TRANSCRIPTIONAL PROPERTIES OF MEOX2

BY

Mona M.T. Friesen

A Thesis submitted to
the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Biochemistry and Medical Genetics
Faculty of Medicine
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Division of Stroke and Vascular Disease
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ABSTRACT

Meox2 induces cell cycle arrest and it is postulated that Meox2 is required for maintaining adult vascular smooth muscle cells (VSMCs) in a quiescent state. Upon vascular injuries, such as atherosclerosis, VSMCs transform from a quiescent, contractile phenotype to a proliferative phenotype. Downregulation of Meox2 expression is observed during this phenotypic transformation.

Currently, direct Meox2 downstream targets are unknown. However, we hypothesize that Meox2 can act as a transcription factor. The aim of this investigation was to examine the transcriptional properties of Meox2.

Our results demonstrated that Meox1 and Meox2 do not require binding to DNA to activate the *p21* promoter. Conversely, *Bapx1* promoter activation is dependent on Meox2-DNA interaction. This study is the first to report that the Meox1 and Meox2 proteins regulate the same downstream target genes and that the mechanism of this regulation is promoter-specific.

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LIST OF ABBREVIATIONS

AD = Activation domain
Ad-*Meox2* = Adenovirus construct encoding Meox2
Ang II = (Angiotensin II)
ASK1 = Apoptosis signalling regulating kinase
BrdU = 5-bromo-2-deoxyuridine
CDK = Cyclin dependent kinase
CDKI = Cyclin dependent kinase inhibitor
Cip1 = Cdk-interacting protein 1
CNP = C-Type Natriuretic Peptide
d.p.c = Days post coitus
DBD = DNA binding domain
EGFP = Enhanced green fluorescent protein
EMSAs = Electrophoretic mobility shift assays
Eng = Engrailed
FACS = Fluorescent activated cell sorting
HEK293 = Human embryonic kidney cells
HUVECs = Human umbilical vein endothelial cells
IPTG = Isopropyl-beta-D-thiogalactopyranoside
MBP = Maltose binding protein
MEFs = Mouse embryonic fibroblasts
Mt = Mutant
NIH/3T3 = Mouse fibroblast cell line
NLS = Nuclear localization sequence
Pax = Paired box homeobox gene
Pax3 = Paired box protein
PBS = Phosphate buffered saline
PBS-T = Phosphate buffered saline with Tween
PCNA = Proliferating cellular nuclear antigen
Rb = Retinoblastoma
RD = Repressor domain
shh = Sonic hedgehog
TBS = Tris buffered saline
TBS-T = Tris buffered saline with Tween
TUNEL = TdT-mediated dUTP-fluorescein nick-end labeling
VEGF = Vascular Endothelial Growth Factor
VSMCs = Vascular smooth muscle cells
WAF1 = Wild-type p53-activated fragment

A) LITERATURE REVIEW

I) Cardiovascular disease

Adult vascular smooth muscle cells (VSMCs) are normally quiescent, contractile cells but these cells can undergo a phenotypic switch and adopt a synthetic (less differentiated), proliferative phenotype. This phenotype switch occurs during natural biological processes such as embryonic development, responses to injury, or vessel remodeling induced by changes in tissue demands [1]. Increases in VSMC proliferation contributes to the progression of many pathological conditions such as atherosclerosis, hypertension, post-angioplasty restenosis and coronary artery transplant vasculopathy [1, 3-6]. In vasculoproliferative diseases such as restenosis, VSMC growth contributes to the stenosis of the blood vessel by forming the neointimal layer [4, 5].

The mechanisms that induce the VSMC phenotypic switch vary due to the heterogeneous nature of blood vessels within the body [1]. Signaling by both autocrine molecules such as platelet derived growth factor, fibroblast growth factor, epidermal growth factors, angiotensin II and paracrine molecules (i.e. nitric oxide and endothelin-1) secreted by adjacent endothelial cells, monocytes, fibroblasts and dendritic cells play a role in mediating the phenotypic switch [1]. As well, stimuli such as hypoxia, mechanical forces and reactive oxygen species are involved in the activation of VSMCs [1, 7-10]. A better understanding of how these signals converge to regulate VSMC cell cycle progression is identifying potential new therapeutic targets for use in the treatment of vasculoproliferative diseases. Conventional therapies largely treat the symptoms which result from an established vascular lesion rather than by targeting the underlying mechanisms involved in lesion formation [3]. Furthermore, understanding how the cell

cycle is regulated in VSMCs may also be a useful approach to prevent the vascularization of tumors which is crucial for both tumor growth and dissemination [11, 12].

Cardiomyocytes proliferate at a very low rate in the healthy adult and this low rate of proliferation cannot be induced sufficiently to regenerate the cardiac muscle lost following insults such as a myocardial infarction [13]. An understanding of how the cell cycle is controlled in cardiomyocytes could provide the basis for the development of therapies aimed at myocardial reconstitution [14-17]. Manipulating the function or level of molecules that either promote cell cycle entry or the down-regulation of molecules that inhibit cell cycle entry would be beneficial for repairing the damaged myocardium [14-17].

II) The cell cycle

1. Five phases

The cell cycle is a tightly regulated process by which a cell grows, duplicates its genomic DNA and separates into two identical daughter cells. The cell cycle includes five stages; three Gap phases (G-phases), the S-phase and the M-phase (Figure 1). Quiescent cells in the G_0 gap phase enter the G_1 phase following stimulation by mitogens or growth factors. During the G_1 phase, cells synthesize the mRNA and proteins required for duplicating their DNA during the S-phase. In the following phase, G_2, cells produce the mRNA and protein necessary for the M-phase (mitosis). Mitosis includes karyokinesis and cytokinesis, the separation of the DNA and the daughter cells respectively.

2. Cell cycle checkpoints

Transitions through the different phases of the cell cycle are regulated by several checkpoints. These checkpoints ensure that the environment is favorable for replication by assessing positive and negative signals. Also, they ensure the sequential order of events by establishing that each step is finished before the next step begins and that DNA is faithfully replicated [18, 19]. Transition past these checkpoints is controlled by positive and negative regulators. Positive regulation is provided by the association of cyclins with, and the activation of, their respective cyclin dependent kinases (CDKs) (see Figure 1). CDK levels are constant during the different phases of the cell cycle. However, the activity of these molecules is regulated by phosphorylation and dephosphorylation by CDK-activating kinases and phosphatases respectively [20-22]. Previously, it was believed that CDKs must bind cyclins in order to be activated. Cyclin protein levels are regulated by their synthesis, degradation and nuclear transport and they exhibit a cyclic expression pattern during the cycle. [3, 23]. The activated cyclin-CDK complexes then phosphorylate other effector molecules such as histones and the retinoblastoma (Rb) pocket-proteins [3]. Recent results suggest that CDKs can also be positively regulated by cell-type specific proteins other than cyclins. Examples of such proteins are the Speedy/Ringo proteins and p35 [24-27]. These proteins do not share any homology with the cyclins but can selectively bind specific CDKs and activate them independent of cyclin binding [25, 28].

For example, the association of cyclinD-CDK4 and cyclinE-CDK2 in G_1 leads to the phosphorylation of hypo-phosphorylated Rb. Hypo-phosphorylated Rb binds to and inactivates the E2F transcription factors. Phosphorylation of Rb disassociates the Rb-E2F

complex and the free E2F then binds and activates the promoters of genes needed for entry into S-phase [29].

Negative regulation of the cell cycle is provided by CDK inhibitors (CDKIs) such as $p21^{WAF1/CIP1}$ (referred to as p21 throughout the remainder of this thesis), $p27^{kip1}$ and $p57^{kip2}$. These molecules bind and inactivate the cyclin-CDK complex [30]. p21 was initially identified using cDNA subtractive hybridization of cells either expressing or not-expressing the transcription factor and tumor suppressor protein p53 [31]. Analysis of the *p21* promoter revealed that it contained two p53 consensus binding sites and electrophoretic mobility shift assays (EMSAs) showed that the p53 protein could indeed bind to these sites [31, 32]. Other transcription factors have also been shown to regulate *p21* expression. GATA-6 and Meox2 induce *p21* and subsequently induce a p21 dependent cell cycle arrest. However, they both act independently of p53 [33-35].

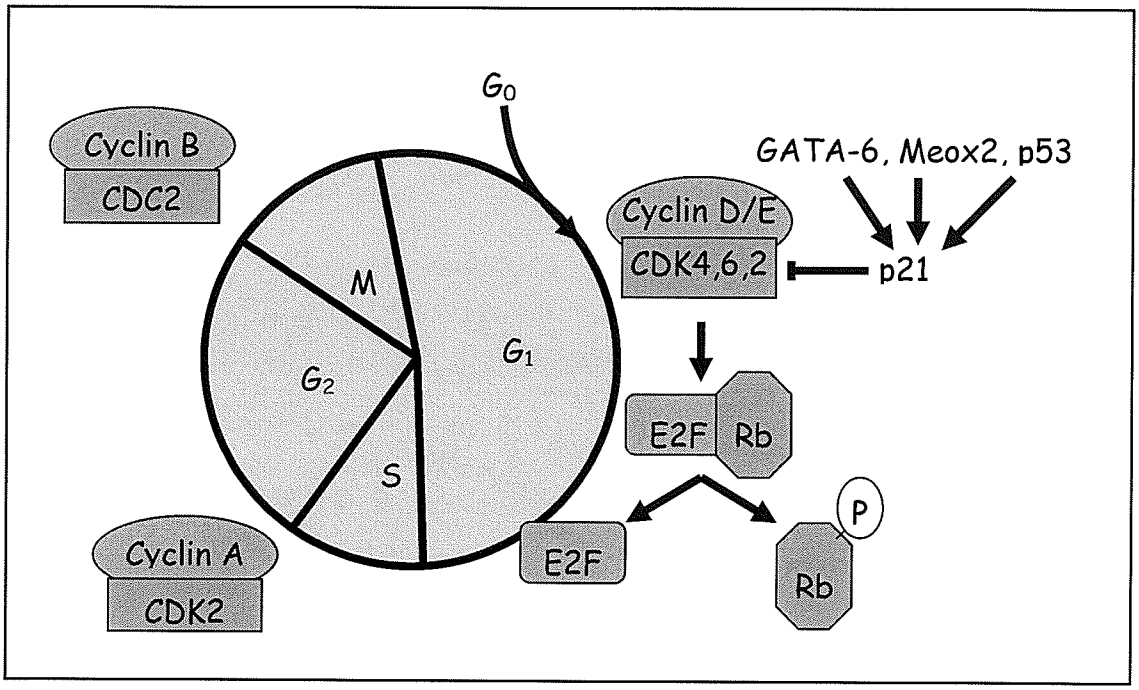

Figure 1: Cyclin regulation of cell cycle progression
Transition through the cell cycle is regulated by the association of specific cyclins with CDKs. Progression through the G_1 phase initially requires the interaction of cyclin D with CDKs 4 and 6 and later the association of cyclin E with CDK2. The p21 CDKI, which is induced by several transcription factors including Meox2, can block transition to the S phase of the cell cycle.

III) Homeobox Genes

1. Isolation and molecular characterization

Several genes, called homeotic genes, were found to cause transformations of one segment or organ to another in insects (homeotic transformations) [36-38]. Subsequent analysis revealed that these genes have a conserved protein coding sequence, the homeobox, which is also well conserved in orthologous genes in other species [39]. The homeobox, first described in 1984, codes for a 60 amino-acid segment (homeodomain) that is able to recognize and bind to specific DNA sequences [2, 39]. This conserved motif is composed of three major helices and a fourth small helix (Figure 2). Helices I and II are separated by a loop whereas helix II and III are separated by a turn [2]. The helix-turn-helix motif, formed by helices II and III, resembles a DNA bindng motif found in many prokaryotic proteins such as the lactose and tryptophan repressors [40, 41]. However, the overall structure of the homeodomain is unique to eukaryotes [42]. Helices III and IV lie within the major groove of the DNA double helix and make intermolecular contacts with the DNA [2, 42]. Conserved residues in this domain are important for the overall three dimensional structure whereas the non-conserved residues convey binding specificity [42]. As a result, homeodomain proteins interact with specific DNA operators through their homeodomain whereas this region and other regions of homeodomain proteins mediate protein-protein interactions with accessory proteins important for transcriptional regulation, nuclear import and protein turnover [42-46].

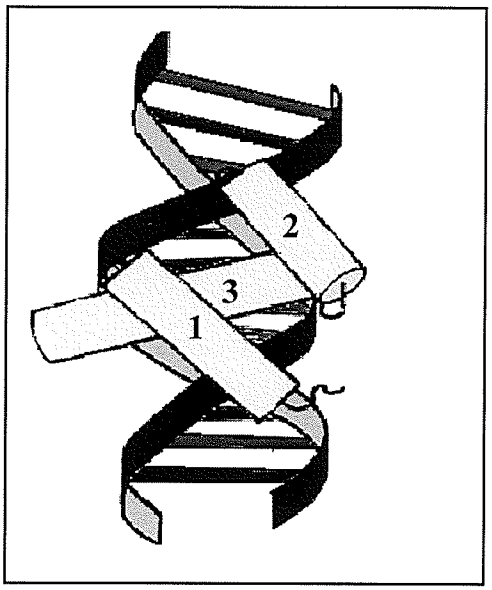

Figure 2: The Homeodomain
The homeodomain is a conserved motif composed of 4 helices. The structure includes a loop between helices 1 and 2 as well as a turn between helices 2 and 3. Helices 3 and 4 are separated by few residues and are often schematically drawn as a single helix, as in the adjacent diagram. Helices 3 and 4 lie in the major groove and interact with the DNA nucleotides [2].
(Image adapted from http://www.uic.edu/classes/bios/bios100/lecturesf04am/lect15.htm University of Illinois at Chicago)

2. Role of homeobox genes in human development and disease

Although homeobox genes were first identified in *Drosophila* [39] they have since been identified in fungi, plants and other eukaryotes [2]. In vertebrates, many homeobox genes are localized in genomic arrays called Hox clusters. Humans and other vertebrate species have four Hox clusters (A-D) that each contain 9-11 genes [47, 48]. The expression pattern of these genes along the anterior-posterior body axis of many different organisms (humans, fly, mice, zebrafish, etc) is collinear with their position in the genome [48]. Non-clustered homeobox genes (Para Hox) form families that are characterized by similarities within their homeodomain sequences as well as the presence of conserved domains flanking the homeodomain [49]. Examples of these families include the *Lim, Pax* and *Six* families that contain LIM, paired box (PAX) and SIX domains, respectively, in addition to their homeodomains [43, 49].

3. Cellular role of homeobox genes

Homeodomain proteins regulate many diverse cellular processes including differentiation, proliferation, apoptosis, migration and overall body patterning [47, 50]. Deregulated homeodomain protein expression has been implicated in different pathological conditions such as vascular disease and the development/progression of cancer [47, 51, 52]. Alterations in homeodomain protein expression can take many forms including the reactivation of developmental genes, the silencing of genes usually expressed in the adult and the activation of genes not normally expressed in a given tissue [49].

IV) Mesenchyme homeobox genes

1. Conservation of *Meox* genes during evolution

Murine *Meox1* (Mesenchyme Homeobox Gene), the first orthologue identified, encodes a 254 amino acid protein [53]. *Meox2,* the only other *Meox* homeobox family member identified, is slightly larger at 303 amino acids and has a predicted molecular weight of 33.6 kDa [54]. These proteins are 95% identical and 100% similar in their homeodomain sequences but have significantly diverged in regions which surround their homeodomains (Figure 3) [53, 54]. The *Meox1/2* genes share a similar genomic organization which includes three exons and two introns and they are not linked to any of the Hox clusters [53-55]. Meox2 also contains a region rich in histidine and glutamine residues known as a CAX repeat (Figure 4) [54, 55]. Similar sequences, also called *opa* sequences, have been shown to be important in the transactivation function of other transcription factors such as HB24, Notch and HOX-11, although its function in Meox2 is still unknown [56-58]. In our lab we have found that Meox2 is a stronger transcriptional