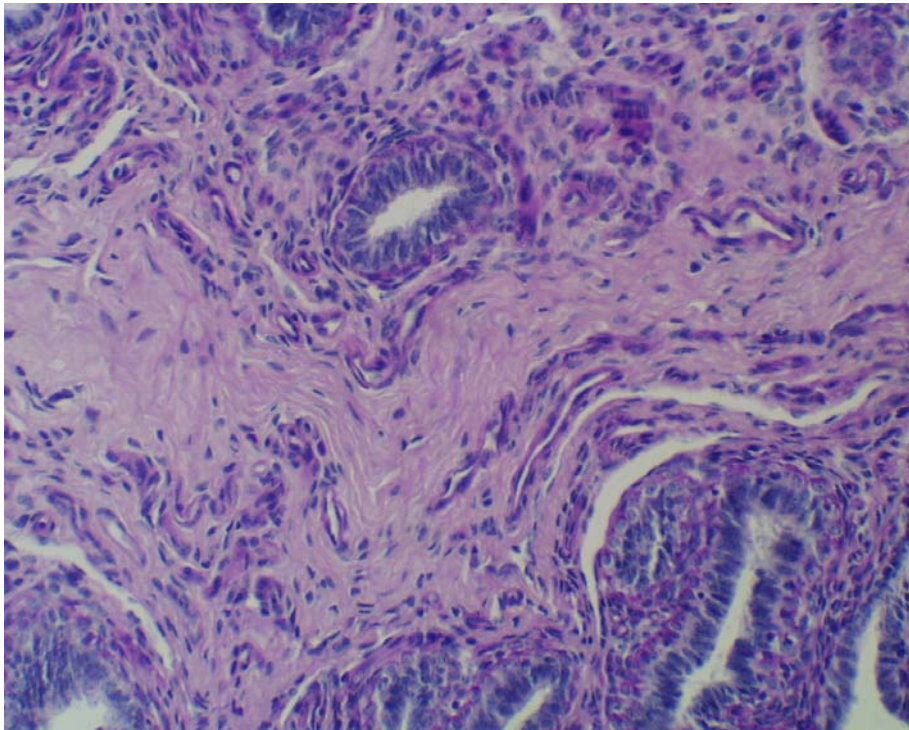


Figure 3.11: Col IV antibody specificity experiment.

Images are from serial sections at 400X magnification. Sections were incubated with Col IV antibody only (A), Col IV antibody and Col IV protein (B), Col IV antibody and Col I protein (C), and Col IV antibody and 1% goat serum (D).

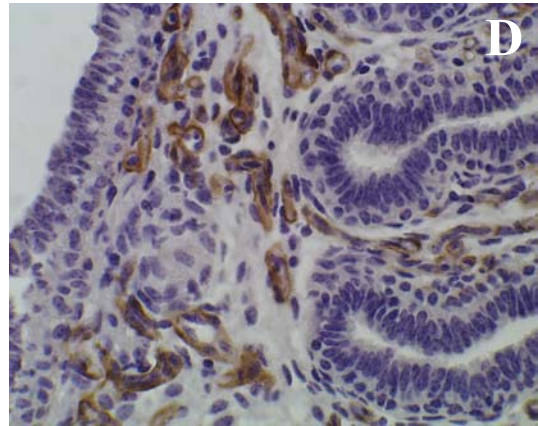
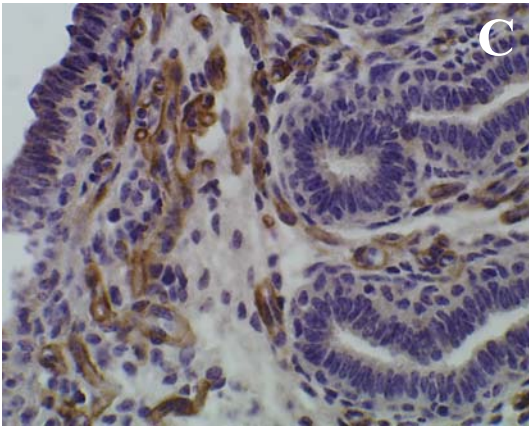
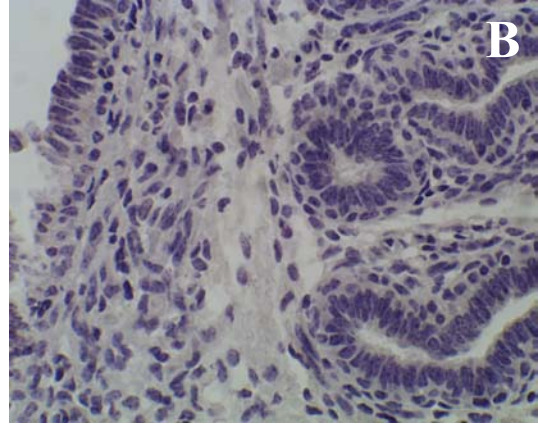
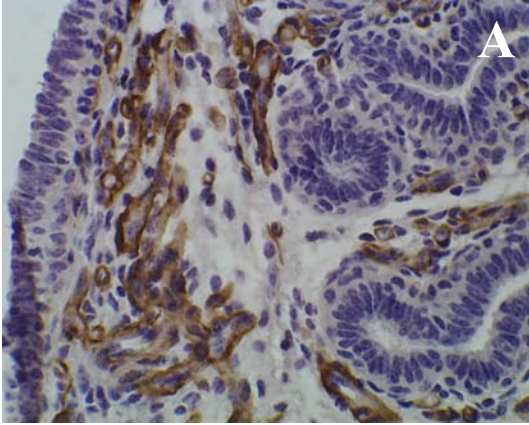


Figure 3.12: LM antibody specificity experiment.

Images A through C and D through E are each from a set of serial sections at 400X magnification. Sections were incubated with LM antibody only (A), LM antibody and LM protein (B), LM antibody and 1% goat serum (C), LM antibody and FN protein (D), and LM antibody only (E).

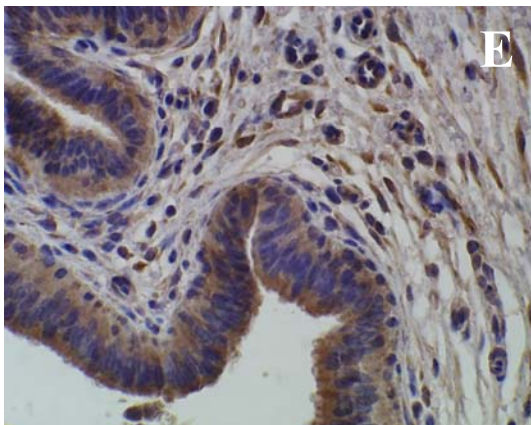
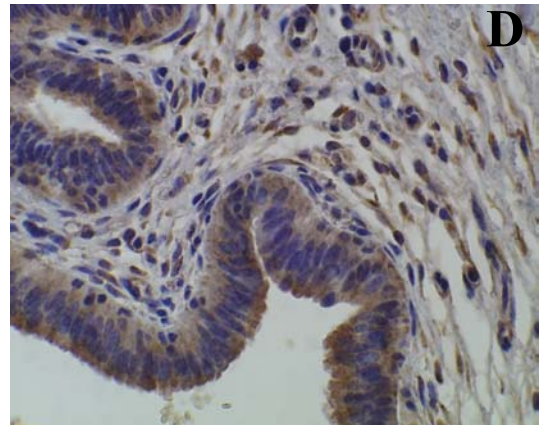
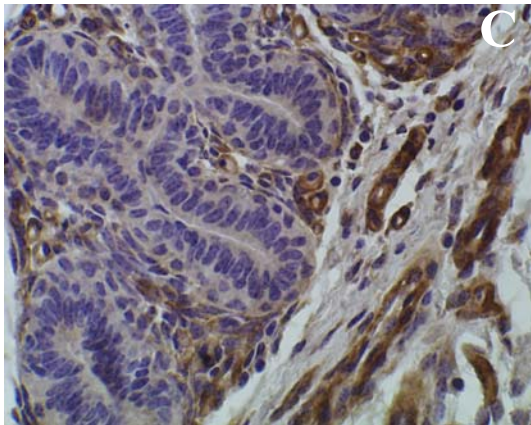
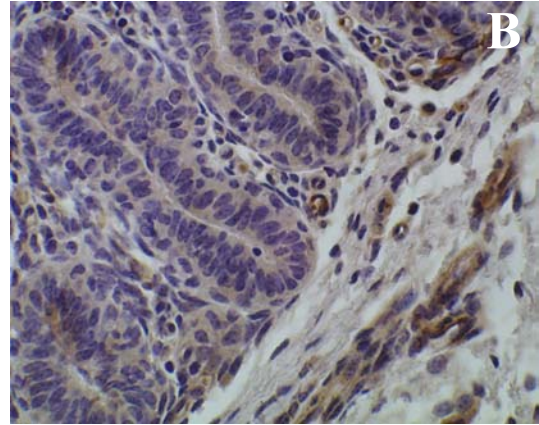
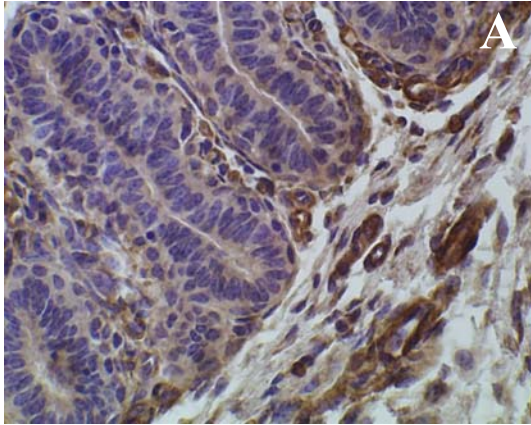
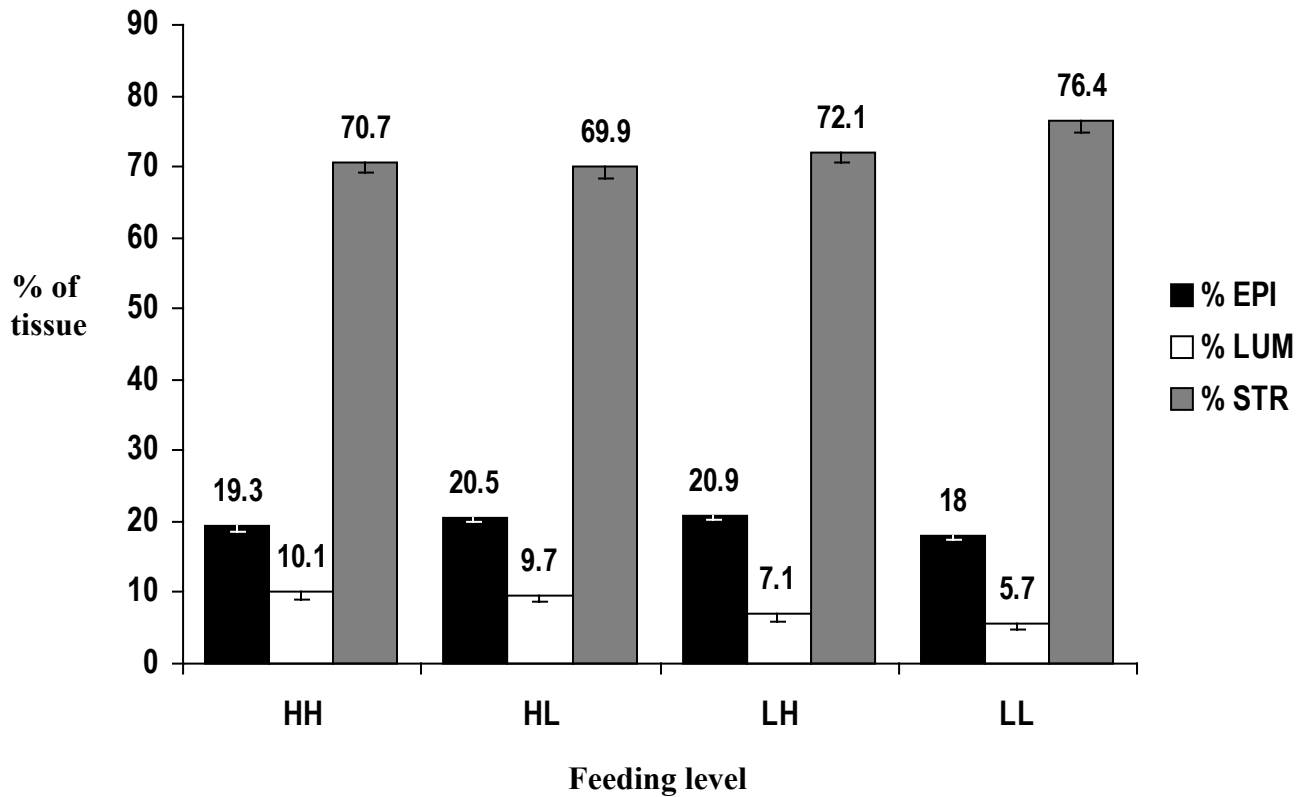


Figure 3.13: FN antibody specificity experiment.

FN (1), LM (2), Col I (3), and Col IV (4) proteins were electrophoresed on a 7.5% SDS-PAGE gel. Incubation with primary antibody for FN demonstrated specificity of the antibody to FN protein.



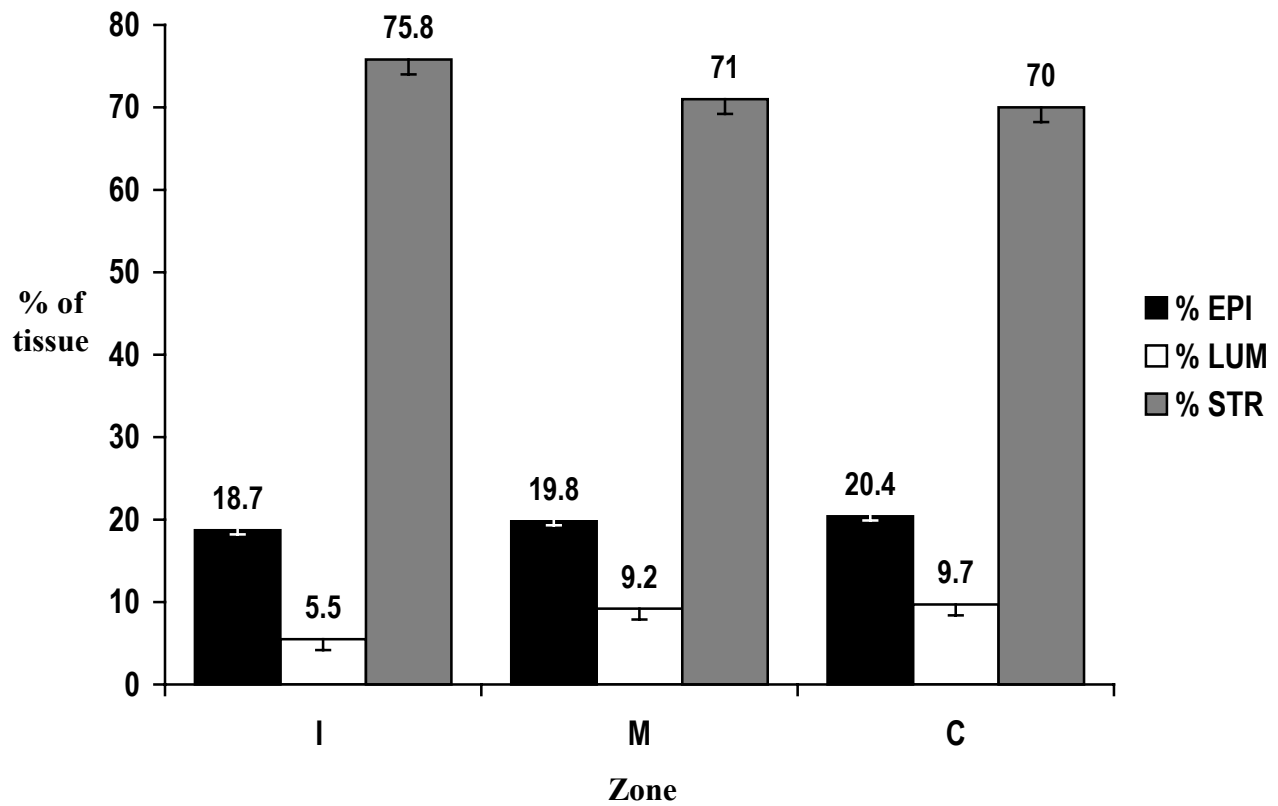
Figure 3.14: Percentages of mammary parenchymal tissue by feeding level.



	Contrast*, p value (SEM)		
	A	B	C
% Epithelium	+0.4, 0.64 (0.9)	+0.9, 0.35 (0.9)	+1.3, 0.29 (1.2)
% Lumen	+3.5, 0.01 (1.4)	+0.9, 0.52 (1.4)	+4.4, 0.02 (1.9)
% Stroma	-4.0, 0.02 (1.7)	-1.7, 0.31 (1.7)	-5.7, 0.01 (2.2)

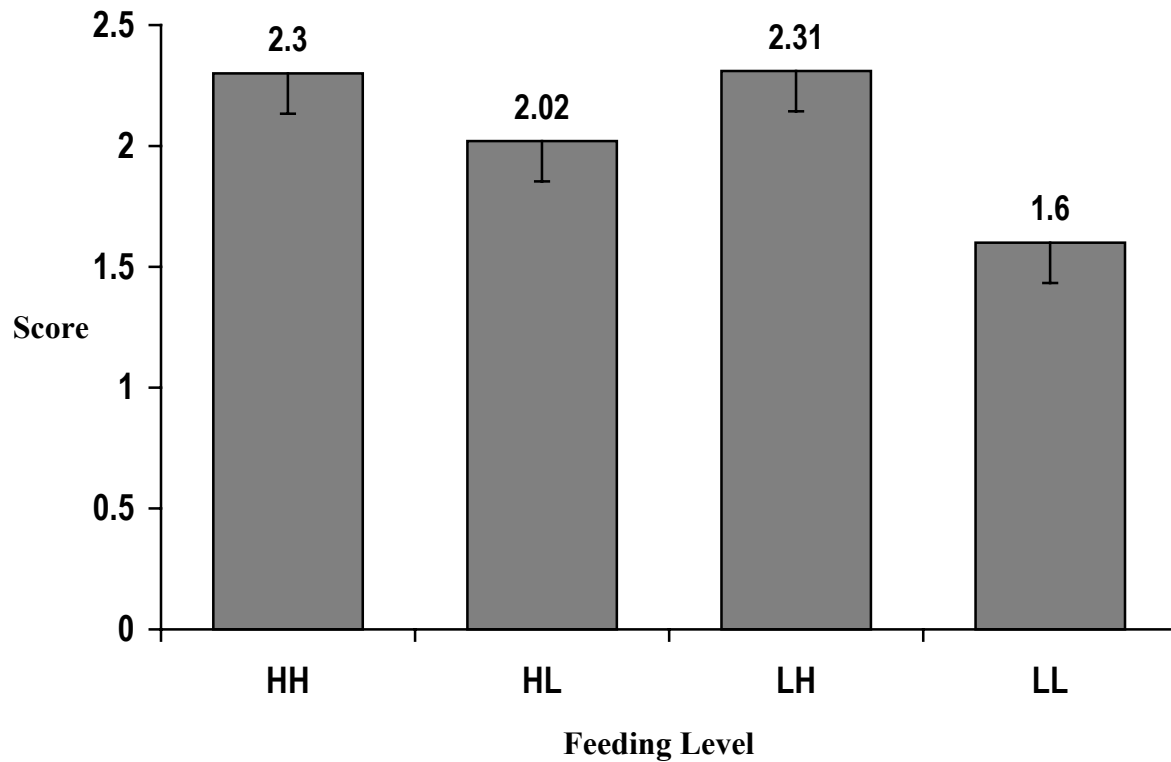
* Contrast A: HH and HL vs. LH and LL, B: HH and LH vs. HL and LL, C: HH vs. LL

Figure 3.15: Percentages of mammary parenchymal tissue by zone.



	Contrast, p value (SEM)	
	I vs. M and C	I vs. C
% Epithelium	-1.4, 0.17 (1.0)	-1.7, 0.15 (1.2)
% Lumen	-3.9, 0.01 (1.5)	-4.1, 0.02 (1.7)
% Stroma	+5.3, 0.004 (1.8)	+5.8, 0.007 (2.1)

Figure 3.16: Developmental score by feeding level.



Odds ratio estimate, p value		
HH vs. LL	HL vs. LL	LH vs. LL
8.34, 0.005	2.25, 0.27	6.80, 0.04

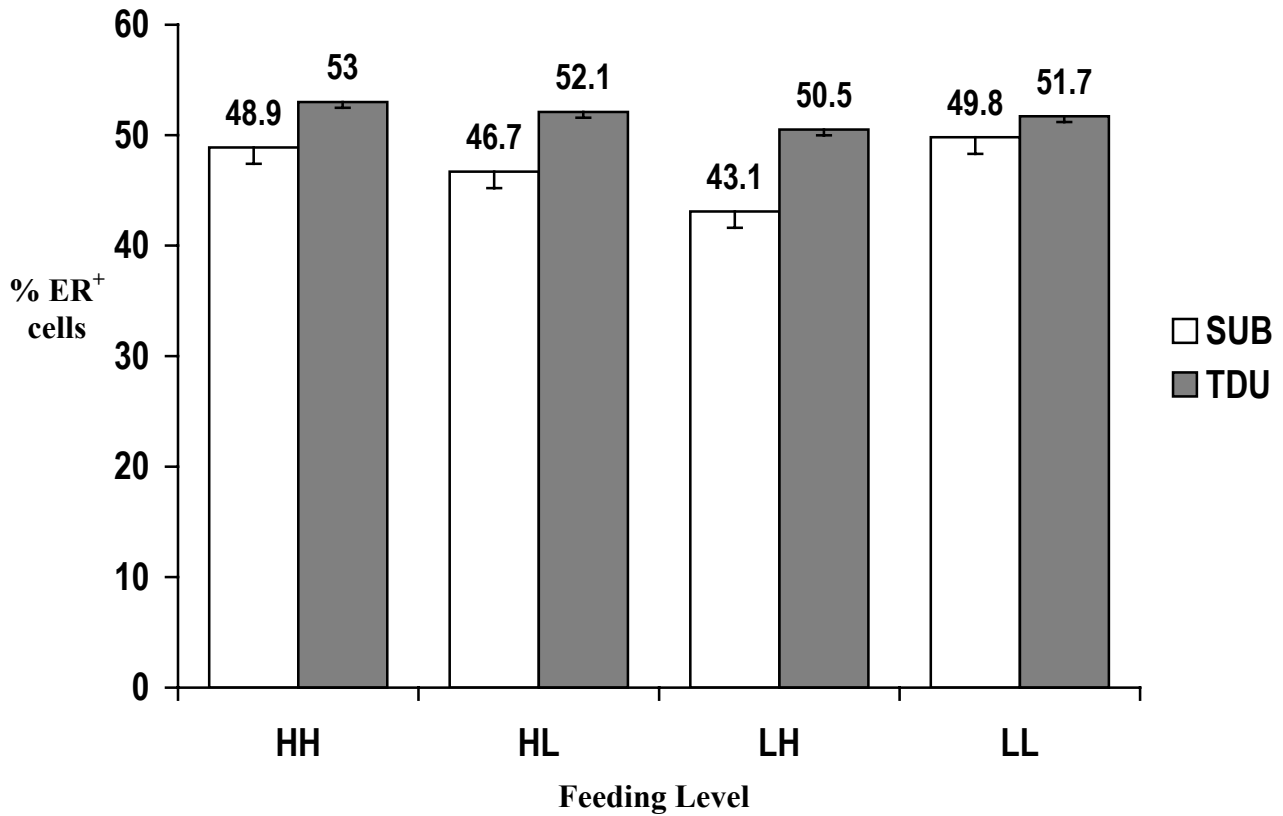
Contrast*, p value (SEM)		
A	B	C
+0.20, 0.15 (0.14)	+0.50, 0.0006 (0.14)	+0.71, 0.0003 (0.18)

* Contrast A: HH and HL vs. LH and LL, B: HH and LH vs. HL and LL, C: HH vs. LL

Table 3.1: Developmental categorization by zone.

	Mean (SEM)			Contrast, p value (SEM)	
	I	M	C	I vs. M and C	I vs. C
Developmental categorization	1.79 (0.12)	2.28 (0.12)	2.10 (0.12)	-0.40, 0.007 (0.15)	-0.31, 0.07 (0.17)

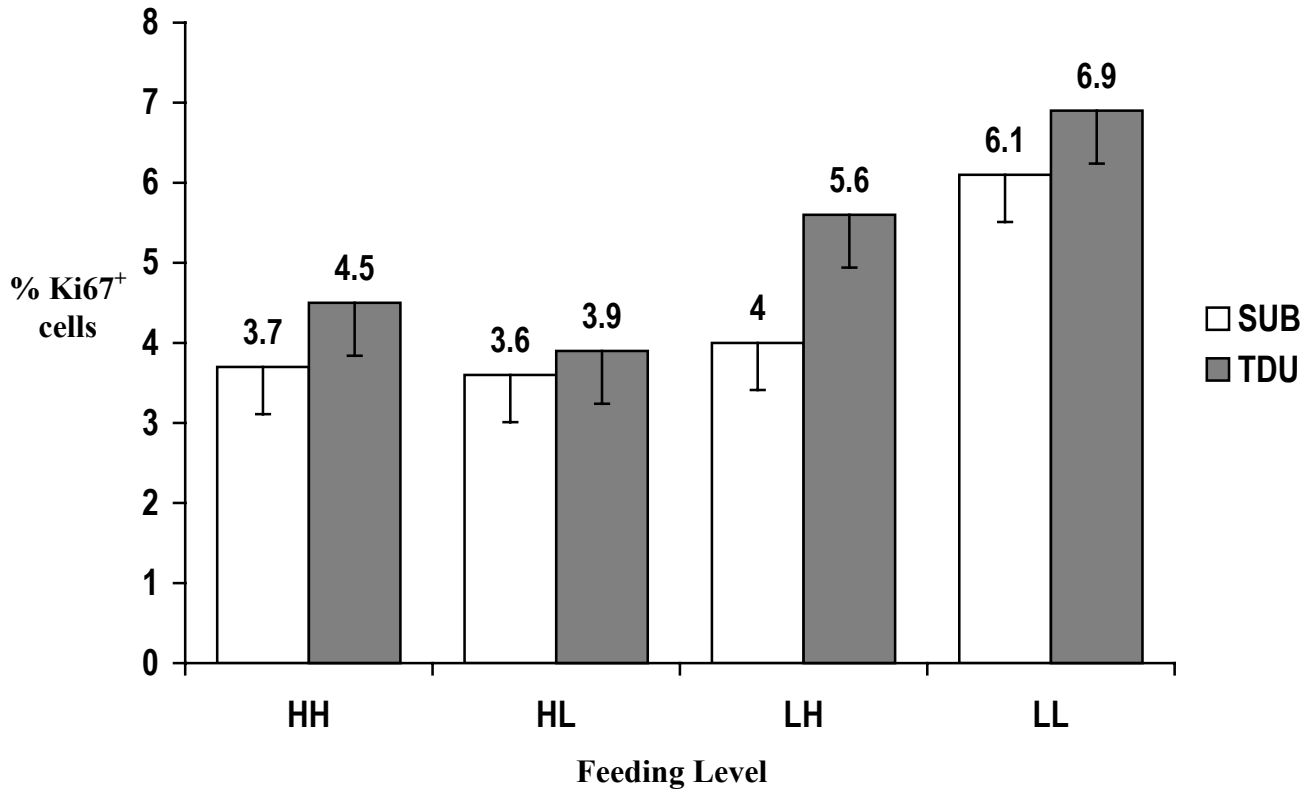
Figure 3.17: Percentage of ER-positive epithelial cells in SUB and TDU by feeding level.



	Contrast*, p value (SEM)		
	A	B	C
% ER ⁺ (SUB)	+1.3, 0.54 (2.2)	-2.2, 0.30 (2.1)	-0.9, 0.76 (2.9)
% ER ⁺ (TDU)	+1.5, 0.53 (2.4)	-0.1, 0.96 (2.4)	+1.4, 0.67 (3.2)

* Contrast A: HH and HL vs. LH and LL, B: HH and LH vs. HL and LL, C: HH vs. LL

Figure 3.18: Percentage of Ki67-positive epithelial cells in SUB and TDU by feeding level.



	Contrast*, p value (SEM)		
	A	B	C
% Ki67 ⁺ (SUB)	-1.4, 0.05 (0.7)	-0.9, 0.18 (0.7)	-2.3, 0.01 (0.9)
% Ki67 ⁺ (TDU)	-2.1, 0.01 (0.8)	-0.3, 0.68 (0.8)	-2.4, 0.03 (1.1)

* Contrast A: HH and HL vs. LH and LL, B: HH and LH vs. HL and LL, C: HH vs. LL

Table 3.2: Percent ER- and Ki67-positive epithelial cells by zone.

	Mean (SEM)			Contrast, p value (SEM)	
	I	M	C	I vs. M and C	I vs. C
% ER ⁺ (SUB)	45.6 (1.9)	48.4 (2.0)	47.3 (2.3)	-2.2, 0.36 (2.4)	-1.7, 0.55 (2.9)
% ER ⁺ (TDU)	49.4 (2.1)	52.7 (2.2)	53.3 (2.5)	-3.6, 0.17 (2.6)	-3.8, 0.22 (3.1)
% Ki67 ⁺ (SUB)	4.8 (0.6)	3.7 (0.6)	4.5 (0.7)	+0.7, 0.36 (0.8)	+0.3, 0.72 (0.9)
% Ki67 ⁺ (TDU)	6.0 (0.7)	4.1 (0.7)	5.5 (0.8)	+1.2, 0.19 (0.9)	+0.4, 0.68 (1.0)

Table 3.3: Percent ER- and Ki67-positive epithelial cells in SUB and TDU.

	Mean (SEM)		Contrast, p value SUB vs. TDU
	SUB	TDU	
% ER ⁺	46.6 (1.2)	52.4 (1.2)	-5.8, <0.0001
% Ki67 ⁺	4.5 (0.4)	5.1 (0.4)	-0.6, 0.19

Table 3.4: Prevalence and intensity of Col IV staining in BM by feeding level.

	Mean (SEM)				Contrast*, p value (SEM)		
	HH	HL	LH	LL	A	B	C
% SUB with a Col IV stained BM	47.9 (5.8)	44.4 (7.1)	41.1 (6.7)	41.7 (6.0)	+4.8, 0.46 (6.4)	+1.5, 0.82 (6.4)	+6.3, 0.46 (8.5)
Intensity of Col IV stained BM in SUB	1.56 (0.10)	1.53 (0.15)	1.34 (0.14)	1.80 (0.13)	-0.03, 0.84 (0.13)	-0.22, 0.09 (0.13)	-0.25, 0.15 (0.17)
% TDU with a Col IV stained BM	9.8 (3.3)	11.3 (4.0)	7.5 (3.8)	8.3 (3.4)	+2.7, 0.47 (3.6)	-1.1, 0.76 (3.6)	+1.5, 0.75 (4.8)
Intensity of Col IV stained BM in TDU	1.49 (0.41)	1.14 (0.49)	1.36 (0.43)	1.46 (0.40)	-0.10, 0.82 (0.41)	+0.13, 0.78 (0.44)	+0.03, 0.96 (0.52)

* Contrast A: HH and HL vs. LH and LL, B: HH and LH vs. HL and LL, C: HH vs. LL

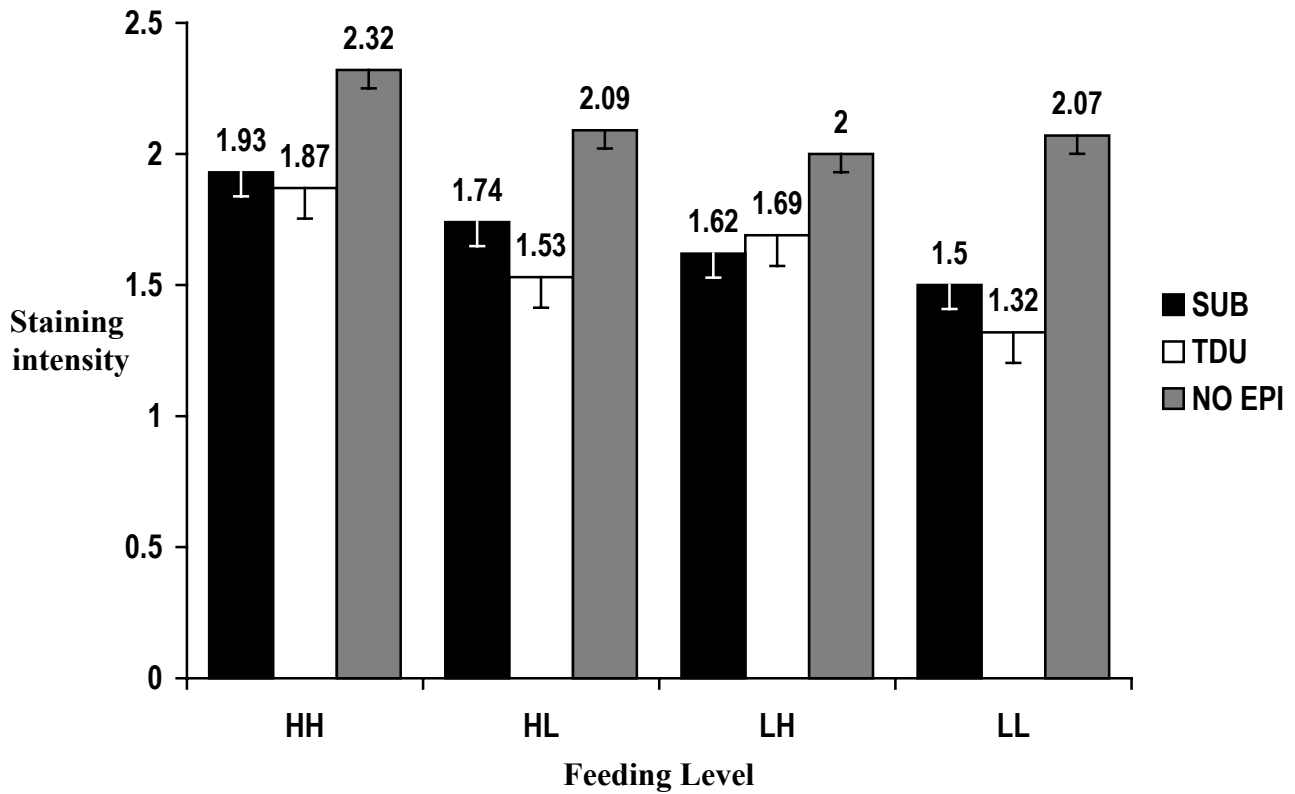
Table 3.5: Prevalence and intensity of Col IV staining in BM by zone.

	Mean (SEM)			Contrast, p value (SEM)	
	I	M	C	I vs. M and C	I vs. C
% SUB with a Col IV stained BM	40.2 (5.5)	44.0 (5.7)	47.1 (5.6)	-5.3, 0.43 (6.7)	-6.9, 0.38 (7.8)
Intensity of Col IV stained BM in SUB	1.66 (0.12)	1.34 (0.11)	1.66 (0.11)	+0.16, 0.28 (0.15)	0.00, 0.99 (0.16)
% TDU with a Col IV stained BM	9.9 (3.1)	11.8 (3.2)	6.0 (3.2)	+1.1, 0.78 (3.8)	+3.9, 0.38 (4.4)
Intensity of Col IV stained BM in TDU	1.31 (0.42)	1.25 (0.37)	1.53 (0.32)	-0.08, 0.90 (0.56)	-0.22, 0.69 (0.53)

Table 3.6: Prevalence and intensity of Col IV staining in the BM of SUB and TDU.

	Mean (SEM)		Contrast, p value SUB vs. TDU
	SUB	TDU	
% with a Col IV stained BM	43.3 (2.5)	9.7 (2.5)	+33.6, <0.0001
Intensity of Col IV stained BM	1.57 (0.06)	1.35 (0.10)	+0.22, 0.06

Figure 3.19: FN staining intensity by feeding level.



	Contrast*, p value (SEM)		
	A	B	C
Intensity of FN staining at SUB	+0.27, 0.05 (0.15)	+0.16, 0.30 (0.15)	+0.43, 0.03 (0.20)
Intensity of FN staining at TDU	+0.19, 0.13 (0.13)	+0.35, 0.008 (0.13)	+0.55, 0.001 (0.17)
Intensity of FN staining at NO EPI	+0.17, 0.26 (0.15)	+0.08, 0.60 (0.15)	+0.24, 0.20 (0.19)

* Contrast A: HH and HL vs. LH and LL, B: HH and LH vs. HL and LL, C: HH vs. LL

Table 3.7: Percentage of samples with a FN stained BM by feeding level.

	Mean			
	HH	HL	LH	LL
% with a FN stained BM in a SUB	18.5	5.3	17.4	11.5
% with a FN stained BM in a TDU	0	0	0	0

Table 3.8: FN staining intensity and percentage of samples with a FN stained BM by zone.

	I	Mean (SEM)		Contrast, p value (SEM)	
		M	C	I vs. M and C	I vs. C
Intensity of FN staining at SUB	1.64 (0.14)	1.83 (0.13)	1.62 (0.13)	-0.08, 0.61 (0.16)	+0.03, 0.88 (0.18)
Intensity of FN staining at TDU	1.41 (0.12)	1.69 (0.12)	1.71 (0.12)	-0.29, 0.04 (0.14)	-0.30, 0.05 (0.16)
Intensity of FN staining at NO EPI	1.98 (0.14)	2.12 (0.13)	2.25 (0.13)	-0.21, 0.19 (0.16)	-0.27, 0.13 (0.18)

	Zone mean		
	I	M	C
% with a FN stained BM in a SUB	9.4	10.3	20.6
% with a FN stained BM in a TDU	0	0	0

Table 3.9: FN staining intensity within different areas of mammary parenchyma.

	Mean (SEM)			Contrast, p value (SEM)	
	At NO EPI	At SUB	At TDU	A	B
Intensity of FN staining	2.22 (0.07)	1.80 (0.07)	1.68 (0.07)	-0.48, <0.0001 (0.08)	+0.12, 0.20 (0.10)

* Contrast A: NO EPI vs. SUB and TDU, B: SUB vs. TDU

Table 3.10: LM staining intensity and percentage of samples with a LM stained BM by feeding level.

	Mean (SEM)				Contrast*, p value (SEM)		
	HH	HL	LH	LL	A	B	C
Intensity of LM staining at SUB	2.42 (0.13)	2.14 (0.15)	2.14 (0.14)	2.48 (0.13)	-0.02, 0.87 (0.14)	-0.03, 0.82 (0.14)	-0.06, 0.77 (0.19)
Intensity of LM staining at TDU	2.11 (0.10)	1.95 (0.12)	1.93 (0.12)	2.17 (0.11)	-0.01, 0.90 (0.11)	-0.04, 0.73 (0.11)	-0.05, 0.72 (0.15)
Intensity of LM staining at NO EPI	2.08 (0.10)	1.75 (0.12)	1.71 (0.11)	2.03 (0.11)	+0.04, 0.70 (0.11)	0.00, 0.99 (0.11)	+0.04, 0.77 (0.15)

* Contrast A: HH and HL vs. LH and LL, B: HH and LH vs. HL and LL, C: HH vs. LL

	Mean				Odds ratio estimate, p value		
	HH	HL	LH	LL	HH vs. LL	HL vs. LL	LH vs. LL
% with a LM stained BM in a SUB	85.7	55	39.1	76	1.71, 0.05	0.40, 0.50	0.16, 0.01
% with a LM stained BM in a TDU	16.7	25	17.4	25.9	N/A	N/A	N/A

Table 3.11: LM staining intensity and percentage of samples with a LM stained BM by zone.

	Mean (SEM)			Contrast, p value (SEM)	
	I	M	C	I vs. M and C	I vs. C
Intensity of LM staining at SUB	2.35 (0.12)	2.20 (0.12)	2.33 (0.12)	+0.09, 0.54 (0.15)	+0.02, 0.90 (0.17)
Intensity of LM staining at TDU	2.09 (0.10)	1.91 (0.10)	2.12 (0.10)	+0.08, 0.49 (0.12)	-0.02, 0.86 (0.14)
Intensity of LM staining at NO EPI	1.88 (0.09)	1.90 (0.10)	1.91 (0.09)	-0.03, 0.83 (0.12)	-0.03, 0.84 (0.13)

	Mean		
	I	M	C
% with a LM stained BM in a SUB	59.4	64.5	72.7
% with a LM stained BM in a TDU	17.6	28.1	17.7

Table 3.12: LM staining intensity within different areas of mammary parenchyma.

	Mean (SEM)			Contrast*, p value (SEM)	
	At NO EPI	At SUB	At TDU	A	B
Intensity of LM staining	1.90 (0.06)	2.29 (0.06)	2.03 (0.06)	+0.26, 0.0002 (0.07)	+0.26, 0.0009 (0.08)

* Contrast A: NO EPI vs. SUB and TDU, B: SUB vs. TDU

DISCUSSION

Mammary Growth and Development

High rates of gain did not alter percent epithelium in mammary parenchymal tissue, which might be a reflection of the method of data collection. There is little variation in epithelial cell layer width from larger to smaller ductular structures. Thus, a wider duct is likely to possess the same amount of epithelial tissue as that of a smaller duct. Based on this assumption, one might expect differences in gland development to manifest in terms of luminal area, and it appears that this was the case (Figure 3.14). Heifers receiving the H diet during the first period of growth and throughout the entire experiment had greater percentages of parenchymal lumen compared to heifers on the L diet from 2 to 8 weeks and heifers that received the L diet for 14 weeks, respectively. These results indicate that rapidly reared heifers had a better developed mammary gland at 14 wk of age. However, larger lumens do not necessarily dictate better mammary development. Recall that prepubertal mammary development is a period of growth characterized by active growth of epithelial cells, which are the precursors of mature secretory cells. Due to the strong correlation between number of secretory cells and milk production (Tucker 1981), epithelial presence is likely a more relevant factor in determining quality of growth. Lack of differences in percent epithelium suggests that none of the dietary treatments led to better mammary development. However, rapid rearing from 2 to 14 wk of age may have impaired development via formation of large ducts with proportionately fewer side branching ductal structures. Although, as discussed previously, larger ducts were not expected to have increased epithelial cell layer width, they should have been associated

with sufficient amount of epithelial support via side-branching structures. Increased luminal space at 14 wk would reduce gland area available for subsequent development of epithelial-rich structures. It is logical to assume that this increased luminal area would be accompanied by a decrease in stroma, which was the case. Furthermore, our characterization of overall tissue development, which was based on developmental scoring (Figure 3.16), supports this idea. Heifers on the H diet early and during the entire treatment demonstrated a reduction in percent parenchymal stroma compared to early L heifers and heifers receiving the L diet for the entire 12 wk (Figure 3.14).

These data and interpretations are supported by previously performed analyses (Brown et al. 2002) on tissue from the same heifers, which showed that parenchymal DNA, parenchymal DNA per BW, and concentration of DNA were all increased in heifers that received the H diet during 2 to 8 wk of age. Because cell density is essentially constant within epithelial structures, this increase in DNA concentration, and thus cell number, is most likely not a result of an increase in epithelial cell density. However, luminal areas are void of cells and stromal areas were reduced in H heifers. Variation in stromal cell density is possible, which suggests that the increased concentration of DNA in H heifers was at least in part due to differences in stromal cell deposition, such as abundance of fibroblasts. The discrepancies between these results and those of others (Sejrsen et al. 1982 and Capuco et al. 1995), which have demonstrated decreases in parenchymal size, DNA, and RNA in rapidly reared heifers, could be due to drastically different treatment ages. These studies began feeding for a high ADG at a much later age, and heifers were not slaughtered until after the onset puberty. Total gland weight per BW, parenchymal

weight per BW, and extra-parenchymal weight per BW were also increased in heifers on the H diet during the earliest phase of growth (Brown et al. 2002), illustrating the larger overall growth of the mammary gland in rapidly reared heifers. However, as alluded to previously, quantity of growth is not the most crucial factor in determining the quality of mammary gland development. A larger 14 wk gland comprised of a high number of disproportionately large ducts devoid of side branching is at risk of reduced subsequent growth potential.

There were no zone differences with respect to percent epithelium in mammary parenchymal tissue. However, percent lumen was increased in zones M and C compared to zone I, and in zone C compared to zone I (Figure 3.15). Larger ducts closer in proximity to the teat are expected when taking into consideration the normal ventral to dorsal route of mammary parenchyma penetration. These results directly support the assumption regarding the constancy in width of epithelial cell layers in larger and smaller ducts. As seen previously, the increase in lumen from zone I to C was accompanied by a decrease in stromal area.

Since the procedure for obtaining tissue composition percentages began with intersection points only contacting parenchymal tissue, this means of data collection might not have reflected obvious differences between tissue sections with varying amounts of overall ductular occupation. Consequently, a semi-quantitative analysis termed “developmental categorization” was designed. Essentially, these results are a reflection of *how much* growth was present in entire tissue sections, whereas tissue composition analyses are

more of a reflection on the *quality* of tissue composition within the parenchymal zone only. Heifers fed the H diet from 8 to 14 wk of age and throughout the entire treatment had increased developmental scores (Figure 3.16). Thus, it is no surprise that these heifers demonstrated higher total gland weights (Brown et al. 2002). However, there were no differences in parenchymal weights between H and L heifers during the 8 to 14 wk period. These results, along with a drastic increase in extra-parenchymal tissue weight for H heifers during the 8 to 14 wk period, suggest that the increase in parenchymal size of rapidly reared heifers was associated with changes during the 2 to 8 wk period of growth. It appears that the majority of the increase in non-parenchymal tissue weight was caused by the H diet during wk 8 to 14 of age. These results are supported by observations made in our lab (Berry 2002) of a substantial, and rather rapid, increase in mammary parenchyma size beginning sometime just after 1 mo of age. In addition, animals ovariectomized between 4 and 6 wk of age were more dramatically affected by ovariectomy than animals that were ovariectomized between 6 and 12 wk of age.

Zones M and C had a greater developmental score than zone I (Table 3.1). As discussed previously, these results can be attributed to the origin and direction of mammary ductal growth. These zone differences provide validity to our developmental categorization scores as a means of measuring the extent of parenchymal occupation of mammary tissue.

Epithelial cell ER expression in neither SUB nor TDU differed in response to dietary regimen (Figure 3.17). Although it has been apparent for some time that estrogen plays a crucial role in mammary development (Wallace 1958), these results suggest mammary growth differences observed between rates of rearing are not due to direct interactions between estrogen and its receptor. This does not imply that estrogen had no effect on mammary developmental variation observed in this study. For example, estrogen is required for normal function of IGF-I, which is a potent mitogen for prepubertal mammary growth (Ruan et al. 1995). These results also do not imply that ER was not responsible for mammary growth differences between feeding treatments. It has been postulated that various growth factors, such as IGF-I and EGF, might directly stimulate the ER in the absence of its natural ligand (Woodward et al. 2000). There were no differences in ER expression with respect to zone of the mammary gland (Table 3.2).

Heifers fed for a low rate of gain between 2 to 8 wk of age, and those heifers receiving the L diet from 2 to 14 wk of age, demonstrated increased epithelial cell proliferation in both SUB and TDU (Figure 3.18). These data are only a reflection of the situation at time of slaughter, and thus cannot be interpreted as accurate during any other time of treatment. However, due to the positive relationship between number of secretory cells and milk production, and considering that epithelial cells are the precursors for alveolar cells that will eventually be involved directly in milk production, the percentage of Ki67-labelled epithelial cells was used to index the quality of mammary growth at 14 wk of age. It is interesting that heifers with the highest proportions of proliferating epithelial cells (LH and LL) also possessed the least amount of luminal space and lowest

developmental scores (Figures 3.14 and 3.16). These results support conclusions drawn from tissue composition data, and indicate that slower rates of rearing between 2 and 14 wk of age resulted in *better*-developed glands at slaughter. There were no differences in epithelial cell proliferation between zones of the mammary gland (Table 3.2), which is surprising. The expectation was that there would be a greater number of proliferating cells at the leading edge of parenchymal penetration into the fat pad (zone I). This suggests that epithelial growth was similar between more mature ductular structures and newer, or more immature, structures at 14 wk of age. Thus, side-branching appears to be as crucial as TDU expansion in terms of ductular growth early in heifer mammary gland development. Direct support for this comes from the finding that SUB, which would be more prevalent in zones closer to the teat, had a proliferative capacity that was similar to TDU.

When ER and Ki67 expression were categorized by structure, TDU were found to have a significantly greater percentage of ER-positive epithelial cells (Table 3.3). Recall that in the pubertal mouse model, disruption in certain proteins led to structure-specific inhibition of ductular growth. For example, blockage of $\beta 1$ integrin, an epithelial cell receptor for the ECM, reduced TEB number, while the remaining gland architecture appeared normal. Our results regarding ER expression in TDU point to a possible structure-specific role for estrogen, or other ER binding proteins, in ductular morphogenesis of the prepubertal heifer mammary gland. Once again, Ki67 results (Table 3.3) were surprising. It was expected that TDU would have greater cell proliferation numbers due to fact that these structures comprise the majority of sites

where active ductular expansion is occurring. Again, this may be a reflection of the relatively young age of these heifers. Capuco et al. (2002) showed that percent BrdU-labelled epithelial cells were progressively increased from the cisternal to the outer regions of the parenchyma in heifers. At young ages, it may be that TDU from throughout the parenchyma are uniformly active.

Extracellular Matrix

It is apparent that ECM proteins, specifically Col IV, FN, and LM, play a role in regulating growth and development of the mammary gland. Mammary epithelial cells grown on a reconstituted BM, of which Col IV and LM are major components, demonstrate increased production of β -casein and resemblance of mature secretory alveoli (Liang Li et al. 1987). These results indicate the direct influence of BM constituents on epithelial cell growth, however, there is also evidence that ECM proteins might mediate the action of known growth factors within the mammary gland. Col IV, LM, and FN, which is not as prominent as the latter two in terms of BM, all promote a highly synergistic proliferative response to both IGF-I and EGF (Woodward et al. 2000). Our heifers showed increased FN deposition adjacent to SUB and TDU in response to a high rate of gain (Figure 3.19). Recall that the H diet was also associated with an increase in luminal area and a decrease in epithelial cell proliferation at 14 wk (Figures 3.14 and 3.18), both of which were interpreted as being associated with poorer mammary development. Alterations in FN deposition appeared to have contributed to the growth differences observed between H and L fed heifers. These results support other work in our lab (Berry 2002) demonstrating greater parenchymal FN in OVX heifers with impaired mammary development. The increase in FN seen with high feeding in our study corresponds with the increase in parenchymal protein from heifers fed the high diet from 2 to 8 wk (Brown et al. 2002), thus furthering support for the speculation made earlier regarding the increase in DNA concentration being stromal rather than epithelial in origin for H heifers in period 1. The frequent lack of FN deposition immediately

adjacent to the leading edge of TDU expansion (Figure 3.5) indicates that this protein must be broken down in order for epithelial penetration to occur. Indeed, FN deposition was lowest at TDU compared to areas adjacent to SUB and areas lacking epithelial occupation (Table 3.9). There is most likely a certain level of ECM protein deposition that is required for support of gland architecture and related biochemical pathways. Levels of deposition beyond this likely interfere with ductal growth via physical inhibition and/or disruption in normal protein signaling cascades.

Unlike FN, which is primarily found in areas of the ECM not associated with a BM, Col IV is a major constituent of the BM and lacks abundance in other stromal areas relative to FN and LM (Alberts et al. 1994). Consequently, Col IV analyses pertained only to deposition within the BM. Col IV and LM staining within BM of SUB and TDU were considerably more prevalent than FN in these structures (Tables 3.6, 3.7, and 3.10), indicating them as critical BM factors for epithelial growth. The frequent co-localization of Col IV and LM (Figures 3.1 and 3.4) suggest a possible synergism between these two ECM proteins, and may implicate similarities in their expression or interaction with other stromal factors. However, neither Col IV nor LM deposition varied in response to rate of gain, suggesting that they were not involved in mediating dietary growth and developmental differences. The expectation was that, especially in LM, there would be a decrease in presence or intensity of protein deposition in faster-growing glands. Breast epithelial cells have been shown to become migratory on laminin-5 following its cleavage with MMP2 (Giannelli et al. 1997). Also, blockage of LM degradation has been

associated with a reduction in both TEB number and extent of mammary ductal branching (Klinowska et al. 1999).

FN deposition at TDU was decreased in zone I (Table 3.8), thus indicating the importance of proper ECM protein regulation in areas where parenchymal tissue is actively penetrating the fat pad. This strengthens support regarding the connection between decreased epithelial proliferation and increased FN deposition in high fed heifers. Blocking ECM protein breakdown via inhibition of MMP results in decreases of both ductal expansion and luminal epithelial cell proliferation in the mouse (Fata et al. 1999). Col IV and LM deposition did not differ by zone (Tables 3.5 and 3.11), which is surprising, but demonstrates again the apparent co-localization of the two proteins.

Considering the association between increased FN deposition and reduced epithelial cell growth, it is logical to assume that stromal areas adjacent to SUB would have more ECM protein deposition than those areas next to TDU. Indeed, deposition of all ECM proteins measured was greater adjacent to more mature ductular structures (Tables 3.6, 3.9, and 3.12). Although deposition of Col IV and LM did not appear to mediate growth differences, decreased staining of these two proteins at TDU, when compared to SUB, does indicate possible structure-specific roles in parenchymal expansion.

From zone C to zone I, there was a decrease in the prevalence of FN-stained BM in SUB (Table 3.8). Although there were no FN-stained BM in TDU, these results reflect the connection between intensity of FN deposition and its presence in BM. These results also suggest that more side-branching of SUB was occurring at the leading edge of

parenchymal expansion, compared to zones M and C, where substantially more gland architecture had most likely been formed at 14 wk of age. If this is an accurate interpretation, then it supports the before-mentioned assumptions regarding high rates of gain and impaired mammary development. Large ducts formed, predominately in zones M and C, due to excessive rates of growth have an inherently lower chance of spontaneous side-branching due to this greater deposition of FN. This illustrates the apparent irreversible damage imposed upon the mammary glands of rapidly reared heifers seen at 14 wk of age.

Staining of tissue sections via PAS reaction did not identify a specific ECM molecule, but did allow visualization of the carbohydrate-containing components of the ECM. Although there was no quantification of the prevalence or intensity of PAS staining, certain observations were made regarding carbohydrates in the ECM and how they might relate to the other ECM proteins tested. Distinct PAS-positive BM were observed on virtually every SUB and very frequently along TDU (Figure 3.8), relative to ECM protein-staining in these structures, indicating the greater overall abundance of carbohydrates in BM. More frequent staining within SUB coincides with quantitative results demonstrated by Col IV, FN, and LM. BM along TDU often appeared “fuzzy” (Figure 3.8B) suggesting a less organized structure compared to those adjacent to SUB. This loss of organization was most likely a result of carbohydrate-components “drifting” away from epithelial structures as BM were degraded. The carbohydrate component of epithelial BM appears to be crucial in terms of ductular expansion, however, regulation by way of degradation also appears essential. These assumptions appear valid when

considering that proteoglycans, which are carbohydrate-rich components of the ECM, have been shown to bind stromal factors such as growth factors, proteolytic enzymes, and protease inhibitors (Alberts et al. 1994). Typically, PAS staining completely encompassed the most basal cell layer of epithelial structures (Figure 3.8A). Staining at the epithelial/stromal interface was expected, and indicates the possible contribution of both epithelial and stromal cells to BM composition. However, equal PAS staining around the basal cell layer suggests that the epithelial population is primarily responsible for producing the carbohydrate components of BM. A lack of carbohydrate staining at epithelial cells closer in proximity to the lumen implies basal-specificity, implicating stromal factors as cues for carbohydrate production.

In more-developed tissue, there was a greater contingency of carbohydrate-containing ECM components in lobules, compared to those areas in between lobules (Figures 3.9 and 3.10). Typically, interlobular staining was characterized by a more “sheet-like” appearance. This is a good indication of the inconsistency in ECM deposition between areas adjacent, and not adjacent, to ductular structures.

SUMMARY AND CONCLUSIONS

Rapid rearing of heifers between 2 and 14 wk of age resulted in increased mammary growth, characterized by greater luminal occupation of gland parenchyma and higher developmental categorization scores. This increase in growth *quantity* was not interpreted as better growth. Lack of epithelial differences between diets suggests that mammary glands of rapidly reared heifers were characterized by increased large ducts lacking sufficient epithelial support with respect to side-branching. Thus, higher rates of gain adversely affected mammary growth in terms of developmental *quality*. In addition, it appears as though rapidly reared heifers were set up for subsequent growth reductions at 14 wk of age, demonstrating reduced epithelial cell proliferation upon slaughter. The increased parenchymal lumen and decreased proliferative activity was accompanied by greater FN deposition adjacent to epithelial structures. Deposition of ECM proteins Col IV and LM were not affected by feeding level, and most likely did not contribute to any differences seen with parameters of growth and development.

Rapid rearing of heifers very early in life increases the quantity of mammary growth, but adversely affects the quality of mammary development via poorer gland architecture and reduced proliferative capacity at 14 wk. Differences in growth and development appear to be due in part to changes in FN deposition.

FUTURE RESEARCH

With respect to animals and experimental design, there are a number of suggestions that could be made to improve this study. If one was interested in determining the effects, if any, that rate of gain during 2 to 14 wk has on subsequent milk production, the obvious suggestion is to examine first lactation results. Heifers could be fed for a high or low rate of gain until 14 wk, and then grouped to achieve similar ADG through lactation. It would be interesting to see if adverse affects present at 14 wk in this study could be overcome by the substantial growth associated with the allometric growth phase beginning later in prepuberty, which is typically the time period of interest in terms of most studies examining effects of nutrition on mammary development and milk production.

The most comprehensive study would involve beginning one high fed and one low fed group of heifers at 2 wk of age, and slaughtering heifers in one or two week increments to assess the effects of nutrition in a chronological manner. This type of design would not only help to elucidate the causes, and extent, of growth differences caused by level of gain, but control heifers could provide a very useful background level of normal mammary development. In terms of determining critical periods associated with normal prepubertal mammary growth, drugs could be used to block proliferation of mammary epithelial cells throughout development, thus allowing the comparison of impairments to development of control glands. This could provide substantial insight into which specific periods of time are most crucial for prepubertal development. We are currently undertaking a study like this in our lab. Beginning at approximately 1 month of age,

heifer calves will be treated with a drug that will abolish cell division in the parenchymal area of the gland.

In terms of tissue composition, improvements could be made regarding method of data collection. To get a more accurate breakdown of parenchymal occupation, computer programs could be used to calculate actual areas for epithelium, lumen, and stroma by way of calibrating a distance measurement and literally outlining each area that contains the tissue of interest. Also, instead of applying tissue composition only to those areas which contained parenchyma, tissue composition could be performed on entire samples.

The semi-quantitative analysis used for determining ECM protein deposition was considered the most accurate method available for my specific samples. However, it is possible to utilize computer programs to fully quantify staining based on pixel intensities of digital images. Admittedly, I attempted this kind of analysis, and found extreme amounts of variation. However, in retrospect, I discovered some ways by which this variation might be reduced, thus making computer analyses plausible. One could make an attempt to subject each sample to the *identical* staining environment, which is especially difficult with large sample numbers. Also, omitting a counter-staining step would virtually eliminate any background intensity that might be associated with colors that are of no interest, such as the bluish-purple caused by hematoxylin and eosin. In these studies, stained sections would have only possessed the brown color produced by the peroxidase/DAB reaction, which was specific to the protein of interest.

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