EPIGENETIC REGULATION MEDIATED BY SNAIL AND ITS IMPLICATION IN TUMOR METASTASIS

By

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To my parents and my beloved wife for always believing in me

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LIST OF ABBREVIATIONS

4-HT	4-Hydroxyltamoxifen
5’-aza	5’-azacytidine
ChIP	Chromatin Immunoprecipitation
CSC	Cancer Stem Cell
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EMT	Epithelial-Mesenchymal Transition
ER	Estrogen Receptor
FGF	Fibroblast Growth Factor
GST	Glutathione-S-Transferase
HDACi	Histone Deacetylase inhibitor
LSD1	Lysine Specific Demethylase 1
NFκB	Nuclear Factor kappa B
PcG	Polycomb Group
PRC2	Polycomb Repressive Complex 2
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SC	Stem Cell
shRNA	short hairpin RNA
TGFβ	Transforming Growth Factor beta
TSS	Transcription Start Site

Abstract of Dissertation Presented to the Graduate School
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Cellular transitions between epithelial and mesenchymal states have crucial roles in embryonic development and carcinoma progression, yet regulation of the morphological plasticity of cells is not well established. Recent studies identified the members of the Snail family of zinc finger transcription factors as central mediators of EMT and induce EMT in part by directly repressing epithelial markers such as E-cadherin, a gatekeeper of the epithelial phenotype and a suppressor of tumor invasion. However, the molecular mechanism underlying Snail-mediated transcriptional repression remains incompletely understood. The goal of my study is to identify the corepressors associated with Snail and characterize their roles in regulating Snail's function. It was shown here that Snail physically interacts with the histone demethylase LSD1 (KDM1A) via the SNAG domain, and recruits LSD1 to epithelial gene promoters. LSD1 then reduces dimethylation of lysine 4 on histone H3 tails (H3K4m2), a covalent modification associated with active chromatin. I further showed that LSD1 is essential for Snail-mediated transcriptional repression and for maintenance of the silenced state of Snail target genes in invasive cancer cells. In the absence of LSD1, Snail fails to repress its targets. In addition, depletion of LSD1 in mesenchymal-like cancer cells

results in partial de-repression of epithelial genes and elevated H3K4m2 levels at the E-cadherin promoter. These results underline the critical role of LSD1 in Snail-dependent transcriptional repression of epithelial markers and suggest that the LSD1 complex could be a potential therapeutic target for prevention of EMT associated tumor invasion.

CHAPTER 1
INTRODUCTION

Cancer Biology

Cancer is the second most common cause of death in the US, surpassed only by heart disease. The most predominant forms of cancer for male and female are prostate cancer and breast cancer respectively, followed by lung cancer for both genders. Lifetime risk of developing various types of cancer among Americans is about 30% with more than one million new cases diagnosed each year (American Cancer Society Atlanta, 2010). The incidence rates of cancer have been increasing over decades, while mortality has decreased, which is mostly due to advances in early detection and diagnosis.

The Properties of Cancer

The transformation of normal cells to malignant cancer cells is a complex multistep process, characterized by a sequence of genetic and epigenetic alterations. Each of these steps confers one or another form of increased physiological fitness to surrounding environments (Foulds, 1954). Genomic instability is proposed as a fundamental characteristic of cancer cells and enables them to develop multiple specific hallmarks, by activating of oncogenes and inactivating of tumor suppressor genes. Cancer cells can acquire the capability to sustain proliferative signaling, to divide despite growth-inhibitory signals, to be resistant to apoptotic events and to replicate immortally. In addition, malignant cells have the unique ability of inducing angiogenesis to provide for their own blood supply, as well as invading the surrounding tissue and metastasizing to distant organs (Hanahan and Weinberg, 2000). Last but not least, two additional emerging hallmarks of cancer have recently been added to the list:

reprogramming of energy metabolism – favoring glycolysis under aerobic conditions in cancer cells, and evading immune destruction (Hanahan and Weinberg, 2011).

Cells of Origin in Cancer

The origin of cancer cell populations has been much debated in cancer biology field for the past decade, as it has been noticed for a long time that tumor cells show remarkable variability in different kind of aspects even within individual tumors, like cellular morphology, proliferative index, genetic lesions and therapeutic response (Axelson et al., 2005; Heppner, 1984). Is the striking heterogeneity originated from a single cell? Or are there many points of origin to account for the various cell types found inside tumors? Understanding these questions could lead to more effective cancer therapies and prevention methods (Marusyk and Polyak, 2010; Visvader, 2011). Currently, two popular conceptual ideas have been put forward to attempt to describe the establishment and maintenance of tumor heterogeneity, the clonal evolution model and the cancer stem cell hypothesis. Although these two theories share some similarities, they are fundamentally different notions with very different implications (Campbell and Polyak, 2007; Polyak, 2007; Shackleton et al., 2009).

Clonal evolution model

The clonal evolution model was first proposed by Nowell in 1976 (Figure 1-1A). It states that cancer originates from a random single cell that over time has acquired various combinations of mutations, providing it with a selective growth advantage over adjacent normal cells (Nowell, 1976). As the tumor progresses, genetic instability and uncontrolled proliferation allow the production of cells with additional mutations and hence new characteristics. Each of these new alterations may provide additional

reproductive advantages over other cancer cells, such as resistant to apoptosis, and lead to a new clonal expansion. Thus, under favorable conditions, new subpopulations of variant cells are born, and other subpopulations may contract, resulting in tumor heterogeneity (Crespi and Summers, 2005; Merlo et al., 2006).

The succession of clonal expansions in part resembles a scheme of Darwinian evolution. According to this model, most aggressive cells drive tumor progression throughout the lifetime of a tumor. Any cancer cell can potentially become invasive and cause metastasis or become resistant to therapies and cause recurrence.

Cancer stem cell hypothesis

The cancer stem cell (CSC) hypothesis (Figure 1-1B), which has received a great deal of attention recently, states that only a particular subset of tumor cells with stem cell-like properties that have the ability to self-renew and drive tumor initiation, progression and recurrence (Pardal et al., 2003; Reya et al., 2001; Visvader and Lindeman, 2008). According to this model, the self-renewal and differentiation potentials of CSCs, which is analogous to somatic stem cells (SC), lead to the production of all cell types inside a tumor, thereby generating tumor heterogeneity (Polyak and Hahn, 2006; Visvader and Lindeman, 2008). These CSCs may be derived from normal SCs, as they are also long-lived, making them more likely than other cells to acquire the multiple mutations needed to become cancer (Miller et al., 2005). In addition, recent evidences showed that the epithelial-mesenchymal transition, a complex developmental process, can also induce non-CSCs to enter into a CSC-like state (Mani et al., 2008; Morel et al., 2008).

Both of the cancer stem cell model and clonal evolution model suggest tumors originate from a single cell that has acquired multiple mutations and has gained

unlimited proliferative potential. However, in the CSC model, normal stem and progenitor cells are considered the most likely targets of transformation, while no normal cells in particular are identified as such by the clonal evolution model. Another difference between these two models is clonal evolution model supposes that any tumor cell has the potential to expand into a subpopulation and to be involved in tumor progression. Nevertheless, the CSC hypothesis indicates only a small pool of cells, with self-renewal and tumor-initiating capability, can contribute to tumor progression. So, based on the CSC model, efficiently eliminating the highly tumorigenic, stem cell-like subpopulation would render the remaining tumor cells more susceptible to standard chemo- or radio-therapy treatment (Tan et al., 2006; Wicha et al., 2006).

Metastasis and Malignant Tumors

Although epithelial cancers deriving from tissues that include breast, lung, colon, prostate and ovary constitute majority of cancers, metastasis is responsible for as much as 90% of cancer-related deaths. Development of distant metastases from the original cancer is an almost incurable illness, yet the mechanism of it remains the most poorly understood component of cancer pathogenesis (Steeg, 2006).

Tumor metastasis consists of a series of discrete biological processes that move tumor cells from the primary site to distant location. The complex metastatic cascade starts with a subset of tumor cells that acquire the ability to migrate and invade, although it is still unaddressed whether this acquisition of malignant traits occurs as an almost-inevitable consequence when tumor reach certain size or as an accidental product thereof (Chaffer and Weinberg, 2011). These cells alter their morphology as well as attachment to neighboring cells and to the extracellular matrix (ECM), degrade surrounding tissue, eventually liberate themselves from the primary tumor and begin to

migrate on their own. Then the disseminated cells invade adjacent stromal compartments and move toward lymphatics or bloodstream. After intravasation, they enter these vessels, survive both shear forces as well as suspension induced cell death (anoikis), and become circulating tumor cells (CTCs). At the distant organ, CTCs exit circulation by extravasation and invade into the microenvironment of the foreign tissue. Eventually, some of these cells successfully adapt to new environment in distant loci and form macrometastases (Joyce and Pollard, 2009; Nguyen et al., 2009; Pantel and Brakenhoff, 2004).

Although metastasis still remains one of the most enigmatic aspects of the disease, a lot of progress has been made in understanding its mechanisms from the past decade. One breakthrough is the recognition of epithelial-mesenchymal transition (EMT) as a prominent regulatory event in the initiation of invasion and metastasis program (Chiang and Massague, 2008; Klymkowsky and Savagner, 2009; Thiery, 2002; Yang and Weinberg, 2008). Through EMT, transformed epithelial cells can acquire the abilities to invade, to resist apoptosis, and to disseminate. Besides, this multifaceted EMT program can be activated transiently or stably, and to different degrees, by carcinoma cells during the course of invasion and metastasis (Figure 1-2) (Barrallo-Gimeno and Nieto, 2005; Polyak and Weinberg, 2009; Thiery, 2009).

Epithelial-Mesenchymal Transition

Overview of EMT

Epithelial and mesenchymal cells exhibit distinct phenotypic and functional characteristics (Thiery and Sleeman, 2006). Epithelial cells establish close contacts with each other and have apical basal polarity through the sequential arrangement of tight junctions, adherens junctions and desmosomes. Mesenchymal cells typically do not

establish stable cell-cell contacts. However, the two cell types are interconvertable under certain circumstances. Epithelial cells can be reprogrammed into mesenchymal cells through a process known as epithelial-mesenchymal transition (EMT) (Hay, 1995). During EMT, epithelial cells downregulate epithelial markers, lose their cell-cell adhesion structures, modulate their polarity and rearrange their cytoskeleton, and concomitantly acquire enhanced migratory and invasive properties. Besides, EMT is not permanent but often reversible. The reverse process is termed mesenchymal-epithelial transition (Tinelli et al., 2009). The interconversion between epithelial and mesenchymal states underscores the enormous phenotypic plasticity of certain embryonic and adult cells, which is believed to be pivotal to many biological processes, such as embryonic morphogenesis, wound healing, organ fibrosis as well as cancer progression (Thiery et al., 2009).

EMT in Development

The phenomenon of EMT was first identified in the studies of the formation of chicken primitive streak and gradually found crucial for embryogenesis (Hay, 1968; Trelstad et al., 1967). The development of metazoan organ systems starts with a single layer of epithelial cells. And the earliest example of an EMT program participating in embryogenesis is the formation of mesoderm. During gastrulation, a small population of epithelial cells at the primitive streak undergoes dramatic morphological changes, loses their epithelial cell-cell contacts, transforms into migratory mesenchymal cells. Subsequently, these cells ingress through the primitive streak, migrate along the narrow extracellular space underneath the ectoderm to form the new mesoderm (Viebahn, 1995).

Besides mesoderm formation, neural crest delamination (Figure 1-3) represents another prototypic developmental EMT event (Yang and Weinberg, 2008). The neural crest is composed of a transient population of stem cell-like progenitors that distinguishes the vertebrates from other metazoans. After gastrulation in vertebrates, the neural plate and the epidermal ectoderm are progressively defined along the rostrocaudal axis, and the neural crest develops at the boundary between these two territories (Kulesa and Gammill, 2010). Through EMT, these multipotent neural crest cells emigrate extensively from the dorsal neural epithelium to sites throughout the embryo where they give rise to a diverse array of derivatives that include craniofacial skeleton, most of the peripheral nervous system, melanocytes, as well as some endocrine cells (Dupin et al., 2006; Taylor and Labonne, 2007).

EMT and Tumor Progression

It took a long time for EMT to be recognized as a potential mechanism for carcinoma progression, although even now, not everyone is convinced about the relevance of this transition in cancer progression. This controversy in part is due to the absence of direct clinical evidence of capturing EMT process in human cancer patients. Besides, clinically, the majority of human metastases resemble primary carcinomas morphologically and retain characteristics of well-differentiated epithelial cells, which raises the question whether EMT indeed occurred during the progression of tumors (Thiery, 2002).

Nevertheless, over the past decade, lines of evidence have emerged in understanding the role of the EMT in enabling metastatic dissemination. It is believed that by activating EMT program carcinoma cells can concomitantly acquire several aspects of malignancy associated properties. The most important one is the enhanced

migratory and invasiveness for the initiation of metastasis. Currently, complete or partial EMT-like processes are documented in breast (Trimboli et al., 2008), ovarian (Vergara et al., 2010), colon (Brabletz et al., 2005), and esophageal (Usami et al., 2008) cancer models. Epithelial cancer cells obtain higher mobility and disseminate from each other by transforming to mesenchymal-like phenotype *in vitro* (Brown et al., 2004; Klymkowsky and Savagner, 2009; Shin et al., 2010). More importantly, increasing number of reports shows that EMT occurs *in vivo* as well while carcinoma progresses. EMT *in vivo* is frequently described as a portion of tumor cells that express low levels of epithelial markers such as E-cadherin or ZO-1, and high levels of mesenchymal markers such as vimentin or fibronectin. In breast cancer, EMT was observed at the margins of cancer cell groups of up to 20% of tumors (Dandachi et al., 2001). Similarly, a colon carcinoma study showed the presence of E-cadherin negative cancer cells at the tumor invasive front, that selectively lost the basement membrane and were invading the surrounding stroma (Brabletz et al., 2001). One technical difficulty in studying EMT *in vivo* is to distinguish mesenchymal cells derived from epithelial tumor cells after EMT from stromal cells or other tumor-associated fibroblasts. To circumvent this problem, cytogenetic analysis was applied in some studies to confirm both the mesenchymal and epithelial compartments were originated from the same precursor cell population (Halachmi et al., 2007).The description of tumor cells that detach from the tumor mass into the adjacent stroma has recently provided morphological evidence of EMT at invasive front of human tumors (Prall, 2007). Besides, direct *in vivo* imaging has also yielded evidence of EMT in cancer progression (Wyckoff et al., 2007).

Besides the modification of the phenotype, EMT could also endow tumor cells with higher resistance to cell death and chemotherapy which is critical for these cells to survive in blood vessels upon detachment and intravasation. TGFβ can prevent tumor progression by directing cells to apoptosis, however it will also promote EMT within certain contexts (Massague, 2008). Interestingly, cells exhibiting a sustained EMT can escape apoptosis after exposure to TGFβ for several weeks (Gal et al., 2008). Members of the Snail family are known to confer resistance to cell death by antagonizing the p53 pathway (Barrallo-Gimeno, 2005; Kurrey et al., 2009b; Wu et al., 2005b). This prosurvival activity can be extended to Twist, as it antagonizes the Myc-mediated proapoptotic effect in neuroblastoma (Puisieux et al., 2006). Similarly, tumors undergoing EMT may resist conventional chemotherapy. For example, colon carcinoma epithelial cell lines made resistant to oxaliplatin exhibit a mesenchymal morphology and express several markers of EMT (Yang et al., 2006a). Both Snail and Twist expressions are found to be associated with resistance to paclitaxel treatment (Kurrey et al., 2009b; Yu et al., 2009a; Yu et al., 2009b). Interestingly, forced overexpression of miR-200c, a negative regulator of EMT, restores chemotherapeutic sensitivity (Cochrane et al., 2010).

Recently, emerging evidence indicates that expression of multiple inducers of EMT in breast cancer cell lines increases the tumor initiating cell population as determined by mammosphere formation and cell surface markers (Mani et al., 2008; Morel et al., 2008). This induced cancer stem cell-like property with self-renewal ability, is likely a critical feature required for ultimate colonization at distant metastatic sites.

Overall, these lines of evidence suggest EMT induces a comprehensive program of properties that are necessary for tumor progression. First, EMT empowers epithelial tumor cells to disseminate from primary tumors and invade into neighboring tissues. Moreover, the heightened resistance to apoptosis that is generated by EMT is critical for circulating tumor cells to survive the voyage to seed in distant sites. Finally, the CSC-like state endows these seeded cells to colonize and form macrometastases.

Regulation of Epithelial-Mesenchymal Transition

Epithelial Junctions and E-cadherin (CDH1)

Epithelial tissues are formed by a single layer of tightly packed, polarized cells that are separated from adjacent tissues by a basal lamina. The structural integrity of epithelium depends upon the establishment and maintenance of stable epithelial junctions. These junctions consist of distinct protein complexes and provide contact with neighboring cells and the extracellular matrix (ECM) (Tyler, 2003). There are four major types of cell junctions in vertebrates serving different functions within the epithelium: tight junctions, adherens junctions, desmosomal junctions and gap junctions (Figure 1-6). The tight junctions contain claudins and occludins, and function as a barrier to prevent passage of particles or solutes across the epithelial layer. The pannexin/ connexin based gap junctions allow epithelial cells to communicate through the direct passage of small molecules between neighboring cells (Shestopalov and Panchin, 2008). The desmosomes protect epithelial cells against shearing forces and contain two nonclassic cadherins: desmocollins and desmogleins. They share conserved extracellular cadherin (EC) domains with classic cadherins but have divergent cytoplasmic structures (Delva et al., 2009; Desai et al., 2009).

The adherens junction is another critical element of the cell-cell junctions. A major function of adherens junctions is to physically tether adjacent cells to one another, as disruption of them causes loosening of cell-cell contacts. Adherens junctions are composed of classical cadherins which comprise approximately 20 members and share a common domain organization (Hulpiau and van Roy, 2009).

E- (epithelial) cadherin has a typical structure as a classical type I cadherin, which is mainly localized at adherens junctions (Nollet et al., 2000). The mature E-cadherin is composed of an extracellular domain that consists of five tandemly repeated cadherin-motif subdomains (EC domains), a single-pass transmembrane domain and a highly conserved carboxyl-terminal cytodomain (Shapiro et al., 1995). The extracellular domain connects two E-cadherin molecules by a calcium-dependent homophilic interaction. And the cytoplasmic domain binds to beta-catenin complex, which is linked to the actin cytoskeleton (Niessen, 2007). Normal E-cadherin expression and function are essential for the induction and maintenance of epithelial morphology (Takeichi, 1991). E-cadherin has also been considered a suppressor of tumor progression (Berx and Roy, 2001; Perl et al., 1998). In breast cancer, loss of E-cadherin correlates with enhanced invasiveness, metastatic potential and poor prognoses (Heimann et al., 2000; Hunt et al., 1997; Siitonen et al., 1996). Aberrant expression of E-cadherin has been frequently observed at the invasive front of human cancers by immunohistochemistry (Wijnhoven et al., 2000). E-cadherin has also been found involved in cell-cell contact inhibition of cell growth by inducing cell cycle arrest (St Croix et al., 1998). Loss of contact inhibition of proliferation allows tumor cells to escape from growth control signals.

EMT Inducing Signals

Progress has been made in understanding the complex mechanism governing EMT. A number of distinct signaling pathways have been unraveled that are common to EMTs in both development and tumor progression, including TGFβ superfamily, Wnts, Notch, NFκB, Tyrosine Kinase Receptors, and many others (Thiery and Sleeman, 2006; Yang and Weinberg, 2008). And vast majority of these signaling pathways known to trigger EMT converge at the induction of the E-cadherin repressors.

The TGFβ superfamily

The transforming growth factor-β (TGFβ) superfamily consists of related multifunctional cytokines, which include TGFβs, activins, and bone morphogenetic proteins (BMPs) (Piek et al., 1999a). Members of the TGFβ superfamily have been well established as potent inducers of EMT. In early stages of development, mesoderm formation, an EMT related event as we previously discussed, is initiated mainly by members of the Nodal subfamily of TGFβ as demonstrated in both *Xenopus* and *Zebrafish* embryos (Kimelman, 2006). Similarly, another TGFβ superfamily member, BMP, is required for neural crest induction (Raible, 2006). In murine NMuMG mammary epithelial cells, TGFβ promotes strong EMT through type I and type II receptor complex and activation of Smads (Brown et al., 2003; Piek et al., 1999b). In cultured canine kidney epithelial MDCK cells, activation of the TGFβ/Smad pathway has been shown to coordinate with Ras activation to promote a full EMT phenotype (Grunert et al., 2003). Similar phenomena were also observed in another mouse mammary epithelial cell line EpH4 (Eger et al., 2004; Janda et al., 2002). Despite the large number of different EMT models regulated by TGFβ, the exact molecular mechanism of this regulation remains

unclear. Some studies suggest TGFβ drive EMT may through transcriptionally upregulation of Snail and SIP1/ZEB2, two negative regulators of E-cadherin (Comijn et al., 2001; Peinado et al., 2003b).

Wnt signaling

The canonical Wnt pathway is implicated in the initiation and maintenance of mesoderm formation. For instantce, Wnt8 is required for the formation of dorsal mesoderm in *Xenopus* and *Zebrafish* (Kelly et al., 1995; Smith and Harland, 1991; Sokol et al., 1991). And, in *Xenopus*, depletion of β-catenin results in failure to form neural crest (Wu et al., 2005a). In avian embryos, Wnt is also necessary and sufficient to induce neural crest cells (Garcia-Castro et al., 2002). In addition, accumulating evidence indicates that hyperactivity of canonical Wnt pathway associated with breast cancer progression by triggering EMT-like programs (Ayyanan et al., 2006; Li et al., 2003; Reya and Clevers, 2005). β-catenin-TCF complex activates EMT in human breast cancer cells in an Axin2 dependent manner by stabilizing Snai1 protein (Yook et al., 2006). A colon cancer study showed platelet-derived growth factor (PDGF) stimulates EMT through the nuclear translocation of β-catenin in a Wnt-independent manner (Yang et al., 2006b).

The Notch pathway

Notch signaling regulates cranial neural crest cells indirectly by inducing the expression of BMPs (Cornell and Eisen, 2005). Overexpression of activated Notch1 induces EMT in immortalized endothelial cells (Grego-Bessa et al., 2004; Timmerman et al., 2004). Furthermore, both Snail and Slug are proposed as Notch targets in several reports (High et al., 2007; Leong et al., 2007; Niessen et al., 2008; Timmerman et al.,

2004). But unlike the TGFβ and Wnt pathways, activation of the Notch signaling pathway is not sufficient to promote EMT. Instead, Notch signaling in many cases needs to be coordinated with additional signaling inputs in order to induce an EMT in development and tumor progression.

The NF-κB pathway

The NF-κB pathway is activated in a range of human cancers and has been implicated in modulating the EMT program, through the induction of Snail transcription and protein stabilization (Julien et al., 2007; Strippoli et al., 2008). Inhibition of NF-κB blocks EMT, and moreover, abrogates the metastatic potential of mammary epithelial cells in a mouse model system (Huber et al., 2004). NF-κB also required for the insulin growth factor receptor (IGFR) pathway-induced EMT by indirectly upregulating ZEB1 in prostate carcinoma cells (Graham et al., 2008).

The tyrosine kinase receptors signal

Signals from the tyrosine kinase receptors are also emerging as important regulators of EMT. For example, FGF controls the EMT and morphogenesis of mesoderm at the primitive streak (Ciruna and Rossant, 2001). Depletion of *HGF* or *Met* genes results in the complete absence of muscle groups that derive from migratory precursor cells during mouse hypaxial skeletal muscle development (Dietrich et al., 1999). Besides, epidermal growth factor (EGF) is known to induce both Snail and Twist expression (Lee et al., 2008; Lo et al., 2007), and also promote E-cadherin endocytosis (Lu et al., 2003). Interestingly, vascular endothelial growth factor (VEGF) forms a regulatory loop with Snail, orchestrating angiogenesis and EMT, two major events in tumor progression (Peinado et al., 2004b; Wanami et al., 2008).

The Snail Family of Transcriptional Repressors

We just discussed numerous EMT-inducing signals. In response to these contextual signals, cells activate expression of certain transcription factors that execute the EMT process, and most of these factors are transcriptional repressors of the E-cadherin gene. Snail/Slug, ZEB1/2, KLF8, and E47 factors bind to and repress the activity of the E-cadherin promoter, whereas factors such as Twist, Goosecoid, FoxC2, and 14-3-3ξ repress E-cadherin indirectly (Thiery et al., 2009). Among all these transcription factors, the Snail family transcriptional repressors, especially Snail, are the most widely characterized effectors of EMT and CDH1 expression.

All members in the Snail family are zinc-finger transcription factors. The first member, dSnail, was described in *Drosophila melanogaster,* where it was shown to be essential for the formation of the mesoderm (Leptin, 1991). Subsequently, dSnail homologues have been found in many species including humans (Paznekas et al., 1999). In vertebrates, there are three members of the Snail family have been indentified to date: Snail (Snai1), Slug (Snai2), and Smuc (Snai3) (Nieto, 2005).

Structure of mammalian Snail and Slug

The basic structures of two major mammalian members of the Snail family, Snail and Slug, are illustrated in Figure 1-5. They share a highly conserved carboxyl-terminus with multiple C2H2 type zinc fingers that function as sequence-specific DNA-binding motif. The consensus binding site for Snail proteins contains a core of six bases, CAGGTG (Batlle et al., 2000a; Cano et al., 2000b; Inukai et al., 1999), which is identical to the so-called E-box, a binding site for basic helix-loop-helix (bHLH) transcription factors. On binding to DNA, Snail proteins are thought to act as transcriptional repressors (Hemavathy et al., 2000a) and their repressor capacity is largely dependent

on the SNAG (Snail/Gfi) domain (Grimes et al., 1996b; Peinado et al., 2004a), which is located at the extremely N-terminal end. The SNAG domain is constituted by the first 20 amino acids of Snail and is required for the binding of corepressors such as Sin3A/HDAC and PRC2 complex (Herranz et al., 2008; Peinado et al., 2004a).The central region of the Snail proteins is serine-proline rich and highly divergent. Slug protein contains the so-called slug domain in this region, but its function remains elusive. In contrast, two functionally different domains have been identified in the central region in Snail: the destruction box domain (Zhou et al., 2004) and the Nuclear Export Signal (NES) domain (Dominguez et al., 2003a). Phosphorylation of serine/proline residues at these domains controls Snail protein stability, subcellular localization and repressor activity.

The role of Snail in development and cancer

Members of the Snail family have been shown to be essential for various important developmental processes, including mesoderm formation, neural differentiation, cell fate and survival decisions, and left-right identities (Hemavathy et al., 2000b). *Snail*-deficient embryos fail to gastrulate, and mesodermal cells are unable to downregulate E-cadherin accumulated at the streak (Carver et al.,2001; Nieto et al., 1994). Snail also has a fundamental role in EMT and metastasis. Aberrant expression of Snail or Slug contributes to the onset of an invasive phenotype in a wide variety of human cancers (Peinado et al., 2007b). Expression of Snail correlates with high tumor grade and nodal metastasis, and is a prognostic marker for breast cancer patients (Blanco et al., 2002; Cheng et al., 2001; Martin et al., 2005). Snail is also associated with tumor recurrence. In a conditional transgenic mouse model for the recurrence of

HER2/neu-induced mammary tumors, the recurrent mammary shows spontaneous upregulation of Snail with an EMT phenotype (Moody et al., 2005).

Recent functional studies reveal E-cadherin as a major target for Snail (Batlle et al., 2000b; Cano et al., 2000a) and Slug (Bolos et al., 2003). There are three consecutive E-boxes at the proximal promoter region of human E-cadherin, and Snail or Slug can directly bind to this region and repress E-cadherin expression. A comparative binding analysis for these E-box elements at the E-cadherin promoter showed that Snail binds with a higher affinity than Slug and E47 (Bolos et al., 2003). More importantly, overexpression of Snail in epithelial cells increases invasiveness in vitro, and coincides with the down-regulation of E-cadherin expression (Batlle et al., 2000b; Cano et al., 2000a). Snail knockout mice died at gastrulation owing to the defects of EMT with sustained expression of E-cadherin (Carver et al., 2001).

In addition to E-cadherin, Snail also prevents the expression of various epithelium-specific genes, such as Occludin and Claudins (Ikenouchi et al., 2003), Cytokeratins (De Craene et al., 2005a), and Mucin1 (Guaita et al., 2002). Given the central role of Snail in EMT, some reports showed Snail not only represses epithelial genes but also stimulates mesenchymal gene transcription, although the mechanism by which Snail functions as an activator is not clear. It has been proposed that the activator effects of Snail are dependent on the repression of epithelial genes (Solanas et al., 2008). Besides, in certain conditions, Snail might work as a direct activator. For example, it has been found that Snail interacts with β-catenin in the nucleus promoting transcriptional activation of Wnt target genes (Stemmer et al., 2008).

In addition to regulating EMT, Snail and Slug can function as anti-apoptotic factors in at least some cellular context. Snail confers resistance to serum depletion-induced and TNF-a-induced cell death in MDCK. In chick and mouse embryos, the expression of Snail genes is inversely correlated with cell death in different developing tissues (Vega et al., 2004). Snail is associated with the inhibition of PTEN phosphatase, a p53 target, and prevents gamma radiation-induced apoptosis (Escriva et al., 2008). The other member, Slug, is also known for antagonizing p53-mediated apoptosis by binding to p53 downstream targets such as puma (Kurrey et al., 2009a; Wu et al., 2005b). Therefore by promoting resistance to apoptosis, Snail family genes provide tumor cells an advantage to invade, migrate to distant tissues, and form metastasis (Barrallo-Gimeno, 2005). Besides, Snail controls bone mass by repressing the transcription of Runx2 and vitamin D receptor (VDR) genes during osteoblast differentiation (Frutos et al., 2009).

Regulation of Snail family function

Numerous lines of evidence have shown that EMT-inducing signaling cascades execute their effect by inducing the expression Snail family transcription repressors (De Craene et al., 2005b). However, there is still limited information available about the factors directly controlling Snail promoter. Comparative analysis of the Snail and Slug promoters reveals the presence of some interesting elements, such as AP1/AP4 sites, SMAD binding sites, LEF1 binding elements and E-boxes (Peinado et al., 2007b). For example, the LEF/beta-catenin complex is a downstream effecter of Wnt signaling, and the presence of a functional LEF/beta-catenin-binding site at xSnail/xSlug promoter has been characterized *in vitro* by electrophoretic mobility shift assay and *in vivo* by deletion studies (Vallin et al., 2001). MyoD, a myogenic regulatory factor can induce the mSlug

promoter, in which the binding was demonstrated with ChIP assays. Moreover, mSlug-deficient mice shows defective muscle regeneration (Zhao et al., 2002). In TGFβ induced EMT, Smad3/4 form complex with the high mobility group A2 (HMGA2). These two cooperatively bind to the Snail promoter and activate Snail transcription (Thuault et al., 2008). Another example is HGF targets the early growth response 1 (Egr1) protein to the Snail promoter and activates its expression in a MAPK1-dependent manner (Grotegut et al., 2006). More recently, ncRNA-a7, a long non-coding RNA with enhancer-like function, is also identified as a cis-element activator of Snail. Depletion of ncRNA-a7 reduces Snail level as well as the migratory ability of A549 cells (Ørom et al., 2010).

Besides transcriptional activation, Snail is also negatively regulated at the transcriptional level. The most interesting example is that Snail can bind to the E-boxes located within its own promoter and repress its own expression, creating a feedback loop. This self-inhibitory effect provides cells with the capability of buffering and ensures a precise control of Snail activity which could be critical during embryonic development (Peiro, 2006). It has been shown that Snail is also negatively regulated by the estrogen receptor (ER) in breast cancer. ER activates transcription of MTA3 in response to estrogen signaling, which in turn recruits the Mi-2/NuRD corepressor complex to Snail promoter regulating Snail by chromatin modifications. The absence of ER or MTA3 leads to aberrant expression of Snail and increased invasive growth of breast cancers (Fujita et al., 2003).

Snail is an unstable protein with a half-life from 20 to 44 minutes. The function of Snail is also regulated at the protein stability and localization levels through the phos-

phorylation of a central portion of Snail protein (Dominguez et al., 2003b). For example, two phosphorylation motifs of GSK-3β have been identified within Snail. Phosphorylation of Snail on Ser 104 and 107 induces its nuclear export. Subsequent phosphorylation on Ser 96 and 100 by GSK-3β facilitates the association of Snail with β-Trcp1 and thus leads to the ubiquitination and degradation of Snail (Zhou et al., 2004). Different from β-Trcp1 that requires the previous phosphorylation of Snail, FBXL14, another E3 ubiquitin ligase, interacts with Snail independently of phosphorylation and promotes its ubiquitination and proteosomal degradation (Vinas-Castells et al., 2009). Curiously, both ubiquitin ligases act through the modification of Lys 138 and 146 of Snail.

Epigenetics and Histone Modifications

Introduction to Epigenetics

The term “epigenetics” was originally coined by the developmental biologist Conrad Waddington in 1940s. To him, epigenetics is “the interactions of genes with their environment, which bring the phenotype into being” (Waddington, 1957). Since then, the concept of epigenetics has evolved dramatically and extensively. Currently, epigenetics is more specifically referred as the study of any stable or heritable changes in phenotype or gene expression independent of changes in underlying DNA sequence (Goldberg et al., 2007). Much of today’s research in epigenetics falls into the study of DNA methylation and histone modifications and the mechanisms by which such changes influence overall chromatin structure and gene expression.

DNA Methylation

DNA methylation in vertebrates occurs almost exclusively at the cytosine within CpG dinucleotides, and most CpGs in the genome are methylated (Bird, 2002; Goll and Bestor, 2005). However, surprisingly, a recent genome-wide DNA methylation profiling identified nearly one-quarter of methylation occurs in non-CpG contexts in embryonic stem cells. And this non-CpG methylation disappears upon differentiation (Lister et al., 2009). CpGs tend to cluster in blocks, termed CpG islands, which are found in 60% of the proximal promoters of the human genes (Strichman-Almashanu et al., 2002; Takai and Jones, 2002). DNA methylation of these islands correlates with transcriptional silencing. The methylation of mammalian genomic DNA is catalyzed by DNA methyltransferases (DNMTs) that can be divided into maintenance and de novo DNMTs (Siedlecki and Zielenkiewicz, 2006; Turkek-Plewa and Jagodzinski, 2005). The presence of DNA methylation has been implicated in various cellular processes, including genomic imprinting, X chromosome inactivation, chromatin condensation, tissue-specific gene expression and cell differentiation (Bird, 2002). DNA methylation is also the most extensively studied epigenetic phenomenon in cancer development. Carcinogenesis can result from aberrations of genomic methylation status of tumor suppressor genes or protooncogenes (El-Osta, 2003; Luczak and Jagodzinski, 2006). For example, DNA hypermethylation at promoter region of CDH1 or BRCA1 gene is frequently observed in different type of tumors (Birgisdottir et al., 2006; Grady et al., 2000).

Histone Modifications

In eukaryotic cells, the nucleosome is the basic structural unit of chromatin. Each nucleosome is composed of 146 base pair of double-stranded DNA wrapped around an

octamer of core histones. The core histones are a group of small, highly conserved, basic proteins and consist of H2A, H2B, H3 and H4 (Kornberg and Lorch, 1999). The N-terminal tails of these histones are accessible to other nuclear proteins and subject to multiple covalent modifications, including methylation, acetylation, phosphorylation and ubiquitination, which are involved in regulation of transcription, DNA repair, genome replication, and chromatin condensation (Jenuwein and Allis, 2001; Strahl and Allis, 2000). For each of these covalent histone modifications, there are enzymes responsible for the dynamic activities that either add or remove the particular chemical residues. The discovery of these enzymes, their substrate specificities and biological significance are of major interest in the field of epigenetics (Kouzarides, 2007).

Acetylation

The acetylation of histones has been known for over forty years and has been correlated to active gene transcription in numerous studies (Clayton et al., 2006). Acetylation generally occurs on the lysine residues at the N-terminal tails of histone H3 and H4 (6 residues on H3 and 5 residues on H4). Several families of enzymes have been identified responsible for writing or erasing of this modification, called histone acetyltransferases (HATs) and histone de-acetylases (HDACs) respectively. HATs have been found as transcriptional coactivators, including GCN5, PCAF, CBP, p300, Tip60 (Yang, 2004), whereas HDACs have been identified as transcriptional corepressors (Sterner and Berger, 2000). Recently, a genome-wide mapping of HATs and HDACs from Keji Zhao's group reveals both are found at active genes with acetylated histones. This suggests HDACs may also play a role at active promoters by controlling acetylation levels and resetting chromatin after transcription (Wang et al., 2009b).

Phosphorylation

Phosphorylation is another well established post-translational histone modification. Phosphates can be added to both serine and threonine residues in each of the core histones and H1 (Nowak and Corces, 2004). Numerous kinases have been identified that are responsible for mediation of this modification. One of great interests in early reports is the phosphorylation of Ser 10 at Histone H3 tail, which has been linked to chromosome condensation and segregation during mitosis and meiosis (Gurley et al., 1978). Members of the aurora kinase family are known to govern this Ser 10 phosphorylation in several organisms (De Souza et al., 2000; Giet and Glover, 2001). Histone phosphorylation also shows a possible role in the induction of transcriptional induction of early-response genes such as c-fos and c-jun, following stimulation of cell proliferation. They showed a conversion of MAP kinase pathways on the aurora B family members MSK1 and MSK2 to induce Ser 10 phosphorylation (Mahadevan et al., 1991). However, the role of histone phosphorylation in gene activation is unclear.

Phosphorylation mediated by histone kinases is counter-balanced by the activity of protein phosphatase. Regulation of the level of histone phosphorylation is carried out via interplay between these two groups of enzymes. Type 1 protein phosphatase (PP1) is responsible for removing the phosphates from Ser 10 of H3 associated with mitosis (Nowak and Corces, 2004). Moreover, evidence showed protein phosphatase type 2A (PP2A) activity is required for dephosphorylating histones involved in transcription regulation (Nowak et al., 2003).

Methylation

Histone methylation has received great attention since the last decade, due to its complexity and biological importance. The methylation can take place on the ε-amino

group of lysine residues (Murray, 1964) and the η-guanidino group of arginine residues on histone tails (Paik and Kim, 1969). Histones H3 and H4 are the primary targets of methylation. Methylations on residues including H3K4, -K9, -K14, -K27, -K36, -K79, -R2, -R17, -R26 as well as H4K20, -K59, -R3 have been studied extensively and linked to chromatin and transcriptional regulation as well as DNA damage response. Lysine methylation can occur at varying degrees either mono-, di-, or tri-methylation; while arginine can be either mono- or di-methylated. The dimethylation of arginine also happens in either symmetric or asymmetric configuration (Margueron et al., 2005; Martin and Zhang, 2005). Of these modifications, methylation at H3K4, H3K36 and H3K79 are associated with active promoters, while methylation at H3K9 and H3K27 are associated with silenced promoters (Zhang and Reinberg, 2001).

The first enzyme with histone methyltransferase activity was identified in the year 2000, which is almost over thirty years later than the first discovery of histone lysine methylation. Thomas Jenuwein's group reported Suv39h1, the human orthologue of Drosophila Su(var)3-9, as a histone lysine methyltransferase with subject specificity towards lysine 9 on histone H3 (Rea et al., 2000). In this study, they also mapped the catalytic motif to the evolutionarily conserved SET domain, which led to the discovery of numerous SET domain containing histone lysine methyltransferases such as Ezh2, MLLs, Nsd1 (Schneider et al., 2002). Each of the SET containing HMTs uses S-adenosyl-L-methionine (SAM) as the methyl group donor. Dot1 is the only identified non-SET containing histone lysine methyltransferase and catalyzes methylation of K79 on histone H3 (van Leeuwen et al., 2002).

Despite remarkable advances made in uncovering enzymes responsible for histone methylation, the biological relevance of these markers ultimately depends on the recruitment of downstream effectors that read these covalent signals and in turn execute specific independent functions on the chromatin template. So far, various histone methyl binding proteins have been identified, many of which belong to distinct protein complexes. For example, BPTF is a component of the NURF complex that is involved in ATP-dependent chromatin remodeling. BPTF binds to tri-methylated H3K4 through its PHD finger and facilitates transcriptional activation by increasing the promoter accessibility (Mizuguchi et al., 1997). Transcription factor TFIID can also directly binds to the H3K4m3 mark via the PHD finger of TAF3 and regulate RNA polymerase II activity at target promoters (Vermeulen et al., 2007). Heterochromatin protein 1 (HP1) is another well-studied histone methyl binding protein. It recognizes both di- and tri-methylated H3K9, the heterochromatin markers, and recruits DNA methyltransferases and Su(var)3-9 to the chromatin template. These enzymes put more repressive markers on neighboring nucleosomes to propagate a heterochromatin epigenetic signature (Bannister et al., 2001; Nielsen et al., 2001).

Unlike histone acetylation and phosphorylation, histone methylation was long considered static and enzymatically irreversible. It was proposed that histone methylation was erased by either passive dilution during replication, replacement of histone subunits, or proteolytic cleavage of modified tails (Ahmad and Henikoff, 2002). The view was changed in 2004, with the discovery of lysine specific demethylase 1 (LSD1) in a study of CtBP corepressor complex (Shi et al., 2004). Immediately after that, numerous families of Jmjc-domain-containing histone lysine demethylases were

also uncovered, demonstrating this modification is dynamically regulated (Shi and Whetstine, 2007).

Lysine Specific Demethylase 1 (LSD1),

LSD1, also named KDM1A, is the first identified lysine demethylase and belongs to the monoamine oxidase superfamily of flavin adenine dinucleotide (FAD)-dependent enzymes. LSD1 contains an N-terminal SWIRM domain commonly found in chromatin-associated proteins. The catalytic activity resides in the carboxyl-terminal amine oxidase-like AOL domain that contains two subdomains: a FAD binding subdomain and a substrate binding subdomain. The central region of LSD1 is the protruding TOWER domain which forms a surface for binding of other partner proteins such as CoREST (Anand and Marmorstein, 2007; Chen et al., 2006; Yang et al., 2006c).

LSD1 mediated demethylation is constrained on converting mono- or di-methylated lys 4 of H3 to unmethylated status, rather than tri-methylated lys 4 of H3. The methylated H3K4 substrate, an amine form, is first oxidized to form an imine intermediated, which is then hydrolyzed to form the formaldehyde and lysine. Two successive rounds of this reaction are required to generate unmodified lysine from its dimethyl form. Based on this chemical characteristic, this reaction requires at least one hydrogen on the amine substrate, which further confirms the inability of LSD1 to act on tri-methylated H3K4 (Shi et al., 2004; Stavropoulos et al., 2006).

LSD1 generally acts as a transcriptional repressor by removing H3K4 dimethylation, which is an active chromatin marker. It was first identified in a study of the CtBP corepressor complex (Shi et al., 2003) and then found in several different protein complexes involved in transcriptional regulation and mediating distinct biological functions. Shi et al. showed LSD1 is associated with the CoREST/HDAC complex, and

further studies indicated CoREST stabilizes LSD1 and facilitates its demethylase activity on native nucleosomes (Lee et al., 2005; Shi et al., 2005). A recent report demonstrated LSD1 is an integral component of the NuRD nucleosome remodeling complex and helps regulate TGFβ signaling pathway. Expression of LSD1 inhibits the invasion of breast cancer cells *in vitro* and suppresses breast cancer metastatic potential *in vivo* (Wang et al., 2009a). Furthermore, LSD1 also forms a complex with SIRT1, an NAD^+-dependent histone deacetylase, and coordinately represses genes regulated by the Notch signaling pathway (Mulligan et al., 2011).

A number of DNA-binding transcription factors have been implicated in recruiting LSD1 to specific genomic locations. For example, Gfi proteins are key transcriptional repressors regulating hematopoiesis. It has been showed that Gfi-1/1b recruits LSD1/CoREST complex to majority of target gene promoters in a lineage-specific pattern during hematopoietic differentiation (Saleque et al., 2007). Another report showed LSD1 interacts with TLX and co-regulates its target genes. TLX is an orphan nuclear receptor and regulates neural stem cell maintenance and self-renewal in both embryonic and adult brains. Inhibition of LSD1 activity leads to reduced neural stem cell proliferation (Sun et al., 2010).

Epigenetic Therapy for Cancer

Over the past two decades, more and more aberrant epigenetic alterations have been linked to cancer progression. This was first evidenced by global changes in DNA methylation. Cancer cells show genome-wide hypomethylation and site-specific CpG island promoter hypermethylation, especially at promoters of tumor suppressors (Esteller, 2008). Besides, misregulations of histone acetylation and methylation are also

frequently observed in different types of cancers. For example, HDAC1/2 has been associated to the etiology of colon cancer. And depletion of both HDAC1 and HDAC2 leads to a complete block of tumor growth in mice (Haberland et al., 2009). LSD1 was found significantly upregulated especially in lung, colon, and bladder cancer samples when compared to adjacent non-cancer tissues, and that knockdown of LSD1 suppressed proliferation of lung and bladder cancer cells (Hayami et al., 2010).

In contrast to genetic mutations, epigenetic changes are potentially reversible. A great effort has been placed on developing drugs that target the enzymes that mediate epigenetic modifications. The prominent examples are 5'-azacytidine (azacytidine, Vidaza) and 2'-deoxy-5'-azacytidine (decitabine, Dacogen), which are potent inhibitors of DNMTs. These two drugs have been approved by the US Food and Drug Administration (FDA) for patients with myelodysplastic syndrome and acute leukemia (Kantarjian et al., 2006; Silverman et al., 2002). Moreover, two HDAC inhibitors, vorinostat and romidepsin, have been approved by FDA for the rare cutaneous T cell lymphoma as well as other hematologic malignancies (O'Connor et al., 2006; Piekarz et al., 2009). Besides these, drugs targeting other epigenetic enzymes, especially histone demethylases, are also under development and received much attention. Inhibitors of monoamine oxidases, such as pargyline and tranylcypromine, have been used as inhibitors of LSD1 (Huang et al., 2007), although no clinical trial has been reported yet.

Summary

Metastases, rather than primary tumors, are responsible for most cancer related deaths. However, mechanisms involved in cancer metastases are still poorly understood. Cumulative evidence demonstrated the developmental process EMT plays

a critical role in promoting metastasis by endowing tumor cells with higher migratory and invasive potential, enhancing resistance to apoptosis, as well as generating tumor stem cell-like properties with self-renewal ability.

The Snail family of zinc finger transcription factors, Snail and Slug, have been identified as direct repressors of a set of epithelial genes (e.g. E-cadherin) and central mediators of EMT. Previous studies have shown that Snail induces repressive histone modifications at target promoters through interactions with histone modifying enzymes, in the histone deacetylases HDACs, the arginine methyltransferase PRMT5, and H3K27 methyltransferase EZH2, a component of the Polycomb repressive complex 2 (PRC2) (Herranz et al., 2008; Hou et al., 2008; Peinado et al., 2004a). These findings improved our understanding of Snail-mediated repression and EMT. However, the significance of H3K4 methylation, which is critically involved in gene regulation, remains elusive in Snail's function. The major goal of my project is to gain a more detailed knowledge of the molecular requirements for Snail-mediated EMT in human cancers, which may offer new targets for the therapeutic intervention and help us design more effective and specific anti-invasive drugs.

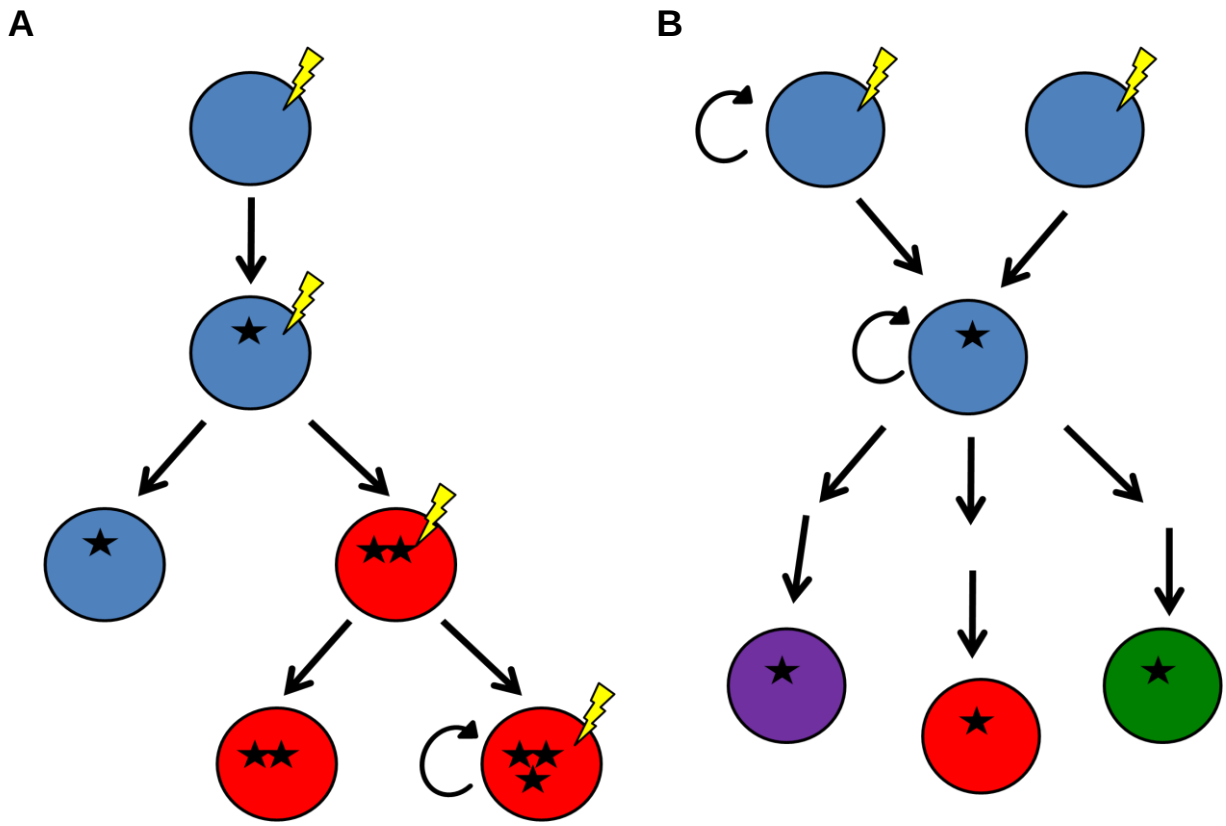


Figure 1-1. Two proposed models for cancer origination and progression. (A) In the clonal evolution model, any normal cell can be a target for transformation. A cancer cell may acquire additional mutations and gain some growth advantage over other cancer cells, leading to a new clonal expansion. (B) In the cancer stem cell model, tumor-initiating mutations likely occur in normal adult stem cells. These cells give rise to 'cancer stem cells' which can self-renew and also differentiate into other types of cells in a tumor. In both (A) and (B), circles represent cells, stars represent mutations, and lightning bolts represent mutagenesis.

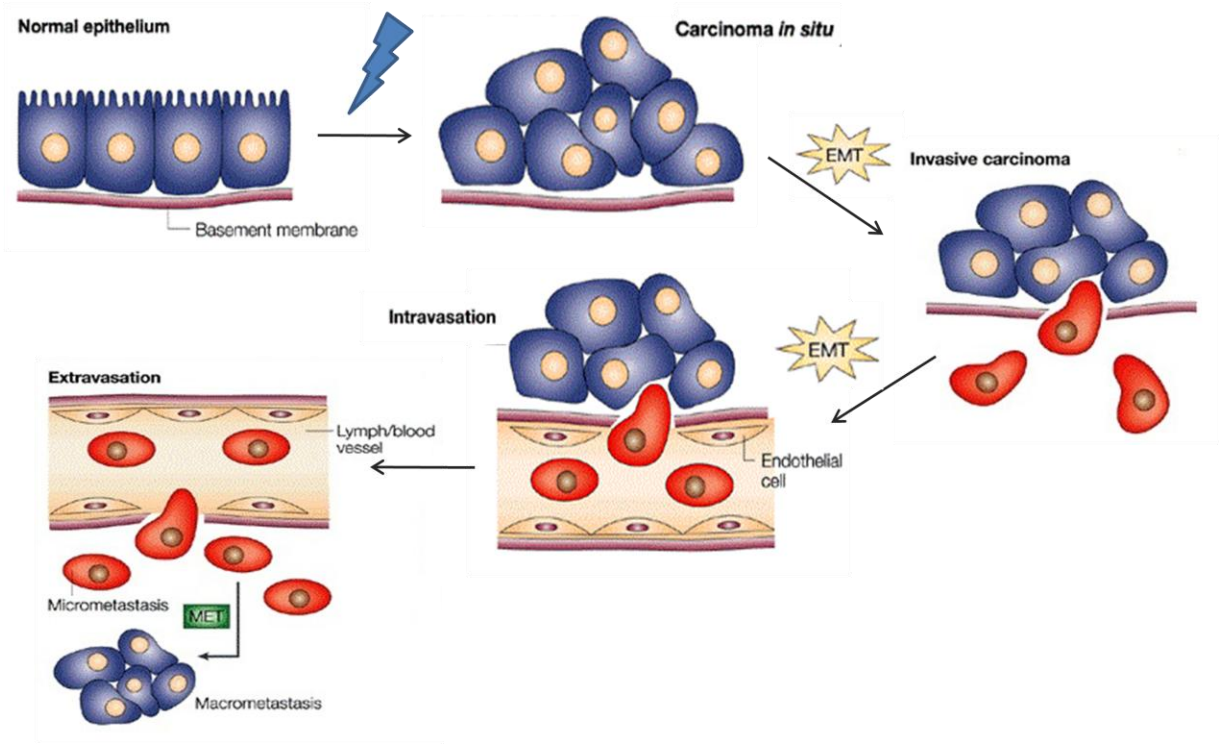


Figure 1-2. The role of EMT and MET in tumor emergence and progression (Thiery, 2002). Normal epithelia lined by a basement membrane can only proliferate locally. Genetic and epigenetic alterations cause carcinoma in situ. Further changes induce the dissemination of tumor cells, probably through EMT. The cells intravasate into lymph or blood vessels and be transported to distant organs. In secondary sites, the circulating tumor cells can extravate and remain dormancy (micrometastasis) or form a new carcinoma through MET.

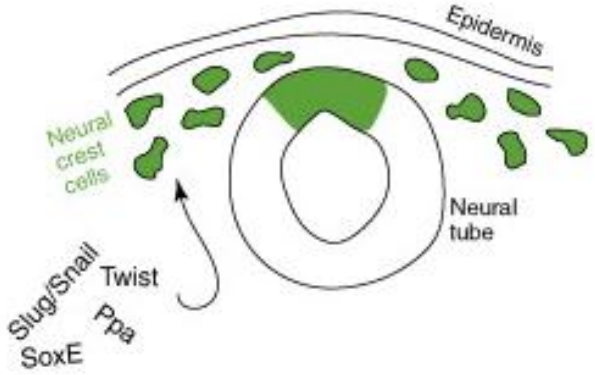


Figure 1-3. Schematic representation of neural crest cell dissemination (Sauka-Spengler and Bronner-Fraser, 2008). After neural tube formed, the neural crest (green) arises on either side of the dorsal aspect of the neural tube. These cells will undergo epithelial to mesenchymal transition and commit migration. Transcription factors such as Snail/Slug, Twist, and SoxE are implicated in the migratory behavior of neural crest.

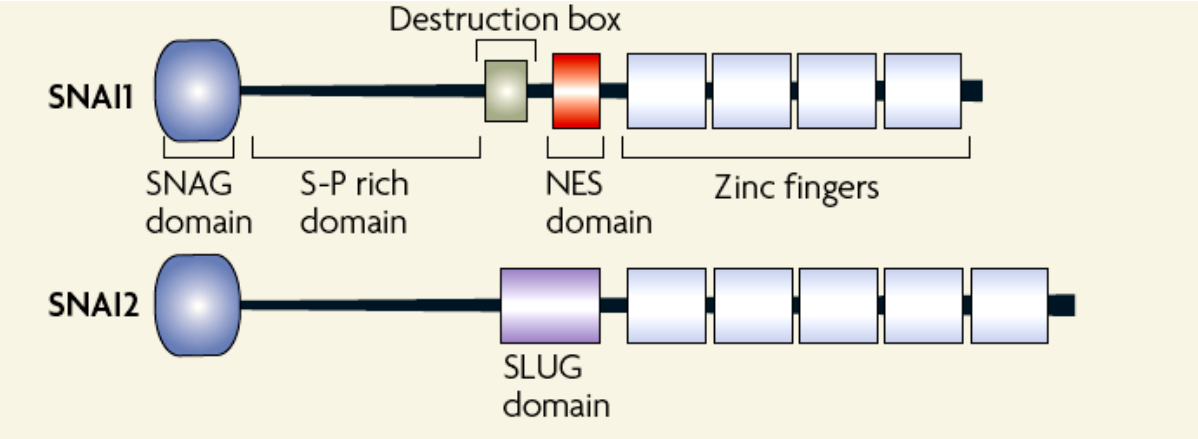


Figure 1-4. Comparative scheme of main structural domains found in mammalian Snail and Slug (Peinado et al., 2007a). Snail and Slug share a conserved carboxyl-terminal zinc finger motif and amino-terminal repressive SNAG domain. The central region is divergent between these two proteins. Snail has the destruction box and NES domain in that region, while Slug contains the SLUG domain.

CHAPTER 2
GENERAL MATERIAL AND METHODS

Cell Culture

MCF7, HEK293 and HEK293FT cells were grown as monolayer culture in DMEM medium (Cellgro), supplemented with 2 mM L-glutamine (Cellgro), 100 μg/mL streptomycin (MediaTech), 100 units/mL penicillin (MediaTech) and 10% (v/v) bovine calf serum (BCS, Hyclone). They were cultured in tissue-culture grade petri dishes.

Tumorigenic MDA-MD-231 cells from ATCC were cultured in DMEM/F12 medium (Cellgro), supplemented with 2 mM L-glutamine (Cellgro), 100 μg/mL streptomycin (MediaTech), 100 units/mL penicillin (MediaTech) and 10% (v/v) BCS (Hyclone).

The immortalized human breast epithelial cell line MCF10A was cultured in DMEM/F12 medium (Cellgro) supplemented with 5% horse serum (Sigma), 20 ng/mL epidermal growth factor (EGF, Sigma), 10 μg/mL insulin (Sigma), 0.5 μg/mL hydrocortisone (Sigma), 100 μg/mL streptomycin and 100 units/mL penicillin. To establish MCF10A-Snail stable cell line, linearized pcDNA3-Snail-Flag plasmid was transfected into MCF10A cells using Lipofectamine2000 reagent. 48 hours later, cells were plated on 10cm petri dishes with low density (100-1000 cells/dish) and selected with 1 ug/mL puromycin (Sigma). Individual clones were isolated 10 days after selection and the overexpression of Snail-Flag was confirmed by immunoblotting with antibody against Flag peptide. All cells were grown in 5% CO_2 at 37^{o}C.

Plasmids Construction

SNAG-GST and GST-ZF

The first 50 amino acids of Snail with SNAG domain was amplified by polymerase chain reaction (PCR) using the pCMV.SPORT6-Snail (purchased from

Invitrogen) construct as template. Phusion™ high-fidelity DNA polymerase (Finnzymes, F-530) was used for PCR and the reaction program was: 98°C X 30 sec initial denaturation; 30 cycles of 98°C X 10 sec, 58°C X 20 sec, 72°C X 30 sec; 72°C X 7 min final extension. The PCR product was subject to restriction enzyme digestion with NdeI and XhoI, and then ligated into the modified pGEX-KG vector (made by the Lu laboratory) which can fuse GST to the C-terminal end of target proteins. The zinc finger region of Snail was cut from pACT-Snail-ZF plasmid, which originally was cloned in the Lu laboratory for the purpose of yeast two-hybrid screening, and then was subcloned into conventional pGEX-KG (GE healthcare) vector.

Snail-Flag and Snail-P2A-Flag

Wildtype Flag-tagged Snail construct was generated by using the full-length Snail cDNA as template with the following PCR primers: 5'-GATTTAGGTGACACTATAG-3' (Forward), and 5'-CCAAGAATTCACTTGTCATCGTCGTCCTTGTAGTCGC GGGGACATCCTGAGCAG-3' (Reverse). DNA sequence coding for the Flag epitope is highlighted with underline. This PCR product was cloned into pcDNA3 expression vector. The P2A mutant was generated by the QuikChange II Site-Directed Mutagenesis Kit from Stratagene with the PCR primers: 5'-CGACCACTATGGCGCGC TCTTTCCTCGTC-3' (Forward), and 5'-AAAGAGCGCGCCATAGTGGTCGAGGCAC-3' (Reverse). Mutation site is indicated underlined.

E-cad-Luciferase Reporter

Human E-cadherin proximal promoter region (-115---+52) that contains the three consecutive E-boxes was amplified by PCR using human genomic DNA as template with the following primers: 5'-GGAATCTAGAGGGGTCCGCGCTGCTGA-3' (Forward), and 5'-GGAACTCGAGTCTGAACTGACTTCCGCA-3' (Reverse). The PCR product then

was digested by XbaI and XhoI, the restriction digest sites of which are highlighted in underline in the primer sequences. And the fragment was cloned into the pGL3 basic vector (Promega).

pCSCGW2-Snail-F Lentivirus Construct

The Snail-F fragment was cut by EcoRI and NheI from pBeta-Snail-F plasmid, and the ends were blunted by using DNA polymerase I, large fragment (Klenow, NEB). The pCSCGW2 lentivirus expression vector, a gift from Dr. Lizi Wu, was digested with XhoI and NheI, and followed by Klenow treatment. Then, these two plasmids were gel purified and ligated with T4 ligase (NEB) at room temperature overnight.

pGIPz-Snail-ER Lentivirus Construct

In order to co-express Snail-ER and shRNAs targeting LSD1, the pGIPz lentivirus shRNA vector (Figure 2-1) containing specific knockdown sequence against endogenous LSD1 was used as the backbone. The estrogen receptor gene was cloned from pBP3-hbER* (Littlewood et al., 1995), which only contains the hormone binding domain of mouse estrogen receptor (amino acides 281-599). Then Snail-ER fusion gene was cut from pcDNA3 together with the CMV promoter by SpeI and NotI, and inserted into pGIPz backbones cut with XbaI and NotI, where the SpeI end matches the XbaI end. Positive clones were selected on LB-agar plates with 50 μg/mL ampicillin and 25 μg/mL zeocin.

All other protein overexpression plasmids were achieved by cloning corresponding cDNA into pcDNA3 mammalian expression vector (Invitrogen).

Protein Isolation and Immunoblotting

Total protein lysates were isolated by first washing cells in cold PBS twice, then adding 50-200 μL lysis buffer (50mM Tris pH7.5, 1mM EDTA, 1% (v/v) SDS, 1% 2-

mercaptoethanol, 20mM dithiothreitol). The samples were boiled for 10 minutes to ensure complete lysis of cells. All protein concentrations were measured by the Bradford protein assay.

Then equal amount of protein lysates were analyzed by immunoblot. The samples were resolved by first adding appropriate amount of 6X loading buffer (4x Tris-SDS pH 6.8, 30% glycerol, 10% SDS, 0.6M dithiothreitol, 0.012% bromophenol blue) and boiled for 5 minutes. The samples were loaded onto polyacrylamide gel for electrophoration in 1X running buffer (25mM Tris, 190mM glycine, 0.2% SDS). The gel was then electrotransferred onto polyvinylidene fluoride (PVDF) membrane using Trans-Blot Semi-Dry Electrophoretic transfer Cell (BioRad) in 1X transfer buffer (20mM Tris, 192mM glycine, 20%methanol). After transfer, membranes were incubated in 5% (w/v) non-fat dry milk in TBST (30mM Tris pH 7.5, 200mM NaCl, 0.05% (v/v) Tween-20) blocking solution for 1 hour at room temperature. Blocked membranes were probed with diluted primary antibody in 3% milk-TBST solution for 1 hour at room temperature, or overnight at 4^{o}C. The membranes were then washed 3 times in TBST at room temperature for 15 minutes each. They were next incubated in 1:10000 diluted peroxidase-conjugated second secondary antibodies in TBST for 1 hour at room temperature. The membranes were then washed 3 times in TBST again. Bound antibodies were detected by applying Pierce ECL substrate solution (Thermo Scientific) and exposing the membrane to X-ray film.

Co-Immunoprecipitation (CoIP) and GST Pull-Down Assay

Transfected HEK293 cells were washed once in PBS and collected in IP buffer (20mM Tris pH 7.4, 1mM EDTA, 150 mM NaCl, 0.5%NP40, 1 X protease inhibitor cocktail (Roche)), then subject to sonication for 15 sec X 3 times at output 2 (Branson,

Sonifier 450). Unlysed cells were separated by centrifuging at 13,000 rpm for 10 minutes. The supernatant, which is the whole cell lysate, was then incubated with 15 μL anti-Flag agarose beads (Sigma) for overnight at 4^{o}C with gentle rocking. The beads were pellet down at 3,000 rpm for 5 minutes and washed for four times with washing buffer (20mM Tris pH 7.4, 1mM EDTA, 200mM NaCl, 0.5% NP40). The bound proteins were stripped off from anti-Flag agarose beads by boiling in loading buffer for 5 minutes and then separated on a 10% polyacrylamide gel and electro-transferred to PVDF membrane for immunoblotting. Antibody against endogenous LSD1 (Millipore) was used to detect the presence of LSD1 protein in the immunoprecipitates. Anti-Flag immunoblotting was also performed to check the immunoprecipitation efficiency.

To detect endogenous Snail-LSD1 interaction, similar Co-immunoprecipitation procedure was applied on whole cell lysates from MDA-MD-231 cells. Two different Snail antibodies (Santa Cruz and Cellsignaling) were used to pull-down endogenous Snail protein, and bound LSD1 protein was detected by a different antibody from Cellsignaling Technology.

To express and purify GST-fusion proteins, BL21 bacteria cells transformed with GST vector, SNAG-GST, or GST-ZF plasmid were first inoculated into 2 mL LB medium containing 50 μg Ampicillin at 37^{o}C with shaking for overnight. Next day, the culture was enlarged to 100 mL and incubated for about additional 3 hours until OD reading reached 0.8-1.0 (600 nm absorbance). The protein expression was induced with 0.1mM IPTG and cultured for additional 4 hours. The cells were harvested and GST proteins were purified using glutathione-agarose beads (Sigma, G4510), according to manufacturer's protocol. Purified proteins were immobilized on the glutathione-agarose beads and

stored at 4 ^{o}C. SDS-PAGE followed by Coomassie staining was performed to determine the quality and amount of fusion protein yield.

Full length LSD1 protein was translated *in vitro* using TNT Quick Coupled Transcription/Translation System (Promega) and labeled with isotope ^{35}S. Final product (10 μL for each pull-down reaction) was incubated with the immobilized GST, SNAG-GST, or GST-ZF fusion protein at 4^{o}C with rotating for 3 hours in IP buffer (20mM Tris pH 7.4, 1mM EDTA, 150mM NaCl, 0.5%NP40, 1 X protease inhibitor cocktail (Roche)). The beads were washed three times with 500 μL of IP buffer. The bound LSD1 protein was eluted by boiling in loading buffer (Tris-HCl, pH7.5, 2% SDS, 50% glycerol, 10% beta-mercaptoethanol), and subjected to SDS-PAGE separation. After electrophoresis, the protein gel was dried and assessed by autoradiography.

RNA Isolation, Reverse Transcription, and Real-Time PCR

Samples were collected and homogenized by vortex in 0.5 mL Trizol reagent (Invitrogen) to obtain total RNA. 0.1 mL of chloroform was added to each homogenized samples. The samples were then centrifuged at 12,000 x g for 15 minutes at 4^{o}C to separate aqueous and phenol-chloroform phases. The aqueous phase was extracted from each sample to a new tube. The RNA was precipitated with the addition of 75% isopropanol (v/v) and centrifuged maximum speed at 4^{o}C for 10 minutes. The RNA pellets were washed with 1 mL of 75% ethanol and centrifuged again to remove supernatant. The pellets were air-dried for 5 minutes before dissolving in sterile-filtered TE (10mM Tris pH 8.0, 1mM EDTA) and stored at -80^{o}C.

Approximately 1 μg total RNA for each sample was added to a reaction cocktail containing DEPC-treated ddH_2O, 2.5 μM dNTP, and 5 nM random primers to 16 μL final

volume. The mixture was incubated at 70°C for 5 minutes, and quenched quickly on ice. 2 μL of 10X RT buffer (NEB M-MuLV), 1 μL RNase inhibitor (Promega), and 1 μL M-MuLV Reverse Transcriptase (NEB) were added to the reaction to a final volume of 20 μL, which was incubated at 42°C for 1 hour. The reaction was heat-inactivated at 65°C for 20 minutes, and was diluted to 200 μL with ddH_2O. 1-2 μL of diluted template was used for real-time PCR.

Each real-time PCR reaction was composed of the following: 1μL cDNA generated from reverse transcription, 1 μL of 5 μM primer mix working solution, 8 μL ddH_2O, and 10 μL of 2X SYBR Green PCR Master Mix (Applied Biosystems). Triplicates were done for each reaction and results were expressed as relative quantitation normalized to endogenous beta-actin expression. Reactions with no template were also included on real-time PCR plate for each set of primers as negative control. Gene expression fold differences were calculated as 2^(ΔΔCt), The thermal cycling parameters were as follows: 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds for denaturing step and 60°C for 60 second for product extension, and a melting curve analysis was performed at the end of each run. StepOne (48-well), or StepOnePlus (96-well) real-time PCR machines (Applied Biosystems) were used for data collection. Primers used were listed in Table 2-2.

Chromatin-Immunoprecipitation (ChIP) Assay

In general, both MCF10A and MCF10A-Snail stable cells were cultured in indicated medium until confluent. Formaldehyde was added to the culture medium at a final concentration of 1% and incubated at room temperature for 10 minutes. Then 2.5M glycine was added to a final concentration of 0.125M to stop the cross-linking reaction.

Cross-liked cells were washed once with cold PBS and scraped off the dish, and then washed with both pellet washing buffer 1 (0.25% TritonX-100, 10mM EDTA, 0.5mM EGTA, 10mM Tris pH8.0) and washing buffer 2 (0.2M NaCl, 1mM EDTA, 0.5mMEGTA, 10mM Tris pH8.0) successively. The cells were then re-suspended in sonication buffer (1mM EDTA, 0.5mM EGTA, 10mM Tris pH8.0) and subjected to sonication to shear the chromatin. The sonication condition is: 20 second at power 5 (Branson, Sonifier 450) with two minutes cooling down on ice, 8 rounds in total. A small aliquot of sonicated chromatin was reverse-crosslinked and run on a 1.5% agarose gel to check the sonication efficiency, and the DNA length should be centered around approximately 500bp, and primarily smaller than 1000bp. The sonicated samples were diluted in ChIP buffer (0.01% SDS, 1.0% TX-100, 1.0mM EDTA, 20mM Tris pH 8.1, 150 mM NaCl) and divided into 1 mL aliquots, each of which represents approximately 2.5 X 10^6 cells, and incubated with specific antibodies at 4^{o}C overnight. Non-immune same species IgG was used as the non-specific control. A 50 μL aliquot of pre-washed Protein A/G slurry (Invitrogen) was added to each aliquot and incubated for additional 2 hours at 4 ^{o}C with rotating. The beads were subjected to a series of washing steps to remove non-specific binding, and finally incubated with elution buffer (1% SDS, 0.1M $NaHCO_3$) at room temperature for 30 minutes to elute the protein-DNA complexes. Supernatant from the non-specific IgG immunoprecipitated sample served as “Input” for all the qPCR analyses in later steps. DNA was released from the complexes by reverse cross-linking at 65^{o}C with 200 mM NaCl for overnight. Reverse cross-linked DNA was incubated with Proteinase K at 45^{o}C for 1 hour to remove proteins, purified by phenol/chloroform extraction. Final DNA sample was subjected to quantitative real-time PCR by using

StepOne PCR system (Applied Biosystem) and SYBR Green dye as detection reagent. ChIP-qPCR primers were listed in Table 2-3. The condition for the PCR was: 94^{o}C X 15 minutes for initial denaturation; 94^{o}C X 15 sec, 60^{o}C X 45 sec for 40 cycles. After PCR, melting curve was added to ensure that a single product is amplified in the reaction. The results were expressed as the ratio to input DNA.

Transfection and Luciferase Reporter Assay

For transfection, cells were seeded at ~60% confluency 18 hours prior to Transfection. They were transfected using TurboFect *in vitro* Transfection Reagent (Fermentas) according to manufacturer's instruction. First, 1 μg of DNA was diluted in 100 μL of serum-free DMEM. Then briefly vortex TurboFect reagent and add 2 uL of it to the diluted DNA, and mixed by pipetting. The whole mix was incubated at room temperature for 20-30 minutes to form a cationic lipid-mediated Transfection complex before adding to cultured cells.

For the luciferase reporter assay, MCF7 cells were seeded in a 12-well plate at a density of 1.0 X 10^5. At 18-24 hours after plating, the cells were transfected as indicated above. 36-48 hr after transfection, cells were collected and assayed with Duo-Glo luciferase assay system (Promega). For each well, 50 μL of cell lysate was mixed with equal volume of Dual-Glo luciferase reagent at room temperature for 15 minutes, and measured for firefly luciferase activity in a luminometer (Pharmingen, Monolight 3010). Then, immediately, 50 μL of Dual-Glo Stop & Glo reagent was added to the mixture and mixed at room temperature for additional 15 minutes, and the Renilla luminescence was measured, which was used as a reference to normalize transfection efficiencies in all

experiments. At least three independent replications were performed for each experiment.

Statistical Analysis

Statistical analyses were derived from at least three independent experiments. Error bars for three independent experiments were presented as the standard deviation of samples, and statistically significant differences were determined using the Student's t-test.

Immunofluorescence Staining

Cells were grown directly onto glass cover slips in 6-well tissue culture plate and transfected with Snail-GFP plasmid 24 hours before Immunofluorescence staining procedure. To stain for the endogenous LSD1, the cover slips were rinsed with PBS twice, and cells were fixed in 3.7% formaldehyde/PBS solution at room temperature for 5 minutes. Then, the cells were rinsed with 0.1% NP-40/PBS 3 times. During the last of wash, the cell membrane was permeablized for 15 minutes at room temperature with 0.1% NP-40/PBS. Before the primary antibody, cells were blocked in 3%BSA/0.1%NP-40/PBS solution for 30 minutes. Next they were incubated with 1:200 diluted LSD1 antibody (Cellsignalling) in 3% BSA/0.1%NP-40/PBS for overnight at 4^{o}C. They were then rinsed three times with 0.1% NP-40/PBS for 5 minutes at room temperature followed by incubating with 1:500 diluted flurophore-conjugated secondary antibodies (Invitrogen) in 3% BSA/0.1% NP-40/PBS solution for 1 hour at room temperature in the dark. Again, the cover slips were washed three times in 0.1% NP-40/PBS for 5 minutes at room temperature, followed by a quick rinse with water, and then counterstained with 200 μg/mL of Hoechst 33342 for 5 minutes at room temperature. The cover slips were rinsed with water, and allowed to air dry in the dark for couple minutes. The cover slips

were mounted onto glass slides using Fluoromount-G (Southern Biotech) and allowed to dry overnight at 4^{o}C in the dark. Glass was stored at 4^{o}C. All images were taken under Leica DM6000B fluorescence microscope (Leica) at the same magnitude.

In Vivo Tumor Xenograft

MDA231-pGIPz or MDA231-shLSD1 cells (5 X 10^{6}) were re-suspended in a mixture of 100 μL of serum free medium and Matrigel (BD Biosciences; 2:1 ratio) and injected into the fourth mammary gland fat pad of severe combined immunodeficient (SCID) mice aged at 6-to-8 weeks. Then the growth of tumors was measured every week with a ruler for total of more than 60 days, and the tumor volumes was calculated with the following formula: volume (mm^3) = (4/3) * π * (length/2)^3. The tumor growth rate was modeled by plotting tumor volumes against corresponding time points. And the difference of final tumor weight between LSD1 knocked down cells and control cells was evaluated by student's t-test with significance level α=0.01.

Lentivirus Production and Infection

To generate lentiviral particles, the shRNA plasmids containing either specific knockdown sequence or non-target sequence as control were transiently transfected into the HEK293FT a transformed HEK293 cell line. Two lentiviral plasmids MD2G (envelope plasmid) and PAX (packing plasmid) were co-transfected to facilitate virus production and packaging. 48 hours after transfection, the virus-containing media was collected and passed through a 45 μm filter to exclude cell debris. The viral media was aliquoted and either used immediately to infect target cells, or stored at -80^{o}C.

Target cells were plated 24 hours prior to lentiviral infection. Adherent cells were infected by replacing culture media with the infection cocktail, which consisted of 1:1

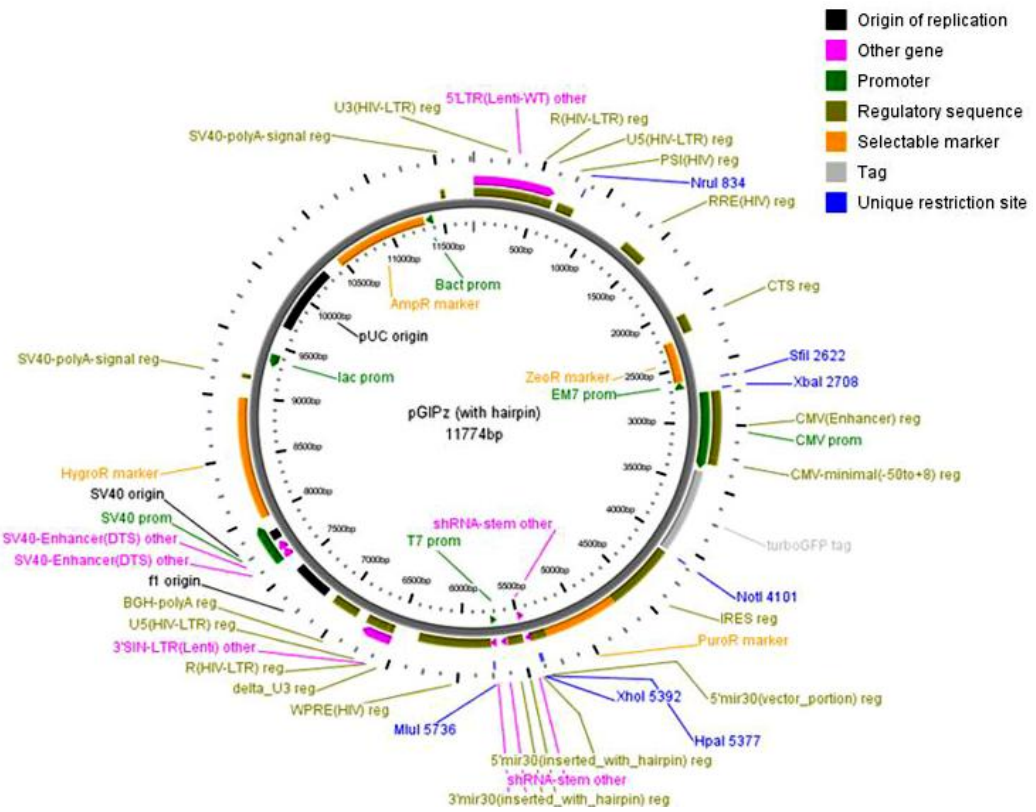
viral media: culture media and 4 μg/mL polybrene. After 24 hours of incubation, the infection cocktail was changed to fresh media for an additional 24 hours of recovery. Then the cells were treated with 1 μg/mL puromycin dihydrochlorid (Cellgro) for a week to eliminate uninfected cells. The media was changed every 2 days. The transformed stable cells were stocked in freezing media (bovine serum albumin containing 10% v/v DMSO at -80^{o}C), or maintained in culture media with lower concentration of puromycin (0.2-0.5 μg/mL).

DNase Accessibility Assay

The DNase accessibility assay was performed as described (Hempel and Ferrier, 2004). 2.5-5.0X10^{5} cells were collected in cold PBS and washed twice by centrifuging at 1000 g for 5 minutes. Then cell pellet was suspended in buffer A (15 mM Tris pH 7.4, 60 mM KCl, 15 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.01% NP40) with protease inhibitor and incubated on ice for 5 minutes. The nuclei were isolated by centrifuging at 1500g at 4^{o}C for 10 minutes. Pelleted nuclei were re-suspended in 200 μL of 1X DNase reaction buffer (40 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$) in preparation for digestion. Chromatin digestion reactions were carried out with 0 U and 5 U/mL DNase (Promega, M6101) at 30^{o}C for 30 minutes. Reactions were stopped by addition of 10 μL of 0.5 M EDTA. After that, samples were incubated with Proteinase K at 45^{o}C for overnight to remove proteins, and DNA was purified by phenol/chloroform extraction. Final DNA sample was subjected to quantitative real-time PCR by using StepOne PCR system (Applied Biosystem) and SYBR Green dye as detection reagent. The PCR program is the same as used in ChIP-qPCR. Primers were designed to amplify the regions flanking

the transcription start sites of CDH1, HBB, or GAPDH. The level of resistance to DNase was calculated as the percentage of corresponding undigested sample.

A.



B.

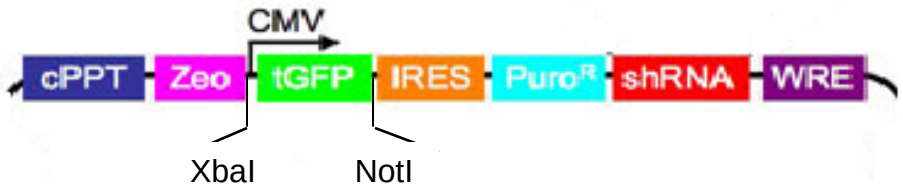


Figure 2-1. Cloning vector information for pGIPz lentiviral vector with mir30RNA. A). Vector map and unique restriction sites of pGIPZ vector. B). XbaI site before CMV promoter and NotI site immediately after GFP gene were used to insert Snail-ER. The whole mRNA was driven by a single CMV promoter ensuring coexpression of Snail-ER and shRNAs. IERS-Puro^r cassette allows for selection of stable integrates. Abbreviations: cPPT is central polypurine tract facilitating lentiviral vector nuclear export, CMV is cytomegaloviral promoter, GFP is green fluorescence protein, IRES is internal ribosome entry site, Puro is puromycin.

Table 2-1. List of primers used for real-time RT-PCR

Target Name	Forward	Reverse	Tm
hSnail	CACTATGCCGCGCTCTTTC	GGTCGTAGGGCTGCTGGAA	60^{o}C
hCDH1	CAGTGAACAACGATGGCATT	CTGGGCAGTGTAGGATGTGA	60^{o}C
hCLDN7	GGATGATGAGCTGCAAAATG	CACCAGGGAGACCACCATTA	60^{o}C
hKRT8	ACCCAGGAGAAGGAGCAGAT	CTCCACTTGGTCTCCAGCAT	60^{o}C
hACTB	AGAAAATCTGGCACCACACC	AGAGGCGTACAGGGATAGCA	60^{o}C
hGAPDH	GACCACAGTCCATGCCATCAC	CATACCAGGAAATGAGCTTGAC	60^{o}C

Table 2-2. List of primers used for other real-time PCR

Target Name	Forward	Reverse	Tm
Ecad_-5kb	CCAGGAGATGAAAGGGACAA	GGATCACAGACTCCAGGTTTC	60^{o}C
Ecad_Pro	AATCAGAACCGTGCAGGTCC	ACAGGTGCTTTGCAGTTCCG	60^{o}C
CLDN7	GGATGATGAGCTGCAAAATG	CACCAGGGAGACCACCATTA	60^{o}C
KRT8	ACCCAGGAGAAGGAGCAGAT	CTCCACTTGGTCTCCAGCAT	60^{o}C
CDH1_TSS	ATTCGAACCCAGTGGAATCA	TCACAGGTGCTTTGCAGTTC	60^{o}C
HBB_TSS	AAGCCAGTGCCAGAAGAGCCAAGGA	CCCACAGGGCAGTAACGGCAGACTT	60^{o}C
GAPDH_TSS	CGGCTACTAGCGGTTTTACG	CTTCAGGCCGTCCCTAGC	60^{o}C

Table 2-3. List of antibodies used for Westernblotting (WB), Immunofluorescence (IF), Immunoprecipitation (IP), and Chromatin-Immunoprecipitation (ChIP)

Antibody	Species	Isotype	Company	Catalog Number	Application
Flag	N/A	Mouse-IgG	Sigma	F-1804	WB, IP, ChIP
Ecadherin	Human	Rabbit-IgG	Cell Signaling	4065	WB
Occludin	Human	Rabbit-IgG	Thermo Scientific	RB-10681	WB
LSD1	Human	Rabbit-IgG	Cell Signaling	C69G12	WB, IF, ChIP
Snail	Human	Mouse-IgG	Cell Signaling	L70G2	WB
Snail	Human	Rabbit-IgG	Cell Signaling	C15D3	WB, IP
Snail	Human	Rabbit-IgG	Santa Cruze	sc-28199	IP
α-Tubulin	Human	Mouse-IgG	Sigma	T-6199	WB
H3K4m2	Human	Rabbit antiserum	Millipore	07-030	WB, ChIP
H3K4m3	Human	Rabbit-IgG	Cell Signaling	C42D8	WB, ChIP
Alexa-Fluor 594	Rabbit	Donkey-IgG	Molecular Probes (Invitrogen)	A21207	IF
HRP-Donkey anti mouse	Mouse	Donkey-IgG	Jackson ImmunoResearch	715-035-150	WB
HRP-Donkey anti rabbit	Rabbit	Donkey-IgG	Jackson ImmunoResearch	715-035-152	WB

CHAPTER 3
SNAIL RECRUITS LSD1 TO EPITHELIAL PROMOTERS DURING EMT

Study Background

Over the last decade, significant progress has been made in understanding the mechanism underlying epithelial-mesenchymal transition. The Snail family of zinc finger transcriptional repressors is known as the master regulator of EMT. Snail is located at the hub of multiple signaling pathways leading to EMT. Upregulation of Snail can induce EMT by downregulating many epithelial cell markers (Nieto, 2002).

Snail family members are C2H2 type zinc finger transcription factors. They all share a highly conserved carboxyl-terminal region with multiple zinc fingers, which are designated for sequence specific DNA binding. The binding motif for Snail members is identical to the so-called E-box, the consensus core binding site of basic helix-loop-helix (bHLH) transcription factors. The amino-terminal region is much more divergent. In vertebrate members, a conserved repression domain termed SNAG is identified. The SNAG domain extends to about 20 amino acids and is present in diverse transcriptional repressors including Gfi-1, IA-1, Gsh-1, and Ovo. The SNAG domain has been shown to be essential for repression mediated by Gfi-1 (Grimes et al., 1996a) and Snail (Batlle et al., 2000b). On binding to DNA, Snail primarily acts as a transcriptional repressor. The repressive activity depends not only on the zinc finger region, but also on the amino-terminal SNAG domain.

Epigenetic regulation has been recognized as a key mechanism controlling gene expression. Histone tails are subject to different types of modifications including phosphorylation, ubiquitination, acetylation and methylation. Enzymes corresponding for these modifications have been found as either co-activators or co-repressors for

numerous transcription factors (Bernstein et al., 2007). Histone lysine methylation is of the most interest for many epigenetic studies, due to its high level of complexity. It was long considered irreversible until the discovery of LSD1 as well as many other Jmjc-domain containing histone demethylases, demonstrating this modification is dynamically regulated (Shi and Whetstine, 2007).

Chromatin-immunoprecipitation (ChIP) assay has been emerging as one of the most powerful methods in the epigenetic field. ChIP has been widely used to capture both direct and indirect association of proteins with specific genomic regions in the context of intact cells (Wells and Farnham, 2002). The original chromatin structure is captured by formaldehyde induced cross-linking. Then the chromosome is broken down into small pieces by either sonication or nuclease digestion. The DNA-protein complex is subject to immunoprecipitation with corresponding antibodies. The abundance of specific proteins bound to interested genomic regions, e.g. promoter or enhancer, is eventually evaluated through quantitative polymerase chain reaction. In this study, I further examined histone lysine methylation status changes induced by Snail at its target promoters, since the epigenetic regulation is expected to be critical in controlling their expression.

The first *in vitro* EMT model was discovered by Stocker and Perryman using the Madin-Darby canine kidney (MDCK) cells. MDCK is a polarized epithelial cell line and can be experimentally converted into migratory fibroblasts in petri dish by incubation with conditioned medium from cultured fibroblasts (Stoker and Perryman, 1985). After that study various EMT systems were established in different epithelial cell lines leading to an exponential discovery of EMT controlling signaling pathways and transcription

factors. MCF10A is one of those excellent EMT model systems. MCF10A is an immortalized, non-transformed epithelial cell line derived from human fibrocystic mammary tissue (Soule et al., 1990). It lacks the ability to either form tumors in nude mice or to grow in an anchorage independent manner. Parental MCF10A cells show classical epithelial morphology and grow as clusters of cells with extensive cell-cell contacts. It also demonstrates substantial phenotypic plasticity that it can be induced to mesenchymal-like phenotype by various EMT regulating factors such as Snail, Slug, Twist and Zeb2 (Gjerdrum et al., 2009). This makes MCF10A easy for manipulating and a popular model in studying EMT.

Results

Snail Directly Represses Epithelial Genes in MCF10A

To gain insight into the mechanism associated with Snail's repressive activity, we intended to map the histone modifications especially the methylation pattern at epithelial promoters in Snail-induced EMT. It has been shown previously that forced expression of Snail drives EMT in several types of epithelial cells concomitantly with downregulation of epithelial markers. We decided to establish a Snail-dependent EMT system in MCF10A cell line, an immortalized human mammary epithelial cell line which has widely been used as an EMT model system. Because it is known that amino-terminal fusions disrupted Snail's repressive activity, we generated a functional Snail-Flag construct in which the Flag epitope tag was fused to the carboxyl terminus of Snail. Linearized plasmid DNA was introduced into MCF10A cells by regular transfection, and stable clones were obtained by puromycin selection for 5-6 consecutive weeks. Parental MCF10A cell shows cobblestone-like morphology with extensive cell-cell contacts (Figure 3-1A). However, the Snail-expressing cells became scattered, contacted their

neighboring cells only focally, and adopted a fibroblast-like appearance typical of mesenchymal cells (Figure 3-1B). This phenotypic change was accompanied with loss of expression of epithelial markers E-cadherin and Occludin as shown by western blotting (Figure 3-1C). These observations indicate an epithelial to mesenchymal morphological transition in Snail-expressing cells.

The E-cadherin proximal promoter contains three consecutive 'E-box' elements recognized by Snail (Figure 3-2A) (Nieto, 2002). Two additional epithelial genes claudin-7 (*CLDN7*) and cytokeratin-8 (*KRT8*) were selected, both of which were reported as direct targets of Snail (De Craene et al., 2005a; Ikenouchi, 2003) and also carry multiple E-boxes at their promoters (Figure 3-2A). I verified whether Snail directly bound to the promoters of these epithelial genes in *vivo* by chromatin immunoprecipitation (ChIP) assays. Chromatin from parental and Snail-Flag-expressing MCF10A cells was immunoprecipitated with a control immunoglobulin (IgG) and anti-Flag antibodies. Then I performed quantitative polymerase chain reaction on the recovered DNA to determine the enrichment of the proximal promoter regions of the epithelial genes as compared with a 5kb upstream region (-5kb) of E-cadherin, which serves as a negative control. Occupancy of Snail was detected specifically in the promoters of E-cadherin, CLDN7 and KRT8 in the Snail-Flag cells (Figure 3-2B).

To confirm that these epithelial genes are indeed inhibited by Snail, we conducted a similar ChIP assay to monitor the binding of RNA polymerase II at their promoters. Consistent with E-cadherin expression, high levels of RNA polymerase II were detected at the E-cadherin promoter in MCF10A cells. In contrast, this binding was largely abolished in cells expressing Snail. Similar pattern was observed for the CLDN7 and

KRT8 promoters (Figure 3-2C). These results suggest that Snail directly represses epithelial markers and induces EMT in MCF10A cells.

Snail Downregulates H3K4m2 Levels at Epithelial Gene Promoters

Having established an EMT model, I applied ChIP analysis to survey potential Snail-induced histone modifications at the target promoters. As previously reported in other studies, I observed a reduction of H4 acetylation level and an induction of H3K27m3 level in the Snail-expressing cells when compared to control MCF10A cells (data not shown). Both di- and tri-methylated H3K4 are associated with active transcription (Kouzarides, 2007; Li et al., 2007). I then assessed the changes of active H3K4 methylation marks at the epithelial gene promoters caused by ectopic Snail expression by using an antibody specific for H3K4m2. Relatively high levels of H3K4m2 were detected at the promoter of the E-cadherin, CLDN7, and KRT8 genes in MCF10A cells. However, this active mark was significantly decreased specifically at the promoter regions in the Snail stable cells (Figure 3-3A). Therefore, the levels of H3K4m2 in the E-cadherin promoter correlated with E-cadherin expression as shown by western blotting. At the same time, I also carried out a similar ChIP assay for the abundance of tri-methylated H3K4 at E-cadherin promoter. Surprisingly, I did not see a significant difference of this mark between parental and Snail-expressing cells (Figure 3-3B). High levels of H3K4m3 were observed at the E-cadherin promoter in both groups of cells, even though E-cadherin is inactive in MCF10A-Snail-Flag stable cells. That indicates expression of Snail only leads to a specific decrease of H3K4m2 at its target promoters.

Snail Interacts with LSD1

Reduction in di-methylated H3K4 mark cannot be mediated directly by any known Snail-associated histone modifying enzymes. There are two types of histone

demethylases are responsible for removing the methyl group from H3K4m2: LSD1, which belongs to the class of flavin adenine dinucleotide (FAD)-dependent amine oxidases, and members of the JARID1 (KDM5) family of Jumonji (JmjC) domain-containing demethylases (Klose and Zhang, 2007; Shi, 2007). LSD1 is found as a co-repressor for many transcription factors, and forms core ternary complex with HDAC1/2 and CoREST (Lan et al., 2008a). This complex, when recruited to chromatin template, can efficiently bind and modify nucleosomal substrates to repress transcription. Unlike JARID1, LSD1 cannot catalyze demethylation on tri-methylated H3K4 (Shi et al., 2004). Because Snail decreases the level of H3K4m2 but not H3K4m3, the LSD1 complex becomes a promising candidate to mediate the repressive function of Snail.

Both of mammalian Snail and Slug share a highly conserved amino-terminal termed SNAG domain. A recent report has demonstrated that Gfi-1, another SNAG domain containing protein, interacts with LSD1 corepressor complex, and a mutation in the SNAG domain of Gfi-1 abolishes this association (Saleque et al., 2007), suggesting that SNAG is necessary for recruitment of the LSD1 complex. This study raises the possibility that SNAG domain might be sufficient for interaction with the LSD1 complex, and thus Snail might regulate gene expression by recruiting the LSD1 complex to its target promoters to remove methyl groups from di-methylated H3 lysine 4.

To test the potential association between the SNAG domain of Snail and the LSD1 complex, we performed the in vitro glutathione S-transferase (GST) pull-down assay. GST pull-down assay is a relatively easy, straightforward method and extensively used to determine physical interaction between two proteins and to map interaction sites. Since the SNAG domain apparently does not tolerate any fusion to its

amino terminus, we specifically placed GST at the carboxyl terminus of the SNAG-GST fusion protein (Figure 3-4A). The recombinant SNAG-GST and GST (control) proteins were produced and affinity-purified from bacteria. Then these fusion proteins were incubated with whole cell lysates prepared from the mammalian HEK293 cells transfected with Flag-LSD1. Unbound proteins were washed away, and the precipitates were subject to Western blotting analysis. Based on Western blotting with the anti-Flag antibody, LSD1 was shown to be associated with SNAG-GST but not GST alone (Figure 3-4C). Moreover, other components of the LSD1 core complex, the endogenous CoREST and HDAC1 specifically bound to SNAG-GST as well (Figure 3-4C). The result suggests that SNAG domain of Snail is sufficient to bind to the LSD1 complex.

To elucidate whether this interaction is direct or not, and which subunit of the LSD1 complex mediates the interaction with Snail, I carried out similar GST binding assays. LSD1, CoREST, and HDAC1 proteins were produced by *in vitro* transcription and translation and labeled with ^{35}S, and then were incubated with GST, SNAG-GST, and GST-ZF. Neither CoREST or HDAC1 showed any signal after washing (data not shown), while LSD1 displayed readily detectable association with SNAG-GST (Figure 3-4B), indicating that LSD1 is responsible for direct interaction with the SNAG domain of Snail and the other components of the core LSD1 complex are recruited to Snail in an in-directed manner. Besides, the specificity of the interaction was further confirmed by lack of binding between LSD1 and the carboxyl-terminal zinc finger motifs of Snail (Figure 3-4B). Furthermore, I also examined which region of LSD1 is involved in binding. A truncated LSD1 mutant that lacks the carboxyl-terminal part of amine oxidase

domain (AOD_C) was generated. And this mutant retains the ability to interact with SNAG domain (Figure 3-4D).

To determine whether Snail and LSD1 might form complex *in vivo*, I carried out co-immunoprecipitation assays. In addition, my previous GST pull-down assay indicated SNAG domain is sufficient to directly interact with LSD1. I want to further test if SNAG is also essential for the interaction. I made a point mutation in the SNAG domain, which changed the second proline to alanine (P2A), since this mutation has been shown to interrupt the repressive capability of SNAG domain in both Snail and Gfi-1. Cellular extracts prepared from HEK293 cells transiently transfected with Flag-tagged wildtype Snail or the P2A mutant form were subjected to immunoprecipitation with anti-Flag antibodies. The presence of endogenous LSD1 was detected in the precipitates obtained only from cells expressing wildtype Snail, but not the P2A mutant (Figure 3-4E). This evidence supports the association between Snail and LSD1 in vivo, and suggests a functional SNAG domain is required for this interaction. 2% of each lysates was saved before immunoprecipitation and loaded as input control, to ensure equal amount of protein was used for co-IP and similar transfection efficiency. Moreover, the ability of Snail to interact with LSD1 was confirmed by immunoprecipitation assays between the endogenous proteins in the highly metastatic MDA-MD-231 breast tumor cells. Endogenous LSD1 was found to be co-immunoprecipitated with endogenous Snail in the immuocomplexes obtained with an anti-Snail antibody but not IgG control (Figure 3-4F).

Finally, Immunofluorescence was used to further confirm the association between Snail and LSD1, and also to monitor the subcellular localization of these two

proteins. I generated a Snail-GFP fusion protein expressing plasmid and transfected it into MCF10A cells. Endogenous LSD1 protein was detected by anti-LSD1 primary antibody with fluor-conjugated anti-rabbit secondary antibody. Snail-GFP exhibited a nuclear speckle pattern and found largely overlapped with LSD1 in nuclei (Figure 3-4G).

Snail Recruits LSD1 to its Target Gene Promoters

We have shown that Snail binds to the E-cadherin promoter and causes reduction in H3K4m2 in Figure 3-1 and Figure 3-2. Given the interaction between Snail and LSD1, it is conceivable that during EMT Snail may recruit LSD1 to the epithelial gene promoters where LSD1 catalyzes demethylation on the H3K4m2 mark. To validate this idea, I performed ChIP analysis to examine the relative fold of enrichment of LSD1 at the Snail target promoters. As expected, I observed a significant increased binding of LSD1 at the E-cadherin, CLDN7, and KRT8 promoters, but not the 5kb upstream control region of E-cadherin promoter in the Snail-expressing cells compared to MCF10A control cells (Figure 3-5A). The levels of LSD1 binding inversely correlated with H3K4m2 (Figure 3-3A). Next, I tested if the occupancy of LSD1 at these promoters is dependent on Snail. Depletion of Snail was based on a lentivirus-mediated RNA interference. MDA-MD-231 cells were infected with lentivirus carrying short hairpin RNA sequence specifically targeting human Snail. The knockdown efficiency of Snail was verified by RT-PCR (Figure 3-5B&C). Then I compared LSD1 occupancy at epithelial gene promoters. Results showed the binding of LSD1 to the E-cadherin and CLDN7 promoters decreased in the Snail-knockdown cells (Figure 3- 5D). The remaining signal of LSD1 might be because of the presence of Slug in these cells (Hajra et al., 2002). Taken together, the present data support of a model that Snail directly recruits LSD1 to

the epithelial promoters via protein interaction and LSD1 in turn epigenetically modifies the promoter chromatin structure by demethylating the active H3K4m2 mark.

Summary

In this study, I used MCF10A as a model system. Ectopically expressing Snail in MCF10A induced epithelial to mesenchymal morphology change accompanied by downregulation of epithelial markers (Figure 3-1). Then I investigated the epigenetic mechanism underlying Snail-mediated transcriptional repression of epithelial genes by surveying repressive histone modifications. It has been recently reported that Snail interacted with the PRC2 complex and Snail-mediated transcriptional repression associated with H3K27m3 (Herranz et al., 2008). Trimethylation of H3K27 by the PRC2 complex enzymatic component EZH2 is related to gene silencing and facultative heterochromatin formation (Schuettengruber et al., 2007). Consistently to previous studies, I detected high levels of H3K27m3 in the E-cadherin promoter specifically in the Snail-expressing cells. In addition to that, I also observed a significant reduction of H3K4m2 at epithelial promoters (Figure 3-3), which indicates LSD1 as a potential candidate corepressor of Snail. To test this idea, I first performed GST pull-down assays. I not only showed Snail physically interacts with LSD1, but also mapped the region of Snail involved in binding down to the amino-terminal SNAG domain. The P2A mutation in SNAG domain disrupted Snail-LSD1 association. Co-immunoprecipitation assays further demonstrated that Snail and LSD1form endogenous complex (Figure 3-4). Furthermore, Chromatin-immunoprecipitation experiments showed LSD1 was recruited to epithelial gene promoters by Snail and inversely correlated with H3K4m2 levels introduced by Snail. Depletion of Snail in MDA-MD-231 cells reduced LSD1 enrichment at Snail target promoters.

LSD1 is the first identified histone demethylase which removes methyl groups from lysine 4 of histone H3. Dimethyl H3K4 is a transcription-activation chromatin mark enriched in the promoter regions of actively transcribed genes and demethylation of this mark by LSD1 thus represses gene expression. Consistently, LSD1 is a component of various transcriptional corepressor complexes that often include HDAC1/2 and CoREST. The latter is a cofactor for LSD1 required for demethylation of nucleosomal substrates. Having established the association between Snail and LSD1, my further studies will involve investigating the biological significance of this association related to Snail's function.

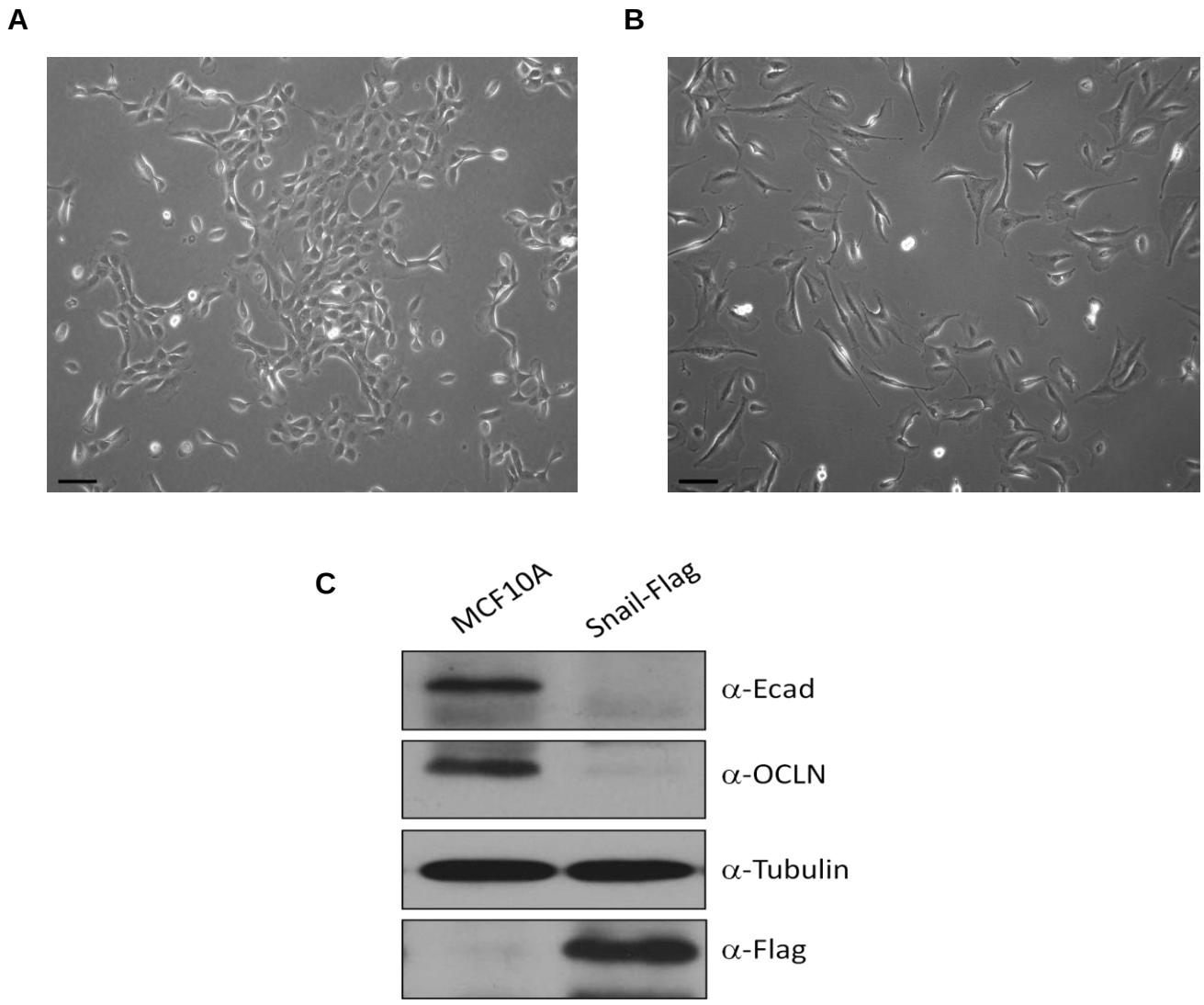


Figure 3-1. Ectopic expression of Flag tagged Snail in MCF10A cells. (A). Phase contrast of wildtype MCF10A under microscope. (B). Phase contrast of MCF10A-Snail-Flag stable cell. (C). Snail downregulates epithelial markers E-cadherin and Occludin. Protein lysates from control and Snail-Flag MCF10A cells were probed by western blotting with indicated antibodies.

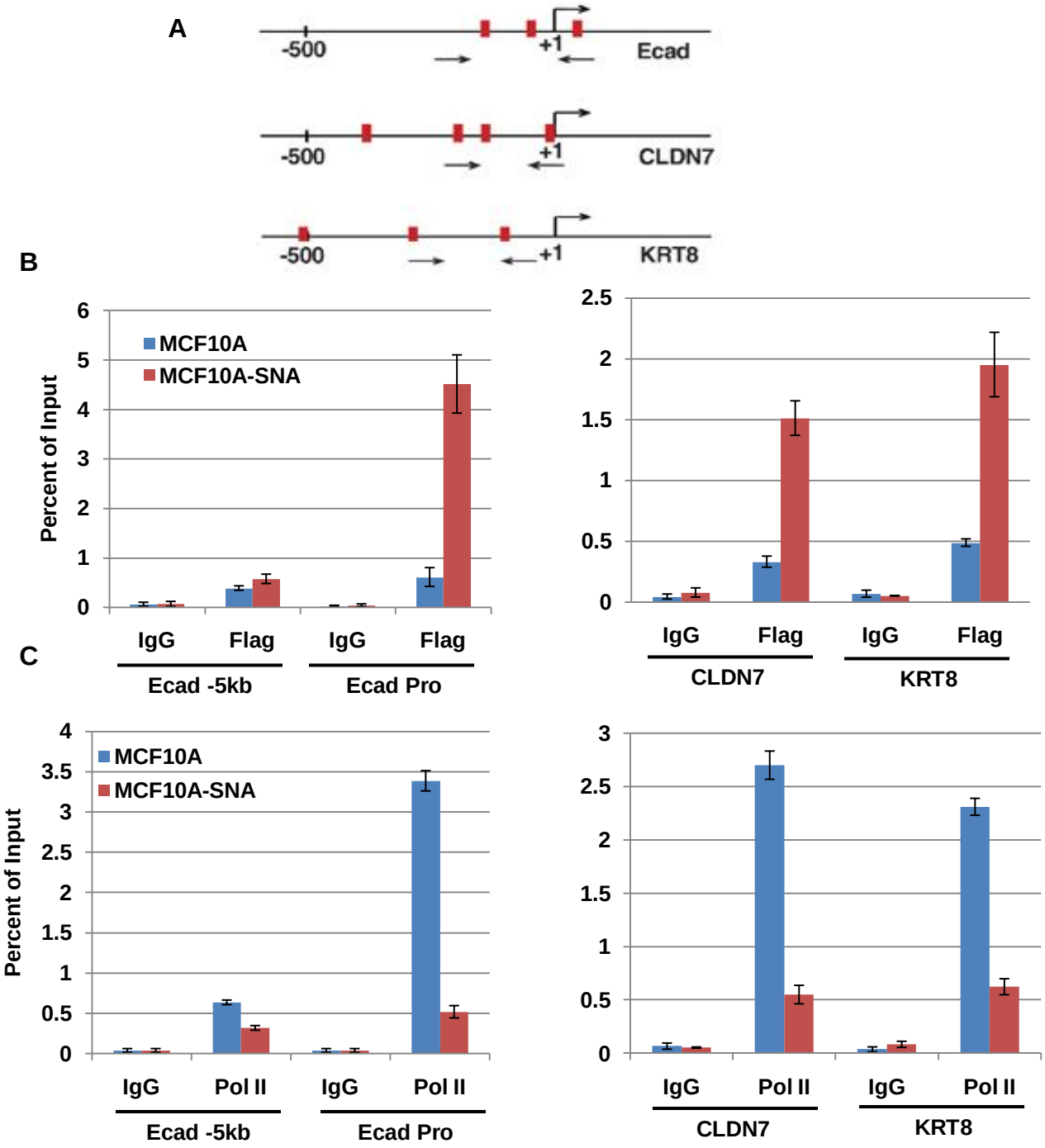


Figure 3-2. Snail binds to epithelial promoters. (A). Diagrams of the proximal promoters of E-cadherin, CLDN7 and KRT8. Vertical bars represent E-boxes. Arrows indicate primers used in chromatin immunoprecipitation (ChIP) qPCR. (B). Snail is specifically enriched at the proximal promoter of E-cadherin, CLDN7 and KRT8 *in vivo* as shown by ChIP analysis. The results are represented as percentage of input chromatin and errors indicated S.D. from triplicate experiments. (C). Expression of Snail in MCF10A dissociates RNA polymerase II from target promoters.

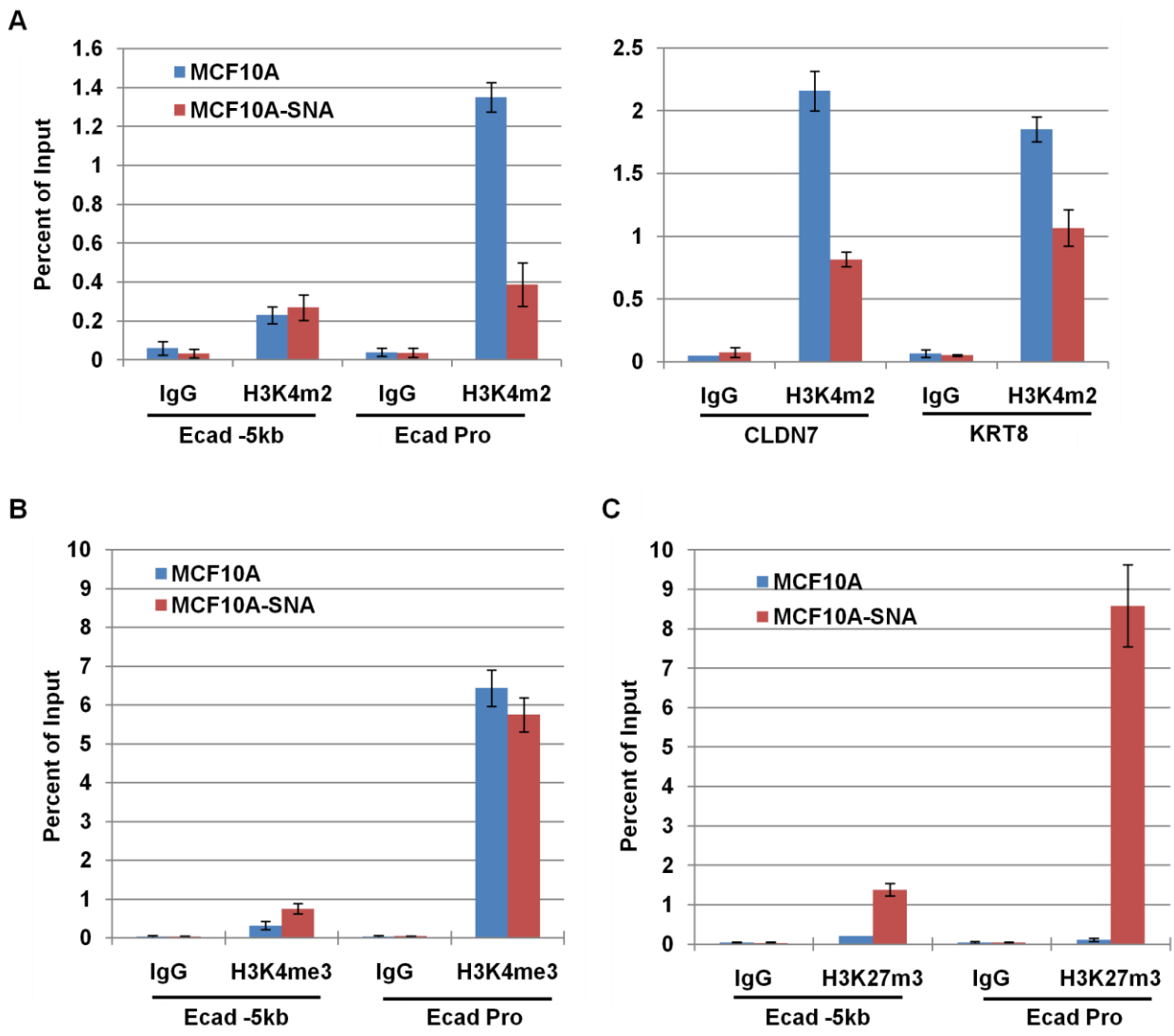


Figure 3-3. Snail reduces H3K4m2 at its target gene promoters. (A). Overexpression of Snail causes a reduction in H3K4m2 levels at the E-cadherin, CLDN7, and KRT8 promoters, but not in the 5kb upstream region of E-cadherin gene. The enrichment was determined by chromatin immunoprecipitation (ChIP) assay. IgG was used as negative control for immunoprecipitation. (B). The H3K4m3 mark at the E-cadherin promoter is not significantly affected by Snail. (C). H3K27m3 is dramatically increased at the E-cadherin promoter after overexpression of Snail.

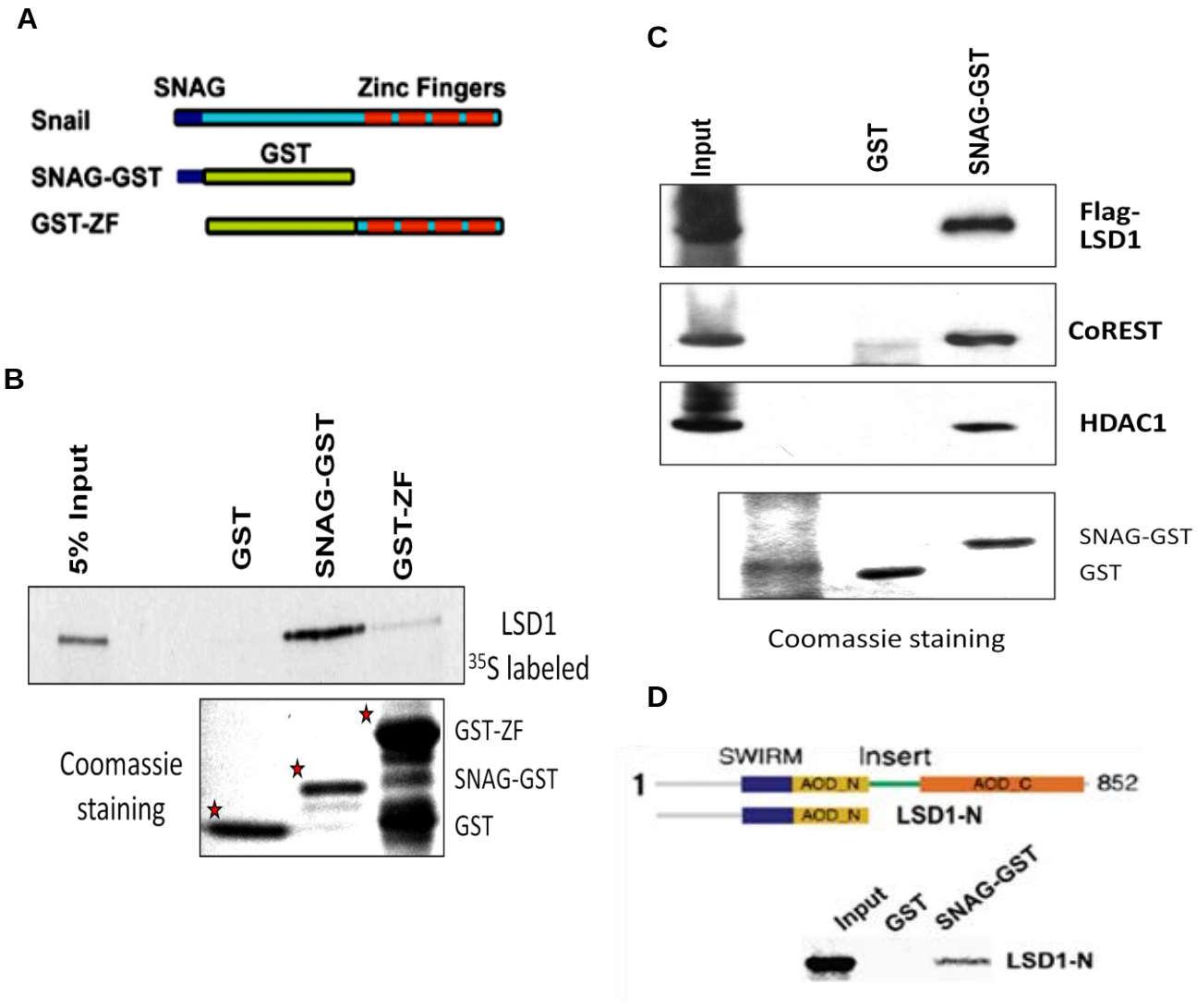


Figure 3-4. Snail physically interacts with histone demethylase LSD1 *in vitro* and *in vivo*. (A). Schematic diagram of SNAG-glutathione-S-transferase (GST) and GST-ZF fusion proteins used in GST pull-down assays. (B). LSD1 directly interacts with the SNAG domain. Full length LSD1 was *in vitro* translated and labeled with ^{35}S. The product was then mixed with GST, SNAG-GST or GST-ZF fusion proteins. Bound LSD1 was then detected by autoradiography after SAS-PAGE. Coomassie staining shows the protein loading of GST, SNAG-GST and ZF-GST. (C). The SNAG domain is sufficient for association with the LSD1 Whole cell lysates prepared from HEK293 cells transfected with Flag-LSD1 was incubated with GST or the SNAG-GST fusion protein and followed by western blotting analysis using anti-Flag, anti-CoREST and anti-HDAC antibodies. Coomassie staining of GST proteins was shown.

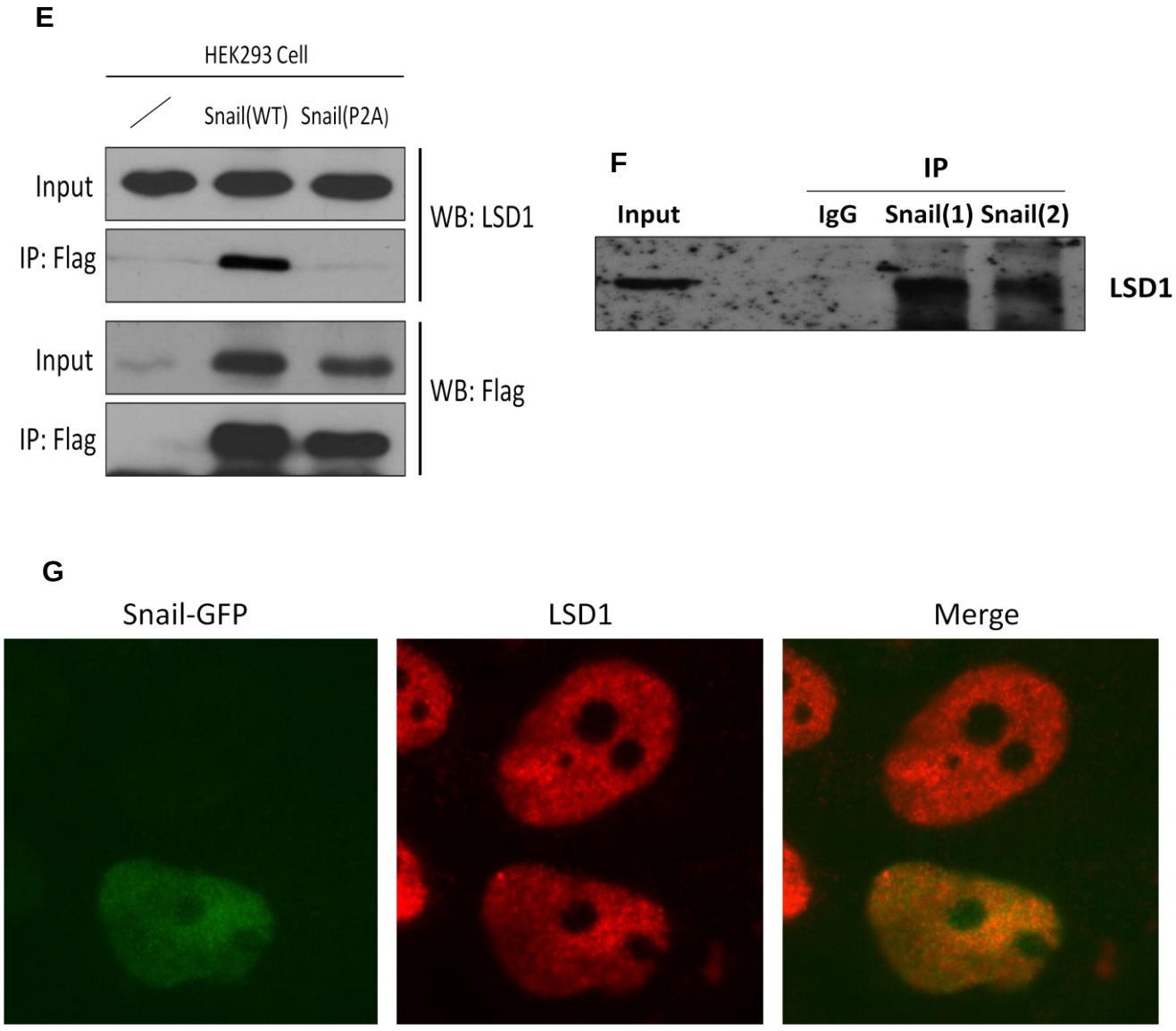
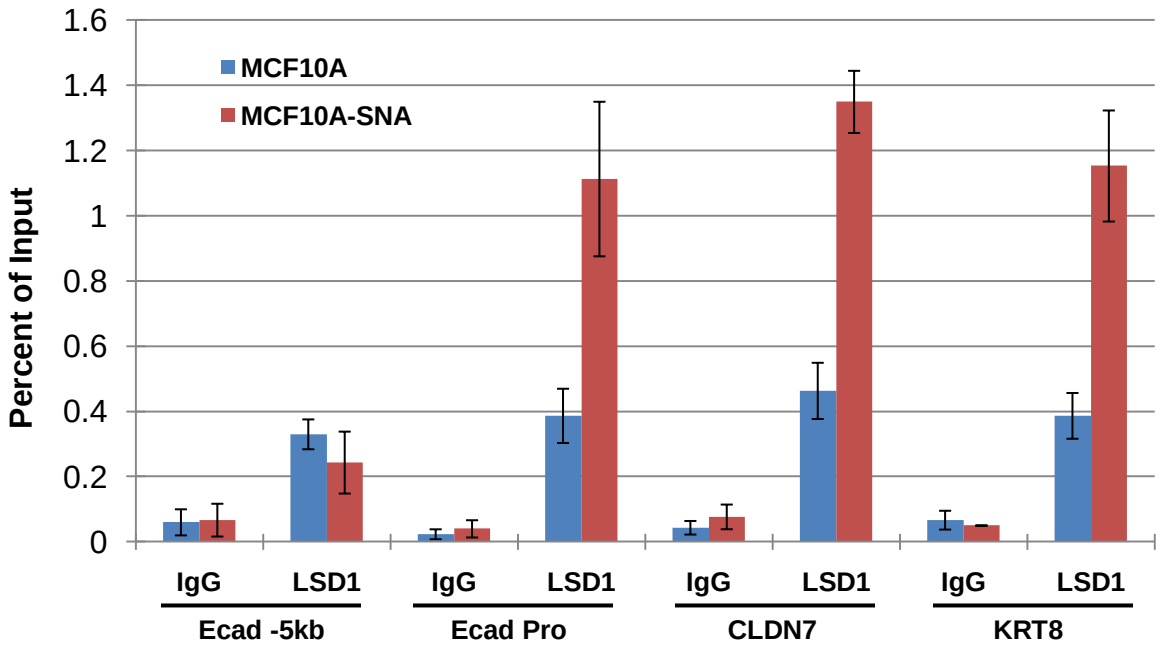


Figure 3-4 continued. (D) The amino-terminus of LSD1 interacts with SNAG. (E) The SNAG domain the required for Snail's association with LSD1in intact cells. Co-immunoprecipitation of whole cell lysates from HEK293 cells overexpressing Flag-tagged wild-type Snail or Snail-P2A mutant was performed with anti-Flag antibody. Western blotting with an anti-LSD1 antibody showed the presence of LSD1 in the precipitates. Anti-Flag western blotting indicates expression of wild-type and mutant Snail. (F) Endogenous Snail and LSD1 form a complex in vivo. MDA-MB-231 cells were lysed and incubated with two anti-Snail antibodies (#1 from Santa Cruz, #2 from Cell Signaling) or control immunoglobulin G, followed by western blotting with the LSD1 antibody. (G) Immunofluorescence for Snail and LSD1 localization in nuclei. Snail was fused with GFP protein, and endogenous LSD1 was detected by anti-LSD1 antibody.

A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1.2
1
0.8
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Percent of Input
MCF10A
MCF10A-SNA
IgG
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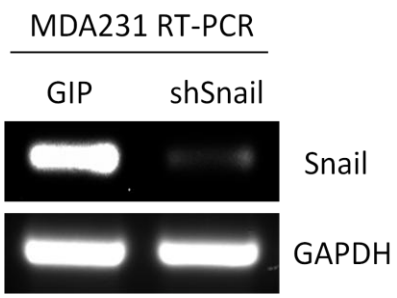
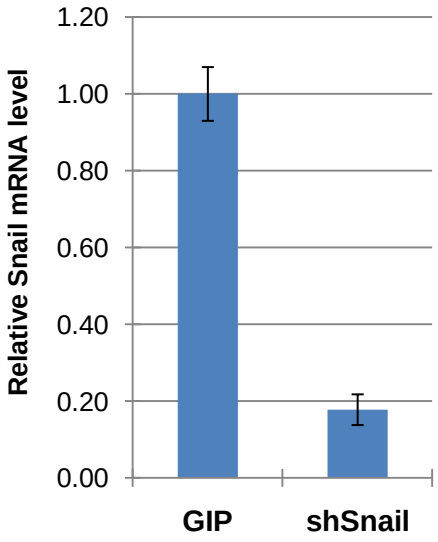
Ecad -5kb
Ecad Pro
CLDN7
KRT8

B


MDA231 RT-PCR
GIP
shSnail
Snail
GAPDH

C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Relative Snail mRNA level
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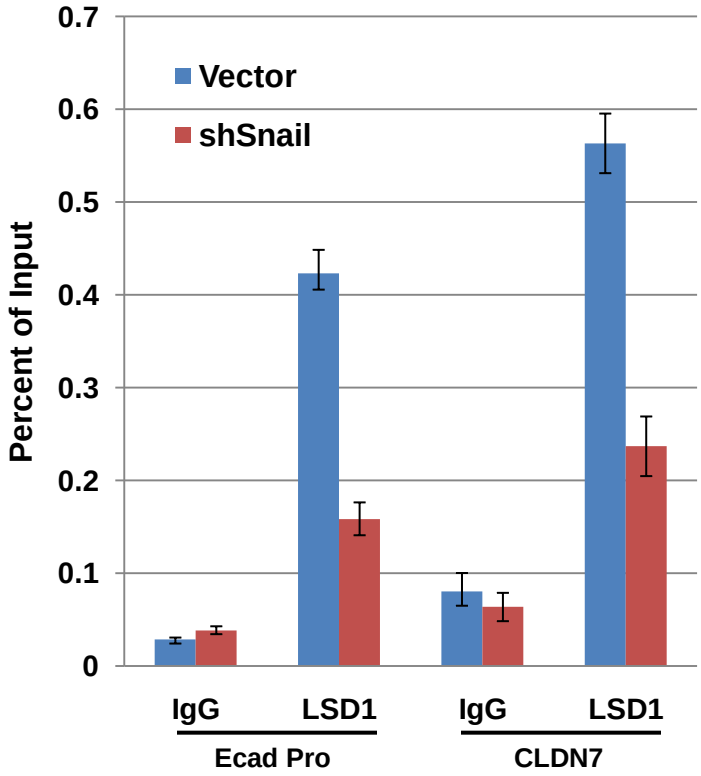
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Percent of Input
Vector
shSnail
IgG
LSD1
IgG
LSD1
Ecad Pro
CLDN7

Figure 3-5. LSD1 is recruited to epithelial gene promoters by Snail. (A). Occupancy of LSD1 at the promoters of E-cadherin, CLDN7, and KRT8 is increased in Snail-expressing cells compared with control MCF10A cells as shown by chromatin immunoprecipitation (ChIP) analysis with the LSD1 antibody. (B). Traditional RT-PCR followed by agarose gel electrophoresis shows reduction of Snail in MDA-MB-231 cells by a lentiviral short hairpin RNA. (C). The efficiency of Snail depletion was quantified by real-time PCR. Relative Snail RNA level was normalized to endogenous beta-actin. (D). The enrichment of LSD1 at the E-cadherin and CLDN7 promoters is reduced in MDA-MB-231 cells depleted of Snail.

CHAPTER 4
LSD1 IS ESSENTIAL FOR SNAIL-MEDIATED TRANSCRIPTIONAL REPRESSION

Study Background

The association of LSD1 to the chromatin template is extensively modulated by its interaction partners and local histone marks. LSD1 forms core complex with CoREST, BHC80 and HDAC1/2. This complex mediates the activation-to-repression transition of target promoters by the deacetylation of H3K9 and demethylation of H3K4 (Lan et al., 2008b). When the complex is recruited to the chromatin, HDACs first remove acetyl group from H3, allowing for CoREST binding of the hypoacetylated tail. Then LSD1 is brought closer to its substrate and demethylates H3K4m2 in a CoREST dependent manner (Lee et al., 2006). Finally, BHC80 binds to unmethylated H3K4, maintaining the complex at the promoter. This on one hand prevents H3K4 remethylation, and on the other hand induces further demethylation of neighboring nucleosomes (Lan et al., 2007).

In Chapter 3, I have shown that Snail directly interacts with LSD1 via the amino-terminal SNAG domain. During EMT, Snail recruits LSD1 to its target promoters and represses gene expression by removing the active H3K4m2 mark. Besides, other evidence shows that LSD1 is highly expressed in clinically advanced breast tumors and in poorly differentiated neuroblastomas (Lim et al., 2010; Schulte et al., 2009). Given the prominent role of Snail family members in initiation of EMT and tumor invasion, I am interested in further exploring the biological functions of LSD1 in Snail-mediated epithelial gene repression and regulation of EMT.

Results

LSD1 is Required for Snail to Repress Epithelial Genes

Luciferase reporter assay has frequently been used to test the activities of transcription factors and their associated partners. Since LSD1 might play a role in Snail-mediated repression, I first built a luciferase reporter by cloning E-cadherin promoter in front of the luciferase gene. This reporter carries three E-boxes recognized by Snail. When transfected into the MCF7 epithelial cells, it could be strongly repressed by expression of exogenous Snail (Figure 4-1A). However, in the presence of LSD1, Snail's repressive activity was further significantly augmented (Figure 4-1A).

Because ectopic expression of Snail potently repressed the E-cadherin promoter in MCF7 cells, I speculated that the endogenous LSD1 complex might have contributed to Snail-mediated gene repression. To examine whether exogenous Snail relies on endogenous LSD1 for its repressive activity, I applied retrovirus-based RNA interference (RNAi) to substantially deplete endogenous LSD1. MCF7 cells were infected with either control retrovirus or retrovirus containing short hairpin RNA against LSD1. Stable knockdown MCF7 cells were selected by puromycin for more than a week. Then both control and LSD1 depleted cells were co-transfected with Snail and reporter genes. As expected, in the absence of sufficient LSD1, Snail-mediated inhibitory effect on the reporter was essentially abolished (Figure 4-1C). Therefore, interference of endogenous LSD1 prevents the ability of Snail to repress E-cadherin promoter.

To further investigate whether inhibition of endogenous epithelial genes by Snail also depended on LSD1, I transduced either control or LSD1-depleted MCF7 cells with lentivirus simultaneously expressing both Snail and GFP. GFP-positive cells were

sorted and purified by flow cytometry, and RNA expression of epithelial genes in these cells was analyzed by real-time PCR. Similarly to luciferase reporter assays, ectopic expression of Snail dramatically reduced endogenous E-cadherin, CLDN7, and KRT8 expression in MCF7 cells (Figure 4-1D). By contrast, the repressive effect of Snail on these genes was significantly diminished in cells depleted of LSD1 (Figure 4-1D). Together these observations suggest that Snail-initiated de novo inhibition of epithelial genes is dependent on LSD1.

LSD1 Mediates Snail-Initiated EMT Process

To further assess the role of LSD1 in Snail-regulated EMT process, I decided to establish an inducible system to control Snail's function in MCF10A cells. There are two inducible systems commonly used for mammalian cells: the Tet-on/off system and the estrogen receptor (ER) fusion system. The Tet-on system permits a tight regulation of gene expression at the transcription level (Gossen et al., 1995). In this system, the expression of target gene is controlled by the tetracycline response element (TRE), an enhancer usually placed immediately upstream of a minimal CMV promoter. In the presence of doxycycline, the transactivator rtTA, which is stably integrated into the genome in advance, binds to the TRE and activates the transcription of target gene.

The Tet system has been shown very tight control of expression. However, it depends on transcription and subsequent translation of the target gene resulting in a slow response to induction. In contrast, the ER system regulates the function of the pre-expressed target protein and has a more immediate effect upon administration compared to the Tet system. The estrogen receptor is a member of nuclear hormone receptor family, and has two major domains called the DNA-binding domain (DBD) and the ligand-binding domain (LBD). The LBD of ER has been widely used to fuse to

different functional types of protein especially transcription factors to generate hormone-dependent inducible systems (Mattioni et al., 1994). In the absence of ligand, the ER-fusion proteins are generally inactivated in the heat shock protein (Hsp) silencing complexes. The binding of ligand induces the conformation change of the LBD and subsequent dissociation of fusion protein from the inhibitory complexes, renders a fully functional fusion protein (Pratt, 1990; Smith and Toft, 1993).

To build a Snail-ER inducible system, I cloned a mutated ligand binding domain of the murine estrogen receptor to the carboxyl-terminal of Snail. This modified LBD is unable to bind and be activated by β-estradiol, yet remains response to activation by 4-hydroxy-tamoxifen (4HT), a synthetic steroid (Littlewood et al., 1995). The ectopic expression of the Snail-ER fusion protein was achieved by the lentiviral infection, and positive cells were sorted out based on the co-expressed GPF signal. Following tamoxifen addition, the MCF10A-Snail-ER cells developed a mesenchymal morphology similar to MCF10A cells stably expressing Snail (Figure 4-2B). Moreover, as anticipated, the Snail-ER expressing MCF10A cells showed decreased E-cadherin expression after tamoxifen treatment for 4 days, while the control MCF10A cells have no response to tamoxifen (Figure 4-2A).

To test if LSD1 is required in this Snail-induced EMT system, I tried to deplete LSD1 in the MCF10A-Snail-ER cells. Since the MCF10A cells are unable tolerate two rounds of virus infection, I could not directly infect the MCF10A-Snail-ER cells with another lentivirus carrying shRNAs against LSD1. To overcome this problem, I build new constructs based on the pGIPz lentiviral plasmids, in which I replaced the original GFP gene on either the control vector or vectors carrying shRNA sequences against

LSD1 with Snail-ER fusion gene. With these constructs, I can express both Snail-ER and shRNAs targeting LSD1 after one lentiviral infection. Figure 4-2C showed all these three plasmids: Snail-ER-control, Snail-ER-shLSD1-F1, and Snail-ER-shLSD1-H6 expressed decent level of Snail-ER fusion protein. In addition to that, the two constructs established on LSD1 knockdown vectors successfully depleted LSD1 in Snail-ER expressing MCF10A cells (Figure 4-2C). Then I treated these cells with 4HT at 200 μM for 4 days. In the presence of endogenous LSD1, induction of Snail function can successfully repress E-cadherin expression (Figure 4-2D) and consistently promote morphological change (data not shown). However, after depletion of LSD1, Snail failed to repress E-cadherin after tamoxifen induction (Figure 4-2D). These data suggest LSD1 is required for the Snail-regulated initiation of EMT process.

LSD1 is Essential for Maintenance of the Silenced State of Snail Target Genes

E-cadherin and other epithelial genes are commonly silenced in highly invasive cancer cells such as MDA-MB-231 or MDA-MB-435. To investigate whether LSD1 is required for maintenance of the silenced status of these genes in MDA-MB-231, I knocked down endogenous LSD1 with the same two lentiviral short hairpin RNAs as used in previous experiment (Figure 4-3A). As I expect, depletion of LSD1 lead to an increase of the E-cadherin and CLDN7 RNA levels as indicated by qRT-PCR (Figure 4-3B), suggesting that disruption of LSD1 activity derepresses these genes. Moreover, treatment of MDA-MB-231 cells with the DNA methylation inhibitor 5'-Aza resulted in re-activation of E-cadherin as detected by RT-PCR. And the upregulation of E-cadherin mRNA level is comparable to the LSD1 knockdown effect (Figure 4-3C). Simultaneous LSD1 knockdown and 5'-Aza treatment apparently had an additive effect on E-cadherin

activation (Figure 4-3C). Nevertheless, these elevated E-cadherin levels in MDA-MD-231 are still much lower than those in typical epithelial cells such as MCF7, indicating that E-cadherin re-activation is incomplete. Similar partial derepression of E-cadherin was reported when the PRC2 repressive complex was inactivated (Herranz et al., 2008). The limited effect is most likely due to multiple repression mechanisms at the E-cadherin promoter in the MDA-MB-231 cells.

Since LSD1 demethylates H3K4m2, I hypothesized that the increased expression of Snail targets could be due to the upregulation of the active H3K4m2 mark. Thus I analyzed H3K4m2 levels at the E-cadherin promoter by ChIP assays in control and LSD1 depleted MDA-MB-231 cells. As a consequence, a dramatic increase of H3K4m2 specifically at the E-cadherin promoter was observed in the LSD1-depleted cells (Figure 4-3D). These results imply that LSD1 is necessary to reinforce silencing of epithelial genes in these cells by consistently erasing the H3K4m2 mark to prevent its accumulation at Snail-targeted promoters.

E-cadherin is Upregulated in LSD1-Depleted Tumors

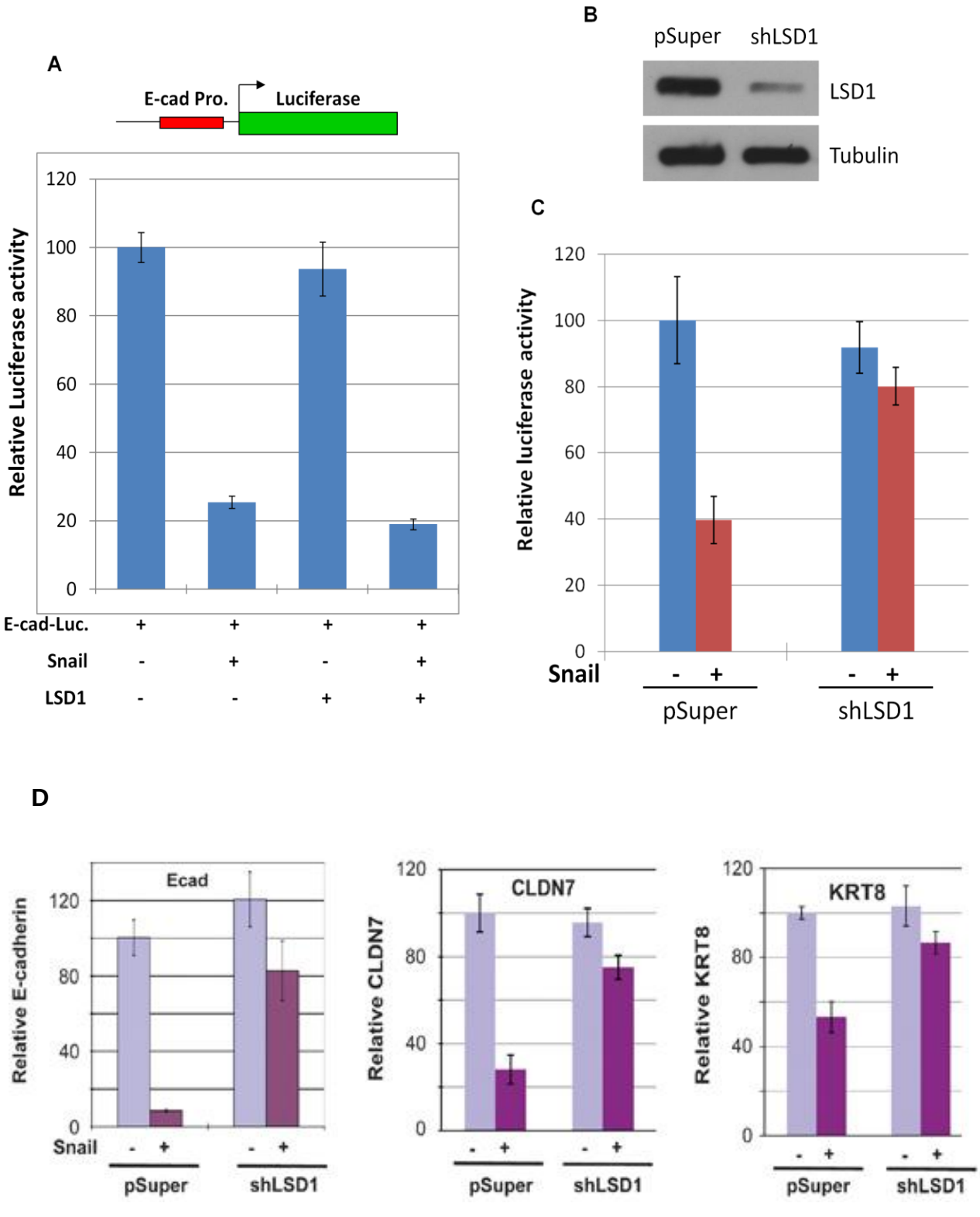
Depletion of LSD1 in MDA-MB-231 cells resulted in partial depression of epithelial genes, but the relative expression levels remained low comparing to typical epithelial cells. The increased enrichment of H3K4m2 at the E-cadherin promoter after LSD1 knockdown may not be sufficient to activate E-cadherin, but rather provide it with a poised status. The full activation of E-cadherin could require additional signals which are missing in the *in vitro* culture system. I hypothesized that mesenchymal invasive tumor cells after depleting of LSD1 might be more readily to activate E-cadherin and to undergo mesenchymal-epithelial transition (MET) *in vivo*. To test this idea, I first generated control and LSD1-depleted MDA-MB-231 cells via lentiviral infection. Cells

were injected into the abdominal mammary fat pad of immunocompromised mice and total five injections for each cell line. MDA-MB-231 has been reported as an aggressive tumorigenic breast cancer cell line (Moody et al., 2005). As expected, the tumors started to be visible after 4-5 weeks of injection. And then the growth of tumors was recorded every week. I found depletion of LSD1 promotes tumor growth *in vivo* (Figure 4-4A). A recent study showed LSD1 negatively regulates TGFβ pathway (Wang et al., 2009a). Depletion of LSD1 in tumor cells might enhance the TGFβ pathway activity which in turns promotes cell proliferation. Next, E-cadherin RNA level in tumor samples was examined by qRT-PCR and knockdown of LSD1 showed roughly 5-fold upregulation of E-cadherin expression (Figure 4-4B). This *in vivo* result indicates that the presence of LSD1 helps maintain the silencing of E-cadherin in tumor cells.

Summary

In this chapter, I have shown that LSD1 serves as an essential effector of Snail-dependent transcriptional regulation of epithelial-mesenchymal transition, as depletion of LSD1 substantially impairs Snail's ability to repress its target promoters as well as to initiate EMT (Figure 4-1 & Figure 4-2). Besides, LSD1 is involved in maintaining the silenced status of these genes in invasive mesenchymal tumor cells (Figure 4-3). However the exact mechanism underlying Snail-mediated transcriptional repression could be complex. I only detected a partial derepression of E-cadherin and have not observed any morphological reversion from mesenchymal phenotype to epithelial phenotype in LSD1-depleted MDA-MB-231 cells. This indicates removing of LSD1 might not be sufficient to fully activate epithelial gene expression program. It is interesting to

investigate other additional epigenetics regulations occurred at these promoters and to test whether they are mediated by Snail genes.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E-cad Pro.
Luciferase
Relative Luciferase activity
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60
40
20
0
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Snail - + - +
LSD1 - - + +
B
pSuper
shLSD1
LSD1
Tubulin
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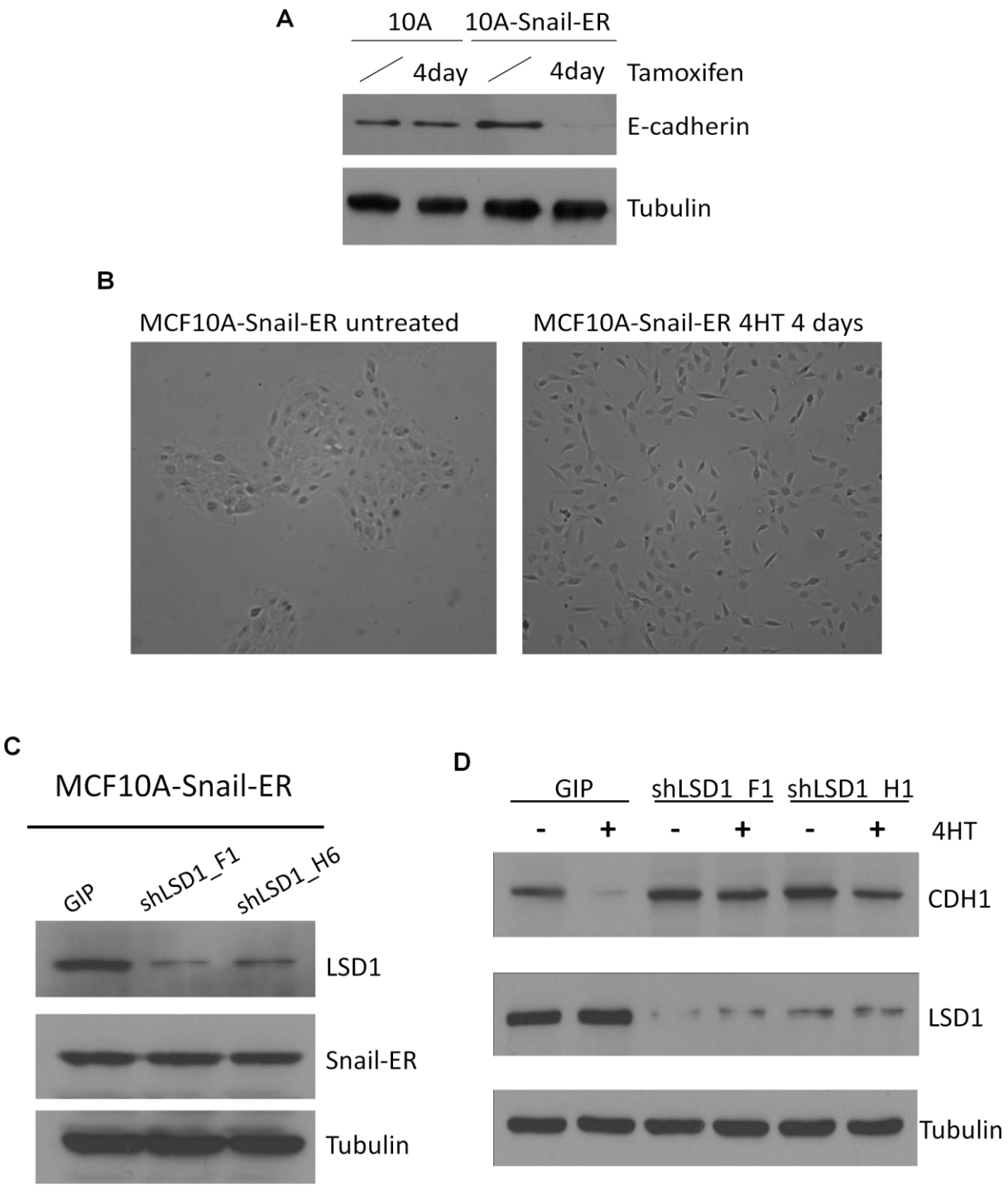
- + - +
pSuper
shLSD1
KRT8
Relative KRT8
120
80
40
0
- + - +
pSuper
shLSD1

Figure 4-1. LSD1 is essential for Snail-mediated repression. (A). LSD1 augments Snail's repressive activity. E-cadherin promoter region containing three E-boxes was cloned before luciferase gene. Luciferase assays were performed. Snail gene was transfected into MCF7 cells with or without LSD1-expressing plasmid. (B). Verification of LSD1 knockdown by western blotting. MCF7 cells were infected with retroviral empty vector pSuper or with short hairpin RNA targeting LSD1, followed by western blotting assays. (C). Depletion of LSD1 impairs the repressive activity of Snail in reporter-based assays. E-cadherin promoter region was cloned into pGL3 reporter vector and co-transfected with Snail-expressing plasmid into control or LSD1-depleted cells. Snail failed to repress the reporter gene in absence of LSD1. Error bars indicated S.D. from three independent experiments. (D). LSD1 is essential for Snail-mediated repression of endogenous epithelial genes. Expression of E-cadherin, CLDN7, and KRT8 was determined by quantitative RT-PCR.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4day
4day
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Tubulin
B
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C
MCF10A-Snail-ER
GIP
shLSD1_F1
shLSD1_H6
LSD1
Snail-ER
Tubulin
D
GIP
shLSD1_F1
shLSD1_H1
- + - + - +
4HT
CDH1
LSD1
Tubulin

Figure 4-2. LSD1 mediates Snail-initiated EMT process. (A). Verification of Snail-ER inducible system in MCF10A cells by western blotting. Both parental MCF10A and Snail-ER expressing MCF10A were treated with 200 nM of 4-hydroxyl-tamoxifen (4HT) for four days before harvested. (B). Phase-contrast image of Snail-ER expressing MCF10A cells before and after induction. (C). Depletion of LSD1 in MCF10A-Snail-ER cells. Western blotting indicates LSD1 knockdown efficiency and Snail-ER expression. Tubulin was probed as loading control. Two independent shRNAs targeting LSD1 were used to exclude non-specific target effects. (D). Depletion of LSD1 abolished Snail induced downregulation of E-cadherin. In control cells, treatment of 4HT can efficiently induce Snail's repressive function on E-cadherin. However, this activity was diminished in LSD1 depleted Snail-ER expressing cells.

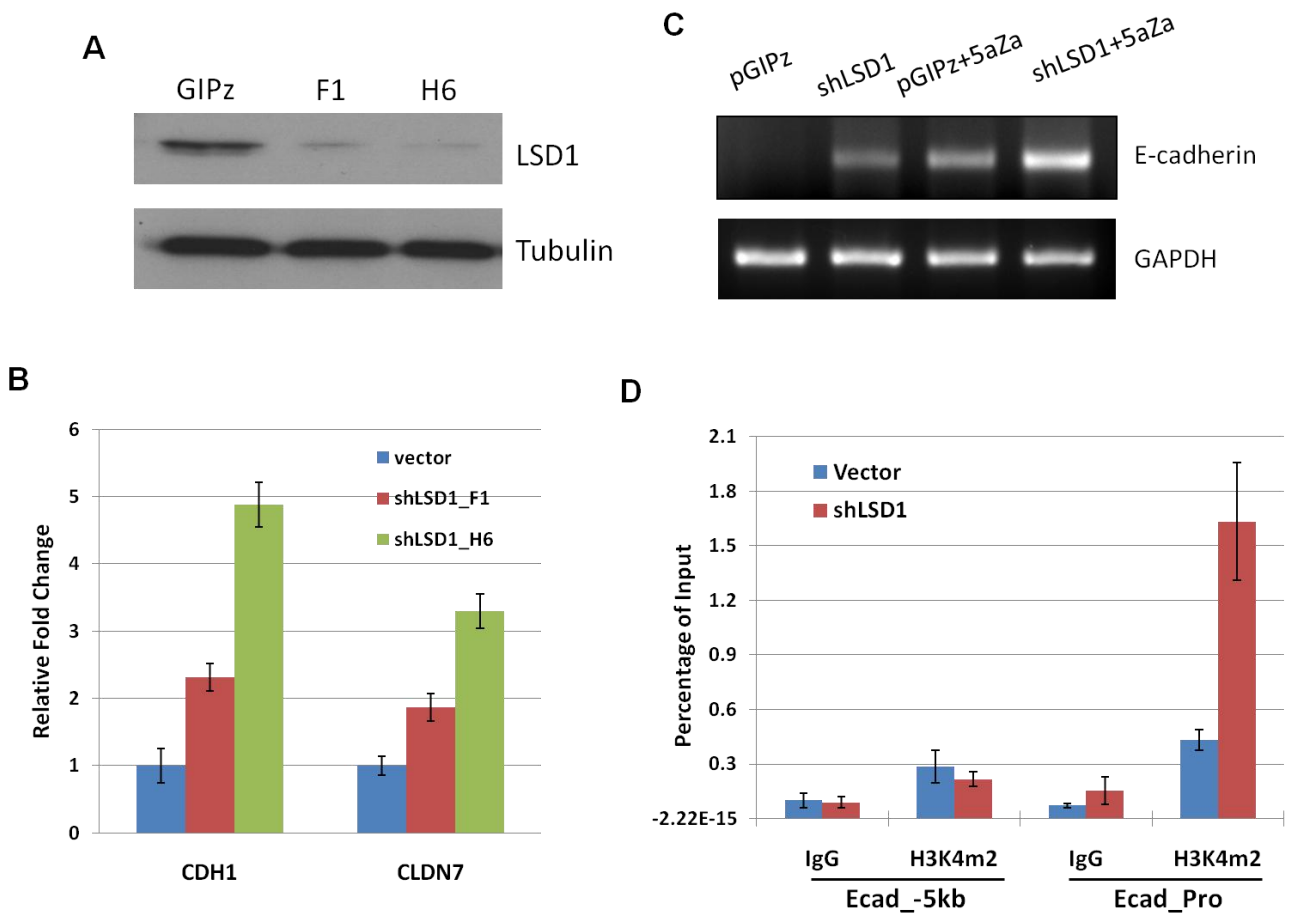


Figure 4-3. LSD1 is required to maintain the silenced status of Snail target genes in invasive cancer cells. (A). Validation of LSD1 depletion in MDA-MD-231 cells with two short hairpin RNAs against LSD1 by western blotting with denoted antibodies. (B). Expression of E-cadherin and CLDN7 is upregulated in MDA-MD-231 cells depleted of LSD1. The RNA levels of the two genes were normalized to GAPDH by quantitative RT-PCR. (C). 5'aZa treatment enhances LSD1's knockdown effect. Both control and LSD1 depleted cells were treated with 5 μM 5'aZa for 48 hours. E-cadherin expression was showed by regular RT-PCR. (D). LSD1 depletion in MDA-MD-231 cells increases H3K4m2 levels specifically at the E-cadherin promoters, as determined by chromatin immunoprecipitation (ChIP) assays.

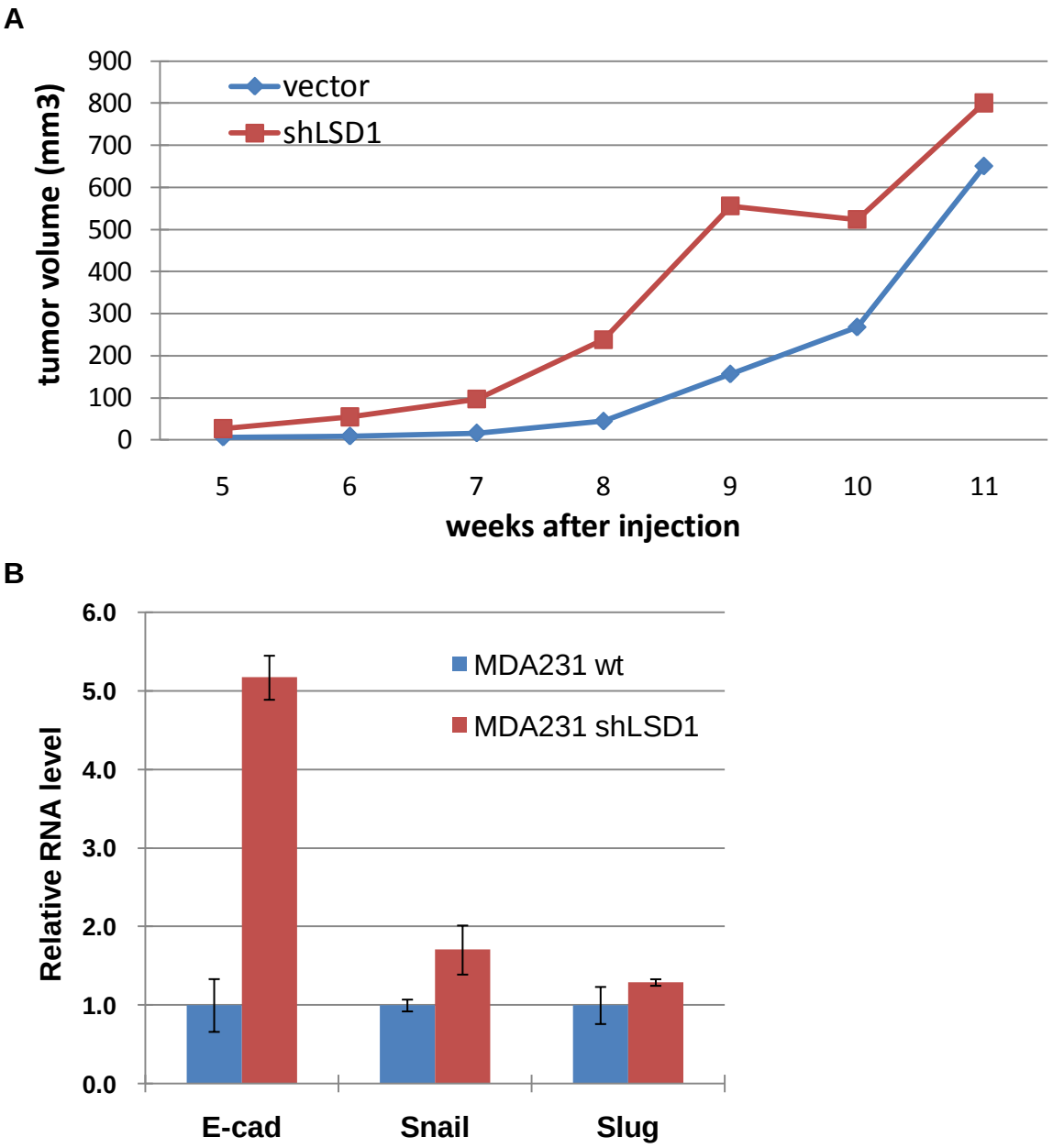


Figure 4-4. Mammary fat pad tumor xenograft assay. (A).Tumor growth curve. $5x10^6$ of control or LSD1-depleted MDA-MB-231 cells were injected into each immunodeficient mice. Tumors started to be visible after five weeks of injection. The size of tumor was measured every week with a rule and the volume was calculated based on the following formula: volume(mm^3) = (4/3)*p*(radius)^3. (B). E-cadherin was upregulated in LSD1-depleted tumors. Mice were sacrificed after 11 weeks of injection. RNAs were extracted from tumor samples by Trizol reagent. Relative RNA levels were measured by real-time RT-PCR and were normalized to β-actin levels. Error bar indicates standard deviation from three independent experiments.

CHAPTER 5
ADDITIONAL EPIGENETIC EVENTS ASSOCIATED WITH SNAIL

Snail and DNA Methylation

In my study, I have highlighted the significance of the LSD1 complex in Snail's function. In addition to that, Snail has been reported to directly or indirectly associate with repressor complexes including HDAC, PRC2, and Ajuba-PRMT5, through its SNAG domain (Herranz et al., 2008; Hou et al., 2008; Peinado et al., 2003a). It is interesting to determine whether all these components are all in the same complex, or they interact with Snail independently, as they are all somewhat essential for Snail's activity. As a consequence of these interactions, Snail leaves multiple repressive histone modifications at its target genes, such as decreased H3K4m2, increased H3K27m3, and histone hypoacetylation.

In addition to these modifications, could there be any more epigenetic event that might be introduced by Snail? One possible candidate is DNA cytosine methylation, a marker of gene silencing. It has been long known that hypermethylation of the promoter sequences of epithelial genes especially E-cadherin occurs frequently in various breast cancer cell lines and primary ductal breast cancers (Graff et al., 1995; Herman et al., 1996). However, in contrast to this well established event, the exact mechanisms that trigger the DNA methylation during cancer progression remains mystery. Interestingly, a study profiled E-cadherin expression in different mammary cell lines. It was found that E-cadherin promoter methylation, but not mutational inactivation, was highly enriched in fibroblastic invasive cell lines, suggesting a potential association between E-cadherin promoter methylation and EMT reprogramming during evolving of tumors (Lombaerts et al., 2006). Moreover, sustained activation of the EMT program by TGFβ in primary

human mammary epithelial cells (HMEC) induces *de novo* DNA methylation at the E-cadherin promoter. And this methylation maintains after withdrawal of TGFβ (Dumont et al., 2008). Given these lines of evidences, it is reasonable to think that Snail might play a role in introducing DNA methylation at the E-cadherin promoter. I then surveyed DNA methylation at the E-cadherin promoter in cells after EMT by bisulfate genomic sequencing with the help from Dr.Lingbao Ai. However, neither the MCF10A-Snail cells nor the MCF10A-Snail-ER cells after tamoxifen treatment showed any significant increase of DNA methylation comparing to their epithelial controls (data not shown). This indicates Snail probably does not have direct impact on DNA methylation. And DNA methylation itself at the E-cadherin promoter could be a late onset and sporadic event as EMT progressed. Besides, *de novo* DNA methylation could also be prevented due to the presence of the H3K4m3 modification (Figure 3-3B) which has been shown to block the binding of the *de novo* DNA methyltransferase subunit DNMT3L to the H3 tail (Ooi et al., 2007).

Identification of the Snail Complex

Purification of Factors Associated with Snail

In searching for more epigenetic factors associated with Snail's function, we undertook a proteomic approach to identify Snail associated proteins. HEK293 cells were stably transfected with the Snail-Flag construct. The stable expression of Snail was much lower than transient transfection and was comparable to endogenous levels of Snail as in other mesenchymal cells. The purification was performed in collaboration with Dr.Huangxuan Shen based on the protocol developed in the laboratory of Dr.Lizi Wu. The Snail-Flag protein and associated polypeptides from nuclear extracts were isolated by anti-Flag affinity chromatography. Then isolated products were visualized by

SDS-PAGE analysis and silver staining (Figure 5-1A). Expectedly, identification of Snail-Flag interacting proteins by mass spectrometry indicated an abundant association with components of the LSD1-CoREST-CtBP corepressor complex (Figure 5-1B), which further confirms our previous discovery about the Snail-LSD1 interaction. In addition to these factors, we also identified two interesting MBT domain containing proteins SFMBT1 and L3MBTL.

SFMBT (known as Scm-like with four mbt domains) is a newly identified polycomb group (PcG) protein. Members of PcG regulate the transcription of developmental-associated genes such as *Hox* genes by creating a repressive chromatin structure (Schwartz and Pirrotta, 2007). Currently, there are three known PcG complexes: two well-characterized classical PcG complexes (PRC1 and PRC2) and the recently recorded PhoRC (Schwartz and Pirrotta, 2008). The main component of the PhoRC complex is Pleiohomeotic (Pho), homologous to the mammalian factor YY1. Pho/YY1 is a sequence specific DNA binding protein and often associated with PcG complexes. It has been shown to mediate the recruitment of PcG complexes to Polycomb Response Elements (PREs) (Mohd-Sarip et al., 2006). Another essential component in the PhoRC complex is the SFMBT protein which forms heterodimer with Pho/YY1 and has been shown required for *Hox* gene silencing (Klymenko et al., 2006). SFMBT is a potent repressor of transcription, however besides this, little is currently known about its biological function. The mammalian version of SFMBT was first cloned in the year 2000 (Usui et al., 2000). So far, two structurally related human homologous (hSFMBT1 and hSFMBT2) have been identified. Both of these two proteins contain four tandem Malignant Brain Tumor (MBT) domains at amino-terminal part and a conserved

protein-interacting Sterile Alpha Motif (SAM) domain near the carboxyl-terminus (Figure 5-2A). The MBT domain is found in several PcG proteins such as the lethal(3) malignant brain tumor-like (L3MBTL) and the sex comb on midleg-like2 (SCML2), and invariably exists in tandem arrays of two to four repeats. The MBT domain is a “chromatin reader”. It recognizes mono- and di-methylated lysines at a number of different positions on histone H3 and H4 tails (Bonasio et al., 2010). In addition, all four MBT domains in SFMBT have been shown required for repressor activity indicating a higher-order structure might be formed by the four MBT repeats (Wu et al., 2007).

Despite the repressive activity of SFMBT proteins in PcG complexes-mediated transcriptional repression, little evidence currently is known about their biological functions in various cellular processes. Some of the indications are from studies in *Drosophila*. For example, *Drosophila Sfmbt* null mutant displays a classic *Polycomb* phenotype (Klymenko et al., 2006). Besides, a genome-wide RNAi screen in cultured *Drosophila* cells identified both dL3MBT and dSFMBT as key regulators of E2F activity. They are recruited to E2F-responsive promoters through physical interaction with E2F and are required for repression of endogenous E2F target genes (Lu et al., 2007).

Validation of Snail and SFMBT1 Association

To confirm physical association between Snail and SFMBT1 or L3MBTL, co-immunoprecipitation assays were performed in HEK293 cells expressing exogenous Snail and Flag epitope-tagged two MBT proteins. Snail was co-immunoprecipitated with both Flag-SFMBT1 and Flag-L3MBTL with anti-Flag antibody (Figure 5-2B). Whole cell lysates from HEK293 cells expressing only exogenous Snail protein were used as control for co-immunoprecipitation. Human Snail has an analog named Slug. These two proteins share high level of structural similarity and functional redundancy (Barrallo-

Gimeno and Nieto, 2009). Similarly, I found exogenous Slug is also associated with both Flag-SFMBT1 and Flag-L3MBTL in co-immunoprecipitation analysis (Figure5-2C). To further confirm the interaction between Snail and SFMBT1, the cell lysates were subject to co-immunoprecipitation with anti-Snail antibody and the presence of Flag-tagged SFMBT1 in the precipitates was further verified by western blotting with anti-Flag antibody. Consistent with previous result, F-SFMBT1 was also found in the immune-precipitated exogenous Snail complex (Figure5-2D).

Snail Reduces the Accessibility of E-cadherin Promoter Region

I have shown that Snail forms complex with SFMBT1, which has been demonstrated as a potent transcriptional co-repressor (Wu et al., 2007). SFMBT1 is a component of the Pho repressor complex (PhoRC). It binds preferentially to mono- or di-methylated lysines on histone tails and is proposed to contribute to repression by compacting nucleosomal arrays (Grimm et al., 2009). To test the idea that Snail may induce the chromatin structure change around the transcription start site of the E-cadherin gene, I measured the relative accessibility to DNase digestion of this region before and after the induction of Snail function. First, MCF10A-Snail-ER cells were treated with 4HT, which consistently resulted in reduced expression of E-cadherin and increased binding of Snail to the E-cadherin promoter (Figure 5-3 A&B).Then nuclei were isolated from MCF10A-Snail-ER cells treated or not with 4HT and incubated with DNase. Digested DNA was extracted by phenol-chloroform and was subjected to q-PCR analysis to quantify the abundance of uncut DNA of the TSS of E-cadherin gene as well as in the TSS sites of GAPDH and HBB. Before the 4HT treatment, the region flanking E-cadherin transcription start site was largely accessible to DNase digestion, similar to the constitutively expressed GAPDH gene. The hemoglobin beta gene that is

silenced in MCF10A showed relative high resistance to the enzyme (Figure 5-3C). Notably, 4HT treatment significantly inhibited the DNase access to the E-cadherin TSS while had no affect on other two control regions. This result suggests that Snail induces a more condensed and closed chromatin structure around the transcription start site of E-cadherin gene.

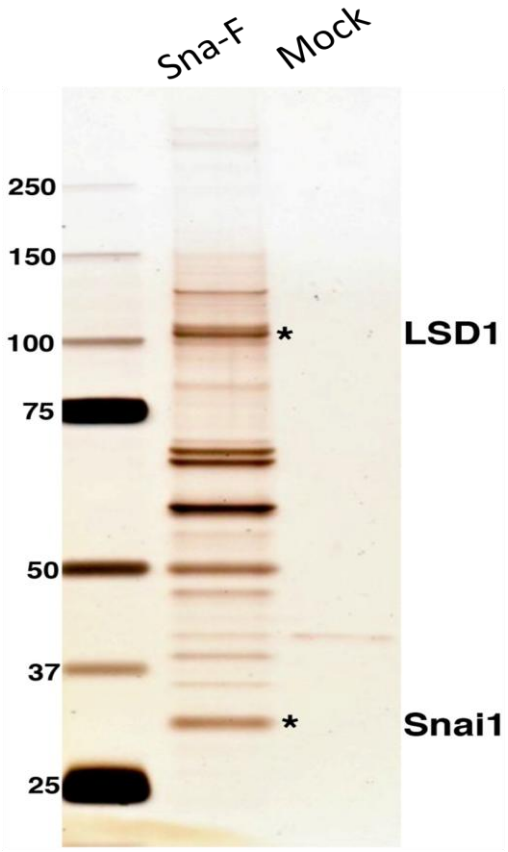
Summary

In this chapter, I tried to identify additional epigenetic events associated with Snail. CpG dinucleotides at the E-cadherin promoter region are frequently methylated in various aggressive cancers (Graff et al., 1995). It has been reported that long term induction of EMT increased DNA methylation level at different epithelial promoters including E-cadherin. I asked whether Snail can contribute to the DNA methylation. However I failed to observe any increase in DNA methylation after overexpression of Snail. Next, we performed a proteomic assay to detect factors present in the Snail complex. Interestingly, in addition to the components of the LSD1 complex, we also identified SFMBT1 and L3MBTL in the immunoprecipitates (Figure 5-1).I further confirmed the interaction between Snail and these two proteins in HEK293 cells (Figure 5-2). Since both of SMBT1 and L3MBTL have nucleosomal compacting activity (Bonasio et al., 2010), I compared the chromatin accessibility flanking the transcription start site of E-cadherin between MCF10A-Snail-ER cells with or without 4HT treatment. Addition of 4HT induced binding of Snail to the E-cadherin promoter, resulting in a relatively closed form at the TSS region (Figure 5-3), which may contribute to the repression of E-cadherin.

For further studies, it is necessary to investigate whether SFMBT1 is recruited by Snail to its targets during EMT, and whether the change in chromatin accessibility is

general to other targets of Snail. Besides, both SFMBT1 and LSD1 were found in Snail immunoprecipitates, it is interesting to resolve the relationship of these proteins, such as whether they form a big complex or SFMBT1 and LSD1 associate with Snail independently. Since the role of SFMBT1 in carcinogenesis is rarely studied, we also plan to test if depletion of SFMBT1 has any effect on the malignant properties of tumors, including uncontrolled growth, invasiveness as well as metastasis.

A



B

Gene Name	Peptide #	MW (KDa)
LSD1	59	93
Rcor1,2,3	92	53,50,56
GSE1	58	136
HDAC1,2	51	55,55
ZNF198	44	155
BHC80	20	79
ZNF217	18	115
CtBP1,2	12	48,49
SFMBT1	7	98
L3MBTL	3	84

Figure 5-1. Identification of the Snail complex. (A). Flag-tagged Snail was stably expressed in HEK293 cells, and nuclear extracts were prepared. Snail-Flag and associated proteins were immunoaffinity purified and eluted with Flag peptide. Silver-stained SDS-PAGE gels showed that multiple polypeptides specifically associated with Snail-Flag as compared to control extracts from untransfected cells. (B). Tandem mass spectrometry (MS-MS) identified numerous interacting proteins, including multiple components of the LSD1/CoREST complex as well as two additional MBT proteins.

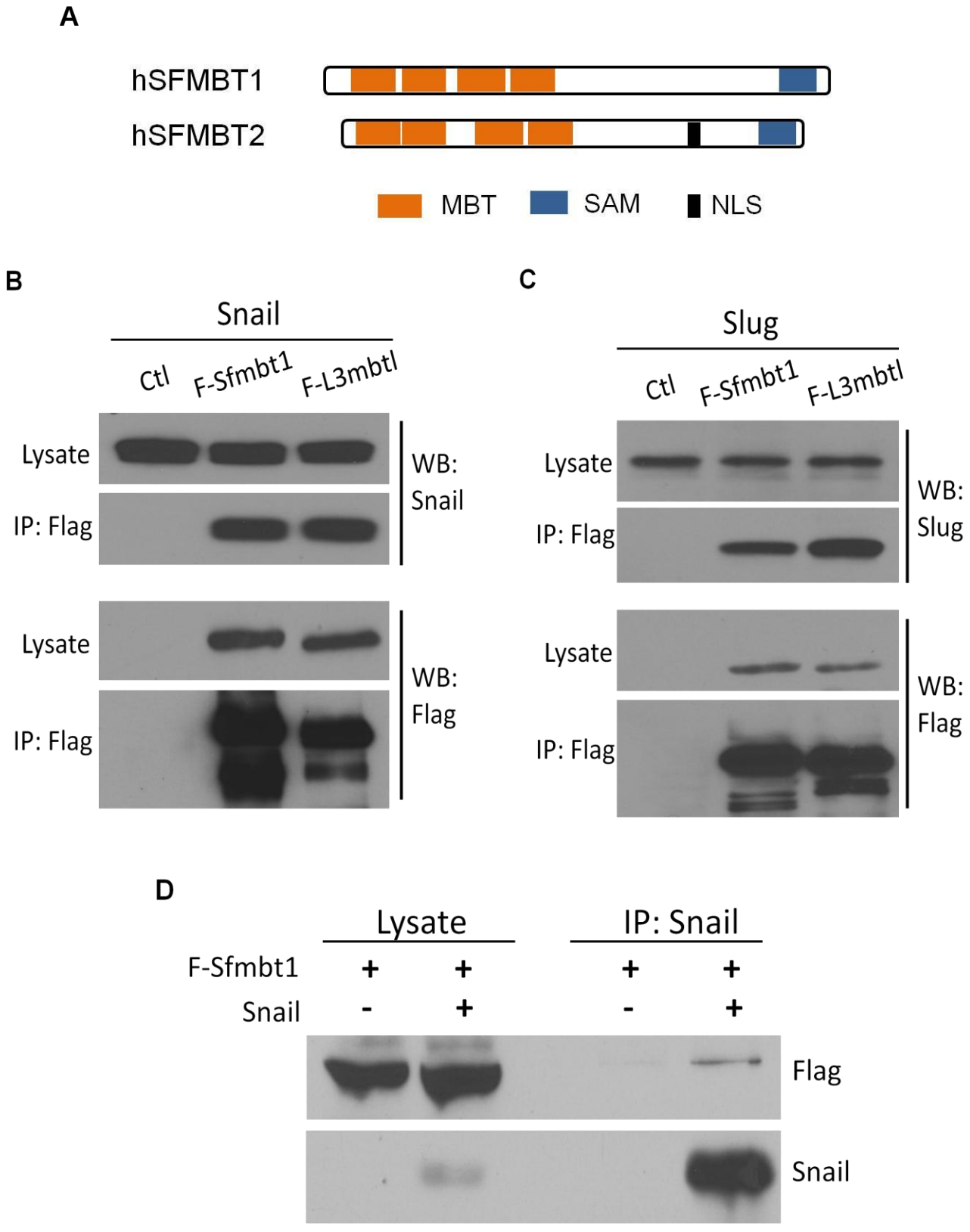

A
hSFMBT1
hSFMBT2
MBT
SAM
NLS
B
Snail
Ctl
F-Sfmbt1
F-L3mbtl
Lysate
IP: Flag
WB: Snail
WB: Flag
C
Slug
D
Lysate
IP: Snail
F-Sfmbt1
Snail
Flag

Figure 5-2. Confirmation of Snail and SFMBT1/L3MBTL interaction. (A). Schematic representative of the structure of two human SFMBT homologous. Both of them have four MBT repeats at the amino-terminal parts and a SAM domain at the carboxyl-terminus, while hSFMBT2 has one nuclear localization signal motif in the middle. (B). Western blotting shows co-immunoprecipitation of Snail with Flag-SFMBT1 and Flag-L3MBTL. Snail was co-transfected with either of the MBT proteins in HEK293 cells. The whole cell lysates were subject to immunoprecipitation with anti-Flag antibody followed by western blotting with indicated antibodies. (C). Similar co-immunoprecipitation assay shows Slug is associated with Flag-SFMBT1 or Flag-L3MBTL as well. (D). Co-immunoprecipitation was performed with anti-Snail antibody. And the presence of Flag-SFMBT1 in the precipitates was verified by anti-Flag antibody. Anti-Snail western blotting indicates the immunoprecipitation efficiency of exogenous Snail.

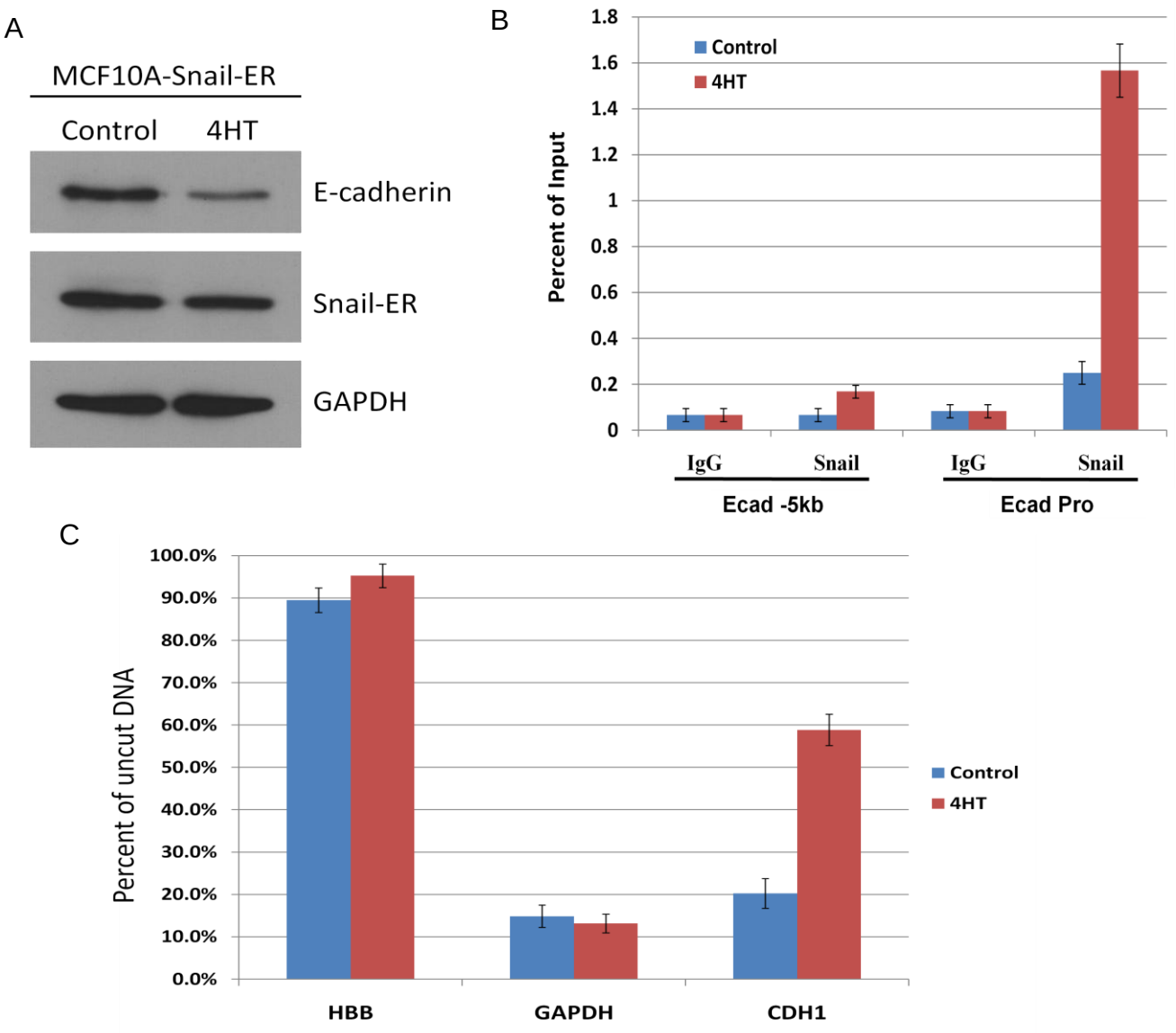


Figure 5-3. Snail reduces the accessibility of E-cadherin promoter region. (A). MCF10A cells ectopically expressing Snail-ER fusion protein were treated with 200 nM of 4HT for five days before harvest. Expression levels of E-cadherin and Snail-ER were verified by western blotting. The housing keeping gene glyceraldedyde-3-phosphate dehydrogenase (GAPDH) was probed as loading control. (B). Snail-ER bound to E-cadherin promoter region after 4HT treatment as shown by chromatin-immunoprecipitation assay. (C). Nuclei were isolated from both untreated and 4HT treated MCF10A-Snail-ER cells and then digested with DNase. DNA was purified and followed by real-time PCR quantification with primers corresponding to the transcriptional start site (TSS) of either HBB, GAPDH or CDH1. Uncut DNA template was shown as percentage of input DNA which was not digested with DNase.

CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

Epithelial-mesenchymal transition (EMT) is a key step during the early stage of tumor progression. Elucidation of the transcriptional and epigenetic regulatory mechanism that controls EMT is crucial for the development of successful therapeutic interventions for metastasis. The Snail family of zinc finger transcriptional repressors is designated as the master regulator of EMT. Snail can induce EMT by downregulating numerous epithelial cell markers (Nieto, 2002). In this dissertation, I have investigated the epigenetic mechanism underlying Snail-mediated transcriptional repression of these epithelial genes. It is shown that ectopically expressing Snail in MCF10A cells promotes epithelial to mesenchymal morphological changes accompanied by the downregulation of several epithelial markers. I discovered the histone demethylase LSD1 as a co-repressor of Snail. During EMT, Snail directly interacts with LSD1 via its amino-terminal SNAG domain and recruits the LSD1 complex to the E-cadherin and other epithelial gene promoters, resulting in downregulation of the active H3K4m2 mark and promoter activity. I further showed depletion of LSD1 in epithelial cells substantially impairs Snail's ability to repress its targets and to induce EMT. LSD1 serves as an essential effector of Snail-dependent transcriptional repression of epithelial genes. Knockdown of LSD1 in invasive tumor cells derepresses epithelial genes. Moreover, recent studies showed that LSD1 is highly expressed in clinically advanced breast tumors (Lim et al., 2009). Together with the prominent role of Snail family members in initiation of EMT and tumor invasion, targeting the enzymatic components of the LSD1 complex by pharmacological interventions may hold a great promise for anti-invasive/metastatic therapy of tumors.

Besides LSD1, we also identified additional co-repressors of Snail by the proteomic approach. The promising candidates include two MBT domain proteins, SFMBT1 and L3MBTL. The MBT domain proteins are chromatin readers that can recognize the mono- or di-methylated histone tails and they can also remodel the chromatin structure by compacting nucleosomes. I first confirmed the presence of SFMBT1 and L3MBTL in the Snail complex. But different from LSD1, which has been shown to directly interact with Snail, I found SFMBT1 protein indirectly associated with Snail in a LSD1-dependent manner. However SFMBT1 seems not essential in maintaining the silenced status of epithelial genes, as depletion of SFMBT1 in MDA-MB-231 cells did not show any significant expression changes of Snail targeted epithelial genes.

LSD1 catalyzes FAD-dependent oxidation of amine-containing substrates and is capable of demethylating mono- and di-methylated H3K4. But LSD1 is unable to catalyze the demethylation of H3K4m3, which has been regarded as a hallmark of active promoters. In my studies, I found in the Snail-expressing MCF10A cells that are negative for E-cadherin the H3K4m3 mark still remains at high levels at the E-cadherin promoter. Therefore, in contrast to H3K4m2, the H3K4m3 mark was not significantly reduced despite Snail expression and E-cadherin repression. This observation is consistent with that no JARID1 family member was identified in purified Snail complex. Trimethylation of H3K27 by the PRC2 complex is associated with gene silencing and facultative heterochromatin formation. Snail was also reported to interact with the enzymatic component Ezh2 of the PRC2 complex and Snail-mediated transcriptional repression associated with H3K27m3 (Herranz et al., 2008). In consistence with this

finding, I detected high levels of H3K27m3 in the E-cadherin promoter specifically in the Snail-expressing cells. Thus, E-cadherin promoter possesses high levels of both the active H3K4m3 and the repressive H3K27m3 mark in Snail-expressing cells. Co-occupancy of such conflicting marks is characterized as “bivalent chromatin pattern.” Surveillance of histone methylations in my study showed Snail induces a bivalent histone modification state at the E-cadherin promoter region.

Bivalent genes are inactive, but are poised for activation. Bivalency is believed to represent a transition state between active and silenced in cells that have not yet committed to a particular development fate. It is noticed that EMT is not permanent but often reversible. The reverse process, known as mesenchymal-epithelial transition (MET), is essential for embryonic development. EMT-derived mesenchymal mesodermal and neural crest cells are multipotent and give rise to diverse embryonic derivatives and cell types, including epithelial tissues (Sauka-Spengler and Bronner-Fraser, 2008). More direct evidence of MET comes from developmental studies of kidney ontogenesis, somitogenesis, and secondary neurulation (Davies, 1996; Lowery and Sive, 2004), during which a mesenchymal cell population aggregates, condenses, develops cell-cell adhesions and reverts to the epithelial state. MET is believed to be critical for cancer metastasis as well. Clinically, majority of human metastases resembles primary carcinomas morphologically and retain characteristics of well-differentiated epithelial cells. This has been explained by a MET process occurring in the disseminated tumor cells (Thiery, 2002), probably due to the lack of EMT-inducing signals or selective advantage of cells with more epithelial properties at ectopic organ microenvironment.

The inter-conversion between epithelial and mesenchymal states is pivotal to embryonic development and malignant progression (Polyak and Weinberg, 2009). However the molecular basis underpinning the phenotypic plasticity of cancer cells at EMT/MET remains largely a mystery. In our study, I found Snail expression in epithelial cells results in a bivalently modified chromatin domain at the E-cadherin promoter, which may resemble euchromatin rather than constitutive heterochromatin. Bivalent genes are poised for activation, suggesting that Snail-mediated repression of E-cadherin is readily reversible. It is interesting to test whether this epigenetic plasticity may constitute the basis for rapid re-activation of E-cadherin during MET and facilitate the seeded tumor cells to exit dormancy status and form macrometastases in distant loci.

Besides, the bivalent chromatin domains with colocalization of active H3K4m3 and repressive H3K27m3 marks are enriched in embryonic stem cell genome. An overwhelming number of developmentally important, lineage-control genes exhibit the bivalent histone modification pattern in ES cells (Azuara et al., 2006; Bernstein et al., 2006). Upon ES differentiation, bivalent domains resolve to monovalent status, that is in differentiated cells, key developmental regulators are marked by either active (H3K4m3) or repressive (H3K27m3) mark. Therefore, it has been proposed that bivalent domains silence developmental genes in stem cells while keeping them poised for activation during later differentiation, providing a basis for cellular plasticity (Mikkelsen et al., 2007). Recently ground-breaking findings suggest that EMT generates stem cell traits, including expression of stem cell markers, formation of spheres, and acquisition of multi-potency (Mani et al., 2008; Morel et al., 2008). These observations have profound

impact on our understanding of cancer metastasis. In particular, Snail is sufficient to reprogram immortalized human mammary epithelial cells into mammary stem cells (Mani et al., 2008), however the underlying molecular mechanism is unknown.

The discovery of a bivalent state at the E-cadherin locus in Snail-expressing cells offers a potential mechanistic explanation for how Snail induces stem cell traits during EMT. And the interpretation can be scaled up to a genome-wide level with ChIP-Chip or ChIP-seq techniques. I hypothesize that Snail induces cell stemness by reprogramming lineage-important genes into a bivalent state. In addition to E-cadherin, I envision that Snail may bind to many lineage-regulating genes, especially those for epithelial differentiation, and impose a bivalent histone modification pattern. This reprogramming process results in de-differentiation of epithelial cells and acquisition of stem cell properties. As the key lineage regulators are poised for activation, the resultant stem-like mesenchymal cells have the potential to revert to epithelial state or differentiate into other cell types, depending on environmental signals. The genome-wide ChIP techniques may be applied to identify H3K3m3/H3K27m3 bivalent genes specifically induced by Snail. The result should offer unprecedented insight into the Snail-mediated reprogramming process and acquisition of stemness. Thus it could have huge implications for illustrating the role and mechanism related to epigenetic control of stem cell feature and cancer metastasis.

Epigenetic changes are increasingly recognized as a major characteristic of human cancers. Great improvement in understanding the epigenetic mechanisms involved in carcinogenesis over the past few years booms the development of epigenetic cancer therapies. Numerous drugs targeting different epigenetic

modifications have shown clinical benefits in treating cancers (Karberg, 2009). However one major issue with these current inhibitors is the lack of specificity which leads to unpredictable side effects. Treatment with these drugs results in global changes of gene expression patterns, not only those aberrantly expressed tumor-related genes. To circumvent this problem, it is important to understand the detailed mechanism that targets these enzymes to their specific chromosomal locations. Here I showed the zinc finger transcription factor Snail recruits histone demethylase LSD1 to its target promoters during EMT. So, in future studies designing small molecules that specifically interrupt the Snail-LSD1 interaction may hold great promise for preventing invasive potential of epithelial tumors while retaining the function of LSD1 related to other regular cellular processes.

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BIOGRAPHICAL SKETCH

Tong Lin was born in 1983 in a beautiful coastal city of Zhejiang Province, China. He grew up close to the seashore for 18 years, where one of his favorites is to take short ferries during weekends to feel the refreshing sea-breezes. In 2002, he attended Fudan University, Shanghai, and spent four years of his college life in this modern and fast paced metropolitan. In his junior year, he joined the laboratory of Dr. Li Jin as an undergraduate research assistant, where he learned human population genetics. His research involved in mapping genes associated with hypertension or heroin addiction in Chinese population. In 2006, he luckily received an offer from the Genetics and Genomics program in University of Florida. He then flew thousands of miles away from home to pursue a graduate degree. After first year's extensive course work, he joined Dr. Jianrong Lu's group, where he studied the epigenetic regulation of tumor metastasis. His work resulted in a publication in *Oncogene*. In August 2011, He received his Ph.D degree from the Genetics and Genomics Program, College of Medicine, University of Florida. Tong intends to keep investigating the role of chromatin structure in gene regulation, especially at genome-wide scale. In the long term, he would like to find a research and development position in the pharmaceutical or biotechnology industry.