The Pennsylvania State University

The Graduate School

Intercollege Graduate Program in Physiology

EFFECT OF RETINOID ANALOGS, BOVINE LACTOFERRIN, AND

RETINOID RECEPTOR EXPRESSION IN BOVINE MAMMARY CELLS

A Thesis in

Physiology

by

Yinglu Wang

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

August 2009

The thesis of Yinglu Wang was reviewed and approved* by the following:

Craig R. Baumrucker
Professor of Animal Nutrition / Physiology
Thesis Adviser

Shannon L. Kelleher
Assistant Professor of Nutrition

Michael H. Green
Professor of Nutrition Science and Physiology

Donna H. Korzick
Associate Professor of Physiology and Kinesiology
Chair, IGDP in Physiology

* Signatures are on file in the Graduate School.

ABSTRACT

The retinoids are a class of substances with similar chemical structure as retinol. Retinoid signaling has been related to a variety of developmental processes including embryo development, cell cycle arrest and apoptosis, normal growth, vision, reproduction and overall survival. The mechanism of this effect in bovine has not been fully studied.

Binding with Retinoic acid receptors (RAR-α, β and γ) or retinoid X receptors (RXR-α, β and γ), retinoids have been shown to induce apoptosis or a G1 arrest and thereby reduce mammary cell number. Our lab was particularly interested in the effect of retinoid signaling in bovine mammary cells. Two cell types, the bovine immortalized (BME-UV1) and primary mammary cells (MeBo), were used as models. Except RXR-γ, all the isotypes of retinoid receptors were expressed in bovine mammary cells. Immortalization of the BME-UV1 cells did not seem to affect retinoid receptor expression, but does affect retinoid effect on cell viability. The effect of natural retinoids (RA), retinoid analogs, and bovine lactoferrin (bLf) on cell viability *in vitro* was evaluated. We conclude that RAR interaction in bovine mammary cell types regulates cell viability *in vitro*. In addition, we conclude retinoids and bLf interact with RAR signaling in bovine mammary cells and regulate cell number *in vitro*, but does not appear to interact with lactogenic hormones.

TABLE OF CONTENTS

List of Figures

List of Tables

List of Abbreviations

CRABP   cellular retinoic acid binding protein

IGF   insulin-like growth factor

LBD   ligand-binding domain

NR   nuclear receptor

PPAR   Peroxisome proliferator-activated receptor

RA   retinoic acid

RAR   retinoic acid receptor

RARE   retinoic acid response element

RXR   retinoid X receptor

THR   thyroid hormone receptors

VDR   vitamin D receptors

ACKNOWLEDGMENTS

First I would like to thank all the help from my advisor, Professor Craig R. Baumrucker. He was really nice and patient. Without his support and guidance, it was absolutely impossible for me to finish my research. Secondly, I am deeply thankful to Dr. Shannon L. Kelleher and Dr. Michael H. Green for serving on my graduate committee. In addition, part of the preliminary studies was conducted by Dr. D. L. Greger and E. Ward.

Chapter 1

Introduction

1.1 Bovine mammary glands and lactation

The study of lactation in milking cows is important for understanding this basic and universal mechanism in mammals. It also provides useful information for the dairy industry. A number of lactogenic hormones, including insulin, hydrocortisol and prolactin, and many other molecules are involved in this complicated process (Akers, 1985; Roh et al., 1994). During the periparturient period, the differentiation of mammary epithelium cells is required for the initiation of lactation. During involution, regression of mammary tissue occurs by apoptosis at the beginning of the dry period (Annen et al., 2007). While retinoids function in differentiation, proliferation, and apoptosis of mammary cells is partially established for human and rodent mammary cells, but the retinoid signaling in bovine mammary cells is poorly understood.

In these studies, the bovine immortalized (BME-UV1) and primary mammary cells (MeBo) were used as *in vitro* models. Bovine Mammary Epithelial Cells (MAC-T) were the precursor of BME-UV1, immortalized by stable transfection with SV-40 large T-antigen (Huynh et al., 1991).

1.2 Retinoids

retinol, retinoids and analogs

Retinol (vitamin A) and its active derivates play indispensible physiological functional roles in vision, early embryonic development, immunity, differentiation, cell cycle arrest and apoptosis (Baumrucker et al., 2005; Donato et al., 2007; Schneider et al., 2000; Toma et al., 1997; Wu et al., 2006; Zhang et al., 1996). Since they exert a wide range of functions, lack of retinol or the inability of producing its active derivates may lead to a series of deficiencies in embryonic development or adults.
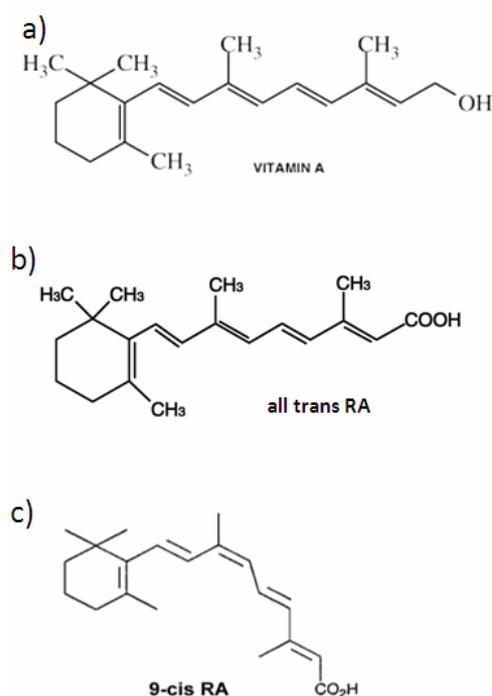
Figure 1-1. Structures of retinol and retinoids. a) retinol; b) all-*trans* retinoic acid is the acid form; c) 9-*cis* retinoic acid is an isomer of all-*trans* retinoic acid.

Figure 1-1 shows the structure of retinol and natural retinoic acid (RA). RA has a similar chemical structure as vitamin A. Vitamin A is converted to retinaldehyde (RAL) by alcohol dehydrogenase (ADH) or short-chain dehydrogenase/reductase (SDR), and RAL is converted to retinoic acid by retinaldehyde dehydrogenase (RALDH) (Romand et al., 2008). Retinoid Acid (RA) is the most active natural form of retinol. *All-trans* retinoic acid (atRA) has the molecular weight of 300.4, soluble in DMSO (25mg/ml) and ethanol (10mg/ml), and light sensitive. It is reported that atRA is one of the many agents that can induce inhibition of cell growth in many normal or malignant cell lines (Mangiarotti et al., 1998; Seewaldt et al., 1997a). *All-trans* RA (atRA) binds to retinoic acid receptors specifically. It binds with the three RARs (α, β, γ) with Kd value of 0.2, 0.4 and 0.2nM respectively. For mammary cancer and normal cells, treatment effects of all-trans RA have been shown by flow cytometry and autoradiographic techniques to arrest the cell in G1 phase (Mangiarotti et al., 1998; Seewaldt et al., 1997b). 9-cis Retinoic acid (9cRA) is naturally produced via isomerization of atRA, and has a molecular weight of 300.4. It is soluble in DMSO (25mg/ml) and ethanol (25mg/ml), and is also light sensitive. 9cRA binds to retinoid X receptors (RXR) specifically, but also there is a small amount of 9cRA that binds to retinoic acid receptors (RAR) (Heyman et al., 1992).

Because of the cell proliferation inhibition effect, retinoids are some of the many biological agents that are used for dermatology and therapy and prevention of and cancer (Altucci and Gronemeyer, 2003; Thielitz et al., 2008). Due to side effects, atRA has been replaced by synthetic N-(4-hydroxyphenyl) retinamide (4-HPR) retinoid (Das et al., 2008; Ponthan et al., 2003). In addition, some studies show the existence of retinoic acid resistance during cancer therapy due to long-time usage of retinoids. However, this

3

resistance may be due to retinoid acid receptor activation of peroxisome proliferator-activated receptor (PPAR) instead of a RAR/RXR interaction (Shaw et al., 2003a).

Because both atRA and 9cRA bind with RARs and RXRs (although with different affinity), it is difficult to study and interpret the interaction between retinoids and their receptors by the natural retinoids. For that reason we and others have utilized the retinoid antagonist and agonist in order to better define the retinoid signaling system. AM 580 (RAR agonist) binds with RARs, but not RXRs. AM has the similar structure as natural retinoids. Ro 41-5253 (RAR antagonist) also binds with RARs specifically, but not RXRs. However, the Ro:RAR complex compound does not bind to RARE, the response elements (Toma et al., 1998). In this way, retinoid signaling pathway is blocked or inhibited by Ro 41-5253.

AM-580

Chemical Name: 4-[(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalen yl)carboxamido]benzoic acid

Ro 41-5253

Figure 1-2. Chemical structure of RAR agonist (AM-580) and antagonist (Ro 41-5253)

Regulation of gene expression

Retinoids, including all-trans retinoic acid (atRA) and 9-cis retinoic acid (9cRA), are small molecules that may be transported to the nucleus of the cell (Figure 1-3). Cellular retinol-binding protein (CRBP) presents retinol to the appropriate enzymes to biosynthesize retinoic acid (RA) (Okuno et al., 1993). Cellular Retinoic Acid Binding Proteins (CRABPs) may act as binding proteins that transport RA from the cytosol to the nucleus. CRABPs may also be involved in the regulation of RA. There are two forms of CRABPs, CRABP I and CRABP II (McPherson et al., 2007).

The specific receptors of atRA consist of the three isotypes of the retinoic acid receptor. The specific receptors of 9cRA composed of the three isotypes of the retinoid X receptor. A small amount of 9cRA binds to retinoic acid receptor as well. The study of the retinoid pathway mechanism was a void prior of the discovery of retinoid receptors. Upon ligand binding, retinoid receptors form dimers in the nucleus. The heterodimeration of RAR and RXR is a key event of this pathway.

With the formation of nuclear RAR/RXR heterodimers, the complex binds to regulatory regions of the chromosome retinoic acid response elements (RARE) of DNA, which leads to the regulation (activate or repress) of a series of downstream genes, and leads to different physiological changes. Studies show that heterodimerization is required for the process of DNA binding (Pfahl, 1993). The "X" of RXRs indicates that this receptor can bind to and form heterodimers with several nuclear receptors in addition to the retinoic acid receptors. The RXRs may form heterodimers with thyroid hormone

receptors (THR), vitamin D receptors (VDR), chicken ovalbumin upstream promoter transcription factor, and peroxisome proliferator-activated receptor (PPAR) (Jimenez-Lara and Aranda, 2000; Shaw et al., 2003b), which are all retinoids-independent. The binding sites of these receptors are similar, with the same pattern in sequence and structure. In this way, all these hormones may be involved in the regulation of cellular differentiation. RXRs and RARs have ligand-binding domain (LBD) and DNA-binding domain (DBD) to carry out the processing of input (ligand binding) and output (transcription) information (Alvarez et al., 2007).
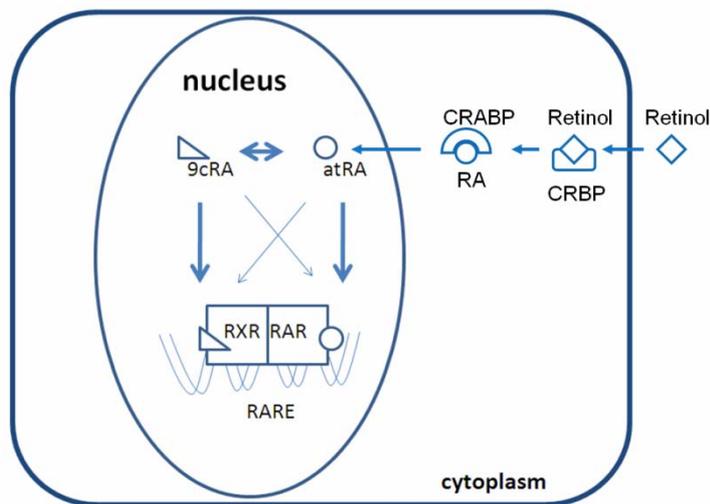
Figure 1-3. A simplified model of the regulation of gene expression by retinoic acid receptors.

RA Response Element and Retinoid X Response Element:

Retinoid receptors are ligand-dependent transcriptional regulators that bind as dimers to specific DNA sequences called retinoic acid response element (RARE) or retinoid X response element (RXRE) within gene promoters and enhancers where they regulate gene expression. The RAR/RXR heterodimers bind and activate RARE, while RXR/RXR homodimers bind and activate RXRE, respectively (Lehmann et al., 1992b). RXR homodimers and RAR-RXR heterodimers can be selectively affected, suggesting that both retinoid response pathways can be independently activated (Lehmann et al., 1992a). Although both RAR and RXR bind with RXRE, studies showed that RAR can not activate RXRE (Xiao et al., 1995). There are a series of RARE and RXRE elements that have been identified, and are consistent with the various functions of retinoids (Ishikawa et al., 1990b; Mangelsdorf et al., 1994). Specific motifs and structures have been identified in RARE or RXRE in which repeats (A/G)G(G/T)TCA separated by a variable number of nucleotides (Ishikawa et al., 1990a). RAR-β is one of the genes that contains RARE at its promoter region.

Retinoic acid receptors and retinoid X receptors

Retinoids exert their important biological functions by binding with two nuclear retinoid receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Raffo et al., 2000). They are both members of the superfamily of steroid/thyroid hormone nuclear

receptors. The nuclear receptor family shares a unique structure. The DNA binding domain (DBD), as its name suggests, binds to the regulatory part of the downstream genes. Another important receptor location is called the ligand binding domain (LBD). Retinoids specifically bind to the LBD of retinoid receptors. Both the DBD and the LBD are conserved, suggesting the subtle functional differences of DNA and ligand binding properties (Chambon, 1996a). The DBD and LBD domains are linked by Hinge region (Figure 1-4).　There are three RARs, RARα, -β and -γ. And there are also three RXRs, RXRα, -β and -γ. These subtypes have distinct expression patterns, which suggest that they may play distinct physiological roles in different tissues (Alvarez et al., 2007).
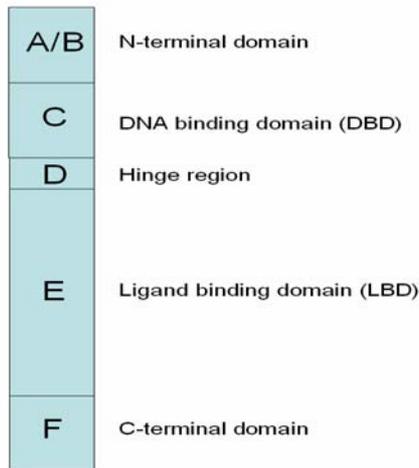
Figure 1-4. Structural Organization of Nuclear Receptors.

Retinoic acid receptor α (RAR-α)

Retinoic acid receptor α (RAR-α), also known as NR1B1 (nuclear receptor subfamily 1, group B, member 1), is a nuclear receptor encoded by the RARA gene. RAR-α is presented in almost all the tissues (Alvarez et al., 2007). It has been reported that there are at least seven kinds of RAR-α forms in the mouse, that comes from the same gene, but

with alternative splicing (Leroy et al., 1991b). The only difference among the seven forms is focused in the N-terminal region (A/B domain) (Leroy et al., 1991a). Several growth-related genes, such as The Wilms' tumor suppressor gene, are the regulatory genes of RAR-α (Goodyer et al., 1995). RAR-α may be phosphorylized by protein kinase A, which is believed to be one of the methods of RAR-α signaling pathway regulation (Rochette-Egly et al., 1995). (Greger et al., 2005) showed that RAR-α mRNA expression was constant in young cattle liver tissue.

Retinoic acid receptor β (RAR-β)

Retinoic acid receptor β (RAR-β), also known as NR1B2, is also a nuclear receptor encoded by the RARB gene. Different from RAR-α, RAR-β is only selectively expressed in heart, lung, spleen (Alvarez et al., 2007), and suggests that RAR-β might be involved in a tissue-dependent manner. RAR-β is different from other isotypes because it was found to not be expressed in many cancerous cells, suggesting that RAR-β may act as a tumor suppressor (Sun et al., 2000). Most transgenic mice (43 out of 57) expressing antisense RAR-β sequences had visible lung tumor (Berard et al., 1996b). A twofold higher incidence was observed in homozygous antisense mice (Berard et al., 1996a). In human and rodent mammary cells, the mRNA expression of RAR-β is significantly lower than the other two RARs, which also suggests that RAR-β might be under more strict regulation and its signaling pathway is more sensitive. Similar to RAR-α, four forms of RAR-β have been identified (Chambon, 1996b). (Shang et al., 1999) proved the expression of RAR-β was blocked when Ro 41-5253, the antagonist of RAR-α, was provided to cultured breast cancerous cells, demonstrating the effect of RAR-α on the induction of RAR-β.

Retinoic acid receptor γ (RAR-γ)

Retinoic acid receptor γ (RAR-γ), also known as NR1B3, is also a nuclear receptor encoded by the *RARG* gene. RAR-γ first identified in mouse (Zelent et al., 1989a). Seven forms (1-7) have been identified and attributed to alternative splicing, while the mRAR-γ1 and mRAR-γ2 are predominant forms (Kastner et al., 1990a). The seven forms are conserved, while the only key difference among them is in the 5' untranslated region (Kastner et al., 1990b). The pattern of expression of RAR-γ suggests that it may be involved in morphogenesis, chondrogenesis, and differentiation of squamous epithelia (Dolle et al., 1990; Krust et al., 1989; Ruberte et al., 1990; Zelent et al., 1989b).

Retinoid X Receptors

Retinoid X receptors (RXR) play an important role in many fundamental physiological processes such as reproduction, cellular differentiation, bone development, hematopoesis and pattern formation during embryogenesis (Mangelsdorf and Evans, 1995). The RXRs were discovered later than RARs. RXRs have different primary structures and ligand specificity when compared to the RARs. Also different from RARs, RXRs can bind many other nuclear receptors besides the RARs, and was the nationale of their name "Retinoid X" receptors. Because RXRs can bind with a large series of nuclear receptors, RXR occupies a central position in multiple signaling pathways as a ubiquitous heterodimerization partner (Egea et al., 2000; Mangelsdorf et al., 1992b). The subtypes of RXRs, α, β and γ, are also expressed in a differential spatio-temporal pattern (Mangelsdorf et al., 1992a). Both the DBD and LDB are conserved among RXR isotypes,

but the apparent dissimilarity of its sequence to that of the RARs was observed (Mangelsdorf et al., 1992c). Multiple subtypes provide the diversity of function possible.

Retinoid X receptor α (RXR-α)

Retinoid X receptor α (RXR-α), also known as NR2B1, is a nuclear receptor encoded by the *RXRA* gene. RXR-α is expressed tissue-dependently (Brocard et al., 1996). Excess retinoic acid expression leads to malformations in limb development in normal mice embryos, while no visible effects were observed in homozygous RXR-α knockout mice embryos, suggesting RXR-α functions as a component in the teratogenic process in the limbs (Sucov et al., 1995b). Results of heterozygous embryos indicate the role RXRA gene plays in limb teratogenesis is dosage dependent (Sucov et al., 1995a).

Retinoid X receptor β (RXR-β)

Retinoid X receptor β (RXR-β), also known as NR2B2, is a nuclear receptor encoded by the RXRB gene. Seven forms have been identified and attributed to different alternative splicing. The N terminal sequence is conserved, while the C terminal does not appear among the seven forms. Two of the seven forms, known as β1 and β2, are dominant comparing with others (Nagata et al., 1994a). The amount of β1 form is higher in fast growing cells than β2 form while in adult tissues the level of these two forms are comparable. The difference between β1 and β2 in primary structure is well conserved among mouse and human (Nagata et al., 1994b).

Retinoid X receptor γ (RXR-γ)

Retinoid X receptor γ (RXR-γ), also known as NR2B3, is a nuclear receptor encoded by the *RXRG* gene. Two forms of RXR-γ have been discovered in mouse with different 5' untranslated regions, suggesting that they may be products from two different promoters (Yu et al., 1991b). The expression pattern of these two forms is restrict and tissue specific: Isoform 1 is expressed in brain and muscle while form 2 is expressed in skeletal and cardiac muscle, but not in liver (Yu et al., 1991a). Two form was also found in chicken with similar pattern (Seleiro et al., 1994).

1.3 Insulin-like Growth Factor Binding Protein 3 (IGFBP3)

With the stimulation of growth hormone (GH), insulin-like growth factor (IGF) is secreted to regulate growth by binding to IGF receptors (Blum and Baumrucker, 2007; Plath-Gabler et al., 2001). There are two IGFs, (IGF-I and IGF-II) with different physiological roles (Blum and Baumrucker, 2008). Insulin-like growth factor binding protein (IGFBP) has specific affinity for IGFs (Paye and Forsten-Williams, 2006). IGFBP functions to regulate the biological effect of IGFs by increasing the half-life of IGF or inhibiting the interaction between IGF and its receptors. Six IGFBPs (1-6) have been identified, ranging in size from 216 to 289 amino acids (Rosenzweig, 2004b). A GCGCCXXC motif and a CWCV sequence were found in all the forms, and analysis of sequence homology suggests that IGFBPs exhibit similar tertiary structures (Rosenzweig, 2004a). The concentration of IGFBP3 is the highest among the six (Ferry et al., 1999).

Recent researches suggest that IGFBP3 may also play its biological role in a IGF-independent fashion (Baumrucker et al., 2006; Liu et al., 2000). The expression of IGFBP2, 3 and 4 is altered by retinoids (Shang et al., 1999). However, only IGFBP3, not any other IGFBPs, was identified as being responsible for the role related to the inhibition of mammary cell proliferation (Shang et al., 1999). Bovine IGFBP3 is located in chromosome 4. IGFBP3 is produced by hepatic Kupffer cells or locally in many tissues (Russell-Jones et al., 1995). The locally tissue expression contributes to paracrine and autocrine roles effects (Huynh et al., 2002). IGFBP3 has a nuclear localization sequence, and is able to enter the nucleus (Jaques et al., 1997), which suggests IGFBP3 may regulate the expression of some downstream genes in the nucleus.

1.4 Lactoferrin

Bovine lactoferrin (bLf) is a glycoprotein with a molecular weight of ~78kDa and a member of transferrin family (Baumrucker et al., 2006). Lf was first identified in cow milk in 1939 (Levay and Viljoen, 1995). Later, it was confirmed that it could also be secreted in tears, saliva, nasal and bronchial secretions (Baggiolini et al., 1970). bLf locates on chromosome 22. Lf has a signal peptide which explains its secretion, and a nuclear localization sequence (NLS) which translocates it to the nuclear compartment. Lf contains two binding domains which bind to Fe^{2+} ions (Levay and Viljoen, 1995). It has been confirmed that Lf has multiple functions, including bactericide and fungicide, and was believed related to innate defense of newborns (Baumrucker et al., 2006; Baveye et al., 1999a; Baveye et al., 1999b). The bactericide and fungicide function was believed to be related to the two iron binding sites, which restrict nutritional iron from microorganisms (Ellison, III, 1994). Some reports found that lactoferrin binds microorganisms and destroys cell membranes (Ellison, III et al., 1988).

In mammary tissue, the physiological concentration of bLf is high before lactation, (around 100 mg/ml), but very low during lactation (Hurley and Rejman, 1993; Welty et al., 1976). Since milk is a good medium for bacteria growth, the change of Lf concentration may be beneficial to prevent mastitis during the dry non-lactating period. Because bovine lactoferrin concentration varies during the lactation cycle in a manner different than other proteins, it may be involved in the regulation of bovine mammary cell proliferation and apoptosis (Baumrucker et al., 2005). Freiburghaus et al. (2009) treated a human colon cancer cell line with a series of concentration of lactoferricin, a peptide

released from Lf, from bovine milk, and showed that the S phase of the cell cycle was prolonged, and therefore cell proliferation was inhibited. Nevertheless, the mechanism of bovine lactoferrin regulation in cell proliferation remains unclear.

Hagiwara et al. (2003b) showed that lactoferrin concentration from cows is related to the age of the cow, the stage of lactation and whether the cow is infected by mastitis. The lactoferrin concentration of mastitic cows tends to be higher than normal cows (Hagiwara et al., 2003a).

1.5 Mutual interaction among retinoids, lactoferrin and IGFBP3

Baumrucker et al. (2003) showed that bovine lactoferrin binds to IGFBP3 and both IGFBP3 expression and RAR/RXR can be activated by retinoids. Lee et al. (1995) demonstrated the existence of a RARE in the human lactoferrin promoter region, but no promoter studies are available for the bovine mammary cells. Baumrucker et al. (1999) demonstrated that both RAR-β and IGFBP3 were blocked by RAR-α antagonist Ro. In addition, the expression of IGFBP3 could be rescued by transfection of RAR-β (Baumrucker et al., 1999). In all, these studies suggested that IGFBP3 is induced by RAR-β activation. The interaction between RXR-α and IGFBP3 was demonstrated with human cells using several independent techniques, including two-hybrid screen (Lehmann et al., 1992d). In addition, the effect of IGFBP3 on cell inhibition is not apparent in RXR-α Knockout mouse (Lehmann et al., 1992c). Bovine Lactoferrin binds rhIGFBP3 competitively with IGF (Baumrucker and Erondu, 2000). Only with the presence of atRA could bovine lactoferrin be translocated into nucleus (Baumrucker et al., 2005).

The mutual interaction among lactoferrin, IGFBP3 and retinoids appears to be important in mammary cells, but extremely complicated in the process of cell differentiation, proliferation and apoptosis in bovine mammary epithelial cells (Puvogel et al., 2005). In an attempt to resolve some of the complications, the research of this thesis focused on the relationship between lactoferrin and retinoids.

Reference List

Akers,R.M. (1985). Lactogenic Hormones: Bindind site, mammary growth,secratory cell differentiation, and milk biosynthesis in ruminanats. *J. Dairy Sci.* **68,** 501-519.

Altucci,L. and Gronemeyer,H. (2003). The promise of retinoids to fight against cancer. *Nature Reviews Cancer* **1,** 181-193.

Alvarez,S., Germain,P., Alvarez,R., Rodriguez-Barrios,F., Gronemeyer,H., and de Lera,A.R. (2007). Structure, function and modulation of retinoic acid receptor beta, a tumor suppressor. *Int. J. Biochem. Cell Biol.* **39,** 1406-1415.

Annen,E.L., Fitzgerald,A.C., Gentry,P.C., McGuire,M.A., Capuco,A.V., Baumgard,L.H., and Collier,R.J. (2007). Effect of continuous milking and bovine somatotropin supplementation on mammary epithelial cell turnover. *J. Dairy Sci* **90,** 165-183.

Baggiolini,M., De,D.C., Masson,P.L., and Heremans,J.F. (1970). Association of lactoferrin with specific granules in rabbit heterophil leukocytes. *J. Exp. Med.* **131,** 559-570.

Baumrucker,C.R. and Erondu,N.E. (2000). Insulin-like growth factor (IGF) system in the bovine mammary gland and milk. *J. Mammary Gland Biol. Neoplasia* **5,** 53-64.

Baumrucker,C.R., Gibson,C.A., and Schanbacher,F.L. (2003). Bovine lactoferrin binds to insulin-like growth factor-binding protein-3. *Domest. Anim. Endocrinol.* **24,** 287-303.

Baumrucker,C.R., Gibson,C.A., Shang,Y., Schanbacher,F.L., and Green,M.H. Lactoferrin specifically binds to IGFBP-3 to cause competitive displacement of IGF from IGF:IGFBP-3 complexes and induces internalization and nuclear localization of lactoferrin:IGFBP-3 complexes in mammary epithelial cells. 81st Endocrine Ann.Meeting San Diego, CA, 404. 1999. Ref Type: Abstract

Baumrucker,C.R., Saurer,S., Blum,J.W., Jungi,T., and Friis,R.R. (2006). Lactoferrin (Lf): Retinoid interactions in the mammary glands of transgenic mice overexpressing human Lf. *Mol. Cell Endocrinol.* **251,** 56-66.

Baumrucker,C.R., Schanbacher,F., Shang,Y., and Green,M.H. (2005). Lactoferrin interaction with retinoid signaling: Cell growth and apoptosis in mammary cells. *Domest. Anim Endocrinol.* **30,** 289-303.

Baveye,S., Elass,E., Mazurier,J., Spik,G., and Legrand,D. (1999a). Lactoferrin: A Mulitfunctional Glycoprotein Involved in the Modulation of the Inflammatory Process. *Clinical Chemistry* **37,** 281-286.

Baveye,S., Elass,E., Mazurier,J., Spik,G., and Legrand,D. (1999b). Lactoferrin: a multifunctional glycoprotein involved in the modulation of the inflammatory process. *Clin. Chem. Lab Med.* **37,** 281-286.

Berard,J., Laboune,F., Mukuna,M., Masse,S., Kothary,R., and Bradley,W.E. (1996a). Lung tumors in mice expressing an antisense RARbeta2 transgene. *FASEB J.* **10,** 1091-1097.

Berard,J., Laboune,F., Mukuna,M., Masse,S., Kothary,R., and Bradley,W.E. (1996b). Lung tumors in mice expressing an antisense RARbeta2 transgene. *FASEB J.* **10,** 1091-1097.

Blum,J.W. and Baumrucker,C.R. (2007). Insulin-like growth factors (IGFs), IGF binding proteins, and other endocrine factors in milk: Role in the Newborn. Springer.

Blum,J.W. and Baumrucker,C.R. (2008). Insulin-like growth factors (IGFs), IGF binding proteins, and other endocrine factors in milk: role in the newborn. *Adv. Exp. Med Biol.* **606,** 397-422.

Brocard,J., Kastner,P., and Chambon,P. (1996). Two novel RXR alpha isoforms from mouse testis. *Biochem. Biophys. Res. Commun.* **229,** 211-218.

Chambon,P. (1996a). A decade of molecular biology of retinoic acid receptors. *FASEB J.* **10,** 940-954.

Chambon,P. (1996b). A decade of molecular biology of retinoic acid receptors. *FASEB J.* **10,** 940-954.

Das,A., Banik,N.L., and Ray,S.K. (2008). N-(4-Hydroxyphenyl) retinamide induced both differentiation and apoptosis in human glioblastoma T98G and U87MG cells. *Brain Res.* **1227,** 207-215.

Dolle,P., Ruberte,E., Leroy,P., Morriss-Kay,G., and Chambon,P. (1990). Retinoic acid receptors and cellular retinoid binding proteins. I. A systematic study of their differential pattern of transcription during mouse organogenesis. *Development* **110,** 1133-1151.

Donato,L.J., Suh,J.H., and Noy,N. (2007). Suppression of mammary carcinoma cell growth by retinoic acid: the cell cycle control gene Btg2 is a direct target for retinoic acid receptor signaling. *Cancer Res* **67,** 609-615.

Egea,P.F., Mitschler,A., Rochel,N., Ruff,M., Chambon,P., and Moras,D. (2000). Crystal structure of the human RXRalpha ligand-binding domain bound to its natural ligand: 9-cis retinoic acid. *EMBO J.* **19,** 2592-2601.

Ellison,R.T., III (1994). The effects of lactoferrin on gram-negative bacteria. *Adv. Exp. Med. Biol.* **357,** 71-90.

Ellison,R.T., III, Giehl,T.J., and LaForce,F.M. (1988). Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. *Infect. Immun.* **56,** 2774-2781.

Ferry,R.J.Jr., Cerri,R.W., and Cohen,P. (1999). Insulin-like growth factor binding proteins: new proteins, new functions. *Horm. Res.* **51,** 53-67.

Freiburghaus,C., Janicke,B., Lindmark-Mansson,H., Oredsson,S.M., and Paulsson,M.A. (2009). Lactoferricin treatment decreases the rate of cell proliferation of a human colon cancer cell line. *J. Dairy Sci.* **92,** 2477-2484.

Goodyer,P., Dehbi,M., Torban,E., Bruening,W., and Pelletier,J. (1995). Repression of the retinoic acid receptor-alpha gene by the Wilms' tumor suppressor gene product, wt1. *Oncogene* **10,** 1125-1129.

Greger,D.L., Philipona,C., and Blum,J.W. (2005). Ontogeny of mRNA abundance of nuclear receptors and nuclear receptor target genes in young cattle. *Domest. Anim Endocrinol.*

Hagiwara,S., Kawai,K., Anri,A., and Nagahata,H. (2003b). Lactoferrin concentrations in milk from normal and subclinical mastitic cows. *J. Vet. Med. Sci.* **65,** 319-323.

Hagiwara,S., Kawai,K., Anri,A., and Nagahata,H. (2003a). Lactoferrin concentrations in milk from normal and subclinical mastitic cows. *J. Vet. Med. Sci.* **65,** 319-323.

Heyman,R.A., Mangelsdorf,D.J., Dyck,J.A., Stein,R.B., Eichele,G., Evans,R.M., and Thaller,C. (1992). 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* **68,** 397-406.

Hurley,W.L. and Rejman,J.J. (1993). Bovine lactoferrin in involuting mammary tissue. *Cell Biol. Int. Rep.* **17,** 283-289.

Huynh,H., Chow,P.K., Ooi,L.L., and Soo,K.C. (2002). A possible role for insulin-like growth factor-binding protein-3 autocrine/paracrine loops in controlling hepatocellular carcinoma cell proliferation. *Cell Growth Differ.* **13,** 115-122.

Huynh,H.T., Robitaille,G., and Turner,J.D. (1991). Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. *Exp. Cell Res.* **197,** 191-199.

Ishikawa,T., Umesono,K., Mangelsdorf,D.J., Aburatani,H., Stanger,B.Z., Shibasaki,Y., Imawari,M., Evans,R.M., and Takaku,F. (1990b). A functional retinoic acid receptor encoded by the gene on human chromosome 12. *Mol. Endocrinol.* **4,** 837-844.

Ishikawa,T., Umesono,K., Mangelsdorf,D.J., Aburatani,H., Stanger,B.Z., Shibasaki,Y., Imawari,M., Evans,R.M., and Takaku,F. (1990a). A functional retinoic acid receptor encoded by the gene on human chromosome 12. *Mol. Endocrinol.* **4,** 837-844.

Jaques,G., Noll,K., Wegmann,B., Witten,S., Kogan,E., Radulescu,R.T., and Havemann,K. (1997). Nuclear localization of insulin-like growth factor binding protein 3 in a lung cancer cell line. *Endocrinology* **138,** 1767-1770.

Jimenez-Lara,A.M. and Aranda,A. (2000). Interaction of vitamin D and retinoid receptors on regulation of gene expression. *Horm. Res.* **54,** 301-305.

Kastner,P., Krust,A., Mendelsohn,C., Garnier,J.M., Zelent,A., Leroy,P., Staub,A., and Chambon,P. (1990b). Murine isoforms of retinoic acid receptor gamma with specific patterns of expression. *Proc. Natl. Acad. Sci. U. S. A* **87,** 2700-2704.

Kastner,P., Krust,A., Mendelsohn,C., Garnier,J.M., Zelent,A., Leroy,P., Staub,A., and Chambon,P. (1990a). Murine isoforms of retinoic acid receptor gamma with specific patterns of expression. *Proc. Natl. Acad. Sci. U. S. A* **87,** 2700-2704.

Krust,A., Kastner,P., Petkovich,M., Zelent,A., and Chambon,P. (1989). A third human retinoic acid receptor, hRAR-gamma. *Proc. Natl. Acad. Sci. U. S. A* **86,** 5310-5314.

Lee,M.O., Liu,Y., and Zhang,X.K. (1995). A retinoic acid response element that overlaps an estrogen response element mediates multihormonal sensitivity in transcriptional activation of the lactoferrin gene. *Mol. Cell Biol.* **15,** 4194-4207.

Lehmann,J.M., Jong,L., Fanjul,A., Cameron,J.F., Lu,X.P., Haefner,P., Dawson,M.I., and Pfahl,M. (1992d). Retinoids selective for retinoid X receptor response pathways. *Science* **258,** 1944-1946.

Lehmann,J.M., Jong,L., Fanjul,A., Cameron,J.F., Lu,X.P., Haefner,P., Dawson,M.I., and Pfahl,M. (1992c). Retinoids selective for retinoid X receptor response pathways. *Science* **258,** 1944-1946.

Lehmann,J.M., Jong,L., Fanjul,A., Cameron,J.F., Lu,X.P., Haefner,P., Dawson,M.I., and Pfahl,M. (1992b). Retinoids selective for retinoid X receptor response pathways. *Science* **258,** 1944-1946.

Lehmann,J.M., Jong,L., Fanjul,A., Cameron,J.F., Lu,X.P., Haefner,P., Dawson,M.I., and Pfahl,M. (1992a). Retinoids selective for retinoid X receptor response pathways. *Science* **258,** 1944-1946.

Leroy,P., Krust,A., Zelent,A., Mendelsohn,C., Garnier,J.M., Kastner,P., Dierich,A., and Chambon,P. (1991b). Multiple isoforms of the mouse retinoic acid receptor alpha are generated by alternative splicing and differential induction by retinoic acid. *EMBO J.* **10,** 59-69.

Leroy,P., Krust,A., Zelent,A., Mendelsohn,C., Garnier,J.M., Kastner,P., Dierich,A., and Chambon,P. (1991a). Multiple isoforms of the mouse retinoic acid receptor alpha are generated by alternative splicing and differential induction by retinoic acid. *EMBO J.* **10,** 59-69.

Levay,P.F. and Viljoen,M. (1995). Lactoferrin: a general review. *Haematologica* **80,** 252-267.

Liu,B., Lee,H.-Y., Weinzimer,S.A., Powell,D.R., Kurie,J.M., and Cohen,P. (2000). Direct functional interactions between IGFBP-3 and RXR- a regulate transcriptional signalling and apoptosis . *J. Biol. Chem.* **275,** 33607-33613.

Mangelsdorf,D., Umesono,K., and Evans,R.M. (1994). The retinoid receptors. In *The Retinoids: biology, chemistry and medicine*. (ed. Sporn,M.B., Roberts,A.B., and Goodman,D.S.), pp. 319-349. New York: Raven Press.

Mangelsdorf,D.J., Borgmeyer,U., Heyman,R.A., Zhou,J.Y., Ong,E.S., Oro,A.E., Kakizuka,A., and Evans,R.M. (1992c). Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. *Genes Dev.* **6,** 329-344.

Mangelsdorf,D.J., Borgmeyer,U., Heyman,R.A., Zhou,J.Y., Ong,E.S., Oro,A.E., Kakizuka,A., and Evans,R.M. (1992a). Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. *Genes Dev.* **6,** 329-344.

Mangelsdorf,D.J., Borgmeyer,U., Heyman,R.A., Zhou,J.Y., Ong,E.S., Oro,A.E., Kakizuka,A., and Evans,R.M. (1992b). Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. *Genes Dev.* **6,** 329-344.

Mangelsdorf,D.J. and Evans,R.M. (1995). The RXR heterodimers and orphan receptors. *Cell* **83,** 841-850.

Mangiarotti,R., Danova,M., Alberrici,R., and Pellicciari,C. (1998). All-trans retinoic acid (ATRA)-induced apoptosis is preceded by G1 arrest in human MCF-7 breat cancer cells. *British Journal of Cancer* **77,** 186-191.

McPherson,L.A., Woodfield,G.W., and Weigel,R.J. (2007). AP2 transcription factors regulate expression of CRABPII in hormone responsive breast carcinoma. *J. Surg. Res* **138,** 71-78.

Nagata,T., Kanno,Y., Ozato,K., and Taketo,M. (1994a). The mouse Rxrb gene encoding RXR beta: genomic organization and two mRNA isoforms generated by alternative splicing of transcripts initiated from CpG island promoters. *Gene* **142,** 183-189.

Nagata,T., Kanno,Y., Ozato,K., and Taketo,M. (1994b). The mouse Rxrb gene encoding RXR beta: genomic organization and two mRNA isoforms generated by alternative splicing of transcripts initiated from CpG island promoters. *Gene* **142,** 183-189.

Okuno,M., Numaguchi,S., Moriwaki,H., and Muto,Y. (1993). [Cellular retinoid-binding proteins]. *Nippon Rinsho* **51,** 879-885.

Paye,J.M. and Forsten-Williams,K. (2006). Regulation of insulin-like growth factor-I (IGF-I) delivery by IGF binding proteins and receptors. *Ann. Biomed. Eng* **34,** 618-632.

Pfahl,M. (1993). Signal transduction by retinoid receptors. *Skin Pharmacol.* **6 Suppl 1,** 8-16.

Plath-Gabler,A., Gabler,C., Sinowatz,F., Berisha,B., and Schams,D. (2001). The expression of the IGF family and GH receptor in the bovine mammary gland. *J Endocrinol.* **168,** 39-48.

Ponthan,F., Lindskog,M., Karnehed,N., Castro,J., and Kogner,P. (2003). Evaluation of anti-tumour effects of oral fenretinide (4-HPR) in rats with human neuroblastoma xenografts. *Oncol. Rep.* **10,** 1587-1592.

Puvogel,G., Baumrucker,C.R., Sauerwein,H., Ruhl,R., Ontsouka,E., Hammon,H.M., and Blum,J.W. (2005). Effects of an enhanced vitamin A intake during the dry period on retinoids, lactoferrin, IGF-system, mammary gland epithelial cell apoptosis and subsequent lactation in dairy cows. *J. Dairy Sci.* **88,** 1785-1800.

Raffo,P., Emionite,L., Colucci,L., Belmondo,F., Moro,M.G., Bollag,W., and Toma,S. (2000). Retinoid receptors: pathways of proliferation inhibition and apoptosis induction in breast cancer cell lines. *Anticancer Res.* **20,** 1535-1543.

Rochette-Egly,C., Oulad-Abdelghani,M., Staub,A., Pfister,V., Scheuer,I., Chambon,P., and Gaub,M.P. (1995). Phosphorylation of the retinoic acid receptor-alpha by protein kinase A. *Mol. Endocrinol.* **9,** 860-871.

Roh,S.G., Baik,M., and Choi,Y.J. (1994). The effect of lactogenic hormones on protein synthesis and amino acid uptake in rat mammary acinar cell culture at various physiological stages. *Int. J. Biochem.* **26,** 479-485.

Romand,R., Kondo,T., Cammas,L., Hashino,E., and Dolle,P. (2008). Dynamic expression of the retinoic acid-synthesizing enzyme retinol dehydrogenase 10 (rdh10) in the developing mouse brain and sensory organs. *J. Comp Neurol.* **508,** 879-892.

Rosenzweig,S.A. (2004a). What's new in the IGF-binding proteins? *Growth Horm. IGF. Res.* **14,** 329-336.

Rosenzweig,S.A. (2004b). What's new in the IGF-binding proteins? *Growth Horm. IGF. Res.* **14,** 329-336.

Ruberte,E., Dolle,P., Krust,A., Zelent,A., Morriss-Kay,G., and Chambon,P. (1990). Specific spatial and temporal distribution of retinoic acid receptor gamma transcripts during mouse embryogenesis. *Development* **108,** 213-222.

Russell-Jones,D.L., Bates,A.T., Umpleby,A.M., Hennessy,T.R., Bowes,S.B., Hopkins,K.D., Jackson,N., Kelly,J., Shojaee-Moradie,F., Jones,R.H. et al. (1995). A comparison of the effects of IGF-I and insulin on glucose metabolism, fat metabolism and the cardiovascular system in normal human volunteers. *Eur. J. Clin. Invest.* **25,** 403-411.

Schneider,S.M., Offterdinger,M., Huber,H., and Grunt,T.W. (2000). Activation of retinoic acid receptor alpha is sufficient for full induction of retinoid responses in SK-BR-3 and T47D human breast cancer cells. *Cancer Res* **60,** 5479-5487.

Seewaldt,V.L., Caldwell,L.E., Johnson,B.S., Swisshelm,K., Collins,S.J., and Tsai,S. (1997a). Inhibition of retinoic acid receptor function in normal human mammary epithelial cells results in increased cellular proliferation and inhibits the formation of a polarized epithelium in vitro. *Exp. Cell Res* **236,** 16-28.

Seewaldt,V.L., Kim,J.H., Caldwell,L.E., Johnson,B.S., Swisshelm,K., and Collins,S.J. (1997b). All-trans-retinoic acid mediates G1 arrest but not apoptosis of normal human mammary epithelial cells. *Cell Growth Differ.* **8,** 631-641.

Seleiro,E.A., Darling,D., and Brickell,P.M. (1994). The chicken retinoid-X-receptor-gamma gene gives rise to two distinct species of mRNA with different patterns of expression. *Biochem. J.* **301 (Pt 1),** 283-288.

Shang,Y., Baumrucker,C.R., and Green,M.H. (1999). Signal relay by retinoic acid receptors a and b in the retinoic acid-induced expression of insulin-like growth factor-binding protein in breast cancer cells. *J. Biol. Chem.* **274,** 18005-18010.

Shaw,N., Elholm,M., and Noy,N. (2003b). Retinoic acid is a high affinity selective ligand for the peroxisome proliferator-activated receptor beta/delta. *J. Biol. Chem.* **278,** 41589-41592.

Shaw,N., Elholm,M., and Noy,N. (2003a). Retinoic acid is a high affinity selective ligand for the peroxisome proliferator-activated receptor beta/delta. *J. Biol. Chem.* **278,** 41589-41592.

Sucov,H.M., Izpisua-Belmonte,J.C., Ganan,Y., and Evans,R.M. (1995a). Mouse embryos lacking RXR alpha are resistant to retinoic-acid-induced limb defects. *Development* **121,** 3997-4003.

Sucov,H.M., Izpisua-Belmonte,J.C., Ganan,Y., and Evans,R.M. (1995b). Mouse embryos lacking RXR alpha are resistant to retinoic-acid-induced limb defects. *Development* **121,** 3997-4003.

Sun,S.Y., Wan,H., Yue,P., Hong,W.K., and Lotan,R. (2000). Evidence that retinoic acid receptor beta induction by retinoids is important for tumor cell growth inhibition. *J. Biol. Chem.* **275,** 17149-17153.

Thielitz,A., bdel-Naser,M.B., Fluhr,J.W., Zouboulis,C.C., and Gollnick,H. (2008). Topical retinoids in acne--an evidence-based overview. *J. Dtsch. Dermatol. Ges.* **6,** 1023-1031.

Toma,S., Isnardi,L., Raffo,P., Dastoli,G., De,F.E., Riccardi,L., Palumbo,R., and Bollag,W. (1997). Effects of all-trans-retinoic acid and 13-cis-retinoic acid on breast-cancer cell lines: growth inhibition and apoptosis induction. *Int. J. Cancer* **70,** 619-627.

Toma,S., Isnardi,L., Raffo,P., Riccardi,L., Dastoli,G., Apfel,C., LeMotte,P., and Bollag,W. (1998). RARalpha antagonist Ro 41-5253 inhibits proliferation and induces apoptosis in breast-cancer cell lines. *Int. J. Cancer* **78,** 86-94.

Welty,F.K., Smith,K.L., and Schanbacher,F.L. (1976). Lactoferrin concentration during involution of the bovine mammary gland. *J. Dairy Sci.* **59,** 224-231.

Wu,K., DuPre,E., Kim,H., Tin,U., Bissonnette,R.P., Lamph,W.W., and Brown,P.H. (2006). Receptor-selective retinoids inhibit the growth of normal and malignant breast cells by inducing G1 cell cycle blockade. *Breast Cancer Res Treat.* **96,** 147-157.

Xiao,J.H., Durand,B., Chambon,P., and Voorhees,J.J. (1995). Endogenous retinoic acid receptor (RAR)-retinoid X receptor (RXR) heterodimers are the major functional forms regulating retinoid-responsive elements in adult human keratinocytes. Binding of ligands to RAR only is sufficient for RAR-RXR heterodimers to confer ligand-dependent activation of hRAR beta 2/RARE (DR5). *J. Biol. Chem.* **270,** 3001-3011.

Yu,V.C., Delsert,C., Andersen,B., Holloway,J.M., Devary,O.V., Naar,A.M., Kim,S.Y., Boutin,J.M., Glass,C.K., and Rosenfeld,M.G. (1991b). RXR beta: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell* **67,** 1251-1266.

Yu,V.C., Delsert,C., Andersen,B., Holloway,J.M., Devary,O.V., Naar,A.M., Kim,S.Y., Boutin,J.M., Glass,C.K., and Rosenfeld,M.G. (1991a). RXR beta: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell* **67,** 1251-1266.

Zelent,A., Krust,A., Petkovich,M., Kastner,P., and Chambon,P. (1989b). Cloning of murine alpha and beta retinoic acid receptors and a novel receptor gamma predominantly expressed in skin. *Nature* **339,** 714-717.

Zelent,A., Krust,A., Petkovich,M., Kastner,P., and Chambon,P. (1989a). Cloning of murine alpha and beta retinoic acid receptors and a novel receptor gamma predominantly expressed in skin. *Nature* **339,** 714-717.

Zhang,X.K., Liu,Y., and Lee,M.O. (1996). Retinoid receptors in human lung cancer and breast cancer. *Mutat. Res* **350,** 267-277.

Chapter 2

Retinoids, retinoid analogs, and lactoferrin interact and differentially affect cell viability of a primary and immortalized bovine mammary cells *in vitro*

2.1 Abstract

Bovine mammary cells were utilized to evaluate the effect of natural retinoids (RA), retinoid analogs, and bovine lactoferrin (bLf) on cell viability *in vitro*. Experiments with Alamar Blue showed a linear relationship between fluorescence and cell viability index. The BME-UV1 cells exhibited twice the metabolic activity while requiring ½ the doubling time of the MeBo cells. The BME-UV1 cells were very sensitive to *all-trans* retinoic acid (atRA) cell viability and exhibited a dose dependent cell viability inhibition with 9-cisRA (9cRA). The MeBo cells exhibited some inhibition with these natural ligands, but they were not as sensitive. The addition of bLf had similar inhibitory effects on cell viability of the two mammary cell types. Applications of RA receptor (RAR) agonist indicated that the stimulation of the RAR in both mammary cell types was highly effective in inhibition of cell viability while the application of a RAR antagonist stimulated MeBo cell viability and inhibited BME-UV1 cell viability. Finally, the use of the RAR antagonist in conjunction with bLf indicated a rescue of the bLf affect in the MeBo cells suggesting that bLf is acting through the RAR receptor. This rescue effect of bLf was not observed with the BME-UV1 cells. We conclude that RAR interaction in bovine mammary cell types regulates cell viability *in vitro* and hypothesize that the natural ligands mediates regulation of bovine mammary cell viability *in vivo* and that bLf obstructs the retinoid induced cell viability inhibitory effect.

2.2 Introduction

The literature ascribes dietary provision of vitamin A equivalents (β-carotene, others) to specific functions, such as vision, immunity, and the regulation of cell growth, differentiation, and apoptosis (Sporn et al. 1994;Napoli 1996). Retinol is transported by retinol binding proteins (Noy 2000;Guo et al. 2004) in the circulation and rentinoic acid is bound to specific cellular retinoic acid binding protein (Newcomer 1995;Okuno et al. 1993). The main molecular mechanism by which retinoids (**RA**) and RA-like molecules exert biological effects is via specific interactions with nuclear retinoid receptors (Napoli 1996). These receptors are ligand-dependent transcriptional regulators that bind as dimers to specific DNA sequences or retinoic acid response elements (**RAREs**) within gene promoters and enhancers, and thereby regulate gene expression. The retinoid receptors currently include two subfamilies with multiple isoforms: the retinoic acid receptors (**RAR**s; RARα, RARβ, RARγ) and the retinoid X receptors (**RXR**s; RXRα, RXRβ, RXRγ) (Sporn et al. 1994). Recent discoveries have shown that RARβ consists of four isoforms (RAR beta 1 to RAR beta 4) that arise from two promoters with alternative slicing (Mendelsohn et al. 1994;Zelent et al. 1991;Alvarez et al. 2007).

Despite two decades of research on the function of RA, the physiological role of RA in mammary gland development is still not understood (Wang et al. 2005). Retinoid activated pathways typically decrease cell viability through apoptosis induction in breast cancer cell lines (Raffo et al. 2000). However, much less is known of the effects and mechanism of action of RA in normal mammary cells (non-cancerous) and the function of RA in bovine mammary cells remains a black box. The best characterization of bovine mammary cells has been conducted with the MAC-T cells that were created by the insertion of the SV-40 large-T antigen (Huynh et al. 1991). Woodward (Woodward et al.

1996) reported retinoid growth inhibition of bovine mammary cells and modulation of the insulin-like growth factor binding proteins (IGFBP). Cohick and Turner (Cohick and Turner 1998) utilized the MAC-T cells to study the insulin-like growth factor system when exposed to retinoids. They demonstrated changes in IGFBP by retinoic acid, specifically identifying an IGFBP-3 decrease. Purup (2001) tested retinoid effects on fresh mammary pieces excised from eight to nine month old Friesian heifers cultured in collagen gels and reported a 50% inhibition of growth at ~8 ng/mL for retinoic acid, the most potent retinoid of those tested. Most recently, Cheli (2003) utilized the BME-UV1, a cell line derived from the MAC-T cells, to confirm the findings of Woodward (1996) and Purup (2001) that retinoids partially inhibited cell viability increases. Administration of retinoids has generally been reported to inhibit proliferation of non-bovine normal mammary epithelial cells when cells are stimulated by growth factors (10 μg/mL insulin and 10 ng/mL EGF) (Lee et al. 1995;Kim et al. 2006). Studies with breast cancer cells show that retinoids may induce apoptosis (Bollag et al. 1997). Gene array expression studies of normal human mammary epithelial cells showed that 187 genes are significantly up-regulated and 109 genes are down-regulated when the cells were treated with an RXR-selective retinoid. These include genes involved in cell proliferation, signaling, apoptosis, and stress response, genes encoding transcription factors, and retinoid-regulated genes (Kim et al. 2006).

The objectives of this study were to evaluate natural retinoids and retinoid analogs for their effect on bovine mammary cells viability *in vitro*. Finally, because we have shown a lactoferrin:retinoid interaction in previous reports (Baumrucker et al. 2003;Baumrucker et al. 2005;Baumrucker et al. 2006), we wished to evaluate lactoferrin effects on cell viability.

2.3 Materials and Methods

Bovine Mammary Cells

All animal-related protocols were approved by an official institutional animal care and use committee. Bovine mammary epithelial cells BME-UV1, obtained from Politus (Zavizion et al. 1996), are immortalized by the insertion of the large T antigen. Primary bovine mammary cells (MeBo) were prepared from mammary tissue of two Penn State University Holstein cows that were collagenase digested and processed as described (O'Brien et al. 1981;Baumrucker et al. 1988) and either directly slow frozen in freezing solution consisting of DMEM/F12 media (Mediatech, Inc; Herndon, VA) with 20% FBS and 10% DMSO or plated in T75 flasks for epithelial growth, subsequent passages, and freezing in freezing solution with recorded passage. Upon culture slow growth after high passages, the initial frozen mammary cells could be plated to obtain fresh low passage cultures. Passages five to twelve were utilized for these experiments.

Bovine mammary cells (BME-UV1) and MeBo (primary culture) were grown in T25 or T75 flasks with DMEM/F12 Media with 10% fetal bovine serum (FBS; VWR International, West Chester, PA) and 1% Penn/Strep/Amphotericin B (Calbiochem/ EMD Chemicals, Inc., La Jolla, CA) until near confluence. The cells were lifted with 1% trypin (VWR International) and centrifuged at 1500 rpm for 10 min to form a pellet. The spent fluid was aspirated and the pellet resuspended in plating media. Cell viability was determined in a hemacytometer after appropriate dilutions in phosphate buffered saline (PBS). For the cell proliferation experiments, cells were plated at 8,000 cells per well in a 96 well plate with 100 μL of media containing 10% FBS media overnight. The following day, media was changed to experimental media containing 2% FBS with antibiotics as described above into which treatments were applied. Two percent FBS was selected as a

control because retinoids require binding to proteins in the circulation to facilitate cellular entry and biological action (Klaassen et al. 1999). The cells were cultured for up to 144 h with 24-h media changes.

Metabolic activity and cell viability

Alamar Blue (AbD Serotec, Ltd. Oxford, UK) was utilized to determine metabolic activity and to relate such activity to cell viability. The assay incorporates an indicator that fluoresces with an excitation of 530-560 nm and emission detected at 590 nm. The indicator is water soluble and non-toxic to the cells and detects mitochondrial redox activity with attached or suspended cells. The fluorescent reagent is reduced by most components of the electron transport chain.

At the time of measure, 10 μL of warmed Alamar Blue solution was added to the control conditioned media (2% FBS) in the 96 well-plate and returned to the 37°C culture incubator for 2h. The developed plate was read in a Perkin-Elmer Victor 3 plate reader (Waltham, MA) with fluorescent detection at the described wave lengths (above). Controls containing no cells were established for the Alamar Blue background and subtracted from all determined values. Fluorescence was always within Alamar Blue fluorescent capacity that was established by autoclaving wells with Alamar Blue without cells added. All experimental treatments were independently repeated three times with four replications for each cell type, treatment, and time period. A standard curve was separately conducted by the plating of different known quantities of cells and immediately, while in suspension, applying Alamar Blue for the 2-h incubation and subsequent reading in the Victor 3 plate reader. For further verification, cells were grown in 6-well plates in order to obtain more cells over similar time periods so that hemacytometer counts and application of Alamar Blue could be evaluated. The results of

these experiments produced a mathematical conversion for fluorescence to cell viability index that was utilized in the following experiments.

Experimental treatments

After establishing the Alamar Blue metabolic and cell viability index parameters, mammary cells were cultured with different treatments of retinoids purchased from Biomol (Biomol Res. Labs, Inc., Plymouth Meeting, PA). These included *all-trans* retinoic acid (atRA) and 9-cis retinoic acid (9cRA) as well as the utilization of specific retinoid receptor agonist (Am-580) and antagonist (Ro 41-5253). It is important to note that atRA while having higher specificity to RAR, it is also a ligand for RXR. Correspondingly, 9cRA has high affinity for RXR (Heyman et al. 1992), may exhibit some capacity to bind the RAR, may destabilize Vitamin D receptor RXR heterodimer-DNA complexes and has been shown to down-regulate both estrogen receptor RNAs (Rubin et al. 1994). The agonist AM-580 acts as a selective RARα agonist with no affinity for RXRs. Within the RARs, AM-580 has the reported Kd's; RARα= 0.036 μM; RARβ = 1.361 μM; RARγ = 3.824 μM. The antagonist Ro 41-5253 is a selective RARα antagonist with Kd's of RARα= 0.060 μM; RARβ = 2.40 μM; RARγ = 3.30 μM. Stock solutions of retinoids were made up in ethanol at 10 mM concentrations and were stored in the dark at -80 °C until utilized for experimental treatments. Dilutions of the stock solutions were made in culture media and retinoid treatment ethanol was < 0.1%. Bovine lactoferrin was purchased ((USB Corp., Cleveland, OH) and stock solution of 130 μM (10 mg/mL) with control culture media was made and alequoted into 1 ml portions and frozen at -80C until experimental use. Treatments were applied to the experimental media (2% FBS+) after overnight cellular attachment. Media was changed at 24 h to ascertain fresh media effects. All media and cell culture treatments were made in protected light environments and were shielded from light for the duration of the experiments.

Statistical Analysis

Alamar Blue proliferation assays were analyzed by ANOVA (SAS 2007) with repeated measures over time. When found to be significant ($P<0.05$), by ANOVA, differences among means were tested with SAS Proc GLM Duncan/Waller means separation analysis. The model included the effects of treatment, time and treatment*time interaction. All analyses showed no treatment*time interactions.

2.4 Results

Alamar Blue and cell viability
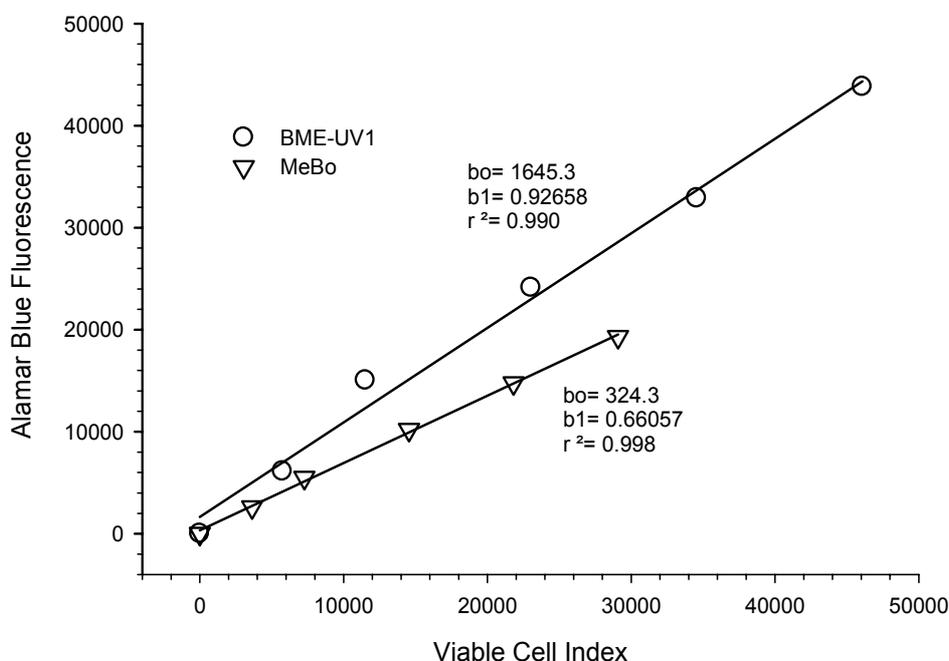
Figure 2-1. Relationship between Alamar Blue fluorescence and bovine mammary cell viability. Cells were lifted from T-75 flasks, counted by hemecytometer and plated at different concentrations shown on the X axis. Alamar Blue was immediately added and the cells incubated for 2 h and fluorescence determined. Regression analysis of each line is shown.

Figure 2-1 shows that relationship of Alamar Blue fluorescence to cell viability index of the two bovine mammary cell types *in vitro*. This figure represents ~80% confluency cells that were lifted and immediately plating at the shown numbers and tested for cell viability (Alamar Blue). It was not surprising to find that the immortalized bovine mammary cells (BME-UV1) showed greater metabolic activity (~1.4x) than the primary mammary cells (MeBo) when cultured at equivalent numbers. This suggests that there are more mitochondria or that they have a greater capacity for energy production in the immortalized cells when compared to the primary cells. Because we were concerned that cell viability index might change with time in culture, separate experiments with cells

grown in 6-well plates to evaluate both hemacytometer counts with Alamar Blue assays after lifting indicated that the cell viability index relationship with fluorescence shown in Figure 2-1 was valid (data not shown). Slope of the lines on Figure 2-1 determined by regression analysis were used to estimate cell viability in subsequent experiments.

Calculations of doubling time (Mai et al. 2007) with hemacytometer counts for both bovine mammary cells showed that the immortalized mammary cell (BME-UV1) had a shorter doubling time and therefore a higher rate of proliferation or lower rate of apoptosis. BME-UV1 doubled in 23.2 h and MeBo doubled in 41.7 h when cultured in control 2% FBS media.

Natural retinoid ligands

Figure 2-2 shows the results of the application of natural retinoid ligands (atRA and 9cRA) to the two bovine mammary cell types. As shown previously in Figure 2-1, the immortalized BME-UV1 cells showed much greater cell viability than that of the primary cells (MeBo) in these assays (Figure 2-2). In addition, the BME-UV1 cells exhibited a more clearly defined cell viability inhibition when the retinoids were applied, perhaps due to their cell doubling response. The cell type BME-UV1 was sensitive to atRA at 0.1 μM concentration that was effective at cell viability inhibition as the higher doses. A weaker effect was achieved with 9cRA. Application of 9cRA showed more clear separation of the dose on inhibitory results. The decline in cell viability at ~70,000 cells may be ascribed to confluency for these cells in a 96 well culture plate. However, the decline in BME-UV1 cell viability at ~50,000 cells per well at >96 h treatment time is currently unknown, but was not due to confluency because these cells reach confluency at >80,000 cells/well. Thus, both atRA and 9cRA have a biphasic action on the BME-UV1 cells. An initial cell viability inhibition followed by a rapid decline that occurs around 50,000 cells. The

primary cells (MeBo) suggested a similar pattern of inhibition with both natural ligands, but these cells do not exhibit the 96 h decline with retinoid treatment. Longer term culture of the MeBo cells (192 h) to obtain more cells did not indicate a change in cellular viability that was similar to the changes that occur with BME-UV1 cells around 96 h (data not shown).
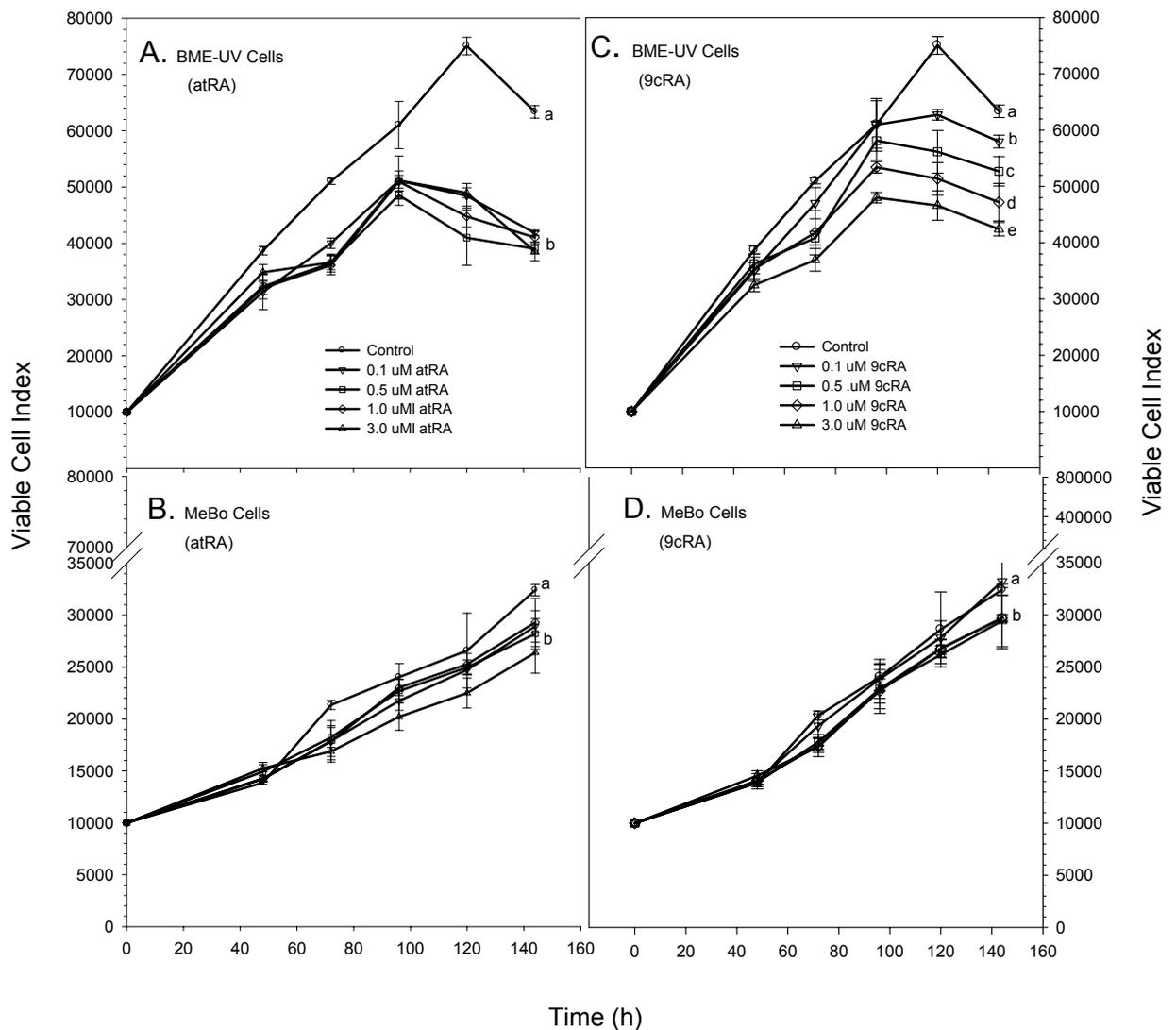
Figure 2-2. Cell viability effects of natural retinoids, including *all-trans* retinoic acid (atRA) and *9-cis* retinoic acid (9cRA), on bovine mammary cell line BME-UV1 and primary cells MeBo *in vitro*. Cells were cultured in 2% FBS and the addition of retinoid as shown in the legend. Treatments with different letters within a sub-figure are significantly different (P<0.05).

Bovine lactoferrin effects

The effect of bovine lactoferrin application on bovine mammary cells *in vitro* are shown in Figure 2-3. Concentrations of bLf from 0.16 to 2.5 μM had cell viability inhibitory effects on both mammary cells. A dose inhibitory effect on both cell types was demonstrated with the BME-UV1 showing greater sensitivity and the occurrence of the later time period decline (> 120 h) that was demonstrated with natural ligands.
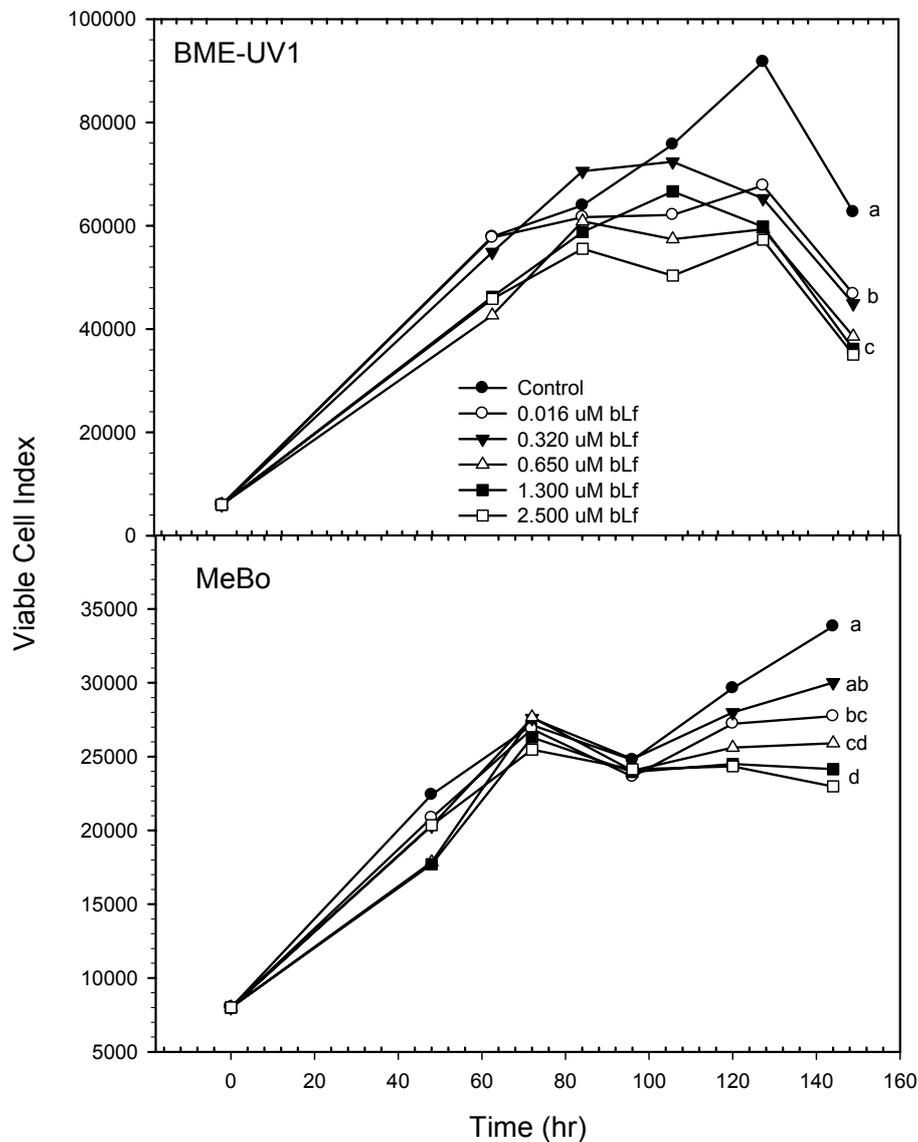
Figure 2-3. Bovine lactoferrin (bLf) effects on cell viability with bovine mammary cells in vitro. Cells were cultured in 2% FBS and the addition of bLf as shown in the legend. Treatment with different letters within each sub-figure are significantly different (P<0.05).

Retinoid analog effects
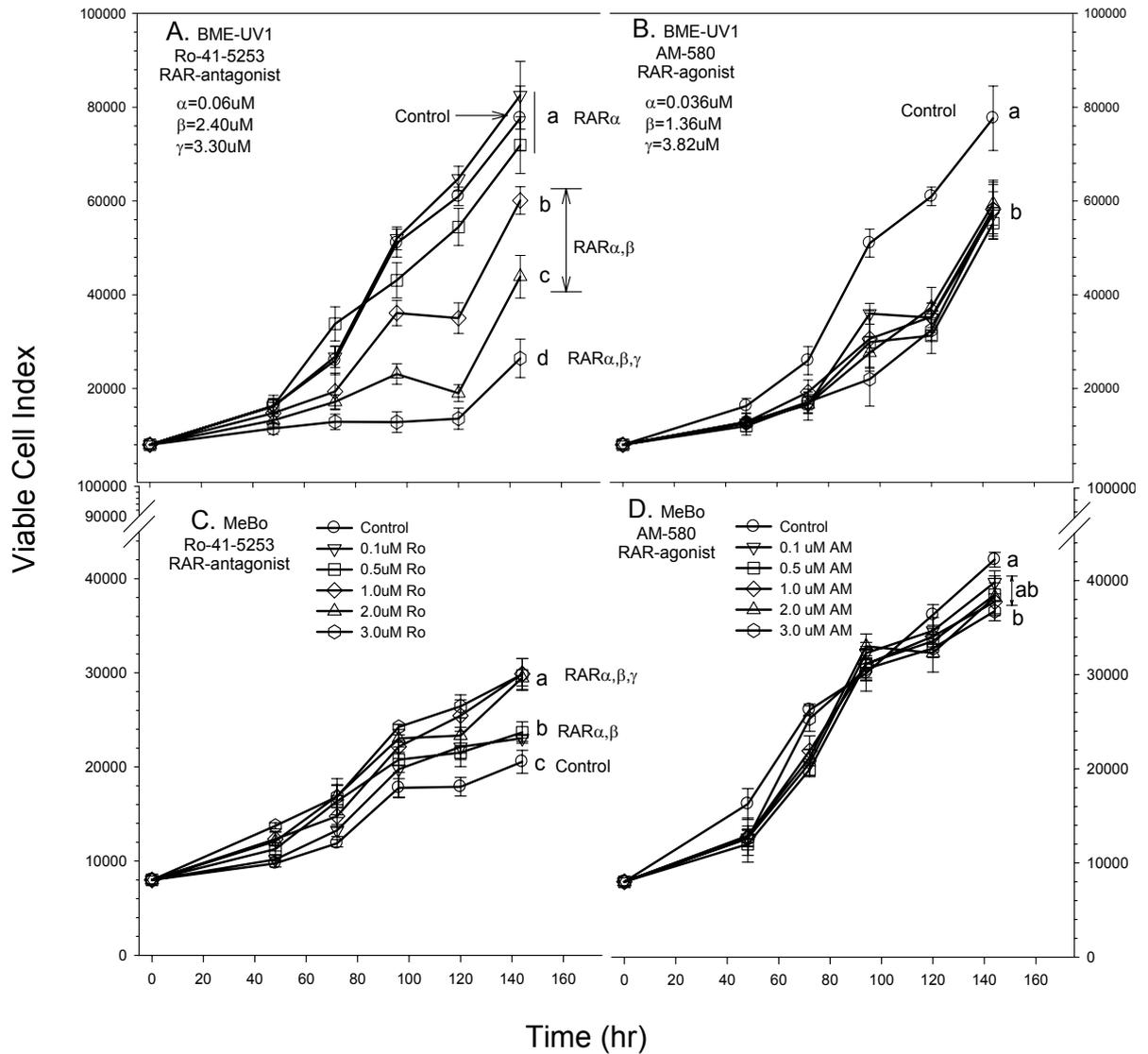
Figure 2-4 Specific retinoic acid receptor (RAR) analog treatment effects on bovine mammary cell viability *in vitro*. Graph A & B are BME-UV1 and C & D are primary cells (MeBo). Agonist and antagonist concentrations that the literature suggests will impact the specific retinoid receptor isotypes are shown in the legend. Different letters (a-d) within each sub-figure are significant (P=<0.05).

Figure 2-4 shows the effect of specific RAR analogs on the two bovine mammary cell types. Ro 41-5253 (Ro) is a specific RAR antagonist that has specificity to the specific RAR receptors (α, β, & γ) in a dose dependent manner (see legend box; Figure 2-4A). It is important to note that while the natural ligand application to the cells had a biphasic effect on the BME-UV1 cells (Figure 2-2A & C), the application of the analogs did not show this response (Figure 2-4A & B). Application of the Ro 41-5253 analog to the BME-UV1 cells (Figure 2-4A) reveals that while a low dose of Ro 41-5253 has no growth inhibiting effect on the cells, the higher doses (1 and 2 μM) significantly affect cell proliferation ($P < 0.05$) and this is likely due to the effect of completely blocking the RARβ receptor (1 μM) and/or blocking the RARγ receptor, as indicated by the antagonist receptor specificity. Much higher concentrations that would be expected to block all RAR receptors (α,β,& γ) caused almost complete blockage of cell viability changes in the BME-UV1 cells. Blocking the RAR receptors was expected to result in less or no cell viability changes because RAR stimulation results in less cell viability (Figure 2-2).

As anticipated, the application of Ro 41-5253 to block the RAR receptors in the MeBo cells (Figure 2-4C) resulted in a stimulated increase in cell viability that occurred at lower doses and showed that at the high concentration (3 μM), when all RAR receptors were thought to be blocked, a significant increase occurring compared to the lower doses. This supports the finding that the natural ligand inhibits cell viability as shown in Figure 2-2. Furthermore, this demonstrates a critical difference between these two mammary cells whereas blocking the RAR receptors in one cell decreased cell viability while the other increases cell viability. Obviously, the retinoid signaling pathways for these cells are different.

Application of the RAR agonist (AM-580) showed that stimulation of the RARα receptor at all doses of AM-580 reduced the cell viability with both cell types. Because the higher doses that would be expected to impact the RARγ were not significantly different than the low dose (0.1μM AM-580) indicates that the RARα is the main contributor of the reduced cell viability effect with the BME-UV1 cells. While a significant reduction of cell viability occurred with the MeBo cells, it was not as pronounced as that of the BME-UV1 cells, but does agree with the application of natural ligands (Figure 2-2).

Retinoid, analogs and lactoferrin interactions
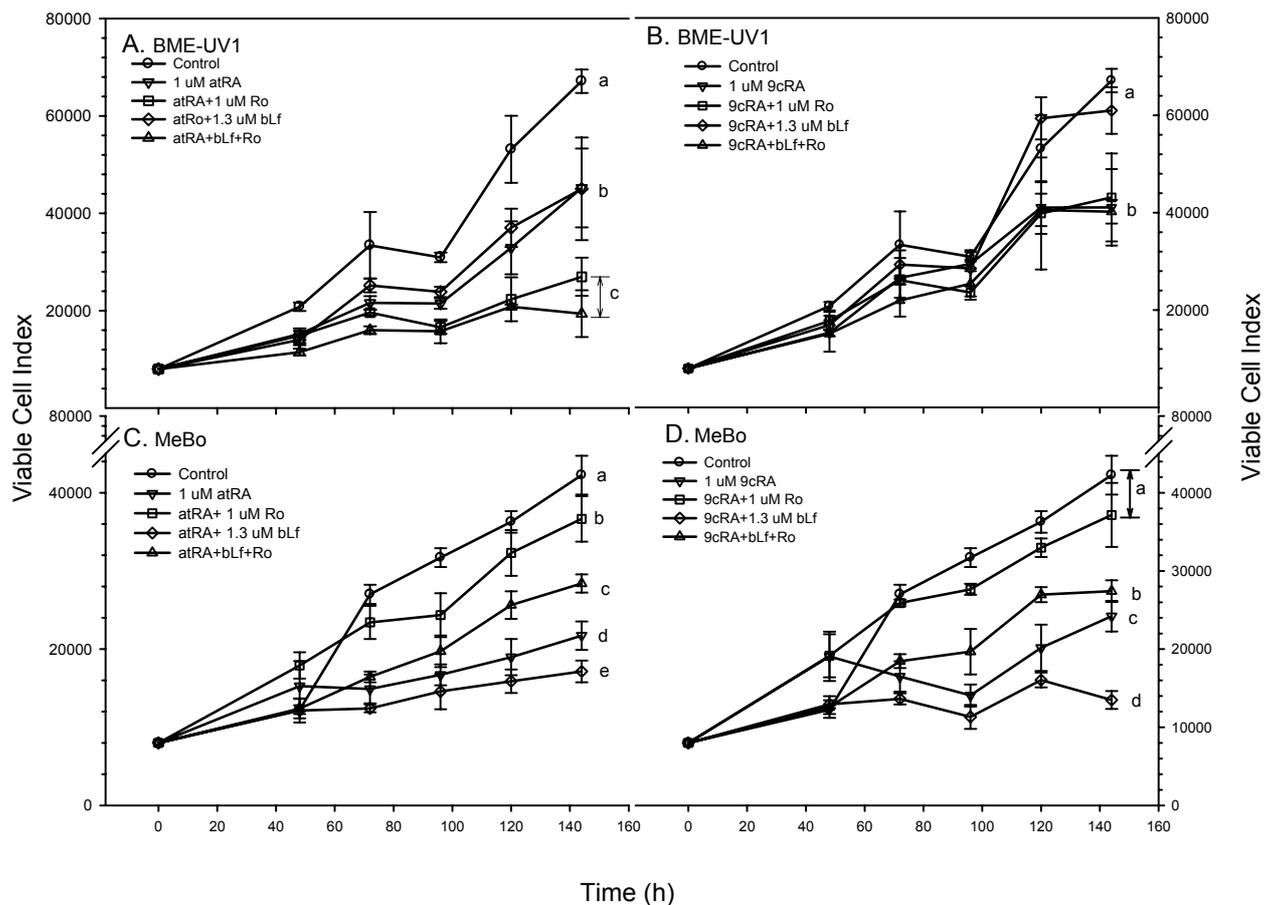
Figure 2-5. Interaction of retinoids, retinoid analogs, and bovine lactoferrin (bLf) with bovine mammary cell viability *in vitro*. Retinoids were added at 1 μM, bLf at 1.3 μM , and analogs at 1 μM. Different letters within each graph are significant ($P<0.05$).

Figure 2-5 shows the effect of exogenous addition of bLf in addition to the application of the RARα antagonist (Ro) to the two bovine mammary cell types *in vitro*. As shown previously in Figure 2-2 with natural ligands at 1 uM concentration, the MeBo cells are inhibited by the application of 1 uM atRA (Figure 2-5A) or 1 uM 9cRA (Figure 2-5C). The addition of bLf to the natural retinoid ligands (atRA and 9cRA) reduced cell viability to a significantly lower level ($P < 0.05$). Adding Ro 41-5253 to the natural ligand alone or the ligand+bLf significantly reversed, prevented, or minimized the decrease cell viability suggesting that bLf is interacting with the RAR receptors.

Figure 2-5 B and D indicate that the application of natural retinoid ligands reduce cell viability with the BME-UV1 cells and while bLf addition independently to these cells inhibits cell viability (Figure 2-3), when added with the atRA, it shows no effect. However, added with 9cRA, it rescues cell viability effects, which is negated when Ro 41-5253 is added to the 9cRA and bLf application.

2.5 Discussion

The literature with human cells and experimental rodent models demonstrate that retinoids have regulatory roles on growth and differentiation (Sporn et al. 1994;Napoli 1996). Mammary epithelial cells respond to retinoids, inducing apoptosis or cell arrest (Seewaldt et al. 1997;Purup et al. 2001). However, though some work with normal mammary epithelial cells has been conducted to define retinoid effects, there are few reports of mechanism of action. Woodward et al. (1996), utilizing a bovine mammary cell line [MAC-T; (Huynh et al. 1991)] that is a precursor of the BME-UV1 (Zavizion et al. 1996) described below, and our preliminary data with primary bovine mammary epithelial cell (MeBo) show that the cells are affected by retinoids. Furthermore, this is supported by a report (Purup et al. 2001) that showed significant reduction in cell proliferation following treatment with RA in primary bovine mammary cells cultured in collagen gels. However, these previous studies utilized high insulin (10 μg/mL) and low IGF-I (50 ng/mL) in the culture media to stimulate high cellular growth and the report of Purup et al.(2001) suggested the retinoid inhibitory effect to be due to IGFs and IGFBP-3 changes. In still another study with RA (atRA; retinol, and a synthetic retinoid) effects on BME-UV1 (Cheli et al. 2003) showed that retinoids are partial inhibitors of the BME-UV1 cell growth. However, this report utilized 1.5×10^5 cells/well in a 96 well culture dish and also utilized 250 μL of culture media that would restrict O_2 availability (it is recommended that 100 μL of media be utilized in 96-well plates). These cells would be confluent at plating and some inhibition may not have been induced by the treatment. Furthermore, any growth potential may have been masked. This manuscript also did not discuss the occurrence or mechanism of retinoid action in the cells.

 Retinoids and mammary cells

Our comparison of cell viability changes of the two bovine mammary cell types validates retinoid inhibitory effects on the BME-UV1 cells (MAC-T) (Purup et al. 2001;Woodward et al. 1996;Cheli et al. 2003) and extends the characterization to show a greater inhibitory sensitivity to atRA over 9cRA. Perhaps this reflects the cross-over nature of atRA to bind both RAR and RXR receptors and the specific action of 9cRA for the RXR and RXR:other ligand interactions. Furthermore, application of the RAR specific analogs at varying concentrations that are known to have RAR receptor isotype specificity differences has demonstrated antagonist blocking and stimulation (agonist) of the RAR receptors α, β, & γ in succession. Surprisingly, both stimulation (agonist) and blocking (antagonist) resulted in a cell viability reduction with the immortalized cell line (BME-UV1). It is noteworthy that the antagonist at the highest concentration (3 μM) almost totally reduced cell viability with the BME-UV1 that far exceeded the inhibition effects of the natural ligand (atRA) shown in Figure 2-2. The demonstration that the application of the RAR antagonist to the primary cells resulted in an increase in the viability of cells was anticipated and would be a more expected outcome in light of the agonist inhibition effects with these MeBo cells. This differential effect between the immortalized and primary bovine mammary cells clearly indicates retinoid signaling mechanisms that exist in the BME-UV1 cells that are not like that of the primary MeBo cells.

Lactoferrin and the mammary cells

The application of bLf to both mammary cells results in decreased cell viability that showed a dose response. Only the MeBo cells show expression of the bLf message (Baumrucker et al. 2005) but the extent of production of the cell product would likely not contribute significantly with daily culture media changes that we employed. How exogenously added bLf may interact with nuclear receptors is currently unknown, but it

has been suggested they enter the cell via the transferrin receptor (Ashida et al. 2004;Suzuki et al. 2005) or still other surface proteins (Spik et al. 1994). Once in the cytoplasm, lactoferrin clearly contains nuclear localization sequences (Baumrucker et al. 2005) that would provide nuclear entrance opportunities that have not been characterized. Previous reports have indicated that bLf interacts with retinoid reporter constructs in human breast cancer cells (Baumrucker et al. 2005) and alters mammary function when Lf is overexpressed in mouse mammary gland when the animals are fed high levels of retinoids (Baumrucker et al. 2006). A recent study by Riley et al (2008) has shown that exogenous addition of bLf on primary bovine mammary epithelial mammospheres decreased casein mRNA expression and reducing mammary cell viability while siRNA suppression of bLf expression increased casein message expression. This finding agrees with the transgenic mouse study (Baumrucker et al. 2006) and would agree with the findings of this report.

Interaction of retinoids, analogs, lactoferrin and mammary cells

Ro 41-5253 selectively binds to RARα, but does not influence RARα/RXRα hertodimerization and DNA binding (Toma et al. 1998a;Toma et al. 1998a). Retinoids have been shown to induce apoptosis in MCF-7 and ZR-75.1 breast cancer cells (Toma et al. 1997;Toma et al. 1998b) and the addition of Ro 41-5253 counteracts this effects in other epithelial cells (Zhang et al. 1995). This was our finding with the primary bovine cells, but not the immortalized BME-UV1 cells. Table 2-1 summary of Figure 2-5 demonstrations that the retinoid antagonist (Ro 41-5253) partially rescues atRA inhibitory effects with MeBo cells and is an indicator that this partial rescue effect is via RAR receptors. However, the blocking of the RAR receptors in atRA treated BME-UV1 cells resulted in a reduction in cell viability. This finding cannot be explained by RAR retinoid receptor interactions. While the application of bLf separately (in 2% FBS) to the

mammary cells was shown to result in a reduction of cell viability (Fig 3), the application of bLf to the cells treated with atRA had no effect on the BME-UV1 cell viability, but decreased cell viability beyond the natural ligand effect with the MeBo cells. Combining atRA+Ro 41-5253+Lf resulted in no additional effect BME-UV1 cells while MeBo cells were partially rescued (Table 2-1).

Table 2-1. Summary of retinoids, retinoid antagonist, and b-lactoferrin on bovine mammary cell viability *in vitro.*

Treatment	MeBo	BME-UV1
atRA	↓↓	↓↓
atRA+Ro	↓↑	↓↓↓
atRA+bLf	↓↓↓	↓↓
atRA+Ro+bLf	↓↓↑	↓↓↓
9cRA	↓↓	↓↓↓
9cRA+Ro	↓↑	↓↓↓
9cRA+bLf	↓↓↓	↔
9cRA+Ro+bLf	↓↓↑	↓↓↓

The application of Ro 41-5253 with 9cRA showed no difference in cell viability with the BME-UV1 cells, but did rescue cell viability with the MeBo cells. The application of bLf with 9cRA to the BME-UV1 totally rescued the 9cRA treatment effect (equal to control), but reduced further cell viability with the MeBo cells. Application of Ro 41-5253 to 9cRA + bLf invokes a partial rescue of MeBo cells that is similar to that of the antagonist when applied with atRA, but completes the rescue of BME-UV1 cells to control levels when 9cRA is the treatment. Application of the three treatments (9cRA, bLf and Ro 41-5253) removes the effect (rescue or decrease) of bLf on the two cells.

Table 2-1 shows the summation of the different effects of treatments shown in Figure 2-5 on the two mammary cells. Arrows up (increase), down (decrease), and horizontal (equal to control) indicate the change in cell viability that occurs with the 4 treatments relative to the control and each other. Bblocking the RAR receptors with Ro 41-5253 partially rescues MeBo from atRA or 9cRA treatments indicating the RAR contribution to retinoid treatment. This is not the case for BME-UV1 where blocking RAR action worsens the effect of atRA and 9cRA in the reduction of cell viability, suggesting action independent of RAR receptors. This result is supported when 9cRA treatment of the BME-UV1 cells is totally rescued by the application of bLf, but did rescue the same event in the MeBo cells.

Conclusion

We show that the retinoid impact upon cell viability between the two mammary cells is different and that the primary mammary cells are likely better models of retinoid signaling that occurs in mammary cells *in vivo*. Furthermore, the data with MeBo shows that bLf interacts with retinoid signaling by decreasing cell viability with both atRA and 9cRA and that this reduction is partially rescued by the application of an RAR antagonist (Ro 41-5253) in a manner that is consistent with the rescue of the retinoid application effect without bLf. The *in vivo* role of bLf during late pregnancy and involution has been ascribed to iron binding and reduced mammary bacterial infections. These studies indicate that Lf has other roles that involve interaction with retinoid receptors and influences in cellular viability that can be either cell cycle or apoptotic events.

Reference List

Alvarez S, Germain P, Alvarez R, Rodriguez-Barrios F, Gronemeyer H, de Lera AR. 2007. Structure, function and modulation of retinoic acid receptor beta, a tumor suppressor. Int J Biochem Cell Biol 39:1406-1415.

Ashida K, Sasaki H, Suzuki YA, Lonnerdal B. 2004. Cellular internalization of lactoferrin in intestinal epithelial cells. Biometals 17:311-315.

Baumrucker CR, Deemer KP, Walsh R, Riss TL, Akers RM. 1988. Primary culture of bovine mammary acini on a collagen matrix. Tissue Cell 20:541-554.

Baumrucker CR, Gibson CA, Schanbacher FL. 2003. Bovine lactoferrin binds to insulin-like growth factor-binding protein-3. Domest Anim Endocrinol 24:287-303.

Baumrucker CR, Saurer S, Blum JW, Jungi T, Friis RR. 2006. Lactoferrin (Lf): Retinoid interactions in the mammary glands of transgenic mice overexpressing human Lf. Mol Cell Endocrinol 251:56-66.

Baumrucker CR, Schanbacher F, Shang Y, Green MH. 2005. Lactoferrin interaction with retinoid signaling: Cell growth and apoptosis in mammary cells. Domest Anim Endocrinol 30:289-303.

Bollag W, Isnardi L, Jablonska S, Klaus M, Majewski S, Pirson W, Toma S. 1997. Links between pharmacological properties of retinoids and nuclear retinoid receptors. Int J Cancer 70:470-472.

Cheli F, Politis I, Rossi L, Fusi E, Baldi A. 2003. Effects of retinoids on proliferation and plasminogen activator expression in a bovine mammary epithelial cell line. J Dairy Res 70:367-372.

Cohick WS, Turner JD. 1998. Regulation of IGF binding protein synthesis by a bovine mammary epithelial cell line. J Endocrinol 157:327-336.

Guo XH, Chu MX, Zhou ZX. 2004. [Molecular biology of the retinol-binding proteins and their genes.]. Yi Chuan 26:257-262.

Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, Thaller C. 1992. 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. Cell 68:397-406.

Huynh HT, Robitaille G, Turner JD. 1991. Establishment of bovine mammary epithelial cells (MAC-T): An *in vitro* model for bovine lactation. Exp Cell Res 197:191-199.

Kim HT, Kong G, Denardo D, Li Y, Uray I, Pal S, Mohsin S, Hilsenbeck SG, Bissonnette R, Lamph WW, Johnson K, Brown PH. 2006. Identification of biomarkers modulated by the rexinoid LGD1069 (bexarotene) in human breast cells using oligonucleotide arrays. Cancer Res 66:12009-12018.

Klaassen I, Brakenhoff RH, Smeets SJ, Snow GB, Braakhuis BJ. 1999. Considerations for in vitro retinoid experiments: importance of protein interaction. Biochim Biophys Acta 1427:265-275.

Lee P-PH, Lee M-T, Darcy KM, Shudo K, Ip MM. 1995. Modulation of normal mammary epithelial cell proliferation, morphogenesis, and functional differentiation by retinoids: A comparison of the retinobenzoic acid derivative RE80 with retinoic acid. Endocrinology 136:1707-1717.

Mai SJ, Ooka T, Li DJ, Zeng MS, Jiang RC, Yu XJ, Zhang RH, Chen SP, Zeng YX. 2007. Functional advantage of NPC-related V-val subtype of Epstein-Barr virus nuclear antigen 1 compared with prototype in epithelial cell line. Oncol Rep 17:141-146.

Mendelsohn C, Larkin S, Mark M, LeMeur M, Clifford J, Zelent A, Chambon P. 1994. RAR beta isoforms: distinct transcriptional control by retinoic acid and specific spatial patterns of promoter activity during mouse embryonic development. Mech Dev 45:227-241.

Napoli JL. 1996. Biochemical pathways of retinoid transport, metabolism, and signal transduction. Clin Immunol Immunopathol 80:S52-S62.

Newcomer ME. 1995. Retinoid-binding proteins: structural determinants important for function. FASEB J 9:229-239.

Noy,N. (1999.) in Handbook of Experimental Pharmacology: The Biochemical and Molecular Basis of Vitamin A and Retinoid Action (Nau, H., and Blaner, W. S., eds), Springer-Verlag, Berlin.

Noy N. 2000. Retinoid-binding proteins: mediators of retinoid action. Biochem J 348 Pt 3:481-495.

O'Brien TL, Riss TL, Baumrucker CR. 1981. Ultrastructure of collagenase-isolated acini (alveoli) from Lactating mammary tissue. Trans Amer Micros Soc 100(3):306-315.

Okuno M, Numaguchi S, Moriwaki H, Muto Y. 1993. [Cellular retinoid-binding proteins]. Nippon Rinsho 51:879-885.

Purup S, Jensen SK, Sejrsen K. 2001. Differential effects of retinoids on proliferation of bovine mammary epithelial cells in collagen gel culture. J Dairy Res 68:157-164.

Raffo P, Emionite L, Colucci L, Belmondo F, Moro MG, Bollag W, Toma S. 2000. Retinoid receptors: pathways of proliferation inhibition and apoptosis induction in breast cancer cell lines. Anticancer Res 20:1535-1543.

Riley LG, Williamson P, Wynn PC, Sheehy PA. 2008. Lactoferrin decreases primary bovine mammary epithelial cell viability and casein expression. J Dairy Res 75:135-141.

Rubin M, Fenig E, Rosenauer A, Menendez-Botet C, Achkar C, Bentel JM, Yahalom J, Mendelsohn J, Miller WH, Jr. 1994. 9-Cis retinoic acid inhibits growth of breast cancer cells and down-regulates estrogen receptor RNA and protein. Cancer Res 54:6549-6556.

SAS. 2007. SAS/STAT Software. Cary, NC.: SAS Inst Inc.

Seewaldt VL, Kim JH, Caldwell LE, Johnson BS, Swisshelm K, Collins SJ. 1997. All-trans-retinoic acid mediates G1 arrest but not apoptosis of normal human mammary epithelial cells. Cell Growth Differ 8:631-641.

Spik G, Legrand D, Leveugle B, Mazurier J, Mikogami T, Montreuil J, Pierce A, Rochard E. 1994. Characterization of Two Kinds of Lactotransferrin (Lactoferrin) Receptors on Different Target Cells. Adv Exp Med Biol13-19.

Sporn MB, Roberts AB, Goodman DS. 1994. The Retinoids; Biology, Chemistry, and Medicine. New York: Raven Press. 679 p.

Suzuki YA, Lopez V, Lonnerdal B. 2005. Mammalian lactoferrin receptors: structure and function. Cell Mol Life Sci 62:2560-2575.

Toma S, Isnardi L, Raffo P, Dastoli G, De Francisci E, Riccardi L, Palumbo R, Bollag W. 1997. Effects of All-*trans*-Retinoic Acid and 13-*cis*-Retinoic Acid on Breast-Cancer cell Lines: Growth Inhibition and Apoptosis Induction. Int J Cancer 70:619-627.

Toma S, Isnardi L, Raffo P, Riccardi L, Dastoli G, Apfel C, LeMotte P, Bollag W. 1998a. RARalpha antagonist Ro 41-5253 inhibits proliferation and induces apoptosis in breast-cancer cell lines. Int J Cancer 78:86-94.

Toma S, Isnardi L, Riccardi L, Bollag W. 1998b. Induction of apoptosis in MCF-7 breast carcinoma cell line by RAR and RXR selective retinoids. Anticancer Res 18:935-942.

Wang YA, Shen K, Wang Y, Brooks SC. 2005. Retinoic acid signaling is required for proper morphogenesis of mammary gland. Dev Dyn 234:892-899.

Woodward TL, Turner JD, Hung HT, Zhao X. 1996. Inhibition of cellular proliferation and modulation of insulin- like growth factor binding proteins by retinoids in a bovine mammary epithelial cell line. J Cell Physiol 167:488-499.

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

Zelent A, Mendelsohn C, Kastner P, Krust A, Garnier JM, Ruffenach F, Leroy P, Chambon P. 1991. Differentially expressed isoforms of the mouse retinoic acid receptor beta generated by usage of two promoters and alternative splicing. EMBO J 10:71-81.

Zhang LX, Mills KJ, Dawson MI, Collins SJ, Jetten AM. 1995. Evidence for the involvement of retinoic acid receptor RAR alpha-dependent signaling pathway in the induction of tissue transglutaminase and apoptosis by retinoids. J Biol Chem 270:6022-6029.

Chapter 3

Retinoid analogs and lactoferrin differentially affect retinoid receptor mRNA experssion in bovine mammary cells *in vitro*

3.1 Abstract

The bovine immortalized (BME-UV1/MAC-T) and primary mammary cells (MeBo) were utilized to evaluate the expression of retinoid receptor mRNA and the outcome of the application of natural retinoids, retinoid analogs, bovine lactoferrin (bLf), and hormones on retinoid mRNA expression *in vitro*. Retinoids have been shown to induce apoptosis or a G1 arrest and thereby reduce mammary cell number. In addition, milligram concentrations of bLf appear in the mammary gland prior to the initiation of lactation and at the de-differentiation of the mammary gland, but remains at extremely low levels during lactation. Lf has a NLS and enters the nuclear compartment with potential for gene regulation. Retinoid receptor subtypes mRNA expression assays were established and quantitative PCR (qPCR) utilized to compare mammary cell receptor expression to bovine liver expression. Under control conditions all RAR receptor mRNAs were expressed in both cells, but RARβ expression was weak and RARγ highly expressed when compared to liver expression. No RXRγ receptor mRNA was detected in the two mammary cells and the RARα and RARβ receptors were weakly expressed (relative to liver) under control conditions. An application of RAR receptor agonist (AM-580) increased RARβ receptor mRNA in MeBo cells, but when bLf was included an additional stimulation of the RARβ in MeBo cells was observed. However, the application of a RAR antagonist (Ro-41-5253) treatment resulted in decreased expression of RARβ receptors and an increase in RXR receptors that was increased further by the application

of bLf to the antagonist. Treatment of the primary MeBo cells with lactogenic hormones induced changes in RAR receptors. The addition of bLf to the lactogenic hormones did not alter RAR receptor expression. We conclude that retinoids and bLf interact with RAR signaling in bovine mammary cells and regulate cell number *in vitro*, but does not appear to interact with lactogenic hormones.

3.2 Introduction

The dietary provision of vitamin A equivalents (β-carotene, others) has generally been linked to regulation of cell growth, differentiation of epithelial cells (Gudas, 1992). Retinol is transported by retinol binding proteins (Guo et al., 2004; Noy, 2000) in the circulation and rentinoic acid is bound to specific cellular retinoic acid binding protein (Newcomer, 1995; Okuno et al., 1993). The retinoids (**RA**) are a class of chemical compounds that are related chemically to vitamin A. The main molecular mechanism by which RA and RA-like molecules exert biological effects is via specific interactions with nuclear retinoid receptors (Napoli, 1996). All-trans retinoic acid (**atRA**) and 9-cis retinoic acid (**9cRA**) are the natural ligands of retinoid receptors. The main molecular mechanism by which retinoids (**RA**) and RA-like molecules exert biological effects is via specific interactions with nuclear retinoid receptors (Napoli, 1996). These receptors are ligand-dependent transcriptional regulators that bind as dimers to specific DNA sequences or retinoid response elements (**RAREs**) within gene promoters and enhancers, and thereby regulate gene expression. The retinoid receptors currently include two subfamilies with multiple isoforms: the retinoic acid receptors (**RAR**s; RARα, RARβ, RARγ) and the retinoid X receptors (**RXR**s; RXRα, RXRβ, RXRγ) (Sporn et al., 1994). The expression of retinoid receptors varies from tissue to tissue (Redfern, 1997; Zhang et al., 1996). RARβ is expressed in normal epithelial cells and has been reported to be induced with the application of retinoic acid (Hoffmann et al., 1990).

Despite two decades of research on the function of retinoic acid, the physiological role of RA in mammary gland development is still not understood (Wang et al., 2005). Retinoid activated pathways typically inhibit proliferation through apoptosis induction in breast cancer cell lines (Raffo et al., 2000). However, in normal mammary cells, all-trans

retinoic acid (atRA) is reported to medicate a G1 cellular arrest (Seewaldt et al., 1999) and to alter the appearance of growth stimulators or growth inhibitors (Yang et al., 1999). While some characterization of bovine mammary cells have appeared (Cohick and Turner, 1998; Purup et al., 2001; Woodward et al., 1996), but none have evaluated retinoid receptor expression. There may be an issue concerning immortilized cells because these altered cells are reported to express low or no RARβ receptor (Swisshelm et al., 1994; Yang et al., 1999).

Administration of retinoids has generally been reported to inhibit proliferation of non-bovine normal mammary epithelial cells when cells are stimulated by growth factors (10 μg/ml insulin and 10 ng/ml EGF) (Kim et al., 2006; Lee et al., 1995). Studies with breast cancer cells show that retinoids may induce apoptosis (Bollag et al., 1997). Gene array expression studies of normal human mammary epithelial cells showed that 187 genes are significantly up-regulated and 109 genes are down-regulated when the cells were treated with an RXR-selective retinoid. These include genes encoding for cell proliferation, cell cycle signaling, cell apoptosis, cell stress response, transcription factors and retinoid-regulated genes (Kim et al., 2006).

The objectives of this study were to characterize the expression of retinoid receptor mRNA and to evaluate natural retinoid and retinoid analogs effect on retinoid receptor mRNA *in vitro*. Finally, because we have shown a lactoferrin:retinoid interaction in previous reports (Baumrucker et al., 2003; Baumrucker et al., 2005; Baumrucker et al., 2006), we wished to evaluate lactoferrin effects on retinoid receptor expression.

3.3 **Materials and Methods:**

Bovine Mammary Cells:

BME-UV1, immortalized bovine mammary epithelial cells (MAC-T subclone) were obtained from Politus (Zavizion, 1996), are immortalized by the insertion of the large T antigen. Primary bovine mammary cells (MeBo) were prepared from two Penn State University Holstein dairy cows mammary tissue that was collagenase digested and processed as described ((Riss et al., 1981)) and either directly slow frozen in freezing solution consisting of DMEM/F12 with 20% FBS and 10% DMSO or platted in T75 flasks for epithelial growth, subsequent passages, and freezing in freezing solution with recorded passage. Subsequent passages (5-12) were utilized for these experiments.

The two bovine mammary cell types were grown in T25 or T75 flasks with DMEM/F12 Media (Mediatech, Inc; Herndon, VA) with 10% FBS and 1% Penn/Strep/Amphotericin B (Calbiochem/ EMD Chemicals, Inc., La Jolla, CA) until near confluence. The cells were lifted with 1% trypin (VWR International) and centrifuged at 1500 rpm for 10 min to form a pellet. The spent fluid was aspirated and the pellet resuspended in platting media. Cell numbers were determined in a hemacytometer after appropriate dilutions in PBS. Cells were platted at $1x10^5$cells per well in a 12 well plate with 200 ìl of platting media containing 10% FBS media overnight. The following day, media was changed to experimental media containing 2% FBS with antibiotics as described above for experimental purposes into which treatments were applied. Two percent FBS was selected as a control because retinoids require binding to proteins in the circulation to facilitate cellular entry and biological action (Klaassen et al., 1999). The cells were cultured for 48 hr with 24 hr media changes.

Bovine mammary tissue was biopsied from German Braunviech cows that were sampled during their transition from lactation into involution and copy DNA (cDNA) was synthesized from extracted total RNA as described (Werner-Misof et al., 2007).

Experimental treatments:

Mammary cells were cultured with different treatments of retinoids purchased from Biomol (Biomol Res. Labs, Inc., Plymouth Meeting, PA). These included *all-trans* retinoic (atRA) and acid 9-cis, retinoic acid (9cRA) as well as the utilization of specific retinoid receptor agonist (Am-580) and antagonist (Ro 41-5253). It is important to note that *all−trans* Retinoic acid (atRA) is a ligand for both the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). However, 9cRA has high affinity for the retinoid X receptor (RXR) (Heyman et al., 1992) and may destabilize Vitamin D receptor RXR heterodimer-DNA complexes and has been shown to down-regulate both estrogen receptor RNA (Rubin et al., 1994). Am-580 acts as a selective RARá agonist with no affinity for RXRs. Within the RARs, AM-580 has the reported Kd's; RARá= 36 nM; RARâ = 1361 nM; RARã = 3824 nM. Ro 41-5253 is a selective RARá antagonist with Kd's of RARá= 60 nM; RARâ = 2400 nM; RARã = 3300 nM. Stock solutions of retinoids were made up in ethanol at 10 mM concentrations and were stored in the dark at -80°C until utilized for experimental treatments. Dilutions of the sock solution were made into the culture media resulting in the ethanol concentration to be less than 0.1%. Bovine lactoferrin was purchased (USB Corp., Cleveland, OH) and stock solution of 130 ìM (10 mg/mL) with control culture media was made and alequoted into 1 ml portions and frozen at -80C until experimental use. Treatments were added to the experimental media (2% FBS+) following the cellular overnight attachment. Media was changed every 24 hr to ascertain fresh media effects. All media and cell culture treatments were made in

protected light environments and were shielded from light during the duration of the experiments.

Total RNA extraction from cultures: Cells were cultured in 12 well plates and seeded at 1×10^5 cells per well. Frozen primary cells (Passage 7-13) were cultured directly from frozen stock. Conditioned media was removed and followed by a 2X PBS rinse of the cells to remove more of the culture media components. Total RNA was isolated from cells (triplicates) and control liquid nitrogen stock cells by the use of a commercial extraction kit that includes a DNase treatment (Promega, WI).

RNA concentration and quality was determined with a NanoDrop ND-1000 spectrophotometer (PeoLab Biotechnologie, Germany). Extracted material was stored at -80C until subsequent use. The first strand cDNA was synthesized according to procedures specified (ProtoScript; New England Biolabs, Boston, MA) commercially available kit and was performed with 500 ng or 1 μg of prepared total RNA. In order to evaluate the specificity and presence of retinoids, fresh bovine liver tissue was obtained at slaughter and immediately frozen in liquid nitrogen, transported to the laboratory and processed as described for mammary cells to obtain liver cDNA.

TABLE 3-1. Primer design for PCR for bovine expression of retinoid receptors.

mRNA	PCR Primers	PCR Program All 4m of 98ºC/35 cycles of:	size/source
RAR-α	F- 5′-CAAGACAAATCCTCCGGCTA-3′ R- 5′-TGTTCCGGTCATTTCTCACA-3′	10s 98ºC, 30s 71ºC, and 30s 72ºC	217bp; BT_021634
RAR-β	F- 5′-ACACCCCAGAGCAAGACACT-3′ R-5′-TCAAGGTCCTGACGATCTCC-3′	98ºC; 30s 72ºC; 30s 72ºC	199bp; DQ_114538
RAR-γ	F- 5′- GGCATGTCCAAGGAAGCTGT -3′ R- 5′- GTTCTCCAGCATCTCTCGGAT-3′	10 s 98ºC; 30 s 70ºC; 30 s 72ºC	221bp; XM_866891
RXR-α	F- 5′- ATTTTCGACAGGGTGCTGAC -3′ R- 5′- GGGACGCATAGACCTTCTCA -3′	10s 98ºC; 30s 62ºC; 30s 72ºC	166bp; DQ_100361
RXR-β	F- 5′ – TGAAGATGTGAAGCC ACCAG-3′ R- 5′- GCTTGAAGAAGCCTTTGCAG-3′	10s 98ºC; 30s 65ºC; 30s 72ºC	164bp; XM_877602
RXR-γ	F- 5′-GCAGGAATGCACAGAGAGCTAT -3′	10s 98ºC; 30s 67.5ºC; 30s 72ºC	179bp;

|	R- 5'-GAAGGCCTGTTTCTGTGTCAT-3'		NM_0010705
bLf	F- 5'-CGGAAATCCTCCAAACACAG -3' R- 5'-GCCTGCAGTCCTGTCCAC -3'	10 98ºC; 30s 60.0ºC; 30s 72ºC	156bp; NM_180998

PCR Assays: Polymerase chain reaction was conducted in a 20 μL volume in a MJ200 cycler. Initial primer sets were tested utilizing a temperature gradient to select the best annealing temperature. The specifics of each developed assay are listed in Table 1. Real time PCR (qPCR) was conducted with SYBR Green PCR Core Reagent kit (Applied Biosystems, Foster City, CA) utilizing their instructions in a MJ200 cycler equipped with real time capabilities. Five reference genenes (housekeeping) were assayed based upon published primer designes (β-actin; MTG1, RPS15A, cyclophin, and PPP11R1 (Piantoni et al., 2008) and geNorm was utilized to establish the best housekeeping gene references for normalization. The geometric mean of cyclophin and PPP11R1 were used to normalize the q PCR results.

Statistical Analysis:

The Real time PCR (qPCR) results were analyzed by ANOVA (SAS, 2007) and the general linear models procedure of SAS (SAS, 2007). Main effects tested were treatment and experimental replicate and their interactions. When found to be significant, by ANOVA, differences among means were tested with SAS Proc GLM Duncan/Waller means separation analysis ($P< 0.05$).

3.4 Results:

Figure 3-1 shows the results of PCR of the RAR receptors with the two bovine mammary cells cultured in 2% FBS when compared to fresh bovine liver expression. Figure 3-1A shows that the RARα receptor is strongly expressed in both mammary cells as well as liver. Figure 3-1B shows that the RARβ receptor is expressed in BME-UV1, MeBo, and liver tissue at what appears to be equivalent expression. The band at <100 bp with BME-UV1 cells is currently unknown. Figure 3-1C shows that RARγ is expressed in all extracts.
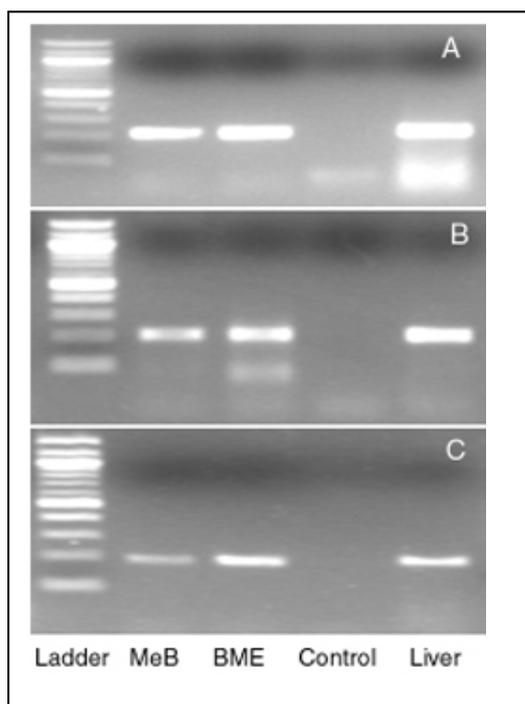
Figure 3-1. Expression of RARα (A), RARβ (B) and RARγ (C) mRNA in total RNA extracts of bovine mammary cells and fresh liver tissue. Culture was in 2% FBS for 4 days. Control is no template. Ladder is 100 bp and gel is 1.8% agarose.

Figure 3-2 shows the results of PCR with the cells and tissues that expresses RXR receptors. Figure 3-2A shows that all cell extracts and liver has strong expression of the

RXRα receptors. Figure 3-2B shows that RXRβ is expressed with all three RNA extracts, with perhaps MeBo exhibiting a stronger signal. Finally, Figure 2C indicates that only liver shows expression of the RARγ receptor isotype with no evidence of mammary cell expression.
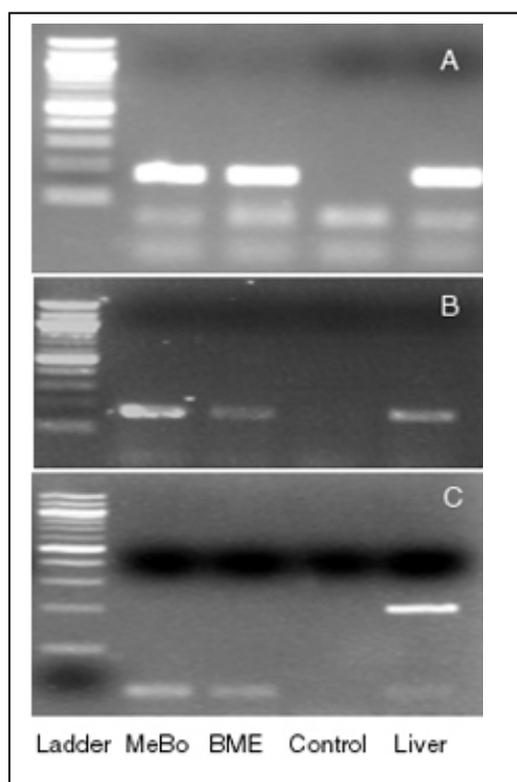

Figure 3-2. Expression of RXRα (A), RXRβ (B) and RXRγ (C) mRNA in total RNA extracts of bovine mammary cells and fresh liver tissue. Culture is in 2% FBS for 4 days. Control is no template. Ladder is 100 bp and gel is 1.8% agarose.

In order to compare expression of retinoid receptor mRNA, we conducted real time PCR (qPCR) with the extracts of the cells shown in Figure 3-1 & 3-2. Figure 3-3 shows that the mammary cells exhibit less RARα, very low RARβ, and very high RARγ receptors relative to bovine liver expression. Furthermore, mammary expressed low RXRα and RXRβ receptors relative to liver expression. No RXRγ was detected by qPCR in mammary cells. To verify the undetectable mRNA for RXRγ, we conducted qPCR on mammary tissue biopsy mRNA that was collected from lactating dairy cows during the transition from lactating to involuting/non-lactating tissue.
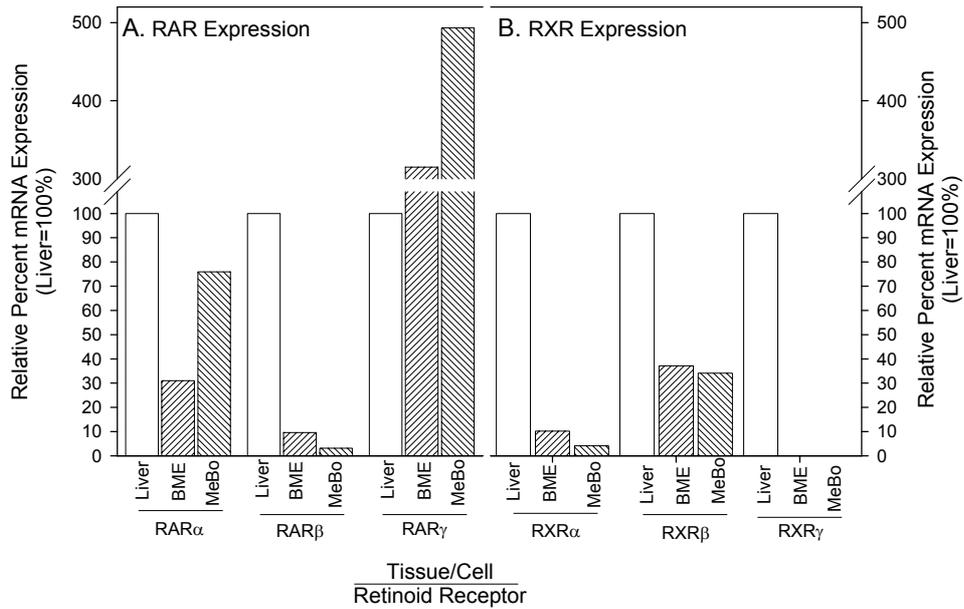
Figure 3-3 Real time PCR (qPCR) analysis of retinoid receptor mRNA expression in the two mammary cells compared to fresh bovine liver expression. Mammary cells were cultured in 2% FBS for 4 days. Data is the mean and SD of 2 separate experiments.
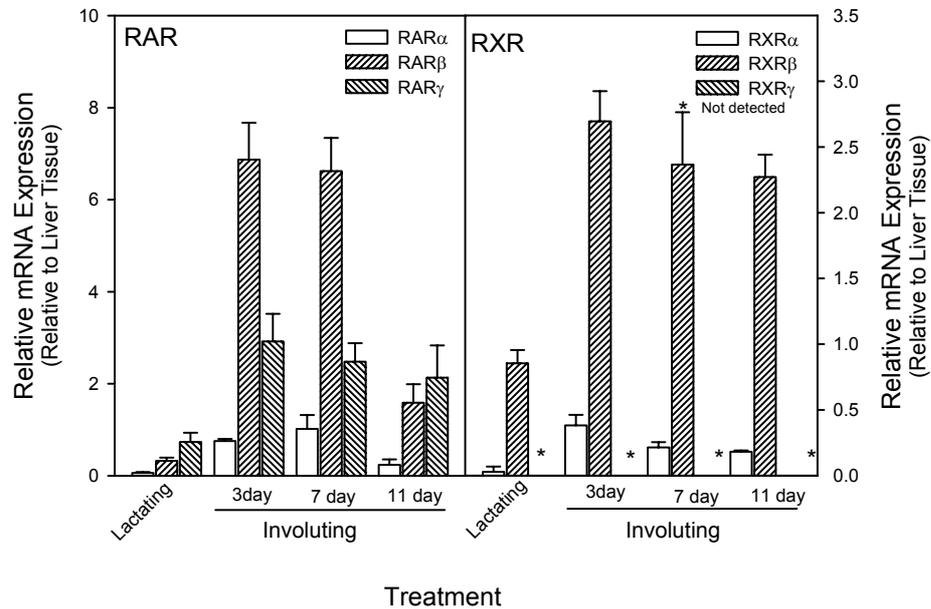
Figure 3-4. qPCR analysis of retinod receptor mRNA expression in total mammary tissue obtained by biopsy at different stages of lactation and involution. Data is the mean and SD of two replications of cDNA obtained from one animal.

Figure 3-4 indicates that RXRγ is not detected in bovine mammary tissue total mRNA, confirming the finding with the cells. Figure 3-4 also indicates that during involution the RARβ & γ and RXRβ appear to be highly increased during the first week of involution.
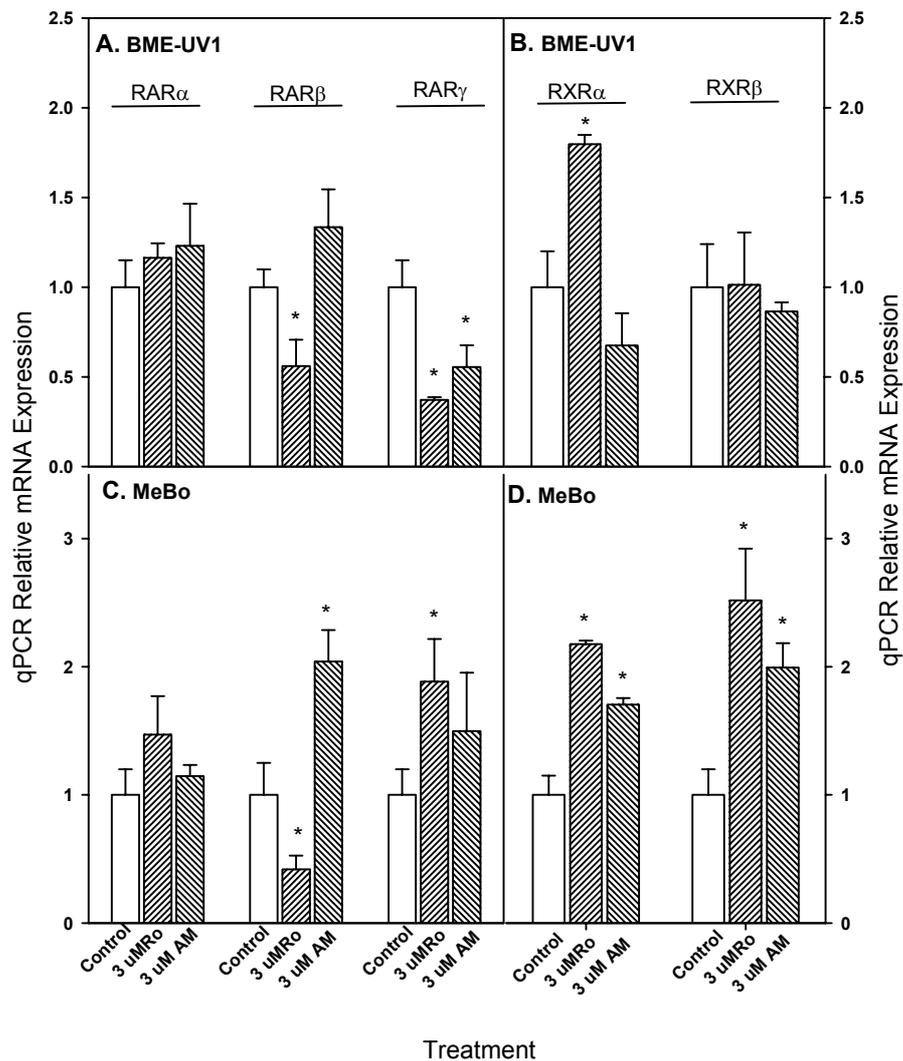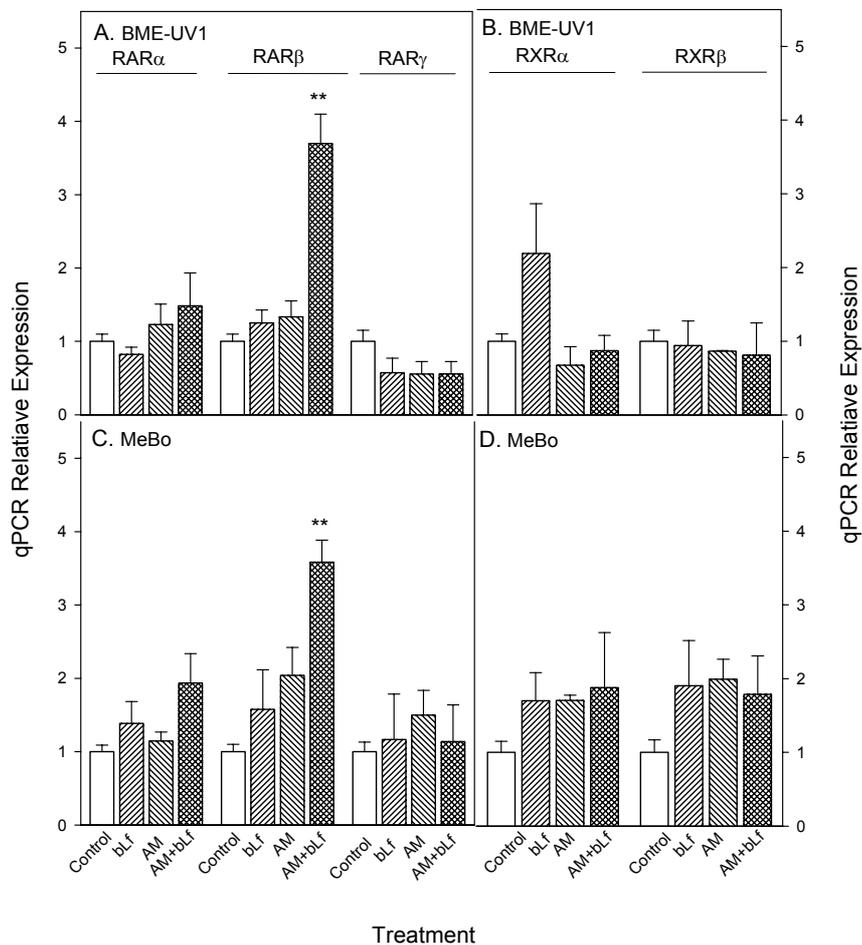

Figure 3-5. qPCR analysis of retinoid receptor mRNA expression when the two mammary cells were cultured in 2% FBS (control), 2% FBS+3 μM Ro-41-5253 (Ro) and 2% FBS+ 3 μM AM-580 (AM). Data is the mean ± the SD of 3 replications. * ($P<0.05$) different than control within each cell and receptor analysis.

Figure 3-6. qPCR analysis of retinoid receptor mRNA expression when the two mammary cells were cultured in 2% FBS (control), 100 ng/ml bLactoferrin (bLf), 2% FBS+3 μM AM 580 (AM) and 2% FBS+ 3 μM AM-580+100 ng/ml bLf (AM+bLf). Data is the mean ± the SD of 2 replications.

Figure 3-5 shows the effect of treatment of the cells with retinoid analogs that are agonists (Am 580) and antagonist (Ro 41-5253) of RAR receptors. At a concentration of 3 μM, all three RAR receptor isotypes are reported to be bound. The RAR antagonist (Ro-41-5253) decreases the RARβ receptor in both cells (Figure 3-5A & C), but decreases RARγ in BME-UV1 cells (Figure 3-5A) while increasing RARγ in MeBo cells (Figure 3-5C). The antagonist also increased RXRα in both cells (Figure 3-5B & D), but increased RXRβ in the MeBo cells alone (Figure 3-5D). Figure 3-5 shows the RAR

agonist (Am-580) decreased the RARγ (Figure 3-5A) and increased the RXRα receptor mRNA (Figure 3-5B) in BME-UV1 cells. In MeBo cells, the agonist increased RARβ (Figure 3-5C), RXRα and RXRβ (Figure 3-5D). Overall, the main differences between the cells were that the agonists and antagonist tended to decrease RARγ in BME-UV1 cells while increasing the RARγ and RXRβ receptors in the MeBo cells.

Figure 3-6 shows the effect of combining treatment of bLf with the retinoid agonist (Am-580). The application of bLf to the BME-UV1 cells caused an increase to the RXRα receptor (Figure 3-6B) and increased the expression of the RXRα and RXRβ in the MeBo cells (Figure 3-6D) that was the equivalent of the agonist (Am-580) alone. Interestingly, the application of the agonist (Am-580) and bLf had a synergistic effect on the RARβ receptors in both cell type (Figure 3-6A & C).

Figure 3-7 shows the effect of combining treatment of bLf with the retinoid antagonist (Ro 41-5253). The application of bLf and Ro 41-5253 has been discussed in Figure 3-5 and 3-6 above. The main new finding with the antagonist and bLf combination was that there was no significant effect of the dual application on any retinoid receptor isotype.

Because the primary bovine mammary epithelial cells appear to be more responsive to hormonal treatments *in vitro*, we focused the remaining experiments on the MeBo cells. Figure 3-8 shows the effect of hormonal treatments that are known to impact MeBo cells *in vitro*. The application of I-F induced a burst of all RAR receptors at 24 h followed by continued increase in RARβ at 48 h and declines in RARα & γ. The addition of prolactin to the treatments appeared to blunt the effect of I-F alone (Figure 3-8). 17β Estradiol (E2) induced increases in all RAR receptors.
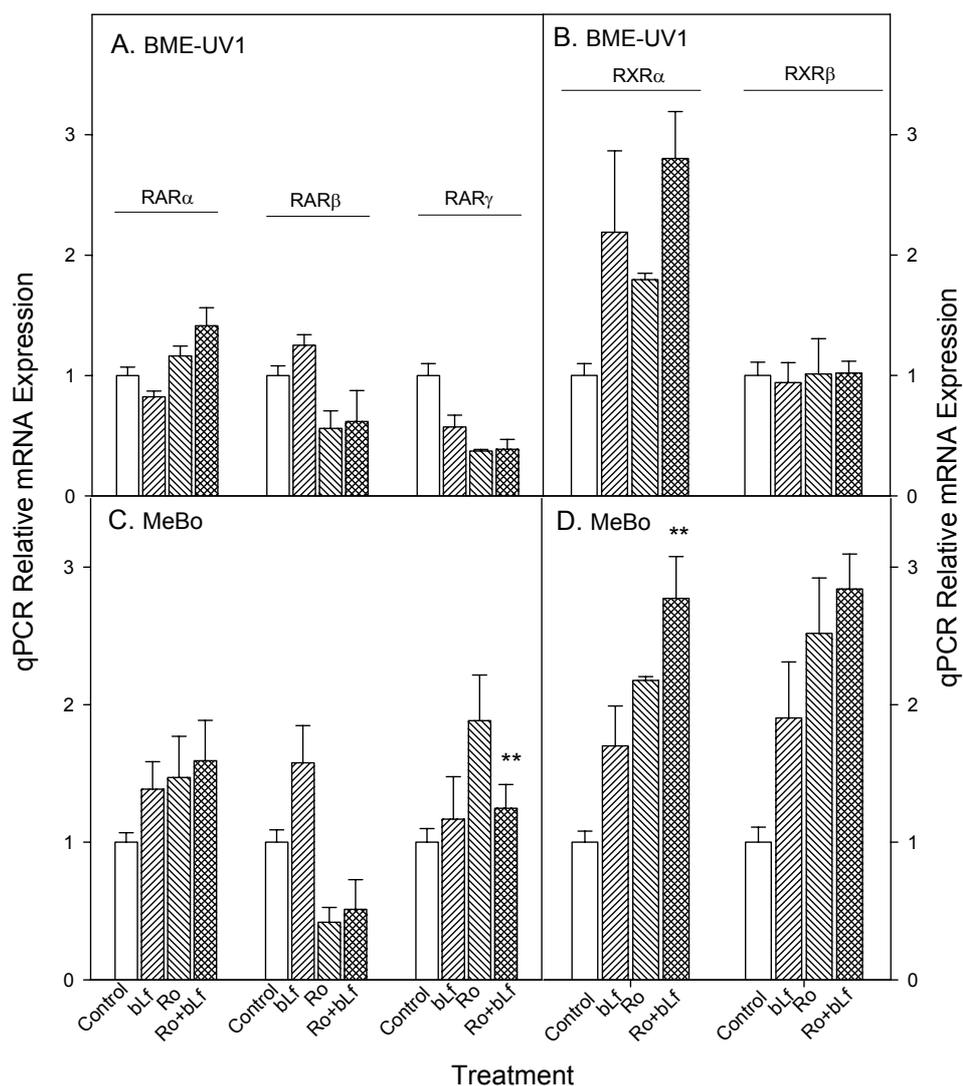
Figure 3-7. qPCR analysis of retinoid receptor mRNA expression when the two mammary cells were cultured in 2% FBS (control), 2% FBS+100 ng/ml bLactoferrin, 2% FBS+3 μM Ro 41-5253 (Ro) and 2% FBS3 μM Ro 41-5253+100 ng/ml bLactoferrin (Ro+bLf). Data is the mean ± the SD of 3 replications. * (P<0.05) different than control within each cell and receptor analysis.

Because the lactogenic hormonal mix of I-F-P was shown to increase the RARβ receptors in MeBo cells (Figure 3-8), we wished to test the hypothesis that bLf would interact with this treatment and change the expression of these receptors. Figure 3-9 shows that the application of bLf to the I-F-P treatment with MeBo cells had no effect on the expression of the RARβ receptors.
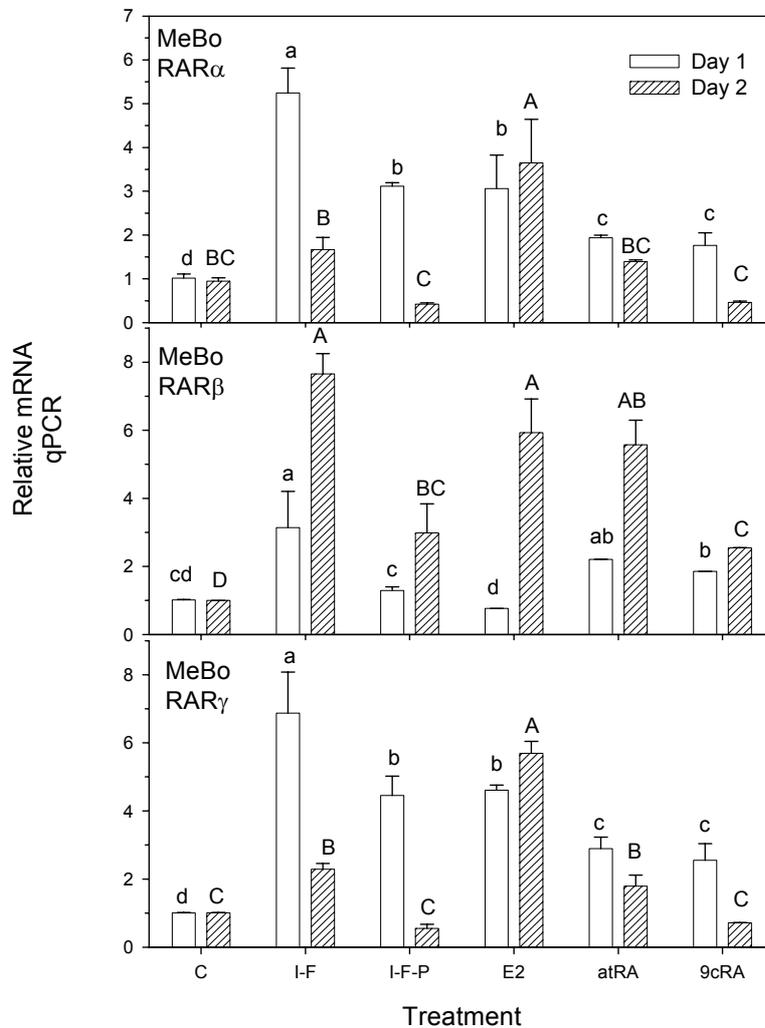
Figure 3-8. qPCR analysis of retinoid receptor mRNA expression in MeBo cells were cultured in media containing lactogenic stimulators, estrogen and natural retinoids. C is control (2% FBS); I-F is 50 ng/ml bInsulin and F is 500 ng/ml hydrocortisol; I-F-P is I-F plus 1 ug/ml bProlactin; E2 is 500 ng/ml 17-beta-estradiol; atRA and 9cRA are the natural retinoids added at 1 μM. Data is the mean ± the SD of 2 replications. Treatments with different letters are significant ((P<0.05) within each receptor type within day.
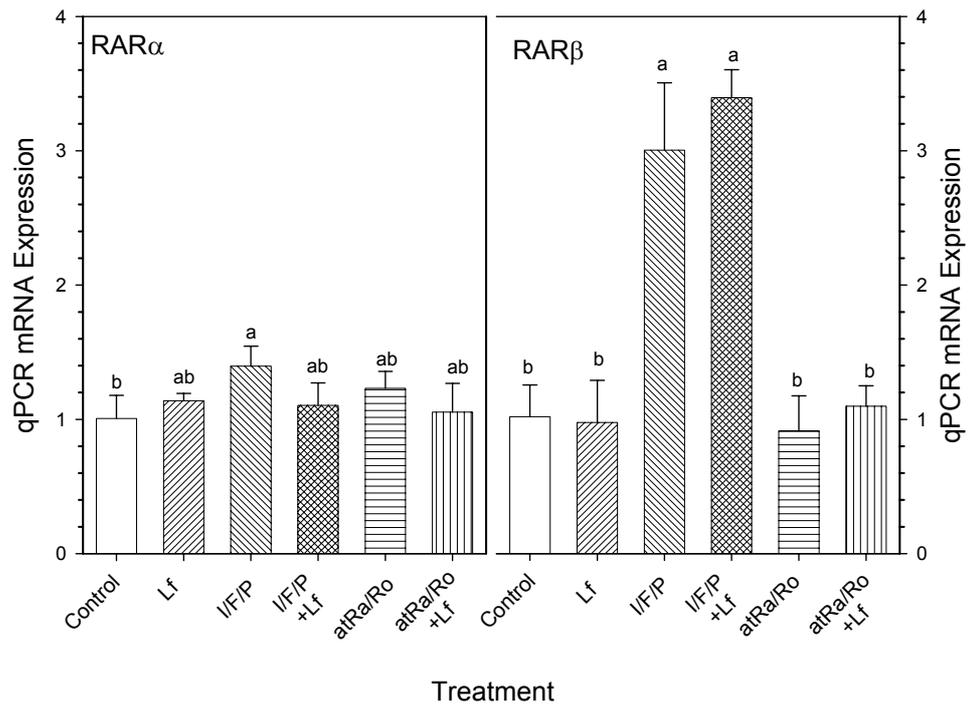
Figure 3-10. qPCR analysis of retinoid receptor mRNA expression in MeBo cells were cultured in media containing lactogenic stimulators, bLf and a retinoid antagonist (Ro-41-5253) . Data is the mean ± the SD of 2 replications. Treatments with different letters are significant ((P<0.05) within each receptor type.

3.5 Discussion

The literature with human cells and experimental animal models (rodents) demonstrate that retinoids have regulatory roles on growth and differentiation (1999; Napoli, 1996; Sporn et al., 1994). Mammary epithelial cells respond to retinoids, inducing either apoptosis or cell arrest (Purup et al., 2001; Seewaldt et al., 1997). However, though some work with normal mammary epithelial cells has been conducted to define retinoid effects, there are few reports of mechanism of action. Reports using the mammary cell line [MAC-T; (Huynh et al., 1991)] that is a precursor of the BME-UV1 (Zavizion et al., 1996) show that the cell numbers are decreased by retinoids (Cheli et al., 2003; 1996). However, these previous studies were conducted with an immortalized mammary cell and other immortalized (cancer) cells have been shown to be lacking the RARβ receptor (Swisshelm et al., 1994; Yang et al., 1999). Nevertheless, work with primary mammary cells (Purup et al., 2001) showed significant reduction in cell proliferation following treatment with RA. However, no reports exist that characterize the presence of retinoid receptors in bovine mammary cells.

Our previous findings showed that lactoferrin bound IGFBP-3 (Baumrucker et al., 2003), it interacted with retinoid signals (Baumrucker et al., 2005), and that transgenic mice over-expressing Lf during lactation exhibited milk production deficiencies when fed a diet with high concentration of retinoids (Baumrucker et al., 2006). These findings led us to characterize the retinoid receptors being expressed in bovine mammary tissue and cells and to investigate bLf interactions with the receptors. A recent study by Riley et al. (2008) has shown that exogenous addition of bLf on primary bovine mammary epithelial

mammospheres decreased casein mRNA expression and reduced mammary cell viability while siRNA suppression of bLf expression increased casein message expression.

To be more selective in retinoid receptor binding capacity than the natural ligands (atRA & 9cRA) we utilized retinoid analogs. Ro 41-5253 selectively binds to RARá, but does not influence RARá/RXRá hertodimerization and DNA binding (Toma et al., 1998). Our use of high concentrations of Ro or Am was aimed at determining the effect of binding all RAR receptors as an antagonist (block) or agonist (stimulate).

Experiments with retinoid receptor mRNA expression indicate that while bovine liver tissue shows expression of all retinoid receptor subtypes (RAR & RXR), the two mammary cells have no detectable expression of RXRγ mRNA. The low expression of most receptor mRNA in the mammary cells and the very high RARγ expression may be the result of the culture under control conditions (2% FBS). When we cultured the primary bovine mammary cells (MeBo) with the addition of hormones, the retinoid receptors were increased 4 to 8 fold. The lack of a RXRγ receptor mRNA expression in the cultured cells was confirmed with the assay of RNA extracted from cow mammary tissue that was lactating and undergoing involution. These assays with involuting mammary tissue in comparison to the same liver expression that was contracted with the cells show the capacity of mammary tissue to changes in retinoid receptor expression relative to liver. While it may be argued that total mammary tissue contains multiple cell types and these changes may be the result of other cell changes, the vast majority of the tissue is epithelial during lactation and the initial phases of involution. Because during the first few weeks of involution the mammary gland is going through extensive remodeling (Capuco and Akers, 1999), the increase in most of the retinoid receptors likely reflects the occurrence of apoptotic mechanisms.

Our comparison of cell viability changes between the primary (MeBo) and immortalized mammary cells (BME-UV1) induced by retinoid, retinoid analogs and bLf on the two bovine mammary cells (Wang and Baumrucker, 2009) validated retinoid inhibitory effects on the BME-UV1 cells (MAC-T) (Cheli et al., 2003; Purup et al., 2001; Woodward et al., 1996). However, in the current study, we show that treatments that stimulate or inhibit RAR signaling result in differential expression of the RAR receptors in the two cells, but with cellular response differences. The BME-UV1 showed a decline in RARγ while the MeBo exhibited increases in RARγ and RXRβ. This differential effect between the immortalized and primary bovine mammary cells clearly indicates retinoid signaling mechanisms that exist in the BME-UV1 cells that are not like that of the primary MeBo. Because stimulation and blocking or RAR receptors resulted in a cell number reduction with the immortalized cell line (BME-UV1), but not with the MeBo cells (Wang and Baumrucker, 2009), the differential response of the receptors are likely part of the signaling dichotomy.

The application of bLf to both mammary cells results in decreased cell viability (Wang and Baumrucker, 2009) and decreased casein mRNA expression while reducing mammary cell viability (2008) shows a biological response of exogenously applied lactoferrin to mammary cells *in vitro*. It is important to note that only the MeBo cells show expression of the bLf message and the extent of production of the cell product would likely not contribute significantly with daily culture media changes (controls) (Baumrucker et al., 2005). How exogenously added bLf interacts with nuclear receptors is currently unknown, but has been suggested as entering via the transferrin receptor (Ashida et al., 2004; Suzuki et al., 2005) or still other surface proteins (Spik et al., 1994). Once in the cytoplasm, lactoferrin contains nuclear localization sequences and nuclear export sequences (Baumrucker et al., 2005) that provide nuclear entrance and exit

opportunities that have not been characterized. Previous reports have indicated that bLf interacts with retinoid reporter constructs in human breast cancer cells (Baumrucker et al., 2005) and alters mammary function when over-expressed in mouse mammary gland when the animals are fed high levels of retinoids (Baumrucker et al., 2006).

The application of an RAR agonist (Am-580) and antagonist (Ro 41 -5253) with the addition of bLf to the cells showed an interaction of Lf with analogs that affected retinoid receptor mRNA expression. The application of Am-580 to the mammary cells increased the RARβ receptor expression in the MeBo cells, but not in the BME-UV1 cells. When bLf was added to the agonist (Am-580), the MeBo cells showed an additional stimulation of the RARβ receptors and the BME-UV1 cells were simulated to express the increased RARβ receptor expression that was not observed when the agonist was independently applied. The lack of an additive effect of bLf upon the antagonist (Ro) strongly suggests that bLf is working through mechanisms that are coupled with RARβ stimulation. Because we have demonstrated that the primary mammary cells (MeBo) are more responsive to retinoids and the application of bLf (Wang and Baumrucker, 2009), we decided to focus our attention on bLf interactions with the primary cells.

We have observed lactoferrin and retinoid interaction effects in mammary tissue from transgenic mice (Baumrucker et al., 2006) and Riley (2008) demonstrating that bLf effects a decrease in cellular milk component synthesis. We now show that lactogenic hormones (Rillema, 1994; Tucker, 1981) affect the expression of RAR receptors. Insulin, hydrocortisol, prolactin and 17β estradiol induce changes in the expression of the RAR receptors in MeBo cells. At 24 h, insulin (I) and hydrocortisol (F) induced a response in all three RAR receptors that declines at 48 h for RARá and RARγ, but continues to increase for RARβ. The addition of prolactin to I and F (I-F-P) reduces the response of I-F to all the receptors. Seventeen-β estradiol induced increase in all three RAR receptors.

In an attempt to demonstrate a bLf interaction with lactogenic signals, we applied bLf with the application of I-F-P. While I-F-P stimulated the expression of RARβ, bLf did not affect this stimulation. This was unexpected in that the application of the RAR analog (Am-580) and bLf showed an interaction.

In conclusion it is evident that the retinoid impact between the two mammary cells is different and that the primary mammary cells are likely better models of retinoid signaling that occurs in mammary cells *in vivo*. Furthermore, the data with MeBo shows that bLf interacts with retinoid signaling by decreasing cell numbers with both natural retinoid ligands, is partially rescued by the application of an RAR antagonist (Ro 41-5253) (Wang and Baumrucker, 2009) and that a retinoid analog and bLf interact to increase the expression of the RARβ receptors.

The *in vivo* role of bLf during late pregnancy and involution has been ascribed to iron binding and reduced mammary bacterial infections (1997; Ward and Conneely, 2004). These studies indicate that lactoferrin has other roles that involve retinoid receptors and that it clearly influences cellular proliferation or apoptosis.

Reference List

Ashida,K., Sasaki,H., Suzuki,Y.A., and Lonnerdal,B. (2004). Cellular internalization of lactoferrin in intestinal epithelial cells. *Biometals* **17,** 311-315.

Baumrucker,C.R., Gibson,C.A., and Schanbacher,F.L. (2003). Bovine lactoferrin binds to insulin-like growth factor-binding protein-3. *Domest. Anim. Endocrinol.* **24,** 287-303.

Baumrucker,C.R., Saurer,S., Blum,J.W., Jungi,T., and Friis,R.R. (2006). Lactoferrin (Lf): Retinoid interactions in the mammary glands of transgenic mice overexpressing human Lf. *Mol. Cell Endocrinol.* **251,** 56-66.

Baumrucker,C.R., Schanbacher,F., Shang,Y., and Green,M.H. (2005). Lactoferrin interaction with retinoid signaling: Cell growth and apoptosis in mammary cells. *Domest. Anim Endocrinol.* **30,** 289-303.

Bollag,W., Isnardi,L., Jablonska,S., Klaus,M., Majewski,S., Pirson,W., and Toma,S. (1997). Links between pharmacological properties of retinoids and nuclear retinoid receptors. *Int. J. Cancer* **70,** 470-472.

Brock, J. H. 1997. Lactoferrin structure-function relationships, p. 3-23. *In* T. W. Hutchens and B. Lönnerdal (ed.), Lactoferrin: interactions and biological functions. Humana Press Inc., Totowa, N.J.

Capuco,A.V. and Akers,R.M. (1999). Mammary Involution in Dairy Animals`. *J. Mammary Gland Biol. Neoplasia* **4,** 137-144.

Cheli,F., Politis,I., Rossi,L., Fusi,E., and Baldi,A. (2003). Effects of retinoids on proliferation and plasminogen activator expression in a bovine mammary epithelial cell line. *J. Dairy Res.* **70,** 367-372.

Cohick,W.S. and Turner,J.D. (1998). Regulation of IGF binding protein synthesis by a bovine mammary epithelial cell line. *J. Endocrinol.* **157,** 327-336.

Gudas,L.J. (1992). Retinoids, retinoid-responsive genes, cell differentiation, and cancer. *Cell Growth Differ.* **3,** 655-662.

Guo,X.H., Chu,M.X., and Zhou,Z.X. (2004). Molecular biology of the retinol-binding proteins and their genes. *Yi Chuan* **26,** 257-262.

Heyman,R.A., Mangelsdorf,D.J., Dyck,J.A., Stein,R.B., Eichele,G., Evans,R.M., and Thaller,C. (1992). 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* **68,** 397-406.

Hoffmann,B., Lehmann,J.M., Zhang,X., Hermann,T., Husmann,M., Graupner.G., and Pfahl,M. (1990). A Retinoic Acid Receptor-Specific Element Controls the Retinoic Acid Receptor-B Promoter. *Mol. Endocrinol.* **4,** 1727-1736.

Huynh,H.T., Robitaille,G., and Turner,J.D. (1991). Establishment of bovine mammary epithelial cells (MAC-T):   An *in vitro* model for bovine lactation. *Exp. Cell Res.* **197,** 191-199.

Kim,H.T., Kong,G., Denardo,D., Li,Y., Uray,I., Pal,S., Mohsin,S., Hilsenbeck,S.G., Bissonnette,R., Lamph,W.W. et al. (2006). Identification of biomarkers modulated by the rexinoid LGD1069 (bexarotene) in human breast cells using oligonucleotide arrays. *Cancer Res* **66,** 12009-12018.

Klaassen,I., Brakenhoff,R.H., Smeets,S.J., Snow,G.B., and Braakhuis,B.J. (1999). Considerations for in vitro retinoid experiments: importance of protein interaction. *Biochim. Biophys. Acta* **1427,** 265-275.

Lee,P.-P.H., Lee,M.-T., Darcy,K.M., Shudo,K., and Ip,M.M. (1995). Modulation of normal mammary epithelial cell proliferation, morphogenesis, and functional differentiation by retinoids: A comparison of the retinobenzoic acid derivative RE80 with retinoic acid. *Endocrinology* **136,** 1707-1717.

Napoli,J.L. (1996). Biochemical pathways of retinoid transport, metabolism, and signal transduction. *Clin. Immunol. Immunopathol.* **80,** S52-S62.

Newcomer,M.E. (1995). Retinoid-binding proteins: structural determinants important for function. *FASEB J.* **9,** 229-239.

Noy,N. (1999.) in Handbook of Experimental Pharmacology: The Biochemical and Molecular Basis of Vitamin A and Retinoid Action (Nau, H., and Blaner, W. S., eds), Springer-Verlag, Berlin.

Noy,N. (2000). Retinoid-binding proteins: mediators of retinoid action. *Biochem. J.* **348 Pt 3,** 481-495.

Okuno,M., Numaguchi,S., Moriwaki,H., and Muto,Y. (1993). [Cellular retinoid-binding proteins]. *Nippon Rinsho* **51,** 879-885.

Piantoni,P., Bionaz,M., Graugnard,D.E., Daniels,K.M., Akers,R.M., and Loor,J.J. (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.

Purup,S., Jensen,S.K., and Sejrsen,K. (2001). Differential effects of retinoids on proliferation of bovine mammary epithelial cells in collagen gel culture. *J. Dairy Res.* **68,** 157-164.

Raffo,P., Emionite,L., Colucci,L., Belmondo,F., Moro,M.G., Bollag,W., and Toma,S. (2000). Retinoid receptors: pathways of proliferation inhibition and apoptosis induction in breast cancer cell lines. *Anticancer Res.* **20,** 1535-1543.

Redfern,C.P.F. (1997). Molecular mechanisms of retinoid function. In *Advances in Organ Biology*. (ed. Bittar,E.E. and Sherbet,G.V.), pp. 35-78. Greenwich, CT: JAI Press, Inc.

Riley,L.G., Williamson,P., Wynn,P.C., and Sheehy,P.A. (2008). Lactoferrin decreases primary bovine mammary epithelial cell viability and casein expression. *J. Dairy Res.* **75,** 135-141.

Rillema,J.A. (1994). Development of the mammary gland and lactation. *Trends Endocrinol. Metab.* **5,** 149-154.

Riss,T.L., O'Brien,T., and Baumrucker,C.R. (1981). Isolation and characterization of acini from cow and rat lactating mammary tissue. *unknown*.

Rubin,M., Fenig,E., Rosenauer,A., Menendez-Botet,C., Achkar,C., Bentel,J.M., Yahalom,J., Mendelsohn,J., and Miller,W.H., Jr. (1994). 9-Cis retinoic acid inhibits growth of breast cancer cells and down-regulates estrogen receptor RNA and protein. *Cancer Res.* **54,** 6549-6556.

SAS (2007). *SAS/STAT Software*. Cary, NC.: SAS Inst Inc.

Seewaldt,V.L., Dietze,E.C., Johnson,B.S., Collins,S.J., and Parker,M.B. (1999). Retinoic acid-mediated G1-S-phase arrest of normal human mammary epithelial cells is independent of the level of p53 protein expression. *Cell Growth Differ.* **10,** 49-59.

Seewaldt,V.L., Kim,J.H., Caldwell,L.E., Johnson,B.S., Swisshelm,K., and Collins,S.J. (1997). All-trans-retinoic acid mediates G1 arrest but not apoptosis of normal human mammary epithelial cells. *Cell Growth Differ.* **8,** 631-641.

Spik,G., Legrand,D., Leveugle,B., Mazurier,J., Mikogami,T., Montreuil,J., Pierce,A., and Rochard,E. (1994). Characterization of Two Kinds of Lactotransferrin (Lactoferrin) Receptors on Different Target Cells. *Adv Exp Med Biol.* 13-19.

Sporn,M.B., Roberts,A.B., and Goodman,D.S. (1994). *The Retinoids; Biology, Chemistry, and Medicine*. New York: Raven Press.

Suzuki,Y.A., Lopez,V., and Lonnerdal,B. (2005). Mammalian lactoferrin receptors: structure and function. *Cell Mol. Life Sci* **62,** 2560-2575.

Swisshelm,K., Ryan,K., Lee,H., Tsou,H.C., Peacocke,M., and Sager,R. (1994). Down-regulation of a retinoic acid receptor- in mammary carcinoma cell lines and its up-regulation in senescing normal mammary epithelial cells. *Cell Growth Diff.* **5,** 133-141.

Toma,S., Isnardi,L., Raffo,P., Riccardi,L., Dastoli,G., Apfel,C., LeMotte,P., and Bollag,W. (1998). RARalpha antagonist Ro 41-5253 inhibits proliferation and induces apoptosis in breast-cancer cell lines. *Int. J. Cancer* **78,** 86-94.

Tucker,H.A. (1981). Physiological control of mammary growth, lactogenesis, and lactation. *J. Dairy Sci* **64,** 1403-1421.

Wang,Y. and Baumrucker,C.R. (2009). Retinoids, retinoid analogs, and lactoferrin interact and differentially affect cell viability of a primary and immortalized bovine mammary cells *in vitro*. *Domes. Anim. Endocrinol.* **Submitted**.

Wang,Y.A., Shen,K., Wang,Y., and Brooks,S.C. (2005). Retinoic acid signaling is required for proper morphogenesis of mammary gland. *Dev. Dyn.* **234,** 892-899.

Ward,P.P. and Conneely,O.M. (2004). Lactoferrin: role in iron homeostasis and host defense against microbial infection. *Biometals* **17,** 203-208.

Werner-Misof,C., Pfaffl,M.W., Meyer,H.H.D., and Bruckmaier,R.M. (2007). The effect of chronic oxytocin-treatment on the ovine mammary gland immune system. *Veterinarni Medicina* **52,** 475-486.

Woodward,T.L., Turner,J.D., Hung,H.T., and Zhao,X. (1996). Inhibition of cellular proliferation and modulation of insulin- like growth factor binding proteins by retinoids in a bovine mammary epithelial cell line. *J. Cell. Physiol.* **167,** 488-499.

Yang,L., Wu,K., Tin-U,C., and Brown,P. (1999). Role of Retinoid Receptors in the Prevention and Treatment of Breast Cancer. *Jornal of Mammary Gland Biology and Neoplasia* **4,** 377-388.

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

Zhang,X.K., Liu,Y., and Lee,M.O. (1996). Retinoid receptors in human lung cancer and breast cancer. *Mutat. Res* **350,** 267-277.

Chapter 4

Final discussion and Future Work

Retinoids have been applied to prevent or reduce cancer proliferation for a long time, especially for breast cancer and lung cancer. Numerous basic experiments and clinical trials have been reported. By the comparison between our results with the two bovine mammary cells types and results published from other labs on human or rodent breast cancer, we found some interesting conclusions.

1. Effect of RAR antagonist (Ro 41-5253)

One of the most significant differences between BME-UV1 and MeBo cell types is their growth pattern when RAR antagonist Ro 41-5253 is added in medium. As an antagonist, Ro 41-5253 has the selectivity of binding of RAR, but has no influence on the heterodimerization of the receptors. In our study, a clear dosage-dependent anti-viability effect was demonstrated with the BME-UV1 cells. In contrast, an increase in cell number was detected in its primary MeBo cells. The anti-viability effect seen in the immortalized cells (BME-UV1) was consistent with the observation in many breast cancer cell lines, such as ZR-75.1 and MCR-7, and is not due to the regulation of RARE, but to the inhibition of AP-1 pathway (Fanjul et al., 1994; Toma et al., 1998a; Toma et al., 2005). No studies on the effect of Ro 41-5253 in mammary primary cells are available. Our results support the concept of Ro 41-5253 as a candidate for cancer therapy of mortalized cells that may inhibit cell proliferation in BME-UV1 cell via AP-1 pathway inhibition, without blocking the viability of noncancerous cell.

2. RARβ mRNA expression

By administration of their specific analogs, at least four retinoids receptors subtypes were identified to be involved in tumor-suppressive activity, and RARβ was believed to be the most important (Xu, 2007). The expression of RARα, RARγ, RXRα and RXRβ genes were similar in six lung cancer cells, but the expression of RARβ varies significantly (Zhang et al., 1996). The absence of RARβ and the abnormal regulation of RARβ may be closely related to the development of cancer (Zhang et al., 1996). In our study, RAR-β expression was confirmed in BME-UV1 cell lines at a very low level, yet its expression was still higher than that of MeBo cells. Clearly, immortalization of the BME-UV1 cells did not seem to affect retinoid receptor expression, but does affect retinoid effect on cell viability.

3. Effect of estradiol on RAR mRNA expression

Estradiol is reported to induce RARα, not RARβ or RARγ, in human breast cancer cells (Roman et al., 1993), However, estradiol increases (4-6 fold) the mRNA expression of all three RARs in MeBo in our study. RARα and RARγ show similar temporal expression patterns, while the induction of increased RARβ expression appears to occur with a different pattern.

4. Activation of RARβ by retinoids

We observed that a retinoid analog (both Ro 41-5253 and AM 580) and bLf treatment resulted in a ~3.5 fold increase in the expression of RARβ mRNA in the MeBo cells, suggestion Lf may interact with RAR in some way.

5. Several new findings

a) In thess studies, we identified the expression pattern of the retinoid receptors in bovine mammary tissues, bovine immortalized mammary cells, and the latter cell

expression corresponds to that of primary cells. b) We showed that 9cRA induced a clearer dose dependent inhibition of cell number than atRA in BME-UV1. c) We identified the inhibition effect of bLf on both of the two mammary cell types in a similar pattern. d) Because of the lactoferrin:retinoid interaction discussed previously (Baumrucker et al., 2002; Baumrucker et al., 2005; Baumrucker et al., 2006), we conducted more Lf experiments in these studies, and conclude that retinoids and bLf interact with RAR signaling in bovine mammary cells hypothesizing that they regulate cell number *in vitro*. This action of lactoferrin does not appear to interact with the lactogenic hormones (insulin, cortosol, prolactin).

Although we investigated the effect of retinoids and bovine lactoferrin on bovine mammary cells, and have provided some clue for the study of mechanistic action, there remains future research needs to establish Lf as a retinoid signaling molecule.

Retinoids have been shown to induce apoptosis (Toma et al., 1997; Toma et al., 1998b) or arrest the cell in the G1 phase of the cell cycle (Seewaldt et al., 1999). Because of time and equipment limits, we have not established a system to evaluate which of the two mechanisms is occurring with the cell viability studies. We may use a Flow Cytometer equipment to test cell arrest. In addition, we may test the mRNA expression of some well identified cell apoptosis related genes in the two cell types by qPCR.

Most of our work focused on RARs and not RXRs because only RAR agonist (AM 580) and RAR antagonist (Ro 41-5253) were commercially available. Other analogs may be available for RXRs for future work. Ro 25-6603, SR11237, and SR11234, Ro 25-5802 were RXR-selective analogs used in other studies (Brooks, III et al., 1997).

Reference List

Baumrucker,C.R., Gibson,C.A., Zavodovskaya,M., Schanbacher,F.L., and Green,M.H. (2002) Interaction of lactoferrin and IGFBP-3 with the retinoid signaling system: Cell growth and apoptosis of mammary cells. 84th Endocrine Ann.Meeting San Francisco, CA, p. 362.

Baumrucker,C.R., Saurer,S., Blum,J.W., Jungi,T., and Friis,R.R. (2006). Lactoferrin (Lf): Retinoid interactions in the mammary glands of transgenic mice overexpressing human Lf. *Mol. Cell Endocrinol.* **251,** 56-66.

Baumrucker,C.R., Schanbacher,F., Shang,Y., and Green,M.H. (2005). Lactoferrin interaction with retinoid signaling: Cell growth and apoptosis in mammary cells. *Domest. Anim Endocrinol.* **30,** 289-303.

Brooks,S.C., III, Sturgill,R., Choi,J., and Yen,A. (1997). An RXR-selective analog attenuates the RAR alpha-selective analog-induced differentiation and non-G1-restricted growth arrest of NB4 cells. *Exp. Cell Res.* **234,** 259-269.

Fanjul,A., Dawson,M.I., Hobbs,P.D., Jong,L., Cameron,J.F., Harlev,E., Graupner,G., Lu,X.P., and Pfahl,M. (1994). A new class of retinoids with selective inhibition of AP-1 inhibits proliferation. *Nature* **372,** 107-111.

Roman,S.D., Ormandy,C.J., Manning,D.L., Blamey,R.W., Nicholson,R.I., Sutherland,R.L., and Clarke,C.L. (1993). Estradiol induction of retinoic acid receptors in human breast cancer cells. *Cancer Res.* **53,** 5940-5945.

Seewaldt,V.L., Dietze,E.C., Johnson,B.S., Collins,S.J., and Parker,M.B. (1999). Retinoic acid-mediated G1-S-phase arrest of normal human mammary epithelial cells is independent of the level of p53 protein expression. *Cell Growth Differ.* **10,** 49-59.

Toma,S., Emionite,L., Scaramuccia,A., Ravera,G., and Scarabelli,L. (2005). Retinoids and human breast cancer: in vivo effects of an antagonist for RAR-alpha. *Cancer Lett.* **219,** 27-31.

Toma,S., Isnardi,L., Raffo,P., Dastoli,G., De,F.E., Riccardi,L., Palumbo,R., and Bollag,W. (1997). Effects of all-trans-retinoic acid and 13-cis-retinoic acid on breast-cancer cell lines: growth inhibition and apoptosis induction. *Int. J. Cancer* **70,** 619-627.

Toma,S., Isnardi,L., Raffo,P., Riccardi,L., Dastoli,G., Apfel,C., LeMotte,P., and Bollag,W. (1998a). RARalpha antagonist Ro 41-5253 inhibits proliferation and induces apoptosis in breast-cancer cell lines. *Int. J. Cancer* **78,** 86-94.

Toma,S., Isnardi,L., Riccardi,L., and Bollag,W. (1998b). Induction of apoptosis in MCF-7 breast carcinoma cell line by RAR and RXR selective retinoids. *Anticancer Res.* **18,** 935-942.

Xu,X.C. (2007). Tumor-suppressive activity of retinoic acid receptor-beta in cancer. *Cancer Lett.* **253,** 14-24.

Zhang,X.K., Liu,Y., and Lee,M.O. (1996). Retinoid receptors in human lung cancer and breast cancer. *Mutat. Res* **350,** 267-277.