2014

Regulation of β-Casein Gene Expression by Octamer Transcription Factors and Utilization of β-Casein Gene Promoter to Produce Recombinant Human Proinsulin in the Transgenic Milk

Xi Qian
University of Vermont, xqian@uvm.edu

Regulation of β-Casein Gene Expression by Octamer Transcription Factors and Utilization of β-Casein Gene Promoter to Produce Recombinant Human Proinsulin in the Transgenic Milk

A Dissertation Presented

by

Xi Qian

To

The Faculty of the Graduate College

Of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Animal Science

October, 2014

Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Doctor of philosophy, specializing in Animal Science.

Dissertation Examination Committee:

_____ Advisor
Feng-Qi Zhao, Ph.D

_____
Bryan A. Ballif, Ph.D.

_____
Robert J. Kelm Jr, Ph.D.

_____
André-Denis G. Wright, Ph.D.

_____ Chairperson
Wolfgang R. Dostmann, Ph.D.

_____ Dean, Graduate College
Cynthia J. Forehand, Ph.D.

August 19, 2014

Abstract

β-Casein is a major milk protein, which is synthesized in mammary alveolar secretory epithelial cells (MECs) upon the stimulation of lactogenic hormones, mainly prolactin and glucocorticoids (HP). Previous studies revealed that the proximal promoter (-258 bp to +7 bp) of the β-casein gene is sufficient for induction of the promoter activity by HP. This proximal region contains the binding sites for the signal transducer and activator of transcription 5 (STAT5), glucocorticoid receptor (GR), and octamer transcription factors (Oct). STAT5 and GR are essential downstream mediators of prolactin and glucocorticoid signaling, respectively. This study investigated the functions of Oct-1 and Oct-2 in HP induction of β-casein gene expression. By transiently transfection experiment, we showed that individual overexpression of Oct-1 and Oct-2 further enhanced HP-induced β-casein promoter activity, respectively, while Oct-1 and Oct-2 knockdown significantly inhibited the HP-induced β-casein promoter activity, respectively. HP rapidly induced the binding of both Oct-1 and Oct-2 to the β-casein promoter, and this induction was not mediated by either increasing their expression or inducing their translocation to the nucleus. In MECs, Oct-2 was found to physically interact with Oct-1 regardless of HP treatment. However, HP induced physical interactions of Oct-1 or Oct-2 with both STAT5 and GR. Although the interaction between Oct-1 and Oct-2 did not synergistically stimulate HP-induced β-casein gene promoter activity, the synergistic effect was observed for the interactions of Oct-1 or Oct-2 with STAT5 and GR. The interactions of Oct-1 with STAT5 and GR enhanced or stabilized the binding of STAT5 and GR to the promoter. Abolishing the interaction between Oct-1 and STAT5 significantly reduced the hormonal induction of β-casein gene transcription. Thus, our study indicates that HP activate β-casein gene expression by inducing the physical interactions of Oct-1 and Oct-2 with STAT5 and GR in mouse MECs.

There is a high and increasing demand for insulin because of the rapid increase in diabetes incidence worldwide. However, the current manufacturing capacities can barely meet the increasing global demand for insulin, and the cost of insulin production keeps rising. The mammary glands of dairy animals have been regarded as ideal bioreactors for mass production of therapeutically important human proteins. We tested the feasibility of producing human proinsulin in the milk of transgenic mice. In this study, four lines of transgenic mice were generated to harbor the human insulin gene driven by the goat β-casein gene promoter. The recombinant human proinsulin was detected in the milk by Western blotting and enzyme-linked immunosorbent assay. The highest expression level of human proinsulin was as high as 8.1 μg/μl in milk of transgenic mice at mid-lactation. The expression of the transgene was only detected in the mammary gland during lactation. The transgene expression profile throughout lactation resembled the milk yield curve, with higher expression level at middle lactation and lower expression level at early and late lactation. The blood glucose and insulin levels and major milk compositions of transgenic mice were not changed. The mature insulin derived from the milk proinsulin retained biological activity. Thus, our study indicates that it is practical to produce high levels of human proinsulin in the milk of dairy animals, such as dairy cattle and goat.

Citations

Materials from this dissertation have been published in the following forms:

Qian, X. and Zhao, F.-Q.. (2013). Interactions of the ubiquitous octamer-binding transcription factor-1 with both the signal transducer and activator of transcription 5 and the glucocorticoid receptor mediate prolactin and glucocorticoid-induced β-casein gene expression in mammary epithelial cells. The International Journal of Biochemistry & Cell Biology, 45, 724-735.

Qian, X. and Zhao, F.-Q.. (2014). Collaborative interaction of Oct-2 with Oct-1 in transactivation of lactogenic hormones-induced β-casein gene expression in mammary epithelial cells. General and Comparative Endocrinology, 204, 185–194.

AND

Materials from this dissertation have been submitted for publication, in the following form:

Qian, X., Kraft, J., Ni, Y., and Zhao F.-Q.. Production of recombinant human proinsulin in the milk of transgenic mice. Scientific Reports.

Acknowledgments

I would like to give my sincere gratitude to Dr. Feng-Qi Zhao for providing me the opportunity of being his student. Over the past five years, his broad vision, logical thinking, and extensive knowledge have inspired me in both my study and research. I wish to express my thanks and appreciation to my academic committee members, Dr. Bryan Ballif, Dr. Wolfgang Dostmann, Dr. Robert Kelm, and Dr. André-Denis Wright for their valuable comments, suggestions, and support for my study and research.

I would like to thank my labmates, Yogi Mishra, Pamela Bentley, Yong Shao, and Shun Kitaoka for their daily support and help. Many thanks go to the ASCI faculty members, staff, and my fellow graduate students for providing me support and making my stay at UVM that much more pleasurable. The warmth that everyone has shown has made up for all the biting-cold winter days in VT. I also wish to thank all my friends for their friendship and encouragement.

I would like to thank my wife with all my heart, Yi, who quit her job and joined me in US three years ago. I could not go this far without her support and understanding. Special thanks go to my daughter, Jasmine. No matter how low my mood is, she manages to cheer me up. The deepest thanks go to my father Zhonggen Qian and my mother Geping Pan. Although they are on the other side of the earth, their love and support are always there for me.

Table of Contents

List of Tables

List of Figures

Chapter 1 Literature review

REGULATION OF MILK PROTEIN GENE EXPRESSION

Milk is the primary source of nutrients for neonates before they are able to consume and digest other types of foods [1]. For hundreds of years, dairy milk and other agricultural products derived from dairy milk (e.g., cheese, butter, and yogurt) have been important foods for humans [2]. Among the many nutrients provided in dairy milk and dairy products, milk protein is an important part of daily protein intake in the human diet. Milk protein provides a high biological value to human health; it is a good source of essential amino acids, and the amino acid composition of major milk proteins is well-balanced for human body utilization [3, 4].

Studies on the regulation of milk protein gene expression in the mammary gland date back to more than a half century ago. Knowledge generated from these studies has not only provided fundamental insight into genetic and nutritional improvement in milk composition and milk production, but it has also elucidated the molecular mechanisms of tissue-specific gene expression. Furthermore, with the development of genetic engineering technology, the promoters of different milk protein genes have been employed for directing the expression of pharmaceutically important proteins in the milk of transgenic livestock, which is the driving force of the emerging “pharming” industry. The studies of the regulation of milk protein gene expression began as a result of the development of endocrine organ surgical ablation, access to pure hormones for

replacement therapy, and the development of mammary explant cultures six decades ago [5]. These early studies demonstrated that basic hormone complexes, namely the lactogenic hormone prolactin (PRL), glucocorticoids (GCs), and insulin (INS), synergistically activate milk protein gene expression [6, 7]. Later studies have focused on the molecular pathways involved in the operations of individual hormones and the interactions of these pathways. The full view of the molecular details of these pathways is emerging: milk protein gene expression is regulated at multiple levels within mammary epithelial cells and depends on concerted actions of hormones, local growth factors, cell-cell interactions, and cell-extracellular matrix (ECM) interactions that modulate the function of specific transcription factors, alter cytoskeletal organization, and change the chromatin state and nuclear structure.

In this review, we aim to provide an overview of the regulation of different milk protein genes (mainly transcriptional regulation) and discuss regulatory mechanisms from the perspective of epigenetics and chromatin. We mainly focus on data that have accumulated since two previous reviews on this subject published more than fifteen years ago [8, 9].

Major milk proteins

Although the proteins in milk can arise from different sources, the focus of this review is on the major proteins that are specifically synthesized in mammary epithelial cells. These mammary-specific proteins in mammals can be grouped into two categories: caseins and whey proteins. The proteins in cow milk contain ~80% caseins and ~20% whey proteins, whereas the proteins in human milk comprise ~40% caseins and ~60%

whey proteins [10]. Caseins are a family of related phosphoproteins, which include αS1-, αS2-, β-, and *κ*-caseins. αS1-, αS2-, and β-casein are characterized as calcium-sensitive caseins because of their quantitative precipitation with calcium chloride, while *κ*-casein does not precipitate with calcium chloride and can quantitatively stabilize calcium-sensitive caseins [11]. The appropriate amino acid composition of caseins and their high digestibility make milk essential for the growth and development of neonates. As a food source, they provide not only large amounts of amino acids but also phosphorus and calcium. In the industry, caseins have a wide variety of applications, ranging from being a major component of cheese, being used as a food additive, and being used in non-food applications, such as casein-based coating or sizing agents [12]. As a result of their relatively hydrophobic nature, individual caseins are not very soluble in aqueous environments; however, caseins can form multi-molecular, spherical casein micelles and thus become colloidally suspended in milk [13]. Caseins can be precipitated from milk by reducing the pH to disturb the charge equilibrium inside colloidal micelles, resulting in a yellow whey solution. Whey is composed of water, lactose, mammary-specific whey proteins [α-lactalbumin, β-lactoglobulin, and whey acidic protein (WAP)], and non-mammary-specific whey proteins (serum albumin, immunoglobulins, growth factors, etc). Whey proteins have been extensively studied for their anti-inflammatory and anti-cancer properties [14] and potential use as a supplementary treatment for diseases [15]. Human mammary-specific whey proteins contain only α-lactalbumin, mouse whey proteins contain α-lactalbumin and WAP, and cow whey proteins contain α-lactalbumin and β-lactoglobulin. α-Lactalbumin serves as a regulatory subunit for lactose synthase, and it is

critical for lactose synthesis [16]. β-Lactoglobulin is a major whey protein in cow milk and an allergen for human infants fed on formula based on cow milk [17]. WAP is important in regulating the proliferation of mammary epithelial cells [18].

The genomic location and organization of the casein and whey protein genes in humans, cattle, and mice are summarized in Table 1.1 and 1.2. As shown in Table 1.2, each of the mammary-specific whey proteins, α-lactalbumin, β-lactoglobulin, and WAP, is encoded by a single-copy gene with relatively small size in different chromosomes. In contrast, caseins are encoded by a cluster of different casein genes on the same chromosome. In humans, the αS1-, β-, and *κ*-casein genes are sequentially clustered in chromosome 4 (Table 1.1). In comparison with the human casein gene locus, there is one αS2-casein gene in cows and two related αS2-casein genes (αS2-casein-like A and αS2-casein-like B) in mice (Table 1.1). The genes encoding the calcium-sensitive caseins (αS1-, β-, and αS2-casein genes) are evolutionarily related, while the *κ*-casein gene is not derived from a common ancestral gene although its expression profile is similar to other casein genes [19].

β-Casein, which is one of the most abundant caseins, accounts for 25-35% of total caseins and ~30% of total milk protein in bovine milk [20]. Secreted bovine β-casein protein is a single polypeptide chain composed of 209 amino acid residues, with a molecular weight of 23983 Da [21]. The β-casein protein can be phosphorylated at multiple positions [22]. The presence of multiphosphorylated forms of β-casein is considered to influence casein micelle stability and the abundance and distribution of calcium in milk [22]. The β-casein gene has been thoroughly studied in different

mammals, and extensive genetic variation has been identified and characterized in bovine [23], goat [24], and camel [25]. Thus far, at least 17 alleles corresponding to 12 β-casein protein variants have been identified in bovine [23]. The most common bovine β-casein protein variants are A1 and A2 [26]. The A1 type β-casein variant evolved from its progenitor A2 type 5000 years ago due to a proline67 to histidine67 point mutation. β-Casein variants have been associated with human health. For example, *in vitro* and animal studies suggest that digesting of A1 but not A2 β-casein influences gastrointestinal inflammation and transit time through the release of beta-casomorphin-7 (BCM-7) [27, 28]. In addition, results from epidemiological studies suggest that consuming A1 type β-casein is statistically associated with higher national mortality rates from ischemic heart disease and with a high incidence of diabetes [26].

Transcriptional regulation of milk protein gene expression

Transcriptional regulation of milk protein gene expression is regulated by *cis*-regulatory regions (promoter and/or enhancer, Fig. 1.1). The *cis*-regulatory region is a stretch of DNA (100-1000 bp) with transcription factor binding sites that are clustered into modular structures. Through protein-DNA and protein-protein interactions, these modularly structured regions integrate positive and negative regulatory signal transduction pathways induced by various extracellular stimuli to regulate gene expression by controlling the initiation and/or stabilization of the transcription complex on gene promoters and enhancers [9]. Previous studies using stable or transiently transfected mammary/non-mammary cells and transgenic mice have established the functional importance of various transcription factors in the regulation of milk protein

genes (Fig. 1.1) [9]. None of these transcription factors are mammary gland specific. Therefore, it is the specific combination of these transcription factors that leads to the unique temporal and spatial expression profiles of milk protein genes.

The β-casein (Fig. 1.1A) and WAP (Fig. 1.1C) gene promoters have been extensively studied for decades as models for hormone signaling control of milk protein gene expression. β-Casein gene regulation involves two principal *cis*-regulatory regions, a proximal promoter and a distal enhancer. The core proximal promoter homologous in humans, cows, and rodents extends ~250 bp upstream from the transcription start site (TSS) of the β-casein gene, and the evolutionally conserved enhancer is located between -1.6 and -6 kb upstream of the 5' of the TSS in different mammalian species (Fig. 1.1A) [19, 29]. The hormone-responsive β-casein proximal promoter has so-called lactogenic response elements that harbor multiple or single binding site(s) for transcription factors, mainly including signal transducer and transcription activator 5 (STAT5) [30, 31], glucocorticoid receptor (GR) [32, 33], CAAT/enhancer binding protein β (C/EBPβ) [34, 35], octamer binding factor-1 (Oct-1) [36, 37], runt-related transcription factor 2 (Runx2) [38], and the repressive transcription factor Yin Yang 1 (YY-1) [39, 40]. The distal enhancer of the β-casein gene, also known as the ECM-responsive element, is responsive to ECM and lactogenic hormones and contains recognition sites for C/EBPβ and STAT5 [29, 41, 42].

The WAP gene promoter (Fig. 1.1C) also contains two regulatory regions, one proximal (-50 to -150 bp in both rat and mouse) and one distal (-720 to -820 bp in rat and -530 to -630 bp in mouse) to the TSS [43, 44]. Both of these regions contain consensus

binding sequences for a number of transcription factors, including nuclear factor 1 (NF-1) [44, 45], GR [46] and STAT5 [44], which have been demonstrated to be responsible for mammary-specific WAP gene expression.

Hormonal regulation of milk protein genes

Prolactin (PRL) and glucocorticoids (GC)

Long before the discovery of the molecular mechanisms of the activation of milk protein gene expression, researchers demonstrated the synergism between PRL and GC [6, 7]. The most extensively studied milk protein gene is the β-casein gene. Using transgenic mouse models [9, 35, 47] and cell culture systems including primary or transformed mammary epithelial cells [48-50], researchers established in the 1990s that STAT5, GR, and C/EBPβ are important signal transducers that mediate PRL and GC synergism in the induction of β-casein gene expression (Fig. 1.2). STAT5, with two closely related protein isotypes STAT5A and STAT5B, is the leading transcription factor responsible for PRL signaling [51]. The binding of PRL to the prolactin receptor (PRLR) triggers activation of Janus kinase 2 (JAK2). Activated JAK2 phosphorylates tyrosine residues on PRLR and creates docking sites for Src homology 2 (SH2) domain-containing proteins. SH2-containing STAT5 is then recruited and phosphorylated by JAK2 at a conserved tyrosine residue within the carboxyl-terminal transcriptional activation domain (Y694 for STAT5A and Y699 for STAT5B) [52, 53]. Phosphorylated STAT5 dimerizes, translocates into the nucleus, and induces β-casein gene transcription by binding to clustered STAT5 binding sites in both promoters and enhancers. Of the two STAT5 protein isotypes, STAT5A is the principal and indispensable mediator of

mammary epithelial cell differentiation and milk protein gene expression [54], whereas STAT5B is more important for growth hormone (GH) signaling in the liver [55]. GC alone can barely activate β-casein gene expression; however, GC potentiates PRL signaling through synergistic protein-protein interactions between GR and STAT5, leading to much more robust induction than PRL alone [32, 33, 56]. A GC potentiation effect occurs in a half glucocorticoid response element (1/2 GRE) binding dependent [32, 57] or independent [58, 59] manner. The interaction between GR and STAT5 promotes sustained STAT5 tyrosine phosphorylation and STAT5 DNA binding [60]. C/EBPβ is another transcription factor that binds to the β-casein proximal promoter and enhancer as a homo- or heterodimer in response to PRL and/or GC [34, 35, 61], and it is implicated in the regulation of milk protein gene expression [35, 47]. Three C/EBPβ protein isotypes, which are translated from a single C/EBPβ mRNA, have been identified, including two transcription-activating isoforms, termed liver-enriched transcriptional activator proteins (LAP and LAP2), and one inhibitory isoform, liver-enriched transcriptional inhibitory protein (LIP) [34, 62, 63]. The LAP C/EBPβ isoform has been shown to synergize with STAT5 and GR during the induction of β-casein gene expression by PRL and GC in a reconstituted COS-7 cell system [56]. The cooperative effects of STAT5 and C/EBPβ for PRL and GC-induced β-casein gene transcription are mediated by GR [56]. All of these transcription factors i.e., STAT5, GR, and C/EBPβ, interact with the p300 coactivator, which remodels the chromatin confirmation via its intrinsic histone acetyltransferase activity to facilitate gene transcription [64-66]. In addition to these positive regulatory transcription factors, YY-1 has been demonstrated to constitutively bind the β-casein

gene proximal promoter in the absence of lactogenic hormones and repress β-casein gene expression [39, 40]. The YY-1 site in the β-casein gene proximal promoter is a low-affinity site, and in response to PRL, its association can readily be interrupted by STAT5 and C/EBPβ binding at adjacent sites [39, 61].

In addition to the transcription factor binding sites mentioned above, there are two highly conserved adjacent binding sites for Oct-1 [36] and Runx2 [38] in casein gene proximal promoters [38]. It has been shown that both Oct-1 [37, 67-69] and Runx2 [38] bind to the endogenous β-casein gene promoter in mammary epithelial cells both *in vivo* and *in vitro*. Oct-1 alone can activate both basal and hormonally induced β-casein gene promoter activity [68], while Runx2 alone cannot [38]. However, Runx2 cooperates with Oct-1, leading to higher activation of the β-casein promoter than Oct-1 alone [38]. This cooperation may be explained by the fact that Oct-1 stimulates the recruitment of Runx2 to the β-casein gene promoter by physically interacting with Runx2 [38]. In addition to Runx2, Oct-1 synergizes with STAT5 and GR through physical interaction to activate β-casein gene expression in response to PRL and GC [69]. Similar to its stimulating effects on Runx2 recruitment, Oct-1 enhances or stabilizes the binding of STAT5 and GR to the β-casein promoter in response to PRL and GC [69]. In addition to the combination of PRL and GC, Oct-1 binding activity can also be induced by progesterone (PG) [37], which is a reproductive hormone that inhibits β-casein gene expression [70]. As Oct-1 has been shown to interact with the progesterone receptor (PR) [71], it is possible that Oct-1 also participates in the repression of β-casein gene expression via interaction with different transcription factors, such as PR.

PRL and GC also regulate milk protein gene expression at other levels e.g., stabilization of milk protein mRNA and milk protein mRNA translation, which have been well reviewed elsewhere [72].

Insulin (INS)

The functional role of INS in regulating milk protein production is supported by *in vitro* and *in vivo* studies. Early *in vitro* studies in mouse [73], rat [74], and bovine [75] mammary explant cultures showed that, in addition to maintaining mammary tissue in culture, INS is required for the maximum induction of the major casein and whey milk protein genes by PRL and hydrocortisone (a type of GC). In *in vivo* studies in cows using a hyperinsulinemic-euglycemic clamp (HEC) approach in which circulating INS levels were elevated four-fold while euglycemia was maintained via the infusion of exogenous glucose, milk protein yield was increased by 15% within four days of HEC treatment [76, 77]. Because the administration of extra glucose to well-fed cows does not increase milk yield [78, 79] and INS does not induce an acute increase in glucose uptake by ruminant mammary glands [80-82], the increase in milk protein yield possibly resulted from an INS stimulatory effect on mammary epithelial cells.

The underlying molecular mechanism of how INS regulates milk protein synthesis is not well understood. However, previous studies suggest that INS may play an important role in milk protein synthesis at multiple levels. First, at the transcriptional level, INS may synergistically cooperate with PRL and hydrocortisone to induce milk protein gene transcription by stimulating the expression of E74-like factor 5 (Elf5) via phosphoinositide 3-kinase (PI3K)/Akt signaling [83, 84], as demonstrated in bovine [85]

and murine [86] mammary explant cultures. Elf5 is a transcription factor that belongs to the E26 transformation-specific (Ets) family, and it regulates mammary epithelium proliferation and differentiation and the transcription of milk protein genes [87-90]. INS-dependent milk protein gene transcription may also be accomplished by INS-induced expression and the activity of STAT5 [9, 85]. Second, in CID-9 mammary epithelial cells, which were derived from the COMMA-D cells originally isolated from the mammary glands of mice in mid-pregnancy [91], INS alone or INS plus PRL increases the rate of milk protein mRNA translation, whereas PRL alone has no effect [92]. INS enhances the translation of β-casein by increasing the initiation of translation and lengthening the mRNA poly(A) tail by cytoplasmic polyadenylation element binding proteins [92]. Finally, more recent studies in cows and mice have suggested that INS may enhance protein synthesis by stimulating genes involved in folate metabolism [85, 86, 93]. Folate metabolism plays an important role in protein synthesis by accepting and releasing one-carbon units, which is also known as the one-carbon pool. A functional role of folate in milk protein synthesis is supported by the fact that folate supplementation in lactating cows results in a significant increase in milk production and milk protein yield [94-96].

Progesterone (PG)

PG is a steroid hormone secreted by the corpus luteum. PG exerts its primary action through the nuclear PR. When bound with PG, the PR dissociates from protein chaperones, dimerizes, and binds to specific binding sites in its target genes, regulating their expression by recruiting its coactivators [97]. Circulating levels of PG rises throughout pregnancy followed by a rapid decline at parturition. In the early pregnancy

stage, PG promotes mammary gland development and functional differentiation. In mid-to-late pregnancy, PG inhibits the production of milk proteins and the closing of the tight junctions until parturition. At parturition, a dramatic decline in circulating PG in combination with decreased PR expression in the mammary gland results in tight junction closure and copious milk protein production [98-100]. The repressive effects of PG on milk protein gene expression were initially observed in an experiment in which ovariectomy led to transient lactogenesis, which is characterized by the transcription of important milk protein genes such as caseins and α-lactalbuminin in late-pregnant mice, and PG but not other hormones (estrogen, PRL, or GC) abolishes the transient lactogenesis triggered by ovariectomy [101, 102].

Only recently have we begun to understand the molecular mechanism of the repression of milk protein gene expression by PG. By using different cell culture systems reconstituted to express the PR, Buser and colleagues found that direct antagonism between activated PR and STAT5/GR signaling contributes to the physiological role of PG in repressing lactogenic hormone-induced β-casein transcription in mammary epithelial cells [70]. However, direct transcriptional repression of milk protein genes by PR is unlikely to be the primary mechanism for PG repression in mammary epithelial cells during pregnancy because PR is expressed only in a scattered subset of mammary epithelial cells during pregnancy [100, 103]. It is possible that the inhibitory effects might be mediated by paracrine factors regulated by PG [104]. Transforming growth factor-β (TGFβ) is likely to be the mediator of PG action in repressing milk protein gene expression [105] because of several lines of evidence: 1) TGFβ antagonizes PRL-induced

signals in mammary epithelial cells [106, 107] and inhibits alveolar formation and the synthesis of milk proteins during pregnancy [108-112], 2) the expression profile of the TGFβ isoforms TGFβ1, TGFβ2, and TGFβ3 in the mammary gland correlates with changes in the level of plasma PG during the transition from pregnancy to lactation [108, 109], and 3) in bovine mammary cells and tissue, PG tends to have increased TGFβ expression [113, 114]. Moreover, PG significantly up-regulates TGFβ expression in normal human osteoblast-like cells [115].

Furthermore, milk protein genes are already transcribed at a considerable level during mid-to-late pregnancy [116] when PG levels are still high, implying that PG may mainly inhibit milk protein synthesis at the post-transcriptional level or milk protein secretion.

Growth hormone (GH) and insulin-like growth factor-1 (IGF-1)

Growth hormone (GH), also known as somatotropin, is a peptide hormone synthesized and secreted by the anterior pituitary. GH stimulates cell growth and changes in protein, carbohydrate, and fat metabolism. Upon binding to its cell membrane-integrated GH receptor (GHR) in target tissues, GH activates various intracellular signaling molecules to regulate gene expression and protein modifications [117]. One well-defined example of GH function is the stimulation of IGF-1 expression [118, 119]. IGF-1 is believed to mediate many of the growth-stimulating and metabolic effects of GH.

GH is well known for its galactopoietic effects on the mammary gland [119, 120]. However, the underlying mechanisms mediating the effects of GH on protein synthesis remain unclear. Early studies employing ligand binding assays failed to detect the GHR

in bovine mammary glands [121, 122], leading to the widely accepted hypothesis that GH acts on the mammary gland through IGF-1 produced locally and by the liver [123]. The direct effects of IGF-1 in the mammary gland are supported by the fact that locally increased IGF-1, either by local arterial IGF-1 infusion in the mammary gland (in lactating goats) [124] or mammary-targeted IGF-1 expression (in mice) [125], increases milk production. This result may be attributable to the ability of IGF-1 to inhibit apoptosis in the mammary gland [126] and stimulate mammary epithelial cell proliferation and glucose transport [127], as shown in cows. However, mammary-specific IGF-1 over-expression in swine [128] and rabbits [129] did not impact milk production and composition. The exact role of IGF in GH-enhanced milk yield requires further investigation.

While it is widely accepted that GH only has indirect action on mammary gland function, the direct role of GH is emerging. Recent studies have shown that both GHR mRNA and protein are found to be expressed in the stromal and epithelial tissues of the bovine mammary gland [130-132]. GHR protein expression in lactating mammary glands was found to be higher than that in non-lactating mammary glands [130]. In addition, GH is capable of stimulating the mRNA expression of milk protein genes, in bovine mammary epithelial cell lines, such as MAC-T [133, 134] and BMEC [135, 136], and bovine mammary explant cultures [137]. Furthermore, it has been recently shown that in bovine [138-141] and swine [142] mammary gland tissues, GH may up-regulate milk protein mRNA translation initiation and elongation via the mammalian target of

rapamycin (mTOR) pathway. Thus, GH may directly stimulate milk protein synthesis at the transcriptional and translational levels.

Extracellular matrix (ECM)

The ECM is a group of filamentous and insoluble proteins that are present between clusters of cells in all tissues [143]. In addition to providing tensile support, the ECM provides channels for the communication of cells in a given tissue. The ECM is categorized into two types: stromal ECM and basement membrane (BM) [143]. The stromal ECM resides in connective tissues, while the BM, also known as the basal lamina, separates the epithelium from the stroma in any given tissue. Representative BM constituents include laminin, type IV collagen, nidogen/entactin, and heparin sulfate [144]. The ECM can alter gene expression profiles by influencing cell morphology and nuclear and chromatin organization [143, 145]. The ECM performs its function by binding to cell-surface integrin receptors and initiating mechanical and chemical signaling [143].

The ECM cooperates with soluble cues, including hormones and growth factors, to guide mammary gland development, functional differentiation, alveolar morphogenesis, lactation, and involution. During lactation, the ECM is needed to induce milk protein gene expression. With the exception of mammary epithelial cell lines such as HC11, which can deposit its own laminin matrix after reaching confluence, for primary mammary epithelial cells and most other mammary epithelial cell lines, endogenous milk protein gene expression can be induced by lactogenic hormones only when they are cultured on a laminin-rich ECM [9]. The ECM has been shown to be required for the

induction of milk protein genes, such as casein [146, 147], β-lactoglobulin [148], and WAP [149] genes, in response to lactogenic hormones. The ECM-dependent regulation of milk protein genes is mediated by certain DNA sequence elements in their promoter regions. For example, promoter truncation analysis revealed an ECM-responsive element, originally named bovine casein enhancer element (BCE-1), ~1.5 kb upstream of the bovine β-casein TSS [41]. This BCE-1 element was found to be highly conserved among different species and was defined to be a β-casein distal enhancer (Fig. 1.1A) that contains binding sites for various mammary transcription factors [19, 29, 41]. More recently, the β-casein proximal promoter was found to be responsive to the ECM as well [147]. ECM-responsive elements in other milk protein genes and other genes are summarized elsewhere [145]. However, exactly how the ECM regulates milk protein genes via these ECM-responsive DNA elements is not well understood. Current evidence suggests several potential mechanisms.

One potential mechanism by which the ECM activates milk protein gene expression is by inducing the binding of mammary transcription factors to ECM-responsive DNA elements. Using primary mammary epithelial cells from mice in mid pregnancy, Streuli et al. demonstrated that in the presence of PRL, only cells cultured on laminin-rich ECM but not cells cultured on collagen I are capable of inducing STAT5 DNA binding activity [148]. These authors also found that the DNA binding activity of NF-1 is only stimulated in cells cultured on a laminin-rich ECM, but this activation is independent of PRL, whereas the DNA binding activity of specificity protein 1 (Sp1) is induced in cells cultured on plastic or collagen I substrata in the presence of PRL [148].

These data suggest that the activities of transcription factors in mammary epithelial cells are differentially regulated by the type of substratum on which the cells are cultured. Subsequently, Edwards et al. found that exposure to a laminin-rich ECM is required for the PRL-induced phosphorylation and nuclear translocation of STAT5 in primary mammary epithelial cells [150]. In EpH4 cells, which were originally isolated from the mammary tissue of a Balb/c mouse in mid pregnancy [151], PRL can induce the transient phosphorylation of STAT5 independent of a laminin-rich ECM, but sustained STAT5 activation, which is necessary for the induction of β-casein transcription, depends on the presence of a laminin-rich ECM [152]. These results suggest that the cross-talk between the ECM and PRL signaling pathway is needed for the induction of milk protein gene expression. In support of this hypothesis, it was found that the ECM cooperates with PRL to induce the binding of STAT5, C/EBP-β, and RNA polymerase II to casein gene promoters, whereas either alone fails to do so [147].

A second potential mechanism is that mediation of milk protein gene transcription by ECM-responsive DNA elements is involved in changes in chromatin structure. Studies using the CID-9 mammary epithelial cell line revealed that BCE-1 must be stably integrated into the genome to become activated by the ECM [41]. Furthermore, inhibitors of histone deacetylase were shown to be sufficient for stimulating the activity of chromatin-integrated BCE-1 in the absence of ECM [41]. Together, these results suggest that epigenetic mechanisms and chromatin remodeling are involved in the ECM-mediated induction of milk protein gene transcription, which will be discussed in the next section.

In addition to regulation through ECM-responsive DNA elements, the ECM can initiate mechanical signals that can induce changes in cell shape and cytoskeletal organization, which may be crucial for the hormonal induction of milk protein genes. For example, when EpH4 cells were cultured in a laminin-rich ECM, they formed into polar, acinar-like structures, and their cytoskeleton reorganized into a cortical network that is required for PRLR/STAT5 signaling [152-154]. During these processes, exposure of mammary epithelial cells to a laminin-rich ECM recruits PRLR to the basal surface of acini, allowing for the binding of PRL and thus the activation of PRLR/STAT5 signaling [152]. Furthermore, increased ECM stiffness can reduce β-casein expression by regulating cellular actin polymerization, implying that matrix compliance is required for cytoskeleton reorganization, a crucial factor for cell-specific gene expression [155].

Epigenetics and chromatin structure in regulation of milk protein gene expression

Casein and WAP gene expression is regarded as a marker for the functional differentiation of mammary epithelial cells, and it has been suggested that epigenetic mechanisms play a key role in mammary gland development and functional differentiation [156]. It is then conceivable that epigenetic mechanisms are implicated in the tissue- and developmental stage-specific regulation of milk protein genes. Epigenetics refers to the study of heritable changes in genome function that occur due to chemical changes in DNA and its surrounding chromatin rather than changes in DNA sequences. Epigenetic regulation is mediated by DNA methylation, histone modifications (acetylation, ubiquitination, methylation, and phosphorylation), and microRNAs, which

modulate the chromatin confirmation and thus gene expression. The development of new technologies, such as chromatin immunoprecipitation (ChIP) followed by quantitative PCR (qChIP), microarrays (ChIP-chip), and more recently DNA sequencing (ChIP-seq) and the availability of complete genomic sequences enable us to quantitatively analyze epigenetic modifications at specific genomic sites as well as on a global scale. Studies investigating epigenetic mechanisms in rodent mammary gland development and functional differentiation [156] and dairy cow milk production [157, 158] have recently been reviewed elsewhere. Here, we only review the involvement of epigenetic and chromatin mechanisms that regulate milk protein gene expression.

DNA methylation

DNA methylation refers to conversion of the cytosine bases in DNA strands into 5-methylcytosine. DNA methylation results in the repression of gene expression perhaps by blocking *cis*-regulating elements where transcription activators should bind [159]. An inverse correlation between DNA methylation status and milk protein gene expression has been documented in many studies. Over three decades ago, Johnson et al. noticed that certain restriction sites in the rat β- and γ-casein genes from lactating mammary glands are readily digested by the methylation-sensitive restriction enzymes *MspI* and *HpaII*, but DNA samples from liver are resistant to digestion at the same restriction sites [160]. Using a similar strategy, the rat *κ*-casein gene was shown to be hypomethylated in lactating mammary glands, but it was hypermethylated in non-mammary tissues and non-lactating mammary glands [161]. The same study also implied the possibility that lactogenic hormonal induction of *κ*-casein gene expression is mediated by reducing DNA

methylation [161]. Hypomethylation during lactation has also been described for three specific sites flanking the bovine αS1-casein gene, and methylation of one of these three sites is inversely correlated with αS1-casein gene expression [162]. In addition to the casein locus, the WAP gene is also specifically hypomethylated in the lactating mammary gland in its coding and 5' flanking regions including the proximal promoter and a hormone-responsive distal site [163, 164]. More recently, the relationship between methylation and bovine αS1-casein gene expression has been explored during different physiological states [165] and during mastitis [166]. DNA methylation at a STAT5-binding enhancer located -10 kb upstream of the TSS can be induced following an 18 h non-milking period, and this induced methylation occurs before the decline in PRL signaling and milk protein gene expression that takes place at 24 to 36 h post-milking [157, 165]. Vanselow and colleagues found that *Escherichia coli*-induced mastitis results in DNA methylation in the same region that is associated with αS1-casein gene silencing [166]. Thus, milk protein gene expression is potentially regulated by DNA methylation, which is influenced by cues not only from mammary gland development and functional differentiation but also from physiological circumstances and health status. However, how these cues change the DNA methylation status and how modified DNA methylation status regulates milk protein gene expression remain unanswered.

Histone modification

Histone modifications have also been implicated in cell differentiation and the transcriptional control of tissue-specific and inducible genes. Histone-modifying complexes catalyze the addition or removal of various chemical elements on histones.

These enzymatic modifications include acetylation, methylation, phosphorylation, and ubiquitination and primarily occur at N-terminal histone tails. Such modifications affect the binding affinity between histones and DNA and loosen or tighten the condensed DNA wrapped around histones, preventing the binding of transcription factors to DNA and leading to gene repression. In contrast, histone acetylation relaxes chromatin condensation and exposes DNA for transcription factor binding, leading to increased gene expression.

There is emerging evidence implying that histone modifications are involved in the regulation of milk protein gene expression. Using ChIP, it has been shown that enrichment in histone H3 acetylation (H3Ac) at proximal promoters and many potential distal regulatory elements in the mouse casein and WAP gene loci occurs specifically in lactating mammary glands but not in the liver [19, 156]. Furthermore, at the cellular level, lactogenic hormones were found to recruit the histone-modifying enzyme p300 to the β-casein promoter in HC11 mouse mammary epithelial cells, which correlated with an increase in histone H3 acetylation and the stable association of RNA polymerase II at promoters and enhancers [61]. Three classical milk transcription factors, STAT5, GR, and C/EBPβ, can interact with p300 [64-66]. Presumably, histone acetylation may contribute to mammary-specific milk protein gene transcription.

In contrast, it has been shown that the ECM and ECM-induced cell shape changes lead to a global deacetylation of histones H3 and H4 and a global reduction in gene expression in HMT-3522-S1 and -T4-2 human mammary epithelial cells [167]. However, these findings do not rule out the possibility that there are locally hyperacetylated regions

involved in tissue-specific gene expression in differentiated cells. For example, the ECM alone or in combination with PRL induces histone acetylation at the promoters of the αS1-casein and β-casein genes in primary rabbit mammary epithelial cells [168] and EpH4 mouse mammary epithelial cells [147].

Chromatin structure

More than a decade ago, researchers noticed that methylated CpG-islands coincide with hypoacetylated histones [169]. Subsequently, it was found that methylated DNA can be bound by methyl-CpG-binding domain proteins (MBDs), which in turn recruit additional proteins, such as histone deacetylases and other chromatin remodeling proteins that modify histones, thereby forming a compact, closed chromatin structure termed heterochromatin [170-173]. DNase I digestion has been widely used to identify open chromatin regions by the presence of DNase I hypersensitive sites. Using this method, DNase I hypersensitive regions have been identified in lactating mammary glands at sites for different milk protein genes, such as ovine β-lactoglobulin [174], rat [45, 46], rabbit [175], and mouse [176] WAP genes, and mouse casein genes [156]. The DNase I hypersensitive sites are usually located upstream of milk protein genes, overlap with regions with DNA hypomethylation and positive histone marks [156], and correlate with transcription factor binding sites, such as binding sites for STAT5 [177, 178], GR [46], and NF-1 [45]. Several lines of evidence indicate that the DNase I hypersensitive sites of milk protein genes appear to be developmentally regulated and potentially activated by lactogenic hormones as indicated by *in vivo* and *in vitro* experiments [175, 178, 179]. This possibility is supported by a recent study using DNase I hypersensitivity,

histone H3 acetylation enrichment, and H3K4-di-methylation enrichment as indicators of open chromatin in which researchers found that milk protein gene loci progressively gain positive chromatin marks from puberty to lactation in conjunction with mouse mammary gland development and differentiation [180]. For example, distal regulatory regions within casein gene loci and the WAP gene region present open chromatin marks after pubertal development, and these open chromatin marks persist after lactation ceases, while proximal promoters only gain an open-chromatin conformation during pregnancy and become closed at the weaning stage [180]. These results suggest a model in which milk protein gene loci achieve a chromatin structure during pubertal development that is poised to be sensitive to lactogenic hormones to achieve the lactation capacity of the mammary gland.

Chromatin structure is influenced not only by histone modifications but also ATP-dependent remodeling. ATP-dependent chromatin remodeling complexes have a common ATPase domain, and energy from the hydrolysis of ATP allows these remodeling complexes to reposition (slide, twist or loop) nucleosomes along DNA, expelling histones from DNA or facilitating the exchange of histone variants, thus creating nucleosome-free DNA regions for gene activation [181]. ATP-dependent chromatin remodeling Switch/Sucrose nonfermentable (SWI/SNF) complexes are implicated in cellular differentiation and tissue-specific gene transcription [182, 183]. Recruitment of SWI/SNF complexes to the *cis*-elements of tissue-specific genes is mediated by association with specific transcription factors, such as GR and C/EBPβ, or binding to acetylated histone tails through their bromodomains [184-187]. For milk

protein gene regulation, Xu et al. showed that laminin-rich ECM and PRL cooperate to recruit the SWI/SNF complex to β-casein and γ-casein promoters via interaction with GR, STAT5, and C/EBPβ, which are needed for stable RNA polymerase II binding and gene transcription [147]. Thus, ECM and PRL may be able to regulate casein gene transcription via ATP-dependent chromatin remodeling.

Aside from the biochemical level, chromatin conformation changes, such as chromatin bending and looping, can occur on a macroscopic scale and lead to the interaction of distantly spaced genomic regions [188, 189]. This possible high order interaction provides a way for transcription factors and other coactivators, associated at proximal promoters and distal enhancers, to cooperate with each other through chromatin looping-mediated protein-protein interaction. It has been demonstrated that lactogenic hormones promote physical interaction between the β-casein gene proximal promoter and an upstream enhancer in HC11 cells and primary three-dimensional mammary acini cultures [190]. This interaction is blocked by PG-induced PR binding to the promoter [190]. Furthermore, developmental regulation of DNA-looping between β-casein regulatory regions was observed in lactating but not virgin mouse mammary glands, and the DNA looping was directly correlated with β-casein gene transcription [156].

POU TRANSCRIPTION FACTOR FAMILY

The octamer motif, ATTTGCAT, and its closely related sequences are in the *cis*-acting regulatory regions of genes that are both ubiquitously expressed and cell type-

specific [191-193]. These sequences can be recognized and bound by a group of *trans*-acting factors, known as octamer transcription factors (Oct). Thus far, eight genes encoding the following Oct proteins have been cloned and characterized: Oct-1, Oct-2, Oct-3/4, Oct-6, Oct-7, Oct-8, Oct-9, and Oct-11 [193]. Apart from Oct-1 [194] and possibly Oct-2 [195], all other Oct proteins are expressed in a tissue-specific and developmental stage-specific manner [193].

Oct factors belong to the POU (Pit-1, Oct and Unc-86) factor family, which is a family of transcription factors that share a characteristic bipartite DNA-binding domain, which is called the POU domain [196, 197]. The POU domain is composed of a conserved amino-terminal-specific domain (POU_S) and a relatively variant carboxy-terminal homeodomain (POU_H), which are tethered by an unconserved linker [193]. Efficient and sequence-specific DNA binding is dependent on the cooperation of POU_H with POU_S [198]. The two subdomains are independently folded and bind to the opposite faces of the DNA in two adjacent major grooves through a helix-turn-helix (HTH) structure [199]. In addition to DNA binding, the POU domain also mediates specific protein-protein interactions between Oct factors or between Oct factors and other transcription factors or cofactors [193].

Oct-1

Oct-1 is one of the most studied Oct transcription factors. This transcription factor is widely expressed in adult and embryonic tissues. Various Oct-1 isoforms have been identified in both humans (at least four) and mice (at least seven) [194]. These isoforms originate from a single-copy Oct-1 gene on chromosome 1 in both humans and mice. In

response to upstream signals, Oct-1 can regulate the expression of a variety of genes either positively or negatively [196]. By regulating target gene expression, Oct-1 is involved in diverse biological processes, such as embryogenesis, organ development, immune responses, and tumorigenicity [196]. Current studies have begun to reveal how Oct-1 mediates the effects of upstream signals on downstream gene expression. Evidence suggests that extracellular signals control target gene expression by modulating Oct-1 DNA binding properties through post-translational modifications, including phosphorylation [200, 201], O-GLcNAcylation [202], and ubiquitylation [203]. For example, following exposure to H_2O_2 and ionizing radiation, Oct-1 is phosphorylated at multiple serines and threonines, and these phosphorylation modifications are able to alter Oct-1 DNA binding properties, resulting in the modulation of targeted gene expression [204]. The subcellular localization of Oct-1 is also finely regulated. For example, in proglucagon-expressing endocrine cells, cAMP elevation results in the nuclear exclusion of Oct-1, thus reducing the interactions between Oct-1 and the Cdx-2 gene promoter and leading to enhanced Cdx-2 expression [205]. Oct-1 also regulates gene expression by interacting with other transcription factors and/or cofactors. For example, the transcriptional synergism between the glucocorticoid receptor (GR) and Oct-1 in mouse mammary tumor virus (MMTV) expression is mediated by direct binding between the GR DNA-binding domain and the POU domain of Oct-1 [71]. Oct-1 binds directly to Oct-1 coactivator in S phase (OCA-S); this complex is selectively recruited to the H2B promoter in S phase, and is essential for S phase-specific H2B transcription *in vivo* and *in vitro* [206].

Oct-2

Oct-2 is generally considered only expressed in the lymphoid and neuronal cells. Oct-2 is encoded by a single gene; however, multiple alternatively spliced isoforms of Oct-2 have been identified [207]. The Oct-2 isoforms present in B lymphocytes play a predominantly *trans*-activating role in gene expression, whereas those Oct-2 isoforms expressed in neuronal cells have a primarily repressive effect [208]. These octamer and octamer-related sequences exist in virtually all Ig variable region promoters and in both the Ig heavy and *k* light chain enhancers [209, 210]. Mutations in these sequences lead to significantly inhibited B cell-specific expression of Ig genes [211, 212]. Because Oct-2 is predominantly expressed in B cells, Oct-2 was believed to play a critical role in determining the B cell-specific expression of Ig genes. However, Oct-2 deficient mice express Ig and other B cell-specific genes tested at the normal level at the pre-B-cell stage of development, indicating that Oct-2 is not essential for Ig gene expression [213]. Then, a model was proposed suggesting that Oct-1 could compensate for Oct-2's function in regulating Ig gene expression. This model is supported by the fact that Oct-1 and Oct-2 have nearly identical DNA binding specificity and that the Ig promoters are equally responsive to both Oct-1 and Oct-2 [214]. The discovery of OCA-B/Bob-1/OBF-1, which is a B cell-specific cofactor, explains the B cell-restricted activity of the octamer element in the promoters of Ig genes [215]. OCA-B interacts with both Oct-1 and Oct-2 and enhances Oct-1- and Oct-2-dependent promoter activity in B cells [216]. Thus, interacting with tissue-specific cofactors confers the B cell-restricted activity of the octamer element and contributes to tissue-specific gene activation. In neuronal cells,

specific Oct-2 isoforms inhibit not only the endogenous tyrosine hydroxylase gene promoter activity [217], but also the expression of the herpes simplex virus immediate-early genes [218]. In addition to lymphocytes and neuronal cells, Oct-2 is also expressed in the testis, kidney, intestine, and mammary gland [195, 219]. Oct-2 can also activate basal mammary-specific β-casein gene promoter activity in mammary epithelial cells [195].

UTILIZATION OF MILK PROTEIN GENE PROMOTERS TO EXPRESS TRANSGENES IN THE MILK OF TRANSGENIC ANIMALS

A number of transgenic animals harboring transgenes containing the 5' and 3' flanking sequences of milk protein genes have been generated. These animals have been used not only for determining the functional importance of *cis*-elements in milk protein gene regulation but also for producing pharmaceutical proteins in the milk of these transgenic animals. Producing pharmaceutical proteins in animal mammary glands has led to the development of a new field of biotechnology known as mammary bioreactor.

Before the development of animal bioreactors, pharmaceutical proteins were either extracted from plants and animals or produced in bacterial or mammalian cell cultures. The transgenic animal bioreactor is superior for its scalability [220]. Because transgenic animals can transmit transgenes to their offspring, productivity can easily be increased by optimizing the breeding program efficiency. On the other side, the large-

scale production of proteins by cell culture is expensive and time-consuming. The cost of building a modern cell culture-based industrial bioreactor is over one hundred million dollars, and it may take several years to build such a facility. Another appeal of genetically engineered animals is the ability to perform many post-translational modifications, such as disulfide bond formation, tyrosine sulfation, glycosylation, and carboxylation, and proper folding of expressed proteins, which occurs in native cells and is required for their biological activity [221]. These features are distinctly superior compared with producing recombinant proteins in prokaryotes such as bacteria because bacteria lack the post-translational machinery found in mammalian cells. In addition, recombinant proteins synthesized in bacteria cannot be secreted into an extracellular environment but often accumulate as insoluble aggregates in inclusion bodies [221]. Producing proteins by mammalian cell culture can overcome the shortages of prokaryotes systems; however, mammalian cells usually require the addition of serum to culture media, and using serum may have contamination from unknown or undetected viruses [220]. Furthermore, expression of recombinant proteins in the mammary gland offers more advantages [222]. The sole function of the mammary gland is to produce milk, which is composed of up to 4% protein (40 g/L). An average dairy cow produces approximately 40 kg milk/day, with up to 1.6 kg of proteins secreted each day. Thus, the mammary gland is a natural protein-secreting organ with high capacity. In addition, milk has only a few main protein components. Thus, recombinant proteins expressed in milk are relatively easier to extract. Extensive studies have shown that the mammary gland has

the ability to synthesize, properly fold, assemble, and secrete complex proteins [221, 222]. These unique properties make the mammary gland the best available bioreactor.

Genetically engineered mammary glands as animal bioreactors have mainly focused on producing biopharmaceuticals. Many different biopharmaceuticals, such as human recombinant erythropoietin [223], human coagulation/clotting factors VIII [224] and IX [225], and human α-1-antitrypsin [226], have been produced in the milk of different transgenic mammals. In 2006, the European Commission approved a biopharmaceutical protein produced in the milk of goats, antithrombin III (commercially named Atryn®), for the treatment of patients with hereditary antithrombin deficiency [227]. Atryn® was then approved by the Food and Drug Administration (FDA) in the United States in 2009 [227]. Atryn® has been the first ever pharmaceutical protein produced in the milk of transgenic animals and the first recombinant antithrombin product approved worldwide. Recently, in our lab, transgenic mice were generated to harbor the human insulin gene driven by a goat β-casein gene promoter [228]. These animals secrete high levels of proinsulin in their milk, and the mature insulin derived from the milk proinsulin retains biological activity [228]. Our study suggests that it may be feasible to produce large amounts of human proinsulin in the milk of dairy animals, such as dairy goats and cows.

Genetically transformed dairy breeds may be or already have been generated to produce milk with modified biochemical composition to meet specific needs. For example, the transgenic approach may be employed to humanize cow milk by overexpressing β- and *κ*-casein variants [227]. The milk casein concentration has already

been increased to enhance cheese-making efficiency [229, 230]. Human lysozyme is being produced in the milk of genetically engineered goats to decrease the rennet-clotting time and increase curd strength, leading to faster cheese making and firmer cheese [231]. It has been shown that increasing the lysozyme concentration in goat milk can extend shelf life by causing spoilage bacteria to grow more slowly [231].

In this study, we first investigated the functional roles of Oct-1 and Oct-2 in lactogenic hormonal regulation of β-casein gene expression. Another aim of this study was to utilize the goat β-casein gene promoter to direct human proinsulin expression in the milk of transgenic mice and to provide a foundation for the potential scale-up of human proinsulin production in the milk of transgenic ruminants.

Table 1.1. Genomic location and organization of the human, cow, and mouse casein genes

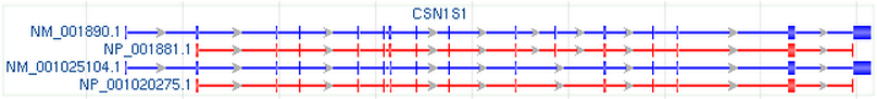
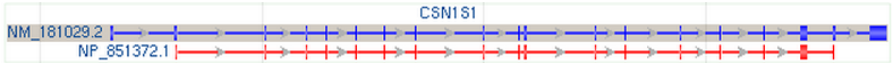
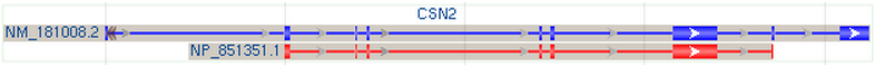
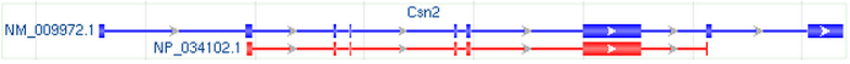
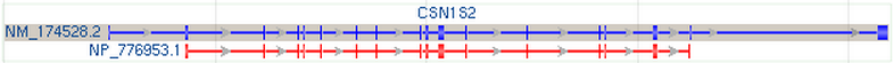
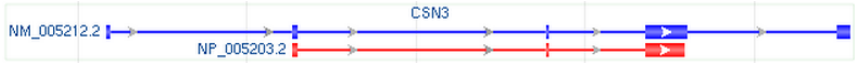
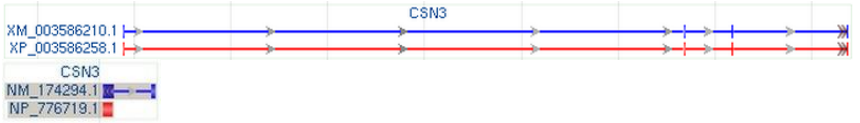
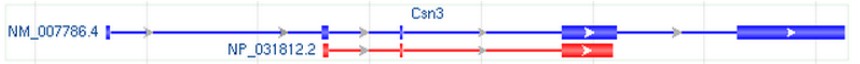
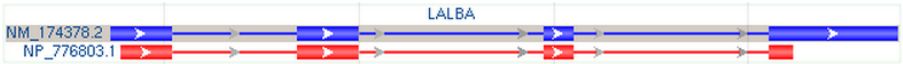
Protein	Gene symbol[1]	Species	Chromosome: location[1]	Transcript and CDS region
αS1-casein	CSN1S1	Human	4: 70,796,799-70,812,289	
		Cattle	6: 87,141,556-87,159,096	
	Csn1s1	Mouse	5: 87,666,224-87,682,573	
β-casein	CSN2	Human	4: 70,820,974-70,826,726	
		Cattle	6: 87,179,499-87,188,004	
	Csn2	Mouse	5: 87,692,624-87,699,421	
αS2-casein	CSN1S2	Cattle	6: 87,262,457-87,280,936	
αS2-casein-like A	Csn1s2a	Mouse	5: 87,774,567-87,788,797	
αS2-casein-like B	Csn1s2b	Mouse	5: 87808122-87824421	

Table 1.1. cont'd….

κ-casein	CSN3	Human	4: 71,108,333-71,117,145	
		Cattle	6: 87,349,410-87,386,900 87,390,197-87,392,750	
	Csn3	Mouse	5: 87,925,633-87,932,264	

[1]Adapted from the NCBI Gene website (http://www.ncbi.nlm.nih.gov/pubmed?Db=gene&Cmd=retrieve&dopt=full_report&list_uids="gene id") version: 12-Jan-2012. In the genomic organization column, each vertical bar represents an exon, and arrows indicate the orientation of the gene. The GenBank Accession Number for each reference sequence is shown.

Table 1.2. Genomic location and organization of the human, cattle, mouse whey protein genes

Protein	Gene symbol[1]	Species	Chromosome: location[1]	Transcript and CDS region
α-Lactalbumin	LALBA	Human	12: 48,961,467-48,963,829	
		Cattle	5: 31,347,861-31,349,882	
	Lalba	Mouse	15: 98,480,400-98,482,683	
β-Lactoglobulin	PAEP	Cattle	11: 103,301,664-103,306,381	
Whey acidic protein	Wap	Mouse	11: 6,635,483-6,638,649	

[1]Adapted from the NCBI Gene website (http://www.ncbi.nlm.nih.gov/pubmed?Db=gene&Cmd=retrieve&dopt=full_report&list_uids="gene id") version: 12-Jan-2012. In the genomic organization column, each vertical bar represents an exon, and arrows indicate the orientation of the gene. The GenBank Accession Number for each reference sequence is shown.

REFERENCES

[1] C.M. Lefèvre, J.A. Sharp, K.R. Nicholas, Evolution of lactation: ancient origin and extreme adaptations of the lactation system, Annu. Rev. Genomics Hum. Genet. 11 (2010) 219-238.

[2] A. Haug, A.T. Høstmark, O.M. Harstad, Bovine milk in human nutrition - a review, Lipids Health Dis. 6 (2007) 25.

[3] R. Jenness, The composition of human milk, Semin. Perinatol. 3 (1979) 225-239.

[4] M.F. Picciano, Nutrient composition of human milk, Pediatr. Clin. North. Am. 48 (2001) 53-67.

[5] I.A. Forsyth, M.C. Neville, Introduction: hormonal regulation of mammary development and milk protein gene expression at the whole animal and molecular levels, J. Mammary Gland Biol. Neoplasia 14 (2009) 317-319.

[6] Y.J. Topper, C.S. Freeman, Multiple hormone interactions in the developmental biology of the mammary gland, Physiol. Rev. 60 (1980) 1049-1106.

[7] B.K. Vonderhaar, S.E. Ziska, Hormonal regulation of milk protein gene expression, Annu. Rev. Physiol. 51 (1989) 641-652.

[8] M.A. Groenen, J.J. van der Poel, Regulation of expression of milk protein genes: a review, Livest. Prod. Sci. 38 (1994) 61-78.

[9] J.M. Rosen, S.L. Wyszomierski, D. Hadsell, Regulation of milk protein gene expression, Annu. Rev. Nutr. 19 (1999) 407-436.

[10] B.L. Luhovyy, T. Akhavan, G.H. Anderson, Whey proteins in the regulation of food intake and satiety, J. Am. Coll. Nutr. 26 (2007) 704S-712S.

[11] C.A. Zittle, Stabilization of calcium-sensitive (α_s) casein by kappa-casein: effect of chymotrypsin and heat on kappa-casein, J. Dairy Sci. 44 (1961) 2101-2103.

[12] J.-L. Audic, B. Chaufer, G. Daufin, Non-food applications of milk components and dairy co-products: A review, Lait 83 (2003) 417-438.

[13] D.S. Horne, Casein interactions: casting light on the black boxes, the structure in dairy products, Int. Dairy J. 8 (1998) 171-177.

[14] R. Hakkak, S. Korourian, M.J. Ronis, J.M. Johnston, T.M. Badger, Dietary whey protein protects against azoxymethane-induced colon tumors in male rats, Cancer Epidemiol. Biomarkers Prev. 10 (2001) 555-558.

[15] G.W. Krissansen, Emerging health properties of whey proteins and their clinical implications, J. Am. Coll. Nutr. 26 (2007) 713S-723S.

[16] U. Brodbeck, W. Denton, N. Tanahashi, K. Ebner, The isolation and identification of the B protein of lactose synthetase as α-lactalbumin, J. Biol. Chem. 242 (1967) 1391-1397.

[17] I. Selo, G. Clement, H. Bernard, J. Chatel, C. Creminon, et al., Allergy to bovine β-lactoglobulin: specificity of human IgE to tryptic peptides, Clin. Exp. Allergy 29 (1999) 1055-1063.

[18] N. Nukumi, T. Iwamori, K. Kano, K. Naito, H. Tojo, Whey acidic protein (WAP) regulates the proliferation of mammary epithelial cells by preventing serine protease from degrading laminin, J. Cell. Physiol. 213 (2007) 793-800.

[19] M. Rijnkels, L. Elnitski, W. Miller, J.M. Rosen, Multispecies comparative analysis of a mammalian-specific genomic domain encoding secretory proteins, Genomics 82 (2003) 417-432.
[20] P. Walstra, R. Jenness, Dairy chemistry and physics, New York: Wiley (1984).
[21] M. Miluchová, M. Gábor, A. Trakovická, J. Hanusova, R. Kasarda, Analysis of beta-casein gene (CSN2) polymorphism in different breeds of cattle, J. Anim. Sci. Biotechnol. 47 (2014) 82-85.
[22] L. Amigo, I. Recio, M. Ramos, Genetic polymorphism of ovine milk proteins: its influence on technological properties of milk - a review, Int. Dairy J. 10 (2000) 135-149.
[23] A. Caroli, S. Chessa, G. Erhardt, Invited review: milk protein polymorphisms in cattle: Effect on animal breeding and human nutrition, J. Dairy Sci. 92 (2009) 5335-5352.
[24] A. Caroli, F. Chiatti, S. Chessa, D. Rignanese, P. Bolla, et al., Focusing on the goat casein complex, J. Dairy Sci. 89 (2006) 3178-3187.
[25] A. Pauciullo, I. Giambra, L. Iannuzzi, G. Erhardt, The β-casein in camels: molecular characterization of the CSN2 gene, promoter analysis and genetic variability, Gene 547 (2014) 159-168.
[26] S. Kamiński, A. Cieślińska, E. Kostyra, Polymorphism of bovine beta-casein and its potential effect on human health, J. Appl. Genet. 48 (2007) 189-198.
[27] M.P. Barnett, W.C. McNabb, N.C. Roy, K.B. Woodford, A.J. Clarke, Dietary A1 β-casein affects gastrointestinal transit time, dipeptidyl peptidase-4 activity, and inflammatory status relative to A2 β-casein in Wistar rats, Int. J. Food Sci. Nutr. (2014) 1-8.
[28] M.R.U. Haq, R. Kapila, R. Sharma, V. Saliganti, S. Kapila, Comparative evaluation of cow β-casein variants (A1/A2) consumption on Th2-mediated inflammatory response in mouse gut, Eur. J. Nutr. (2013) 1039-1049.
[29] P. Winklehner-Jennewein, S. Geymayer, J. Lechner, T. Welte, L. Hansson, et al., A distal enhancer region in the human β-casein gene mediates the response to prolactin and glucocorticoid hormones, Gene 217 (1998) 127-139.
[30] H. Wakao, F. Gouilleux, B. Groner, Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response, EMBO J. 13 (1994) 2182-2191.
[31] M. Schmitt-Ney, W. Doppler, R.K. Ball, B. Groner, β-Casein gene promoter activity is regulated by the hormone-mediated relief of transcriptional repression and a mammary-gland-specific nuclear factor, Mol. Cell. Biol. 11 (1991) 3745-3755.
[32] J. Lechner, T. Welte, J.K. Tomasi, P. Bruno, C. Cairns, et al., Promoter-dependent synergy between glucocorticoid receptor and Stat5 in the activation of β-casein gene transcription, J. Biol. Chem. 272 (1997) 20954-20960.
[33] J. Lechner, T. Welte, W. Doppler, Mechanism of interaction between the glucocorticoid receptor and Stat5: role of DNA-binding, Immunobiology 198 (1997) 112-123.

[34] W. Doppler, T. Welte, S. Philipp, CCAAT/enhancer-binding protein isoforms and are expressed in mammary epithelial cells and bind to multiple sites in the-casein gene promoter, J. Biol. Chem. 270 (1995) 17962-17969.
[35] T.N. Seagroves, S. Krnacik, B. Raught, J. Gay, B. Burgess-Beusse, et al., C/EBPβ, but not C/EBPα, is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland, Genes Dev. 12 (1998) 1917-1928.
[36] H. Saito, T. Oka, Hormonally regulated double-and single-stranded DNA-binding complexes involved in mouse-casein gene transcription, J. Biol. Chem. 271 (1996) 8911-8918.
[37] F.-Q. Zhao, K. Adachi, T. Oka, Involvement of Oct-1 in transcriptional regulation of β-casein gene expression in mouse mammary gland, BBA-GENE STRUCT. EXPR. 1577 (2002) 27-37.
[38] C.K. Inman, N. Li, P. Shore, Oct-1 counteracts autoinhibition of Runx2 DNA binding to form a novel Runx2/Oct-1 complex on the promoter of the mammary gland-specific gene β-casein, Mol. Cell. Biol. 25 (2005) 3182-3193.
[39] V.S. Meier, B. Groner, The nuclear factor YY1 participates in repression of the β-casein gene promoter in mammary epithelial cells and is counteracted by mammary gland factor during lactogenic hormone induction, Mol. Cell. Biol. 14 (1994) 128-137.
[40] B. Raught, B. Khursheed, A. Kazansky, J. Rosen, YY1 represses β-casein gene expression by preventing the formation of a lactation-associated complex, Mol. Cell. Biol. 14 (1994) 1752-1763.
[41] C.A. Myers, C. Schmidhauser, J. Mellentin-Michelotti, G. Fragoso, C.D. Roskelley, et al., Characterization of BCE-1, a transcriptional enhancer regulated by prolactin and extracellular matrix and modulated by the state of histone acetylation, Mol. Cell. Biol. 18 (1998) 2184-2195.
[42] C. Schmidhauser, G.F. Casperson, C. Myers, K. Sanzo, S. Bolten, et al., A novel transcriptional enhancer is involved in the prolactin-and extracellular matrix-dependent regulation of beta-casein gene expression, Mol. Biol. Cell. 3 (1992) 699-709.
[43] F. Öztürk-Winder, M. Renner, D. Klein, M. Müller, B. Salmons, et al., The murine whey acidic protein promoter directs expression to human mammary tumors after retroviral transduction, Cancer Gene Ther. 9 (2002) 421-431.
[44] S. Li, J.M. Rosen, Nuclear factor I and mammary gland factor (STAT5) play a critical role in regulating rat whey acidic protein gene expression in transgenic mice, Mol. Cell Biol. 15 (1995) 2063-2070.
[45] S. Li, J.M. Rosen, Distal regulatory elements required for rat whey acidic protein gene expression in transgenic mice, J. Biol. Chem. 269 (1994) 14235-14243.
[46] S. Li, J.M. Rosen, Glucocorticoid regulation of rat whey acidic protein gene expression involves hormone-induced alterations of chromatin structure in the distal promoter region, Mol. Endocrinol. 8 (1994) 1328-1335.

[47] G.W. Robinson, P.F. Johnson, L. Hennighausen, E. Sterneck, The C/EBPβ transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland, Genes Dev. 12 (1998) 1907-1916.
[48] R. Ball, R. Friis, C. Schoenenberger, W. Doppler, B. Groner, Prolactin regulation of beta-casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line, EMBO J. 7 (1988) 2089-2095.
[49] K.G. Danielson, C.J. Oborn, E.M. Durban, J.S. Butel, D. Medina, Epithelial mouse mammary cell line exhibiting normal morphogenesis *in vivo* and functional differentiation *in vitro*, Proc. Natl. Acad. Sci. U. S. A. 81 (1984) 3756-3760.
[50] W. Salmon Jr, W. Daughaday, A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage *in vitro*, J. Lab Clin. Med. 49 (1957) 825.
[51] C. Bole-Feysot, V. Goffin, M. Edery, N. Binart, P.A. Kelly, Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice, Endocr. Rev. 19 (1998) 225-268.
[52] X. LIu, G.W. Robinson, F. Gouilleux, B. Groner, L. Hennighausen, Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 8831-8835.
[53] F. Gouilleux, H. Wakao, M. Mundt, B. Groner, Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription, EMBO J. 13 (1994) 4361.
[54] X. Liu, G.W. Robinson, K.-U. Wagner, L. Garrett, A. Wynshaw-Boris, et al., Stat5a is mandatory for adult mammary gland development and lactogenesis, Genes Dev. 11 (1997) 179-186.
[55] G.B. Udy, R.P. Towers, R.G. Snell, R.J. Wilkins, S.-H. Park, et al., Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 7239-7244.
[56] S.L. Wyszomierski, J.M. Rosen, Cooperative Effects of STAT5 (Signal Transducer and Activator of Transcription 5) and C/EBP β (CCAAT/Enhancer-Binding Protein-β) onβ-Casein Gene Transcription Are Mediated by the Glucocorticoid Receptor, Mol. Endocrinol. 15 (2001) 228-240.
[57] W. Doppler, M. Windegger, C. Soratroi, J. Tomasi, J. Lechner, et al., Expression level-dependent contribution of glucocorticoid receptor domains for functional interaction with STAT5, Mol. Cell. Biol. 21 (2001) 3266-3279.
[58] E. Stoecklin, M. Wissler, R. Moriggl, B. Groner, Specific DNA binding of Stat5, but not of glucocorticoid receptor, is required for their functional cooperation in the regulation of gene transcription, Mol. Cell. Biol. 17 (1997) 6708-6716.
[59] E. Stöcklin, M. Wissler, F. Gouilleux, B. Groner, Functional interactions between Stat5 and the glucocorticoid receptor, Nature 383 (1996) 726-728.
[60] S.L. Wyszomierski, J. Yeh, J.M. Rosen, Glucocorticoid receptor/signal transducer and activator of transcription 5 (STAT5) interactions enhance STAT5 activation by prolonging STAT5 DNA binding and tyrosine phosphorylation, Mol. Endocrinol. 13 (1999) 330-343.

[61] E.B. Kabotyanski, M. Huetter, W. Xian, M. Rijnkels, J.M. Rosen, Integration of prolactin and glucocorticoid signaling at the β-casein promoter and enhancer by ordered recruitment of specific transcription factors and chromatin modifiers, Mol. Endocrinol. 20 (2006) 2355-2368.
[62] P. Descombes, U. Schibler, A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the sam mRNA, Cell 67 (1991) 569-579.
[63] B. Raught, W.-L. Liao, J.M. Rosen, Developmentally and hormonally regulated CCAAT/enhancer-binding protein isoforms influence beta-casein gene expression, Mol. Endocrinol. 9 (1995) 1223-1232.
[64] E. Pfitzner, R. Jahne, M. Wissler, E. Stoecklin, B. Groner, p300/CREB-binding protein enhances the prolactin-mediated transcriptional induction through direct interaction with the transactivation domain of Stat5, but does not participate in the Stat5-mediated suppression of the glucocorticoid response, Mol. Endocrinol. 12 (1998) 1582-1593.
[65] S. Mink, B. Haenig, K.-H. Klempnauer, Interaction and functional collaboration of p300 and C/EBPbeta, Mol. Cell. Biol. 17 (1997) 6609-6617.
[66] C. Schwartz, K. Beck, S. Mink, M. Schmolke, B. Budde, et al., Recruitment of p300 by C/EBPβ triggers phosphorylation of p300 and modulates coactivator activity, EMBO J. 22 (2003) 882-892.
[67] B. Dong, C. Huang, D. Li, F.-Q. Zhao, Oct-1 functions as a transactivator in the hormonal induction of β-casein gene expression, Mol. Cell. Biochem. 328 (2009) 93-99.
[68] B. Dong, F.-Q. Zhao, Involvement of the ubiquitous Oct-1 transcription factor in hormonal induction of beta-casein gene expression, Biochem. J. 401 (2007) 57-64.
[69] X. Qian, F.-Q. Zhao, Interactions of the ubiquitous octamer-binding transcription factor-1 with both the signal transducer and activator of transcription 5 and the glucocorticoid receptor mediate prolactin and glucocorticoid-induced β-casein gene expression in mammary epithelial cells, Int. J. Biochem. Cell Biol. 45 (2013) 724-735.
[70] A.C. Buser, E.K. Gass-Handel, S.L. Wyszomierski, W. Doppler, S.A. Leonhardt, et al., Progesterone receptor repression of prolactin/signal transducer and activator of transcription 5-mediated transcription of the β-casein gene in mammary epithelial cells, Mol. Endocrinol. 21 (2007) 106-125.
[71] G.G. Préfontaine, R. Walther, W. Giffin, M.E. Lemieux, L. Pope, et al., Selective binding of steroid hormone receptors to octamer transcription factors determines transcriptional synergism at the mouse mammary tumor virus promoter, J. Biol. Chem. 274 (1999) 26713-26719.
[72] R.E. Rhoads, E. Grudzien-Nogalska, Translational regulation of milk protein synthesis at secretory activation, J. Mammary Gland Biol. Neoplasia 12 (2007) 283-292.
[73] F.F. Bolander, K.R. Nicholas, J.J. Van Wyk, Y.J. Topper, Insulin is essential for accumulation of casein mRNA in mouse mammary epithelial cells, Proc. Natl. Acad. Sci. U. S. A. 78 (1981) 5682-5684.

[74] J.K. Kulski, K.R. Nicholas, Y.J. Topper, P. Qasba, Essentiality of insulin and prolactin for accumulation of rat casein mRNAs, Biochem. Biophys. Res. Commun. 116 (1983) 994-999.
[75] C.R. Andersen, B. Larson, Comparative maintenance of function in dispersed cell and organ cultures of bovine mammary tissue, Exp. Cell Res. 61 (1970) 24-30.
[76] T. Mackle, D. Dwyer, K.L. Ingvartsen, P. Chouinard, J. Lynch, et al., Effects of insulin and amino acids on milk protein concentration and yield from dairy cows, J. Dairy Sci. 82 (1999) 1512-1524.
[77] T. Mackle, D. Dwyer, K.L. Ingvartsen, P. Chouinard, D. Ross, et al. Effects of insulin and postruminal supply of protein on use of amino acids by the mammary gland for milk protein synthesis, J. Dairy Sci. 83 (2000) 93-105.
[78] J.H. Clark, H.R. Spires, R.G. Derrig, M.R. Bennink, Milk production, nitrogen utilization and glucose synthesis in lactating cows infused postruminally with sodium caseinate and glucose, J. Nutr. 107 (1977) 631-644.
[79] M. Léonard, E. Block, Effects on nutrient and hormonal profile of long-term infusions of glucose or insulin plus glucose in cows treated with recombinant bovine somatotropin before peak milk yield, J. Dairy Sci. 80 (1997) 127-143.
[80] K. Hove, Effects of hyperinsulinemia on lactose secretion and glucose uptake by the goat mammary gland, Acta Physiol. Scand. 104 (1978) 422-430.
[81] B. Laarveld, D. Christensen, R. Brockman, The effect of insulin on net metabolism of glucose and amino acids by the bovine mammary gland, Endocrinology 108 (1981) 2217-2221.
[82] S. Tesseraud, J. Grizard, B. Makarski, E. Debras, G. Bayle, et al., Effect of insulin in conjunction with glucose, amino acids and potassium on net metabolism of glucose and amino acids in the goat mammary gland, J. Dairy Sci. 59 (1992) 135-149.
[83] A.R. Saltiel, J.E. Pessin, Insulin signaling pathways in time and space, Trends in cell biology, 12 (2002) 65-71.
[84] D.G. Lemay, M.C. Neville, M.C. Rudolph, K.S. Pollard, J.B. German, Gene regulatory networks in lactation: identification of global principles using bioinformatics, BMC Syst. Biol. 1 (2007) 56.
[85] K.K. Menzies, C. Lefèvre, K.L. Macmillan, K.R. Nicholas, Insulin regulates milk protein synthesis at multiple levels in the bovine mammary gland, Funct. Integr. Genomics 9 (2009) 197-217.
[86] K.K. Menzies, H.J. Lee, C. Lefèvre, C.J. Ormandy, K.L. Macmillan, et al., Insulin, a key regulator of hormone responsive milk protein synthesis during lactogenesis in murine mammary explants, Funct. Integr. Genomics 10 (2010) 87-95.
[87] J. Harris, P.M. Stanford, K. Sutherland, S.R. Oakes, M.J. Naylor, et al., Socs2 and elf5 mediate prolactin-induced mammary gland development, Mol. Endocrinol. 20 (2006) 1177-1187.
[88] S.R. Oakes, M.J. Naylor, M.-L. Asselin-Labat, K.D. Blazek, M. Gardiner-Garden, et al., The Ets transcription factor Elf5 specifies mammary alveolar cell fate, Genes Dev. 22 (2008) 581-586.

[89] R.S. Thomas, A.N. Ng, J. Zhou, M.J. Tymms, W. Doppler, et al., The Elf group of Ets-related transcription factors . ELF3 and ELF5, Adv. Exp. Med. Biol. 480 (2000) 123-128.
[90] J. Zhou, R. Chehab, J. Tkalcevic, M.J. Naylor, J. Harris, et al., Elf5 is essential for early embryogenesis and mammary gland development during pregnancy and lactation, EMBO J. 24 (2005) 635-644.
[91] C. Schmidhauser, M.J. Bissell, C.A. Myers, G.F. Casperson, Extracellular matrix and hormones transcriptionally regulate bovine beta-casein 5'sequences in stably transfected mouse mammary cells, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 9118-9122.
[92] K.M. Choi, I. Barash, R.E. Rhoads, Insulin and prolactin synergistically stimulate β-casein messenger ribonucleic acid translation by cytoplasmic polyadenylation, Mol. Endocrinol. 18 (2004) 1670-1686.
[93] K.K. Menzies, C. Lefèvre, J.A. Sharp, K.L. Macmillan, P.A. Sheehy, et al., A novel approach identified the FOLR1 gene, a putative regulator of milk protein synthesis, Mamm. Genome 20 (2009) 498-503.
[94] C. Girard, J. Matte, Dietary supplements of folic acid during lactation: effects on the performance of dairy cows, J. Dairy Sci. 81 (1998) 1412-1419.
[95] B. Graulet, J. Matte, A. Desrochers, L. Doepel, M.-F. Palin, et al., Effects of Dietary Supplements of Folic Acid and Vitamin B_{12} on Metabolism of Dairy Cows in Early Lactation, J. Dairy Sci. 90 (2007) 3442-3455.
[96] C. Girard, J. Matte, Folic acid and vitamin B_{12} requirements of dairy cows: A concept to be revised, Livest. Prod. Sci. 98 (2005) 123-133.
[97] X. Li, B.W. O'Malley, Unfolding the action of progesterone receptors, J. Biol. Chem. 278 (2003) 39261-39264.
[98] B.B. Virgo, G.D. Bellward, Serum progesterone levels in the pregnant and postpartum laboratory mouse, Endocrinology 95 (1974) 1486-1490.
[99] N. Kuhn, Progesterone withdrawal as the lactogenic trigger in the rat, J. Endocrinol. 44 (1969) 39-54.
[100] P.M. Ismail, J. Li, F.J. DeMayo, B.W. O'Malley, J.P. Lydon, A novel LacZ reporter mouse reveals complex regulation of the progesterone receptor promoter during mammary gland development, Mol. Endocrinol. 16 (2002) 2475-2489.
[101] R. Deis, C. Delouis, Lactogenesis induced by ovariectomy in pregnant rats and its regulation by oestrogen and progesterone, J. Steroid Biochem. 18 (1983) 687-690.
[102] L. Assairi, C. Delouis, P. Gaye, L. Houdebine, M. Bousquet, et al., Inhibition by progesterone of the lactogenic effect of prolactin in the pseudopregnant rabbit, Biochem. J. 144 (1974) 245.
[103] C. Brisken, Hormonal control of alveolar development and its implications for breast carcinogenesis, J. Mammary Gland Biol. Neoplasia 7 (2002) 39-48.
[104] R.D. Rajaram, C. Brisken, Paracrine signaling by progesterone, Mol. Cell Endocrinol. 357 (2012) 80-90.
[105] J. Monks, TGFβ as a potential mediator of progesterone action in the mammary gland of pregnancy, J. Mammary Gland Biol. Neoplasia 12 (2007) 249-257.

[106] C. Jhappan, A. Geiser, E. Kordon, D. Bagheri, L. Hennighausen, et al., Targeting expression of a transforming growth factor beta 1 transgene to the pregnant mammary gland inhibits alveolar development and lactation, EMBO J. 12 (1993) 1835.
[107] D. Pierce, M.D. Johnson, Y. Matsui, S.D. Robinson, L.I. Gold, et al., Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-beta 1, Genes Dev. 7 (1993) 2308-2317.
[108] S.D. Robinson, A.B. Roberts, C.W. Daniel, TGF beta suppresses casein synthesis in mouse mammary explants and may play a role in controlling milk levels during pregnancy, J. Cell Biol. 120 (1993) 245-251.
[109] S. Robinson, G. Silberstein, A. Roberts, K. Flanders, C. Daniel, Regulated expression and growth inhibitory effects of transforming growth factor-beta isoforms in mouse mammary gland development, Development, 113 (1991) 867-878.
[110] M. Mieth, F.-D. Boehmer, R. Ball, B. Groner, R. Grosse, Transforming growth factor-β inhibits lactogenic hormone induction of β-casein expression in HC11 mouse mammary epithelial cells, Growth Factors, 4 (1990) 9-15.
[111] A. Sudlow, C. Wilde, R. Burgoyne, Transforming growth factor-beta 1 inhibits casein secretion from differentiating mammary-gland explants but not from lactating mammary cells, Biochem. J. 304 (1994) 333-336.
[112] E. Cocolakis, M. Dai, L. Drevet, J. Ho, E. Haines, et al., Smad signaling antagonizes STAT5-mediated gene transcription and mammary epithelial cell differentiation, J. Biol. Chem. 283 (2008) 1293-1307.
[113] A. Plath, R. Einspanier, F. Peters, F. Sinowatz, D. Schams, Expression of transforming growth factors alpha and beta-1 messenger RNA in the bovine mammary gland during different stages of development and lactation, J. Endocrinol. 155 (1997) 501-511.
[114] J. Zarzynska, M. Gajewska, T. Motyl, Effects of hormones and growth factors on TGF-β1 expression in bovine mammary epithelial cells, J. Dairy Res. 72 (2005) 39-48.
[115] X.-H. Luo, E.-Y. Liao, X. Su, Progesterone upregulates TGF-b isoforms (b1, b2, and b3) expression in normal human osteoblast-like cells, Calcif. Tissue Int. 71 (2002) 335-343.
[116] G.W. Robinson, R.A. McKnight, G.H. Smith, L. Hennighausen, Mammary epithelial cells undergo secretory differentiation in cycling virgins but require pregnancy for the establishment of terminal differentiation, Development 121 (1995) 2079-2090.
[117] T. Zhu, E.L. Goh, R. Graichen, L. Ling, P.E. Lobie, Signal transduction via the growth hormone receptor, Cell Signal. 13 (2001) 599-616.
[118] G.J. Allan, E. Tonner, M.C. Barber, M.T. Travers, J.H. Shand, et al., Growth hormone, acting in part through the insulin-like growth factor axis, rescues developmental, but not metabolic, activity in the mammary gland of mice expressing a single allele of the prolactin receptor, Endocrinology, 143 (2002) 4310-4319.

[119] D. Bauman, Bovine somatotropin and lactation: from basic science to commercial application, Domest. Anim. Endocrinol. 17 (1999) 101-116.
[120] I.R. Dohoo, K. Leslie, L. DesCôteaux, A. Fredeen, P. Dowling, et al., A meta-analysis review of the effects of recombinant bovine somatotropin: 1. Methodology and effects on production, Can. J. Vet. Res. 67 (2003) 241-251.
[121] A. Gertler, A. Ashkenazi, Z. Madar, Binding sites of human growth hormone and ovine and bovine prolactins in the mammary gland and the liver of lactating dairy cow, Mol. Cell. Endocrinol. 34 (1984) 51-57.
[122] J. Keys, J. Djiane, Prolactin and growth hormone binding in mammary and liver tissue of lactating cows, J. Recept. Res. 8 (1988) 731-750.
[123] R. Akers, Major advances associated with hormone and growth factor regulation of mammary growth and lactation in dairy cows, J. Dairy Sci. 89 (2006) 1222-1234.
[124] C. Prosser, S. Davis, V. Farr, L. Moore, P. Gluckman, Effects of close-arterial (external pudic) infusion of insulin-like growth factor-II on milk yield and mammary blood flow in lactating goats, J. Endocrinol. 142 (1994) 93-99.
[125] H.-Y. Su, W. Cheng, Increased milk yield in transgenic mice expressing insulin-like growth factor 1, Anim. Biotechnol. 15 (2004) 9-19.
[126] I.A. Forsyth, The insulin-like growth factor and epidermal growth factor families in mammary cell growth in ruminants: action and interaction with hormones, J. Dairy Sci. 79 (1996) 1085-1096.
[127] C. Baumrucker, B. Stemberger, Insulin and insulin-like growth factor-I stimulate DNA synthesis in bovine mammary tissue *in vitro*, J. Animal Sci. 67 (1989) 3503-3514.
[128] M.H. Monaco, D.E. Gronlund, G.T. Bleck, W.L. Hurley, M.B. Wheeler, et al, Mammary specific transgenic over-expression of insulin-like growth factor-I (IGF-I) increases pig milk IGF-I and IGF binding proteins, with no effect on milk composition or yield, Transgenic Res. 14 (2005) 761-773.
[129] E. Wolf, P.M. Jehle, M.M. Weber, H. Sauerwein, A. Daxenberger, et al., Human insulin-like growth factor I (IGF-I) produced in the mammary glands of transgenic rabbits: yield, receptor binding, mitogenic activity, and effects on IGF-binding proteins, Endocrinology, 138 (1997) 307-313.
[130] F. Sinowatz, D. Schams, S. Kolle, A. Plath, D. Lincoln, et al., Cellular localisation of GH receptor in the bovine mammary gland during mammogenesis, lactation and involution, J. Endocrinol. 166 (2000) 503-510.
[131] A. Plath-Gabler, C. Gabler, F. Sinowatz, B. Berisha, D. Schams, The expression of the IGF family and GH receptor in the bovine mammary gland, J. Endocrinol. 168 (2001) 39-48.
[132] H. Jiang, C.S. Okamura, M.C. Lucy, Isolation and characterization of a novel promoter for the bovine growth hormone receptor gene, J. Biol. Chem. 274 (1999) 7893-7900.
[133] T.L. Johnson, B.A. Fujimoto, R. Jiménez-Flores, D.G. Peterson, Growth hormone alters lipid composition and increases the abundance of casein and lactalbumin mRNA in the MAC-T cell line, J. Dairy Res. 77 (2010) 199-204.

[134] Y. Zhou, R. Akers, H. Jiang, Growth hormone can induce expression of four major milk protein genes in transfected MAC-T cells, J. Dairy Sci. 91 (2008) 100-108.
[135] T. Komatsu, M.T. Rose, T. Kobayashi, Y. Obara, A. Hagino, et al., Growth hormone acts on the synthesis and secretion of alpha-casein in bovine mammary epithelial cells, J. Dairy Res. 72 (2005) 264-270.
[136] S. Yonekura, K. Sakamoto, T. Komatsu, A. Hagino, K. Katoh, et al., Growth hormone and lactogenic hormones can reduce the leptin mRNA expression in bovine mammary epithelial cells, Domest. Anim. Endocrinol. 31 (2006) 88-96.
[137] J. Yang, B. Zhao, V. Baracos, J. Kennelly, Effects of bovine somatotropin on β-casein mrna levels in mammary tissue of lactating cows, J. Dairy Sci. 88 (2005) 2806-2812.
[138] A. Hayashi, K. Nones, N. Roy, W. McNabb, D. Mackenzie, et al., Initiation and elongation steps of mRNA translation are involved in the increase in milk protein yield caused by growth hormone administration during lactation, J. Dairy Sci. 92 (2009) 1889-1899.
[139] A.A. Hayashi, C.G. Proud, The rapid activation of protein synthesis by growth hormone requires signaling through mTOR, Am. J. Physiol. Endocrinol. Metab. 292 (2007) E1647-E1655.
[140] S. Burgos, M. Dai, J. Cant, Nutrient availability and lactogenic hormones regulate mammary protein synthesis through the mammalian target of rapamycin signaling pathway, J. Dairy Sci. 93 (2010) 153-161.
[141] C. Toerien, J. Cant, Abundance and phosphorylation state of translation initiation factors in mammary glands of lactating and nonlactating dairy cows, J. Dairy Sci. 90 (2007) 2726-2734.
[142] R. Manjarín, J. Steibel, R. Kirkwood, N. Taylor, N. Trottier, Transcript abundance of hormone receptors, mammalian target of rapamycin pathway-related kinases, insulin-like growth factor I, and milk proteins in porcine mammary tissue, J. Anim. Sci. 90 (2012) 221-230.
[143] W. Guo, F.G. Giancotti, Integrin signalling during tumour progression, Nat. Rev. Mol. Cell Biol. 5 (2004) 816-826.
[144] R. Kalluri, Basement membranes: structure, assembly and role in tumour angiogenesis, Nat. Rev. Cancer 3 (2003) 422-433.
[145] V.A. Spencer, R. Xu, M.J. Bissell, Extracellular matrix, nuclear and chromatin structure, and gene expression in normal tissues and malignant tumors: a work in progress, Adv. Cancer Res. 97 (2007) 275-294.
[146] C.H. Streuli, C. Schmidhauser, N. Bailey, P. Yurchenco, A. Skubitz, et al., Laminin mediates tissue-specific gene expression in mammary epithelia, J. Cell Biol. 129 (1995) 591-603.
[147] R. Xu, V.A. Spencer, M.J. Bissell, Extracellular matrix-regulated gene expression requires cooperation of SWI/SNF and transcription factors, J. Biol. Chem. 282 (2007) 14992-14999.

[148] C.H. Streuli, G.M. Edwards, M. Delcommenne, C.B.A. Whitelaw, T.G. Burdon, et al., Stat5 as a target for regulation by extracellular matrix, J. Biol. Chem. 270 (1995) 21639-21644.
[149] C.Q. Lin, P.J. Dempsey, R.J. Coffey, M.J. Bissell, Extracellular matrix regulates whey acidic protein gene expression by suppression of TGF-alpha in mouse mammary epithelial cells: studies in culture and in transgenic mice, J. Cell Biol. 129 (1995) 1115-1126.
[150] G.M. Edwards, F.H. Wilford, X. Liu, L. Hennighausen, J. Djiane, et al., Regulation of mammary differentiation by extracellular matrix involves protein-tyrosine phosphatases, J. Biol. Chem. 273 (1998) 9495-9500.
[151] E. Reichmann, R. Ball, B. Groner, R.R. Friis, New mammary epithelial and fibroblastic cell clones in coculture form structures competent to differentiate functionally, J. Cell Biol. 108 (1989) 1127-1138.
[152] R. Xu, C.M. Nelson, J.L. Muschler, M. Veiseh, B.K. Vonderhaar, et al., Sustained activation of STAT5 is essential for chromatin remodeling and maintenance of mammary-specific function, J. Cell Biol. 184 (2009) 57-66.
[153] G.S. Zoubiane, A. Valentijn, E.T. Lowe, N. Akhtar, S. Bagley, et al., A role for the cytoskeleton in prolactin-dependent mammary epithelial cell differentiation, J. Cell Sci. 117 (2004) 271-280.
[154] C. Roskelley, P. Desprez, M. Bissell, Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 12378-12382.
[155] J. Alcaraz, R. Xu, H. Mori, C.M. Nelson, R. Mroue, et al., Laminin and biomimetic extracellular elasticity enhance functional differentiation in mammary epithelia, EMBO J. 27 (2008) 2829-2838.
[156] M. Rijnkels, E. Kabotyanski, M.B. Montazer-Torbati, C.H. Beauvais, Y. Vassetzky, et al., The epigenetic landscape of mammary gland development and functional differentiation, J. Mammary Gland Biol. Neoplasia 15 (2010) 85-100.
[157] K. Singh, A. Molenaar, K. Swanson, B. Gudex, J. Arias, et al., Epigenetics: a possible role in acute and transgenerational regulation of dairy cow milk production, Animal 6 (2012) 375-381.
[158] K. Singh, R.A. Erdman, K.M. Swanson, A.J. Molenaar, N.J. Maqbool, et al., Epigenetic regulation of milk production in dairy cows, J. Mammary Gland Biol. Neoplasia 15 (2010) 101-112.
[159] R.J. Klose, A.P. Bird, Genomic DNA methylation: the mark and its mediators, Trends Biochem. Sci. 31 (2006) 89-97.
[160] M.L. Johnson, J. Levy, S. Supowit, L. Yu-Lee, J. Rosen, Tissue-and cell-specific casein gene expression. II. Relationship to site-specific DNA methylation, J. Biol. Chem. 258 (1983) 10805-10811.
[161] M.D. Thompson, H.L. Nakhasi, Methylation and expression of rat κ-casein gene in normal and neoplastic rat mammary gland, Cancer Res. 45 (1985) 1291-1295.

[162] G.J. Platenburg, E.J. Vollebregt, C.N. Karatzas, E.P. Kootwijk, H.A. De Boer, et al., Mammary gland-specific hypomethylation of Hpa II sites flanking the bovine αS1-casein gene, Transgenic Res. 5 (1996) 421-431.
[163] A.M. Dandekar, E.A. Robinson, E. Appella, P.K. Qasba, Complete sequence analysis of cDNA clones encoding rat whey phosphoprotein: homology to a protease inhibitor, Proc. Natl. Acad. Sci. U. S. A. 79 (1982) 3987-3991.
[164] M.B. Montazer-Torbati, C. Hue-Beauvais, S. Droineau, M. Ballester, N. Coant, et al., Epigenetic modifications and chromatin loop organization explain the different expression profiles of the Tbrg4, WAP and Ramp3 genes, Exp. Cell Res.314 (2008) 975-987.
[165] K. Singh, K. Swanson, C. Couldrey, H. Seyfert, K. Stelwagen, DNA methylation events associated with the suppression of milk protein gene expression during involution of the bovine mammary gland, in: Proceedings of the New Zealand Society of Animal Production, vol. 69, New Zealand Society of Animal Production, 2009, pp. 57-59.
[166] J. Vanselow, W. Yang, J. Herrmann, H. Zerbe, H.-J. Schuberth, et al., DNA-remethylation around a STAT5-binding enhancer in the αS1-casein promoter is associated with abrupt shutdown of αS1-casein synthesis during acute mastitis, J. Mol. Endocrinol. 37 (2006) 463-477.
[167] J. Le Beyec, R. Xu, S.-Y. Lee, C.M. Nelson, A. Rizki, et al., Cell shape regulates global histone acetylation in human mammary epithelial cells, Exp. Cell Res. 313 (2007) 3066-3075.
[168] G. Jolivet, T. Pantano, L.M. Houdebine, Regulation by the extracellular matrix (ECM) of prolactin-induced αs1-casein gene expression in rabbit primary mammary cells: Role of STAT5, C/EBP, and chromatin structure, J. Cell. Biochem. 95 (2005) 313-327.
[169] J. Dobosy, E. Selker, Emerging connections between DNA methylation and histone acetylation, Cell. Mol. Life Sci. 58 (2001) 721-727.
[170] P.J. Skene, R.S. Illingworth, S. Webb, A.R. Kerr, K.D. James, et al., Neuronal MeCP2 is expressed at near histone-octamer levels and globally alters the chromatin state, Mol. Cell. 37 (2010) 457-468.
[171] M.C. Lorincz, D.R. Dickerson, M. Schmitt, M. Groudine, Intragenic DNA methylation alters chromatin structure and elongation efficiency in mammalian cells, Nat. Struct. Mol. Biol. 11 (2004) 1068-1075.
[172] M.C. Lorincz, D. Schübeler, M. Groudine, Methylation-mediated proviral silencing is associated with MeCP2 recruitment and localized histone H3 deacetylation, Mol. Cell. Biol. 21 (2001) 7913-7922.
[173] J.P. Thomson, P.J. Skene, J. Selfridge, T. Clouaire, J. Guy, S. et al., CpG islands influence chromatin structure via the CpG-binding protein Cfp1, Nature 464 (2010) 1082-1086.
[174] C. Whitelaw, S. Harris, M. McClenaghan, J. Simons, A. Clark, Position-independent expression of the ovine beta-lactoglobulin gene in transgenic mice, Biochem. J. 286 (1992) 31-39.

[175] B. Millot, M. Fontaine, D. Thepot, E. Devinoy, A distal region, hypersensitive to DNase I, plays a key role in regulating rabbit whey acidic protein gene expression, Biochem. J. 359 (2001) 557-565.
[176] B. Millot, L. Montoliu, M. Fontaine, T. Mata, E. Devinoy, Hormone-induced modifications of the chromatin structure surrounding upstream regulatory regions conserved between the mouse and rabbit whey acidic protein genes, Biochem. J. 372 (2003) 41-52.
[177] C.B. Whitelaw, Nucleosome organisation of the beta-lactoglobulin gene. Transcription complex formation, Adv. Exp. Med. Biol. 480 (2000) 147-153.
[178] C.B. Whitelaw, Hormonal influences on β-lactoglobulin transgene expression inferred from chromatin structure, Biochem. Biophys. Res. Commun. 224 (1996) 121-125.
[179] C.B. Whitelaw, J. Webster, Temporal profiles of appearance of DNase I hypersensitive sites associated with the ovine beta-lactoglobulin gene differ in sheep and transgenic mice, Mol. Gen. Genet. 257 (1998) 649-654.
[180] M. Rijnkels, C. Freeman-Zadrowski, J. Hernandez, V. Potluri, L. Wang, et al., Epigenetic modifications unlock the milk protein gene loci during mouse mammary gland development and differentiation, PloS One 8 (2013) e53270.
[181] G.G. Wang, C.D. Allis, P. Chi, Chromatin remodeling and cancer, Part II: ATP-dependent chromatin remodeling, Trends Mol. Med. 13 (2007) 373-380.
[182] I.L. de la Serna, K.A. Carlson, A.N. Imbalzano, Mammalian SWI/SNF complexes promote MyoD-mediated muscle differentiation, Nat. Genet. 27 (2001) 187-190.
[183] G.J. Narlikar, H.-Y. Fan, R.E. Kingston, Cooperation between complexes that regulate chromatin structure and transcription, Cell, 108 (2002) 475-487.
[184] E. Kowenz-Leutz, A. Leutz, AC/EBPβ isoform recruits the SWI/SNF complex to activate myeloid genes, Mol. Cell 4 (1999) 735-743.
[185] J.A. Martens, F. Winston, Recent advances in understanding chromatin remodeling by Swi/Snf complexes, Curr. Opin. Genet. Dev. 13 (2003) 136-142.
[186] J.G. McNally, W.G. Müller, D. Walker, R. Wolford, G.L. Hager, The glucocorticoid receptor: rapid exchange with regulatory sites in living cells, Science 287 (2000) 1262-1265.
[187] C. Dhalluin, J.E. Carlson, L. Zeng, C. He, A.K. Aggarwal, et al., Structure and ligand of a histone acetyltransferase bromodomain, Nature 399 (1999) 491-496.
[188] B. Tolhuis, R.-J. Palstra, E. Splinter, F. Grosveld, W. de Laat, Looping and interaction between hypersensitive sites in the active β-globin locus, Mol. Cell 10 (2002) 1453-1465.
[189] W. de Laat, F. Grosveld, Spatial organization of gene expression: the active chromatin hub, Chromosome Res. 11 (2003) 447-459.
[190] E.B. Kabotyanski, M. Rijnkels, C. Freeman-Zadrowski, A.C. Buser, D.P. Edwards, et al., Lactogenic hormonal induction of long distance interactions between β-casein gene regulatory elements, J. Biol. Chem. 284 (2009) 22815-22824.

[191] H. Schöler, A.K. Hatzopoulos, R. Balling, N. Suzuki, P. Gruss, A family of octamer-specific proteins present during mouse embryogenesis: evidence for germline-specific expression of an Oct factor, EMBO J. 8 (1989) 2543-2550.
[192] H.R. Schöler, Octamania: the POU factors in murine development, Trends in Genet. 7 (1991) 323-329.
[193] F.-Q. Zhao, Octamer-binding transcription factors: genomics and functions, Front. Biosci. (Landmark Ed), 18 (2013) 1051-1071.
[194] F.-Q. Zhao, Y. Zheng, B. Dong, T. Oka, Cloning, genomic organization, expression, and effect on β-casein promoter activity of a novel isoform of the mouse Oct-1 transcription factor, Gene. 326 (2004) 175-187.
[195] B. Dong, F.-Q. Zhao, Expression of the Oct-2 transcription factor in mouse mammary gland and cloning and characterization of a novel Oct-2 isoform, Cell Tissue Res. 328 (2007) 595-606.
[196] J. Kang, A. Shakya, D. Tantin, Stem cells, stress, metabolism and cancer: a drama in two Octs, Trends Biochem. Sci. 34 (2009) 491-499.
[197] C.P. Verrijzer, P.C. Van der Vliet, POU domain transcription factors, Biochem. Biophys. Acta. 1173 (1993) 1-21.
[198] C.P. Verrijzer, M. Alkema, W.W. van Weperen, H.C. Van Leeuwen, M. Strating, et al., The DNA binding specificity of the bipartite POU domain and its subdomains, EMBO J. 11 (1992) 4993-5003..
[199] K. Phillips, B. Luisi, The virtuoso of versatility: POU proteins that flex to fit, J. Mol. Biol. 302 (2000) 1023-1039.
[200] C. Schild-Poulter, A. Shih, D. Tantin, N. Yarymowich, S. Soubeyrand, et al., DNA-PK phosphorylation sites on Oct-1 promote cell survival following DNA damage, Oncogene 26 (2007) 3980-3988.
[201] N. Segil, S.B. Roberts, N. Heintz, Mitotic phosphorylation of the Oct-1 homeodomain and regulation of Oct-1 DNA binding activity, Science 254 (1991) 1814-1816.
[202] J. Kang, Z. Shen, J.-M. Lim, H. Handa, L. Wells, et al., Regulation of Oct1/Pou2f1 transcription activity by O-GlcNAcylation, FASEB J. 27 (2013) 2807-2817.
[203] J. Kang, B. Goodman, Y. Zheng, D. Tantin, Dynamic regulation of Oct1 during mitosis by phosphorylation and ubiquitination, PloS One 6 (2011) e23872.
[204] J. Kang, M. Gemberling, M. Nakamura, F.G. Whitby, H. Handa, et al., A general mechanism for transcription regulation by Oct1 and Oct4 in response to genotoxic and oxidative stress, Genes Dev 23 (2009) 208-222.
[205] P. Wang, Q. Wang, J. Sun, J. Wu, H. Li, et al., POU homeodomain protein Oct-1 functions as a sensor for cyclic AMP, J. Biol. Chem. 284 (2009) 26456-26465.
[206] L. Zheng, R.G. Roeder, Y. Luo, S phase activation of the histone H2B promoter by OCA-S, a coactivator complex that contains GAPDH as a key component, Cell 114 (2003) 255-266.
[207] T. Wirth, A. Priess, A. Annweiler, S. Zwilling, B. Oeler, Multiple Oct2 isoforms are generated by alternative splicing, Nucleic Acids Res. 19 (1991) 43-51.

[208] D. Latchman, The Oct-2 transcription factor, Int. J. Biochem. Cell Biol. 28 (1996) 1081-1083.
[209] K. Nelms, B. Van Ness, Identification of an octamer-binding site in the human kappa light-chain enhancer, Mol. Cell. Biol. 10 (1990) 3843-3846.
[210] T.G. Parslow, D.L. Blair, W.J. Murphy, D.K. Granner, Structure of the 5'ends of immunoglobulin genes: a novel conserved sequence, Proc. Natl. Acad. Sci. U. S. A. 81 (1984) 2650-2654.
[211] L. Poellinger, B.K. Yoza, R.G. Roeder, Functional cooperativity between protein molecules bound at two distinct sequence elements of the immunoglobulin heavy-chain promoter, Nature 337 (1989) 573-576.
[212] L.M. Staudt, M.J. Lenardo, Immunoglobulin gene transcription, Annu. Rev. Immunol. 9 (1991) 373-398.
[213] L. Corcoran, M. Karvelas, G. Nossal, Z.-S. Ye, T. Jacks, et al., Oct-2, although not required for early B-cell development, is critical for later B-cell maturation and for postnatal survival, Genes Dev. 7 (1993) 570-582.
[214] I. Kemler, E. Bucher, K. Seipel, M.M. Müller-lmmerglück, W. Schaffner, Promoters with the octamer DNA motif (ATGCAAAT) can be ubiquitous or cell type-specific depending on binding affinity of the octamer site and Oct-factor concentration, Nucleic Acids Res. 19 (1991) 237-242.
[215] M. Gstaiger, L. Knoepfel, O. Georgiev, W. Schaffner, C.M. Hovens, A B-cell coactivator of octamer-binding transcription factors, Nature 373 (1995) 360-362.
[216] M. Strubin, J.W. Newell, P. Matthias, OBF-1, a novel B cell-specific coactivator that stimulates immunoglobulin promoter activity through association with octamer-binding proteins, Cell 80 (1995) 497-506.
[217] S.J. Dawson, S.O. Yoon, D.M. Chikaraishi, K.A. Lillycrop, D.S. Latchman, The Oct-2 transcription factor represses tyrosine hydroxylase expression via a heptamer TAATGARAT-like motif in the gene promoter, Nucleic Acids Res. 22 (1994) 1023-1028.
[218] K.A. Lillycrop, S.J. Dawson, J.K. Estridge, T. Gerster, P. Matthias, et al., Repression of a herpes simplex virus immediate-early promoter by the Oct-2 transcription factor is dependent on an inhibitory region at the N terminus of the protein, Mol. Cell. Biol. 14 (1994) 7633-7642.
[219] A.K. Hatzopoulos, A.S. Stoykova, J.R. Erselius, M. Goulding, T. Neuman, et al., Structure and expression of the mouse Oct2a and Oct2b, two differentially spliced products of the same gene, Development 109 (1990) 349-362.
[220] D. Bauman, I. Mather, R. Wall, A. Lock, Major advances associated with the biosynthesis of milk, J. Dairy Sci. 89 (2006) 1235-1243.
[221] R. Montesino, J.R. Toledo, The mammary gland: bioreactor for the production of recombinant proteins, Biotecnol. Apl. 23 (2006) 279-286.
[222] A.J. Clark, The mammary gland as a bioreactor: expression, processing, and production of recombinant proteins, J. Mammary Gland Biol. Neoplasia. 3 (1998) 337-350.
[223] Y. Cheng, Y. Wang, J. Luo, Y. Shen, Y. Yang, et al., Cloned goats produced from the somatic cells of an adult transgenic goat, Chin. J. Biotechnol. 18 (2002) 79-83.

[224] C.-M. Chen, C.-H. Wang, S.-C. Wu, C.-C. Lin, S.-H. Lin, et al., Temporal and spatial expression of biologically active human factor VIII in the milk of transgenic mice driven by mammary-specific bovine α-lactalbumin regulation sequences, Transgenic Res. 11 (2002) 257-268.
[225] G.-C. Gil, W.H. Velander, K.E. Van Cott, Analysis of the N-glycans of recombinant human Factor IX purified from transgenic pig milk, Glycobiology 18 (2008) 526-539.
[226] A.L. Archibald, M. McClenaghan, V. Hornsey, J.P. Simons, A.J. Clark, High-level expression of biologically active human alpha 1-antitrypsin in the milk of transgenic mice, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 5178-5182.
[227] M. Samiec, M. Skrzyszowska, Transgenic mammalian species, generated by somatic cell cloning, in biomedicine, biopharmaceutical industry and human nutrition/dietetics-recent achievements, Pol. J. Vet. Sci. 14 (2011) 317-328.
[228] X. Qian, J. Kraft, Y. Ni, F.-Q. Zhao, Production of recombinant human proinsulin in the milk of transgenic mice, Sci. Rep. (2014).
[229] M. Baranyi, L. Hiripi, L. Szabó, A.P. Catunda, I. Harsányi, et al., Isolation and some effects of functional, low-phenylalanine κ-casein expressed in the milk of transgenic rabbits, J. Biotechnol. 128 (2007) 383-392.
[230] L.W. Pampel, R. Boushaba, N.J. Titchener-Hooker, A methodical approach to ultra-scale-down of process sequences: application to casein removal from the milk of transgenic animals, Biotechnol. Prog. 24 (2008) 192-201.
[231] E. Scharfen, D. Mills, E. Maga, Use of human lysozyme transgenic goat milk in cheese making: effects on lactic acid bacteria performance, J. Dairy Sci. 90 (2007) 4084-4091.
[232] C.J. Waston, K.E. Gordon, M. Robertson, A.J. Clark, Interaction of DNA-binding proteins with a milk protein gene promoter *in vitro*: identification of a mammary gland-specific factor, Nucleic Acids Res. 19 (1991) 6603-6610.
[233] A.V. Kazansky, B. Raught, S.M. Lindsey, Y. Wang, J.M. Rosen, Regulation of mammary gland factor/Stat5a during mammary gland development, Mol. Endocrinol. 9 (1995) 1598-1609.
[234] H. Wakao, M. Schmitt-Ney, B. Groner, Mammary gland-specific nuclear factor is present in lactating rodent and bovine mammary tissue and composed of a single polypeptide of 89 kDa, J. Biol. Chem. 267 (1992) 16365-16370.
[235] T. Welte, S. Philipp, C. Cairns, J.-Å. Gustafsson, W. Doppler, Glucocorticoid receptor binding sites in the promoter region of milk protein genes, J. Steroid Biochem. Mol. Biol. 47 (1993) 75-81.
[236] X. Qian, F.-Q. Zhao, Collaborative interaction of Oct-2 with Oct-1 in transactivation of lactogenic hormones-induced β-casein gene expression in mammary epithelial cells, Gen. Comp. Endocrinol. 204 (2014) 185-194.
[237] A.K. Patel, M. Singh, V. Suryanarayana, Buffalo alpha S1-casein gene 5'-flanking region and its interspecies comparison, J. Appl. Genet. 55 (2014) 75-87.
[238] T.G. Burdon, K.A. Maitland, A.J. Clark, R. Wallace, C.J. Watson, Regulation of the sheep beta-lactoglobulin gene by lactogenic hormones is mediated by a

transcription factor that binds an interferon-gamma activation site-related element, Mol. Endocrinol. 8 (1994) 1528-1536.
[239] A. Kuss, J. Gogol, H. Geldermann, Associations of a polymorphic AP-2 binding site in the 5'-flanking region of the bovine β-lactoglobulin gene with milk proteins, J. Dairy Sci. 86 (2003) 2213-2218.

FIGURE LEGENDS

Fig. 1.1. Schematic representation of the *cis*-regulatory regions of milk protein genes. DNA binding sites for transcription factors are shown in different shapes. **A**) Transcription factor binding sites mapped in the promoter and enhancer of the β-casein gene. The β-casein gene enhancer was originally identified in the bovine species; thus, it was named bovine casein enhancer element (BCE-1) [41]. These binding sites are highly conserved in β-casein gene promoters and enhancers in rabbits, rats, mice, goats, sheep, and cows [29]. Signal transducer and activator of transcription 5 (STAT5) [30, 41, 52, 53, 61, 232-234], CCAAT/enhancer binding protein (C/EBP) [35, 41, 47, 61, 63], and Yin Yang 1 (YY-1) [39, 40, 61] binding sites and half glucocorticoid response elements (½ GREs) for glucocorticoid receptor (GR) [32, 61, 235] in the β-casein proximal promoter and/or distal enhancer have been functionally verified and extensively studied. The binding sites for octamer factors (Oct) [37, 68, 69, 236] and runt-related transcription factor 2 (Runx2) [38] have recently been characterized. The E26 transformation-specific (Ets) site and nuclear factor 1 (NF-1) binding site are predicted based on sequence identity [29]. **B**) Transcription factor binding sites mapped in the promoter of the αS1-casein gene promoter. These binding sites are putative based on sequence identity, and they are highly conserved in αS1-casein gene promoters in buffalo, yak, cow, sheep, goat, camel, and human [237]. AP-1: activator protein 1. **C**) Structural organization of the transcription binding sites in the two highly conserved *cis*-regulatory regions of the rodent whey acidic protein (WAP) promoters. These binding sites are highly conserved in rats and mice [44]. NF-1 [44-46] and STAT5 [44] binding sites and ½ GREs [46, 235] have been functionally characterized in rats and/or mice. **D**) Schematic representation of the transcription factor binding sites in the β-lactoglobulin proximal promoter. STAT5 [238], NF-1 [232], and activator protein 2 (AP-2) [239] sites have been verified in the β-lactoglobulin gene promoters of sheep and/or cows. The numbers indicate positions relative to the transcription start sites (TSSs, +1).

Fig. 1.2. Induction of β-casein gene transcription by lactogenic hormones is mediated by synergism between signal transducer and activator of transcription 5 (STAT5) and glucocorticoid receptor (GR). The binding of prolactin (PRL) to the PRL receptor (PRLR) on the cell membrane of mammary epithelial cells (MECs) triggers activation of Janus kinase 2 (JAK2). Activated JAK2 phosphorylates tyrosine residues on PRLR and creates docking sites for Src homology 2 (SH2) domain-containing proteins. SH2-containing STAT5 is then recruited and phosphorylated by JAK2 at a conserved tyrosine residue within the carboxyl-terminal transcriptional activation domain. Phosphorylated STAT5 dimerizes, translocates into the nucleus, and induces β-casein gene transcription at a minimal level by binding to clustered STAT5 binding sites. In the presence of glucocorticoids (GC), GC pass through the cell membrane, bind to, and activate GR by releasing GR from heat shock complexes. Activated GR dimerizes, translocates into nucleus, binds to glucocorticoid response element (GRE) half-sites, and physically interacts with STAT5. GC and PRL stimulation also activates C/EBPβ binding to its response elements at β-casein regulatory regions. The synergistic interactions among STAT5, GR, and C/EBPβ results in much more robust induction of β-casein gene transcription than PRL alone by recruiting the p300, a coactivator with histone acetylase (HAT) activity, and stabilizing the basal transcription complex.

Fig. 1.1.

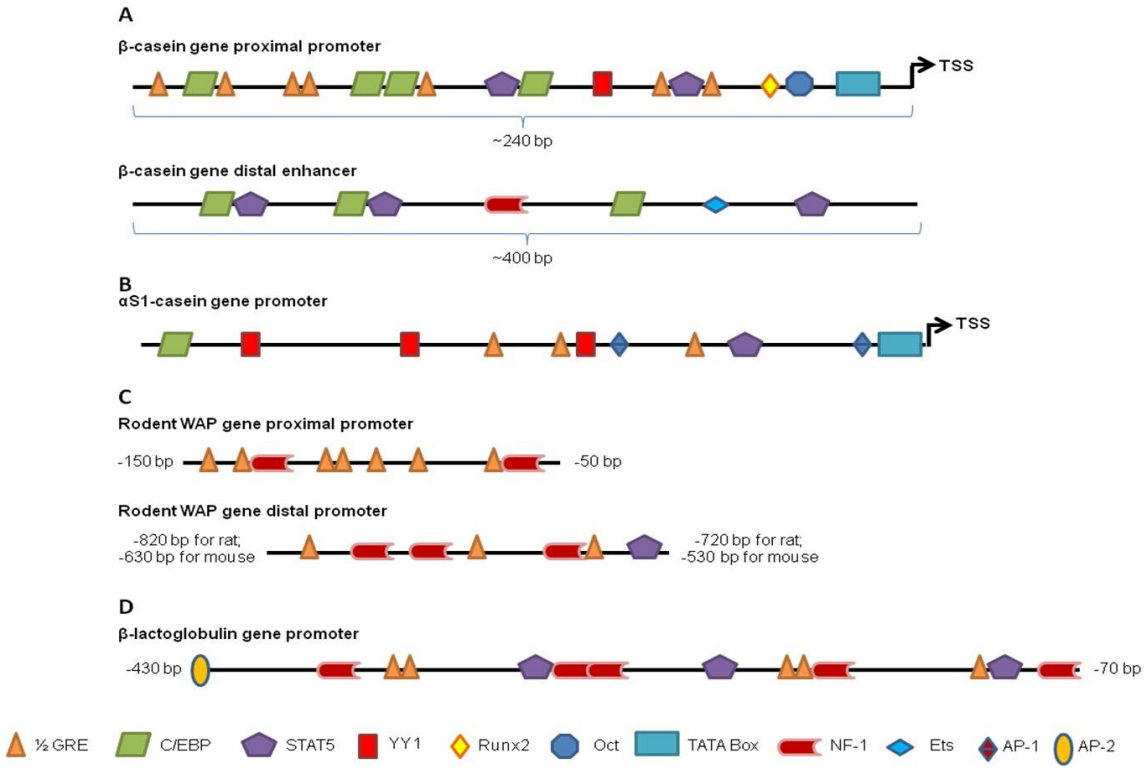
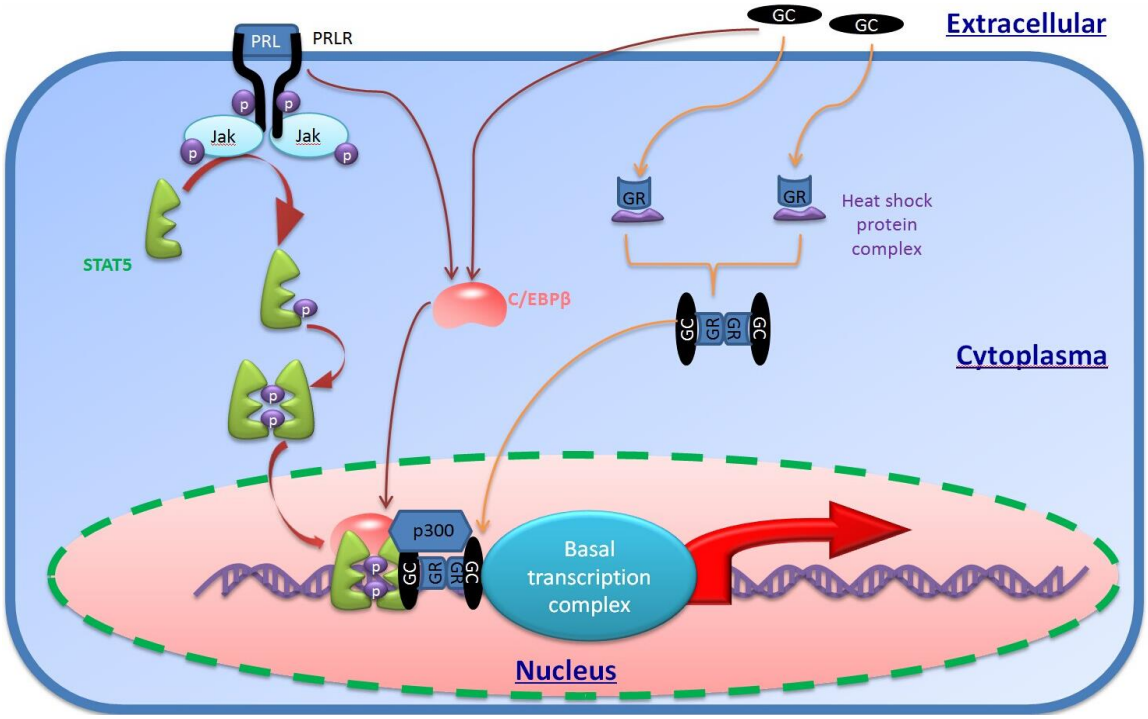

A
β-casein gene proximal promoter
TSS
~240 bp
β-casein gene distal enhancer
~400 bp
B
αS1-casein gene promoter
TSS
C
Rodent WAP gene proximal promoter
-150 bp
-50 bp
Rodent WAP gene distal promoter
-820 bp for rat;
-630 bp for mouse
-720 bp for rat;
-530 bp for mouse
D
β-lactoglobulin gene promoter
-430 bp
-70 bp
½ GRE
C/EBP
STAT5
YY1
Runx2
Oct
TATA Box
NF-1
Ets
AP-1
AP-2

Fig. 1.2.


GC
GC
Extracellular
PRL
PRLR
P
P
Jak
Jak
P
P
GR
GR
Heat shock
protein
complex
STAT5
P
C/EBPβ
GC
GR
GR
GC
Cytoplasma
P
P
p300
GC
GR
GR
GC
P
P
Basal
transcription
complex
Nucleus

Chapter 2 Interactions of the ubiquitous octamer-binding transcription factor-1 with both the signal transducer and activator of transcription 5 and the glucocorticoid receptor mediate prolactin and glucocorticoid-induced β-casein gene expression in mammary epithelial cells*

Xi Qian[1] and Feng-Qi Zhao[1,£]

[1]From the Laboratory of Lactation and Metabolic Physiology, Department of Animal Science, University of Vermont, Burlington, VT 05405

[£]To whom correspondence should be addressed: Feng-Qi Zhao, Department of Animal Science, University of Vermont, 211 Terrill Building, 570 Main Street, Burlington, VT 05405, USA, Tel.: (802) 656-0786; Fax: (802) 656-8196; E-mail: fzhao@uvm.edu

*This chapter has been published in the following form: Qian, X., and Zhao, F.-Q.. (2013). Interactions of the ubiquitous octamer-binding transcription factor-1 with both the signal transducer and activator of transcription 5 and the glucocorticoid receptor mediate prolactin and glucocorticoid-induced β-casein gene expression in mammary epithelial cells. The International Journal of Biochemistry & Cell Biology, 45, 724-735.

ABSTRACT

Regulation of milk protein gene expression by lactogenic hormones (prolactin and glucocorticoids) provides an attractive model for studying the mechanisms by which protein and steroid hormones synergistically regulate gene expression. β-Casein is one of the major milk proteins and its expression in mammary epithelial cells is stimulated by lactogenic hormones. The signal transducer and activator of transcription 5 and glucocorticoid receptor are essential downstream mediators of prolactin and glucocorticoid signaling, respectively. Previous studies have shown that mutating the octamer-binding site of the β-casein gene proximal promoter dramatically reduces the hormonal induction of the promoter activity. However, little is known about the underlying molecular mechanisms. In this report, we show that lactogenic hormones rapidly induce the binding of octamer-binding transcription factor-1 to the β-casein promoter and this induction is not mediated by either increasing the expression of octamer-binding transcription factor-1 or inducing its translocation to the nucleus. Rather, lactogenic hormones induce physical interactions between the octamer-binding transcription factor-1, signal transducer and activator of transcription 5, and glucocorticoid receptor to form a ternary complex, and these interactions enhance or stabilize the binding of these transcription factors to the promoter. Abolishing these interactions significantly reduces the hormonal induction of β-casein gene transcription. Thus, our study indicates that octamer-binding transcription factor-1 may serve as a master regulator that facilitates the DNA binding of both signal transducer and activator of transcription 5 and glucocorticoid receptor in hormone-induced β-casein expression,

and defines a novel mechanism of regulation of tissue-specific gene expression by the ubiquitous octamer-binding transcription factor-1.

Keywords: Gene expression; Hormonal regulation; Milk protein; Protein-protein interactions; Octamer binding transcription factor; Transcriptional regulation

Abbreviations: DTT, dithiothreitol; EGF, epidermal growth factor; EMSA, electrophoresis mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; GRE, glucocorticoid response elements; HP, hydrocortisone (glucocorticoids) and prolactin; IP, immunoprecipitation; MECs, mammary epithelial cells; Oct-1, octamer-binding transcription factor-1; PMSF, phenylmethylsulfonyl fluoride; POU, Pit-1, Oct, and Unc-86; POU_H, POU homeodomain; POU_S, POU-specific domain; PrlR, prolactin receptor; qChIP, quantitative chromatin immunoprecipitation; qPCR, quantitative PCR; qRT-PCR, quantitative reverse transcription PCR; snRNA, small nuclear RNA; STAT5, signal transducer and activator of transcription 5; TBP, TATA box-binding protein; WT, wild-type.

INTRODUCTION

Transcriptional regulation of gene expression is largely dependent on the interactions of transcription factors with the corresponding *cis*-DNA elements located in the promoter or enhancer region of a gene. Octamer-binding transcription factor-1 (Oct-1) was originally discovered for its ability to bind the conserved octamer motif

(ATGCAAAT), which is located in the promoter and enhancer sequences of the histone H2B, immunoglobulin, and U2 small nuclear RNA (snRNA) genes (Sive and Roeder, 1986). As a member of the POU (Pit-1, Oct and Unc-86) family of homeodomain transcription factors, Oct-1 contains a POU specific domain (POU_S) in addition to a POU homeodomain (POU_H), which is distantly related to the classic homeodomain encoded by homeobox genes (Kang et al., 2009b, Zhao, 2013). Oct-1 has been implicated in many important biological processes, including embryogenesis (Range and Lepage, 2011; Sebastiano et al., 2010), immune/inflammatory responses (Cheng et al., 2012; Ren et al., 2011), metabolic responses to stress (Goettsch et al., 2011; Malhas et al., 2009; Wang et al., 2009), and tumorigenicity (Kang et al., 2009b; Shakya et al., 2009). The genes regulated by Oct-1 include a wide variety of both ubiquitously expressed genes and tissue-specific genes. Oct-1 regulates these genes via DNA binding-dependent or -independent mechanisms. Both of the POU-domains are required for the high-affinity, site-specific binding to the octamer motif and are involved in protein-protein interactions with other transcription factors and co-factors (Kang et al., 2009b; Ren et al., 2011; Robinson et al., 2011).

β-Casein is a major milk protein, that is expressed via stimulation by lactogenic hormones, including prolactin and glucocorticoids (HP) (Rosen et al., 1999). There are three highly conserved regions in the proximal promoter of the casein genes, which are referred to as blocks A, B, and C (Yoshimura and Oka, 1990). Blocks A and B have been intensively studied and have been shown to be the binding sites of HP downstream molecules, signal transducer and activator of transcription 5 (STAT5) and glucocorticoid

receptor (GR) (Groner et al., 1994). Following mammary epithelial cell stimulation with lactogenic hormones, both STAT5 and GR are phosphorylated, translocate from the cytoplasm to the nucleus, recognize and bind to the corresponding binding sites in blocks A and B, and synergistically stimulate β-casein gene transcription (Lechner et al., 1997). Less is known about the mechanisms by which block C contributes to β-casein gene regulation. We have previously demonstrated that block C contains an octamer-binding site and that both its integrity and orientation are critical for the hormonal induction of β-casein gene promoter activity (Dong and Zhao, 2007; Dong et al., 2009).

In this study, we explored the molecular mechanisms by which Oct-1 participates in the hormonal induction of β-casein gene expression in mammary epithelial cells. Quantitative chromatin immunoprecipitation (qChIP) experiments indicated that Oct-1 indeed binds to the β-casein gene promoter in mammary epithelial cells and that this binding activity is hormonally regulated. Transfection experiments revealed that Oct-1 knockdown inhibits while overexpression stimulates β-casein gene expression induced by lactogenic hormones. Additionally, we demonstrated that in response to lactogenic hormones, Oct-1 physically interacts with STAT5 and GR, which facilitates the DNA binding of both STAT5 and GR to the β-casein gene promoter. Our data provide new insight into the molecular mechanisms by which the ubiquitously expressed Oct-1 contributes to the hormonal regulation of mammary epithelial cell-specific β-casein gene expression.

MATERIALS AND METHODS

Materials

Prolactin (L6520), hydrocortisone (one of glucocorticoids, H6909), insulin (I0516), and murine epidermal growth factor (EGF) (E4127) were purchased from Sigma (St. Louis, MO). Heat-inactivated fetal calf serum (1082-147), RPMI 1640 medium (31800-022), gentamicin (15750-060), and antibiotic-antimycotic solution (15240-062) were purchased from Invitrogen (Carlsbad, CA). Dynabeads® Protein A (100-01D) for ChIPs and immunoprecipitations (IPs) and Dynabeads® M-280 Streptavidin (112-05D) for DNA pull-down assays were also obtained from Invitrogen. Charcoal-stripped horse serum (52-0745) was purchased from Cocalico Biologicals (Reams town, PA). Growth factor reduced matrigel (354230) and dispase (354235) were obtained from BD Biosciences (Franklin Lakes, NJ). The mouse Oct-1B (mOct-1B/pcDNA3.1), GR (mGR/pcDNA3.1), STAT5a (mSTAT5a/pcDNA3.1), and prolactin receptor (PrlR) expression plasmids as well as the wild-type (WT) mouse β-casein promoter (-258/+7)/luciferase construct (LHRRWT/pGL3) have been described previously (Dong and Zhao, 2007). The *Renilla* luciferase control plasmid (phRL-CMV) was purchased from Promega (Madison, WI). The anti-TATA box binding protein (TBP) (sc-273), anti-actin (sc-1615-R), anti-STAT5 (sc-1081), and anti-GR (sc-1004) antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The anti-Oct-1 (A310-610A) antibody was provided by Bethyl Laboratories (Montgomery, TX). Normal rabbit IgG (10500C) was obtained from Invitrogen.

Cell Cultures, transfection, and luciferase assays

The murine mammary epithelial cell line, HC11, was cultured as previously described (Kabotyanski et al., 2006). HC11 Lux cells, which are HC11 cells stably transfected with a β-casein promoter luciferase construct (p-344/-1βc-Lux), were obtained from Dr. Hynes (Friedrich Miescher Institute, Switzerland) (Wartmann et al., 1996) and cultured as described for HC11 cells. Primary mouse mammary epithelial cells (MECs) were isolated following the procedures described by Watkin and Streuli (2002). Briefly, the mammary glands from mid-pregnant C57BL/6 mice were pooled, minced, and digested by collagenase. Next, the epithelial cells were enriched via centrifugation, plated on 60-mm dishes that were pre-coated with matrigel, and cultured in complete growth medium (D-MEM/F-12 supplemented with 10% fetal calf serum, 5 μg/ml bovine insulin, 10 ng/ml EGF, 1 μg/ml hydrocortisone, 1× antibiotic-antimycotic solution, and 50 μg/ml gentamicin). After 2 days of confluence, the cells were incubated in hormone-priming medium (D-MEM/F-12 medium supplemented with 10% charcoal-treated horse serum, 5 μg/ml bovine insulin, 1× antibiotic-antimycotic solution, and 50 μg/ml gentamicin) for 24 h and then incubated for 24 h in hormone-treatment medium (priming medium supplemented with 1 μg/ml hydrocortisone and 5 μg/ml prolactin).

The methods applied for the transfection and luciferase assays have been described previously (Dong and Zhao, 2007). In the Oct-1-overexpression studies, HC11 cells were transfected with either 0.2 pmol of pcDNA3.1 or mOct-1B/pcDNA3.1, 0.2 pmol of LHRRWT/pGL3, and 0.004 pmol of phRL-CMV using Lipofectamine 2000 (Invitrogen). In the siRNA transfection experiments, HC11 Lux cells were transfected with either 40 pmol of Oct-1 siRNA #1 (Santa Cruz Biotechnologies, siRNA #sc-36120),

Oct-1 siRNA #2 [Ambion (Austin, TX), siRNA #68842], or control siRNA (Ambion, siRNA #4611). In the co-transfection studies, HC11 cells were transfected with 0.07 pmol of the Oct-1B, GR, or STAT5 expression plasmid or various combinations of these constructs along with 0.2 pmol of LHRRWT/pGL3 and 0.004 pmol of phRL-CMV. In all groups, the total molar amount of DNA was balanced using pcDNA3.1. After 10-12 h, the transfection medium was replaced with hormone medium (RPMI1640 supplemented with 10% charcoal-treated horse serum, 50 µg/ml gentamicin, 1 µg/ml hydrocortisone, 5 µg/ml bovine insulin, and 5 µg/ml prolactin). Luciferase activities were examined after 24 h of hormone treatment. The *Renilla* luciferase control plasmid was used to normalize transfection efficiency. In HC11 Lux cells, the luciferase activity levels were normalized to protein concentrations.

qChIP

ChIP was performed as described previously (Kabotyanski et al., 2006) with a few modifications. Formaldehyde was added to the growth medium at a final concentration of 1% to crosslink the chromatin and interacting proteins. After sonication, the chromatin suspension was precleared with Dynabeads® Protein A. Before performing the IP, 1% of the total sheared chromatin was kept as a total input control. Next, the designated antibody was added to precipitate the sheared chromatin. The immuno-complexes were then captured with Dynabeads® Protein A. After reverse cross-linking and DNA purification, 2 µl of the final precipitated DNA was used in each PCR with SsoFast EvaGreen Supermixes (Bio-Rad, Hercules, CA). The primer sequences used for the ChIP assays are as follows: forward, 5'-GCTTCTGAATTGCTGCCTTG-3', and

reverse, 5'-GTCCTATCAGACTCTGTGACCGTA-3'. The PCR efficiency of the primers was verified. The IP data were normalized to the input DNA. For the primary MECs, cells cultured on matrigel were released with dispase reagent at 37 °C followed by fixation with formaldehyde, and the fixation was stopped by adding 10 mM EDTA.

Quantitative reverse transcription PCR (qRT-PCR)

RNA was isolated from HC11 cells using Trizol reagent (Invitrogen). Reverse transcription was performed using SuperScript II reverse transcriptase (Invitrogen) per the manufacturer's protocol. TaqMan gene expression assays were used to quantify the mRNA expression levels of Oct-1 [Applied Biosystems (Foster City, CA), Mm00448332_m1], β-casein (Mm00839664_m1), β-actin (Mm01205647_g1), and GAPDH (Mm99999915_g1). The PCRs were performed in duplicate in a 10 µl volume containing 5 µl Universal PCR Master Mix (Applied Biosystems, #4364338), 0.5 µl TaqMan assay, and 4.5 µl diluted cDNA (50 ng reverse-transcribed RNA). The relative expression levels of the target genes were normalized with the β-actin expression levels and calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Cell lysis and Western blot

Nuclear and cytoplasmic proteins were extracted based on the method described by Schreiber et al. (1989). Briefly, the collected cells were resuspended in 500 µl of cold hypotonic buffer A [10 mM HEPES (pH 7.4), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), proteinase inhibitor cocktail (Sigma), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] and incubated on ice for 15 min. After the addition of 32 µl of 10% Nonidet P40 (NP40), the cells were vigorously vortexed for 10 s.

After centrifugation for 30 s, the supernatant was collected and treated as the cytoplasmic fraction. The nuclear pellet was then resuspended in 150 µl of ice-cold hypertonic buffer [20 mM HEPES (pH 7.4), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, proteinase inhibitor cocktail, and 1 mM PMSF], and the tube was vigorously rocked at 4 °C for 15 min. After a 5-min centrifugation at 4 °C, the supernatant was isolated as the nuclear portion.

Whole cell protein lysates were prepared by adding NP40 lysis buffer (Invitrogen) consisting of 50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1% NP40, and 0.02% NaN_3 with freshly added protease inhibitor cocktail and PMSF.

The protein concentrations were determined using the Microplate BCA protein assay kit (Thermo Scientific, Rockford, IL). Equal amounts of protein from each treatment were analyzed via Western blotting with specific antibodies against Oct-1, STAT5, and GR, as described previously (Zhao et al., 2002).

IPs

The IPs were carried out according to the instructions provided with the Relia BLOT® IP/Western Blot kit (Bethyl Laboratories). In general, 1 mg of cell lysate was incubated overnight with 3 µg of the corresponding antibodies at 4 °C with rotation. The immune complexes were captured using Dynabeads® Protein A and analyzed via Western blotting.

DNA pull-down assays

DNA pull-down assays were carried out as previously reported by Magné et al. (2003). The biotinylated oligonucleotides used in the DNA pull-down assays are as follows: BK_C_WT, 5'-biotin-CCACAAAATTAGCATGTCATTA-3'; BK_C_MT, 5'-biotin-CCACAAATAATCCATGTCATTA-3'; and BK_B_WT, 5'-biotin-CACGTAGACTTCTTGGAATTGAAGGGACTTTTTGA-3'. Next, 1 mg of nuclear extract was incubated with 1 µg of the biotinylated oligonucleotides in binding buffer (10 mM HEPES, 100 µM EDTA, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 4 mM spermidine, 1 mM DTT, 0.1 mg/ml bovine serum albumin, 2.5% glycerol, proteinase inhibitor cocktail, and 1 mM PMSF) overnight at 4 °C. Dynabeads® M-280 Streptavidin was then added to pull down the oligonucleotide-protein complexes. The proteins pulled down were boiled in SDS-PAGE loading buffer and then analyzed via Western blotting.

Site-directed mutagenesis of STAT5

Site-directed mutagenesis was performed using the GeneArt® Site-Directed Mutagenesis System (Invitrogen). The leucine (Leu) 767 residue of STAT5 was mutated to proline (Pro), as this single mutation was shown to abolish the STAT5-Oct-1 interaction (Magné et al., 2003). The following pair of primers was used: mSTAT5-L767P-Forward, 5'-GGCACGTGGAAGAACTTCCACGCCGGCCCATGGACAG-3', and mSTAT5-L767P-Reverse, 5'-CTGTCCATGGGCCGGCGTGGAAGTTCTTCCACGTGCC-3' (the mutations are underlined).

Electrophoresis mobility shift assays (EMSAs)

EMSAs were performed using the 5'-end biotin labeled probe, Bio_βCP_STAT5 (5'-biotin-AGACTTCTTGGAATTGAAGGGA-3'), which corresponds to a portion of the mouse β-casein promoter (the STAT5 binding motif is underlined). Twenty femtomoles of the probe was incubated with 6 μg of nuclear extract for 20 min at room temperature. The remaining steps were performed according to the instructions of the Light Shift Chemiluminescent EMSA Kit protocol (Pierce, Rockford, IL).

Statistical analysis

All statistical analyses were carried out using JMP statistical software (SAS, Cary, NC). The comparisons between two groups were performed using the *t*-test. A one-way ANOVA test with Turkey's post hoc analysis was performed when comparisons were performed between more than two groups.

RESULTS

Lactogenic hormones, HP, rapidly induce Oct-1 binding to the β-casein gene promoter

We have previously demonstrated by using EMSA that Oct-1 binds to the octamer motif in the β-casein promoter (Zhao et al., 2002). To determine whether this binding occurs in mammary epithelial cells and whether the binding activity is responsive to HP treatment, we performed ChIPs in HC11 cells using anti-Oct-1 antibody at different time points after HP treatment. The primers were designed to amplify the β-casein gene proximal promoter, which contains the binding sites for Oct-1, STAT5, and other

transcription factors (Fig. 2.1A). STAT5 binding was measured as a positive control. The binding activity of Oct-1 and STAT5 to the β-casein gene promoter was relatively low in the absence of HP (Fig. 2.1B) but increased dramatically at 30 min of HP treatment followed by an appreciable decrease at 24 h (Fig. 2.1B). Normal rabbit IgG, the negative control, was unable to immunoprecipitate Oct-1-DNA complexes (Fig. 2.1B). To quantify the effects of HP on Oct-1 binding activity, chromatin DNA obtained via ChIP was analyzed using qPCR. As shown in Fig. 2.1C, Oct-1 binding activity increased approximately 4-fold at 30 min after HP treatment and then decreased to only an ~2-fold increase compared with the levels detected in the untreated cells. STAT5, the positive control, displayed similar binding dynamics, which correlated with previously published results (Fig. 2.1D) (Kabotyanski et al., 2006). HP-induced Oct-1 binding activity was also confirmed in primary MEC cultures (Fig. 2.1E, bottom). The HP-induced β-casein mRNA expression levels observed in primary cells were verified using RT-PCR (Fig. 2.1E, top). These results demonstrate that Oct-1 binds to the β-casein gene promoter in the intact HC11 cells and primary MECs, which is regulated by HP, with a dynamic binding profile similar to that of STAT5.

Oct-1 serves as a transcriptional activator of the β-casein gene

To test the effect of Oct-1 on HP-induced β-casein gene expression in mammary epithelial cells, we examined the effect of Oct-1 overexpression on HP-induced β-casein promoter activity and endogenous β-casein expression in HC11 cells. First, HC11 cells were co-transfected with the β-casein (-258/+7) *firefly* luciferase reporter plasmid (LHRRWT/pGL3) and the *Renilla* luciferase reporter vector (phRL-CMV) along with

either the Oct-1 expression plasmid or the empty vector followed by HP treatment. Oct-1 overexpression further enhanced HP induction of β-casein promoter activity by 80% compared with the vector control group (Fig. 2.2A, left). Fig. 2.2A (right) shows that the quantity of Oct-1 protein in the cells transfected with the Oct-1 expression plasmid was substantially higher than that of the cells transfected with the empty vector. Second, overexpression of Oct-1 also increased endogenous HP-induced β-casein expression levels by approximately 3-fold in HC11 cells (Fig. 2.2B).

To further verify the transactivator function of Oct-1 in the HP induction of β-casein gene expression, we utilized siRNA to knockdown Oct-1 expression in HC11 Lux cells, which are stably transfected with a luciferase reporter driven by the β-casein gene promoter. Two different Oct-1 siRNAs, Oct-1 siRNA #1 and Oct-1 siRNA #2, were tested and both of them successfully repressed Oct-1 expression (Fig. 2.3A, middle and bottom) and were able to significantly inhibit the HP-induced luciferase activity in HC11 Lux cells by approximately 30% compared with the cells transfected with control siRNA (Fig. 2.3A, top). We also examined the effect of the Oct-1 siRNA #1 on endogenous HP-induced β-casein expression in HC11 cells. As shown in Fig. 2.3B, endogenous HP-induced β-casein expression in HC11 cells was reduced by approximately 65% in response to Oct-1 knockdown. Thus, our observations indicated that Oct-1 functions as a transactivator in HP-regulated β-casein gene expression.

HP do not affect Oct-1 expression nor Oct-1 subcellular localization in mammary epithelial cells

HP may affect the binding of Oct-1 to the β-casein gene promoter by increasing either Oct-1 expression levels or Oct-1 levels in the nucleus. To test these possibilities, we investigated the effects of HP on Oct-1 mRNA and protein expression levels in HC11 cells. HC11 cells were treated either with or without HP for various time periods. Oct-1 mRNA levels were assessed using qRT-PCR. The mRNA levels of β-casein and GAPDH were also examined as positive and negative controls, respectively. As shown in Fig. 2.4A, a dramatic HP-mediated induction of β-casein mRNA expression was observed at 3-24 h of the HP treatment, while the GAPDH mRNA levels were not affected by HP treatment (Fig. 2.4B). The mRNA and protein levels of Oct-1 were unchanged by HP treatment (Fig. 2.4C and D).

Next, to determine whether HP induces Oct-1 translocation from the cytoplasm to the nucleus, HC11 cells were treated with HP for various time periods. The cytoplasmic and nuclear fractions were then extracted and analyzed via Western blotting using an anti-Oct-1 antibody. As shown in Fig. 2.5A, Oct-1 was primarily localized to the nucleus regardless of the hormone treatment, while HP rapidly induced the translocation of STAT5 and GR to the nucleus within 5 min (Fig. 2.5A), as previously reported (Lechner et al., 1997). These results were also confirmed by immunofluorescence staining as shown in Fig. 2.5B. These observations suggest that HP induces Oct-1 binding activity via mechanisms other than enhancing Oct-1 expression and translocation.

HP induce the formation of a ternary complex of Oct-1 with STAT5 and GR at the β-casein gene promoter

Transcription of the β-casein gene in the mammary gland is triggered by HP, mediated through the interaction between STAT5 and GR (Lechner et al., 1997). To study the role of Oct-1 in this process, co-IPs were performed. Whole cell lysates were prepared from HP-treated and HP-untreated HC11 cells and then immunoprecipitated with an anti-Oct-1, anti-GR, or anti-STAT5 antibody. As shown in Fig. 2.6A, the anti-Oct-1 antibody co-immunoprecipitated STAT5 and GR in the cells treated with HP for only 30 min (Fig. 2.6A, top, lane 4), whereas STAT5 and GR were not associated with Oct-1 in the absence of HP (Fig. 2.6A, top, lane 3). As expected, normal rabbit IgG failed to co-immunoprecipitate STAT5 and GR (Fig. 2.6A, top, lanes 1 and 2). In Fig. 2.6A (top), lanes 5-8 show that the same amounts of proteins were used for each IP. The IPs with the anti-STAT5 and anti-GR antibodies revealed the same results (Fig. 2.6A, middle and bottom). These results demonstrated that HP induced rapid physical interactions between Oct-1, STAT5, and GR in HC11 cells. The same results were also observed in primary MECs (Fig. 2.6B). The physical interactions of Oct-1 with STAT5 and GR were also verified with DNA pull-down assays. A biotinylated, wild-type β-casein proximal promoter block C oligonucleotide (BK_C_WT) (Fig. 2.6C), which contains the Oct-1 binding site, was incubated with nuclear extracts isolated from HC11 cells either with or without HP treatment and then immobilized on streptavidin-coated Dynabeads. The streptavidin-bead-bound complexes were then analyzed via Western blotting using the anti-Oct-1, anti-STAT5, and anti-GR antibodies. As shown in Fig. 2.6D, HP induced an increase in the quantity of Oct-1 molecules bound to wild-type BK_C_WT oligonucleotide (lanes 1 and 2), which agrees with our ChIP results. Additionally, the

BK_C_WT oligonucleotide pulled down STAT5 and GR (Fig. 2.6D, comparing lanes 1 and 2), while the mutated BK_C_MT oligonucleotide (Fig. 2.6C), in which the sequence is identical to the BK_C_WT oligonucleotide except that the octamer-binding site was mutated so that Oct-1 is unable to bind (Zhao et al., 2002), failed to pull down Oct-1, STAT5, or GR (Fig. 2.6D, lanes 3 and 4). Thus, these data indicate that HP induce the formation of an Oct-1-STAT5-GR ternary complex in both HC11 cells and primary MECs.

Previous reports have shown that the stable recruitment of STAT5 to the STAT5-binding site at the cyclin D1 promoter depends on the presence of Oct-1 at the adjacent octamer motif (Brockman and Schuler, 2005). Therefore, we hypothesized that Oct-1 facilitates STAT5 and GR binding to the β-casein gene promoter. To test this hypothesis, we performed DNA pull-down assays using a biotinylated β-casein gene promoter block B oligonucleotide, BK_B_WT, which contains a STAT5-binding site and two flanking half glucocorticoid response elements (1/2 GREs) (as shown in Fig. 2.7A) and has been previously shown to be bound by STAT5 and GR in EMSAs (Préfontaine et al., 1998). The biotinylated BK_B_WT oligonucleotide was incubated with nuclear extracts isolated from HP-treated or HP-untreated HC11 cells, which were transfected with either an Oct-1 siRNA or a control siRNA. The oligonucleotide-captured protein complexes were then analyzed via Western blotting. As shown in Fig. 2.7B, BK_B_WT pulled down the Oct-1-STAT5-GR ternary complex upon HP treatment (lanes 1-4). Oct-1 knockdown both decreased the relative quantity of Oct-1 pulled down by BK_B_WT (Fig. 2.7B, the top gel, lanes 3 and 4 and lanes 5-8) and diminished the binding activity of STAT5 and GR

(Fig. 2.7B, two middle gels, lanes 3 and 4). The same experiment was performed using the biotinylated BK_C_WT oligonucleotide (Fig. 2.7C) in HC11 cells transfected with a STAT5 siRNA. STAT5 knockdown resulted in a decrease in the quantity of STAT5 in the complex pulled down by BK_C_WT following HP treatment (Fig. 2.7C, the second gel, comparing lanes 3 with 4) but did not alter the quantities of Oct-1 and GR that were pulled down (Fig. 2.7C, the first and third gel, lanes 3 and 4). These data indicate that Oct-1 may facilitate or stabilize the binding activities of STAT5 and GR at block B of the β-casein gene promoter, while HP-induced Oct-1 binding to block C is independent of STAT5.

Oct-1 synergistically interacts with both STAT5 and GR in HP-mediated induction of β-casein promoter activity

To examine the interactions of Oct-1, STAT5, and GR in the process of HP induction of β-casein expression, co-transfection experiments were performed. HC11 cells were co-transfected with the Oct-1, STAT5, or GR expression plasmids or various combinations of these plasmids followed by HP treatment. As shown in Fig. 2.8, HP only marginally induced β-casein promoter activity when the individual plasmids were transfected. However, when two of the Oct-1, STAT5, and GR plasmids were co-transfected, HP induction of the promoter activity increased significantly (Fig. 2.8, groups 4, 5, and 6), and the highest promoter activity was achieved only when all three plasmids were transfected (Fig. 2.8, group 7). Thus, the interactions of Oct-1 with both STAT5 and GR are critical for the full induction of β-casein promoter activity in response to HP.

A mutation that impairs the Oct-1 and STAT5 interaction significantly reduces the HP-mediated induction of β-casein gene promoter activity

To further study the critical importance of the interaction between Oct-1 and STAT5 in the HP-mediated induction of β-casein gene expression, we mutated the Leu 767 residue of STAT5 to Pro (MT STAT5) because this single mutation has been shown to impair the STAT5-Oct-1 interaction without jeopardizing STAT5 DNA binding (Magné et al., 2003). COS-7 cells were made to be HP sensitive by transfecting the cells with a pcDNA3.1, MT STAT5, or WT STAT5 plasmid along with the Oct-1, GR, PrlR, LHRRWT/pGL3, and phRL-CMV expression plasmids followed by a 24-h HP treatment. As shown in Fig. 2.9A, the MT STAT5 group showed only approximately 30% of the HP-induced β-casein gene promoter activity of the wild-type STAT5 group, and the pcDNA3.1 group showed the lowest activity. To verify the interaction between Oct-1 and MT STAT5, an IP was performed using the anti-Oct-1 antibody. As shown in Fig. 2.9B (lanes 3 and 4 of the second gel), the Leu/Pro single mutation greatly diminished the HP-induced interaction between STAT5 and Oct-1, while the interaction between Oct-1 and GR was not disturbed (Fig. 2.9B, the third gel, lanes 3 and 4). Additionally, to rule out the possibility that the Leu/Pro mutation of STAT5 impairs its DNA binding ability, an EMSA was performed using a biotinylated STAT5-binding site probe, Bio_βCP_STAT5, of the β-casein gene promoter (Fig. 2.9C). The experiment confirmed that the Leu/Pro mutation did not affect the binding activity of STAT5 (Fig. 2.9C, compare lanes 2 and 3).

The protein-DNA complexes were abolished using an anti-STAT5 antibody but not normal rabbit IgG. In short, these data suggest that impairing the Oct-1-STAT5 interaction significantly diminishes HP-induced β-casein gene promoter activity.

DISCUSSION

Previous studies have indicated that Oct-1 may play a role in the hormonal regulation of β-casein gene expression (Zhao et al., 2002; Dong and Zhao, 2007). In this study, we demonstrated that Oct-1 activates the hormonal induction of β-casein gene expression via physical interactions with STAT5 and GR and that interrupting these interactions significantly diminishes the hormonal induction.

In this report, we show that Oct-1 is a downstream signaling molecule of lactogenic hormones in mammary epithelial cells. Although Oct-1 binds to the β-casein gene promoter without lactogenic hormone stimulation and this binding is important for the basal promoter activity of β-casein gene (Zhao et al., 2002, 2004), lactogenic hormones further induce Oct-1 binding to the promoter. These data are correlated with previous findings that have demonstrated that the Oct-1 DNA-binding ability is developmentally regulated in mouse mammary tissue and that its expression pattern correlates with β-casein expression (Saito and Oka, 1996). We also show that the increase in Oct-1-binding activity by lactogenic hormones does not result from the regulation of Oct-1 mRNA and protein expression or from the induction of Oct-1 nuclear translocation in mammary epithelial cells. One of the possible mechanisms for the binding activity

increase may be recruitment of Oct-1 to the DNA via the interactions with STAT5 and GR.

STAT5 and GR play an essential role in mediating the induction of β-casein gene expression via prolactin and glucocorticoid signaling, respectively. The STAT5 and GR proteins physically interact with each other and synergistically stimulate β-casein gene transcription upon hormonal induction (Stöcklin et al., 1996; Wyszomierski et al., 1999). This interaction activates STAT5 by prolonging STAT5 DNA-binding and tyrosine phosphorylation (Wyszomierski et al., 1999) and enhances the binding of GR to the half-GREs (Cella et al., 1998; Stöcklin et al., 1996). Previous studies have also shown that Oct-1 physically interacts with STAT5 or GR in a promoter-specific manner. For example, activation of the MMTV promoter has been shown to be highly dependent on the GR-Oct-1 interaction (Préfontaine et al., 1998). The cytokine-activated STAT5 and Oct-1 molecules form a stable complex in the transcriptional activation of Cyclin D1 (Brockman and Schuler, 2005; Magné et al., 2003). In this study, using co-IP and DNA pull-down assays, we demonstrate for the first time that Oct-1, STAT5, and GR form a ternary complex upon stimulation with lactogenic hormones. This complex may stabilize Oct-1-binding at the β-casein gene promoter.

Our DNA pull-down assays (Fig. 2.7), however, showed that the relative quantities of STAT5 and GR molecules pulled down by the β-casein promoter block B oligonucleotide were much less in Oct-1 knockdown cells, while surprisingly, STAT5 knockdown in the cells had no effect on the quantity of either Oct-1 or GR protein pulled down by the β-casein promoter block C oligonucleotide. This result indicates that Oct-1

plays a central role in either facilitating or stabilizing STAT5 and GR bindings at the β-casein promoter in response to lactogenic hormone signaling. Our data also show that the interactions between Oct-1, STAT5, and GR do not require the bindings of all of these factors to the corresponding DNA-binding sites of the promoter, as the oligonucleotides used in the pull down experiments did not contain the binding sites for all three factors. However, the maximal transcriptional activation of the β-casein promoter must require the binding activity of these factors, especially STAT5, because mutations in each of these sites at the β-casein promoter (especially the STAT5 site) dramatically reduced the hormonal induction of the promoter activity (Dong and Zhao, 2007).

The importance of the Oct-1-STAT5-GR interaction in the hormonal induction of the β-casein gene was demonstrated by our co-transfection experiment in HC11 cells, which showed that the maximal transcriptional induction of the β-casein gene promoter by lactogenic hormones is achieved only in the presence of Oct-1, STAT5, and GR. These results agree with our previous study in COS-7 cells which are reconstituted to be lactogenic hormone- responsive (Dong and Zhao, 2007; Dong et al., 2009). Moreover, we employed a Leu/Pro-mutated form of STAT5, which showed diminished interaction with Oct-1 when stimulated with lactogenic hormones in a co-transfection experiment in COS-7 cells. This experiment showed that the mutated form of STAT5 was not able to activate the β-casein gene promoter as efficiently as the WT STAT5. The reduced efficiency is mainly due to the impaired interaction of the mutated STAT5 molecule with Oct-1, as this mutation does not affect the binding activity of STAT5 to the β-casein gene promoter.

Overall, it is conceivable that the Oct-1, STAT5, and GR interactions are critical for the lactogenic hormone-mediated induction of the β-casein gene.

Another mechanism by which lactogenic hormones enhance the DNA-binding activity of Oct-1 may be via post-translational modification of Oct-1 protein, such as protein phosphorylation. Oct-1 has been shown to be phosphorylated by several kinases, such as protein kinase A (PKA) (Caelles et al., 1995; Roberts et al., 1991), cyclic GMP-dependent kinase (Belsham and Mellon, 2000), and DNA-dependent protein kinase (DNA-PK) (Kang et al., 2009a; Schild-Poulter et al., 2007). In mammary epithelial cells, prolactin rapidly induces the phosphorylation of STAT5 through Janus kinase 2 (JAK2), which then dimerizes, translocates to nucleus, and binds to the β-casein promoter (Wyszomierski and Rosen, 2001). Prolactin may also phosphorylate Oct-1 by activating downstream kinases. Aside from JAK2, the potential downstream protein kinases may include protein kinase B (PKB), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) (Goffin et al., 2002; Yu-Lee, 2002). Additionally, evidence has shown that Oct-1 DNA-binding activity can be modulated via phosphorylation. For example, Oct-1 is phosphorylated at both serine and threonine residues *in vivo* upon oxidative stress, and this phosphorylation at two conserved DNA-binding domain serine residues regulates the binding of Oct-1 to DNA (Kang et al., 2009a). The role of Oct-1 phosphorylation in the hormonal induction of β-casein gene expression requires further study.

Our previous study showed that Oct-1-binding activity in virgin mouse mammary glands is also induced by progesterone (Zhao et al., 2002), a reproductive hormone that

inhibits β-casein gene expression (Buser et al., 2007). The specific mechanism by which progesterone inhibits β-casein gene expression is unknown. As Oct-1 has been shown to interact with the progesterone receptor (PR) (Préfontaine et al., 1999), it is possible that Oct-1 also participates in the inhibition of β-casein gene expression via interactions with different factors, such as PR.

Oct-1 has been reported to interact with basal transcription factors, such as TBP and TFIIB, at both the small nuclear RNA gene promoter (Zwilling et al., 1994) and the lipoprotein lipase promoter (Nakshatri et al., 1995). Although the interaction of Oct-1 with TBP was not observed in our DNA pull-down assays performed in this study, the interaction may require the DNA binding of TBP. In the proximal β-casein gene promoter, the octamer motif is only 20 base pairs upstream of the TATA box. Thus, Oct-1 may potentially bind to the transcription initiation complex on the β-casein gene promoter. We hypothesize that lactogenic-hormone-activated Oct-1 (via phosphorylation) recruits and tethers other lactogenic hormone signaling molecules, including STAT5 and GR, to the basal transcription machinery to form and stabilize the active transcription complex at the β-casein promoter.

In conclusion, we have demonstrated for the first time that Oct-1 forms a ternary complex with STAT5 and GR upon the stimulation with lactogenic hormones. Additionally, these interactions enhance or stabilize the binding of these transcription factors to the β-casein gene promoter and mediate the hormonal induction of β-casein gene expression.

Acknowledgements—We thank Dr. Bryan Ballif for his assistance with the project and for reviewing the manuscript. We also thank Dr. Margaret Neville for providing the murine β-casein antibody.

REFERENCES

Belsham DD, Mellon PL. Transcription factors Oct-1 and C/EBPβ (CCAAT/enhancer-binding protein-β) are involved in the glutamate/nitric oxide/cyclic-guanosine 5′-monophosphate-mediated repression of gonadotropin-releasing hormone gene expression. Molecular Endocrinology 2000;14:212-28.

Brockman JL, Schuler LA. Prolactin signals via Stat5 and Oct-1 to the proximal cyclin D1 promoter. Molecular and Cellular Endocrinology 2005;239:45-53.

Buser AC, Gass-Handel EK, Wyszomierski SL, Doppler W, Leonhardt SA, Schaack J, et al. Progesterone receptor repression of prolactin/signal transducer and activator of transcription 5-mediated transcription of the β-casein gene in mammary epithelial cells. Molecular Endocrinology 2007;21:106-25.

Caelles C, Hennemann H, Karin M. M-phase-specific phosphorylation of the POU transcription factor GHF-1 by a cell cycle-regulated protein kinase inhibits DNA binding. Molecular and Cellular Biology 1995;15:6694-701.

Cella N, Groner B, Hynes NE. Characterization of Stat5a and Stat5b homodimers and heterodimers and their association with the glucocorticoid receptor in mammary cells. Molecular and Cellular Biology 1998;18:1783-92.

Cheng CC, Yang SP, Lin WS, Ho LJ, Lai JH, Cheng SM, et al. Magnesium lithospermate B mediates anti-inflammation targeting activator protein-1 and nuclear factor-kappa B signaling pathways in human peripheral T lymphocytes. International Immunopharmacology 2012;13:354-61.

Dong B, Huang C, Li D, Zhao FQ. Oct-1 functions as a transactivator in the hormonal induction of β-casein gene expression. Molecular and Cellular Biochemistry 2009;328:93-9.

Dong B, Zhao FQ. Involvement of the ubiquitous Oct-1 transcription factor in hormonal induction of β-casein gene expression. Biochemical Journal 2007;401:57-64.

Goettsch C, Goettsch W, Brux M, Haschke C, Brunssen C, Muller G, et al. Arterial flow reduces oxidative stress via an antioxidant response element and Oct-1 binding site within the NADPH oxidase 4 promoter in endothelial cells. Basic Research in Cardiology 2011;106:551-61.

Goffin V, Binart N, Touraine P, Kelly PA. Prolactin: the new biology of an old hormone. Annual Review of Physiology 2002;64:47-67.

Groner B, Altiok S, Meier V. Hormonal regulation of transcription factor activity in mammary epithelial cells. Molecular and Cellular Endocrinology 1994;100:109-14.

Kabotyanski EB, Huetter M, Xian W, Rijnkels M, Rosen JM. Integration of prolactin and glucocorticoid signaling at the β-casein promoter and enhancer by ordered recruitment of specific transcription factors and chromatin modifiers. Molecular Endocrinology 2006;20:2355-68.

Kang J, Gemberling M, Nakamura M, Whitby FG, Handa H, Fairbrother WG, et al. A general mechanism for transcription regulation by Oct1 and Oct4 in response to genotoxic and oxidative stress. Genes and Development 2009a;23:208-22.

Kang J, Shakya A, Tantin D. Stem cells, stress, metabolism and cancer: a drama in two Octs. Trends in Biochemical Sciences 2009b;34:491-9.

Lechner J, Welte T, Tomasi JK, Bruno P, Cairns C, Gustafsson J, et al. Promoter-dependent synergy between glucocorticoid receptor and Stat5 in the activation of β-casein gene transcription. Journal of Biological Chemistry 1997;272:20954-60.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 2001;25:402-8.

Magné S, Caron S, Charon M, Rouyez MC, Dusanter-Fourt I. STAT5 and Oct-1 form a stable complex that modulates cyclin D1 expression. Molecular and Cellular Biology 2003;23:8934-45.

Malhas AN, Lee CF, Vaux DJ. Lamin B1 controls oxidative stress responses via Oct-1. Journal of Cell Biology 2009;184:45-55.

Nakshatri H, Nakshatri P, Currie RA. Interaction of Oct-1 with TFIIB. Implications for a novel response elicited through the proximal octamer site of the lipoprotein lipase promoter. Journal of Biological Chemistry1995;270:19613-23.

Préfontaine GG, Lemieux ME, Giffin W, Schild-Poulter C, Pope L, LaCasse E, et al. Recruitment of octamer transcription factors to DNA by glucocorticoid receptor. Molecular and Cellular Biology 1998;18:3416-30.

Préfontaine GG, Walther R, Giffin W, Lemieux ME, Pope L, Haché RJ. Selective binding of steroid hormone receptors to octamer transcription factors determines transcriptional synergism at the mouse mammary tumor virus promoter. Journal of Biological Chemistry 1999;274:26713-9.

Range R, Lepage T. Maternal Oct1/2 is required for Nodal and Vg1/Univin expression during dorsal-ventral axis specification in the sea urchin embryo. Developmental Biology 2011;357:440-9.

Ren X, Siegel R, Kim U, Roeder RG. Direct interactions of OCA-B and TFII-I regulate immunoglobulin heavy-chain gene transcription by facilitating enhancer-promoter communication. Molecular Cell 2011;42:342-55.

Roberts SB, Segil N, Heintz N. Differential phosphorylation of the transcription factor Oct1 during the cell cycle. Science 1991;253:1022-6.

Robinson AR, Kwek SS, Hagemeier SR, Wille CK, Kenney SC. Cellular transcription factor Oct-1 interacts with the Epstein-Barr virus BRLF1 protein to promote disruption of viral latency. Journal of Virology 2011;85:8940-53.

Rosen JM, Wyszomierski SL, Hadsell D. Regulation of milk protein gene expression. Annual Review of Nutrition 1999;19:407-36.

Saito H, Oka T. Hormonally regulated double-and single-stranded DNA-binding complexes involved in mouse-casein gene transcription. Journal of Biological Chemistry 1996;271:8911-8.

Schild-Poulter C, Shih A, Tantin D, Yarymowich NC, Soubeyrand S, Sharp PA, et al. DNA-PK phosphorylation sites on Oct-1 promote cell survival following DNA damage. Oncogene 2007;26:3980-8.

Schreiber E, Matthias P, Müller MM, Schaffner W. Rapid detection of octamer binding proteins with ‘mini extracts’, prepared from a small number of cells. Nucleic Acids Research 1989;17:6419.

Sebastiano V, Dalvai M, Gentile L, Schubart K, Sutter J, Wu GM, et al. Oct1 regulates trophoblast development during early mouse embryogenesis. Development 2010;137:3551-60.

Shakya A, Cooksey R, Cox JE, Wang V, McClain DA, Tantin D. Oct1 loss of function induces a coordinate metabolic shift that opposes tumorigenicity. Nature Cell Biology 2009;11:320-7.

Sive HL, Roeder RG. Interaction of a common factor with conserved promoter and enhancer sequences in histone H2B, immunoglobulin, and U2 small nuclear RNA (snRNA) genes. Proceedings of National Academy of Sciences of the United States of America 1986;83:6382-6.

Stöcklin E, Wissler M, Gouilleux F, Groner B. Functional interactions between Stat5 and the glucocorticoid receptor. Nature 1996;383:726-8.

Wang P, Wang Q, Sun J, Wu J, Li H, Zhang N, et al. POU homeodomain protein Oct-1 functions as a sensor for cyclic AMP. Journal of Biological Chemistry 2009;284:26456-65.

Wartmann M, Cella N, Hofer P, Groner B, Liu X, Hennighausen L, et al. Lactogenic hormone activation of Stat5 and transcription of the β-casein gene in mammary epithelial cells is independent of p42 ERK2 mitogen-activated protein kinase activity. Journal of Biological Chemistry 1996;271:31863-8.

Watkin H, Streuli CH. Adenoviral-mediated gene transfer in two-dimensional and three-dimensional cultures of mammary epithelial cells. Methods in Cell Biology 2002;69:403-23.

Wyszomierski SL, Rosen JM. Cooperative effects of STAT5 (signal transducer and activator of transcription 5) and C/EBPβ (CCAAT/enhancer-binding protein-β) on β-casein gene transcription are mediated by the glucocorticoid receptor. Molecular Endocrinology 2001;15:228-40.

Wyszomierski SL, Yeh J, Rosen JM. Glucocorticoid receptor/signal transducer and activator of transcription 5 (STAT5) interactions enhance STAT5 activation by prolonging STAT5 DNA binding and tyrosine phosphorylation. Molecular Endocrinology 1999;13:330-43.

Yoshimura M, Oka T. Transfection of β-casein chimeric gene and hormonal induction of its expression in primary murine mammary epithelial cells. Proceedings of National Academy of Sciences of the United States of America 1990;87:3670-4.

Yu-Lee LY. Prolactin modulation of immune and inflammatory responses. Recent Progress in Hormone Research 2002;57:435-55.

Zhao F-Q. Octamer-binding transcription factors: genomics and functions (review). Frontiers in Bioscience 2013, in press.

Zhao F-Q, Adachi K, Oka T. Involvement of Oct-1 in transcriptional regulation of β-casein gene expression in mouse mammary gland. Biochimica Biophysica Acta 2002;1577:27-37.

Zhao F-Q, Zheng Y, Dong B, Oka T. Cloning, genomic organization, expression, and effect on β-casein promoter activity of a novel isoform of the mouse Oct-1 transcription factor. Gene 2004;326:175-87.

Zwilling S, Annweiler A, Wirth T. The POU domains of the Oct1 and Oct2 transcription factors mediate specific interaction with TBP. Nucleic Acids Research 1994;22:1655-62.

FIGURE LEGENDS

Fig. 2.1. Lactogenic hormones induce the binding of Oct-1 to the β-casein gene promoter *in vitro*. (A) A schematic representation of the putative transcription factor-binding sites in the murine β-casein gene proximal promoter. The primers used for the qChIP assay (Primer F and Primer TR) are indicated. The abbreviations used are as follows: C/EBP, CCAAT/enhancer-binding protein; Runx2, Runt-related transcription factor 2; and TATA, TATA box. (B) ChIP assays were performed on chromatin prepared from the HC11 cells treated with HP for 0 min, 30 min, or 24 h using either anti-Oct-1 or anti-STAT5 antibodies or normal rabbit IgG (Ctrl Ab). PCR was performed using primer F and Primer TR. (C and D) qPCR was performed to measure the binding dynamics of Oct-1 (C) and STAT5 (D) at the β-casein gene promoter in HC11 cells treated with HP for the indicated time periods. The IP data were normalized to the input DNA, and the quantity of the precipitated DNA is expressed as the fold change in the hormone-treated cells relative to the untreated cells. Three independent experiments were performed. The values are the mean ± SE. $*P<0.05$, $**P<0.01$, and n.s. = no significant difference. (E) Primary MECs were isolated from mid-pregnant mice and grown on matrigel until confluent. Two days later, the cells were primed followed by treatment either with or without HP stimulation for 24 hrs. β-Casein gene expression was analyzed by RT-PCR (top). The binding activity of Oct-1 at the β-casein gene promoter was measured using qChIP (bottom). The data represent the mean of three independent experiments ± SE. $*P< 0.05$.

Fig. 2.2. Oct-1 overexpression enhances HP-induced β-casein gene expression. (A) HC11 cells were co-transfected with the pcDNA3.1 vector (V) or an Oct-1 expression plasmid (Oct-1B) along with a *firefly* luciferase reporter construct driven by the β-casein promoter and a *Renilla* luciferase control plasmid (phRL-CMV) followed by HP treatment for 24 h. The reporter luciferase activity levels are expressed as the mean values ± SE (left). The data were combined from three independent experiments. **P*< 0.05. The Oct-1 expression levels in transfected cells were monitored via Western blot analysis of the whole cell lysates using an anti-Oct-1 antibody (right). β-Actin was used as a loading control. (B) Western blot analysis was performed to measure the relative quantity of β-casein protein in the whole cell lysates of HP-treated HC11 cells transfected with either the pcDNA3.1 (V) or Oct-1B plasmid (left). The densitometric analysis of β-casein expression shown represents the data of three independent experiments (right). The values are the mean ± SE. **P*< 0.05.

Fig. 2.3. Oct-1 knockdown inhibits HP-induced β-casein gene expression. (A) HC11 Lux cells, which are stably transfected with the mouse β-casein promoter (-344/-1)/luciferase reporter, were transfected with either Oct-1 siRNA #1 (Oct-1_#1), Oct-1 siRNA #2 (Oct-1_#2), or control siRNA (Ctrl) followed by HP treatment for 24 h. The luciferase activity levels were then assayed and normalized by the protein concentrations (top). The relative luciferase activity levels are expressed as the mean values ± SE from three independent experiments (**P*< 0.05). Oct-1 knockdown efficiencies in the cells were monitored via qRT-PCR and Western blot analysis (middle and bottom). β-Actin was used as a loading

control. (B) Western blot analysis was performed to measure the relative quantity of endogenous β-casein protein in whole cell lysates from HP-treated HC11 cells transfected with either Oct-1 siRNA #1 or Ctrl siRNA (top). The densitometric analysis of the β-casein expression shown represents three independent experiments (bottom). The values represent the mean ± SE. *$P< 0.05$.

Fig. 2.4. The effects of HP treatment on Oct-1 mRNA and protein expression levels in HC11 cells. (A-C) HC11 cells were treated with HP for 0, 1, 3, 6, 12, 24, or 48 h, and the total RNA isolated from the cells treated or untreated with HP was then analyzed via qRT-PCR for β-casein (A, positive control), GAPDH (B, negative control), and Oct-1 (C) mRNA expression. The data are expressed as the mean of three independent experiments ± SE. **$P<0.01$ and ***$P<0.001$. (D) Western blot analysis was performed to examine Oct-1 protein expression in HC11 cells treated with HP for 0, 1, 6, 24, or 48 h. β-Actin was used as a loading control.

Fig. 2.5. HP induce the translocation of STAT5 and GR but not Oct-1. (A) HC11 cells were treated with HP for the indicated time periods, and the cytoplasmic and nuclear fractions were subsequently isolated. Western blot analyses were performed using specific antibodies against Oct-1, STAT5, GR, TBP (nuclear loading control), and β-actin (cytoplasmic loading control). (B) HC11 cells were treated with or without HP for 30 min. The intracellular localization of Oct-1 was examined by immunofluorescence staining.

Intracellular localization of STAT5 was also studied as a positive control. DAPI = 4',6-diamidino-2-phenylindole (nuclear staining).

Fig. 2.6. HP induce the formation of the Oct-1-STAT5-GR ternary complex. (A) Whole cell lysates of HC11 cells treated either with or without HP for 30 min were immunoprecipitated using antibodies against Oct-1 (top), STAT5 (middle), and GR (bottom) and were subsequently analyzed via Western blot analyses with anti-Oct-1, anti-STAT5, anti-GR, and anti-β-actin (control) antibodies. A normal rabbit IgG (Ctrl Ab) was used in IP assays as an antibody-specificity control. Five percent of each whole cell lysate was stored before IP and was used as an input control. (B) IP assays with the anti-Oct-1 antibody were also performed in primary MECs to examine the association of Oct-1 with STAT5 and GR in response to HP stimulation. (C) A schematic view of the biotinylated oligonucleotides used in the DNA pull-down assay in (D). The Oct-1-binding motif is printed in boldface for the BK_C_WT sequence. The mutated Oct-1-binding site is underlined for the BK_C_MT sequence. (D) Nuclear extracts isolated from the HP-treated and HP-untreated HC11 cells were incubated with either the BK_C_WT (lanes 1 and 2) or BK_C_MT (lanes 3 and 4) biotinylated oligonucleotides. DNA-protein complexes were then captured using streptavidin-coated Dynabeads. The captured proteins were analyzed via Western blot analyses using the indicated antibodies.

Fig. 2.7. Oct-1 facilitates STAT5 and GR binding at the β-casein promoter following HP stimulation. (A) A schematic view of the biotinylated oligonucleotides used in the DNA

pull-down assays in (B) and (C). BK_B_WT comprises a STAT5 binding site and two half GREs, which are indicated with boldface and underlined, respectively. (B and C) HC11 cells transfected with either Oct-1 siRNA #1 (B) or STAT5 siRNA (C) were treated either with or without HP, and nuclear extracts were then prepared for DNA pull-down assays. The captured proteins were analyzed via Western blot analyses using the antibodies indicated on the right side of each panel.

Fig. 2.8. Oct-1 synergistically interacts with STAT5 and GR in the HP-mediated induction of β-casein gene promoter activity. In 12-well plates, HC11 cells were transfected with a *firefly* luciferase reporter construct driven by the β-casein promoter; a *Renilla* luciferase control plasmid (phRL-CMV); and Oct-1, STAT5, and GR expression plasmids or various combinations of these plasmids, followed by HP treatment for 24 h. In all groups, the total amount of DNA transfected was balanced with the corresponding vector DNA on a molar basis. The relative luciferase activity levels are expressed as the mean values ± SE (n=10). Three independent experiments were carried out. $*P<0.05$, $**P<0.01$, and n.s. = no significant difference.

Fig. 2.9. Impaired Oct-1-STAT5 interaction significantly diminishes HP-induced β-casein gene promoter activity. (A) COS-7 cells were transfected with PrlR, Oct-1, and GR expression plasmids; a *firefly* luciferase reporter construct driven by the β-casein promoter; and a *Renilla* luciferase control plasmid (phRL-CMV) along with pcDNA3.1, mutated STAT5 (MT STAT5), or wild-type STAT5 (WT STAT5). Following HP

treatment for 24 h, the cells were lysed and the luciferase activity levels were analyzed. *P<0.05 and **P<0.01. (B) The cell lysates from (A) were used in IP analyses to examine the interactions of Oct-1 with STAT5 and GR. The immunoprecipitated proteins were analyzed via Western blot analyses using the antibodies indicated on the right side of the panel. (C) The transfected cells from (A) were also used in EMSA analyses to examine the binding activity of the mutated and wild-type forms of STAT5 using a biotinylated STAT5-binding-site-containing oligonucleotide probe corresponding to the β-casein gene promoter. Nuclear extracts isolated from pcDNA3.1-transfected cells were used as a negative control. In lanes 4-7, either the anti-STAT5 antibody or the normal rabbit IgG was added to verify the specific binding.

Fig. 2.1

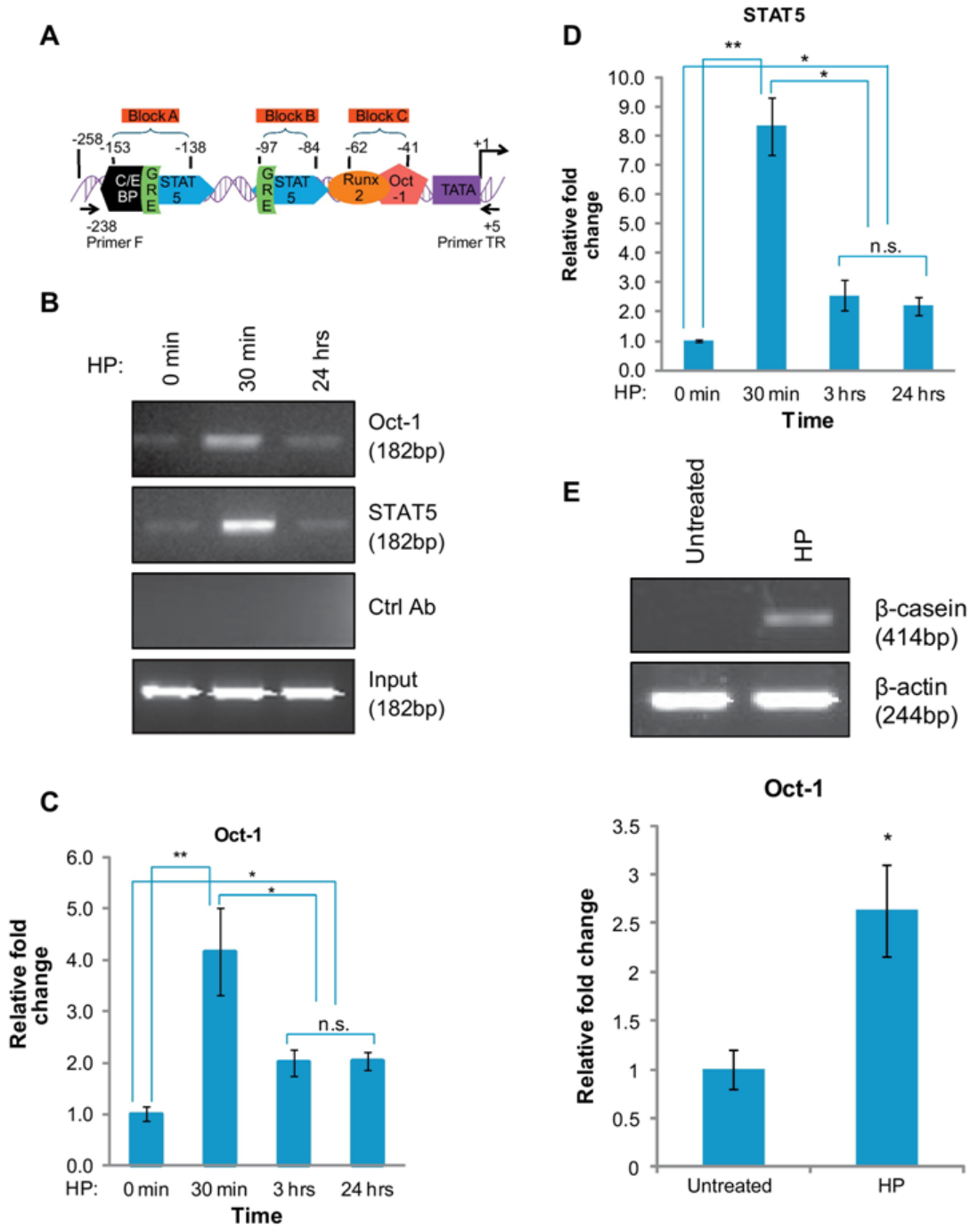

A
Block A
Block B
Block C
-258
-153
-138
-97
-84
-62
-41
+1
C/EBP
GRE
STAT 5
GRE
STAT 5
Runx 2
Oct -1
TATA
-238
Primer F
+5
Primer TR
B
HP:
0 min
30 min
24 hrs
Oct-1 (182bp)
STAT5 (182bp)
Ctrl Ab
Input (182bp)
C
Oct-1
Relative fold change
HP:
0 min
30 min
3 hrs
24 hrs
Time
n.s.
D
STAT5
Relative fold change
n.s.
E
Untreated
HP
β-casein (414bp)
β-actin (244bp)
Oct-1
Relative fold change
Untreated
HP

Fig. 2.2

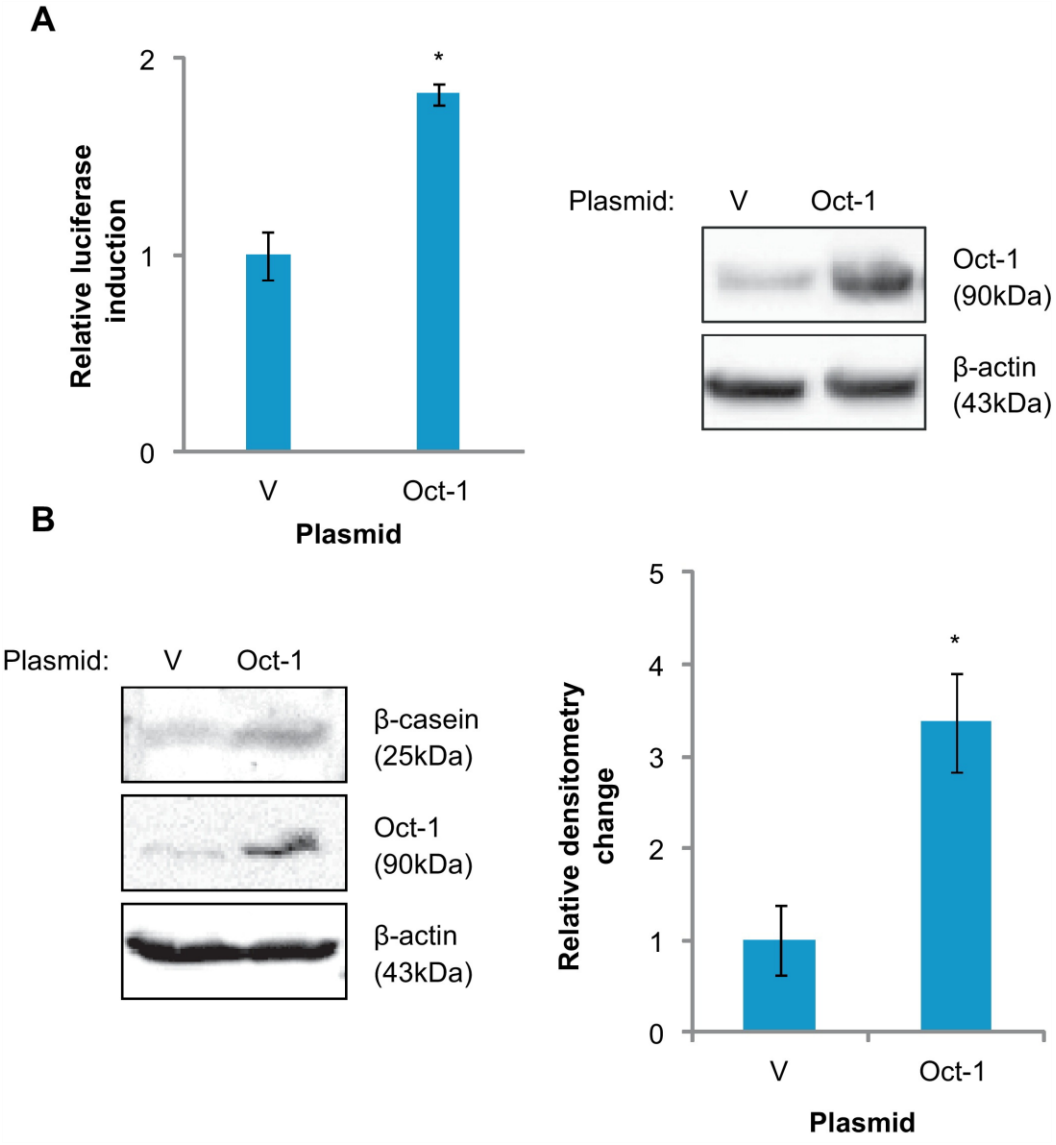

A
Relative luciferase induction
2
1
0
*
V
Oct-1
Plasmid
Plasmid: V Oct-1
Oct-1 (90kDa)
β-actin (43kDa)
B
Plasmid: V Oct-1
β-casein (25kDa)
Oct-1 (90kDa)
β-actin (43kDa)
Relative densitometry change
5
4
3
2
1
0
*
V
Oct-1
Plasmid

Fig. 2.3

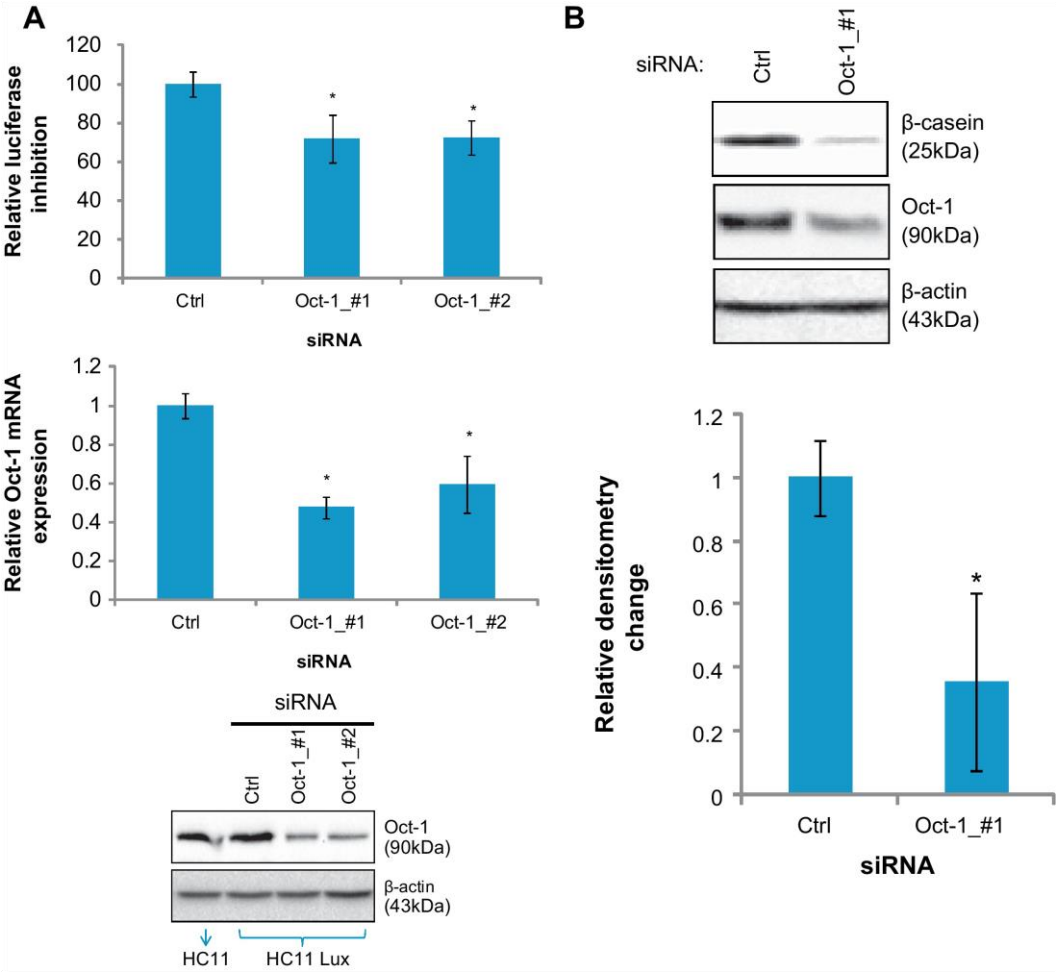

A
Relative luciferase inhibition
120
100
80
60
40
20
0
Ctrl
Oct-1_#1
Oct-1_#2
siRNA
Relative Oct-1 mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
Ctrl
Oct-1_#1
Oct-1_#2
siRNA
siRNA
Ctrl
Oct-1_#1
Oct-1_#2
Oct-1 (90kDa)
β-actin (43kDa)
HC11
HC11 Lux
B
siRNA:
Ctrl
Oct-1_#1
β-casein (25kDa)
Oct-1 (90kDa)
β-actin (43kDa)
Relative densitometry change
1.2
1
0.8
0.6
0.4
0.2
0
Ctrl
Oct-1_#1
siRNA

Fig. 2.4

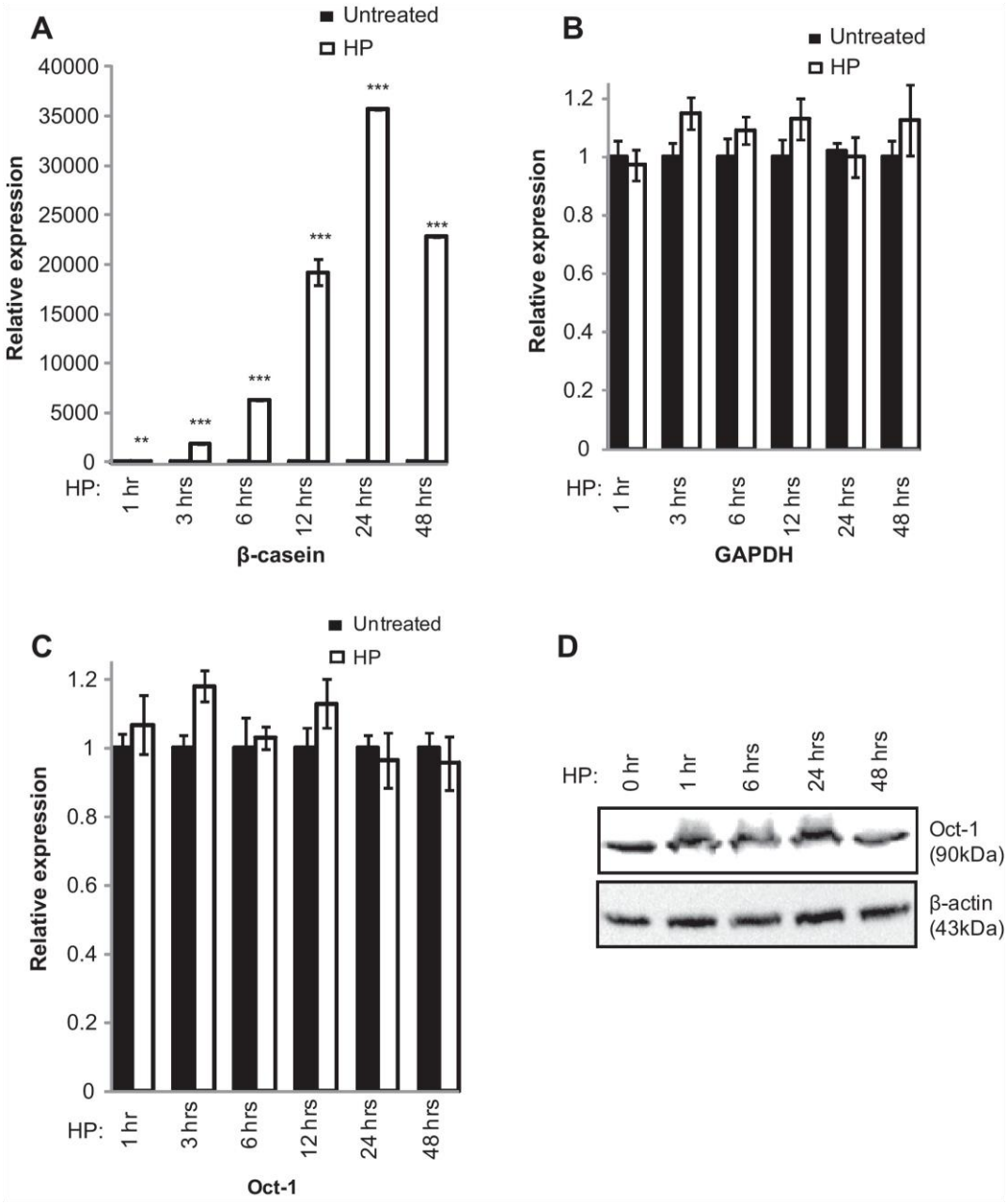

A
Untreated
HP
Relative expression
40000
35000
30000
25000
20000
15000
10000
5000
0
**
***
***
***
***
HP:
1 hr
3 hrs
6 hrs
12 hrs
24 hrs
48 hrs
β-casein
B
Untreated
HP
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
HP:
1 hr
3 hrs
6 hrs
12 hrs
24 hrs
48 hrs
GAPDH
C
Untreated
HP
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
HP:
1 hr
3 hrs
6 hrs
12 hrs
24 hrs
48 hrs
Oct-1
D
HP:
0 hr
1 hr
6 hrs
24 hrs
48 hrs
Oct-1
(90kDa)
β-actin
(43kDa)

Fig. 2.5

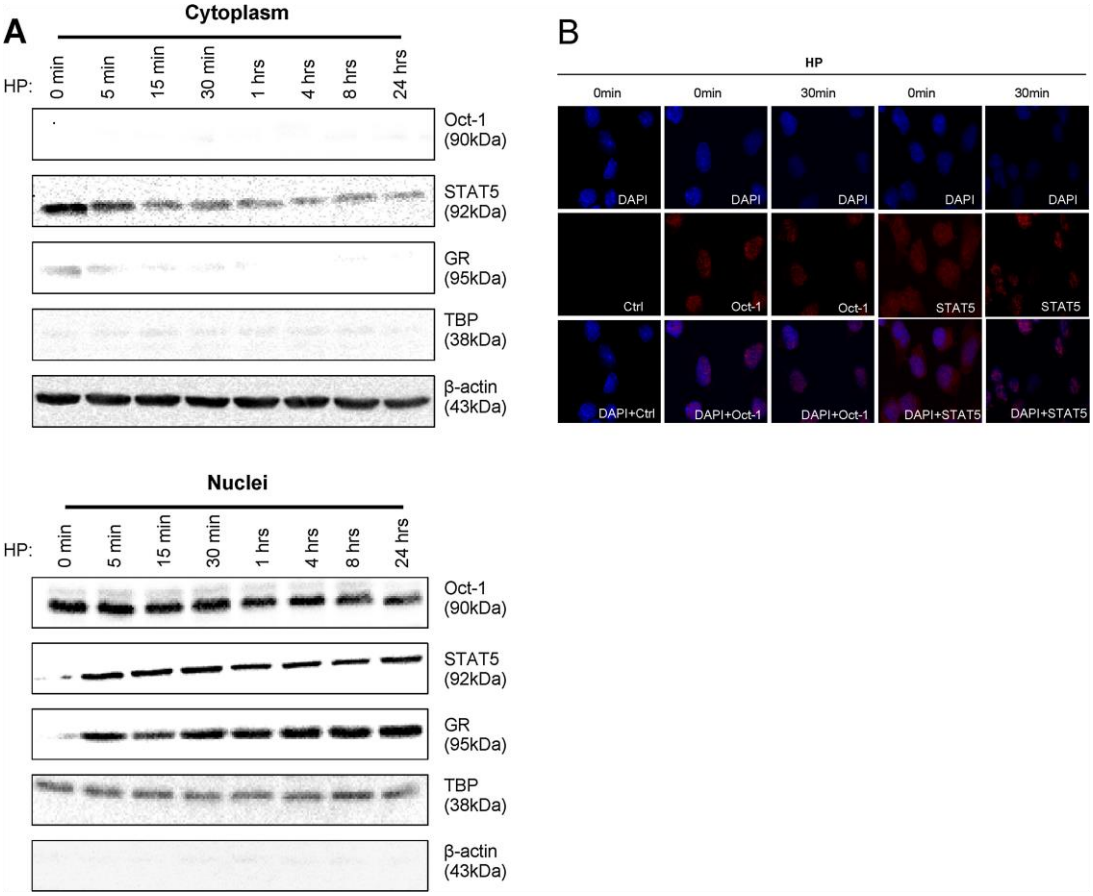

A
Cytoplasm
HP:
0 min
5 min
15 min
30 min
1 hrs
4 hrs
8 hrs
24 hrs
Oct-1 (90kDa)
STAT5 (92kDa)
GR (95kDa)
TBP (38kDa)
β-actin (43kDa)
Nuclei
HP:
0 min
5 min
15 min
30 min
1 hrs
4 hrs
8 hrs
24 hrs
Oct-1 (90kDa)
STAT5 (92kDa)
GR (95kDa)
TBP (38kDa)
β-actin (43kDa)
B
HP
0min
0min
30min
0min
30min
DAPI
Ctrl
Oct-1
STAT5
DAPI+Ctrl
DAPI+Oct-1
DAPI+STAT5

Fig. 2.6

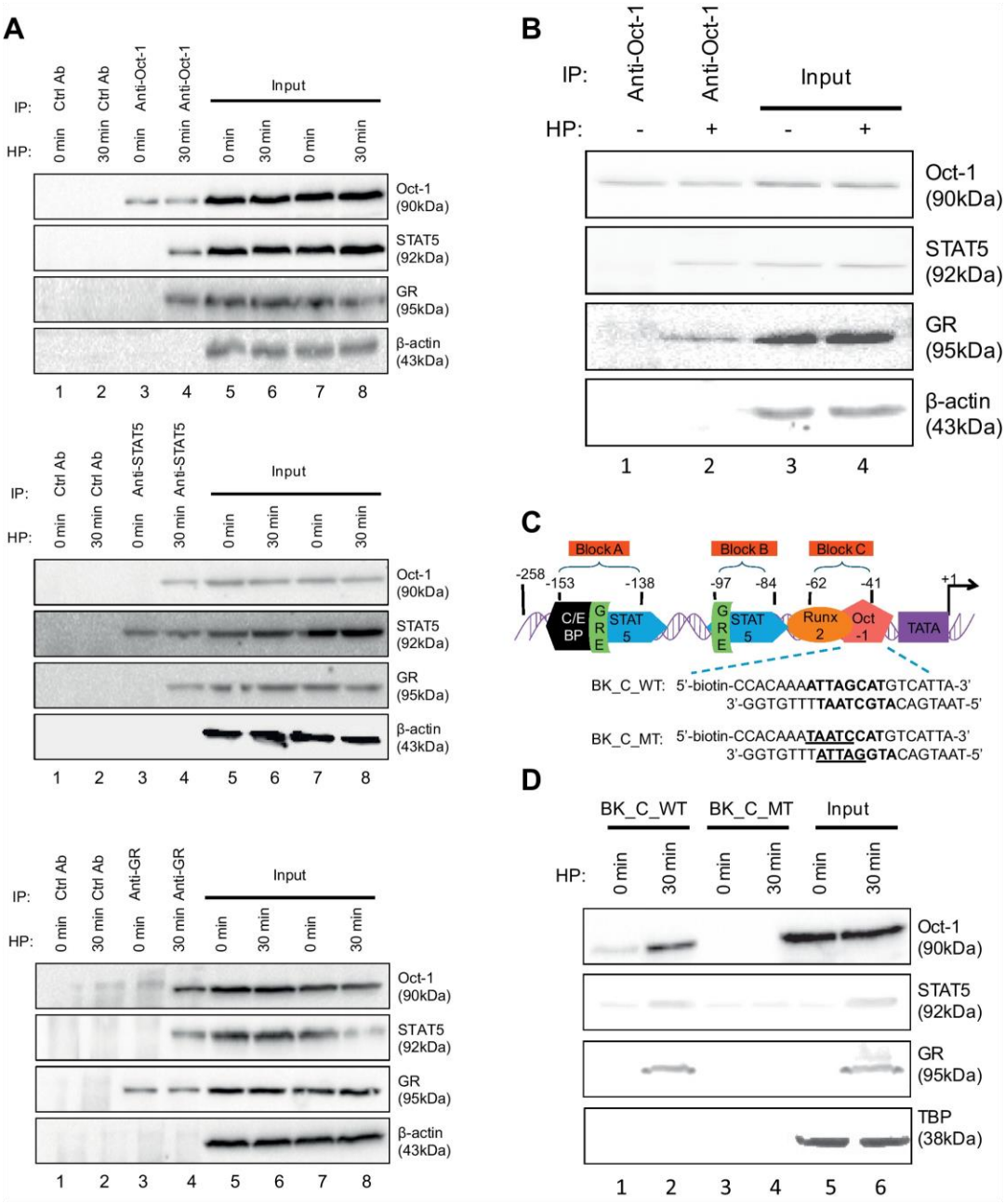

A
IP:
HP:
Ctrl Ab
Anti-Oct-1
Anti-STAT5
Anti-GR
Input
0 min
30 min
Oct-1 (90kDa)
STAT5 (92kDa)
GR (95kDa)
β-actin (43kDa)
1 2 3 4 5 6 7 8
B
IP:
HP:
Anti-Oct-1
Input
Oct-1 (90kDa)
STAT5 (92kDa)
GR (95kDa)
β-actin (43kDa)
1 2 3 4
C
Block A
Block B
Block C
-258
-153
-138
-97
-84
-62
-41
+1
C/EBP
GRE
STAT 5
Runx 2
Oct -1
TATA
BK_C_WT: 5'-biotin-CCACAAAATTAGCATGTCATTA-3'
3'-GGTGTTTTAATCGTACAGTAAT-5'
BK_C_MT: 5'-biotin-CCACAAATAATCCATGTCATTA-3'
3'-GGTGTTTATTAGGTACAGTAAT-5'
D
BK_C_WT
BK_C_MT
Input
HP:
0 min
30 min
Oct-1 (90kDa)
STAT5 (92kDa)
GR (95kDa)
TBP (38kDa)
1 2 3 4 5 6

Fig. 2.7

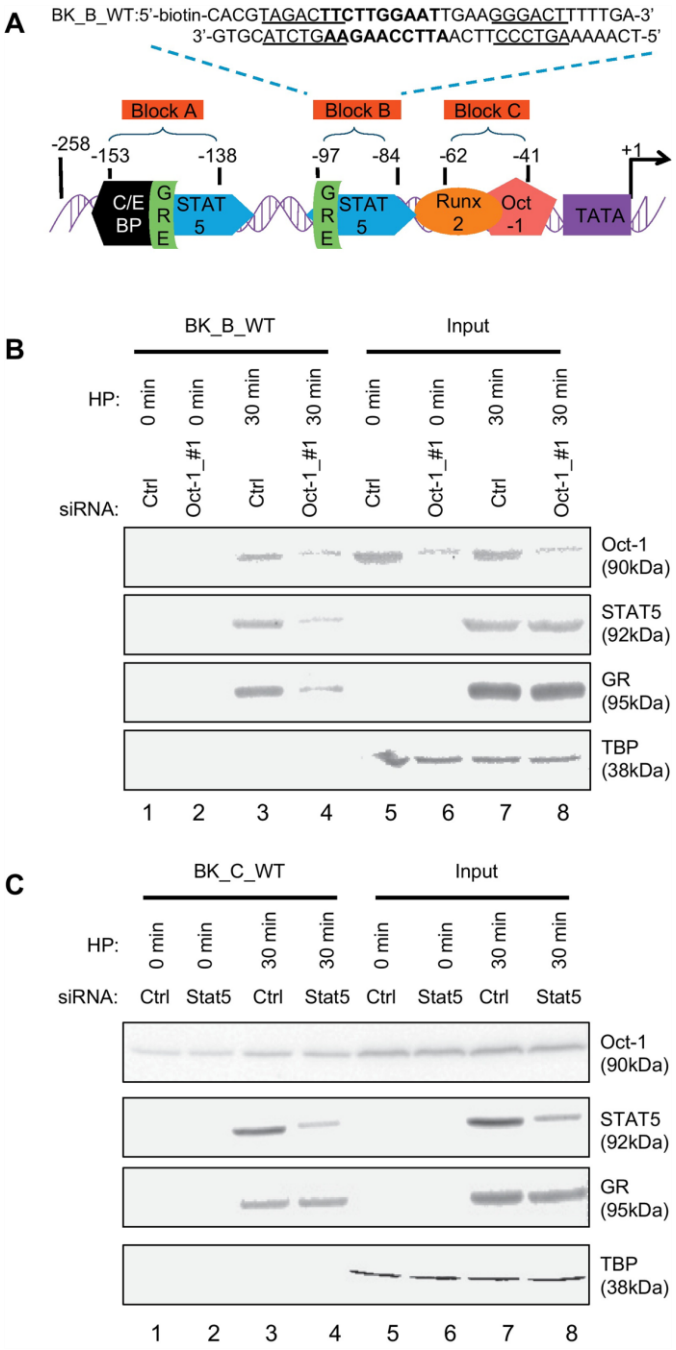
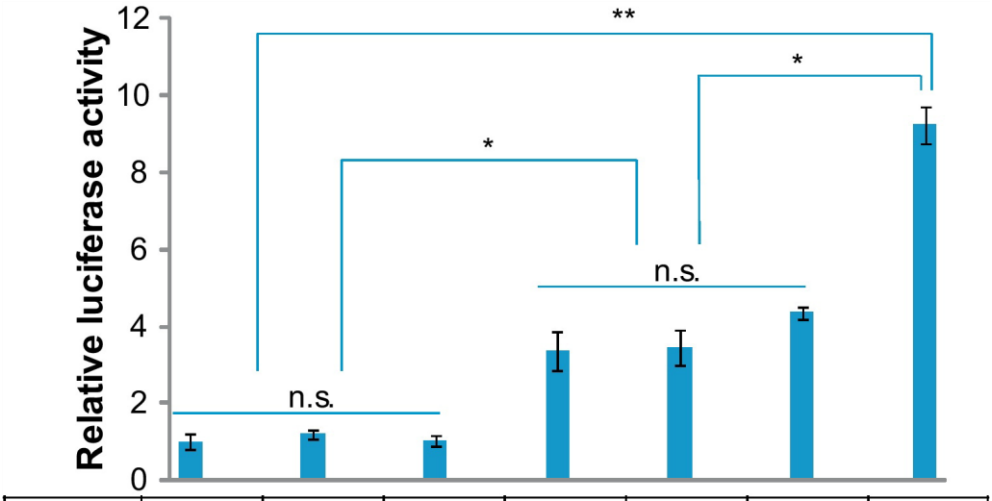
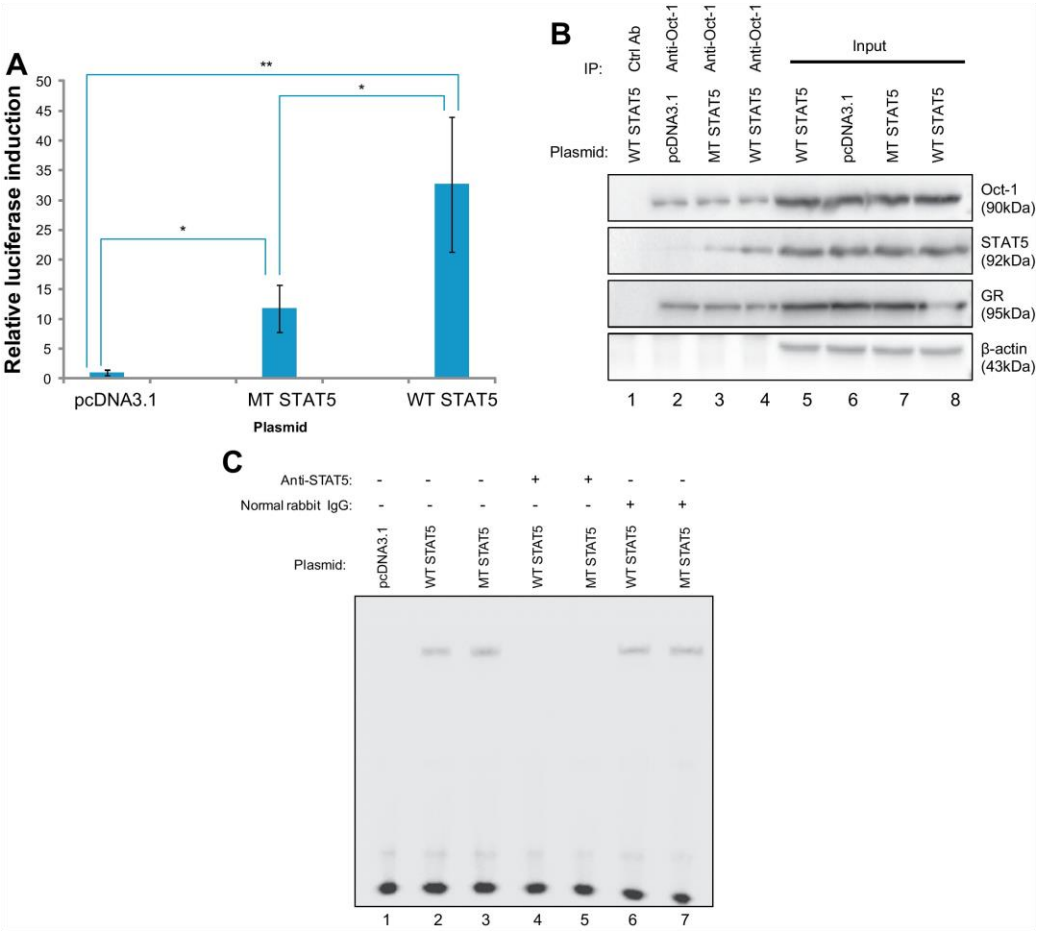

A
BK_B_WT:5'-biotin-CACGTAGACTTCTTGGAATTGAAGGGACTTTTTGA-3'
3'-GTGCATCTGAAGAACCTTAACTTCCCTGAAAAACT-5'
Block A
Block B
Block C
-258
-153
-138
-97
-84
-62
-41
+1
C/EBP
GRE
STAT5
GRE
STAT5
Runx2
Oct-1
TATA
B
BK_B_WT
Input
HP:
0 min
0 min
30 min
30 min
0 min
0 min
30 min
30 min
siRNA:
Ctrl
Oct-1_#1
Ctrl
Oct-1_#1
Ctrl
Oct-1_#1
Ctrl
Oct-1_#1
Oct-1 (90kDa)
STAT5 (92kDa)
GR (95kDa)
TBP (38kDa)
1 2 3 4 5 6 7 8
C
BK_C_WT
Input
HP:
0 min
0 min
30 min
30 min
0 min
0 min
30 min
30 min
siRNA:
Ctrl
Stat5
Ctrl
Stat5
Ctrl
Stat5
Ctrl
Stat5
Oct-1 (90kDa)
STAT5 (92kDa)
GR (95kDa)
TBP (38kDa)
1 2 3 4 5 6 7 8

Fig. 2.8



	1	2	3	4	5	6	7
Oct-1	+	-	-	+	+	-	+
STAT5	-	+	-	+	-	+	+
GR	-	-	+	-	+	+	+

Fig. 2.9


A
Relative luciferase induction
50
45
40
35
30
25
20
15
10
5
0
**
*
*
pcDNA3.1
MT STAT5
WT STAT5
Plasmid
B
IP:
Ctrl Ab
Anti-Oct-1
Anti-Oct-1
Anti-Oct-1
Input
Plasmid:
WT STAT5
pcDNA3.1
MT STAT5
WT STAT5
WT STAT5
pcDNA3.1
MT STAT5
WT STAT5
Oct-1 (90kDa)
STAT5 (92kDa)
GR (95kDa)
β-actin (43kDa)
1 2 3 4 5 6 7 8
C
Anti-STAT5: - - - + + - -
Normal rabbit IgG: - - - - - + +
Plasmid:
pcDNA3.1
WT STAT5
MT STAT5
WT STAT5
MT STAT5
WT STAT5
MT STAT5
1 2 3 4 5 6 7

Chapter 3 Collaborative interaction of Oct-2 with Oct-1 in transactivation of lactogenic hormones-induced β-casein gene expression in mammary epithelial cells[*]

Xi Qian and Feng-Qi Zhao[*]

Laboratory of Lactation and Metabolic Physiology, Department of Animal Science, University of Vermont, Burlington, VT 05405

[*]To whom correspondence should be addressed: Feng-Qi Zhao, Department of Animal Science, University of Vermont, 211 Terrill Building, 570 Main Street, Burlington, VT 05405, USA, Tel.: +1 802 656 0786; Fax: +1 802 656 8196;

E-mail address: fzhao@uvm.edu

[*]This chapter has been published in the following form: Qian, X., and Zhao, F.-Q.. (2014). Collaborative interaction of Oct-2 with Oct-1 in transactivation of lactogenic hormones-induced β-casein gene expression in mammary epithelial cells. General and Comparative Endocrinology, 204, 185–194.

ABSTRACT

Octamer-binding transcription factor-1 (Oct-1) is found to mediate lactogenic hormones (prolactin and glucocorticoids, HP)-induced β-casein gene expression in mammary alveolar secretory epithelial cells (MECs). The mammary gland also expresses Oct-2 isoform. In this study, we show that Oct-2 is also involved in HP-induced β-casein expression. Oct-2 endogenously binds to the β-casein promoter in MECs, and HP induce Oct-2 binding activity via mechanisms other than increasing Oct-2 expression or inducing Oct-2 translocation to the nucleus. Oct-2 transactivates HP-induced β-casein gene expression, and this function is exchangeable with Oct-1. In MECs, Oct-2 is found to physically interact with Oct-1 regardless of HP treatment. However, HP induce physical interactions of Oct-2 with both signal transducer and activator of transcription 5 (STAT5) and glucocorticoid receptor (GR). These results provided biochemical evidence that Oct-2 may form a heteromer with Oct-1 in induction of β-casein gene expression by HP in MECs.

Abbreviations: EGF, epidermal growth factor; EMSA, electrophoresis mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; HP, hydrocortisone (glucocorticoids) and prolactin; IP, immunoprecipitation; MEC, mammary alveolar secretory epithelial cell; Oct, octamer-binding transcription factor; PMSF, phenylmethylsulfonyl fluoride; POU, Pit-1, Oct, and Unc86; POU_H, POU homeodomain; POU_S, POU-specific domain; PrlR, prolactin receptor; qChIP, quantitative chromatin immunoprecipitation; qPCR, quantitative PCR; qRT-PCR,

quantitative reverse transcription PCR; snRNA, small nuclear RNA; STAT5, signal transducer and activator of transcription 5; TBP, TATA box-binding protein

Keywords: Gene expression; Hormonal regulation; Milk protein; Octamer binding transcription factor; Transcriptional regulation

INTRODUCTION

β-Casein gene encodes one of the major milk proteins, and its expression has been widely used as a marker of functional differentiation of mammary alveolar secretory epithelial cells (MECs). Studies of the expression of β-casein and other milk protein genes have led to the discovery of the prolactin receptor (PrlR)/Janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) signaling pathway, which is implicated in normal mammary gland development, lactation, and breast tumorigenesis (Anderson et al., 2007, Furth et al., 2011 and Hennighausen and Robinson, 2008). The transcription of the β-casein gene is controlled by modular promoter regions, termed blocks A, B, and C, which contain multiple binding sites for different transcription factors. Binding of transcription factors to these blocks either induces or suppresses β-casein gene expression, with varying combinations acting synergistically to stimulate or inhibit transcription (Rijnkels et al., 2010, Rosen et al., 1999 and Yoshimura and Oka, 1990). For example, the lactogenic hormone complex of prolactin and glucocorticoids (HP) respectively activate STAT5 and glucocorticoid receptor (GR) transcription factors. In turn, STAT5 and GR synergistically stimulate β-casein gene

expression through binding at A and B promoter blocks and protein–protein interactions between each other and other transcription factors (Lechner et al., 1997).

Our previous studies showed that the octamer-binding transcription factor-1 (Oct-1) binds to the block C at the β-casein gene promoter and also participates in HP-induced β-casein gene expression by forming a ternary protein complex with STAT5 and GR in MECs (Dong and Zhao, 2007a, Qian and Zhao, 2013 and Zhao et al., 2002). Oct-1 belongs to a group of highly conserved transcription factors that specifically bind to the octamer motif (ATGCAAAT) and closely related sequences that are found in promoters and enhancers of a wide variety of both ubiquitously expressed and cell type-specific genes (Zhao, 2013). To date, eight genes that encode these Oct proteins, Oct-1, Oct-2, Oct-3/4, Oct-6, Oct-7, Oct-8, Oct-9, and Oct-11, have been cloned and characterized (Zhao, 2013). All Oct proteins contain a POU specific domain (POU_S) in addition to a POU homeodomain (POU_H), which is distantly related to the classic homeodomain encoded by homeobox genes (Kang et al., 2009). Except for the ubiquitous expression pattern of Oct-1, all other members of the Oct factors are thought to be expressed in a developmental stage-dependent or tissue-restricted manner. However, Oct-2, which is thought to be expressed only in B lymphocytes and neuronal cells and to be mainly involved in immunoglobulin gene expression, may also be ubiquitously expressed in a variety of tissues including the mammary gland (Dong and Zhao, 2007b). Oct-1 and Oct-2 recognize their target sequences in an identical fashion, and their optimal recognition site is the canonical octamer motif (Herr and Cleary, 1995). Additionally, Oct-1 and Oct-2 can cooperatively bind to the IgH promoter and form a heteromeric complex *in vitro*

(Herr and Cleary, 1995). These previous findings led to our hypothesis that Oct-2 is involved in lactogenic hormones-induced β-casein gene expression in MECs.

MATERIALS AND METHODS

Materials

Prolactin (L6520), hydrocortisone (one of glucocorticoids, H6909), insulin (I0516), and murine epidermal growth factor (EGF, E4127) were obtained from Sigma (St. Louis, MO). Heat-inactivated fetal calf serum (1082-147), RPMI 1640 medium (31800-022), gentamicin (15750-060), antibiotic-antimycotic solution (15240-062), normal rabbit IgG (10500C), and Dynabeads® Protein A (100-01D) were purchased from Invitrogen (Carlsbad, CA). Charcoal-stripped horse serum (52-0745) was purchased from Cocalico Biologicals (Reams town, PA). The mouse Oct-2 (mOct-2/pcDNA3.1), Oct-1B (mOct-1B/pcDNA3.1), GR (mGR/pcDNA3.1), STAT5a (mSTAT5a/pcDNA3.1), and PrlR (mPrlR/pcDNA3.1) expression plasmids as well as the wild-type mouse β-casein promoter (−258/+7)/luciferase construct (LHRRWT/pGL3) have been described previously (Dong and Zhao, 2007a). The Renilla luciferase control plasmid (phRL-CMV) was purchased from Promega (Madison, WI). The anti-Oct-2 (sc-233), anti-Oct-4 (sc-5279), anti-TATA box binding protein (TBP) (sc-273), anti-actin (sc-1615-R), anti-STAT5 (sc-1081), and anti-GR (sc-1004) antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The anti-Oct-1 (A310-610A) antibody was provided by Bethyl Laboratories (Montgomery, TX).

Animals

All of the animal work and handling was carried out in accordance with institutional policies and federal guidelines and approved by the University of Vermont Instituitional Animal Care and Use Committee (IACUC). C57BL/6 mice were purchased from Jackson laboratory (Bar Harbor, ME). Mice were housed in air- and temperature-controlled cage shelves on a 12 h light–dark cycle and were fed mouse chow (#5015, Lab Diet, St. Louis, MO) and water ad libitum.

Cell culture, transfection, and luciferase assays

The murine MEC line, HC11, was cultured as previously described (Qian and Zhao, 2013). HC11 Lux cells, which are HC11 cells stably transfected with a β-casein promoter/luciferase construct (p-344/-1βc-Lux), were obtained from Dr. Hynes (Friedrich Miescher Institute, Switzerland) (Wartmann et al., 1996) and cultured as described for HC11 cells. Primary mouse MECs were isolated following the procedures reported previously (Watkin and Streuli, 2002). Briefly, the mammary glands from mid-pregnant (10-12 d) mice were pooled, minced, and digested by collagenase. Next, the epithelial cells were enriched via centrifugation and cultured in complete growth medium (D-MEM/F-12 supplemented with 10% fetal calf serum, 5 μg/ml bovine insulin, 10 ng/ml EGF, 1 μg/ml hydrocortisone, 1× antibiotic-antimycotic solution, and 50 μg/ml gentamicin). COS-7 cells were grown in D-MEM medium containing 10% fetal calf serum, 1× antibiotic–antimycotic solution, and 50 μg/ml gentamicin.

The methods for the transfection and luciferase assay have been described previously (Qian and Zhao, 2013). In the Oct-1-overexpression studies, HC11 cells were

transfected with either 0.2 pmol of pcDNA3.1 or mOct-2/pcDNA3.1, 0.2 pmol of LHRRWT/pGL3, and 0.004 pmol of phRL-CMV using Lipofectamine 2000 (Invitrogen). In the siRNA transfection experiments, HC11 Lux cells were transfected with either 40 pmol of an Oct-2 siRNA [siRNA #151207, Ambion (Austin, TX)], or a control siRNA (siRNA #4611, Ambion). In the plasmid and siRNA co-transfection experiment, HC11 Lux cells were transfected with 0.1 pmol of plasmid (Oct-2 plasmid or pcDNA3.1) and 20 pmol of siRNA (Oct-2 siRNA or control siRNA). For the co-transfection experiments in COS-7 cells, COS-7 cells were transfected with 0.05 pmol of the PrlR, Oct-1B, Oct-2, GR, or STAT5 expression plasmid or various combinations of these constructs along with 0.2 pmol of LHRRWT/pGL3 and 0.004 pmol of phRL-CMV. In all transfection experiments, the total molar amount of DNA was balanced using pcDNA3.1 or the control siRNA. After 10-12 h, the transfection medium was replaced with hormone medium containing 50 μg/ml gentamicin, 1 μg/ml hydrocortisone, 5 μg/ml bovine insulin, and 5 μg/ml sheep prolactin. Luciferase activities were examined after 24 h of hormone treatment. The Renilla luciferase control plasmid was used to normalize transfection efficiency. In HC11 Lux cells, the luciferase activity levels were normalized to protein concentrations.

Western blotting

Nuclear and cytoplasmic proteins were extracted as the method described by Schreiber et al. (1989). Whole cell protein lysates were prepared by adding NP40 lysis buffer (Invitrogen) consisting of 50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50

mM NaF, 1 mM Na_3VO_4, 1% NP40, and 0.02% NaN_3 with freshly added protease inhibitor cocktail and PMSF.

The protein concentrations were determined using the Microplate BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Equal amounts of protein from each treatment were analyzed via Western blotting with specific antibodies against Oct-1, Oct-2, STAT5, GR, TBP, β-actin, and β-casein, as described previously (Zhao et al., 2002).

Quantitative chromatin immunoprecipitation (ChIP) assay

ChIP was performed as described previously by Kabotyanski et al. (2006) with a few modifications. Briefly, cells were incubated with 1% formaldehyde for 10 min at 37 °C to cross-link proteins to DNA. Cell lysates were sonicated on ice to shear chromatin to an average DNA length of 200-1000 bp as verified by agarose gel electrophoresis. The sheared chromatin was pre-cleared with Protein A. Before performing the IP, 1% of the total sheared chromatin was saved as a total input control. Next, the sheared chromatin was incubated with either an anti-Oct-2 antibody or the normal rabbit IgG (2 μg, negative control) overnight at 4 °C with continual rotation. The cross-links of immunoprecipitated DNA-protein complexes were reversed, and the DNA was finally purified by phenol/chloroform extraction and ethanol precipitation. After purification, 2 μl of the final precipitated DNA was examined by quantitative real-time PCR using SsoFast EvaGreen supermix (Bio-Rad, Hercules, CA) with the forward primer 5'-TAGAATTTCTTGGGAAAGAC-3' and the reverse primer 5'-CTTTAGTGGAGGACAAGAGA-3' for the β-casein promoter. The PCR efficiency of the primers was verified and the IP data were normalized to the input DNA.

Quantitative reverse transcription PCR (qRT-PCR)

RNA was isolated from HC11 cells using Trizol reagent (Invitrogen). Reverse transcription was performed using the SuperScript II reverse transcriptase (Invitrogen) per the manufacturer's protocol. TaqMan gene expression assays were used to quantify the mRNA expression levels of Oct-2 [mm00448353_m1, Applied Biosystems (Foster City, CA)] and β-actin (Mm01205647_g1)]. The PCR reactions were performed in duplicate in a 10 μl volume containing 5 μl Universal PCR Master Mix (#4364338, Applied Biosystems), 0.5 μl TaqMan assay, and 4.5 μl diluted cDNA (50 ng reverse-transcribed RNA). The relative expression levels of the target genes were normalized with the β-actin expression levels and calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Immunoprecipitation (IP)

IPs were carried out according to the instructions provided with the Relia BLOTTING® IP/Western Blotting Kit (Bethyl Laboratories). In general, 1 mg of cell extract was incubated overnight with 3 μg of anti-Oct-2 antibody at 4 °C with rotation. The immune complexes were captured using Dynabeads® Protein A and analyzed via Western blotting.

Electrophoresis mobility shift assays (EMSAs)

EMSAs were carried out as described previously (Zhao et al., 2002). The oligonucleotide used in EMSAs was the -69/-38 region (block C) of mouse β-casein gene promoter, 5'-ATCTTACAAACCACAAA**ATTAGCAT**GTCATTA-3' (Oct binding motif

is printed as boldface). Nuclear proteins (5 μg) from the mammary gland of late pregnant mice (17-19 d) or HeLa cells or *in vitro* translated Oct-1 or/and Oct-2 products (0.5-2 μl) were incubated for 30 min with the block C oligonucleotide probe, end labeled with [γ-^{32}P] ATP. Nuclear extracts of HeLa cells were purchased from Santa Cruz Biotechnology. The mouse Oct-2 (mOct-2/pcDNA3.1) and Oct-1B (mOct-1B/pcDNA3.1) expression plasmids were transcribed and translated *in vitro* following the technical manual of the TNT quick coupled transcription/translation system (Promega). For the binding reactions with antibody, antibody was added to the binding reaction and incubated on ice for 30-60 min before addition of labeled probe. Incubation was subsequently continued for an additional 20 min at room temperature.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as previously described (Dong and Zhao, 2007a). HC11 cells were grown on glass coverslips to confluency and then treated with or without 1 μg/ml hydrocortisone and 5 μg/ml prolactin for 30 min. The cells were subsequently fixed with 4% paraformaldehyde in PBS, pH 7.4 (Invitrogen) at room temperature for 20 min. Next, the cells were washed twice in PBS and permeabilized in 0.1% Triton X-100 in PBS (pH 7.4) with 0.5% BSA for 15 min at room temperature, followed by washing twice in PBS, pH 7.4 with 1% BSA for 10 min. The immunofluorescence staining of the cells was carried out using an anti-Oct-2 antibody (2 μg/ml in PBS, pH 7.4 with 1% BSA) or normal rabbit IgG followed by an Alexa Fluor® 568-conjugated secondary antibody (1:1000 dilution in PBS, pH 7.4 with 1% BSA).

Finally, the coverslips were washed and examined under a confocal microscope (Bio-Rad, Hercules, CA).

Statistical analysis

All statistical analyses were carried out using JMP statistical software (SAS, Cary, NC). The comparisons between two groups were performed using *t*-test. A one-way ANOVA test with Turkey's post hoc analysis was performed when comparisons were performed between more than two groups. A $P < 0.05$ is declared as a significant difference.

RESULTS

Both Oct-1 and Oct-2 bind to the block C of the β-casein promoter, and the binding activity can be induced by HP

The expression of Oct-2 in mouse MECs was confirmed by Western blotting (Fig. 3.1A). To further confirm that Oct-2 binds to the β-casein gene promoter *in vivo* and to determine whether the binding activity is responsive to HP treatment as that of Oct-1 (Qian and Zhao, 2013) in MECs, we performed ChIP assays in HC11 cells using the anti-Oct-2 antibody at different time points after HP treatment. The primers were designed to amplify the β-casein gene proximal promoter, which contains the binding sites for Oct-2. The binding activity of Oct-2 to the β-casein gene promoter was relatively low in the absence of HP but increased dramatically at 30 min of HP treatment followed by an appreciable decrease at 3 and 24 h (Fig. 3.1B, left panel). Normal rabbit IgG (Ctrl AB), a

negative control, was unable to immunoprecipitate Oct-2-DNA complex (Fig. 3.1B, left panel). The right panel of Fig. 3.1B shows the quantitative effects of HP on Oct-2 binding activity on the β-casein gene proximal promoter as determined by qPCR. These results demonstrated that Oct-2 binds to the β-casein gene promoter in MECs and that this binding is regulated by HP.

HP do not influence Oct-2 expression nor its subcellular localization in MECs

HP may affect the binding of Oct-2 to the β-casein gene promoter by increasing either Oct-2 expression levels or Oct-2 amount in the nucleus. To test these possibilities, HC11 cells were cultured with or without HP for different time periods. Oct-2 mRNA and protein expression levels were then measured via qRT-PCR and Western blotting, respectively. As shown in Fig. 3.2A and B, the mRNA and protein levels of Oct-2 were not influenced by HP. The expression levels of β-casein gene and GAPDH gene were also examined as a positive or negative control, respectively. A significant HP-induction of β-casein mRNA expression was detected while the GAPDH mRNA expression was not affected by HP treatment (data not shown).

Since previous results have showed that HP could induce the translocation of downstream signaling molecules, like STAT5 and GR, from the cytoplasm to the nucleus within minutes (Lechner et al., 1997), we investigated whether HP treatment affects Oct-2 subcelluar localization. HC11 cells were treated with or without HP followed by immunofluorescence staining. As shown in Fig. 3.2C, Oct-2 was primarily localized in the nucleus regardless of HP treatment.

Oct-2 serves as a transcriptional activator of the β-casein gene expression

To examine the effect of Oct-2 on HP-induced β-casein gene expression in MECs, we first examined the effects of Oct-2 over-expression on HP-induced β-casein gene promoter activity and endogenous β-casein gene expression in HC11 cells. HC11 cells were co-transfected with the β-casein gene promoter (-258/+7)/*firefly* luciferase reporter plasmid (LHRRWT/pGL3) and the *Renilla* luciferase reporter vector (phRL-CMV, a transfection control) along with either an Oct-2 expression plasmid or the empty vector pcDNA3.1, followed by HP treatment. Fig. 3.3A (left) shows that over-expression of Oct-2 further dramatically stimulated HP-induced β-casein gene promoter activity by ~3.5 fold in comparison with the empty vector control group. Fig. 3.3A (right) demonstrates the over-expression of Oct-2 protein in Oct-2 plasmid-transfected cells. In addition, Oct-2 over-expression also resulted in a ~2.5-fold increase in the HP-induced endogenous β-casein gene expression in HC11 cells (Fig. 3.3B).

To further confirm the transactivator role of Oct-2 in the HP induction of β-casein gene expression, an Oct-2 siRNA was employed to knockdown Oct-2 expression in HC11 Lux cells, which are stably transfected with a luciferase reporter flanked with the β-casein gene proximal promoter at the 5' end. The Oct-2 siRNA resulted in an appreciable decrease in the amount of Oct-2 protein in HC11 Lux cells (Fig. 3.3C, right) and meanwhile significantly inhibited the HP-induced β-casein gene promoter activity by ~60% compared with the cells transfected with a control siRNA (Fig. 3.3C, left). Oct-2 knockdown also led to a ~80% inhibition in the HP-induced endogenous β-casein gene

expression (Fig. 3.3D). Finally, co-transfection of the Oct-2 expression plasmid along with the Oct-2 siRNA in HC11 Lux cells rescued the inhibitory effect of Oct-2 siRNA (Fig. 3.3E). Therefore, these results clearly indicated that Oct-2 serves as a transactivator in HP-induced β-casein gene expression.

Oct-2 interacts with Oct-1 in mammary epithelial cells, and this interaction does not depend on HP

To examine if there is an interaction between Oct-2 and Oct-1, co-IPs were performed. Nuclear protein samples were prepared from HP-treated and -untreated HC11 cells transfected with both Oct-1 and Oct-2 plasmids and then immunoprecipitated with an anti-Oct-2 antibody. As shown in Fig. 3.4A, lanes 5 and 6, the anti-Oct-2 antibody co-immunoprecipitated Oct-1 regardless of HP treatment, while the normal rabbit IgG (Ctrl AB) failed to co-immunoprecipitate Oct-1 (Fig. 3.4A, lane 4).

To examine the effects of interaction between Oct-2 and Oct-1 on HP-induction of β-casein gene expression, co-transfection experiments were performed in HC11 cells with Oct-1 and Oct-2 expression plasmids. As shown in Fig. 3.4B, transfection with either Oct-1 or Oct-2 plasmid significantly induced the β-casein gene promoter activity, but co-transfection of both plasmids together did not further increase the promoter activity. In addition, it appeared that Oct-2 had a more potent effect than Oct-1. Co-transfection with Oct-1 and Oct-2 siRNAs showed the similar, but inhibitive effects in HC11 Lux cells (Fig. 3.4C).

Oct-2 synergistically interacts with both STAT5 and GR in HP-induction of β-casein promoter activity

Induction of β-casein transcription by HP in the mammary gland is mediated through the synergistic interaction of GR and STAT5. To test whether Oct-2 can functionally interact with these two factors, COS-7 cells were reconstituted to be lactogenic hormone-responsive by co-transfection with the Oct-2, STAT5, or GR expression plasmids or various combinations of these plasmids along with PrlR expression plasmid, β-casein promoter/*firefly* luciferase construct, and *Renilla* luciferase control plasmid, followed by HP treatment. As shown in Fig. 3.5A, HP induction of β-casein promoter activity was low when the individual plasmids were transfected. When two of the Oct-2, STAT5, and GR plasmids were co-transfected, however, HP induction of the promoter activity increased significantly (Fig. 3.5A, groups 4, 5, and 6), and the highest promoter activity was achieved only in the presence of all three plasmids (Fig. 3.5A, group 7). To examine whether Oct-2 and Oct-1 synergistically interact in the presence of STAT5 and GR, the expression plasmids of STAT5 and GR were co-transfected with the Oct-1 or Oct-2 plasmid or both. As shown in Fig. 3.5B, either Oct-2 or Oct-1 showed synergistic activation with GR and STAT5, however, co-transfection of both plasmids did not further increase the promoter activity, consistent with the results reported in Fig. 3.4B and C.

To test whether Oct-2 physically interacts with GR and STAT5, nuclear extracts prepared from HP-treated or -untreated HC11 cells were immunoprecipiated with an anti-Oct-2 antibody. As shown in Fig. 3.5C, the anti-Oct-2 antibody co-immunoprecipitated

STAT5 and GR in the cells treated with HP for 30 min (Fig. 3.5C, the third and fourth gel, lane 3), whereas STAT5 and GR were not associated with Oct-2 in the absence of HP (Fig. 3.5C, the third and fourth gel, lane 2). Oct-1 and Oct-2 were associated together independent of HP treatment (Fig. 3.5C, the first and second gel, lanes 2 and 3), consistent with the data in Fig. 3.4A. As expected, normal rabbit IgG (Ctrl Ab) did not co-immunoprecipitate Oct-1, Oct-2, STAT5, and GR. These results demonstrated that HP induced the physical interactions of Oct-2 with STAT5 and GR.

DISCUSSION

Oct-2 has been considered to be B cell- and neuronal cell-specific (Latchman, 1996). This study provided further evidence to support our previous finding that Oct-2 is also expressed in mouse mammary gland (Dong and Zhao, 2007b). These evidence include the Western blotting and immunofluorescence staining of Oct-2 in mammary epithelial cells (Figs. 3.1A and 3.2C). Furthermore, this study provided functional evidence of Oct-2 and its interactions with other proteins in MECs.

In this study, we demonstrated that, like its counterpart Oct-1 (Qian and Zhao, 2013), Oct-2 is involved in the hormonal induction of β-casein gene expression by HP in mouse MECs. This involvement is supported by two lines of evidence: 1) The endogenous binding of Oct-2 on β-casein promoter was showed by our ChIP assay, and this binding activity was induced by HP (Fig. 3.1B); and 2) Overexpression of Oct-2 (Fig. 3.3A and B) or knockdown of endogenous Oct-2 expression (Fig. 3.3C and D)

dramatically increased or inhibited β-casein promoter activity and endogenous β-casein expression, respectively.

The physical interaction between Oct-1 and Oct-2 is supported by our IP results (Figs. 3.4A and 3.5C). These two proteins could be co-immunoprecipitated together, and this association was independent of HP treatment. These results correlate well with previous published results, which showed that Oct-1 and Oct-2 form cooperative homo- or heterodimer on a regulatory site in immunoglobulin heavy-chain (IgH) promoters (Herr and Cleary, 1995), and the formation of the homo-or hetero-dimer is mediated by both POU homeodomain and specific domain (Verrijzer et al., 1992). However, the interaction between Oct-1 and Oct-2 does not synergistically stimulate HP-induced β-casein gene expression because the effects of over-expression or knockdown of both Oct-1 and Oct-2 together on the β-casein promoter activity were not more than the sum of the over-expression or knockdown of individual factors (Figs. 3.4B, C and 3.5B). It appears that Oct-2 and Oct-1 are functionally interchangeable in the regulation of β-casein gene. This observation is consistent with previous studies in Oct-1 or Oct-2 knockout mice in which Oct-1 and Oct-2 operate redundantly in regulating B cell development and IgG transcription (Wang et al., 2004). Aside from IgG gene, U2 snRNA (Ström et al., 1996; Tanaka and Herr, 1990), U6 snRNA (Murphy et al., 1992), and H2B genes (Hinkley and Perry, 1992) are also regulated by both Oct-1 and Oct-2 interchangeably.

Additionally, this study showed that like Oct-1 (Qian and Zhao, 2013), Oct-2 is also induced by HP to physically interact with both STAT5 and GR (Fig. 3.5C), and the interactions of Oct-2 with these two factors together play a synergistic role in the process

of HP-induction of β-casein gene expression (Fig. 3.5A). STAT5 and GR are the essential downstream signaling molecules of prolactin and glucocorticoids, respectively (Lechner et al., 1997a, 1997b). It is well established that synergistic interaction between these two factors mediates induction of β-casein gene expression by HP (Rijnkels et al., 2010; Rosen et al., 1999). Because we have shown that Oct-1 physically interacts with STAT5 and GR on the β-casein promoter in our previous study (Qian and Zhao, 2013) and this study showed the physical interaction of Oct-2 with Oct-1 on the same promoter, these four factors may form a hetero-complex together in HP-induced β-casein expression.

In this study, Oct-2 binding to block C of the β-casein promoter was induced by HP in mammary epithelial cells (Fig. 3.1B). This induction did not result from the stimulation of Oct-2 expression or the induction of Oct-2 nuclear translocation by HP (Fig. 3.2). One of the possible mechanisms for this induction may be the recruitment of Oct-2 to the DNA via its interactions with Oct-1, STAT5, and GR because HP induces Oct-1, STAT5, and GR binding to the β-casein promoter (Kabotyanski et al., 2006; Qian and Zhao, 2013). Another possible mechanism is that HP may induce post-translational modifications of Oct-2, such as phosphorylation, glycosylation, and sumoylation. These modifications may enhance its DNA binding activity. For example, phosphorylation of Oct-2 by protein kinase A, protein kinase C, and casein kinase 2 *in vitro* regulates its DNA binding specificity (Grenfell et al, 1996); alternative phosphorylation and glycosylation of several residues of Oct-2 are involved in differential binding behaviors of Oct-2 to the octamer motif (Ahmad et al., 2006).

In summary, results from this study showed for the first time that the lactogenic hormones, prolaction and glucocorticoids, induce physical interactions of Oct-2 with Oct-1, STAT5 and GR in activation of β-casein gene expression in mouse MECs.

Acknowledgements: We thank Dr. Margaret Neville for providing the murine β-casein antibody, Dr. Nancy Hynes for providing HC11 Lux cells, and Drs. John Cidlowski, Wolfgang Doppler, Russell Hovey, and Jeff Rosen for providing expression plasmids of PrlR, STAT5 and GR. This work was supported by a USDA Vermont Experimental Station Hatch Grant (VT-HO1508).

REFERENCES

Ahmad, I., Hoessli, D.C., Walker-Nasir, E., Rafik, S.M., Shakoori, A.R. Nasir-ud-Din, 2006. Oct-2 DNA binding transcription factor: functional consequences of phosphorylation and glycosylation. Nucleic Acids Res. 34, 175-184.

Anderson, S.M., Rudolph, M.C., McManaman, J.L., Neville M.C., 2007. Key stages in mammary gland development. Secretory activation in the mammary gland: it's not just about milk protein synthesis. Breast Cancer Res. 9, 204-217.

Dong, B., Zhao, F.Q., 2007a. Involvement of the ubiquitous Oct-1 transcription factor in hormonal induction of β-casein gene expression. Biochem. J. 401, 57-64.

Dong, B., Zhao, F.Q., 2007b. Expression of the Oct-2 transcription factor in mouse mammary gland and cloning and characterization of a novel Oct-2 isoform. Cell Tissue Res. 328, 595-606.

Furth, P.A., Nakles, R.E., Millman, S., Diaz-Cruz, E.S., Cabrera, M.C., 2011. Signal transducer and activator of transcription 5 as a key signaling pathway in normal mammary gland developmental biology and breast cancer. Breast Cancer Res. 13, 220-233.

Grenfell, S.J., Latchman, D.S., Thomas, N., 1996. Oct-1 and Oct-2 DNA-binding site specificity is regulated *in vitro* by different kinases. Biochem. J. 315, 889-893.

Hennighausen, L., Robinson, G.W., 2008. Interpretation of cytokine signaling through the transcription factors STAT5A and STAT5B. Genes Dev. 22, 711-721.

Herr, W., Cleary, M.A., 1995. The POU domain: versatility in transcriptional regulation by a flexible two-in-one DNA-binding domain. Genes Dev. 9, 1679-1693.

Hinkley, C., Perry, M., 1992. Histone H2B gene transcription during *Xenopus* early development requires functional cooperation between proteins bound to the CCAAT and octamer motifs. Mol. Cell. Biol. 12, 4400-4411.

Kabotyanski, E.B., Huetter, M., Xian, W., Rijnkels, M., Rosen, J.M., 2006. Integration of prolactin and glucocorticoid signaling at the β-casein promoter and enhancer by ordered recruitment of specific transcription factors and chromatin modifiers. Mol. Endocrinol. 20, 2355-2368.

Kang, J., Shakya, A., Tantin, D., 2009. Stem cells, stress, metabolism and cancer: a drama in two Octs. Trends Biochem. Sci. 34, 491-499.

Latchman, D., 1996. The Oct-2 transcription factor. Int. J. Biochem. Cell Biol. 28, 1081-1083.

Lechner, J., Welte, T., Tomasi, J.K., Bruno, P., Cairns, C., Gustafsson, J., Doppler, W., 1997. Promoter-dependent synergy between glucocorticoid receptor and Stat5 in the activation of β-casein gene transcription. J. Biol. Chem. 272, 20954-20960.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402-408.

Murphy, S., Yoon, J., Gerster, T., Roeder, R.G., 1992. Oct-1 and Oct-2 potentiate functional interactions of a transcription factor with the proximal sequence element of small nuclear RNA genes. Mol. Cell. Biol. 12, 3247-3261.

Qian, X., and Zhao, F.Q., 2013. Interactions of the ubiquitous octamer-binding transcription factor-1 with both the signal transducer and activator of transcription 5 and the glucocorticoid receptor mediate prolactin and glucocorticoid-induced β-casein gene expression in mammary epithelial cells. Int. J. Biochem. Cell Biol. 45, 724-735.

Rijnkels, M., Kabotyanski, E., Montazer-Torbati, M.B., Hue Beauvais, C., Vassetzky, Y., Rosen, J. M., Devinoy, E., 2010. The epigenetic landscape of mammary gland development and functional differentiation. J. Mammary Gland Biol. Neoplasia 15, 85-100.

Rosen, J.M., Wyszomierski, S.L. Hadsell, D., 1999. Regulation of milk protein gene expression. Annu. Rev. Nutr. 19, 407-436.

Saito, H., Oka, T., 1996. Hormonally regulated double- and single-stranded DNA-binding complexes involved in mouse beta-casein gene transcription. J. Biol. Chem. 271, 8911-8918.

Schreiber, E., Matthias, P., Müller, M.M., 1989. Rapid detection of octamer binding proteins with 'mini extracts', prepared from a small number of cells. Nucleic Acids Res. 17, 6419.

Ström, A.C., Forsberg, M., Lillhager, P., Westin, G., 1996. The transcription factors Sp1 and Oct-1 interact physically to regulate human U2 snRNA gene expression. Nucleic Acids Res. 24, 1981-1986.

Tanaka, M., Herr, W., 1990. Differential transcriptional activation by Oct-1 and Oct-2: interdependent activation domains induce Oct-2 phosphorylation. Cell 60, 375-386.

Verrijzer, C.P., van Oosterhout, J., van der Vliet, P.C., 1992. The Oct-1 POU domain mediates interactions between Oct-1 and other POU proteins. Mol. Cell. Biol. 12, 542-551.

Wang, V.E., Tantin, D., Chen, J., 2004. B cell development and immunoglobulin transcription in Oct-1-deficient mice. Proc. Natl. Acad. Sci. U. S. A. 101, 2005-2010.

Wartmann, M., Cella, N., Hofer, P., Groner, B., Liu, X., Hennighausen, L., Hynes, N.E., 1996. Lactogenic hormone activation of Stat5 and transcription of the β-casein gene in mammary epithelial cells is independent of p42 ERK2 mitogen-activated protein kinase activity. J. Biol. Chem. 271, 31863-31868.

Watkin, H., Streuli, C.H., 2002. Adenoviral-mediated gene transfer in two-dimensional and three-dimensional cultures of mammary epithelial cells. Methods Cell Biol. 69, 403-423.

Yoshimura, M., Oka, T., 1990. Transfection of beta-casein chimeric gene and hormonal induction of its expression in primary murine mammary epithelial cells. Proc. Natl. Acad. Sci. USA. 87, 3670-3674.

Zhao, F.Q., Adachi, K., Oka, T., 2002. Involvement of Oct-1 in transcriptional regulation of β-casein gene expression in mouse mammary gland. Biochim. Biophys. Acta. 1577, 27-37.

Zhao, F.Q., 2013. Octamer-binding transcription factors: genomics and functions. Front. Biosci. 18, 1051-1071.

FIGURE LEGENDS

Fig. 3.1. Both Oct-1 and Oct-2 bind to the block C region of the β-casein promoter and the binding activity of Oct-2 can be induced by prolactin and hydrocortisone (HP) in mammary epithelial cells. (A) Western blotting analysis of Oct-2 and TATA-binding protein (TBP) expression in nuclear extracts of HC11 cells and primary mouse mammary epithelial cells (PMEC). PC= positive control (*in vitro* translated Oct-2). (B) Chromatin immunoprecipitation (ChIP) analyses of Oct-2 binding activity at the β-casein promoter in HC11 cells treated with or without HP for indicated time periods using either an anti-Oct-2 antibody or normal rabbit IgG (Ctrl AB) (left panel). The quantitative data obtained by quantitative PCR (right panel) represent the mean of three independent experiments ± SE. Bars with different letters are significantly different ($P< 0.05$).

Fig. 3.2. Effects of prolactin and hydrocortisone (HP) treatment on Oct-2 expression and subcellular localization in HC11 cells. (A and B) Quantitative reverse transcription PCR (qRT-PCR) analysis of Oct-2 mRNA expression (A) and Western blotting analysis of Oct-2 protein expression (B) in HC11 cells treated with or without HP for 48 h. The data represent the mean of three independent experiments ± SE. (C) Immunofluorescence staining of Oct-2 in HC11cells treated with or without HP for 30 min.

Fig. 3.3. Oct-2 functions as a transactivator in hormonal induction of β-casein gene expression by prolactin and hydrocortisone (HP). (A) Relative luciferase activity of β-casein promoter luciferase construct (right) and Western blotting analysis of Oct-2 and β-

actin proteins in HC11 cells treated with HP for 24 h and transiently transfected with an Oct-2 expression plasmid or pcDNA3.1 vector plasmid. (B and D) Western blotting analyses of the endogenous expression of β-casein and β-actin proteins as well as Oct-2 protein levels in HC11 cells treated with HP and transiently transfected with an Oct-2 expression plasmid (B) or an Oct-2 siRNA (D) in comparisons with the pcDNA3.1 vector plasmid or a control siRNA (Ctrl) (left). The relative intensities of endogenous β-casein expression in these cells were quantified and showed (right). (C) Relative luciferase activity of β-casein promoter luciferase construct (left) and Western blotting analysis of Oct-2 and β-actin proteins in HC11 Lux cells treated with HP and transiently transfected with an Oct-2 siRNA or a control siRNA (Ctrl) (right). (E) Relative luciferase activity of β-casein promoter luciferase construct in HC11 cells treated with HP and transiently transfected with an Oct-2 siRNA, Oct-2 expression plasmid or their combination. The data represent the mean of three independent experiments ± SE. Bars with different letters or with "*" are significantly different ($P< 0.05$).

Fig. 3.4. Interaction of Oct-2 with Oct-1. (A) The interaction between Oct-1 and Oct-2 in HC11 cells was examined by immunocoprecipitations. TBP = TATA-binding protein (loading control). (B and C) The effects of co-transfection of Oct-1 and Oct-2 expression plasmids in HC11 cells (B) or siRNAs in HC11 Lux cells (C) on the induction of the β-casein gene promoter activity by prolactin and hydrocortisone (HP). The bottom panels are Western blotting analyses of Oct-2 levels in cells transfected with Oct-2 expression

plasmid (B) or Oct-2 siRNA (C). The data represent the mean of three independent experiments ± SE. Bars with different letters are significantly different ($P < 0.05$).

Fig. 3.5. Interactions of Oct-2 with STAT5 and GR in the induction of β-casein gene promoter activity by prolactin and hydrocortisone (HP). (A and B) Relative luciferase activity of β-casein promoter luciferase construct in COS-7 cells treated with HP for 24 h and transiently transfected with Oct-1, Oct-2, STAT5, or GR expression plasmid or various combinations of these constructs along with prolactin receptor plasmid. The data represent the mean of three independent experiments ± SE. Bars with different letters are significantly different ($P < 0.05$). (C) Co-immunoprecipitations of Oct-2 with Oct-1, STAT5, and GR in HC11 cells treated either with or without HP for 30 min. Normal rabbit IgG (Ctrl Ab) was used as an antibody-specificity control.

Fig. 3.1

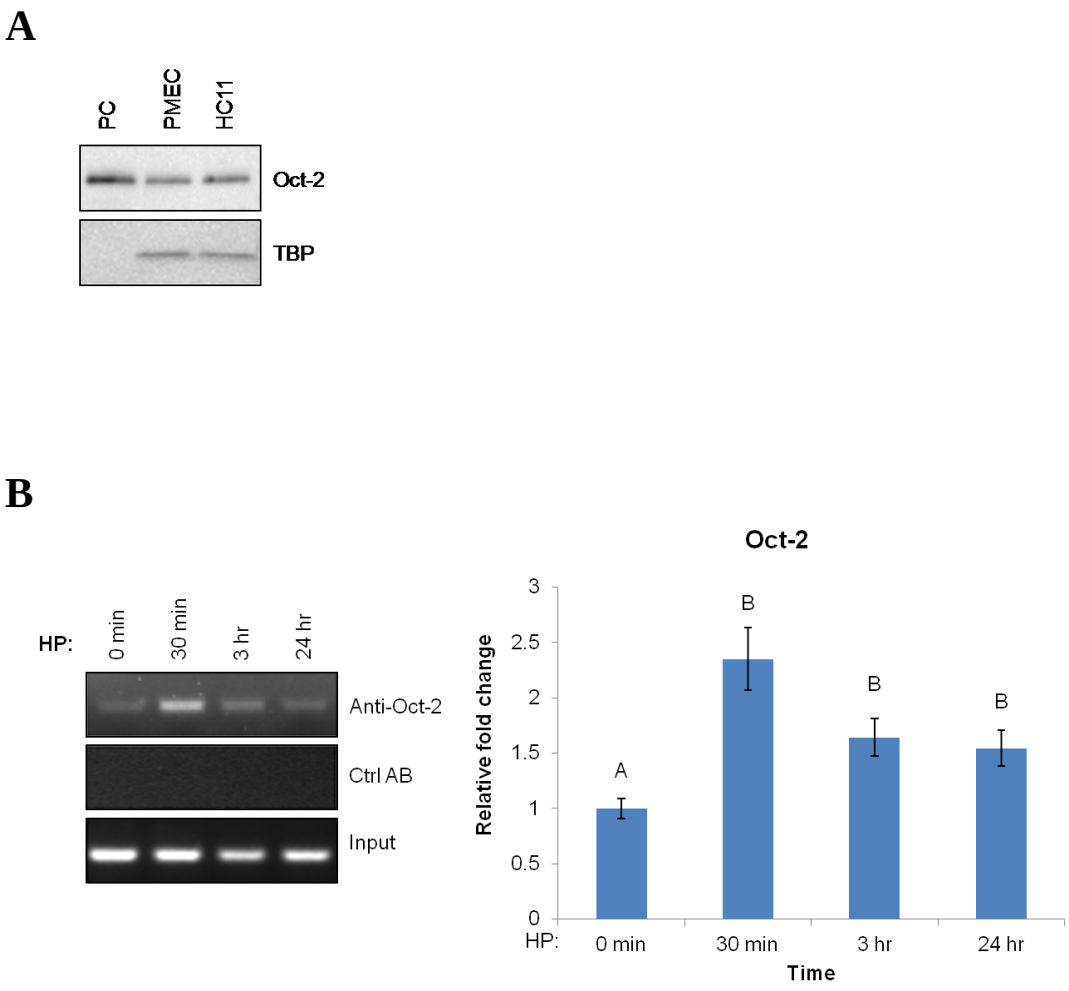

A
PC
PMEC
HC11
Oct-2
TBP
B
HP:
0 min
30 min
3 hr
24 hr
Anti-Oct-2
Ctrl AB
Input
Oct-2
Relative fold change
3
2.5
2
1.5
1
0.5
0
A
B
B
B
HP:
0 min
30 min
3 hr
24 hr
Time

Fig. 3.2

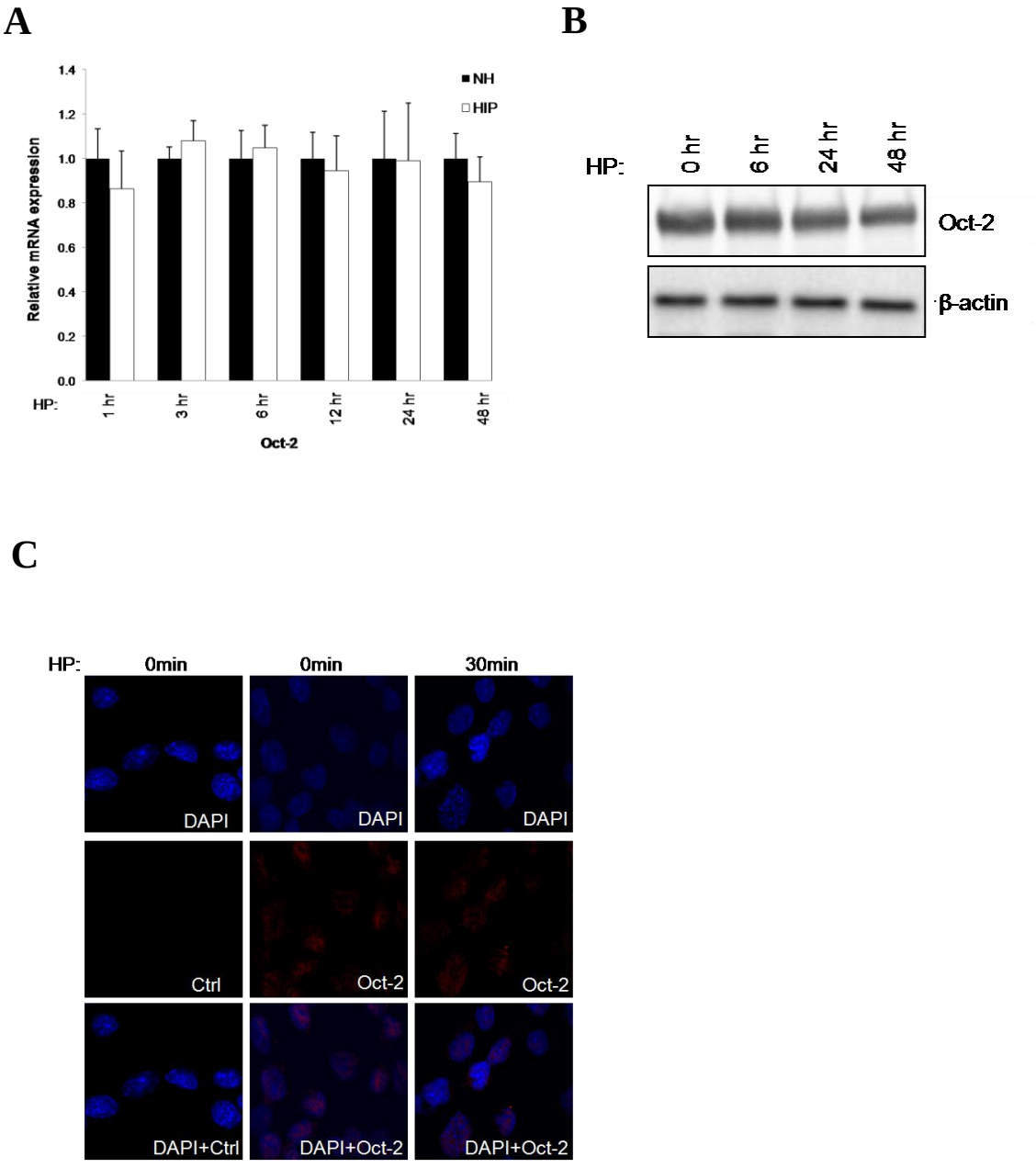
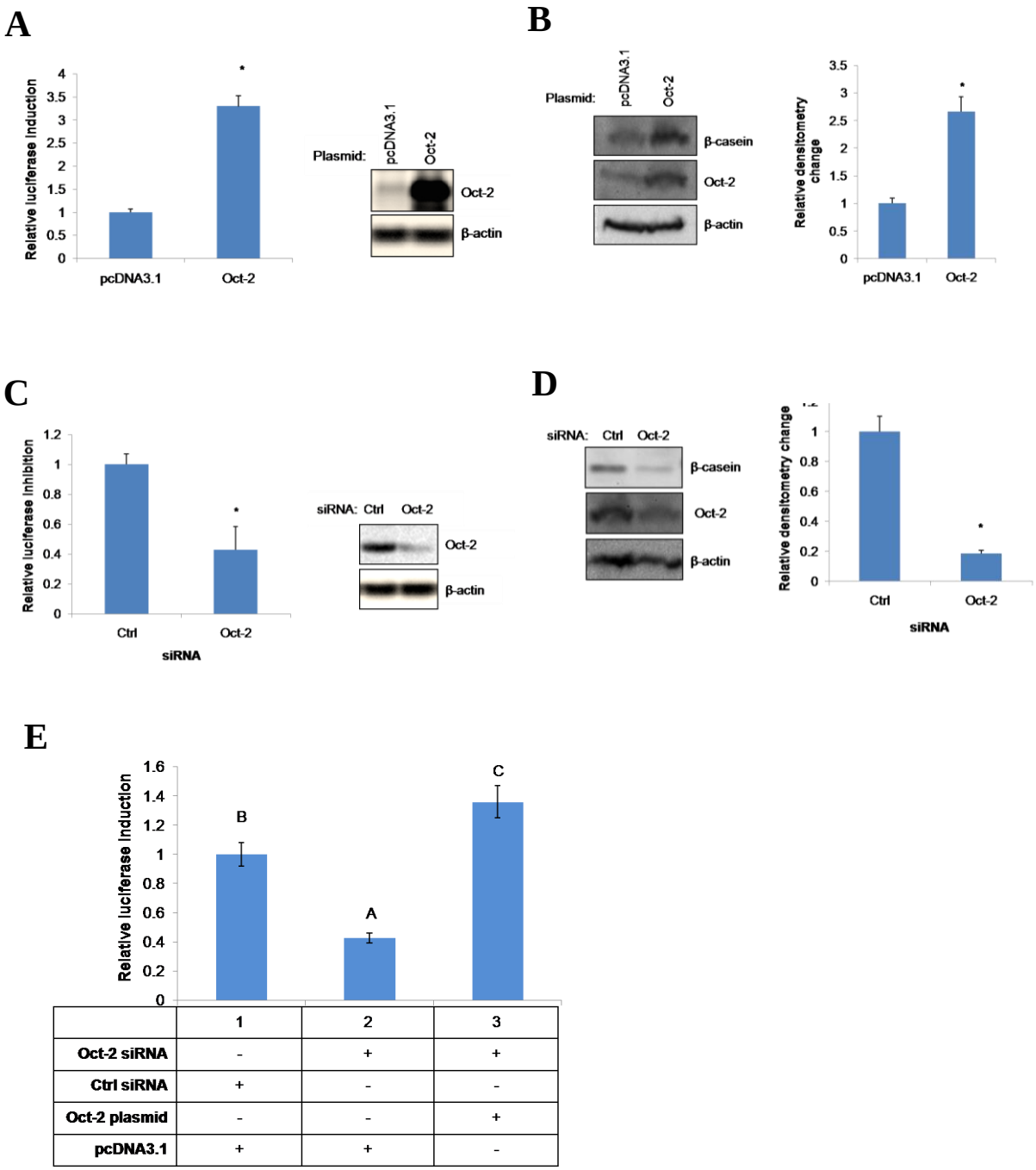

A
B
C
NH
HIP
Relative mRNA expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
HP:
1 hr
3 hr
6 hr
12 hr
24 hr
48 hr
Oct-2
HP:
0 hr
6 hr
24 hr
48 hr
Oct-2
β-actin
HP:
0min
0min
30min
DAPI
DAPI
DAPI
Ctrl
Oct-2
Oct-2
DAPI+Ctrl
DAPI+Oct-2
DAPI+Oct-2

Fig. 3.3



	1	2	3
Oct-2 siRNA	-	+	+
Ctrl siRNA	+	-	-
Oct-2 plasmid	-	-	+
pcDNA3.1	+	+	-

Fig. 3.4

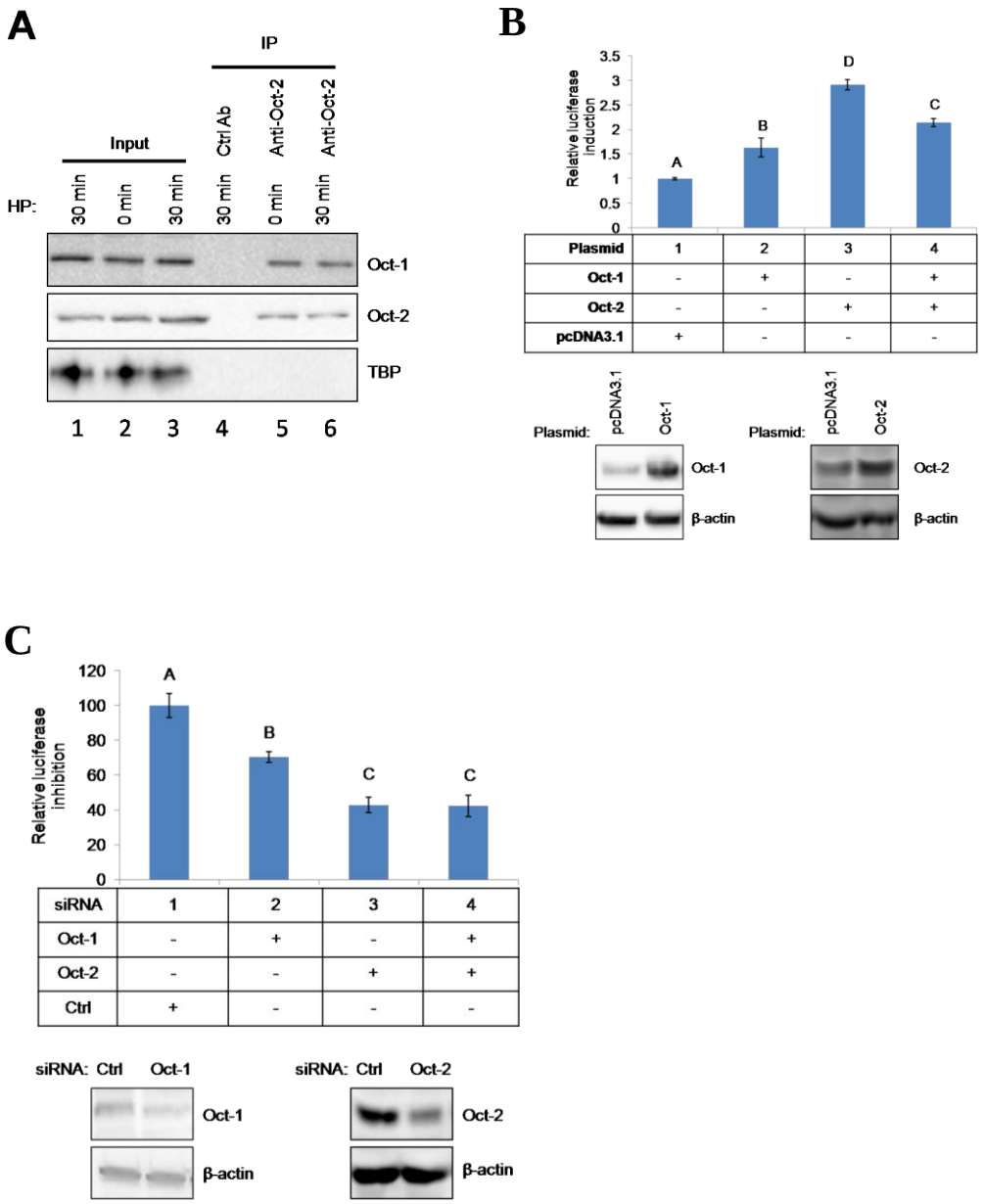
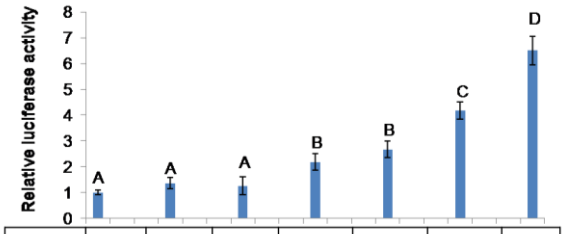

A
IP
Input
Ctrl Ab
Anti-Oct-2
Anti-Oct-2
HP:
30 min
0 min
30 min
30 min
0 min
30 min
Oct-1
Oct-2
TBP
1 2 3 4 5 6
B
Relative luciferase induction
3.5
3
2.5
2
1.5
1
0.5
0
A
B
D
C
Plasmid 1 2 3 4
Oct-1 - + - +
Oct-2 - - + +
pcDNA3.1 + - - -
Plasmid: pcDNA3.1 Oct-1
Oct-1
β-actin
Plasmid: pcDNA3.1 Oct-2
Oct-2
β-actin
C
Relative luciferase inhibition
120
100
80
60
40
20
0
A
B
C
C
siRNA 1 2 3 4
Oct-1 - + - +
Oct-2 - - + +
Ctrl + - - -
siRNA: Ctrl Oct-1
Oct-1
β-actin
siRNA: Ctrl Oct-2
Oct-2
β-actin

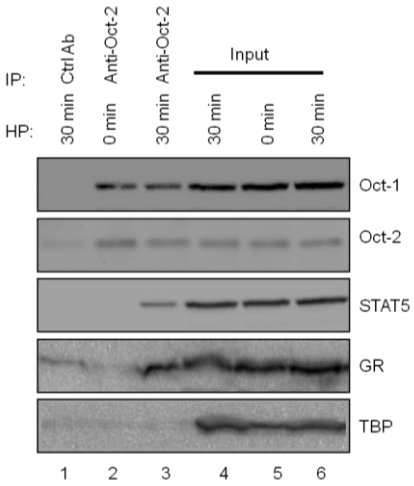
Fig. 3.5

A

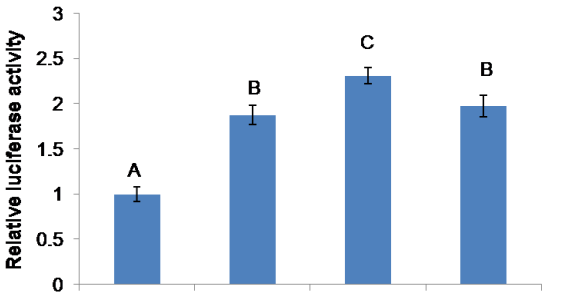


	1	2	3	4	5	6	7
Oct-2	+	-	-	+	+	-	+
STAT5	-	+	-	+	-	+	+
GR	-	-	+	-	+	+	+

C



B



Plasmid	1	2	3	4
Oct-1	-	+	-	+
Oct-2	-	-	+	+
STAT5	+	+	+	+
GR	+	+	+	+

Chapter 4 Production of recombinant human proinsulin in the milk of transgenic mice*

Xi Qian[1], Jana Kraft[2], Yingdong Ni[3], and Feng-Qi Zhao[1*]

[1]Department of Animal Science, University of Vermont, Burlington, VT 05405, USA

*To whom correspondence should be addressed: Feng-Qi Zhao, Department of Animal Science, University of Vermont, 211 Terrill Building, 570 Main Street, Burlington, VT 05405, USA, Tel.: (802) 656-0786; Fax: (802) 656-8196; E-mail address: fzhao@uvm.edu

*This chapter has been submitted for publication, in the following form: Qian, X., Kraft, J., Ni, Y., and Zhao F.-Q.. Production of recombinant human proinsulin in the milk of transgenic mice. Scientific Reports.

ABSTRACT

There is a steady increasing demand for insulin worldwide. Current insulin manufacturing capacities can barely meet this increasing demand. The purpose of this study was to test the feasibility of producing human proinsulin in the milk of transgenic animals. Four lines of transgenic mice harboring a human insulin cDNA with expression driven by the goat β-casein gene promoter were generated. The expression level of human proinsulin in transgenic milk was as high as 8.1 μg/μl at mid-lactation. The expression of the transgene was only detected in the mammary gland during lactation, with higher levels at mid-lactation and lower levels at early and late lactation. The blood glucose and insulin levels and the major milk compositions were unchanged. The mature insulin derived from the milk proinsulin retained its biological activity. In conclusion, our study provides supporting evidence to explore the production of high levels of human proinsulin in the milk of dairy animals.

Keywords: Bioreactor; Dairy pharming; Milk; Recombinant human insulin; Transgenic mouse

INTRODUCTION

Diabetes is a disease characterized by high blood sugar (glucose) levels, which can lead to a number of serious complications, including heart disease, stroke, kidney failure, blindness, nerve damage and foot problem[1, 2]. The number of people diagnosed with diabetes has continued to increase worldwide [366 million people had diabetes in

2011; by 2030 this number is projected to rise to 552 million according to the International Diabetes Federation (http://www.idf.org/media-events/press-releases/2011/diabetes-atlas-5th-edition), 2013]. Diabetes occurs either when the pancreas does not produce enough insulin (Type I) or when the body cannot effectively use the insulin it produces (Type II)[2, 3]. Insulin is used clinically to treat both Type I and Type II diabetes[4, 5]. In humans *in vivo*, the single insulin gene (*INS*) is first transcribed and translated to a single chain precursor called preproinsulin [(110 amino acids (aa)] in the β-cells of the islets of Langerhans in the pancreas[6]. The signal peptide (the first 23-24 aa at the N-terminus) is removed during insertion into the endoplasmic reticulum, resulting in proinsulin (86 aa, ~9.5 kDa). Proinsulin consists of three domains: an amino-terminal B chain (30 aa, ~3.4 kDa), a carboxy-terminal A chain (21 aa, ~2.4 kDa), and a connecting C chain (34 aa, ~3.0 kDa)[6, 7]. Within the endoplasmic reticulum, proinsulin is cut by neuroendocrine-cell-specific prohormone convertases (PC1 and PC2) to excise the C chain. The remaining B- and A-chains are bound together by disulfide bonds, resulting in the mature form of insulin (~5.8 kDa)[6-8].

Currently, biosynthetic human insulin is manufactured for widespread clinical use employing recombinant DNA technology[9]. In early days, A chain and B chain of human insulin are produced in separate bacterial strains, and, after separate purification, they are joined by air oxidation[10]. At present, clinical insulin and its analogs are primarily produced in yeast as the inactive precursor, proinsulin, which must then undergo enzymatic cleavage of the C chain using trypsin and carboxypeptidase B to obtain full potency[8, 9, 11]. However, both bacteria and yeast have inherent limitations in productivity

and secretion efficiency for the production of high-volume therapeutic insulin[12]. Additionally, the International Diabetes Federation predicts that worldwide, by 2030, one in ten people will suffer from diabetes, giving rise to a large demand for insulin, which is expected to grow from US$12B in 2011 to more than US$32B by 2018[13]. Current insulin production methods are insufficient to meet this rapidly increasing demand.

The production of biopharmaceutical proteins in the mammary glands of genetically modified dairy animals ("dairy pharming") is currently under extensive exploration because it promises to provide high-quality therapeutic medicine for humans at an acceptable cost[14]. The overall objective of this study was to test the technical and health feasibility of producing human proinsulin in the milk of transgenic mice and provide a foundation for the potential scale-up of human proinsulin production in the milk of transgenic dairy animals.

MATERIALS AND METHODS

Generation of transgenic mice that express human proinsulin in milk

This study was approved by the University of Vermont Animal Care and Use Committee, and all of the animal work and handling was carried out in accordance with institutional polices and federal guidelines. Full-length human INS cDNA was amplified by PCR from the plasmid PCMV6-XL5-INS-cDNA (Origene, Rockville, MD) using the primers pCMV-INS-F and pCMV-INS-R (Table 4.1). The PCR product (495 bp) was

cloned into a commercial mammary gland expression vector (pBC1, Invitrogen, Grand Island, NY) at the Xho I site to generate the pBC1-INS plasmid via blunt cloning using the Quick Blunting and Quick Ligation Kit (New England Biolabs, Ipswich, MA) (Fig. 4.1A). The pBC1-INS plasmid was digested with the restriction enzymes Not I and Sal I to release a 16.3 kb linear DNA fragment that contained human insulin cDNA flanked by the goat β-casein gene promoter and 3' and 5' untranslated sequences (Fig. 4.1A). The released transgene DNA fragment was then purified by agarose gel electrophoresis and electro-elution before being microinjected into the pronuclei of fertilized C57BL/6 oocytes to generate transgenic mice at the Transgenic Mouse Facility of the University of Vermont following standard procedures. The mice were maintained on a C57BL/6 background.

Screening of transgenic animals by PCR

Genomic DNA was isolated from the tail tips of 2- to 3-week-old mice. The identification of transgenic mice carrying the pBC1-INS transgene construct was carried out by PCR with two pairs of primers: Pr1F, Pr1R, Pr2F, and Pr2R (Table 4.1). Primers Pr1F and Pr1R were complementary to the 5'-flanking sequence of the goat β-casein gene and to the human INS cDNA, respectively, whereas Pr2F and Pr2R were complementary to the human INS cDNA and the 3'-flanking sequence of the goat β-casein gene, respectively (Fig. 4.1A). To verify that the same amount of genomic DNA was used in each PCR reaction, mouse β-actin genome DNA was also amplified with mβActin-F1 and mβActin-F1 (Table 4.1). The PCR conditions were as follows: an initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 68°C for 1

min. The final PCR products were visualized by electrophoresis in a 3% agarose gel in Tris-acetate-EDTA (TAE, 40 mM Tris, 20 mM acetate, and 1 mM EDTA, pH 7.6) buffer.

Evaluation of the transgene copy number by real-time PCR (qPCR)

The transgene copy number in the transgenic mice was determined by qPCR as described previously[15] with primers designed for a single copy control gene (lymphotoxin B gene: Ltb) and the pBC1-INS transgene. The primer sequences for Ltb have been described previously[15], and the primer sequences for the pBC1-INS were pBC1-INS-F and pBC1-INS-R (Table 4.1). The PCR products for Ltb were linked to those of pBC1-INS, and the resulting Ltb-pBC1-INS DNA fragment was cloned into the pCR-Blunt II-TOPO vector (Invitrogen) and transformed to TOP10 Competent Cells (Invitrogen). Subsequently, the Ltb-pBC1-INS plasmid was isolated and used as a calibration sample with a known pBC1-INS/Ltb ratio (1:1). qPCR assays were performed on a CFX96 Real-Time PCR system (Bio-Rad, Hercules, CA) using a 20 µl reaction mixture containing 10 µl SsoFast EvaGreen supermix (Bio-Rad), 500 nM forward and reverse primers, and 20 ng of genomic DNA. The PCR cycling conditions were as follows: one cycle at 98°C for 2 min and 40 cycles of 98°C for 5 s and 65°C for 5 s. Using the $2^{-\Delta\Delta Ct}$ method[27], the relative copy number of the transgene was determined with respect to the calibration sample.

Blood glucose level measurement

Blood was obtained by nicking the lateral tail vein using a sterile scalpel blade and immediately measured with a FreeStyle Lite Blood Glucose Monitoring System (FreeStyle, Alameda, CA). The measurements were carried out at 9 to 10 am, and no pre-fasting was performed.

Western blotting

Mammary gland tissues were collected from transgenic mice at pre-pregnancy, pregnancy (pregnant for 16-18 d), mid-lactation (lactating for 10-12 d), and involution (5 d after lactation) time points. The total protein was extracted from mammary gland tissue that had been homogenized in NP40 lysis buffer [50 mM Tris (pH 7.4), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1% Nonidet P40, proteinase inhibitor cocktail (Sigma, St. Louis, MO), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] using a Dounce homogenizer. The homogenate was then vigorously rocked at 4°C for 30 min, followed by a 10 min centrifugation at 4°C. The supernatant was saved, and the protein concentrations were determined using the Microplate BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). The protein extracts were boiled for 5 min in 1 × Laemmli sample buffer [62.5 mM Tris-Cl (pH 6.8), 2.5% SDS, 0.002% bromophenol blue, 10% glycerol, and 710 mM β-mercaptoethanol]. Equal amounts of protein from each developmental stage were then analyzed via Western blotting with specific antibodies against human proinsulin (Abcam, Cambridge, MA), as described previously[28].

Milk and blood sample collection and ELISA

Milk samples from the transgenic mice and non-transgenic mice were collected during early (3-5 d), middle (9-11 d), and late (15-17 d) lactation using a Medela

Freestyle pump (McHenry, IL). Just prior to milking, 5 I.U. of oxytocin was injected intraperitoneally to the mice. The milk samples were defatted by centrifugation at 4°C for 15 min at 10,000 *g*. The resulting skim milk was diluted one million fold, and the human proinsulin concentrations were then determined with a Human Insulin ELISA Kit (RAB0327) from Sigma according to the manufacturer's instructions. Tail blood samples (40 µl) were drained into heparinized microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA), transferred to centrifuge tubes, and centrifuged at 4°C and 2,000 *g* for 10 min. The resulting supernatant (plasma) was saved and used for ELISA analysis. Two different ELISA kits were used: 1) a Human Insulin ELISA Kit (RAB0327, Sigma), which is specifically used to measure human insulin and proinsulin; and 2) a mouse Ultrasensitive Insulin ELISA kit (80-INSMSU-E10, Alpco, Salem, NH), which has 147% and 0.27% cross-reactivity against human insulin and proinsulin, respectively.

RT-PCR and qRT-PCR

Total RNA was isolated from various tissues (mammary gland, kidney, spleen, lung, thymus, salivary gland, ovary, liver, blood, muscle, and heart) of the transgenic and non-transgenic mice using Trizol reagent (Invitrogen) and digested by RNase-free DNase I (Invitrogen). A total of 5 µg of each DNase I-treated RNA sample was used to synthesize the first-strand cDNA using the Reverse SuperScript II reverse transcriptase (Invitrogen) and oligo (dT) primer per the manufacturer's protocol. The primers for amplification of the human proinsulin transcript were ProINS-F and ProINS-R (Table 4.1). The primers for mouse β-actin were mβActin-F2 and mβActin-R2 (Table 4.1). An initial reaction of 5 min at 95°C was followed by 32 cycles (28 cycles for β-actin) of 30 s

denaturation at 95°C, 30 s of annealing at 62°C, and 1 min of extension at 68°C. A total of 10 µl of each PCR reaction was resolved on 1% agarose gels via electrophoresis. For qRT-PCR, the reactions were performed in duplicate in a 20-µl volume containing 10 µl of SsoFast EvaGreen Supermix (Bio-Rad), 500 nM forward and reverse primers (1 µl each), and 8 µl of diluted cDNA (corresponding to 25 ng of reverse-transcribed total RNA). The relative expression of the transgene was normalized to β-actin and calculated by the $2^{-\Delta\Delta Ct}$ method[28].

Milk composition analyses

The milk samples were diluted 5-fold with distilled water. The milk triacylglyceride concentration was measured by a Colorimetric Assay Kit provided by Cayman Chemical (Ann Arbor, MI), and the milk protein concentration was measured using a BCA Protein Assay Kit provided by Pierce (Rockford, IL).

Conversion of proinsulin to mature insulin

The conversion of the proinsulin in the transgenic milk to insulin was carried out by proteolysis using trypsin (Roche, Indianapolis, IN) and carboxypeptidase B (Roche), as described previously[29]. Specifically, wild-type (WT) milk and transgenic milk were digested with trypsin and carboxypeptidase B with proinsulin to enzyme ratios of 300:1 (w/w) and 600:1 (w/w), respectively. The amount of transgenic milk added to the reaction was calculated based on the proinsulin concentration determined by ELISA, and WT milk containing the same amount of protein as the transgenic milk was used. The digestion was carried out in a buffer (pH 7.5) with 0.1 M Tris-HCl and 1 mM $MgCl_2$ at 37°C for 1 h.

Assay of insulin receptor autophosphorylation

Assays for the autocatalytic activity of the insulin receptor were performed as described[30, 31]. Chinese hamster ovary (CHO) cells were maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum plus 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 25 µg/ml of Fungizone. Before transfection, CHO cells were seeded at 0.1×10^6 cells/well in 12-well plates and grown in a humidified incubator at 37°C and 5% CO_2 overnight to 70-80% confluence. Then, the cells were transfected with 1.25 µg/well of human insulin receptor expression plasmid (#24049, Addgene, Cambridge MA) using Lipofectamine (Invitrogen) according to the manufacturer's instructions. After transfection, the cells were made quiescent by serum starvation for 12 h, then treated with 1 µM commercial insulin (Sigma), with digested and undigested transgenic milk (with equivalent insulin concentration as the commercial insulin used), as well as with digested and undigested WT milk samples (with the same protein concentration as the transgenic milk used) for 1 h. The CHO cells were then lysed in 50 mM Tris (pH 7.4) containing 130 mM NaCl, 5 mM EDTA, 1.0% Nonidet P-40, 1 × proteinase inhibitor cocktail (Sigma), 100 mM NaF, 50 mM β-glycerophosphate, and 100 µM Na_3VO_4. Equal amounts of cell lysates (20 µg of protein) were applied to 96-well ELISA and analyzed for tyrosine phosphorylation of the insulin receptor using the Phospho-IR ELISA Kit (Millipore, Billerica, MA).

Statistical analysis

All statistical analyses were carried out using JMP statistical software (SAS, Cary, NC). The comparisons between two groups were performed using the *t*-test, and the

comparisons between more than two groups were analyzed with one-way ANOVA followed by post hoc Dunnett's multiple comparison test.

RESULTS

Generation and characterization of the transgenic mice

To generate transgenic mice expressing human proinsulin in milk, we inserted the full-length human insulin cDNA into the mammary gland-specific expression vector pBC1, in which the human insulin cDNA is controlled by the goat β-casein promoter and flanked by the 5' and 3' untranslated sequences of the goat β-casein gene (Fig. 4.1A). The transgene was linearized from the vector and injected into the fertilized mouse eggs, which were then transferred into recipients. Thirty pups were obtained. Among them, one male (#24) and 3 female (#5, #12, and #15) transgenic founders were identified (Fig. 4.1B and C). The four transgenic founders were mated with wild type mice, and all of them transmitted the transgenes to their offspring. A total of 16 F1 transgenic mice were identified among the 36 offspring.

We used a qPCR technique to estimate the copy number of the transgene in each line of the transgenic mice[15]. The results indicated that the transgene copy numbers in the founders were different, ranging from 7 to 20. Additionally, transgene loss was observed when comparing the transgene copy numbers between the founders and their offspring, in agreement with previously published results[16-18] (Table 4.2).

Expression of human proinsulin in transgenic mouse tissues

To examine the stage-specific expression of human insulin in the mammary gland of the transgenic mice, mammary gland tissues were collected at the pre-pregnancy, pregnancy (pregnant for 16-18 d), lactation (lactating for 10-12 d), and involution (5 d after lactation) stages and analyzed for human insulin mRNA by qRT-PCR. As shown in Fig. 4.2A (upper panel), the expression of the human proinsulin transcript increased approximately 40-fold at the late pregnant stage compared to the virgin stage, reaching a peak (~100-fold) at mid-lactation, and became undetectable at the involution stage. This expression pattern is consistent with the endogenous mRNA expression profile of mouse β-casein (Fig. 4.2A, bottom panel), a major milk protein[19]. In addition, the developmental expression profiles of human proinsulin protein and endogenous mouse β-casein protein in the mammary glands of the transgenic mice were similar to those of their mRNAs (Fig. 4.2B).

To examine the mammary tissue-specific expression of human insulin in the transgenic mice, the kidney, spleen, lung, thymus, salivary gland, ovary, liver, blood, muscle, heart, and mammary glands were collected from the transgenic founders (#5, #12, and #15) at the lactation stage and analyzed for the expression of human insulin mRNA by RT-PCR. As shown in Fig. 4.2C, the human proinsulin transcripts were found only in the mammary gland but not in the other tested tissues of the transgenic mice, except that weak expression was observed in the blood sample of #12. In addition, no human insulin mRNA was detected in the mammary gland of the non-transgenic litter mates.

Expression of human proinsulin in the milk of transgenic mice

To examine the presence of human proinsulin in the milk of the transgenic mice, Western blot analysis was performed using an antibody specific for human proinsulin (Fig. 4.3A). As shown in Fig. 4.3A, milk samples from #5 (F0), #12 (F0), #15 (F0), and #5's F1 offspring, #5_1 and #5_2, showed a strong band of human proinsulin at approximately 9.5 kDa, as observed in a commercial recombinant human proinsulin positive control (lanes 1, 2, 3, and 8). Western blotting also showed that the concentrations of human proinsulin in the milk of #5 and its F1 offspring ranged from 1.0 to 3.0 µg/µl based on the amounts of recombinant human proinsulin applied in lanes 1 to 3. No proinsulin band was detected in the milk of the wild-type mice (lanes 7 and 9).

To quantitatively measure the human proinsulin expression levels in the milk of all the transgenic founder mice and their F1 females at different lactation stages, milk samples were collected at early (3-5 d), mid- (9-11 d), and late (15-17 d) lactation stages and analyzed for human proinsulin by ELISA. As shown in Fig. 4.3B, the concentrations of human proinsulin in the transgenic milk samples were relatively low at early lactation in all lines but increased at mid-lactation, followed by a decrease at late lactation, except in #12, where the milk at late lactation had the highest level of human proinsulin. Table 4.2 lists the proinsulin concentrations (ranging from 1.4 to 8.1 µg/µl) in the milk samples of the transgenic mice at mid-lactation. The milk proinsulin concentrations of #5, #5_1, and #5_2 were 1.4, 1.2, and 2.0 µg/µl, respectively, which correlated well with their concentration range determined by the titration in Western blotting (Fig. 4.3A). Human proinsulin was not detectable in the milk samples from the non-transgenic littermates by

ELISA. Notably, the human proinsulin concentrations in the transgenic milk were not correlated with the transgene copy number (r=0.39, *P*=0.71).

Blood metabolic profiles of the transgenic mice

To test whether human proinsulin was secreted into the blood streams of the transgenic mice, we first measured the blood levels of human insulin in the transgenic mice (3 founders and 6 offspring) at mid-lactation using an ELISA kit (RAB0327, Sigma) specific for human insulin and proinsulin. No human proinsulin or insulin was detected in the blood samples from either the transgenic or the non-transgenic animals. We also used another mouse Ultrasensitive Insulin ELISA kit (Alpco, 80-INSMSU-E10), which has 147% and 0.27% cross-reactivity to human insulin and proinsulin, respectively, in our assay. No differences in blood insulin levels were observed between the transgenic and non-transgenic mice (Fig. 4.4A), and the detected insulin levels were approximately 5.6 ng/ml for both the transgenic and non-transgenic mice, consistent with the normal physiological plasma insulin levels in a previous study[20].

In addition, the blood glucose levels in these animals were also measured with a glucose meter. No differences in the plasma glucose levels were observed between the transgenic and non-transgenic mice, and the average glucose level was approximately 110 mg/dl for both the transgenic and non-transgenic mice (Fig. 4.4B).

Major milk compositions in the transgenic mice

Milk samples collected from the transgenic mice at the early, mid-, and late lactation stages were analyzed for their triacylglyceride and total protein levels. In

comparison with the milk samples from the WT mice, no significant differences were observed for these compositions at any stages (Table 4.3).

Bioactivity of proinsulin in transgenic milk

The proinsulin in the transgenic milk was converted to mature insulin via *in vitro* enzymatic digestion with trypsin and carboxypeptidase B. The digested transgenic milk was used to treat CHO cells that over-expressed the human insulin receptor, and then the tyrosine phosphorylation of the insulin receptor in the CHO cells was measured. Commercial insulin, digested non-transgenic milk, and undigested transgenic milk were used as controls. As observed in Fig. 4.5, both commercial insulin and the digested transgenic milk could phosphorylate the insulin receptor, whereas the undigested transgenic milk and the digested and undigested non-transgenic milk did not show any detectable activity.

DISCUSSION

The mammary gland-specific expression vector pBC1 was used in this study to generate the transgene construct for producing transgenic mice that expressed human proinsulin in milk. In the construct, the full-length human insulin cDNA was flanked by the 2×β-globin insulator, the goat β-casein promoter and the untranslated exons E1 and E2 in the 5' region, and the untranslated goat β-casein exons E7, E8, and E9 and the 3' genomic DNA sequence in the 3' region. β-Casein is one of the major milk proteins and accounts for approximately 28% of the total milk protein in mice[21] and 37% in goats[22].

Thus, the β-casein promoter has been widely used to drive the high level expression of foreign transgenes in the mammary gland[23]. The other untranslated sequences of the β-casein genes in the construct are considered to enhance the stability of the mRNA of the transgene in the mammary gland. The effectiveness of this vector and the goat gene sequences in mouse were confirmed in our study by the high levels of human proinsulin in the milk of all transgenic mouse lines. In addition, our study also confirmed the mammary-specific and lactation stage-specific expression of the transgene. The expression of human proinsulin followed the profile of endogenous β-casein in the transgenic mice. Although our RT-PCR results indicated that there was a weak band for the human proinsulin transcript expressed in the blood sample of one of the transgenic mice (#12, Fig. 4.2C), we could not detect any human proinsulin protein in the blood of the other animals by ELISA. Thus, the transcript in the blood sample from #12 might be a result of RNA sample contamination. It is important to note that our sequencing analysis discovered that the goat β-casein promoter sequence in pBC1 misses 31 bp of the block B sequence of the β-casein proximal promoter (data not shown). This block B sequence has been well-identified to contain the binding sites for the signal transducer and activator of transcription 5 (STAT5) and for the glucocorticoid receptor; it is also essential for the induction of β-casein proximal promoter activity by the lactogenic hormones prolactin and glucocorticoids in *in vitro* analyses[24, 25]. Our study indicated that the block B sequence may not be as important in a genomic context as in *in vitro* analysis of the proximal promoter.

Transgene loss during animal passage is a common phenomenon[16-18]. In this study, only approximately half of the transgene copies were transmitted from the transgenic founders to their offspring. Transgenes are exogenous fragments of DNA that are introduced into the genome at random sites, and they are usually concatamerized into a tandem array[16]. Due to a lack of balancing transgenes on the paired chromosome during meiosis, transgenes may become unstable and rearrange, perhaps gradually, causing copy number loss from generation to generation[16-18].

Several lines of evidence indicated that the form of insulin in the transgenic milk was proinsulin rather than mature insulin. First, the proinsulin detected in the milk by Western blotting showed the same size as the commercial proinsulin product (Fig. 4.3A). Second, no insulin bioactivity was detected in the transgenic milk without enzymatic digestion, even though the concentration of insulin product in the transgenic milk was high, whereas following endopeptidase digestion of the transgenic milk, insulin bioactivity was detected (Fig. 4.5). These results indicated that the mammary gland could not process the maturation of insulin during the secretion process as the pancreas does. The mammary gland may not express PC1 and PC2, the enzymes required to release peptide C from proinsulin. However, the mammary gland can recognize the insulin signal peptide and secrete the proinsulin protein into milk. The insulin bioactivity detected in the enzymatically digested and renatured transgenic milk may also suggest that the mammary gland is able to carry out the correct post-translational modifications of insulin to retain its bioactivity.

The global insulin market is expected to triple by 2018, and current insulin production practices will face rising difficulty in meeting this rapidly rising demand[13]. Currently, almost all clinical insulin is the recombinant protein or "analog" produced in yeast. The secretion level of recombinant proteins in yeast is still at the magnitude of mg/L (approximately 80 mg/L for human proinsulin)[13, 17]. To meet the increasing demand for human insulin, our study confirmed that it may be feasible to produce high amounts of human proinsulin in the milk of transgenic animals, an emerging biotechnology called "dairy pharming". In this study, the concentrations of human proinsulin in transgenic milk samples collected at mid-lactation ranged from 1.2 to 8.1 µg/µl. Even if the lowest level of human proinsulin (1.2 µg/µl) were expressed in the milk of transgenic goats or cows, the average production by goat (3 kg milk/day) or cow (40 kg milk/day) would produce 3 g or 48 g of proinsulin per day or 0.9 and 13 kg per 9 month lactation, respectively. This rate of production would provide an unlimited human insulin supply to treat diabetes using a limited number of animals.

The expression levels of recombinant human proinsulin in the transgenic milk were shown to range from 1.2 to 8.1 µg/µl (Table 4.2). The expression levels determined by ELISA were in agreement with those titrated by Western blotting. However, the expression levels were not correlated with the transgene copy numbers, suggesting that the expression was still position dependent, although the pBC1 vector was intentionally designed to minimize the position effect of the transgene by incorporating a segment of insulating sequences, namely, the chicken β-globin insulator (Fig. 4.1A)[26].

No apparent health side effects were observed in the transgenic mice. No human proinsulin was detected in the blood of these animals, and the animals had normal blood insulin and glucose levels. In addition, the total levels of milk protein and triacylglycerol were unchanged in these animals.

In conclusion, we successfully generated transgenic mice expressing high levels of human proinsulin in their milk. Our results suggest that it is feasible to produce large amounts of human proinsulin in the milk of dairy animals, such as dairy goats and cows.

Conflict of interest: The authors declare no conflict of interest.

Acknowledgements: We are grateful to Dr. Mercedes Rincon and Mr. John Dodge at the University of Vermont Transgenic Facility for their service in producing the transgenic mice. We thank the staff of the Small Animal Facility of the University of Vermont for the excellent animal care. We also thank Dr. Frederick Stanley for providing the human insulin receptor plasmid. This work was supported by a Hatch grant (VT-H01508) from the USDA National Institute of Food and Agriculture (to FQZ).

Table 4.1. Sequences of primers used in this study.

Forward	Sequence (5'-3')	Reverse	Sequence (5'-3')
pCMV-INS-F	GGCCGCGAATTCGGCCATT	**pCMV-INS-R**	TTGTTGGTTCAAGGGCTTTATTC
mβActin-F1	TAGACTTCGAGCAGGAGATG	**mβActin-R1**	CCACCAGACAGCACTGTGTT
Pr1F	ACCAGGGATCAAACCTGCAC	**Pr1R**	ACGCTTCTGCAGGGACCCCT
Pr2F	TTGTGAACCAACACCTGTGC	**Pr2R**	TGCTGAGAATCATTAATCTCAGC
ProINS-F	CAACACCTGTGCGGCTCACA	**ProINS-R**	CACAATGCCACGCTTCTGCA
mβActin-F2	TAGACTTCGAGCAGGAGATG	**mβActin-R2**	CCACCAGACAGCACTGTGTT
mCSN2-F	AGAGGATGTGCTCCAGGCTA	**mCSN2-R**	TAAGGAGGGGCATCTGTTTG
pBC1-INS-F	CAGGAATCGCGGATCCTC	**pBC1-INS-R**	CCATGGCAGAAGGACAGTGAT

Table 4.2. Transgene copy numbers and human proinsulin expression levels in milk of multiple lines of transgenic mice

Line F0 (gender[a])	Offspring F1 (gender[a])	Transgene copy number	Expression level in milk[b] (µg/µl)
5 (F)		13	1.4
	5_1 (F)	7	1.2
	5_2 (F)	6	2.0
12 (F)		20	7.8
	12_1 (F)	10	7.2
	12_2 (F)	6	8.1
15 (F)		7	4.1
	15_1 (F)	5	2.1
	15_2 (F)	3	1.6
24 (M)		9	NA
	24_1 (F)	5	5.7

[a]F=female, M=male. [b]Milk samples were collected at mid-lactation, and the concentration of human proinsulin was quantified by ELISA.

Table 4.3. Total protein and triglyceride levels of milk from transgenic and wild-type mice during different lactation stages

		Transgenic (n=6)	**Wild-type** (n=6)	**P value** (t test)
Protein (g/L)	Early lactation	102.5 ± 5.1	100.8 ± 6.4	0.541
	Mid-lactation	109.0 ± 8.4	106.9 ± 8.9	0.636
	Late Lactation	99.2 ± 4.5	97.5 ± 7.6	0.483
Triglycerides (g/L)	Early lactation	335.7 ± 39.5	345.8 ±42.1	0.604
	Mid-lactation	320.6 ± 33.1	309.1 ± 30.0	0.538
	Late Lactation	301.9 ± 30.9	290.8 ± 23.1	0.371

REFERENCES

1. Beckman, J. A., Creager, M. A. & Libby, P. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *Jama* **287**, 2570-2581. (2002).
2. Deshpande, A. D., Harris-Hayes, M. & Schootman, M. Epidemiology of diabetes and diabetes-related complications. *Phys. Ther.* **88**, 1254-1264. (2008).
3. Muller, L. *et al.* Increased risk of common infections in patients with type 1 and type 2 diabetes mellitus. *Clin. Infect. Dis.* **41**, 281-288. (2005).
4. Rosenstock, J., Dailey, G., Massi-Benedetti, M., Fritsche, A., Lin, Z. & Salzman, A. Reduced Hypoglycemia Risk With Insulin Glargine A meta-analysis comparing insulin glargine with human NPH insulin in type 2 diabetes. *Diabetes Care* **28**, 950-955. (2005).
5. Turner, R. 1998. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). Lancet 352, 837-853.
6. Steiner, D. The biosynthesis of insulin, in Seino, S., Bell. G.I. (Eds.), Beta cell in health and Disease. Springer Inc., Japan, pp. 31-49. (1990).
7. Bell, G. I., Pictet, R. L., Rutter, W. J., Cordell, B., Tischer, E. & Goodman, H.M. Sequence of the human insulin gene. *Nature* **284**, 26-32. (1980).
8. Thim, L. *et al.* Secretion and processing of insulin precursors in yeast. *Proc. Natl. Acad. Sci. U. S. A.* **83**, 6766-6770. (1986).
9. Kjeldsen, T. Yeast secretory expression of insulin precursors. *Appl. Microbiol. Biotechnol.* **54**, 277-286. (2000).
10. Riggs, A. D. Bacterial production of human insulin. *Diabetes Care* **4**, 64-68. (1981).
11. Chance, R. E. & Frank, B. H. Research, development, production, and safety of biosynthetic human insulin. *Diabetes Care* **16**, 133-142. (1993).
12. Schmidt, F. Recombinant expression systems in the pharmaceutical industry. *Appl. Microbiol. Biotechnol.* **65**, 363-372. (2004).
13. Nielsen, J. Production of biopharmaceutical proteins by yeast: advances through metabolic engineering. *Bioengineered* **4**, 207-211. (2013).
14. Wall, R., Kerr, D. & Bondioli, K. Transgenic dairy cattle: genetic engineering on a large scale. *J. Dairy Sci.* **80**, 2213-2224. (1997).
15. Serova, I. A. *et al.* A 3,387 bp 5'-flanking sequence of the goat alpha-S1-casein gene provides correct tissue-specific expression of human granulocyte colony-stimulating factor (hG-CSF) in the mammary gland of transgenic mice. *Transgenic Res.* **21**, 485-498. (2012).
16. Kong, Q. *et al.* Transgene expression is associated with copy number and cytomegalovirus promoter methylation in transgenic pigs. *PLoS One* **4**, e6679. (2009).

17. Melo, E. O. Canavessi, A. M., Franco, M. M. & Rumpf, R. Animal transgenesis: state of the art and applications. *J. Appl. Genet.* **48**, 47-61. (2007).
18. Nakanishi, T. *et al.* FISH analysis of 142 EGFP transgene integration sites into the mouse genome. *Genomics* **80**, 564-574. (2002).
19. Robinson, G. W., McKnight, R. A., Smith, G. H. & Hennighausen, L. Mammary epithelial cells undergo secretory differentiation in cycling virgins but require pregnancy for the establishment of terminal differentiation. *Development* **121**, 2079-2090. (1995).
20. Kido, Y., Philippe, N., Schaeffer, A. & Accili, D. Genetic modifiers of the insulin resistance phenotype in mice. *Diabetes* **49**, 589-596. (2000).
21. Boumahrou, N. *et al.* The major protein fraction of mouse milk revisited using proven proteomic tools. *J. Physiol. Pharmacol.* **60**, 113-118. (2009).
22. Greppi, G., Roncada, P. & Fortin, R. Protein Components of Goat's Milk, in: Cannas, A., Pulina, G. (Eds.), Dairy goats feeding and nutrition. CAB International Inc., Massachusetts, pp. 71-94. (2008).
23. Dalrymple, M. A. & Garner, I. Genetically modified livestock for the production of human proteins in milk. Biotechnol. *Genet. Eng. Rev.* **15**, 33-50. (1998).
24. Dong, B. & Zhao, F. Involvement of the ubiquitous Oct-1 transcription factor in hormonal induction of beta-casein gene expression. *Biochem. J.* **401**, 57-64. (2007).
25. Qian, X. & Zhao, F.-Q. Interactions of the ubiquitous octamer-binding transcription factor-1 with both the signal transducer and activator of transcription 5 and the glucocorticoid receptor mediate prolactin and glucocorticoid-induced β-casein gene expression in mammary epithelial cells. *Int. J. Biochem. Cell. Biol.* **45**, 724-735. (2013).
26. Talbot, D., Collis, P., Antoniou, M., Vidal, M., Grosveld, F. & Greaves, D. R. A dominant control region from the human β-globin locus conferring integration site-independent gene expression. *Nature* **338**, 352-355. (1989).
27. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402-408. (2001).
28. Zhao, F.-Q., Adachi, K. & Oka, T. Involvement of Oct-1 in transcriptional regulation of β-casein gene expression in mouse mammary gland. *Biochim. Biophys. Acta.* **1577**, 27-37. (2002).
29. Kemmler, W., Peterson, J. D. & Steiner, D. F. Studies on the conversion of proinsulin to insulin I. Conversion *in vitro* with trypsin and carboxypeptidase B. *J. Biol. Chem.* **246**, 6786-6791. (1971).
30. Jacob, K. K., Whittaker, J. & Stanley, F. M. Insulin receptor tyrosine kinase activity and phosphorylation of tyrosines 1162 and 1163 are required for insulin-increased prolactin gene expression. *Mol. Cell. Endocrinol.* **186**, 7-16. (2002).
31. Pender, C. *et al.* Regulation of insulin receptor function by a small molecule insulin receptor activator. *J. Biol. Chem.* **277**, 43565-43571. (2002).

FIGURE LEGENDS

Fig. 4.1. Transgene construction and the identification of transgenic mice. (A) Schematic representation of the transgene construction. The full length of insulin cDNA in the pCMV6-XL5-INS-cDNA was amplified by PCR and inserted into the pBC1 vector at the *Xho* I site, generating the pBC1-INS construct. Before microinjection, the pBC1-INS construct was excised with Sal I and Not I. From left to right, the linearized pBC1-INS comprises the 2×β-globin insulator; the goat β-casein promoter and untranslated exons E1 and E2; human insulin cDNA; untranslated goat β-casein exons E7, E8, and E9; and 3' genomic DNA. Pr1F, Pr1R, Pr2F, and Pr2R primers were used in PCR for the identification of the transgenic mice. (B&C) Identification of the transgenic mice by PCR using the Pr1 primer pair (B) and Pr2 primer pair (C). Non-transgenic wild-type (WT) mouse DNA was used as a negative control, and the DNA used for microinjection served as a positive control. β-Actin was amplified to show the same amount of DNA used in each genomic DNA sample.

Fig. 4.2. Expression of the human insulin transgene in transgenic mouse tissues. (A) qRT-PCR analysis of human proinsulin transcripts in the mammary gland tissues of transgenic F1 mice at the virgin (NP), pregnancy (P), lactation (L), and involution (I) stages (upper panel). β-Actin was used as an internal control. Three mice in each stage were included for the analysis. The data are expressed as the mean ± SE.***, $P < 0.001$ when compared to the NP group. The endogenous β-casein gene expression was measured as a positive control (bottom panel). (B) Western blot analysis of the protein

expression of human proinsulin and endogenous β-casein in mammary gland tissues at the different developmental stages depicted in (A). The same amount of protein was applied in each lane, and each lane used the mammary tissue sample pooled from three transgenic mice at the same stage. β-Actin was used as a loading control. (C) RT-PCR analysis of human proinsulin transcripts in various tissues of transgenic females #5, #12, and #15 at the mid-lactation stage. 1, mammary gland; 2, kidney, 3, spleen; 4, lung; 5, thymus; 6, salivary gland; 7, ovary; 8; liver, 9, blood; 10, muscle; 11, heart; 12, mammary gland from non-transgenic mice; 13, ddH_20. β-Actin was used as a loading control.

Fig. 4.3. Detection of human proinsulin in the milk of transgenic mice. (A) Western blot analysis of human proinsulin in milk samples from transgenic mice. Commercial recombinant human proinsulin at concentrations of 0.5, 1.0, and 3 μg/μl (lanes 1-3, respectively) was used as a positive control (PC). Lanes 4, 5, and 6 are milk samples from transgenic founder #5 and its F1 offspring, #5_1 and #5_2, respectively. Lanes 10 to 12 are the milk of #15, #12, and #5, respectively. Milk from wild-type (WT) mouse (lanes 7 & 9) was used as a negative control. The same volume of milk samples was loaded in each well. (B) Expression profiles of human proinsulin in the milk of transgenic mice throughout lactation. Milk samples from three transgenic lines were collected at early, mid-, and late lactation and measured for human proinsulin concentrations by ELISA. The presence of proinsulin was barely detectable in the corresponding transgenic littermates (negative controls).

Fig. 4.4. Blood insulin and glucose levels in transgenic mice at mid-lactation. (A) Blood insulin concentrations in transgenic (T) and non-transgenic (NT) mice at mid-lactation as analyzed by ELISA. (B) Blood glucose concentrations in transgenic (T) and non-transgenic (NT) mice at mid-lactation. Nine transgenic-positive mice and nine transgenic-negative littermates in 3 transgenic lines were analyzed. The data are expressed as mean ± SE.

Fig. 4.5. Insulin receptor autophosphorylation in CHO cells treated with enzymatically digested transgenic milk. The proinsulin (100 µg) in transgenic milk was converted to mature insulin via *in vitro* enzymatic digestion with trypsin and carboxypeptidase B (DTM) and then used to treat CHO cells that over-expressed the human insulin receptor. After 1 h of treatment, the cells were lysed, and 20 µg of protein lysate was used in ELISA assays for tyrosine phosphorylation. Commercial insulin (PC), undigested transgenic milk (UTM), and digested (DWD) and undigested (UWD) non-transgenic milk were used as controls. Three experiments were repeated, and representative results are shown here. In the first two experiments, the transgenic milk samples from #5 (F0) and its offspring, #5_1(F1) and #5_2 (F1), were combined to obtain 100 µg of proinsulin. In the third experiment, the transgenic milk samples from #12 (F0) and its offspring, #12_1 (F1), were combined.

Fig. 4.1

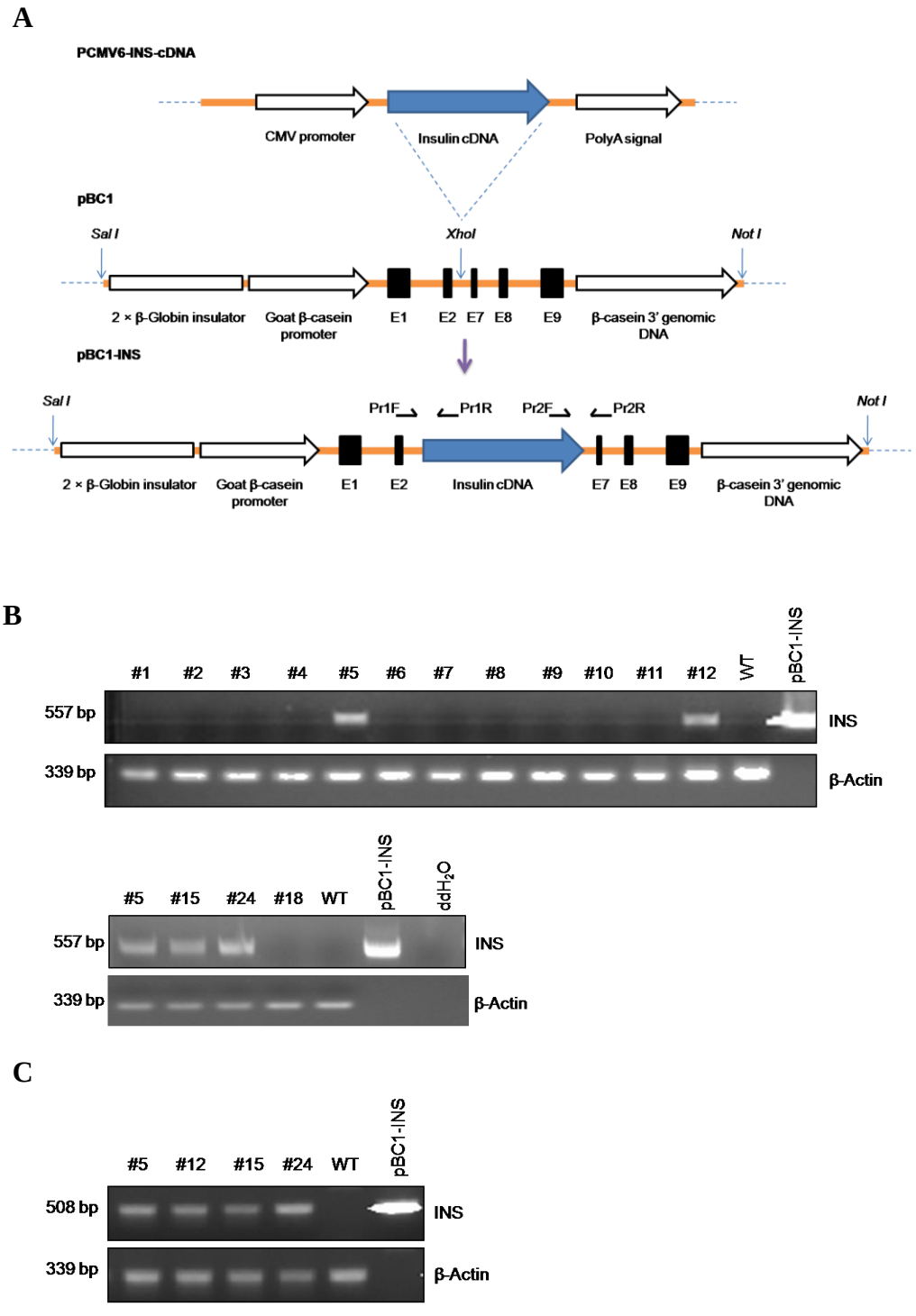

A
PCMV6-INS-cDNA
CMV promoter
Insulin cDNA
PolyA signal
pBC1
Sal I
XhoI
Not I
2 × β-Globin insulator
Goat β-casein promoter
E1
E2
E7
E8
E9
β-casein 3' genomic DNA
pBC1-INS
Pr1F
Pr1R
Pr2F
Pr2R
B
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
#11
#12
WT
pBC1-INS
557 bp
339 bp
INS
β-Actin
#5
#15
#24
#18
WT
pBC1-INS
ddH2O
C
#5
#12
#15
#24
WT
pBC1-INS
508 bp

Fig. 4.2

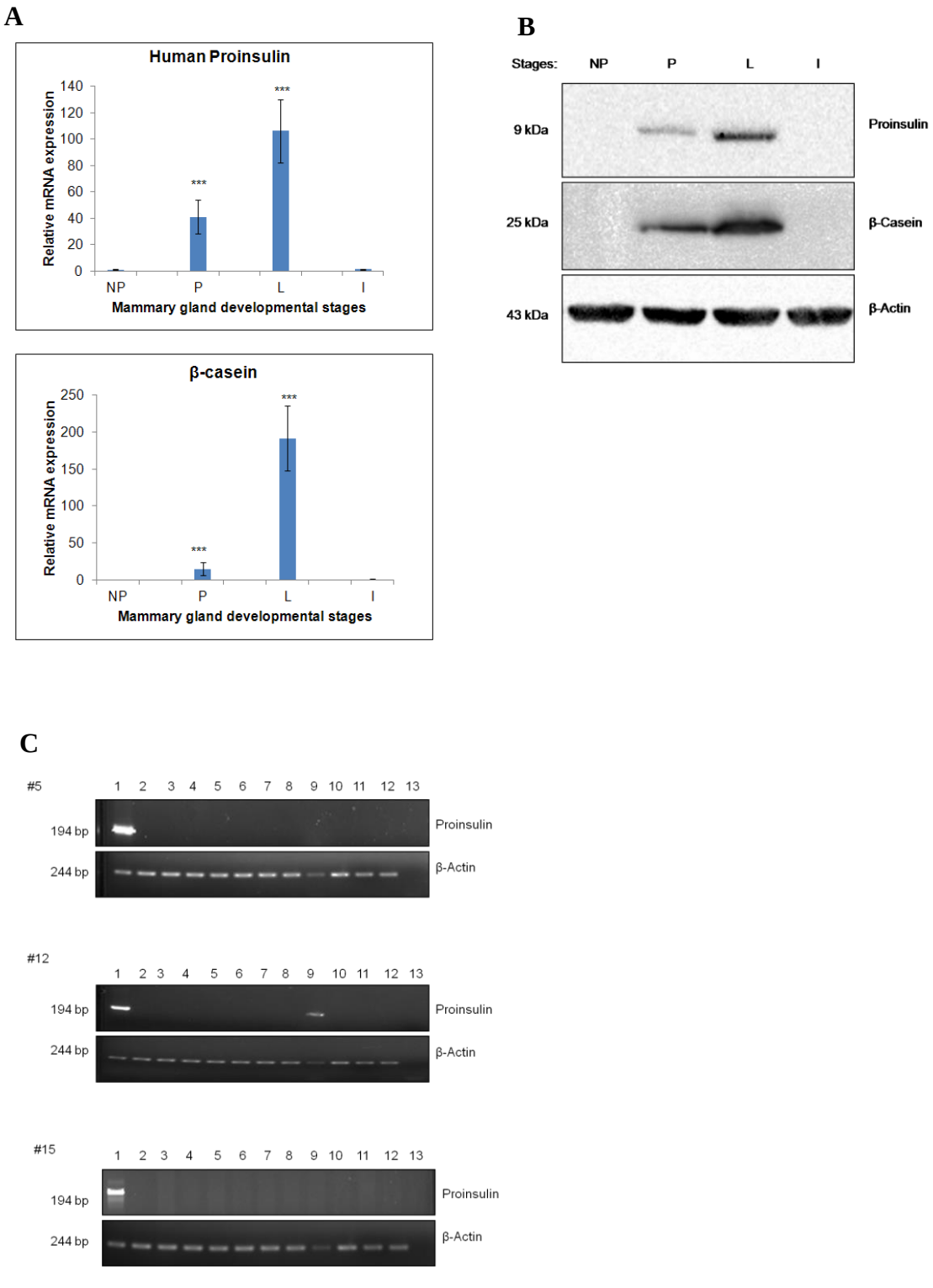
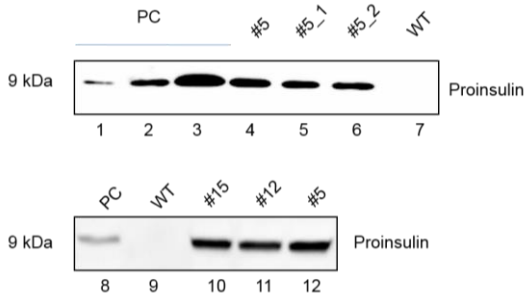
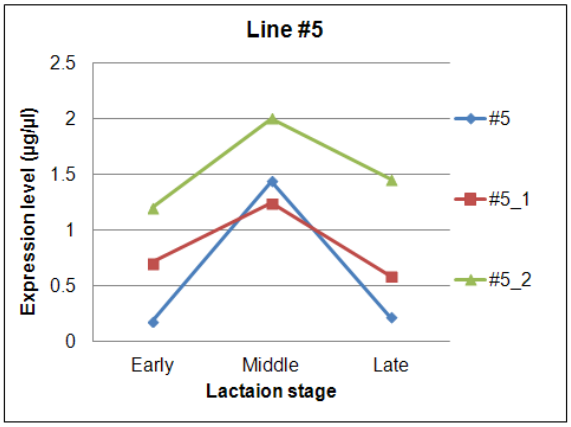

A
Human Proinsulin
Relative mRNA expression
140
120
100
80
60
40
20
0
***
***
NP
P
L
I
Mammary gland developmental stages
β-casein
Relative mRNA expression
250
200
150
100
50
0
***
***
NP
P
L
I
Mammary gland developmental stages
B
Stages: NP P L I
9 kDa
Proinsulin
25 kDa
β-Casein
43 kDa
β-Actin
C
#5
1 2 3 4 5 6 7 8 9 10 11 12 13
194 bp
Proinsulin
244 bp
β-Actin
#12
1 2 3 4 5 6 7 8 9 10 11 12 13
194 bp
Proinsulin
244 bp
β-Actin
#15
1 2 3 4 5 6 7 8 9 10 11 12 13
194 bp
Proinsulin
244 bp
β-Actin

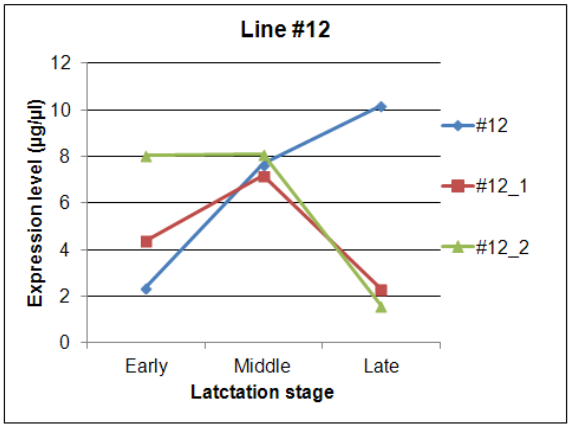
Fig. 4.3

A


PC
#5
#5_1
#5_2
WT
9 kDa
Proinsulin
1 2 3 4 5 6 7
PC
WT
#15
#12
#5
9 kDa
Proinsulin
8 9 10 11 12

B


Line #5
Expression level (µg/µl)
2.5
2
1.5
1
0.5
0
Early
Middle
Late
Lactaion stage
#5
#5_1
#5_2


Line #12
Expression level (µg/µl)
12
10
8
6
4
2
0
Early
Middle
Late
Latctation stage
#12
#12_1
#12_2

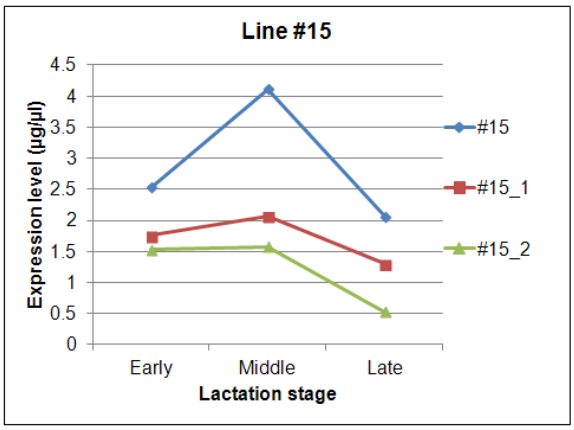
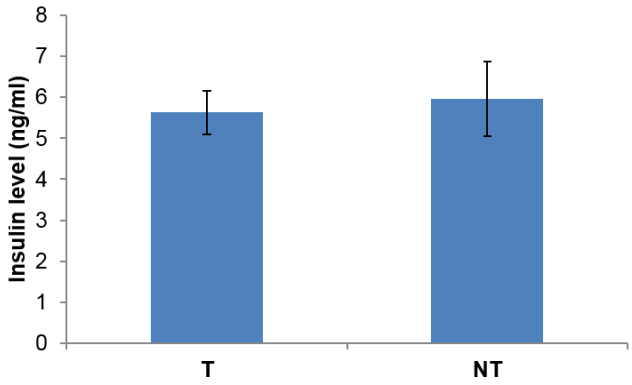

Line #15
Expression level (µg/µl)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Early
Middle
Late
Lactation stage
#15
#15_1
#15_2

Fig. 4.4

A


Insulin level (ng/ml)
8
7
6
5
4
3
2
1
0
T
NT

B

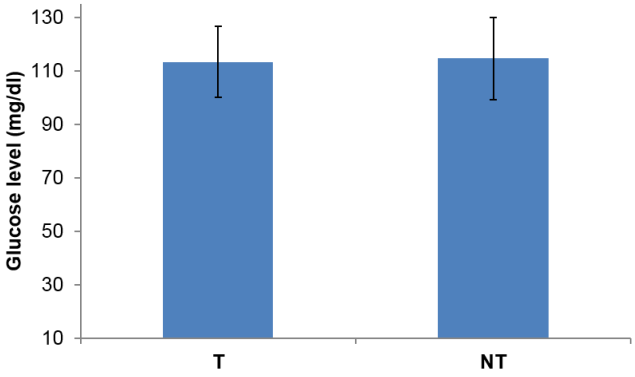
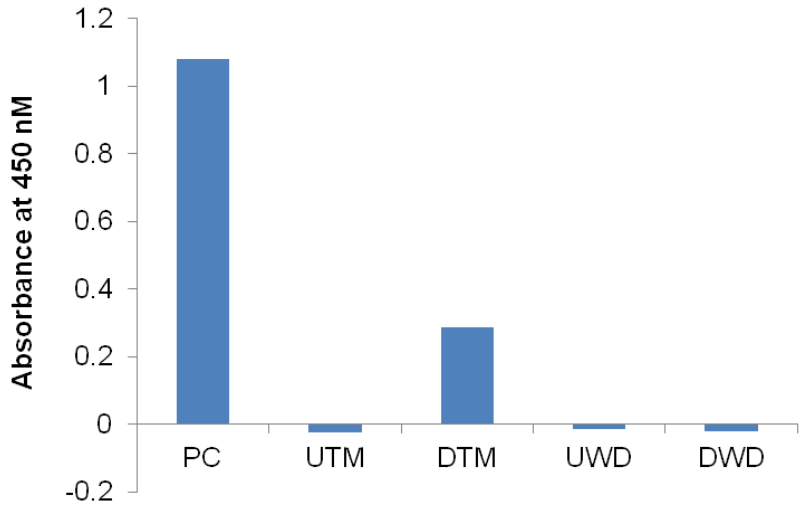

Glucose level (mg/dl)
130
110
90
70
50
30
10
T
NT

Fig. 4.5



Chapter 5 General discussion

OCT-1 AND OCT-2 ARE INVOLVED IN THE INDUCTION OF β-CASEIN GENE EXPRESSION BY LACTOGENIC HORMONES

Lactogenic hormones, HP, synergistically induce mammary-specific β-casein gene expression. The sequence alignment of the 5′ flanking sequences of human, rat, cow, sheep, and goat β-casein gene reveals two evolutionarily conserved regions, the proximal promoter and the distal enhancer (Rijnkels et al., 2003; Winklehner-Jennewein et al., 1998). These two regions contain composite binding sites for different transcription factors, including STAT5, GR, C/EBPβ, and YY-1 (Rosen et al., 1999). The importance of these transcription factors in regulating β-casein gene expression has been established by studies using transient transfection experiments and transgenic and knockout mouse models (Rosen et al., 1999). Because none of these transcription factors is mammary-specific, mammary-specific β-casein gene expression is believed to be dependent on the combinatorial protein-protein interactions among these transcription factors and on their binding at the composite regulatory elements present in both the proximal promoter and the enhancer in response to lactogenic hormones. A previous study using chromatin immunoprecipitation has shown that signaling transduction pathways regulated by lactogenic hormones induce similar kinetics of assembly and disassembly of different

transcription factors, the coactivator p300, histone modifiers, and RNA polymerase II at the proximal promoter and at the distal enhancer of the β-casein gene (Kabotyanski et al., 2006). This result suggests that these two regulatory regions may interact with one another through chromatin looping. Later, Kabotyanski and colleagues confirmed this hypothesis using a chromosome conformation capture assay. These researchers found that lactogenic hormones induce the physical interaction between the β-casein gene proximal promoter and the distal enhancer (Kabotyanski et al., 2009) and that withdrawal of lactogenic hormones results in disruption of this interaction, accompanied by a decrease in β-casein gene expression (Rijnkels et al., 2010).

Based on the previous results, a working model has been proposed to elucidate how the signaling pathways regulated by lactogenic hormones are integrated to activate β-casein expression (Rijnkels et al., 2012). In this model, YY-1 binds to the proximal promoter of the β-casein gene in the absence of lactogenic hormones and forms a repressive complex by recruiting histone deacetylase 3 (HDAC3) and LIP (inhibitory isoform of C/EBPβ) (Bauknecht et al., 1996; Meier and Groner, 1994; Yang et al., 1996; Yang et al., 1997), promoting a transcriptionally negative chromatin in association with deacetylated histone H3 (Kabotyanski et al., 2006) and dimethylated lysine 9 of histone H3 (H3K9) (Buser et al., 2011). Deacetylated H3 is possibly promoted by HDAC3 (Kabotyanski et al., 2009), although the mechanism by which dimethylated H3K9 is maintained is unknown. After stimulation with lactogenic hormones, the transcription factors STAT5, GR, and LAP (activating isoform of C/EBPβ) rapidly bind to their respective binding sites at the β-casein gene proximal promoter and distal enhancer

(Kabotyanski et al., 2006), displace the repressive complex, and, subsequently, recruit nuclear co-activator p300 via a protein-protein interaction (Mink et al., 1997; Pfitzner et al., 1998). The recruited coactivator p300 facilitates histone H3 acetylation, thus relaxing the chromatin structure at the β-casein *cis*-regulatory regions through its intrinsic histone acetyltransferase (HAT) activity. Lactogenic hormones also promote open chromatin structure by inducing H3K9 demethylation via undiscovered mechanisms. Interactions among these activated transcription factors and the co-activator result in physical contact between the proximal promoter and the distal enhancer through DNA looping, which facilitates the recruitment of the preinitiation complex and β-casein transcription (Kabotyanski et al., 2009).

In this study, we report that Oct-1 and Oct-2 are also the downstream signaling molecules of lactogenic hormones in mammary epithelial cells. Although either Oct-1 or Oct-2 constitutively binds to and induces the basal activity of β-casein gene promoter without the treatment of lactogenic hormones (Dong and Zhao, 2007a; Zhao et al., 2002; Zhao et al., 2004), lactogenic hormones further increase their binding to the promoter (Qian and Zhao, 2013, 2014). Our transfection experiments indicated that Oct-1 and Oct-2 function as transactivators in lactogenic hormones-regulated β-casein gene expression in mammary epithelial cells (Qian and Zhao, 2013, 2014). Oct-1 has recently been shown to interact with and to facilitate the binding of Runx2, which is a transcription factor with the binding site adjacent to octamer motif at the β-casein gene promoter. Additionally, overexpression of Oct-1 and Runx2 together leads to a higher basal β-casein gene promoter than does that of either protein alone (Inman et al., 2005). In this study, we

demonstrated that lactogenic hormones induce physical interactions of either Oct-1 or Oct-2 or both with both STAT5 and GR and increase β-casein gene promoter activity (Qian and Zhao, 2013, 2014). In addition, we showed that Oct-1 facilitates the binding of both STAT5 and GR at the β-casein gene promoter, similar to the effect of Oct-1 on Runx2 binding (Qian and Zhao, 2013). Progesterone is a reproductive hormone that inhibits β-casein gene expression by inducing the binding of the progesterone receptor (PR) at the β-casein promoter (Buser et al., 2007; Buser et al., 2011). Oct-1 binding activity at the β-casein gene promoter can be induced by progesterone in the mammary gland of virgin mice (Zhao et al., 2002). Coincidentally, progesterone has also been shown to recruit unphosphorylated (unactivated) STAT5 to the β-casein promoter, potentially contributing to the inhibition of β-casein by progesterone (Buser et al., 2007). Because Oct-1 can interact with the PR (Préfontaine et al., 1999), Oct-1 may also play a role in the inhibition of β-casein gene expression by interacting with other transcription factors, such as PR. Collectively, we expand Rijnkels's model (Rijnkels et al., 2012) and propose a working model of the involvements of Oct-1 and Oct-2 in regulating β-casein gene expression (Fig. 5.1). Depending on upstream signaling, Oct-1 and Oct-2 can act as either a transactivator or a repressor and switch β-casein gene expression between the inducible or repressive states in mammary epithelial cells as follow: 1) In the quiescent state, Oct-1 and possibly Oct-2 promote an inhibitory state for the β-casein gene by interactions with YY1, LIP, and HDAC3. 2) Following progesterone treatment, PR joins in and stabilizes the repressive complex, further inhibiting β-casein gene expression. 3) Following lactogenic hormones treatment, however, Oct-1 and Oct-2 switch the β-casein

gene to a permissive state and interact with STAT5, GR, LAP, and Runx2, promoting pre-initiation complex formation and inducing β-casein gene expression.

Supporting this hypothesis, previous studies have shown that Oct-1 and other Oct proteins serve as switchable regulators in controlling their target gene expression. Depending on upstream signaling, the Oct protein-targeted genes can either be rapidly induced or become stably repressed. One excellent example is the role of Oct-1 in regulating the interleukin 2 gene in CD4 T lymphocytes (Shakya et al., 2011). In naïve T lymphocytes, Oct-1 recruits the Mi-2/NuRD chromatin-remodeling complex to the interleukin 2 gene, inducing heavy methylation at the CpG site proximal to the transcription start site and, in turn, mediating gene repression. In contrast, upon T lymphocyte activation, Oct-1 loses its ability to associate with Mi-2/NuRD, instead indirectly or directly recruiting Jmjd1a/KDM3A histone demethylase to the interleukin 2 gene and, in turn, blocking gene repression by opposing inhibitory histone methylation (Shakya et al., 2011). The switch of the interleukin 2 gene from a repressive to a permissive state is regulated by the MAPK signaling pathway (Shakya et al., 2011). Oct-1 may have a similar function in regulating the MMTV promoter (Åstrand et al., 2009) and *Polr2a*, *Ahcy*, and *Cdx2* genes (Shakya et al., 2011). Oct-4, which is another member of the Oct family, also has a bi-potential function in regulating target genes involved in embryonic stem cell (ESC) development, such as *Hoxa5*, *Otx2*, *Pou4f1*, *Hoxc6*, and *Pax6* (Bernstein et al., 2006; Chen et al., 2008). Further investigation is required to determine whether Oct-1 and Oct-2 have this bi-potential or switchable role in β-casein gene regulation in response to hormonal treatments. Specifically, the presence of Oct-

interacting co-factors and the chromatin status of the β-casein gene promoter must be investigated under different hormonal treatments. One possibility is that Oct-1 and Oct-2 may recruit histone-lysine N-methyltransferase SETDB1 (Yeap et al., 2009; Yuan et al., 2009), which was found to be associated with Oct-4, to promote the observed formation of dimethylated H3K9 at the β-casein proximal promoter in the absence of lactogenic hormones (Buser et al., 2011). However, after lactogenic hormones treatment, Oct-1 and Oct-2 may recruit Jmjd1a/KDM3A histone demethylase to the β-casein gene, in turn, removing inhibitory histone methylation.

The following question is how Oct proteins respond to upstream signals and to hormones and interact with other proteins to regulate their target gene transcription. Previous studies have revealed that Oct proteins can integrate various upstream signals, which precisely regulate Oct proteins at multiple levels, including expression level (Karwacki-Neisius et al., 2013; Nichols et al., 1998), localization (Malhas et al., 2009; Tolkunova et al., 2007; Wang et al., 2009), and activity (Shakya et al., 2011). Most Oct proteins are subjected to post-transcriptional modifications, including phosphorylation (Lin et al., 2012; Nieto et al., 2007; Pevzner et al., 2000; Schild-Poulter et al., 2007; Segil et al., 1991; Tanaka and Herr, 1990), O-GlcNAcylation (Jang et al., 2012; Kang et al., 2013; Webster et al., 2009), SUMOylation (Wei et al., 2007; Zhang et al., 2007), and ubiquitylation (Kang et al., 2011; Xu et al., 2004). These post-transcriptional modifications are most likely responsible for many of Oct protein changes in protein stability, localization, and activity (Kang et al., 2013; Nieto et al., 2007; Schild-Poulter et al., 2007; Segil et al., 1991; Wang et al., 2009; Wei et al., 2007). In this study, we report

that the binding activities of both Oct-1 and Oct-2 to the β-casein promoter can be induced by lactogenic hormones and that this induction was not mediated by either increasing their expression or inducing their translocation to the nucleus. Therefore, lactogenic hormones most likely enhance Oct-1 and Oct-2 DNA-binding activity via mechanisms of post-translational modification. These post-translational modifications, particularly phosphorylation, may also be able to mediate the assembly of multiprotein transcriptional regulatory complexes (Holmberg et al., 2002; Whitmarsh and Davis, 2000).

The results from this study indicate that Oct-2 interacts with Oct-1 regardless of HP treatment, but that the interaction between Oct-1 and Oct-2 does not synergistically stimulate β-casein gene expression in response to lactogenic hormones. Oct-1 and Oct-2 appear to be functionally redundant in the hormonal regulation of the β-casein gene. This observation is consistent with previous studies in Oct-1 and Oct-2 knockout mice in which Oct-1 and Oct-2 operate redundantly in regulating B cell development and IgG transcription (Wang et al., 2004). These data also imply that Oct-1 and Oct-2 may form heteromers at the β-casein gene promoter. This possibility is supported by the fact that Oct proteins interact not only with various transcription factors and co-activators (Kang et al., 2009b), but also with themselves to form homo/hetero-dimers or even higher-order complexes (Nieto et al., 2007; Reményi et al., 2001; Verrijzer et al., 1992).

THE MAMMARY GLAND AS A BIOREACTOR

Proteins are the building blocks of life. Some proteins, called enzymes, catalyze metabolic reactions, and some proteins are involved in the process of cell signaling, whereas other proteins serve structural functions. Malfunctions or deficiencies of certain proteins cause life-threatening diseases. With the aid of genetic engineering, pharmaceutical companies now produce these proteins in a recombinant way to save people's lives. To produce a recombinant protein, a transgene construct containing a protein-of-interest-coding DNA sequence must be generated first. Then, the recombinant protein is produced in a chosen expression system. Commonly used protein expression systems include bacteria, yeasts, insects, mammalian cells, transgenic plants, and transgenic animals (Wang et al., 2013). Of these systems, transgenic animals are currently under extensive exploration, and different transgenic animals have been generated to produce recombinant proteins secreted into a specific fluid, such as milk, blood, urine, plasma, and egg white (Wang et al., 2013). Thus far, producing foreign proteins in the mammary glands of transgenic animals seems to be best approach. This approach, which is also known as a mammary bioreactor system, is superior to producing recombinant proteins in other body fluids of transgenic animals and in other expression systems in one or more aspects. For example, compared with producing a recombinant protein in blood, expressing of recombinant proteins in milk does not compromise the host animal's own health and survival, and extracting the recombinant proteins from milk does not involve animal sacrifice (Clark, 1998; Montesino and Toledo, 2006). The mammary bioreactor system offers better approach than other expression systems

because of its scalability, its ability to perform post-translational modifications, and its ease of milk harvest (Clark, 1998; Montesino and Toledo, 2006).

Because the incidence of diabetes is rapidly increasing worldwide, the global insulin market is expected to triple by 2018 (Nielsen, 2013). The current insulin production practices will face difficulty in meeting this rapidly rising demand. Using a mammary bioreactor system to produce human insulin is an attractive approach to meet this demand. In this study, we successfully generated transgenic mice expressing high levels of human proinsulin (1.2-8.1 μg/μl) in their milk. In addition, our study also confirmed the mammary-specific and lactation stage-specific expression of the human insulin transgene and demonstrated that the transgenic animals had no apparent health defects. These results suggest that producing much human proinsulin in the milk of dairy animals, such as dairy goats and cows, is feasible. If a transgenic cow produces 1 g of insulin per liter of milk, which is an extremely safe target based on our study in transgenic mice, then this transgenic cow would produce 10 kg insulin per year with an average annual milk production of 10,000 kg. A ton of insulin can be produced by approximately 100 cows. If we can make this possibility a reality, then this approach will greatly relieve the urgent demand for insulin worldwide.

However, producing transgenic dairy animals is not as simple as producing transgenic mice. At this time, the cost of producing transgenic farm animals remains extremely high, and the efficiency is low (Houdebine, 2009). Producing a transgenic farm animal has been estimated to cost hundreds of thousands of dollars, without any guarantee of success (Houdebine, 2009). Many efforts have already been or can be made

to improve the efficiency and to reduce the cost of producing transgenic dairy animals. The primary barrier for transgenic animal production remains identifying more efficient systems of transgene delivery. To ensure that the transgene is present in every cell in the animal's body, all genetic-engineering techniques attempt to introduce the transgene at the time of fertilization or at the earliest stage of embryo development. Although pronuclear microinjection has been used for more than two decades, the inherent inefficiency of transgene delivery, variable transgene expression patterns, and uncertain transmission through generations preclude the wide-spread application of this technology (Niemann and Kues, 2007; Thomson et al., 2003). In recent years, several alternatives to pronuclear microinjection have been developed to improve transgene-delivery efficiency for generating transgenic dairy animals, including injection or infection of oocytes and/or embryos by lentiviral vectors (Hofmann et al., 2003), by sperm-mediated DNA transfer (Smith and Spadafora, 2005), and by transfection of cultured differentiated cells combined with somatic cell nuclear transfer (Samiec and Skrzyszowska, 2011). In addition, a great savings can be achieved if the transgene-delivered embryos can be subject to screening for transgene incorporation, gene construct number, transgene copy number, and transgene chromosome integration sites before embryo implantation in recipient animals (Samiec and Skrzyszowska, 2011). These selections ensure that nearly all the produced progeny will be transgenic. Moreover, the number of male transgenic animals can be greatly reduced if only X chromosome-containing embryos are used for implantation. For instance, for the method of sperm-mediated DNA transfer, sperm sexing for X and Y chromosomes can be performed to ensure only X chromosome sperm

are used in the artificial fertilization. Sperm sexing has been used commercially to produce sexed offspring with ~90% accuracy (Seidel, 2009).

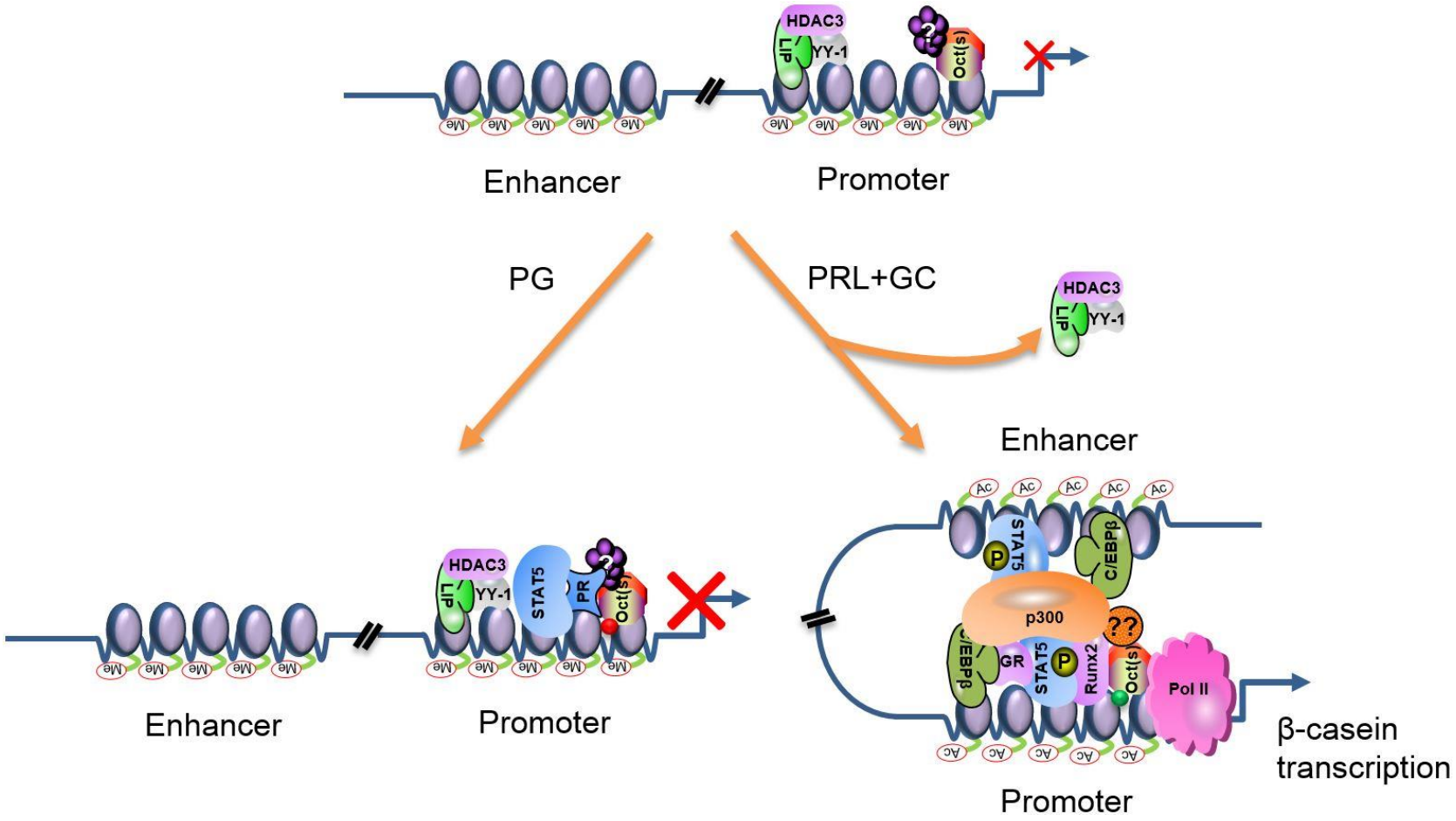
In conclusion, the biochemical and molecular information generated by this study defines a novel mechanism of how Oct factors are involved in mediating lactogenic hormone-regulated tissue-specific gene expression. Our exploration of mammary gland bioreactor with the transgenic mice model indicates that it may be feasible to apply our transgene construct (pBC1-INS-cDNA) to produce recombinant human proinsulin in the milk of dairy animals, such as dairy cows and goats, in industrial scale.

FIGURE LEGENDS

Fig. 5.1. A working model illustrating the hormonal regulation of β-casein gene transcription in mammary epithelial cells. This model was developed from Rijnkels's new model (Rijnkels et al., 2012). In the absence of hormones, Yin Yang 1 (YY-1) binds to the β-casein proximal promoter and presumably forms a repressive complex by recruiting histone deacetylase 3 (HDAC3) and liver-enriched transcriptional inhibitory protein (LIP, a dominant negative isoform of CAAT/enhancer binding protein β, C/EBP β), resulting in the formation of a negative histone marker, deacetylated histone H3, at the proximal promoter of the β-casein gene (Kabotyanski et al., 2009). Oct-1 and/or Oct-2 may bind to the octamer motif at the β-casein gene proximal promoter as a monomer or even as a high-order complex, such as a heterodimer or as a homodimer. Oct(s) may recruit its cofactors, possibly histone-lysine N-methyltransferase SETDB1, leading to the formation of dimethylated lysine 9 of histone H3 (H3K9). After progesterone (PG) treatment, the progesterone receptor (PR) binds to half glucocorticoid response element (½ GRE) at the β-casein gene promoter (Buser et al., 2007). In turn, the bound PR may further stabilize the repressive complex at the β-casein gene promoter by enhancing Oct(s) binding and by recruiting the unactivated (unphosphorylated) signal transducer and transcription activator 5 (STAT5). When the mammary epithelial cells are treated with prolactin (PRL) and glucocorticoids (GC), Oct(s) may be activated by post-translational modification and switch to function as a transactivator. Activated Oct-1 may recruit its coactivator, such as Jmjd1a/KDM3A histone demethylase, to the β-casein gene, in turn removing inhibitory histone methylation. PRL and GC result in STAT5, glucocorticoid

receptor (GR), and C/EBP β (positive isoform) rapidly binding to their respective response elements within β-casein regulatory regions, recruiting p300 through protein-protein interactions and, in turn, facilitating histone acetylation (Kabotyanski et al., 2009). Additionally, the interactions of Oct-1 with STAT5 and GR can stabilize the binding of STAT5 and GR to the β-casein promoter (Qian and Zhao, 2013). Interactions between the promoter and enhancer, which are mediated through these transcription factors and co-activators, enable DNA looping. The formation of the active chromatin loop between distant regulatory elements facilitates binding of the basal transcriptional machinery to the DNA template and initiates transcription.

Fig. 5.1


HDAC3
LIP
YY-1
Oct(s)
Me
Enhancer
Promoter
PG
PRL+GC
HDAC3
LIP
YY-1
Enhancer
HDAC3
LIP
YY-1
STAT5
PR
Oct(s)
Enhancer
Promoter
Ac
STAT5
P
C/EBPβ
p300
??
GR
STAT5
P
Runx2
Oct(s)
Pol II
β-casein
transcription
Promoter

Abbreviations

BCE-1, bovine casein enhancer element;

BCM-7, beta-casomorphin-7;

BM, basement membrane;

C/EBPβ, CAAT/enhancer binding protein β;

ChIP, chromatin immunoprecipitation;

ChIP-chip, chromatin immunoprecipitation (ChIP) followed by microarrays;

ChIP-seq, immunoprecipitation (ChIP) followed by DNA sequencing;

CHO, Chinese hamster ovary;

DTT, dithiothreitol;

ECM, extracellular matrix;

EGF, epidermal growth factor;

Elf5, E74-like factor 5;

EMSA, electrophoresis mobility shift assay;

ESC, embryonic stem cell;

Ets, E26 transformation-specific;

FDA, Food and Drug Administration;

GAPDH, glyceraldehydes-3-phosphate dehydrogenase;

GCs, glucocorticoids;

GH, growth hormone;

GHR, growth hormone receptor;

GR, glucocorticoid receptor;

GRE, glucocorticoid response elements;

1/2 GRE, half glucocorticoid response element;

H3Ac, histone H3 acetylation;

HAT, histone acetyltransferase;

HEC, hyperinsulinemic-euglycemic clamp;

HDAC3, histone deacetylase 3;

H3K9, lysine 9 of histone H3;

HP, hydrocortisone (glucocorticoids) and prolactin;

HTH, helix-turn-helix;

IGF-1, insulin-like growth factor-1;

IP, immunoprecipitation;

INS, insulin;

JAK2, Janus kinase 2;

LAP, liver-enriched transcriptional activator proteins;

LIP, liver-enriched transcriptional inhibitory protein;

MBDs, methyl-CpG-binding domain proteins;

MECs, mammary epithelial cells;

MEC, mammary alveolar secretory epithelial cell;

MMTV, mouse mammary tumor virus;

mTOR, mammalian target of rapamycin;

NF-1, nuclear factor 1;

OCA-S, Oct-1 coactivator in S phase;

Oct, octamer-binding transcription factor;

Oct-1, octamer-binding transcription factor-1;

PC, prohormone convertase;

PG, progesterone;

PI3K, phosphoinositide 3-kinase;

PMSF, phenylmethylsulfonyl fluoride;

POU, Pit-1, Oct, and Unc-86;

POU_H, POU homeodomain;

POU_S, POU-specific domain;

PR, progesterone receptor;

PRL, prolactin;

PrlR, prolactin receptor;

PRLR, prolactin receptor;

qChIP, quantitative chromatin immunoprecipitation;

qPCR, quantitative PCR;

qRT-PCR, quantitative reverse transcription PCR;

Runx2, runt-related transcription factor 2;

snRNA, small nuclear RNA;

SH2, Src homology 2;

Sp1, specificity protein 1;

STAT5, signal transducer and activator of transcription 5;

SWI/SNF, Switch/Sucrose nonfermentable;

TAE, Tris-acetate-EDTA;

TBP, TATA box-binding protein;

TGFβ, transforming growth factor-β;

TSS, transcription start site;

WAP, whey acidic protein;

WT, wild-type;

YY-1, Yin Yang 1;

Bibliography

Ahmad, I., Hoessli, D.C., Walker-Nasir, E., Rafik, S.M., Shakoori, A.R., and Nasir-ud-Din. (2006). Oct-2 DNA binding transcription factor: functional consequences of phosphorylation and glycosylation. Nucleic Acids Research 34, 175-184.

Akers, R. (2006). Major advances associated with hormone and growth factor regulation of mammary growth and lactation in dairy cows. Journal of Dairy Science 89, 1222-1234.

Alcaraz, J., Xu, R., Mori, H., Nelson, C.M., Mroue, R., Spencer, V.A., Brownfield, D., Radisky, D.C., Bustamante, C., and Bissell, M.J. (2008). Laminin and biomimetic extracellular elasticity enhance functional differentiation in mammary epithelia. The EMBO Journal 27, 2829-2838.

Allan, G.J., Tonner, E., Barber, M.C., Travers, M.T., Shand, J.H., Vernon, R.G., Kelly, P.A., Binart, N., and Flint, D.J. (2002). Growth hormone, acting in part through the insulin-like growth factor axis, rescues developmental, but not metabolic, activity in the mammary gland of mice expressing a single allele of the prolactin receptor. Endocrinology 143, 4310-4319.

Andersen, C.R., and Larson, B. (1970). Comparative maintenance of function in dispersed cell and organ cultures of bovine mammary tissue. Experimental Cell Research 61, 24-30.

Anderson, S.M., Rudolph, M.C., McManaman, J.L., and Neville M.C. (2007). Key stages in mammary gland development. Secretory activation in the mammary gland: it's not just about milk protein synthesis. Breast Cancer Research 9, 204-217.

Archibald, A.L., McClenaghan, M., Hornsey, V., Simons, J.P., and Clark, A.J. (1990). High-level expression of biologically active human alpha 1-antitrypsin in the milk of transgenic mice. Proceedings of the National Academy of Sciences of the United States of America 87, 5178-5182.

Assairi, L., Delouis, C., Gaye, P., Houdebine, L., Bousquet, M., and Denamur, R. (1974). Inhibition by progesterone of the lactogenic effect of prolactin in the pseudopregnant rabbit. Biochemical Journal 144, 245-252.

Åstrand, C., Belikov, S., and Wrange, Ö. (2009). Histone acetylation characterizes chromatin presetting by NF1 and Oct1 and enhances glucocorticoid receptor binding to the MMTV promoter. Experimental Cell Research 315, 2604-2615.

Audic, J.-L., Chaufer, B., and Daufin, G. (2003). Non-food applications of milk components and dairy co-products: A review. Le Lait 83, 417-438.

Ball, R., Friis, R., Schoenenberger, C., Doppler, W., and Groner, B. (1988). Prolactin regulation of β-casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line. The EMBO Journal 7, 2089-2095.

Baranyi, M., Hiripi, L., Szabó, L., Catunda, A.P., Harsányi, I., Komáromy, P., and Bősze, Z. (2007). Isolation and some effects of functional, low-phenylalanine κ-casein expressed in the milk of transgenic rabbits. Journal of Biotechnology 128, 383-392.

Bauknecht, T., See, R.H., and Shi, Y. (1996). A novel C/EBP β-YY1 complex controls the cell-type-specific activity of the human papillomavirus type 18 upstream regulatory region. Journal of Virology 70, 7695-7705.

Bauman, D. (1999). Bovine somatotropin and lactation: from basic science to commercial application. Domestic Animal Endocrinology 17, 101-116.

Bauman, D., Mather, I., Wall, R., and Lock, A. (2006). Major advances associated with the biosynthesis of milk. Journal of Dairy Science 89, 1235-1243.

Baumrucker, C., and Stemberger, B. (1989). Insulin and insulin-like growth factor-I stimulate DNA synthesis in bovine mammary tissue in vitro. Journal of Animal Science 67, 3503-3514.

Beckman, J.A., Creager, M.A., and Libby, P. (2002). Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. JAMA 287, 2570-2581.

Bell, G.I., Pictet, R.L., Rutter, W.J., Cordell, B., Tischer, E., and Goodman, H.M. (1980). Sequence of the human insulin gene. Nature 284, 26-32.

Belsham, D.D., and Mellon, P.L. (2000). Transcription factors Oct-1 and C/EBPβ (CCAAT/enhancer-binding protein-β) are involved in the glutamate/nitric oxide/cyclic-guanosine 5′-monophosphate-mediated repression of gonadotropin-releasing hormone gene expression. Molecular Endocrinology 14, 212-228.

Bernstein, B.E., Mikkelsen, T.S., Xie, X., Kamal, M., Huebert, D.J., Cuff, J., Fry, B., Meissner, A., Wernig, M., and Plath, K. (2006). A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125, 315-326.

Bolander, F.F., Nicholas, K.R., Van Wyk, J.J., and Topper, Y.J. (1981). Insulin is essential for accumulation of casein mRNA in mouse mammary epithelial cells. Proceedings of the National Academy of Sciences of the United States of America 78, 5682-5684.

Bole-Feysot, C., Goffin, V., Edery, M., Binart, N., and Kelly, P.A. (1998). Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocrine Reviews 19, 225-268.

Boumahrou, N., Andrei, S., Miranda, G., Henry, C., Panthier, J.J., Martin, P., and Bellier, S. (2009). The major protein fraction of mouse milk revisited using proven proteomic tools. Journal of Physiology and Pharmacology 60, 113-118.

Brisken, C. (2002). Hormonal control of alveolar development and its implications for breast carcinogenesis. Journal of Mammary Gland Biology and Neoplasia 7, 39-48.

Brockman, J.L., and Schuler, L.A. (2005). Prolactin signals via Stat5 and Oct-1 to the proximal cyclin D1 promoter. Molecular and Cellular Endocrinology 239, 45-53.

Brodbeck, U., Denton, W., Tanahashi, N., and Ebner, K. (1967). The isolation and identification of the B protein of lactose synthetase as α-lactalbumin. Journal of Biological Chemistry 242, 1391-1397.

Burdon, T.G., Maitland, K.A., Clark, A.J., Wallace, R., and Watson, C.J. (1994). Regulation of the sheep β-lactoglobulin gene by lactogenic hormones is mediated by a transcription factor that binds an interferon-gamma activation site-related element. Molecular Endocrinology 8, 1528-1536.

Burgos, S., Dai, M., and Cant, J. (2010). Nutrient availability and lactogenic hormones regulate mammary protein synthesis through the mammalian target of rapamycin signaling pathway. Journal of Dairy Science 93, 153-161.

Buser, A.C., Gass-Handel, E.K., Wyszomierski, S.L., Doppler, W., Leonhardt, S.A., Schaack, J., Rosen, J.M., Watkin, H., Anderson, S.M., and Edwards, D.P. (2007). Progesterone receptor repression of prolactin/signal transducer and activator of transcription 5-mediated transcription of the β-casein gene in mammary epithelial cells. Molecular Endocrinology 21, 106-125.

Buser, A.C., Obr, A.E., Kabotyanski, E.B., Grimm, S.L., Rosen, J.M., and Edwards, D.P. (2011). Progesterone receptor directly inhibits β-casein gene transcription in mammary epithelial cells through promoting promoter and enhancer repressive chromatin modifications. Molecular Endocrinology 25, 955-968.

Caelles, C., Hennemann, H., and Karin, M. (1995). M-phase-specific phosphorylation of the POU transcription factor GHF-1 by a cell cycle-regulated protein kinase inhibits DNA binding. Molecular and Cellular Biology 15, 6694-6701.

Cella, N., Groner, B., and Hynes, N.E. Characterization of Stat5a and Stat5b homodimers and heterodimers and their association with the glucocorticoid receptor in mammary cells. Molecular and Cellular Biology 18, 1783-1792.

Chance, R.E., and Frank, B.H. (1993). Research, development, production, and safety of biosynthetic human insulin. Diabetes Care 16, 133-142.

Chen, C.M., Wang, C.H., Wu, S.C., Lin, C.C., Lin, S.H., and Cheng, W.T. (2002). Temporal and spatial expression of biologically active human factor VIII in the milk of transgenic mice driven by mammary-specific bovine α-lactalbumin regulation sequences. Transgenic Research 11, 257-268.

Chen, X., Xu, H., Yuan, P., Fang, F., Huss, M., Vega, V.B., Wong, E., Orlov, Y.L., Zhang, W., and Jiang, J. (2008). Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. Cell 133, 1106-1117.

Cheng, C.C., Yang, S.P., Lin, W.S., Ho, L.J., Lai, J.H., Cheng, S.M., and Lin, W.Y. (2012). Magnesium lithospermate B mediates anti-inflammation targeting activator protein-1 and nuclear factor-kappa B signaling pathways in human peripheral T lymphocytes. International Immunopharmacology 13, 354-61

Cheng, Y., Wang, Y., Luo, J., Shen, Y., Yang, Y., Ju, H., Zou, X., Xu, S., Lao, W., and Du, M. (2002). Cloned goats produced from the somatic cells of an adult transgenic goat. Chinese Journal of Biotechnology 18, 79-83.

Choi, K.M., Barash, I., and Rhoads, R.E. (2004). Insulin and prolactin synergistically stimulate β-casein messenger ribonucleic acid translation by cytoplasmic polyadenylation. Molecular Endocrinology 18, 1670-1686.

Clark, A.J. (1998). The mammary gland as a bioreactor: expression, processing, and production of recombinant proteins. Journal of Mammary Gland Biology and Neoplasia 3, 337-350.

Clark, J.H., Spires, H.R., Derrig, R.G., and Bennink, M.R. (1977). Milk production, nitrogen utilization and glucose synthesis in lactating cows infused postruminally with sodium caseinate and glucose. The Journal of Nutrition 107, 631-644.

Cocolakis, E., Dai, M., Drevet, L., Ho, J., Haines, E., Ali, S., and Lebrun, J.-J. (2008). Smad signaling antagonizes STAT5-mediated gene transcription and mammary epithelial cell differentiation. Journal of Biological Chemistry 283, 1293-1307.

Dalrymple, M.A., and Garner, I. (1998). Genetically modified livestock for the production of human proteins in milk. Biotechnology and Genetic Engineering Reviews 15, 33-50.

Dandekar, A.M., Robinson, E.A., Appella, E., and Qasba, P.K. (1982). Complete sequence analysis of cDNA clones encoding rat whey phosphoprotein: homology to a protease inhibitor. Proceedings of the National Academy of Sciences of the United States of America 79, 3987-3991.

Danielson, K.G., Oborn, C.J., Durban, E.M., Butel, J.S., and Medina, D. (1984). Epithelial mouse mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. Proceedings of the National Academy of Sciences of the United States of America 81, 3756-3760.

de la Serna, I.L., Carlson, K.A., and Imbalzano, A.N. (2001). Mammalian SWI/SNF complexes promote MyoD-mediated muscle differentiation. Nature Genetics 27, 187-190.

de Laat, W., and Grosveld, F. (2003). Spatial organization of gene expression: the active chromatin hub. Chromosome Research 11, 447-459.

Deis, R., and Delouis, C. (1983). Lactogenesis induced by ovariectomy in pregnant rats and its regulation by oestrogen and progesterone. Journal of Steroid Biochemistry 18, 687-690.

Descombes, P., and Schibler, U. (1991). A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the sam mRNA. Cell 67, 569-579.

Deshpande, A.D., Harris-Hayes, M., and Schootman, M. (2008). Epidemiology of diabetes and diabetes-related complications. Physical Therapy 88, 1254-1264.

Dhalluin, C., Carlson, J.E., Zeng, L., He, C., Aggarwal, A.K., Zhou, M.-M., and Zhou, M.-M. (1999). Structure and ligand of a histone acetyltransferase bromodomain. Nature 399, 491-496.

Dobosy, J., and Selker, E. (2001). Emerging connections between DNA methylation and histone acetylation. Cellular and Molecular Life Sciences CMLS 58, 721-727.

Dohoo, I.R., Leslie, K., DesCôteaux, L., Fredeen, A., Dowling, P., Preston, A., and Shewfelt, W. (2003). A meta-analysis review of the effects of recombinant bovine somatotropin: 1. Methodology and effects on production. Canadian Journal of Veterinary Research 67, 241-251.

Dong, B., Huang, C., Li, D., and Zhao, F.-Q. (2009). Oct-1 functions as a transactivator in the hormonal induction of β-casein gene expression. Molecular and Cellular Biochemistry 328, 93-99.

Dong, B., and Zhao, F.-Q. (2007a). Involvement of the ubiquitous Oct-1 transcription factor in hormonal induction of β-casein gene expression. Biochemical Journal 401, 57-64.

Dong, B., and Zhao, F.Q. (2007b). Expression of the Oct-2 transcription factor in mouse mammary gland and cloning and characterization of a novel Oct-2 isoform. Cell and Tissue Research. 328, 595-606.

Doppler, W., Welte, T., and Philipp, S. (1995). CCAAT/enhancer-binding protein isoforms and are expressed in mammary epithelial cells and bind to multiple sites in the-casein gene promoter. Journal of Biological Chemistry 270, 17962-17969.

Doppler, W., Windegger, M., Soratroi, C., Tomasi, J., Lechner, J., Rusconi, S., Cato, A.C., Almlöf, T., Liden, J., and Okret, S. (2001). Expression level-dependent contribution of glucocorticoid receptor domains for functional interaction with STAT5. Molecular and Cellular Biology 21, 3266-3279.

Edwards, G.M., Wilford, F.H., Liu, X., Hennighausen, L., Djiane, J., and Streuli, C.H. (1998). Regulation of mammary differentiation by extracellular matrix involves protein-tyrosine phosphatases. Journal of Biological Chemistry 273, 9495-9500.

Forsyth, I.A. (1996). The insulin-like growth factor and epidermal growth factor families in mammary cell growth in ruminants: action and interaction with hormones. Journal of Dairy Science 79, 1085-1096.

Forsyth, I.A., and Neville, M.C. (2009). Introduction: hormonal regulation of mammary development and milk protein gene expression at the whole animal and molecular levels. Journal of Mammary Gland Biology and Neoplasia 14, 317-319.

Furth, P.A., Nakles, R.E., Millman, S., Diaz-Cruz, E.S., and Cabrera, M.C. (2011). Signal transducer and activator of transcription 5 as a key signaling pathway in normal mammary gland developmental biology and breast cancer. Breast Cancer Research 13, 220-233.

Gertler, A., Ashkenazi, A., and Madar, Z. (1984). Binding sites of human growth hormone and ovine and bovine prolactins in the mammary gland and the liver of lactating dairy cow. Molecular and Cellular Endocrinology 34, 51-57.

Gil, G.-C., Velander, W.H., and Van Cott, K.E. (2008). Analysis of the N-glycans of recombinant human Factor IX purified from transgenic pig milk. Glycobiology 18, 526-539.

Girard, C., and Matte, J. (1998). Dietary supplements of folic acid during lactation: effects on the performance of dairy cows. Journal of Dairy Science 81, 1412-1419.

Girard, C., and Matte, J. (2005). Folic acid and vitamin B_{12} requirements of dairy cows: A concept to be revised. Livestock Production Science 98, 123-133.

Goettsch, C., Goettsch, W., Brux, M., Haschke, C., Brunssen, C., Muller, G., Bornstein, S.R., Duerrschmidt, N., Wagner, A.H., and Morawietz, H. (2011). Arterial flow reduces oxidative stress via an antioxidant response element and Oct-1 binding site within the NADPH oxidase 4 promoter in endothelial cells. Basic Research in Cardiology 106, 551-61.

Goffin, V., Binart, N., Touraine, P., and Kelly, P.A. (2002). Prolactin: the new biology of an old hormone. Annual Review of Physiology 64, 47-67.

Gouilleux, F., Wakao, H., Mundt, M., and Groner, B. (1994). Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. The EMBO Journal 13, 4361-4369.

Graulet, B., Matte, J., Desrochers, A., Doepel, L., Palin, M.-F., and Girard, C. (2007). Effects of dietary supplements of folic acid and vitamin B_{12} on metabolism of dairy cows in early lactation. Journal of Dairy Science 90, 3442-3455.

Grenfell, S.J., Latchman, D.S., and Thomas, N. (1996). Oct-1 and Oct-2 DNA-binding site specificity is regulated in vitro by different kinases. Biochemical Journal 315, 889-893.

Greppi, G., Roncada, P., and Fortin, R. (2008). 4 Protein components of goat's milk. Dairy Goats Feeding and Nutrition 71-94.

Groenen, M.A., and van der Poel, J.J. (1994). Regulation of expression of milk protein genes: a review. Livestock Production Science 38, 61-78.

Groner, B., Altiok, S., and Meier, V. (1994). Hormonal regulation of transcription factor activity in mammary epithelial cells. Molecular and Cellular Endocrinology 100, 109-114.

Guo, W., and Giancotti, F.G. (2004). Integrin signalling during tumour progression. Nature Reviews Molecular cell biology 5, 816-826.

Hakkak, R., Korourian, S., Ronis, M.J., Johnston, J.M., and Badger, T.M. (2001). Dietary whey protein protects against azoxymethane-induced colon tumors in male rats. Cancer Epidemiology Biomarkers & Prevention 10, 555-558.

Harris, J., Stanford, P.M., Sutherland, K., Oakes, S.R., Naylor, M.J., Robertson, F.G., Blazek, K.D., Kazlauskas, M., Hilton, H.N., and Wittlin, S. (2006). Socs2 and elf5 mediate prolactin-induced mammary gland development. Molecular Endocrinology 20, 1177-1187.

Haug, A., Hostmark, A.T., and Harstad, O.M. (2007). Bovine milk in human nutrition - a review. Lipids in Health and Disease 6, 25.

Hayashi, A., Nones, K., Roy, N., McNabb, W., Mackenzie, D., Pacheco, D., and McCoard, S. (2009). Initiation and elongation steps of mRNA translation are involved in the increase in milk protein yield caused by growth hormone administration during lactation. Journal of Dairy Science 92, 1889-1899.

Hayashi, A.A., and Proud, C.G. (2007). The rapid activation of protein synthesis by growth hormone requires signaling through mTOR. American Journal of Physiology-Endocrinology and Metabolism 292, E1647-E1655.

Hennighausen, L., and Robinson, G.W. (2008). Interpretation of cytokine signaling through the transcription factors STAT5A and STAT5B. Genes & Development 22, 711-721.

Herr, W., and Cleary, M.A. (1995). The POU domain: versatility in transcriptional regulation by a flexible two-in-one DNA-binding domain. Genes & Development 9, 1679-1693.

Hinkley, C., and Perry, M. (1992). Histone H2B gene transcription during Xenopus early development requires functional cooperation between proteins bound to the CCAAT and octamer motifs. Molecular and Cellular Biology 12, 4400-4411.

Hofmann, A., Kessler, B., Ewerling, S., Weppert, M., Vogg, B., Ludwig, H., Stojkovic, M., Boelhauve, M., Brem, G., and Wolf, E. (2003). Efficient transgenesis in farm animals by lentiviral vectors. EMBO Reports 4, 1054-1058.

Holmberg, C.I., Tran, S.E., Eriksson, J.E., and Sistonen, L. (2002). Multisite phosphorylation provides sophisticated regulation of transcription factors. Trends in Biochemical Sciences 27, 619-627.

Horne, D.S. (1998). Casein interactions: casting light on the black boxes, the structure in dairy products. International Dairy Journal 8, 171-177.

Houdebine, L. M. (2009). Production of pharmaceutical proteins by transgenic animals. Comparative Immunology, Microbiology & Infectious Diseases 32, 107-121.

Hove, K. (1978). Effects of hyperinsulinemia on lactose secretion and glucose uptake by the goat mammary gland. Acta Physiologica Scandinavica 104, 422-430.

Inman, C.K., Li, N., and Shore, P. (2005). Oct-1 counteracts autoinhibition of Runx2 DNA binding to form a novel Runx2/Oct-1 complex on the promoter of the mammary gland-specific gene β-casein. Molecular and Cellular Biology 25, 3182-3193.

Ismail, P.M., Li, J., DeMayo, F.J., O'malley, B.W., and Lydon, J.P. (2002). A novel LacZ reporter mouse reveals complex regulation of the progesterone receptor promoter during mammary gland development. Molecular Endocrinology 16, 2475-2489.

Jacob, K.K., Whittaker, J. and Stanley, F.M. (2002). Insulin receptor tyrosine kinase activity and phosphorylation of tyrosines 1162 and 1163 are required for insulin-increased prolactin gene expression. Molecular and Cellular Endocrinology 186, 7-16.

Jang, H., Kim, T.W., Yoon, S., Choi, S.-Y., Kang, T.-W., Kim, S.-Y., Kwon, Y.-W., Cho, E.-J., and Youn, H.-D. (2012). O-GlcNAc regulates pluripotency and reprogramming by directly acting on core components of the pluripotency network. Cell Stem Cell 11, 62-74.

Jenness, R. (1979). The composition of human milk. Seminars in Perinatology 3, 225-239.

Jhappan, C., Geiser, A., Kordon, E., Bagheri, D., Hennighausen, L., Roberts, A., Smith, G., and Merlino, G. (1993). Targeting expression of a transforming growth factor β 1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. The EMBO Journal 12, 1835-1845.

Jiang, H., Okamura, C.S., and Lucy, M.C. (1999). Isolation and characterization of a novel promoter for the bovine growth hormone receptor gene. Journal of Biological Chemistry 274, 7893-7900.

Johnson, M.L., Levy, J., Supowit, S., Yu-Lee, L., and Rosen, J. (1983). Tissue- and cell-specific casein gene expression. II. Relationship to site-specific DNA methylation. Journal of Biological Chemistry 258, 10805-10811.

Johnson, T.L., Fujimoto, B.A., Jiménez-Flores, R., and Peterson, D.G. (2010). Growth hormone alters lipid composition and increases the abundance of casein and lactalbumin mRNA in the MAC-T cell line. Journal of Dairy Research 77, 199-204.

Jolivet, G., Pantano, T., and Houdebine, L.M. (2005). Regulation by the extracellular matrix (ECM) of prolactin-induced αs1-casein gene expression in rabbit primary

mammary cells: Role of STAT5, C/EBP, and chromatin structure. Journal of Cellular Biochemistry 95, 313-327.
Kabotyanski, E.B., Huetter, M., Xian, W., Rijnkels, M., and Rosen, J.M. (2006). Integration of prolactin and glucocorticoid signaling at the β-casein promoter and enhancer by ordered recruitment of specific transcription factors and chromatin modifiers. Molecular Endocrinology 20, 2355-2368.
Kabotyanski, E.B., Rijnkels, M., Freeman-Zadrowski, C., Buser, A.C., Edwards, D.P., and Rosen, J.M. (2009). Lactogenic hormonal induction of long distance interactions between β-casein gene regulatory elements. Journal of Biological Chemistry 284, 22815-22824.
Kalluri, R. (2003). Basement membranes: structure, assembly and role in tumour angiogenesis. Nature Reviews Cancer 3, 422-433.
Kang, J., Gemberling, M., Nakamura, M., Whitby, F.G., Handa, H., Fairbrother, W.G., and Tantin, D. (2009a). A general mechanism for transcription regulation by Oct1 and Oct4 in response to genotoxic and oxidative stress. Genes & Development 23, 208-222.
Kang, J., Goodman, B., Zheng, Y., and Tantin, D. (2011). Dynamic regulation of Oct1 during mitosis by phosphorylation and ubiquitination. PloS One 6, e23872.
Kang, J., Shakya, A., and Tantin, D. (2009b). Stem cells, stress, metabolism and cancer: a drama in two Octs. Trends in Biochemical Sciences 34, 491-499.
Kang, J., Shen, Z., Lim, J.-M., Handa, H., Wells, L., and Tantin, D. (2013). Regulation of Oct1/Pou2f1 transcription activity by O-GlcNAcylation. The FASEB Journal 27, 2807-2817.
Karwacki-Neisius, V., Göke, J., Osorno, R., Halbritter, F., Ng, J.H., Weiße, A.Y., Wong, F.C., Gagliardi, A., Mullin, N.P., and Festuccia, N. (2013). Reduced Oct4 expression directs a robust pluripotent state with distinct signaling activity and increased enhancer occupancy by Oct4 and Nanog. Cell Stem Cell 12, 531-545.
Kazansky, A.V., Raught, B., Lindsey, S.M., Wang, Y., and Rosen, J.M. (1995). Regulation of mammary gland factor/Stat5a during mammary gland development. Molecular Endocrinology 9, 1598-1609.
Kemmler, W., Peterson, J.D., and Steiner, D.F. (1971). Studies on the conversion of proinsulin to insulin I. Conversion in vitro with trypsin and carboxypeptidase B. Journal of Biological Chemistry 246, 6786-6791.
Keys, J., and Djiane, J. (1988). Prolactin and growth hormone binding in mammary and liver tissue of lactating cows. Journal of Receptors and Signal Transduction 8, 731-750.
Kido, Y., Philippe, N., Schaeffer, A., and Accili, D. (2000). Genetic modifiers of the insulin resistance phenotype in mice. Diabetes 49, 589-596.
Kjeldsen, T. (2000). Yeast secretory expression of insulin precursors. Applied Microbiology and Biotechnology 54, 277-286.
Klose, R.J., and Bird, A.P. (2006). Genomic DNA methylation: the mark and its mediators. Trends in Biochemical Sciences 31, 89-97.

Komatsu, T., Rose, M.T., Kobayashi, T., Obara, Y., Hagino, A., Sakamoto, K., and Aso, H. (2005). Growth hormone acts on the synthesis and secretion of alpha-casein in bovine mammary epithelial cells. Journal of Dairy Research 72, 264-270.

Kong, Q., Wu, M., Huan, Y., Zhang, L., Liu, H., Bou, G., Luo, Y., Mu, Y., and Liu, Z. (2009). Transgene expression is associated with copy number and cytomegalovirus promoter methylation in transgenic pigs. PLoS One 4, e6679.

Kowenz-Leutz, E., and Leutz, A. (1999). AC/EBPβ isoform recruits the SWI/SNF complex to activate myeloid genes. Molecular Cell 4, 735-743.

Krissansen, G.W. (2007). Emerging health properties of whey proteins and their clinical implications. Journal of the American College of Nutrition 26, 713S-723S.

Kuhn, N. (1969). Progesterone withdrawal as the lactogenic trigger in the rat. Journal of Endocrinology 44, 39-54.

Kulski, J.K., Nicholas, K.R., Topper, Y.J., and Qasba, P. (1983). Essentiality of insulin and prolactin for accumulation of rat casein mRNAs. Biochemical and Biophysical Research Communications 116, 994-999.

Kuss, A., Gogol, J., and Geldermann, H. (2003). Associations of a polymorphic AP-2 binding site in the 5′-flanking region of the bovine β-lactoglobulin gene with milk proteins. Journal of Dairy Science 86, 2213-2218.

Laarveld, B., Christensen, D., and Brockman, R. (1981). The effect of insulin on net metabolism of glucose and amino acids by the bovine mammary gland. Endocrinology 108, 2217-2221.

Latchman, D. (1996). The Oct-2 transcription factor. The International Journal of Biochemistry & Cell Biology 28, 1081-1083.

Le Beyec, J., Xu, R., Lee, S.-Y., Nelson, C.M., Rizki, A., Alcaraz, J., and Bissell, M.J. (2007). Cell shape regulates global histone acetylation in human mammary epithelial cells. Experimental Cell Research 313, 3066-3075.

Lechner, J., Welte, T., and Doppler, W. (1997a). Mechanism of interaction between the glucocorticoid receptor and Stat5: role of DNA-binding. Immunobiology 198, 112-123.

Lechner, J., Welte, T., Tomasi, J.K., Bruno, P., Cairns, C., Gustafsson, J.-Å., and Doppler, W. (1997b). Promoter-dependent synergy between glucocorticoid receptor and Stat5 in the activation of β-casein gene transcription. Journal of Biological Chemistry 272, 20954-20960.

Lefèvre, C.M., Sharp, J.A., and Nicholas, K.R. (2010). Evolution of lactation: ancient origin and extreme adaptations of the lactation system. Annual Review of Genomics and Human Genetics 11, 219-238.

Lemay, D.G., Neville, M.C., Rudolph, M.C., Pollard, K.S., and German, J.B. (2007). Gene regulatory networks in lactation: identification of global principles using bioinformatics. BMC Systems Biology 1, 56.

Léonard, M., and Block, E. (1997). Effects on nutrient and hormonal profile of long-term infusions of glucose or insulin plus glucose in cows treated with recombinant bovine somatotropin before peak milk yield. Journal of Dairy Science 80, 127-143.

Li, S., and Rosen, J.M. (1994a). Distal regulatory elements required for rat whey acidic protein gene expression in transgenic mice. Journal of Biological Chemistry 269, 14235-14243.

Li, S., and Rosen, J.M. (1994b). Glucocorticoid regulation of rat whey acidic protein gene expression involves hormone-induced alterations of chromatin structure in the distal promoter region. Molecular Endocrinology 8, 1328-1335.

Li, S., and Rosen, J.M. (1995). Nuclear factor I and mammary gland factor (STAT5) play a critical role in regulating rat whey acidic protein gene expression in transgenic mice. Molecular and cellular biology 15, 2063-2070.

Li, X., and O'Malley, B.W. (2003). Unfolding the action of progesterone receptors. Journal of Biological Chemistry 278, 39261-39264.

Lin, C.Q., Dempsey, P.J., Coffey, R.J., and Bissell, M.J. (1995). Extracellular matrix regulates whey acidic protein gene expression by suppression of TGF-alpha in mouse mammary epithelial cells: studies in culture and in transgenic mice. The Journal of Cell Biology 129, 1115-1126.

Lin, Y., Yang, Y., Li, W., Chen, Q., Li, J., Pan, X., Zhou, L., Liu, C., Chen, C., and He, J. (2012). Reciprocal regulation of Akt and Oct4 promotes the self-renewal and survival of embryonal carcinoma cells. Molecular Cell 48, 627-640.

LIu, X., Robinson, G.W., Gouilleux, F., Groner, B., and Hennighausen, L. (1995). Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue. Proceedings of the National Academy of Sciences of the United States of America 92, 8831-8835.

Liu, X., Robinson, G.W., Wagner, K.-U., Garrett, L., Wynshaw-Boris, A., and Hennighausen, L. (1997). Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes & Development 11, 179-186.

Livak, K.J., Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402-408.

Lorincz, M.C., Dickerson, D.R., Schmitt, M., and Groudine, M. (2004). Intragenic DNA methylation alters chromatin structure and elongation efficiency in mammalian cells. Nature Structural & Molecular Biology 11, 1068-1075.

Lorincz, M.C., Schübeler, D., and Groudine, M. (2001). Methylation-mediated proviral silencing is associated with MeCP2 recruitment and localized histone H3 deacetylation. Molecular and cellular biology 21, 7913-7922.

Luhovyy, B.L., Akhavan, T., and Anderson, G.H. (2007). Whey proteins in the regulation of food intake and satiety. Journal of the American College of Nutrition 26, 704S-712S.

Luo, X.-H., Liao, E.-Y., and Su, X. (2002). Progesterone upregulates TGF-b isoforms (b1, b2, and b3) expression in normal human osteoblast-like cells. Calcified Tissue International 71, 335-343.

Mackle, T., Dwyer, D., Ingvartsen, K.L., Chouinard, P., Lynch, J., Barbano, D., and Bauman, D. (1999). Effects of insulin and amino acids on milk protein concentration and yield from dairy cows. Journal of Dairy Science 82, 1512-1524.

Mackle, T., Dwyer, D., Ingvartsen, K.L., Chouinard, P., Ross, D., and Bauman, D. (2000). Effects of insulin and postruminal supply of protein on use of amino acids by the mammary gland for milk protein synthesis. Journal of Dairy Science 83, 93-105.

Magné, S., Caron, S., Charon, M., Rouyez, M.C., and Dusanter-Fourt, I. (2003). STAT5 and Oct-1 form a stable complex that modulates cyclin D1 expression. Molecular and Cellular Biology 23, 8934-8945.

Malhas, A.N., Lee, C.F., and Vaux, D.J. (2009). Lamin B1 controls oxidative stress responses via Oct-1. Journal of Cell Biology 184, 45-55.

Manjarín, R., Steibel, J., Kirkwood, R., Taylor, N., and Trottier, N. (2012). Transcript abundance of hormone receptors, mammalian target of rapamycin pathway-related kinases, insulin-like growth factor I, and milk proteins in porcine mammary tissue. Journal of Animal Science 90, 221-230.

Martens, J.A., and Winston, F. (2003). Recent advances in understanding chromatin remodeling by Swi/Snf complexes. Current Opinion in Genetics & Development 13, 136-142.

McNally, J.G., Müller, W.G., Walker, D., Wolford, R., and Hager, G.L. (2000). The glucocorticoid receptor: rapid exchange with regulatory sites in living cells. Science 287, 1262-1265.

Meier, V.S., and Groner, B. (1994). The nuclear factor YY1 participates in repression of the β-casein gene promoter in mammary epithelial cells and is counteracted by mammary gland factor during lactogenic hormone induction. Molecular and Cellular Biology 14, 128-137.

Melo, E.O., Canavessi, A.M., Franco, M.M., and Rumpf, R. (2007). Animal transgenesis: state of the art and applications. Journal of Applied Genetics 48, 47-61.

Menzies, K.K., Lee, H.J., Lefèvre, C., Ormandy, C.J., Macmillan, K.L., and Nicholas, K.R. (2010). Insulin, a key regulator of hormone responsive milk protein synthesis during lactogenesis in murine mammary explants. Functional & Integrative Genomics 10, 87-95.

Menzies, K.K., Lefèvre, C., Macmillan, K.L., and Nicholas, K.R. (2009a). Insulin regulates milk protein synthesis at multiple levels in the bovine mammary gland. Functional & Integrative Genomics 9, 197-217.

Menzies, K.K., Lefèvre, C., Sharp, J.A., Macmillan, K.L., Sheehy, P.A., and Nicholas, K.R. (2009b). A novel approach identified the FOLR1 gene, a putative regulator of milk protein synthesis. Mammalian Genome 20, 498-503.

Mieth, M., Boehmer, F.-D., Ball, R., Groner, B., and Grosse, R. (1990). Transforming growth factor-β inhibits lactogenic hormone induction of β-casein expression in HC11 mouse mammary epithelial cells. Growth Factors 4, 9-15.

Millot, B., Fontaine, M., Thepot, D., and Devinoy, E. (2001). A distal region, hypersensitive to DNase I, plays a key role in regulating rabbit whey acidic protein gene expression. Biochemical Journal 359, 557-565.

Millot, B., Montoliu, L., Fontaine, M., Mata, T., and Devinoy, E. (2003). Hormone-induced modifications of the chromatin structure surrounding upstream regulatory regions conserved between the mouse and rabbit whey acidic protein genes. Biochemical Journal 372, 41-52.

Mink, S., Haenig, B., and Klempnauer, K.-H. (1997). Interaction and functional collaboration of p300 and C/EBPβ. Molecular and Cellular Biology 17, 6609-6617.

Monaco, M.H., Gronlund, D.E., Bleck, G.T., Hurley, W.L., Wheeler, M.B., and Donovan, S.M. (2005). Mammary specific transgenic over-expression of insulin-like growth factor-I (IGF-I) increases pig milk IGF-I and IGF binding proteins, with no effect on milk composition or yield. Transgenic Research 14, 761-773.

Monks, J. (2007). TGFβ as a potential mediator of progesterone action in the mammary gland of pregnancy. Journal of Mammary Gland Biology and Neoplasia 12, 249-257.

Montazer-Torbati, M.B., Hue-Beauvais, C., Droineau, S., Ballester, M., Coant, N., Aujean, E., Petitbarat, M., Rijnkels, M., and Devinoy, E. (2008). Epigenetic modifications and chromatin loop organization explain the different expression profiles of the Tbrg4, WAP and Ramp3 genes. Experimental Cell Research 314, 975-987.

Montesino, R., and Toledo, J.R. (2006). The mammary gland: bioreactor for the production of recombinant proteins. Biotecnología Aplicada 23, 279-286.

Muller, L.M., Gorter, K.J., Hak, E., Goudzwaard, W.L., Schellevis, F.G., Hoepelman, A.I., and Rutten, G.E. (2005). Increased risk of common infections in patients with type 1 and type 2 diabetes mellitus. Clinical Infectious Diseases 41, 281-288.

Murphy, S., Yoon, J., Gerster, T., and Roeder, R.G. (1992). Oct-1 and Oct-2 potentiate functional interactions of a transcription factor with the proximal sequence element of small nuclear RNA genes. Molecular and Cellular Biology 12, 3247-3261.

Myers, C.A., Schmidhauser, C., Mellentin-Michelotti, J., Fragoso, G., Roskelley, C.D., Casperson, G., Mossi, R., Pujuguet, P., Hager, G., and Bissell, M.J. (1998). Characterization of BCE-1, a transcriptional enhancer regulated by prolactin and extracellular matrix and modulated by the state of histone acetylation. Molecular and Cellular Biology 18, 2184-2195.

Nakanishi, T., Kuroiwa, A., Yamada, S., Isotani, A., Yamashita, A., Tairaka, A., Hayashi, T., Takagi, T., Ikawa, M., Matsuda, Y., Okabe, M. (2002). FISH analysis of 142 EGFP transgene integration sites into the mouse genome. Genomics 80, 564-574.

Nakshatri, H., Nakshatri, P., and Currie, R.A. (1995). Interaction of Oct-1 with TFIIB. Implications for a novel response elicited through the proximal octamer site of the lipoprotein lipase promoter. Journal of Biological Chemistry 270, 19613-19623.

Narlikar, G.J., Fan, H.-Y., and Kingston, R.E. (2002). Cooperation between complexes that regulate chromatin structure and transcription. Cell 108, 475-487.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Schöler, H., and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95, 379-391.

Nielsen, J. (2013). Production of biopharmaceutical proteins by yeast: advances through metabolic engineering. Bioengineered 4, 207-211.

Niemann, H., and Kues, W.A. (2007). Transgenic farm animals: an update. Reproduction, Fertility and Development 19, 762-770.

Nieto, L., Joseph, G., Stella, A., Henri, P., Burlet-Schiltz, O., Monsarrat, B., Clottes, E., and Erard, M. (2007). Differential effects of phosphorylation on DNA binding properties of N Oct-3 are dictated by protein/DNA complex structures. Journal of Molecular Biology 370, 687-700.

Nukumi, N., Iwamori, T., Kano, K., Naito, K., and Tojo, H. (2007). Whey acidic protein (WAP) regulates the proliferation of mammary epithelial cells by preventing serine protease from degrading laminin. Journal of Cellular Physiology 213, 793-800.

Oakes, S.R., Naylor, M.J., Asselin-Labat, M.-L., Blazek, K.D., Gardiner-Garden, M., Hilton, H.N., Kazlauskas, M., Pritchard, M.A., Chodosh, L.A., and Pfeffer, P.L. (2008). The Ets transcription factor Elf5 specifies mammary alveolar cell fate. Genes & development 22, 581-586.

Öztürk-Winder, F., Renner, M., Klein, D., Müller, M., Salmons, B., and Günzburg, W.H. (2002). The murine whey acidic protein promoter directs expression to human mammary tumors after retroviral transduction. Cancer Gene Therapy 9, 421-431.

Pampel, L.W., Boushaba, R., and Titchener-Hooker, N.J. (2008). A methodical approach to ultra-scale-down of process sequences: application to casein removal from the milk of transgenic animals. Biotechnology Progress 24, 192-201.

Patel, A.K., Singh, M., and Suryanarayana, V. (2014). Buffalo alpha S1-casein gene 5′-flanking region and its interspecies comparison. Journal of Applied Genetics 55, 75-87.

Pender, C., Goldfine, I.D., Manchem, V.P., Evans, J.L., Spevak, W.R., Shi, S., Rao, S., Bajjalieh, S., Maddux, B.A., and Youngren, J.F. (2002). Regulation of insulin receptor function by a small molecule insulin receptor activator. Journal of Biological Chemistry 277, 43565-43571.

Pevzner, V., Kraft, R., Kostka, S., and Lipp, M. (2000). Phosphorylation of Oct-2 at sites located in the POU domain induces differential down-regulation of Oct-2 DNA-binding ability. Biochemical Journal 347, 29-35.

Pfitzner, E., Jahne, R., Wissler, M., Stoecklin, E., and Groner, B. (1998). p300/CREB-binding protein enhances the prolactin-mediated transcriptional induction through direct interaction with the transactivation domain of Stat5, but does not participate in the Stat5-mediated suppression of the glucocorticoid response. Molecular Endocrinology 12, 1582-1593.

Picciano, M.F. (2001). Nutrient composition of human milk. Pediatric Clinics of North America 48, 53-67.

Pierce, D., Johnson, M.D., Matsui, Y., Robinson, S.D., Gold, L.I., Purchio, A.F., Daniel, C.W., Hogan, B., and Moses, H.L. (1993). Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-β 1. Genes & Development 7, 2308-2317.

Platenburg, G.J., Vollebregt, E.J., Karatzas, C.N., Kootwijk, E.P., De Boer, H.A., and Strijker, R. (1996). Mammary gland-specific hypomethylation of Hpa II sites flanking the bovine αS1-casein gene. Transgenic Research 5, 421-431.

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

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

Préfontaine, G.G., Lemieux, M.E., Giffin, W., Schild-Poulter, C., Pope, L., LaCasse, E., Walker, P., Hache, R.J.G. (1998). Recruitment of octamer transcription factors to DNA by glucocorticoid receptor. Molecular and Cellular Biology 18, 3416-3430.

Préfontaine, G.G., Walther, R., Giffin, W., Lemieux, M.E., Pope, L., and Haché, R.J. (1999). Selective binding of steroid hormone receptors to octamer transcription factors determines transcriptional synergism at the mouse mammary tumor virus promoter. Journal of Biological Chemistry 274, 26713-26719.

Prosser, C., Davis, S., Farr, V., Moore, L., and Gluckman, P. (1994). Effects of close-arterial (external pudic) infusion of insulin-like growth factor-II on milk yield and mammary blood flow in lactating goats. Journal of Endocrinology 142, 93-99.

Qian, X., Kraft, J., Ni, Y., and Zhao, F.-Q. (2014). Production of recombinant human proinsulin in the milk of transgenic mice. Scientific Reports.

Qian, X., and Zhao, F.-Q. (2013). Interactions of the ubiquitous octamer-binding transcription factor-1 with both the signal transducer and activator of transcription 5 and the glucocorticoid receptor mediate prolactin and glucocorticoid-induced β-casein gene expression in mammary epithelial cells. The International Journal of Biochemistry & Cell Biology 45, 724-735.

Qian, X., and Zhao, F.-Q. (2014). Collaborative interaction of Oct-2 with Oct-1 in transactivation of lactogenic hormones-induced β-casein gene expression in mammary epithelial cells. General and Comparative Endocrinology 204, 185-194.

Rajaram, R.D., and Brisken, C. (2012). Paracrine signaling by progesterone. Molecular and Cellular Endocrinology 357, 80-90.

Range, R., and Lepage, T. (2011). Maternal Oct1/2 is required for Nodal and Vg1/Univin expression during dorsal-ventral axis specification in the sea urchin embryo. Developmental Biology 357, 440-449.

Raught, B., Khursheed, B., Kazansky, A., and Rosen, J. (1994). YY1 represses β-casein gene expression by preventing the formation of a lactation-associated complex. Molecular and Cellular Biology 14, 1752-1763.

Raught, B., Liao, W.-L., and Rosen, J.M. (1995). Developmentally and hormonally regulated CCAAT/enhancer-binding protein isoforms influence β-casein gene expression. Molecular Endocrinology 9, 1223-1232.

Reichmann, E., Ball, R., Groner, B., and Friis, R.R. (1989). New mammary epithelial and fibroblastic cell clones in coculture form structures competent to differentiate functionally. The Journal of Cell Biology 108, 1127-1138.

Reményi, A., Tomilin, A., Pohl, E., Lins, K., Philippsen, A., Reinbold, R., Schöler, H.R., and Wilmanns, M. (2001). Differential dimer activities of the transcription factor Oct-1 by DNA-induced interface swapping. Molecular Cell 8, 569-580.

Ren, X., Siegel, R., Kim, U., and Roeder, R.G. (2011). Direct interactions of OCA-B and TFII-I regulate immunoglobulin heavy-chain gene transcription by facilitating enhancer-promoter communication. Molecular Cell 42, 342-355.

Rhoads, R.E., and Grudzien-Nogalska, E. (2007). Translational regulation of milk protein synthesis at secretory activation. Journal of Mammary Gland Biology and Neoplasia 12, 283-292.

Riggs, A. D. (1981). Bacterial production of human insulin. Diabetes Care 4, 64-68.

Rijnkels, M., Elnitski, L., Miller, W., and Rosen, J.M. (2003). Multispecies comparative analysis of a mammalian-specific genomic domain encoding secretory proteins. Genomics 82, 417-432.

Rijnkels, M., Freeman-Zadrowski, C., Hernandez, J., Potluri, V., Wang, L., Li, W., and Lemay, D.G. (2013). Epigenetic modifications unlock the milk protein gene loci during mouse mammary gland development and differentiation. PloS One 8, e53270.

Rijnkels, M., Kabotyanski, E., Montazer-Torbati, M.B., Beauvais, C.H., Vassetzky, Y., Rosen, J.M., and Devinoy, E. (2010). The epigenetic landscape of mammary gland development and functional differentiation. Journal of Mammary Gland Biology and Neoplasia 15, 85-100.

Rijnkels, M., Kabotyanski, E., Shore, A., and Rosen, J.M. (2012). The chromatin landscape of the casein gene locus. Hormone Molecular Biology and Clinical Investigation 10, 201-205.

Roberts, S.B., Segil, N., Heintz, N. (1991). Differential phosphorylation of the transcription factor Oct1 during the cell cycle. Science 253, 1022-1026.

Robinson, A.R., Kwek, S.S., Hagemeier, S.R., Wille, C.K., Kenney, S.C. (2011). Cellular transcription factor Oct-1 interacts with the Epstein-Barr virus BRLF1 protein to promote disruption of viral latency. Journal of Virology 85, 8940-8953.

Robinson, G.W., Johnson, P.F., Hennighausen, L., and Sterneck, E. (1998). The C/EBPβ transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. Genes & Development 12, 1907-1916.

Robinson, G.W., McKnight, R.A., Smith, G.H., and Hennighausen, L. (1995). Mammary epithelial cells undergo secretory differentiation in cycling virgins but require pregnancy for the establishment of terminal differentiation. Development 121, 2079-2090.

Robinson, S., Silberstein, G., Roberts, A., Flanders, K., and Daniel, C. (1991). Regulated expression and growth inhibitory effects of transforming growth factor-β isoforms in mouse mammary gland development. Development 113, 867-878.

Robinson, S.D., Roberts, A.B., and Daniel, C.W. (1993). TGF β suppresses casein synthesis in mouse mammary explants and may play a role in controlling milk levels during pregnancy. The Journal of Cell Biology 120, 245-251.

Rosen, J.M., Wyszomierski, S.L., and Hadsell, D. (1999). Regulation of milk protein gene expression. Annual Review of Nutrition 19, 407-436.

Rosenstock, J., Dailey, G., Massi-Benedetti, M., Fritsche, A., Lin, Z. and Salzman, A. (2005). Reduced Hypoglycemia Risk With Insulin Glargine A meta-analysis

comparing insulin glargine with human NPH insulin in type 2 diabetes. Diabetes Care 28, 950-955.
Roskelley, C., Desprez, P., and Bissell, M. (1994). Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction. Proceedings of the National Academy of Sciences of the United States of America 91, 12378-12382.
Saito, H., and Oka, T. (1996). Hormonally regulated double-and single-stranded DNA-binding complexes involved in mouse-casein gene transcription. Journal of Biological Chemistry 271, 8911-8918.
Salmon Jr, W.D., and Daughaday, W.H. (1957). A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. The Journal of Laboratory and Clinical Medicine 49, 825-836.
Saltiel, A.R., and Pessin, J.E. (2002). Insulin signaling pathways in time and space. Trends in Cell Biology 12, 65-71.
Samiec, M., and Skrzyszowska, M. (2011). Transgenic mammalian species, generated by somatic cell cloning, in biomedicine, biopharmaceutical industry and human nutrition/dietetics-recent achievements. Polish Journal of Veterinary Sciences 14, 317-328.
Scharfen, E., Mills, D., and Maga, E. (2007). Use of human lysozyme transgenic goat milk in cheese making: effects on lactic acid bacteria performance. Journal of Dairy Science 90, 4084-4091.
Schild-Poulter, C., Shih, A., Tantin, D., Yarymowich, N.C., Soubeyrand, S., Sharp, P.A., Haché, R.J. (2007). DNA-PK phosphorylation sites on Oct-1 promote cell survival following DNA damage. Oncogene 26, 3980-3988.
Schmidhauser, C., Bissell, M.J., Myers, C.A., and Casperson, G.F. (1990). Extracellular matrix and hormones transcriptionally regulate bovine β-casein 5'sequences in stably transfected mouse mammary cells. Proceedings of the National Academy of Sciences of the United States 87, 9118-9122.
Schmidhauser, C., Casperson, G.F., Myers, C., Sanzo, K., Bolten, S., and Bissell, M. (1992). A novel transcriptional enhancer is involved in the prolactin-and extracellular matrix-dependent regulation of β-casein gene expression. Molecular Biology of the Cell 3, 699-709.
Schmidt, F. (2004). Recombinant expression systems in the pharmaceutical industry. Applied Microbiology and Biotechnology 65, 363-372.
Schmitt-Ney, M., Doppler, W., Ball, R.K., and Groner, B. (1991). β-Casein gene promoter activity is regulated by the hormone-mediated relief of transcriptional repression and a mammary-gland-specific nuclear factor. Molecular and Cellular Biology 11, 3745-3755.
Schreiber, E., Matthias, P., Müller, M.M., Schaffner, W. (1989). Rapid detection of octamer binding proteins with ‘mini extracts’, prepared from a small number of cells. Nucleic Acids Research 17, 6419.
Schwartz, C., Beck, K., Mink, S., Schmolke, M., Budde, B., Wenning, D., and Klempnauer, K.H. (2003). Recruitment of p300 by C/EBPβ triggers

phosphorylation of p300 and modulates coactivator activity. The EMBO Journal 22, 882-892.

Seagroves, T.N., Krnacik, S., Raught, B., Gay, J., Burgess-Beusse, B., Darlington, G.J., and Rosen, J.M. (1998). C/EBPβ, but not C/EBPα, is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland. Genes & Development 12, 1917-1928.

Sebastiano, V., Dalvai, M., Gentile, L., Schubart, K., Sutter, J., Wu, G.M., Tapia, N., Esch, D., Ju, J.Y., Hübner, K., Bravo, M.J., Schöler, H.R., Cavaleri, F., and Matthias, P. (2010) Oct1 regulates trophoblast development during early mouse embryogenesis. Development 137, 3551-3560.

Segil, N., Roberts, S.B., and Heintz, N. (1991). Mitotic phosphorylation of the Oct-1 homeodomain and regulation of Oct-1 DNA binding activity. Science 254, 1814-1816.

Seidel Jr, G.E. (2009). Sperm sexing technology—The transition to commercial application: An introduction to the symposium "Update on sexing mammalian sperm". Theriogenology 71, 1-3.

Selo, I., Clement, G., Bernard, H., Chatel, J., Creminon, C., Peltre, G., and Wal, J. (1999). Allergy to bovine b-lactoglobulin: specificity of human IgE to tryptic peptides. Clinical and Experimental Allergy 29, 1055-1063.

Serova, I.A., Dvoryanchikov, G.A., Andreeva, L.E., Burkov, I.A., Dias, L.P., Battulin, N.R., Smirnov, A.V., and Serov, O.L. (2012). A 3,387 bp 5'-flanking sequence of the goat alpha-S1-casein gene provides correct tissue-specific expression of human granulocyte colony-stimulating factor (hG-CSF) in the mammary gland of transgenic mice. Transgenic Research 21, 485-498.

Shakya, A., Cooksey, R., Cox, J.E., Wang, V., McClain, D.A., and Tantin, D. (2009). Oct1 loss of function induces a coordinate metabolic shift that opposes tumorigenicity. Nature Cell Biology11, 320-327.

Shakya, A., Kang, J., Chumley, J., Williams, M.A., and Tantin, D. (2011). Oct1 is a switchable, bipotential stabilizer of repressed and inducible transcriptional states. Journal of Biological Chemistry 286, 450-459.

Singh, K., Erdman, R.A., Swanson, K.M., Molenaar, A.J., Maqbool, N.J., Wheeler, T.T., Arias, J.A., Quinn-Walsh, E.C., and Stelwagen, K. (2010). Epigenetic regulation of milk production in dairy cows. Journal of Mammary Gland Biology and Neoplasia 15, 101-112.

Singh, K., Molenaar, A., Swanson, K., Gudex, B., Arias, J., Erdman, R., and Stelwagen, K. (2012). Epigenetics: a possible role in acute and transgenerational regulation of dairy cow milk production. Animal 6, 375-381.

Singh, K., Swanson, K., Couldrey, C., Seyfert, H., and Stelwagen, K. (2009). DNA methylation events associated with the suppression of milk protein gene expression during involution of the bovine mammary gland. Proceedings of the New Zealand Society of Animal Production, 69, 57-59.

Sive, H.L., and Roeder, R.G. (1986). Interaction of a common factor with conserved promoter and enhancer sequences in histone H2B, immunoglobulin, and U2 small

nuclear RNA (snRNA) genes. Proceedings of National Academy of Sciences of the United States of America 83, 6382-6386.
Sinowatz, F., Schams, D., Kolle, S., Plath, A., Lincoln, D., and Waters, M. (2000). Cellular localisation of GH receptor in the bovine mammary gland during mammogenesis, lactation and involution. Journal of Endocrinology 166, 503-510.
Skene, P.J., Illingworth, R.S., Webb, S., Kerr, A.R., James, K.D., Turner, D.J., Andrews, R., and Bird, A.P. (2010). Neuronal MeCP2 is expressed at near histone-octamer levels and globally alters the chromatin state. Molecular Cell 37, 457-468.
Smith, K., and Spadafora, C. (2005). Sperm-mediated gene transfer: Applications and implications. Bioessays 27, 551-562.
Spencer, V.A., Xu, R., and Bissell, M.J. (2007). Extracellular matrix, nuclear and chromatin structure, and gene expression in normal tissues and malignant tumors: a work in progress. Advances in Cancer Research 97, 275-294.
Steiner, D. (2008). The biosynthesis of insulin. Pancreatic Beta Cell in Health and Disease 31-49.
Stöcklin, E., Wissler, M., Gouilleux, F., and Groner, B. (1996). Functional interactions between Stat5 and the glucocorticoid receptor. Nature 383, 726-728.
Stoecklin, E., Wissler, M., Moriggl, R., and Groner, B. (1997). Specific DNA binding of Stat5, but not of glucocorticoid receptor, is required for their functional cooperation in the regulation of gene transcription. Molecular and Cellular Biology 17, 6708-6716.
Streuli, C.H., Edwards, G.M., Delcommenne, M., Whitelaw, C.B.A., Burdon, T.G., Schindler, C., and Watson, C.J. (1995a). Stat5 as a target for regulation by extracellular matrix. Journal of Biological Chemistry 270, 21639-21644.
Streuli, C.H., Schmidhauser, C., Bailey, N., Yurchenco, P., Skubitz, A., Roskelley, C., and Bissell, M.J. (1995b). Laminin mediates tissue-specific gene expression in mammary epithelia. The Journal of Cell Biology 129, 591-603.
Ström, A.C., Forsberg, M., Lillhager, P., and Westin, G. (1996). The transcription factors Sp1 and Oct-1 interact physically to regulate human U2 snRNA gene expression. Nucleic Acids Research 24, 1981-1986.
Su, H.-Y., and Cheng, W. (2004). Increased milk yield in transgenic mice expressing insulin-like growth factor 1. Animal Biotechnology 15, 9-19.
Sudlow, A., Wilde, C., and Burgoyne, R. (1994). Transforming growth factor-β 1 inhibits casein secretion from differentiating mammary-gland explants but not from lactating mammary cells. Biochemical Journal 304, 333-336.
Talbot, D., Collis, P., Antoniou, M., Vidal, M., Grosveld, F., and Greaves, D.R. (1989). A dominant control region from the human β-globin locus conferring integration site-independent gene expression. Nature 338, 352-355.
Tanaka, M., and Herr, W. (1990). Differential transcriptional activation by Oct-1 and Oct-2: interdependent activation domains induce Oct-2 phosphorylation. Cell 60, 375-386.
Tesseraud, S., Grizard, J., Makarski, B., Debras, E., Bayle, G., and Champredon, C. (1992). Effect of insulin in conjunction with glucose, amino acids and potassium

on net metabolism of glucose and amino acids in the goat mammary gland. Journal of Dairy Research 59, 135-149.

Thim, L., Hansen, M.T., Norris, K., Hoegh, I., Boel, E., Forstrom, J., Ammerer, G., and Fiil, N.P. (1986). Secretion and processing of insulin precursors in yeast. Proceeding of National Academy of Sciences of the United States of America 83, 6766-6770.

Thomas, R.S., Ng, A.N., Zhou, J., Tymms, M.J., Doppler, W., and Kola, I. (2002). The Elf group of Ets-related transcription factors. Biology of the Mammary Gland 123-128.

Thompson, M.D., and Nakhasi, H.L. (1985). Methylation and expression of rat κ-casein gene in normal and neoplastic rat mammary gland. Cancer Research 45, 1291-1295.

Thomson, A.J., Marques, M.M., and McWhir, J. (2003). Gene targeting in livestock. Reproduction in domestic ruminants V. Proceedings of the Sixth International Symposium on Reproduction in Domestic Ruminant 495-508.

Thomson, J.P., Skene, P.J., Selfridge, J., Clouaire, T., Guy, J., Webb, S., Kerr, A.R., Deaton, A., Andrews, R., and James, K.D. (2010). CpG islands influence chromatin structure via the CpG-binding protein Cfp1. Nature 464, 1082-1086.

Toerien, C., and Cant, J. (2007). Abundance and phosphorylation state of translation initiation factors in mammary glands of lactating and nonlactating dairy cows. Journal of Dairy Science 90, 2726-2734.

Tolhuis, B., Palstra, R.-J., Splinter, E., Grosveld, F., and de Laat, W. (2002). Looping and interaction between hypersensitive sites in the active β-globin locus. Molecular Cell 10, 1453-1465.

Tolkunova, E., Malashicheva, A., Parfenov, V.N., Sustmann, C., Grosschedl, R., and Tomilin, A. (2007). PIAS proteins as repressors of Oct4 function. Journal of Molecular Biology 374, 1200-1212.

Topper, Y.J., and Freeman, C.S. (1980). Multiple hormone interactions in the developmental biology of the mammary gland. Physiological Reviews 60, 1049-1106.

Turner, R. (1998). Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). Lancet 352, 837-853.

Udy, G.B., Towers, R.P., Snell, R.G., Wilkins, R.J., Park, S.-H., Ram, P.A., Waxman, D.J., and Davey, H.W. (1997). Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. Proceedings of the National Academy of Sciences of the United States of America 94, 7239-7244.

Vanselow, J., Yang, W., Herrmann, J., Zerbe, H., Schuberth, H.-J., Petzl, W., Tomek, W., and Seyfert, H.-M. (2006). DNA-remethylation around a STAT5-binding enhancer in the αS1-casein promoter is associated with abrupt shutdown of αS1-casein synthesis during acute mastitis. Journal of Molecular Endocrinology 37, 463-477.

Verrijzer, C.P., van Oosterhout, J., and van der Vliet, P.C., (1992). The Oct-1 POU domain mediates interactions between Oct-1 and other POU proteins. Molecular and Cellular Biology 12, 542-551.

Virgo, B.B., and Bellward, G.D. (1974). Serum progesterone levels in the pregnant and postpartum laboratory mouse. Endocrinology 95, 1486-1490.

Vonderhaar, B.K., and Ziska, S.E. (1989). Hormonal regulation of milk protein gene expression. Annual Review of Physiology 51, 641-652.

Wakao, H., Gouilleux, F., and Groner, B. (1994). Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. The EMBO Journal 13, 2182-2191.

Wakao, H., Schmitt-Ney, M., and Groner, B. (1992). Mammary gland-specific nuclear factor is present in lactating rodent and bovine mammary tissue and composed of a single polypeptide of 89 kDa. Journal of Biological Chemistry 267, 16365-16370.

Wall, R., Kerr, D., and Bondioli, K. (1997). Transgenic dairy cattle: genetic engineering on a large scale. Journal of Dairy Science 80, 2213-2224.

Wang, G.G., Allis, C.D., and Chi, P. (2007). Chromatin remodeling and cancer, Part II: ATP-dependent chromatin remodeling. Trends in Molecular Medicine 13, 373-380.

Wang, P., Wang, Q., Sun, J., Wu, J., Li, H., Zhang, N., Huang, Y., Su, B., Li, R.K., Liu, L., Zhang, Y., Elsholtz, H.P., Hu, J., Gaisano, H.Y., and Jin, T. (2009). POU homeodomain protein Oct-1 functions as a sensor for cyclic AMP. Journal of Biological Chemistry 284, 26456-65.

Wang, V.E., Tantin, D., and Chen, J. (2004). B cell development and immunoglobulin transcription in Oct-1-deficient mice. Proceedings of the National Academy of Sciences of the United States of America 101, 2005-2010.

Wang, Y., Zhao, S., Bai, L., Fan, J., and Liu, E. (2013). Expression systems and species used for transgenic animal bioreactors. BioMed Research International 580463.

Wartmann, M., Cella, N., Hofer, P., Groner, B., Liu, X., Hennighausen, L., and Hynes, N.E. (1996). Lactogenic hormone activation of Stat5 and transcription of the β-casein gene in mammary epithelial cells is independent of p42 ERK2 mitogen-activated protein kinase activity. Journal of Biological Chemistry 271, 31863-31868.

Waston, C.J., Gordon, K.E., Robertson, M., and Clark, A.J. (1991). Interaction of DNA-binding proteins with a milk protein gene promoter in vitro: identification of a mammary gland-specific factor. Nucleic Acids Research 19, 6603-6610.

Watkin, H., and Streuli, C.H. (2002). Adenoviral-mediated gene transfer in two-dimensional and three-dimensional cultures of mammary epithelial cells. Methods in Cell Biology 69, 403-423.

Webster, D.M., Teo, C.F., Sun, Y., Wloga, D., Gay, S., Klonowski, K.D., Wells, L., and Dougan, S.T. (2009). O-GlcNAc modifications regulate cell survival and epiboly during zebrafish development. BMC Developmental Biology 9, 28.

Wei, F., Schöler, H.R., and Atchison, M.L. (2007). Sumoylation of Oct4 enhances its stability, DNA binding, and transactivation. Journal of Biological Chemistry 282, 21551-21560.

Welte, T., Philipp, S., Cairns, C., Gustafsson, J.-Å., and Doppler, W. (1993). Glucocorticoid receptor binding sites in the promoter region of milk protein genes. The Journal of Steroid Biochemistry and Molecular Biology 47, 75-81.

Whitelaw, C., Harris, S., McClenaghan, M., Simons, J., and Clark, A. (1992). Position-independent expression of the ovine β-lactoglobulin gene in transgenic mice. Biochemical Journal 286, 31-39.

Whitelaw, C.B.A. (2000). Nucleosome organisation of the β-lactoglobulin gene. Transcription complex formation. Advances in Experimental Medicine and Biology 480, 147-153.

Whitelaw, C.B.A., and Webster, J. (1998). Temporal profiles of appearance of DNase I hypersensitive sites associated with the ovine β-lactoglobulin gene differ in sheep and transgenic mice. Molecular and General Genetics MGG 257, 649-654.

Whitelaw, C.B.A. (1996). Hormonal influences on β-lactoglobulin transgene expression inferred from chromatin structure. Biochemical and Biophysical Research communications 224, 121-125.

Whitmarsh, A., and Davis, R. (2000). Regulation of transcription factor function by phosphorylation. Cellular and Molecular Life Sciences CMLS 57, 1172-1183.

Winklehner-Jennewein, P., Geymayer, S., Lechner, J., Welte, T., Hansson, L., Geley, S., and Doppler, W. (1998). A distal enhancer region in the human β-casein gene mediates the response to prolactin and glucocorticoid hormones. Gene 217, 127-139.

Wolf, E., Jehle, P.M., Weber, M.M., Sauerwein, H., Daxenberger, A., Breier, B.H., Besenfelder, U., Frenyo, L., and Brem, G. (1997). Human insulin-like growth factor I (IGF-I) produced in the mammary glands of transgenic rabbits: yield, receptor binding, mitogenic activity, and effects on IGF-binding proteins. Endocrinology 138, 307-313.

Wyszomierski, S.L., and Rosen, J.M. (2001). Cooperative Effects of STAT5 (Signal Transducer and Activator of Transcription 5) and C/EBP β (CCAAT/Enhancer-Binding Protein-β) onβ-Casein Gene Transcription Are Mediated by the Glucocorticoid Receptor. Molecular Endocrinology 15, 228-240.

Wyszomierski, S.L., Yeh, J., and Rosen, J.M. (1999). Glucocorticoid receptor/signal transducer and activator of transcription 5 (STAT5) interactions enhance STAT5 activation by prolonging STAT5 DNA binding and tyrosine phosphorylation. Molecular Endocrinology 13, 330-343.

Xu, H.M., Liao, B., Zhang, Q.J., Wang, B.B., Li, H., Zhong, X.M., Sheng, H.Z., Zhao, Y.X., Zhao, Y.M., and Jin, Y. (2004). Wwp2, an E3 ubiquitin ligase that targets transcription factor Oct-4 for ubiquitination. Journal of Biological Chemistry 279, 23495-23503.

Xu, R., Nelson, C.M., Muschler, J.L., Veiseh, M., Vonderhaar, B.K., and Bissell, M.J. (2009). Sustained activation of STAT5 is essential for chromatin remodeling and

maintenance of mammary-specific function. The Journal of Cell Biology 184, 57-66.

Xu, R., Spencer, V.A., and Bissell, M.J. (2007). Extracellular matrix-regulated gene expression requires cooperation of SWI/SNF and transcription factors. Journal of Biological Chemistry 282, 14992-14999.

Yang, W.-M., Inouye, C., Zeng, Y., Bearss, D., and Seto, E. (1996). Transcriptional repression by YY1 is mediated by interaction with a mammalian homolog of the yeast global regulator RPD3. Proceedings of the National Academy of Sciences of the United States of America 93, 12845-12850.

Yang, W.-M., Yao, Y.-L., Sun, J.-M., Davie, J.R., and Seto, E. (1997). Isolation and characterization of cDNAs corresponding to an additional member of the human histone deacetylase gene family. Journal of Biological Chemistry 272, 28001-28007.

Yang, J., Zhao, B., Baracos, V., and Kennelly, J. (2005). Effects of bovine somatotropin on β-casein mRNA levels in mammary tissue of lactating cows. Journal of Dairy Science 88, 2806-2812.

Yeap, L.-S., Hayashi, K., and Surani, M.A. (2009). ERG-associated protein with SET domain (ESET)-Oct4 interaction regulates pluripotency and represses the trophectoderm lineage. Epigenetics & Chromatin 2, 12.

Yonekura, S., Sakamoto, K., Komatsu, T., Hagino, A., Katoh, K., and Obara, Y. (2006). Growth hormone and lactogenic hormones can reduce the leptin mRNA expression in bovine mammary epithelial cells. Domestic Animal Endocrinology 31, 88-96.

Yoshimura M, and Oka T. (1990). Transfection of β-casein chimeric gene and hormonal induction of its expression in primary murine mammary epithelial cells. Proceedings of National Academy of Sciences of the United States of America 87, 3670-3674.

Yuan, P., Han, J., Guo, G., Orlov, Y.L., Huss, M., Loh, Y.-H., Yaw, L.-P., Robson, P., Lim, B., and Ng, H.-H. (2009). Eset partners with Oct4 to restrict extraembryonic trophoblast lineage potential in embryonic stem cells. Genes & Development 23, 2507-2520.

Yu-Lee LY. (2002). Prolactin modulation of immune and inflammatory responses. Recent Progress in Hormone Research 57, 435-455.

Zarzynska, J., Gajewska, M., and Motyl, T. (2005). Effects of hormones and growth factors on TGF-β1 expression in bovine mammary epithelial cells. Journal of Dairy Research 72, 39-48.

Zhang, Z., Liao, B., Xu, M., and Jin, Y. (2007). Post-translational modification of POU domain transcription factor Oct-4 by SUMO-1. The FASEB Journal 21, 3042-3051.

Zhao, F.Q. (2013). Octamer-binding transcription factors: genomics and functions. Frontier Bioscience 18, 1051-1071.

Zhao, F.-Q., Adachi, K., and Oka, T. (2002). Involvement of Oct-1 in transcriptional regulation of β-casein gene expression in mouse mammary gland. Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression 1577, 27-37.

Zhao F-Q, Zheng Y, Dong B, and Oka T. (2004). Cloning, genomic organization, expression, and effect on β-casein promoter activity of a novel isoform of the mouse Oct-1 transcription factor. Gene 326, 175-187.

Zhou, J., Chehab, R., Tkalcevic, J., Naylor, M.J., Harris, J., Wilson, T.J., Tsao, S., Tellis, I., Zavarsek, S., and Xu, D. (2005). Elf5 is essential for early embryogenesis and mammary gland development during pregnancy and lactation. The EMBO Journal 24, 635-644.

Zhou, Y., Akers, R., and Jiang, H. (2008). Growth hormone can induce expression of four major milk protein genes in transfected MAC-T cells. Journal of Dairy Science 91, 100-108.

Zhu, T., Goh, E.L., Graichen, R., Ling, L., and Lobie, P.E. (2001). Signal transduction via the growth hormone receptor. Cellular Signalling 13, 599-616.

Zittle, C.A. (1961). Stabilization of calcium-sensitive (α_s) casein by kappa-casein: effect of chymotrypsin and heat on kappa-casein. Journal of Dairy Science 44, 2101-2103.

Zoubiane, G.S., Valentijn, A., Lowe, E.T., Akhtar, N., Bagley, S., Gilmore, A.P., and Streuli, C.H. (2004). A role for the cytoskeleton in prolactin-dependent mammary epithelial cell differentiation. Journal of Cell Science 117, 271-280.

Zwilling, S., Annweiler, A., and Wirth, T. (1994). The POU domains of the Oct1 and Oct2 transcription factors mediate specific interaction with TBP. Nucleic Acids Research 22, 1655-1662.