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Lineage Specific Methylation of the *Elf5* Promoter in Mammary Epithelial Cells

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ABSTRACT

Recent characterization of mammary stem and progenitor cells has improved our understanding of the transcriptional network that coordinates mammary development; however, little is known about the mechanisms that enforce lineage commitment and prevent transdifferentiation in the mammary gland. The E-twenty six transcription factor Elf5 forces the differentiation of mammary luminal progenitor cells to establish the milk producing alveolar lineage. Methylation of the *Elf5* promoter has been proposed to act as a lineage gatekeeper during embryonic development. We used bisulphite sequencing to investigate in detail whether *Elf5* promoter methylation plays a role in lineage commitment during mammary development. An increase in *Elf5* expression was associated with decreasing *Elf5* promoter methylation in differentiating HC11 mammary cells. Similarly, purified mammary epithelial cells from mice had increased *Elf5* expression and decreased promoter methylation during pregnancy. Finally, analysis of epithelial subpopulations revealed that the *Elf5* promoter is methylated and silenced in the basal, stem cell-containing population relative to luminal cells. These results demonstrate that *Elf5* promoter methylation is lineage-specific and developmentally regulated in the mammary gland in vivo, and suggest that loss of *Elf5* methylation specifies the mammary luminal lineage, while continued *Elf5* methylation maintains the stem cell and myoepithelial lineages. STEM CELLS *2011;29:1611–1619*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The mammary epithelial hierarchy is hypothesized to consist of multipotent stem cells, lineage committed progenitor cells, and mature terminally differentiated cells. Regulation of this hierarchy by hormones and transcription factors underpins the morphological changes seen during postnatal mammary development [1]. In mice, mammary stem cell activity is maximal at mid pregnancy when the gland is undergoing alveolar proliferation in preparation for lactation. Hormonal signals received by mature luminal cells induce proliferation via paracrine feedback to basally located stem cells [2–4]. Transcription factors are then required for differentiation of mammary stem cells toward the luminal lineage.

We have previously identified the E-twenty six transcription factor, Elf5, as an important regulator of mammary alveolar development [5, 6]. Elf5 is not expressed in the stem cell enriched subpopulation of the mammary gland, but is expressed in both luminal progenitor and mature luminal cells. During pregnancy, *Elf5* deficient mammary glands fail to undergo alveolar morphogenesis and accumulate luminal progenitor cells. Conversely, forced expression of *Elf5* in virgin mice causes the formation of alveolar structures, milk production, and erosion of the luminal progenitor population. Together, these results demonstrate that Elf5 is required for the differentiation of luminal progenitor cells toward the alveolar lineage.

While transcription factors drive cellular differentiation, epigenetic modifications are hypothesized to maintain lineage commitment and prevent transdifferentiation [7]. An example of this principle is the specification of the trophectoderm (TE) and inner cell mass (ICM) at the early blastocyst stage of the embryo. Lineage determination is achieved by transcription factors that form a complex regulatory network with positive and negative feedback loops [8]. Stabilization of this transcriptional network is followed by epigenetic modifications to prevent transdifferentiation. For example, global de novo DNA methylation occurs in the ICM, while the TE remains hypomethylated. Elf5 also plays an essential role in this differentiation event, acting in the transcriptional network to maintain the TE

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lineage [9–11]. Interestingly, in a genome-wide screen for genes with differential methylation between the TE and ICM, *Elf5* was the only gene identified. *Elf5* is heavily methylated in the ICM preventing these cells from crossing into the TE lineage. Thus, methylation of the *Elf5* promoter appears to act as a unique gatekeeper of lineage determination in the blastocyst by providing a barrier to transdifferentiation.

Little is known about the role of epigenetic regulation in normal mammary development [12]. Bloushtain-Qimron et al. [13] have described cell type-specific expression and methylation patterns in normal human breast tissue, and the epigenetic modifiers, Pygo2 [14] and Bmi-1 [15], have been shown to influence the epithelial hierarchy. However, no studies have examined epigenetic control of transcription factors known to regulate mammary development. Therefore, we used bisulphite sequencing to determine whether *Elf5* promoter methylation is regulated during mammary gland development. Our results provide the first association between modifications in DNA methylation and changes in expression of a transcription factor that drives mammary development.

Materials and Methods

HC11 Cell Model

Cell culture reagents were obtained from Invitrogen (Carlsbad, CA, www.invitrogen.com) unless otherwise stated. HC11 cells (Nancy Hynes, Friedrich Miescher Institute, Basel Switzerland) were maintained in RPMI 1640 containing 10% fetal calf serum (FCS; heat inactivated 30 minutes at 50°C), 20 mM HEPES, 6 mM L-Glutamine, 5 μg/ml insulin (Sigma-Aldrich, St. Louis, MO, www.sigmaaldrich.com), 10 ng/ml epidermal growth factor (EGF; BioScientific, Gymea, Australia, www.biosci.com.au), 0.1125% $Na(CO_3)_2$, 50 U/ml penicillin, and 50 μg/ml streptomycin. HC11 cells were seeded in six-well plates at 1×10^5 cells per well (Day 0) and grown to 70%–80% confluence. On day 3, EGF was removed and on day 4 dexamethasone (dex; 0.5 μM; Sigma-Aldrich) and prolactin (Prl; 5 μg/ml; National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) were added. This treatment was repeated daily until day 8.

Transfection of short interfering RNA

HC11 cells were transfected with short interfering RNA (siRNA) targeting *Elf5* using Lipofectamine reagent (Invitrogen) on day 1 of the differentiation protocol (described above). siRNA molecules targeting *Elf5* were synthesized using the Silencer siRNA Construction Kit (Ambion, Austin TX, www.ambion.com), or were purchased from Dharmacon (*mElf5* On-Target plus #12; Lafayette CO, www.dharmacon.com). Control siRNA was either an siRNA molecule directed against green fluorescent protein (GFP), or the siGENOME RNA induced silencing complex (RISC)-free control from Dharmacon. A Mock transfection without siRNA was also included as a control.

Animals

All experiments involving mice were performed under the supervision of either the Garvan/St. Vincent's Animal Experimentation Committee or the Melbourne Health Research Directorate Animal Ethics Committee. Timed-mating was used to study animals at different stages of pregnancy. Pairs were cohoused in the afternoon and females were checked for a vaginal plug the following morning. In virgin animals oestrous staging was determined by vaginal smears stained using Diff Quick (Lab Aids, Narrabeen, New South Wales, Australia). Thoracic and inguinal mammary glands were dissected from virgin or pregnant mice at 8–12 weeks of age.

Preparation of Purified MECs

Diced mammary glands were subjected to three to four rounds of digestion with Collagenase Type L (Sigma-Aldrich) in RPMI/FCS medium (RPMI 1640, 10 mM HEPES buffer, 2.5% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 20 μg/ml gentamycin). The epithelial pellet was then filtered through sterile wire gauze and stored at −80°C until required for processing.

Cell Sorting

Mammary epithelial cell (MEC) suspensions were prepared as described previously [16]. For flow cytometry, antibodies against mouse antigens were purchased from BD Biosciences (San Jose, CA, www.bdbiosciences.com) unless otherwise specified. These included CD24-PE, biotinylated CD31, CD45, CD29-FITC (Chemicon, Temecula, CA, www.chemicon.com), CD61-APC (Caltag Laboratories, Burlingame, CA, www.caltagmedsystems.co.uk/caltag), and streptavidin-APC-Cy7. Cell sorting was performed using a FACS Aria (BD Biosciences). For immunohistochemistry (IHC) analysis of sorted cell populations, slides were prepared using a Shandon CytoSpin 4 Centrifuge (ThermoFisher Scientific, Waltham, MA, www.thermofisher.com). These cells were permeabilized in 0.2% Triton X-100 for 5 minutes at room temperature before proceeding with antigen retrieval and IHC staining as described below.

Western Blot Analysis

For Western blot analyses, cells were solubilized in lysis buffer (50 mM HEPES pH7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM pyrophosphate, and 100 mM NaF) containing protease inhibitor cocktail (Roche Castle Hill, Australia, www.roche.com). Protein concentration was determined using Protein Assay Dye Reagent (Bio-Rad, Hercules CA, www.bio-rad.com) before lysates were resolved by SDS/polyacrylamide gel electrophoresis using the NuPage precast gel system from Invitrogen. Proteins were transferred to polyvinylidene fluoride membranes, which were then blocked for 1 hour at room temperature with 1% bovine serum albumin (Sigma-Aldrich). Membranes were incubated with anti-milk (1:10,000; Accurate Chemical, Westbury, NY, www.accuratechemical.com) or anti-β-actin (1:40000; Sigma-Aldrich) primary antibodies overnight at 4°C. Specific binding was detected following 2 hours incubation with horseradish peroxidase conjugated secondary antibodies.

RNA Extraction and Real Time PCR Analysis

RNA was extracted using TRIZOL Reagent (Invitrogen) and purified using RNeasy Mini or Micro Spin columns with DNAse treatment (QIAGEN, Doncaster, Australia, www.qiagen.com). Single-stranded cDNA was produced using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, www.appliedbiosystems.com) or AMV reverse transcriptase (Promega, Madison, WI, www.promega.com). Quantitative polymerase chain reaction (PCR) was performed using TaqMan Gene Expression Assays (*Elf5* Mm00468732_m; *β2M* Mm00437764_m1; *keratin 18* Mm01601702_g1; *WAP* Mm00839913_m1; *β-casein* Mm00839664_m1; *Eomes* Mm01351985_m1) and the Prism 7900HT Sequence Detection System from Applied Biosystems or FastStart DNA master SYBR Green I enzyme mix and a Light Cycler instrument from Roche. The data were analyzed according to the $2^{-\Delta\Delta Ct}$ method [17] and are presented as fold change or $Log_{10}RQ$ (Relative Quantity). Statistical significance was determined using single-tailed Student's *t* tests or one-way analysis of variance (ANOVA) with Bonferroni or Tukey comparison, as appropriate.

DNA Extraction and Bisulphite Clonal Sequencing

The *Elf5* promoter is CpG rich, but does not satisfy the criteria for a CpG island (http://cpgislands.usc.edu). Bisulphite clonal sequencing was used to analyze the methylation status of four neighboring regions of the *Elf5* promoter. Genomic DNA was

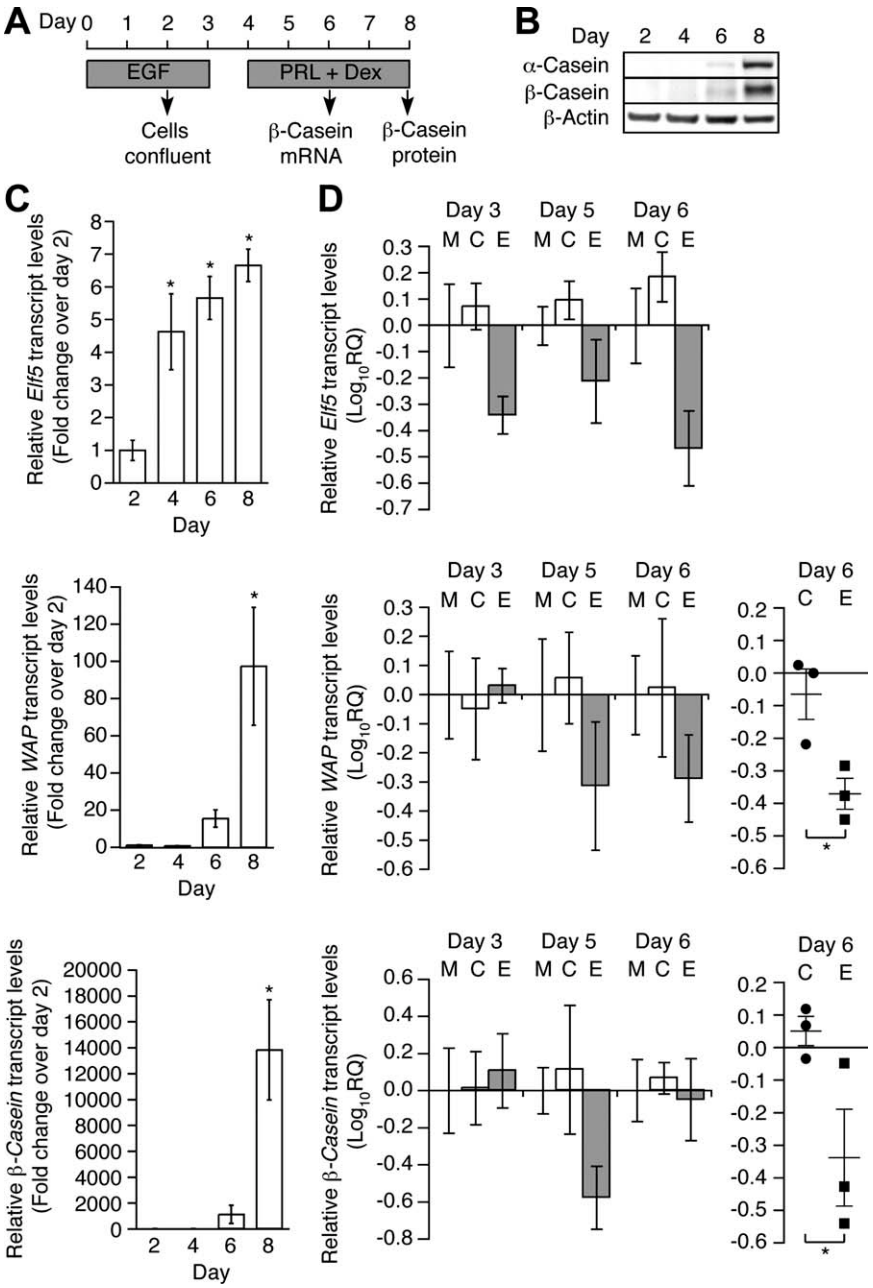

Figure 1. *Elf5* expression increases during, and contributes to, HC11 cell differentiation. **(A):** HC11 cells were treated as described in Materials and Methods. **(B):** Differentiation was confirmed by Western blot analysis of milk protein expression. **(C):** *β-casein, WAP*, and *Elf5* expression were analyzed using *β2M* as an internal control. Data represent means ± SEM for triplicate experiments; *, $p < .05$ (Bonferroni comparison following repeated measure analysis of variance). **(D):** HC11 cells were transfected with *Elf5* (E) or control (C) short interfering RNA on day 1 of the differentiation protocol. *β-casein, WAP*, and *Elf5* expression were analyzed using *β2M* or *ALAS1* as an internal control. Left panels: data (means with 95% confidence intervals for triplicate polymerase chain reaction reactions) from a representative experiment are presented relative to the mock transfected control (M) at each time point. Right panels: data are presented as means ± SEM for three independent experiments; *, $p < .05$ (*t* test). Abbreviations: Dex, dexamethasone; EGF, epidermal growth factor; PRL, prolactin.

extracted as described previously [18]. The bisulphite reaction was carried out for 6 hours at 55°C, under conditions described previously [18–21].

Bisulphite converted DNA was analyzed by bisulphite PCR analysis. Duplicate or triplicate PCR amplifications were performed using seminested bisulphite conversion specific primers listed in Supporting Information Table 1. The locations of the bisulphite PCR amplicons relative to the transcription start site (TSS) are summarized in Figures 2, 3, and 5. PCR amplifications were performed in a final volume of 25 μl containing 1–2 μl of bisulphite treated DNA, 200 μM deoxyribonucleotide triphosphate mix, 100 ng of each primer, 10x PCR Buffer without $MgCl_2$, 1.5 mM $MgCl_2$, 1.5 units of Platinum *Taq* DNA polymerase (Invitrogen) under the following conditions: 95°C for 4 minutes x one cycle; 95°C for 45 seconds, 57.3°C for 90 seconds, 72°C for 2 minutes x five cycles; 95°C for 45 seconds, 57.3°C for 90 seconds, 72°C for 90 seconds x 25 cycles; 72°C for 4 minutes x one cycle; hold at 4°C. The methylation status of the PCR amplicons was determined by bisulphite sequencing of the pooled PCR products to ensure representative clonal analysis.

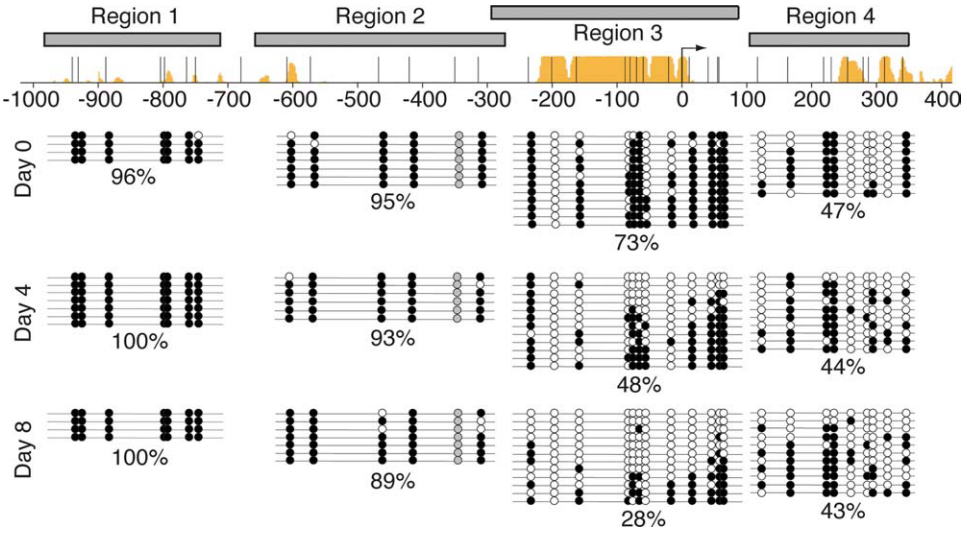

Figure 2. *Elf5* promoter methylation decreases during HC11 cell differentiation. Clonal bisulphite sequencing of the *Elf5* promoter. Vertical lines mark each CpG dinucleotide and the conservation score for 20 species of placental mammals (obtained using the UCSC genome browser; http://genome.ucsc.edu/) is superimposed in yellow. Each line represents an individual clone with open circles depicting unmethylated sites and solid circles indicating methylated sites. The CpG dinucleotide at −355 bp is polymorphic and absent where shaded gray.

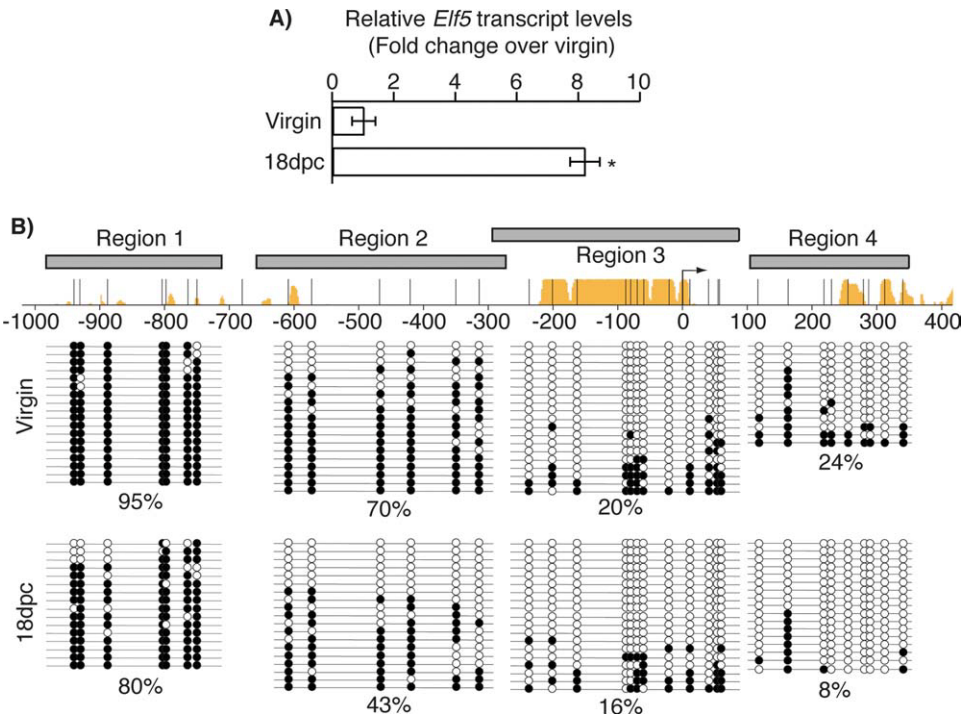

Figure 3. *Elf5* promoter methylation decreases during pregnancy. Epithelial cells were purified from mouse mammary glands as described in Materials and Methods. **(A):** *Elf5* expression was determined using *Keratin 18* as an internal control. Data are presented as means ± SEM for three independent experiments; *, $p < .001$ (*t* test). **(B):** Clonal bisulphite sequencing of the *Elf5* promoter. Vertical lines marks each CpG dinucleotide and the conservation score for 20 species of placental mammals (obtained using the UCSC genome browser; http://genome.ucsc.edu/) is superimposed in yellow. Each line represents an individual clone with open circles depicting unmethylated sites and solid circles indicating methylated sites.

Immunohistochemistry

Immunocytochemistry reagents were purchased from Dako (Produktionsvej, Denmark, www.dako.com) unless otherwise stated. Antigen retrieval was performed using pH9 target retrieval solution (S2367) at 125°C for 30 seconds. Slides were blocked in endogenous enzyme blocking solution and 2.5% horse serum prior to 1 hour incubation with goat anti-Elf5 primary antibody (1:600; Santa Cruz Biotechnology, Santa Cruz, CA, www.scbt.com) and 30 minutes application of ImmPress Goat (Vector Laboratories, Burlingame, CA, www.vectorlabs.com) secondary reagent. Visualization was via diaminobenzidine, and hematoxylin was used as a counter stain. All sections were imaged on a DMRB light microscope and DC200 camera from Leica Microsystems (Wetzlar, Germany, www.leica-microsystems.com).

RESULTS

The HC11 cell line represents an in vitro model of mammary cell differentiation where milk production can be induced by lactogenic hormones [22]. HC11 cells were treated as described in Materials and Methods (Fig. 1A), and cellular differentiation was confirmed by increased expression of the milk proteins, α-casein, and β-casein, from day 6 of the protocol (Fig. 1B). An increase in *Elf5* expression was evident by day 4 of the protocol, preceding induction of *β-casein* and whey acidic protein (*WAP*) expression (Fig. 1C). To determine whether Elf5 regulates HC11 differentiation, control (C) or *Elf5*-targeting (E) siRNA oligonucleotides were transfected at day 1 of the protocol. Reduced *Elf5* expression was accompanied by decreased *WAP* and *β-casein* expression in differentiating HC11 cells (Fig. 1D). This effect was statistically significant at day 6 despite the technical difficulties associated with variable and incomplete *Elf5* knockdown. Conversely, forced expression of Elf5 leads to increased *WAP* expression in differentiating HC11 cells (manuscript in preparation). Thus, *Elf5* expression increases during, and contributes to, HC11 cell differentiation. We next used HC11 cells to determine whether *Elf5* promoter methylation is present in mammary cells and regulated during functional differentiation. In undifferentiated HC11 cells, there was extensive methylation of the *Elf5* promoter region from −1000 bp to +400 bp with respect to the TSS (Fig. 2). Consistent with the rising levels of *Elf5* expression, there was a notable reduction in *Elf5* promoter methylation by day 4 of the protocol and an even more marked decrease at day 8. Region 3, which spans the TSS, showed the greatest decrease with the proportion of CpG dinucleotides that were methylated being 73%, 48%, and 28% at days 0, 4, and 8, respectively.

To determine whether *Elf5* promoter methylation is regulated during mammary differentiation in vivo, MECs were purified from virgin and pregnant mice. *Elf5* expression was significantly increased at 18 days postcoitus (dpc) relative to virgin levels in 12–13 week old mice (Fig. 3A). The extent of *Elf5* promoter methylation in purified MECs was more heterogeneous and not as extensive as in HC11 cells; however, a subtle difference was observed between the two samples (Fig. 3B). Region 3, which showed the greatest loss of methylation in HC11 cells, was poorly methylated in MECs with the virgin sample having only 20% methylation. Very little change was observed in this region in the differentiated mammary gland sample with 16% of CpG sites being methylated. In contrast, regions 1, 2, and 4 had decreased methylation in the pregnant sample with the total proportion of methylated CpG sites in these regions dropping from 64% to 40%.

A possible explanation for the mixed pattern of *Elf5* promoter methylation seen in the differentiating mammary gland could be the heterogeneity of epithelial cells present. The purification technique used excludes stromal cells but includes both luminal and myoepithelial cells. There have been no detailed studies of the Elf5 expression pattern throughout mammary development; therefore, we performed a detailed IHC analysis as shown in Figure 4. Approximately half of the luminal epithelial cells stained positive for Elf5 in virgin mice at both oestrus and dioestrus. By 4 dpc the majority of luminal cells stained positive for Elf5, while the myoepithelial cells remained negative. This pattern was maintained throughout pregnancy to 1 day post partum (dpp). By late pregnancy, the Elf5 positive luminal cells greatly outnumbered the Elf5 negative myoepithelial cells. Therefore, the decrease in *Elf5* promoter methylation seen at 18 dpc (Fig. 3B) may reflect a change in the cell types present.

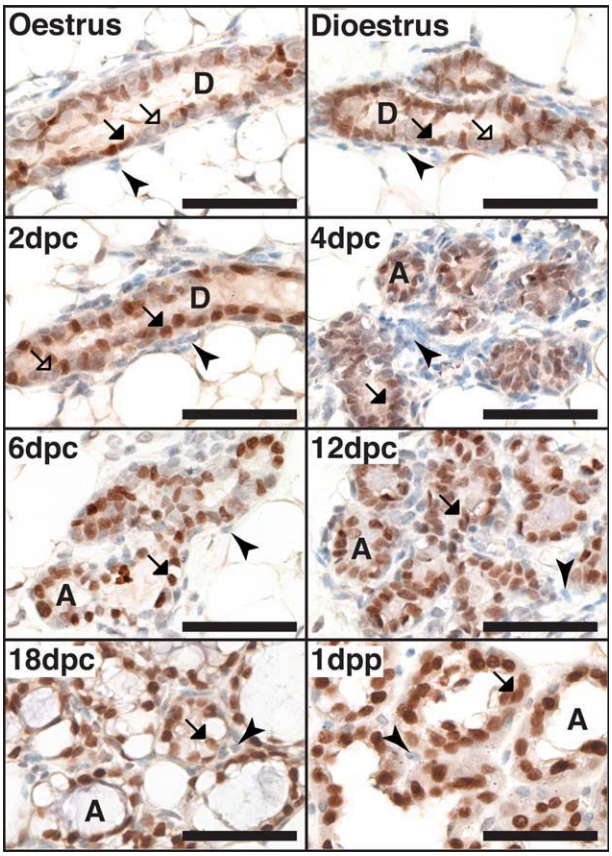

Figure 4. Elf5 expression in the mammary gland during pregnancy. Elf5 immunohistochemistry was performed on sections collected at different developmental stages. Examples of $Elf5^{+}$ luminal cells (solid arrows), $Elf5^{-}$ luminal cells (hollow arrows), and basal cells (solid arrowheads) are indicated. Scale bars = 50 μm. Abbreviations: A, alveolar lumen; D, duct lumen; dpc, days post-coitus; dpp, days post-partum.

To determine the cell type-specific pattern of *Elf5* promoter methylation, fluorescence activated cell sorting was used. Basal cells, including myoepithelial and stem cells ($CD24^{+}CD29^{hi}$), luminal progenitor cells ($CD24^{+}CD29^{lo}CD61^{+}$), and mature luminal cells ($CD24^{+}CD29^{lo}CD61^{-}$), were purified from virgin mouse mammary tissue (Fig. 5A). Real time PCR analysis demonstrated that *Elf5* expression is low in the basal cell subset (Fig. 5B), and IHC analysis confirmed that these cells do not express Elf5 (Fig. 5C). *Elf5* expression was greater in mature luminal cells compared with basal cells, and was significantly increased in the luminal progenitor population (Fig. 5B). Both luminal populations consisted of a heterogeneous mix of Elf5 positive and negative cells (Fig. 5C), consistent with the results shown in Figure 4. The *Elf5* promoter was more extensively methylated in basal cells than in the two luminal subpopulations (Fig. 5D). Region 2 showed the greatest difference with 88% methylation in basal cells compared with 29% and 21% in the luminal progenitor and mature luminal cells, respectively. The significant difference in *Elf5* expression between luminal progenitor and mature luminal cells was not associated with dramatic changes in the proportion of Elf5 positive cells or in the level of *Elf5* promoter methylation. This result suggests that promoter methylation is not the only regulator of *Elf5* expression, and that other factors may modulate the magnitude of Elf5 expression within the luminal epithelial population.

As luminal progenitor cells are scarce during pregnancy [23], basal ($CD24^{+}CD29^{hi}$) and total luminal ($CD24^{+}CD29^{lo}$)

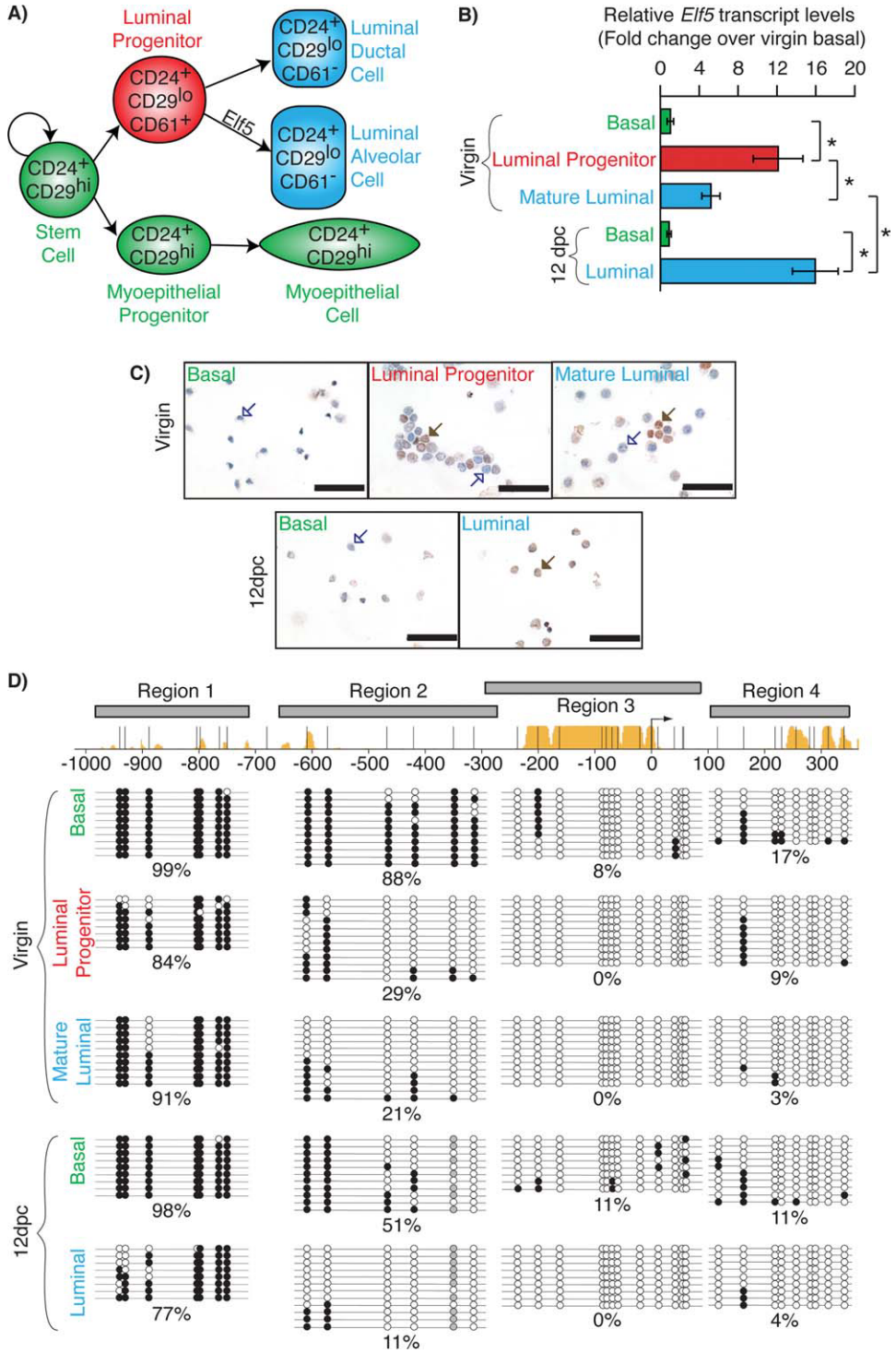

Figure 5. *Elf5* promoter methylation is lineage specific in the mammary epithelium. Luminal progenitor cell, mature luminal cell, and basal cell subpopulations were purified from mouse mammary glands using the cell surface markers shown in **(A)**. **(B):** *Elf5* expression was determined using *β2M* as an internal control. Data are presented as means ± SEM for at least three independent experiments; *, $p < .05$ (Tukey comparison following one-way analysis of variance). **(C):** Elf5 immunohistochemistry was performed on cytospins of sorted cell populations. Solid brown arrows indicate Elf5 positive cells and hollow blue arrows indicate Elf5 negative cells. Scale bars = 50 μm. **(D):** Clonal bisulphite sequencing of the *Elf5* promoter. Vertical lines mark each CpG dinucleotide and the conservation score for 20 species of placental mammals (obtained using the UCSC genome browser; http://genome.ucsc.edu/) is superimposed in yellow. Each line represents an individual clone with open circles depicting unmethylated sites and solid circles indicating methylated sites. The CpG dinucleotide at −355 bp is polymorphic and absent where shaded gray.

cells were collected from glands at 12 dpc. As in the virgin sample, *Elf5* was low in the basal cell population, but was strongly expressed in the luminal cell subset (Fig. 5B). This corresponded to an absence of Elf5 in the basal population and Elf5 expression in the vast majority of luminal cells (Fig. 5C), consistent with the results shown in Figure 4. *Elf5*

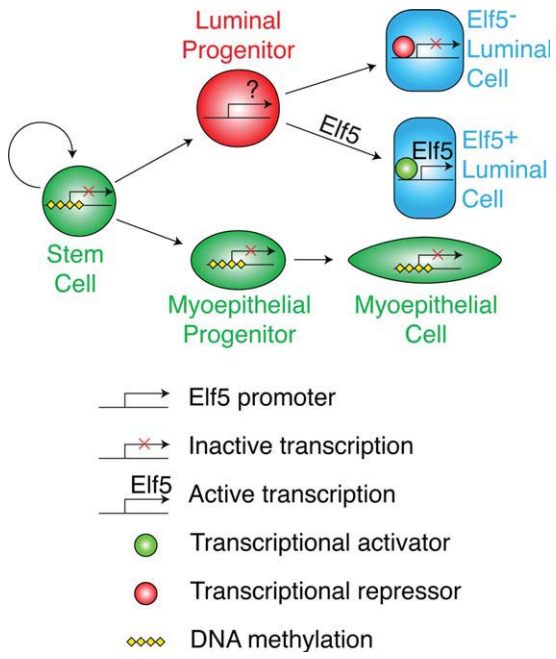

Figure 6. Lineage specific *Elf5* promoter methylation and the mammary epithelial hierarchy. The *Elf5* promoter is methylated in the stem cell containing basal fraction. Mammary stem cells must downregulate *Elf5* methylation to differentiate into luminal progenitor cells. In mature luminal cells, transcriptional activators and repressors mediate hormonal regulation of *Elf5* expression.

methylation was also reduced in luminal as compared with basal cells in the pregnant sample (Fig. 5D). Once more, region 2 showed the greatest difference with 51% methylation in basal cells and 11% methylation in luminal cells.

Elf5 expression was significantly higher in luminal cells during pregnancy than in the mature luminal cells from virgin mice. This increase is consistent with the results shown in Figure 3A and reflects the increased proportion of luminal cells expressing Elf5 during pregnancy (Figs. 4, 5C). There was no change in the level of *Elf5* expression in the basal compartment during pregnancy, but there was a decrease in *Elf5* methylation in this population. Within region 2 methylation dropped from 88% in the virgin sample to 51% at 12 dpc (Fig. 5D). There was also a decrease in *Elf5* methylation in luminal cells during pregnancy with methylation of region 2 being 21% in the virgin sample and 11% at 12dpc.

Discussion

Methylation of the *Elf5* promoter has been proposed to act as a lineage gatekeeper during embryonic development [11], and Elf5 acts in the mammary gland to specify the alveolar lineage [5]. Using bisulphite sequencing, we have investigated *Elf5* promoter methylation in the context of mammary development. We first used the HC11 in vitro model to demonstrate that *Elf5* expression increases during, and contributes to MEC differentiation (Fig. 1). The increase in *Elf5* expression during HC11 differentiation was associated with a loss of *Elf5* promoter methylation (Fig. 2). Primary MECs were then purified from virgin and pregnant mice to study *Elf5* methylation during mammary development in vivo. We observed an overall increase in *Elf5* expression and decrease in promoter methylation in MECs during pregnancy (Fig. 3), consistent with an increased proportion of luminal epithelial cells expressing Elf5 (Fig. 4). Finally, we examined sorted epithelial subpopulations to reveal that *Elf5* is methylated and silenced in the basal, stem cell-containing population relative to luminal cells (Fig. 5).

Together, these results demonstrate that *Elf5* promoter methylation is lineage-specific and developmentally regulated in the mammary gland in vivo. The basal cell fraction exhibits higher promoter methylation and lower *Elf5* expression than luminal cells (Fig. 5). During pregnancy, the proportion of *Elf5* expressing luminal cells increases relative to the basal population, so there is an overall decrease in *Elf5* promoter methylation (Fig. 4). These findings may have important implications for the MEC hierarchy. We speculate that *Elf5* promoter methylation established in embryonic stem cells [11] is carried through to mammary stem cells residing in the basal compartment. Mammary stem cells must then downregulate *Elf5* promoter methylation to differentiate towards the luminal lineage (Fig. 6). Meanwhile, continued methylation of the *Elf5* promoter may maintain the myoepithelial and stem cell lineages. As stem cell activity is maximal at mid pregnancy [2], the decreased *Elf5* promoter methylation we observed in basal cells during pregnancy (Fig. 5D) may reflect an increase in the proportion of cells transitioning toward the luminal lineage. Further experiments are required to demonstrate that *Elf5* promoter methylation directly prevents differentiation of mammary stem cells into the luminal lineage.

It is also evident from these studies that promoter methylation is not the sole determinant of *Elf5* expression. The increase in *Elf5* expression in mature luminal cells during pregnancy was associated with a moderate decrease in *Elf5* promoter methylation (Fig. 5B, 5D). However, there was no difference in *Elf5* methylation between the luminal progenitor and mature luminal cells from virgin mice despite statistically significant differences in *Elf5* expression levels (Fig. 5B, 5D). Furthermore, both the luminal progenitor and mature luminal populations had mosaic Elf5 expression in virgin mice, despite their homogeneous patterns of *Elf5* promoter methylation (Fig. 5C, 5D). These results suggest that transcriptional mechanisms act in luminal epithelial cells to determine which cells express *Elf5* and to modulate the level of *Elf5* expression. We have demonstrated previously that hormonal stimuli can induce *Elf5* expression in luminal epithelial cells, but the transcriptional mechanisms underlying these effects remain to be elucidated [24–26]. In Elf5 negative luminal cells, transcriptional repressors may suppress *Elf5* expression in the absence of promoter methylation. A potential candidate is the estrogen receptor (ER), as Elf5 is expressed in ER negative luminal epithelial cells [5, 27], and a potential DNA binding site for Elf5 has been identified near the *ER* gene [28]. In summary, loss of promoter methylation appears to be a prerequisite for transcriptional induction of *Elf5* expression in a subset of luminal epithelial cells (Fig. 5). Loss of *Elf5* promoter methylation may specify the luminal lineage, while *Elf5* expression drives alveolar differentiation.

It is interesting to note that the pattern of *Elf5* promoter methylation differs between HC11 cells and primary mouse MECs. HC11 cells possess extensive methylation across all four regions, with region 3 (spanning the TSS) exhibiting the greatest change in methylation upon cellular differentiation (Fig. 2). In comparison, MECs are predominantly methylated at region 1, lack methylation at regions 3 and 4, but exhibit differential methylation at region 2 (Figs. 3B, 5D). The biological significance of differences between

HC11 and primary cells remains unclear. One possibility is that HC11 cells may have acquired increased methylation of the *Elf5* promoter during adaptation to growth in tissue culture. Methylation at region 3 may be regulated during HC11 cell differentiation simply because these cells begin with a higher level of overall baseline methylation. In MECs the absence of methylation at region 3 may make region 2 more susceptible to alterations in DNA methylation. To explore this possibility, we have performed a direct comparison of *Elf5* expression in HC11 and primary cells. *Elf5* is expressed at similar levels in HC11 cells and sorted basal MECs, with expression being substantially higher in luminal MECs (Supporting Information Fig. 1). Thus, the relatively high level of *Elf5* promoter methylation in HC11 cells is consistent with their relatively low level of *Elf5* expression.

Our findings also demonstrate that processes involved in embryonic development can be adapted for later reuse in specific organs. A gain in *Elf5* methylation in the ICM of the blastocyst is proposed to prevent transdifferentiation to the TE. In the mammary gland, sustained *Elf5* methylation in myoepithelial and stem cells may prevent their transdifferentiation to the luminal lineage. During TE specification Elf5 cooperates with Cdx2 and Eomes in a transcriptional network. Cdx2 activates the *Elf5* promoter, and Elf5 can in turn bind and activate the *Cdx2* and *Eomes* promoters in a positive feedback loop [11]. This transcriptional network does not appear to be active in the mammary gland, however, as *Cdx2* is not expressed in mammary cells [29] (data not shown) and *Eomes* is not enriched in mammary luminal cells [30] (Supporting Information Fig. 2). Further work is required to determine whether Elf5 forms a positive feedback loop with other transcription factors to enforce alveolar cell fate in the mammary gland. A likely candidate is signal transducer and activator of transcription 5 (STAT5), which, like Elf5, is essential for alveolar development during pregnancy [31, 32]. STAT5a/b deficient mammary glands display depleted luminal progenitor cells in virgin animals, and Elf5 expression in luminal progenitor cells is STAT5 dependent [33]. In addition, Elf5 has been shown to bind the *STAT5* promoter [6], suggesting that a positive feedback loop may exist between the two transcription factors.

Conclusion

In conclusion, the *Elf5* promoter displays lineage specific methylation during mammary development. This is the first example of a lineage specific epigenetic mark to be associated with a transcription factor that governs mammary cell fate. Further experiments are required to delineate the direct and indirect mechanisms linking DNA methylation, Elf5 expression, and luminal cell differentiation. We propose that loss of *Elf5* methylation specifies the mammary luminal lineage while continued *Elf5* methylation maintains the myoepithelial and stem cell lineages.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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