Epigenetic gene regulation

of differentiation and epithelial-mesenchymal transition

in mammary epithelial cells

Dissertation

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Summary

Mammary epithelial cells can differentiate to serve the epithelial function, or undergo epithelial-mesenchymal transition (EMT) to lose function under certain circumstances, respectively. Both processes correlate closely to breast cancer, i.e., differentiation reduces the risk of tumorigenesis while EMT is correlated to tumor metastasis. In this dissertation, both processes were analyzed with respect to the gene regulation by epigenetic modifications.

The regulation of beta-casein gene expression in HC11 mammary epithelial cells provides a model to study the mechanism by which mammary epithelial cell differentiation is controlled. In this part, the chromatin accessibility at the beta-casein promoter and the role of H3K9me2 in transcriptional regulation of beta-casein gene in HC11 cells have been studied. Moreover, the function of the JmjC domain-containing demethylase Jmjd1a in beta-casein expression was also analyzed. The results revealed that the beta-casein promoter is inaccessible in HC11 cells, and the inaccessibility is independent of hormone treatment. Moreover, the repressive histone mark H3K9me2 is associated at beta-casein promoter. Since the associated H3K9me2 is correlated to chromatin inaccessibility, it probably represses the beta-casein gene. Additionally, other H3K9me2 interplay partners can be also involved in the repression. Although the overexpression of Jmjd1a essential domains reduces global H3K9me2 level, beta-casein expression is still not upregulated. This is, at least in part, due to the inhibitory effect of Jmjd1a domains on transcription machinery, or due to the inhibitory effect of the interplay partners on chromatin structure. These results also suggest that approaches to identify the elicitors of the H3K9me2 modification in the HC11 cells might contribute to the understanding how differentiation is regulated in mammary epithelial cells and breast cancer cells by epigenetic mechanisms.

EMT is regulated by molecular networks of extracellular stimuli, signaling factors and inducers. Research on the transcriptional regulation of EMT had been focused on inducers like Snail until the identification of CBF-A/KAP-1/FTS-1 complex as a master regulator. However, the epigenetic regulation of this master regulator and inducer Snail remains unclear. In this part, the role of H3K27me3 and the histone methylation inhibitor DZNep in regulation of EMT was analyzed. DZNep was identified to be a novel EMT inducer and the inductive effect results, at least in part, from the activation of the CBF-A/KAP-1/FTS-1 complex. This finding also revealed that H3K27me3 regulates EMT at levels of marker genes, Snail, and most probably also the master regulator. The promoters of both the epithelial marker Cdh1 and the mesenchymal marker Acta2 are associated with H3K27me3 and the associations correlate to gene transcription, but those associations were not affected by DZNep. In addition, Snail might be a

target of H3K27me3, since removal of H3K27me3 by both DZNep and Suz12 knockdown can equally enhance the TGFβ3 mediated induction of Snail mRNA. Finally, although DZNep activates the CBF-A/KAP-1/FTS-1 complex to induce EMT, the H3K27me3 association at FTS-1 element is not involved in FSP1 upregulation. DZNep might affect some unknown histone or non-histone proteins, which are essential for the activation of the CBF-A/KAP-1/FTS-1 complex. Since DZNep has been considered to be a potential anti-breast cancer drug (Hayden, Johnson et al. 2009), and EMT correlates closely to tumor invasion and metastasis, DZNep might potentially enhance the risk of breast cancer metastasis by inducing EMT in cancer cells.

Zusammenfassung

Brustepithelzellen können neben der Differenzierung um epitheliale Funktionen wahrzunehmen unter bestimmten Umständen auch die Epithelial-Mesenchymale Transition (EMT) durchlaufen und sich so ihrer Funktion entziehen. Diese Differenzierungsvorgänge spielen eine wichtige Rolle bei der Ausbildung von Brustkrebs, da die Differenzierung der Brustepithelzellen das Risiko der Tumorgenese reduziert, während die EMT eine wichtige Rolle bei der Tumormetastasierung spielt. In dieser Arbeit wurde der Einfluß von epigenetischen Modifikationen in Hinblick auf die Genexpression in diesem Zusammenhang untersucht.

Zur Untersuchung dieser Vorgänge wurde die Beta-Casein Expression in HC11 Zellen als Modellsystem für die Untersuchung der Differenzierung von Brustepithelzellen verwendet. Unter anderem wurde die Funktion der Chromatinzugänglichkeit und die H3K9 Dimethylierung am Beta-Casein Promotor in Bezug auf die Regulation der Transkription in HC11 Zellen untersucht. Es wurde gezeigt, daß die H3K9 Dimethylierung mit einer Verringerung der Chromatinzugänglichkeit einhergeht und dies wahrscheinlich für die Repression der Expression des Beta-Casein Gens verantwortlich ist. Trotz Verringerung der H3K9 Dimethylierung kann durch Überexpression der JmjC-Domäne der Histondemethylase Jmjd1 die Repression der Transkription nicht aufgehoben werden. Hieraus wird ersichtlich, daß in diesem Zusammenhang sowohl epigenetische, als auch nicht-epigenetische Phänomene für die Differenzierung essentiell sind. Weiter wird deutlich, daß Ansätze zur Entfernung der H3K9 Dimethylierung möglicherweise die Differenzierung von Brustepithelzellen zu Krebszellen verstärkt und diese Modifikation als möglicher therapeutischer Ansatz zur Behandlung von Brustkrebs weiter untersucht werden muß.

Die EMT wird durch eine Vielzahl molekularer Netzwerke extrazellulärer Stimuli und Signalmoleküle reguliert. Zunächst war die Regulation der EMT durch Transkriptionsregulation nur durch den Induktor Snail charakterisiert worden, bis darüber hinaus auch der CBF-A/KAP-1/FTS-1 Komplex als Masterregulator identifiziert werden konnte. In einem weiteren Ansatz konnte in dieser Arbeit gezeigt werden, daß der Histonmethylierungsinhibitor DZNep als EMT Induktor durch Aktivierung des CBF-A/KAP-1/FTS-1 Komplexes fungiert. Diese Erkenntnisse verdeutlichen, daß die Trimethylierung von H3K27 die EMT durch die Regulation der Expression der Markergene, Snail, und evtl. den Masterregulator beeinflusst. Die Promotoren sowie epithelialer (Cdh1), als auch mesenchymaler (Acta2) Marker weisen die H3K27 Trimethylierung auf und diese beeinflusst die Expression der untersuchten Gene. Die Behandlung mit DZNep hatte allerdings keinen Einfluß auf deren Expression. Darüber hinaus ist Snail möglicherweise ein Ziel der Regulation durch die H3K27 Trimethylierung, da sowohl

deren Verringerung durch DZNep, als auch durch Suz12 Knockdown einen Einfluß auf die TGFß3 vermittelte Snail-Expression zeigen. Die H3K27 Trimethylierung am FTS-1 Element scheint aber nicht an der EMT beteiligt zu sein, da die FSP-1 Expression nicht durch diese Histonmodifikation reguliert wird. Alternativ induziert DZNep die EMT durch die indirekte Aktivierung des CBF-A/KAP-1/FTS-1 Komplexes durch bislang unbekannte Elemente (Histone oder nicht-Histon Proteine). Da die EMT mit der Invasivität und Metastasierung von Krebszellen korreliert und DZNep die EMT von Brustepithelzellen induziert, sollte die Wirksamkeit von DZNep als Substanz zur Behandlung von Krebs in Frage gestellt und weiter untersucht werden.

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Abbreviations

aa	amino acid
AdoHcy	S-Adenosyl-homocysteine (SAH)
AdoMet	S-Adenosyl methionine (SAM)
ATP	Adenosine-5'-triphosphate
bp	base pair
BSA	Bovine serum albumin
cDNA	complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
C-terminus	Carboxyl-terminus
DIP	Dexamethasone, Insulin, Prolactin
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNMT	DNA methyltransferase
dNTP	Deoxyribonucleoside triphosphate
E. coli	*Escherichia coli*
EDTA	Ethlylene diamine tetraacetic acid
EED	Embryonic ectoderm development
EMT	Epithelial-mesenchymal transition
EZH2	Enhancer of Zeste homolog 2
FAD	Flavin adenine dinucleotide
FCS	Fetal calf serum
g	gram
GAS	Gamma interferon activated sites
h	hour
H3	Histone H3
H3K9me2	Dimethylated Lys 9 of histone 3
H3K27me3	Trimethylated Lys 27 of histone 3
H4	Histone H4
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HMT	Histone methyltransferase
HP1	heterochromatin protein 1
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
JmjC	Jumonji C
KDa	Kilo Dalton
KDM	Lysine demethylase
l	liter
LB medium	Luria-bertani-broth medium
LSD1	Lysine-specific demethylase 1
M	Molar
m-, µ-, n-	milli-, micro-, nano-
min	minute
mRNA	Messenger RNA

N-terminus	Ami o-terminus
PAGE	Polyacrylamide gel electro horesis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PIC	Pre-initiation complex
Pol II	RNA polymerase II
PRL	prolactin
PRLR	Prolactin receptor
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	radiations per minute
rRNA	Ribosomal RNA
RT	room temperature
RT-PCR	Reverse transcription PCR
SDS	Sodium dedocyl sulphate
sec	second
shRNA	short hairpin RNA
siRNA	small interfering RNA
SUZ12	Suppressor of zeste-12
TBE	Tris-EDTA-borate buffer
TE	Tris-EDTA buffer
UV	Ultraviolet light

1 Introduction

1.1 Epigenetic regulation of gene expression

1.1.1 Epigenetics and chromatin

The term “epigenetics” was first proposed by C. Waddington and developed later to describe an interaction between genetic materials with its surroundings to create a phenotype (Waddington 1942; Goldberg, Allis et al. 2007). Currently, “epigenetics” means an event of a cell or organism to external signal, in which gene expression patterns are modified without altering the DNA sequences. Those epigenetic events can be maintained through mitoses or meioses and transferred to the next generation (Devinoy and Rijnkels 2010). Epigenetic mechanism contributes not only to the phenotypic production by interactions between DNA and external environment, but also to the fate of any given cell. Each cell type has an epigenetic signature of its own that represents genotype, development, and environmental influences. This signature finally gives rise to the phenotype of the cell and organism (Morgan, Santos et al. 2005). By the original sense, the word “epigenetic” refers to anything “in addition” to the genetic information encoded in the DNA. At the molecular level, epigenetic events are associated to the chromatin state and include both DNA methylation and of histone modification (Jones and Baylin 2002; Egger, Liang et al. 2004).

Eukaryotic nucleus hosts approximately 2 meters of genomic DNA in a space of 10μm in diameter. In order to be stored in a way that the space is efficiently minimized and its readout can be controlled, DNA is packaged with associated proteins into chromatin (Felsenfeld and Groudine 2003). The basic, repetitive unit of chromatin is the nucleosome, which comprises 146 bp of DNA wrapped around a histone octamer. Each histone octamer consists of two subunits of four types of histones: H3, H4, H2A and H2B (Kornberg and Lorch 1999). Nucleosome can be further packaged into higher order structure by means of association with the linker histone H1 and nonhistone proteins as well as looping and folding of the chromatin fiber.

There are two types of chromatin: heterochromatin and euchromatin. Heterochromatin refers to the cytologically condensed regions throughout the cell cycle, whereas euchromatin readily decondenses during interphase (Maison and Almouzni 2004). For the influence on transcription, heterochromatin usually shows repression, whereas euchromatin can be either actively or inactively transcribed (Grewal and Jia 2007). Heterochromatin can be further divided into constitutive and facultative (developmentally regulated) heterochromatin. Constitutive heterochromatin remains condensed throughout the cell cycle and thereby is

permanently silenced. Facultative heterochromatin is transcriptionally repressed based on developmental state and still has the potential to convert between heterochromatin and euchromatin (Craig 2005).

1.1.2 Histone modifications

The four core histones are composed of an N-terminal tail, a C-terminal tail and a globular domain (Luger, Mader et al. 1997). The unstructured N-terminal tails are easily accessible as they are projecting outward from the nucleosome (Fig. 1-1), therefore they can be post-translationally modified by phosphorylation, ubiquitylation, sumoylation, acetylation and methylation (Kouzarides 2007).

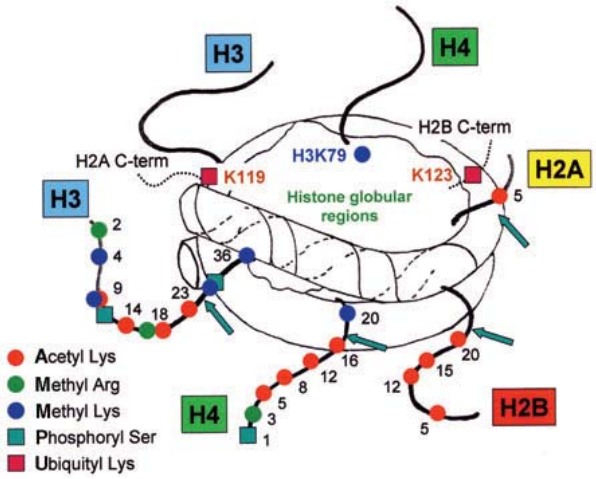

Figure 1-1 Post-translational modifications of histone

Sites of modification with corresponding residues are indicated. The arrows demonstrate the N-terminal tails of the four core histones. Figure from Turner 2005.

These modifications are involved in the regulation of gene expression by altering chromatin structure. For instance, acetylation is generally related to active euchromatin, as it can alter the chromatin structure to a more accessible state by neutralizing the positive charge of the histone and therefore weaken the DNA-histone interaction to allow transcription factors to bind (Imhof, Yang et al. 1997). Moreover, histone modifications can also recruit chromatin remodeling enzymes or other effector proteins, which in turn modify chromatin structure, to regulate transcription. One example of effecter protein is heterochromatin protein 1 (HP1), which recognizes and binds to methylated H3K9 to form heterochromatin, and therefore inhibits transcription (Grewal and Jia 2007).

1.1.3 Histone lysine methylation

Histone lysine methylation plays essential roles in heterochromatin formation, X-chromosome inactivation, and regulation of transcription (Martin and Zhang 2005). Each lysine residue can be mono-, di- or tri-methylated (Fig. 1-2). The methyl group is provided by

S-Adenosyl methionine (AdoMet), which is then converted into S-Adenosyl homocysteine (AdoHcy). Histone lysine methylation is catalyzed by histone methyltransferases (HMTs).

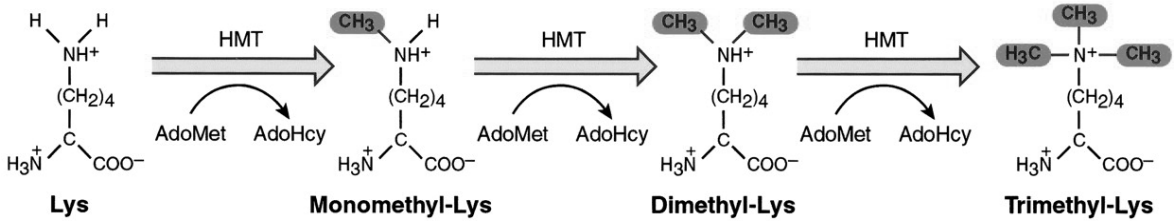

Figure 1-2 Chemistry of lysine methylation

Molecular structures of lysine and mono-, di-, and tri-methyl-lysine are shown. HMT: histone methyltransferase. Figure from Zhang and Reinberg 2001.

Most analyzed histone lysine methylations are histone 3 lysines 4, 9, 27, 36 and 79, and histone 4 lysine 20 (Martin and Zhang 2005). Histone methylation can either activate or repress transcription, depending on the methylated site of lysine residue (Jenuwein and Allis 2001). In general, methylations on H3K4, H3K36, and H3K79 is associated with active transcription, whereas those on H3K9, H3K27, and H4K20 are related to transcriptional silencing (Ebert, Lein et al. 2006).

1.1.3.1 Trimethylated Lys 27 of histone 3 (H3K27me3)

H3K27me3 is the epigenetic mark of transcriptional silencing. It is catalyzed by the Polycomb repressive complex 2 (PRC2), which consists of three core components: EZH2, SUZ12, and EED. EZH2 is the catalytic component with HMT activity, whereas SUZ12 and EED are essential co-factors.

The transcriptional repression mediated by H3K27me3 results from cooperation of PRC2, H3K27me3, and another complex Polycomb repressive complex 1 (PRC1), as indicated by a genome-wide study in that more than 1000 silenced genes are co-occupied by PRC1, PRC2 and H3K27me3 (Bracken, Dietrich et al. 2006). Once H3K27me3 is formed by PRC2, PRC1 binds to it and leads to transcription silencing by several mechanisms including spread of methylation to more chromosomal regions, direct inhibition of the transcriptional machinery, PRC1-mediated ubiquitylation of H2AK119, chromatin compaction, and recruitment of DNA methyltransferases (DNMT) to target gene (Sparmann and van Lohuizen 2006) (Fig. 1-3A).

1.1.3.2 Dimethylated Lys 9 of histone 3 (H3K9me2)

Methylation of H3K9 is basically related to transcriptional repression and heterochromatin formation. HMTs are divided to two types: heterochromatic HMT Suv39h mostly methylates H3K9 on heterochromatin, and euchromatic HMT such as G9a and Riz, which catalyzes methylation reaction on euchromatin (Shinkai 2007). Different methylation states influence functions of methylated H3K9, respectively.

Both H3K9me2 and H3K9me3 play a role in formation of constitutive heterochromatin (Ebert, Lein et al. 2006). HP1 binds to H3K9me2/3 via its chromodomain and subsequently induces the formation of heterochromatin (Fig. 1-3B) (Bannister, Zegerman et al. 2001). Furthermore, the HP1accumulation can be positively controlled by both H3K9me and the HP1-Suv39h interaction (Stewart, Li et al. 2005). Besides heterochromatin formation, H3K9me2 is involved in transcriptional regulation as well. For instance, it can cooperate with DNMT to repress tumor suppressor genes (Jenuwein 2006; McGarvey, Fahrner et al. 2006).

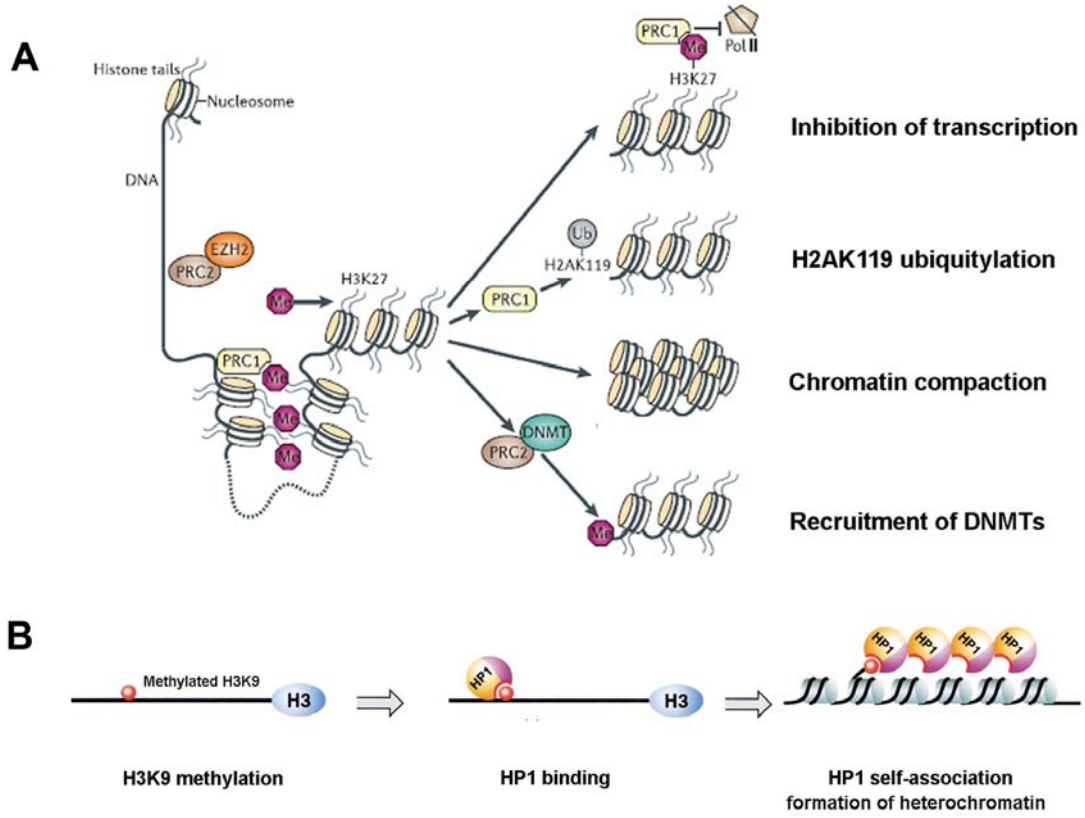

Figure 1-3 Transcriptional repression mediated by H3K27me3 and H3K9me2/3.

(A) Gene silencing mediated by H3K27me3. Catalytic subunit EZH2 of PRC2 methylates H3K27 to H3K27me3, which is recognized and bound by PRC1. The binding might induce transcriptional repression by indicated mechanisms. Ub: ubiquitylation; DNMTs: DNA methyltransferases; Pol II: RNA polymerase II. Figure from Sparmann and van Lohuizen 2006.

(B) Formation of heterochromatin via methylated H3K9. HP1 recognizes and binds to H3K9me2/3 to initiate heterochromatin formation. The heterochromatin is propagated by self-association of HP1. Figure modified from Zhang and Reinberg 2001.

1.1.4 Histone lysine demethylases

Histone methylation has been long considered to be a static epigenetic mark. However, several recently identified histone demethylases demonstrated that this modification is in fact reversible. There are at least two groups of lysine demethylases discovered. As the first identified demethylase, lysine specific demethylase 1 (LSD1) can eliminate methyl group by using flavin adenine dinucleotide (FAD) as a cofactor. It demethylates only mono- and di-

methylated lysines (Shi, Lan et al. 2004). Another group JmjC-domain-containing histone demethylases (JHDMs) contain a Jumonji C (JmjC) domain and catalyze lysine demethylation through an oxidative reaction by cofactors iron Fe(II) and alpha-ketoglutarate (αKG) . Unlike LSD1, the JHDMs can demethylate all mono-, di-, and trimethylated lysines (Tsukada, Fang et al. 2005) (Fig. 1-4).

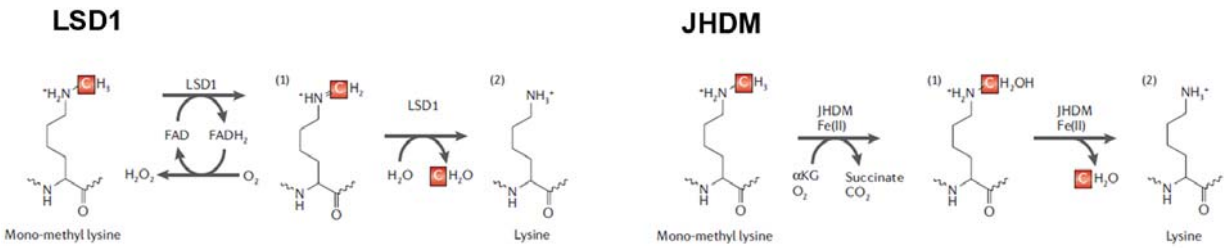

Figure 1-4 Demethylations mediated by LSD1 and JHDM.

LSD1 eliminates methyl group by using FAD as a cofactor. JHDM requires cofactors iron Fe(II) and αKG. Figure from Klose, Kallin et al. 2006.

There are approximately 27 different JHDMs encoded in the human genome, 15 of which have been revealed to be active histone demethylases on the H3 tail. A phylogenetic tree of the Jumonji proteins is described based on an alignment of JmjC domains (Cloos, Christensen et al. 2008). Among all proteins, JMJD1A und JMJD2C are involved in many essential biological events such as regulation of cell signaling, differentiation, renewal, and chromatin structure by demethylation of methylated H3K9 (Fig. 1-5).

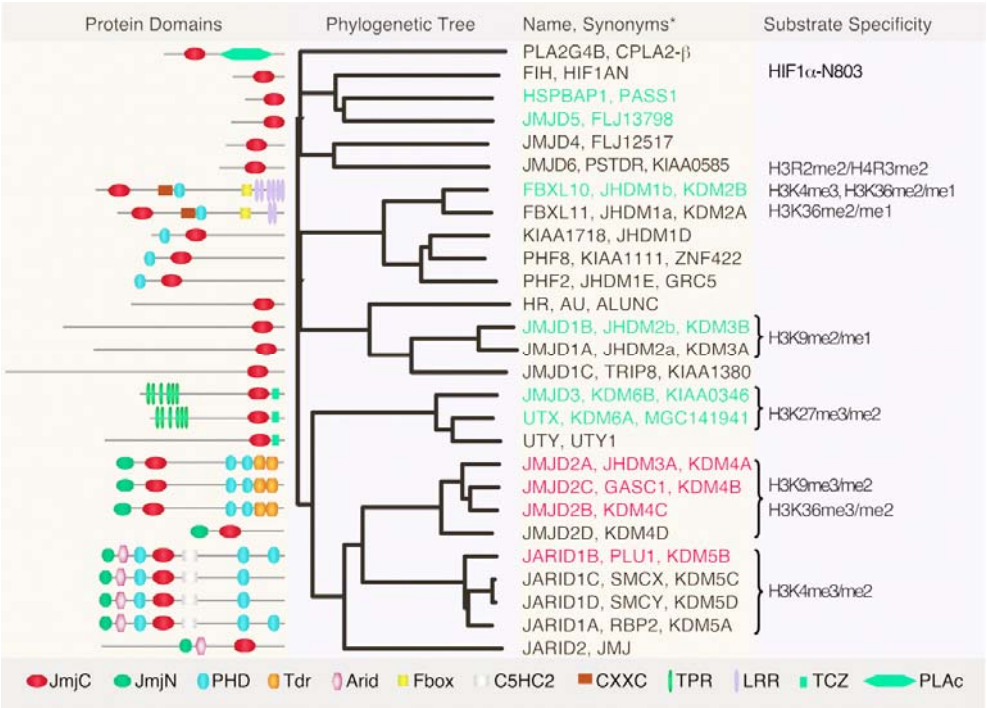

Figure 1-5 JmjC family of lysine demethylases

The domain structures, names, synonyms, and substrate specificities are shown. Figure from Cloos, Christensen et al. 2008.

JMJD1A mainly demethylates H3K9me/me2. It can function as a coactivator of androgen receptor (AR)-mediated transcription by removing H3K9me2 out of target genes (Yamane, Toumazou et al. 2006). In addition to regulation of signaling, JMJD1A also plays a role in cell differentiation and spermatogenesis. JMJD1A regulates TGFβ-mediated smooth muscle cell differentiation by cooperating with specific transcription factor myocardin and binding to target gene promoter (Lockman, Taylor et al. 2007). The role in spermatogenesis is shown by its direct binding and regulating the transcription of two important genes required for packaging and condensation of sperm chromatin (Okada, Scott et al. 2007).

Unlike JMJD1A, JMJD2C targets both H3K9me2/3 and H3K36me2/3 (Whetstine, Nottke et al. 2006). By demethylating H3K9me2/3, it could delocalize HP1 and thereby inhibit the formation of heterochromatin *in vivo* (Cloos, Christensen et al. 2006). JMJD2C is a potential oncogene in squamous cell carcinoma, and the oncogenic effect might result from demethylation of H3K9me3/me2 (Yang, Imoto et al. 2001). In line with JMJD1A, JMJD2C controls transcriptional regulation. For example, it can bind to the AR and act as a coactivator of AR-mediated transcription and cellular growth (Wissmann, Yin et al. 2007).

Both JMJD1A and JMJD2C show regulation of cell self-renewal in embryonic stem cells, in that JMJD1A demethylates H3K9me2 at several pluripotency-related gene promoters, while JMJD2C positively controls the key transcription factor Nanog by demethylating H3K9me3 at promoter (Loh, Zhang et al. 2007).

1.2 Mammary epithelial cell differentiation

1.2.1 Mammary gland development and functional differentiation

The mammary gland is composed of two tissue compartments: the epithelium and the stroma. The epithelium comprises three epithelial cell types: ductal and alveolar luminal cells, and myoepithelial cells. The ductal and alveolar cells form the inner layer of ducts and the lobuloalveolar units, respectively, and they are encased by a mesh-like system of basal, myoepithelial cells, which participate in the delivery of milk. The extensive system of ducts and alveoli is embedded in the stroma (Hennighausen and Robinson 2005; Asselin-Labat, Sutherland et al. 2007).

Mammary gland development proceeds postnatally through puberty, pregnancy, and lactation phases (Fig. 1-6) (Topper and Freeman 1980). During pregnancy the alveolar epithelial cells undergo three temporally coordinated events: proliferation, differentiation, and survival, which are initiated and regulated by diverse hormones, such as prolactin (PRL), receptor activator of NF-κB Ligand (RANK-L) (Fata, Kong et al. 2000) and ligands of the epidermal growth factor (EGF) family (Long, Wagner et al. 2003).

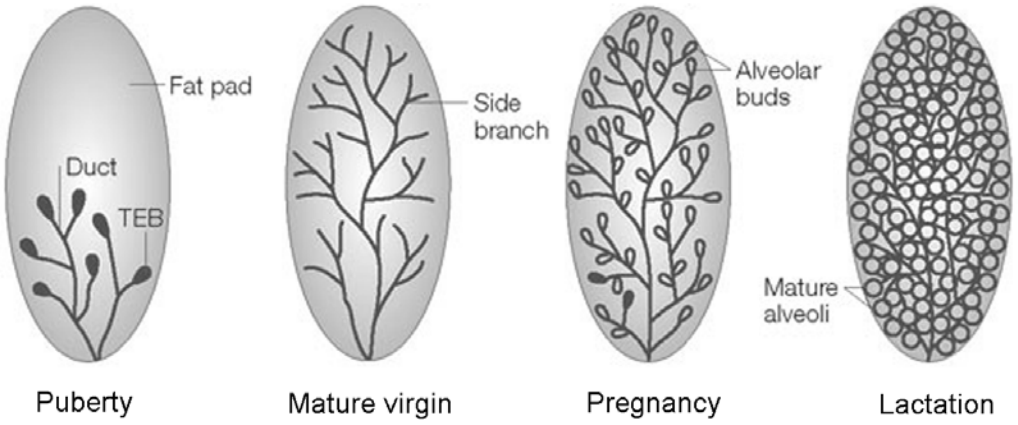

Figure 1-6 Schematic presentation of mammary gland development

During puberty, ductal growth and formation of terminal end buds (TEB) are promoted by hormones such as estrogen and progesterone. In the stage of mature virgin, the entire fat pad is filled with primary and secondary ducts. During pregnancy, the release of hormones like prolactin, placental lactogens and progesterone increase cell proliferation and the formation of alveolar buds, which differentiate into alveoli at the end of pregnancy. During lactation, alveoli are fully matured and the luminal cells synthesize and secrete milk components. Figure from Hennighausen and Robinson 2005.

Functional epithelial cell differentiation of the mammary gland begins around mid-pregnancy and is divided into initiation and activation phases. The initiation phase is characterized by enhanced expression of some milk protein genes and biosynthetic enzymes, and accumulation of neutral lipid droplets by alveolar cells; during the activation phase, alveolar cells illustrate increased expression of milk protein genes, for example beta-casein and whey acidic protein (WAP) (Robinson, McKnight et al. 1995), activation of additional milk protein genes and biosynthetic enzyme expression, polarization of organelles, expansion of mitochondria and rough endoplasmic reticulum, maturation of the Golgi apparatus, and closure of tight-junction complexes (McManaman and Neville 2003).

1.2.2 A protective role of mammary epithelial differentiation in breast tumorigenesis

Accumulating evidence indicates that mammary epithelial cell differentiation plays a protective role in breast cancer. Epidemiological and clinical findings suggest that women who delivered when they were younger than 24 years of age showed a reduction of breast tumorigenesis, and more protection could be achieved by additional pregnancy (Lambe, Hsieh et al. 1996). Moreover, similar protective effects were displayed by experimental rodent models as well (Russo, Moral et al. 2005).

Many explanations have been proposed to clarify the mechanism by which mammary differentiation reduces risk of breast cancer. Although some thought the protection might not result directly from mammary gland (Sinha, Pazik et al. 1988), the most convincing understanding declared that the protective effect is indeed mediated by the mammary epithelial

differentiation. During differentiation, the altered expression patterns of biomarker genes finally protects the mammary gland from tumorigenesis (Russo, Mailo et al. 2005). Therefore, approaches which can alter mammary cell differentiation might influence the fate of breast cancer cells accordingly.

1.2.3 Regulation of mammary epithelial cell differentiation

1.2.3.1 Stat5a is required for mammary epithelial cells differentiation

Previously named as Mammary Gland Factor (MGF), Stat5a was identified as a transcription factor promoting prolactin induced transcription of milk protein genes in mammary epithelium of lactating sheep mammary tissue (Wakao, Gouilleux et al. 1994). Later, Stat5b was discovered in the mammary gland as a signaling factor to mediate the biological function of growth hormone (GH) (Liu, Robinson et al. 1995; Lin, Mietz et al. 1996)

Stat5a and Stat5b are two closely related members of the family of Stat proteins (Fig. 1-7A). They have 96% homology at the protein level, and the highest difference is located at the C-terminal transactivation domain. Stat5a and Stat5b proteins are encoded by two genes located on chromosome 11 in mouse and chromosome 17 in humans, and the transcriptional start sites are within 10 kb of each other. While Stat5a is the prevalent Stat5 protein in mammary tissue, Stat5b is more abundant in muscle and liver. Other tissues, such as heart and salivary gland, have an equivalent distribution of Stat5a and Stat5b (Hennighausen and Robinson 2005).

Stat5 mediated transcription is triggered by binding of either PRL as well as placental lactogen to the PRLR or neuregulin1/2 to ERBB4. Stat5 is phosphorylated by the Jak2 kinase, then dimerizes and translocates into the nucleus, where it binds to the gamma interferon activation site (GAS) of promoter and induce transcription of the target genes such as milk proteins, Elf5, and suppressors-of-cytokine-signaling (Socs/Cish) family proteins. Some target genes are essential for normal mammary function. For example, milk proteins like beta-casein and WAP are critical components of milk. The transcription factor Elf-5 further activates additional genes required for mammary function. On the other hand, other target genes function to regulate the activated Stat5 signaling. As feedback inhibitors, Socs/Cish proteins are recruited to the receptor and inhibit Stat5 signaling. In addition, the membrane-associated caveolin-1 modulates Stat5 activation by regulating JAK2 accessibility. The phosphatase SHP-2 binds to the receptor and Stat5, leading to the inhibition of Stat5 phosphorylation as well (Fig. 1-7B) (Hennighausen and Robinson 2005).

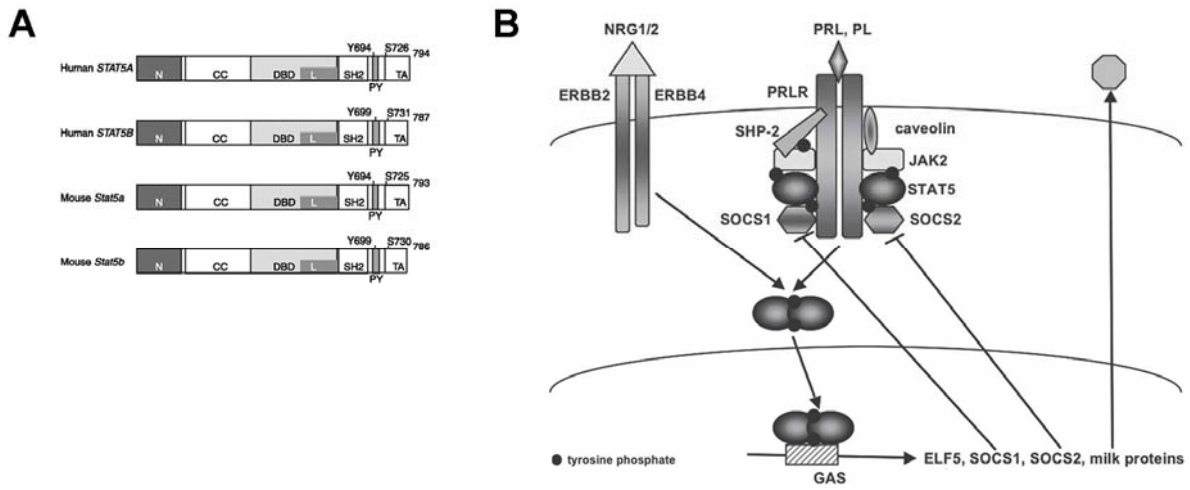

Figure 1-7 Stat5 mediated signaling pathway in mammary epithelial cells.

(A) The structural domains of human and mouse Stat5a and Stat5b proteins.
The functional domains are displayed: N-terminal domain (N), coiled-coil domain (CC), DNA-binding domain (DBD), linker domain (L), SH2 domain (SH2), and transactivation domain (TA). A highly conserved tyrosine residue is labeled as the phosphorylated state (PY). Figure from Tan and Nevalainen 2008.

(B) Regulation of Stat5 signaling in mammary epithelial cells.
PRL: prolactin; PL: placental lactogen; NRG1/2: neuregulin1/neuregulin2; SOCS: suppressors-of-cytokine-signaling; GAS: gamma interferon activation site. Figure from Hennighausen and Robinson 2005.

The essential function of Stat5 in mammary epithelial cells was investigated by the phenotypes of knockout mice. Stat5a loss caused a deficiency in mammary alveolar differentiation and milk secretion during pregnancy (Liu, Robinson et al. 1997). In Stat5-null mice, the epithelial cells could form simple ducts, but cell proliferation and alveolar development during pregnancy were greatly inhibited. A conditional Stat5 knockout gave rise to a phenotype similar to what is observed in Stat5-null epithelia, namely, females were able to form ducts in puberty, but alveolar development in pregnancy was attenuated, and dams were unable to rear offspring. These findings show Stat5 is required for stage-specific proliferation, differentiation, and maintenance of secretory mammary epithelial cells (Cui, Riedlinger et al. 2004).

1.2.3.2 Transcription factors control the mammary luminal cell fate

All three epithelial cell types (ductal, alveolar, and myoepithelial cells) are originated from a common multipotential mammary stem cell (Shackleton, Vaillant et al. 2006; Stingl, Eirew et al. 2006). It is assumed that this stem cell contributes to a common progenitor, which subsequently splits into two lineages: the luminal progenitors that generate both ductal and alveolar luminal epithelial cells and the myoepithelial progenitors that produce myoepithelial cells (Fig. 1-8). The differentiation of luminal progenitors into ductal or alveolar cells is regulated by diverse transcription factors (Siegel and Muller 2010).

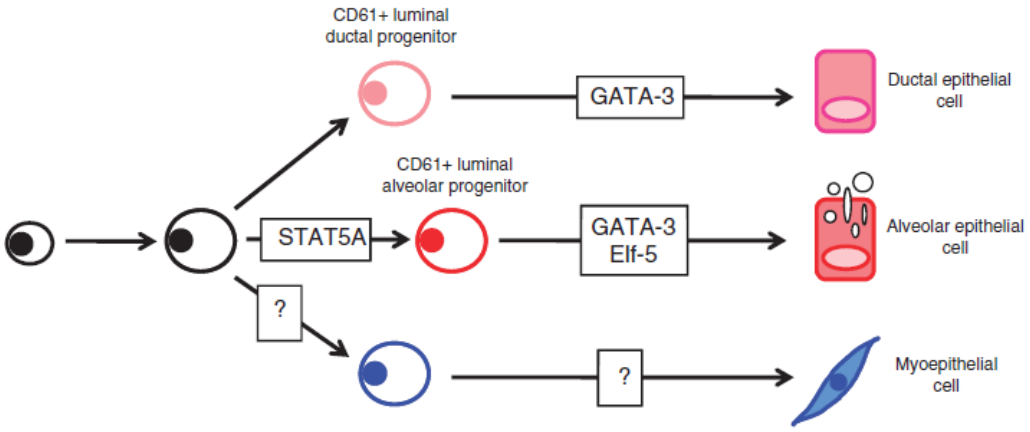

Figure 1-8 Transcription factors in regulation of mammary epithelial cells differentiation

GATA-3 is involved in genesis of both ductal and alveolar epithelial cells. Stat5 determines CD61+ alveolar progenitors and Elf-5 plays a role in the induction of the terminally differentiated alveolar epithelial cells. Figure from Siegel and Muller 2010.

Stat5a is both necessary and sufficient for specification of luminal alveolar progenitors from stem cells. Stat5-null epithelium reduced CD61+ luminal alveolar progenitor cells without altering the population of CD61+ luminal ductal progenitors, therefore, transplants of Stat5-deficient stem cell-enriched populations could form normal duct but failed to create functional alveolar development. Controlled expression of Stat5a could restore the luminal progenitors and rescued alveolar development (Yamaji, Na et al. 2009).

Other transcription factors like GATA3 and Elf-5 display critical function in determination of luminal epithelial cell differentiation in mammary gland. GATA-3 is the key regulatory factor in the differentiation of mammary progenitor cells. It regulates both the formation of mature ductal epithelium and alveolar differentiation during pregnancy (Kouros-Mehr, Slorach et al. 2006; Asselin-Labat, Sutherland et al. 2007). Elf-5 is considered to be a master regulator of alveolar differentiation during pregnancy. Like Stat5a -deficient mammary glands, mammary-specific knockout of Elf-5 led to a complete block in alveolar differentiation. However, unlike GATA-3, loss of Elf-5 has no influence on ductal elongation and branching in mammary epithelial, meaning it only controls alveolar differentiation (Oakes, Naylor et al. 2008; Choi, Chakrabarti et al. 2009)

1.2.4 Regulation of beta-casein gene expression in mammary epithelial cells

Beta-casein gene expression is regarded as a marker for function of mammary epithelial cells. The beta-casein gene promoter has been investigated as a model for differentiation regulation of target gene. The beta-casein gene expression is regulated by cooperative network of transcription factors, regulatory elements, extracellular matrix, chromatin structure and nuclear organization.

Transcription factors Stat5, CCAAT/enhancer-binding protein β (C/EBP-β), and the glucocorticoid receptor (GR) regulate the beta-casein gene expression by binding to the promoter upon the treatment of cells with the lactogenic hormones dexamethasone, insulin and prolactin (Rosen, Wyszomierski et al. 1999; Doppler, Geymayer et al. 2000; Groner 2002). Other cofactors YY-1, Histone deacetylase 1 (HDAC1) and p300 also play roles in regulation of beta-casein gene expression and possessing posttranslational modifications of histone (Meier and Groner 1994; Pfitzner, Jahne et al. 1998; Xu, Nie et al. 2003) (Fig. 1-9).

In addition to promoter, a distal enhancer which is located in the mouse approximately -6 kb upstream of the transcription start site, is involved in lactogenic hormones induced beta-casein gene expression as well. Upon the lactogenic treatment, a chromatin loop structure is formed by those two elements. This in turn recruits Stat5 and HDAC1 to promoter and enhancer; the latter contributes to the deacetylation of C/EBP-β and allows transcriptional activation (Xu, Nie et al. 2003). With the reciprocal dissociation of YY1 from the promoter, RNA polymerase II binds to promoter and enhancer and induces beta-casein transcription (Kabotyanski, Huetter et al. 2006). By removal of hormones, the chromatin loop is disrupted and beta-casein expression is inhibited consequently (Kabotyanski, Rijnkels et al. 2009).

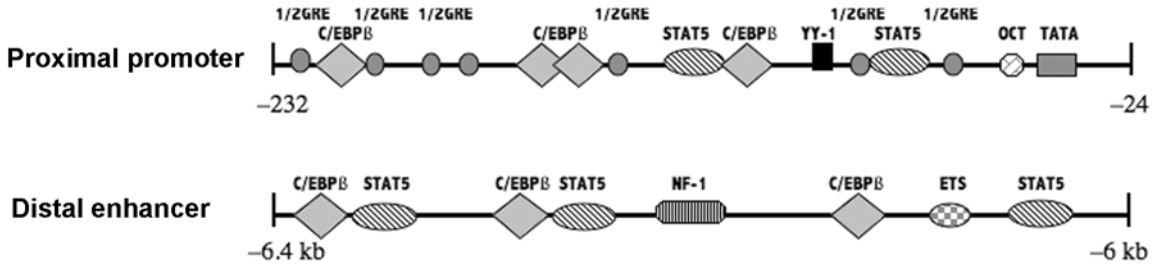

Figure 1-9 Schematic representation of mouse beta-casein regulatory elements.

DNA binding sites of indicated transcription factors are shown. Figure from Kabotyanski, Huetter et al. 2006.

Extracellular matrix plays a crucial role in regulation of beta-casein transcription. Laminin-111, in cooperation with prolactin, induces histone acetylation and recruits transcription factors as well as ATP-dependent remodeling complex SWI/SNF to the beta-casein promoter. This results in chromatin remodeling in an ATP dependent manner, and probably the exposure of a core promoter for binding of pre-initiation complex (PIC) (Xu, Spencer et al. 2007). Furthermore, laminin-111 also can help to induce mammary functional differentiation by reorganizing cells from flat monolayers into polarized acini, leading to both the exposures of the prolactin receptor and sustained activation of Stat5 (Xu, Nelson et al. 2009).

Nuclear organization of the casein gene locus is involved in the mammary epithelial cell differentiation as well. The redistribution of heterochromatin was found in mammary gland during development. In the lactating mammary gland, heterochromatic regions are mostly

located at the nuclear periphery, whereas in case of virgin gland, heterochromatin is distributed throughout the nucleus (Kress, Ballester et al. 2010). The localization of casein gene also varies in HC11 cells upon stimulation. At untreated state, the casein locus stays inside its chromosome territory and is predominantly positioned at the nuclear periphery in the vicinity to the envelope, namely, the heterochromatic region. Once treated with lactogenic hormone, the chromatin loop containing casein locus slightly moves towards the centre of the nucleus, becomes unfolded, and finally is relocated outside its chromosome territory. This correlates with active transcription (Ballester, Kress et al. 2008; Kress, Ballester et al. 2010). Those findings show that the repositioning of beta locus out of heterochromatin region plays a role of cell differentiation.

1.2.5 The epigenetic status of beta-casein promoter during differentiation

The epigenetic status of a DNA sequence based on DNA methylation or histone modification determines the chromatin status, nuclear organization, the accessibility to transcription factors, and finally regulation of gene expression.

1.2.5.1 DNA methylation at beta-casein promoter

The beta-casein promoter is hypomethylated during differentiation. Hypomethylation was found from the lactating mammary gland compared to the liver, which correlates to gene expression (Johnson, Levy et al. 1983). Recently, a study of the tissue and developmental stage-specific DNA methylation status in the mouse casein gene cluster region showed that the casein gene promoters demonstrate lower levels of DNA methylation in lactating mammary gland compared to non-mammary tissue, and similar finding is also found from mammary epithelial cells isolated from mammary gland at different stages of development (Rijnkels, Kabotyanski et al. 2010). Those data suggested that the chromatin across beta-casein promoter is decondensed and transcriptionally active (Devinoy and Rijnkels 2010).

1.2.5.2 Histone modification at beta-casein promoter

Beta-casein promoter can be modified by both active and repressive histone markers. In HC11 cells, histone H3 acetylation associated with open chromatin occurs rapidly upon the lactogenic treatment at promoter to enhance the gene transcription (Kabotyanski, Huetter et al. 2006). As mentioned above, repressive modifications H4K20me3 and H3K9me3 might contribute to the constitutive heterochromatin, where the casein locus is located in untreated condition (Kress, Ballester et al. 2010). So far two other main repressive modifications H3K9me2 and H3K27me3, which lead to facultative heterochromatin and silenced genes, are not well analyzed yet.

1.3 Mammary epithelial cell transition of mesenchymal cell aspect

1.3.1 Epithelial-mesenchymal transition (EMT)

Epithelial cells contribute to the cavities of body and many glands. They form layers of polarized cells connected with their neighbors by specific membrane structures including adherens junctions, desmosomes, gap junctions and tight junctions. On the other hand, mesenchymal cells do not have apical-basolatarel polarization, and they are only loosely organized in a three-dimensional extracellular matrix and function to form connective tissues adjacent to epithelia. In spite of differences in phenotypes and functions, the two cell types are able to convert one another under special circumstances. The conversion of epithelial cells to mesenchymal cells is known as EMT, and the reverse process is mesenchymal–epithelial transition (MET) (Thiery and Sleeman 2006).

EMT process is a complex series of morphological and cellular events, in which epithelial cells gradually lose many of their epithelial properties and acquire lots of mesenchymal features (Hay 1995). Hallmarks of EMT include: (a) loss of cell surface proteins, (b) gain of cytoskeletal molecules, (c) actin reorganization, and (d) activation of transcription factors (Teng, Zeisberg et al. 2007) (Fig. 1-10)

(a) The downregulation of cell surface molecules like E-Cadherin (Cdh1) and Integrin. Cdh1 is a prototypical epithelial cell marker, and its decreased expression was found during EMT in many procedures such as embryonic development, tissue fibrosis, and cancer (Hay and Zuk 1995). Furthermore, a cadherin switch from Cdh1 to mesenchymal marker N-cadherin (Cdh2) is shown in EMT, indicating loss of epithelial marker is accompanied simultaneously with the gain of mesenchymal marker. In addition, due to an intensive cross regulation between Cdh1 and Integrin (Li, Yang et al. 2003; Li, Yang et al. 2007), Integrin expressions are altered as well in EMT. However, since different integrins are expressed in both epithelial and mesenchymal cells, this effect is context-dependent (Zeisberg and Neilson 2009).

(b) The upregulation of cytoskeletal markers and extracellular proteins. Fibroblast-specific protein-1 (FSP1) is a member of the family of Ca^{2+}-binding S100 proteins (Strutz, Okada et al. 1995). As a fibroblast marker, it has been employed to detect EMT in the formations of cancer and fibrosis (Iwano, Plieth et al. 2002; Xue, Plieth et al. 2003). Furthermore, FSP1 has been found expressed by most epithelial cells during early stage of EMT in tissue fibrosis. Lineage tagging in transgenic reporter mice showed that EMT leads to more than one-third of all $FSP1^+$ fibroblasts in fibrotic kidneys and livers (Iwano, Plieth et al. 2002; Zeisberg, Yang et al. 2007). Another cytoskeletal molecule is smooth-muscle-actin (Acta2). It belongs to actin family, and it is generally expressed in vascular smooth muscle cells and myoepithe-

lial cells (Gabbiani, Kapanci et al. 1981). It has been found upregulated in many EMT processes for example embryonic development and fibrosis (Ramsdell and Markwald 1997).

(c) The activation of small GTPases with the cytoskeletal rearrangement. During EMT, the remodeling of actin cytoskeleton from cortical actin to stress fibers is accompanied by loss of polarity. Disruption of intercellular junctions leads to enhanced motility, which is another feature of EMT. The process is mediated by GTPases, which are activated by guanine nucleotide exchange factors (GEFs) upon stimuli. Besides reorganization, the activated Rho can also give rise to *de novo* formation of stress fibers (Zavadil and Boettinger 2005).

(d) Activated transcription factors. Many transcription factors are activated and in turn regulate EMT. CArG box–binding factor–A (CBF-A) and KRAB-associated protein 1 (KAP-1) form a complex with fibroblast transcription site–1 (FTS-1), which is a regulatory element present in the promoter of various EMT target genes and inducers, to regulate EMT (Venkov, Link et al. 2007). In addition, Snail and Slug regulate various aspects of EMT as well (Barrallo-Gimeno and Nieto 2005). Other factors involved include Twist and FOXC2 (Yang, Mani et al. 2004; Mani, Yang et al. 2007).

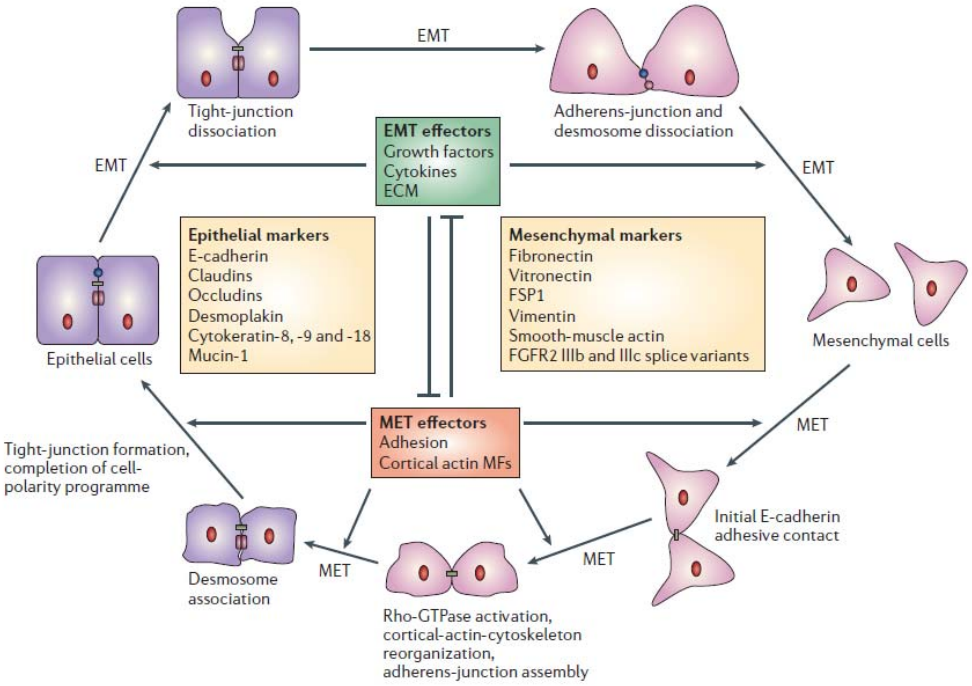

Figure 1-10 Overview of EMT program

The diagram shows the cycle of events of EMT and MET. Typical epithelial and mesenchymal marker genes are indicated. ECM: extracellular matrix; FGFR2: fibroblast-growth-factor receptor-2; MFs: microfilaments. Figure from Thiery and Sleeman 2006.

1.3.2 EMT in physiological and pathological events

1.3.2.1 EMT in embryonic development and wound healing

EMT plays a fundamental role in normal embryonic development, for example the formation of mesoderm and the derivation of neural crest cells. During gastrulation, epithelial cells need to undergo EMT prior to migration to finally form the endodermal and mesodermal embryonic tissues (Bellairs 1986). Similar process occurs by neural crest development where epithelial cells acquire mesenchymal phenotypes through EMT (Duband, Monier et al. 1995). EMT also contributes to other procedures like formation of cardic valve, palastal fusion, and branching morphogenesis during mammary gland development (Hollier, Evans et al. 2009).

Moreover, being involved in wound healing and tissue regeneration, EMT acts as a physiological response to injury. During wound healing, keratinocytes at the surrounding region undergo part of the EMT process, in which they seem to obtain an intermediate phenotype known as the “metastable” state. This allows them to move while maintaining loose contacts rather than to migrate as individual cells and to facilitate the regeneration of the original tissue structure (Schafer and Werner 2008).

1.3.2.2 EMT in fibrosis and cancer metastasis

In addition to embryonic development, EMT also gives rise to many pathological processes such as organ fibrosis. In case of tissue wound, inflammation associated with injury process let cells secrete several cytokines like TGFβ, EGF, and fibroblast growth factor 2 (FGF2), which in turn induce epithelial cells to undergo EMT. Several analyses confirm that EMT is involved in fibrosis in many human organs like kidney, lung and liver (Chilosi, Poletti et al. 2003; Kalluri and Neilson 2003).

EMT is indicated to play a role in cancer metastasis as well. During the cancer progress, carcinoma cells acquire mesenchymal properties via EMT resulting in altered adhesion ability as well as enhanced proteolysis and mobility. Thereby, they could first detach from the primary tumor, then adhere to and invade into the surrounding stroma, enter blood vessels, and finally disseminate at distant tissues and organs where they settle and form the secondary tumors (Sleeman 2000; Tomaskovic-Crook, Thompson et al. 2009).

1.3.2.3 EMT plays a special role in breast cancer metastasis

EMT plays a major role in breast cancers which are mainly derived from epithelial cells. This is supported by *in-vitro* data. For example, EMT is able to drive both normal and malignant mammary epithelial cells to gain invasive and migratory phenotypes (Blick, Widodo et al. 2008). Moreover, EMT has a clear involvement in breast cancer cell lines with high metastatic potential (Hollier, Evans et al. 2009). Most importantly, induced by EMT, cells isolated

from breast carcinoma are more related to breast cancer stem cells, therefore they have ability to reestablish the second tumor, and enhance resistance to drugs and radiation (Santisteban, Reiman et al. 2009).

In addition, clinical data demonstrate the involvement of EMT in breast cancer as well. Microarray analysis identified a correlation of EMT with malignance from patients with breast cancer. Particularly, upregulation of mesenchymal marker genes is related to poorly differentiated cancers (Teschendorff, Journee et al. 2007). Similar results were also obtained by a microarray–based immunohistochemical study using 28 different markers, in that EMT is shown to preferentially occur in breast tumors with one specific phenotype. Moreover, the tendency of EMT may be related to the high aggressiveness and determine metastatic spread of these tumors (Sarrio, Rodriguez-Pinilla et al. 2008).

1.3.3 Regulation of EMT

1.3.3.1 EMT is regulated by molecular networks

Triggered by extracellular stimuli, EMT program is regulated by molecular networks of signaling factors and inducers which control target gene expression.

Triggering signals include components of the extracellular matrix (ECM), such as collagen and hyaluronic acid, soluble growth factors, like members of transforming growth factor β (TGFβ), fibroblast growth factor (FGF), epidermal growth factor (EGF), and Scatter factor/hepatocyte growth factor (SF/HGF) (Tab.1).

Factors involved in EMT regulation

Extracellular stimuli	Signaling factors	Inducer
TGFβ	Smad3	Snail/Slug
HGF	Smad7	Id2
EGF	Rho GTPases	Twist
FGF2	Ras/Raf	Hey1, Hes1
BMP7	PI3K	δEF1
Jagged	Notch	SIP1
Wnt	GSK3β	Fos
	NF-κB	LEF1
	P38 MAPK	E2A

Table 1 Factors involved in EMT regulation

Table from Zavadil and Boettinger 2005

Association of ligands with receptors starts the activation of many intracellular signaling factors such as small GTPase family, Ras, Rho and Rac, and members of the Src tyrosine-kinase family (Fig. 1-11). These factors contribute to disassembly junctional complexes and reorganize cytoskeletal (Thiery and Sleeman 2006). Another important signaling factor is

Smad family. Smad3 is essential in EMT regulation and its activation accounts for majority of TGFβ target genes (Yang, Piek et al. 2003). Once being phosphorylated, Smad2/3 translocate to the nucleus with cytoplasmic Smad4, in which they bind to regulatory regions of target genes to control transcription (Derynck and Zhang 2003).

Besides signaling factors, transcriptional inducers such as Snail (now known as Snai1) and Slug (now known as Snai2) are activated as well and in turn regulate EMT target genes. The Snail/Slug zinc-finger proteins act as transcriptional repressor by association to E-box elements in target promoters. It represses transcription of the CDH1 gene in cultured cells and during mesoderm formation during early embryonic development. Like Snail, Slug is also rapidly induced by ligand stimulation, and leads to disassembly of desmosomes (Zavadil and Boettinger 2005).

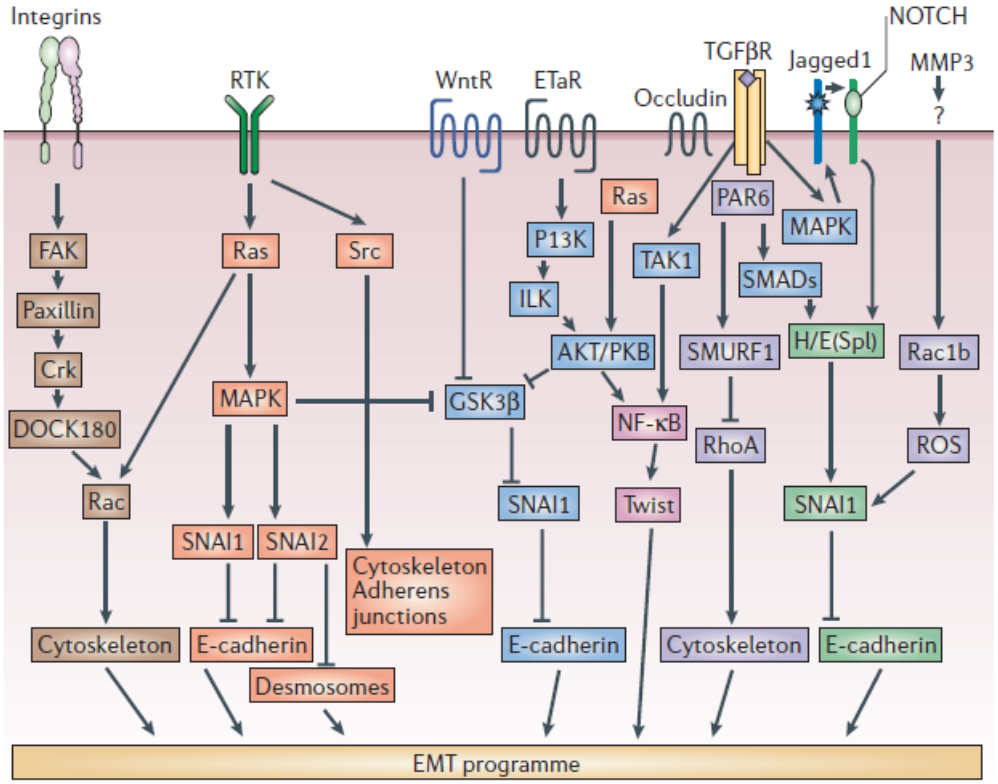

Figure 1-11 EMT program is regulated by molecular networks

MMPs: Matrix metalloproteinases; ETaR: endothelin-A receptor; FAK: focal adhesion kinase; GSK3β: glycogen-synthase kinase-3β; H/E(Spl): hairy/enhancer of split; ILK: integrin-linked kinase; MAPK: mitogen-activated protein kinase; NF-κB: nuclear factor-κB; PAR6: partitioning-defective protein-6; PI3K: phosphatidyl-inositol 3-kinase; PKB: protein kinase-B; ROS: reactive oxygen species; TAK1: TGFβ-activated kinase-1; TGFβR: TGF β receptor; WntR: Wnt receptor. Figure from Thiery and Sleeman 2006.

1.3.3.2 CBF-A/KAP-1/FTS-1 complex acts as EMT master regulator.

Recently, CBF-A/KAP-1/FTS-1 complex, consisting of two transcription factors associated with one DNA element FTS-1, is identified as EMT master regulator. CBF-A can function as

either a transcriptional activator or a repressor dependent on distinct genomic loci, whereas KAP-1 is usually considered to be a transcriptional repressor. It interacts with the nucleosome remodeling and deacetylase (NuRD) complex. The direct transcription target of this complex is FSP1, which is a crucial facilitator of EMT and its expression is a marker of early stage of EMT (Venkov, Link et al. 2007).

The regulation of EMT by the CBF-A/KAP-1/FTS-1 complex is triggered by CBF-A. CBF-A is generally localized in the cytoplasm in a latent form. Upon the external stimuli, it is activated and migrates into the nucleus, in which CBF-A/KAP-1/FTS-1 binding complex is formed. As a master regulator, this complex subsequently activates and drives known EMT transcriptional inducers such as Snail, Twist, HMGA2, LEF-1, and Ets-1 to control EMT target genes. Therefore, the regulation is based on the modulation of the whole molecular networks. In addition, the complex itself can also directly control EMT target genes (Fig.1-12) (Teng, Zeisberg et al. 2007; Venkov, Link et al. 2007).

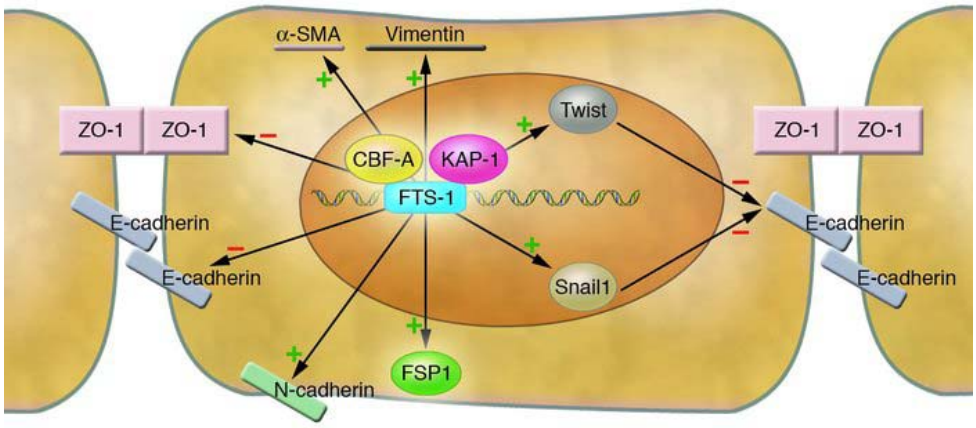

Figure 1-12 The CBF-A/KAP-1/FTS-1 complex is a master regulator of EMT

During EMT, the CBF-A and KAP-1 proteins recognize and bind to the FTS-1 element to form the CBF-A/KAP-1/FTS-1 complex and therefore regulate EMT. Arrows with +/– indicate up or down-regulation, respectively. ZO-1: zona occludens 1. Figure from Teng, Zeisberg et al. 2007.

1.3.4 Epigenetic modifications involved in EMT

So far only few data showed that epigenetic modifications, especially repressive histone marks, are involved in EMT. PRC2 contributes to repressions of some epithelial marker genes in EMT. SUZ12 is required for downregulation of CDH1 by Snail and the repression is mediated by H3K27me3 (Herranz, Pasini et al. 2008). Similarly, H3K27me3 represses β4 integrin as well (Yang, Pursell et al. 2009). In addition, the induction of mesenchymal marker genes can be mediated by replacement of methylated H3K27 to histone variant H3.3B. Upon treatment with TGFβ, Hairy and Enhancer-of-split (H/E(Spl)) related repressors are activated via Smad3/4, and subsequently repress EZH1 and EZH2 genes. Displacement of PRC com-

plex to histone variant H3.3B releases the repressive chromatin and therefore induce mesenchymal gene expression (Blumenberg, Gao et al. 2007).

Transcription of the EMT inducer Snail can be enhanced by the binding of transcription factor YY1 to the 3′ enhancer. Even though the detailed mechanism is still unclear yet, It is assumed that YY1 can recruit histone modification enzymes to alter the chromatin structure of the 3′ enhancer, since YY1 is reported to recruit CBP/p300 acetyltransferases and the arginine methyltransferase PRMT1 (Palmer, Majumder et al. 2009).

1.4 Aims

Mammary epithelial cells can undergo differentiation to serve the epithelial function, or undergo EMT (also called dedifferentiation) to lose their epithelial function under certain circumstances, respectively. Both processes not only control the physiological function of epithelial cells but also correlate closely to breast cancer, i.e., differentiation plays a protective role in breast tumorigenesis while EMT is correlated to tumor invasion and metastasis. Therefore, the mechanisms by which they are regulated might have clinical applications in related pathological events. In this dissertation, both processes were analyzed in separate parts with respect to the gene regulation by epigenetic modifications.

Part of mammary epithelial cell differentiation

The analysis of marker gene beta-casein in HC11 cells provides a model to study mammary epithelial cell differentiation. Cytological studies revealed that casein gene locus is associated with heterochromatin during differentiation in both tissue and HC11 cells (Ballester, Kress et al. 2008). Furthermore, the binding of Stat5a to endogenous beta-casein promoter upon lactogenic treatment in HC11 cells so far has not been convincingly demonstrated. Thus, the aims of this part were:

1. to analyze chromatin accessibility status of beta-casein promoter in HC11 cells.
2. to study whether epigenetic modification is associated at beta-casein promoter.
3. to regulate the transcription of beta-casein by altering the epigenetic modifications with epigenetic inhibitors or enzymes.

Part of mammary epithelial cell EMT

Triggered by extracellular stimuli, EMT program is regulated by molecular networks of signaling factors and inducers. However, little is known about the role of epigenetic modifications, especially repressive histone marks, in transcriptional regulation of EMT. Thus, the objectives in this part were:

1. to investigate whether repressive histone mark is involved in regulation of EMT by treatment of the cells with histone methylation inhibitors.
2. to elucidate how those epigenetic modifications regulate EMT.

2 Materials and Methods

2.1 Materials

2.1.1 Antibodies

2.1.1.1 Primary antibodies

Name	Type	Source	Application
Stat5a	rabbit antiserum	R&D Systems (Cat. PA-ST5A)	WB: 1:2000 ChIP: 2.5 µg
Phospho-Stat5	rabbit polyclonal	Cell signaling (Cat. 9351)	WB: 1:1000
RNA POL II P-CTD clone CTD4H8	mouse monoclonal	Millipore (Cat. 05-623)	ChIP: 2.5 µg
H3K27me3	rabbit polyclonal	Millipore (Cat. 07-449)	WB: 1:1000 ChIP: 2.5 µg
H3K9me2	mouse monoclonal	Abcam (Cat. Ab1220)	WB: 1:500 ChIP: 2.5 µg
H3	rabbit polyclonal	Abcam (Cat. ab1791)	ChIP: 2.5 µg
SUZ12	mouse monoclonal	Millipore (Cat. 04-046)	WB: 1:1000
IgG	normal rabbit IgG	Santa Cruz (Cat. sc-2027)	ChIP: 2.5 µg
Actin	rabbit polyclonal	Sigma (Cat. A2066)	WB: 1:1000
Tubulin	mouse monoclonal	Sigma (Cat. T5168)	WB: 1:50000
Gapdh	mouse monoclonal	Santa Cruz (Cat. sc-137179)	WB: 1:1000
Myc-Tag (9B11)	mouse monoclonal	Cell signaling (Cat. 2276)	WB: 1:2000
AlexaFluor488 phalloidin	conjugated	Invitrogen (Cat. A12379)	IF: 1:40

2.1.1.2 Secondary antibodies

Name	Type	Source	Application
Goat anti-mouse HRP-conjugated	IgG (H+L)	Pierce (Cat. 31160)	WB: 1:10000
Goat anti-rabbit HRP-conjugated	IgG (H+L)	Pierce (Cat. 31210)	WB: 1:10000

2.1.2 Primers for quantitative PCR

Gene	Region	Sequence 5'-3'	application

beta-casein	promoter	Forward	GCTTCTGAATTGCTGCCTTG	ChIP
		Reverse	CAAAAAGTCCCTTCAATTCCA	
Socs3	promoter	Forward	CACAGCCTTCAGTGCAGAG	ChIP
		Reverse	AGAGACAGCGGTCGTAGGAG	
Cish	promoter	Forward	GTTCGCACCACAGCCTTTCAGTCC	ChIP
		Reverse	GTCCAGGGGTGCGAAGCTCAGG	
Gapdh	promoter	Forward	CACCCTGGCATTTTCTTCCA	ChIP
		Reverse	GACCCAGAGACCTGAATGCTG	
MageA2	promoter	Forward	TTGGTGGACAGGGAAGCTAGGGGA	ChIP
		Reverse	CGCTCCAGAACAAAATGGCGCAGA	
Chd1	promoter	Forward	AGACAGGGGTGGAGGAAGTT	ChIP
		Reverse	ACCAGTGAGCAGCGCAGAG	
Acta2	promoter	Forward	CAGTTGTTCTGAGGGCTTAGGATGTTTATC	ChIP
		Reverse	ACAAGGAGCAAAGACGGGCTGAAGCTGGCC	
Krt17	promoter	Forward	ACCATCCGCCAGTTTACCTC	ChIP
		Reverse	CTACCCAGGCCACTAGCTGA	
CBF-A	promoter	Forward	CGGCTCATTCTCCTCTTTTG	ChIP
		Reverse	CGGGCTTTGTCCTCATTTTA	
FTS-1	element	Forward	GGGGAGGTGAATTGATTCCT	ChIP
		Reverse	AACTCCTTGAGCTCCGACTG	
beta-casein		Forward	TCACTCCAGCATCCAGTCACA	RT-PCR
		Reverse	GGCCCAAGAGATGGCACCA	
18S rRNA		Forward	CGGCTACCACATCCAAGGA	RT-PCR
		Reverse	CCAATTACAGGGCCTCGAAA	
Cdh1		Forward	CAGGTCTCCTCATGGCTTTGC	RT-PCR
		Reverse	CTTCCGAAAAGAAGGCTGTCC	
Acta2		Forward	GGACGTACAACTGGTATTGTGC	RT-PCR
		Reverse	CGGCAGTAGTCACGAAGGAAT	
Snail		Forward	CACACGCTGCCTTGTGTCT	RT-PCR
		Reverse	GGTCAGCAAAAGCACGGTT	
CBF-A		Forward	GCGGGAAAAATGTTCGTTGGT	RT-PCR
		Reverse	CCTCTTGATCGTCCAGTGTTG	
KAP-1		Forward	CGGCGCTATGGTGGATTGT	RT-PCR
		Reverse	GGTTAGCATCCTGGGAATCAGAA	
FSP-1		Forward	TGAGCAACTTGGACAGCAACA	RT-PCR
		Reverse	TTCCGGGGCTCCTTATCTGGG	
beta-casein	promoter	Forward	TGGAATTGAAGGGACTTTTTG	CHART-PCR
		Reverse	AGGAGGTGAAGCTGAAAGGA	
beta-casein	promoter	Forward	CGGTCCTCTCACTTGGCTGGAGGAA	Chromatin lockdown assay
		Reverse	GGAGGACAAGAGAGGAGGTGAAGCTGA	
Cish	promoter	Forward	GATAAGCGCACCCATCCCCAAAGAA	Chromatin lockdown assay
		Reverse	CAGGCGTCTAGTGCTTTGGACCGAGA	
Jmjd1a	600-	Forward	CGGGATCCATTGGCTTGTGGCTGCCT	cloning

	1323 aa	Reverse	CCGCTCGAGTTAAGGTTTGCCCAAACT	
Jmjd2c	2-300 aa	Forward	CGGGATCCGAGGTGGTGGAGGTGGAG	cloning
		Reverse	ACGCGTCGACCTAACAAGTGCACAACTTAGC	
Acta2	promoter	Forward	CCCCCCGGGCCCAGTGTCTGGGCATTTGA	cloning
		Reverse	CCCCCCGGGGCTGAACGCTGAAGGGTTAT	

2.1.3 Plasmids

Name	Application	gene	Reference/Source
pGL3-basic-beta-casein-promoter	luciferase assay	beta-casein promoter –344 to –1	(Litterst, Kliem et al. 2003)
pXM-Stat5a	expression	mouse Stat5a	(Moriggl, Gouilleux-Gruart et al. 1996)
pXM	expression	backbone	(Moriggl, Gouilleux-Gruart et al. 1996)
PRL receptor	expression	prolactin receptor	(Litterst, Kliem et al. 2003)
pCMV-*LacZ*	expression	*LacZ*	(Litterst, Kliem et al. 2003)
pGL3-basic-Acta2 promoter	luciferase assay	Acta2 promoter	This work
pGL3-basic-Cdh1 promoter	luciferase assay	Cdh1 promoter	André Brandl, Jena
pVig-Flag-Myc-jmjd1a_600-1323	lentiviral expression	mouse Jmjd1a 600-1323 aa	This work
pVig-Flag-Myc-jmjd2c_2-310	lentiviral expression	mouse jmjd2c 2-310 aa	This work
pVig-Flag	lentiviral expression	backbone	K Überla, Bochum
Δsp2	lentiviral packaging		K Brocke-Heidrich, Leipzig
pHIT-G	lentiviral packaging		K Brocke-Heidrich, Leipzig
pVig-siSuz12	lentiviral siRNA	Suz12	F Nonnenmacher, Jena
pVig-scr.	control scrambled		K Überla, Bochum

2.1.4 Buffers and solutions

Buffer	
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4
SDS running buffer (10×)	250 mM Tris, 1% SDS, 1.9 M Glycine
WB transfer buffer	25 mM Tris, 192 mM Glycine, 0.1% SDS, 20% Ethanol
TE buffer	10 mM Tris-HCl pH 8.0, 1 mM EDTA
TBE buffer	90 mM Tris-HCl pH 8.3, 2.5 mM EDTA, 90 mM Boric acid
TBST buffer	10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20

2.1.5 Enzymes and kits

All restriction endonucleases and corresponding buffers were obtained from New England Biolabs (Frankfurt am Main).

Enzyme/Kit	Company
T4 DNA Ligase	NEB, Frankfurt am Main
Taq DNA polymerase	Invitrogen, Karlsruhe

Alkaline Phosphatase, Calf Intestinal (CIP)	NEB, Frankfurt am Main
QIAquick Gel Extraction Kit	Qiagen, Hilden
QIAquick Plasmid Maxi Kit	Qiagen, Hilden
QIAquick PCR Purification Kit	Qiagen, Hilden
peqGOLD TriFast	Peqlab, Erlangen
RNeasy Mini Kit	Qiagen, Hilden
RevertAid First Strand cDNA Synthesis Kit	Fermentas, St. Leon-Rot
Chromatin Lockdown Assay	Biorad
High Pure PCR Template Preparation Kit	Roche, Mannheim
TransFectin reagent	Biorad

2.1.6 Epigenetic inhibitors

Name	Chemical name	Inhibition of	Concentr.
DZNep	3-deazaneplanocin A	S-adenosylhomocysteine hydrolase	5 µM
BIX-01294	trihydrochloride hydrate	G9a HMT	4 µM
Chaetocin	Chaetocin from *Chaetomium minutum*	SUV39H1 HMT	25 nM

2.1.7 Hormones and cytokines

Name	Concentr.	Company
TGFβ3	5 ng/ml	Immunotools (Cat. 11343153)
dexamethasone	1 µM	Sigma (Cat. D4902)
insulin	5 µg/ml	Sigma (Cat. I0516)
Prolactin	1 µg/ml	Sigma (Cat.L6250)

2.1.8 Bacteria

E.coli host strain	Genotype
TOP10	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G

2.1.9 Cell lines

Cell line	Cell type	ATCC number/source	media
HC11	mouse mammary epithelial cell	(Ball, Friis et al. 1988)	RPMI 1640, L-glutamine 10% FCS, 10 ng/ml EGF 5 g/ml insulin
NMuMG (clone 18)	mouse mammary epithelial cell	(Deckers, van Dinther et al. 2006)	DMEM, L-glutamine 10% FCS, 10 g/ml insulin
HEK 293	human embryonic kidney cell	CRL-1573	DMEM, L-glutamine 10% FCS

2.2 Methods

2.2.1 Microbiology methods

2.2.1.1 Transformation of bacteria

Competent *E.coli* cells TOP10 were used to amplify plasmid DNA. Fifty µl of *E.coli* were thawed on ice, mixed with 50 ng of plasmid or 10 µl of ligation mixture in KCM buffer (100 mM KCl, 30 mM $CaCl_2$, 50 mM $MgCl_2$). The mixture was incubated on ice for 30 min followed by 10 min at RT. The cells were then mixed with 1 ml of LB medium and incubated at 37°C for 1 h with shaking. The transformed cells were then plated on LB agar plates containing appropriate antibiotics and incubated at 37°C overnight.

2.2.1.2 Isolation of plasmid DNA (Mini preparation)

The mini preparation of plasmid DNA is based on the alkaline-detergent lysis method. Briefly, 1 ml of bacterial culture was pelleted by centrifugation at 6000 x g for 1 min. The pellet was resuspended in P1 buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/ml RNase A) followed by 100 µl of P2 buffer (200 mM NaOH, 1% SDS) and inversion 5 times. After incubation at RT for 5 min, 75 µl of P3 buffer (3 M potassium acetate, pH 5.5) was added and the tubes were inverted again 5 times. After incubation for 5 min on ice, the sample was centrifuged at 20000 x g for 10 min. The supernatant was then transferred to a new tube and DNA was precipitated by 1ml of absolute ethanol. After washing by 70% ethanol twice the pellet was air dried. The isolated plasmid DNA was resuspended in 50 µl TE buffer.

2.2.1.3 Isolation of plasmid DNA (Maxi preparation)

QIAquick Plasmid Maxi kit was used according to the manufacturer's instruction. The isolated plasmid DNA was resuspended in TE buffer.

2.2.2 DNA methods

2.2.2.1 Measurement of DNA concentration

The DNA concentration was measured by the absorbance at the wavelength of 260 nm using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington). One unit of absorbance at 260 nm corresponds to 50 ng/µl double stranded DNA. The concentration of DNA (ng /µl) = reading at 260 nm x dilution factor x 50.

2.2.2.2 Restriction digestion of DNA

Briefly, 1 µl of restriction endonuclease digests 1 µg of DNA in 50µl of reaction with appropriated buffer according to the manufacturer's instruction.

2.2.2.3 5'-dephosphorylation of DNA

Dephosphorylation was performed by incubation of 1 µg of linearized vector with 0.5 U of calf intestinal phosphatase at 37°C for 1 h. Finally the reaction was terminated by heating at 70°C for 5 min.

2.2.2.4 Ligation of DNA

Approximately 50-100 ng of linearized and 5'-dephosphorylated vector were used for ligation. The molar ratio of vector to insert was 1:10. One µl of T4 DNA ligase was used according to the manufacturer's instruction in a final volume of 15 µl and incubated at 16°C overnight. Finally the reaction was terminated by heating at 70°C for 5 min.

2.2.2.5 PCR

For a standard reaction, 50 ng of template DNA, 20 pmol of each primer, 200 µM dNTPs, 1 Unit of the Taq DNA polymerase and the adequate 10x buffer were mixed to a final volume of 25 µl. The thermal program included an initialization at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 58-63°C for 30 sec, and extension at 72°C for 1 min/kb amplicon, with a final extension at 72°C for 10 min. The amplification of the DNA fragments was controlled by agarose gel electrophoresis.

2.2.2.6 Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was performed to separate DNA fragments by size. The concentration of agarose ranged 0.8 - 2% in TBE buffer. Ethidium bromide was added to a final concentration of 1 µg/ml. The DNA samples were mixed with H_2O and 6X loading dye (0.25% bromophenol blue, 0.25% xylene xyanol, 40% sucrose) and loaded onto the gel. The electrophoresis was performed in TBE buffer with 10 V/cm gel length. One kb plus ladder served as a size standard. The DNA was visualized by UV light (254-366 nm) and documented by gel documentary system.

2.2.2.7 Isolation of DNA from agarose gels

DNA fragments separated by agarose gel electrophoresis were extracted and purified using the QIAquick Gel Extraction kit according to the manufacturer's instructions. Finally the isolated DNA was eluted with 30 µl of H_2O.

2.2.3 RNA methods

2.2.3.1 RNA extraction

Isolation of total RNA was performed using the peqGOLD TriFast and the RNeasy Mini Kit. Cells were harvested and lysed in 1 ml of TriFast. Then the cell suspension was mixed with 200 µl of chloroform, shaken vigorously, and incubated at RT for 10 min. After centrifugation at 14000 rpm for 15 min at 4°C, the aqueous phase was transferred to a fresh tube and mixed 1:1 with 100% Ethanol. The purification was then continued using the RNeasy Mini Kit. Finally the isolated total RNA was eluted with 30 µl of RNase-free H_2O.

2.2.3.2 Measurement of RNA concentration

The total RNA concentration was measured by the absorbance at the wavelength of 260 nm using the NanoDrop ND-1000 spectrophotometer. One unit of absorbance at 260 nm corresponds to 40 ng/µl single-stranded RNA.

The concentration of RNA (ng /µl) = reading at 260 nm x dilution factor × 40

2.2.3.3 First-strand cDNA synthesis

Two µg of total RNA were subjected to first-strand cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit according to the manufacturer's instructions. Briefly, 2 µg of total RNA were mixed with 1 µl of random hexamer primer and H_2O in a final volume of 11 µl, and incubated at 65°C for 5 min. Then 4 µl of 5X reaction buffer, 1 µl of RNase Inhibitor, 2 µl of 10 mM dNTP Mix, and 2 µl of M-MuLV Reverse Transcriptase (20 U/µl) were added. The mixture was incubated at 25°C for 5 min followed at 37°C for 60 min. Finally the reaction was terminated by heating at 70°C for 5 min. The resulting cDNA was 1:2 diluted with H_2O. In subsequent quantitative RT-PCR 3 µl of this cDNA were used for each reaction.

2.2.3.4 Quantitative RT-PCR

Three µl of DNA template together with 10 pmol of each primer and 10 µl of SYBR Green Mix (Abgene, Epsom, UK) were mixed in a final volume of 20 µl. This mixture was distributed into 96-well plates and covered with clear optical seal. All reactions were performed in triplicate. The quantitative RT-PCR reaction was performed by i-Cycler PCR machine (Biorad). The following thermal program was used: 95°C for 15 min to activate the DNA polymerase, followed by 45 cycles of denaturation at 95°C for 30 sec, annealing at 58-63°C for 30 sec, and extension at 72°C for 1 min/kb amplicon. All measurements were demonstrated by threshold cycles (Ct) values by the Biorad MyiQ software.

Melting curve analysis was performed to characterizing amplicons by heating the PCR reaction to 99.5°C with a temperature transition rate of 0.05°C/sec. A single narrow peak

obtained by melting curve analysis at a specific melting temperature indicated specific amplification. Additionally, the PCR product was characterized again by agarose gel electrophoresis.

2.2.3.5 Analysis of the quantitative PCR Data

For RT-PCR: The Ct of the gene of interest (GOI) and the housekeeping gene (HKG) were used to calculate their relative expression levels in different samples (for example untreated example versus treated sample). The relative expression level was calculated as:

Relative expression = $2^{-(S\Delta Ct-C\Delta Ct)}$

ΔCt is the difference of the Ct values between GOI and HKG, i.e., Ct_{GOI}- Ct_{HKG}. SΔCt - CΔCt is the difference between the sample ΔCt and the control ΔCt. The relative expression level of the control sample was set to 1.

For ChIP: Data were displayed as % of input DNA representing the % of DNA occupancy.

% Input DNA = $2^{(Ct\ Input-\ Ct\ IP)}$ × (Input ratio/IP ratio) x 100

Input ratio = (µl of Input DNA used per PCR reaction / µl final volume Input was resuspended in after DNA purification) x µl of pre-cleared lysate saved for Input; IP ratio = (µl of IP DNA used per PCR reaction / µl final volume IP was resuspended in after DNA purification) × µl of pre-cleared lysate used per IP.

2.2.4 Tissue culture methods

2.2.4.1 Cultivation and treatment of cells

HC11 cells were maintained in growth medium (mentioned in 2.1.9). For differentiation analysis, confluent HC11 cells were starved in RPMI 1640 medium supplemented with 2% FCS and 5 µg/ml insulin for 2 days. HC11 cells were then treated with 1 µM dexamethasone, 5 µg/ml insulin, and 5 µg/ml PRL.

NMuMG cells (clone NM18) were maintained in growth medium (mentioned in 2.1.9). To investigate EMT, 7.5×10^4 cells were seeded in 6-well plate 1 day before the treatment. After treatment with 5 ng/ml TGFβ3, morphological images were obtained after 24 and 48 h. Finally the cells were lysed for mRNA, protein, and chromatin analyses, or analyzed for redistribution of actin structure by immunofluorescence.

2.2.4.2 Passaging of cells

Adherent cells were washed one time with PBS and incubated with Trypsin/EDTA for 5 min at RT or 37°C. Once detached, the cells were suspended in the corresponding growth media and seeded out onto dishes at the desired dilution.

2.2.4.3 Storage and thawing of mammalian cells

DMSO was added to 5×10^6 cells in 1ml FCS to a final concentration of 10%. The cell suspension was transferred to 1.5 ml freezing vials and gradually cooled down to - 80°C using a freezing box encased with an isopropanol jacket. To utilize cells in culture, they were quickly thawn at 37°C, washed and seeded for further culturing.

2.2.4.4 Determination of cell numbers

The cells were diluted 1:20 with Isotone and the number of particles was measured by the Coulter counter (Beckman Coulter, Krefeld). Only particles between 5-18 µm in size were to be counted.

2.2.4.5 Transient transfection

HEK 293 cells were transfected using Polyethylenimine (PEI). Cells were seeded 1 d before. Plasmids were diluted by PBS and mixed with PEI followed by incubation for 20 min at RT. The mixture was applied to the cells in serum-free-DMEM medium and incubated for 3 h in incubator. Finally the normal growth medium was refreshed. Cells were harvested 24-48 h after transfection. The ratio of DNA to PBS and PEI was: 1 µg DNA: 27 µl PBS: 3 µl PEI.

NMuMG cells were transfected using TransFectin according to the manufacturer's manual.

2.2.4.6 Lentivirus production

HEK 293 cells were transiently transfected with the lentiviral vector and the packaging plasmids Δsp2 and pHIT-G using PEI. Medium was replaced after 24 h. Forty eight hours after transfection, virus supernatants were collected and filtered. Virus was concentrated by centrifugation at 3600 rpm for 16 h at 4°C, and the pellet was resuspended with medium.

2.2.4.7 Virus transduction

HC11 and NMuMG cells were transduced in the presence of 4 µg/ml polybrene (Sigma) with a multiplicity of infection (MOI) of 1-2. After two rounds of transduction, the transduction efficiency was determined by global level of corresponding protein (by gene knockdown) or expression of fused Tag (by gene overexpression) by western blot. Afterwards, the stable cells were maintained for experiments.

2.2.5 Protein methods

2.2.5.1 Cellular extract preparation

Cells were harvested and lysed through sonication for 10 sec at 20% amplitude in NETN2 buffer (20 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, 10% Glycerol, complete protease and phosphatase inhibitors). The supernatant was separated by centrifugation at 13000 rpm for 10 min. Protein concentration was determined by Bradford assay.

2.2.5.2 Bradford assay

Bradford assay was performed in 96-well plate. Two µl of extract were mixed with 1:4 diluted Bradford reagents, incubated at RT for 5 min, and measured by the absorbance at the wavelength of 595 nm using the ELx 808 Photometer. Standard curve was obtained by serial dilution of bovine serum albumin (BSA) with concentration of 0, 1, 2, 4, 6, and 10 µg/µl.

2.2.5.3 SDS-PAGE and Western blot

Ten µg of extract were used for SDS-PAGE to detect histone modifications, while 60 µg for detection of non-histone proteins. Proteins were denatured for 5 min at 95°C in sample buffer (0.125 M Tris-HCl pH 6.8, 0.5% SDS, 10% glycerol, and 0.0001% bromophenol blue), separated by SDS-PAGE using the Mini protein gel system and transferred to nitrocellulose membrane using the Mini Trans-Blot System (Biorad). The blots were blocked in blocking solution (5% non-fat milk in TBST) at RT for 1 hour and incubated with primary antibodies at 4°C overnight. After washing three times for 5 min in TBST buffer, the blots were incubated with secondary antibody coupled to HRP for 1 hour at RT followed by washing three times in TBST for 5 min. Proteins were detected by using Immobilon Western Chemiluminescent HRP Substrate (Millipore).

2.2.5.4 Blot stripping

Blot stripping was conducted by membrane incubation in stripping buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 100 mM beta-Mercaptoethanol) at RT for 60 min followed by washing with H_2O and TBST buffer.

2.2.6 Immunofluorescence

For filamentous actin (F-actin) staining, AlexaFluor488 phalloidin (Invitrogen) was used. NMuMG cells grown on coverslips were fixed with 2% paraformaldehyde in PBS for 10 min and subsequently permeabilized with 0.15% Triton X-100 in PBS for 5 min at RT. After washing three times with PBS, cells were incubated with AlexaFluor488 phalloidin for 30 min at

RT. The coverslips were rinsed in PBS and mounted onto slides with DAPI containing mounting medium. The stained cells were analyzed by confocal microscopy LSM 510 (Carl Zeiss).

2.2.7 Luciferase assay

2.2.7.1 Cell lysates preparation for luciferase assay

HEK 293 cells were harvested and lysed in 50 µl of lysis buffer (25 mM Glycylglycine pH 7.8, 8 mM $MgSO_4$, 1 mM EDTA, 1 mM DTT, 15% Glycerol, 1% Triton X-100) and incubated for 10 min on ice. The cell lysates were centrifuged at 13000 rpm for 10 min and the supernatant was used directly for measurement or stored at -20°C.

2.2.7.2 Determination of luciferase and β-galactosidase activity

Ten µl of cell lysates were pipetted into a white 96-well plate and measured at the wavelength of 562 nm for 30 sec by FLUOstar Optima Photometer after the addition of 50 µl Luciferin reaction buffer (20 mM Tricine/NaOH, pH 7.8, 1.02 mM $[MgCO_3]_4$•$Mg[OH]_2$•H_2O, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33 mM DTT, 270 µM Coenzyme A, 470 µM luciferin, 530 µM ATP). For the determination of the β-galactosidase activity, 3.3 µl of the cell lysates and 33.3 µl of β-galactosidase reaction buffer (1 mM $MgCl_2$, 100 mM Phosphate buffer pH 8.0, 1% Galacton-Tropix, Bedford) were mixed in a white 96-well plate and incubated at RT for 45 min under agitation. The light emission was measured under the same condition as the luciferase activity after the addition of 50 µl of enhancer solution (200 mM NaOH, 10% Emerald chemiluminescent amplifier).

The normalized luciferase value was calculated by multiplying the measured luciferase value with the quotient of the average β-galactosidase value and the measured corresponding β-galactosidase value.

2.2.8 Chromatin immunoprecipitation (ChIP) assay

For each condition, 3×10^6 cells were used. After cross-linking with 1% formaldehyde for 10 min at RT followed by a quenching of formaldehyde using 0.125 M glycine, cells were harvested, resuspended in 150µl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, complete protease and phosphatase inhibitors), and sonicated 4 times for 10 sec of each at 50% amplitude. After centrifugation at 12000 rpm for 10 min at 4°C, the supernatants were diluted 1:10 in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1) and subjected to preclear with 2 µg of sheared salmon sperm DNA and 20 µl of protein A/G-plus beads for 1 h at 4°C. Immunoprecipitation was performed with corresponding antibodies. After overnight incubation, 2 µg of sheared salmon sperm DNA and 25 µl of protein A/G-plus beads were again added for 1 h at 4°C. Beads were washed sequentially for 5

min each in low salt buffer (0.1% SDS, 1% Triton X-100. 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and TE buffer. Elution was done in 250 µl of elution buffer (1% SDS, 0.1 M $NaHCO_3$). Eluates were heated at 65°C overnight to reverse the cross-linking. Supernatants were then incubated with proteinase K for 1 h at 45°C, and DNA was purified using QIAquick PCR Purification Kit. The DNA was analyzed by qPCR. Data were displayed as % of input DNA.

2.2.9 Chromatin accessibility assay

2.2.9.1 Chromatin accessibility by real-time PCR (CHART-PCR) assay

CHART-PCR assay was performed as described by Rao, Procko et al. 2001. For each condition, 5×10^6 cells were used. Cells were harvested and resuspended in 1 ml ice-cold Nonidet P-40 lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, 0.15 mM spermine, 0.5 mM spermidine) and incubated on ice for 5 min. The suspension was centrifuged at 3000 rpm for 5 min to pellet the nuclei. The nuclei were resuspended in NEB buffer 4 and subsequently treated with 50 units of HinfI enzyme at 37°C overnight. The genomic DNA was isolated using High Pure PCR Template Preparation Kit (Roche). Sixty ng of genomic DNA was analyzed by qPCR. The accessibility was plotted as a percentage of the undigested sample for each treatment.

2.2.9.2 Chromatin lockdown assay

The chromatin lockdown assays were performed using chromatin lockdown kit (Biorad) according to the manufacturer's instructions. Rhodopsin (Rho) acts as reference gene with locked promoter. The chromatin inaccessibility is displayed as percentages of Rho, whose value was set as 100%.

3 Results

3.1 Regulation of chromatin accessibility at the beta-casein promoter in HC11 cells

3.1.1 Hormone treatment activates Stat5a and enhances beta-casein mRNA

HC11 cells were used in this work to analyze the epigenetic regulation of differentiation marker gene beta-casein. This cell line has been reported to undergo differentiation after treatment with lactogenic hormones dexamethasone, insulin, and prolactin (DIP), and thus has been widely used as a model of mammary cell differentiation both *in vitro* and *in vivo* (Merlo, Graus-Porta et al. 1996).

Base on the classical mechanism of Stat5a-mediated gene regulation, after DIP treatment, phosphorylated Stat5a then dimerizes and translocates into the nucleus, in which it binds to GAS elements at target gene promoters and in turn regulates transcription (Ball, Friis et al. 1988). To first confirm the activation of Stat5a upon DIP treatment, western blots were performed to detect phosphorylated Stat5a. As shown in Fig. 3-1A, Stat5a was endogenously expressed and became phosphorylated in response to DIP. No phosphorylated Stat5a was detected in untreated cells, indicating DIP indeed results in robust activation of Stat5a in HC11 cells. Therefore, this cell model was used in this work to investigate Stat5a-responsive activation of beta-casein.

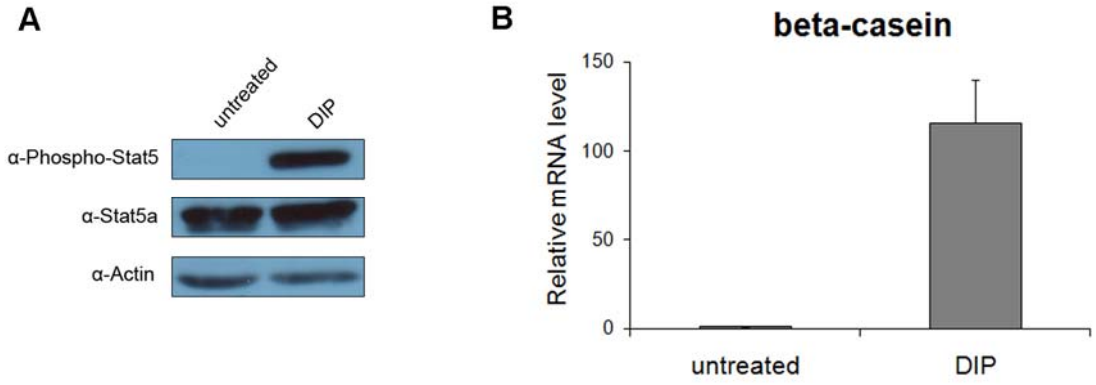

Figure 3-1 DIP treatment activates Stat5a and enhances beta-casein mRNA.

(A) DIP treatment activates Stat5a in HC11 cells. HC11 cells were starved for 3 days and then treated with DIP for 15 min or left untreated. Cell extracts were analyzed by western blots using the indicated antibodies. Actin was the loading control.
(B) DIP treatment enhances beta-casein mRNA. HC11 cells were starved for 3 days and then treated with DIP for 6 h or left untreated. Beta-casein mRNA was measured by real-time RT-PCR and normalized to cytokeratin 18 (Krt18) mRNA. Data are demonstrated as fold change of DIP-treated to untreated cells. The mRNA level from untreated cells was set as 1. Error bars represent standard error of three independent experiments.

To test whether the phosphorylated Stat5a could induce beta-casein gene expression, real time RT-PCR was performed. DIP treatment resulted in a 100-fold induction of beta-casein mRNA when compared to untreated cells, suggesting that beta-casein transcript is really induced by hormone stimulation and Stat5a-mediated regulation in HC11 cells (Fig. 3-1B).

3.1.2 Beta-casein promoter is inaccessible to Stat5a binding in HC11 cells

At the transcriptional level, the beta-casein gene is mainly regulated by binding of hormone induced transcription factors to regulatory elements. In order to analyze whether activated Stat5a is able to bind to beta-casein promoter and thereby upregulate transcription, ChIP assays were carried out. The binding was compared to the known Stat5a responsive genes such as Socs3 and Cish.

Although two GAS elements are present at promoter (Kabotyanski, Huetter et al. 2006), Stat5a did not bind to beta-casein promoter regardless of phosphorylation, revealing this region is inaccessible to Stat5a and DIP treatment fails to improve the accessibility (Fig. 3-2). On the other hand, Stat5a bindings were detectable at Socs3 and Cish promoters, and DIP treatment enhanced the bindings, showing activated Stat5a accounts for the majority of binding in spite of basal binding by unphosphorylated Stat5a.

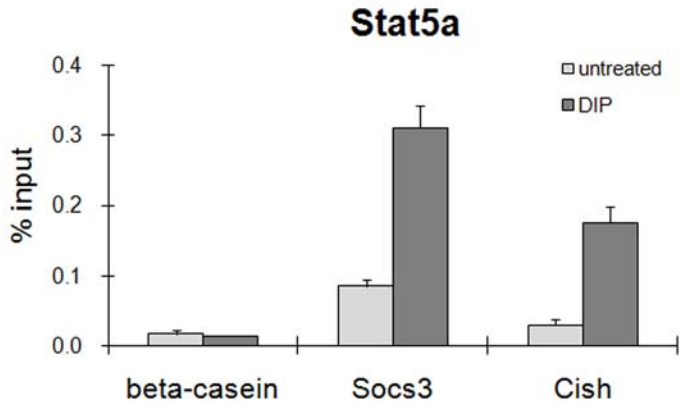

Figure 3-2 Activated Stat5a does not bind to beta-casein promoter in HC11 cells.

HC11 cells were starved for 3 days and then treated with DIP for 15 min or left untreated. ChIP assays were performed using Stat5a antibody as well as nonspecific anti-rabbit IgG. The precipitated DNA was analyzed by real-time PCR using primers flanking promoters of the indicated genes, respectively. The enrichment of Stat5a is demonstrated as the percentage of input DNA. Error bars represent standard error of two independent experiments.

3.1.3 Chromatin accessibility analyses at beta-casein promoter *in vivo*

3.1.3.1 Chromatin accessibility assays

In order to confirm the chromatin inaccessibility of the beta-casein promoter, assays were undertaken to study the chromatin accessibility status across the promoter. Two assays with digestion by either restriction enzyme HinfI or DNase I were performed. Based on the fact that condensed or nucleosome-associated DNA is less or not accessible, this repressive chromatin region is protected to the digestion with the enzymes. The cleavage is detected by real-time PCR with primers flanking the region which is cut. The chromatin accessibility is inversely proportional to the amount of PCR product (Fig. 3-3).

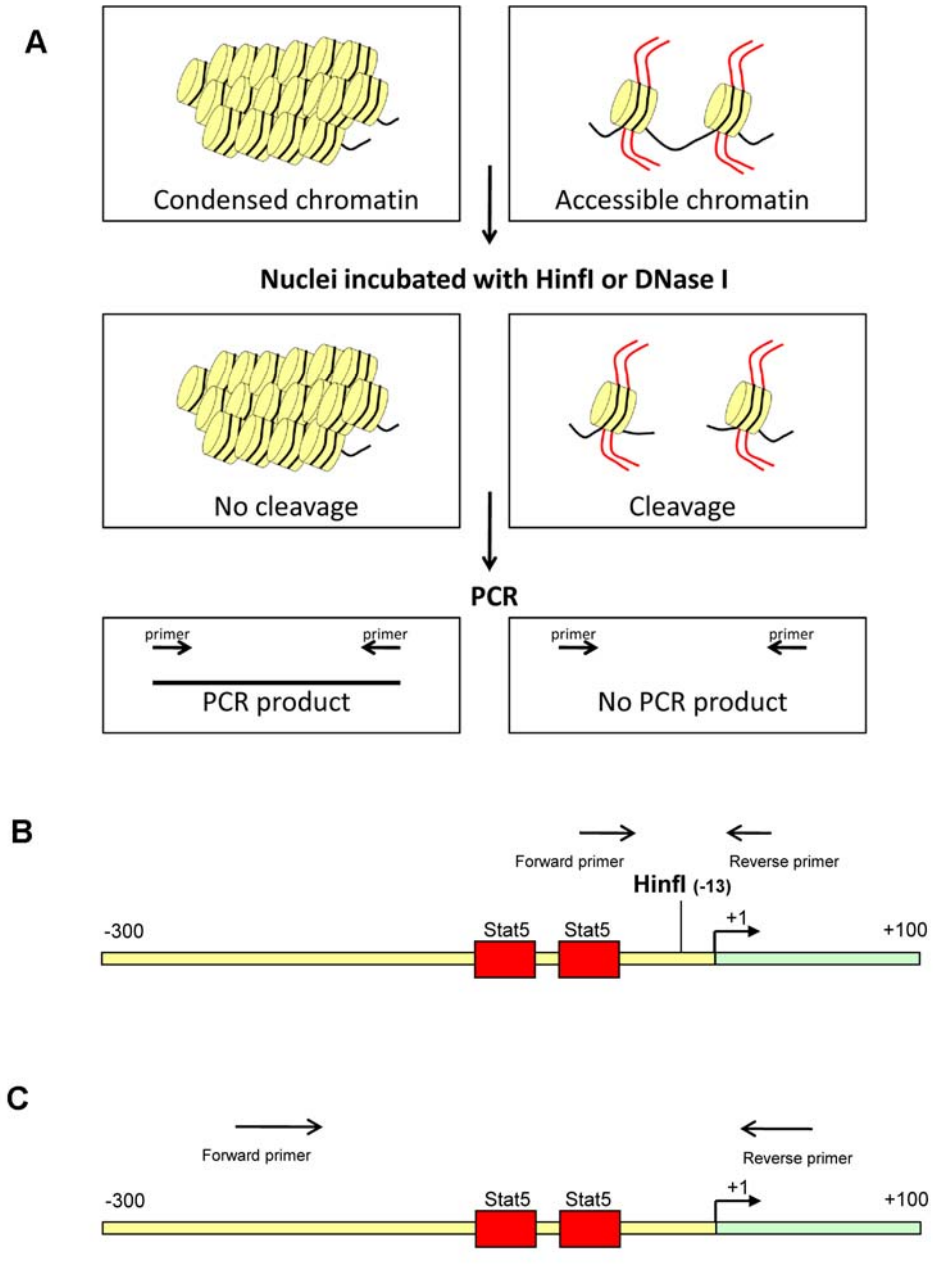

Figure 3-3 Schematic representation of chromatin accessibility assays and mouse beta-casein promoter

(A) The scheme outlines the principle of chromatin accessibility assay. The different cleavage resulted from chromatin structure by restriction enzyme or DNase I is analyzed by PCR. The chromatin accessibility is inversely proportional to the amount of PCR product. Figure from Rao, Procko et al. 2001.
(B) Structure of beta-casein promoter showing the positions of Stat5 binding sites, HinfI restriction site and primers for accessibility assay with HinfI by Chromatin Accessibility by real-time PCR (CHART-PCR) assay.
(C) Structure of beta-casein promoter showing positions of Stat5 binding sites and primers for accessibility assay with DNase I by chromatin lockdown assay.

3.1.3.2 Inaccessible beta-casein promoter cannot be opened by DIP treatment.

3.1.3.2.1 Beta-casein promoter is inaccessible to HinfI digestion by CHART-PCR assay

CHART-PCR assay was conducted by incubating nuclei, prepared from untreated and DIP treated HC11 cells, with HinfI for 1h, 4h, and 16h, respectively. Purified naked (genomic) DNA acted as control. In order to exclude false negative results raised from different digestion efficiencies between nuclei and naked DNA, purified DNAs were analyzed on a 1% agarose gel to ensure that HinfI functions equally. One hundred ng of DNA purified from nuclei was subjected to both classical and real-time PCR analyses.

The complete digestion of DNA by HinfI was determined by DNA smear on the gel. Nuclei showed complete cleavage after 16-h digestion, whereas naked DNA was completely cleaved even 1 h after digestion (Fig. 3-4A). Since these two samples showed identical DNA smears, HinfI functioned indeed equally. Therefore the difference of HinfI cleavage across beta-casein promoter between two samples results only from chromatin structure.

By classical PCR, PCR products were seen by 16-h HinfI digestion in both untreated and DIP treated HC11 cells, indicating the chromatin structure across promoter is closed and not released upon DIP treatment. As a control, no PCR product was observed by 1-h HinfI digestion of naked DNA, confirming that cleavage at promoter really occurred by naked DNA (Fig. 3-4B). Similar results were found by quantitative measurement by real-time PCR. Beta-casein promoter showed approximately 60% inaccessibility to HinfI, and the inaccessibility were not improved upon DIP treatment, indicating DIP was unable to alter the chromatin accessibility of beta-casein promoter. In line with naked DNA control from classical PCR, beta-casein promoter was totally cut by HinfI, leading to an inaccessibility of 0.3% (Fig. 3-4C). These results showed that beta-casein promoter is inaccessible and lactogenic hormones DIP cannot alter this state.

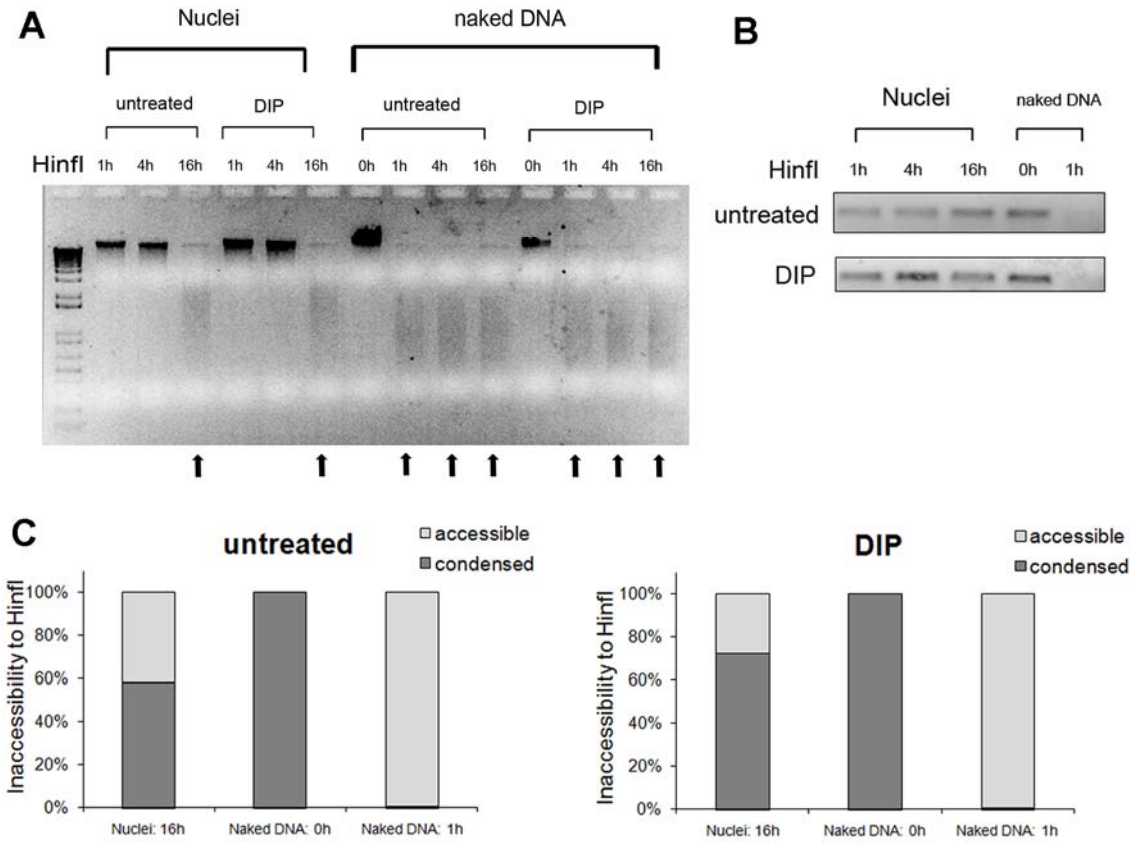

Figure 3-4 Beta-casein promoter is inaccessible to HinfI digestion.

HC11 cells were starved for 3 days and then treated with DIP for 15 min or left untreated. Nuclei were treated with HinfI (1h, 4h and 16h) and analyzed by CHART-PCR. Purified naked (genomic) DNA was used to control the efficiency of HinfI

(A) The digestion efficiencies of HinfI in nuclei and naked DNA. Arrows represent complete cleavage indicated by DNA smear.
(B) Assay was analyzed by classical PCR. PCR products were analyzed on a 1% agarose gel.
(C) Assay was analyzed by real-time PCR. The inaccessibility is expressed as percentages of undigested naked DNA, whose value was set as 100%.

3.1.3.2.2 Beta-casein promoter is inaccessible to DNase I by chromatin lockdown assay

The inaccessibility of beta-casein promoter seems to inhibit the binding of activated Stat5a. To confirm the inaccessibility, another assay, known as chromatin lockdown assay, was performed. This assay is based on DNase I hypersensitivity site. DNase I hypersensitivity I sites are gaps in the nucleosomal array that leave 200–400 bp of sequence susceptible to be digested by DNase I (McArthur, Gerum et al. 2001). They are related to accessible region such as enhancer or promoter (Reinke and Hoerz 2004). Thus, this assay can also indicate whether beta-casein promoter is located at the nucleosomal array or condensed chromatin.

Besides beta-casein gene, in order to ensure the specificity of the results, three control genes were applied in this assay. Rhodopsin (Rho) acts as reference gene, to which data of other genes are normalized, because it is not expressed in most cells and its promoter is al-

ways inaccessible. Its inaccessibility to DNaseI was set as 100%. Both as accessible controls, housekeeping gene Gapdh has an open chromatin at promoter in most cells, whereas Cish is a specifically responsive gene of Stat5a.

In untreated cells, Gapdh and Cish promoters showed roughly 10% protection of digestion, indicating open chromatin structures. The accessibility status of Cish can therefore account for the binding of Stat5a by ChIP assay. In contrast, beta-casein promoter showed 110% protection, indicating an even more condensed structure than that of Rho (Fig. 3-5). As there was no difference observed between untreated and DIP-treated cells, accessibility status of all tested promoters were independent of DIP treatment.

In line with the results from CHART-PCR assay, the inaccessibility of beta-casein promoter was confirmed by chromatin lockdown assay, and this finding implied that the protected, repressed chromatin structure probably accounts for the binding failure of Stat5a to the beta-casein promoter. Furthermore, since the chromatin lockdown assay is based on DNase I hypersensitive sites, beta-casein promoter is indeed located at the nucleosomal array or condensed chromatin region, which hence causes the chromatin inaccessibility.

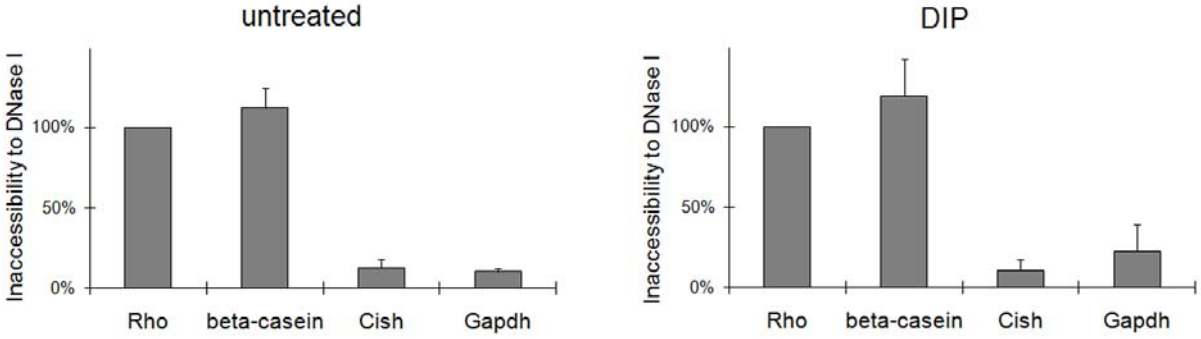

Figure 3-5 Chromatin inaccessibility at the beta-casein promoter to DNase I digestion

HC11 cells were starved for 3 days and then treated with DIP for 15 min or left untreated. After *in situ* DNase I digestion, Nuclei from both cells were purified and analyzed by real-time PCR with primers flanking promoters. Rho is the reference gene for inaccessible promoter; Gapdh is the reference gene for accessible promoter. Inaccessibility is expressed as percentages of Rho, whose value was set as 100%. Error bars represent standard error of two independent experiments.

3.1.4 H3K9me2 association at beta-casein promoter

Given that the chromatin at beta-casein promoter is in fact not accessible to enzymes and transcription factors, the question arose as to whether and which epigenetic repressive marks contribute to this inaccessible status. As DNA methylation might not play a role in mammary epithelial cell differentiation (Rijnkels, Kabotyanski et al. 2010), histone modifications, especially repressive lysine methylations, were investigated. ChIP assays were performed with antibodies against several known lysine methylations to monitor the histone marks at beta-casein promoter. As demonstrated in Fig. 3-6, H3K9me2 was massively asso-

ciated at beta-casein promoter, and this association was independent of DIP treatment. In contrast, other marks such as H3K27me3, H3K9me3, and H4K20me3 were not detectable (data not shown).

Two Stat5 responsive genes Socs3 and Cish, as well as one housekeep gene Gapdh, were analyzed as controls. Fairly low H3K9me2 occupancies were observed at all three promoters. For these genes, at which Stat5a bindings were detected, transcription can be induced by DIP. Conversely, Magea2, a G9a HMT target gene, displayed strong H3K9me2 association at its promoter, implying a constitutively repressed promoter mediated by H3K9me2 (Tachibana, Sugimoto et al. 2002).

The association of H3K9me2 at beta-casein promoter is considered to be robust, because its occupancy is comparable to that of Magea2. Furthermore, the H3K9me2 association remained unaffected upon DIP-treatment, which was related to the persisted inaccessible status at that promoter as well. Therefore, as the only repressive mark observed, the massive H3K9me2 modification might account for the promoter inaccessibility and the subsequent inhibition of Stat5a binding.

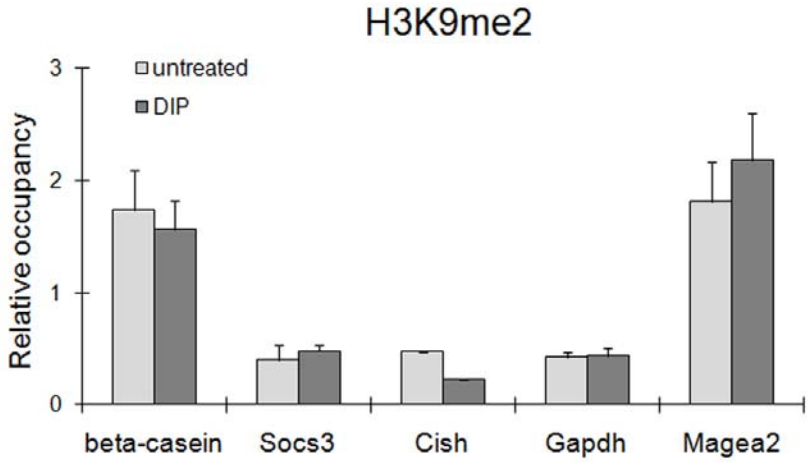

Figure 3-6 H3K9me2 associated at beta-casein promoter

HC11 cells were starved for 3 days followed by DIP treatment for 15 min or left untreated. ChIP was performed with antibodies against H3K9me2 and H3. The precipitated DNA was analyzed by real-time PCR using primer pairs flanking promoters of indicated genes, respectively. The relative occupancy of H3K9me2 is demonstrated as the percentage of input DNA and normalized to H3 occupancy. Error bars represent standard error of three independent experiments.

3.1.5 H3K9me2 association inversely correlates with binding of RNA polymerase II

Provided the binding of Stat5a is impaired due to chromatin inaccessibility mediated by H3K9me2, the Pol II transcription machinery might be inhibited as well. ChIP assays were undertaken to analyze the associations of RNA polymerase II at tested promoters. Specifical-

ly, phosphorylated C-terminal domain RNA polymerase II (p-CTD RNA Pol II) was used since it is essential for both transcription and mRNA splicing process (Hirose and Ohkuma 2007).

The bindings of Pol II are inversely correlated to the associations of H3K9me2 at all tested promoters. Only extremely slight Pol II bindings, as well as weak induction upon DIP, were found by beta-casein and Magea2 promoters, indicating that the Pol II occupancies at beta-casein promoter are truly impaired. On the contrary, all control promoters presented strong bindings of Pol II, which could be further dramatically improved by DIP (Fig. 3-7). Additionally, a rather high basal binding was observed at the Socs3 promoter, suggesting a basal PIC binding to the accessible promoter. The enhanced binding of Pol II to the Gapdh promoter in response to DIP treatment, supports that Gapdh expression can be influenced by lactogenic hormones (personal remark of Stefanie Kliem). The correlation of associated H3K9me2 to the inaccessible promoter and the inhibited Pol II binding suggests that H3K9me2 might play a major inhibitory role in beta-casein RNA polymerase II transcription.

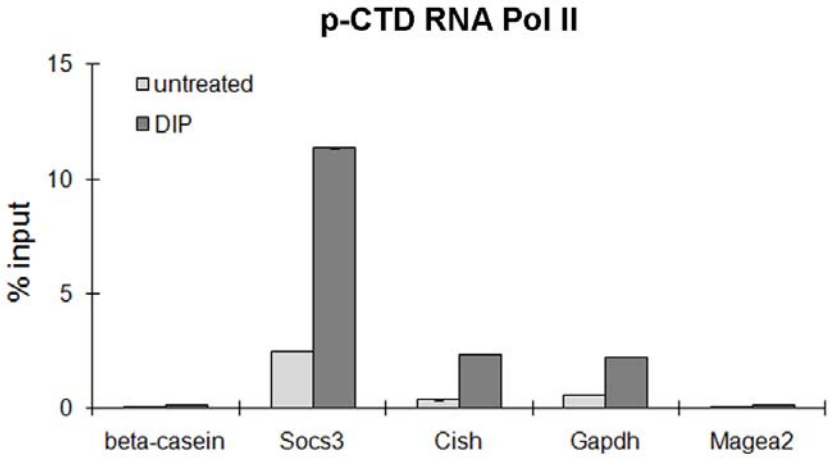

Figure 3-7 RNA polymerase II does not associate with beta-casein promoter

HC11 cells were starved for 3 days followed by treatment of DIP for 15 min or untreated control. ChIP was performed with antibodies against p-CTD RNA Pol II. The precipitated DNA was analyzed by real-time PCR using primer pairs flanking promoters of indicated genes, respectively. The occupancy of Pol II is demonstrated as the percentage of input DNA.

3.1.6 Upregulation of beta-casein gene by removal of H3K9me2

As repressive mark, H3K9me2 inhibits gene expression either by contribution to heterochromatin formation or direct regulating transcription in cooperation with other factors (McGarvey, Fahrner et al. 2006). Given that H3K9me2 correlates to beta-casein transcriptional repression, approaches were tried to abolish this modification so as to upregulate transcription.

3.1.6.1 Removal of H3K9me2 by G9a inhibitor BIX-01294

BIX-01294 (diazepin-quinazolin-amine derivative) was chosen since it selectively inhibits the G9a HMT without competing with the cofactor AdoMet (Fig. 3-8A); hence the generation

of H3K9me2 can be specifically impaired both *in vitro* and *in vivo* (Kubicek, O'Sullivan et al. 2007).

To know whether BIX-01294 is capable to influence the chromatin accessibility of beta-casein promoter through elimination of H3K9me2 occupancy, HC11 cells were treated with BIX-01294 for 1d and chromatin lockdown assay was performed. A 10% enhancement of promoter accessibility was observed, suggesting that it is able, but only slightly, to open the inaccessible chromatin structure at beta-casein promoter (Fig. 3-8B). One explanation might be that G9a is not the main HMT responsible for H3K9me2 at beta-casein promoter, for example Suv39h might also play a role. Alternatively, if the promoter-associated H3K9me2 from previously synthesized methylation persists, reduction of new generation of H3K9me2 by BIX-01294 probably only shows little effect.

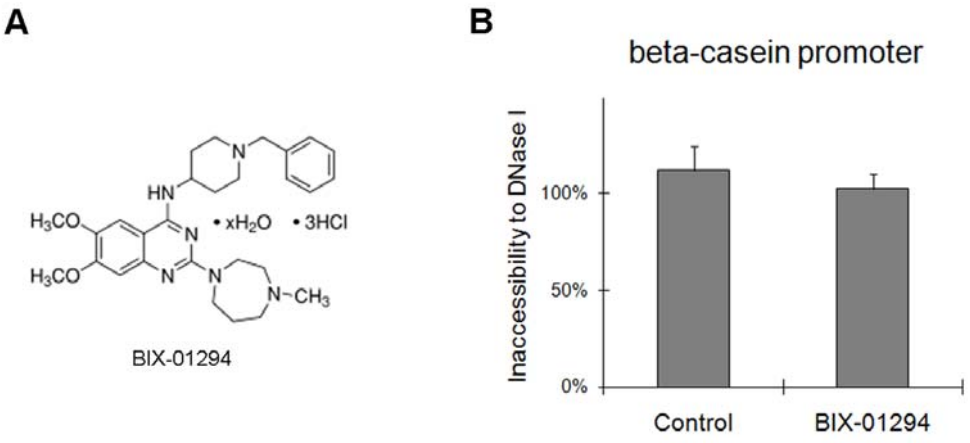

Figure 3-8 BIX-01294 slightly improved the promoter accessibility

(A) Chemical structure of compound BIX-01294.
(B) BIX-01294 slightly improved the promoter accessibility. HC11 cells were starved for 2 days and left treated for 1 day with 4μM BIX-01294. After *in situ* DNase I digestion, Nuclei were purified and analyzed by real-time PCR with primers flanking beta-casein promoter. Chromatin inaccessibility is expressed as percentages of Rho and then normalized to untreated control, whose value was set as 100%. Error bars represent standard error of two independent experiments.

I further analyzed whether the effect of promoter accessibility mediated by BIX-01294 can induce beta-casein transcription. In untreated cells, BIX-01294 contributed to 3-fold induction of beta-casein mRNA, suggesting the increased promoter accessibility by BIX-01294 could enhance the basal transcription (Fig. 3-9A). Based on this finding, a further mRNA enhancement was expected when the cells were treated with both BIX-01294 and DIP. However, BIX-01294 failed to further induce transcription based on DIP treatment (Fig. 3-9B). Probably it simultaneously downregulates some proteins, which depend on DIP to upregulate beta-casein transcription, thus the induced transcription is eliminated. Moreover, as the mRNA amount upon DIP treatment results from a combination of new mRNA generation and pro-

longed mRNA stability by prolactin (Guyette, Matusik et al. 1979), BIX-01294 might also function to increase beta-casein mRNA degradation.

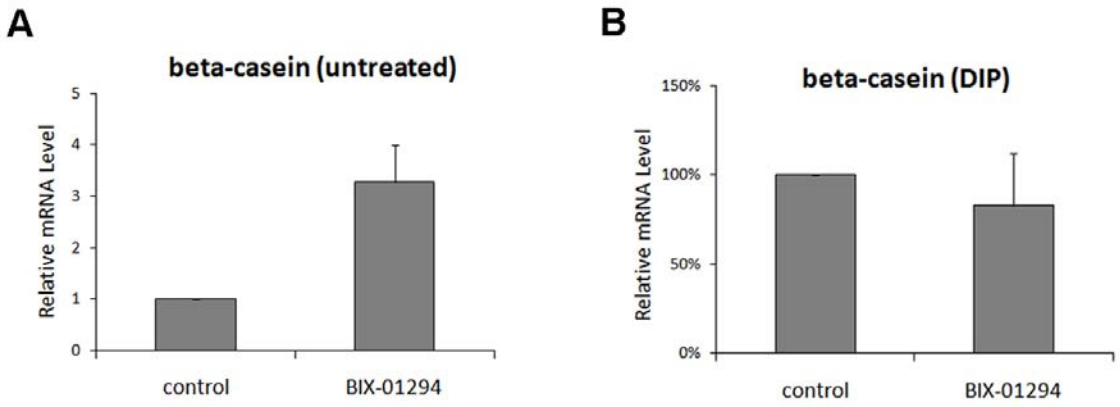

Figure 3-9 BIX-01294 induces basal transcription of beta-casein gene.

HC11 cells were starved for 2 days and incubated for 1 day with 4μM BIX-01294 followed by DIP treatment for 6 h or non-treatment. Beta-casein mRNA was measured by real-time RT-PCR and normalized to Krt18 mRNA. Data are demonstrated as fold change of mRNA level from BIX-01294 incubated cells in comparison to control cells. Error bars represent standard error of three independent experiments.

(A) BIX-01294 induces beta-casein basal transcription in untreated cells. The mRNA level from control cells was set as 1.

(B) BIX-01294 fails to further enhance beta-casein transcription in DIP-treated cells. The mRNA level from control cells was set as 100%.

3.1.7 The role of lysine demethylases Jmjd1a and Jmjd2c in transcriptional regulation of beta-casein

By inhibiting G9a, BIX-01294 can only reduce the new generation of methylation, but it is unable to eliminate the promoter-associated H3K9me2 from previously synthesized methylation. Therefore, another approach to degrade H3K9me2 is to carry out *in vivo* demethylation reaction by specific lysine demethylases.

3.1.7.1 Cloning of essential domains of Jmjd1a and Jmjd2c

JmjC-domain-containing histone demethylases Jmjd1a and Jmjd2c were chosen because both are able to demethylate H3K9me2, and in turn involved in many cellular processes, such as signaling, cell differentiation, and stem cell renewal. Since the essential domains were described to be sufficient to mediate the demethylation reaction as efficiently as full-length proteins, only these domains were cloned for overexpression experiments. As stable expression is required for analysis of mammary differentiation in HC11 cells, virus-based expression system was used.

The insert of Jmjd1a ranging from 600 to 1323 aa flanks JmjC and Zinc-finger domains (Yamane, Toumazou et al. 2006), while that of Jmjd2c ranging 2 to 310 aa flanks JmjC and

JmjN domains (Whetstine, Nottke et al. 2006) (Fig. 3-10A). Inserts were fused downstream with Myc-Tag in lentiviral vector pVig-Myc-IRES-EGFP (Fig.3-10B).

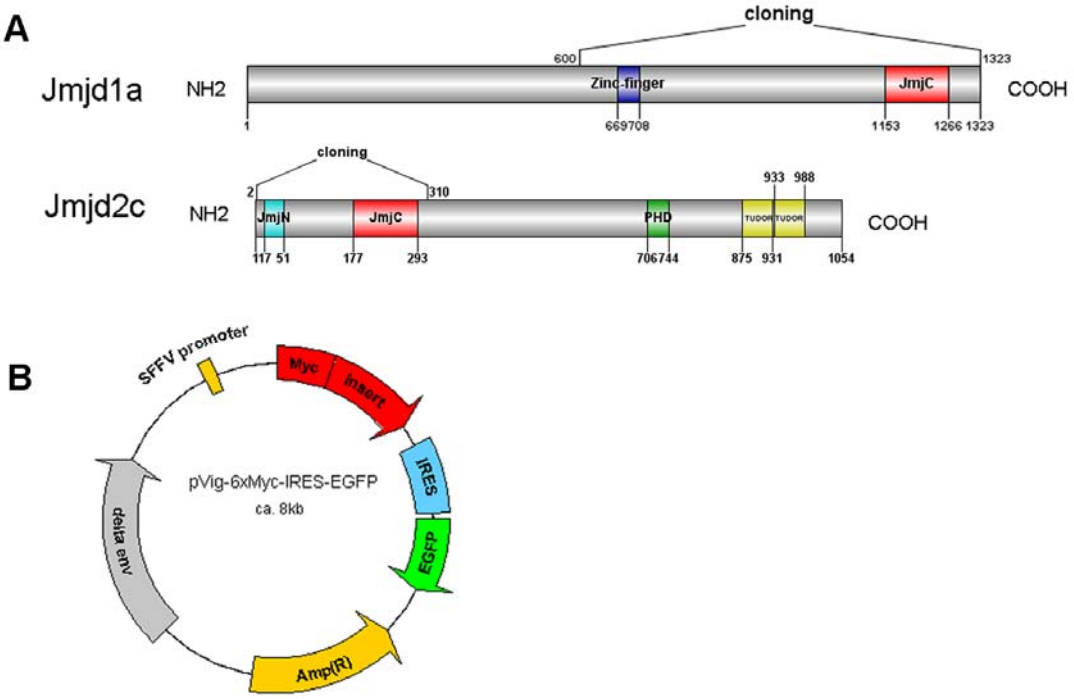

Figure 3-10 Cloning of essential domains of Jmjd1a and Jmjd2c

(A) Schematic representation of Jmjd1a and Jmjd2c proteins with their domains. Essential domains required for histone demethylase activity were labeled by “cloning”.
(B) Parental vector for cloning pVig-Myc-IRES-EGFP was modified from lentiviral vector pVig. Location of insert as mentioned.

3.1.7.2 Jmjd1a domains demethylate H3K9me2 in HC11 cells

Lentiviruses stably expressing essential domains of Jmjd1a and Jmjd2c were produced by transfecting HEK 293 cells with constructed lentiviral vectors and corresponding packaging vectors, as described in section of Materials and Method. HC11 cells were then transduced with MOI of 1 and 2. To monitor the appropriate expression of the essential domains, western blots were performed using anti-Myc antibody. The essential domains of both demethylases were of the proper molecular weights, suggesting proper expressions (Fig. 3-11A). Moreover, a comparison of transduction efficiency between different MOI revealed that Jmjd1a domains were equally expressed, whereas Jmjd2c domains with MOI of 2 showed better expression than those with MOI of 1. Finally, the transduced HC11 cells with MOI of 2 were maintained for further analysis.

To confirm that the essential domains of Jmjd1a and Jmjd2c stably expressed in HC11 cells can in fact reduce H3K9me2 level *in vivo*, demethylase activities were determined by western blots. Jmjd1a domains reduced global H3K9me2, but the effect was not strong (Fig. 3-11B). This suggests that the JmjC and Zinc-finger domains of Jmjd1a are able to, but still

not efficiently, mediate demethylation reaction in HC11 cells. However, Jmjd2c domains did not show demethylation effect. In addition to H3K9me2, H3K9me3 can be also demethylated by Jmjd2c resulting in H3K9me2. Therefore, the decreased level by H2K9me2 demethylation might be balanced by the accumulating H2K9me2 as a product from H3K9me3 demethylation, leading to a constant H3K9me2 level on western blots (Shin and Janknecht 2007). Finally, HC11 cells stably expressing the essential domains of Jmjd1a were subjected to further experiments.

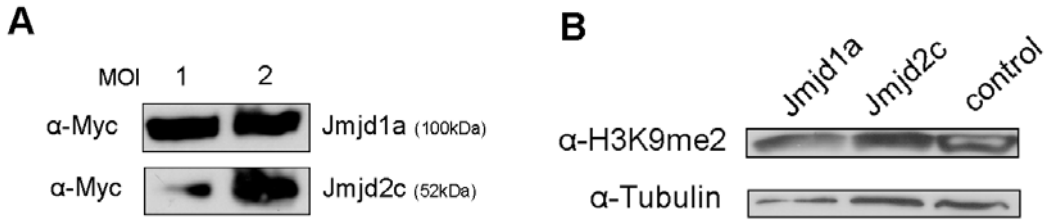

Figure 3-11 Stable expressions of essential domains of Jmjd1a and Jmjd2c in HC11 cells

(A) Essential domains of Jmjd1a and Jmjd2c are stably expressed in HC11 cells. HC11 cells were transduced by lentivirus of Jmjd1a and Jmjd2c with MOI of 1 and 2. Cell extracts were detected by western blots using anti-Myc antibody.
(B) Jmjd1a domains demethylate H3K9me2 *in vivo*. Cell extracts of HC11 cells with MOI of 2 were detected by western blots using indicated antibodies. Tubulin was the loading control.

Given the JmjC and Zinc-finger domains of Jmjd1a reduced the global H3K9me2 in HC11 cells, it was investigated whether the abolished H3K9me2 can in turn upregulate transcription of beta-casein, the beta-casein mRNA levels in both untreated and DIP treated cells were measured. Unexpectedly, Jmjd1a domains downregulated the beta-casein transcription in both untreated (Fig. 3-12A) and DIP treated HC11 cells (Fig. 3-12B).

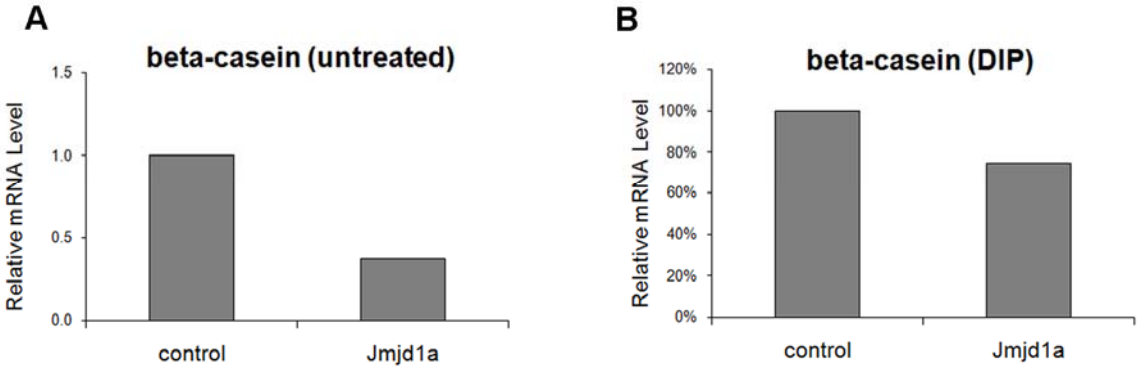

Figure 3-12 Jmjd1a domains inhibit beta-casein transcription.

Transduced HC11 cells by Jmjd1a domains were starved for 3 days followed by DIP treatment for 6 h or non-treatment. Beta-casein mRNA was measured by real-time RT-PCR and normalized to Krt18 mRNA. Data are demonstrated as fold change of mRNA level.
(A) Jmjd1a domains downregulate beta-casein basal transcription in untreated HC11 cells. The mRNA level from control cells was set as 1.
(B) Jmjd1a domains fail to enhance beta-casein mRNA in DIP-treated HC11 cells. The mRNA level from control cells was set as 100%.

Although the global H3K9me2 level was reduced by expression of Jmjd1a domains, beta-casein mRNA was not enhanced accordingly. Given that the inhibitor BIX-01294 did not enhance beta-casein transcription either, the associated H3K9me2 might really interplay with other unknown factors to regulate beta-casein gene expression and probably H3K9me2 only has a “fine-tuning” function in the interplay. Alternatively, as shown in Fig. 3-11B, overexpression of JmjC and Zinc-finger domains of Jmjd1a were not sufficient to completely demethylate global H3K9me2 in HC11 cells, therefore probably to simultaneously express both demethylases might solve this problem. This should be proven in future experiments. Since instead of an enhancement a reduction of the beta-casein transcription was observed in Jmjd1a overexpressing HC11 cells, it could be possible that the activity of Pol II transcription machinery or other essential transcription factors are inhibited by Jmjd1a domains. To address this question, luciferase assays were done to analyze the role of Jmjd1a domains in regulation of beta-casein promoter activity.

3.1.7.3 Jmjd1a domains eliminate beta-casein promoter activity

To measure the transcriptional activity of beta-casein promoter, HEK 293 cells were transiently transfected with beta-casein luciferase reporter construct, STAT5a, and the prolactin receptor. This heterologous cell system allows study of prolactin-mediated signaling and transcription regulation of the beta-casein promoter (Pfitzner, Jahne et al. 1998). In the presence of Stat5a, a 25-fold enhancement of reporter gene activity was obtained upon prolactin treatment, indicating the Stat5a induced transcriptional activity of beta-casein promoter in response to prolactin (Fig. 3-13).

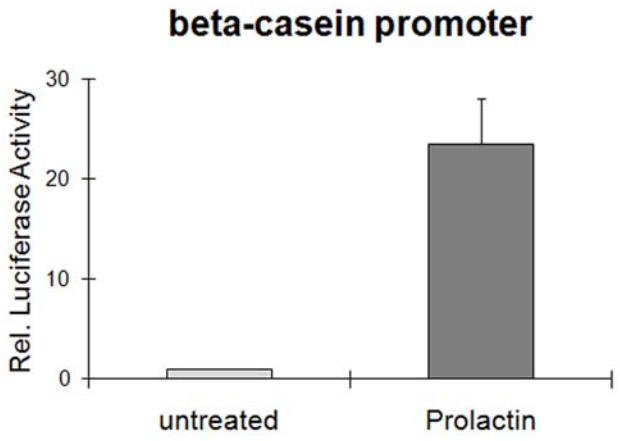

Figure 3-13 Stat5a and Prolactin induce beta-casein promoter activity

HEK 293 cells were transiently transfected with beta-casein luciferase reporter construct, STAT5a, and the prolactin receptor. *LacZ* plasmid was cotransfected as normalization control. Transfected cells were treated with prolactin for 16 h or left untreated. The luciferase activities were determined and the relative activities were normalized to the β-galactosidase activities. The data were demonstrated as fold change compared to the untreated control, whose level was set as 1. Error bars represent standard error of three independent experiments.

Next I analyzed whether Jmjd1a domains can regulate the transcriptional activity of beta-casein promoter. HEK 293 cells were transiently transfected with the indicated lentivirus constructs of Jmjd1a and Jmjd2c domains. The domains of both demethylases were appropriately expressed, indicated by molecular weights in western blots using anti-Myc antibody, respectively (Fig. 3-14A). To further examine the corresponding *in vivo* demethylase activities in HEK 293 cells, western blots were performed. Consistent with the findings in HC11 cells, Jmjd1a domains reduced global H3K9me2, whereas Jmjd2c domains failed to demonstrate demethylation effect (Fig. 3-14B), most probably due the same reason as HC11 cells. Therefore, only the construct of Jmjd1a domains was taken for luciferase assay.

PGl3-promoter empty vector containing a SV-40 minimal promoter was used in luciferase assay to exclude the general effects on all promoters. As shown in Fig. 3-14C, Jmjd1a domains downregulated beta-casein promoter activity. Furthermore, the downregulation was specific to beta-casein promoter since no effect was observed at the control promoter, suggesting Jmjd1a domains might downregulate some factors essential for prolactin-mediated transactivation activity of beta-casein promoter. This effect can in turn contribute to, at least in part, the downregulated mRNA level, as observed in HC11 cells. However, the inhibitory effect from the potential interplay partners of H3K9me2 on chromatin structure can be also involved.

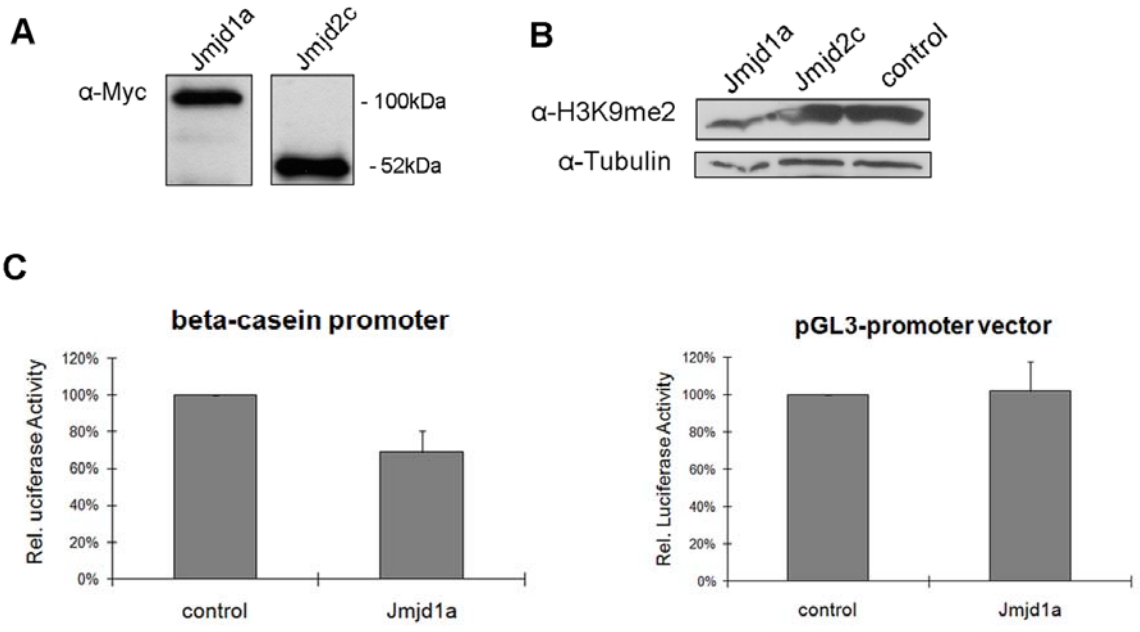

Figure 3-14 Jmjd1a domains depletes beta-casein promoter activity

(A) Myc-fused Jmjd1a and Jmjd2c domains were expressed in HEK 293 cells. Cells were transiently transfected by indicated lentiviral constructs. Proper expression of Myc-fused Jmjd1a and Jmjd2c domains were confirmed by western blots using anti-Myc antibody.

(B) Jmjd1a demonstrated H3K9me2 demethylase activity. Demethylase activity in 293 cells was analyzed by western blots using anti-H3K9me2 antibody. Tubulin was the loading control.

(C) Jmjd1a domains reduce beta-casein promoter activity. The construct of Jmjd1a domains was cotransfected in luciferase assay. Only prolactin treated cells were subjected to analysis. The data were demonstrated as fold change of Jmjd1a domains compared to the mock control, whose level was set as 100%. Error bars represent standard error of three independent experiments.

3.2 Regulation of EMT by the histone methylation inhibitor DZNep

3.2.1 TGFβ3 induces EMT in NMuMG cells

NMuMG cells represent a cell line derived from mammary gland epithelium. Cells from clone NM18 undergo EMT upon TGFβ treatment and thus have been used for *in vivo* analysis of EMT (Deckers, van Dinther et al. 2006). As shown in Fig. 3-15A, the cells demonstrated morphological changes typical for EMT upon treatment with TGFβ3. Quantitative RT-PCR showed characteristic expression pattern of EMT marker genes: eliminated epithelial marker Cdh1 as well as elevated mesenchymal marker Acta2. Moreover, the EMT inducer Snail was upregulated as well (Fig. 3-15B). These findings revealed that this cell line can indeed undergo EMT in response to TGFβ treatment.

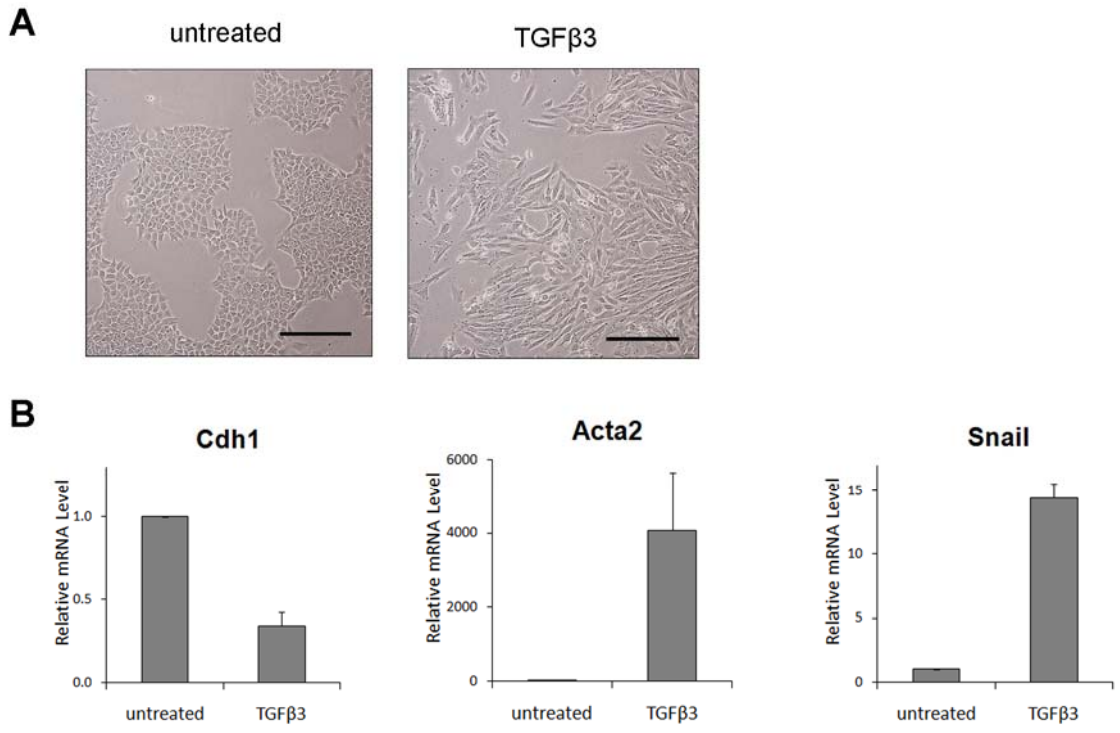

Figure 3-15 TGFβ3 induces EMT in NMuMG cells.

(A) TGFβ3 induces a mesenchymal morphology in NMuMG cells. NMuMG cells (clone NM18) treated with 5 ng/ml TGFβ3 for 2 days exhibited EMT morphology. Scale bars: 250 µm.
(B) TGFβ3 regulates EMT marker genes and inducer. The mRNA levels of indicated EMT marker genes and inducer were measured by quantitative RT-PCR and normalized to 18S rRNA. Data are demonstrated as fold change of mRNA level in comparison to untreated cells. Error bars represent standard error of three independent experiments.

3.2.2 The effect of DZNep on EMT induction in NMuMG cells

3.2.2.1 The role of histone methylations in regulating EMT

In order to investigate the role of epigenetic modifications, especially histone lysine methylations, in regulating EMT, NMuMG cells were treated with histone methylation inhibitors DZNep, BIX-01294, and Chaetocin for 2 days. DZNep depletes H3K27me3, while BIX-01294 and Chaetocin abolish methylated H3K9 by inhibiting G9a and Suv39h HMTs, respectively. The contributions of inhibitors to EMT were first examined by morphological change.

Untreated cells displayed typical epithelial morphology. Among treatments with three inhibitors, only DZNep showed an EMT-like effect, namely, cells lost cell-cell contact, were detached from each other, and gained fibroblast-like phenotypes. This indicated that DZNep might induce, entirely or in part, an EMT-like process in NMuMG cells. BIX-01294 and Chaetocin did not display typical morphological changes. Once induced with TGFβ3, control cells underwent EMT. All inhibitors, when supplied together with TGFβ3, also showed similar EMT procedures, indicating that the inhibitors do not interfere with the TGFβ3 induced EMT (Fig. 3-16). Since DZNep alone could potentially induce EMT-like process, its role in regulating EMT was examined.

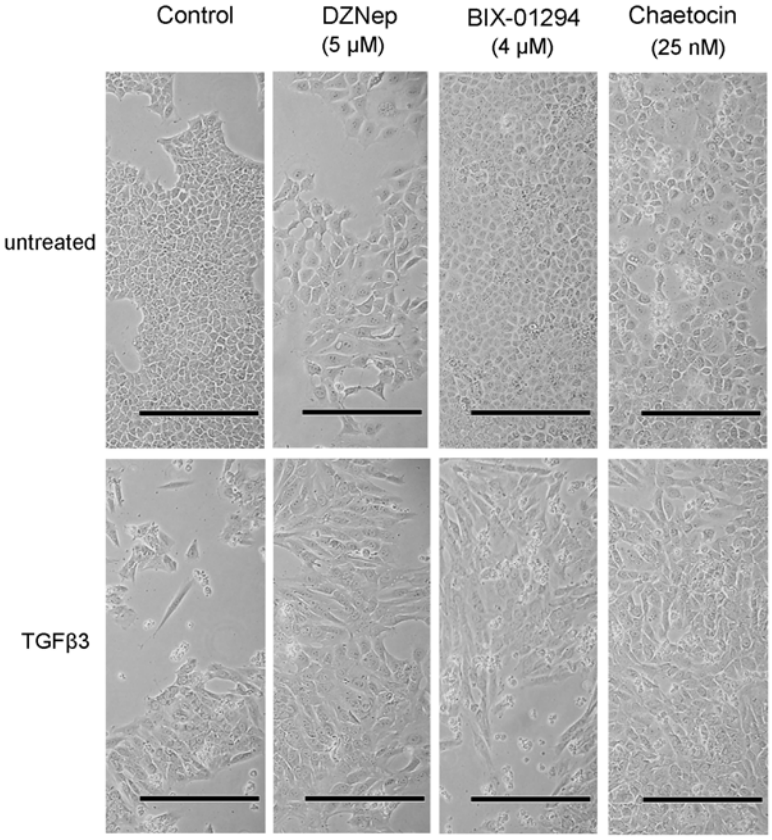

Figure 3-16 Regulation of EMT by epigenetic inhibitors

NMuMG cells were treated with indicated inhibitors or in combination with 5 ng/ml TGFβ3 for 2 days. Scale bars: 250 μm.

3.2.2.2 DZNep induces EMT

DZNep inhibits AdoHcy hydrolase, resulting in an indirect inhibition of AdoMet-dependent reactions carried out by many methyltransferases (Borchardt, Keller et al. 1984; Chiang 1998) (Fig. 3-17A and B). It was described to abolish global H3K27me3, therefore it is able to reactivate silenced genes in cancer cells (Tan, Yang et al. 2007).

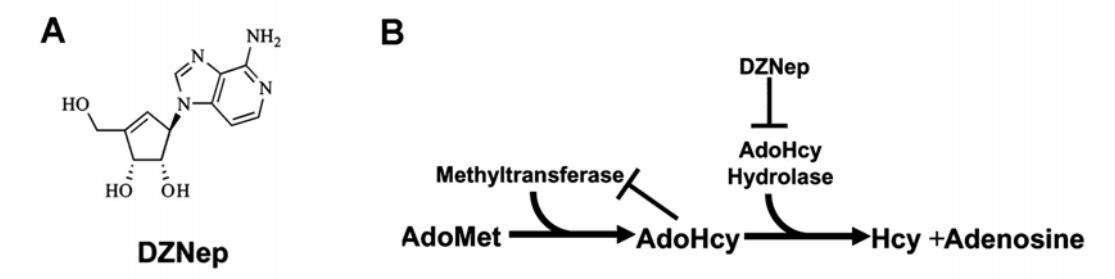

Figure 3-17 DZNep indirectly inhibits the methyltransferases.

(A) Chemical structure of DZNep.
(B) DZNep indirectly inhibits the methyltransferases. DZNep inhibits AdoHcy hydrolase, leading to enhanced AdoHcy levels, which subsequently inhibits the methyltransferases. Figure from Miranda, Cortez et al. 2009.

DZNep was described to deplete H3K27me3 by reducing global levels of Ezh2, Suz12, and Eed of PRC2 in a cell-dependent manner (Tan, Yang et al. 2007). Having shown that DZNep could induce an EMT-like process in NMuMG cells, western blots were carried out to determine its effect on expression level of Suz12 as a component of PRC2 and global H3K27me3 level. DZNep depleted global H3K27me3 level in a time-dependent manner (Fig. 3-18A). The marked decrease in H3K27me3 level was observed after 24 h treatment, but the level was completely recovered after 48 h treatment, indicating the reduced H3K27me3 level resulted from 24 h treatment might in turn alters EMT process. However, the global level of Suz12 was not reduced accordingly, suggesting in NMuMG cells DZNep depletes H3K27me3 level via different mechanisms (Fig. 3-18B).

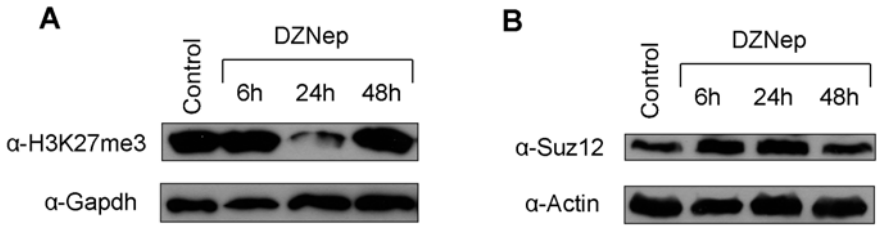

Figure 3-18 DZNep eliminates global level of H3K27me3.

NMuMG cells were treated with 5 μM DZNep for 6 h, 24 h, and 48 h. Cell extracts were analyzed by western blots using antibodies against H3K27me3 (A) and Suz12 (B). Gapdh and Actin were the loading controls, respectively.

In order to confirm the morphological change induced by DZNep corresponds indeed to EMT, quantitative RT-PCR analyses of epithelial marker Cdh1 and mesenchymal marker Ac-

ta2 were performed. DZNep alone repressed Cdh1, and led to more downregulation when combined with TGFβ3, implying DZNep and TGFβ3 synergistically downregulate Cdh1. For expression pattern of Acta2, DZNep alone elevated Acta2 mRNA level up to 30 folds, and an additive effect was observed in combination of TGFβ3 leading to a dramatic enhancement (Fig. 3-19). Taken together, these results support that the morphological change induced by DZNep resulted indeed from EMT, because the typical expression patterns of EMT marker genes regulated by DZNep are in the same direction as those by TGFβ3.

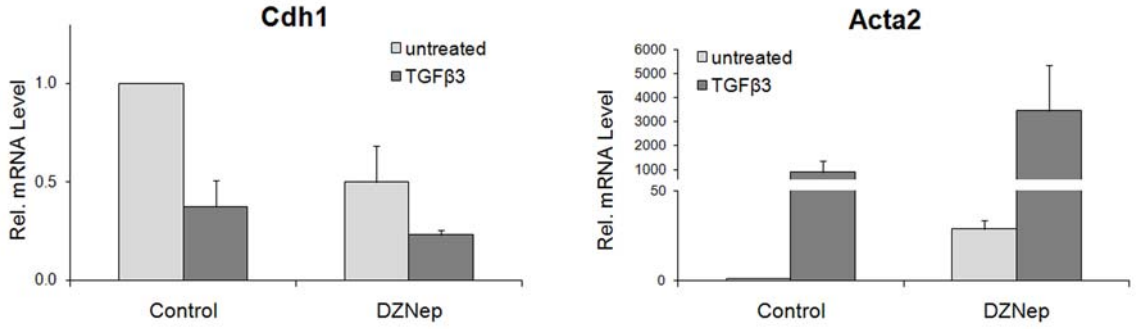

Figure 3-19 DZNep affects EMT marker genes.

NMuMG cells were treated with 5 μM DZNep in combination with 5 ng/ml TGFβ3 for 2 days. Cdh1 and Acta2 mRNA levels were measured by real-time RT-PCR and normalized to 18S rRNA. Data are demonstrated as fold change of mRNA level in comparison to untreated control cells. Error bars represent standard error of two independent experiments.

As another hallmark of EMT, actin structure is rearranged from sub-plasmalemmal cortical actin network to stress fiber, which contributes to increased cell motility (Zavadil and Boettinger 2005). To further confirm that DZNep alone or in combination with TGFβ3 indeed promotes EMT, immunofluorescence experiments were performed to detect the actin fiber reorganization in NMuMG cells. F-actin was detected by phalloidin, a fungal toxin specific for F-actin (Bettinger, Gilbert et al. 2004), while nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI).

Induced by TGFβ3, actin structure was rearranged from cortical network to stress fiber (Fig. 3-20). Treatment with DZNep alone did not lead to a typical pattern of actin redistribution, but the enhanced staining in cytoplasm probably indicated that the stress fiber was being generated. The cotreatment of DZNep and TGFβ3 only slightly enhanced the formation of stress fiber induced by TGFβ3, suggesting only a minute synergistic effect occurred. Together, these findings confirmed that DZNep alone indeed induces EMT, and enhances EMT when combined with TGFβ3.

Taken together, the association of the morphological changes induce by DZNep with expression pattern of marker genes as well as the reorganization of actin structure revealed that DZNep alone at least partially induces EMT and could enhance the EMT process in

combination with TGFβ3. The mechanism by which DZNep induces EMT was therefore investigated.

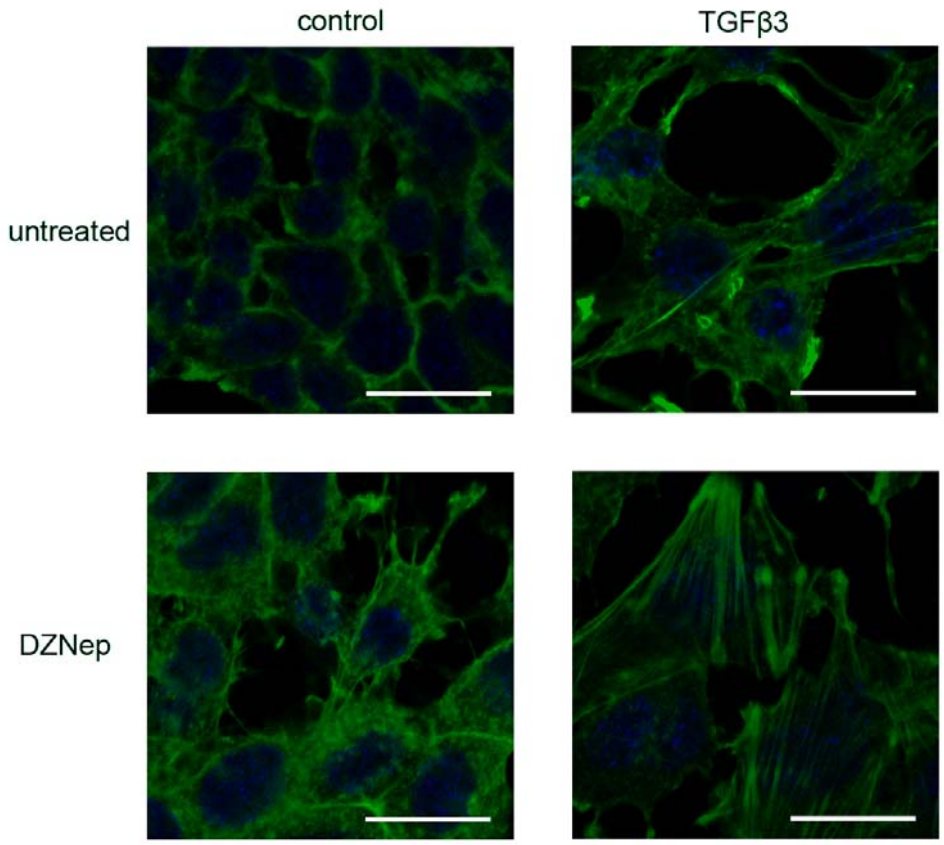

Figure 3-20 DZNep induces actin fiber reorganization

NMuMG cells were treated with 5 μM DZNep or in combination with 5 ng/ml TGFβ3 for 2 days. F-actin was stained with AlexaFluor488 phalloidin. The cells were counterstained with phalloidin (green) and DAPI (blue). Scale bars: 20 μm.

3.2.3 DZNep does not deplete H3K27me3 associations at marker gene promoters

Given that DZNep functions by depleting H3K27me3, the question arose whether DZNep induces EMT by directly altering the associated H3K27me3 at promoters of EMT marker genes. So far H3K27me3 has been only described to be involved in downregulation of epithelial marker genes CDH1 and β4 integrin (Herranz, Pasini et al. 2008; Yang, Pursell et al. 2009). In order to detect the H3K27me3 association at Cdh1 and Acta2 promoters, ChIP assays were performed.

3.2.3.1 H3K27me3 is associated at Cdh1 and Acta2 promoters

H3K27me3 associations were detected at Cdh1 and Acta2 promoters, and both associations correlated to gene transcription. In agreement of the results from Herrenz, Pasini and coworkers, the H3K27me3 was associated at Cdh1 promoter. Importantly, in this study 3-fold induction of H3K27me3 association was observed upon treatment with TGFβ3, suggesting

PRC2 is recruited and thereby represses the Cdh1 promoter. At Acta2 promoter, the H3K27me3 association was decreased in response to TGFβ3, revealing that the reduction of H3K27me3 contributes to, at least in part, the upregulation of Acta2 gene during EMT (Fig. 3-21).

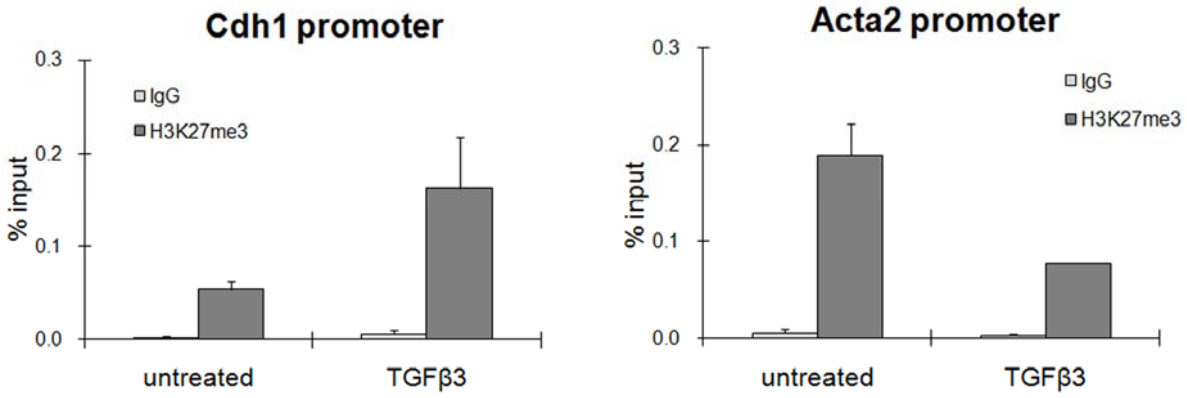

Figure 3-21 H3K27me3 is associated with Cdh1 and Acta2 promoters.

NMuMG cells were treated with 5 ng/ml TGFβ3 for 3 days or left untreated. ChIP assays were performed with H3K27me3 antibody as well as nonspecific anti-rabbit IgG. The precipitated DNA was analyzed by real-time PCR using primer pairs flanking promoters of Cdh1 and Acta2 genes, respectively. The association of H3K27me3 is reflected as the percentage of input DNA. Error bars represent standard error of three (Cdh1) or two (Acta2) independent experiments.

3.2.3.2 DZNep does not alter H3K27me3 associations at Acta2 promoter

Given that H3K27me3 is associated at Acta2 promoter, and the association correlates to transcription during EMT, I further analyzed whether the effect of DZNep on Acta2 upregulation results directly from the alteration of associated H3K27me3 at promoter. NMuMG cells were treated with DZNep in a time-dependent manner and ChIP assays were performed to detect the H3K27me3 at Acta2 promoter.

To control the effect of DZNep, keratin 17 (Krt17) was chosen because it is a PRC2 target gene and shows persistent H3K27me3 association at its promoter (Tan, Yang et al. 2007). Krt17 promoter displayed a reduction of H3K27me3 by DZNep treatment at 6 h and 24 h, implying that DZNep indeed functions properly. DZNep treatment at 48 h caused even higher H3K27me3 association at the Krt17 promoter (Fig. 3-22A). This finding only in part correlated to global H3K27me3 level observed by western blots, because 6 h-treatment did not significantly influence the global H3K27me3 level, but it still decreased promoter-associated H3K27me3. This might indicate that slight depletion in global H3K27me3 level is sufficient to reduce the promoter-associated H3K27me3, or the effect of DZNep on promoter-associated H3K27me3 is promoter dependent.

Unexpectedly, no depletion of H3K27me3 at Acta2 promoter was detected at all time points after DZNep treatment (Fig. 3-22B). As shown by western blots, DZNep reduced

global level of H3K27me3 mostly after treatment for 24 h, but this global reduction did not give rise to decreased H3K27me3 at Acta2 promoter. This suggests that the associated H3K27me3 at Acta2 promoter is independent of the global amount, or like the finding of Krt17, the effect of DZNep on promoter-associated H3K27me3 is promoter dependent. Taken together, although H3K27me3 is associated at Acta2 promoter and correlates to transcription, DZNep does not directly deplete H3K27me3 at the promoter to upregulate Acta2 gene.

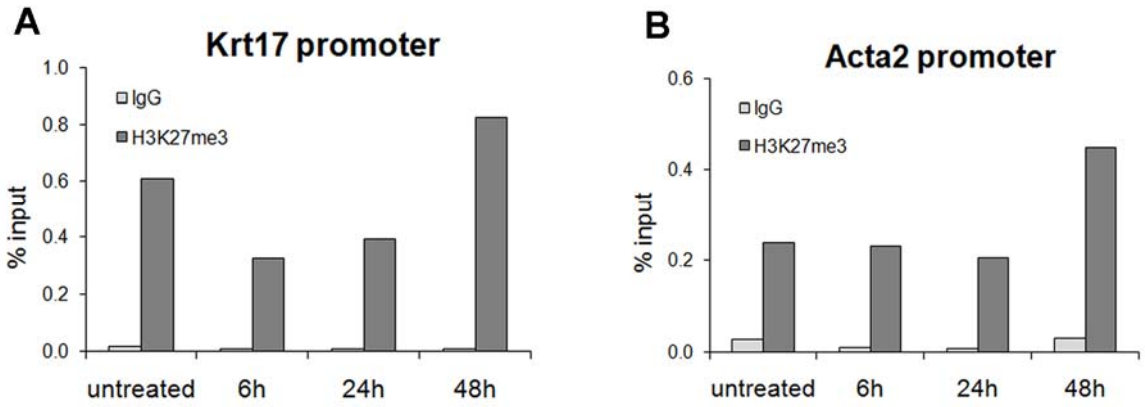

Figure 3-22 DZNep does not alter H3K27me3 at Acta2 promoter.

NMuMG cells were treated with 5 μM DZNep for 6 h, 24 h, and 48 h or left untreated. ChIP assays were performed with H3K27me3 antibody as well as nonspecific anti-rabbit IgG. The precipitated DNA was analyzed by real-time PCR using primer pairs flanking promoters of indicated genes, respectively. The association of H3K27me3 is reflected as the percentage of input DNA. Krt17: keratin 17.

3.2.4 DZNep induces EMT via modulating inducer and regulators

3.2.4.1 DZNep upregulates TGFβ3 induced Snail transcription

Since DZNep does not directly eliminate the associated H3K27me3 at Acta2 promoter to enhance gene expression, the effect of DZNep might result from regulating the expression of EMT inducer such as Snail instead of direct target genes. In addition, the downregulation of Cdh1 could also be mediated by such effect.

To test the regulation of Snail gene expression by DZNep, real time RT-PCR was performed. Once induced by TGFβ3, NMuMG cells demonstrated a robust induction of Snail mRNA amount, which confirms the role of Snail as an EMT inducer. Although DZNep alone showed no effect, cotreatment of DZNep and TGFβ3 caused a higher mRNA induction, suggesting DZNep could enhance the TGFβ3 induced upregulation of Snail transcription, which finally results in the enhanced EMT process (Fig. 3-23A).

3.2.4.2 DZNep enhances EMT-master regulator CBF-A/KAP-1/FTS-1 complex

Without TGFβ3, DZNep is sufficient to induce EMT, proven by morphological changes as well as the expression patterns of marker genes Cdh1 and Acta2. However, since Snail was

upregulated only by cotreatment with both DZNep and TGFβ3, the effect by DZNep alone could not be explained by contribution from Snail mRNA amount. Thus, an inducer or regulator might also account for the effect of DZNep on inducing EMT.

The CBF-A/KAP-1/FTS-1 complex was recently identified as an EMT master regulator. As the direct transcript of this complex, FSP1 is a crucial facilitator of EMT and its expression is a marker of an early stage of EMT. Triggered by CBF-A, the complex is activated and subsequently induces EMT by both upregulating EMT inducers such as Snail, Twist and controlling directly EMT target genes. (Venkov, Link et al. 2007). To test the contribution of DZNep to the regulation of this complex, real time RT-PCR was performed to measure the mRNA amounts of CBF-A, KAP-1, and FSP1.

TGFβ3 treatment slightly increased mRNA amounts of CBF-A and KAP-1, but decreased FSP1 amount in control cells, indicating TGFβ3 is not sufficient to activate this complex (Fig. 3-23B). In contrast, DZNep alone indeed activated CBF-A/KAP-1/FTS-1 complex. DZNep caused 2.5-fold induction of mRNA amounts of CBF-A and KAP-1, implying that DZNep leads to even more inductive effect on CBF-A and KAP-1 than TGFβ3 does. Most importantly, a 5-fold induction of FSP-1 was achieved by DZNep alone, suggesting the upregulated CBF-A and KAP-1 might in turn result in more binding to FTS-1 element, leading to enhanced FSP-1 transcription. These data revealed that the activated CBF-A/KAP-1/FTS-1 complex probably contributes to the EMT-inductive effect induced by DZNep, at least DZNep alone without TGF β3. However, treatment with TGFβ3 abolished the enhanced FSP-1 mRNA by DZNep. This suggests that TGFβ3 might interfere with effect of DZNep on the activation of this master regulator.

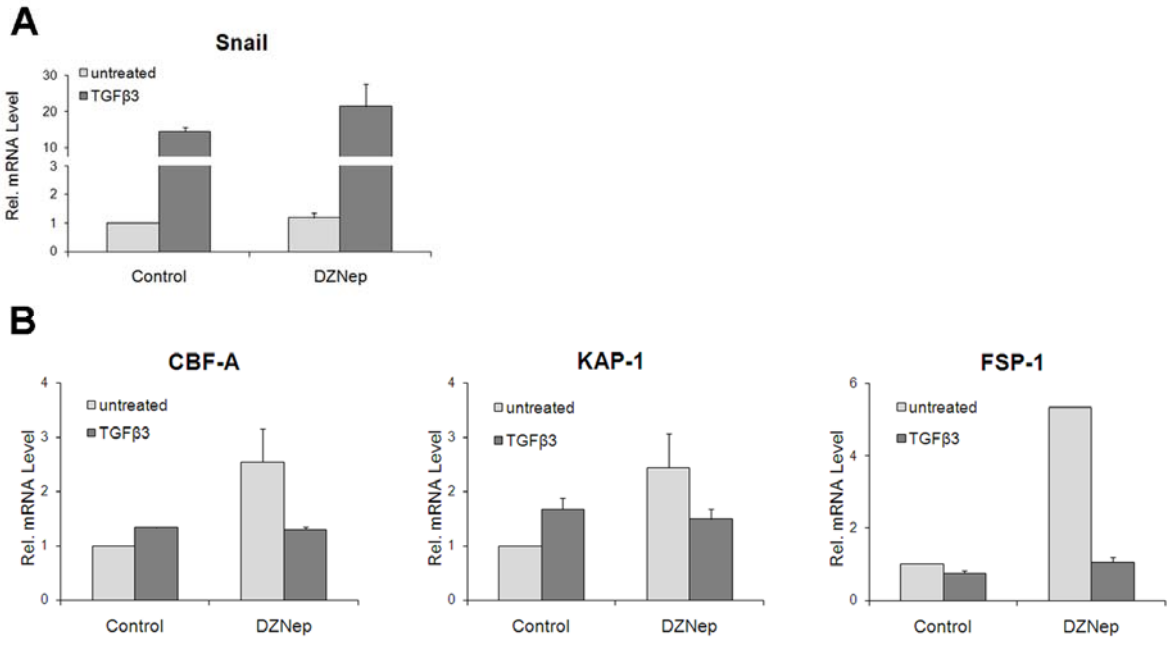

Figure 3-23 DZNep regulates expressions of Snail and CBF-A/KAP-1/FTS-1 complex.

NMuMG cells were treated with 5 ng/ml TGFβ3 or in combination with 5 μM DZNep for 2 days. mRNA levels of indicated genes were measured by real-time RT-PCR and normalized to 18S rRNA. Data are demonstrated as fold change of mRNA level in comparison to untreated control cells. Error bars represent standard error of two independent experiments.
(A) DZNep enhances TGFβ3 induced Snail transcript.
(B) DZNep alone activates the CBF-A/KAP-1/FTS-1 complex.

3.2.4.3 DZNep does not abolish H3K27me3 at FTS-1 element

The regulation of EMT by the CBF-A/KAP-1/FTS-1 complex is triggered by CBF-A. Once upregulated, CBF-A and KAP-1 bind to the FTS-1element, contributing to the formation of the complex, which is critical for transcription of FSP1and sufficient to induce early EMT events (Venkov, Link et al. 2007). Given that the activation of CBF-A/KAP-1/FTS-1 complex by DZNep can be due to the release of PRC2/H3K27me3-mediated transcription repression, ChIP assays were performed to investigate the association of H3K27me3, and further to examine the corresponding influence by DZNep.

TGFβ3 slightly enhanced the association of H3K27me3 at FTS-1 element (Fig.3-24). This finding correlated to the expression patterns, since TGFβ3 did not significantly influence the mRNA inductions of FSP-1(Fig. 3-23B). These data again confirm that TGFβ3 might have no significant effect on the activation of CBF-A/KAP-1/FTS-1 complex. Treatment with DZNep alone had no effect, but cotreatment with TGFβ3 even elevated H3K27me3 association. These results imply that the upregulated FSP1 did not result from H3K27me3 depletion by DZNep at FTS-1 element. Probably the binding of DZNep-induced CBF-A and KAP-1 to FTS-1 element simply enhances FSP1 expression. Alternatively, DZNep influences some unknown histone or non-histone proteins, which are essential for the activation of the CBF-A/KAP-1/FTS-1 complex.

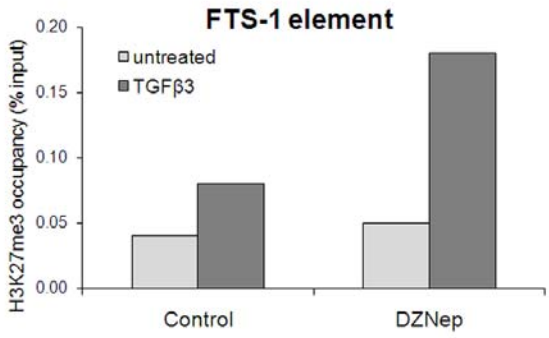

Figure 3-24 DZNep does not deplete H3K27me3 at FTS-1 element.

NMuMG cells were treated with 5 ng/ml TGFβ3 or in combination with 5 μM DZNep for 3 days. ChIP assays were performed with H3K27me3 antibody as well as nonspecific anti-rabbit IgG. The precipitated DNA was analyzed by real-time PCR using primer pairs flanking CBF-A promoter and FTS-1 element. The association of H3K27me3 is reflected as the percentage of input DNA.

3.2.4.4 DZNep downregulates specifically the promoter activity of Cdh1

DZNep induces the TGFβ3 induced Snail mRNA (Fig. 3-23A), which could result from either direct effect of DZNep on Snail gene or the positive regulation by activated CBF-A/KAP-1/FTS-1 complex. Since Snail represses Cdh1 transcription by binding to the elements at promoter (Cano, Perez-Moreno et al. 2000), in order to know whether the enhanced Snail can in turn regulate the promoter activity of Chd1, luciferase assays were performed. Moreover, pGl3-promoter empty vector containing a SV-40 minimal promoter was used to control the specific effect on Cdh1 promoter. Besides HEK 293 cells as a general model for testing promoter activity, NMuMG cells were used as the cellular system where factors essential for EMT should be active.

Similar effects were observed in both cellular systems (Fig. 3-25). DZNep displayed a repressive effect on both promoters, suggesting DZNep might affect some factors essential for transcription machinery. However, the inhibitory effect on the Cdh1 promoter was stronger, indicating a specific inhibition in addition to the unspecific effect by DZNep. This finding confirms that the downregulation of Cdh1 induced by DZNep might really result from enhancement of EMT factors like CBF-A, KAP-1, FSP-1 or Snail.

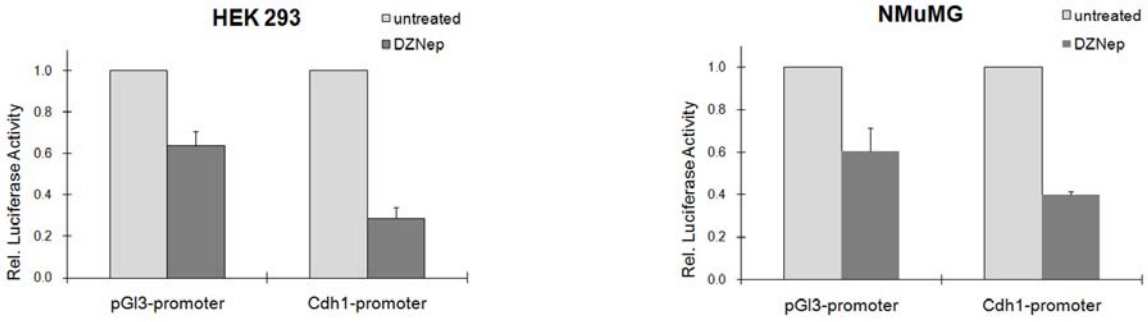

Figure 3-25 DZNep downregulates Cdh1 promoter activity

HEK 293 cells were transfected using PEI whereas NMuMG cells using Transfectin in 24-well-plate. For each well, cells were transiently transfected with 500 ng of luciferase reporter plasmid and 6 ng of *LacZ* expression plasmid. Twenty-four hours after transfection, cells were treated with 5 μM DZNep for 1 d or left untreated. Cells were harvested and luciferase and β-galactosidase activities were assayed. Luciferase activities were normalized to the *LacZ* expression and demonstrated as fold change to untreated cells. Error bars represent standard error of three (HEK 293 cells) or two (NMuMG cells) independent experiments.

3.2.5 Knockdown of Suz12 enhances EMT by regulating Snail

Besides H3K27me3, DZNep inhibits H4K20me3 as well. Moreover, as DZNep inhibits the methyltransferases via an indirect mechanism (Miranda, Cortez et al. 2009), the effect on EMT by DZNep could be independent of H3K27me3. In order to prove the H3K27me3-dependent effect of DZNep, knockdown of the PRC2 core component Suz12 was performed in NMuMG cells and the effect was compared to that of DZNep.

3.2.5.1 Knockdown of Suz12 enhances EMT morphology

The NMuMG cells stably expressing siSuz12 were established by lentiviral transduction. Nontargeting scrambled siRNA was used as a control. Knockdown efficiency was determined by reduced global level of Suz12 by western blot (Fig. 3-26A). In addition, as a component of PRC2, knockdown of Suz12 led to a decreased global level of H3K27me3. However, the global H3K27me3 level was not totally abolished, probably because the knockdown of Suz12 was not complete, or alternatively, the knockdown of other subunits of PRC2 such as Ezh2 and Eed were also required. The influence of H3K27me3 depletion mediated by Suz12 knockdown on EMT was first analyzed by morphological change.

Without TGFβ3 treatment, both cells displayed typical epithelial morphology (Fig. 3-26B). When treated with TGFβ3, only a small population of control cells displayed a typical EMT morphology, whereas the majority still maintained epithelial phenotypes. This suggests that the EMT process in transduced cells was generally weaker than that in wide type cells, probably due to some unspecific inhibitory effects from viral transduction. On the other hand, cells stably expressing siSuz12 still demonstrated typical EMT morphology when treated with TGFβ3. This revealed that although EMT was somehow inhibited by viral transduction, Suz12 knockdown in combination with TGFβ3 still showed a stronger EMT induction than control. Taken together, only combined with TGFβ3 treatment, Suz12 knockdown could enhance EMT morphology. This finding is different from what was observed by DZNep treatment, where DZNep alone is sufficient to induce EMT morphology (Fig. 3-16).

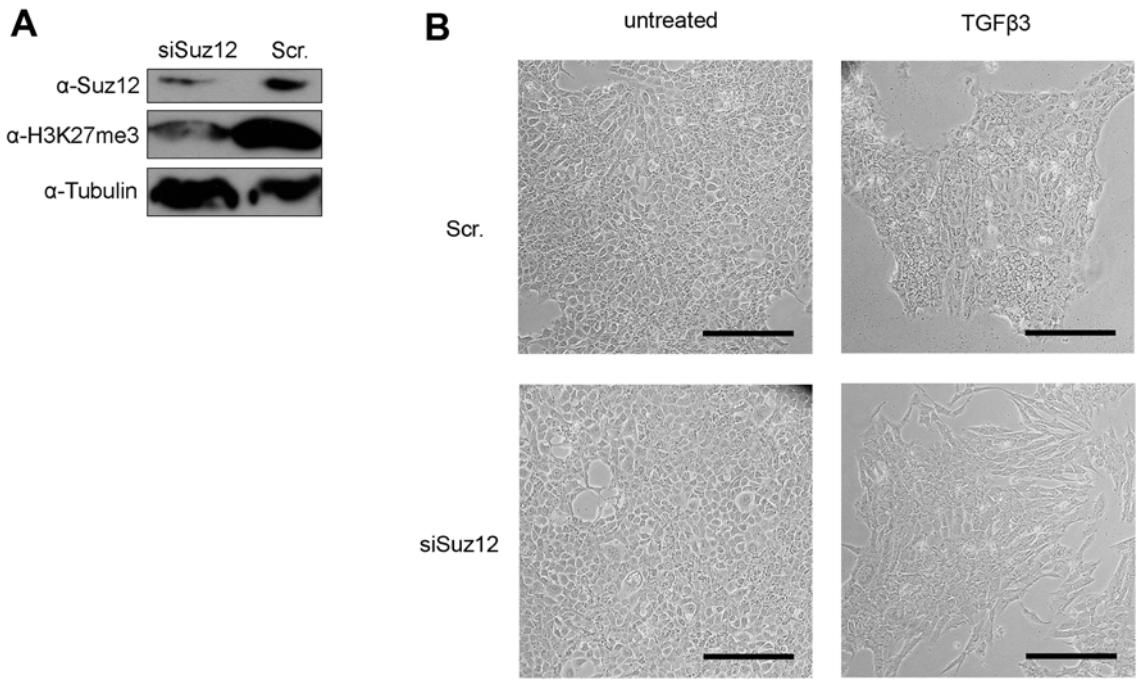

Figure 3-26 Knockdown of Suz12 enhances EMT morphology.

(A) Knockdown of Suz12 reduced global H3K27me3 level. NMuMG cells were transduced by lentivirus expressing siRNA against Suz12. Cell extracts were analyzed by western blots using indicated antibodies. Control cells stably express nontargeting scrambled siRNA. Scr: scrambled siRNA.
(B) Suz12 knockdown enhances EMT morphology. NMuMG cells were treated with 5 ng/ml TGFβ3 or left untreated for 3 days. Scale bars: 250 μm.

3.2.5.2 Knockdown of Suz12 regulates marker genes

Having shown that Suz12 knockdown only enhanced the TGFβ3 induced EMT morphology, the question arose whether it also affects EMT marker genes. The expression patterns of maker genes were measured by quantitative RT-PCR. Both Cdh1 and Acta2 in control cells showed weaker typical EMT expression patterns than those in wide type cells (Fig. 3-27). This was in agreement with the untypical morphological changes in those cells (Fig. 3-26B). Knockdown of Suz12 downregulated Cdh1 expression, and slight synergistic effect was observed when combined with TGFβ3. On the other hand, Suz12 knockdown even reduced Acta2 mRNA in both untreated and TGFβ3 induced cells. This finding implied that the Suz12 knockdown contributes to EMT, but probably in a gene dependent manner, namely, it affects Cdh1 other than Acta2. Alternatively, the unspecific EMT-inhibitory effect from viral transduction is also marker gene dependent, thus more inhibition occurs in Acta2 gene. Taken together, the EMT-inductive effect mediated by Suz12 knockdown is weaker than that observed by DZNep treatment.

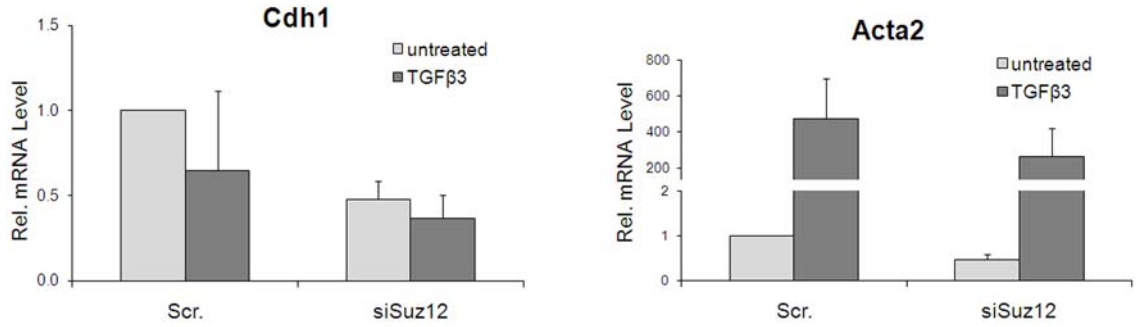

Figure 3-27 Knockdown of Suz12 regulates EMT marker genes.

The NMuMG cells stably expressing siRNA against Suz12 were treated with 5 ng/ml TGFβ3 for 2 days. Cdh1 and Acta2 mRNA levels were measured by real-time RT-PCR and normalized to 18S rRNA. Data are demonstrated as fold change of mRNA level in comparison to untreated control cells stably expressing nontargeting scrambled siRNA. Error bars represent standard error of two independent experiments.

3.2.5.3 Knockdown of Suz12 enhances the TGFβ3 induced Snail expression

Treated with TGFβ3, Suz12 knockdown could enhance EMT morphology. Moreover, Suz12 knockdown alone, or combined with TGFβ3, downregulates Cdh1. In order to analyze whether these inductive effects also result from enhanced Snail as DZNep does, real time RT-PCR was performed. Interestingly, like DZNep, Suz12 knockdown alone did not affect

Snail mRNA, but it enhanced Snail transcription when treated with TGFβ3 (Fig.3-28A). This indicates that the depletion of H3K27me3, mediated from DZNep or Suz12 knockdown, contributes equally to the TGFβ3 induced Snail mRNA level, and this upregulation probably in turn enhances the EMT process.

3.2.5.4 Knockdown of Suz12 fails to activate CBF-A/KAP-1/FTS-1 complex

Given that knockdown of Suz12 led to elevation of Snail mRNA level induced by TGFβ3, the contribution of Suz12 knockdown to the regulation of CBF-A/KAP-1/FTS-1 complex was analyzed by real time RT-PCR. The mRNA amounts of CBF-A and KAP-1 as well as the product FSP1 were measured. Consistent with DZNep, TGFβ3 treatment slightly increased mRNA amounts of CBF-A and KAP-1, but decreased FSP1 amount in control cells (Fig. 3-28B). This again implies that TGFβ3 is unable to activate this complex. Importantly, knockdown of Suz12 downregulated all three genes of this complex, suggesting the Suz12 knockdown did not activate, and even inactivated the CBF-A/KAP-1/FTS-1 complex. This is probably done by altered mRNA stability mediated by Suz12 knockdown. Most importantly, this finding confirms that the activation of the complex by DZNep is not dependent on H3K27me3, and might in fact results from DZNep's effect on some other histone or non-histone proteins, which are essential for the activation of the CBF-A/KAP-1/FTS-1 complex. Therefore, although both DZNep and Suz12 knockdown showed H3K27me3 depletion and EMT induction similarly, they function differently. DZNep functions by activating the master regulator or with Snail, whereas Suz12 knockdown only enhances TGFβ3 induced Snail expression.

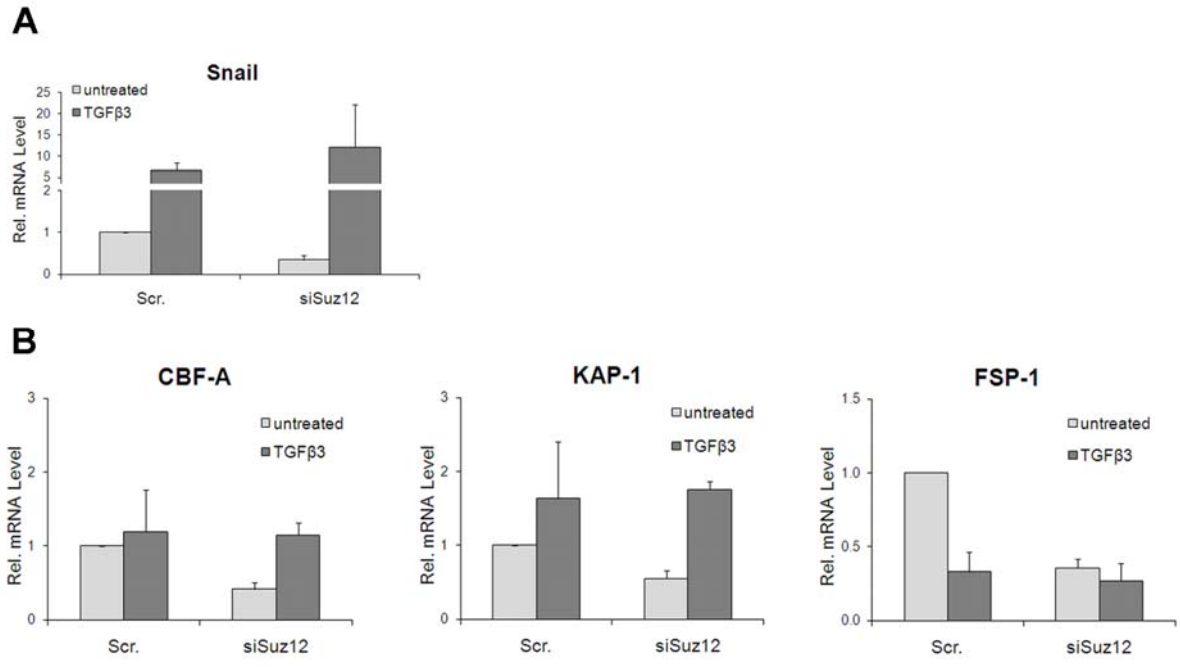

Figure 3-28 The effects of Suz12 knockdown on Snail and the CBF-A/KAP-1/FTS-1 complex.

NMuMG cells stably expressing siRNA against Suz12 were treated with 5 ng/ml TGFβ3 for 2 days. The mRNA levels of indicated genes were measured by real-time RT-PCR and normalized to 18S rRNA. Data are demonstrated as fold change of mRNA level in comparison to untreated cells stably expressing scrambled siRNA. Error bars represent standard error of two independent experiments.
(A) Suz12 knockdown enhances TGFβ3 induced Snail expression.
(B) Suz12 knockdown inactivates the CBF-A/KAP-1/FTS-1 complex.

3.2.5.5 Suz12 knockdown represses Cdh1 promoter activity

DZNep represses the Cdh1 promoter activity (Fig. 3-25). In order to examine whether Suz12 knockdown has the same effect on Cdh1 promoter activity as DZNep does, luciferase assays were performed. Pronounced downregulation of Suz12 was obtained in HEK 293 cells by transient transfection (Fig. 3-29A). Like DZNep, Suz12 knockdown repressed Cdh1 promoter activity too, and this effect was also in correlation to the enhanced Snail mRNA (Fig. 3-29B). In addition, this repressive effect was weaker than that by DZNep.

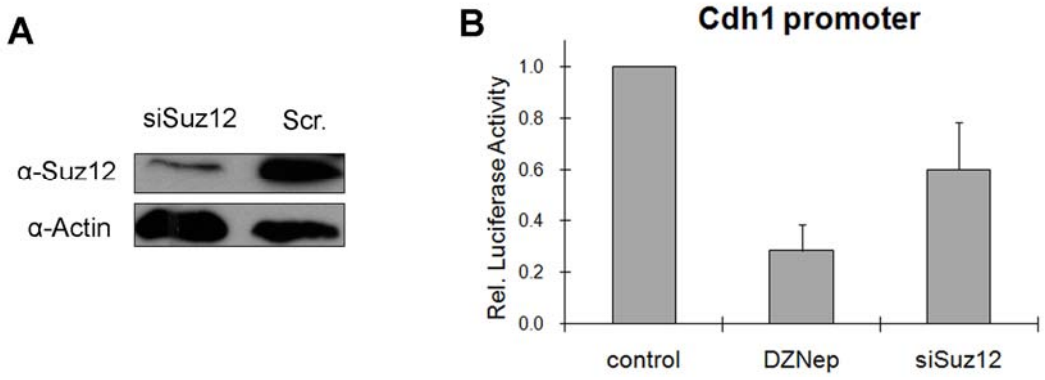

Figure 3-29 Downregulation of Cdh1 promoter activity by DZNep and siSuz12

(A) Knockdown of Suz12 in HEK-293 cells. Cells were transiently transfected with siSuz12 plasmid using PEI. Cell extracts were analyzed by western blots using the indicated antibodies.
(B) Knockdown of Suz12 showed less repressive effect on Cdh1 promoter than DZNep did. Cells were transfected with 500 ng of luciferase reporter plasmid, 25 ng of siSuz12 plasmid, and 6 ng of *LacZ* expression plasmid. Twenty-four hours after transfection, cells were treated with 5 μM DZNep for 1 d or left untreated. Luciferase activities were normalized to the *LacZ* expression and demonstrated as fold change to untreated cells. Error bars represent standard error of three independent experiments.

4 Discussion

4.1 Regulation of chromatin accessibility at the beta-casein promoter in HC11 cells

The regulation of beta-casein gene expression in HC11 mammary epithelial cells provides a model to study the mechanism by which mammary epithelial cell differentiation is controlled. At the transcriptional level, the beta-casein gene is mainly regulated by binding of hormone induced transcription factors to regulatory elements. However, the binding of Stat5a, the key transcription factor during mammary differentiation, to endogenous beta-casein promoter upon lactogenic treatment in HC11 cells has not been convincingly demonstrated yet, indicating an inaccessible chromatin structure might exist. Moreover, other findings showed that the beta-casein locus is associated with heterochromatin. These results taken together suggest that although beta-casein is the differentiation marker gene of mammary epithelial cells, it might be somehow repressed in this specific cell line. Little is known about the molecular mechanism involved and whether epigenetic modifications, especially repressive histone marks, contribute to these phenomena. To address these questions, the chromatin accessibility at the beta-casein promoter and the role of H3K9me2 in transcriptional regulation of beta-casein gene in HC11 cells have been studied. Moreover, the function of the JmjC domain-containing demethylase Jmjd1a in beta-casein expression was also analyzed.

The results revealed that the beta-casein promoter is inaccessible in HC11 cells, and the inaccessibility could not be reverted by hormone treatment. Moreover, the repressive histone mark H3K9me2 is associated at beta-casein promoter. Since the associated H3K9me2 correlates to chromatin inaccessibility, it probably represses the beta-casein gene. Additionally, other interplay partners can be also involved in the repression. Although the overexpression of Jmjd1a essential domains reduces global H3K9me2 level, beta-casein expression is still not upregulated. This is, at least in part, due to the inhibitory effect of Jmjd1a domains on transcription machinery, or due to the inhibitory effect of the interplay partners on chromatin structure. Finally, because mammary differentiation reduces risk of breast cancer, approaches to remove the associated H3K9me2 might enhance differentiation and therefore reduce tumorigenesis of breast cancer.

4.1.1 Beta-casein promoter is inaccessible in HC11 cells

At the transcriptional level, the beta-casein gene is mainly regulated by binding of hormone induced transcription factors to regulatory elements. However, in this cell line, the binding of

Stat5a to the endogenous beta-casein promoter upon lactogenic treatment has not been convincingly demonstrated yet. In this work the phenomenon was also observed. The activated Stat5a did not bind to beta-casein promoter after DIP treatment (Fig. 3-2). This appears to be a controversial finding, because beta-casein mRNA was induced by DIP (Fig. 3-1B) and binding of Stat5a to target gene promoter is required for hormone-regulated transcription. Several controls ensure the reliability of this result. Stat5a became phosphorylated in response to DIP, suggesting an activated status (Fig. 3-1A). Moreover, Stat5a did bind to Socs3 and Cish promoters and this binding was enhanced upon DIP treatment, confirming that Stat5a has truly the binding capability (Fig. 3-2).

Having shown that Stat5a did not get access to beta-casein promoter, the inaccessibility in this region was further proven by CHART-PCR and chromatin lockdown assays. Both assays confirmed that the beta-casein promoter is indeed locked (Fig. 3-4 and 3-5). Moreover, since the chromatin lockdown assay is based on the analysis of DNase I hypersensitive sites, beta-casein promoter is therefore located at the nucleosomal array or condensed chromatin region, which can thus explain why Stat5a does not bind. In addition, both assays showed that lactogenic hormones failed to improve the locked chromatin, indicating that some events keep locking this region, and DIP treatment is not sufficient to open it.

As mentioned, although the HC11 cell line has been widely used, the binding of Stat5a to endogenous beta-casein promoter upon lactogenic treatment *in vivo* has not been convincingly demonstrated. Some available data might result from the unspecificity of antibody used in ChIP assay (Kabotyanski, Huetter et al. 2006). Moreover, the enhancement of mRNA might not always represent newly synthesized transcript. Because prolactin can increase beta-casein mRNA stability up to17-25 folds (Guyette, Matusik et al. 1979), the 100-fold induction can result from an increased mRNA stability of few newly synthesized transcript. Provided this was really the case, the new transcript could be only in the range of 4-6 folds, indicating a rather weak induction concerning the function of beta-casein in differentiation. Furthermore, one finding also found that the beta-casein locus is associated with heterochromatin (Ballester, Kress et al. 2008; Kress, Ballester et al. 2010). Taken together, the correlation of inaccessible beta-casein promoter to the association of gene loci with heterochromatin implied that this gene might be indeed repressed. The mechanisms involved were analyzed.

4.1.2 H3K9me2 at beta-casein promoter might inhibit transcription

Chromatin inaccessibility is determined by epigenetic processes such as DNA methylation and histone modifications. Since DNA methylation may not be involved in mammary epithelial cell differentiation (Rijnkels, Kabotyanski et al. 2010), histone modifications, especially repressive lysine methylations, were investigated. The occupancies of those marks at beta-

casein promoter were examined by ChIP assays. Finally, a massive association of H3K9me2 was observed, at the beta-casein but not at the Cish and Gapdh promoters (Fig. 3-6). In contrast, other marks such as H3K27me3, H3K9me3, and H4K20me3 were not detectable.

The association of H3K9me2 at beta-casein promoter is considered to be robust, because its enrichment was comparable to that of Magea2, a G9a HMT target gene displaying strong H3K9me2 association at its promoter (Tachibana, Sugimoto et al. 2002). Most importantly, the associations were in coincidence with chromatin inaccessibilities, respectively. The massive H3K9me2 modification is correlated to the chromatin inaccessibility and thereby might inhibit the Stat5a binding at the beta-casein promoter.

As H3K9me2 represses transcription mostly by formation of heterochromatin via HP1 (Jenuwein 2006), the region at beta-casein promoter can be heterochromatic. A recent study showed a correlation of casein gene loci to heterochromatin in undifferentiated HC11 cells. The casein locus stays predominantly at the heterochromatic region in untreated cells. Hormone treatment results in the formation of a chromatin loop containing casein locus which moves outside this chromosome territory to euchromatic region. This reorganization correlates with active transcription (Ballester, Kress et al. 2008; Kress, Ballester et al. 2010). Thus, the associated H3K9me2 at promoter observed in this work is probably the molecular mechanism of the nuclear organization, at least for untreated cells. Alternatively, H3K9me2 can directly lock the beta-casein promoter in cooperation with DNMT as well (McGarvey, Fahrner et al. 2006).

Provided the binding of Stat5a is impaired due to chromatin inaccessibility mediated by H3K9me2, the Pol II transcription machinery might be inhibited as well. ChIP data revealed that the associated H3K9me2 is reversely correlated to binding of Pol II (Fig. 3-7). Only extremely slight Pol II binding was observed at beta-casein promoter, indicating a trivial basal transcription. Importantly, DIP treatment only achieved very weak enhancement of Pol II binding. Since p-CTD RNA Pol II presents both transcription and mRNA splicing processes (Hirose and Ohkuma 2007), DIP treatment might only give rise to few syntheses of beta-casein mRNA. Low binding of Pol II in combination with undetectable Stat5a binding supports the hypothesis that the observed fairly strong induction of beta-casein mRNA is actually obtained from only few newly synthesized mRNA molecules, which are strongly enhanced in their stabilities by DIP treatment. Therefore, the correlation of associated H3K9me2 to the inaccessible promoter and the inhibited Pol II binding suggests that the beta-casein gene is repressed in this cell line and H3K9me2 might play a major inhibitory role.

In addition, lack of 3D architecture in HC11 cells culture condition can also result in weak induction of beta-casein transcription. ECM plays a crucial role in regulation of beta-casein transcription via chromatin remodeling and reorganizing cells from monolayers into polarized

acini (Xu, Spencer et al. 2007; Xu, Nelson et al. 2009). Without ECM, HC11 cells only form flat monolayers in 2D culture. This might cause epithelial cells to rapidly lose the functions to differentiate upon hormone treatment. However, since HC11 cells are able to produce their own matrix required for differentiation (Chammas, Taverna et al. 1994), this can only in part account for the weak transcriptional induction.

As a functional marker, beta-casein gene regulation can give an insight into the regulation of mammary epithelial cell differentiation. The finding that beta-casein transcription is probably inhibited by H3K9me2 reveals that HC11 cells only in part or even not undergo efficiently functional differentiation after DIP treatment, thus this cell line is probably not perfect for investigations of mammary epithelial cell differentiation although widely used. Moreover, since mammary differentiation reduces breast tumorigenesis, approaches to remove H3K9me2 might enhance differentiation of breast cancer cells and might be further proven as concepts for cancer therapy.

4.1.3 Methylation inhibitor BIX-01294 only induces beta-casein basal transcription

Specific inhibitors can be used to analyze the functional effect of epigenetic modifications in regulation of gene expression. G9a specific inhibitor BIX-01294 was described to efficiently reduce H3K9me2 (Kubicek, O'Sullivan et al. 2007). However, it only slightly opened the inaccessible beta-casein promoter (Fig. 3-8B). This indicates that if the promoter-associated H3K9me2 from previously synthesized methylation persists, reduction of new generation of H3K9me2 by BIX-01294 probably only can show little effect. Alternatively, another HMT such as Suv39h might also be responsible for H3K9me2 at beta-casein promoter. Furthermore, other epigenetic modifications, which were not examined in this work, might also interplay with H3K9me2, thus removal of only one modification such as H3K9me2 is probably not sufficient to completely open the chromatin structure.

Interestingly, beta-casein basal transcription was induced to 3 folds by BIX-01294 (Fig. 3-9A). This suggests that BIX-01294 makes the promoter a bit accessible and therefore PIC can bind and induce basal mRNA without binding of Stat5a. Moreover, this result also supports that H3K9me2 might only play a “fine tuning” role when interplayed with other partners. However, treatment with both BIX-01294 and DIP failed to further induce transcription. No synergistic effect of DIP and BIX-01294 was obtained (Fig. 3-9B). Some reasons could account for this phenomenon. BIX-01294 might downregulate some proteins, which are essential for DIP induced beta-casein transcription, hence it finally eliminates the induction of transcription. Moreover, given the mRNA induction upon DIP treatment results from a combination of new mRNA generation and prolonged mRNA stability, BIX-01294 might also function to increase beta-casein mRNA degradation.

4.1.4 Jmjd1a reduces H3K9me2, but fails to enhance beta-casein transcription

BIX-01294 can only reduce the new production of methylation by inhibiting G9a, but it cannot abolish the previously synthesized methylation. Therefore, another approach to degrade H3K9me2 is to carry out *in vivo* demethylation reaction by specific lysine demethylases. JmjC domain-containing demethylases Jmjd1a and Jmjd2c were chosen to eliminate H3K9me2 in HC11 cells, because both regulate self-renewal in embryonic stem cells (Loh, Zhang et al. 2007), and HC11 have characteristics of a mammary-specific stem cell (Cella, Cornejo Uribe et al. 1996).

Only Jmjd1a essential domains demonstrated *in vivo* demethylase activity in HC11 cells (Fig.3-11B). However, the reduction was not massive, suggesting the overexpression of JmjC and Zinc-finger domains of Jmjd1a are able to, but still not efficiently, mediate demethylation reaction in HC11 cells. In contrast, Jmjd2c domains did not show demethylation effect. Because in addition to H3K9me2, H3K9me3 can be demethylated by Jmjd2c resulting in H3K9me2. The decreased level by H2K9me2 demethylation might be balanced by the accumulating H2K9me2 as a product from H3K9me3 demethylation, leading to a constant H3K9me2 level on western blots (Shin and Janknecht 2007).

Unexpectedly, although expression of Jmjd1a domains reduced the global H3K9me2 level, it downregulated the beta-casein transcription in both untreated and DIP treated HC11 cells (Fig. 3-12A and B). Given that the inhibitor BIX-01294 did not enhance beta-casein transcription either, probably the associated H3K9me2 indeed interplays with other unknown modifications to regulate beta-casein gene expression and H3K9me2 might only play a “fine-tuning” role in the interplay. Therefore, the inhibition to H3K9me2 alone is not sufficient to upregulate the repressed beta-casein gene. Furthermore, as shown in Fig. 3-11B, overexpression of JmjC and Zinc-finger domains of Jmjd1a are not sufficient to completely demethylate global H3K9me2 in HC11 cells. Probably simultaneous expression of both demethylases might solve this problem. Importantly, it is also possible that Jmjd1a domains might downregulate some factors specifically essential for prolactin-induced Stat5a-mediated transcriptional activation. This hypothesis was confirmed by reporter gene assay. The beta-casein promoter activity was indeed downregulated by Jmjd1a domains (Fig. 3-14C). Thus, this inhibitory effect on promoter activity might, at least in part, account for the downregulation of mRNA level. In addition, the inhibitory effect from the potential interplay partners of H3K9me2 on chromatin structure can also be involved.

Taken together, based on the finding that the association of H3K9me2 at beta-casein promoter is correlated to the chromatin inaccessibility and probably represses beta-casein transcription, a new mechanism is proposed by which beta-casein gene is regulated in HC11 cells (Fig. 4-1). In HC11 cells, the beta-casein promoter is inaccessible, probably due to the

association of H3K9me2, which represses this gene either by formation of heterochromatin via HP1 or by direct cooperation with DNMT. Furthermore, H3K9me2 might also interplay with other partners, and H3K9me2 might only play a “fine tuning” role. The overexpression of Jmjd1a essential domains reduces global H3K9me2 level, but the beta-casein expression is still not upregulated. This is, at least in part, due to the inhibitory effect of Jmjd1a domains on transcription machinery, or due to the inhibitory effect of the interplay partners on chromatin structure.

This mechanism could help to understand how epigenetic modification regulates mammary epithelial cell differentiation. In addition, since mammary differentiation reduces risk of breast cancer, approaches to remove the associated H3K9me2 might enhance differentiation and therefore reduce tumorigenesis of breast cancer. In addition, since DIP treatment does not improve the promoter accessibility, functional differentiation in HC11 cells can be only in part or even not achieved. Therefore, evaluation should be subjected to this cell line as a model of mammary cell differentiation.

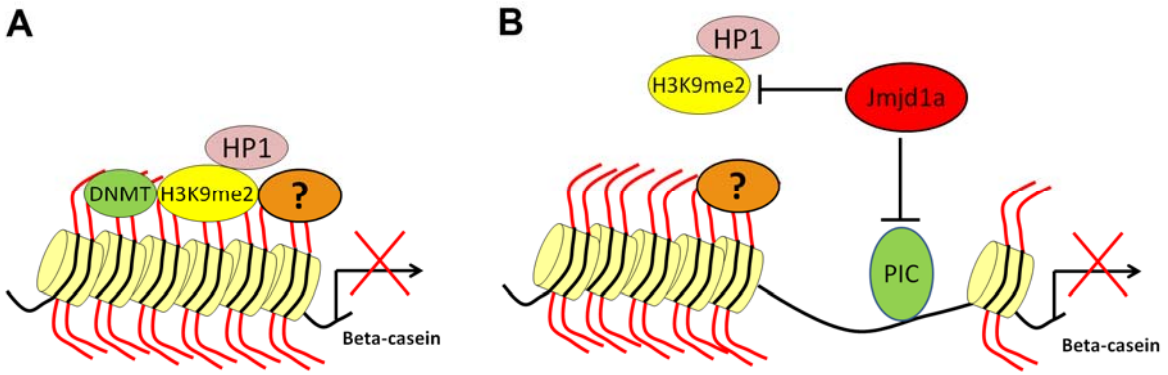

Figure 4-1 H3K9me2 association at beta-casein promoter in HC11 cells

(A) Associated H3K9me2 is correlated to chromatin inaccessibility at beta-casein promoter and probably thereby represses beta-casein transcription. This repression is mediated by formation of heterochromatin via HP1 or direct cooperation with DNMT. Furthermore, some other unknown interplay partners labeled as “question mark” might also be involved.

(B) The essential domains of Jmjd1a reduce global H3K9me2. However, the beta-casein transcription is still not upregulated, at least in part due to the inhibitory effect on some essential factors of the transcription machinery. Moreover, the inhibitory effect from the potential interplay partners on chromatin structure might be also involved and therefore keep locking the chromatin structure. PIC: pre-initiation complex.

4.2 Regulation of EMT by DZNep

EMT is regulated by molecular networks of extracellular stimuli, signaling factors and inducers. In contrast to regulations directly applied to individual target gene, the regulation by inducers or regulators might affect the entire EMT process. The research of transcriptional regulation of EMT had been focused mainly on the inducer Snail until the identification of CBF-A/KAP-1/FTS-1 complex as a master regulator. This complex not only activates EMT

inducers such as Snail and Twist, but also directly regulates EMT target genes (Teng, Zeisberg et al. 2007; Venkov, Link et al. 2007). However, the regulation of the complex still remains unknown.

Epigenetic modifications were described to be involved in the regulation of EMT. PRC2 contributes to repression of some epithelial marker genes. For example, SUZ12 is required for downregulation of CDH1 by Snail and the repression is mediated by H3K27me3 (Herranz, Pasini et al. 2008). Similarly, H3K27me3 represses β4 integrin as well (Yang, Pursell et al. 2009). In addition, PRC is involved in the induction of mesenchymal marker genes. Upon treatment with TGFβ, Hairy and Enhancer-of-split (H/E(Spl)) related repressors are activated via Smad3/4, and subsequently repress EZH1 and EZH2 genes. Displacement of PRC complex to histone variant H3.3B releases the repressive chromatin and therefore induce mesenchymal gene expression (Blumenberg, Gao et al. 2007). In addition to marker genes, the EMT inducer Snail is also associated with epigenetic modification. Snail transcription can be enhanced by the binding of transcription factor YY1 to the 3′ enhancer. YY1 can presumably recruit histone modification enzymes to alter the chromatin structure of the 3′ enhancer, for example CBP/p300 acetyltransferases and the arginine methyltransferase PRMT1 (Palmer, Majumder et al. 2009). However, the role of repressive histone marks in regulation of EMT inducers was still unknown.

In this part, the role of epigenetic modifications in regulation of EMT was studied by treating cells with different histone methylation inhibitors. Moreover, the role of H3K27me3 depletion by knockdown of Suz12, a core component of the PRC2, in regulating EMT was also analyzed. Data indicate that H3K27me3 regulates EMT at levels of marker genes, Snail, and most probably also the master regulator. The histone methylation inhibitor DZNep was identified to be a novel EMT inducer. The inductive effect, at least in part, results from the activation of the CBF-A/KAP-1/FTS-1 complex, an EMT master regulator. However, the activation is independent of the associated H3K27me3 at FTS-1 element, and might result from the effect of DZNep on other factors. TGFβ3 plays no role in the activation of the CBF-A/KAP-1/FTS-1 complex. Furthermore, Snail might be a target of H3K27me3, since depletion of H3K27me3 by either DZNep or Suz12 knockdown could enhance Snail upregulation by TGFβ3. In addition, both epithelial marker Cdh1 and mesenchymal marker Acta2 promoters are associated with H3K27me3 and the associations correlate to gene transcription. However, DZNep does not affect those associations. Importantly, as EMT correlates closely to tumor invasion and metastasis, DZNep might potentially enhance the risk of breast cancer metastasis via inducing EMT in mammary epithelial cells.

4.2.1 Identification of DZNep as a novel EMT inducer

In order to investigate the role of epigenetic modifications, especially histone lysine methylations, in regulating EMT, different histone methylation inhibitors were applied to NMuMG cells and their influences on EMT were monitored. Among these inhibitors, DZNep depletes H3K27me3, while BIX-01294 and Chaetocin abolish methylated H3K9 by inhibiting G9a and Suv39h HMTs, respectively (Kubicek, O'Sullivan et al. 2007; Tan, Yang et al. 2007; Lakshmikuttyamma, Scott et al. 2009). To identify *in vivo* EMT process, the typical mesenchymal morphology and four essential molecular marks as follows were analyzed: loss of epithelial marker Cdh1, gain of mesenchymal marker Acta2, actin fiber reorganization, and induction of transcription factor Snail (Teng, Zeisberg et al. 2007).

Only DZNep led to an EMT-like morphological change in NMuMG cells, and TGFβ3 treatment was not required (Fig. 3-16). This event was further proven to be indeed a kind of EMT by hallmarks. DZNep alone was sufficient to regulate Cdh1 and Acta2 transcriptions, and synergistic effects occurred when combined with TGFβ3 (Fig. 3-19). Similar finding was also observed by reorganized actin structure (Fig. 3-20). Although DZNep alone did not lead to a typical pattern of actin redistribution, the enhanced staining in cytoplasm probably indicated that the stress fiber was being generated. The cotreatment of DZNep and TGFβ3 slightly enhanced the formation of stress fiber induced by TGFβ3, suggesting a synergistic effect occurred. Moreover, Snail mRNA was also enhanced, but only by combination of DZNep and TGFβ3 (Fig.3-23A). Taken together, this confirmed that DZNep in fact induces some important events in the process of EMT induction. It also indicated that some processes still require TGFβ3.

The action of DZNep is still not clear and seems to be context dependent. By inhibiting AdoHcy hydrolase, DZNep disturbs AdoMet-AdoHcy metabolism and leads to enhanced AdoHcy level, which subsequently inhibits the methyltransferases. It abolishes global H3K27me3 level, but the downregulation of PRC2 components is not required (Borchardt, Keller et al. 1984; Chiang 1998), even though DZNep can in fact abolish all three components of PRC2 (Tan, Yang et al. 2007). In this work, the marked decrease in H3K27me3 level was observed after 24 h treatment, but was completely recovered after 48 h treatment, indicating the reduced H3K27me3 level resulted from 24 h treatment might in turn alter EMT process (Fig. 3-18A). The global level of Suz12 was not reduced accordingly (Fig. 3-18B), probably due to the instability of the entire PRC2 instead of downregulation (Tan, Yang et al. 2007).

The identification of DZNep as an EMT inducer indicates that H3K27me3 is involved in EMT regulation. The mechanisms involved, which is probably also the target of DZNep, were

further analyzed. The effect of DZNep on histone methylation seems to be indirect and other histone modifications can also be involved.

This finding also can have clinical relevance. As EMT correlates closely to tumor invasion and metastasis, the application of DZNep to tumor patients might potentially enhance the risk of breast cancer metastasis via inducing EMT in cancer cells. Therefore, more valuation of DZNep for cancer therapy should be done.

4.2.2 DZNep induces EMT by activating the CBF-A/KAP-1/FTS-1 complex

In order to analyze whether the inductive effect of DZNep results directly from H3K27me3 depletion at marker gene promoters, the associations of H3K27me3 at Cdh1 and Acta2 promoters were analyzed by ChIP assay. Interestingly, both promoters displayed associations of H3K27me3 (Fig. 3-21). Importantly, EMT induction by TGFβ3 correlated to reduction of associated H3K27me3 at promoters and thus probably contributes, at least in part, to the upregulation of Acta2 gene during EMT. However, although global H3K27me3 level was decreased, DZNep did not deplete H3K27me3 association at Acta2 promoter (Fig. 3-22B). Since H3K27me3 enhancement should contribute to loss of Cdh1 expression, further loss of Cdh1 by H3K27me3 abolishment via DZNep was not expected. Therefore, H3K27me3 modification at the promoters of Cdh1 and Acta2 are not the direct targets of DZNep.

Because DZNep alone, or combined with TGFβ3, can induce all EMT hallmarks, the inducers were expected to be most probably the targets. In addition, the downregulation of Cdh1 could also be mediated by inducers. This was confirmed by Snail mRNA analysis. Although DZNep alone showed no effect, cotreatment of DZNep and TGFβ3 caused mRNA upregulation, suggesting DZNep could enhance the TGFβ3 induced Snail transcription, which probably results in the enhanced EMT process (Fig. 3-23A). Moreover, since Snail represses Cdh1 transcription by binding to elements at promoter (Cano, Perez-Moreno et al. 2000), the enhanced Snail can in turn repress the promoter activity of Chd1, as confirmed by luciferase assays (Fig. 3-25). The upregulated Snail can explain why DZNep enhances EMT based on TGFβ3, but still fails to explain why DZNep alone is sufficient to regulate Cdh1 and Acta2. Thus, in addition to Snail, another inducer or regulator was also expected to be the target of DZNep.

The transcriptional regulation of EMT has been so far only focused on inducers like Snail and Twist. The CBF-A/KAP-1/FTS-1 complex was recently identified as a master regulator, which not only activates EMT inducers such as Snail and Twist, but also directly regulates EMT target genes (Teng, Zeisberg et al. 2007; Venkov, Link et al. 2007). Being the transcription product of this complex, FSP-1 is a crucial EMT facilitator and its expression is a marker of early EMT (Venkov, Link et al. 2007).This complex was interesting for further studies because it can regulate the entire EMT process.

TGFβ3 treatment slightly increased mRNA amounts of CBF-A and KAP-1, but decreased FSP1 amount in control cells, indicating TGFβ3 is not sufficient to activate this complex (Fig. 3-23B). In contrast, DZNep alone indeed activated CBF-A/KAP-1/FTS-1 complex. DZNep caused 2.5-fold induction of mRNA amounts of CBF-A and KAP-1, implying that DZNep leads to even more inductive effect on CBF-A and KAP-1 than TGFβ3 does. Most importantly, a 5-fold induction of FSP-1 was achieved by DZNep alone, suggesting the upregulated CBF-A and KAP-1 might in turn result in more binding to FTS-1 element, leading to enhanced FSP-1 transcription. These data revealed that the activated CBF-A/KAP-1/FTS-1 complex, at least in part, contributes to DZNep induced EMT. However, treatment with TGFβ3 abolished the enhanced FSP-1 mRNA by DZNep. This suggests that TGFβ3 might interfere with effect of DZNep on the activation of this master regulator.

Although DZNep activates the CBF-A/KAP-1/FTS-1 complex, the H3K27me3 association at FTS-1 element is not involved in the upregulation of FSP1, since DZNep does not affect the H3K27me3 association at FTS-1 element (Fig.3-24). These results imply that probably the binding of DZNep-induced CBF-A and KAP-1 to FTS-1 element simply enhances FSP1 expression. Furthermore, DZNep might affect some other histone or non-histone proteins, which are essential for the activation of the CBF-A/KAP-1/FTS-1 complex. It might affect, for example associated H4K20 methylations at different regulatory elements (Tan, Yang et al. 2007) or the methylation of non-histone proteins. Furthermore, during the process of this project, one publication was released describing DZNep as a global histone inhibitor (Miranda, Cortez et al. 2009). According to this finding, in addition to H3K27me3, DZNep also inhibits another repressive mark H3K9me2, which is mediated by G9a or Suv39h. Importantly, DZNep even inhibits active histone marks such as H3K4me3. These data indicate that the effect of DZNep on the CBF-A/KAP-1/FTS-1 complex could also result from those modifications on histone or non-histone proteins.

4.2.3 Knockdown of Suz12 enhances EMT by regulating Snail

As mentioned before, the pharmacologic effect of DZNep is still unclear. In order to prove the H3K27me3-dependent effect of DZNep, knockdown of the PRC2 core component Suz12 was performed in NMuMG cells and the effect was compared to that of DZNep. Suz12 knockdown was determined by reduced global level of Suz12 (3-26A). In addition, as a component of PRC2, knockdown of Suz12 led to a decreased global level of H3K27me3. However, the global H3K27me3 level was not totally abolished, probably because the knockdown of Suz12 was not complete, or the knockdown of other subunits of PRC2 such as Ezh2 and Eed were also required.

The effect of Suz12 knockdown was first analyzed by morphological change and marker genes. Unlike DZNep, the typical EMT morphology was only achieved when treated with

TGFβ3 (Fig. 3-26B). Moreover, Suz12 knockdown only reduces Cdh1, but does not regulate the upregulation of Acta2 gene expression (Fig. 3-27). These results demonstrate that Suz12 knockdown also enhances EMT, but the effect is weaker than that observed by DZNep treatment.

Consistent with DZNep, Snail mRNA was also elevated in Suz12 knockdown cells when treated with TGFβ3 (Fig. 3-28A). This indicates that the depletion of H3K27me3, mediated from DZNep or Suz12 knockdown, contributes equally to the TGFβ3 induced Snail mRNA level, and this upregulation probably in turn enhances the EMT process. This finding also suggests that PRC2 involvement in Snail transcription is expected. However, ChIP assay required to prove this hypothesis was not performed because no appropriate primers could be designed. A repressive effect of Suz12 knockdown on Cdh1 promoter activity was also in correlation to DZNep (Fig. 3-29B). But, this repressive effect was weaker than that by DZNep. This is also in agreement with the less Snail induction observed by Suz12 knockdown than DZNep treatment when cells were treated with TGFβ3 (Fig. 3-23A and 3-28A).

Importantly, knockdown of Suz12 downregulated all three genes of the CBF-A/KAP-1/FTS-1 complex, suggesting the Suz12 knockdown did not activate, and even inactivated the master regulator. This is probably mediated via altered mRNA stability by Suz12 knockdown. Most importantly, this finding confirms that the activation of the complex by DZNep is not dependent on H3K27me3, and might in fact results from DZNep's effect on some other histone or non-histone proteins, which are essential for the activation of the CBF-A/KAP-1/FTS-1 complex. Therefore, although both DZNep and Suz12 knockdown similarly showed H3K27me3 depletion and EMT induction, they function differently. DZNep functions by activating the master regulator or with Snail, whereas Suz12 knockdown only enhances TGFβ3 induced Snail expression.

Taken together, results from this part proposed the role of repressive mark H3K27me3 and inhibitor DZNep in regulation of EMT. H3K27me3 regulates EMT at levels of marker genes, Snail, and most probably also the master regulator (Fig. 4-2). Firstly, both epithelial marker Cdh1 and mesenchymal marker Acta2 promoters are associated with H3K27me3 and the associations correlate to gene transcription. However, DZNep does not affect those associations. Secondly, Snail might be a target of H3K27me3, since removal of H3K27me3 by both DZNep and Suz12 knockdown can equally enhance the TGFβ3 induced Snail mRNA. In addition, the repressive effect on Cdh1 promoter activity is confirmed by luciferase assay, and the effects are also in correlation to the enhanced Snail mRNA levels. The role of H3K27me3 on Snail transcription was not confirmed by ChIP assay. Furthermore, the enhancement of the Snail mRNA by DZNep could at least in part result from the positive regulation by the activated CBF-A/KAP-1/FTS-1 complex. DZNep obviously affects some unknown histone or non-histone proteins, which are essential for the activation of the CBF-A/KAP-1/FTS-1 com-

plex, to induce EMT, since the H3K27me3 association at FTS-1 element is not involved in upregulated FSP1. Finally, this complex cannot be activated by TGFβ3.

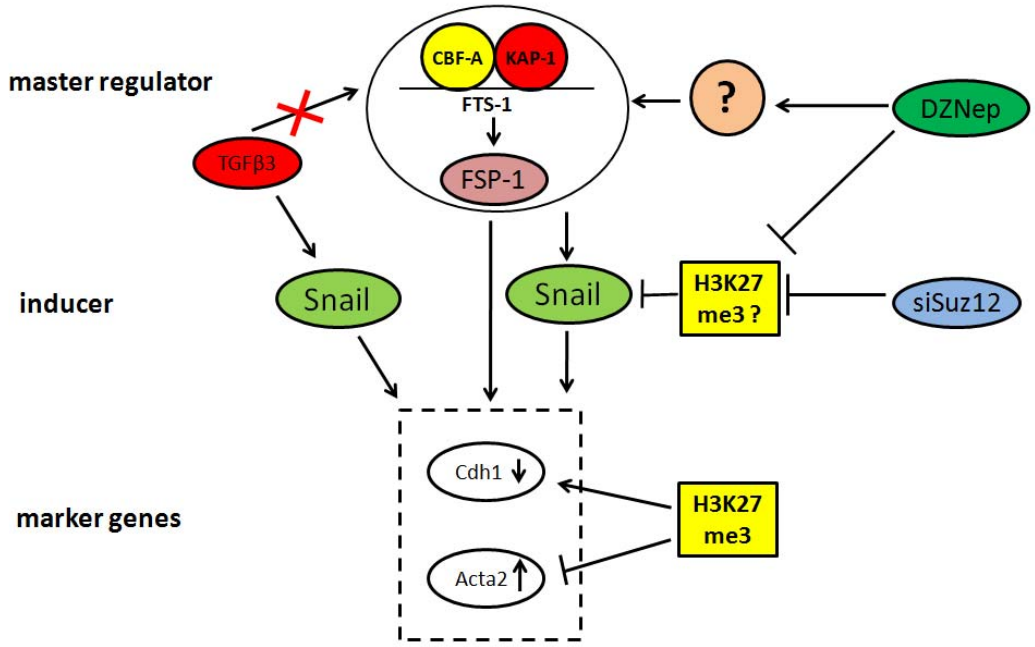

Figure 4-2 The role of H3K27me3 and DZNep in regulating EMT

During EMT, the expressions of marker genes are regulated by inducers such as Snail and the master regulator CBF-A/KAP-1/FTS-1 complex. H3K27me3 regulates EMT at levels of marker genes, Snail, and most probably also the master regulator. DZNep regulates EMT induction via the activation of CBF-A/KAP-1/FTS-1 complex, probably via influencing some histone or non-histone proteins, which are essential for the activation of the CBF-A/KAP-1/FTS-1 complex. TGFβ3 is not sufficient to activate this complex. Snail might be a target of H3K27me3. Moreover, Snail might also be positively regulated by the CBF-A/KAP-1/FTS-1 complex. Although Cdh1 and Acta2 promoters are modified by H3K27me3 and the associations are correlated to transcription, these associations are not involved in DZNep induced EMT.

4.2.4 Potential use of DZNep for clinical purposes

The finding presented here might provide some reservation to use DZNep for new clinical application in cancer treatment. However, it might have positive effect for treatment of other diseases. As an EMT inducer, it can enhance EMT-related physiological events, for example wound healing and tissue regeneration. Moreover, since EMT also contributes to other procedures like formation of cardic valve, palastal fusion, and branching morphogenesis during mammary gland development (Hollier, Evans et al. 2009), those processes might be also accelerated by DZNep.

On the other hand, since DZNep has been considered to be a potential anti-breast cancer drug (Hayden, Johnson et al. 2009), and EMT correlates closely to tumor invasion and metastasis, DZNep might potentially enhance the risk of breast cancer metastasis by inducing EMT in cancer cells. Therefore, reevaluation of DZNep for cancer therapy is required.

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- **Fu X,** Pfitzner E. H3K9me2 correlates to chromatin inaccessibility at beta-casein promoter in mammary epithelial cells.

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- H3K9me2 contributes to chromatin inaccessibility at beta-casein promoter in HC11 mammary epithelial cells. Nov. 2010, Institute of Molecular Medicine and Cell Research, Albert-Ludwigs University Freiburg.
- H3K9me2 contributes to chromatin inaccessibility at beta-casein promoter in HC11 mammary epithelial cells. Nov. 2010, Max Delbrück Center for Molecular Medicine (MDC), Berlin
- Regulation of mammary epithelial-mesenchymal transition by the histone methylation inhibitor DZNep. Nov. 2010, Institute of Experimental Internal Medicine, Otto von Guericke University Magdeburg

Selbstständigkeitserklärung

Hiermit erkläre ich, Xin Fu, geboren am 26.05.1973, das mir die für die Biologisch-Pharmazeutische Fakultät geltende Promotionsordnung bekannt ist. Ich habe die vorliegende Dissertation selbstständig angefertigt und auβer den angegebenen keine Hilfsmittel, persönliche Mitteilungen oder Quellen eingesetzt. Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen. Es wurden von mir keine geldwerten Leistungen erbracht, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Diese Dissertation wurde nur dem Fakultätsrat der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena und keiner anderen Hochschule zur wissenschaftlichen Prüfung oder zur Dissertation eingereicht. Diese Arbeit ist weder identisch noch teilidentisch mit einer Arbeit, welche der Friedrich-Schiller-Universität Jena oder einer anderen Hochschule zur Dissertation vorgelegt worden ist.