EXTRACELLULAR MATRIX-REGULATED GENE EXPRESSION REQUIRES COOPERATION OF SWI/SNF AND TRANSCRIPTION FACTORS

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Running Title: SWI/SNF Regulates Casein Expression

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Extracellular cues play crucial roles in the transcriptional regulation of tissue-specific genes, but whether and how these signals lead to chromatin remodeling is not understood and subject to debate. Using chromatin immunoprecipitation (ChIP) assays and mammary-specific genes as models, we show here that extracellular matrix (ECM) molecules and prolactin cooperate to induce histone acetylation and binding of transcription factors and the SWI/SNF complex to the ß- and ?-casein promoters. Introduction of a dominant negative Brg1, an ATPase subunit of SWI/SNF complex, significantly reduced both ß- and ?-casein expression, suggesting that SWI/SNF-dependent chromatin remodeling is required for transcription of mammary-specific genes. ChIP analyses demonstrated that the ATPase activity of SWI/SNF is necessary for recruitment of RNA transcriptional machinery, but not for binding of transcription factors or for histone acetylation. Co-immunoprecipitation analyses showed that the SWI/SNF complex is associated with STAT5, C/EBPß, and glucocorticoid receptor (GR). Thus, ECM- and prolactin-regulated transcription of the mammary-specific casein genes requires the concerted action of chromatin remodeling enzymes and transcription factors.

Differentiated function of mammary epithelial cells is regulated by signals from both ECM and lactogenic hormones (1-3). The gene encoding the milk protein, ß-casein, has been used widely as a marker for functional differentiation of MECs. We and others have shown that in both primary mouse mammary epithelial cells and immortalized mammary epithelial cell lines (4-6), transcription of ß-casein requires signals from both laminin-111 (previously referred to as laminin-1) and prolactin (1,2,7-10). A number of transcription factors, including STAT5, C/EBPß, and GR, have been shown to be involved in this process [reviewed in (7)].

Modulation of chromatin structure by histone modifications and ATP-dependent remodeling has been implicated in cell differentiation and transcriptional control of tissue-specific and inducible genes (11-13). Histone modifying enzymes are believed to be recruited to promoter regions through their association with transcription factors, and are critical for tissue-specific gene expression and functional differentiation of specific cell types (14,15). Histone acetylation is a dynamic process and is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) (16). The mapping of global histone acetylation patterns has demonstrated that chromatin accessibility and gene expression are correlated with histone hyperacetylation of

promoters and other cis-elements (17,18). The p300 HAT cooperates with STAT5 to enhance exogenous ß-casein promoter activity in COS cells, indicating that histone acetylation may play a role in ß-casein transcription (19). Trichostatin A (TSA), an inhibitor of HDAC, was shown to activate the bovine casein ECM-response element (BCE-1) in an ECM-independent fashion in a mouse epithelial cell line in tissue culture plastic, but surprisingly the same treatment inhibited the endogenous ß-casein transcription (20,21). Therefore, the role of histone acetylation in mammary-specific gene transcription has not been elucidated.

ATP-dependent chromatin remodeling SWI/SNF complexes are involved in cellular differentiation and tissue-specific transcription (14,22,23). Mammalian cells contain at least two SWI/SNF-like complexes that share a number of subunits, but are distinguished from one another by their ATPase subunits, Brg1 and Brm1 (24). Introducing a dominant negative Brg1 (DN-Brg1) or Brm1 into mouse NIH-3T3 fibroblasts completely abrogated MyoD-mediated muscle differentiation, and failure to induce transcription of the muscle-specific myogenin gene was correlated with inhibition of chromatin remodeling in the promoter region (14). To date, two different mechanisms have been described for recruiting SWI/SNF complexes to tissue-specific genes. Transcription factors, such as GR and C/EBPß, have been shown in mammalian cells to recruit the SWI/SNF complex to cis-elements to activate specific gene transcription (23,25-27). Alternatively, the ATPase subunits of SWI/SNF contain bromodomains, which can bind directly to acetylated histone tails in vitro (28,29). Thus, acetylated histones in a particular chromatin region may contribute to the recruitment of SWI/SNF complexes to specific genes.

Using the β- and γ-casein genes as models, here we investigate how ECM and prolactin regulate the activity of STAT5 and C/EBPß, and we elucidate the roles of histone acetylation and ATP-dependent chromatin remodeling in expression of these mammary specific genes. The findings from this study indicate that the precise regulation of mammary-specific gene transcription depends not only on transcription factor activation and histone modifications, but also on ATP-dependent chromatin remodeling.

EXPERIMENTAL PROCEDURES

Reagents and antibodies - Antibodies against acetylated H4 and acetylated H3 were from Upstate Biotechnology. The H3 antibody was from Abcam. The STAT5 antibody was from R&D Systems, and those against C/EBPß, RNA polymerase II, GR, and Brg1 were from Santa Cruz Biotechnology. Anti-Flag antibody (M2) was from Sigma. Protein A agarose beads were obtained from Upstate Biotechnology. Phosphatase inhibitor cocktail and protease inhibitor cocktail were from Calbiochem.

Cell culture and transfections - EpH4 cells were derived from IM-2 cells, originally isolated from the mammary tissue of a mid-pregnant mouse (4,5). EpH4 cells were maintained in growth medium consisting of DMEM/F12 (UCSF cell culture facility) supplemented with 2% fetal bovine serum (GIBCO-BRL), 50 μg/ml gentamycin (UCSF cell culture facility), and 5 μg/ml insulin (Sigma). Cells were plated at a density of 10000/cm^2 in growth medium and allowed to attach for 16-24 hours. The cells were cultured in DMEM/F12 medium supplemented with 5 μg/ml insulin and 1 μg/ml hydrocortisone (Sigma) (GIH medium). In other experiments, 3 μg/ml prolactin, 2% laminin-rich ECM (lrECM; Matrigel®, BD Biosciences), 3 μg/ml prolactin plus 2% lrECM [or 100 μg/ml

laminin-111 (Trevigen)] were added in the GIH medium (1).

EpH4 cells were seeded onto 35 mm dishes and transfected using Lipofectamine 2000 (Invitrogen) with the following plasmids: 3 μg of DN-Brg1 plasmid (14) (a kind gift from Dr. Anthony N. Imbalzano, University of Massachusetts Medical School, MA, USA.), 1 μg of pTet-tak, which encodes the tet-VP16 regulator, and 0.4 μg of pNeo plasmid. Twenty four hours after transfection, 400 μg/ml geneticin (Sigma) was added to the media, which were changed every 48 hours for 10 days. The resulting stably-transfected clones were washed twice with growth medium and incubated in the presence or absence of 0.5 μg/ml tetracycline for four days. Positive clones expressing a 200 kDa Flag-tag protein in the absence, but not presence of, tetracycline were identified by western blots analysis.

Promoter reporter plasmid construction and luciferase assays - A 340 bp DNA fragment containing the ß-casein promoter region was amplified from mouse genomic DNA using the following primer sequences: forward primer 5'CGA GGT ACC TTC ATA ACT GAG GTT AAA GCC-3'; reverse primer 5'CAG AAG CTT GTC CTA TCA GAC TCT GTG AC-3'. PCR product was digested with *Hind* III and *Kpn* I, and subsequently cloned into a reporter vector pGL3 (Promega). EpH4 cells were co-transfected with pGL-casein and pNeo plasmid (1:10). Stably transfected cells were isolated by G418 selection and cultured in GIH medium for 2 days in the presence of different inducer. Following induction, equal amounts of cell lysates were assayed for luciferase activity.

Western blot and co-immunoprecipitation (co-IP) -Western blot experiments were performed as previously described (30). Total and nuclear protein were extracted from EpH4 cells or nuclei using radio-immunoprecipitation buffer [50 mM Tris (pH 7.4), 30 mM NaCl, 1% (v/v) NP40, 1% (w/v) deoxycholate, 0.1% (w/v) SDS, protease inhibitor cocktail, and phosphatase inhibitor cocktail]. After sonication, insoluble material was removed by centrifugation at 15,000 g for 10 min. Proteins (20 μg) from each sample were subjected to SDS gel electrophoresis and then transferred to nitrocellulose membrane (Schleicher & Schuell). The membrane was subsequently blocked in TBST buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% (v/v) Tween 20] containing 5% Carnation nonfat dried milk, and incubated in blocking buffer containing primary antibody. All blots were further incubated in blocking buffer containing horseradish peroxidase-conjugated secondary antibodies and subjected to enhanced chemiluminescence (ECL) using the SuperSignal chemiluminescent substrate (Pierce, Rockford, IL).

Cells transfected with DN-Brg1 were cultured in the presence or absence of tetracycline for 2 days in GIH medium containing prolactin and lrECM, and nuclei were isolated using a nucleus isolation kit (Sigma). The nuclei were resuspended and sonicated in lysis buffer (10 mM Tris-HCl, pH7.5; 150 mM NaCl; 2 mM EDTA; 0.5% Triton X-100; protease inhibitor cocktail) and centrifuged at 15000 g for 10 min. After centrifugation, 40 μl of agarose beads conjugated to an anti-Flag M2 antibody were added to the supernatant of each sample and incubated with shaking at 4°C for 4 hours The agarose beads were washed with rinsing buffer (50 mM Tris-HCl, pH7.5; 150 mM NaCl; 0.05% Triton X-100). Agarose-associated protein complexes were eluted using SDS loading buffer and analyzed by western blots.

RT-PCR and Real Time PCR - Total RNA was extracted from cells with Trizol reagent (Invitrogen). cDNA was synthesized using

Superscript first strand synthesis kit (Invitrogen) from 1 µg RNA samples. One microliter of cDNA was used as a template for PCR and real time PCR. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified and used as a loading control. The following primers were used to amplify ß-casein, ?-casein, lactoferrin and GAPDH cDNA sequences: forward primer of the ß-casein gene 5'-GCT CAG GCT CAA ACC ATC TC-3' and reverse primer 5'-TGT GGA AGG AAG GGT GCT AC-3'; forward primer of the ?-casein gene: 5'-CCC AGG AGT CTT CCT TTT CC-3' and reverse primer 5'-GGA AAC CAC GAA GAA ACC AA-3'; forward primer of the lactoferrin gene: 5'-AGT GAG GAG AAG CGC AAG TGT G-3' and reverse primer 5'-AGC CCC AGT GTA GCC TTG GTA T-3'; forward primer for GAPDH gene 5'CCC CTG GCC AAG GTC ATC CAT GAC-3' and reverse primer 5'CAT ACC AGG AAA TGA GCT TGA CAA AG-3'. Quantitative real-time PCR analysis was performed with the Lightcycler System (Roche) using the Lightcycler FastStart DNA Master SYBR Green I kit (Roche) (31). The following Lightcycler PCR amplification protocol was used: 95°C for 10 min (initial denaturation), and 45 amplification cycles (95°C for 5 s, 60°C for 10 s, 72°C for 5 s). Amplification was followed by melting curve analysis to verify the presence of a single PCR product.

Chromatin immunoprecipitation - The ChIP assay was performed based on the Upstate Biotechnology ChIP protocol (32) with a few modifications. Cellular components were cross-linked by adding formaldehyde to a final concentration of 1% and incubated at room temperature for 10 minutes. The cross-linking reaction was stopped by adding glycine to a final concentration of 125 mM. Nuclei were isolated with a nucleus isolation kit and resuspended in ChIP lysis buffer (1% SDS, 10mM EDTA, 50 mM Tris-HCl pH8.0) containing protease inhibitor cocktail. The nuclei were then sonicated to shear DNA to lengths between 200 and 1000 bp. The sonicated lysates were diluted to an OD_{260} of 2 units per ml with ChIP dilution buffer and incubated with 60 µl of protein A-conjugated agarose beads to reduce non-specific binding. Primary antibodies were added to the pre-cleared supernatant fraction and incubated from 5 hour to overnight at 4ºC with rotation. Protein A-conjugated agarose beads (50 µl) were then added to the samples for one hour, and the protein-DNA complexes were eluted from the protein A agarose by incubation in 250 µl elution buffer (1% SDS, 0.1M $NaHCO_3$). Protein-DNA cross links were reversed by heating at 65ºC for 5 hours. The immunoprecipitated DNA was phenol/chloroform extracted and ethanol-precipitated in the presence of 15 µg of linear polyacrylamide, an inert carrier. The isolated DNA was then analyzed by semi-quantitative PCR using the following primers: ß-casein promoter forward primer 5'GTC CTC TCA CTT GGC TGG AG-3' and reverse primer 5'GTG GAG GAC AAG AGA GGA GGT-3'; Amylase promoter forward primer 5' TCA GTT GTA ATT CTC CTT GTA CGG-3' and reverse primer 5'CCT CCC ATC TGA AGT ATG TGG GTC-3'; ?-casein promoter forward primer 5'AAA CAG GTG AGT CTG CCT TCA-3' and reverse primer 5'CCA AAT GGA AGA CGA GAG GA-3'.

Statistics - All data analysis was performed using Sigma Plot. Bar graphs represent mean ± standard error of the mean (SEM).

RESULTS

Expression of mammary-specific genes depends on signals from both lactogenic hormones and ECM molecules; for ß-casein, the relevant ECM molecule is laminin-111 (2,9,10). Using EpH4, an epithelial cell line derived from normal mouse mammary gland

(4,5), we observed that both ß- and ?-casein mRNA levels were highly upregulated in response to prolactin and lrECM treatment (Fig. 1A); expression correlated with significant changes in cellular morphology as shown previously for ß-casein (3). Furthermore, consistent with previous studies (1,30), we established that laminin-111 was indeed the lrECM constituent that induced ß-casein expression in EpH4 cells (data not shown).

The mouse ß-casein promoter contains binding sites for STAT5 and C/EBPß, and half-sites of the palindromic glucocorticoid response element (GRE) (33-35). To determine whether ECM and prolactin directly induce transcriptional activation of the ß-casein promoter, we amplified and cloned the promoter region from -340 to -1 into a luciferase reporter vector and stably transfected the reporter plasmid into EpH4 cells. Luciferase activity was dramatically induced in the transfected cells after treatment with lrECM and prolactin (Fig. 1B), indicating that the ß-casein promoter is transcriptionally activated in these cells. Consistent with the PCR results, neither prolactin nor lrECM alone could appreciably enhance promoter activity. STAT5 and C/EBPß binding sites were also identified in the bovine ß-casein ECM-response element, BCE-1 (20). To determine whether these two factors regulate ECM- and prolactin- induced expression of the endogenous ß-casein gene, total and nuclear lysates of EpH4 cells were analyzed by western blotting. Although the total level of STAT5 did not change, the levels of phosphorylated STAT5 and its nuclear translocation increased after combined treatment with lrECM and prolactin. However, neither treatment alone could induce these changes (Fig. 1C, 1D). Total cell and nuclear levels of C/EBPß remained unchanged after the treatments (Fig. 1C, 1D).

To determine whether STAT5 and C/EBPß become associated with the ß-casein promoter after ECM and prolactin treatment, we performed ChIP assays. Addition of these two ligands significantly increased the association of STAT5 and C/EBPß with the ß-casein promoter, whereas the interaction between the promoter and GR remained at the control level (Fig. 2A). The promoter of the β-amylase gene, which is not expressed in mammary epithelial cells, was included as a negative control and was not detected in any of the ChIP samples (data not shown). Thus, exposure of EpH4 cells to lrECM and prolactin increases both STAT5 levels in the nucleus, and the binding of this factor and C/EBPß to the ß-casein promoter. We also found that treatment with prolactin and lrECM moderately enhanced the binding of Brg1, the ATPase subunit of SWI/SNF complex, to the ß-casein promoter in EpH4 and primary mammary epithelial cells (Figure 2A and supplemental Figure 1). Analysis of the DNA immunoprecipitated with a RNA polymerase II antibody showed an increased association of this protein with the ß-casein promoter in response to treatment with lrECM and prolactin (Fig 2A).

Binding sites of STAT5 and C/EBPß were identified in promoters of other milk protein genes, such as ?-casein (36). We asked whether ?-casein wae regulated similarly to β-casein. The association of these factors with the ?-casein promoter was determined by ChIP assays. We found that treatment with prolactin and lrECM enhanced binding of STAT5 and C/EBPß, and increased Brg1 and RNA Polymerase II levels in the promoter region of ?-casein gene (Fig. 2B). The mouse casein genes cluster at a single gene locus on chromosome 5 in this order: α, β, ?, d, and ? (37), and the expression of casein genes is coordinately regulated during pregnancy and lactation (38). Thus, the binding of these transcription factors and the chromatin

remodeling complex together appears to activate the entire gene locus.

We showed above that treatment with lrECM and prolactin induced the recruitment of transcription factors and the SWI/SNF complex to the ß-casein promoter. To determine whether ECM and prolactin control these events separately or cooperatively, we performed ChIP analysis after cells were treated either singly or with both agents. We found that STAT5 bound to the ß-casein promoter in cells treated with both lrECM and prolactin, but treatment with either component alone failed to induce appreciable binding (Fig. 2C). These results are consistent with the western data showing that nuclear translocation of STAT5 depends on both the ECM and hormonal signals (Fig. 1D). Combined lrECM and prolactin treatment also induced binding of C/EBPß to the ß-casein promoter (Fig. 2C). Recruitment of Brg1 and RNA polymerase II in the ß-casein promoter required also both lrECM and prolactin (Fig. 2C). These results establish that ECM cooperates with prolactin to induce the binding of transcription factors as well as the transcriptional machinery to the ß-casein promoter.

Previously, we showed that treatment with histone deacetylase inhibitors could partially substitute for lrECM in activating a stably integrated bovine ECM-response element (BCE-1) in a mammary epithelial cell line (CID-9), suggesting that histone acetylation may play a role in transcriptional regulation of this enhancer (20). Surprisingly, however, the same treatment was later shown to inhibit transcriptional activation of the endogenous ß-casein gene (21). Here we sought to determine whether histone acetylation is involved in transcriptional regulation of the endogenous ß-casein gene. ChIP assays using antibodies against acetylated histone H3 and H4 demonstrated enhanced histone acetylation in the ß-casein promoter, but not the ß-amylase promoter, in response to treatment with lrECM and prolactin (Fig 3A). In addition, neither lrECM nor prolactin alone induced histone acetylation in the ß-casein promoter (data not shown), confirming that the cooperation between the two signals is important.

To determine whether the increase of acetylated histone in the ß-casein promoter was sufficient to induce transcription of the endogenous gene, EpH4 cells were treated with TSA in the presence or absence of ECM and prolactin. ChIP data showed that the levels of acetylated histone H4 (AcH4) appreciably increased in the ß-casein promoter (Fig. 3B). Quantitative PCR showed, however, that the level of ß-casein mRNA was increased by only 1.6 fold in undifferentiated cells after TSA treatment; the levels of both total and phosphorylated STAT5, C/EBPß, and GR did not change (Fig. 3C, 3D). In the functionally differentiated cells that were cultured with prolactin and lrECM, TSA treatment significantly suppressed the induction of ß-casein expression. Western blot analysis showed that phosphorylated STAT5 levels decreased in TSA treated cells, suggesting that this inhibition may be due to an indirect effect of TSA on STAT5 phosphorylation (Fig. 3C, 3D). These results now clarify previous contradictions and indicate that histone acetylation alone is not sufficient to induce transcription of the endogenous ß-casein gene above the basal level.

A point mutation in the ATP-binding site of Brg1 was shown to abolish its ATPase activity, and produce a dominant negative effect on the chromatin remodeling function of the SWI/SNF complex (39). To examine whether this ATPase was indeed the additional factor required for ß-casein expression, we generated a mammary cell line that conditionally

expressed Flag-tagged DN-Brg1 under the control of a tetracycline-repressible transactivator. Withdrawal of tetracycline from culture medium for 2 days caused a dramatic induction of DN-Brg1 expression (Fig. 4A) and a significant repression in ß- and ?-casein transcription (Fig. 4B, 4C), but lactoferrin and GAPDH transcription remained unchanged. Thus, the ATPase activity of SWI/SNF is necessary for transcriptional activation of casein genes. The binding of STAT5 and C/EBPß, as well as the levels of AcH4 in the ß-casein promoter in ECM- and prolactin- treated cells did not change significantly in response to DN-Brg1 expression (Fig. 4D). Western blot analysis revealed that DN-Brg1 expression also did not affect the nuclear levels of STAT5, C/EBPß and GR (data not shown). These results rule out the possibility that expression of DN-Brg1 inhibited ß-casein transcription indirectly by repressing the activity of transcription factors, and suggest that transcription factor binding and histone acetylation in the ß-casein promoter are events that take place upstream of Brg1 ATPase activity and are not dependent on SWI/SNF. In contrast, recruitment of RNA polymerase II to the ß-casein promoter was inhibited by DN-Brg1 expression, suggesting that its function in mediating transcription of casein genes occurs downstream of SWI/SNF, and therefore, is dependent on ATP-dependent chromatin remodeling (Fig. 4D).

Formation of the SWI/SNF complex was shown to occur independently of its ATPase activity in NIH 3T3 cells (40,41). We asked whether the SWI/SNF complex interacts with STAT5, C/EBPß and GR, and whether the ATPase activity of Brg1 is necessary for this interaction in mammary epithelial cells. Protein complexes from ‘tet+’ (no DN-Brg1 expression) and ‘tet-’ (with DN-Brg1 expression) cells were immunoprecipitated with agarose beads conjugated with anti-FLAG M2 antibody. The immunoprecipitated protein complexes were analyzed by western blot using antibodies against STAT5, C/EBPß, GR, and lamin B. STAT5 co-immunoprecipitated with the DN-Brg1 in the ‘tet-’ cells, but it was absent from the immunoprecipitate of ‘tet+’ cells (Fig. 5A). We also detected interactions between DN-Brg1 and GR, and DN-Brg1 and C/EBPß in lysates from the ‘tet-’ cells but not ‘tet+’ cells (Fig. 5A). However, lamin B was not enriched in the co-IP samples from ‘tet-’ cells comparing to control (tet+) cells, suggesting that the interaction between STAT5, C/EBPβ, GR and the SWI/SNF complex is specific (Fig. 5A). That SWI/SNF is associated with GR and C/EBPβ has been shown (23,25), but the interaction between SWI/SNF and STAT5 has not been reported previously. To confirm the co-IP results in DN-Brg1-expressing cells, we performed a co-IP experiment using the parental cells. The results showed that endogenous wild type SWI/SNF was bound to STAT5 in lrECM- and prolactin-treated EpH4 cells but not in control cells (Fig. 5B). Therefore, the SWI/SNF chromatin remodeling complex may be recruited to the ß-casein promoter by STAT5, C/EBPß, and/or GR. Several other milk proteins, including a-casein, ?-casein, whey acidic protein (WAP), and ß-lactoglobulin have been shown to be regulated by ECM and lactogenic hormones (7,42-44). The promoter or enhancer elements of these genes contain binding sites for STAT5 and GR (7,36,45). Interestingly, DN-Brg1 expression inhibited ?-casein transcription significantly, but had no detectable effect on transcription of the lactoferrin gene (Fig 4B). These data are consistent with the finding that the expression of lactoferrin is not dependent on the cooperation of ECM and prolactin signals in MECs, and that basal transcriptional regulation may be different for lactoferrin expression (46). These results indicate that transcription factors such as STAT5 determine

recruitment of the SWI/SNF complex by binding to specific promoters to allow expression of milk protein genes.

DISCUSSION

By necessity, lactation is a tightly regulated process during mammary gland development. Extracellular and intracellular signals must be coordinated precisely and rapidly to ensure that milk is produced and delivered in a timely fashion after parturition and during suckling. Using mammary-specific casein genes as markers, we show that laminin and prolactin regulate casein expression by inducing binding of STAT5 and C/EBPß to their promoters, and that both histone acetylation and ATP-dependent chromatin remodeling are involved in this process (Fig. 5C).

Previous studies have shown that ECM cooperates with prolactin to control ß-lactoglobulin expression by activating the JAK2/STAT5 signal transduction pathway to induce STAT5 phosphorylation and its nuclear translocation in primary MECs (42,47). Here we demonstrate that laminin and prolactin cooperatively regulate the binding of STAT5 to the ß-casein promoter. Upon addition of prolactin and lrECM, C/EBPß becomes bound to the ß-casein promoter. How these extracellular signals regulate C/EBPß activity still remains to be determined, but one report showed that the nuclear levels of C/EBPß in primary rabbit mammary epithelial cells increased when these cells were plated on a collagen gel (48). We did not observe a significant change in the nuclear level of C/EBPß in EpH4 cells in response to laminin and prolactin treatment. Indeed a luciferase reporter gene fused to a C/EBPß-response element was not activated by laminin and prolactin (data not shown), suggesting that C/EBPß DNA-binding activity to the ß-casein promoter is enhanced selectively. The binding sites of STAT5, C/EBPß, and GR are in close proximity to one another within the ß-casein promoter, and our co-IP experiments showed that these three factors are all associated with the SWI/SNF complex. It has been reported that STAT5 cooperats with C/EBPß to regulate ß-casein promoter activity, and that this cooperation is mediated by GR (33). Therefore, these factors most likely form a protein complex with chromatin remodeling enzymes on the ß-casein promoter, and the binding of STAT5 may enhance the interaction of C/EBPß with the promoter.

The tight link between eukaryotic gene transcription and histone acetylation is now firmly established (49,50). Using footprinting analysis, previously we showed that whereas binding of transcription factors was not sufficient to activate the BCE-1 element in another mouse epithelial cell line, the enhancer element could be activated in the absence of ECM upon TSA treatment (20). Surprisingly however, the expression of the endogenous ß-casein was inhibited by the same treatment (21). Using ChIP assays which can directly detect the level of histone acetylation in a specific chromatin region, we demonstrated here that histone acetylation was indeed involved in transcriptional activation of the endogenous ß-casein gene in functional MECs, which is consistent with a recent study in HC11 cells (51). We show that TSA treatment only slightly increased the basal level of ß-casein transcription in undifferentiated cells, indicating that histone acetylation contributes to, but is not sufficient for, induction of endogenous mammary-specific gene transcription. The controversial effects of TSA on the activity of endogenous ß-casein gene and exogenous BCE-1 element may be due to differences in the nuclear environment surrounding these sequences and the manner with which each sequence is packaged into chromatin. Analysis of global histone acetylation patterns has demonstrated that gene expression is correlated with histone

hyperacetylation in specific chromatin regions (17,18). However, treatment with HDAC inhibitors only induced expression of less than 3% of genes in cultured cells (52,53). These findings imply that transcription of other tissue-specific genes and inducible genes may require different types of chromatin remodeling.

ATP-dependent chromatin remodeling conducted by the SWI/SNF complex increases nucleosome mobility and may uncover a core promoter for assembly of a preinitiation complex (54). Studies with ATPase-deficient Brg1 have demonstrated that the ATPase activity of SWI/SNF is required for expression of tissue-specific genes in muscle and adipose tissues (14,55,56). However, whether the expression of mammary-specific genes depends on SWI/SNF function was not addressed previously. The ChIP assays show that the SWI/SNF complex is associated with the ß-casein promoter in EpH4 cells upon transcriptional activation. This association is detected also in primary mammary epithelial cells (supplemental Figure 1). Furthermore, the ATPase activity of SWI/SNF is necessary for transcription of the ß-casein gene. DN-Brg1 expression leads to a reduction in binding of RNA polymerase II, but not the levels of STAT5, C/EBPß, and AcH4 in the promoter region. Together with the data generated from TSA experiments, these results suggest that chromatin remodeling induced by histone acetylation is not sufficient for assembly or stabilization of the RNA transcriptional machinery on the ß-casein promoter, and that this process depends on ATP-dependent chromatin remodeling (Fig. 5C). Such precise regulation from extracellular signals to chromatin structure is most likely fundamental to mammary gland development and function to ensure control of milk protein gene expression during lactation, a process that is vital to the offspring's survival.

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FOOTNOTES

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The abbreviates used are: ECM, extracellular matrix; ChIP, chromatin immunoprecipitation; co-IP, co-immune precipitation; GR, glucocorticoid receptor; HAT, histone acetyltransferases;

HDAC, histone deacetylase; TSA, trichostatin A; MEC, mammary epithelial cell; DN-Brg1, dominant negative Brg1.

FIGURE LEGENDS

Fig 1. Stat5 phosphorylation and nuclear translocation are activated in EpH4 cells in response to ECM and prolactin treatment. (A) ß- and γ-casein expression was determined by RT-PCR in EpH4 cells cultured for 2 days in GIH (DMEM/F12 supplemented with 5 µg/ml insulin and 1 µg/ml hydrocortisone). Media alone (Ctrl), GIH + 3 µg/µl prolactin (PRL), GIH + 2% lrECM (ECM), and GIH + 3 µg/µl prolactin + 2% lrECM (ECM+PRL). GAPDH cDNA was used as a loading control. (B) The ß-casein promoter was cloned into pGL3 luciferase vector, and stably transfected into EpH4 cells. The ß-casein promoter activity was determined by luciferase assays. (C, D) Transcription factor levels in the cell lysates (C) and nuclear lysates (D) of EpH4 cells were determined by western blotting.

Fig 2. Binding of transcription factors and the SWI/SNF complex to the ß-casein promoter is regulated by ECM and prolactin. (A, B) ChIP assays followed by PCR analysis to detect the binding of STAT5, C/EBPß, GR, Brg1, and RNA Polymerase II in the ß-casein (A; n=4) and γ-casein (B; n=2) promoters. The PCR results were quantified by AlphaEaseFC software, and the values of bound DNA were normalized to input DNA. Fold enrichments were determined by dividing the normalized values from treated cells by that of untreated cells, * $p<0.05$. (C) Quantification of ChIP results in EpH4 cells treated with prolactin (PRL), lrECM (ECM), or prolactin plus lrECM (PRL+ECM). Graph displays the mean of two experiments.

Fig 3. Histone acetylation contributes to, but is not sufficient to induce ß-casein expression. (A) The levels of total histone H3, as well as acetylated histone H4 and H3 in the ß-casein promoter were measured by ChIP analysis. The ß-Amylase promoter was used as a control, * $p<0.05$ (n=3). (B) The levels of AcH4 in the ß-casein promoter were determined by ChIP assays in control and TSA (80 nM)-treated cells. (C) The ß-casein mRNA levels in undifferentiated EpH4 cells (in GIH medium) and differentiated cells (in GIH plus prolactin and lrECM) were measured by quantitative RT-PCR after TSA treatment. Graph displays average ± SEM; * $p<0.05$, *** $p<0.01$ (n=4). (D) Protein levels of phosphorylated STAT5, total cell STAT5, C/EBPß and GR were analyzed by western blotting after TSA treatment.

Fig 4. DN-Brg1 expression in EpH4 cells suppresses transcription of the ß-casein gene. (A) Western blot analysis of DN-Brg1 expression in stably transfected EpH4 cells. (B, C) RT-PCR (B) and quantitative PCR (C) analysis of the levels of ß- and γ-casein genes in DN-Brg1-expressing (tet-) and non-expressing (tet+) cells. Graph displays average ± SEM; *** $p<0.01$ (n=4). (D) ChIP assays showing the levels of AcH4 and the binding activity of STAT5 and C/EBPß in the ß-casein promoter in DN-Brg1-expressing cells. Graph displays mean of three experiments, * $p<0.05$.

Fig 5. STAT5, C/EBPß, and GR interact with DN-Brg1 in EpH4 cells. (A) Interaction between transcription factors and DN-Brg1 was determined by co-IP analysis. Total lysates before immunoprecipitation were used as input control. (B) Interaction between endogenous Brg1 and

STAT5 was detected by co-IP analysis. (C) Model displaying how exposure of mammary epithelial cells to ECM and prolactin may induce the recruitment of transcription factors and chromatin remodeling enzymes to the ß-casein promoter, and how aberrations in SWI/SNF function interfere with RNA polymerase II recruitment.

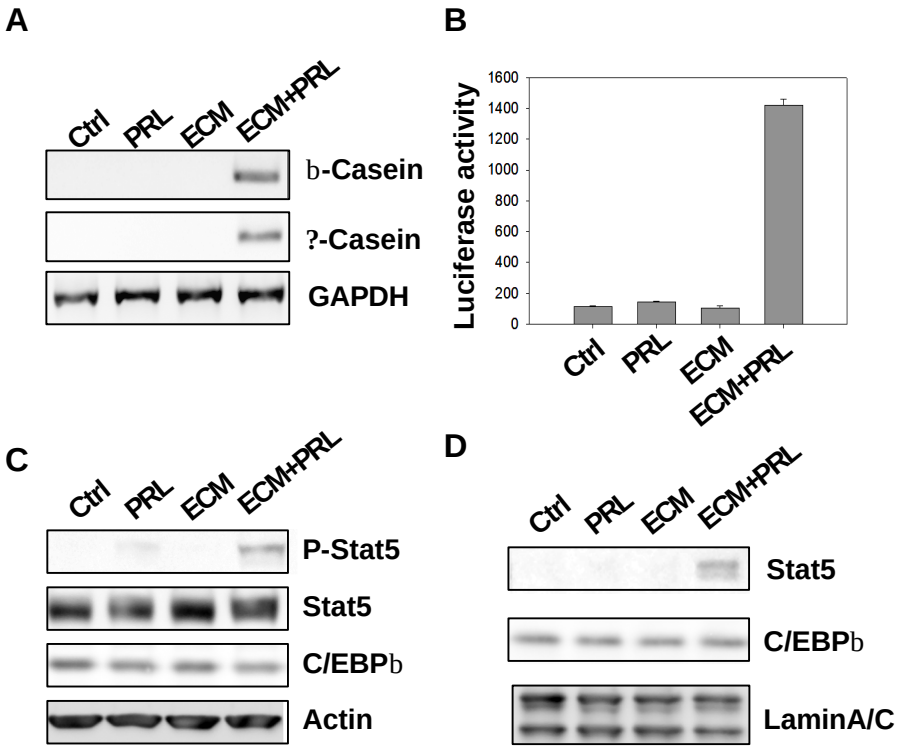

A
Ctrl
PRL
ECM
ECM+PRL
b-Casein
?-Casein
GAPDH
B
Luciferase activity
1600
1400
1200
1000
800
600
400
200
0
Ctrl
PRL
ECM
ECM+PRL
C
Ctrl
PRL
ECM
ECM+PRL
P-Stat5
Stat5
C/EBPb
Actin
D
Ctrl
PRL
ECM
ECM+PRL
Stat5
C/EBPb
LaminA/C

Figure 1

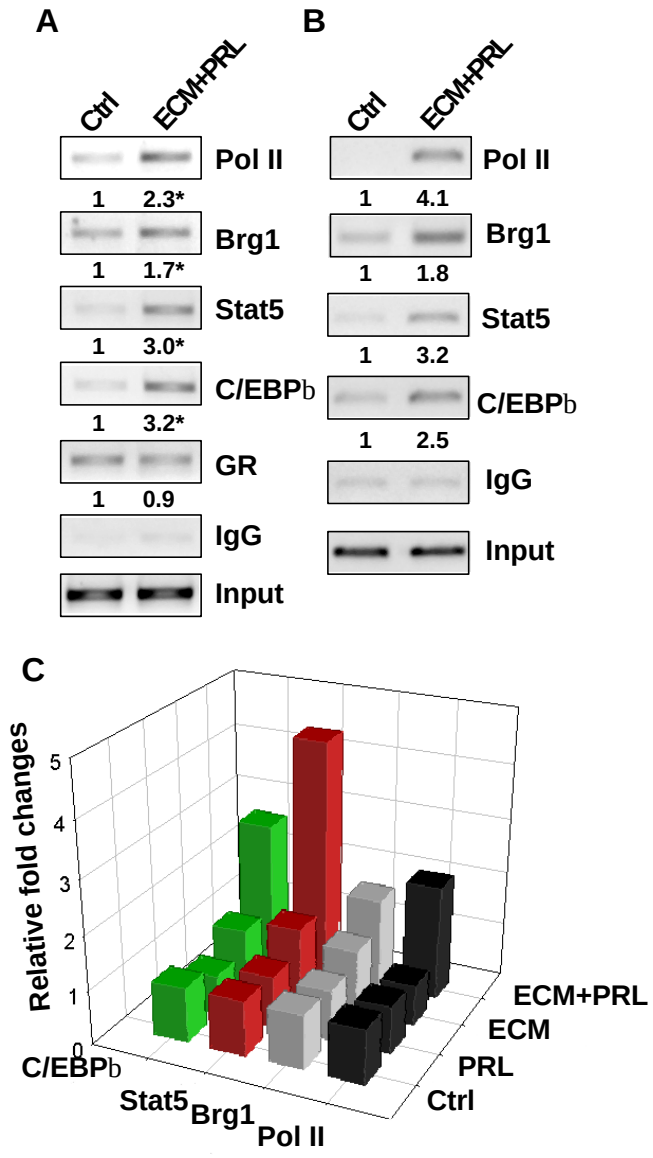

A
Ctrl
ECM+PRL
Pol II
1 2.3*
Brg1
1 1.7*
Stat5
1 3.0*
C/EBPb
1 3.2*
GR
1 0.9
IgG
Input
B
Ctrl
ECM+PRL
Pol II
1 4.1
Brg1
1 1.8
Stat5
1 3.2
C/EBPb
1 2.5
IgG
Input
C
Relative fold changes
0
1
2
3
4
5
C/EBPb
Stat5
Brg1
Pol II
Ctrl
PRL
ECM
ECM+PRL

Figure 2

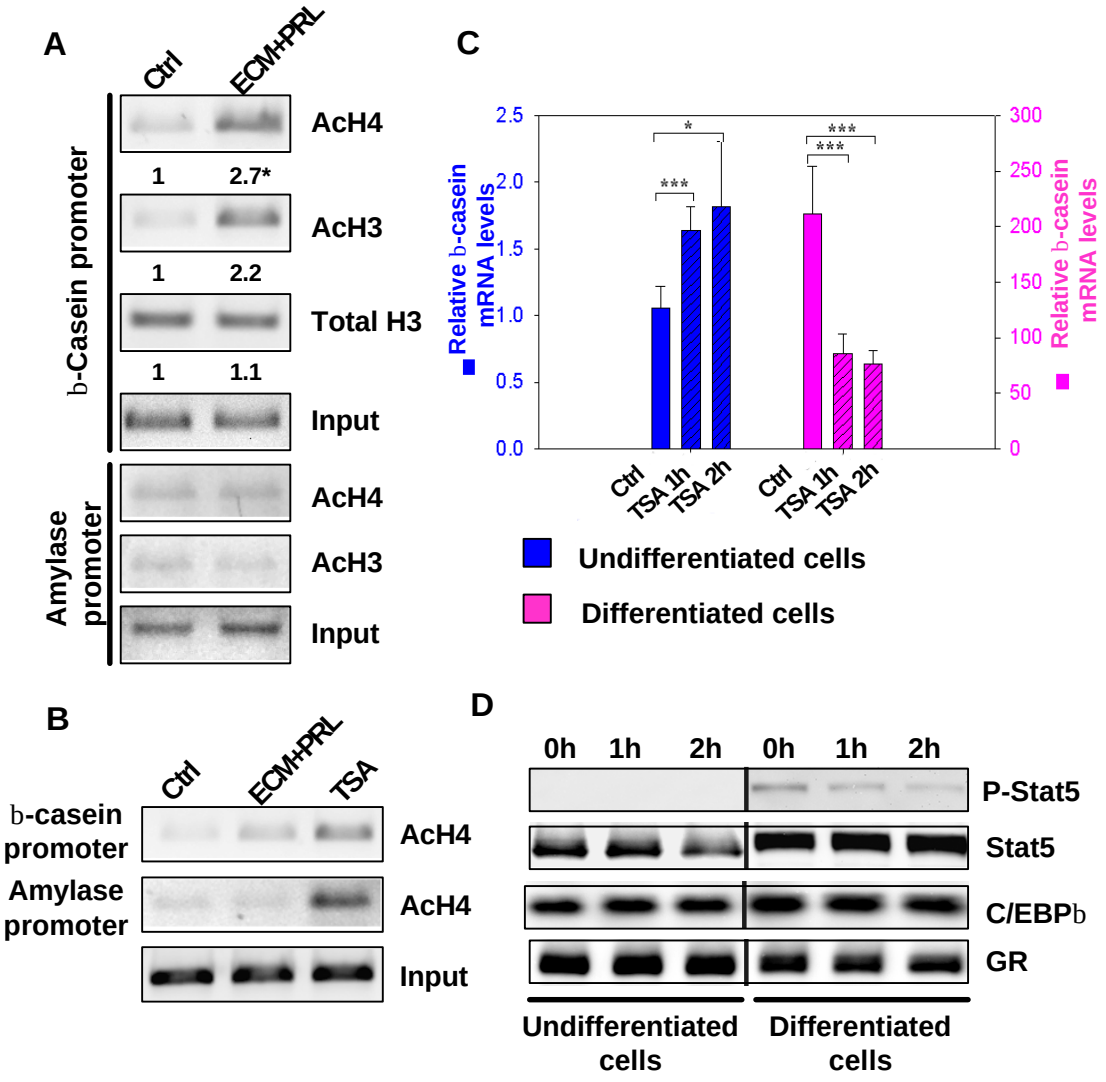

A
Ctrl
ECM+PRL
b-Casein promoter
AcH4
1
2.7*
AcH3
1
2.2
Total H3
1
1.1
Input
Amylase promoter
AcH4
AcH3
Input
C
Relative b-casein mRNA levels
2.5
2.0
1.5
1.0
0.5
0.0
300
250
200
150
100
50
0
*
***
***
***
Ctrl
TSA 1h
TSA 2h
Ctrl
TSA 1h
TSA 2h
Undifferentiated cells
Differentiated cells
B
Ctrl
ECM+PRL
TSA
b-casein promoter
AcH4
Amylase promoter
AcH4
Input
D
0h
1h
2h
0h
1h
2h
P-Stat5
Stat5
C/EBPb
GR
Undifferentiated cells
Differentiated cells

Figure 3

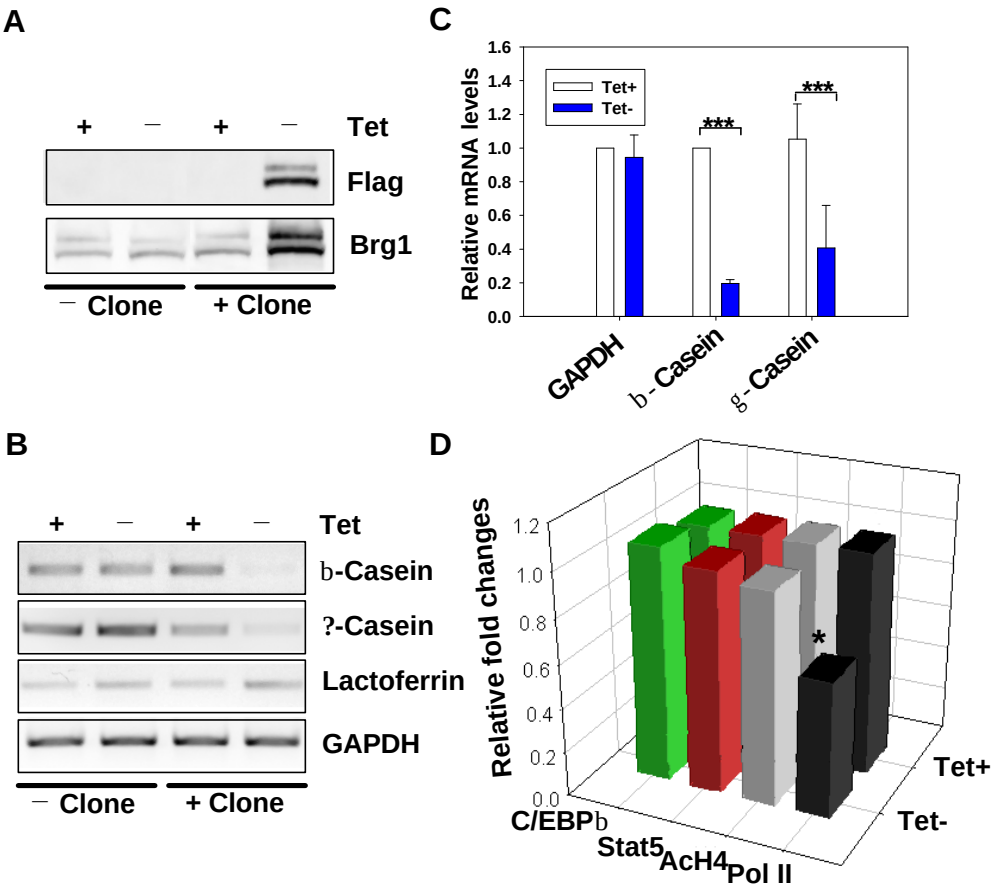

A
+ – + – Tet
Flag
Brg1
– Clone
+ Clone
C
Relative mRNA levels
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Tet+
Tet-
***
***
GAPDH
b-Casein
g-Casein
B
+ – + – Tet
b-Casein
?-Casein
Lactoferrin
GAPDH
– Clone
+ Clone
D
Relative fold changes
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Tet+
Tet-
C/EBPb
Stat5
AcH4
Pol II

Figure 4

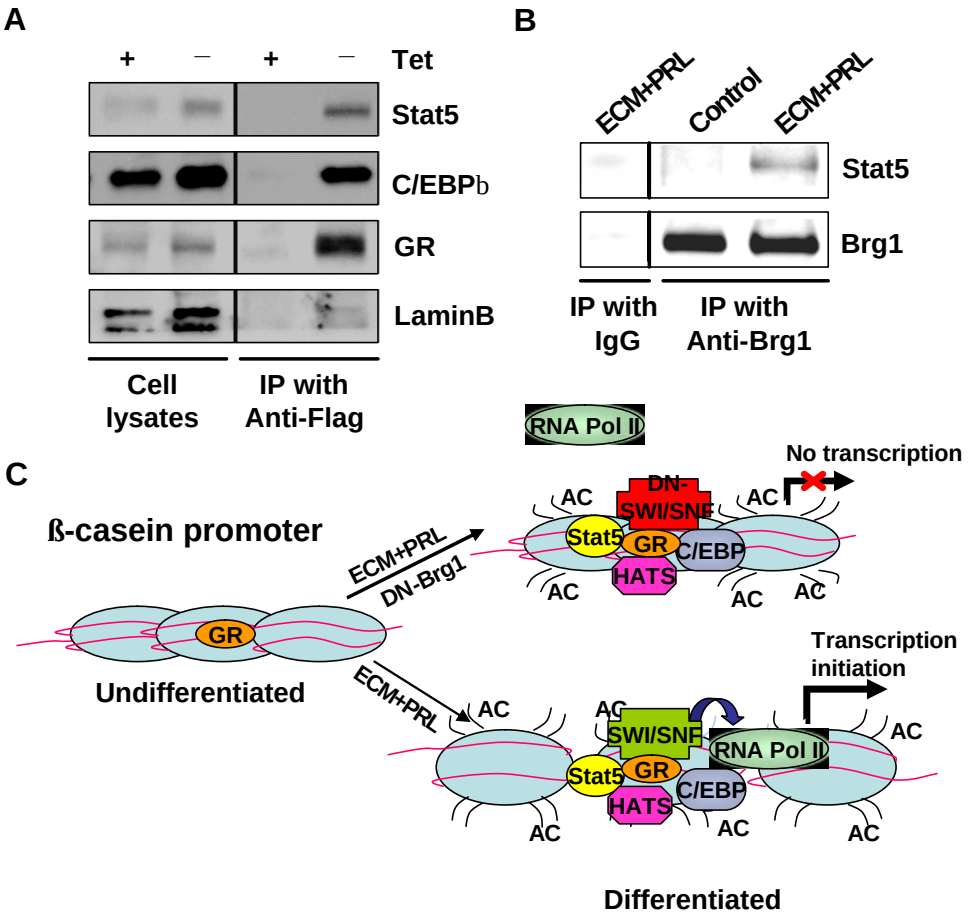
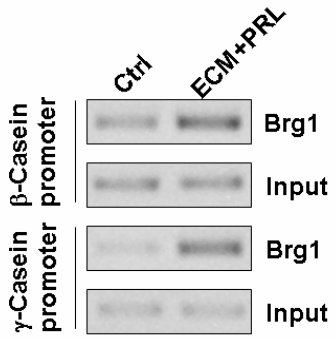

A
+
–
+
–
Tet
Stat5
C/EBPb
GR
LaminB
Cell lysates
IP with Anti-Flag
B
ECM+PRL
Control
ECM+PRL
Stat5
Brg1
IP with IgG
IP with Anti-Brg1
C
ß-casein promoter
GR
Undifferentiated
ECM+PRL
DN-Brg1
ECM+PRL
RNA Pol II
No transcription
AC
DN-SWI/SNF
Stat5
GR
C/EBP
HATS
Transcription initiation
SWI/SNF
RNA Pol II
Differentiated

Figure 5

Supplemental Figure 1



SFig 1: Binding of SWI/SNF complex to the β- and γ-casein promoters in primary mammary epithelial cells. Primary mammary epithelial cells were isolated from virgin CD-1 mice, and then were treated with prolactin and 2 % lrECM for 24 hours. ChIP assay followed by PCR to detect the binding of Brg1 in the ß-casein and γ-casein promoters.