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# 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

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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.



## 1. 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

*Abbreviations:* DTT, dithiothreitol; EGF, epidermal growth factor; EMSA, electrophoresis mobility shift assay; GAPDH, glyceraldehydes-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.

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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.

## 2. Materials and methods

### 2.1. 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.

### 2.2. 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.

### 2.3. 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. 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.

### 2.4. 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).

### 2.5. 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).

### 2.6. 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.

### 2.7. 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.

### 2.8. Site-directed mutagenesis of STAT5

Site-directed mutagenesis was performed using the GeneArt® Site-Directed Mutagenesis System (Invitrogen). The leucine (Leu) 767 residue of mouse 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′-CTGTCCATGGGCCGGCGTGGAAGTTC-TTCCACGTGCC-3′ (the mutations are underlined).

### 2.9. 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).

### 2.10. 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.

## 3. Results

### 3.1. 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. 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. 1B) but increased dramatically at 30 min of HP treatment followed by an appreciable decrease at 24 h (Fig. 1B). Normal rabbit IgG, the negative control, was unable to immunoprecipitate Oct-1–DNA complexes (Fig. 1B). To quantify the effects of HP on Oct-1 binding activity, chromatin DNA obtained via ChIP was analyzed using

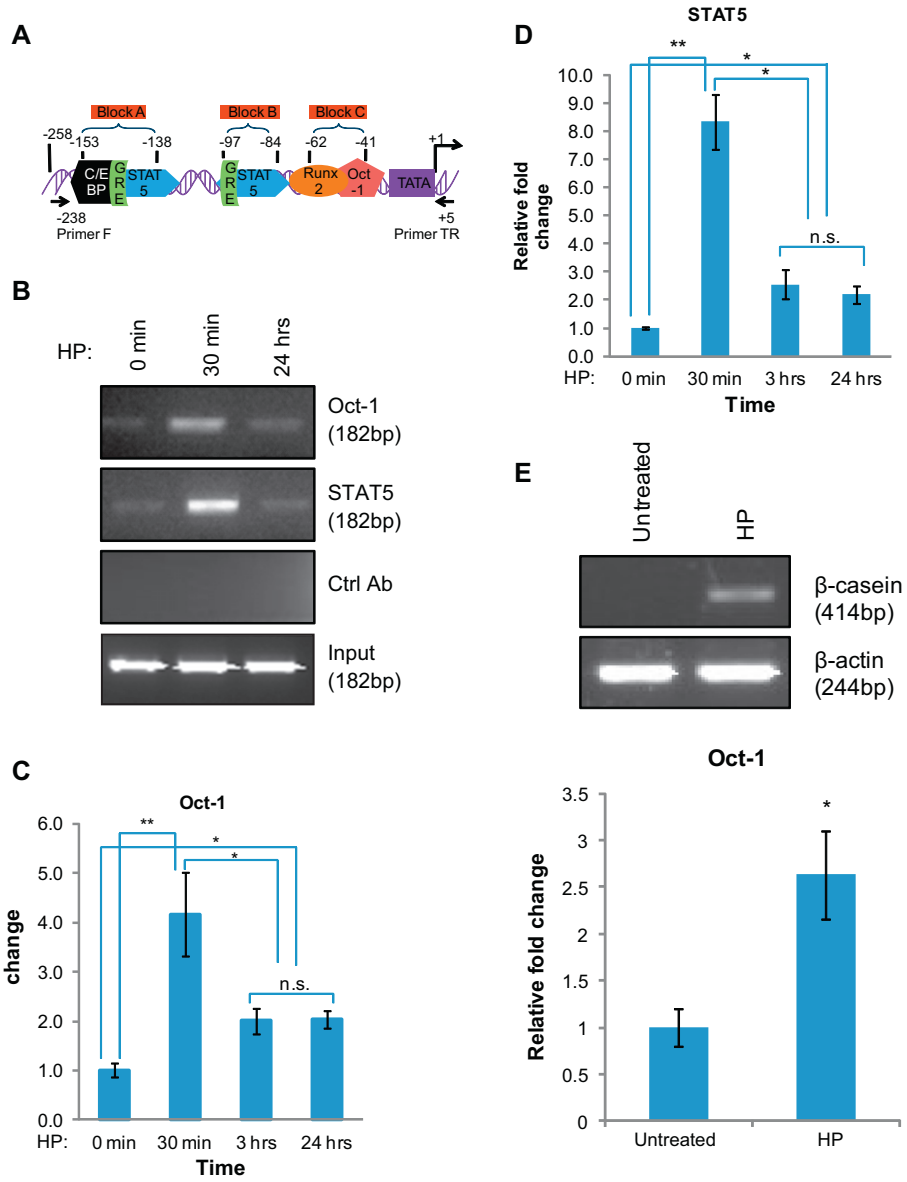



**Fig. 1.** Lactogenic hormones induce the binding of Oct-1 to the β-casein gene promoter in vitro. (A) A schematic representation of the putative transcription factor-binding sites in the murine β-casein gene proximal promoter. The primers used for the qChIP assay (Primer F and Primer TR) are indicated. 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 h. 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. $^{*}P < 0.05$.

qPCR. As shown in Fig. 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. 1D) (Kabotyanski et al., 2006). HP-induced Oct-1 binding activity was also confirmed in primary MEC cultures (Fig. 1E, bottom). The HP-induced β-casein mRNA expression levels observed in primary cells were verified using RT-PCR (Fig. 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 binding dynamic profile similar to that of STAT5.

### 3.2. Oct-1 servers 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)

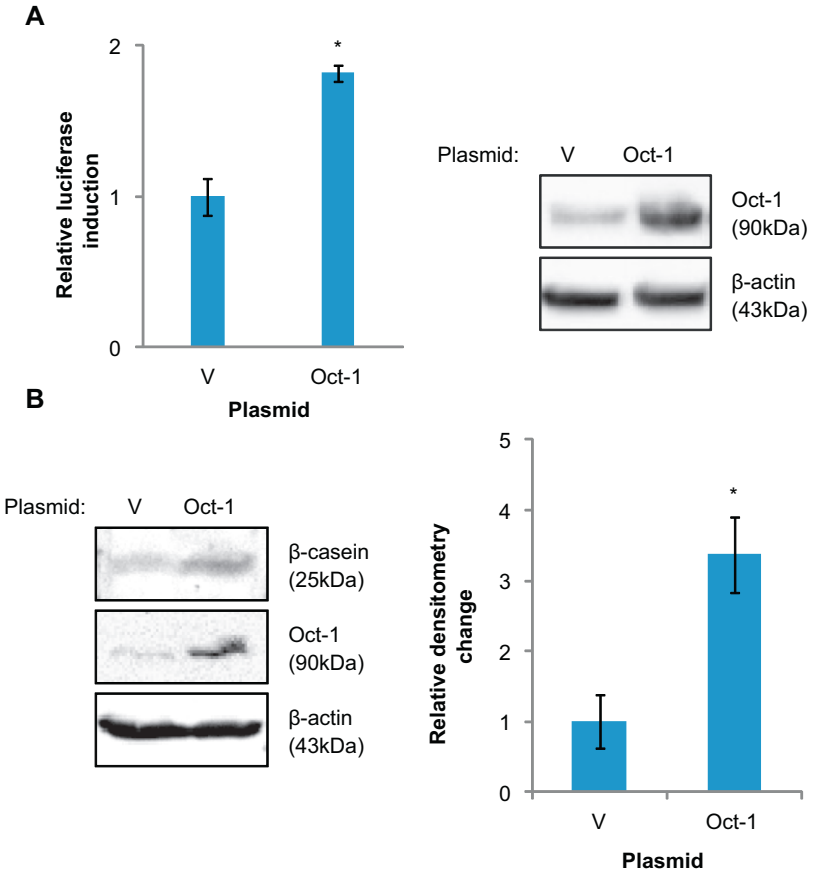



**Fig. 2.** Oct-1 overexpression enhances HP-induced β-casein gene expression. (A) HC11 cells were co-transfected with the pcDNA3.1 vector (V) or an Oct-1 expression plasmid (Oct-1B) along with a *firefly* luciferase reporter construct driven by the β-casein promoter and a *Renilla* luciferase control 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. $^{*}P < 0.05$.

*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. 2A, left). Fig. 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. 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. 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. 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. 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.

### 3.3. 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. 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. 4B). The mRNA and protein levels of Oct-1 were unchanged by HP treatment (Fig. 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. 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

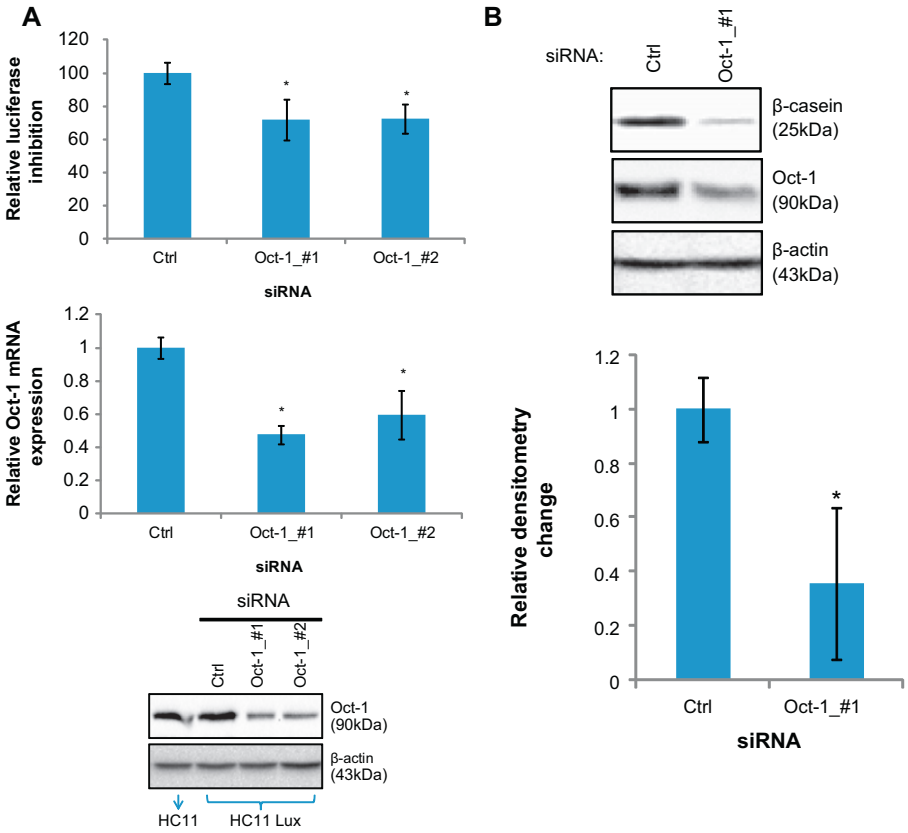



**Fig. 3.** Oct-1 knockdown inhibits HP-induced β-casein gene expression. (A) HC11 Lux cells, which are stably transfected with the mouse β-casein promoter (−344/−1)/luciferase reporter, were transfected with either Oct-1 siRNA #1 (Oct-1_#1), Oct-1 siRNA #2 (Oct-1_#2), or control siRNA (Ctrl) followed 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. *$P < 0.05$.

within 5 min (Fig. 5A), as previously reported (Lechner et al., 1997). These results were also confirmed by immunofluorescence staining as shown in Fig. 5B. These observations suggest that HP induces Oct-1 binding activity via mechanisms other than enhancing Oct-1 expression and translocation.

### 3.4. *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. 6A, the anti-Oct-1 antibody co-immunoprecipitated STAT5 and GR in the cells treated with HP for only 30 min (Fig. 6A, top, lane 4), whereas STAT5 and GR were not associated with Oct-1 in the absence of HP (Fig. 6A, top, lane 3). As expected, normal rabbit IgG failed to co-immunoprecipitate STAT5 and GR (Fig. 6A, top, lanes 1 and 2). In Fig. 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. 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. 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. 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. 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. 6D, comparing lanes 1 and 2), while the mutated BK_C_MT oligonucleotide (Fig. 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. 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

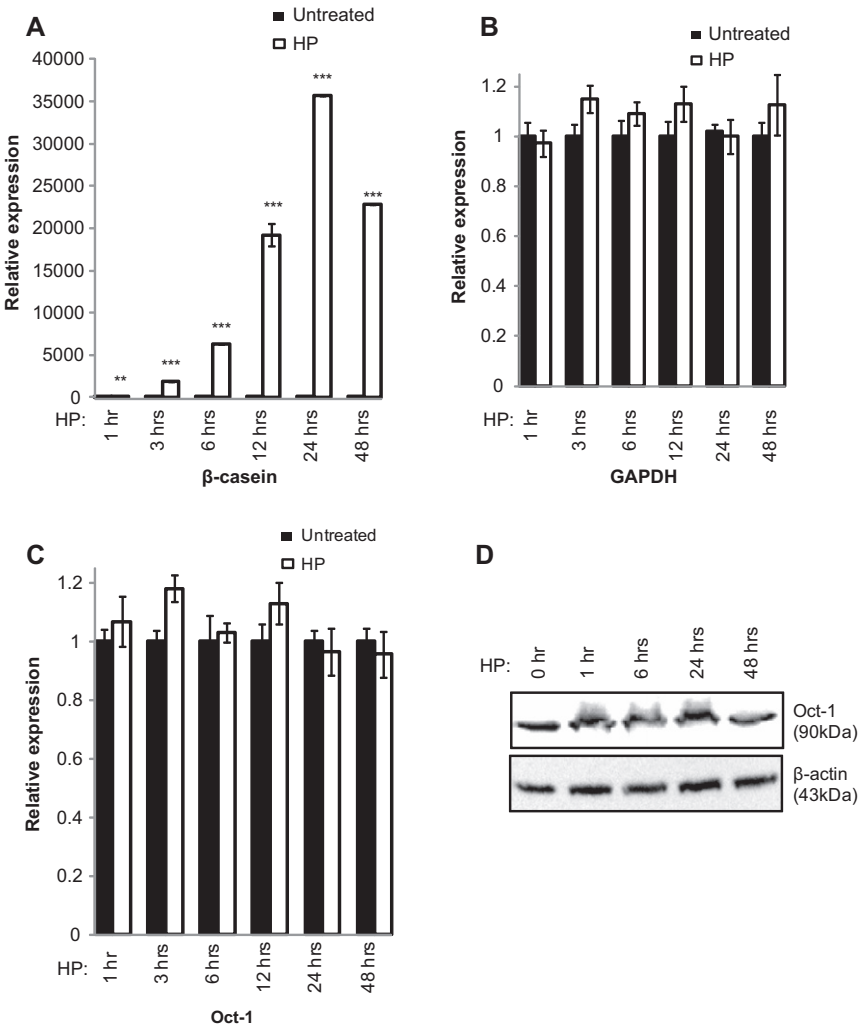



**Fig. 4.** The effects of HP treatment on Oct-1 mRNA and protein expression levels in HC11 cells. (A–C) HC11 cells were treated with HP for 0, 1, 3, 6, 12, 24, or 48 h, and the total RNA isolated from the cells treated or untreated with HP was then analyzed via qRT-PCR for β-casein (A, positive control), 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.

half glucocorticoid response elements (1/2 GREs) (as shown in Fig. 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. 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. 7B, the top gel, lanes 3 and 4 and lanes 5–8) and diminished the binding activity of STAT5 and GR (Fig. 7B, two middle gels, lanes 3 and 4). The same experiment was performed using the biotinylated BK_C_WT oligonucleotide (Fig. 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. 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. 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.

### 3.5. 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. 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. 8, groups 4, 5, and 6), and the highest promoter activity was achieved only when all three plasmids were transfected (Fig. 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.

### 3.6. 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

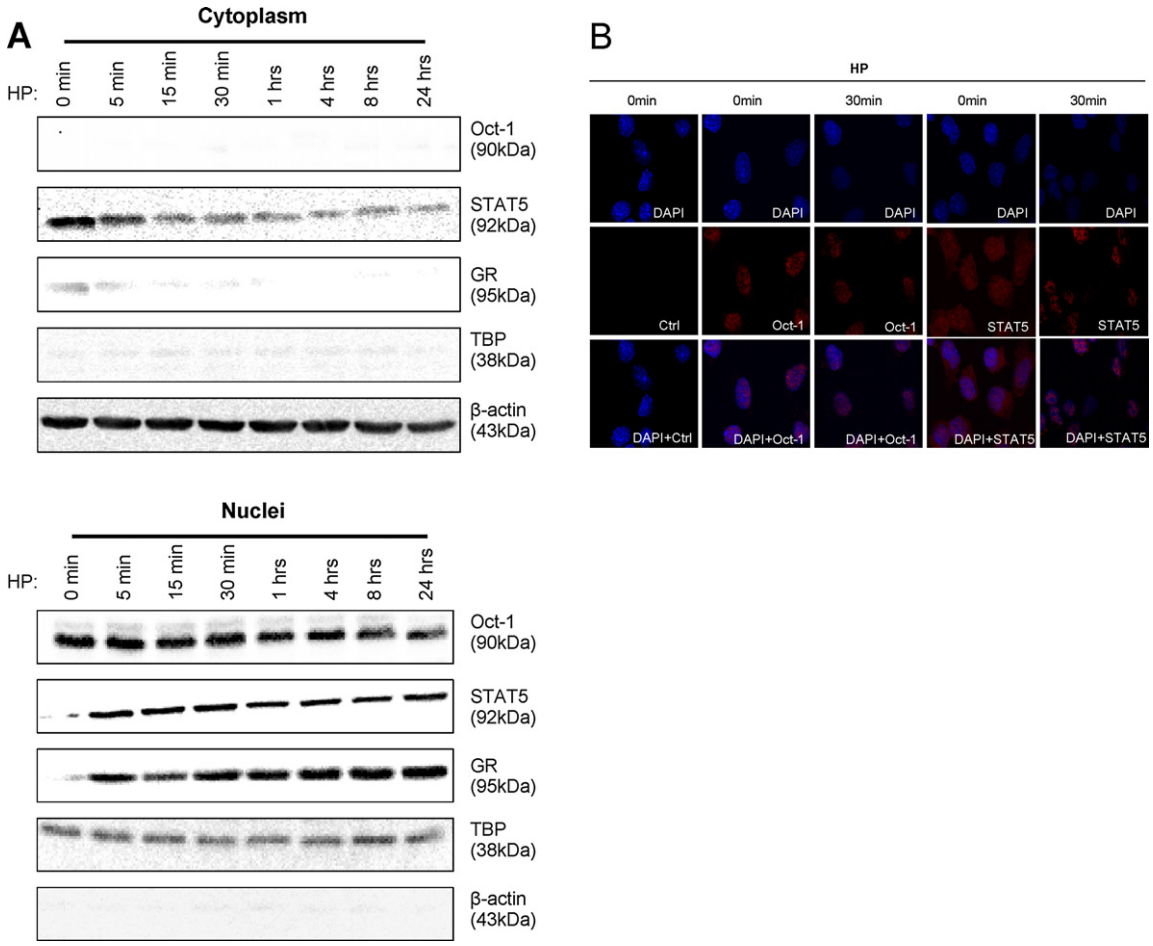



**Fig. 5.** HP induce the translocation of STAT5 and GR but not Oct-1. (A) HC11 cells were treated with HP for the indicated time periods, and the cytoplasmic and nuclear fractions were subsequently isolated. Western blot analyses were performed using specific antibodies against Oct-1, STAT5, GR, TBP (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).

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. 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. 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. 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. 9C). The experiment confirmed that the Leu/Pro mutation did not affect the binding activity of STAT5 (Fig. 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.

## 4. 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.

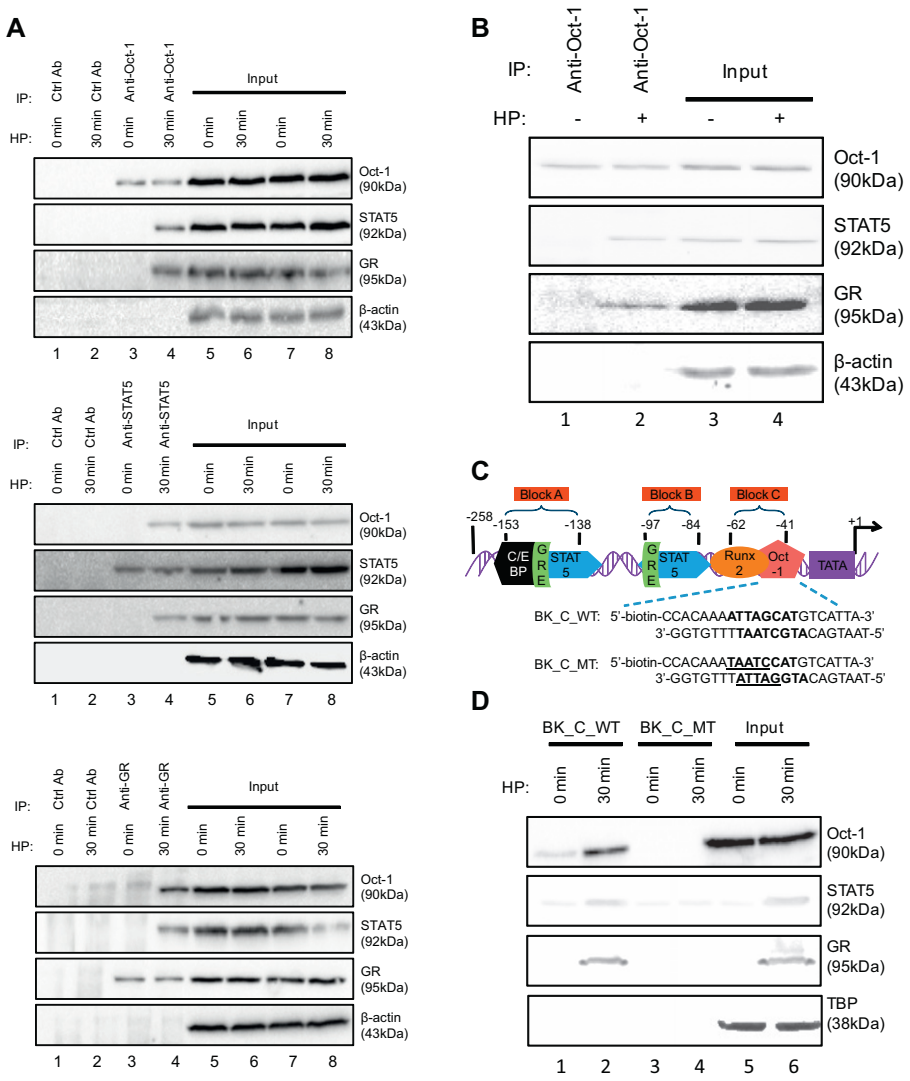



**Fig. 6.** HP induce the formation of the Oct-1–STAT5–GR ternary complex. (A) Whole cell lysates of HC11 cells treated either with or without HP for 30 min were immunoprecipitated using antibodies against Oct-1 (top), STAT5 (middle), and GR (bottom) and were subsequently analyzed via Western blot analyses 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.

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. 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

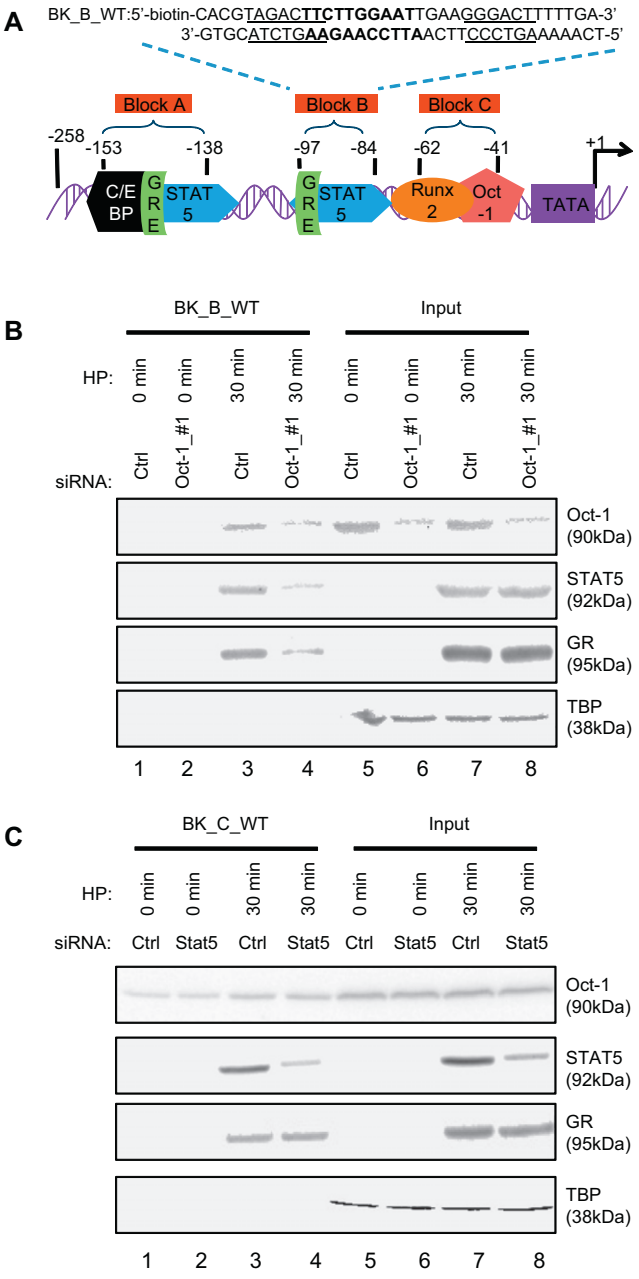
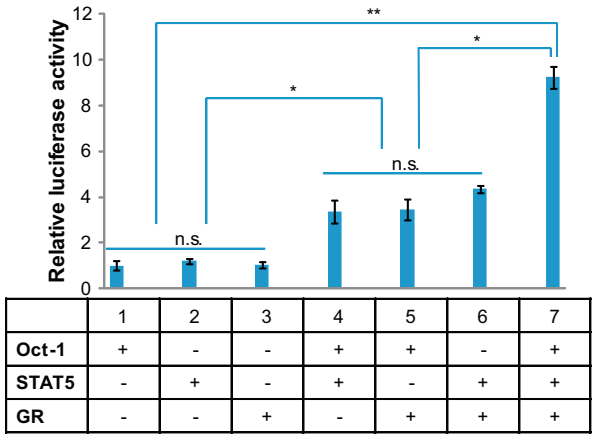



**Fig. 7.** Oct-1 facilitates STAT5 and GR binding at the β-casein promoter following HP stimulation. (A) A schematic view of the biotinylated oligonucleotides used in the DNA pull-down assays in (B) and (C). BK_B_WT comprises a STAT5 binding site and two half GREs, which are indicated with boldface 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.




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

**Fig. 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.

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

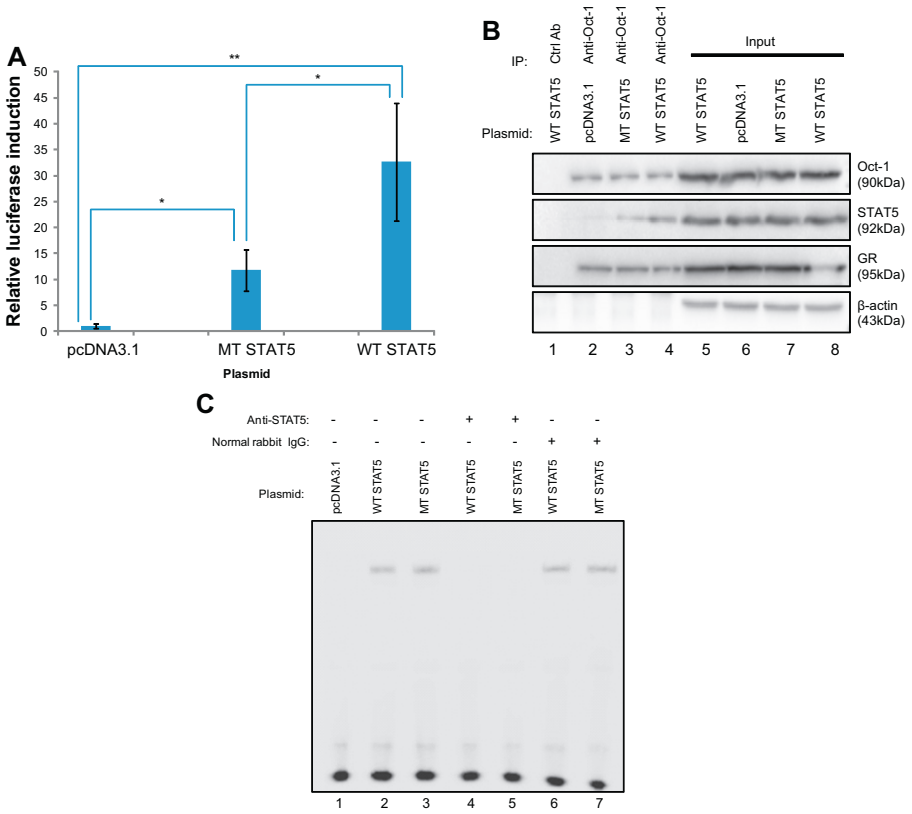



**Fig. 9.** Impaired Oct-1–STAT5 interaction significantly diminishes HP-induced β-casein gene promoter activity. (A) COS-7 cells were transfected with PrlR, Oct-1, and GR expression plasmids; a *firefly* luciferase reporter construct driven by the β-casein promoter; and a *Renilla* luciferase control 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.

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.

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