6-2016

Effects of Neonatal Dexamethasone on the Maturation and Endowment of Cardiomyocytes

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School of Medicine
in conjunction with the
Faculty of Graduate Studies

Effects of Neonatal Dexamethasone on the Maturation and Endowment of Cardiomyocytes

by

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

June 2016

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.

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ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Dr. Lubo Zhang for his support, time and mentoring throughout my graduate career. Being placed in such a nurturing and forgiving environment was such a blessing. I am truly thankful for this opportunity I was given.

To my committee members, Dr. Blood, Dr. Buchholz, Dr. Ducsay and Dr. Mata-Greenwood, thank you for your expert advice, innovative thoughts and kind words. You have enriched my research and my skill set, I am forever thankful for this. I would also like to thank my colleagues in the program and in my lab. I appreciate the time you dedicated to suggesting alternate approaches, troubleshooting, critiquing my work, and brainstorming.

A special thank you to my family, my mother and siblings who have been supportive and understanding throughout this entire process. Also, thank you to the Charles family, and of course, Dwane for the encouragement, support and prayers. You each have made a positive impact and contributed to my progress and success. Finally, I would like to thank God for giving me an undeserved opportunity to study and learn more about His creations.

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ABBREVIATIONS

5-AZA	5-Aza-2'-deoxycytidine
5mC	5-Methyl Cytosine
11β-HSD2	11β-Hydroxysteroid dehydrogenase-2
CDK	Cyclin-Dependent Kinases
CDKi	Cyclin Dependent Kinase Inhibitors
CEBPα	CCAAT/enhancer-binging protein alpha
CEBPβ	CCAAT/enhancer-binging protein beta
D2SV	D2 splice variant
DEX	Dexamethasone
G0	Gap 0
G1	Gap Phase 1
G2	Gap Phase 2
GC	Glucocorticoid
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
DNMT3a	DNA methyltransferases 3a
DNMT3b	DNA methyltransferases 3b
HAT	Histone Acetyl Transferase
FRNK	FAK-related non-kinase
HDAC	Histone deacetylase
hGRα	Human GR α
hGRβ	Human GR β

H3K27me3	Histone H3 at lysine 27
HSP	Heat Shock Protein
HPA	Hypothalamic-Pituitary- Adrenal Axis
IUGR	Intrauterine Growth Restriction
M	Mitosis
MR	Mineralocorticoid Receptor
nGRE	Negative GRE
NLS	Nuclear Localization Symbol
P4, P7 and P14	Postnatal Day 4, 7, 14, respectively
PCNA	Proliferating Cell Nuclear Antigen
PRC2	Polycomb Repressive Complex 2
S	Synthesis phase
SDS	Sodium Dodecyl Sulfate
YAP1	Yes-Associated Protein 1

ABSTRACT OF THE DISSERTATION

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by

Maresha Gay

Doctor of Philosophy, Graduate Program in Pharmacology
Loma Linda University, June 2016
Dr. Lubo Zhang, Chairperson

Each year 1 in 9 infants are born preterm in the United States. Preterm infants have a host of complications associated with prematurity, including respiratory distress syndrome. Dexamethasone, a synthetic glucocorticoid is the 'gold standard' intervention in the treatment of preterm infants and mothers at risk of preterm birth because it promotes organ maturation, particularly the lungs and aids in surfactant production. However, a growing body of evidence suggests perinatal dexamethasone exposure is associated with health consequences later in life including increased risk of cardiovascular disease. Cardiomyocytes are the functional unit of the heart and are unique in that they don't have an infinite proliferative capacity. In fact, soon after birth myocytes exit the cell cycle and become terminally differentiated. It is therefore crucial that sufficient proliferation occurs before terminal differentiation takes place to ensure adequate cardiomyocyte endowment in the mature heart. The purpose of this study is to test the hypothesis that neonatal dexamethasone exposure causes premature cardiomyocyte terminal differentiation, to elucidate potential molecular targets, and evaluate the role of epigenetic modifications. In newborn rats, dexamethasone was administered in tapered, clinically relevant doses during the first three days of life. We

found that newborn dexamethasone treatment induced premature terminal differentiation in cardiomyocytes resulting in reduced cardiomyocyte number in the mature heart, in a glucocorticoid receptor-dependent manner. In addition we demonstrated that an increase in DNA methylation is of importance in dexamethasone-mediated cardiomyocyte terminal differentiation. To further elucidate the molecular mechanisms involved, the effect of dexamethasone was determined in freshly isolated cardiomyocytes from newborn rats. We demonstrated that dexamethasone has a direct effect and induces hypermethylation of cyclin D2 gene promoter, resulting in epigenetic repression of cyclin D2 protein and mRNA expression in cardiomyocytes. Of importance, inhibition of DNA methylation reversed dexamethasone-mediated down-regulation of cyclin D2 expression and blocked dexamethasone-induced cardiomyocyte terminal differentiation. Furthermore, overexpression of cyclin D2 gene in cardiomyocyte rescued dexamethasone-mediated phenotype. Thus, we demonstrated a cause and effect relation of epigenetic repression of cyclin D2 gene and dexamethasone-mediated cardiomyocyte terminal differentiation in newborn rat hearts. These findings provided new insights in understanding the potential harmful effects of perinatal glucocorticoid treatment on reducing cardiomyocyte endowment in the heart and possible long-term adverse cardiovascular consequences.

CHAPTER ONE

INTRODUCTION

Developmental Origins of Adult Diseases

Large-scale experimental and epidemiological studies have identified a clear association between an unfavorable developmental environment and increased risk of disease later in life. An adverse developmental environment is a major risk factor in developing chronic illness such as diabetes, hypertension and heart disease (Barker 1990, Barker 2004, Barker, Gelow et al. 2010). The developmental environment is therefore not only vital for an organism's immediate structural and functional maturation but also later health.

During development an organism is plastic, that is, able to adapt to the environment and make changes to ensure immediate survival. In development, narrow windows exist, where an organism is especially sensitive to changes. Changes during these periods can have considerable impact on the organism by altering the developmental trajectory of the affected system. If these physiological and biochemical modifications persist the function of the affected structure may be permanently compromised, termed programming. Adverse environmental exposure, inadequate maternal nutrition and hypoxia have all been implicated as precursors to remodeling events that result in permanent neurologic, metabolic and cardiac dysfunction (Welberg and Seckl 2001, Meaney, Szyf et al. 2007).

The developmental origin of adult disease hypothesis seeks to explain how acute exposure to environmental stress produces a 'memory', which continues throughout the life of an organism. Figure 1.1 illustrates the concept of the developmental origins of

adult disease hypothesis. A central theory is that glucocorticoid signaling is instrumental in modulating pathology either directly or indirectly (Edwards, Benediktsson et al. 1993, Welberg, Seckl et al. 2000, O'Regan, Kenyon et al. 2004). Consistent with this theory developmental stress that increases glucocorticoid exposure in an organism is associated with increased pathology risk (Kapoor, Dunn et al. 2006, Cottrell and Seckl 2009). Yet, synthetic glucocorticoids are routinely administered as therapeutics in the treatment of preterm infants and mothers at risk of preterm birth.

According to the Center for Disease Control, cardiovascular disease is the number one cause of death in the United States. Like other chronic diseases, the prevalence and progression of cardiovascular diseases may have developmental origins. For adequate cardiac function the heart must be endowed with an optimal myocyte number. Cardiomyocyte endowment is limited and should be taken into consideration since glucocorticoids are known to regulate the cell cycle (Goya, Maiyar et al. 1993, Kim, Cheng et al. 1999, Crochemore, Michaelidis et al. 2002). Perinatal glucocorticoid exposure has been shown to reduce total cardiomyocyte number, and induces physiological hypertrophy in the adult heart (Bal, de Vries et al. 2008). It is therefore probable that premature glucocorticoid exposure may have negative implication on the adult heart by altering the developmental trajectory, contributing to cardiac vulnerability and dysfunction.

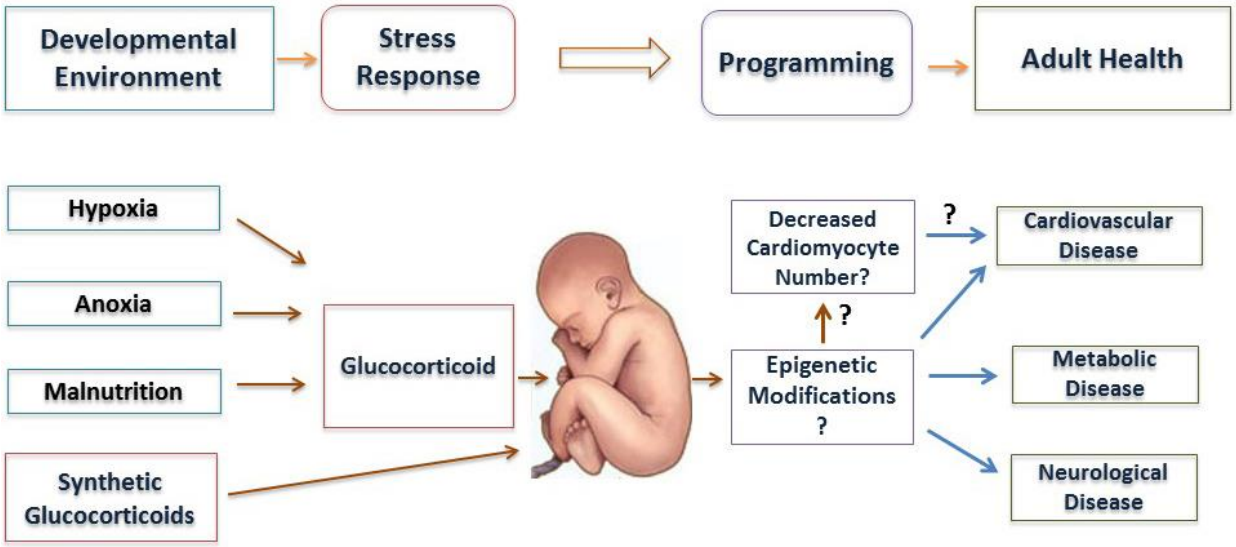

Figure 1.1. Developmental programming of adult health and disease. Stress during development can result in long term health challenges such as cardiovascular, metabolic and neurological diseases. A critical component in stress response is glucocorticoids. However excess or premature glucocorticoid exposure may also contribute to disease vulnerability. The role of epigenetic modifications in modulating adult diseases is still being investigated.

Glucocorticoid and the Glucocorticoid Receptor during Development

Glucocorticoids (GCs) are a class of steroid hormones produced in the zona fasciculate of the adrenal cortex, which functions during stress response. The glucocorticoid receptor (GR) is a member of the super family of the ligand activated nuclear receptors, encoded by the NR3C1 gene. Both the human (cortisol) and rodents (corticosterone) glucocorticoids are derived from cholesterol and the synthesis is regulated by the hypothalamic-pituitary- adrenal axis (HPA).

During development GCs are vital for functional and structural organ maturation. This is especially true for the lungs, in which GCs stimulate surfactant production in late gestation (Bolt, van Weissenbruch et al. 2001, Manwani, Gagnon et al. 2010). However, the timing of exposure is important to health outcome, and hence requires strict regulation. Too frequent or excess exposure is associated with negative implications on the brain including delayed maturation of astrocytes (Huang, Harper et al. 2001), and altered glucocorticoid receptor expression (Okret, Poellinger et al. 1986). Studies also reported that fetal glucocorticoid exposure adversely impacts growth and development (Mulder, Robles de Medina et al. 2002). For instance, elevated cortisol is associated with growth restriction in the fetus (Goland, Jozak et al. 1993). Also, glucocorticoid exposure is implicated in altered HPA axis sensitivity and altered stress response capability (Welberg and Seckl 2001).

Several preventative mechanisms exist to minimize untimely exposure during the fetal development such as corticosteroid-binding globulin and the enzyme 11β-hydroxysteroid dehydrogenase-2 (11β-HSD2). These mechanisms ensure glucocorticoid availability is regulated both in the circulation and locally. Glucocorticoids are lipophilic

and can easily cross the placenta and other biological membranes. To prevent circulating glucocorticoids from entering fetal circulation the enzyme 11β-hydroxysteroid-dehydrogenase 2 (11β-HSD2) catalyzes cortisol and corticosterone to the biologically inert 11-ketometabolites cortisone and 11-dehydrocorticosterone, respectively (Seckl 1997). Synthetic glucocorticoids such as dexamethasone are poor substrates of 11β-HSD2, which allows them to bypass the protective border of the placenta and enter fetal circulation. Once in the circulation glucocorticoids, both natural and synthetic, bind to the glucocorticoid receptor (GR) in the target organs/tissues where they are able to induce genomic effects such as transactivation and transrepression (Beato, Herrlich et al. 1995).

The glucocorticoid receptor pathway is shown in figure 1.2. GRs are ubiquitously expressed and the magnitude and nature of response are organ specific (Oakley and Cidlowski 2011). When unbound to a ligand the GR is found in the cytoplasm as a multiprotein complex bound to HSP90 and other chaperone proteins (Pratt and Toft 1997, Grad and Picard 2007). Upon ligand activation the GR undergoes conformational changes exposing the nuclear localization signal. The GR then translocates to the nucleus where it forms a homodimer that can then bind the glucocorticoid response element (GRE) to exert a genomic effect.

GREs are regions containing a pair of imperfect palindrome sequences separated by three nucleotides. Upon GR binding to the GRE coactivators are recruited along with other factors, which initiates transcription (Lonard and O'Malley B 2007). In terms of transrepression binding to negative GRE or nGREs are responsible for recruiting corepressors along with histone deacetylases (HDACs) (Newton and Holden 2007, Kadmiel and Cidlowski 2013), making DNA inaccessible for transcription.

The glucocorticoid receptor has several domains including a DNA binding domain, a ligand-binding domain and two domains involved in transactivation. The GR (NR3C1) gene contains 9 exons of which only exons 2-9 codes the GR protein. In humans, alternate splicing of these exons gives rise to two isoforms of the GR, hGRα and hGRβ (human GR α and β, respectively) differing only in the C-terminus. Yet, hGRβ antagonizes and represses the hGRα isoform, providing another means of regulation (Bamberger, Bamberger et al. 1995, Charmandari, Chrousos et al. 2005).

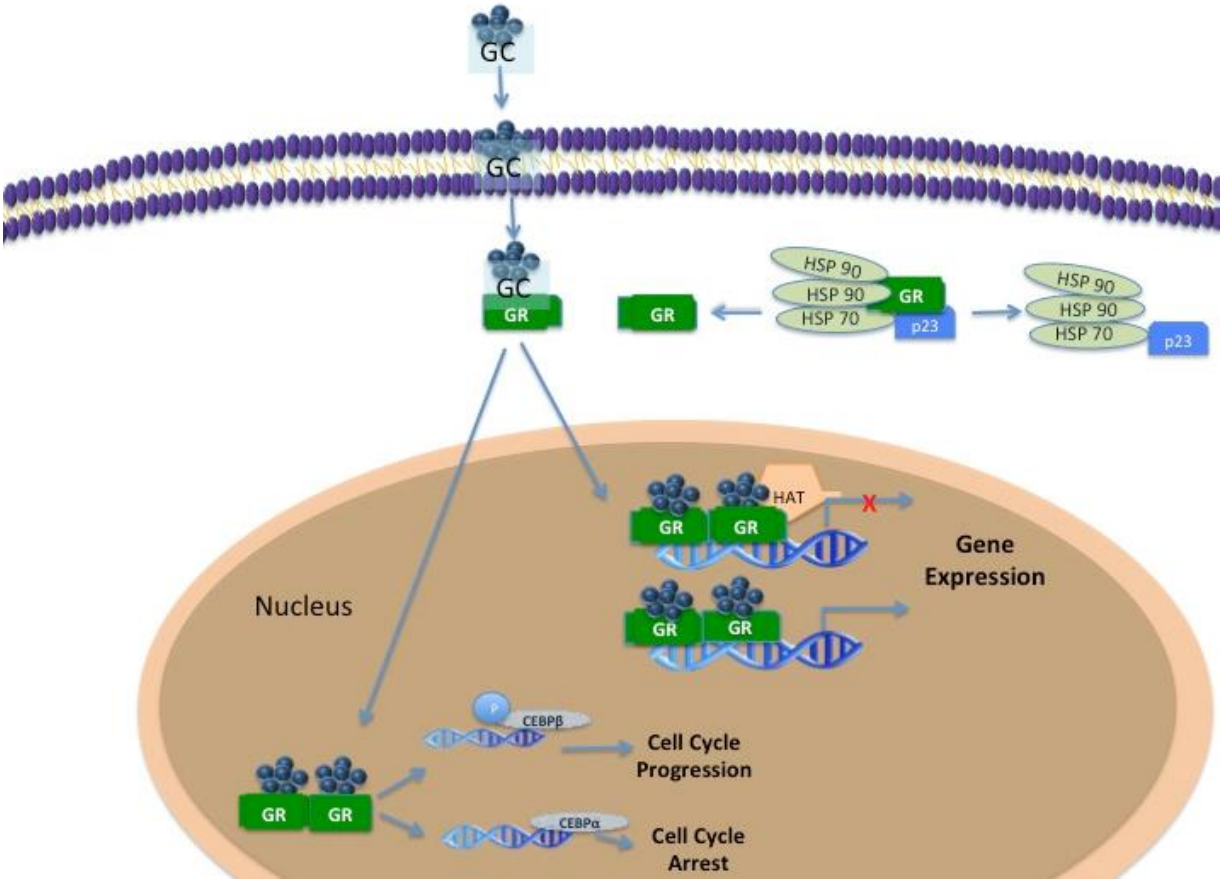

Figure 1.2. Glucocorticoid receptor pathway. Glucocorticoids enter the cell and bind to the glucocorticoid receptor. When unbound to a ligand the glucocorticoid receptor is in a multi-protein complex. Once bound the ligand displaces the multiprotein complex, binds the glucocorticoid receptor and enters the nucleus from the cytosol. In the nucleus a homodimer is formed which is able to bind to the glucocorticoid response element (GRE) of DNA. The ligand-receptor complex binds the GRE and induces genomic effects such as transactivation or transrepression. Glucocorticoids are also able to induce cell cycle progression or arrest.
GC-glucocorticoid, GR-glucocorticoid receptor, HSP- heat shock protein, HAT- histone acetyl transferase, CEBPβ-CCAAT/enhancer-binging protein beta, and CEBPα-CCAAT/enhancer-binding protein alpha.

Glucocorticoid Treatment and Cardiovascular Disease

The heart is influenced by glucocorticoids both directly and indirectly. Perinatal glucocorticoid exposure has been shown to alter cardiac structure and function. Such changes include hemodynamic modifications and dysregulation of the HPA axis, resulting in abnormal endogenous glucocorticoid expression, and blood pressure.

Premature glucocorticoid exposure permanently alters the HPA axis resulting in aberrant circulating endogenous glucocorticoids. For instance human infants born to high anxiety-mothers are reported to have dysregulated cortisol levels during adolescence (Van den Bergh, Van Calster et al. 2008). In the same study, the exposure during week 12-22 of pregnancy was especially consequential, resulting in abnormal cortisol production in offspring (Van den Bergh, Van Calster et al. 2008). Similar results were reported in guinea pigs (McCabe, Marash et al. 2001). Maternal administration of glucocorticoids during pregnancy reduced circulating cortisol levels (McCabe, Marash et al. 2001). Additionally, *in utero* glucocorticoid exposure hindered growth, resulting in smaller animals at birth (McCabe, Marash et al. 2001). While controversial, a correlation between birth weight and adult cortisol levels has been observed, that is, smaller babies at birth have higher fasting cortisol levels (Phillips, Walker et al. 2000). These results seem to be species specific and may reflect experimental differences such as time of measurement. Nonetheless, it is apparent that a relationship exists between glucocorticoid exposure and stress response, on the growth and HPA axis function.

Dexamethasone exposure is also associated with changes in expression of essential proteins. The hippocampus of dexamethasone treated animals expressed higher mRNA levels of 11β HSD-1(Shoener, Baig et al. 2006), an enzyme that converts the

inactive glucocorticoid into the active form. The increase in 11β HSD-1 would therefore increase the bioavailability of circulating glucocorticoids, though potentially harmful.

GCs also influences blood pressure. O'Regan and colleagues determined prenatal dexamethasone elevated systolic blood pressure in a sex specific manner, with females having higher blood pressures than their male counterparts (O'Regan, Kenyon et al. 2004). Phillips *et. al.* observed a direct correlation between circulating cortisol levels and systolic blood pressure elevation (Phillips, Walker et al. 2000). This is supported by the cortisol induced hypertension characteristic in Cushing syndrome patients (Whitworth, Brown et al. 1995, Whitworth, Mangos et al. 2000).

In the heart, animals exposed to glucocorticoids during the neonatal phase had major histopathological changes during adulthood (Bal, de Vries et al. 2008, Niu, Herrera et al. 2013). A 3-day neonatal tapered dose of dexamethasone in rodents was used to model the physiological effects on the heart (Bal, de Vries et al. 2008, Niu, Herrera et al. 2013). Dexamethasone treatment resulted in increased cardiomyocyte hypertrophy, collagen deposition and suppression of proliferation (de Vries, Bal et al. 2006, Bal, de Vries et al. 2009). These findings were not without functional consequences, Bal *et. al.* reports a decrease in systolic function as a result of dexamethasone treatment, characterized by reduced ejection fraction (Bal, de Vries et al. 2008). In a similar study dexamethasone exposure resulted in an impaired ability to respond to cardiac challenges later in life (Niu, Herrera et al. 2013). Taken together these studies indicate an important link between glucocorticoid exposure and cardiac function.

Cardiomyocyte Development and 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, Bhardwaj 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 (Clubb and Bishop 1984, Burrell, Boyn et al. 2003, Ahuja, Sdek et al. 2007). 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. 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 (Figure 1.3). The uncoupling of cytokinesis from karyokinesis and ultimate exit of the cell cycle characterize the transition, resulting in binucleation (Li, Wang et al. 1996). Subsequent increases in heart size are independent of proliferation and the result of increases in individual cell size termed hypertrophy.

In humans, the fetal heart consists of mainly mononucleate cardiomyocytes and thus is the time point in which most proliferation occurs. Just before birth, binucleation begins and can extend into early neonatal life. 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 (Li, Wang 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, Wang 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, Wang et al. 2012). Rodents and sheep, on the other hand, have approximately 90 percent of the cardiomyocyte population binucleated (Botting, Wang et al. 2012).

In rats, cardiomyocyte development is only completed following birth. After birth, proliferation is still robust but slowly begins to decline at neonatal day 4 until day 14, when it ceases. During this time binucleation increases until it reaches its peak at day 14. The neonatal rat therefore provides an adequate model of cardiomyocyte development and represents a near term human heart. While a neonatal rat model of human prematurity is a good model of the heart it does have limitations. For instance, preterm infants are born before partition, where considerable biological changes occur (Gibbs, Romero et al. 1992). One of which is the natural glucocorticoid surge, which stimulates organ maturation. Glucocorticoids also stimulate the downregulation of 11β-HSD2 allowing the biologically active glucocorticoids to be readily available.

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 and 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).

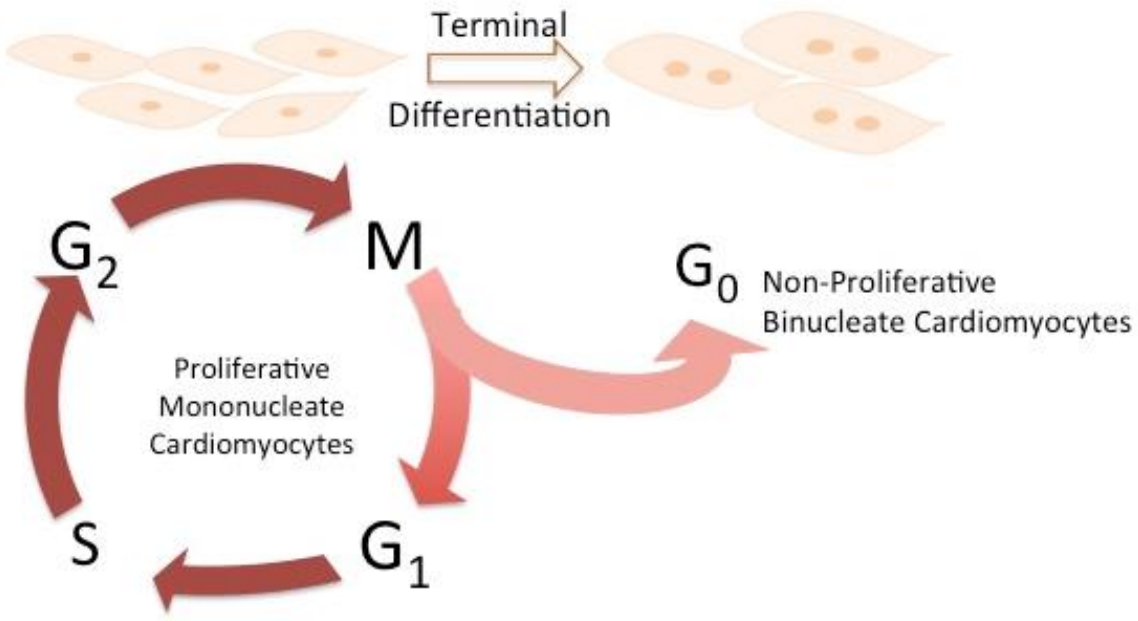

Figure 1.3. Cardiomyocyte terminal differentiation. Cardiomyocyte growth occurs during two phases, hyperplasic and hypertrophic phase. During hyperplasia cardiomyocytes undergo several rounds of replication and cell division. Soon after birth myocytes undergo one final round of cell division and karyokinesis occurs without cytokinesis, resulting in a binucleate terminally differentiated cell. Subsequent growth is by increase in cell size, without changing number of cardiomyocytes, termed hypertrophy.

Glucocorticoids and Cardiomyocyte Terminal Differentiation

The intrauterine environment is highly influential on the health of an individual. Its influence can lead to structural and functional adaptations of several organs, including the heart. Persistence of these adaptations can increase vulnerability to diseases later in life, including metabolic syndrome and cardiovascular disease (Barker 1990, Barker 2004, Botting, Wang et al. 2012). Altered cardiomyocyte number may be responsible for this increased susceptibility. In support, animal studies provide evidence that fetal stress caused by hypoxia (Bae, Xiao et al. 2003), glucocorticoids (Giraud, Louey et al. 2006) or maternal malnutrition (Corstius, Zimanyi et al. 2005, Bubb, Cock et al. 2007) affects both the number of cardiomyocytes and the ability of the heart to cope with stress later in life.

Evidence exists for a role of glucocorticoids in regulating the cardiomyocyte development. Early studies by Rudolph *et al.* reported a reduction in cardiomyocyte proliferation after fetal sheep cortisol infusion, associated with hypertrophic growth (Rudolph, Roman et al. 1999). However, more recently, studies in fetal sheep revealed increased proliferation without an increase in cardiomyocyte size after cortisol infusion (Giraud, Louey et al. 2006). In this latter study no differences in length, width, and overall percent binucleation of cardiomyocytes were observed between cortisol-treated and non-treated groups. In addition, the cortisol treatment did not drive the maturation of cardiomyocytes but rather stimulated their entry into the cell cycle suggesting cortisol is associated with hyperplastic growth.

In the fetal rat, low-dose dexamethasone, was found to decrease fetal body weight when administered prenatally by Torres *et al.* (Torres, Belser et al. 1997). In this study the dexamethasone-treated group was found to have increased cardiomyocyte

proliferation in the fetal heart as compared to control. In addition, a sex-dependent component of cardiomyocyte proliferation was observed, with females having significantly more DNA synthesis as compared to males. Taken together, these findings provide evidence for premature glucocorticoid exposure associated with a developmental delay of the heart maturation.

In neonates dexamethasone treatment has been found to decrease total cardiomyocyte number (de Vries, Bal et al. 2006) possibly by decreasing proliferation (de Vries, Bal et al. 2006, Bal, de Vries et al. 2009). De Vries *et al.* reported reduced proliferation during neonatal day 2-4 with no subsequent changes. This study also noted no changes in the level of apoptosis, supporting suppressed proliferation as the cause of lower cardiomyocyte number. These reductions in cardiomyocyte number were noted to continue into adulthood, associated with reduced systolic function (Bal, de Vries et al. 2008). It is evident that the effect of glucocorticoid treatment is dependent on the time of exposure. Fetal exposure to dexamethasone results in increased cardiomyocyte proliferation while neonatal exposure has an opposite effect. This provides evidence for a time-dependent mechanism of glucocorticoid action, highlighting the importance of monitoring perinatal circulating glucocorticoid levels due to the diverse impact on the heart development.

Cell Cycle Regulation During 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 hormones.

Cell cycle regulators are differentially expressed within the mononucleate versus binucleate state. Cardiomyocytes exit the cell cycle as they become binucleated and as such are terminally differentiated. Cell cycle phases include gap phase 1 (G1), synthesis phase (S), gap phase 2 (G2), and mitosis (M). The G0 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 et al. 1998). During the fetal development, CDKs are highly expressed within the heart and become down-regulated in adulthood. Conversely, the negative regulators of cell cycle, such as CDKIs, are then up-regulated in the adult heart (Pasumarthi and 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 by (Brooks, Poolman 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 et al. 2011).

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 et al.

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 (Liu, Yue et al. 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 (Liu, Yue 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) (Li, Wang et al. 1996). A recent study identified a potential candidate regulator involved in this process, FAK-related non-kinase (FRNK) (O'Neill, Mack et al. 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, Mack 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 (Xin, Kim et al. 2011, von Gise, Lin et al. 2012). 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, Lin et al. 2012).

It is evident that considerable research has been done on cell cycle regulation and terminal differentiation but much is still necessary. While pathways and mechanisms have been proposed conclusive studies are yet to be established.

Epigenetic Regulation of Terminal Differentiation

Epigenetic modifications refer to changes in the expression of genes independent of the DNA sequence. Changes in the intrauterine environment are associated with long-term adverse effects, known as fetal programming (Barker 2004). 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, Alexander 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 et al. 2010, Stein, Jones et al. 2011), however the specific details of this regulation of cardiomyocyte maturation are lacking.

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, Lau et al. 2010), the same time frame 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, while 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 may be associated with methylation-induced suppression of proliferation.

Normal development of the heart is characterized by a transition from mononucleate to binucleate cardiomyocytes. This conversion is associated with a decrease in the proliferative capacity of the heart, as terminal differentiation

occurs. Cardiomyocyte number in the adult heart is therefore determined at an early developmental stage; demonstrating the importance of protecting cardiomyocyte number in fetal and neonatal developmental period to optimize cardiac function. Stimuli such as glucocorticoids may alter cardiomyocyte number and has been shown to act by changing the timing and percentages of proliferation and/or binucleation (de Vries, Bal et al. 2006, Bal, de Vries et al. 2008, Bal, de Vries et al. 2009). The mechanisms by which these changes occur may involve cell cycle molecules and may be induced by epigenetic cues such as DNA methylation.

Central Hypothesis

The central hypothesis of this project is that dexamethasone exposure in newborn rats induces epigenetic repression of cyclin D2 gene in cardiomyocytes resulting in premature cardiomyocyte terminal differentiation and a reduction in myocyte endowment in the heart.

Significance

Each year 1 out of 9 babies are born preterm in the United States. Preterm infants have a risk of complications generally due to prematurity. One such complication is respiratory distress syndrome due to immature lungs. Dexamethasone aids in maturation of the lungs and surfactant production (Jobe 2010) however, off target effects of dexamethasone on vital organs have also been reported (Dodic, Abouantoun et al. 2002, Chang, Yeh et al. 2013). Conclusive studies in humans and animals report neonatal glucocorticoid exposure increases the risk of long-term health consequences such as diabetes, cardiovascular

disease and premature death (Ng and Celermajer 2004, O'Regan, Kenyon et al. 2004, Walker 2007). Yet, dexamethasone treatment is still the gold standard intervention for preterm infants. Glucocorticoid exposure seems to be an important physiological mediator during stress response (Raff, Hong et al. 2003). It has been observed that the timing of glucocorticoid exposure can have detrimental consequences on the developmental trajectory and growth of the organism (Lumbers, Boyce et al. 2005, Bal, de Vries et al. 2009, Feng, Reini et al. 2013). However, a mechanism of dexamethasone action in long-term programming has not yet been delineated. Cardiomyocyte proliferation is limited and occurs only during a narrow developmental window (Li, Wang et al. 1996, Poolman and Brooks 1998). Environmental factors that compromise the proliferative period may result in few cardiomyocytes in the adult heart. This is not without consequences, as myocytes are the functional unit of the heart, without adequate numbers the heart is vulnerable to subsequent stresses later in life. It is therefore possible that premature terminal differentiation is a mechanism, which contributes to risk of cardiovascular disease. Glucocorticoids are known to alter the cell cycle in multiple cell types. Since terminal differentiation is tightly associated with cell cycle withdrawal this presents a plausible mechanism (Rogatsky, Trowbridge et al. 1997, Smith, Redman et al. 2000). We expect the findings in this study to reveal dexamethasone exposure results in premature cardiomyocyte terminal differentiation. Additionally, considering the long-term consequences of antenatal dexamethasone on adult health epigenetic mechanisms may provide insight on this programming phenomenon at the molecular level.

CHAPTER TWO

DEXAMETHASONE TREATMENT OF NEWBORN RATS DECREASE CARDIOMYOCYTE ENDOWMENT IN THE DEVELOPING HEART THROUGH EPIGENETIC MODIFICATIONS

By

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This paper has been published by PLoS One: 25923220, 2015

Abstract

The potential adverse effect of synthetic glucocorticoid, dexamethasone therapy on the developing heart remains unknown. The present study investigated the effects of dexamethasone on cardiomyocyte proliferation and binucleation in the developing heart of newborn rats and evaluated DNA methylation as a potential mechanism. Dexamethasone was administered intraperitoneally in a three day tapered dose on postnatal day 1 (P1), 2 and 3 to rat pups in the absence or presence of a glucocorticoid receptor antagonist Ru486, given 30 minutes prior to dexamethasone. Cardiomyocytes from P4, P7 or P14 animals were analyzed for proliferation, binucleation and cell number. Dexamethasone treatment significantly increased the percentage of binucleated cardiomyocytes in the hearts of P4 pups, decreased myocyte proliferation in P4 and P7 pups, reduced cardiomyocyte number and increased the heart to body weight ratio in P14 pups. Ru486 abrogated the effects of dexamethasone. In addition, 5-aza-2'-deoxycytidine (5-AZA) blocked the effects of dexamethasone on binucleation in P4 animals and proliferation at P7, leading to recovered cardiomyocyte number in P14 hearts. 5-AZA alone promoted cardiomyocyte proliferation at P7 and resulted in a higher number of cardiomyocytes in P14 hearts. Dexamethasone significantly decreased cyclin D2, but not p27 expression in P4 hearts. 5-AZA inhibited global DNA methylation and blocked dexamethasone-mediated down-regulation of cyclin D2 in the heart of P4 pups. The findings suggest that dexamethasone acting on glucocorticoid receptors inhibits proliferation and stimulates premature terminal differentiation of cardiomyocytes in the developing heart *via* increased DNA methylation in a gene specific manner.

Introduction

The synthesized glucocorticoid dexamethasone is routinely administered to pregnant women at high risk of preterm delivery or to preterm infants to reduce the incidence and severity of respiratory distress syndrome (Garland, Alex et al. 1999, Jobe 2010). Despite this beneficial effect of dexamethasone treatment, possible long-term adverse effects, including altered cardiovascular and neurological function, have been observed later in life in the exposed individual (Barrington 2001, Ortiz, Quan et al. 2003, Kamphuis, de Vries et al. 2007, Bal, de Vries et al. 2008). With regard to the cardiovascular system, negative effects of perinatal glucocorticoid exposure on the development of organs such as the heart and kidney have been noted, including premature death, hypertrophic cardiomyopathy and hypertension (Werner, Sicard et al. 1992, Ortiz, Quan et al. 2003, Kamphuis, de Vries et al. 2007, Bal, de Vries et al. 2008).

Cardiomyocyte terminal differentiation occurs in late fetal development and around birth, and this crucial period of terminal differentiation dictates cardiomyocyte endowment in the heart for life (Paradis, Gay et al. 2013). In humans, cardiomyocytes in term newborn hearts are largely terminal differentiated and the heart grows by enlargement of cardiomyocytes but not proliferation during the postnatal developmental period. In rodents, cardiomyocyte terminal differentiation starts and continues within the first two weeks of postnatal life (Clubb and Bishop 1984, Li, Wang et al. 1996), which is an equivalent timeframe in the heart development to the late fetal stage in third trimester of human gestation (Ahuja, Sdek et al. 2007). Thus, neonatal rats provide a reasonable animal model to study the effect of glucocorticoid treatment on preterm infants at the critical window of the heart development. Glucocorticoids are established cell cycle

regulators known to repress the cell cycle, and the glucocorticoid receptor is believed to be the predominant mediators of this effect (Smith, Redman et al. 2000, Crochemore, Michaelidis et al. 2002). However, the mechanisms remain largely unknown.

Epigenetic regulation plays an important role during the development. Strong evidence suggests that dynamic DNA methylation orchestrates cardiomyocyte development, maturation and disease (Patterson, Chen et al. 2010, Gilsbach, Preissl et al. 2014). It has been shown that dexamethasone has differential effects in the regulation of DNA methylation, which is organ- as well as gene-dependent (Crudo, Petropoulos et al. 2012, Crudo, Petropoulos et al. 2013). Thus, the present study sought to determine the effect of dexamethasone treatment in newborn rats on the developing heart and the mechanism of DNA methylation. The hearts were studied at three postnatal development ages of neonatal rats, day 4 (the beginning of binucleation), day 7, and day 14 (the maximum of binucleation). Day 14 represents a mature heart in terms of cardiomyocyte binucleation and terminal differentiation of the heart. The heart to body weight ratio, binucleation, proliferation, cardiomyocyte number, cell size and protein abundance of cyclin D2 and p27 were analyzed.

Materials and Methods

Experimental Animals

Time-dated pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI). After birth on postnatal day 1 (P1), pups were randomly divided into two groups: 1) saline control, and 2) dexamethasone groups. Newborn rats were treated with tapered doses of dexamethasone (Sigma, 0.5, 0.3, 0.1 mg/kg on P1, P2,

and P3 pups, respectively) in the absence or presence of Ru486 (Sigma, 25 mg/kg, P1) . Dexamethasone was administered by intraperitoneal injection, and Ru486 was given 30 minutes prior to dexamethasone. Some animals were treated with 5-aza-2'-deoxycytidine (5-AZA, 3 mg/kg) on P1, P2, and P3 through intraperitoneal injection, followed by either saline or dexamethasone (0.5, 0.3, 0.1 mg/kg on P1, P2, and P3, respectively). After treatments, animals were anesthetized using isoflurane and hearts were removed for analyses in P4, P7, or P14 pups. All procedures and protocols in the present study were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines by US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Primary Cardiomyocyte Isolation and Culture.

Cardiomyocytes were isolated from neonatal rat hearts by enzymatic (0.1% trypsin and 0.5 mg/ml type II collagenase) digestion as previously described (Xiao, He et al. 2000). Cells were cultured in Hyclone Medium 199 (Thermo Scientific) containing 10% fetal bovine serum (Gemini Bio-Products) and 1% antibiotics (10,000 I.U./mL penicillin, 10,000 μg/mL streptomycin) at 37 ºC in 95% air/5% CO_2.

Immunocytochemistry

Primary cardiomyocytes were double stained with α-actinin, a cardiomyocyte marker, and Ki-67, a proliferation marker. Cell proliferation was also examined by 5-bromo-2-deoxyuridine (BrdU) incorporation (Patterson, Chen et al. 2010). Briefly, cardiomyocytes isolated from P7 pups were plated on coverslips and allowed 24 hours

for attachment. Culture media was then replaced with media containing BrdU (Sigma, 5 μM) for 24 hours. Cardiomyocytes plated on coverslips were fixed with 3.7% paraformaldehyde and permeabilized with 0.5% Triton-X100. The cells were blocked with 1% bovine serum albumin for 1 hour at room temperature before incubation with primary antibodies: mouse anti-α-actinin (Sigma) (1:200), rabbit anti-Ki-67 (Abcam, Cambridge, MA) (1:100), or rabbit anti-BrdU (Abcam) (1:100) at 4 ºC overnight. Samples were incubated with the secondary antibodies: anti-rabbit AlexaFluor 647 conjugated (1:400; Life Tech.) and anti-mouse AlexaFlour 488 conjugated (1:400; Life Tech.) for 1 hour at room temperature. Nuclei were stained with Hoechst (Sigma) for 1 minute. The immunofluorescence staining was assessed using a Zeiss Axio Imager. All microscope and quantitative analysis was carried out using Image J software.

Cell Number Counting

Cardiomyocytes were counted using a hemocytometer. To correct for absolute cell number, cardiomyocyte purity was factored in. Estimates of cardiomyocyte purity were generated using the percent of cardiomyocytes stained with α-actinin from immunocytochemistry results. The hemocytometer values were multiplied by cardiomyocytes fraction calculated using immunocytochemistry, resulting in cardiomyocyte number. This value is expressed as cell number per gram heart weight to account for variations in heart size.

Western Immunoblotting

Hearts of P4 pups 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 10% 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 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 Hyperfilm. To assure equal loading and minimize variability among gels, samples were normalized to GAPDH.

5-mC DNA ELISA.

DNA methylation in hearts on P4 and P7 pups 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 hearts 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

Each experimental group contains a minimum of 4 animals. Data are expressed as mean ± SEM obtained from the number of experimental animals given (*n*). 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

Dexamethasone Affects Heart Development in Neonatal Rats

Newborn pups of P1 to P3 were treated with tapered dose of dexamethasone or saline. At P4, P7 and P14, heart weight, body weight and the heart to body weight ratio were evaluated. As shown in Figure 1, in the saline control animals, heart and body weight increased as pups grew, albeit the heart to body weight ratio stayed relatively constant. The dexamethasone treatment increased heart weight in P7 and P14 pups (Fig. 2.1A), but body weight only at P7 pups (Fig. 2.1B), resulting in a significant increase in the heart to body ratio in P14 pups (Fig. 2.1C). To determine the role of glucocorticoid receptor (GR) in the dexamethasone-mediated effects, Ru486 was administered 30 minutes prior to dexamethasone. While Ru486 treatment alone had no significant effects on heart weight, body weight or heart to body weight ratio in P4 and P7 pups, it caused a significant decrease in both heart and body weight in P14 pups (Fig. 2.1). In the presence

of Ru486, the effects of dexamethasone were abrogated (Fig. 2.1), suggesting a GR-mediated effect of dexamethasone.

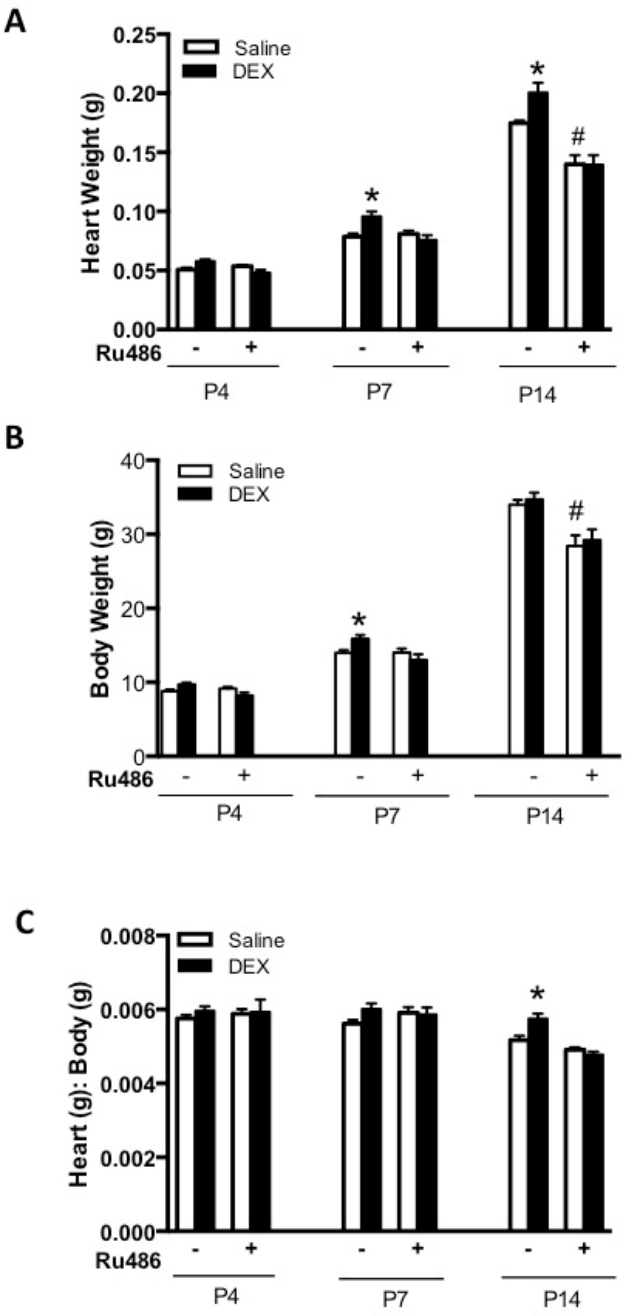

Figure 2.1. Effect of dexamethasone (DEX) on heart development in neonatal rats. Newborn rats were treated with tapered dose of DEX in the absence or presence of Ru486 during the first three days of postnatal life. Ru486 was administered 30 minutes prior to the DEX treatment. Heart and body weights were determined in day 4 (P4), day 7 (P7) and day 14 (P14) neonatal rats. Data are mean ± SEM, n = 10-21. * $p<0.05$, DEX *vs*. Saline; # $p<0.05$, +Ru486 *vs*. -Ru486.

Dexamethasone Induces Premature Cardiomyocyte Binucleation and Suppresses Proliferation

Binucleation is one of the characteristics of cardiomyocyte maturation and terminal differentiation. Thus, we determined whether dexamethasone treatment influenced cardiomyocyte binucleation in the developing heart. Cardiomyocytes isolated from P4 and P7 pups were stained with a cardiomyocyte marker α-actinin and nuclei were labeled with Hoechst. The percentage of binucleation was scored by counting the number of mononucleated and binucleated cells using microscopy. As shown in Figure 2.2, in control animals, there was a development-dependent increase in percent binucleated cells in the hearts of P4 and P7 pups. The dexamethasone treatment resulted in a significant increase of percent binucleated cells in the hearts of early developmental age of P4 pups, which was blocked by Ru486 (Fig. 2.2). Ru486 treatment alone, while it had no significant effect on cardiomyocyte binucleation in P4 pups, significantly decreased percent binucleated cells in the hearts of P7 pups (Fig. 2.2). Because binucleation of cardiomyocytes is an early indicator showing the transformation of hyperplasia to hypertrophy growth and suggesting cell destiny from proliferation to differentiation, the proliferation of cardiomyocytes was determined by double staining of α-actinin and Ki67, a marker for cellular proliferation. The dexamethasone treatment showed a tendency in decreasing percentage of Ki67-positive cardiomyocytes in P4 pups, and it significantly decreased Ki67-positive cardiomyocytes in P7 pups (Fig. 2.3A). Ru486 treatment alone had no significant effect on cardiomyocyte proliferation in either P4 or P7 pups, but abrogated the dexamethasone-induced inhibitory effect on myocyte proliferation (Fig. 2.3A). To confirm the proliferation data observed with Ki67, BrdU

incorporation was also examined in P7 cardiomyocytes. As shown in Figure 2.3B, the dexamethasone treatment significantly decreased BrdU incorporation in P7 cardiomyocytes, which was blocked by Ru486.

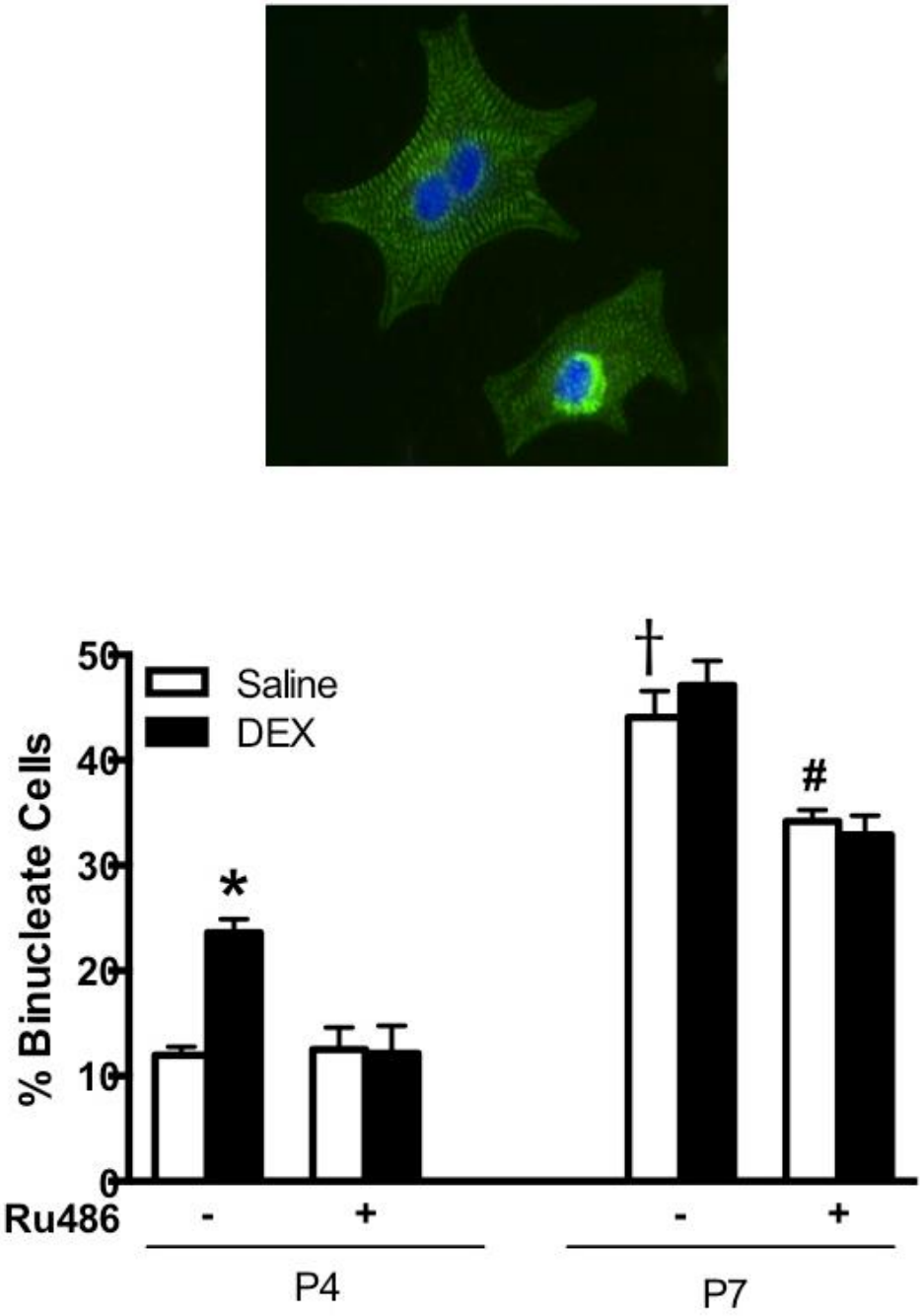

Figure 2.2. Effect of dexamethasone (DEX) on cardiomyocyte binucleation in neonatal rats. Newborn rats were treated with tapered dose of DEX in the absence or presence of Ru486 during the first three days of postnatal life. Ru486 was administered 30 minutes prior to the DEX treatment. Cardiomyocytes isolated from day 4 (P4) and day 7 (P7) neonatal hearts were stained with α-actinin, and nuclei stained with Hoechst. Representative staining of mononucleated and binucleated cells were shown in the upper panel. Data are mean ± SEM, n = 6-14. * $p<0.05$, DEX *vs*. Saline; # $p<0.05$, +Ru486 *vs*. -Ru486; † $p<0.05$, P7 *vs*. P4.

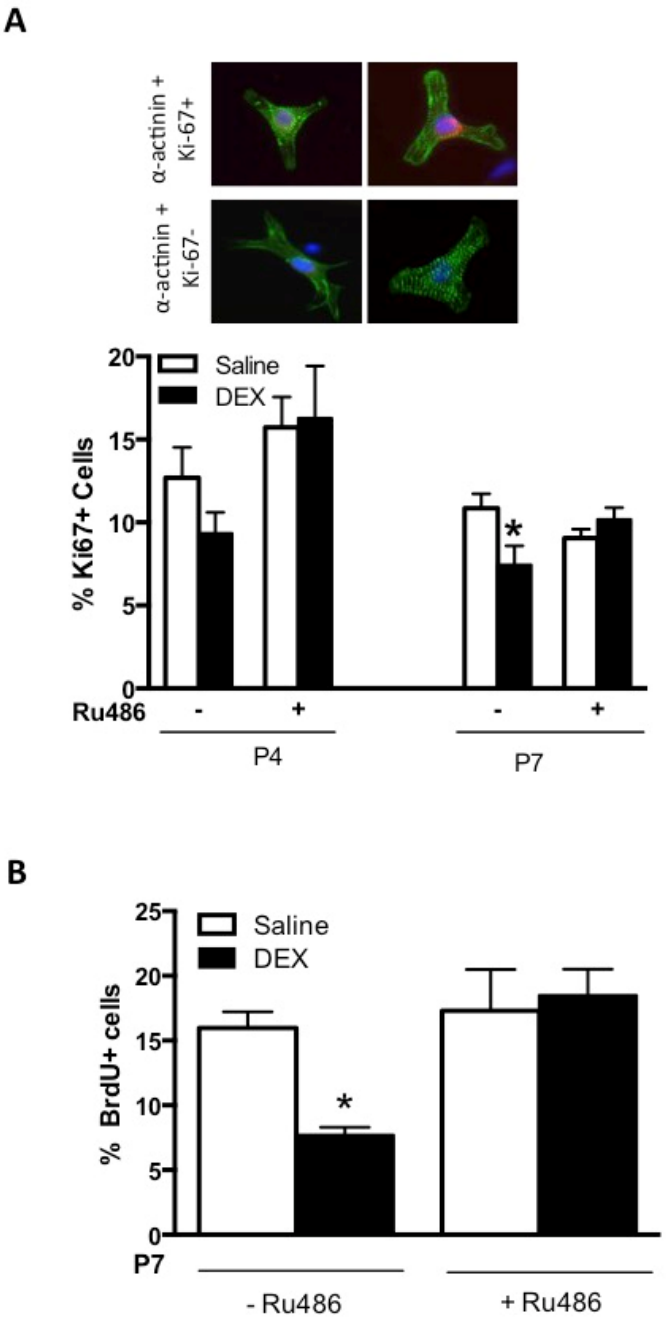

Figure 2.3. Effect of dexamethasone (DEX) on cardiomyocyte proliferation in neonatal rats. Newborn rats were treated with tapered dose of DEX in the absence or presence of Ru486 during the first three days of postnatal life. Ru486 was administered 30 minutes prior to the DEX treatment. Panel **A**: Cardiomyocytes isolated from day 4 (P4) and day 7 (P7) neonatal hearts were double stained with α-actinin and Ki67, and nuclei were stained with Hoechst. Representative staining of α-actinin and Ki67 co-localization was shown in the upper panel. Panel **B**: Cardiomyocytes isolated from P7 neonatal hearts were examined for BrdU incorporation. Data are mean ± SEM, n = 4-14. * $p<0.05$, DEX *vs*. Saline.

Dexamethasone Decreases Cardiomyocyte Endowment

Because cardiomyocytes are largely non-proliferative in the matured heart, early endowment can potentially dictate adult cardiac function. To examine whether dexamethasone treatment influences cardiomyocyte number, we counted cardiomyocytes and normalized it to heart weights for P4, P7 and P14 animals. Figure 4 shows the effect of dexamethasone on cardiomyocyte number in the developing heart. In control animals, cardiomyocyte number per heart weight increased from P4 to P7 pups, with no further increase in P14 pups. The dexamethasone treatment had no significant effect on cardiomyocyte number in P4 and P7 pups, but significantly decreased myocyte number in the heart of P14 pups (Fig. 2.4). This effect of dexamethasone was blocked by Ru486 (Fig. 2.4). We also evaluated whether dexamethasone impacted cardiomyocyte size. The mononucleate and binucleate cells were scored separately with immunocytochemistry staining. Binucleated cardiomyocytes were significantly larger than mononucleate cells, as expected. During the early developmental ages of P4 and P7 pups, neither mononucleate nor binucleate cardiomyocytes increased in cell size (data not shown). In addition, neither dexamethasone nor Ru486 had significant effects on the cell size of mononucleated or binucleated cardiomyocytes (data not shown).

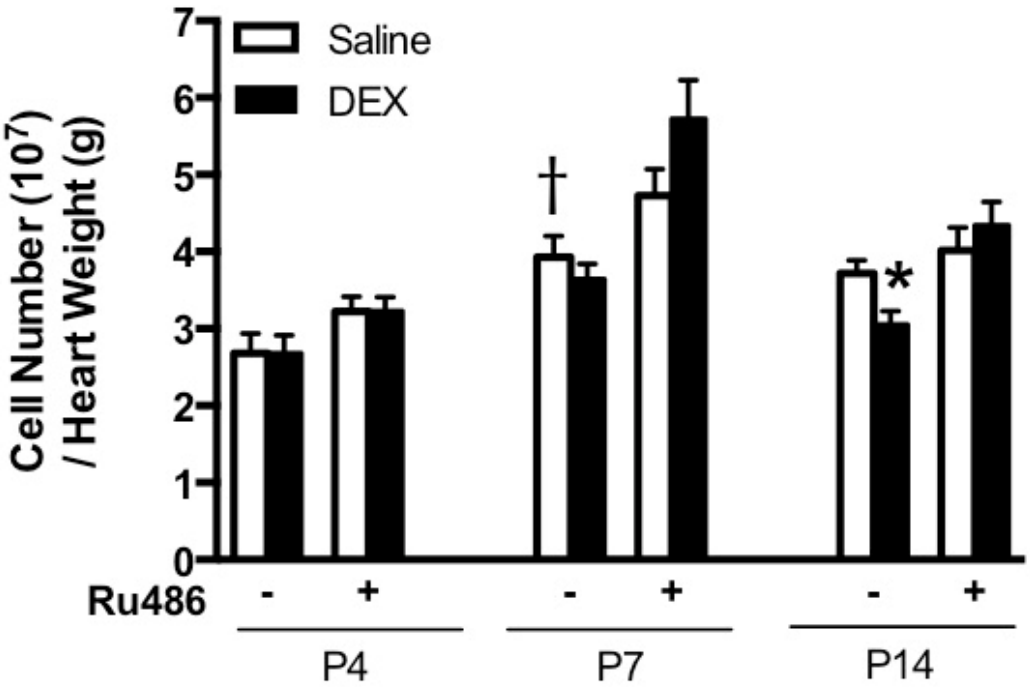

Figure 2.4. Effect of dexamethasone (DEX) on cardiomyocyte number in neonatal rats. Newborn rats were treated with tapered dose of DEX in the absence or presence of Ru486 during the first three days of postnatal life. Ru486 was administered 30 minutes prior to the DEX treatment. Cardiomyocytes isolated from day 4 (P4), day 7 (P7) and day 14 (P14) neonatal hearts were counted and normalized to per gram of heart weight. Data are mean ± SEM, n = 6-20. * $p<0.05$, DEX *vs*. Saline; † $p<0.05$, P7 *vs*. P4.

5-AZA Inhibits Dexamethasone-Induced Effects on Heart Development in Neonatal Rats

Given that epigenetic modifications play an important role in the heart development and the activation of GR has been noted to alter the methylation pattern of multiple genes, we determined whether heightened methylation played a role in dexamethasone-induced effects on the heart development in neonatal rats. The effects of dexamethasone was evaluated in the absence or presence of a methylation inhibitor 5-AZA. Although 5-AZA treatment alone had no significant effects on either heart weight or body weight in P4 and P7 pups, it caused a significant but symmetric decrease in both heart and body weight in P14 pups (Figs. 2.5A and 2.5B). Interestingly, although it had no significant effects on either heart or body weight in P7 pups, it significantly increased the heart to body ratio in P7 pups (Fig. 2.5C). Of importance, in the presence of 5-AZA, effects of dexamethasone on heart weight, body weight and the heart to body weight ration were abrogated (Fig. 2.5), suggesting DNA methylation as a critical mechanism of dexamethasone effects.

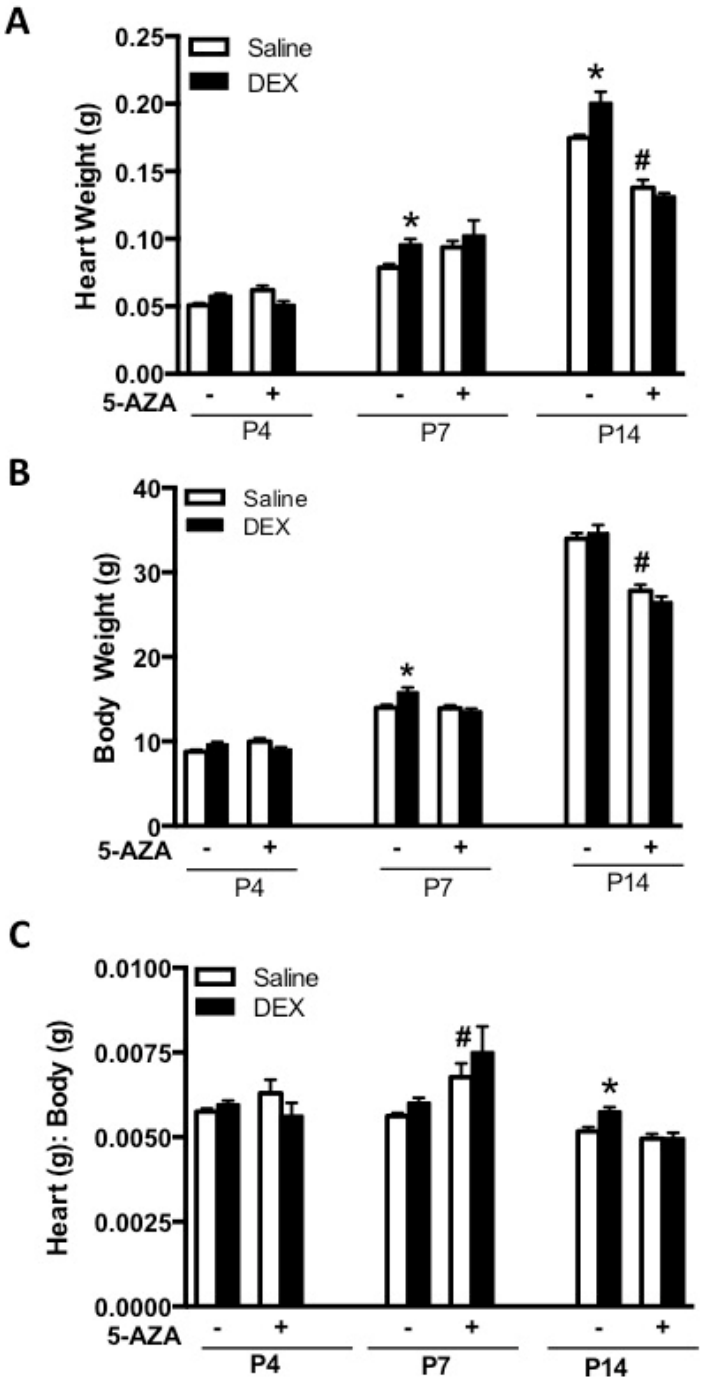

Figure 2.5. 5-AZA inhibits dexamethasone (DEX)-mediated effects on heart development in neonatal rats. Newborn rats were treated with tapered dose of DEX in the absence or presence of 5-AZA during the first three days of postnatal life. 5-AZA was administered 30 minutes prior to the DEX treatment. Heart and body weights were determined in day 4 (P4), day 7 (P7) and day 14 (P14) neonatal rats. Data are mean ± SEM, n = 5-21. * $p<0.05$, DEX *vs*. Saline; # $p<0.05$, +5-AZA *vs*. -5-AZA.

5-AZA Blocks Dexamethasone-Mediated Premature Cardiomyocyte Terminal Differentiation

The effects of 5-AZA on dexamethasone-induced premature cardiomyocyte binucleation and inhibition of proliferation were further studied. As shown in Figure 2.6A, the dexamethasone-induced decrease of Ki67-positive cardiomyocytes in P7 pups was blocked by 5-AZA. Similarly, dexamethasone-mediated suppression of BrdU incorporation was inhibited by 5-AZA (Figure 2.6B), suggesting a methylation-dependent mechanism in dexamethasone-induced decease in cardiomyoctye proliferation. Of interest, 5-AZA alone significantly increased Ki67-positive cardiomyocytes, despite the effects of dexamethasone (Fig. 2.6A). Although 5-AZA alone had no significant effect on cardiomyocyte binucleation, it inhibited the dexamethasone-induced increase of percent binucleated cells in the hearts of P4 pups (Fig. 2.6C).

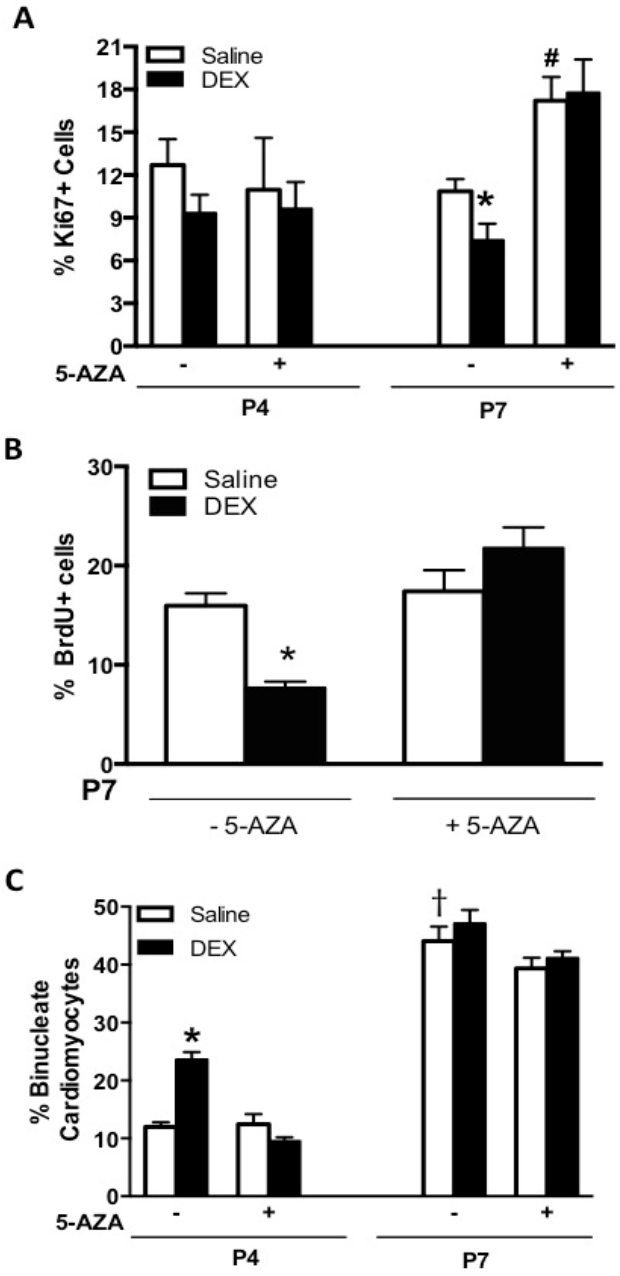

Figure 2.6. 5-AZA blocks dexamethasone (DEX)-induced effects on cardiomyocyte proliferation and binucleation in neonatal rats. Newborn rats were treated with tapered dose of DEX in the absence or presence of 5-AZA during the first three days of postnatal life. 5-AZA was administered 30 minutes prior to the DEX treatment. Panel **A**: Cardiomyocytes isolated from day 4 (P4) and day 7 (P7) neonatal hearts were double stained with α-actinin and Ki67, nuclei were stained with Hoechst. Panel **B**: Cardiomyocytes isolated from P7 neonatal hearts were examined for BrdU incorporation. Panel **C**: Cardiomyocytes isolated from P4 and P7 neonatal hearts were stained with α-actinin and Hoechst, and mononucleated and binucleated cells were determined. Data are mean ± SEM, n = 4-14. * $p<0.05$, DEX *vs*. Saline; # $p<0.05$, +5-AZA *vs*. -5-AZA; † $p<0.05$, P7

5-AZA Abrogates the Effects of Dexamethasone on Cardiomyocyte Endowment

As shown in Figure 2.7, in P4 pups, 5-AZA treatment did not influence cardiomyocyte numbers in either saline or dexamethasone group. In P7 pups, a significant reduction of cardiomyocyte number was induced by 5-AZA alone. In contrast, in P14 pups, 5-AZA treatment resulted in a significant increase of cardiomyocyte number in both saline and dexamethasone groups. Of importance, the dexamethasone-induced decrease of cardiomyocyte number in P14 pups was abrogated by 5-AZA (Fig. 2.7). These data provide novel evidence of developmental specific effect of DNA methylation on cardiomyocyte number in the critical window of the heart development. The results may be interpreted as evidence for a role of DNA methylation in regulating cardiomyocyte proliferation. Inhibition of methylation may potentially affect key regulatory proteins essential to proliferation in the developing heart.

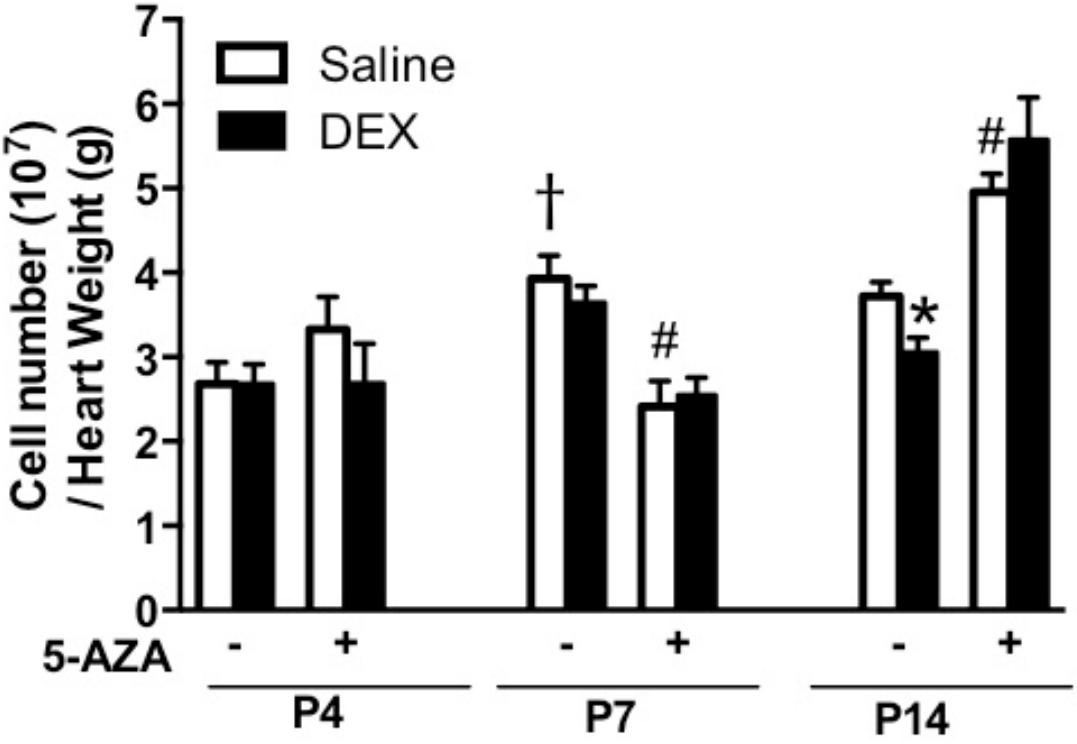

Figure 2.7. 5-AZA abrogates dexamethasone (DEX)-mediated effects on cardiomyocyte number in neonatal rats. Newborn rats were treated with tapered dose of DEX in the absence or presence of 5-AZA during the first three days of postnatal life. 5-AZA was administered 30 minutes prior to the DEX treatment. Cardiomyocytes isolated from day 4 (P4), day 7 (P7) and day 14 (P14) neonatal hearts were counted and normalized to per gram of heart weight. Data are mean ± SEM, n = 5-20. * $p<0.05$, DEX *vs*. Saline; # $p<0.05$, +5-AZA *vs*. -5-AZA; † $p<0.05$, P7 *vs*. P4.

5-AZA Inhibits Dexamethasone-Induced Down-Regulation of Cyclin D2 in the Heart

To examine potential target genes involved in dexamethasone-mediated regulation of the proliferation and binucleation of cardiomyocyte, we determined the protein abundance of cyclin D2 and p27 in the hearts of P4 pups, which play an important role in the regulation of cell cycle activity. As shown in Figure 2.8A, the dexamethasone treatment significantly decreased cyclin D2 expression in the heart, which was blocked by 5-AZA. In contrast, dexamethasone had no significant effect on p27 expression either in absence or the presence of 5-AZA (Fig. 2.8B).

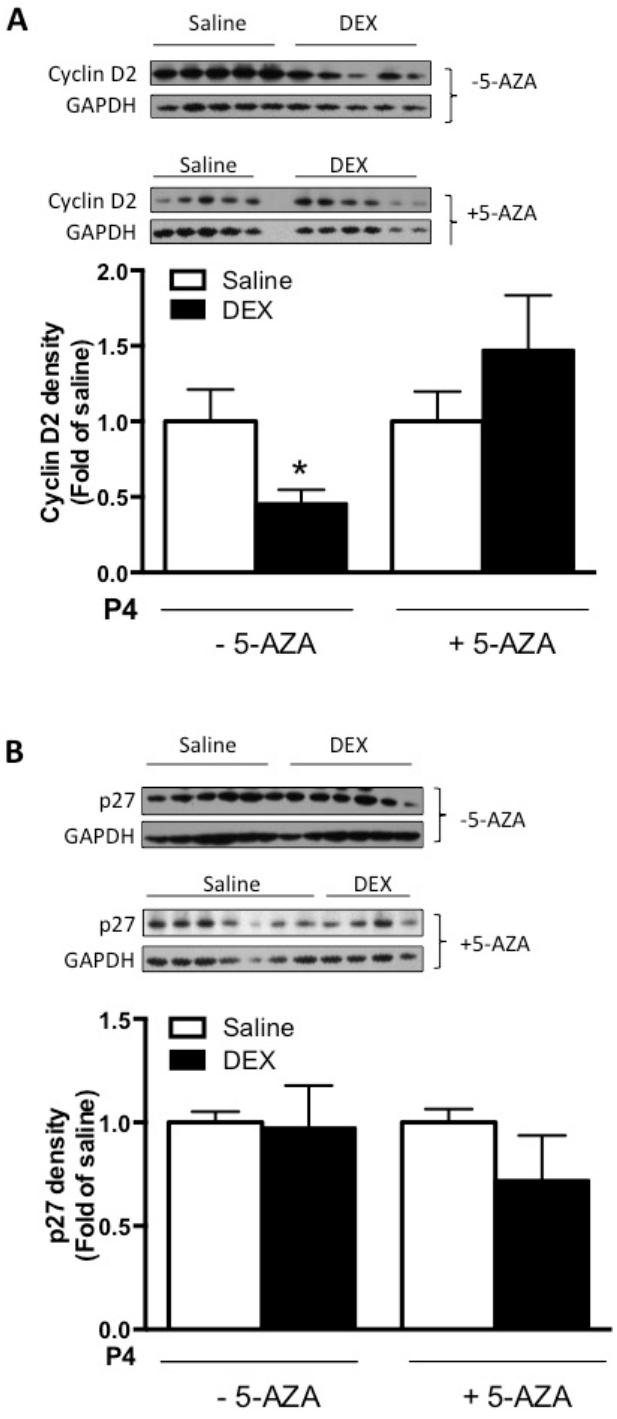

Figure 2.8. 5-AZA blocks dexamethasone (DEX)-induced down-regulation of cyclin D2 in the heart. Newborn rats were treated with tapered dose of dexamethasone (DEX) in the absence or presence of 5-AZA during the first three days of postnatal life. 5-AZA was administered 30 minutes prior to the DEX treatment. Protein was isolated from day 4 (P4) neonatal hearts and protein abundance of cyclin D2 (**A**) and p27 (**B**) was determined by Western blot. Data are mean ± SEM, n = 5-6 * $p<0.05$, DEX *vs*. Saline.

Dexamethasone does not Change Global Methylation of the Heart

Genomic DNA was extracted from the whole hearts, and a 5-methyl cytosine detection kit was used to analyze global methylation levels of DNA from each group. In saline control animals, there was a development-dependent decrease in global methylation in the heart, and DNA methylation levels were significantly reduced in P7 pup hearts as compared with P4 pups (Fig. 2.9). In P4 pups, 5-AZA significantly decreased the DNA methylation level in the heart, albeit the dexamethasone treatment had no significant effect on the level of methylated 5-methyl cytosine (Fig. 2.9). In P7 pups with reduced DNA methylation levels in the heart, 5-AZA had no further effect on the methylation level, regardless of saline or dexamethasone treatments (Fig. 2.9).

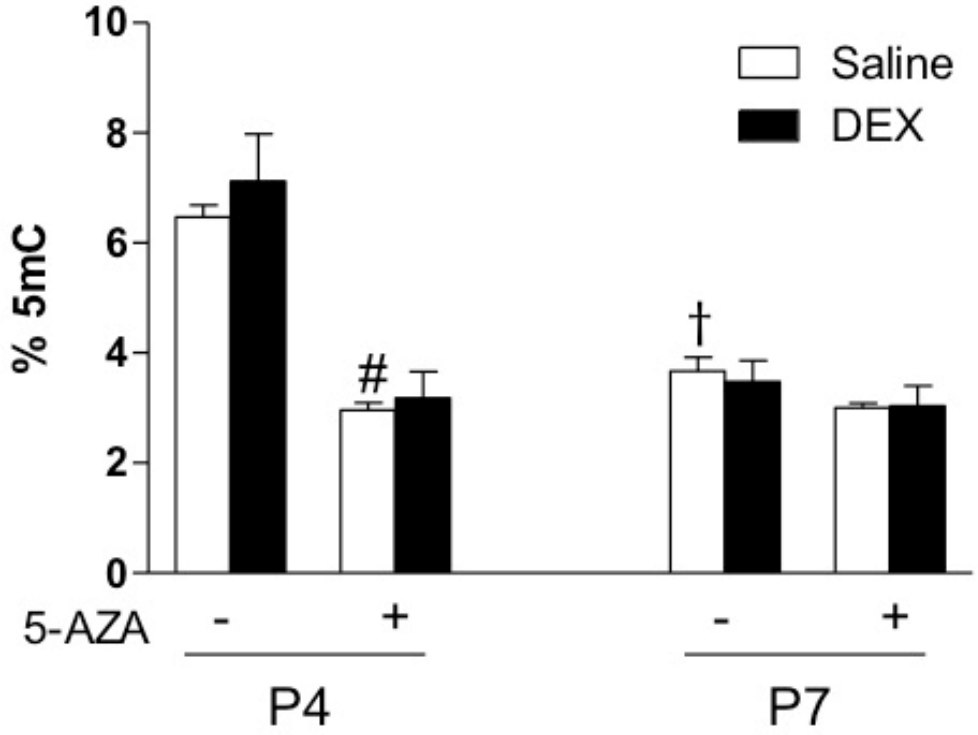

Figure 2.9. 5-AZA decreases DNA methylation levels in neonatal hearts. Newborn rats were treated with tapered dose of dexamethasone (DEX) in the absence or presence of 5-AZA during the first three days of postnatal life. 5-AZA was administered 30 minutes prior to the DEX treatment. Genomic DNA was extracted from day 4 (P4) and day 7 (P7) neonatal hearts, and methylation levels were measured using a 5-mC ELISA kit. Data are mean ± SEM, n = 5-6. # $p<0.05$, +5-AZA *vs*. -5-AZA; † $p<0.05$, P7 *vs*. P4.

Discussion

The synthetic glucocorticoid dexamethasone is commonly used to reduce the morbidity of respiratory complications in preterm infants. Yet, the potential adverse effects of dexamethasone therapy on the developing heart remain unknown. In the present study, we examined the impact of clinically relevant neonatal doses of dexamethasone on cardiomyocyte proliferation and binucleation in the developing heart. The results provided evidence of glucocorticoid-mediated stimulation of premature cardiomyocyte binucleation, inhibition of myocyte proliferation, and reduction in total cardiomyocyte number during the critical window of the heart development. We demonstrated that the dexamethasone-induced effects were abrogated by a GR antagonist Ru486, and thus revealed the GR-mediated effect on premature heart development in newborns. In addition, we provide novel evidence of a potential mechanism of DNA methylation in GR-mediated effects in the developing heart. The results provided insights in the regulation of cardiomyocyte maturation by endogenous glucocorticoids and the underlying mechanisms that may be involved.

Dexamethasone has been widely used in clinic to prevent the morbidity of chronic lung disease in preterm infants. In addition to its effect on the lung, glucocorticoids are also essential regulators of the development of other organs such as the brain and heart (La Mear, MacGilvray et al. 1997, Chang, Yeh et al. 2013). Given that the developmental stage of hearts and brains in newborn rats is somewhat equivalent to that of the fetal development in the third trimester of human gestation, they provide a model in studying the effect of dexamethasone therapy in preterm infants on the heart and brain development. Recently, Chang and colleagues uncovered that neonatal dexamethasone

treatment altered the susceptibility of the immature brain to hypoxic-ischemic brain injury (Chang, Yeh et al. 2013). Studies also have provided evidence for negative occurrences with the dexamethasone treatment including myocardial hypertrophy (La Mear, MacGilvray et al. 1997) and premature death (Kamphuis, de Vries et al. 2007). De Vries and colleagues investigated the long-term effect of neonatal dexamethasone therapy on cardiac function, and showed that the heart to body weight ratio was increased in P7 pups but decreased in 45-week-old rats (de Vries, van der Leij et al. 2002). Similar to this study, our results also showed an increase in the heart to body weight ratio in P14 pups.

It has been reported that dexamethasone treatment increased cardiomyocyte length, width and volume at 45 week (de Vries, van der Leij et al. 2002, Bal, de Vries et al. 2009), suggesting neonatal dexamethasone therapy had a long-term effect on the heart and may be responsible for the cardiomyocyte hypertrophy later in life in adulthood. However, the underlying mechanisms at the cellular and molecular levels remain elusive. The present study provided a mechanism in the understanding of this long-term effect of neonatal dexamethasone therapy, and demonstrated that dexamethasone treatment significantly suppressed proliferation and promoted premature maturation of cardiomyocytes, resulting in a decreased total cardiomyocyte number in the developing heart. The finding that dexamethasone-mediated stimulation of premature cardiomyocyte binucleation and inhibition of myocyte proliferation were blocked by Ru486 demonstrates the GR-mediated effects during this critical window of the heart development.

In the developing heart, cardiomyocytes are initially mononucleate and proliferative, but proliferation capacity is soon limited as mature cardiomyocytes undergo

binucleation and hence are non-proliferative (Clubb and Bishop 1984, Li, Wang et al. 1996). Fetal exposure to glucocorticoids has been shown to stimulate binucleation in preterm piglets (Kim, Eiby et al. 2014) , but has no overall effects in fetal lambs (Giraud, Louey et al. 2006). Because rodent cardiomyocyte terminal differentiation starts and continues within the first two weeks of postnatal life, which is equivalent to human heart development in late fetal stage of third trimester, neonatal rats provide a reasonable animal model to study the effect of glucocorticoid treatment on preterm infants at the critical window of the heart development. The present study demonstrates that dexamethasone treatment of newborn rats stimulates premature cardiomyocyte binucleation in day 4 pups. The transition to binucleation is a critical time in the heart development because of the limited proliferative capacity thereafter. Therefore, premature binucleation can potentially depress cardiomyocyte endowment. Since cardiomyocytes are the functional unit of the heart, adequate numbers are essential for cardiac function in adaption to physiological changes. Indeed, our results showed decreased cardiomyocyte proliferation on P4 and P7 pups, as well as a significantly lower cardiomyocyte number in P14 pups. Since the majority of cardiomyocytes are binucleated and terminally differentiated at this developmental age, it is likely that the dexamethasone-induced reduction of cardiomyocyte number will persist into adulthood and contribute to heart dysfunction later in life. The depressed cardiomyocyte number was also reported with other stressor such as intrauterine growth restriction (Corstius, Zimanyi et al. 2005). This raises a major concern, when cardiomyocyte numbers are lowered in the heart, the remaining cardiomyocytes may undergo cellular hypertrophy to generate sufficient contractile forces. This compensation is not without consequence in

increased risk of ischemic heart disease. Although the present study focuses on the effect of glucocorticoid on the critical window of the heart development in cardiomyocyte maturation and binucleation, which is of clinical relevance in the glucocorticoid therapy in preterm infants, the potential effect of glucocorticoid on the heart development in the earlier embryonic stage remains an interesting question for further investigation.

Heart growth occurs by one of two methods, hyperplasia or hypertrophy, and fetal glucocorticoid exposure was noted to induce both hyperplasic (Torres, Belser et al. 1997, Giraud, Louey et al. 2006) and hypertrophic growth (Rudolph, Roman et al. 1999). In neonates, complicated and sometimes contradictory accounts have also been noted. Studies have revealed that the glucocorticoid treatment is associated with hyperplasic (Sato, Sheppard et al. 1996), hypertrophic (Whitehurst, Zhang et al. 1999, Lister, Autelitano et al. 2006) or no effect on the growth (Porrello, Meeker et al. 2010) of cardiomyocytes, as analyzed by the protein to DNA ratio. Formation of binucleated myocardial cells is regarded as an early indicator of transition of hyperplasia to hypertrophic growth (Clubb and Bishop 1984), indicating ceasing of proliferation of cardiomyocytes. Mounting evidence has shown that arrest of the cell cycle, and thereby locking of cells in either G1 or G0 phase stop myocyte proliferation(Capasso, Bruno et al. 1992, McGill and Brooks 1995). Poolman and colleagues studied the profile of the cell cycle in cardiomyocytes in postnatal rats from P2 to P5, and found that the percentage of G0/G1 phase cells was increased during the early neonatal development (Poolman and Brooks 1998). These studies suggested that cell cycle dependent molecules, such as cyclins, cyclin-dependent kinase (CDK) and CDK inhibitors (CDI), may control the transition from hyperplasia to hypertrophic growth of myocytes (Brooks, Poolman et al.

1997, Horky, Kuchtickova et al. 1997). Indeed, CDI p21 was up-regulated during the switch of cardiac myocyte from hyperplasia to hypertrophic growth in rats (Horky, Kuchtickova et al. 1997). G1 phase acting proteins, CDK4 and CDK6 were also up-regulated in this process and were associated with hypertrophic growth of myocytes (Poolman and Brooks 1998, Nagai, Takano et al. 2001).

It is possible that increased exposure to glucocorticoids during the critical window of the heart development may alter the expression or activities of these key regulating proteins of the cell cycle. The primary mechanism of glucocorticoid activity is *via* the GR, an intracellular ligand dependent transcription factor. The GR plays a critical role in fetal heart maturation and cardiovascular health and disease. Knockout of the GR gene in cardiomyocytes results in impaired cardiac structure and function that can be observed at embryonic day 17.5 (Rog-Zielinska, Thomson et al. 2013), and the offspring die prematurely from spontaneous cardiovascular disease (Oakley, Ren et al. 2013). In glucocorticoid null fetal mice hyper-proliferation was reported, implicating the GR as an important regulator of cell proliferation (Bird, Tan et al. 2007). Several lines of evidence suggest the role of glucocorticoids in regulating the cell cycle. It has been shown that dexamethasone suppresses rat epithelial cell growth by blocking a specific cell cycle of either G1 or G0 phase, and dexamethasone withdrawal increases the expression of G1 marker genes, including c-Myc and cyclin D1 (Goya, Maiyar et al. 1993). In addition, glucocorticoid signaling arrests cell cycle activity by either transcriptional repression of G1 phase kinases CDK4/6 or enhanced transcription of CDK endogenous inhibitor, CDIs p21 and p27 (Oakley, Ren et al. 2013). Moreover, a recent study has shown that dexamethasone up-regulates CDI p21 and inhibits osteoblastic cell proliferation and these

effects are blocked by the GR antagonist Ru486 or specific silencing of GR, demonstrating a GR-dependent mechanism (Li, Qian et al. 2012). Further studies are needed to investigate the effect of dexamethasone on the cell cycle regulating molecules in cardiomyocytes of the development heart.

Although the mechanisms by which perinatal dexamethasone treatment causes long-term effects are still not known, increasing evidence indicates that epigenetic modifications, such as DNA methylation, play an important role. Accumulating evidence indicates interaction between GR and proteins involved in methylation, such as DNMT3b and MeCP2, leading to promoter methylation of genes (Sharma, Bhave et al. 2013). In a recent study, Crudo and colleagues found that prenatal exposure to synthetic glucocorticoids resulted in an altered global DNA methylation pattern in an organ and development dependent manner, and these changes were also observed in the next generation, indicating dexamethasone-caused methylation as a long-term manipulation in the gene regulation (Crudo, Petropoulos et al. 2012). Many mammalian genes possesses glucocorticoid-response element (GRE), and the expression is subjected to regulation by GR activation. GR binding to targets genes leads to change of methylation status on the gene promoters (Grange, Cappabianca et al. 2001, Crudo, Petropoulos et al. 2013). In the present study, inhibition of DNA methylation by 5-AZA blocked the dexamethasone-induced changes in cardiomyocyte proliferation and reversed the dexamethasone-induced decrease of cardiomyocyte number, providing novel evidence of DNA methylation in dexamethasone-induced change of gene regulation and heart development. Of importance, these findings suggest a potential strategy to abrogate or reverse the dexamethasone-induced adverse effects, considering the routine administration of

dexamethasone in clinical practice to deal with preterm risks. Despite the effect of dexamethasone, the finding that 5-AZA alone promoted cardiomyocyte proliferation at P7 pups resulting in a significant increase in cardiomyocyte number in P14 hearts is intriguing and suggests an important role of DNA methylation in the heart development. This is in agreement with the findings in a study by Kou *et al*., which showed that DNA synthesis was increased in cardiomyocytes treated with 5-AZA (Kou, Lau et al. 2010).

Evidence from recent studies indicates that DNA methylation may be an important mechanism in cell cycle regulation. In Hela cells, multiple DNA sequences are found differentially methylated between G0 and S phase, suggesting dynamic methylation is involved in control of cell cycle (Brown, Fraga et al. 2007). Indeed, cell cycle regulating proteins are targets of epigenetic regulation and one example is the CDI protein P16, which inhibits CDK4/CDK6 activity and leads to G1-cell cycle arrest. Extensive methylation of the p16 promoter results in inactivation of P16 and cell proliferation (Matsuda, Ichida et al. 1999). In the present study, we found that the dexamethasone treatment had no significant effect on global methylation levels in the hearts of P4 and P7 pups. Given the finding that inhibition of methylation by 5-AZA abrogated dexamethasone-induced effects on cardiomyocyte proliferation and binucleation, it is possible that instead of a genome-wide effect, GR-mediated methylation may be gene specific in the developing heart. It has been shown that dexamethasone has differential effects in the regulation of methylation that is organ-dependent and gene-dependent in an organ (Crudo, Petropoulos et al. 2012, Crudo, Petropoulos et al. 2013). Indeed, the present study demonstrated that dexamethasone significantly decreased cyclin D2 (a cell cycle promoter), but not p27 (a cell cycle

inhibitor), in the heart of P4 pups, which was blocked by 5-AZA. This suggests that methylation-dependent down-regulation of cyclin D2 may play a role in dexamethasone-induced decrease in cardiomyocyte proliferation. The finding of a development-dependent decrease in global methylation from P4 to P7 hearts is intriguing given that a study conducted by Kou and colleagues reported that global methylation increased progressively with age in cardiomyocytes (Kou, Lau et al. 2010). A possible reason of this inconsistency between the two studies may be the difference in sample source. In the present study we isolated DNA from the whole heart that is composed of many cell types including fibroblasts, endothelial cells, smooth muscle cells and cardiomyocytes, whereas Kou *et al.* analyzed global DNA methylation in isolated and cultured cardiomyocytes (Kou, Lau et al. 2010). Nonetheless, alteration of methylation during development or aging has been noted in a variety of organs, including the heart (Vanyushin, Nemirovsky et al. 1973, Horvath 2013).

The present study provides novel evidence of dexamethasone-mediated premature terminal differentiation of cardiomyocytes at the critical window of heart development during early postnatal life. Dexamethasone promotes premature exit of the cell cycle and cardiomyocyte binucleation, leading to a significant decrease of proliferating cells. These effects result in a decrease in cardiomyocyte endowment in the heart. Although the present finding suggests an important role of DNA methylation in dexamethasone-mediated regulation of cardiomyocyte proliferation and binucleation in the developing heart, other mechanisms, e.g. histone modifications may not be excluded. Whereas protein methylation and DNA methylation are mediated by different mechanisms, it

remains to be explored whether histone methylation is also involved in the dexamethasone-induced effects.

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 and the Loma Linda University School of Medicine. The authors thank Dr. Christopher Wilson for providing some of the animals in the studies.

CHAPTER THREE

EPIGENETIC REPRESSION OF CYCLIN D2 MEDIATES DEXAMETHASONE-INDUCED CARDIOMYOCYTE TERMINAL DIFFERENTIATION

By

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This paper has been accepted by the Journal of Pharmacology and Experimental Therapy, and is in press.

Abstract

The previous study demonstrated that *in vivo* treatment of newborn rats with dexamethasone inhibited cardiomyocyte proliferation and stimulated premature terminal differentiation of the cardiomyocyte in the developing heart. Yet, mechanisms remain undetermined. The present study tested the hypothesis that the direct effect of glucocorticoid receptor-mediated epigenetic repression of cyclin D2 gene in the cardiomyocyte plays a key role in the dexamethasone-mediated effects in the developing heart. Treatment of cardiomyocytes isolated from newborn rats with dexamethasone for 48 h significantly inhibited cardiomyocyte proliferation with increased binucleation, and decreased cyclin D2 protein abundance. These effects were blocked by Ru486. In addition, the dexamethasone treatment significantly increased cyclin D2 gene promoter methylation in newborn rat cardiomyocytes. 5-Aza-2'-deoxycytidine inhibited dexamethasone-mediated promoter methylation, recovered dexamethasone-induced cyclin D2 gene repression, and blocked the dexamethasone-elicited effects on cardiomyocyte proliferation and binucleation. In addition, the overexpression of cyclin D2 restored the dexamethasone-mediated inhibition of proliferation and increase in binucleation in newborn rat cardiomyocytes. The results demonstrate that dexamethasone acting on glucocorticoid receptors has a direct effect and inhibits proliferation and stimulates premature terminal differentiation of cardiomyocytes in the developing heart *via* epigenetic repression of cyclin D2 gene.

Introduction

Glucocorticoids are essential in the tissue and organ development. In lung development, glucocorticoids play a critical role and stimulate the maturation, which is the basis for wide-spread use of synthetic glucocorticoids, *e.g.*, dexamethasone, in clinics. Dexamethasone is used to treat preterm infants and mothers at risk of preterm birth to reduce the incidence and severity of respiratory distress syndrome (Liggins and Howie 1972, 1995). Yet in other tissues and organs, synthetic glucocorticoid exposure may be detrimental (Ortiz, Quan et al. 2003, Shoener, Baig et al. 2006, Kamphuis, de Vries et al. 2007, Bal, de Vries et al. 2008). Compelling evidence exists for deleterious effects of glucocorticoid treatment on the brain and HPA axis, glucose metabolism, and aortic function (Davis, Waffarn et al. 2011, Kelly, Lewandowski et al. 2012). In our recent study, we examined the impact of clinically relevant neonatal doses of dexamethasone on cardiomyocyte proliferation and binucleation in the developing heart. We provided evidence that treatment of newborn rats with dexamethasone during a critical window of the heart development inhibited cardiomyocyte proliferation, stimulated premature cardiomyocyte binucleation and reduced the total cardiomyocyte number in the heart (Gay, Li et al. 2015). These findings provided new insights in the regulation of cardiomyocyte maturation by glucocorticoids, yet the underlying mechanisms remain largely elusive.

It has been well established that during the heart development cardiomyocyte growth occurs in two phases, hyperplasia and hypertrophy (Li, Wang et al. 1996, Poolman and Brooks 1998). Early cardiac growth is by hyperplasia, in which cardiomyocytes proliferate and endow the heart with adequate amount of myocytes.

However, cardiomyocyte proliferation is not indefinite. In rodents, during late gestation and within the first 2 weeks of life cardiomyocyte proliferative growth is progressively replaced by hypertrophic growth as myocytes exit the cell cycle and lose the ability to divide, resulting in binucleated cells (Clubb and Bishop 1984, Li, Wang et al. 1996). Subsequent growth is mainly by increasing the myocyte size. Binucleated cardiomyocytes are believed to have exited the cell cycle, and therefore represent a terminally differentiated state. As binucleation is occurring the expression of genes for mitosis, cytokinesis and cell cycle reentry declines, resulting in loss of the proliferative capacity (Brooks, Poolman et al. 1997, Kang and Koh 1997, Brooks, Poolman et al. 1998). The critical widow during the heart development when myocyte proliferation is still possible is therefore an especially influential time on the cardiomyocyte developmental trajectory.

Although much is still unknown about the mechanisms underlying the transition of cardiomyocytes from proliferative to terminally differentiated binucleation, many studies have been focused on molecules involved in cell cycle regulation and cytokinesis as well as epigenetic modifications that can occur during this transition. Cyclin D2 is a cell cycle promoter that plays an important role in the regulation of cardiomyocyte proliferation and terminal differentiation (McGill and Brooks 1995, Brooks, Poolman et al. 1997, Poolman and Brooks 1998, Nagai, Takano et al. 2001, Paradis, Gay et al. 2013). Glucocorticoids are known to influence the cell cycle and proliferation in a variety of cell types including the heart (de Vries, Bal et al. 2006, Sundberg, Savola et al. 2006, Bird, Tan et al. 2007). Of importance, cyclin D proteins are established targets of glucocorticoids (Fernandes, Guida et al. 1999, Sundberg, Savola et al. 2006, Gay, Li et al.

2015). In rodent hearts, we have demonstrated that hypoxia and dexamethasone treatments significantly decrease cyclin D2 protein abundance (Tong, Xiong et al. 2013, Gay, Li et al. 2015, Paradis, Gay et al. 2015), suggesting a role of cyclin D2 in dexamethasone-induced inhibition of cardiomyocyte proliferation in the developing heart. In the present study, we sought to test the hypothesis that dexamethasone has a direct effect on newborn rat cardiomyocytes in repressing the cyclin D2 gene *via* increasing promoter methylation, and the down-regulation of cyclin D2 expression plays a causal role in dexamethasone-mediated transition of cardiomyocyte proliferation to terminal differentiation in the developing heart.

Materials and Methods

Experimental Animals

All procedures and protocols in the present study were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines by US National Institutes of Health Guide for the Care and Use of Laboratory Animals. Time-dated pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI). Postnatal day 2 pups were anesthetized using isoflurane and hearts were removed for isolation of cardiomyocytes. The adequacy of anesthesia was monitored by foot withdrawal reflex.

Cardiomyocyte Isolation and Culture

Cardiomyocytes were isolated from hearts by enzymatic digestion (0.1% trypsin and 0.5 mg/ml type II collagenase), as previously described (Xiao, He et al. 2000). Cells

were cultured in Hyclone media 199 (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Sacramento, CA) and 1% antibiotics (10,000 I.U./mL penicillin, 10,000 μg/mL streptomycin) at 37 °C in 95% air/5% CO_2. Myocytes were plated on 6-well plates, some wells contained poly-L-lysine (Sigma, St. Louis, MO) coated coverslips. The seeding density was about 0.67×10^6 cells per well. 5-Bromo-2'-deoxyuridine (BrdU) was used to enrich the population of cardiomyocytes by limiting the proliferation of non-myocytes. After 24 hours of attachment, the media was changed and replaced with Hyclone media 199 containing BrdU (0.1 mM Sigma) and allowed to grow for 24 hours.

Cell Treatments

Cardiomyocytes were treated for 48 h with dexamethasone (100 nM, Sigma) or media alone, in the presence or absence of glucocorticoid receptor inhibitor Ru486 (1 μM, Sigma). In addition, a methylation inhibitor, 5-aza-2'-deoxycytidine (5-AZA, 10 μM, Sigma) was used to determine the effect of DNA methylation in dexamethasone-mediated effects. Thus, there were six treatment groups: 1) control, 2) dexamethasone, 3) Ru486, 4) Ru486+dexamethasone, 5) 5-AZA, and 6) 5-AZA+dexamethasone.

Immunocytochemistry

After treatments, cells were washed twice with PBS and fixed with paraformaldehyde (3.7%) followed by permeabilization with Triton X-100 (0.5%). Cells were then incubated with 1% bovine serum albumin for 1 h, followed by incubation with primary antibodies for 1 h at room temperature, mouse anti-α actinin (1:200, Sigma) and

rabbit anti-Ki-67 (1:100 Abcam, Cambridge, MA). Next, cells were incubated for 1 h at room temperature with secondary anti-rabbit AlexaFluor 647 conjugated antibody (1:400; Life Technologies Carlsbad, CA) and anti-mouse AlexaFluor 488 conjugated antibody (1:400; Life Technologies). The nuclei were stained with Hoechst. A Zeiss Axio imager was used for all immunofluorescent imaging and the analysis was performed using Image J software.

Western Immunoblotting

After treatments, cardiomyocytes were washed with PBS, and RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) was added to each well on ice. Cells were scraped and total protein was determined using BCA protein assay (Thermo Fisher Scientific). Equal amounts of protein were loaded onto a 10% polyacrylamide gel with 0.1% sodium dodecyl sulfate (SDS). Samples were transferred to PVDF membrane and were blocked with milk for 1 h at room temperature. Membranes were incubated with anti-cyclin D2 (1:1000, Abcam) overnight at 4 °C followed by washing. Secondary antibody was applied for 1 h at room temperature. Proteins were visualized with Western blot chemiluminescence reagent and hyperfilm. β-actin was used as the housekeeping protein to assure equal loading.

Real-Time PCR

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) extraction protocol. SuperScript III first strand synthesis system for RT-PCR (Invitrogen) was used for reverse transcription of cDNA. The abundance of cyclin D2 mRNA was determined

by quantitative real-time PCR with IQ5 (Bio-Rad, Irvine, CA) using 2× SYBR green PCR mix (Biotool). The sequences of two primers used for cyclin D2 mRNA quantification were: forward: 5'-CCTCACGACTTCATTGAGCA and reverse: 5'-GGTAGCACACAGAGCGATGA. β-actin (forward: 5'-TCAGGTCATCACTATCGGCAAT; reverse: 5'-ACTGTGTTGGCATAGAGGTC TT) served as an internal control. Each PCR was of 25 µl volume and contained 500 nM each primer, 1× SYBR green master mix and nuclease free water (Qiagen). The real time-PCR protocol was: 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, anneal at 56 °C for 15 s, extension 72 °C for 15 s. All samples were analyzed in triplicate and threshold cycle number for each sample (Ct) was averaged. Real time quantitation was done by $2^{-\triangle\triangle Ct}$ method, as described by Livak *et. al*. (Livak and Schmittgen 2001).

Methylated DNA Immunoprecipitation (MeDIP) Assay

Rat cyclin D2 gene (CcnD2) is located on rat chromosome 4 (http://www.ncbi.nlm.nih.gov/gene/64033). To assess if dexamethasone induced methylation of this promoter, a 920-bp proximal promoter region of CcnD2 was evaluated by MeDIP assay using the MeDIP kit (Active Motif). Briefly, genomic DNA was purified from rat cardiomyocytes using FlexiGene DNA Kit (Qiagen). 20 µg genomic DNA from each sample were fragmented to 200-600 bp range and confirmed by 2% agarose gel electrophoresis. 800 ng of each fragmented DNA was next denatured at 95 °C and subjected to immunoprecipitation (IP). Briefly, methylated DNA fragments were pulled down and purified by 5-methylcytosine (5-mC) antibody and protein G magnetic beads (Active Motif). The immunoprecipitated DNA fragments were phenol-

chloroform-isoamyl alcohol extracted and ethanol precipitated along with 20 µl glycogen as inert carrier. The precipitate was washed, dried and reconstituted in 50µl of 10 mM Tris-HCl buffer, pH 7.5. 2 µl of the 5-mC antibody-enriched DNA fragments were next subjected to quantitative real-time PCR with IQ5 (Bio-Rad), using four pairs of primers targeting on the CcnD2 promoter: zone 1 (most proximal), forward: 5'-GCGAA GCAGTTCAGAGGGAAGG, reverse: 5'-CAGCACAGCAGCTCCATAGC; zone 2, forward: 5'-TATCCGGGGCCCCTAGCATG, reverse: 5'-CCTTCCCTCTGAACTGCTTCGC; zone 3, forward: 5'-CTTATTCTTCCTTCAGGGGTC, reverse: 5'-AACCCTCAAAACCCACGG; zone 4 (most distal), forward: 5'-TTC CGCACGAGGGTCATATT, reverse: 5'-AAGGGAAGGCTGA TTTGAGAA. Parallel to the enrichment of 5-mC antibody-pulled DNA fragments, a second aliquot of each fragmented genomic DNA sample (80 ng) were first heat denatured for 10 min, processed the same way as the immunoprecipitated DNA without 5-mC antibody/protein G to generate the input DNA, and was in parallel subjected to quantitative real-time PCR using the same PCR primers. In each 5-mC antibody-enriched DNA sample, the abundance of methylated DNA was first calculated as fold of corresponding input DNA and expressed as % of control samples.

Overexpression of Cyclin D2

Total RNA was isolated from the rat heart by Trizol reagent (Invitrogen) and cDNA was synthesized using SuperScript III first strand synthesis system for RT-PCR (Invitrogen). Using 100 ng of cDNA as template, the protein-coding domain of rat cyclin D2 (Genbank accession number: NM_022267) was PCR amplified as an 898-bp

amplicon, with the help of following PCR primers: forward: 5'-GAGACCC**GCGATCGCC**ATGGAGCTGCT GTGCTGTGAGG, reverse: 5'-GAGACCC**GTTTAAAC**TCACAGGTCAACATCCCGCACG. To facilitate cloning, the forward and reverse PCR primers respectively contained SgfI (*isoschizomer*: AsiSI was used) and PmeI cloning sites (bold). The 898-bp CcnD2 amplicon was next cloned in pF4A CMV Flexi vector (Promega) between SgfI (5') and Pme I (3') orientation and sequence confirmed. This CcnD2 overexpressing clone was named *FlexiCcnD2*. To generate corresponding negative control (for transfection experiments), the pF4A CMV Flexi vector was double digested with above two restriction enzymes to remove the cytotoxic *Barnase* sequence, blunt-ended, 5'-phosphorylated using the *Quick blunting kit* (New England Biolab), and blunt end ligated with T4 DNA ligase enzyme. These constructs, namely, *FlexiCcnD2* and *Flexi empty construct* were transfected into H9C2 cells using the X-treme GENE HP DNA Transfection reagent (Roche) for 24 h followed by recovery for another 24 h in fresh medium without transfection reagent. Whole cell lysates in RIPA buffer containing protease inhibitors were next made and protein concentration determined. 50 µg total proteins were electrophoresed by SDS-PAGE, followed by immunoblotting with rat cyclin D2 antibody (Abcam). In subsequent experiments, the same CcnD2 expression constructs were used to successfully overexpress CcnD2 in isolated rat cardiomyocytes.

5-mC DNA ELISA

Global CpG methylation in 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 were denatured and used to coat the plate wells with 5-mC coating buffer. After incubation at 37 °C for 1 h, 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 h. After washing 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 h. 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 significance ($P<0.05$) was determined by analysis of variance (ANOVA) followed by Neuman-Keuls post hoc testing or Student's t test, where appropriate.

Results

Dexamethasone Inhibited Proliferation and Increased Binucleation in Newborn Rat Cardiomyocytes

The direct effect of dexamethasone on cardiomyocyte proliferation and binucleation was determined by immunocytochemistry staining with the cardiomyocyte

marker and α-actinin and Ki67, a marker of proliferation. Ki67 is a protein present during all phases of the cell cycle but absent during G0 phase. Hence, terminally differentiated cardiomyocytes were determined based on the presence two nuclei and the absence of Ki67 protein. As shown in Figure 3.1A, quantification of co-localized Ki67 and α-actinin demonstrated about a 60% decrease in proliferating cardiomyocytes as a result of dexamethasone treatment, which was blocked by Ru486, a glucocorticoid receptor antagonist. We further evaluated the effect of dexamethasone treatment on cardiomyocyte binucleation and terminal differentiation. As shown in Figure 3.1B, dexamethasone induced an approximate 50% increase in binucleated cardiomyocytes. Consistent with the findings of proliferation, blocking the glucocorticoid receptor with Ru486 reversed the dexamethasone-mediated effect (Figure 3.1B). These findings suggest a direct effect of dexamethasone in increasing the transition of cardiomyocyte proliferation to terminal differentiation in a glucocorticoid receptor-dependent manner.

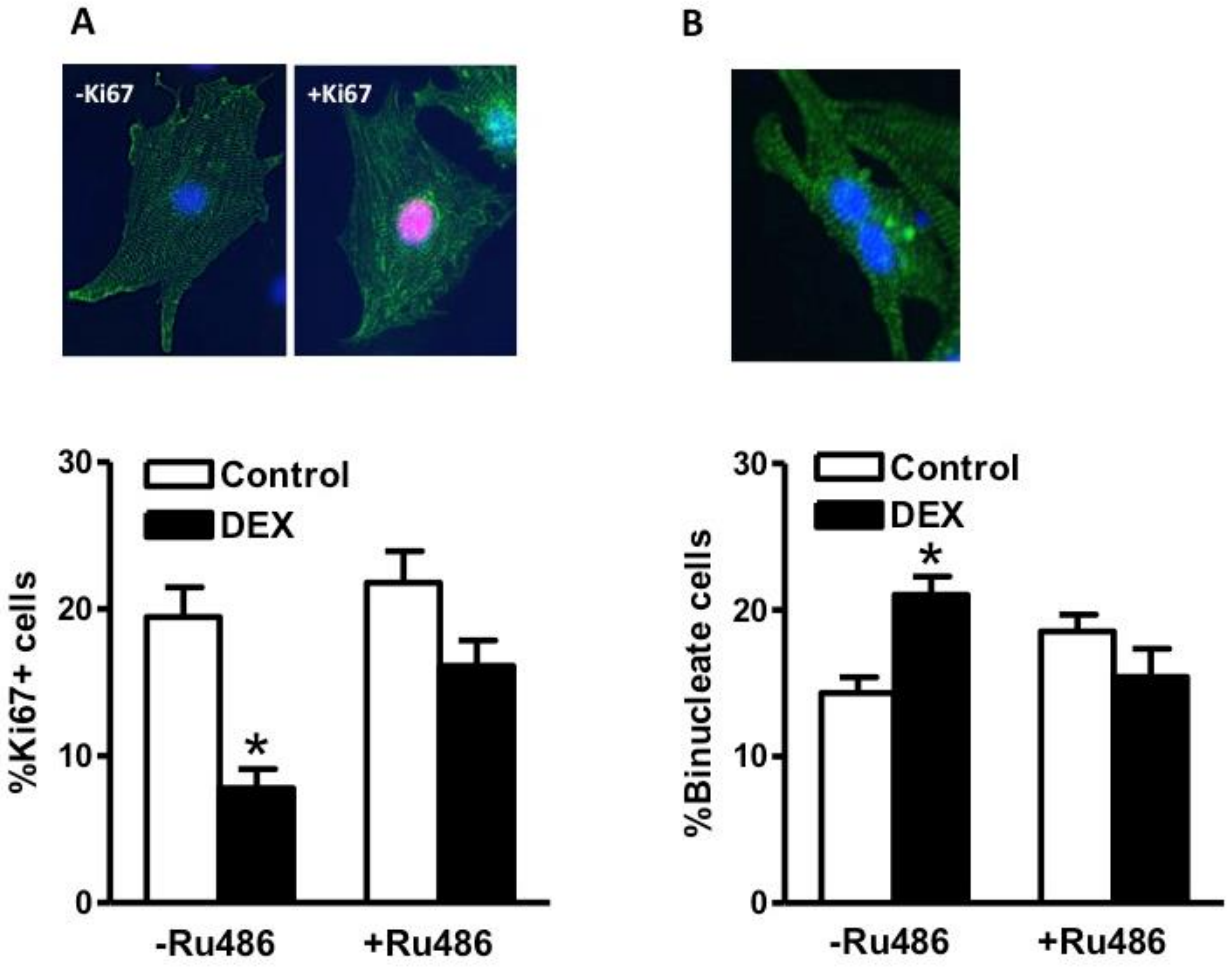

Figure 3.1. Dexamethasone decreased proliferation and increased binucleation in newborn cardiomyocytes. Cardiomyocytes were isolated from 2-day old rats and treated with dexamethasone (DEX) in the absence or presence of Ru486 for 48 hours. Cells were then fixed and stained with Ki67 and α-actinin. The nuclei were stained with Hoechst. **A**. Ki67 positive cardiomyocytes. **B**. Binucleate cardiomyocytes. Data are mean ± SEM, n = 7-9. * $p<0.05$, DEX *vs*. Control.

Dexamethasone Down-Regulated Cyclin D2 Expression in Newborn Rat Cardiomyocytes

The expression level of cell cycle regulators varies over the developmental stage of an organism. Cyclin D2 is a protein that is implicated in cardiomyocyte proliferation G1 phase progression. We therefore evaluated whether dexamethasone down-regulated cyclin D2 expression in newborn rat cardiomyocytes. Consistent with the decrease in proliferating cardiomyocytes caused by dexamethasone treatment, dexamethasone induced a 65% decline in cyclin D2 protein abundance in cardiomyocytes (Figure 3.2). To confirm the involvement of the glucocorticoid receptor, cardiomyocytes were treated with dexamethasone in the presence of Ru486. As shown in Figure 3.2, Ru486 blocked the effect of dexamethasone and restored cyclin D2 protein expression, further implicating the glucocorticoid receptor in the dexamethasone-mediated effects.

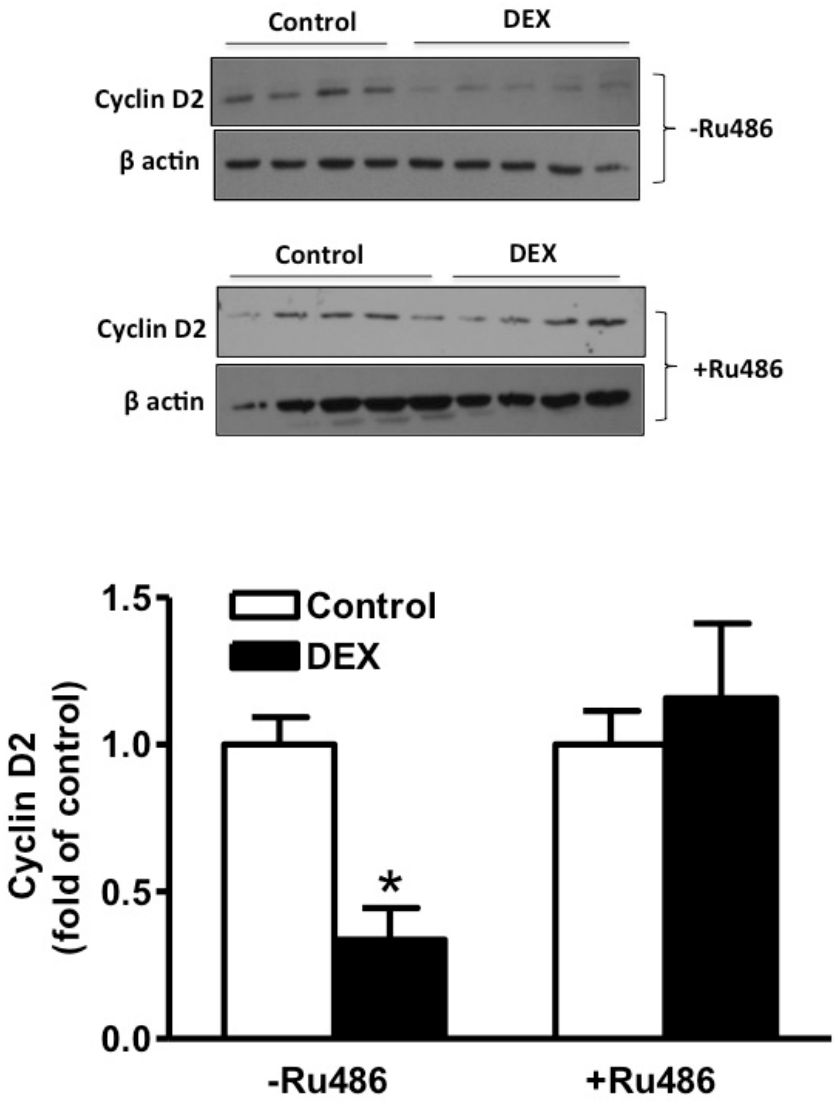

Figure 3.2. Dexamethasone down-regulated cyclin D2 protein abundance in newborn cardiomyocytes. Cardiomyocytes were isolated from 2-day old rats and treated with dexamethasone (DEX) in the absence or presence of Ru486 for 48 hours. Cyclin D2 protein abundance was determined by Western blot. Data are mean ± SEM, n = 4-5 * $p<0.05$, DEX *vs*. Control.

Dexamethasone Increased Promoter Methylation in the CcnD2 Gene in Newborn Rat

Dexamethasone treatment had no significant effect on global DNA methylation in cardiomyocytes (Figure 3.3). We further evaluated the effect of dexamethasone on promoter methylation of the CcnD2 gene. The proximal CcnD2 gene promoter was divided into 4 regions denoted as zones based on the proximity to the transcription start site. The dexamethasone treatment significantly increased methylation in the two zones most proximal to the transcription start site, zones 1 and 2 (Figure 3.4). In contrast, methylation levels of zones 3 and 4 of the CcnD2 gene promoter were not affected by dexamethasone (Figure 3.4). 5-AZA treatment decreased global DNA methylation in cardiomyocytes (Figure 3.3), but did not significantly altered promoter methylation in the CcnD2 gene (Figure 3.4). Of importance, 5-AZA blocked dexamethasone-induced methylation in zones 1 and 2 of the CcnD2 gene promoter (Figure 3.4).

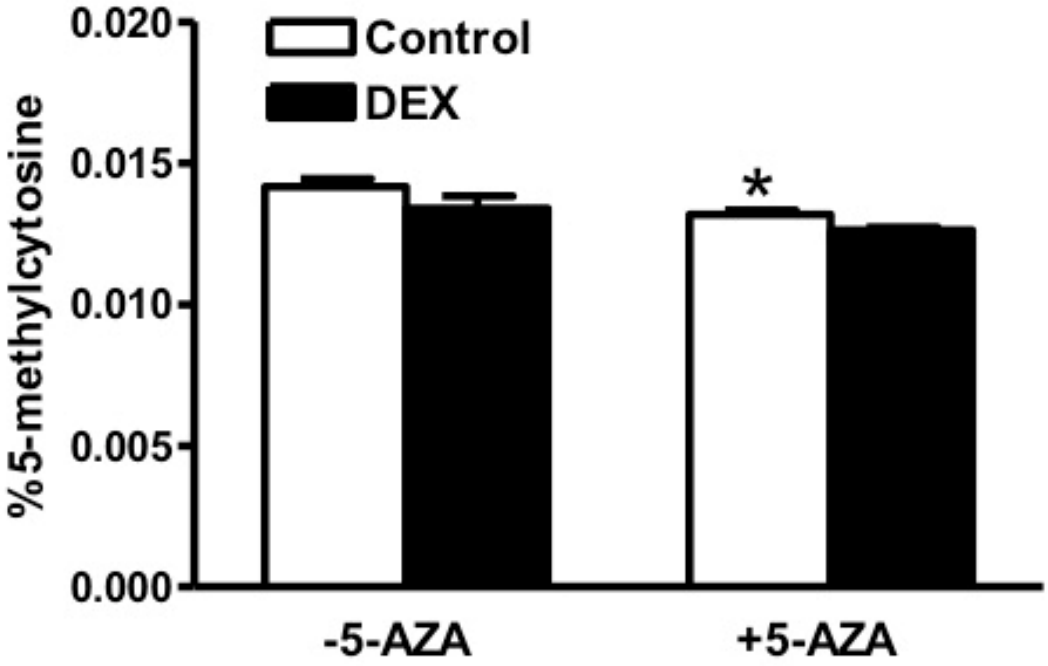

Figure 3.3. Effect of dexamethasone on global methylation in newborn cardiomyocytes. Cardiomyocytes were isolated from 2-day old rats and treated with dexamethasone (DEX) in the absence or presence of 5-aza-2'-deoxycytidine (5-AZA) for 48 hours. Genomic DNA was extracted and 5-methylcytosine was determined using a 5-mC ELISA kit. Data are mean ± SEM, n = 4. * $p<0.05$, +5-AZA *vs*. -5-AZA.

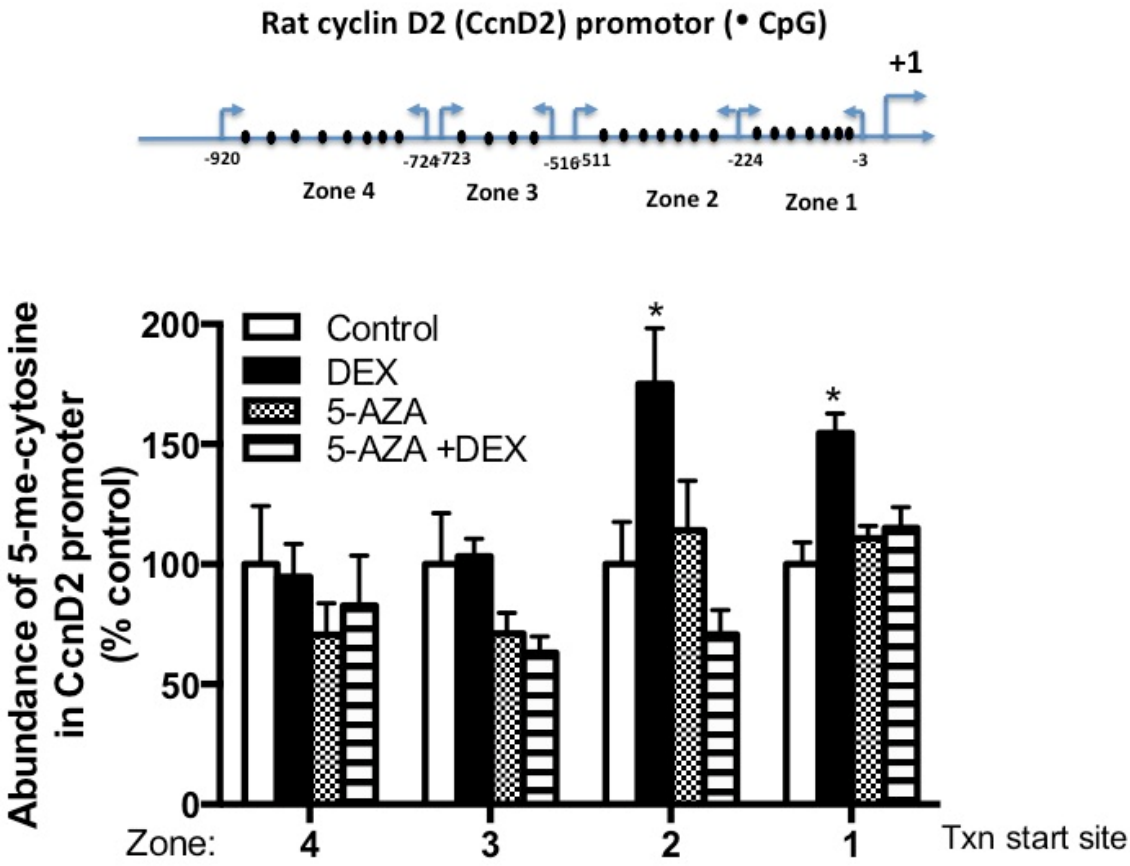

Figure 3.4. Dexamethasone increased cyclin D2 promoter methylation in newborn cardiomyocytes. Cardiomyocytes were isolated from 2-day old rats and treated with dexamethasone (DEX) in the absence or presence of 5-aza-2'-deoxycytidine (5-AZA) for 48 hours. Genomic DNA was extracted and 5-methylcytosine in CcnD2 proximal promoter was determined by MeDIP assays. Data are mean ± SEM, n= 4. * P<0.05, DEX *vs*. control.

5-AZA Reversed Dexamethasone-Induced Down-Regulation of Cyclin D2 Expression, Decrease in Proliferation and Increase in Binucleation in Newborn Rat Cardiomyocytes

To establish whether or not methylation plays a causal role in the dexamethasone-induced reduction of cyclin D2 expression in cardiomyocytes, we evaluated the effect of 5-AZA treatment on cyclin D2 protein and mRNA abundance. As shown in Figure 3.5, 5-AZA blocked the dexamethasone-mediated effects and restored cyclin D2 protein (Figure 3.5A) and mRNA (Figure 3.5B) abundance to values comparable to the control. The functional significance of restoration of cyclin D2 by 5-AZA was determined by examining cardiomyocyte proliferation and binucleation. As shown in Figure 3.6, 5-AZA alone had no significant effect on cardiomyocyte proliferation and binucleation, but blocked dexamethasone-induced decrease in proliferation and increase in binucleation in newborn rat cardiomyocytes.

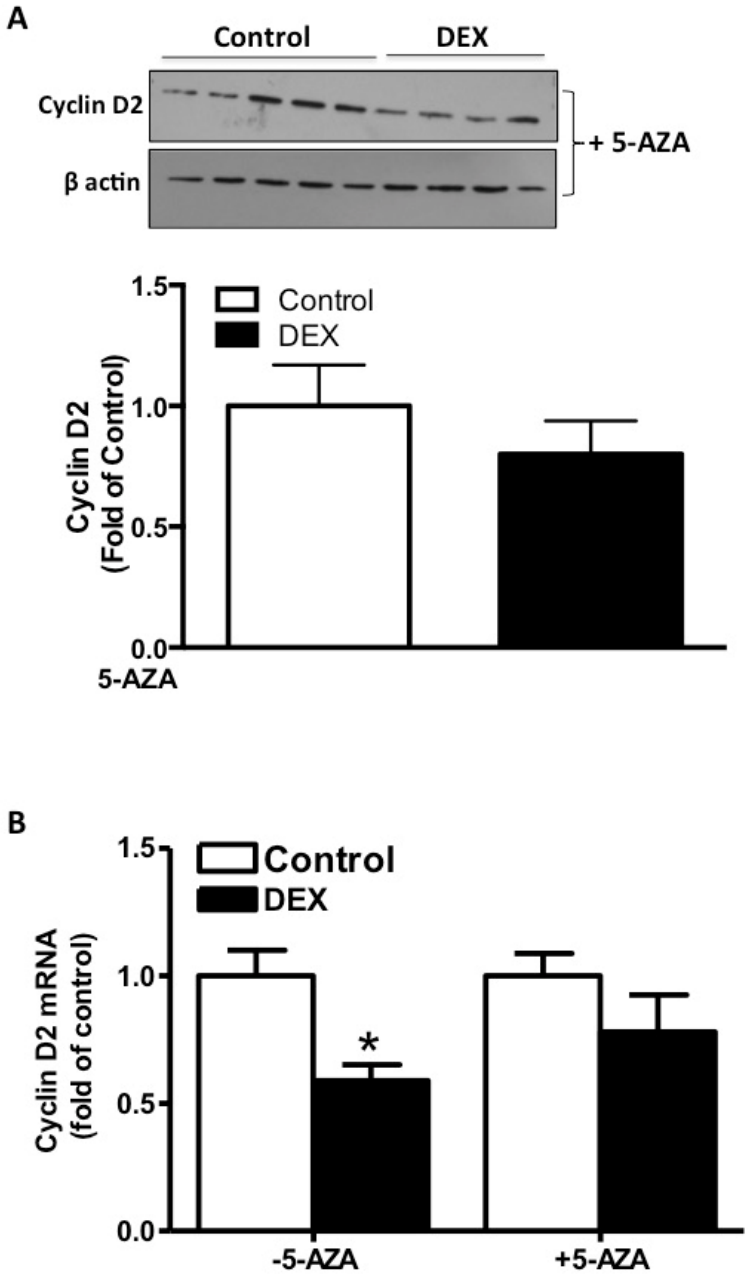

Figure 3.5. 5-AZA blocked dexamethasone-induced down-regulation of cyclin D2 protein and mRNA abundance in newborn cardiomyocytes. Cardiomyocytes were isolated from 2-day old rats and treated with dexamethasone (DEX) in the absence or presence of 5-aza-2'-deoxycytidine (5-AZA) for 48 hours. Cyclin D2 protein and mRNA abundance was determined by Western blot and quantitative real-time PCR, respectively. Data are mean ± SEM, n = 4-5. * $p<0.05$, DEX *vs*. Control.

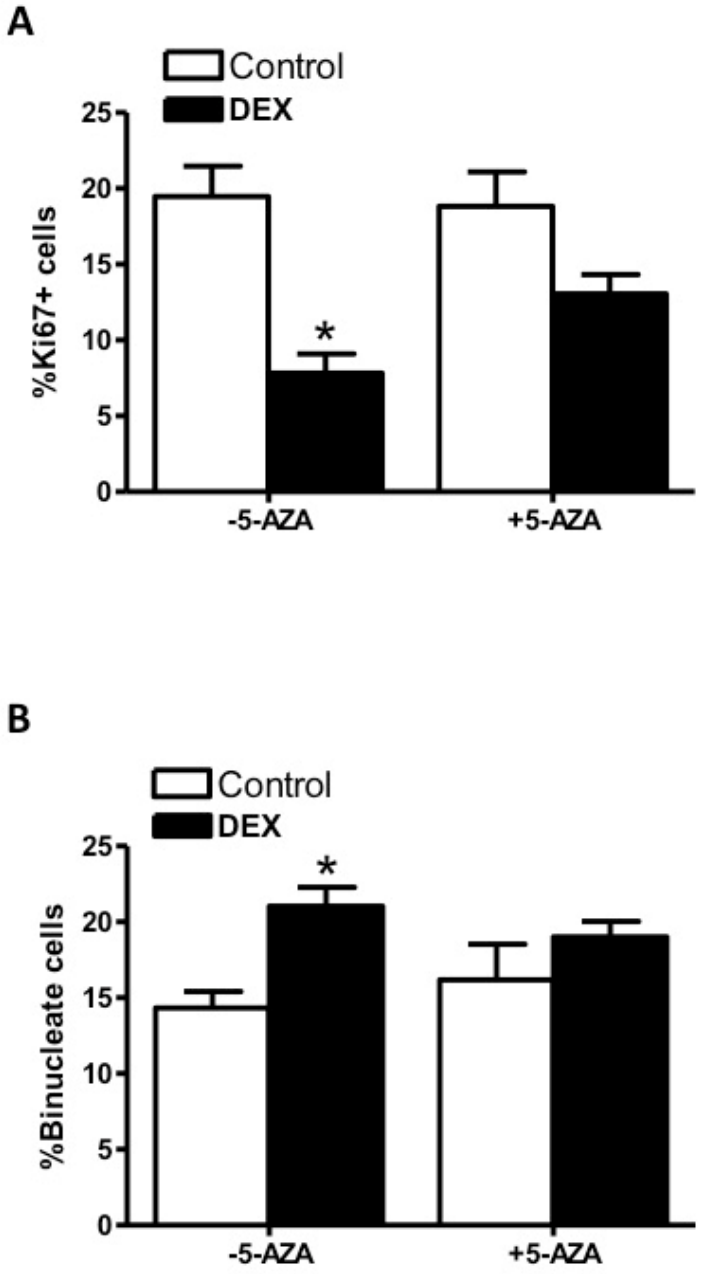

Figure 3.6. 5-AZA inhibited dexamethasone-induced terminal differentiation in newborn cardiomyocytes. Cardiomyocytes were isolated from 2-day old rats and treated with dexamethasone (DEX) in the absence or presence of 5-aza-2'-deoxycytidine (5-AZA) for 48 hours. Cells were then fixed and stained with Ki67 and α-actinin. The nuclei were stained with Hoechst. **A**. Ki67 positive cardiomyocytes. **B**. Binucleate cardiomyocytes. Data are mean ± SEM, n = 6-9. * $p<0.05$, DEX *vs*. Control.

Overexpression of Cyclin D2 Rescued Dexamethasone-Mediated Decrease in Proliferation and Increase in Binucleation in Newborn Rat Cardiomyocytes

A transfection protocol was established by transfecting H9C2 cells for 24 hours with either Flexi-empty (-CcnD2, control) or Flexi-CcnD2 (+CcnD2, S and M) constructs followed by recovery in fresh medium without transfection reagent for another 24 hours. As shown in Figure 3.7, very low level of expression of CcnD2 was seen in cells transfected with *Flexi empty construct* (-CcnD2, control), and robust expression of the rat cyclin D2 protein was confirmed in cells transfected with *FlexiCcnD2* (+CcnD2, S and M). To further establish a cause and effect relation between dexamethasone-induced reduction of cyclin D2 expression and the transition of cardiomyocytes from proliferation to terminal differentiation of binucleation, we overexpressed the cyclin D2 gene in newborn rat cardiomyocytes. To this end we established a protocol where the direct effect of cyclin D2 on proliferation and binucleation may be determined. Thus, cyclin D2 protein expression level was measured in empty vector, *Flexi-empty* (-CCnD2) and *Flexi-CcnD2* expression vector (+CCnD2) transfected cardiomyocytes. As shown in Figure 3.7, cardiomyocytes transfected with an empty vector, *Flexi-empty* (-CCnD2) followed by the treatment with dexamethasone tended to express less cyclin D2 protein. *Flexi-CcnD2* expression vector (+CCnD2)-transfected cardiomyocytes expressed about 3-fold more cyclin D2 protein compared to empty vector-transfected control, demonstrating the efficacy of transfection. The effect of cyclin D2 overexpression on dexamethasone-mediated myocyte terminal differentiation was then evaluated. As shown in Figure 3.8, similar to the findings above, in *Flexi-empty* (-CCnD2)-transfected cardiomyocytes dexamethasone decreased proliferation (Figure 3.8A) and increased binucleation (Figure

3.8B). Of importance, in *Flexi-CcnD2* expression vector (+CcnD2)-transfected cardiomyocytes, dexamethasone had no significant effect on cardiomyocyte proliferation and binucleation (Figure 3.8), suggesting that overexpression of cyclin D2 protein in cardiomyocytes is sufficient to rescue dexamethasone-mediated effects on proliferation and binucleation, and thus demonstrating a causal role of reduced cyclin D2 in dexamethasone-induced transition of proliferation to terminal differentiation of binucleation in newborn rat cardiomyocytes.

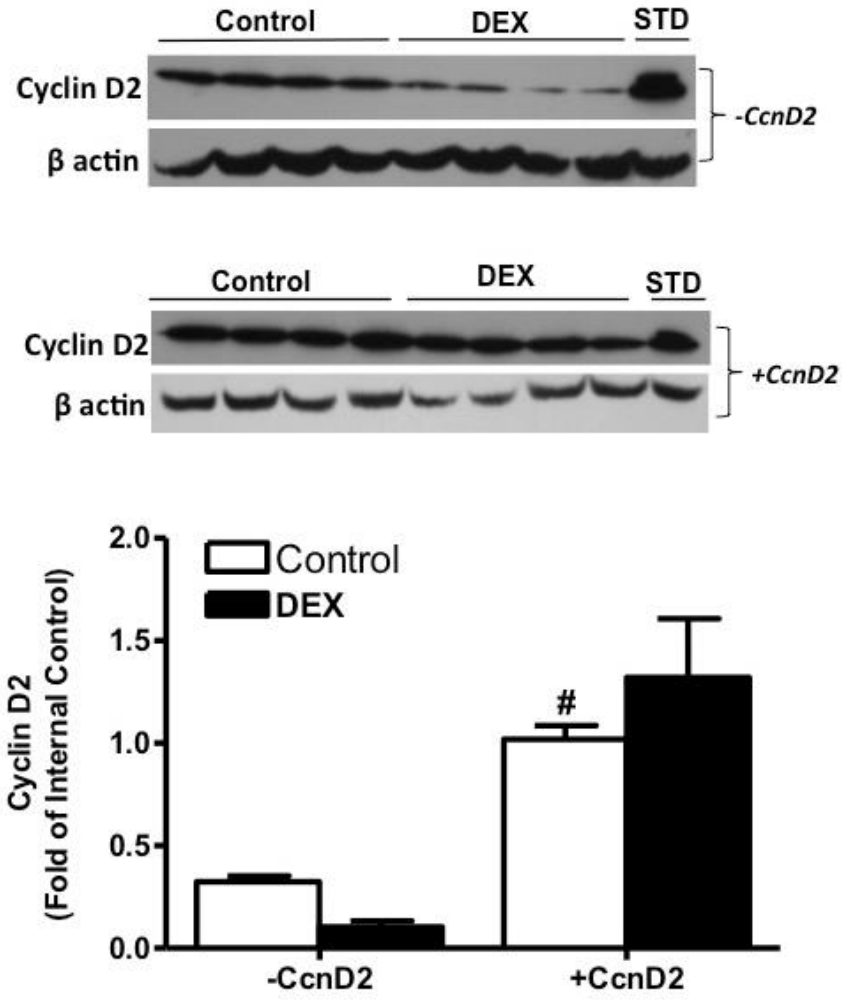

Figure 3.7. Overexpression of cyclin D2 in newborn cardiomyocytes. Cardiomyocytes were isolated from 2-day old rats and transfected with *FlexiCcnD2* (+CcnD2) or *Flexi empty construct* (-CcnD4). Myocytes were then treated with DEX for 48 hours. Cyclin D2 protein abundance was determined by Western blot. STD: internal standard. Data are mean ± SEM, n = 4. # $p<0.05$, *+CcnD2 vs. -CcnD2.*

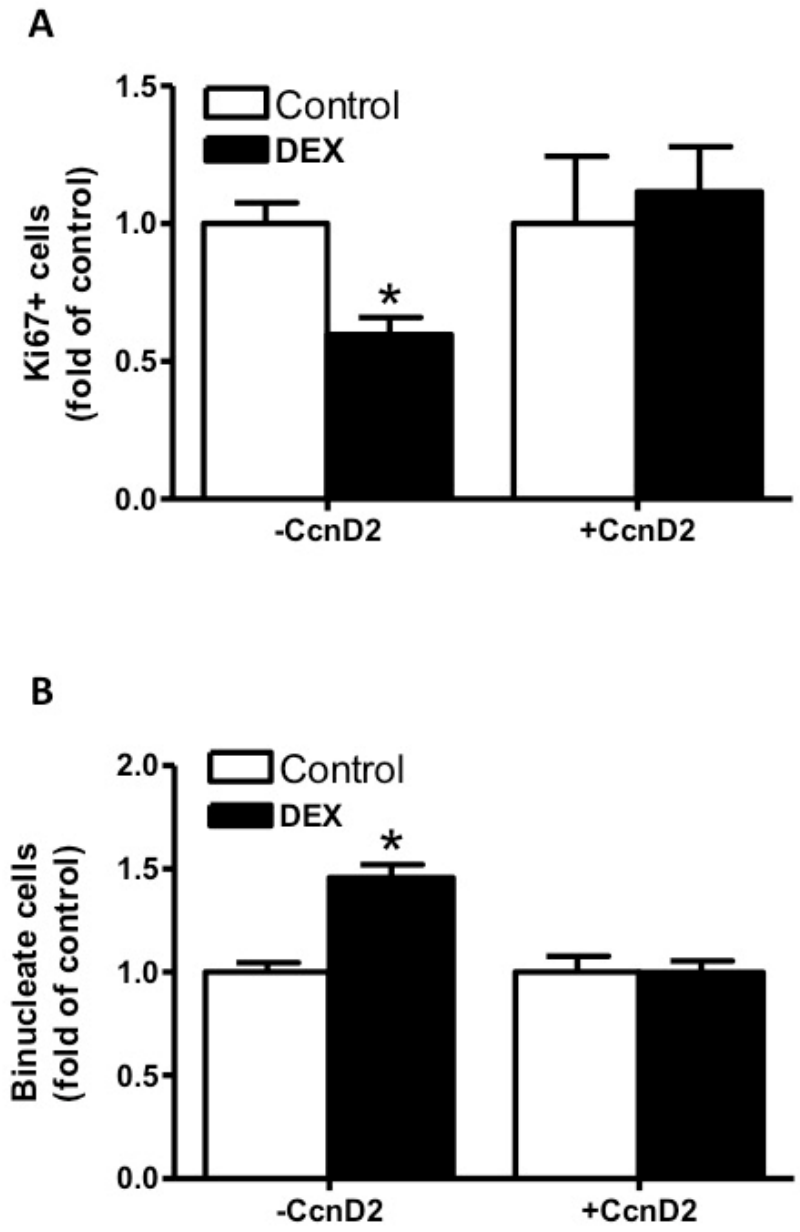

Figure 3.8. Overexpression of cyclin D2 abrogated dexamethasone-induced terminal differentiation in newborn cardiomyocytes. Cardiomyocytes were isolated from 2-day old rats and transfected with *FlexiCcnD2* (+CcnD2) or *Flexi empty construct* (-CcnD4). Myocytes were then treated with DEX for 48 hours. Cells were fixed and stained with Ki67 and α-actinin. The nuclei were stained with Hoechst. **A**. Ki67 positive cardiomyocytes. **B**. Binucleate cardiomyocytes. Data are mean ± SEM, n = 5-6. * $p<0.05$, DEX *vs*. Control.

Discussion

Our previous study demonstrated that *in vivo* treatment of newborn rats with clinically relevant doses of dexamethasone stimulated premature terminal differentiation and maturation of cardiomyocytes and decreased myocyte endowment in the developing heart (Gay, Li et al. 2015) . In the present study, we provided novel evidence of a direct effect of dexamethasone in inhibiting cardiomyocyte proliferation and stimulating myocyte binucleation in a glucocorticoid receptor-dependent manner. Of importance, we demonstrated a cause and effect relation between dexamethasone-mediated epigenetic repression of cyclin D2 gene and dexamethasone-induced transition of proliferation to terminal differentiation of binucleation in newborn rat cardiomyocytes.

In cardiomyocytes shortly after birth a rapid switch to terminal differentiation occurs, which is characterized by the loss of proliferative potential, cell cycle exit and a transition to binucleate phenotype (Li, Wang et al. 1996, Soonpaa, Kim et al. 1996). It is evident that cardiomyocyte terminal differentiation is closely associated with the cell cycle as the vast majority of adult cardiomyocytes are arrested in the G0/G1 phase (Capasso, Bruno et al. 1992, Walsh, Ponten et al. 2010). Previous studies by Poolman and colleagues observed a marked increase in G0/G1 phase cardiomyocytes as the switch from hyperplasic to hypertrophic growth occurs between neonatal day 2 and 5 in rodents (Poolman and Brooks 1998). Additionally, positive cell cycle regulators progressively decline and the expression levels of cell cycle inhibitors increases during the terminal differentiation (Brooks, Poolman et al. 1997, Horky, Kuchtickova et al. 1997, Li, Poolman et al. 1998, Poolman and Brooks 1998), suggesting an intrinsic loss of proliferative capacity in terminally differentiated cardiomyocytes. Analysis of G1 phase

cell cycle regulators identified a marked decrease in cyclin D2, from the fetal phase to barely detectable in adult cardiomyocytes (Brooks, Poolman et al. 1997), further supporting a role of cell cycle regulation in the terminal differentiation. The cell cycle is influenced by glucocorticoids that have been shown to inhibit proliferation and result in cellular arrest in the G0 or G1 phase (Goya, Maiyar et al. 1993, Rogatsky, Trowbridge et al. 1997, Zou, Yang et al. 2015). Yet, in fetal sheep cortisol infusion did not influence the rate of proliferation or the expression of MAPK signaling proteins (Lumbers, Boyce et al. 2005). This likely illustrates the effects of glucocorticoid are dependent on timing and duration of exposure. The effects of glucocorticoids on proliferation are believed to be closely related to transcriptional regulation of gene expression of G1 activators such as c-Myc and cyclin D1 (Goya, Maiyar et al. 1993). Additionally, glucocorticoid signaling is instrumental in regulating the expression of cell cycle inhibitors including p21 (Bird, Tan et al. 2007) and p53 (Li, Qian et al. 2012). These studies indicate that glucocorticoids induce similar changes in gene expression, as is characteristic of the terminal differentiation. It is therefore possible that glucocorticoid exposure matures cardiomyocytes by mechanisms similar to that of terminal differentiation, accelerating cardiomyocyte maturation. In the present study, we determined the direct effects of dexamethasone are mediated by the glucocorticoid receptor. While terminal differentiation is normal during cardiomyocyte development dexamethasone appears to accelerate the process by reducing proliferation and increasing binucleation of cardiomyocytes prematurely. Additionally, cyclin D2 that is instrumental in cellular progression through the G1 phase of the cell cycle was down-regulated as a result of dexamethasone treatment. Similarly, previous studies have shown that stresses such as

hypoxia and anoxia also decrease cyclin D2 protein expression and proliferation of cardiomyocytes (Tong, Xiong et al. 2013, Paradis, Gay et al. 2015). During hypoxia and other physiological stresses glucocorticoids are increased and are believed to be important physiological mediators during stress response (Raff, Hong et al. 2003). In terms of gene regulation there seems to be a consistent mechanism initiated independent of glucocorticoid source, whether endogenous or exogenous. Interestingly, in our previous study neonatal dexamethasone exposure was not associated with changes in the cell cycle inhibitor p27 (Gay, Li et al. 2015), yet hypoxia and anoxia treatment resulted in a significant increase in p27 (Tong, Xiong et al. 2013, Paradis, Gay et al. 2015). This highlights an important difference between hypoxic/anoxic stress (which stimulates endogenous glucocorticoid release) and exogenous glucocorticoid exposure, potentially indicating alternate pathways.

Substantial evidence indicates the importance of epigenetic modifications including DNA methylation in glucocorticoid signaling (Crudo, Suderman et al. 2013, Sharma, Bhave et al. 2013, Petropoulos, Matthews et al. 2014). The glucocorticoid receptor is influential in the DNA methylation process, interactions between the glucocorticoid receptor and methylation specific proteins such as DNMT3b and MeCP2 are instrumental in epigenetic repression (Sharma, Bhave et al. 2013). Furthermore glucocorticoid exposure is associated with both methylation and demethylation of promoter and enhancer regions illustrating a potentially complex regulatory pathway (Thomassin, Flavin et al. 2001, Crudo, Suderman et al. 2013). Crudo and colleagues have demonstrated that the endogenous glucocorticoid surge alters the expression of methylation specific genes such as DNMT3b and Mbd2 and is instrumental in defining

DNA methylation patterns in an organ specific manner (Crudo, Petropoulos et al. 2012). The effects of glucocorticoids on methylation patterns are long lasting and are sustained into adulthood and even in subsequent generations (Crudo, Petropoulos et al. 2012, Crudo, Suderman et al. 2013). In the present study, we found that treatment with dexamethasone was not associated with changes in global methylation. Yet, dexamethasone induced hypermethylation of the cyclin D2 gene promoter, which was consistent with the decline in cyclin D2 protein and mRNA expression. Interestingly, inhibition of methylation by 5-AZA blocked dexamethasone-mediated cyclin D2 promoter hypermethylation and restored the expression of cyclin D2 protein and mRNA in cardiomyocytes. Of importance, 5-AZA also blocked the dexamethasone-induced effects in inhibiting cardiomyocyte proliferation and increasing myocyte binucleation. These findings illustrate an important role of DNA methylation in cardiomyocyte terminal differentiation and further implicate methylation as a vital factor in the induction of dexamethasone-mediated effects. Other studies also support the importance of methylation in perpetuating cardiomyocyte terminal differentiation (Kou, Lau et al. 2010, Gilsbach, Preissl et al. 2014). For instance, Kou *et. al.* found that inhibition of DNA methylation in cardiomyocytes using 5-Azacytidine resulted in increased DNA synthesis and delayed terminal differentiation during the development of postnatal day 7 and 10 rats (Kou, Lau et al. 2010). This is consistent with the present findings that dexamethasone treatment induced terminal differentiation prematurely and was associated with hypermethylation of the *CcnD2* gene promoter. Inhibition of methylation restored the developmental trajectory in myocytes treated with dexamethasone, suggesting that hypermethylation is a contributing factor in terminal differentiation. It is

apparent that methylation is also important in cell cycle regulation, studies in HeLa cells showed dynamic DNA methylation patterns within a single cell cycle with differential methylation occurring between the G1 and S phase (Brown, Fraga et al. 2007). The methylation of specific G1 proteins such as P16 resulted in transcriptional loss of protein expression (Matsuda, Ichida et al. 1999) and the cyclin D2 promoter was also a target of aberrant hypermethylation (Matsubayashi, Sato et al. 2003), indicating that methylation is of importance in cell cycle control and transition between the various stages.

In cardiomyocytes, cyclin D2 has been identified as an essential protein related to cardiomyocyte proliferative potential (McGill and Brooks 1995, Brooks, Poolman et al. 1997, Poolman and Brooks 1998, Nagai, Takano et al. 2001, Paradis, Gay et al. 2013). Considering that most adult cardiomyocytes are locked in the G1/G0 phase of the cell cycle (Capasso, Bruno et al. 1992, Walsh, Ponten et al. 2010) it is likely that decreased expression of the G1 cell cycle promoter, cyclin D2 contributes to the permanent cell cycle exit characteristic of terminal differentiation. Pasumarthi *et. al.* demonstrated that reestablishing cyclin D2 protein expression levels resulted in DNA synthesis in adult mitotically inactive cardiomyocytes (Pasumarthi, Nakajima et al. 2005). Studies in transgenic mice expressing cyclin D2 in the heart demonstrated a robust increase in cardiomyocyte DNA synthesis in the infarct border zone 7 days after myocardial injury, which was sustained up to 150 days after the injury (Pasumarthi, Nakajima et al. 2005). This was associated with an increase in cardiomyocyte number (Pasumarthi, Nakajima et al. 2005). Additionally, the regenerated myocardium of cyclin D2 expressing animals was associated with functional improvements of the heart (Hassink, Pasumarthi et al. 2008). Consistent with these findings, the present study demonstrated that overexpression of

cyclin D2 rescued the effects of dexamethasone on cardiomyocyte terminal differentiation, providing evidence of cause and effect relation between dexamethasone-induced down-regulation of cyclin D2 and the acceleration of terminal differentiation in cardiomyocytes.

In summary, the present study revealed a novel mechanism by which dexamethasone stimulates premature terminal differentiation in newborn cardiomyocytes. We demonstrated a causal role of down-regulating cyclin D2 in the direct effect of dexamethasone in inhibiting cardiomyocyte proliferation and increasing myocyte binucleation. While this present study established a key role of promoter methylation in dexamethasone-mediated epigenetic repression of cyclin D2 gene in cardiomyocytes other mechanisms may also be involved. Given the clinical importance of perinatal dexamethasone treatment in preterm infants and mothers at risk of preterm birth, and the role of endogenous glucocorticoids in mediating physiological stresses such as hypoxia, the present study provides a critical insight into the mechanisms of glucocorticoid-mediated harmful effects that may negatively impact in cardiomyocyte endowment in the heart and cardiac function later in life.

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 and the Loma Linda University School of Medicine.

CHAPTER FOUR

GENERAL DISCUSSION

Dexamethasone, Growth and Long Term Programming

This study presents compelling evidence for the detrimental effects of glucocorticoid therapy during cardiomyocyte development. While much is still to be elucidated about the mechanisms involved in cardiomyocyte terminal differentiation, this study identifies a role of glucocorticoid signaling and methylation during this process. Our findings suggest glucocorticoids, such as dexamethasone prematurely matures the heart by stimulating cell cycle exit, decreasing proliferation and increasing binucleate cardiomyocytes, resulting in less total cardiomyocytes in the adult heart. The cell cycle protein, cyclin D2 was found to be essential for normal terminal differentiation to occur and dexamethasone treatment resulted in down-regulation of its expression. In addition, the glucocorticoid receptor (GR) was identified as an essential component in dexamethasone signaling, since blocking the GR abrogates the effects of dexamethasone. Restoration of cyclin D2 levels abrogated the effects of dexamethasone, establishing a relationship between its decline and cell cycle withdrawal. Taken together, this study presents novel findings supporting dexamethasone-induced cardiomyocyte maturation and presents a potential mechanism, which involves methylation and glucocorticoid receptor activation.

Endogenous GCs are essential during development and normal maturation. During late gestation in mammals, a surge of glucocorticoids occurs, increasing the bioavailability in circulation. This surge stimulates maturation of the lungs and surfactant production; yet other organs are also sensitive to GCs (Fowden, Li et al. 1998).

Excessive, or poor timing of GC exposure is associated with increased disease risk, including cardiovascular disease (Cottrell and Seckl 2009, Harris and Seckl 2011). Considering the multi-target potential of GCS, its regulation is important. As described in chapter two, dexamethasone treatment results in altered growth of both the heart and body in rodents. Large-scale epidemiological studies report a correlation between birth weight, growth rate and adult health. In fact, low birth weight is associated with increased risk of cardiovascular disease (Barker, Gluckman et al. 1993), hypertension (Kwong, Wild et al. 2000) and diabetes (Barker 2004). The rate of growth is generally inversely proportional to disease risk, that is, compromised growth rate is associated with higher disease likelihood (Phillips, Walker et al. 2000, Osmond, Kajantie et al. 2007). While growth rate alone is an inadequate marker for disease outcome it does shed light on disease predisposition. When combined with other factors that further contribute to the development of diseases such as adult lifestyle, obesity, stress and the environment, the likelihood of pathology increases. In chapter two, neonatal exposure to dexamethasone during the first three days of life resulted in differential changes in growth rate. Heart weight significantly increased on neonatal day 7 and 14, accompanied by increased body weight at day 7. In terms of heart to body ratio day 14 animals treated with dexamethasone exhibited significantly larger heart to body ratios. Interestingly, other studies report the timing of glucocorticoid exposure can have differing results on growth, for instance, fetal exposure results in lower birth weights (Bloom, Sheffield et al. 2001) and in this study we report neonatal treatment accelerates growth, illustrating a time dependent mechanism of glucocorticoid action.

During development an organism is plastic, and can respond to the environment. Adaptation is essential to ensure the immediate survival of the organism, by ensuring synthesis of essential proteins and making metabolic or structural changes, if necessary (Barker 2004). However, during development there exist periods when an organism is especially vulnerable, in which small changes can have major life-long effects on health. Modifications that result in suboptimal function or altered cell numbers contribute to disease risk (Bae, Xiao et al. 2003, Bubb, Cock et al. 2007, Thornburg, Jonker et al. 2011). When glucocorticoids are given during periods of enhanced sensitivity, immediate changes including altered growth, morphology or organ function may occur. For instance, fetal exposure to the synthetic glucocorticoid bethamethasone results in physiological changes in the brain, such as decreased expression of synapsis promoting proteins (Antonow-Schlorke, Schwab et al. 2003). Also, offspring exposed to glucocorticoids in maternal circulation displayed abnormal prefrontal neuron morphology (Murmu, Salomon et al. 2006). In fact, in the brain, the complexity of structure and the density of dendritic spines decreased in animals exposed to glucocorticoids (Murmu, Salomon et al. 2006). In the heart, dexamethasone induces physiological changes such as suppressed proliferation and morphological changes (de Vries, Bal et al. 2006). This is in agreement with chapters two and three of this study; here we present data indicating dexamethasone treatment impairs proliferation both *in vivo* and *ex vivo*. Dexamethasone treatment inhibits the expression of the positive cell cycle protein, cyclin D2. In the heart, an important determining phase of adult cardiac health is when proliferation is still possible. The majority of proliferation occurs exclusively during the fetal and early neonatal phase with negligible proliferation thereafter. Hence, modifications in

proliferation during this time are especially consequential on overall cardiomyocyte number.

Consistent with proliferation changes, the percent of binucleate cardiomyocytes increased after dexamethasone treatment in chapter two and three, both *in vivo* and *ex vivo*. Binucleation is an important physiological marker of terminal differentiation. In chapters two and three, dexamethasone was found to induce similar changes *ex vivo* and *in vivo*. As such, these studies support a direct mechanism which dexamethasone matures the cardiomyocyte population. As one can expect, this abrupt maturation is not without consequences. In chapter two we report the mature hearts (neonatal day 14) of dexamethasone treated pups though larger than control hearts, contained less cardiomyocytes. This in agreement with the study by De Vries *et. al.* which found the suppression of proliferation in dexamethasone treated pups reduces the number of cardiomyocytes in the adult heart (de Vries, Bal et al. 2006). Since the number of viable cardiomyocytes influences the ability of the heart to respond to subsequent stress adequate cardiomyocyte endowment is essential for optimal cardiac function, a poorly endowed heart may be ill equipped to respond to insult. Here, interference of cellular proliferation during development was shown to result in fewer cardiomyocytes in the adult heart. This may potentially contribute to the altered cardiac function (Bal, de Vries et al. 2008), hypertrophic cardiomyopathy (Werner, Sicard et al. 1992) and reduced life expectancy (Kamphuis, de Vries et al. 2007), reported in other studies after antenatal and neonatal glucocorticoid exposure.

In this study we determined dexamethasone treatment during development accelerated cardiomyocyte maturation and terminal differentiation, consequentially

resulting in fewer myocytes in the adult heart. Neonatal dexamethasone exposure alters growth rate, proliferation, binucleation and cyclin D2 protein expression.

Potential Molecular Mechanisms of Premature Cardiomyocyte Terminal Differentiation

The present study identifies two essential mechanisms during dexamethasone-induced terminal differentiation, glucocorticoid receptor (GR) activation and the epigenetic changes in DNA methylation pattern. A growing body of evidence indicates DNA methylation regulates normal cellular events, including growth and the cell cycle. It is becoming clear that glucocorticoid receptor activation also influences growth (Katz, Penefsky et al. 1988, Porrello, Meeker et al. 2010), the cell cycle (Crochemore, Michaelidis et al. 2002, Li, Qian et al. 2012) and DNA methylation profile (Crudo, Petropoulos et al. 2013). Here we present compelling data indicating aberrant DNA methylation and GR activation are essential in dexamethasone signaling.

Glucocorticoids exert their effects by binding to two receptors, the glucocorticoid or mineralocorticoid receptor (MR). The MR has a higher intrinsic affinity for glucocorticoids than the GR, and synthetic glucocorticoids such as dexamethasone poorly activate the mineralocorticoid receptor. While both receptors are important, the glucocorticoid receptor has been identified as an essential component for heart maturation (Katz, Penefsky et al. 1988, Kayes-Wandover and White 2000, Rog-Zielinska, Thomson et al. 2013), but its role in cardiomyocyte development and more specifically terminal differentiation is still poorly understood. In chapters two and three, evidence is presented for the role of the GR in dexamethasone induced premature

terminal differentiation. Inhibition of the GR abrogated the dexamethasone-induced effects on heart and body weight, proliferation, binucleation, cyclin D2 protein and mRNA expression and cardiomyocyte number. Similarly, Tronche *et. al.* report GR deficient mice displayed altered growth resulting in smaller, less developed animals, indicating glucocorticoid signaling is important during growth (Tronche, Opherk et al. 2004). In GR null mice, marked increase in cellular proliferation occurs at day 17.5, supporting the influence of the GR on proliferation rate (Bird, Tan et al. 2007). Our *ex vivo* model indicates similar findings as our *in vivo* model, supporting the role of GR activation in dexamethasone-dependent maturation, terminal differentiation and cell cycle regulation.

Epigenetic modifications are important in cardiomyocytes. Chim *et. al.* determined on the first 7 days of life methylation specific proteins such as Methyl-CpG-binding protein 2 expression changes in the heart (Chim, Cheung et al. 2000). During normal rodent cardiomyocyte development DNA methyl transferase 1 (DNMT1), which ensures methylation patterns are copied to newly synthesized DNA, and methyl binding proteins increases gradually from neonatal day 1 to 14 (Kou, Lau et al. 2010). The developmental increase in DNA methylation and methylation regulators (Kou, Lau et al. 2010) coincides with the transition from hyperplasic to hypertrophic growth. Also, inhibition of methylation reversed terminal differentiation in cardiomyocytes, resulting in restoration of proliferation (Kou, Lau et al. 2010). Additionally, cardiomyocytes undergo dramatic changes in methylation patterns between developmental stages (Gilsbach, Preissl et al. 2014). Suggesting DNA methylation may at least, in part, regulate cardiomyocyte terminal differentiation.

DNA methylation is also important during glucocorticoid signaling (Crudo, Petropoulos et al. 2012, Crudo, Suderman et al. 2013). The normal glucocorticoid surge in near term mothers alters gene global methylation and expression in a variety of organs including the liver, kidney, adrenal glands and placenta (Crudo, Petropoulos et al. 2012). Synthetic glucocorticoids treatment also modifies DNA methylation patterns (Crudo, Petropoulos et al. 2012); the glucocorticoid receptor has been shown to interact with methylation specific proteins such a as MeCP2 and DnMT3, which increases methylation of specific gene promoters (Sharma, Bhave et al. 2013). A large body of evidence is laid out in chapters two and three, which evaluates whether methylation is a mediator in the effects of dexamethasone. To illustrate the importance of methylation throughout cardiomyocyte development and during dexamethasone treatment 5-aza-2'-deoxycytidine, a methylation inhibitor was used.

In chapters two and three we found inhibition of methylation blocked all effects of dexamethasone on growth, proliferation, binucleation and cyclin D2 protein and mRNA, suggesting methylation plays a role in potentiating the effects of dexamethasone. Furthermore, inhibition of methylation in vivo (Chapter two) resulted in the significant increase in proliferation, after dexamethasone treatment. This is in agreement with Kou *et. al.* that found inhibition of methylation restores cardiomyocyte proliferation and delays terminal differentiation, implicating methylation in the terminal differentiation process (Kou, Lau et al. 2010).

In chapter two of this study 5-aza-2'-deoxycytidine decreased global methylation in the heart, however no changes in global DNA methylation occurred with dexamethasone treatment. We also found global methylation declines in saline control

animals between neonatal day 4 and 7 as terminal differentiation is occurring in the heart. This is contrary to what was expected since Kou *et. al* suggest a developmental increase of DNA methylation during the neonatal phase (Kou, Lau et al. 2010). This discrepancy is likely due to the sample source. Kou and colleagues measured methylation from isolated cardiomyocytes while our studies utilized the whole heart. As expected the whole heart is not a homogenous sample but represents a collection of cells, all of which contributes to the methylation patterns reported here. Interestingly, another study reports a decline in the DNA methylation pattern in cardiomyocytes during the neonatal phase similar to our findings (Gilsbach, Preissl et al. 2014). Gilsbach et. al. identified large scale demethylation from embryonic stem cells to neonatal cardiomyocytes (Gilsbach, Preissl et al. 2014). Gene ontology analysis determined during postnatal development genes involved in cardiac contraction and mitochondrial function was further demethylated. While the majority of genes in neonatal cardiomyocytes were hypomethylated a small amount of genes were differentially hypermethylated (Gilsbach, Preissl et al. 2014). Taken together these studies support the dynamic methylation of cardiomyocyte genes. Our study and that of Kou and colleagues suggests specific genes involved in terminal differentiation may be hypermethylated since inhibition of methylation reverses terminal differentiation (Kou, Lau et al. 2010). Thus necessitating gene specific methylation studies.

Considering that dexamethasone exposure in rodents results in down regulation of cyclin D2 expression we examined the DNA methylation pattern of the cyclin D2 gene (*CcnD2*) promoter. In this study we determined the two regions most proximal to the transcription start site was hypermethylated. This is in agreement with the decrease

expression of cyclin D2 protein and mRNA observed in chapter 3. 5-AZA rescued mRNA and protein expression after dexamethasone treatment in chapter three. These studies describe a novel mechanism involving methylation of the CcnD2 gene promoter and GR activation to propagate the effects of dexamethasone on cardiomyocytes.

Potential Molecular Targets of Premature Terminal Differentiation

It is clear from this study and others that dexamethasone treatment alters cardiomyocyte maturation (Rog-Zielinska, Craig et al. 2014). Yet, the exact mechanism and potential molecular targets remain elusive. A plethora of potential contributing factors exist, varying from cell cycle proteins to epigenetic changes (Engel, Schebesta et al. 2006, Di Stefano, Giacca et al. 2011, Porrello, Johnson et al. 2011, Beigi, Schmeckpeper et al. 2013). In this study we identified the importance of cell cycle proteins during terminal differentiation.

It is evident that terminal differentiation is closely related to the cell cycle since myocytes lose proliferative capacity and the majority becomes arrested in the G0/G1 phase (Capasso, Bruno et al. 1992, Brooks, Poolman et al. 1998, Walsh, Ponten et al. 2010). Terminal differentiation is also associated with the coordinated loss of pro-proliferative factors and an increase in cell cycle inhibitors (Brooks, Poolman et al. 1998, Pasumarthi and Field 2002). Cell cycle progression is tightly regulated by cellular checkpoints to ensure proper progression. The regulators of the late G1 phase checkpoint include the D cyclins (D1, D2, or D3) and the cyclin dependent kinases (CDK4 and CDK6). When complexed CDKs and their respective cyclins are able to phosphorylate

members of the Rb family, resulting in the release of transcription factor E2F. This then promotes exit from the G1 phase.

While cyclin D2 is very important for G1 cell cycle exit, its expression decreases during myocyte development, becoming relatively undetectable in adults (Brooks, Poolman et al. 1997). Physiological stresses also influence the expression of cyclin D2. As discussed in chapters two and three, *in vivo* and *ex vivo* dexamethasone treatment resulted in a significant decrease in cyclin D2 protein expression, which was rescued by inhibition of methylation and blockade of the GR. Consistent with this hypoxic and anoxic episodes in the fetus (Tong, Xiong et al. 2013) and neonate (Paradis, Gay et al. 2015) decreases cyclin D2 expression. During physiological stresses such as hypoxia, circulating glucocorticoid levels increase and is believed to mediate stress response. Thus, the similarities in gene regulation between hypoxia/anoxia episodes and dexamethasone exposure may indicate a common response between endogenous and exogenous glucocorticoid exposure. In chapter two of this study no changes in p27 (a negative cell cycle regulator) occurred after dexamethasone treatment, yet p27 significantly increases after hypoxia and anoxia episodes (Tong, Xiong et al. 2013, Paradis, Gay et al. 2015). This discovery suggests an important difference between hypoxic/anoxic stress and exogenous glucocorticoid exposure and may indicate an alternate pathway or mechanism.

Pasumarthi *et. al*. demonstrated reestablishing cyclin D2 expression levels is beneficial for cell cycle reentry, even in mitotically inactive cardiomyocytes (Pasumarthi and Field 2002, Hassink, Pasumarthi et al. 2008). Transgenic expression of cyclin D2 after myocardial injury induces a two-fold increase in DNA synthesis along with the

regression of infarct size, and cardiomyocyte number increase(Pasumarthi, Nakajima et al. 2005). DNA synthesis in the myocardial injury border was sustained up to 150 days after the injury(Pasumarthi, Nakajima et al. 2005). Additionally, functional improvements in the heart were also evident in cyclin D2 expressing mice after injury (Hassink, Pasumarthi et al. 2008). Indicating that targeted expression of cyclin D2 in cardiomyocytes can overcome cell cycle blockage and stimulate proliferation even in adult, mitotically inactive cardiomyocytes. We therefore overexpressed cyclin D2 in cardiomyocytes and treated with dexamethasone to evaluate whether rescuing cyclin D2 expression will inhibit the effects of dexamethasone and reestablish the normal terminal differentiation trajectory. Indeed, overexpression of cyclin D2 *(+CcnD2*) was sufficient to block all effects of dexamethasone, even on cyclin D2 protein levels, which remained comparable to control. Restoring cyclin D2 protein expression abrogated the effects of dexamethasone on proliferation, binucleation and cyclin D2 expression, establishing a direct cause and effect relationship between cyclin D2 down-regulation and acceleration of terminal differentiation.

Other Potential Mediators of Terminal Differentiation

While this study presents novel and cogent evidence supporting cyclin D2 as a target of dexamethasone and identifies hypermethylation of the CcnD2 promoter as a molecular target, other mechanisms and regulators are yet to be elucidated. For instance, GATA4 is an established transcriptional regulator of numerous cell cycle genes, including cyclin D2 (Rojas, Kong et al. 2008, Yamak, Temsah et al. 2012). Rojas *et. al.* determined GATA4 binds to the cyclin D2 and Cdk4 promoters directly regulating their

expression, the inactivation of GATA4 results in right ventricle hypoplasia, and proliferative defects (Rojas, Kong et al. 2008). This illustrates GATA4 is instrumental in establishing cellular number. Glucocorticoids such as beclomethasone are shown to down-regulate GATA4 expression resulting in inhibition of normal regeneration of the injured myocardium (Huang, Yang et al. 2013). While research is limited in rodents it is possible that the GATA4 may be an upstream target of dexamethasone, resulting in cyclin D2 down-regulation.

Alternatively, in terms of cell cycle regulation considerable research has recently been dedicated to the phosphoinositide 3-kinase (PI3K) and AKT pathway, which may present another potential target during cardiomyocyte terminal differentiation. In non-cardiomyocytes glucocorticoids are identified as suppressors of the PI3K/AKT pathway resulting in cell cycle arrest (Zou, Yang et al. 2015). The phosphoinositide 3-kinase (PI3K) and AKT pathways seems to be beneficial in cardiomyocyte cell cycle activation. C3orf58, a novel secreted paracrine factor termed hypoxia and Akt induced stem cell factor (HASF) is a stimulator of DNA synthesis and promoter of mitosis and cell division dependent on PI3k and AKT pathway (Beigi, Schmeckpeper et al. 2013). Overexpression of HASF stimulates DNA synthesis in adult hearts in response to injury by increasing the expression of several cyclins (Beigi, Schmeckpeper et al. 2013). The transcriptional co-activator yes-associated protein (YAP) has been classified as essential for myocyte proliferation, survival and as an upstream activator of the PI3K-AKT pathway(Del Re, Yang et al. 2013, Xin, Kim et al. 2013). Del Re and colleagues identified the homozygous deletion of *YAP1* in cardiomyocytes resulted in cardiomyopathy and premature death (Del Re, Yang et al. 2013). This was measured by increased fibrosis,

apoptosis and attenuation of proliferation. During neonatal development YAP expression decreases and seems to be associated with the up-regulation of cell cycle inhibitors such as p27 (Lin, Zhou et al. 2015). The targeting of *Yap1* and upstream regulation of the kinase pathway leading to its activation appear to be involved in cardiomyocyte terminal differentiation. These studies provide insights into alternate pathways that may be activated during cell cycle exit. However, the role of glucocorticoids in inhibition of the PI3K/AKT pathway in cardiomyocytes has not been well characterized.

Additionally, epigenetic modifications such as histone modifications and microRNAs (miRNA) may also play a role in cardiomyocyte terminal differentiation and should also be considered. Several miRNAs in the miR-15 family are identified as important physiological regulators of cardiomyocyte proliferation and the cell cycle (Porrello, Johnson et al. 2011). MicroRNAs are small non-coding RNAs that recognizes and binds complementary mRNA sequences primarily in the 3' untranslated region of target genes, inducing gene silencing. The microRNAs involved in the onset of cardiomyocyte binucleation and cell cycle withdrawal include miR-15a, miR-15b, miR-16-1, miR-16-2, miR-497 and miR-195, with miR-195 being the most highly expressed (Porrello, Johnson et al. 2011). Interestingly, miR-195, miR-497, miR-15a and miR-16 are up regulated during neonatal day 7-14, when the majority of cardiomyocytes are binucleate (Porrello, Johnson et al. 2011). Between P7-P14, miR-195 expression in the heart is inversely correlated with the expression of several genes for mitosis, cell division and the cell cycle such as *Chek1*, *Cdc2a*, *Birc5*, *Nusap1*, and *Spag* (Porrello, Johnson et al. 2011). The expression of the miR-15 family of miRNAs is closely associated with cell cycle regulation (Bandi, Zbinden et al. 2009, Klein, Lia et al. 2010), overexpression of

miR-15a and miR-16 induces cell cycle arrest in the G1/G0 phase of the cell cycle (Bandi, Zbinden et al. 2009). The inhibition of the miR-15 family led to an increase in mitotic entry and progression of cardiomyocytes in the cell cycle (Porrello, Johnson et al. 2011). However, inhibition of the miR-15 family was insufficient to stimulate cytokinesis, as Aurora B kinase expression was still repressed (Porrello, Johnson et al. 2011).

Histone modifications may also play a contributing role in terminal differentiation. Eukaryotic DNA is wrapped around an octomer of histones that are organized into chromatin structures. Epigenetic modification of histones dictates the accessibility. The loosely packed euchromatin is transcriptionally active while heterochromatin is tightly coiled making the gene inaccessible for transcription. Histone methylation typically results in a heterochromatin structure and occurs in terminally differentiated cells (Brero, Easwaran et al. 2005, Sdek, Zhao et al. 2011). In adult cardiomyocytes modifications of histones H3K9me2/3 and H3K27me are associated with repression of transcription and heterochromatin configuration (Sdek, Zhao et al. 2011). Cell cycle genes are identified as targets of histone modification. Rb is a cell cycle repressor that induces repression by two methods, inhibition of E2f or epigenetic remodeling. Members of the retinoblastoma family (Rb, p107, and p30) are involved in orchestrating and targeting histone modification of E2F-dependent genes (Sdek, Zhao et al. 2011). Rb proteins recruit enzymes such as histone deacetylase 1 (HDAC1), which promote gene repression (Chung, Cho et al. 2002). Polycomb Repressive Complex 2 (PRC2) is known to be involved in the trimethylation of histone H3 at lysine 27 (H3K27me3). A component of the PRC2 complex, Enhancer of Zeste 2 (Ezh2) is

believed to ensure normal cardiac growth and adult activity (Delgado-Olguin, Huang et al. 2012). Ezh2 has also been shown to repress negative regulators of the cell cycle such as Ink4a and Ink4b. He and colleagues inactivated the Ezh2 subunit of PRC2 and noted hypoplasia and upregulation of Ink4a/b (He, Ma et al. 2012). This data potentially implicates Ezh2 as a regulator of proliferation in the heart possibly via methylation. The discovery of the epigenome has expanded the possibilities of biological regulation. It is evident that epigenetic modifications are involved in regulating cardiomyocyte proliferation and maturation. This complex regulation appears to include many epigenetic modifications including microRNAs, DNA methylation, and histone modifications.

Perspective on Neonatal Dexamethasone Therapy

Corticosteroid treatment was first used in preterm infants for lung maturation in 1972 (Liggins and Howie 1972, 1995). Since then corticosteroids have been administered to women at risk of preterm delivery and in high-risk pregnancies, and is a very successful intervention (1995, Tyson, Parikh et al. 2008). However, glucocorticoid treatment during periods of heightened sensitivity is associated with growth, neuronal, and HPA-axis defects(Torres, Belser et al. 1997, McCabe, Marash et al. 2001, Bal, de Vries et al. 2008). Here data is presented indicating glucocorticoid exposure during the neonatal phase is associated with cardiac consequences. In our present study we demonstrate that neonatal dexamethasone exposure stimulates cell cycle withdrawal prematurely, resulting in fewer cardiomyocytes in the adult heart. We identified dexamethasone as being influential on heart and body growth, with differing effects based on timing and duration of exposure (Torres, Belser et al. 1997, Bal, de Vries et al.

2008). Also, glucocorticoid receptor activation and DNA methylation were identified as essential components in perpetuating the effects of dexamethasone. Considering the negative consequences of glucocorticoids on the heart and the importance of DNA methylation in developmental disease programming, further studies are necessary to determine mechanisms that may limit the deleterious effects of dexamethasone and should be further evaluated.

Much research has been dedicated to understanding cardiomyocytes and attempting to restore regenerative potential (Poss, Wilson et al. 2002, Bergmann, Bhardwaj et al. 2009, Jopling, Sleep et al. 2010, Porrello, Mahmoud et al. 2013). The adult heart is largely believed to be a post-mitotic organ and thus only capable of modest regeneration. Our study identified DNA methylation as a factor that limits the capability of cardiomyocytes to proliferate. It may therefore present a potential therapeutic target that may be exploited to regain proliferation. Additionally, selective activation of the cell cycle is also promising. In this study we identified the target down-regulation of cyclin D2 as a mechanism used by dexamethasone to achieve cell cycle withdrawal and terminal differentiation. It is evident that reestablishing cell cycle protein expression may also be of value as a therapeutic. Thus, this study enriches our understanding of the pathology risk associated with dexamethasone treatment in the heart and the mechanisms involved.

Conclusion

The developmental environment may arguably be the most important influence on adult health. Glucocorticoids are essential for normal development and promote organ maturation. Due to the stimulatory nature of glucocorticoids, such as dexamethasone,

regulation is important. Yet, during development glucocorticoid exposure is common in stressed infants or as a pharmacologic intervention for preterm infants. Other studies report neonatal glucocorticoid exposure is associated with elevated cardiovascular disease risk. In this study we demonstrate clinically relevant doses of dexamethasone accelerates cardiomyocyte maturation. Cardiomyocytes have a limited proliferative potential, the inability of cardiomyocytes to reenter the cell cycle makes the proliferation period a critical window in cardiomyocyte development. In fact, a consequence of dexamethasone reported in this study is the significant reduction of the cardiomyocyte population. We further report the effects of dexamethasone are modulated by DNA methylation and require glucocorticoid receptor activation. In terms of molecular targets, cell cycle proteins seem to be closely associated, the G1 phase promoter cyclin D2 protein and mRNA expression declined as a result of dexamethasone treatment. This down-regulation of cyclin D2 protein is probably due to hypermethylation of the cyclin D2 promoter, observed here. Furthermore, all effects of dexamethasone were abrogated by overexpression of cyclin D2, further implicating cell cycle dysregulation in dexamethasone mechanism of action. This study provides strong evidence for the effects of dexamethasone and the cardiovascular consequences associated with exposure. Also, this study highlights regulatory mechanisms involved in both premature and normal terminal differentiation. Finally, we delineated a cause and effect relationship between decreased cyclin D2 expression and terminal differentiation. However, it is likely that many other molecules and pathways are involved. It is therefore paramount that further studies are conducted, considering the clinical importance and implications of this study.

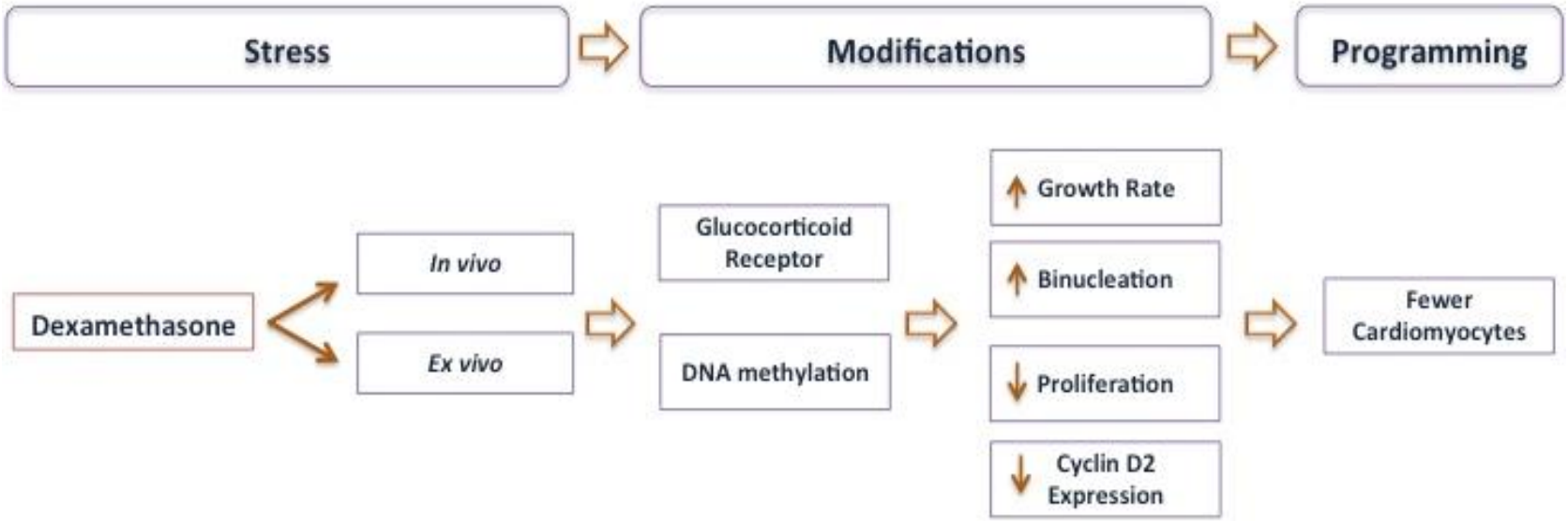

Figure 4.1. Proposed effects of dexamethasone on cardiomyocyte maturation. Dexamethasone accelerates the maturation of cardiomyocytes by increasing growth rate, and binucleate cardiomyocyte population and decreasing the rate of proliferation and cyclin D2 expression, resulting in fewer cardiomyocytes in the mature heart. The effects of dexamethasone appear to be glucocorticoid receptor dependent and methylation mediated.

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