MESENCHYME HOMEOBOX 2 REGULATION OF FETAL ENDOTHELIAL PROGENITOR CELL FUNCTION

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DEDICATION

"Here's to strong women. May we know them. May we be them. May we raise them." –Unknown

To my mother, Cynthia J. Bender Palmer, PhD, for instilling in me from an early age that women can be successful scientists, and supporting me on my journey to become one. From playing dress-up in lab coats and measuring birdseed consumption to defending my doctoral dissertation, you have believed in and encouraged me along each step of the way. While others may cringe at turning out *just* like their mother, I could not be more proud or feel more fortunate to be following in your footsteps. With all of my love and admiration, I dedicate this work to you.

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Cassandra R. Gohn

MESENCHYME HOMEOBOX 2 REGULATION OF FETAL ENDOTHELIAL PROGENITOR CELL FUNCTION

In the United States, 10% of pregnancies are complicated by diabetes mellitus (DM). Intrauterine DM exposure can have long-lasting implications for the fetus, including cardiovascular morbidity. Previously, we showed that fetal endothelial colony forming cells (ECFCs) from DM pregnancies have decreased vessel-forming ability and increased senescence. However, the molecular mechanisms responsible for this dysfunction remain largely unknown. The objective of this thesis was to understand how Mesenchyme Homeobox 2 (MEOX2) interacts with pathways that regulate cell cycle progression and migration, and how this interaction results in impaired vasculogenesis in DM-exposed ECFCs.

We tested the hypothesis that upregulated MEOX2 in DM-exposed ECFCs decreases network formation through impairments in senescence, cell cycle progression, migration, and adhesion. MEOX2 is a transcription factor which inhibits angiogenesis by upregulating cyclin dependent kinase inhibitors. Here, data show that nuclear MEOX2 is increased in DM-exposed ECFCs. Lentiviral-mediated overexpression of MEOX2 in control ECFCs increased network formation, altered cell cycle progression, increased senescence, and enhanced migration. In contrast, MEOX2-knockdown in DM-exposed ECFCs

decreased network formation and migration, while cell cycle progression and senescence were unchanged.

Adhesion and integrin expression defects were evaluated as mechanisms by which MEOX2 altered ECFC migration. While MEOX2-overexpression did not alter adhesion or cell surface integrin levels in control cells, MEOX2 overexpression in DM-exposed ECFCs resulted in an increase in α6 integrin surface expression. Similarly, MEOX2-knockdown in DM-exposed ECFCs did not alter adhesion, though did reduce α6 integrin surface expression and total cellular α6 mRNA and protein levels.

Together, these data suggest that alterations in cell cycle progression and senescence are not responsible for the disrupted vasculogenesis of DM-exposed ECFCs. Importantly, these data suggest that altered migration may be a key mechanism involved and that altered cell surface levels of the α6 integrin may modify migratory capacity. Moreover, these data suggest that the α6 integrin may be a previously unrecognized transcriptional target of MEOX2. Ultimately, while initially believed to be maladaptive, these data suggest that increased nuclear MEOX2 in DM-exposed ECFCs may serve a protective role, enabling vessel formation despite exposure to a DM intrauterine environment.

Laura S. Haneline, MD, Chair

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LIST OF ABBREVIATIONS

BEC	Brain endothelial cell
bFGF	Basic fibroblast growth factor
BMI	Body mass index
Cdc42	Cell division cycle protein 42
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
ChIP	Chromatin immunoprecipitation
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle Medium
EBM-2	Endothelial Basal Media-2
ECFC	Endothelial colony forming cell
ECM	Extracellular matrix
EGM-2	Endothelial Growth Media-2
FCS	Fetal calf serum
GFP	Green fluorescent protein
HPRT	Hypoxanthine phosphoribosyltransferase
HUVEC	Human umbilical vein endothelial cell
MEOX2	Mesenchyme homeobox 2
MNC	Mononuclear cell
MOI	Multiplicity of infection
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

PBS	Phosphate buffered saline
PLAC8	Placenta-specific 8
Rac-1	Ras-related C3 botulinum toxin substrate 1
Rb	Retinoblastoma
RIPA	Radio-immunoprecipitation assay
RT-PCR	Real-time reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
shRNA	Short hairpin RNA
siRNA	Short-interfering RNA
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
UCB	Umbilical cord blood
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

CHAPTER 1: INTRODUCTION

Overview of Diabetes Mellitus

Obesity and diabetes epidemic

Currently, the United States and several other countries worldwide are suffering from an obesity epidemic (Fig. 1.1). This trend toward increased body mass index (BMI) affects individuals of all ages (1). Further, this increase in obesity is tied to an increase in the occurrence of type 2 diabetes mellitus (DM) (2). The American Diabetes Association defines diabetes as “a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (3).” According to the Center for Disease Control’s National DM Statistics Report for 2014, 29.1 million Americans, or 9.3% of the American population, have DM. This percentage can be further broken down into the 21 million diagnosed patients (Fig. 1.2), and the perhaps more alarming, 8.1 million undiagnosed cases. Additionally, it is estimated that in 2012, 86 million Americans 20 years and older suffered from prediabetes. In 2012, there were 1.7 million new DM cases diagnosed, demonstrating that the occurrence of this disease continues to increase at an alarming rate (4).

Clinically, there are two common types of DM, which account for the majority of American cases. Type 1 DM (T1DM) makes up 5-10% of cases and is characterized by an autoimmune destruction of pancreatic β-cells. Without these insulin-producing cells, insulin production ceases, and patients ultimately become dependent upon exogenous insulin for survival. Type 2 DM (T2DM), accounts for 90-95% of cases, and results from the progressive loss of insulin sensitivity in

liver, adipose, and skeletal muscle tissues. This loss of insulin sensitivity results in elevated fasting plasma glucose levels. To compensate for this hyperglycemic state, pancreatic β-cells increase insulin secretion, often resulting in a hyperinsulinemic state, which is characteristic of T2DM patients. Over time, this increased demand for insulin secretion results in β-cell exhaustion, and a partial, or often complete, failure of insulin production (3, 5, 6).

Clinical complications of DM extend far beyond states of hyperglycemia and hyperinsulinemia. DM patients are predisposed to developing microvascular and macrovascular complications, which often manifest in conditions of hypertension, heart disease, stroke, retinopathy, and kidney disease, among other complications (4, 7). Additionally, in 2012, an estimated 246,000 American deaths were attributed to DM (8). The economic burden of these complications is substantial. In 2012, an estimated $245 billion dollars were spent as either direct or indirect costs of DM (4, 8). It is evident that this disease poses numerous life-threatening risks to patients, and that this epidemic is of great clinical and economic distress.

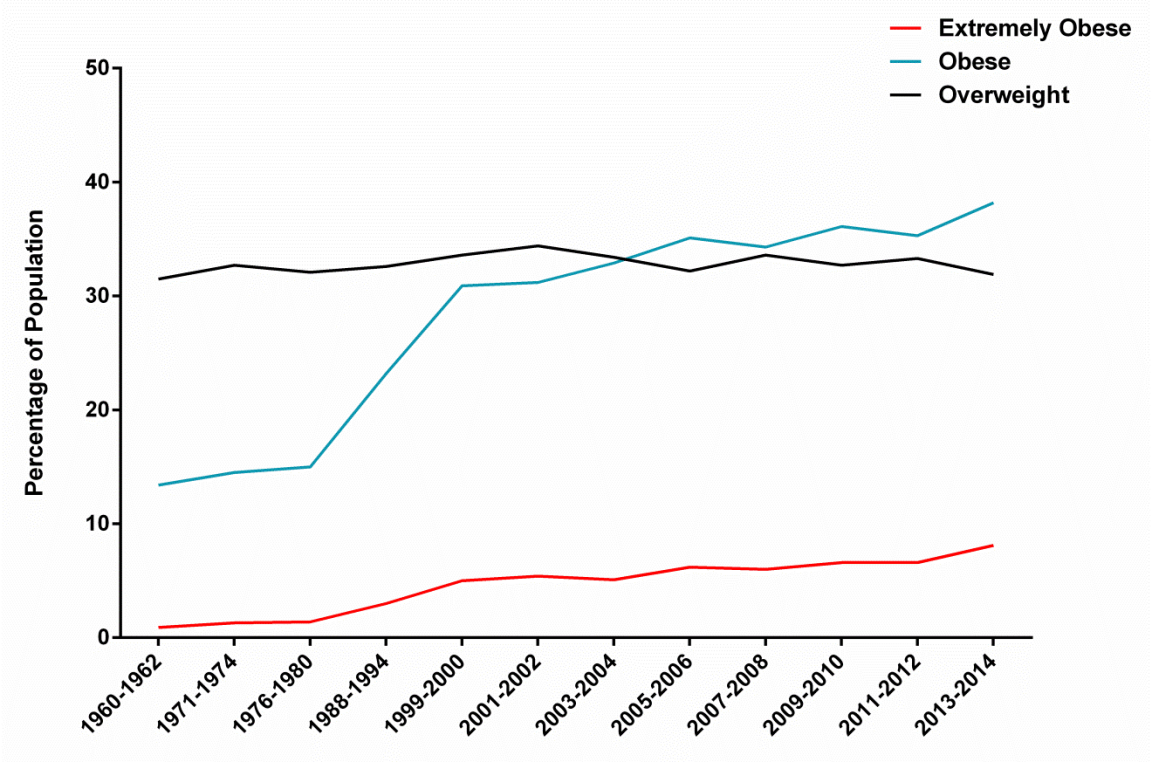


Figure 1.1. The rate of overweight and obese Americans is increasing. There has been an increase in both overweight and obese Americans aged 20-74 years between 1960-2014. Notably, obesity increased from 15% in 1976-1989, to 30.9% in 1999-2000. Red line indicates extremely obese individuals (BMI ≥ 40.0). Blue line indicates obese individuals (BMI ≥ 30.0). Black line indicates overweight individuals (BMI 25.0-29.9). Adapted from Fryar et al. (9).

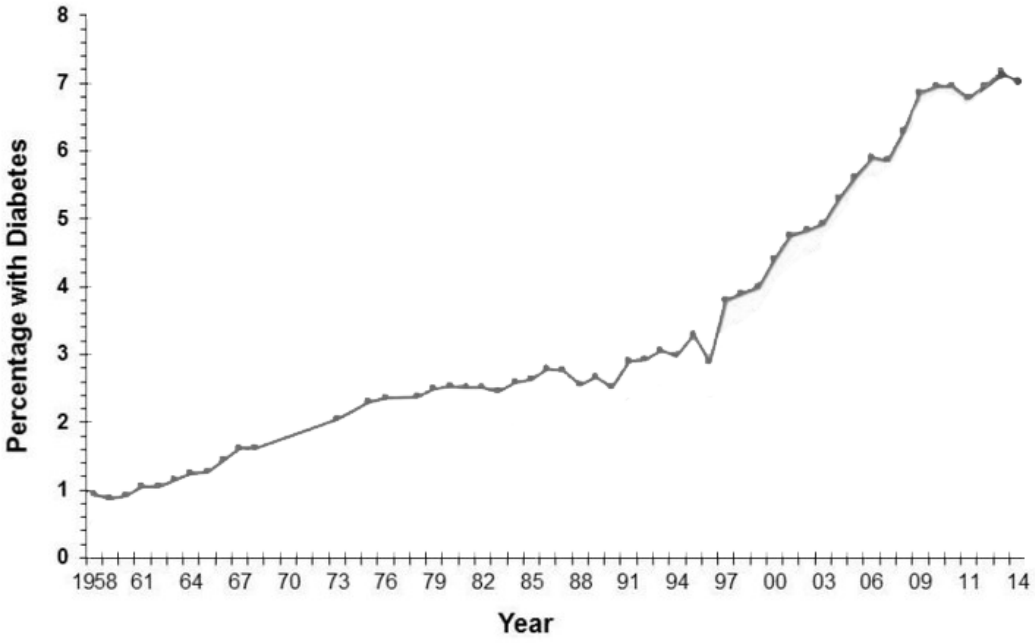


Figure 1.2. The rate of DM is increasing in the United States. There has been an increase in the percentage of the American population diagnosed with DM between 1958-2014. The rate of diagnosed diabetes increased from 0.93% in 1958 to 7.02% in 2014. Adapted from the National Diabetes Statistics Report 2014 (4).

Adverse fetal effects caused by maternal DM during pregnancy

Obesity and DM in women of childbearing years is of particular concern. It is now estimated that 33% of pregnant mothers are obese (1). Further, as many as 10% of pregnancies are complicated by DM (10). Hyperglycemia associated with this disease poses a risk to not only afflicted mothers, but children as well. DM during pregnancy often results in fetal exposure to a hyperglycemic intrauterine environment, as glucose readily crosses the placenta, but maternal insulin is unable to traverse this barrier (10). The fuel-mediated toxicity hypothesis is now widely accepted and explains that when increased metabolic nutrients (fuels) are passed from the mother to the fetus, there can be devastating teratogenic effects (11). Specifically with intrauterine exposure to hyperglycemia, the fetal pancreas produces additional insulin to handle the excess glucose loads (10). States of hyperglycemia, followed by hyperinsulinemia can lead to a multitude of adverse fetal effects that are immediately recognized upon delivery. Depending upon the developmental stage at exposure, the fetus can suffer consequences including congenital anomalies (12-15), prematurity (14, 16, 17), macrosomia (14, 18-21), respiratory distress (14, 22, 23), and hypoglycemia (14, 24, 25).

Extending beyond the immediate neonatal effects, there can be long-term consequences of a DM intrauterine environment (26-33). According to the Barker hypothesis, also termed developmental programming, changes in the intrauterine environment during crucial developmental timeframes can lead to alterations in anatomical structure and physiological responses to stimuli (34, 35). These

alterations, in turn, can result in a predisposition to disease development throughout the lifespan of offspring from DM pregnancies (36). Studies show that children of DM mothers are more likely to develop the metabolic syndrome, insulin resistance, type 2 DM, obesity, and high blood pressure (28, 37-46). The long-term effects of intrauterine exposure to diabetes is now also an area with increasing research interest, as well as clinical concern, as both the immediate and long-term consequences of fetal exposure to DM contribute to poor prognoses and increased chronic diseases that significantly impede the quality of life for afflicted individuals.

Some of the most convincing evidence regarding the adverse effects of exposure to a DM environment *in utero* stems from studies conducted in the Pima Indian population. Interestingly, this population has an exceptionally high rate of T2DM. Studies revealed that offspring born to mothers suffering from DM during pregnancy had a six-fold increased risk of developing T2DM during adolescence when compared to individuals born to non-DM mothers. In contrast, the rates of juvenile T2DM among children born to non-DM were negligible (Fig. 1.3) (44). Additionally, children born to DM mothers had an increased prevalence of obesity and higher systolic arterial blood pressure (39, 45) compared to individuals born to non-DM mothers. Apart from the DM intrauterine environment, some have argued that these adverse effects could be linked to genetics. However, evidence from the Pima Indians seems to argue in favor of the intrauterine environment. This population has a very homogenous genetic background. Further, the rates of DM are higher in offspring of DM mothers when

compared to offspring whose fathers had DM (44). Although the existence of a genetic predisposition for DM has been well established (47), these results suggest that environmental factors also play a critical role in the development of DM in the Pima Indian population.

There is also compelling evidence regarding the devastating effects of intrauterine exposure to DM outside of the Pima population. A longitudinal study examining white children at 6, 7, 9, and 11 years of age found that large for gestational age offspring of DM mothers are predisposed to develop the metabolic syndrome. Among these children, 50% were at risk for developing the metabolic syndrome, and an additional 15% met diagnostic criteria for the disease (38). Further, a record-linkage study of Swedish men reported that *in utero* exposure to DM resulted in higher offspring BMI at age 18 years. However, siblings born during healthy pregnancies did not have this increased BMI, suggesting that the intrauterine environment strongly affects long-term health outcomes (48). Finally, the relationship between a DM intrauterine environment and blood pressure in a mixed population of European, African, and Hispanic descent at 10-16 years of age was examined. Results indicated that the offspring of DM mothers have both higher systolic and mean arterial blood pressure, while diastolic blood pressure remained unchanged (40). Taken together, these studies suggest that the intrauterine environment impacts long-term offspring health. Further, exposure to a DM intrauterine environment predisposes offspring to develop chronic metabolic and cardiovascular complications, which can be recognized during adolescence. While there is compelling evidence of the origin

of these diseases, the molecular mechanisms responsible for the pathophysiology, remain largely unknown.

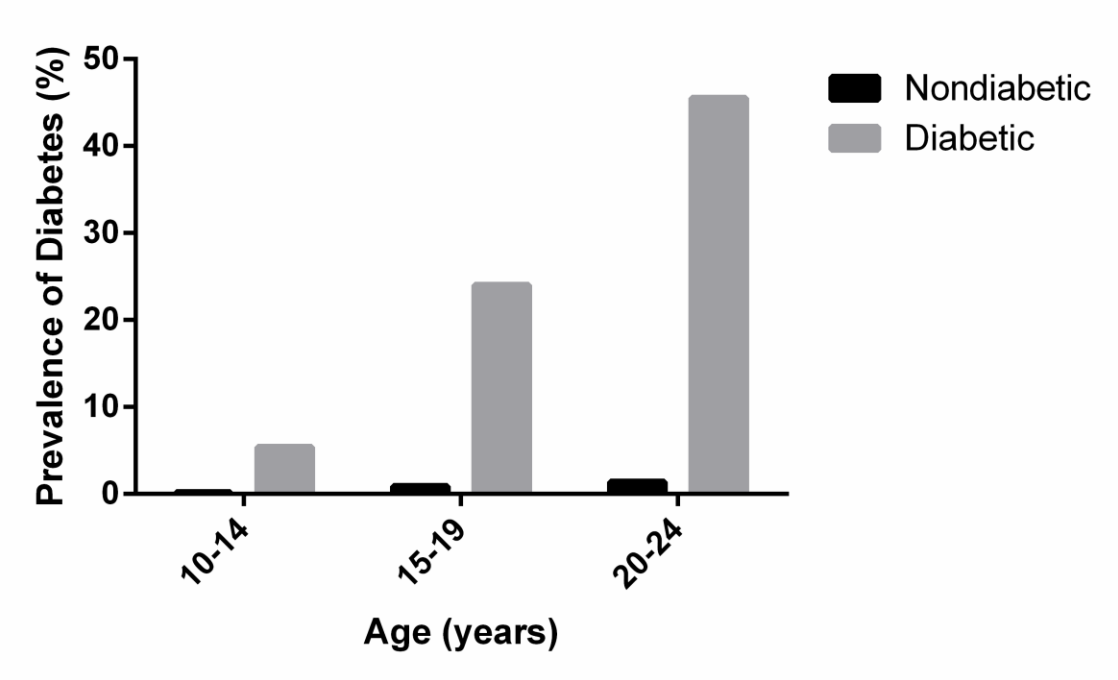


Figure 1.3. Pima Indian children born to DM mothers have a greater prevalence of DM than those born to non-DM mothers. Children born to diabetic mothers are more likely to be diagnosed with T2DM at each age range than those born to non-DM mothers. Black bars represent non-DM children. Grey bars represent DM children. Adapted from Pettitt et al. (44).

Endothelial Colony Forming Cell Model

Endothelial colony forming cells

The overarching hypothesis of this thesis is that many of the cardiovascular complications experienced by children of DM mothers stem from the inability of the child's stem and progenitor cells to maintain vascular health either by efficiently repairing damaged blood vessels or forming new vessels. Endothelial colony forming cells (ECFCs) are one of the progenitor cell subsets involved in these processes (49, 50). ECFCs are an endothelial progenitor population that circulate in peripheral and umbilical cord blood (UCB) (49-51). While this population of cells may be harvested from either source, ECFCs are enriched in UCB at 33-36 weeks gestational age, and this enrichment is maintained throughout the remainder of the pregnancy (51). Additionally, higher numbers of endothelial progenitors are found in UCB compared to adult peripheral blood (49, 52, 53). A hierarchy of ECFCs exists, and UCB has a larger proportion of high proliferative potential ECFCs (49, 54, 55). This population allows ECFCs from UCB to expand *ex vivo*, forming larger colonies with a higher proliferation rate, and forming network structures on matrigel matrix more quickly than ECFCs isolated from adult peripheral blood (49, 55). Given these data, UCB is an ideal source from which to harvest ECFCs. While ECFCs are utilized as a form of regenerative therapy (56, 57), it is our objective to extend their use. We aim to use these cells to better understand the pathophysiology behind the maladaptive phenotypes observed in children exposed to intrauterine DM.

Negative impact of a DM intrauterine environment on fetal ECFCs

Upon recognition that exposure to a DM intrauterine environment resulted in several adverse long-term clinical manifestations, research studies began to focus on the cellular mechanisms involved in these disorders. Of particular interest to our laboratory is the predisposition for hypertension experienced by individuals whose mothers had T2DM during pregnancy (39, 40, 45). Early studies from the Haneline laboratory indicate that ECFCs from uncomplicated pregnancies exposed to hyperglycemic conditions (10 and 15mM dextrose) display a dysfunctional phenotype (58). Specifically, when grown *in vitro* for 7 days under hyperglycemic conditions, ECFCs exhibit diminished colony formation resulting from increased senescence and decreased proliferation (58). Additionally, hyperglycemia also decreased *in vitro* network formation on a matrigel matrix composed largely of laminin and collagen IV, indicating reduced ECFC functional capacity (58).

Because ECFCs exposed to acute hyperglycemic conditions exhibited profound dysfunctional phenotypes, the Haneline laboratory speculated that ECFCs exposed to intrauterine DM over the course of many months would display a similar, if not more severe pathophysiology. To test this hypothesis, ECFCs from DM human pregnancies were used to examine senescence, proliferation, and network-forming ability. Interestingly, ECFCs from DM pregnancies have decreased proliferation and increased senescence *in vitro* (58), suggesting DM exposure alters ECFC cell cycle progression. Further, ECFCs from DM pregnancies also exhibited reduced network formation *in vitro*

(59). Subsequent studies evaluated ECFC functional capacity *in vivo*. Using a xenograft transplantation assay, cellularized gel plugs containing either ECFCs from control or DM pregnancies were implanted into the flanks of immunodeficient mice. After 14 days, the plugs were removed and analyzed for vessel formation using anti-human CD31 staining. These studies indicated that ECFCs from DM pregnancies formed two-fold fewer chimeric vessels compared to ECFCs from uncomplicated pregnancies. Together these data demonstrated that ECFCs from DM pregnancies have significantly impaired vasculogenic function (58).

Due to the numerous long-term adverse effects of intrauterine exposure to DM, a need exists to elucidate the molecular mechanisms involved. Currently, our laboratory aims to identify cellular and molecular alterations in ECFCs from DM pregnancies, which have the potential to be used as biomarkers to identify at-risk individuals or therapeutic targets to correct clinical ailments.

The role of integrins in cellular adhesion and migration

Cellular migration is a result of complex, direct interactions between a cell and the extracellular matrix (ECM) and environmental cues such as cytokines and chemokines (60). This physical contact between a cell and the ECM occurs through dynamic signaling proteins known as integrins (60-62). Integrins are composed of heterodimers that contain an α-subunit and a β-subunit. Each subunit has an extracellular and a cytoplasmic domain (Fig. 1.4) (61, 63, 64). The extracellular domain binds specific motifs within ECM proteins, allowing the cell to adhere to the ECM. The cytoplasmic domain is responsible for signaling to

cytoskeletal proteins and inducing changes in signaling cascades to regulate actin polymerization and initiate migration (61-63).

Integrin-mediated adhesion is a dynamic process. For an endothelial cell to migrate, adhesion complexes must temporarily form, but also break down, allowing for motility through detachment from the ECM (60, 61, 65-67). In endothelial cells, this process is largely regulated by the activation of small Rho GTPases. During a process known as cellular polarization, chemotactic and cytokine stimuli initiate a local activation of signaling cascades to establish a leading edge of a cell (60, 68). In endothelial cells, these stimuli include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiopoietins, fibroblast growth factor-2, hepatocyte growth factor, platelet-derived growth factor, epidermal growth factor, transforming growth factor-β (TGF-β), interleukins, tumor necrosis factor-α (TNF-α), platelet-activating factor, ephrins, soluble adhesion molecules, endoglin, and angiogenin (60). At the leading edge of a polarized cell, active cell division cycle protein 42 (Cdc42) induces the formation of filipodia, which sense the above listed migration stimuli (60, 61, 65-67). The cell next sends out extensions through active Ras-related C3 botulinum toxin substrate 1 (Rac-1)-dependent formation of lamellipodia (60, 61, 65-67). These protrusions attach to the ECM through integrin interactions, leading to contraction of the cell body (60, 61, 65-67). Phosphatases then cause the trailing edge of the cell to detach from the ECM, and the cell moves forward (Fig. 1.5) (60, 61, 65-67).

Migration is a critical step in establishing vascular networks. Migration of endothelial cells is essential during developmental vasculogenesis, but also for blood vessel repair and angiogenesis (56). During wound repair, endothelial cell monolayers compose the luminal surface of blood vessels. When this cell layer is disrupted, new endothelial cells migrate to repair the damaged area. Often, this process is mediated by growth factors such as VEGF and bFGF. Release of these growth factors at the site of injury results in endothelial cells bordering the wound to migrate into the injured area. This migration is a result of the growth factors binding directly to the integrins present on the endothelial cells, initiating the process of cellular polarization (60, 61, 65-67, 69, 70). Additionally, the growth factors may also bind to their specialized receptors present on the endothelial cell, leading to activation of intracellular signaling cascades. These growth factor mediated-cascades cross-talk with the integrin signaling cascades, resulting in polarization and cellular migration as described above (69, 70). These leading endothelial cells transmit the migratory signal to cells located further from the leading edge, and effectively direct a sheet migration (68, 71).

In normal physiology, most endothelial cells are quiescent (60, 68). However, when a tissue is deprived of nutritional content or exposed to hypoxic conditions, and therefore not adequately supported by pre-existing blood vessels, new vessels form (60, 68). During this process of angiogenesis, VEGF is produced by endothelial and vascular smooth muscle cells. This potent chemokine modulates the Notch/Dll4 pathway and directs endothelial cell migration via paracrine pathways. Ultimately, activation of the Notch/Dll4

pathway leads to the initiation of a tip cell, which directs the newly sprouting vessel. Other stalk cells follow behind the tip cell, creating a mature vessel (60, 68).

Endothelial cells express a wide variety of laminin-binding integrins which enable cellular adhesion and migration, including α1, α2, α3, α6, α7, β1, and β4 (64, 72). Each heterodimer has a specific location and function within the vasculature. For example, endothelial cells located in large blood vessels and the microvasculature express α2β1,α3β1, and α6β1, while α1β1 is only found in small vessels (72-77). The α6β4 heterodimer is located at the basement membrane of small vessels (72, 77, 78), while the α2β1 heterodimer is located on the cellular borders of the endothelium, and is believed to be responsible for regulating endothelial lining permeability (72, 75). The heterodimers α1β1, α2β1, and α6β1 have been implicated in *in vitro* network formation and *in vivo* angiogenesis (72, 79-82).

While each integrin subunit is of great importance to endothelial physiology, of particular interest is α6, which is known to participate in endothelial cell adhesion, migration, and network formation (83-85). Specifically, in ECFCs, a reduction in α6 expression decreased adhesion, migration, and network formation *in vitro*, as well as reduced reperfusion, capillary density, and ECFC incorporation into microvasculature in a murine hind limb ischemia model (85). Further, expression of the α6 integrin in endothelial cells is known to be regulated by transcription factors, as well as growth factors and inflammatory cytokines (86, 87). In CD34+ endothelial progenitor cells, α6 expression was upregulated by

VEGF (84). Conversely, in mature endothelial cells, TNF-α and interleukin 1β reduced α6 integrin expression (83). Taken together, these data demonstrate a critical role of α6 integrin expression in endothelial cell function.

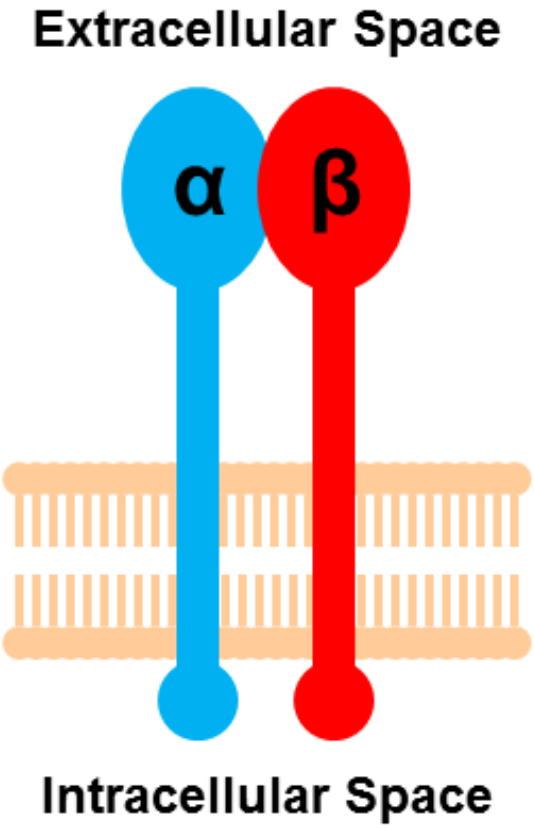


Figure 1.4. Integrins are composed of heterodimers. Each integrin heterodimer contains an α-subunit and a β-subunit. Each subunit contains an extracellular domain, which binds ECM proteins, and a cytoplasmic domain, which is responsible for directing intracellular signaling cascades.

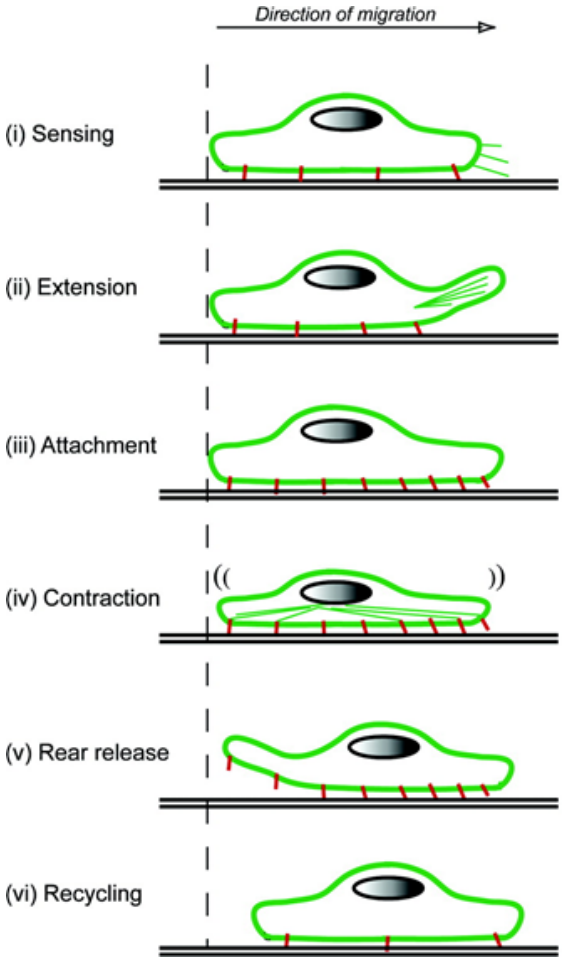


Figure 1.5. Major steps of endothelial cell migration. Endothelial cell migration occurs in 6 sequential events: i) Cdc42-dependent filipodia sense stimuli; ii) Rac1-dependent formation of lamellipodia resulting in forward cell extension; iii) Attachment of the lamellipodia to the ECM; iv) Contraction of the cell body enabling forward momentum; v) Release of ECM at rear of the cell; and vi) Recycling of the adhesive and signaling structures. Adapted from Lamalice et al. (60).

The role of Mesenchyme Homeobox 2 (MEOX2) as a Transcription Factor

MEOX2 regulates cell cycle progression

RNA microarray data from the Haneline laboratory suggested that the gene *MEOX2* was differentially expressed in ECFCs from control and T2DM pregnancies. Following literature review, MEOX2 was considered a potentially interesting protein for subsequent studies. MEOX2 is a transcription factor that promotes senescence through both direct binding to the promoter regions of cyclin dependent kinase inhibitors (CDKIs), such as p16 and p21, and DNA-binding independent mechanisms (88, 89). Cellular senescence is a state in which dividing cells exit the cell cycle and proliferation ceases. Normal cell cycle progression is regulated by phosphorylation events mediated by cyclin dependent kinases (CDKs) (90). However, during cellular senescence, CDKIs bind to the CDKs and prevent them from associating with cyclin proteins (91). This results in the inhibition of cyclin dependent kinase activation, preventing the phosphorylation of the retinoblastoma (Rb) protein (90, 91). Without this crucial phosphorylation step, G1/S phase progression is prevented (90, 91). As a result, cell cycle progression is halted and cells are unable to proliferate (Fig. 1.6) (91).

When MEOX2 was overexpressed in human umbilical vein endothelial cells (HUVECS), the homeodomain region of MEOX2 bound directly to the homeodomain binding site in the p16 promoter (88). The mechanism by which MEOX2 regulates p21 is not as clear. One study showed that MEOX2 upregulation of p21 occurs in a DNA binding-independent manner (88). However, other studies demonstrated that when MEOX2 was overexpressed in HUVECs

that MEOX2 bound directly to an AT-rich site in the p21 promoter (92). Irrespective of the mechanism, however increased MEOX2 expression enhanced senescence (88) and decreased proliferation (89) in these studies.

Another study sought to identify novel downstream targets in MEOX2-overexpressing HUVECs by conducting an RNA microarray. This study revealed that MEOX2 regulates a multitude of downstream proteins, including additional CDKIs, p19 and p57 (93). While these targets have yet to be verified, they are known to act in a similar manner as p16 and p21 (94). Further, the microarray data revealed that MEOX2 also regulates genes involved in cell adhesion, chemotaxis, and signal transduction, including β integrin subunits (93). Verification of these targets could lead to the identification of novel pathways regulated by MEOX2 and enable a more complete understanding of the mechanistic function of this protein.

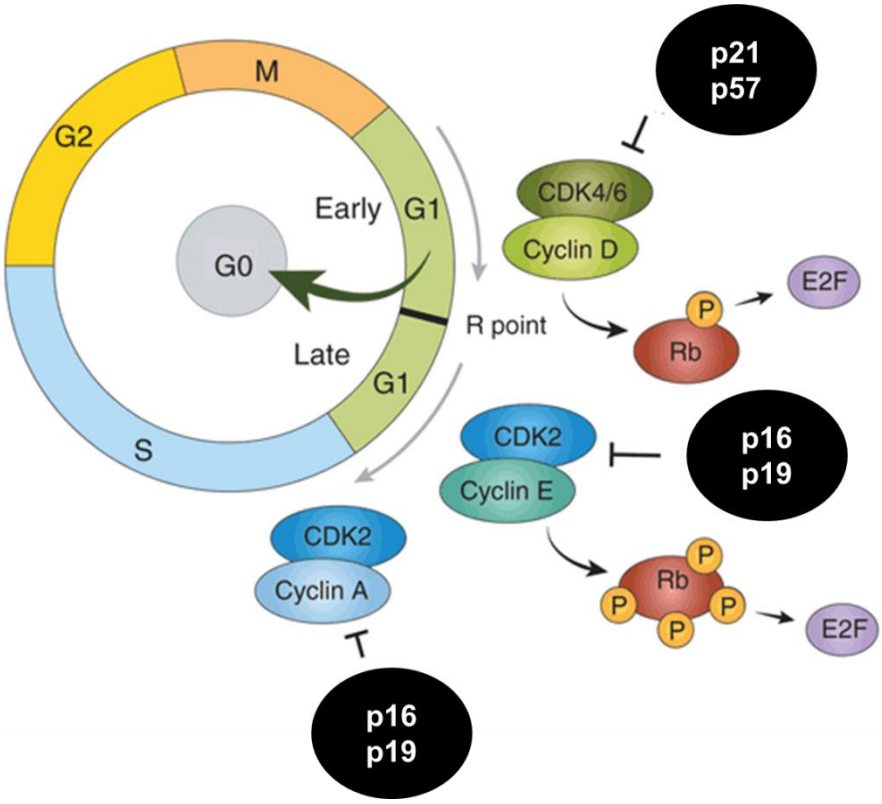


Figure 1.6. Regulation of the cell cycle. Cells enter S phase by activating CDK4/6 and CDK2. Activation occurs when CDKs complex with cyclin proteins. In G1 phase, CDK4/6-cyclin D complex is activated, and thus deactivates Rb by phosphorylation. Next, E2F factors regulate expression of pro-proliferative signals including cyclin D, and allow cells to enter S phase. Activation of CDK2-cyclin E complex causes the hyperphosphorylation of Rb, resulting in a full release of E2F. Expression of the E2F target gene, cyclin A, mediates progression through S phase. CDKIs including p16, p19, p21, and p57 inhibit the activity of CDKs. Adapted from Strauss et al. (91).

MEOX2 regulates migration

In addition to cell cycle regulation, a limited number of studies have examined the role of MEOX2 in cellular migration. Some evidence in vascular smooth muscle cells (VSMCs) demonstrated that MEOX2 inhibits migration in a dose-dependent manner, though the molecular mechanism was not investigated in detail. In VSMCs from both rabbits and rats, a higher multiplicity of infection (MOI) of viral particles containing a MEOX2 construct led to decreased migration in scratch wound healing and transwell migration assays (95, 96). Further, in HUVECs, increasing adenoviral transduction of both human and rat MEOX2 constructs decreased migration at increasing MOIs (93). While these studies provided interesting initial insights into a potential link between MEOX2 expression and migration, limitations exist. Neither study presented data confirming MEOX2 overexpression at the protein level. While these studies did utilize viral infection methods, without confirmation of protein overexpression, it is difficult to determine the merit of the functional readouts presented in the later assays. It is evident that additional studies of both MEOX2 overexpression and knockdown must be conducted to assess the regulatory role of MEOX2 in migration. Work in this thesis directly addresses this current knowledge gap.

MEOX2 regulates vessel-formation

Reports in the literature show mixed results regarding the role of MEOX2 in vessel-formation. In HUVECs, MEOX2 overexpression was reported to be antiangiogenic (89, 92, 93, 97). For example, when HUVECs were transduced with an adenovirus expressing MEOX2, VEGF-induced network formation

decreased in a dose-dependent manner with increasing MOI compared to GFP-expressing controls. These data suggest that higher levels of MEOX2 result in reductions in network formation (89). Additional studies in HUVEC revealed that MEOX2 inhibits nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling via direct interaction with p65, a subunit of NF-κB. These studies demonstrated that MEOX2 binds to p65 in the nucleus through its homeodomain, and prevents p65 from binding to its consensus sequence. This repression of the NF- κB signaling cascade leads to decreased network formation in response to growth stimuli (92, 93).

Conversely, in human and mouse brain endothelial cells (BECs), MEOX2 overexpression achieved by adenoviral infection up to 100 MOI positively correlated with network formation in an MOI dose dependent manner. At higher levels (500 MOI), MEOX2 had no effect on network formation. Further, MEOX2 depletion in human BECs reduced network formation *in vitro*, while *MEOX2* heterozygous mice displayed reduced capillary length and hypoxia-induced angiogenesis *in vivo*. In each of these studies, MEOX2 expression was confirmed at the protein level (98). Taken together, these data suggest that there are differences in the function of MEOX2 across cells types. Additionally, dosage affects the function of MEOX2. However, it is evident that MEOX2 does participate in regulating angiogenesis both *in vitro* and *in vivo*.

Finally, MEOX2 impacts angiogenesis and vascular remodeling in a variety of additional cell types. In adventitial fibroblasts, MEOX2 overexpression reduced vascular remodeling through decreased inflammatory cytokine

expression (99). MEOX2 expression also reduced perivascular adipocyte proliferation and differentiation through decreased focal adhesion kinase and extracellular signal-related kinase 1/2 activities and increased p53 signaling, inducing apoptosis (100, 101). In epithelial cells, MEOX2 interacted with TGF-β signaling molecules to contribute to the antiangiogenic response of this pathway. Specifically, MEOX2 and TGF-β synergized to maximally inhibit proliferation, an effect that was greater than that of either protein individually (102).

MEOX2 involvement in a multitude of other processes

In addition to participating in cell cycle regulation and vessel-formation, MEOX2 is involved in a myriad of other processes. Early studies focused on the role of MEOX2 in development and found a requirement for MEOX2 in palate development (103, 104) and limb myogenesis (105, 106).

The role of MEOX2 in cancer progression seems to be tissue-specific. High MEOX2 expression is linked to poor survival in individuals with non-small cell lung cancer. Specifically, in these lung tumors, the promoter sequence of MEOX2 displayed lower expression of repressive histone markers despite comparable levels of DNA methylation in noncancerous tissues. Additionally, lung tumors had higher levels of MEOX2 protein. Although the exact mechanism is still uncharacterized, MEOX2 expression correlated with increased chemoresistance and ultimately decreased survival (107). However, in hepatocellular carcinoma, MEOX2 expression had the opposite effect on survival. Analysis of cancerous tissues revealed that MEOX2 mRNA and protein expression were lower than in noncancerous samples. Further, correlation

analysis of MEOX2 expression and clinical features revealed that lower MEOX2 levels were associated with increased tumor invasion and ultimately poor survival rates (108). Together, these data suggest that although MEOX2 is involved in multiple physiological and pathophysiological processes the specific role of MEOX2 varies across cell and tissue type.

Hypothesis and Aims

Hypothesis: Upregulated MEOX2 in ECFCs from DM pregnancies decreases network formation through impairments in senescence, cell cycle progression, migration, adhesion, and integrin signaling.

Aim 1: Determine whether MEOX2 is increased in ECFCs from DM pregnancies.

Aim 2: Determine whether MEOX2 regulates senescence, cell cycle progression, network formation, and migration of ECFCs.

Aim 3: Determine whether MEOX2 regulates migration through differential integrin expression in ECFCs from DM pregnancies.

CHAPTER 2: MATERIALS AND METHODS

Umbilical Cord Blood Collection

Following informed consent, umbilical cord blood samples were collected from healthy pregnancies and pregnancies complicated by T1DM and T2DM. All pregnancies were singleton gestations. Women with preeclampsia, hypertension, or other illnesses known to affect glucose metabolism were excluded. Infants born with identified chromosomal abnormalities were excluded. This protocol was approved by the Institutional Review Board at the Indiana University School of Medicine.

Cell Culture

ECFCs were cultured from the umbilical cord blood by the Angio BioCore at the Indiana University Simon Cancer Center as previously described (58). Briefly, following harvest, umbilical cord blood was diluted 1:1 with phosphate buffered saline (PBS) and underlaid with an equal volume of Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ). Blood was centrifuged for 30 min at room temperature at 740*g.* Mononuclear cells (MNCs) were harvested and washed with Endothelial Basal Media-2 (EBM-2, Lonza, Walkersville, MD) containing an additional 10% fetal calf serum (FCS, Atlanta Biologicals, Flowery Branch, GA) and antibiotic-antimycotic solution (Corning, Manassas, VA).

MNCs were resuspended in Endothelial Growth Media-2 (EGM-2, Lonza) and plated in six-well dishes, which were pre-coated with type I collagen (Corning) at $5x10^6$ cells per well. After 24 hr, adherent cells were washed with EGM-2. EGM-2 was changed daily for 7 days, and then every other day until the

first passage. Colonies of endothelial cells began to arise between days 5 and 8 of culture. Cells were harvested using trypsin (Corning) and passaged when they reached confluency.

Early passage ECFCs (≤ passage 6) were used for experiments. For routine culture, ECFCs were grown in EGM-2 containing an additional 10% FCS and antibiotic-antimycotic solution. HeLa (ATCC, Manassas, VA) and Lenti-X 293T cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Corning) containing 10% FCS and antibiotic-antimycotic solution.

Western Blotting

Nuclear lysates were obtained using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (ThermoFisher, Waltham, MA). Whole cell lysates were obtained by lysing cells in radio-immunoprecipitation assay (RIPA) buffer containing Complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN). Equal amounts of lysate were loaded on precast 4-12% bis-tris gels (Life Technologies, Grand Island, NY), separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were immunoblotted with the following primary antibodies: mouse anti-Lamin A/C (Cell Signaling Technology, Danvers, MA), mouse anti-MEOX-2 [JJ-7] (Santa Cruz Biotechnology, Dallas, TX), rabbit anti-CDKN2A/p16INK4a [EP4353Y3] (Abcam, Cambridge, MA), rabbit anti-SP-1 (Cell Signaling Technology), rabbit anti-α6 integrin (Cell Signaling Technology), and mouse anti-vinculin (Sigma Aldrich, St. Louis, MO). Membranes were incubated in secondary antibodies conjugated to

horseradish peroxidase (Bio-Rad). Blots were developed with Supersignal West Femto (ThermoFisher), exposed to film, and compiled in Adobe Photoshop CS6. Band intensity was quantified using ImageJ 1.45s.

Generation of Lentivirus Encoding MEOX2 cDNA and short hairpin (sh) MEOX2 Constructs

The lentiviral vector plasmid (pUC2CL6IPwo), packaging accessory plasmids (pCD/NL2 and pCD/NL-BH), and envelope plasmid (pVSVG) were generous gifts from Helmut Hanenberg (Heinrich Heine University School of Medicine, Düsseldorf, Germany) (109, 110). MEOX2 cDNA (RC501948, OriGene Technologies Inc., Rockville, MD) was subcloned into pUC2CL6IPwo.The lentiviral vector plasmid (pGIPZ) containing an shMEOX2 construct was obtained from GE Dharmacon (Lafayette, CO) (RHS4430-200162184; Clone ID: V2LHS_207280). Both lentiviral vectors contain a puromycin resistance cassette, which enabled selection of transduced cells. Lentiviral particles were produced by transfection of Lenti-X 293T cells with the appropriate lentiviral vector (1.16 µg/ml), a packaging accessory plasmid (1.16 µg/ml), and a VSVG envelope plasmid (1.67 µg/ml) using Fugene 6 (Roche Applied Science). The pUC2CL6IPwo vector required the use of the pCD/NL2 packaging plasmid, while the pGIPZ vector was *tat*-dependent and required the pCD/NL-BH packaging plasmid. Lentiviral supernatants were collected and filtered through a 0.45-µm asymmetric polyethersulfone filter unit (ThermoFisher). Supernatants were used immediately or stored at −80 °C for future use.

Lentiviral Transduction of ECFCs

ECFCs were plated at 250,000 cells per dish in 100mm type I collagen-coated tissue culture dishes. The following day, lentiviral supernatant was added to each dish at dilutions of 1:2 to 1:10. The media was changed 24 hr after transduction, and cells were incubated overnight at 37°C. Transduced cells were selected in media containing 1 μg/ml puromycin (ThermoFisher) for 2 days. MEOX2 expression was evaluated by western blotting to confirm overexpression or knockdown.

siRNA Transfection

ECFCs were transfected with short-interfering RNAs (siRNAs) (20 μM) using Lipofectamine RNAiMAX (ThermoFisher) according to the manufacturer's instructions. ECFCs were transfected with either a nontargeting smart-pool siRNA (siControl) (D-001810-10-05, ON-TARGETplus, GE Dharmacon) or human MEOX2 siRNA (siMEOX2) (J-012176-08, ON-TARGETplus, GE Dharmacon). Media was changed after 24 hr. Cells were passaged 48 hr following transfection and plated for cell-cycle analysis, matrigel network formation, and transwell migration assays. MEOX2 expression was examined by western blotting to confirm knockdown 3 days following transfection.

Flow Cytometric Analysis of p16 Expression

ECFCs were harvested using trypsin, and permeabilized and fixed with Cytofix/Cytoperm (BD Biosciences, San Jose, CA). Cells were stained with PE-conjugated, mouse anti-p16 (BD Biosciences), and analyzed on an LSRII (Becton Dickinson, San Jose, CA) in the Indiana University Simon Cancer Center Flow Cytometry Core. HeLa cells, which have detectable p16 at baseline, were used as a positive control. A minimum of 10,000 events was recorded per sample. Mean fluorescence intensity was quantified using FlowJo Single Cell Analysis Software vX.0.6.

Senescence Assays

ECFCs were plated in type I collagen-coated six-well tissue culture plates at 10,000 cells per well. After 3 days, senescence-associated β-galactosidase staining was performed as previously described (58, 109). Briefly, cells were washed with PBS and fixed with 2% formaldehyde and 0.2% gluteraldehyde for 5 min at room temperature. Cells were washed with PBS and stained for 24 hr with 150mmol/l β-galactosidase solution, 2mmol/l $MgCl_2$, 40 mmol/l trisodium citrate, 5mmol/l potassium ferricyanide, 5 mmol/l potassium ferrocyanide, at pH 6, and containing X-Gal dye (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) (Invitrogen, Grand Island, NY). β-galactosidase positive staining was determined by visual scoring of deep blue stained cells. At least 100 total cells per well were scored, and the percentage of β-galactosidase positive cells was determined.

Cell Cycle Analysis

ECFCs were plated in 100mm type I collagen-coated tissue culture dishes at 400,000 cells per dish. After 24 hr, cells were incubated with BrdU labeling reagent (Invitrogen) for 1 hr. Cells were stained as previously described (111). Briefly, cells were trypsinized, resuspended in 70% ice-cold ethanol, and placed at -20°C. After 16 hr, cells were centrifuged, resuspended in 2N hydrochloric acid, and incubated at room temperature for 25 min. Following an additional centrifugation, cells were resuspended in 50mM sodium borate pH 8.5 and incubated for 2 min at room temperature. Cells were stained using Alexa Flour 488 mouse anti-BrdU (Invitrogen) for 90 min at room temperature and 7-AAD (Life Technologies) for 15 min at room temperature. Samples were analyzed by flow cytometry on the LSRII 407nm laser and a minimum of 10,000 events was recorded per sample. Analysis was performed using FlowJo Single Cell Analysis Software vX.0.6.

Matrigel Network Formation Assay

ECFCs were plated in 100mm type I collagen-coated tissue culture dishes at 400,000 cells per dish. After 1 day in culture, cells were trypsinized and plated at 4,500 cells per well in triplicate in angiogenesis 15µ-slides (Ibidi USA, Inc., Madison, WI). Wells were coated with 10µl matrigel matrix (Corning) and cells were plated in EGM2. Phase contrast images were obtained using a Spot camera (Spot Imaging, Sterling Heights, MI) on an Axiovert 35 microscope (Zeiss, Thornwood, NY) at times indicated. The number of closed networks per well was scored and averaged for each condition.

Transwell Migration Assays

ECFCs were plated in 100mm dishes at 400,000 cells per dish. After 1 day in culture, cells were serum-starved in EBM-2 for 1 hr and then trypsinized. Cells were plated on type I collagen-coated 8.0μm pore size transwell inserts (Corning). ECFCs were plated at 3-5x10^4 cells per insert. The number of cells was kept constant for each replicate. Inserts were placed in 24-well dishes containing EGM-2, 10% FCS, and antibiotic-antimycotic solution, and incubated for 4 hr at 37°C. Transwell surfaces were wiped with cotton swabs to remove non-migrated cells and then fixed with cold 100% methanol for 15 min. After fixation, cells were washed with PBS and stained with 1% crystal violet in 10% acetic acid for 10 min at room temperature. After brief destaining, phase contrast images were obtained using a Spot camera on an Axiovert 35 microscope, and the number of migrated cells was scored.

Adhesion Assays

ECFCs were plated in 100mm dishes at 400,000 cells per dish. After 1 day in culture, cells were trypsinized and plated in 96-well dishes coated with type I collagen (0.05mg/ml), type IV collagen (0.1 mg/ml) or laminin (0.1 mg/ml) (Sigma Aldrich) at 5,000 cells per well. Cells were fixed with 4% paraformaldehyde for 15 min at 37°C, at time points denoted in the figure legends. After fixation, cells were washed with phosphate buffered saline and stained with 1% crystal violet in distilled water for 10 min. Cells were washed with distilled water, and the crystal violet stain was dissolved in 10% acetic acid. Plates were placed on a plate

rocker for 5 min. Absorbance was quantified using a VersaMax Tunable microplate reader (Molecular Devices, Sunnyvale, CA) at 590 nm.

Flow Cytometric Analysis of Integrin Surface Expression

ECFCs were plated in 100mm type I collagen-coated tissue culture dishes at 400,000 cells per dish. After 1 day in culture, cells were serum-starved in EBM-2 for 1 hr and then stimulated for 4 hr in EGM-2 containing an additional 10% FCS and antibiotic-antimycotic solution. Cells were harvested by scraping using versene (Life Technologies) and resuspended in ice cold PBS containing 2% FCS. Cells were triple-stained in two groups for 30 min in the dark at 4°C. Group 1 was stained with FITC-conjugated, anti-α1 integrin (BioLegend, San Diego, CA), APC-conjugated, anti-α3 integrin (BioLegend), and PE-conjugated, anti-α6 integrin (BioLegend). Group 2 was stained with PE-conjugated, anti-α2 integrin (BioLegend), anti-α7 integrin (Abcam), and APC/Cy7-conjugated, anti-β1 integrin (BioLegend). Following staining with anti-α7 integrin (Abcam) cells were stained with Alexa Fluor 488 goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA). Samples were analyzed by flow cytometry on the LSRII 407nm laser and a minimum of 10,000 events was recorded per sample. Analysis was performed using FlowJo Single Cell Analysis Software vX.0.6.

Real-Time Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

ECFCs were lysed in Qiazol (Qiagen, Valencia, CA), and RNA was purified using the miRNeasy kit (Qiagen). RNA was reverse transcribed using a qScript cDNA Master Mix Kit (Quanta Biosciences, Beverly, MA). Reverse-transcriptase PCR was performed on a Lightcycler 480 (Roche). Detection of α6 integrin,

hypoxanthine phosphoribosyltransferase (HPRT), and MEOX2 was performed using Lightcycler 480 SYBR Green I Master Mix (Roche). HPRT was used to normalize α6 integrin values using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used are provided in Table 2.1.

Table 2.1. RT-PCR Primers

Gene	*Primer Sequences*
α6 integrin	5′-TTTGAAGATGGGCCTTATGAA-3′ 5′-CCCTGAGTCCAAAGAAAAACC-3′
HPRT	5′-CCTTGGTCAGGCAGTATAATCCA-3′ 5′-GGTCCTTTTCACCAGCAAGCT-3′
MEOX2	5′-AGAGGAAAAGCGACAGCTCA-3′ 5′-AAGTTCTCTGATTTGCTCTTTGGT-3′

Chromatin Immunoprecipitation (ChIP) Assay

ECFCs were plated in 100mm type 1 collagen-coated tissue culture dishes at $1x10^6$ cells per dish. After 24 hr, cell extracts were prepared by crosslinking DNA-protein complexes and isolating nuclei using the SimpleChIP Enzymatic Chromatin Immunoprecipitation Kit (Cell Signaling) according to the manufacturer's protocol. Lysates were sonicated (4 cycles, 30 seconds on, 30 seconds off) at 4°C using the Bioruptur Plus (Diagenode, Seraing, Belgium). Lysates were centrifuged to remove cell debris. Samples were incubated overnight with the following antibodies: mouse anti-flag M2 (Sigma Aldrich) or mouse anti-IgG isotype control (Cell Signaling Technologies). Two percent of the samples were used as input. DNA-protein complexes were collected with ChIP-grade Protein G magnetic beads (Cell Signaling Technologies) for 2 hr at 4°C and washed 4 times with SimpleChIP Enzymatic Chromatin IP Kit wash buffers for 5 min at 4°C. DNA was eluted in SimpleChIP Enzymatic Chromatin IP Kit elution buffer by shaking for 30 min at 65°C. DNA was purified using the DNA Clean and Concentrator-5 Kit (Zymo Research, Irvine, CA) and analyzed by RT-PCR, performed on a Lightcycler 480. The primer sequences used are provided in Table 2.2.

Table 2.2. ChIP Primers

Gene	*Primer Sequences*
α6 integrin	5′-ACAACCCATCCTTGACTTGC-3′
	5′-CCTTGTCCCCAGATCACCTA-3′
p16	5-AGGATTCCTTTTGGAGAGTCG-3′
	5′-CCCAGACAGCCGTTTTACAC-3′

Statistical Analysis

Data illustrated are mean ± SEM. Paired and unpaired *t*-tests and repeated measures 2-way ANOVAs were conducted when appropriate, as denoted in the figure legends. Graphpad Prism 6 was used for all statistical analyses, and significance was noted when $p<0.05$.

CHAPTER 3: RESULTS

Aim 1: Determine whether MEOX2 is increased in ECFCs from DM pregnancies.

MEOX2 RNA and total protein is not increased in ECFCs from DM pregnancies

Preliminary RNA microarray studies indicated that ECFCs from DM pregnancies had increased MEOX2 levels. To further validate this result in a larger population, RNA and protein were isolated from ECFCs from control and DM pregnancies followed by RT-PCR and western blotting, respectively. Surprisingly, no differences were detected in MEOX2 RNA between ECFCs from control and DM pregnancies (Fig. 3.1). Further, total protein was comparable between samples from control and DM pregnancies (Fig. 3.2).

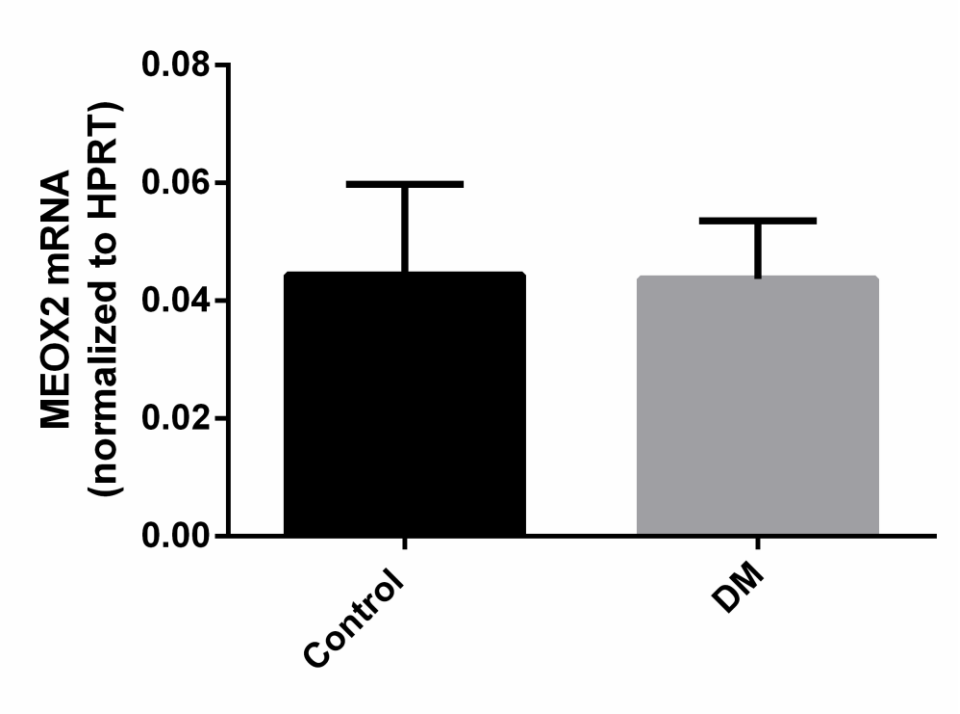


Figure 3.1. MEOX2 RNA is not increased in ECFCs from DM pregnancies. RNA was harvested from ECFCs from control and DM pregnancies and RT-PCR was performed. Results were normalized to HPRT and to the mean control expression for MEOX2. n=15 control, n=17 DM, $p>0.05$ by unpaired *t*-test.

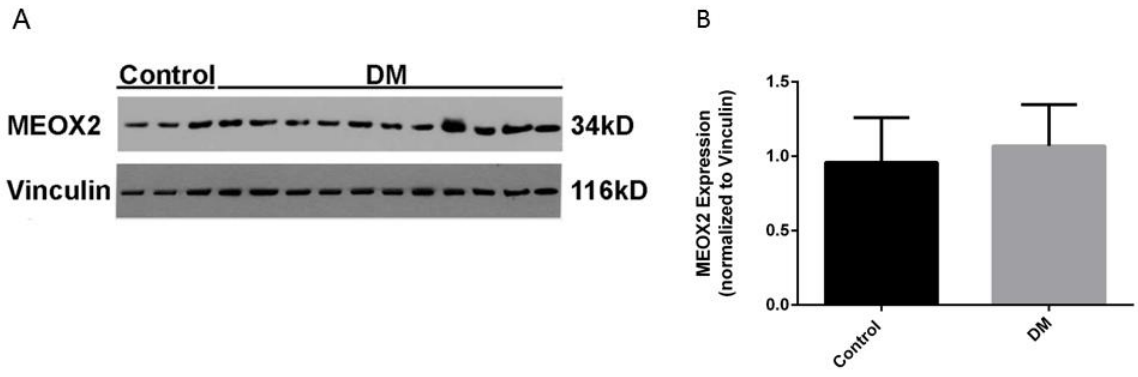


Figure 3.2. MEOX2 total protein is not increased in DM-exposed ECFCs. (A) Representative western blot. Whole cell lysates from ECFCs from control and DM pregnancies were separated by SDS-PAGE. Blots were probed for MEOX2 and vinculin, as a loading control. **(B)** Quantitation of total MEOX2 expression. n=14 control, n=17 DM, $p>0.05$ by unpaired *t*-test.

Nuclear MEOX2 is increased in ECFCs from DM pregnancies

Although MEOX2 RNA and total protein did not differ between ECFCs from control and DM pregnancies, we sought to determine whether there was a difference in MEOX2 protein subcellular localization. Because MEOX2 is a transcription factor (88, 89) whose functional role occurs in the nucleus, we hypothesized that there would be increased nuclear MEOX2 in ECFCs from DM pregnancies. To determine whether nuclear MEOX2 is increased in ECFCs from DM pregnancies, western blotting analyses were conducted using nuclear lysates. As recently published, these data confirmed increased nuclear MEOX2 in ECFCs from DM pregnancies compared to control samples (Fig. 3.3) (112). While some heterogeneity in MEOX2 levels was evident in DM-exposed samples, nuclear MEOX2 was consistently higher in these samples compared to controls. We speculate this heterogeneity may be due to the heterogeneity of the disease, as management of DM during pregnancy can vary drastically in an individual and across different pregnancies (113).

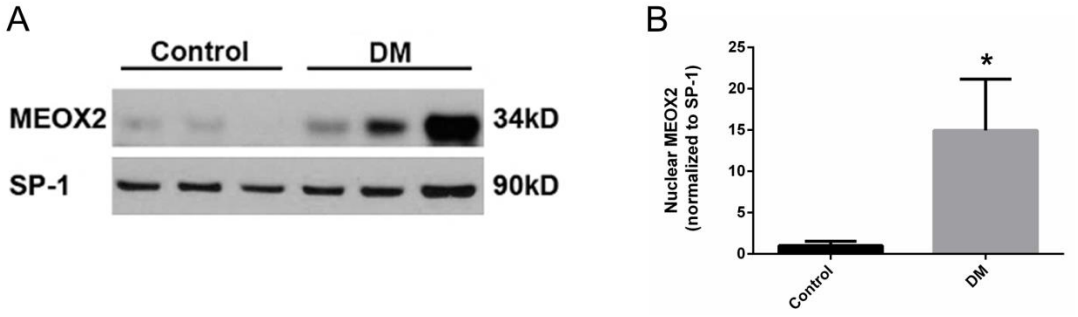


Figure 3.3. ECFCs from DM pregnancies have increased nuclear MEOX2. **(A)** Representative western blot. Nuclear lysates from ECFCs were separated by SDS-PAGE. Blots were probed for MEOX2 and SP-1, as a loading control. **(B)** Quantitation of nuclear MEOX2 expression. n=8 control, n=11 DM, *$p<0.05$ by unpaired *t*-test.

Aim 2: Determine whether MEOX2 regulates senescence, cell cycle progression, network formation, and migration of ECFCs.

MEOX2 overexpression is sufficient to alter functional capacity of ECFCs

MEOX2 is implicated in the upregulation of p16 to induce senescence in HUVECS (88). Previously, the Haneline laboratory showed that ECFCs from DM pregnancies have increased senescence, decreased cell cycle progression, and decreased network formation (58). Therefore, we questioned whether increased MEOX2 expression in control ECFCs was sufficient to induce impaired function. To test this hypothesis, MEOX2 was overexpressed in ECFCs from control pregnancies using a lentiviral vector. ECFCs transduced with an empty lentiviral vector were used as a control. Overexpression was confirmed by western blotting (Fig. 3.4). The data from these studies were recently published (112).

Because p16 is a known mediator of senescence (94, 114), p16 expression was examined using two methods. Initially, p16 expression was measured using western blotting techniques with nuclear lysates. MEOX2 overexpression in control ECFCs resulted in an increase in p16 compared to ECFCs transduced with the empty vector (Fig. 3.4). While this result provided initial insight into p16 levels in a population of cells, it did not elucidate p16 expression on a cell by cell basis. To further validate this result on a single-cell basis, flow cytometric analysis was employed to detect intracellular p16. The results indicated that control ECFCs transduced with the empty vector control have very low levels of p16 staining (Fig. 3.5). In contrast, a subset of ECFCs transduced with the lentivirus containing the MEOX2 cDNA have higher p16

levels. These data confirmed an increase in p16 in a subset of ECFCs that overexpress MEOX2. Additionally, β-galactosidase senescence assays showed that MEOX2 overexpression increased the number of senescent cells (Fig. 3.6) (112). Interestingly, when MEOX2 was overexpressed, similar levels of p16 staining and senescent ECFCs were observed. We speculate that ECFCs with high p16 expression undergo senescence as a result of MEOX2 overexpression.

Our previous data demonstrate that ECFCs from DM pregnancies have reduced proliferation (58). Therefore, we questioned whether increased MEOX2 was sufficient to alter cell cycle progression of control ECFCs. To examine cell cycle progression, cells were pulsed with BrdU for 1 hr and levels of BrdU and 7-AAD were detected using flow cytometry. In control ECFCs with increased MEOX2 expression, an increase in the proportion of cells in the G1 phase and a decrease in the proportion of cells in the G2 phase were observed compared to control ECFCs (Fig. 3.7) (112). Surprisingly, the proportion of ECFCs in S phase was unaltered. Because MEOX2 overexpression increased senescence (Fig. 3.6), a subsequent reduction in proliferation, as indicated by a reduction in the proportion of ECFCs in S phase was expected.

Given that MEOX2 overexpression in control cells led to increased senescence and altered cell cycle progression, it was predicted that overexpression of MEOX2 in control ECFCs would reduce network formation *in vitro*. Surprisingly, MEOX2 overexpression in control ECFCs increased the number of closed networks compared to the empty vector controls (Fig. 3.8 A and B). To verify that the alterations in network formation were due to MEOX2

protein levels, and not an inherent characteristic of the control ECFC phenotype, MEOX2 was also overexpressed in ECFCs from DM pregnancies. Data from these studies showed that MEOX2 overexpression in DM samples also increased the number of closed networks compared to the empty vector controls (Fig. 3.8 C and D) (112). Together, these data suggest that MEOX2 overexpression is sufficient to induce changes in senescence, cell cycle progression, and network formation. However, there is a disconnect between the observed phenotypes. It was expected that the increased senescence and altered cell cycle progression observed with MEOX2 overexpression would result in reduced network formation. Because there were fewer cells progressing through the cell cycle, it was expected that there would be fewer actively proliferating cells available to form network structures, ultimately resulting in deficits in network formation. However, the data instead suggest that MEOX2 regulation of senescence and cell cycle progression does not negatively impact overall vasculogenic function.

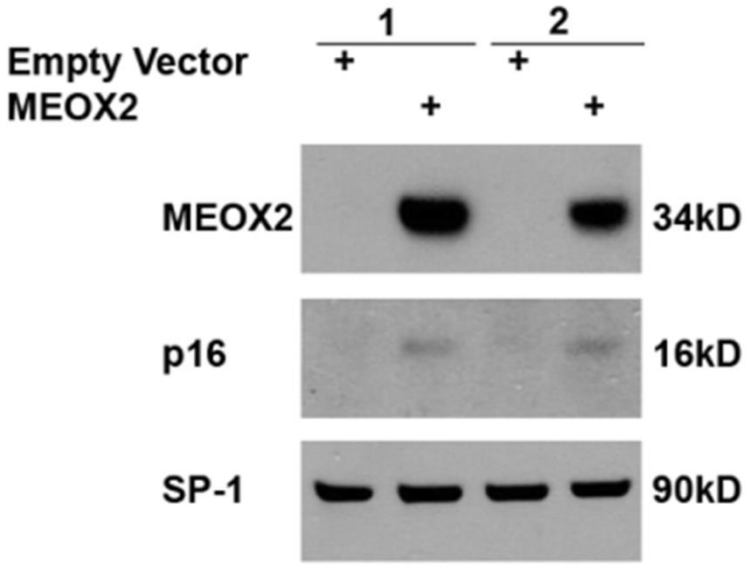


Figure 3.4. MEOX2 was overexpressed in control ECFCs using lentiviral transduction techniques. Representative western blot illustrating MEOX2 and p16 expression. Nuclear lysates were analyzed for MEOX2, p16, and SP-1 as a loading control. Numbers represent separate transductions of ECFCs from different pregnancies.

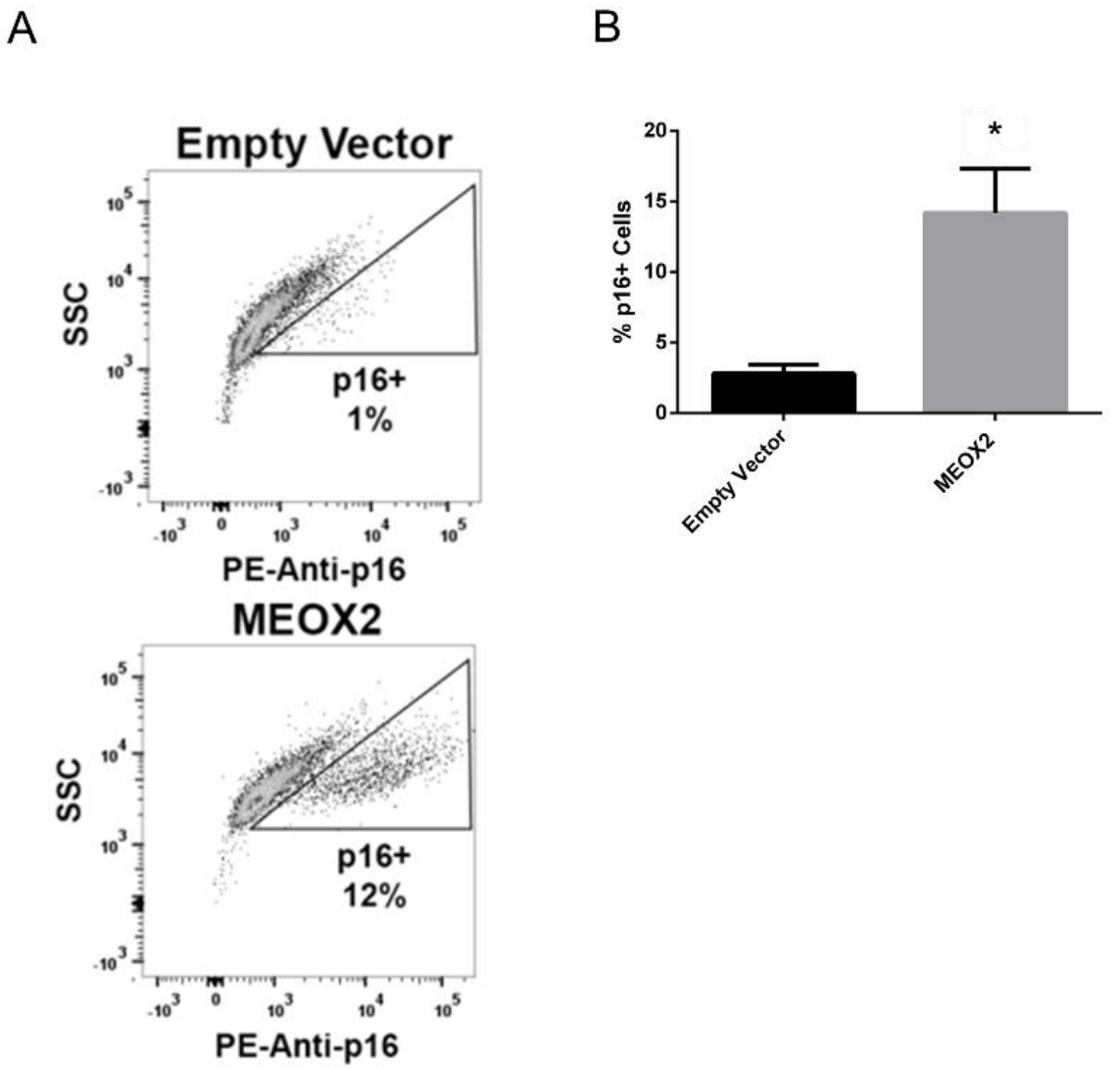


Figure 3.5. MEOX2 overexpression in control ECFCs increased p16 expression. (A) Representative gating strategy of intracellular p16 expression in transduced ECFCs. p16 expression was measured by intracellular staining followed by flow cytometry. **(B)** Quantitation of p16+ cells by flow cytometry. n=4 transductions, *p<0.001 by paired *t*-test.

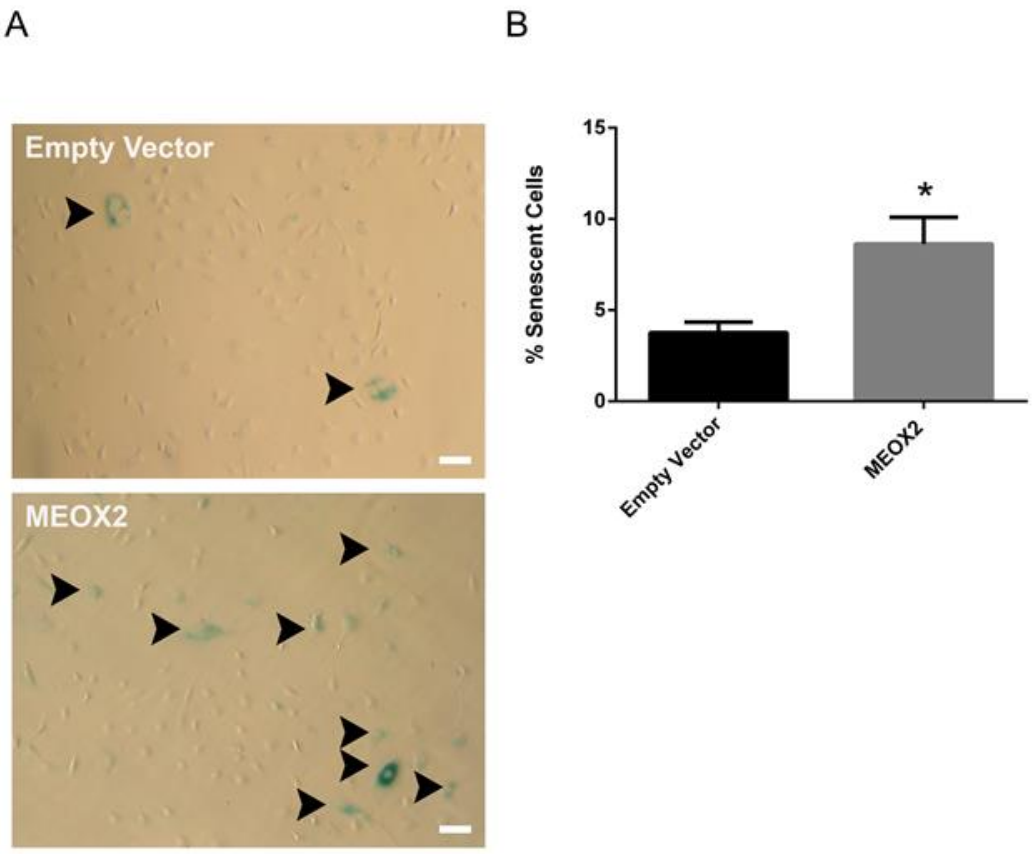


Figure 3.6. MEOX2 overexpression in control ECFCs increased senescence. (A) Representative images from 3-day senescence-associated-β-galactosidase assays with transduced ECFCs (100x magnification). Arrows denote location of senescent cells. Scale bars represent 100μm. **(B)** Quantitation of 3-day senescence-associated-β-galactosidase assays, n=4 transductions, *p<0.05 by paired *t*-test.

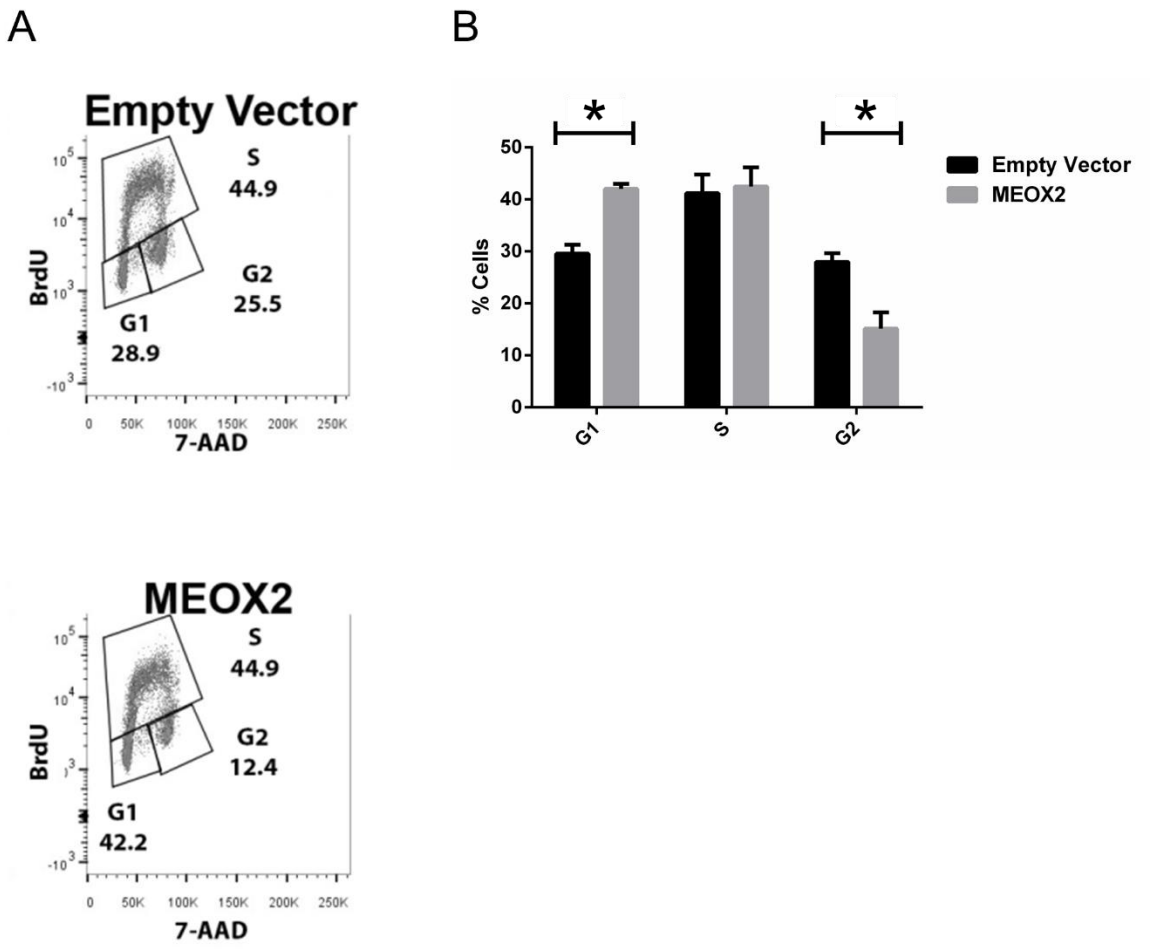


Figure 3.7. MEOX2 overexpression in control ECFCs altered cell cycle progression. (A) Representative gating strategy to measure cell cycle progression of transduced ECFCs. Cell cycle analysis was conducted using flow cytometric analysis of BrdU and 7-AAD staining. **(B)** Quantitation of cell cycle analysis of transduced ECFCs, n=3 transductions, *p<0.001 by repeated measures 2-way ANOVA.

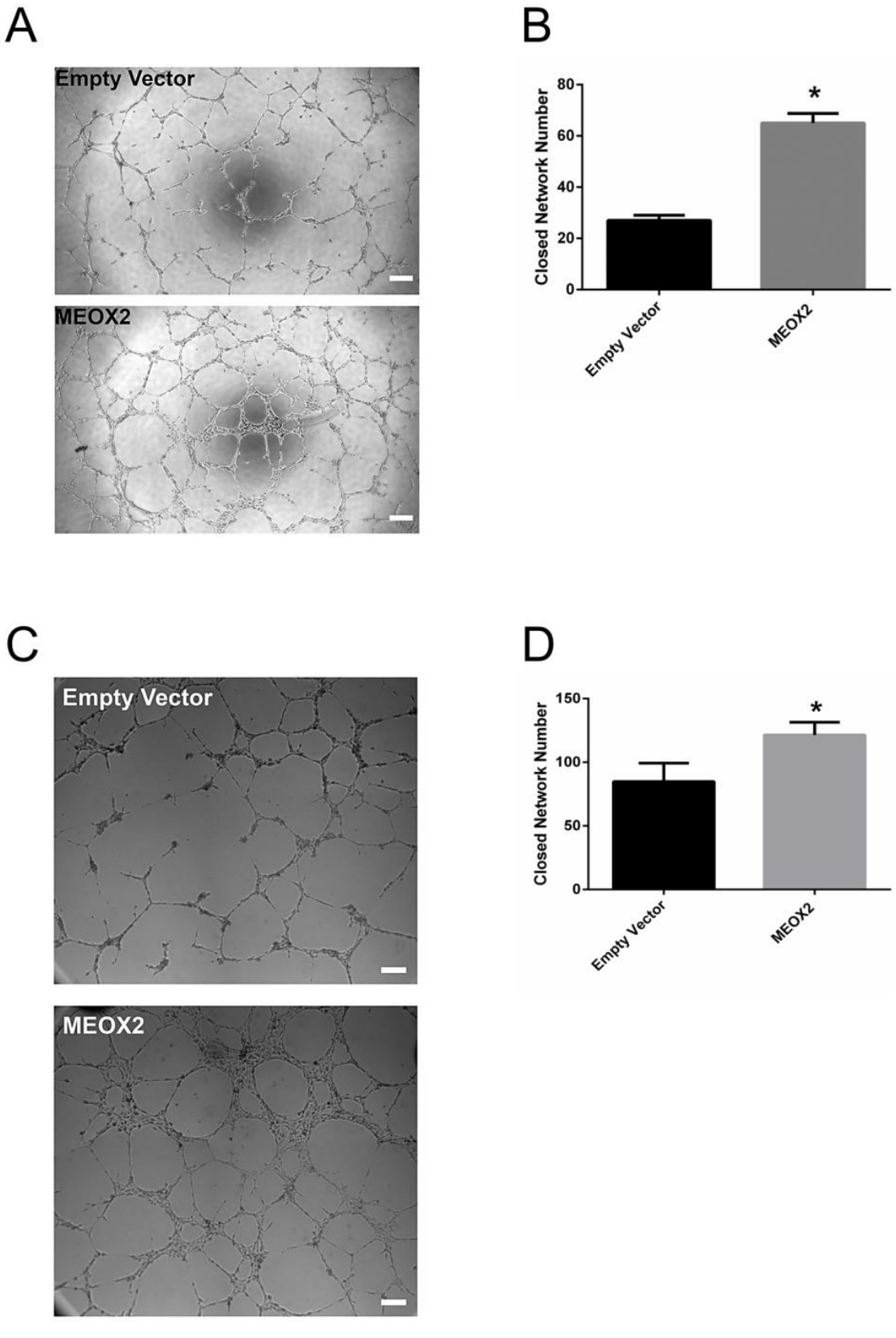

A
Empty Vector
MEOX2
B
Closed Network Number
80
60
40
20
0
*
Empty Vector
MEOX2
C
Empty Vector
MEOX2
D
Closed Network Number
150
100
50
0
*
Empty Vector
MEOX2

Figure 3.8. MEOX2 overexpression in control and DM-exposed ECFCs enhanced network formation. (A) Representative images from matrigel network formation assay with transduced control ECFCs at 8 hr post-plating (50x magnification). Scale bar represents 200μm. **(B)** Quantitation of matrigel network formation assay with transduced control ECFCs at 8 hr post-plating, n=3 transductions, *p<0.05 by paired *t*-test. **(C**) Representative images from matrigel network formation assay with transduced DM-exposed ECFCs at 8 hr post-plating (50x magnification). Scale bar represents 200μm. **(D)** Quantitation of matrigel network formation assay with transduced DM-exposed ECFCs at 8 hr post-plating, n=6 transductions, *p<0.05 by paired *t*-test.

MEOX2 is required to alter network formation, but not senescence or cell cycle progression

To determine whether MEOX2 was required to induce changes in ECFCs from DM pregnancies, MEOX2 was depleted using either an shRNA or siRNA approach. shRNA methods were utilized when assays required long-term, stable knockdown of MEOX2, such as 3-day senescence assays. However, the shRNA transduction process was more labor intensive and was not as efficient as the transient siRNA knockdown methods. Therefore, when assays required only short-term knockdown of MEOX2, siRNA approaches were used. Nevertheless, knockdown was confirmed by western blotting for both approaches (Fig. 3.9). To determine the time of optimal MEOX2 knockdown by siRNA, time course experiments were conducted. Western blotting experiments revealed that optimal MEOX2 knockdown was achieved between days 3 and 4 following siRNA transfection (Fig. 3.10). Data from these studies were recently published (112).

Following confirmed MEOX2 knockdown, ECFCs from DM and control pregnancies were analyzed for alterations in cellular and functional phenotypes. Despite average MEOX2 knockdown of greater than 90%, senescence of DM-exposed ECFCs was no different to ECFCs transduced with the shControl vector (Fig. 3.11). Senescence assays were not conducted in control ECFCs because at baseline, these cells have negligible senescent cells. We speculated that detection below this already low threshold would be unlikely. Similarly, flow cytometric cell cycle analysis indicated that MEOX2 knockdown in ECFCs from

DM (Fig. 3 .12 A and B) and control (Fig. 3.12 C and D) pregnancies did not alter cell cycle progression (112).

To examine whether MEOX2 knockdown improved network formation, matrigel assays were conducted. Because MEOX2 overexpression in control ECFCs resulted in an increase network formation, we hypothesized that MEOX2 knockdown would lead to decreased vasculogenesis *in vitro.* Further, we expected to see a similar decrease in network formation upon MEOX2 knockdown in ECFCs from DM pregnancies. Although MEOX2 knockdown in control ECFCs did not affect network formation (Fig. 3.13 A and B), MEOX2 knockdown in ECFCs from DM pregnancies resulted in decreased network formation compared to siControls (Fig. 3.13 C and D), complimentary to the MEOX2 overexpression assays (Fig. 3.8) (112). We speculate that differences were not observed in the control ECFCs, because of the low baseline levels of MEOX2 protein, whereas ECFCs from DM pregnancies had higher MEOX2 protein levels at baseline.

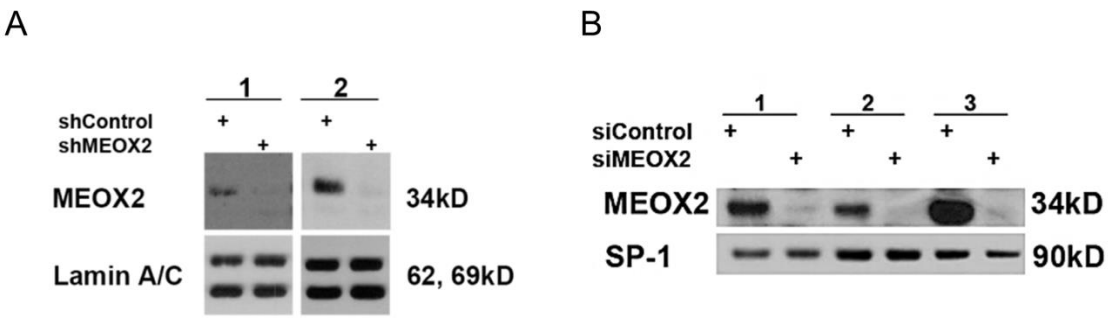


Figure 3.9. MEOX2 was knocked down in cells from DM pregnancies using shRNA or siRNA techniques. (A) Representative western blot depicting MEOX2 expression when knocked down by shRNA. Lamin A/C was used as a loading control. Numbers represent separate transductions of ECFCs from different pregnancies. **(B)** Representative western blot showing MEOX2 expression when knocked down by siRNA. SP-1 was used as a loading control. Numbers represent separate transfections of ECFCs from different pregnancies.

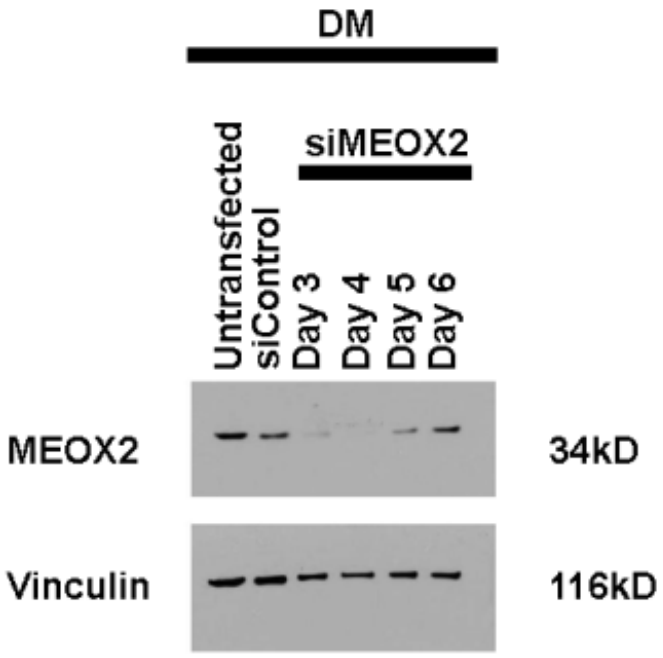


Figure 3.10. siMEOX2 reduced MEOX2 protein levels following transfection. Representative western blot depicting MEOX2 knockdown in whole cell lysates from ECFCs from DM pregnancies on days 3, 4, 5, and 6 following transfection. Vinculin was used as a loading control.

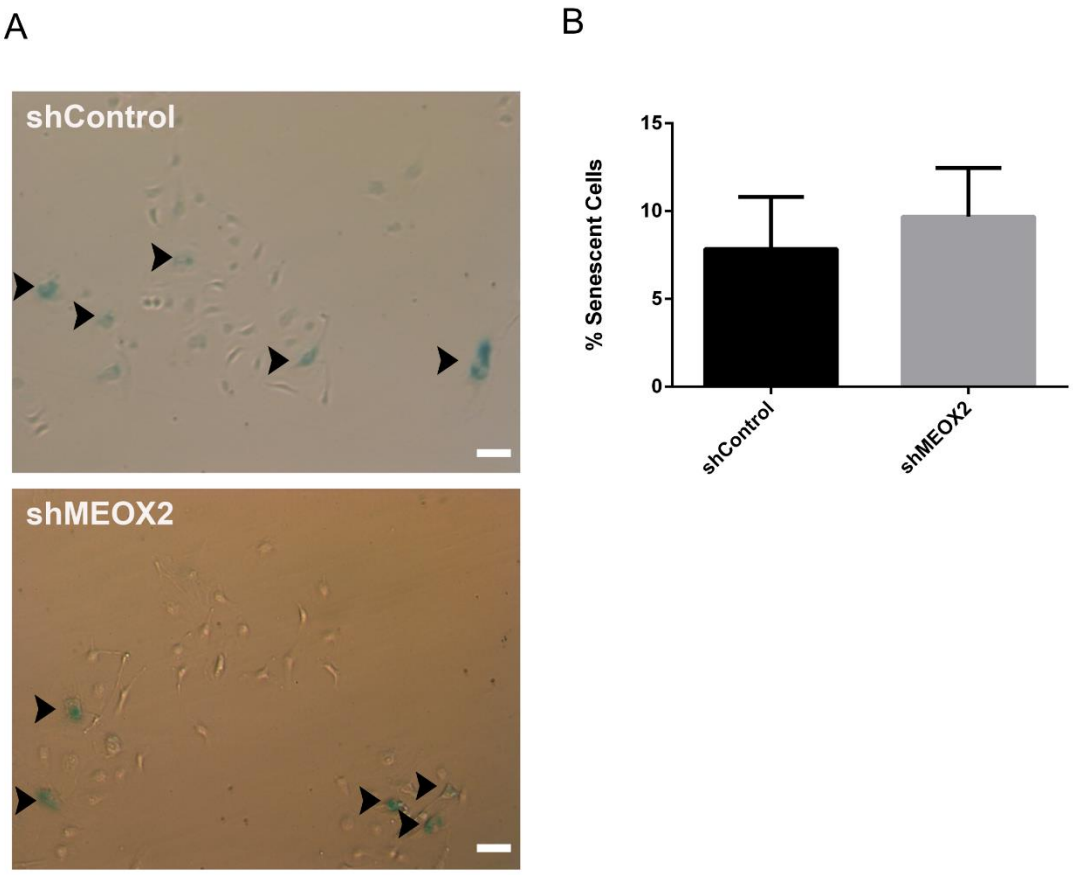


Figure 3.11. MEOX2 knockdown in ECFCs from DM pregnancies did not alter senescence. (A) Representative images from 3-day senescence-associated-β-galactosidase assays with transduced ECFCs (100x magnification). Arrows denote location of senescent cells. Scale bars represent 100μm. **(B)** Quantitation of 3-day senescence-associated-β-galactosidase assays, n=9 transductions, p>0.05 by paired *t*-test.

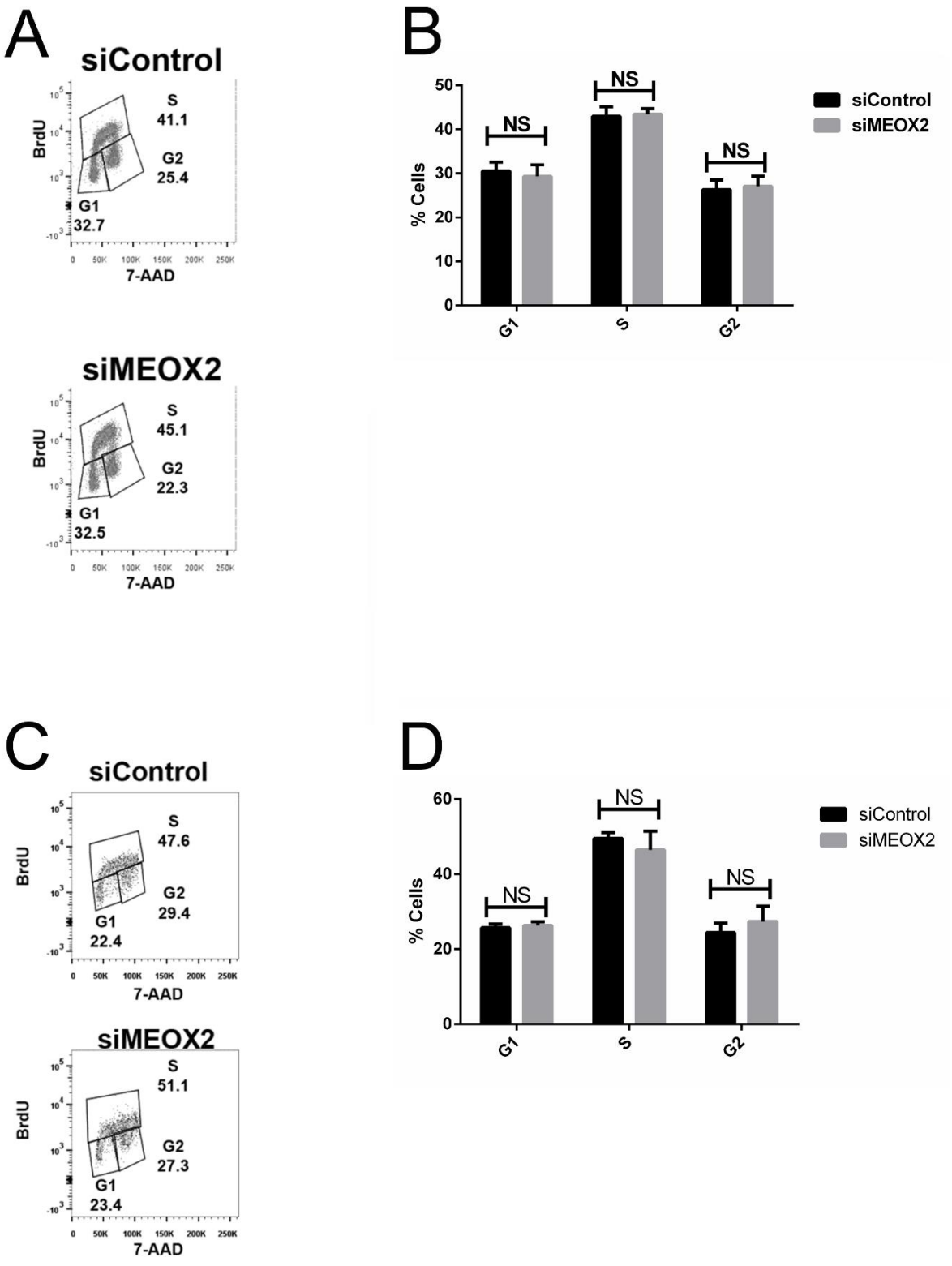
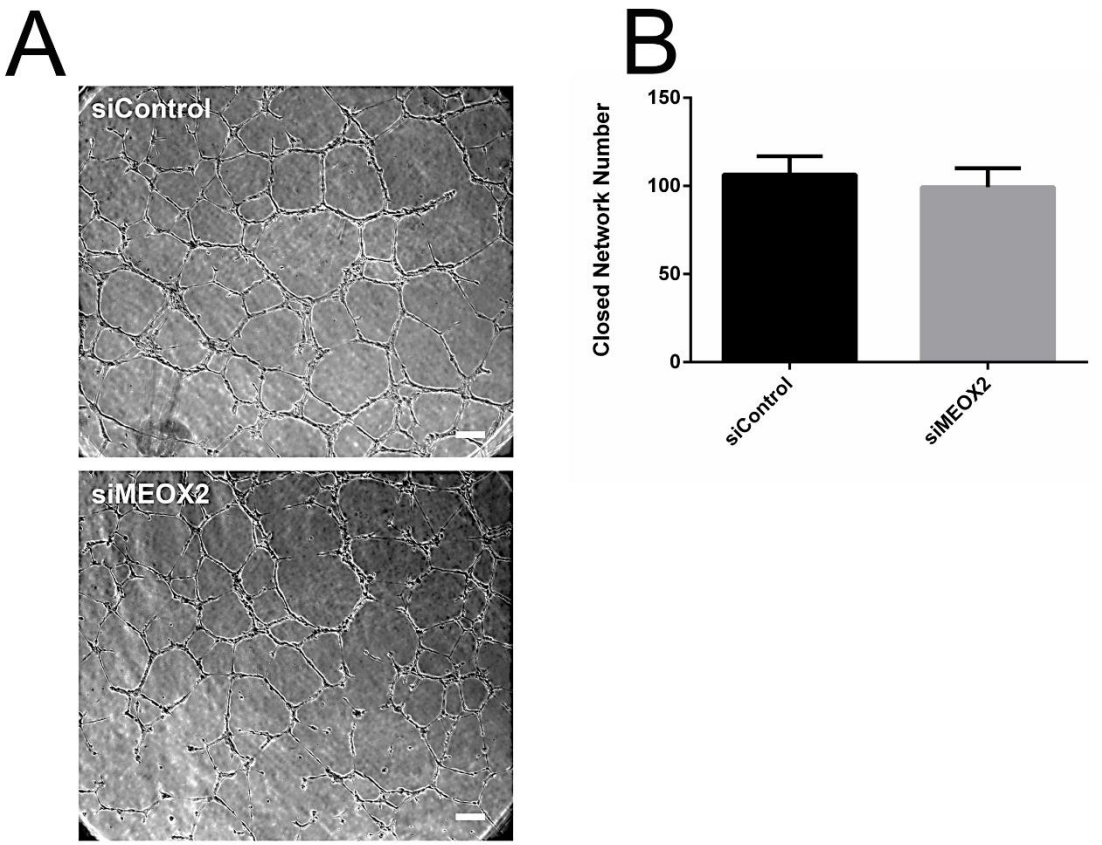

A
siControl
BrdU
S
41.1
G2
25.4
G1
32.7
7-AAD
siMEOX2
S
45.1
G2
22.3
G1
32.5
7-AAD
B
NS
NS
NS
% Cells
G1
S
G2
siControl
siMEOX2
C
siControl
S
47.6
G2
29.4
G1
22.4
7-AAD
siMEOX2
S
51.1
G2
27.3
G1
23.4
7-AAD
D
NS
NS
NS
% Cells
G1
S
G2
siControl
siMEOX2

Figure 3.12. MEOX2 knockdown in ECFCs from DM and control pregnancies did not alter cell cycle progression. (A) Representative gating strategy to measure cell cycle progression of transfected DM-exposed ECFCs. Cell cycle analysis was conducted using flow cytometric evaluation of BrdU and 7-AAD staining. **(B)** Quantitation of cell cycle analysis of transfected DM-exposed ECFCs, n=9 transfections, $p>0.05$ by repeated measures 2-way ANOVA. **(C)** Representative gating strategy to measure cell cycle progression of transfected control ECFCs. Cell cycle analysis was conducted using flow cytometric evaluation of BrdU and 7-AAD staining. **(D)** Quantitation of cell cycle analysis of transfected control ECFCs, n=5 transfections, $p>0.05$ by repeated measures 2-way ANOVA.


A
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siMEOX2
B
Closed Network Number
150
100
50
0
siControl
siMEOX2
C
siControl
siMEOX2
D
Closed Network Number
50
40
30
20
10
0
*
siControl
siMEOX2

Figure 3.13. MEOX2 knockdown in ECFCs from DM pregnancies altered network formation. (A) Representative images from matrigel assay with transfected control ECFCs at 8 hr post-plating (50x magnification). Scale bar represents 200μm. **(B)** Quantitation of matrigel assay with transfected control ECFCs at 8 hr post-plating, n=5 transfections, $p>0.05$ by paired *t*-test. **(C)** Representative images from matrigel assay with transfected DM-exposed ECFCs at 8 hr post-plating (50x magnification). Scale bar represents 200μm. **(D)** Quantitation of matrigel assay with transfected DM-exposed ECFCs at 8 hr post-plating, n=12 transfections, $*p<0.01$ by paired *t*-test.

MEOX2 alters migration in ECFCs

Together, the data indicate that MEOX2 is both sufficient and required to alter network formation, supporting a regulatory role in vasculogenesis. Surprisingly, however, the data also indicate that this regulation is independent of alterations to senescence and cell cycle progression. Inasmuch, other possible mechanisms by which MEOX2 regulates vasculogenesis in ECFCs were examined. Migration is a critical step in establishing vascular networks (56). Therefore, ECFC migratory capacity was examined in ECFCs from control and DM pregnancies. As presented in our recent publication, a 50% reduction in migration was observed in ECFCs from DM pregnancies when plated on collagen I (Fig. 3.14), congruent with our previously published network formation data (58). However, ECFCs from DM pregnancies had normal migration on collagen IV and laminin (Fig. 3.14) (112).

To determine if MEOX2 is sufficient to alter migration, MEOX2 was overexpressed in ECFCs from control and DM pregnancies. Interestingly, MEOX2 overexpression in ECFCs from uncomplicated pregnancies increased migration on collagen I (112) and laminin (Fig. 3.15), consistent with the observed increases in network formation (Fig. 3.8 A and B). In contrast, MEOX2 overexpression in control cells did not affect migration on collagen IV (Fig. 3.15). Similarly, MEOX2 overexpression in DM-exposed ECFCs also increased migration on collagen I (Fig. 3.16), consistent with the observed increases in network formation (Fig. 3.8 C and D) (112).

To determine if MEOX2 is required to alter migration, MEOX2 was knocked down in ECFCs from DM pregnancies. MEOX2 knockdown in DM-exposed ECFCs decreased migration on collagen I (112) and laminin (Fig. 3.17), congruent with decreased network formation (Fig. 3.13 C and D). However, MEOX knockdown in DM-exposed ECFCs did not alter migration on collagen IV (Fig. 3.17).

Taken together, these data suggest that ECFCs from DM pregnancies have reduced migratory capacity on collagen I. Further, these data suggest that MEOX2 is both sufficient and required to alter migration on collagen I and laminin, but not on collagen IV. Although deficits in migratory capacity on laminin were not observed in DM-exposed ECFCs, we speculate MEOX2 is involved in regulating migration on this ECM. Specifically, we observed that MEOX2 overexpression in control ECFCs increased migration on laminin, whereas MEOX2 knockdown in DM-exposed ECFCs decreased migration on laminin. It is conceivable that increased nuclear MEOX2 may enable maintained migration of these ECFCs despite exposure to a DM environment *in utero*.

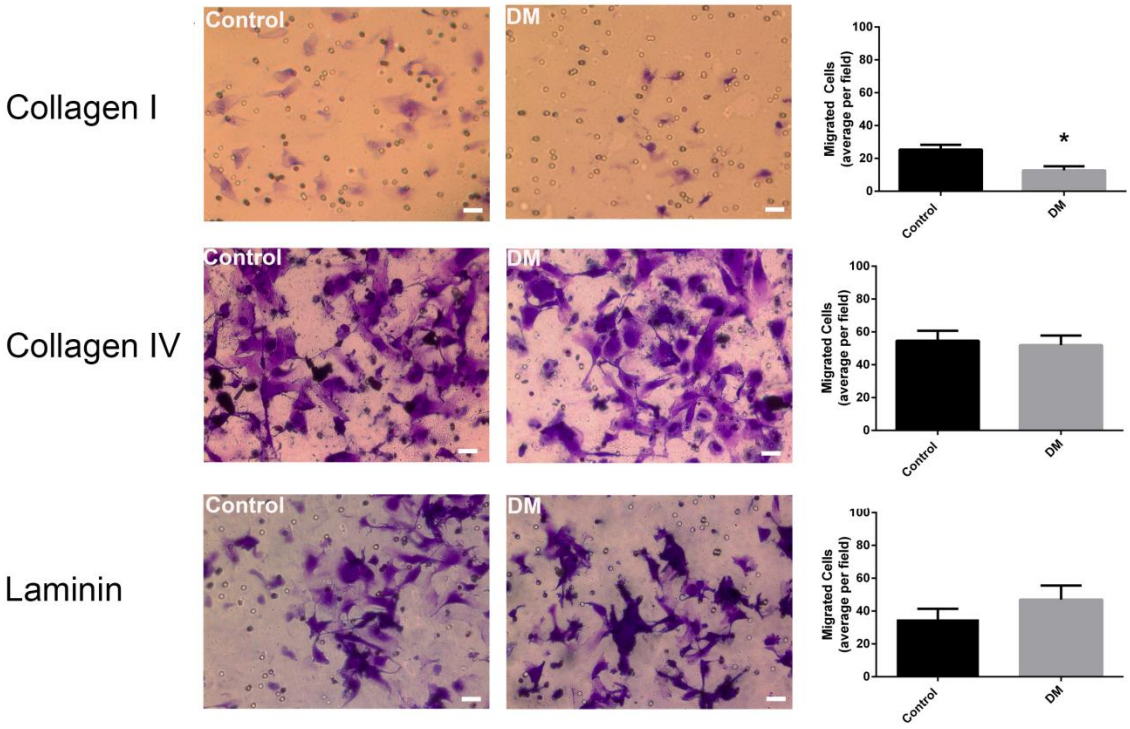

Figure 3.14. ECFCs from DM pregnancies had reduced migration on collagen I, but normal migration on collagen IV and laminin. Representative images and quantitation of transwell migration assay with ECFCs from control and DM pregnancies (320x magnification). Scale bars represent 30μm, n=6 control, 6 DM, *p<0.01 by unpaired *t*-test.

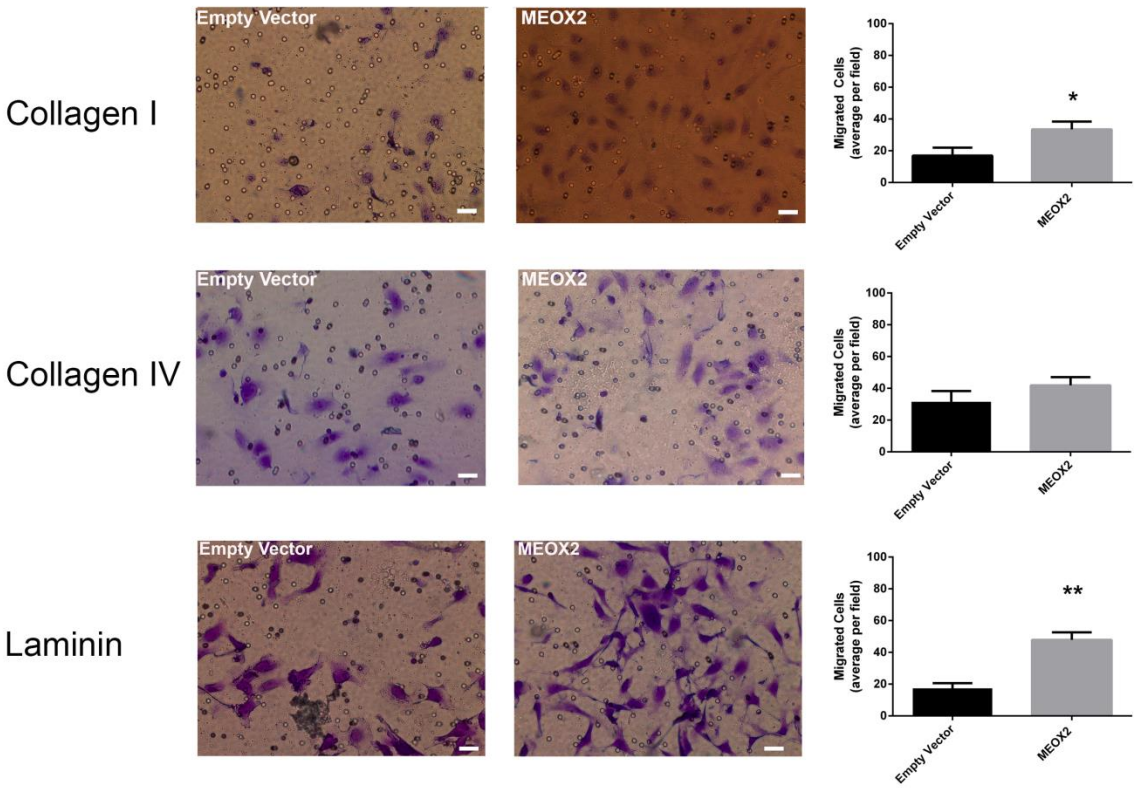

Figure 3.15. MEOX2 overexpression in ECFCs from uncomplicated pregnancies increased migration on collagen I and laminin. Representative images and quantitation of transwell migration assay with transduced ECFCs (320x magnification). Scale bars represent 30μm, n=5 transductions, *p<0.05, **p<0.005 by paired *t*-test.

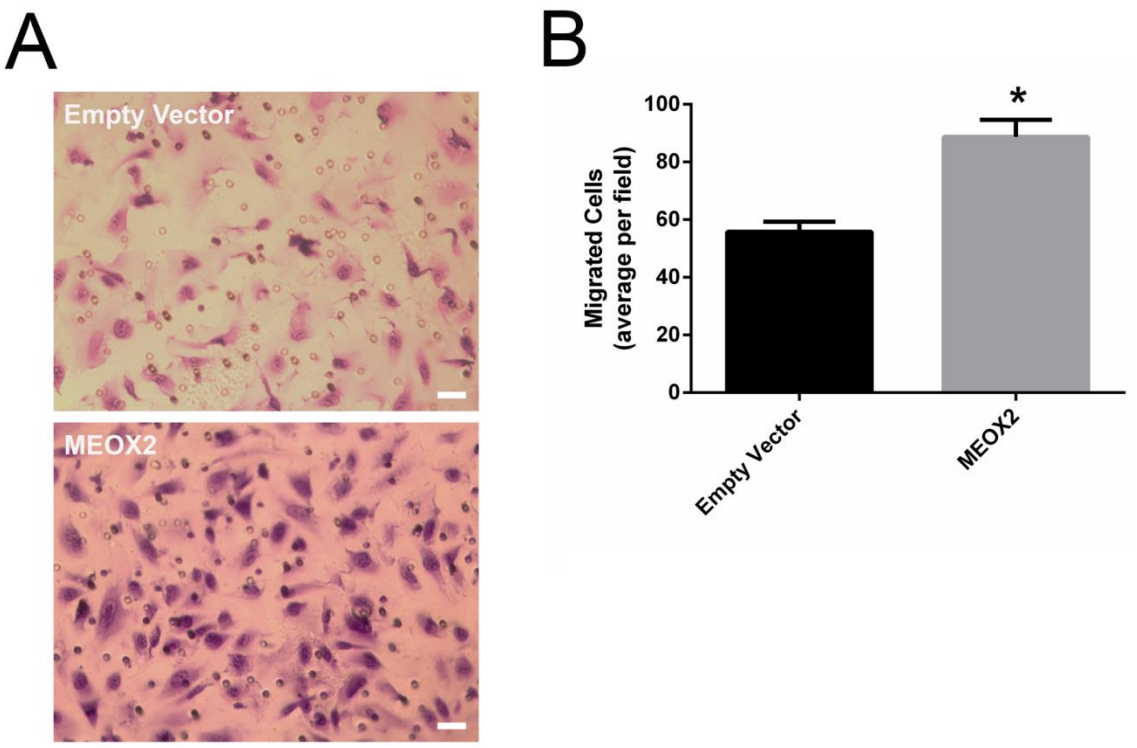

Figure 3.16. MEOX2 overexpression in ECFCs from DM pregnancies increased migration on collagen I. (A) Representative images of transwell migration assay with transduced ECFCs (320x magnification). Scale bars represent 30μm. **(B)** Quantitation of transwell migration assay with transduced ECFCs, n=5 transductions, *p<0.05 by paired *t*-test.

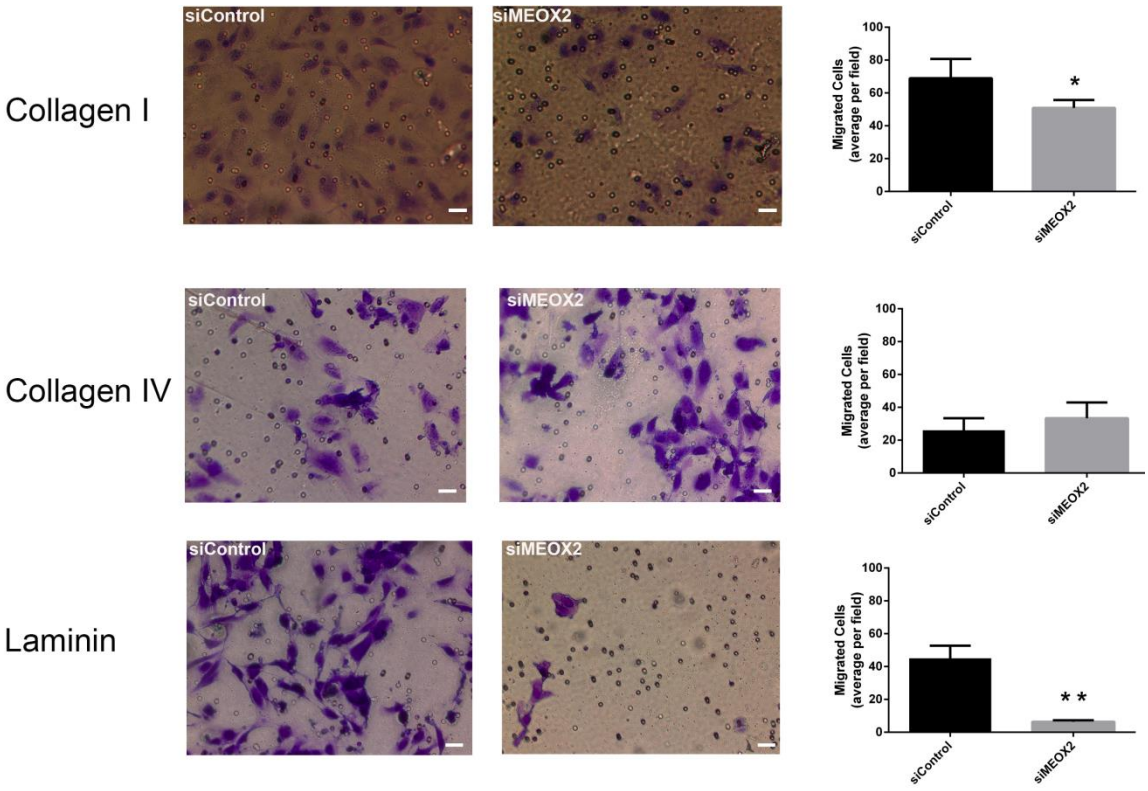

Figure 3.17. MEOX2 knockdown in ECFCs from DM pregnancies decreased migration on collagen I and laminin. Representative images and quantitation of transwell migration assay with transfected ECFCs (320x magnification). Scale bars represent 30μm, n=6 transfections, *p<0.01, **p<0.005 by paired *t*-test.

Aim 3: Determine whether MEOX2 regulates migration through differential integrin expression in ECFCs from DM pregnancies.

MEOX2 regulation of adhesion is not responsible for differences in migratory capacity

Results from the transwell migration studies indicated that MEOX2 regulates migration on both collagen I and laminin. To form traction enabling forward momentum and ultimately cellular migration, a cell must first adhere to the ECM (27, 29-32). Therefore, it was hypothesized that the differences seen in the migration studies could be attributed to differences in ECM adhesion. Specifically, MEOX2 overexpression in ECFCs from control pregnancies would increase adhesion on collagen I and laminin, while MEOX2 knockdown in ECFCs from DM pregnancies would decrease adhesion on these ECMs.

To test these hypotheses, ECFCs were plated on either collagen I or laminin, and adhesion was measured using crystal violet staining at multiple time points. MEOX2 overexpression increased adhesion on collagen I at both the 15 and 60 min time points (Fig. 3.18 A). However, adhesion was comparable at the 30 and 45 minute time points. Because adhesion was increased at the earliest and latest time points, it was expected to also be increased at the intermediate time points. However, because adhesion did not differ at the intermediate time points, it is not believed that these data is physiologically relevant, but instead an experimental artifact. It is unlikely that cells would adhere to the ECM, release, and then adhere again at a later time point. Therefore, we interpret the data as displaying no relevant difference in adhesion on collagen I. Further, MEOX2

overexpression did not alter adhesion on laminin (Fig. 3.18 B). Similarly, MEOX2 knockdown did not alter adhesion on either ECM (Fig. 3.19 A and B). Importantly, no differences in adhesion between ECFCs from control and DM pregnancies were observed (Fig. 3.20). Taken together these data suggest that altered adhesion does not account for the observed differences in migratory capacity *in vitro*.

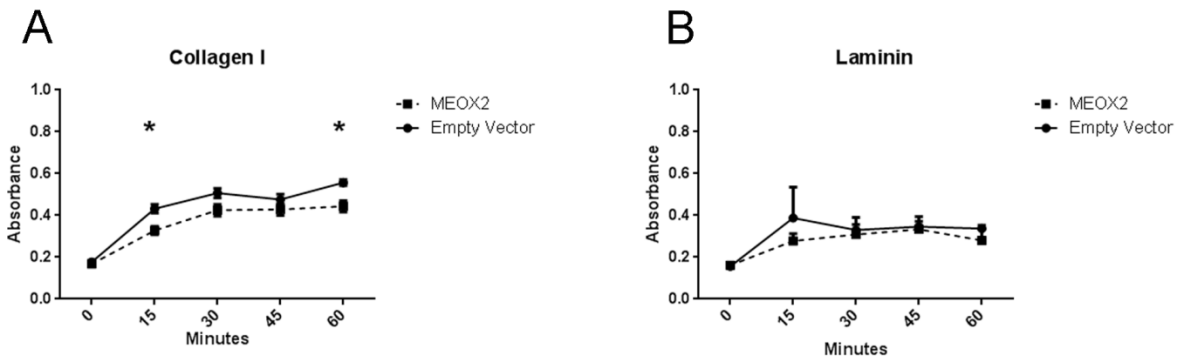

Figure 3.18. MEOX2 overexpression in control ECFCs did not alter adhesion. ECFCs were plated on collagen I or laminin in 15 min intervals for 60 min. Absorbance was quantified on a plate reader as a measure of adhesion. **(A)** Quantitation of absorbance with transduced control ECFCs plated on collagen I, n=5 transductions, *p<0.05 by 2-way ANOVA. **(B)** Quantitation of absorbance with transduced control ECFCs plated on laminin, n=3 transductions, p>0.05 by 2-way ANOVA.

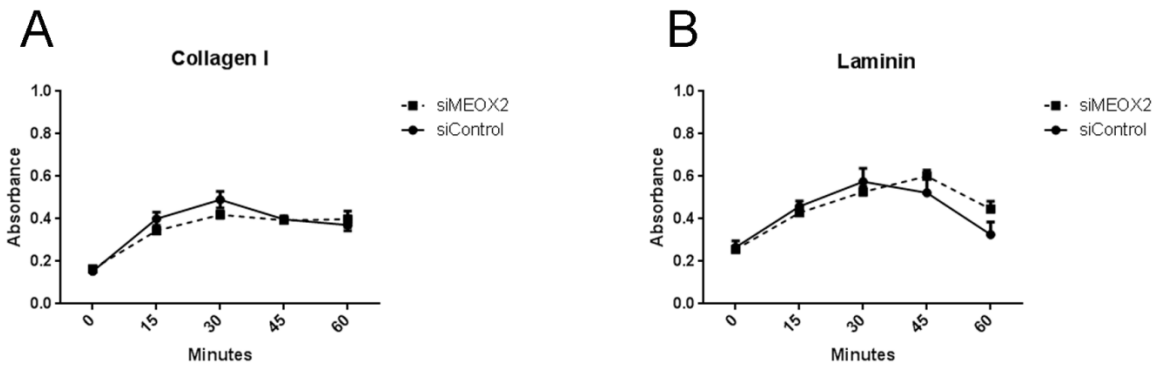

Figure 3.19. MEOX2 knockdown in DM-exposed ECFCs did not alter adhesion. ECFCs were plated on collagen I or laminin in 15 min intervals for 60 min. Absorbance was quantified on a plate reader as measure of adhesion. **(A)** Quantitation of absorbance with transfected DM-exposed ECFCs plated on collagen I, n=3 transfections, $p>0.05$ by 2-way ANOVA. **(B)** Quantitation of absorbance with transfected DM-exposed ECFCs plated on laminin, n=3 transfections, $p>0.05$ by 2-way ANOVA.

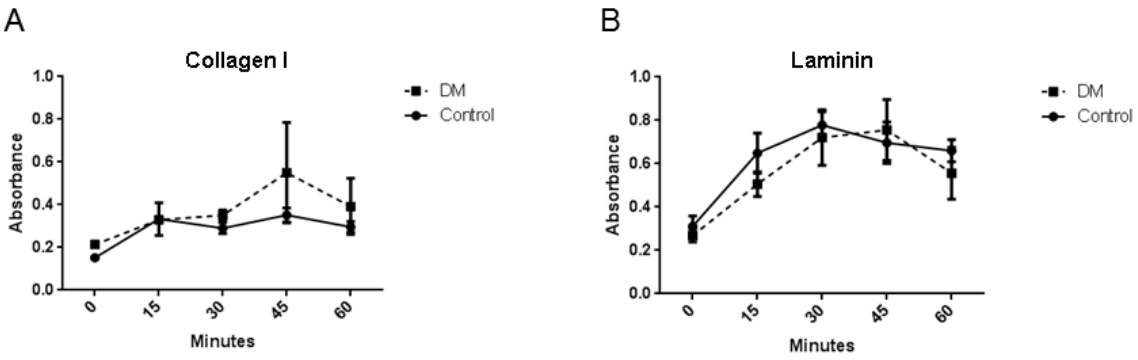

Figure 3.20. ECFCs from DM-exposed pregnancies had normal adhesion. ECFCs were plated on collagen I or laminin in 15 min intervals for 60 min. Absorbance was quantified on a plate reader as measure of adhesion. **(A)** Quantitation of absorbance of ECFCs on collagen I, n=3 control, 3 DM, $p>0.05$ by 2-way ANOVA. **(B)** Quantitation of absorbance of ECFCs on laminin, n=6 control, 6 DM, $p>0.05$ by 2-way ANOVA.

MEOX2 regulation of α2β1 heterodimer is not responsible for differences in migratory capacity

Because integrins are known to play a pivotal role in actin polymerization, which is required for normal cellular migration (61-63), integrin expression was examined. Integrins exists as heterodimers composed of an α-subunit and a β-subunit. The combination of these subunits confers binding specificity for certain ECM proteins, including, but not limited to collagens, fibronectins, and laminins (61, 63, 64). Because MEOX2 regulates migration on both collagen I and laminin, it was hypothesized that a heterodimer that serves as a common receptor for these two ECMs may exist. Importantly, the α2β1 heterodimer is a common receptor for both collagen I and laminin, though it does not bind collagen IV (76, 115-117). Because we observed alterations in migration on both collagen I and laminin, but not collagen IV, we speculated that MEOX2 expression may impact surface levels of α2β1. We hypothesized that MEOX2 overexpression in control ECFCs would increase α2β1 heterodimer levels, while MEOX2 knockdown in ECFCs from DM pregnancies would decrease α2β1 heterodimer levels.

To examine for the α2β1heterodimer, flow cytometric techniques were employed. ECFCs were starved for 1 hr in serum-free media, and subsequently stimulated for 4 hr in media containing serum and growth factors. Then, ECFCs were stained with an antibody that specifically detects the α2β1 heterodimer to quantify this receptor on a single-cell basis. The data demonstrated that MEOX2 overexpression in control ECFCs decreased α2β1 heterodimer levels (Fig. 3.21 A). However, MEOX2 knockdown in ECFCs from DM pregnancies did not alter

α2β1 heterodimer levels (Fig. 3.21 B). These data suggest that MEOX2 is sufficient to alter α2β1 heterodimer levels in control ECFCs, however MEOX2 is not required for cell surface levels of the α2β1 heterodimer in DM-exposed ECFCs.

While these data enabled an examination α2β1 heterodimer levels, there are limitations to this approach. The antibody used in these studies specifically recognized the α2β1 heterodimer, but did not give any insight into the levels of each subunit individually. Integrin heterodimers are formed intracellularly and subsequently transported to the cell surface (118, 119). Although α and β-subunits are not present individually on the cell surface, multiple α and β-subunits can combine to form 24 distinct heterodimers (118, 119). The presence or absence of surface expression of specific subunits, and thus heterodimers, is known to alter multiple cellular processes including migration (85, 118, 119). Therefore, examination of surface levels of individual α and β-subunits may provide additional insight into MEOX2 regulation of migration in ECFCs.

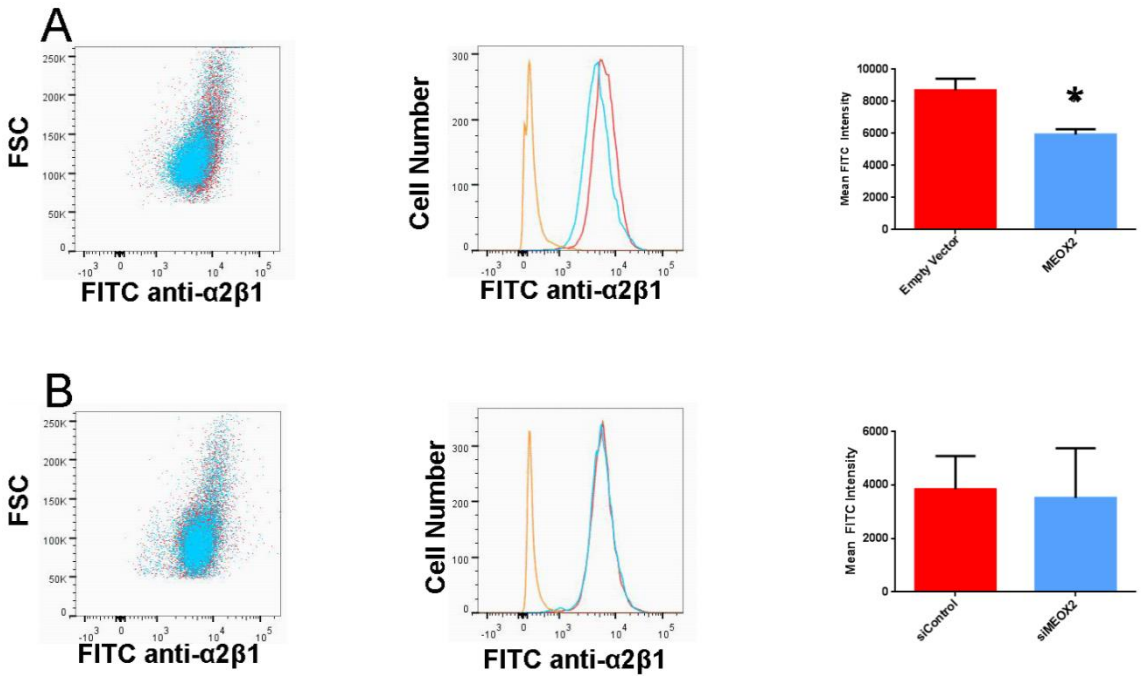

Figure 3.21. MEOX2 overexpression in control ECFCs reduced α2β1 heterodimers, but MEOX2 knockdown in ECFCs from DM pregnancies did not alter α2β1 levels. (A) Representative dot plot (left panel), histogram (middle panel), and quantitation of mean fluorescence intensity (right panel) depicting α2β1 heterodimer levels in transduced ECFCs from control pregnancies. Empty vector-transduced ECFCs are depicted in red, MEOX2-overexpressing ECFCs are depicted in blue, and unstained negative controls are depicted in orange, n=3 transductions, *p<0.05 by paired *t*-test. **(B)** Representative dot plot (left panel), histogram (middle panel), and quantitation of mean fluorescence intensity (right panel) depicting α2β1 heterodimer levels in transfected ECFCs from DM pregnancies. siControl-transfected ECFCs are depicted in red, siMEOX2-transfected ECFCs are depicted in blue, and unstained negative controls are depicted in orange, n=3 transfections, p>0.05 by paired *t*-test.

MEOX2 alters α6 integrin subunit surface expression

Multiple integrin subunits, both α and β, are expressed in endothelial cells (35, 36). Studies in Aim 2 demonstrated that MEOX2 altered migration on both collagen I and laminin ECMs. Therefore, it was hypothesized that alterations in migration are the result of differential surface expression of integrin subunits.

Studies in Aim 2 suggest a correlation between MEOX2 regulation of migration and network formation. Broadly, we hypothesize that the observed differences in network formation are attributed to differential migration. These network formation assays were conducted on matrigel matrix, which is composed of 60% laminin, 30% collagen IV, and 10% enactin for structural support. Taking the matrigel composition into account, we focused our integrin studies on the laminin-binding integrins α1, α2, α3, α6, α7, and β1.

To examine ECFCs for differential surface expression of the above integrins, cells were stained with antibodies against each of the identified laminin-binding integrins, and single-cell integrin surface expression was quantified by flow cytometry. In transduced control ECFCs, there was relatively low surface expression of α1 and α7, moderate surface expression of α3, and high surface expression of α2, α6, and β1 integrins. Further MEOX2 overexpression in ECFCs from control pregnancies did not alter surface levels of any of the measured integrins (Fig. 3.22). Conversely, in transfected ECFCs from DM pregnancies, there was relatively low surface expression of α1, moderate surface expression of α3, α6, and α7, and high surface expression of α2 and β1. Interestingly, MEOX2 knockdown in DM-exposed ECFCs decreased α6 surface levels (Fig.

3.23). Further these data support the hypothesis that MEXO2 regulates surface levels of the α6 integrin subunit as a compensatory response. It is conceivable that this would account for the observed differences in migration. Interestingly, these data also suggest that there may be differences in laminin-binding integrin surface expression between control and DM ECFCs. Notably in these studies, surface levels of the α7 integrin were relatively low in transduced control ECFCs, but markedly higher in transfected DM-exposed ECFCs. Future studies are needed to examine this potential difference in integrin surface expression, which could serve to elucidate the mechanism responsible for observed differences in migration between control and DM-exposed ECFCs.

Interestingly, others have reported that α6 is involved in the regulation of endothelial cell migration, network formation *in vitro*, and vessel formation *in vivo* (85). As noted previously, increased nuclear MEOX2 in DM-exposed ECFCs appears to serve a compensatory role allowing for improved network formation. While an inherent difference in α6 surface levels between control and DM-exposed ECFCs was not observed (Fig. 3.24), it is conceivable that the high levels of MEOX2 in ECFCs from DM pregnancies may maintain α6 integrin surface levels to allow for migration and network formation despite prior exposure to a DM intrauterine environment.

To further examine MEOX2 regulation of α6 surface expression, MEOX2 was overexpressed in ECFCs from DM pregnancies. MEOX2 overexpression in DM-exposed ECFCs resulted in a modest increase in α6 surface levels (Fig. 3.25). These data are complementary the MEOX2 knockdown experiments, also

performed in ECFCs from DM-pregnancies. Taken together, these data further reinforce the hypothesis that MEOX2 regulates α6 integrin surface expression in ECFCs from DM pregnancies. Future studies to understand why MEOX2 overexpression impacts α6 integrin surface expression differently in control versus DM-exposed ECFCs may provide new insights into the effect of prior intrauterine exposure to the DM environment on ECFCs.

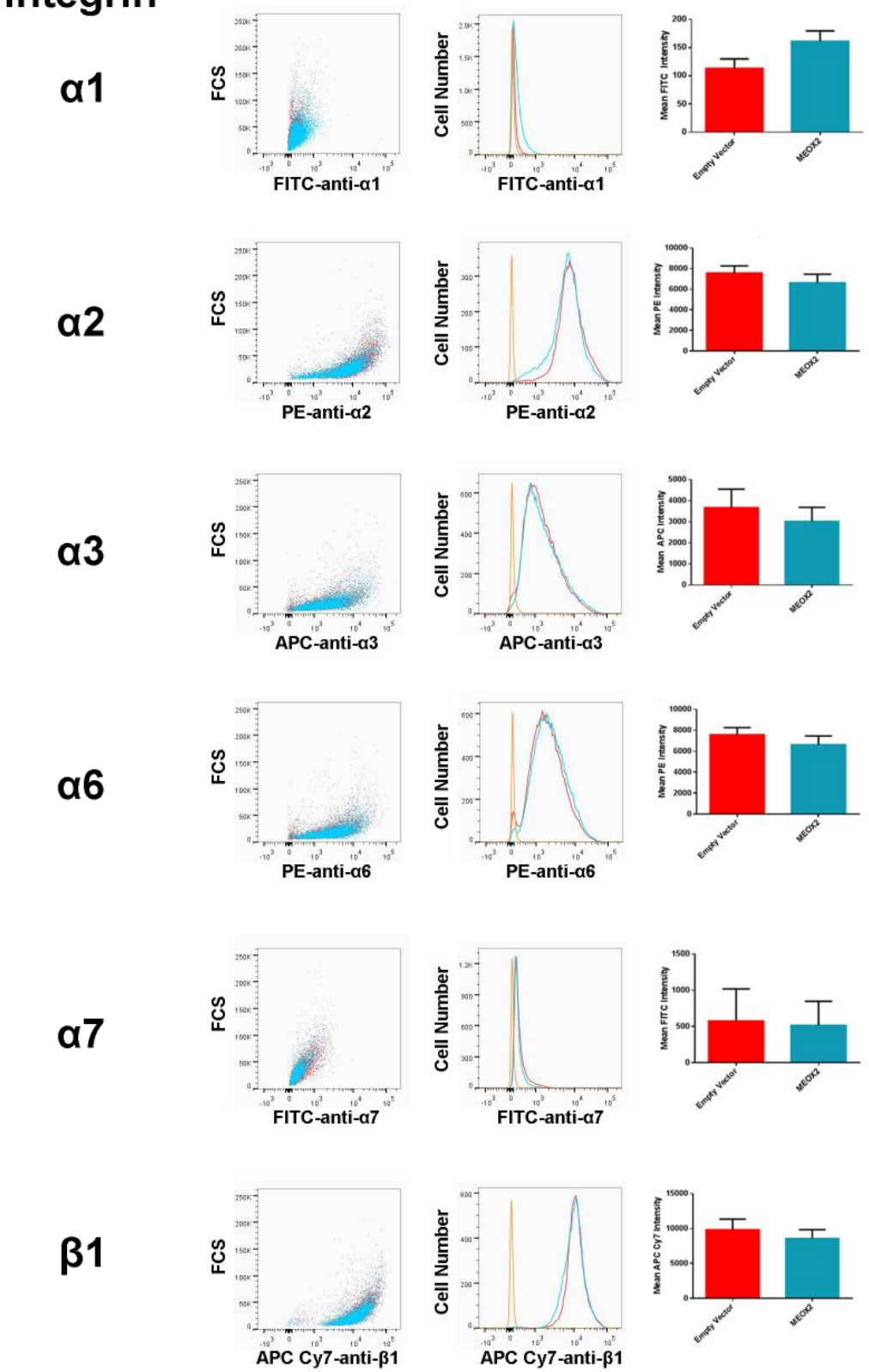
Integrin
α1
FCS
FITC-anti-α1
Cell Number
FITC-anti-α1
Mean FITC Intensity
Empty Vector
MEOX2
α2
FCS
PE-anti-α2
Cell Number
PE-anti-α2
Mean PE Intensity
Empty Vector
MEOX2
α3
FCS
APC-anti-α3
Cell Number
APC-anti-α3
Mean APC Intensity
Empty Vector
MEOX2
α6
FCS
PE-anti-α6
Cell Number
PE-anti-α6
Mean PE Intensity
Empty Vector
MEOX2
α7
FCS
FITC-anti-α7
Cell Number
FITC-anti-α7
Mean FITC Intensity
Empty Vector
MEOX2
β1
FCS
APC Cy7-anti-β1
Cell Number
APC Cy7-anti-β1
Mean APC Cy7 Intensity
Empty Vector
MEOX2

Figure 3.22. MEOX2 overexpression in ECFCs from control pregnancies did not alter integrin surface levels. Representative dot plot (left panels), histogram (middle panels), and quantitation of mean fluorescence intensity as a measure of integrin surface levels (right panels). Empty vector-transduced ECFCs are depicted in red, MEOX2-overexpressing ECFCs are depicted in blue, and unstained negative controls are depicted in orange, n=7 transductions, $p>0.05$ by paired *t*-test.

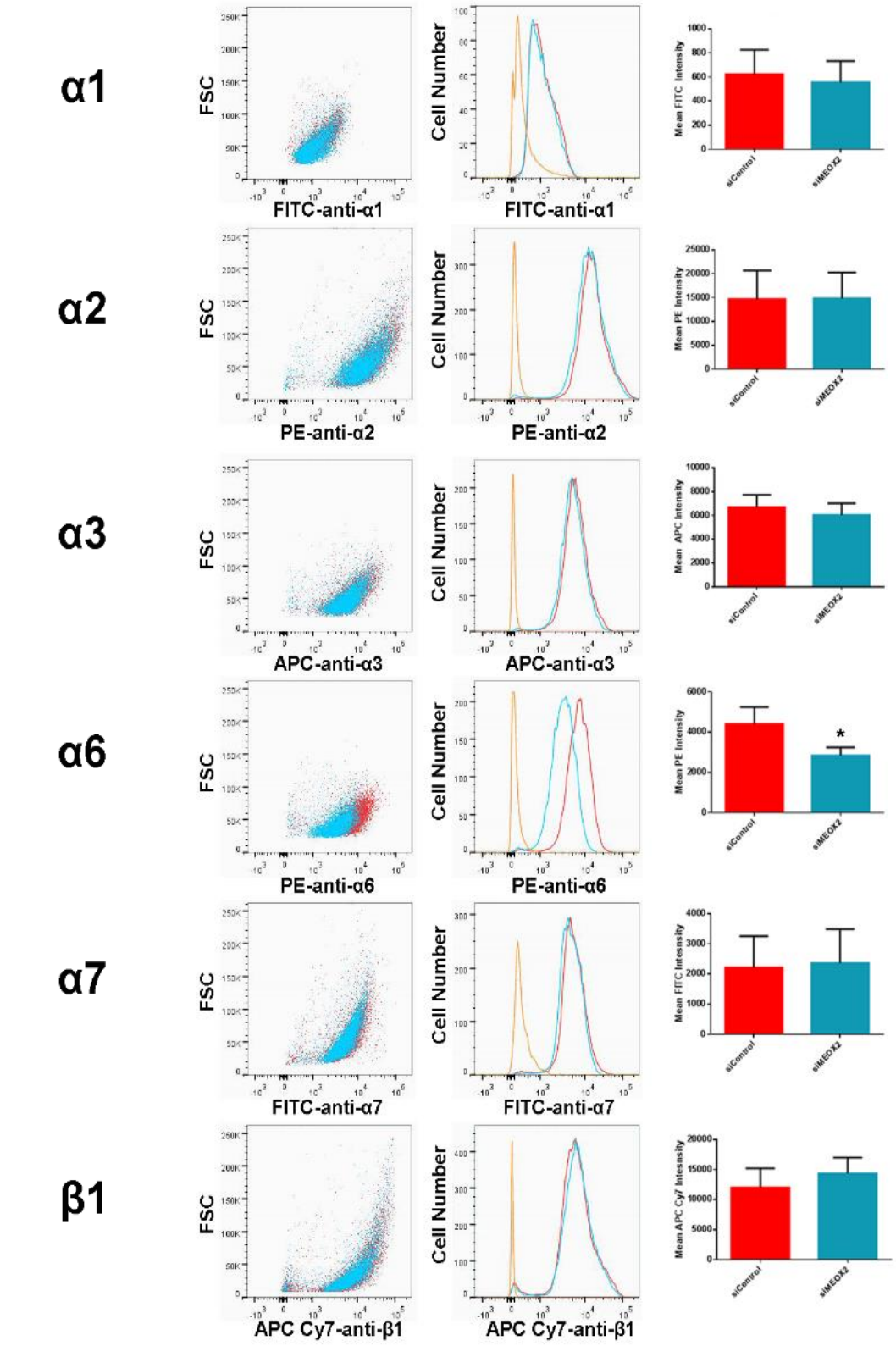
Integrin
α1
α2
α3
α6
α7
β1
FSC
FITC-anti-α1
Cell Number
Mean FITC Intensity
siControl
siMEOX2
PE-anti-α2
Mean PE Intensity
APC-anti-α3
Mean APC Intensity
PE-anti-α6
*
FITC-anti-α7
APC Cy7-anti-β1
Mean APC Cy7 Intensity

Figure 3.23. MEOX2 knockdown in ECFCs from DM pregnancies reduced α6 integrin surface levels. Representative dot plot (left panels), histogram (middle panels), and quantitation of mean fluorescence intensity as a measure of integrin surface levels (right panels). siControl-transfected ECFCs are depicted in red, siMEOX2-transfected ECFCs are depicted in blue, and unstained negative controls are depicted in orange, n=8 transfections, *p<0.05 by paired *t*-test.

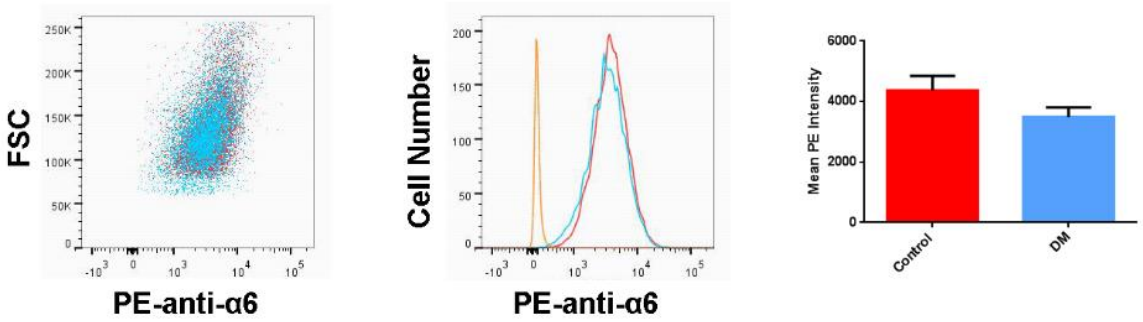

Figure 3.24. ECFCs from DM pregnancies did not have altered α6 integrin surface levels. Representative dot plot (left panel), histogram (middle panel), and quantitation of mean fluorescence intensity as a measure of α6 surface levels (right panels). ECFCs from control pregnancies are depicted in red, ECFCs from DM pregnancies are depicted in blue, and unstained negative controls are depicted in orange, n= 9 control, 9 DM, $p>0.05$ by unpaired *t*-test.

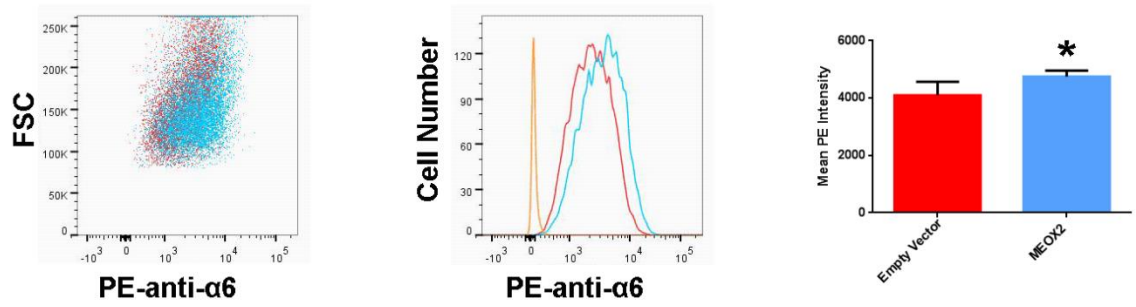

Figure 3.25. MEOX2 overexpression in ECFCs from DM pregnancies increased α6 integrin surface levels. Representative dot plot (left panel), histogram (middle panel), and quantitation of mean fluorescence intensity as a measure of α6 surface levels (right panels). Empty vector-transduced ECFCs are depicted in red, MEOX2-overexpressing ECFCs are depicted in blue, and unstained negative controls are depicted in orange, n= 9 transductions, *p<0.05 by paired *t*-test.

MEOX2 regulates α6 integrin mRNA and total protein expression

The finding that modifying MEOX2 expression levels alters surface expression of the α6 integrin was exciting in that MEOX2 is a transcription factor that directly regulates the expression of several genes (88, 89). Though the α6 subunit has not been reported as a transcriptional target of MEOX2, previous studies have shown that multiple transcription factors, including SP-1, SP-3, and NFI, regulate α6 integrin expression (37, 38). Additionally, the α6 integrin is known to express two isoforms, α6A and α6B. Each of these isoforms has a distinct mRNA transcript, which is generated by alternative splicing (120, 121). The α6A isoform contains 25 exons, while the α6B isoform only contains the first 24 exons, which ultimately produce distinct proteins (120, 121). These protein isoforms differ in their cytoplasmic region (120), however both isoforms form heterodimers with the β1 and β4 subunits, and are present at cell surfaces (122).

Aim 1 studies indicated that MEOX2 was increased in the nucleus of ECFCs from DM pregnancies. Further, Aim 2 studies revealed a correlation between MEOX2 protein and α6 integrin surface levels in ECFCs from DM pregnancies. Given these data, we hypothesized that the α6 integrin may be a transcriptional target of MEOX2. To initially evaluate this possibility, α6 RNA and total protein were examined in ECFCs from control and DM-exposed ECFCs. RT-PCR primers were designed to span exons 5 and 6, enabling detection of both mRNA transcripts. Using RT-PCR, no inherent differences were detected in α6 integrin RNA levels between ECFCs from control and DM pregnancies (Fig. 3.26 A). In the future, it would be interesting to examine mRNA transcript levels

of α6A and α6B individually by designing primers that span exon 25, which is present in the α6A transcript, but not the α6B transcript. Western blotting revealed that ECFCs express both isoforms of the α6 integrin, as indicated by separate protein bands. The upper band of the doublet represents α6A, while the lower band represents α6B (122). Band intensity was quantified for each isoform individually. Interestingly, ECFCs from DM pregnancies had higher α6A total protein compared to ECFCs from control pregnancies, while α6B total protein levels were unaltered (Fig. 3.26 B-D). To test whether reducing MEOX2 expression levels correlated with reductions in α6 integrin RNA and total protein levels, MEOX2 knockdown studies were conducted in DM-exposed ECFCs, as previously described. Reduction of MEOX2 expression in DM-exposed ECFCs decreased α6 RNA as well as the α6A and α6B protein isoforms (Fig. 3.27). These findings are consistent with the hypothesis that MEOX2 may transcriptionally regulate α6 integrin expression, ultimately resulting in differences in mRNA and protein.

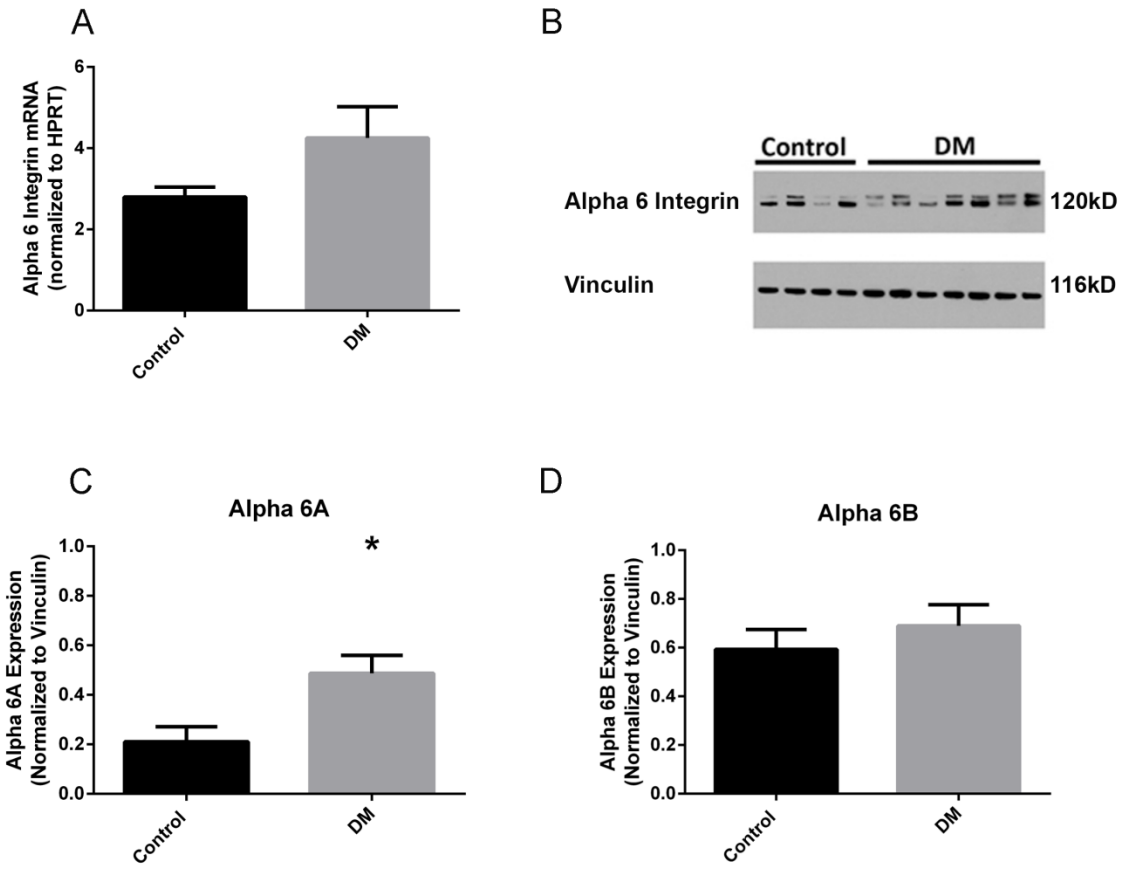

Figure 3.26. ECFCs from DM pregnancies had normal α6 integrin RNA levels, but had increased α6A isoform total protein. (A) Quantitation of α6 integrin RNA in ECFCs from control and DM pregnancies, n=8 control, 11 DM, p>0.05 by unpaired *t*-test. **(B)** Representative western blot. Whole cell lysates from control and DM-exposed ECFCs were separated by SDS-PAGE. Blots were probed for α6 integrin and vinculin, as a loading control. **(C)** Quantification of α6A isoform expression in ECFCs from control and DM pregnancies, n= 11 control, 13 DM, *p<0.05 by unpaired *t*-test. **(C)** Quantification of α6B isoform expression in ECFCs from control and DM pregnancies, n= 11 control, 13 DM, p>0.05 by unpaired *t*-test.

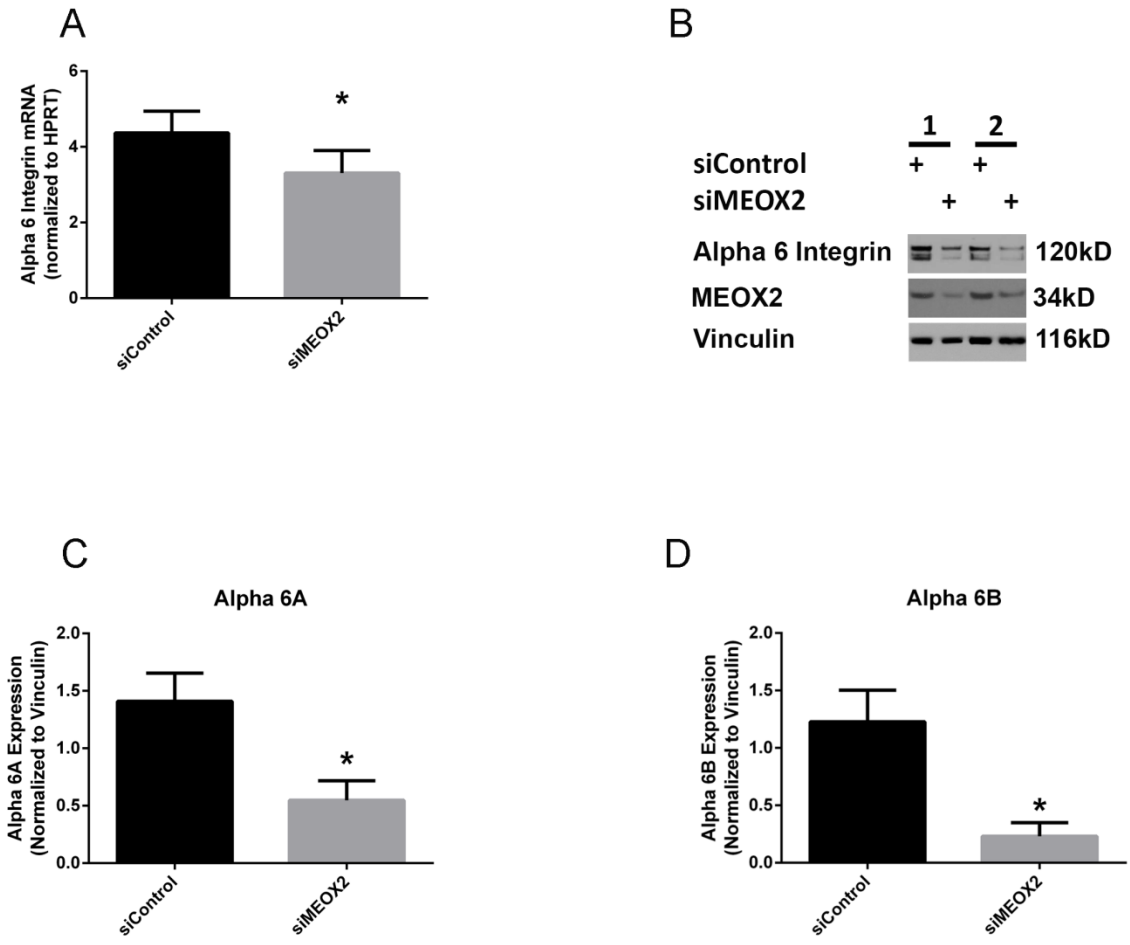

Figure 3.27. MEOX2 knockdown in ECFCs from DM-exposed pregnancies decreased α6 integrin RNA and α6 integrin total protein. (A) Quantitation of α6 integrin RNA in transfected ECFCs from DM pregnancies, n=6 transfections, *p<0.05 by paired *t*-test. **(B)** Representative western blot. Whole cell lysates from transfected ECFCs from DM pregnancies were separated by SDS-PAGE. Blots were probed for α6 integrin, MEOX2, and vinculin, as a loading control. **(C)** Quantification of α6A isoform expression in transfected ECFCs from DM pregnancies, n=6 transfections, *p<0.05 by paired *t*-test. **(C)** Quantification of α6B isoform expression in transfected ECFCs from DM pregnancies, n=6 transfections, *p<0.05 by paired *t*-test.

CHAPTER 4: DISCUSSION

Fetal exposure to a diabetic intrauterine environment can lead to a multitude of adverse effects throughout the life of a child (28, 37-46). While the predisposition for the development of chronic conditions is becoming widely accepted as a point of great clinical concern, the underlying molecular mechanisms contributing to the development of these disorders are largely unknown. In these studies, we found evidence that the transcription factor, MEOX2, is likely involved in regulating vascular network formation.

Previously, in HUVECs MEOX2 was shown to increase cellular senescence, via upregulation of the cyclin dependent kinase inhibitors p16 and p21, and decrease proliferation, suggesting that MEOX2 serves a maladaptive role (88). In control ECFCs, MEOX2 overexpression increased p16 expression and senescence and decreased cell cycle progression. These findings are consistent with data reported in the literature (88). However, knockdown of MEOX2 in ECFCs from DM pregnancies did not alter senescence or cell cycle progression, suggesting that MEOX2 alone is not sufficient to alter senescence or proliferation in cells from DM pregnancies. ECFCs from DM pregnancies were exposed to long-term dynamic metabolic perturbations including hyperglycemia, hyperinsulinemia, and dyslipidemia over the course of 9 months. It is likely that these exposures caused alterations in the expression and/or function of additional proteins, which may also participate in the regulation of senescence and proliferation. Therefore, we speculate that altering MEOX2 levels independently of these additional proteins is not sufficient to induce changes in

the senescent or proliferation phenotypes. Nevertheless, MEOX2 impacted network formation. Specifically, overexpression of MEOX2 in ECFCs from control and DM pregnancies increased network formation. Conversely, MEOX2 knockdown in ECFCs from DM pregnancies resulted in decreased network formation. These data suggest that MEOX2 does regulate ECFC network formation, but this regulation is independent of alterations in senescence or cell cycle progression. Moreover, instead of serving a maladaptive role in vasculogenesis, increased MEOX2 expression in ECFCs from DM pregnancies serves a compensatory mechanism to enhance network formation.

Research addressing the role of MEOX2 in vasculogenesis is limited. Consistent with the data presented here, moderate MEOX2 overexpression positively correlated with network formation in human and mouse BECs (123). Further, MEOX2 depletion in human BECs and *MEOX2* heterozygous mice reduced network formation *in vitro* and *in vivo*, respectively (98, 123). Taken together, these physiologically relevant data also suggest a protective role of MEOX2 in regulating vasculogenesis.

Because our data suggest that MEOX2 regulation of DM-exposed ECFC network formation was not due to alterations in senescence or cell cycle progression, ECFCs were examined for migration differences. Migration is a critical step in establishing vascular networks (56). However, few studies examine the role of MEOX2 in cellular migration. In HUVECs, adenoviral infection with vectors containing either human or rat MEOX2 cDNAs decreased migration, though this phenotype was only observed at high concentrations of

viral particles per cell (93). In contrast, we show that MEOX2 overexpression in ECFCs from control and DM pregnancies increased migration, while MEOX2 knockdown in ECFCs from DM pregnancies reduced migratory function. These data correlate with the observed effects of MEOX2 on ECFC network formation, suggesting a possible connection between MEOX2-regulation of these two phenotypes. In ECFCs, MEOX2 appears to enhance network formation in matrigel by increasing the migratory capacity of ECFCs. Given the apparent discrepancy between previous findings in HUVECs and our data, we speculate that MEOX2 function may differ across cell types (93) or under different experimental conditions. While HUVECs do contain ECFCs (50), the enrichment for ECFCs in the total cell population is highly variable between samples and dependent on culture methods. HUVECs are routinely studied as a source of differentiated endothelial cells, raising the possibility that differentiation status of endothelial cells may affect the role of MEOX2. Here, primary human ECFCs isolated from multiple uncomplicated and DM pregnancies were utilized to ensure reproducibility and to enhance the validity of the results. Given our previous data demonstrating that later passage ECFCs exhibit alterations in function (109), we consistently utilized early passage cells (<6). It is unclear whether a similar approach was taken in prior studies (93). Nevertheless, the consistency of our findings in numerous control and DM-exposed ECFCs support the supposition that MEOX2 has an important role in the regulation of migration and vasculogenesis.

To address the mechanisms responsible for the differences detected in migratory capacity, MEOX2 regulation of adhesion was examined. However, neither MEOX2 overexpression in control ECFCs, nor knockdown in ECFCs from DM pregnancies resulted in differential adhesion on collagen I or laminin. Taken together, these data suggest that differential adhesion is not responsible for the observed differences in ECFC migratory capacity *in vitro.* However, these studies have limitations. Adhesion studies monitored ECFC adhesion on a single ECM in a 2-dimentional setting, without any disturbance of flow. Thus, the results from this experimental setting may not directly correlate and recapitulate an *in vivo* vascular environment. It is conceivable that ECFC adhesion in a 3-dimensional matrix, which is impacted by several ECM proteins, as well as blood flow, would yield differing results. Therefore, we speculate that MEOX2 may regulate adhesion, but we were unable to detect differences due to our experimental system. Future analyses which more closely recapitulates the *in vivo* setting are needed to confirm our studies.

To further explore the mechanism responsible for MEOX2 regulation of migration, integrin expression was examined. Interestingly, MEOX2 knockdown in ECFCs from DM pregnancies resulted in a decrease in α6 integrin surface levels. Further, following MEOX2 knockdown, α6 integrin RNA and total protein levels were reduced, suggesting that MEOX2 may regulate α6 integrin expression in ECFCs.

To examine if MEOX2 was sufficient to alter α6 integrin expression, MEOX2 was overexpressed in ECFCs from control and DM pregnancies.

Interestingly, MEOX2 overexpression in control ECFCs did not alter α6 integrin surface levels. However, MEOX2 overexpression in ECFCs from DM pregnancies increased α6 integrin surface expression. Again, we suspect this apparent discrepancy in MEOX2 overexpression data may be attributed to the fact that ECFCs from DM pregnancies were exposed to many perturbations *in utero*. This chronic environmental stress likely alters the expression and function of additional proteins, potentially including transcription factor binding partners of MEOX2. Additionally, differences in the ratio of heterochromatin versus euchromatin in ECFCs from control and DM pregnancies may exist, thereby altering the availability of the α6 integrin promoter region for binding by transcription factors, including MEOX2. Importantly, a prior study from our lab reported alterations in DNA methylation of regulatory regions of the placenta-specific 8 (*PLAC8*) gene in DM-exposed ECFCs (111). These data suggest that impaired epigenetic regulation in DM-exposed ECFCs may modify transcription factor functionality and gene expression. This same mechanism may explain why MEOX2 overexpression in control versus DM-exposed ECFCs have different effects on α6 integrin expression. Future studies to test this mechanism will be important.

Previous studies in mature endothelial cells demonstrated that reduced α6 integrin expression decreased migration and network formation *in vitro* (85). These phenotypes align with those that we observe upon knockdown of MEOX2 in ECFCs from DM pregnancies. Ultimately, detailed studies that directly assess

ECFC migration and network formation within the context of α6 integrin reduction are warranted.

Importantly, differential MEOX2 expression in humans has important clinical implications for diseases associated with vascular dysfunction (124-126). For example, reduced MEOX2 in heart capillary ECs, human coronary artery ECs, and brain ECs, is associated with increased cardiac dysfunction, coronary heart disease, and Alzheimer's disease, respectively (98, 123, 127, 128). In addition to MEOX2, integrins also play an essential role in the development of these diseases. A loss of β1 integrin in cardiac myocytes can result in cardiomyopathy (129, 130). Furthermore, in Alzheimer's disease, extracellular amyloid plaque composed largely of amyloid-β peptide binds to the α2β1 and αVβ1 heterodimers, promoting neurotoxicity and endothelial cell toxicity (131-133). Because these disorders often result in poor quality of life, enhanced morbidities, and increased mortality, an improved understanding of whether MEOX2 has a direct pathologic role in the development of these diseases is of utmost importance. Our studies in DM-exposed ECFCs provide insight into the role of MEOX2 in the regulation of migration and network formation, as well as α6 integrin expression. It is plausible that MEOX2 may regulate expression and/or function of other integrins in different disease states and cell types. Future mechanistic studies could be applied to other diseases resulting in vascular dysfunction from oxidative stress enabling a more complete understanding of these pathologic conditions, and ultimately resulting in future therapeutic advances.

The overall objective of this thesis was to understand how MEOX2 regulates cell cycle progression and migration, and how this interaction results in impaired vasculogenesis in ECFCs from T2DM pregnancies. Using primary human cells, key etiological factors that contribute to the pathophysiology of cellular dysfunction of ECFCs were examined. The use of primary human cells provides an important translational approach to understand the pathophysiology behind the impaired phenotypes of ECFCs from DM pregnancies. While initially believed to be maladaptive, MEOX2 may serve a protective mechanism, which enables increased vessel formation despite prior exposure to a diabetic intrauterine environment. Ultimately, the overarching objective of futures studies would be to evaluate MEOX2 as a potential therapeutic target to restore vasculogenesis, which would be of tremendous clinical value in preventative care measures.

CHAPTER 5: FUTURE DIRECTIONS

Determine if MEOX2 alters the kinetics of vasculogenesis

The studies presented demonstrate that MEOX2 is both sufficient and required for network formation of ECFCs *in vitro* at an 8 hr time point. While these data provide exciting new insight into a role of MEOX2 during vasculogenesis, significant gaps in knowledge exist regarding the molecular mechanisms responsible. Recently, our laboratory developed a novel software program that enables kinetic analysis of vasculogenesis *in vitro* (59). Using this software, we identified two distinct phases of network formation. Specifically, during Phase 1 (0-5 hr post-plating), ECFCs exhibit enhanced movement to form increased network structures. During Phase 2 (5-10 hr post-plating), network structures stabilize (59). Additionally, we identified a novel measure of network connectivity, the branch-to-node ratio (59).

In the future, more detailed mechanistic analysis of MEOX2 regulation of vasculogenesis could be conducted by utilizing these novel tools. We propose to use time-lapsed video microscopy combined with this software program to determine whether MEOX2 is sufficient and/or required to alter both Phase 1 (early) and 2 (late) of vasculogenesis. Further, we aim to quantify network connectivity using the branch-to node measurement. Distinguishing between Phase 1 and Phase 2 network formation will further elucidate how MEOX2 regulates migration and/or network stability pathways, and provide new mechanistic targets to examine. These, in combination with other measurements,

including the number of closed networks, will enable us to gain a more comprehensive understanding of the role of MEOX2 during vasculogenesis.

Determine whether MEOX2 is sufficient and/or required to alter vessel formation *in vivo*

Here we present compelling data indicating that MEOX2 is both sufficient and required to induce changes in network formation i*n vitro*. However, to further address the potential of MEOX2 to be utilized as a therapeutic target to restore vessel formation, the role of MEOX2 in *in vivo* vasculogenesis must be examined. We hypothesize that MEOX2 overexpression in control ECFCs will result in increased vessel formation *in vivo*, while MEOX2-knockdown in ECFCs from DM pregnancies will decease vessel formation.

To investigate whether MEOX2 expression alters vessel formation *in vivo,* we propose to utilize xenograft transplantation assays. Xenograft transplants of transduced control and DM-exposed ECFCs will be conducted in NOD/SCID mice as previously described (54, 58, 134-137). Transduced ECFCs will be suspended in collagen/fibronectin gel plugs. After polymerization, the gels will be coated with EGM2 media, and then subcutaneously implanted into the flanks of NOD/SCID mice. The right flank of each mouse will be implanted with plugs containing either MEOX2-overexpressing control ECFCs or MEOX2-knockdown DM-exposed ECFCs. The left flank of each mouse will be implanted with plugs containing ECFCs that have been transduced with empty vector controls. On day 14, plugs will be removed, fixed with formalin, sectioned, and stained with mouse anti-human CD31.

Significant progress was made toward achieving these studies. An animal protocol was approved by the Indiana University Institutional Animal Care and Use Committee. Additionally, preliminary studies were conducted to optimize culture and staining conditions.

Determine whether MEOX2 regulates GTPase activity

Data in this thesis suggest that MEOX2 regulates migration of ECFCs. In order for an endothelial cell to migrate, adhesion complexes are dynamically formed and broken down to allow for motility through detachment from the ECM (60, 61, 65-67). This process is largely regulated by the phosphorylation and subsequent activation of small Rho GTPases (Fig. 1.5) (60, 68). We hypothesize that observed differences in migration may be attributed to differential activation of small Rho GTPases. Specifically, MEOX2 overexpression would increase active Cdc42 and Rac. Conversely, MEOX2 knockdown would decrease active Cdc42 and Rac. To test these hypotheses, an immunoprecipitation assay for GTP-bound Cdc42 and Rac in MEOX2 overexpressing and knockdown ECFCs will be conducted. These experiments will further elucidate MEOX2 regulation of migration and may explain the discrepancy between the observed differences in migration, but lack of differences in adhesion.

Determine the mechanism by which nuclear MEOX2 is increased in ECFCs from DM pregnancies

In addition to understanding the role of MEOX2 in regulating the inherent functional differences between ECFCs from control and DM pregnancies, we are also interested in identifying the mechanism behind the upregulation of nuclear

MEOX2 in samples from diabetic pregnancies. The data presented in this thesis suggest that subcellular localization of MEOX2 is altered following exposure to DM *in utero*. Specifically, ECFCs from DM pregnancies have increased nuclear MEOX2 protein compared to ECFCs from control pregnancies (Fig. 3.2). We hypothesize that alterations from the DM intrauterine environment signal for a shuttling of MEOX2 from the cytoplasm to the nucleus. However, the precise stimulus for this movement remains unknown. To address this, we propose to expose ECFCs from control pregnancies to individual conditions routinely found in states of DM, including, hyperglycemia, hyperinsulinemia, hyperlipidemia, inflammatory cytokines, and reactive oxygen species. Following treatment with the aforementioned stimuli, protein will be harvested, and subcellular localization of MEOX2 will be examined. We propose that testing these conditions associated with DM will further elucidate a pathway by which MEOX2 is translocated to the nucleus, and provide a future targeted approach by which to conduct further mechanistic studies.

Determine whether α6 integrin regulates migration and network formation of ECFCs

The data presented suggest that the α6 integrin may be a novel transcriptional target of MEOX2. Previously, α6 integrin was implicated in *in vitro* migration and network formation in endothelial cells (41). Additionally, we also showed that MEOX2 is involved in the regulation of these *in vitro* migration and network formation of ECFCs. In order to deterimine whether the observed

differences in migration and network formation of ECFCs are attributed to α6 integrin levels, we propose to manipulate MEOX2 and α6 integrin protein level.

Initially, we propose to examine the role of the α6 integrin in ECFC migration and network formation. To address this, the α6 integrin will be knocked down in ECFCs from DM pregnancies. Following α6 knockdown, migration and network formation assay will be conducted. We hypothesize that a reduction of the α6 integrin will result in decreased migration and network formation *in vitro*.

If the α6 integrin is found to alter network formation in ECFCs, we next propose to examine the potential role of MEOX2 in regulating the effect of α6 on migration and network formation. For these studies, MEOX2 will be overexpressed in ECFCs from DM pregnancies using a lentiviral approach, leading to high levels of the α6 integrin, as shown in Fig. 3.25. Following MEOX2 overexpression, DM-exposed ECFCs will be transfected with either a non-targeting control or an si-α6 integrin construct to reduce α6 integrin expression. Then, migration and network formation assays will be conducted. We hypothesize this reduction of α6 integrin will result in decreased migration and network formation *in vitro*, providing further confirmation of the role of α6 integrin in MEOX2 regulation of vasculogenesis.

The data presented indicate that MEOX2 knockdown does not result in a complete loss in α6 integrin surface expression, but rather a modest reduction of less than 50%. Therefore, it is critical to account for the degree of α6 integrin knockdown in these studies. Initially, an siRNA approach will be utilized, and several different siRNA constructs against the α6 integrin will be tested. If of the

α6 integrin knockdown using siRNA is not consistent with levels observed in the MEOX2 knockdown studies, an shRNA approach will be used. Using an shRNA which contains a green fluorescent protein (GFP) construct would allow for sorting of low, medium, and high-expressing cells. GFP expression will inversely correlate with α6 integrin expression, and allow for selection of cells with a modest, but not complete reduction of the α6 integrin.

Determine if MEOX2 binds to the α6 integrin promoter

The data in this thesis indicate that MEOX2 regulates expression of the α6 integrin subunit in ECFCs. Because MEOX2 is a known transcription factor, it is interesting to speculate that the α6 integrin may be a previously unrecognized target of MEOX2. In order to determine if MEOX2 binds to the promoter region of the α6 integrin subunit, preliminary ChIP studies were conducted. Potential binding sites with the sequence, ATTA, were identified within the α6 integrin promoter, as this sequence is a known consensus sequence for MEOX2 (88). Additionally, MEOX2 is known to bind to the p16 promoter (88), which was selected as a positive control. DM-exposed ECFCs exhibit reduced proliferation (58), limiting large-scale *in vitro* expansion. To overcome this barrier, MEOX2 overexpression was conducted in control ECFCs for assay optimization. Because ChIP experiments with MEOX2 have not previously been conducted, antibody optimization was required. In these studies, a flag-tagged MEOX2 construct was overexpressed in ECFCs from control pregnancies, enabling the potential for optimization of both a MEOX2-specific and a flag-specific antibody. While the MEOX2-specific antibody used in previous western blotting experiments was not

successful in immunoprecipitating the MEOX2 protein, an antibody against the flag-tag was successful for the MEOX2 immunoprecipitation.

Surprisingly, preliminary studies demonstrate a trend towards increased MEOX2 binding to the α6 integrin promoter following MEOX2 overexpression in control ECFCs (Fig. 5.1 A). Similarly, p16 also indicated a trend toward increased MEOX2 binding to the promoter region (Fig. 5.1 B). These pilot studies provide promising data supporting the possibility that the α6 integrin is a transcriptional target of MEOX2. Future studies will serve to increase the sample size with ECFCs from additional control pregnancies as well as DM-exposed ECFCs to determine if there is significant MEOX2 binding to the α6 integrin promoter. Further, additional sites within the α6 integrin promoter will be examined to determine if MEOX2 binding is specific to one site. The results of these studies will serve to further elucidate the mechanistic role of MEOX2 in regulating α6 integrin expression.

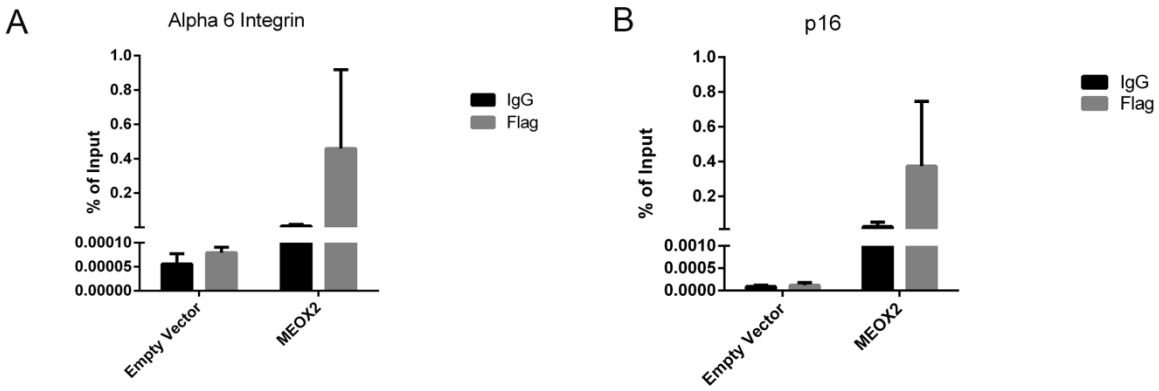

Figure 5.1. MEOX2 overexpression in control ECFCs may increase MEOX2 binding to the α6 integrin and p16 promoters. A flag-tagged MEOX2 construct was overexpressed in ECFCs from control pregnancies. **(A)** Quantitation of MEOX2 binding to the α6 integrin promoter (-650 to -568 upstream of transcription start site), n=3 transductions, p>0.05 by repeated measures 2-way ANOVA. **(B)** Quantitation of MEOX2 binding to the p16 promoter (-508 to -432 upstream of transcription start site), n=3 transductions, p>0.05 by repeated measures 2-way ANOVA.

Identify additional potential transcriptional targets of MEOX2

The studies presented in this thesis indicate that the α6 integrin may be a previoulsy unrecognized transcriptional target of MEOX2. However, it is plausible to suspect that α6 integrin is not the only currently unidentified target of MEOX2. Given our preliminary success in utilizing ChIP methods to verify the α6 integrin as a potential transcriptional target of MEOX2, we propose to utilize ChIP sequencing to identify additional transcriptional targets. For these studies, we propose to utilize lentiviral transduction methods to overexpress a flag-tagged MEOX2 and an empty vector construct in ECFCs from control and DM pregnancies, which will enable immunoprecipitation with an antibody directed against the flag tag, as previously conducted.

Using a ChIP sequencing approach will enable unbiased sequencing to identify numerous potential transcriptional targets of MEOX2. However, in order to verify and narrow these targets, we also propose to utilize a microarray to determine whether expression levels of potential transcriptional targets are altered. We will utilize the same empty vector and flag-tagged MEOX2 transduced ECFCs from control and DM pregnancies. During analysis, we will overlay our findings from our ChIP sequencing studies on the microarry results, to enable identification of genes that have both altered expression and binding of MEOX2. The expression of these targets will then be verified at a protein level. Following verification, these targets will be examined for a funcitonal role in migration and network formation using transwell and matrigel assays.

Using these combined high throughput sequencing approaches will provide invaluable insight of additional transcriptional targets of MEOX2. Overexpressing MEOX2 in both control and DM-exposed ECFCs will enable comparisions of inherent differences in MEOX2 regulation of gene expression given prior exposure to intrauterine DM. Further, it is expected that this workflow will identify additional transcritpional targets of MEOX2, which may be responsible for differences in migration and network formation. In all, this process could reveal additional information regarding the mechanism by which MEOX2 regulates vascuolgenesis in ECFCs.

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2012/01/01. doi: 10.3727/215517911X617888. PubMed PMID: 27004134;

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CURRICULUM VITAE

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EDUCATION

2012	B.S. Biology, Summa Cum Laude Minors: Chemistry, Sociology	Saint Mary's College Notre Dame, IN
2017	Ph.D. Physiology Minor: Life Sciences	Indiana University Indianapolis, IN

AWARDS AND FELLOWSHIPS

2008 Granger Business Association College Scholarship

2008 Senior Year 4-H Scholarship, Indiana 4-H Foundation

2008-2012 Presidential Scholarship, Saint Mary's College

2011 Inducted into Tri Beta National Biological Honor Society

2012 Inducted into Kappa Gamma Pi National Catholic College Graduate Honor Society

2012 Awarded membership to Sigma Xi Scientific Research Society

2012 Indiana University School of Medicine Graduate Division University Fellowship

2015 Indiana Physiological Society Presentation Award

2015 Eli Lilly and Company Science Day Selected Poster

2015 Indiana Clinical and Translational Sciences Predoctoral Fellowship in Translational Research

2016 Indiana Clinical and Translational Sciences Predoctoral Fellowship in Translational Research, Renewal

2016 National Science Foundation Portal to the Public STEM Communications Fellowship

2016 Integrating Women Leaders Conference Scholarship

2016 Association of Women in Science Regional Conference Travel Grant

2016 Indiana University School of Medicine Office of Diversity Affairs Travel Grant

2016 Sigma Xi Graduate Student Biomedical Research Competition 3rd Place

PROFESSIONAL MEMBERSHIPS

2011-Present	Tri Beta National Biological Honor Society
2012-Present	Sigma Xi Scientific Research Society
2014-Present	Indiana Physiological Society
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2015-Present	American Physiological Society

ORAL PRESENTATIONS

Gohn CR and Paetkau D. The effect of *Morinda citrifolia* on the prenatal development of Sprague Dawley rats. Oral presentation, Saint Mary's College Belle Biology Day, April 2012, Notre Dame, IN.

Gohn CR and Haneline LS. MEOX2 regulation of fetal endothelial progenitor cell function. Oral presentation, 6th Annual Meeting of the Indiana Physiological Society, February 2016, Greencastle, IN.

Gohn CR and Haneline LS. MEOX2 regulation of fetal endothelial progenitor cell function. Oral presentation, University of Toledo Graduate Student Association Symposium, April 2016, Toledo, OH.

Gohn CR and Haneline LS. MEOX2 regulation of fetal endothelial progenitor cell function. Oral presentation, Association for Women in Science Regional Conference, September 2016, Notre Dame, IN.

Gohn CR, Blue EK, Varberg KM, and Haneline LS. MEOX2 regulation of fetal endothelial progenitor cell function. Oral presentation, American Developmental Origins of Health and Disease Society Inaugural Meeting, October 2016, Detroit, MI.

POSTER PRESENTATIONS

Gohn CR and Paetkau D. The effect of *Morinda citrifolia* on the prenatal development of Sprague Dawley rats. Poster presentation, Saint Mary's College Belle Biology Day, April 2012, Notre Dame, IN.

Gohn CR and Haneline LS. MEOX2 regulation of fetal endothelial progenitor cell senescence. Poster presentation, 5th Annual Meeting of the Indiana Physiological Society, February 2015, Indianapolis, IN.

Gohn CR and Haneline LS. MEOX2 regulation of fetal endothelial progenitor cell senescence. Poster presentation, North American Vascular Biology Organization Vasculata, August 2015, Charlottesville, VA.

Gohn CR and Haneline LS. MEOX2 regulation of fetal endothelial progenitor cell senescence. Poster presentation, Indiana University Center for Diabetes and Metabolic Diseases Symposium, August 2015, Indianapolis, IN.

Gohn CR and Haneline LS. MEOX2 regulation of fetal endothelial progenitor cell senescence. Poster presentation, 7th Annual Meeting of the Indiana Clinical and Translational Sciences Institute, September 2015, Indianapolis, IN.

Gohn CR and Haneline LS. MEOX2 regulation of fetal endothelial progenitor cell senescence. Poster presentation, Eli Lilly Science Day, September 2015, Indianapolis, IN.

Gohn CR and Haneline LS. MEOX2 regulation of fetal endothelial progenitor cell function. Poster presentation, Indiana Clinical and Translational Sciences Institute Purdue University Retreat, January 2016, West Lafayette, IN.

Gohn CR and Haneline LS. MEOX2 regulation of fetal endothelial progenitor cell function. Poster presentation, Translational Science, April 2016, Washington, D.C.

Gohn CR and Haneline LS. MEOX2 regulation of fetal endothelial progenitor cell function. Poster presentation, Indiana University Pediatric Scholar's Day, April 2016, Indianapolis, IN.

Gohn CR and Haneline LS. MEOX2 regulation of fetal endothelial progenitor cell function. 7th Annual Meeting of the Indiana Physiological Society, February 2017, Indianapolis, IN.

Gohn CR and Haneline LS. MEOX2 regulation of fetal endothelial progenitor cell function. Poster presentation, Translational Science, April 2017, Washington, D.C.

MANUSCRIPTS

Blue EK, Sheehan BM, Nuss ZV, Boyle FA, Hocutt CM, **Gohn CR**, Varberg KM, McClintick JN, and Haneline LS. Epigenetic regulation of Placenta-Specific 8 contributes to altered function of endothelial colony forming cells exposed to intrauterine gestational diabetes mellitus. *Diabetes*, 2015 Jul; 64(7):2664-75. doi: 10.2337/db14-1709. Epub 2015 Feb 26.

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Varberg KM, Naida RO, Blue EK, Chu C, **Gohn CR**, and Haneline LS. Elevated Transgelin 1 contributes to dysfunction in endothelial colony forming cells from gestational diabetic pregnancies. *In preparation*.

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