EPIGENETICS OF HOXC13 ENDOCRINE REGULATION
AND ITS ROLE IN GENE EXPRESSION
AND TUMORIGENESIS

by

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ABSTRACT

EPIGENETICS OF HOXC13 ENDOCRINE REGULATION
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HOX genes are a subgroup of homeobox containing gene superfamily and have crucial roles in cell proliferation, cell differentiation, and embryonic development. HOXC13, one of 39 identified human HOX genes, is involved in hair development and human leukemia. The regulatory mechanism that drives HOXC13 expression is mostly unknown. Our studies demonstrated that HOXC13 is transcriptionally activated by steroid hormone estrogen (17β-estradiol; E2). The HOXC13 promoter contains several estrogen responsive elements (EREs), including ERE1 and ERE2, which are close to the transcription start site, and are associated with E2-mediated activation of HOXC13. Knockdown of estrogen receptors (ERs) ERα and ERβ suppressed E2-mediated activation of HOXC13. Similarly, knockdown of mixed lineage leukemia histone methylase (MLL)3 suppressed E2-induced activation of HOXC13. MLLs (MLL1-MLL4) were bound to the HOXC13 promoter in an E2-dependent manner. Knockdown of either ERα or ERβ affected the E2-dependent binding of MLLs (MLL1-MLL4) into HOXC13 EREs, suggesting critical roles of ERs in recruiting MLLs in the HOXC13 promoter. Overall, our studies have demonstrated that HOXC13
transcriptionally regulated by E2, and MLLs, which, in coordination with ERα and ERβ, play critical roles in this process. Although MLLs are known to regulate HOX genes, the roles of MLLs in hormone-mediated regulation of HOX genes are unknown. Herein, we demonstrated that MLLs are critical players in E2-dependent regulation of the HOX gene.

We also investigated the biochemical functions of HOXC13 and explored its potential roles in tumor cell viability. We have designed a phosphorothioate based antisense oligonucleotide that specifically knockdown HOXC13 in cultured cells. Cell viability and cytotoxicity assays demonstrated that HOXC13 is essential for cell growth and viability. Antisense mediated knockdown of HOXC13 affected the cell viability and induced apoptosis in cultured tumor cells. HOXC13 regulates the expression of cyclins and antisense-mediated knockdown of HOXC13 resulted in cell cycle arrest and apoptosis in colon cancer cells. Intriguingly, application of HOXC13 antisense oligonucleotide suppressed the growth of xenografted colorectal tumor implanted in nude mice.

Our further investigations demonstrated that HOXC13 is overexpressed in some cases of breast and colon carcinoma tissues in comparison to their corresponding surrounding normal tissues. We also showed that overexpression of HOXC13 induced the level of tumor growth factors bFGF (basic fibroblast growth factor) and TGFβ1 (transforming growth factor), while HOXC13 knockdown, down regulated these genes. Interestingly, HOXC13 overexpressed cells facilitated 3-D colony formation soft-agar assay while the HOXC13 homeodomain deleted overexpressed cells did not have such ability. This further indicates the potential role of HOXC13 and its homeodomain in cell proliferation and tumorigenesis.
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<tr>
<td>AP1</td>
<td>Activator protein</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-Bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinases</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CGBP</td>
<td>CpG dinucleotide binding protein</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DSG</td>
<td>Desmoglein</td>
</tr>
<tr>
<td>Dpy30</td>
<td>Dpy30 domain containing protein</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen response element</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Foxn1</td>
<td>Forkhead box protein n1</td>
</tr>
<tr>
<td>Foxq</td>
<td>Forkhead box protein q</td>
</tr>
<tr>
<td>HMT</td>
<td>Histone methyltransferase</td>
</tr>
<tr>
<td>HOX</td>
<td>Homeobox</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor-binding protein</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth medium</td>
</tr>
<tr>
<td>MLL</td>
<td>Mixed lineage leukemia</td>
</tr>
<tr>
<td>MMLV</td>
<td>Moloney murine leukemia virus</td>
</tr>
<tr>
<td>MTT</td>
<td>(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NTB</td>
<td>Nitro blue tetrazolium chloride</td>
</tr>
<tr>
<td>NR</td>
<td>Nuclear receptor</td>
</tr>
<tr>
<td>ORC</td>
<td>Origin recognition complex</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
</tr>
<tr>
<td>RC</td>
<td>Replication complex</td>
</tr>
<tr>
<td>Rbpb5</td>
<td>Retinoblastoma binding protein 5</td>
</tr>
<tr>
<td>RNAPII</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
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<td>SAM</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
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<td>Description</td>
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<tr>
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</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA buffer</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Transforming growth factor beta 1</td>
</tr>
<tr>
<td>SET</td>
<td>Su(var) 3-9, enhancer of zeste and trithorax</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetra methyl Rhodamine isothiocyanate</td>
</tr>
<tr>
<td>TTBS</td>
<td>Tween-20 tris buffer saline</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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CHAPTER 1
MLL FAMILY OF HISTONE METHYL TRANSFERASES IN HOX GENE REGULATION, HORMONE SIGNALING, AND DISEASES

1.1 Introduction

The genome contains hereditary information of an organism, which is encoded in DNA or RNA (in some type of viruses) (1, 2). In eukaryotic cells, DNA is packaged in a structure known as chromatin, which is a DNA and protein complex (2, 3). Chromatin is composed of repetitive units called nucleosomes; in each nucleosome 145-147 base pairs of the DNA are wrapped as super helix around a core protein complex that is made of histone octamer (4). Histone octamer contains two copy of each histone protein H2A, H2B, H3, and H4. The N-terminal tail of these histone proteins undergoes various kinds of posttranslational modifications such as acetylation, methylation, phosphorylation, ubiquitylation, simulation, and ADP-ribosylation that can affect gene activity, stability, compactness, and flexibility of chromatin (5-8). Among all histone modifications, histone acetylation, in general, transforms chromatin into a more relaxed structure that leads to higher levels of associated gene transcription. On the other hand, histone methylation is associated with both gene activation and silencing that depends on the location of modification (9).

Histone methyl transferases (HMTs) are responsible for methylation of histones. Mixed lineage leukemias (MLLs) are a group of HMTs that specifically methylate lysine 4 of histone 3 (H3K4), which represents gene activation. Some recent studies demonstrated that MLLs can interact with nuclear hormone receptors such as estrogen receptors (ERs) (10, 11).

It is well known that MLLs are key regulators of homeobox (HOX) genes, which are
a group of homeodomain containing genes that are revolutionary conserved and involved in embryonic development and cell differentiation\(^{(3, 12, 13)}\). Transcription of several HOX
genes is influenced by steroid hormones such as estrogen (14-17). Several studies demonstrated that ERs and MLLs interactions have important role in estrogenic regulation of HOX genes (17, 18).

### 1.2 Histone methylation

Histone methylation is carried by variety of histone methyl transferases (HMTs) in lysine as well as arginine residues of N-terminal tail of histones. Lysine methylation can occur in the form of mono-, di-, or tri-methylation while arginine methylation is in the form of either mono or di. The methyl group donor in lysine methylation is S-adenosyl methionine (SAM) that is further converted to S-adenosyl homocysteine (SAH) (9).

![Diagram of histone methylation](image)

Figure 1.2 Lysine residues can be methylated to make mono-, di-, tri-methylated lysine. Tri-methylated form is the sign of gene activity while di-methylated form represents either activation or repression. HMTs catalyze the methylation of lysine. Conversion of SAM to SAH in this process is providing the methyl group.

Methylation in lysine 4 of histone 3 (H3K4) and also in lysine 20 of histone 4 (H3K20) is a marker for transcriptional activation while methylation in lysine 9, 27, 36, 79 of histone 3 (H3K9, 27, 36, 79) cause gene silencing and chromatin condensation (9). The methylation on a specific residue can change the modification markers on the other residues of the same histone or even other neighboring histones. The number of methyl group on one single lysine or arginine residues also plays a crucial role in the function of these histone modifications. For instance, The tri-methylated H3K4 is a mark for gene activation while the
di-methylated form of H3K4 can be involved in activation as well as inactivation of a gene (19).

1.3 H3K4 methylation by MLL methyl-transferases

H3K4 methylation is a specific marker for gene activation that can provide a landscape for transcription factors to modulate gene activity (20). In yeast, the only H3K4-specific HMT is Set1 (9, 20, 21). In mammals, HMT activity is more complicated and carried by multiple enzymes. Human encodes at least seven different MLL homologs such as MLL1, MLL2, MLL3, MLL4, MLL5, Set1A and Set1B that are known as MLL family of histone methyl transferases (11). They all have similar enzymatic activity but their amino acid sequence comparison shows less than 30% similarity (3). Although, these enzymes are isolated from human cells and many of them are found to be associated with human diseases, the exact mode of action and the molecular mechanism still remain elusive.

1.3.1 The role of different domain structures of MLLs

Regardless of low sequence homology between different MLLs, they all have a highly evolutionarily conserved domain called SET domain. SET domain is the primary cause of HMT capability of MLLs (22). Each member of MLL family contains various domain structures that along with SET domain give different functionalities (Figure 1.3). Each one of MLL1-MLL4 contains 1 to 5 LXXLL domains (NR boxes) that are responsible for interaction with nuclear receptors (NRs) which might be the main cause of MLLs involvement in hormonal signaling and gene activation (23, 24). Several AT-hook motifs exist in MLL1, MLL3 and MLL4 structures. They are known to interact with AT rich region of DNA, Cysteine rich CXXC Zn-finger motif, another DNA binding element, just exist in MLL1 and MLL4. MLL2 and MLL3 contain one HMG domain (high-mobility group domain) that binds to DNA with lower sequence specificity. These DNA binding motifs make MLLs capable of binding to promoter of target genes. PHD (plant homeodomain) domains and RING fingers are protein-protein interaction domains. For instance, PHD domains in MLLs have been shown
to interact with polycomb group proteins and histone deacetylases (HDACs), which may have important roles in regulation of MLLs target genes activity (25, 26). MLL1 contains two Taspase1 proteolytic sites that are known as cleavage sites by Taspase1. Taspase1 cleaves MLL1 to two separate fragments (27, 28). MLL1-MLL4 contain one of each FYRN and FYRC domains that along with SET domain are responsible for MLL C- and N-terminal fragments heterodimerization (29). Despite existence of highly conserved SET domain in all MLLs that is the main reason of their HMT activities, there are various differences including amino acid sequences and motif structures (3). More studies are needed to explore detail information about other possible unique functions of each MLLs and their domain structures.

Figure 1.3 Schematic views of domain structures of MLLs. All MLLs contain conserved SET domain that is vital for methyl transferase activity. MLL1-4 possess one or more LXXLL domains that are recognized by nuclear receptors. PHD and RING domains are known to be involved in protein-protein interactions. AT-hook and CXXC zinc finger domains are DNA binding domains. FYRC and FYRN along with SET domain are involved in C- and N-terminal of MLLs fragments heterodimerization. Taspase sites are cleavage locations by Taspase 1 protease enzyme (3).

1.3.2 MLLs interaction with NRs through their NR boxes

Nuclear receptors (NRs) are large classes of proteins that are capable of sensing hormones and transducing the hormone signal to regulate transcription of hormone sensitive
genes (23, 30). NRs also have the ability to interact with DNA directly and therefore have been categorized as a group of transcription factors (30, 31). In general, NRs have a DNA binding domain (DBD) that targets specific DNA sequences known as response element and a ligand binding domain (LBD), which is targeted by a specific hormone or any other hormone like chemicals. Upon interaction of hormone with LBD, NR undergoes conformational changes that can be identified by LXXLL motifs (NR box) of transcription co-factors and also makes NR as a functionally active transcription factor (10, 24). As mentioned in section 1.3.1, MLLs have one or more LXXLL domains. It has been shown that MLLs, as co-activators, are involved in hormonal regulation of various genes. As an example, MLL2 binds to estrogen receptor alpha (ERα) via its LXXLL domain and plays an important role in estrogentic activation of cathepsin D (32). Knockdown of MLL2 inhibited the estrogentic activation of cathepsin D suggesting the critical role of MLL2 in hormonal activation of this estrogen sensitive gene (32). MLLs, in addition to direct binding, can also bind to NRs through activating signal cointegrator-2 (ASC2). ASC2 can act as a bridge to connect MLLs to their target nuclear receptors (33, 34). ASC2 is known as a co-activator for several NRs such as ERs and retinoic acid receptors (RAR). It can interact with NRs via its two LXXLL domains (10, 35). ASC2 is a member of ASCOM multi-protein complex that also may contains MLLs (MLL3 and MLL4) and MLL interacting proteins such as Wdr5, Rbbp5, and Ash2 (23, 36). In transactivation of RAR-β2 (retinoic acid receptor beta 2) by retinoic acid, ASC2 along with other components of ASCOM multi-protein complex such as MLL3 and MLL4 is recruited to the promoter of RAR-β2 (23, 24). It has been shown that the H3K4 tri-methylation of RAR-β2 promoter was reduced upon Knockdown of MLL3 and MLL4 independently but the reduction was not severe. In the case of both MLL3 and MLL4 knockdown, the retinoic acid mediated transcription of RAR-β2 was significantly down. These results show the existence of two independent ASCOM complexes that contain either MLL3 or MLL4 (24). Further analysis demonstrated that recruitment of both MLL3 and MLL4
in the promoter of RAR-ß2 were compromised upon siRNA mediated knockdown of ASC2, which suggested the crucial role of ASC2 in H3K4 tri-methylation of RAR-ß2 promoter by both MLL3 and MLL4 (24).

Menin, one of the essential components of MLL1 and MLL2, contains two LXXLL domains and it can bind to ERα by these NR boxes (24, 35). It has been shown that menin was recruited in the promoter of TFF1 (pS2) in estrogen mediated activation of this gene. Menin Knock down caused down regulation of TFF1 and reduction of H3K4 tri-methylation in its promoter (35). Similar to ASC2 role for MLL3 and MLL4, menin also connects MLL1 and MLL2 complexes to activated NRs (37).

1.3.3 MLLs as multi complex proteins

In yeast, the sole H3K4-specific HMT is Set1, which is a part of COMPASS multi-protein complex. COMPASS contains Set1, Spp1, Sdc1, Swd1, Swd2, Swd3, and Bre2. It has been shown that Set1 can be an active HMT only as a part of the above protein complex (9).

In human, purification and characterization of each MLL demonstrated that each of them exist in a multi-protein complex. Similar to yeast Set1, MLLs are active HMTs only as a part of multi-protein complex (38). These complexes have common subunits such as Wdr5 (WD repeat-containing protein 5), Dpy30 (a dosage compensation protein), Rbbp5 (Retinoblastoma-binding protein 5), Ash2, and Menin (3). It has been shown that, CGBP (human CpG binding protein) is associated with MLL1, MLL2, and Set1 and therefore interacts with promoter of HOXA7 which is known as a MLL target gene (39). MLL1 recruitment in promoter of HOXA7 was diminished by CGBP knockdown. This confirmed the significance of CGBP in regulation of MLL target genes (39). Sequence analysis revealed that Set1 complex components Bre2, Spp1, Swd1, Swd3, and Sdc1 are yeast homolog of human MLLs components Ash2, Dpy30, Rbbp5, Wdr5 and CGBP respectively (40). Each one of MLL complex components has different roles in either its DNA binding or H3K4 HMT
activity. For example, Wdr5 subunit is essential for H3K4 tri-methylation activity. Knockdown of Wdr5 will down regulate MLL1 target genes, HOXA9 and HOXC8 (38). Despite transcription reduction of HOXA9 and HOXC8, knockdown of Wdr5 had no effect on recruitment of MLL1 in those target genes (41). MLL interacting proteins have also important role in HMT activity and product specificity. For instance, The purified MLL1 from other component of its complex has only H3K4 mono methyltransferase activity and upon adding Rbbp5, Ash2, Wdr5 and Dpy30 the H3K4 di-methyltransferase activity of MLL1 is increased about 600 fold (42).

Since these results obtained in vitro with purified proteins, there is a possibility that other unidentified components in the complex are responsible for tri-methyltransferase activity of MLL1 complex in vivo and more studies are needed to reveal more detailed information about MLL complexes and their functions.

1.4 MLLs are important regulators of HOX genes

The key role of MLL1 in regulation of HOX genes has been shown in several independent studies (13, 43). The lethality of MLL1 disruption has been reported by generation of homozygous MLL1 knockout mice. This knockout mutation caused several patterning defects in mouse embryo neuronal system (44). MLL1 knockout in mouse also down regulated a series of Hox genes such as Hoxa4, a7, a9, and a10 (44). Further investigations demonstrated that MLL1 deletion mutation resulted reduced level of H3K4 mono methylation in promoter of several Hox genes (45). Deletion mutation of Taspase 1, which cleaves pre MLL1 to active MLL1 fragments, also had negative regulatory effect on some members of HOXA cluster (46).

1.4.1 HOX genes are members of homeobox genes

HOX genes are a group of homeobox containing genes that encode short transcription factor to control embryonic development, differentiation and proliferation both in invertebrates and vertebrates(47, 48). For the first time, homeobox genes were studied in
Figure 1.4 Schematic comparison of human HOX gene clusters with Drosophila homeobox genes. It has been believed that mammalian HOX genes are evolutionary derived by cis-amplification and trans-duplication (paralogues) from Drosophila homeobox genes. Human trans-paralogues genes have higher level of sequence similarities compared with the cis-paralogues genes. Each group of trans-paralogues is responsible for specific body section development of embryo. The 3’-end genes are more involved in anterior while 5’-end genes are more involved in posterior sections development (49).
Drosophila melanogaster (fruit fly). Fruit fly contains minimum of eight homeobox genes that are responsible for thoracic and abdominal segments development (50). They all contain a highly evolutionary conserved 60 amino acid long motif, which is responsible for binding of homeodomain proteins to the specific sequences of DNA to mediate transcription regulatory effect on their target genes (51, 52). Vertebrates also have several homeobox genes that are Hox genes in non-human vertebrates and HOX genes in human. Mutation of each Hox genes in vertebrates resulted in change in the identity of specific vertebral body part. More studies have been performed on Hox genes that were responsible for more significant anatomical changes. Based on these studies each specific Hox gene found to be responsible for identity of a particular segment of a vertebrate (Figure 1.4) (53, 54).

In human, there are 39 HOX genes that are categorized to four different clusters (A to D) based on their locations in different chromosomes (chromosomes 7, 17, 12, 2 respectively) (Figure 1.4) (55). It has been shown that these genes are evolutionary created by both cis-amplification and trans-duplication from Drosophila homeobox genes (56). The trans-paralogues genes are located in the same parallel position along the 5'-3' coordination direction and labeled with the same number. These trans-paralogues genes have higher level of sequence similarities compared with cis-paralogues genes that are located along 5'-3' directions and have the same alphabetical code but different numbers. Although functional similarities exist between some trans-paralogues, each one of trans-paralogues genes has distinct biological functions in most cases (57, 58).

1.4.2 Roles of HOX cofactors

The 60 amino acid long highly conserved homeodomain of Hox proteins is made of a helix-turn-helix motif that identifies specific nucleotide sequences (5'-TAAT-3') (59). Although each Hox proteins binds to DNA with high specificity in vivo, in vitro experiments using purified Hox proteins showed very weak DNA binding with very low sequence specificity (59). These results lead to the question of what makes specificity of in vivo Hox
binding possible. It is now well known that this specificity is due to interaction with other DNA-binding proteins that known as Hox cofactor proteins (60, 61). Drosophila Extradenticle (known as Exd) was the first discovered Hox cofactor and its mutation causes specific segment transformation (62). Pbx1 protein is vertebrate homolog of Exd. Both Exd and Pbx are members of PBC subclass of TALE (three amino acid loop extension) homeodomain proteins. *In vitro* experiments demonstrated that Pbx/Exd cooperation with various Hox proteins increased DNA binding specificity (63). The Pbx/Hox or Exd/Hox complexes can have both activation and repression effect on their target genes depending on other various cofactors interaction with complex or the kind of extracellular signals (64). The level of both Hox DNA-binding affinity and Hox DNA-binding specificity upon Hox and Pbx protein interaction is highly different for different Hox proteins (65).

In vertebrate, Hox gene transcription can be auto- or cross-controlled. For instance, Hoxb1 is transcriptionally regulated by Hoxa1 (66). Independent studies showed that Hox regulatory elements in their promoter require both Pbx and Hox binding sites. Independent mutation of Hox or Pbx binding site in the promoter of Hox genes showed prevention of regulatory effect by other Hox genes (67, 68).

Vertebrates encode different Pbx proteins including Pbx1-Pbx4. Interestingly, mutation of each one of Pbx causes some morphology effects similar to effects upon mutation of Hox genes (69, 70). These results are additional proofs for crucial role of Pbx family proteins in Hox protein functions.

Hox genes require a specific conserved hexapeptide motif in the upstream of their DNA binding domain and those Hox/HOX genes that are homologues of Abd-B homeobox gene don’t possess such specific sequences; therefore, they are not able to interact with Pbx/PBX cofactors (63). There are several studies that show some members of MEIS homeodomain proteins act as cofactor for Hox proteins and are able to interact without requiring the conserved hexapeptide motif, which is necessary for Pbx/PBX interaction
Table 1.1 Selected examples of mammalian Hox target genes

<table>
<thead>
<tr>
<th>HOX gene</th>
<th>+/-</th>
<th>Target gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoxa2</td>
<td>-</td>
<td>Six2</td>
</tr>
<tr>
<td>Hoxa5</td>
<td>+</td>
<td>p53</td>
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<td>+</td>
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</tr>
<tr>
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<td>+</td>
<td>Osteopontin</td>
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<td>HOXA9</td>
<td>-</td>
<td>BRCA1, EphB4</td>
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<tr>
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<td>+</td>
<td>p21, β3-Integrin, IGFBP1</td>
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<tr>
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<td>-</td>
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<tr>
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<td>+</td>
<td>IGFBP-1</td>
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<td>+</td>
<td>Epha7</td>
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<tr>
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<td>+</td>
<td>COL5A2</td>
</tr>
<tr>
<td>Hoxb3</td>
<td>+</td>
<td>TTF-1</td>
</tr>
<tr>
<td>HOXB5</td>
<td>+</td>
<td>Thrombospondin-2, Angiopoietin</td>
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<td>+</td>
<td>BFGF, VEGFA, MMP2, WNT5A, PDGFA</td>
</tr>
<tr>
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<td>-</td>
<td>N-CAM</td>
</tr>
<tr>
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<td>+</td>
<td>N-CAM</td>
</tr>
<tr>
<td>HOXC6</td>
<td>+</td>
<td>N-CAM, BMP7, FGFR2, PDGFRA, IGFBP3</td>
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<td>IGFBP-1, NEP/MME</td>
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<td>-</td>
<td>Foxq1</td>
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<td>HOXC13</td>
<td>+/-</td>
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<td>β1-Integrin</td>
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<tr>
<td>HOXD3</td>
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<td>β3-Integrin</td>
</tr>
<tr>
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<td>+</td>
<td>Renin</td>
</tr>
<tr>
<td>HOXB9</td>
<td>+</td>
<td>VEGF, bFGF, TGFβ1, NRG</td>
</tr>
<tr>
<td>Hoxd13</td>
<td>+</td>
<td>Epha7</td>
</tr>
</tbody>
</table>

Above table shows some of Hox/HOX gene targets. The + or - signs are representing weather corresponding Hox/HOX gene act as a activator or repressor for its target genes. HOX and Hox represent human and rat/mouse genes respectively (48, 71-78).

(63, 79, 80). MEIS subfamily of TALE homeodomain proteins includes Hth for Drosophila and Meis and Prep for vertebrates. Both Meis and Prep are able to bind Pbx and Hox complex and enhance nuclear localization and stability of the Hox complex (81-83).
I-kappa B proteins are inhibitory factors for NF-kappa B family transcription factors that are critically involved in different extracellular signal responses including necrosis factors and UV irradiation (84). It has been shown that HOXB7 directly interacts with I-kappa B-α, one member of I-kappa B proteins, and this interaction enhanced the transcriptional activity of HOXB7. This shows I-kappa B-α, previously known as inhibitor factor, act as the activator for HOXB7. Other studies showed the direct interaction of HOXB7 with CBP (CREB-binding protein) both in vitro and in vivo. Since CBP can recruit P/CAF that has histone acetyl-transferase activity, it has been that CBP along with P/CAF in HOXB7 complex provides chromatin remodeling activity in the HOXB7 binding site and enhances HOXB7 binding affinity in that region (85).

1.4.3 HOX target genes

Hox/HOX proteins as crucial transcription factors have important roles in controlling cell proliferation and differentiation as well as embryogenesis (86, 87). In the recent years, numerous Hox/HOX target genes have been identified in both in vitro and in vivo studies (table 1.1). Mouse neural cell adhesion molecule (N-CAM) was the very first identified Hox target that is responsible for adhesion in between neuronal cells (88).

Previously, Hox genes were known as transcription factors to precede cell differentiations involved in embryo development, but numerous studies demonstrated that Hox/HOX genes are active in adult cells and various different functions such as cell division, adhesion and migration, and apoptosis(48). Hox/HOX proteins have both positive and negative regulatory effects depend on the identity of their target gene and also the related cofactors (89). As an example, both Hoxa9 and Hoxc8 down-regulate intact mouse Opn by direct interaction with Opn promoter. OPN, product of Opn gene, controls both adhesion and cell migration in angiogenesis, bone remodeling, inflammation, and apoptosis (90, 91). Another study shows that HOA10 is positively regulating IGFBP-1 (Insulin-like growth factor binding protein-1) that is highly involved in endometrium formation(92) HOXA5 also activates
IGFBP-1 that leads to embryo implantation (93).

Several Hox/HOX genes are involved in cell cycle regulation and apoptosis. HOXA5 acts as an activator for tumor suppressor gene p53 by direct interaction in promoter of p53. HOXA10 has been shown to activate p21 (a CDK inhibitor).

Godwin et al. demonstrated that homozygous Hoxc13 mutant mouse has defective hair as well as abnormality in vibrissae, nail, filiform papilla, and caudal vertebrae whereas heterozygous mutation in Hoxc13 showed no significant morphological effect. These observations showed that in addition to roles of Hox genes in differentiation and cell proliferation, they can be responsible for synthesis of structural proteins. These findings suggested that Hoxc13 might be involved in regulation of approximately 60 hair keratin genes(94). Another independent study demonstrated that HOXC13 is highly expressed in human anagen hair follicles. Sequence analysis on some hair keratin promoters showed several TTAT, TAAT, and TTAC that are known to be potential HOX binding sites.

Figure 1.5 Lack of hair in Hoxc13 mutant mouse. (A) Control mouse with Hoxc13 wild type (B) One of Hoxc13 mutant allele (C&D) Both Hoxc13 mutant alleles (homozygous)(94).
Electrophoretic mobility shift assay demonstrated that HOXC13 interacts with TAAT and TTAT sites on the promoter of three hair keratins (Keratin 32, 35, and 37). In the β-galactosidase reporter gene experiment, homeodomain truncated HOXC13 did not bind to the same motifs mentioned above (72). Desmoglein 4 (DSG4), which is one of major hair shaft cortex proteins, also has been reported to be regulated by HOXC13. Reporter gene assay revealed down regulatory effect of HOXC13 on DSG4 (74). In another study, transient co-transfection of HOXC13 and Foxn1 and also chromatin immunoprecipitation assay (ChIP) demonstrated that Foxn1 is a target regulatory of HOXC13 (74).

1.4.4 Endocrine regulation of HOX genes

HOX/Hox gene expressions are highly regulated throughout embryogenesis and differentiation (95). Hormonal regulation of Hox genes is critical in their roles in hematopoiesis and reproduction. Any disruption in endocrine regulation of Hox/HOX genes such as exposure to endocrine disrupting chemicals leads to misregulation of HOX/Hox genes in both developing and adult tissues. Hormones that are critically regulating HOX/Hox genes are retinoic acid, sex hormones such as progesterone, testosterone, and estrogen (96).

1.4.4.1 Retinoic acid

Retinoic acid is well known as a crucial factor in neuronal system development in early stages of embryo development. Retinoic acid mediates its effect by interacting with its specific receptors. There are two groups of retinoic acid receptors RAR and RXR, which belong to the nuclear receptor family. Retinoic acid is recognizing its receptors in the form of homo- or heterodimer. The retinoic acid /receptor complex binds to the specific sequence enhancers in the promoter of target genes known as RARE/RXRE (retinoic acid response element) and mediates its transactivation effect on the target genes (97, 98).

Retinoic acid treatment resulted in up regulation of some Hox genes such as Hoxa1 and Hoxb1. These two genes are involved in anterior sections development suggesting the
regulatory effect of retinoic acid in anterior sections development including central nervous system. Disruption of retinoic acid signaling by RAR mutation resulted the same phenotypic transformation as 3’ end Hox genes suggesting regulatory effect of retinoic acid on 3’ end HOX/Hox genes (99).

1.4.4.2 Progesterone

Progesterone is one of the female steroid hormones involved in menstrual cycle, pregnancy, and embryogenesis (96, 100). It is secreted from corpus luteum in second half of the menstrual cycle and is involved in fertilized ovum attachment to uterus (101). In human, progesterone regulates its target genes via two isoforms of progesterone receptor, PR-A and PR-B. Both of these isoforms are produced by a single gene (hPR) that is under influence of two separate estrogen regulated promoters (102). Some HOX genes are regulated by endogenous progesterone. As an example, the transcription level of both HOXA10 and HOXA11 are higher in endometrial tissue samples obtained during latter half of menstrual cycles (96). In vitro studies on primary endometrial stromal cells demonstrated that both HOXA10 and HOXA11 are up regulated by medroxyprogesterone acetate (MPA) (103, 104). In contrast to HOXA10 transcription elevation under MPA treatment, in myometrial cells, HOXA10 mRNA level was decreased upon MPA treatment. This will prevent any further differentiation in myocytes and allow them for more proliferation that is vital for placental growth in pregnancy (105). Long term MPA treatment on endometrial cells caused HOXA10 down regulation showing that effect of progesterone is likely time dependent (103). Overall, endometrial differentiation is under the influence of HOXA10 and HOXA11 that are highly regulated by progesterone.

1.4.4.3 Testosterone

Testosterone is a member of androgen steroid family that is known to be responsible for male reproductive tissue development and all other male morphogenic characteristics in human and non-human vertebrates (106). In mammals, testicles in males, ovaries in
females, and adrenal glands (in much lower amounts) are secretion organs for testosterone (107). Polycystic ovary syndrome (PCOS) in women is the result of high secretion level of androgen (108). The level of endometrium HOXA10 expression is decreased in PCOS patients. Interestingly, only high level of testosterone or di-hydrotestosterone and no other androgens up regulates HOXA10 in endometrial tissues (96). These observations show the negative regulatory effect of testosterone on HOXA10. In vitro studies in Ishikawa cells demonstrated that testosterone down regulates HOXA10 via an androgen receptor-mediated mechanism which can be inhibited by a known androgen receptor antagonist such as flutamide (109).

1.4.4.4 Estrogen

Estrogen (E2 or 17β-estradiol) is mainly secreted in ovary tissues and has crucial role in embryonic development of female reproductive tract and also reproductive functions in adult females (110). The effect of estrogen is via two estrogen receptors ERα and ERβ. ERs are two important members of steroid/thyroid hormone superfamily of nuclear receptors that have similar structural architectures (111). ERα, compared to ERβ, is found to be more involved in estrogentic gene transcriptional regulation (112). In general, ERs and estrogen interaction promotes ERs dimerization (as a homo- or hetero-dimer) and translocation to the nucleus where they are targeted to estrogen responsive elements (EREs) present in the promoter of estrogen responsive genes (Figure 1.6) (113). Although ERE was shown to have consensus sequence 5’-GGTCAnnnTGACC-3’, only a few known estrogen target genes have perfect full ERE. Other estrogen target genes have EREs with one or more sequence differences from consensus (114, 115). In general, interaction of ERs with EREs that have two or more sequence changes from optimal consensus sequence requires either higher concentration of ERs or presence of other transcription factors or cofactors (116). There are several evidences that show activated ERs are able to interact with half-EREs (5’-GGTCA-3’ or 5’-TGACC-3’) and transactivate certain target genes (117-119). ERs can also
regulate the target gene expression without direct DNA binding. Both ERα and ERβ have been shown to physically interact with Sp1. The ER-Sp1 complex is able to interact with Sp1 site in retinoic acid receptor α 1 (RAR-1) and elevate RAR-1 transcription \((120)\). ERs can also interact with fos/jun factors and can be recruited on AP1 site in the promoter of target genes \((121)\). During estrogen-mediated gene activation, various co-activators or co-repressors coordinate with ER to carry out estrogen-mediated gene activation. Several HOX genes have been reported to be targeted by estrogen. Estrogen treatment to cells has been shown to up regulate HOXA10 transcription both \textit{in vitro} and \textit{in vivo} \((103)\).

We (Dr. Mandal’s laboratory team) showed HOXB9, HOXC6, and HOXC10 are transcriptionally activated upon estrogen treatment \((15, 16, 18)\). HOXB9 transcription was induced in both Jar (human choriocarcinoma placenta cells) and MCF7 (human breast cancer cells) upon estrogen application. Estrogenic induction of HOXB9 was via both ERs interaction with Half-EREs in the promoter of HOXB9. MLLs, which are known as the key regulators of HOX gens, have critical roles in this regulation. Chromatin immuno-precipitation assay (ChIP) revealed that MLL1 and MLL3 recruitment along with ERs were significantly higher in estrogen treated cells and antisense mediated knockout of each MLL1 and MLL3 had inhibitory effect on estrogenic activation of HOXB9 \((18)\). Similarly, both HOXC6 and HOXC10 mRNA level induction have been observed in treated Jar cells with estrogen and interaction of both ERs with half or imperfect EREs were involved. Interestingly, in HOXC6 estrogenic regulation, MLL2 and MLL3 had important role while in HOXC10 up regulation by estrogen, MLL3 and MLL4 were critical player \((15, 16)\).

Estrogen can have negative regulatory effects on some HOX genes. Overexpression of HOXB13 has been reported in some cases of ERα positive breast cancer patients treated with tamoxifen. Down regulation of HOXB13 has also been shown in MCF7 cells upon estrogen treatment that further indicate down regulatory effect of estrogen on HOXB13 \((17)\).
Figure 1.6 The genomic mechanism of estrogen signaling. Estrogen (ligand) enters into the cell and interacts with ERs. Upon this interaction, ERs undergo dimerization. The dimerized and activated ERs are deposited to the promoter EREs of estrogen responsive gene leading to its transactivation.

1.4.5 HOX gene in tumorigenesis

HOX proteins are members of homeodomain containing superfamily of transcription factors and have critical roles in embryonic differentiation and development (57). It is well known that intricate pattern of HOX genes expression continues in adult tissues, specifically in those tissues that undergo continuous differentiation and proliferation (122). Expressions
of these genes are tightly regulated and their abnormal activities are shown to be associated with defective development and ontogenesis, also in some cases, tumor suppression (123).

Table 1.2 **HOX genes are involved in tumorigenesis** (123).

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<th>Expression change</th>
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<td>Oesophageal squamous cell carcinoma</td>
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<td>5' HOXA, 5'HOXD, HOXA1, HOXC5</td>
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<td>Lung carcinoma</td>
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<td>Neuroblastoma</td>
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</tbody>
</table>

In general tumorigenesis is a consequence of altered activity of oncogenes, tumor suppressor genes, or non-coding RNAs (124). There are numerous reports of abnormal expression of HOX genes in malignant tissues. Misregulation of HOX genes in malignant tissues can be classified into three categories, elevated expression and down regulation of these genes compared with normal level of activity in the same type of tissues and
temporospatial misregulation that expression pattern of a group of HOX genes in a specific tumor tissue is different from nonmalignant cells of the same type of tissue (Table 1.2) (125).

It has been reported that MLL translocations in lymphoblastic leukemias might be related to up the regulation of some HOX genes such as HOXA4, HOXA5, HOXA7, and HOXA9. Further studies in some cases of those leukemias demonstrated the High expression level of above HOX genes, specifically HOXA9, which is critical for leukaemic cells survival (126, 127). Interestingly, both HOXA9 and HOXC13 fusion with NUP98, a nucleoporin protein, have been observed, which shows oncogenic potential of these genes (128, 129).

As mentioned above, down regulation of some HOX genes can promotes malignancy. As an example, HOXB13 down regulation in distal colon and rectum is the cause of abnormal proliferation (130). An in vitro study demonstrated that exogenous overexpression of HOXB13 can suppress the growth of LNCaP prostate cancer cells (131). Similarly, several HOX genes such as HOXC6, HOXD1, and HOXD8 expression were in a very low level in the neuroblastoma cells and overexpression of these genes by retinoic acid caused differentiation and conversion to normal cells (132).

An excellent example for temporospatial misregulation of HOX genes is expression of HOXA5 that is only observable in basal cells of normal tissues while it express in nearly all tumors (123).

As the investigation of HOX genes in tumorogenesis is continuing, the idea of targeting specific HOX genes for cancer therapy is becoming more apparent. In several studies HOX genes have been studied as potential biomarkers such as HOXB13 in breast cancer and HOXA9 in leukemia (133, 134).
CHAPTER 2
MIXED LINEAGE LEUKEMIA HISTONE METHYLASES PLAY CRITICAL
ROLES IN ESTROGEN-MEDIATED REGULATION OF HOXC13

2.1 Introduction

Homeobox containing genes are key players in embryogenesis and development (86, 87). Misregulation of homeobox genes is associated with tumorigenesis. There are more than 200 homeobox containing genes identified in vertebrates and they have been classified into two major groups, class I and II. Class I homeobox containing genes share high degree of identity (more than 80 %) and are called HOX genes. In human there are 39 different HOX genes that are clustered in four different groups called HOXA, HOXB, HOXC, and HOXD located in chromosomes 7, 17, 12, and 2, respectively (86, 87). Each of these HOX genes plays critical roles in embryogenesis and organogenesis. The nature of body structures depend on the specific combination of HOX gene products and expression of specific HOX gene vary at different stages of development. Therefore, proper regulation and maintenance of HOX genes are essential for normal physiological functions and growth.

HOXC13 is a critical gene involved in regulation of hair keratin gene cluster and alopecia (135-137). Transgenic mice over-expressing HoxC13 in differentiating keratinocytes of hair follicles develops alopecia, accompanied by a progressive pathological skin condition that resembles ichthyosis (136, 137). HoxC13 mutant mice lack external hair suggesting their critical roles in hair development (137). HOXC13 is also found to be a fusion partner of NUP98 in adult acute myeloid leukemia (129, 138). This protein also binds to ETS family transcription factor PU.1 and affect differentiation of murine erythroleukemia (139). Although HOX13 is critical player in hair development and disease, little is known about its own regulation. Notably, steroid hormones are critical players in sexual differentiation. Steroid
hormones such as estrogen and androgens are also linked with hair follicle growth and
difference in hair patterning in males and females (140, 141). However, the molecular
mechanism showing the roles of steroid hormones in hair development is poorly understood.
Herein, we have investigated if HOXC13, a critical player in hair follicle development, is
regulated by steroid hormones.

Mixed lineage leukemias (MLLs) are human histone H3 lysine 4 (H3K4) specific
methyl-transferases (HMT) that play critical roles in gene activation. MLLs are key players in
HOX genes regulation (13, 39, 43, 142-150). MLLs are also well known to be rearranged in
acute lymphoblastic and myeloid leukemias (142, 145). In human, there are several MLL
families of proteins such as MLL1, MLL2, MLL3, MLL4, etc. Each of them possesses H3K4
specific HMT activity and exists as multi-protein complex with several common protein
subunits (142, 151, 152). Recently, we demonstrated that human CpG binding protein
(CGBP) interacts with MLL1, MLL2, and hSet1 and regulates the expression of MLL target
HOX genes (39). Studies from our laboratory (and others) demonstrated that MLLs are
important players in cell cycle regulation and stress response (27, 28, 153-159). Knockdown
of MLL1 resulted in cell cycle arrest at G2/M phase (160).

Recent studies demonstrated that several MLLs (MLL2, MLL3 and MLL4) act as
coregulators for estrogen-mediated activation of estrogen sensitive genes (36, 142, 161-
163). MLL2 interacts with estrogen receptor (ER) in an estrogen dependent manner and
regulate activation of cathepsin D (161, 163). MLL3 and MLL4 regulate estrogen sensitive
gene liver X-receptor (LXR) (23, 36, 164). Although MLLs are recognized as major regulator
of HOX genes during embryogenesis, they are not implicated in steroid hormone-mediated
HOX gene regulation. Herein, we have investigated the roles of MLL family of HMTs in
estrogen-mediated regulation of HOXC13. Our results demonstrated that HOXC13 is
transcriptionally regulated by estrogen (17β-estradiol or E2) and MLL histone methylases in
coordination with estrogen receptors (ERs), regulate E2-induced activation of HOXC13.
2.2 Experimental procedures

2.2.1 Cell culture, estrogen treatment and antisense oligonucleotide experiments

Human choriocarcinoma placenta (JAR) cell obtained from ATCC was maintained in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and penicillin/streptomycin (100 units and 0.1 mg/mL, respectively) in a humidified CO₂ incubator as described previously (39, 165, 166). Prior to estrogen treatment, JAR cells were grown in phenol red free DMEM F-12 (Sigma), containing 10% activated charcoal stripped FBS at least for three generations. The final round of the cells were grown up to 70% confluency and treated with different concentrations (0 – 1000 nM) of estrogen (17β-estradiol or E2) for varying time periods. Stock solution of estrogen in ethanol or DMSO was made in 100 μM concentration. The cells were then harvested and subjected to RNA and proteins extraction or chromatin immuno-precipitation assay (ChIP).

To treat JAR cells with antisense oligo nucleotides, cells were grown up to 60% confluency in 60mm culture plate and transfected with varying amounts (3– 9 μg) of different antisense oligonucleotides. Briefly, cocktails of different amounts of antisense and transfection reagents (iFect, K.D. medical) were made in presence of 300 μl culture media (without supplements). First appropriate volume of antisense solution (from 1μg/μL stock concentration) was added to 100 μL of culture media (without supplements) in a 1.5 mL centrifuge tube. 12 μL of the iFect transfection reagent added to 200 μL of culture media (without supplements) separately and drop wise in a way that drops of reagents did not touch the tube side wall. Both of the mixtures were incubated for 5 min in dark (transfection reagent is light sensitive). The mixture of antisense and culture media (about 100 μL volume) was added to transfection reagent mixture drop wise. The cocktails were incubated for 40 min in dark before application to the cell culture plate. Cells were washed twice with supplement free culture media and finally submerged in 1.7 mL of media (without
supplements). The antisense-transfection reagents cocktail was applied to the cells drop wise and incubated for 7 h before addition of 2 mL of culture media with all supplements and 20% activated charcoal stripped FBS. The cells were then incubated for additional 24h before estrogen treatment.

2.2.2 Preparation of RNA and protein extract

The cells were harvested from culture plates and collected by centrifugation at 500 g for 5 min at 4 °C. The cells were then resuspended in diethyl pyrocarbonate (DEPC) treated buffer A (20 mM Tris-HCl, pH 7.9; 1.5 mM MgCl₂; 10 mM KCl and 1 mM EDTA; 0.5 mM Dithiothreitol (DTT)) for 10 min on ice and then centrifuged at 3500 g for 5 min at 4 °C. The supernatant was subjected to phenol-chloroform extraction. Equal volume of 50% phenol and chloroform was added to each sample and after vortexing for at least 10 s, samples were centrifuged at 10000 g for 10 min at room temperature. The supernatants were transferred to fresh tubes and equal volume of chloroform were added to each and then subjected to vortexing and centrifugation as performed for phenol treatment. For precipitation of cytoplasmic mRNA, 2.5 times more volume of absolute ethanol added to each transferred supernatant and then incubated at least 3-4 h at -80 °C. All samples were centrifuged at 13000g for 30 min at 4 °C. The supernatants were discarded and the mRNA pellets were washed with 70 % ethanol and DEPC treated water, air dried and dissolved in DEPC treated water containing 0.5 mM EDTA (155).

For preparation of protein extract, harvested cells were resuspended and incubated with whole cell extract buffer (50 mm Tris-HCl, pH 8.0; 150 mM NaCl; 5 mM EDTA; 0.05% NP-40; 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 1x protease inhibitors) for 20 min on ice followed by centrifugation at 10000 g (4 °C) for 15 min. The supernatant containing the whole cell protein extract was stored at -80 °C until further analysis.
2.2.3 Reverse transcriptase PCR (RT-PCR)

In order to quantify concentration of each mRNA sample, Nano Drop 1000 Spectrophotometer (Thermo Scientific) was used. All mRNA solutions were diluted with DEPC treated water to the same final concentration. cDNA synthesis was performed in two steps. For the first step, a 10 µL reaction mix containing 500 ng of RNA, 2.4 µM of oligo dT (Promega) was made in PCR tubes (96 well PCR plate) for each individual sample and then incubated in thermocycler (mastercycler, Eppendorf) at 70 ºC for 10 min and then in 4 ºC while preparing the second reaction mix. For the second reaction mix, 100 units of MMLV reverse transcriptase, 1X first strand buffer (Promega), 100 µM of each dATP, dGTP, dCTP and dTTP; 1 mM dithiothreitol (DTT) (Invitrogen, Carlsbad, CA, USA) and 20 units of RNaseOut (Invitrogen) were mixed and the volume was made to 15 µL for each sample. For dNTPs components a 10 mM stock solution of mixture of all four nucleotides was used. The second reaction mix was added to the first step product and mixed well. The final solution (25µL) was incubated in the thermocycler at 37 ºC for 1 h and then reaction was quenched at 4 ºC. The cDNA product was diluted to 100 µL with water and 5 µL of that cDNA was used in PCR.

PCR reactions performed in 10 µL reaction mix volume containing 5 µL of the diluted cDNA, 2 µM of desired primer pair (from 100 µM premixed stock solution of both forward and reverse primers), 125 µM of each dATP, dGTP, dCTP and dTTP (Invitrogen) (1.25 mM stock solution of mixture of all four dNTPs), 0.5 unit of Taq DNA Polymerase (GenScript) and 1X reaction buffer (GenScript). Each reaction mixture was covered by 8 µL of mineral oil (Sigma) to prevent evaporation. Reactions were done in 96 well PCR plates (VWR) and using thermocycler (mastercycler, Eppendorf) by following programing for each cycle, 30 s at 94 ºC denaturation, 30 s at 60 ºC annealing, 45 s at 72 ºC elongation for 30 to 32 cycles. At the end of the cycles, a 10 min elongation at 72 ºC was performed in order to complete all polymerizations and then 4 ºC to quench the reactions. The PCR product could be kept in -
20 °C before subjecting to electrophoresis by 1.5% agarose gel. 1X TAE buffer was used as running buffer and for preparing agarose gel. All the gene specific primer pairs that were used for PCR are described in Table 2.1. Each experiment was repeated three times. The normality of the data was analyzed by using t-test and ANOVAs were performed at 5% level of significance.

2.2.4 Western blot Analysis

For Western blot analysis, 25 μg protein extracts were electrophoresed through SDS-PAGE (10%) and transferred to nitrocellulose membrane. For transferring, 0.8 mA current was applied per cm² area of the membrane for 3 hr. The membranes were then incubated in solution of dry skim milk and TTBS buffer (50 mM Tris-HCl; 150 mM NaCl and 0.1% Tween 20, pH 7.5) for 45 min on shaker/rocker at room temperature then washed with TTBS buffer 3 times for 5 min each time. Membranes were probed with anti-MLL1 (Bethyl laboratory), anti-MLL2 (Bethyl laboratory), anti-MLL3 (Abgent), anti-MLL4 (Sigma), anti-ERα (Santa Cruz), anti- ERβ (Santa Cruz), and anti-β-actin (Sigma) antibodies. To make antibody solutions 3-5 µL (depending on the quality and concentration) of antibody solution added to 10 mL of TTBS buffer containing 75 mg of bovine serum albumin (BSA). For immuno-probing, membrane were first incubated in the primary antibody solution for 2-3 hr and washed with TTBS buffer 3 times for 5 min each time. Membranes were then incubated with appropriate secondary antibody solution for 1 hr and washed with TTBS again 3 times for 5 min each time before development.

To develop immunostained membrane using alkaline phosphatase method, developing buffer containing 75 µL of 50 mg/mL stock solution of nitro blue tetrazolium chloride (NTB) in 70% dimethylformamide (DMF) and 30 µL of 50mg/mL stock solution of 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) in DMF added to 10 mL of AP buffer (100 mM Tris-HCl; 100 mM NaCl and 5 mM MgCl₂, pH 9.5). This buffer has to be prepared just before
developing procedure. The washed membranes were incubated in this buffer on the shaker until appearance of desire signals.

2.2.5 Chromatin immuno-precipitation (ChIP) experiment

ChIP assays were performed by using EZ Chip™ Chromatin immuno-precipitation kit (Upstate, Billerica, MA, USA) as described previously (160). Cells were fixed in 4 % formaldehyde/PBS for at least 15 min in room temperature. For neutralizing excess formaldehyde, 125 mM glycine was added to each fixed cell culture and incubated for 15 min. All of the media was discarded and cells were washed with cold PBS that had treated with protease inhibitor cocktail (2-3 µL per ml of PBS). 1.2 mL of cold SDS lysis buffer added (50 mM Tris-HCl; 10 mM EDTA and 1% SDS, pH 8.1) and cells were scraped and transferred to 1.5 ml centrifuge tube. In order to shear the chromatin, all samples were sonicated using microprobe 1/8’ (VWR) by the power of 10 watts for 4 times, 2 min each, on ice. To check the size of fragments, 10 µL of each sample diluted by 40 µl water and 5 µl of 5M NaCl and incubated at 65 ºC water bath for 2-3 hr. All samples were subjected to Phenol-chloroform as mentioned in section 2.2.2. Purified DNAs were analyzed by agarose gel electrophoresis. The size of fragments adjusted below 400 bp. To pre-clean the fragmented chromatins, 60 µl protein G agarose (Upstate) was added to each sample and rotated at 4ºC for 1 hr and then centrifuged at 500g for 5 min at 4ºC and transferred all the supernatants to new tubes. The pre-cleaning procedure repeated one more time. 100 µl of each sample were kept for input and another100 µl of each pre-cleaned chromatin sample was used for each of ERα, ERβ, MLL1, MLL2, MLL3 and MLL4 antibodies immune-staining. Samples, except the input, were diluted to 1-1.2 mL by ChIP dilution buffer (16.7 mM Tris-HCl; 167 mM NaCl; 1.2 mM EDTA; 0.01% SDS and 1.1% Triton X100, pH 8.1). 3-4 µg of antibody for each staining added to diluted chromatins and rotated at 4 ºC overnight. The next day, 65 µl of protein G agarose was added to each sample and rotated at 4 ºC for additional of 5 hr. All samples were centrifuged at 500 g for 5 min at 4 ºC and supernatant
was discarded. To wash the protein G agarose and antibody complexes, 1 mL of low salt buffer (20 mM Tris-HCl; 150 mM NaCl; 2 mM EDTA; 0.1% SDS and 1 % Triton X100, pH 8.1) were added, rotated for 5 min at 4 °C (or 3 min at room temperature) and centrifuged at 500 g. All the supernatants were discarded, and the same wash procedures performed using high salt buffer (20 mM Tris-HCl; 500 mM NaCl; 2 mM EDTA; 0.1% SDS and 1 % Triton x100, pH 8.1) then LiCl buffer (10 mM Tris-HCl; 1 mM EDTA; 250 mM LiCl; 1% Deoxycolic acid and 1 % IGPAL CA630, pH 8.1) and then 3 times with TE buffer (10mM Tris-HCl and 1 mM EDTA). After washing steps, agarose bid pellets were eluted by adding 350 µl of ChIP elution buffer (0.1 N sodium bicarbonate and 1% SDS) and kept at 4 °C for overnight. For de-crosslinking, 20 µL of 5M NaCl added to each eluted sample and incubated at 65 °C for 5 h and then 10 µL of each 2.5 M Tris-HCl pH 8.1, 0.25M EDTA pH 8.0, and proteinase K (Sigma) solution (10mg/mL) were added and incubated at 45 °C for 2 h. in order to purify the DNA, phenol-chloroform procedure following by ethanol precipitation was performed as mentioned before (overnight ethanol precipitation at -80 °C). DNA pellets were dissolved in 100-120 µL water and 3-5 µL of this purified DNA fragments were used as templates in PCR amplification of four ERE regions of HOXC13 promoter using primer pairs listed in Table 2.1.

2.2.6 Co-immunoprecipitation of MLL-ER complexes

In order to confirm physical interaction of MLLs with ERα and ERβ, We performed co-immunoprecipitation from JAR cells in the absence and presence of E2. In brief, cells were treated with 100 nM E2 for 6 h, harvested for preparation of nuclear extract. Cell pellets were resuspended and incubated with buffer A as mentioned in section 2.2.2 in order to separate cytoplasmic extract from nuclear pellet. After discarding the cytoplasm fraction, the nuclear pellets were resuspended and incubated with buffer C (20 mM Tris-HCl, pH 7.9; 1.5 mM MgCl₂; 420 mM NaCl; 1 mM EDTA; 0.5 mM DTT and 0.2 mM PMSF). E2-treated and untreated nuclear extracts were incubated overnight at 4°C with MLL3 antibodies bound to the protein-G agarose beads. Beads were separated, washed with buffer C in the presence
of 0.1% NP 40. The affinity bound proteins were eluted from the beads using 0.2 M glycine, pH 2.9 and analyzed by western blot for the presence of ERα, ERβ, and MLL3 by using specific antibodies. Western blot were developed using ECL-Plus (GE Healthcare) and detected by using Phosphorimager (Storm840).

Table 2.1 Primers used for RT-PCR, ChIP and antisense experiments

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>CTCTTCCAGCCTTCTCTCCT</td>
<td>AGCAGCTGTGTGGCGGTACAG</td>
</tr>
<tr>
<td>HOXC13-ORF</td>
<td>GGAAGTCTCCCCTCCAGAC</td>
<td>CGATTGCTGACCACCTCTCT</td>
</tr>
<tr>
<td>MLL1</td>
<td>GAGGACGGCAGATGTTCAAACT</td>
<td>GGAGCAAGAGGTTCAAGATCAAAT</td>
</tr>
<tr>
<td>MLL2</td>
<td>GTGCAAGAAAGATGTTCAAGA</td>
<td>GCACATGCTGCTCAAGAGGA</td>
</tr>
<tr>
<td>MLL3</td>
<td>AAGCAAAAGGACTCAAGAGGA</td>
<td>ACAAGGCAATAGGAGGGGTGG</td>
</tr>
<tr>
<td>MLL4</td>
<td>GTCTATGCGCAGTGGAGAC</td>
<td>AGTCTGCGATCCCCGATTTTG</td>
</tr>
<tr>
<td>HOXC13-ERE1</td>
<td>GCGCTTCCGTGTCCTCTTA</td>
<td>CAGGCTTCCTGCGGTGTC</td>
</tr>
<tr>
<td>HOXC13-ERE2</td>
<td>TTTGCCGAGTATATTCCAATGGG</td>
<td>TCTGCTTTCATCCGGCTGGAT</td>
</tr>
<tr>
<td>HOXC13-ERE3</td>
<td>TTTCAGGCGCTTCTCTCTC</td>
<td>CGCGGGTGAAGAAGTTGGA</td>
</tr>
<tr>
<td>HOXC13-ERE4</td>
<td>TGCCCTCATATAACCTCGGA</td>
<td>AGGCTTTGGGAGTAGGAACC</td>
</tr>
<tr>
<td>ERα antisense</td>
<td>CATGGTCACTGCGCAG</td>
<td>CAGGCTTCCTGCGGTGTC</td>
</tr>
<tr>
<td>ERβ antisense</td>
<td>GAATGCTCATAGCTGA</td>
<td>CAGGCTTCCTGCGGTGTC</td>
</tr>
<tr>
<td>MLL1 antisense</td>
<td>TGCCAGTCGTTCCCTCTCCAC</td>
<td>TCTGCTTTCATCCGGCTGGAT</td>
</tr>
<tr>
<td>MLL2 antisense</td>
<td>ACTCTGCACTCCCTCCCGCTCA</td>
<td>CGCGGGTGAAGAAGTTGGA</td>
</tr>
<tr>
<td>MLL3 antisense</td>
<td>CACACTGCTTCCCTCCAGCTCC</td>
<td>AGGCTTTGGGAGTAGGAACC</td>
</tr>
<tr>
<td>MLL4 antisense</td>
<td>CACACTGCTTCCCTCCAGCTCC</td>
<td>AGGCTTTGGGAGTAGGAACC</td>
</tr>
<tr>
<td>Scramble antisense</td>
<td>CGTTTGTCCCTCCAGCATCT</td>
<td>AGGCTTTGGGAGTAGGAACC</td>
</tr>
</tbody>
</table>

aPhosphorothioate antisense oligonucleotide.

2.3 Results

2.3.1 HOXC13 gene is transcriptionally regulated by estrogen

Estrogen receptors (ERs) are major players in estrogen-mediated regulation of estrogen responsive genes (110, 167). In general, upon binding to estrogen, ERs get activated. The activated ERs bind to estrogen response elements (EREs) present in the promoter of estrogen responsive genes leading to their transcriptional activation (168). Herein, prior getting into examining the estrogen-mediated regulation of HOXC13, we analyzed its promoter for the presence of any ERE sequences. Our results demonstrated
that HOXC13 promoter contains six putative EREs (ERE1/2 sites) within -1 to -3000 bp upstream of the transcription start site (Figure 2.1). All the EREs show 100% homology with ERE1/2 sites (GGTCA or TGACC) but not with consensus full ERE sequence (GGTCA\textsubscript{nnn}TGACC). The presence of these EREs in the close proximity of the transcription start site indicated that HOXC13 might be potentially regulated by estrogen via involvement of estrogen receptors.

![Figure 2.1 Schematic diagram showing different EREs located at the HOXC13 promoter. All the EREs analyzed in HOXC13 promoter are ERE1/2 sites (GGTCA or TGACC)](image)

In order to examine if HOXC13 gene is regulated by estrogen, we treated a steroidogenic human cell line (JAR cells, choriocarcinoma placental origin, cultured in phenol red free media containing activated charcoal treated FBS) with varying concentrations (1-1000 nM) of estrogen (17β-estradiol or E2) for 8 h. The RNA was isolated from the E2 treated cells and analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) using HOXC13 specific primers (Figure 2.2, Table 2.1). Interestingly, our results demonstrated that HOXC13 was over-expressed upon exposure to E2 in a concentration dependent manner (Figures 2.2A, B). In comparison to the control, HOXC13 expression was 4 to 5 fold higher in presence of 100 and 1000 nM E2 (compare lanes 1 with 5 and 6, Figures 2.2A & B). The stimulation of HOXC13 gene expression upon exposure to E2 demonstrated that HOXC13 is transcriptionally regulated by E2. Time-dependent experiments demonstrated that HOXC13 activation was maximum at 6 to 8 h of E2-treatment (Figures 2.2C and D; with 100 nM E2, lanes 4 and 5).
Figure 2.2 Effect of estrogen (E2) on HOXC13 gene expression. (A & B) JAR cells were initially grown in phenol red free media, treated with varying concentration (0 – 1000 nM) of 17β-estradiol (estrogen) for 8 h. The total RNA was isolated and analyzed by RT-PCR using primers specific to HOXC13. β-actin was used as control. The quantification RT-PCR products are shown in figure B. (C & D): JAR cells were treated with 100 nM of 17β-estradiol for varying time periods (0-24 h). The total RNA was isolated and analyzed by RT-PCR using primers specific to HOXC13. β-actin was used as control. The RT-PCR products were quantified, the relative expression of HOXC13 (relative to actin) are shown in figure D. Each of these experiments were repeated thrice (n = 3) and values were assumed significantly different at $p \leq 0.05$. 
2.3.2 Estrogen receptors (ERs) play critical role in E2-induced HOXC13 expression

In order to examine any role of ERs in E2-induced activation of HOXC13, we knocked down ERα and ERβ separately using specific antisense oligonucleotides in JAR cells and exposed the cells with 100 nM E2 for additional 8 h. A scramble antisense (with no homology to ERs) was used as negative control. Our results demonstrated that application of ERα or ERβ antisense, knocked down the respective genes efficiently in mRNA level (lanes 4-6, Figures 2.3A and B, the quantifications are shown in respective bottom panels). After confirming the effective knocked down, we analyzed the RNA from these ER knocked down and E2-treated cells for the expression levels of HOXC13 using RT-PCR. As seen in figure 2.3A-B, HOXC13 expression was increased upon exposure to estrogen (compare lanes 1 and 2) and application of scramble antisense did not have any significant effect on E2-mediated activation of HOXC13. Interestingly, upon knockdown of either ERα or ERβ, the E2-dependent activation of HOXC13 was suppressed almost to the basal level (compare lanes 5 and 6 with lanes 1 and 2, Figures 2.3A and B, quantifications are shown in respective bottom panels). These results demonstrated that both ERα and ERβ are essential for E2-mediated transcriptional activation of HOXC13.

2.3.3 MLLs play critical roles in E2-induced HOXC13 expression

As MLLs are well known as master regulator of HOX genes and several MLLs are implicated in estrogen signaling, we examined whether any of the MLLs are involved in E2-dependent stimulation of HOXC13 expression. We knocked down different MLL genes (MLL1, MLL2, MLL3, and MLL4) separately by using specific phosphorothioate antisense oligonucleotides and then exposed the cells to E2 (100 nM for 8 h). Before performing E2 related experiments, we examined the efficacies of different MLL (MLL1- MLL4) specific antisense oligonucleotides and their most effective doses. The specific MLL knockdowns were confirmed by analyzing their respective gene expression at the mRNA levels. On the basis of these initial experiments, we applied specific concentration of each of the MLLs
Figure 2.3 Effect of depletion of ERα and ERβ on estrogen induced expression of HOXC13. JAR cells were grown up to 60% confluency prior to treatment with different concentration of ERα and ERβ specific phosphorothioate oligo nucleotides by using iFect transfection reagent (K.D medical). Control cells were treated with a scramble antisense with no homology with ERα and ERβ gene. The antisense transfected cell were incubated for 24 h and then treated with estrogen (100 nM) for additional 8 h. Cells were harvested and subjected to RNA preparation. The mRNA was subjected to RT-PCR analysis by using primer specific to HOXC13 along with ERα and ERβ. β-actin was used as control. The RT-PCR products were analyzed in agarose gel. Quantification of transcript accumulation based on RT-PCR products (average of 3 replicates, n =3) is shown beneath the respective gel images. Bars indicate standard errors. Values were assumed significantly different at p ≤ 0.05 levels. The results of experiment associated with ERα and ERβ are shown in figure A and B respectively.
antisense oligonucleotides that showed the most effective knockdown of respective genes and then exposed the cells with E2 (100 nM for 8 h) in an MLL knocked down environment. In parallel, we also applied a scramble antisense (no homology to any of the MLLs) as negative control. As seen in figure 2.4A, upon application of MLL1 antisense oligonucleotide followed by exposure to E2, MLL1 was efficiently knocked down, whereas scramble antisense had no significant effect on level of MLL1 mRNA (Figure 2.4A). Interestingly, upon down regulation of MLL1, E2-mediated up regulation of HOXC13 was slightly decreased (Figure 2.4A, lane 3). Similar results were also observed for MLL2 and MLL4 down-regulation (Figures 4B, D). The knockdown of MLL3 almost abolished the E2-mediated activation of HOXC13 (Figure 2.4C). These results demonstrated that MLL family of HMTs, especially MLL3, play critical roles in E2-mediated regulation of HOXC13.

2.3.4 E2-induced recruitment of ERs and MLLs in the HOXC13 promoter

As HOXC13 promoter contains several ERE1/2 regions within first 3000 nucleotides upstream of transcription start site, we analyzed the involvement of some of these EREs (ERE1-ERE4, located at -234, -1260, -1788 and -2000 bp upstream) by analyzing the in vivo binding of ERs and MLLs. We analyzed the in vivo binding of the different factors in the absence and presence of E2, using chromatin immuno-precipitation (ChIP) assay (160), using antibodies against ERs and MLLs. ChIP experiments were also performed in parallel with anti-actin antibody as nonspecific negative control. In brief, JAR cells were treated with 100 nM E2 for 6 hr and then control and E2-treated cells were subjected to ChIP analysis. The immuno-precipitated DNA fragments were PCR amplified using primers specific to ERE1, ERE2, ERE3, and ERE4 regions of HOXC13 promoter. As seen in figure 2.5A, no significant binding of actin was observed in any of the ERE regions irrespective of the absence and presence of E2. The binding of ERα and ERβ were increased in both ERE1 and ERE2 regions of HOXC13 promoter (lanes 1-4, Figure 2.5A). The level of E2-induced binding of ERα and ERβ were more in the ERE2 region in comparison to ERE1. ERE3 and
Figure 2.4 Effect of depletion of MLL1, MLL2, MLL3, and MLL4 on estrogen induced expression of HOXC13. JAR cells were grown up to 60% confluency and then transfected with MLL1 (panel A), MLL2 (panel B), MLL3 (panel C), and MLL4 (panel D) specific phosphorothioate oligonucleotides separately by using iFect transfection reagent. Control cells were treated with a scramble antisense with no homology with MLL1, MLL2, MLL3 and MLL4 gene. The antisense oligonucleotide treated cells were incubated for 24 h and the treated with estrogen (100 nM) for 8h and subjected to RNA preparation. The mRNA was analyzed by RT-PCR, using primers specific to HOXC13 along with respective MLLs (MLL1- MLL4). β-Actin was used as loading control. The RT-PCR products were analyzed in agarose gel. Quantification of transcript accumulation based on RT-PCR product (average of 3 replicates) is shown at the bottom of respective gel. Bars indicate standard errors. Values were assumed to be significantly different at $p \leq 0.05$. 
ERE4 regions were not sensitive to ER binding as a function of E2, probably because of their distance from transcription start site, although some amount of constitutive binding were observed in the both the regions.

The binding profiles of different MLLs were interesting. First of all, although some amount of binding of MLL1 was observed in ERE3, no significant E2-dependent binding of any of the MLLs was observed in the ERE3 and ERE4 (Figure. 2.5A lanes 5-8). Significant amount of constitutive binding of MLL1, MLL3 and MLL4 were observed in ERE1 even in the absence of E2 (Figure 2.5A lanes 1 and 2). However, MLL2 binding to ERE1 was enhanced upon addition of E2 (Figure 2.5A, lanes 1 and 2). Interestingly, binding of all of the MLLs (MLL1–MLL4) was greatly enhanced upon addition of E2 in ERE2 (Figure 2.5A, lanes 3 and 4). These results demonstrated that ERE1 (-234 bp) and ERE2 (-1260 bp), which are close to the transcription start site, are mostly responsible for estrogen dependent binding of ERs and MLLs and hence regulation of HOXC13. ERE2 appeared to have more critical roles (sensitivity to estrogen) among all other EREs examined. ERE3 and ERE4, which are located far upstream (-1788 bp or further), were not sensitive to E2-dependent binding of any of the MLLs/ERs, indicating no significant roles of these EREs in HOXC13 activation (Figure 2.5A).

To further confirm the E2-dependent binding of ERs and MLLs to the HOXC13 promoter, we analyzed their binding profiles in a time-dependent manner in the ERE1 and ERE2 (Figure 2.5B). In agreement with the above findings, binding of ERα and ERβ were increased in both ERE1 and ERE2 in the presence of E2. Interestingly, the kinetics of E2-dependent binding of ERα and ERβ to both ERE1 and ERE2 are different. The binding of ERα is very low in the absence of E2, and is significantly enhanced in the presence of E2 in both ERE1 and ERE2. However, in the case of ERβ, some constitutive binding was observed in ERE2 even in the absence of E2 and this binding was increased in the presence
**Figure 2.5** E2-dependent recruitment of ERα, ERβ and MLLs (MLL1-MLL4) in ERE1, ERE2, ERE3, and ERE4 of HOXC13 promoter. (A) Estrogen (100 nM for 6 h) treated and untreated JAR cells were subjected to ChIP assay, using antibodies against ERα, ERβ, MLL1, MLL2, MLL3 and MLL4. β-actin antibody was used as control IgG. The immunoprecipitated DNA fragments were PCR amplified using primers specific to ERE1, ERE2, ERE3 and ERE4 of the HOXC13 promoter. (B) Dynamics of recruitments of ERα, ERβ and MLLs (MLL1-MLL4), H3K4-trimethyl and RNAPII in ERE1 and ERE2 of HOXC13 promoter under E2 treatment using ChIP assay. JAR cells were treated with 100 nM estrogen for different time periods (0-8 h) and then subjected to ChIP assay using different antibodies. Immuno-precipitated DNA fragments were PCR amplified using primers specific to ERE1 and ERE2 of HOXC13 promoter. These results were kindly provided by Dr. Khairul Ansari.
of E2 (Figure 2.5B; compare 0 h and 6-8 h time points). These differences in kinetic profiles of binding of ERα and ERβ suggest that they have distinct modes of action in regulating target gene activation. It is Important to mention that, although it is poorly understood, the difference in the kinetics of binding of ERα and ERβ to the target gene promoters has been previously observed by other laboratories (169).

E2-dependent binding of MLLs (MLL1-MLL4) was primarily observed in the ERE2 region (Figure 2.5B). Again, as seen above, MLL2 binding was observed in the ERE1 region as a function of E2 (left panel, Figure 2.5B). The E2-dependent increase in binding of MLLs to the EREs were observed at as early 30 min post estrogen exposure and increased with time reaching a maximum at ~6 h (Figure 2.5B). The binding of MLL3 to the ERE2 region appeared to be the most prominent, although E2-induced binding of other MLLs (MLL1, MLL2 and MLL3) were also significant (Figure 2.5B). In addition, we also analyzed the status of RNA polymerase II (RNAPII) and H3K4-trimethylation level in the ERE1 and ERE2 regions. Our results demonstrated that in both ERE1 and ERE2, the level of RNAPII and H3K4-trimethylation were increased in presence of E2 (Figure 2.5B). These results demonstrated that both ERE1 and ERE2 (especially ERE2) coordinate the binding of ERs and MLLs coregulators as well as RNAP II and regulate E2-mediated transcriptional activation of HOXC13. Important to note that though ERE2 is located far upstream (1260 bp away from the transcription start site), we still observed significant transcription dependent increase in RNAP II binding to these ERE regions. These observations suggest that likely there is a looping of the large promoter regions so that far upstream cis-elements could be reached closer to the promoter proximal sites and coordinate with RNAP II and other transcription factors during transcription initiation (170, 171).

In addition, binding of some amounts of MLLs to certain EREs even prior to the addition of E2, suggests that this binding might be linked with the basal transcriptional regulation of the gene. Furthermore, we also observed that the recruitment of MLL2 is
induced by E2 at both ERE1 and ERE2. However, the recruitment of other MLLs (i.e., MLL1, MLL3 and MLL4) at ERE1 is not induced by E2 (Figure 2.5). These differences in recruitment profiles can be attributed to different possibilities. Once of the possibility is, even if there is an ERE, all the time it may not be responsive (not participating in the activation), likely because of the presence of other EREs which are more appropriately positioned to coordinate with transcription factors and coactivators to initiate efficient transcription. The other possibility is that, in addition to ERE1/2 sites, other neighboring promoter elements coordinate with it and that ultimately drives the assembly of the MLLs and other coregulator complexes around the specific ERE.

2.3.5 Recruitment of MLL histone methylases to the HOXC13 EREs is mediated via ERs

ERs are well known to bind directly to the EREs of the estrogen responsive genes via their DNA binding domain. MLLs (MLL1-MLL4) also have DNA binding domain that might be involved in direct binding with promoters. However, this binding may be critical for regulation of basal transcription of the target genes. On the other hand, MLLs might be recruited to the HOXC13 promoter via protein-protein interaction (direct or indirect) with ERs. Amino acid sequence analysis demonstrated that MLL1- MLL4, all have LXXLL domains (also called NR box) that are known to be involved in estrogen dependent interaction with ERs (142). MLL1 has only one LXXLL domain whereas MLL2, MLL3 and MLL4 have multiple (142). In fact, MLL2, MLL3 and MLL4 are recently shown to interact with ER and are involved in E2-mediated activation of estrogen responsive genes (36, 142, 161-163). Herein, we examined if all of these MLLs that are involved in E2-mediated activation of HOXC13 are directly binding to the EREs or they are recruited to EREs via interactions with ERs in an E2-dependent manner. To examine this, we knocked down both ERα and ERβ separately, then exposed the cell with 100 nM E2 for 6 h and analyzed the binding status of all the MLLs to the ERE1 and ERE2 regions of HOXC13 promoter (Figure 2.6). As expected, our results demonstrated that binding of each of the MLLs (MLL1- MLL4) was increased in ERE2 of
Figure 2.6 (A) Roles of ERα and ERβ on estrogen-dependent recruitment of MLLs (MLL1-MLL4) into the ERE1 and ERE2 regions of HOXC13 promoter. JAR cells were grown up to 60% confluency and transfected with ERα and ERβ antisense oligonucleotides for 24 h followed by exposure to estrogen (100 nM) for additional 6 h. Cells were harvested and subjected to ChIP assay using antibodies against, MLL1, MLL2, MLL3 and MLL4. The immuno-precipitated DNA fragments were PCR amplified using primer specific to ERE1 and ERE2 regions of HOXC13 promoter. (B) Interaction of MLL3 with ERs. JAR cells were treated with 100 nM estrogen for 6 h before harvesting for preparation of nuclear extract. The extracts were immuno-precipitated by using MLL3 antibody. The immuno-precipitated MLL3 complexes were then analyzed by western blot and using ERα and ERβ antibodies. Immuno-precipitation with protein-G agarose beads were used as negative control. These results were kindly provided by Dr. Khairul Ansari.

HOXC13 promoter in presence of E2 in the cells that were treated with scramble antisense (Figure 2.6A, lanes 5 and 6). However, knockdown of either ERα or ERβ, significantly
decreased (or even abolished) the recruitment of MLLs especially into ERE2 (Figure 2.6A, lanes 3 and 4, and 7 and 8). These results demonstrated that E2-induced binding of each of the MLLs to the HOXC13 promoter were mediated via interaction (direct or indirect via other MLL interacting proteins) with ERα and ERβ.

The physical interactions of MLLs with ERs were further conformed by using co-immunoprecipitation (co-IP) experiments. As MLL3 showed the most potent activity towards E2-dependent HOXC13 regulation, we analyze the interaction of MLL3 with both ERα and ERβ separately. In brief, JAR cells were treated with 100 nM E2 for 6 hr. Nuclear extracts were prepared from these E2-treated and untreated cells and were incubated with MLL3 antibody (bound to the protein-G agarose beads) for overnight at 4 °C. Protein bound to the MLL3-attached and control beads were analyzed by western blotting using ERα, ERβ, and MLL3 specific antibodies. Our results demonstrated that the interaction of both ERα and ERβ with MLL3 were increased in presence of E2 (Figure 2.6B). The direct physical interaction between MLL2- ERα, MLL3-ERα, and MLL4-ERα are previously shown by other laboratories. Thus, our results, in agreement with other reported data, demonstrated that MLLs are recruited to the HOXC13 promoter via interactions (direct or indirect) with ERs.

**2.4 Discussion**

HOX genes play major role in embryonic development where they determine the antero-posterior body axis (86). Hox genes are also expressed in adult tissues where they are necessary for functional differentiation (96). In general, HOX gene products act as transcription factors that regulate critical genes necessary for cell differentiation and development (86, 87). Despite their critical and well characterized functions, the regulatory mechanisms that drive HOX gene expression are mostly unknown. Although mechanism is unclear, recently, several hormones have been shown to regulate HOX gene expression and endocrine regulation of HOX genes appear to allow generation of structural and functional diversity in both developing and adult tissues (96).
HOXC13 is a homeobox containing gene that plays critical roles in hair development. Hair follicle development, male and female specific hair patterning, and sexual differentiation are strongly dependent on steroid hormones like estrogen, progesterone and androgens (135-137, 141). Herein, our studies demonstrated that HOXC13 gene is transcriptionally regulated by estrogen (E2). ERα and ERβ are two major players in estrogen dependent gene activation (110). Our studies demonstrated that antisense-mediated knockdown of either ERα or ERβ down regulated the E2-mediated activation of HOXC13 indicating their critical roles in the process. ER-mediated regulation of estrogen sensitive genes is a complicated process (168). In presence of estrogen, ERs get activated and bind to the EREs of estrogen responsive genes and eventually results in transcription activation (110). In addition to ERs, estrogen-mediated gene activation requires various other coregulators and coactivators that results in chromatin modification and remodeling (23, 172). Herein, our results demonstrated that MLL histone methylases and ERs play crucial roles in E2-mediated regulation of HOXC13. Knockdown of MLLs (especially MLL3) suppressed the E2-mediated activation of HOXC13.

In general, ERs along with various coregulators are recruited to estrogen responsive elements (EREs) present in the promoter of estrogen responsive genes (110). Our sequence analysis demonstrated that HOXC13 promoter contains at least six EREs within -3000 bp upstream of the transcription start site. In vivo binding analysis (ChIP) demonstrated that, in presence of E2, ERs bind primarily to the ERE1 (-234 bp) and ERE2 (-1260 bp) regions that are closer to the transcription start site. These results suggest that ERE1 and ERE2 of HOXC13 promoter are primarily responsible for E2 mediated gene activation.

ChIP analysis also demonstrated that MLL histone methylases (MLL1-MLL4) were bound to the responsible EREs in an E2-dependent manner. Knockdown of ERα and ERβ down regulated the recruitment of MLLs into the HOXC13 EREs demonstrating important roles of ER in recruiting MLL histone methylases into the HOXC13 promoter. Furthermore,
our coimmunoprecipitation experiments demonstrated that MLL3 interacts with both ERα and ERβ in an E2-dependent manner. Consistent with our observations, MLL2, MLL3 and MLL4 are previously shown to interact with ERα in an estrogen-dependent manner (36, 142, 161-163).

Importantly, there are several MLLs (MLL1-MLL5) with similar enzymatic functions (H3K4 specific HMT activity) and they are likely involved in regulating different target genes. Because of the difference in promoter cis-elements and their organization, different genes require different activators and coactivators. Based on our knockdown experiments, MLL3 is the most important MLL coactivator for HOXC13 expression. However, we observed that other MLLs (MLL1, MLL2, MLL4) are also involved in HOXC13 regulation, though weaker effects (knockdown experiments) than MLL3. As MLL1, MLL2 and MLL4 are involved in E2-mediated HOXC13 expression, we expected (as observed, Figure 2.5A) that they should be binding to HOXC13 EREs as a function of E2. However, irrespective of the relative importance of the MLLs (MLL1-MLL4), ChIP analysis (Figure 2.5A) showed efficient E2-dependent binding of all the MLLs in the ERE2 region. Notably, ChIP assay do not provide truly a quantitative measurement in terms of activity of the enzyme, though this provides important information about relative binding efficiency. This might explain the difference in MLL binding profile (ChIP data) versus their activity in knockdown experiments.

Our studies demonstrated that in addition to MLL2-MLL4, MLL1 is also recruited to the ERE2 of HOXC13 promoter in an E2-dependent manner. Amino acid sequence analysis demonstrated that each MLLs (MLL1- MLL4) contains one or more LXXLL domains (NR box) that are known to interact with nuclear receptors (NR) and mediate ligand dependent gene activation (142). MLL1 contains one NR box, whereas MLL2-MLL4 contain several (three to four) NR boxes which indicate that each of the MLLs have the potential to interact with ERs and be involved in estrogen mediated gene activation (142). Although further studies are needed to understand the detail roles of different MLLs and their coordination
with ERs, our studies demonstrated that MLL1-MLL4 are involved in E2-mediated HOXC13 regulation. Furthermore, E2-dependent increase in histone H3K4 trimethylation level suggested that some of the MLLs might be critical in regulating histone H3K4 methylation in HOXC13 promoter that is crucial for gene activation. Although MLLs are well known as major regulator of HOX genes, their roles in endocrine regulation of HOX genes are unknown. Our results demonstrated that MLLs play critical role in estrogen dependent regulation of HOX gene expression. Steroid hormone has been linked with hair growth, sex differentiation and difference in hair patterning in male and female. Our studies provide a molecular link between steroid hormone and regulation of HOXC13 that may have an implication in understanding the mechanism of sex specific hair development. In addition, our results demonstrated that HOXC13 expression is induced by steroid hormones estrogen in JAR cells which has a placental origin. Although at this time, it is not clear about the roles of HOX genes in placental function, this particular organ is critical in embryogenesis and fetal development. It is well known that placenta produces several steroid hormones that are circulated maternally and to the fetus and play critical roles in pregnancy and fetal growth (173). A significant amount of these hormones remains in the placental tissue that may regulate placental genes, development and function. Based on our observations, we hypothesize that estrogen-mediated expression of HOXC13 and possibly various other HOX genes may have crucial roles in placental function and this aspects needs further investigation.
CHAPTER 3

ANTISENSE OLIGONUCLEOTIDE MEDIATED KNOCKDOWN OF HOXC13 AFFECTS CELL GROWTH AND INDUCES APOPTOSIS IN TUMOR CELLS

3.1 Introduction

Antisense-oligonucleotide mediated gene targeting has been a promising strategy for down regulation of various genes for understanding their biochemical functions and in developing potential therapy (174, 175). The sequence specific binding of an antisense-oligonucleotide to a target mRNA results in degradation of the target mRNA and prevent gene translation. Various genes associated with apoptosis, cell growth, metastasis, and angiogenesis are potential targets for antisense therapy (176-178). For example, antisense against Bcl-2, which is an anti-apoptotic gene, is a target for cancer therapy and in clinical trial (179). Herein, we have developed an antisense oligonucleotide against a homeobox containing gene, HOXC13, and examined the roles of HOXC13 in growth and viability of tumor cells and explored its potential in novel cancer therapy.

Homeobox containing genes (HOX genes) are evolutionary conserved family of genes that play crucial roles during embryogenesis and development (86, 87). There are 39 different HOX genes in human that are clustered in four different groups HOXA-D. HOX genes act as transcription factors and they bind to their target gene promoters regulating target gene expression (180, 181). Colinear expression of different HOX genes is crucial for proper cell differentiation and development (182, 183). In addition to their crucial roles in embryonic development, HOX gene, appeared to play important roles in postnatal development (184). Various HOX genes such as HOXA5, HOXA7, HOXC10 etc are shown to be important for cell cycle regulation (185). Studies from our laboratory and others
demonstrated that HOX genes are misregulated in various cancer including breast and prostate cancer (15, 186). For example, HOXC6, a gene which is important for mammary gland development, is closely associated with prostate cancer and branching morphogenesis (187, 188). HOXB9, that is crucial for mammary gland development, is important for angiogenesis and breast cancer (189). Similarly, HOXC10, that is critical for spinal cord development, is transcriptionally regulated by estradiol and is over expressed in breast cancer tissues (15). Thus increasing amount of studies indicate that HOX genes are misregulated in various human diseases including cancer and are potential targets for novel cancer therapy.

Herein, we have investigated the function of HOXC13 in cell cycle progression, growth and tumorigenesis and explored its therapeutic potential. It is well recognized that HOXC13 is an important regulator of hair keratin gene cluster (135-137). Over expression of HoxC13 in transgenic mice develops alopecia, accompanied by a progressive pathological skin condition (136, 137). HoxC13 knockout mice lack external hair indicating its important roles in hair development (137). In addition to its developmental function, recent study demonstrated that HOXC13 is a member of human DNA replication complex, interacts with ORC2, binds to the origin of replication, and involves in DNA replication (190-192). HOXC13 is also shown to be fused with NUP98 and associated with acute myeloid leukemia (193). Recently we demonstrated that HOXC13 is transcriptionally regulated by estradiol in placental choriocarcinoma cells (JAR) and mixed lineage leukemia (MLL) family of histone methylases, that are key players in gene activation, coordinate with estrogen receptors (ERs), and regulate estradiol-induced activation of HOXC13 (119). In this study, we have shown that HOXC13 is a critical player in cell viability, cell cycle progression and its over expression induces three-dimensional (3D) colony formation in soft agar media indicating its potential role in tumorigenesis.
3.2 Experimental procedures

3.2.1 Cell culture and antisense transfection

Human colorectal adenocarcinoma (SW480), human breast adenocarcinoma (MCF7), human lung metastasized prostate carcinoma (PC3ML), human cervical cancer (HeLa), non-malignant colon fibroblast (CCD-18Co), human bronchioalveolar carcinoma (H358) and human embryonic kidney (HEK293) cell lines were obtained from American Type Cell Culture Collection (ATCC). Cells were grown and maintained in DMEM (Dulbecco’s modified Eagle’s media; Sigma) supplemented with 10 % heat inactivated fetal bovine serum, 1 % L-glutamine, 100 units/ml penicillin and 0.1 mg streptomycin/ml. All cell lines were maintained in a humidified incubator with 5 % CO$_2$ and 95 % air at 37 °C (16).

For antisense transfection, cells were grown up to 60% confluency in 60 mm culture plates and then transfected with HOXC13 antisense oligonucleotide (Anti-HOXC13: 5'-GCTGACCACCTTCTTCTTT-3') using iFect transfection reagent (K.D medical) as previously described (chapter 2, section 2.2.1) (16). Briefly, cocktails of different amounts of antisense (3-7 µg) and transfection reagents were made in 300 µL DMEM (without supplements) by incubating 45 min in dark. Cells were washed twice with supplement free DMEM and then 1.7 mL of supplement free DMEM was added to each cell culture plate. Antisense-transfection reagents cocktail was applied to the cell plates and incubated for 24 h, then 2 mL of DMEM with all supplements including 20% FBS was added to all cell culture plates. The cells were incubated for additional 24 h then harvested for RNA/protein extraction or fixed for microscopic analysis.

3.2.2 RNA and protein extraction

The cells were harvested and centrifuged at 500g for 5 min at 4 °C, then resuspended in diethyl pyrocarbonate (DEPC) treated buffer A (20 mM Tris-HCl, pH 7.9; 1.5 mM MgCl$_2$; 10 mM KCl and 0.5 mM dithiothreitol (DTT)) for 10 min on ice and then centrifuged at 3500g for 5 min at 4 °C. Supernatant was subjected to phenol-chloroform
extraction followed by ethanol precipitation (16). The RNA pellets were air dried and dissolved in DEPC treated water containing 0.5 mM EDTA.

For preparation of protein extracts, antisense-treated and control cells were resuspended in whole cell extraction buffer (50 mm Tri s/HCl, pH 8.0; 150 mM NaCl; 1 mM EDTA; 0.05% NP-40; 0.2 mM PMSF; 0.5 mM DTT; and 1x protease inhibitors cocktail) for 25 min on ice followed by centrifugation at 10000 g for 20 min at 4 ºC. The supernatant containing the nuclear protein extract and stored at -80 ºC prior to Western blot analysis as previously described (chapter 2, section 2.2.2) (16).

3.2.3 RT-PCR and Western blot analysis

For cDNA synthesis, a cocktail of 2.4 µM of oligo dT (Promega), 100 units of MMLV reverse transcriptase, 1X first strand buffer (Promega), 100 µM each dNTPs (dATP, dGTP, dCTP and dTTP, Promega), 1 mM DTT and 20 units of RNaseOut (Invitrogen) were added to 500 ng of the RNA extract for each sample and the volume was made up to 25 µL and reverse transcription reaction was performed. Each cDNA product was diluted to 100 µL, and 5 µL of the diluted cDNA was subjected to PCR as described previously (Chapter 2, section 2.2.3) using specific primer pairs described in Table 3.1. Real time PCR was performed by using Sso EvaGreen supermix and CFX96 real-time detection system (Bio-Rad). For data analysis CFX manager software (Bio-Rad) was used. Each experiment was repeated three times with three replicates each time.

For western blot analysis, 30µg protein extract from each sample were electrophoresed in SDS-PAGE (10%), transferred to nitrocellulose membrane and then subjected to western blotting with anti-HOXC13 (Abcam) and anti-actin (Sigma) antibodies. Western blot was developed using alkaline phosphatase method as described before (chapter 2, section 2.2.4)
3.2.4 Cytotoxicity (MTT assay) and growth rate measurement

MTT assay was performed as described before (166, 194). SW480 cells were seeded in 96 micro titer plates and incubated in the normal growth condition to reach 60% confluency. Cells were transfected by HOXC13 antisense. For transfection, a cocktail of antisense oligonucleotide and transfection reagent with 23 µg/mL of antisense oligonucleotide was prepared as explained before (chapter 2, section 2.2.1). Cells were washed twice with media without supplement and then submerged with 85 µL of media without supplements for each well of 96 well plates, then 15 µL of the cocktail was added to each well. After 24 h of incubation 100 µL of complete growth media containing 20% FBS was added to each well. After 48 h of treatment 30 µL of 5 mg/mL (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (in PBS) was added to each well and all samples were incubated in standard cell culture condition for 4 h. Supernatant from each well was removed and replaced by 100 µL of DMSO, incubated at room temperature on a shaker for 1 h. All plates were analyzed by micro plate reader (Fluostar-omega, BMG Labtech) at 560 nm. Each experiment was performed in 4 replicates and experiments were repeated at least two times.

For growth rate measurement, SW480 cells were grown in 60 mm cell culture plates and transfected with HOXC13 and scramble antisenses separately, incubated for varying time periods. Cells were harvested, stained with trypan blue and counted using hemocytometer and plotted. Each experiment was performed in 3 replicates and experiments were repeated at least two times.

3.2.5 Flow cytometry analysis

SW480 cells were transfected with varying concentrations of HOXC13 and scramble antisense for 48 h, then harvested by trypsinization, washed twice with cold PBS. For fixation step, cell pellets were resuspended with 300 µL of cold PBS and then 700 µL of cold absolute ethanol was added to each sample drop wise in order to have 70% ethanol
concentration in cell suspension. Fixed cells were incubated for 1h in 4 °C then stored in -20 °C overnight (or longer up to a week). Fixed cells were washed with cold PBS and resuspended with propidium iodide (PI) solution (0.5 µg/mL) for 2 h. Stained cells were subjected to FACS analysis (Beckman Coulter, Fullerton, CA, USA)(195)

3.2.6 Cytochrome c immunostaining

SW480 cells were grown on cover slips (placed inside tissue culture plate), transfected with HOXC13 or scramble antisenses for 48 h and subjected to immunostaining with anti-cytochrome c antibody (196). Treated and control cells were fixed by 4% formaldehyde for about 15 min in room temperature. Fixed cells can be stored in 4 °C for overnight while they are under 4% formaldehyde/PBS solution. Cells were then washed with cold PBS twice, permeabilized by 0.2% Triton X-100/PBS for 15 min on rocker, washed twice with PBS, and blocked with goat serum. For blocking step, 30 µL of normal goat serum (Sigma) was added on each coverslip and covered it with parafilm to avoid evaporation and incubated for 45 min in room temperature. After blocking step, 30 µL of primary antibody solution (0.5 µg cytochrome c (Upstate)) in 30 µL of 10% goat serum in PBS) was added on each cover slip and covered it with the same parafilm. Samples were incubated for 2 h in room temperature and then washed twice with 0.2% Tween 20 in PBS. For secondary antibody staining, 30 µL of secondary antibody (3 µg fluorescein isothiocyanate (FITC) conjugated in 10% goat serum in PBS) was added on each cover slip and covered with a new piece of parafilm then incubated in dark for 1 h. All samples were washed twice with 0.2% Tween 20 in PBS. In order to visualize nuclear integrity, cells were stained with 4', 6-diamidino-2-phenylindole (DAPI). For DAPI staining, 20 µL of 0.5 mg/mL DAPI in water added on each cover slip and incubated for a few seconds, briefly washed with PBS, mounted on the microscope slide and subjected to fluorescence microscopy (Nikon Eclipse TE2000-U, Japan).
3.2.7 TUNEL assay

The TUNEL (terminal dUTP nicked end labeling) assay was performed using ApoAlert DNA fragmentation assay kit (Clontech) (197). Briefly, SW480 cells were grown up to 60% confluency in 60 mm culture plates and then transfected with HOXC13 and scramble-antisense oligonucleotides separately for 48 h. Cells were fixed by 4% formaldehyde for at least 25 min at 4˚C, harvested and washed twice with cold PBS. All samples were permeabilized with 0.2% Triton X-100/PBS for 15 min on ice, washed twice with cold PBS, equilibrated each sample in 100 μL equilibration buffer for 15 min and then centrifuged (500 g). Each sample was resuspended with 50 μL of reaction mix containing nucleotides and TdT enzyme for 60 min at 37 °C. To stop the reaction, each sample was mixed with 1 ml of 2x SSC buffer for 15 min, washed twice with PBS and then stained with PI (propidium iodide, 0.5 mg/mL) and DAPI (5 mg/mL) for 5 min, then briefly washed with PBS, and resuspended in 10-20 μL PBS and spread on the microscope slide, mounted with DPX mounting solution (Sigma) under a cover slip and on microscope slide and then analyzed under fluorescence microscope.

3.3 Results and discussion

3.3.1 Design and characterization of knockdown efficacy of HOXC13 antisense-oligonucleotide

To understand the biochemical functions of HOXC13, we knocked it down in cultured human cells using an antisense oligonucleotide. HOXC13 antisense oligonucleotide, (5'-GCTGACCACCTTCTTCTTT-3') was designed to bind at 940-960 nt of HOXC13 mRNA (Figure 3.1A, Table 3.1). Importantly, to enhance the in vivo stability of the HOXC13 antisense, we introduced phosphorothioate linkages instead of regular phosphodiester bonds throughout the antisense oligonucleotide (Figure 3.1A).

To assess the efficiency of the antisense we transfected human colon cancer cells (SW480) with varying concentrations (3-7μg) of HOXC13 antisense using iFect transfection
reagent and incubated for 48 h. A scramble antisense (with no homology to HOXC13) was also transfected in parallel as a negative control (Table 3.1). RNA was isolated from the control and antisense-treated cells, reverse-transcribed into cDNA and subjected to PCR-amplification using real-time PCR and as well as regular PCR. Our analysis demonstrated that transfection with HOXC13 antisense efficiently knocked down HOXC13 both at mRNA (Figure 3.1B, real-time quantification is in figure 3.1C) and protein level (Figure 3.1D).

Figure 3.1 HOXC13 antisense oligonucleotide design and characterization. (A) Oligonucleotide sequences of HOXC13 antisense. Nucleotides are linked by phosphorothioate linkages instead of phosphodiester bonds. (B-D) Antisense mediated of knockdown of HOXC13 and scramble antisense for 48 h. RNA was extracted, reverse-transcribed and subjected to PCR with primers specific to HOXC13 and GAPDH (control) (in panel B). Real-time PCR for quantification of HOXC13 knockdown in control and antisense treated cells are shown in panel C (GAPDH was used as control). Bars are indicating standard error (n=6, p<0.05). (D) Protein extracts from the control and antisense-treated cells were analyzed by western blotting using actin (control) and HOXC13 antibodies.
HOXC13 knockdown was highest at 7μg antisense concentration (Figure 3.1B-D). GAPDH level remained mostly unaffected upon HOXC13 antisense transfection (Figures 3.1B-C). Scramble antisense had no significant impact on HOXC13 expression (Figures 3.1B-D). These analyses demonstrated that HOXC13 antisense effectively and specifically knocked down HOXC13 in SW480 cells.

3.3.2 Antisense-mediated knockdown of HOXC13 affects cell growth and viability

To examine the impact of HOXC13 knockdown on cell viability, we transfected different types of cancer and normal cells with HOXC13 antisense for 48h and then examined its impact on cell viability using microscopic analysis and MTT assay. The cell lines include MCF7 (human breast adenocarcinoma), H358 (human bronchioalveolar carcinoma), SW480 (human colorectal adenocarcinoma), CCD-18Co (non-malignant colon fibroblast), PC3ML (human lung metastasized prostate carcinoma), and HeLa (human cervical cancer). Microscopic analysis showed that transfection with scramble antisense had no significant impact on cellular morphologies and appearance (Figure 3.2). However, upon transfection with HOXC13 antisense, growth of most cells was impaired though to a different extent. SW480 (colon cancer) and MCF7 cells (human breast cancer) were more sensitive to HOXC13 knockdown compared with the other cancer cells (PC3ML, HeLa, and H358) (Figure 3.2). Non-malignant colon epithelial cells (CCD-18Co) appeared to be not significantly affected upon HOXC13 knockdown indicating preferential sensitivity of colon tumor cells over normal cells. MTT assay also demonstrated that HOXC13 knockdown significantly affected the viability of colon cancer (~ 90% cell death) and breast cancer cell (>55 % cell death) (Figure 3.3A). These results demonstrated that HOXC13 is critical for viability of most cell types and its knockdown induced death in cultured tumor cells.

To examine further the impact of HOXC13 knockdown on cellular growth, we transfected SW480 cells with 7 μg of HOXC13 antisense oligonucleotide for varying time periods and analyzed its impact on cell growth. In brief, control and antisense treated cells
were harvested at different time points and counted under a microscope. Cell numbers were plotted against time. This analysis showed that control as well as the scramble antisense-

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward primer (5′- 3′)</th>
<th>Reverse primer (5′- 3′)</th>
</tr>
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<tbody>
<tr>
<td>GAPDH</td>
<td>CAATGACCCCTTCATTGACC</td>
<td>GACAAGCTCCGTTCCTCAG</td>
</tr>
<tr>
<td>HOXC13</td>
<td>GGAAGTCTCCCCTCCCAGAC</td>
<td>CGATTGCTGACACCTTTCT</td>
</tr>
<tr>
<td>Cyclin A</td>
<td>AAGAAGCAGCCAGACATCAGGGA</td>
<td>AGCTGCTTCTCCTCAGAAC</td>
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<tr>
<td>Cyclin B</td>
<td>TTGATACTGCTCTCCAAGCCCAA</td>
<td>TTGGTCTGACTGCTTGCTTCT</td>
</tr>
<tr>
<td>Cyclin D</td>
<td>AGAAGCTGTGACGCTACACCGACA</td>
<td>AGAAGCTGTGACGCTACACCGACA</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>TTTCAAGGTATCAGGCTTGCGACA</td>
<td>ACAACATGGCTTTCTTGCTC</td>
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<tr>
<td>HOXC13 antisense</td>
<td>GCTGACCACCTTTCTTCTTT</td>
<td>a</td>
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<tr>
<td>Scramble antisense</td>
<td>CGTTGTCCCTCAGCATCT</td>
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a Phosphorothioate antisense oligonucleotide

treated cells grew exponentially with similar fashion and almost tripled in 72 h (Figure 3.3B), while under HOXC13 antisense treatment condition, cells did not grow further (up to 48 h) and upon longer time (72 h) incubation were started to die (Figure 3.3B). These results demonstrated that HOXC13 expression is critical for cell growth and viability and HOXC13 knockdown induced cell death in colon cancer cells.

3.3.3 HOXC13 knockdown affects cell cycle progression

To examine further the impact of HOXC13 knockdown on cellular function, we examined the impact of HOXC13 knockdown on cell cycle progression. We transfected SW480 cells with varying concentrations of HOXC13 antisense for 48 h, stained with propidium iodide (PI) and analyzed by flow cytometry. Transfection with scramble antisense was performed in parallel as a negative control. Our analysis demonstrated that, transfection
Figure 3.2 Effect of HOXC13 knockdown on cell viability. Microscopic analysis of different cell lines, MCF7 (human breast adenocarcinoma), H358 (human bronchioalveolar carcinoma), SW480 (human colorectal adenocarcinoma), CCD-18Co (non-malignant colon fibroblast), PC3ML (human lung metastasized prostate carcinoma), and HeLa (human cervical cancer) were transfected with HOXC13 and scramble antisenses separately for 48 h and visualized under a microscope.
Figure 3.3 *Cytotoxicity of HOXC13 knockdown and its impact on cell growth* (A) Cytotoxicity analysis: Different cell lines were transfected with 7µg of HOXC13 and scramble antisenses for 48 h and then subjected to MTT assay. The percent viability of HOXC13 antisense versus scramble antisense samples were plotted for different cell types. Bars indicate standard errors (n=8, p<0.05). (C) Analysis of cell growth: SW480 cells were transfected with 7µg HOXC13 and scramble antisenses and harvested at different times (24, 48 and 72 h), stained with trypan blue and counted under microscope and plotted. Three replicates of each experiment were repeated for at least two times. Bars indicate standard errors.
with the scramble antisense increased the amount of apoptotic cell population (from 9.5 % (control) to 28 % (scramble) and also effected cell populations at G0/G1 phase (decreased in comparison to control) and these changes are likely associated with toxic effect of the transfection reagent used (Figure 3.4). Interestingly however, transfection with HOXC13 antisense drastically decreased the cell population at G0/G1 and S phase, while apoptotic cells were significantly increased, in comparison to the control or scramble antisense treated cells (Figure 3.4). Upon transfection with 3μg of HOXC13 antisense, about 50% cell underwent apoptosis, while G0/G1 population dropped from 63 % to 32.3 %, S-phase population dropped from 19.6 % to 11.6 % (Figure 3.4). G2/M-phase population was not significantly affected (Figure 3.4). Increased HOXC13 antisense concentration (5 and 7μg) further increased the percent apoptotic cell death (Figure 3.4). These results demonstrated that HOXC13 is a key player in cell cycle regulation and growth and knockdown of HOXC13 affected the cell cycle progression and ultimately induced apoptosis in SW480 cells.

3.3.4 HOXC13 knockdown induced nuclear fragmentation and cytochrome c release and misregulated cyclin expression resulting in apoptosis

Apoptosis is a programmed cell death mechanism by which body eliminates unwanted cells and can be induced by various external or internal stimuli. Nuclear fragmentation, changes the mitochondrial membrane potential, caspase activation etc are different signatures of apoptotic cells (165, 198, 199). Herein, to examine the nature of cell death under HOXC13 knockdown environment, we transfected SW480 cells with HOXC13 antisense and subjected to TUNEL assay, DAPI (nuclear staining dye) staining, and cytochrome c immunostaining analysis.

DAPI staining showed that knockdown of HOXC13 induced nuclear fragmentation and condensation (intense DAPI staining) in SW480 cells (Figure 3.5A, bottom panel). TUNEL assay demonstrated that transfection with HOXC13 antisense resulted in DNA fragmentation (dUTP labeling at the nicked DNA sites, shown by green colored nuclei
Figure 3.4 HOXC13 knockdown affects cell cycle progression. SW480 cells were transfected with varying concentrations of HOXC13 and scramble antisenses for 48 h. Cells were harvested, fixed with 70% ethanol/PBS, stained with PI and subjected to flow cytometry analysis. Cell population percentage at each stages of the cell cycle are shown in the bottom panel.
Propidium iodide (that stains dead cells, red color) staining showed that most of the cells that were stained green with dUTP, were also stained with PI, indicating cell death (Figure 3.5A). Scramble antisense had no significant impact on nuclear staining and morphologies and most cells remained healthy (Figure 3.5A).

Mitochondrial membrane potential is known to be perturbed upon apoptotic cell death and these results in release of cytochrome c from the mitochondria to cytosol. To examine the impacts of HOXC13 knockdown on mitochondrial membrane potential and integrity, we have immunostained HOXC13 antisense treated cells with cytochrome c antibody and subjected to immunofluorescence analysis. DAPI staining was performed to stain the nuclei. This analysis demonstrated that cytochrome c is located inside the mitochondria (green speckles outside the nucleus) in both control and scramble antisense-treated cells (Figure 3.5B). However upon transfection with HOXC13 antisense, cytochrome-c was released from mitochondria to cytosol in most cells indicating perturbation in mitochondrial membrane potential and apoptotic cell death induced by HOXC13 knockdown (images of single cell are shown in figure 3.5B).

To understand the potential mechanism of cell cycle arrest and cell death induced by HOXC13 knockdown, we examined the expression of various cell cycle regulatory genes such as cyclins in the absence and presence of HOXC13 knockdown environment. Notably, the changes in expression levels of cyclins are associated with normal cell cycle progression (200, 201). For example, cyclin D and E are required for progression through G1 phase, cyclin A is produced in late G1 and it accumulates during S and G2 phase, while cyclin B is typically expressed during the G2 to M phase transition (200, 201). Our results showed that HOXC13 knockdown down-regulated the expression of cyclin B, D and E, while cyclin A was mostly unaffected (Figure 3.6A). The misregulation of cyclin expression is likely the cause of observed cell cycle arrest and induced apoptosis upon knockdown of HOXC13 in SW480 cells.
Figure 3.5 HOXC13 knockdown affects cell viability and nuclear integrity. (A) TUNEL assay: SW480 cells were transfected with 7µg of HOXC13 and scramble antisense separately for 48 h, fixed with 4% formaldehyde and then subjected to terminal dUTP nicked end labeling and stained with DAPI (blue staining) and PI (red staining). The green speckles are showing apoptotic cells containing fragmented DNA. (B) Cytochrome c release assay: HOXC13 knocked down and control cells were immuno-stained with cytochrome c antibody and then FITC-labeled secondary antibody and visualized under fluorescence microscope. Nuclear staining was performed with DAPI.
3.4 Conclusion

In this study we have developed a phosphorothioate based antisense oligonucleotide for HOXC13 that specifically and efficiently knocked down HOXC13 in cultured human cells. Our studies demonstrated that HOXC13 is a key player in cell growth and viability. Antisense-mediated knockdown of HOXC13 affects the cell viability and apoptosis in cultured tumor cells. HOXC13 regulates the expression of cyclins that play crucial role in cell cycle progression. Antisense-mediated knockdown of HOXC13 resulted in cell cycle arrest and ultimately led to apoptosis in colon cancer cells. Overall, we showed that HOXC13 is a critical player in tumor cell viability and can be targeted for developing novel cancer therapy.
CHPTER 4
HOXC13 IS ASSOCIATED WITH TUMORIGENSIS VIA INVOLVEMENT OF ITS HOMEODOMAIN AND ITS KNOCKDOWN SUPPRESSES XENOGRAFTED TUMOR IN VIVO

4.1 Introduction

HOX genes consist one of the two subgroups of homeobox containing genes that produce transcription factors containing highly conserved homeodomain (48, 49). The homeodomain is a highly conserved 60 amino acid long region that is involved in HOX proteins-DNA interactions. Homeodomain interacts with specific short nucleotide sequence region (5’TAA-3’), which is known as HOX binding motif (51, 52). Although this interaction is weak and with very low specificity in vitro, it is much stronger and more specific in vivo due to the HOX protein interaction with other DNA binding proteins, which are known as HOX cofactors (59, 61). 39 different HOX genes have been identified in human; these genes are categorized into four clusters (A, B, C, and D) located on chromosomes 7, 17, 12, and 2 respectively (55, 202). Trans-paralogues HOX genes, which have the same numbers, have high sequence similarity and are responsible for the development of a specific segment of a growing embryo (57, 58). Although HOX genes have key roles in embryogenesis, their expressions continue in adult tissues, specifically those tissues that have ongoing growth and differentiation (203). Misregulation of HOX genes in adult is found to be associated with abnormalities such as cancer. Numerous studies have been shown overexpression of specific HOX genes in malignant tissues and their critical roles in survival of cancerous cells (47, 123).

HOXC13 is known to be involved in hair follicle development via regulation of several hair keratin gene expressions (72, 135, 204). HOXC13 homozygous knockout
mouse has been shown to cause lack of external hair (94, 137). HOXC13 overexpression also causes alopecia (hair loss) and ichthyosis (a type of skin disorder) (94, 136). We previously showed that HOXC13 is up regulated upon estrogen treatment in Jar cells and MLLs had important role in that transactivation(119). We also showed that antisense oligonucleotide mediated knockdown of HOXC13 has down regulated some of cell cycle regulatory genes and induced cell growth arrest and apoptosis in colon cancer cells in vitro (Chapter 3). Recent studies demonstrated that HOXC13 protein might have some cellular functions other than being a transcription factor. HOXC13 protein is found to interact with lamin B2 origin of replication in early S phase (205). Further investigation showed that HOXC13 protein interacts with some pre-replicative complex (pre-RC) subunits such as ORC1, ORC2, and Cdc6 (190). These findings indicated that HOXC13 is a potential member of pre-RC complex and may have a role in replication initiation process. HOXC13 is shown to be a common fusion partner of nucleoprotein NUP98 in acute myeloid leukemia (138, 206). Expression of HOXC13 was detected in some breast cancer tissues, whereas no detectable expression was observed in normal breast tissues (47). Although some of HOXC13 cellular functions along with its estrogenic regulation in cancer cells have been explained, its role in tumorigenesis is mostly unknown. Here we investigated the roles of HOXC13 and its conserved homeodomain in tumorigenesis. Our results showed that HOXC13 regulates some of tumor growth factors and its overexpression induces three-dimensional cell colony formation. We also demonstrated that antisense oligonucleotide knock down of HOXC13 suppressed implanted colorectal carcinoma xenograft in mice.

4.2 Experimental procedures

4.2.1 Immunohistological staining of cancer tissues microarray

Both colon and breast cancer tissue microarray slides that each includes 6 individual cases (two of each) of colon and breast tumors and their corresponding surrounding normal tissues obtained from US Biomax Inc and subjected to DAB immunohistological staining (75). In brief, slides were deparafinized by first immersing twice in xylene for 10 min and then
sequentially immersed in 100%, 95% and 70% ethanol for 5 min in each step. Slides were incubated in 0.1 M sodium citrate solution at 95 °C for 15 min in order to retrieve antigens, then incubated with 3% H$_2$O$_2$ for 15 min, washed with PBS for three times. For blocking step PBS buffer containing 10% donkey serum was used. Each slide was incubated with 3 μg of HOXC13 (Abcam) antibody in PBS containing 10% donkey serum overnight, then washed three times with PBS, and then incubated with biotin conjugated secondary antibody that raised in donkey for 1.5 h. The slides were washed with PBS three times and then incubated with avidin–biotin complexes for 1.5 h, washed twice with PBS and then twice with 0.1 M tris-HCl buffer (pH 7.4). The samples were incubated with a DAB substrate (kit, Vector Laboratories) for peroxidase labeling. In order to dehydrate the tissues, both slides sequentially immersed in 70%, 95%, and then 100% ethanol. After dehydration, the samples sequentially incubated in CitriSolv cleaning agent (Fischer Scientific). Tissue sections were fixed with DPX mounting solution, and analyzed using a light microscope (Nikon Eclipse TE2000-U, Japan).

4.2.2 Cell culture and antisense transfection

SW480 (Human colorectal adenocarcinoma) and HEK293 (human embryonic kidney) cell lines were obtained from American Type Cell Culture Collection (ATCC). Cells were grown and maintained in DMEM (Dulbecco’s modified Eagle’s media; Sigma) supplemented with 10 % heat inactivated FBS, 1 % L-glutamine, 100 units/ml penicillin and 0.1 mg streptomycin/ml. Cells were maintained in a humidified incubator as previously described in chapter 2, section 2.2.1.

Antisense transfection was performed on 60% confluent SW480 in 60 mm culture plates with HOXC13 antisense oligonucleotide (5'-GCTGACCACCTTCTTCTTT-3') using iFect transfection reagent (K.D medical) as previously described in chapter 2, section 2.2.1.
4.2.3 RNA extraction and RT-PCR

Cytoplasmic RNA was extracted and deproteinized as previously described in chapter 2, section 2.2.2. Briefly, harvested cells were centrifuged at 500g for 5 min at 4 °C, then resuspended in diethyl pyrocarbonate (DEPC) treated buffer A then centrifuged at 3500g for 5 min at 4 °C. Supernatant was subjected to phenol-chloroform extraction followed by ethanol precipitation. The RNA pellets were air dried and dissolved in DEPC treated water containing 0.5 mM EDTA.

RT-PCR was performed on RNA solution as described before (Chapter 2, section 2.2.3) PCR reactions performed using specific primers in table 4.1. Real time PCR was performed by using Sso EvaGreen supermix and CFX96 real-time detection system (Bio-Rad) as explained previously (Chapter 3, section 3.2.3). Each experiment was done 3 times and with 3 replicates.

4.2.4 Protein extraction and western blot analysis

In order to fractionate cytoplasmic and nuclear proteins, harvested cells were resuspended in 200 µL (for each 100 mm cell culture plate) buffer A (20 mM Tris-HCl, pH 7.9; 1.5 mM MgCl₂; 10 mM KCl and 0.5 mM dithiothreitol (DTT) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)) for 10 min on ice, then centrifuged at 3500g for 5 min at 4 °C. The supernatant that contained cytoplasmic proteins was stored in -80 °C for later analysis. The remaining nuclear pellets were resuspended in 200 µL buffer C (20 mM Tris-HCl, pH 7.9; 1.5 mM MgCl₂; 420 mM NaCl; 0.5 mM EDTA; 0.5 mM dithiothreitol (DTT) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)) for 25 min on ice and then centrifuged at 13000g for 10 min at 4 °C. The supernatant containing nuclear proteins was stored in -80 °C for later experiments. 30µg protein extract from each fraction were electrophoresed in 10% SDS-PAGE, transferred to nitrocellulose membrane and subjected to western blotting with anti-Flag antibody (Sigma). Membranes were developed using alkaline phosphatase method as described before (chapter 2, section 2.2.4).
4.2.5 Immunofluorescence microscopy

Flag tagged HOXC13 and HOXC13-ΔHD stable cell lines along with HEK293 cells were cultured on coverslip overnight and subjected to immunofluorescence staining as described before (Chapter 3, section 3.2.7). Briefly, all cells were fixed by 4% formaldehyde, washed with cold PBS twice, permeabilized by 0.2% Triton X-100/PBS, blocked, and then incubated with primary antibodies specific to FLAG (Sigma) and RNAPII (Abcam). Cells were washed twice with 0.2% Tween 20 in PBS and incubated with fluorescein isothiocyanate (FITC) and rhodamine conjugated secondary antibodies (Jackson Immuno Research Laboratories), washed with PBS, stained with DAPI, and analyzed under a fluorescence microscope (Nikon Eclipse TE2000-U, Japan).

4.2.6 Plasmid construction

In order to make Flag-HOXC13 and Flag-HOXC13-ΔHD constructs, DNA sequences of Human full length HOXC13 and HOXC13 homeodomain deletion mutant (HOXC13-ΔHD) were generated by PCR using HOXC13 cDNA from MGC cDNA library (Mammalian Gene Collection, Thermo Scientific)(Figure 4.5). The forward primers flanked by HindIII restriction site sequences 5'-AAGCTTACGACTTCGCTGCTCCTGC-3' for both HOXC13 full length and HOXC13-ΔHD. The reverse primers flanked by BamH1 restriction site sequences at their 5' end, 5'-GGATCCTCAGGTGGAGTGGAGATGAGGC-3' for full length HOXC13, and 5'-GGATCCTCACCACGCCGGTAGCTGCTCA-3' for HOXC13-ΔHD. For the PCR reaction, Elongase Enzyme Mix kit (Invitrogen) was used. Since the size of the PCR product was about 1Kb, elongation period for each cycle was set for 80 s and 45 s period was set for each of denaturation and annealing step. Reactions were performed using thermocycler (Eppendorf mastercycler) for 29 cycles. All PCR products were purified by 1.5% gel electrophoresis followed by extraction using E.Z.N.A. Gel Extraction Kit (Omega Bio-tek). In order to make sticky ends, all PCR products cloned to pGEM-T vector using pGEM-T Easy Vector System I (Promega) in 5 µL volume (0.5 µL DNA ligase; 0.5 µL pGEM-T easy vector; 2.5 µL of 2x buffer and appropriate amount of PCR product) for each reaction. The reaction
mixes were incubated at 4°C for at least 24h. For amplification of constructs, all products were transformed to JM109 competent cells. 150 μL of competent cell suspension added to each mixture and incubated on ice for 20 min. For transformation, heat shock method was used. After ice incubation, tubes were incubated in 42 °C water bath for exact period of 60 s and then immediately kept on ice for 5 min. 0.5 mL of SOC media (sterilized LB media containing 20 mM glucose) added to each sample and incubated at 37 °C for 1 h. Each mixture was briefly centrifuged. All bacterial pellets were resuspended with 200 μL of fresh SOC media and plated on agar culture plates (100 μg/mL ampicillin sodium, 0.1 mM IPTG, 40 μg/mL Xgal) and incubated at 37 °C overnight. 5 to 10 distinct White colonies were selected (Blue white screening) (Figure 4.1). Selected colonies were separately cultured in 5 mL LB media containing 100 μg/mL ampicillin sodium and incubated in 37 °C shaker incubator for 16 h. Plasmid construct from each overnight cultured suspension were extracted and purified using E.Z.N.A. Plasmid Isolation Kits (Omega Bio-Tek) and purified DNA was eluted in 50 μL of elution buffer. 5 μL of each product was subjected to restriction site analysis to ensure having the right construct. Digestions were performed in 10 μL volume reaction mix containing both HindIII and BamH1 enzymes (Promega) and compatible buffer for both enzymes following Promega instructions. Reaction mixtures were incubated at 37 °C for 2-3 h and then gel electrophoresed to observe released digested insert in the positive samples. Selected samples subjected to DNA sequencing analysis for final selection. Both final selected construct plasmids (HOXC13 and HOXC13-ΔHD) and pFLAG-CMV4 vector was subjected to restriction digestion by HindIII and BamH1 as described before. Digested products were purified by gel electrophoresis and E.Z.N.A. Plasmid Isolation Kit. Each digested insert was cloned in digested pFLAG-CMV4 vector in 10μL reaction mix containing 1μ of T4 DNA Ligase (Roche Diagnostics), 0.4 μg ATP (Promega), appropriate buffer, and both digested vector and insert. All mixtures were incubated at 4°C
for at least 24 h. Each ligated product was transformed to JM109 competent cells and plated to select individual colonies. Cultured selected colonies were subjected to Mini Prep plasmid extraction followed by restriction enzyme digestion and DNA sequencing analysis to select the final sample constructs.

4.2.7 Generation of HOXC13 overexpressed stable cell line

HEK293 cells were grown up to 80% confluency in 60 mm culture plates. In order to transfect the constructed plasmid to human cell lines, plasmids must be free of bacterial endotoxins. Therefore, transformed JM109 bacteria with confirmed construct were subjected to endotoxin free extraction using E.Z.N.A. Endo-Free Plasmid Mini Prep kit (Omega Bio-tek). Each constructed plasmid was transfected into HEK293 cells using Lipofectamine 2000 Transfection Reagent (Invitrogen). A cocktail of plasmid and transfection reagent made in 500 μL of culture media (without supplements). First, 5μg of plasmid and 8 μL of transfection
reagent were separately added to 250 μL culture media (without supplements). The mixtures were incubated at room temperature for 5 min. The plasmid mixture was added to reagent mixture drop wise and the final mixture was incubated at room temperature for 30 min in dark. During cocktail incubation, HEK293 cells were washed by supplement free media twice and submerged in 3.5 mL media supplemented with 10% FBS without any antibiotics (due to sensitivity of Lipofectamine to antibiotics). All treated cells were incubated for 30 h and then washed with PBS, trypsinized and transferred into the large cell culture plates (145x20mm) containing 16 mL of cell culture media supplemented with 500 μg/mL G418 disulfate (Sigma) for selective propagation of transfected cells. These large plates were maintained up to four weeks, until individual colonies were appeared. 10 to 15 colonies of each sample were selected and transferred separately to 6 well cell culture plates where cells maintained in growth media supplemented with 500 μg/mL G418. Positive colonies were confirmed by western blot analysis using anti-Flag antibody (Sigma).

4.2.8 Soft agar assay for colony formation

Colony forming ability of HEK293 and Flag-HOXC13 stable cell lines were assessed using soft agar method as described previously (207, 208). 1% noble agar (BD diagnostic systems) was dissolved in PBS, autoclaved and cooled down in water bath to approximately 40 °C and then mixed with equal volume of DMEM containing 20 % FBS, 4 mM L-glutamine and 2% Penicillin/Streptomycin (200 unit and 0.2 mg/mL respectively), which also held in 40 °C water bath. 1 mL of the mixture was plated on each wells of 6 well culture plates and allowed to cool down to solidify as the base layer. For the top layer, 0.7% noble agar in PBS, autoclaved and kept in 40 °C water bath. Approximately, 5000 HEK293, Flag-HOXC13, and HOXC13- ΔHD stable cells were added to a mixture of 0.5 mL DMEM and 0.5 ml of 0.7% agar and then plated on top of the base agar layer. The plates were cooled to room temperature for about 15 min and then placed in the tissue culture incubator. The cells were fed with 0.5 mL of normal growth media, for HEK293, and media containing 500 μg/mL G418 for stable cells, at 2 days interval for 4-5 weeks until colonies were visible under a light
microscope. Colonies were stained with 0.005 % crystal violet for 2 h and then examined under light microscope.

4.2.9 Xenograft generation in mice and antisense treatment

All animal experiments were performed following the IACUC approved protocol and appropriate precautions were taken to minimize any potential pain and stress. Six weeks old athymic nude mice purchased from Harlan (Indianapolis, IN) and then hosted in UT Arlington animal care facility located in life science building.

To generate xenograft, SW480 cells were grown up to 80% confluency, trypsinized, washed with PBS, and counted using a hemocytometer. Approximately $2 \times 10^6$ cells were resuspended in 100 μL PBS for each injection. Cell suspensions were injected subcutaneously and near the right back limb of the animal (209). Animals were monitored daily for their health and to check the tumor growth. After each tumor size reached about 22 mm$^2$ (approximately 3 weeks after injection), HOXC13 antisense solution in PBS (350 μg/20 g body weight) was injected to each animal subcutaneously and in opposite side of the tumor location in 4 days interval. Control mice were injected by equal volume of PBS. The size of tumors was measured every 4 days using calipers and cross-sectional area of tumors was plotted. The experiments were repeated 3 times.

4.3 Results and Discussion

4.3.1 HOXC13 is overexpressed in colon and breast carcinomas

In our recent study we demonstrated that HOXC13 is transcriptionally regulated by estradiol (119). HOXC13 is a key player in cell cycle progression and regulates various cell cycle regulatory factors. Knockdown of HOXC13 is resulted in cell cycle arrest and apoptosis in cultured tumor cells (Chapter 2). To further understand the potential roles of HOXC13 in tumorigenesis, we examined its expression level in various carcinomas. Here we have
Figure 4.2 Expression of HOXC13 in colon cancer tissue. Human colon cancer tissue microarray slide, which contains 6 cases of colon carcinoma along with their surrounding adjacent normal colon tissue, was subjected to immunohistochemical staining (DAB staining) using HOXC13 antibody and then analyzed under a light microscope. Microscopic view of tissue histology representing HOXC13 expression in all samples are showed in panel B and further magnification of case 2 samples are presented in panel C.
Figure 4.3 Expression of HOXC13 in breast cancer tissue. Human breast cancer tissue microarray slide, which contains 6 cases of breast cancer tumor along with their corresponding adjacent normal breast tissue, was subjected to DAB immunohistochemical staining using HOXC13 antibody and then photographed under a light microscope. Microscopic view of tissue histology representing HOXC13 expression in all samples are presented in panel B and further magnification of case 1 tissues are showed in panel C.
immunostained two different human carcinoma tissues, colon and breast carcinomas, using HOXC13 antibodies and examined the level of HOXC13 expression.

We purchased colon and breast cancer tissue microarrays from commercial sources that contain 6 cases of breast or colon cancer (in duplicates) along with corresponding adjacent normal tissues. Tissue microarrays were deparafinized and then subjected to immunohistochemical staining (DAB staining) using HOXC13 antibody and then examined under light microscope and photographed. These immunohistological analysis showed that HOXC13 expression was relatively higher (more intense DAB staining) in some of the cases of colon cancer in comparison to their adjacent normal colon tissues (such as case 2 figure 4.2, the microscopic images of these cases are shown in two different level of magnifications in figures 4.2 B-C). However in other remaining cases, the level of HOXC13 expression was similar or slightly higher in cancerous in comparison to their surrounding normal tissues. Notably, in all of the cases, the normal colon tissue shows the distinct signatures of differentiated tissues and epithelial microstructures while these microstructures were lost in carcinoma tissues. Similarly, cell densities in each case of colon cancer tissue were much higher in comparison to the surrounding normal tissues (Figures 4.2B-C). In the breast cancer tissue microarray analysis, we observed that HOXC13 is significantly overexpressed in the most cancerous tissues in comparison to their corresponding surrounding normal tissues (Figure 4.3A-C). Breast tissue microstructures were lost in the cancerous tissues and cell densities were increased. These analyses suggested that HOXC13 may be overexpressed in some of the colon carcinomas, though it is overexpressed in more cases of breast cancers.

4.3.2 HOXC13 is a nuclear protein that facilitates cell proliferation and 3D-growth via involvement of its homeodomain

As HOXC13 is appeared to be overexpressed in selected breast and colon cancer tissues, we aimed to examine its potential roles in cell proliferation and tumor growth.
Toward this goal, we generated a stable transfected cell line that overexpressed HOXC13. Notably, HOXC13 is a homeodomain containing protein and proteins containing homeodomain usually bind to DNA and promoter of target genes regulating target gene expression (51, 205). HOXC13 homeodomain is located in carboxyl-terminus side (260aa-322aa) that also contains major part of a nuclear localization signal (NLS) (Figure 4.4).

![Figure 4.4 Schematic view of HOXC13 protein structure. HOXC13 protein contains 330 amino acids and possesses a conserved homeodomain between amino acids 260 and 322. A nuclear localization signal motif (NLS) is located between 258 and 266 amino acids that overlaps with homeodomain.](image)

To examine the potential function of HOXC13 as well as its homeodomain contribution, we generated another stable transfected cell line overexpressing the homeodomain truncated HOXC13 (Figure 4.5). Initially, we cloned full-length HOXC13 and HOXC13 with homeodomain deletion (HOXC13-ΔHD) in a pFlag-CMV4 human expression construct and then transfected into HEK293 cells (Figure 4.5). Transfected cells were grown and maintained in a media containing G418 antibiotic. The stably transfected cell colonies were isolated and individual colonies were grown and maintained in G418 containing media.

To examine the HOXC13 expression levels in the stable transfected cell lines, we fractionated the cytoplasm and nuclear extracts from Flag-HOXC13 and Flag-HOXC13-ΔHD stable cell lines and analyzed by western blot using anti-Flag antibody. As seen in figure 4.6A, Flag-HOXC13 (full length) is overexpressed in the stable cell line and primarily enriched in the nuclear fraction in comparison to cytoplasmic fraction (lanes 1-2, figure 4.6A). Interestingly, upon deletion of homeodomain, HOXC13-ΔHD lost its nuclear
Figure 4.5 Generation of flag tagged HOXC13 and HOXC13-ΔHD (homeodomain truncated) overexpressed cell lines. HOXC13 and HOXC13-ΔHD cDNAs were generated by PCR using specific primers that include appropriate restriction sites. PCR product was cloned to pFlag-CMV4 human expression vector separately. Each construct were then transfected to HEK293 cells. Transfected cells were grown and maintained with media containing G418 antibiotic and stably transfected cells were isolated, grown, and maintained in G418 containing growth media.

Localization and enriched primarily in cytoplasm (compare lanes 3 and 4, Figure 4.6A). HOXC13 as well as HOXC13-ΔHD overexpression in the respective stable cell lines were also evident in RNA level (RT-PCR analysis is shown in Figure 4.6B).

To further confirm the subcellular distribution of HOXC13, we performed immunostaining of HOXC13 and HOXC13-ΔHD stable cell lines using anti-flag and then
Figure 4.6 *The role of homeodomain in distribution of HOXC13 protein between cytoplasm and nucleus regions.* (A) Western blot analysis of protein extract from cytoplasmic and nuclear fractions using Flag-antibody. Cytoplasmic and nuclear proteins of Flag-HOXC13 were loaded in lines 1 and 2. Respective protein fractions for HOXC13-ΔHD were loaded in lines 3 and 4. (B) HOXC13 RNA level was analyzed by reverse transcriptase PCR (RT-PCR) using primers specific for HOXC13. 28S and 18S rRNA as well as GAPDH (using specific primers in table 4.1) were used as loading controls.
examined under a fluorescence microscope. Cells were also co-immunostained with RNA polymerase II (RNAP II) antibody as control. Nucleus was visualized by DAPI staining. This analysis demonstrated that Flag-HOXC13 is primarily localized in the nucleus (Figure 4.7A), while Flag-HOXC13-ΔHD protein was spread all over the cells beyond the nucleus (Figure 4.7B). As expected RNAPII was primarily localized in cell nuclei especially in the euchromatic regions (2). Our analysis showed that HOXC13 is mostly a nuclear protein and its nuclear localization is associated with homeodomain that also contains a nuclear localization signal.

Notably, while examining the fluorescence microscopy of the HOXC13 stable cells, we observed that upon overexpression of HOXC13-full length protein, HEK293 cells were appeared preferably clumped and eventually formed three-dimensional colonies in comparison to the individually separated with widespread nature of the HEK293 cells (Figure 4.8). Interestingly, however the homeodomain deleted HOXC13 stable cells (Flag-HOXC13-ΔHD) lost this 3D-colony forming tendency and grew more like the untransfected HEK293 cells (Figure 4.8). These observations indicated that HOXC13 is potentially associated with 3-dimensional cellular growth and colony formation and its homeodomain play crucial roles in this process.

4.3.3 Homeodomain of HOXC13 controls 3D-colony formations in soft-agar assay

As HOXC13 overexpressed cells appeared to facilitate the three-dimensional cellular growth in tissue culture plate, we further examined if it truly induces 3D-colony formation using a soft-agar based assay. Briefly, we plated HEK293 cells, Flag-HOXC13 and Flag-HOXC13-ΔHD stable cells in soft agar and then cells were allowed to grow for about 4 weeks, until distinct visible colonies were formed. Our analysis showed that there were plenty of distinct visible (in naked eye) cell colonies in the Flag-HOXC13 stable cell containing soft-agar plates, while there were very few (or none) such colonies in the control HEK293 cells (Figure 4.9A). Colonies at different layers were counted under microscope and
Figure 4.7 HOXC13 is a nuclear protein. Fluorescence immunostaining performed in Flag-HOXC13 (A), Flag-HOXC13-ΔHD (B) overexpressed stable cells and HEK293 cells (C). To examine HOXC13 localization, Flag antibody for stable cell lines and HOXC13 antibody for HEK293 cell line were used. All cells also co-immunostained with RNA polymerase II (RNAPII) antibody as control. Nucleus was stained by DAPI staining.
Figure 4.8 Three-dimensional cellular growths of Flag-HOXC13 overexpressed cells. Flag-HOXC13 and Flag-HOXC13-ΔHD overexpressed cells were grown and maintained in normal growth media containing G418 antibiotic. HEK293 cells were used as control. Cells were observed and photographed using a light microscope (Nikon Eclipse TE2000-U, Japan).

Plotted (Figure 4.9B). Interestingly, we also found that there were very few small colonies of cells in the plates containing Flag-HOXC13-ΔHD stable cells (Figure 4.9A-B). The number of colonies in the HOXC13-full-length overexpressed cells was about 5 fold higher in comparison to the Flag-HOXC13-ΔHD or the empty HEK293 cells. These observations demonstrated that HOXC13 overexpression contributed towards the formation of large 3D-colonies and that is mediated via its homeodomain.

4.3.4 HOXC13 regulates the expression of tumor growth factors via its homeodomain

To understand the potential mechanism of tumor growth and cell proliferation associated with HOXC13, we examined the impact of HOXC13 overexpression in expression level of various tumor growth factors that include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and transforming growth factor beta 1 (TGFβ1)(75). We isolated RNA from the control HEK293 cells, HOXC13-full length and HOXC13-ΔHD stable cells. RNA was subjected to RT-PCR and real-time PCR analysis.
Figure 4.9 HOXC13 over expression induces tumorigenesis. (A) 3D-colony formation ability of HOXC13 over expressed cells. HEK293, Flag-HOXC13, and Flag-HOXC13-ΔHD stable cells were incubated in soft agar separately for about 4 weeks, stained with 0.005% crystal violet, and analyzed under microscope. Magnified view of one HOXC13 over expressed colony is shown on the right side. (B) Number of colonies grown in soft agar cultures were counted under light microscope and plotted. Bars indicate standard errors.

using various target gene primers. These analyses showed that HOXC13 overexpression has no significant impact on the VEGF expression, though bFGF and TGFβ1 were significantly up regulated. This overexpression of bFGF and TGFβ1 were diminished in the HOXC13-ΔHD stable cells, indicating crucial role of HOXC13 homeodomain in
Figure 4.10 HOXC13 regulates the expression of tumor growth factors. (A) The expression level of tumor growth factor genes upon HOXC13 overexpression. Cytoplasmic RNA extracts from HEK293 (control), Flag-HOXC13, and Flag-HOXC13-ΔHD stable cells subjected to reverse transcriptase PCR (RT-PCR) using specific primers listed in table 4.1. (B) Real time quantification of gene expression relative to GAPDH was performed in both stable cells as well as HEK293. Bars indicate standard errors (n = 3). (C) The expression level of TGFβ1 and bFGF upon HOXC13 down regulation. SW480 cells were treated with 5 and 7 µg of HOXC13 and 5 µg of scramble antisense for 48 h and cytoplasmic RNA extracts were analyzed by RT-PCR. (D) Real time quantification of gene expression relative to GAPDH. Bars are showing standard errors (n = 3).
Figure 4.11 Suppression of colorectal adenocarcinoma cells xenograft by HOXC13 antisense. SW480 cells were subcutaneously injected into the right back limb of six weeks old athymic nude mice. Mice were monitored daily for appearance and the size of tumors. Once the tumor size reached ~20 mm² (cross-sectional area), mice were administered with HOXC13 antisense (350mg/20g body weight) into the left back limb (away from tumor) at 4 days interval for 28 days. Control mice were treated with the same dose of scramble antisense. Tumor sizes were measured using a slide caliper. (A) Tumor cross-sectional areas for both HOXC13 and scramble antisense treated mice were plotted versus times. (B) Pictures represent mice xenograft at different times during HOXC13 and scramble antisense treatments. (C) Xenografted tumors from the control and HOXC13 antisense treated animals were excised out. Bars indicate standard errors (n=3).
transcriptional regulation of various tumor growth factors (Figures 4.10A-B). Importantly, antisense-mediated HOXC13 knockdown down regulated the expression of bFGF and TGFβ2 further demonstrating potential roles of HOXC13 in regulating these tumor growth factors (Figures 4.10C-D).

4.3.5 Antisense-mediated knockdown of HOXC13 suppresses the growth of colon cancer xenograft in vivo

As HOXC13 is found to be important for cell viability and 3D-colony formation, we further examined its potential roles in tumor growth in vivo using a human colon cancer xenograft (210, 211). In brief, we implanted a colon cancer xenograft in nude mice by injecting SW480 cells near the right back limb. Once the tumor size reached ~20 mm², we administered HOXC13 antisense oligonucleotide intraperitoneally (350 µg/20g body weight, in three parallel replicates) on a different spatial location (left back limb) away from the xenografted tumor. Control mice were injected with equal amount of scramble antisense. Growth of tumor (tumor size) was monitored over time and plotted (Figure 4.11A). As seen in figure 4.11A, the sizes of the control or scramble antisense treated tumors were increased exponentially over time. Interestingly, application of HOXC13 antisense suppressed the growth of colon cancer xenograft significantly (Figure 4.11A). Representative pictures of mice bearing tumors are shown in figure 4.11B. Xenografted tumors from the control and HOXC13 antisense treated animals were excised out and shown in figure 4.11C. Overall, these results demonstrated that HOXC13 is critical for colon tumor growth in vivo.

4.4 Conclusion

In this investigation, we demonstrated that HOXC13 is overexpressed in some cases of breast as well as colon carcinoma tissues in comparison to corresponding surrounding normal tissues. Our further studies showed that HOXC13 is primarily enriched in the cell nucleus while upon deletion of its homeodomain, which also contains major part of
Table 4.1 Primers used for RT-PCR, cloning, and antisense experiments

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward primer (5′- 3′)</th>
<th>Reverse primer (5′ - 3′)</th>
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<tr>
<td>ORF primers</td>
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<tr>
<td>GAPDH</td>
<td>CAATGACCCCTTCATTGACC</td>
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<td>HOXC13</td>
<td>AAGGAGTTCGCTTCTACCC</td>
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<td>TGFβ1</td>
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<td>bFGF</td>
<td>TTCTTCTGCGCATCCAC</td>
<td>CGGTTAGCACACACTCCTTTGT</td>
</tr>
<tr>
<td>VEGF</td>
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<td>TCTGCTGGTCCAAATCC</td>
</tr>
<tr>
<td>Cloning Primers</td>
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<tr>
<td>HOXC13-Flag-CMV4</td>
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</tr>
<tr>
<td>HOXC13-ΔHD Flag-CMV4</td>
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<td>Antisense oligonucleotides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOXC13 antisense</td>
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<td></td>
</tr>
<tr>
<td>Scramble Antisense</td>
<td>CGTTGTCCCTCCACATCT</td>
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Flanked by appropriate restriction sites
Phosphorothioate antisense oligonucleotide.

the nuclear localization signal (NLS), it lost nuclear localization and enriched mostly in cytoplasm. We also demonstrated that HOXC13 overexpression significantly induced the expression level of bFGF and TGFβ1, which are closely involved in cancer proliferation. HOXC13-ΔHD overexpression had no effect on the activity of bFGF and TGFβ1 showing the importance of homeodomain in those processes.

We performed in vitro colony formation assay to examine the roles of HOXC13 and its homeodomain in ability of HEK293 cells to form three-dimensional colonies. This experiment demonstrated that overexpression of HOXC13 resulted in about 5 fold higher number of 3D-colonies in soft-agar media compared with normal HEK293 and HOXC13-
ΔHD under the same condition. Application of HOXC13 antisense oligonucleotide suppressed the growth of the colorectal cancer xenograft that was implanted in nude mice. Overall, these results further indicate the importance of HOXC13 in tumor growth and viability and the fact that can be a therapeutic target gene.
REFERENCES


The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions, Mol. Endocrinol. 13, 1672-1685.


BIOGRAPHICAL INFORMATION

Sahba Kasiri is a PhD candidate graduate student in the Department of Chemistry and Biochemistry at the University of Texas at Arlington (UTA). He earned his bachelor’s degree in Pharmacy in 2001 from the Baha’i Institute for Higher Education (BIHE), Tehran, Iran. He joined Dr. Mandal’s laboratory at UTA in 2007. His research project was about endocrine regulation of HOXC13 and also the role of this gene in cell proliferation, viability, and tumorigenesis. His future plan is to continue his studies in cancer epigenetics and novel gene therapy.