Extracellular Proteoglycan Decorin in Bovine Mammary Physiology

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Abstract

The majority of bovine mammary gland research focuses on the main cell types - mammary epithelial cells and fibroblasts. However, the extracellular matrix (**ECM**) within the mammary gland is also of importance for its ability to regulate cell shape, proliferation, polarity, differentiation, gene transcription, protein synthesis, and secretion. Decorin is an ECM proteoglycan known to impact mammary cell proliferation in humans and rodents. Prior to this work, very little was known about decorin in bovine mammary biology. A series of bovine mammary cell culture experiments was conducted. The first experiment demonstrated existence of decorin pathway molecules in immortalized bovine mammary cells, but stopped short of demonstrating mature decorin proteoglycan deposition into the extracellular space. During the investigation it was noted that when cultured under basal conditions, intracellular decorin core protein (**DCP**) localization patterns appeared to be coordinated with specific phases of the cell cycle. Therefore, the objective of the second set of experiments was to characterize DCP localization patterns in bovine mammary epithelial cells (**BME**) at known phases of the cell cycle. The work was carried out in two sequential experiments. The hypothesis of the first experiment was that DCP accumulates in BME during S-phase of the cell cycle; the research rejected this hypothesis. The hypothesis of the second experiment, formulated after completion of the first experiment for this objective, was that DCP accumulates in BME during metaphase of the cell cycle. However, the experiment was unable to confirm of reject this hypothesis. Major findings were that both BME and mammary fibroblasts produce DCP and known decorin pathway molecules. BME produce intracellular DCP, but it is not accumulated during the S-phase of the cell cycle. However, it is still unknown if DCP is accumulated in BME during metaphase. Future research should focus on further characterization of decorin and its associated pathway molecules to learn if decorin induces proliferation or apoptosis of bovine mammary epithelial cells. This is important because number and activity of mammary epithelial cells ultimately determine milk yield in dairy cows. Fundamental knowledge gained in this research area may one day be applied at the animal-level and lead to gains in milk production efficiency by altering the cellular composition of mammary glands.

General Audience Abstract

The mammary gland is a heterogeneous tissue with two main cell types - mammary epithelial cells and fibroblasts. These two cells types produce the majority of the extracellular matrix (**ECM**) within the mammary gland. The ECM is important for regulation of cell shape, proliferation, polarity, differentiation, gene transcription, protein synthesis, and secretion. Decorin is an ECM proteoglycan known to impact mammary cell proliferation in humans and rodents. Prior to this work, very little was known about decorin in bovine mammary biology. A series of bovine mammary cell culture experiments was conducted. The first experiment demonstrated existence of decorin pathway molecules in bovine mammary cells, but stopped short of demonstrating mature decorin proteoglycan deposition into the extracellular space. During the investigation it was noted that when cultured under basal conditions, intracellular decorin core protein (**DCP**) localization patterns appeared to be coordinated with specific phases of the cell cycle. Therefore, the objective of the second set of experiments was to characterize DCP localization patterns in bovine mammary epithelial cells (**BME**) at known phases of the cell cycle. The work was carried out in two sequential experiments. The hypothesis of the first experiment was that DCP accumulates in BME during S-phase of the cell cycle; the research rejected this hypothesis. The hypothesis of the second experiment, formulated after completion of the first experiment for this objective, was that DCP accumulates in BME during metaphase of the cell cycle. Major findings were that both BME and mammary fibroblasts produce DCP and known decorin pathway molecules. BME produce intracellular DCP, but it is not accumulated during the S-phase of the cell cycle. However, it is still unknown if DCP is accumulated in BME during metaphase. Future research should focus on further characterization of decorin and its associated pathway molecules to learn if decorin induces proliferation or apoptosis of bovine mammary cells. This is important because number and activity of mammary epithelial cells ultimately determine milk yield in dairy cows. Fundamental knowledge gained in this research area may one day be applied at the animal-level and lead to gains in milk production efficiency by altering the cellular composition of mammary glands.

Dedication

This body of work is dedicated to the community of people that made my PhD work possible by supporting me.

I need to thank my family, who always encouraged me to seek out the answers to all of my off the wall questions. Their encouragement made it possible for me to stay in school for so long.

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Table 1: Abbreviations used throughout dissertation

Abbreviation	Definition
BME-UV1	Bovine Mammary Epithelial Cells
bp	Base Pair
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine Serum Albumin
CSN2	Beta-casein
DAPI	4',6-diamidino-2-phenylindole
DCN	Decorin
DCP	Decorin Core Protein
DEAE	Diethylaminoethyl
CHO	Chondroitinase ABC
DIC	Differential Interference Contrast
DMEM	Dulbecco's Modified Eagle Media
DPBS	Dulbecco’s Phosphate Buffered Saline
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
FBS	Fetal Bovine Serum
GAG	Glycosaminoglycan
GDP	Guanosine diphosphate
GTP	Guanosine-5'-triphosphate
HGF	Hepatocyte Growth Factor
HGFR	Hepatocyte Growth Factor Receptor
ICC	Immunocytochemistry
IGF1	Insulin Like Growth Factor 1
IGF1R	Insulin Like Growth Factor 1 Receptor
IHC	Immunohistochemistry
INS	Insulin
INSR	Insulin Receptor
IU	International Unit
kDa	Kilodalton
LALBA	Alpha-Lactalbumin
L-TGFB	Latent Transforming Growth Factor Beta
MEC	Mammary Epithelial Cell
MF-T2	Bovine Mammary Fibroblast
MG	Mammary Gland
MTG1	Mitochondrial GTPase 1
NC	Negative Control
PAC	Paclitaxel
PPP1R11	Protein Phosphatase 1 Regulatory (Inhibitor) Subunit 11
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction

rER	Rough Endoplasmic Reticulum
RPS15	Ribosomal Protein S15 A
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SLRP	Small Leucine Rich Proteoglycan
STIM	Stimulatory Media
SV-40	Simian Vacuolating Virus 40
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline Tween 20
TGFB1	Transforming Growth Factor Beta 1
TGFB1R	Transforming Growth Factor Beta 1 Receptor

Chapter 1: Literature Review

MAMMARY DEVELOPMENT

Mammary growth and differentiation in dairy heifers can be described as a continuum of events that begin in utero and extends throughout the animal's productive life. At birth, mammary glands of cattle are rudimentary, consisting only of a few ducts branching from a cistern at the apex of the teat (Sejrsen, 1994). At two months of age, during the prepubertal period, cattle will experience allometric growth in the mammary gland, a faster rate of growth compared to the body (Sinha and Tucker, 1969). The mechanisms for this accelerated growth are not fully understood. However, it is known that early mammary gland development is the foundation for milk secreting tissue that will be formed (Akers, 2000).

Mammary glands of all mammals are skin glands that contain epithelial and stromal structures in addition to myoepithelial cells (not discussed here). Epithelial structures include ducts and alveoli; cells that comprise these structures are derived from the embryonic ectoderm. Stromal structures are mainly connective tissue (adipose, blood and lymph vessels, connective tissue proper) and are derived from the embryonic mesoderm. Thus, any given mammary gland contains two major types of tissue that serve different functions. Epithelial structures have the capacity to make and secrete milk whereas stromal tissues primarily provide support and contribute to glandular morphology. Mammary growth includes expansion of both of these tissue "compartments". Mammary differentiation mainly involves a change in function of mammary epithelial cells (milk component biosynthesis and secretion) and requires action by the hormones of pregnancy. Mammary growth is called mammogenesis and mammary differentiation occurs during the two stages of lactogenesis. Mammogenesis occurs in embryonic, fetal, prepubertal, pubertal, and pregnant lifecycle stages, with the most mammogenesis occurring during pregnancy. Lactogenesis occurs during the final trimester of pregnancy; the process is complete by parturition. Lactogenesis is followed by galactopoesis, lactation, and finally involution, which completes a given period of lactation. Each new pregnancy that a mammal experiences initiates mammogenesis and the other stages associated with the process of lactation and involution (Akers, 2002).

EXTRACELLULAR MATRIX

The extracellular matrix (**ECM**) is a dynamic, structural component of all organs, including the mammary gland; organ-and tissue-specific ECM are made and secreted by resident

cells. In general, the ECM is composed of a diverse group of macromolecules, including collagens, non-collagenous glycoproteins, and proteoglycans (Kierszenbaum and Tres, 2012). The ECM is formed during embryogenesis, but is subject to developmental changes throughout an animal's lifetime (Vracko, 1974). The ECM regulates shape, proliferation, polarity, differentiation, gene transcription, protein synthesis, and secretion for many cell types (Scott, 1995; Alberts, 2002; Rozario and DeSimone, 2010).

Bovine Mammary ECM

There is little literature on the ECM of the bovine mammary gland; the majority of bovine mammary biology literature focuses on the cells within the gland, not their ECM. This omission limits full comprehension of bovine mammary physiology. For instance, it is well established in humans and in mice that the mammary ECM influences mammary epithelial cell (**MEC**) function (Keely et al., 1995; Kefalides et al., 1979). It is therefore not unreasonable to suspect that the bovine mammary ECM serves a similar functional role. Bovine mammary glands are not of static composition. Number of cells, type of cells, and degree of cellular differentiation are dynamic throughout life, as discussed above.

To completely understand the changing dynamics of the bovine mammary gland we must broaden our working knowledge of the ECM, especially the ability of the ECM to regulate the number or function of MEC. The interaction of the ECM and MEC has the potential to be the next avenue researchers pursue to be able to change milk production efficiency in dairy cattle; this, in essence, is why the bovine mammary ECM is presently of research interest.

Mammary Epithelial Cell ECM

Mammary epithelial cells sit atop an extracellular basement membrane that is manufactured and secreted into the local environment by MEC (Riley et al., 2010; Theocharis et al., 2016; **Figure 1**). This placement under MEC, allows the basement membrane to indirectly and directly affect MEC. Indirectly, the basement membrane is able to act as a semipermeable filter that can stop molecules from reaching MEC (Kefalides et al., 1979; Halfter et al., 2015). The basement membrane also acts as a boundary past which epithelial cells are prohibited from growing (Kefalides et al., 1979). The term "basement membrane" is defined as: the pericellular interface between the epithelial cells and connective tissue. The basement membrane is composed of sheet-like arrangements of ECM proteins. There are three layers to the basement membrane: lamina lucida, lamina densa, and lamina fibroreticular. However, older literature

refers to the basement membrane as the basal lamina because this was prior to the discovery of the three layers of the basement membrane (Timpl and Brown, 1996; Young, 2006; Maller et al., 2010).

In lactating rat mammary glands, major components of the basement membrane include: collagen IV, laminin, fibronectin, and heparan sulfate proteoglycans (Monaghan et al., 1983). While these major components are always found in the basement membrane, the formation of the specific structure of the basement membrane depends on the proportion of each component found in the basement membrane. Different components, such as collagen IV and heparan sulfate, attach to each other and form into 3-D structures naturally without aid from enzymes in the mammary gland (Grant et al., 1989). The plastic nature of the basement membrane allows the environment of MEC to vary depending on the requirements of the MEC.

Collagen

The most abundant protein, collagen, includes 28 different types. Collagen is defined as having a structure consisting of three parallel polypeptide strands in a left handed polyproline II-type helical conformation coiled around each other with a one residue stagger to form a right-handed triple helix (Shoulders and Raines, 2009). The classification categories of collagen are: (1) fibrillar, which encompasses collagen found in the basement membrane; (2) fibril-associated collagens with interrupted triple helices; (3) long chain; (4) filamentous; (5) short chain; (6) multiplexin; and (7) membrane-associated collagens with interrupted triple helices (Mouw et al., 2014). Collagen forms polymeric structures that make up tissue scaffolds that include fibrils, microfibrils, filaments, and collagen networks. Examples of each of structures appear in the basement membrane surrounding the alveoli in the mammary gland (The Extracellular Matrix: an Overview, 2011).

Genes for collagen are transcribed in the nucleus of the cell to form mRNA. Depending on the type of collagen being produced, the peptides could be from the same gene, 3 different genes, or 2 from the same gene and 1 from another gene (The Extracellular Matrix: an Overview, 2011). Pre-pro collagen is translated from mRNA to protein in the rough endoplasmic reticulum (**rER**). Next, in the rER the peptides undergo alignment where disulfide bonds form with cystine. Also in the rER, helix formation occurs. Helix formation involves 3 strands of the collagen peptides self-assemble to form a soluble precursor of collagen (procollagen). Upon leaving the rER and flowing into the Golgi apparatus, the helix undergoes post translational

modifications. During this sugars can become attached to the structure, and glycosylation, and hydroxylation can also occur (The Extracellular Matrix: an Overview, 2011). Cellular membranes derived from the Golgi apparatus envelope the modified triple helix collagen molecules (procollagen), forming vesicle. These vesicles undergo exocytosis, a form of intracellular transport (Myllyharju, 2008). Once the procollagen is secreted into the extracellular space, processing continues. Collagen peptidases remove the loose ends of the procollagen molecule; the molecule then starts to form a collagen fiber (The Extracellular Matrix: an Overview, 2011). Crosslinks between collagen molecules increase stability of the fibrillar structure and create less elasticity within the structure. The structures formed will first be small, protofibrils. After combining protofibrils end to end the structure mature collagen fibril is formed (Shoulders and Raines, 2009).

Basement Membrane Collagen IV

Collagen type IV has a flexible helical structure that can interact with other collagen molecules and other proteins to form an extensive network (Timpl and Brown, 1996; Theocharis et al., 2016). Typically, collagen IV is found in the basement membrane of large and small ducts and alveoli and is thought to play an important role in molecular filtration, typically in the lamina densa (Warburton et al., 1982; Keely et al., 1995; Theocharis et al., 2016). After birth, collagen IV deposition increases dramatically and is in high abundance in the adult mammary gland, throughout cycles of regression and lactation (Keely et al., 1995). Collagen IV is a stable molecule that persists over time (Laurie et al., 1980). In a mature bovine mammary gland, the highest amount of collagen IV is found during the dry period, but collagen IV is also present throughout the lactation period (Capuco et al., 1997). The increase in collagen IV during the dry period is probably due to ability of collagen to increase MEC differentiation, which is required to happen before onset of lactation (Emerman et al., 1977). Due to the stability of the molecule, constant presence, and location in the bovine mammary gland, collagen IV is an important part of the basement membrane.

Laminin in the Basement Membrane

Within the basement membrane components, laminins are the largest family (Ekblom et al., 2003). Laminins are large heterotrimeric cross-shaped glycoproteins, that are assembled along the basement membrane adjacent to epithelial cells (Keely et al., 1995; Theocharis et al., 2016). The laminin cross-shape is from three short arms and one long arm with the short arms

consisting of at least one α, one β, and one γ chain. The laminin chains are able to interact with other ECM components, such as: collagen IV, fibronectin, and nidogen (Aumailley and Smyth, 1998; Theocharis et al., 2016). During lactation in mice, laminin is abundant in the basement membrane surrounding MEC, specifically in the lamina lucida (Warburton et al., 1982; Keely et al., 1995). The abundance of laminin helps organize the basement membrane by initiating self-assembly of components of the basement membrane, such as, nidogen and collagen IV (McKee et al., 2009; Pozzi et al., 2017). Based on the interactions of laminin and its integrin receptors, laminin can influence differentiation, adhesion, polarity, and migration of cells and are therefore important components of tissue formation and survival (Aumailley et al., 2005; Taddei et al., 2003; Theocharis et al., 2016).

Fibronectin in the Basement Membrane

A single gene encodes each of the two fibronectin subunits. Each subunit is approximately 250 kDa and are bound together at their C-terminals with a disulfide bond (Hynes, 1990). There are multiple variations of each subunit, due to alternative splicing, and the variety made depends on the type of cell or tissue where the fibronectin is found (Theocharis et al., 2016). In lactating rat mammary gland, fibronectin appeared prominently in the basement membrane bound to MEC via integrin receptors (Warburton et al., 1982; Theocharis et al., 2016). Fibronectin binds to integrins via the RGD region (Arginine-Glycine-Aspartic Acid; Rozario and DeSimone, 2010).

Enactin in the Basement Membrane

Enactin, also known as nidogen, is a small glycoprotein of 150 kDa with two amino terminal globular domains that are separated by short stretches of amino acids. Enactin is approximately 30-40 nm in length with three globular domains separated by two rod-like segments (Fox et al., 1991). The rod-like segments are attached together via disulfide bonds (Timpl et al., 1983). The two supramolecular networks of laminin and collagen IV are bridged together by enactin (Mak and Mei, 2017). Enactin binds laminin at a higher affinity (1-10 nM) than it does collagen IV (greater than 100 nM; Aumailley et al., 1989).

Heparan Sulfate Proteoglycans in the Basement Membrane

Heparan sulfate proteoglycans are found in the basement membrane usually anchored to the MEC via cell surface receptors in the lamina lucida (Warburton et al., 1982). The primary heparan sulfate proteoglycans found in the mammary gland are perlecan, agrin, collagen XVIII,

and collagen XV (Iozzo and Schaefer, 2015; Theocharis et al., 2016). Large aggregating heparan sulfate proteoglycans are able to regulate the hydration status of the basement membrane, which changes the rigidity and size of the basement membrane (Halfter et al., 2015). Perlecan and agrin are pericellular proteoglycans (Iozzo and Schaefer, 2015).

Cell Attachments to the Basement Membrane

Mammary epithelial cells are known to attach to the underlying basement membrane components and each other through membrane proteins known as integrins (**Figure 1**). Two types of integrins, α and β, are associated with MEC attachment to the basement membrane (Boisvert et al., 2010). Integrins act to mediate cell adhesion by linking the actin cytoskeleton of the MEC with the ECM, specifically fibronectin, collagen IV, and laminin (Schatzmann et al., 2003; Glukhova and Streuli, 2013; Theocharis et al., 2016). Through these links, integrins act to anchor MEC and provide sensory input via mechanotransduction. Further, integrins can synergize with signaling pathways and their presence provides the cells with the conditions necessary for cell adhesion, spreading and migration, cellular differentiation, cell survival, and cell cycle progression (Schatzmann et al., 2003; Glukhova and Streuli, 2013; Theocharis et al., 2016).

Due to the placement of the basement membrane, directly under MEC, the basement membrane affects how MEC interact with their environment, the ECM. The basement membrane functions as a semipermeable filter that can stop molecules from reaching MEC (Kefalides et al., 1979). The basement membrane also acts as a boundary that the MEC use as support (Kefalides et al., 1979). The aforementioned abilities of the basement membrane allow the basement membrane and hormones to act in concert on MEC. In mice, MEC produce casein only in the combined presence of lactogenic hormones and collagen I, a component of the ECM that MEC can bind to via integrins (Emerman et al., 1977; Levine and Stockdale, 1985). Casein production is an indicator of differentiation of MEC, which is a key marker for the production of milk (Lee et al., 1985). Overall, without the basement membrane the MEC would not fully differentiate and produce specific milk components.

Mammary Stromal Cell ECM

Because fibroblasts are the predominant cell type of bovine mammary stroma, which is formed from mesenchymal tissue, this discussion of mammary stromal cell ECM is focused on mammary fibroblasts (Vracko, 1974; Larson, 1985; **Figure 2**). Unlike MEC, mammary

fibroblasts do not reside on a true basement membrane and instead form an interstitial ECM that is not as consistent as the basement membrane (Theocharis et al., 2016). This contributes to fibroblast organization i.e. mammary fibroblasts are not organized into a specific structure and are not polarized like many epithelial tissues (**Figure 1**). However, fibroblasts and the stromal compartment control morphogenesis of the glandular epithelium (Sakakura et al., 1976; Howlett and Bissell, 1993).

The primary component of bovine mammary gland stroma is collagen (Larson, 1985). In humans, rats, and Mongolian gerbils collagen I is the main type of collagen found in the stroma (Warburton et al., 1982; Marotta et al., 1985; Leonel et al., 2017). Fibronectin is an abundant ECM molecule that is present in bovine mammary stroma of cows in many physiological stages (Musters et al., 2004; De Vries et al., 2010). In the rat mammary gland, fibronectin is found in both the basement membrane and stroma depending on the stage of lactation. However, during involution and mammogenesis fibronectin is only found in the stroma (Warburton et al., 1982).

BIDIRECTIONAL TALK

When the calf is first born, the mammary parenchyma tissue is surrounded by the mammary fat pad that it will eventually be invaded by the parenchyma as the calf grows (Swett and Matthews, 1934). The mammary fat pad will be become the stroma of a mature bovine mammary gland. The stroma is next to the parenchyma tissue that is sectioned into lobules by the stroma (Weber et al., 1955). With such close proximity to each other there is going to be crosstalk between the stroma and parenchyma tissue throughout the gland's development. The crosstalk will define how the gland develops and the "talk" will be in the form of messages sent via hormones or the ECM (Ellis et al., 2000; Dhimolea et al., 2012).

The documented biological importance of components of the ECM leads to the hypothesized notion of bidirectional talk between adjacent cells and the ECM (Bissell et al., 1982). The bidirectional talk theory involves intricate signaling pathways that have to be set up perfectly to have the optimal response. For instance, the responsiveness of MEC to hormones depends on the composition of the ECM. However, the composition of the ECM also depends on the amount and variety of hormones present, thus supporting the notion that bidirectional crosstalk is important for mammary gland function (Emerman et al., 1977). An example of a feedback loop is the ability of the ECM to sequester growth factors away from cells and the ability of growth factors to increase synthesis or degradation of ECM molecules that sequester

the growth factors. Optimization of the feedback loop does not depend on the number of growth factor receptors present on the MEC (Salomon et al., 1981). Therefore, broader understanding of the ECM in the mammary gland also requires understanding how growth factor abundance changes ECM molecule abundance.

The responsiveness of MEC to hormones depends on the ECM; however, the structure of the ECM is also hormone dependent. The affect observed on either side of this dependency loop is not dependent on the number of receptors present (Salomon et al., 1981). The ECM is able to sequester hormones away from the cells and hormones are able to increase synthesis or degradation of the ECM molecules. The dependency of cells on the ECM make it difficult to research the exact mechanisms of each component of the ECM on the cells.

SMALL LEUCINE-RICH PROTEOGLYCANS

Small leucine-rich proteoglycans (**SLRP**) located within interstitial ECM interact with other proteins, such as, collagens, growth factors, and cell surface receptors. Like the ECM, SLRP are dynamic molecules that are synthesized, secreted, deposited, and degraded throughout all tissues (Chen and Birk, 2013). The basic structure consists of a core protein with a covalently attached glycosaminoglycan (**GAG**) chain or charged amino acid at the N terminus outside the leucine-rich repeats (The Extracellular Matrix: an Overview, 2011). There are four types of GAG: heparin sulfate, chondroitin sulfate, keratin sulfate, and hyaluronic acid (Ruoslahti, 1988). There are five classes (I-V) of SLRP. Classes are based on gene location on the chromosomes, conservation and homology at both DNA and protein levels, and the presence of characteristic N-terminal cysteine rich clusters with defined spacing (Schaefer and Iozzo, 2008). Classes I-III are canonical and classes IV-V are non-canonical groupings (Schaefer and Iozzo, 2008). A distinctive feature of class I-III is an “ear” repeat region that varies in length, but is always located in the leucine rich repeat (Scott et al., 2004; Schaefer and Iozzo, 2008; Chen and Birk, 2013). Each SLRP has different types of binding abilities related to ligands bound to the SLRP (Chen and Birk, 2013). Small leucine-rich proteoglycan are involved in inflammation, diabetes, fibrosis, and metabolic pathways (The Extracellular Matrix: an Overview, 2011). The focus of this research was on decorin (**DCN**), a class I SLRP (Iozzo, 1998; Schaefer and Iozzo, 2008). Other class I SLRP that have similar exon organization and highly conserved intron/econ junctions include biglycan, asporin, extracellular matrix protein 2, and extracellular matrix protein x (Schaefer and Iozzo, 2008; Iozzo and Schaefer, 2015).

DECORIN

Decorin was named by Ruoslahti in 1988 (Ruoslahti, 1988); prior to that, DCN was alternatively known as proteoglycan 40 (pg40), corresponding to its approximate molecular weight (Krusius and Ruoslahti, 1986; Stanescu, 1990; Hanemann et al., 1993), proteoglycan II (pgII) (Stanescu, 1990; Hanemann et al., 1993), or proteoglycan S2 (PG-S2) (Hanemann et al., 1993). Decorin derived its name from its function that it can "decorate" fibrillar collagen, and is able to regulate fibrillogenesis, a key process in matrix assembly and homeostasis (Schaefer and Iozzo, 2008; The Extracellular Matrix: an Overview, 2011).

The decorin core protein (**DCP**) is a gene product that is transcribed and translated in the usual manner. Post-translational modifications of DCP occur in the Golgi apparatus. In the case of DCP, the main modification is the covalent linkage of GAG chains; specifically, the addition of a single chondroitin or dermatan sulfate side chain on a serine residue on the N-terminal side of DCP. This forms mature decorin, which is then translocated to the extracellular space (Moses et al., 1997)

The structure of mature decorin is summarized next. Mature decorin has a molecular weight of 80-140 kDa (Cöster and Fransson, 1981; Bratt et al., 1992); DCP constitutes 45 kDa of this and the single glycosaminoglycan (**GAG**) chain comprises the remaining portion. The DCP shape is generally described as being either horseshoe- (Weber et al., 1996) or banana-like (Scott et al., 2006). The C-terminal region of DCP is cysteine-rich, the central region is made of twelve leucine-rich repeats and is generally recognized as a ligand-binding domain (discussed in further detail below), and the N-terminal region contains the single a single chondroitin/dermatan sulfate side chain and a distinct pattern of cystine residues ($C_{X3}C_XC_{X6}C$) (Reed and Iozzo, 2002; Scott et al., 2004).

Debate exists on the biological form of mature extracellular DCN. At least one group believes that DCN primarily exists in dimer form (Scott et al., 2006). X-ray crystallography of DCN showed distinct dimers with the concave ligand binding domains of each molecule interacting with the other (Scott et al., 2006). Other groups contend that decorin, when present, exists primarily in monomeric form (Weber et al., 1996). This distinction has important consequences. Existence of monomeric but not dimeric decorin would allow for interaction of the concave central ligand binding domain of decorin with ligands (fibrillar collagen, fibronectin, transforming growth factor beta, etc). This is the prevailing stance; however, there is also the

possibility there are two biological forms (monomer and dimer) of DCN that are both available depending on the function needed by the tissue.

STUDYING THE MAMMARY ECM WITH CELL CULTURE EXPERIMENTS

To study the ECM, most research has been conducted in cell culture. Cell culture has the ability to allow researchers to slowly unravel the function of individual molecules; however, there are difficulties in providing the environment that MEC would be exposed to in vivo. Another difficulties is having the proper comparison of MEC, the nominal automatic control would be a lack of any type of environment i.e. a plastic dish. However, it has been shown that when primary MEC are exposed to plastic dishes they lose their specificity of function (Mackenzie et al., 1982).

One strategy is to provide a layer of collagen I that can mimic the major component of the stromal ECM. When this is accomplished the MEC are able to start producing and forming basement membrane (David and Bernfield, 1979; Howlett and Bissell, 1993). Collagen I could be required to be able to form and maintain the integrity of the basement membrane (David and Bernfield, 1979; Howlett and Bissell, 1993).

Another benefit of adding collagen is the ability to examine differentiation in MEC, similar to what is observed during lactogenesis there more developed rER and Golgi in the MEC (Emerman et al., 1977). The addition of lactogenic hormones, prolactin and insulin, to the cell culture media allowed the MEC grown on basement membrane components to become at least partially differentiated by producing casein, a marker of differentiation (Emerman et al., 1977; Lee et al., 1985; Li et al., 1987). However, only heparan sulfate is able to specifically increase casein production, while other components, such as, collagen IV and fibronectin does not increase casein production (Li et al., 1987).

Another important marker of differentiated MEC is polarity. With the addition of collagen I that completely surrounds MEC, there is an induction of polarity that is indicated by the formation of lumens within the cell culture (Hall et al., 1982). However, this does not work on all types of MEC, so it may be dependent on the ability of MEC to produce a “basement membrane”, which is known to induce polarity in MEC (Parry et al., 1987). Polarity is dependent on the ability of MEC to undergo morphological changes. A combination of hormones and ECM molecules in vitro can alter the morphology of MEC and induce polarity and cell differentiation (Mackenzie et al., 1982; Li et al., 1987).

DECORIN SIGNALING PATHWAYS

Decorin is able to bind to many ligands and in so doing can positively or negatively affect cell proliferation. Proliferative and anti-proliferative pathways involving decorin will be discussed next (**Figure 3**).

Proliferative Pathways Involving Decorin

Decorin Signaling Through Insulin Like Growth Factor 1 Receptor

Insulin-like Growth Factor 1 Receptor (**IGF1R**) is an important part of the growth of organisms. Mouse pups with IGF1 gene knockouts do not survive (Liu et al., 1993). Increased IGF1 signaling is associated with mammary growth in dairy cattle (McGrath et al., 1991; Ellis et al., 1991; Berry et al., 2001). However, IGF1 detection in or near MEC occurs weakly during mammogenesis and is more highly concentrated during involution (Sinowatz et al., 2000). IGF1R are present in MEC (Forsyth et al., 1999; Purup et al., 1993), IGF1 is stimulatory to bovine MEC (Weber et al., 1999), and the proliferative effect of serum on mammary cells in vitro is closely related to the concentration of IGF1 in serum (Weber et al., 2000). Decorin has been shown to bind and activate IGF1R and its ligand IGF1 (K_d = 2.7 and 1.5 n*M*, respectively), leading to cell proliferation (Iozzo et al., 2011). With the knowledge that IGF1R is important for mammary epithelial cell proliferation and that DCN can bind IGF1R to promote cell proliferation makes decorin/IGF1R pathway a potentially important pathway to interrogate for its potential to promote proliferation of MEC.

Decorin Sequestration of Transforming Growth Factor beta 1 and Blockage of Downstream Pathway

The biologically active form of transforming growth factor beta 1 (**TGFB1**) is an inhibitor of epithelial cell proliferation and regulator of bovine mammary involution (Knabbe et al., 1987; Kolek et al., 2003). When added to cell cultures of BME, TGFB1 treatment resulted in a decreased number of cells in the S and G2/M phases of the cell cycle and a corresponding increase in the number of cells in the G1 phase of the cell cycle (Kolek et al., 2003). Whereas the biologically active form of TGFB1 is anti-proliferative for mammary epithelial cells (Knabbe et al., 1987; Kolek et al., 2003), it is proliferative for stromal cells (Woodward et al., 1995; Musters et al., 2004). Mammary stromal cell proliferation was increased in TGFB1 treated glands and mammary epithelial cell proliferation and apoptosis were not affected (Musters et al., 2004).

Decorin binds all three TGFB isoforms (TGFB1, TGFB2, TGFB3) through interaction with DCP, not the GAG chain (Yamaguchi et al., 1990; Hildebrand et al., 1994). Through these

interactions, DCN is able to physically sequester TGFB1 away from transforming growth factor beta 1 receptor (**TGFB1R**) and in this manner, promote cell proliferation in several different cell types (Yamaguchi et al., 1990). It has been shown that TGFB1 and TGFB2 can regulate the amount of DCN produced (Bassols and Massague, 1988). However, it is also unknown how fluctuating quantities of TGFB1 affect DCN abundance in the bovine mammary gland, or vice versa.

Anti-proliferative Pathways Involving Decorin

Decorin Signaling Through Hepatocyte Growth Factor Receptor

Hepatocyte Growth Factor Receptor (**HGFR**) is normally found in epithelial cells in both normal and cancerous tissue. HGFR aids cancerous cells by maintaining their ability to proliferate via Hepatocyte Growth Factor (**HGF**), a ligand that binds to HGFR. Hepatocyte growth factor may also sequester a cell surface death receptor molecule, Fas, which allows continued proliferation. Hepatocyte growth factor is also able to induce epithelial cell scatter by disrupting cell-to-cell adhesions. While this process occurs in normal tissue, the problem occurs when in cancerous tissue. The ability to cells to disassociate and migrate "helps" to spread the cancerous tissue, which makes the cancer harder to treat (Ma et al., 2003). While the HGF/HGFR axis is important in vivo to maintain the cell number, the balance of the axis is also important.

Decorin is a high affinity ligand for HGFR. When DCN is bound to HGFR the kinase domain of the receptor becomes phosphorylated and leads to the recruitment of growth factor receptor-bound protein 2, which can lead to the degradation of HGFR. Degradation can occur with the internalization of HGFR via Cbl mediated ubiquitination. DCN is also able to decrease the levels of β-catenin, which also induces HGFR internalization and cell growth arrest (Goldoni et al., 2009). If DCN is able to work through the HGFR in MEC there could be an overall loss in MEC numbers, which would decrease milk production.

Decorin Independent Pathway Through TGFB1R

Transforming growth factor beta is a growth factor that has previously been studied in cattle mammary development (Maier et al., 1991; Plath et al., 1997; Musters et al., 2004). All three isoforms of TGFB are present in lactating and non-lactating bovine mammary gland (Maier et al., 1991). TGFB1 is the predominant form in the mammary gland and is known to increase fibronectin and other ECM proteins in a multitude of species and tissue types (Jin et al., 1991).

TGFB1 is expressed throughout the entire gland with the majority of expression being found in the alveolar epithelium (Maier et al., 1991). However, the amount of TGFB1 changes during different time periods of mammary development. During pregnancy and lactogenesis TGFB1 mRNA abundance is low in the parenchyma in comparison to amounts found during mammogenesis, in virgin, and involuting glands (Plath et al., 1997). The different amounts of TGFB1 indicate TGFB1 has different roles in the mammary gland depending on the time in development the mammary gland is experiencing. When TGFB1 is in high abundance, it is known to bind to TGFB1R on MEC and reduce proliferation (Zarzynska et al., 2005).

Decorin Signaling Through Epidermal Growth Factor Receptor

The Epidermal Growth Factor Receptor (**EGFR**) consists of an extracellular domain, a hydrophobic transmembrane domain, and intracellular catalytic tyrosine kinase domain, and several intracellular tyrosine residues which become phosphorylated to couple with downstream effectors (Foley et al., 2010). Decorin does not contain a region that is homologous to EGFR ligand epidermal growth factor (**EGF**). Decorin binds to EGFR within its leucine rich regions, specifically the sixth region. This region is near where DCN binds to TGFB and collagen (Santra et al., 2002). Binding of a receptor does not always induce a signal; however, DCN was able to dimerize EGFR and cause downstream changes within the cell. Decorin was able to have prolonged and rapid activation of the MAP kinase pathway, which leads to long activation of p21 that will halt the cells in G_1 (Moscatello et al., 1998; Iozzo et al., 1999). Decorin is also able to down regulate protein levels of EGFR without influencing the mRNA levels of EGFR, with the lowered amount of receptors there is a decrease ability for the cells to bind to other ligands via EGFR for increased proliferation (Csordas et al., 2000). The theory is that DCN is able to bind to EGFR long term and induce the receptor to undergo endocytosis.

RATIONALE AND SIGNIFICANCE

The majority of published DCN research relating to the mammary gland describes DCN purported anti-cancer properties in human mammary glands (Grant et al., 2002; Cabello-Verrugio and Brandan, 2007). During invasive breast carcinoma when human mammary stroma lose DCN there is correlation with poor survival in patients (Troup et al., 2003; Goldoni et al., 2008). There is no known literature on DCN impacts in the bovine mammary gland.

Based on previous work with DCN and known components of the bovine mammary gland there would appear to be untapped research ripe for exploration. The proteins within the

ECM that DCN is able to bind to are within the basement membrane and stroma of the mammary gland. Fibronectin is found in both compartments and DCN is able to bind to fibronectin, which can interfere with fibronectin's ability to bind to cells (Lewandowska et al., 1987; Schmidt et al., 1987). The other molecule in the bovine mammary gland stroma DCN can bind to is collagen I (Keene et al., 2000).

Decorin is of specific interest to us for three main reasons. First, the expression of DCN in murine mammary epithelial cells changes with increasing age, suggesting different roles for decorin during various physiological stages (Gu et al., 2010). In our own preliminary studies, we too have observed DCN abundance to change with physiological stage in bovine mammary tissue (O'Diam et al., 2012). The majority of the DCN staining was observed in the interlobular stromal compartment, especially during late gestation and early lactation. However, there was also staining near the MEC during prepubertal mammary gland development (O'Diam et al., 2012). Second, DCN can upregulate proliferation of cells depending on the pathway DCN activates. If DCN is able to increase proliferation of MEC, this could positively affect milk yield per cow because milk yield is partially a function of cell number. Third, DCN expression is upregulated during quiescence, and suppressed in most actively dividing normal cells (Moscatello et al., 1998; Santra et al., 2002). For each lactation a cow undergoes, the mammary gland will experience a quiescent period (dry period) that might involve DCN activity (increase the number of MEC) and ECM remodeling. A goal of this research is to resolve if DCN works through a proliferative or anti-proliferative pathway when interacting with bovine MEC.

We choose a cell culture approach with immortalized cell monocultures, allowing for the study of regulatory effects of decorin on cell proliferation in a controlled cell culture environment. The Daniels lab has access to low passage number stocks of a bovine mammary epithelial **BME** (Zavizion et al., 1996), and one bovine mammary fibroblast cell line **MF-T2** (Woodward et al., 1995). The bovine mammary epithelial cells are an immortalized cell line known as "BME-UV1". These cells got their name because they were bovine mammary epithelial cells from a lactating pregnant Holstein cow from the University of Vermont (Zavizion et al., 1996). To confer immortalization, BME were microinjected with the Simian Vacuolating Virus 40 (**SV-40**) Large-T-antigen from the plasmid pZipSVtsa58 (Zavizion et al., 1996). The immortalized mammary fibroblast cell line MF-T2, was originally described by Woodward and colleagues (1995). They too were stably transfected cells from a non-pregnant heifer with the SV-40 large T

antigen (Woodward et al., 1995).

The focus of this research will be on the ECM proteoglycan DCN and the cellular signaling pathways that DCN is known to affect. No literature exists regarding DCN in bovine mammary biology. It is presently unknown which major cell type within mammary glands, epithelial cells, fibroblasts, or both, is responsible for synthesizing, processing, and secreting DCN in to the ECM. Knowing this is the first step to improve our understanding of the biological importance of the ECM molecule DCN to bovine mammary biology.

DCN is able to bind to a multitude of targets and activate or antagonize cellular pathways (**Figure 3**). Decorin can increase cell proliferation by signaling through the IGF1R pathway or by sequestering TGFB1 away from the TGFB1R and preventing downstream effects. Decorin can decrease cell proliferation by a decorin independent pathway in which TGFB1 binds and activates TGFB1R leading to an increased abundance of p15. Decorin can induce internalization of HGFR, which will induce cell cycle arrest and decrease β-catenin. Lastly, decorin can decrease cell proliferation by signaling through the EGFR pathway.

The basic work proposed here addresses cellular and molecular aspects of mammary ECM biology. The ECM has been found to regulate proliferation and functions of many cell types. The ECM is important for mammary gland development but is not fully characterized, representing a large gap in knowledge. To more fully understand dynamics of mammary gland structure and function, working knowledge of the ECM must be broadened. The focus is on the ECM proteoglycan DCN in bovine MEC. Decorin has numerous growth and regulatory roles; however to date, the Daniels lab is the only known lab studying decorin bovine mammary biology. Regulatory effects of decorin on cell proliferation are mediated through known cellular pathways. Here, these pathways will be investigated through the use of cell culture experiments with monocultures of bovine mammary epithelial and fibroblast cell lines. The expected major outcome of the work will be demonstration of a mechanistic link between decorin and bovine mammary cell proliferation. Number and activity of mammary epithelial cells ultimately determine milk yield in dairy cows, so fundamental knowledge gained here may one day be applied at the animal-level and lead to gains in milk production efficiency.

Hypotheses

This work addressed cellular and molecular aspects of decorin mammary ECM biology. Monocultures of bovine mammary epithelial (BME) and fibroblast (MF-T2) cell lines were used.

Our objective of Chapter 2 was to characterize relative gene transcript abundance of known decorin pathway molecules and decorin core protein abundance in two immortalized bovine mammary cell lines grown under basal conditions. The hypothesis was that under basal culture conditions, MF-T2 would produce relatively more decorin core protein and decorin pathway genes than BME.

The objective of the second set of experiments, reported in Chapter 3, was to characterize decorin localization patterns in BME at known phases of the cell cycle. Two hypotheses were tested. The first was that intracellular decorin core protein accumulates in BME during the S-phase of the cell cycle. The second hypothesis tested in Chapter 3 was that intracellular decorin core protein accumulates in BME during metaphase of the cell cycle.

WORKS CITED

Akers, R. M. 2000. Selection for milk production from a lactation biology viewpoint. Journal of dairy science. 83:1151-1158.

Akers, R. M. 2002. Lactation and the mammary gland. 1st ed. Iowa State University Press, Ames, Iowa.

Alberts, B. 2002. Molecular biology of the cell. 4th ed. Garland Science, New York.

Aumailley, M., L. Bruckner-Tuderman, W. G. Carter, R. Deutzmann, D. Edgar, P. Ekblom, J. Engel, E. Engvall, E. Hohenester, J. C. Jones, H. K. Kleinman, M. P. Marinkovich, G. R. Martin, U. Mayer, G. Meneguzzi, J. H. Miner, K. Miyazaki, M. Patarroyo, M. Paulsson, V. Quaranta, J. R. Sanes, T. Sasaki, K. Sekiguchi, L. M. Sorokin, J. F. Talts, K. Tryggvason, J. Uitto, I. Virtanen, K. von der Mark, U. M. Wewer, Y. Yamada, and P. D. Yurchenco. 2005. A simplified laminin nomenclature. Matrix Biol. 24:326-332.

Aumailley, M. and N. Smyth. 1998. The role of laminins in basement membrane function. J. Anat. 193 (Pt 1):1-21.

Aumailley, M., H. Wiedemann, K. Mann, and R. Timpl. 1989. Binding of nidogen and the laminin-nidogen complex to basement membrane collagen type IV. Eur. J. Biochem. 184:241-248.

Bassols, A. and J. Massague. 1988. Transforming growth factor beta regulates the expression and structure of extracellular matrix chondroitin/dermatan sulfate proteoglycans. J. Biol. Chem. 263:3039-3045.

Berry, S. D., T. B. McFadden, R. E. Pearson, and R. M. Akers. 2001. A local increase in the mammary IGF-1: IGFBP-3 ratio mediates the mammogenic effects of estrogen and growth hormone. Domest Anim Endocrinol. 21:39-53.

Bissell, M. J., H. G. Hall, and G. Parry. 1982. How does the extracellular matrix direct gene expression? J. Theor. Biol. 99:31-68.

Boisvert, M., N. Chetoui, S. Gendron, and F. Aoudjit. 2010. Alpha2beta1 integrin is the major collagen-binding integrin expressed on human Th17 cells. Eur. J. Immunol. 40:2710-2719.

Cabello-Verrugio, C. and E. Brandan. 2007. A novel modulatory mechanism of transforming growth factor-beta signaling through decorin and LRP-1. J. Biol. Chem. 282:18842-18850.

Capuco, A. V., R. M. Akers, and J. J. Smith. 1997. Mammary growth in Holstein cows during the dry period: quantification of nucleic acids and histology. J Dairy Sci. 80:477-487.

Chen, S. and D. E. Birk. 2013. The regulatory roles of small leucine-rich proteoglycans in extracellular matrix assembly. FEBS J. 280:2120-2137.

Csordas, G., M. Santra, C. C. Reed, I. Eichstetter, D. J. McQuillan, D. Gross, M. A. Nugent, G. Hajnoczky, and R. V. Iozzo. 2000. Sustained down-regulation of the epidermal growth factor receptor by decorin. A mechanism for controlling tumor growth in vivo. J. Biol. Chem. 275:32879-32887.

Daniels, K. M. unpublished data. Decorin in bovine mammary tissue.

Danielson, K. G., H. Baribault, D. F. Holmes, H. Graham, K. E. Kadler, and R. V. Iozzo. 1997. Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. J Cell Biol. 136:729-743.

David, G. and M. R. Bernfield. 1979. Collagen reduces glycosaminoglycan degradation by cultured mammary epithelial cells: possible mechanism for basal lamina formation. Proc Natl Acad Sci U S A. 76:786-790.

De Vries, L. D., H. Dover, T. Casey, M. J. VandeHaar, and K. Plaut. 2010. Characterization of mammary stromal remodeling during the dry period. J Dairy Sci. 93:2433-2443.

Dhimolea, E., A. M. Soto, and C. Sonnenschein. 2012. Breast epithelial tissue morphology is affected in 3D cultures by species-specific collagen-based extracellular matrix. J Biomed Mater Res A. 100:2905-2912.

Emerman, J. T., J. Enami, D. R. Pitelka, and S. Nandi. 1977. Hormonal effects on intracellular and secreted casein in cultures of mouse mammary epithelial cells on floating collagen membranes. Proc Natl Acad Sci U S A. 74:4466-4470.

Ellis, S., S. Purup, K. Sejrsen, and R. M. Akers. 2000. Growth and morphogenesis of epithelial cell organoids from peripheral and medial mammary parenchyma of prepubertal heifers. J Dairy Sci. 83:952-961.

Ekblom, P., P. Lonai, and J. F. Talts. 2003. Expression and biological role of laminin-1. Matrix Biol. 22:35-47.

The Extracellular Matrix: an Overview. 2011. Biology of the Extracellular Matrix. Springer Berlin Heidelberg. Berlin, Germany.

Foley, J., N. K. Nickerson, S. Nam, K. T. Allen, J. L. Gilmore, K. P. Nephew, and D. J. Riese, 2nd. 2010. EGFR signaling in breast cancer: bad to the bone. Semin. Cell Dev. Biol. 21:951-960.

Fox, J. W., U. Mayer, R. Nischt, M. Aumailley, D. Reinhardt, H. Wiedemann, K. Mann, R. Timpl, T. Krieg, J. Engel, and et al. 1991. Recombinant nidogen consists of three globular domains and mediates binding of laminin to collagen type IV. EMBO J. 10:3137-3146.

Glukhova, M. A. and C. H. Streuli. 2013. How integrins control breast biology. Curr. Opin. Cell Biol. 25:633-641.

Goldoni, S., A. Humphries, A. Nystrom, S. Sattar, R. T. Owens, D. J. McQuillan, K. Ireton, and R. V. Iozzo. 2009. Decorin is a novel antagonistic ligand of the Met receptor. J Cell Biol. 185:743-754.

Goldoni, S., D. G. Seidler, J. Heath, M. Fassan, R. Baffa, M. L. Thakur, R. T. Owens, D. J. McQuillan, and R. V. Iozzo. 2008. An antimetastatic role for decorin in breast cancer. Am. J. Pathol. 173:844-855.

Grant, D. S., C. P. Leblond, H. K. Kleinman, S. Inoue, and J. R. Hassell. 1989. The incubation of laminin, collagen IV, and heparan sulfate proteoglycan at 35 degrees C yields basement membrane-like structures. J Cell Biol. 108:1567-1574.

Grant, D. S., C. Yenisey, R. W. Rose, M. Tootell, M. Santra, and R. V. Iozzo. 2002. Decorin suppresses tumor cell-mediated angiogenesis. Oncogene. 21:4765-4777.

Gu, Y., S. Zhang, Q. Wu, S. Xu, Y. Cui, Z. Yang, X. Zhao, and B. Sun. 2010. Differential expression of decorin, EGFR and cyclin D1 during mammary gland carcinogenesis in TA2 mice with spontaneous breast cancer. J. Exp. Clin. Cancer Res. 29:6.

Halfter, W., P. Oertle, C. A. Monnier, L. Camenzind, M. Reyes-Lua, H. Hu, J. Candiello, A. Labilloy, M. Balasubramani, P. B. Henrich, and M. Plodinec. 2015. New concepts in basement membrane biology. FEBS J. 282:4466-4479.

Hall, H. G., D. A. Farson, and M. J. Bissell. 1982. Lumen formation by epithelial cell lines in response to collagen overlay: a morphogenetic model in culture. Proc Natl Acad Sci U S A. 79:4672-4676.

Hildebrand, A., M. Romaris, L. M. Rasmussen, D. Heinegard, D. R. Twardzik, W. A. Border, and E. Ruoslahti. 1994. Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta. Biochem. J. 302 (Pt 2):527-534.

Howlett, A. R. and M. J. Bissell. 1993. The influence of tissue microenvironment (stroma and extracellular matrix) on the development and function of mammary epithelium. Epithelial Cell Biol. 2:79-89.

Hynes, R. O. 1990. Fibronectins. Springer series in molecular biology. Springer-Verlag, New York.

Iozzo, R. V. 1998. Matrix proteoglycans: from molecular design to cellular function. Annu Rev Biochem. 67:609-652.

Iozzo, R. V. 1999. The biology of the small leucine-rich proteoglycans. Functional network of interactive proteins. J. Biol. Chem. 274:18843-18846.

Iozzo, R. V., S. Buraschi, M. Genua, S. Q. Xu, C. C. Solomides, S. C. Peiper, L. G. Gomella, R.

C. Owens, and A. Morrione. 2011. Decorin antagonizes IGF receptor I (IGF-IR) function by interfering with IGF-IR activity and attenuating downstream signaling. J. Biol. Chem. 286:34712-34721.

Iozzo, R. V. and L. Schaefer. 2015. Proteoglycan form and function: A comprehensive nomenclature of proteoglycans. Matrix Biol. 42:11-55.

Jin, Y., D. A. Cox, R. Knecht, F. Raschdorf, and N. Cerletti. 1991. Separation, purification, and sequence identification of TGF-beta 1 and TGF-beta 2 from bovine milk. J. Protein Chem. 10:565-575.

Keely, P. J., J. E. Wu, and S. A. Santoro. 1995. The spatial and temporal expression of the alpha 2 beta 1 integrin and its ligands, collagen I, collagen IV, and laminin, suggest important roles in mouse mammary morphogenesis. Differentiation. 59:1-13.

Keene, D. R., J. D. San Antonio, R. Mayne, D. J. McQuillan, G. Sarris, S. A. Santoro, and R. V. Iozzo. 2000. Decorin binds near the C terminus of type I collagen. J. Biol. Chem. 275:21801-21804.

Kefalides, N. A., R. Alper, and C. C. Clark. 1979. Biochemistry and metabolism of basement membranes. Int. Rev. Cytol. 61:167-228.

Kierszenbaum, A. L. and L. L. Tres. 2012. Histology and cell biology : an introduction to pathology. 3rd ed. Saunders, Philadelphia, PA.

Knabbe, C., M. E. Lippman, L. M. Wakefield, K. C. Flanders, A. Kasid, R. Derynck, and R. B. Dickson. 1987. Evidence that transforming growth factor-beta is a hormonally regulated negative growth factor in human breast cancer cells. Cell. 48:417-428.

Kolek, O., B. Gajkowska, M. M. Godlewski, and T. Motyl. 2003. Antiproliferative and apoptotic effect of TGF-beta 1 in bovine mammary epithelial BME-UV1 cells. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 134:417-430.

Larson, B. L. 1985. Lactation. Iowa State University Press, Ames, IA.

Laurie, G. W., C. P. Leblond, I. Cournil, and G. R. Martin. 1980. Immunohistochemical evidence for the intracellular formation of basement membrane collagen (type IV) in developing tissues. J. Histochem. Cytochem. 28:1267-1274.

Lee, E. Y., W. H. Lee, C. S. Kaetzel, G. Parry, and M. J. Bissell. 1985. Interaction of mouse mammary epithelial cells with collagen substrata: regulation of casein gene expression and secretion. Proc Natl Acad Sci U S A. 82:1419-1423.

Leonel, E. C. R., L. R. J. Falleiros, S. G. P. Campos, and S. R. Taboga. 2017. Histological and immunohistochemical characterization of the Mongolian gerbil's mammary gland during gestation, lactation and involution. Acta Histochem. 119:273-283.

Levine, J. F. and F. E. Stockdale. 1985. Cell-cell interactions promote mammary epithelial cell differentiation. J Cell Biol. 100:1415-1422.

Lewandowska, K., H. U. Choi, L. C. Rosenberg, L. Zardi, and L. A. Culp. 1987. Fibronectin-mediated adhesion of fibroblasts: inhibition by dermatan sulfate proteoglycan and evidence for a cryptic glycosaminoglycan-binding domain. J Cell Biol. 105:1443-1454.

Li, M. L., J. Aggeler, D. A. Farson, C. Hatier, J. Hassell, and M. J. Bissell. 1987. Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. Proc Natl Acad Sci U S A. 84:136-140.

Liu, J. P., J. Baker, A. S. Perkins, E. J. Robertson, and A. Efstratiadis. 1993. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell. 75:59-72.

Mackenzie, D. D., I. A. Forsyth, B. E. Brooker, and A. Turvey. 1982. Culture of bovine mammary epithelial cells on collagen gels. Tissue Cell. 14:231-241.

Maier, R., P. Schmid, D. Cox, G. Bilbe, and G. K. McMaster. 1991. Localization of transforming growth factor-beta 1, -beta 2 and -beta 3 gene expression in bovine mammary gland. Mol. Cell. Endocrinol. 82:191-198.

Mak, K. M. and R. Mei. 2017. Basement Membrane Type IV Collagen and Laminin: An Overview of Their Biology and Value as Fibrosis Biomarkers of Liver Disease. Anat Rec (Hoboken).

Maller, O., H. Martinson, and P. Schedin. 2010. Extracellular matrix composition reveals complex and dynamic stromal-epithelial interactions in the mammary gland. J. Mammary Gland Biol. Neoplasia. 15:301-318.

Ma, P. C., G. Maulik, J. Christensen, and R. Salgia. 2003. c-Met: structure, functions and potential for therapeutic inhibition. Cancer Metastasis Rev. 22:309-325.

Marotta, M., G. Martino, F. P. D'Armiento, A. M. Aiello, M. L. Roberti, R. Vecchione, and P. Rosati. 1985. Collagen in human breast cancer: morphological and biochemical study. Appl. Pathol. 3:170-178.

McGrath, M. F., R. J. Collier, D. R. Clemmons, W. H. Busby, C. A. Sweeny, and G. G. Krivi. 1991. The direct in vitro effect of insulin-like growth factors (IGFs) on normal bovine mammary cell proliferation and production of IGF binding proteins. Endocrinology. 129:671-678.

McKee, K. K., S. Capizzi, and P. D. Yurchenco. 2009. Scaffold-forming and Adhesive Contributions of Synthetic Laminin-binding Proteins to Basement Membrane Assembly. J. Biol. Chem. 284:8984-8994.

Monaghan, P., M. J. Warburton, N. Perusinghe, and P. S. Rudland. 1983. Topographical arrangement of basement membrane proteins in lactating rat mammary gland: comparison of the distribution of type IV collagen, laminin, fibronectin, and Thy-1 at the ultrastructural level. Proc Natl Acad Sci U S A. 80:3344-3348.

Moscatello, D. K., M. Santra, D. M. Mann, D. J. McQuillan, A. J. Wong, and R. V. Iozzo. 1998. Decorin suppresses tumor cell growth by activating the epidermal growth factor receptor. J. Clin. Invest. 101:406-412.

Mouw, J. K., G. Ou, and V. M. Weaver. 2014. Extracellular matrix assembly: a multiscale deconstruction. Nat. Rev. Mol. Cell Biol. 15:771-785.

Musters, S., K. Coughlan, T. McFadden, R. Maple, T. Mulvey, and K. Plaut. 2004. Exogenous TGF-beta1 promotes stromal development in the heifer mammary gland. J Dairy Sci. 87:896-904.

Myllyharju, J. 2008. Prolyl 4-hydroxylases, key enzymes in the synthesis of collagens and regulation of the response to hypoxia, and their roles as treatment targets. Ann Med. 40:402-417.

O'Diam, K. M., S. G. Velleman, V. A Swank, S. Ellis, A. V. Capuco, and K. M. Daniels. 2012. First demonstration of decorin an extracellular matrix molecule in bovine mammary tissue. Proc. of 2012 JAM. Phoenix, AZ.

Parry, G., B. Cullen, C. S. Kaetzel, R. Kramer, and L. Moss. 1987. Regulation of differentiation and polarized secretion in mammary epithelial cells maintained in culture: extracellular matrix and membrane polarity influences. J Cell Biol. 105:2043-2051.

Plath, A., R. Einspanier, F. Peters, F. Sinowatz, and D. Schams. 1997. Expression of transforming growth factors alpha and beta-1 messenger RNA in the bovine mammary gland during different stages of development and lactation. J. Endocrinol. 155:501-511.

Pozzi, A., P. D. Yurchenco, and R. V. Iozzo. 2017. The nature and biology of basement membranes. Matrix Biol. 57-58:1-11.

Reed, C. C. and R. V. Iozzo. 2002. The role of decorin in collagen fibrillogenesis and skin homeostasis. Glycoconj. J. 19:249-255.

Riley, L. G., M. Gardiner-Garden, P. C. Thomson, P. C. Wynn, P. Williamson, H. W. Raadsma, and P. A. Sheehy. 2010. The influence of extracellular matrix and prolactin on global gene expression profiles of primary bovine mammary epithelial cells in vitro. Anim. Genet. 41:55-63.

Roberts, A. B., K. C. Flanders, U. I. Heine, S. Jakowlew, P. Kondaiah, S. J. Kim, and M. B. Sporn. 1990. Transforming growth factor-beta: multifunctional regulator of differentiation and development. Philos. Trans. R. Soc. Lond. B Biol. Sci. 327:145-154.

Rozario, T. and D. W. DeSimone. 2010. The extracellular matrix in development and

morphogenesis: a dynamic view. Dev. Biol. 341:126-140.

Ruoslahti, E. 1988. Structure and biology of proteoglycans. Annu. Rev. Cell Biol. 4:229-255.

Sakakura, T., Y. Nishizuka, and C. J. Dawe. 1976. Mesenchyme-dependent morphogenesis and epithelium-specific cytodifferentiation in mouse mammary gland. Science. 194:1439-1441.

Salomon, D. S., L. A. Liotta, and W. R. Kidwell. 1981. Differential response to growth factor by rat mammary epithelium plated on different collagen substrata in serum-free medium. Proc Natl Acad Sci U S A. 78:382-386.

Santra, M., C. C. Reed, and R. V. Iozzo. 2002. Decorin binds to a narrow region of the epidermal growth factor (EGF) receptor, partially overlapping but distinct from the EGF-binding epitope. J. Biol. Chem. 277:35671-35681.

Schaefer, L. and R. V. Iozzo. 2008. Biological functions of the small leucine-rich proteoglycans: from genetics to signal transduction. J. Biol. Chem. 283:21305-21309.

Schatzmann, F., R. Marlow, and C. H. Streuli. 2003. Integrin signaling and mammary cell function. J. Mammary Gland Biol. Neoplasia. 8:395-408.

Schmidt, G., H. Robenek, B. Harrach, J. Glossl, V. Nolte, H. Hormann, H. Richter, and H. Kresse. 1987. Interaction of small dermatan sulfate proteoglycan from fibroblasts with fibronectin. J Cell Biol. 104:1683-1691.

Scott, J. E. 1995. Extracellular matrix, supramolecular organisation and shape. J. Anat. 187 (Pt 2):259-269.

Scott, P. G., P. N. Bishop, and J. Bella. 2006. On the calculation of the binding force between decorin and collagen. J. Biomech. 39:1159-1160; author reply 1160-1152.

Scott, P. G., P. A. McEwan, C. M. Dodd, E. M. Bergmann, P. N. Bishop, and J. Bella. 2004. Crystal structure of the dimeric protein core of decorin, the archetypal small leucine-rich repeat proteoglycan. Proc Natl Acad Sci U S A. 101:15633-15638.

Sejrsen, K. 1994. Relationships between nutrition, puberty and mammary development in cattle. The Proceedings of the Nutrition Society. 53:103-111.

Shoulders, M. D. and R. T. Raines. 2009. Collagen structure and stability. Annu Rev Biochem. 78:929-958.

Silberstein, G. B., P. Strickland, S. Coleman, and C. W. Daniel. 1990. Epithelium-dependent extracellular matrix synthesis in transforming growth factor-beta 1-growth-inhibited mouse mammary gland. J Cell Biol. 110:2209-2219.

Sinha, Y. N. and H. A. Tucker. 1969. Mammary development and pituitary prolactin level of

heifers from birth through puberty and during the estrous cycle. Journal of dairy science. 52:507-512.

Sinowatz, F., D. Schams, A. Plath, and S. Kolle. 2000. Expression and localization of growth factors during mammary gland development. Adv. Exp. Med. Biol. 480:19-25.

Stanescu, V. 1990. The small proteoglycans of cartilage matrix. Semin. Arthritis Rheum. 20:51-64.

Swett, W. W. and C. A. Matthews. 1934. Dairy Cow's Udder Studied to Establish Development Standards. Yearbook of Agriculture. 175-181.

Taddei, I., M. M. Faraldo, J. Teuliere, M. A. Deugnier, J. P. Thiery, and M. A. Glukhova. 2003. Integrins in mammary gland development and differentiation of mammary epithelium. J. Mammary Gland Biol. Neoplasia. 8:383-394.

Theocharis, A. D., S. S. Skandalis, C. Gialeli, and N. K. Karamanos. 2016. Extracellular matrix structure. Adv Drug Deliv Rev. 97:4-27.

Timpl, R., M. Dziadek, S. Fujiwara, H. Nowack, and G. Wick. 1983. Nidogen: a new, self-aggregating basement membrane protein. Eur. J. Biochem. 137:455-465.

Timpl, R. and J. C. Brown. 1996. Supramolecular assembly of basement membranes. Bioessays. 18:123-132.

Troup, S., C. Njue, E. V. Kliewer, M. Parisien, C. Roskelley, S. Chakravarti, P. J. Roughley, L. C. Murphy, and P. H. Watson. 2003. Reduced expression of the small leucine-rich proteoglycans, lumican, and decorin is associated with poor outcome in node-negative invasive breast cancer. Clin. Cancer Res. 9:207-214.

Vracko, R. 1974. Basal lamina scaffold-anatomy and significance for maintenance of orderly tissue structure. Am. J. Pathol. 77:314-346.

Warburton, M. J., D. Mitchell, E. J. Ormerod, and P. Rudland. 1982. Distribution of myoepithelial cells and basement membrane proteins in the resting, pregnant, lactating, and involuting rat mammary gland. J. Histochem. Cytochem. 30:667-676.

Weber, A. F., R. L. Kitchell, and R. L. Sautter. 1955. Mammary gland studies. I. The identity and characterization of the smallest lobule unit in the udder of the dairy cow. Am. J. Vet. Res. 16:255-263.

Weber, I. T., R. W. Harrison, and R. V. Iozzo. 1996. Model structure of decorin and implications for collagen fibrillogenesis. J. Biol. Chem. 271:31767-31770.

Wheelock, J., A. Smith, F. Dodd, and R. Lyster. 1967. Changes in the quantity and composition of mammary gland secretion in the dry period between lactations: I. The beginning of the dry

period. Journal of Dairy Research. 34:1-12.

Woodward, T. L., N. Dumont, M. O'Connor-McCourt, J. D. Turner, and A. Philip. 1995. Characterization of transforming growth factor-beta growth regulatory effects and receptors on bovine mammary cells. J. Cell. Physiol. 165:339-348.

Yamaguchi, Y., D. M. Mann, and E. Ruoslahti. 1990. Negative regulation of transforming growth factor-beta by the proteoglycan decorin. Nature. 346:281-284.

Young, B. 2006. Wheater's functional histology : a text and colour atlas. 5th ed. Churchill Livingstone/Elsevier, Edinburgh.

Zarzynska, J., M. Gajewska, andT. Motyl. 2005. Effects of hormones and growth factors on TGF-beta1 expression in bovine mammary epithelial cells. J. Dairy Res. 72:39-48.

Zavizion, B., M. van Duffelen, W. Schaeffer, and I. Politis. 1996. Establishment and characterization of a bovine mammary epithelial cell line with unique properties. In Vitro Cell. Dev. Biol. Anim. 32:138-148.

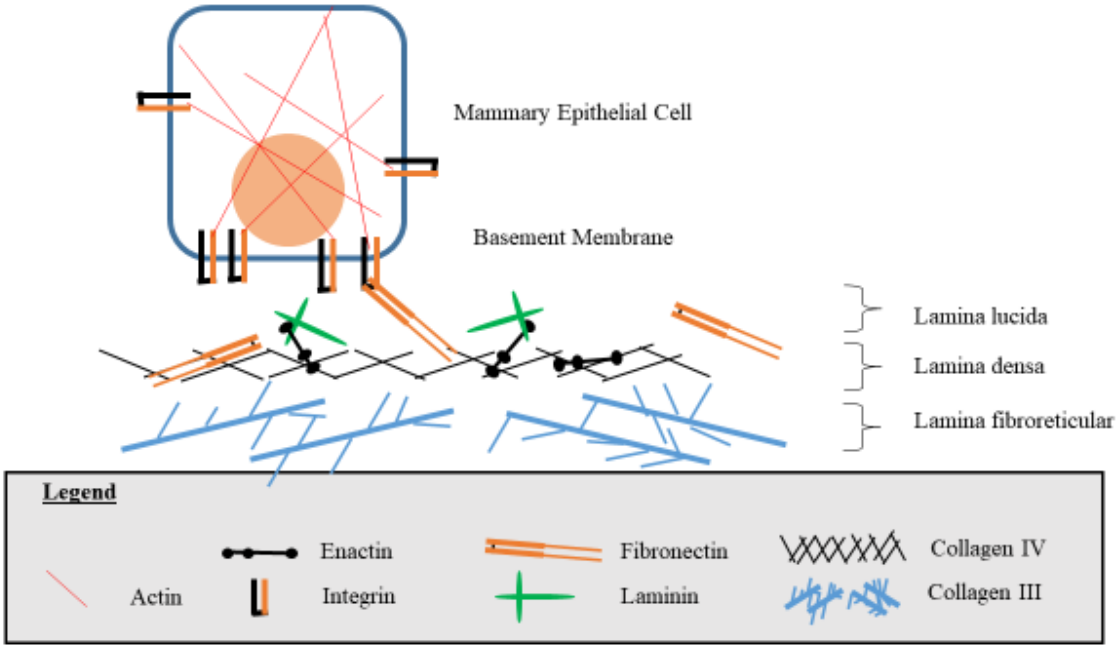


Figure 1: in vivo extracellular matrix molecules and their organization in bovine mammary epithelial cells. Molecules are not to scale and represent their general structure and interaction amongst other molecules. Myoepithelial cells are not included in the figure.

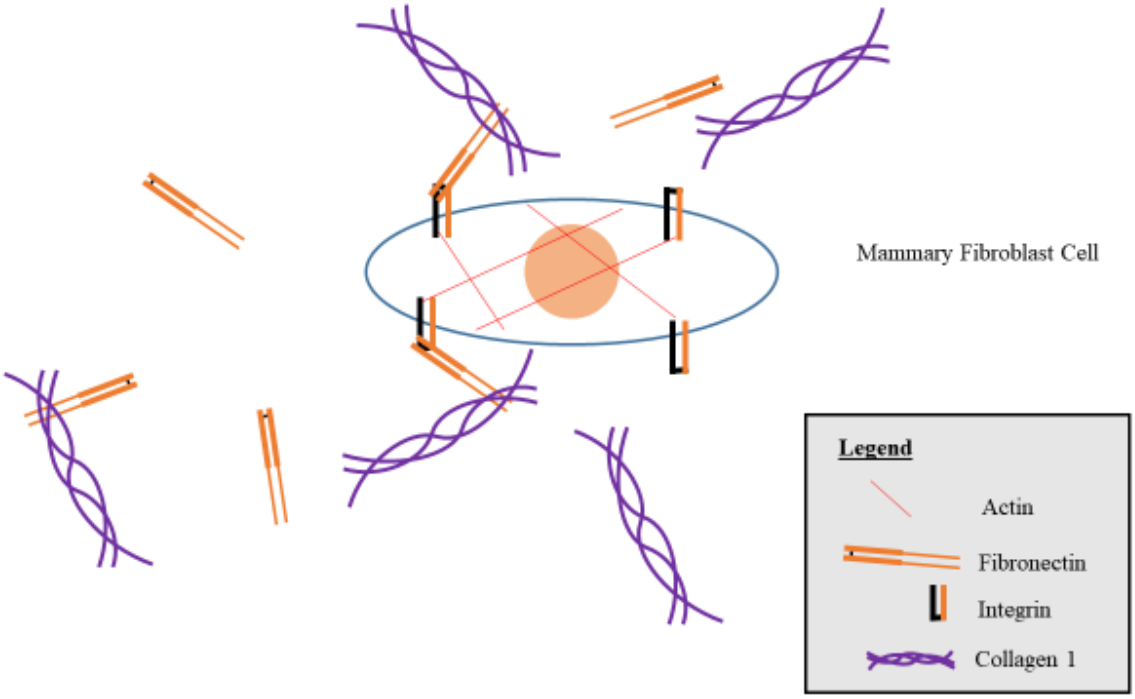


Figure 2: in vivo extracellular matrix molecules and their organization in bovine fibroblast cells. Molecules are not to scale and represent their general structure and interaction amongst other molecules.

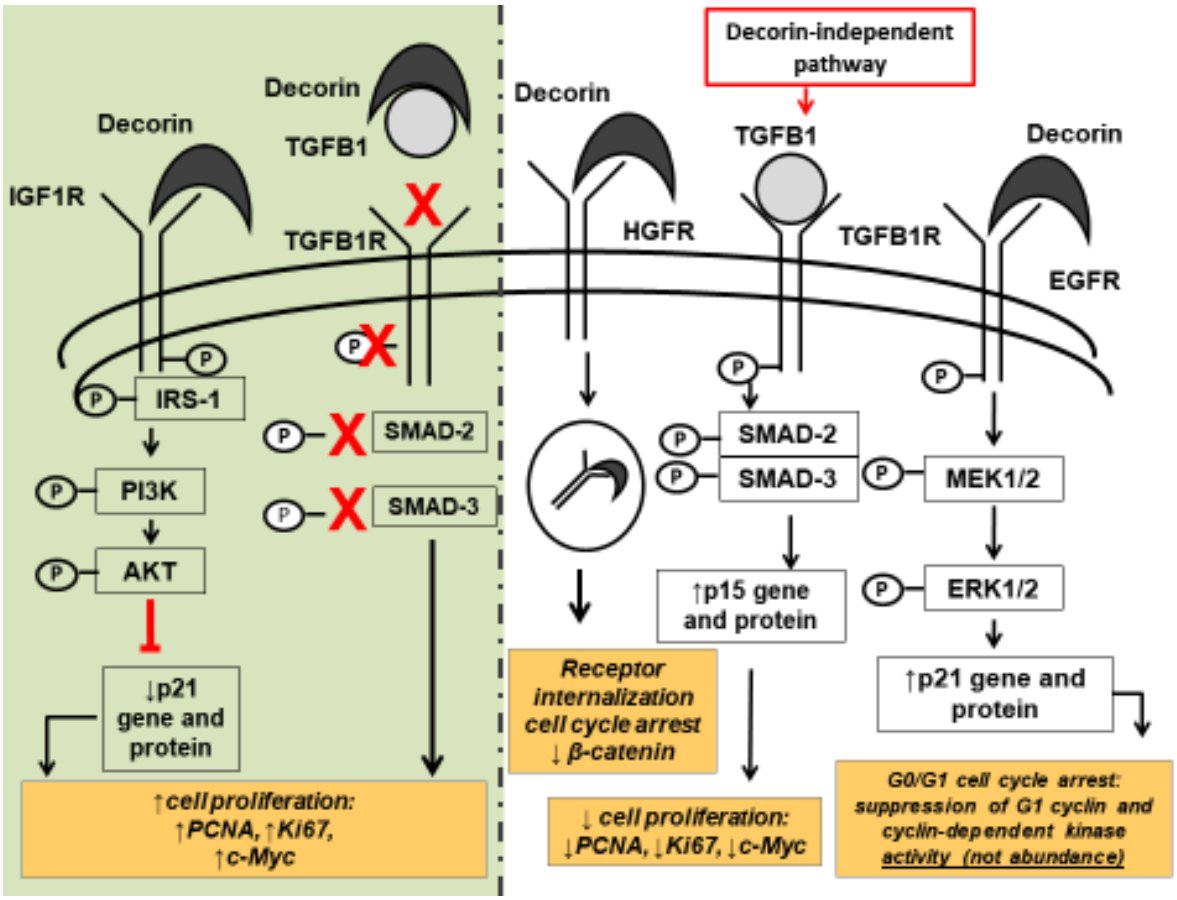


Figure 3: Three known decorin signaling pathways, and one related decorin independent pathway with resulting downstream cellular effects are shown. Decorin can increase cell proliferation by signaling through the insulin-like growth factor receptor (IGF1R) pathway or by sequestering transforming growth factor beta 1 (TGFB1) away from the transforming growth factor beta 1 receptor (TGFB1R) and preventing downstream effects. Decorin can decrease cell proliferation by a decorin independent pathway in which TGFB1 binds and activates TGFB1R leading to an increased abundance of p15. Decorin can induce internalization of hepatocyte growth factor receptor (HGFR), which will induce cell cycle arrest and decrease β-catenin. Lastly, decorin can decrease cell proliferation by signaling through the epidermal growth factor receptor (EGFR) pathway. Decorin is a member of the small leucine-rich proteoglycans that has an N-terminal glycosaminoglycan chain. The concave portion of decorin is what interacts with the different receptor ligands.

Chapter 2: Comparative Decorin Pathway Gene Abundance Analysis in Cultured Bovine Mammary Epithelial and Fibroblast Cells

ABSTRACT

The mammary gland is a heterogeneous tissue with two main cell types - mammary epithelial cells and fibroblasts. These two cells types produce the majority of the extracellular matrix (**ECM**) within the mammary gland. The ECM is important for regulation of cell shape, proliferation, polarity, differentiation, gene transcription, protein synthesis, and secretion. Decorin is an ECM proteoglycan known to impact mammary cell proliferation in humans and rodents. In dairy cattle, the primary decorin producing cell populations and primary decorin responsive cell populations have not yet been identified. Knowing this will advance our understanding of ECM regulation of bovine mammary growth and development. Our objective was to characterize relative gene transcript abundance of known decorin pathway molecules and decorin core protein abundance in two immortalized bovine mammary cell lines grown under basal conditions. Our hypothesis was that under basal culture conditions mammary fibroblasts (**MF-T2**) would produce relatively more decorin core protein and decorin pathway genes than bovine mammary epithelial cells (**BME**). Bovine mammary epithelial cells and MF-T2 were each initially grown in 6 well plastic dishes with Dulbecco's Modified Eagle Medium (**DMEM**) plus 10% fetal bovine serum. Initial densities were 5×10^5 cells/well for BME and 1×10^6 cells/well for MF-T2. After 16 h, media was changed to 100 % DMEM. Cell lysates were collected after 16 h of DMEM incubation; total RNA and DNA were quantified. Total RNA was converted into cDNA by reverse transcription expression and quantified by real time quantitative PCR (**RT-qPCR**). Decorin pathway genes of interest were: *decorin core protein, transforming growth factor beta 1, transforming growth factor 1 receptor, insulin-like growth factor 1, insulin-like growth factor receptor 1, epidermal growth factor, epidermal growth factor receptor, hepatocyte growth factor, hepatocyte growth factor receptor,* and *insulin receptor*. Prior to data analyses, genes of interest were normalized to the average of three internal control genes and the final DNA content of each cell culture well. MF-T2 produced relatively more (31x more abundant) decorin core protein mRNA when both cell types were cultured on under basal conditions. All other genes of interest were detected in both cell types, with transforming growth factor beta 1 mRNA also being relatively more abundant in MF-T2 (9x more abundant). At the transcript level, components of known decorin signaling pathways are present in BME and MF-T2 grown under basal conditions suggesting but not proving, that decorin is an important ECM

proteoglycan in bovine mammary glands. As a second objective, immunocytochemistry was used to demonstrate that decorin core protein (rabbit polyclonal decorin primary antibody (1:50; SC22753) and AlexaFluor 594 goat anti-rabbit secondary antibody (1:200; A11012) is present in BME; no data for MF-T2 were generated. While existence of decorin pathway molecules was demonstrated in bovine mammary cells, these experiments stopped short of demonstrating mature decorin proteoglycan deposition into the extracellular space. However, because decorin has known growth regulatory properties, resultant findings can be used in the future to design mechanistic cell culture studies.

INTRODUCTION

At birth, the mammary gland of the bovine is rudimentary consisting only of a few ducts branching from a cistern at the top of the teat that is surrounded by a mammary fat pad (Swett and Matthews, 1934; Sejrsen, 1994). In dairy cattle, this basic arrangement serves as the architectural foundation of the entire udder, components of which will eventually undergo mammogenesis, lactogenesis, galactopoesis, involution and tissue remodeling during the dry period (Akers, 2000). Existence of these developmental stages highlights mammary dynamism, meaning that deconstruction and construction of cellular and non-cellular materials within the udder both occur often.

The major anatomical features of the mammary gland are epithelial and stromal elements. Ducts and alveoli are composed of epithelial structures; cells that comprise these structures are derived from the embryonic ectoderm. The other main structure in the bovine mammary gland, stroma, is mainly connective tissue (adipose, blood and lymph vessels, connective tissue proper) and is derived from the embryonic mesoderm. Epithelial and stromal tissue types work in concert, but have different functions. Sheet-like epithelial structures have the capacity to make and secrete milk whereas stromal tissues primarily provide support and contribute to gland shape. Mammary growth includes expansion of both of these tissue “compartments” (Akers, 2002). Within these two compartments are two main cell types - mammary epithelial cells and mammary fibroblasts.

The majority of research in the bovine mammary gland focuses on the mammary epithelial cells because of their milk production significance; however, to more completely understand mammary gland structure and function, both cells types should be investigated. Cell

culture experiments represent a unique opportunity to examine characteristics of monocultures of cells. Our lab has low passage number stocks of a bovine mammary epithelial (**BME**; Woodward et al., 1995), and a bovine mammary fibroblast cell line (**MF-T2**; Zavizion et al., 1996). Both cell types used were immortalized using Simian Vacuolating Virus 40.

Besides ensured cell type homogeneity, another advantage of cell culture experiments is that, researchers can control the environment that surrounds the cultured cells by changing components of the media, altering the surface upon which cells are grown, or both. In vivo, the environment that surrounds the cells is called the extracellular matrix (**ECM**). The ECM has been found to regulate shape, proliferation, polarity, differentiation, gene transcription, protein synthesis, and secretion for many cell types (Scott, 1995; Alberts, 2002; Rozario and DeSimone, 2010). Decorin is able to bind to a multitude of targets and activate or antagonize cellular pathways (**Figure 4**).

The focus of this research was on an ECM molecule, decorin (**DCN**), a class I small leucine-rich proteoglycan, and the cellular signaling pathways that DCN is known to affect (Iozzo, 1998; Schaefer and Iozzo, 2008). Decorin consists of a 45 kDa core protein (**DCP**) and the single glycosaminoglycan (**GAG**) chain that is covalently linked to DCP in the Golgi apparatus (Moses et al., 1997). To our knowledge, no literature exists regarding DCN in bovine mammary biology. Decorin is of interest because of the potential ability to increase proliferation of mammary epithelial cells (Yamaguchi et al., 1990; Iozzo et al., 2011). Milk yield is partially a function of cell number, so the increase in mammary epithelial cells could increase milk yield (Akers, 2000).

The objective was to characterize relative gene transcript abundance of known decorin pathway molecules and DCP abundance in two immortalized bovine mammary cell lines grown under basal conditions. Our preliminary work with DCP immunohistochemistry (**IHC**) in bovine tissue led us to hypothesize that, under basal culture conditions, MF-T2 would produce relatively more *DCP* and DCN pathways genes than BME (O'Diam et al., 2012; **Figure 4**).

MATERIALS AND METHODS

Cell Culture BME and MF-T2 Decorin Pathway Genes

Prior to use, all media and Dulbecco's Phosphate Buffered Saline (**DPBS;** Mediatech Inc.; Manassas, VA; catalog # 55031 PB) were prewarmed in a warm water bath. All media were supplemented with 100 IU/mL penicillin, 100 IU/mL streptomycin (Fisher Scientific; catalog # SV3007901), and 0.25 µg/mL Gentamicin Sulfate (Fisher Scientific; catalog # BW17518Z).

Initial plating density (cells per well of 6 well dishes; Fisher Scientific; Waltham, MA; catalog # 08777233) for BME was 5.0 x 10^4 cells/well and for MF-T2, 1.0 x 10^5 cells/well. Each cell type was grown in duplicate plastic wells concurrently and the duplicate wells were grown three times giving each cell type an n = 6. All cells were cultured in Dulbecco's Modified Eagle Media (**DMEM**; Mediatech Inc.; catalog # 50003 PB) plus 10 % fetal bovine serum (**FBS;** Atlanta Biologicals; Flowery Branch, GA; S11550) in an incubator set at 37ºC in 5 % CO_2. After 16 h cells were washed with 1 mL DPBS and then 2 mL of fresh DMEM were added. After 16 h, cells and media were collected.

Cell Culture for Decorin Core Protein Identification

Bovine Mammary Epithelial cells at 5 x 10^5 were grown on six sterilized glass coverslips (22 mm^2; Fisher Scientific; catalog # 12540B) that were inserted singly into individual wells of a six well plate (1 coverslip/well). The cell culture plates were plastic and did not contain an artificial matrix (Fisher Scientific; catalog # 08777233). For the plating and attachment phase, individual wells were filled with 2 mL of DMEM plus 10 % FBS for 16 h. The media was then aspirated and each well was washed with 2 mL of DPBS. Cells then underwent a serum starvation phase where each well received 2 mL of DMEM for 16 h. The wells were then washed with 2 mL of DPBS and then received 2 mL of fresh DMEM; plates were incubated for an additional 16 h. Coverslips were collected after 16 h for immunocytochemistry (**ICC**), as described below.

RNA, DNA, and Protein Isolation from Cultured Cells

Total RNA, DNA, and total protein were sequentially extracted using the reagents from the RNA/DNA/Protein Purification Kit (Norgen Biotek Corp.; Ontario, Canada; catalog # 24000). The manufacturer's protocol for cells growing in a monolayer was used, as summarized next. To wash the cells, the media was aspirated and then the cells were washed with DPBS. To lyse the cells, 350 µL of Buffer Sk were added to the plate and were manually swirled for 5 min. The lysate was then transferred to a new microcentrifuge tube, 200 µL of 100 % ethanol were then added, and the tube was vortexed for 10 s.

To extract the total RNA, a spin column was assembled and 600 µL of the lysate plus ethanol was added to the column. All centrifugation for this kit was conducted at room temperature. The column was centrifuged for 1 min at 4,000 x *g* (Eppendorf Centrifuge 5430R) and the flowthrough was retained on ice for further use (see protein extraction below). The column was then reassembled with a new collection tube, 400 µL of Wash Solution A were

added, and the column was centrifuged for 1 min at 12,000 x *g*. The flowthrough was discarded. This wash step was repeated. The column was then reassembled and underwent a drying step by centrifuging for 2 min at 12,000 x *g*. A new microcentrifuge tube was added to the column and 50 µL of Elution A Solution were added to the column. To elute the total RNA, the column was centrifuged for 1 min at 14,000 x *g*.

DNA was sequentially extracted from the same column. For this, the column was assembled with a new collection tube and 500 µL of Wash Solution El were added. The column was centrifuged for 2 min at 12,000 x *g* and the flowthrough was discarded. The column was then reassembled with a new collection tube and dried by centrifugation for 2 min at 12,000 x *g*. The column was then placed into a new microcentrifuge tube and 100 µL of Elution F Buffer were added. The column was first centrifuged for 2 min at 200 x *g* then, without removal from the centrifuge, centrifuged for 1 min at 14,000 x *g*.

DNA and RNA purity and quantity were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Rockland, DE). The resulting DNA and total RNA were stored at -80 ºC.

Finally, protein was extracted from the same column using a new collection tube. Three hundred µL of the column flowthrough from the beginning of the RNA extraction were transferred to a new microcentrifuge tube. Another 300 µL of DNase/RNase free water were added to the microcentrifuge tube containing the flowthrough. Then 24 µL of Binding Buffer A were added to the microcentrifuge tube and the contents were mixed by inversion. Next, 650 µL of the flowthrough plus water plus Binding Buffer A solution were added to the column. The column was centrifuged for 2 min at 5,200 x *g*. The flowthrough was then discarded and the column reassembled. The column received 500 µL of Wash Solution C and was centrifuged for 2 min at 5,200 x *g*. The flowthrough was discarded and the column reassembled to be dried via centrifugation for 1 min at 12,000 x *g*. In a new microcentrifuge tube, 9.3 µL of Protein Neutralizer were added and the column was assembled with the new microcentrifuge tube with Protein Neutralizer. Then 100 µL of Elution Buffer C were added to the column and centrifuged for 2 min at 5,200 x *g*. The eluted protein was stored at -80 ºC until further processing.

Quantification of Gene Expression

Real time quantitative PCR (**RT-qPCR**) was used to quantify the expression of the following genes: *decorin core protein (**DCP**), transforming growth factor beta 1 (**TGFB1**),*

*transforming growth factor beta 1 receptor (**TGFB1R**), insulin like growth factor 1 (**IGF1**), insulin like growth factor 1 receptor (**IGF1R**), epidermal growth factor (**EGF**), epidermal growth factor receptor (**EGFR**), hepatocyte growth factor (**HGF**), hepatocyte growth factor receptor (**HGFR**),* and *insulin receptor (**INSR**). Mitochondrial ribosome associated GTPase* (***MTG1***), *ribosomal protein S15a* (***RPS15A***), and *protein phosphate 1 regulatory inhibitor subunit 11* (***PPP1R11***) were used as endogenous control genes as described below. Genes of interest were selected based on their known involvement in decorin signaling in other species and tissue types (**Figure 4**; **Table 2**).

Single-stranded cDNA was synthesized from each RNA sample according to the method of Swank et al. (2013). For each BME and MF-T2 sample, 2.0 and 0.5 μg of RNA, respectively, (contained in 11.3 μL) were first denatured in DNase/RNase free water for 12 min, at 70°C; samples were then placed on ice for 3 min. Reverse transcription was performed afterward. Reverse transcription master mix (8.7 μL) contained: 4 μL of M-MLV RT 5X buffer (Promega; Madison, WI), 2 μL of 0.1 *M* dithiothreitol, 1 μL 10 m*M* dNTP mix (Promega), 1 μL of oligo(dT)20 primer, 0.5 μL M-MLV reverse transcriptase (Promega), and 0.2 μL of RNase inhibitor (Promega). The 8.7 μL of master mix was added to each well of denatured RNA (11.3 μL) in a 0.2 mL thin wall PCR 8-strip well tube (World Wide Medical Services Inc.; Tampa, FL) for final reaction volume of 20 μL. Reverse transcription was carried out in an Arktik Thermal Cycler (Thermo Scientific; Waltham, MA) with cycle conditions at 40 °C for 1 h, followed by 95 °C for 10 min.

Resultant cDNA was then diluted 1:1 with addition of 20 μL of DNase/RNase free water. The cDNA was vortexed, aliquoted (5 μL each) into strip well tubes, and stored at -20°C until further use. Later, RT-qPCR assays were performed on all samples in duplicate, with each reaction mixture (10 μL) containing 0.25 μL each forward and reverse primer, 4.75 μL of PowerUp SYBR Green Master Mix (Life Technologies, Grand Island, NY), 3.75 μL of RNase/DNase free water, and 1 μL of cDNA (1:1 stock). The RT-qPCR assays were performed using a QuantStudio 6 Flex Real-Time PCR system (Life Technologies) with the following cycling conditions: 95 °C for 3 min (heat activation of polymerase); 45 repeating cycles of: 94 °C for 15 s (denaturation), primer-specific annealing temperature for 30 s (**Table 2**), and 72 °C for 30 s (extension). Each RT-qPCR run culminated with a melt curve analysis with the following settings: 95.0 °C for 2 min, 55.0 °C for 30 s, and then a temperature increase of 0.1

°C/s until 95.0 °C was reached.

The purpose of the melt curve analyses was to verify RT-qPCR product purity (absence of genomic DNA contamination and primer dimers). Confirming absence of genomic DNA contamination was of particular interest here due to the sequential nucleic acid and protein extraction kit that was used, which did not include a genomic DNA digestion step (common in total RNA extraction kits). RT-qPCR product size was verified by electrophoresing representative samples of RT-qPCR products for each primer pair on a 3.5 % agarose gel; dsDNA was labeled with SYBR Safe DNA gel stain (Life Technologies, catalog # S33102) and the gel was visualized on a ChemiDoc XRS (Bio-Rad Laboratories, Inc.; Hercules, CA). All RT-qPCR products were visualized as single bands that corresponded with predicted product size (**Table 2**; **Figure 5**; **Figure 6**).

Each RT-qPCR assay included no template and no reverse-transcriptase controls. The no template control received 1 μL of RNase/DNase free water instead of cDNA, and the no reverse-transcriptase control received 1 μL of the reverse transcription product to which no reverse-transcriptase was added. Individual samples were repeated when the resulting coefficient of variation for duplicate samples was greater than 5 %.

Primer sequences for *EGF*, *EGFR*, *HGF*, and *HGFR* (**Table 2**) were designed using Primer-Blast that is maintained by the National Center for Biotechnology Information (NCBI). Primers were designed to be between 18 and 22 bp in length, have between 50 and 60% guanine to cytosine content, have an optimal product size of 150 bp, and have a preference of crossing an intron. However, not all bovine gene sequences that were searched have known introns (e.g., *EGF* and *EGFR*). Primer sequences that matched these criteria were queried using the basic local alignment search tool (BLAST) in the Bos taurus genome database that is maintained by the National Center for Biotechnology Information (NCBI). The results from the query that returned a 100% identity for the gene of interest were then used for RT-qPCR analyses. Primer sequences for were *MTG1*, *RPS15A*, and *PPP1R11* obtained from Piantoni (2008). Primer sequences for *TGFB1* were obtained from Plath et al. (1997). Primer sequences for *IGF1* were obtained from Velayudhan et al. (2008).

Target genes of interest were normalized to the geometric mean of *MTG1*, *RPS15A*, and *PPP1R11* (target gene Ct – reference genes Ct = ΔCt) (Vandesompele et al., 2002). Delta Ct data were transformed to $2^{(-\Delta Ct)}$ to represent fold differences relative to the reference genes

(Velayudhan et al., 2008).

Primer efficiencies (**Table 3**) were determined for each gene and tissue type by the use of serial dilutions of cDNA (1:1, 1:10, 1:100, 1:1000), using the following equation: $[(10^{(-1/slope)}-1)*100]$ (Bustin et al., 2009).

Purification of RT-qPCR Products

RT-qPCR products from representative RT-qPCR reactions (*TGFB1R, IGF1R, EGF, EGFR, METR, INSR,* and *DCP*) were electrophoresed on a 3.5 % agarose gel; individual bands were excised with a clean scalpel blade. A QIAquick Gel Extraction Kit (Qiagen Hilden, Germany; catalog # 28704) was used to purify DNA fragments from each excised gel band. Briefly, gel fragments were weighed in a 15 mL conical tube and 3 volumes of Buffer QG were added to each sample. For the entire protocol, all centrifugation steps were conducted at room temperature. Samples were then incubated at 50 ºC for 15 min, with vortexing every 2-3 min. One volume of isopropanol was then added to each sample and tubes were inverted to mix. Samples were added to the upper chambers of assembled QIAquick spin columns; columns were spun at 18,000 *x g* for 1 min. The flowthrough was discarded. Not all of the initial sample could fit on to each column at one time, so the rest of the sample was added to the column and centrifuged for 1 min under the previous conditions. The flowthrough was again discarded. Then 500 µL of Buffer PE was added to each column. Columns were allowed to stand for 5 minutes at room temperature and were then centrifuged as before. The flowthrough was discarded. Each column was then placed into a clean microcentrifuge tube. To elute the purified DNA, 15 µL of DNase/RNase free water were added to each column. Columns were incubated for 1 min at room temperature and then centrifuged as before. The flowthrough, containing purified DNA, was retained.

DNA concentration and purity of each sample were measured on a NanoDrop spectrophotometer ND1000 (Thermo Fisher Scientific). Purified DNA samples were stored at -20 ºC until DNA sequencing. DNA sequencing was conducted at the Virginia Bioinformatics Institute.

Cell Fixation and Immunocytochemistry

While in the wells, the coverslips were rinsed twice with 2 mL of DPBS. The final DPBS wash was aspirated and to fix the cells, 3 mL of 3.7 % formaldehyde (Fisher Scientific; catalog # SF98-4) were added to each well. The formaldehyde was aspirated after 10 min of incubation at room temperature and wells were washed three times with 2 mL of DPBS. For antigen retrieval,

the coverslips were placed in a 30 mL beaker and incubated in 15 mL of 10 m*M* citrate buffer, pH 6.0 at 90 º C for 30 min. The citrate was aspirated off and then the cells on the coverslips were permeabilized by adding 2 mL of 0.1 % Triton- X-100 in DPBS for 5 min at room temperature. The coverslips were washed two times with DPBS and then blocked with CAS Block for 30 min at room temperature. Coverslips were then incubated with DCP primary antibody (rabbit IgG 1:50; Santa Cruz; catalog number 22753) overnight in a humidified container at 4 º C. Negative control coverslips were run concurrently; instead of primary antibody CAS Block was added to the coverslip (**Figure 8**). The coverslips were washed with DPBS four times for 3 min each. Coverslips were incubated with a goat anti rabbit 594 secondary antibody (1:200; Invitrogen; catalog number A11012) for 1 h at room temperature in the dark. Coverslips were then washed four times with DPBS for 2 min each. After the final wash, one drop of SlowFade Gold antifade mounting medium (Invitrogen Corp; catalog # S36939), containing the nuclear counterstain DAPI, was added to positively charged glass microscope slides and individual coverslips were flipped onto the slides for permanent mounting.

Digital Image Capture and Image Interpretation

Photomicrographs were taken within 24 h of finishing the staining procedures. For each section, at least six images were taken at each objective. Images were acquired using a Nuance FX multispectral imaging system (Perkin Elmer, Inc., Waltham, MA) mounted on a Nikon Eclipse 800 epi-fluorescence microscope that was fitted with Plan Fluor 20x and 40x objectives. Excitation light was generated using a mercury lamp light source and standard filter cubes fitted with long pass emission filters. The Nuance system was configured to use multiple customized emission filter sets (filter cube combinations).

A single observer looked at all composite images and documented DCP staining patterns in relation to nuclear features for each image.

Statistical Analysis

All statistical analyses were carried out using the mixed procedure of SAS 9.3 (Cary, N.C.). Results are expressed as lsmeans ± standard error of the mean. Differences were considered statistically significant at $P \leq 0.05$. The experiment was carried out in duplicate cell culture wells and repeated three times. The experimental unit was cell culture well. Fixed model effect was cell type; the residual was the sole random effect.

RESULTS

Both *DCP* and relevant *DCN* pathway genes are transcribed in BME and MF-T2 grown

under basal conditions (**Figure 4**). MF-T2 expressed more *DCP* than BME when both cell types were grown under basal conditions (**Figure 7**). In addition, MF-T2 also produced more *TGFB1* than BME. Relative abundance of *EGFR, IGF1R, INSR, HGF, HGFR,* and *TGFB1R* did not differ by cell type when cells were grown under basal conditions (**Figure 7**). In this experiment, *IGF1* was undetectable in BME and MF-T2. All PCR products were visualized on an agarose gel and were found to be single bands of the anticipated size (**Figure 5**; **Figure 6**).

While gene expression data for MF-T2 are discussed, no protein-level data are reported or discussed. This is because the MF-T2 auto-senesced during routine culturing and eventually died. Additional low-passage stocks of MF-T2 were not available.

Using ICC, DCP was detected in BME grown in DMEM treatment media for 16 h. With this approach, only intracellular DCP can be visualized. Decorin core protein appeared to be located in the perinuclear area of the cytoplasm. It was also evident that DCP was not constitutively expressed in cultured BME (**Figure 8**). Roughly 50 % of the BME within a given microscopic field appeared to contain DCP, whereas the other 50 % appeared not to have intracellular DCP despite all cells being treated identically to that point. Where DCP was most abundant, the BME appeared to have just gone through mitosis or were preparing for mitosis, based on the orientation, shape and size of nuclei (**Figure 8**).

DISCUSSION

The overall objective was to characterize relative gene transcript abundance of known DCN pathway molecules and DCP abundance in two immortalized bovine mammary cell lines (BME or MF-T2) grown under basal conditions. *DCP* and *TGFB1* were both expressed in greater abundance in MF-T2 than BME when both cell types were grown under basal conditions (**Figure 7**). Relative abundance of *EGFR, IGF1R, INSR, HGF, HGFR,* and *TGFB1R* did not differ by cell type when MF-T2 and BME were grown under basal conditions (**Figure 7**).

DCP

When grown under basal conditions on plastic, MF-T2 may simply have constitutively more *DCP* and the mature proteoglycan than BME; this could contribute to the fibroblast phenotype. A similar, but not identical, situation is known to occur in epidermal cells; when trying to identify a cell type cytokeratin 18 is a specific protein marker of epidermal cells and is not observed in fibroblasts (Hu et al., 2009). While our gene data are supportive of increased *DCP* potentially representing a fibroblast phenotype, we were not able to compare DCP abundance between MF-T2 and BME at the protein level as we had planned due to MF-T2 cell

death. Nonetheless, using ICC we observed DCP in the perinuclear area of BME grown on glass coverslips and incubated with basal media for 16 h – showing that transcription and translation of DCP occurs in BME (**Figure 8**). This finding alone is important because it shows that DCN exists in bovine mammary cells and may be important for bovine mammary biology.

TGFB1

Our study is the only one we are aware of that isolated *TGFB1* gene expression responses of BME and MF-T2 in an identical cell culture environment. When cultured under basal conditions (no FBS), MF-T2 produced relatively more *TGFB1* than BME. Zarzyska and colleagues (2005) cultured BME and MAC-T and found that FBS deficiency (10% to 2% or 0.5% FBS; said to mimic the naturally occurring decline in the access of bioactive compounds and nutrients at the end of lactation and dry period) was associated with increased TGFB1 expression at the level of transcript and protein. Zarzyska and colleagues (2005) did not use fibroblasts in their experiments so it is difficult to compare our findings to theirs; both groups do however agree that BME produce *TGFB1* when cultured under basal conditions.

Efforts to recapitulate biology from cell culture experiments are difficult. However, extrapolating our findings back to the level of the cow at dry-off (the start of mammary involution), it is possible that an increased relative amount of *TGFB1* in fibroblasts compared to epithelial cells may represent the starting point for a differential response to the cellular environment. We suggest that in an environment of defined nutrient availability that fibroblasts and epithelial cells must compete for, such as that present for both cell types at dry-off, TGFB1 may represent an autocrine-paracrine regulatory point. It is possible that transcribed TGFB1, once it reaches protein form, could act in an autocrine manner on fibroblasts to have a net proliferative effect. Alternatively, it is also possible that the transcribed TGFB1 once it reaches protein form acts in a paracrine manner on neighboring MEC to blunt their proliferation. In these scenarios, fibroblasts would outcompete the epithelial cells for the fixed nutrient resources. This could be the basis of mammary tissue turnover during the dry period. In support of this, biologically active TGFB1 (25 kDa homodimer) can stimulate or inhibit cell growth, depending upon the target cells and other growth factors present in the local environment (Plaut, 1993). As reviewed in Plaut (1997), biologically active TGFB1 has a short (2.7 min) half-life and must act near its site of production. The biologically active form of TGFB1 is a recognized inhibitor of epithelial cell proliferation and regulator of bovine mammary involution (Knabbe et al., 1987;

Kolek et al., 2003). When added to cell cultures of BME, TGFB1 treatment resulted in a decreased number of cells in the S and G2/M phases of the cell cycle and a corresponding increase in the number of cells in the G1 phase of the cell cycle (Kolek et al., 2003). Whereas the biologically active form of TGFB1 is anti-proliferative for mammary epithelial cells (Knabbe et al., 1987; Kolek et al., 2003), it is proliferative for stromal cells (Woodward et al., 1995; Musters et al., 2004). Musters and others (2004) implanted either a single slow-release TGFB1 impregnated plastic pellet (5 μg of TGFB1) or a placebo into mammary glands of 9-mo-old prepubertal heifers. Mammary stromal cell proliferation was increased in TGFB1 treated glands and mammary epithelial cell proliferation and apoptosis were not affected (Musters et al., 2004).

In further demonstration of the role of TGFB1 in bovine mammary glands, Plath and others (1997) excised glandular mammary tissue from Brown Swiss dairy cattle in the following physiological phases of development: mammogenesis during pregnancy, lactogenesis, galactopoesis, and involution. Total RNA was extracted from mammary tissues and relative abundance of *TGFB1* was assayed in RT-qPCR experiments (Plath et al., 1997). *TGFB1* concentrations were highest in involution, the most quiescent phase tested (Plath et al., 1997). However, due to the way the mammary tissue samples were processed, it cannot be said which mammary cell type (epithelial cells or fibroblasts) were responsible for the increased production of *TGFB1* during involution.

Transforming growth factor beta 1 is able to influence the development of the mammary gland by altering the composition of the ECM, which can change the communication that occurs between mammary fibroblast and MEC (Sporn et al., 1986; Massague, 1990; Roberts et al., 1990). Transforming growth factor beta 1 is known to positively regulate expression of ECM proteins such as fibronectin, collagen IV, and laminin as determined with human MEC (Stampfer et al., 1993). Localized release of TGFB1 from a pellet implanted in the stromal compartment induced intense collagen I gene expression (Silberstein et al., 1990). TGFB1, through signaling pathways, is known to increase RNA abundance of the ECM component fibronectin, which results in increased fibronectin biosynthesis (Ignotz and Massague, 1986; Dean et al., 1988). TGFB1 also shifts the type of integrins found on cells (upregulation of α_2 and a downregulation of α_3), which alters the type ECM components that can bind to the cells (Heino and Massague, 1989; Santala and Heino, 1991). Because these items are main components of the bovine mammary gland ECM, an increase in cellular production of TGFB1 may represent a key

regulatory starting point for allowing mammary fibroblasts to begin composing the ECM (Larson, 1985; Keely et al., 1995; De Vries et al., 2010). Work by Bassols and Massague (1988) is supportive of this general concept because they showed that TGFB1 added to cell culture media of NRF-49F, MV1LU, and W138 (rat kidney fibroblast, mink lung epithelial, and human lung epithelial cell types, respectively) caused the cells to increase production of *DCP*. However, it should be noted that TGFB1 added to cultured primary human skin fibroblast cells resulted in decreased DCN and *DCP* (Kahari et al., 1995). An important follow-up experiment for bovine mammary biologists would be to study the effects of increasing concentrations of TGFB1 in basal media in primary cell culture. Based on our work, gene and protein abundance of DCP in bovine mammary fibroblasts and bovine mammary epithelial cells would be the principal focus. Gene expression could be analyzed by collecting cell lysates, harvesting their total RNA, and using them in RT-qPCR experiments. Analysis of the abundance of DCP will likely require collection and concentration of conditioned cell culture media, enzymatic removal of the single GAG chain attached to DCP, and western blotting with a DCP-specific antibody (Yamagata et al., 1968; Braunewell et al., 1995; Li and Velleman, 2009).

DCP and TGFB1

In our experiment, we showed that *DCN* and *TGBF1* gene production were both increased in MF-T2 compared to BME. Although our experiment does not provide direct proof, mature decorin proteoglycan in the ECM of quiescent mammary fibroblasts might bind and sequester active TGFB1 protein away from its receptors as suggested in **Figure 4**. The result of this would support proliferation of mammary fibroblasts. Quiescent MEC, on the other hand, would not need to make and secrete DCN into the ECM for the specific purpose of sequestering TGFB1 because, for them, it would be fruitful if the TGFB1 bound to and activated their surface TGFB1R because in that case, receptor activation would be anti-proliferative, as shown in **Figure 4**.

A better experiment than the one we performed would have included measurement of TGFB1 pathway genes, phosphorylated and non-phosphorylated forms of SMAD-2 and SMAD-3 as well as the proliferation marker c-Myc to better understand the effects of a quiescent cell environment on DCN pathway genes, especially those related to TGFB1, in these two mammary cell types.

TGFB1R

One curiosity of our findings is that despite increased *TGFB1* in MF-T2, there was no

corresponding up- or down-regulation of *TGFB1R*, the receptor for *TGFB1,* in MF-T2 or BME. A few explanations are presented. First, despite observed effects at the mRNA level, these may have not carried through to the level of TGFB1 translation into protein. This is a common drawback in most RT-qPCR experiments. Second, at least 3 types of receptors for TGFB1 exist (Cheifetz et al., 1987; Massague et al., 1992a). We only measured relative abundance of the most common form present in bovine mammary tissue; perhaps mRNA for the other receptor subtypes was affected. This was beyond the scope of our work. Third, even if TGFB1 protein was translated and secreted into the extracellular space, there is evidence that TGFB1 is commonly secreted from cells in latent form, complexed to a 75 kDa glycoprotein called latent TGFB (**L-TGFB**); the 100 kDa complex is unable to bind to TGFB1R (Wakefield et al., 1987). The ECM protease plasmin is known to liberate TGFB1 from L-TGFB (Massague et al., 1992b). Our experimental design did not measure TGFB1 complexes or presence of plasmin in conditioned cell culture media so we cannot say if TGFB1 was present or if present, what form it was present in. Because we cannot provide information on this, we are likewise unable to report on whether or not TGFB1 bound to and activated TGFB1R in either cell type. All we can report is that BME and MF-T2 grown under basal conditions did not exhibit up- or down-regulation of *TGFB1R* despite a noted increase in *TGFB1* in MF-T2 compared to BME. Our findings leave open-the-door that TGFB1 protein produced by mammary fibroblasts in response to exposure to a quiescent environment is not destined to activate autocrine TGFB1 signaling, but perhaps is destined for paracrine signaling with nearby mammary epithelial cells, where the end result would be anti-proliferative. More research is needed to address this potentiality.

IGF1 and IGF1R Expression

IGF1 is the natural ligand for IGF1R. Decorin can also serve as a ligand for IGF1R pathway activation (**Figure 4**; Iozzo et al., 2011) Signaling through both pathways yields a proliferative response (McGrath et al., 1991; Ellis et al., 2000; Berry et al., 2001; Iozzo et al., 2011). In addition to *DCP* and *TGFB1* (discussed above), we wished to assess relative abundance of *IGF1* and *IGF1R* in two immortalized bovine mammary cell lines (BME or MF-T2) grown under basal conditions. Both of these genes are reported in the bovine mammary literature (Pfaffl et al., 2002; Boutinaud et al., 2004; Daniels et al., 2009). Historically, while known to be present in both mammary parenchyma and mammary fat pad, relative abundance of *IGF1* is dependent on the stage of development with the highest amount being found during

mammogenesis and involution (Sinowatz et al., 2000). Despite known involvement in bovine mammary biology, in this experiment, *IGF1* was undetectable in BME and MF-T2. To verify that the observed responses were not artificial, *IGF1* primers were tested against positive control liver tissue and these samples amplified as expected (data not shown). Therefore, the interpretation of our findings is that, for both mammary cell types, cell culture conditions did not elicit local production of *IGF1* or result in net cell proliferation. This finding makes biological sense.

Whereas *IGF1* was undetectable in both cell types, *IGF1R* was detectable (**Figure 7**). However, the relative abundance of *IGF1R* did not differ between treatments. This is at odds with the in vivo literature regarding *IGF1R*, which reports mammary parenchyma having more *IGF1R* than mammary fat pad when equal amounts of total RNA are used for analysis (Daniels et al., 2009). Our cell culture experiment used more homogenously sourced total RNA (immortalized mammary cell lines) than did Daniels and others (2009), who used homogenized tissue. This might be the reason for a lack of cell type response for *IGF1R*. As with the *IGF1* ligand data though, our *IGF1R* findings make biological sense and the amount of *IGF1R* observed is likely reflective of basal metabolism in BME and MF-T2. In our experiment, the increased *DCP* produced by MF-T2 (compared to BME) did not lead to coordinated receptor up- or down-regulation of *IGF1R* in MF-T2. Under basal cell conditions, one might expect *IGF1R* receptor down-regulation but not up-regulation, as the latter would generally be supportive of cell proliferation. When considered in this light, our observation of no effect on relative abundance of *IGF1R* receptor is plausible. Perhaps with more time in culture we would have observed *IGFR1R* receptor down-regulation.

HGF and HGFR Expression

Hepatocyte growth factor is a natural ligand for HGFR, a receptor found on epithelial cells (Ma et al., 2003). DCN and DCP are high affinity ligands for HGFR with K_d of 1.5 – 2.2 n*M* (Goldoni et al., 2009). When DCN binds to HGFR under serum starved cell culture conditions, Tyr1356 becomes phosphorylated and leads to the recruitment of growth factor receptor-bound protein 2, which can lead to the internalization and degradation of HGFR or shedding of the ectodomain of HGFR (**Figure 4**). DCN is also able to decrease β-catenin, which also induces HGFR internalization (Goldoni et al., 2009). HGFR internalization and degradation leads to cell growth arrest, an increase in caspase 3/7 activity, and eventually to cell death

(**Figure 4**). Indirect evidence from our experiment does not appear to support the notion that HGFR internalization, from binding either HGF or DCN (**Figure 4**) occurred at different rates in BME and MF-T2 (**Figure 7**) because there was no overall loss of BME or MF-T2 during the experimental period. If our work is to be repeated, it is advised that researchers include a longer incubation period than the one used here to better study cell growth arrest and death.

Another inferred possibility from our data is that, of available DCN binding partners, the binding affinity of HGFR for DCN might be low in BME and MF-T2 grown under basal cell culture conditions. If true, this would mean that under basal conditions, HGFR pathway activation through DCN binding is not likely the main action of DCN (**Figure 4**). We recognize that this idea is currently only speculative. A more thorough investigation of this might have included measurement of β-catenin levels to better understand if DCN did indeed lower the levels of β-catenin, which would lead to internalization of HGFR.

EGF and EGFR

Epidermal growth factor receptor consists of an extracellular domain, a hydrophobic transmembrane domain, and intracellular catalytic tyrosine kinase domain, and several intracellular tyrosine residues which become phosphorylated to couple with downstream effectors (Foley et al., 2010). Decorin does not contain a region that is homologous to EGFR ligand EGF; however, DCN is able to bind EGFR within its leucine rich region (Santra et al., 2002). Decorin is able to dimerize EGFR and cause downstream changes within the cell. Decorin acts in a prolonged and rapid activation of the MAP kinase pathway, which leads to long term activation of p21 that will halt the cells in G_1 (**Figure 4**; Moscatello 1998; Iozzo et al., 1999). As stated previously, DCN is also able to downregulate protein levels of EGFR without influencing the mRNA levels of EGFR; therefore, the lack of significance found in this work can make biological sense (Csordas et al., 2000). However, the absence of protein data makes us unable to compare our results to that of Csordas and colleagues (2000). In the future, it would be prudent to have protein data for each receptor and ligand.

Decorin Core Protein Immunocytochemistry

We observed that where DCP was most abundant, the BME appeared to have just gone through mitosis or were preparing for mitosis, based on the orientation, shape and size of nuclei (**Figure 8**). Based on this observation a literature search was conducted to evaluate if there were any known instances of ECM molecules affecting or being affected by the cell cycle while inside

the cell. While there are many accounts of ECM molecules working to affect the cell cycle from the outside, especially as adhesion points of contact for the cells (Zhao et al., 1998; Charnley et al., 2013; Gerard and Goldbeter, 2014), there was no mention of ECM molecules impacting (or alternatively being affected by) the cell cycle while inside the cell. In sum and to our great interest, observations suggested an association between intracellular DCP abundance and times within the cell cycle.

Conclusions

The existence of DCN pathway molecules was demonstrated in bovine mammary cells. However, the experiments stopped short of demonstrating mature DCN proteoglycan being deposited into the ECM. These experiments were meant to be initial characterizations that can be used for designing future mechanistic cell culture studies. For instance, gene knock-down experiments involving DCN signaling in bovine mammary cells similar to Zafiropoulos and colleagues (2008), who investigated DCN role on MG-63 (human osteosarcoma cells) cell growth and motility by silencing DCN.

Going forward it will be important for us to show two things with respect to DCN in bovine mammary biology. The first is that we should show that not only is the *DCP* gene transcribed in bovine mammary cells but that it is also translated and processed into a mature proteoglycan that can be released from a given cell and deposited in its ECM. Secondly, it will be important for us to show that within our cell culture systems, abundance of the *DCP* gene, DCP, or both, is dynamic and responsive to various stimuli in the cell culture media. The implications of DCN in the bovine mammary gland are still far from known based on this body of work. In the future, decorin-signaling pathways may be manipulated in vivo in efforts to increase milk production efficiency via increased epithelial cell proliferation or decreased apoptosis during growth and involution.

WORKS CITED

Akers, R. M. 2002. Lactation and the mammary gland. 1st ed. Iowa State University Press, Ames, Iowa.

Akers, R. M. 2000. Selection for milk production from a lactation biology viewpoint. Journal of dairy science. 83:1151-1158.

Alberts, B. 2002. Molecular biology of the cell. 4th ed. Garland Science, New York.

Bassols, A. and J. Massague. 1988. Transforming growth factor beta regulates the expression and structure of extracellular matrix chondroitin/dermatan sulfate proteoglycans. J. Biol. Chem. 263:3039-3045.

Berry, S. D., T. B. McFadden, R. E. Pearson, and R. M. Akers. 2001. A local increase in the mammary IGF-1: IGFBP-3 ratio mediates the mammogenic effects of estrogen and growth hormone. Domest Anim Endocrinol. 21:39-53.

Boutinaud, M., J. H. Shand, M. A. Park, K. Phillips, J. Beattie, D. J. Flint, and G. J. Allan. 2004. A quantitative RT-PCR study of the mRNA expression profile of the IGF axis during mammary gland development. J. Mol. Endocrinol. 33:195-207.

Braunewell, K. H., R. Martini, R. LeBaron, H. Kresse, A. Faissner, B. Schmitz, and M. Schachner. 1995. Up-regulation of a chondroitin sulphate epitope during regeneration of mouse sciatic nerve: evidence that the immunoreactive molecules are related to the chondroitin sulphate proteoglycans decorin and versican. Eur. J. Neurosci. 7:792-804.

Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele, and C. T. Wittwer. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55:611-622.

Charnley, M., F. Anderegg, R. Holtackers, M. Textor, and P. Meraldi. 2013. Effect of Cell Shape and Dimensionality on Spindle Orientation and Mitotic Timing. PLoS One. 8:e66918.

Cheifetz, S., J. A. Weatherbee, M. L. Tsang, J. K. Anderson, J. E. Mole, R. Lucas, and J. Massague. 1987. The transforming growth factor-beta system, a complex pattern of cross-reactive ligands and receptors. Cell. 48:409-415.

Csordas, G., M. Santra, C. C. Reed, I. Eichstetter, D. J. McQuillan, D. Gross, M. A. Nugent, G. Hajnoczky, and R. V. Iozzo. 2000. Sustained down-regulation of the epidermal growth factor receptor by decorin. A mechanism for controlling tumor growth in vivo. J. Biol. Chem. 275:32879-32887.

Daniels, K. M., A. V. Capuco, M. L. McGilliard, R. E. James, and R. M. Akers. 2009. Effects of milk replacer formulation on measures of mammary growth and composition in Holstein heifers. J Dairy Sci. 92:5937-5950.

Daniels, K. M., M. L. McGilliard, M. J. Meyer, M. E. Van Amburgh, A. V. Capuco, and R. M. Akers. 2009. Effects of body weight and nutrition on histological mammary development in Holstein heifers. J Dairy Sci. 92:499-505.

Dean, D. C., R. F. Newby, and S. Bourgeois. 1988. Regulation of fibronectin biosynthesis by dexamethasone, transforming growth factor beta, and cAMP in human cell lines. J Cell Biol. 106:2159-2170.

De Vries, L. D., H. Dover, T. Casey, M. J. VandeHaar, and K. Plaut. 2010. Characterization of mammary stromal remodeling during the dry period. J Dairy Sci. 93:2433-2443.

Ellis, S., S. Purup, K. Sejrsen, and R. M. Akers. 2000. Growth and morphogenesis of epithelial cell organoids from peripheral and medial mammary parenchyma of prepubertal heifers. J Dairy Sci. 83:952-961.

Gerard, C. and A. Goldbeter. 2014. The balance between cell cycle arrest and cell proliferation: control by the extracellular matrix and by contact inhibition. Interface Focus. 4:20130075.

Goldoni, S., A. Humphries, A. Nystrom, S. Sattar, R. T. Owens, D. J. McQuillan, K. Ireton, and R. V. Iozzo. 2009. Decorin is a novel antagonistic ligand of the Met receptor. J Cell Biol. 185:743-754.

Heino, J. and J. Massague. 1989. Transforming growth factor-beta switches the pattern of integrins expressed in MG-63 human osteosarcoma cells and causes a selective loss of cell adhesion to laminin. J. Biol. Chem. 264:21806-21811.

Hu, H., J. Wang, D. Bu, H. Wei, L. Zhou, F. Li, and J. J. Loor. 2009. In vitro culture and characterization of a mammary epithelial cell line from Chinese Holstein dairy cow. PLoS One. 4:e7636.

Ignotz, R. A. and J. Massague. 1986. Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J. Biol. Chem. 261:4337-4345.

Iozzo, R. V. 1998. Matrix proteoglycans: from molecular design to cellular function. Annu Rev Biochem. 67:609-652.

Iozzo, R. V., S. Buraschi, M. Genua, S. Q. Xu, C. C. Solomides, S. C. Peiper, L. G. Gomella, R. C. Owens, and A. Morrione. 2011. Decorin antagonizes IGF receptor I (IGF-IR) function by interfering with IGF-IR activity and attenuating downstream signaling. J. Biol. Chem. 286:34712-34721.

Iozzo, R. V., D. K. Moscatello, D. J. McQuillan, and I. Eichstetter. 1999. Decorin is a biological ligand for the epidermal growth factor receptor. J. Biol. Chem. 274:4489-4492.

Iozzo, R. V. and L. Schaefer. 2015. Proteoglycan form and function: A comprehensive nomenclature of proteoglycans. Matrix Biol. 42:11-55.

Kahari, V. M., L. Hakkinen, J. Westermarck, and H. Larjava. 1995. Differential regulation of decorin and biglycan gene expression by dexamethasone and retinoic acid in cultured human skin fibroblasts. J. Invest. Dermatol. 104:503-508.

Keely, P. J., J. E. Wu, and S. A. Santoro. 1995. The spatial and temporal expression of the alpha 2 beta 1 integrin and its ligands, collagen I, collagen IV, and laminin, suggest important roles in mouse mammary morphogenesis. Differentiation. 59:1-13.

Knabbe, C., M. E. Lippman, L. M. Wakefield, K. C. Flanders, A. Kasid, R. Derynck, and R. B. Dickson. 1987. Evidence that transforming growth factor-beta is a hormonally regulated negative growth factor in human breast cancer cells. Cell. 48:417-428.

Kolek, O., B. Gajkowska, M. M. Godlewski, and T. Motyl. 2003. Antiproliferative and apoptotic effect of TGF-beta 1 in bovine mammary epithelial BME-UV1 cells. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 134:417-430.

Larson, B. L. 1985. Lactation. Iowa State University Press, Ames, IA.

Li, X. and S. G. Velleman. 2009. Effect of transforming growth factor-beta1 on decorin expression and muscle morphology during chicken embryonic and posthatch growth and development. Poult. Sci. 88:387-397.

Ma, P. C., G. Maulik, J. Christensen, and R. Salgia. 2003. c-Met: structure, functions and potential for therapeutic inhibition. Cancer Metastasis Rev. 22:309-325.

Massague, J. 1990. The transforming growth factor-beta family. Annu. Rev. Cell Biol. 6:597-641.

Massague, J., J. Andres, L. Attisano, S. Cheifetz, F. Lopez-Casillas, M. Ohtsuki, and J. L. Wrana. 1992. TGF-beta receptors. Mol. Reprod. Dev. 32:99-104.

Massague, J., S. Cheifetz, M. Laiho, D. A. Ralph, F. M. Weis, and A. Zentella. 1992. Transforming growth factor-beta. Cancer Surv. 12:81-103.

Mauviel, A., M. Santra, Y. Q. Chen, J. Uitto, and R. V. Iozzo. 1995. Transcriptional regulation of decorin gene expression. Induction by quiescence and repression by tumor necrosis factor-alpha. J. Biol. Chem. 270:11692-11700.

McGrath, M. F., R. J. Collier, D. R. Clemmons, W. H. Busby, C. A. Sweeny, and G. G. Krivi. 1991. The direct in vitro effect of insulin-like growth factors (IGFs) on normal bovine mammary cell proliferation and production of IGF binding proteins. Endocrinology. 129:671-678.

Moscatello, D. K., M. Santra, D. M. Mann, D. J. McQuillan, A. J. Wong, and R. V. Iozzo. 1998.

Decorin suppresses tumor cell growth by activating the epidermal growth factor receptor. J. Clin. Invest. 101:406-412.

Moses, J., A. Oldberg, E. Eklund, and L. A. Fransson. 1997. Biosynthesis of the proteoglycan decorin -- identification of intermediates in galactosaminoglycan assembly. Eur. J. Biochem. 248:767-774.

Musters, S., K. Coughlan, T. McFadden, R. Maple, T. Mulvey, and K. Plaut. 2004. Exogenous TGF-beta1 promotes stromal development in the heifer mammary gland. J Dairy Sci. 87:896-904.

O'Diam, K. M., S. G. Velleman, V. A Swank, S. Ellis, A. V. Capuco, and K. M. Daniels. 2012. First demonstration of decorin an extracellular matrix molecule in bovine mammary tissue. Proc. of 2012 JAM. Phoenix, AZ.

Pfaffl, M. W., T. M. Georgieva, I. P. Georgiev, E. Ontsouka, M. Hageleit, and J. W. Blum. 2002. Real-time RT-PCR quantification of insulin-like growth factor (IGF)-1, IGF-1 receptor, IGF-2, IGF-2 receptor, insulin receptor, growth hormone receptor, IGF-binding proteins 1, 2 and 3 in the bovine species. Domest Anim Endocrinol. 22:91-102.

Piantoni, P., M. Bionaz, D. E. Graugnard, K. M. Daniels, R. M. Akers, and J. J. Loor. 2008. Gene expression ratio stability evaluation in prepubertal bovine mammary tissue from calves fed different milk replacers reveals novel internal controls for quantitative polymerase chain reaction. J. Nutr. 138:1158-1164.

Plath, A., R. Einspanier, F. Peters, F. Sinowatz, and D. Schams. 1997. Expression of transforming growth factors alpha and beta-1 messenger RNA in the bovine mammary gland during different stages of development and lactation. J. Endocrinol. 155:501-511.

Plaut, K. 1993. Role of epidermal growth factor and transforming growth factors in mammary development and lactation. J Dairy Sci. 76:1526-1538.

Roberts, A. B., K. C. Flanders, U. I. Heine, S. Jakowlew, P. Kondaiah, S. J. Kim, and M. B. Sporn. 1990. Transforming growth factor-beta: multifunctional regulator of differentiation and development. Philos. Trans. R. Soc. Lond. B Biol. Sci. 327:145-154.

Rozario, T. and D. W. DeSimone. 2010. The extracellular matrix in development and morphogenesis: a dynamic view. Dev. Biol. 341:126-140.

Santala, P. and J. Heino. 1991. Regulation of integrin-type cell adhesion receptors by cytokines. J. Biol. Chem. 266:23505-23509.

Santra, M., C. C. Reed, and R. V. Iozzo. 2002. Decorin binds to a narrow region of the epidermal growth factor (EGF) receptor, partially overlapping but distinct from the EGF-binding epitope. J. Biol. Chem. 277:35671-35681.

Scott, J. E. 1995. Extracellular matrix, supramolecular organisation and shape. J. Anat. 187 (Pt 2):259-269.

Sejrsen, K. 1994. Relationships between nutrition, puberty and mammary development in cattle. Proc. Nutr. Soc. 53:103-111.

Silberstein, G. B., P. Strickland, S. Coleman, and C. W. Daniel. 1990. Epithelium-dependent extracellular matrix synthesis in transforming growth factor-beta 1-growth-inhibited mouse mammary gland. J Cell Biol. 110:2209-2219.

Sinowatz, F., D. Schams, A. Plath, and S. Kolle. 2000. Expression and localization of growth factors during mammary gland development. Adv. Exp. Med. Biol. 480:19-25.

Sjogren, K., J. L. Liu, K. Blad, S. Skrtic, O. Vidal, V. Wallenius, D. LeRoith, J. Tornell, O. G. Isaksson, J. O. Jansson, and C. Ohlsson. 1999. Liver-derived insulin-like growth factor I (IGF-I) is the principal source of IGF-I in blood but is not required for postnatal body growth in mice. Proc Natl Acad Sci U S A. 96:7088-7092.

Sporn, M. B., A. B. Roberts, L. M. Wakefield, and R. K. Assoian. 1986. Transforming growth factor-beta: biological function and chemical structure. Science. 233:532-534.

Stampfer, M. R., P. Yaswen, M. Alhadeff, and J. Hosoda. 1993. TGF beta induction of extracellular matrix associated proteins in normal and transformed human mammary epithelial cells in culture is independent of growth effects. J. Cell. Physiol. 155:210-221.

Swett, W. W. and C. A. Matthews. 1934. Dairy Cow's Udder Studied to Establish Development Standards. Yearbook of Agriculture. 175-181.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3:RESEARCH0034.

Varshney, N., A. K. Mohanty, S. Kumar, J. K. Kaushik, A. K. Dang, M. Mukesh, B. P. Mishra, R. Kataria, S. P. Kimothi, T. K. Mukhopadhyay, D. Malakar, B. S. Prakash, S. Grover, and V. K. Batish. 2012. Selection of suitable reference genes for quantitative gene expression studies in milk somatic cells of lactating cows (Bos indicus). J Dairy Sci. 95:2935-2945.

Velayudhan, B. T., K. M. Daniels, D. P. Horrell, S. R. Hill, M. L. McGilliard, B. A. Corl, H. Jiang, and R. M. Akers. 2008. Developmental histology, segmental expression, and nutritional regulation of somatotropic axis genes in small intestine of preweaned dairy heifers. J Dairy Sci. 91:3343-3352.

Wakefield, L. M., D. M. Smith, T. Masui, C. C. Harris, and M. B. Sporn. 1987. Distribution and modulation of the cellular receptor for transforming growth factor-beta. J Cell Biol. 105:965-975.

Woodward, T. L., N. Dumont, M. O'Connor-McCourt, J. D. Turner, and A. Philip. 1995. Characterization of transforming growth factor-beta growth regulatory effects and receptors on bovine mammary cells. J. Cell. Physiol. 165:339-348.

Yamagata, T., H. Saito, O. Habuchi, and S. Suzuki. 1968. Purification and properties of bacterial chondroitinases and chondrosulfatases. J. Biol. Chem. 243:1523-1535.

Yamaguchi, Y., D. M. Mann, and E. Ruoslahti. 1990. Negative regulation of transforming growth factor-beta by the proteoglycan decorin. Nature. 346:281-284.

Zafiropoulos, A., D. Nikitovic, P. Katonis, A. Tsatsakis, N. K. Karamanos, and G. N. Tzanakakis. 2008. Decorin-induced growth inhibition is overcome through protracted expression and activation of epidermal growth factor receptors in osteosarcoma cells. Mol. Cancer Res. 6:785-794.

Zarzynska, J., M. Gajewska, and T. Motyl. 2005. Effects of hormones and growth factors on TGF-beta1 expression in bovine mammary epithelial cells. J. Dairy Res. 72:39-48.

Zavizion, B., M. van Duffelen, W. Schaeffer, and I. Politis. 1996. Establishment and characterization of a bovine mammary epithelial cell line with unique properties. In Vitro Cell. Dev. Biol. Anim. 32:138-148.

Zhao, J. H., H. Reiske, and J. L. Guan. 1998. Regulation of the cell cycle by focal adhesion kinase. J Cell Biol. 143:1997-2008.

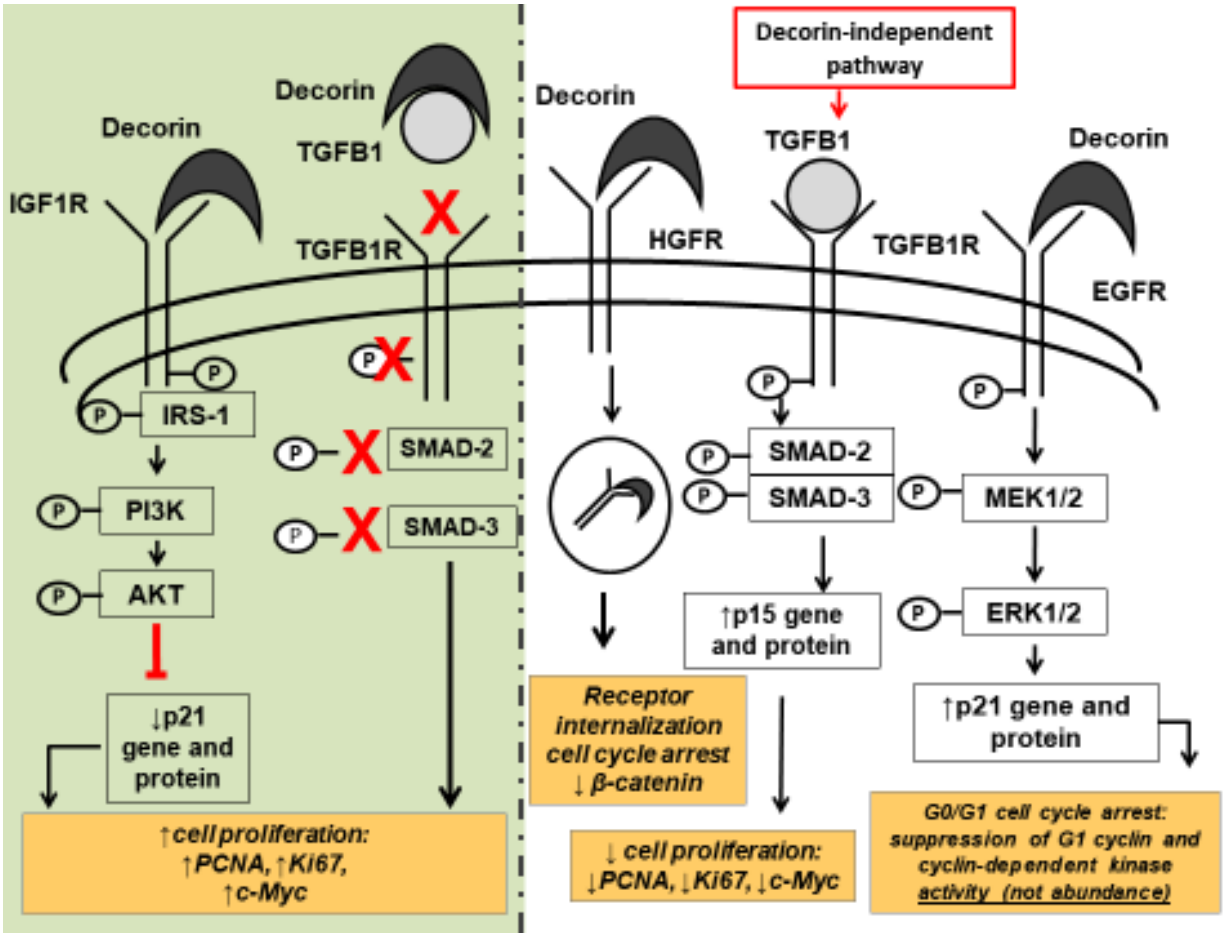


Figure 4: Three known decorin signaling pathways, and one related decorin independent pathway with resulting downstream cellular effects are shown. Decorin can increase cell proliferation by signaling through the insulin-like growth factor receptor (IGF1R) pathway or by sequestering transforming growth factor beta 1 (TGFB1) away from the transforming growth factor beta 1 receptor (TGFB1R) and preventing downstream effects. Decorin can decrease cell proliferation by a decorin independent pathway in which TGFB1 binds and activates TGFB1R leading to an increased abundance of p15. Decorin can induce internalization of hepatocyte growth factor receptor (HGFR), which will induce cell cycle arrest and decrease β-catenin. Lastly, decorin can decrease cell proliferation by signaling through the epidermal growth factor receptor (EGFR) pathway. Decorin is a member of the small leucine-rich proteoglycans that has an N-terminal glycosaminoglycan chain. The concave portion of decorin is what interacts with the different receptor ligands.

Table 2: Primer gene name used for RT-qPCR, forward and reverse sequence, annealing temperature, product size, GenBank Accession Number

Gene Name[1]	Sequences (5'-3') Forward/Reverse	Annealing Temp (°C)	Product Size (bp)	GenBank Accession Number
DCP	ACC ATC CCT CAA GGT CTT CC / CAT TGT CAA CCG CAG AGA TG	60.4	148	XM_005206039.1
EGF	GCT CTC CCT CTG GAA ATG GG / GGA ACG AAC AGG TAT CAG AAG GT	62.0	144	AY192564.1
EGFR	ACG ACT GAA GTT CTG GCA GG / TGA AAA ACA GTG CAA GGC CG	59.0	109	XR_814374.1
IGF1	GTT GGT GGA TGC TCT CCA GT / CTC CAG CCT CCT CAG ATC AC	62.4	148	NM_001077828
IGF1R	GTG ACG GGC TAC GTG AAG AT / GTT CCC CTC TAG CTG CTC CT	62.3	102	NM_001244612
INSR	GTT TTC ATC CCC AGG CCT TC / GCT CGA TGC GAT AGC CAG TA	64.5	211	AY574999.1
HGF	ACA GCT TTT TGC CTT CGA GCT ATC GGG GTA AAG ACC TAC AGG / CAT CAA AGC CCT TGT CGG GAT A	62.0	292	NM_001012999.2
HGFR	GCG TTG ACT TAT TCA CGG GC / TCG AGA AAC CAC AAC CTG CAT	60.4	131	AB057406.1
MTG1	CTT GGA ATC CGA GGA GCC A / CCT GGG ATC ACC AGA GCT GT	62.3	101	XM_010819872.1
PPP1R11	CCA TCA AAC TTC GGA AAC GG / ACA GCA GCA TTT TGA TGA GCG	60.4	101	XP_005223660.1
RPS15A	GAA TGG TGC GCA TGA ATG TC / GAC TTT GGA GCA CGG CCT AA	60.4	101	NM_001024541.2
TGFB1	GGA CCT GGG CTG GAA GTG / GGC CAG GAC CTT GCT GTA CT	64.5	112	NM_001166068.1
TGFB1R	ATG TGA AGA TGG GCA AGT CC / TCA GCT CTG GTT GGT GTC AG	60.4	132	XM_005210201.2

[1] *CSN2* = beta-casein, *DCP* = decorin core protein, *EGF* = epidermal growth factor, *EGFR* = epidermal growth factor receptor, *IGF1* = insulin-like growth factor 1, *IGF1R* = insulin-like growth factor 1 Receptor, *INSR* = insulin receptor, *HGFR* = hepatocyte growth factor receptor, *LALBA* = alpha-lactalbumin, *MTG1* = mitochondrial ribosome associated GTPase, *PPP1R11* = protein phosphate 1 regulatory inhibitor subunit 11, *RPS15A* = ribosomal protein S15A, *TGFB1* = transforming growth factor beta 1, *TGFB1R* = transforming growth factor beta 1 receptor.

Table 3: Efficiency table of genes used for RT-qPCR with different cell types

Gene[1]	Cell Type[2]	Media[3]	Slope[4]	R^{2} [5]	Efficiency Percentage[6]
DCN	BME	FBS	-3.75	0.94	85
EGFR	BME	FBS	-2.02	0.99	213
IGF1	BME	FBS	.	.	.
IGF1R	BME	FBS	-3.3	0.95	101
INSR	BME	FBS	-2.9	0.99	121
HGFR	BME	FBS	-3.18	0.99	106
MTG1	BME	FBS	-3.2	0.99	105
PPP1R11	BME	FBS	-4.02	0.99	77
RPS15A	BME	FBS	-4.1	0.92	75
TGFB1	BME	FBS	-2.72	0.99	133
TGFB1R	BME	FBS	-2.93	0.99	119
DCN	MF-T2	FBS	-3.77	0.99	84
EGFR	MF-T2	FBS	-3.34	0.99	99
IGF1	MF-T2	FBS	.	.	.
IGF1R	MF-T2	FBS	-2.56	0.99	146
INSR	MF-T2	FBS	-3.97	0.96	79
HGF	MF-T2	FBS	-3.05	0.97	113
HGFR	MF-T2	FBS	-3.33	0.99	100
MTG1	MF-T2	FBS	-2.92	0.99	120
PPP1R11	MF-T2	FBS	-3.5	0.95	93
RPS15A	MF-T2	FBS	-3.87	0.98	81
TGFB1	MF-T2	FBS	-2.76	0.99	130
TGFB1R	MF-T2	FBS	-3.46	0.99	95

[1] *CSN2* = beta-casein, *DCN* = decorin, *EGF* = epidermal growth factor, *EGFR* = epidermal growth factor receptor, *IGF1* = insulin-like growth factor 1, *IGF1R* = insulin-like growth factor 1 Receptor, *INSR* = insulin receptor, *HGFR* = hepatocyte growth factor receptor, *LALBA* = alpha-lactalbumin, *MTG1* = mitochondrial ribosome associated GTPase, *PPP1R11* = protein phosphate 1 regulatory inhibitor subunit 11, *RPS15A* = ribosomal protein S15A, *TGFB1* = transforming growth factor beta 1, *TGFB1R* = transforming growth factor beta 1 receptor.

[2] Cell types used for the efficiency immortalized bovine mammary epithelial cells (BME) and bovine mammary fibroblast cells (MF-T2). The liver samples were from tissue of 8 wk old Holstein calves.

[3] Media type that the cells were collected in; fetal bovine serum (10 % FBS plus Dulbecco's modified Eagle's medium [DMEM], DMEM, or stimulatory media (STIM, DMEM plus Hydrocortisone 1 µg/mL, Retinoic Acid 1 µg/mL [R2625], Insulin 5 µg/mL [I4011], Prolactin 1 IU/mL[L6520]).

[4] Slope of the standard curve; dilutions were 1:1, 1:10, 1:100, and 1:1000.

[5] R^2 = coefficient of determination of the standard curve.

[6] Efficiency is calculated as $[(10^{(-1/slope)}-1)*100]$ (Bustin et al., 2009).

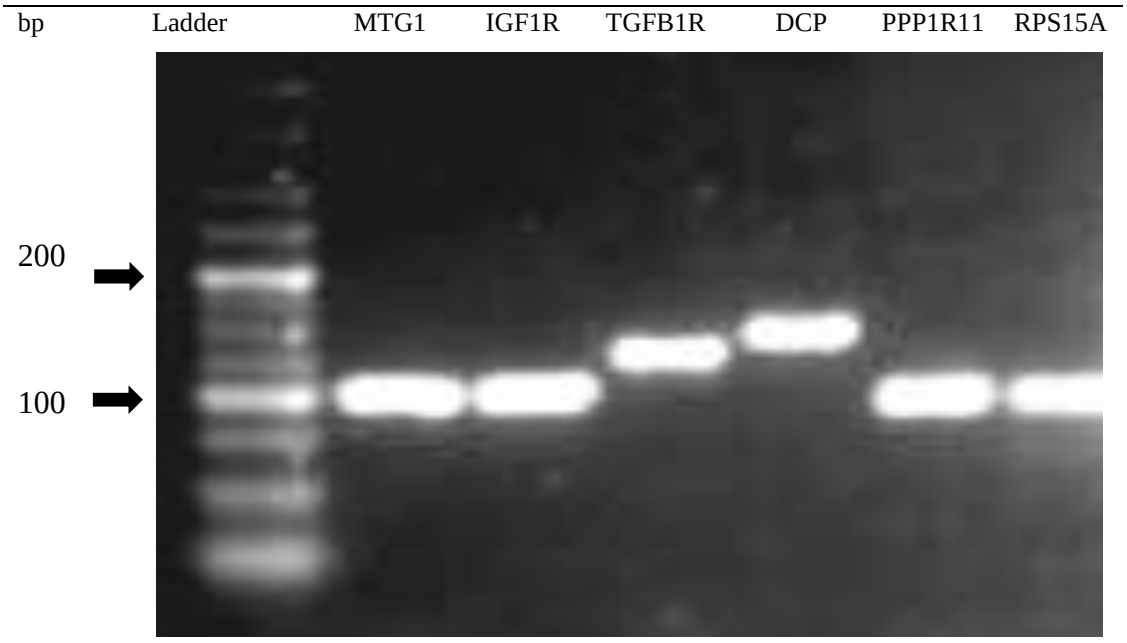


Figure 5: Verification of cell culture RT-qPCR products for *DCP* = decorin core protein, *IGF1R* = insulin-like growth factor 1 receptor, *MTG1* = mitochondrial ribosome associated GTPase 1, *PPP1R11* = protein phosphate 1 regulatory inhibitor subunit 11, *RPS15A* = ribosomal protein S15A, *TGFB1R* = transforming growth factor beta 1 receptor using a 3.5 % agarose gel.

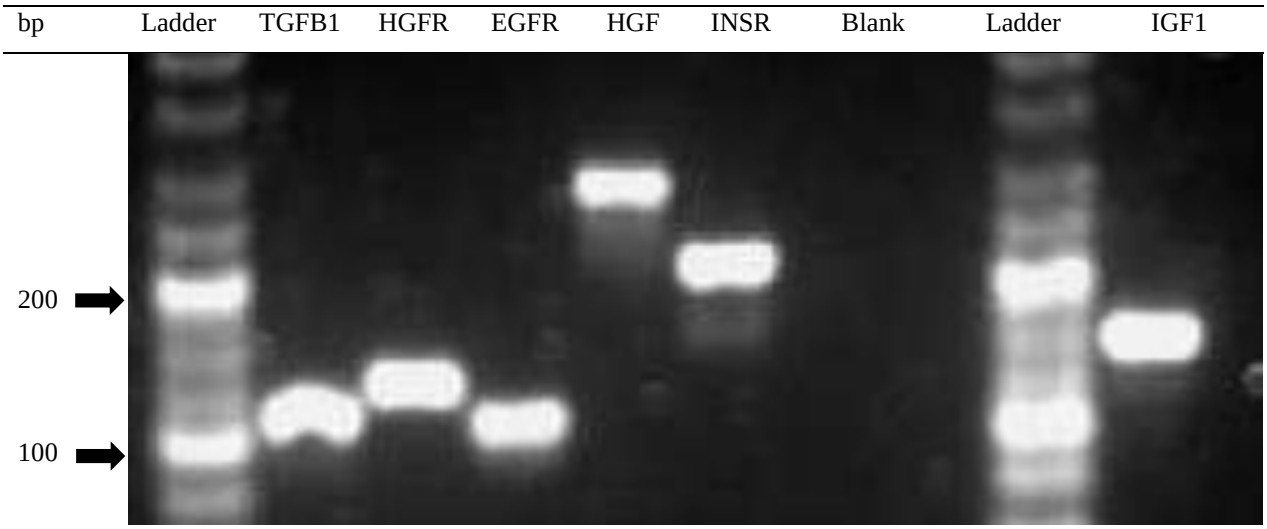


Figure 6: Verification of cell culture RT-qPCR products for *EGFR* = epidermal growth factor receptor, *IGF1* = insulin-like growth factor 1, *INSR* = insulin receptor, *HGF* = hepatocyte growth factor, *HGFR* = hepatocyte growth factor receptor, *TGFB1* = transforming growth factor beta 1 using a 3.5 % agarose gel.

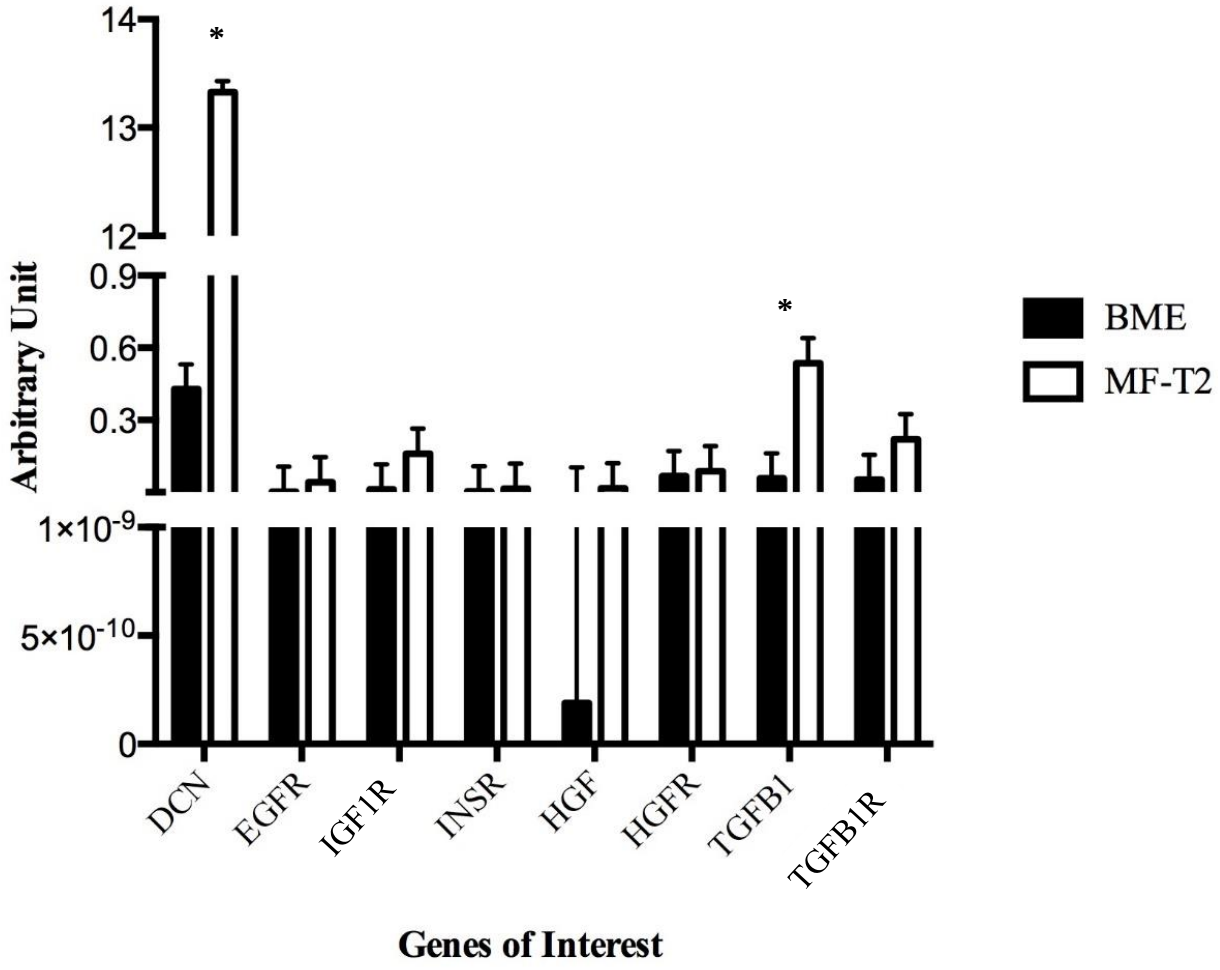


Figure 7: Comparison of bovine mammary epithelial cells (BME, black) versus bovine mammary fibroblast cells (MF-T2, white) mRNA levels of decorin and genes known to interact with *decorin* (*DCP*): *epidermal growth factor receptor* (*EGFR*), *insulin-like growth factor 1 receptor* (*IGF1R*), *insulin receptor (INSR), hepatocyte growth factor* (*HGF*), and *hepatocyte growth factor receptor* (*HGFR*), *transforming growth factor-beta 1* (*TGFB1),and transforming growth factor beta 1 receptor* (*TGFB1R*). *IGF1* was undetectable in BME and MF-T2. The arbitrary unit is the relative abundance of the genes of interest divided by the total DNA per a well. Each bar represents n = 6 for each cell type. * $P < 0.05$.

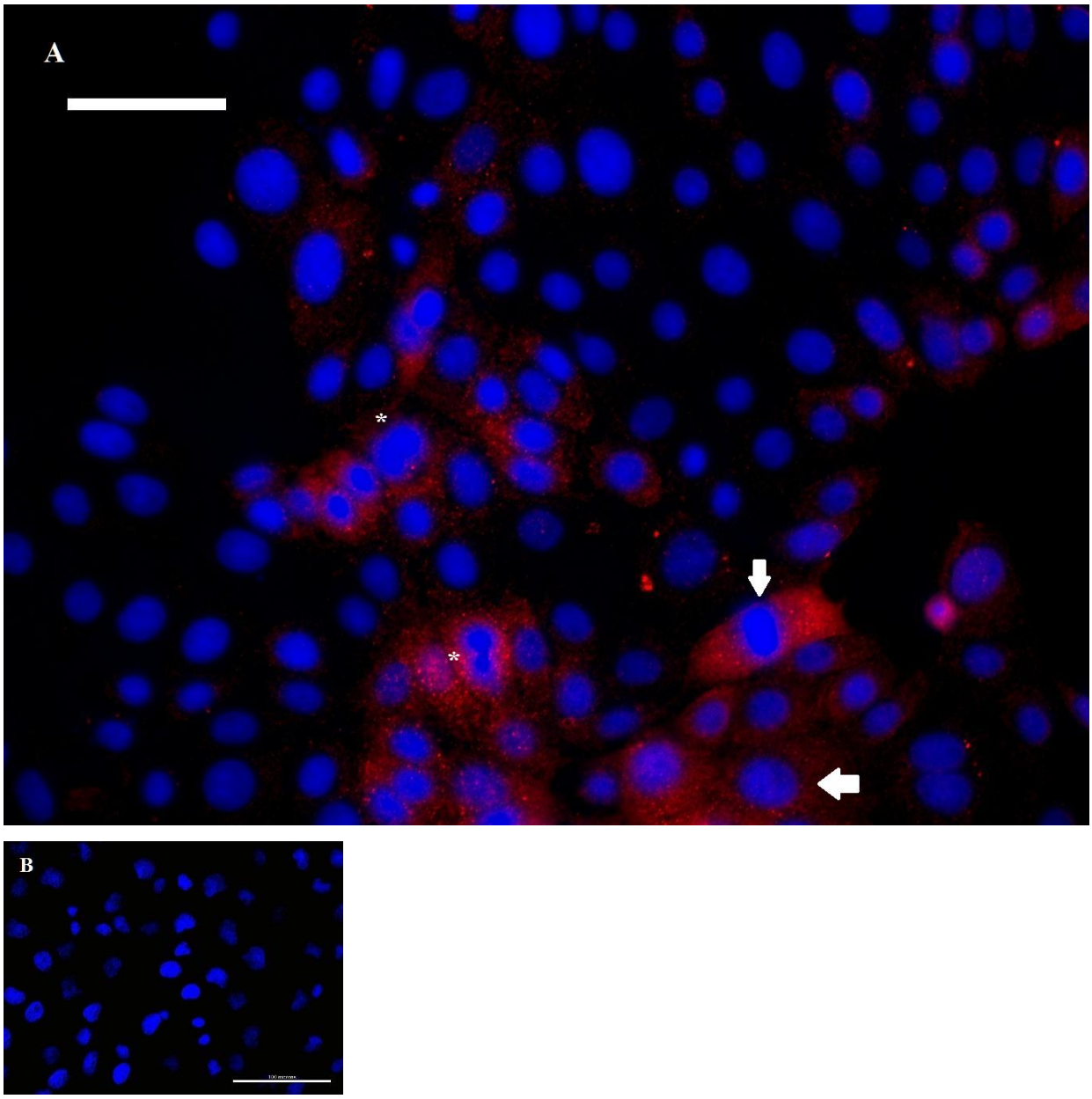


Figure 8: Fluorescent immunocytochemistry of bovine mammary epithelial cells (BME). A) Decorin (1:50; Rabbit polycloncal 22753) = red and DNA = blue. Images obtained at 40x objective lens magnification; scale bar = 50µm. * indicate cells that appear to be dividing. The arrows indicate enlarged nuclei. B) Negative control component images of bovine mammary epithelial cells on coverslips incubated with CAS Block instead of the decorin core protein primary antibody. Scale bars represent 100 µm.

CHAPTER 3: INVESTIGATION OF DECORIN CORE PROTEIN GENE ABUNDANCE AND PROTEIN LOCALIZATION AT TWO PHASES OF THE CELL CYCLE IN BOVINE MAMMARY EPITHELIAL CELLS

ABSTRACT

The initial hypothesis was that intracellular decorin core protein (**DCP**) accumulates in bovine mammary epithelial cells (**BME**) during the S-phase of the cell cycle. To investigate if DCP was accumulating during the S-phase, BME were cultured for 16 h in Dulbecco's Modified Eagle Media either in the absence or presence of 10% fetal bovine serum (**DMEM** or 10% **FBS** in DMEM, respectively), with 5-bromo-2′-deoxyuridine (**BrdU**; a specific marker for S-phase) added for the final 2 h of culture. Dual-label immunocytochemistry (**ICC**) was used to concurrently detect BrdU and DCP in BME. Bovine mammary epithelial cells incubated with FBS had a greater number of BrdU+/DCP- cells compared to BME incubated with DMEM (P = 0.03). Media type did not affect percent of BrdU+/DCP+ cells (1.05 ± 1.09 for DMEM and 5.95 ± 1.03 for FBS, respectively). Media type did not affect percent of BrdU-/DCP+ cells (14.41 ± 3.70 for DMEM and 11.15 ± 3.51 for FBS, respectively). Percentage of BrdU-/DCP- cells was affected by media type; cells grown in DMEM had nearly 20% more BrdU-/DCP- cells than cells grown in FBS. Hypothesis testing allowed for the rejection of the null hypothesis. The hypothesis of the second experiment, formulated after completion of the first experiment, was that DCP accumulates in BME during metaphase of the cell cycle. For the metaphase restriction experiment, 16 h after initial plating a 2 x 2 factorial combination of media (basal or stimulatory) and metaphase restriction agent (20 n*M* paclitaxel [**PAC**] or 0 n*M* PAC) were applied. The basal media consisted of DMEM alone and was coded as **DMEM**. The stimulatory media (**STIM**) consisted of DMEM plus 1 µg/mL hydrocortisone, 1 µg/mL retinoic acid, 5 µg/mL insulin and 1 IU/mL prolactin. The four treatments used were: **DMEM, DMEM plus PAC, STIM,** or **STIM plus PAC.** Representative cell culture wells were harvested at 0, 24, or 48 h of incubation with treatments for DNA, gene expression of DCP, and ICC analysis for cells in metaphase with or without DCP. DNA concentrations were not affected by the two- and three-way fixed model terms. DNA concentration increased over time between 0, 24 and 48 h (P < 0.0001). Irrespective of time and restriction agent, cells grown in FBS had more DNA than cells grown in DMEM (P = 0.003). The restriction agent PAC had no effect on DNA concentration (P = 0.251). Gene expression of DCP was unaffected by fixed model effects. Quantification of cells in the metaphase restriction experiment proved difficult. Upon inspection, cells in metaphase tended to

be split between two different focal planes. This made digital image acquisition of areas containing metaphase-restricted cells and cells not in metaphase difficult. Technical difficulties with PAC, along with imaging difficulties between cells in metaphase and not in metaphase prevented successful performance of the experiment. Therefore, the null hypothesis regarding DCP accumulation during metaphase in BME was not rejected. Additional follow-up work with: improved imaging techniques, incorporation of cell counting (and perhaps analysis of cell ploidy by flow cytometry), and increased concentrations of PAC are warranted.

INTRODUCTION

In bovine mammary glands, sheet-like epithelial structures have the capacity to make and secrete milk. Additionally, mammary epithelial cells make and secrete an extracellular matrix (**ECM**). The ECM is important for regulation of cell shape, proliferation, polarity, differentiation, gene transcription, protein synthesis, and secretion (Scott, 1995; Alberts, 2002; Rozario and DeSimone, 2010). Decorin (**DCN**) is an ECM proteoglycan known to impact mammary cell proliferation in humans and rodents (Ferdous et al., 2009; Iozzo et al., 2011). Decorin is made inside cells and secreted into the ECM. In our previous work with an immortalized bovine mammary epithelial cell line (**BME**; Woodward et al., 1995), we observed that when cultured under basal conditions, intracellular decorin core protein (**DCP**) was not constitutively expressed and its localization patterns appeared to be coordinated with specific phases of the cell cycle (see Chapter 2). Visual inspection gave the impression that intracellular DCP was most abundant in BME that appeared to be enlarging for cell division, undergoing S-phase, or dividing during mitosis, based on the orientation, shape and size of nuclei. To follow-up on previous observations, the objectives of the following research were to characterize DCP localization patterns in BME at known phases of the cell cycle. The work was carried out in two separate experiments.

The hypothesis of the first experiment was that DCP accumulates in BME during S-phase of the cell cycle. The hypothesis of the second experiment, formulated after completion of the first experiment, was that DCP accumulates in BME during metaphase of the cell cycle.

MATERIALS AND METHODS

Cells and Routine Cell Culture Conditions

BME were used for two experiments. The first experiment evaluated the appearance of DCP during the S-phase of the cell cycle. The second experiment analyzed the presence of DCP

during metaphase. In both experiments, BME were grown in six-well plastic dishes with no additional substratum (Fisher Scientific; catalog # 08777233). Initial plating density was 5.0 x 10^5 cells per well. For cytology, at plating one sterilized glass coverslip was inserted at the bottom of the designated wells (described below). Cells were grown in an incubator set at 37 ºC in 5 % CO_2.

All cells were initially cultured in Dulbecco's Modified Eagle Media (**DMEM**) plus 10 % fetal bovine serum (**FBS**). All media were supplemented with 100 IU/mL penicillin, 100 IU/mL streptomycin, and 0.25 µg/mL Gentamicin Sulfate. After a 16 h attachment period, cells were washed with 1 mL Dulbecco's phosphate buffered saline (**DPBS**) and then treatment media were added as described for each experiment.

Cell Culture Conditions for S-Phase Experiment

Sixteen h after initial plating, DMEM was aspirated and wells were washed with 1 mL DPBS. Cell culture wells then received treatment media of either 2 mL of DMEM (n = 4 wells) or 2 mL of DMEM and 10 % FBS (n = 4 wells). To do this, 14 h after application of treatment media, 20 µ*M* per a well of 5-Bromo-2′-deoxyuridine (**BrdU**; Alfa Aesar; Haverhill, MA; catalog # H2760-06) were applied to each well. BrdU is a thymidine analog that incorporates into the DNA of dividing cells exclusively during the S-phase of the cell cycle (Kee et al., 2002; Taupin, 2007). Cells were harvested 2 h after BrdU application (total of 16 h after treatment media application). BrdU powder was mixed into a stock solution of 0.9 % sterile saline at 20 mg/mL (65 m*M*). **Figure 9** depicts the entirety of the S-phase experiment from initial plating of BME to harvest. A minimum of 6 six-well plastic dishes were used for the BrdU experiment. Cells harvested in the S-phase experiment were used for BrdU immunocytochemistry (described below).

Cell Culture Conditions for Metaphase Restriction Experiment

There are currently no known antibodies that cross-react with bovine cells that singularly denote metaphase. However, microtubules (hollow tubes that are composed of tubulin dimers) undergo fluctuations in appearance such as alignment and extension during various phases of the cell cycle. Chromosomal DNA also undergoes characteristic changes in visual appearance during phases of the cell cycle. During metaphase, condensed chromosomes centrally align in the middle of mitotic spindles, which are composed of microtubules (Cooper, 2000). Presence of centrally aligned condensed chromosomal DNA along with alpha-tubulin, the main component of microtubules, can be used to identify cells in metaphase. This method was used in the

metaphase restriction experiment, as further described below.

Paclitaxel (**PAC;** trade name Taxol) stabilizes microtubules by decreasing their ability to change in length, which prevents the progression of cells from metaphase into anaphase (Yvon et al., 1999; Choi and Yoo, 2012; Hu et al., 2014).

For the metaphase restriction experiment, 16 h after initial plating DMEM was aspirated and wells were washed with 1 mL DPBS. Treatment medias were arranged in a 2 x 2 factorial combination of media (basal or stimulatory) and metaphase restriction agent (20 n*M* paclitaxel or 0 n*M* paclitaxel). The basal media consisted of DMEM alone and was coded as **DMEM**. The stimulatory media consisted of DMEM plus hydrocortisone 1 µg/mL [Sigma-Aldrich Co. LLC.; catalog # H4001], retinoic acid 1 µg/mL [Sigma-Aldrich Co. LLC.; catalog # R2625], insulin 5 µg/mL [Sigma-Aldrich Co. LLC.; catalog # I4011], and prolactin 1 IU/mL[Sigma-Aldrich Co. LLC.; catalog #L6520]); stimulatory media is coded as **STIM**. Stock solutions were made prior to incorporation into STIM media (hydrocortisone 1 mg/2 mL of 100 % ethanol; retinoic acid 5 mg/mL in dimethyl sulfoxide; insulin 1,000 µg/mL in ddH_2O (pH 2.0); and prolactin 6 IU/mL in 0.005 *M* NaOH). The working concentration of 20 n*M* PAC was chosen for its ability to block progression to anaphase in BME grown in DMEM (data not shown). The four treatments used were therefore: **DMEM, DMEM plus PAC, STIM,** or **STIM plus PAC.**

Individual cell culture wells were harvested after either 0, 24, or 48 h of treatment media incubation. Media was not changed during the experimental period. A schematic of the entire experiment from initial plating of BME to harvest is found in **Figure 10**. Cells harvested in the metaphase restriction experiment were used for real time quantitative PCR (**RT-qPCR**), DNA analysis (described below), and immunocytochemistry (**ICC**; described below). The experiment was carried out in duplicate wells and repeated at least two times (minimum of 4 wells for each media and time point combination for RT-qPCR).

RNA, DNA, and Protein Isolation from BME

Where indicated, total RNA, DNA, and total protein were sequentially extracted using the reagents from the RNA/DNA/Protein Purification Kit (Norgen Biotek Corp.; Ontario, Canada; catalog # 24000). The manufacturer's protocol for cells growing in a monolayer was used, as summarized next. To wash the cells, the media was aspirated and then the cells were washed with 1 mL DPBS. To lyse the cells, 350 µL of Buffer Sk were added to the plate and were manually swirled for 5 min. The lysate was then transferred to a new microcentrifuge tube,

200 µL of 100 % ethanol were then added, and the tube was vortexed for 10 s.

To extract the total RNA (for RT-qPCR), a spin column was assembled and 600 µL of the lysate plus ethanol was added to the column. All centrifugation for this kit was conducted at room temperature. The column was centrifuged for 1 min at 4,000 x *g* (Eppendorf Centrifuge 5430R) and the flowthrough was retained on ice for further use (see protein extraction below). The column was then reassembled with a new collection tube, 400 µL of Wash Solution A were added, and the column was centrifuged for 1 min at 12,000 x *g*. The flowthrough was discarded. This wash step was repeated. The column was then reassembled and underwent a drying step by centrifuging for 2 min at 12,000 x *g*. A new microcentrifuge tube was added to the column and 50 µL of Elution A Solution were added to the column. To elute the total RNA, the column was centrifuged for 1 min at 14,000 x *g*.

DNA (assayed to approximate cell number) was sequentially extracted from the same column. For this, the column was assembled with a new collection tube and 500 µL of Wash Solution El were added. The column was centrifuged for 2 min at 12,000 x *g* and the flowthrough was discarded. The column was then reassembled with a new collection tube and dried by centrifugation for 2 min at 12,000 x *g*. The column was then placed into a new microcentrifuge tube and 100 µL of Elution F Buffer were added. The column was first centrifuged for 2 min at 200 x *g* then, without removal from the centrifuge, centrifuged for 1 min at 14,000 x *g*.

DNA and RNA purity and quantity were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Rockland, DE). The resulting DNA and total RNA were stored at -80 ºC.

Finally, protein was extracted from the same column using a new collection tube. Three hundred µL of the column flowthrough from the beginning of the RNA extraction were transferred to a new microcentrifuge tube. Another 300 µL of DNase/RNase free water were added to the microcentrifuge tube containing the flowthrough. Then 24 µL of Binding Buffer A were added to the microcentrifuge tube and the contents were mixed by inversion. Next, 650 µL of the flowthrough plus water plus Binding Buffer A solution were added to the column. The column was centrifuged for 2 min at 5,200 x *g*. The flowthrough was then discarded and the column reassembled. The column received 500 µL of Wash Solution C and was centrifuged for 2 min at 5,200 x *g*. The flowthrough was discarded and the column reassembled to be dried via

centrifugation for 1 min at 12,000 x *g*. In a new microcentrifuge tube 9.3 µL of Protein Neutralizer were added and the column was assembled with the new microcentrifuge tube with Protein Neutralizer. Then 100 µL of Elution Buffer C were added to the column and centrifuged for 2 min at 5,200 x *g*. The eluted protein was stored at -80 ºC and further work with this fraction is not discussed.

Quantification of Gene Expression in BME

In the metaphase restriction experiment, real time quantitative PCR (**RT-qPCR**) was used to quantify the expression of *DCP*. The two milk protein genes *beta casein (**CSN2**) and alpha lactalbumin (**LALBA**)* were used as markers of terminally differentiated bovine mammary epithelial cells (Lee et al., 1985; Sigl et al., 2013). *Mitochondrial ribosome associated GTPase* (***MTG1***)*, ribosomal protein S15A* (***RPS15A***)*,* and p*rotein phosphate 1 regulatory inhibitor subunit 11* (***PPP1R11***) were used as endogenous control genes as described below.

Single-stranded cDNA was synthesized from each total RNA sample according to the method of Swank et al. (2013). For each BME sample, 2.0 µg of RNA, (contained in 11.3 µL) were first denatured in DNase/RNase free water for 12 min, at 70°C; samples were then placed on ice for 3 min. Reverse transcription was performed afterward. Reverse transcription master mix (8.7 µL) contained: 4 µL of M-MLV RT 5X buffer (Promega; Madison, WI), 2 µL of 0.1 *M* dithiothreitol, 1 µL 10 m*M* dNTP mix (Promega), 1 µL of oligo(dT)20 primer, 0.5 µL M-MLV reverse transcriptase (Promega), and 0.2 µL of RNase inhibitor (Promega). The 8.7 µL of master mix was added to each well of denatured RNA (11.3 µL) in a 0.2 mL thin wall PCR 8-strip well tube (World Wide Medical Services Inc.; Tampa, FL) for final reaction volume of 20 µL. Reverse transcription was carried out in an Arktik Thermal Cycler (Thermo Scientific; Waltham, MA) with cycle conditions at 40 °C for 1 h, followed by 95 °C for 10 min.

Resultant cDNA was then diluted 1:1 with addition of 20 µL of DNase/RNase free water. The cDNA was vortexed, aliquoted (5 µL each) into strip well tubes, and stored at -20°C until further use. Later, RT-qPCR assays were performed on all samples in duplicate, with each reaction mixture (10 µL) containing 0.25 µL each forward and reverse primer, 4.75 µL of PowerUp SYBR Green Master Mix (Life Technologies, Grand Island, NY), 3.75 µL of RNase/DNase free water, and 1 µL of cDNA (1:1 stock). The RT-qPCR assays were performed using a QuantStudio 6 Flex Real-Time PCR system (Life Technologies) with the following cycling conditions: 95 °C for 3 min (heat activation of polymerase); 45 repeating cycles of: 94

°C for 15 s (denaturation), primer-specific annealing temperature for 30 s (**Table 4**), and 72 °C for 30 s (extension). Each RT-qPCR run culminated with a melt curve analysis with the following settings: 95.0 °C for 2 min, 55.0 °C for 30 s, and then a temperature increase of 0.1 °C/s until 95.0 °C was reached.

The purpose of the melt curve analyses was to verify RT-qPCR product purity (absence of genomic DNA contamination and primer dimers). Confirming absence of genomic DNA contamination was of particular interest here due to the sequential nucleic acid and protein extraction kit that was used, which did not include a genomic DNA digestion step (common in total RNA extraction kits). RT-qPCR product size was verified by electrophoresing representative samples of RT-qPCR products for each primer pair on a 3.5 % agarose gel; dsDNA was labeled with SYBR Safe DNA gel stain (Life Technologies, catalog # S33102) and the gel was visualized on a ChemiDoc XRS (Bio-Rad Laboratories, Inc.; Hercules, CA). All RT-qPCR products were visualized as single bands that corresponded with predicted product size.

Each RT-qPCR assay included no template and no reverse-transcriptase controls. The no template control received 1 μL of RNase/DNase free water instead of cDNA, and the no reverse-transcriptase control received 1 μL of the reverse transcription product to which no reverse-transcriptase was added. Individual samples were repeated when the resulting coefficient of variation for duplicate samples was greater than 5 %.

Target genes of interest were normalized to the geometric mean of *MTG1*, *RPS15A*, and *PPP1R11* (target gene Ct – reference genes Ct = ΔCt) (Vandesompele et al., 2002). Delta Ct data were transformed to $2^{(-\Delta Ct)}$ to represent fold differences relative to the reference genes (Velayudhan et al., 2008).

Primer efficiencies (**Table 5**) were determined for each gene and media type by the use of serial dilutions of cDNA (1:1, 1:10, 1:100, 1:1000), using the following equation: $[(10^{(-1/slope)}-1)*100]$ (Bustin et al., 2009).

Cell Fixation and Immunocytochemistry in BME

For cells grown on glass coverslips in both the S-phase and metaphase restriction experiments, at harvest, the final DPBS wash was aspirated and cells were fixed with 3 mL of 3.7 % formaldehyde per well (Fisher Scientific; catalog # SF98-4). The formaldehyde was aspirated after 10 min of incubation at room temperature and wells were washed three times with 2 mL of DPBS. For antigen retrieval, the coverslips were placed in a 30 mL beaker and

incubated in 15 mL of 10 m*M* citrate buffer, pH 6.0 at 90 º C for 30 min. The citrate was aspirated and then the cells on the coverslips were permeabilized by adding 2 mL of 0.1 % Triton- X-100 in DPBS for 5 min at room temperature. The coverslips were then washed two times with DPBS and blocked with CAS Block for 30 min at room temperature. Coverslips were incubated with primary antibody solutions that varied by experiment (**Table 6**) overnight in a humidified container at 4 º C. Negative control coverslips were included; instead of primary antibody CAS Block was added to the coverslip. After primary antibody incubation, coverslips were washed with DPBS four times for 3 min each. Coverslips were next incubated with a secondary antibody solution that varied by experiment (**Table 6**) for 1 h at room temperature in the dark. Coverslips were then washed four times with DPBS for 2 min each. After the final wash, one drop of SlowFade Gold antifade mounting medium (Invitrogen Corp; catalog # S36939), containing the nuclear counterstain DAPI, was added to positively charged glass microscope slides and individual coverslips were flipped onto the slides for permanent mounting.

Microscopy, Imaging, and Image Interpretation

Digital image capture was the same in both the S-phase and metaphase restriction experiments. Briefly, photomicrographs of immunostained cells attached to glass coverslips were taken within 24 h of coverslipping. For each coverslip, at least six images were taken at 40x and 100x objective lens magnification. Images were acquired using a Nuance FX multispectral imaging system (Perkin Elmer, Inc., Waltham, MA) mounted on a Nikon Eclipse 800 epi-fluorescence microscope that was fitted with Plan Fluor 40x, and 100x objectives. Excitation light was generated using a mercury lamp light source and standard filter cubes fitted with long pass emission filters. The Nuance system was configured to use multiple customized emission filter sets (filter cube combinations).

For quantification of cells in the S-phase experiment, a minimum of 350 cells per treatment media were counted by a single observer and classified into one of four groups: BrdU+/DCP+, BrdU+/DCP-, BrdU-/DCP+, or BrdU-/DCP-.

Quantification of cells in the metaphase restriction experiment proved difficult. Upon inspection, cells in metaphase tended to be split between two different focal planes. This made digital image acquisition of areas containing metaphase-restricted cells and cells not in metaphase difficult; an example image is shown in **Table 9** (white arrow in the first row). Had image acquisition gone as planned, each image would have been analyzed as follows:

- Total number of cells in image;
- Within image, number of cells in metaphase;
    - Within metaphase cells, number of these that are DCP+ and DCP- ;
    - Within metaphase cells, intensity of DCP staining;
- Within image, number of cells in all other phases of the cell cycle;
    - Within non-metaphase cells, number of these that are DCP+ and DCP- ;
    - Within non-metaphase cells, intensity of DCP staining.

In light of the technical hindrance in imaging, general impressions of the cells (both those in metaphase and not in metaphase) within a given microscopic field were documented at the microscope in real-time. This approach is similar to that of a pathologist. Briefly, a single observer blind to treatment scanned each microscope slide at 40x magnification. Areas for examination were selected randomly by blurring the fine focus and manually moving the microscope stage. After moving to a new area of interest, the observer used the fine-focus knob to move through the varied focal planes and recorded observations in a laboratory notebook. Observations included things like: "number of metaphase cells is (equal-to, greater than, or less-than) number of non-metaphase cells"; "number of DCP+ cells in metaphase is (low or high)" "intensity of DCP in metaphase cells is (low or high)". The same options were repeated for non-metaphase cells. This process was repeated approximately 4 times per microscope slide.

Statistical Analysis

Statistical analyses were carried out using the mixed procedures of SAS 9.3 (Cary, N.C.). Results are expressed as lsmeans ± standard error of the mean. Differences were considered statistically significant at $P \leq 0.05$.

For the S-phase experiment, differences in mean percentages of labeled BrdU and DCP cells were analyzed within group (BrdU+/DCP+; BrdU+/DCP-; BrdU-/DCP+; or BrdU-/DCP-) using a student's t-test. The model accounted for the effect of media (DMEM or FBS).

For the metaphase restriction experiment, DNA and RT-qPCR data were analyzed with a model that included the fixed effects of: media (DMEM or FBS), restriction agent (presence or absence of PAC), time (0, 24, or 48 h), all two-way interactions, and the three-way interaction. A minimum of four wells per a treatment were used for the RT-qPCR and DNA data. Values used in the RT-qPCR data were $2^{-\Delta Ct}$ values divided by the DNA content of the well from which the RNA was extracted. Where applicable, contrasts statements were used for means separation, they

were: linear contrast for DMEM across time points, quadratic contrast for DMEM across time points, linear contrast for DMEM + PAC across time points, linear contrast for STIM across time points, linear contrast for STIM + PAC across time points, linear contrast for DMEM and STIM without PAC across time points, and linear contrast for DMEM and STIM with PAC across time points. The ICC images compiled in the metaphase restriction experiment were not statistically evaluated.

RESULTS

Intracellular BrdU and Decorin Localization in the S-phase Experiment

Use of a dual staining immunocytochemistry (**ICC**) approach allowed for visualization of both DCP and cells that were in the S-phase of the cell cycle (BrdU positive) in BME cells grown under basal (DMEM) and stimulatory (FBS) conditions. Primary localization of DCP in the BME treated with DMEM and FBS was the peri-nuclear area of BME (**Figure 11**; **Table 7**). The percentage of BME cells in each of the 4 groups shown in **Figure 12** are summarized as follows for DMEM: BrdU+/DCP- 29.07 ± 4.17, BrdU+/DCP+ 1.05 ± 1.09, BrdU-/DCP+ 14.41 ± 3.70, or BrdU-/DCP- 55.44 ± 4.99. For FBS, the profile was: BrdU+/DCP- 43.47 ± 3.95, BrdU+/DCP+ 5.95 ± 1.03, BrdU-/DCP+ 11.15 ± 3.51, or BrdU-/DCP- 39.39 ± 4.74. BME incubated with FBS had a greater number of BrdU+/DCP- cells compared to BME incubated with DMEM ($P = 0.03$; **Figure 12**). Media type did not affect percent of BrdU+/DCP+ cells (1.05 ± 1.09 for DMEM and 5.95 ± 1.03 for FBS, respectively; **Figure 12**). Media type did not affect percent of BrdU-/DCP+ cells (14.41 ± 3.70 for DMEM and 11.15 ± 3.51 for FBS, respectively; **Figure 12**). Percentage of BrdU-/DCP- cells was affected by media type; cells grown in DMEM had nearly 20% more BrdU-/DCP- cells than cells grown in FBS (**Figure 12**).

DNA Content of Cells in the Metaphase Restriction Experiment

DNA concentrations were not affected by the two- and three-way fixed model terms. DNA concentration increased over time between 0, 24 and 48 h ($P = 0.008$; **Figure 13**). Irrespective of time and restriction agent, cells grown in FBS had more DNA than cells grown in DMEM ($P = 0.028$; **Figure 13**). The main effect of time was also significant ($P = 0.008$; **Figure 13**). When contrasts were run there was a linear contrast found to be significant between the sample given STIM + PAC with the amount of DNA content increasing across time ($P = 0.035$; 17.020 ± 5.828 versus 43.225 ± 6.515). The restriction agent PAC had no effect on DNA concentration ($P = 0.906$; **Figure 13**).

Relative Gene Expression of Cells in the Metaphase Restriction Experiment

Relative abundance of *DCN* and *LALBA* were not dependent on any model terms (**Table 8**). Relative abundance of *CSN2* differed based on the main effect of media and the interaction of restriction agent and time ($P = 0.031$; $P = 0.007$; **Table 8**). Pre-planned contrasts for the interaction of restriction agent and time were not significant.

Immunocytochemistry Analysis of Cells in the Metaphase Restriction Experiment

As noted earlier, quantification of cells in the metaphase restriction experiment proved difficult and results were not analyzed statistically. However, after viewing, some general trends were noted. As expected at time 0 h, total number of cells and percentage of cells in metaphase appeared equal across all time 0 h wells.

Compared to the 0 h samples, impressions of the 24 h samples were as follows. More cells were present per microscopic field at 24 h. BME were most abundant when cells were incubated with STIM media (STIM and STIM plus PAC; **Table 9**) and less abundant in BME incubated with basal media (DMEM and DMEM plus PAC; **Table 9**). At 24 h, the addition of PAC to both DMEM and FBS appeared to result in more cells in metaphase compared to other cell-cycle phases (**Table 9**). Differences in number of DCP+ cells, intensity of DCP staining, and preferential localization of DCP (metaphase restricted versus non-metaphase restricted cells) were not appreciable between treatments at 24 h.

Compared to the 0 h and 24 h samples, impression of the 48 h samples were as follows. The images that came out clear were in areas that did not have many cells. However, the images are not indicative of what was observed through the eyepieces of the microscope. More cells were present per microscopic field at 48 h compared to 0 and 24 h time points. Further, at 48 h, it was difficult to assess number of cells in metaphase compared to number of cells not in metaphase. There did appear to be differences in the number of DCP+ cells and intensity of DCP staining between BME undergoing both DMEM (DMEM and DMEM plus PAC) treatments versus both STIM (STIM and STIM plus PAC) treatments. BME treated with DMEM appeared to have more DCP staining at a higher intensity compared to STIM treated BME (**Table 9**).

DISCUSSION

In both of our experiments, primary localization of DCP in the BME treated with DMEM and FBS was the peri-nuclear area of BME (**Figure 9**). This is consistent with knowledge of DCN being post-translationally modified (glycosaminoglycan chain addition) and packaged in the Golgi prior to delivery of the mature DCN proteoglycan to the extracellular space (Moses et

al., 1997).

BME in S-Phase Do Not Preferentially Accumulate DCP

To follow-up on previous observations, the objectives of the following research were to characterize DCP localization patterns in BME at known phases of the cell cycle. The work was carried out in two separate experiments. The hypothesis of the first experiment was that DCP accumulates in BME during S-phase of the cell cycle. In our experiment, supportive findings would manifest as higher proportions of BME positive for DCP and also labelled with BrdU. However, number of DCP+ cells was not proportionally higher in cells in the S-phase of the cell cycle (BrdU+ cells) compared to cells not in S-phase (BrdU- cells) (**Figure 12**). This was true when cells were incubated in DMEM or FBS (**Table 7**). These findings led us to reject our hypothesis and conclude that DCP does not accumulate in BME during S-phase of the cell cycle.

Examination of nuclear features and DCP staining patterns in our first experiment led us to our second hypothesis, which was that DCP accumulates in BME during metaphase of the cell cycle. Our experiment used a metaphase restriction agent, PAC, to preferentially accumulate cells in metaphase.

PAC was not Effective at Restricting Cells to Metaphase

Paclitaxel has been used successfully by other research groups to restrict cultured cells to metaphase when used in 100 and 30 n*M* concentrations in A-498 and ATCC HTB75 cells (epithelial-like human kidney carcinoma cells and epithelial-like human ovarian adenocarcinoma cells), respectively (Yvon et al., 1999). Our initial testing with PAC over a range of concentrations (0 to 120 n*M*) indicated that 20 n*M* of PAC was the minimum effective concentration for restricting BME grown in DMEM to metaphase (data not shown).

The typical cell cycle for BME is 19.5 h long (Zavizion et al., 1998). Our experiment was conducted in such a way that the majority of cells were in the G0-phase of the cell cycle at time point 0 (**Figure 9**). In the G0-phase of the cell cycle, cells are known to be N with regard to ploidy. The G1-phase is also a monoploid phase (N), the S-phase is a diploid phase (2N), as is G-2, and the first part of the M-phase. At the end of mitosis, which results in the formation of 2 new daughter cells, each cell returns to monoploid status (N). Mention of ploidy is relevant for this part of the discussion because it directly relates to DNA content, which we measured by spectrofluorometry. Monoploid bovine cells typically have 6.6 pg of DNA whereas diploid mammalian cells typically have 13.2 pg of DNA. As an example, if all cells within a given cell

culture of BME were to undergo a complete "turn" of the cell cycle in a 24 h period, the number of cells and the DNA content of the well would both be expected to double. The chemical agent PAC blocks the progression of cells from metaphase to anaphase, thereby suspending cells in a 2N ploidy with no increase in cell number.

Given the method of DNA quantification we used, at 24 h of culture, we would not expect to detect differences in DNA content between the four treatments but might expect to observe a difference in cell number. Our DNA data are supportive of this (**Figure 13**); we did not, however, perform a direct cell count at any time point beyond initial plating. In retrospect, this would have been helpful. By 48 h of culture, had PAC been effective, the expected results for DNA content ranked highest to lowest should be: STIM > STIM+PAC > DMEM+PAC = DMEM. Our DNA data showed no difference in DNA content at 48 h, leading us to conclude that the PAC did not work as expected in this experiment. Similarly, by 48 h of culture, had PAC been effective, the expected results for cell number ranked highest to lowest should be: STIM > DMEM > DMEM+PAC = STIM+PAC. Our general observations indicated that cell numbers, while not directly counted, appeared uniformly high across treatments at 48 h providing additional indication that the PAC did not work as expected; this observation is reflected in our DNA data (**Figure 13**). A possible explanation as to why PAC did not work in BME could be the limited initial testing at 24 h in just DMEM (data not shown). Retrospectively, it would have been beneficial to have included more time points and media conditions at the time of initial testing.

Stimulatory Media Increased Cell Numbers as Expected

The hormones added to the STIM treatment media are known to cause proliferation and differentiation in BME (Lee et al., 2013). Regardless of use of restriction agent PAC or time, the DNA data support that STIM increased proliferation amongst the BME ($P = 0.028$; **Figure 13**). In our experiment, increase in cell number appeared to occur in the absence of differentiation, as assessed here by measurement of relative abundance of the two milk protein genes *CSN2* and *LALBA* (**Table 8**). Other groups have reported *CSN2* and *LALBA* increases in bovine mammary epithelial cells as markers of terminal differentiation (Lee et al., 1985; Sigl et al., 2013). Potentially, our experiment did not provide the proper substrate for the mammary epithelial cells to up regulate production of *CSN2* and *LALBA.*

DCP in the Metaphase Restriction Experiment

In further support of PAC not working as expected, no main effect of PAC was noted for

relative gene abundance of *DCP* (**Table 8**). Similarly, media type (DMEM or FBS) did not affect relative abundance of *DCP* in this experiment (**Table 8**). In other cell types, *DCN* accumulation is upregulated during quiescence (Coppock et al., 2000). However, gene data do not always correlate with protein data. As noted in the results, DCP abundance at 48 h was most evident in cells grown in DMEM and DMEM+PAC as opposed to STIM or STIM+PAC (**Table 9**). The visual observation of increased DCP in BME incubated with DMEM would suggest that there is more intracellular DCP during times of quiescence in bovine mammary epithelial cells. It must be reiterated though that ICC data were not analyzed statistically. Had we been able to statistically detect more intracellular DCP in cells grown in DMEM as opposed to FBS, the following interpretation could be put forth: increase in DCP is due to the translation and posttranslational modification of DCP and does not occur at the gene level.

Conclusions

Overall, findings led us to reject our first hypothesis and conclude that DCP does not accumulate in BME during S-phase of the cell cycle. We next wished to test whether or not DCP accumulated in BME during metaphase. Technical difficulties with use of the metaphase restriction agent PAC, along with imaging difficulties between cells in metaphase and not in metaphase prevented us from successfully performing our experiment. Therefore, we are not yet comfortable rejecting or not rejecting our hypothesis about DCP and its association with metaphase in BME. Additional follow-up work with: improved imaging techniques, incorporation of cell counting (and perhaps analysis of cell ploidy by flow cytometry), and increased concentrations of PAC are warranted.

WORKS CITED

Akers, R. M. 2002. Lactation and the mammary gland. 1st ed. Iowa State University Press, Ames, Iowa.

Alberts, B. 2002. Molecular biology of the cell. 4th ed. Garland Science, New York.

Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele, and C. T. Wittwer. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55:611-622.

Choi, Y. H. and Y. H. Yoo. 2012. Taxol-induced growth arrest and apoptosis is associated with the upregulation of the Cdk inhibitor, p21WAF1/CIP1, in human breast cancer cells. Oncol. Rep. 28:2163-2169.

Cooper, G. M. 2000. The Cell: A Molecular Approach. 2nd ed. Sinauer Associates, Sunderland, MA.

Coppock, D., C. Kopman, J. Gudas, and D. A. Cina-Poppe. 2000. Regulation of the quiescence-induced genes: quiescin Q6, decorin, and ribosomal protein S29. Biochem. Biophys. Res. Commun. 269:604-610.

Ferdous, Z., S. B. Peterson, H. Tseng, D. K. Anderson, R. V. Iozzo, and K. J. Grande-Allen. 2010. A role for decorin in controlling proliferation, adhesion, and migration of murine embryonic fibroblasts. J Biomed Mater Res A. 93:419-428.

Hu, P., Q. Huang, Z. Li, X. Wu, Q. Ouyang, J. Chen, and Y. Cao. 2014. Silencing MAP3K1 expression through RNA interference enhances paclitaxel-induced cell cycle arrest in human breast cancer cells. Mol. Biol. Rep. 41:19-24.

Iozzo, R. V., S. Buraschi, M. Genua, S. Q. Xu, C. C. Solomides, S. C. Peiper, L. G. Gomella, R. C. Owens, and A. Morrione. 2011. Decorin antagonizes IGF receptor I (IGF-IR) function by interfering with IGF-IR activity and attenuating downstream signaling. J. Biol. Chem. 286:34712-34721.

Kee, N., S. Sivalingam, R. Boonstra, and J. M. Wojtowicz. 2002. The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. J. Neurosci. Methods. 115:97-105.

Lee, E. Y., W. H. Lee, C. S. Kaetzel, G. Parry, and M. J. Bissell. 1985. Interaction of mouse mammary epithelial cells with collagen substrata: regulation of casein gene expression and secretion. Proc Natl Acad Sci U S A. 82:1419-1423.

Lee, H. Y., Y. T. Heo, S. E. Lee, K. C. Hwang, H. G. Lee, S. H. Choi, and N. H. Kim. 2013. Short communication: retinoic acid plus prolactin to synergistically increase specific casein gene expression in MAC-T cells. J Dairy Sci. 96:3835-3839.

Moses, J., A. Oldberg, E. Eklund, and L. A. Fransson. 1997. Biosynthesis of the proteoglycan decorin -- identification of intermediates in galactosaminoglycan assembly. Eur. J. Biochem. 248:767-774.

Piantoni, P., M. Bionaz, D. E. Graugnard, K. M. Daniels, R. M. Akers, and J. J. Loor. 2008. Gene expression ratio stability evaluation in prepubertal bovine mammary tissue from calves fed different milk replacers reveals novel internal controls for quantitative polymerase chain reaction. J. Nutr. 138:1158-1164.

Rozario, T. and D. W. DeSimone. 2010. The extracellular matrix in development and morphogenesis: a dynamic view. Dev. Biol. 341:126-140.

Santra, M., C. C. Reed, and R. V. Iozzo. 2002. Decorin binds to a narrow region of the epidermal growth factor (EGF) receptor, partially overlapping but distinct from the EGF-binding epitope. J. Biol. Chem. 277:35671-35681.

Scott, J. E. 1995. Extracellular matrix, supramolecular organisation and shape. J. Anat. 187 (Pt 2):259-269.

Sigl, T., K. Gellrich, H. H. Meyer, M. Kaske, and S. Wiedemann. 2013. Multiparous cows categorized by milk protein concentration and energy-corrected milk yield during early lactation--metabolism, productivity and effect of a short-term feed restriction. J. Anim. Physiol. Anim. Nutr. (Berl.). 97:278-296.

Swank, V. A., W. S. Yoho, K. M. O'Diam, M. L. Eastridge, A. J. Niehaus, and K. M. Daniels. 2013. Jersey calf performance in response to high-protein, high-fat liquid feeds with varied fatty acid profiles: blood metabolites and liver gene expression. J Dairy Sci. 96:3845-3856.

Swett, W. W. and C. A. Matthews. 1934. Dairy Cow's Udder Studied to Establish Development Standards. Yearbook of Agriculture. 175-181.

Taupin, P. 2007. BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation. Brain Res Rev. 53:198-214.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3:RESEARCH0034.

Varshney, N., A. K. Mohanty, S. Kumar, J. K. Kaushik, A. K. Dang, M. Mukesh, B. P. Mishra, R. Kataria, S. P. Kimothi, T. K. Mukhopadhyay, D. Malakar, B. S. Prakash, S. Grover, and V. K. Batish. 2012. Selection of suitable reference genes for quantitative gene expression studies in milk somatic cells of lactating cows (Bos indicus). J Dairy Sci. 95:2935-2945.

Velayudhan, B. T., K. M. Daniels, D. P. Horrell, S. R. Hill, M. L. McGilliard, B. A. Corl, H.

Jiang, and R. M. Akers. 2008. Developmental histology, segmental expression, and nutritional regulation of somatotropic axis genes in small intestine of preweaned dairy heifers. J Dairy Sci. 91:3343-3352.

Woodward, T. L., N. Dumont, M. O'Connor-McCourt, J. D. Turner, and A. Philip. 1995. Characterization of transforming growth factor-beta growth regulatory effects and receptors on bovine mammary cells. J. Cell. Physiol. 165:339-348.

Yamaguchi, Y., D. M. Mann, and E. Ruoslahti. 1990. Negative regulation of transforming growth factor-beta by the proteoglycan decorin. Nature. 346:281-284.

Yvon, A. M., P. Wadsworth, and M. A. Jordan. 1999. Taxol suppresses dynamics of individual microtubules in living human tumor cells. Mol Biol Cell. 10:947-959.

Zavizion, B., J. H. White, and A. J. Bramley. 1998. Cell cycle-dependent fluctuation of urokinase-type plasminogen activator, its receptor, and inhibitors in cultured bovine mammary epithelial and myoepithelial cells. Biochim. Biophys. Acta. 1403:141-150.

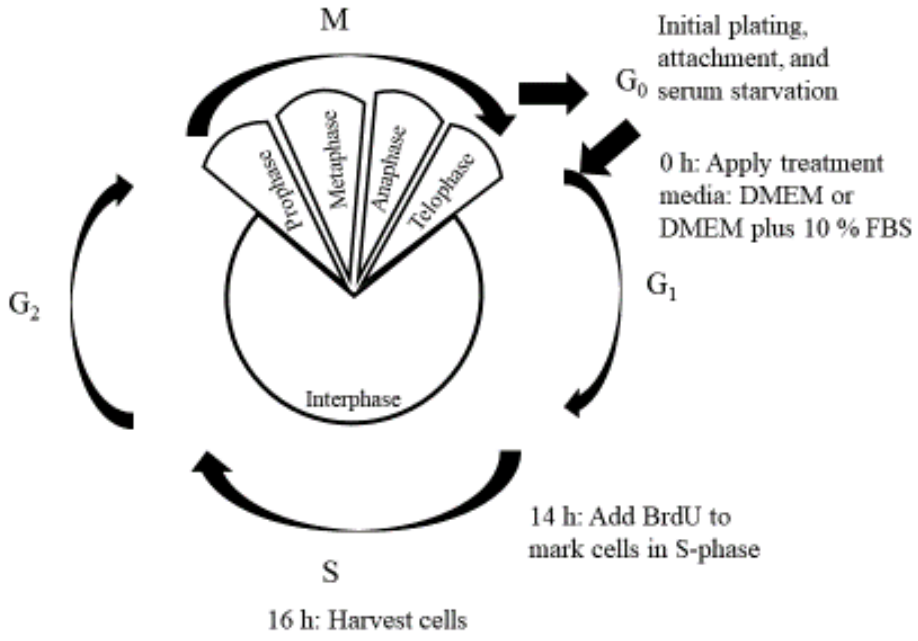


Figure 9: Schematic of cell culture component of the S-phase experiment. The complete cell cycle is represented for individual cells. The timing for the experiment is specific to bovine mammary epithelial cells and showcases that at the time of harvest the majority of cells should have been undergoing S-phase.

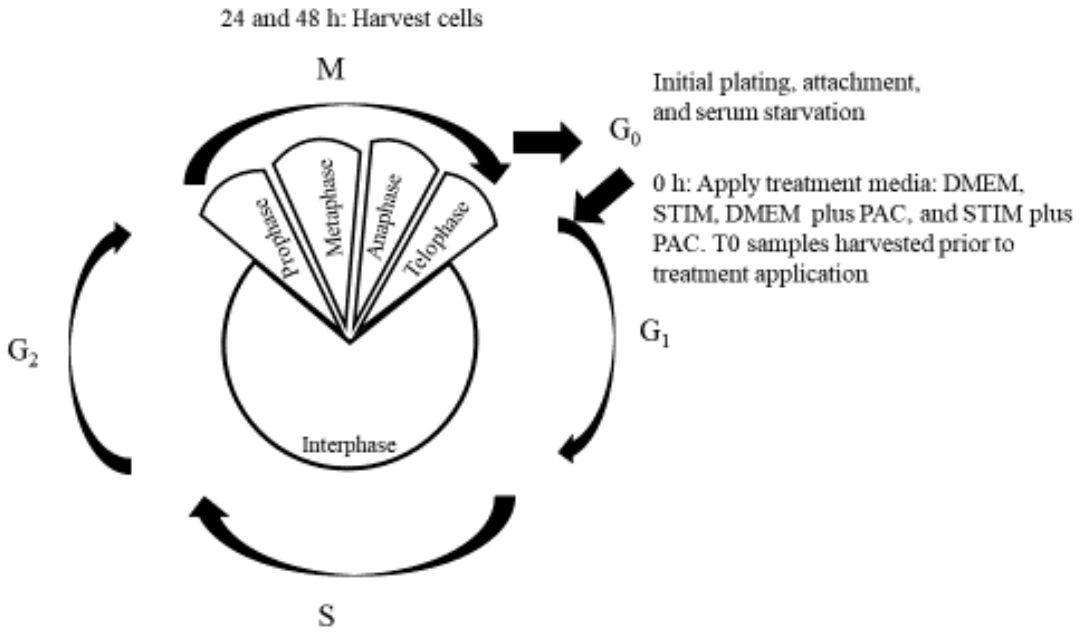


Figure 10: Schematic of cell culture component of the metaphase restriction experiment. The complete cell cycle is represented for individual cells. The timing for the experiment is specific to bovine mammary epithelial cells.

Table 4: Primer gene name used for RT-qPCR during metaphase restriction experiment, forward and reverse sequence, annealing temperature, product size, GenBank Accession Number

Gene Name[1]	Sequences (5'-3') Forward/Reverse	Annealing Temp (°C)	Product Size (bp)	GenBank Accession Number
CSN2	CTC TGC CTC TGC TCC AGT CT / GAT AGG GCA CTG CTT TCT GG	62.4	135	M55158.1
DCN	ACC ATC CCT CAA GGT CTT CC / CAT TGT CAA CCG CAG AGA TG	60.4	148	XM_005206039.1
LALBA	TGG GTC TGT ACC ACG TTT CA / TGA GGG TTC TGG TCG TCT TT	62.4	128	NM_174378.2
MTG1	CTT GGA ATC CGA GGA GCC A / CCT GGG ATC ACC AGA GCT GT	62.3	101	XM_010819872.1
PPP1R11	CCA TCA AAC TTC GGA AAC GG / ACA GCA GCA TTT TGA TGA GCG	60.4	101	XP_005223660.1
RPS15A	GAA TGG TGC GCA TGA ATG TC / GAC TTT GGA GCA CGG CCT AA	60.4	101	NM_001024541.2

[1] CSN2 = beta-casein, DCN = decorin, LALBA = alpha-lactalbumin, MTG1 = mitochondrial ribosome associated GTPase, PPP1R11 = protein phosphate 1 regulatory inhibitor subunit 11, RPS15A = ribosomal protein S15A.

Table 5: Efficiency table for genes used for RT-qPCR during metaphase restriction experiment with different media types

Gene[1]	Tissue[2]	Media[3]	Slope[4]	R^{2} [5]	Efficiency Percentage[6]
CSN2	BME	DMEM	-3.06	0.99	112
DCN	BME	DMEM	-3.10	0.98	110
LALBA	BME	DMEM	-3.32	0.98	100
MTG1	BME	DMEM	-3.35	0.98	99
RPS115A	BME	DMEM	-3.07	0.98	112
CSN2	BME	STIM	-3.08	0.88	111
DCN	BME	STIM	-3.06	0.95	112
LALBA	BME	STIM	-2.67	0.98	88
MTG1	BME	STIM	-2.78	0.98	129
PPP1R11	BME	STIM	-3.38	0.99	126
RPS115A	BME	STIM	-3.34	0.99	99

[1] *CSN2* = beta-casein, *DCN* = decorin, *LALBA* = alpha-lactalbumin, *MTG1* = mitochondrial ribosome associated GTPase, *PPP1R11* = protein phosphate 1 regulatory inhibitor subunit 11, *RPS15A* = ribosomal protein S15A.

[2] Cell types used for the efficiency immortalized bovine mammary epithelial cells (BME) and bovine mammary fibroblast cells (MF-T2). The liver samples were from tissue of 8 wk old Holstein calves.

[3] Media type that the cells were collected in; fetal bovine serum (10 % FBS plus Dulbecco's modified Eagle's medium [DMEM], DMEM, or stimulatory media (STIM, DMEM plus Hydrocortisone 1 µg/mL, Retinoic Acid 1 µg/mL [R2625], Insulin 5 µg/mL [I4011], Prolactin 1 IU/mL[L6520]).

[4] Slope of the standard curve; dilutions were 1:1, 1:10, 1:100, and 1:1000.

[5] R^{2} = coefficient of determination of the standard curve.

[6] Efficiency is calculated as $[(10^{(-1/\text{slope})}-1)*100]$ (Bustin et al., 2009).

Table 6: Antibodies used in immunocytochemistry separated by experiment with fluorophore, host, serotype, catalog, source, and dilution used for different cell culture experiments

Antibody	Fluorophore	Host and Serotype	Catalog (Source)	Dilution
S-phase Experiment				
Primary Antibody				
BrdU	--	Mouse IgG_1	17058 (Abcam)	1:200
DCP	--	Rabbit IgG	22753 (Santa Cruz)	1:50
Secondary Antibody				
BrdU	488	Goat anti-Mouse IgG_1	A21121 (Invitrogen)	1:200
DCP	594	Goat anti-Rabbit IgG	A11012 (Invitrogen)	1:200
Metaphase Restriction Experiment				
Primary Antibody				
Alpha Tubulin	--	Mouse IgG_1	T9026 (Sigma-Aldrich Co. LLC.)	1:200
DCP	--	Rabbit IgG	22753 (Santa Cruz)	1:50
Secondary Antibody				
Alpha Tubulin	488	Goat anti-Mouse IgG_1	A21121 (Invitrogen)	1:200
DCP	594	Goat anti-Rabbit IgG	A11012 (Invitrogen)	1:200

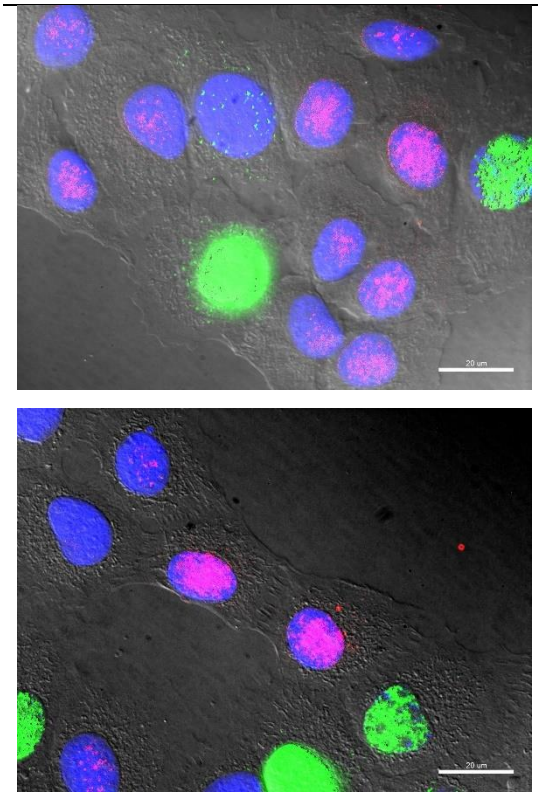
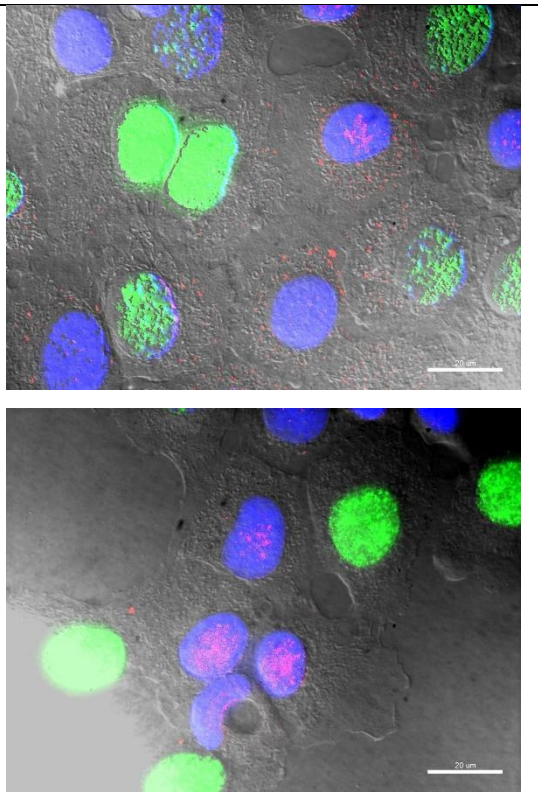
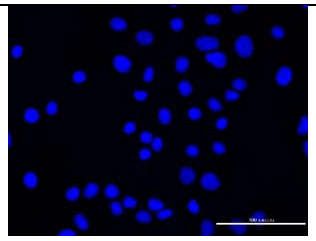
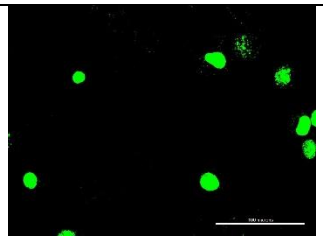
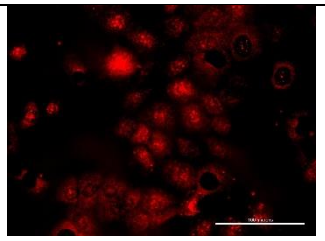
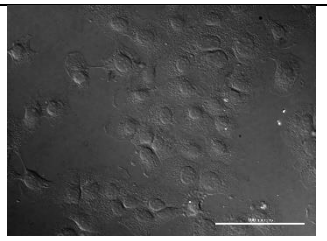
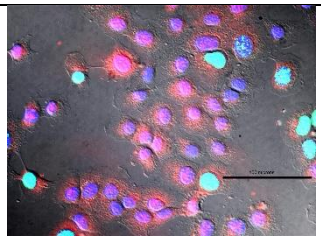
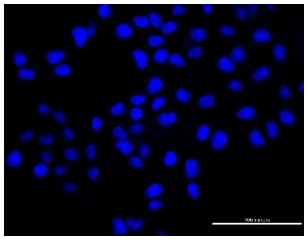
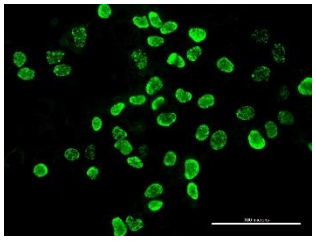
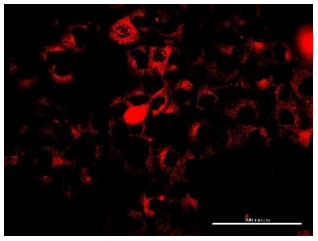
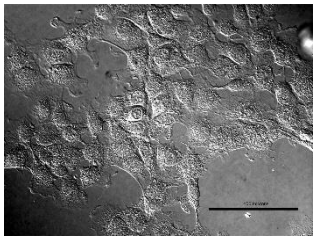
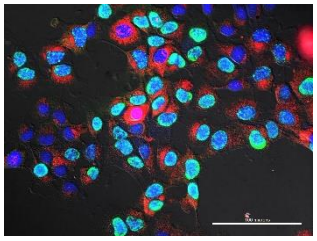
DMEM	FBS
	

Figure 11: In the S-phase experiment, serum-starved bovine mammary epithelial cells were incubated with either a basal medium DMEM (Dulbecco's Modified Eagle's Medium) or DMEM plus 10 % fetal bovine serum (FBS) for 16 h. BrdU, a thymidine analog and marker of the S-phase of the cell cycle, was added to culture media at 14 h. Decorin core protein = red, BrdU = green, DNA = blue. Images were obtained at 100x objective lens magnification; scale bars represent 20 µm.

Table 7: S-phase experiment component images of immortalized bovine mammary cells (BME). BME were incubated for 16 h with one of two media: DMEM (Dulbecco's modified Eagle's medium) or 10 % FBS (fetal bovine serum) with DMEM. 5-bromo-2'-deoxyuridine in green (BrdU), a thymidine analog and marker of the S-phase of the cell cycle was added at hour 14. Decorin core protein = red, BrdU = green, DNA = blue. Images were obtained at 40x objective lens magnification; scale bars = 100 µm

Media	DAPI	BrdU	Decorin	DIC	Composite
DMEM					
FBS					

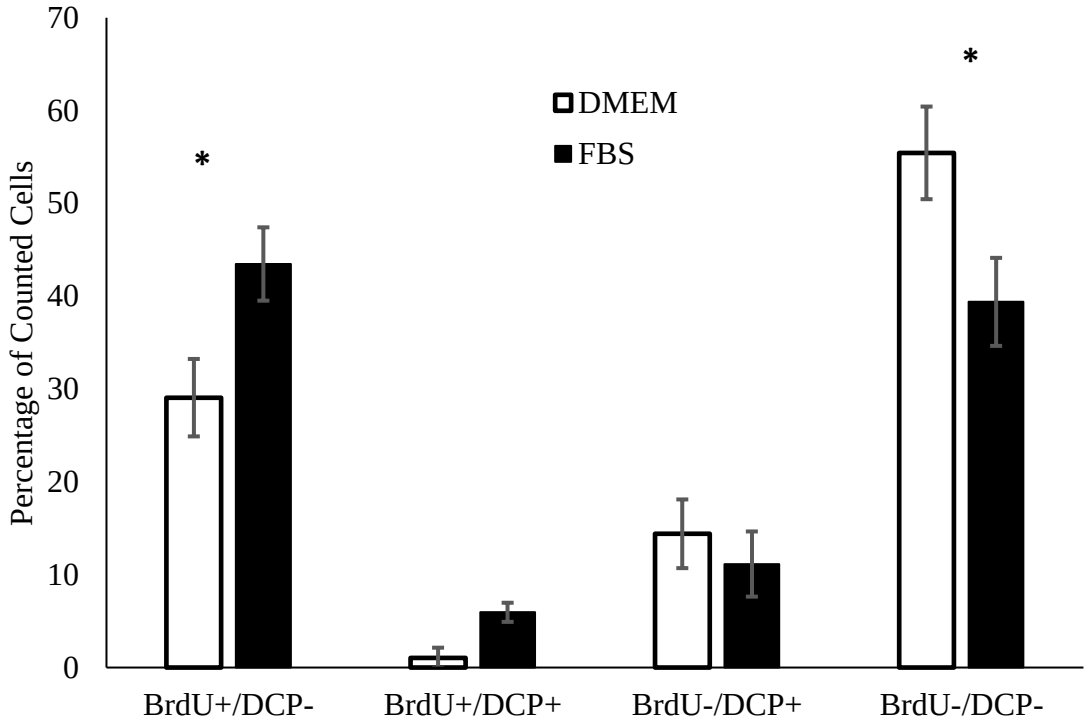


Figure 12: In S-phase experiment, bovine mammary epithelial cells were dually stained for 5-bromo-2'-deoxyuridine (BrdU) and decorin core protein (DCP). A minimum of 350 cells per treatment were counted by a single observer and classified into one of four groups: $BrdU^{+}/DCP^{+}$, $BrdU^{+}/DCP^{-}$, $BrdU^{-}/DCP^{+}$, or $BrdU^{-}/DCP^{-}$. The cell categories were divided by the total number of cells (4',6-diamidino-2-phenylindole labeled cells) to be put on a percentage basis. * $P < 0.05$ within group.

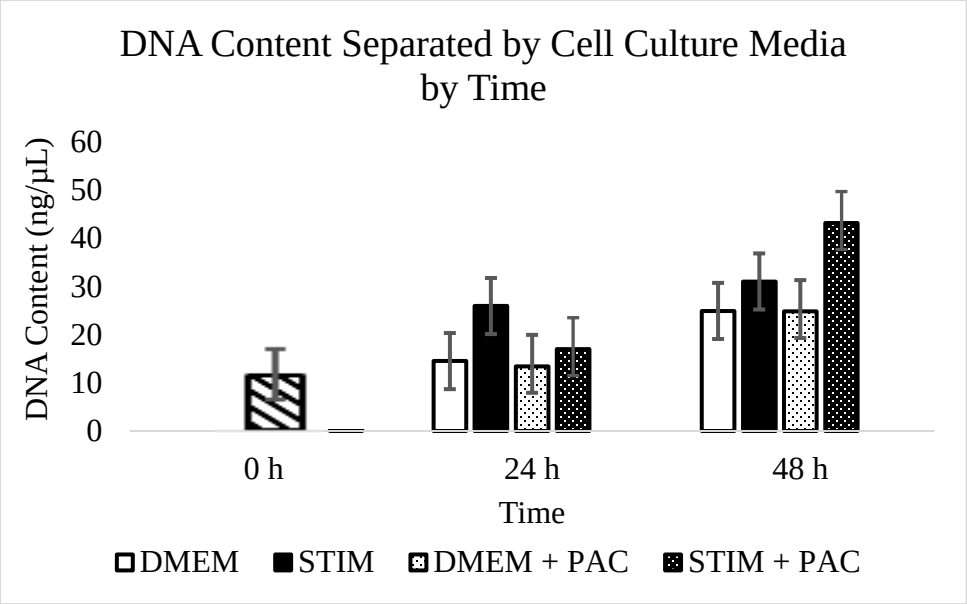


Figure 13: Metaphase restriction experiment DNA content (ng/µL) in immortalized bovine mammary epithelial cells treated with one of four different cell culture medias: Dulbecco's modfied Eagle's medium (DMEM), stimulatory media (STIM; DMEM plus Hydrocortisone 1 µg/mL, Retinoic Acid 1 µg/mL, Insulin 5 µg/mL, Prolactin 1 IU/mL), DMEM plus paclitaxel (PAC), or STIM plus PAC at two time points (0, 24, or 48 h). Each bar represents a minimum of four and a maximum of six wells. Time 0 samples were harvested before treatments were added; therefore, there is only one "treatment". Tests of fixed effects were: media ($P = 0.028$); time, ($P = 0.008$); restriction agent, ($P = 0.906$); two and three-way interactions were not significant.

Table 8: In the metaphase restriction experiment, statistically analyzed gene data from RT-qPCR analysis ($2^{-\Delta Ct}$) was divided by DNA content. Data were separated by the two factorials that were implemented during the experiment on the immortalized bovine mammary epithelial cells (BME) time and media. Fixed effects *P* values for all main effects and there interactions

	Time																
	0 h[2]	24 h				48 h											
		Media[1]									Fixed Effects, *P* =						
Gene[3]		DMEM	STIM	DMEM plus PAC	STIM plus PAC	DMEM	STIM	DMEM plus PAC	STIM plus PAC	SEM	Media	Agent[4]	Time	Media x Agent	Media x Time	Agent x Time	Media x Agent x Time
CSN2	0.002	0.008	0.0004	0.0021	0.0013	0.0148	0.0037	0.0023	0.0009	0.0044	0.031	0.078	0.185	0.393	0.539	0.007[5]	0.408
DCN	0.425	0.414	0.3072	0.5288	0.4478	0.1589	0.1848	0.3333	0.2532	0.0977	0.331	0.330	0.289	0.330	0.331	0.331	0.331
LALBA	0.001	0.019	0.0009	0.0033	0.0022	0.0096	0.0032	0.0032	0.0021	0.0060	0.070	0.223	0.145	0.218	0.442	0.097	0.616

[1] The different media types used in the experiment Dulbecco's modified Eagle media (DMEM), stimulatory media (STIM; DMEM plus Hydrocortisone 1 µg/mL [Sigma-Aldrich Co. LLC.; catalog # H4001], Retinoic Acid 1 µg/mL [Sigma-Aldrich Co. LLC.; catalog # R2625], Insulin 5 µg/mL [Sigma-Aldrich Co. LLC.; catalog # I4011], Prolactin 1 IU/mL[Sigma-Aldrich Co. LLC.; catalog #L6520]), DMEM plus Paclitaxel (PAC), and STIM plus PAC.

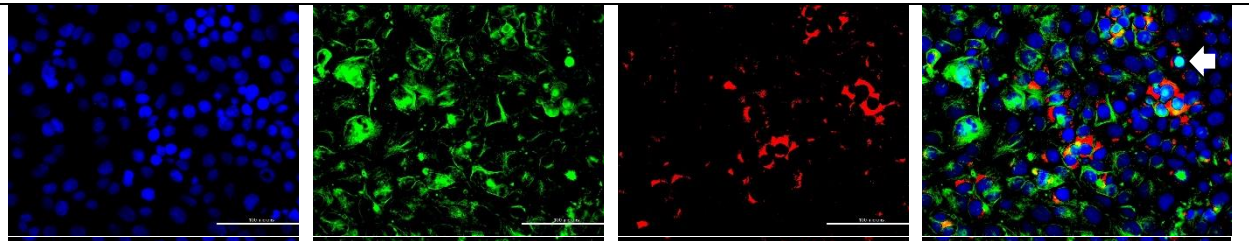
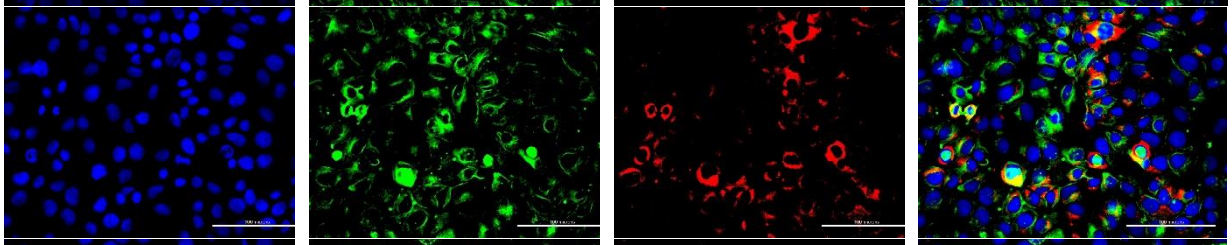
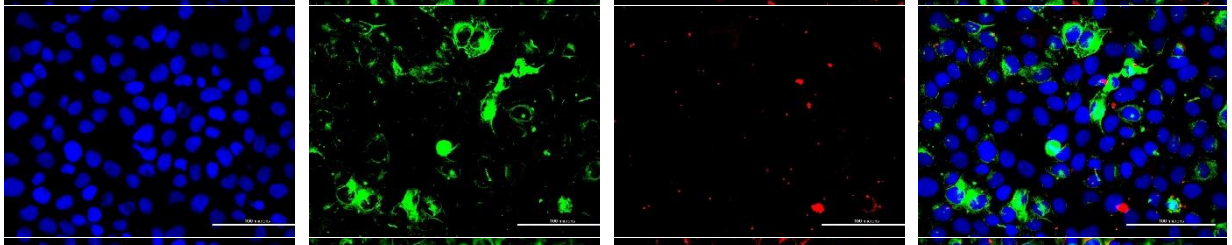
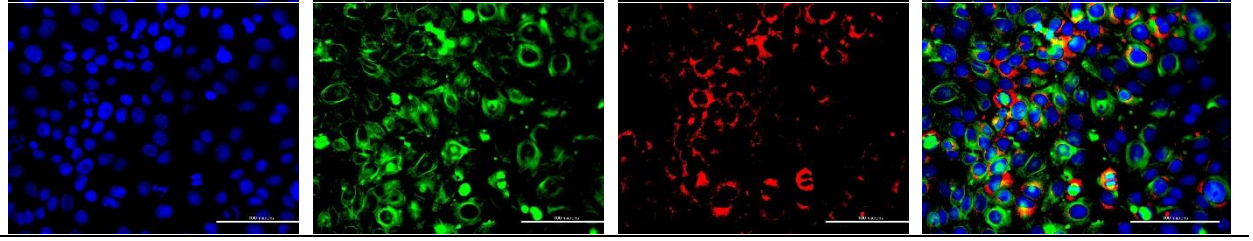
[2] 0 h samples did not receive a treatment.

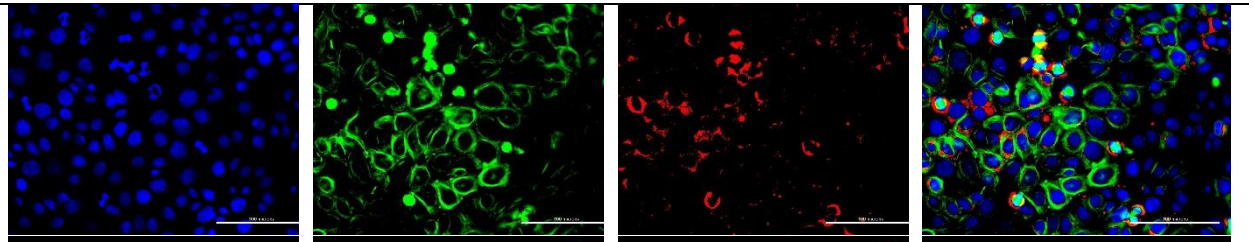
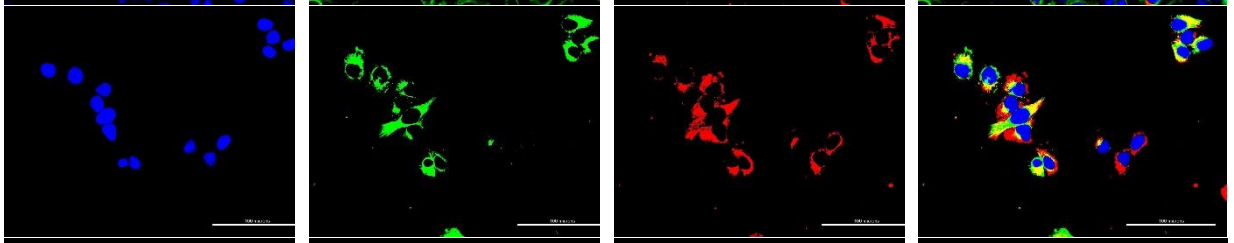
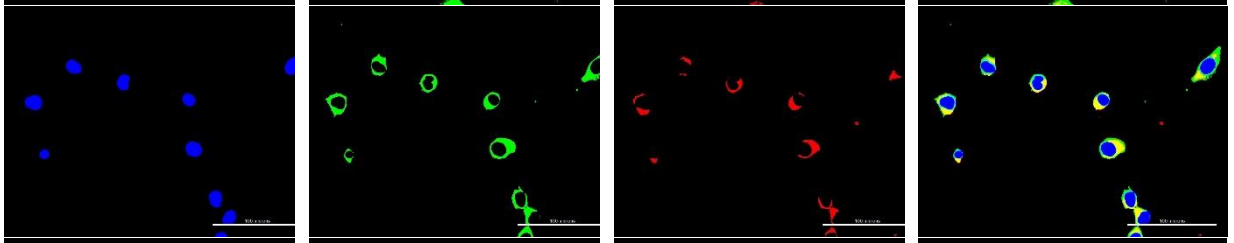
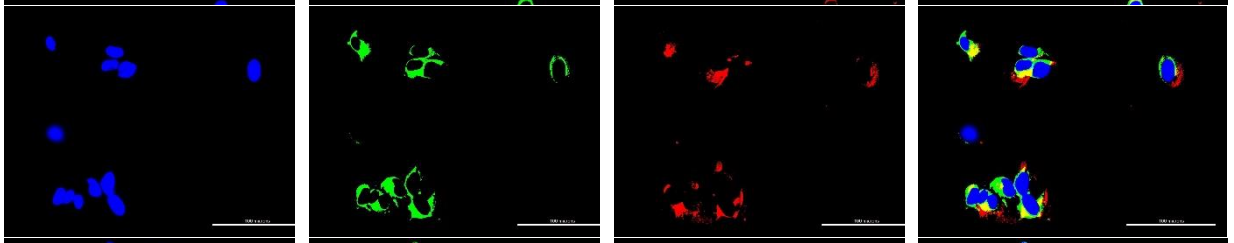
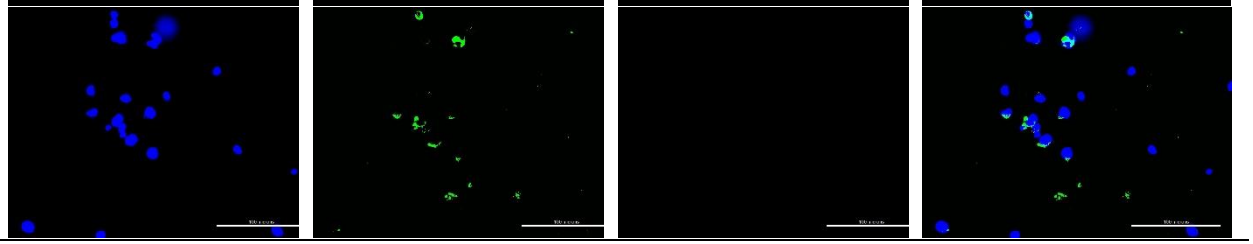
[3] *CSN2* = beta-casein, *DCN* = decorin, *LALBA* = alpha lactalbumin.

[4] Agent = Restriction agent.

[5] When contrasts were run to test for differences across time no significance was found between any of the contrasts.

Table 9: Metaphase restriction experiment component images of BME on coverslips. Each coverslip was stained for α tubulin in green, decorin core protein in red (DCP), and 4',6-diamidino-2-phenylindole (DAPI a nuclear counterstain). The primary antibodies used were α tubulin T9026 (Sigma-Aldrich Co. LLC.; 1:200) and decorin 22753 (Santa Cruz; 1:50). The white arrow indicates a cell that is out of focus due to the difference in the focal planes of the image. The images were taken at 40x with the scale bars equal to 100 µm

Media[1]	Time	DAPI	α Tubulin	DCP	Composite
DMEM	0				
DMEM	24				
STIM	24				
DMEM plus PAC	24				

STIM plus PAC	24	
DMEM	48	
STIM	48	
DMEM plus PAC	48	
STIM plus PAC	48	

[1] The different media types used in the experiment Dulbecco's modified Eagle media (DMEM), stimulatory media (STIM; DMEM plus Hydrocortisone 1 µg/mL [Sigma-Aldrich Co. LLC.; catalog # H4001], Retinoic Acid 1 µg/mL [Sigma-Aldrich Co. LLC.; catalog

R2625], Insulin 5 µg/mL [Sigma-Aldrich Co. LLC.; catalog # I4011], Prolactin 1 IU/mL[Sigma-Aldrich Co. LLC.; catalog #L6520]), DMEM plus Paclitaxel (PAC), and STIM plus PAC.

Chapter 4: Overall Conclusions and Future Outlook

This work addressed cellular and molecular aspects of bovine mammary extracellular matrix (**ECM**) biology. Mammary gland growth and involution are based on a dynamic equilibrium between proliferation and apoptosis of mammary epithelial cells (**MEC**) and fibroblasts. MEC and fibroblasts are the two major cells types found in the mammary gland and they are both known to produce a distinct ECM (Vracko, 1974; Sakakura et al., 1976; Larson, 1985). The focus of this dissertation was on the ECM proteoglycan decorin (**DCN**) and its potential role in bovine mammary biology. Decorin has numerous growth and regulatory roles. Regulatory effects of DCN on cell proliferation are mediated through known cellular pathways. Here, these pathways were investigated with cell culture experiments with monocultures of bovine mammary epithelial and fibroblast cell lines. The expected major outcome of the work was to be demonstration of a mechanistic link between DCN and bovine mammary cell proliferation. We fell short of that goal. However, many new pieces of information regarding DCN in bovine mammary biology were found.

As reported in Chapter 2, pathways related to DCN signaling were investigated with cell culture experiments using monocultures of bovine mammary epithelial and fibroblast cell lines. While existence of decorin pathway molecules was demonstrated in both types of bovine mammary cells (with relatively more produced by fibroblasts), experiments stopped short of demonstrating mature DCN proteoglycan deposition into the extracellular space. However, because DCN has known growth regulatory properties, resultant findings can be used in the future to design mechanistic cell culture studies. For instance, information generated here will be helpful in designing gene knock-down experiments involving DCN signaling in bovine mammary cells. Additionally, experimental evaluation of downstream DCN pathway genes such as, p21, Ki67, PCNA, and cMyc should be included. In humans and rodents, PCR arrays that encompass complete pathways (e.g. EGFR pathway) are available. However, to-date there are only two known types of bovine PCR arrays that include the WNT pathway and a collection of different cytokines, respectively (Abcam; Qiagen), neither of which would have aided in the examination of decorin core protein (**DCP**) pathways.

In Chapter 3, our overall findings led us to reject our null hypothesis and conclude that **DCP** does not accumulate in BME during S-phase of the cell cycle. We next wished to test whether or not DCP accumulated in BME during metaphase. Technical difficulties with use of

the metaphase restriction agent paclitaxel (**PAC**), along with imaging difficulties between cells in metaphase and not in metaphase prevented us from successfully performing our experiment. Therefore, we are not yet comfortable rejecting or not rejecting our hypothesis about DCP and its association with metaphase in BME. Additional follow-up work with: improved imaging techniques, incorporation of cell counting (and perhaps analysis of cell ploidy by flow cytometry), and increased concentrations of PAC are warranted.

Future research should focus on further characterization of DCN and its associated pathway molecules to learn if DCN induces proliferation or apoptosis of bovine mammary cells. This is important because number and activity of mammary epithelial cells ultimately determine milk yield in dairy cows. Fundamental knowledge gained in this research area may one day be applied at the animal-level and lead to gains in milk production efficiency by altering the cellular composition of mammary glands. Thinking beyond the work contained in this dissertation, it would be of interest to examine decorin in the following scenarios.

Decorin in Bovine Mammary Fibroblasts

It is unfortunate that our cultures with immortalized bovine mammary fibroblasts died. Generation and use of primary mammary fibroblast cultures may prove useful for the study of decorin because, at the gene level, they produced proportionally more DCP than bovine mammary epithelial cells. Our lab has recently isolated bovine mammary fibroblasts from virgin heifers using a protocol adapted from Barrington et al. (1997).

Exogenous TGFB1: Effects on Decorin in Bovine Mammary Cells

Others have shown that transforming growth factor beta-1 (**TGFB1)** and TGFB2 can regulate the amount of DCN produced (Bassols and Massague, 1988). We speculate that TGFB1 is able to alter the amount of DCN in bovine mammary cells, potentially providing a mechanism for how TGFB1 is able to regulate anti proliferative and proliferative effects on mammary epithelial and fibroblast cells, respectively (Knabbe et al., 1987; Woodward et al., 1995). By adding differing levels of exogenous TGFB1 to cultured bovine mammary cells, we would be able to test the impact of TGFB1 on the abundance of DCN and cell proliferation.

Exogenous Decorin: Effects on Bovine Mammary Cells

To better learn how DCN works in bovine mammary cells, one potential methods would be to first silence DCN production with siRNA, followed by administration of exogenous DCN at different concentrations. This might allow us to know if the various DCN pathways are differentially activated by varied concentrations of DCN. Another method would be to

overexpress DCN in cell culture to observe if increased production of DCN induces differences in bovine mammary cells, potentially through changes in cell numbers.

Decorin in Cells Grown on Different Strata

In the experiments performed in this dissertation, all cells were grown on plastic substrata. Many other cell culture plate coatings exist. Popular options include matrigel and collagen I. These alternate strata are popular because the cells of interest are able to start producing and forming a true basement membrane (David and Bernfield, 1979; Howlett and Bissell, 1993). Primary MEC grown on plastic dishes they lose their specificity of function, which hampers the ability to use these cells as a model of the mammary gland (Mackenzie et al., 1982)

Decorin in Co-cultured Bovine Mammary Cells

After completion of some of the experiments outlined about, a next possible step would to be to investigate the interaction of DCN produced by one cell type acting on the other cell type. Strategies that could be implemented would be the use of conditioned media or co-culturing of the cells. For conditioned media, cells would be grown in culture and their media would be aspirated and used as a treatment on another type of cell. This strategy does not allow direct contact between cell types but allows for communication through soluble factors. This type of "communication" occurs in vivo. Co-culturing on the other hand, allows for both cell types to be either next to or on top of each other and is therefore even closer to an in vivo model. Both strategies have pros and cons, but together they can be used to develop a model that is a closer representation of the bovine mammary gland.

Decorin and the Cell Cycle

The next step to investigate the interaction of DCN with the cell cycle is to conduct the experiment in chapter 3 with the noted refinements and the added factor of siRNA. By silencing *DCP* we would be able to observe if the loss of DCN would halt the cell's ability to proceed through the cell cycle. This experiment would answer the question: Is DCN a requirement for mitosis to occur in cell cultured BME? It is known that an organism can survive with *DCN* null mice, so it is implied that DCN is not required for cells to undergo the cell cycle (Danielson et al., 1997). However, within an organism there is known to be redundancy of molecules, so there could be another molecule that could replace DCN within the cell cycle that would not be present in our cell culture model. There is the possibility that there is no correlation between the ability of the cell to pass through the cell cycle and the presence of DCN. If this is true, in the above

experiment the cells would continue to cycle normally.

WORKS CITED

Barrington, G. M., T. E. Besser, W. C. Davis, C. C. Gay, J. J. Reeves, and T. B. McFadden. 1997. Expression of immunoglobulin G1 receptors by bovine mammary epithelial cells and mammary leukocytes. J Dairy Sci. 80:86-93.

Bassols, A. and J. Massague. 1988. Transforming growth factor beta regulates the expression and structure of extracellular matrix chondroitin/dermatan sulfate proteoglycans. J. Biol. Chem. 263:3039-3045.

Danielson, K. G., H. Baribault, D. F. Holmes, H. Graham, K. E. Kadler, and R. V. Iozzo. 1997. Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. J Cell Biol. 136:729-743.

David, G. and M. R. Bernfield. 1979. Collagen reduces glycosaminoglycan degradation by cultured mammary epithelial cells: possible mechanism for basal lamina formation. Proc Natl Acad Sci U S A. 76:786-790.

Howlett, A. R. and M. J. Bissell. 1993. The influence of tissue microenvironment (stroma and extracellular matrix) on the development and function of mammary epithelium. Epithelial Cell Biol. 2:79-89.

Knabbe, C., M. E. Lippman, L. M. Wakefield, K. C. Flanders, A. Kasid, R. Derynck, and R. B. Dickson. 1987. Evidence that transforming growth factor-beta is a hormonally regulated negative growth factor in human breast cancer cells. Cell. 48:417-428.

Larson, B. L. 1985. Lactation. Iowa State University Press, Ames, IA.

Mackenzie, D. D., I. A. Forsyth, B. E. Brooker, and A. Turvey. 1982. Culture of bovine mammary epithelial cells on collagen gels. Tissue Cell. 14:231-241.

Sakakura, T., Y. Nishizuka, and C. J. Dawe. 1976. Mesenchyme-dependent morphogenesis and epithelium-specific cytodifferentiation in mouse mammary gland. Science. 194:1439-1441.

Vracko, R. 1974. Basal lamina scaffold-anatomy and significance for maintenance of orderly tissue structure. Am. J. Pathol. 77:314-346.

Woodward, T. L., N. Dumont, M. O'Connor-McCourt, J. D. Turner, and A. Philip. 1995. Characterization of transforming growth factor-beta growth regulatory effects and receptors on bovine mammary cells. J. Cell. Physiol. 165:339-348.

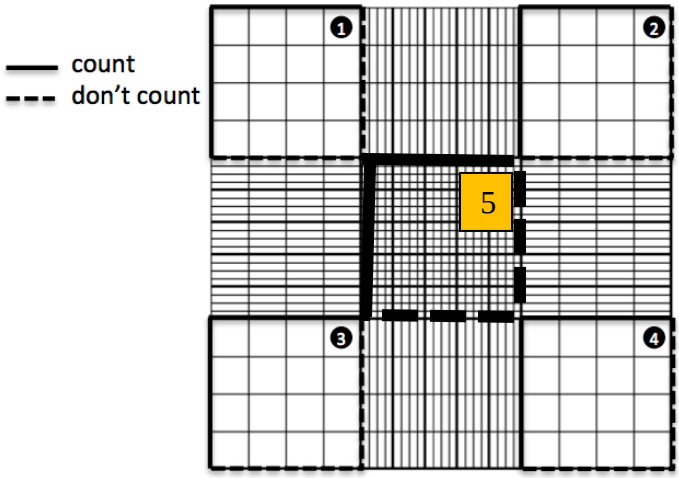
Appendix

CELL CULTURE: PASSING CELLS

Check CO_2 Tank

Check H_2O container in incubator

1. Wipe down hood with 70 % ethanol and turn on hood
2. Take DMEM, DPBS, Waste container, and Trypan Blue out of the fridge. Take the trypsin out of the freezer.
3. Turn on water bath and put in DMEM, DPBS, and trypsin
    a. Let hood and water bath warm up for 20 min
4. Spray everything with 70 % ethanol before putting into the hood
5. Aspirate off media in petri dishes and put into waste container
6. Rinse the petri dishes with 5 mL of DPBS
7. Add 1 mL of trypsin to petri dish
    a. Incubate dish with trypsin in incubator for 10 min
        i. While dish is incubating set up the hemocytometer by wiping it down with a kimwipe
        ii. While dish is incubating set up petri dishes by writing date, cell type, passage number, media type on bottom of petri dish. Add 12 mL of desired media type and put into incubator when taking out cells with trypsin.
    b. Check cells detached from dish under microscope (might need to tap petri dish to help detach cells
8. Add 5 mL of DPBS
9. Put into 15 mL falcon tube
10. Take 50 µL of cell suspension and put into a microcentrifuge tube
11. Add 50 µL of trypan blue to microcentrifuge tube
12. Count cells on hemocytometer



    a. Do math: number of cells counted/10 * volume cells are suspended in * 2 * 10000 aka area of hemocytometer
13. Centrifuge falcon tube for 10 min

14. Decant off liquid on top of cell pellet
15. Resuspend cell pellet in media for desired cell/mL amount
16. Add to petri dishes premade in step 7ii
17. Put petri dish back in the incubator
18. Wipe down hood with 70 % ethanol and turn off water bath

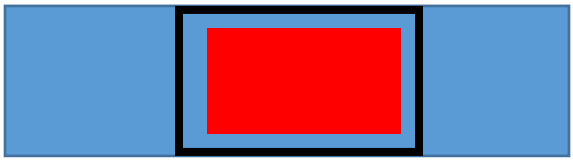
CELL CULTURE: CHANGING MEDIA

1. Wipe down hood with 70 % ethanol and turn on hood
2. Take DMEM, DPBS, and Waste container out of the fridge.
3. Turn on water bath and put in DMEM, DPBS, and trypsin
    a. Let hood and water bath warm up for 20 min
4. Spray everything with 70 % ethanol before putting into the hood
5. Aspirate off media in petri dishes and put into waste container
6. Rinse the petri dishes with 5 mL of DPBS
7. Add 12 mL of desired media
8. Put petri dish back in the incubator
9. Wipe down hood and turn off water bath

ICC PROTOCOL: FOR CELLS GROWN ON GLASS COVERSLIPS (6 WELL PLATE)

1. Turn on 10 m*M* citrate buffer, pH 6.0 on heated plate
2. Aspirate off media
3. Wash with prewarmed DPBS 2x
4. Fix in 3 mL of 3.7 % formaldehyde for 10 min at room temperature
   ***Use 50 mL beakers if not using the entire 6 well plate
5. Wash with 3 mL of DPBS 3x
6. Antigen Retrieval: Incubate in citrate buffer heated to 90 °C for 30 min
7. Permeabilize cells: Incubate in 0.1 % Triton X in PBS for 5 min at room temperature
8. Wash with 3 mL of DPBS 2x
9. Move coverslips onto slides
10. Make a box with the PAP pen on the slide and put the coverslip on the slide with cells facing up (Blue box = slide, Black outline = PAP pen outline, red box = coverslip)

Example:



11. Block with CAS Block for 30 min at room temperature
12. Incubate with primary antibody overnight at 4 ° C in black low profile container
13. Wash coverslips with DPBS for 3 min 4x
14. Spin down secondary antibody dilution for 10 min at 10,000 *x g* at room temperature
15. Incubate with secondary antibody for 1 h in dark at RT
16. Wash coverslips with DPBS for 2 min 4x
17. Add nuclear counterstain (aka Slowfade with DAPI) to slide
18. Flip coverslip onto a new slide
19. Take out bubbles as much as possible using back of tweezers

20. Let sit for ~ 1 h
21. Image slides

RNA/DNA/PROTEIN PURIFICATION KIT

Catalog number 24000

Norgen Biotek Corp.

Protocol for monolayer cells

Set Up of Components

Centrifuge steps are all carried out in a benchtop microcentrifuge at 14,000 *x g* at Room Temperature *except where noted.*

All components are stored at room temperature *except working concentration of protein loading dye (contains DL-Dithiothreitol/DTT) store at -20 ° C.*

1. Wash Solution A add 90 mL of 96 – 100 % ethanol to supplied bottle containing the Concentrated Wash Solution A. Final volume of 128 mL
2. Wash Solution El add 15 mL of isopropanol to supplied bottle containing the Concentrated Wash Solution El. Final volume 30 mL
3. Protein Loading Dye add 93 mg of DL-Dithiothreitol (DTT). Store at -20 ° C

Lysis of Cells

Max 3 x 10^6 cells, optimal 1 x 10^6 cells (helps to mitigate cross contamination of RNA fraction in the genomic DNA fraction)

1. Aspirate media
2. Wash with 1x DPBS
3. Aspirate 1x DPBS
4. Add 350 µL of Buffer SK to culture plate
5. Swirl buffer around plate for 5 minutes
6. Transfer lysate to microcentrifuge tube
7. Add 200 µL of 96 – 100 % ethanol to lysate
8. Vortex for 10 s

Total RNA Purification from Lysate

1. Assemble column with collection tube
2. Apply 600 µL of lysate + ethanol onto the column
3. Centrifuge for 1 min at ≥ 3,500 *x g*
4. Retain flowthrough for Protein Purification, store on ice or -20 ° C
5. If lysate volume larger than 600 µL, repeat steps 3-4
    a. Combine flowthrough
6. Reassemble the spin column with a new/clean collection tube
7. Apply 400 µL of Wash Solution A to the column
8. Centrifuge for 1 min

9. Discard flowthrough
10. Reassemble the column
11. Wash a column (second time), add 400 µL of Wash Solution A
12. Centrifuge for 1 min
13. Discard flowthrough
14. Reassemble the column
15. To dry column, centrifuge for 2 min
16. Discard collection tube
17. Place the column into a new 1.7 mL microcentrifuge tube
18. Add 50 µL of Elution Solution A to the column
19. Centrifuge for 1 min at 14,000 *x g*
20. Retain the column for Genomic DNA Isolation
21. Store RNA -20 ° C (Short term) -70 ° C (Long term)

Genomic DNA Purification from Lysate

Use column from step 20 of total RNA Purification from Lysate

1. Assemble the column with a new collection tube
2. Apply 500 µL of Wash Solution El to column
3. Centrifuge for 2 min
4. Discard the flowthrough
5. Reassemble the column with a new collection tube
6. To dry column, centrifuge column for 2 min
7. Discard collection tube
8. Place column into a new 1.7 mL microcentrifuge tube
9. Add 100 µL of Elution Buffer F to the column
10. Centrifuge for 2 min at 200 *x g*
11. Centrifuge for 1 min at 14,000 *x g*
12. Retain the column for Total Protein
13. Store DNA 4 ° C (Short term) -20 ° C (Long term)

Isolate Total Proteins

Use flowthrough from step 4 of Total RNA Purification from Lysate and column from step 15 of Genomic DNA Purification from Lysate

1. Assemble column with new collection tube
2. Transfer 300 µL of flowthrough from the RNA binding step 4 to a separate microcentrifuge
3. Adjust the volume to be a total of 600 µL with molecular biology grade water
    a. To purify the entire flowthrough adjust the volume to 1.2 mL with molecular biology grade water
4. Add 24 µL (or 48 µL for the entire flowthrough) of Binding Buffer A. Mix contents
5. Apply 650 µL of the flowthrough + Binding Buffer A onto the column
6. Centrifuge for 2 min at 5,200 *x g*

7. Discard flowthrough
8. Reassemble spin column
9. Repeat steps 5 - 8 as needed
10. Apply 500 µL of Wash Solution C to the column
11. Centrifuge for 2 min at 5,200 *x g*
12. Discard the flowthrough
13. Reassemble spin column
    a. Can spin for an additional min to dry column
14. Add 9.3 µL of Protein Neutralizer to a new 1.7 mL microcentrifuge tube
15. Transfer the spin column to the microcentrifuge tube
16. Apply 100 µL of the Elution Buffer C to the column
17. Centrifuge for 2 min at 5,200 *x g*
    a. 95 % of bound protein is recovered in the first elution
18. Store DNA 4 ° C (Short term) -20 ° C (Long term)

PROTOCOL FOR EXTRACTING PROTEOGLYCANS USING DEAE BEADS (NON-COLUMN)

Based on:

Gubbiotti, M. A., T. Neill, H. Frey, L. Schaefer, and R. V. Iozzo. 2015. Decorin is an autophagy inducible proteoglycan and is required for proper in vivo autophagy. Matrix Biol. 48: 14-25.

1. Incubate media overnight at 4 º C with 100 µL/mL of **Buffer DEAE**
2. Centrifuge and take off supernatant
3. Wash pellets with 1 mL of **WB1**
4. Centrifuge and take off supernatant
5. Wash pellets with 1 mL of **WB1**
6. Centrifuge and take off supernatant
7. Wash pellets with l mL of **WB2**
8. Centrifuge and take off supernatant
9. Wash pellets with 1 mL of **WB2**
10. Centrifuge and take off supernatant
11. Add 50 µL of **SDS Buffer** to 50 µL of sample. Mix by pippetting up and down (this amount is for duplicate samples in SDS Page Gel).
12. Boil for 5 min at 90 º C
13. Centrifuge and take off supernatant
14. Supernatant will be loaded into SDS Page Gel

*****All centrifuge steps were conducted at RT with a table top centrifuge for ~30 s

-Continue with SDS Page Gel and Western Blotting

Recipes

TrisHCl 200 mM (pH 7.5)

Makes 500 mL

MW TrisHCl: 157.6

1. Weight out 15.76 g TrisHCl
2. Add 250 mL of ddH_2O
3. pH to 7.5 (use 1 M NaOH)
4. q.s. to 500 mL

NaCl 1 M

Makes 500 mL

MW NaCl: 58.44

1. Weigh out 29.22 g of NaCl
2. Add 250 mL ddH_2O
3. Stir
4. q.s to 500 mL

Sodium Phosphate 100 mM (pH 7.0)

Makes 500 mL

MW Sodium Phosphate: 119.98

1. Weigh out 5.999 g of sodium monophosphate
2. Add 250 mL ddH_2O
3. pH to 7.0 (use 1 M NaOH)
4. q.s. to 500 mL

Buffer DEAE (40 mL) [50 % (w/v) Diethylaminoethyl Sephacel (*DEAE*), in 20 mM Tris-HCl (pH 7.5), 0.1% (w/v) Triton X-100, and 0.15 M NaCl]

DEAE: Sigma, ca number I6505

Makes 40 mL

**Easiest container to make buffer in was a graduate cylinder

1. Shake stock container of DEAE
2. Pour DEAE Beads (20 mL of beads) into a new container
3. Let Beads Settle
4. Pour off ethanol from DEAE Beads
5. Add 6 mL of 1 M NaCl
6. Add 0.04 mL of Triton X-100
7. Add 4 mL of 0.2 M TrisHCl
8. Stir
9. q.s. to 40 mL

WB 1: Buffer **0.15 M** NaCl **[**20 mM Tris-HCl (pH 7.5), 0.1% (w/v) Triton X-100, and 0.15 M NaCl]

Makes 100 mL

1. Measure out 15 mL of 1 M NaCl
2. Add 0.1 mL of Triton X-100
3. Add 10 mL of 0.2 M TrisHCl
4. Stir
5. q.s. to 100 mL

WB 2: Buffer **0.2 M** NaCl [20 mM Tris-HCl (pH 7.5), 0.1% (w/v) Triton X-100, and 0.2 NaCl]

Makes 100 mL

1. Measure out 20 mL of NaCl 1 M
2. Add 0.1 mL of Triton X-100
3. Add 10 mL of 0.2 M TrisHCl
4. Stir
5. q.s. to 100 mL

SDS Buffer [10 mM sodium phosphate (pH 7.0), 2 % (w/v) SDS, 10 % (w/v) glycerol, and 0.003 % (w/v) Bromphenol Blue]

Makes 100 mL

1. Add 10 mL of 0.2 M Sodium Phosphate
2. Weigh out 2 g of SDS
3. Measure out 10 mL of glycerol
4. Measure out 0.003 g of Bromophenol Blue
5. Add half of 50 mL ddH_2O
6. Stir
7. q.s. to 100 mL