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IGF-I AND LEPTIN HORMONAL INFUSIONS ALTER THE CELL PROLIFERATION AND TRANSCRIPTIONAL PROFILE OF THE PREPUBERTAL BOVINE MAMMARY GLAND

By

Brett Eric Etchebarne

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Animal Science

2006

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ABSTRACT

IGF-I AND LEPTIN HORMONAL INFUSIONS ALTER THE CELL PROLIFERATION AND TRANSCRIPTIONAL PROFILE OF THE PREPUBERTAL BOVINE MAMMARY GLAND

by

Brett Eric Etchebarne

High-energy diets promoting body growth rates >1 kg/d impair mammary development in prepubertal dairy heifers. Biological mechanisms to explain this result are not well understood, but insulin-like growth factor-I (IGF-I) and leptin likely play a role. Adipocytes produce the protein leptin, and leptin concentrations increase with increased fat deposition in the body and mammary gland. IGF-I stimulates and leptin inhibits proliferation of mammary epithelial cells (MEC) *in vitro* and *in vivo* in cattle, in contrast to human and mouse MEC, which proliferate in response to leptin. We hypothesize that leptin inhibits the mammogenic action of IGF-I, and diets that promote rapid prepubertal body growth inhibit mammary development if fat deposition is increased. Our objectives were to elucidate the effects of IGF-I and leptin infusion on cell cycling in MEC, and identify key genes controlling the interaction of these two hormones in bovine parenchya. A functional genomics approach was used to analyze mammary tissue collected from six prepubertal Holstein heifers after 7 d of intramammary hormone infusions of IGF-I, leptin, IGF-I plus leptin, or saline control. Mammary parenchyma tissue was collected, fixed, and immunostained for proliferation markers (BrdU and Ki-67), and an apoptotic marker (caspase-3).

Infusion of IGF-I increased the percentage of BrdU-labelled MEC in the S-phase of the cell cycle 52% (Silva, 2002). Leptin infusion decreased the percentage of BrdU-labeled MEC 48% in IGF-I-treated quarters, and 19% in control quarters (Silva, 2002). No differences were observed in the total cells within the cell cycle (by Ki-67 immunostaining). A numerically-insignificant increase in caspase-3 immunostaining was seen in IGF-I and leptin treated quarters relative to control. We conclude that increased concentrations of IGF-I promote, and leptin inhibit, MEC proliferation by increasing the progression of cells from the G1 into the S-phase of the cell cycle. Gene expression profiles of total parenchyma mRNA from each quarter of the same six animals (Silva, 2002) were examined using bovine-specific microarrays. mRNA expression of genes known to mediate cell cycling and proliferation within specific pathways in the mammary gland, including the JAK/STAT pathway, Ras/Raf pathway, PI3K pathway, and Bad/Bcl pathway were altered by IGF-I and leptin treatments. Interestingly, addition of leptin to IGF-I treated quarters increased the mRNA of one of these genes, the suppressor of cytokine signaling (SOCS)-3, 2.3-fold relative to IGF-I-infused quarters. Assuming protein changes follow mRNA expression, increased SOCS3, which signals within the JAK/STAT cell proliferation pathway, could possibly explain the inhibition of IGF-I-induced mammary epithelial cell proliferation. Overall, this study has demonstrated that IGF-I and leptin intramammary infusions in prepubertal heifers alter the cell cycling and mRNA profiles within the mammary gland to affect MEC proliferation.

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Brett E. Etchebarne

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LIST OF ABBREVIATIONS

14-3-3 eta	14-3-3 protein eta
ANOVA	Analysis of variance
BDNF	Brain derived neurotrophic factor
BLAST	Basic local alignment search tool
BMET	Bovine metabolism
BOTL	Bovine total leukocyte
BrdU	Bromodeoxyuridine
BW	Body weight
CASP8	Caspase 8
cDNA	Complementary DNA
ChREBP	Carbohydrate responsive element binding protein
CISH	Cytokine-inducible SH2 domain containing protein
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CT	Cycles to threshold
Cy3	Cytidine 3, green fluorescent dye
Cy5	Cytidine 5, red fluorescent dye
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
dUTP	2'-Deoxyuridine 5'-Triphosphate
E2	Estradiol, estrogen
ERK	Extracellular signal-regulated kinase
ExPASy	Expert Protein Analysis System
FABP	Fatty acid binding protein
FADS	Fatty acid desaturase
FBS	Fetal bovine serum
FDR	False discovery rate
FRAP1	FK506 binding protein 12-rapamycin associated protein 1
GALT	Lactose-1-phosphate uridyltransferase
GAPDH	Glyceraldehyde phosphate dehydrogenase
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GO	Gene Ontology
HGF	Hematopoietic growth factor
IFNA2	Interferon alpha 2
IGF+LEP	IGF-I plus leptin
IGFBP	Insulin-like growth factor binding protein
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II
IGF-IR	Insulin-like growth factor receptor
IL	Interleukin
IR	Insulin receptor

IRS1	Insulin receptor substrate 1
JAK	Janus kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
Ki-67	Proliferating cell nuclear antigen Ki-67
LEP	Saline plus leptin
MAPK	Mitogen activated protein kinase
MEC	Mammary epithelial cells
MGE	Mammary gland extract
MM	Mismatch probe
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
NF-kappa B	Nuclear factor kappa B
OB-R	Leptin receptor
OB-Rb	Leptin receptor, long form
oLeptin	Recombinant ovine leptin
OMIM	Online Mendelian Inheritance in Man
PCR	Pyruvate dehydrogenase kinase 1
PFKL	Phosphofructokinase, liver isoform
PI3K	Phosphoinositide 3'-kinase
PIGF	Phosphatidylinositol glycan, class F
PKB	Protein kinase B
PKU	Phenylketonuria
PM	Perfect match probe
PPAR	Peroxisome proliferator-activated receptor
PRL	Prolactin
PTP	Protein tyrosine phosphatase
Q-RT-PCR	Quantitative real-time reverse-transcriptase PCR
RefSeq	NCBI reference sequence
RELA	NF-kappa B p65 subunit
RNA	Ribonucleic acid
RPL-19	Ribosomal protien L-19
RXR	Retinoic acid receptor
SAL	Saline
SCD	Stearoyl CoA desaturase
SOCS	Suppressor of cytokine signaling
SREBP	Sterol regulatory element binding protein
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
TIGR	The Institute for Genome Research
TNF	Tumor necrosis factor
TUNEL	Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling

CHAPTER 1

Statement of Problem

Feeding high-energy diets in the prepubertal growth period in dairy cattle impairs mammary gland development. Biological mechanisms to explain this result are not well understood, but insulin-like growth factor-I (IGF-I) and leptin likely play a role. Adipocytes produce the protein leptin, and leptin concentrations increase in response to increased fat deposition in the body and mammary gland. IGF-I stimulates and leptin inhibits proliferation of bovine mammary epithelial cells *in vitro* and *in vivo*.

My objectives were the following:

1) To elucidate the effects of IGF-I and leptin infusion on cell cycling in mammary epithelial cells using immunohistochemical labeling of cell cycle markers.

2) Identify key genes controlling the interaction of these two hormones in bovine mammary tissue employing a functional genomics approach.

3) Determine if leptin alters IGF-I-induced expression of genes involved in the major IGF-I signaling pathways using Q-RT-PCR.

Crosstalk between hormones is a critical area of research geared toward the understanding of the endocrine feedback loop regulating body energy stores. "Adiposity signals", which are putative molecular substrates present in the circulation that reveal information about body condition to the brain in order to

help regulate energy intake, could possibly be present in the body in the form of the hormones insulin and leptin. Both of these hormones are known to circulate in the body in proportion to body fat mass in the physiologically normal mammalian organism, and can act upon the CNS to inhibit food intake through their ability to signal within the brain (5;61). The suggested physiological and intracellular signaling interaction between insulin and leptin at the level of the CNS has subsequently been extended to other specific tissues, including the mammary gland. It has been well established that high energy diets increase fat deposition within peripheral tissue stores, including the mammary gland (293). Local concentrations of IGF-I and leptin are known to be increased in the mammary gland with increased energy intake (242). However, although IGF-I has been shown to have a stimulatory effect on mammary epithelial cell proliferation, mammary gland development is inhibited by high energy diets (275;278). Thus, I believe that a hereto unknown factor is increased in concentration with the feeding of high energy diets which interferes with the proliferative action of IGF-I on mammogenesis. It is here proposed that leptin, a hormone produced by adipocytes, could potentially mediate the inhibition of IGF-I-stimulated mammary parenchymal development through crosstalk between the IGF-I and leptin receptors. Evidence is presented supporting SOCS3 as a potential mediator of the negative feedback on IGF-IR activation promoted by leptin intracellular signaling.

REVIEW OF LITERATURE

Introduction

Mammary gland development in the prepubertal phase of growth in the dairy heifer is crucial to determining future milk production (267). Genetic selection for higher milk yield in dairy cattle is correlated to growth capacity of heifers and milk yield of cows which gives rise to a positive relationship between milk yield and body weight at calving (141). This relationship suggests a positive relationship between rate of gain of heifers in the prepubertal stage and subsequent milk yield potential. However, numerous studies have established that high energy intake and subsequent high rate of gain can lead to reduced mammary development and impaired future milk yield in dairy cattle. This window of time for mammary developmental sensitivity to energy intake is isolated to the prepubertal phase, with post-pubertal and pregnancy periods being unaffected. Milk yield capacity is largely influenced by the number of mammary epithelial cells (MEC) present at puberty, which will form the foundation for the future milk producing cell population (306). The negative relationship between prepubertal growth rate and milk yield capacity suggests that nutritional regimes can influence the MEC population and have a negative effect on mammary development. Numerous hormones act to guide mammary development including estrogen, progesterone, growth hormone (GH), insulin-like growth factor-I (IGF-I), and leptin (107;165;194;278). Studies have shown that GH levels are altered by nutrient availability and that impaired mammary development may be due to alterations in GH action (236;237). This review aims to investigate the

effect of energy intake on mammary gland development and discuss potential physiological regulators mediating this effect on future milk production potential in dairy cows.

Mammary gland development

Postnatal bovine mammary gland development can be divided into four distinct stages of development as first described by Tucker (307). These include periods between birth and conception (the prepubertal phase), between conception and the first parturition, during the first lactation, and the dry period prior to a subsequent pregnancy. Of critical importance to future milk yield considerations is the prepubertal phase in which mammary development is most dramatically affected by energy intake (267). This phase of growth prior to puberty will be the area of focus for this review.

Development of the mammary gland and its internal structures is referred to as mammogenesis. While mammogenesis is recognized as being most extensive and dramatic during pregnancy, mammary development really begins in the fetus and proceeds beyond initiation of lactation (306). One remarkable feature of the mammary gland is that it can repeatedly undergo periods of growth and functional differentiation during pregnancy and upon initiation of lactation, and regression during the dry period (307). The mammary gland contains both parenchymal and extraparenchymal structures. The parenchymal tissue is composed of epithelial structures such as alveoli and ducts, and associated stromal connective tissue (8). The stromal tissue provides a support system

surrounding the epithelium containing cellular components such as fibroblasts, endothelial cells associated with blood vessels, and leukocytes localized within the tissue, and non-cellular components including collagen and other connective tissue proteins (8). The extraparenchymal region of the gland includes a considerable white adipose tissue region called the 'fat pad' which is large during early phases of development and largely disappears during pregnancy (8).

A critical period of mammogenesis exists between 3-9 months of age in the growing dairy heifer. During this period the mammary gland grows at a faster rate compared with the rest of the body (allometric growth) (282). Puberty in well managed heifers occurs between 7-9 months of age, and after reaching puberty, the mammary growth rate decelerates and grows at a rate equal to that of other tissues (isometric growth) after a few estrous cycles (153;282). Quantitative measures of the total numbers of parenchymal cells in the mammary gland and thus mammary development include deoxyribonucleic acid (DNA) concentration in the parenchymal tissue (8). The amount of DNA in the gland has been shown to increase 1.6 times faster than body weight between birth and 2 months of age. Between 5-9 months of age this measurement increases to 3.5 times faster than that of body weight (BW), and from 9-12 months of age this value returns to 1.5 (282). The allometric growth phase brings about rapid growth of the mammary fat pad and ducts, but no alveoli are yet formed. This phase is tied to reproductive development of the animal, and ovariectomy in the first weeks of life almost completely abolishes mammary development (236;313). Upon initiation of puberty, the mammary glands of heifers weigh 2-3 kg of which only 0.5-1 kg is

parenchymal tissue (269). This parenchymal tissue contains approximately 10-20% epithelial cells, 40-50% stromal tissue, and 30-40% fat cells (8).

The number of alveolar structures in the mammary gland is a basic element limiting milk production (305). Milk yield during lactation will be determined by the number of epithelial cells present and by the secretory activity per cell (106;167). Of primary importance to an animal's lactation potential is cell number, because cell activity is generally not a limiting factor (201). Correlation between total mammary cell numbers and milk yield is generally high, with estimates ranging from 0.50 to 0.85 (304). It thus becomes clear that to maximize milk production potential, the development of an adequate prepubertal ductal network is of great importance to establish subsequent alveolar growth and differentiation prior to lactation.

Nutrition and mammogenesis

Herman and Ragsdale were among the first to observe that mammary development was stunted in cows raised on a high energy diets (137). Soon after, this assertion was confirmed by Swanson (293), Little and Kay (185), and Harrison et al. (131). Of particular importance to studies of mammogenesis were the observations that glands from cows raised on high energy diets differed in both size and shape from those of cows raised under moderate feeding regimes (137).

Upon examination of a number of studies focusing on the effects of nutrition during the prepubertal phase of mammary development, it becomes clear that

this period was especially critical to the subsequent milk yield capacity of heifers. Numerous separate experiments have confirmed that high energy feeding levels during the prepubertal period reduce mammary development (196;265;266). To investigate the influence of plane of nutrition on mammary development in Holstein heifers, Sejrsen et al. (1982) fed pre- and postpubertal heifers a ration containing 60% concentrate and 40% forage in restricted or ad libitum amounts (267). They found that ad libitum feeding during the prepubertal period lowered parenchyma weights 23% and parenchyma DNA concentration 32% compared with restricted feeding. Parenchyma composed 38% of the total mammary gland mass in heifers receiving the restricted feeding level compared with 23% of total mass of the heifers fed ad libitum. Feeding different diets after puberty, however, did not alter parenchymal growth (266;267). The high intensity feeding after puberty tended to increase the amount of adipose tissue, but total fat-free parenchyma was only 3% lower than for heifers fed the restricted diet (267).

Two studies comparing the effect of feeding level from 300-450 kg of body weight in unbred heifers (267) and from 300 kg to 3 months prior to calving (104) showed a lack of effect of feeding level on mammary growth in the postpubertal period. Studies examining the effect of diet in the early prepubertal phase on mammary gland development are equivocal. While a study by Sejrsen et al. (1998) suggests that mammary growth is unaffected by plane of nutrition prior to 90 kg live body weight (268), Brown al. (2005) reported that increasing energy and protein intake from 2 to 8 wk of age increased mammary parenchymal mass, DNA and RNA of Holstein heifer calves (37). This study suggests that increasing

protein and energy intake in Holstein heifer calves prior to the 3-9 months of age allometric growth phase can increase the rate of development of mammary parenchyma without detrimental effects on potential future milk yield.

Specific factors present within a diet type fed at different levels have also been examined to determine effects on mammary development. In one study, alteration of the forage to concentrate ratio in two isoenergetic diets did not alter mammary gland growth (263). However, Capuco et al. (1995) reported that a high energy corn-based diet produced a more dramatic decrease in mammary development than a high energy alfalfa-based diet (43). Here it was suggested that the higher protein supply in the alfalfa-based diet could partially compensate for the negative effects of the high energy level on mammary development. Similarly, Radcliff et al. (1997) proposed that the minor negative effect of feeding a high energy diet on mammary development seen in their study may have been related to dietary protein levels (242). The authors suggested that high protein levels in high energy diets may provide a protective effect against inhibition of mammary gland development. The importance of ruminal protein degradability was investigated by Mantysaari et al. (1995) and the negative effect of feeding level on prepubertal mammogenesis was unaffected by the amount of by-pass protein present in the diet (196). In a study in which heifers were fed a high energy diet at three levels of protein (14, 16 and 19% of diet), those heifers on a low protein diet that attained puberty early had a 33% greater impairment of mammary parenchymal development compared to those fed a high protein diet (324). This study showed once again that while dietary protein does not have a

major effect on mammary development of rapidly grown prepubertal heifers, feeding low-protein diets increases the risk of impaired mammary development when heifers are fed for rapid growth and attain puberty early.

Dietary fat has also been shown to have effects on prepubertal mammary growth in ruminants. Sheep reared on diets higher in polyunsaturated fat had increased mammary parenchymal development (200). In a recent study in which diets differing in linoleic acid concentrations at a moderate energy level (~800 g/d) were fed to growing heifers over the first 12 months of age, a tendency for increased mammary parenchymal development at 12 months of age on the lower linoleic acid concentration diet was observed which did not translate to differences in first lactation milk yield, likely because of the small magnitude of difference between diets (299).

Physiological basis for effect of feeding level on mammary gland development

Bovine somatotropin (bST) or growth hormone (GH), is a growth factor that is elevated in prepubertal dairy heifers fed restricted diets compared with those fed a high plane of nutrition (143;265;266). In an early study monitoring mammary development at increased dietary energy levels, prepubertal and postpubertal heifers fed either an ad libitum or 60% of ad libitum diet had different responses for average daily gain and mammary parenchymal development. Parenchymal weight was significantly lower in the ad libitum fed prepubertal group (265). In this study restricted heifers gained 613 g/d, the ad libitum group gained nearly

double this at 1218 g/d over the allometric growth period (265). The feeding level in this study did not affect mammary development of postpubertal heifers and mammary parenchyma fat versus protein composition was unaffected in all treatments. Concentrations of prolactin, insulin, and glucocorticoids were higher in blood in the ad libitum fed group for both pre- and postpubertal heifers (265). Interestingly, GH was increased in the restricted feeding prepubertal heifer group compared with the ad libitum fed group. No difference in GH levels was detected in the postpubertal groups. The amount of mammary parenchymal tissue was significantly positively correlated with GH levels. Additionally, it was also found to be negatively correlated with mammary adipose tissue concentration. It was therefore hypothesized that GH may be a major factor in the control of mammary development during the prepubertal period (265). These data were supported by Capuco et al. (1995) in a study starting heifers at a body weight of 175 kg on diets set to gain 0.725 or 0.95 kg/d until puberty (43). In the high rate of gain group, GH was significantly lower and mammary parenchymal tissue was reduced compared to the lower rate of gain group. It was then hypothesized that GH may be a limiting factor in mammogenesis of rapidly gaining heifers and studies then began to focus on supplemental exogenous bST (43).

Daily GH injections in heifers from 8 to 15.6 mo of age increased the amount of mammary parenchyma and decreased extraparenchymal tissue compared to control (264). Interestingly, however, this GH administration does not seem to increase milk yield during the first lactation (123;211). Studies focusing on the manipulation of heifer growth rates through nutrition during certain prepubertal

developmental periods either with or without GH administration have shown that the effects of diet and GH are not additive (45;228). In a study in which GH was administered to heifers on a high energy diet, the hormone mitigated the negative effects on milk production by the high plane of nutrition and reduced age at first calving (240).

Mammary gland development is under the control of complex hormonal mechanisms involving numerous factors, and many facets of this process are not clearly understood. Though certain aspects of developmental processes are often species specific, much of the study of mammary gland endocrinology and development still finds its roots in the classical rat studies (194). Based on these early studies it has become clear that certain hormones are involved in specific phases of mammary development (107). A number of hormones are required for allometric ductal growth and end bud formation during the peripubertal period in rats. These hormones were largely determined using adrenalectomized, ovariectomized and hypophysectomized rats to identify estrogen, adrenal corticoid and somatotropin as basal factors needed for normal development (194). Studies by Kleinberg and colleagues (165) in the rat repopularized and extended the findings of Lyons. Their research showed that both lactogenic (human GH) and non-lactogenic (rat GH) GHs were more potent than prolactins (PRLs) in furthering the process of mammary development. Using GH receptor and PRL receptor mutations it was shown that binding of the GH receptor mediated mammary gland differentiation, and development was mediated by the actions of estradiol, PRL and placental lactogen by PRL receptor activation

(101). More recently the importance of other hormones including the growth factors epidermal growth factor, platelet-derived growth factor, transforming growth factors, fibroblast growth factors, mammary derived growth factors, and the insulin-like growth factors (IGF) have come to the forefront as possible mediators of parenchymal development (107;234;274).

Key Mammary Gland Growth factors

Growth Hormone and IGF-I

Bovine somatotropin (GH) and insulin-like growth factor-I (IGF-I) are the most studied effectors of bovine mammary gland development and numerous experiments have focused on elucidating the mechanism by which GH mediates its effects. Growth hormone is both required for prepubertal development and is affected by feeding level (265). High feeding levels have been shown to depress circulating GH levels, yet GH is positively correlated with mammary development (265) and bST administration increases mammary growth (264). Some debate exists, however, about whether GH itself has a direct action in mammary tissue. It does not appear that somatotropin binds to its receptor in the prepubertal mammary gland (202), and GH does not stimulate mammary epithelial cells in vitro though GH-receptor mRNA is present (133).

Insulin-like growth factor-I is likely the mediator of GH effects on mammary growth in ruminants (Figure 1). IGF-I is widely expressed across tissues and is a peptide of 70 amino acids with a molecular weight of 7.6 kDa. IGF-I is known to elicit numerous biologic actions on cell growth and death, linear growth,

embryonic and postnatal growth, ovarian function, and protein and carbohydrate metabolism (8;154;325). Bovine, human and porcine IGF-I are identical in amino acid sequence, and murine IGF-I differs by 5 amino acid residues (325). Three related ligands, insulin, IGF-I, and IGF-II make up the IGF family. Growth hormone treatment stimulates the liver to produce IGF-I, which then enters the circulation and can act on peripheral tissues (253). In addition to this endocrine action, IGF-I can be produced locally within a tissue (126;192;210) and thus may act in this autocrine and/or paracrine fashion in the mammary gland (203). Therefore, the presence of locally acting IGF-I within peripheral tissues could cooperate with pituitary-derived GH to stimulate a biological response. In addition, liver-derived IGF-I can serve as an endocrine feedback regulator of GH secretion, which may also act to stimulate peripheral tissue IGF-I production (178;193;283).

Both IGF-I and IGF-II receptors are present in the bovine mammary gland (40;80;125) and IGF-I has been shown to act locally in the mammary gland to induce mammary cell proliferation both in vitro at physiological concentrations found in blood and serum (236), and in vivo (275) in a study in which supraphysiological levels of IGF-I were directly infused into the mammary gland via the teat canal. Somewhat paradoxically, however, IGF-I levels are increased, not decreased like GH at high levels of feeding. These results are thus apparently conflicting with in vivo data in which mammary development is positively correlated with serum GH and negatively correlated with serum IGF-I (267).

IGF-I and II are found in the circulation and extracellular fluids. They can be conjugated to any one of a number of different IGF binding proteins (IGFBP) (56), or bound to binding proteins together with a ternary complex (30). Free IGF-I in the circulation has a half-life of only 10 minutes, which can be extended to 30 to 90 minutes when associated with binding proteins and to more than 12 hours when bound to the ternary complex (30). The IGF binding proteins have been proposed as mediators of the paradoxical in vivo and in vitro effects of IGF-I on mammary development. However, binding protein levels present in heifers on elevated feeding regimes do not seem sufficiently different to account for the decreased mitogenic capacity of IGF-I. It has thus been suggested that a decrease in sensitivity of the mammary gland to IGF-I is the reason for reduced mammary growth at high levels of feeding (267). In vitro results support this hypothesis. Mammary explants removed from heifers fed at a high level show a lower mitogenic response to IGF-I than tissue from heifers on a moderate feeding level (238). In addition, neither IGF-receptor mRNA nor IGF-I binding to its receptor were reduced in these tissues, and mammary gland extracts (MGE) from heifers fed at a high level of feeding were unable to stimulate cell proliferation at the level of MGE from heifers at a moderate level of feeding (320). This action suggested that an additional factor(s) present in the extracts may be interfering with IGF-I signaling and thus reducing the mitogenic response (267). The addition of IGF-I in vitro induces mammary cells to produce their own binding proteins (202). The IGF binding proteins are high affinity, soluble carrier proteins that circulate in blood and extracellular fluids and appear to control IGF blood

transport and association with the IGF surface receptors (57). One such binding protein, IGFBP-3 has been suggested as a possible mediator of the reduced effect of IGF-I at high levels of feeding in dairy heifers. IGFBP-3 levels were found to be higher in MGE from heifers on the high feeding level. Mammary explant and organoid culture studies also support this assertion, because addition of IGFBP-3 inhibited the IGF-I mitogenic response in vitro (238;320).

While these data support the hypothesis that IGFBP-3 may be the factor inhibiting mammary growth at high levels of intake, no mechanism to date has been implicated in the regulation of IGFBP-3 levels. Other factors have recently been suggested as potential mediators, including locally produced growth factors and cytokines, including TGF-beta (238) and leptin (276;278).

Regulation of mammary ductal development is largely regulated by IGF-I (163). Using the rodent model, Ruan and colleagues (1992) showed that localized IGF-I administration in the mammary gland of estrogen-treated hypophysectomized and ovariectomized rats was sufficient to stimulate ductal development (250). A critical requirement for estrogen (estradiol, E2) has been demonstrated in the analysis of GH and IGF-I action in the rodent and bovine mammary gland within the IGF-I axis (9;248). In addition, Kleinberg et al., (2000) demonstrated in rodents that when various elements of the IGF-I axis were deleted, IGF-I and the IGF-I receptor (IGF-R) were essential to normal mammary development (164). In addition, neither estradiol nor GH alone or in combination had any effect on prepubertal mammary development in IGF-I-knockout mice (164). Normal development of the ductal system in this IGF-I-knockout was

restored following administration of exogenous estrogen and IGF-I in this model (249).

Much evidence indicates that a local effect of GH exists on IGF-I action on development in the mammary gland of peripubertal rodents (164). Growth hormone first binds to GH receptors in the stromal tissue of the gland which induces production of IGF-I, which then promotes the development of terminal end buds (9). In ruminants, evidence for a similar action exists (108;144;240;271). In sheep, IGF-I expression is predominantly found in the stromal tissue of the mammary parenchyma which is likely the mediator of allometric growth of parenchyma tissue (144). The fetal ovine mammary gland has also been examined for IGF-I production, and in situ hybridization indicated that IGF-I production is relegated to stromal cells (108). Similar evidence for action of IGF-I promoting prepubertal mammary gland development in ruminants has been shown using in vitro bovine mammary epithelial cell culture (133;271). In vivo, Radcliff and colleagues (2000) showed that administration of exogenous GH increases both serum IGF-I levels and prepubertal mammary gland development (240). In addition, Collier et al. (1993) infused IGF-I for 10 days via the teat canal of pregnant beef heifers and noted a trend indicating a 17% increase in dry fat-free parenchyma mass without a corresponding increase in parenchyma DNA (59).

IGF-I is secreted locally in the bovine mammary gland by stromal cells, but not epithelial cells (125). In addition, the IGF-I receptor is found abundantly on mammary epithelial cells (122;236). The presence of both a local IGF-I producing

source and target within the mammary parenchyma reinforces the role of locally-produced IGF-I as a mediator of ductal development. Some debate exists as to whether this locally produced IGF-I is responsible for its physiological action in the mammary gland, and Hodgkinson and colleagues (1991), have suggested that circulating IGF-I is the major source of IGF-I delivered to the mammary epithelium (140).

Signaling in the IGF-I Family

Normal development of the mammary gland is contingent upon the interactions of a wide range of signaling pathways activated in response to endocrine factors signaling in a tissue-specific manner. Prolactin, estrogen, GH and progesterone have all been known to be crucial hormones guiding mammary gland development for nearly half a century (215). The mechanisms by which a number of these factors elicit their actions, however, have only recently been discovered.

Up to five different classes of membrane bound receptors may interact with the ligands of the IGF family. Two splice variants of the insulin receptor exist (IR-A and IR-B), the type I IGF receptor (IGF-IR), and two potential hybrid receptors based on the dimerization of one subunit of the IGF-IR with either of the two IR splice variants (14;111). The biological actions of insulin, IGF-I and IGF-II are believed to be mediated through IR-A, IR-B, and IGF-IR, respectively (124;206;262). The IGF-IR is a transmembrane tyrosine kinase receptor with structural similarity to the IR that can bind IGF-I, and IGF-II and insulin with 99%

and 99.8% lower affinities, respectively. The Type II IGF receptor is also known as the mannose-6-phosphate receptor. Many investigators believe that the receptor's role in IGF-II function is to moderate IGF-II action by mediating its endocytosis and degradation (172).

A ligand-binding domain is present on each of the two extracellular alpha subunits of the IGF-IR. Each alpha subunit is linked to a beta subunit containing both the transmembrane domain and intracellular tyrosine kinase catalytic domain. Upon ligand binding, autophosphorylation on tyrosine residues of the catalytic domain propagate cytoplasmic signals (300).

Stimulation of postnatal body growth is the predominant physiological action of IGF-I, this was shown by targeted mutations within the IGF-I and IGF-II genes, which disrupted body weight gains in growing mice (79;124;186). IGF-I's action on cell proliferation is its most frequently studied action. IGF-I has been shown to be necessary for progression of cells through both the G1 and G2/M phases of the cell cycle (2;291). In addition, IGF-I has been shown to protect cells from death by its actions on apoptotic and necrotic pathways (63;309). Mammary cells are among the various cell types that have been shown to be protected from apoptosis by IGF-I. IGF-I-dependent signaling pathways dictating cell survival are well studied and defined and are highly cell-type specific (124). The effects of IGF-I on cell signaling in the mammary gland have recently been extensively outlined in a review by Hadsell and colleagues (124).

Four distinct, but interacting, signaling pathways are now known to be engaged by the action of the IGF-IR in the mammary gland (Figure 2). Signaling

through the tyrosine phosphorylation of the scaffolding protein insulin receptor substrate 1 (IRS1) is known to enhance cell survival via IGF-I binding (223). The IRS-1 protein mediates IGF-I –dependent cell survival through activation of phosphatidylinositol 3'-kinase (PI3K) and the serine threonine kinase Akt (75). A second IGF-I-stimulated pathway acts to induce cell proliferation and involves activation of ras, raf, and mitogen activated protein kinase-1 (MAPK1) and MAPK3 (230;247). In this pathway, cell cycle progression is stimulated through the regulation of the cell cycle regulator cyclin D1 and the cyclin-dependent kinase inhibitor 1B (10). Another proliferative pathway has been shown to involve phosphorylation of beta-catenin, which is able to stimulate expression of cyclin D1 and the transcription factor c-myc (60;148;235). A fourth pathway promotes cell survival and is mediated through the interaction of IGF-IR with the 14-3-3 family of proteins (70;114). In this pathway, the serine threonine kinase raf1 translocates to the mitochondria, resulting in the phosphorylation and inactivation of the BCL2-antagonist of cell death, BAD (232).

Although these pathway interactions are presented independently for simplicity, much evidence supports cross-talk among the different pathways that add additional levels of regulation, redundancy and amplification of signaling. Numerous other growth factor receptors are also known to activate these pathways, so a coordination of signaling between varieties of factors is also present depending on cell type and physiological context (124). In addition, much of the work done to elucidate these signaling pathways has used in vitro cell lines or transgenic animals. Thus, the complexity of signaling is likely much higher

when numerous interacting cell types are present in a wild-type animal. Much remains to be elucidated regarding how these IGF-IR-dependent signaling molecules interact in an intact animal to shape normal mammary gland development.

Biological actions of leptin

Leptin is a hormone secreted by fat cells (112;113;336) that mediates central hormonal and metabolic responses to the nutritional state of mammalian species. The prototypical leptin protein is a 16 kDa product of the murine obesity (ob) gene and orthologs have been identified in many mammals including sheep, pigs and cattle (91;151;180;243). This protein is highly conserved across species with the cattle amino acid sequence being 99% similar to that of sheep and 89% similar to that of mouse and human sequences (120;149;151;336).

Circulating blood leptin concentrations vary with the amount of adipose tissue in the body, suggesting a role for leptin in signaling adiposity (41;113;323). Blood leptin concentrations have been shown to increase with body fatness in cattle, sheep, rodents and humans (81;93;112;113). In addition, acute alterations of energy intake have been shown to affect plasma leptin concentrations (149;177). Blood concentrations of leptin were shown to be high in subjects on a high energy diet independent of body fatness level, and fasting rapidly decreased blood leptin concentrations (28;51;81;197;207). Although several other tissue types, including the stomach, skeletal muscle, mammary gland and placenta

have been shown to secrete leptin in the body, adipocytes are believed to be the major source of leptin production (5;164).

In humans and rodents, leptin secretion occurs in a pulsatile manner and is subject to diurnal changes (281). Pulsatile regulation of leptin secretion has also been observed in sheep, however, in both cows and sheep diurnal changes do not seem to be present (27;30;301).

A number of hormones and factors have been shown to promote leptin secretion including insulin, glucocorticoids and cytokines. Testosterone, peroxisome proliferators-activator receptor (PPAR) gamma agonists, IFN-gamma, and sympathetic nervous activity mediated by catecholamines have been shown to inhibit leptin secretion (5;6;130;245;279). The pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-alpha), interleukin-1-beta (IL-1beta), and transforming growth factor-beta (TGF-beta) were able to stimulate leptin production from precursor preadipocytes in the absence of mature adipocytes (279). Growth hormone appears to have differing effects on leptin production in human and rat adipocytes, increasing synthesis in the former while decreasing it in the latter (6).

Leptin Receptor

The biological actions of leptin are elicited by the binding of leptin to the leptin receptor (OB-Rb). OB-Rb is the long isoform of the leptin receptor which contains a transmembrane intracellular tail that is required for endocrine function and signaling (32;34;62;100;116;175;176;208;296;297;334). Alternative splicing

of the primary LR transcript can result in the production of multiple isoforms (OB-Ra to -e) that contain a common extracellular domain but lack transmembrane signaling capacity (100;175;176). OB-Rb is a member of the interleukin-6 family of class I cytokine receptors. Though they contain no intrinsic enzymatic activity, cytokine receptors are able to transmit signals via noncovalently associated Janus kinase (JAK) family tyrosine kinases (Figure 3). Ligand binding to OB-Rb elicits the activation and tyrosine phosphorylation of Jak2 and the subsequent tyrosine phosphorylation of two tyrosine residues, Tyr985 and Tyr1138 on the OB-Rb (16;121;294). This then initiates the activation of intracellular signals by recruiting specific signaling proteins with phosphotyrosine binding domains leading to the subsequent activation of downstream signaling proteins along a number of pathways. One specific protein recruited by the phosphorylation of Tyr1138 of the OB-Rb is the SH2 domain-containing protein signal transducer and activator of transcription-3 (STAT3). Upon its phosphorylation and activation dimerized STAT3 then translocates to the nucleus and mediates gene transcription, including that of the suppressor of cytokine signaling, SOCS3, which is able to attenuate OB-Rb signaling in a feedback manner by interfering with further Jak2 activation (16;19;25;26;44;179;289;322).

Crosstalk between hormones is a critical area of research geared toward the understanding of the endocrine feedback loop regulating body energy stores. "Adiposity signals", which are putative molecular substrates present in the circulation that can reveal information about body condition to the brain in order to help regulate energy intake, could possibly be present in the body in the form

of the hormones insulin and leptin (5). Both of these hormones are known to circulate in the body in proportion to body fat mass in the physiologically normal mammalian organism, and can act upon the central nervous system (CNS) to inhibit food intake through their ability to signal within the brain (5). The suggested physiological and intracellular signaling interaction between insulin and leptin at the level of the CNS has subsequently been extended to other specific tissues, including the mammary gland. It has been well established that high energy diets increase fat deposition within peripheral tissue stores, including the mammary gland. Local concentrations of IGF-I and leptin are known to be increased locally in the mammary gland with increased energy intake. However, although IGF-I has been shown to have a stimulatory effect on mammary epithelial cell proliferation, mammary gland development is inhibited by high energy diets. Thus, it is here hypothesized that a hereto unknown factor is increased in concentration with the feeding of high energy diets which interferes with the proliferative action of IGF-I on mammogenesis. Our laboratory has proposed that leptin, a hormone produced by adipocytes, could potentially mediate the inhibition of IGF-I-stimulated mammary parenchymal development via crosstalk between the IGF-I and leptin receptors. Evidence is presented supporting SOCS3 as a potential mediator of the negative feedback on IGF-IR activation promoted by leptin intracellular signaling.

Suppressor of Cytokine Signaling (SOCS) Proteins

Recently a new family of cytokine-inducible proteins called the "suppressors of cytokine signaling (SOCS)" were discovered which function as negative regulators of signaling pathways involved in the cellular actions of numerous cytokines, growth factors, and hormones (310). The SOCS proteins share a homologous structure with an NH2-terminal region of variable length, a central SH2-domain, and a COOH-terminal SOCS-box (310). While a number of SOCS genes are constitutively expressed in certain tissues, others have been shown to be highly regulated and act as temporal and spatial modulators of cytokine and growth hormone signaling in tissues such as the mammary gland. The JAK/STAT pathway is one specific signaling network that has been identified as being negatively regulated by the SOCS proteins. Both IGF-I and leptin are known to signal via the JAK/STAT pathway, which may indicate that the SOCS proteins may mediate signaling interactions and cross-talk between these two hormones which are both known to function locally within the mammary gland. Briefly, the binding of a cytokine or growth factor to its receptor triggers tyrosine phosphorylation and activation of receptor-associated JAKs. These kinases then act to phosphorylate multiple target proteins, including tyrosine residues in the cytoplasmic domains of the receptor, and receptor-associated STAT monomers. Phosphorylated STAT monomers form dimers that then translocate to the nucleus and bind to specific DNA target sequences, modulating gene transcription (315).

Cytokines and growth factors have a plethora of biological effects and a great potency in most tissues, which necessitates a high degree of control to avoid

potentially damaging consequences. Soluble receptors, binding proteins, and counter-regulatory factors such as TGF-beta exist in the extracellular milieu to provide one level of control. A greater amount of control, however, may arise from the actions of intracellular molecules such as phosphatases to dephosphorylate and inactivate specific proteins (198). Similarly, the SOCS proteins, which are known to be induced by cytokine activation of the JAK/STAT signaling pathway, can act to bind specific proteins and downregulate pathway activation (315).

Cytokine-inducible SH2 domain protein (CISH) was the first member of the SOCS family to be identified. CISH was found to be an immediate early-response gene whose expression was induced by several cytokines (331). The protein "suppressor of cytokine signaling 1" (SOCS1) was the second member to be identified, discovered independently in three separate laboratories (96;213;290). Currently eight members of the SOCS family have been identified, SOCS1-SOCS7 and CISH. Based on database sequence mining at least 20 SOCS-related proteins exist in humans and mice, each containing a 40-residue C-terminal motif, the "SOCS box", but differ in their individual types of protein-protein interaction motifs (138;290;315).

SOCS genes vary in expression level across tissues, particularly SOCS1 and SOCS3. This expression is often not constitutive and can be rapidly regulated by various cytokines as well as GH, prolactin and leptin (315). The promoters of many SOCS genes contain STAT-responsive DNA elements, consistent with their expression dependence on the JAK/STAT signaling pathway (213;290).

However, JAK/STAT-independent regulation of SOCS expression has also been described for SOCS3 in neutrophils stimulated by IL-10 (49).

Inhibition of the JAK/STAT signaling pathway by the SOCS proteins can arise from several distinct molecular interactions. This inhibition can work by SOCS proteins binding directly to the SH2 domain of the tyrosine phosphorylated cytokine receptors or by directly binding to the JAK tyrosine kinases which also interact with the receptors. For example, SOCS1 can act by directly binding to all four JAK molecules, and inhibiting their catalytic ability. The association between SOCS1 and JAK2 occurs at the SOCS1 SH2 domain, with the N-terminal amino acid region providing kinase inhibitory activity (96;213;218;329). SOCS3 also binds to JAKs, but with lower affinity than SOCS1 and a weaker inhibition of catalytic activity. SOCS3 may, however, be more effective at interacting with activated cytokine receptors. The SOCS3 protein has been shown to associate with the activated GH receptor to inhibit STAT5 signaling. Here the mode of SOCS action is not by prevention of the docking of STAT5 to its JAK receptor but still results in the inhibition of JAK2 activation (127).

Various members of the SOCS family have been shown to have actions in the lactating rodent mammary gland (135). Expression levels of SOCS1-3 were examined in the postpartum mammary glands in a study by Helman and colleagues (135). While SOCS1 expression did not change over the course of 3 days, SOCS2 and SOCS3 expression gradually increased during the three days postpartum. It is likely that protein expression levels in the mammary gland were induced by an increase in circulating prolactin in response to suckling and that

SOCS3 acts as a physiological inhibitor of prolactin (PRL) receptor signaling. PRL signaling is mediated through STAT5 and MAP kinase pathways and promotes lactogenesis (119;136). To further this work, Tam et al. (2001) showed that PRL is able to induce expression of SOCS1-3 and CISH in the mammary gland (295). In addition, it was shown that the mammary gland is rendered unresponsive to PRL following pup withdrawal, which is likely because of increased levels of SOCS3 (295).

SOCS1 signaling has been implicated in the negative regulation of PRL action in the developing mammary gland of the mouse (181). While PRL has been intensely studied in relation to lactation and mammary growth, its role in mammary development is less clear (8). Species variation in the role of PRL in the process of mammogenesis has been demonstrated. In the virgin mouse, PRL induces the regression of fully elongated terminal end buds and promotes the appearance of ductal side branches (74). PRL-deficient mice have markedly reduced ductal branching with arrest of mammogenesis at puberty (142). STAT5a activation has been implicated in the PRL signaling cascade linking the transcriptional activator to lobulo-alveolar development during pregnancy and lactation (147) and STAT5a-null mice show a mammary phenotype very similar to PRLR+/- females, with impaired differentiation of lobulo-alveolar units and the inability to lactate (187;298). In cattle, however, PRL seems to be less important, especially prior to lobulo-alveolar formation during pregnancy. The large natural fluctuations seen in blood PRL levels in response to environmental conditions do

not accompany changes in mammary gland development in growing calves, indicating that PRL concentrations have little impact on mammogenesis (8).

In the pubertal, developing, rodent mammary gland, a critical role for SOCS1 in mammary cell proliferation and differentiation was demonstrated by Lindeman et al. (181). When the SOCS1 gene was knocked out in mice, it was shown that lobulo-alveolar development was accelerated during pregnancy thus establishing this protein as an inhibitor of PRL-signaling during normal development. Tonko-Geymayer et al., (2002) examined SOCS3 and CISH expression patterns in the mouse mammary gland over the course of pregnancy, lactation and involution, as well as in cultured mouse HC11 mammary epithelial cells treated with glucocorticoids (302). It was found that these SOCS protein family members inhibited PRL receptor-mediated activation of STAT5 in vitro, as expected and that glucocorticoids inhibited expression of both proteins (302). In addition, CISH and SOCS3 were differentially expressed in the mammary gland during different physiological states: CISH levels were high during the second half of pregnancy and SOCS3 expression was elevated during the first 12 days post conceptum. During involution, SOCS3 levels increased, whereas CISH levels decreased, lactation initiation coincided with high expression of both inhibitory factors (302). Epidermal growth factor was implicated as a possible mediator of SOCS expression in the mammary gland in this study in a manner independent of PRL signaling through STAT5 activation based on differential expression of SOCS3 and CISH in HC11 cells stimulated with either EGF or PRL (302).

The SOCS proteins have also been implicated in the control of insulin signaling in adipocytes in the mouse. CIS, SOCS2 and SOCS3 were transiently increased in adipose tissues taken from female mice stimulated with ovine PRL for 0-24 hours (183). It is believed that PRL-induced SOCS3 expression could play a role in the negative regulation of insulin signaling in adipose tissue, as PRL exposure inhibited insulin-stimulated leptin production in these adipocytes. This inhibitory effect was accompanied by increased SOCS2 in adipose tissue of the PRL-null transgenic mice, possibly indicating a mechanism for decreased leptin secretion. In addition, in the absence of insulin, PRL was shown to have no effect on leptin production (183).

The SOCS proteins have been shown to interact directly with the activated members of the IGF family of receptors. Dey and colleagues were able to demonstrate binding of the negative regulators of cytokine signaling SOCS2 (84) and SOCS3 (83) to the activated IGF-IR and IR using a yeast two-hybrid screen to detect binding of the receptor and SOCS substrate both in vitro and in intact cells. It was also shown that in cells overexpressing IGF-IR and SOCS3, SOCS3 is phosphorylated on tyrosine residues after treatment of the cells with IGF-I, suggesting that SOCS3 is indeed a substrate for the receptor tyrosine kinase (83). A role for SOCS proteins in the inhibition of the GH tyrosine kinase receptor has also been demonstrated. In Chinese hamster ovary cells transfected with both the GH receptor and SOCS cDNAs, SOCS1 and SOCS3 were able to block GH-induced transactivation of the GH-responsive serine protease inhibitor 2.1 gene promoter (1).

A role for the SOCS proteins in control of signaling by cytokines of the interleukin-6 (IL-6) family has been well established (78;256). Bjorbaek and colleagues (1999) showed that in addition to mediating SHP-2 binding and ERK activation during acute stimulation, a specific tyrosine residue of the leptin receptor mediates feedback inhibition of leptin receptor signaling by binding to activated leptin receptor-induced SOCS3 (23). Thus, the leptin receptor is able to autoregulate itself using the SOCS3 protein as a signaling inhibitor. In addition, using degenerate phosphopeptide libraries screened against recombinantly produced SH2 domains to determine the sequences of optimal phosphopeptide ligands, De Souza et al. (2002) predicted that SOCS3 would have a high affinity for the interleukin-6 family (78). This prediction was validated by binding SOCS3 to the phosphopeptides derived from the putative docking sites of the IL-6 family receptors gp130, leptin and erythropoietin. SOCS3 showed the highest affinity for the gp130 receptor, which has only one phosphopeptide docking site, and lower affinities for leptin and erythropoietin, which can compensate for the weaker affinity with the binding of SOCS3 to multiple individual sites on the receptor (78;99).

Peripheral administration of leptin to ob/ob mice rapidly induced SOCS3 mRNA in the hypothalamus but had no effect on CIS, SOCS1 and SOCS2 (25). In mammalian cell lines, SOCS-3, but not CIS or SOCS2, blocked leptin-induced signal transduction. Peripheral ciliary neurotrophic factor (CNTF) administration to ob/ob mice rapidly induced SOCS3 mRNA in areas of the hypothalamus that

are both overlapping and distinct from those in which SOCS3 was induced by leptin (24).

FIGURES

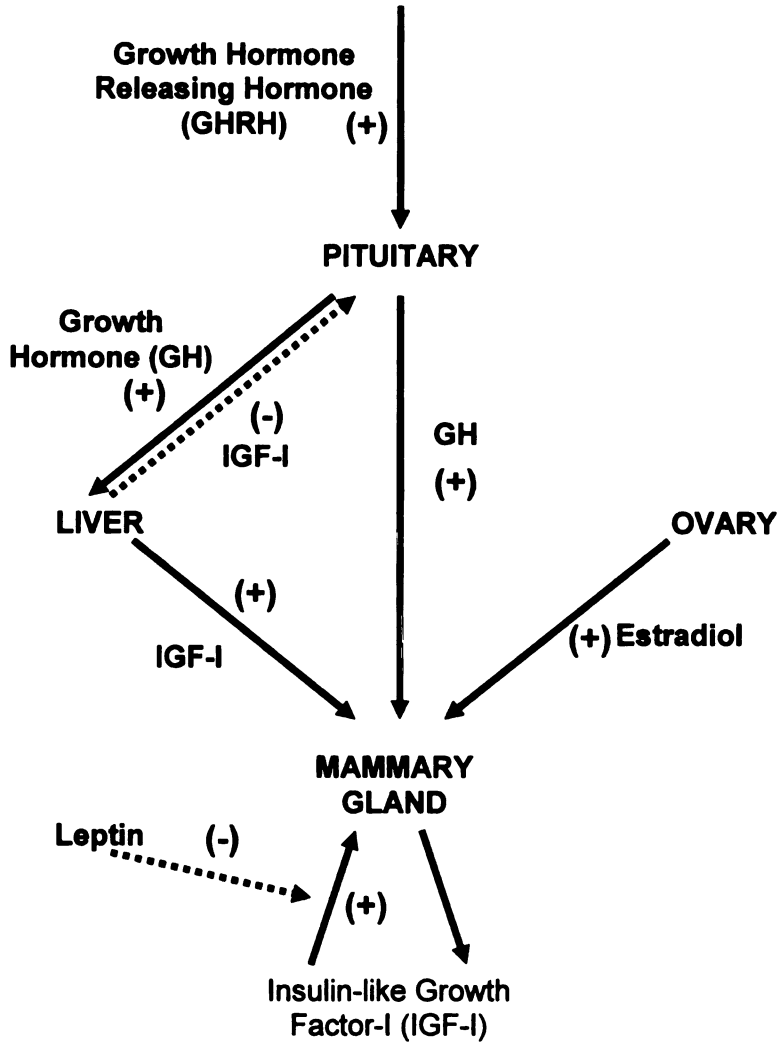

Figure 1: The growth hormone/insulin-like growth factor-I (GH/IGF-I) axis and the bovine mammary gland. Estrogen, growth hormone (GH), and insulin-like growth factor-I (IGF-I) have stimulatory effects on mammary development. IGF-I is produced by the liver in response to GH and has a negative feedback effect on GH secretion by the pituitary gland. IGF-I also has a stimulatory effect within the mammary gland with an autocrine/paracrine mode of action. Recent evidence from Silva (2002) supports a role for leptin in the inhibition of IGF-I action in the mammary gland. Adapted from Hadsell et al. (124).

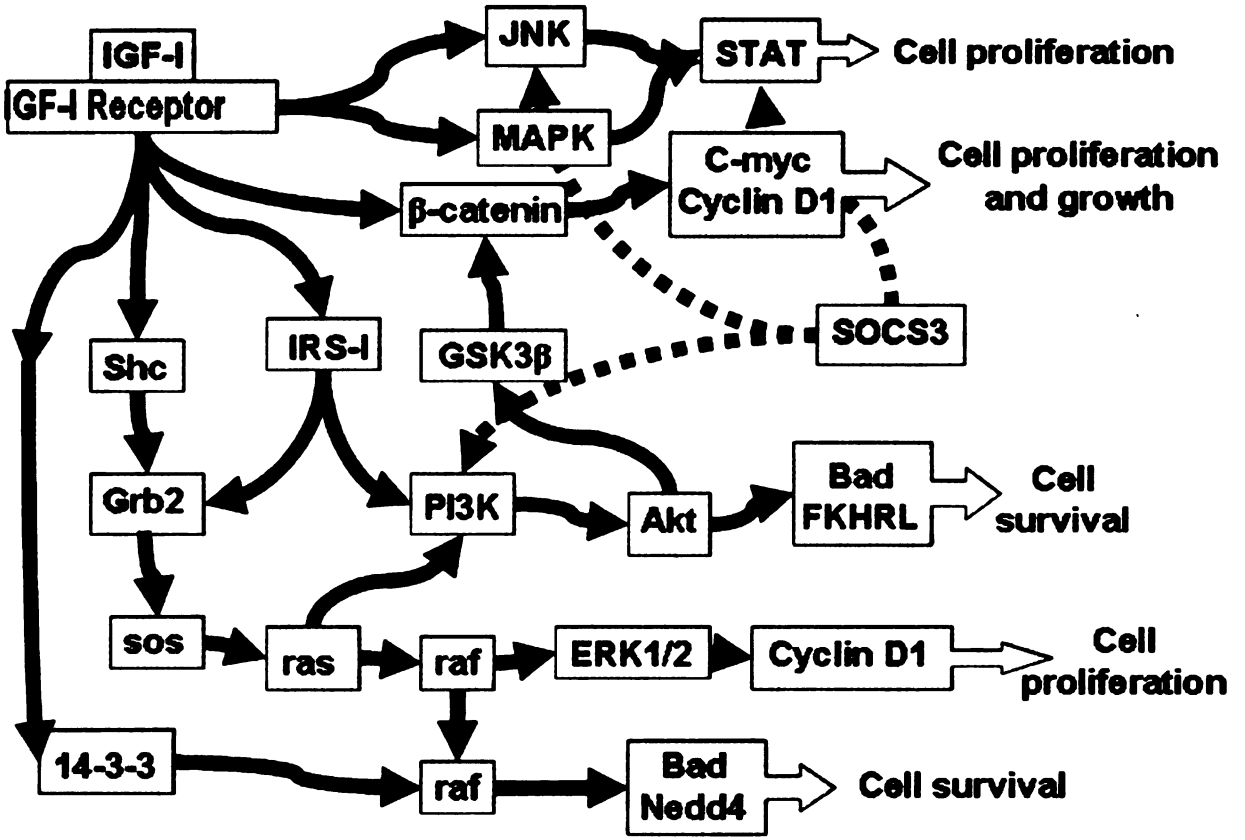

Figure 2: Insulin-like growth factor I receptor (IGF-IR) signal transduction pathways. Five major pathways exist by which the IGF-IR mediates its effects on cell survival, growth and proliferation. IRS1 = insulin receptor substrate 1, PI3K = phosphatidylinositol 3'-kinase, JNK = janus kinase, MAPK = mitogen activated protein kinase, STAT = signal transducer and activator of transcription, GSK3β = glycogen synthase kinase 3β. SOCS3 = suppressor of cytokine signaling-3. Adapted from Hadsell et al. (124).

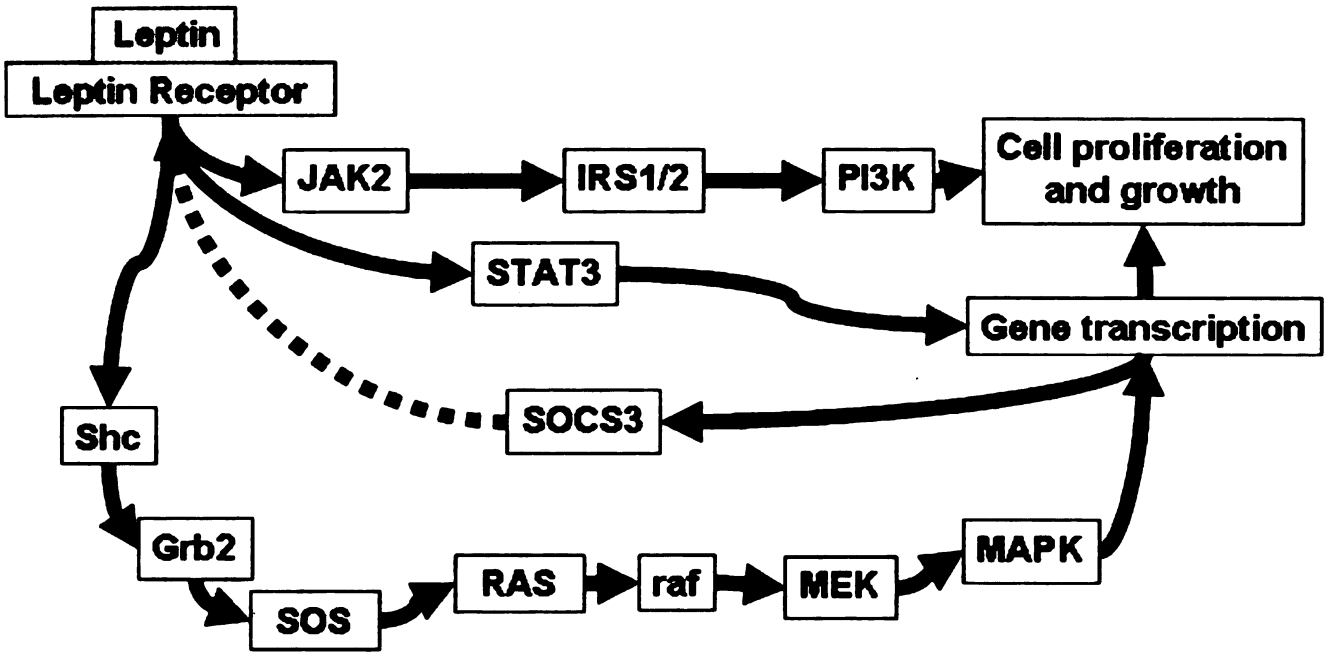

Figure 3: Leptin receptor signaling pathways. Leptin receptor activation stimulates STAT1, STAT3 and STAT5 tyrosine phosphorylation *in vitro* and STAT3 tyrosine phosphorylation *in vivo* (132). The Ob-Rb isoform acts as a potent JAK/STAT signaling activator. Leptin's ability to activate STAT3 is decreased in the hypothalamus when mice are subjected to high fat diets (103). Leptin deficiency occurs through two mechanisms, either by a defect in leptin transport across the blood brain barrier or by suppression of leptin signaling (103). Leptin signaling is blocked by suppressors of cytokine signaling-3 (SOCS-3) and by dephosphorylation by protein tyrosine phosphatase 1B (PTP-1B). SOCS-3 and PTP-1B deficient in mice exhibit increased sensitivity to leptin and resistance to obesity (103). The phosphatidylinositol 3-kinase (PI3K) and Ras-mitogen activated protein kinase (MAPK) signaling pathways, both of which are downstream of JAK, are activated by leptin (132). Adapted from Harvey and Ashford (132).

CHAPTER 2

Design and application of a bovine metabolism long oligonucleotide microarray

Abstract

The Human Genome Project provided a catalyst for the development of extensive publicly available databases housing genome sequence information for many species of research interest, including production animals. When coupled with advancements in oligonucleotide synthesis, these bioinformatics resources provide a powerful tool for in-house generation of custom oligonucleotide microarrays designed to test specific hypotheses. Nutritional genomics is the integration of genomics into the nutritional sciences that has been used to elucidate the functions and interactions of genes and nutrients which can then be used to design diet-based therapeutic interventions to avoid disease manifestation. We have designed a bovine metabolism-focused microarray containing known genes using publicly available genomic internet database resources provided by NCBI, TIGR, KEGG, Swiss-Prot and BioCarta. 70mer oligonucleotides for each gene were designed and spotted on glass slides at sufficient replication to facilitate accurate detection of changes in gene expression. A microarray containing a robust collection of genes known to be involved in metabolism and regulation of metabolism will provide a better understanding of the etiology of common metabolic diseases in cattle.

Keywords: metabolism, gene expression, microarray, disease

Introduction

Nutrition and functional genomics

Nutritional genomics is a new tool to study the interactions between dietary nutrients, environmental factors and cellular and genetic processes (156;209;292). Advances in a number of scientific fields have allowed for parallel assessment of expression of thousands of messenger RNAs (mRNA) to be performed in a single experiment. Oligonucleotide production has improved for both yield and quality of gene probes. Robotics technology now allows the arraying of hundreds or thousands of PCR amplified complementary DNA (cDNA) clones or genes at high density on derivatized glass slides or chip substrates. Genetics research has provided identification of a large fraction of the total gene products present in mammalian systems. Mathematical aspects of computational biology have made analysis of data from large scale genomics research increasingly possible. These advances will accelerate discovery of genes responsible for various nutritionally-related diseases and syndromes (18;118).

A complete understanding of nutrition requires research in many facets of biology. Chemical analysis techniques must be performed to isolate and characterize the structures of essential nutrients. Biochemical and physiological experiments must be performed to determine nutrient metabolic and signaling pathways responsible for homeostasis in the organism of interest. In addition,

research on inborn errors of metabolism or other genetic studies is used to identify gene-nutrient interactions. Examples of this include the human recessive defect galactosemia, in which defective lactose-1-phosphate uridyltransferase (GALT) causes galactose to accumulate in the blood, causing health problems including mental retardation, and phenylketonuria (PKU), a trait that results in accumulation of phenylalanine in the blood that can cause neurological damage (156). One limitation of this approach is that one is unable to sufficiently predict and quantify interactions among dietary components and polymorphic alleles. Microarray technology provides a high-throughput functional genomics approach to begin to provide a greater understanding of the complex and reciprocal interactions within the genome at the molecular level (292). Both genetic and dietary variables can be manipulated to design an effective nutritional functional genomics study to examine gene interactions affecting normal and diseased physiological states.

Developing a microarray

Advances in robotics and biotechnology in the past decade have made possible the fabrication of microarrays for expression screening of tens-of-thousands of genes in a single experiment (128;184;257;259;287;318). Microarrays are hundreds to thousands of DNA fragments (probes) precisely positioned at a high density on a solid support where they can act as molecular detectors to measure relative mRNA abundance between biological samples (Figure 1). Microarrays vary in the type of solid support used (glass slides or filters), the type of probe

used (cDNA or oligonucleotides) and the manner in which the probe is deposited on the array (synthesized in situ or spotted).

The first-generation arrays were built by spotting cDNAs amplified from clone libraries onto nylon membranes (129;258). A cDNA microarray is generated by amplifying gene specific sequences using polymerase chain reaction (PCR). This approach requires development of a clone library and thus is limited by the presence or absence of genes within the clone library. A clone library is generated by extracting mRNA from a tissue or organism of interest, converting this mRNA into cDNA, fragmenting the cDNA using restriction enzymes, and then "cloning" the cDNA fragments into plasmid vectors. Once inserted into the plasmid vector the sequence can be amplified by growing it in competent bacteria which are able to take up the plasmid. These competent bacteria also contain DNA coding for antibiotic resistance so that clone containing colonies can be selected in bacterial culture. Antibiotic resistant colonies now each contain different plasmids (and thus gene sequence fragments) and can be collected and sequenced to create a gene library. One limitation of cDNA microarrays is that cDNA clones and PCR amplicons must be tracked, which can lead to misidentification of up to 10-30% of clones (314;318). Nylon membrane arrays are limited in their spot density but development of spotting procedures for modified glass slides increased the maximal spot density and allowed addition of more genes probes to an array.

Oligonucleotide arrays are a more recent advance that allows designing of probes to ensure unique probe sequences, optimal melting temperature, and

similar hybridization efficiency between probes (Figure 1). Control of these factors standardizes hybridization conditions from gene to gene to improve expression data. This approach is not limited by the genes present in clone libraries and requires only a gene sequence from a database. Although the probes used in oligonucleotide arrays are shorter in length than cDNA probes, they experience less problems with secondary structural formation (probes annealing to themselves), low hybridization efficiency, and non-specific hybridization. Oligonucleotide microarrays can be composed of short oligonucleotides (25 bases) synthesized directly onto a solid support using photolithographic technology (Affymetrix arrays) (Figure 2) (184;189) or constructed from long oligonucleotides (55-130 bases) spotted onto glass slides (39;155;338). Large differences in expression data among genes have been reported across microarray platforms (cDNA, oligonucleotide, and Affymetrix arrays), but this variation may be due to probe sequence and annotation (31;169;332). However, data from one study directly comparing gene expression data from a spotted long (80mer) oligonucleotide array to those on an Affymetrix 25mer chip showed that for the majority of genes (92%), no significant effect of platform on gene expression was detected (171). Here, treatment effects were stronger than platform effects. Only 8% of genes showed divergent results between the two platforms, and this divergence may represent incorrect probe identification on the array, incorrect gene identification, or alternative splicing (171). Medium length (70mer) oligonucleotide probes spotted onto glass slides

at high density are an excellent and cost-effective microarray platform increasingly used by researchers in many disciplines (314).

Conducting a microarray experiment consists of four steps: 1) generation of the array, 2) sample mRNA isolation, conversion to cDNA, amplification and labeling with fluorescence dye, 3) hybridization of labeled sample to the array and measurement of resulting hybridization, and 4) analysis and interpretation of the data (54;89;94;134) (Figures 1, 2).

The first step in developing a microarray is to generate a set of sequence-validated probes, each with a unique sequence that has little cross-hybridization to other probes. The gene set should contain a comprehensive representation of the expressed fraction of the genome or a collection of genes specifically tailored to an experimental system of interest (i.e. a set specific to a research field such as metabolism, or type of tissue such as the mammary gland). Following collection of the genes of interest, microarrays can be fabricated in a number of ways (89;94;134;184). In general there are two main types of microarrays: either the oligonucleotides are synthesized on the area in situ (Affymetrix arrays) or they are printed or deposited onto their solid supports.

Microarrays rely on a labeled representation of mRNA generated using reverse transcriptase to make a single-stranded cDNA. Following cDNA synthesis different colored fluorescent labels (Cy3 = red, and Cy5 = green) are attached to the aminoallyl modified cDNA targets in the experiment (control or treated) and hybridized to the array. Labeled Cy3 and Cy5 fluoresce at different wavelengths and are detected using a confocal laser scanner. The signal presented by each

fluor indicates relative mRNA abundance. To analyze expression, data spots must be correctly identified and aligned to a grid specifying spot location. Background and signal intensities are calculated based on the expected position, size and shape of each spot. To standardize hybridization across an array, normalization procedures are applied to analysis. Normalization procedures may make use of genes that are known to be unaffected by treatments and remain constant in their expression (housekeeping genes), or the average spot intensity across the entire slide, based on the assumption that the majority of genes are not modified between treatments (239). A local weighted linear regression procedure (lowess) is considered the most robust normalization and is often used for data normalization (58). Fold change cutoffs (i.e. greater than 1.5 fold) are a rudimentary criteria for detecting differentially expressed genes still used by some researchers (58). More recently, linear and mixed models allow analysis of variance and determination of statistically significant genes (i.e. $P<0.05$) (15;190). In addition, many data visualization techniques have been developed for microarray data analysis including clustering and principle component analysis. These methods provide deductive insight especially in data across multiple treatment levels for time sequences (95). Known information on gene activity and relationship to other genes should be used to analyze microarray data in a biological context (239). Recent approaches include determination of biochemical and cell signaling pathways that are coordinately regulated between experimental conditions. Bayesian approaches also allow integration of previously described biological data. Statistical and bioinformatics applications to

microarray data analysis is a quickly progressing field and the interested reader is directed to the many reviews of this topic (15;53;190).

A number of different designs may be employed in microarray experiments, including direct comparison, dye-flip, reference, and loop designs (52;158;326) (Figure 3). Often the type of comparison being made in the study as well as availability of biological replicates and funding will determine the type of design used. Although biological replicates (an increased number of individual animals included for cDNA comparisons) are preferred to technical replicates (repeated sampling of a single or pooled sample over a number of different microarrays), the cost of each sample will often determine the number of samples analyzed. Dye-flip experiments, in which two samples are compared on two different microarrays with the labeling dye reversed for each microarray, are preferred to account for preferential binding of either the Cy3 or Cy5 toward given genes (50). Dye-flips, however, are technical replicates, not biological replicates.

Nutritional genomics and animal production diseases

Discovery of specific genes responsible for disease phenotype will enable improved prediction of disease risk and development of novel methods to prevent and treat diseases specific to an animal's genotype (293). Diet has been identified as a causative agent for a number of diseases in dairy cattle including ketosis, hepatic lipidosis, ruminal acidosis, laminitis, and periparturient paresis. Each of these diseases is incompletely understood, and prevention solely through careful formulation of diets specific to the metabolic status of the animal

is difficult. The precise molecular mechanisms causing diseased phenotypes are not clear. It is highly likely that some diet-regulated genes, including their normal, common splice-variants (genes with DNA sequence rearrangements), play a part in dictating the onset, incidence, progression and severity of each disease. One major factor determining disease susceptibility lies in individual genetic makeup, as many animals in the group will be phenotypically normal with others failing in health. It is possible, however, that dietary intervention based on knowledge of nutritional requirement, nutritional status and genotype can be used to prevent or mitigate these diseases by improving group diet formulation.

Dietary factors influence gene expression

Multiple mechanisms exist by which dietary factors can elicit cellular responses in biological systems (Figure 4). Nutrients may directly modify gene expression as ligands for transcription factors or stimulate signal transduction pathways (109;156;191;209). Nutrients may also indirectly modify gene expression via their metabolites or through the modification of other cellular pathways (156). One example of a dietary factor acting as a transcription factor ligand (Figure 4A) is illustrated in fatty acid metabolism, which is known to be regulated by the peroxisome proliferator-activated receptor (PPAR) family of transcription factors. The fatty acids palmitic, linoleic and arachidonic acid and eicosinoids have been shown to be ligands for the nuclear receptors of the PPARs, which act as fatty acid sensors (3;12;105;166). The PPAR lipid sensors heterodimerize with the retinoic acid receptor (RXR), and RXR expression can be regulated by dietary

retinol (76). In a similar manner, the sterol regulatory element binding proteins (SREBP) are activated by protease cleavage and are regulated by low oxysterol concentrations and changes in insulin/glucose and polyunsaturated fatty acids (92), and the carbohydrate responsive element binding proteins (ChREBP) are proteins activated in response to high glucose and regulated by phosphorylation events (156;308).

Dietary chemicals can be metabolically converted to ligands that alter gene expression (Figure 4B). One example of this is seen in steroid biosyntheis. Concentrations of steroid hormones derived from cholesterol are regulated by 10 steps in their biosynthetic pathway. The various intermediates produced along this pathway are also available to branch into other metabolic pathways (1) (222;229). Specific combinations of alleles regulating the enzymatic steps in these assorted pathways will regulate the concentration of any given ligand branching off of steroid biosynthesis and thus potentially link nutrient intake and gene expression.

Dietary chemicals and nutrients have been linked to effects on numerous established signal transduction pathways (Figure 4C). These include polyphenols (PI3K/Akt pathway) (3), resveratrol (MAPK pathway) (13), phenethyl isothiocyanate (NF-kappa B pathway) (150), genistein (NF-kappa B and PI3K/Akt pathways) (254), and retinoids (PI3K/Akt pathway) (219).

Nutrition and genome interactions

Selection pressures exerted on the bovine species in the processes of gene mutation, purifying selection and random drift as well as human selection have given rise to the primary sequence of the bovine genome as well as the genetic variation that exists today within the species (90). One of the most persistent and variable environmental pressures working to shape an organism's genome is nutrition, which can contribute greatly to genetic variation (102;292). Nutrients and environmental factors can heavily influence the development and viability of a fetus and a growing portion of literature indicate that gene imprinting is an important factor in the penetrance of deleterious genetic flaws (33;159). The ability of dietary components to affect postnatal growth and gene imprinting shows that nutrition is an important *in utero* selection pressure that may contribute to fixation of altered phenotypes and genetic defects in populations (33;159). Genomic responses to nutritional insults can work at the level of DNA transcription, mRNA translation, and protein and mRNA stability to maintain homeostasis (156). Maternal nutritional status can alter the epigenetic state of the fetal genome and imprint gene expression levels with lifelong consequences (317). These epigenetic alterations of the fetal genome do not alter the primary DNA sequence, but affect gene expression. This could explain the subtle differences observed in monozygotic twins (82). Methylation of DNA has been shown to be a major factor in regulating gene expression. These methylation patterns are established early in life and remain stable throughout life (139;159). Cells can thus use genomic adaptation to regulate nutrient transport and nutrient status, alter nutrient storage capacity, alter the flux of intermediates through

metabolic branch points, and restructure the cellular transcriptome and proteome to initiate cellular differentiation, proliferation or apoptosis.

Cattle metabolic disease and nutritional genomics

Similar limitations exist for assessing the regulation of individual or multiple genes by dietary factors in cattle. One must first separate cause from effect for each gene to evaluate which genes are responsible for a given phenotype. Next, one must determine if expression patterns for one strain or genotype are particular to that genotype. Based on inbred mouse studies, different breeds of cattle may indeed have unique patterns of gene expression based on diet and genotype (110;156;182). This could also help explain differences in metabolic disease susceptibility seen in different breeds of cattle. Increased susceptibility to copper toxicity and periparturient paresis by the Jersey breed, relative to the Holstein would be an example of this difference (88;195).

Directed dietary intervention to treat or prevent diseases can be a great challenge. It is difficult to determine the precise way to confront a disease state when multiple genes and environmental variables interact to cause the phenotype. Identifying the genes with the greatest contribution to the disease state must first be performed and then linked to their regulation by dietary variables. To use the example of hepatic lipidosis, a disorder which can arise in overconditioned lactating dairy cows in the immediate postpartum period, much is known about the etiology of the disease, but we do not have a true understanding of the genes responsible. A bovine specific microarray will

facilitate the discovery of genes regulating the diseased condition in dairy cattle and forego the need to use model organism microarrays (i.e. mouse) to study these problems.

RESULTS

Development of the bovine metabolism (BMET) microarray

First generation microarrays employing extensive cDNA libraries have allowed high numbers of both known and unidentified genes to be surveyed. Many of these arrays have no or limited spot replication, impeding observation of within-plate variance and increasing the technical variance of expression observations (29). The Human Genome Project has added significantly to databases such as Entrez Gene, the Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), The Institute for Genomics Research (TIGR), and BioCarta. These publicly available resources, paired with recent price reductions in oligonucleotide synthesis, allow researchers to feasibly design and produce microarrays with gene sets tailored to specific research areas.

To build a comprehensive metabolism directed gene database, a directed search to collect metabolic enzymes and signal transduction related genes was performed using internet database resources and nucleotide sequences gathered from United States Department of Agriculture expressed sequence tag projects. Genes were collected from several functional categories: metabolic enzymes, mitogen and mitogen binding proteins, growth factors and cytokines, intracellular signaling proteins, transcriptional control regulatory proteins,

apoptotic regulators, and cell-cycle regulatory proteins. A gene list of human DNA sequences pertaining to metabolic genes and genes specific to pathways of interest were extracted from the Swiss-Prot Metabolic Pathway website (2), the Kyoto Encyclopedia of Genes and Genomes (KEGG) (3), and BioCarta (4) websites. The resulting genes from this search were annotated using NCBI Gene (5), Swiss-Prot, and Online Mendelian Inheritance in Man (OMIM) (6), along with their KEGG, BioCarta and Gene Ontology (7) classifications. The human sequences from metabolism related genes were searched against bovine sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) (11). Those genes with an NCBI Reference Sequence (RefSeq), complete sequence, or 3'-end sequence were saved for oligonucleotide design. Next, we used keywords corresponding to categories of interest to search the Gene Ontology, UniGene and Swiss-Prot databases to gather human sequences of known genes and collected their bovine sequences in the same manner. This process generated a large list of genes corresponding to a UniGene cluster unique ID number with a specific accession number as an identifier (Figure 5). A cluster is a compilation of sequences of overlapping DNA that represent one gene (8). Only those bovine genes with a RefSeq link, complete sequence, 3'-end sequencing, or strong TIGR (9) cluster match were used to facilitate oligonucleotide probe design. All Perl scripts written to generate this data are available upon request under an open source license.

From these databases 4,010 human RefSeq genes were identified. From the nucleotide sequences of these 4,010 genes 2,371 matching bovine sequence

homologues with an expectation value (e-value) of $1e10^{-35}$ or smaller were found. The expectation value is a measurement of sequence similarity, and values below $1e10^{-4}$-$1e10^{-15}$ tend to show strong sequence homology between two sequences being compared. A very low e-value score was chosen to ensure correct gene identification. This gene set included 513 GenBank RefSeq genes, 109 completely sequenced genes, and 901 3'-end United States Department of Agriculture sequenced genes and all genes had a TIGR cluster match. Of the 1207 KEGG metabolism genes extracted, greater than 1,100 are represented in this dataset.

Using the Oligopicker program (316) 70mer oligonucleotide probes were successfully designed from 2,349 of the genes and splice variants. Each oligonucleotide was designed within specific parameters to standardize hybridization behavior. These 70mers were then synthesized at the Massachusetts General Hospital Oligonucleotide Synthesis Facility (10) and spotted four times per gene on microarray slides at the Massachusetts General Hospital Microarray Core Facility (11) on Poly-L-lysine coated glass slides. Replicate oligonucleotide spots were printed on each array in a global fashion (spot replicates are distributed across the slide to account for spatial variation in hybridization conditions) to ensure adequate spot replication for downstream data analysis. The BMET array contains 9,582 spots including housekeeping (including GAPDH and Beta-actin) and negative (sequences not found in the bovine genome) control genes (10 *Arabidopsis thaliana* metabolic genes) (314).

As part of our effort to build a strong bovine metabolic genome resource, we are currently developing a web-based analytical tool to help with downstream analysis of the BMET array. Known human metabolic and cell regulatory pathways represented within KEGG and BioCarta will be adapted to this bovine-specific gene set using the GenMAPP tool (12) and a GeneLink resource (Figure 5). This is housed at our websites http://www.nutri-genomics.org (13) and http://nbfgc.msu.edu (14).

Spot replication improves within-array quality control and increases the statistical power of accurately detecting small changes in expression at a lower cost than slide replication (29). Reduction in technical error to increase statistical power is especially important for metabolic research, in which changes in gene expression are often subtle and the cost per experimental unit is very high. In addition, our focus on only those genes that are relevant to metabolism increases the efficiency of downstream bioinformatics and data analysis for integration of metabolic gene networks. Because all genes included in this design are annotated with corresponding human homologues, the design can be applied to other species to promote our understanding of comparative metabolism. Our design of a focused oligonucleotide microarray with multiple spots per gene will facilitate research in the study of cattle metabolic disease genomics of cattle and can be easily applied to other species and disciplines.

Conclusions

The merging of nutritional and genomics approaches to the understanding of interactions between diet and genotype is revealing a system of great complexity. Nutritional genomics studies have given researchers the opportunity to target specific dietary interventions to avoid and treat metabolic diseases in humans and cattle. Mechanisms underlying the effects of dietary nutrients on genome stability, imprinting, expression and signaling may become more clear in the near future through the employment of targeted microarrays and experimental design to answer questions remaining on specific metabolic diseases encountered in modern animal production.

The design of a microarray containing a large fraction of the genome with known links to metabolism and signal transduction will increase the quality and utility of gene expression data that can be acquired from microarray technology and lead to advancements in the field of nutritionally related production animal diseases.

Acknowledgments

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Website References

1) http://www.genome.jp/dbget-bin/show_pathway?hsa00100+3156; Kyoto Encyclopedia of Genes and Genomes, Steroid Biosynthesis Pathway.

2) http://us.expasy.org/; The ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics.

3) http://www.genome.jp/kegg/pathway.html; Kyoto Encyclopedia of Genes and Genomes pathways website

4) http://cgap.nci.nih.gov/Pathways/BioCarta; Pathways obtained from BioCarta via the Cancer Genome Anatomy Project.

5) http://www.ncbi.nih.gov/entrez/query.fcgi?db=gene; Entrez Gene.

6) http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM; The Online Mendelian Inheritance in Man database, a catalog of human genes and genetic disorders.

7) http://www.geneontology.org/; The Gene Ontology consortium website.

8) http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene; NCBI's UniGene link. This system automatically partitions GenBank sequences into a non-redundant set of gene-oriented clusters.

9) http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=cattle; The Institute for Genome Research's bovine gene index.

10) https://dnacore.mgh.harvard.edu/synthesis/index.shtml; Massachusetts General Hospital Oligonucleotide Synthesis Facility.

11) https://dnacore.mgh.harvard.edu/microarray/index.shtml; Massachusetts General Hospital Microarray Core Facility.

12) http://www.genmapp.org; GenMAPP is a free computer application designed to visualize gene expression data on maps representing biological pathways and groupings of genes.

13) http://www.nutri-genomics.org/; Home of the BMET microarray database.

14) http://nbfgc.msu.edu/; Michigan State University Center for Animal Functional Genomics database.

FIGURES

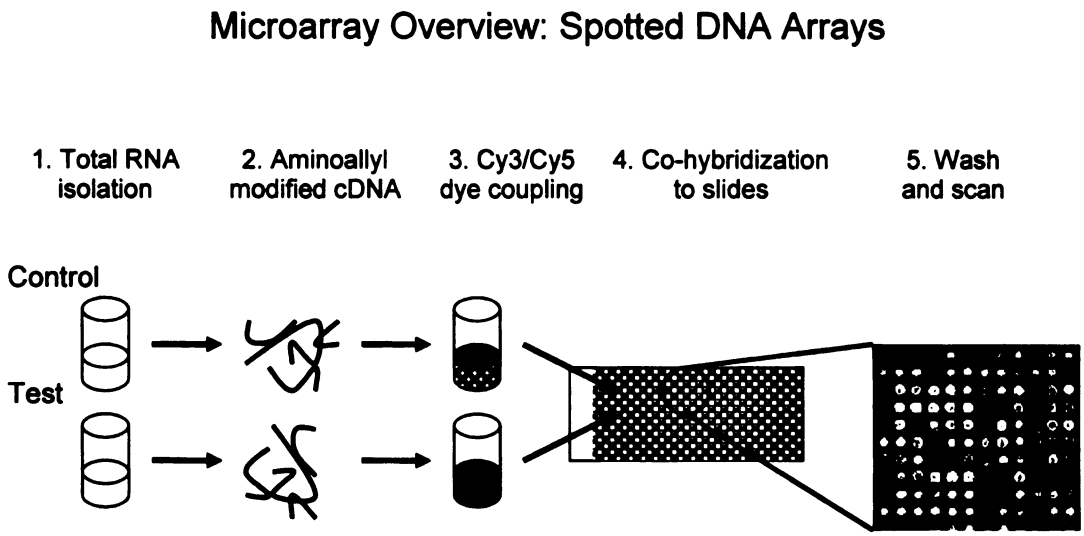

Figure 1. Spotted DNA Arrays. Plasmid clones are propagated in bacteria, and the cloned inserts are amplified by polymerase chain reaction (PCR) and then purified. These purified PCR products are robotically printed onto solid supports. Alternatively, oligonucleotides can be used instead of PCR products. Synthesized oligonucleotides are printed directly onto the glass support. Fluorescently labeled control and test samples are fluorescently labeled with Cy3 or Cy5 dyes and hybridized simultaneously onto the glass slide array. Following washes, hybridization is detected by phosphorimaging or measurement of the excitation of the two fluors at the appropriate wavelengths by a laser scanner.

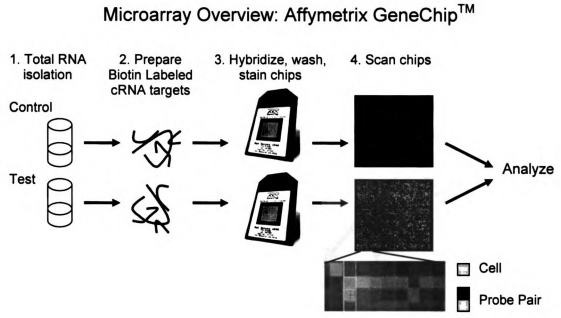

Figure 2. Affymetrix Arrays. The GeneChip gene "probes" are synthesized in situ on the chip using photolithographic techniques. Gene probes are made up of 25mer oligonucleotides called perfect match probes (PM), and each is paired with a "mismatch" probe (MM) that contains a base pair substitution to interfere with hybridization between the probe and its "target", present in the sample. Test and control samples are labeled with biotin and hybridized to separate chips. The hybridized "target" probes are then stained with streptavidin phycoerythrin and scanned for light emitted at 570 nm, which indicates the sample's signal. Match and mismatch probe intensities can be used for expression data analysis (PM-MM), or match pairs can be compared between the control and test samples alone (PM only) (270).

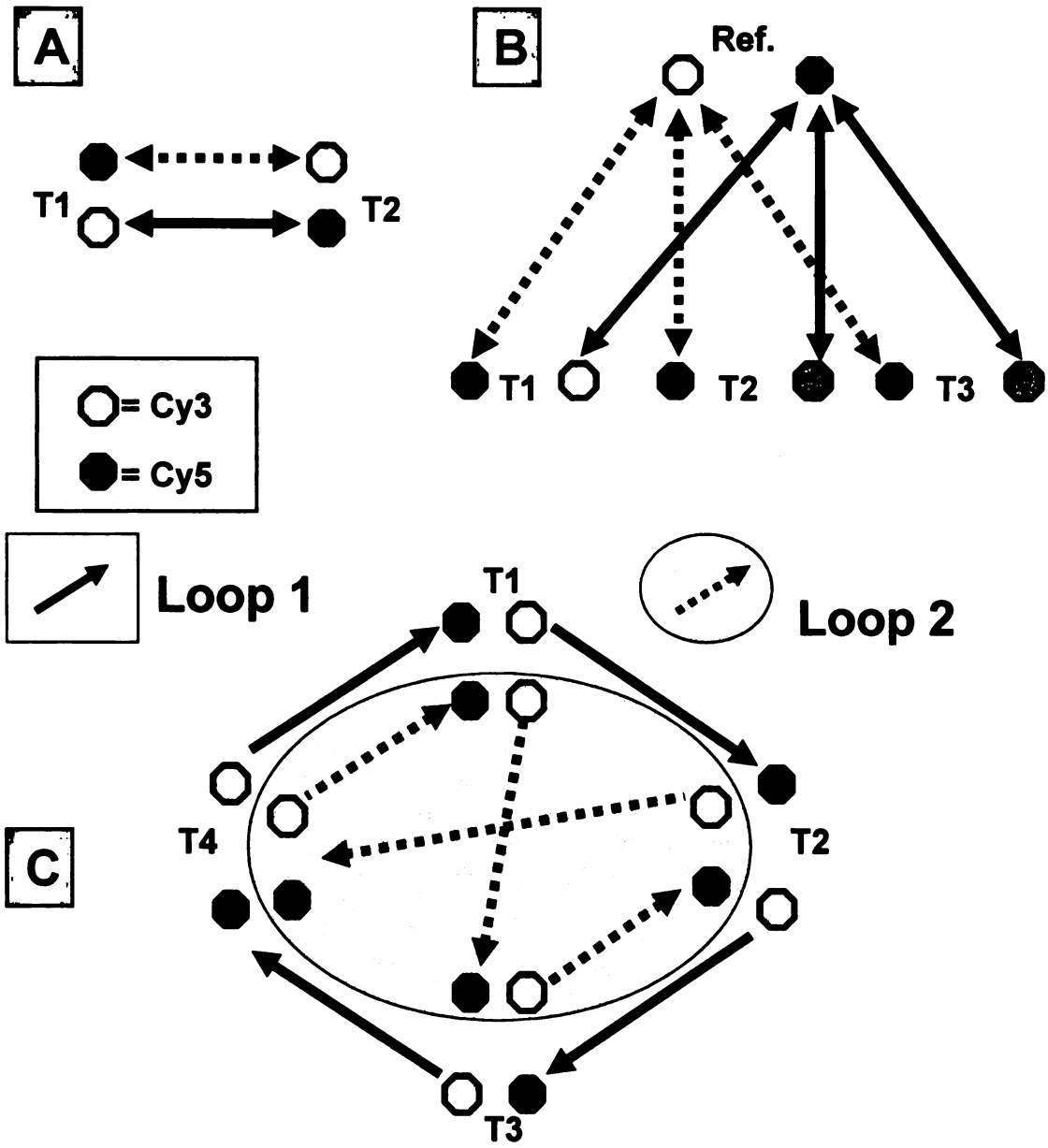

Figure 3. Microarray experimental designs for two-color arrays. A) Direct comparison with dye-flip; B) Reference design for three treatments with dye-flip; C) Augmented loop design for four treatments.
All Affymetrix GeneChips are scanned individually based on a one color system and all chips can be directly compared to each other.

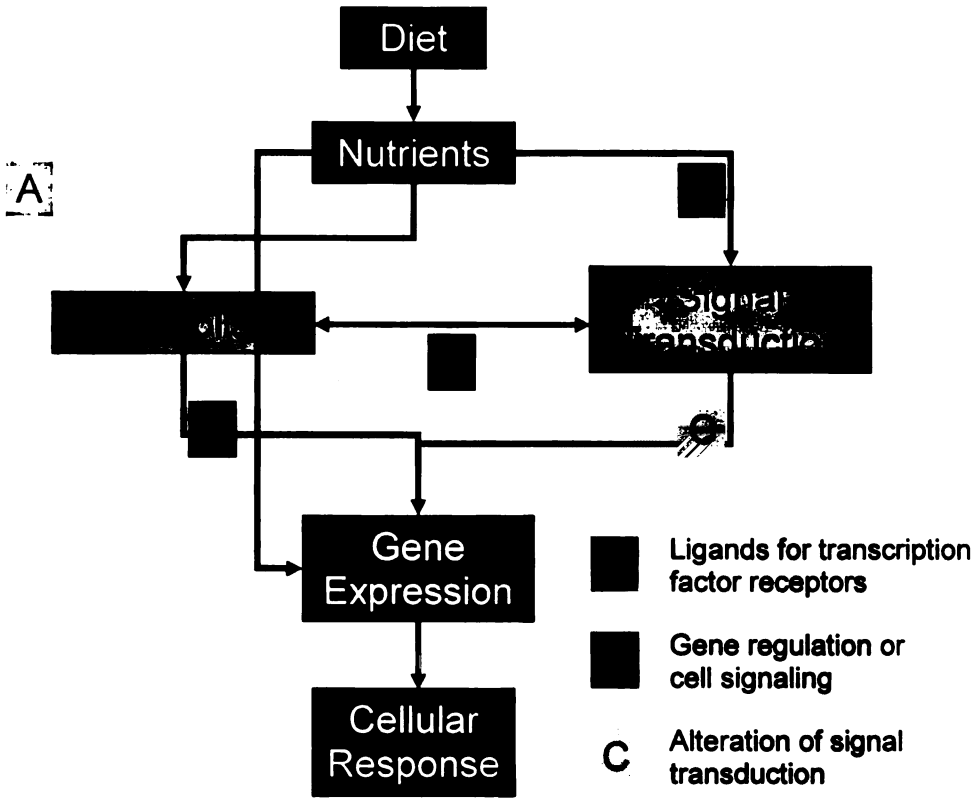

Figure 4. Nutritional genomics model. Nutrients can act directly as ligands for transcription factor receptors (*A*); may be metabolized by primary or secondary metabolic pathways to alter concentrations of substrates or intermediates involved in gene regulation or cell signaling (*B*); or might alter signal transduction pathways and signaling (*C*).

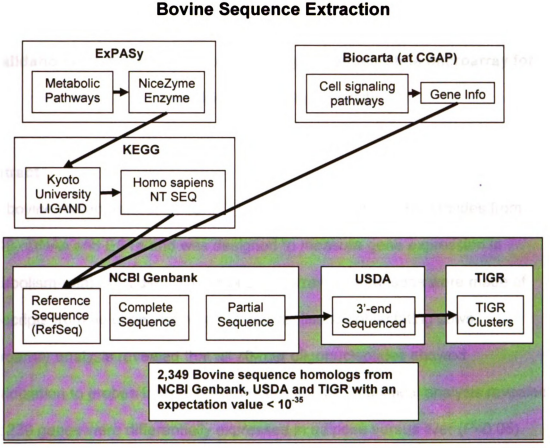

Figure 5. Metabolic gene database assembly. Using database resources such as ExPASy (2), the Kyoto Encyclopedia of Genes and Genomes (3), BioCarta (4), and NCBI GenBank (5) 2320 human genomic sequences were identified. Following a BLAST search against bovine sequences provided by NCBI, the United States Department of Agriculture, and The Institute for Genomic Research (9), 2349 bovine sequence homologues were detected at an expectation value better than $1e10^{-35}$.

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CHAPTER 3

Validation of a metabolism-specific long-oligonucleotide microarray for transcription profiling in cattle

Abstract

The bovine metabolism array (BMET) contains spots of oligonucleotides from 2,348 cDNAs and ESTs and was designed to measure gene expression in metabolism-related studies. To validate this array, comparisons were made of transcripts from adipose, mammary, and liver tissues of lactating cows. Microarray analysis revealed that all 70-mer oligonucleotides showed hybridization to probes from at least one tissue type. Statistical analysis revealed that 239 genes were differentially expressed in adipose versus liver ($P<0.05$). The adipose versus mammary comparison revealed 365 differentially expressed genes, and 125 genes were different in liver vs. mammary. Quantitative real-time PCR confirmed expression patterns for 7 of 8 genes tested. Our results demonstrate that the BMET array is informative for studies measuring changes in the bovine metabolic transcriptome for a number of tissue types.

Keywords: metabolism, gene expression, microarray, Q-RT-PCR

Introduction

Advances in a number of scientific fields have allowed for parallel assessment of expression of thousands of messenger RNAs (mRNA) to be performed in a single experiment. Since their inception in 1995 (258), high-density microarrays have been widely used in genomics research in organisms with sufficient genomic resources. With the growing amount of genomic sequence information and improved annotation methods, "boutique" microarrays, which contain cDNA or oligonucleotide probes tailored to include only genes with specific ontological links, are becoming increasingly popular. In an effort to enhance our understanding of metabolic processes achieved by all the chemical compounds, including metabolic enzymes, a set of cDNA sequences encoding a large fraction of the bovine metabolome as well as interacting signal transduction pathway genes was collected (Chapter 2). From this sequence library, 70-mer oligonucleotide probes were designed, synthesized, and hybridized to derivatized glass slides to produce the bovine metabolism (BMET) microarray.

To assess the utility of the BMET array, hybridization tests of glass microarray slides containing the 2,348 oligonucleotides were conducted by using target RNA from three tissues (adipose, mammary, and liver) from three lactating cows (9 tissue samples).

MATERIALS AND METHODS

Animals. Animals used in this study were three primiparous lactating Holstein cows described by VanderKooi et al. (311). These cows were housed in

individual tie stalls and exposed to 24 h/d of light and were fed an ad libitum mixed diet of alfalfa haylage, corn silage and grain containing ~1.7 Mcal of NEL/kg and 18% crude protein with free access to water. Feed was offered at 0330 and 1630 h daily. Cows were milked three times daily at 0545 1400 and 2200 h. The Michigan State University All-University Committee on Animal Use and Care approved experimental procedures (311).

Sample collection and tissue processing.

Cows were killed by captive bolt and exsanguination 181 d post-calving. Liver and mammary tissue was harvested within 20 min of death (311). A 10-g piece of liver tissue, 10 g of adipose tissue taken from the omasal fat deposit, and several 100-g slices of mammary tissue taken from the left hemigland were removed, immediately placed in liquid nitrogen, and stored at -80°C until future use (22;311).

RNA isolation procedure. Total RNA was extracted from frozen mammary, liver, and adipose tissues collected from tissue samples (after homogenization) with Trizol reagent (Invitrogen Life Technologies Corp., Carlsbad, CA) essentially as recommended by the manufacturer (Invitrogen Life Technologies Corp., Carlsbad, CA). Tissue was kept cold using dry ice and ~200 mg of mammary tissue was weighed and directly added to 3 mL of Trizol reagent in a 15 ml culture tube. Tissue was homogenized using a Polytron for ~30 s. Between homogenization of individual samples, the Polytron bit was rinsed with diethyl

pyrocarbonate- (DEPC, Sigma) treated water, and RNAse Away (Molecular BioProducts, San Diego,

CA). Samples were split into three, 1-mL samples and incubated at room temperature for 5 min. Chloroform (200 µL) was added to each microcentrifuge tube and samples were manually shaken, incubated for 3 min at room temperature, and centrifuged at 10,500 x g for 15 min at 4°C. The upper phase was transferred to a clean tube. Isopropanol (500 µL) was added to the precipitated RNA. The tube was vortexed, incubated at room temperature for 10 min, and centrifuged at 10,500 x g for 10 min at 4°C. The isopropanol was decanted and the remaining pellet was washed with 75% ethanol, centrifuged at 8500 x g for 5 min at 4°C, decanted, and dried. RNA was then subjected to DNase treatment (RQ1 RNase-free DNase; Promega, Madison, WI) following the manufacturer's protocol (Promega, Madison, WI). Nuclease free water (52 µL), DNase buffer (10 µL of 10X; Promega), and DNase (1 µL of 2 U/µL; Promega) were added to the pellet and then incubated at 37°C for 30 min. A phenol/chloroform separation step was performed by adding RNase-free water (37 µL) and phenol/chloroform (100 µL). The tube was shaken and centrifuged for 2 min at 14,000 x g. The upper phase (~90 µL) was transferred to a fresh tube, sodium acetate (3 M, 9 µL) and ethanol (250 µL) were added to this phase, and the mixture was stored overnight at -20°C. The following day, microcentrifuge tubes were centrifuged at 14,000 x g at 4°C for 20 min. The liquid was decanted and the pellet was washed with ethanol (75%, 500 µL). The tube was centrifuged at 14,000 x g at 4°C for 10 min. The ethanol was decanted

and the pellet was dried in the hood for 15 min. The pellet was resuspended in 50 µL of nuclease-free water and incubated at 60°C for 10 min. The quantity of RNA isolated was determined using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE), and quality was checked using the using the RNA 6000 Nano LabChip kit and Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA).

BMET microarray experimental design. The BMET microarray was used to compare gene expression profiles of adipose, mammary, and liver samples. The BMET array was described previously (Chapter 2) (97) and contains 2,348 spots representing genes involved with bovine metabolism and signal transduction. This array includs 10 metabolic housekeeping genes from *Arabidopsis thaliana* used for spike-in controls, 1320 blank spots (printing buffer only spotted) and 64 empty spots (nothing spotted).

Complete annotation of the BMET gene set can be found at http://www.nutri-genomics.org/data.html. The oligonucleotides were synthesized and the BMET microarrays were printed at the Massachusetts General Hospital Microarray Core Facility at Harvard University (https://dnacore.mgh.harvard.edu/microarray/protocol-printing.shtml). Briefly, cDNA oligonucleotides (70mers) were suspended in water to 20 µM and rearrayed into Genetix polystyrene V-bottom plates (cat# X6004). Before printing, 5 µl of 1X Printing Buffer (150 mM sodium phosphate, 0.0005% sarcosyl, (pH 8.5) was added to each well. Plates were then sealed using Corning seals and

incubated at 37°C for 30 minutes to help resuspend DNA. Plates were shaken near maximum rotational speed on a flat-bed shaker for 1 min and then centrifuged at 2000 x g for 3 min. Each gene was then spotted four times on a Codelink glass slide (Amersham Pharmacia Biotech; Piscataway, NJ) (10816 total spots) across the array using the Genomic Solutions Omnigrid 100 printer (Genomic Solutions, Harvard Biosciences, Inc., Holliston, MA). Humidity was kept between 30-40% during the print run using a sensor-activated humidifier and dehumidifier in conjunction. Following a print run, slides were sealed with moist paper towels in a chamber containing supersaturated NaCl overnight. Slides were then washed in Blocking Solution (50 mM 2-aminoethanol, 0.1 M Tris (pH 9.0), 0.1% N-Lauroyl sarcosine) prewarmed to 55°C for 15 min, rinsed twice in deionized water, washed again for 30 min in 55°C Washing Solution (4X SSC, 0.1% N-lauroyl sarcosine), rinsed in deionized water 2x and centrifuged at 800 x g for 5 minutes to dry.

Screening for genes differentially expressed between adipose, mammary, and liver was performed on three animals using a loop design (158) balanced for Cy3 (green) and Cy5 (red) fluorescent dye labeling (Amersham Pharmacia Biotech; Piscataway, NJ) with dye-flip (Figure 1) for each of the three tissue types (18 arrays total).

Preparation of labeled cDNA for BMET hybridization. For cDNA synthesis, 15 µg of sample RNA was used as a template in reverse transcription reactions (*SuperScript III* Fluorescent Labeling Kit; Invitrogen Life Technologies Corp., Carlsbad, CA) in which oligo(dT)15-18 was used as a primer. In the Superscript

III system, cDNA is prepared with a randomly incorporated amino-modified dUTP. Following first-strand synthesis, cDNAs for each tissue within an animal were differentially labeled using *N*-hydroxysuccinimide-derivatized Cy3 and Cy5 dyes (Amersham Pharmacia, Ltd., Piscataway, NJ). Differentially labeled cDNAs were combined and concentrated to 10 µl by using Microcon 30 spin concentrators (Millipore Corp., Bedford, MA). Microarray hybridization was performed after addition of 100 µl of SlideHyb-3 hybridization buffer (Ambion Inc., Alameda, CA) to the concentrated Cy3–Cy5-labeled probe cDNAs (7). The labeled cDNAs were incubated at 70°C for 5 min just prior to 18-hour array hybridization in Corning Microarray Hybridization Chambers (Fisher Scientific) placed in water baths set to 42°C. Following hybridization, microarray slides were washed with 2X SSC saline sodium citrate (0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS for 4 min at 42°C, 2X SSC for 4 min at room temperature, 0.2X SSC for 4 min at room temperature, 0.06X SSC for 10 sec at room temperature and once in double-distilled H_2O. Microarrays were dried by centrifugation in a cushioned 50-ml conical tube and immediately scanned with a GeneTAC LS IV microarray scanner (version 3.01). GeneTAC LS software version 3.3.0 (Genomic Solutions, Inc., Ann Arbor, MI) was used to process microarray images, find spots, integrate robot spotting files with the microarray image, and finally to create reports of raw spot intensities. Total intensity values for each dye channel were converted to comma-delimited data files, exported into Excel spreadsheets, and imported into SAS (255) for data normalization and analysis.

Quantitative real-time PCR validation of microarray gene expression changes. The expression differences of certain genes found to be significant by microarray analysis were checked using quantitative real-time reverse transcription-PCR (Q-RT-PCR) using an Applied Biosystems 7000 DNA Sequence detection system (Perkin Elmer Corp., Foster City, CA). Total RNA was extracted from each tissue from each of the three cows, quantified and quality checked as described previously. The PCR system used was the ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

RNA was converted into first-strand cDNA by adding 1 µg of total RNA to a 12 µl reaction containing 10 mM oligo$(dT)_{18}$ primer. Following a 5 min incubation at 70°C, the reaction was chilled on ice and pH adjusted by addition of 4 µl of a buffer supplied by the reverse transcriptase manufacturer (Invitrogen Life Technologies). The final reagent concentrations were 50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates, and 200 units of Superscript II RNase H reverse transcriptase (Invitrogen Life Technologies). Incubation at 37°C continued for 30 min in the presence of RNase H at 42°C to remove the original RNA templates. Heating at 70°C for 15 min subsequently inactivated RNase H. The reaction tubes were removed from the thermocycler and 0.2 µL of 0.5 M EDTA was added and mixed. Then, 25 µL of water, 5 µL 3M sodium acetate, and 125 µL of ethanol (-20°C) were added to the tube. Tubes were then allowed to precipitate overnight at -20°C. The reaction mixture containing the newly-synthesized cDNA was transferred to a new, clean

microcentrifuge tube. These tubes were then centrifuged at 14,000 x g and 4°C for 20 min and the supernatant was decanted. Pellets were washed with 250 µL of ethanol (75%, -20°C) and the tube was centrifuged at 14,000 x g and 4°C for 6 min. The supernatant was decanted and the pellet was allowed to dry for 15 min. The pellet was resuspended in 50 µL of water and incubated at 60°C for 5 min. The cDNA concentration was analyzed using a spectrophotometer (NanoDrop), and was then diluted to a final concentration of 10 ng/µL and stored at -80°C until the Q-RT-PCR reaction was initiated.

SYBR Green PCR Master Mix (Perkin Elmer Corp.) and gene-specific primers were used to perform Q-RT-PCR reactions. Primer Express Software (Perkin Elmer Corp.) was used to design all primers, which were then synthesized by a commercial facility (Invitrogen Life Technologies). Primer sequences for genes analyzed in this report are included in Table 1.

The amount of primer used was determined by performing an optimization matrix for each primer using three concentrations of primers: 50:50 nM, 300:300 nM, 900:900 nM. Dissociation curves were similar for all concentrations and the 300:300 nM matrix was chosen, thus 3 µL of primer was used for all experiments. Each gene of interest and the control gene were measured in duplicate. Within each well of a 96-well reaction plate (MicroAmp Optical, Applied Biosystems), 30 ng of sample cDNA (3 µL), 6.5 µL DEPC water, 3 µL of each primer, and 12.5 µL Sybr Green (Applied Biosystems) were added.

To determine an appropriate reference control gene by which relative mRNA abundances for genes of interest could be measured, a number of potential

housekeeping genes were screened based on microarray results. These candidate control genes included β-actin (B-actin), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hydroxymethyl-bilane synthase (HMBS), hypoxanthine phosphoribosyltransferase 1 (HPRT1), TATA box binding protein (TBP), and succinate dehydrogenase complex, subunit A (SDHA). Q-RT-PCR threshold cycle (C_T) values for GAPDH for all tissues were consistent among animals and this gene was deemed a suitable reference control.

The $2^{-\Delta\Delta CT}$ method of Q-RT-PCR analysis was performed as previously described (7;65;188). This method enabled relative gene expression changes across treatments based on quantitative differences in the PCR amplified target reaching a fixed C_T number at a set treatment versus other treatments (Table 6). Gene-specific standard errors were estimated using independent analyses of variance (ANOVA). All analyses were performed using the SAS System (255).

Statistical analysis of microarray data. *M* vs. *A* scatter plots were constructed for each array to visualize potential dye intensity biases in the microarray data sets. Here, log intensity ratios $M = \log(Cy3/Cy5) = \log Cy3 - \log Cy5$ were plotted against mean log intensities $A = (\log Cy3 + \log Cy5)/2$ for each array spot as previously described (9, 10). Array specific normalization was performed using a robust local regression technique (Cleveland 1979 #660), LOESS, of SAS (255). Monitoring *M-A* plots for data from each array before and after LOESS normalization assessed efficiency of LOESS normalization. Normalized data were then back-transformed prior to further statistical analyses using the

following formulas: logCy3' = $A + M/2$ and logCy5' = $A - M/2$, where logCy3' and logCy5' are the normalized log intensities. Here, *M* represents each of the normalized *M* values with *M* being the LOESS predicted value for each spot.

Statistical analysis of LOESS-adjusted log intensities was performed using a mixed model approach consisting of two steps (9, 11). The first step involved an array-specific spatial variability normalization model:

yadj = **dye** array day*array block(array) dye*block(array) + e

All terms are random except dye.

The second step involved gene-specific analyses for the estimated residuals obtained from the normalization approach obtained from step one to test the effect of treatment on expression profiles for individual genes. These models were:

resid = **heifer** + treat + treat*heifer + **dye** + array(heifer) + **pin** + spot(pin*array*heifer)

All terms are random except heifer, dye and pin.

These analyses were computed using the MIXED procedure of SAS (255). In the gene-specific analysis, genes were considered significantly different if $P<0.05$.

Spotted cDNA sequences representing genes whose expression profiles were significantly altered by hormonal infusion in this analysis were subjected to BLASTn analysis to reveal identities. The biological functions and ontologies of these genes were determined through an extensive Entrez-Gene (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=Search&DB=gene) and

PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?) literature search. This information was used to establish clusters of affected genes within broad functional categories for subsequent expression validation and downstream analysis.

RESULTS

Identification of genes differentially expressed in mammary and liver of lactating cows. The BMET microarray comparison between mammary and liver tissues revealed significant differential expression ($P<0.05$) of 125 of 2348 genes measured (5.3%) (Supplemental Table 1). The relative mRNA abundance of 78 of these genes was found to be higher in mammary than liver tissue. Genes significantly different in mRNA abundance between the two tissues were grouped within a number of gene ontologies and pathways using the GenMAPP MAPPFinder 2 program (72;87). This analysis indicated overrepresentation of liver specific expression within a number of pathways including alcohol and carbohydrate metabolism (Table 1). Mammary specific ontology overrepresentation was clustered within intracellular signaling and transcription factor binding ontologies (Table 1). Alpha-lactalbumin was the most significantly underexpressed gene in liver relative to mammary tissue (fold-change = 0.16).

Identification of genes differentially expressed in adipose and mammary of lactating cows. The BMET microarray comparison between mammary and adipose tissues revealed significant differential expression ($P<0.05$) of 365 of

2348 genes measured (15.5%) (Supplemental Table 2). The relative mRNA abundance of 170 of these genes was found to be higher in mammary than adipose tissue. Significantly altered genes could be broadly classified within a number of gene ontologies and pathways. GenMAPP MAPPFinder 2 pathway analysis (72;87) indicated significant differential expression of 11 genes in mammary tissue (PRLR, MVD, NSDHL, SEC14L2, SC5DL, SC4MOL, NMNAT2, PISD, SCD, FADS1 and MGST2), and three genes in adipose tissue (CMAS, ELOVL1, and STAR) within the lipid biosynthesis ontology (Table 2). Within the insulin signaling pathway eight genes were more highly expressed in adipose than mammary tissue (SLC2A4, GSK3A, GYS1, MAP3K3, MAPK10, MAP2K1, MAP3K11, and MAP3K12). Genes significantly different between the two tissues and involved with RNA polymerase II transcription factor activity tended to cluster within adipose tissue (ATF2, BTF3, HOXC10, IRF4, SF01, TEF, and SOX9) more than in mammary tissue (FOXA3 and MYOD1).

Identification of genes differentially expressed in adipose and liver of lactating cows. The BMET microarray comparison between adipose and liver tissues revealed significant differential expression ($P<0.05$) of 238 of 2348 genes measured (10.1%) (Supplemental Table 3). The relative mRNA abundance of 91 of these genes was found to be higher in adipose than liver tissue. Genes significantly different between the two tissue types were clustered within a number of specific gene ontologies and pathways relevant to tissue-specific expression in the lactating cow as indicated by GenMAPP pathway analysis

(Table 3) (72;87). Three genes involved in carboxy-lyase activity (ODC1, PISD, and SGPL1A) were found to be more abundant in liver tissue than adipose. As with the adipose versus mammary tissue comparison, genes falling within the RNA pol II transcription factor activity gene ontology tended to be more highly expressed in adipose tissue (ATF2, HOXC10, SREBF1, SREBF2, TEF, THRAP6, GTF2, and IRD1) rather than liver (MYOD). Three genes involved with hepatocyte growth factor (HGF) signaling (MAP2K1, STAT3, RASA1) were found to be more abundant in adipose than liver tissue. The adipocyte-specific fatty acid binding protein FABP4 was found to be very highly expressed in adipose tissue relative to liver (3.42 fold difference). Two other genes involved in fatty acid binding (FABP2, ANXA2) were also more abundant in adipose than liver tissue.

Q-RT-PCR confirms IGF-I induced expression changes in 7 of 8 genes significantly altered by microarray analysis. Significant differential expression of six of seven genes between liver and mammary tissue, five of six genes between adipose and liver, and five of six genes between adipose and mammary tissue as indicated by BMET microarray analysis were confirmed by Q-RT-PCR (Table 5). For all genes tested, magnitude of fold-change was much larger by Q-RT-PCR analysis than by the BMET microarray. For example mRNA coding for suppressor of cytokine signaling 3 (SOCS3) was confirmed as more abundant in mammary than liver tissue. Microarray analysis generated a ratio of 0.85 for SOCS3, indicating a slight increase in mRNA abundance in mammary tissue

relative to liver. However, Q-RT-PCR analysis revealed a much larger magnitude in relative abundance with SOCS3 upregulated 5.4-fold in mammary relative to liver. Similarly, the cytokine inducible SH2-containing protein (CISH) was confirmed as overexpressed in mammary relative to liver tissue. CISH was 9-fold more abundant in mammary than liver by Q-RT-PCR, though microarray analysis predicted a 0.65 ratio of mammary to liver. In the adipose versus mammary comparison, interferon alpha 2 (IFNA2) gene expression was predicted to be 1.55-fold higher in adipose versus mammary tissue (P=0.058). This fold-change difference was magnified to 75-fold higher in adipose than mammary by Q-RT-PCR analysis. Only one gene, Absent, small, or homeotic-like 2 (ASH2L) was invalidated by Q-RT-PCR analysis. All three tissue mRNA abundance comparisons for this gene were significant in the opposite direction indicated by BMET microarray analysis. In all, 14 of 17 Q-RT-PCR tissue comparison mRNA abundance comparisons were in accordance with the predicted directionality of microarray relative abundance (Table 5).

DISCUSSION

Tissue specificity of expression

One critical test for the value of the BMET microarray lies in its ability to detect differences in expression of genes known to be differentially expressed in different tissues. Overall, the BMET performed well at achieving this goal. For example, genes involved in coagulation, such as plasminogen and fibrinogen, were both significantly more abundant in liver than adipose and mammary, as

expected (337). Also expressed in a liver-specific manner included a number of genes involved with alcohol metabolism, derivation of energy from oxidation of organic compounds, carbohydrate metabolism and carboxy-lyase activity (Tables 1 and 3). All of these gene ontologies are consistent with processes known to be highly regulated within the liver. The liver specific isoforms of phosphofructokinase (PFKL) and fatty acid binding protein 1 (FABP1) were significantly overexpressed in liver compared to adipose tissue.

Genes expressed in a greater abundance in adipose tissue were identified and seemed to fit well with adipose-tissue related physiological processes. Specifically, the significant differences in expression of in FABP5 in adipose vs. liver, FABP4 in both adipose vs. liver and mammary comparisons stand out. The signaling activity of adipose tissue through the mitogen activated protein kinase (MAPK) cascade seems to be quite high based on the frequency of genes highly expressed within this ontology. The growth factor pathways for insulin and hematopoietic growth factor (HGF) and a number of intracellular signaling components were also highly represented in the significantly expressed adipose gene group (Tables 2 and 3).

Mammary tissue gene expression in the lactating dairy cow is highly dynamic. As might be expected, the most significantly overexpressed genes in mammary compared to adipose tissue were alpha-lactalbumin and beta-lactoglobulin. These genes code for two proteins produced in great abundance in mammary tissue during lactation. Similarly, 11 genes involved with lipid biosynthesis were found to be highly expressed in mammary relative to adipose tissue, including a

number of genes known to be regulated during milk fat production such as stearoyl coA desaturase (SCD), and fatty acid desaturase 1 (FADS1) (20;244;280). A number of genes involved with intracellular signaling were also found to be predominantly expressed in mammary tissue (Table 1). Of these, CISH and SOCS3, two genes which are known to inhibit prolactin (PRL) receptor-mediated activation of STAT5 in the mammary gland, were confirmed as overrepresented in mammary versus liver tissue (302). It is possible that these genes are expressed to a greater degree late in lactation (181 days in milk) to promote mammary gland involution by increasing apoptosis and inhibiting proliferation of mammary epithelial cells (174;295;302).

In conclusion, the use of microarrays to help understand complex gene expression patterns in various tissue types in cattle is becoming increasingly helpful in understanding phenotype and genotype interactions. The BMET microarray should provide a useful platform for helping to understand bovine disease states, and nutritional and hormonal regulation of gene expression to improve the production and quality of food in the future.

FIGURE

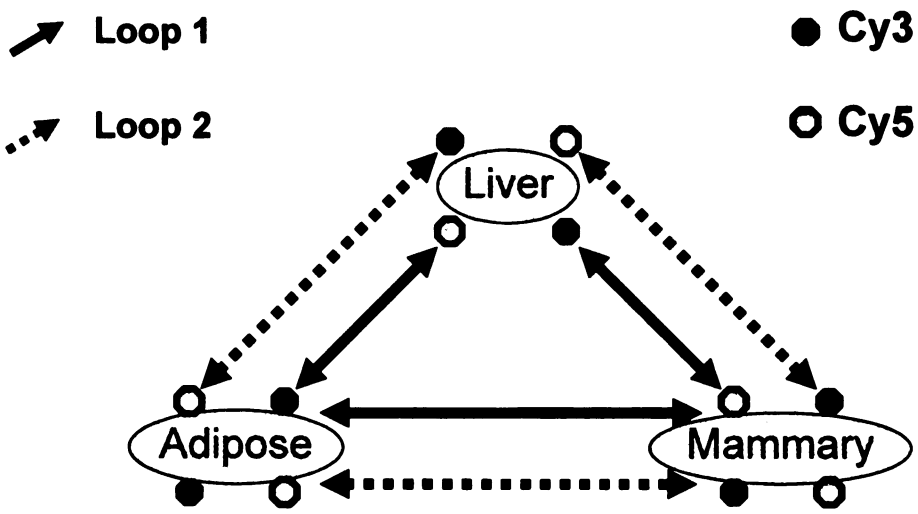

Figure 1: Tissue comparison microarray loop design. Each of three tissues for three animals with flip dye labeling was compared using the BMET microarray (18 arrays total).

TABLES

Table 1.

Liver vs. Mammary Tissue GenMAPP Analysis

Gene	Pathway/Ontology	Liver	Mammary
	Intracellular signaling cascade		
Interleukin-8 (IL8)			X
Cytokine inducible SH2-containing protein (CISH)*			X
Natriuretic peptide receptor B/guanylate cyclase B (NPR2)			X
Suppressor of cytokine signaling-3 (SOCS3)*			X
Signal transducer and activator of transcription 3 (STAT3)			X
	Carbohydrate metabolism		
Fucosyltransferase 1 (FUT1)		X	
Isocitrate dehydrogenase 3 (NAD+) alpha (IDH3A)		X	
Sucrase-isomaltase (alpha-glucosidase) (SI)		X	
Solute carrier family 5 (sodium/glucose cotransporter), member 2 (SLC5A2)		X	
	Energy derivation by oxidation of organic compounds		
Hexokinase 3 (white cell) (HK3)		X	
Phosphoglycerate mutase 1 (brain) (PGAM1)		X	
Glucose 6-phosphate translocase (SLC37A1)		X	
Isocitrate dehydrogenase 3 (NAD+) alpha (IDH3A)		X	
	Alcohol catabolism		
Fucosyltransferase 1 (FUT1)		X	
Phosphoglycerate mutase 1 (brain) (PGAM1)		X	

Hexokinase 3 (white cell) (HK3)		X	
Alcohol dehydrogenase 6 (class V) (ADH6)		X	
Glucose 6-phosphate translocase (SLC37A1)		X	
Gene	**Pathway/Ontology**	**Liver**	**Mammary**
	Oxidoreductase activity		
Alcohol dehydrogenase 6 (class V) (ADH6)		X	
Isocitrate dehydrogenase 3 (NAD+) alpha (IDH3A)		X	
Acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase) (ACAA2)		X	
Apurinic/apyrimidinic exonuclease, class I (APEX1)		X	
NAD(P) dependent steroid dehydrogenase-like (NSDHL)			X
Glutaryl-CoA dehydrogenase (GCDH)			X
Nephrocystin 3 (NPHP3)			X
	Immune cell activation		
Histone deacetylase 4 (HDAC4)			X
Histone deacetylase 5 (HDAC5)			X

X – indicates significantly higher expression in tissue
*** Validated by Q-RT-PCR**

Table 2.

Adipose vs. Mammary Tissue GenMAPP Analysis

Gene	Pathway/Ontology	Mammary	Adipose
	Lipid biosynthesis		
Cytidine monophosphate N-acetylneuraminic acid synthetase (CMAS)			X
Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 1 (ELOVL1)			X
Steroidogenic acute regulator (STAR)			X
Delta 4-delta 7/11 truncated prolactin receptor (PRLR)		X	
Mevalonate (diphospho) decarboxylase (MVD)		X	
NAD(P) dependent steroid dehydrogenase-like (NSDHL)		X	
Squalene epoxidase SEC14-like 2 (SEC14L2)		X	
Sterol-C5-desaturase (ERG3 delta-5-desaturase homolog, fungal)-like (SC5DL)		X	
Sterol-C4-methyl oxidase-like (SC4MOL)		X	
Nicotinamide nucleotide adenylyltransferase 2 (NMNAT2)		X	
Phosphatidylserine decarboxylase (PISD)		X	
Stearoyl-CoA desaturase (delta-9-desaturase) (SCD)		X	
Delta-5 fatty acid desaturase (FADS1)		X	
Microsomal glutathione S-transferase 2 (MGST2)		X	
	Regulation of actin cytoskeleton		
Mitogen activated protein kinase kinase 1 (MAP2K1)			X
Fibroblast growth factor 1 (FGF1)		X	
Vav 1 oncogene (VAV1)		X	
Mitogen-activated protein kinase 6 (MAPK6)		X	
Fibroblast growth factor receptor 4 (FGFR4)		X	

Gene	Pathway/Ontology	Mammary	Adipose
Basic transcription factor 3 (BTF3)	**Transcription from RNA pol II promoter**		X
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105) (NFKB1)	**Transcription from RNA pol II promoter**	X	
Kruppel-like factor 11 (KLF11)		X	
CCR4 carbon catabolite repression 4-like (CCRN4L)		X	
E74-like factor 5 (ets domain transcription factor) (ELF5)		X	
E1A-associated protein p300; E1A-binding protein, 300kD (EP300)		X	
Eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)		X	
Tyrosine hydroxylase (TH)	**Oxidoreductase activity**		X
Stearoyl-CoA desaturase (SCD)		X	
Sterol-C4-methyl oxidase-like (SC4MOL)		X	
Fatty acid desaturase 1 (FADS1)		X	
Dopamine beta-hydroxylase (dopamine beta-monooxygenase) (DBH)		X	
Kruppel-like factor 10 (KLF10)	**Regulation of cell proliferation**		X
MAX dimerization protein 4 (MXD4)		X	
Jagged 2 (JAG2)		X	
Kruppel-like factor 11 (KLF11)		X	
Solute carrier family 2 (facilitated glucose transporter), member 4 (SLC2A4)	**Insulin signaling**	X	

Gene	Pathway/Ontology	Mammary	Adipose
Glycogen synthase kinase 3 alpha (GSK3A)		X	
Glycogen synthase 1 (muscle) (GYS1)		X	
Mitogen-activated protein kinase kinase kinase 3 (MAP3K3)		X	
Mitogen-activated protein kinase 10 (MAPK10)		X	
Mitogen activated protein kinase kinase 1 (MAP2K1)		X	
Mitogen-activated protein kinase kinase kinase 11 (MAP3K11)		X	
	RNA pol II transcription factor activity		
Mitogen-activated protein kinase kinase kinase 12 (MAP3K12)		X	
Mitogen-activated protein kinase 6 (MAPK6)			X
	RNA pol II transcription factor activity		
Activating transcription factor 2 (ATF2)		X	
Basic transcription factor 3 (BTF3)		X	
Homeo box C10 (HOXC10)		X	
Interferon regulatory factor 4 (IRF4)		X	
steroidogenic factor-1 SF-1 (SF1)		X	
Thyrotrophic embryonic factor (TEF)		X	
SRY (sex determining region Y)-box 9 (SOX9)		X	
Hepatocyte nuclear factor 3 gamma (FOXA3)			X
myogenic differentiation 1 (MYOD1)			X
	Acyltransferase activity		
Arylalkylamine N-acetyltransferase (AANAT)		X	
Carnitine O-octanoyltransferase (CROT)		X	
Monoacylglycerol O-acyltransferase 2 (MOGAT2)		X	
Nuclear receptor coactivator 1 (NCOA1)		X	
N-myristoyltransferase 1 (NMT1)		X	
Lysophospholipase 3 (lysosomal phospholipase A2) (LYPLA3)			X
E1A-associated protein p300; E1A-binding protein, 300kD (EP300)			X

Gene	Pathway/Ontology	Mammary	Adipose
	Protein tyrosine kinase activity		
Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1B (DYRK1B)		X	
Mitogen activated protein kinase kinase 1 (MAP2K1)		X	
Mitogen-activated protein kinase kinase kinase 11 (MAP3K11)		X	
Mitogen-activated protein kinase kinase kinase 12 (MAP3K12)		X	
Cyclin-dependent kinase (CDC2-like) 10 (CDK10)		X	
v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (ERBB3)		X	
	Hexose metabolism		
Fibroblast growth factor receptor 4 (FGFR4)			X
v-yes-1 Yamaguchi sarcoma viral related oncogene homolog (LYN)			X
	Hexose metabolism		
Mannose phosphate isomerase (MPI)		X	
Protein O-fucosyltransferase 1 (POFUT1)		X	
Fucosyltransferase 1 (galactoside 2-alpha-L-fucosyltransferase) (FUT1)		X	
Hexokinase 3 (HK3)		X	
Lactate dehydrogenase B (LDHB)		X	
Solute carrier family 35 (UDP-galactose transporter), member A2 (SLC35A2)			X
Phosphoenolpyruvate carboxykinase 2 (PCK2)			X

X – indicates significantly higher expression in tissue

Table 3.

Adipose vs. Liver Tissue GenMAPP Analysis

Gene	Pathway/Ontology	Liver	Adipose
	Lipid Binding		
Fatty acid binding protein 2 (FABP2)			X
Fatty acid binding protein 4 (FABP4)*			X
Annexin A2 (ANXA2)			X
	Acyltransferase activity		
Arylalkylamine N-acetyltransferase (AANAT)			X
Carnitine O-octanoyltransferase (CROT)			X
N-myristoyltransferase 1 (NMT1)			X
E1A binding protein p300 (EP300)			X
	Alcohol catabolism, glycolysis		
lactate dehydrogenase B (LDHB)			X
Glucose phosphate isomerase (GPI)			X
Hexokinase 3 (HK3)			X
	Carboxy-lyase activity		
Ornithine decarboxylase 1 (ODC1)		X	
Phosphatidylserine decarboxylase (PISD)		X	
Sphingosine-1-phosphate lyase 1 (SGPL1)		X	
	Hepatocyte growth factor activity		
Mitogen activated protein kinase kinase 1 (MAP2K1)		X	
Signal transducer and activator of transcription 3 (STAT3)		X	
RAS p21 protein activator 1 (RASA1)		X	
	MAPK activity		

Mitogen-activated protein kinase kinase kinase 3 (MAP3K3)			X
Mitogen-activated protein kinase 10 (MAPK10)			X
Gene	**Pathway/Ontology**	**Liver**	**Adipose**
	RNA pol II transcription factor activity		
Activating transcription factor 2 (ATF2)		X	
Homeo box C10 (HOXC10)		X	
Sterol regulatory element binding factor 1 (SREBF1)		X	
Sterol regulatory element binding transcription factor 2 (SREBF		X	
Thyrotrophic embryonic factor (TEF)		X	
Thyroid hormone receptor associated protein 6 (THRAP6)		X	
General transcription factor II I repeat domain-containing 1 (GTF2IRD1)		X	
Myogenic differentiation 1 (MYOD)			X
	Insulin Signaling		
Solute carrier family 2 (facilitated glucose transporter), member 4 (SLC2A4)		X	
Mitogen-activated protein kinase kinase kinase 3 (MAP3K3)		X	
Mitogen-activated protein kinase 10 (MAPK10)		X	
Mitogen activated protein kinase kinase 1 (MAP2K1)		X	
Suppressor of cytokine signaling 3 (SOCS3)		X	
Mitogen-activated protein kinase 6 (MAPK6)			X
	Fatty Acid Metabolism		
Acyl-CoA thioesterase 3 (ACOT3)			X
Lysophospholipase II (LYPLA2)			X
Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 1 (ELOVL1)			X
Carnitine O-octanoyltransferase (CROT)			X
Prostaglandin E synthase (PTGES)			X

Gene	Pathway/Ontology	Liver	Adipose
Acyl-CoA synthetase medium-chain family member 1 (ACSM1)		X	
	MAPK signaling pathway		
RAS p21 protein activator (GTPase activating protein) 1 (RASA1)			X
Mitogen activated protein kinase kinase 1 (MAP2K1)			X
	Carbohydrate metabolism		
Mitogen-activated protein kinase 10 (MAPK10)			X
Signaling intermediate in Toll pathway, evolutionarily conserved (SITPEC)			X
Activating transcription factor 2 (ATF2)			X
Mitogen-activated protein kinase 6 (MAPK6)		X	
ER degradation-enhancing-mannosidase-like protein 2 (C20orf31)			X
Glucose phosphate isomerase (GPI)			X
Glyoxalase I (GLO1)			X
Solute carrier family 2 (facilitated glucose transporter), member 4 (SLC2A4)			X
Solute carrier family 5 (sodium/glucose cotransporter), member 2 (SLC5A2)			X
Isocitrate dehydrogenase 3 (NAD+) alpha (IDH3A)		X	
	Lipid Biosynthesis		
Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 1 (ELOVL1)			X
Steroidogenic acute regulatory protein (STAR)			X
Phosphatidylserine decarboxylase (PISD)		X	
Prolactin receptor (PRLR)		X	
Sterol-C5-desaturase (ERG3 delta-5-desaturase homolog, fungal)-like (SC5DL)		X	
	Oxidoreductase activity		

IMP (inosine monophosphate) dehydrogenase 2 (IMPDH4)			X
Actate dehydrogenase B (LDHB)			X
Aldehyde oxidase 1 (AOX1)			X
Alcohol dehydrogenase 6 (class V) (ADH6)		X	
Dehydrogenase/reductase (SDR family) member 4 (DHRS4)		X	
Isocitrate dehydrogenase 3 (NAD+) alpha (IDH3A)		X	

Gene	**Pathway/Ontology**	**Liver**	**Adipose**
	Lyase activity		
Carbonic anhydrase VI (CA6)			X
Carbonic anhydrase IX (CA9)			X
Glyoxalase 1 (GLO1)			X
Guanylate cyclase 2C (heat stable enterotoxin receptor) (GUC'			X
6-pyruvoyltetrahydropterin synthase (PTS)			X
Adenylate cyclase 3 (ADCY3)		X	
Ornithine decarboxylase 1 (ODC1)		X	
Phosphatidylserine decarboxylase (PISD)		X	
Sphingosine phosphate lyase 1 (SGPL1)		X	

X – indicates significantly higher expression in tissue
*** Validated by Q-RT-PCR**

Table 4:

Gene specific-Q-RT-PCR primer pairs.

Primer sequences for internal control genes and genes of interest. All primer pairs were optimized at a ratio of 300:300 nM for forward and reverse using of 30 ng cDNA template per reaction.

Gene Name	Primer sequences (5' to 3')	
CISH	Forward:	TGTGCATAGCCAAGACCTTCTC
	Reverse:	CCTCACTGGCCGTAATGGA
GAPDH	Forward:	GCATCGTGGAGGGACTTATGA
	Reverse:	GGGCCATCCACAGTCTTCTG
IFNA2	Forward:	GCCACCATGTGGGATGAGA
	Reverse:	CAGACAGAATTGCAGGTCAGTGA
SOCS3	Forward:	GAGAACTGCCGGGAATCTTTG
	Reverse:	GAGGTTTAAAGCTCTGTTTTAAACCAA
S100CBP	Forward:	CTGGCCGTGGACAAAATAATG
	Reverse:	GAAGCTCTGGAAGCCCACTTT
FABP4	Forward:	GCGTGGGCTTTGCTACCA
	Reverse:	CCCCATTCAAACTGATGATCAA
ASH2	Forward:	TCCGCCTTCCATCCACTTC
	Reverse:	GCCGTGGTGGAGCACACT
APOII	Forward:	GCCTTGGCTTGGGTTTGAA
	Reverse:	GGCCGACTATGGCAAGGA

Table 5.

Tissue comparison Q-RT-PCR Results.

Gene	Adipose vs. Liver	Adipose vs. Mammary	Liver vs. Mammary
Alpha-lactalbumin (LALBA)	Z	X	X
Apolipoprotein A-II (ApoA2)	Z	Z	X
Ash2 (absent, small, or homeotic)-like (ASH2L)	Ø	Ø	Ø
Cytokine inducible SH2-containing protein (CISH)	Y		X
Fatty acid binding protein 4 (FABP4)	X	X	Z
Interferon, alpha 2 (IFNA2)		X	
S100 calcium binding protein A10 (S100A10)		X	X
Suppressor of cytokine signaling-3 (SOCS3)	Y		X

X = validated by Q-RT-PCR ($P < 0.05$)
Y = message not detected in adipose;
Z = not significant on microarrays, sig. Q-RT-PCR
Ø = invalidated (inverted)

CHAPTER 4

IGF-I and leptin hormonal infusions alter the cell proliferation and transcriptional profile of the prepubertal bovine mammary gland

ABSTRACT

In dairy cattle high-energy diets before puberty promoting body growth rates above 1 kg/d impair mammary development. Rapid growth regimens enable earlier calving but also lead to reduced milk production. Biological mechanisms to explain this result are not well understood, but insulin-like growth factor-I (IGF-I) and leptin likely play a role. Adipocytes produce the protein leptin, and local leptin concentrations in the mammary gland increase in response to increased fat deposition. IGF-I stimulates and leptin inhibits proliferation of bovine mammary epithelial cells *in vitro* and *in vivo*. This is in contrast to human and mouse studies, which show a proliferatory effect of leptin on mammary epithelial cells. Our objective was to elucidate the effects of IGF-I and leptin infusion on cell cycling in mammary epithelial cells, identify key genes controlling the interaction of these two hormones in bovine mammary tissue, and determine if leptin alters IGF-I-induced expression of genes involved in the major IGF-I signaling pathways. A functional genomics approach was used to analyze parenchyma tissue collected from prepubertal Holstein heifers after 7 d of intramammary hormone infusions. With this model Silva (2002) previously showed that IGF-I stimulated proliferation of epithelial cells 60% and leptin

blocked this mitogenic effect of IGF-I (278). Each mammary quarter of six heifers received infusions via the streak canal of one of four treatments: IGF-I, leptin, IGF-I plus leptin, or saline plus BSA control. Intramammary infusion of IGF-I increased the percentage of BrdU-labeled mammary epithelial cells in the S-phase of the cell cycle by 52%. Intramammary infusion of leptin decreased BrdU-labeling 48% in IGF-I-treated quarters, and 19% in saline-treated quarters. No differences were observed in the number of cells within the cell cycle, as indicated by the Ki-67 labeling index. A significant increase in apoptosis, as marked by caspase-3 immunostaining, was seen in IGF-I and leptin treated quarters relative to saline control. One possibility is that increased concentrations of IGF-I may promote the proliferation of mammary epithelial cells by increasing the progression of cells into the S-phase of the cell cycle *in vivo* in mammary epithelial cells in prepubertal heifers. Conversely, increasing the leptin concentration above normal may reduce epithelial cell proliferation by limiting the progression of cells into the S-phase of the cell cycle and stalling them within the G1 phase of the cell cycle. We hypothesized that changes in cell proliferation should be accompanied by alteration of the profile of transcribed genes involved in cell proliferation and survival. Gene expression profiles of total parenchyma mRNA from each quarter of the six animals were examined using bovine-specific cDNA and oligonucleotide microarrays. Numerous genes mediating intracellular signaling were significantly altered ($P<0.05$) by the treatments, all with small fold changes (< 1.5-fold up- or down-regulation). IGF-I induced changes within the JAK/STAT pathway, the Ras/Raf pathway, the PI3K pathway, and the Bad/Bcl

pathway. Similarly, leptin altered expression within the JAK/STAT pathway, the Ras/Raf pathway, and the PI3K pathway including increased expression of the suppressor of cytokine signaling protein SOCS3. These data suggest that leptin may counter-regulate IGF-I action through IGF and cytokine receptor families' downstream signaling pathways. Overall, this study has demonstrated that IGF-I and leptin intramammary infusions in prepubertal heifers can serve as a useful model for study of hormonal regulation of cell cycle progression and gene transcription in the developing gland.

Introduction

Mammary growth and development are precisely controlled processes that are governed by the complex interplay of circulating hormones and locally acting factors. Classic studies in the laboratory and in domestic species established that normal development of the mammary gland is contingent upon the interactions of a wide range of circulating hormones secreted from the ovary and hypothalamus (69;194;214). The importance of tissue-derived factors in promoting mammogenesis has more recently been investigated (9;68;237;327). Mammary epithelial cells form the foundation for future lactation potential, and numerous studies have indicated that high planes of nutrition have a negative impact on this cell population in ruminants (38;43;117;265;267;324). High energy diets increase the serum concentrations of both IGF-I and leptin (28;241). IGF-I has a well-established role in promoting mammary epithelial cell proliferation and survival, mediated by the IGF-I receptor (IGF-IR), which is able to activate

several distinct signaling pathways (124). Leptin is a hormone secreted by fat cells (112;113;336) that mediates central hormonal and metabolic responses to the nutritional state of mammalian species. Leptin has been shown to be synthesized in mammary tissue (285) and can also be found in the circulation, suggesting that either locally or extra-mammary-produced leptin may influence the milieu surrounding mammary epithelial cells (278). Recently our lab has shown that exogenous bovine and ovine leptin inhibit the IGF-I stimulated proliferation of bovine mammary cells *in vitro* in the MAC-T cell line (276) and *in vivo* in a mammary infusion study (278). It is believed that leptin acts in the mammary gland by signaling through the long form of the leptin receptor (Ob-Rb), which is expressed by mammary epithelial cells (Silva, VandeHaar, et al. 2002 #560).

Interaction between members of the IGF family and interleukin-6 family of class I cytokine receptors has been demonstrated within the central nervous system (47;48;260;261), skeletal muscle (303), adipose tissue (246), ovary (4;288;333), liver (46), and mammary epithelial cells (284). In accordance with these results, it is here proposed that leptin, a hormone produced by adipocytes, could potentially mediate the inhibition of IGF-I-stimulated mammary parenchymal development through crosstalk between the IGF-I and leptin receptors. Evidence supporting distinct interactions between IGF-I and leptin on cell proliferation both in the mammary gland and other tissues supports the hypothesis that these factors may act synergistically to alter the profile of

transcribed genes in the prepubertal bovine mammary gland to affect mammogenesis.

It is believed that intramammary infusions of the hormones IGF-I and leptin alter the profile of transcribed genes mediating crosstalk between the IGF-I and leptin and cell proliferation of mammary epithelial cells. To test this hypothesis the expression profiles of genes likely to control mammogenesis from quarters infused with IGF-I and or leptin hormones in prepubertal heifers was measured using a functional genomics approach. The first objective of this study was to extend the analysis of Silva (2002) on the effects of *in vivo* hormonal infusion on mammary epithelial cell proliferation (278). This was done by measuring the total number of cells committed to the cell cycle and apoptosis using immunohistochemical analysis of Ki-67 and Caspase-3, respectively. The second objective of this study was to examine global expression of transcribed genes using cDNA and oligonucleotide microarray platforms. Thirdly, quantitative RT-PCR was used to analyze the mRNA expression profile of major components of the IGF-I and leptin signaling pathways likely to mediate crosstalk between the two hormones.

MATERIALS AND METHODS

Animals. Six prepubertal Holstein heifers weighing 209±10 kg were purchased and housed in individual stalls at the Beef Cattle Research Center of Michigan State University. Heifers were fed once a day a corn silage-based diet at restricted intake formulated to achieve a body weight gain of 700 g/d, according

to standard recommendations (216;293). This growth rate was selected to promote normal mammary development (Sejrsen, Purup, et al. 2000 #5150). Heifers had free access to water. The Michigan State University All-University Committee on Animal Use and Care approved experimental procedures.

Infusion procedure. Intramammary infusions of hormones were performed as described in Silva (2002). Six heifers were used to test the effects of intramammary infusion of leptin on proliferation of mammary epithelial cells. Heifers were adapted to diet and environment for 10 d before the start of infusions. The front or rear halves of the udders were treated as separate strips and randomly assigned to receive intramammary infusions of either IGF-I or saline (278). To balance for the effects of strip, two heifers were infused at each time, one with IGF-I infusions in the front half, and the other with IGF-I infusions in the rear half. The left and right side of each strip were assigned randomly to receive intramammary infusions of either recombinant ovine leptin (oLeptin) or saline over a 7 d infusion period. Treatments were then defined as IGF-I (IGF), IGF-I + oLeptin (IGF+LEP), saline (SAL) and saline + oLeptin (LEP) (Silva 2002 #5810). Thus, each treatment was applied to a total of six quarters, one in each heifer, and the effect of oLeptin always was compared against its contralateral quarter, which was not infused with leptin (278).

Lyophilized recombinant human IGF-I (GroPep Pty Ltd, North Adelaide, Australia), which is identical to bovine IGF-I, was dissolved in 10 mM HCl, neutralized and diluted in sterile, physiological saline at a concentration of 1 μg

IGF-I/ml. The saline contained 1 mg of bovine serum albumin/ml (Invitrogen, Carlsbad, CA). Ten micrograms of IGF-I were infused daily using a 12-ml syringe with an intramammary infusion cannula (278). Recombinant ovine leptin was purchased from Dr. Arieh Gertler of the Hebrew University, Jerusalem, Israel, and was produced as described by Gertler et al. (120). Lyophilized leptin was dissolved in saline at a concentration of 10 μg oLeptin/ml. Leptin-treated quarters were infused daily with 100 μg of oLeptin. Solutions were infused at 0800 h each day (278). On the last day of treatment, additional infusions were given at 2000 h to maximize the effect of the infused hormones on BrdU incorporation the following morning. Heifers were killed 14 h after the last infusion. To avoid any microbial contamination, all materials used during infusion were sterile. The teats were cleaned with an iodine solution and the teat ends scrubbed with ethanol before infusion (278). The solutions infused were tested for the presence of endotoxins using a commercial kit with sensitivity to detect 0.006 ng of endotoxin/ml (LAL, BioWhittaker, Walkersville, MD) (278).

Sample collection and tissue processing.

Mammary parenchymal tissue was harvested from the heifers as described previously (278). At slaughter, mammary glands were removed within 5 min of death and samples from three regions of the mammary parenchyma (proximal, intermediate, and distal to the teat) were collected and fixed in neutral buffered formalin for 18 h (Sigma). At this time mammary samples (~5 g each) from each of the three regions of the mammary parenchyma were collected for

RNA extraction, sealed individually and snap frozen in liquid nitrogen for storage at -80°C until use (278).

BrdU immunohistochemistry

Bromodeoxyuride-incorporation immunohistochemistry was performed by Silva (2002). Total epithelial cells and BrdU-labeled epithelial cells were quantified in three microscopic fields from each of three slide sections (Figure 1A). Two independent evaluators blinded to the identity of the samples enumerated cells, and an average of 2935 epithelial cells were counted in each quarter to calculate the percentage of cells labeled with BrdU (278).

Ki-67 immunohistochemistry

Immunohistochemical analysis of Ki-67 labeling was performed by Matti Kiupel, Michigan State University Department of Pathology and Diagnostic Investigation, as described by Kiupel et al. (162). Room temperature paraffin-embedded tissue samples were incubated in 10 mM HCl/citrate buffer (pH 6.0) and heated in a microwave oven (600 W) for 25 minutes. Slides were transferred to 100% ethanol and immersed in methanol containing 1% hydrogen peroxide for 30 min to block endogenous peroxidase activity. Sections were then washed in distilled water and PBS (pH 7.6) containing normal equine serum for 15 min to block nonspecific antibody binding. A mouse anti-Ki-67 antibody was used for immunostaining (Immunotech S.A., Marseille, France) at a dilution of 1:200 in 10% normal equine serum for 60 minutes. Antibody binding was visualized with

the Vector ABC Elite kit (Vector PK4000) with 3,3'-diaminobenzidine substrate (Vector SK4100). Final washing was performed in distilled, deionized water. Sections were then counterstained with Mayer's hematoxylin, then dehydrated, cleared, and mounted in DPX (BDH). Equine lymphoid tissue was used as a positive control (162). Total epithelial cells and Ki-67-labeled epithelial cells were quantified in three microscopic fields from each of three slide sections (Figure 1B). Two independent evaluators blinded to the identity of the samples determined the percentage of Ki-67 labeling, counting an average of 1710 epithelial cells per quarter.

Caspase-3 immunohistochemistry

Cell death analysis was performed by Matti Kiupel essentially as described by Kiupel et al. (161). Tissue sections were deparaffinized and incubated in target retrieval solution (Dako Corporation, Santa Barbara, CA) for 15 min at 95-99C. Immunostaining was performed using the Dako Autostainer (Dako Corporation, Santa Barbara, CA) by incubation with a polyclonal anti-mouse anti-activated caspase-3 antibody (R&D Systems, Minneapolis, MN) at a dilution of 1:100 at -4C overnight, followed by a 1:500 dilution of a biotinylated goat anti-rabbit secondary antibody (Dako Corporation, Santa Barbara, CA). A peroxidase labeled streptavidin-biotin complex (Dako Corporation, Santa Barbara, CA) was used for antibody binding localization, after which antibody binding was visualized with a 3,3'-diaminobenzidine as a chromogen substrate. After a final wash in automation buffer, sections were counterstained with

Lerner's hematoxylin. Sections incubated with isotype control antibodies were used as negative controls (161). Total epithelial cells and caspase-3-labeled epithelial cells were quantified in three microscopic fields from each of three slide sections (Figure 1C). Two independent evaluators blinded to the identity of the samples determined the percentage of caspase-3 labeling, with an average of 3136 epithelial cells counted per quarter.

Infusion study statistical analyses. Data from the IGF-I and leptin infusion study were analyzed by least squares ANOVA using the following model, where IGF is saline or IGF-I infusion, leptin is saline or leptin infusion and region is the parenchymal regions sampled (proximal, intermediate and distal to the teat), as described by Silva (2002). The model was:

$$Y = \mu + \text{heifer} + IGF + LEP + IGF \times LEP + e$$

The MIXED procedure of SAS (2000) was utilized with the data log-transformed to remove heterogeneity of variance (255). Data are presented as back-transformed. Estimates of variance were calculated as confidence intervals from the transformed data. BrdU-labeling among parenchymal regions was compared by Bonferroni t-test adjusted for 3 comparisons (217).

RNA isolation procedure. Total RNA was extracted from frozen mammary tissues from intermediate tissue samples (after homogenization) with Trizol reagent (Invitrogen Life Technologies Corp., Carlsbad, CA) essentially as recommended by the manufacturer (Invitrogen Life Technologies Corp.,

Carlsbad, CA). Tissue was kept cold using dry ice and ~200 mg of mammary tissue was weighed and directly added to 3 mL of Trizol reagent in a 15 ml culture tube. Tissue was homogenized using a Polytron for ~30 s. Between homogenization of individual samples, the Polytron bit was rinsed with diethyl pyrocarbonate (DEPC, Sigma) treated water, and RNAse Away (Molecular BioProducts, San Diego, CA). Samples were aliquotted into three 1.5-mL centrifuge tubes and incubated at room temperature for 5 min. Chloroform (200 µL) was added to each tube and samples were manually shaken, incubated for 3 min at room temperature, and centrifuged at 10,500 x g for 15 min at 4°C. The upper phase was transferred to a clean tube. Isopropanol (500 µL) was added to precipitate RNA. The tube was vortexed, incubated at room temperature for 10 min, and centrifuged at 10,500 x g for 10 min at 4°C. The isopropanol was decanted and the remaining pellet was washed with 75% ethanol, centrifuged at 8500 x g for 5 min at 4°C, decanted, and dried. RNA was then subjected to DNase treatment (RQ1 RNase-free DNase; Promega, Madison, WI) following the manufacturer's protocol (Promega, Madison, WI). Nuclease free water (52 µL), DNase buffer (10 µL of 10X; Promega), and DNase (1 µL of 2U/µL; Promega) were added to the pellet and then incubated at 37°C for 30 min. A phenol/chloroform separation step was performed by adding RNase-free water (37 µL) and phenol/chloroform (100 µL). The tube was shaken and centrifuged for 2 min at 14,000 x g. The upper phase (~90 µL) was transferred to a fresh tube, sodium acetate (3 M, 9 µL) and ethanol (250 µL) were added to this phase, and the mixture was stored overnight at -20°C. The following day,

microcentrifuge tubes were centrifuged at 14,000 x g at 4°C for 20 min. The liquid was decanted and the pellet was washed with ethanol (75%, 500 µL). The tube was centrifuged at 14,000 x g at 4°C for 10 min. The ethanol was decanted and the pellet was dried under air for 15 min. The pellet was resuspended in 50 µL of nuclease-free water and incubated at 60°C for 10 min. RNA used in the BOTL4 microarray study was quantified using spectrophotometry at 260- and 280-nm and quality was checked by 28S and 18S rRNA band visualization following gel electrophoresis and ethidium bromide staining. The quantity of RNA isolated for the BMET microarray study was determined using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and quality was checked with a RNA 6000 Nano LabChip kit and Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA).

BOTL4 microarray experimental design. The BOTL4 microarray used is an expanded version of those previously described (7;67;98;328). The BOTL4 microarray contains 4800 total spots consisting of 709 bovine EST clone inserts developed from a normalized total leukocyte (BOTL) cDNA library (328) and an additional 627 amplicons representing additional genes including cytokines, growth factor associated proteins, receptors and intracellular signaling components (67). A list of known genes represented on the BOTL4 micorarray and their sequences can be viewed at http://gowhite.ans.msu.edu/public_php/gd-bovine-immunology.php. Amplicons were suspended in 50% DMSO and spotted in triplicate on glass slides in a 4 x 12 pattern of 48 patches with 100 spots per

patch. This array included 144 spots representing GAPDH (three spots in each of 48 patches), 75 spots representing β-actin, 78 spots representing ribosomal protein L-19 (RPL-19), 48 synthetic Lambda Q gene spots (1 per patch), 303 negative control spots (DMSO only), and 144 blank spots (nothing spotted).

Screening for leptin-, IGF-I- and IGF-I + leptin-induced mammary parenchymal gene expression changes was performed on all six animals using a loop design (157;158) balanced for Cy3 (green) and Cy5 (red) fluorescent dye labeling (Amersham Pharmacia Biotech; Piscataway, NJ) for each of the four treatments (24 arrays total). RNA pools of 20 μg per sample were used for cDNA synthesis and the two dye coupling reactions; the pools were separated into two aliquots of 10 μg each for reverse transcription and dye-labeling. Within a four comparison loop, the first comparison made was between SAL vs. IGF, the second comparison was between IGF and IGF+LEP, the third comparison was between IGF+LEP and LEP, and the fourth comparison was between LEP and SAL. Three loops were run in the forward direction and three in reverse (i.e. in array no. 1 three animals had Cy3 dye-labeling of the IGF-I treatment and three had Cy5). Comparisons included within the loop were designed to test the major comparisons of interest with regard to the BrdU incorporation results, in which the most significant results were found between the SAL and IGF (array no. 1) and IGF and IGF+LEP (array no. 4) treated quarters.

BMET microarray experimental design. The BMET microarray was used to compare gene expression profiles of SAL, IGF and IGF+LEP infused quarters.

The BMET array was described previously (Chapter 2) (97) and contains 2,348 spots representing genes involved with bovine metabolism and signal transduction. This array included 10 metabolic housekeeping genes from *Arabidopsis thaliana* used for spike-in controls, 1320 blank spots (printing buffer only spotted) and 64 empty spots (nothing spotted).

Complete annotation of the BMET gene set can be found at http://www.nutri-genomics.org/data.html. The BMET microarrays were printed at the Massachusetts General Hospital Microarray Core Facility at Harvard University (https://dnacore.mgh.harvard.edu/microarray/protocol-printing.shtml) as described in Chapter 3 of this thesis (Materials and Methods).

Two microarray comparisons, SAL versus IGF, and IGF versus IGF+LEP, were screened directly for all six animals with the design balanced for Cy3 (green) and Cy5 (red) fluorescent dye labeling (Amersham Pharmacia Biotech; Piscataway, NJ) (12 arrays total). Total RNA (15 ug) from each quarter of the three treatments of interest was used for cDNA synthesis and dye coupling reactions. In the first comparison (SAL vs. IGF), SAL cDNA was labeled with the Cy3 dye (Amersham Biosciences, Piscataway, NJ) for three heifers (20, 28, and 29) and IGF-I was labeled with Cy5 (Amersham Biosciences, Piscataway, NJ); SAL was labeled with the Cy5 dye in the other three heifers (35, 36 and 88) with IGF-I samples labeled with the Cy3 dye. This reciprocal dye design was repeated for the second comparison (IGF vs. IGF+LEP).

Preparation of labeled cDNA for BOTL4 hybridization. Gene expression profiles from SAL, IGF, LEP, and IGF+LEP treated quarters of all six heifers were evaluated on cDNA microarrays (24 microarrays total). Synthetic lambda Q gene containing an engineered poly-A tail was spiked into each cDNA synthesis reaction (650 pg) to provide a control for cDNA synthesis and dye labeling efficiency. For cDNA synthesis 8 µg of sample RNA was converted to cDNA using the Atlas Powerscript fluorescent labeling kit (BD Biosciences, Alameda, CA). First strand cDNA synthesis, RNase-H cleanup of template RNA, and sample purification were performed according to procedures outlined by Aho et al. (7).

Resulting cDNAs were coupled to Cy3 and Cy5 dyes using the Atlas Powerscript fluorescent labeling kit (BD Biosciences, Alameda, CA) using the manufacturer's protocol. Labeled cDNAs were purified to remove unincorporated dyes, combined, and concentrated according to procedures outlined previously (7). The labeled cDNAs were incubated at 70°C for 5 min just prior to an 18-hour array hybridization using a GeneTAC Hybridization Station (Genomic Solutions, Ann Arbor, MI) with a step-down hybridization protocol as previously described (64;66). Following hybridization, microarray slides were washed twice in low stringency buffer and once in high stringency buffer in the Hybstation unit (Genomic Solutions, Inc., Ann Arbor, MI). Finally, microarrays were rinsed once in 2X SSC (saline sodium citrate, 0.15 M NaCl plus 0.015 M sodium citrate) and once in double-distilled H_2O. Microarrays were dried by centrifugation in a cushioned 50-ml conical tube and immediately scanned with a GeneTAC LS IV

microarray scanner (version 3.01). GeneTAC LS software version 3.3.0 (Genomic Solutions, Inc., Ann Arbor, MI) was used to process microarray images, find spots, integrate robot spotting files with the microarray image, and to create reports of raw spot intensities. Total intensity values for each dye channel were converted to comma-separated value data files and exported into Excel spreadsheets and loaded into SAS (255) for data normalization and analysis.

Preparation of labeled cDNA for BMET hybridization. Gene expression profiles from SAL, IGF, and IGF+LEP treated quarters of all six heifers were evaluated on oligonucleotide microarrays (12 microarrays total). For cDNA synthesis 15 µg of sample RNA was used as a template in reverse transcription reactions (*SuperScript III* Fluorescent Labeling Kit; Invitrogen Life Technologies Corp., Carlsbad, CA) in which random hexamers were used as a primer. In the Superscript III system, cDNA is prepared with a randomly incorporated amino-modified dUTP. Following first-strand synthesis, cDNAs for each tissue within an animal were differentially labeled using *N*-hydroxysuccinimide-derivatized Cy3 and Cy5 dyes (Amersham Pharmacia, Ltd., Piscataway, NJ). Differentially labeled were combined and concentrated to 10 µl by using Microcon 30 spin concentrators (Millipore Corp., Bedford, MA). Microarray hybridization was performed after addition of 100 µl of SlideHyb-3 (Ambion Inc., Alameda, CA) to the concentrated Cy3–Cy5-labeled probe cDNAs (7). The labeled cDNAs were incubated at 70°C for 5 min just prior to 18-hour array hybridization in Corning Microarray Hybridization Chambers (Fisher Scientific) placed in water baths set

to 42°C. Following hybridization, microarray slides were washed with 2xSSC saline sodium citrate (0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS for 4 min at 42 C, 2X SSC for 4 min at room temperature, 0.2XSSC for 4 min at room temperature, 0.06X SSC for 10 sec at room temperature and once in double-distilled H_2O. Microarrays were dried by centrifugation in a cushioned 50-ml conical tube and immediately scanned with a GeneTAC LS IV microarray scanner (version 3.01). Genepix Pro software version 6.0 (Molecular Devices Corporation, Sunnyvale, CA) was used to process microarray images, find spots, integrate robot spotting files with the microarray image, and to create reports of raw spot intensities. Total intensity values for each dye channel were converted to comma-separated value data files, exported into Excel spreadsheets, and loaded into SAS (255) for data normalization and analysis.

Statistical analysis of microarray data. Microarray M vs. A scatterplots to detect and account for potential dye intensity biases analysis was performed essentially as described in Chapter 3 of this thesis.

Statistical analysis of LOESS-adjusted log intensities was performed using a mixed model approach consisting of two steps (9, 11). For the BOTL4 microarray, the first step involved an array-specific spatial variability normalization model:

$$\log(y_{gijklmn}) = \mu + Gene_g + Treatment_i + Dye_j + Animal_k +$$

$$Slide(Array)_{kloop} + Patch(Slide)_{klm} + \varepsilon_{gijklmn}$$

Where $y_{gijklmn}$ represents each observed fluorescent intensity signal; μ is an overall mean value; T_i is the main effect of treatment i (SAL, IGF-I, IGF+LEP, LEP); D_j is the main effect of dye j; A_k is the main effect of animal k; $S(A)_{kl}$ is the effect of slide k within array l; $P(S)_{klm}$ is the effect of patch m within slide k within loop l; and $e_{gijklmn}$ is a stochastic error (assumed to be normally distributed with mean 0 and variance σ^2).

The second step involved gene-specific analyses for the estimated residuals (e_{ijklmn}) obtained from the normalization approach obtained from step one to test the effect of treatment on expression profiles for individual genes. These models were:

$$\varepsilon_{gijklmn} = m_g + T_{gi} + D_{gj} + T^*A_{gik} + A_{gk} + S(A)_{gkl} + Sp(S)_{gkln} + e_{gijklmn}$$

Where all the effects have the same definitions as for the normalization model, except that $P(S)_{gkln}$ represents the random effect of each spot, and g index is added to account for effects now being gene specific. The error terms e_{ijklmn} were assumed to have independent normal distributions with gene-specific variances σ_g^2.

Similarly, for the BMET microarray analysis data were analyzed first with the following model, where all terms are random except dye:

yadj = **dye** + array + day*array + block(array) + dye*block(array) + e

Next, residuals calculated from step one were analyzed in a second step according to the following model:

resid = heifer + **treat** + treat*heifer + **dye** spot(heifer) + e

Where heifer and array are confounded, and treat was tested by treat*heifer.

These analyses were computed using the MIXED procedure of SAS (255). In the gene-specific analysis, genes were considered significantly different if $P<0.05$.

Quantitative RT-PCR validation of microarray gene expression changes. The expression differences of certain genes found to be significant by microarray analysis were checked using quantitative real-time reverse transcription-PCR (Q-RT-PCR) using an Applied Biosystems 7000 DNA Sequence detection system (Perkin Elmer Corp., Foster City, CA). Total RNA was extracted from each quarter from each of the six heifers, quantified and checked for quality as described previously. The PCR system used was the ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

RNA was converted into first-strand cDNA by adding 1 µg of total RNA to a 12-µl reaction containing 10 mM oligo$(dT)_{18}$ primer. Following a 5-min incubation at 70°C, the reaction was chilled on ice and pH adjusted by addition of 4 µl of a buffer supplied by the reverse transcriptase manufacturer (final reagent concentrations were 50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$), 1 mM deoxynucleoside triphosphates, and 200 units of Superscript II RNase H reverse transcriptase (Invitrogen Life Technologies). Incubation at 37°C continued for 30 min in the presence of RNase-H to remove the original RNA templates. Heating at 70°C for 15 min subsequently inactivated RNase-H. The

reaction tubes were removed from the thermocycler and 0.2 µL of 0.5 M EDTA was added and mixed. Then, 25 µL of water, 5 µL of 3M sodium acetate, and 125 µL of ethanol (-20°C) were added to the tube. Tubes were then allowed to precipitate overnight at -20°C. The reaction mixture containing the newly-synthesized cDNA was transferred to a new, clean microcentrifuge tube. These tubes were then centrifuged at 14,000 x g and 4°C for 20 min and the supernatant was decanted. Pellets were washed with 250 µL of ethanol (75%, -20°C) and the tube was centrifuged at 14,000 x g and 4°C for 6 min. The supernatant was decanted and the pellet was allowed to dry for 15 min. The pellet was resuspended in 50 µL of water and incubated at 60°C for 5 min. The cDNA concentration was analyzed using a spectrophotometer (NanoDrop), and was then diluted to a final concentration of 10 ng/µL and stored at -80°C until the Q-RT-PCR reaction was initiated.

SYBR Green PCR Master Mix (Perkin Elmer Corp.) and gene-specific primers were used to perform Q-RT-PCR reactions. Primer Express Software (Perkin Elmer Corp.) was used to design all primers, which were then synthesized by a commercial facility (Invitrogen Life Technologies). Primer sequences for genes analyzed in this report are included in Table 1.
The amount of primer used was determined by performing an optimization matrix for each primer using three concentrations of primers: 50:50 nM, 300:300 nM, 900:900 nM. Dissociation curves were similar for all concentrations and the 300:300 nM matrix was chosen, thus 3 µL of primer was used for all experiments. Each gene of interest and the control gene were measured in duplicate. Within

each well of a 96-well reaction plate (MicroAmp Optical, Applied Biosystems), 30 ng of sample cDNA (3 µL), 6.5 µL DEPC water, 3 µL primer, and 12.5 µL Sybr Green (Applied Biosystems) were added.

To determine an appropriate reference control gene by which relative mRNA abundances for genes of interest could be measured, a number of potential housekeeping genes were screened. These candidate control genes included β-actin (B-actin), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hydroxymethyl-bilane synthase (HMBS), hypoxanthine phosphoribosyltransferase 1 (HPRT1), TATA box binding protein (TBP), and succinate dehydrogenase complex, subunit A (SDHA) (Table 6). The variation in gene expression across all treatments for these six genes was tested using the methods of Vandesompele et al. (312). The expression of GAPDH and HMBS were not significantly different from 1 nor each other across all treatments (SAL, IGF, LEP, IGF+LEP) and were therefore suitable reference controls.

The $2^{-\Delta\Delta CT}$ method of Q-RT-PCR analysis was performed as previously described (7;65;188). In this study analysis GAPDH was used instead of HMBS to serve as the control gene to calculate initial ΔC_T values, as it has previously been shown to be an appropriate control gene in experiments with mammary tissue (284;286). This method allows for the determination of relative gene expression changes across treatments based on quantitative differences in the PCR amplified target reaching a fixed threshold cycle (C_T) number for a specific treatment versus other treatments (Table 7). In our $2^{-\Delta\Delta CT}$ analysis, the C_T for SAL was the calibrator used to determine relative expression changes for IGF,

LEP, and IGF+LEP treated quarters for each GAPDH-normalized test gene. Gene-specific standard errors were estimated using independent analyses of variance (ANOVA), which included the effects of quarter and treatment. All analyses were performed using the SAS System (255).

RESULTS

Intramammary infusion of oLeptin decreased BrdU-labeling of mammary epithelial cells.

As reported by Silva (2002), in both saline- and IGF-I-treated quarters, infusion of leptin decreased the percentage of cells in the S-phase of the cell cycle (Figure 2B) (278). Leptin decreased ($P < 0.001$) BrdU-labeling by 48% in IGF-I-treated quarters (4.1 vs. 7.9 %) and decreased ($P = 0.01$) BrdU-labeling only 19% (5.0 vs. 6.2 %) in saline-treated quarters (Figure 2). In contrast to the significant increase in BrdU labeling in IGF-I-treated quarters relative to SAL reported in a previous study (275), no differences were detected between IGF and SAL in this study.

Cell cycle commitment is unaffected by treatment.

The number of cells committed to the cell cycle was unaffected by hormonal infusion as measured by the percentage of cells labeled by the nuclear cell proliferation antigen Ki-67 (Table 1). The percentage of Ki-67 positive mammary epithelial cells ranged from 8.5-11.7%, but did not differ significantly across SAL, IGF, LEP, and IGF+LEP treatments (Figure 3).

The percentage of proliferating cells occupying the S-phase of the cell cycle was significantly reduced by leptin (Table 1). This was calculated by dividing the percentage of BrdU-labeled cells by the percentage of Ki-67-labeled cells. Both the main effect of leptin ((LEP + IGF+LEP) – (SAL + IGF)) (P=0.015) and the IGF versus IGF+LEP contrast (P=0.02) were highly significant (Figure 5).

Apoptosis in prepubertal mammary epithelial cells is low.

Using caspase-3 immunohistochemical staining as a marker of cell apoptosis (Figure 4), the pattern of programmed cell death in each quarter was measured. The overall percentage of apoptotic cells was very low (less than 0.25%). Although apoptosis was significantly increased in IGF, IGF+LEP and LEP treated quarters relative to SAL (Figure 4). The low numerical percentages of caspase-3 immunostaining in each quarter do not indicate a large biological significance due to any treatment.

BOTL4 microarray SAL vs. IGF differentially expressed genes. IGF-I infusion significantly increased the expression of 31 genes and decreased 34 genes relative to SAL treatment on the BOTL4 cDNA microarray (P<0.05) (Supplemental Table 1). Of these 65 genes altered by IGF-I treatment, 15 were without known function according to BLASTn searches and were ignored for subsequent analysis. Gene ontology analysis revealed that IGF-I treatment induced changes in 38 genes that could be divided into four broad ontologies (Table 2). These included genes involved with extracellular receptor mediated

signal transduction (n=18), cell proliferation and apoptosis (n=7), transcriptional regulation (n=6), and cell structure and metabolism (n=8).

BOTL4 microarray IGF-I vs. IGF-I + leptin differentially expressed genes. The comparison between IGF-I and IGF+LEP treated quarters revealed expression changes with 65 transcripts ($P<0.05$) (Supplemental Table 1). Eighteen genes were without known function, 34 were downregulated and 31 upregulated by IGF+LEP treatment relative to IGF. Genes of interest with regard to IGF and leptin signaling were involved with extracellular receptor mediated signal transduction (n=17), cell proliferation and apoptosis (n=9), and cell structure and metabolism (n=2) (Table 3).

BOTL4 microarray SAL vs. LEP differentially expressed genes. Leptin infusion altered the expression of 92 mRNAs relative to SAL treatment ($P<0.05$) (Supplemental Table 1). Of these, 25 had no known function according to BLASTn searches, 32 were downregulated and 60 upregulated. As with the IGF treatment results, genes of particular interest could be broadly categorized into four major categories (Table 4). Included were genes implicated in extracellular receptor mediated signal transduction (n=18), cell proliferation and apoptosis (n=7), transcriptional regulation (n=9), and cell structure and metabolism (n=16).

BMET microarray SAL vs. IGF differentially expressed genes. Sufficient data for statistical analysis was not found for 25 of 2,348 bovine genes in the IGF vs.

SAL BMET microarray comparison. IGF infusion significantly altered the expression of 135 (5.8%) genes compared to SAL control on the BMET array ($P<0.05$) (Supplemental Table 2). This included the upregulation of 102 genes by IGF-I and downregulation of 33 mRNAs relative to control treatment (Supplemental Table 2). Gene ontology analysis also revealed that IGF treatment induced changes in 39 genes of interest that could be divided into four broad categories related to IGF and leptin action in the mammary gland (Table 5). These included genes involved with receptor-mediated signal transduction (n=19), cell proliferation and apoptosis (n=10), transcriptional regulation (n=6), and cell structure and metabolism (n=4).

Significantly altered genes grouped within a number of pathways and gene ontologies according to GenMAPP analysis (72;87). These included the insulin signaling pathway, which included upregulation of the mRNAs encoding FOXO1A, IRS1, RAD, RAF1, PKCalpha, MAP3K4, MAPK13, MAP2K5, and downregulation of TSC2, EGR1 (Figure 9). Seven genes found within the TGF-beta signaling pathway were significantly upregulated by IGF-I treatment including TGFBR1, TGFBR3, FST, MADH2, STAT1, and EGF (Figure 8). Genes falling within the pathway regulating G1 to S phase cell cycle control were also significantly overrepresented in the analysis of control vs. IGF-I treatments. These included upregulation of mRNAs coding for MYC, CCNE1 ($P=0.06$), and E2F4 ($P=0.07$), and downregulation of CCND2, PCNA ($P=0.07$), and CREB3L1 (Figure 10).

BMET microarray IGF-I vs. IGF+LEP differentially expressed genes. The BMET microarray comparison between IGF and IGF+LEP treated quarters revealed significant differential expression ($P<0.05$) of 182 of 2323 genes measured (7.8%) (Supplemental Table 3). The IGF+LEP treatment significantly increased the expression of 108 and decreased 73 mRNAs relative to IGF-I treatment. Significantly altered genes could be broadly classified within a number of gene ontologies relevant to IGF-I- and leptin-related phenotype within the mammary gland (Table 6). These included receptor-mediated signal transduction genes (n=28), genes involved in cell proliferation and apoptosis (n=5), regulators of transcription (n=3), and cell structure and metabolism (n=2).
GenMAPP pathway analysis (72;87) indicated overrepresentation of significantly altered genes within the insulin signaling pathway, including the leptin-induced upregulation of GSK3A, CAP1, FRAP1, GYS1, MAP3K11 ($P=0.06$), and SHIP2, and downregulation of PI3KA ($P=0.06$), SRF, MAP2K4, and PFKL (Figure 12). The TGF-beta signaling pathway was also significantly overrepresented in IGF-I plus leptin versus IGF-I treatment comparison when a genes tending to be altered by treatment ($P<0.1$) were also included in the GenMAPP analysis, including upregulation of ENG, MADH3 ($P<0.05$), TNF, CTNNB1, MADH7, and MADH2, and downregulation of NFKB1 ($P<0.05$) (Figure 11).

Q-RT-PCR confirms IGF-I induced expression changes in 4 of 21 genes significantly altered by microarray analysis. As shown in Figure 6, IGF-I-induced expression changes indicated by BOTL4 and BMET microarray

analysis were confirmed by Q-RT-PCR for four tested genes (Table 8). FK506 binding protein 12-rapamycin associated protein 1 (FRAP1) was confirmed as upregulated 56% with Q-RT-PCR analysis, closely mirroring its predicted change by BOTL4 microarray analysis. Although the BOTL4 microarray analyses predicted a 26% upregulation of the apoptosis-related cysteine peptidase caspase-8 (CASP8) by IGF-I treatment relative to SAL infusion, a 110% upregulation was observed with Q-RT-PCR. However, although microarray analysis predicted that this gene was additionally downregulated by IGF+LEP relative to IGF-I treatment, no significant differences were detected. 14-3-3-protein, eta isoform (YWHAH) similarly was confirmed for only one of two comparisons deemed significant by microarray analysis, showing a downregulation of 55% by IGF-I treatment relative to control. For a number of genes, a reversal of the direction of fold change was seen using Q-RT-PCR compared to that of microarrays. Although microarrays indicated an upregulation of NF-KB p65 by 17%, Q-RT-PCR showed a 47% increase in expression in the IGF+LEP treatment compared to IGF, but no significant difference in the SAL vs. IGF contrast. Similar disparities in comparing microarray and Q-RT-PCR results for genes found to be significantly altered by treatment for both platforms were seen with brain derived neurotrophic factor (BDNF) (upregulated 2.86 in IGF relative to SAL); mitogen activated protein kinase kinase kinase 1 (MAP3K1) (upregulated in IGF relative to SAL); NF-kappa B p65 (RELA) (upregulated 1.40 in IGF+LEP relative to IGF), and phosphatidylinositol glycan, class F (PIGF) (upregulated 1.52 in IGF relative to IGF+LEP). In three cases, a false-negative

gene was reported based on microarray analysis. While the BMET microarrays predicted a non-significant increase in SOCS3 mRNA of 1.13 in IGF+LEP relative to IGF (p=0.21), this gene was found to be upregulated 3.5-fold as measured by Q-RT-PCR. In addition, FRAP1, though found to be significantly upregulated by IGF-I relative to control for both the BOTL4 microarray and Q-RT-PCR, was not significantly upregulated (p=0.16) by 1.07 by the BMET microarray. YWHAH was also not predicted to be significantly different in the SAL vs. IGF comparison by the BMET array (0.98 fold, p=0.83) though this change was detected by the BOTL4 microarray and Q-RT-PCR. Expression changes for eight genes expected to be differentially expressed by BOTL4 microarrays were not significantly different by any treatment comparison when checked by Q-RT-PCR (OB-R, v-Jun, TLE1, IL8RB, SAP18, MAD2L1, FAK2, and BCAR1) (Table 2-4). Similarly, the BMET genes ALG12, CYCD2, IFNA2, MYC, PRKD2, PTCH, and SST were not found to be significantly changed in any treatment contrast.

Q-RT-PCR analysis reveals potential IGF-I and leptin signaling pathway interaction. Seven genes believed to be potentially involved with IGF-I and leptin signaling in the mammary gland, signal transducer and activator of transcription 3 (STAT3), STAT5A, STAT5B, cytokine inducible SH2-containing protein (CISH), suppressor of cytokine signaling 1 (SOCS1), SOCS3 and SOCS5, were measured by Q-RT-PCR. No significant differences in any treatment contrasts were detected for any of these genes except SOCS3. Expression of SOCS3 was shown to be significantly downregulated in IGF and in

LEP quarters relative to IGF+LEP (Figure 6). Leptin upregulated SOCS3 mRNA expression 3.4-fold in IGF-treated quarters ($P<0.05$), and IGF-I increased leptin-SOCS3 expression 2.4-fold in leptin treated quarters ($P<0.05$).

DISCUSSION

Silva (2002) found that an elevated concentration of leptin in the mammary gland in vivo can decelerate IGF-I stimulated epithelial cell proliferation (278). The reduction in the number of cells in the S-phase of the cell cycle was consistent with our previous work showing that leptin inhibits proliferation of the bovine mammary epithelial cell line MAC-T (276). The current study is the first to provide a possible intracellular mechanism by which leptin might inhibit proliferation of bovine mammary epithelial cells at a supraphysiological dose. Studies examining the effects of leptin on human breast cell lines have shown stimulation of cell proliferation by leptin in three studies (85;145;173), but inhibition at elevated doses (17). It is possible that this is a species-specific difference between humans and cattle.

A number of lines of evidence suggest that an interaction between the hormones IGF-I and leptin likely plays a role in the nutritional inhibition of mammogenesis in prepubertal ruminants. A definitive molecular mechanism linking high energy diets, which are known to increase circulating but not local mammary IGF-I concentrations, and mammary parenchymal development is still unknown (269;320;321). Paradoxically, increased serum IGF-I levels are correlated with high planes of nutrition but do not promote mammogenesis even

though IGF-I is a potent stimulator of mammary epithelial cells in culture (269). Proliferation of primary bovine mammary epithelial cells is maximized with ~25 ng of IGF-I/ml, a concentration much lower than that found in serum (231;319;320). Although the presence of IGF binding proteins in the extracellular milieu can reduce the amount of free IGF-I available to bind its receptor, the results shown in this and our previous study (275) indicate that the bovine mammary gland is responsive to an increase in IGF-I above the normal physiological concentration found in heifers fed for moderate growth. A similar effect was seen in a study using pregnant beef heifers in which the intramammary infusion of IGF-I for 10 d increased the mass of mammary parenchyma (59). In line with this local effect of IGF-I on mammogenesis, transgenic mice overexpressing mammary IGF-I showed stimulated development of mammary alveolar buds (319). Yet the fact that high energy intake in ruminants decreases mammary growth while increasing body growth suggests that factors other than IGF-I must be involved in the control of mammary epithelial cell proliferation.

Our lab has recently proposed that leptin mediates the nutritional impairment of mammary development in ruminants based on a number of lines of evidence (28;225;276;277). Serum leptin concentrations are elevated in high energy diets (28) and decreased mammogenesis in prepubertal heifers is associated with increased body fat mass (277). In a study by McFadden and Cockrell (1993), the presence of adipose tissue in mammary explant cultures inhibited proliferation of bovine mammary epithelial cells (199). The signaling form of the leptin receptor, Ob-Rb, is expressed in the epithelial cells of bovine

mammary tissue (276). Leptin inhibits proliferation *in vitro* of the bovine mammary epithelial cell line MAC-T (276). And finally, Ob-Rb is a member of the interleukin-6 (IL-6) receptor family, and may share similar signaling capabilities with the IL-6 receptor in the mammary gland (19;225). Interleukin-6 has been shown to inhibit bovine mammary epithelial cell proliferation (225).

In our study, leptin decreased the fraction of cells in the S-phase of the cell cycle to a greater extent in the IGF-I-treated quarters than in saline-treated quarters. This inhibition of cell proliferation agrees with our *in vitro* experiments in which leptin decreased DNA synthesis of MAC-T cells when they were stimulated with IGF-I or fetal bovine serum (FBS), but not when they were grown in serum free basal media (276;277). Leptin was likely able to reduce the percentage of cells in the S-phase of the cell cycle in saline-treated quarters *in vivo* by inhibiting the mitogenic effects of endogenous IGF-I or other growth factors present in the intact mammary gland. In culture, doses of leptin from 16 to 1,000 ng/ml decreased IGF-I- or FBS- stimulated DNA synthesis 10 to 30% (276).

The dose of leptin infused in this experiment was likely supraphysiological. As reported by Silva (2002), leptin concentrations in the saline and IGF-I infused quarters were 6 ng leptin/g of tissue, while leptin-infused quarters reached 170 ng/g of parenchymal tissue 14 h after the final leptin infusion (278). The mass of mammary parenchyma in a 200 kg heifer is ~100 g / quarter (unpublished data); therefore, control quarters contained ~600 ng of leptin and this was increased temporarily to 100 μg, or 1000 ng/g, in leptin-treated quarters (Silva 2002 #5810).

Thus, the half-life of infused leptin was 5 to 6 h, and the leptin concentration would have been ~50 ng/g of tissue at 24-h after infusion, which is at least 8-fold above normal during the entire 7-d treatment period (278). Mammary leptin concentrations elicited by either high energy intake or obesity are likely much lower, and whether these smaller increases in mammary leptin also would decrease proliferation of mammary epithelial cells remains to be determined.

Although a number of studies in humans and mice have described a proliferative response of mammary gland to leptin expression, the physiological state of the tissue used may be significantly different from the bovine mammary tissue in our studies. Our conclusion that leptin inhibits bovine mammogenesis is based on intramammary infusions of leptin in physiologically normal heifers, whereas the reports showing that leptin stimulates mammogenesis in rodents are based on mice with genetic deficiencies of leptin or the leptin receptor. Three studies showed an increase in cell proliferation in human tumor cells in response to leptin added in the range of 50 ng to 16 ug in two different cell lines (85;173;226), and a fourth study reported no change in proliferation when 100 ng of leptin were added to HTB26 tumor cells (224). However, in normal mammary cell lines the effect of leptin on mammary epithelial cell proliferation is conflicting. Hu and colleagues (2002) reported an increase in mammary epithelial cell proliferation at when 100 ng of leptin was added to human HBL100 cells (145), but Baratta et al, (2003) showed that mammary epithelial cell growth was inhibited by high concentrations of leptin (1.5 and 15 uM) in the mouse HC11 cell line (17). This report is consistent with our laboratory's study showing a decrease

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in bovine mammary epithelial cell proliferation in the MAC-T cell in response to 25 ng of leptin (276;277).

Another possible explanation for the apparent discrepancy of ruminant, rodent and human results is the leptin dose. It is possible that leptin exhibits a biphasic effect on mammary cell proliferation, with low doses of leptin stimulating, and high doses inhibiting mammogenesis. Such a biphasic effect of leptin has been reported previously, for granulosa cells (251) and human mammary epithelial cells (17). At such high leptin concentrations, it is possible that leptin is eliciting its action by binding to a cognate receptor within the interleukin-6 family of cytokines.

The biological actions of leptin are elicited by the binding of leptin to the leptin receptor (Ob-Rb). Ob-Rb is the long isoform form of the leptin receptor which contains a transmembrane intracellular tail that is required for endocrine function and signaling (32;34;62;100;116;175;176;208;296;297;334). Alternative splicing of the primary Ob-R transcript can result in the production of multiple isoforms (Ob-Ra to -e) that contain a common extracellular domain but lack transmembrane signaling capacity (100;175;176). Only the activation of Ob-Rb leads to activation of STAT3 (26).

The binding of leptin and IGF-I to their respective receptors induces a cascade of intracellular signaling events that regulate key cellular functions. Leptin is a member of the gp130 family of membrane bound receptors, a group that includes oncostatin M, IL-6, IL-11, LIF, and ciliary neurotrophic factor (146). These cytokines can signal through a common signaling subunit, glycoprotein

130, to produce a variety of responses, including growth arrest of mammary epithelial cells (146). Insulin-like growth factor-I is a key regulator of cellular proliferation, survival and differentiation in numerous cell types, and the activation of the IGF-I receptor (IGF-IR) is now known to generate signals within at least four distinct signal transduction pathways (124). These pathways are not necessarily specific to IGF-I, but can be activated by a number of growth factor receptors to allow for significant signaling complexity (124). Increasingly, studies are revealing that insulin and leptin signal transduction pathways show significant amounts of crosstalk across multiple tissue types (21;46-48;168;220;221). Intracellular signaling for insulin and IGF are similar, and signaling interactions between IGF-I and leptin in the developing mammary gland seem likely.

Our analysis of cell cycle regulation by leptin and IGF-I indicate that leptin may inhibit mammary epithelial cell proliferation by altering cell cycle kinetics. Immunohistochemical labeling of the proliferating cell nuclear antigen Ki-67 indicated that the number of cells committed to the cell cycle was unaffected by treatment. The percentage of Ki-67 labeled cells ranged from 8.5-11.7%, which strongly agrees with work by Capuco et al. (42) examining cycling in prepubertal mammary epithelial cells. Less than 0.25% of mammary epithelial cells across all treatments in our study were apoptotic as measured by caspase-3 labeling. This value is in line with the fraction of cells found to be apoptotic in the prepubertal mammary glands of heifers as measured by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) (212). A smaller fraction of cells in the leptin-treated quarters occupied the S-phase of the

cell cycle, indicated by the relative labeling between the percentage of cells labeled by Ki-67 and BrdU. A number of studies have shown that IGF-I stimulates cyclin-dependent kinases and cyclins during the late G1 phase to advance cells into the S-phase of the cell cycle and accelerate proliferation (115;227). In addition, although the increase in BrdU labeling by IGF-I was not significant in this study, we have previously shown that IGF-I intramammary infusion does increase the fraction of cells in the S-phase of the cell cycle in a study designed to specifically test the IGF vs. SAL comparison with higher statistical power (275). The results presented in this study extend this information and indicate that leptin may inhibit the recruitment of mammary epithelial cells into the S-phase of the cell cycle and leave them stranded in the G1-phase, thereby slowing proliferation. Such an effect of leptin on decreased cell-cycling could be mediated through the inhibition of the STAT3 and ERK signaling pathways, known to control proliferation in mammary epithelial cells (205;233;272). Although no studies have shown a direct inhibitory effect of leptin on cell cycle progression, it is possible that the interference with IGF-I-stimulated proliferation could be mediated through a member of the suppressor of cytokine signaling (SOCS) family.

Links between the interleukin-6 class I cytokine receptors, including leptin, as well as the IGF family, and the SOCS families have been well documented (19). Inhibition of the JAK/STAT signaling pathway by the SOCS proteins can arise from several distinct molecular interactions. SOCS proteins are able to bind directly to the SH2 domain of the tyrosine phosphorylated cytokine and IGF-I

receptors, or bind directly to the janus kinase (JAK) tyrosine kinases, which also interact with the IL-6 and IGF receptor families (84;96;213;218;310;329). Activation of these cytokines can lead to the activation of STAT3. Upon its phosphorylation and activation, STAT3 translocates to the nucleus and mediates gene transcription, including that of SOCS3, which is able to attenuate leptin receptor signaling in a feedback manner by interfering with further JAK2 activation (16;19;25;26;44;179;289;322;339). SOCS genes vary in expression level across tissues, particularly SOCS1 and SOCS3. This expression is often not constitutive and can be rapidly regulated by various cytokines as well as GH, prolactin and leptin (315). The promoters of many SOCS genes contain STAT-responsive DNA elements, consistent with their expression dependence on the JAK/STAT signaling pathway (213;290). However, JAK/STAT-independent regulation of SOCS expression has also been described for SOCS3 in neutrophils stimulated by IL-10 (49). The SOCS3 protein has been shown to bind to JAKs, but with lower affinity than SOCS1 and with a weaker inhibition of catalytic activity (127). SOCS3 may, however, be more effective at interacting with activated cytokine receptors. Hansen et al., (1999) showed that SOCS3 associates with the tyrosine-activated GH receptor, thereby inhibiting GH-activated STAT5 signaling. Here the mode of SOCS action is not by prevention of the docking of STAT5 to its JAK, but the result is still the inhibition of JAK2 activation (127).

In this study, it was shown that SOCS3 mRNA is upregulated 3.4-fold in quarters infused with IGF-I plus leptin relative to IGF-I alone. A leptin-induced

increase in SOCS3 protein level when the IGF-I receptor is activated, assuming protein changes mirror those of mRNA, could possibly explain the inhibition of proliferation in mammary epithelial cells (Figure 7). A SOCS-mediated feedback mechanism may exist that works to decrease mammary epithelial cell sensitivity to the IGF-I mitogenic effect and thereby inhibits proliferation during periods of high energy feeding or increased adiposity in the ruminant. Decreased signaling through the IGF-I receptor would reduce progression to the S-phase of the cell cycle and slow proliferation. Future experiments measuring SOCS3 protein levels and activation of critical components of the IGF-I and leptin signaling pathways, including STAT3, insulin receptor substrate 1 (IRS1), STAT5A and B, and JAK2 will be necessary to elucidate such a mechanism.

Microarray gene expression study

The experimental design put forth in this study is well-suited for studying the individual and counter-regulatory effects of the hormones IGF-I and leptin on mammary parenchyma gene expression. The within-animal comparisons employed by infusion of different hormonal treatments to adjacent quarters within the same animal contribute to increased power in testing the interactions of these hormones, as animal variability is removed.

Alteration of expression profiles of genes regulating proliferation and apoptosis, intracellular signaling, and cell morphogenesis and metabolism by IGF-I and leptin were of particular interest in this study given the BrdU incorporation results. Microarray results for each treatment comparison revealed

numerous candidates for mediation of the cell proliferation response by IGF-I and its abolishment by leptin. However, many of these changes in gene expression were invalidated by Q-RT-PCR. To date, real-time PCR has been performed on 21 genes of interest deemed significant by analysis with the BOTL4 and BMET microarrays (Table 8). Of these 21 genes, four were found to be in agreement with microarray results with regard to up- and down-regulation direction within the appropriate treatment contrast. An additional four genes found to be significant by microarray analysis were also found to be significant by Q-RT-PCR but were either reversed in their fold-change directionality or were significant for a different treatment contrast (Figure 6). At this time, it seems that the use of the BOTL4 and BMET microarrays were not well-suited for detecting differences in relative gene expression in the tissues of study.

The FK506 binding protein 12-rapamycin associated protein (FRAP1) gene was upregulated 56% in IGF-I treated tissues relative to saline infused control. Similarly, caspase-8 mRNA was increased by IGF-I by over 2-fold. The 14-3-3 protein eta isoform (YWHAH) gene was significantly downregulated in IGF-I treated tissues relative to saline control. Although BOTL4 microarray analysis predicted very small fold changes due to the treatments (all <1.5-fold at $P<0.05$), Q-RT-PCR showed greater relative differences for each of these genes. This fold-change magnification is likely correlated with the inherent muted dynamic range of microarrays in measuring relative expression changes.

The FRAP1 gene encodes the protein belonging to a family of phosphatidylinositol kinase-related kinases. It is also commonly referred to as

mTOR (mammalian target of rapamycin), and has numerous links to the IGF-I signaling pathways PI3K/akt, raf/ERK, and MEK/MAPK in various cell types including mammary epithelial cells (71). Studies in neuroblastoma cells have shown that maintenance of IGF-I-induced proliferation requires mTOR function along the MAPK pathway (204). The well established role for FRAP1 in IGF-I signaling supports a possible role in regulation of mammary epithelial cell proliferation as observed in this infusion study.

The 14-3-3 genes code for a family of proteins that have previously been shown to bind to and regulate other proteins, including the IGF-IR, to modulate neurodevelopment, cell-division, signal transduction and gene transcription (Wong, Likhodi, et al. 2005 #5720). These proteins are phosphoserine (pSer)-binding proteins that have been shown to interact directly with oncogenic products and components of mitogenic and apoptotic signaling pathways (114). The 14-3-3 eta isoform has most commonly been linked with brain functions, and one study showed that it was downregulated by IGF-I in Purkinje cells (335). The downregulation of 14-3-3 eta observed in this experiment may contribute to the increased mammary epithelial cell proliferation observed by interacting with cyclins and cyclin dependent kinases to modify cell cycling.

Caspase-8 is known to be involved with antiproliferative and apoptotic signaling in mammary epithelial cells (252). However, although a significant 2-fold increase in caspase-8 mRNA expression was observed in IGF-I tissues relative to control, no only minor differences in apoptosis were observed in our tissues based on caspase-3 immunostaining (Figure 4). It seems unlikely that

caspase-8 regulation provides a proliferative stimulus in the mammary gland, and this change in gene expression can not be explained at the moment.

Quantitative-RT-PCR on microarray genes of interest revealed significant differences in expression of mitogen activated protein kinase kinase kinase 1 (MAP3K1), brain derived neurotrophic factor (BDNF), NF-kappa B p65 (RELA), and phosphatidylinositol glycan, class F (PIGF) in this mammary gland infusion model. Each of these genes was significantly altered in at least one treatment contrast not predicted by either BOTL4 or BMET microarray analysis.

MAP3K1 is a mitogen activated protein kinase (MAPK) that is activated in normal mouse mammary and human breast cancer cells in response to prolactin (73). Our results seem to follow with this study and the upregulation of MAP3K1 in IGF-I quarters relative to control could indicate amplified signal transduction through the MAPK pathway to enhance mitogenesis in mammary epithelial cells.

BDNF is a member of the nerve growth factor family and is necessary for survival of neurons in the brain. This growth factor has been shown to interact with IGF-I in the brain to mediate neuronal plasticity by amplifying IGF-I signaling through the extracellular signal-regulated kinase (ERK) pathway (152). Although BDNF mRNA has not been previously found in the mammary tissue, its upregulation in IGF-I treated quarters relative to control could indicate some mitogenic function for this growth factor in the gland.

NF-κB activity in normal mammogenesis in humans and mice has been previously reported in numerous studies (36;55;160). These studies implicate this transcription factor in a role in cell survival, protecting cells that have

received an apoptotic stimulus. Immunological functions of high NF-κB levels have been reported in bovine mammary glands infected with mastitis (35). A study by Lappas et al., (2005) showed that the NF-κB transcription pathway is important to regulation of IL-6 in adipose tissue (170). The upregulation of NF-κB p65 mRNA in IGF-I plus leptin treated quarters relative to IGF-I could possibly indicate a role for this gene in the negative regulation of leptin secretion.

PIGF is a protein involved in glycosylphosphatidylinositol (GPI)-anchor biosynthesis and modification (273). Blood cells use the GPI anchor to attach proteins to the cell surface. Currently, no literature exists linking PIGF to either IGF-I, leptin or the mammary gland, and the biological relevance of changes in PIGF gene expression in our study cannot be explained at this time.

The inconsistency between microarray and Q-RT-PCR results revealed in this study are somewhat puzzling. Two major reasons behind the weak correlation between the two techniques are apparent. First of all, the experimental design used in this study to measure changes in gene expression in response to the hormones IGF-I and leptin could also be improved for the application of a functional genomics approach. Samples were harvested at day 7 of this infusion trial; this time point may be unsuitable for functional genomics analysis, as mRNA expression changes could have occurred early in response to the hormonal infusions and reached a lower, steady state by day 7. Tissue harvested at different time points, especially interspersed within the first 24 hr of infusion may likely be better suited for mRNA analysis.

Secondly, it is possible that the changes in gene expression within the mammary parenchyma in our study are quite small and difficult to detect using the microarrays because of the inherent limitations of sensitivity of the platform. Prepubertal bovine mammary parenchymal tissue consists of approximately 10-20% epithelial cells, 40-50% stromal tissue and 30-40% fat cells at the initiation of puberty (265;266). Although approximately 30% of the cells in our parenchymal tissue appear to be epithelial (unpublished observations), Ki-67-labeling shows that only 8-12% of these epithelial cells are proliferating. It is therefore possible that the gene expression profile of our target cell group is highly diluted by stromal and fat cell mRNA. Following this logic, the large fold change revealed for SOCS3 may indicate that it is regulated in stromal cells rather than epithelial cells. However, the fact that a 3.4-fold change was detected for SOCS3 gene mRNA that was undetected by microarray analysis is puzzling. Microarray sensitivity or analysis must surely be improved here to reveal such obvious differences in expression. The use of false discovery rate (FDR) analysis may help to explain the failure of validation of microarray results. Although FDR analysis was not applied to the BOTL4 microarrays, it was used to determine the probability of validation for genes measured in the BMET microarray. In the IGF-I vs. IGF+LEP treatment comparison only 15 genes were found to have an FDR value below P=0.3. This indicates a 30% probability of validation for each of these genes. Expression of the most highly ranked gene (MYC) and 15th gene (IFNA2) were measured by Q-RT-PCR and validation failed. Further Q-RT-PCR analysis of the remaining 13 genes could lead to validation, though all predicted

fold-changes for these genes were less than 16% up- or down-regulated. Similarly, only three genes in the control versus IGF-I microarray comparison had FDR values below P=0.41.

The problem of false-negative generation by microarray analysis was also revealed in this study. Based on a comparison of the two microarray platforms used with Q-RT-PCR, three genes were found to be false negatives in BMET microarray analysis. Though the BMET microarrays predicted a non-significant increase in SOCS3 mRNA of 13% in IGF+LEP relative to IGF (P=0.21), this gene was found to be significantly upregulated by Q-RT-PCR measurements. FRAP1 was found to be significantly upregulated by IGF relative to SAL for both the BOTL4 microarray and Q-RT-PCR, but was insignificant in BMET analysis. Similarly, YWHAH was also not detected as differentially expressed in the SAL vs. IGF comparison by the BMET array (0.98 fold, p=0.83), but a significant BOTL4 microarray change was confirmed by Q-RT-PCR. Therefore, the reasons behind the inversion of fold change by real-time PCR relative to microarray predicted changes, differences in significant contrasts, and false negative prediction are hereto unclear and will require further analysis of the microarray platforms used in this study.

In conclusion, increased concentrations of IGF-I promote the proliferation of mammary epithelial cells by increasing the progression of cells into the S-phase of the cell cycle *in vivo* in mammary epithelial cells in prepubertal heifers. Conversely, increasing the leptin concentration above normal reduces proliferation by limiting the progression of cells into the S-phase of the cell cycle

and stalls them within the G1 phase of the cell cycle. These effects on cell proliferation are likely regulated by changes in gene expression induced by IGF-I and leptin within the mammary parenchymal tissue. The SOCS3 could potentially be a primary leptin-induced inhibitor of IGF-I signaling and cell proliferation to mediate this effect. Overall, this study has demonstrated that IGF-I and leptin intramammary infusions in prepubertal heifers can serve as a useful model for the study of hormonal regulation of cell cycle progression and gene transcription in the developing gland.

FIGURES

A)

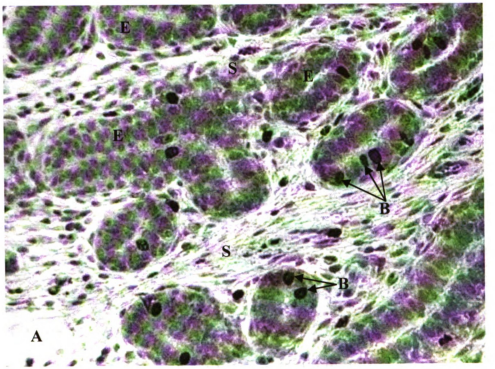


Figure 1A. BrdU labeling.

B)

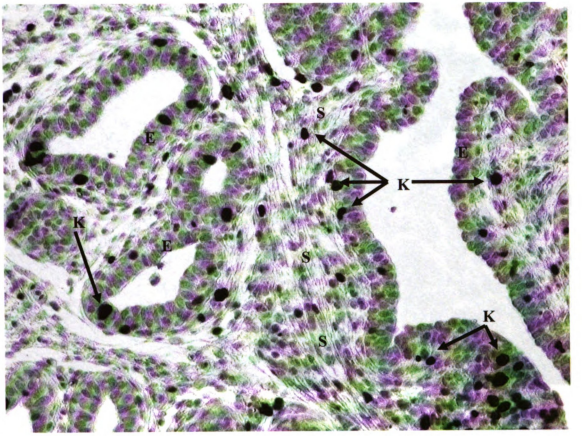


Figure 1B. Ki-67 labeling.

C)

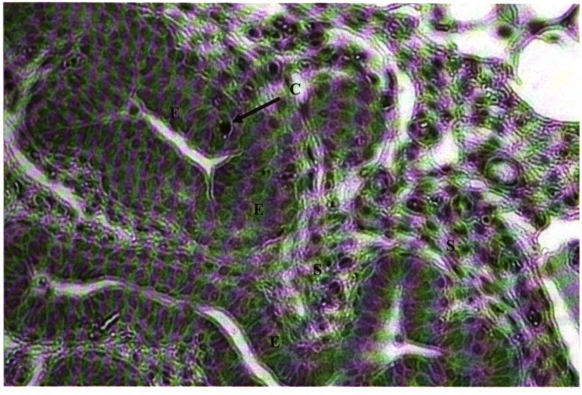


Figure 1C. Caspase-3 labeling.

Figure 1. Representative mammary parenchymal sections immunostained for bromodeoxyuridine (BrdU), proliferating cell nuclear antigen (Ki-67) and caspase-3.
Panel A) B = BrdU-positive cells (darker), E=epithelial cells, S=stromal cells, and A=adipocytes. BrdU-labeling was calculated by dividing the number of BrdU-positive epithelial cells by the total number of epithelial cells (278).
Panel B) K = Ki-67-positive cells (darker), E = epithelial cells, S = stromal cells, and A = adipocytes. Ki-67-labeling percentage equals the number of Ki-67-positive epithelial cells divided by the total number of epithelial cells.
Panel C) C = Caspase-3-positive cells (darker), E = epithelial cells, S = stromal cells, and A = adipocytes. Caspase-3-labeling percentage equals the number of caspase-3-positive epithelial cells divided by the total number of epithelial cells.

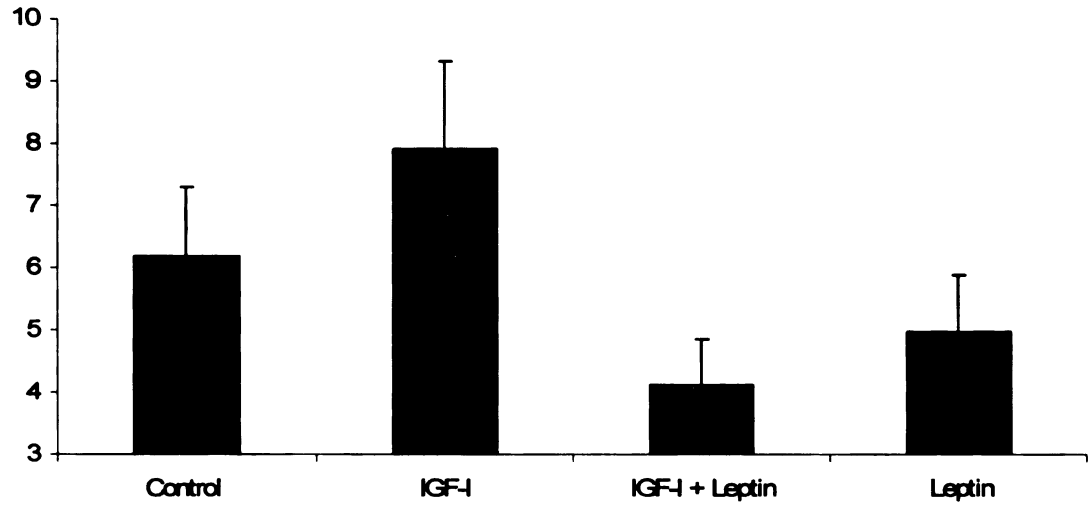


Figure 2. Effects of intramammary infusion of ovine leptin and IGF-I on bromodeoxyuridine- (BrdU) labeling of mammary epithelial cells in prepubertal heifers. Individual mammary glands of six heifers were infused for 7 d with either saline or IGF-I (10 μg/d). Contralateral quarters were infused with either saline+leptin (100 μg leptin/d) or IGF-I + leptin (100 μg leptin/d). BrdU was infused intravenously about 2 to 3 h before slaughter. Leptin decreased ($P<0.02$) mammary epithelial cell proliferation in both saline- and IGF-I-treated quarters. Results are back-transformed. Estimates of variance were calculated as confidence intervals from the transformed data for comparisons of leptin effect within IGF-I treatments (Data taken from Silva (278)).

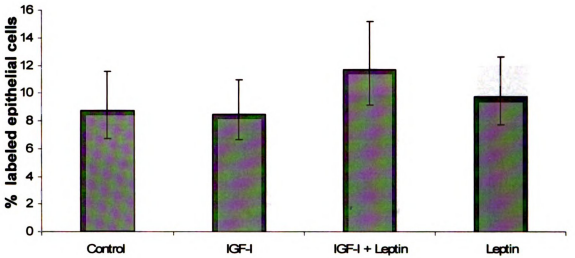


Figure 3. Effects of intramammary infusion of ovine leptin and IGF-I on proliferating cell nuclear antigen (Ki-67) labeling in prepubertal heifers. The number of cells committed to the cell cycle was measured by the percentage of proliferating cell nuclear antigen (Ki-67) labeling. No significant differences in the number of cycling cells were found among the four treatments.

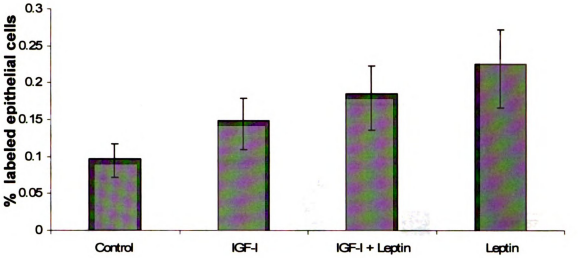


Figure 4. Caspase-3 immunostaining indicates apoptosis in the mammary gland. Immunohistochemical staining of caspase-3 was performed on sections taken from all treated quarters. Two contrasts were significantly different ($P<0.05$): LEP vs. SAL, IGF vs. LEP and one contrast showed a tendency toward a difference ($P<0.07$): IGF-LEP vs. SAL. Although statistical differences exist, there is very little evidence of apoptosis plays a large role in shaping the mammary gland, as numerically all caspase-3 percentages across treatments were very small (<0.25%).

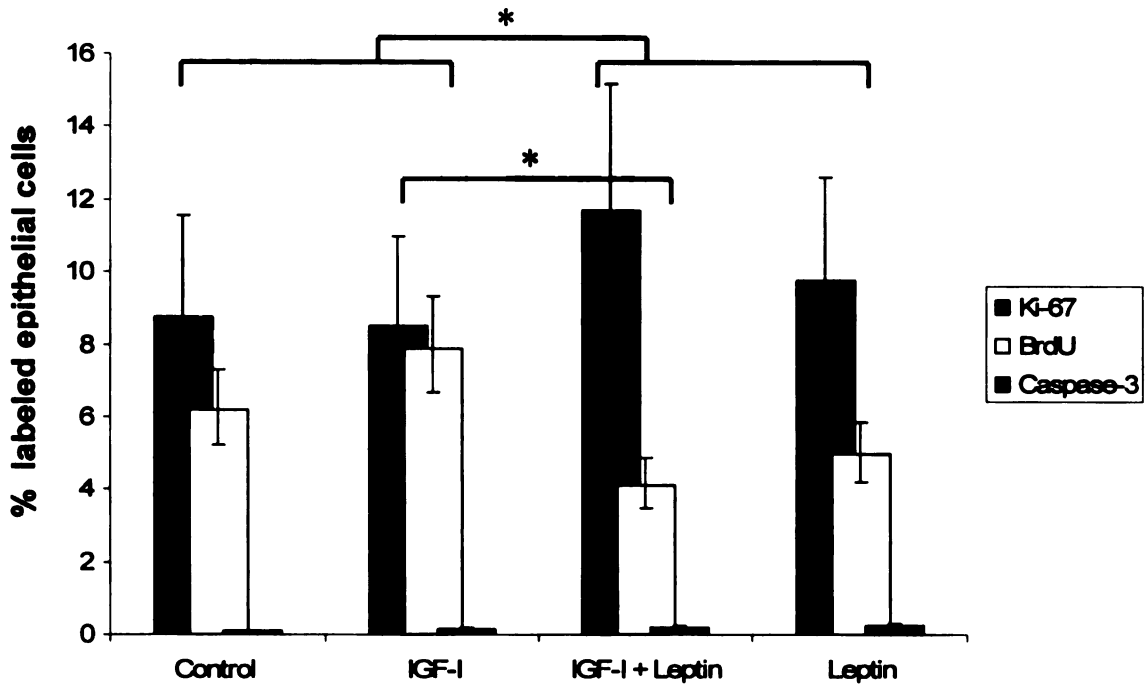
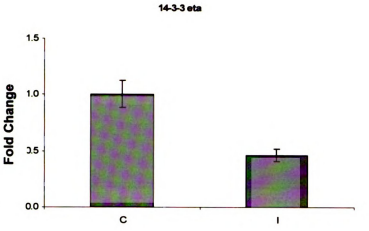
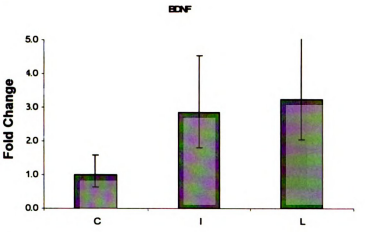


Figure 5. Effects of ovine leptin and IGF-I on the percentage of cells committed to the S-phase of the cell cycle. Leptin treatment significantly reduced the number of proliferating cells occupying the S-phase of the cell cycle. The fraction of proliferating cells within the S-phase of the cell cycle was determined by the dividing the percentage of BrdU-labeled cells by the percentage of Ki-67-labeled cells. The main effect of leptin ((LEP + IGF+LEP) – (SAL + IGF)) (P=0.015) and the IGF versus IGF+LEP contrast (P=0.02) were both significant.

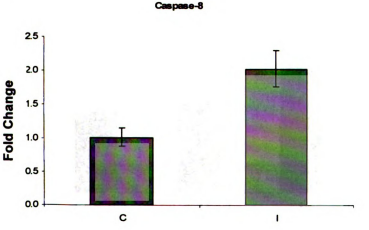
A)


14-3-3 eta
Fold Change
1.5
1.0
3.5
0.0
C
I

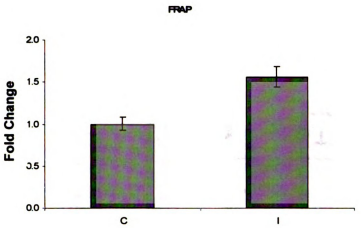
B)


BDNF
Fold Change
5.0
4.0
3.0
2.0
1.0
0.0
C
I
L

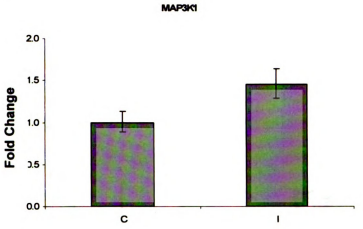
C)


Caspase-8
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
C
I

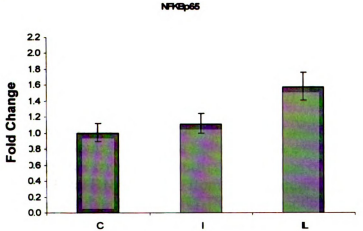
D)


FRAP
Fold Change
2.0
1.5
1.0
0.5
0.0
C
I

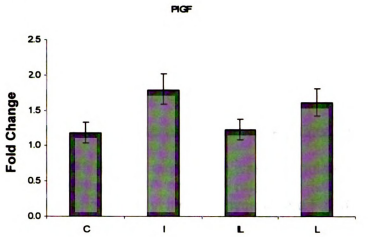
E)


MAP3K1
Fold Change
2.0
1.5
1.0
0.5
0.0
C
I

F)


NFKBp65
Fold Change
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C
I
L

G)



H)

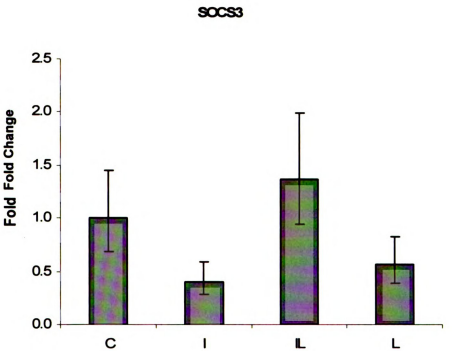


Figure 6. Genes found to be significantly altered by treatment in mammary tissue from six prepubertal bovine heifers. Data were derived from Q-RT-PCR assay of RNA from six cows and expressed as ratios of expression relative to SAL infused control. Total RNA isolated from treated quarters was converted to first-strand cDNA and subjected to Q-RT-PCR as described previously and in Materials and Methods. Data presented are the mean +/- standard error of the mean relative expression levels of each gene within group, calculated as $2^{-\Delta\Delta Ct}$, as described, with GAPDH as a control gene and the mean ΔCt value for each gene in the control samples as the calibrator.

A) IGF-I treatment significantly decreased the expression of 14-3-3 protein, eta isoform, 55% relative to SAL control.
B) Brain derived neurotophic factor (BDNF) was significantly upregulated 2.86-fold by in IGF-I treated quarters and 3.25-fold by leptin relative to SAL control.
C) Caspase-8 mRNA was significantly upregulated 2-fold in IGF relative to SAL quarters.
D) IGF-I treatment significantly increased the expression of FRAP1 56% relative to SAL control.
E) MAP3K1 mRNA expression was upregulated 46% by IGF-I treatment relative to SAL.
F) NF-kB p65 (RELA) expression was upregulated 34% in IGF-LEP treated quarters relative to IGF, and 47% relative to SAL.
G) For the phosphatidyl inositol glycan, type F (PIGF) gene IGF quarters were significantly different than both SAL (52%) and IGF-LEP (46%). LEP tended to be different from SAL ($P<0.08$) (37%). Heifer 88 was an outlier for this gene and was deleted from analysis.
H) Suppressor of cytokine signaling-3 (SOCS3) mRNA was significantly upregulated 3.4-fold in IGF-LEP quarters relative to IGF.

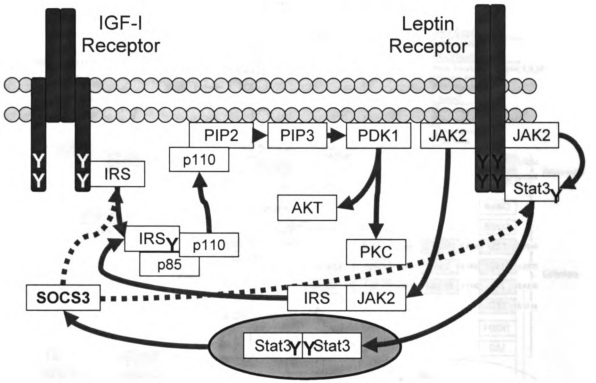


Figure 7. A schematic of IGF-I and leptin intracellular signal transduction. Binding of IGF-I to its receptor induces intrinsic tyrosine kinase activation and receptor autophosphorylation. The insulin receptor substrate (IRS) molecules are then recruited to the receptor, are activated by tyrosine phosphorylation, and are then able to activate downstream targets such as the regulatory subunit (p85) of phosphatidylinositol 3-kinase (PI3K), which also contains a catalytic (p110) subunit. Phosphatidylinositol triphosphate is activated by the p110 subunit of PI3K to signal downstream to the phosphor-inositide dependent kinase (PDK1), protein kinase B (PKB/Akt) and protein kinase C (PKC).
Leptin binding to its receptor induces activation of the janus kinase (JAK) proteins, dimerization of its receptor, and JAK-mediated phosphorylation of the intracellular portion of the leptin receptor. JAK2 activation leads to recruitment of the signal transducers and activators of transcription-3 (STAT3) protein, which binds to tyrosine phosphorylated SH2 proteins on the receptor and is activated by phosphorylation. Phosphorylated STAT3 translocates to the nucleus and induces the transcription of target genes such as the suppressor of cytokine signaling-3 (SOCS3) protein. SOCS3 has the ability to interfere with signaling along both of these pathways. It binds directly to the SH2 docking sites on the IGF-I receptor (83) as well as IRS substrates (86). SOCS3 can directly mediate feedback inhibition of the leptin receptor by preventing the docking of STAT3 (25) and STAT5 genes (135;302), or by directly interfering with JAK2 activation (330). Thus, I hypothesize that the effects of IGF-I and leptin in the prepubertal mammary gland may result from crosstalk mediated by this common signaling pathway and SOCS3 (Adapted from Niswender and Schwartz (221)).

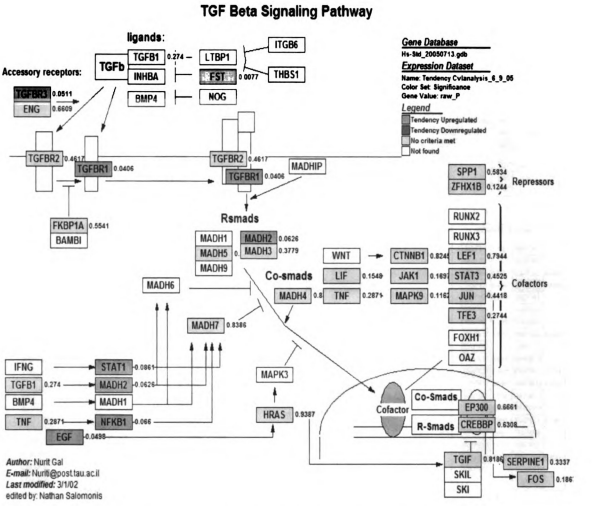


Figure 8. Genes differentially expressed in the SAL vs. IGF treatment comparison are significantly overrepresented in the TGF-beta signaling pathway. GenMAPP analysis indicated that seven genes significant at $P<0.1$ were altered in the SAL vs. IGF comparison within the TGF-beta signaling pathway. Gene significance is indicated by the number next to the gene on the pathway map. Unaffected genes are indicated in light gray and genes absent from the BMET microarray are indicated by white boxes.

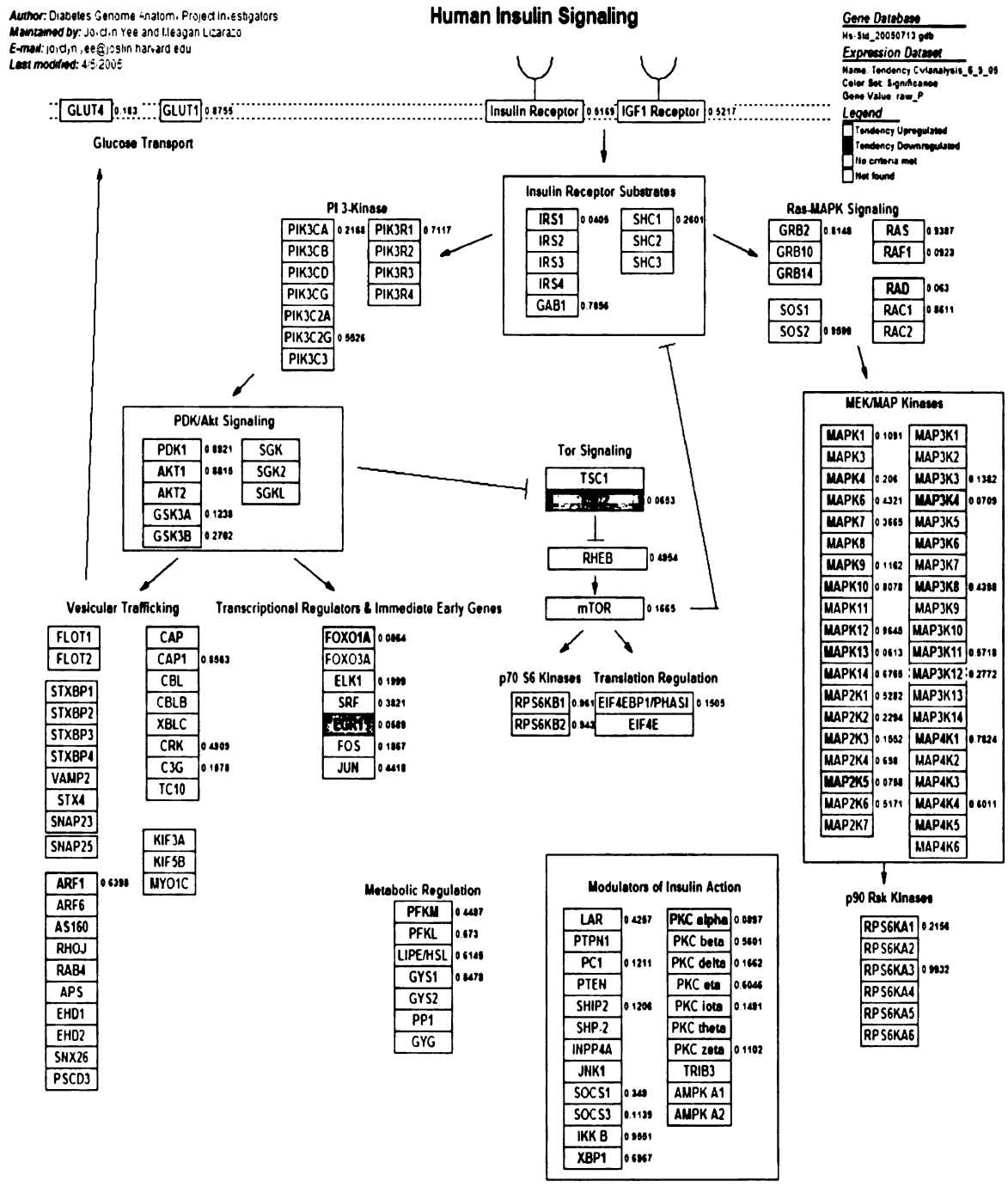


Figure 9: Genes differentially expressed in the SAL vs. IGF treatment comparison are significantly overrepresented in the insulin signaling pathway. Nine genes within the insulin signaling pathway were different in this treatment comparison at $P<0.1$ according to GenMAPP analysis. Upregulated genes are indicated in red, and downregulated genes are indicated in blue. Unaffected genes are light gray on this map. White boxes indicate genes not present on the BMET microarray.

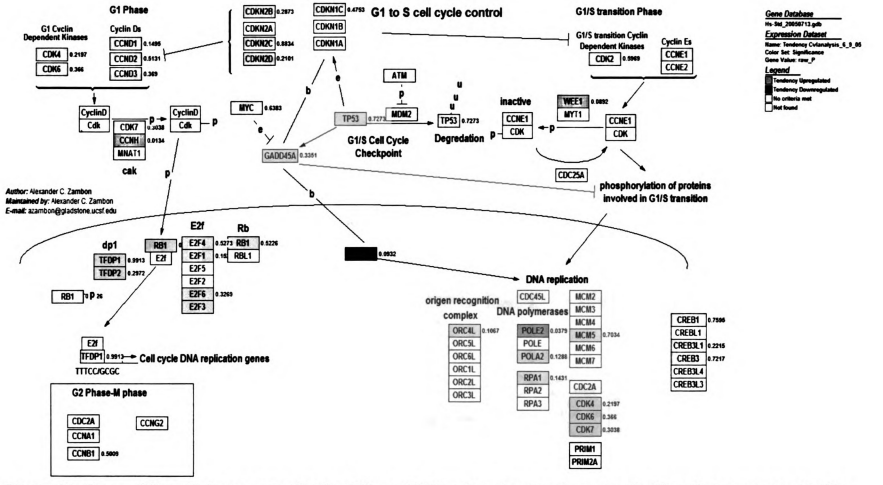


Figure 10. Genes differentially expressed in the SAL vs. IGF treatment comparison are significantly overrepresented in the G1 to S Cell Cycle Control Pathway. Seven genes involved with the G1 to S transition in the cell cycle were affected by IGF-I compared to SAL ($P<0.1$) by GenMAPP analysis. Blue indicates downregulation in IGF compared to SAL, and red indicates upregulation. Light gray squares are genes unaffected by treatment, and white squares indicate genes not present on the BMET microarray.

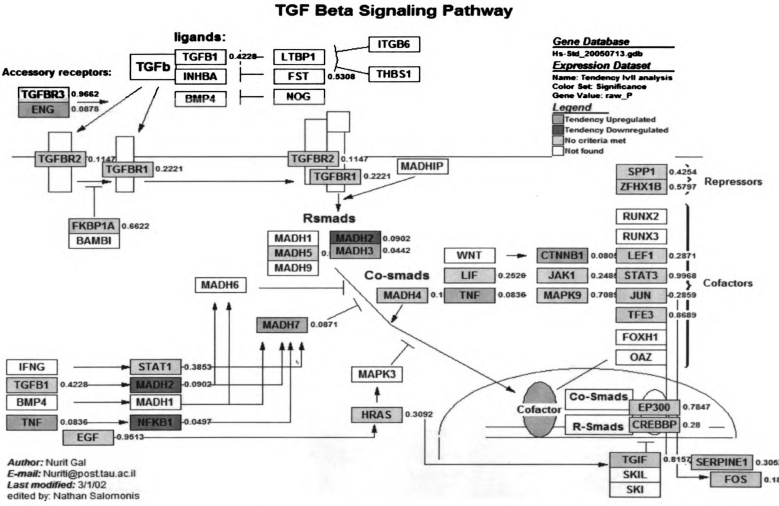


Figure 11. Genes differentially expressed in the IGF vs. IGF-LEP treatment comparison are significantly overrepresented in the TGF-beta signaling pathway. GenMAPP analysis reveals that the members of the TGF-beta signaling pathway are overrepresented in the genes affected in the IGF vs. IGF-LEP treatment comparison (P<0.1). Blue indicates

downregulation in IGF-LEP compared to IGF, and red indicates upregulation. Light gray squares are genes unaffected by treatment, and white squares indicate genes not present on the BMET microarray.

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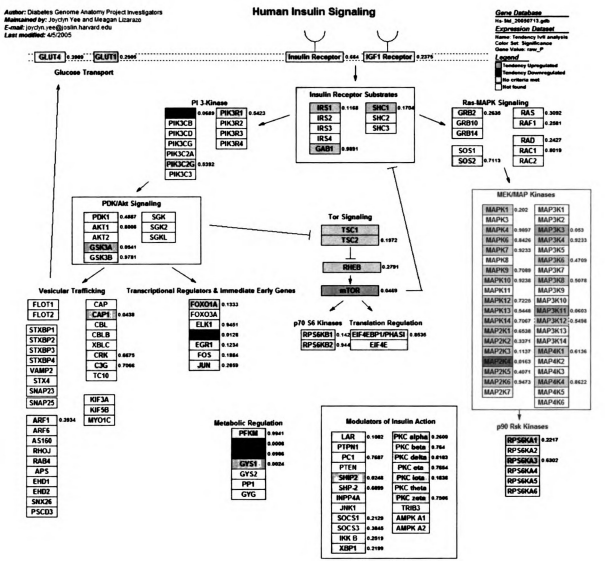
Author: Diabetes Genome Anatomy Project Investigators
Maintained by: Joychi Yee and Meagan Lizarazo
E-mail: joychi.yee@stjohn.harvard.edu
Last modified: 4/5/2005
Human Insulin Signaling
Gene Database
Expression Dataset
Legend
Tendency Upregulated
Tendency Downregulated
No criteria met
Not found
GLUT4 0.2000
GLUT5 0.2000
Glucose Transport
Insulin Receptor 0.584
IGF1 Receptor 0.2274
Insulin Receptor Substrates
IRS1 0.1168
IRS2
IRS3
IRS4
GAB1 0.0991
SHC1 0.1704
SHC2
SHC3
PI 3-Kinase
0.0681
PIK3CB
PIK3CD
PIK3CG
PIK3C3A
PIK3C2G 0.5392
PIK3C3
PIK3R1 0.5423
PIK3R2
PIK3R3
PIK3R4
Ras-MAPK Signaling
GRB2 0.2638
GRB10
GRB14
SOS1
SOS2 0.7113
RAS 0.3092
RAF1 0.2581
RAD 0.2427
RAC1 0.8019
RAC2
PDK/Akt Signaling
PDK1 0.4867
AKT1 0.8066
AKT2
GSK3A 0.0641
GSK3B 0.0781
SGK
SGK2
SGKL
Tor Signaling
TSC1
TSC2 0.1972
RHEB 0.2791
mTOR 0.5469
MEK/MAP Kinases
MAPK1 0.202
MAPK3
MAPK4 0.9697
MAPK6 0.8426
MAPK7 0.9233
MAPK8
MAPK9 0.7089
MAPK10 0.9238
MAPK11
MAPK12 0.7225
MAPK13 0.8446
MAPK14 0.7067
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MAP2K4
MAP2K5 0.6871
MAP2K6 0.8473
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MAP3K2
MAP3K3 0.003
MAP3K4 0.9233
MAP3K5
MAP3K6 0.4709
MAP3K7
MAP3K8 0.5078
MAP3K9
MAP3K10
MAP3K11 0.0603
MAP3K12 0.6456
MAP3K13
MAP3K14
MAP4K1 0.6136
MAP4K2
MAP4K3
MAP4K4 0.8622
MAP4K5
MAP4K6
Vesicular Trafficking
FLOT1
FLOT2
STXBP1
STXBP2
STXBP3
STXBP4
VAMP2
STX4
SNAP23
SNAP25
ARF1 0.3934
ARF6
AS160
RHOJ
RAB4
AP5
EHD1
EHD2
SNX26
PSCD3
Transcriptional Regulators & Immediate Early Genes
CAP
CAP1 0.6438
CBL
CBLB
XBLC
CRK 0.6875
CRKL 0.7066
TC10
KIF3A
KIF5B
MYO1C
FOXO1A 0.1533
FOXO3A
ELK1 0.9451
0.9129
EGR1 0.1234
FOS 0.1884
JUN 0.2600
p70 S6 Kinases
Translation Regulation
RPS6KB1 0.142
RPS6KB2 0.544
EIF4EBP1/PHAS I 0.6836
EIF4E
p90 Rsk Kinases
RPS6KA1 0.2217
RPS6KA2
RPS6KA3 0.6302
RPS6KA4
RPS6KA5
RPS6KA6
Metabolic Regulation
PFKM 0.9941
0.8006
0.9906
GYS1 0.9824
GYS2
PP1
GYG
Modulators of Insulin Action
LAR 0.1062
PTPN1
PC1 0.7687
PTEN
SHIP2 0.0346
SHP-2 0.6808
INPP4A
JNK1
SOCS1 0.2129
SOCS3 0.3645
IKK B 0.2019
XBP1 0.2199
PKC alpha 0.2649
PKC beta 0.764
PKC delta 0.6183
PKC eta 0.7884
PKC iota 0.1838
PKC theta
PKC zeta 0.7586
TRB3
AMPK A1
AMPK A2

Figure 12. Genes differentially expressed in the IGF vs. IGF-LEP treatment comparison are significantly overrepresented within the insulin signaling pathway. GenMAPP analysis indicates a number of genes falling within the insulin signaling pathway are significantly altered in this treatment comparison ($P<0.1$). Blue indicates downregulation in IGF-LEP compared to IGF, and red indicates upregulation. Light gray squares are genes unaffected by treatment, and white squares indicate genes not present on the BMET microarray.

TABLES

Table 1. Ki-67 and BrdU labeling comparison

Ki-67 Labeling

Treatment	Labeling %	S.E.	Pr > |t|
SAL	8.7336	2.9049	*
IGF	8.4869	2.9049	*
IGF+LEP	11.6778	2.9049	*
LEP	9.7680	2.9049	*

BrdU Labeling (From Silva, 2002)

Treatment	Labeling %	S.E.	Pr > |t|
SAL	6.8915	0.9737	*
IGF	9.1526	0.9737	*
IGF+LEP	4.9540	0.9737	*
LEP	5.3680	0.9737	*

Ki-67 and BrdU Difference

Treatment	Difference	S.E.	Pr > |t|
SAL	1.8421	2.7042	0.51
IGF	-0.6658	2.7042	0.81
IGF+LEP	6.7238	2.7042	**
LEP	4.3999	2.7042	0.12

* = Treatment labeling percentage is different than 0 ($P<0.01$)

** = Difference between Ki-67 and BrdU is significant ($P<0.05$)

Table 2:

Genes altered by IGF-I versus SAL treatment detected by BOTL4 microarray analysis

Upregulated genes (IGF-I relative to control)

Gene name	Fold Change	*P*<	Function
			Receptor-Mediated Signal Transduction Genes
p21-activated protein kinase I (PakI)	1.12	0.001	Serine/Threonine Protein Kinase
Focal adhesion kinase 2 (FAK2)	1.27	0.01	Protein tyrosine kinase activity, MAPK pathway activation
SMAD 7 MAD-related gene	1.22	0.01	Transcription Regulation, TGF-β Signal Transduction
Dual Specificity Phosphatase 4 (DUSP4)	1.29	0.01	MAPK/JNK Pathway Negative Regulator
Leptin receptor (OBR)	1.29[Ø]	0.01	GP130 Receptor Family Signal Transduction
Chemokine (C-C motif) receptor 7 (CCR7)	1.19	0.01	G-protein Coupled Receptor Protein Signaling Pathway
FKBP-rapamycin associated protein (FRAP1)	1.34*	0.02	PI3K Signal Transduction
MAIL	1.15	0.03	Toll/Interleukin-1 Signal Transduction
Copine III (CPNE3)	1.11	0.03	Protein Serine/Threonine Kinase Activity
Interleukin-1 (IL-1)	1.17	0.04	Toll-like Receptor Signaling
CC-chemokine receptor (CCR-5) gene delta-32	1.16	0.04	G-protein Coupled Receptor Protein Signaling Pathway
Serine/Threonine Kinase 17b (DRAK2)	1.15	0.04	Induction of Apoptosis
G protein alpha inhibiting activity polypeptide (GNAI2)	1.13	0.04	G-protein Coupled Receptor Protein Signaling Pathway
Interleukin-8 Receptor Type B (IL8RB)	1.16[Ø]	0.05	G-protein Signaling, Cell Proliferation
			Cell Proliferation/Apoptosis Genes
Ki-67 (MKI67)	1.16	0.02	Regulation of Cell Cycle
Breast Cancer Anti-estrogen Resistance 1 (BCAR1)	1.16[Ø]	0.04	Cell Proliferation Signal Transduction
BTG family, member 3 (BTG3)	1.11	0.04	Negative Regulation of Cell Proliferation
			Transcriptional Regulation Genes
Transducin-like enhancer 3 (Esp3)	1.23	0.001	Transcription Regulation
NF-κB transcription factor p65 subunit (RELA)	1.1[Ø]	0.005	Transcription Factor Anti-apoptotic
Apoptotic cysteine protease Mch5 (Caspase 8)	1.26*	0.01	Apoptosis Regulation
Upstream stimulatory factor 1b, 1a	1.18	0.01	Transcription Regulation

			Cell Structure and Metabolism Genes
Pyruvate dehydrogenase kinase isoenzyme 3 (PDK3)	1.15	0.01	Glucose Metabolism
Stearoyl-CoA desaturase	1.26	0.02	Lipid Transport and Metabolism
Matrix Metalloproteinase 3 (MMP3)	1.19	0.03	Collagen Catabolism
MMP2	1.16	0.03	Collagen Catabolism
Drosophila lipid desaturase (DEGS)	1.14	0.04	Lipid Transport and Metabolism
MMP7	1.14	0.05	Collagen Catabolism

Downregulated genes (IGF-I relative to control)

Gene name	Fold Change	*P*<	Function
			Receptor-Mediated Signal Transduction Genes
Urokinase-type plasminogen activator receptor	0.84	0.01	Cell Surface Receptor Signal Transduction
14-3-3 protein eta chain (YWHAH)	0.87*	0.03	Phosphoserine Binding Signal Transduction
GSK-3β	0.88	0.04	Cell Proliferation Signal Transduction
Adenylate kinase isozyme 3 (AK3)	0.87	0.03	Adenylate Kinase Activity
Mitogen-activating protein 3K1 (MAP3K1)	0.90[Ø]	0.05	Protein Serine/threonine Kinase Activity
			Cell Proliferation and Apoptosis Genes
Cyclin-dependent kinase inhibitor 1A (CDKN1A)	0.84	0.02	Intracellular Signaling for Cell Cycle Arrest
Kinase suppressor of ras-1 (KSR1)	0.85	0.04	Intracellular Signaling Cascade
Thyroid hormone receptor, alpha	0.85	0.05	Cell Cycle Regulation
			Transcriptional Regulation Genes
Sin3-associated polypeptide, 18 (SAP18)	0.86[Ø]	0.001	Transcription Repression
Interferon-gamma receptor mRNA (IFN-gamma-R1)	0.81	0.02	Regulation of Cell Growth, Transcription
			Cell Structure and Metabolism Genes
Endoglin (endoglin)	0.78	0.05	Cell Adhesion
Decorin (DCN)	0.85	0.05	Organogenesis

* Validated by Q-RT-PCR (Figure 6)
[Ø] Invalidated by Q-RT-PCR (Table 8)

Table 3:

Genes altered by IGF-LEP versus IGF-I treatment detected by BOTL4 microarray analysis

Upregulated Genes (IGF-I + leptin relative to IGF-I)

Gene name	Fold Change	P<	Function
			Receptor-Mediated Signal Transduction Genes
Transducin-like enhancer protein (TLE1)	1.26[Ø]	0.001	GP130 Receptor Family Receptor Signal Transduction
CD8 antigen alpha polypeptide (p32) (CD8A)	1.19	0.01	Protein Tyrosine Kinase Signaling Pathway
Interleukin 2 receptor beta chain (p70-75) mRNA (IL-2R beta)	1.26	0.02	Cytokine Signal Transduction
Janus kinase 1 (JAK1)	1.18	0.04	Protein Tyrosine Kinase, Oncostatin M Regulation
			Cell Proliferation and Apoptosis Genes
Heparin-binding vascular endothelial growth factor (VEGF)	1.23	0.01	Positive Regulation of Cell Proliferation
CD30 ligand mRNA complete cds (CD30L)	1.19	0.03	Apoptosis Induction
Defender Against Death (DAD-1)	1.17	0.03	Anti-apoptosis

Downregulated genes (IGF-I + leptin relative to IGF-I)

Gene name	Fold Change	P<	Function
			Receptor-Mediated Signal Transduction Genes
Seven transmembrane domain orphan receptor (TPRA40)	0.85	0.01	G-protein Coupled Receptor, Lipid Metabolism
Brain-Derived Neurotrophic Factor (BDNF)	0.75	0.02	Growth Factor
Insulin-like growth factor II (IGF-II)	0.78[Ø]	0.02	Cell Proliferation
Insulin-like 3 (INSL3)	0.83	0.02	Growth Factor
Leptin receptor	0.79[Ø]	0.02	GP130 Receptor Family Receptor Signal Transduction
cAMP specific phosphodiesterase 7B (PDE7B gene)	0.89	0.02	cAMP-Specific Phosphodiesterase Activity
MAIL	0.86	0.02	Toll/Interleukin-1 Signal Transduction
Interleukin-1 (IL-1)	0.86	0.03	Toll-like Receptor Signaling
Wee1 mitotic inhibitor kinase	0.84	0.04	Protein Serine/threonine Kinase Activity
Interleukin-8 Receptor Type B (IL8RB)	0.85[Ø]	0.04	Cytokine Signal Transduction
JNK3 alpha2 protein kinase (MAPK10)	0.87	0.04	JNK Cascade
Small inducible cytokine A1 (CCL1)	0.83	0.05	Cell Signal Transduction

Casein kinase 1 alpha1 (CSNK1A)	0.84	0.04	Protein Kinase Activity
			Cell Proliferation and Apoptosis Genes
Death Associated Protein (DAP)	0.83	0.001	Apoptosis Induction
Apoptotic Cysteine Protease Mch5 isoform alpha (Caspase 8)	0.81[ø]	0.01	Apoptosis Regulation
Serine protease-like protein (Granzyme-B)	0.83	0.03	Apoptosis Induction
Inhibin A subunit	0.85	0.03	Cell Cycle Arrest
Nuclear distribution protein (NUDC)	0.87	0.03	Cell Proliferation
Candidate tumor suppressor protein DICE1	0.87	0.04	Cell Growth and Division Regulation
			Cell Structure and Metabolism
Tissue Inhibitor of Metalloproteinase 4 (TIMP4)	0.90	0.04	Metalloendopeptidase Inhibitor
Cyclophilin-40 (PPID)	0.88	0.05	Protein Folding

[ø] Invalidated by Q-RT-PCR (Table 8)

Table 4:

Genes altered by LEP versus SAL treatment detected by BOTL4 microarray analysis

Upregulated Genes (Leptin relative to control)

Gene name	Fold Change	P<	Function
			Receptor-Mediated Signal Transduction Genes
Transducin-like enhancer protein (TLE1)	1.19[ø]	0.01	GP130 Receptor Family Receptor Signal Transduction
MALT1	1.16	0.01	Activation of NF-κB-inducing Kinase
Copine III (CPNE3)	1.14	0.01	Protein Serine/Threonine Kinase Activity
Oncostatin-M specific receptor beta subunit (OSMRB)	1.25	0.02	GP130 Receptor Linked Signal Transduction
Janus kinase 1 (JAK1)	1.20	0.03	Intracellular Signaling Cascade
Ran GTPase activating protein 1 (RANGAP1)	1.13	0.03	Ran GTPase Signal Transduction
Chemokine (C-C motif) receptor 7 (CCR7)	1.14	0.04	G-protein Coupled Receptor Protein Signaling Pathway
Chemokine Ligand 20 (MIP-3a)	1.18	0.05	Signal Transduction
			Cell Proliferation and Apoptosis Genes
MAD2L1	1.24	0.01	Mitotic Checkpoint
Allograft inflammatory factor-1 (AIF)	1.23	0.01	Negative Regulation of Cell Proliferation
Deleted in split-hand/split-foot 1 region (DSS1)	1.15	0.01	Cell Proliferation
BTG family, member 3 (BTG3)	1.11	0.05	Negative Regulation of Cell Proliferation
			Transcriptional Regulation Genes
Nuclear antigen Sp100 (SP100)	1.21	0.001	Transcription Regulation
Transducin-like enhancer 3 (Esp3)	1.18	0.01	Transcription Regulation
TAT-SF1 (HIV-1 transcript)	1.18	0.02	Transcriptional Elongation Regulator Activity
TAR DNA binding protein (TARDBP)	1.17	0.03	Transcription Factor Activity
c-Jun Proto Oncogene (JUN)	1.10	0.03	Cell Growth and Maintenance, Transcription Factor
E2F transcription factor 1 (E2F1)	1.14	0.05	Transcriptional Co-repressor, Apoptosis
			Cell Structure and Metabolism Genes
Blood-brain barrier large neutral amino acid transporter	1.20	0.001	Amino Acid Transporter
Calpain 2, (m/II) large subunit (CAPN2)	1.21	0.01	Proteolysis
Alpha 1 type XXI collagen precursor (COL21A1)	1.16	0.01	Cell Adhesion
Drosophila; lipid desaturase (DEGS)	1.16	0.02	Lipid Transport and Metabolism
Hexokinase 3 (white cell) (HK3)	1.14	0.02	Kinase Activity
Myeloid/lymphoid or Mixed-Lineage Leukemia	1.12	0.03	Cell Growth and Maintenance

Matrix Metalloproteinase 15 (MMP15)	1.15	0.04	Metallopeptidase Activity
Low density lipoprotein B (Ldlb)	1.12	0.05	Intracellular Protein Transport
Desmoplakin I (DPI)	1.09	0.05	Epidermal Differentiation

Downregulated genes (Leptin relative to control)

Gene name	Fold Change	P<	Function
			Receptor-Mediated Signal Transduction Genes
Insulin-like growth factor II (IGF-II)	0.74[Ø]	0.01	Growth Factor; Cell Proliferation
Bone Morphogenetic protein 2 (BMP2)	0.81	0.02	Cytokine/Growth Factor Activity
14-3-3 protein eta chain	0.86[Ø]	0.02	Phosphoserine Binding Signal Transduction
Protein tyrosine phosphatase, receptor type, F (PTPRF)	0.86	0.02	Receptor Protein Tyrosine Phosphatase Signaling Pathway
Kinase Suppressor of Ras-1 (KSR1)	0.83	0.03	Intracellular Signaling Cascade
Interleukin-12 (IL-12)	0.83	0.03	Cell Surface Receptor Linked Signal Transduction
Protein Tyrosine Phosphatase RCAP	0.85	0.03	Protein Tyrosine Phosphatase
Protein phosphatase 2A alpha	0.85	0.04	Protein Serine/threonine Phosphatase Complex
Mitogen-activating protein 3K1 (MAP3K1)	0.86[Ø]	0.04	Protein Serine/threonine Kinase Activity
GRO-alpha (CXCL1)	0.85	0.05	Intracellular Signaling Cascade
			Cell Proliferation and Apoptosis Genes
TNF receptor-1 associated protein (TRADD)	0.83	0.02	Apoptosis Regulation
Geminin	0.85	0.02	Cell Cycle Arrest
Nuclear distribution protein C homolog (NUDC)	0.88	0.04	Cell Proliferation
			Transcriptional Regulation Genes
SMAD 7 MAD-related protein	0.78	0.01	Transcription Regulation, TGF-beta Signal Transduction
Sin3-associated polypeptide, 18 (SAP18)	0.87[Ø]	0.01	Transcription Corepressor
Max-Associated Protein (MAD4)	0.90	0.04	Transcription Regulation
			Cell Structure and Metabolism Genes
Decorin (DCN)	0.77	0.001	Organogenesis
Inosine monophosphate dehydrogenase type II gene	0.84	0.001	Purine Nucleotide Biosynthesis
Chaperonin-like protein (HTR3)	0.88	0.01	Protein Folding
Matrix Metalloproteinase 13 (MMP-13)	0.81	0.02	Collagen Catabolism
MMP9	0.84	0.04	Collagen Catabolism

MMP9B	0.89	0.04	Collagen Catabolism
Heparan sulfate 3-O-sulfotransferase-1 (3OST1)	0.86	0.04	Transferase Activity

[ø] Invalidated by Q-RT-PCR (Table 8)

Table 5:

Genes altered by IGF-I versus SAL Treatment detected by BMET microarray analysis

Upregulated genes (IGF-I relative to control)

Gene name	Fold Change	P<	Function
			Receptor-Mediated Signal Transduction Genes
TGF-beta receptor type I (TGFBR3)	1.39	0.05	TGF-beta capture and presentation to signaling receptors
Adenylate cyclase type III (CYA3)	1.36	0.04	Calmodulin regulated intracellular signaling cascade
v-kit feline sarcoma viral oncogene homolog (KIT)	1.21	0.04	Protooncogene controlling cytokine-cyokine receptor interaction
Guanine nucleotide-binding protein alpha-1 subunit	1.20	0.005	G protein adenylate cyclase inhibiting activity
cAMP-specific cyclic phosphodiesterase 7B (CN7B)	1.16	0.003	cAMP-mediated signal transduction
cAMP-dependent protein kinase beta (PRKACB)	1.16	0.02	Activation of signal transduction through AMPK
Toll-like receptor 6 (TLR6)	1.16	0.04	Toll-like receptor signaling pathway
Adenylate cyclase, type I (CYA1)	1.15	0.03	Calmodulin regulated intracellular signaling cascade
Follistatin precursor (FS)	1.14	0.007	Direct activin antagonist
Receptor-interacting serine threonine kinase 2 (RIPK2)	1.12	0.04	Positive regulation of NF-kappaB signaling cascade
Guanine nucleotide-binding protein, alpha-13 subunit	1.11	0.03	Transmembrane G-protein receptor signaling protein activity
Suppressor of cytokine signaling 2 (SOCS2)	1.1	0.04	Negative regulation of JAK-STAT signaling pathway
Epidermal growth factor (EGF)	1.09	0.04	Epithelial tissue growth factor
TGF-beta receptor type I (TGFBR1)	1.08	0.04	TGF-beta cell surface signal transduction
Insulin receptor substrate 1 (IRS1)	1.08	0.04	PI3K pathway signal transduction
			Cell Proliferation/Apoptosis Genes
DNA polymerase epsilon subunit B (POLE2)	1.40	0.03	DNA repair and chromosomal DNA replication
Platelet-derived growth factor receptor (PDGFRA)	1.27	0.03	Controls growth factor activity for cells of mesenchymal origin
Interleukin-17 (IL-17)	1.20	0.03	Proinflammatory cytokine can stimulate IL6 expression
Cyclin H (CCNH)	1.15	0.01	Regulation of Cell Cycle
Cell division cycle 7-related protein kinase (CDC7)	1.14	0.04	Regulation of G1/S phase transition
Neuropeptide Y (NPY)	1.13	0.02	Neuroactive ligand-receptor interaction, proliferation regulation
Tumor necrosis factor ligand member 5 (TNFSF5)	1.10	0.01	Anti-apoptosis signal transduction
			Transcriptional Regulation Genes
Protein kinase DNA-activated polypeptide (PRKDC)	1.24	0.04	Protein serine/threonine kinase involved in transcription
MAX interactor 1 (MXI1)	1.17	0.04	Transcriptional repressor inhibits MYC

Transcription elongation factor A2 (TCEA2)	1.16	0.01	Elongation factor that releases RNA pol II complexes from arrest
General transcription factor II, I (GTF2I)	1.11	0.02	Phosphoprotein involved in transcription and signal transduction
			Cell Structure and Metabolism Genes
Somatostatin receptor 1 (SSR1)	1.10	0.009	Inhibition of release of hormones and secretory proteins
Cut-like 1, CCAAT displacement protein (CUTL1)	1.14	0.02	Regulates gene expression, morphogenesis and differentiation

Downregulated genes (IGF-I relative to control)

Gene name	Fold Change	*P*<	Function
			Receptor-Mediated Signal Transduction Genes
Dual specificity phosphatases 1 (DUSP1)	0.90	0.03	Inactivates mitogen-activated protein (MAP) kinase
Guanylate cyclase 2C (GUCY2C)	0.86	0.004	cGMP intracellular signaling cascade
Natriuretic peptide receptor B (NPR2)	0.05	0.02	G-protein coupled receptor signaling
Stathmin/oncoprotein 18 (STMN1)	0.87	0.03	Intracellular relay of cell signals
			Cell Proliferation and Apoptosis Genes
Tumor necrosis factor receptor member 5 (TNFRSF5)	0.92	0.04	Anti-apoptosis signal transduction
Nucleolar phosphoprotein B23 (NPM1)	0.77	0.02	Negative regulation of the cell cycle
Apoptosis-related cysteine protease (CASP3)	0.87	0.02	Central regulator of cell apoptosis
			Transcriptional Regulation Genes
Zinc finger protein 134 (ZNF134)	0.80	0.03	Regulation of transcription
RAR-related orphan receptor C (RORC)	0.85	0.05	Regulation of transcription
			Cell Structure and Metabolism Genes
Dihydrolipoamide branched chain transacylase (DBT)	0.80	0.02	Branched-chain amino acid catabolism
Enolase 1, alpha (ENO1)	0.87	0.01	Glycolysis and gluconeogeneis; amino acid biosynthesis

Table 6:

Genes altered by IGF-LEP versus IGF-I treatment detected by BMET microarray analysis

Upregulated genes (IGF-I + leptin relative to IGF-I)

Gene name	Fold Change	P<	Function
			Receptor-Mediated Signal Transduction Genes
Axin 1 (AXIN1)	1.32	0.01	Wnt signaling pathway mediator; controls development
Mitogen-activated protein kinase kinase kinase 11 (MAP3K11)	1.25	0.04	MAPK signaling pathway; involved with NFKB signaling
Interferon, alpha 2 (IFNA2)	1.24[Ø]	0.01	JAK-STAT pathway interaction
Hepatocyte growth factor (HGF)	1.24	0.02	Tyrosine kinase signaling mediated growth factor
Arenergic, alpha-1B-, receptor (ADRA1B)	1.23	0.04	Cell proliferation regulator through G protein receptor
Interleukin-1, beta (IL-1B)	1.22	0.03	Proinflammatory cytokine signaling through JAK-STAT
Toll-like receptor 4 (TLR4)	1.22	0.03	Toll-like receptor signaling pathway activator
Colony stimulating factor 2 (CSF2)	1.21	0.05	Cytokine signaling through JAK-STAT pathway
Erythropoietin (EPO)	1.18	0.04	IL-6 family member signals through JAK-STAT pathway
FK506 binding protein 12 (FRAP1)	1.16	0.03	Mediates signal transduction in the PI3K pathway
G protein-coupled receptor kinase (GIT2)	1.16	0.04	G protein coupled receptor activity
Tumor necrosis factor superfamily, member 2 (TNF)	1.13	0.05	Proinflammatory cytokine; signals in MAPK pathway
Protein phosphatases 5, catalytic subunit (PP5C)	1.13	0.02	Positive regulation of NF-kappaB cascade
Erythropoietin receptor (EPOR)	1.11	0.01	IL-6 receptor family signal transduction
Interleukin-10 (IL-10)	1.12	0.04	Regulation of JAK-STAT pathway activity
v-Myc myelocytomatosis viral oncogene homologue (MYC)	1.12[Ø]	0.01	MAPK signaling pathway control of cell proliferation
v-Myc myelocytomatosis viral related oncogene (MYCN)	1.10	0.03	Cell growth and maintenance; transcription factor
			Cell Proliferation and Apoptosis Genes
Cell division cycle 42, GTP binding protein 25kDa (CDC42)	1.17	0.001	Control of cell cycle via MAPK pathway activity
Polo-like kinase 1 (PLK1)	1.16	0.04	Regulation of G2/M phase transition in cell cycle
Pituitary tumor-transforming 1 (PTTG1)	1.14	0.01	Tumorigenic activity; stimulates cell cycle progression
			Regulation of Transcription
Upstream transcription factor 1 (USF1)	1.19	0.02	Transcriptional activator
E2F transcription factor 4, p107/p130-binding (E2F4)	1.17	0.04	Control of cell cycle via the TGF beta pathway
Peroxisome proliferative activated receptor, delta (PPARD)	1.16	0.04	Involved in fatty acid metabolism; transcription repressor

TGF beta 1 induced transcript 1 (TGFB1i1)	1.13	0.04	Positive regulation of transcription
			Cell Structure and Metabolism
Matrix metalloproteinase 14 (MMP14)	1.29	0.02	Extracellular matrix breakdown; tissue remodeling

Downregulated genes (IGF-I + leptin relative to IGF-I)

Gene name	Fold Change	*P*<	Function
			Receptor-Mediated Signal Transduction Genes
cAMP responsive element binding protein 3-like 1 (CREB3L1)	0.81	0.01	cAMP-mediated signal transduction
Serum response element-binding transcription factor (SRF)	0.83	0.01	MAPK pathway signal transduction
Nuclear receptor subfamily 3, group C, member 1 (NR3C1)	0.83	0.05	Neuroactive ligand-receptor signal transduction
p21/Cdc42/Rac1-activated kinase 1 (PAK1)	0.84	0.02	MAPK signaling pathway regulating cell morphology
NF-kappa B light polypeptide enhancer p105 (NFKB1)	0.84	0.03	MAPK regulation of proinflammatory reaction
NF-kappa B light polypeptide enhancer inhibitor beta (NFKBIB)	0.87	0.03	Inhibitor of the NFKB complex
TNF receptor-associated factor 6 (TRAF6)	0.88	0.03	Mediates signal transduction NF-kappaB pathway
Ran GTPase activating protein 1 (RANGAP1)	0.88	0.05	Induces GTPase activity of RAN but not RAS
Mitogen-activated protein kinase kinase 4 (MAP2K4)	0.89	0.02	Serine/threonine kinase signaling in the MAPK pathway
Transforming growth factor, beta (TGFB2)	0.89	0.02	TGF-beta and MAPK signaling pathway mediator
Casein kinase 1, epsilon (CSNK1E)	0.86	0.01	Serine/threonine protein kinase
			Cell Proliferation and Apoptosis Genes
Cyclin D2 (CCND2)	0.82 [ø]	0.03	Regulation of C1/S transition of the cell cycle
DNA fragmentation factor, 45kDa alpha polypeptide (DFFA)	0.87	0.03	Associates with caspase-3 during apoptosis
			Cell Structure and Metabolism
Somatostatin (SST)	0.82 [ø]	0.001	Regulation of endocrine and nervous system function
Somatostatin receptor 2 (SSTR2)	0.84	0.02	Regulation of endocrine and nervous system function

[ø] Invalidated by Q-RT-PCR (Table 8)

Table 7:

Gene specific-Q-RT-PCR primer pairs.

Primer sequences for internal control genes and genes of interest. All primer pairs were optimized at a ratio of 300:300 nM for forward and reverse using of 30 ng cDNA template per reaction.

Gene Name	Primer sequences (5' to 3')	
14-3-3 eta	Forward:	CAGGCAAGCAAACGACAATG
	Reverse:	TGGACTGACGGTTGCTTTGAG
ALG12	Forward:	TGGCCGTGGACTCCTATTTC
	Reverse:	ACTCTTGTTCAGGACGGTGTTGTA
B-actin	Forward:	CGCCATGGATGATGATATTGC
	Reverse:	AAGCCGGCCTTGCACAT
BCAR1	Forward:	TCCTCTCAGAGCGATATCTTAACCA
	Reverse:	GCTCCCGTGCCGCTCTA
BDNF	Forward:	GTGACAGCATCAGCGAATGG
	Reverse:	CGTCCCGCCCGACAT
Caspase-8	Forward:	CCGGGACCACATCCCATATAG
	Reverse:	TTCTTGGAGCCTCTGGAATAGC
CISH	Forward:	TGTGCATAGCCAAGACCTTCTC
	Reverse:	CCTCACTGGCCGTAATGGA
MYC	Forward:	CGGACACACAACGTTTTGGA
	Reverse:	TCTGGTCACGAAGAGCAAAAAAG
CYCD2	Forward:	GCTCTGTGCGCCACTGACT
	Reverse:	CCCCCACGCTTCCAGTT
FAK2	Forward:	AGGGACAAGCTATTGGGAGTTG
	Reverse:	TCCAGTGTGGCCTTGTTTCTC
FRAP1	Forward:	ACCATCTCCGATGAAAGAATGAA
	Reverse:	GGGAACCAGCCCATAAGAAAA

GAPDH	Forward:	GCATCGTGGAGGGACTTATGA
	Reverse:	GGGCCATCCACAGTCTTCTG
HMBS	Forward:	CTACATCCCAGCCCTCTGCTA
	Reverse:	GGCCCCTCGGTGAGACA
HPRT	Forward:	GCTCGAGATGTGATGAAGGAGAT
	Reverse:	ACAGGTCGGCAAAGAACTTATAGC
IFNA2	Forward:	GCCACCATGTGGGATGAGA
	Reverse:	CAGACAGAATTGCAGGTCAGTGA
IGF-II	Forward:	CGTTCCTTTTGGATCTGGACTTA
	Reverse:	GGACAAGCCTACCTGCAAATTT
IL8RB	Forward:	GGATGGCAGGCCTTCCTT
	Reverse:	GGTGTGGAGTCTCAGAGGGTAGTAG
Lep R	Forward:	GGGCACATCCAAGCATTAAAA
	Reverse:	GGCCGGCATCAAAGCTTT
MAD2L1	Forward:	CACCTATGGTTACTACCTCCAAAGG
	Reverse:	GTTCCGTTTTTGGTACCTATCTGACT
MAP3K1	Forward:	CTGCCTCTAGATGTCAAAATCCATT
	Reverse:	AACACCACAATTACCAGCAAGCT
NFKB	Forward:	CCTGCCCATCTCTCTAGGAGAGT
	Reverse:	CGGCTCCAGGCTCTACAGAA
PIGF	Forward:	GCCTTTCCTATTCCACTGGATTG
	Reverse:	CCACGTAGCCAAAGGTTGCT
PRKD2	Forward:	GTCGGTGGGCGTGATCAT
	Reverse:	TTTGATGTCCTCGTCCTCGTT
PTCH	Forward:	GGACAGCTGGGAGGAAATGTT
	Reverse:	GCGGGACAGTCTGGATCAA
SAP18	Forward:	TGCCGTCTCGTCCACAGAA
	Reverse:	CAAAAGAATGAAGGTAATAGGGCTTAA
SDHA	Forward:	GCCCGGGTTCAGGTCATC
	Reverse:	GGCCTCTGGTAGGAATCTCTATCA
SOCS1	Forward:	CCCACATGGTCCAGGAAAAT

	Reverse:	GCTCCTTCCCCTTCCAGATC
SOCS3	Forward:	GAGAACTGCCGGGAATCTTTG
	Reverse:	GAGGTTTAAAGCTCTGTTTTAAACCAA
SOCS5	Forward:	GCCCGAATTGAACAGTGGAA
	Reverse:	TTACAGTGGAGGAGTGAAACACACA
STAT5A	Forward:	AAGAGCTCACTGGGTCTGTCTGT
	Reverse:	GCCTGCCTGGCGTACCT
STAT5B	Forward:	ATTGCACGTTGTGCTGCTTCT
	Reverse:	CTGTTTGTGGCCCCAGTTCT
SST	Forward:	TGTTGCCCATATAAGACCTCTGATT
	Reverse:	CGAGGGTCTTATTGAGGATTGG
TBP	Forward:	GTGCGGCACCAGGTGAAG
	Reverse:	TACAGTGACGCTCTGGTGTTCTC
TLE1	Forward:	TGCGTTTATCTGTGCCTCTTAGACT
	Reverse:	ACGATTCAGAGCACCACAGAGA
v-JUN	Forward:	CCCACAGTTATTTTAATTGACACTCAA
	Reverse:	CCCCAACCTCTTTTCTGCATT

Table 8:

Microarray versus Q-RT-PCR analysis

	BOTL4 Microarray			BMET Microarray			Q-RT-PCR Analysis		
Gene name	**Contrast**	**Fold Change**	**P-value**	**Contrast**	**Fold Change**	**P-value**	**Contrast**	**Fold-change**	**P-value**
14-3-3 eta	C vs. I	0.87	*	C vs. I	0.98	0.89	C vs. I	0.46	**
	I vs. IL	1.20	0.06	I vs. IL	0.83	0.17	I vs. IL	-	NS
BDNF	C vs. I		NS	ND	-	-	C vs. I	2.86	*
	I vs. IL	0.75	*	ND	-	-	I vs. IL	-	NS
Caspase-8	C vs. I	1.26	**	C vs. I	1.18	*	C vs. I	2.01	**
	I vs. IL	0.81	**	I vs. IL	0.89	0.10	I vs. IL	ND	-
FRAP1	C vs. I	1.36	*	C vs. I	1.07	0.16	C vs. I	1.56	**
	I vs. IL		NS	I vs. IL	1.11	0.04	C vs. IL	-	-
MAP3K1	C vs. I	0.90	*	C vs. I	-	-	C vs. I	1.45	*
	I vs. IL	-	NS	I vs. IL	ND	-	I vs. IL	ND	-
NFKB p65	C vs. I	1.19	**	C vs. I	1.24	0.16	C vs. I	-	NS
	I vs. IL		NS	I vs. IL	0.98	0.89	I vs. IL	1.41	*
PIGF	ND	-	-	C vs. I	0.91	0.22	C vs. I	1.52	*
				I vs. IL	0.86	**	I vs. IL	0.68[Ŧ]	*
SOCS3	ND	-	-	I vs. IL	1.13	0.21	I vs. IL	3.42	*
FAK2	C vs. I	1.27	**	C vs. I	0.97	0.59	C vs. I	-	NS
LEPR	C vs. I	1.29	**	C vs. I	1.14	0.28	C vs. I	-	NS
	I vs. IL	0.79	*	I vs. IL	0.97	0.79	I vs. IL	-	NS
TLE1	I vs. IL	1.26	**	I vs. IL	1.00	0.90	I vs. IL	-	NS

	BOTL4 Microarray			BMET Microarray			Q-RT-PCR Analysis		
Gene name	**Contrast**	**Fold Change**	**P-value**	**Contrast**	**Fold Change**	**P-value**	**Contrast**	**Fold-change**	**P-value**
IL8RB	C vs. I	1.16	*	C vs. I	-	-	C vs. I	-	NS
	I vs. IL	0.85	*	I vs. IL	-	-	I vs. IL	-	NS
SAP18	C vs. I	0.86	**	C vs. I	1.01	0.76	C vs. I	-	NS
ALG12	I vs. IL	-	-	I vs. IL	0.86	**	I vs. IL	-	NS
BCAR1	C vs. I	1.16	*	C vs. I	-	-	C vs. I	-	NS
CCND2	I vs. IL	-	-	I vs. IL	0.82	*	I vs. IL	-	NS
IFNA2	ND	-	-	I vs. IL	1.2	**	I vs. IL	-	NS
MYC	C vs. I	-	NS	I vs. IL	1.08	**	I vs. IL	-	NS
PRKD2	ND	-	-	I vs. IL	0.89	**	I vs. IL	-	NS
PTCH	ND	-	-	I vs. IL	1.12	**	I vs. IL	-	NS
SST	ND	-	-	I vs. IL	0.86	*	I vs. IL	-	NS

* $P < 0.05$
** $P < 0.01$
ND = Not determined
NS = Not significant
Ŧ Heifer 88 deleted from analysis (found to be an outlier)

CHAPTER FIVE

SUMMARY AND CONCLUSIONS

It has been well established that raising dairy heifers on high energy diets before puberty impairs mammary development and reduces future milk production. The overall goal of my research was to help understand the mechanisms by which this depression of mammary development is mediated. Previous studies by Silva (275;278) established that insulin-like growth factor-I (IGF-I) is a potent mitogen of mammary epithelial cells both in vivo and in vitro and that leptin can inhibit the IGF-I-induced proliferation of epithelial cells, also in vivo and in vitro (276;278). I hypothesized that this interaction between IGF-I and leptin in the mammary gland that alters epithelial cell proliferation is mediated by changes in the transcriptional profile of genes mediating cell proliferation and apoptosis. To test this hypothesis, cDNA microarray technology was employed to measure the expression profile of RNA isolated from each of four quarters infused with saline control (SAL), IGF-I (IGF), leptin (LEP), or IGF-I plus leptin (IGF-LEP). Although results presented in Chapter 4 indicate that a number of genes were identified as differentially expressed between quarters infused with these hormonal treatments using the Bovine Total Leukocyte 4 (BOTL4) microarray, follow-up studies using quantitative real-time PCR (Q-RT-PCR) did not validate the changes in expression of many of these genes. A high percentage of genes tested with PCR showed no difference in gene expression

in the contrasts deemed significant by microarray analysis, thereby invalidating these results.

There are many potential reasons behind the failure of the BOTL4 microarray to accurately detect significant differences in the relative abundance of mRNAs between treatments in this study. The first possibility is that RNA of inferior quality was used to produce cDNA for fluorescent dye labeling and hybridization to the microarray slides in our study. The BOTL4 microarray studies were completed before the Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA) and NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) units were available in the Michigan State University Center for Animal Functional Genomics. With the arrival of the NanoDrop Spectrophotometer, the ability to accurately quantify mRNA and cDNA was drastically improved. It is possible that initial total RNA measurements for reverse transcriptase reactions were inaccurate during this time and less RNA was loaded than was intended, thereby decreasing the signal of each fluorescent dye-labeled sample. This may account for the generally weak hybridization signals detected in the BOTL4 microarray experiments. The ability to measure the quality of RNA used in the microarray studies was much lower prior to the use of the Agilent 2100 BioAnalyzer. Quality of RNA in this study was checked by 28S and 18S rRNA band visualization following gel electrophoresis and ethidium bromide staining. This is a much less sensitive measurement of RNA quality than with the results provided by the BioAnalyzer RNA 6000 Nano LabChip kit. Numerous studies have shown that RNA quality is of utmost importance in determining

microarray hybridization quality, and the arrival of the BioAnalyzer significantly improved the frequency of production of "good" microarrays.

The BOTL4 microarray was generated from a cDNA library containing 1,334 unique expressed sequence tags and amplicons representing known genes. This design of this microarray and inclusion of its gene set was tailored to studies in bovine immunology with a high percentage of known genes annotated within immune function ontologies. This made for a suitable, but not ideal, platform for the study of mammary gland development, with many genes known to be involved with IGF-I and leptin signaling and physiological function absent from the gene library. A small number of genes were significantly altered by BOTL4 microarray analysis in the SAL vs. IGF treatment comparison. At a significance level of $P<0.1$, 84 genes (6.3%) were significantly difference by this comparison. Of these 84, only 46 had a known gene function or ontological association based on BLASTn sequence searches against human gene databases. Of these 46 genes, 27 had a documented link with IGF-I in the literature. Similar results were obtained in the IGF vs. IGF-LEP microarray comparison. In this comparison 94 genes (7.0%) were significantly altered at $P<0.1$; 55 had a known gene function or ontology and 31 of these 55 had a documented link with leptin or IGF-I in the literature. This small percentage of significantly altered genes with relevant literature associations, low fold change differences detected (all significant differences had fold changes below 37%), and overall disappointing BOTL4 results led us to believe that perhaps a gene library containing a greater amount of IGF-I, leptin, and receptor tyrosine kinase-

mediated signal transduction genes would be more appropriate to this study. These factors, as well as the paucity of positive Q-RT-PCR microarray follow-up data (Chapter 4, Table 8) led to the generation of the Bovine Metabolism (BMET) microarray. However, results really were not improved when the BMET array was used.

The BMET library contains 2,348 70-mer oligonucleotides representing known genes related to bovine metabolism and signal transduction (Chapter 2). Microarray comparisons of SAL vs. IGF and IGF vs. IGF-LEP were performed using BMET microarray slides produced in the first print run by the Massachusetts General Hospital Microarray Core Facility at Harvard University. In the SAL vs. IGF comparison 129 genes (5.5%) were found to be significantly different between treatments ($P<0.05$) (Chapter 4, Supplementary Table 2). Although the percentage of genes altered by treatment was greater than the 5% expected by chance, improving upon the BOTL4 differences, these results remained underwhelming. In addition, all fold changes detected in this comparison were below 40% upregulation or downregulation. Similarly, in the IGF vs. IGF-LEP microarray comparison 150 genes were significantly different between treatments (6.5%), all with fold changes below 37% (Chapter 4, Supplementary Table 3). To date, Q-RT-PCR has validated the changes predicted by BMET microarray result for 1 of 8 genes tested in the IGF vs. IGF-LEP comparison (Chapter 4, Table 8).

A number of possibilities exist for the failure of the BMET microarray to accurately predict differences in gene expression within tissues in this study. The

first of these is microarray print quality. Microarrays used in the intramammary hormonal infusion study (Chapter 4) were from the first print run by Harvard University. Microarrays used in the BMET validation study (Chapter 3) were from the third print run. A noticeable difference in spot quality and hybridization efficiency was detectable between these two print runs (unpublished data) using either the cover-slip and hybridization chamber method or the GeneTAC Hybridization Station (Genomic Solutions, Ann Arbor, MI) (unpublished results). This could explain the poor Q-RT-PCR follow-up of microarray results. Secondly, the actual hybridization method could account for the failure of the BOTL4 and BMET microarrays. The high frequency of unequal hybridization of dyes across microarray slides, high background, and variability in spot signal intensities in microarrays hybridized in the GeneTAC Hybridization Station for both BOTL4 and BMET microarrays led me to switch to the cover-slip method of microarray hybridization. This technique is employed by Dr. John Quackenbush at The Institute of Genome Research (TIGR), and I learned of it while taking the Advances in Genome Technology and Bioinformatics short course at Woods Hole Marine Biological Laboratory in October, 2004. The cover slip method improved the signal-background ratios and seemed to improve the ability of microarrays to detect significant differences between labeled cDNA samples (Chapter 3), as seven of eight genes tested to date with this new method have been validated using Q-RT-PCR. However, the expected magnitudes of gene expression differences used in the tissue comparison study are obviously much larger than those expected in the infusion study, which confounds these results.

It has been suggested that the cover slip method is not suitable for detection of small fold-changes between samples because of over-hybridization of spots seen as a result of this procedure (Najib Elmassadi, MGH Microarray Facility Chief Technician, personal communication).

Microarray spot alignment, quality control, and statistical analysis are also major areas of importance in generating quality data. The choice of spot alignment programs and ability to detect and remove bad spots from analysis is critical to improving the reliability of microarray data. GeneTAC LS software version 3.3.0 (Genomic Solutions, Inc., Ann Arbor, MI) was used to process microarray images, find spots, integrate robot spotting files with the microarray image, and to create reports of raw spot intensities in the BOTL4 study. I believe that, while adequate, this program is certainly not optimal for microarray spot analysis. Spot alignment was improved greatly by the use of Genepix Pro software version 6.0 (Molecular Devices Corporation, Sunnyvale, CA). Ease of detection of spots, the ability to detect and flag good, bad, and absent spots, and to output data were all improved with this software package. The statistical models used to analyze microarray data in this study were also very important in generating reliable results. As a quick method of analyzing the success of microarray experiments, the p-value frequency plot seemed to be very useful in showing if data seemed to be good or bad. For example, the detection of 7% of genes significantly altered at $P<0.1$ between IGF vs. IGF+LEP in the BOTL4 microarray experiment indicates a violation of the major assumptions of *p*-value distribution in an experiment. One would expect at least 10% of the genes in this

study to be different simply by chance. This deflation of significant *p*-values cannot be explained, but perhaps lies in the model used to test for significant differences. None of the *p*-value frequency plots seen in the infusion study comparisons were significantly biased toward low ($P<0.05$) *p*-values, which may help to explain my difficulty in validating microarray results by Q-RT-PCR. These signfificance level frequency distributions were improved in the tissue comparison study, as would be expected in the comparison of very different tissue types rather than hormonally-treated quarters within the same animal's mammary gland. The addition of spike-in controls to microarray hybridizations in future experiments should also help in detection of significant fold changes, and microarray quality control in future studies. Ten metabolic genes specific to Arabidopsis thaliana are present on the BMET microarray slide, and cRNA probes to be spiked in at known ratios are now available for use for future microarray experiments (a gift from Dr. Norman Lee, TIGR). At the time of microarray hybridization in the infusion experiment these positive control genes were not yet available.

In conclusion, I believe that with continued improvement of the BMET microarray hybridization protocol, including the use of spike-in positive control genes and improved hybridization station utility, the intramammary infusion comparisons will yield useful and significant data that correlates well with Q-RT-PCR results. Currently, adipose tissue samples taken from control and growth hormone treated cattle which hybridized to a microarray of acceptable quality are being hybridized at Harvard University to directly compare the results generated

at MSU. Preliminary results indicate that the hybridization protocol and hybridization station used at Harvard has greatly improved the quality of the microarray, indicating that procedural issues, rather than the quality of tissue samples or the BMET array, are responsible for microarray hybridization quality (unpublished data).

The use of Q-RT-PCR to examine gene expression levels of genes known to mediate signaling within the IGF and interleukin-6 receptor families was fruitful. This study revealed that the addition of leptin to IGF-I treated quarters induced the upregulation of cytokine signaling-3 (SOCS3) mRNA levels (Chapter 4, Figure 6). I initially hypothesized that transcriptional regulation of genes which mediated crosstalk between the IGF-I and leptin receptors could likely mediate the effect of leptin inhibition of IGF-I-induced cell proliferation in mammary epithelial cells. The SOCS3 protein qualifies as a potential mediator of a feedback mechanism by which the mitogenic activity elicited by the binding of IGF-I to its receptor (IGF-IR) is inhibited by a protein that is upregulated in response to leptin signaling through the leptin receptor (OB-Rb). This effect would be promoted by physiological conditions in which both IGF-I and leptin levels are increased, as is seen when prepubertal diets promoting high rates of gain and adiposity are fed. However, future experiments confirming the upregulation of the SOCS3 protein, and not just mRNA will be necessary. Also helpful would be studies focused on elucidating the mode of action of SOCS3 in mammary epithelial cells based on its interaction with the IGF-IR and/or its signal transduction mediators insulin receptor substrate-1 (IRS1), the signal transducer

and activator of transcription-5 (STAT5A) and janus kinase 2 (JAK2) (See Chapter 6).

Also important in my study was the addition of two immunohistochemical markers of cell proliferation and apoptosis to the analysis of cell cycling in mammary epithelial cells in the infusion study. Immunostaining of the proliferating cell nuclear antigen Ki-67, when combined with the BrdU labeling results of Silva (2002) showed that although leptin treatment does not change the percentage of mammary epithelial cells that are in the cell cycle, it decreases the fraction of cells within the S-phase of the cell cycle. These results support a role for IGF-I as a cell cycle progression factor, whereby it pushes cells through the G1-S transition phase and thereby accelerates cell proliferation. While caspase-3 labeling was significantly increased in all hormonally-infused quarters relative to saline-infused control, numerical values showed that a very tiny fraction of the total epithelial cell population is undergoing programmed cell death. This result seems consistent with expected results on a number of levels. In a prepubertal heifer in the allometric growth phase of the mammary gland one would not expect to see much cell death compared to proliferation (Dr. Tony Capuco, personal communication, (42)). In addition, the actions of hormones known to alter cell cycling such as IGF-I and leptin should also accompany an increase in cell turnover, albeit small, as checkpoint damage repair mechanisms are in place to eliminate rapidly dividing cells with errors in DNA replication.

In conclusion, this study was able to demonstrate that increased concentrations of IGF-I promote the proliferation of mammary epithelial cells by

increasing the progression of cells into the S-phase of the cell cycle *in vivo* in mammary epithelial cells in prepubertal heifers. Increasing the leptin concentration above normal reduces this IGF-I-induced proliferation by limiting the progression of cells into the S-phase of the cell cycle and stalling them within the G1 phase of the cell cycle. These effects on cell proliferation are likely mediated by gene expression changes induced by IGF-I and leptin within mammary parenchymal tissue. One such gene, SOCS3 could potentially be a primary leptin-induced inhibitor of IGF-I signaling and cell proliferation. Although microarray analysis was generally unsuccessful in accurately measuring gene expression differences between treated mammary gland quarters, the potential for this technology in achieving this goal still exists. Future studies aimed at elucidating the mechanism of SOCS3 action in mammary epithelial cells and improving microarray analysis of mammary parenchymal tissue expression patterns should reveal more potential answers to the question of why feeding high energy diets to prepubertal heifers impairs mammogenesis.

CHAPTER SIX

FUTURE RESEARCH

The tissues used in this study are useful for a variety of studies to confirm and extend the hypotheses presented in this thesis:

1.) I measured the gene expression in this study using total RNA isolated from complete parenchymal tissue. I have also isolated RNA from mammary epithelial cells removed from the whole parenchymal tissue using laser capture microdissection and the Ambion RNAqueous kit. To increase the amount of RNA samples were amplified with two rounds of amplification using the Arcturus RiboAmp RNA Amplification kit to produce ~60ug of RNA for each tissue. I have successfully measured expression of GAPDH, STAT3 and leptin receptor for all tissues and found no significant differences across all treatment contrasts. Future studies may be useful to measure global gene expression patterns using the BMET microarray or the specific genes, such as SOCS3. The use of an enriched epithelial cell population could reveal interesting clues into the mechanisms of IGF-I and leptin actions in mammary parenchyma. In addition, stromal cell populations could be isolated to compare this with whole tissue and epithelial cell gene expression patterns.

2.) One important study to be done is the measurement of SOCS3 protein in all tissues. This is necessary to confirm that the change in mRNA expression measured between IGF, IGF+LEP and LEP is carried through translation into increased protein abundance. This can be performed for the other genes significantly altered by treatment as measured by Q-RT-PCR as well.

3.) It would be very interesting to determine the mechanism by which SOCS3 works in mammary tissue. SOCS proteins have been shown to alter receptor tyrosine kinase activity by binding to the growth hormone receptor, the IGF-I receptor, the leptin receptor, STAT5A and B, STAT3, and JAK2. Measuring the activation of each of these proteins by their tyrosine phosphorylation state in all tissues used in this study would be instrumental in determining signaling interactions between IGF-I and leptin in the mammary gland.

4.) Immunohistochemical staining of SOCS3 in the mammary gland would be useful in determining the site of action. The large fold change difference (3.4-fold) between SOCS3 mRNA abundance in IGF and IGF-LEP tissues may indicate that this gene is upregulated in the more abundant stromal cell population. This could be confirmed with in situ hybridization or immunohistochemistry.

5.) A yeast two-hybrid screen of SOCS3 could show whether it binds to the IGF-IR and/or leptin receptor in the mammary gland. This information would be useful in establishing a potential direct action of SOCS3 on these signaling proteins in the bovine mammary gland. This information, when paired with tyrosine phosphorylation of various tyrosine residues on the IGF-IR and OBR as well as JAK2, STAT5A, STAT5B, and STAT3 could be very interesting.

Other studies not directly related to tissues used in this dissertation:

6.) Genes found to be different between SAL, IGF and IGF-LEP in this study could also be measured in mammary tissue taken from animals grown on high energy diets in other studies, such as those of Davis Rincker (77). Davis Rincker found that prepubertal Holstein heifers grown on a high energy diet for 12 weeks showed an increase in leptin, IGF-I and an inhibition of mammary growth relative to heifers grown on high energy diets for shorter durations. Once again, the SOCS3 protein could potentially mediate this inhibitory effect on mammogenesis, and it would be interesting to measure its expression in mammary tissue from animals reared on high planes of nutrition for different lengths of time.

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APPENDIX

Final BMET Perl Script

```
#!/bin/sh
echo MODIFIED.1
exit
#!/usr/bin/perl

##########################################################################
##########################################################################
##GeneLink.pl version 1.0 April 2nd, 2004
##Bill Nobis, Michigan State University
##nobiswil@msu.edu
##
##Use of this script:
##To provide identification and annotation of ESTs using NCBI's refseq
and LocusLink projects
##
##How it works:
##After performing a BLAST with your ESTs or clusters against the
refseq database*
##(*see accompanying readme file for instructions on how to do this)
##use the BLAST output as input in this script, which will output a
"GeneLink" file by using LocusLink.
##The output will be a tab delimited file in the following format:
##clone name, clone indentifier, e-value of top refseq hit, locuslink
id, gene symbol, gene name, gi # of refseq hit,
##accesion # of refseq hit, omim number, summary of gene function,
##KEGG pathways gene is involved in, any GOslim terms associated with
the gene
##
##This tab delimited file can be opened in your favorite spreadsheet
program, dropped easily into your favorite SQL database,
##or this script can also output an html file if you'd like
##
###**For the intitial blast, you can download the standalone blast
program from NCBI at ftp://ftp.ncbi.nih.gov/blast/executables/LATEST-
BLAST
##    or use a similiar program such as WashU BLAST.  You may download
the refseq database to BLAST your ESTs or clusters to at
##    ftp://ftp.ncbi.nih.gov/refseq/release/vertebrate_mammalian
###**You also must download the latest LocusLink and loc2ref files from
NCBI and put it in the same directory as the script
##    You can download locuslink at
ftp://ftp.ncbi.nih.gov/refseq/LocusLink/
##    You want the file that will start LL_tmpl, download this, rename
it locuslink.txt and put it in the same directory as the script
##    You also want the file named loc2ref, download this and put it in
the same directory as the script
###**Even with the loc2ref file, due to discrepencies in the refseq and
LocusLink releases there are some ids that won't be in the loc2ref file
##      you can get theese locus ids from this script via the
LWP::simple module, to do this
```

```
##    you also must have an internet connection open when running the
script this way.  You may choose not to extract missing ids from the
web.
##    If you are using a unix or linux computer the easiest way to
install a module is to go to the CPAN shell
##    by typing " perl -MCPAN -e shell ".  Then just type " install
LWP::Simple "
##    If you are using the ActiveState perl for windows, just type "ppm
install LWP::Simple"
##**WARNING!! Depending on the number of queries in your BLAST output
and your CPU this script could take 20 mins (minimum) to hours to run!!
##
##For more explanation on anything please see the accompanying readme
file
##
##Feel free to do whatever you want with the source, just give proper
credit
##Sorry for the lack of comments in the script....if you any questions
on the code (not usage) please email me
##########################################################################
#####################################################################


use strict;
use Cwd;

my $dir = getcwd();
my (@parseout, @locusinput, @locusinput2);
print "\n-----------------------------------------------------------------
-------";
print "\nGeneLink.pl script v1.0 April 2, 2004\nBill Nobis
(nobiswil\@msu.edu)\n";
print "-----------------------------------------------------------------
-----\n";
open (LOCUSLINK, "</home/bill/Projects/BOTL/Final/locuslink.txt") ||
die ("FATAL ERROR!\nLocuslink file missing, please download from NCBI,
rename file locuslink.txt, and place in $dir\n");  #<LL3_040211.txt
close LOCUSLINK;
open (LOC2REF, "</home/bill/Projects/BOTL/Final/loc2ref") || die
("FATAL ERROR!\nLoc2ref file missing, please download from NCBI, rename
file loc2ref.txt, and place in $dir\n");  #<LL3_040211.txt
close LOC2REF;


print "\nEnter the file name and location of your BLAST output file\n";
print "Current working directory is: $dir\n";
my $blast = <STDIN>;
chomp ($blast);

print "\nWhat format is the BLAST output in? (1=xml 2=html)\n";
my $format = <STDIN>;
chomp ($format);

if ($format == 1) {
      print "\nParsing BLAST output......\n";
      @parseout = ParseblastXML ($blast);
      }
```

```
if ($format == 2) {
      print "\nParsing BLAST output......\n";
      @parseout = ParseblastHTML ($blast);
      }
if ($format > 2) {
      print "PLEASE ENTER A VALID SELECTION\n";
      die;
}
if ($format =~ /\D/g) {
      print "PLEASE ENTER A VALID SELECTION\n";
      die;
}

$/ = "\n";
print "\nWould you like to print out a FASTA file of sequences that did
not return a hit?\n";
print "Another BLAST could be peformed with this set.\n";
print "Perhaps with less stringent conditions.\n\n";
print "If you answer yes, you will need the original FASTA file used
for input\n";
print "(1=yes 2=no)\n";
my $nohit = <STDIN>;
chomp ($nohit);
if ($nohit == 1){
      NoHits();
}
if ($nohit == 2) {
      print "You entered no, continuing......\n";
}
if ($nohit > 2) {
      print "PLEASE ENTER A VALID SELECTION\n";
      die;
}
if ($nohit =~ /\D/g) {
      print "PLEASE ENTER A VALID SELECTION\n";
      die;
}
############Ask if you want to get missing ones from internet
print "There are often sequences for whose locuslink IDs could not be
extracted from loc2ref file\n";
print "Would you like to extract these IDs from the internet? (requires
open connection and LWP::Simple module)\n";
print "\n(1=yes 2=no)\n";

$/ = "\n";
my $ID = <STDIN>;
chomp ($ID);
if ($ID > 2) {
      print "PLEASE ENTER A VALID SELECTION\n";
      die;
}
if ($ID =~ /\D/g) {
      print "PLEASE ENTER A VALID SELECTION\n";
      die;
}

$/ = "\n";
```

```
print "\nEnter the desired output filename\n";
print "Ouput file will be saved in :\n$dir\n";
my $outfile = <STDIN>;
chomp ($outfile);


print "Would you like to create an html file output in addition to the
tab delimited one?\n";
print "(1=yes 2=no)\n";

my $htm = <STDIN>;
chomp ($htm);

my $html;
if ($htm == 1){
      print "\nEnter the desired name of the HTML output file\n";
      print "It will be saved in $dir\n";
      $html = <STDIN>;
      chomp ($html);
}
if ($htm > 2) {
      print "PLEASE ENTER A VALID SELECTION\n";
      die;
}
if ($htm =~ /\D/g) {
      print "PLEASE ENTER A VALID SELECTION\n";
      die;
}

print "Running the script with the desired options, (script can take
30min to several hours to run)\n";
print "Retreiving Locuslink IDs, please wait, this can take a
while....\n";
@locusinput = GetlocusIDs (@parseout);

#################Find ones that are missing
my %hits;
foreach (@parseout){
      my ($name, $gi, $acc, $def, $score, $evalue) = split "\t";
      $hits{$name} = $evalue;
}
my %linksfound;
foreach (@locusinput){
      my ($clone, $locusID, $evalue) = split "\t";
      $linksfound{$clone} = $evalue;
}
my @missing = ();
foreach (keys %hits) {
    push(@missing, $_) unless exists $linksfound{$_};
}

print "\nDone\n";

if ($ID == 1){
      print "Extracting locuslink IDs from the internet...\n";
      my @missinginput = AlternateGetlocusIDs(@parseout);
      @locusinput = (@locusinput, @missinginput);
```

```
      print "Web extraction complete\n";
}
if ($ID == 2) {
      print "You did not want to extract missing IDs from the web,
continuing......\n";
}

##################################################

print "\nParsing LocusLink Database file....\n";
my @locusoutput1 = Locuslinkparse (@locusinput);


Locuslinkparse2 (@locusoutput1);

print "\nDone writing to tab delimited file\n";
$/ = "\n";

if ($htm == 1){
      csv2HTML($outfile);
      print "\nDone writing to html file\n";
}
if ($htm == 2){
      print "\a\a\a\nScript complete, check output file\n";
}

unlink "locustemp.txt";
unlink "nohits.txt";

print "\a\a\a\nScript complete, check output files\n";


sub ParseblastXML {

      my $blast = shift @_;
      my ($queryname, $gi, $acc, $def, $score, $evalue, $nohits,
@parse);
      $/ ='<?xml version="1.0"?>';
      open (NOHITS, ">nohits.txt");
      print "$blast\n";
      open (BLAST, "<$blast") || die ("Error Opening $blast \n");

      while (<BLAST>) {

            ($queryname, $gi, $acc, $def, $score, $evalue, $nohits) =
"";

            if ($_ =~ /<BlastOutput_query-
def>(.+?)<\/BlastOutput_query-def>/g) {
                  $queryname = $1;
#                 if ($queryname =~ /\#/g) {
#                       $queryname =~ s/\#/\t/g;
#                 }
#                 else{
#                       $queryname = "$queryname\t";
#                 }
```

```
            }

            if ($_ =~ /<Hit_id>gi\|(.+?)\|ref\|(.+?)\|<\/Hit_id>/g){
                  $gi = $1;
                  $acc = $2;
            }

            if ($_ =~ /<Hit_def>(.+?)<\/Hit_def>/g) {
                  $def = $1;
                  if ($def =~ /gi\|(.+?)\|.+?\|(.+?)\|.+?/g) {
                        $gi = $1;
                        $acc = $2;
                  }
            }

            if ($_ =~ /<Hsp_bit-score>(.+?)<\/Hsp_bit-score>/g) {
                  $score = $1;
            }

            if ($_ =~ /<Hsp_evalue>(.+?)<\/Hsp_evalue>/g) {
                  $evalue = $1;

            }
            if ($_ =~
/<Iteration_message>(.+?)<\/Iteration+_message>/g) {
                  $nohits = $1;
            }
            if ($nohits =~ /No Hits Found/ig) {
                  print NOHITS "$queryname\n";
                  ($queryname, $gi, $acc, $def, $score, $evalue,
$nohits) = "";
                  next;
            }
            else {
                  push @parse,
"$queryname\t$gi\t$acc\t$def\t$score\t$evalue\n";
                  ($queryname, $gi, $acc, $def, $score, $evalue,
$nohits) = "";
                  next;
            }
      }

      close NOHITS;
      close BLAST;
      return @parse;

}

sub NoHits {

      open NOHITS, "<nohits.txt";
      print "\nEnter the file name of the FASTA file used for BLAST
input\n";
      print "Current working directory is:\n$dir\n";
      my $original = <STDIN>;
      chomp ($original);
      open (FASTA, "<$original") || die ("Error Opening $original \n");
```

```
	print "\nEnter the desired file name of the FASTA ouptut file\n";
	print "Current working directory is:\n$dir\n";
	my $nohits_out = <STDIN>;
	chomp ($nohits_out);
	open (FASTA_OUT, ">$nohits_out") || die ("Error Opening $original
\n");

	my (@clone_with_hits, @clones);
	$/ = ">";
	<FASTA>;
	while (<FASTA>) {
		my ($name, $sequence) = split "\n";
		#my ($clone, $TC) = split "\t", $name;
		push @clone_with_hits, "$name\t$sequence";
	}

	$/ = "\n";

	while (<NOHITS>) {
		$_ =~ s/>//g;
		$_ =~ s/\n//g;
		$_ =~ s/\r//g;
		push @clones, "$_";
	}

	foreach my $clone_name (@clones) {

		foreach  (@clone_with_hits) {
			my ($clone, $sequence) = split "\t";
			if ($clone =~ /$clone_name/i) {
				print FASTA_OUT ">$clone\n$sequence\n";
			}
			else {
				next;
			}
		}
	}
	print "FASTA file complete\n";
	close FASTA_OUT;
	close FASTA;
	close NOHITS;
}

sub AlternateGetlocusIDs {
	use LWP::Simple qw($ua get);
	$ua->agent("Mozilla"); # hide the fact that this is libwww-perl

	my @missinginput;
	my $locusID;

	$/ = "\n";
	foreach  (@_) {
		chomp;
		my ($name, $gi, $acc, $def, $score, $evalue) = split "\t";
		foreach (@missing){
			if ($_ eq $name){
```

```
                        my $url =
"http\:\/\/www\.ncbi\.nlm\.nih\.gov\/entrez\/viewer\.fcgi\?val\=$gi\&db
\=Nucleotide\&dopt\=GenBank";
                        my $ncbi_text = get($url);
                              if ($ncbi_text =~
m/LocusID:<a.+?>(.+?)<\/a>/g) {
                                    $locusID = $1;
                                    push @missinginput,
"$name\t$locusID\t$evalue\n";
                                    print ".";
                                    next;
                              }
                        }
                  else{
                        next;
                  }
            }
      }
      return @missinginput;
}


sub GetlocusIDs {
      open (LOC2REF, "</home/bill/Projects/BOTL/Final/loc2ref");
      $/ = "\n";
      my $blank = "";
      my (@loc2ref, @locusinput);
      while (<LOC2REF>) {
            my ($id, $accession, $number, $status, $protacc, $num) =
split "\t";
            $accession =~ s/\..+//g;
            push @loc2ref, "$accession\t$id";
            next;
      }

      foreach (@_) {
            chomp;
            my ($name, $gi, $acc, $def, $score, $evalue) = split "\t";
            $acc =~ s/\..+//g;
            foreach (@loc2ref) {
                  my ($accession, $id) = split "\t";
                  if ($accession =~ /$acc/ig and $evalue =~ /\d/g) {
                        print ".";
                        push @locusinput2, "$name\t$id\t$evalue\n";
                        next;
                  }
                  else {
                        next;
                  }
            next;
            }
      }

      close LOC2REF;
      return @locusinput2;
}
```

```
sub Locuslinkparse {
      $/ = "\n";
      open (LOCUSPARSE, ">>locustemp.txt");
      my (@locuslinkIDs, @locuslinkparse);

      foreach (@_) {
            my ($clone, $locusID, $evalue) = split "\t";
            push @locuslinkIDs, "$locusID\t$clone\t$evalue";
      }

      foreach  (@locuslinkIDs) {
            $_ =~ s/\n//g;
            $_ =~ s/\r//g;
      }

      open (LOCUSLINK,
"</home/bill/Projects/BOTL/Final/locuslink.txt");  #<LL3_040211.txt

      $/ = ">>";
      <LOCUSLINK>;
      while (<LOCUSLINK>) {
            my ($id, $refseq, $symbol, $name, $summary, $omim, @DBKEGG,
@GO, @locuslinkparse);
            if ($_ =~ /LOCUSID: (.+?)\n/g) {
                  $id  = $1;
                  $id =~ s/\r//g;
                  }
            if ($_ =~ /NM: (.+?)\n/g) {
                  $refseq = $1;
                  $refseq =~ s/\r//g;
            }
            if ($_ =~ /OFFICIAL_SYMBOL: (.+?)\n/g) {
                  $symbol = $1;
                  $symbol =~ s/\r//g;
            }
            if ($_ =~ /OFFICIAL_GENE_NAME: (.+?)\n/g) {
                  $name = $1;
                  $name =~ s/\r//g;
            }
            if ($_ =~ /PREFERRED_SYMBOL: (.+?)\n/g) {
                  $symbol = $1;
                  $symbol =~ s/\r//g;
            }
            if ($_ =~ /PREFERRED_GENE_NAME: (.+?)\n/g) {
                  $name = $1;
                  $name =~ s/\r//g;
            }
            if ($_ =~ /SUMMARY: (.+?)\n/g) {
                  $summary = $1;
                  $summary =~ s/\r//g;
            }
            if ($_ =~ /OMIM: (.+?)\n/g) {
                  $omim = $1;
                  $omim =~ s/\r//g;
            }
            @DBKEGG = m/(DB_DESCR: KEGG pathway.+?DB_LINK: .+?)\n/sg;
            foreach (@DBKEGG) {
```

```
                $_ =~ s/DB_DESCR: /  /g;
                $_ =~ s/DB_LINK: /<>/g;
                $_ =~ s/\r//g;
                $_ =~ s/\n//g;
        }
        @GO = m/(GO: .+?)\n/sg;
        foreach  (@GO) {
                $_ =~ s/GO: /  /g;
                $_ =~ s/\r//g;
                $_ =~ s/\n//g;
        }
        push @locuslinkparse,
"$id\t$symbol\t$name\t$refseq\t$omim\t$summary\t@DBKEGG\t@GO";  #print
it out in tab delimited format
        print LOCUSPARSE
"$id\t$symbol\t$name\t$refseq\t$omim\t$summary\t@DBKEGG\t@GO\n";
        ($id, $symbol, $name, $refseq, $omim, $summary, @DBKEGG,
@GO) = "";
    }
    close LOCUSLINK;
    close LOCUSPARSE;
    return @locuslinkIDs;
}

sub Locuslinkparse2{
    my @GOslimbio = ("behavior    GO:0007610", "biological_process
unknown     GO:0000004", "cell communication    GO:0007154", "cell
recognition GO:0008037", "cell-cell signaling   GO:0007267", "host-
pathogen interaction    GO:0030383", "response to endogenous stimulus
    GO:0009719", "response to external stimulus     GO:0009605",
"response to abiotic stimulus GO:0009628", "response to biotic stimulus
    GO:0009607", "signal transduction   GO:0007165", "cell growth and
or maintenance    GO:0008151", "cell cycle      GO:0007049", "cell
growth      GO:0016049", "cell organization and biogenesis
    GO:0016043", "cytoplasm organization and biogenesis
    GO:0007028", "organelle organization and biogenesis
    GO:0006996", "mitochondrion organization and biogenesis
    GO:0007005", "cytoskeleton organization and biogenesis
    GO:0007010", "cell proliferation    GO:0008283", "chemi-
mechanical coupling     GO:0006943", "cell homeostasis
    GO:0019725", "metabolism      GO:0008152", "amino acid and
derivative metabolism   GO:0006519", "biosynthesis    GO:0009058",
"carbohydrate metabolism      GO:0005975", "catabolism
    GO:0009056", "coenzymes and prosthetic group metabolism
    GO:0006731", "electron transport    GO:0006118", "energy pathways
    GO:0006091", "lipid metabolism      GO:0006629", "nucleobase
nucleoside nucleotide and nucleic acid metabolism     GO:0006139", "DNA
metabolism  GO:0006259", "transcription   GO:0006350", "protein
metabolism  GO:0019538", "protein biosynthesis  GO:0006412", "protein
biosynthesis      GO:0006416", "protein biosynthesis  GO:0006453",
"protein modification   GO:0006464", "secondary metabolism
    GO:0019748", "response to stress    GO:0006950", "transport
    GO:0006810", "ion transport   GO:0006811", "protein transport
    GO:0015031", "death     GO:0016265", "cell death
    GO:0008219", "development     GO:0007275", "cell differentiation
    GO:0030154", "embryonic development GO:0009790", "morphogenesis
    GO:0009653", "regulation of gene expression epigenetic
```

```
      GO:0040029", "growth    GO:0040007", "reproduction
      GO:0000003", "physiological processes     GO:0007582", "viral
life cycle  GO:0016032");
      my @GOslimcell = ("cell GO:0005623", "intracellular
      GO:0005622", "chromosome      GO:0005694", "cilium
      GO:0005929", "cytoplasm GO:0005737", "cytoplasmic chromosome
      GO:0000229", "cytoplasmic vesicle   GO:0016023", "cytoskeleton
      GO:0005856", "cytosol   GO:0005829", "endoplasmic reticulum
      GO:0005783", "endosome  GO:0005768", "Golgi apparatus
      GO:0005794", "lipid particle  GO:0005811", "microtubule
organizing center GO:0005815", "mitochondrion   GO:0005739",
"peroxisome GO:0005777", "plastid   GO:0009536", "ribosome
      GO:0005840", "vacuole   GO:0005773", "lysosome  GO:0005764",
"nucleus    GO:0005634", "nuclear chromosome    GO:0000228", "nuclear
membrane    GO:0005635", "nucleolus GO:0005730", "nucleoplasm
      GO:0005654", "plasma membrane GO:0005886", "thylakoid
      GO:0009579", "cellular_component unknown  GO:0008372", "external
encapsulating structure GO:0030312", "cell envelope   GO:0030313",
"cell wall  GO:0005618", "extracellular   GO:0005576", "extracellular
matrix      GO:0005578", "extracellular space   GO:0005615",
"unlocalized      GO:0005941");
      my @GOslimmolec = ("antioxidant activity  GO:0016209", "apoptosis
regulator activity      GO:0016329", "binding   GO:0005488", "calcium
ion binding GO:0005509", "carbohydrate binding  GO:0030246", "lipid
binding     GO:0008289", "nucleic acid binding  GO:0003676", "DNA
binding     GO:0003677", "chromatin binding     GO:0003682",
"transcription factor activity      GO:0003700", "transcription factor
activity    GO:0000130", "nuclease activity     GO:0004518", "RNA
binding     GO:0003723", "translation factor activity nucleic acid
binding     GO:0008135", "nucleotide binding    GO:0000166", "oxygen
binding     GO:0019825", "protein binding GO:0005515", "cytoskeletal
protein binding   GO:0008092", "actin binding   GO:0003779", "cell
adhesion molecule activity    GO:0005194", "chaperone activity
      GO:0003754", "chaperone activity    GO:0003757", "chaperone
activity    GO:0003758", "chaperone activity    GO:0003760", "chaperone
activity    GO:0003761", "chaperone regulator activity
      GO:0030188", "defense and immunity protein activity
      GO:0003793", "catalytic activity    GO:0003824", "kinase
      GO:0016301", "protein kinase activity     GO:0004672", "hydrolase
      GO:0016787", "peptidase activity    GO:0008233", "protein
phosphatase activity    GO:0004721", "transferase     GO:0016740",
"enzyme regulator activity    GO:0030234", "molecular_function unknown
      GO:0005554", "motor activity  GO:0003774", "protein stabilization
activity    GO:0017028", "protein tagging activity    GO:0008638",
"signal transducer activity   GO:0004871", "receptor binding
      GO:0005102", "receptor activity     GO:0004872", "nutrient
reservoir activity      GO:0045735", "structural molecule activity
      GO:0005198", "transcription regulator activity  GO:0030528",
"translation regulator  GO:0045182", "transporter activity
      GO:0005215", "electron transporter activity     GO:0005489", "ion
channel activity  GO:0005216", "neurotransmitter transporter activity
      GO:0005326", "triplet codon-amino acid adaptor activity
      GO:0030533");
      $/ = "\n";
      open (OUT, ">$outfile") || die ("Error Opening $outfile \n$!\n");
```

```
      print "Finding matches in LocusLink, compiling GeneLink file,
this may take quite a while (about 25 mins, but could be hours) and use
a lot of CPU resources....\n";
      open LOCUSPARSE, "<locustemp.txt";
      my @output;
      while (<LOCUSPARSE>) {
            my ($id, $symbol, $name, $refseq, $omim, $summary, $DBKEGG,
$GO) = split "\t";
            my ($acc, $gi, $type) = split (/\|/, $refseq);
            my ($GObio, $GOcell, $GOmolec);

            foreach (@GOslimbio){
                  my ($name, $GOnum) = split "\t";
                  if ($GO =~ /$GOnum/g){
                        $GObio = "$name\|$GOnum";
                  }
            }

                  foreach (@GOslimcell){
                  my ($name, $GOnum) = split "\t";
                  if ($GO =~ /$GOnum/g){
                        $GOcell = "$name\|$GOnum";
                  }
            }

                  foreach (@GOslimmolec){
                  my ($name, $GOnum) = split "\t";
                  if ($GO =~ /$GOnum/g){
                        $GOmolec = "$name\|$GOnum";
                  }
            }

            foreach (@_){
                  my ($llID, $clone, $evalue) = split "\t";
                  if ($llID == $id) {
                        #print OUT
"$clone\t$TC\t$evalue\t$id\t$name\t$symbol\t$gi\t$acc\t$omim\t$summary\
t$DBKEGG\t$GObio\t$GOcell\t$GOmolec\n";
                        print ".";
                        push @output,
"$clone\t$evalue\t$id\t$name\t$symbol\t$summary\t$omim\t$DBKEGG\t$GObio
\t$GOcell\t$GOmolec";
                        next;
                  }
            }
            next;
      }
      #make sure there are only unique entries in the output
      my @uniq;
      my %seen = ();
      foreach my $item (@output) {
            push(@uniq, $item) unless $seen{$item}++;
      }

      foreach (@uniq){
            print OUT "$_\n";
      }
```

```
	$/ = "\n";
	open (NOHITS, "<nohits.txt") || die ("Error\n");
	while (<NOHITS>) {
		chomp;
		#my ($clonename, $TCnumber) = split "\t";
		print OUT "$_\n";
	}
	close NOHITS;
	close OUT;
}

sub csv2HTML {

	my $csv = shift @_;
	my $blank = "";
	my @out;

	$/ = "\n";
	open (HTML, ">$html") || die ("Error Opening $html \n");

	print HTML "<HTML>\n<HEAD>\n";
	print HTML "</HEAD>\n<BODY MODIFIED_BGCOLOR='\"#FFFFFF\"'>\n\n";
	print HTML "<TABLE CELLPADDING=4 CELLSPACING=0 BORDER=1>\n";
	print HTML "<TR MODIFIED_BGCOLOR='\"#EEEEEE\"'><TH>Sequence
name</TH>\n";
	print HTML "<TH>E-value</TH>\n";
	print HTML "<TH>LocusLink ID</TH>\n";
	print HTML "<TH>Official Gene Name</TH>\n";
	print HTML "<TH>Official Symbol</TH>\n";
	########re-ordering edit
	print HTML "<TH>Summary of Gene Function</TH>\n";
	print HTML "<TH>OMIM</TH>\n";
	print HTML "<TH>Bibliography</TH>\n";
	###################
	##print HTML "<TH>Accession</TH>\n";
	##print HTML "<TH>OMIM</TH>\n";
	##print HTML "<TH>Bibliography</TH>\n";
	##print HTML "<TH>Summary of Gene Function</TH>\n";
	###################
	print HTML "<TH>KEGG Pathways</TH>\n";
	print HTML "<TH>GO Biological Process</TH>\n";
	print HTML "<TH>GO Cellular Component</TH>\n";
	print HTML "<TH>GO Molecular Function</TH></TR>\n";

	open (CSV, "<$csv") || die ("Can not open file $csv\n");
	while (<MODIFIED_CSV>){
		##my ($clone, $TC, $evalue, $id, $name, $symbol, $gi, $acc,
$omim, $summary, $DBKEGG, $GObio, $GOcell, $GOmolec) = split "\t";
		my ($clone, $evalue, $id, $name, $symbol, $summary, $omim,
$DBKEGG, $GObio, $GOcell, $GOmolec) = split "\t";
		my ($pubmed, $pubmedsearch);
		if ($id != $blank) {
			$pubmed = "<A HREF=
\"http\:\/\/web\.ncbi\.nlm\.nih\.gov\/entrez\/query\.fcgi\?db\=gene\&cm
d\=Display\&dopt\=gene\_pubmed\&from\_uid\=$id\">Pubmed Links<\/A>";
```

```
                $pubmedsearch = "<A HREF=
\"http\:\/\/www\.ncbi\.nlm\.nih\.gov\/entrez\/query\.fcgi\?db\=PubMed\&
cmd\=search\&term\=$name\">Pubmed Search<\/A>";
            }
            #$acc = "<A HREF=
\"http\:\/\/www\.ncbi\.nlm\.nih\.gov\/entrez\/viewer\.fcgi\?val\=$gi\&d
b\=Nucleotide\&dopt\=GenBank\">$acc<\/A>";
            $id = "<A HREF=
\"http\:\/\/www\.ncbi\.nlm\.nih\.gov\/LocusLink\/LocRpt\.cgi\?l\=$id\">
$id<\/A>";
            if ($omim != $blank) {
                $omim = "<A HREF=
\"http\:\/\/www\.ncbi\.nlm\.nih\.gov\/entrez\/dispomim\.cgi\?id\=$omim\
">$omim<\/A>";
            }
            my ($GOnamebio, $GOnumbio) = split /\|/, $GObio;
            my ($GOnamecell, $GOnumcell) = split /\|/, $GOcell;
            my ($GOnamemolec, $GOnummolec) = split /\|/, $GOmolec;
            if ($GObio =~ /\D+/g) {
                $GObio = "<A HREF= \"http\:\/\/godatabase\.org\/cgi-
bin\/go\.cgi\?query\=$GOnumbio\&view\=details\&search\_constraint\=term
s\&depth\=0\">$GObio<\/A>";
            }
            if ($GOcell =~ /\D+/g) {
                $GOcell = "<A HREF= \"http\:\/\/godatabase\.org\/cgi-
bin\/go\.cgi\?query\=$GOnumcell\&view\=details\&search\_constraint\=ter
ms\&depth\=0\">$GOcell<\/A>";
            }
            if ($GOmolec =~ /\D+/g) {
                $GOmolec = "<A HREF=
\"http\:\/\/godatabase\.org\/cgi-
bin\/go\.cgi\?query\=$GOnummolec\&view\=details\&search\_constraint\=te
rms\&depth\=0\">$GOmolec<\/A>";
            }

            my @KEGG;
            my @pathways = split /   /, $DBKEGG;
            foreach (@pathways){
                my ($KEGGname, $KEGGurl) = split "<>";
                push @KEGG, "<A HREF\=\"$KEGGurl\">$KEGGname<\/A>";
            }


            ##my $line =
"$clone\t$TC\t$evalue\t$id\t$symbol\t$name\t$acc\t$omim\t$pubmed
$pubmedsearch\t$summary\t@KEGG\t$GObio\t$GOcell\t$GOmolec\n";
            my $line =
"$clone\t$evalue\t$id\t$name\t$symbol\t$summary\t$omim\t$pubmed
$pubmedsearch\t@KEGG\t$GObio\t$GOcell\t$GOmolec\n";

            print HTML "<TR><TD>";
            $line =~ s/\t/<\/TD><TD VALIGN=TOP>/g;
            print HTML "$line";
              print HTML "</TD></TR>\n";
      }

      print HTML "</TABLE>\n";
```

```
	print HTML "</BODY>\n</HTML>";
	close CSV;
	close HTML;
}

sub ParseblastHTML{
	print "Not included in genelink.pl yet, please try to do your
BLAST output in XML\n";
	die;
}
```

Supplementary Table 1: Significant BOTL4 microarray genes

Gene or clone name	Gene name	Trt Comparison	Fold Change	StdErr	*P*-value
NBFGC_AW657120	SAP18 sin3-associated polypeptide, 18	SAL vs IGF	0.86	0.06	0.004
BOTL0100003XG08R	Transducin-like enhancer 3 (Esp3)	SAL vs IGF	1.23	0.09	0.005
BOTL0100004XG09R	p21-activated protein kinase I (Pakl)	SAL vs IGF	1.12	0.05	0.005
BOTL0100006XH02R	NF-kappa-B transcription factor p65 subunit	SAL vs IGF	1.17	0.07	0.005
BOTL0100011_F08	Unknown	SAL vs IGF	1.16	0.07	0.005
NBFGC_BF773771	FAK2 focal adhesion kinase	SAL vs IGF	1.27	0.11	0.007
apoptotic cysteine protease Mch5 isoform alpha (Mch5)	Apoptotic cysteine protease Mch5 isoform alpha (Mch5)	SAL vs IGF	1.26	0.11	0.008
BOTL0100008_F06	Unknown	SAL vs IGF	1.12	0.05	0.008
NBFGC_BF773656	SMAD 7 MAD-related gene	SAL vs IGF	1.22	0.09	0.008
uPAR	urokinase-type plasminogen activator receptor	SAL vs IGF	0.84	0.08	0.009
NBFGC_BE721857	Chemokine (C-C motif) receptor 7 (CCR7)	SAL vs IGF	1.19	0.09	0.010
BOTL0100003XH07R	Similar to goliath protein	SAL vs IGF	0.86	0.07	0.010
BOTL0100002XF12R	Unknown	SAL vs IGF	0.88	0.06	0.010
DUSP4	DUSP4	SAL vs IGF	1.29	0.13	0.012
BOTL0100009_H06	Similar to WW domain-containing oxidoreductase	SAL vs IGF	0.86	0.08	0.012
BOTL0100009_D12	Thymopoietin (TMPO) gene, exons 4 and 5, and complete cds for thymopoietin alpha	SAL vs IGF	1.15	0.07	0.013
BOTL0100012_E02	Unknown	SAL vs IGF	1.15	0.07	0.014
NBFGC_BE757495	Upstream stimulatory factor 1b, 1a	SAL vs IGF	1.18	0.09	0.014
NBFGC_BF602343 MY047	Leptin receptor	SAL vs IGF	1.29	0.13	0.014
NBFGC_AW632336	PDK3 pyruvate dehydrogenase kinase isoenzyme	SAL vs IGF	1.15	0.08	0.015
BOTL0100011_F05	Ki-67 (MKI67)	SAL vs IGF	1.16	0.08	0.015
interferon-gamma receptor mRNA (IFN-gamma-R1)	interferon-gamma receptor mRNA (IFN-gamma-R1)	SAL vs IGF	0.81	0.11	0.015

NBFGC_BE684567	cyclin-dependent kinase inhibitor 1A Cip1	SAL vs IGF	0.84	0.09	0.016
FKBP-rapamycin associated protein mRNA, complete cds (FRAP)	FKBP-rapamycin associated protein (FRAP)	SAL vs IGF	1.34	0.16	0.016
scavenger R	scavenger R	SAL vs IGF	1.15	0.08	0.022
BOTL0100006XB04R	Unknown	SAL vs IGF	1.13	0.07	0.023
BOTL0100004XC05R	Squamous cell carcinoma antigen recognised by T cells (SART1)	SAL vs IGF	1.14	0.07	0.023
BOTL0100008_G10	Stearoyl-CoA desaturase	SAL vs IGF	1.26	0.13	0.025
BOTL0100001XH09R	14-3-3 protein eta chain	SAL vs IGF	0.87	0.08	0.026
BOTL0100008_F08	Mitochondrial adenylate kinase isozyme 3 (AK3), exon 1	SAL vs IGF	0.87	0.08	0.026
SAM 3-10lb	SAM 3-10lb	SAL vs IGF	0.86	0.09	0.027
MMP2short	MMP2short	SAL vs IGF	1.16	0.09	0.027
BOTL0100013_A04	Unknown	SAL vs IGF	1.15	0.08	0.027
transcobalamine	transcobalamine	SAL vs IGF	1.20	0.11	0.028
MMP3	MMP3	SAL vs IGF	1.19	0.10	0.028
BOTL0100011_E03	Copine III (CPNE3)	SAL vs IGF	1.11	0.06	0.029
BOTL0100010_F09	Unknown	SAL vs IGF	1.13	0.07	0.029
glycoprotein IIa (LAMP)	glycoprotein IIa (LAMP)	SAL vs IGF	1.17	0.09	0.030
BOTL0100011_G05	Unknown	SAL vs IGF	0.89	0.07	0.031
BOTL0100006XC08R	weakly similar to ZINC FINGER PROTEIN 135	SAL vs IGF	0.85	0.10	0.032
NBFGC_BG689033	MAIL: an inflammation-associated gene	SAL vs IGF	1.15	0.08	0.032
BOTL0100003XG07R	Unknown	SAL vs IGF	1.10	0.06	0.033
BOTL0100006XF09R	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide ...	SAL vs IGF	1.13	0.07	0.035
IL-1	IL-1	SAL vs IGF	1.17	0.10	0.036
NBFGC_BF076028	GSK-3beta	SAL vs IGF	0.88	0.08	0.037
breast cancer anti-estrogen resistance 1, mRNA (BCAR1)	breast cancer anti-estrogen resistance 1 (BCAR1)	SAL vs IGF	1.16	0.10	0.037
BOTL0100009_F08	Unknown	SAL vs IGF	1.11	0.07	0.037
BOTL0100011_E04	Cell cycle-regulated factor p78	SAL vs IGF	0.87	0.09	0.038

BOTL0100012_B01	Degenerative spermatocyte (homolog Drosophila; lipid desaturase) (DEGS)	SAL vs IGF	1.14	0.08	0.039
CC-chemokine receptor (CCR-5) gene delta-32 allele complete	CC-chemokine receptor (CCR-5) gene delta-32	SAL vs IGF	1.16	0.10	0.039
BOTL0100008_D09	Unknown	SAL vs IGF	1.10	0.06	0.040
NBFGC_AW670373	KSR1 kinase suppressor of ras-1	SAL vs IGF	0.85	0.11	0.041
BOTL0100004XC04R	Unknown	SAL vs IGF	1.12	0.07	0.042
BOTL0100010_E07	BTG family, member 3 (BTG3)	SAL vs IGF	1.11	0.07	0.042
BOTL0100010_C10	Unknown	SAL vs IGF	1.15	0.09	0.044
BOTL0100009_F07	Signal recognition particle 19kD (SRP19)	SAL vs IGF	1.08	0.05	0.045
mRNA for DRAK2 (DRAK-2)	DRAK2 (DRAK-2)	SAL vs IGF	1.15	0.09	0.045
BOTL0100013_A03	Unknown	SAL vs IGF	1.08	0.05	0.045
S.scrofa endoglin mRNA	Endoglin (endoglin)	SAL vs IGF	0.78	0.16	0.046
NBFGC_BE754163	Thyroid hormone receptor, alpha	SAL vs IGF	0.85	0.10	0.047
BOTL0100003XB12R	Unknown	SAL vs IGF	1.13	0.08	0.047
BOTL0100010_E04	Unknown	SAL vs IGF	1.16	0.10	0.048
decorin (DCN) mRNA	decorin (DCN)	SAL vs IGF	0.85	0.11	0.049
MMP7	MMP7	SAL vs IGF	1.14	0.09	0.050
interleukin-8 receptor type B (IL8RB)	interleukin-8 receptor type B (IL8RB)	SAL vs IGF	1.16	0.10	0.050
BOTL0100008_C10	Inosine monophosphate dehydrogenase type II gene	SAL vs LEP	0.84	0.07	0.002
BOTL0100010_H09	Unknown	SAL vs LEP	1.19	0.06	0.002
BOTL0100012_C05	Homo sapiens nuclear antigen Sp100 (SP100)	SAL vs LEP	1.21	0.07	0.002
BOTL0100010_H10	Unknown	SAL vs LEP	1.26	0.09	0.002
BOTL0100012_B12	Blood-brain barrier large neutral amino acid transporter (LAT)	SAL vs LEP	1.20	0.07	0.003
decorin (DCN) mRNA	Decorin (DCN)	SAL vs LEP	0.77	0.11	0.003
BOTL0100013_A04	Unknown	SAL vs LEP	1.22	0.08	0.003
BOTL0100012_E02	Unknown	SAL vs LEP	1.19	0.07	0.003
BOTL0100011_E02	Unknown	SAL vs LEP	1.21	0.08	0.004
BOTL0100012_B07	Unknown	SAL vs LEP	0.82	0.09	0.005
BOTL0100011_G09	Transducin-like enhancer protein (TLE1)	SAL vs LEP	1.19	0.08	0.005

BOTL0100011_A04	Alpha 1 type XXI collagen precursor (COL21A1)	SAL vs LEP	1.16	0.07	0.006
BOTL0100006XG03R	Chaperonin-like protein (HTR3)	SAL vs LEP	0.88	0.06	0.006
NBFGC_AW657120	SAP18 sin3-associated polypeptide, 18	SAL vs LEP	0.87	0.06	0.006
BOTL0100008_D05	Unknown	SAL vs LEP	1.20	0.08	0.007
BOTL0100011_B08	Transducin-like enhancer 3 (Esp3)	SAL vs LEP	1.18	0.07	0.007
Ovine mRNA for insulin-like growth factor II (IGF-II)	Insulin-like growth factor II (IGF-II)	SAL vs LEP	0.74	0.14	0.007
NBFGC_BF773656	SMAD 7 MAD-related	SAL vs LEP	0.78	0.12	0.008
BOTL0100012_E12	MAD2 (mitotic arrest deficient, yeast, homolog)-like 1 (MAD2L1)	SAL vs LEP	1.24	0.10	0.009
BOTL0100004XG10R	Deleted in split-hand/split-foot 1 region (DSS1)	SAL vs LEP	1.15	0.07	0.009
BOTL0100007_D07	Unknown	SAL vs LEP	1.18	0.08	0.010
M class I lymphocyte antigen (A-A 0201) mRNA	M class I lymphocyte antigen (A-A 0201)	SAL vs LEP	0.81	0.11	0.011
BOTL0100012_G01	Vacuolar ATPase isoform VA68	SAL vs LEP	1.15	0.07	0.011
BOTL0100011_E03	Copine III (CPNE3)	SAL vs LEP	1.14	0.06	0.011
BOTL0100010_H08	Calpain 2, (m/II) large subunit (CAPN2)	SAL vs LEP	1.21	0.10	0.012
BOTL0100011_G12	Unknown	SAL vs LEP	1.18	0.08	0.012
BOTL0100009_D12	Thymopoietin (TMPO) gene, exons 4 and 5, and complete cds for thymopoietin alpha	SAL vs LEP	1.15	0.07	0.012
BOTL0100009_F10	Mucosa associated lymphoid tissue lymphoma translocation gene 1 (MALT1)	SAL vs LEP	1.16	0.08	0.014
BOTL0100010_F01	Unknown	SAL vs LEP	1.14	0.07	0.014
BOTL0100013_E05	Blood-brain barrier large neutral amino acid transporter (LAT)	SAL vs LEP	1.15	0.07	0.014
Bos taurus allograft inflammatory factor-1 (AIF) mRNA	Allograft inflammatory factor-1 (AIF)	SAL vs LEP	1.23	0.11	0.014
oncostatin-M specific receptor beta subunit (OSMRB) mRNA	oncostatin-M specific receptor beta subunit (OSMRB)	SAL vs LEP	1.25	0.12	0.016
BOTL0100009_H10	V beta T-cell receptor (TCRBV)	SAL vs LEP	1.15	0.07	0.016
bone morphogenetic protein 2 (BMP2)	bone morphogenetic protein 2 (BMP2)	SAL vs LEP	0.81	0.11	0.016
BOTL0100004XG11R	HARP (HARP)	SAL vs LEP	1.09	0.05	0.017

BOTL0100012_B01	degenerative spermatocyte (homolog Drosophila; lipid desaturase) (DEGS)	SAL vs LEP	1.16	0.08	0.017
MMP-13 protein	MMP-13 protein	SAL vs LEP	0.81	0.11	0.018
BOTL0100001XH09R	14-3-3 protein eta chain	SAL vs LEP	0.86	0.08	0.018
BOTL0100003XF09R	Protein tyrosine phosphatase, receptor type, F (PTPRF)	SAL vs LEP	0.86	0.09	0.018
BOTL0100006XB01R	Geminin	SAL vs LEP	0.85	0.09	0.019
BOTL0100009_B07	Midnolin	SAL vs LEP	1.15	0.08	0.019
BOTL0100013_B10	Unknown	SAL vs LEP	1.11	0.06	0.019
BOTL0100001XD02R	Hexokinase 3 (white cell) (HK3)	SAL vs LEP	1.14	0.07	0.020
BOTL0100009_D10	Unknown	SAL vs LEP	1.16	0.08	0.020
BOTL0100009_G10	Unknown	SAL vs LEP	1.14	0.07	0.021
BOTL0100010_G02	Unknown	SAL vs LEP	1.13	0.07	0.022
alpha mac	alpha mac	SAL vs LEP	0.78	0.14	0.022
BOTL0100011_F09	TAT-SF1 (HIV-1 transcript)	SAL vs LEP	1.18	0.09	0.023
TNF receptor-1 associated protein (TRADD)	TNF receptor-1 associated protein (TRADD)	SAL vs LEP	0.83	0.10	0.024
TRP	TRP	SAL vs LEP	0.88	0.08	0.024
Janus kinase 1, mRNA (JAK1)	Janus kinase 1 (JAK1)	SAL vs LEP	1.20	0.11	0.025
BOTL0100008_A11	c-jun proto oncogene (JUN), clone hCJ-1	SAL vs LEP	1.10	0.05	0.025
BOTL0100012_H09	Unknown	SAL vs LEP	1.15	0.08	0.026
BOTL0100008_A05	Unknown	SAL vs LEP	1.14	0.08	0.027
BOTL0100007_C10	TAR DNA binding protein (TARDBP)	SAL vs LEP	1.17	0.09	0.027
NBFGC_AW670373	KSR1 kinase suppressor of ras-1	SAL vs LEP	0.83	0.11	0.027
BOTL0100008_A10	Protein tyrosine phosphatase, receptor type, C-associated protein (PTPRCAP)	SAL vs LEP	0.85	0.10	0.029
BOTL0100009_H11	Myeloid/lymphoid or mixed-lineage leukemia (trithorax (Drosophila) homolog)	SAL vs LEP	1.12	0.07	0.030
BOTL0100013_F07	Unknown	SAL vs LEP	1.15	0.08	0.030
BOTL0100011_C09	Epidermal growth factor receptor pathway substrate 15, related sequence (Eps15-rs)	SAL vs LEP	0.85	0.10	0.030
BOTL0100009_A05	Unknown	SAL vs LEP	1.14	0.08	0.031

retinoic acid X receptor b (RxR-beta)	retinoic acid X receptor b (RxR-beta)	SAL vs LEP	0.86	0.09	0.034
BOTL0100013_G10	Ran GTPase activating protein 1 (RANGAP1)	SAL vs LEP	1.13	0.07	0.034
ICAD-S	(NSD) ICAD-S	SAL vs LEP	0.81	0.13	0.034
BOTL0100009_F07	Signal recognition particle 19kD (SRP19), mRNA	SAL vs LEP	1.09	0.05	0.035
IL-12	IL-12	SAL vs LEP	0.83	0.11	0.035
max-associated protein (MAD4)	max-associated protein (MAD4)	SAL vs LEP	0.90	0.07	0.036
MMP9	MMP9	SAL vs LEP	0.84	0.11	0.036
MMP9B	MMP9B	SAL vs LEP	0.89	0.08	0.036
MMP15	MMP15	SAL vs LEP	1.15	0.09	0.036
BOTL0100013_F12	Unknown	SAL vs LEP	1.10	0.06	0.037
BOTL0100013_G03	Partial heparan sulfate 3-O-sulfotransferase-1 precursor (3OST1 gene)	SAL vs LEP	0.86	0.10	0.037
BOTL0100004XC05R	Squamous cell carcinoma antigen recognised by T cells (SART1)	SAL vs LEP	1.12	0.07	0.037
BOTL0100001XA06R	Protein phosphatase 2A alpha	SAL vs LEP	0.85	0.10	0.038
BOTL0100008_E02	Unknown	SAL vs LEP	0.87	0.09	0.038
SAM 2-14la	SAM 2-14la	SAL vs LEP	1.16	0.10	0.039
BOTL0100006XB08R	PDGF associated protein (HASPP28)	SAL vs LEP	1.09	0.05	0.039
BOTL0100013_E11	Unknown	SAL vs LEP	1.12	0.08	0.040
BOTL0100012_F03	Unknown	SAL vs LEP	0.89	0.08	0.041
mitogen-activated protein kinase kinase kinase (MAP3K1)	mitogen-activated protein kinase kinase kinase (MAP3K1)	SAL vs LEP	0.86	0.10	0.041
BOTL0100005XA11R	Unknown	SAL vs LEP	1.08	0.05	0.043
NBFGC_BE721857	Chemokine (C-C motif) receptor 7 (CCR7)	SAL vs LEP	1.14	0.09	0.043
BOTL0100006XD12R	Unknown	SAL vs LEP	1.10	0.06	0.044
BOTL0100004XB04R	Nuclear distribution protein C homolog (NUDC)	SAL vs LEP	0.88	0.08	0.045
BOTL0100012_C08	Unknown	SAL vs LEP	1.14	0.09	0.047
BOTL0100005XD03R	Unknown	SAL vs LEP	1.10	0.07	0.047
BOTL0100012_H10	Contains the Desmoplakin I (DPI) gene...	SAL vs LEP	1.09	0.06	0.048

E2F transcription factor 1, mRNA (E2F1)	E2F transcription factor 1 (E2F1)	SAL vs LEP	1.14	0.09	0.048
BOTL0100008_E09	low density lipoprotein B (Ldlb)	SAL vs LEP	1.12	0.07	0.049
macrophage inflammatory protein 3 alpha (MIP-3a) mRNA	macrophage inflammatory protein 3 alpha (MIP-3a)	SAL vs LEP	1.18	0.11	0.049
GRO-alpha	GRO-alpha	SAL vs LEP	0.85	0.11	0.049
BOTL0100010_E07	BTG family, member 3 (BTG3)	SAL vs LEP	1.11	0.07	0.050
BOTL0100011_G09	Transducin-like enhancer protein (TLE1)	IGF vs IGF+LEP	1.26	0.08	0.001
BOTL0100009_B07	Midnolin	IGF vs IGF+LEP	1.23	0.08	0.001
DAP	DAP	IGF vs IGF+LEP	0.83	0.07	0.002
BOTL0100009_H10	V beta T-cell receptor (TCRBV)	IGF vs IGF+LEP	1.20	0.07	0.003
BOTL0100011_G08	Unknown	IGF vs IGF+LEP	1.26	0.09	0.003
heparin-binding vascular endothelial growth factor (VEGF)	Heparin-binding vascular endothelial growth factor (VEGF)	IGF vs IGF+LEP	1.23	0.09	0.006
BOTL0100012_D12	Unknown	IGF vs IGF+LEP	1.19	0.08	0.006
BOTL0100004XG11R	HARP (HARP)	IGF vs IGF+LEP	1.11	0.05	0.006
BOTL0100013_F12	Unknown	IGF vs IGF+LEP	1.15	0.06	0.006
BOTL0100003XG06R	Seven transmembrane domain orphan receptor	IGF vs IGF+LEP	0.85	0.07	0.008
BOTL0100011_D04	Unknown	IGF vs IGF+LEP	0.86	0.07	0.008
TRP	TRP	IGF vs IGF+LEP	0.85	0.08	0.008
brain-derived neurotrophic factor (BDNF)	brain-derived neurotrophic factor (BDNF)	IGF vs IGF+LEP	0.80	0.11	0.011
apoptotic cysteine protease Mch5 isoform alpha (Mch5)	apoptotic cysteine protease Mch5 isoform alpha (Mch5)	IGF vs IGF+LEP	0.81	0.11	0.012
BOTL0100009_A05	Unknown	IGF vs IGF+LEP	1.17	0.08	0.013
BOTL0100005XD03R	Unknown	IGF vs	1.14	0.07	0.013

CD8 antigen alpha polypeptide (p32) (CD8A)	CD8 antigen alpha polypeptide (p32) (CD8A)	IGF+LEP IGF vs IGF+LEP	1.19	0.09	0.013
BOTL0100009_D03	Unknown	IGF vs IGF+LEP	1.18	0.08	0.014
macrophage inflammatory protein-1, alpha	macrophage inflammatory protein-1, alpha	IGF vs IGF+LEP	1.16	0.08	0.014
BOTL0100003XA05R	Unknown	IGF vs IGF+LEP	0.86	0.08	0.015
(NSD) insulin-like 3, mRNA (INSL3)	Insulin-like 3 (INSL3)	IGF vs IGF+LEP	0.83	0.10	0.015
BOTL0100002XB11R	Unknown	IGF vs IGF+LEP	0.89	0.06	0.016
SCC	SCC	IGF vs IGF+LEP	1.19	0.09	0.016
steroid 5-alpha-reductase	steroid 5-alpha-reductase (5-alpha reductase)	IGF vs IGF+LEP	0.82	0.11	0.016
NBFGC_BF602343 MY047	Leptin receptor	IGF vs IGF+LEP	0.79	0.13	0.018
BOTL0100001XA04R	cAMP specific phosphodiesterase 7B (PDE7B gene)	IGF vs IGF+LEP	0.89	0.07	0.018
Ovine mRNA for insulin-like growth factor II (IGF-II)	Insulin-like growth factor II (IGF-II)	IGF vs IGF+LEP	0.78	0.14	0.019
NBFGC_BG689033	MAIL: an inflammation-associated gene	IGF vs IGF+LEP	0.86	0.08	0.020
interleukin 2 receptor beta chain (p70-75) (IL-2R beta)	interleukin 2 receptor beta chain (p70-75) mRNA (IL-2R beta)	IGF vs IGF+LEP	1.26	0.13	0.020
PHE	PHE	IGF vs IGF+LEP	0.88	0.07	0.022
BOTL0100011_H09	sema domain, immunoglobulin domain (Ig)	IGF vs IGF+LEP	1.19	0.10	0.023
BOTL0100008_F01	Unknown	IGF vs IGF+LEP	1.13	0.07	0.023
NBFGC_BG690285	NBFGC_BG690285 vinexin beta (SH3-containing adaptor molecule)	IGF vs IGF+LEP	0.90	0.06	0.023
BOTL0100012_C05	Nuclear antigen Sp100 (SP100)	IGF vs IGF+LEP	1.14	0.07	0.024

BOTL0100011_A04	Alpha 1 type XXI collagen precursor (COL21A1)	IGF vs IGF+LEP	1.12	0.07	0.025
BOTL0100004XB04R	Nuclear distribution protein (NUDC)	IGF vs IGF+LEP	0.87	0.08	0.025
CD30 ligand mRNA complete cds (CD30L)	CD30 ligand mRNA complete cds (CD30L)	IGF vs IGF+LEP	1.19	0.10	0.027
BOTL0100003XG07R	Unknown	IGF vs IGF+LEP	0.91	0.06	0.028
BOTL0100007_D07	Unknown	IGF vs IGF+LEP	1.15	0.08	0.029
BOTL0100008_D05	Unknown	IGF vs IGF+LEP	1.15	0.08	0.030
inhibin A subunit	inhibin A subunit	IGF vs IGF+LEP	0.85	0.10	0.030
serine protease-like protein mRNA (Granzyme-B)	serine protease-like protein mRNA (Granzyme-B)	IGF vs IGF+LEP	0.83	0.11	0.031
BOTL0100011_G10	Human V beta T-cell receptor (TCRBV)	IGF vs IGF+LEP	1.22	0.12	0.031
DAD-1 complete cds (DAD-1)	DAD-1 complete cds (DAD-1)	IGF vs IGF+LEP	1.17	0.10	0.034
IL-1	IL-1	IGF vs IGF+LEP	0.86	0.10	0.034
SAM 7-20R	SAM 7-20R	IGF vs IGF+LEP	1.14	0.08	0.036
BOTL0100009_H11	Myeloid/lymphoid or mixed-lineage leukemia (trithorax (Drosophila) homolog)	IGF vs IGF+LEP	1.12	0.07	0.036
AW653810	Wee1 mitotic inhibitor kinase	IGF vs IGF+LEP	0.84	0.11	0.037
BOTL0100004XH11R	Candidate tumor suppressor protein DICE1	IGF vs IGF+LEP	0.87	0.09	0.039
Janus kinase 1, mRNA (JAK1)	Janus kinase 1 (JAK1)	IGF vs IGF+LEP	1.18	0.11	0.039
BOTL0100007_A09	Unknown	IGF vs IGF+LEP	0.90	0.07	0.040
B-actin	B-actin	IGF vs IGF+LEP	0.86	0.10	0.041

interleukin-8 receptor type B (IL8RB)	interleukin-8 receptor type B (IL8RB)	IGF vs IGF+LEP	0.85	0.10	0.041
NBFGC_BE754483 casein kinase 1 alpha1	Casein kinase 1 alpha1	IGF vs IGF+LEP	0.84	0.12	0.042
BOTL0100009_B10	Unknown	IGF vs IGF+LEP	1.15	0.09	0.043
BOTL0100013_C02	Unknown	IGF vs IGF+LEP	1.10	0.06	0.043
BOTL0100013_B11	Helicase-MOI	IGF vs IGF+LEP	1.16	0.09	0.043
BOTL0100008_F07	Unknown	IGF vs IGF+LEP	0.92	0.05	0.044
(NSD) NBFGC_BE752838 JNK3A2 JNK3 alpha2 protein kinase	JNK3A2 JNK3 alpha2 protein kinase	IGF vs IGF+LEP	0.87	0.10	0.044
beta chemokine mRNA complete cds (6CKine)	beta chemokine (6CKine)	IGF vs IGF+LEP	0.83	0.12	0.045
TIMP4	TIMP4	IGF vs IGF+LEP	0.90	0.07	0.045
small inducible cytokine A1 (I-309 homologous to mouse Tca-3)	Small inducible cytokine A1 (I-309 homologous to mouse Tca-3)	IGF vs IGF+LEP	0.83	0.12	0.046
TLH29 protein precursor (TLH29), interferon, alpha-inducible	TLH29 protein precursor (TLH29), interferon, alpha-inducible	IGF vs IGF+LEP	1.19	0.11	0.047
NBFGC_AW669767	Ribosomal protein S6, 70 kD, polypeptide 2	IGF vs IGF+LEP	0.89	0.08	0.048
BOTL0100002XD02R	Cyclophilin-40	IGF vs IGF+LEP	0.88	0.08	0.048

Supplementary Table 2: Significant BMET microarray genes: SAL vs. IGF-I

BMET ID	Gene Name	Symbol	Fold Change	Raw P	P-FDR	HarvardID
NG_002443.1	glutamate-ammonia ligase (glutamine synthase)	GLUL	1.23	0.00	0.34	MSU_Bt_01_91769
AF184162.1	MyoD family inhibitor	MDFI	1.12	0.00	0.34	MSU_Bt_01_93478
CB168744	adenylosuccinate synthase	ADSS	1.18	0.00	0.41	MSU_Bt_01_92984
CB421649	thyrotrophic embryonic factor	TEF	1.09	0.00	0.44	MSU_Bt_01_92937
AW652347	solute carrier family 23 (nucleobase transporters), member 2	SLC23A2	1.15	0.00	0.44	MSU_Bt_01_93348
CK958507	phosphate cytidylyltransferase 2, ethanolamine	PCYT2	0.83	0.00	0.44	MSU_Bt_01_91972
CK771096	protein geranylgeranyltransferase type I, beta subunit	PGGT1B	1.28	0.00	0.44	MSU_Bt_01_92617
CK849082	PR domain containing 4	PRDM4	0.85	0.00	0.44	MSU_Bt_01_92177
NM_174547.1	guanylate cyclase 2C (heat stable enterotoxin receptor)	GUCY2C	0.87	0.00	0.44	MSU_Bt_01_91510
BM366162	valyl-tRNA synthetase 2-like	VARS2L	1.14	0.00	0.44	MSU_Bt_01_93061
CK849696	glyoxylate reductase/hydroxypyruvate reductase	GRHPR	1.11	0.01	0.44	MSU_Bt_01_92155
NM_174324.2	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1	GNAI1	1.20	0.01	0.44	MSU_Bt_01_91589
AB006985.1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	ABCB1	1.17	0.01	0.44	MSU_Bt_01_93525
CB534880	phosphodiesterase 7B	PDE7B	1.16	0.01	0.44	MSU_Bt_01_92729
NM_174609.2	solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	SLC6A4	1.09	0.01	0.44	MSU_Bt_01_91472
NM_173949.2	protein phosphatase 2C, magnesium-dependent, catalytic subunit	PPM2C	1.11	0.01	0.44	MSU_Bt_01_91736
NM_174108.2	melanocortin 1 receptor (alpha melanocyte stimulating hormone receptor)	MC1R	0.90	0.01	0.44	MSU_Bt_01_91671
NM_175801.1	follistatin	FST	1.14	0.01	0.44	MSU_Bt_01_91381
NM_174603.2	solute carrier family 2 (facilitated glucose transporter), member 3	SLC2A3	1.13	0.01	0.44	MSU_Bt_01_91476
BE217451	solute carrier family 39 (zinc transporter), member 6	SLC39A6	1.23	0.01	0.44	MSU_Bt_01_93281
CK956377	somatostatin receptor 1	SSTR1	1.10	0.01	0.44	MSU_Bt_01_91986
CK771613	uroporphyrinogen decarboxylase	UROD	1.12	0.01	0.44	MSU_Bt_01_92602
CK774204	alanine-glyoxylate aminotransferase 2-like 1	AGXT2L1	1.10	0.01	0.44	MSU_Bt_01_92493

NM_174049.1	enolase 1, (alpha)	ENO1	0.87	0.01	0.44	MSU_Bt_01_91695
NM_174139.2	prodynorphin	PDYN	0.88	0.01	0.44	MSU_Bt_01_91655
BE721495	cyclin H	CCNH	1.15	0.01	0.44	MSU_Bt_01_93253
Bauman411	isovaleryl Coenzyme A dehydrogenase	IVD	1.13	0.01	0.44	MSU_Bt_01_93579
BM251767	uridine-cytidine kinase 2	UCK2	1.36	0.01	0.44	MSU_Bt_01_93090
CK770186	sal-like 2 (Drosophila)	SALL2	0.82	0.01	0.44	MSU_Bt_01_92659
CK848805	ribosomal protein L28	RPL28	1.13	0.02	0.44	MSU_Bt_01_92191
NM_174534.2	deoxyribonuclease I	DNASE1	1.33	0.02	0.44	MSU_Bt_01_91513
M37384.1	aldehyde dehydrogenase 3 family, memberA1	ALDH3A1	0.92	0.02	0.44	MSU_Bt_01_91802
NM_182989.1	phosphatidylethanolamine N-methyltransferase	PEMT	0.89	0.02	0.44	MSU_Bt_01_91272
NM_174580.1	POU domain, class 5, transcription factor 1	POU5F1	0.93	0.02	0.44	MSU_Bt_01_91490
CB426537	elongation factor RNA polymerase II	ELL	1.21	0.02	0.44	MSU_Bt_01_92915
CK777978	phosphoribosyl pyrophosphate synthetase 1	PRPS1	1.11	0.02	0.44	MSU_Bt_01_92358
CK777226	transcription elongation factor A (SII), 2	TCEA2	1.16	0.02	0.44	MSU_Bt_01_92384
CK969386	folylpolyglutamate synthase	FPGS	1.12	0.02	0.44	MSU_Bt_01_91908
NM_174599.2	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1	SLC1A1	1.11	0.02	0.44	MSU_Bt_01_91480
CK846163	serine hydroxymethyltransferase 1 (soluble)	SHMT1	0.90	0.02	0.44	MSU_Bt_01_92282
NM_174624.2	tumor necrosis factor (ligand) superfamily, member 5 (hyper-IgM syndrome)	TNFSF5	1.10	0.02	0.44	MSU_Bt_01_91466
CB166882	nucleophosmin (nucleolar phosphoprotein B23, numatrin)	NPM1	0.78	0.02	0.44	MSU_Bt_01_92997
X56062.1			1.38	0.02	0.44	MSU_Bt_01_93506
Bauman3162	ectonucleoside triphosphate diphosphohydrolase 2	ENTPD2	1.12	0.02	0.44	MSU_Bt_01_93585
NM_177943.1	SEC14-like 2 (S. cerevisiae)	SEC14L2	1.32	0.02	0.44	MSU_Bt_01_91308
CK849194	glutathione S-transferase A4	GSTA4	1.14	0.02	0.44	MSU_Bt_01_92171
NM_181001.2	tyrosinase (oculocutaneous albinism IA)	TYR	0.80	0.02	0.44	MSU_Bt_01_91294
CK770398	aldehyde dehydrogenase 4 family, member A1	ALDH4A1	1.13	0.02	0.44	MSU_Bt_01_92650
CK959627	propionyl Coenzyme A carboxylase, alpha polypeptide	PCCA	1.06	0.02	0.44	MSU_Bt_01_91964
BE808500	phosphoglucomutase 1	PGM1	1.11	0.02	0.44	MSU_Bt_01_93217
BE663394	distal-less homeo box 3	DLX3	1.13	0.03	0.44	MSU_Bt_01_93274
NM_174808.1	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa	NDUFV1	1.15	0.03	0.44	MSU_Bt_01_91411

CK958538	caspase 3, apoptosis-related cysteine protease	CASP3	0.87	0.03	0.44	MSU_Bt_01_91971
NM_174585.2	protein kinase, cAMP-dependent, catalytic, beta	PRKACB	1.16	0.03	0.44	MSU_Bt_01_91485
BF601020	CASP8 and FADD-like apoptosis regulator	CFLAR	1.18	0.03	0.44	MSU_Bt_01_93186
NM_173905.2	dihydrolipoamide branched chain transacylase (E2 component of branched chain keto acid dehydrogenase complex; maple syrup urine disease)	DBT	0.81	0.03	0.44	MSU_Bt_01_91755
CK770704	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	ABCC3	1.16	0.03	0.44	MSU_Bt_01_92636
BE487204	cut-like 1, CCAAT displacement protein (Drosophila)	CUTL1	1.14	0.03	0.44	MSU_Bt_01_93276
CB531176	general transcription factor II, i	GTF2I	1.12	0.03	0.44	MSU_Bt_01_92748
AY491054.1	neuropeptide Y	NPY	1.14	0.03	0.44	MSU_Bt_01_93290
CK770945	mannosyl (alpha-1,6-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase	MGAT2	1.16	0.03	0.44	MSU_Bt_01_92627
BF776344	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	PFKFB4	1.22	0.03	0.44	MSU_Bt_01_93164
NM_174216.1	vascular endothelial growth factor	VEGF	1.10	0.03	0.44	MSU_Bt_01_91629
CK954566	isocitrate dehydrogenase 3 (NAD+) gamma	IDH3G	0.94	0.03	0.44	MSU_Bt_01_92000
CB170514	ribosomal protein L7	RPL7	1.17	0.03	0.44	MSU_Bt_01_92972
NM_174126.2	natriuretic peptide receptor B/guanylate cyclase B (atrionatriuretic peptide receptor B)	NPR2	0.86	0.03	0.44	MSU_Bt_01_91661
NM_174516.2	cholinergic receptor, nicotinic, beta polypeptide 1 (muscle)	CHRNB1	0.81	0.03	0.44	MSU_Bt_01_91516
CK769938	quinoid dihydropteridine reductase	QDPR	0.92	0.03	0.44	MSU_Bt_01_92669
NM_174129.2	5'-nucleotidase, ecto (CD73)	NT5E	0.76	0.03	0.44	MSU_Bt_01_91660
CK944455	zinc finger protein 134 (clone pHZ-15)	ZNF134	0.81	0.03	0.44	MSU_Bt_01_92105
CB172413	aconitase 1, soluble	ACO1	1.05	0.03	0.44	MSU_Bt_01_92959
NM_173951.2	plasminogen	PLG	1.09	0.03	0.44	MSU_Bt_01_91735
CK775178	K562 cell-derived leucine-zipper-like protein 1	KLP1	0.95	0.03	0.44	MSU_Bt_01_92463
U91966.1			0.85	0.03	0.44	MSU_Bt_01_91778
NM_174229.2	adenylate cyclase 1 (brain)	ADCY1	1.15	0.03	0.44	MSU_Bt_01_91621
CK770926	UDP-N-acetyl-alpha-D-galactosamine:(N-acetylneuraminyl)-galactosylglucosylceramide N-acetylgalactosaminyltransferase (GalNAc-T)	GALGT	1.18	0.03	0.44	MSU_Bt_01_92629
CK771914	dual specificity phosphatase 1	DUSP1	0.90	0.03	0.44	MSU_Bt_01_92588
AY486456.1	platelet-derived growth factor receptor, alpha	PDGFRA	1.27	0.03	0.44	MSU_Bt_01_93291

	polypeptide					
CK968451	glycyl-tRNA synthetase	GARS	1.24	0.03	0.44	MSU_Bt_01_91916
CK954510	carbonyl reductase 1	CBR1	1.12	0.03	0.44	MSU_Bt_01_92003
NM_174237.2	adenylate kinase 3 like 1	AK3L1	1.18	0.03	0.44	MSU_Bt_01_91617
CK777870	arachidonate 5-lipoxygenase	ALOX5	1.38	0.04	0.44	MSU_Bt_01_92365
NM_174682.2	butyryl Coenzyme A synthetase 1	BUCS1	1.24	0.04	0.44	MSU_Bt_01_91441
AW464251	solute carrier family 7 (cationic amino acid transporter, y+ system), member 8	SLC7A8	1.11	0.04	0.44	MSU_Bt_01_93358
AY486451.1	embryonic ectoderm development	EED	1.12	0.04	0.44	MSU_Bt_01_93294
CB530559	guanine nucleotide binding protein (G protein), alpha 13	GNA13	1.11	0.04	0.44	MSU_Bt_01_92753
NM_173877.1	coagulation factor II (thrombin)	F2	1.16	0.04	0.44	MSU_Bt_01_91768
CK776094	syndecan 1	SDC1	0.86	0.04	0.44	MSU_Bt_01_92423
CK846301	polymerase (DNA directed), epsilon 2 (p59 subunit)	POLE2	1.40	0.04	0.44	MSU_Bt_01_92277
NM_181012.2	isocitrate dehydrogenase 1 (NADP+), soluble	IDH1	1.19	0.04	0.44	MSU_Bt_01_91290
CK972197	ribonucleotide reductase M1 polypeptide	RRM1	1.15	0.04	0.44	MSU_Bt_01_91889
NM_173898.2	carbonic anhydrase VI	CA6	0.90	0.04	0.44	MSU_Bt_01_91757
BF605641	zinc ribbon domain containing, 1	ZNRD1	1.16	0.04	0.44	MSU_Bt_01_93182
NM_173881.1	myoglobin	MB	1.13	0.04	0.44	MSU_Bt_01_91765
NM_173934.1	lumican	LUM	0.80	0.04	0.44	MSU_Bt_01_91740
CK848121	stathmin 1/oncoprotein 18	STMN1	0.88	0.04	0.44	MSU_Bt_01_92218
CB439768	pseudouridylate synthase 1	PUS1	1.12	0.04	0.44	MSU_Bt_01_92871
CK847702			1.21	0.04	0.44	MSU_Bt_01_92229
AF416586.1	interleukin 17 (cytotoxic T-lymphocyte-associated serine esterase 8)	IL17	1.20	0.04	0.44	MSU_Bt_01_93454
CB530939	CDC7 cell division cycle 7 (S. cerevisiae)	CDC7	1.14	0.04	0.44	MSU_Bt_01_92750
AV610239	insulin receptor substrate 1	IRS1	1.08	0.04	0.44	MSU_Bt_01_93401
NM_174621.2	transforming growth factor, beta receptor I (activin A receptor type II-like kinase, 53kDa)	TGFBR1	1.08	0.04	0.44	MSU_Bt_01_91467
CL7064Contig1	dihydrofolate reductase	DHFR	1.16	0.04	0.44	MSU_Bt_01_93542
CB420483	MAX interactor 1	MXI1	1.18	0.04	0.44	MSU_Bt_01_92942
CK943738	TAF5 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 100kDa	TAF5	1.22	0.04	0.44	MSU_Bt_01_92114
AF263826.1	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	KIT	1.22	0.04	0.44	MSU_Bt_01_93466

AV600312	gamma-glutamyltransferase-like activity 1	GGTLA1	1.15	0.04	0.44	MSU_Bt_01_93417
CB444358	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 4	ELOVL4	1.29	0.04	0.44	MSU_Bt_01_92851
NM_177523.2	suppressor of cytokine signaling 2	SOCS2	1.11	0.04	0.44	MSU_Bt_01_91309
CK944989	solute carrier family 27 (fatty acid transporter), member 2	SLC27A2	1.16	0.04	0.44	MSU_Bt_01_92100
U57745.1	tumor necrosis factor receptor superfamily, member 5	TNFRSF5	0.92	0.04	0.44	MSU_Bt_01_91789
NM_180995.2	dopamine beta-hydroxylase (dopamine beta-monooxygenase)	DBH	0.69	0.04	0.44	MSU_Bt_01_91298
AY436318.1	adiponectin receptor 1	ADIPOR1	1.08	0.05	0.44	MSU_Bt_01_93296
AJ618974.1	toll-like receptor 6	TLR6	1.17	0.05	0.44	MSU_Bt_01_93432
BF606787	protein kinase, DNA-activated, catalytic polypeptide	PRKDC	1.24	0.05	0.44	MSU_Bt_01_93178
CK846424	pancreatic lipase	PNLIP	1.18	0.05	0.44	MSU_Bt_01_92270
CB167755	adenylate cyclase 3	ADCY3	1.36	0.05	0.44	MSU_Bt_01_92989
AF229356.1	peroxisome proliferative activated receptor, alpha	PPARA	1.08	0.05	0.44	MSU_Bt_01_93471
NM_174319.1	hexokinase 1	HK1	1.15	0.05	0.44	MSU_Bt_01_91591
AJ277986.1	angiotensin II receptor, type 2	AGTR2	1.28	0.05	0.44	MSU_Bt_01_93439
NM_174178.2	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	SDHA	1.14	0.05	0.44	MSU_Bt_01_91644
CK775448	receptor-interacting serine-threonine kinase 2	RIPK2	1.12	0.05	0.44	MSU_Bt_01_92450
CK965464	elongation factor, RNA polymerase II, 2	ELL2	1.07	0.05	0.44	MSU_Bt_01_91934
AY191360.2	epidermal growth factor (beta-urogastrone)	EGF	1.09	0.05	0.44	MSU_Bt_01_93315
CK955814	ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	ENPP2	0.84	0.05	0.44	MSU_Bt_01_91989
NM_175805.2	farnesyltransferase, CAAX box, beta	FNTB	1.15	0.05	0.44	MSU_Bt_01_91379
CL10813Contig1	transforming growth factor, beta receptor III (betaglycan, 300kDa)	TGFBR3	1.39	0.05	0.44	MSU_Bt_01_93568
BE667266	solute carrier family 20 (phosphate transporter), member 1	SLC20A1	0.80	0.05	0.44	MSU_Bt_01_93270
BE685496	phenylalanine-tRNA synthetase 1 (mitochondrial)	FARS1	1.29	0.05	0.44	MSU_Bt_01_93256
AF095463.1	fibrinogen, A alpha polypeptide	FGA	1.29	0.05	0.44	MSU_Bt_01_93491
BP106073	lipase, hepatic	LIPC	1.17	0.05	0.44	MSU_Bt_01_93033
CK836343	RAR-related orphan receptor C	RORC	0.85	0.05	0.44	MSU_Bt_01_92309
CK968409	microsomal glutathione S-transferase 3	MGST3	1.09	0.05	0.44	MSU_Bt_01_91917

NM_174110.1	melanocortin 4 receptor	MC4R	1.22	0.05	0.44	MSU_Bt_01_91670
CK778464	homeodomain interacting protein kinase 2	HIPK2	1.14	0.05	0.44	MSU_Bt_01_92343
NM_174601.2	solute carrier family 1 (neutral amino acid transporter), member 5	SLC1A5	1.14	0.05	0.44	MSU_Bt_01_91478

Supplementary Table 3: Significant BMET microarray genes: IGF vs. IGF-I+LEP

BMET ID	Gene Name	Symbol	Fold Change	Raw P-value	P-FDR	HarvardID
CK774383	protein kinase D2	PRKD2	0.90	0.00	0.15	MSU_Bt_01_92489
AF519455.1	v-myc myelocytomatosis viral oncogene homolog (avian)	MYC	1.09	0.00	0.15	MSU_Bt_01_93445
CK849107	mitochondrial ribosomal protein L11	MRPL11	0.88	0.00	0.26	MSU_Bt_01_92175
CB463726	microsomal glutathione S-transferase 1	MGST1	1.17	0.00	0.26	MSU_Bt_01_92777
CK775118	phosphofructokinase, liver	PFKL	0.79	0.00	0.26	MSU_Bt_01_92466
BM254199	asparagine-linked glycosylation 12 homolog (yeast, alpha-1,6-mannosyltransferase)	ALG12	0.87	0.00	0.26	MSU_Bt_01_93085
CK975157	glutathione S-transferase kappa 1	GSTK1	0.88	0.00	0.29	MSU_Bt_01_91859
CF930077	sine oculis homeobox homolog 1 (Drosophila)	SIX1	0.88	0.00	0.29	MSU_Bt_01_92716
CK964253	phosphatidylinositol glycan, class F	PIGF	0.87	0.00	0.29	MSU_Bt_01_91939
CL6141Contig1	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	EHHADH	1.15	0.00	0.29	MSU_Bt_01_93550
CK953248	UDP glycosyltransferase 2 family, polypeptide B17	UGT2B17	1.12	0.00	0.29	MSU_Bt_01_92024
CF615578	glycogen synthase 1 (muscle)	GYS1	1.18	0.00	0.29	MSU_Bt_01_92720
BM089871	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	ETS1	1.08	0.00	0.29	MSU_Bt_01_93100
CK772384	patched homolog (Drosophila)	PTCH	1.12	0.00	0.29	MSU_Bt_01_92566
NM_174085.1	interferon, alpha 2	IFNA2	1.20	0.00	0.29	MSU_Bt_01_91684
CK976481	thymidylate synthetase	TYMS	1.25	0.00	0.45	MSU_Bt_01_91850
CK971125	solute carrier family 35, member B2	SLC35B2	0.89	0.00	0.46	MSU_Bt_01_91900
NM_174613.2	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	SLC7A5	1.03	0.01	0.57	MSU_Bt_01_91470
CB534532	steroid receptor RNA activator 1	SRA1	0.82	0.01	0.57	MSU_Bt_01_92733
CK967279	nuclear transcription factor Y, beta	NFYB	0.95	0.01	0.57	MSU_Bt_01_91921
NM_174367.1	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	ITGAV	0.89	0.01	0.57	MSU_Bt_01_91569
CK774167	methyl CpG binding protein 2 (Rett syndrome)	MECP2	1.09	0.01	0.57	MSU_Bt_01_92496
NM_173977.2	aconitase 2, mitochondrial	ACO2	0.92	0.01	0.57	MSU_Bt_01_91726

NM_177958.1	endothelin converting enzyme 2	ECE2	1.13	0.01	0.57	MSU_Bt_01_91304
BE754109	ash1 (absent, small, or homeotic)-like (Drosophila)	ASH1L	0.90	0.01	0.57	MSU_Bt_01_93232
BE758175	axin 1	AXIN1	1.27	0.01	0.57	MSU_Bt_01_93221
CK941897	low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor)	LRP1	0.91	0.01	0.57	MSU_Bt_01_92127
M76545.1	casein kinase 1, epsilon	CSNK1E	0.85	0.01	0.57	MSU_Bt_01_91800
BP109690	serum response factor (c-fos serum response element-binding transcription factor)	SRF	0.83	0.01	0.57	MSU_Bt_01_93025
CL6665Contig1	cell division cycle 42 (GTP binding protein, 25kDa)	CDC42	1.12	0.01	0.57	MSU_Bt_01_93544
AH006932.1	acetylcholinesterase (YT blood group)	ACHE	0.89	0.01	0.57	MSU_Bt_01_93442
NM_181028.2	cellular retinoic acid binding protein 1	CRABP1	0.85	0.01	0.57	MSU_Bt_01_91284
CK770847	heme oxygenase (decycling) 1	HMOX1	1.07	0.01	0.57	MSU_Bt_01_92632
AV601057	activating transcription factor 4 (tax-responsive enhancer element B67)	ATF4	1.09	0.01	0.57	MSU_Bt_01_93412
BE754636	phosphatidylinositol glycan, class T	PIGT	1.10	0.02	0.57	MSU_Bt_01_93230
NM_174041.2	dihydropyrimidine dehydrogenase	DPYD	0.88	0.02	0.57	MSU_Bt_01_91698
AB110822.1	hepatocyte growth factor (hepapoietin A; scatter factor)	HGF	1.18	0.02	0.57	MSU_Bt_01_93515
BP110719	transforming growth factor, beta 2	TGFB2	0.90	0.02	0.57	MSU_Bt_01_93023
CB430950	hypothetical protein MGC2744	MGC2744	0.91	0.02	0.57	MSU_Bt_01_92896
CK769924	mitogen-activated protein kinase kinase 4	MAP2K4	0.86	0.02	0.57	MSU_Bt_01_92671
NM_174239.2	aldehyde dehydrogenase 1 family, member A1	ALDH1A1	1.21	0.02	0.57	MSU_Bt_01_91616
NM_174198.3	toll-like receptor 4	TLR4	1.18	0.02	0.57	MSU_Bt_01_91636
BI898840	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	MYCN	1.05	0.02	0.57	MSU_Bt_01_93111
CK770119	pyrroline-5-carboxylate reductase family, member 2	PYCR2	1.15	0.02	0.57	MSU_Bt_01_92662
NM_173970.2	vasoactive intestinal peptide	VIP	1.10	0.02	0.57	MSU_Bt_01_91728
CK773032	phosphodiesterase 4C, cAMP-specific (phosphodiesterase E1 dunce homolog, Drosophila)	PDE4C	0.90	0.02	0.57	MSU_Bt_01_92545
CK769918	proprotein convertase subtilisin/kexin type 7	PCSK7	1.24	0.02	0.57	MSU_Bt_01_92672
NM_173929.2			1.22	0.02	0.57	MSU_Bt_01_91742
AW483044	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	NFKBIB	0.86	0.02	0.57	MSU_Bt_01_93352
CK954815	amiloride binding protein 1 (amine oxidase (copper-	ABP1	0.85	0.02	0.57	MSU_Bt_01_91997

	containing))					
CK771947	inositol 1,4,5-trisphosphate 3-kinase A	ITPKA	0.87	0.02	0.57	MSU_Bt_01_92587
CK940515	likely ortholog of mouse signaling intermediate in Toll pathway-evolutionarily conserved	SITPEC	1.07	0.02	0.57	MSU_Bt_01_92137
CK773068	thrombopoietin (myeloproliferative leukemia virus oncogene ligand, megakaryocyte growth and development factor)	THPO	0.85	0.02	0.57	MSU_Bt_01_92540
CB420822	basic helix-loop-helix domain containing, class B, 2	BHLHB2	0.87	0.02	0.57	MSU_Bt_01_92940
CK770077	cAMP responsive element binding protein 3-like 1	CREB3L1	0.82	0.02	0.57	MSU_Bt_01_92663
BI535045	arginase, type II	ARG2	1.08	0.02	0.57	MSU_Bt_01_93152
CB168874	ectonucleoside triphosphate diphosphohydrolase 4	ENTPD4	1.12	0.02	0.57	MSU_Bt_01_92983
CK950615	RAB2, member RAS oncogene family	RAB2	0.91	0.02	0.57	MSU_Bt_01_92056
NM_174378.2	lactalbumin, alpha-	LALBA	1.21	0.02	0.57	MSU_Bt_01_91566
NM_175826.2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8, 19kDa	NDUFA8	0.95	0.02	0.57	MSU_Bt_01_91365
CB171300	egl nine homolog 1 (C. elegans)	EGLN1	0.95	0.02	0.57	MSU_Bt_01_92967
NM_174844.1	glycine cleavage system protein H (aminomethyl carrier)	GCSH	0.92	0.02	0.57	MSU_Bt_01_91399
NM_173923.2	interleukin 6 (interferon, beta 2)	IL6	1.11	0.02	0.57	MSU_Bt_01_91746
CB422062	inositol polyphosphate phosphatase-like 1	INPPL1	1.10	0.02	0.57	MSU_Bt_01_92934
CK848538	heat shock 70kDa protein 4	HSPA4	0.84	0.02	0.57	MSU_Bt_01_92202
NM_174390.2	matrix metalloproteinase 14 (membrane-inserted)	MMP14	1.25	0.03	0.57	MSU_Bt_01_91563
NM_174767.2	visual system homeobox 1 homolog, CHX10-like (zebrafish)	VSX1	0.86	0.03	0.57	MSU_Bt_01_91419
AY309439.1	metal-regulatory transcription factor 1	MTF1	1.11	0.03	0.57	MSU_Bt_01_93301
NM_180997.1			1.17	0.03	0.57	MSU_Bt_01_91296
NM_174537.1	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	FCER1G	1.09	0.03	0.57	MSU_Bt_01_91511
NM_174822.2	dehydrogenase/reductase (SDR family) member 4	DHRS4	0.86	0.03	0.57	MSU_Bt_01_91401
CK959659	uronyl-2-sulfotransferase	UST	1.19	0.03	0.57	MSU_Bt_01_91962
AY238475.2	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	NR3C1	0.86	0.03	0.57	MSU_Bt_01_93309
CB168917	pituitary tumor-transforming 1	PTTG1	1.11	0.03	0.57	MSU_Bt_01_92982
CB460441	GDP-mannose pyrophosphorylase A	GMPPA	0.87	0.03	0.57	MSU_Bt_01_92793

CK773329	iduronidase, alpha-L-	IDUA	0.85	0.03	0.57	MSU_Bt_01_92531
AB110600.1	myogenin (myogenic factor 4)	MYOG	1.11	0.03	0.57	MSU_Bt_01_93516
NM_174076.2	glutathione peroxidase 1	GPX1	0.93	0.03	0.57	MSU_Bt_01_91688
NM_174304.1	cytochrome P450, family 17, subfamily A, polypeptide 1	CYP17A1	1.17	0.03	0.57	MSU_Bt_01_91601
CK835459	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 2	B4GALT2	1.08	0.03	0.57	MSU_Bt_01_92310
Bauman677	achaete-scute complex-like 2 (Drosophila)	ASCL2	1.18	0.03	0.57	MSU_Bt_01_93576
CK773664	early growth response 4	EGR4	1.12	0.03	0.57	MSU_Bt_01_92518
NM_174067.2			1.11	0.03	0.57	MSU_Bt_01_91689
NM_173990.1	adenosylmethionine decarboxylase 1	AMD1	0.93	0.03	0.57	MSU_Bt_01_91721
NM_174660.1	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6	SLC25A6	0.92	0.03	0.57	MSU_Bt_01_91450
NM_174742.2	adipocyte, C1Q and collagen domain containing	ACDC	1.10	0.03	0.57	MSU_Bt_01_91427
AY436318.1	adiponectin receptor 1	ADIPOR1	0.92	0.03	0.57	MSU_Bt_01_93296
AY241931.1	upstream transcription factor 1	USF1	1.15	0.03	0.57	MSU_Bt_01_93307
Bauman1497	KIAA0669 gene product	KIAA0669	0.84	0.03	0.57	MSU_Bt_01_93612
CK849436	nucleoside phosphorylase	NP	0.83	0.03	0.57	MSU_Bt_01_92165
CK775577	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7	HSD3B7	1.15	0.03	0.57	MSU_Bt_01_92438
CB464145	TNF receptor-associated factor 3	TRAF3	0.93	0.03	0.57	MSU_Bt_01_92773
NM_174027.2	colony stimulating factor 2 (granulocyte-macrophage)	CSF2	1.18	0.03	0.57	MSU_Bt_01_91704
CK970235	Kruppel-like factor 2 (lung)	KLF2	0.91	0.04	0.57	MSU_Bt_01_91903
CK774197	glutaminase 2 (liver, mitochondrial)	GLS2	0.84	0.04	0.57	MSU_Bt_01_92495
CB223228	pogo transposable element with ZNF domain	POGZ	1.07	0.04	0.57	MSU_Bt_01_92953
BM364844	zinc finger protein 174	ZNF174	1.15	0.04	0.57	MSU_Bt_01_93065
CK949072	chemokine (C-C motif) receptor 3	CCR3	0.85	0.04	0.57	MSU_Bt_01_92065
CK773713	chromosome 20 open reading frame 17	C20orf17	0.90	0.04	0.57	MSU_Bt_01_92515
CK848612	aminolevulinate, delta-, synthase 1	ALAS1	0.91	0.04	0.57	MSU_Bt_01_92199
CB456647			0.89	0.04	0.57	MSU_Bt_01_92806
BI540229	interferon regulatory factor 5	IRF5	1.09	0.04	0.57	MSU_Bt_01_93133
NM_181002.1	amine oxidase, copper containing 3 (vascular adhesion protein 1)	AOC3	0.96	0.04	0.57	MSU_Bt_01_91293

NM_174808.1	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa	NDUFV1	0.88	0.04	0.57	MSU_Bt_01_91411
CK774362	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6	ARHGEF6	0.90	0.04	0.57	MSU_Bt_01_92491
NM_174088.1	interleukin 10	IL10	1.07	0.04	0.57	MSU_Bt_01_91682
BF077122	TAF6 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 80kDa	TAF6	0.87	0.04	0.57	MSU_Bt_01_93193
BE752621	G protein-coupled receptor kinase interactor 2	GIT2	1.11	0.04	0.57	MSU_Bt_01_93237
AY318752.1	hepatocyte nuclear factor 4, alpha	HNF4A	0.92	0.04	0.57	MSU_Bt_01_93300
CK769843	activating signal cointegrator 1 complex subunit 3-like 1	ASCC3L1	1.18	0.04	0.57	MSU_Bt_01_92677
L29425.1	protein tyrosine phosphatase, receptor-type, Z polypeptide 1	PTPRZ1	0.94	0.04	0.57	MSU_Bt_01_91809
NM_174475.2	three prime repair exonuclease 1	TREX1	1.10	0.04	0.57	MSU_Bt_01_91524
CK849717	aldolase C, fructose-bisphosphate	ALDOC	0.81	0.04	0.57	MSU_Bt_01_92152
NM_173960.1	somatostatin	SST	0.86	0.04	0.57	MSU_Bt_01_91731
CK954611	phosphatidylinositol glycan, class Q	PIGQ	1.12	0.04	0.57	MSU_Bt_01_91998
CK848838	CAP, adenylate cyclase-associated protein 1 (yeast)	CAP1	1.14	0.04	0.57	MSU_Bt_01_92190
CK777467	pepsinogen 5, group I (pepsinogen A)	PGA5	1.14	0.04	0.57	MSU_Bt_01_92378
CB166648	SMAD, mothers against DPP homolog 3 (Drosophila)	SMAD3	1.06	0.04	0.57	MSU_Bt_01_93000
NM_174365.2			1.11	0.04	0.57	MSU_Bt_01_91570
CB445614	neuromedin B	NMB	1.05	0.04	0.57	MSU_Bt_01_92845
CK771135	adrenergic, alpha-1B-, receptor	ADRA1B	1.18	0.04	0.57	MSU_Bt_01_92616
NM_174352.2	insulin-like growth factor 2 receptor	IGF2R	0.91	0.04	0.57	MSU_Bt_01_91579
CK953200	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	PCK2	0.89	0.04	0.57	MSU_Bt_01_92025
CK951562	MAX dimerization protein 1	MAD	0.92	0.05	0.57	MSU_Bt_01_92041
AV612120	cyclin D2	CCND2	0.83	0.05	0.57	MSU_Bt_01_93398
CK846743	aldehyde reductase (aldose reductase) like 6	ALDRL6	1.14	0.05	0.57	MSU_Bt_01_92255
CB456064	Ran GTPase activating protein 1	RANGAP1	0.84	0.05	0.57	MSU_Bt_01_92809
CK846035	FK506 binding protein 12-rapamycin associated protein 1	FRAP1	1.11	0.05	0.57	MSU_Bt_01_92292
NM_174124.1	natriuretic peptide precursor A	NPPA	1.07	0.05	0.57	MSU_Bt_01_91662
CK771188	calcineurin binding protein 1	CABIN1	1.14	0.05	0.57	MSU_Bt_01_92613
NM_174173.2	G protein-coupled receptor kinase 1	GRK1	1.05	0.05	0.57	MSU_Bt_01_91646
NM_177485.2	aminomethyltransferase (glycine cleavage system protein T)	AMT	0.89	0.05	0.57	MSU_Bt_01_91337

NM_174808.1	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa	NDUFV1	0.88	0.04	0.57	MSU_Bt_01_91411
CK774362	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6	ARHGEF6	0.90	0.04	0.57	MSU_Bt_01_92491
NM_174088.1	interleukin 10	IL10	1.07	0.04	0.57	MSU_Bt_01_91682
BF077122	TAF6 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 80kDa	TAF6	0.87	0.04	0.57	MSU_Bt_01_93193
BE752621	G protein-coupled receptor kinase interactor 2	GIT2	1.11	0.04	0.57	MSU_Bt_01_93237
AY318752.1	hepatocyte nuclear factor 4, alpha	HNF4A	0.92	0.04	0.57	MSU_Bt_01_93300
CK769843	activating signal cointegrator 1 complex subunit 3-like 1	ASCC3L1	1.18	0.04	0.57	MSU_Bt_01_92677
L29425.1	protein tyrosine phosphatase, receptor-type, Z polypeptide 1	PTPRZ1	0.94	0.04	0.57	MSU_Bt_01_91809
NM_174475.2	three prime repair exonuclease 1	TREX1	1.10	0.04	0.57	MSU_Bt_01_91524
CK849717	aldolase C, fructose-bisphosphate	ALDOC	0.81	0.04	0.57	MSU_Bt_01_92152
NM_173960.1	somatostatin	SST	0.86	0.04	0.57	MSU_Bt_01_91731
CK954611	phosphatidylinositol glycan, class Q	PIGQ	1.12	0.04	0.57	MSU_Bt_01_91998
CK848838	CAP, adenylate cyclase-associated protein 1 (yeast)	CAP1	1.14	0.04	0.57	MSU_Bt_01_92190
CK777467	pepsinogen 5, group I (pepsinogen A)	PGA5	1.14	0.04	0.57	MSU_Bt_01_92378
CB166648	SMAD, mothers against DPP homolog 3 (Drosophila)	SMAD3	1.06	0.04	0.57	MSU_Bt_01_93000
NM_174365.2			1.11	0.04	0.57	MSU_Bt_01_91570
CB445614	neuromedin B	NMB	1.05	0.04	0.57	MSU_Bt_01_92845
CK771135	adrenergic, alpha-1B-, receptor	ADRA1B	1.18	0.04	0.57	MSU_Bt_01_92616
NM_174352.2	insulin-like growth factor 2 receptor	IGF2R	0.91	0.04	0.57	MSU_Bt_01_91579
CK953200	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	PCK2	0.89	0.04	0.57	MSU_Bt_01_92025
CK951562	MAX dimerization protein 1	MAD	0.92	0.05	0.57	MSU_Bt_01_92041
AV612120	cyclin D2	CCND2	0.83	0.05	0.57	MSU_Bt_01_93398
CK846743	aldehyde reductase (aldose reductase) like 6	ALDRL6	1.14	0.05	0.57	MSU_Bt_01_92255
CB456064	Ran GTPase activating protein 1	RANGAP1	0.84	0.05	0.57	MSU_Bt_01_92809
CK846035	FK506 binding protein 12-rapamycin associated protein 1	FRAP1	1.11	0.05	0.57	MSU_Bt_01_92292
NM_174124.1	natriuretic peptide precursor A	NPPA	1.07	0.05	0.57	MSU_Bt_01_91662
CK771188	calcineurin binding protein 1	CABIN1	1.14	0.05	0.57	MSU_Bt_01_92613
NM_174173.2	G protein-coupled receptor kinase 1	GRK1	1.05	0.05	0.57	MSU_Bt_01_91646
NM_177485.2	aminomethyltransferase (glycine cleavage system protein T)	AMT	0.89	0.05	0.57	MSU_Bt_01_91337

BF074766	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1B	DYRK1B	1.11	0.05	0.57	MSU_Bt_01_93197
CK772526	phosphatidylserine synthase 1	PTDSS1	0.79	0.05	0.57	MSU_Bt_01_92562
CK848974	UDP-N-acteylglucosamine pyrophosphorylase 1	UAP1	0.85	0.05	0.57	MSU_Bt_01_92183
CL513Contig1	chemokine (C-C motif) ligand 4-like	CCL4L	1.15	0.05	0.57	MSU_Bt_01_93554
NM_174093.1	interleukin 1, beta	IL1B	1.18	0.05	0.57	MSU_Bt_01_91677
CK771728	glyoxalase I	GLO1	1.12	0.05	0.57	MSU_Bt_01_92593
CK770969	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	NFKB1	0.82	0.05	0.57	MSU_Bt_01_92625
CK775768	v-myb myeloblastosis viral oncogene homolog (avian)-like 2	MYBL2	1.22	0.05	0.57	MSU_Bt_01_92435
BF043952	membrane metallo-endopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10)	MME	0.93	0.05	0.57	MSU_Bt_01_93203
NM_174218.1	tryptophanyl-tRNA synthetase	WARS	1.10	0.05	0.57	MSU_Bt_01_91627
CK948095	hydroxysteroid (17-beta) dehydrogenase 2	HSD17B2	1.09	0.05	0.57	MSU_Bt_01_92080
CL5741Contig1	phospholipase A2, group IVB (cytosolic)	PLA2G4B	1.24	0.05	0.57	MSU_Bt_01_93551
NM_174243.2	arrestin, beta 1	ARRB1	0.90	0.05	0.57	MSU_Bt_01_91614
NM_174018.2	cystic fibrosis transmembrane conductance regulator, ATP-binding cassette (sub-family C, member 7)	CFTR	1.07	0.05	0.57	MSU_Bt_01_91708
NM_174585.2	protein kinase, cAMP-dependent, catalytic, beta	PRKACB	0.96	0.05	0.57	MSU_Bt_01_91485
BI898520	solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulator 1	SLC9A3R1	1.15	0.05	0.57	MSU_Bt_01_93113
NM_173907.2	dopa decarboxylase (aromatic L-amino acid decarboxylase)	DDC	0.84	0.05	0.57	MSU_Bt_01_91753
D50045.1	major histocompatibility complex, class II, DQ alpha 1	HLA-DQA1	0.91	0.05	0.57	MSU_Bt_01_93508
M15886.1	diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein)	DBI	0.94	0.05	0.57	MSU_Bt_01_91806
CB419791	pantothenate kinase 1	PANK1	0.90	0.05	0.57	MSU_Bt_01_92949
AW461340	mitogen-activated protein kinase kinase kinase 3	MAP3K3	1.22	0.05	0.57	MSU_Bt_01_93365
AV589230	SREBP CLEAVAGE-ACTIVATING PROTEIN	SCAP	1.20	0.05	0.57	MSU_Bt_01_93422
NM_173878.2	coagulation factor III (thromboplastin, tissue factor)	F3	1.16	0.05	0.57	MSU_Bt_01_91767
BI535743	glycogen synthase kinase 3 alpha	GSK3A	1.23	0.05	0.57	MSU_Bt_01_93149
CK979058	chromosome condensation-related SMC-associated protein 1	CNAP1	1.16	0.05	0.57	MSU_Bt_01_91829

CK770419	RuvB-like 1 (E. coli)	RUVBL1	0.94	0.05	0.57	MSU_Bt_01_92648
CK771154	eukaryotic elongation factor-2 kinase	EEF2K	0.88	0.05	0.57	MSU_Bt_01_92614