MIXED LINEAGE LUEKEMIA HISTONE METHYLTRANSFERASES IN HORMONAL REGULATION OF HOXB9 AND TARGET GENE REGULATION

by

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ABSTRACT

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Homeobox gene (HOX) genes are evolutionary conserved genes that play important roles in cell differentiation, cell proliferation and embryogenesis. HOX genes bind to the DNA via their homeodomain and act as transcription factors. Of the 39 HOX genes present in human, HOXB9 is known to be a critical player in skeletal and mammary gland development. HOXB9 also regulates renin gene expression which is a critical player in Renin-Angiotensin system.

Recent studies also demonstrate that HOXB9 is critical for angiogenesis. I have investigated transcriptional regulation of HOXB9 and its potential biochemical function during cell cycle progression and tumorigenesis. My studies demonstrate that HOXB9 is an estrogen responsive gene. HOXB9 promoter contains multiple estrogen response elements through which they interact with estrogen-receptors and regulate gene expression in presence of estrogen. Mixed lineage leukemia (MLL) family of histone methylases that are key players in

gene activation and epigenetics, coordinate with estrogen-receptors during transcriptional activation of HOXB9 in presence of estrogen.

Studies also demonstrate that HOXB9 is overexpressed in breast cancer. HOXB9 regulates various cell cycle regulatory genes that includes various Cyclins and p-proteins and regulates cell cycle progression. Overexpression of HOXB9 induces cell cycle arrest in G0/G1 phase and ultimately induces apoptosis. Homeodomain of HOXB9 plays critical roles in transcriptional regulation of cell cycle regulatory genes and cell cycle progression. Further studies demonstrated that HOXB9 overexpression stimulates three-dimensional growth of tumor in colony formation assay. HOXB9 controls the expression of various tumor growth and angiogenic factors via involvement of its homeodomain and thus influences tumor growth. In conclusion, HOXB9 is an estrogen responsive gene and is overexpressed in breast cancer. HOXB9 is a crucial player in cell cycle regulation and tumorigenesis.

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LIST OF ABBREVIATIONS

MLL	Mixed lineage leukemia
HMT	Histone methyl transferase
RNAP II	RNA polymerase II
SET	Su[1] 3-9, enhancer of zeste and trithorax
Cgbp	CpG dinucleotide binding protein
SAM	S- adenosyl methionine
HOX	Homeobox domain containing gene
Wdr5	WD repeats containing protein 5
Rbbp5	Retinoblastoma binding protein 5
Dpy 30	DPY 30 domain containing protein
ChIP	Chromatin immune-precipitation
RT-PCR	Reverse transcriptase polymerase chain reaction
FBS	Fetal bovine serum
DMEM	Dulbecco's modified eagle's medium
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
LB	Lysogeny broth medium
IPTG	Isopropyl-D-1-thiogalactopyranoside
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraaceticacid
PBS	Phosphate buffered saline
FITC	Fluorescein isothiocyanate
DAPI	4', 6-diamidino-2-phenylindole
DEPC	Diethyl pyrocarbonate
MMLV	Moloney murine leukemia virus
Tris	Tris (hydroxymethyl) aminomethane
PMSF	Phenylmethanesulfonylfluoride
ALL	Acute lymphoblastic leukemia
AML	Acute myelogenous leukemia
NR	Nuclear receptor
ER	Estrogen receptor
PR	Progesterone Receptor
ERE	Estrogen response element
RA	Retinoic acid
RARE	Retinoic acid response element
VEGF	Vascular endothelial growth factor
bFGF	Basic fibroblast growth factor
TGFβ1	Transforming growth factor beta 1
NRG	Neuregulin
WD	Tryptophan-aspartic acid motif

CHAPTER 1

MIXED LINEAGE LEUKEMIAS IN HISTONE METHYLATION, GENE EXPRESSION, AND HORMONE SIGNALING

1.1 Introduction

Eukaryotic gene expression is a highly complex and tightly regulated process including transcription initiation and promoter clearance, elongation, termination, splicing, maturation, and export [1-4]. These processes are often coupled together and co-transcriptional and may be controlled in different stages. RNA polymerase II (RNAP II) is the key player in transcription of protein coding genes. RNAP II, along with various general transcription factors (GTFs such as TFIID, TFIIA, TFIIB, TFIIF, TFIIE and TFIIH) bind to the promoter of the genes. In presence of ribonucleotide triphosphates (rNTPs), transcription begins with the formation of the first phosphodiester bond. Then RNAP II proceeds through transcription elongation phase where longer and longer transcripts are synthesized until it reaches to the end of the gene where transcription termination takes place. The nascent RNA is then processed and transported to the cytoplasm for translation.

Importantly, in eukaryotes, DNA which carries the genetic information is complexed with various histone proteins and packaged in the form of a complex chromatin [4, 5]. These complex chromatin needs to be remodeled (opened up) prior to getting the access to the transcription machineries for transcription [4, 6]. The repeating unit of chromatin is called nucleosome where DNA is wrapped tightly around the histone proteins. There are four different types of histone proteins present inside the nucleosome core. These include the histones H2A,

H2B, H3 and H4. Each histone is present in two copies forming a histone octamer [4, 5]. Recent studies demonstrate that the N-terminal tails of histones that are protruding out undergo various types of covalent modification that dictates the states of chromatin such as gene activation and silencing.

1.2 Histone modification and histone code hypothesis

Histones can undergo a variety of post-translational modifications which include acetylation, methylation, phosphorylation and ubiquitination. Histone acetylation is a well characterized post translational modification and is known to alter the chromatin structure and regulate gene expression patterns. In general histone acetylation is associated with transcription activation. Histone methyl transferases (HMT) are key enzymes that mediate methylation of basic residues, lysine and arginine on histone tails and impose both gene activation as well as gene silencing. Histones can undergo methylation on lysine # 4, 9, 27, 36, 79 in H3 and on lysine # 20 in H4 and also at the H1 amino terminus [4, 6]. Many different HMTs have been identified in living organisms to carry out the mono-, di-, and tri- methylation at these different lysine residues. A list of histone lysine methyltransferases identified to date has been listed in the table below [5, 7-11].

Table 1.1 Histone lysine methylation and different HMTs involved and their functions.

Histone lysine	Histone methyl transferase	Function
H1K26	Hs EZH2	Transcriptional silencing
H3K4	Dm Trx; Hs SMYD3, Hs MLL1, MLL2, MLL3, MLL4, MLL5	Transcriptional activation Transcriptional activation and elongation
	Hs SET1; Sc Set1	Transcriptional activation
	Hs SET 7/9	Transcriptional activation (along with H3K9 and H4K20 methylation by Dm ASH1)
	Dm Ash1	Transcriptional activation
	Meisetz	
H3K9	Dm Su [1] 3-9; Hs SUVAR39H1 and SUVAR39H2; SpClr4	Heterochromatin formation and Euchromatic silencing, DNA methylation
	Hs G9a, Hs GLP1, ESET	Euchromatic silencing, DNA methylation
H3K27	Dm E(Z), Hs EZH1, EZH2; Hs and Mm G9a Hs SET7/9	Euchromatic silencing
H3K36	Sc/Sp Set2	Transcriptional elongation and silencing
	Mm NSD1, Hs SYMD2	Transcriptional regulation
H3K79	Hs DOT1L; Sc Dot1p	Transcriptional silencing
H4K20	Hs and Dm SET8	Cell cycle dependent silencing and mitosis
	Dm SUV4-20	Heterochromatic silencing
	Mm and Hs SUV4-20H1 and SUV4-20H2	Transcriptional regulation
	Mm NSD1	Transcriptional activation (along with H3K4 and H4K20 methylation by Dm ASH1)
	Dm ASH1,Sp Set9	

Abbreviations used: Dm, Drosophila melanogaster; Hs, Homo sapiens, Mm, Mus musculus; Sc, Saccharomyces cerevisiae; and Sp, Saccharomyces pombe.

Since histones can undergo different modifications and depending on the different types of modifications, different effector proteins are recruited [4, 6]. The chromodomain, the tudor domain and the WD40 repeat (tryptophan-aspartic acid containing motif) domain containing

proteins have been identified to interact with the methylated lysine residues [6]. It is very likely that a single histone modification doesn't work alone. Possibly a combination of multiple histone modifications are interdependent on one another and can work together to act as receptors for recruiting multi-protein complexes that signify downstream functions and mediate unique cellular responses.

1.3 MLLs are H3K4 specific histone methyl transferases

H3K4 trimethylation are evolutionarily conserved mark closely associated with actively transcribing genes. MLLs which are responsible for H3K4 trimethylation are known to be direct regulators of HOX genes. HOX genes are critical players in development and disease. They act as transcription factors and bind to the promoter of the genes involved in cell differentiation, cell cycle, angiogenesis and cardiovascular disease [12]. HOX genes are known to interact with other HOX collaborators such as Pbx1 and Mesi1 and directly or indirectly influence the expression of its target genes [13, 14]. Roles of histone methylation in maintaining epigenetic memory, gene regulation and development are largely conserved in mammals [15-17]. The H3K4 trimethylation pattern in human are similar to yeast and are associated with actively transcribing regions [15]. In yeast, Set1 is the sole histone H3K4-specific HMT present in a multiprotein complex called COMPASS and is linked with gene activation [18]. Set1 is a unique methyltransferase present in yeast since upon its deletion, H3K4 methylation is completely lost [19]. It is associated with the coding regions of highly transcribed genes and the occupancy of Set1 correlates with the presence of H3K4 trimethylation levels. Unlike in yeast, human encode at least ten different H3K4-specific HMTs [7, 18]. These include MLL1 (mixed lineage leukemia 1), MLL2, MLL3, MLL4, MLL5, hSet1A and hSet1B, ASH1, SET 7/9, SMYD3, and a meiosis specific factor Meisetz [18]. MLLs are well known to be often rearranged, amplified or deleted in different types of cancer. MLLs are also known as master regulators of homeobox (HOX) genes that play key roles in embryogenesis, development and adult tissue homeostasis [20]. MLLs

have been isolated from human cells and their protein-protein interaction profiles and their enzymatic activities have been characterized in detail. These studies demonstrated that MLL1, MLL2, MLL3, MLL4, MLL5, hSet1A and hSet1B exist as distinct multiprotein complexes and all contain a SET domain in common which is responsible for their HMT activity [21]. MLLs transfer the methyl group from the cofactor S-adenosyl-L- methionine (SAM) to the ε -nitrogen atom on lysine with the help of the SET domain [19].

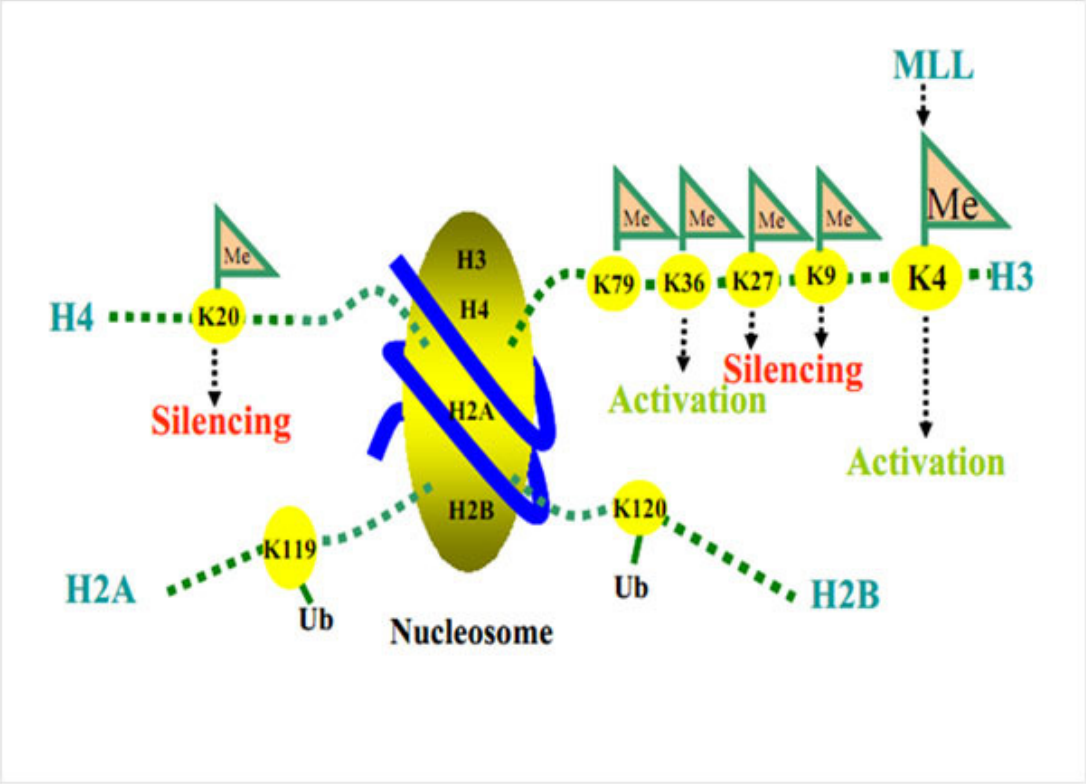

Figure 1.1 Lysine 4 methylation on histone H3 by MLLs. H3K4 methylation is associated with gene activation whereas H3K9 methylation is associated with gene silencing. MLLs carry out H3K4 methylation.

1.4 MLLs contain various functional domains

In general, MLL (MLL1) undergoes proteolytic cleavage shortly after translation and this cleavage divides the protein into a larger N-terminal portion 320 kDa and a smaller C-terminal part 180 kDa by the enzyme Taspase [22]. This proteolytic cleaving of MLL1 by Taspase 1 has been reported to be essential for proper Hox gene function [23].

MLLs contain diverse types of functional domains that include SET domain, DNA binding AT hooks, a cysteine rich CXXC motif, plant homeodomains (PHD) finger motifs, a bromodomain, a transcription activation domain (TAD), the WDR5 interaction motif, LXXLL domains (also called NR-boxes) etc [21, 22, 24, 25]. The SET domain is an evolutionarily conserved domain and is responsible for their histone H3K4-specific HMT activity. It is named for its presence in Drosophila chromatin regulators Su (Var) 3-9, E (z), and Trx [21, 25]. MLL1 belongs to the SET1 family of SET domain proteins such as SUV39, SET1, SET2, E (z), Riz, SMYD and SUV2-20 families [18]. The SET domain is responsible for transferring the methyl group from SAM to the ε-nitrogen atom on the lysine with the help of adjacent cysteine-rich domains [15, 19, 24, 26]. FYRN, FYRC and the SET domains are involved in the heterodimerization between MLL-N and MLL-C terminal fragments by mediating the interactions between N320 and C180 subunits of MLL [27]. The RING fingers are also involved in protein-protein interactions [26].

MLL1 contains three AT-hook motifs which have DNA binding activity. The AT-hooks bind to AT-rich DNA, and show no preference for a particular sequence but to the bends or cruciforms in DNA and help MLLs in targeting to specific genomic regions by recognizing these structures [22]. MLL1 contains four PHD fingers which bear homology to bromodomains and are the second most conserved domain between MLL and trithorax (trx) [20, 22] The PHD fingers have been shown to be involved in protein-protein interactions [20]. The second PHD finger is required for homodimerization and the third one is known to interact with Cyclophillin Cyp33

[28]. MLL1 is known to co-localize with Cyp33 and over expression of Cyp33 is known to affect the expression of MLL downstream HOX target genes. CXXC domain of MLL1 helps it bind to unmethylated CpG islands and is responsible for target gene recognition, transactivation and myeloid transformation in MLL-fusion protein related leukemogenesis [18, 24]. The promoters of most of the active genes are usually hypomethylated and it is therefore hypothesized that the CXXC domain of MLL1 helps in directing it to the unmethylated promoters of active genes. Although, the CXXC domain of MLL1 helps it bind to the unmethylated CpG islands, several genes that are not regulated by MLL1 also contain CpG islands thus suggesting the possibility of several other mechanisms of MLL1 gene targeting. The CXXC domain bears homology to the DNA methyl transferase (DNMT) and also interacts with histone deacetylases (HDACs) and was shown to recruit polycomb proteins HPC2 and BMI-1 and the co-repressor protein CtBP [22, 24] . The repression domain is retained in the MLL leukemic fusion proteins while the activation domain and the SET domain are lost which possibly accounts for the aberrant MLL functions eventually leading to leukemic transformations.

A more recent structure of the TAD of MLL1 bound to the CBP protein describes one such additional mechanism that could also be involved in targeting MLL1 to specific loci [22]. The transcription activating domain of MLL1 has been found to interact with the CBP protein in a yeast three hybrid screen using CREB-CBP as a bait and was shown to be essential for MLL1 mediated transcriptional activation. MLL1 recruitment to chromatin was shown to increase H3K4 trimethylation by the SET domain of MLL1 and was shown to be present in a multi-protein complex consisting of Wdr5, Rbbp5, Ash2L and Dpy30 [17, 25]. Wdr5 is a conserved component of Set1 family complexes and is conserved from yeast to humans [17]. Wdr5 has been reported to be essential for H3K4 trimethylation by MLL1 and has been reported to be a histone binding module that presents histone H3 for further methylation by MLL1. The Wdr5 binding surface in MLL1 has been reported to be a short six residue conserved sequence in the

N-flanking region of the MLL1 SET domain. This Wdr5 interaction motif or Win motif is highly conserved among metazoan MLL1 orthologs and other SET1 family members [19]. MLL contains “LXXLL domain” or NR-box which helps it bind to the Nuclear Receptors (NRs) [29]. Upon ligand binding, the conserved C-terminal ligand binding domain of the NRs undergoes a conformational change that releases it from any repressors bound to it. This change in confirmation is then recognized by an α-helical LXXLL motif which mediates the association with the NRs [29]. Several coactivators like SRC-1, GRIP-1, TIF-1α, RIP-140 have been shown to contain the NR-box. MLL1 contains 1 NR-box, MLL2 contains 5, MLL3 contains 3 and MLL4 contains 4 NR-boxes [29, 30]. The surrounding sequences around the LXXLL domain also play a vital role in the interaction between NR boxes and its coactivators.

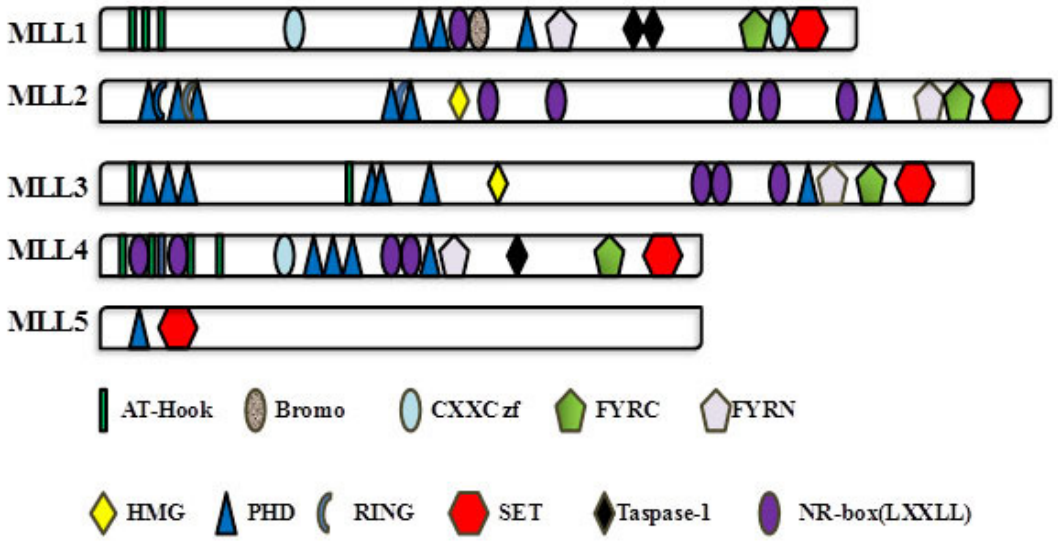

Figure 1.2 Domain structures in MLL. AT-Hook is a DNA binding domain. Bromo domains are involved in the recognition of acetylated lysine residues. CXXC-zf is a zinc finger domain that binds to unmethylated CpG islands in the DNA. FYRC and FYRN domains are involved in the heterodimerization between MLL^N and MLL^C terminal fragments. HMG domain is also a DNA binding domain that binds DNA with low specificity. PHD (plant homeodomain) are involved in protein-protein interactions; RING fingers are also involved in protein-protein interactions. SET domain is responsible for histone lysine methylation. Taspase 1 marks the proteolotyic site for cleavage by the protease Taspase 1.

1.5 MLL1 is rearranged in leukemia

The mixed lineage leukemia-1 gene located on 11q23 was initially recognized as a recurrent locus of chromosomal translocation in acute myeloid leukemia (AML) and also acute lymphoblastic leukemia (ALL) [28, 31]. During MLL-fusion partner formation so far, MLL has been found in >60 different translocation partners. Most of these translocation partners are expressed in a variety of adult tissues including hematopoietic cells [32]. MLL translocation partners appear to fall into two functional categories, they act either as signaling molecules that normally localize to the cytoplasm/cell junctions or nuclear factors that have intrinsic transactivation properties. Almost all of the MLL partner proteins that contribute to the majority of ALL/AML have been identified and several MLL partner proteins like AF3p21, AF5q31,

AF9q34, AF15q14, LAF4, Gas-7, GRAF, GMPS, GEPHYRIN and LARG have been isolated from rare, infrequent translocations [32-34]. Up-regulation of A cluster homeobox (HOX) genes and the HOX cofactor MEIS1 is pivotal for MLL-fusion protein mediated leukemogenesis [34]. MLL translocations are found in de novo ALL in infants and they are also found in therapy or treatment related AML (t-AML) in infants, children and adults. ALL and AML show similar pathogenesis but the breakpoints in MLL translocations may vary slightly. The survival rates of the patients suffering from t-AML or ALL show a 5 year overall survival rate of < 35%. Two rarer cases of luekemogenic MLL mutants have been described so far. These include: 1) various partial tandem duplication of MLL and 2) deletions of MLL exon 8 that encodes critical cysteine residues within the first PHD finger of MLL. MLL mutants promote leukemogenesis without immediate need of MLL for a fusion partner suggesting that alterations of MLL function alone can promote leukemogenesis. MLL partial tandem duplications (PTD) are found in AML with a normal karyotype or with trisomy 11. The majority of MLL-PTD observed in AML cases are without chromosomal aberrations and in less than one-third of cases with aberrations. The only exception was AML with trisomy 11, of which 25% of 16 samples were MLL-PTD positive. MLL-PTD have also been reported in healthy individuals and also in the cord-blood of newly born, although the observed exon fusions were unusual than the fusions observed in patients suffering from AML. Neither the pathological mechanism of MLL-PTD nor its direct targets has been well characterized till date.

1.6 MLLs exist as multi-subunit complexes

Biochemical studies have shown that MLL family of HMTs shares many proteins in common: Rbbp5, Ash2L, Wdr5, human CpG binding protein (Cgbp) and Dpy30 [18]. The presence of three such subunits in common in MLL/SET1 family of HMTs implies common regulatory mechanism for the enzymatic activities and/or substrate specificities. For the optimal methyltransferase activity, the presence and the interaction of all the subunits are required. By

in vitro assays as well as in vivo assays, the importance of all these subunits for the full methyltransferase activity of MLL has already been known. One of the crucial components of the MLL1 core complex, Wdr5 has been shown to interact with MLL1, MLL3, MLL4, and Set1A/Set1B. While, Wdr5 has been reported to be essential for the stable interaction with the SET domain for proper methyl transferase activity, the presence of Ash2L and Rbbp5 cannot be neglected and are essential for the full methyltransferase activity both in vivo and in vitro [35]. Wdr5 alone is not sufficient for in-vitro methyl transferase activity of MLL1. However, Ash2L and Rbbp5 are not able to stably interact with MLL1 even though both the proteins are capable of stimulating MLL1 methyl transferase activity [36]. Recently, Dou et al. have shown that Ash2L/Rbbp5 heterodimer has a weak intrinsic H3K4 methyltransferase activity and that Ash2L is able to interact with histone H3 and SAM. This catalytic activity requires the highly conserved DPRY domain of Ash2L as well as its interaction with Rbbp5 [36]. However, the importance of Ash2L in the methyltransferase activity of the two of the most predominant H3K4 methyltransferase in humans Set1A/Set1B remains to be tested yet [37].

Recent studies from Dr. Mandal laboratory have shown that CpG binding protein Cgbp interacts with MLL1, MLL2 and hSet1. Cgbp is a CXXC finger domain DNA binding protein that binds to unmethylated CpG islands and plays a critical role in gene expression and mammalian development [18]. Immuno-precipitation experiments showed that Flag-Cgbp-IP pulled down MLL1, MLL2 and hSET1 HMTs along with their associated components Ash2L, Wdr5, Rbbp5 and Menin. The recruitment of Cgbp into the promoter of HOXA7 was performed by ChIP assay. These observations were further supported by the immuno-colocalization experiments performed on FLAG-Cgbp stable cells with anti-FLAG, anti-MLL1, anti-MLL2 and anti-hSET1 antibodies wherein Cgbp co localized with MLL1, MLL2 and partially with hSet1. Furthermore, the functional significance of Cgbp was asserted when the antisense mediated knockdown of

Cgbp diminished the recruitment of MLL1 and down regulated the level of H3K4 trimethylation affecting the expression of HoxA7.

1.7 MLLs are master regulators of HOX genes

Various studies have reported that *Mll1* is essential for appropriate development of tissues during embryogenesis and is required for successful skeletal, hematopoietic, cranial and neurofacial development [28]. Homozygous *Mll1* mutant mice that die during embryogenesis and lethality at embryonic day 10.5 is associated with multiple patterning defects in neural-crest derived structures of the brachial arches. *Mll1* mutant mice demonstrated decrease in the number of hematopoietic cells and also exhibited abnormalities with decreased number of expression of Hox genes in the fetal liver [38]. Mice homozygous and heterozygous for *Mll* and also *Mll1* mutant mice showed decreased expression of evolutionary conserved Hox genes: Hoxb5, Hoxb8, Hoxb6 followed by no expression of Hoxc9 and Hoxb4. Hoxa7 regulation undergoes activation between E7.5 and E8.5 followed by a later stage of tissue specific maintenance. Hoxc8 which is regulated by early and late response elements also failed to maintain expression beyond E9 in *Mll1* mutant mice [38].

1.7.1 HOX gene structure and function

Homeobox (HOX) genes are evolutionary conserved group of genes which are necessary for body axis patterning during embryogenesis [11]. These homeobox genes were first described in *Drosophila melanogaster* and were found to provide developmental identity to various segments of the fly [12, 39]. Drosophila has eight homeobox genes clustered in one region of the genome and they are collectively known as the homeotic complex, HOM-C. Vertebrates also possess these homeobox genes and they are referred to as Hox genes in nonhuman vertebrates and HOX genes in human. The homeobox genes are present in a cluster and the 3' to 5' chromosomal alignment of the homeobox genes parallels their spatio-temporal pattern of expression along the anterior-posterior axis in the developing embryo. The

expression of a particular set of homeobox genes regulates development in a segment specific manner and plays a well characterized role in cell fate specification and differentiation.

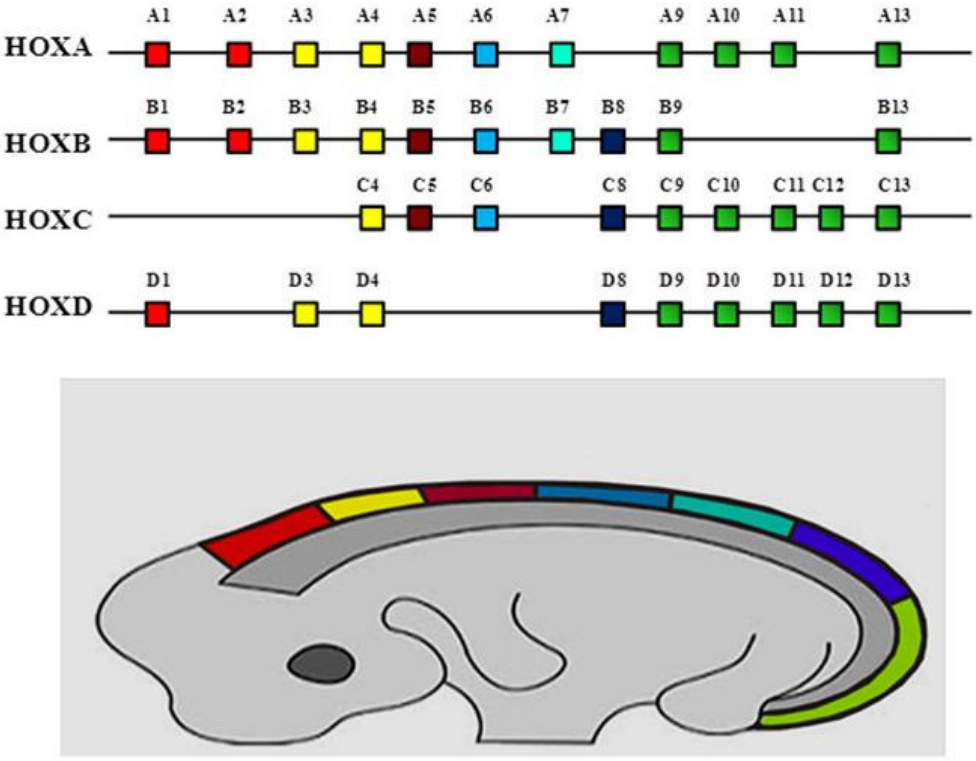

Figure 1.3 Distribution of HOX genes in embryonic development. In the developing embryos, the pattern of expression of HOX genes in human correlates with its chromosomal positioning.

In humans there are 39 different HOX genes organized in four different clusters: A, B, C and D. These are located on chromosomes # 7, 17, 12, and 2 respectively [30]. All the HOX genes contain a 183 bp sequence known as the homeobox which encodes a 61 amino acid DNA binding domain known as the homeodomain. The homeodomain contains a helix-turn-helix motif and recognizes highly similar nucleotide sequences containing a 5'-TAAT-3' core [12]. HOX genes encode transcription factors and are known to cause activation or repression of target genes although its target genes have yet not been properly identified [39, 40]. More than

one HOX protein is expressed in a given cell at any one point of time and these HOX proteins compete for regulatory sequences within a given target site. Depending upon the tissue context and the physiological context of the cell, the HOX proteins bind to DNA and interact with various other cofactors to regulate different downstream genes which might provide enhanced DNA specificity or differential binding affinity [41].

1.7.2 HOX genes and its interacting partners

Various HOX genes are known to interact with a variety of other proteins, also known as “collaborators” which provide differential tissue specificity and differential binding. The first group of proteins that were found to interact with the HOX proteins was the PBC containing proteins which include the Pbx proteins in vertebrates and the extradenticle proteins in Drosophila [41]. A conserved pentapeptide located upstream from the DNA-binding domain of most HOX proteins was found responsible for its interaction with PBX and this interaction contributed to the in-vivo specificity of most of the HOX proteins by generating multiple specific HOX/PBX heterodimer at HOX binding sites. It is assumed that HOX/PBX proteins interact with other proteins and confer DNA binding selectivity of HOX proteins on their target genes. However, not all HOX proteins can interact with PBX as they do not contain the conserved pentapeptide motif required for their interaction [41, 42]. In Drosophila, heterodimeric complexes between AbdB like Hox and Meis1 proteins have also been described in in-vitro experiments but there have been few reports that show its existence in vivo. A complex involving HOXA9-PBX and MEIS1 has been found in vitro and in myeloid cells [42]. MEIS1 is also known to interact with PREP1, another homeodomain containing proteins belonging to the TALE (three amino acid loop extension) family of amino acids. Nuclear localization of PBC complexes is thus hypothesized to be dependent upon its interacting partners like MEIS1 and PREP1 and this interaction is also thought to provide target gene specificity to the HOX proteins [14].

It has recently been demonstrated that HOXB7 interacts with various other transcription factors including NF-κB both in vitro and in vivo to modulate the activity of its target genes. NF-κB plays a central role in cellular defense against stress, cytokines and pathogens and also plays a central role in limb morphogenesis [43, 44]. HOXB7 also interacts with IκB-α, which is the first identified inhibitor of NF-κB [43]. Although it is not clear whether IκB-α also regulates HOXB7, the N-terminal domains of HOXB7 and both the ankyrin repeats and the C-terminal region of IκB-α were required for physical interaction [43]. A possible crosstalk between NF-κB/ IκB-α proteins and HOXB7 would provide a very interesting investigation as each of these transcription factors are required for distinct physiological processes.

Recent studies have also demonstrated physical interaction between HOXB7 and the coactivator CBP both in vivo and in vitro. This interaction led to enhanced transcription activation by HOXB7 and required the N-terminal region of HOXB7 as well as the two C-terminal domains of CBP [45]. One possible mechanism is that CBP can act as a bridge between HOXB7 and the basal transcription apparatus for its interaction with other transcription factors. Another possible mechanism is that CBP can interact with pCAF, another histone acetyl transferase and can modify the chromatin located at the HOX binding site due to acetylation either alone or in conjunction with pCAF thus enabling the access of HOXB7 to its target protein sites [45].

1.7.3 Specificity in HOX target gene regulation

The DNA binding domain of HOX proteins is highly conserved over species. The detailed study of the three dimensional structure of the homeodomain reveals that this homeodomain is highly conserved among all the HOX proteins [41]. Because of its highly conserved molecular structure, the majority of Hox proteins and its paralogous genes within one species, preferentially recognize a conserved but unspecific ATTA core motif [12]. The low DNA binding specificity of the HOX/Hox proteins, however sharply contrasts with the highly specific

effects these transcription factors exert on distinct and different sets of target genes in vivo. Hox proteins have been found to exert high development specificity in vivo and this has been attributed to the influence of other proteins on the Hox proteins [12]. All these studies suggested that multiple domains within Hox proteins are essential for in vivo specificity. Based upon these findings the idea emerged that Hox proteins would homodimerize or heterodimerize with many other factors, so called cofactors, which would subsequently enhance their sequence selectivity and binding specificity. In vitro studies have shown that the human HOXD9 protein activates transcription of the HOXD9 promoter by interacting with the Hox crosstalk region and this activity is antagonized by the HOXD8 protein. Although, HOXD8 in itself isnot a inhibitory protein, the amino terminus/ helix 1 region and an additional effector domain located at the amino terminus end is required for its inhibitory function. The deletion of the homeo domain helix 2/3 region did not seem to have an effect on the inhibitory effect of HOXD8 on HOXD9 promoter activity. This study therefore suggests that this activity is DNA binding independent and very likely mediated by protein-protein interactions [41].

1.7.4 HOX genes and its downstream target genes

HOX genes have been identified as key players in cell differentiation, cell proliferation, cell adhesion and migration, angiogenesis and apoptosis in addition to their roles during embryogenesis [12, 46]. The ability of HOX genes to act as both activators and repressors in expression of its downstream target genes are being studied by genome wide approaches in the recent years. In its monomeric state, the HOX transcription factors are reported to bind DNA weakly whereas when bound with other cofactors, they are reported to act as strong activators. In the recent years, a number of HOX target genes have been identified that play distinct roles in cellular identity and cellular function by both in vitro and in vivo experiments. The experimental procedures used to verify the HOX target genes have varied greatly making it difficult to state if some of them are direct targets and some of the Hox binding sites have not

been experimentally confirmed [40]. The sequences surrounding the HOX binding sites have also been reported to be essential for target gene regulation and tissue specific gene activation. A list of current likely targets of HOX genes are listed below utilizing the current information so far.

Table 1.2 List of mammalian Hox target genes

HOX gene	+/-	Target gene	Species
Hoxa2	-	Six2	Mouse
Hoxa5	+	p53	Mouse
HOXA5			Human
HOXA5	+	p53, Progesterone receptor, Pleiotrophin, IGFBP-1	Human
Hoxa9	-	Osteopontin	Mouse
HOXA9	-	BRCA1, EphB4	Human
HOXA10	+	p21	Human
HOXA10	+	β3-Integrin, IGFBP1	Human
HOXA10	-	CDX4, EMX2	Human
Hoxa10	+	IGFBP-1	Mouse
Hox a13	+	Epha7	Mouse
Hoxd13	+	Epha7	Mouse
HOXB1	+	COL5A2	Human
Hoxb3	+	TTF-1	Mouse
Hoxb5	+	SPI3, Flk1	Mouse
HOXB7	+	BFGF, VEGFA, MMP2, WNT5A, PDGFA	Human
HOXB5	+	Thrombospoindin-2, Angiopoietin	Human
Hoxb8	-	N-CAM	Human
Hoxb9	+	N-CAM	Human
HOXC6	-	N-CAM	Human
Hoxc8	-	IGFBP-1, NEP/MME	Human
HOXD1	-	Integrinβ1	Human
HOXD3	+	Integrinβ3	Human
Hoxb6,b7,b8,c8, d10	+	Renin	Mouse

1.8 Endocrine Regulation of HOX genes and its target genes

1.8.1 Retinoic acid and its influence on HOX gene expression

Endocrine regulation of developmental gene expression plays a critical role in the segmental expression of specific development genes necessary for the development of various organ systems. Retinoic acid has a well characterized role in the embryogenesis wherein it controls the expression of the anterior most Hox genes [46, 47]. In humans, oversupply of retinoic acid or Vitamin A leads to birth defects and in pregnant mice retinoic acid treatment causes homeotic transformations. It has been demonstrated that Retinoic acid receptors (RAR and RXR) upon ligand binding target retinoic acid response element (RARE and RXRE) of Hoxa1, Hoxb1 and Hoxd4 and regulate its expression. Retinoic acid regulation of Hox genes has been known to be limited to the chromosomal 3' located Hox paralogous group of genes which are expressed early in embryogenesis in relatively anterior regions of the body such as the head and the cervix. It is however less known to regulate the 5' paralogous Hox genes [47]. While retinoic acid has been known to up regulate the expression of the 3' paralogous genes Hoxa1 and Hoxb1, at the same time is known to suppress the expression of Otx2, a non clustered homeobox gene which is expressed throughout in the early murine embryo. Otx2 expression then becomes restricted to the most anterior segment that is associated with future brain development. The paralogous Hox genes Hoxa1 and Hoxb1 are expressed more posteriorly in the region associated with midbrain and hindbrain development. In Xenopus embryos, retinoic acid treatment preferentially initiates the development of more posterior structures which is observed by a reduction in forebrain volume but accompanied by a compensatory increase in hindbrain volume. A feedback system between Hox genes and RARs exists which is essential for the proper development of anterior and posterior segments [48].

1.8.2 Estrogen and its influence on HOX gene expression

Estrogen is essential for the development of the female reproductive tract. Estrogen exerts its impact via the estrogen receptors ERα and ERβ and plays an important role in the estrogen mediated regulation of hormone responsive genes. Estrogen receptors bind to the estrogen responsive elements on the promoter and are also known to interact with various other partner proteins called as coactivators and corepressors and influence the recruitment of RNA PII on the target gene promoter [49, 50]. In vivo studies demonstrate that ERα deficient mice display normal appearing although functionally deficient uteri that are unresponsive to 17-β-estradiol result in inability to functionally differentiate adult endometrium, causing sterility[49, 50]. Similarly, ERβ deficient mice also display morphologically normal and also have normal responses to estradiol, although female mice are sub fertile [50].

In female reproductive mice, Hox gene expression shows a varied pattern. Hoxa9 has been reported to be expressed in the developing oviduct; Hoxa11 is expressed in the uterine and cervical analgen and Hoxa13 in the developing vagina [51]. Hoxa10, a gene that has been studied in detail for the past few years is reported to show a dose dependent response to physiological levels of estrogen ranging from 10^{-6} nM to 10^{-10} nM in adult endometrial cells [46, 52]. Moreover, up-regulation of HOXA10 mRNA levels is also seen in Ishikawa cells, a well differentiated endometrial adenocarcinoma cell line, which is known to express the estrogen and progesterone receptors [46, 52]. Similarly, HOXC13 and HOXB9 has been shown to be an estrogen responsive gene in JAR cells (placental choriocarcinoma cell line) [53]. HOXC13 targets keratin gene expression and plays a role in difference in hair patterning in males and females [11]. Upon knockdown of estrogen receptors, ERα and ERβ, E2-mediated activation of HOXC13 is suppressed. Similarly, knockdown of histone methylase MLL3 suppressed E2-induced activation of HOXC13 in JAR cells [54].

The molecular mechanism involved in estrogen-Hox signaling is ligand specific. In embryonic development, diethylstilbestrol (DES) but not estrogen causes a postal shift in expression of Hoxa9, Hoxa10, Hoxa11 genes. DES not only alters the spatial domains of expression of Hox genes but also lowers reporter gene expression of HOXA10 ERE compared with treatment with 17-β-Estradiol [49].

1.8.3 Progesterone and its influence on HOX gene expression

In the adult tissues, progesterone is secreted by the corpus luteum and directs the endometrium to undergo terminal differentiation necessary for embryo receptivity. Progesterone receptor A knockout mice are infertile and demonstrate failed implantation of artificially transferred blastocyst [47-49]. Artificial injections of progesterone in these mice do not show differentiation but show proliferation and inflammation of the tissues in the secretory phase. HOX genes like HOXA10 shows increased up regulation upon medroxy progesterone acetate treatment in the concentration range of 10^{-9} M to 10^{-6} M [48]. It has been observed that the progesteronal regulation of HOXA10 occurs via the progesterone receptor and is blocked upon treatment with RU486 both in primary endometrial stromal cells as well as in murine uteri. HOXA11 mRNA levels are also affected by progesterone levels and shows a similar regulation by estrogen and progesterone to HOXA10 throughout the estrus/menstrus cycle [55]. Progesterone is the major stimulus for cyclic endometrial terminal differentiation and is necessary for embryo implantation and infertility. This functional differentiation is carried out by high endometrial levels of the HOX genes, HOXA10 and HOXA11 driven by progesterone [55, 56]. Both progesterone and HOXA10 induce similar phenotypic effects characteristics of terminal differentiation in adult endometrial cells. It is likely that homeotic genes function downstream of progesterone to mediate cyclic endometrial functional differentiation [57, 58].

1.8.4 Testosterone and its influence on HOX gene expression

Testosterone is necessary for mesonephric duct differentiation in male embryos and is necessary for the proper male reproductive tract development. A coordinated expression of estrogen and progesterone are required for ovulation and proper menstrual cyclicity in women and both hormones differentially regulate HOX gene expression in adult female reproductive tract throughout the reproductive cycle [51, 73]. Testosterone blocks estradiol, progesterone or combined estradiol or progesterone mediated up regulation of HOXA10 mRNA levels in vitro in Ishikawa cells. Lower testosterone levels are associated with higher HOXA10 expression and increased receptivity to embryo implantation whereas higher testosterone levels are associated with menstrual anomalies, hirsuitism and anovulation [46, 55, 57, 58]. Androgens have their own physiological roles in adult females but their increased levels are also associated with disease and pathogenesis in females. A disorder commonly known as Polycystic Ovarian Syndrome (PCOS) that results in infertility, unsuccessful implantation and miscarriages in pregnant women has been associated with increased testosterone levels [59, 60]. PCOS is often characterized by excessive hair growth, insulin resistance and obesity in adult females. While the exact cause of PCOS in women is yet largely unknown, it is widely diagnosed with elevated androgen levels [55, 59].

Expressions of the HOX genes are tightly regulated throughout development. While retinoic acid regulates the expression of most of the anterior 3' to 5' homeobox genes, estrogen and progesterone are known to regulate most of the abdominal homeobox genes. Similar to retinoic acid, estrogen regulates the 5' paralogous group of genes, Hoxa9, Hoxa10 and Hoxa11 which are expressed in posterior and distal domains of the body axis. Even though the roles of MLLs in regulation of Hox genes are largely known and homeobox genes play a critical role in the developmental processes and are hugely affected by hormones, the role of MLLs in the

hormonal regulation of Hox genes and its involvement of coactivators and corepressors in a cell specific environment have not yet been fully understood.

1.9 Discussion

Histone modifications are associated with changes to the chromatin DNA template which can lead to significant biological consequences many of which can lead to disease. Histone H3 methylation at lysine 4 is known to be actively associated with gene activation. In human there are seven HMTs that are responsible for H3K4 trimethylation namely: MLL1, MLL2, MLL3, MLL4, MLL5, hSET1A, hSET1B etc. [11]. Many of these histone modifications can work in conjunction with one another and affect biological processes. Recent discoveries of histone methyl transferases and other chromatin modifiers have enabled us to understand the mechanisms involved in the control of key physiological and pathological processes. As alterations in these enzymes disrupt the chromatin marks and deregulate the processes that are associated with chromatin modification, such changes are closely associated with tumorigenesis and disease progression.

Any changes in these chromatin modifiers can affect the downstream processes inside a cell. MLLs which are responsible for H3K4 trimethylation are known to be direct regulators of HOX genes. HOX genes are critical players in development and disease. They act as transcription factors and bind to the promoter of the genes involved in cell differentiation, cell cycle, angiogenesis and cardiovascular disease. HOX genes are known to interact with other HOX collaborators such as Pbx1 and Mesi1 and directly or indirectly influence the expression of its target genes [11, 13]. Some of these target genes have already been largely studied while recent studies carried out in different laboratories have elaborated on the HOX target genes. Studies done in the recent years also show that some of the HOX genes possess redundant functions and knockdown of one of these paralogous group of genes also affects the expression of its target genes. Previous studies in our laboratory have already demonstrated that MLLs are

involved in the estrogen mediated activation of HOXC6, HOXB9 and HOXC13 genes [30, 53, 54]. In the future, it would be of great interest to study the role of MLL family of HMTs in the regulation of the paralogous HOX genes and HOX target genes in a hormonal environment. In a similar fashion, further studies on the role of other co-activators and co-repressors which interact with estrogen receptors and HMTs in a hormone mediated, cell specific context to drive the gene expression will bring in meaningful insight into the underlying complex biological pathway.

CHAPTER 2

HISTONE METHYLASE MLL1 AND MLL3 COORDINATE WITH ESTROGEN RECEPTORS IN ESTROGEN-MEDIATED HOXB9 EXPRESSION

2.1 Introduction

Post-translational modification of histone plays critical roles in gene expression and silencing [8, 23]. Mixed lineage leukemias (MLLs) are highly evolutionarily conserved human histone methylating enzymes that specially methylate histone H3 at lysine 4 (H3K4) and regulate gene activation [17, 19, 20, 59-67]. Set1 is the sole MLL homologue and H3K4-specific histone methylase present in yeast [61]. In higher eukaryotes, H3K4-specific HMTs are diverged with increased structural and functional complexity. In human, there are at least eight H3K4-specific HMTs that include MLL1, MLL2, MLL3, MLL4, MLL5, hSet1A, hSet1B, and ASH1 [62]. The high conservation and multiplicity of MLLs suggest that they have crucial and distinct functions in the cell, though their detailed mechanisms of action are largely unknown [35, 62, 63]. Each possesses a SET domain that is responsible for their histone H3K4-specific methylation activity and is linked with gene activation. MLL1 is often rearranged in human leukemias [64]. During rearrangement, MLL1 gets fused with various fusion proteins (called MLL-fusion partners) in frame leading to generation of chimeric proteins and oncogenic transformation. MLLs are also well known as master regulators of HOX genes that play key roles in embryonic development [65]. Mll1 knockout (mice) is embryonic lethal. Mll1 mutant mice also exhibit hematopoietic abnormalities and are associated with decreased expression of a number of Hox genes (Hoxa7, a9, a10, a4) in the Mll1 mutant fetal liver [66, 67]. Biochemical

studies demonstrated that MLLs (MLL1-4 and Set1A-B) exist as multiprotein complexes with several common core protein subunits including Ash2, Wdr5, Rbbp5, Cgbp and Dpy30 [6, 18, 20]. MLL core proteins are involved in their recruitment in specific promoter and in regulation of di- and trimethylation states of histone H3K4 [7, 8, 68]. In addition to core subunits MLLs also associate with distinct proteins and that might be linked with their distinct functions in cells [7, 18]. Recent studies demonstrated, in addition to their general roles in gene activation, MLLs (MLL1-4) participate in hormone mediated gene activation and signaling [54, 69-71]. MLLs interact with nuclear hormone receptors and act as novel coregulators of hormone dependent gene activation.

HOX genes are evolutionarily conserved homeobox containing genes that play critical roles during embryonic development [72, 73]. There are 39 HOX genes in human that are arranged in four different clusters (HOXA-D) and each plays unique function during development [74, 75]. Recent studies demonstrate that several HOX genes are associated in various types of diseases including cardiovascular diseases and cancer [76]. In particular relevance to our study, HOXB9 is a critical player in skeletal and mammary gland development (Figure 2.1). HOXB9 is involved in regulation of Renin, an aspartyl protease that cleaves Angiotensinogen into angiotensin I [77]. Angiotensin I is further cleaved into an octapeptide angiotensin II with the help of angiotensinogen converting enzyme and ultimately influences blood pressure, fluid homeostasis and electrolyte balance [34]. HOXB9 along with PBX (another homeodomain containing protein) bind to the proximal promoter of Renin gene regulating its expression [34]. Independent studies showed that Angiotensinogen and Renin gene expression are stimulated by estrogen [78].

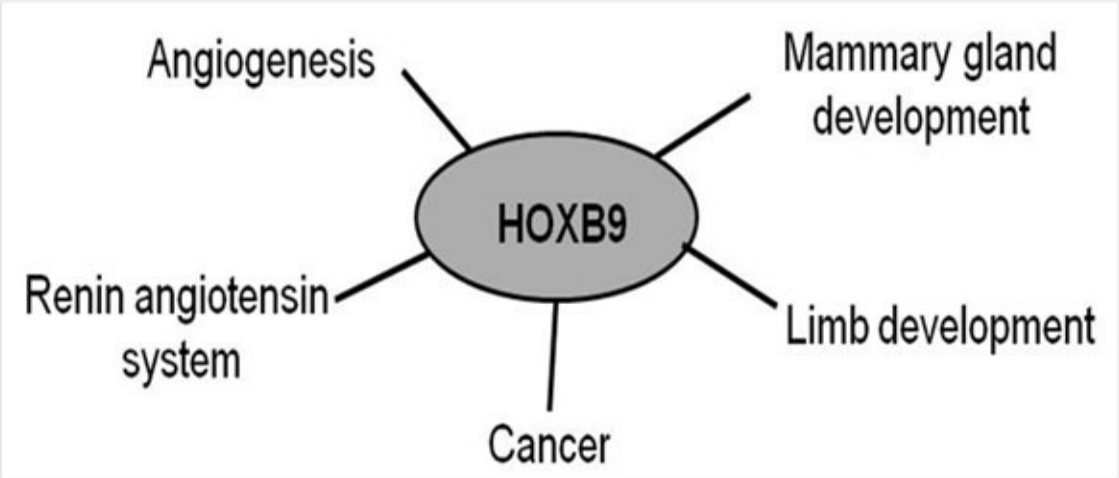

Figure 2.1 HOXB9 as a central player in mammary gland development, limb development, renin angiotensin system, angiogenesis and cancer. HOXB9 affects different physiological pathways via target gene regulation.

As HOXB9 is expressed in hormone sensitive mammary gland development and is linked with control of steroid hormone regulated Renin-Angiotensin system, we examined if HOXB9 is also transcriptionally regulated by steroid hormone such as estrogen. Although, it is well recognized that MLLs are master players in HOX gene regulation, little is known about their roles in hormonal regulation of HOX genes. Our studies demonstrated that HOXB9 is an estrogen-responsive gene. Estrogen-receptors and MLL histone methylases play key roles in estrogen-dependent activation of HOXB9.

2.2 Materials and methods

2.2.1 Cell culture, estrogen treatment and antisense–mediated knockdown experiments

In general, JAR (human choriocarcinoma placenta cells, ATCC) and MCF7 (human breast cancer cells, ATCC) were grown and maintained in RPMI-1640 and DMEM media respectively, supplemented with 10% FBS, 2 mM L-glutamine and penicillin/streptomycin (100 unit and 0.1

mg mL^{-1} respectively) in presence of 5% CO_2 at 37 ^{0}C [79-81]. For the estrogen treatment, cells were initially grown in phenol red-free media containing charcoal stripped FBS for three rounds and then treated with varying concentrations of E2 followed by incubation for varying time periods as needed.

For antisense-mediated knockdown experiments, JAR cells were grown up to 60% confluency and transfected with varying amounts (0.6 to 1.8 µg mL^{-1}) of different antisense oligonucleotides (custom synthesized from IDT-DNA) in FBS-free media using iFECT transfection reagent (MoleculA) and following manufacturer's instruction. In brief, antisense and iFECT transfection reagents were mixed in 300 µL DMEM-F-12 was applied to cells (60 mm plate) in presence of 1.7 mL supplement free medium. The cells were incubated for 7 h followed by addition of 2 mL media containing all supplements and 20% charcoal stripped FBS. Cells were then incubated for additional 48 h. Depending on need, antisense-treated cells may have been exposed to 100 nM E2 and incubated for additional 6 h and then harvested for RNA/protein extraction.

2.2.2 RNA extraction, Reverse transcription and Real Time PCR

The cells were harvested by centrifugation at 500g. The total RNA was extracted by using RNAGEM tissue plus RNA extraction kit (ZyGEM) following manufacturer's instruction. The reverse transcription reactions were performed with 1 µg of total RNA in a 25 µL reaction cocktail containing 2.4 µM of oligo dT (Promega), 100 units of MMLV reverse transcriptase, 1 x first strand buffer (Promega), 100 µM each of dNTPs (Invitrogen), 1 mM dithiothreitol (DTT), and 20 units of RNaseOut (Invitrogen). The cDNA was diluted to 100 µL final volume. For semi-quantitative PCR the cDNA was PCR amplified by using Taq DNA Polymerase (Genscript) and primers as described Table 2.1. For real-time PCR analysis, the cDNA was amplified using SsoFast EvaGreen supermix (Bio-Rad) using CFX96 real-time PCR detection system. The real-

time PCR results were analyzed using the CFX Manager software. The experiments were repeated at least twice with three replicates each time.

2.2.3 Protein extraction and western blotting

Whole cell protein extracts from E2 and/or antisense-treated and control cells were prepared in whole cell extract buffer (50 mm Tris/HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.05% NP-40, 0.2 mM PMSF, 1X protease inhibitors) as described previously. The protein extract was analyzed by western blotting using antibodies specific to MLL1 (Upstate), MLL2 (Bethyl laboratory), MLL3 (Abgent), MLL4 (Sigma), ERα (Santa Cruz), ERβ (Santa Cruz), and β-actin (Sigma). Western blots were developed using alkaline phosphatase method.

2.2.4 Chromatin Immuno-precipitation (ChIP) experiment

ChIP assays were performed on JAR cells using EZ Chip chromatin immuno-precipitation kit (Upstate) as described previously [4, 13, 38]. For ChIP assay, JAR cells were treated with 100 nM of E2 for varying time points, fixed in 4% formaldehyde, lysed in lysis buffer and sonicated to shear the chromatin to 150-450 bp in length (see Figure 2.6). The sonicated chromatin was then run on 1% Agarose gel to confirm the fragment size. The fragmented chromatin was then diluted in ChIP dilution buffer containing 0.01% SDS, 1.10% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl. It was then pre-cleaned using 60 µL of protein G-agarose (Millipore) beads for 5 h at 4 ^{0}C. Protein G-agarose was then removed by centrifugation at 500g for 7 min and the chromatin containing supernatant was carefully taken out and aliquoted for immuno-precipitation with different antibodies. The pre-cleaned supernatant was subjected to immuno-precipitation using different antibodies specific to ERα (Santa Cruz), ERβ (Santa Cruz), MLL1 (Upstate), MLL2 (Bethyl laboratory), MLL3 (Abgent), and MLL4 (Sigma), RNA polymerase II (abcam, 8WG16 clone), and H3K4-trimethyl (Upstate) with an overnight incubation at 4 ^{0}C. The supernatant was then carefully discarded after centrifugation at 500g for 5 min. The protein G agarose beads were then subjected through

different wash steps with 1 ml each of low salt immune complex buffer containing 0.10% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl; high salt immune complex buffer containing 0.10% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl; LiCl Immune complex wash buffer containing 250 mM LiCl, 1% IGEPAL CA-630, 1% Deoxycholic acid, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1; and twice with 1X TE wash buffer containing 10 mM Tris-HCl, 1mM EDTA, pH 8.0. In each wash step, 1 mL of the above wash buffers were used and rotated with a rotor in 4 ^{0}C for 5 min, centrifuged at 500 g for 7 min in 4 ^{0}C and the supernatant carefully removed. The immuno-precipitated chromatin was eluted out with 250 µL of elution buffer containing 0.1 N $NaHCO_3$ and 1% SDS. The immuno-precipitated chromatin was then subjected to de-cross linking with 20 µL of 5M NaCl at 65 ^{0}C for 5 h and then de-proteinized using 20 µL of 1 mg mL^{-1} proteinase K (Roche) at 45 ^{0}C for 2 h (Figure 2.6). The purified DNA fragments were obtained after phenol-chloroform extraction and overnight precipitation in ethanol at -80 ^{0}C. The DNA fragments were then used for semi quantitative and/or real-time PCR amplifications using promoter specific primers as shown in Table 2.1.

2.2.5 Luciferase reporter assay

Four $ERE_{1/2}$ along with their flanking regions (350-400 nt in total) were cloned and inserted upstream of luciferase gene in pGL3-promoter vector (Promega) (primers used for Luciferase reporter assay are listed in supplementary Table 2.1). JAR cells were transfected with these ERE-containing luciferase reporters constructs using FUGENE 6 transfection reagent. Control transfections were done using empty pGL3 promoter vector without any ERE insertion or with a luciferase construct-containing segment of HOXB9 promoter containing no ERE (non specific control, non-ERE). At 24 h post-transfection, cells were treated with 100 nM E2 and incubated for additional 6 h. Cells were then subjected to luciferase assay using ONE-Glo Luciferase Assay System (Promega) as instructed and detected using a micro plate reader (Flowstar-

Omega). Each treatment was done in four replicates and the experiment was repeated at least twice (n = 8).

2.3 Results

2.3.1 Effects of estrogen (E2) on HOXB9 gene expression

As HOXB9 is linked with various hormonally regulated physiological processes including mammary gland development, Renin gene expression and cardiovascular function, we examined if its expression is influenced by steroid hormones such as estrogen [33-35]. To examine steroid hormone-mediated regulation of HOXB9, we treated two different steroidogenic human cell lines, JAR (Placental choriocarcinoma origin and MCF7 (human breast cancer), with 17β-estradiol (E2) and analyzed its impact on HOXB9 expression [82]. Initially cells were grown in phenol-red free media containing charcoal stripped fetal bovine serum and then treated with varying concentrations of E2 for 8 h. RNA was isolated from these E2-treated cells, reverse transcribed into cDNA and analyzed by regular PCR as well as real-time PCR using primers specific to HOXB9 (Figure 2.2A-B). Beta-actin was used as loading control. Real-time PCR quantification of HOXB9 expression (relative to β-actin) as a function of E2-treatment are shown in the respective bottom panels of Figures 2.2A and B. Interestingly, in JAR cells, HOXB9 expression was induced upon treatment with E2 in a dose dependent manner (Figure 2.2A). HOXB9 expression was increased by ~4 fold in 10-100 nM E2-treatment in comparison to control (compare lane 1 with 4 and 5, Figure 2.2A). Treatment with E2 also induced HOXB9 expression in MCF7 cells, though the level of induction was less in comparison to JAR cells (Figure 2.2B). The highest expression of HOXB9 was observed at 1 nM of E2 in MCF7 cells (Figure 2.2B). Time-dependent studies (in JAR cells) demonstrated that E2-induced transcriptional activation of HOXB9 reached to maxima at about 4-8 h post E2-treatment

(Figure 2.2C, real-time PCR quantification is shown in right panel). The induction of HOXB9 expression in two independent steroidogenic cell lines demonstrated that HOXB9 is an estrogen-responsive gene.

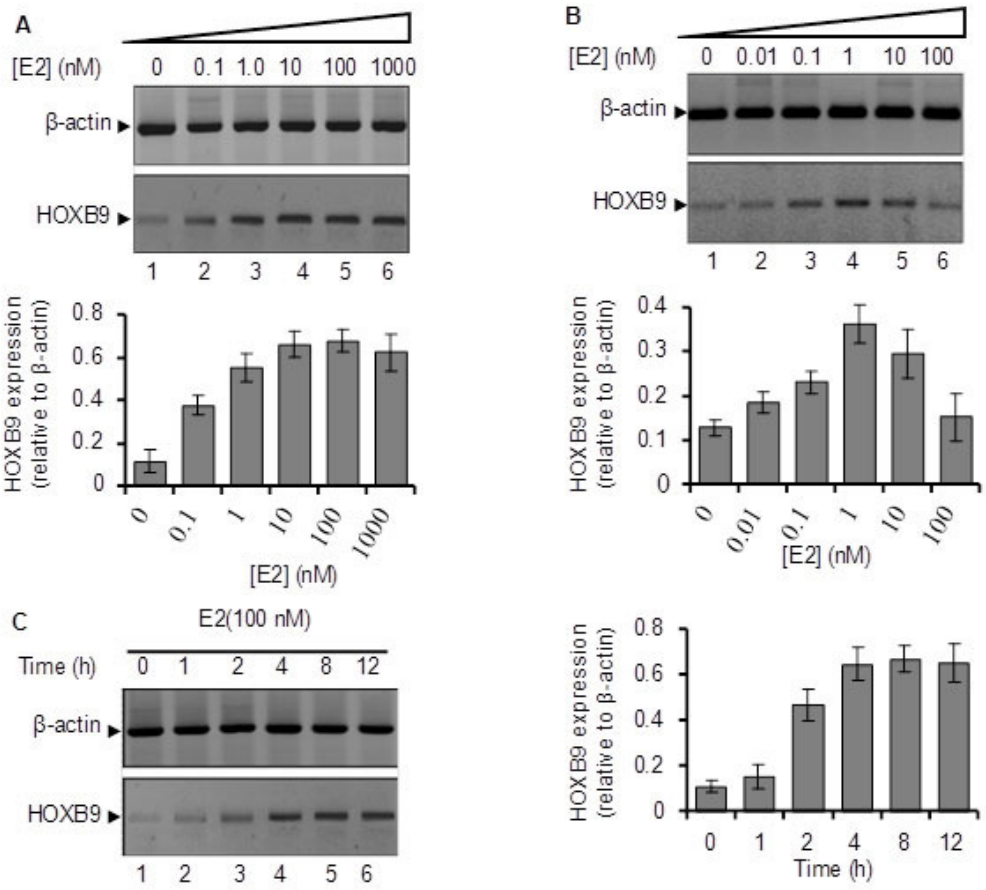

Figure 2.2 Effect of E2 on HOXB9 expression. (A) Effect of E2 on HOXB9 expression in JAR cells. JAR cells (grown in phenol-red free media) were treated with varying concentrations of E2. RNA was isolated, reverse transcribed, and subjected to regular PCR (top panel) and real-time PCR (bottom panel) using primers specific to HOXB9. β-actin was used as a loading control. Each experiment was repeated at least thrice. Bars indicate standard errors ($p < 0.05$). (B) Effect of E2 on HOXB9 expression in MCF7 cells. This effect was analyzed in a similar way as described for JAR cells in Figure 2.2. Top panel shows the agarose gel analysis of the PCR products and bottom panel shows the real-time PCR data. (C) Time dependent HOXB9 expression in JAR cells upon E2 treatment. JAR cells were treated with 100 nM E2 for varying time periods (0 -12 h) and RNA was reverse transcribed and analyzed by regular PCR (left panel) and real-time PCR (right panel) using HOXB9 and β-actin primers. Each experiment was repeated at least thrice ($n = 3$).

2.3.2 HOXB9 promoter contains potential estrogen-response elements

In order to examine mechanism by which HOXB9 may be transcriptionally activated upon exposure to E2, we analyzed its promoter sequence for presence of any putative estrogen-response elements [83]. These analyses revealed that there are four $ERE_{1/2}$ sites (GGTCA or TGACC, termed as ERE1- ERE4) present within first 3000 nt upstream of HOXB9 transcription start site (Figure 2.4A). Analysis of the neighboring sequences of $ERE_{1/2}$ sites did not show presence of any typical consensus full ERE (GGTCAnnnTGACC). However, ERE4 (TGTCCnnnGGTCA) appears to have very close homology (with one nucleotide difference) with a consensus full ERE (Figure 2.4A) [84, 85]. These analyses suggested that ERE4 might be a potential imperfect full ERE while others (ERE1-3) are just $ERE_{1/2}$ sites (Figure 2.4A).

Irrespective of their sequence homology with consensus full ERE, we examined the estrogen-responsiveness of each $ERE_{1/2}$ sites using a luciferase based reporter assay [86]. We cloned each ERE along with ~150 nt flanking sequences on both side into a luciferase expression construct pGL3 (Figure 2. 4A).

The ERE sequences along with 150 nt flanking sequences were PCR amplified from genomic DNA using promoter primers respective to that ERE. The promoter primers were designed with XhoI and KpnI restriction sites, with KpnI site in the forward primer and XhoI site in the reverse primer. The PCR product was then run on 1.0% Agarose gel and gel purified using gel extraction kit (Qiagen) and then ligated with pGEM-T vector (Promega) using 2X Rapid ligation buffer (Promega) for overnight at 4 ^{0}C. The ligated product was then transformed into JM 109 competent cells and plated into IPTG/X-Gal/ampicillin plates for blue/white selection. The white colonies that were obtained upon successful ligation into pGEM-T and transformed into JM 109 cells were then further picked and grown into 5 mL LB broth containing 5 µg of ampicillin. The presence of insert in each of the white colonies was confirmed after plasmid digestion using XhoI and KpnI restriction enzymes. The inserts released were then

further gel purified using gel extraction kit, ligated into pGL3 vector that had been cut open with XhoI and KpnI restriction enzymes The ligated product was then transformed into JM109 competent cells and plated into LB plates containing 5 µg of Ampicillin. The presences of the inserts in the colonies were confirmed by insert release after digestion with XhoI and KpnI restriction enzymes. Positive colonies were further confirmed by sequencing the plasmids for the presence of the EREs. A stepwise process of the cloning of the EREs into pGEM-T and pGL3 is outlined in the diagram Below (Figure 2.3)

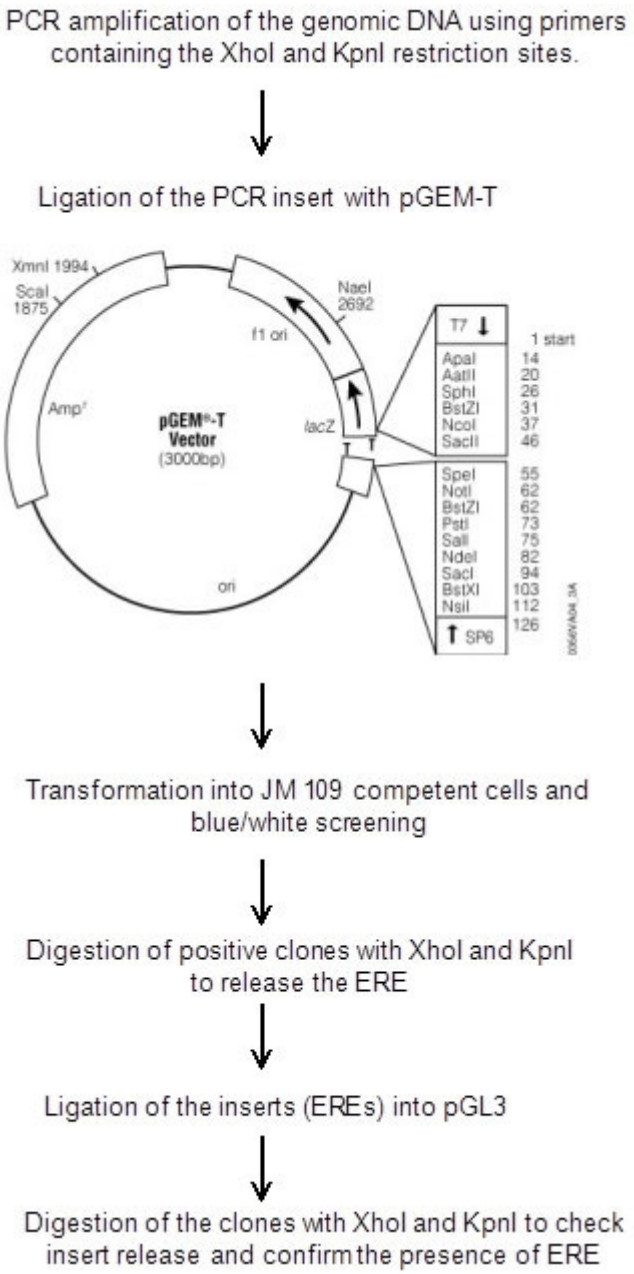

Figure 2.3 Flowchart showing the stepwise process for cloning of HOXB9 EREs. Above diagram shows the process of cloning HOXB9 EREs into pGEM-T and subsequently into pGL3.

A promoter region containing no ERE (non-ERE) sequences was also cloned as negative control. Each ERE-pGL3 construct was transfected into JAR and MCF7 cells

separately, and then cells were exposed to E2 for 6 h and subjected to luciferase analysis using a commercial luciferase detection kit (Figure 2.4B-C). Our results demonstrated that transfection with empty pGL3 construct did affect luciferase activity upon E2-treatment in both JAR and MCF7 cell lines (Figures 2.4B and C). However transfection with ERE1-pGL3, ERE3-pGL3 and ERE4-pGL3 followed by E2-exposure increased the luciferase activity by 2.1, 2.9, and 2.6 fold respectively in JAR cells (Figure 2.4B). Transfection with either ERE2-pGL3 or non-ERE-pGL3 did not have any significant effect on luciferase activity (Figures 2.4B). In contrast to JAR cells, E2-dependent luciferase induction was observed only for ERE4-pGL3 in MCF7 cells (Figure 2.4C). No E2-response was observed for ERE1-, ERE2- and ERE3-pGL3 in this cell line (Figure 2.4C). These results suggested that ERE4 which is an imperfect full ERE, is likely an E2-responsive ERE and may be associated with E2-mediated activation of HOXB9. As ERE1 and ERE3 also showed E2-responses in JAR cells, they may also be involved in E2-mediated transcriptional regulation of HOXB9 in this cell line. Nevertheless, as E2-induced expression of HOXB9 was more robust in JAR cells in comparison to MCF7 cells, and ERE1, ERE3 and ERE4 were E2-responsive in JAR cells, we investigated the mechanism of E2-dependent HOXB9 expression in JAR cells.

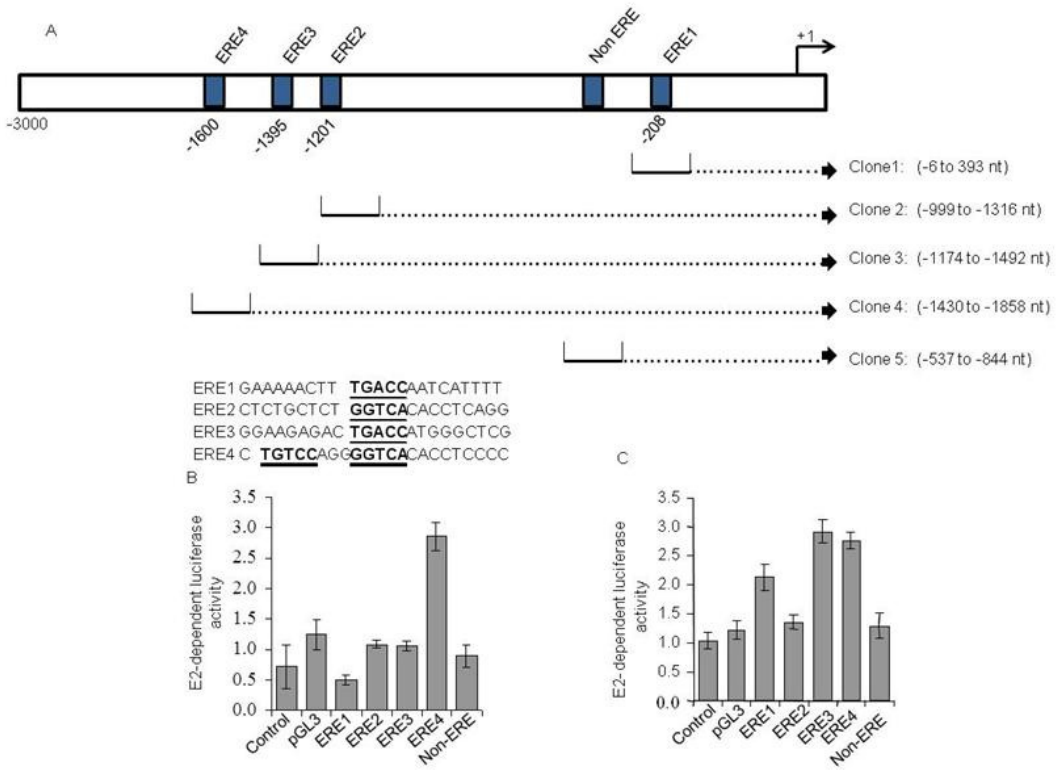

Figure 2.4 Analysis of HOXB9 promoter EREs and their E2-response using luciferase based reporter assay. (A) HOXB9 promoter (up to -3000 nt) contains three $ERE_{1/2}$ sites named as ERE1 to ERE3 and an imperfect full EREs (ERE4). The promoter segments containing these $ERE_{1/2}$ sites along with ~150 nt on both sides were cloned into luciferase based reporter construct pGL3 and used for transfection. (B) E2-response of HOXB9 promoter EREs in JAR cells. The ERE containing pGL3 constructs were transfected into JAR cells for 24 h. Control cells were treated with either none (no construct transfected, control lane), or empty pGL3 vector or non-ERE-pGL3 (non-ERE). Both control as well as plasmid transfected cells were then treated with 100 nM E2 for 6 h and subjected to luciferase assay by using ONE-Glo Luciferase Assay System. The ratios of E2-induced luciferase activities over corresponding E2-untreated samples were plotted. The experiment with four replicate treatments was repeated at least twice. Bars indicate standard errors. (C) E2-response of HOXB9 promoter EREs in MCF7 cells. Experiments were performed in MCF7 cells in the same way as described in Figure B.

2.3.3 Estrogen receptors play critical roles in E2-mediated transcriptional activation.

As HOXB9 is an E2-responsive gene and its promoter contains multiple estrogen-response elements, we hypothesized that estrogen-receptors (ERs) are involved in E2-mediated transcriptional regulation of HOXB9 [87]. To examine the roles of ERs, we initially knocked down ERα and ERβ separately in JAR cells using specific antisense oligonucleotides (Table 2.1) and then treated with E2 (100 nM for 6 h). RNA and protein from ER knocked down and E2-treated cells were analyzed for the expression of ERs as well as HOXB9. A scramble antisense (with no homology to ERs) was used as a negative control. Our results demonstrated that ERα and ERβ were knocked down effectively and specifically by respective antisenses (Figures 5A-B and data not shown). As seen in Figure 2.5A, treatment with E2 increased the expression of HOXB9 (lane 2, Figure 2.5A). Interestingly, upon knockdown of ERα, E2-dependent activation of HOXB9 was decreased (lane 4, Figure 2.5A, real-time PCR data in right panel). Similarly, knockdown of ERβ also suppressed E2-dependent induction of HOXB9 (lanes 2 and 4, Figure 2.5B, real-time PCR data in the right panel). We also performed the combined knockdown of ERα and ERβ in JAR cells and found that E2-dependent activation of HOXB9 was suppressed almost to the basal level which is more than that observed under individual knockdown conditions. These results demonstrated that both ERα and ERβ play critical roles in E2-mediated transcriptional activation of HOXB9.

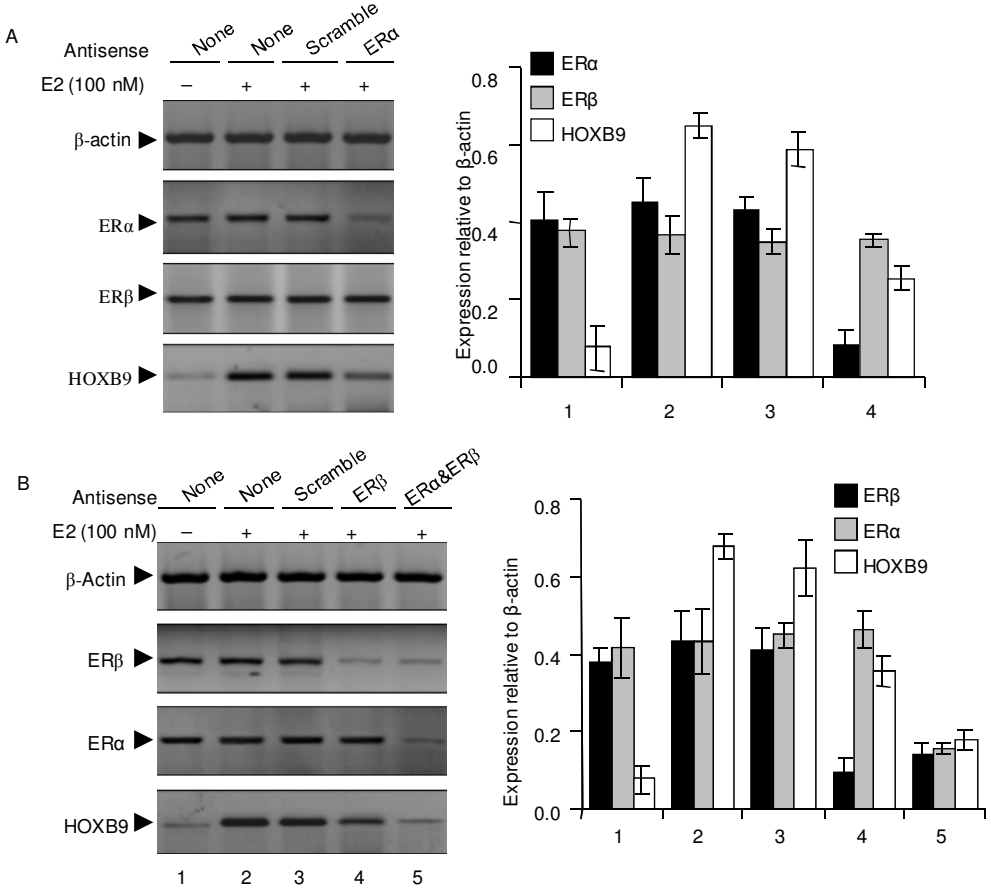

Figure 2.5 Roles of ERα and ERβ in E2-induced expression of HOXB9. (A) ERα knockdown affects HOXB9 expression in JAR cells: 9 µg each of ERα and a scramble antisense were used to transfect JAR cells for 20 h. 2 mL of media containing an additional 100 µL/mL of charcoal stripped FBS was then added to the plates for 28 h. Antisense transfected cells were then treated with 100 nM of E2 for 6 h. RNA was isolated and subjected to reverse transcriptase-PCR analysis by using primers specific to ERα and HOXB9. The specificity of ERα antisense was confirmed by PCR with ERβ with primers specific to ERβ. β-actin was used as a quantitative control. PCR products were analyzed in agarose gel. Lane 1: control cells (no E2 control). Lane 2: cells were treated with 100 nM E2. Lane 3: cells were initially transfected with 9 µg of scramble antisense followed by exposure to E2. Lane 4: cells were initially transfected with 9 µg of ERα antisense and then treated with E2. Real-time PCR analysis of ERα, ERβ and HOXB9 relative to β-actin is plotted. Each experiment was repeated at least thrice (n = 3). Bars indicate standard errors. (B) Effect of ERβ knockdown or a combined knockdown of ERα and ERβ on E2-induced HOXB9 expression. Experiments and analysis were performed in the same way as shown in panel A. Experiments were partially contributed by Dr. Khairul Ansari.

2.3.4 ERα and ERβ bind to HOXB9 promoter in presence of E2

As both ERα and ERβ played essential roles in HOXB9 gene expression, we examined their E2-dependent binding in HOXB9 promoter using chromatin immuno-precipitation (ChIP) assay [13, 88]. JAR cells were treated with E2 (100 nM for 6 h), fixed with formaldehyde, sonicated to shear the chromatin and then subjected to immuno-precipitation with ERα and ERβ specific antibodies. ChIP experiment was performed in parallel with β-actin antibody as non-specific antibody control. A diagrammatic outline of the ChIP assay performed with with ERα, ERβ and β-actin specific antibodies is outlined below (Figure 2.6)

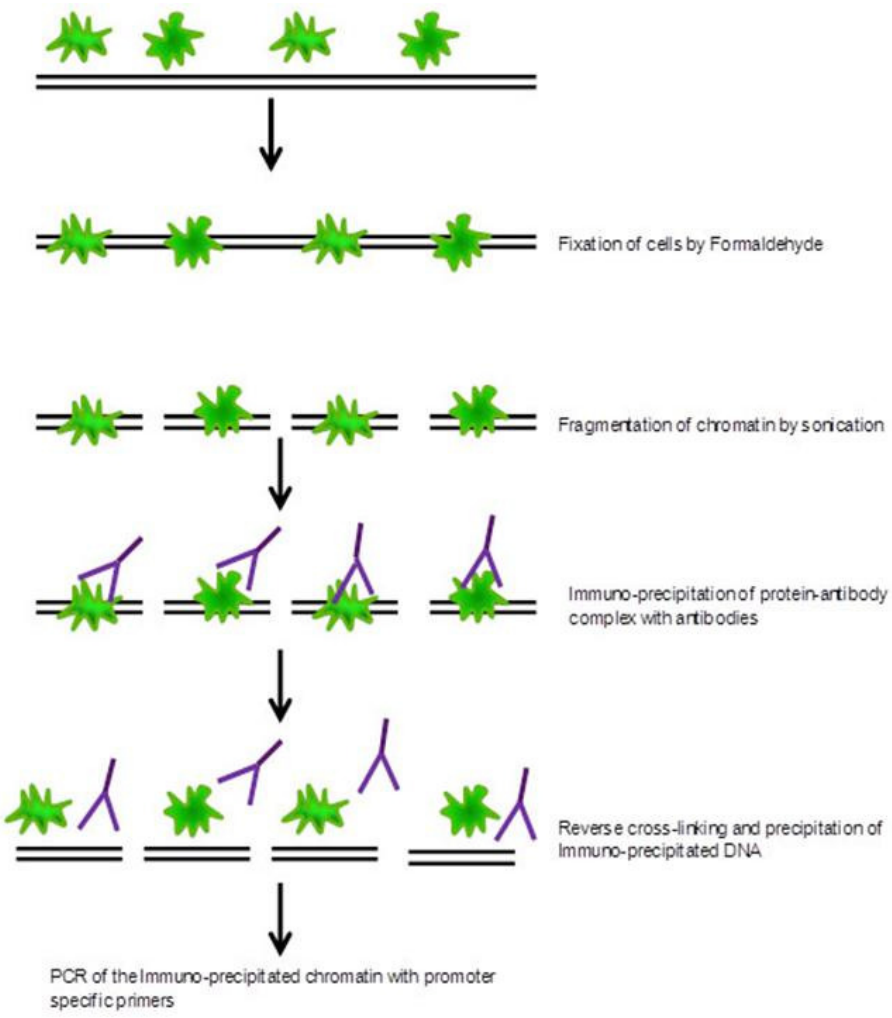

Figure 2.6 A flowchart representing Chromatin immuno-precipitation assay with ERα, ERβ and β-actin antibodies.

The immuno-precipitated DNA fragments were PCR-amplified using primers spanning different ERE regions (ERE1-ERE4) of HOXB9 promoter (Figure 2.7A). The real-time PCR quantifications of the ChIP DNA fragments are shown in Figure 2.7B. A promoter segment (-660 to -791 nt) containing no ERE site was used as a negative control (non-ERE). ChIP analysis demonstrated that β-actin was not bound to any EREs both in absence and presence of E2 (Figure 2.7A). However, the binding of ERα was increased in ERE1, ERE3, and ERE4 regions in presence of E2-treatment (Figures 2.7A-B). Similarly, increased binding of ERβ was

observed in all ERE regions (ERE1- ERE4, but not in non-ERE regions). These observations demonstrated that ERα and ERβ were bound to HOXB9 EREs especially ERE1, ERE3 and ERE4 regions upon treatment with E2. Some amounts of constitutive binding of ERs was observed in the ERE regions prior to addition of E2 and this might be associated with basal transcription of the gene in absence of external stimuli. Notably, the same set of EREs (ERE1, ERE3 and ERE4) showed E2-dependent luciferase response in reporter assay in JAR cells (see Figure 2.4) further indicating the involvement of these EREs in E2-dependent transcriptional regulation of HOXB9.

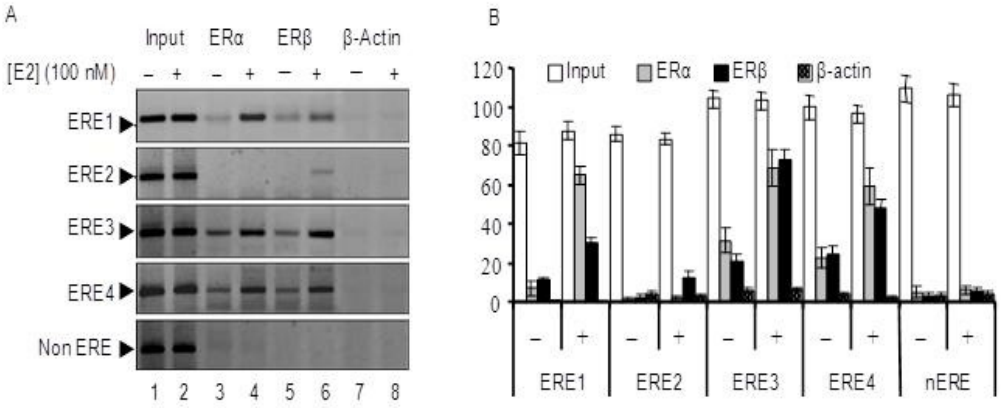

Figure 2.7 E2-dependent recruitment of ERα and ERβ in the ERE and non-ERE regions of HOXB9 promoter. (A-B) ERα and ERβ are recruited to the different HOXB9 ERE regions. JAR cells were treated with 100 nM E2 for 6 h and subjected to ChIP assay using antibodies specific to ERα and ERβ. β-actin antibody was used as control IgG. The immuno-precipitated DNA fragments were PCR-amplified using primers specific to ERE1-4 of HOXB9 promoter. Primers specific to a promoter sequence containing no ERE (non-ERE) were used as control. Lanes 1, 3, 5 and 7 are no-E2 controls. Lanes 2, 4, 6 and 8, were E2-treated samples. ChIP DNA fragments were analyzed by real-time PCR with primers specific to the different ERE and non-ERE regions and shown in panel B. Each experiment was repeated at least thrice. Bars indicate standard errors.

2.3.5 Roles of MLLs in E2-mediated regulation of HOXB9

ER-mediated gene activation requires various ER-coregulators. Recent studies showed that MLL histone methylases interact with ERs in an E2-dependent manner and act as ER-coregulators in E2-dependent transcriptional activation of estrogen responsive genes [89, 90] [91]. To examine if MLLs are associated with E2-dependent activation of HOXB9, we knocked down different MLLs (MLL1, MLL2, MLL3, and MLL4) separately by using specific antisense oligonucleotides (Table 2.1), then exposed the MLL-knocked down cells to E2 and analyzed its impact on E2-dependent activation of HOXB9. MLL-specific knockdowns were confirmed by analyzing their respective gene expression both at RNA (compare lane 3 with lane 1, Figures 8A-D for MLL1 to MLL4, respectively) and protein levels. A scramble antisense with no homology to MLLs was used as negative control. Our results demonstrated that application of MLL1-specific antisense specifically knocked down MLL1 but not MLL2 or β-actin, and the knockdown of MLL1 resulted in decrease of E2-dependent activation of HOXB9 (Figure 2.8A). Knockdown of MLL3 also significantly affected the E2-dependent expression of HOXB9 (Figure 2.8C). In contrast, knockdown of MLL2 did not affect E2-induced HOXB9 expression, whereas MLL4-knockdown showed a modest suppression (Figures 2.8B-D). These results demonstrated that MLL3 and MLL1 play major roles in E2-mediated activation of HOXB9 while MLL2 and MLL4 may not be involved in the E2-mediated activation of HOXB9. Notably, knockdown of either MLL1 or MLL3 did not have any significant effect on the expression of ERα and ERβ, suggesting direct roles of these MLLs in E2-induced HOXB9 expression.

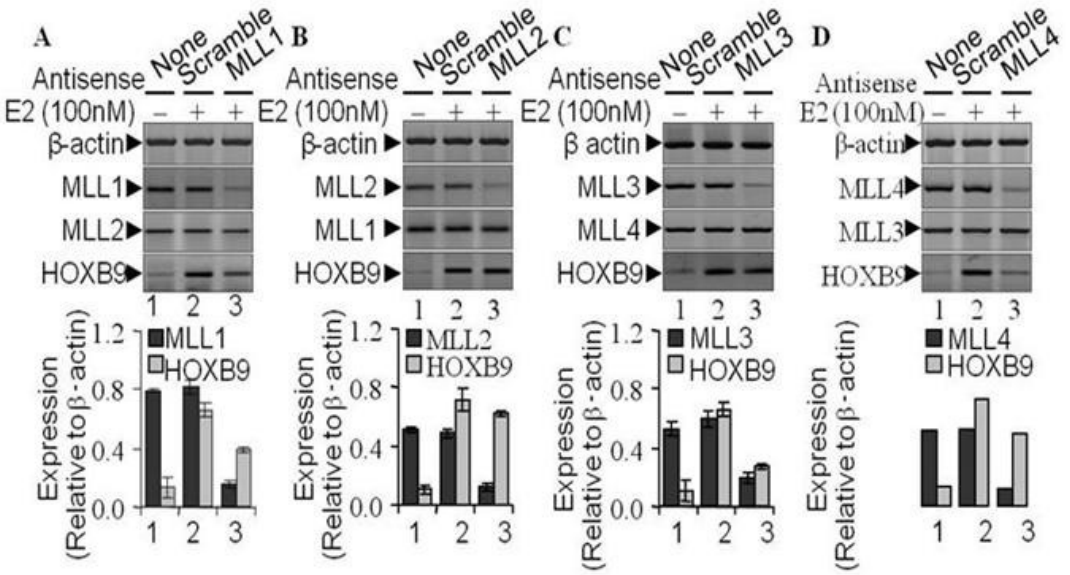

Figure 2.8 Effect of knockdown of MLL1, MLL2, MLL3, and MLL4 on E2-induced expression of HOXB9. 5 µg of MLL1, MLL2, MLL3, and MLL4 specific antisense oligonucleotides was used to transfect JAR cells separately. Scramble antisense with no homology with MLLs was used as a control. The antisense-treated cells were incubated for 48 h followed by treatment with 100 nM E2 for 6 h. RNA was isolated from treated and control cells and subjected to reverse transcriptase-PCR by using primers specific to HOXB9 along with MLL1, MLL2, MLL3, and MLL4. β-actin was used as control. The PCR products were analyzed by agarose gel. Quantification of transcript accumulation was done by using real-time PCR (shown in respective bottom panel). (A) Effect of MLL1 knockdown. Lane 1: control cells (no E2-treatment); Lane 2: cells that were initially transfected with scramble antisense followed by exposure to E2. Lane 3: cells were initially transfected with MLL1 antisense and then treated with E2. Real-time PCR analysis of the expression profiles of MLL1 and HOXB9 (relative to β-actin, average of three replicate experiments, n = 3) were quantified and plotted in the bottom panel. MLL2 was used as control to determine target specificity of MLL1 antisense. (B-D) Effect of MLL2-4 knockdown. These Figures show the effects of knockdown of MLL2 (MLL1 as control), MLL3 (MLL4 as control), and MLL4 (MLL3 as control), respectively, in the similar manner as shown for MLL1 in panel A.

2.3.6 MLL histone methylases bind to HOXB9 promoter in presence of E2

As MLL1 and MLL3 are essential in E2-mediated HOXB9 activation, we examined their bindings to the EREs of HOXB9 promoter in presence of E2. We performed ChIP assays in E2-treated and control cells using antibodies specific to different MLLs and β-actin (as non-specific control) and ChIP DNA fragment were PCR-amplified using primers specific to ERE1, ERE3 and ERE4 (Figure 2.9A). The ChIP DNA was also quantified using real-time PCR (Figure 2.9B). These analyses demonstrated that binding of MLL1 was increased in ERE3 region in presence of E2 (lane 4, Figure 9A-B). However, binding of MLL3 was significantly increased in all the EREs (ERE1, ERE3 and ERE4) in presence of E2 (Figure 2.9A-B). No significant E2-dependent binding was observed for MLL2 and MLL4 (Figure 2.9A-B). Time-dependent ChIP analysis demonstrated that MLL3 recruitment was increased in ERE1, ERE3, and ERE4 in presence of E2. Similarly, E2-dependent MLL1 binding was also observed in the ERE3 region. These results, in agreement with knockdown experiments (Figure 2.8) further demonstrated that MLL1 and MLL3 are involved in regulation of HOXB9 gene expression under E2 environment.

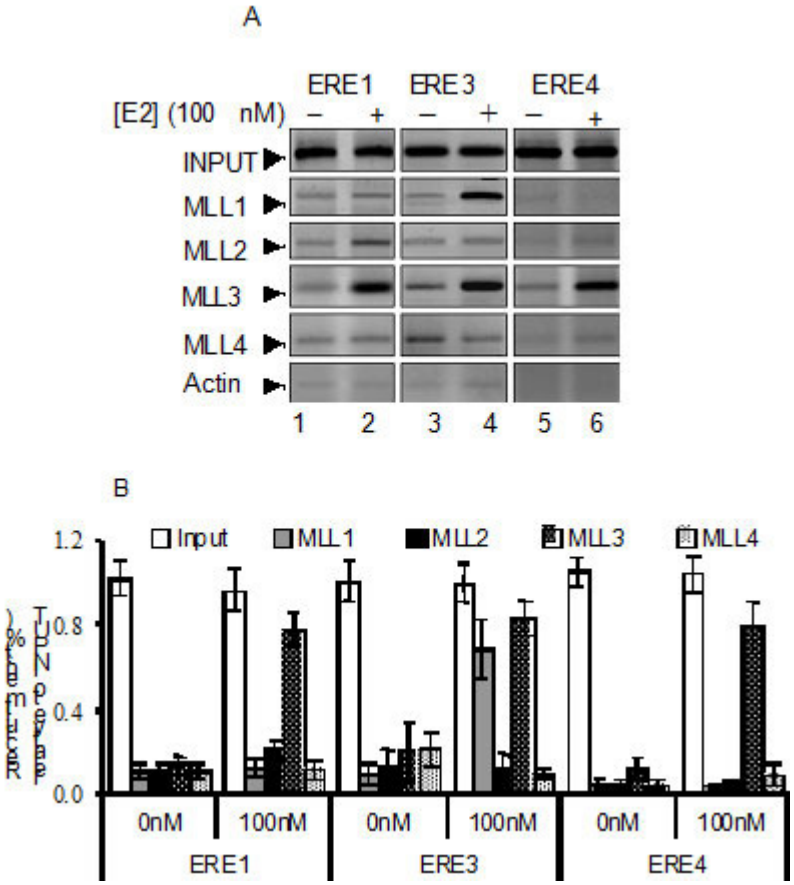

Figure 2.9 E2-dependent recruitment of MLLs (MLL1-4) in ERE1, ERE3, and ERE4 of HOXB9 promoter. (A-B) Recruitment of MLLs on different HOXB9 ERE regions.100 nM E2 was used to treat JAR cells for 6 h and subjected to ChIP assay. Antibodies specific to MLL1, MLL2, MLL3 and MLL4 were used for ChIP assay. β-actin antibody was used as control IgG. The immuno-precipitated DNA fragments were PCR-amplified using primers specific to ERE1, ERE3 and ERE4 of HOXB9 promoter. Lanes 1, 3, and 5 are no-E2 controls. Lanes 2, 4, and 6 were E2-treated samples. ChIP DNA fragments were analyzed by real-time PCR and shown in panel B. Each experiment was repeated at least thrice. Bars indicate standard errors.

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

Primers	Forward primer (5′ - 3′)	Reverse primer (5′ - 3′)
PCR primer		
β-actin	AGAGCTACGAGCTGCCTGAC	GTACTTGCGCTCAGGAGGAG
HOXB9	TGGGACGCTTAGCAGCTATT	CGTACTGGCCAGAAGGAAAC
HOXB9-ERE1	GTCGGAGCTGACGCTTTATC	GCTCAACTTCTCAGCCAACA
HOXB9-ERE2	ACAGGAGGGCTGAAAACTCA	CCCTAAATTTGCCCTGATGA
HOXB9-ERE3	AAGCTCCAATGAAGGGGTCT	CTGAGTTTTCAGCCCTCCTG
HOXB9-ERE4	CCAGAGGAGAACTGGGTCTG	TCTATTCTGTGGCCCTGCAC
HOXB9-non-ERE	AAAGCGGACAACGTCCTAAA	GGCCCTCATTTCCTCTTCTC
MLL1	GAGGACCCCGGATTAAACAT	GGAGCAAGAGGTTCAGCATC
MLL2	AGGAGCTGCAGAAGAAGCA	CAGCCAAACTGGGAGAAGAG
MLL3	CATATGCACGACCCTTGTTG	ACTGCTGGATGTGGGGTAAG
MLL4	CCCTCCTACCTCAGTCGTCA	CAGCGGCTACAATCTCTTCC
ERα	AGCACCCTGAAGTCTCTGGA	GATGTGGGAGAGGATGAGGA
ERβ	AAGAAGATTCCCGGCTTTGT	TCTACGCATTTCCCCTCATC
Cloning primer		
HOXB9-ERE1	GTAGCTGGGGCTGAGGTTA	ACATTATCCGGGCGCTTG*
HOXB9-ERE2	ACAGGAGGGCTGAAAACTC*	GAACCAGAGCGCCTTTACAT*
HOXB9-ERE3	TGCAGGGCCACAGAATAGAT	AAGGGTTAAGGCCACTTTCC*
HOXB9-ERE4	ACGAGATGGCTTCATTTGGA*	AGACCCCTTCATTGGAGCTT*
HOXB9-Non-ERE	GGAAGCTCGCAGTCATGTAA*	GAGGGAGGGAGAGCAAGG*
Antisense		
MLL1 antisense	TGCCAGTCGTTCCTCTCCAC**	
MLL2 antisense	ACTCTGCCACTTCCCGCTCA**	
MLL3 antisense	CCATCTGTTCCTTCCACTCCC**	
MLL4 antisense	CCTTCTCTTCTCCCTCCTTGT**	
ERα antisense	TCCCACCTTTCATCATTCCC **	
ERβ antisense	GCCACACTTCACCATTCCCA **	
Scramble antisense	CGTTTGTCCCTCCAGCATCT**	

*Flanked by appropriate restriction sites; **Phosphorothioate antisense oligonucleotides.

2.4 Discussion

HOX genes are critical developmental regulators and therefore their expression is likely to be influenced by various types of hormones [30]. For example, retinoic acid (RA) influences HOX gene expression controlling the developmental process [92-94]. In case of embryonic stem cells, RA influences HOX gene expression in a collinear fashion (3'-5' direction) [48, 49]. However, in matured tissues, the expression and regulation of different HOX genes appear to be independent and tissue specific [48]. Steroid hormones are also implicated in regulation of different HOX genes *in vivo*, though their detailed mechanism of action is mostly unexplored. Recent studies demonstrated that HOXA10 is transcriptionally regulated by steroid hormones and their expression is misregulated by endocrine disrupting chemicals [95-97]. Our studies demonstrated that HOXB9, a homeobox containing gene that is implicated in regulation of Renin-Angiotensinogen system and cardiovascular function, is transcriptionally regulated by estrogen. Renin-Angiotensinogen system which is closely linked with control of hypertension, blood pressure and plasma volume, is also controlled by steroid hormones [98]. Thus, our studies showing the estrogen-mediated regulation of HOXB9, extends the mechanistic implication of estrogen signaling in Renin-Angiotensinogen system and cardiovascular function.

Estrogen is a critical player in female reproduction. In addition, estrogen also plays critical roles in bone strength, controlling blood cholesterol, hypertension and other cardiovascular functions in both male and female. Abnormal estrogen signaling leads to critical human disease including cardiovascular disease and cancer [83, 99-104]. Estrogen signaling follows diverse pathways including estrogen-dependent activation of estrogen-responsive genes [43-46]. In general, during estrogen-mediated gene activation, estrogen binds to its cognate receptors (ERα and ERβ) and activates them [44]. Activated ERs migrate to nucleus and bind to estrogen-response elements [83] present in the promoters of estrogen responsive genes resulting in their transactivation. Sequence analysis demonstrated that HOXB9 promoter contains multiple

$ERE_{1/2}$ sites (ERE1-3) and a potential imperfect full ERE (ERE4) [40]. Luciferase based reporter assay demonstrated that ERE1, ERE3 and ERE4 are responsive to E2-exposure in JAR cells suggesting their potential involvement in E2-dependent activation of HOXB9. However, in MCF7 cells, only ERE4 showed estrogen response in luciferase assay which further indicate that ERE4 is likely an imperfect full ERE. The difference in luciferase response in two different cell lines may lie in the difference in cell lines. Antisense-mediated knockdown and ChIP analysis demonstrated that both ERα and ERβ are essential for E2-mediated activation of HOXB9. Both ERα and ERβ bind to ERE1, ERE3 and ERE4 of HOXB9 promoter as a function of E2, suggesting the involvement of these EREs in HOXB9 regulation. As seen in Figure 2.5, the independent knockdown of ERα and ERβ affected E2-dependent activation of HOXB9. However, upon combined knockdown of ERα and ERβ, the effects were more severe resulting in complete suppression of E2-dependent HOXB9 activation. These observations suggest that both ERα and ERβ are critical in E2-mediated regulation of HOXB9 and it is possible that they form heterodimer and bind to HOXB9 EREs and modulate transcription.

Importantly, during estrogen-mediated gene activation, along with ERs, several ER-coregulators that interact with ERs play essential roles in transactivation of the target gene [43]. These coregulators usually possess enzymatic activity, modify chromatin and bridge ERs with transcription machinery [105]. Many ER-coactivators have been identified including SRC-1 family of protein, CREB-binding protein (CBP/p300), p/CAF, ASCOM (activating signal cointegrator-2 that also contains MLLs) etc [105-112]. Recent studies demonstrated that MLL family of histone methylases, interact with ERs in an estrogen-dependent manner and bind to ERE regions of ER-target genes regulating their expression under hormonal environment [25 44, 27 44, 28 44, 45 44]. Our analysis demonstrated that histone methylase MLL1 and MLL3, play critical roles in HOXB9 gene regulation under estrogen environment [28 44, 45 44]. Knockdown of MLL1 and MLL3 resulted in suppression of E2-dependent activation of HOXB9.

MLL2 and MLL4 have little or no impact on E2-induced HOXB9 expression. ChIP analysis demonstrated that like ERs, recruitment of MLL3 increased in all the E2-responsive HOXB9 ERE regions (ERE1, ERE3 and ERE4) in presence of E2. MLL1 was also bound to ERE3 in an E2-dependent manner. These results further supported that MLL1 and MLL3 are important players in E2-mediated transcriptional regulation of HOXB9. Notably, like MLL3, MLL2 and MLL4 have several NR-boxes (nuclear receptor boxes) and hence are capable of interacting with ERs, and are also implicated in E2-mediated activation of different estrogen responsive genes [25, 27, 28, 45]. The involvement of MLL1 and MLL3 but not MLL2 and MLL4 in E2-induced HOXB9 expression suggested that different estrogen-responsive genes require different MLL-coregulators and MLL3 and MLL1 are specific for HOXB9 regulation.

It is well known that MLLs are chromatin modifying enzymes that methylate histone H3 at lysine 4 (H3K4) and play critical roles in gene activation [113-115] . As MLL1 and MLL3 act as coactivators of ER and is essential for E2-induced HOXB9 expression, recruitment of MLL1 and MLL3 in different ERE regions of HOXB9 may result in histone H3K4-methylation and facilitates recruitment of RNA polymerase II (RNAP II) and enhanced transcription. Indeed, ChIP analysis showed that level of H3K4-trimethylation and recruitment of RNAPII was increased in a time-dependent manner in ERE1, ERE3 and ERE4 regions of HOXB9 promoter under E2-treatment. This result indicated that MLLs plays a critical role in histone methylation and HOXB9 gene activation. Though under normal condition the recruitments of MLLs in the promoter of their target gene are mediated either by their own DNA binding domains or via interacting partners, recruitment of MLL1 and MLL3 under E2 environment may be mediated by ERs [116, 117]. Our analysis of E2-dependent recruitment of MLL1 and MLL3 in ERE1, ERE3 and ERE4 of HOXB9 in ER depleted cells indicated that recruitment of MLL1 and MLL3 are indeed dependent on by both ERα and ERβ. Notably, E2-dependent interaction between MLLs and nuclear receptors (including ER) are well recognized. For example, histone methylase

MLL2 has been shown to directly interact with ERα, via its LXXLL domains and regulate E2 dependent activation of cathepsin D [27]. Similarly, MLL-interacting protein, menin that also has LXXLL domains, interacts with ERα and regulates E2-dependent activation TFF1 (pS2) [25]. MLL3 and MLL4 complexes are shown to interact with multiple nuclear receptors including ER, RAR and LXR in ligand dependent manner and regulate ligand dependent gene activation.

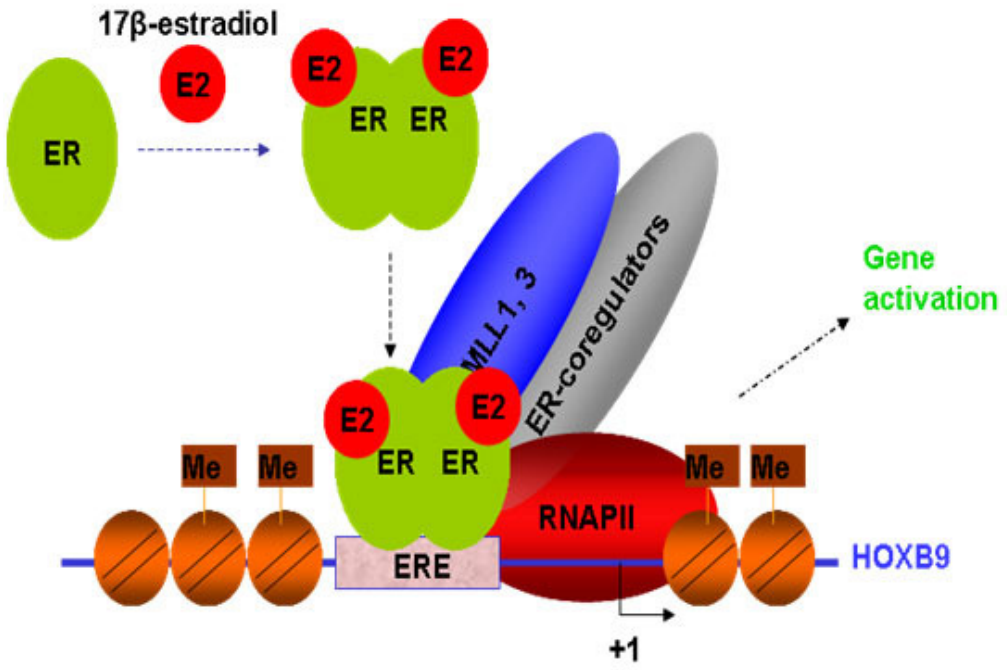

Figure 2.10 Graphical model of the involvement of MLL1, MLL3 and ER-coregulators in the estrogen mediated up regulation of HOXB9.

Our studies further demonstrated that MLL1 and MLL3 functionally interact with ERs and regulate E2-induced HOXB9 expression. Similar to HOXB9, HOXC13 is also transcriptionally activated by estrogen via coordination of MLLs and ERs [28]. However, the mechanism of transcriptional regulation and coregulators requirement between these HOX

genes appear to be different. MLL1 and MLL3 are primary ER-coregulators in HOXB9 regulation, while MLL1-4 participate in E2-induced HOXC13 expression. Furthermore, there is no apparent conservation in organization of the promoter elements between HOXB9 and HOXC13 genes indicating their distinct mechanism of transcriptional regulation. The cell type as well as tissue specific distinct mechanism of HOXB9 and HOXC13 gene expression under retinoic acid exposure was also observed previously [48]. As MLLs are acting as coactivators of ERs, it is likely that different HOX genes are controlled by different MLL coactivators and in case of HOXB9 they are primarily MLL1 and MLL3 (see Figure 2.10). Analyzing the roles of E2 and other steroid hormones in regulation of all 39 HOX genes and elucidating their mechanism is critical in detailed understanding of the steroid hormone mediated regulation of HOX genes.

Overall, our studies demonstrated that HOXB9 is an E2-responsive gene. As estrogen and ERs are closely associated with cardiovascular disease, HOXB9 is closely linked with regulation of Renin-Angiotensinogen system and regulation of blood pressure, plasma volume, electrolyte balance, and our studies revealed a novel epigenetic mechanism by which MLL-histone methylases coordinate with ERs to regulate HOXB9 gene expression. Our studies also implicated potential involvement of MLL histone methylases in regulation of cardiovascular functions such as blood pressure, fluid homeostasis and electrolyte balance.

CHAPTER 3

HOMEODOMAIN CONTAINING PROTEIN HOXB9 PLAYS CRITICAL ROLES IN CELL CYCLE PROGRESSION, TUMORIGENESIS, AND IS OVEREXPRESSED IN BREAST CANCER

3.1 Introduction

Homeobox containing genes or HOX genes are a family of transcription factors that play pivotal roles in embryogenesis, cell proliferation and oncogenesis [42, 118]. HOX genes bind to DNA via homeodomain, a conserved domain present in all the 39 HOX genes [12]. The homeodomain binds to 5′-TAAT-3′ core sequence present in the promoter of target genes [119]. The surrounding sequences around the TAAT motif also play a essential role in recognition of target motif by HOX protein [119]. Each HOX gene has a specific function and has its own set of target genes some of which could also be regulated by other HOX genes [120]. Such a multiple regulation of target genes by different HOX genes denotes the importance of direct and indirect function of HOX genes in gene transcription. The HOX genes bind to the DNA via their homeodomain and are known to facilitate subsequent recruitment of histone acetyl transferases as well as other general transcription factors on the promoter of the genes [45].

Recent studies from our laboratory demonstrated that HOXB9 is a hormone responsive gene and its transcription is coordinated via involvement of MLL1 and MLL3 histone methyl-transferases and ERs in presence of estrogen [121]. Recently, HOXB9 has also been found to play an active role in angiogenesis [122]. Although HOXB9 is itself not associated with oncogenic transformation, it promotes tumor progression and distal metastasis when co-expressed with transforming oncogene such as activated rat sarcoma viral oncogene (Ras)[123]. HOXB9 is known to transactivate several angiogenic as well as erythroblastic

leukemia viral gene homologs (ErbB) and TGFβ ligands[122]. The activation of these genes leads to epithelial to mesenchymal transition (EMT), and increases angiogenesis and distal metastasis [123]. EMT transition is characterized by loss of cell to cell contact, invasion and is associated with resistance to chemotherapeutic drugs and radiation. Recent reports highlight the involvement of HOXB9 in DNA damage response and radiation resistance through transactivation of TGF-β1 in breast cancer cells [122, 123].

In this study, we have explored the biochemical function of HOXB9 and its association with tumorigenesis and cell cycle progression. HOXB9 is homeodomain containing proteins. It is a well known player in development of mammary gland, sternum and in regulation of Renin which is closely linked with blood pressure control [124, 125]. Though, HOXB9 is well known to be associated with steroid hormone regulated organogenesis, limb development and is transcriptionally regulated by estrogen, the biochemical mechanism of action and roles of HOXB9 in tumorigenesis is mostly unknown. Similarly, the role of HOXB9 homeodomain on regulation of gene expression is also remained to be explored. Herein, we invested the roles of HOXB9 and its homeodomain in cell cycle progression and tumorigenesis. Our studies demonstrated that HOXB9 controls the expression of various cell cycle regulatory genes and tumor growth factors via its homeodomain and ultimately affects cell cycle progression and tumor growth.

3.2 Experimental section

3.2.1 Cell culture and cell synchronization

HeLa (human cervical cancer), HEK 293 (human embryonic kidney), SW-480 (human colorectal adenocarcinoma), HEPG2 (hepatocellular carcinoma), H358 (bronchioalveolar carcinoma), JAR (Human choriocarcinoma placenta), MCF7 (human adenocarcinoma mammary), T47D (ductal carcinoma mammary), MDA-MB-231 (ER negative human adenocarcinoma mammary), K562 (chronic myelogenous leukemia), SY-SY5Y (human

neuroblastoma), Hs 798.Pl (normal lung fibroblast) cells were grown and maintained in DMEM containing 10% FBS, 2 mM L-glutamine and 1% Penicillin/Streptomycin (100 unit and 0.1 mg mL^{-1} respectively) in presence of 5% CO_2 at 37 ^0C.

For cell synchronization using double thymidine block method, HeLa cells were grown to 60% confluency and treated with 10 mM thymidine for 16 h. It was then washed twice with 1X phosphate buffered saline (PBS, pH 7.4) and released in fresh media for 8 h. Cells were then treated with 10 mM thymidine for additional 18 h, released into the fresh media after washing twice with PBS. Cells were then harvested every 2.5 h and subjected to RNA and protein extraction.

3.2.2 Immuno-histological analysis of breast cancer tissue microarray

The breast cancer tissue microarray slide containing 6 different cases (duplicates of each) of breast cancer and their corresponding adjacent normal tissue were purchased from US Biomax Inc and subjected to immuno-histological staining. For staining, the paraffin embedded tissue microarray slide was immersed twice in xylene (for 10 min) and then sequentially immerged in 100%, 95% and 70% ethanol (5 min each) to deparaffinize the tissue. Antigen retrieval was done by incubating the slide in 0.01M sodium citrate buffer at 95 ^0C for 15 min following supplier's instruction. For immuno-histological staining, the tissue microarray slide was incubated with 3% H_2O_2 for 15 min, washed with PBS thrice, and then blocked with blocking buffer containing donkey serum. The slide was then incubated with *HOXB9* antibody overnight, washed three time in PBS, and then incubated with biotinylated donkey secondary antibody for 1.5 h. The slide was washed thrice with PBS, incubated with Avidin-Biotin Complex (ABC, Vector laboratories) for 1.5 h, washed twice with PBS and then twice with 0.1 M tris-HCl (pH 7.4). Slide was incubated with diaminobenzidine (DAB) substrate (Vector Laboratories) for peroxidase labeling. The tissue microarray slide was dehydrated with sequential immersion under 70%, 95%, and 100% ethanol and then cleaned by sequentially incubation (1, 5 and 10

min) in CitriSolv clearing agent (Fisherbrand). Tissue sections were finally mounted with DPX mounting solution (Sigma), photographed, and examined under microscope (Nikon Eclipse TE2000-U, Japan).

3.2.3 Establishment of HOXB9 (full length) and HOXB9 homeodomain stably transfected cell line

Human full length HOXB9 and homeodomain deleted HOXB9 were cloned and inserted downstream of a gene encoding Flag peptide in human expression vector pFLAG-CMV4. The Flag tagged HOXB9 (Flag-HOXB9) and Flag tagged homeodomain deleted HOXB9 (Flag-HOXB9-ΔHD) constructs were transfected into HEK 293 cells using Lipofectamine transfection reagent following manufacturer's instruction. In brief, 2 µg of the Flag-tagged constructs were incubated with 8 µL of Lipofectamine transfection reagent for 30 min in 500 µL DMEM at room temperature and the transfection mix was added to HEK 293 cells (at 80% confluency). After 30 h, cells were washed with PBS, trypsinized and re-plated in medium supplemented with 500 µg mL^{-1} of G418 disulfate (Sigma) allowing the selective propagation of transfected cells in culture. The cells were maintained in media supplemented with 500 µg $mL^{-1}$of G418 disulfate for 13-15 days, until individual colonies were picked up and grown separately. The individual clones were grown and maintained in selective media prior to analysis by western blotting and immuno-staining with anti-FLAG antibody (Sigma). Positive colonies were further confirmed by western blotting using anti-FLAG antibody (Sigma).

3.2.4 Antisense-mediated knockdown experiments

For antisense mediated knockdown experiments, HeLa cells were grown up to 60% confluency and transfected with varying concentrations (0.75 to 2.25 µg mL^{-1}) of HOXB9 antisense oligonucleotides (IDT-DNA) with iFECT transfection reagent (Molecul A) following manufacturer's protocol in FBS free media. Transfection with a scramble antisense with no homology with HOXB9 gene was done in parallel and used as antisense control. In brief, antisense oligonucleotides along with the transfection reagent were mixed in 300 µL of blank

DMEM and incubated for a period of 45 min at room temperature before applying directly into 4 mL plates containing 1.7 mL of blank DMEM. Prior additions of the transfection reagent-antisense mixture, cells were washed twice with 2 mL of blank DMEM. After 20 h of antisense application, the cells were treated with 2 mL of media containing all the supplements and an additional 20% FBS. Cells were then harvested after 72 h for RNA/protein extraction or fixed in 4% formaldehyde for Chromatin Immuno-precipitation (ChIP) assay with anti-HOXB9 (Santacruz), anti-RNAP II (Sigma) and anti-H3K4 trimethyl (Upstate) antibodies.

3.2.5 RNA extraction, reverse transcription and real-time PCR

For RNA extraction, the cells were harvested by centrifugation at 500 g and resuspended in Buffer A (20 mM Tris.HCl pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.2 mM PMSF) and incubated for 10 min on ice. The cells were then centrifuged at 3500 g for 5 min and the supernatant was subjected to phenol-chloroform and chloroform extraction, and ethanol precipitation by incubation overnight at - 80 ^{0}C. For cDNA synthesis, 500 ng of RNA was mixed with 2.5 µM of oligo dTs (Promega) and incubated at 70 ^{0}C for 10 min. A reaction cocktail containing 2.5 unit MMLV Reverse Transcriptase (Promega), 1X first strand buffer (Promega), 100 µM of dNTPs (Invitrogen), 1 mM DTT and 20 units of RNaseOut (Invitrogen) was added to the RNA and incubated 1 h at 37 ^{0}C. The resulting cDNA was diluted to 100 µL final volume. The cDNA was subjected to PCR amplification with forward and reverse gene-specific primer (described in table 3.1) and the program constituted 32 cycles of 94 ^{0}C for 30 s, 60 ^{0}C for 4 s and 72 ^{0}C for 45s, with a final extension at 72 ^{0}C for 5 min. PCR products were electrophoresed through 1.5% (w v^{-1}) agarose gels containing 0.5 µg mL^{-1} ethidium bromide and visualized using alpha imager.

For real-time PCR analysis, the cDNA was amplified using SsoFast Eva Green super mix (Bio-Rad) using CFX96 real-time PCR detection system. The real-time PCR results were

analyzed using the CFX Manager software. The experiments were repeated at least twice with three replicates each time.

3.2.6 Protein extraction and western blotting

Cells were harvested by centrifugation at 500 g, resuspended in whole cell extract buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.05% NP-40, 0.2 mM PMSF and 1X protease inhibitors (1 mg mL^{-1}) and incubated at least 20 min on ice for whole cell protein extraction. For extraction of nuclear protein, cells were resuspended in buffer A for 10 min on ice, centrifuged at 3500 g for 5 min. The nuclear pellet was resuspended in Buffer C (20 mM Tris-HCl pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.5 mM DTT, 0.2 mM PMSF and 0.2 mM EDTA) for at least 20 min on ice, centrifuged at 13000g for 10 min. The supernatant containing the nuclear protein was separated. Both whole cell protein extract and nuclear protein extracts were analyzed by Western blotting using antibodies specific to β-actin (Sigma), Cyclin B1 (Santa Cruz), Cyclin E (Santa Cruz), HOXB9 (Santa Cruz), FLAG (Sigma). Secondary antibodies were purchased from: anti-Rabbit (Promega), anti-Mouse (Promega). The Western blots were developed by using alkaline phosphatase method.

3.2.7 Chromatin immuno-precipitation assay

ChIP assays were performed on HeLa and HEK 293 cells using EZ Chip chromatin immuno-precipitation kit (Upstate) as described previously. For ChIP assay, 1 X 10^6 cells were fixed in 4% formaldehyde, collected with 1X PBS containing protease inhibitor (5 µL mL^{-1}) and PMSF (5 µL mL^{-1}). The cells were then lysed in lysis buffer and sonicated to shear the chromatin to 150-450 bp in length. The sonicated chromatin was pre-cleaned using protein G agarose (Millipore) beads and subjected to immuno-precipitation using antibodies specific to FLAG (Sigma), H3K4 trimethyl (Upstate), HOXB9 (Santacruz). The immuno-precipitated chromatin was then deproteinized by incubating at 65 ^{0}C in presence of NaCl, followed by incubation in presence of proteinase K (Sigma) to obtain purified DNA fragments. The immuno-

precipitated DNA was then PCR amplified using promoter specific primers (shown in table 3.1) to analyze the recruitment of different proteins.

3.2.8 Immuno-fluorescence microscopy

HEK 293, Flag-HOXB9-FL and Flag-HOXB9-ΔHD stable cell lines were grown overnight on cover slips and then subjected to cell synchronization using the protocol described above. The cells were fixed in 4% formaldehyde for 20 min, washed with PBS and permeabilized with 0.2% Triton X-100 for 15 min. It was then washed with PBS, blocked with 30 µL of goat serum for 1 h and immuno-stained with the primary antibodies specific to FLAG (Sigma), RNAP II (Abcam) and Lamin B (Abcam). After incubation in the primary antibodies for 2 h, the cells were washed twice with PBS for 5 min each and incubated with fluorescein isothiocyanate (FITC) or rhodamine (Jackson Immuno Research Laboratories, West Grove, PA USA) conjugated secondary antibodies in 30 µL of goat serum for 1 h. The cells were washed 3 times with 1X PBS for 5 min, stained with DAPI for nuclear staining and visualized under a fluorescence microscope (Nikon Eclipse TE2000-U, Nikon, Melville, NY USA).

3.2.9 FACS analysis

FACS analysis of HEK 293, Flag-HOXB9-FL and Flag-HOXB9-ΔHD cells were performed in a Fusion Beckman Coulter (Fullerton, CA USA) Cytomics FC500 Flow Cytometry Analyzer. Cells were grown up to 60% confluency and harvested by spinning it at 500 g for 5 min. Cells were washed three times with PBS, fixed in 70% ethanol overnight at -20 ^{0}C. It was then washed twice with PBS and were treated with 50 µL of 5 mg mL^{-1} of RNase A solution. The fixed cells were then resuspended in 700 µL of PBS and stained with 0.5 µg mL^{-1} of propidium iodide solution and then subjected to flow cytometry.

3.2.10 Colony formation assay

Colony forming ability of Flag-HOXB9 and Flag-HOXB9-ΔHD stably expressing cells was accessed using soft agar method. In brief, 1.2% agar was made in PBS, autoclaved, cool down (to approximately 40 ^{0}C). An equal volume of 2X DMEM with 20% FBS, 4 mM L-glutamine and 2% Penicillin/Streptomycin (100 unit and 0.1 mg mL^{-1} respectively) was added to the agar and the mixture was plated on 60 mm culture plate, and kept in room temperature to allow the base agar to solidify. Approximately 10,000 HEK 293, Flag-HOXB9 and Flag-HOXB9-ΔHD cells were added to a mixture of 1.75 mL 2X DMEM and of 1.75 mL 1.2% agar at room temperature and then plated on top of the base agar layer layer. The dishes were then kept in room temperature for 45 min to allow the top agar to solidify and then kept in cell culture incubator under normal growth conditions. The cells were fed with 0.5 mL of normal growth media at 2 days interval for 21 days. Prior to counting the colonies, the media was removed from the plates and rinsed with PBS. The plates were then stained with 0.005% crystal violet for 2 h and the plates were washed to remove crystal violet solution by immersing in PBS.

Table 3.1 List of primers used for PCR and cloning

Gene	Forward primer (3´-5´)	Reverse primer (3´-5´)
GAPDH	CAATGACCCCTTCATTGACC	GACAAGCTTCCCGTTCTCAG
HOXB9	CTACGGTCCCTGGTGAGGTA	TAATCAAAGACCCGGCTACG
Cyclin A	AAGAAGCAGCCAGACATCACGG	AGCRGCAGTTTCCCTCTCAGAAC
Cyclin B	TTGATACTGCCTCTCCAAGCCC	TTGGTCTGACTGCTTGCTCTTCC
Cyclin D	AGAAGCTGTGCATCTACACCGA	TGATCTGTTTGTTCTCCTCCGCCT
Cyclin E	TTTCAGGGTATCAGTGGTGCGA	ACAACATGGCTTTCTTTGCTCGGG
p16	TGCCCAACGCACCGAATAGTTA	ATCTATGCGGGCATGGTTACTGC
p18	TGCGCTGCAGGTTATGAAACTT	AGGGCAGGTTCCCTTCATTATCCT
p21	GACACCACTGGAGGGTGACT	CAGGTCCACATGGTCTTCT
p27	ACTTGGAGAAGCACTGCAGAGA	GTCGCTTCCTTATTCCTGCGCATT
p57	ATCCACGATGGAGCGTCTT	CCTGCTGGAAGTCGTAATCC
VEGF	TCCACCATGCCAAGTGGTCCC	TGGATGGCAGTAGCTGCGCT
bFGF	TTCTTCCTGCGCATCCAC	CGGTTAGCACACACTCCTTTGAT
NRG	CGGAGGAGCTGTACCAGAAG	GCTTTTTCCGCTGTTTCTTG
TGFβ1	TCTCGGAAGAGAAAGGGCCCTG	GGACATTTCTCACAGTGTGGCCC
Cyclin A (P)	TCCACACTCAGTTTGCTTGG	AGGTGACTGACAAGCCAGGT
Cyclin B (P)	ACAATTGGCCTTGGGAAACTGG	AGATGACTTTGCCAGGGTCACAC
Cyclin D (P)	CCGACTGGTCAAGGTAGGAA	ACAACCCCTGTGCAAGTTTC
Cyclin E (P)	TGCCTCCCTGGTTCAAGTGATT	AGTTCCCTGTCTTCATGCCCTTCA
bFGF-(P)	ATCTGGAGTTAAAGCCTTCTTG	TAATGTTGAGTGTGTGGGAGATG
VEGF-(P)	TCCGGGTTTTATCCCTCTTC	TCTGCTGGTTTCCAAAATCC
NRG-(P)	CCCGACCACAACACAAGAGTA	TGAAACCTACTCCTTCCCTTGTC
TGFβ1-(P)	GGAAAGGGTGGGAGTCCAA	TTGCTCCAAACGCCAACC
HOXB9-FLAG-CMV4	AAGCTTTCCATTTCTGGGACGC TTAGCAG	GGATCCTTACTCTTTGCCCTGCTC CTTATTC

3.3 Results

3.3.1 HOXB9 is overexpressed in breast cancer tissue

As HOXB9 is known to be associated with mammary gland development and our previous studies demonstrated that HOXB9 is transcriptionally regulated by estrogen, we examined its expression in different malignant and non-malignant human cells such as MCF7 (breast cancer), T47D (breast cancer), MDAMB231 (human ER-negative breast cancer cell), HEPG2 (hepatocellular carcinoma), JAR (placental choriocarcinoma), HeLa (cervical cancer), SW480 (colorectal adenocarcinoma), K562 (chronic myelogenous leukemia), H358

(bronchioalveolar carcinoma), SY-SY5Y (nueroblastoma), and Hs798.Pl (normal placenta). RNA was isolated from each cell line, reverse-transcribed and subjected to real-time-PCR (qPCR) analysis for the expression of HOXB9. GAPDH was used as control. Our analysis showed that HOXB9 is differentially expressed in various cell lines and its expression was higher in MCF7, and HeLa cells in comparison to others other cell lines (Figure 3.1A). Notably, MCF7 is estrogen-receptor positive breast cancer cell line and previous studies demonstrated that HOXB9 is transcriptionally induced upon exposure to estrogen in MCF7 cells [121].

To further examine the potential link between HOXB9 and estrogen-signaling, we examined its expression in human breast cancer tissue by immuno-histological staining. We obtained breast cancer tissue microarray from commercial source that contains 6 cases of breast cancer (in duplicates) along with corresponding adjacent normal tissue and subjected to DAB staining using HOXB9 antibody. Analysis of immuno-histological staining of tissue microarray showed that HOXB9 expression was relatively higher (more intense DAB staining) in most cases of breast cancer tissue in comparison to its corresponding adjacent breast normal tissues (Figure 3.1B, a magnified view of case-6 is shown in Figure 3.1C, quantification is shown in Figure 3.1D). In combination cell cultures analysis in Figure 3.1A, the increased level of HOXB9 protein in breast cancer tissue compared to its corresponding normal healthy tissue indicated that HOXB9 is upregulated in breast cancer.

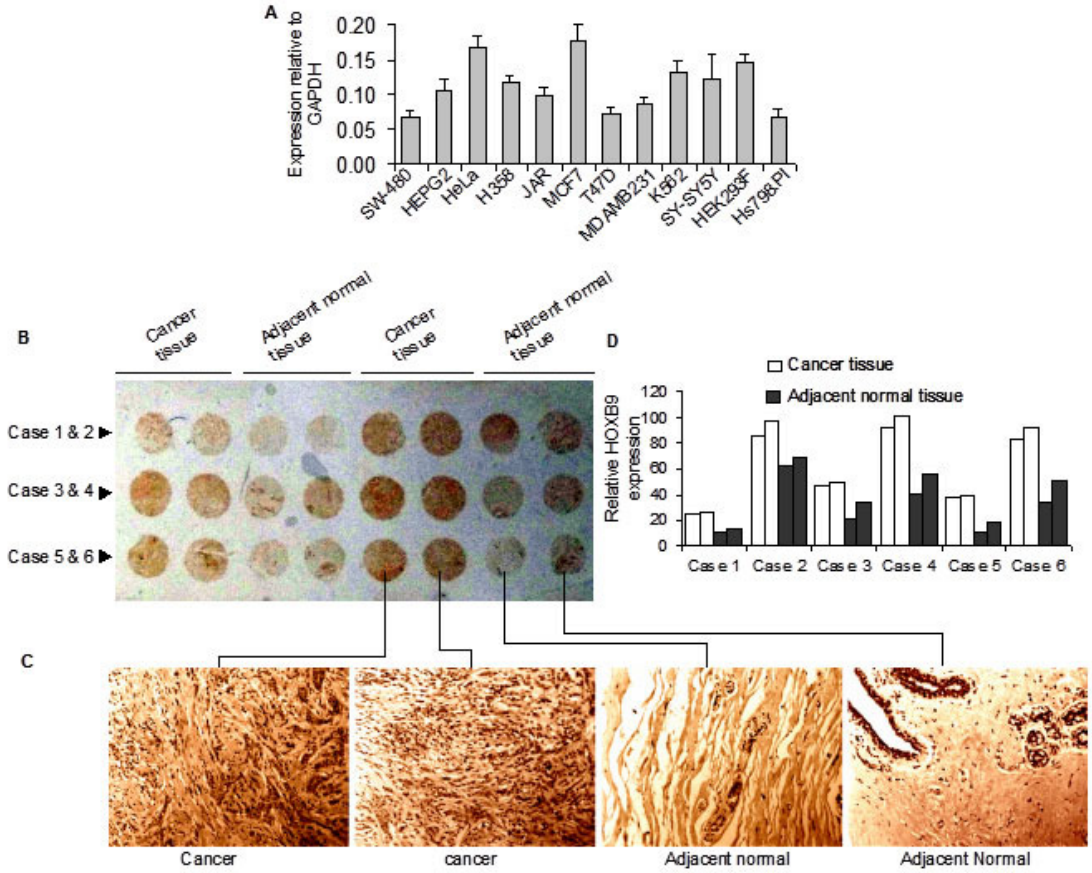

Figure 3.1. Expression of HOXB9 in different malignant and nonmalignant human cell lines and human breast cancer tissue. (A) Expression of HOXB9 in different cell lines. The total RNA was isolated from SW-480 (human colorectal adenocarcinoma), HEPG2 (hepatocellular carcinoma), HeLa (human cervical cancer), H358 (bronchioalveolar carcinoma), JAR (Human choriocarcinoma placenta), MCF7 (human adenocarcinoma mammary), T47D (ductal carcinoma mammary), MDA-MB-231 (ER negative human adenocarcinoma mammary), K562 (chronic myelogenous leukemia), SY-SY5Y (human neuroblastoma), HEK 293 (human embryonic kidney), Hs 798.PI (normal lung fibroblast) cell lines, reverse transcribed to cDNA and analyzed by real-time PCR using primers specific to HOXB9. GAPDH was used as a loading control. HOXB9 expression relative to GAPDH is plotted. Each experiment was repeated at least thrice (n = 3). Bars indicate standard errors. (B) Immuno-histological analysis of HOXB9 expression in breast cancer tissue: Human breast cancer tissue microarray (6 cases of breast cancer along with their matched adjacent normal breast tissue) was obtained from US Biomax and immuno-stained with HOXB9 antibody followed by peroxidase labeling with DAB substrate kit. Magnified view of tissue histology showing HOXB9 expression in case 6 is shown in panel C. Relative quantification of HOXB9 expression. The relative quantification of HOXB9 expression within the tissue section (assuming highest expression as 100%) is presented in panel D. Experiments were partially performed by Dr. Khairul Ansari.

3.3.2 HOXB9 homeodomain is critical for its nuclear localization and DNA binding

To understand the potential roles of HOXB9 in tumorigenesis, we analyzed its biochemical function. The HOX genes are well known to act as transcription factor where they bind to DNA via their homeodomain and manipulate gene expression. To examine the importance of HOXB9 homeodomain, we generated two stable cell lines over expressing either full length HOXB9 or homeodomain deleted HOXB9 (Figure 3.2A-B). The full length HOXB9 and HOXB9 with homeodomain deletion were cloned in a Flag-CMV4 human expression construct, transfected to HEK 293 cells and stable transformants were selected using G418 antibiotic selection procedure. Individual colonies expressing the Flag-tagged HOXB9 (Flag-HOXB9) or homeodomain deleted HOXB9 (Flag-HOXB9-ΔHD) were isolated and maintained separately under G418 containing media. To examine the expression level as well as distribution of HOXB9 in nucleus and cytoplasm, we fractionated the cytoplasm and nuclear extracts from Flag-HOXB9 and Flag-HOXB9-ΔHD stable cell lines and analyzed by western blot using anti-Flag antibody. These analyses demonstrated that Flag-HOXB9 is overexpressed in the stable cell line and enriched in nuclear fraction in comparison to cytoplasmic fraction. However, upon deletion of homeodomain, it is fractionated more in the cytoplasmic extract (Figure 3.2B).

We further examined the cellular distribution of HOXB9 using immuno-fluorescence staining and microscopy. We performed coimmuno-staining of Flag-HOXB9 and Flag-HOXB9-ΔHD stable cell lines using anti-flag antibody and RNA polymerase II (RNAP II) antibody. Nucleus was visualized by DAPI staining. Fluorescence microscopic analysis of the immuno-stained cells showed that HOXB9 (Flag-HOXB9) is primarily localized in the nucleus at G1 phase (Figure 3.3A). Partial co-localization with RNAP II was observed in the euchromatic region, though HOXB9 is primarily distributed all over the nucleus (Figure 3.3A). As the cell undergoes mitotic phases where DNA is much condensed, RNAP II was mostly dissociated

from condensed DNA. However some amount of HOXB9 still remained associated with mitotic chromatin and also dispersed in cytoplasm (Figure 3.3A).

Interestingly, upon deletion of homeodomain (Flag-HOXB9-ΔHD) of HOXB9, the nuclear localization is mostly lost and homeodomain deleted HOXB9 was spread all over the cells (G1-phase) (Figure 3.3B). In the mitotic phase HOXB9 immuno-staining showed a distinct gap in the place of condensed mitotic chromatin demonstrating no significant affinity for mitotic chromatin (Figure 3.3B). These observations demonstrated that HOXB9 is primarily a nuclear protein and its homeodomain is critical for binding to target DNA motif. The cell cycle progression in both Flag-HOXB9 and Flag-HOXB9-ΔHD was monitored by using lamin-B immuno-staining that is well known to be localized in the nuclear membrane (Figures 3.4A-B).

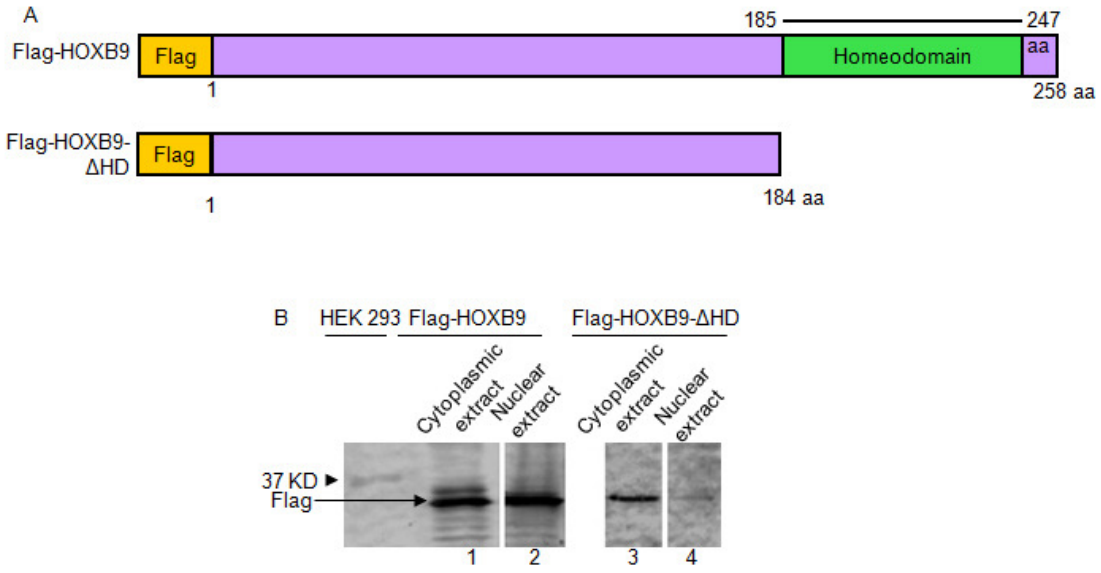

Figure 3.2 Expression of HOXB9 full length and homeodomain protein in HEK 293 cells. (A) Diagrammatic representation of Flag tagged HOXB9 full length (Flag-HOXB9) and Flag HOXB9 homeodomain deleted (Flag-HOXB9-ΔHD) protein. (B) Western blotting of HOXB9 full length and homeodomain deleted protein. Flag-HOXB9 and Flag-HOXB9ΔHD were fractionated to cytoplasmic and nuclear extracts and subjected to western blot analysis using Flag antibody.

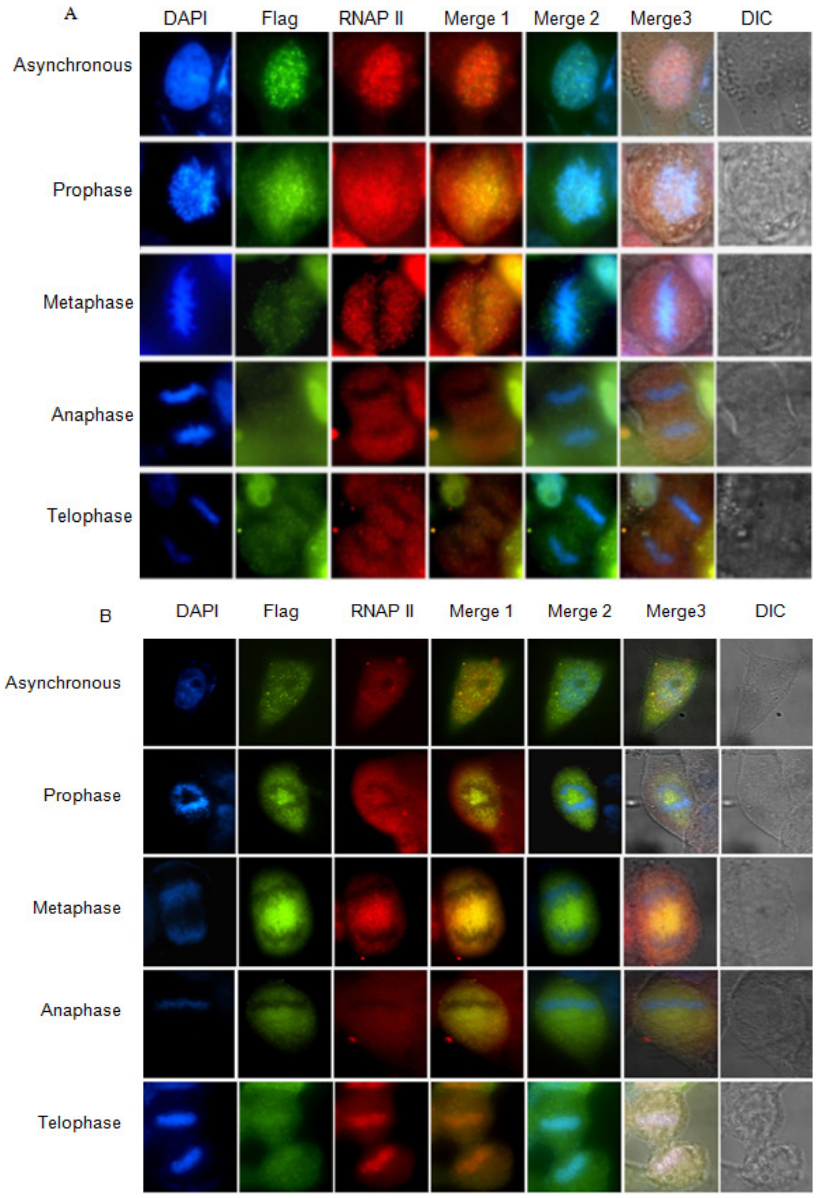

Figure 3.3 HOXB9 homeodomain is critical for its nuclear DNA binding. (A) HOXB9 binds to the nuclear DNA via its homeodomain. Stable cells expressing Flag-HOXB9 cells were fixed with 4% formaldehyde. Immuno-staining was performed with RNAP II and Flag primary antibodies. Secondary antibodies conjugated to FITC or TRITC were used to stain them. DAPI was used to stain the nucleus. Merge 1 is overlay between Flag and RNAP II, Merge 2 is overlay between DAPI and FLAG, Merge 3 is overlay between DAPI, FLAG, RNAP II and DIC. Only DIC images are shown on the right. (B) HOXB9 nuclear localization signal is lost upon homeodomain deletion. Staining with RNAP II and Flag primary antibodies were performed as described above on Flag-HOXB9-ΔHD cells.

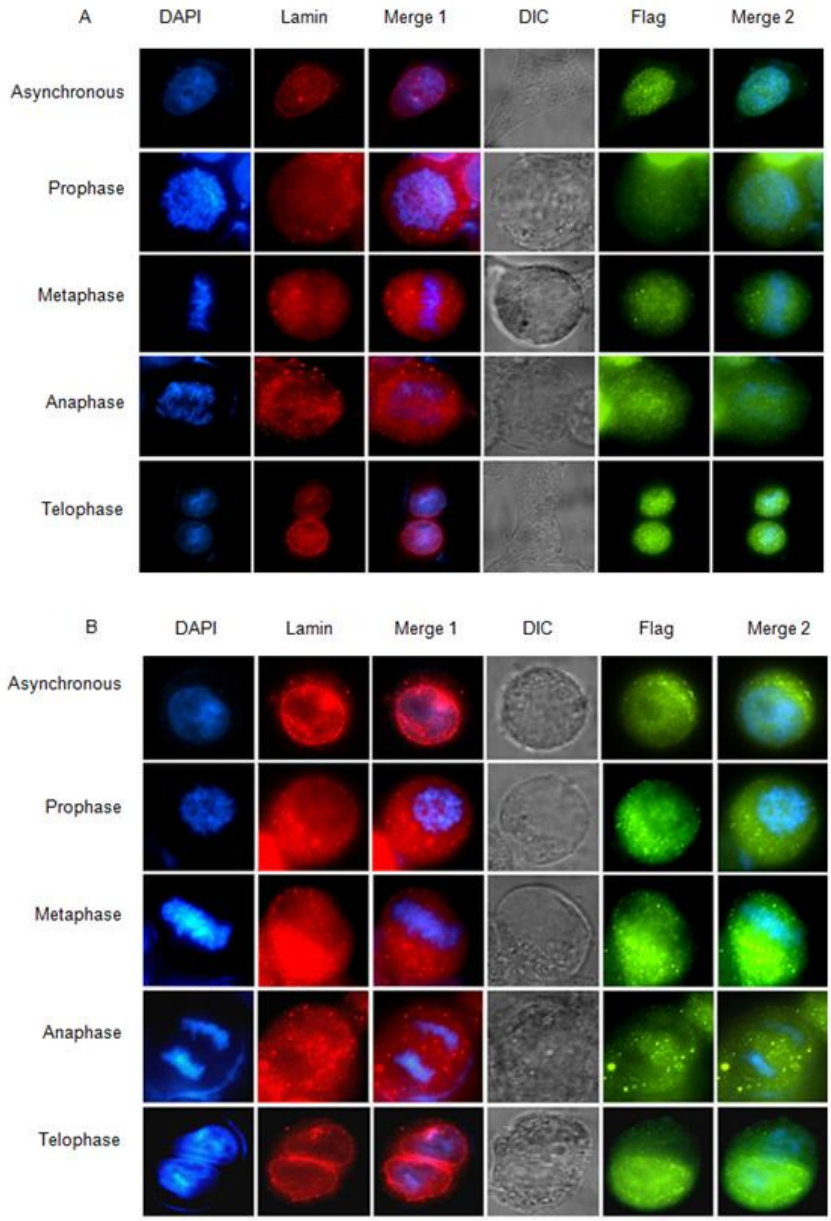

Figure 3.4 Nuclear staining with Lamin-B. (A) Nuclear staining with Lamin on the Flag-HOXB9 stable cells. Flag-HOXB9 stable cell lines were immuno-stained with Lamin-B. Merge 1 is overlay between DAPI and Lamin-B. Merge 2 is overlay between DAPI and FLAG. (B) Nuclear staining with Lamin-B on the Flag-HOXB9 ΔHD stable cells. Stable cells expressing Flag-HOXB9-ΔHD stable cell lines were immuno-stained with Lamin-B. Merge 1 is overlay between DAPI and Lamin images. Merge 2 is overlay between Flag and DAPI images.

3.3.3 HOXB9 influences cell cycle progression via involvement of its homeodomain

Several HOX genes are implicated in cell cycle regulation. To further explore the cellular functions of HOXB9, we examined its potential roles in cell cycle progression. We examined the distribution of cell population at different phases of cell cycle in both Flag-HOXB9 and Flag-HOXB9-ΔHD stable cell lines using flow cytometry and compared with untransfected control (as well as transfected with empty vector pFlag-CMV4) HEK 293 cells. These analyses demonstrated that HOXB9 overexpression has dramatic effects on cell cycle progression in HEK 293 cells. Upon HOXB9 (Flag-HOXB9) overexpression cell population at G0/G1 phase shifted from 53% to 17%, (Figure 3.5A). HOXB9 overexpression also reduced the mitotic (G2/M) and S-phase cells populations (Figure 3.5A). Most cells underwent apoptosis as evidenced by increase from 21% to 66% (Figure 3.5A). Interestingly, upon deletion of HOXB9 homeodomain (Flag-HOXB9-ΔHD), the dramatic defects in cell cycle progression was mostly rescued with increase in G0/G1 (to 45%) and G2/M (to 20%) population. These observations demonstrated that HOXB9 is a critical player during cell cycle progression and HOXB9 homeodomain is crucial for cell cycle regulatory function. The overexpression of HOXB9 most likely led to cell cycle arrest in G0/G1 phase.

We examined the status of cell cycle regulatory genes in the Flag-HOXB9 and Flag-HOXB9-ΔHD stable cell lines. We isolated the RNA from the control HEK 293, Flag-HOXB9 and Flag-HOXB9-ΔHD stable cell lines, reverse transcribed into cDNA, and analyzed the expression of various cell cycle regulatory genes such as Cyclin A-D, p21, p53 and p57. Our results demonstrated that indeed, several cell cycle regulatory Cyclins (Cyclin A) and p-proteins (p27, p53, p57) were upregulated upon overexpression of HOXB9 full length protein and their expression were decreased to normal levels in the Flag-HOXB9-ΔHD stable cell line (Figure 3.6A, qPCR data shown in right panel). Cyclin E expression was downregulated in both Flag-HOXB9 and Flag-HOXB9-ΔHD stable cell lines in comparison to the untransfected HEK 293

cells. Taken together, these observations further demonstrated that HOXB9 plays critical roles in regulation of cell cycle regulatory genes via involvement of homeodomain and this is reflected in the drastic changes in cell cycle distribution in the Flag-HOXB9 and Flag-HOXB9-ΔHD stable cell lines. HOXB9 homeodomain deleted fragment has also some impact on cell cycle progression as evidenced in both (Figure 3.5A and Figure 3.6A).

To further confirm the potential roles of HOXB9, we examined its expression pattern throughout the cell cycle. For this purpose, we synchronized HeLa cells using double thymidine treatment as described by our laboratory previously [126]. In brief, cells were treated with 10 mM thymidine (18 h), released into fresh media (9 h), and then blocked again by addition of 10 mM thymidine (17 h) and finally released into fresh media at G_1/S boundary. Cyclins B and E were used as markers for cell cycle synchronization. As reported previously, Cyclin B was expressed prominently in the G2/M phase while Cyclin E expression was high in S and G1 phase but low in G2/M phase (Figure 3.5B) [126]. Interestingly, HOXB9 also expressed differentially during cell cycle progression in HeLa cells. HOXB9 was expressed high in the S-phase and then decreased throughout the cell cycle (Figure 3.5B). The differential expression of HOXB9 indicates further its potential roles in cell cycle progression.

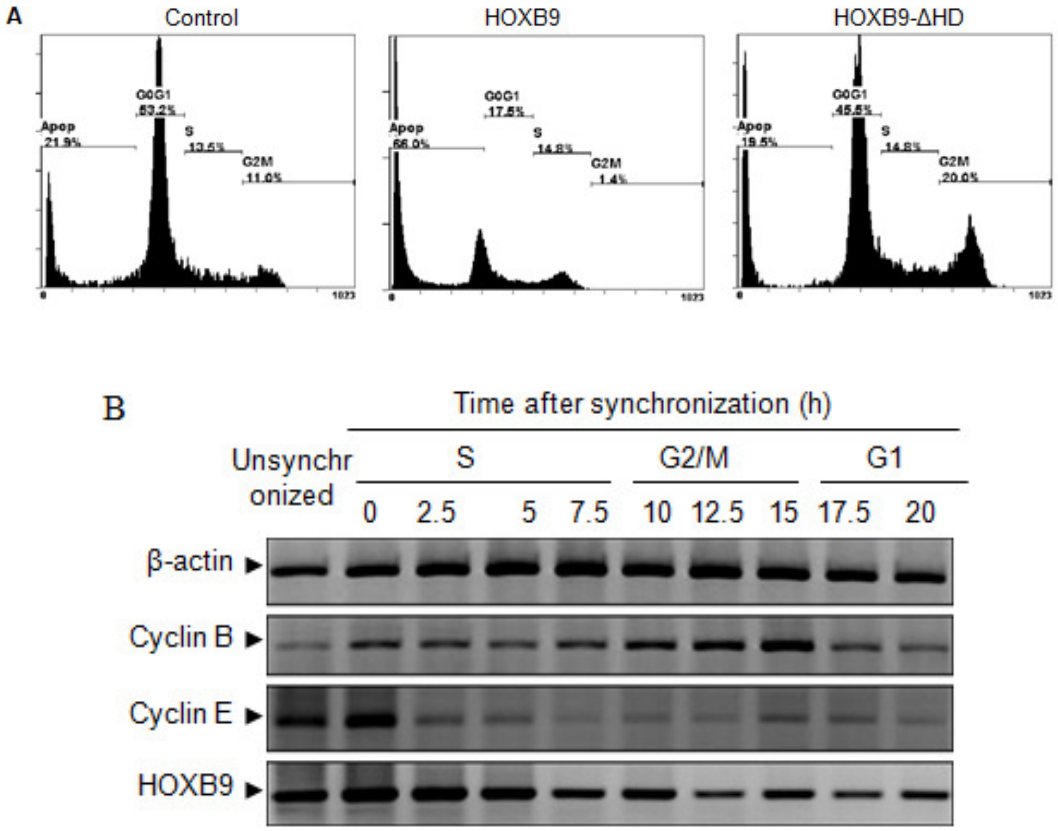

Figure 3.5 Effect of HOXB9 on cell cycle progression and on expression of cell cycle regulatory genes. (A) Effect of homeodomain on cell cycle progression. HEK 293 cells, stable cells expressing Flag-HOXB9 and Flag-HOXB9-ΔHD were grown in G418 containing media and were subjected to FACS analysis. (B) Expression of HOXB9 during cell cycle progression in HeLa cells. HeLa cells were synchronized using double thymidine block method. RNA was isolated, reverse-transcribed and subjected to PCR using primers specific to Cyclin B, Cyclin E, HOXB9 and actin. The amplified DNA was run on agarose gel.

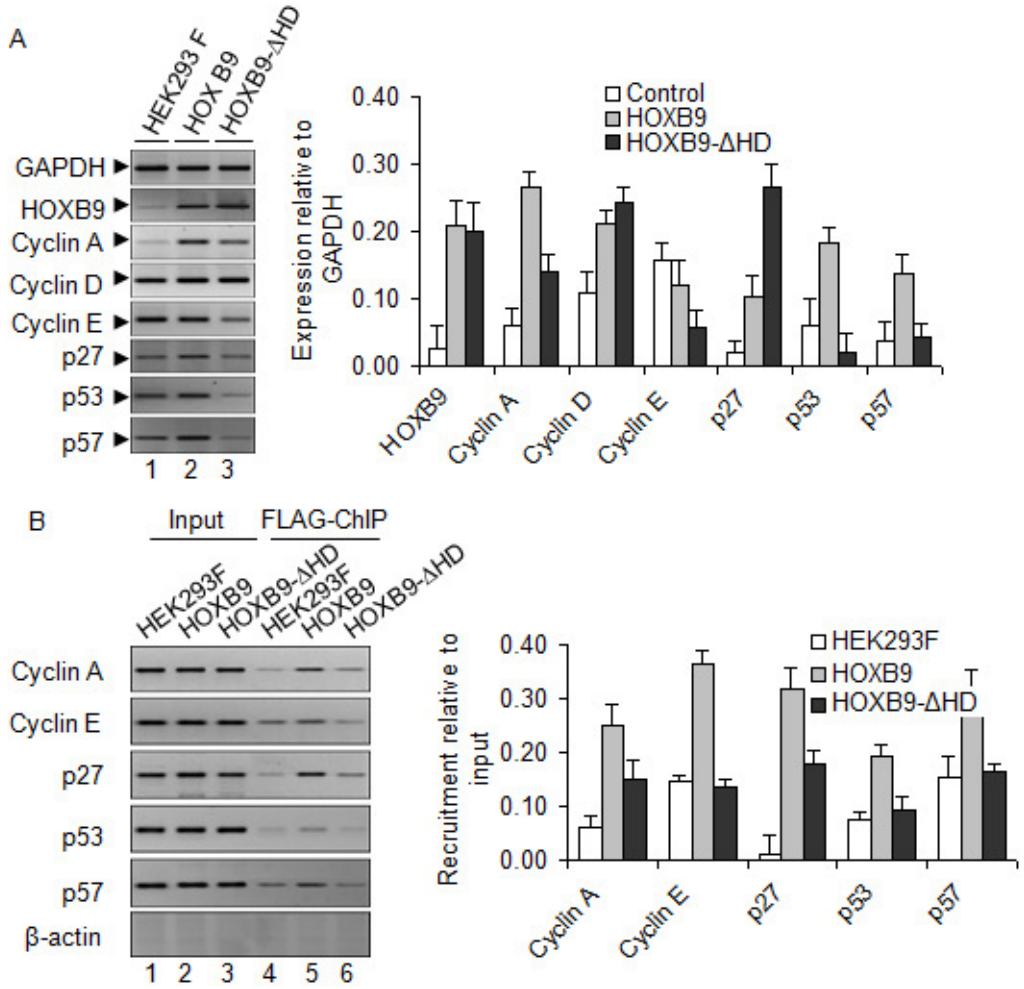

Figure 3.6 (A) Overexpression of HOXB9 affects cell cycle regulatory genes. Reverse transcriptase PCR was performed with GAPDH, HOXB9, Cyclin A, Cyclin D, Cyclin E, p27, p53 and p57 primers and analyzed on agarose gel. Lanes 1-3 shows target gene expression on HEK 293 cells, Flag-HOXB9 and Flag-HOXB9-ΔHD cells respectively. Right panel shows real time PCR results. (B) Recruitment of HOXB9 on the target gene promoters. Chromatin immuno-precipitation assay was performed on HEK 293, Flag-HOXB9, and Flag-HOXB9-ΔHD cells with anti-FLAG antibody. The immuno-precipitated DNA was PCR amplified to analyze the recruitment of HOXB9 on the promoters of Cyclin A, Cyclin E, p27, p53 and p57. Lane 1 & 4 is HEK 293 cells as control. Lane 2 & 5 is Flag-HOXB9 expressing HEK 293 cells. Lane 3 & 6 is Flag-HOXB9-ΔHD over expressing HEK 293 cells. Real time PCR was performed on the target gene promoters. Results are shown on the right.

We examined the binding of HOXB9 in the promoter of affected cell cycle regulatory genes using chromatin immuno-precipitation (ChIP) assay (Figure 3.6B, real-time quantification in the right panel). Briefly, we grew HEK 293 cells, Flag-HOXB9 and Flag-HOXB9-ΔHD stable cell lines separately, fixed with formaldehyde, sonicated to shear the chromatin (~3-500 bp) and subjected to immuno-precipitation using flag antibody. Anti-actin antibody was used as negative control (data not shown). The immuno-precipitated chromatins were reverse-cross-linked and subjected to PCR analysis using primers specific to promoters of different target genes. These analyses demonstrated that HOXB9 binding was enriched in the promoter of target cell cycle regulatory genes (Cyclin A, Cyclin E, p27, p53 and p57) and that binding was reduced upon deletion of HOXB9 homeodomain (Figure 3.6B, real time quantification of the ChIP DNA is shown in the right panel). These analyses demonstrated that HOXB9 is directly involved in controlling these target cell cycle regulatory genes via interaction with its homeodomain.

3.3.4 HOXB9 homeodomain is critical for tumor growth and regulation of genes associated with tumor growth and angiogenesis

As our studies showed that HOXB9 is over expressed in breast cancer, we examined if it has any roles in tumorigenesis. We used a 3-dimentional colony formation assay in soft-agar as described previously [127-129]. We plated HEK 293, Flag-HOXB9 and Flag-HOXB9-ΔHD cells in soft agar, fed with 2X FBS containing media and allowed to grow for 4-6 weeks, until visible colonies were formed. Colonies at different layers were counted under microscope and plotted. Our results demonstrated that in 4-6 weeks, plenty of distinctly visible colonies were formed in different layers of soft agar in the plate containing Flag-HOXB9 cells (Figure 3.7A). Expanded views of colonies are shown on the bottom panel which demonstrated clearly a three-dimensional cellular growth of Flag-HOXB9 stable cell line (Figure 3.7A, quantification showing the number colonies in different stable cell lines are shown 3.7B). Interestingly, there were very few small colonies of cells in the plates containing Flag-HOXB9-ΔHD stable cell line and almost no colonies were observed in the untransfected HEK 293 cells (Figure 3.7A-B). These

observations demonstrated that full-length HOXB9 overexpression contributed towards the formation of large 3D-growth of cell colonies indicating potential roles of HOXB9 in tumorigenesis. The reduction in number of colonies and sizes of the 3D-growth in the homeodomain deleted HOXB9 strain (Flag-HOXB9-ΔHD) demonstrated that homeodomain of HOXB9 is crucial for tumorigenesis.

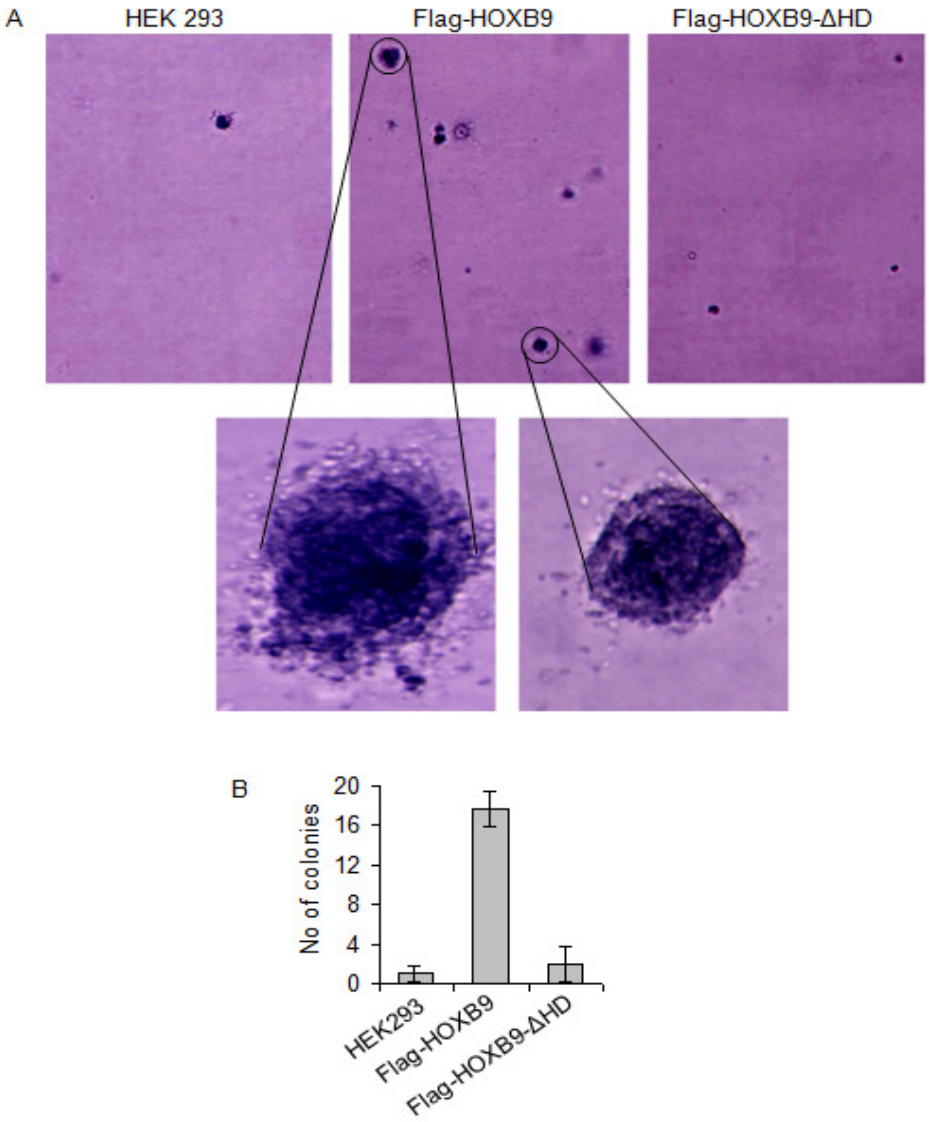

Figure 3.7 Colony forming ability of HOXB9 full length and homeodomain deleted cells. (A) Colony formation of HOXB9 full length and homeodomain deleted cells in vitro. Top panel shows HEK 293 cells used as control. Middle panel shows colony forming ability of HOXB9 in HEK 293 cells. Flag-HOXB9 overexpressed cells were grown in soft agar for 21 days and fed with media every 2 days. The numbers of colonies formed in soft agar were counted after staining with 0.005% crystal violet solution. Magnified colonies are shown on the right. Bottom panel shows colonies from Flag-HOXB9-ΔHD overexpressed cells grown in soft agar. (B) Number of colonies grown in soft agar for each of the cells. Colonies formed in HEK 293, Flag-HOXB9 and Flag-HOXB9-ΔHD were counted under light microscope and plotted. Bars represent standard errors.
Experiments were partially contributed by Sahba Kasiri.

To further examine potential mechanism of tumor growth mediated by HOXB9, we examined its roles in transcriptional regulation of various growth and angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor (TGFβ1) and neuregulin (NRG) under both HOXB9-overexpressed and homeodomain deleted environment [130, 131]. Our results demonstrated that overexpression of HOXB9 (Flag-HOXB9 stable cell line) resulted in upregulation of VEGF, bFGF, TGFβ1 and NRG genes (Figure 3.8A, quantification are shown in the bottom panel). The deletion of homeodomain of HOXB9 (Flag-HOXB9-ΔHD) resulted in decreased expression of above genes further indicating critical roles of HOXB9 and its homeodomain in their transcriptional regulation. ChIP assay demonstrated that level of HOXB9 recruitment were increased in the promoter of VEGF, bFGF, TGFβ1 and NRG genes when they were overexpressed under HOXB9 overexpressed condition (Flag-HOXB9 stable cell line) and decreased under homeodomain deletion environment (Flag-HOXB9-ΔHD stable cell line). The binding of HOXB9 at the promoter of these genes (Figure 3.8B, quantification are shown in the bottom panel), indicates critical roles of HOXB9 in transcriptional activation of tumor growth factors.

To further confirm the roles of HOXB9 in controlling tumor growth factors, we knocked down HOXB9 in HEK 293 cells using HOXB9 specific antisense-oligonucleotide and examined its impact on VEGF, bFGF, TGFβ1 and NRG genes expression. We transfected HEK 293 cells using HOXB9 antisense (and scramble antisense with no homology to HOXB9) using lipofectamine transfection reagent for 72 h, isolated the RNA, reverse transcribed and subjected to real-time PCR analysis. Our results demonstrated that HOXB9 antisense efficiently knocked down HOXB9 expression both at the mRNA and protein level (Figures 3.9A and B). Scramble antisense has no significant impact on HOXB9 expression (Figures 3.9A and B). Interestingly, upon knockdown of HOXB9, VEGF, bFGF, TGFβ1, and NRG genes were downregulated (Figures 3.9A, quantifications on the right panel). ChIP analysis showed that HOXB9

knockdown also downregulated the recruitment of HOXB9, RNAP II and level of H3K4-trimethylation at the promoter of above target genes (Figure 3.10). These results further demonstrated that HOXB9 plays critical roles in transcriptional regulation of key genes associated with tumor growth and angiogenesis.

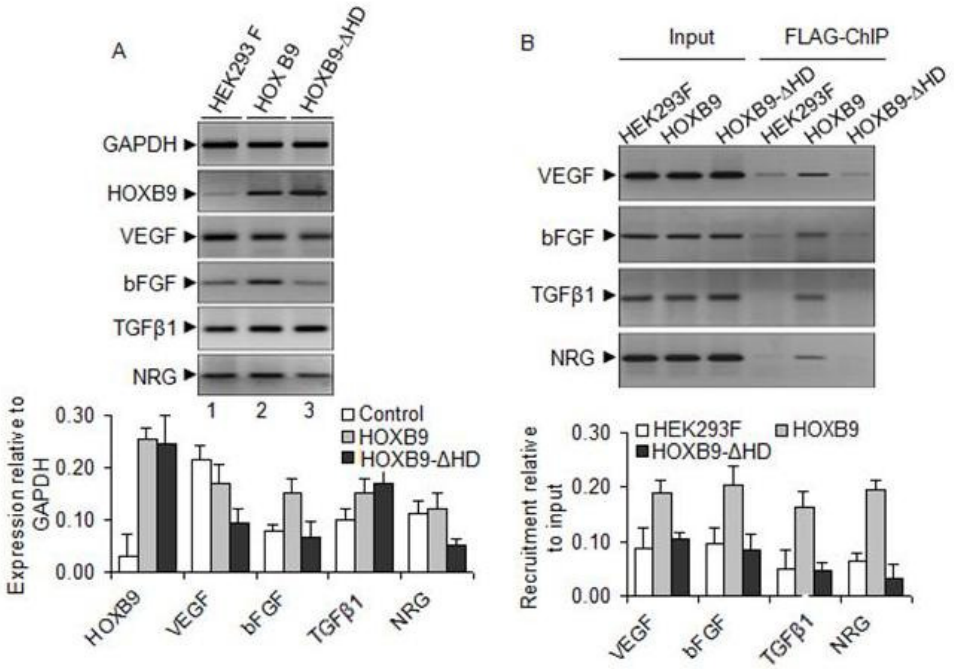

Figure 3.8 HOXB9 controls the expression of tumor growth and angiogenic factors. (A) Expression of the angiogenic genes in HEK 293 cells, Flag-HOXB9 and Flag-HOXB9-ΔHD cells. Reverse transcriptase PCR was performed with primers specific to GAPDH, HOXB9, VEGF, bFGF, TGFβ1 and NRG. Lane1 is HEK 293 cells as control. Lane 2 is Flag-HOXB9 overexpressing HEK 293 cells. Lane 3 is Flag-HOXB9-ΔHD overexpressing HEK 293 cells. Real time quantification of gene expression relative to GAPDH is shown in bottom panel. Bars indicate standard errors (n =3). (B) Recruitment of HOXB9 on the HEK 293 cells and stable cells expressing Flag-HOXB9 and Flag-HOXB9-ΔHD. Antibodies specific to FLAG was used for performing ChIP assay on the target gene promoters. The immuno-precipitated DNA was used to analyze the recruitment of HOXB9 on the promoters of VEGF, bFGF, TGFβ1 and NRG using promoter specific primers. Lane1 is HEK 293 cells used as control. Lane 2 is ChIP DNA from Flag-HOXB9 expressing HEK 293 cells. Lane 3 is ChIP DNA from Flag-HOXB9-ΔHD over expressing HEK 293 cells. Real time quantification of recruitment level of Flag tagged HOXB9 (% relative to Input) on the target gene promoters are shown on the right. Bars indicate standard errors (n= 3).

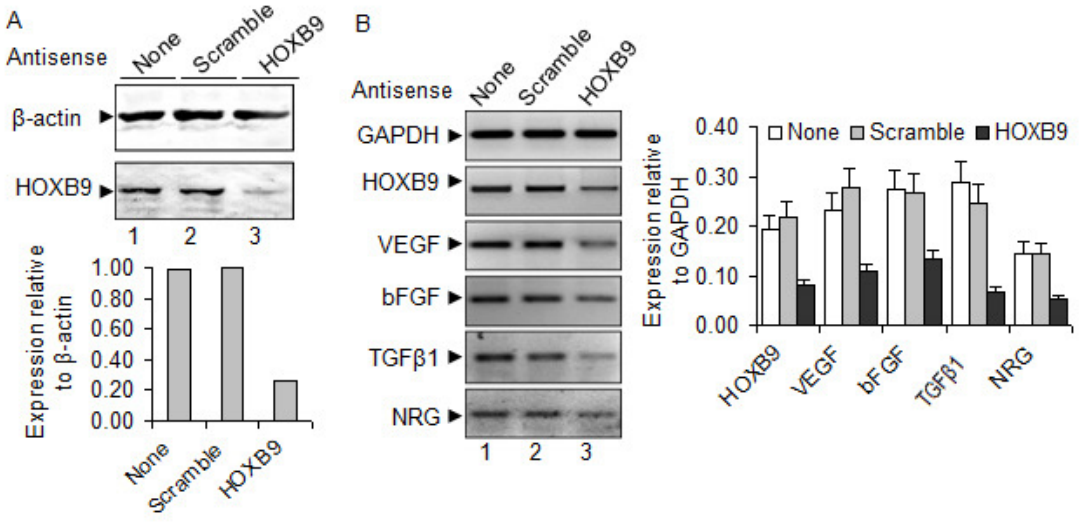

Figure 3.9 HOXB9 knockdown affects expression of angiogenic factors. (A) Western blotting of HOXB9 knockdown HeLa cells. The protein extract of control, scramble antisense and HOXB9 antisense treated HeLa cells were subjected to Western blotting with antibodies specific to HOXB9. β-actin antibody is used as a loading control. (B) HOXB9 knockdown down regulates the expression of tumor growth and angiogenic factors. HOXB9 was knocked down in HeLa cells using antisense oligonucleotides specific to HOXB9. A scramble antisense with no homology to HOXB9 was used as a control. The antisense treated cells were incubated for 72 h and were used for analysis. The RNA extract of control, scramble antisense and HOXB9 antisense treated HeLa cells were subjected to reverse transcriptase-PCR with primers specific to HOXB9, VEGF, bFGF, TGFβ1 and NRG. GAPDH was used as a control. Lane 1 is control cells. Lane 2 is cells treated with scramble antisense. Lane 3 is cells treated withHOXB9 antisense. Real time quantification of expression relative to GAPDH is shown in right panel. Bars indicate standard errors (n = 3).

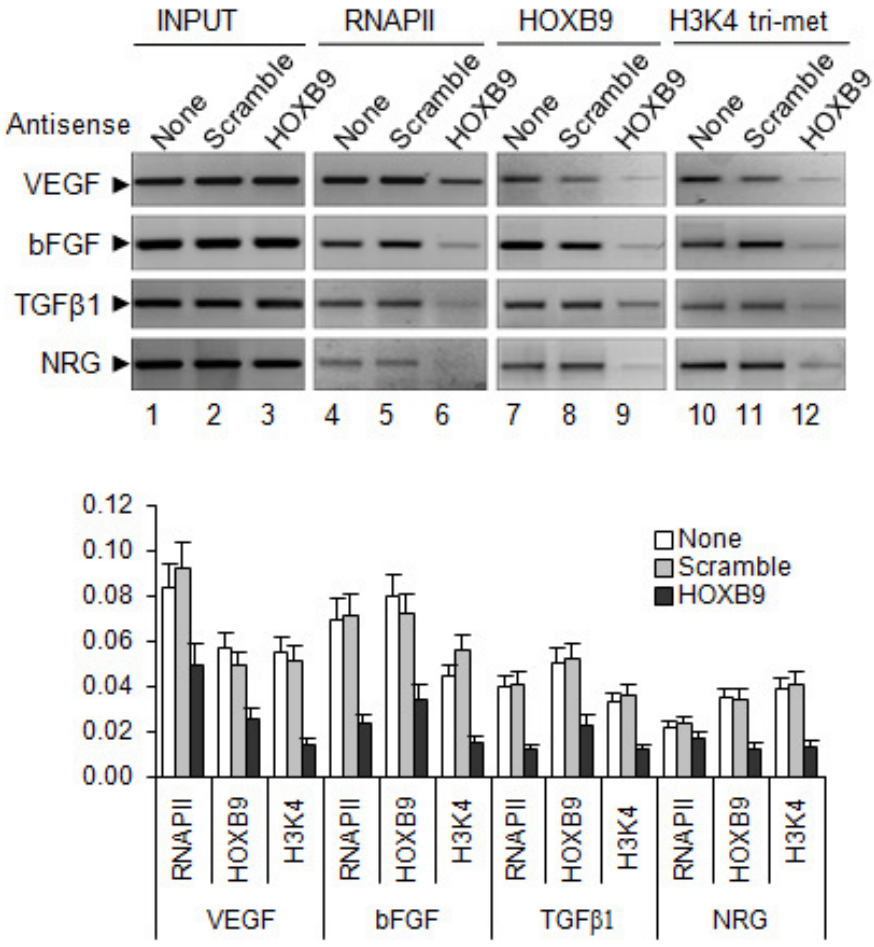

Figure 3.10 Recruitment of HOXB9, RNAP II and H3K4 on HOXB9 knockdown cells. Chromatin immuno-precipitation (ChIP) assay was performed with HOXB9, RNAP II and H3K4 antibodies on HOXB9 knockdown cells. Control, scramble and HOXB9 antisense treated HeLa cells were subjected to ChIP assay using antibodies specific to RNAP II, HOXB9 and H3K4 trimethyl. The immuno-precipitated chromatin DNA fragments was PCR analyzed by using primers specific to VEGF, bFGF, TGFβ1 and NRG. Lanes 1-3 are INPUT. Lanes 4-6 are RNAP II immuno-precipitated DNA from control, scramble antisense and HOXB9 antisense treated cells respectively. Lanes 7-9 are HOXB9 immuno-precipitated DNA. Lanes 10-12 are H3K4 trimethyl immuno-precipitated DNA. Quantification of recruitment levels relative to INPUT is shown below. Bars indicate standard errors (n = 3).

3.4 Discussion

HOX genes are well known as key players during embryonic development, hematopoietic stem cell differentiation and also in adult tissue homeostasis [124, 132-134]. HOX genes are highly conserved throughout eukaryotes and HOX proteins contain a conserved motif called homeodomain. In general, homeodomain fold consists of a 60-amino acid helix-turn-helix structure, through which they recognize specific DNA sequences present in the promoter of various genes and modulate their transcriptional states [124, 132-134]. Thus HOX genes are crucial transcription factors that controls cell differentiation, organogenesis and development. Misregulation of HOX genes and their mutation cause several developmental defects and various human diseases including cancer. HOX gene expressions are known to be regulated by various hormones such as retinoic acids, steroid hormones, vitamins and others. Recent studies from our laboratory demonstrated that several HOX genes such as HOXC6, HOXC13, HOXC10 and HOXB9 are transcriptionally regulated by steroid hormone estrogen, via coordination of ERs and MLL histone methylases [20, 30, 53, 54, 135-139].

In this study, we demonstrated that HOXB9, which is well known to be a critical player in mammary gland development, regulation of renin-angiotensin system and is regulated by estrogen, is upregulated in breast cancer tissue. HOXB9 expression was also observed higher in various tumor cells including estrogen-receptor positive MCF7 breast cancer cells [123, 131]. The transcriptional regulation of HOXB9 by estrogen, its association with steroid responsive organogenesis and over-expression in breast cancer tissues suggest that it is an estrogen-regulated gene and its overexpression in breast cancer tissue is likely due to higher estrogenic activity in these tissues.

Our studies demonstrated that HOXB9 overexpression induced severe cell cycle defect. Cells were arrested at G0/G1 and ultimately underwent apoptosis. Interestingly, the deletion of

homeodomain from C-terminus of HOXB9 and overexpression of the remaining N-terminus, rescued the defected cell population almost to the normal level. These observations support the strong contribution of HOXB9 homeodomain in cell cycle regulation. Importantly, our studies also demonstrated that HOXB9 affected the expression of various cell cycle regulatory genes including Cyclin A, Cyclin E, p27, p53 and p57, indicating possible mechanism of HOXB9 and its homeodomain in cycle progression and apoptosis. Notably, the misregulation of most of the cell cycle regulatory genes was reversed upon deletion of homeobox domain of HOXB9. Notably, HOX genes are previously reported to be involved in cell cycle progression via modulating the expression of Cyclins and p-proteins [126, 136, 140-142]. For example, HOXA5 is known to activate p53, which is known to regulate the expression of p21, an inhibitor of CDK enzymes that is a critical player for cell cycle progression [143-145]. HOXA10 directly upregulates the expression of p21 leading to cell cycle arrest at the G1 phase in both monocytic and fibroblast cell lines [146]. Both p21 and p53 plays vital roles in cell cycle regulation. p21 is a CDK inhibitor and p53 is responsible for holding cells at G1/S transition by regulating the expression of p21 [147-150]. Furthermore, HOXA10 expression is known to be influenced by p57, another critical regulator of cell cycle progression and knockdown of HOXC10 induces cell cycle arrest at G2/M phase. Thus our data demonstrating the critical roles of HOXB9, especially via its homeodomain in transcriptional regulation of Cyclins and p-proteins and cell cycle progression further demonstrated that HOX genes are key regulators of cell cycle progression and homeodomain plays critical roles in these processes most likely via binding to the promoter of cell cycle regulatory genes and modulating their expression.

Increasing amount of studies demonstrated that HOX genes are critical in adult tissue and often some HOX genes are reported to be overexpressed in various types of cancer. For example, HOXC6 a critical player in mammary gland development and milk production is over

expressed in breast and prostate cancer [151]. HOXA10 expression is associated with ovarian cancer [152]. Our studies demonstrated that HOXB9 is overexpressed in breast cancer.

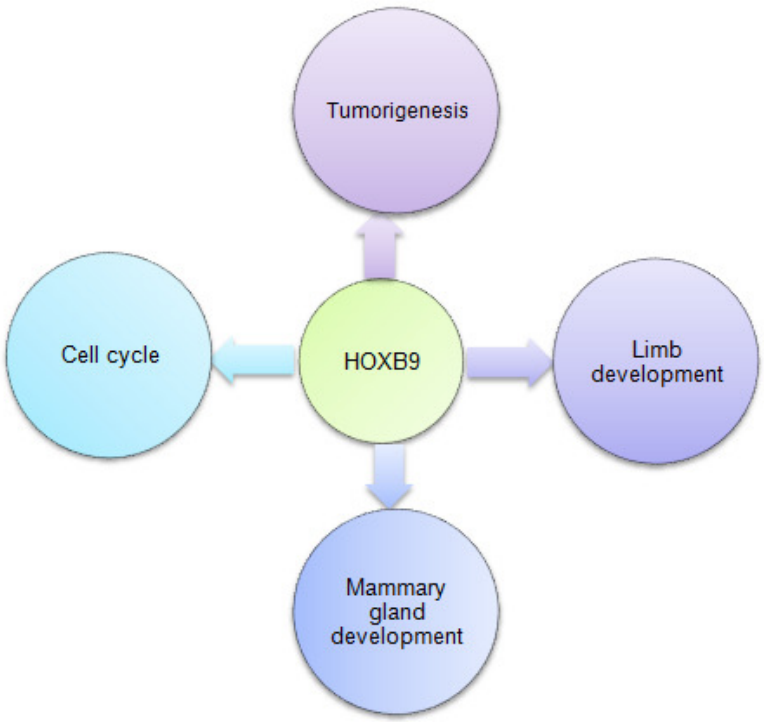

Figure 3.11 HOXB9 as a central player in key physiological processes. HOXB9 plays a direct/indirect role in cell cycle progression, tumorigenesis, mammary gland development and thoracic cage development.

Recent studies also demonstrated that HOXB9 is a critical player in angiogenesis and tumor growth (see Figure 3.11). Herein, using *in vitro* colony formation assay we examined the roles of HOXB9 and its homeodomain in three-dimensional growth and colony formation of HEK 293 cells. Our studies demonstrated that overexpression of full-length HOXB9 resulted in formation of large number (20 fold) of 3D-colonies in soft-agar media in comparison to the untransfected normal HEK 293 cells under similar condition. Sizes of the colonies were big enough to visualize under naked eye. Interestingly, overexpression of homeodomain truncated

HOXB9 in HEK 293 cells reduced the number of cell colonies almost to the untransfected cell level. These observations demonstrated that HOXB9 overexpression induced colony formation and 3D-growth of cells and hence may be critical in tumorigenesis and this role of HOXB9 is mediated via coordination of its homeodomain. Analysis of gene expression status demonstrated that HOXB9 overexpression as well as knockdown affected the level of expression of various tumor growth and angiogenic factors. HOXB9 binds to the promoter of these target genes via its homeodomain and regulate their expression. Thus, our biochemical studies demonstrated that HOXB9 plays potential roles in tumor growth via regulation of tumor growth and angiogenic factors.

Overall our studies demonstrated that HOXB9, a gene which is critical in hormone regulated organogenesis and development, is overexpressed in breast cancer. HOXB9 controls the cell cycle progression via involvement of its homeodomain and regulates various cell cycle regulatory Cyclins and p-proteins. HOXB9 also potentially influences tumor growth via modulation of the expression of tumor growth and angiogenic factors. Our studies revealed novel function of HOX genes in breast cancer, angiogenesis and cell cycle progression (Figure 3.11).

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