Published in final edited form as:

J Mammary Gland Biol Neoplasia. 2010 March ; 15(1): 85–100. doi:10.1007/s10911-010-9170-4.

The Epigenetic Landscape of Mammary Gland Development and Functional Differentiation

Monique Rijnkels

USDA/ARS Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, TX, USA

Elena Kabotyanski

Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA

Mohamad B. Montazer-Torbati

Department of Animal Science, Faculty of Agriculture, University of Birjand, Birjand, Iran

C. Hue Beauvais

INRA, UR1196 Génomique et Physiologie de la Lactation, 78352 Jouy-en-Josas, France

Yegor Vassetzky

Université Paris-Sud 11 CNRS UMR 8126, Institut de Cancérologie Gustave-Roussy, 94805 Villejuif cedex, France

Jeffrey M. Rosen

Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA

Eve Devinoy

INRA, UR1196 Génomique et Physiologie de la Lactation, 78352 Jouy-en-Josas, France

Abstract

Most of the development and functional differentiation in the mammary gland occur after birth. Epigenetics is defined as the stable alterations in gene expression potential that arise during development and proliferation. Epigenetic changes are mediated at the biochemical level by the chromatin conformation initiated by DNA methylation, histone variants, post-translational modifications of histones, non-histone chromatin proteins, and non-coding RNAs. Epigenetics plays a key role in development. However, very little is known about its role in the developing mammary gland or how it might integrate the many signalling pathways involved in mammary gland development and function that have been discovered during the past few decades. An inverse relationship between marks of closed (DNA methylation) or open chromatin (DnaseI hypersensitivity, certain histone modifications) and milk protein gene expression has been documented. Recent studies have shown that during development and functional differentiation, both global and local chromatin changes occur. Locally, chromatin at distal regulatory elements and promoters of milk protein genes gains a more open conformation. Furthermore, changes occur both in looping between regulatory elements and attachment to nuclear matrix. These changes are induced by developmental signals and environmental conditions. Additionally, distinct epigenetic patterns have been identified in mammary gland stem and progenitor cell sub-populations. Together, these findings suggest that epigenetics plays a role in mammary development and function. With the new tools for epigenomics developed in recent years, we now can begin to establish a framework for the role of epigenetics in mammary gland development and disease.

Keywords

Mammary gland; Epigenetic; Milk protein genes; Chromatin; Development

Introduction

Mammary gland morphogenesis begins during embryonic development and proceeds postnatally through puberty, pregnancy, lactation, and subsequent involution. Most of the development and functional differentiation in the mammary gland, therefore, occurs after birth. During three major developmental windows—puberty, pregnancy, and involution—the gland undergoes profound morphological and functional changes [1]. These changes correspond to periods of cell proliferation, apoptosis, and differentiation in conjunction with changes in gene expression patterns [2–8] and are regarded as a succession of cell fate determinations [9]. During the past decades, we have gained knowledge about the numerous signaling pathways involved in establishing these expression patterns and morphological changes, which have been reviewed by Watson and Khaled [10].

Epigenetics has been defined as the “stable alterations in gene expression potential that arise during development and proliferation” [11]. These alterations have been shown among others to be involved in development of the central nervous system [12], the pancreas [13], the liver [14], and the male or female reproductive organs, [15] and during differentiation of hematopoietic progenitors and T-helper-cells [16–20]. Therefore, such epigenetic modifications can be expected to play a role during mammary gland development, as well. Furthermore, epigenetics also may be defined as “the manifestation of a phenotype, which can be transmitted to the next generation of cells or individual, without alterations to the DNA sequence (genotype)” [21]. In general, epigenetics has been interpreted in the context of changes to the chromatin but could be interpreted more widely to include any external effect on the phenotype (epigenator).

Mammary gland development enables lactation to occur after parturition, and lactation performances in domestic animals have been largely improved in ruminants by genetic selection [22]. However, the environment during mammary gland development from fetal life to pregnancy and lactation, also can influence lactation in genetically selected animals, thus altering the expected performances of an animal [23]. The resulting phenotype is, therefore, not only related to the genotype of the animal but might be related to epigenetic modifications of the genome, resulting in a specific epigenotype.

At the biochemical level, epigenetic changes lead to alterations in chromatin conformation. These changes in chromatin are brought about by DNA methylation (DNAm) [24], histone variants [25], post-translational modifications of the core and N-terminal tails of histones [26,27], non-histone chromatin proteins [27–29], and non-coding RNAs (nc RNA) [30].

Large-scale chromatin conformation represents another level of epigenetic regulation. Experimental evidence in eukaryotic cells suggests that bending and looping of chromatin facilitates specific genomic interactions over distance [31,32]. These interactions may occur between transcription activators bound to enhancers and transcription machinery at the promoter, they can also insulate a gene domain from the action of a repressive chromatin environment.

The mechanisms involved in epigenetics can be summarized in several steps [21]. First, influences coming from outside the cell, such as a differentiation signal, environmental influences, and nutrition, can be considered as the “epigenator signal,” which is defined by

protein-protein interactions. These external signals could generate an “epigenetic initiator signal,” which will determine where the modification will occur. The epigenetic initiator signal can be DNA-binding factors or ncRNAs. The epigenetic state then will be sustained through cell divisions, with the contribution of an “epigenetic maintainer signal” such as histone/DNA modifiers (enzymatic activities that convey modifications) or histone variants. Based on this paradigm, we can see how direct transcriptional regulation and cell memory regulation use similar mechanisms to change chromatin status (chromatin conformation) and affect gene transcription, both in the short term and in the long term.

Chromatin conformation is expected to play a key role in transcriptional regulation during mammary gland development. However, the precise changes in chromatin conformation/compaction involved in mammary gland development and differentiation are not well known. The mammary gland is an excellent model to study these processes because of its postnatal development and differentiation. Its easy access and the possibility of performing tissue reconstitution experiments also offer distinct advantages. Finally, the availability of numerous genetically engineered mouse models renders it an especially attractive model for studying specific changes in chromatin conformation. Despite these advantages, very little is known about chromatin status in the developing mammary gland and how it might integrate the many signaling pathways discovered during the past few decades, which are involved in mammary gland development and function.

Almost four decades ago, Marzluff and McCarty [33] reported that the acetylation of what we now know as Histone H3 and H4 in mammary tissue was influenced by hormones and that this process was reversible and correlated with RNA transcription. They postulated that the reversible acetylation of histones in mouse mammary explants could play a role in transcriptional regulation by modifying DNA-histone interactions. Similarly, Hohmann and Cole [34] observed differential lysine incorporation into histone fractions under the influence of lactogenic hormones. They hypothesized that these hormones regulate intracellularly the structure of new chromatin as it is being formed, a process that has been well established in non-mammary developmental systems.

It has taken 3 decades to return our attention to the changes in histone modifications and chromatin during mammary gland development and functional differentiation. This renewed interest is attributable in part to new technologies and the availability of complete genome sequences. These technologies enable us to study chromatin compaction, DNA methylation, and histone post-transcriptional modifications at specific genomic locations and, more recently, on a genome wide scale in more detail and more quantitatively than previously was possible [35–42].

In this review, we summarize what is known about chromatin conformation and epigenetic modifications during normal mammary gland development and functional differentiation as marked by the expression of milk protein genes, such as casein and WAP. Furthermore, we address emerging data on epigenetics in mammary stem and progenitor cells.

Changes in Epigenetic Marks and Chromatin Conformation During Mammary Gland Development

DNAse and DNaseI hypersensitivity (DHS) have been used since the 1980s to assess the correlation between chromatin conformation and gene expression [43–47]. Several researchers have investigated these aspects of chromatin conformation in the mammary gland in relation to the expression of individual milk protein genes. Lately, with the availability of more advanced tools, there is a renewed interest.

DNA Methylation

Global DNAm has been associated with mammary cell differentiation in vitro; its inhibition by 5-aza-2'deoxyctidine during cell replication prevented the maximal differentiation of the cell normally observed in the absence of the drug [48]. Several studies have reported the inverse correlation between expression of individual genes and DNAm in the lactating mammary gland and other tissues. Johnson and colleagues [49] showed that certain DNAm sensitive restriction sites in the rat β - and γ -casein genes from the lactating mammary gland are readily digested, whereas in liver DNA these sites resisted digestion, indicating hypomethylation of the DNA in the lactating mammary gland compared to the liver. This finding correlated with gene expression. They also showed a similar inverse correlation with casein expression in tumor sub-populations. They raised the question whether lactogenic hormones might alter the DNAm status of these genes. Similarly, the mouse kappa-casein gene is hypomethylated in lactating mammary glands but is hypermethylated in non-mammary tissue and non-lactating tissue [50]. Platenburg and colleagues [51] showed that sites flanking the bovine α s1-casein genes were specifically hypomethylated in the mammary gland and that hypomethylation of one site in the promoter region was associated with gene expression. Vanselow and others [52] further showed that a site located further upstream in a regulatory region ~10 kb distal to the bovine α s1-casein gene was hypomethylated with mammary gland development and showed hypermethylation upon mastitis. Singh and colleagues (this issue [53]) report the hypermethylation of this site and closely associated sites upon involution.

Recently, we examined the tissue- and developmental stage-specific DNAm status in the mouse casein gene cluster region (Fig. 1a) and found that in addition to the promoters of the casein genes, potential distal regulatory elements (DRE) show lower levels of DNAm in lactating mammary gland compared to non-mammary tissue (Fig. 1c). Upon further analysis of mammary epithelial cells (MEC) isolated from mammary gland at different stages of development and differentiation, we found that for the promoters these lower DNAm levels correlated with the major induction of gene expression during pregnancy, whereas several potential DRE were either hypomethylated at any stage in MEC compared to non-MEC or already acquired lower methylation levels during pubertal development (Rijnkels et al in preparation [54]). A similar relationship between the methylation profile of a gene and its expression has been described for other major milk protein genes.

Dandekar and colleagues [55] demonstrated hypomethylation of CpGs within the coding region of what is now known to be the WAP gene [56] in lactating mammary gland, whereas these sites were hypermethylated in a tumor cell line that does not express the gene. More recently, Montazer-Torbati and colleagues [57] studied larger regions around the rabbit WAP gene compared to the mouse gene (Fig. 2a,b) and showed that regulatory regions flanking the gene have lower methylation levels in the lactating mammary gland compared to liver (Fig. 2d). These regions with lower DNAm levels include the promoter and a hormone-responsive distal hypersensitive site (HSS2) located at -6 kb from the transcription start site (Fig. 2c). Based on preliminary results, the hypomethylated profile of the gene seems to exist already after the first trimester of pregnancy (day 8 in the rabbit) (Montazer-Torbati unpublished data).

Several studies indicate that DNAm levels of genes expressed in the mammary gland decrease in conjunction with functional differentiation of the gland ([49,50,52,54,55,57]; Rijnkels et al in preparation). However, in most experiments, except in our more recent studies, whole mammary tissue was used, which could skew the data as a result of the cell heterogeneity in early mammary gland developmental stages. However, the fact that hypomethylation of milk protein gene regions is observed only in the mammary gland and that it is specific to lactation or earlier developmental stages, whereas hypermethylation is

observed in other tissues and other stages, confirms that the hypomethylated DNA has a mammary epithelial origin. Furthermore, this finding suggests that the extent of hypomethylation often is underestimated.

Milk protein gene expression seems to occur after a progressive demethylation of their regulatory elements, which occurs in pregnancy and puberty. The existence of active demethylation mechanisms is still a matter of debate [58]. In the mammary gland, the extensive cell proliferation, which occurs during puberty, pregnancy, and early lactation, [59–61] could lead to passive demethylation of milk protein gene regulatory elements.

Chromatin Conformation Inferred from DNA Sensitivity to DNaseI

Chromatin conformation can be evaluated by the use of small molecules, which can penetrate open chromatin conformation and reach the DNA. DNaseI has been widely used in such experiments and has allowed researchers to identify open chromatin conformation by the presence of DNA cleavage sites, which are hypersensitive to the digestion. Several investigators have used DNaseI to study chromatin conformation in the mammary gland during lactation. Whitelaw and colleagues [62] have reported the presence of a strong mammary-specific hypersensitive site in the proximal regulatory region of the ovine β -lactoglobulin (BLG) gene. Later studies have shown that this proximal site appears early in pregnancy, whereas a second, much weaker DHS located around -2 kb from the transcriptional start site is detected only during lactation [63]. Interestingly, the proximal DHS overlaps with Stat5 binding sites. However, even though the appearance of this DHS correlates with Stat5 activation, its presence was not dependent on the interaction of Stat5 with the underlying Stat5 binding site [64]. Two other, even weaker DHS, located within the first 2 introns of the gene, were detected only in virgin animals [65].

In the ovine Acetyl-CoA Carboxylase- α (ACC) [66] gene, two DHS have been detected during lactation within a 1.6 kb region upstream from the transcription start site and have been associated with the recruitment of both Stat5 and SREBP1.

Similar results obtained for the rat, rabbit, and mouse WAP gene have revealed the presence of sites in distal as well as in proximal regulatory regions [67–69], respectively. These sites are located much further upstream than in the BLG or ACC genes, up to -7 kb from the transcription start sites (Fig. 2c). Three of them are not detected in a tissue that does not express WAP. In the rabbit mammary gland, the different sites progressively appear during pregnancy and the most distal site disappears after weaning (Fig. 2c). Their appearance can be induced ex vivo in the mammary gland of pregnant rabbits by lactogenic hormones.

In the casein gene cluster, we have shown the co-localization of DHS (Fig. 1c) with evolutionary conserved regions (ECR, Fig. 1b)—potential DRE—and promoters of the casein genes in late pregnant and lactating mammary gland tissue [54,70] (and Rijnkels in preparation). Furthermore, some ECR/DRE already display DHS in MECs in mature virgin tissue. This finding indicates that an open chromatin conformation at these regions in the lactating mammary gland corresponds to gene expression and would indicate a functional role for the DRE.

The above results obtained for different milk protein genes indicate that lactogenic hormones can induce an open chromatin conformation at regulatory regions, which correlates with gene expression. However, some regions attain an open conformation earlier in mammary gland development (Figs. 1 and 2). DHS often overlap with binding sites for transcription factors such as STAT5, known to be important for mammary gene expression, even though their binding is not necessarily required for DHS formation. This finding suggests that DHS formation might facilitate binding of such factors once activated or,

alternatively, that a low level of activated transcription factors can bind to high affinity binding sites and induce an opening of the chromatin, which then may extend to neighboring low affinity binding sites. It also indicates that during development, different chromatin conformations at different potential regulatory regions contribute to gene regulation through either repression or activation.

Post Translational Modifications of Histones

Histone acetylation was one of the first modifications to be shown many decades ago to correlate with active gene transcription and so-called open chromatin [71]. More recently, histone acetylation has been shown to correlate with active regulatory elements and actively transcribed genes [72–74]. Since changes of histone acetylation during mammary gland development were first observed, a full battery of antibodies has been developed to study specific histone modifications. Chromatin ImmunoPrecipitation (ChIP) now enables us to identify specific genomic regions that are enriched for such modifications.

Using these new tools, we investigated the presence of histone H3 acetylation (H3Ac) at the promoters and a number of potential DREs / ECRs [70] in the mouse casein gene cluster in lactating mammary gland tissue and liver. We found a positive correlation of enrichment of H3Ac at both gene promoters and several ECRs with gene expression (Fig. 1e), [54] (Rijnkels et al in preparation). Similar results were obtained for the mouse WAP promoter and HSS2 (Fig. 2e). Currently, these studies are being extended to whole genome analysis for several different histone modifications using ChIP-sequencing. This research should provide new insight into the genome-wide changes in histone modifications and their correlation with gene expression patterns in order to develop a framework for the contribution of chromatin changes to gene transcription and development.

Jolivet and colleagues [75] showed in rabbit primary MEC histone H4 hyperacetylation on a distal regulatory region (–3.4 kb) of the α s1-casein gene under the influence of extracellular matrix (ECM). However, in human S1 cells, functional differentiation associated with the formation of polarized 3D structures actually showed a decrease in global H4 acetylation [48]. This finding was associated with a reduction in overall gene transcripts and probably illustrated the global silencing of genes not needed for functional differentiation status and lactation. These results do not preclude local hyperacetylation at regions involved in tissue-specific gene regulation as found for the milk protein genes.

Chromatin and Gene Transcription in Mammary Epithelial Cell Lines

Chromatin Loop Domains and Attachment to Nuclear Structure

The three-dimensional interaction of chromatin loops is another way chromatin conformation can influence the transcriptional potential of a gene or larger genomic region [32]. The development of the chromatin conformation capture (3C) [76] technique and high-throughput variations of this technique (4C [77,78], 5C [79]) has enabled the study of such higher order interactions in the nuclei of cells. Several studies have shown inter-chromosomal and intrachromosomal interactions between different regions in the genome during development [77,79–82].

Kabotyanski and colleagues [83] showed that lactogenic hormones induce the physical interaction between the β -casein gene promoter and the β -casein upstream enhancer (BCE) [70,84–86]. This interaction as well as gene transcription could be inhibited by progesterone-induced PR binding to the promoter [83,87]. These findings in HC11 cells and primary MECs were substantiated further by the fact that this interaction was much more prevalent in MECs isolated from lactating mammary gland than in those isolated from virgin mice. We now have shown in HC11 cells that this interaction is lost upon withdrawal of

lactogenic hormones in concordance with the decrease in β -casein gene expression, indicating that this reversible interaction is directly correlated with gene transcription (Fig. 3).

On a more global scale, the chromatin loops are packed into the nucleus, and their attachment to nuclear structures changes with both development and transcription potential [88,89]. Although the existence of a nuclear matrix per se remains a matter of debate, nuclear structure may play an important role in this process [90]. The attachment of the chromatin fiber to nuclear structures can be visualized under experimental conditions when nuclei are incubated in hypertonic media in the presence of mild detergents. Soluble proteins, which maintain the chromatin structure in its native state, then are extracted and the chromatin fiber unfolds. Loops then can be visualized using fluorescent in situ hybridization [90].

Using this approach on mammary gland nuclei extracted from mouse mammary cells (HC11) with or without lactogenic induction of milk protein genes, Ballester and colleagues [91] concluded that the size of chromatin loops decreased when casein and WAP gene expression were induced. We have recently confirmed this result by a biochemical assay [92] that allowed us to visualize the regions of the WAP locus interacting with the nuclear matrix (see Table 1, Fig. 4, Devinoy unpublished data). Altogether, those results show that along chromosomes, not only the genome is organized in domains with different chromatin conformation, but that the folding of this chromatin fiber in chromatin loops also plays a key role in the regulation of gene expression.

Modifications of Epigenetic Marks and Binding of Transcription Factors

Marzluff and colleagues [33] and Hohmann and Cole [34] suggested in the early 1970s that chromatin changed under influences of lactogenic hormones. Johnson and colleagues [49] expressed in the early 1980s that it would be of great interest to determine if any of the actions of lactogenic hormones is to alter the DNAm status of the casein genes. Since then we have learned more about how lactogenic hormones regulate gene transcription [93–95], and recently we have been able to study how they specifically direct recruitment of their respective signal transducers to regulatory elements in the DNA and study associated changes in histone modifications [75,83,87,96–98].

Beta-casein gene expression is regarded as a marker for functional differentiation of mammary epithelial cells. Furthermore, the β -casein gene promoter has been studied for many years as a model for hormonal gene induction and milk protein gene regulation. In these studies, researchers have established that STAT5, C/EBP- β , and the glucocorticoid receptor (GR) are important factors in the transcriptional regulation of β -casein gene expression [94,95,99]. The casein gene promoters and several other milk protein genes have so-called lactogenic response elements that harbor recognition sites for these factors. The β -casein gene promoter is associated with the BCE, which is responsive to both ECM and lactogenic hormones [84–86]. These effects require stable genomic integration of reporter constructs, indicating the importance of chromatin environment [84,85].

Using ChIP, we showed that glucocorticoid (Gc) treatment recruits the GR to the mouse β -casein promoter and BCE in HC11 cells [96]. It also results in histone H3 hyperacetylation of these sites but alone does not induce gene transcription [96]. Prolactin did not have these effects, but the combination of both Gc and Prl stabilizes GR occupation while attenuating hyperacetylation. In turn, Prl alone enhances STAT5 occupancy on both the promoter and BCE, without any appreciable induction of transcription, and this occupancy is further stabilized by the addition of Gc. Induction with both hormones results in the recruitment of C/EBP β . However, because different isoforms of C/EBP β are indistinguishable with current

antibodies and are associated with both activation and repression of casein gene transcription [100–102], it is not possible to determine which isoforms are bound by ChIP assays. Furthermore, histone modifiers like p300 and HDAC1 have been demonstrated to be recruited upon lactogenic stimulation, and they most likely also are involved in modifying the chromatin and other proteins present [96,98].

Recently, we also showed that induction with Prl displaces factors thought to be part of a repressive complex; YY1 and HDAC3 [83,98]. Lactogenic induction abolished H3K9me2, a histone modification associated with repressive chromatin state (D. Edwards personal communication, Weston Porter personal communication). Xu and colleagues [98] observed similar results for H3Ac and H4Ac at the β - and γ -casein gene promoter in their studies of EpH4 MECs. They also showed that lactogenic stimulation in combination with laminin-rich ECM resulted in the recruitment of the ATP-dependent SWI/SNF chromatin-modifying complex, which was needed for RNA-Pol-II recruitment and transcription. Their results indicated that the SWI/SNF complex is being recruited to the β -casein gene promoter through its interactions with GR, STAT5A, and C/EBP β in a Prl- and ECM-specific manner. In further studies [97], these investigators showed that the sustained activation of STAT5A is needed for chromatin remodeling and maintenance of casein expression in Ep4H cells. These changes were induced by proper polarization of the cell in a 3D structure through interactions with a laminin-rich ECM.

The findings by Kabotyanski and colleagues [83] that lactogenic hormones induce the physical interaction between the β -casein gene promoter and the BCE (described above) put these results further in a chromatin perspective.

In addition to recognition sites for STAT5, C/EBP β , and GR, the casein gene promoters also have closely associated recognition sites for Oct1 and Runx2. These sites are present in most mammals, including opossum and platypus [103] (and Rijnkels unpublished observations) and are the most conserved sites in β -casein promoter (Rijnkels unpublished observations). Dong and Zhao [104] showed that Oct1 is important for the lactogenic induction of gene expression from the β -casein gene promoter. By ChIP analysis, they observed that Oct1 is bound to the endogenous β -casein gene promoter, although this binding is not hormone-dependent. They suggested that this finding might be due to a transient increase caused by lactogenic induction that is not captured at the 48-hour time point analyzed [104]. However, it is possible that Oct1 binding is not directly dependent on Prl or Gc but depends on other signaling pathways. In earlier work by Zhao and colleagues [105], findings suggested that Oct1 binding activity is estrogen and progesterone-responsive in virgin mammary gland, indicating that Oct1 binding could have an initiator function. These findings were based on electromobility shift assay (EMSA), an in vitro assay, and need to be substantiated using tissue ChIP assays.

Interestingly, Oct1 has been shown to have such an initiator function in the hormonally regulated MMTV promoter [106] and the IL2 promoter [107]. At the MMTV promoter, the constitutive interaction of Oct1–NF1 presets the chromatin; exhibited by increased histone acetylation in the continued presence of linker-histone H1 binding, and shows enhanced and prolonged GR binding upon hormonal stimulation [106]. Stimulation of naïve CD4⁺ cells results in the binding of Oct1 and nuclear factor of activated T cells (NFAT) and induces histone acetylation at the IL2 promoter and the demethylation of a specific CpG site [107]. In resting CD4⁺ cells, after withdrawal of this stimulation, H3Ac and bound Oct1 remain but NFAT binding is lost, resulting in a poised chromatin state that possibly is more responsive in a secondary immune memory response. Such an initiator function could fit with the fact that Inman and colleagues [103] have shown that Oct1 interacts with Runx2 and that this interaction is also needed for β -casein gene induction. These investigators also

showed that Oct1 and Runx2 are present at the β -casein gene promoter in HC11 cells in the presence of lactogenic hormones, but they did not investigate hormonal regulation. Because Runx2 is a nuclear matrix-binding protein, it is conceivable that Oct1–Runx2 interaction recruits the casein gene promoters to a transcriptionally active nuclear subdomain [103], facilitating induction upon lactogenic stimulation. Alternatively, the Oct1–Runx2 complex might also be involved in recruiting other factors such as GR and STAT5 to the promoter. Both Oct1 and Runx2 have been shown to interact with GR [108,109]. Both also have been shown to interact with STAT factors [110–112]. It is not known if the previously reported Gc induced H3Ac on the β -casein promoter, and BCE is dependent on Oct1–Runx2 binding. Furthermore, it would be interesting to know if the physical interaction between the promoter and the BCE is influenced by the Oct1–Runx2 complex. An interesting note is that Runx2 also can participate in repressive complexes that include HDAC3 and/or Sin3a [113]. One could speculate that Runx2 could be involved in switching between a repressive state—the β -casein promoter bound to a repressive complex containing YY1, HDAC3, C/EBP β -LIP (liver inhibiting protein), and other factors—and a permissive state interacting with Oct1, GR, STAT5, C/EBP β -LAP (liver activating protein), inhibiting or facilitating the 3D interaction of the promoter and BCE, respectively.

Epigenetics and Stem Cells in the Mammary Gland

In previous sections, we described epigenetic changes in the context of functional differentiation and expression of milk protein genes. This section examines what is known about epigenetics in the different stem and progenitor cell populations in the mammary gland. As alluded to earlier, epigenetic regulation is thought to play a major role in stem cell differentiation and lineage determination [114,115]. Most studies have been performed in an ES cell context, but recent studies in adult tissue have substantiated the importance of DNase, histone modifications, and their respective modifying enzymes in maintenance and differentiation of stem cells. [16,18,116–118].

Changes in gene expression patterns during mammary gland development [2–6] and its stem and progenitor cell compartments have been established [7,8,119]. Models for the cell fate decisions that eventually lead to a fully differentiated cell that is capable of making milk have been proposed [9,120,121]. Numerous factors that are important for these cell fates have been identified; they include Bmi1 [122], Notch [123,124], Wnt-signalling pathways [125–127], PML [128], Pygo-2 [129], Gata-3 [130,131], Elf5 [132], Stat5a [133], Pae3 [134], RankL [135], Amphiregulin [136]. The question remains regarding how epigenetics is involved in cell identification and differentiation in the mammary gland. Bloushtain-Qimron [119] analyzed DNase and gene expression in human MEC subpopulations. They identified discrete cell-type and differentiation state-specific DNase and gene expression patterns [119]. They found a high degree of similarity between the progenitor-cell phenotype defining epigenetic programs in mammary and embryonic stem cells. Furthermore, their results suggest that epigenetic control of transcription factors helps define the phenotype of progenitor and differentiated cells. They demonstrated that FOXC1—hypomethylated and highly expressed in progenitor-like cells—induced a progenitor-like phenotype in differentiated MECs. Interestingly, researchers recently reported that in embryonic cells, ELF5—in MEC, a determinant of luminal cell differentiation—is epigenetically controlled and plays a role in lineage fate restriction [137]. In the mammary gland, expression of ELF5 was detected in luminal progenitor cells but not in the stem cells enriched population [133]. Whether epigenetic regulation of ELF5 occurs in the mammary gland remains to be determined.

Preliminary analysis of MECs isolated from mature virgin mice and sorted for differentiation and progenitor markers [138] indicates that DNase in the casein gene locus

does not differ among different mammary subpopulations (Rijnkels et al unpublished observations).

Regulation of Epigenetic Changes

Although the first observations of changes in chromatin conformation correlating with different stages of mammary gland development were made decades ago, the main questions are still: “What are the mechanisms and signaling pathways that regulate these changes?” What are the “Epigenators” and “epigenetic initiators” of chromatin conformation described by Berger and colleagues [21] in the mammary gland?

Epigenators

Clearly, the hormones and signaling pathways of puberty and lactation come to mind as signals inducing epigenetic changes. But also the influence of the ECM and cell-shape and -context play an important role, as is discussed in more detail in the contributions in this issue from Mina Bissell's and Sophie Lelièvre's laboratories.

Studies describing interactions between ECM and chromatin conformation, as well as the reciprocal action of signals sent by the nucleus to the ECM, suggest that ECM might be a true epigenator [139–141]. Recent studies demonstrated that ECM component laminin-1 can mediate epigenetic changes at the E-Cadherin promoter in human breast cancer cells, possibly by reducing Dnmt1 levels [142], and β 4-integrin, a receptor for laminin and mediator of signaling pathways involved in β -casein expression [143], has been shown to be under epigenetic control in the mammary gland [144].

Some inroads, therefore, have been made in the elucidation of the intersection of cell signaling pathways and chromatin change using cell culture models. It could be argued that most of these effects on chromatin status are of the direct transcriptional kind and not necessarily truly epigenetic, as in most cases cell division is not occurring upon lactogenic hormone treatment or formation of 3D structures. However, the fact that these pathways also are involved in mammary gland development and differentiation suggests that certain traditional epigenetic changes also are under their control.

External factors such as nutrition, inflammation, and other exogenous exposures acting during late as well as early development may alter changes signaled by hormones, growth factors, and ECM and influence the epigenetic state of the gland. For example, after acute mastitis during lactation, DNase of the bovine α s1-casein regulatory region is induced in the infected quarter, leading to a decrease in gene expression [52]. Perturbed intra-uterine environments and deleterious environment exposures in early stages of development may result in abnormal mammary phenotypes and breast cancer detected later in life [145–150]. Epigenetic effects of such exposures have been described for other organs [151]. However, effects on the epigenetic state of the mammary gland are not known yet.

Factors that Target Epigenetic Modifications; Epigenetic Initiators

Major advances have been made in determining the distribution of chromatin marks in relation to gene expression in different tissues and developmental stages. Yet, very little is known about the tissue- and locus-specific targeting of epigenetic modifications in general. Certain transcription- and co-factors have been suggested to be involved, as has ncRNA. Some of these factors are known to be present in the mammary gland during development and differentiation.

Transcription Factors—YY1 can be part of the polycomb repressive complex 2 (PRC2) [152] and other repressive or activating complexes [153]. In the PRC2 complex, YY1 is

suggested to play a role in targeting the complex to certain sites in the genome, resulting in a repressive chromatin mark characterized by H3K27me3 and DNAm. In the mammary gland, YY1 is known to be involved in β -casein gene repression [154,155] and possibly other milk protein genes [156]. The C/EBP β -LIP isoform and HDAC3 might be part of such a repressive complex [83,102]. However, the exact nature of this complex and its effects on chromatin have yet to be determined.

SNAIL1 is another transcription factor that can interact with components of PRC2, and it has been shown to recruit PRC2 to the E-cadherin gene (CDH1), resulting in repression [157]. In the mammary gland SNAIL1 is the mediator of epithelial-mesenchymal transition (EMT) through repression of E-cadherin.

The homeo-domain containing factor Pygo-2 has been shown to play a role in the expansion of mammary progenitor-like cells [129]. It binds directly to H3K4me and facilitates trimethylation globally and at Wnt/beta-catenin target loci by recruiting H3K4-methyltransferase complexes. Optimal expansive self-renewal depends on this chromatin function of Pygo-2.

Nuclear hormone receptors regulate gene transcription through the recruitment of numerous co-factors, several of which have chromatin-modifying capacities [158,159]. Hyperacetylation at the β -casein promoter upon Gc signaling suggests that GR might be involved in recruiting histone acetyl transferases (HATs) to the promoter [96].

Non-protein-coding RNA (ncRNA)—It is now well recognized that most of the genome is transcribed, producing a large number of ncRNA [160,161]. It has become clear that ncRNAs are involved in the regulation of gene expression at many levels during development [161]. The regulation of micro-(mi)RNAs and other ncRNAs is under epigenetic control by histone modifications and DNAm, similar to protein coding genes [162]. However, mounting evidence suggests that ncRNAs play a role in regulation and targeting of epigenetic events [30,163,164].

Mammary expressed miRNAs have been identified in human [165] mouse [166–170] and cow [171]. Recent studies in the mouse show differential expression of miRNA during mammary gland development. Expression appears to be co-regulated for certain miRNAs [166]. A miRNA signature for mammary progenitor cells was identified and indicates a role in MEC cell fate specification [167]. Several miRNAs have been found to regulate directly factors important for stem cell function, such as BMI1 (miR200c, [172]) and cell proliferation Cox2 (miR101a [173]) and pTEN (miRNA205 [174]). Most miRNAs are involved in post-transcriptional regulation; yet, some are involved in expression enhancement (RNAa) at promoters (e.g., MiR-373 upregulates E-Cadherin) [175]. RNAa could function in part by inducing loss of the repressive histone modification H3K9me3, even though DNAm is not necessarily affected by RNAa and may even interfere with it [176]. However, the exact mechanisms of RNAa action have not been elucidated.

Other roles for ncRNAs are in the targeting of epigenetic events to specific loci in the genome. Several large ncRNAs have been associated with chromatin modifying complexes (HotAir, [177]; RepA, [178]; Air, [179]; Kcnq1ot1, [180]). Recent genome wide analyses of histone modifications indicative for active gene transcription [69] have uncovered a great number of large intervening non-coding RNAs (Linc RNA) in the human [181] and mouse [182] genome, and many of them appear to associate with chromatin complexes and affect gene expression [181].

In the mammary gland, many ncRNAs are regulated during development (J. Mattick and J. Rosen, unpublished observations), but only one Linc RNA has been characterized in detail thus far: pregnancy induced non-coding RNA (Pinc) [183], the expression of which is persistently upregulated after pregnancy. Pinc is temporally and spatially regulated in response to developmental stimuli, and its expression is detected in the terminal ductal-lobular-unit-like structures of the parous gland. The different splice forms of Pinc might have different functions in cell-cycle progression and survival, which could contribute to the developmentally mediated changes to the terminal ductal-lobular-unit-like structures observed after pregnancy and lactation [183]. In light of the findings described above, it is tempting to speculate about the function of Pinc. Pinc could be part of a PRC-like complex similar to Hotair [177] or other chromatin-modifying complexes [181] and exert its function through mammary-specific chromatin changes that permanently alter the epigenetic state of the gland. Furthermore, Pinc expression itself might in part be epigenetically regulated; preliminary data from our laboratory indicate that the Pinc promoter is hypomethylated in MEC compared to non-MEC cells (Fig. 5). This hypomethylation is already detected in MEC derived from 6-week-old virgin animals, indicating a mammary intrinsic hypomethylation.

Conclusions

Most of the chromatin changes summarized above have been observed in cell culture systems and are involved in direct-acute gene transcription regulation. The changes that we observed in potential DREs in the casein gene cluster during pubertal development and in the WAP region during pregnancy suggest that there is also a layer of changes that could be classified as a more classical epigenetic mechanism. As such, these appear to be involved in the establishment and maintenance of an epigenetic identity or memory of the cells. Whole genome analyses will now need to be applied to establish the network of genes that are epigenetically regulated and in order to help determine the global pathways of mammary gland development and functional differentiation. Establishing epigenetic marks at defined developmental stages and MEC sub-populations will also help elucidate the changes in epigenetic regulation that occur in cancer. Bloushtain-Qimron et al [119,184] reported the characterization of in vivo cell type-specific DNA methylation patterns with clinical relevance. Their results suggest an important role for epigenetic regulation in stem cell self-renewal, pluripotency and differentiation as well as a role of abnormalities in these processes in tumor initiation and progression. The development of ChIP procedures for small numbers of cells [185,186] should enable the analysis of histone modifications and transcription factor occupancy in MEC sub-populations.

No doubt the next few years will provide us with many new insights into the role of epigenetic regulation in mammary gland development and disease.

Acknowledgments

USDA/ARS 6250-51000-048-00, NIH 1R21HD053762, and NIH 5R03HD56090 to MR; NIH R37-CA16303-35 to JMR; Iranian Ministry of Science, Research and Technology to MBMT and INRA-292 and P00258 to ED

Abbreviations

BCE	beta casein enhancer
C/EBPβ	CCAAT-enhancer-binding proteins
ChIP	chromatin immunoprecipitation

DNAme	DNA methylation
DHS	DNaseI hypersensitivity
DRE	distal regulatory elements
ECR	evolutionary conserved regions
EMSA	Electro Mobility Shift Assay
Gc	glucocorticoid
GR	glucocorticoid receptor
MEC	mammary epithelial cell
Pg	progesterone
PR	progesterone receptor
Prl	prolactin
ncRNA	non-coding RNA
STAT5	signal transducers and activators of transcription

References

1. Topper YJ, Freeman CS. Multiple interactions in the developmental biology of the mammary gland. *Physiol Rev* 1980;80:1049–56. [PubMed: 7001510]
2. Stein T, Morris JS, Davies CR, et al. Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3. *Breast Cancer Res* 2004;6:R75–91. [PubMed: 14979920]
3. Master SR, Stoddard AJ, Bailey LC, Pan TC, Dugan KD, Chodosh LA. Genomic analysis of early murine mammary gland development using novel probe-level algorithms. *Genome Biol* 2005;6:R20. [PubMed: 15693949]
4. McBryan J, Howlin J, Kenny PA, Shioda T, Martin F. ERalpha-CITED1 co-regulated genes expressed during pubertal mammary gland development: implications for breast cancer prognosis. *Oncogene* 2007;26:6406–19. [PubMed: 17486082]
5. Rudolph MC, McManaman JL, Phang T, et al. Metabolic regulation in the lactating mammary gland: a lipid synthesizing machine. *Physiol Genomic* 2007;28:323–36.
6. Clarkson RW, Wayland MT, Lee J, Freeman T, Watson CJ. Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression. *Breast Cancer Res* 2004;6:R92–109. [PubMed: 14979921]
7. Kendrick H, Regan JL, Magnay FA, et al. Transcriptome analysis of mammary epithelial subpopulations identifies novel determinants of lineage commitment and cell fate. *BMC Genomics* 2008;9:591. [PubMed: 19063729]
8. Raouf A, Zhao Y, To K, et al. Transcriptome analysis of the normal human mammary cell commitment and differentiation process. *Cell Stem Cell* 2008;3:109–18. [PubMed: 18593563]
9. Visvader JE. Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. *Genes Dev* 2009;23:2563–77. [PubMed: 19933147]
10. Watson CJ, Khaled WT. Mammary development in the embryo and adult: a journey of morphogenesis and commitment. *Development* 2008;135:995–1003. [PubMed: 18296651]
11. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* 2003;33(Suppl):245–54. [PubMed: 12610534]
12. Okano H, Temple S. Cell types to order: temporal specification of CNS stem cells. *Curr Opin Neurobiol* 2009;19:112–9. [PubMed: 19427192]

13. Haumaitre C, Lenoir O, Scharfmann R. Directing cell differentiation with small-molecule histone deacetylase inhibitors: the example of promoting pancreatic endocrine cells. *Cell Cycle* 2009;8:536–44. [PubMed: 19197155]
14. Waterland RA, Kellermayer R, Rached MT, et al. Epigenomic profiling indicates a role for DNA methylation in early postnatal liver development. *Hum Mol Genet* 2009;18:3026–38. [PubMed: 19457928]
15. Bromfield J, Messamore W, Albertini DF. Epigenetic regulation during mammalian oogenesis. *Reprod Fertil Dev* 2008;20:74–80. [PubMed: 18154701]
16. Broske AM, Vockentanz L, Kharazi S, et al. DNA methylation protects hematopoietic stem cell multipotency from myeloerythroid restriction. *Nat Genet* 2009;41:1207–15. [PubMed: 19801979]
17. Cui K, Zang C, Roh TY, et al. Chromatin signatures in multipotent human hematopoietic stem cells indicate the fate of bivalent genes during differentiation. *Cell Stem Cell* 2009;4:80–93. [PubMed: 19128795]
18. Wilson CB, Rowell E, Sekimata M. Epigenetic control of T-helper-cell differentiation. *Nat Rev Immunol* 2009;9:91–105. [PubMed: 19151746]
19. Reiner SL. Epigenetic control in the immune response. *Hum Mol Genet* 2005;14(Spec No 1):R41–6. [PubMed: 15809272]
20. Janson PC, Winerdal ME, Winqvist O. At the crossroads of T helper lineage commitment-epigenetics points the way. *Biochim Biophys Acta* 2009;1790:906–19. [PubMed: 19162128]
21. Berger SL, Kouzarides T, Shiekhata R, Shilatifard A. An operational definition of epigenetics. *Genes Dev* 2009;23:781–3. [PubMed: 19339683]
22. Dobson H, Smith R, Royal M, Knight C, Sheldon I. The high-producing dairy cow and its reproductive performance. *Reprod Domest Anim* 2007;42(Suppl 2):17–23. [PubMed: 17688598]
23. Bobe G, Lindberg GL, Reutzel LF, Hanigan MD. Effects of lipid supplementation on the yield and composition of milk from cows with different beta-lactoglobulin phenotypes. *J Dairy Sci* 2009;92:197–203. [PubMed: 19109279]
24. Bestor TH. The DNA methyltransferases of mammals. *Hum Mol Genet* 2000;9:2395–402. [PubMed: 11005794]
25. Bernstein E, Hake SB. The nucleosome: a little variation goes a long way. *Biochem Cell Biol* 2006;84:505–17. [PubMed: 16936823]
26. Kouzarides T. Chromatin modifications and their function. *Cell* 2007;128:693–705. [PubMed: 17320507]
27. Campos EI, Reinberg D. Histones: annotating chromatin. *Annu Rev Genet* 2009;43:559–99. [PubMed: 19886812]
28. Dhasarathy A, Wade PA. The MBD protein family-reading an epigenetic mark? *Mutat Res* 2008;647:39–43. [PubMed: 18692077]
29. Postnikov Y, Bustin M. Regulation of chromatin structure and function by HMGN proteins. *Biochim Biophys Acta*. 2009
30. Mattick JS, Amaral PP, Dinger ME, Mercer TR, Mehler MF. RNA regulation of epigenetic processes. *Bioessays* 2009;31:51–9. [PubMed: 19154003]
31. Tolhuis B, Palstra RJ, Splinter E, Grosveld F, de Laat W. Looping and interaction between hypersensitive sites in the active beta-globin locus. *Mol Cell* 2002;10:1453–65. [PubMed: 12504019]
32. de Laat W, Grosveld F. Spatial organization of gene expression: the active chromatin hub. *Chromosome Res* 2003;11:447–59. [PubMed: 12971721]
33. Marzluff WF Jr, McCarty KS. Two classes of histone acetylation in developing mouse mammary gland. *J Biol Chem* 1970;245:5635–42. [PubMed: 5472362]
34. Hohmann P, Cole RD. Hormonal effects on amino acid incorporation into lysine-rich histones in the mouse mammary gland. *J Mol Biol* 1971;58:33–540.
35. Crawford GE, Davis S, Scacheri PC, et al. DNase-chip: a high-resolution method to identify DNase I hypersensitive sites using tiled microarrays. *Nat Methods* 2006;3:503–9. [PubMed: 16791207]

36. Sabo PJ, Kuehn MS, Thurman R, et al. Genome-scale mapping of DNase I sensitivity in vivo using tiling DNA microarrays. *Nat Methods* 2006;3:511–8. [PubMed: 16791208]
37. Clark SJ, Harrison J, Paul CL, Frommer M. High sensitivity mapping of methylated cytosines. *Nucleic Acids Res* 1994;22:2990–7. [PubMed: 8065911]
38. Wilson IM, Davies JJ, Weber M, et al. Epigenomics: mapping the methylome. *Cell Cycle* 2006;5:155–8. [PubMed: 16397413]
39. Lister R, Ecker JR. Finding the fifth base: genome-wide sequencing of cytosine methylation. *Genome Res* 2009;19:959–66. [PubMed: 19273618]
40. Johnson KD, Bresnick EH. Dissecting long-range transcriptional mechanisms by chromatin immunoprecipitation. *Methods* 2002;26:27–36. [PubMed: 12054902]
41. Kirmizis A, Bartley SM, Kuzmichev A, et al. Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27. *Genes Dev* 2004;18:1592–605. [PubMed: 15231737]
42. Barski A, Zhao K. Genomic location analysis by ChIP-Seq. *J Cell Biochem* 2009;107:11–8. [PubMed: 19173299]
43. Waalwijk C, Flavell RA. DNA methylation at a CCGG sequence in the large intron of the rabbit beta-globin gene: tissue-specific variations. *Nucleic Acids Res* 1978;5:4631–4. [PubMed: 745990]
44. Waalwijk C, Flavell RA. MspI, an isoschizomer of hpaII which cleaves both unmethylated and methylated hpaII sites. *Nucleic Acids Res* 1978;5:3231–6. [PubMed: 704354]
45. Holliday R, Pugh JE. DNA modification mechanisms and gene activity during development. *Science* 1975;187:226–32. [PubMed: 1111098]
46. Groudine M, Kohwi-Shigematsu T, Gelinis R, Stamatoyannopoulos G, Papayannopoulou T. Human fetal to adult hemoglobin switching: changes in chromatin structure of the beta-globin gene locus. *Proc Natl Acad Sci U S A* 1983;80:7551–5. [PubMed: 6200877]
47. Forrester WC, Thompson C, Elder JT, Groudine M. A developmentally stable chromatin structure in the human beta-globin gene cluster. *Proc Natl Acad Sci* 1986;83:1359–63. [PubMed: 3456593]
48. Plachot C, Lelievre SA. DNA methylation control of tissue polarity and cellular differentiation in the mammary epithelium. *Exp Cell Res* 2004;298:122–32. [PubMed: 15242767]
49. Johnson ML, Levy J, Supowit SC, Yu-Lee LY, Rosen JM. Tissue- and cell-specific casein gene expression. II. Relationship to site-specific DNA methylation. *J Biol Chem* 1983;258:10805–11. [PubMed: 6411721]
50. Thompson MD, Nakhasi HL. Methylation and expression of rat kappa-casein gene in normal and neoplastic rat mammary gland. *Cancer Res* 1985;45:1291–5. [PubMed: 3971374]
51. Platenburg GJ, Vollebregt EJ, Karatzas CN, Kootwijk EPA, de Boer HA, Strijker R. Mammary gland-specific hypomethylation of hpaII sites flanking the bovine alpha-s1-casein gene. *Transgenic Res* 1996;5:421–31. [PubMed: 8840525]
52. Vanselow J, Yang W, Herrmann J, et al. DNA-remethylation around a STAT5-binding enhancer in the alphaS1-casein promoter is associated with abrupt shutdown of alphaS1-casein synthesis during acute mastitis. *J Mol Endocrinol* 2006;37:463–77. [PubMed: 17170087]
53. Singh K, Swanson K, Couldrey C, Seyfert H-M, Stelwagen K. Suppression of bovine α S1-casein gene expression during involution of the mammary gland is associated with increased DNA methylation at a STAT5-binding site in the α S1-casein promoter. *J Dairy Sci* 2008;91:378.
54. Rijnkels M, Freeman-Zadrowski C, Hernandez J. Epigenetic changes during functional differentiation of the mammary gland. *J Dairy Sci* 2009;92:325–6.
55. Dandekar AM, Robinson EA, Appella E, Qasba PK. Complete sequence analysis of cDNA clones encoding rat whey phosphoprotein: homology to a protease inhibitor. *Proc Natl Acad Sci U S A* 1982;79:3987–91. [PubMed: 6955785]
56. Hennighausen LG, Sippel AE. Characterization and cloning of the mRNAs specific for the lactating mouse mammary gland. *Eur J Biochem* 1982;125:131–41. [PubMed: 6896688]
57. Montazer-Torbati MB, Hue-Beauvais C, Droineau S, et al. Epigenetic modifications and chromatin loop organization explain the different expression profiles of the Tbrg4, WAP and Ramp3 genes. *Exp Cell Res* 2008;314:975–87. [PubMed: 18255060]

58. Ooi SK, Bestor TH. The colorful history of active DNA demethylation. *Cell* 2008;133:1145–8. [PubMed: 18585349]
59. Denamur R. Nucleic acids of the mammary gland during gestation and lactation in the rabbit. *C R Hebd Seances Acad Sci* 1963;256:4748–50.
60. Traurig HH. Cell proliferation in the mammary gland during late pregnancy and lactation. *Anat Rec* 1967;157:489–503.
61. Traurig HH. A radioautographic study of cell proliferation in the mammary gland of the pregnant mouse. *Anat Rec* 1967;159:239–47. [PubMed: 6082276]
62. Whitelaw CBA, Harris S, McClenaghan M, Simons JP, Clark AJ. Position-independent expression of the ovine beta-lactoglobulin gene in transgenic mice. *Biochem J* 1992;286:31–9. [PubMed: 1520282]
63. Whitelaw C. Hormonal influences on beta-lactoglobulin transgene expression inferred from chromatin structure. *Biochem Biophys Res Commun* 1996;224:121–5. [PubMed: 8694798]
64. Whitelaw CB. Nucleosome organisation of the beta-lactoglobulin gene. Transcription complex formation. *Adv Exp Med Biol* 2000;480:147–53. [PubMed: 10959421]
65. Whitelaw CB, Webster J. Temporal profiles of appearance of DNase I hypersensitive sites associated with the ovine beta-lactoglobulin gene differ in sheep and transgenic mice. *Mol Gen Genet* 1998;257:649–54. [PubMed: 9604888]
66. Barber MC, Vallance AJ, Kennedy HT, Travers MT. Induction of transcripts derived from promoter III of the acetyl-CoA carboxylase- α gene in mammary gland is associated with recruitment of SREBP-1 to a region of the proximal promoter defined by a DNase I hypersensitive site. *Biochem J* 2003;375:489–501. [PubMed: 12871210]
67. Li S, Rosen JM. Glucocorticoid regulation of rat whey acidic protein gene expression involves hormone-induced alterations of chromatin structure in the distal promoter region. *Mol Endocrinol* 1994;8:1328–35. [PubMed: 7854350]
68. Millot B, Fontaine ML, Thepot D, Devinoy E. A distal region, hypersensitive to DNase I, plays a key role in regulating rabbit whey acidic protein gene expression. *Biochem J* 2001;359:557–65. [PubMed: 11672429]
69. Millot B, Montoliu L, Fontaine ML, Mata T, Devinoy E. Hormone-induced modifications of the chromatin structure surrounding upstream regulatory regions conserved between the mouse and rabbit whey acidic protein genes. *Biochem J* 2003;372:41–52. [PubMed: 12580766]
70. Rijnkels M, Elnitski L, Miller W, Rosen JM. Multi-species comparative analysis of a mammalian specific genomic domain encoding secretory proteins. *Genomics* 2003;82:417–32. [PubMed: 13679022]
71. Hebbes TR, Thorne AW, Crane-Robinson C. A direct link between core histone acetylation and transcriptionally active chromatin. *EMBO J* 1988;7:1395–402. [PubMed: 3409869]
72. Roh TY, Ngau WC, Cui K, Landsman D, Zhao K. High-resolution genome-wide mapping of histone modifications. *Nat Biotechnol* 2004;22:1013–6. [PubMed: 15235610]
73. Heintzman ND, Hon GC, Hawkins RD, et al. Histone modifications at human enhancers reflect global cell-type-specific gene expression. *Nature* 2009;459:108–12. [PubMed: 19295514]
74. Heintzman ND, Stuart RK, Hon G, et al. Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. *Nat Genet* 2007;39:311–8. [PubMed: 17277777]
75. Jolivet G, Pantano T, Houdebine LM. Regulation by the extracellular matrix (ECM) of prolactin-induced α casein gene expression in rabbit primary mammary cells: Role of STAT5, C/EBP, and chromatin structure. *J Cell Biochem* 2005;95:313–27. [PubMed: 15778982]
76. Dekker J, Rippe K, Dekker M, Kleckner N. Capturing chromosome conformation. *Science* 2002;295:1306–11. [PubMed: 11847345]
77. Simonis M, Klous P, Splinter E, et al. Nuclear organization of active and inactive chromatin domains uncovered by chromosome conformation capture-on-chip (4C). *Nat Genet* 2006;38:1348–54. [PubMed: 17033623]
78. Zhao Z, Tavoosidana G, Sjolinder M, et al. Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions. *Nat Genet* 2006;38:1341–7. [PubMed: 17033624]

79. Dostie J, Richmond TA, Arnaout RA, et al. Chromosome Conformation Capture Carbon Copy (5C): a massively parallel solution for mapping interactions between genomic elements. *Genome Res* 2006;16:1299–309. [PubMed: 16954542]
80. Palstra RJ, Tolhuis B, Splinter E, Nijmeijer R, Grosveld F, de Laat W. The beta-globin nuclear compartment in development and erythroid differentiation. *Nat Genet* 2003;35:190–4. [PubMed: 14517543]
81. Jiang H, Peterlin BM. Differential chromatin looping regulates CD4 expression in immature thymocytes. *Mol Cell Biol* 2008;28:907–12. [PubMed: 18039856]
82. Vernimmen D, De Gobbi M, Sloane-Stanley JA, Wood WG, Higgs DR. Long-range chromosomal interactions regulate the timing of the transition between poised and active gene expression. *EMBO J* 2007;26:2041–51. [PubMed: 17380126]
83. Kabotyanski EB, Rijnkels M, Freeman-Zadrowski C, Buser AC, Edwards DP, Rosen JM. Lactogenic hormonal induction of long-distance interactions between {beta}-casein gene regulatory elements. *J Biol Chem* 2009;284:22815–24. [PubMed: 19542223]
84. Myers CA, Schmidhauser C, Mellentin-Michelotti J, et al. Characterization of BCE-1, a transcriptional enhancer regulated by prolactin and extracellular matrix and modulated by the state of histone acetylation. *Mol Cell Biol* 1998;18:2184–95. [PubMed: 9528790]
85. Schmidhauser C, Casperson GF, Myers CA, Sanzo KT, Bolten S, Bissell MJ. A novel transcriptional enhancer is involved in the prolactin- and extracellular matrix-dependent regulation of beta-casein gene expression. *Mol Biol Cell* 1992;3:699–709. [PubMed: 1498370]
86. Winklehner-Jenneweit P, Geymayer S, Lechner J, et al. A distal enhancer region in the human beta-casein gene mediates the response to prolactin and glucocorticoid hormones. *Gene* 1998;217:127–39. [PubMed: 9795185]
87. Buser AC, Gass-Handel EK, Wyszomierski SL, et al. Progesterone receptor repression of prolactin/signal transducer and activator of transcription 5-mediated transcription of the beta-casein gene in mammary epithelial cells. *Mol Endocrinol* 2007;21:106–25. [PubMed: 16973758]
88. Vassetzky Y, Lemaitre JM, Mechali M. Specification of chromatin domains and regulation of replication and transcription during development. *Crit Rev Eukaryot Gene Exp* 2000;10:31–8.
89. Eivazova ER, Gavrillov A, Pirozhkova I, et al. Interaction in vivo between the two matrix attachment regions flanking a single chromatin loop. *J Mol Biol* 2009;386:929–37. [PubMed: 19118562]
90. Razin SV, Iarovaia OV, Sjakste N, et al. Chromatin domains and regulation of transcription. *J Mol Biol* 2007;369:597–607. [PubMed: 17466329]
91. Ballester M, Kress C, Hue-Beauvais C, et al. The nuclear localization of WAP and CSN genes is modified by lactogenic hormones in HC11 cells. *J Cell Biochem* 2008;105:262–70. [PubMed: 18500724]
92. Ioudinkova E, Petrov A, Razin SV, Vassetzky YS. Mapping long-range chromatin organization within the chicken alpha-globin gene domain using oligonucleotide DNA arrays. *Genomics* 2005;85:143–51. [PubMed: 15607430]
93. Briskin C, Rajaram RD. Alveolar and lactogenic differentiation. *J Mammary Gland Biol Neoplasia* 2006;11:239–48. [PubMed: 17111223]
94. Groner B. Transcription factor regulation in mammary epithelial cells. *Domest Anim Endocrinol* 2002;23:25–32. [PubMed: 12142223]
95. Doppler W, Geymayer S, Weirich HG. Synergistic and antagonistic interactions of transcription factors in the regulation of milk protein gene expression. Mechanisms of cross-talk between signalling pathways. *Adv Exp Med Biol* 2000;480:139–46. [PubMed: 10959420]
96. Kabotyanski EB, Huetter M, Xian W, Rijnkels M, Rosen JM. Integration of prolactin and glucocorticoid signaling at the {beta}-casein promoter and enhancer by ordered recruitment of specific transcription factors and chromatin modifiers. *Mol Endocrinol* 2006;20:2355–68. [PubMed: 16772529]
97. Xu R, Nelson CM, Muschler JL, Veisoh M, Vonderhaar BK, Bissell MJ. Sustained activation of STAT5 is essential for chromatin remodeling and maintenance of mammary-specific function. *J Cell Biol* 2009;184:57–66. [PubMed: 19139262]

98. Xu R, Spencer VA, Bissell MJ. Extracellular matrix-regulated gene expression requires cooperation of SWI/SNF and transcription factors. *J Biol Chem* 2007;282:14992–9. [PubMed: 17387179]
99. Rosen JM, Wyszomierski SL, Hadsell D. Regulation of milk protein gene expression. *Annu Rev Nutr* 1999;19:407–36. [PubMed: 10448531]
100. Wyszomierski SL, Rosen JM. Cooperative effects of STAT5 (signal transducer and activator of transcription 5) and C/EBPbeta (CCAAT/enhancer-binding protein-beta) on beta-casein gene transcription are mediated by the glucocorticoid receptor. *Mol Endocrinol* 2001;15:228–40. [PubMed: 11158330]
101. Doppler W, Welte T, Philipp S. CCAAT/enhancer-binding protein isoforms beta and delta are expressed in mammary epithelial cells and bind to multiple sites in the beta-casein gene promoter. *J Biol Chem* 1995;270:17962–9. [PubMed: 7629103]
102. Raught B, Liau S-L, Rosen JM. Developmentally and hormonally regulated CCAAT/enhancer-binding protein isoforms influence beta-casein gene expression. *Mol Endocrinol* 1995;9:1223–32. [PubMed: 7491114]
103. Inman CK, Li N, Shore P. Oct-1 counteracts autoinhibition of Runx2 DNA binding to form a novel Runx2/Oct-1 complex on the promoter of the mammary gland-specific gene beta-casein. *Mol Cell Biol* 2005;25:3182–93. [PubMed: 15798204]
104. Dong B, Zhao FQ. Involvement of the ubiquitous Oct-1 transcription factor in hormonal induction of beta-casein gene expression. *Biochem J* 2007;401:57–64. [PubMed: 16965262]
105. Zhao FQ, Adachi K, Oka T. Involvement of Oct-1 in transcriptional regulation of beta-casein gene expression in mouse mammary gland. *Biochim Biophys Acta* 2002;1577:27–37. [PubMed: 12151092]
106. Astrand C, Belikov S, Wrangé O. Histone acetylation characterizes chromatin presetting by NF1 and Oct1 and enhances glucocorticoid receptor binding to the MMTV promoter. *Exp Cell Res* 2009;315:2604–15. [PubMed: 19463811]
107. Murayama A, Sakura K, Nakama M, et al. A specific CpG site demethylation in the human interleukin 2 gene promoter is an epigenetic memory. *EMBO J* 2006;25:1081–92. [PubMed: 16498406]
108. Ning YM, Robins DM. AML3/CBFalpha1 is required for androgen-specific activation of the enhancer of the mouse sex-limited protein (Slp) gene. *J Biol Chem* 1999;274:30624–30. [PubMed: 10521447]
109. Prefontaine GG, Walther R, Giffin W, Lemieux ME, Pope L, Hache RJ. Selective binding of steroid hormone receptors to octamer transcription factors determines transcriptional synergism at the mouse mammary tumor virus promoter. *J Biol Chem* 1999;274:26713–9. [PubMed: 10480874]
110. Brockman JL, Schuler LA. Prolactin signals via Stat5 and Oct-1 to the proximal cyclin D1 promoter. *Mol Cell Endocrinol* 2005;239:45–53. [PubMed: 15885880]
111. Magne S, Caron S, Charon M, Rouyez MC, Dusanter-Fourt I. STAT5 and Oct-1 form a stable complex that modulates cyclin D1 expression. *Mol Cell Biol* 2003;23:8934–45. [PubMed: 14645506]
112. Kim S, Koga T, Isobe M, et al. Stat1 functions as a cytoplasmic attenuator of Runx2 in the transcriptional program of osteoblast differentiation. *Genes Dev* 2003;17:1979–91. [PubMed: 12923053]
113. Westendorf JJ. Transcriptional co-repressors of Runx2. *J Cell Biochem* 2006;98:54–64. [PubMed: 16440320]
114. Hemberger M, Dean W, Reik W. Epigenetic dynamics of stem cells and cell lineage commitment: digging Waddington's canal. *Nat Rev Mol Cell Biol* 2009;10:526–37. [PubMed: 19603040]
115. Schuettengruber B, Cavalli G. Recruitment of polycomb group complexes and their role in the dynamic regulation of cell fate choice. *Development* 2009;136:3531–42. [PubMed: 19820181]
116. Trowbridge JJ, Snow JW, Kim J, Orkin SH. DNA methyltransferase 1 is essential for and uniquely regulates hematopoietic stem and progenitor cells. *Cell Stem Cell* 2009;5:442–9. [PubMed: 19796624]

117. Fan G, Beard C, Chen RZ, et al. DNA hypomethylation perturbs the function and survival of CNS neurons in postnatal animals. *J Neurosci* 2001;21:788–97. [PubMed: 11157065]
118. Lim DA, Huang YC, Swigut T, et al. Chromatin remodelling factor Mll1 is essential for neurogenesis from postnatal neural stem cells. *Nature* 2009;458:529–33. [PubMed: 19212323]
119. Bloushtain-Qimron N, Yao J, Snyder EL, et al. Cell type-specific DNA methylation patterns in the human breast. *Proc Natl Acad Sci U S A* 2008;105:14076–81. [PubMed: 18780791]
120. Hennighausen L, Robinson GW. Information networks in the mammary gland. *Nat Rev Mol Cell Biol* 2005;6:715–25. [PubMed: 16231422]
121. LaMarca HL, Rosen JM. Minireview: hormones and mammary cell fate—what will I become when I grow up? *Endocrinology* 2008;149:4317–21. [PubMed: 18556345]
122. Pietersen AM, Evers B, Prasad AA, et al. Bmi1 regulates stem cells and proliferation and differentiation of committed cells in mammary epithelium. *Curr Biol* 2008;18:1094–9. [PubMed: 18635350]
123. Bouras T, Pal B, Vaillant F, et al. Notch signaling regulates mammary stem cell function and luminal cell-fate commitment. *Cell Stem Cell* 2008;3:429–41. [PubMed: 18940734]
124. Buono KD, Robinson GW, Martin C, et al. The canonical Notch/RBP-J signaling pathway controls the balance of cell lineages in mammary epithelium during pregnancy. *Dev Biol* 2006;293:565–80. [PubMed: 16581056]
125. Boras-Granic K, Wysolmerski JJ. Wnt signaling in breast organogenesis. *Organogenesis* 2008;4:116–22. [PubMed: 19279723]
126. Badders NM, Goel S, Clark RJ, et al. The Wnt receptor, Lrp5, is expressed by mouse mammary stem cells and is required to maintain the basal lineage. *PLoS One* 2009;4:e6594. [PubMed: 19672307]
127. Lindvall C, Zylstra CR, Evans N, et al. The Wnt co-receptor Lrp6 is required for normal mouse mammary gland development. *PLoS One* 2009;4:e5813. [PubMed: 19503830]
128. Li W, Ferguson BJ, Khaled WT, et al. PML depletion disrupts normal mammary gland development and skews the composition of the mammary luminal cell progenitor pool. *Proc Natl Acad Sci U S A* 2009;106:4725–30. [PubMed: 19261859]
129. Gu B, Sun P, Yuan Y, et al. Pygo2 expands mammary progenitor cells by facilitating histone H3 K4 methylation. *J Cell Biol* 2009;185:811–26. [PubMed: 19487454]
130. Asselin-Labat ML, Sutherland KD, Barker H, et al. Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation. *Nat Cell Biol* 2007;9:201–9. [PubMed: 17187062]
131. Kouros-Mehr H, Slorach EM, Sternlicht MD, Werb Z. GATA-3 maintains the differentiation of the luminal cell fate in the mammary gland. *Cell* 2006;127:1041–55. [PubMed: 17129787]
132. Oakes SR, Naylor MJ, Asselin-Labat ML, et al. The Ets transcription factor Elf5 specifies mammary alveolar cell fate. *Genes Dev* 2008;22:581–6. [PubMed: 18316476]
133. Yamaji D, Na R, Feuermann Y, et al. Development of mammary luminal progenitor cells is controlled by the transcription factor STAT5A. *Genes Dev* 2009;23:2382–7. [PubMed: 19833766]
134. Kurpios NA, MacNeil L, Shepherd TG, Gludish DW, Giacomelli AO, Hassell JA. The Pea3 Ets transcription factor regulates differentiation of multipotent progenitor cells during mammary gland development. *Dev Biol* 2009;325:106–21. [PubMed: 18977342]
135. Fernandez-Valdivia R, Mukherjee A, Ying Y, et al. The RANKL signaling axis is sufficient to elicit ductal side-branching and alveologenesis in the mammary gland of the virgin mouse. *Dev Biol* 2009;328:127–39. [PubMed: 19298785]
136. Booth BW, Boulanger CA, Anderson LH, Jimenez-Rojo L, Briskin C, Smith GH. Amphiregulin mediates self-renewal in an immortal mammary epithelial cell line with stem cell characteristics. *Exp Cell Res*. 2009
137. Ng RK, Dean W, Dawson C, et al. Epigenetic restriction of embryonic cell lineage fate by methylation of Elf5. *Nat Cell Biol* 2008;10:1280–90. [PubMed: 18836439]
138. Sleeman KE, Kendrick H, Robertson D, Isacke CM, Ashworth A, Smalley MJ. Dissociation of estrogen receptor expression and in vivo stem cell activity in the mammary gland. *J Cell Biol* 2007;176:19–26. [PubMed: 17190790]

139. Xu R, Boudreau A, Bissell MJ. Tissue architecture and function: dynamic reciprocity via extra- and intra-cellular matrices. *Cancer Metastasis Rev* 2009;28:167–76. [PubMed: 19160017]
140. Lelievre SA. Contributions of extracellular matrix signaling and tissue architecture to nuclear mechanisms and spatial organization of gene expression control. *Biochim Biophys Acta* 2009;1790:925–35. [PubMed: 19328836]
141. Le Beyec J, Xu R, Lee SY, et al. Cell shape regulates global histone acetylation in human mammary epithelial cells. *Exp Cell Res* 2007;313:3066–75. [PubMed: 17524393]
142. Benton G, Crooke E, George J. Laminin-1 induces E-cadherin expression in 3-dimensional cultured breast cancer cells by inhibiting DNA methyltransferase 1 and reversing promoter methylation status. *FASEB J* 2009;23:3884–95. [PubMed: 19635753]
143. Muschler J, Lochter A, Roskelley CD, Yurchenco P, Bissell MJ. Division of labor among the $\alpha 6 \beta 4$ integrin, $\beta 1$ integrins, and an E3 laminin receptor to signal morphogenesis and beta-casein expression in mammary epithelial cells. *Mol Biol Cell* 1999;10:2817–28. [PubMed: 10473629]
144. Yang X, Pursell B, Lu S, Chang TK, Mercurio AM. Regulation of beta 4-integrin expression by epigenetic modifications in the mammary gland and during the epithelial-to-mesenchymal transition. *J Cell Sci* 2009;122:2473–80. [PubMed: 19549682]
145. Kyle UG, Pichard C. The Dutch Famine of 1944–1945: a pathophysiological model of long-term consequences of wasting disease. *Curr Opin Clin Nutr Metab Care* 2006;9:388–94. [PubMed: 16778567]
146. Heijmans BT, Tobi EW, Stein AD, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci U S A* 2008;105:17046–9. [PubMed: 18955703]
147. De Assis S, Hilakivi-Clarke L. Timing of dietary estrogenic exposures and breast cancer risk. *Ann N Y Acad Sci* 2006;1089:14–35. [PubMed: 17261753]
148. Hilakivi-Clarke L, de Assis S. Fetal origins of breast cancer. *Trends Endocrinol Metab* 2006;17:340–8. [PubMed: 16997567]
149. Soto AM, Vandenberg LN, Maffini MV, Sonnenschein C. Does breast cancer start in the womb? *Basic Clin Pharmacol Toxicol* 2008;102:125–33. [PubMed: 18226065]
150. Fernandez-Twinn DS, Ekizoglou S, Gusterson BA, Luan J, Ozanne SE. Compensatory mammary growth following protein restriction during pregnancy and lactation increases early-onset mammary tumor incidence in rats. *Carcinogenesis* 2007;28:545–52. [PubMed: 16952910]
151. Burdge GC, Lillycrop KA, Jackson AA. Nutrition in early life, and risk of cancer and metabolic disease: alternative endings in an epigenetic tale? *Br J Nutr* 2009;101:619–30. [PubMed: 19079817]
152. Caretti G, Di Padova M, Micales B, Lyons GE, Sartorelli V. The Polycomb Ezh2 methyltransferase regulates muscle gene expression and skeletal muscle differentiation. *Genes Dev* 2004;18:2627–38. [PubMed: 15520282]
153. Thomas MJ, Seto E. Unlocking the mechanisms of transcription factor YY1: are chromatin modifying enzymes the key? *Gene* 1999;236:197–208. [PubMed: 10452940]
154. Meier VS, Groner B. The nuclear factor YY1 participates in repression of the beta-casein gene promoter in mammary epithelial cells and is counteracted by mammary gland factor during lactogenic hormone induction. *Mol Cell Biol* 1994;14:128–37. [PubMed: 8264581]
155. Raught B, Khursheed B, Kazansky A, Rosen J. YY1 represses beta-casein gene expression by preventing the formation of a lactation-associated complex. *Mol Cell Biol* 1994;14:1752–63. [PubMed: 8114709]
156. Rosen JM, Zahnow C, Kazansky A, Raught B. Composite response elements mediate hormonal and developmental regulation of milk protein gene expression. *Biochem Soc Symp* 1998;63:101–13. [PubMed: 9513715]
157. Herranz N, Pasini D, Diaz VM, et al. Polycomb complex 2 is required for E-cadherin repression by the Snail1 transcription factor. *Mol Cell Biol* 2008;28:4772–81. [PubMed: 18519590]
158. Kishimoto M, Fujiki R, Takezawa S, et al. Nuclear receptor mediated gene regulation through chromatin remodeling and histone modifications. *Endocr J* 2006;53:157–72. [PubMed: 16618973]

159. Li X, Wong J, Tsai SY, Tsai MJ, O'Malley BW. Progesterone and glucocorticoid receptors recruit distinct coactivator complexes and promote distinct patterns of local chromatin modification. *Mol Cell Biol* 2003;23:3763–73. [PubMed: 12748280]
160. Kapranov P, Cheng J, Dike S, et al. RNA maps reveal new RNA classes and a possible function for pervasive transcription. *Science* 2007;316:1484–8. [PubMed: 17510325]
161. Amaral PP, Mattick JS. Noncoding RNA in development. *Mamm Genome* 2008;19:454–92. [PubMed: 18839252]
162. Barski A, Jothi R, Cuddapah S, et al. Chromatin poises miRNA-and protein-coding genes for expression. *Genome Res* 2009;19:1742–51. [PubMed: 19713549]
163. Mayer C, Schmitz KM, Li J, Grummt I, Santoro R. Intergenic transcripts regulate the epigenetic state of rRNA genes. *Mol Cell* 2006;22:351–61. [PubMed: 16678107]
164. Guil S, Esteller M. DNA methylomes, histone codes and miRNAs: tying it all together. *Int J Biochem Cell Biol* 2009;41:87–95. [PubMed: 18834952]
165. Liu CG, Calin GA, Meloon B, et al. An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. *Proc Natl Acad Sci U S A* 2004;101:9740–4. [PubMed: 15210942]
166. Avril-Sassen S, Goldstein LD, Stingl J, et al. Characterisation of microRNA expression in post-natal mouse mammary gland development. *BMC Genomics* 2009;10:548. [PubMed: 19930549]
167. Ibarra I, Erlich Y, Muthuswamy SK, Sachidanandam R, Hannon GJ. A role for microRNAs in maintenance of mouse mammary epithelial progenitor cells. *Genes Dev* 2007;21:3238–43. [PubMed: 18079172]
168. Sdassi N, Silveri L, Laubier J, et al. Identification and characterization of new miRNAs cloned from normal mouse mammary gland. *BMC Genomics* 2009;10:149. [PubMed: 19351399]
169. Silveri L, Tilly G, Vilotte JL, Le Provost F. MicroRNA involvement in mammary gland development and breast cancer. *Reprod Nutr Dev* 2006;46:549–56. [PubMed: 17107644]
170. Wang C, Li Q. Identification of differentially expressed micro-RNAs during the development of Chinese murine mammary gland. *J Genet Genomics* 2007;34:966–73. [PubMed: 18037133]
171. Gu Z, Eleswarapu S, Jiang H. Identification and characterization of microRNAs from the bovine adipose tissue and mammary gland. *FEBS Lett* 2007;581:981–8. [PubMed: 17306260]
172. Shimono Y, Zabala M, Cho RW, et al. Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. *Cell* 2009;138:592–603. [PubMed: 19665978]
173. Tanaka T, Haneda S, Imakawa K, Sakai S, Nagaoka K. A microRNA, miR-101a, controls mammary gland development by regulating cyclooxygenase-2 expression. *Differentiation* 2009;77:181–7. [PubMed: 19281778]
174. Greene SB, Gunaratne PH, Hammond SH, Rosen JM. A putative role for microRNA-205 in progenitors of mammary epithelial cells. *J Cell Sci*. 2010 in press.
175. Place RF, Li LC, Pookot D, Noonan EJ, Dahiya R. MicroRNA-373 induces expression of genes with complementary promoter sequences. *Proc Natl Acad Sci U S A* 2008;105:1608–13. [PubMed: 18227514]
176. Li LC, Okino ST, Zhao H, et al. Small dsRNAs induce transcriptional activation in human cells. *Proc Natl Acad Sci U S A* 2006;103:17337–42. [PubMed: 17085592]
177. Rinn JL, Kertesz M, Wang JK, et al. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* 2007;129:1311–23. [PubMed: 17604720]
178. Zhao J, Sun BK, Erwin JA, Song JJ, Lee JT. Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. *Science* 2008;322:750–6. [PubMed: 18974356]
179. Nagano T, Mitchell JA, Sanz LA, et al. The Air noncoding RNA epigenetically silences transcription by targeting G9a to chromatin. *Science* 2008;322:1717–20. [PubMed: 18988810]
180. Pandey RR, Mondal T, Mohammad F, et al. Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. *Mol Cell* 2008;32:232–46. [PubMed: 18951091]
181. Khalil AM, Guttman M, Huarte M, et al. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. *Proc Natl Acad Sci U S A* 2009;106:11667–72. [PubMed: 19571010]

182. Guttman M, Amit I, Garber M, et al. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. *Nature* 2009;458:223–7. [PubMed: 19182780]
183. Ginger MR, Shore AN, Contreras A, et al. A noncoding RNA is a potential marker of cell fate during mammary gland development. *Proc Natl Acad Sci U S A* 2006;103:5781–6. [PubMed: 16574773]
184. Bloushtain-Qimron N, Yao J, Shipitsin M, Maruyama R, Polyak K. Epigenetic patterns of embryonic and adult stem cells. *Cell Cycle* 2009;8:809–17. [PubMed: 19229128]
185. Dahl JA, Collas P. MicroChIP-a rapid micro chromatin immuno-precipitation assay for small cell samples and biopsies. *Nucleic Acids Res* 2008;36:e15. [PubMed: 18202078]
186. Goren A, Ozsolak F, Shores N, et al. Chromatin profiling by directly sequencing small quantities of immunoprecipitated DNA. *Nat Methods* 2009;7:47–9. [PubMed: 19946276]
187. Elnitski L, Riemer C, Burhans R, Hardison R, Miller W. MultiPipMaker: comparative alignment server for multiple DNA sequences. *Curr Protoc Bioinformatics* 2005;Chapter 10(Unit10):14.
188. Wen B, Wu H, Shinkai Y, Irizarry RA, Feinberg AP. Large histone H3 lysine 9 dimethylated chromatin blocks distinguish differentiated from embryonic stem cells. *Nat Genet* 2009;41:246–50. [PubMed: 19151716]

Figure 1. Tissue specific epigenetic marks in the mouse casein gene cluster. **a** Graphic depiction of the mouse casein gene region. Indicating genes in the region: Csn1s1 (alpha S1 casein), Csn2 (beta), Csn1s2a (alpha S2a), Csn1s2b (alpha S2b), AK015291 (EST), Odam, Fdc-Sp, Csn3 (kappa casein) location and direction of transcription are indicated. **b** Location of ECRs base on multi-species comparative sequence analysis of 14 mammalian species (Human, chimpanzee, macaque, marmoset, galago, rabbit, mouse, rat, cow, dog, shrew, armadillo, elephant and opossum) using MultiPipMaker [187]. Red indicates analyzed ECR (c) Summary of preliminary results of DNaseI Hypersensitive site mapping of lactating/late-pregnant mammary gland and liver tissue. HS are indicated with an arrow, – no HS. **d** Preliminary results of DNA methylation analysis of HpaII sites and/or bisulfite sequencing: presence of DNA methylation is indicated with a +, – no methylation with –. **e** Graphical depiction of Histone H3 acetylation on selected regions of the casein locus based on ChIP of lactating mammary gland (lavender box) and liver (purple box) analyzed by real-time PCR or regular PCR. *H3K9Me2 LOCK in liver based on data from [188].

Figure 2. Tissue and developmental stage specific epigenetic marks in the WAP region: a Graphic depiction of the WAP genomic region in rabbit chromosome 10 (genbank: CM000799)(top) and mouse chromosome 11 (UCSC browser) (bottom) location and direction of transcriptional are indicated. *Note:* the Ramp3 gene has not been identified in rabbit. **b** Location of conserved regions based on mouse and rabbit comparative sequence analysis (from Millot et al 2003 [69]) numbers indicate DNaseI hypersensitive sites [57,69]). **c** Summary results of DNaseI Hypersensitive site mapping of lactating mammary gland and liver tissue in rabbit [57,69]). DHS are indicated with an arrow, – no DHS. **d** Results of DNA methylation analysis using methylation sensitive restriction enzyme. Presence of DNA methylation is indicated with a +, no methylation with–, intermediate methylation with ± [57]. **e** Graphical depiction of Histone H3 acetylation (IP/input) on selected regions of the WAP locus (promoter and lactation specific HSS2) based on ChIP of lactating mammary gland (lavender box) and liver (purple box) analyzed by real-time PCR.

Figure 3.

Detection of loop formations between beta casein gene regulatory elements in HC11 cells induced with lactogenic hormones and after withdrawal of hormones. **a** Beta casein gene expression induction 24 hrs after addition of lactogenic hormones and 96 hrs after subsequent withdrawal of Prl, determined by Q-RT-PCR as described in Kabotyanski et al 2009 [83]. **b** Location (Kb) of HindIII sites and primers used to determine looping in 3C assay from [83]. **c** Fold increase of looping between the β -casein gene promoter and the BCE after addition of Prl and subsequent withdrawal of Prl. **d** As in C for the β -casein promoter and far upstream control, C, region.

Figure 4.

Linear representation of chromatin loop interactions with the nuclear matrix around the WAP gene. In mouse mammary 4T1 cells (ATCC®: CRL-2539™) or in mouse mammary HC11 cells incubated in the presence (+) or absence (–) of lactogenic hormones [154], expressed genes are depicted in green, gene which are not expressed are in red. The interactions with the nuclear matrix listed in Table I or with type II topoisomerase are indicated by grey (nuclear matrix) or red (type II topoisomerase) symbols.

Figure 5.

Methylation status of Pinc during mammary gland development: Pinc is a pregnancy upregulated long-non-coding RNA. DNA was isolated from MEC preparations or non-MEC (or fatpad) for 3 week and 6-week-old virgin animals, 8 day lactating mammary gland and liver. DNA was treated with Bisulfite [37], PCR amplified with primers specific for Pinc promoter region and PCR fragments were directly sequenced. Presence of a T-peak in chromatogram at location of CpG after BS treatment and sequencing indicates hypomethylation (no fill) while presence of a C-peak indicates hypermethylation (*dark gray fill*).

Table 1

Interactions between the mouse WAP locus and nuclear structures.

Position relative to the initiation of transcription of the WAP gene (kb)	-13.8	-10.3	-2.9	+2.9	+8.9	+13
Nuclear matrix from 4T1	-	+	-	-	+	+
Nuclear matrix from HC11 w/o IPD	+	+	-	-	+	-
Nuclear matrix from HC11 with IPD	+	+	+	+	+	-
Position of near by AT-rich regions (kb)	-14.5	-11	-2.2	+3	+8.5	+13

Mouse mammary cells (HC11 and 4T1) were grown to confluency and treated with or without lactogenic hormones as previously described [95]. Nuclear matrix was prepared, DNA extracted, labelled and hybridized with a membrane carrying a series of oligonucleotides, as previously described [92]. Fifty-three oligonucleotides had been designed regularly positioned over a 46 kb region covering from -22 to +24 kb around the WAP gene transcription start site (TSS). After several washes, signals were quantified. Two oligonucleotides located at -10.3 and +8.9 from the TSS of the WAP gene gave strong signals in HC11 cells treated with or without hormones as well as in 4T1 cells. One oligonucleotide located at -13.8 kb gave a low but significant signal with nuclear matrix from HC11 cells treated with or without lactogenic hormones but this signal was not observed for 4T1 cells. Two oligonucleotides corresponding to -2.9 and +2.9 gave low but significant signals with nuclear matrix from HC11 cells treated with lactogenic hormones. These signals are specific to these cells and not observed in HC11 cells in the absence of hormones or in 4T1 cells. One oligonucleotide located at +13.0, only gave a signal in the 4T1 cells. All oligonucleotides, which gave signals in one of above conditions, are located within or close to AT-rich regions. AT-rich regions have been predicted to be potential MAR using the MAR Wiz software. However, we could not detect an interaction for oligonucleotides corresponding to such regions located around -16 kb and -7.4 kb in the mammary cells we studied.