3-2015

Perinatal Hypoxia Exposure on the Developing Heart: The Role of Endothelin-1

Alexandra Paradis

LOMA LINDA UNIVERSITY
School of Medicine
in conjunction with the
Faculty of Graduate Studies

Perinatal Hypoxia Exposure on the Developing Heart: the Role of Endothelin-1

by

Alexandra Paradis

A Dissertation submitted in partial satisfaction of
the requirements for the degree
Doctor of Philosophy in Pharmacology

March 2015

Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

_____, Chairperson
Lubo Zhang, Professor of Pharmacology

_____
Arlin B. Blood, Associate Professor of Pediatrics, Associate Professor of Physiology

_____
John N. Buchholz, Professor of Pharmacology

_____
Charles A. Ducsay, Professor of Physiology, Gynecology and Obstetrics

_____
Penelope Duerksen-Hughes, Professor of Biochemistry

ACKNOWLEDGEMENTS

I would like to deeply thank Dr. Lubo Zhang for his guidance, support, patience, and investment in me throughout my graduate career. I am grateful for all the opportunities I was given.

I would like to thank my committee members, Dr. Blood, Dr. Buchholz, Dr. Ducsay, Dr. Duerksen-Hughes, and Dr. Zhang for their role in my graduate career. Their guidance and direction has strengthened my research work.

I must thank all my colleagues in the program, especially those in Dr. Zhang's lab, who have been instrumental to my graduate career. I am thankful for their willingness to brainstorm, troubleshoot, and share their expertise with me. I am also grateful for their friendship and encouragement. I will sincerely miss our daily interactions.

Thank you to my parents, my brother and sisters, and of course Samuel, for your encouragement, constant support, and unconditional love. This journey would have been far too daunting without you all to stand by me.

CONTENT

FIGURES

TABLES

ABBREVIATIONS

5-Aza	5-Aza-2'-deoxycytidine
5-mC	5-methylcytosine
Akt	Protein kinase B
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
D2SV	Cyclin D2 splice variant
DNMT	DNA methyltransferase
E18, 21	Embryonic day 18, 21
ECE-	Endothelin converting enzyme
EED	Embryonic ectoderm development
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
ET-1	Endothelin-1
ET_AR	Endothelin receptor subtype A
ET_BR	Endothelin receptor subtype B
Ezh2	Enhancer of zeste 2
FAK	Focal adhesion kinase
FRNK	FAK-related non-kinase
G0	G zero phase, resting phase
G1	Gap 1 phase
G2	Gap 2 phase
HIF-1α	Hypoxia inducible factor 1 α subunit

IUGR	Intrauterine growth restriction
MAPK	Mitogen-activated protein kinase
MMP-	Matrix metalloproteinase
NAC	N-acetyl cysteine
P4, 7, 14	Postnatal day 4, 7, 14
p21, p27	Cyclin-dependent kinase inhibitor 1, 1B
PCNA	Proliferating cell nuclear antigen
PD145065	Non-selective ET-receptor antagonist
PD156707	Selective ET_A-receptor antagonist
PI3K	Phosphoinositide 3-kinase
PRC2	Polycomb repressive complex 2
ROS	Reactive oxygen species
siRNA	Small interfering RNA
TIMP	Tissue inhibitor of metalloproteinase
UBC	Ubiquitin protein C
YAP1	Yes-associated protein 1

ABSTRACT OF THE DISSERTATION

Perinatal Hypoxia Exposure on the Developing Heart: the Role of Endothelin-1

Alexandra Paradis

Doctor of Philosophy, Graduate Program in Pharmacology
Loma Linda University, March 2015
Dr. Lubo Zhang, Chairperson

Heart disease is the leading cause of death worldwide. Numerous epidemiological and animal studies have indicated that an adverse intrauterine environment is associated with increased risk for cardiovascular disease. Therefore proper cardiac development is imperative in optimizing cardiac function throughout life. A key process in determining cardiomyocyte endowment, and thus cardiac function, is the period of terminal differentiation. This involves the maturation of cardiomyocytes and is essential to heart development, however acceleration of this process may alter cardiomyocyte endowment. Hypoxia/anoxia is a major perinatal stressor that often afflicts the fetus as well as the premature infant, and leads to the production of endothelin-1. Our study aims to test the hypothesis that perinatal hypoxia exposure induces a premature terminal differentiation of cardiomyocytes, focusing on the role of endothelin-1 and the underlying epigenetic and molecular mechanisms. We established two rat models for this study: 1) *ex vivo* endothelin-1 treatment of fetal cardiomyocytes, and 2) *in vivo* anoxia episodic treatment of neonatal rats. In the first part of our study, we demonstrated that endothelin-1 exposure promoted premature terminal differentiation of cardiomyocytes. Furthermore, this effect was associated with an increase in global DNA methylation. Our next section of the study simulated the major clinical problem of premature birth and the anoxic episodes that

often accompany it. We demonstrated that early neonatal anoxic episodes decrease cardiomyocyte proliferation during the first two weeks of life. Moreover, this loss of proliferation ultimately resulted in a decrease in cardiomyocyte endowment by day 14 when the heart is essentially mature. Furthermore, the ET_A-receptor appears to be a key mediator of these effects. Lastly, our third section identified several proteins in the fetal cardiomyocyte that were altered due to endothelin-1. Many of the proteins are associated with proliferation and survival and may help elucidate a molecular mechanism for endothelin-1-induced cardiomyocyte maturation. These findings provide new insights in the understanding of hypoxia-induced terminal differentiation of cardiomyocytes and the role of endothelin-1 as well as the epigenetic and molecular mechanisms involved. This study provides supporting evidence of the detrimental effects of perinatal hypoxia/anoxia on cardiac development, and thus function for a lifetime.

CHAPTER ONE

INTRODUCTION

Developmental Programming of Health and Disease

It has been well established that an adverse intrauterine environment is associated with an increased predisposition to cardiovascular disease (Barker, 1995, 1997, 2004). Therefore this timeframe and critical environment is highly influential on the health of an individual across their lifetime. Its influence can lead to structural and functional adaptations of several organs, including the heart. Persistence of these adaptations can increase vulnerability to disease later in life (Barker, 1990, 2004; Botting et al., 2012). A study has shown that adult rats, which were exposed to an adverse environment *in utero*, were more susceptible to ischemic injury when encountering a stress later in life (G. Li et al., 2003).

In terms of the developing heart, an adverse environment may alter cardiomyocyte number and be responsible for this increased susceptibility to cardiovascular disease. In support, animal studies provide evidence that fetal stress caused by hypoxia (Bae, Xiao, Li, Casiano, & Zhang, 2003; Botting, McMillen, Forbes, Nyengaard, & Morrison, 2014), as well as other factors (Bubb et al., 2007; Corstius et al., 2005; Giraud, Louey, Jonker, Schultz, & Thornburg, 2006), affects both the number of cardiomyocytes and the ability of the heart to cope with stress later in life.

Heart Development and Cardiomyocyte Terminal Differentiation

Cardiomyocytes are the functional unit of the heart; therefore the number of viable myocytes dictates cardiac function. The total cardiomyocyte population is

determined early in life during the fetal development and around birth, with negligible increases thereafter (Bergmann et al., 2009). Hence, preservation of cardiomyocyte number will fortify the heart and allow adequate response to stress later in life.

It has long been held that the heart loses proliferative capacity soon after birth in most mammals (Ahuja, Sdek, & MacLellan, 2007; Burrell et al., 2003; Clubb & Bishop, 1984). This timeframe is consistent with the conversion of cardiomyocytes from a mononucleate to binucleate phenotype. Binucleation is a characteristic of terminally differentiated cells that are unable to proliferate, whereas mononucleate cells continue to cycle (Figure 1.1). Early in normal fetal development the majority of cardiomyocytes are mononucleate, allowing growth to be achieved by proliferation. In the timeframe surrounding birth, the heart maturation occurs in which mononucleate cells begin the transition to a binucleate phenotype. The uncoupling of cytokinesis from karyokinesis and ultimate exit of the cell cycle characterize the transition, resulting in binucleation (F. Li, Wang, Capasso, & Gerdes, 1996). Subsequent increases in heart size are independent of proliferation and the result of increases in individual cell size termed hypertrophy.

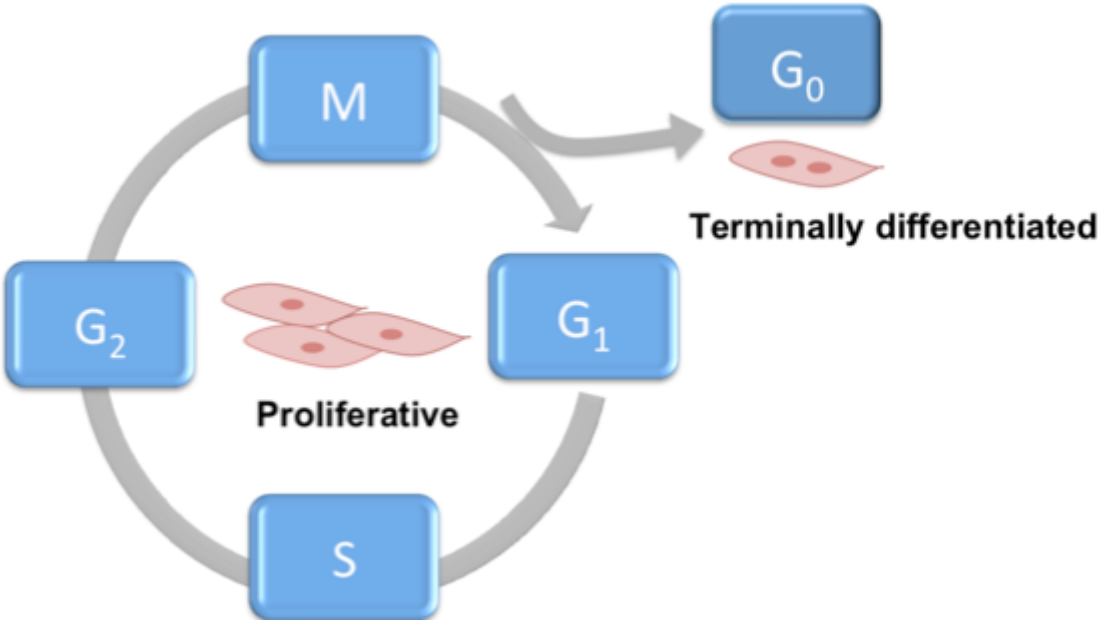

Figure 1.1. Cardiomyocyte terminal differentiation. Early in development, cardiomyocytes are mononucleate and exhibit cell cycle activity. As cardiomyocytes become terminally differentiated, they progressively exit the cell cycle and the percentage of binucleate cardiomyocytes is increased.

In humans, the fetal heart consists of mainly mononucleate cardiomyocytes and thus is the time point in which most proliferation occurs. Prior to birth, binucleation begins and by birth the heart is essentially mature. Similarly, sheep follow this pattern of development, providing a close model for studying the heart. Rodents are another commonly used model however it is to be noted that cardiomyocyte binucleation in rodents begins and ends within the first two weeks after birth (F. Li et al., 1996). In all these species, the adult heart contains the greatest amount of binucleate cells when compared to the fetal and neonatal stage. However the percentage of binucleate cells within the adult heart varies among species, as reviewed by Botting *et. al.* (Botting et al., 2012). In humans, there is considerable debate on the amount of binucleate cells present in the adult heart, with percentages ranging from 25 to 60 (Botting et al., 2012). Rodents and sheep, on the other hand, have approximately 90 percent of the cardiomyocyte population binucleated (Botting et al., 2012).

A rodent model is advantageous in studying heart development in that it allows interventions to be performed after birth but while the heart is still immature. In this way, a more direct effect of treatment on the individual newborn rats can be observed as opposed to treatment performed *in utero.* The rodent heart reaches full maturation at postnatal day 14 thus providing a two-week window for both performing treatment and observing its effects on heart development and maturation. Therefore studying the neonatal rat heart provides a model to study the equivalent maturation process that occurs in both the fetal and preterm human heart.

The loss of proliferation and the increase of binucleate cardiomyocytes characterize this transition to a terminally differentiated state. In the adult heart, the

proliferative capacity of cardiomyocytes is lost, and the heart is known to exhibit only negligible increases in cell number (Bergmann et al., 2009). Thus once this transition occurs, the number of cardiomyocytes that will reside in the heart throughout the lifetime of that individual is determined. While this is a normal transition, if it occurs too early or at an accelerated pace, it may ultimately affect the number of cardiomyocytes endowed in the heart.

At this point, the physiological importance of binucleation is still poorly understood. A plausible explanation is that multinucleation optimizes cellular response, enhancing cell survival when coping with stress (Anatskaya & Vinogradov, 2007). Another argument is that binucleation occurs in order to meet the high metabolic demand of cardiomyocytes. As such binucleation plays an advantageous role in allowing the cell to generate twice the amount of RNA in order to synthesize proteins (Ahuja, Sdek, et al., 2007). Although the role of binucleation is unclear, the percentage of binucleation can be used as a parameter to identify mature cardiomyocytes.

Potential Mechanisms of Terminal Differentiation

The molecular mechanisms responsible for cardiomyocyte binucleation remain unknown. Considering the distinct characteristics between the two cardiomyocyte phenotypes, it is apparent that a marked change in cell cycle activity must occur to achieve binucleation. The process appears to be tightly associated with regulation of the cell cycle, cytokinesis, and epigenetic mechanisms.

Cell Cycle Regulation

Cell cycle regulators are differentially expressed within the mononucleate versus binucleate state. Cardiomyocytes exit the cell cycle following binucleation and as such are terminally differentiated. Cell cycle phases include gap phase 1 (G_1), synthesis phase (S), gap phase 2 (G_2), and mitosis (M). The G_0 phase provides an exit route from the cell cycle in which the cells remain in an indefinite quiescent state. Molecules that determine the rate of growth and proliferation include cyclin-dependent kinases (CDKs) and their inhibitors (CDKIs). CDKs promote the cell cycle whereas CDKIs are known to inhibit the cell cycle (Brooks, Poolman, & Li, 1998). During the fetal development, CDKs are highly expressed within the heart and become downregulated in adulthood. Conversely, the negative regulators of cell cycle, such as CDKIs, are then upregulated in the adult heart (Pasumarthi & Field, 2002). The prominent CDKIs, such as p21, p27, and p57, appear to play a role in the cardiomyocyte arrest of the cell cycle during development (as reviewed in (Brooks et al., 1998)). In neonatal cardiomyocytes, targeting p21 and p27 *via* siRNA knockdown promoted proliferation and progression of cells into the S phase. Furthermore, the proliferation of adult cardiomyocytes was induced with the knockdown of the three CDKIs: p21, p27, and p57 (Di Stefano, Giacca, Capogrossi, Crescenzi, & Martelli, 2011). Maternal hypoxia has also been shown to downregulate cyclin D2 and upregulate p27 expression, associated with a decrease in proliferation of fetal cardiomyocytes (Tong, Xiong, Li, & Zhang, 2013).

A conserved splice variant of cyclin D2, D2SV, has been shown to induce embryonic cardiomyocytes to exit the cell cycle while reducing the capacity to enter the cell cycle. D2SV forms micro-aggregates that sequester cell cycle promoting proteins

such as CDK4, cyclin D2, and cyclin B1, leading to cell cycle exit (Sun, Zhang, Wafa, Baptist, & Pasumarthi, 2009). D2SV expression in the embryonic heart is higher than the adult, contrary to expectations. The role of D2SV in negatively regulating proliferation, underlines the inherent ability of the heart to autoregulate cell cycle activity. This mechanism appears to be essential in optimizing cardiomyocyte number. Maintenance of the balance between promotion and inhibition of the cell cycle is necessary to obtain the full potential of the heart.

Liu *et al.* found that cyclin G1 expression in the mouse heart was low during fetal (E18) and postnatal day 2, and was increased from day 4 on (Z. Liu, Yue, Chen, Kubin, & Braun, 2010). The expression of this cell cycle protein corresponds with the polyploidization of cardiomyocytes. This study demonstrated that overexpression of cyclin G1 stimulated S-phase entry but blocked cytokinesis, the latter exhibiting a stronger effect. By knocking-out cyclin G1, several pro-proliferative factors such as proliferating cell nuclear antigen (PCNA), survivin, aurora B, and mad2 were downregulated, suggesting that cardiomyocytes exited the cell cycle (Z. Liu et al., 2010). Altogether, cyclin G1 expression is associated with cardiomyocyte transition and increases multi-nucleation of these cells.

In rodents, cardiomyocyte transition occurs during the first two weeks of postnatal life. The majority of myocytes are binucleate by postnatal day 7 (P7) (F. Li et al., 1996). A recent study identified a potential candidate regulator involved in this process, FAK-related non-kinase (FRNK) (O'Neill, Mack, & Taylor, 2012). FRNK is an endogenous inhibitor of a major factor in cardiac growth, the focal adhesion kinase (FAK). FRNK expression is increased during the first postnatal week, peaking at P7

through P14. Together with the finding that bromodeoxyuridine uptake is higher in hearts of FRNK null mice, these data implicate the role of FRNK in the suppression of cardiac DNA synthesis in postnatal life. The FRNK null mouse hearts from P14 and P21 also showed significantly elevated levels of Aurora-B, a protein necessary for cytokinesis (O'Neill et al., 2012). The peak expression of this factor is consistent with the time frame in which the majority of cardiomyocyte terminal differentiation occurs, providing evidence that FRNK is a regulatory factor in the maturation of postnatal cardiomyocytes.

In addition, YAP1 is a main target for the Hippo kinase cascade, a key pathway in regulating organ growth. When *Yap1* is inactivated in the fetal heart, lethal hypoplasia and decreased proliferation results (von Gise et al., 2012; Xin et al., 2011). In turn, YAP1 activation promotes proliferation of both fetal and postnatal cardiomyocytes while also activating several cell cycle genes, such as cyclin A2, cyclin B1, and cyclin-dependent kinase 1. Furthermore, the YAP1-induced cardiomyocyte proliferation requires interaction with TEAD transcription factors (von Gise et al., 2012). The targeting of Yap1 and upstream regulation of the kinase pathway leading to its activation appear to be involved in cardiomyocyte terminal differentiation.

Oxidative stress can be induced by hypoxia; the role of reactive oxygen species (ROS) on cardiomyocyte transition was evaluated using the scavenger, N-acetyl-L-cysteine (NAC). *In vivo* treatment of dams with NAC followed by *in vitro* treatment of isolated cardiomyocytes resulted in increased proliferating cell nuclear antigen (PCNA) expression and decreased binucleation. In addition, these NAC-treated cardiomyocytes had decreased expression of p38 MAPK and Connexin43 (Cx43), whereas ROS was

shown to activate p38 MAPK and increase expression of Cx43 (Matsuyama & Kawahara, 2011).

The regulation of cardiomyocyte transition is intricate and subjected to a complex molecular mechanism. The involvement of numerous factors is necessary in maintaining tight control of this significant event in the heart development. Notably, at any time point, both stimulators and inhibitors are modulating the overall cardiomyocyte population. While myocytes are actively proliferating in the developing heart it is important to maintain mechanisms that will prevent excessive hyperplasia. As cardiomyocytes become binucleate, a gradual decrease in proliferative factors and simultaneous increase in inhibitors occurs. This extensive regulation illustrates the significance of maintaining optimal cardiomyocyte number.

Epigenetic Regulation

Epigenetic modifications refer to changes in the expression of genes independent of the DNA sequence. The intrauterine environment has been shown to play an active role in affecting development via epigenetic mechanisms (Barker, 1990, 2004; Webster & Abela, 2007; L. Zhang, 2005) and attributing to long-term adverse effects, known as fetal programming. Initially, an organ can adapt to facilitate immediate survival and functional compensation. However, sustained stress may result in compromised physiology and/or tissue remodeling of an organ.

The epigenetic mechanisms involved in differentiation from progenitor cells to cardiomyocytes have been investigated (Wamstad et al., 2012). However, few studies have focused on the final step, *i.e.* terminal differentiation. It is known that the heart

responds to environmental cues by modifying the epigenome (Patterson, Chen, Xue, Xiao, & Zhang, 2010; Stein et al., 2011), however the specific details of this regulation of cardiomyocyte maturation are lacking.

Polycomb Repressive Complex (PRC)2 is known to be involved in the trimethylation of histone H3 at lysine 27 (H3K27me3). This complex is important in regulating developmental processes and it is involved in suppressing genes leading to cellular differentiation (T. I. Lee et al., 2006). A component of this complex, enhancer of zeste (Ezh)2 is believed to ensure normal cardiac growth and adult activity (Delgado-Olguin et al., 2012). Ezh2 has also been shown to repress negative regulators of the cell cycle such as Ink4a and Ink4b. He *et al.* inactivated the Ezh2 subunit of PRC2 and noted hypoplasia and upregulation of Ink4a/b (He et al., 2012). Another PRC2 component, EED (embryonic ectoderm development) is important for heart development. In fetal cardiomyocytes, inactivation of EED results in perinatal lethal heart defects as well as an upregulation of Ink4a, Ink4b, and other key developmental regulators (He et al., 2012). This data, taken together, implicates the PRC2 complex and its components (Ezh2 and EED) as regulators of proliferation in the heart, potentially *via* epigenetic modifications.

Kou and colleagues investigated other epigenetic mechanisms involved in the maturation of cardiomyocytes (Kou et al., 2010). As noted before, terminal differentiation and binucleation are inversely correlated to proliferation. A significant increase in global methylation in the heart occurs during the neonatal period (Kou et al., 2010), the same time frame for which binucleation occurs. Furthermore, expression of DNA methyltransferases involved in *de novo* DNA methylation (DNMT3a and DNMT3b) was significantly increased during the first 90 days of postnatal life. Inhibition

of methylation with 5-azacytidine during neonatal day 7 and 10 resulted in a marked increase in DNA synthesis and delayed maturation. Histone modifications were also noted. Altogether these changes are associated with the terminally differentiated form of cardiomyocytes, whereas a DNA methylation inhibitor reverted the myocytes to a less differentiated state. This study provides evidence for a role of methylation in both the reduction of proliferation and progression of cardiomyocytes to terminal differentiation. Binucleate myocytes are both non-proliferative and terminally differentiated. Therefore, it is plausible to hypothesize that binucleation might be associated with methylation-induced suppression of proliferation.

The discovery of the epigenome has expanded the possibilities of biological regulation. Epigenetic modifications are employed in a variety of biological processes including fetal programming of disease state as well as normal development. This role in terminal differentiation of cardiomyocytes is of particular interest. These studies provide evidence for the involvement of the epigenome in regulating cardiomyocyte proliferation and maturation. This complex regulation appears to include DNA methylation and histone modifications. With the observation that epigenetics is key in cardiomyocyte maturation the focus should be on further elucidating these intricate mechanisms.

Perinatal Hypoxia and Heart Development

The intrauterine environment is physiologically "hypoxic" and necessary for the development and function of organs in the fetal stage (Giaccia, Simon, & Johnson, 2004; Ream, Ray, Chandra, & Chikaraishi, 2008; Webster & Abela, 2007). However severe hypoxia can be detrimental to organ development (Webster & Abela, 2007), particularly

an essential organ such as the heart. In the light of the field of fetal programming, this perinatal stress may yield life-long detrimental effects on the heart.

Hypoxia is induced under a variety of conditions including nicotine exposure, drug abuse, high altitude pregnancy, preeclampsia, anemia, and placental insufficiency (L. Zhang, 2005). Reduced oxygen to the fetus can affect a number of developmental processes and result in growth restriction (Jensen & Moore, 1997; McCullough, Reeves, & Liljegren, 1977). Previous studies have shown that hypoxia directly reduces proliferation in fetal rat cardiomyocytes, marked by reduced Ki-67 expression, a proliferation marker (Tong et al., 2013). The downregulation of cyclin D2, a cell cycle activator, and upregulation of p27, a cell cycle inhibitor, were associated with reduced proliferation in hypoxia. Hypoxic conditions also upregulated the tissue inhibitor of metalloproteinases, TIMP-3 and -4 (Tong et al., 2013). Upon knockdown, TIMP-3 increased cyclin D2 and Ki-67 in control cardiomyocytes, whereas TIMP-4 had no effect. However, the hypoxia-mediated effects were blocked completely by TIMP-4 and only partially by TIMP-3. This data implicates these inhibitors as potential candidates for enhanced cardiac remodeling and reduced proliferation (Tong et al., 2013). A maternal hypoxia model was also found to increase size and percent of binucleate cardiomyocytes (Bae et al., 2003), as well as induce remodeling of the fetal and neonatal rat heart (Tong, Xue, Li, & Zhang, 2011). A study by Jonker *et al.* using a fetal anemia sheep model reported larger, more mature cardiomyocytes and a marked increase in binucleation compared to control (Jonker et al., 2010).

Several studies have also shown an increase in cell size of neonatal rat cardiomyocytes via hypoxia-induced hypertrophy both *in vitro* (Chu et al., 2012; Ito et

al., 1996) and *in vivo* (Radom-Aizik et al., 2013). Furthermore *in vitro* hypoxia followed by reoxygenation led to premature senescence and reduced proliferation of neonatal rat cardiomyocytes (F. X. Zhang et al., 2007). Altogether these studies suggest that hypoxia stimulates the transition of cardiomyocytes to a terminally differentiated form.

On the contrary, there are a number of studies in support of hypoxia-induced proliferation of cardiomyocytes. In adult zebrafish, hypoxia induced by ventricular amputation has been shown to mediate heart regeneration (Jopling, Sune, Faucherre, Fabregat, & Izpisua Belmonte, 2012), which occurs through the proliferation and dedifferentiation of cardiomyocytes (Jopling et al., 2010). Furthermore, transgenic mice overexpressing hypoxia-inducible factor 1alpha (HIF1α) had reduced infarct size and enhanced cardiac function at 4 weeks after injury with myocardial infarction (Kido et al., 2005). Additionally, neonatal cardiomyocytes treated with C3orf58, a hypoxia and Akt induced stem cell factor (HASF) exhibited increased DNA synthesis and number of cells in mitosis and cytokinesis. Altogether this demonstrates that HASF can induce proliferation of cardiomyocytes, specifically via the PI3K-AKT-CDK7 pathway (Beigi et al., 2013). Therefore these studies indicate a possible dual role of hypoxia in regulating cardiomyocyte proliferation.

Growth restriction *in utero* can also lead to reduced oxygen supply to the fetus. Intrauterine growth restriction (IUGR) is associated with hypoxia (Bae et al., 2003; Moore, 2003; D. Xiao, Ducsay, & Zhang, 2000; L. Zhang, 2005) and has been shown to reduce the percentage of binucleate cardiomyocytes (Bubb et al., 2007). Furthermore, Morrison *et al.* showed that placental restriction leads to chronic fetal hypoxia, which results in increased percentage of mononucleate cardiomyocytes in the fetal sheep. The

restricted fetuses have smaller individual cardiomyocytes but when compared relative to heart weight, the cells were larger (Morrison et al., 2007). The results of this study suggest that placental restriction-induced hypoxia reduces the total number of cardiomyocytes in the heart. Other studies demonstrate that after 20 days of uteroplacental insufficiency, the percentage of binucleate cardiomyocytes as well as cell cycle activity decreased. In addition, fewer mononucleate cardiomyocytes expressed Ki-67, indicating reduced proliferative capacity of these hearts (Louey, Jonker, Giraud, & Thornburg, 2007).

Therefore studies show support for both a positive and negative effect of hypoxia on cardiomyocyte proliferation. This could be due to the differences in the age and species of animal used, as well as methods of hypoxia induction and proliferation quantification. One possible explanation for hypoxia's effect on binucleation is that cardiomyocytes respond by initiating proliferation but are unable to complete cell division and thus become binucleate. Altogether, studies give evidence that hypoxia is a major fetal stressor capable of modulating cardiomyocyte endowment in the heart—ultimately influencing cardiac health throughout life. However the downstream regulators and molecular mechanism of hypoxia-induced terminal differentiation are not yet known.

Endothelin-1

Endothelins and Endothelin Receptors

Endothelin (ET) is a 21-amino acid peptide involved in regulating vascular homeostasis. The endothelium-derived peptide was originally isolated from porcine aortic endothelial cells in 1988 (Yanagisawa, Inoue, et al., 1988). This original peptide was

identified as endothelin-1 with the subsequent discoveries of two other isoforms, endothelin-2 (ET-2) and endothelin-3 (ET-3) (Inoue et al., 1989). Endothelin-1 (ET-1) is currently the most potent vasoconstrictor known (Kawanabe & Nauli, 2011; Yanagisawa, Inoue, et al., 1988; Yanagisawa, Kurihara, et al., 1988) and is mainly secreted from vascular endothelial cells (Agapitov & Haynes, 2002). The ET-2 peptide differs from ET-1 by two amino acids whereas the ET-3 peptide differs by six amino acids (Kedzierski & Yanagisawa, 2001).

As shown in Figure 1.2, the endothelin synthesis pathway begins with the transcription of pre-proendothelin mRNA that is then translated into pre-proendothelin, a 212-residue peptide (Agapitov & Haynes, 2002; Barton & Yanagisawa, 2008). A short secretary sequence is cleaved before furin-like proteases process the prepro-ET into the 38 to 39-residue peptide, big-endothelin (Agapitov & Haynes, 2002; Barton & Yanagisawa, 2008). A number of endothelin converting enzyme isoforms (ECE-1, ECE-2, ECE-3) cleave the big-ET into the functional 21-residue peptide, endothelin (Agapitov & Haynes, 2002; Barton & Yanagisawa, 2008; Gao & Raj, 2010). ECE-1 is mainly found in endothelial cells and ECE-2 is often found in neurons. These two converting enzymes have a higher affinity for big ET-1 versus big ET-2 or big ET-3 (Kedzierski & Yanagisawa, 2001). In addition to ECE, it has been shown that chymase and neprilysin can convert big-ET-1 into its active form ET-1 (Simard et al., 2009). Furthermore, matrix metalloproteinase-2 (MMP-2, gelatinase A) can also convert human big ET-1 (1-38) to ET-1 (1-32), which preferentially acts on ET_BR (Fernandez-Patron, Radomski, & Davidge, 1999; Jeyabalan et al., 2003).

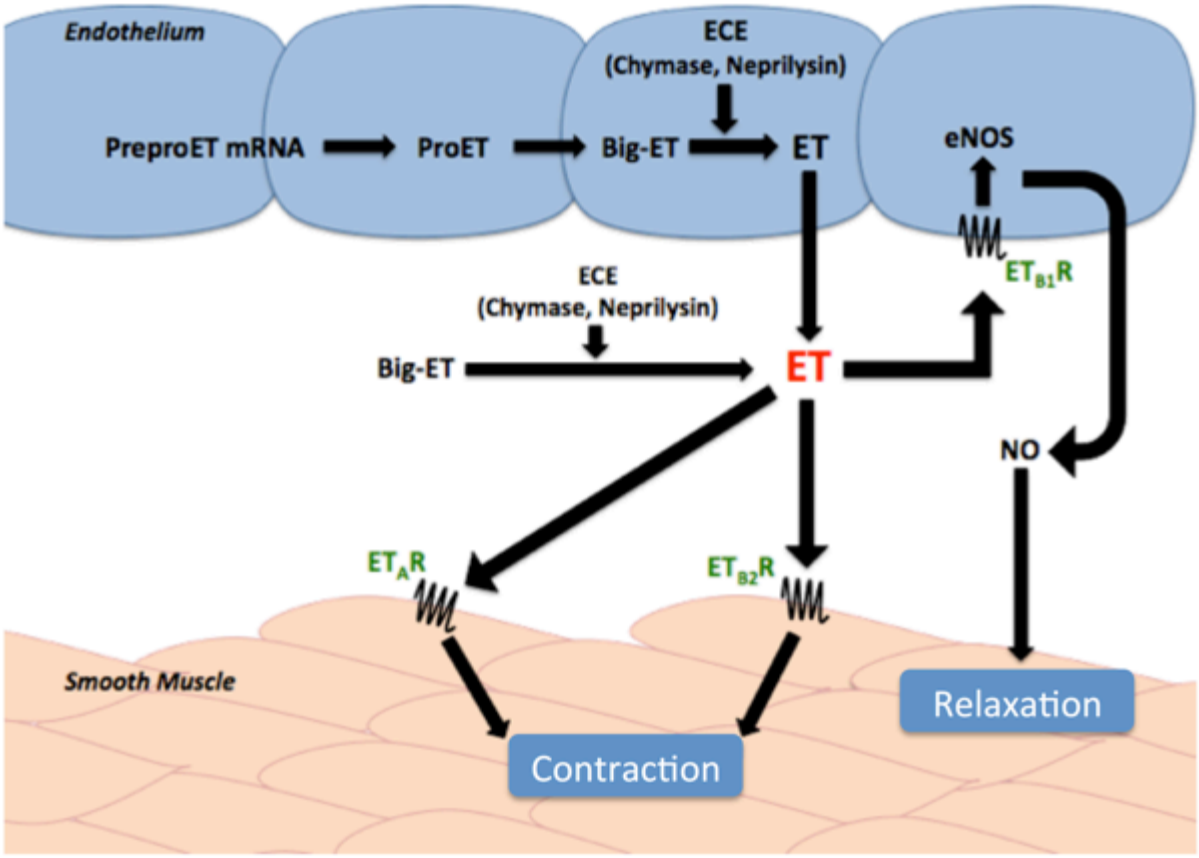

Figure 1.2. Endothelin synthesis and action pathway. Endothelin is mainly secreted by endothelial cells and can act on either the ET_A- or ET_B- receptor to elicit a given response. ET: endothelin; ECE: endothelin-converting enzyme; NOS: nitric oxide synthase; NO: nitric oxide; ET_AR: endothelin A receptor; $ET_{B1}R$: endothelin receptor B subtype 1; $ET_{B2}R$: endothelin receptor B subtype 2.

The ET peptides are strong vasoactive substances that can bind one of two types of G-protein coupled receptors, the ET_A- or ET_B-receptor. The ET_A-receptor (ET_AR) is found in vascular smooth muscle (Hosoda et al., 1991) and therefore associated with the vasoconstrictor effects of ET. It has the highest binding affinity for ET-1 followed by ET-2 and then ET-3 (Arai, Hori, Aramori, Ohkubo, & Nakanishi, 1990; Kawanabe & Nauli, 2011; Sakurai et al., 1990; Yanagisawa, 1994). All three isoforms have equal binding affinity for the ET_B-receptor (ET_BR), which is involved in both vasoconstriction and vasodilatation effects (Arai et al., 1990; Kawanabe & Nauli, 2011; Perreault & Coceani, 2003; Sakurai et al., 1990; Yanagisawa, 1994). Due to the ET_BR's involvement in constriction and dilatation, it has been categorized into two subtypes, ET_B1 and ET_B2 (Kedzierski & Yanagisawa, 2001; Warner, Allcock, Corder, & Vane, 1993). The ET_B1 subtype is mainly found in endothelial cells (Sakurai et al., 1990) regulating the vasodilator effect and ET_B2 is found predominantly in vascular smooth muscle cells (Arai et al., 1990) exerting vasoconstrictor effects along with ET_AR. The ET_BR is also involved in the clearance of endothelins from tissues (Wilkes, Susin, & Mento, 1993). In cardiomyocytes, the ET_A-receptor is the predominant subtype (Kohan, Rossi, Inscho, & Pollock, 2011), and has been implicated to play a role in regulating proliferation (Agapitov & Haynes, 2002; Goldie, 1999; Komuro et al., 1988). In cardiomyocytes, the ET_A-receptor is the predominant subtype (Kohan et al., 2011), and has been implicated to play a role in regulating proliferation (Agapitov & Haynes, 2002; Goldie, 1999; Komuro et al., 1988).

The ET-1 peptide is an autocoid (Wilkes et al., 1993) being that it acts in an autocrine or paracrine fashion rather than as an endocrine (Agapitov & Haynes, 2002;

Rubanyi & Polokoff, 1994; Wagner et al., 1992). Therefore the local tissue concentrations are much higher than the low picomolar concentration typically found in circulation. The endothelial cell is the main site of ET-1 production (Inoue et al., 1989), but cardiomyocytes are also known to secrete ET-1 (Kedzierski & Yanagisawa, 2001). ET-2 is secreted in endothelial cells, heart and kidney (Agapitov & Haynes, 2002), while ET-3 is not produced in endothelial cells (Howard, Plumpton, & Davenport, 1992) but rather in the endocrine, gastrointestinal, and central nervous systems (Agapitov & Haynes, 2002). Endothelin is important in normal physiological processes, especially during growth and development. The general effect of ET is an increase in both blood pressure and vascular tone (Agapitov & Haynes, 2002; Kelly & Whitworth, 1999), as well as reducing cardiac output and heart rate (Kelly & Whitworth, 1999). Although essential to the maintenance of vascular homeostasis, ET-1 can be upregulated in several pathophysiological conditions, such as hypertension, preeclampsia, and heart failure (George & Granger, 2011; Rautureau & Schiffrin, 2012; Wei et al., 1994; Zolk et al., 1999). ET-1 can act as a growth factor and play a key role in tissue development and differentiation and induce proliferation and vascular smooth muscle cell growth (Goldie, 1999). An upregulation of ET-1 (Ponicke et al., 1998; Zolk et al., 1999) and ET_AR and a downregulation of ET_BR were observed in the myocardium of human end-stage heart failure patients (Zolk et al., 1999). There are many stimuli for the secretion of ET-1 including: vasoactive hormones, growth factors, hypoxia, shear stress, ischemia, lipoproteins, free radicals, endotoxin and cyclosporine (Gao & Raj, 2010). Endothelium-derived NO, vasodilators, natriuretic peptides, heparin and prostaglandins, can inhibit ET-1 production (Agapitov & Haynes, 2002).

Hypoxia Induction of Endothelin-1

Hypoxia is a strong inducer of ET expression *via* hypoxia-inducible factor 1-alpha (HIF-1α) (Yamashita, Discher, Hu, Bishopric, & Webster, 2001). HIF-1α is expressed constitutively but becomes degraded in a normoxic environment. However, in a low-oxygen environment, the enzymes leading to its degradation are inhibited and thus HIF-1α can bind to response elements on genes.

The ET-1 gene promoter contains one such hypoxia response element (Hu, Discher, Bishopric, & Webster, 1998; Kakinuma et al., 2001; Minchenko & Caro, 2000). Additionally, previous studies demonstrated a negative correlation between pO_2 and plasma ET-1 levels in fetal goats (Yamada et al., 2001) as well as pO_2 and ET-1 levels in human amniotic fluid (Ostlund, Lindholm, Hemsen, & Fried, 2000). ET-1 mRNA levels were significantly increased in rat lung (H. Li et al., 1994) and placentas (Thaete, Jilling, Synowiec, Khan, & Neerhof, 2007) exposed to hypoxia. The cardiomyocyte is both a site of synthesis and action of ET-1 (Kedzierski & Yanagisawa, 2001; Kohan et al., 2011), suggesting a localized role for hypoxia-induced ET-1 action in the heart.

Central Hypothesis

The central hypothesis of our project is that hypoxia induces ET-1 production, which stimulates a premature terminal differentiation of cardiomyocytes in the developing heart *via* epigenetic modifications.

Significance

It has been well established that an adverse intrauterine environment predisposes

an individual to an increased risk of cardiovascular disease (Barker & Osmond, 1986; Bateson et al., 2004; Gluckman, Hanson, Cooper, & Thornburg, 2008). Hypoxia is a major environmental stress to the developing heart and fetus. A low oxygen environment is a foremost cause of intrauterine growth restriction (Jensen & Moore, 1997; McCullough et al., 1977) and poor organ development. The full impact and mechanism by which hypoxia alters heart development is not yet known. However it is known that the number of cardiomyocytes endowed in the heart is determined during fetal and neonatal development. As the heart matures, the cardiomyocytes undergo a terminal differentiation process and lose their proliferative capacity. Thus the number of cardiomyocytes in the heart is determined during the first two weeks of neonatal life in rats (Botting et al., 2012), a number that persists throughout adulthood. Cardiomyocytes are the functional unit of the heart, and therefore the number of viable myocytes will dictate cardiac function. By modifying cardiomyocyte endowment *via* a premature terminal differentiation, overall cardiac function may be compromised. We expect findings from our study to reveal a novel role of ET-1 in the hypoxia-induced premature cardiomyocyte maturation. Moreover, considering the crucial role the intrauterine environment plays in altering development, the possibility that epigenetic mechanisms are involved in the premature transition provides a mechanistic understanding worthy of future investigation.

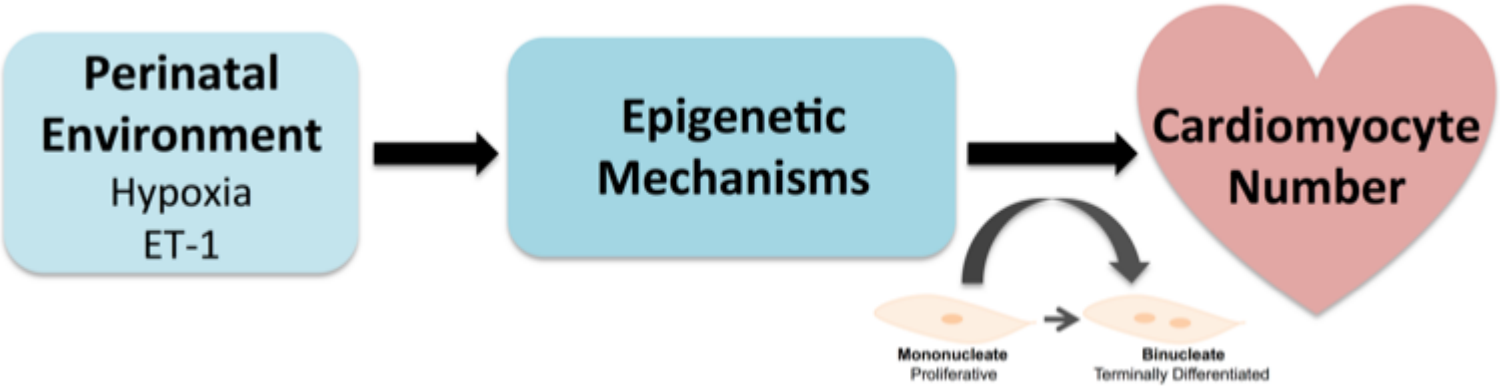

Figure 1.3. Proposed model. Proposed sequence of events in which an adverse perinatal environment induces epigenetic mechanisms, which in turn, alter cardiomyocyte endowment.

CHAPTER TWO

ENDOTHELIN-1 PROMOTES CARDIOMYOCYTE TERMINAL DIFFERENTIATION IN THE DEVELOPING HEART *VIA* HEIGHTENED DNA METHYLATION

By

Alexandra Paradis, Daliao Xiao, Jianjun Zhou, and Lubo Zhang

This paper has been published by International Journal of Medical Sciences. 2014 Feb 20;11(4):373-80.

Abstract

Hypoxia is a major stress on fetal development and leads to induction of endothelin-1 (ET-1) expression. We tested the hypothesis that ET-1 stimulates the terminal differentiation of cardiomyocytes from mononucleate to binucleate in the developing heart. Hypoxia (10.5% O_2) treatment of pregnant rats from day 15 to day 21 resulted in a significant increase in prepro-ET-1 mRNA expression in fetal hearts. ET-1 *ex vivo* treatment of fetal rat cardiomyocytes increased percent binucleate cells and decreased Ki-67 expression, a marker for proliferation, under both control and hypoxic conditions. Hypoxia alone decreased Ki-67 expression and in conjunction with ET-1 treatment decreased cardiomyocyte size. PD145065, a non-selective ET-receptor antagonist, blocked the changes in binucleation and proliferation caused by ET-1. DNA methylation in fetal cardiomyocytes was significantly increased with ET-1 treatment, which was blocked by 5-aza-2'-deoxycytidine, a DNA methylation inhibitor. In addition, 5-aza-2'-deoxycytidine treatment abrogated the increase in binucleation and decrease in proliferation induced by ET-1. Hypoxic stress and synthesis of ET-1 increases DNA methylation and promotes terminal differentiation of cardiomyocytes in the developing heart. This premature exit of the cell cycle may lead to a reduced cardiomyocyte endowment in the heart and have a negative impact on cardiac function.

Introduction

Heart disease is the leading cause of death in the United States. It has been well established that an adverse intrauterine environment increases vulnerability to cardiovascular disease later in life (Barker, 1995; Barker & Osmond, 1986).

Environmental factors during the critical period of fetal development can influence the maturation of organs, such as the heart. Involved in this maturation is a transition of cardiomyocytes from a mononucleate to a binucleate phenotype. This normal transition occurs during fetal and early postnatal life, and is attributed to the uncoupling of cytokinesis from karyokinesis (Clubb & Bishop, 1984). Mononucleate cardiomyocytes retain the ability to proliferate whereas the binucleate cells do not, and this is because they have exited the cell cycle and become terminally differentiated (Ahuja, Sdek, et al., 2007). Alterations to the timing of this critical transition may have long-term consequences on heart development and function throughout life.

Hypoxia is a major stress to the fetal development. Our previous studies have shown that an *in vivo* rat model of maternal hypoxia results in fetal cardiomyocytes prematurely exiting the cell cycle (Bae et al., 2003; Tong et al., 2013; Tong et al., 2011). This early-onset transition leads to fewer but larger cardiomyocytes as a result of increased binucleation and hypertrophy, and decreased proliferation of the cells. The timing of this transition is critical in determining the number of cardiomyocytes endowed in the heart for a lifetime. Whereas these *in vivo* studies showed the effect of hypoxia on fetal heart development, the mechanisms remain unknown.

Hypoxia is a known inducer of endothelin-1 (ET-1) expression (Hashiguchi et al., 1991; Ostlund et al., 2000; Yamada et al., 2001; Yamashita et al., 2001). ET-1 plays an important role in regulating cell cycle, and the cardiomyocyte is both a site of synthesis and action of ET-1 (Kedzierski & Yanagisawa, 2001; Kohan et al., 2011), suggesting a localized role for hypoxia-induced ET-1 action in the heart. Thus, the present study tested the hypothesis that ET-1 induces a premature cardiomyocyte transition in the developing

heart. Given a recent finding that the terminal differentiation of cardiomyocytes is characterized by a hypermethylated genome and compact chromatin (Kou et al., 2010), we further tested the hypothesis that ET-1 promotes cardiomyocyte terminal differentiation by an increase in DNA methylation. Herein, we present evidence that ET-1 *via* action of ET-1 receptors stimulates the premature transition of fetal cardiomyocytes, characterized by increased binucleation and decreased proliferation. DNA methylation of fetal cardiomyocytes is increased with ET-1 treatment, and the ET-1-induced changes in binucleation and proliferation are blocked by a DNA methylation inhibitor 5-aza-2'–deoxycytidine. Altogether the results suggest that epigenetic regulation *via* DNA methylation is involved in the cardiomyocyte transition stimulated by increased synthesis of ET-1.

Materials and Methods

Experimental Animals

Time-dated pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI) and divided into two groups: (i) normoxic control and (ii) 10.5% O_2 hypoxia treatment from gestational day 15 to 21, as previously described (Patterson, Xiao, Xiong, Dixon, & Zhang, 2012; Xue, Dasgupta, Chen, & Zhang, 2011). Hearts were isolated from day 21 fetuses. To isolate hearts, pregnant rats were anesthetized with isoflurane, and adequate anesthesia was determined by loss of pedal withdrawal reflex. Fetuses were removed and pregnant rats killed by removing the hearts. Fetal hearts were isolated for the studies. All procedures and protocols were approved by

the Institutional Animal Care and Use Committee and followed the guidelines by US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Primary Cardiomyocyte Culture and Treatment

Cardiomyocytes were isolated from normoxic day 21 fetal rats as previously described (Y. Xiao, He, Gilbert, & Zhang, 2000). Cells were cultured in Hyclone Medium 199 (Thermo Scientific) supplemented with 10% fetal bovine serum (Gemini Bio-Products) and 1% antibiotics (10,000 I.U./mL penicillin, 10,000 µg/mL streptomycin) at 37°C in 95% air/5% CO_2. BrdU (0.1mM) was added to the medium to prevent fibroblast proliferation. Within three days of culture, the cells formed a monolayer with synchronized beating, characteristic of viable cardiomyocytes. Experiments were performed at 70-80% confluency. Cells were treated under normoxia (21% O_2) or hypoxia (1% O_2) for 24 hours, in the absence or presence of ET-1 (Sigma; 10 nM), PD145065 (Calbiochem; 10 nM), or 5-aza-2'-deoxycytidine (Sigma; 10 µM).

Real-Time Reverse Transcription-Polymerase Chain Reaction

RNA was isolated from the fetal hearts and prepro-ET-1 mRNA abundance was determined by real-time RT-PCR using Icycler Thermal cycler (Bio-Rad), as described previously (Xue et al., 2011). Reverse transcription and cDNA synthesis was performed using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). The primers are 5'-CTAGGTCTAAGCGATCCTTGAA-3' (forward) and 5'-CTTGATGCTGTTGCTGATGG-3' (reverse). PCR was performed in triplicate, and threshold cycle numbers were averaged.

Immunocytochemistry

Primary cardiomyocytes were double stained with alpha-actinin, a cardiomyocyte marker, and Ki-67, a proliferation marker as described previously (Tong et al., 2013). Cardiomyocytes were plated on coverslips and fixed with acetone for 10 minutes. The cells were blocked with 1% bovine serum albumin for 1 hour at room temperature before incubation with the primary antibodies: mouse anti-α-sarcomeric actinin (Sigma, St.Louis, MO) (1:200) and rabbit anti-Ki-67 (Abcam, Cambridge, MA) (1:100) in 4°C overnight. The samples were incubated with the secondary antibodies: anti-mouse FITC-conjugated and anti-rabbit Texas Red-conjugated antibodies for 1 hour at room temperature. Nuclei were stained with Hoescht (Sigma) for 1 minute. The immunofluorescence staining was assessed using a Zeiss Axio Imager.A1 microscope and quantitative analysis was carried out using Image J software. Percent binucleation, Ki-67 expression, and cell size were measured.

5-mC DNA Enzyme-linked Immunosorbent Assay (ELISA)

DNA methylation in primary fetal cardiomyocytes was determined by measuring 5-methylcytosine (5-mC) using a 5-mC DNA ELISA kit (Zymo Research). The kit features a unique anti-5-mC monoclonal antibody that is both sensitive and specific for 5-mC. The protocol for measurement of 5-mC level is described in the manufacturer's instruction. Briefly, 100 ng of genomic DNA from cardiomyocytes and standard controls provided by the kit was denatured and used to coat the plate wells with 5-mC coating buffer. After incubation at 37°C for 1 hour, the wells were washed with 5-mC ELISA buffer and then an antibody mix consisting of anti-5-mC and a secondary antibody was

added to each well. The plate was covered with foil and incubated at 37°C for 1 hour. After washed out the antibody mix from the wells with 5-mC ELISA buffer, a HRP developer was added to each well and incubated at room temperature for 1 hour. The absorbance at 405 nm was measured using an ELISA plate reader. The percent 5-mC was calculated using the second-order regression equation of the standard curve that was constructed with negative control and positive controls in the same experiment.

Statistical Analysis

Data are expressed as means ± SEM. Statistical analysis ($p < 0.05$) was determined by analysis of variance followed by Neuman-Keuls *post hoc* test or Student's t test, where appropriate.

Results

Maternal Hypoxia Increased Prepro-ET-1 mRNA in Fetal Hearts

Animals were exposed to maternal hypoxia from gestational day 15-21; at the end of treatment hearts were isolated from day 21 fetal rats. Figure 2.1 demonstrated a significant increase in prepro-ET-1 mRNA abundance in fetal hearts exposed to 10.5% O_2, as compared to the normoxic control (21% O_2).

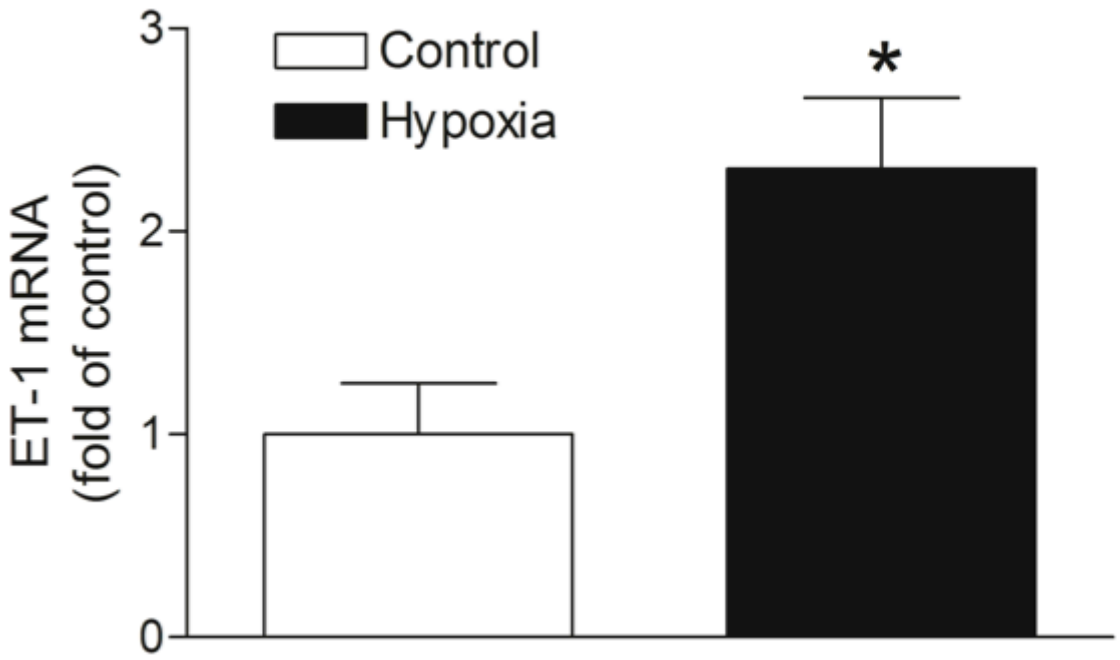

Figure 2.1. Effect of hypoxia on prepro-ET1 mRNA in the fetal heart. Hearts were isolated from near-term fetuses of pregnant rats treated with control or hypoxia. mRNA abundance of prepro-ET-1 was determined by real-time RT-PCR. Data are means ± SEM. * $P < 0.05$, hypoxia *vs*. control. n = 7-8

ET-1 Stimulated Binucleation and Inhibited Proliferation of Fetal Cardiomyocytes

The morphology of mononucleate cells and binucleate cells of primary fetal cardiomyocytes are shown in Figure 2.2A. Whereas the basal levels of binucleate cardiomyocytes in fetal hearts were low, the treatment of cardiomyocytes with ET-1 resulted in a significant increase in percent binucleation, as compared to the control in both normoxic and hypoxic conditions (Figure 2.2A). Hypoxia alone in the absence of ET-1 had a slight increase in percent binucleation but it did not reach a significant level, as compared to the normoxic control. Percent Ki-67 positive cells, indicating proliferation, in ET-1 treated cardiomyocytes were significantly decreased in both normoxia and hypoxia (Figure 2.2B). Unlike the effect of binucleation, hypoxia alone in the absence of ET-1 significantly decreased percent of Ki-67 positive cells (Figure 2.2B).

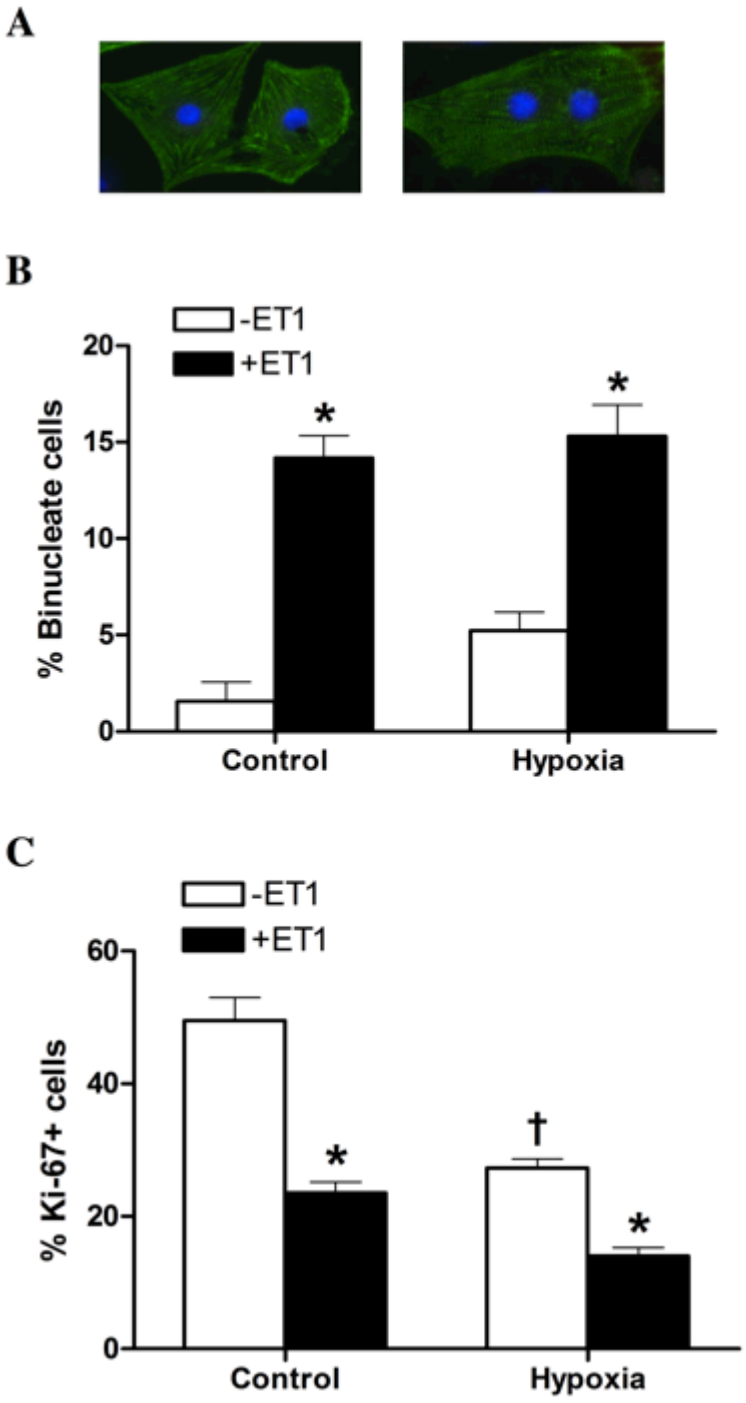

Figure 2.2. Effect of ET-1 on binucleation and proliferation of fetal cardiomyocytes. Cardiomyocytes isolated from fetal hearts were treated with ET-1 (10 nM) under normoxic control (21% O_2) or hypoxic (1% O_2) conditions for 24 h. **A**. Morphology of mononucleate and binucleate fetal cardiomyocytes. **B**. Binucleation result. **C**. Proliferation result. Data are means ± SEM. * $P < 0.05$, +ET-1 *vs*. -ET-1; † $P < 0.05$, hypoxia *vs*. control. n = 5

Interaction of ET-1 and Hypoxia Decreased Cardiomyocyte Size

Neither ET-1 nor hypoxia alone had a significant effect on cardiomyocyte size (Figure 2.3). However, cardiomyocyte size was significantly decreased with the ET-1 treatment under the hypoxic condition (Figure 2.3).

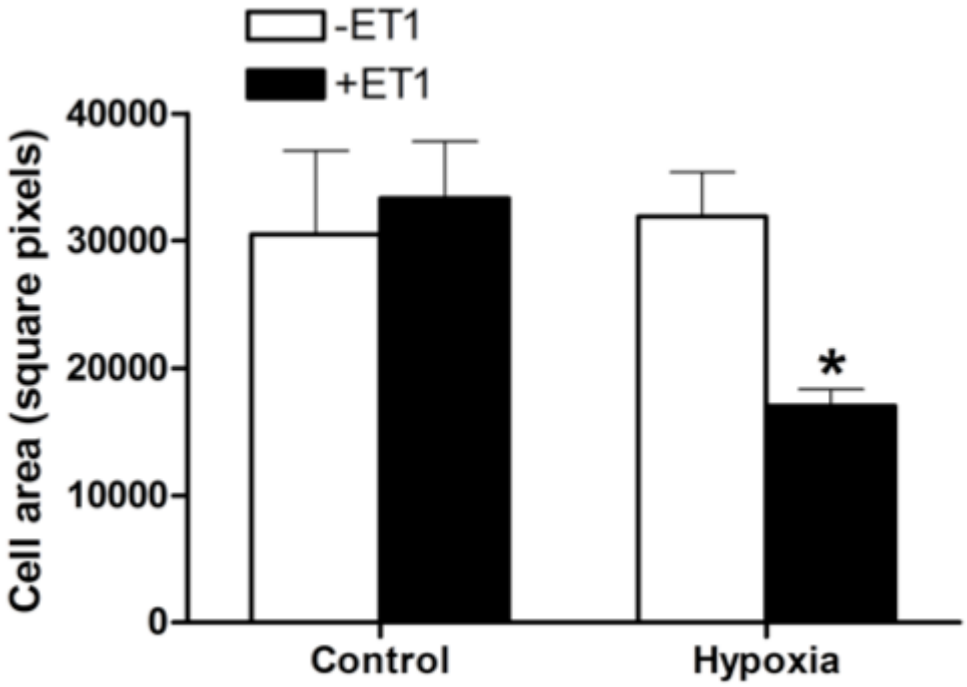

Figure 2.3. Effect of ET-1 on fetal cardiomyocyte size. Cardiomyocytes isolated from fetal hearts were treated with ET-1 (10 nM) under normoxic control (21% O_2) or hypoxic (1% O_2) conditions for 24 h. Data are means ± SEM. * $P < 0.05$, +ET-1 *vs*. -ET-1. n = 7-10

PD145065 Inhibited the Effects of ET-1

PD145065, a non-selective ET-receptor antagonist, blocked the effects of ET-1 on percent binucleation (Figure 2.4A) and Ki-67 expression (Figure 2.4B) in fetal cardiomyocytes. PD145065 in the absence of ET-1 had no significant effect on either binucleation or proliferation of cardiomyocytes.

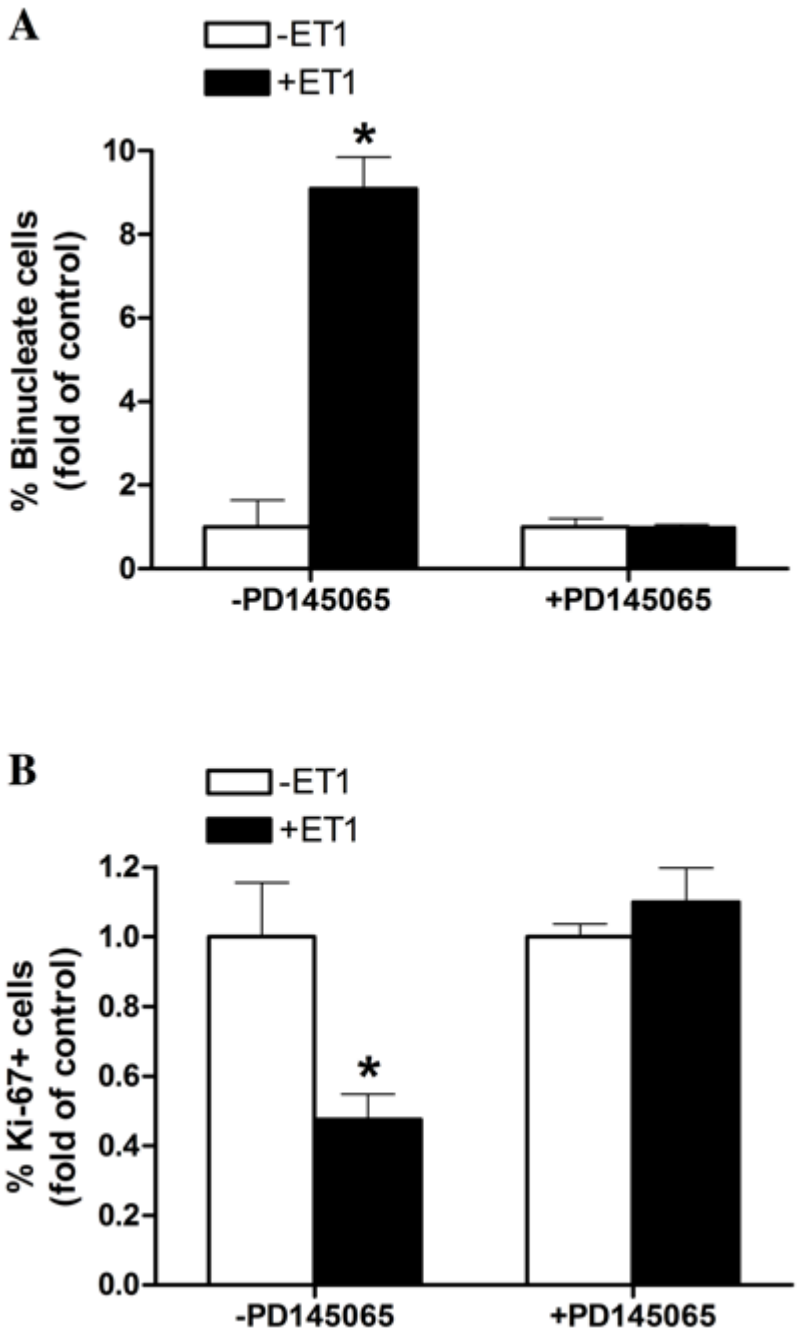

Figure 2.4. PD145065 abrogates ET-1-mediated effects on binucleation and proliferation of fetal cardiomyocytes. Cardiomyocytes isolated from fetal hearts were treated with ET-1 (10 nM) for 24 h in the absence or presence of PD145065 (10 nM). **A**. Binucleation result. **B**. Proliferation result. * $P < 0.05$, ET-1 *vs*. control. n = 5

ET-1 Increased DNA Methylation in Fetal Cardiomyocytes

ET-1 treatment of fetal cardiomyocytes resulted in a significant increase in DNA methylation, seen as increased percent 5-mC in Figure 2.5. In the presence of 5-aza-2'-deoxycytidine, a DNA methylation inhibitor, the effects of ET-1 were blocked (Figure 2.5).

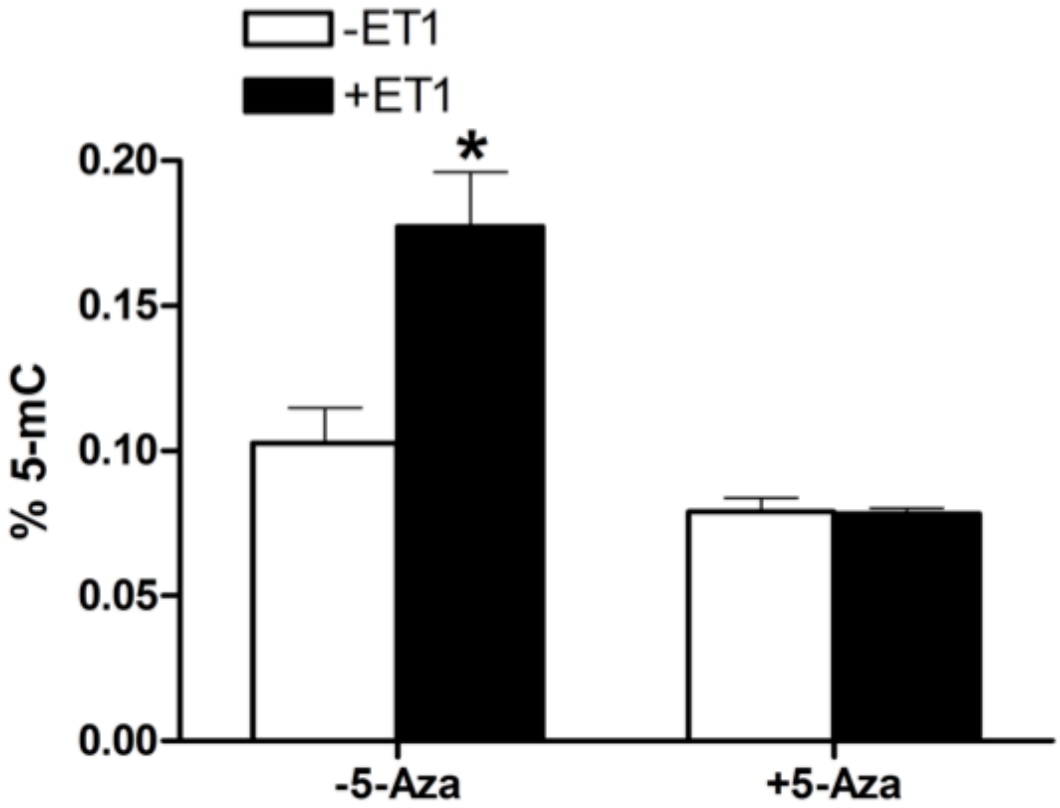

Figure 2.5. 5-Aza-2'-deoxycytidine blocks ET-1-increased DNA methylation in fetal cardiomyocytes. Cardiomyocytes isolated from fetal hearts were treated with ET-1 (10 nM) for 24 h in the absence or presence of 5-aza-2'-deoxycytidine (5-Aza, 10 μM). * $P < 0.05$, ET-1 *vs*. control. n = 5

5-Aza-2'-deoxycytidine Abrogated the Effects of ET-1 on Fetal Cardiomyocytes

In the presence of 5-aza-2'-deoxycytidine, ET-1-induced stimulation of binucleation (Figure 2.6A) and inhibition of Ki-67 expression (Figure 2.6B) in fetal cardiomyocytes were blocked. Whereas 5-aza-2'-deoxycytidine alone in the absence of ET-1 had a tendency to increase cardiomyocyte binucleation, this effect did not reach the significant level (Figure 2.6A).

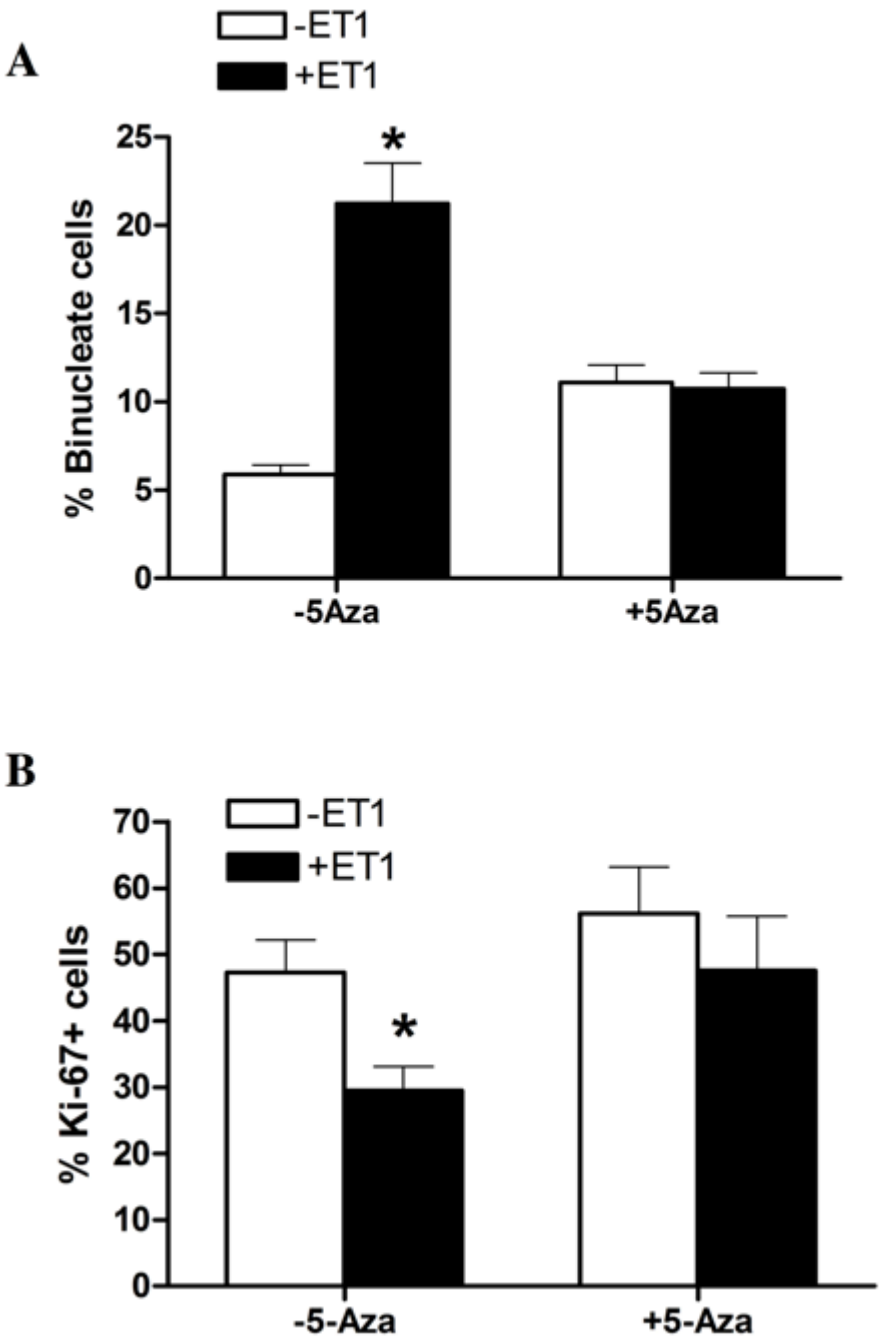

Figure 2.6. 5-Aza-2'-deoxycytidine abrogates ET-1-mediated effects on binucleation and proliferation of fetal cardiomyocytes. Cardiomyocytes isolated from fetal hearts were treated with ET-1 (10 nM) for 24 h in the absence or presence of 5-aza-2'-deoxycytidine (5-Aza, 10 μM). **A**. Binucleation result. **B**. Proliferation result. * $P < 0.05$, ET-1 *vs*. control. n = 5

Discussion

The present study provides evidence that ET-1 inhibited proliferation and induced the premature transition of fetal cardiomyocytes from a mononucleate to a binucleate phenotype, indicative of terminally differentiated cardiomyocytes. Hypoxia alone did not elicit the same effects as ET-1. An ET-receptor antagonist, PD145065 blocked the ET-1-induced increase in binucleation and decrease in proliferation. Additionally, we demonstrated that ET-1 treatment increased DNA methylation in fetal cardiomyocytes, and a DNA methylation inhibitor, 5-aza-2'-deoxycytidine abrogated ET-1-induced DNA methylation and terminal differentiation of cardiomyocytes.

The ET-1 synthesis pathway begins with the transcription of prepro-ET-1 mRNA, which is translated into prepro-ET-1. A series of enzymatic cleavages produce Big-ET-1 and the matured ET-1, a 21-amino acid peptide (Barton & Yanagisawa, 2008). Several studies have reported the regulation of ET-1 synthesis by hypoxia, including the identification of a HIF-1 binding site on the prepro-ET-1 gene promoter (Hu et al., 1998; Minchenko & Caro, 2000). Specifically in cardiomyocytes, a HIF-1α binding site has been identified on the 5'-promoter region of the prepro-ET-1 gene (Kakinuma et al., 2001). Additionally, previous studies demonstrated a negative correlation between pO_2 and plasma ET-1 levels in fetal goats (Yamada et al., 2001) as well as pO_2 and ET-1 levels in human amniotic fluid (Ostlund et al., 2000). ET-1 mRNA levels were significantly increased in rat lung (H. Li et al., 1994) and placentas (Thaete et al., 2007) exposed to hypoxia. In agreement with these findings, the present study demonstrated a significant increase in prepro-ET-1 mRNA in the fetal rat heart resulting from *in utero*

hypoxia, suggesting a local paracrine action of ET-1 in hypoxia-mediated effect on the fetal heart.

Physiological circulating levels of ET-1 are in the low picomolar range (Kohan et al., 2011; Nakas-Icindic, Zaciragic, Hadzovic, & Avdagic, 2004) and may be significantly upregulated in pathophysiological conditions, such as hypoxia, heart failure, hypertension, and preeclampsia (George & Granger, 2011; Rautureau & Schiffrin, 2012; Wei et al., 1994; Zolk et al., 1999). ET-1 acts in a paracrine and/or autocrine fashion and therefore tissue concentrations are significantly higher than those in the circulation (Rubanyi & Polokoff, 1994). The concentration of ET-1 (10 nM) was chosen based on other studies (Ito et al., 1991; X. Li et al., 2009; Majumdar et al., 2009; Yu et al., 2013) and the rationale that ET-1 levels in the local tissue are much greater than in circulation. In the present study, we found that fetal rat cardiomyocytes exposed to elevated levels of ET-1 exhibited both increased binucleation and decreased proliferation. The binucleate cells are unable to proliferate and thus become terminally differentiated. In the rat heart, normal transition of cardiomyocytes to the binucleate form starts at birth and lasts during the first two weeks of postnatal life (Clubb & Bishop, 1984). Therefore, the accelerated transition in the fetal heart due to increased levels of ET-1 has long-term implications. A premature transition of terminal differentiation may result in a reduced number of cardiomyocytes and altered cardiac growth after birth. As previous work has shown, hypoxia causes a premature exit of cell cycle in fetal cardiomyocytes (Bae et al., 2003), but the downstream regulators are not known.

The present study demonstrated that ET-1 increased the percent of binucleate cells independent of hypoxia, and hypoxia alone had no significant effect on the

binucleation of cardiomyocytes. These findings suggest a lack of direct effect of hypoxia on the cardiomyocyte transition and provide evidence that ET-1 is a key downstream regulator of the premature exit of cell cycle in the fetal heart, observed *in vivo* in fetal hypoxia (Bae et al., 2003). It is important to note that while cardiomyocytes have the ability to produce ET-1, endothelial cells contribute a large portion of its production. The isolated cardiomyocytes may not provide the full mechanism for hypoxia to produce a sufficient ET-1 response and the subsequent effects on binucleation and cell size. The finding that PD145065 blocked the ET-1-induced increase in binucleation and decrease in proliferation of fetal cardiomyocytes indicates the ET-1 receptor-mediated effects. PD145065 is a non-selective ET_A- and ET_B-receptor antagonist and has been shown to block the effect of ET-1 *via* the ET-receptors (Ceccarelli et al., 2003; Doherty et al., 1993; Drimal et al., 2003).

The finding that hypoxia decreased proliferation of fetal cardiomyocytes is in agreement with previous reports (Bae et al., 2003; Tong et al., 2013; Tong et al., 2011). Prior studies have shown that hypoxia promotes HIF-1α association with HIF-1β and enhances the expression of cyclin-dependent kinase inhibitors (CKIs), which in turn inhibits cell cycle proteins and decreases cell proliferation (Goda, Ryan, et al., 2003). It is also suggested that HIF-1 regulates the G1/S phase transition by regulating the expression of cyclin E, a required factor for the transition (Goda, Dozier, & Johnson, 2003). Hypoxia has also been shown to induce expression of metalloproteinase inhibitors (TIMPs) that may have an inhibitory or stimulatory effect on cellular proliferation depending on the subtype and tissue involved (Tong & Zhang, 2012). In the heart, TIMP-3 is highly expressed and shown to inhibit proliferation in neonatal mouse cardiomyocytes

(Hammoud et al., 2007); a result of up-regulated p27 expression *via* the EGFR-JNK-SP-1 mediated pathway (Hammoud, Burger, Lu, & Feng, 2009). Although TIMP-3 and -4 are upregulated by hypoxia, their promoters do not contain HIF-responsive elements. Thus the regulation of these inhibitors and the subsequent effects on proliferation appear to be mediated by other genes that contain HIF-responsive elements.

ET-1 is one possible candidate considering it both contains a HIF-response element in its promoter (Hu et al., 1998; Minchenko & Caro, 2000) and has been shown to regulate proliferation (Bae et al., 2003; Tong et al., 2013; Tong et al., 2011; F. X. Zhang et al., 2007). The finding that, unlike ET-1, hypoxia had no significant effect on binucleation suggests that hypoxia-induced effect on proliferation was not mediated by ET-1, but rather by an independent and direct effect of hypoxia. The synergistic effect of hypoxia and ET-1 more closely mimics the physiological system as a whole. Ki-67 expression and cell size were significantly decreased by hypoxia and ET-1 treatment together. These results agree with previous reports, from our lab and others, that hypoxia alone had a direct effect in decreasing proliferation of cardiomyocytes (Bae et al., 2003; Tong et al., 2013; Tong et al., 2011; F. X. Zhang et al., 2007).

A change in cell size was only observed with the addition of both ET-1 and hypoxia. Both ET-1 and hypoxia are known hypertrophic factors (Ito et al., 1996; Shubeita et al., 1990; Suzuki, Hoshi, & Mitsui, 1990). In neonatal rat cardiomyocytes, mild hypoxia (10% O_2) has been found to induce hypertrophy (Chu et al., 2012; Ito et al., 1996). However more severe hypoxia (1% O_2), as was done in our study, appears to elicit the opposite effect leading to a reduction in cell size. Thus the severity at which hypoxia is induced likely has a differential effect on changes in cellular size. ET-1 has also been

shown to stimulate cardiomyocytes to proliferate, and in the case of terminally differentiated cells it leads to hypertrophy (Cullingford et al., 2008; Ito et al., 1991). Furthermore previous studies have found that hypertrophic growth is initially observed in the first week of postnatal life (Clubb & Bishop, 1984; F. Li et al., 1996). Given that fetal and neonatal hearts are at very different developmental stages and experience very different oxygen tensions, it is possible that cardiomyocytes of the fetal heart respond differently to environmental cues such as hypoxia as that seen in the neonatal cardiomyocytes.

The finding that 5-aza-2'-deoxycytidine blocked the ET-1-induced increase in binucleation and decrease in proliferation is intriguing and suggests that ET-1 induces methylation of DNA as a means of involvement in cardiomyocyte terminal differentiation and suppression of proliferation. Whereas the present study focused on the downstream mechanisms of ET-1 in regulating terminal differentiation of cardiomyocytes, whether DNA methylation plays a role in the hypoxia-mediated direct effect on proliferation remains to be determined. 5-Aza-2'-deoxycytidine has been widely used as a DNA methylation inhibitor, and in the concentration range of 1 to 30 μM it inhibits DNA methylation both globally and at specific sites of DNA (Meyer, Zhang, & Zhang, 2009; Patterson et al., 2010; Vallender & Lahn, 2006; F. Xiong, Xiao, & Zhang, 2012). In the present study, we found that ET-1 significantly increased global DNA methylation in cardiomyocytes and this was blocked by 5-aza-2'-deoxycytidine. Epigenetic mechanism of DNA methylation acts to silence gene transcription, typically at cytosine residues within CpG dinucleotides. A previous study showed that methylation gradually increases over the course of development in neonatal cardiomyocytes (Kou et al., 2010), the same

time frame for which binucleation occurs. Furthermore, expression of DNA methyltransferases involved in *de novo* DNA methylation (DNMT3a and DNMT3b) was significantly increased during the first 90 days of postnatal life. Inhibition of methylation with 5-aza-2'-deoxycytidine during neonatal day 7 and 10 resulted in a marked increase in DNA synthesis and delayed maturation (Kou et al., 2010). It is well known that environmental cues during fetal development can profoundly alter the structure and function of an organ *via* epigenetic regulation. Particularly in the heart, cardiac function is dependent in part on cardiomyocyte number. Thus hypoxia-mediated ET-1 may signal through epigenetic mechanisms to negatively impact cardiomyocyte development. The present study suggests that DNA methylation is an epigenetic mechanism through which ET-1 stimulates cardiomyocyte transition of terminal differentiation. Ultimately, this may lead to reduced total cardiomyocyte number in the heart. Many studies have demonstrated that genes associated with the cell cycle and cytokinesis are involved in this transition process (Ahuja, Perriard, et al., 2007; Chen et al., 2004; Engel, Schebesta, & Keating, 2006; Y. Liu et al., 2007; Sdek et al., 2011). Adult cardiomyocytes from knockout mice lacking Rb and p130 show a decrease in heterochromatin and an increase in proliferation associated with derepression of cell cycle genes (Sdek et al., 2011). These genes may be differentially regulated by changes in methylation patterns thus altering the cell cycle and cytokinesis. Future studies will have to elucidate the methylation status of specific genes during this transition phase.

The present study identifies a novel mechanism of ET-1-induced hypermethylation as a downstream regulator of hypoxia-mediated cardiomyocyte transition from mononucleate to binucleate cells in the developing heart. Cardiomyocyte

endowment is determined during fetal and early postnatal development, when most cardiomyocytes become binucleate and cease to proliferate (Botting et al., 2012; Thornburg et al., 2011). Given that hypoxia is one of the most important and clinically relevant stresses to the fetal development, and that fetal hypoxia results in fewer but larger cardiomyocytes and increases the susceptibility of heart to ischemic injury in offspring (Bae et al., 2003; G. Li, Bae, & Zhang, 2004; G. Li et al., 2003; Xu, Williams, O'Brien, & Davidge, 2006), the present study provides a mechanistic understanding worthy of further investigation in humans.

Acknowledgements

A portion of this research used the Loma Linda University School of Medicine Advanced Imaging and Microscopy Core, a facility supported in part by the National Science Foundation through the Major Research Instrumentation program of the Division of Biological Infrastructure Grant No. 0923559 and the Loma Linda University School of Medicine.

CHAPTER THREE

NEWBORN HYPOXIA/ANOXIA INHIBITS CARDIOMYOCYTE PROLIFERATION AND DECREASES ENDOWMENT IN THE DEVELOPING HEART: ROLE OF ENDOTHELIN-1

By

Alexandra Paradis, Maresha Gay, Christopher Wilson, and Lubo Zhang

This paper has been published by PLoS One. 2015 Feb 18;10(2):e0116600

Abstract

In the developing heart, cardiomyocytes undergo terminal differentiation during a critical window around birth. Hypoxia is a major stress to preterm infants, yet its effect on the development and maturation of the heart remains unknown. We tested the hypothesis in a rat model that newborn anoxia accelerates cardiomyocyte terminal differentiation and results in reduced cardiomyocyte endowment in the developing heart *via* an endothelin-1-dependent mechanism. Newborn rats were exposed to anoxia twice daily from postnatal day 1 to 3, and hearts were isolated and studied at postnatal day 4 (P4), 7 (P7), and 14 (P14). Anoxia significantly increased HIF-1α protein expression and pre-proET-1 mRNA abundance in P4 neonatal hearts. Cardiomyocyte proliferation was significantly decreased by anoxia in P4 and P7, resulting in a significant reduction of cardiomyocyte number per heart weight in the P14 neonates. Furthermore, the expression of cyclin D2 was significantly decreased due to anoxia, while p27 expression was increased. Anoxia has no significant effect on cardiomyocyte binucleation or myocyte size. Consistently, prenatal hypoxia significantly decreased cardiomyocyte proliferation but had no effect on binucleation in the fetal heart. Newborn administration of PD156707, an ET_A-receptor antagonist, significantly increased cardiomyocyte proliferation at P4 and cell size at P7, resulting in an increase in the heart to body weight ratio in P7 neonates. In addition, PD156707 abrogated the anoxia-mediated effects. The results suggest that hypoxia and anoxia *via* activation of endothelin-1 at the critical window of heart development inhibits cardiomyocyte proliferation and decreases myocyte endowment in the developing heart, which may negatively impact cardiac function later in life.

Introduction

The intrauterine environment plays a well-established role in predisposition to cardiovascular disease later in life (Barker, 1995). Environmental factors during the critical period of heart development may alter the maturation of the heart and thus potentially its life-long function. Cardiomyocytes are the functional contractile units of the heart that undergo a normal maturation process in which terminal differentiation is the final outcome. As the cardiomyocytes terminally differentiate and exit the cell cycle, they lose their proliferative capacity (Ahuja, Sdek, et al., 2007). Cardiomyocyte growth then transitions from hyperplastic to hypertrophic, in which the cells can only increase in size rather than number (Bugaisky & Zak, 1979; F. Li et al., 1996). Ultimately the proliferative capacity of cardiomyocytes is lost and the adult heart is known to exhibit negligible increases in cell number (Bergmann et al., 2009). Therefore the timing of this transition is pivotal in determining cardiomyocyte endowment in the heart for the rest of the animal's life.

Hypoxia is a major stress to preterm infants, yet its effect on the development and maturation of the heart remains unknown. Given that the transition of cardiomyocyte terminal differentiation occurs in rodents during the first two weeks of neonatal life (Clubb & Bishop, 1984; F. Li et al., 1996), which is an equivalent timeframe to the late fetal stage in third trimester of human gestation (Ahuja, Sdek, et al., 2007), they provide a reasonable animal model to study the effect of anoxia on preterm infants at the critical window of the heart development. This process of terminal differentiation begins in the rat heart around postnatal day 4 (F. Li et al., 1996) and progresses until day 14 when the heart is essentially mature, thus three time-points within this period were evaluated in this

study. Previous studies in rats have shown that maternal hypoxia (10.5 % O_2) leads to a premature exit from the cell cycle in fetal cardiomyocytes (Bae et al., 2003; Tong et al., 2013; Tong et al., 2011). Additionally, neonatal cardiomyocytes have been shown to decrease proliferation when exposed to hypoxic conditions (F. X. Zhang et al., 2007). Studies have also been performed in sheep in which placental restriction is induced, resulting in reduced cardiomyocyte maturation (Bubb et al., 2007) and proliferation (Louey et al., 2007), increased proportion of mononucleate cardiomyocytes (Morrison et al., 2007), and decreased cardiomyocyte endowment (Botting et al., 2014). However, the *in vivo* effects of anoxia, as a preterm model, on cardiomyocyte proliferation and endowment in the developing rat heart are, as of yet, not known. Additionally, the downstream regulators of cardiomyocyte proliferation and maturation are unknown.

Endothelin-1 (ET-1) expression is induced by hypoxia (Hashiguchi et al., 1991; Ostlund et al., 2000; Yamada et al., 2001; Yamashita et al., 2001). Studies performed in endothelial cells (Hu et al., 1998; Minchenko & Caro, 2000) and cardiomyocytes (Kakinuma et al., 2001) have identified a HIF-1α binding site in the prepro-ET-1 gene. Furthermore, the cardiomyocyte is both a site of synthesis and action for ET-1 (Kedzierski & Yanagisawa, 2001; Kohan et al., 2011), as it acts mainly at the paracrine or autocrine level (Agapitov & Haynes, 2002; Rubanyi & Polokoff, 1994). Our recent work showed that *ex vivo* ET-1 treatment promoted terminal differentiation of fetal cardiomyocytes, *via* an increase in DNA methylation (Paradis, Xiao, Zhou, & Zhang, 2014). The predominant ET-1 receptor subtype in cardiomyocytes is the ET_A-receptor (Kohan et al., 2011), which is thought to be involved in regulating proliferation (Agapitov & Haynes, 2002; Goldie, 1999; Komuro et al., 1988). Currently, little is

known about the role that basal ET-1 plays in the terminal differentiation of cardiomyocytes, as well as the effect of hypoxia/anoxia-induced ET-1 production on this process.

Therefore, in the present study we tested the hypothesis that *in vivo* neonatal anoxia decreases proliferation of cardiomyocytes *via* the ET_A-receptor-dependent mechanisms, resulting in reduced cardiomyocyte endowment in the developing heart. Herein, we provide evidence that the ET_A-receptor mediates the anoxia-induced decrease in cardiomyocyte proliferation. Furthermore, cardiomyocyte endowment in the developing heart was decreased by anoxia and restored with PD156707, a selective ET_A-receptor antagonist.

Methods

Experimental Animals

Time-dated pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI) and allowed to give birth. Neonatal pups from 7 litters were used and divided into the treatment groups. Data from pups of multiple litters were pooled. Starting at postnatal day 1, newborn rats were placed in a temperature-controlled (37˚C) anoxia chamber. Nitrogen was infused into the chamber for 10 minutes and an oxygen sensor was used to verify the level of oxygen in the chamber being < 0.2%. Control animals were placed in a chamber with oxygen maintained at 21%. Anoxia treatments were performed twice a day with 8 hours in between, from postnatal day 1 until postnatal day 3. A group of animals was treated with intraperitoneal injections of an ET_A-receptor antagonist, PD156707 (2 mg/kg), prior to each episode of anoxia, twice a

day for the first 3 postnatal days. Neonatal pups were anesthetized with isoflurane and hearts isolated for studies on postnatal day 4, 7, and 14. To investigate the comparative effect of prenatal hypoxia, some of the time-dated pregnant Sprague-Dawley rats were treated with either normoxic control or 10.5% O_2 from gestational day 15 to 21, as previously described (Patterson et al., 2012; Xue et al., 2011). Following the hypoxia treatment, pregnant rats were allowed to give birth. Hearts were isolated from postnatal day 4 and 7 neonatal rats. All procedures and protocols were approved by the Loma Linda University Institutional Animal Care and Use Committee (IACUC) and all procedure adhered to the guidelines by US National Institutes of Health Guide for the Care and Use of Laboratory Animals (http://grants.nih.gov/grants/olaw/Guide-for-the-care-and-use-of-laboratory-animals.pdf).

Measurement of Cardiomyocyte Number

Hearts from day 4, 7, and 14 neonatal pups were isolated and the atria excised. The hearts were then completely enzymatically digested to yield primary cardiomyocytes, as previously described (Paradis et al., 2014; Y. Xiao et al., 2000). A pre-plate step was performed to enrich the cardiomyocyte population. This is a commonly used method (Chlopcikova, Psotova, & Miketova, 2001) that is based on the differential attachment of cardiomyocytes and non-myocyte cells of the heart. Cardiomyocytes take approximately 24 hours to fully attach to the plate while non-myocytes attach within a couple hours. After a 2-hour pre-plate step to remove attached non-myocytes, cardiomyocytes in the media were collected and used for counting cardiomyocyte number *via* hemacytometer. Briefly, an aliquot of cardiomyocytes was

counted using a hemacytometer and the counts were multiplied by the total volume of cell suspension and normalized according to the heart weight, to yield the number of cardiomyocytes per heart weight.

Immunocytochemistry

To perform immunocytochemical staining, cardiomyocytes isolated from day 4 and 7 hearts were allowed to attach to plates in Hyclone Medium 199 (Thermo Scientific) supplemented with 10% fetal bovine serum (Gemini Bio-Products) and 1% antibiotics (10,000 I.U./mL penicillin, 10,000 µg/mL streptomycin) at 37°C in 95% air/5% CO_2. After 24 hours, cardiomyocytes were fully attached and were double stained with alpha-actinin, a cardiomyocyte marker, and Ki-67, a proliferation marker as described previously (Paradis et al., 2014; Tong et al., 2013). Cardiomyocytes were plated on coverslips and fixed with 4% paraformaldehyde (ThermoScientific) for 15 minutes followed by permeabilization with Triton X-100 (Fisher) for 10 minutes. The cells were blocked with 1% bovine serum albumin for 1 hour at room temperature before incubation with the primary antibodies: mouse anti-α-sarcomeric actinin (A7811, Sigma) (1:200) and rabbit anti-Ki-67 (ab16667, Abcam) (1:100) at room temperature for 1 hour. The samples were incubated with the secondary antibodies: anti-mouse Alexa Fluor 488 (A21202, Life Technologies) and anti-rabbit Alexa Fluor 647 antibodies (A21244, Life Technologies) for 1 hour at room temperature. Nuclei were stained with Hoescht (Sigma) for less than 1 minute. The immunofluorescence staining was assessed using a Zeiss Axio Imager.A1 microscope and quantitative analysis was carried out using *ImageJ* software (http://imagej.nih.gov/ij/). Ki-67 expression, binucleation, and cell size were measured.

Flow Cytometry

Primary cardiomyocytes isolated from day 14 neonatal rats were stained for analysis by flow cytometry. Cells were washed in staining buffer (PBS + 5% FBS), spun down, and re-suspended in 4% paraformaldehyde for 20 minutes at room temperature in the dark. The fixed cells were then washed in permeabilization wash buffer (eBioscience) and supernatant discarded. Cells were stained with antibodies for the cardiomyocyte marker, Troponin T (ab10214, Abcam) (1:200), and proliferation marker, Ki-67-conjugated to allophycocyanin (APC) (eBioscience) (50-5698, 1:200). After incubation and washing, cells were incubated with the secondary antibody for Troponin T, fluorescein isothiocyanate (FITC) (555988, BD Pharmingen) (1:100). Finally cells were washed and resuspended in 1% paraformaldehyde to be run on a FACSAria (BD Biosciences) and analyzed via FACSDiva software (BD Biosciences) for percentage of Ki-67 expressing cardiomyocytes.

Real-Time Reverse Transcription-Polymerase Chain Reaction

RNA was isolated from the postnatal day 4 (P4) hearts and prepro-ET-1 mRNA abundance was determined by real-time RT-PCR using Icycler Thermal cycler (Bio-Rad), as described previously (Xue et al., 2011). Reverse transcription and cDNA synthesis was performed using SuperScript III First-Strand Synthesis Supermix for RT-PCR (Life Technologies). The primers are 5'-CTAGGTCTAAGCGATCCTTGAA-3' (forward) and 5'-CTTGATGCTGTTGCTGATGG-3' (reverse). PCR was performed in triplicate, and threshold cycle numbers were averaged.

Western Immunoblotting

HIF-1α, ET_A-receptor (ET_AR), and ET_B-receptor (ET_BR) protein abundance in the P4 heart was measured from control and anoxia groups. The protein abundance of cyclin D2 and p27 was measured in P4 hearts from control and anoxia groups as well as in the presence and absence of PD156707. Tissues were homogenized and protein isolated using the RIPA lysis buffer system (Santa Cruz Biotechnology). Protein concentrations were quantified using the BCA protein assay (ThermoScientific) and all samples were loaded with equal protein onto 7.5% (HIF-1α) or 10% (ET_AR, and ET_BR, cyclin D2, and p27) polyacrylamide gel with 0.1% sodium dodecyl sulfate (SDS). Proteins were then separated by electrophoresis and transferred onto nitrocellulose membranes. Non-specific binding sites were blocked with Tris-buffered saline solution (TBS) containing 5% dry milk. The membranes were incubated with primary antibodies against HIF-1α (sc10790, Santa Cruz Biotechnology; 1:500), ET_AR (sc33536, Santa Cruz Biotechnology; 1:500), ET_BR (sc33538, Santa Cruz Biotechnology; 1:500), cyclin D2 (ab3085, Abcam; 1:1000), and p27 (ab7961, Abcam; 1:1000). After washing, membranes were incubated with secondary antibodies. Proteins were visualized with enhanced chemiluminescence reagents and western blots were exposed to Hyper film. Kodak image software was used to quantify all results.

Statistical Analysis

Data are expressed as means ± SEM. Statistical analysis ($p < 0.05$) was determined by two-way analysis of variance (ANOVA) followed by Neuman-Keuls *post hoc* test or Student's t test, where appropriate, using GraphPad Prism software. The two-

way ANOVA was performed to evaluate the effects of two factors, within each age group: (1) control versus anoxia, and (2) in the presence and absence of PD156707.

Results

Newborn Anoxia Treatment Increased Pre-proET-1 mRNA in the Heart

Neonatal rats were exposed to anoxia twice a day from postnatal day 1 to 3, and hearts were isolated at P4. As seen in Figure 3.1, there was a significant increase in prepro-ET-1 mRNA abundance in neonatal hearts exposed to anoxia (< 0.2% O_2), as compared to the normoxic control (21% O_2).

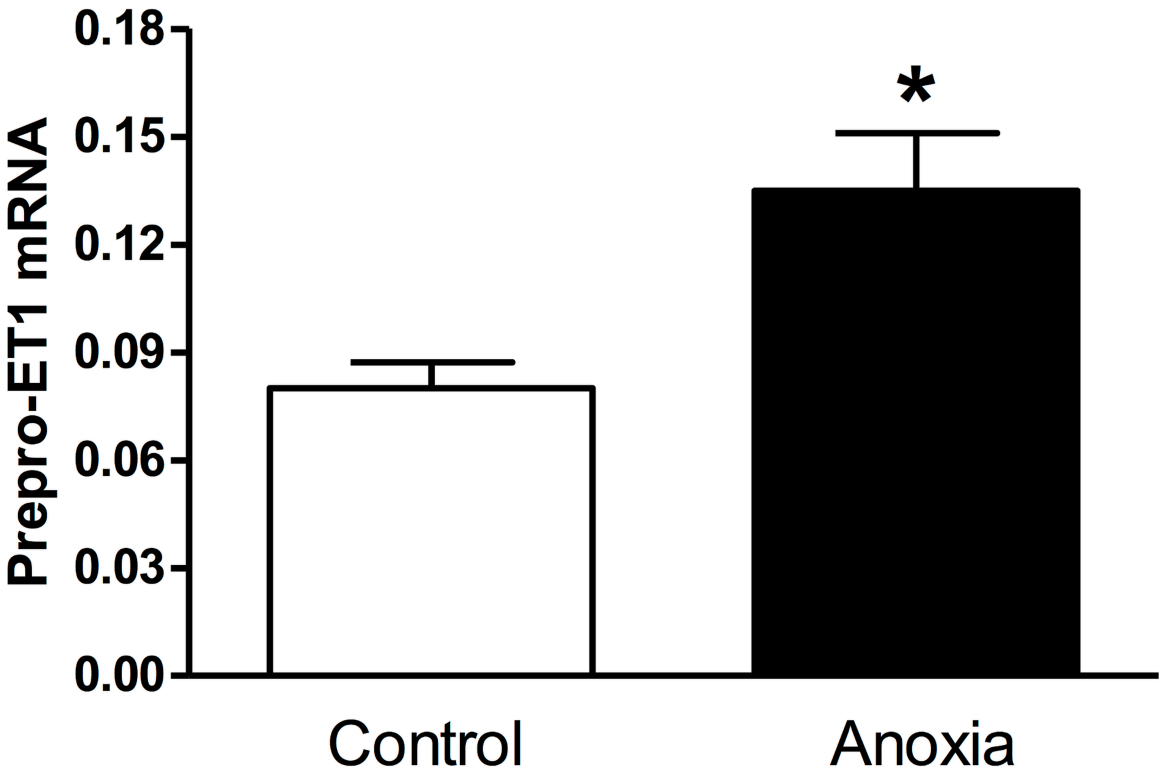

Figure 3.1. Effect of newborn anoxia on prepro-ET-1 mRNA in the neonatal heart. Hearts were isolated from day 4 neonatal rats treated with control or anoxia. mRNA abundance of prepro-ET-1 was determined by real-time RT-PCR. Data are means ± SEM. * $P < 0.05$, anoxia *vs*. control. n = 3–5

Newborn Anoxia Treatment Increased HIF-1α Protein Abundance in the Heart

Hearts from P4 rats treated with anoxia were collected and protein isolated. Neonatal hearts exposed to anoxia had significantly increased levels of the HIF-1α protein (Figure 3.2).

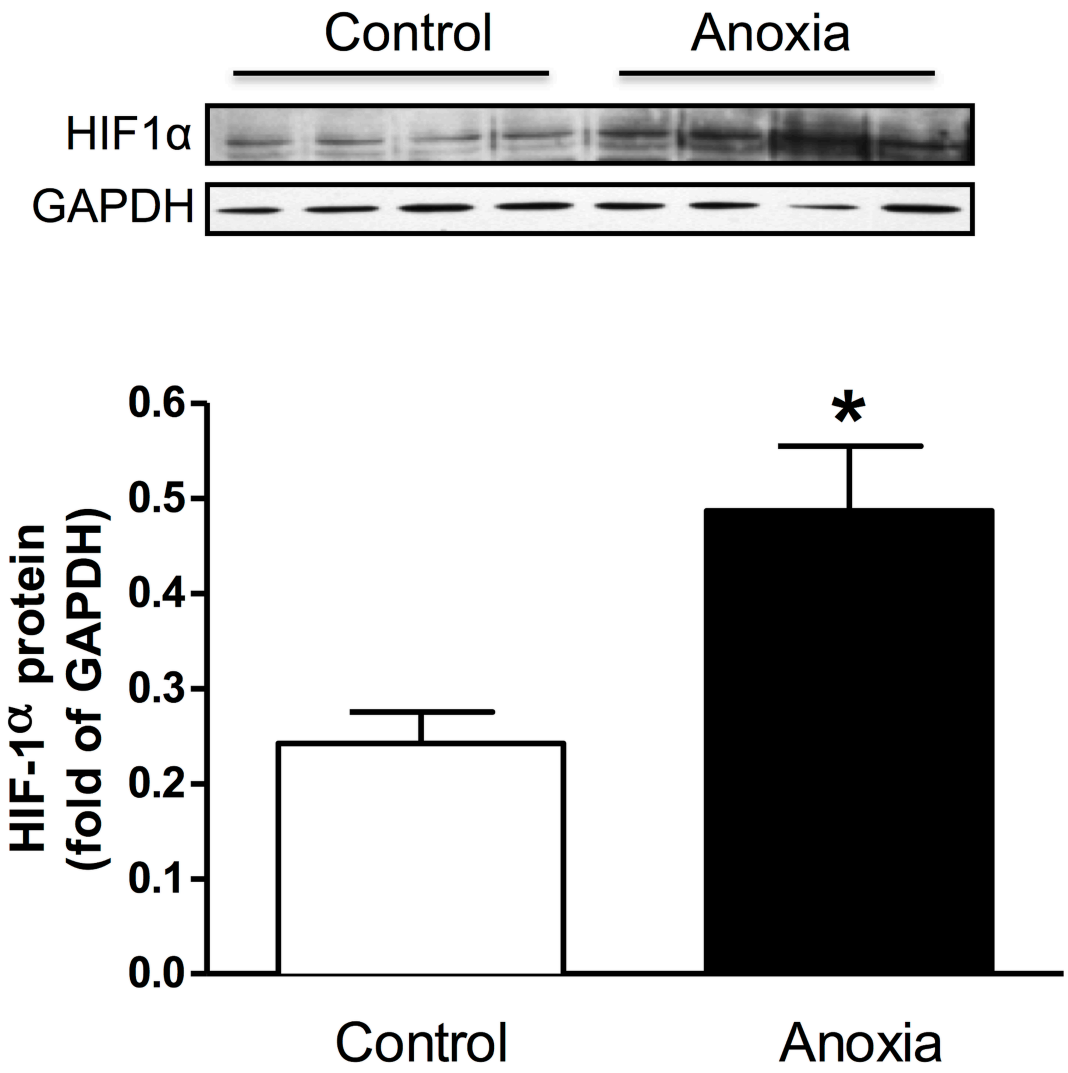

Figure 3.2. Effect of newborn anoxia on HIF-1⟨ protein abundance in the neonatal heart. Hearts were isolated from day 4 neonatal rats treated with control or anoxia. Protein abundance of HIF-1⟨ was determined by Western immunoblotting. Data are means ± SEM. * $P < 0.05$, anoxia *vs.* control. n = 4

Newborn Anoxia Treatment Decreased Cardiomyocyte Proliferation

As shown in Figure 3.3, there is a development-dependent decrease in cardiomyocyte proliferation at the critical window of the heart development during the first two weeks of life in rodents, and myocyte proliferation reduces to minimal levels at postnatal day 14. Anoxia treatment of newborns caused a significant decrease in the proliferation of neonatal cardiomyocytes at both postnatal days 4 and 7 (Figure 3.3B). Treatment of newborn rats with a selective ET_A-receptor antagonist, PD156707, caused a significant increase in cardiomyocyte proliferation in P4 neonatal rats (Figure 3.3B). In addition, PD156707 abrogated the anoxia-induced effects in the developing hearts (Figure 3.3B). In contrast to proliferation, there is a development-dependent increase in cardiomyocyte binucleation in the developing heart (Figure 3.3C). Neither anoxia nor PD156707 treatments had significant effects on cardiomyocyte binucleation (Figure 3.3C).

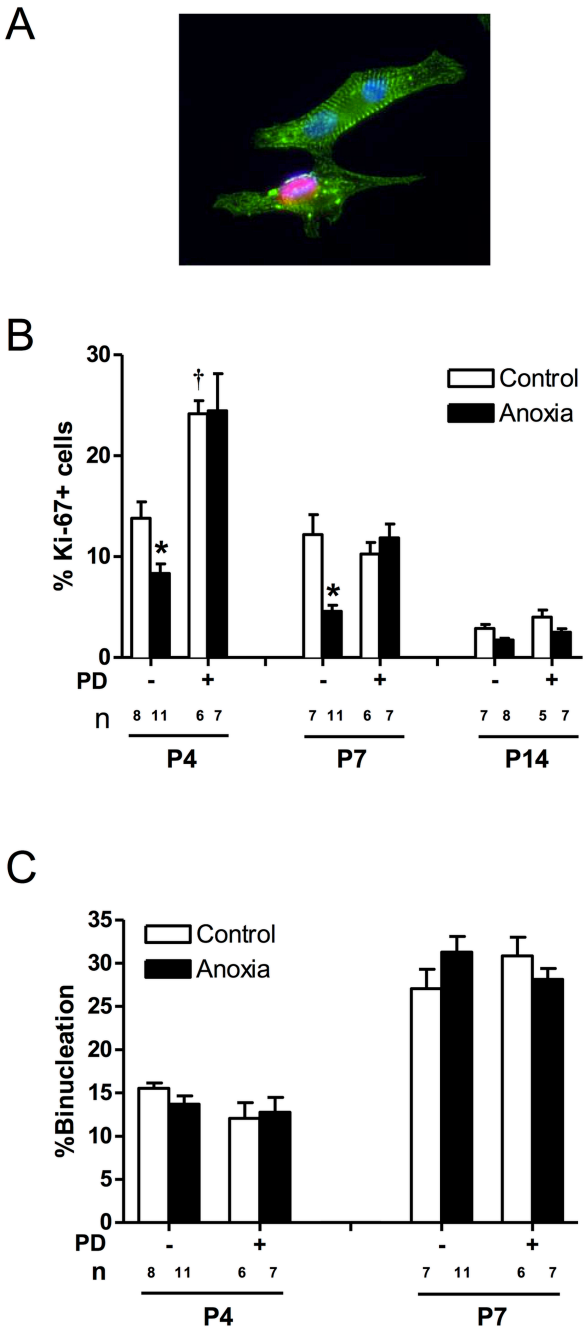

Figure 3.3. Effect of newborn anoxia and PD156707 on proliferation and binucleation of neonatal cardiomyocytes. Cardiomyocytes were isolated from P4, P7, and P14 neonatal rats that were treated with control or anoxia, in the absence or presence of PD156707. Cells from P4 and P7 rats were stained with α-actinin and Ki-67, and nuclei were stained using Hoechst staining. P14 cardiomyocytes were stained with Ki-67 and analyzed *via* FACS. **Panel A** shows a representative image of cardiomyocytes stained with alpha-actinin (green), Ki-67 (red), and Hoescht (blue). **Panel B** shows percent of Ki-67 expressing cells. **Panel C** shows percent of binucleate cells. Data are means ± SEM. * $P < 0.05$, anoxia *vs*. control. † $P < 0.05$, -PD156707 *vs*. +PD156707. PD: PD156707; n: animal numbers.

Newborn Anoxia Treatment Decreased Cyclin D2 and Increased p27 Expression

The protein expression of cyclin D2 was decreased due to anoxia treatment and this effect was abolished in the presence of PD156707 (Figure 3.4A). On the contrary, p27 expression in the neonatal heart was significantly increased due to anoxia treatment, and PD156707 blocked the effect of anoxia (Figure 3.4B).

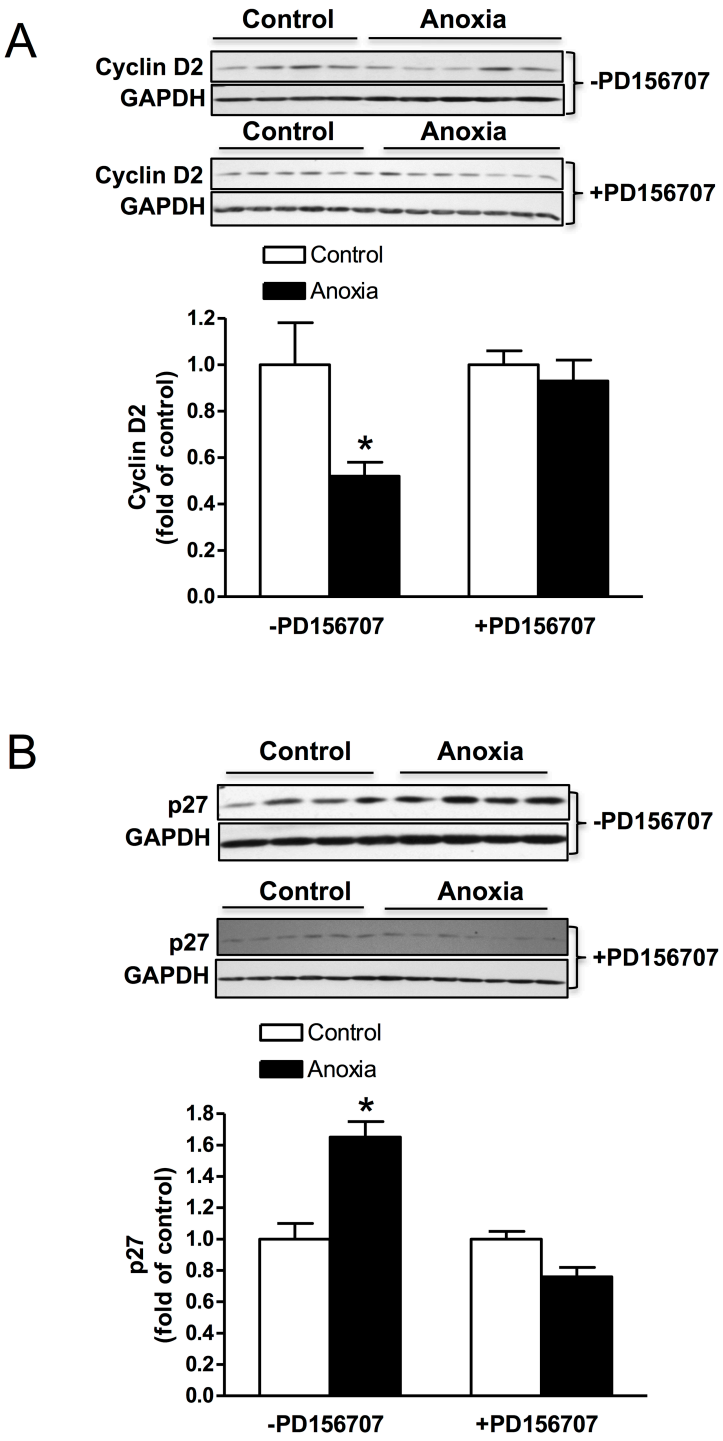

Figure 3.4. Effect of newborn anoxia on cyclin D2 and p27 protein expression in the cardiomyocyte. Hearts were isolated from day 4 neonatal rats treated with control or anoxia in the presence (n = 6-7) or absence (n = 4) of PD156707. Protein abundance of cyclin D2 in the absence and presence of PD156707 (A), and p27 in the absence and presence of PD156707 (B) was determined by Western immunoblotting. Data are means ± SEM. * $P < 0.05$, anoxia *vs.* control.

Newborn Anoxia Treatment Decreased Cardiomyocyte Number by Day 14

There was no significant change in cardiomyocyte number due to newborn anoxia treatment at day 4 and 7. However, results for day 14 show that anoxia leads to a significant decrease in cardiomyocyte number per heart weight (Figure 3.5). PD156707 alone caused a significant increase in cardiomyocyte number in the day 7 neonate (Figure 3.5). In the presence of PD156707, the anoxia-mediated effects at day 14 were blocked (Figure 3.5).

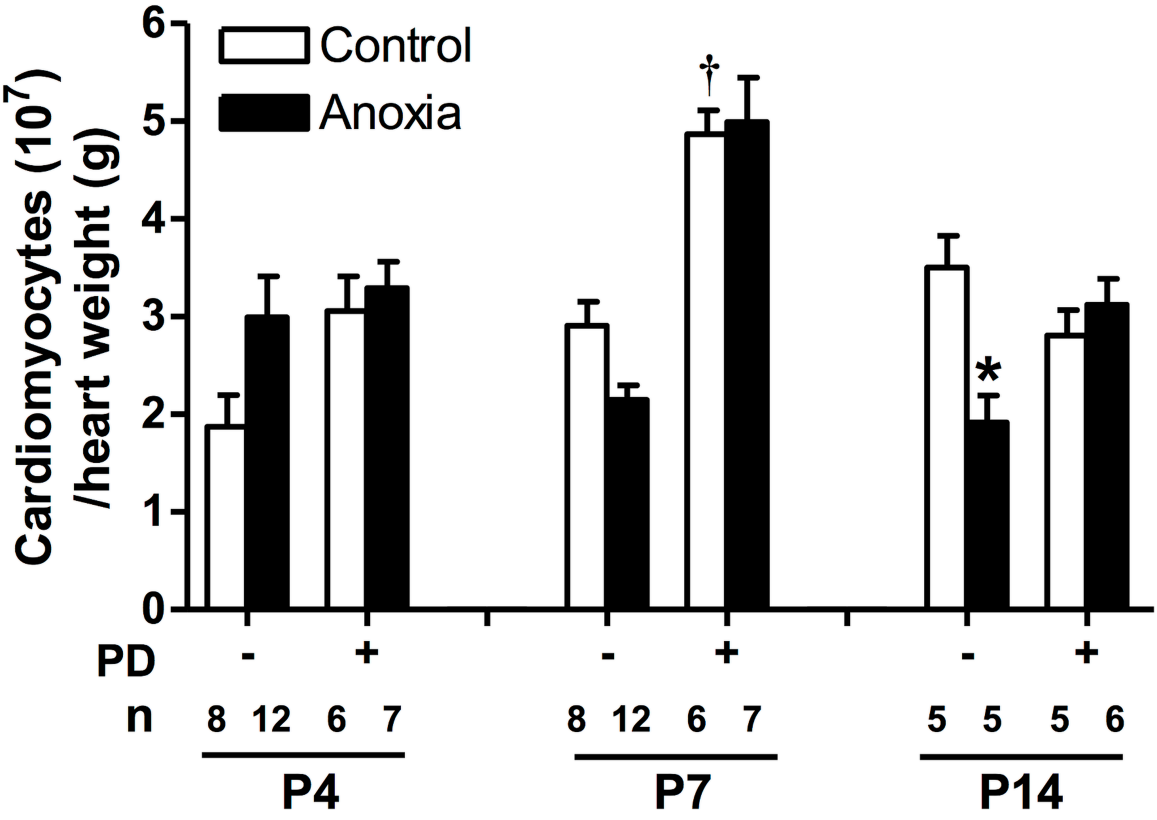

Figure 3.5. Effect of newborn anoxia and PD156707 on number of cardiomyocytes per heart weight. Cardiomyocytes were isolated from day 4, 7, and 14 neonatal rats that were treated with control or anoxia, in the absence or presence of PD156707. Hearts were weighed and cardiomyocytes counted by hemacytometer (day 4 and 7) and FACS (day 14). Data are expressed as cardiomyocyte number/g heart weight, and are means ± SEM. * $P < 0.05$, anoxia *vs*. control. † $P < 0.05$, -PD156707 *vs*. +PD156707. PD: PD156707; n: animal numbers.

Cell Size was Increased in the Presence of PD156707

Anoxia had no effect on mononucleate or binucleate cell size at both day 4 and 7 (Figure 3.6). However, PD156707 treatment was able to increase both mononucleate and binucleate cell size at postnatal day 7 (Figure 3.6).

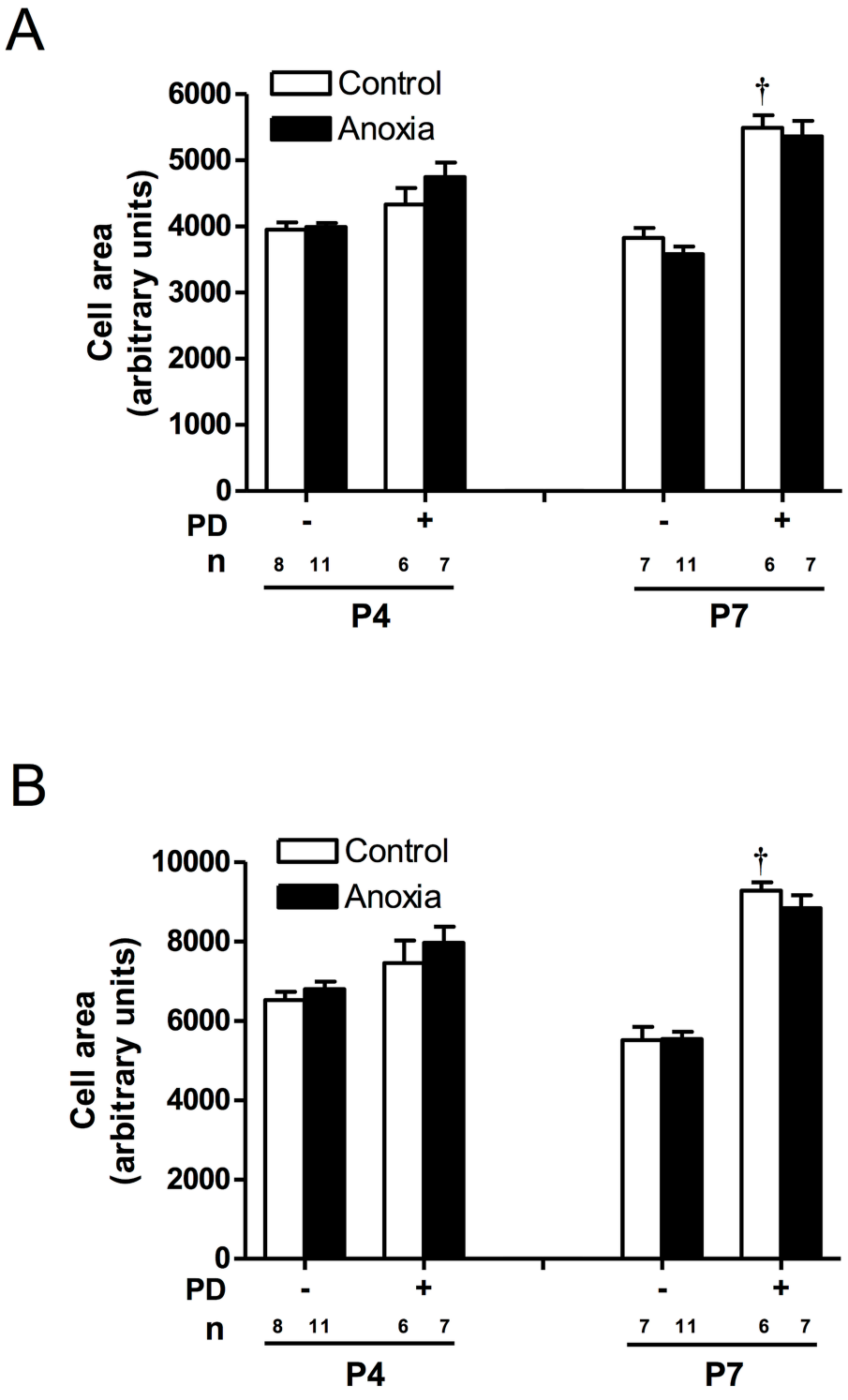

Figure 3.6. Effect of newborn anoxia and PD156707 on cardiomyocyte size in the neonatal heart. Cardiomyocytes were isolated from day 4, 7, and 14 neonatal rats that were treated with control or anoxia, in the absence or presence of PD156707. Mononucleate (A) and binucleate (B) cell size was measured using *ImageJ*. Data are means ± SEM. † $P < 0.05$, -PD156707 *vs.* +PD156707. PD: PD156707; n: animal numbers.

PD156707 Increased Heart to Body Weight Ratio

There was no significant effect of anoxia on the heart to body weight ratio for any day (Figure 3.7). However, PD156707 treatment significantly increased the heart to body weight ratio in day 4 and 7 neonates (Figure 3.7). Heart and body weight averages in the presence and absence of anoxia and PD156707 are listed in Table 3.1.

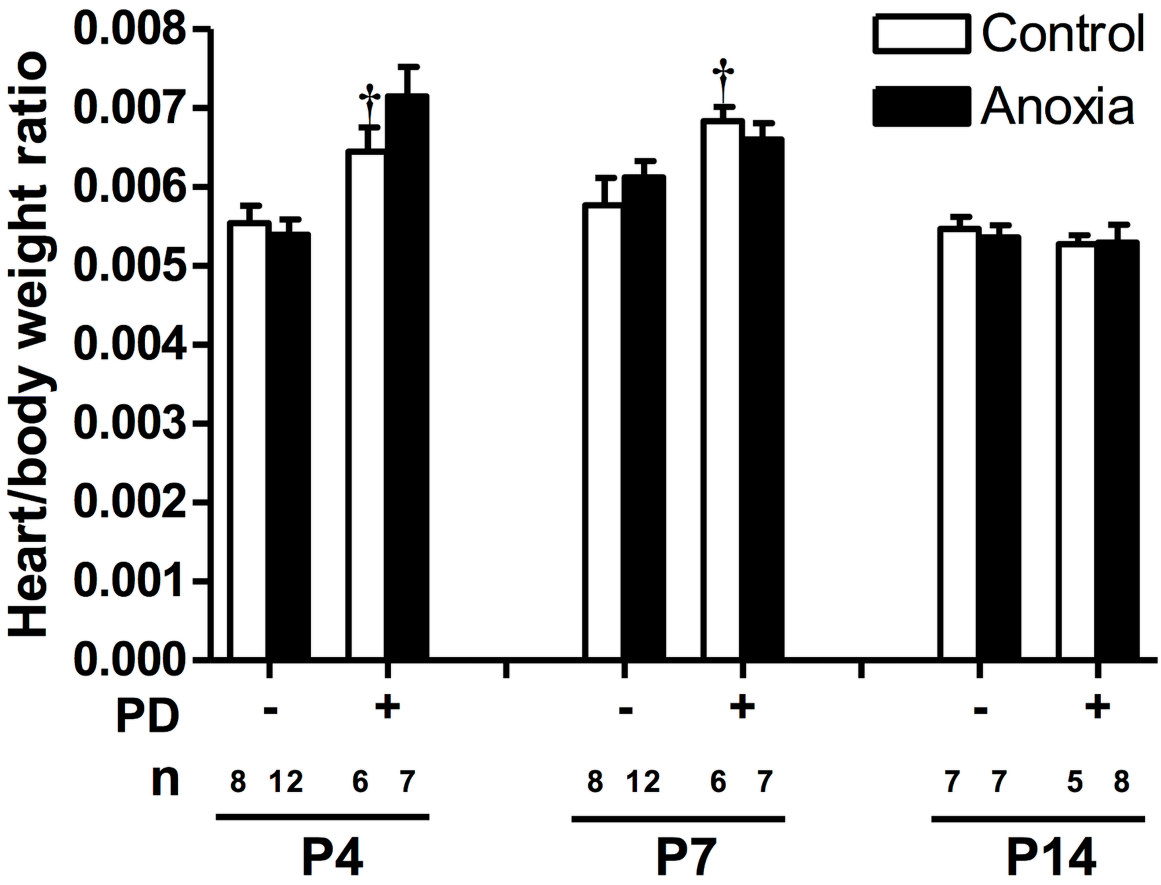

Figure 3.7. Effect of newborn anoxia and PD156707 on heart to body weight ratio of neonatal rats. Body and heart weights were taken from day 4, 7, and 14 neonatal rats that were treated with control or anoxia, in the absence or presence of PD156707. Data are means ± SEM. † $P < 0.05$, -PD156707 *vs.* +PD156707. PD: PD156707; n: animal numbers.

Table 3.1. Effect of newborn anoxia and PD156707 on body and heart weight of neonatal rats. Body and isolated hearts were weighed from day 4, 7, and 14 neonatal rats that were treated with control or anoxia, in the absence or presence of PD156707. Heart to body weight ratio values are also represented. Data are means ± SEM**.** * $P < 0.05$, control -PD156707 *vs*. +PD156707. Number of animals is represented in parentheses.

		Body weight (grams)			Heart weight (grams)			Heart to Body weight ratio		
		P4	**P7**	**P14**	**P4**	**P7**	**P14**	**P4**	**P7**	**P14**
-PD156707	**Control**	8.51 ± 0.374 (8)	14.44 ± 0.550 (8)	22.45 ± 1.090 (7)	0.047 ± 0.0023 (8)	0.083 ± 0.0046 (8)	0.123 ± 0.0076 (7)	0.0055 ± 0.0002 (8)	0.0058 ± 0.0003 (8)	0.0055 ± 0.0002 (7)
	Anoxia	8.06 ± 0.317 (12)	13.65 ± 0.472 (12)	22.28 ± 0.879 (7)	0.044 ± 0.0023 (12)	0.083 ± 0.0025 (12)	0.120 ± 0.0072 (7)	0.0054 ± 0.0002 (12)	0.0061 ± 0.0002 (12)	0.0054 ± 0.0002 (7)
+PD156707	**Control**	9.57 ± 0.186 (6)	15.05 ± 0.289 (6)	21.68 ± 0.960 (5)	0.062 ± 0.0029 (6)	0.103 ± 0.0024* (6)	0.114 ± 0.0053 (5)	0.0065 ± 0.0003 (6)	0.0068 ± 0.0002* (6)	0.0053 ± 0.0001 (5)
	Anoxia	7.57 ± 0.498 (7)	12.75 ± 0.302 (7)	23.90 ± 0.779 (8)	0.054 ± 0.0042 (7)	0.084 ± 0.0034 (7)	0.127 ± 0.0081 (8)	0.0072 ± 0.0004 (7)	0.0066 ± 0.0002 (7)	0.0053 ± 0.0002 (8)

Neonatal Anoxia Had No Effect on ET-Receptor Density

Hearts from postnatal day 4 rats that were treated with anoxia were collected and protein isolated. There was no significant change in protein abundance of either ET_AR or ET_BR, due to anoxia treatment (Figure 3.8).

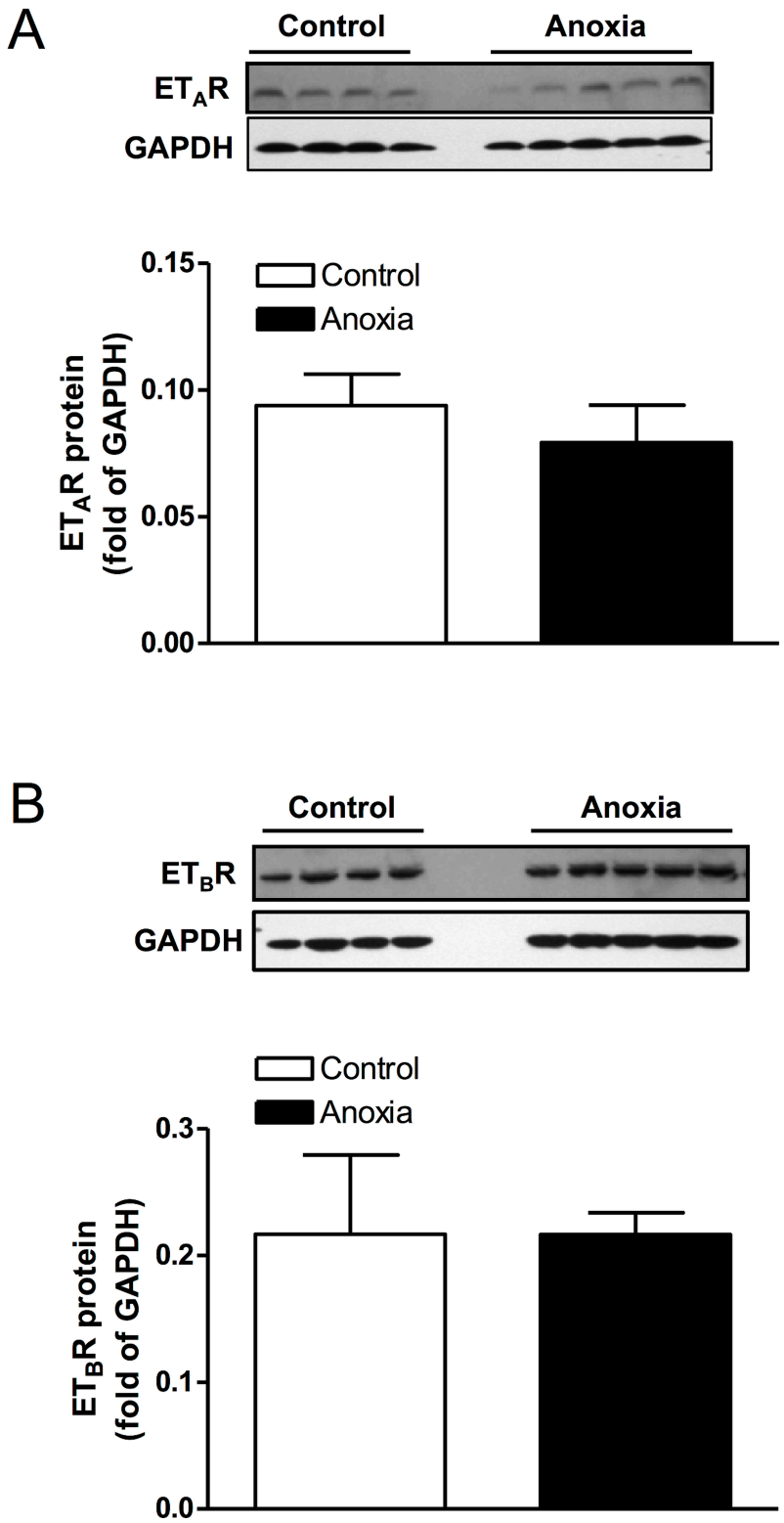

Figure 3.8. Effect of newborn anoxia on ET_A- and ET_B-receptor protein abundance in the neonatal heart. Hearts were isolated from day 4 neonatal rats treated with control or anoxia. Protein abundance of ET_AR (A) and ET_BR (B) was determined by Western immunoblotting. Data are means ± SEM. n = 4-5

Prenatal Hypoxia Decreased Cardiomyocyte Proliferation in the Fetal Heart

To investigate the comparative effect of prenatal hypoxia, pregnant rats were treated with either normoxic control or 10.5% O_2 from gestational day 15 to 21, and hearts were isolated from postnatal day 4 and 7 neonatal rats. Similar to the findings in newborn anoxia treatment, prenatal hypoxia resulted in a significant decrease in the proliferation of cardiomyocytes at postnatal day 7 (Figure 3.9A), but had no significant effects on percent binucleation (Figure 3.9B) or the heart to body weight ratio (Figure 3.9C).

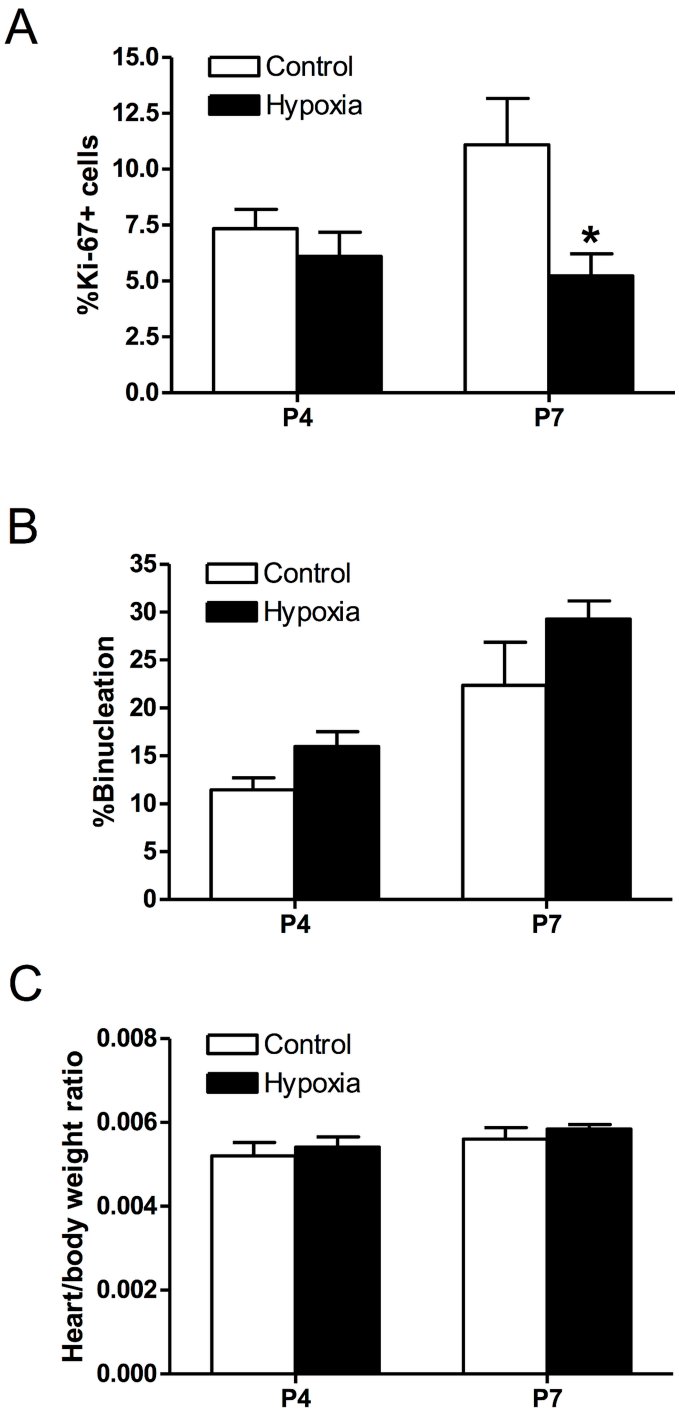

Figure 3.9. Effect of prenatal hypoxia on neonatal cardiomyocyte proliferation, binucleation, and heart to body weight ratio. Cardiomyocytes were isolated from day 4 and 7 neonatal rats that were treated with control or maternal hypoxia. Cells were stained with α-actinin and Ki-67, and nuclei stained with Hoechst. **Panel A** shows percent of Ki-67 expressing cells (n = 3-4). **Panel B** shows percent of binucleate cells (n = 4). **Panel C** shows the heart to body weight ratio (n = 8-9). Data are means ± SEM. * $P < 0.05$, hypoxia *vs.* control.

Discussion

In the present study, we provide evidence showing that *in vivo* newborn anoxia leads to a decrease in the proliferation of cardiomyocytes in the developing heart. Furthermore, our results suggest that anoxia treatment leads to a significant reduction in number of cardiomyocytes per heart weight of the day 14 neonate, which is terminally differentiated. The findings that anoxia increased ET-1 production in the heart and the anoxia-induced changes in proliferation and cardiomyocyte number were reversed with PD156707, suggest a mechanism mediated by the ET_A-receptor. In addition, basal ET-1 was also found to play a role in cardiomyocyte proliferation, as well as the heart to body weight ratio.

Cardiomyocytes undergo a terminal differentiation process that reaches completion by the first two weeks of neonatal life in rats (Ahuja, Sdek, et al., 2007; Clubb & Bishop, 1984). After this, cardiomyocytes in the heart have negligible proliferative capacity and further growth is mainly *via* hypertrophy. Thus the number of cardiomyocytes that will reside in the adult heart is determined during this early stage and if altered may result in life-long consequences. Hypoxic stress during perinatal development has been shown by previous studies to diminish the proliferation of cardiomyocytes (Paradis et al., 2014; Tong et al., 2013; Tong et al., 2011). Furthermore, fetal hearts exposed to hypoxia have fewer (Bae et al., 2003; Botting et al., 2014) and larger cardiomyocytes (Bae et al., 2003), and adult male rats that were exposed to hypoxia *in utero* were more susceptible to ischemic injury as seen by increased myocardial infarction and reduced recovery (G. Li et al., 2003).

Preterm birth is a complex clinical problem that is highly associated with episodes of severe hypoxia and even anoxia, which can be so severe that the infant must be mechanically ventilated (Bolivar et al., 1995). Preterm infants have an immature respiratory system (Martin, Wang, Koroglu, Di Fiore, & Kc, 2011) that is unable to provide adequate oxygen at times and thus ventilatory support is frequently needed. However, several studies have shown that episodic airway obstruction and hypoxemia commonly occur in these infants (Dimaguila, Di Fiore, Martin, & Miller, 1997; Dransfield, Spitzer, & Fox, 1983). Given that the rodent heart is relatively immature at birth, the present study with episodic anoxia treatments of newborn rats provides a reasonable animal model to study the effects of anoxia/hypoxia on the heart development in preterm infants. Anoxia itself has been shown to alter proliferation, and, in rat fibroblasts, leads to arrest of the cell cycle at the G1 and S phase (Gardner, Li, Yang, & Dang, 2003).

To confirm the extent of hypoxic exposure to the neonatal hearts used in our study, the protein levels of hypoxia-inducible factor 1 alpha (HIF-1α) were evaluated. The results show that HIF-1α protein abundance is significantly increased in neonatal hearts exposed to *in vivo* anoxia; furthermore increased levels of HIF-1α in the heart have also been observed in the prenatal hypoxia model (Bae et al., 2003). In agreement with previous work, we showed that cardiomyocyte proliferation was decreased following *in vivo* neonatal anoxia treatment at postnatal day 4 and 7. The cardiomyocytes take approximately 24 hours for them to fully attach to the plate before the immunocytochemical staining of Ki-67 can be performed. While the potential effect of this attachment process on the rate of proliferation may not be excluded in the present

study, the same procedure applied to all treatment groups. By postnatal day 14, there was a trend for anoxia to decrease proliferation however this trend was not significant in our data. The heart is thought to be fully mature and essentially an adult phenotype of cardiomyocytes by day 14 in rats, therefore the rate of myocyte proliferation is normally very low at this point and anoxia had no significant effect on lowering it further. Similarly, our results from the prenatal hypoxia model showed a decrease in the proliferation of neonatal cardiomyocytes at postnatal day 7. Interestingly, newborn anoxia had no significant effect on the binucleation of cardiomyocytes. Previous work has shown that maternal hypoxia leads to an increase in the amount of binucleate cardiomyocytes in the fetal heart (Bae et al., 2003), thus indicating a development stage-specific effect.

Furthermore, we investigated two proteins that are closely involved in the regulation of the cell cycle: cyclin D2 and p27. These proteins have previously been studied and found to be differentially expressed in the hypoxia-treated fetal heart (Tong et al., 2013). Cyclin D2 is associated with other cell cycle regulators that work to promote cell cycle activity, while p27 is a cyclin-dependent kinase inhibitor and thus inhibits cell cycle activity. Therefore the expression of these two proteins should be inversely related, as our results indicate. Cyclin D2, a cell cycle promoter, is significantly decreased during anoxia treatment, while the cell cycle inhibitor, p27, is upregulated under these conditions. These results are consistent with our finding that anoxia treatment decreases cardiomyocyte proliferation. In addition, we tested the role of ET-1 acting through its ET_AR on the expression of cyclin D2 and p27. In the presence of PD156707, an ET_AR antagonist, anoxia had no effect on cyclin D2 expression. However, p27 expression was

significantly decreased in the presence of PD156707 compared to control conditions. These findings suggest that ET-1 and the ET_AR are key mediators in the anoxia-induced effects on cyclin D2 and p27. Ultimately, these results may help to explain the overall decrease in cardiomyocyte proliferation due to anoxia treatment.

Although a gradual decrease in proliferation in the critical window of the heart development is a normal developmental process, hypoxia and anoxia appear to accelerate this progression, particularly during the early development. The endpoint of cardiomyocyte number is a metric to measure the consequence of altering the proliferative capacity. Our results suggest that anoxia reduces cardiomyocyte endowment at postnatal day 14, when the heart is presumed to be fully mature and cardiomyocytes have terminally differentiated. Anoxia reduced proliferation at days 4 and 7, resulting in fewer cardiomyocytes in the differentiated heart seen at day 14. Given that cardiomyocytes are the functional contractile units of the heart, this decreased cardiomyocyte endowment in the heart may have negative impact in cardiac function and become more susceptible to injury later in life. While our results suggest a significant reduction in cardiomyocyte endowment due to anoxia at the critical window of the heart development, future studies using unbiased and random stereology will be needed to provide conclusive evidence of this effect.

Previous studies from our laboratory and others have shown that hypoxia regulates proliferation of cardiomyocytes and vascular muscle (Cooper & Beasley, 1999; Paradis et al., 2014; Tong et al., 2013; F. X. Zhang et al., 2007). However the downstream regulators of this response have yet to be identified. Our previous work in an *ex vivo* model showed that primary fetal cardiomyocytes exhibited a similar decrease in

proliferation when treated with endothelin-1 (ET-1) (Paradis et al., 2014). It is known that ET-1 expression is induced under hypoxic conditions *via* a HIF-binding site on its promoter (Hashiguchi et al., 1991; Hu et al., 1998; Minchenko & Caro, 2000; Ostlund et al., 2000; Yamada et al., 2001; Yamashita et al., 2001), specifically in cardiomyocytes (Kakinuma et al., 2001). ET-1 itself has also been shown to regulate proliferation, having a mitogenic effect on vascular smooth muscle (Agapitov & Haynes, 2002; Goldie, 1999; Komuro et al., 1988). Moreover our results showed an increase in prepro-ET-1 mRNA in the P4 neonatal heart when exposed to anoxia. Previous work has also shown an increase in prepro-ET-1 mRNA in the fetal heart exposed to maternal hypoxia (Paradis et al., 2014). These studies taken together implicate a role for ET-1 in mediating the hypoxia- and anoxia-induced decrease in cardiomyocyte proliferation.

A selective ET-receptor antagonist was used to study the role of both basal and anoxia-induced ET-1 in the present study. ET-1 can activate two receptor subtypes: the ET_A- and ET_B-receptor. Activation of the ET_A-receptor leads to vasoconstriction and is primarily found in vascular muscle (Hosoda et al., 1991). In contrast, the ET_B-receptor can provide a vasodilation effect as well as vasoconstriction depending on the receptor location, in endothelial cells (Sakurai et al., 1990) or vascular muscle (Arai et al., 1990; Kawanabe & Nauli, 2011; Sakurai et al., 1990; Yanagisawa, 1994), respectively. The ET_B-receptor also plays a role in the clearance of endothelin from tissues (Wilkes et al., 1993). In cardiomyocytes, the ET_A-receptor is the predominant subtype (Kohan et al., 2011), and has been implicated in regulating proliferation (Agapitov & Haynes, 2002; Goldie, 1999; Komuro et al., 1988). Therefore, our study evaluated the effects of PD156707, a selective antagonist for the ET_A-receptor (Reynolds et al., 1995), on

cardiomyocyte proliferation. Due to the short half-life of PD156707 of about one hour (Coe, Haleen, Welch, Liu, & Coceani, 2002), it was given twice a day just prior to anoxia exposure in the present study. We also evaluated the protein expression of the ET-receptors, both ET_AR and ET_BR. Interestingly, the results showed no change in the expression of either receptors due to anoxia treatment, suggesting that a change in receptor density is not contributing to the effects of anoxia or ET-1. The finding that PD156707 ameliorated the anoxia-induced decrease in proliferation of cardiomyocytes at day 4 and 7 implicates the ET_A-receptor as a key mediator. Furthermore, the addition of PD156707 alone elicited an increase in proliferation at day 4 beyond that of the control. This observation was not seen at day 7 or day 14, suggesting that the regulation of basal ET-1 function in the heart is dependent on the stage of development. At an earlier stage, basal ET-1 levels play a key role in regulating cardiomyocyte proliferation. The effect of basal ET-1 in regulating cardiomyocyte endowment in the developing heart is intriguing. The treatment of newborn rats with ET_A-receptor antagonist led to an increase in cardiomyocyte number per heart weight at day 7, suggesting that an appropriate level of basal ET-1 is necessary to optimize cardiomyocyte endowment in the heart.

Anoxia treatment had no significant effect on mononucleate and binucleate cell size, however inhibition of ET_AR by PD156707 caused an increase in cell size at day 7. This may suggest that basal ET-1 plays a role in maintaining cell size and, and if activation of the ET_A-receptor is blocked, the cell undergoes hypertrophy. The change in binucleate cell size is likely more relevant because the mononucleate cells still have the capacity to divide and are not yet terminally differentiated.

The heart to body weight ratio was unchanged with anoxia treatment for all age groups. However by blocking basal ET-1 with PD156707, the heart to body weight ratio was increased at postnatal day 7. These results suggest that the heart is increasing in size, which agrees with the results of increased cell size, proliferation, and cardiomyocyte number in the presence of PD156707. In the present study, the cardiomyocyte number were counted in freshly isolated myocytes, and the *in vivo* PD156707 treatment increased the cardiomyocyte number by about 65% in day 7 hearts. It has been previously demonstrated in neonatal rats that the heart contains approximately 25% cardiomyocytes (Banerjee, Fuseler, Price, Borg, & Baudino, 2007; Walsh, Ponten, Fleischmann, & Jovinge, 2010). If the PD156707 treatment induced proportional changes in the non-myocyte composition of the heart, it might increase the cardiomyocyte composition in the heart to around 42%, albeit the proliferation of non-myocyte cells in the heart could be differentially regulated. It is important to note that although changes in cardiomyocyte size measured in cells that were attached to plates suggest a physiological difference due to the PD156707 treatment, they are not necessarily representative of what's happening *in vivo*. It is likely that the increases of both cardiomyocyte number and cell size contribute to the increased heart weight observed at day 7 neonatal rats. The finding that the heart to body weight ratio is unchanged at day 14 even though anoxia treatment decreases cardiomyocyte endowment implies that the cardiomyocytes may increase their size to compensate for the loss of cells and maintain the size of the heart. However, we were unable to measure cardiomyocyte size from the day 14 hearts due to technical limitations and their poor attachment to the culture plate at this stage. Another possibility includes an increase in non-cardiomyocyte cell number and size in the heart after anoxia treatment.

The present study evaluated not only the effects of newborn anoxia treatment on the terminal differentiation of neonatal cardiomyocytes but also the role of basal ET-1 on this process. We identified a mechanism through which neonatal anoxia exposure induces an accelerated loss of cardiomyocyte proliferation *via* the ET_A-receptor, which subsequently results in reduced cardiomyocyte endowment in the fully differentiated heart. Our study also demonstrated the role that basal ET-1 plays in regulating cardiomyocyte size, proliferation, and number in the developing heart. Given the clinical implications of these findings in understanding the effects of hypoxia on the heart development in preterm infants, further investigation into the mechanisms involved is needed.

Acknowledgements

The authors would like to acknowledge Dr. David Baylink and Dr. Xiaobing Zhang for the use of the FACSAria, as well as Amanda Neises for her technical assistance.

This study was supported by the National Institutes of Health grants HL118861 (to L. Zhang). A portion of this research used the Loma Linda University School of Medicine Advanced Imaging and Microscopy Core, a facility supported in part by the National Science Foundation through the Major Research Instrumentation program of the Division of Biological Infrastructure Grant No. 0923559 and the Loma Linda University School of Medicine.

CHAPTER FOUR

ENDOTHELIN-1-INDUCED CHANGES IN THE FETAL CARDIOMYOCYTE PROTEOME

By

Alexandra Paradis, Chiranjib Dasgupta, and Lubo Zhang

Abstract

Hypoxia is a fetal stressor that is known to lead to the production of endothelin-1 (ET-1). Previous work has shown that ET-1 treatment leads to the premature terminal differentiation of fetal cardiomyocytes, however the mechanism is, as of yet, unknown. We tested the hypothesis that the fetal cardiomyocyte proteome will be greatly altered due to ET-1-treatment, revealing a potential molecular mechanism of ET-1-induced terminal differentiation. Over a thousand proteins were detected in the fetal cardiomyocytes and of that 75 proteins were significantly altered due to ET-1 treatment. Following pathway analysis, the merged network depicted several key proteins that appear to be involved in regulating proliferation, including: EED, UBC, ERK1/2, MAPK, Akt, and EGFR. Of particular interest is the EED protein, which is associated with regulating proliferation *via* epigenetic mechanisms. Herein we propose a model of the molecular mechanism by which ET-1 induced cardiomyocyte terminal differentiation occurs.

Introduction

There is compelling evidence indicating that an adverse intrauterine environment can result in an increased vulnerability to cardiovascular disease later in adult life (Barker, 1995; Barker & Osmond, 1986). Environmental stress factors during fetal development can impact the growth and maturation of critical organs in the developing fetus, such as the heart. Hypoxia is one major stress factor to the developing fetus. Previous studies have shown that a hypoxic environment results in the premature terminal differentiation of fetal cardiomyocytes (Bae et al., 2003; Paradis et al., 2014; Tong et al.,

2013; Tong et al., 2011), characterized by decreased proliferative capacity. Terminal differentiation is the final step in the maturation of cardiomyocytes and it occurs in a timeframe surrounding birth. Cardiomyocytes undergo cell-cycle withdrawal soon after birth, and the majority of postnatal cardiac growth is due to cardiomyocytes growing in size with very little proliferation (Bergmann et al., 2009). Studies have shown that hypoxia accelerates this maturation process and can ultimately lead to fewer cardiomyocytes endowed within the heart (Bae et al., 2003). The heart is fully mature shortly after birth and thus the timing of this transition is crucial in determining the number of cardiomyocytes that will reside in the heart for a lifetime. If this timeline is perturbed, it may lead to long-term detrimental consequences on heart development and function throughout life.

Several *in vivo* and *in vitro* fetal studies have shown the effect of hypoxia on fetal heart development, however the mechanisms remain largely unknown. Our recent studies have suggested a role for endothelin-1 (ET-1) in hypoxia-induced terminal differentiation of cardiomyocytes (Paradis et al., 2014). Hypoxia is known to induce ET-1 expression (Hashiguchi et al., 1991; Ostlund et al., 2000; Yamada et al., 2001; Yamashita et al., 2001), and the cardiomyocyte is both a site of synthesis and action of ET-1 (Kedzierski & Yanagisawa, 2001; Kohan et al., 2011). Although ET-1 has been implicated in the accelerated terminal differentiation process, the protein regulators downstream to ET-1 are unknown.

Therefore, in the present study we tested the hypothesis that ET-1 alters the expression of key regulatory proteins involved in cardiomyocyte terminal differentiation. Herein, we provide evidence that ET-1 treatment differentially regulates expression of

key proteins involved in the maturation and proliferation of fetal cardiomyocytes. Our proteomics experiment identified seventy-five (75) proteins that were previously unknown to be differentially modulated by ET-1 treatment in fetal cardiomyocytes. Collectively, the effect of these proteins may describe the molecular mechanism(s) that dictate(s) accelerated terminal differentiation of cardiomyocytes.

Materials and Methods

Experimental Animals

Time-dated pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI). Hearts were isolated from day 21 fetuses, as previously described (Paradis et al., 2014). To isolate hearts, pregnant rats were anesthetized with isoflurane, and adequate anesthesia was determined by loss of pedal withdrawal reflex. Fetuses were removed and pregnant rats killed by removing the hearts. Fetal hearts were isolated for the studies. All procedures and protocols were approved by the Institutional Animal Care and Use Committee and followed the guidelines by US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Primary Cardiomyocyte Culture and Treatment

Cardiomyocytes were isolated from day 21 fetal rats as previously described (Paradis et al., 2014; Y. Xiao et al., 2000). Briefly, hearts were enzymatically digested then preplated to further enrich in cardiomyocytes. Cells were cultured in Hyclone Medium 199 (Thermo Scientific) supplemented with 10% fetal bovine serum (Gemini Bio-Products) and 1% antibiotics (10,000 I.U./mL penicillin, 10,000 µg/mL

streptomycin) at 37°C in 95% air/5% CO_2. BrdU (0.1mM) was added to the medium to prevent fibroblast proliferation. Within three days of culture, the cells formed a monolayer with synchronized beating, characteristic of viable cardiomyocytes. Cells were treated in the absence or presence of ET-1 (Sigma; 10 nM) in the growth media at 70-80% confluency for 24 hours.

Proteomic Analysis

Analysis of the isolated primary cardiomyocytes proteome was done before and after ET-1 treatment by Tandem Mass Tag (TMT) labeling of the peptides followed by tandem mass tags (TMTs)-LC-MS/MS analysis with an LTQ-Orbitrap-Pro instrument as previously described (D. Xiao et al., 2014; L. Xiong et al., 2011; K. Zhang et al., 2012). TMT reagents belong to a family of reagents known as isobaric mass tags, which provide a high throughput alternative to immunoblot based quantitation.

Primary fetal cardiomyocytes were homogenized in RIPA lysis buffer (Santa Cruz Biotechnology) containing protease inhibitors and protein concentrations were determined using the BCA assay (ThermoScientific). Proteins were reduced by 50 mM DTT in triethyl ammonium bicarbonate buffer (TEAB) at 55°C for 30 minutes and then alkylated with 50 mM 2-iodoacetamide (IAA) for 45 minutes at room temperature in dark. Next, proteins were precipitated with acetone (80% v/v of acetone) and reconstituted in 90 µl TEAB. An aliquot of 100 µg proteins was digested with 4 µg of freshly prepared trypsin (Sigma) at 37°C for at least 10 hours. Resulting peptides were TMT labeled at their N-term end by TMT duplex kit (Thermoscientific) according to the manufacture's instructions. Samples from each treatment group were TMT-labeled

with one of the respective reporters at *m/z*= 126.1 (control) and 127.1 (ET-1, 10nM). Each TMT-labeled protein pool was acidified with 0.1% formic acid (FA) and fractionated with strong cation-exchange (SCX) chromatography on a Toptip column (Poly LC, MD). For fractionation, the matrix was equilibrated with 0.1% FA in 20% acetonitrile (ACN) to facilitate peptide binding. After collection of the flow-through (FT), 1 mL of each subfraction was sequentially eluted with solvents, 50 mM KCl, 200 mM KCl, and 5% ammonium hydroxide in 20% ACN. Next, the fractions were dried under vacuum to remove ACN, reconstituted in 1% FA, and then desalted using a Toptip column with C18/hypercarb mixed materials (Poly LC, MD). The eluted peptides were once again vacuum-dried, reconstituted in 30 µl of 0.1% FA, and then subjected to LC-MS/MS analysis. The LC-MS/MS analysis was performed as described previously (K. Zhang et al., 2012). Quantitation of SCX fractionated TMT-labeled peptides was carried out on the Thermo LTQ-Orbitrap Pro mass spectrometer. Peptides were separated by online reversed phase liquid chromatography (RPLC) using an Easy-nLC that is equipped with an autosampler (Thermo Scientific). A 10 cm, 75 µm id, 3 µm particle size, C18-A2 analytical column (Thermo) was used for the RPLC separations. Approximately 3.2 µg of peptide sample was injected. A *pre-column* (Thermo, 0.1 x 2 cm, 5 µm C18-A1) connected in line preceding the *analytical* column and a 200-min gradient (solvent A, 0.1% FA in water; solvent B, 0.1% FA in ACN) from 5-30% solvent B was used for separating the peptides. The Orbitrap mass analyzer was set to acquire data at 60,000 resolution for the parent full-scan mass spectrum followed by data-dependent high collision-energy dissociation (HCD) MS/MS spectra for the top 10 most abundant ions acquired at 7500 resolution. Mass Spectrometry data were processed and searched against

rat protein database through the Thermo Scientific Proteome Discoverer 1.3 platform using SEQUEST search engine with parameters as previously described (L. Xiong et al., 2011). Protein expression levels (*i.e.,* fold change values of protein expression in ET-1 treated group relative to control) were expressed as the ratios of the intensities of reporter ions (127/126). We applied the following criteria for data analysis. For a protein expression fold change to be considered significant, a fold change cut-off value of 1.146 (more than 14.6% up-or down-regulation) was used. Though our previous work (D. Xiao et al., 2014) along with others (L. Xiong et al., 2011; K. Zhang et al., 2012) used a cut-off value of 1.2 for similar isobaric TMT labeling experiments, in this study we used a slightly relaxed cut-off value to include changes in expression those proteins that would otherwise fall just short of being significant and that these proteins may also be of physiological significance. For a protein to be considered correctly detected with unique peptides, respective MS/MS spectrum was checked on Proteome Discoverer (version 1.3) and only accepted if the major product ions matched the theoretically predicted product ions from the database. Subsequently, Ingenuity Pathway Analysis (IPA) tool (Qiagen) was used to decipher molecular pathway relationships between significantly modulated proteins in the form of network maps.

Results

Endothelin-1 Alters the Fetal Cardiomyocyte Proteome

A total of more than a thousand proteins were identified in the fetal cardiomyocytes by one-dimensional LC-MS/MS approach. Of these proteins, the expression of 75 proteins was differentially modulated due to endothelin-1 treatment

compared to untreated control groups (Table 4.1). IPA pathway analysis was performed using stringent filter (rodent) to decipher relationships amongst the modulated proteins. This analysis revealed that the modulated proteins were distributed into five discrete networks 1 thru 5 corresponding to Figures 2 thru 6, which are presented here with their sub-cellular distribution. Figure 4.1A depicts the merged network map, whose keys are presented in Figure 4.1B. The proteins associated with each network as well as their known functions are depicted in Table 4.2. Figures 4.7, 4.8, 4.9, and 4.10 are network maps of those proteins that are interacting with the four major convergence points (proteins): 1) EED, 2) UBC, 3) ERK/p38 MAPK/Akt/RhoA, and 4) EGFR respectively.

Figure 4.1. Merged network map showing endothelin-1-induced changes in the fetal cardiomyocyte proteome. Primary fetal cardiomyocytes were treated with control or endothelin-1 (10 nM) for 24 hours. Protein expression analysis was performed in fetal cardiomyocytes by TMT-LC-MS/MS analysis with a LTQ-Orbitrap-Velos instrument. IPA pathway analysis using the stringent filter (rodent) revealed five separate sub-networks for endothelin-1-modulated proteins. **Panel A** depicts a merged network of all the proteins detected. **Panel B** is the key. The network is represented based on sub-cellular distribution.

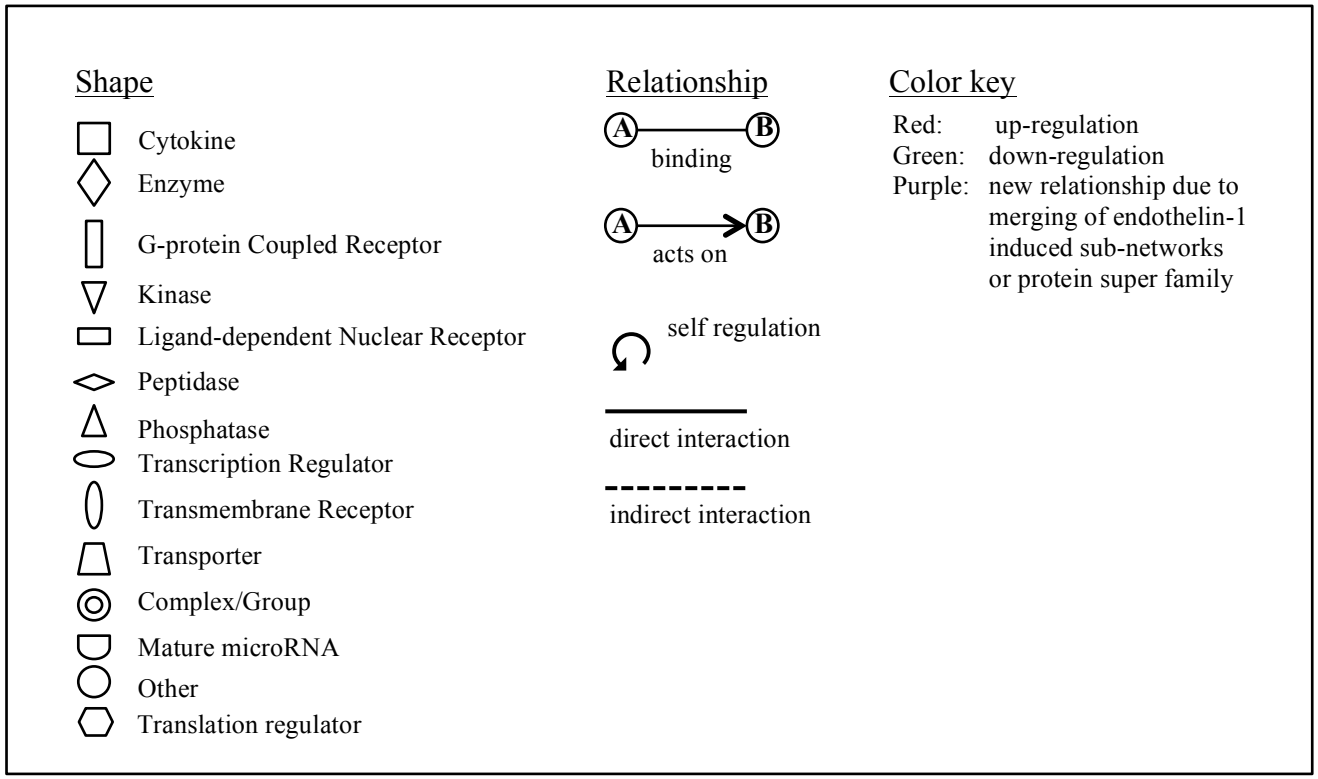

Figure 4.1B. IPA network keys

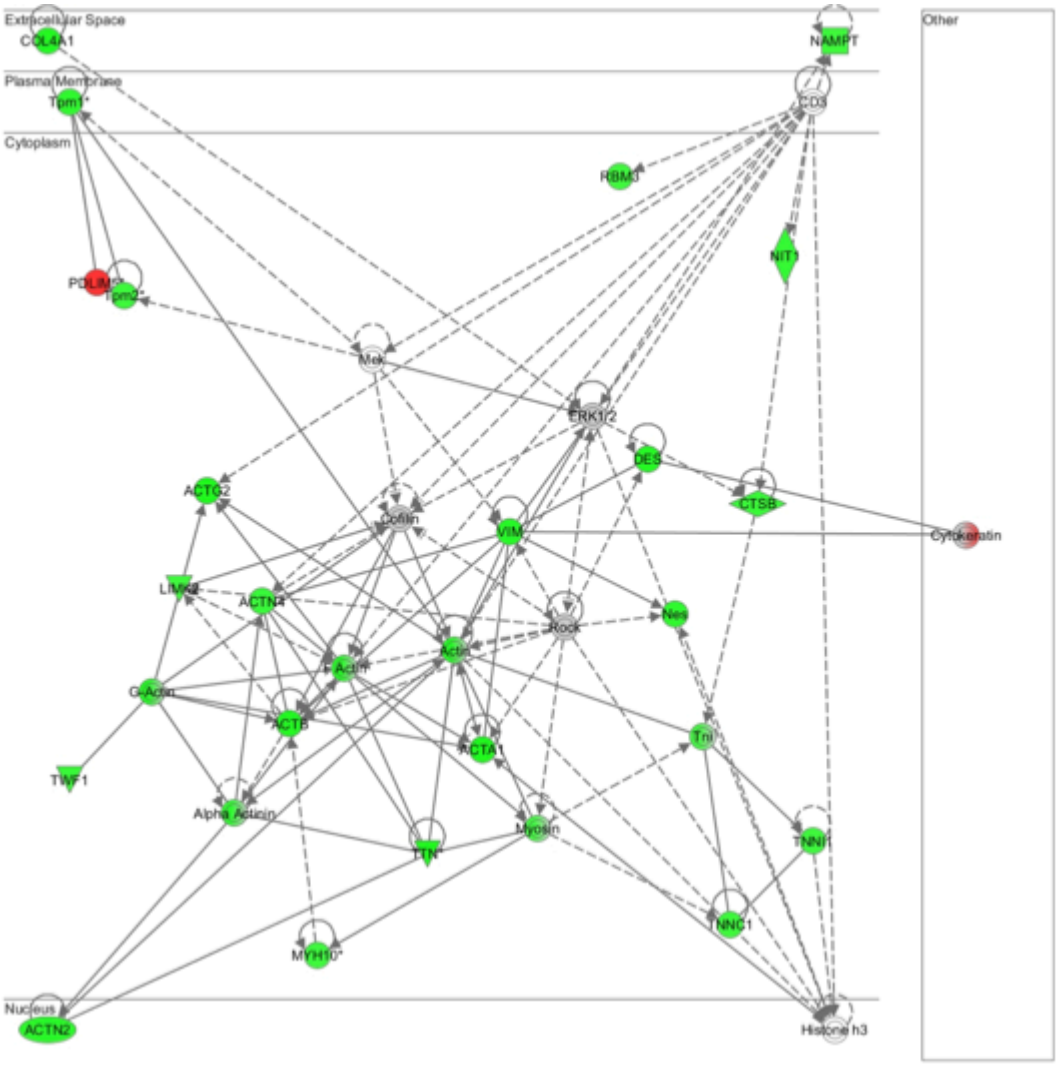

Figure 4.2. Network 1. ET-1 induced proteins, which are known to be involved in organ morphology, skeletal and muscular system development and function, and embryonic development. The network is represented based on sub-cellular distribution.

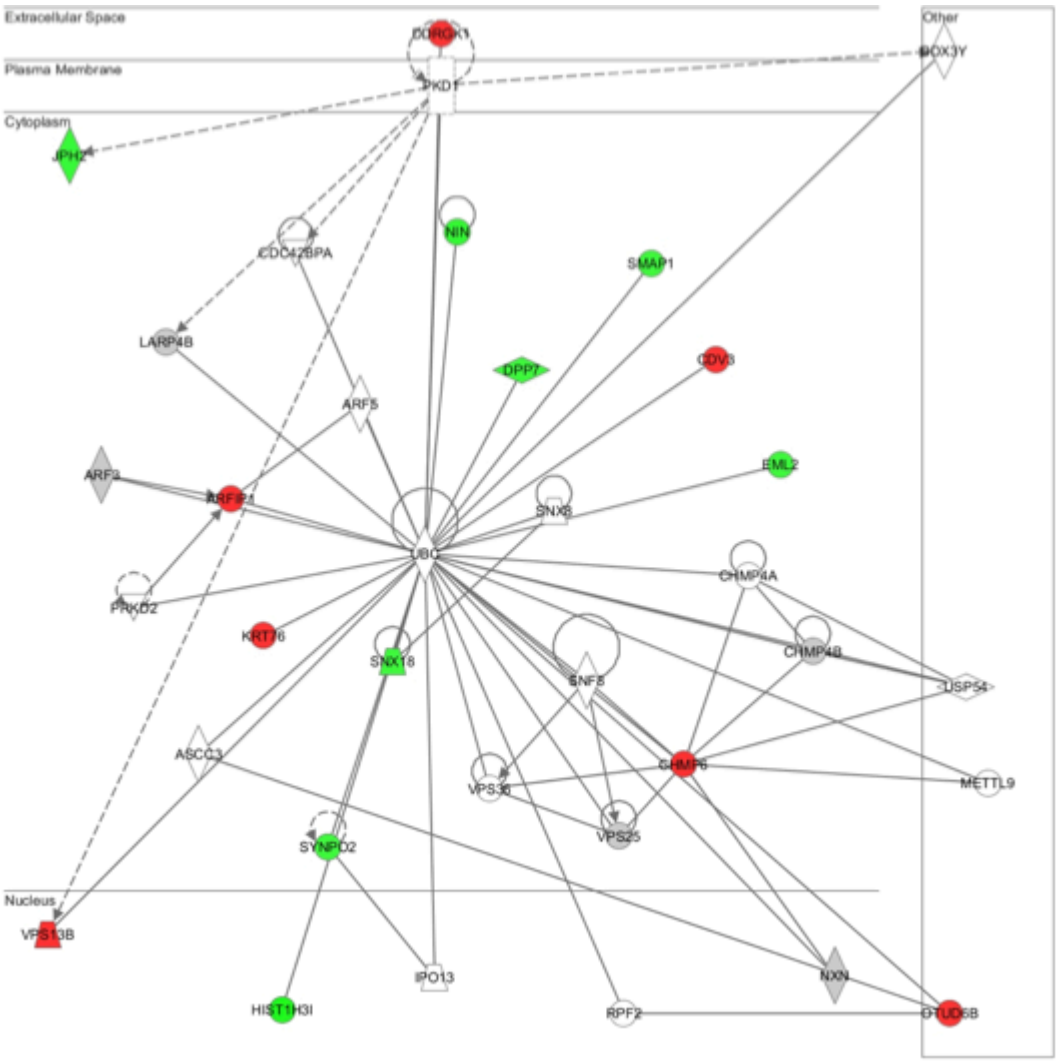

Figure 4.3. Network 2. ET-1 induced proteins, which are known to be involved in cancer and infectious diseases. The network is represented based on sub-cellular distribution.

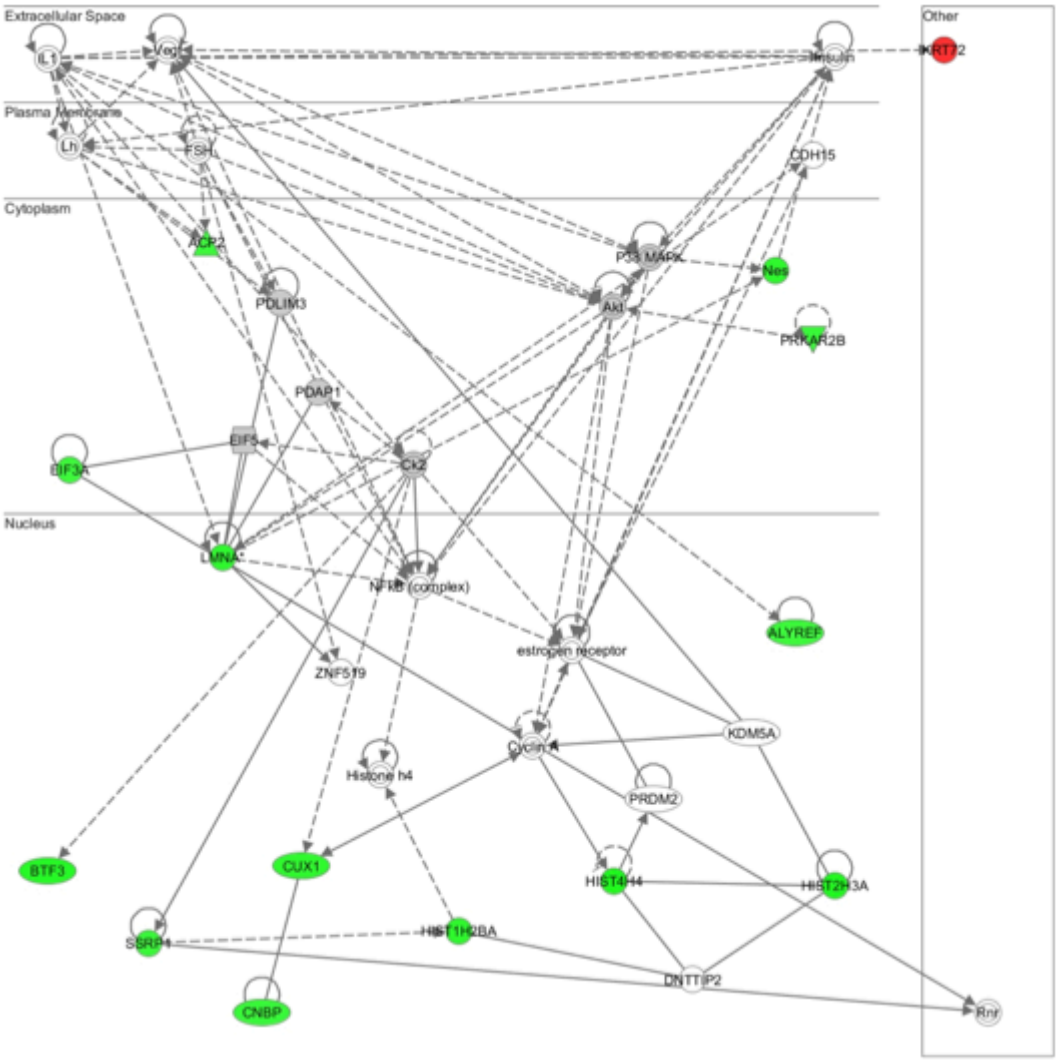

Figure 4.4. Network 3. ET-1 induced proteins, which are known to be involved in gene expression and organismal injury and abnormalities. The network is represented based on sub-cellular distribution.

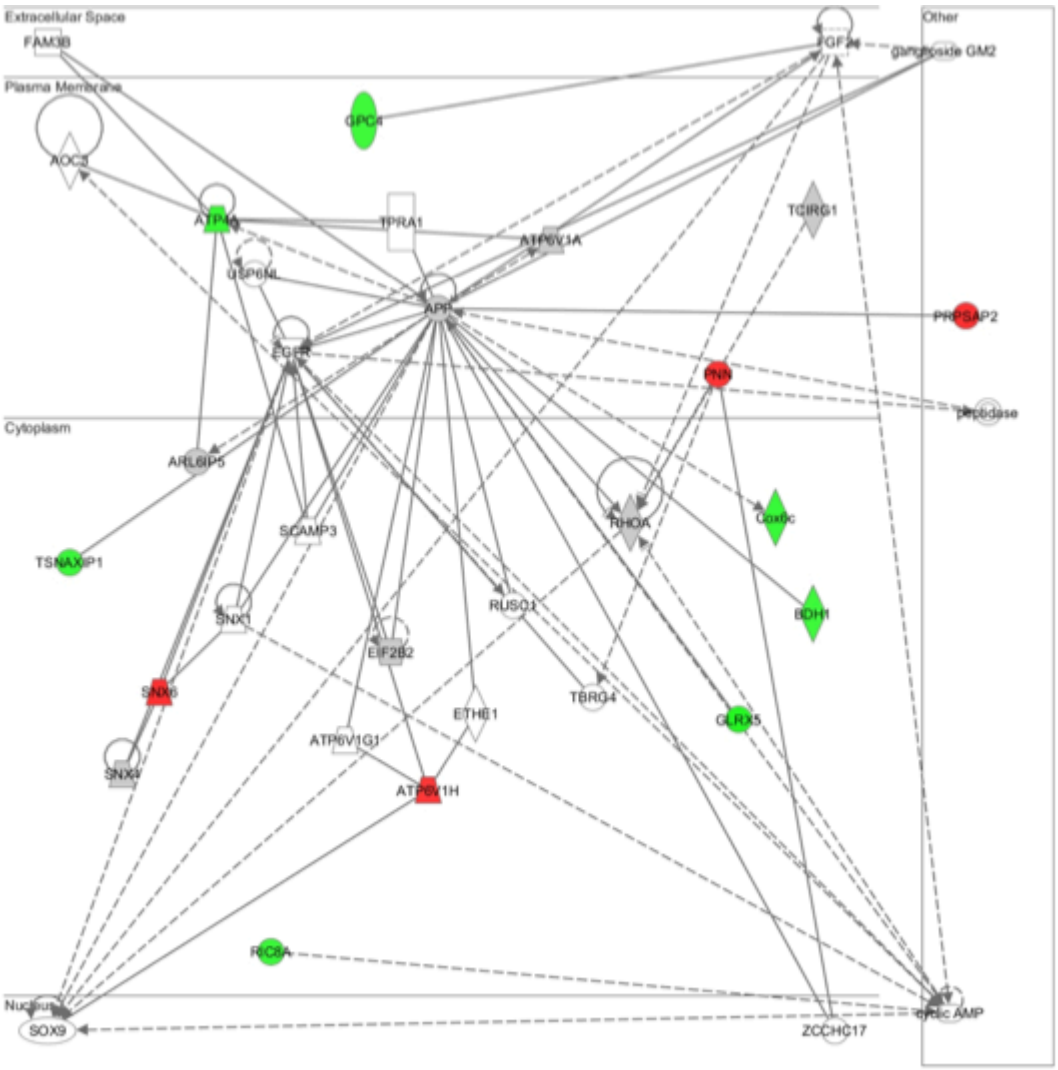

Figure 4.5. Network 4. ET-1 induced proteins, which are known to be involved in cellular assembly and organization, tissue development, and molecular transport. The network is represented based on sub-cellular distribution.

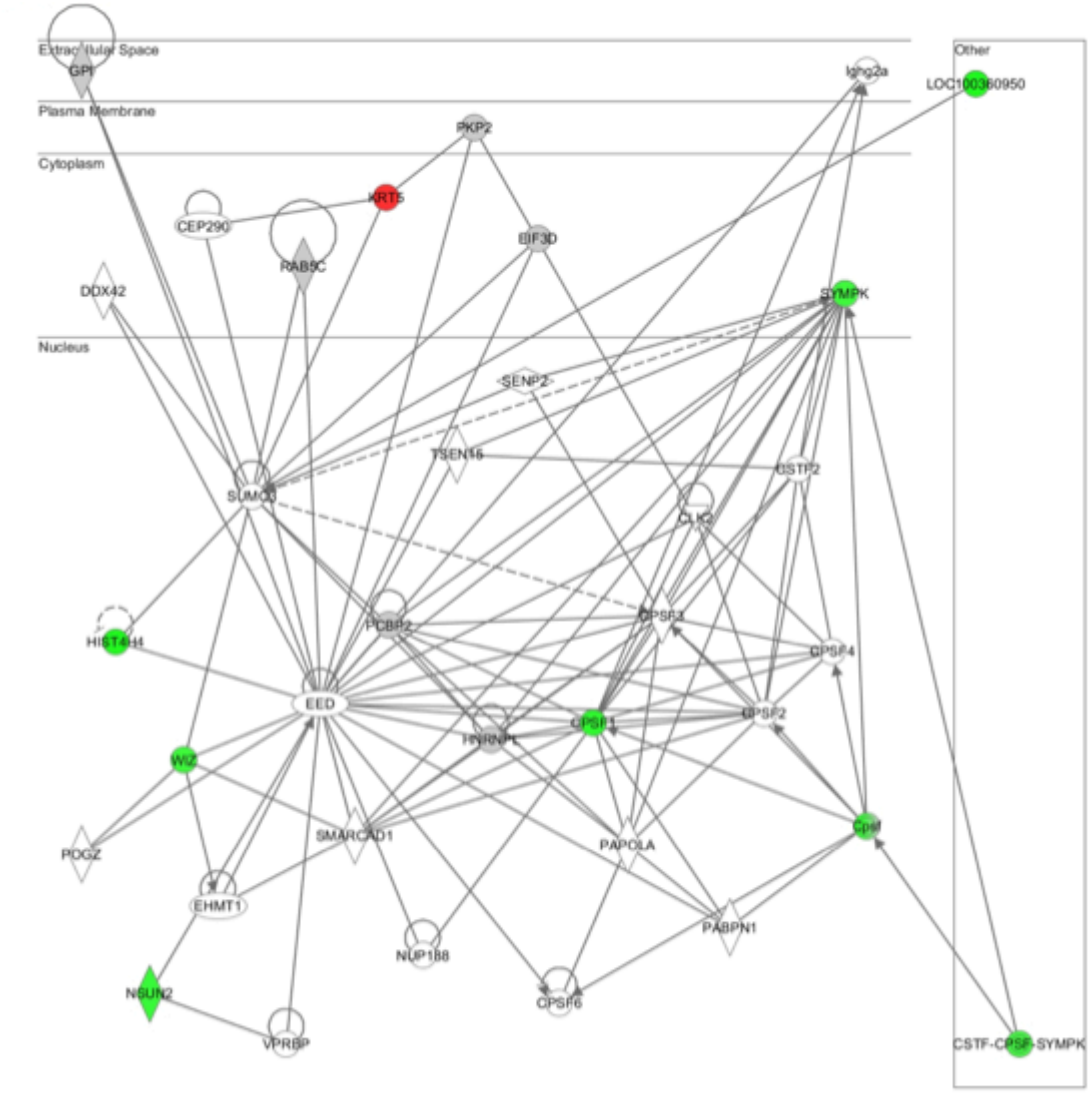

Figure 4.6. Network 5. ET-1 induced proteins, which are known to be involved in RNA post-transcriptional modification, post-translational modification, and connective tissue disorders. The network is represented based on sub-cellular distribution.

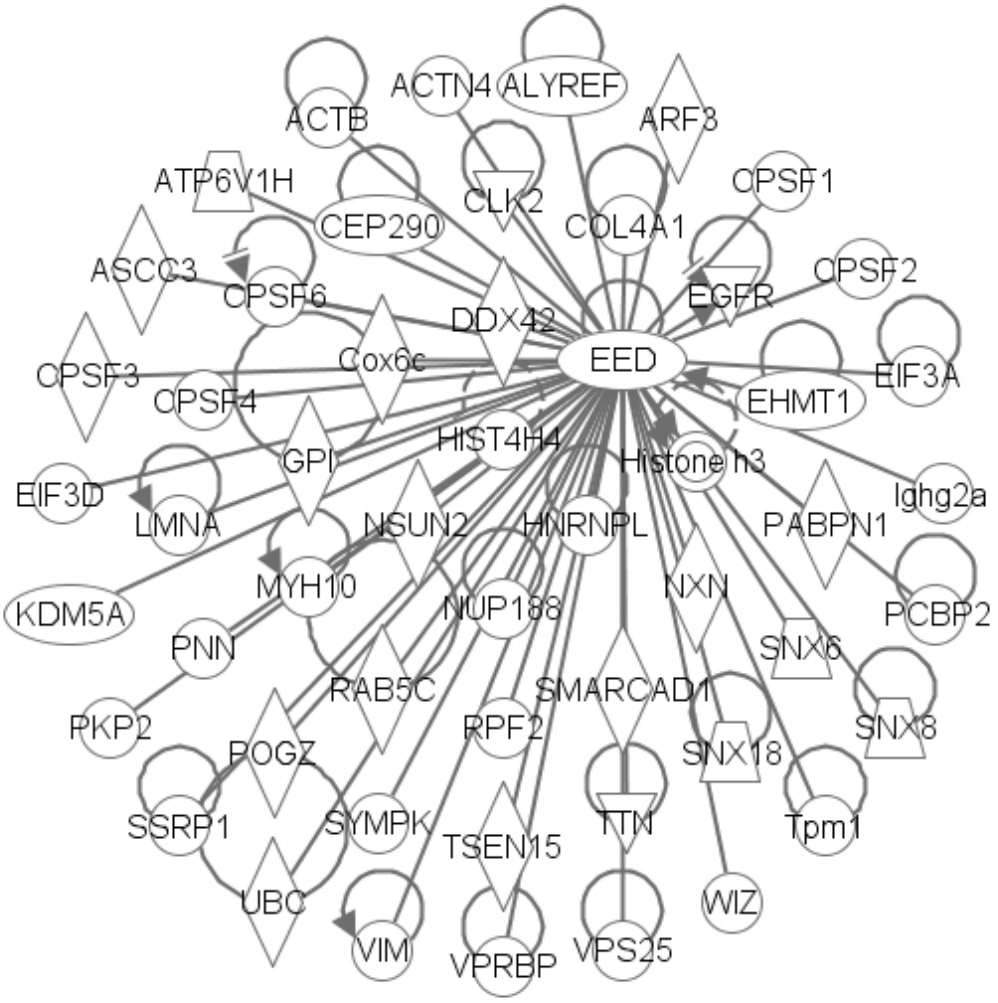

Figure 4.7. EED interacting proteins. The ET-1 induced cardiomyocyte proteome reveals a large number of proteins that are interacting with EED (embryonic ectoderm development), a potential key player in the ET-1 induced effects on the cardiomyocyte.

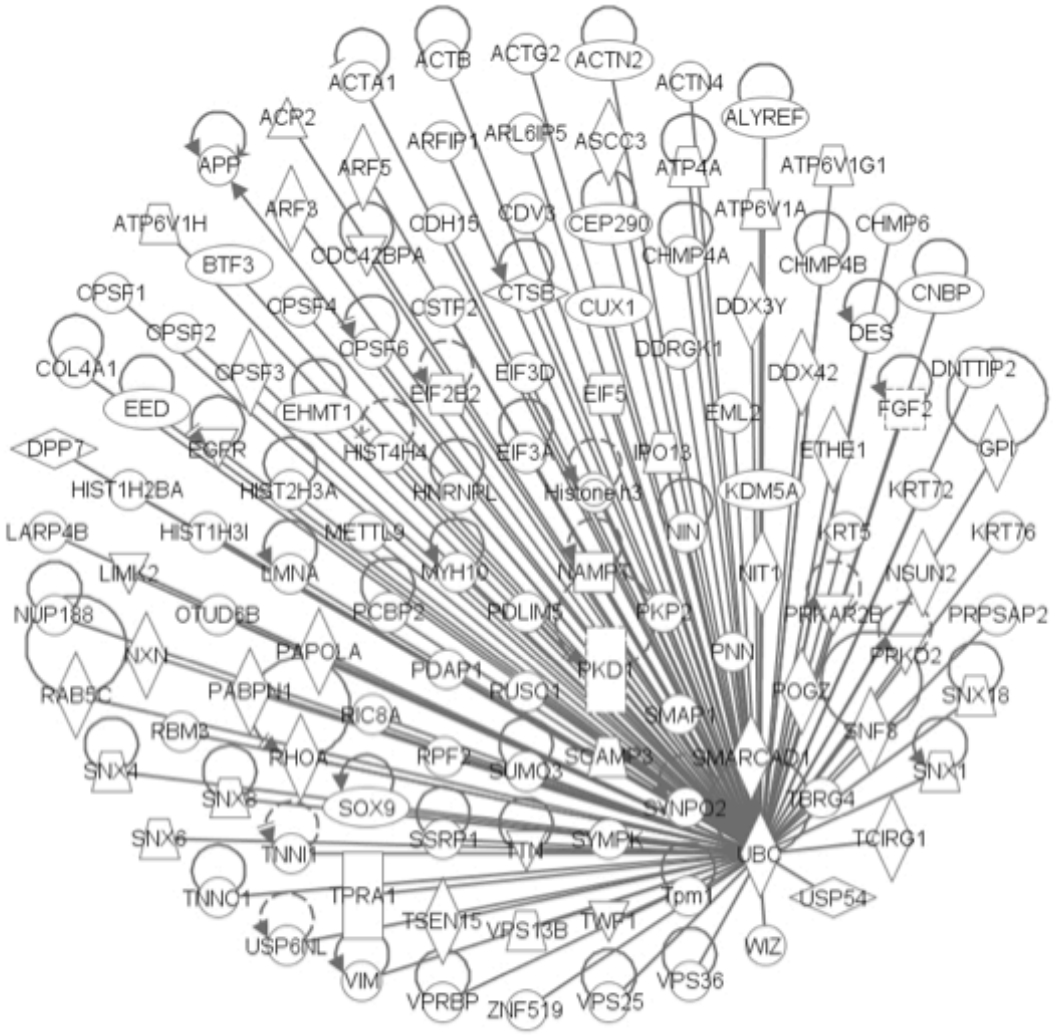

Figure 4.8. UBC interacting proteins. The ET-1 induced cardiomyocyte proteome reveals a large number of proteins that are interacting with UBC (ubiquitin protein C), one of the major convergence points in the merged network map.

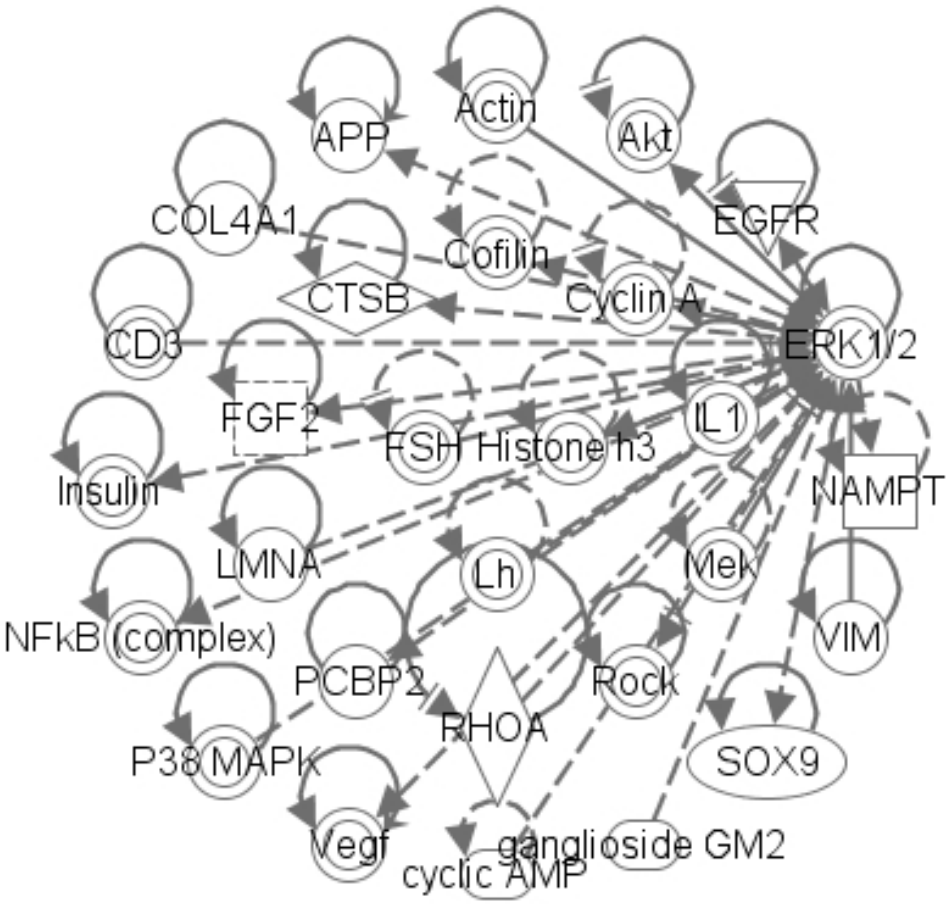

Figure 4.9. ERK1/2 interacting proteins. The ET-1 induced cardiomyocyte proteome reveals a large number of proteins that are interacting with ERK1/2, a key protein involved in cell survival and proliferation.

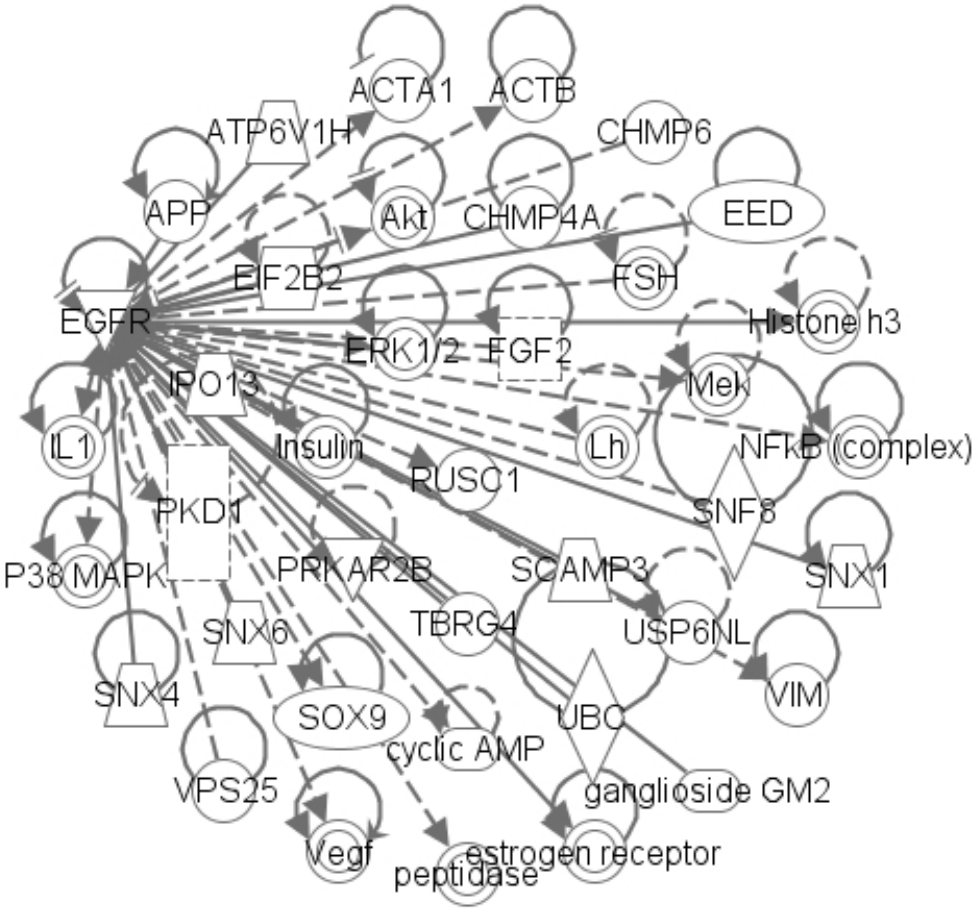

Figure 4.10. EGFR interacting proteins. The ET-1 induced cardiomyocyte proteome reveals a large number of proteins that are interacting with EGFR (epidermal growth factor receptor), another major convergence points in the merged network map.

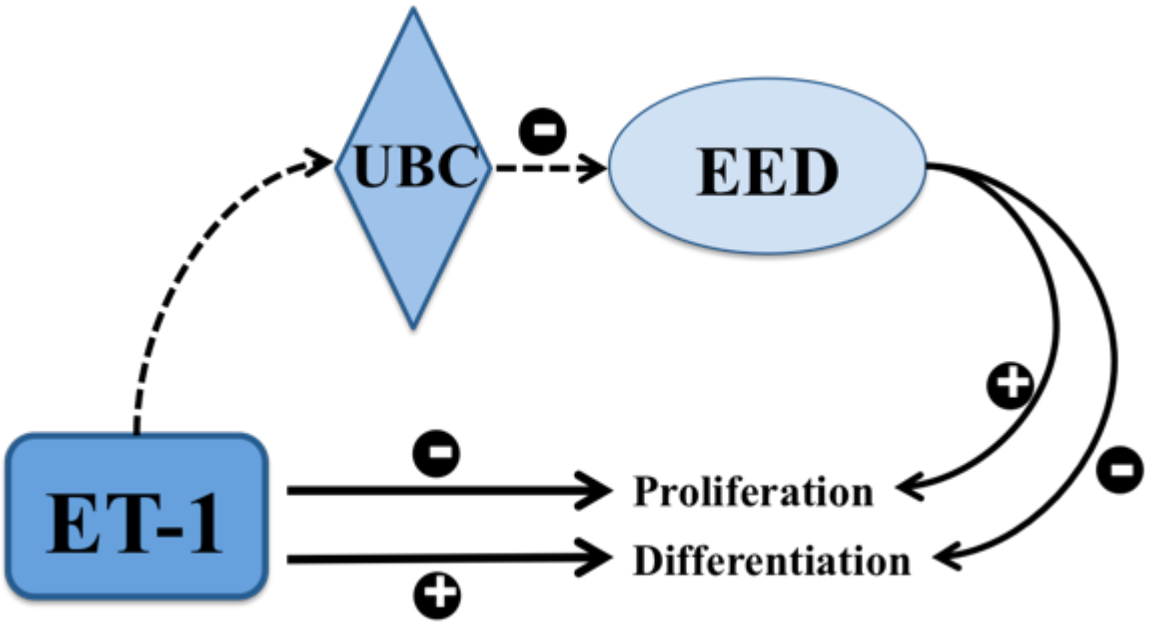

Figure 4.11. Proposed mode of action of ET-1. Endothelin-1 stimulates cardiomyocyte terminal differentiation and decreases proliferation, whereas the EED protein is known to do the opposite. The proposed model illustrates a mechanism by which ET-1 induces UBC, which may lead to a downregulation of EED and thus inhibit its effect on proliferation and differentiation.

Table 4.1. Endothelin-1-mediated changes in the fetal cardiomyocyte proteome. Represented is a list of 75 proteins whose expression was altered by ET-1 treatment of the fetal cardiomyocyte. The fold change (126/127) refers to the change in protein expression in cardiomyocytes treated with 10nM ET-1 compared to control.

Accession #	Entrez Gene Name	Location	127/126 ± SEM	Fold Change
IPI00777957.2	acid phosphatase 2, lysosomal	Cytoplasm	0.859 ± 0.014	-1.164
IPI00189813.1	actin, alpha 1, skeletal muscle	Cytoplasm	0.736 ± 0.039	-1.358
IPI00189819.1	actin, beta	Cytoplasm	0.759 ± 0.022	-1.317
IPI00200455.1	actin, gamma 2, smooth muscle, enteric	Cytoplasm	0.746 ± 0.016	-1.340
IPI00363022.4	actinin, alpha 2	Nucleus	0.795± 0.050	-1.258
IPI00213463.2	actinin, alpha 4	Cytoplasm & Nucleus	0.846 ± 0.048	-1.182
IPI00763263.2	Aly/REF export factor	Nucleus	0.863 ± 0.029	-1.158
IPI00199911.2	ADP-ribosylation factor interacting protein 1	Cytoplasm	2.465 ± 0.058	2.465
IPI00365705.6	ATPase, H+/K+ exchanging, alpha polypeptide	Plasma Membrane	0.843 ± 0.021	-1.186
IPI00952436.1	ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H	Cytoplasm	1.148 ± 0.056	1.148
IPI00480620.1	3-hydroxybutyrate dehydrogenase, type 1	Cytoplasm	0.866 ± 0.106	-1.155
IPI00372004.3	basic transcription factor 3	Nucleus	0.781 ± 0.017	-1.282
IPI00565758.1	CDV3 homolog (mouse)	Cytoplasm	1.157 ± 0.010	1.157
IPI00364337.3	charged multivesicular body protein 6	Cytoplasm	1.224 ± 0.044	1.224
IPI00201505.3	CCHC-type zinc finger, nucleic acid binding protein	Nucleus	0.846 ± 0.104	-1.182
IPI00780087.2	collagen, type IV, alpha 1	Extracellular Space	0.771 ± 0.086	-1.305
IPI00230832.7	cytochrome c oxidase subunit VIc	Cytoplasm	0.847 ± 0.045	-1.181
IPI00370154.3	cleavage and polyadenylation specific factor 1, 160kDa	Nucleus	0.804 ± 0.013	-1.243

IPI00562653.2	cathepsin B	Cytoplasm	0.839 ± 0.016	-1.192
IPI00370330.4	cut-like homeobox 1	Nucleus	0.806 ± 0.030	-1.241
IPI00373131.3	DDRGK domain containing 1	Extracellular Space	1.167 ± 0.009	1.167
IPI00421517.7	desmin	Cytoplasm	1.296 ± 0.030	-1.296
IPI00230946.4	dipeptidyl-peptidase 7	Cytoplasm	0.861 ± 0.083	-1.162
IPI00372810.5	eukaryotic translation initiation factor 3, subunit A	Cytoplasm	0.866 ± 0.029	-1.155
IPI00947800.1	echinoderm microtubule associated protein like 2	Cytoplasm	0.867 ± 0.005	-1.153
IPI00365904.4	glutaredoxin 5	Cytoplasm	0.765 ± 0.045	-1.306
IPI00471669.1	glypican 4	Plasma Membrane	0.868 ± 0.060	-1.151
IPI00231976.7	histone cluster 1, H2ba	Nucleus	0.801 ± 0.036	-1.248
IPI00393508.3	histone cluster 1, H2bb	Nucleus	0.486 ± 0.054	-2.058
IPI00767428.2	histone cluster 1, H3i	Nucleus	0.57 ± 0.033	-1.754
IPI00476722.4	histone cluster 2, H3a	Nucleus	0.518 ± 0.058	-1.931
IPI00960040.1	histone cluster 4, H4	Nucleus	0.646 ± 0.047	-1.549
IPI00199887.1	junctophilin 2	Cytoplasm	0.85 ± 0.028	-1.177
IPI00382153.4	keratin 5	Cytoplasm	1.233 ± 0.072	1.233
IPI00421781.1	keratin 72	Other	1.328 ± 0.052	1.328
IPI00421812.1	keratin 76	Cytoplasm	1.178 ± 0.066	1.178
IPI00231014.1	LIM domain kinase 2	Cytoplasm	0.87 ± 0.015	-1.149
IPI00201060.4	lamin A/C	Nucleus	0.81 ± 0.059	-1.235

IPI00958096.1	GF20391-like	Other	0.734 ± 0.065	-1.362
IPI00959660.1	RAB1B, member RAS oncogene family-like	Other	0.829 ± 0.076	-1.207
IPI00957208.1	keratin 6A-like	Other	1.179 ± 0.086	1.179
IPI00203211.4	leucine rich repeat (in FLII) interacting protein 1	Other	1.195 ± 0.030	1.195
IPI00211813.2	myosin, heavy chain 10, non-muscle	Cytoplasm	0.872 ± 0.035	-1.146
IPI00230979.1	nicotinamide phosphoribosyltransferase	Extracellular Space	0.863 ± 0.101	-1.158
IPI00201261.2	nestin	Cytoplasm	0.806 ± 0.033	-1.240
IPI00365822.3	ninein (GSK3B interacting protein)	Cytoplasm	0.822 ± 0.072	-1.216
IPI00831725.1	nitrilase 1	Cytoplasm	0.834 ± 0.053	-1.200
IPI00364693.3	NOP2/Sun RNA methyltransferase family, member 2	Nucleus	0.85 ± 0.060	-1.176
IPI00366831.3	OTU domain containing 6B	Other	1.163 ± 0.078	1.163
IPI00949808.1	PDZ and LIM domain 5	Cytoplasm	1.164 ± 0.055	1.164
IPI00370658.1	pinin, desmosome associated protein	Plasma Membrane	1.178 ± 0.092	1.178
IPI00365600.3	protein kinase, cAMP-dependent, regulatory, type II, beta	Cytoplasm	0.846 ± 0.028	-1.181
IPI00194524.1	phosphoribosyl pyrophosphate synthetase-associated protein 2	Other	1.59 ± 0.170	1.590
IPI00367437.5	RNA binding motif (RNP1, RRM) protein 3	Cytoplasm	0.869 ± 0.016	-1.151
IPI00565999.1	similar to H3 histone, family 3B	Other	0.752 ± 0.090	-1.329
IPI00567981.3	similar to Histone H3.3	Other	0.805 ± 0.017	-1.243
IPI00777683.2	similar to pyridoxal (pyridoxine, vitamin B6) kinase	Other	0.83 ± 0.037	-1.204
IPI00196562.3	RIC8 guanine nucleotide exchange factor A	Cytoplasm	0.85 ± 0.005	-1.176

IPI00764478.2	small ArfGAP 1	Cytoplasm	0.865 ± 0.035	-1.156
IPI00949260.1	sorting nexin 18	Cytoplasm	0.867 ± 0.021	-1.153
IPI00365613.2	sorting nexin 6	Cytoplasm	1.186 ± 0.157	1.186
IPI00231263.3	structure specific recognition protein 1	Nucleus	0.857 ± 0.114	-1.166
IPI00364043.4	symplekin	Cytoplasm	0.87 ± 0.059	-1.149
IPI00205262.5	synaptopodin 2	Cytoplasm	0.87 ± 0.053	-1.149
IPI00362425.1	troponin C type 1 (slow)	Cytoplasm	0.861 ± 0.005	-1.162
IPI00231699.5	troponin I type 1 (skeletal, slow)	Cytoplasm	0.869 ± 0.053	-1.151
IPI00197888.2	tropomyosin 1, alpha	Plasma Membrane	0.813 ± 0.039	-1.230
IPI00187731.4	tropomyosin 2, beta	Cytoplasm	0.86 ± 0.059	-1.163
IPI00196557.4	translin-associated factor X interacting protein 1	Cytoplasm	0.853 ± 0.096	-1.172
IPI00554003.3	titin	Cytoplasm	0.73 ± 0.047	-1.370
IPI00361693.1	twinfilin actin-binding protein 1	Cytoplasm	0.847 ± 0.063	-1.180
IPI00957757.1	unc-80 homolog (C. elegans)	Cytoplasm	0.839 ± 0.019	-1.192
IPI00230941.5	vimentin	Cytoplasm	0.78 ± 0.039	-1.282
IPI00400610.4	vacuolar protein sorting 13 homolog B (yeast)	Nucleus	1.196 ± 0.011	1.196
IPI00564521.2	widely interspaced zinc finger motifs	Nucleus	0.86 ± 0.021	-1.162

Data are mean ± SEM.

Table 4.2. Top diseases and functions associated with each network. The proteins are categorized into 5 networks and pathway analysis reveals the major functions and diseases that these groups of proteins are known to be associated with.

Network	Top Diseases and Functions	Focus Molecules	Molecules in Each Network
1	Organ Morphology, Skeletal and Muscular System Development and Function, Embryonic Development	22	ACTA1, ACTB, ACTG2, Actin, ACTN2, ACTN4, Alpha Actinin, CD3, Cofilin, COL4A1, CTSB, Cytokeratin, DES, ERK1/2, F Actin, G-Actin, Histone h3, LIMK2, Mek, MYH10, Myosin, NAMPT, Nes, NIT1, PDLIM5, RBM3, Rock, Tni, TNNC1, TNNI1, Tpm1, Tpm2, TTN, TWF1, VIM
2	Cancer, Gastrointestinal Disease, Infectious Disease	15	ARF3, ARF5, ARFIP1, ASCC3, CDC42BPA, CDV3, CHMP6, CHMP4A, CHMP4B, DDRGK1, DDX3Y, DPP7, EML2, HIST1H3I, IPO13, JPH2, KRT76, LARP4B, METTL9, NIN, NXN, OTUD6B, PKD1, PRKD2, RPF2, SMAP1, SNF8, SNX8, SNX18, SYNPO2, UBC, USP54,V PS25, VPS36, VPS13B
3	Gene Expression, Cancer, Organismal Injury and Abnormalities	14	ACP2, Akt, ALYREF, BTF3, CDH15, Ck2, CNBP, CUX1, Cyclin A, DNTTIP2, EIF5, EIF3A, estrogen receptor, FSH, HIST1H2BA, HIST2H3A, HIST4H4, Histone h4, IL1, Insulin, KDM5A, KRT72, Lh, LMNA, Nes, NFkB (complex), P38 MAPK, PDAP1, PDLIM3, PRDM2, PRKAR2B, Rnr, SSRP1, Vegf, ZNF519
4	Cellular Assembly and Organization, Tissue Development, Molecular Transport	11	AOC3, APP, ARL6IP5, ATP4A, ATP6V1A, ATP6V1G1, ATP6V1H, BDH1, Cox6c, cyclic AMP, EGFR, EIF2B2, ETHE1, FAM3B, FGF2, ganglioside GM2, GLRX5, GPC4, peptidase, PNN, PRPSAP2, RHOA, RIC8A, RUSC1, SCAMP3, SNX1, SNX4, SNX6, SOX9, TBRG4, TCIRG1, TPRA1, TSNAXIP1, USP6NL, ZCCHC17
5	RNA Post-Transcriptional Modification, Post-Translational Modification, Connective Tissue Disorders	7	CEP290, CLK2, Cpsf, CPSF1,C PSF2, CPSF3, CPSF4, CPSF6, CSTF2, CSTF-CPSF-SYMPK, DDX42, EED, EHMT1, EIF3D, GPI, HIST4H4, HNRNPL, Ighg2a, KRT5, LOC100360950, NSUN2, NUP188, PABPN1, PAPOLA, PCBP2, PKP2, POGZ, RAB5C, SENP2, SMARCAD1, SUMO3, SYMPK, TSEN15, VPRBP, WIZ

Discussion

This present study identified proteins that were modulated due to ET-1 treatment of the fetal cardiomyocyte. Most of these proteins were found to be significantly downregulated (green color) with some that were significantly upregulated (red color). The proteins involved in each of the 5 networks are depicted in Table 4.2. When multiple pathways are merged, several convergence points (proteins) were identified. These proteins may indicate key regulators of the fetal cardiomyocyte proteome due to ET-1 treatment (Figure 4.1A).

Of particular interest is the convergence of numerous interacting proteins (Figure 4.7) with EED (embryonic ectoderm development), which is located at the bottom left of the merged network map (Figure 4.1). EED is a member of the Polycomb group (PcG) proteins, which are important transcriptional repressors (Boyer et al., 2006). These proteins repress transcription of several key developmental regulators that would otherwise promote cell differentiation. It is a key component of the PRC2 complex, along with EZH2 and SUZ12, which is involved in histone modification and subsequent gene silencing (T. I. Lee et al., 2006). Though EED *per se*, did not show any significant changes in our study, several interacting protein partners of EED, e.g., LMNA, VIM, TTN, PNN, ACTB, ACTN 4, COL4A1, Histone cluster 2 were down-regulated (Figure 4.7). Consequently and cumulatively this situation may compromise the function of EED as well as of PRC2 complex. Our IPA analyses mapped all modulated proteins that interact with EED (Figure 4.7). These findings are in agreement with Biogrid database (http://thebiogrid.org).

Several studies have demonstrated the important role of the PRC2 complex in development. For example, the deletion of EED results in perinatal lethal heart defects and thinned myocardium (He et al., 2012). Inactivation of EED in fetal cardiomyocytes led to an upregulation in developmental and cell cycle regulators (He et al., 2012). Additionally, it was shown that Ezh2 promotes cardiomyocyte proliferation by suppressing the cell cycle inhibitors, *Ink4a* and Ink4b *(He et al., 2012)*.

The results of this present study implicate EED as a potential key regulator of endothelin-1-induced effects. Figure 4.7 illustrates a large number of interactions that EED has with various identified proteins following ET-1 treatment. Considering the role EED plays within the PRC2 complex to suppress gene transcription, it is possible that this protein is regulating the expression of several of the other proteins identified in this study. Most of the proteins revealed in this study were downregulated due to ET-1 treatment, several of which are involved in organ morphology and development. Our previous work (Paradis et al., 2014) demonstrated that ET-1 treatment of fetal cardiomyocytes resulted in increased terminal differentiation, characterized by increased binucleation and decreased proliferation. These morphological changes are crucial to the overall development of the heart and its function throughout a life. This present study reveals that the protein EED may be a key regulator of the morphological and functional changes previously observed by ET-1 treatment.

The second convergence point is found in the center of the merged network map (Figure 4.1), surrounding UBC (ubiquitin C). This protein is depicted as interacting with numerous other proteins that were identified *via* the pathway analysis (Figure 4.8). UBC is an ubiquitin precursor and a source of ubiquitin proteins. Ubiquitin can be conjugated

to other various proteins and in turn targets them for proteasomal degradation. Based on the network map, it appears that UBC is interacting with the majority of proteins identified in this study. That being said, it may be possible that UBC is involved in the downregulation of many of the mapped proteins, including EED. A study has shown that the level of PRC2 can be regulated by ubiquitination (Zoabi, Sadeh, de Bie, Marquez, & Ciechanover, 2011). This complex is actually dissociated into the individual units (EED, Ezh2, and SUZ12), which are then ubiquitinated and degraded (Zoabi et al., 2011). Therefore this study provides evidence that ubiquitin can be conjugated to EED, leading to its degradation. The pathway analysis suggests UBC is a key regulator of the protein changes observed following ET-1 treatment. Of particular interest is the likelihood that UBC is regulating another key player, EED, involved in the ET-1-induced fetal cardiomyocyte proteome (Figure 4.11).

The two proteins, EED and UBC, are associated with epigenetic mechanisms, for example histone modifications. Our study identified several histone proteins were also significantly downregulated in the ET-1 treated fetal cardiomyocytes. These proteins include: HIST1H2BA, HIST1H2BB, HIST1H3I, HIST2H3A, and HIST4H4. Histone proteins are an essential component of chromatin. The biosynthesis of these proteins is tightly linked to the cell cycle (Ewen, 2000; Ma et al., 2000; Zhao et al., 2000); as DNA replicates, more histone proteins must also be produced (Osley, 1991). The ET-1 treatment induced decrease in histone protein expression supports our previous (Paradis et al., 2014) evidence that ET-1 treated fetal cardiomyocytes prematurely exit the cell cycle. If these cells are no longer actively cycling, then reduced histone production may occur.

A third convergence point of interactions between proteins is found in the upper left corner of the merged network map (Figure 4.1). These proteins are key players involved in proliferation and the cell cycle, including: ERK1/2, p38 MAPK, Akt, and RhoA. These proteins are shown to have many interactions with others in the network (Figure 4.9). One such protein is vimentin, a key structural protein that is associated with RhoA signaling. Eriksson *et al.* reviews the topic of vimentin and RhoA interaction and suggests that the outcome of RhoA-mediated signaling is dependent on the presence and organization of vimentin (Eriksson et al., 2009). Furthermore, several diseases caused by mutations in lamin A/C are shown to have defective MAPK and Akt signaling cascades (Carmosino et al., 2014). It is possible that changes in lamin A/C expression would alter the structure of the nuclear envelope and thus disrupt ERK1/2 activity and its downstream cascade (Carmosino et al., 2014). Considering the crucial role that ERK1/2, MAPK, and Akt play in cell survival and proliferation, it is likely that the dysregulation of structural proteins such as lamin A/C can alter the signaling pathways of the before mentioned proteins and thus decrease proliferation. Moreover, our previous work has found that ET-1 induces a decrease in proliferation of fetal cardiomyocytes (Paradis et al., 2014). Therefore these proliferation-involved proteins may provide a mechanism through which ET-1 induces its functional changes.

A fourth convergence point on the merged map (Figure 4.1) is localized to the plasma membrane, surrounding EGFR (epidermal growth factor receptor). The proteins that are interacting with EGFR are depicted in Figure 4.10. Activation of the epidermal growth factor receptor results in a signaling cascade that leads to cell proliferation. Particularly in the cardiomyocyte, this pathway is crucial to their function and survival

(Sridharan et al., 2013). Mutations in this receptor as well as its closely related family members, has been shown to result in dilated cardiomyopathy (Crone et al., 2002; Garcia-Rivello et al., 2005; Ozcelik et al., 2002). Studies evaluating the developmental role of the EGFR family of receptors have found that it is essential for cardiac development and muscle differentiation (Garcia-Rivello et al., 2005; Gassmann et al., 1995). Mutations in this family of receptors cause lethality during mid-embryogenesis most likely due to a lack of cardiac trabeculae (Gassmann et al., 1995; K. F. Lee et al., 1995). These studies suggest that EGFR and its family of receptors are essential to cardiac development and may implicate this pathway in the regulation of the ET-1-induced effects on the cardiomyocyte.

The expression of several proteins involved in post-transcriptional and post-translational modifications were also differentially regulated by ET-1 treatment. Our previous work has shown that ET-1 treatment leads to an increase in global DNA methylation of fetal cardiomyocytes and in the presence of a DNA methylation inhibitor the ET-1 induced premature terminal differentiation was also blocked (Paradis et al., 2014). Together these findings suggest that several routes of epigenetic mechanisms may be involved in cardiomyocyte maturation. This finding may further implicate the involvement of the EED protein considering its role in transcriptional regulation *via* epigenetic modifications. As part of the PRC2 complex, EED has been shown to utilize histone modifications in order to suppress various developmental regulators that would otherwise promote cell differentiation (T. I. Lee et al., 2006). In the context of the developing heart, this may suggest that the EED protein is increased during early heart development when proliferation is high. However as the heart nears maturation, it is

likely that EED becomes downregulated to allow for differentiation to occur. ET-1 is shown to accelerate terminal differentiation (Paradis et al., 2014), and based on our current data ET-1 treatment may decrease EED levels in order to promote differentiation and maturation of the heart.

ET-1 treatment of fetal cardiomyocytes also led to a downregulation of cytoskeletal and contractile proteins, many of which have been associated with cardiac disease. Several of the proteins identified in this present study are known to be associated with heart disease such as, 1) hypertrophic cardiomyopathy (cardiac troponin I, titin, α-tropomyosin, and α-actin) and 2) dilated cardiomyopathy (actin, tropomyosin, troponin I and C, titin, desmin, and LIM protein) (Harvey & Leinwand, 2011; Wieczorek, Jagatheesan, & Rajan, 2008).

Our present work found that tropomyosin alpha and beta were both downregulated due to ET-1. Previous work has shown that a mutation in α-tropomyosin leads to hypertrophic cardiomyopathy and cardiac dysfunction (Coviello et al., 1997; Wieczorek et al., 2008). Another form of heart disease, dilated cardiomyopathy, can also result from a mutation in tropomyosin (Rajan et al., 2010; Wieczorek et al., 2008). Likewise, the loss of actin has been implicated in heart disease and failure (Olson, Michels, Thibodeau, Tai, & Keating, 1998; Stefani et al., 2008). Thus, the downregulation of these proteins due to ET-1 treatment may predispose the heart to cardiovascular dysfunction and disease.

Mutations in titin, a large sarcomeric protein, have been associated with increased incidence of dilated cardiomyopathy (Herman et al., 2012; McNally, 2012). Therefore a decrease in titin function is implicated in contractile dysfunction and disease. Our study

also revealed that vimentin, a cytoskeletal component, was significantly downregulated due to ET-1 treatment. This protein is detected in developing muscle however not in muscle that has become terminally differentiated (Eriksson et al., 2009; Gard, Bell, & Lazarides, 1979; Granger & Lazarides, 1979), which supports our previous work (Paradis et al., 2014) showing ET-1's ability to promote terminal differentiation of cardiomyocytes.

Another structural protein identified in our study, which plays a crucial role in cardiomyocyte function, is Lamin A/C. Dilated cardiomyopathy has been observed in mice with lamin A/C mutations (Mounkes, Kozlov, Rottman, & Stewart, 2005; Nikolova et al., 2004; van Berlo, Duboc, & Pinto, 2004) and the cardiomyocytes of these hearts have increased fragility and abnormal nuclear structure (Nikolova et al., 2004). Along with dilated cardiomyopathy, a specific lamin A/C mutation resulted in early death due to arrhythmia (Mounkes et al., 2005). A review of the literature by Carmosino *et al.* on the role of lamin A/C in cardiomyocyte functions, implies that a decrease in lamin A/C expression is key in promoting cardiomyocyte senescence (Carmosino et al., 2014). Altogether these studies imply that lower levels of lamin A/C can result in cardiac disease (Carmosino et al., 2014). Therefore, our present work suggests that ET-1 treatment results in a significant decrease in lamin A/C expression and may result in a predisposition to cardiovascular disease.

The major intermediate filament in cardiomyocytes (Tokuyasu, Maher, Dutton, & Singer, 1985), desmin, has been shown to interact with lamin A/C (Carmosino et al., 2014). Increased disorganization of desmin filaments is observed in lamin A/C mutations of the heart (Nikolova et al., 2004; Sebillon et al., 2003). Desmin mutations have also

been associated with dilated cardiomyopathy (Wang et al., 2001). Desmin-related myopathies are characterized by desmin aggregates (Abraham et al., 1998) and this aberrant aggregation is shown to lead to desmin network disruption, abnormal nuclear shape, and cardiac dysfunction (Wang et al., 2001; Wang, Osinska, Gerdes, & Robbins, 2002).

The present study examined the ET-1 induced changes in the fetal cardiomyocyte proteome. Our proteomic analyses revealed a number of proteins that may be involved in the ET-1-induced terminal differentiation process previously described (Paradis et al., 2014). Based on our pathway analysis, many of these proteins are associated with pathways that regulate proliferation and survival of cardiomyocytes. Furthermore, many of the proteins identified are known to be downregulated in heart disease, implying that these young hearts may be more susceptible to disease. The results of this study provide supporting evidence to pursue several avenues of protein interactions in order to identify the molecular mechanism behind the ET-1-induced premature terminal differentiation of cardiomyocytes.

Acknowledgments

This study was supported by the National Institutes of Health grants HL118861 (to L. Zhang). A portion of this research used the Loma Linda University School of Medicine Mass Spectrometry Core.

CHAPTER FIVE

GENERAL DISCUSSION

Potential Mediators of Premature Terminal Differentiation

Hypoxia is a far too common stress to the developing fetus. A key mediator of the effects of hypoxia is endothelin-1 (ET-1). The gene for this small peptide contains a hypoxia-response element (Hu et al., 1998; Kakinuma et al., 2001; Minchenko & Caro, 2000) and thus becomes quickly elevated under a low oxygen environment. Therefore, this present work hypothesized that ET-1 is intermediating the hypoxia/anoxia-induced terminal differentiation of cardiomyocytes. Prepro-ET-1 is significantly increased under hypoxia and anoxia as was shown in chapters two and three. Furthermore by blocking the ET-receptors (both selectively and non-selectively), the hypoxia/anoxia-induced effects were abrogated, providing further evidence for ET-1 as a key downstream regulator.

As described in chapters two and three, hypoxia and anoxia treatment lead to a significant decrease in the proliferation of cardiomyocytes. The endowment of this functional unit of the heart during the critical stage of development can have a major impact on the overall function of the heart. Both *in vitro* hypoxia and *in vivo* anoxia treatment significantly reduced the percentage of proliferating cardiomyocytes. Similarly, treatment of fetal cardiomyocytes versus neonatal rats yielded the same effects on cardiomyocyte proliferation. Together, this suggests a true effect of hypoxia on cardiomyocyte proliferation, regardless of the method of treatment used.

Interestingly, the *in vitro* and *in vivo* model of hypoxia/anoxia had different results in regards to the percent of binucleation. The *in vitro* treatment of fetal cardiomyocytes with hypoxia or ET-1 led to a significant increase in binucleation.

However the *in vivo* anoxia model did not yield the same result; the percent of binucleation was not changed in this case. This difference may be due to a variety of differences in experimental design. Other studies have also shown an increase in binucleation of fetal cardiomyocytes (Bae et al., 2003; Jonker et al., 2010) however studies have not been performed in neonatal cardiomyocytes previously. Furthermore, the *in vitro* culture treatment versus *in vivo* treatment may explain the differential binucleation results. These two forms of treatment may elicit very different effects on the cardiomyocyte. The *in vitro* treatment showed the direct effect of ET-1 on the maturation of fetal cardiomyocytes, which lead to increased binucleation. However, the *in vivo* model was utilized to simulate the anoxic episodes that often occur in preterm newborns. In this model, there are many other factors other than ET-1 involved and thus these factors may be counteracting ET-1's direct effect on binucleation.

Much evidence from the studies laid out in chapters two and three indicate that the endothelin-receptor is a key mediator of the hypoxia/anoxia-induced effects. A non-selective ET-receptor antagonist (PD145065) was utilized in the in vitro studies and it was found to block the effects of the ET-1 treatment. Subsequently, the selective ET_A-receptor (PD156707) was able to abrogate the anoxia-induced effects on proliferation. The ET_A-receptor is the predominant subtype found in cardiomyocytes (Kohan et al., 2011) and thus of particular interest for these studies. Although the receptor density was unchanged in the neonatal heart treated with anoxia, the inhibition of this receptor abrogated the anoxia-induced effects on the heart.

In chapter three, the results showed that anoxia treatment led to an increase in p27 expression and decrease in cyclin D2 expression. The p27 protein associated with cyclin

and cyclin-dependent kinase complexes and inhibits their activity. In this way, p27 works to regulate the cell cycle and control cell proliferation (Ishida, Kitagawa, Hatakeyama, & Nakayama, 2000). Normally, the amount of p27 is increased during G_0-G_1 phase, when p27 translation and protein stability is maximal (Wander, Zhao, & Slingerland, 2011), but then decreases upon entry into S phase (Nourse et al., 1994). This cell cycle inhibitor, p27, is predominantly regulated by post-translational modifications (Hengst & Reed, 1996; Pagano et al., 1995). One common modification is phosphorylation, which regulates the stability, localization, and function of p27 (Wander et al., 2011). Phosphorylation at a certain residue can stabilize the p27 protein and is more commonly observed in cells found in the G_0 or G_1 phase of the cell cycle (Ishida et al., 2000). Although the expression of phosphorylated p27 protein was not measured in the present studies, it is likely that the expression of this form would also be significantly altered in mature cardiomyocytes. Terminally differentiated cardiomyocytes have significantly decreased expression of Ki-67, a protein that is expressed in every phase of the cell cycle except for the G_0 phase. Therefore these mature cardiomyocytes are not actively dividing and have exited the cell cycle and now reside in the G_0 phase. Seeing that adult cardiomyocytes are terminally differentiated and that there is an increase in p27 expression, it leads us to presume that the levels of the phosphorylated form would also be altered.

Another post-translational modification that modulates p27 expression is ubiquitination. The degradation of p27 can be achieved through both the ubiquitin-proteasome pathway and ubiquitin-independent proteolytic cleavage (Shirane et al., 1999). The ubiquitination of p27 has been observed to be lower during the G_0/G_1 phase as

compared to the G_1/S transition (Shirane et al., 1999). Thus it is possible that an increase in terminal differentiation of cardiomyocytes may coincide with a decrease in p27 ubiquitination. Ubiquitin protein C (UBC) is a key protein that interacted with numerous other proteins identified in the proteomics study outlined in chapter four. UBC appears to interact with a majority of the proteins identified in the pathway analysis, suggesting that ubiquitination is a key mechanism involved in the differential expression of these proteins following ET-1 treatment. UBC expression itself was not measured in the present studies but it is likely that UBC's interaction with p27 may be reduced while its interaction with other proteins may increase, resulting in terminally differentiated cardiomyocytes.

The expression and role of p27 and cyclin D2 is inversely correlated, as implied by the results in chapter three. In fact, it has been suggested that cyclin D2 plays a key role in p27 downregulation during the transition from the G_0 to G_1 phase (Susaki, Nakayama, & Nakayama, 2007). Cyclin D2 can mediate the translocation of p27 from the nucleus to the cytoplasm were p27 degradation then takes place (Susaki et al., 2007). Our results show that p27 expression is increased while cyclin D2 expression is decreased due to anoxia treatment. This may suggest that an alternative explanation for increased p27 expression is due to the corresponding decrease in cyclin D2. Without as much cyclin D2 present, the p27 is not rapidly translocated to the cytoplasm for degradation and thus it can elicit its inhibitory effects on the cell cycle.

The proteomics analysis in chapter four also revealed an interaction between a number of proteins involved in proliferation and cell survival, such as Akt, ERK1//2, and p38 MAPK. The proliferation pathway in cardiomyocytes is initiated by several signaling

mechanisms involving the PI3K/Akt and Ras/ERK pathways (Ahuja, Perriard, et al., 2007; Kang & Sucov, 2005; Sucov, Gu, Thomas, Li, & Pashmforoush, 2009; Tseng et al., 2005). Data from Lee *et al.* suggests that activation of ERK and Akt pathways can lead to the phosphorylation and degradation of p27 (J. G. Lee & Kay, 2011). The expression of p27 was found to be downstream of the ERK pathway (Gysin, Lee, Dean, & McMahon, 2005), and the inhibition of p27 would lead to greater cell proliferation. Inhibition of the ERK pathway thus results in greater expression of the cell cycle inhibitor, p27 (Gysin et al., 2005). In the light of our present work, these studies may suggest that the ERK pathway is diminished due to hypoxia/anoxia treatment.

Several studies have shown that Akt can phosphorylate p27 at several residues and subsequently abolish the function of the cell cycle inhibitor (Fujita, Sato, Katayama, & Tsuruo, 2002; Liang et al., 2002). Akt activation can also lead to increased FOXO (Forkhead O) transcription factors, followed by decreased p27 expression and increased cardiomyocyte proliferation (Evans-Anderson, Alfieri, & Yutzey, 2008). Together, these studies imply that the Akt pathway is closely involved in the proliferation of cardiomyocytes *via* the regulation of the cell cycle inhibitor p27. Chapters two and three of this present study showed that cardiomyocyte proliferation is decreased while p27 expression is increased. Furthermore, chapter four provided evidence that both the ERK and Akt pathways may be key players in the mechanism leading to ET-1-induced premature terminal differentiation.

Implications of an Altered Cardiomyocyte Population

Proper cardiomyocyte endowment is a crucial component in establishing a

fortified heart that can function properly for a lifetime. The present work provides evidence that an anoxia-induced premature terminal differentiation may reduce cardiomyocyte endowment. Based on the field of Developmental Origins of Health and Disease, it is well established that stresses *in utero* can alter the structure and function of organs, predisposing them to disease later in life. The developing heart is no exception. The functional unit of the heart, the cardiomyocyte, is necessary in providing the contractile force to pump blood throughout the body. Therefore if the structure and function of the cardiomyocytes become altered, then the overall function of the heart would consequentially be affected. Hearts of these individuals may have decreased cardiac function and be unable to cope with stress later in life.

Porello *et al.* have shown that a decrease in the population of cardiomyocytes in the neonate has a great impact on the adult heart. Later in life, the less populated hearts may be more vulnerable to situations where there is increased stress or workload (Porrello et al., 2009; Porrello, Widdop, & Delbridge, 2008). Studies by this group have also suggested that adult cardiac hypertrophy originates in early neonatal development when cardiomyocyte growth is altered (Porrello et al., 2009). Dysregulated trophic signaling, such as through increased PI3K or suppressed MAPK activation, have been associated with the establishment of cardiac hypertrophy (Porrello et al., 2009). Previous work from our lab has shown that prenatal hypoxia increases the susceptibility of the heart to ischemia-reperfusion injury in adulthood (G. Li et al., 2003). Together these studies suggest that the cardiomyocyte population determined during the perinatal period is essential to the proper structure and function of the adult heart. A reduced cardiomyocyte population will increase the vulnerability of that heart to disease

throughout its life, especially if it encounters a secondary stress in adulthood.

There are a number of factors that can determine cardiomyocyte endowment, including: hormones and signaling pathways related to proliferation, apoptosis, and of course the timing of terminal differentiation (Botting et al., 2012). Based on the present study, hypoxia/anoxia and the downstream ET-1 appear to also be involved in regulating the endowment of cardiomyocytes. This work found that anoxia treatment leads to a significant decrease in proliferation during early neonatal time-points followed by an ultimate reduction in cardiomyocyte endowment by neonatal day 14. By the end of the first two neonatal weeks, the heart is presumed to be fully mature and like that of an adult heart. Therefore, neonatal anoxia treatment alters the rate of proliferation, which ultimately alters the number of cardiomyocytes per weight of the adult heart.

Potential Epigenetic Mechanisms Involved in Premature Terminal Differentiation

The perinatal environment can elicit its effects on the development of an organ *via* epigenetic mechanisms. The work presented here provides evidence that two of the major epigenetic mechanisms, DNA methylation and histone modifications, are involved in the premature terminal differentiation of cardiomyocytes. ET-1 treated fetal cardiomyocytes had a significant increase in global DNA methylation, as shown in chapter two. The increase in global DNA methylation was associated with an accelerated cardiomyocyte terminal differentiation. Previous work by Kou et al. has shown that the first two weeks of neonatal life when cardiomyocytes normally transition to the terminally differentiated form is correlated with an increase in DNA methylation (Kou et

al., 2010). Altogether suggesting that a hypermethylated genome is associated with the terminal differentiation of cardiomyocytes (Kou et al., 2010). Our results using the DNA methylation inhibitor, 5-aza-2'-deoxycytidine, provide further evidence for this phenomenon. Following the addition of 5-aza-2'-deoxycytidine, ET-1's effect on cardiomyocyte binucleation and proliferation were also blocked. Therefore suggesting that DNA methylation is a mechanism by which ET-1 induces a premature terminal differentiation of cardiomyocytes. Although global DNA methylation was not measured in chapter three, we presume that the anoxia-induced premature terminal differentiation would be associated with a hypermethylated genome. Anoxia is shown to induce ET-1 and if this peptide acts through DNA methylation to alter terminal differentiation, then we presume simulating anoxia itself would result in the same effect.

At this point, no specific methylation studies have been done however it is possible that two of the cell cycle genes, p27 and cyclin D2, are being differentially methylated. The expression of these two proteins are inversely correlated and tightly involved in regulating the cell cycle and hence proliferation and terminal differentiation. Studies have found that the p27 and cyclin D2 genes are located in the same gene cluster (Ansari-Lari et al., 1998; Qian, Jin, & Lloyd, 2000). This gene cluster consists of many regulatory genes including those involved in cell proliferation, and it is found on human chromosome 12p13 as well as its syntenic regions on mouse and rat chromosomes (Ansari-Lari et al., 1998). Due to the location of these genes in the same gene cluster, it has been suggested that there is a common regulatory mechanism due to shared regulatory elements (Qian et al., 2000). One possible regulatory mechanism may be DNA methylation or histone modifications modulating the expression of these two genes.

However, considering the fact that these two cell cycle genes are inversely correlated, it is possible that different regulatory mechanisms are involved. The expression of p27 appears to be increased under circumstances that promote terminal differentiation while the expression of cyclin D2 is decreased. Therefore, differential methylation of these two regions may be occurring. It is also possible that histone modifications are differentially activating the transcription of p27 and repressing the transcription of cyclin D2 under hypoxic/anoxic conditions that stimulate terminal differentiation.

Interestingly, the expression of several histone proteins was decreased in ET-1 treated fetal cardiomyocytes, as described in chapter four. This finding may suggest that the DNA is more unwound and less compact following ET-1 treatment. Histones play a key role in winding DNA to condense and compact the genome. In order for transcription to occur, the DNA must be unwound from the histones so that the transcription machinery can access the genes. Therefore a decrease in the expression of histones may suggest that the DNA is unwound and allowing greater access for transcription to occur. Terminal differentiation is likely associated with a decrease in the transcription of several proliferative and cell cycle genes while the transcription of genes associated with differentiation is increased. Therefore certain regions of the genome may be less compact due to a loss of histone proteins and result in increased transcription of those regions. Subsequently, while a decrease in histone proteins may result in a less compacted genome, there is also an increase in global DNA methylation. In this way certain genes, particularly those related to proliferation, may still be "turned off" to allow terminal differentiation to occur.

Regardless of the expression of histones, modification of these proteins may also be regulating the period of terminal differentiation. The PRC2 complex, of which EED is a crucial subunit, is involved in histone modifications and subsequent gene silencing (T. I. Lee et al., 2006). EED was identified in the proteomics analysis to be a key mediator in the ET-1 induced terminal differentiation of cardiomyocyte. The PRC2 complex is particularly involved in retaining the cell in a proliferative state by silencing genes related to cell differentiation. Although the expression of EED was not measured, it is likely that its expression is decreased as the cell transitions to a terminally differentiated form.

Overall, the present study suggests that many epigenetic mechanisms are involved in regulating this critical period of terminal differentiation. Future studies will be needed to elucidate this intricate mechanism further.

Conclusion

The perinatal environment is one that is experienced by every single individual. One of the most detrimental and far too common insults during this time is hypoxia. This environmental stress during the crucial period of development can have lasting effects on the structure and function of an individual throughout life. This present study provides evidence for the damaging effects of hypoxia/anoxia on the cardiomyocyte population in the heart. Hypoxia/anoxia treatment decreases cardiomyocyte proliferation and ultimately leads to premature terminal differentiation and reduced cardiomyocyte endowment in the adult heart. This present work identified a novel mechanism by which ET-1 is a key mediator of the effects of hypoxia/anoxia on cardiomyocyte development, more specifically *via* the ET_A-receptor. Furthermore, this work illuminates several regulatory

mechanisms and potential molecular pathways involved in the process of premature terminal differentiation. This process appears to be linked to cell cycle genes, such as p27 and cyclin D2, as well as several epigenetic mechanisms. Global DNA methylation is enhanced due to ET-1 treatment and when this mechanism becomes abrogated, the ET-1 induced terminal differentiation is also blocked. The findings presented here also implicate the expression and epigenetic modification of histone proteins as potential mechanisms in the hypoxia/ET-1-induced premature terminal differentiation of cardiomyocytes. In addition, a proteomics analysis of proteins differentially regulated and potentially involved in the ET-1 induced terminal differentiation will allow future studies to further elucidate the mechanism behind this process. Future studies will be imperative considering the importance of optimizing the development and endowment of cardiomyocytes in the heart at an early stage.

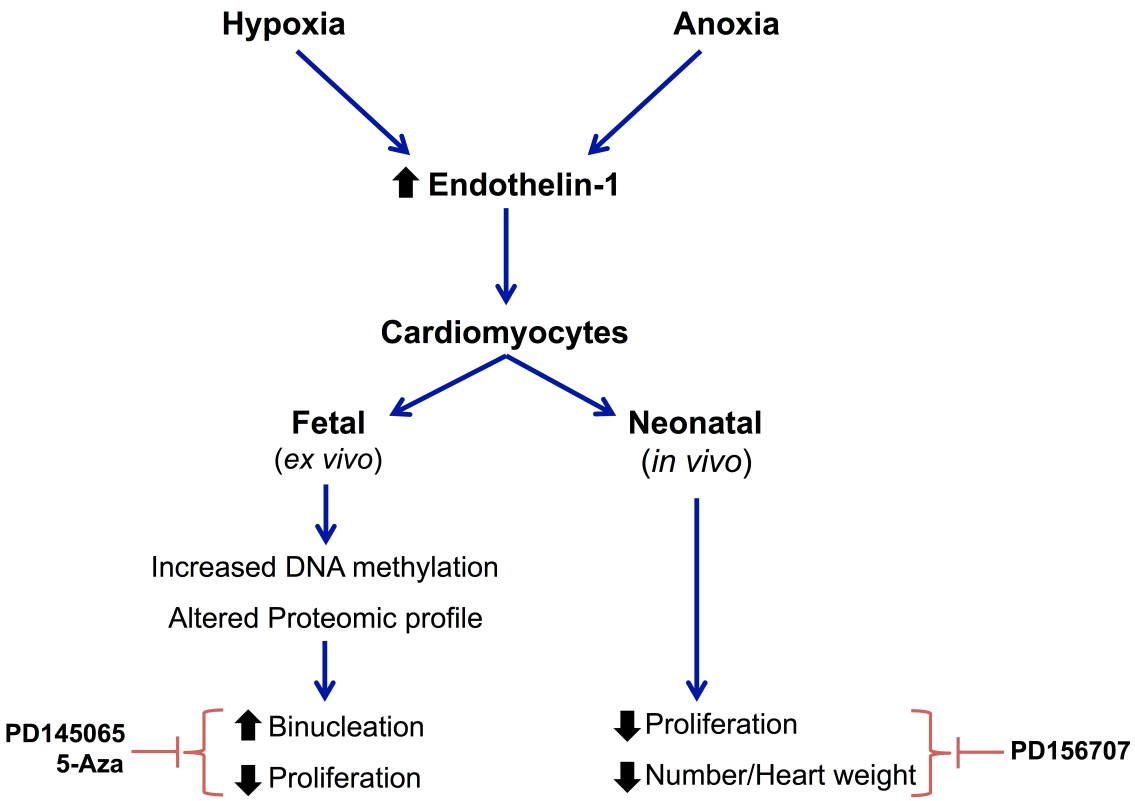

Figure 5.1. Summary of the effects of ET-1 on cardiomyocyte maturation. The effect of hypoxia/anoxia exposure on cardiomyocytes is mediated by endothelin-1 (ET-1). Varying effects were observed in fetal cardiomyocytes treated *ex vivo* and neonatal cardiomyocytes treated *in vivo*. ET-1-treated fetal cardiomyocytes had increased binucleation, decreased proliferation, increased global DNA methylation, and a significantly altered proteomic profile. The *in vivo* neonatal anoxia model also resulted in a decrease in proliferation as well as a significant reduction in cardiomyocyte endowment in the day 14 heart.

REFERENCES

Abraham, S. C., DeNofrio, D., Loh, E., Minda, J. M., Tomaszewski, J. E., Pietra, G. G., & Reynolds, C. (1998). Desmin myopathy involving cardiac, skeletal, and vascular smooth muscle: report of a case with immunoelectron microscopy. *Hum Pathol, 29*(8), 876-882.

Agapitov, A. V., & Haynes, W. G. (2002). Role of endothelin in cardiovascular disease. *J Renin Angiotensin Aldosterone Syst, 3*(1), 1-15. doi: 10.3317/jraas.2002.001

Ahuja, P., Perriard, E., Pedrazzini, T., Satoh, S., Perriard, J. C., & Ehler, E. (2007). Re-expression of proteins involved in cytokinesis during cardiac hypertrophy. *Exp Cell Res, 313*(6), 1270-1283. doi: 10.1016/j.yexcr.2007.01.009

Ahuja, P., Sdek, P., & MacLellan, W. R. (2007). Cardiac myocyte cell cycle control in development, disease, and regeneration. *Physiol Rev, 87*(2), 521-544. doi: 10.1152/physrev.00032.2006

Anatskaya, O. V., & Vinogradov, A. E. (2007). Genome multiplication as adaptation to tissue survival: evidence from gene expression in mammalian heart and liver. *Genomics, 89*(1), 70-80. doi: 10.1016/j.ygeno.2006.08.014

Ansari-Lari, M. A., Oeltjen, J. C., Schwartz, S., Zhang, Z., Muzny, D. M., Lu, J., . . . Gibbs, R. A. (1998). Comparative sequence analysis of a gene-rich cluster at human chromosome 12p13 and its syntenic region in mouse chromosome 6. *Genome Res, 8*(1), 29-40.

Arai, H., Hori, S., Aramori, I., Ohkubo, H., & Nakanishi, S. (1990). Cloning and expression of a cDNA encoding an endothelin receptor. *Nature, 348*(6303), 730-732. doi: 10.1038/348730a0

Bae, S., Xiao, Y., Li, G., Casiano, C. A., & Zhang, L. (2003). Effect of maternal chronic hypoxic exposure during gestation on apoptosis in fetal rat heart. *Am J Physiol Heart Circ Physiol, 285*(3), H983-990. doi: 10.1152/ajpheart.00005.2003

Banerjee, I., Fuseler, J. W., Price, R. L., Borg, T. K., & Baudino, T. A. (2007). Determination of cell types and numbers during cardiac development in the neonatal and adult rat and mouse. *Am J Physiol Heart Circ Physiol, 293*(3), H1883-1891. doi: 10.1152/ajpheart.00514.2007

Barker, D. J. (1990). The fetal and infant origins of adult disease. *BMJ, 301*(6761), 1111.

Barker, D. J. (1995). Fetal origins of coronary heart disease. *BMJ, 311*(6998), 171-174.

Barker, D. J. (1997). Fetal nutrition and cardiovascular disease in later life. *Br Med Bull, 53*(1), 96-108.

Barker, D. J. (2004). The developmental origins of chronic adult disease. *Acta Paediatr Suppl, 93*(446), 26-33.

Barker, D. J., & Osmond, C. (1986). Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet, 1*(8489), 1077-1081.

Barton, M., & Yanagisawa, M. (2008). Endothelin: 20 years from discovery to therapy. *Can J Physiol Pharmacol, 86*(8), 485-498. doi: 10.1139/y08-059

Bateson, P., Barker, D., Clutton-Brock, T., Deb, D., D'Udine, B., Foley, R. A., . . . Sultan, S. E. (2004). Developmental plasticity and human health. *Nature, 430*(6998), 419-421. doi: 10.1038/nature02725

Beigi, F., Schmeckpeper, J., Pow-Anpongkul, P., Payne, J. A., Zhang, L., Zhang, Z., . . . Dzau, V. J. (2013). C3orf58, a Novel Paracrine Protein, Stimulates Cardiomyocyte Cell-Cycle Progression Through the PI3K-AKT-CDK7 Pathway. *Circ Res, 113*(4), 372-380. doi: 10.1161/circresaha.113.301075

Bergmann, O., Bhardwaj, R. D., Bernard, S., Zdunek, S., Barnabe-Heider, F., Walsh, S., . . . Frisen, J. (2009). Evidence for cardiomyocyte renewal in humans. *Science, 324*(5923), 98-102. doi: 10.1126/science.1164680

Bolivar, J. M., Gerhardt, T., Gonzalez, A., Hummler, H., Claure, N., Everett, R., & Bancalari, E. (1995). Mechanisms for episodes of hypoxemia in preterm infants undergoing mechanical ventilation. *J Pediatr, 127*(5), 767-773.

Botting, K. J., McMillen, I. C., Forbes, H., Nyengaard, J. R., & Morrison, J. L. (2014). Chronic hypoxemia in late gestation decreases cardiomyocyte number but does not change expression of hypoxia-responsive genes. *J Am Heart Assoc, 3*(4). doi: 10.1161/jaha.113.000531

Botting, K. J., Wang, K. C., Padhee, M., McMillen, I. C., Summers-Pearce, B., Rattanatray, L., . . . Morrison, J. L. (2012). Early origins of heart disease: low birth weight and determinants of cardiomyocyte endowment. *Clin Exp Pharmacol Physiol, 39*(9), 814-823. doi: 10.1111/j.1440-1681.2011.05649.x

Boyer, L. A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L. A., Lee, T. I., . . . Jaenisch, R. (2006). Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature, 441*(7091), 349-353. doi: 10.1038/nature04733

Brooks, G., Poolman, R. A., & Li, J. M. (1998). Arresting developments in the cardiac myocyte cell cycle: role of cyclin-dependent kinase inhibitors. *Cardiovasc Res, 39*(2), 301-311.

Bubb, K. J., Cock, M. L., Black, M. J., Dodic, M., Boon, W. M., Parkington, H. C., . . . Tare, M. (2007). Intrauterine growth restriction delays cardiomyocyte maturation

and alters coronary artery function in the fetal sheep. *J Physiol, 578*(Pt 3), 871-881. doi: 10.1113/jphysiol.2006.121160

Bugaisky, L., & Zak, R. (1979). Cellular growth of cardiac muscle after birth. *Tex Rep Biol Med, 39*, 123-138.

Burrell, J. H., Boyn, A. M., Kumarasamy, V., Hsieh, A., Head, S. I., & Lumbers, E. R. (2003). Growth and maturation of cardiac myocytes in fetal sheep in the second half of gestation. *Anat Rec A Discov Mol Cell Evol Biol, 274*(2), 952-961. doi: 10.1002/ar.a.10110

Carmosino, M., Torretta, S., Procino, G., Gerbino, A., Forleo, C., Favale, S., & Svelto, M. (2014). Role of nuclear Lamin A/C in cardiomyocyte functions. *Biol Cell, 106*(10), 346-358. doi: 10.1111/boc.201400033

Ceccarelli, F., Scavuzzo, M. C., Giusti, L., Bigini, G., Costa, B., Carnicelli, V., . . . Mazzoni, M. R. (2003). ETA receptor-mediated Ca2+ mobilisation in H9c2 cardiac cells. *Biochem Pharmacol, 65*(5), 783-793.

Chen, H. W., Yu, S. L., Chen, W. J., Yang, P. C., Chien, C. T., Chou, H. Y., . . . Lee, Y. T. (2004). Dynamic changes of gene expression profiles during postnatal development of the heart in mice. *Heart, 90*(8), 927-934. doi: 10.1136/hrt.2002.006734

Chlopcikova, S., Psotova, J., & Miketova, P. (2001). Neonatal rat cardiomyocytes--a model for the study of morphological, biochemical and electrophysiological characteristics of the heart. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub, 145*(2), 49-55.

Chu, W., Wan, L., Zhao, D., Qu, X., Cai, F., Huo, R., . . . Yang, B. (2012). Mild hypoxia-induced cardiomyocyte hypertrophy via up-regulation of HIF-1alpha-mediated TRPC signalling. *J Cell Mol Med, 16*(9), 2022-2034. doi: 10.1111/j.1582-4934.2011.01497.x

Clubb, F. J., Jr., & Bishop, S. P. (1984). Formation of binucleated myocardial cells in the neonatal rat. An index for growth hypertrophy. *Lab Invest, 50*(5), 571-577.

Coe, Y., Haleen, S. J., Welch, K. M., Liu, Y. A., & Coceani, F. (2002). The endothelin A receptor antagonists PD 156707 (CI-1020) and PD 180988 (CI-1034) reverse the hypoxic pulmonary vasoconstriction in the perinatal lamb. *J Pharmacol Exp Ther, 302*(2), 672-680.

Cooper, A. L., & Beasley, D. (1999). Hypoxia stimulates proliferation and interleukin-1alpha production in human vascular smooth muscle cells. *Am J Physiol, 277*(4 Pt 2), H1326-1337.

Corstius, H. B., Zimanyi, M. A., Maka, N., Herath, T., Thomas, W., van der Laarse, A., . . . Black, M. J. (2005). Effect of intrauterine growth restriction on the number of

cardiomyocytes in rat hearts. *Pediatr Res, 57*(6), 796-800. doi: 10.1203/01.pdr.0000157726.65492.cd

Coviello, D. A., Maron, B. J., Spirito, P., Watkins, H., Vosberg, H. P., Thierfelder, L., . . . Seidman, C. E. (1997). Clinical features of hypertrophic cardiomyopathy caused by mutation of a "hot spot" in the alpha-tropomyosin gene. *J Am Coll Cardiol, 29*(3), 635-640.

Crone, S. A., Zhao, Y. Y., Fan, L., Gu, Y., Minamisawa, S., Liu, Y., . . . Lee, K. F. (2002). ErbB2 is essential in the prevention of dilated cardiomyopathy. *Nat Med, 8*(5), 459-465. doi: 10.1038/nm0502-459

Cullingford, T. E., Markou, T., Fuller, S. J., Giraldo, A., Pikkarainen, S., Zoumpoulidou, G., . . . Clerk, A. (2008). Temporal regulation of expression of immediate early and second phase transcripts by endothelin-1 in cardiomyocytes. *Genome Biol, 9*(2), R32. doi: 10.1186/gb-2008-9-2-r32

Delgado-Olguin, P., Huang, Y., Li, X., Christodoulou, D., Seidman, C. E., Seidman, J. G., . . . Bruneau, B. G. (2012). Epigenetic repression of cardiac progenitor gene expression by Ezh2 is required for postnatal cardiac homeostasis. *Nat Genet, 44*(3), 343-347. doi: 10.1038/ng.1068

Di Stefano, V., Giacca, M., Capogrossi, M. C., Crescenzi, M., & Martelli, F. (2011). Knockdown of cyclin-dependent kinase inhibitors induces cardiomyocyte re-entry in the cell cycle. *J Biol Chem, 286*(10), 8644-8654. doi: 10.1074/jbc.M110.184549

Dimaguila, M. A., Di Fiore, J. M., Martin, R. J., & Miller, M. J. (1997). Characteristics of hypoxemic episodes in very low birth weight infants on ventilatory support. *J Pediatr, 130*(4), 577-583.

Doherty, A. M., Cody, W. L., He, J. X., DePue, P. L., Cheng, X. M., Welch, K. M., . . . et al. (1993). In vitro and in vivo studies with a series of hexapeptide endothelin antagonists. *J Cardiovasc Pharmacol, 22 Suppl 8*, S98-102.

Dransfield, D. A., Spitzer, A. R., & Fox, W. W. (1983). Episodic airway obstruction in premature infants. *Am J Dis Child, 137*(5), 441-443.

Drimal, J., Knezl, V., Drimal, J., Jr., Drimal, D., Bauerova, K., Kettmann, V., . . . Stefek, M. (2003). Cardiac effects of endothelin-1 (ET-1) and related C terminal peptide fragment: increased inotropy or contribution to heart failure? *Physiol Res, 52*(6), 701-708.

Engel, F. B., Schebesta, M., & Keating, M. T. (2006). Anillin localization defect in cardiomyocyte binucleation. *J Mol Cell Cardiol, 41*(4), 601-612. doi: 10.1016/j.yjmcc.2006.06.012

Eriksson, J. E., Dechat, T., Grin, B., Helfand, B., Mendez, M., Pallari, H. M., & Goldman, R. D. (2009). Introducing intermediate filaments: from discovery to disease. *J Clin Invest, 119*(7), 1763-1771. doi: 10.1172/jci38339

Evans-Anderson, H. J., Alfieri, C. M., & Yutzey, K. E. (2008). Regulation of cardiomyocyte proliferation and myocardial growth during development by FOXO transcription factors. *Circ Res, 102*(6), 686-694. doi: 10.1161/circresaha.107.163428

Ewen, M. E. (2000). Where the cell cycle and histones meet. *Genes Dev, 14*(18), 2265-2270.

Fernandez-Patron, C., Radomski, M. W., & Davidge, S. T. (1999). Vascular matrix metalloproteinase-2 cleaves big endothelin-1 yielding a novel vasoconstrictor. *Circ Res, 85*(10), 906-911.

Fujita, N., Sato, S., Katayama, K., & Tsuruo, T. (2002). Akt-dependent phosphorylation of p27Kip1 promotes binding to 14-3-3 and cytoplasmic localization. *J Biol Chem, 277*(32), 28706-28713. doi: 10.1074/jbc.M203668200

Gao, Y., & Raj, J. U. (2010). Regulation of the pulmonary circulation in the fetus and newborn. *Physiol Rev, 90*(4), 1291-1335. doi: 10.1152/physrev.00032.2009

Garcia-Rivello, H., Taranda, J., Said, M., Cabeza-Meckert, P., Vila-Petroff, M., Scaglione, J., . . . Hertig, C. M. (2005). Dilated cardiomyopathy in Erb-b4-deficient ventricular muscle. *Am J Physiol Heart Circ Physiol, 289*(3), H1153-1160. doi: 10.1152/ajpheart.00048.2005

Gard, D. L., Bell, P. B., & Lazarides, E. (1979). Coexistence of desmin and the fibroblastic intermediate filament subunit in muscle and nonmuscle cells: identification and comparative peptide analysis. *Proc Natl Acad Sci U S A, 76*(8), 3894-3898.

Gardner, L. B., Li, F., Yang, X., & Dang, C. V. (2003). Anoxic fibroblasts activate a replication checkpoint that is bypassed by E1a. *Mol Cell Biol, 23*(24), 9032-9045.

Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R., & Lemke, G. (1995). Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature, 378*(6555), 390-394. doi: 10.1038/378390a0

George, E. M., & Granger, J. P. (2011). Endothelin: key mediator of hypertension in preeclampsia. *Am J Hypertens, 24*(9), 964-969. doi: 10.1038/ajh.2011.99

Giaccia, A. J., Simon, M. C., & Johnson, R. (2004). The biology of hypoxia: the role of oxygen sensing in development, normal function, and disease. *Genes Dev, 18*(18), 2183-2194. doi: 10.1101/gad.1243304

Giraud, G. D., Louey, S., Jonker, S., Schultz, J., & Thornburg, K. L. (2006). Cortisol stimulates cell cycle activity in the cardiomyocyte of the sheep fetus. *Endocrinology, 147*(8), 3643-3649. doi: 10.1210/en.2006-0061

Gluckman, P. D., Hanson, M. A., Cooper, C., & Thornburg, K. L. (2008). Effect of in utero and early-life conditions on adult health and disease. *N Engl J Med, 359*(1), 61-73. doi: 10.1056/NEJMra0708473

Goda, N., Dozier, S. J., & Johnson, R. S. (2003). HIF-1 in cell cycle regulation, apoptosis, and tumor progression. *Antioxid Redox Signal, 5*(4), 467-473. doi: 10.1089/152308603768295212

Goda, N., Ryan, H. E., Khadivi, B., McNulty, W., Rickert, R. C., & Johnson, R. S. (2003). Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia. *Mol Cell Biol, 23*(1), 359-369.

Goldie, R. G. (1999). Endothelins in health and disease: an overview. *Clin Exp Pharmacol Physiol, 26*(2), 145-148.

Granger, B. L., & Lazarides, E. (1979). Desmin and vimentin coexist at the periphery of the myofibril Z disc. *Cell, 18*(4), 1053-1063.

Gysin, S., Lee, S. H., Dean, N. M., & McMahon, M. (2005). Pharmacologic inhibition of RAF-->MEK-->ERK signaling elicits pancreatic cancer cell cycle arrest through induced expression of p27Kip1. *Cancer Res, 65*(11), 4870-4880. doi: 10.1158/0008-5472.can-04-2848

Hammoud, L., Burger, D. E., Lu, X., & Feng, Q. (2009). Tissue inhibitor of metalloproteinase-3 inhibits neonatal mouse cardiomyocyte proliferation via EGFR/JNK/SP-1 signaling. *Am J Physiol Cell Physiol, 296*(4), C735-745. doi: 10.1152/ajpcell.00246.2008

Hammoud, L., Xiang, F., Lu, X., Brunner, F., Leco, K., & Feng, Q. (2007). Endothelial nitric oxide synthase promotes neonatal cardiomyocyte proliferation by inhibiting tissue inhibitor of metalloproteinase-3 expression. *Cardiovasc Res, 75*(2), 359-368. doi: 10.1016/j.cardiores.2007.05.006

Harvey, P. A., & Leinwand, L. A. (2011). The cell biology of disease: cellular mechanisms of cardiomyopathy. *J Cell Biol, 194*(3), 355-365. doi: 10.1083/jcb.201101100

Hashiguchi, K., Takagi, K., Nakabayashi, M., Takeda, Y., Sakamoto, S., Naruse, M., . . . Demura, H. (1991). Relationship between fetal hypoxia and endothelin-1 in fetal circulation. *J Cardiovasc Pharmacol, 17 Suppl 7*, S509-510.

He, A., Ma, Q., Cao, J., von Gise, A., Zhou, P., Xie, H., . . . Pu, W. T. (2012). Polycomb repressive complex 2 regulates normal development of the mouse heart. *Circ Res, 110*(3), 406-415. doi: 10.1161/circresaha.111.252205

Hengst, L., & Reed, S. I. (1996). Translational control of p27Kip1 accumulation during the cell cycle. *Science, 271*(5257), 1861-1864.

Herman, D. S., Lam, L., Taylor, M. R., Wang, L., Teekakirikul, P., Christodoulou, D., . . . Seidman, C. E. (2012). Truncations of titin causing dilated cardiomyopathy. *N Engl J Med, 366*(7), 619-628. doi: 10.1056/NEJMoa1110186

Hosoda, K., Nakao, K., Hiroshi, A., Suga, S., Ogawa, Y., Mukoyama, M., . . . Imura, H. (1991). Cloning and expression of human endothelin-1 receptor cDNA. *FEBS Lett, 287*(1-2), 23-26.

Howard, P. G., Plumpton, C., & Davenport, A. P. (1992). Anatomical localization and pharmacological activity of mature endothelins and their precursors in human vascular tissue. *J Hypertens, 10*(11), 1379-1386.

Hu, J., Discher, D. J., Bishopric, N. H., & Webster, K. A. (1998). Hypoxia regulates expression of the endothelin-1 gene through a proximal hypoxia-inducible factor-1 binding site on the antisense strand. *Biochem Biophys Res Commun, 245*(3), 894-899. doi: 10.1006/bbrc.1998.8543

Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K., & Masaki, T. (1989). The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc Natl Acad Sci U S A, 86*(8), 2863-2867.

Ishida, N., Kitagawa, M., Hatakeyama, S., & Nakayama, K. (2000). Phosphorylation at serine 10, a major phosphorylation site of p27(Kip1), increases its protein stability. *J Biol Chem, 275*(33), 25146-25154. doi: 10.1074/jbc.M001144200

Ito, H., Adachi, S., Tamamori, M., Fujisaki, H., Tanaka, M., Lin, M., . . . Hiroe, M. (1996). Mild hypoxia induces hypertrophy of cultured neonatal rat cardiomyocytes: a possible endogenous endothelin-1-mediated mechanism. *J Mol Cell Cardiol, 28*(6), 1271-1277. doi: 10.1006/jmcc.1996.0117

Ito, H., Hirata, Y., Hiroe, M., Tsujino, M., Adachi, S., Takamoto, T., . . . Marumo, F. (1991). Endothelin-1 induces hypertrophy with enhanced expression of muscle-specific genes in cultured neonatal rat cardiomyocytes. *Circ Res, 69*(1), 209-215.

Jensen, G. M., & Moore, L. G. (1997). The effect of high altitude and other risk factors on birthweight: independent or interactive effects? *Am J Public Health, 87*(6), 1003-1007.

Jeyabalan, A., Novak, J., Danielson, L. A., Kerchner, L. J., Opett, S. L., & Conrad, K. P. (2003). Essential role for vascular gelatinase activity in relaxin-induced renal vasodilation, hyperfiltration, and reduced myogenic reactivity of small arteries. *Circ Res, 93*(12), 1249-1257. doi: 10.1161/01.res.0000104086.43830.6c

Jonker, S. S., Giraud, M. K., Giraud, G. D., Chattergoon, N. N., Louey, S., Davis, L. E., . . . Thornburg, K. L. (2010). Cardiomyocyte enlargement, proliferation and maturation during chronic fetal anaemia in sheep. *Exp Physiol, 95*(1), 131-139. doi: 10.1113/expphysiol.2009.049379

Jopling, C., Sleep, E., Raya, M., Marti, M., Raya, A., & Izpisua Belmonte, J. C. (2010). Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature, 464*(7288), 606-609. doi: 10.1038/nature08899

Jopling, C., Sune, G., Faucherre, A., Fabregat, C., & Izpisua Belmonte, J. C. (2012). Hypoxia induces myocardial regeneration in zebrafish. *Circulation, 126*(25), 3017-3027. doi: 10.1161/circulationaha.112.107888

Kakinuma, Y., Miyauchi, T., Yuki, K., Murakoshi, N., Goto, K., & Yamaguchi, I. (2001). Novel molecular mechanism of increased myocardial endothelin-1 expression in the failing heart involving the transcriptional factor hypoxia-inducible factor-1alpha induced for impaired myocardial energy metabolism. *Circulation, 103*(19), 2387-2394.

Kang, J. O., & Sucov, H. M. (2005). Convergent proliferative response and divergent morphogenic pathways induced by epicardial and endocardial signaling in fetal heart development. *Mech Dev, 122*(1), 57-65. doi: 10.1016/j.mod.2004.09.001

Kawanabe, Y., & Nauli, S. M. (2011). Endothelin. *Cell Mol Life Sci, 68*(2), 195-203. doi: 10.1007/s00018-010-0518-0

Kedzierski, R. M., & Yanagisawa, M. (2001). Endothelin system: the double-edged sword in health and disease. *Annu Rev Pharmacol Toxicol, 41*, 851-876. doi: 10.1146/annurev.pharmtox.41.1.851

Kelly, J. J., & Whitworth, J. A. (1999). Endothelin-1 as a mediator in cardiovascular disease. *Clin Exp Pharmacol Physiol, 26*(2), 158-161.

Kido, M., Du, L., Sullivan, C. C., Li, X., Deutsch, R., Jamieson, S. W., & Thistlethwaite, P. A. (2005). Hypoxia-inducible factor 1-alpha reduces infarction and attenuates progression of cardiac dysfunction after myocardial infarction in the mouse. *J Am Coll Cardiol, 46*(11), 2116-2124. doi: 10.1016/j.jacc.2005.08.045

Kohan, D. E., Rossi, N. F., Inscho, E. W., & Pollock, D. M. (2011). Regulation of blood pressure and salt homeostasis by endothelin. *Physiol Rev, 91*(1), 1-77. doi: 10.1152/physrev.00060.2009

Komuro, I., Kurihara, H., Sugiyama, T., Yoshizumi, M., Takaku, F., & Yazaki, Y. (1988). Endothelin stimulates c-fos and c-myc expression and proliferation of vascular smooth muscle cells. *FEBS Lett, 238*(2), 249-252.

Kou, C. Y., Lau, S. L., Au, K. W., Leung, P. Y., Chim, S. S., Fung, K. P., . . . Tsui, S. K. (2010). Epigenetic regulation of neonatal cardiomyocytes differentiation. *Biochem Biophys Res Commun, 400*(2), 278-283. doi: 10.1016/j.bbrc.2010.08.064

Lee, J. G., & Kay, E. P. (2011). PI 3-kinase/Rac1 and ERK1/2 regulate FGF-2-mediated cell proliferation through phosphorylation of p27 at Ser10 by KIS and at Thr187 by Cdc25A/Cdk2. *Invest Ophthalmol Vis Sci, 52*(1), 417-426. doi: 10.1167/iovs.10-6140

Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C., & Hauser, C. (1995). Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature, 378*(6555), 394-398. doi: 10.1038/378394a0

Lee, T. I., Jenner, R. G., Boyer, L. A., Guenther, M. G., Levine, S. S., Kumar, R. M., . . . Young, R. A. (2006). Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell, 125*(2), 301-313. doi: 10.1016/j.cell.2006.02.043

Li, F., Wang, X., Capasso, J. M., & Gerdes, A. M. (1996). Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. *J Mol Cell Cardiol, 28*(8), 1737-1746. doi: 10.1006/jmcc.1996.0163

Li, G., Bae, S., & Zhang, L. (2004). Effect of prenatal hypoxia on heat stress-mediated cardioprotection in adult rat heart. *Am J Physiol Heart Circ Physiol, 286*(5), H1712-1719. doi: 10.1152/ajpheart.00898.2003

Li, G., Xiao, Y., Estrella, J. L., Ducsay, C. A., Gilbert, R. D., & Zhang, L. (2003). Effect of fetal hypoxia on heart susceptibility to ischemia and reperfusion injury in the adult rat. *J Soc Gynecol Investig, 10*(5), 265-274.

Li, H., Chen, S. J., Chen, Y. F., Meng, Q. C., Durand, J., Oparil, S., & Elton, T. S. (1994). Enhanced endothelin-1 and endothelin receptor gene expression in chronic hypoxia. *J Appl Physiol, 77*(3), 1451-1459.

Li, X., Wei, X. L., Meng, L. L., Chi, M. G., Yan, J. Q., Ma, X. Y., . . . Zheng, J. Q. (2009). Involvement of tissue transglutaminase in endothelin 1-induced hypertrophy in cultured neonatal rat cardiomyocytes. *Hypertension, 54*(4), 839-844. doi: 10.1161/hypertensionaha.109.130161

Liang, J., Zubovitz, J., Petrocelli, T., Kotchetkov, R., Connor, M. K., Han, K., . . . Slingerland, J. M. (2002). PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nat Med, 8*(10), 1153-1160. doi: 10.1038/nm761

Liu, Y., Tang, M. K., Cai, D. Q., Li, M., Wong, W. M., Chow, P. H., & Lee, K. K. (2007). Cyclin I and p53 are differentially expressed during the terminal differentiation of the postnatal mouse heart. *Proteomics, 7*(1), 23-32. doi: 10.1002/pmic.200600456

Liu, Z., Yue, S., Chen, X., Kubin, T., & Braun, T. (2010). Regulation of cardiomyocyte polyploidy and multinucleation by CyclinG1. *Circ Res, 106*(9), 1498-1506. doi: 10.1161/circresaha.109.211888

Louey, S., Jonker, S. S., Giraud, G. D., & Thornburg, K. L. (2007). Placental insufficiency decreases cell cycle activity and terminal maturation in fetal sheep cardiomyocytes. *J Physiol, 580*(Pt. 2), 639-648. doi: 10.1113/jphysiol.2006.122200

Ma, T., Van Tine, B. A., Wei, Y., Garrett, M. D., Nelson, D., Adams, P. D., . . . Harper, J. W. (2000). Cell cycle-regulated phosphorylation of p220(NPAT) by cyclin E/Cdk2 in Cajal bodies promotes histone gene transcription. *Genes Dev, 14*(18), 2298-2313.

Majumdar, P., Chen, S., George, B., Sen, S., Karmazyn, M., & Chakrabarti, S. (2009). Leptin and endothelin-1 mediated increased extracellular matrix protein production and cardiomyocyte hypertrophy in diabetic heart disease. *Diabetes Metab Res Rev, 25*(5), 452-463. doi: 10.1002/dmrr.964

Martin, R. J., Wang, K., Koroglu, O., Di Fiore, J., & Kc, P. (2011). Intermittent hypoxic episodes in preterm infants: do they matter? *Neonatology, 100*(3), 303-310. doi: 10.1159/000329922

Matsuyama, D., & Kawahara, K. (2011). Oxidative stress-induced formation of a positive-feedback loop for the sustained activation of p38 MAPK leading to the loss of cell division in cardiomyocytes soon after birth. *Basic Res Cardiol, 106*(5), 815-828. doi: 10.1007/s00395-011-0178-8

McCullough, R. E., Reeves, J. T., & Liljegren, R. L. (1977). Fetal growth retardation and increased infant mortality at high altitude. *Obstet Gynecol Surv, 32*(7), 596-598.

McNally, E. M. (2012). Genetics: broken giant linked to heart failure. *Nature, 483*(7389), 281-282. doi: 10.1038/483281a

Meyer, K., Zhang, H., & Zhang, L. (2009). Direct effect of cocaine on epigenetic regulation of PKCepsilon gene repression in the fetal rat heart. *J Mol Cell Cardiol, 47*(4), 504-511. doi: 10.1016/j.yjmcc.2009.06.004

Minchenko, A., & Caro, J. (2000). Regulation of endothelin-1 gene expression in human microvascular endothelial cells by hypoxia and cobalt: role of hypoxia responsive element. *Mol Cell Biochem, 208*(1-2), 53-62.

Moore, L. G. (2003). Fetal growth restriction and maternal oxygen transport during high altitude pregnancy. *High Alt Med Biol, 4*(2), 141-156. doi: 10.1089/152702903322022767

Morrison, J. L., Botting, K. J., Dyer, J. L., Williams, S. J., Thornburg, K. L., & McMillen, I. C. (2007). Restriction of placental function alters heart development

in the sheep fetus. *Am J Physiol Regul Integr Comp Physiol, 293*(1), R306-313. doi: 10.1152/ajpregu.00798.2006

Mounkes, L. C., Kozlov, S. V., Rottman, J. N., & Stewart, C. L. (2005). Expression of an LMNA-N195K variant of A-type lamins results in cardiac conduction defects and death in mice. *Hum Mol Genet, 14*(15), 2167-2180. doi: 10.1093/hmg/ddi221

Nakas-Icindic, E., Zaciragic, A., Hadzovic, A., & Avdagic, N. (2004). Endothelin in health and disease. *Bosn J Basic Med Sci, 4*(3), 31-34.

Nikolova, V., Leimena, C., McMahon, A. C., Tan, J. C., Chandar, S., Jogia, D., . . . Fatkin, D. (2004). Defects in nuclear structure and function promote dilated cardiomyopathy in lamin A/C-deficient mice. *J Clin Invest, 113*(3), 357-369. doi: 10.1172/jci19448

Nourse, J., Firpo, E., Flanagan, W. M., Coats, S., Polyak, K., Lee, M. H., . . . Roberts, J. M. (1994). Interleukin-2-mediated elimination of the p27Kip1 cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature, 372*(6506), 570-573. doi: 10.1038/372570a0

O'Neill, T. J. t., Mack, C. P., & Taylor, J. M. (2012). Germline deletion of FAK-related non-kinase delays post-natal cardiomyocyte mitotic arrest. *J Mol Cell Cardiol, 53*(2), 156-164. doi: 10.1016/j.yjmcc.2012.04.007

Olson, T. M., Michels, V. V., Thibodeau, S. N., Tai, Y. S., & Keating, M. T. (1998). Actin mutations in dilated cardiomyopathy, a heritable form of heart failure. *Science, 280*(5364), 750-752.

Osley, M. A. (1991). The regulation of histone synthesis in the cell cycle. *Annu Rev Biochem, 60*, 827-861. doi: 10.1146/annurev.bi.60.070191.004143

Ostlund, E., Lindholm, H., Hemsen, A., & Fried, G. (2000). Fetal erythropoietin and endothelin-1: relation to hypoxia and intrauterine growth retardation. *Acta Obstet Gynecol Scand, 79*(4), 276-282.

Ozcelik, C., Erdmann, B., Pilz, B., Wettschureck, N., Britsch, S., Hubner, N., . . . Garratt, A. N. (2002). Conditional mutation of the ErbB2 (HER2) receptor in cardiomyocytes leads to dilated cardiomyopathy. *Proc Natl Acad Sci U S A, 99*(13), 8880-8885. doi: 10.1073/pnas.122249299

Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romero, P., Del Sal, G., Chau, V., . . . Rolfe, M. (1995). Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science, 269*(5224), 682-685.

Paradis, A., Xiao, D., Zhou, J., & Zhang, L. (2014). Endothelin-1 Promotes Cardiomyocyte Terminal Differentiation in the Developing Heart via Heightened DNA Methylation. *Int J Med Sci, 11*(4), 373-380. doi: 10.7150/ijms.7802

Pasumarthi, K. B., & Field, L. J. (2002). Cardiomyocyte cell cycle regulation. *Circ Res, 90*(10), 1044-1054.

Patterson, A. J., Chen, M., Xue, Q., Xiao, D., & Zhang, L. (2010). Chronic prenatal hypoxia induces epigenetic programming of PKC{epsilon} gene repression in rat hearts. *Circ Res, 107*(3), 365-373. doi: 10.1161/circresaha.110.221259

Patterson, A. J., Xiao, D., Xiong, F., Dixon, B., & Zhang, L. (2012). Hypoxia-derived oxidative stress mediates epigenetic repression of PKCepsilon gene in foetal rat hearts. *Cardiovasc Res, 93*(2), 302-310. doi: 10.1093/cvr/cvr322

Perreault, T., & Coceani, F. (2003). Endothelin in the perinatal circulation. *Can J Physiol Pharmacol, 81*(6), 644-653. doi: 10.1139/y03-013

Ponicke, K., Vogelsang, M., Heinroth, M., Becker, K., Zolk, O., Bohm, M., . . . Brodde, O. E. (1998). Endothelin receptors in the failing and nonfailing human heart. *Circulation, 97*(8), 744-751.

Porrello, E. R., Bell, J. R., Schertzer, J. D., Curl, C. L., McMullen, J. R., Mellor, K. M., . . . Delbridge, L. M. (2009). Heritable pathologic cardiac hypertrophy in adulthood is preceded by neonatal cardiac growth restriction. *Am J Physiol Regul Integr Comp Physiol, 296*(3), R672-680. doi: 10.1152/ajpregu.90919.2008

Porrello, E. R., Widdop, R. E., & Delbridge, L. M. (2008). Early origins of cardiac hypertrophy: does cardiomyocyte attrition programme for pathological 'catch-up' growth of the heart? *Clin Exp Pharmacol Physiol, 35*(11), 1358-1364. doi: 10.1111/j.1440-1681.2008.05036.x

Qian, X., Jin, L., & Lloyd, R. V. (2000). Aberrant DNA methylation of cyclin D2 and p27 genes in rodent pituitary tumor cell lines correlates with specific gene expression. *Endocr Pathol, 11*(1), 85-96.

Radom-Aizik, S., Zaldivar, F. P., Nance, D. M., Haddad, F., Cooper, D. M., & Adams, G. R. (2013). Growth inhibition and compensation in response to neonatal hypoxia in rats. *Pediatr Res, 74*(2), 111-120. doi: 10.1038/pr.2013.80

Rajan, S., Jagatheesan, G., Karam, C. N., Alves, M. L., Bodi, I., Schwartz, A., . . . Wieczorek, D. F. (2010). Molecular and functional characterization of a novel cardiac-specific human tropomyosin isoform. *Circulation, 121*(3), 410-418. doi: 10.1161/circulationaha.109.889725

Rautureau, Y., & Schiffrin, E. L. (2012). Endothelin in hypertension: an update. *Curr Opin Nephrol Hypertens, 21*(2), 128-136. doi: 10.1097/MNH.0b013e32834f0092

Ream, M., Ray, A. M., Chandra, R., & Chikaraishi, D. M. (2008). Early fetal hypoxia leads to growth restriction and myocardial thinning. *Am J Physiol Regul Integr Comp Physiol, 295*(2), R583-595. doi: 10.1152/ajpregu.00771.2007

Reynolds, E. E., Keiser, J. A., Haleen, S. J., Walker, D. M., Olszewski, B., Schroeder, R. L., . . . et al. (1995). Pharmacological characterization of PD 156707, an orally active ETA receptor antagonist. *J Pharmacol Exp Ther, 273*(3), 1410-1417.

Rubanyi, G. M., & Polokoff, M. A. (1994). Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacol Rev, 46*(3), 325-415.

Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K., & Masaki, T. (1990). Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature, 348*(6303), 732-735. doi: 10.1038/348732a0

Sdek, P., Zhao, P., Wang, Y., Huang, C. J., Ko, C. Y., Butler, P. C., . . . Maclellan, W. R. (2011). Rb and p130 control cell cycle gene silencing to maintain the postmitotic phenotype in cardiac myocytes. *J Cell Biol, 194*(3), 407-423. doi: 10.1083/jcb.201012049

Sebillon, P., Bouchier, C., Bidot, L. D., Bonne, G., Ahamed, K., Charron, P., . . . Komajda, M. (2003). Expanding the phenotype of LMNA mutations in dilated cardiomyopathy and functional consequences of these mutations. *J Med Genet, 40*(8), 560-567.

Shirane, M., Harumiya, Y., Ishida, N., Hirai, A., Miyamoto, C., Hatakeyama, S., . . . Kitagawa, M. (1999). Down-regulation of p27(Kip1) by two mechanisms, ubiquitin-mediated degradation and proteolytic processing. *J Biol Chem, 274*(20), 13886-13893.

Shubeita, H. E., McDonough, P. M., Harris, A. N., Knowlton, K. U., Glembotski, C. C., Brown, J. H., & Chien, K. R. (1990). Endothelin induction of inositol phospholipid hydrolysis, sarcomere assembly, and cardiac gene expression in ventricular myocytes. A paracrine mechanism for myocardial cell hypertrophy. *J Biol Chem, 265*(33), 20555-20562.

Simard, E., Jin, D., Takai, S., Miyazaki, M., Brochu, I., & D'Orleans-Juste, P. (2009). Chymase-dependent conversion of Big endothelin-1 in the mouse in vivo. *J Pharmacol Exp Ther, 328*(2), 540-548. doi: 10.1124/jpet.108.142992

Sridharan, V., Sharma, S. K., Moros, E. G., Corry, P. M., Tripathi, P., Lieblong, B. J., . . . Boerma, M. (2013). Effects of radiation on the epidermal growth factor receptor pathway in the heart. *Int J Radiat Biol, 89*(7), 539-547. doi: 10.3109/09553002.2013.782110

Stefani, M., Tsubakihara, M., Hambly, B., Liew, C., Allen, P., Macdonald, P., & dos Remedios, C. (2008). Actin and Its Binding Proteins in Heart Failure. In C. dos Remedios & D. Chhabra (Eds.), *Actin-Binding Proteins and Disease* (Vol. 8, pp. 318-334): Springer New York.

Stein, A. B., Jones, T. A., Herron, T. J., Patel, S. R., Day, S. M., Noujaim, S. F., . . . Dressler, G. R. (2011). Loss of H3K4 methylation destabilizes gene expression patterns and physiological functions in adult murine cardiomyocytes. *J Clin Invest, 121*(7), 2641-2650. doi: 10.1172/jci44641

Sucov, H. M., Gu, Y., Thomas, S., Li, P., & Pashmforoush, M. (2009). Epicardial control of myocardial proliferation and morphogenesis. *Pediatr Cardiol, 30*(5), 617-625. doi: 10.1007/s00246-009-9391-8

Sun, Q., Zhang, F., Wafa, K., Baptist, T., & Pasumarthi, K. B. (2009). A splice variant of cyclin D2 regulates cardiomyocyte cell cycle through a novel protein aggregation pathway. *J Cell Sci, 122*(Pt 10), 1563-1573. doi: 10.1242/jcs.047738

Susaki, E., Nakayama, K., & Nakayama, K. I. (2007). Cyclin D2 translocates p27 out of the nucleus and promotes its degradation at the G0-G1 transition. *Mol Cell Biol, 27*(13), 4626-4640. doi: 10.1128/mcb.00862-06

Suzuki, T., Hoshi, H., & Mitsui, Y. (1990). Endothelin stimulates hypertrophy and contractility of neonatal rat cardiac myocytes in a serum-free medium. *FEBS Lett, 268*(1), 149-151.

Thaete, L. G., Jilling, T., Synowiec, S., Khan, S., & Neerhof, M. G. (2007). Expression of endothelin 1 and its receptors in the hypoxic pregnant rat. *Biol Reprod, 77*(3), 526-532. doi: 10.1095/biolreprod.107.061820

Thornburg, K., Jonker, S., O'Tierney, P., Chattergoon, N., Louey, S., Faber, J., & Giraud, G. (2011). Regulation of the cardiomyocyte population in the developing heart. *Prog Biophys Mol Biol, 106*(1), 289-299. doi: 10.1016/j.pbiomolbio.2010.11.010

Tokuyasu, K. T., Maher, P. A., Dutton, A. H., & Singer, S. J. (1985). Intermediate filaments in skeletal and cardiac muscle tissue in embryonic and adult chicken. *Ann N Y Acad Sci, 455*, 200-212.

Tong, W., Xiong, F., Li, Y., & Zhang, L. (2013). Hypoxia inhibits cardiomyocyte proliferation in fetal rat hearts via upregulating TIMP-4. *Am J Physiol Regul Integr Comp Physiol*. doi: 10.1152/ajpregu.00515.2012

Tong, W., Xue, Q., Li, Y., & Zhang, L. (2011). Maternal hypoxia alters matrix metalloproteinase expression patterns and causes cardiac remodeling in fetal and neonatal rats. *Am J Physiol Heart Circ Physiol, 301*(5), H2113-2121. doi: 10.1152/ajpheart.00356.2011

Tong, W., & Zhang, L. (2012). Fetal hypoxia and programming of matrix metalloproteinases. *Drug Discov Today, 17*(3-4), 124-134. doi: 10.1016/j.drudis.2011.09.011

Tseng, Y. T., Yano, N., Rojan, A., Stabila, J. P., McGonnigal, B. G., Ianus, V., . . . Padbury, J. F. (2005). Ontogeny of phosphoinositide 3-kinase signaling in

developing heart: effect of acute beta-adrenergic stimulation. *Am J Physiol Heart Circ Physiol, 289*(5), H1834-1842. doi: 10.1152/ajpheart.00435.2005

Vallender, T. W., & Lahn, B. T. (2006). Localized methylation in the key regulator gene endothelin-1 is associated with cell type-specific transcriptional silencing. *FEBS Lett, 580*(18), 4560-4566. doi: 10.1016/j.febslet.2006.07.017

van Berlo, J. H., Duboc, D., & Pinto, Y. M. (2004). Often seen but rarely recognised: cardiac complications of lamin A/C mutations. *Eur Heart J, 25*(10), 812-814. doi: 10.1016/j.ehj.2004.03.007

von Gise, A., Lin, Z., Schlegelmilch, K., Honor, L. B., Pan, G. M., Buck, J. N., . . . Pu, W. T. (2012). YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy. *Proc Natl Acad Sci U S A, 109*(7), 2394-2399. doi: 10.1073/pnas.1116136109

Wagner, O. F., Christ, G., Wojta, J., Vierhapper, H., Parzer, S., Nowotny, P. J., . . . Binder, B. R. (1992). Polar secretion of endothelin-1 by cultured endothelial cells. *J Biol Chem, 267*(23), 16066-16068.

Walsh, S., Ponten, A., Fleischmann, B. K., & Jovinge, S. (2010). Cardiomyocyte cell cycle control and growth estimation in vivo--an analysis based on cardiomyocyte nuclei. *Cardiovasc Res, 86*(3), 365-373. doi: 10.1093/cvr/cvq005

Wamstad, J. A., Alexander, J. M., Truty, R. M., Shrikumar, A., Li, F., Eilertson, K. E., . . . Bruneau, B. G. (2012). Dynamic and coordinated epigenetic regulation of developmental transitions in the cardiac lineage. *Cell, 151*(1), 206-220. doi: 10.1016/j.cell.2012.07.035

Wander, S. A., Zhao, D., & Slingerland, J. M. (2011). p27: a barometer of signaling deregulation and potential predictor of response to targeted therapies. *Clin Cancer Res, 17*(1), 12-18. doi: 10.1158/1078-0432.ccr-10-0752

Wang, X., Osinska, H., Dorn, G. W., 2nd, Nieman, M., Lorenz, J. N., Gerdes, A. M., . . . Robbins, J. (2001). Mouse model of desmin-related cardiomyopathy. *Circulation, 103*(19), 2402-2407.

Wang, X., Osinska, H., Gerdes, A. M., & Robbins, J. (2002). Desmin filaments and cardiac disease: establishing causality. *J Card Fail, 8*(6 Suppl), S287-292. doi: 10.1054/jcaf.2002.129279

Warner, T. D., Allcock, G. H., Corder, R., & Vane, J. R. (1993). Use of the endothelin antagonists BQ-123 and PD 142893 to reveal three endothelin receptors mediating smooth muscle contraction and the release of EDRF. *Br J Pharmacol, 110*(2), 777-782.

Webster, W. S., & Abela, D. (2007). The effect of hypoxia in development. *Birth Defects Res C Embryo Today, 81*(3), 215-228. doi: 10.1002/bdrc.20102

Wei, C. M., Lerman, A., Rodeheffer, R. J., McGregor, C. G., Brandt, R. R., Wright, S., . . . Burnett, J. C., Jr. (1994). Endothelin in human congestive heart failure. *Circulation, 89*(4), 1580-1586.

Wieczorek, D. F., Jagatheesan, G., & Rajan, S. (2008). The role of tropomyosin in heart disease. *Adv Exp Med Biol, 644*, 132-142.

Wilkes, B. M., Susin, M., & Mento, P. F. (1993). Localization of endothelin-1-like immunoreactivity in human placenta. *J Histochem Cytochem, 41*(4), 535-541.

Xiao, D., Dasgupta, C., Chen, M., Zhang, K., Buchholz, J., Xu, Z., & Zhang, L. (2014). Inhibition of DNA methylation reverses norepinephrine-induced cardiac hypertrophy in rats. *Cardiovasc Res, 101*(3), 373-382. doi: 10.1093/cvr/cvt264

Xiao, D., Ducsay, C. A., & Zhang, L. (2000). Chronic hypoxia and developmental regulation of cytochrome c expression in rats. *J Soc Gynecol Investig, 7*(5), 279-283.

Xiao, Y., He, J., Gilbert, R. D., & Zhang, L. (2000). Cocaine induces apoptosis in fetal myocardial cells through a mitochondria-dependent pathway. *J Pharmacol Exp Ther, 292*(1), 8-14.

Xin, M., Kim, Y., Sutherland, L. B., Qi, X., McAnally, J., Schwartz, R. J., . . . Olson, E. N. (2011). Regulation of insulin-like growth factor signaling by Yap governs cardiomyocyte proliferation and embryonic heart size. *Sci Signal, 4*(196), ra70. doi: 10.1126/scisignal.2002278

Xiong, F., Xiao, D., & Zhang, L. (2012). Norepinephrine causes epigenetic repression of PKC{varepsilon} gene in rodent hearts by activating Nox1-dependent reactive oxygen species production. *FASEB J, 26*(7), 2753-2763. doi: 10.1096/fj.11-199422

Xiong, L., Darwanto, A., Sharma, S., Herring, J., Hu, S., Filippova, M., . . . Zhang, K. (2011). Mass spectrometric studies on epigenetic interaction networks in cell differentiation. *J Biol Chem, 286*(15), 13657-13668. doi: 10.1074/jbc.M110.204800

Xu, Y., Williams, S. J., O'Brien, D., & Davidge, S. T. (2006). Hypoxia or nutrient restriction during pregnancy in rats leads to progressive cardiac remodeling and impairs postischemic recovery in adult male offspring. *FASEB J, 20*(8), 1251-1253. doi: 10.1096/fj.05-4917fje

Xue, Q., Dasgupta, C., Chen, M., & Zhang, L. (2011). Foetal hypoxia increases cardiac AT(2)R expression and subsequent vulnerability to adult ischaemic injury. *Cardiovasc Res, 89*(2), 300-308. doi: 10.1093/cvr/cvq303

Yamada, J., Fujimori, K., Ishida, T., Sanpei, M., Honda, S., & Sato, A. (2001). Plasma endothelin-1 and atrial natriuretic peptide levels during prolonged (24-h) non-acidemic hypoxemia in fetal goats. *J Matern Fetal Med, 10*(6), 409-413.

Yamashita, K., Discher, D. J., Hu, J., Bishopric, N. H., & Webster, K. A. (2001). Molecular regulation of the endothelin-1 gene by hypoxia. Contributions of hypoxia-inducible factor-1, activator protein-1, GATA-2, AND p300/CBP. *J Biol Chem, 276*(16), 12645-12653. doi: 10.1074/jbc.M011344200

Yanagisawa, M. (1994). The endothelin system. A new target for therapeutic intervention. *Circulation, 89*(3), 1320-1322.

Yanagisawa, M., Inoue, A., Ishikawa, T., Kasuya, Y., Kimura, S., Kumagaye, S., . . . et al. (1988). Primary structure, synthesis, and biological activity of rat endothelin, an endothelium-derived vasoconstrictor peptide. *Proc Natl Acad Sci U S A, 85*(18), 6964-6967.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., . . . Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature, 332*(6163), 411-415. doi: 10.1038/332411a0

Yu, L., Li, M., She, T., Shi, C., Meng, W., Wang, B., & Cheng, M. (2013). Endothelin-1 Stimulates the Expression of L-Type Ca(2+) Channels in Neonatal Rat Cardiomyocytes via the Extracellular Signal-Regulated Kinase 1/2 Pathway. *J Membr Biol, 246*(4), 343-353. doi: 10.1007/s00232-013-9538-7

Zhang, F. X., Chen, M. L., Shan, Q. J., Zou, J. G., Chen, C., Yang, B., . . . Cao, K. J. (2007). Hypoxia reoxygenation induces premature senescence in neonatal SD rat cardiomyocytes. *Acta Pharmacol Sin, 28*(1), 44-51. doi: 10.1111/j.1745-7254.2007.00488.x

Zhang, K., Schrag, M., Crofton, A., Trivedi, R., Vinters, H., & Kirsch, W. (2012). Targeted proteomics for quantification of histone acetylation in Alzheimer's disease. *Proteomics, 12*(8), 1261-1268. doi: 10.1002/pmic.201200010

Zhang, L. (2005). Prenatal hypoxia and cardiac programming. *J Soc Gynecol Investig, 12*(1), 2-13. doi: 10.1016/j.jsgi.2004.09.004

Zhao, J., Kennedy, B. K., Lawrence, B. D., Barbie, D. A., Matera, A. G., Fletcher, J. A., & Harlow, E. (2000). NPAT links cyclin E-Cdk2 to the regulation of replication-dependent histone gene transcription. *Genes Dev, 14*(18), 2283-2297.

Zoabi, M., Sadeh, R., de Bie, P., Marquez, V. E., & Ciechanover, A. (2011). PRAJA1 is a ubiquitin ligase for the polycomb repressive complex 2 proteins. *Biochem Biophys Res Commun, 408*(3), 393-398. doi: 10.1016/j.bbrc.2011.04.025

Zolk, O., Quattek, J., Sitzler, G., Schrader, T., Nickenig, G., Schnabel, P., . . . Bohm, M. (1999). Expression of endothelin-1, endothelin-converting enzyme, and endothelin receptors in chronic heart failure. *Circulation, 99*(16), 2118-2123.