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Effect of Maternal Hypoxia on the Cerebral and Cardiac Tissue Remodeling

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The Effect of Maternal Hypoxia on the Cerebral and Cardiac Tissue Remodeling

by

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ABBREVIATIONS

PO_2	Arterial blood partial pressure of oxygen
MMP	Matrix metalloproteinase
TIMP	Tissue inhibitors of metalloproteinase
ECM	Extracellular matrix
TNF-α	Tumor necrosis factor-alpha
STAT	Signal transducers and activators of transcription
HIF	Hypoxia inducible factor
LV	Left ventricular
TGF-β	Transforming growth factor beta
ROS	Reactive oxygen species
MT-MMP	Membrane type matrix metalloproteinase
IL	Interleukin
BBB	Blood brain barrier
MI	Myocardial infarction
NF-κB	Nuclear factor kappa B
AP-1	Activating protein 1
DDR	Discoidin domain receptor
siRNA	Small interfering RNA
BrdU	Bromodeoxyuridine

ABSTRACT OF THE DISSERTATION

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Dr. Lubo Zhang, Chairperson

Large epidemiological studies and animal studies have indicated a clear association of adverse intrauterine environment with an increased incidence of low birth weight, perinatal mortality, and cardiovascular disease in later adult life. In humans, one of the most common stresses in utero is hypoxia. The goal of this project is to test the hypothesis that fetal hypoxia causes reprogramming of MMPs and TIMPs expression patterns and activities leading to aberrant brain and heart development in the fetuses and neonates. In the first part of project, we tested the hypothesis that maternal hypoxia increases the activity of MMPs and decreases the expression of TIMPs in the brain of neonatal rats. This was accomplished by using a rat model. Time-dated pregnant rats were divided between normoxic and hypoxia (10.5% O_2 on days 15-21 of gestation). Our studies demonstrated that fetal hypoxia impaired the proteolytic balance by increasing the activity of MMPs and decreasing the expression of TIMPs in the brain of neonatal rats and that unopposed extracellular matrix degradation was likely to contribute to the brain injury and growth restriction observed in the present study. The second part of project focused on the effect of fetal hypoxia on heart development. We demonstrated that maternal hypoxia during gestation altered MMPs and TIMPs expression patterns in the developing heart and resulted in the abnormal cardiac growth pattern and cardiomyocyte

proliferation with the aberrant content of fibrillar collagen network in fetal and neonatal hearts. We further explored the direct effect of hypoxia on cardiomyocyte proliferation by using intact fetal hearts ex vivo model and H9c2 cells in vitro model. Our collective observations indicated an inhibitory effect on cell proliferation in the developing hearts following hypoxia, which can be mediated by upregulation of TIMP-4. These findings provide a mechanistic understanding worth of investigation in humans in fetal origins of neurovascular and cardiovascular disease caused by intrauterine adverse environment.

CHAPTER ONE

INTRODUCTION

Mammalian embryos grow in a state of relatively low partial oxygen tension (as compared with the adult), and this state is necessary for vasculogenesis, angiogenesis, hematopoiesis and chondrogenesis during fetal development (Ream et al., 2008). The partial oxygen tension of a developing embryo is below 10 mmHg and the fetal arterial blood partial pressure of oxygen (PO_2) is approximately 20 mmHg, both of which are regarded as being hypoxic compared to normal tissue with an oxygen tension of 20–40 mmHg and normal arterial blood partial pressure of oxygen of 100 mmHg (Webster and Abela 2007). This suggests that, compared to the adult, the fetus is persistently relative hypoxic during organ formation, growth and maturation, and that fetal tissues have a lower threshold at which they reach a state of oxygen insufficiency (Patterson and Zhang 2010). Although fetal PO_2 is low in comparison to the adult value, adequate delivery of oxygen to the fetal tissue is still maintained by several important mechanisms. Fetal cardiac output is greater than that of adult, which facilitates oxygen transportation to fetal tissues. In addition, higher hemoglobin concentration and affinity for oxygen in the fetus can also increase the oxygen carrying capacity of fetal blood. Finally, the fetal organs are relative overperfused compared with their oxygen requirements. Thus, the oxygen concentration in fetal blood is only slightly less than that in maternal blood, and oxygen delivery to some organs even exceed their adult counterparts (Richardson and Bocking, 1998).

Although the restricted oxygen supply is essential for intrauterine growth, excessive or severe hypoxia might compromise normal development and can adversely affect the fetus in various ways (Thornburg et al., 2008). Large epidemiological studies and animal studies have indicated a clear association of adverse intrauterine environment with an increased incidence of low birth weight, perinatal morbidity and mortality, neurological deficits, and cardiovascular disease in later adult life (Barker and Osmond, 1986; Bateson et al., 2004; Gluckman et al., 2008; McMillen and Robinson, 2005). This finding implies adaptations made by the fetus in response to intrauterine stresses leads to permanent changes in postnatal physiology, namely "fetal programming". The prenatal adaptive change in gene expression patterns and phenotype modifies the growth of specific organs during the critical period of development and predisposes the body to heightened susceptibility of cardiovascular and neurovascular disease in its adult life. In humans, one of the common stresses in utero is hypoxia, which may occur under many conditions, including pregnancy at high altitude, pregnancy with cigarette smoke, drug abuse, anemia, pulmonary disease and hypertension (Zhang, 2005). However, little is known about the fetal adaptive mechanisms involved and potential therapeutic approaches. Elucidating the various molecular mechanisms underlying the structure and functional changes in cardiac and cerebral growth is an area of ongoing research.

Fetal hypoxia activates various cardiovascular, endocrine and metabolic responses. For example, it alters heart rate, increases arterial blood pressure, and causes a redistribution of cardiac output to the vital organs, such as the brain and heart (Arbeille et al., 1995; Teitel and Rudolph, 1985). In the brain, the autoregulation of middle cerebral artery and other cerebral arteries allows vasoconstriction or vasodilatation, attempting to

maintain the delivery of oxygen and other nutrients in the cerebral tissue (Salihagić-Kadić et al., 2006). However, in the presence of prolonged hypoxia, the loss of cerebrovascular autoregulation occurs, which may lead to adverse fetal outcome eventually. This can be the result of following events. The cerebral vessels may reach their maximal dilatation, or the vessel dilatation is limited due to the formation of brain edema in the presence of prolonged hypoxia. Moreover, hypoxia may induce lesions of the cerebral tissue, which impairs the autonomous cerebral flow regulation (Salihagić-Kadić et al., 2006). In the heart, the occurrence of cardiac remodeling modifies the structure, function and gene expression in the fetal heart to attempt to compensate for the hypoxic stress. Cardiac hypertrophy and fibrosis are the major processes during the heart remodeling in the adaptive response to fetal hypoxia. Hypertrophy owing to the cardiomyocyte enlargement and hyperplasia might occur as a result of an increased myocardial workload (Dasgupta and Zhang, 2011; Zhang, 2005; Sundgren et al., 2003 and 2003; Giraud et al., 1993; Jonker et al., 2010). Fibrosis is characterized by a disproportionate accumulation of fibrillar collagen, which stiffens the ventricles and causes the loss of compliance and the impairment of contraction and relaxation (Manabe et al., 2002). The adaptive alteration of the fetal heart might not change the basal cardiovascular function but might cause heightened vulnerability to ischemic injury in adulthood (Patterson et al., 2010; Xue et al., 2011; Xue and Zhang, 2009). Fetal hypoxia also increases the risk of heart failure and other cardiovascular disease in later postnatal life (Tintu et al., 2007). In addition to the adverse effect on heart development, numerous studies have demonstrated that fetal hypoxia is one of the major causes of neurodevelopmental impairment and neurological deficits in the offspring (Louzoun-

Kaplan et al., 2008; Haramati et al., 2010; Golan et al., 2009; Sullivan et al., 2010; Miles and Kernie, 2008).

Recent studies indicate that the timely breakdown of extracellular matrix (ECM) is crucial for normal fetal development (Nagase and Woessner, 1999). ECM is a complicated microenvironment that includes a range of matrix proteins, signaling molecules, proteases and cell types involved in the tissue remodeling process (Spinale, 2007). Various factors that participate in the cardiac and cerebral remodeling have been revealed and matrix metalloproteinases (MMPs) are one of the most significant mediators in ECM turnover. MMPs are a family of zinc-dependent proteases. Together with tissue inhibitors of metalloproteinases (TIMPs), they have been implicated in a variety of physiological and pathological processes in the cardiovascular and central nervous systems, including the modulation of fibrillar collagen structure and deposition, and the regulation of cell proliferation and cell death. MMPs can be regulated at the transcriptional level and their activities can be inhibited by their endogenous inhibitors, the TIMPs. Nevertheless, whether and to what extent MMPs and TIMPs expression patterns in the developing brain and heart are altered by in utero hypoxia remain to be elusive.

Fetal Hypoxia and Brain Development

Fetal hypoxia has been shown to link to brain growth restriction and neurological deficits during development. When pathophysiological hypoxia occurs, the brains cells can reduce non-obligatory energy consumption by initially switching to lower energy requiring state and may completely inhibiting neuronal activity if the hypoxic insult becomes more severe. The brain cells can also use anaerobic metabolism to maintain

their production of high-energy metabolites for a time. However, the use of anaerobic metabolism is very inefficient as anaerobic glycolysis produces lactate and only 2 ATP. As the glucose is consumed rapidly, the excessive lactic acid production causes metabolic acidosis, along with other local or systemic consequences such as impaired cardiac contractility and vascular tone (Gunn and Bennet, 2009).

Evidence indicating the detrimental effects of in utero hypoxia on the fetal brain is also well documented. The reduced fetal cerebral oxygenation could result from a decrease in fetal cerebral perfusion owing to a failure of cerebral–hypoxic vasodilatation, cardiac decompensation or perinatal stroke (Rees et al., 2008). Low brain oxygenation in fetuses is associated with abnormal neurovascular development and an increased risk of brain injury, such as cerebral palsy or periventricular leukomalacia (PVL) in newborns (Hallak et al., 2000; Volpe, 2001). It has been shown that 2 h of transient hypoxia (9% oxygen) during late pregnancy reduced the level of brain-derived neurotrophic factor (BDNF) in the fetal brain, probably in relation to the impaired morphology of the hippocampus and cerebellum during development (Golan et al., 2004). Hypoxia did not stimulate inflammation or cell death, but delayed neuronal migration by diminishing the proteins involved in the migration, such as Reelin, Disabled1 and amyloid precursor protein, in the fetal brain (Golan et al., 2009). Hypoxia might regulate cell proliferation by altering the fetal cerebella gene expression, which is evidenced by a trend of upregulation in cell cycle-related genes seen 2 h after fetal hypoxia, followed by downregulation 24 h or 20 days after hypoxia (Haramati et al., 2010). In addition, key proteins in the gamma-aminobutyric acid (GABA) pathway were immediate repressed by transient hypoxia in the fetal cerebral cortex, which might result in an increased

susceptibility to seizures and epilepsy in the offspring (Louzoun-Kaplan et al., 2008). It is possible that hypoxia-induced fetal programming in the brain development is responsible for the increased vulnerability to cerebral insults in adulthood (Herlenius and Largercrantz, 2001). Meanwhile, GABA has a trophic effect during early brain development, and hypoxia might alter the function of GABAergic transmission during this period, thus compromising the development of neuronal wiring, plasticity of the neuronal network, and affecting the neural organization (Herlenius and Largercrantz, 2001).

Interestingly, chronic fetal hypoxia appears to regulate brain tissue remodeling by a similar pathway. In a guinea pig model, the enhancement of oxidative stress and apoptosis caused by chronic fetal hypoxia is mediated by inflammatory cytokines activation in the fetal brain (Guo et al., 2010); this finding is also supported by our own study that downregulation of TIMPs by chronic hypoxia might participate in increased apoptosis in the neonatal rat brain (Tong et al., 2010). Taken together, insufficient oxygen alters fetal brain growth, resulting in abnormalities in fetal and neonatal cerebral structure. This abnormality probably involves a sustained reduction in neuronal proliferation and increased cell death.

Fetal Hypoxia and Cardiac Remodeling

Many studies have demonstrated that hypoxia results in a decrease in fetal body weight, but an increase in the heart:body weight ratio (Bae et al., 2003; Camm et al., 2010; Xu et al., 2006; Wang et al., 2009; Xiao et al., 2000). During fetal hypoxia, there is a redistribution of fetal cardiac output from the periphery to essential organs such as the brain and heart. This is induced by carotid chemoreflex and sustained by the local

vasodilators nitric oxide and adenosine in the essential circulation and the peripheral vasoconstriction factors, catecholamines and neuropeptide Y (Camm et al., 2010). This causes the retarded growth of nonessential organs and tissues, but maintains growth of the heart and brain. Nevertheless, fetal hypoxia causes apoptotic cell death in the heart and increases the number and size of binucleated cardiomyocytes (Bae et al., 2003). In normal fetal heart development, cardiomyocytes first undergo hyperplasia before midgestation. In rats, the cardiomyocytes become binucleated and terminally differentiated in the first two weeks after birth (Louey and Thornburg, 2005). The binucleated cardiomyocytes are no longer capable of proliferation and division. Fetal hypoxia interrupts the proliferation of myocytes prematurely and cardiomyocytes undergo hypertrophic growth to compensate for the reduced number of myocytes. In fact, the enlargement of the fetal heart following hypoxia has been demonstrated (Bae et al., 2003).

Although the mechanism of cardiac hypertrophy resulting from chronic hypoxia has not been well established, a study using long-term intermittent hypoxia has demonstrated that tumor necrosis factor-alpha (TNF-α), insulin-like growth factor II (IGF-II), phosphorylated p38 mitogen-activated protein kinase (p38 MAPK), signal transducers and activators of transcription-1 (STAT)-1 and STAT-3 are involved in 4-week hypoxia-induced hypertrophic myocardium and increased interstitial space (Chen et al., 2007). In addition, interleukin-6 (IL-6), mitogen-activated protein kinase 5 (MEK5) and extracellular signal-regulated kinase 5 (ERK5) are activated with 8-week-hypoxia exposure (Chen et al., 2007), suggesting that a pro-inflammatory cytokine pathway is involved in cardiac hypertrophy triggered by long-term hypoxia. Studies of chronic

anemic fetal sheep have illustrated increases in biventricular cardiac output and myocardial blood flow accompanied by fetal heart hypertrophy (Mascio et al., 2005; Martin et al., 1998). This is not unexpected because there is an association between prenatal hypoxia and the development of primary pulmonary hypertension in the offspring (Rueda-Clausen et al., 2009). In this case, increased capillary blood supply and ventricular work are required to maintain cardiac function, and increasing myocardial vessel growth might be an adaptation to anemia–hypoxia insult.

It has been shown in fetal lambs that anemia and/or hypoxia increase hypoxia inducible factor 1 (HIF-1) and vascular endothelial growth factor (VEGF) and cause capillary coronary vascular growth (Martin et al., 1998; Cartwright et al., 2007). Additionally, HIF-1 also induces the transcription of glycolytic enzymes to maintain myocardial use of peripherally generated lactate as an energy substrate in the hypoxic condition (Martin et al., 1998). Thereby, cardiac hypertrophy with angiogenesis stimulated by the pro-inflammatory cytokines, HIF-1 and VEGF, has an essential role in the adaptive mechanisms in response to chronic hypoxia. Interestingly, hypoxia during the early fetal development stage resulted in myocardial thinning in a rat model, which is different from the increased heart:body weight ratio or hypertrophy that occurred with hypoxia at late gestation (Ream et al., 2008). This finding implies that the distinct duration and gestational periods of hypoxia might determine the nature and severity of abnormal heart development.

In addition to cardiomyocyte hypertrophy, alteration of ECM components, particularly interstitial collagens in the heart, is seen in cardiac remodeling caused by hypoxia. Type I (85% of cardiac interstitium) and type III (11% of cardiac interstitium)

collagens are the major collagens in the connective tissue network of the vertebrate heart and form several distinct organized layers in the heart walls to provide rigidity and elasticity (Carver et al., 1993; Weber, 1989). Human fetal heart expresses collagen III before collagen I during the second trimester and collagen III forms a major component of the collagen network in the fetal heart (Jackson et al., 1993). After birth, the ratios of total collagen to total protein as well as that of collagens I to III are high in neonatal hearts and they gradually decrease with age, which explains the relatively rigid and less compliant heart in the neonate as compared with that in the adult (Marijianowski et al., 1994). The ECM network of the heart is formed by a complex three-dimensional arrangement of glycoproteins and proteoglycans and is closely linked to cardiac function (Carver et al., 1991). This elastic and stress-tolerant network is responsive to multiple pathophysiological signals, for example, myocardial hypoxia that stimulates the synthesis of collagens (Carver et al., 1991; Wikman-Coffelt et al., 1979).

Generally, ECM turnover during development under both normoxic and hypoxic conditions is tightly controlled by coordinated degradation and synthesis of collagens and other ECM components. Excessive amounts of collagens released from cardiac fibroblasts might contribute to ventricular stiffness and impairment of diastolic filling after cardiac insults (Kania et al., 2009). A study by Xu et al. confirmed that fetal hypoxia significantly enhances beta and/or alpha myosin heavy chain (MHC) isoform ratio and collagens I and III accumulation, yet reduces MMP-2 activity in the left ventricular (LV) of adult rat offspring; this suggests that impaired fetal development leads to dysregulated collagen deposition in the heart and alters the susceptibility of the heart to ischemia and/or reperfusion injury (Xu et al., 2006). Fetal hypoxia alters the expression patterns of

many genes, including the 70-kd heat shock protein (HSP70), endothelial nitric oxide synthase (eNOS), beta2-adrenoreceptor, protein kinase C epsilon isozyme and type 2 angiotensin II (AT2) receptors in the heart, and causes LV remodeling (Patterson et al., 2010; Xue et al., 2011; Xu et al., 2006; Li et al., 2004; Li et al., 2003; Thornburg, 2011). In vitro studies of cultured cardiac fibroblasts have revealed that angiotensin II directly stimulates collagen synthesis and the expression of ECM proteins via type 1 angiotensin II (AT1) receptors, and indirectly stimulates collagen deposition via induction of transforming growth factor beta (TGF-β), endothelin 1 (ET-1), IL-6 and osteopontin (Manabe et al., 2002). Additionally, a study using knockout mice reported that angiotensin II failed to promote fibrosis in TGF-β1-deficient mice (Schultz et al., 2002). The sustained activation of TGF-β in mice overexpressing TGF-β can induce ventricular fibrosis in the heart (Sakata et al., 2008). Interestingly, collagen I in mouse cardiomyocytes was enhanced after hypoxia reoxygenation, and the release of reactive oxygen species (ROS) appeared to induce the genes for collagen (Hu et al., 2007). The overexpression of TGF-β1 can block ROS and therefore inhibit collagen accumulation, implying an antifibrotic role for TGF-β1 (Hu et al., 2007). Collectively, TGF-β1 might act as both a pro- and anti-fibrotic factor upon various stimuli and upon short-term or long-term stimuli. The primary effect of TGF-β1 in the fetal and neonatal heart following chronic hypoxia remains unclear. More studies demonstrating the direct effect of hypoxia on abnormal development of fetal and neonatal heart are therefore warranted.

The Role of MMPs in the Heart and Brain

The MMP family includes at least 25 members (Klein and Bischoff, 2011), which are generally classified into several classes according to their specific substrates:

collagenases (MMP-1, -8, -13 and -18) are capable of degrading insoluble fibrillar collagens, especially collagens I and III; gelatinases (MMP-2 and -9) can digest gelatins as well as collagen IV in the basement membranes; membrane type 1–6 MMPs (MT1–MT6 MMPs) can digest collagens and activate other MMPs; stromelysins (MMP-3, -10 and -11) are active against some ECM components; and a heterogeneous group that includes MMP-7, -12, -20, -26 and -28 (Phatharajaree et al., 2007; D'Armiento, 2002). For most MMPs, their expression level is generally low in normal adults. Growth factors such as platelet-derived growth factor and epidermal growth factor, as well as proinflammatory cytokines such as interleukin (IL)-1 and -6, TGF-b, TNF-a have been shown to induce MMP synthesis (Creemers et al., 2001). IL-4, heparin, corticosteroids can repress MMP gene expression (Creemers et al., 2001). The regulation of MMP expression by those factors depends on different types of cells and MMPs. An important control of MMP activity is through a group of specific MMP endogenous inhibitors, the TIMPs. There are four TIMPs identified so far, termed TIMP-1, -2, -3 and -4; these can bind noncovalently with high efficiency to active MMPs in a 1:1 molar ratio, therefore preventing access of the MMP catalytic domains to their substrates (Spinale, 2007). The most studied are TIMP-1 and TIMP-2, which bind MMP-9 and MMP-2 with a high affinity, respectively (Vaillant et al., 1999). TIMP-3 was first proposed by Yang and Hawkes in 1992, and can bind directly to ECM proteins (Spinale, 2007; Yang and Hawkes, 1992). TIMP-4, as a recently identified member of TIMP family, was first discovered in the human heart in 1996, and has a high binding affinity to proMMP-2 (Fig. 1) (Greene et al., 1996; Bigg et al., 1997).

MMPs have been shown to degrade components of the extracellular matrix and of basement membranes in the brain. The protection of the normal brain function is provided by blood brain barrier (BBB) that consists of astrocytic endfeet, pericytes, capillary basement membranes and brain microvascular endothelial cells (Iadecola and Nedergaard, 2007). Additionally, tight junctions (TJs) and adherens junctions also maintain the barrier property by limiting paracellular diffusion (Pun et al., 2009). Most of MMPs are synthesized and transported into ECM as inactive proenzymes, and then can be activated by proteinases and other mediators in the tissues. The inhibition of MMPs activity can prevent BBB degradation (Chen et al., 2009), possibly by preserving one of the tight junction protein occludin and reducing endothelial gap formation (Reijerkerk et al., 2006). Previous studies have demonstrated knockout of superoxide dismutase (SOD) enhances MMP expression and deteriorates BBB dysfunction (Maier et al., 2006), indicating ROS is involved in triggering MMP activation. This is further supported by another study that has shown the overexpression of SOD reduces MMP-9 activation (Morita-Fujimura et al., 2000). The activation of MMPs can lead to the degradation of endothelium basement membrane and damage the BBB integrity (Yang and Rosenberg, 2011). In addition, hypoxia inducible factor (HIF)-1 appears to regulate the expression of furin, and in turn activates several MMPs such as MT1-MMP and MMP-3. Both of them are required for the activation of the major types of MMPs, MMP-2 and MMP-9, in the brain, respectively (McMahon et al., 2005; Svineng et al., 2008).

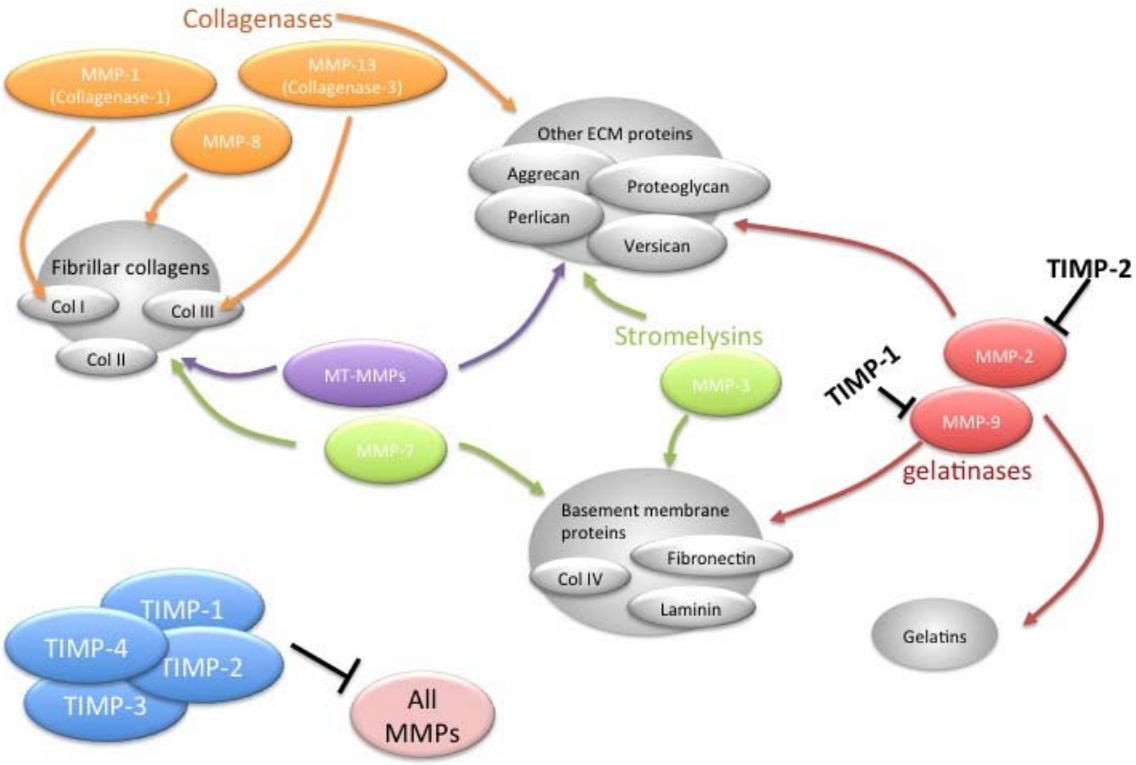

Figure 1. MMPs and TIMPs in myocardium. So far 25 matrix metalloproteinases (MMPs) have been identified. According to their digesting substrates, there are three kinds of MMPs in the hearts - collagenase, stromelysin and gelatinase. Membrane type (MT)-MMPs are a unique subfamily of MMPs. They become fully active enzymes once inserted into the cell membrane, rather than being secreted in the proenzyme form. Six types of MT-MMPs, MT1-MMP is highly expressed and is a primary enzyme that digests fibrillar collagens in the heart in addition to collagenases. The major types of MMPs in the heart and brain are gelatinases, MMP-2 and MMP-9, and tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 have high affinity to inhibit MMP-9 and MMP-2 expression, respectively. TIMP-3 and TIMP-4 are predominantly expressed in the heart.

In the heart, approximate two thirds of the total cardiac tissue is myocytes, and one third consists of fibroblasts, endothelial cells, other cells, extracellular tissue fluid and ECM (Tyagi, 1997). Slow turnover of ECM are characterized by that synthesis and degradation are in a dynamic balance, which is essential for normal cardiac tissue development. The structural backbone myocardial ECM is formed by a fibrillar collagenous network that contains mainly collagen type I and III, and other components in lower quantity, as well as various groups of enzymes such as MMPs (Cleutjens, 1996). Pathological changes at the molecular level include the abnormal alteration in qualitative and quantitative constitution of myocytes and constitution of ECM, and these changes are the result of cardiac tissue remodeling in multiple myocardium diseases (Maisch, 1996). For example, an acute ischemic insult can activates MMPs and then impairs the ECM at the site of ischemic injury, causing dilatation and systolic failure. As the compensatory synthesis of ECM at sites distal from infarcted area initiates, the fibrosis and diastolic failure may occur (Tyagi et al., 1996). Due to pressure or volume overload of heart muscle, the structural remodeling of heart tissue after the myocardial infarction (MI) induces the development of hypertrophy, as well as fibrosis in the non-infarcted region of the heart, which may increase the risk of cardiac failure. A range of animal studies have investigated the profile of MMPs after MI. Herzog et al first demonstrated the early increased activity of MMP-1 and MMP-2 in both infarcted and non-infarcted area 1 h after ischemic injury in rats. MMP-9 was increased in the infarcted region 2 h after MI (Herzog et al., 1998). Chen et al demonstrated that MMP-1 and MMP-9 levels were enhanced in the infarcted region two days after MI (Chen et al., 2005). The increase of MMP-9 was also seen in the non-infarct region 4 days after the infarction. Additionally,

the rise of MMP-2 was only observed in the infarcted zone one week after MI, and reached a maximum level two weeks after the infarction in mice model (Chen et al., 2005). Previous study has demonstrated the deletion of MMP-2 reduced LV dilation and the incidence of LV rupture after MI (Hayashidani et al., 2003), while the knockout of MMP-9 significantly attenuated LV remodeling, LV enlargement, collagen accumulation and cardiac injury after MI (Ducharme et al., 2000). Collectively, these data indicated that MMPs are one of the major factors responsible for modulating matrix turnover and maintaining the structural and functional integrity of myocardium under normal conditions, and that the abnormal activation of MMPs induced by MI might break the balance between synthesis and degradation of ECM, causing maladaptive LV remodeling in response to the severe insults.

Central Hypothesis

The **central hypothesis** of my project is that fetal hypoxia causes reprogramming of MMPs and TIMPs expression patterns and activities leading to aberrant brain and heart development in the fetuses and neonates.

Significance

Epidemiological evidence has indicated that intrauterine growth restriction is a risk factor that is associated with an increased incidence of low birth weight, perinatal mortality, neurological deficits and cardiovascular disease in later adult life (Barker and Osmond, 1986; Bateson et al., 2004; Gluckman et al., 2008; McMillen and Robinson, 2005). In humans, fetal hypoxia is one of the major causes of intrauterine growth restriction, fetal brain injury and neurological deficits (Jensen and Moore, 1997;

Prandota, 2004). Insufficient delivery of oxygen to fetuses contributes to remarkable pathophysiological changes in fetal tissue remodeling. However, little is known about the fetal adaptive mechanisms involved. Elucidating the various molecular mechanisms underlying the structure and functional changes in cardiac and cerebral growth, particularly understanding the role of MMPs and TIMPs in the immature heart and brain tissue remodeling after hypoxia, is an area of ongoing research. We expect finding from our study to reveal a novel role of MMPs and TIMPs in maternal hypoxia-induced retarded growth in the offspring. Moreover, considering that hypoxia is one of the most important and clinical relevant stresses to the fetus and neonates, the possibility that fetal hypoxia may result in reprogramming of matrix-related proteins in the offspring with a consequence of abnormal cardiac or cerebral structure and function may provide a mechanistic understanding worthy of investigation in human. These findings also could have significant implications for other adverse intrauterine environment and will give a better understanding of how intrauterine environment can impact the developmental process of the fetus.

CHAPTER TWO

MATERNAL HYPOXIA INCREASES THE ACTIVITY OF MMPS AND DECREASES THE EXPRESSION OF TIMPS IN THE BRAIN OF NEONATAL RATS

By

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Abstract

A recent study has shown that increased activity of matrix metalloproteinases-2 and -9(MMP-2 & -9) has detrimental effect on the brain after neonatal hypoxia. The present study determined the effect of maternal hypoxia on neuronal survivability and the activity of MMP-2 and -9, as well as the expression of tissue inhibitors of metalloproteinase 1 and 2 (TIMP-1 & -2) in the brain of neonatal rats. Pregnant rats were exposed to 10.5% oxygen for 6 days from the gestation day 15 to day 21. Pups were sacrificed at day 0, 4, 7, 14, and 21 after birth. Body weight and brain weight of the pups were measured at each time point. The activity of MMP-2 & -9 and the protein abundance of TIMP-1 & -2 were determined by zymography and Western blotting, respectively. The tissue distribution of MMPs was examined by immunofluorescence staining. The neuronal death was detected by Nissl staining. Maternal hypoxia caused significant decreases in body and brain size, increased activity of MMP-2 at day 0 and increased MMP-9 at day 0 and 4. The increased activity of the MMPs was accompanied by an overall tendency towards a reduced expression of TIMPs at all ages with the significance observed for TIMPs at day 0, 4, and 7. Immunofluorescence analysis showed an increased expression of MMP-2, -9 in the hippocampus at day 0 and 4. Nissl staining revealed significant cell death in the hippocampus at day 0, 4, 7. Functional tests showed worse neurobehavioral outcomes in the hypoxic animals.

Introduction

Chronic maternal hypoxia remains a primary contributor to intrauterine growth restriction and continues to be a major clinical problem for fetal development (Lueder et al., 1994). The occurrence of the maternal hypoxia can be a result of smoking, drug use or living at high altitude during pregnancy. The insufficient oxygen supply from mother during pregnancy is one of the major causes for the fetal brain injury, mental retardation and neurological deficits, and one of the most likely triggers of sudden infant death syndrome (Prandota, 2004). It may also delay the development of motor reflexes in the newborn (Golan et al., 2004). Mental retardation, cerebral palsy, and seizures all have been shown to be the consequences of the prenatal hypoxic insult (Hallak et al., 2000). Maternal hypoxia may have a devastating impact on the mother and fetus, and after parturition the health of the offspring is often permanently affected (Schneider et al., 2008, Lueder et al., 1994).

Matrix metalloproteinases (MMPs) comprise a family of zinc-binding proteolytic enzymes that degrade the extracellular matrix during physiological and pathological tissue remodeling (Sifringer et al., 2007; Buss et al., 2007, Badier-Commander et al., 2000; Tian et al., 2007). MMP-2 and MMP-9 (gelatinases) are secreted principally by astrocytes and are opposed by their endogenous inhibitors TIMP-2 and TIMP-1 (Vaillant et al., 1999). Gelatinases are the most investigated MMPs in the brain because numerous neurovascular matrix and cell surface proteins have been identified as their substrates (Tian et al., 2007; Sulik and Chyczewski, 2008). MMPs and TIMPs have been implicated in extracellular matrix (ECM) remodeling during brain development (Wojcik et al., 2009); however, MMPs may also have detrimental effects after brain injury. For example,

MMPs trigger neuronal cell death after hypoxia-reoxygenation (Lee and Lo, 2004), and impair the blood brain barrier integrity after focal ischemia (Rosenberg et al., 1998).

Earlier authors reported that gestational hypoxia could alter neural development and one of the mechanism involved is a defect of brain energetic adaptation. The absence of response of Glut3 and hypoxia inducible factor-1α (HIF-1α) at E19 in the hypoxic fetal brains might attribute to insufficient energetic adaptation (Royer et al., 2000). On the other hand, extracellular matrix proteins such as MMP-2 and MMP-9 may play a critical role in the neonatal brain, as tissue remodeling is predominating and long-lasting process during brain maturation (Cockle et al., 2007). There is an indication that hypoxia can cause the increased levels of reactive oxygen species (ROS) that activate NF-κB transcription pathways, including that of genes encoding MMPs (Cockle et al, 2007). The resultant imbalance between MMP-9 and TIMP-1 (tissue inhibitor of metalloproteinase -1; the endogenous inhibitor of MMP-9) may exacerbate the neuronal loss (Rosenberg et al., 1996). Sunagawa and colleagues have demonstrated that the ratio of MMP-9 to TIMP-1 correlates with the severity of neurological sequelae after acute perinatal asphyxia (Sunagawa et al., 2009). However, whether chronic maternal hypoxia will have an effect on the brain MMPs and TIMPs in the neonatal offspring has not been determined. Thus, the present study was designed to investigate the effect of maternal hypoxia on neonatal brain injury and brain expression of MMP-2, -9 and TIMP-1, -2 at postnatal ages from day 0 to day 21. We hypothesize that maternal hypoxia increases the activity of MMPs and decreases the expression of TIMPs in the brain of neonatal rats.

Materials and Methods

Experimental Animals

All animal research protocols have been approved by Loma Linda University Institutional Animal Care and Use Committee (IACUC). Time-dated Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI) and housed in light and temperature controlled environment with food and water ad libitum for the duration of the study. Animals were assigned into the normoxic group and maternal hypoxic group (10.5% oxygen) from day 15 to day 21 of gestation. Hypoxia was induced with a mixture of nitrogen gas and air as described previously (Li et al., 2003). The normoxic control group was housed identically, except the room air was flowing through the chambers. Pups were delivered on gestational day 22.

Sample Collecting

12 pregnant rats were randomly assigned for normoxic and maternal hypoxic group. 8-13 pups from each litter were delivered. A total of 12 unsexed rat pups were randomly chosen from normoxic or hypoxic litter and euthanized at postnatal day 0, 4, 7, 14, 21. Transcardial perfusion was performed as previously described (Hu et al., 2000). Briefly, under anesthesia with 3.0% isoflurane pups were thoracotomized. A catheter was placed in the apex of the left ventricle and an incision was made on the right atrium. The pups were perfused with 40 ml of ice-cold phosphate buffered saline (PBS). The brain tissue was then collected and stored at -80°C for zymography and Western blotting analysis. For immunohistochemical analysis, the pups were first perfused with 40 ml of PBS followed by 40 ml of 10% buffered formalin. Collected brains were post fixed in

formalin at 4°C overnight followed by cryoprotection in 30% sucrose. Upon euthanization, both length and weight of the brain and body were measured respectively.

Western Blotting

Protein was extracted from cerebral tissues of the right hemisphere by gentle homogenization in lysis buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% Na deoxycholate, 1 mM EDTA, and 0.1% sodium dodecyl sulfate (SDS)], containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO), followed by centrifugation at 15,000 g at 4°C for 20 min. The supernatant was used as a whole cell protein extract and the protein concentration was determined by using a detergent compatible assay (Bio-Rad). Equal amounts of protein (30 μg) were loaded on an SDS-PAGE gel. The protein samples were electrophoresed and transferred onto a nitrocellulose membrane, which was then blocked and incubated with the primary antibodies at 4°C overnight. The primary antibodies used were mouse monoclonal anti-TIMP-1 and anti-TIMP-2 (Millipore, MAB3300, MAB3310, 1:1000). Nitrocellulose membranes were incubated with secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. Immunoblots were then visualized with an ECL Plus chemiluminescence reagent kit (Amersham Biosciences, Arlington Heights, IL). The optical densities of the bands were calculated with Image J software, version 1.0 and normalized to β-actin.

Gel Zymography

The brain samples were homogenized in lysis buffer including protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). After centrifugation, the supernatant was collected and the protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of protein (120 μg) were loaded and separated by 10% Tris-glycine gel with 0.1% gelatin as substrate. The gel was renatured and then incubated with a developing buffer at 37 °C for 48 h. The gel was stained with 0.5% Coomassie blue R-250 for 1 h and then destained. Data were analyzed with the Image J software, version 1.0. The sample for positive control was collected in the severe HI neonatal injury group of an unrelated study, and was loaded with equal amount of the protein into different gels. When quantifying MMP-2 and MMP-9 activity, the density of each band in different gels was first normalized with respect to the positive control, and then all the normalized densities from different time points or different groups were normalized with the density from normoxic day 0, which was set as 100% OD baseline.

Nissl Staining

At postnatal day 0, 4, 7, 14, and 21, animals were anesthetized and transcardially perfused with ice-cold PBS followed by 10% buffered formalin. Brains were collected, postfixed overnight and cryoprotected in 30% sucrose/PBS. Coronal brain sections of 10 μm thicknesses were cut in a cryostat and Nissl staining was performed as previously described (Ostrowski et al., 2006). Six animals per group were used for Nissl Staining.

Immunofluorescence Staining

The tissue expression of matrix metalloproteinases and glial fibrillary acidic protein (GFAP; astrocytic marker) were assessed in the hippocampus by using double immunofluorescence staining. First, the antigen retrieval was done by microwaving sections in a citrate buffer for 10 min. After blocking with 5% donkey serum (1 h at room temperature), the sections were incubated with the following primary antibodies (Millipore): rabbit anti MMP-9 or anti MMP-2, in combination with mouse anti GFAP antibody (1:100, 4°C, overnight). The sections were then incubated with the secondary antibodies –anti-mouse FITC-conjugated and anti-rabbit Texas Red- conjugated donkey antibodies (1:200, 2 h at room temperature). After three washes, the sections were cover slipped with SlowFade reagent (Invitrogen) and observed under the fluorescent microscope with a digital camera (OLYMPUS BX51). Microphotographs were taken separately for each stain and merged by means of the Magna Fire software.

Functional Tests

All neurobehavioral tests were performed in a blinded set-up.

T-maze Test

The T-maze spontaneous alternation task has been used to test the impairment of cognitive ability and working memory caused by hippocampal dysfunction (Matchett et al., 2007). At 4-week-old, rats were tested for a spontaneous alternation on a T-shaped maze as previously described (Matchett et al., 2007). Rats were placed in the stem of T-maze and allowed to freely explore the two arms of the maze. After an arm of maze was

chosen, animals were placed again in the stem, and the trial was repeated for a total of ten times. Data are expressed as the rate of spontaneous alteration.

Wire Hanging

The wire hanging test was conducted on day 10, 14, and 22 rats to evaluate the neuromuscular and locomotor development as previously described (Fan et al., 2005). Pups suspended by their forelimbs from a horizontal rod (5mmx5mm area, 35cm long, between two poles 50cm high) tended to support themselves with their hind limbs, preventing from falling. Suspension latencies were recorded.

Data analysis

Data are expressed as means ± standard deviation (SD). The statistical analysis of differences between individual groups was performed by using *t*-test or ANOVA test. Differences for which $p<0.05$ were considered significant.

Results

Body/Brain Weight and Length

As shown in Figure 2, the treatment of maternal hypoxia in the rats for six days resulted in a significant decrease in the size and weight of body and brain (Figure 2A-B), at postnatal ages of 0, 4, 7, 14, and 21 days.

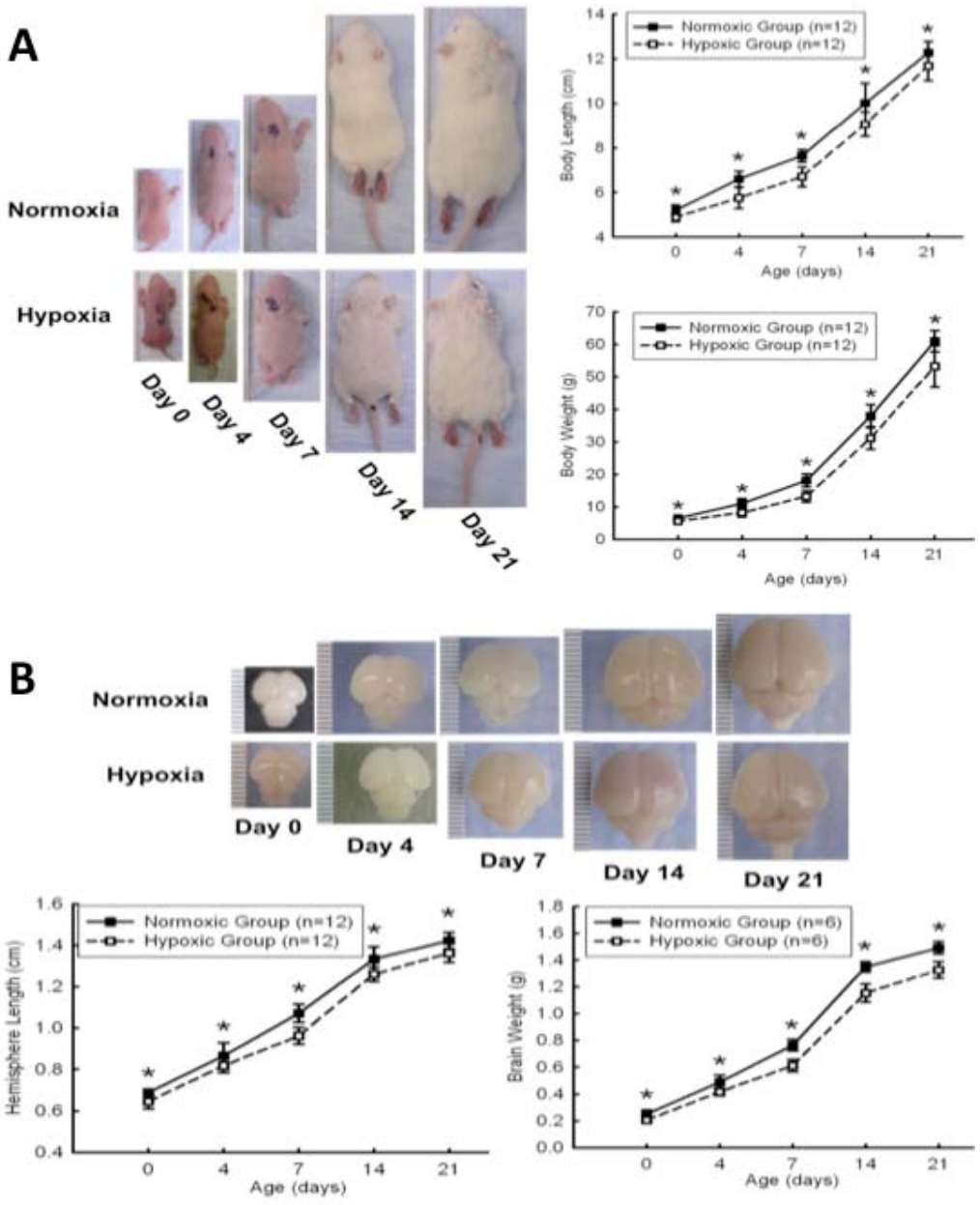

Figure 2. Chronic hypoxia on the body and brain weight and length in animals. Pups from maternal hypoxia dams were sacrificed at postnatal day 0, 4, 7, 14, and 21. Body and brain size and weight was measured at each time point. Chronic prenatal hypoxia significantly reduced body (A) and brain size (B) in maternal hypoxia offspring. *p<0.05, N=12 per group.

Profiles of MMPs and TIMPs

Profiles of MMP-2 and MMP-9 activity in neonates following 6-day of maternal hypoxia were determined at postnatal day 0(n=6), 4(n=6), 7(n=6), 14(n=6) and 21(n=6). Gel zymography in the brain extracts from right hemisphere showed that late maternal hypoxia triggered significant increase of MMP-2 activity at day 0. It also significantly increased MMP-9 activity at both day 0 and 4 (Figure 3). At day 0, 4, and 7, the protein abundance of TIMP-1 and TIMP-2 in the right hemisphere was significantly decreased in the hypoxic-treated pups, as compared with the normoxic controls (Figure 4A-D), resulting in a significant increase in the ratio of TMIP-1/MMP-9 and TIMP-2/MMP-2 in the pups of hypoxic treatment (Figure 4E-F).

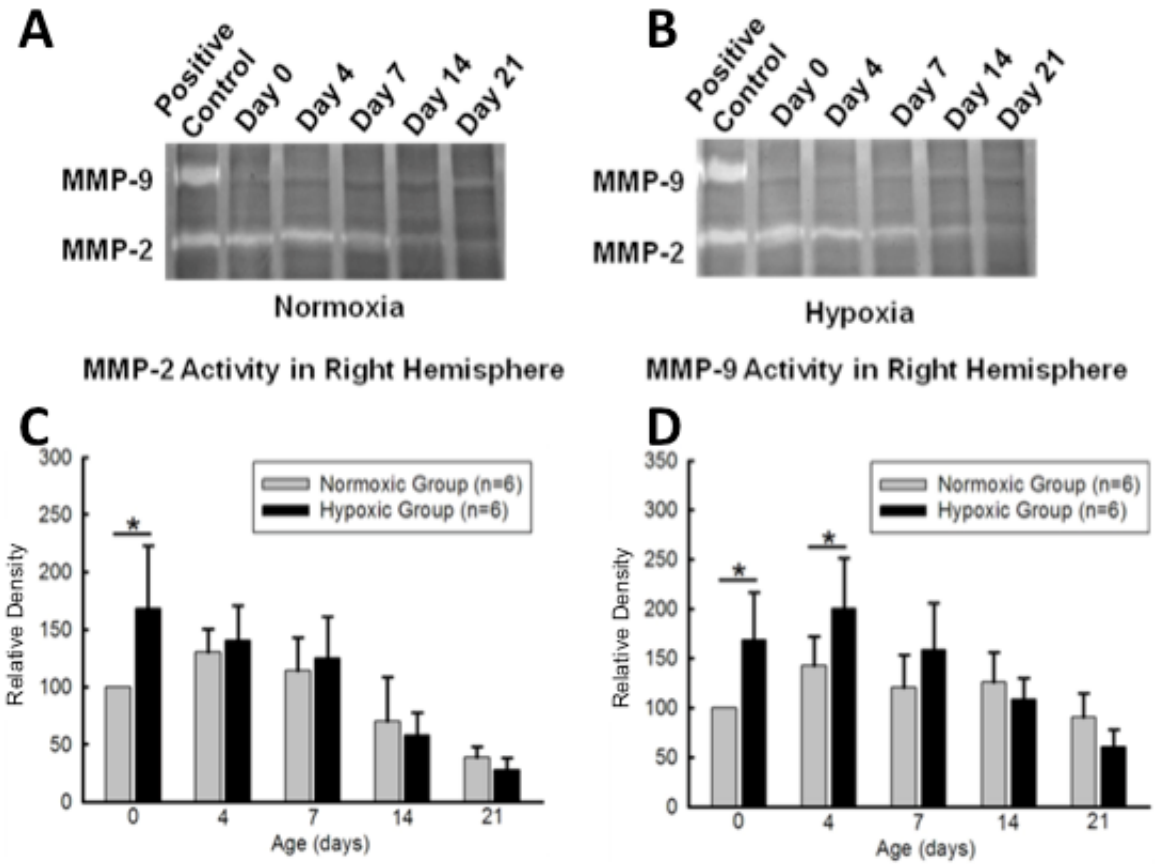

Figure 3. Gel zymography of right hemisphere homogenates at postnatal day 0, 4, 7, 14, and 21. Top photographs show representative zymogram gels (A, B). Bar graphs show quantified densitometry data. MMP-2 activity was increased in hypoxic animals compared to control animals at day 0 (C). MMP-9 activity was also increased in hypoxic animals at day 0 and 4 (D). No significant differences in MMP-2, -9 activities were detected at other time points. *$p<0.05$, N=6 per group.

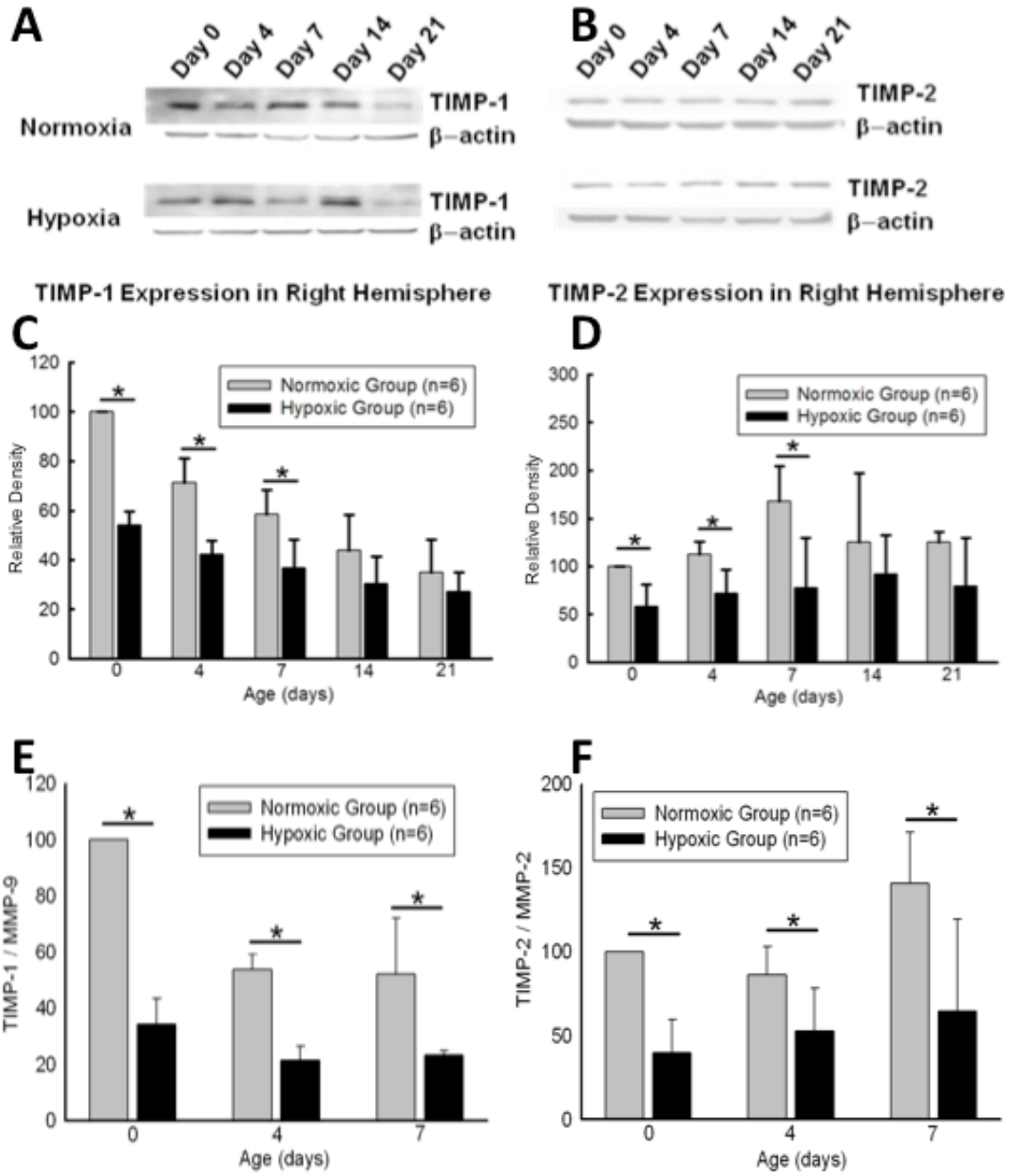

Figure 4. (A) Western blot analysis of right hemisphere homogenates at postnatal day 0, 4, 7, 14, and 21. Upper panel of A and B shows representative immunoblots. Lower panel of C and D shows quantified densitometry data. TIMP-2 and TIMP-9 expression was decreased in hypoxic animals compared to control animals at day 0, 4, and 7. No significant difference in TIMP-1, -2 expressions was detected at other time points. (E and F) Ratios of TIMP-1/MMP-9 and TIMP-2/MMP-2 were analyzed. The significant decrease in both ratios was detected at day 0, 4, and 7 in the hypoxic group. *$p<0.05$, N=6 per group.

Morphological Changes

Morphological changes following prenatal hypoxia were exhibited by Nissl staining in the coronal brain sections (Figure 5A). Neurons in the CA1 hippocampal region of normoxic animals appeared normal morphologically on day 0, 4, and 7 after birth. In contrast the pyramidal cells with condensation of cytoplasm and karyoplasms were found in CA1 following prenatal hypoxia. These neurons, as presented in the lower panels of the figure 5A, were dark stained and sometimes triangular in shape. Additionally, small foci of cell loss could occasionally be seen. As shown in Figure 5B, the number of dead cells in the CA1 area of hypoxic hippocampus was significantly higher than in the normoxia group on day 0, 4, and 7 after birth (t-test, $p<0.05$). The graph also demonstrates a tendency towards a decrease in numbers of dead cells as compared between day 0 and later time points.

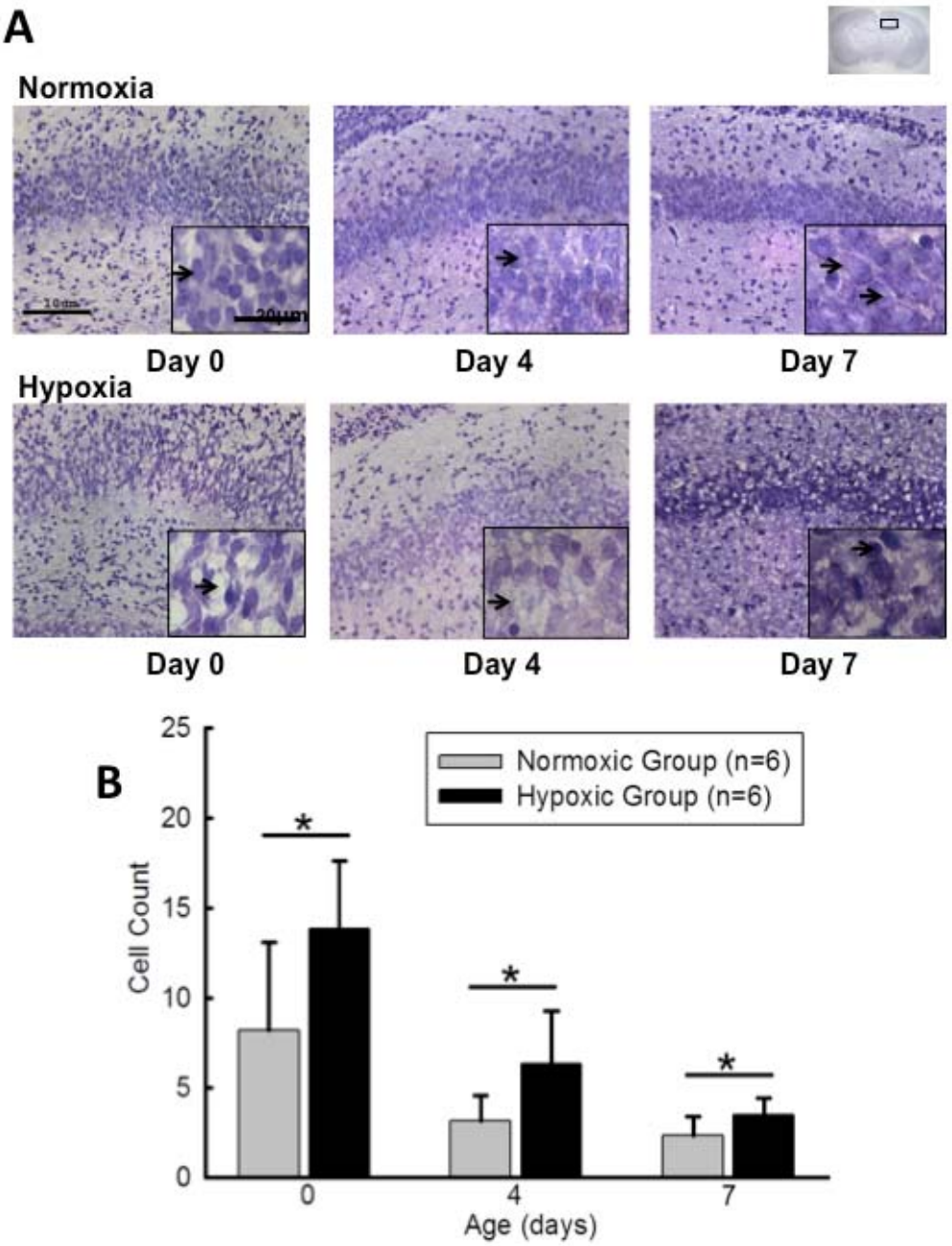

Figure 5. Effect of maternal hypoxia on morphological changes and cell death. (A) Representative Nissl stained CA1 hippocampal area in coronal brain sections from normoxic and maternal hypoxic groups at day 0, 4, and 7 are shown. Scale bars indicate 10μm (20 μm in the insets). At higher magnifications in hypoxia animals, the number of cells in hypoxia animals was markedly reduced and arrows indicate dying cells or empty spaces after cells died. Normal neurons are indicated by the arrows in the normoxia slides. (B) Cell death counting revealed a significant cell loss after maternal hypoxia at day 0, 4, and 7. There were no detectable differences in the number of dead cells at day 14 and 21 (data not shown). *$p<0.05$, N=6 rats per group (three counting areas in each slide from each animal). The inserted brain slide photo indicates the areas that samples were taken.

Distribution of MMP-2 and MMP-9

Double immunofluorescence analysis of the brain sections revealed a predominant distribution of MMP-2, and MMP-9 in the astroglial elements found within CA1 hippocampal region (Figure 6, 7). Only a weak MMP-9 immunoreactivity was observed in the normoxia group at days 0, 4, and 7. A mild tendency of increase in MMP-9 immunoreactivity was seen on day 14 and 21 after birth in the normoxia group. Interestingly, MMP-9 epitopes only partially co localized with GFAP in the normoxic group (day 14 and 21). Following prenatal hypoxia a tremendous induction of MMP-9 colocalizing with astrocytic marker GFAP could be seen in CA1 sector. This increased MMP-9 immunostaining was observed at day 0, reached its maximum on day 4 and started to decline on day 7.

The normoxic group exhibited a very low level of MMP-2 tissue expression in the brain region encompassing CA1 at all time points (Figure 7) with enhanced immunoreactivity observed on day 7. However in the hypoxic animals strong MMP-2 immunoreactivity was detected on the postnatal day 0, 4, and 7 (to a lesser degree) as compared with the normoxia group. Noticeably, MMP-2 stain was only partially colocalized with GFAP. MMP-2 immunostaining was reduced at day 14. On day 21 there was no detectable MMP-2 immunoreactivity in both hypoxia and normoxia groups.

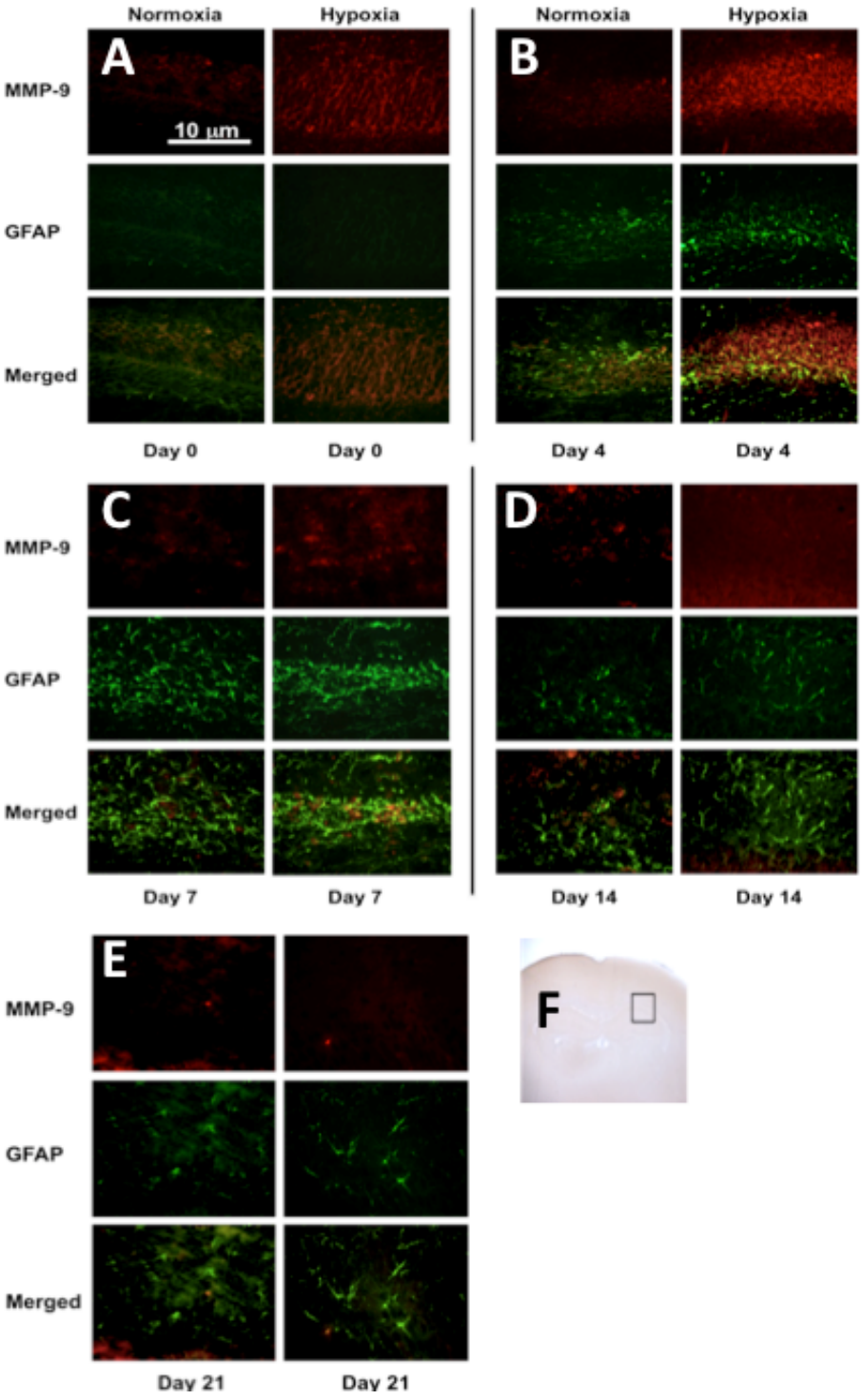

Figure 6. Double-labeling fluorescent immunohistochemistry for MMP-9 and GFAP in hypoxic pups. Red and green stain shows MMP-9s and GFAP expression, respectively. The photographs show that the expression of MMP-9 in astrocytes in CA1 region was increased in hypoxic animals at day 0 and 4. Scale bar = 10µm. The inserted brain slide photo (F) indicates the areas that samples were taken.

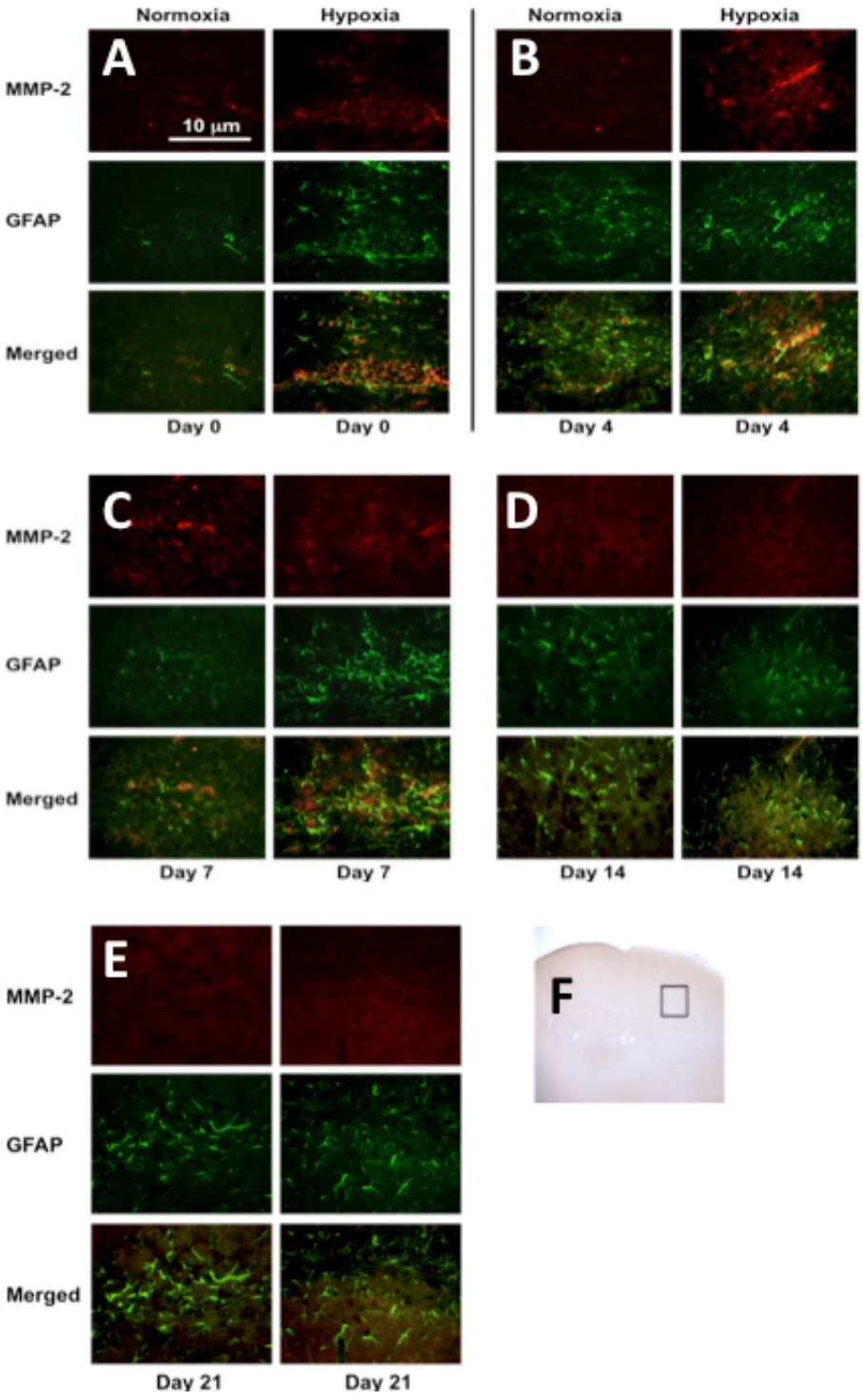

Figure 7. Double-labeling fluorescent immunohistochemistry for MMP-2 and GFAP in hypoxic pups. Red and green stain shows MMP-2 and GFAP expression, respectively. The photographs show that the expression of MMP-2 in astrocytes in CA1 region was increased in hypoxic animals at day 0 and 4. Scale bar = 10μm. The inserted brain slide photo (F) indicates the areas that samples were taken.

Neurobehavioral Tests

To assess the effect of maternal hypoxia on postnatal neurological function, the T-maze test was performed at day 28 and the wire hanging test at day 10, 14, and 22. The results of T-maze testing for the spontaneous alternation and cognitive ability do not show significant difference between the normoxic and hypoxic groups. Moreover, hypoxic group has tendency to show more spontaneous alternation in the test (Figure 8A, n=5 for each group). However, the wire hanging test demonstrated that the duration of wire hanging in the hypoxic groups was significantly shorter than that in the control groups at all three ages determined (Figure 8B, n=5 for each group, $p<0.05$).

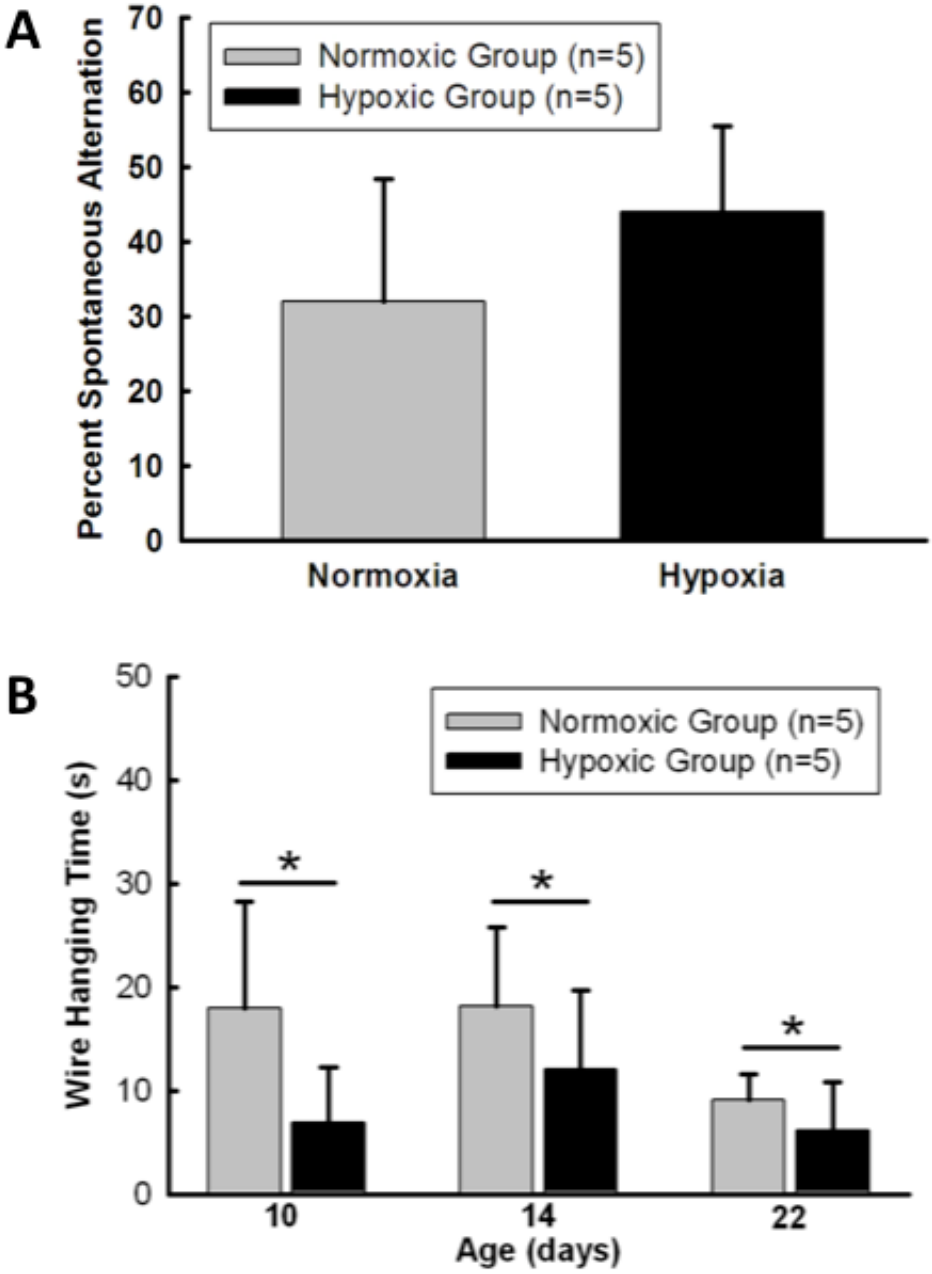

Figure 8. The effect of chronic maternal hypoxia on changes in functional performance. (A) Two groups of animals did not show difference in % alternations when presented with two choices in the T-maze. (B) Wire hanging analysis showed hypoxic animals performed worse on locomotors functional test than control animals. *$p<0.05$, N=5 per group.

Discussion

The present study has examined the effect of chronic maternal hypoxia on neonatal growth and the neuronal survivability in the immature brain. Consistent with previous findings, chronic hypoxia during late pregnancy caused a significant reduction in the body and brain size of rat pups, indicating a developmental retardation. The previous study demonstrated that acute hypoxia down-regulated TIMP-2 mRNA levels and up-regulated MMP-2 mRNA and protein expression in vitro (Ben-Yosef et al., 2002). In acute asphyxiated neonates, the presence of neurological sequelae strongly correlated with a high serum MMP-9/TIMP-1 ratio (Sunagawa et al., 2008). The present study extends the investigations into the role of TIMPs and MMPs in the neonatal brain injury and growth retardation following chronic maternal hypoxia. We are unaware of other studies that demonstrate an imbalance of the TIMPs and MMPs in chronic maternal hypoxia.

The mechanism of brain damage caused by maternal hypoxia in offspring is not clear. However, the observed pattern of injury in the present study is consistent with the increased vulnerability of CA1 neurons to hypoxia (Hallak et al., 2000). The morphological changes detected in the CA1 included more cell death in maternal hypoxic rats than that in normoxic animals. Other authors reported that the acute prenatal hypoxia induced neuronal loss in the hippocampus, causing a reduction in CA1 cell layer width (Golan et al., 2004). This raises a possibility that neonatal growth retardation may occur due to cell death or inhibition of cell proliferation. This notion is in accordance with the results of a previous study demonstrating that acute perinatal hypoxia altered the structural and functional properties of cell membranes and initiated apoptotic genes transcription (Zhuravin et al., 2004).

Although the growth restriction may also occur due to maternal nutrition restriction induced by hypoxic insult, Ream and colleagues have demonstrated that maternal nutrition is not a critical factor in prenatal hypoxia-induced growth restriction (Ream et al., 2008). Another study suggested that hypoxia-induced reduction of appetite did not contribute to ventricular hypertrophy in offspring (Xu et al., 2006). Taken together with our data it suggests that chronic or acute fetal hypoxia causes the brain growth restriction *via* the activation of cell death pathways. Consistent with the present finding, previous studies in the same animal model demonstrated that maternal hypoxia resulted in the increased apoptosis in the heart of fetal rats (Bae et al., 2003). Interestingly, Golan et al. have shown hypoxia did not affect cell death in the fetal brain, which suggested hypoxia might have delayed effect on neuronal death in neonatal brains (Golan et al., 2009).

To investigate the changes of TIMPs and MMPs in the development of brain injury caused by prenatal hypoxia, we compared the time course of the changes in the ratio of TIMPs and MMPs in hypoxic versus normoxic rats. Our data have shown (i) a significant decrease in this ratio in the brain of newborn and neonates up to one week old in hypoxic treated animals compared with controls, (ii) a consistent time course of the changes in the TIMPs expression and in the cell death between normoxic and hypoxic animals. Previous studies have reported that both TIMP-1 and TIMP-2 promote the cell division *in vitro* (Hayakawa et al., 1994, Hayakawa et al., 1992), and that TIMP-1 has an anti-apoptosis effect *in vitro* (Guedez et al., 1998). The present findings suggest that maternal hypoxia alters the ratio of TIMPs and MMPs by suppressing the TIMPs expression, which is likely to contribute to the reduced cell growth and proliferation and

the increased neuronal death in the developing brain. These findings also imply the possibility that raising TIMPs levels may restore the balance of TIMPs and MMPs and improve the neural cell survivability and proliferation, preventing the development impairment of the brain associated with fetal hypoxia. This strategy is however technically challenging to either directly and selectively inhibit MMPs or increase TIMPs in fetus in a pregnant mother rat. Our study has also implied that the extracellular matrix proteolysis dysfunction and disassociation of neurovascular unit might attribute to the pathophysiology of maternal hypoxia, which requires further investigations.

The present finding that maternal hypoxic-induced morphological changes in the CA1 region and the decreased TIMPs were not associated with a worsened performance in the T-Maze test is somewhat surprising, given that uncompensated maternal hypoxia has been shown to trigger significant alterations in fetal brain function (Pearce, 2006). In addition, a previous study demonstrated that short- and long-term memory was impaired in offspring rats following the maternal hypoxic treatment (Golan et al., 2004). Several factors may explain this discrepancy. The first possible reason relates to the timing of hypoxia. In the present study, pregnant rats were subjected to chronic hypoxia during the late phase of pregnancy when neurula is fully-formed and encephalon is well-developed. Therefore, the brain is not as vulnerable as during the mid-phase of pregnancy, especially between embryonic days 6 and 13. This time period includes the events leading to neurulation and encephalization along with intense cell proliferation and glial lineage commitment (Gressens et al., 1997). The quantitative image analysis showed more dead cells in hypoxic than in normoxic group, however there was no obvious cell loss in the CA1 area in both groups. Since the hippocampus is critical for the cognitive ability, it is

possible that our chronic hypoxia model may still be too mild to cause severe functional deficits. The second possible reason relates to the maternal or fetal compensation. There is no difference for the ratio of brain to body size between normoxic and hypoxic animals at postnatal day 0 (data not shown), which may be due to a compensation of cardiac output redistribution to preserve the fetal brain in the hypoxic condition. This compensation protects the immature brain from severe damage (Williams et al., 2005).

In the present study, we have demonstrated that maternal hypoxia causes a decrease in neonatal brain TIMPs levels and developmental retardation through neuronal death, with no changes in the short-term cognitive ability. Future studies are needed to investigate 1) other than MMPs/TIMPs hypoxia-related factors involved in the neonatal brain injury following late maternal hypoxia 2) the potential functional deficit of the brain in a long term in offspring after prenatal hypoxia. Additionally, the question whether pharmacological intervention directed at the increased TIMPs levels selectively attenuates developmental retardation of the brain in pups needs to be addressed. Studies from our laboratory have shown that erythropoietin (EPO) indirectly increases TIMP-1 levels *in vitro* (unpublished data). However, there is a limitation for elevated TIMPs levels. Over-suppressing MMPs may be deleterious considering that MMPs play an important role in tissue modulation and organ development (Matrisian and Hogan, 1990; Talhouk et al., 1992). Our study has identified differences in MMPs and TIPMs immunoreactivity mainly in the hippocampus. However, since the significant changes of MMPs and TIPMs detected by Western blotting were sampled from right hemisphere, this sampling might dilute changes of those proteins in local tissue. In the future study, to determine brain region- and cell type-specific distribution of these molecules, in situ

MMP zymography and immunostaining of specific cell type markers would be needed. We acknowledge that our study is correlational in nature and as such it does not allow us to establish causal relationship between alteration of MMPs,TIMPs and hypoxic brain injury. However the results of this study support the argument that hypoxia induced changes in MMPs and TIMPs may be involved in neonatal brain injury following late maternal hypoxia.

In the present study the pups were not selected by their gender and the gender effect was not examined. Previous study, however, has indicated no gender differences following severe or moderate hypoxic-ischemic insult from postnatal day 5 to day 21. More extensive brain injury in males than females was found only at postnatal day 60 in moderate hypoxic-ischemic group (Zhu et al., 2006). Although it has been reported that the effects of circulating estrogens and progestins are likely involved in the greater neuroprotection afforded to adult females, Hurn and his colleagues have shown no differences in functional outcomes between male and female neonates, which indicates that gender differences may not be a major factor affecting brain injury after neonatal hypoxia (Hurn and Macrae, 2000; Hurn et al. 2005).

Overall, we conclude that chronic maternal hypoxia impairs the proteolytic balance by increasing the activity of MMPs and decreasing the expression of TIMPs in the brain of neonatal rats. Unopposed extracellular matrix degradation is likely to contribute to the brain injury and growth retardation observed in the present study. These results may provide a molecular basis for novel therapies targeting extracellular proteolysis in neonatal brain affected by maternal hypoxia in the future.

Acknowledgments

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CHAPTER THREE

MATERNAL HYPOXIA ALTERS MATRIX METALLOPROTEINASE EXPRESSION PATTERNS AND CAUSES CARDIAC REMODELING IN FETAL AND NEONATAL RATS

By

Wenni Tong, Qin Xue, Yong Li and Lubo Zhang

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Abstract

Fetal hypoxia leads to progressive cardiac remodeling in rat offspring. The present study tested the hypothesis that maternal hypoxia results in reprogramming of matrix metalloproteinase (MMP) expression patterns and fibrillar collagen matrix in the developing heart. Pregnant rats were treated with normoxia or hypoxia (10.5% O_2) from day 15 to 21 of gestation. Hearts were isolated from 21-day fetuses (E21) and postnatal day 7 pups (PD7). Maternal hypoxia caused a decrease in the body weight of both E21 and PD7. The heart to body weight ratio was increased in E21 but not in PD7. Left ventricular myocardium wall thickness and cardiomyocyte proliferation were significantly decreased in both fetal and neonatal hearts. Hypoxia had no effect on fibrillar collagen content in the fetal heart, but significantly increased the collagen content in the neonatal heart. Western blotting revealed that maternal hypoxia significantly increased collagen I, but not collagen III, levels in the neonatal heart. Maternal hypoxia decreased MMP-1 but increased MMP-13 and MT1-MMP in the fetal heart. In the neonatal heart, MMP-1 and MMP-13 were significantly increased. Active MMP-2 and MMP-9 levels and activities were not altered in either fetal or neonatal hearts. Hypoxia significantly increased tissue inhibitors of metalloproteinase (TIMP)-3 and TIMP-4 in both fetal and neonatal hearts. In contrast, TIMP-1 and TIMP-2 were not affected. The results demonstrate that *in utero* hypoxia reprograms the expression patterns of MMPs and TIMPs and causes cardiac tissue remodeling with the increased collagen deposition in the developing heart.

Introduction

Substantial evidence has shown a clear association of adverse intrauterine environment with an increased incidence of cardiovascular disease and hypertension later in the life (Barker and Osmond, 1986; Bateson et al., 2004; Cluckman et al., 2008; McMillen and Robinson, 2005), suggesting that the prenatal environment can change the postnatal physiology, namely "programming". Programming is a result of adaptive alterations in gene expression patterns and phenotype in response to the *in utero* stresses, which modify the growth of specific organs during the critical period of development in early life (Louey and Thornburg, 2005). This may predispose the organism to a heightened susceptibility of cardiovascular disease in its adult life. Multiple stimuli have been identified as being capable of inducing fetal programming in animal models, including malnourishment, exposure to hypoxia, cocaine, nicotine or glucocorticoid during the pregnancy (Bae et al., 2005; Gluckman et al., 2008; Kwak et al., 2011; McMillen and Robinson, 2005; Patterson et al., 2010; Xue et al., 2011; Xue and Zhang, 2009). Recent animal studies have demonstrated that fetal hypoxia is linked to early changes in the developing cardiovascular system (Camm et al., 2010; Patterson et al., 2010; Ream et al., 2008). In fact, “physiological hypoxia” is a normal part of fetal life for all vertebrates and it plays an active role in vasculogenesis, angiogenesis, hematopoeisis and chondrogenesis during the fetal development (Ream et al., 2008). The partial oxygen tension of embryo is below 10 mmHg, which is regarded as being hypoxic compared with normal adult tissues with the oxygen tension of 20-40 mmHg (Webster and Abela, 2007), indicating that the fetus is persistently hypoxic during the organ formation, growth and maturation, and that fetal tissues have a lower threshold to reach a state of oxygen insufficiency (Patterson and Zhang, 2010). Although a restricted oxygen supply is

essential for intrauterine growth, excessive or severe hypoxia may compromise the normal fetal or neonatal development. The fetus may experience prolonged hypoxia under various conditions, such as pregnancy at high altitude, pregnancy with smoke, drug abuse, anemia, pulmonary disease, hypertension, etc. Fetal exposure to pathophysiological hypoxia results in the redistribution of blood flow to facilitate oxygen delivery to the vital organs, such as the brain and heart (Teitel and Rudolph, 1985). In rodents, the heart is particularly vulnerable to stressors, such as hypoxia, during the late fetal development and early postnatal life when it undergoes rapid growth and maturation (Louey and Thornburg, 2005). The maturation process of cardiomyocytes in rats occurs over postnatal day 4 to 12, which is marked by binucleation and escape from the cell cycle (Zhang, 2005). Nonetheless, little is known about cardiac remodeling and related genes expression patterns in the heart during the critical developmental stages of the fetus and neonate in response to fetal hypoxia.

It has been demonstrated that the timely breakdown and restructure of extracellular matrix (ECM) are critical for the normal fetal organ development (Nagase and Woessner, 1999). ECM is a complicated microenvironment including numerous matrix proteins (such as collagens), signaling molecules, proteases, and all of them contribute to the tissue remodeling process (Spinale, 2007). The functional integrity of myocardium depends largely on the extracellular collagenous matrix (Marijianowski et al., 1994). Aberrant amount, distribution or organization of fibrillar collagens in the myocardium is associated with various pathophysiological changes in the heart (Heeneman et al., 2003). Many factors that participate in the cardiac tissue remodeling have been revealed and matrix metalloproteinases (MMPs) are the one of the most

significant mediators in the ECM turnover. MMPs are a family of zinc-dependent proteases that consist of at least 25 different MMPs in vertebrate (Fanjul-Fernández et al., 2010). Although the most studied MMPs in the heart are gelatinases, MMP-2 and MMP-9 that are capable of degrading type I and IV collagens, the major types of fibrillar collagens in the heart are type I and III collagens that are digested mainly by collagenase-1 (MMP-1) and collagenase-3 (MMP-13), respectively (Spinale, 2007; Vanhoutte and Heymans, 2010; Weber, 1989). In addition to the secreted MMPs, there is a unique subfamily of MMPs, namely membrane type- (MT-) MMPs. MT-MMPs (MT1-MT6 MMPs) have been identified to be fully active enzymes once inserted into the cell membrane, rather than being secreted in the proenzyme form. Among the six types of MT-MMPs, MT1-MMP is highly expressed and is a primary enzyme that digests fibrillar collagens in the heart in addition to collagenases (D'Armiento, 2002; Ohuchi et al., 1997; Spinale, 2007). Together with the four types of tissue inhibitors of metalloproteinases (TIMPs) identified, MMPs have been implicated in a variety of physiological and pathological processes in the cardiovascular system, ranging from fibrillar collagen digesting in the heart formation and growth to cardiac ischemia-reperfusion injury (Kandasamy et al., 2010). Yet whether and to what extent MMPs and TIMPs expression patterns in the fetal heart are altered by *in utero* hypoxia remain to be elusive. Herein, we present evidence in a rat model that maternal hypoxia during gestation alters MMPs and TIMPs expression patterns in the developing heart and results in the abnormal cardiac growth pattern and cardiomyocyte proliferation with the aberrant content of fibrillar collagen network in fetal and neonatal hearts.

Materials and Methods

Experimental Animals and Hypoxic Exposure

Time-dated pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI) and were randomly divided into two groups: 1) normoxic control, and 2) hypoxic treatment of 10.5% oxygen from gestational day 15 to day 21, as described previously (Xue and Zhang, 2009). Hearts were obtained from day 21 fetal and day 7 neonatal rats of mixed sex. The sample size was 4 or 5 pups per group. For Western immunoblots, hearts were flash frozen in liquid nitrogen and stored at -80°C until analysis. For the tissue slide preparation, hearts were fixed in 10% buffered formalin and embedded in paraffin. All procedures and protocols used in the present study were approved by the Institutional Animal Care and Use Committee, and followed the guidelines by US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Myocardial Morphometry

Transverse sections of 5 μm prepared from the middle portion of each heart were mounted and stained with hematoxylin and eosin. Sections were viewed at 20× or 40× magnification. The images were digitized and analyzed by the Image-Pro Plus image analysis software. The left ventricular wall thickness was determined in the anterior wall (AW), posterior wall (PW), septal wall (SW), and free wall (FW).

Collagen Measurement

Fibrillar collagen structure and composition in fetal and neonatal hearts were examined by scanning electron microscopy (SEM), as described previously (Ohtani et al., 1988). Briefly, the fetal and neonatal hearts were fixed in 2.5% glutaraldehyde and stored at 4°C until processing. Hearts were immersed in 10% NaOH for 2-4 days at room temperature, and then rinsed in distilled water for several times until the heart became transparent. Hearts were then treated with 1% aqueous solution of tannic acid for 2-3 h, rinsed in distilled water for overnight, and postfixed in 1% aqueous solution of OsO_4 for 1-2 h. The specimens were dehydrated in a series of increasing ethanol concentrations (50, 80, and 100%) and further dehydrated by critical point drying at 31°C for 5–10 min. The samples were then mounted on a specimen holder for drying overnight in a desiccator, coated with gold and examined with a Philips XL-20 SEM. High-resolution digital images were acquired directly to a computer. The collagen deposition in the heart was also determined by collagen staining using Picrosirius Red that binds specifically to collagens. Paraffin sections were first dewaxed, then further deparaffinized by xylene and rehydrated sequentially in ethanol. Rehydrated sections were hematoxylin stained and washed for 10 min in water. The sections were stained in 0.1% picrosirius red for 1 h followed by washing in two changes of acidified water (5 ml glacial acetic acid in 1 liter of water). Slides were then dehydrated again in increasing concentrations of ethanol up to 100%, followed by washing in xylene and mounted. Sections were viewed at 20 or 40× magnification, and the images were digitized and analyzed by the Image-Pro Plus image analysis software. Soluble collagen content was determined using the QuickZyme collagen assay kit (QuickZyme Biosciences, Netherlands) according to the

instructions of the manufacturer. Briefly, hearts were homogenized in 0.5 M acetic acid and pepsin (1:10 weight/tissue wet weight) and incubated overnight at 4 °C. The samples were then centrifuged (10 min at 14,000 rpm), the supernatant was collected and total protein was quantified. Samples (200 µg) and the collagen standard were added to assigned wells in a 96-well plate, and the dilution buffer was added to each well to a final volume of 140 µL/well. The Sirius Red dye solution was added to each well, and the plate was sealed and incubated on ice with gently shaking for 20 min. The plate was then centrifuged at 3000 ×g at 4 °C. The collagen fiber that binds to Sirius Red dye forms a pellet at the bottom of the well. The pellets were washed 3 times with the washing buffer. The detection buffer was added to the pellets and mixed thoroughly and the signal was read at 540 nm. A standard curve was generated using the collagen standard provided by the kit and collagen content (µg) per well was determined. All steps were performed on ice to avoid degradation of the collagen fibers.

Ki-67 Staining

The application of the nuclear protein, Ki-67, was used to determine the cell proliferation of cardiomyocytes, as described previously (Xue et al., 2008). Hearts were fixed in 10% neutral buffered formalin and embedded in paraffin. Immunohistochemical detection of proliferation marker Ki-67 was performed using BD Pharmingen anti-Ig HRP detection kit. Briefly, transverse slices of hearts were first deparaffinized in xylene and rehydrated with a series of decreased concentrations of alcohol (100%, 95%, 90%, 75%). To block the endogenous peroxidase activity, the slices were incubated with 0.3% H_2O_2 for 10 min. Nonspecific binding sites was blocked for 1 h at room temperature in a

Tris-buffered saline solution containing 5% bovine serum albumin. The slices were then incubated with mouse monoclonal antibody against Ki-67 (1:50, Abcam Inc, Cambridge, MA) for overnight at 4 °C. The slices were rinsed three times in phosphate-buffered saline for 5 min each time, followed by incubation with biotinylated goat anti-mouse IgG (1:50, BD Pharmingen) for 60 min at room temperature. The samples were then exposed to streptravidin-HRP and reacted with diaminobenzidine substrate solution according to the manufacture's recommendations. The slices were viewed with a Zeiss microscope, and images were captured with an attached SPOT digital camera imaging system.

Western Blot Analysis

Hearts were homogenized in a lysis buffer containing 150 mM NaCl, 50 mM Tris.HCl, 10 mM EDTA, 0.1% Tween-20, 1% Triton, 0.1% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 µg/ml leupeptin, and 5 µg/ml aprotinin, pH 7.4 and allowed to incubate for 1 h on ice. Homogenates were then centrifuged at 4 °C for 10 min at 10,000 ×g, and supernatants collected. Protein concentrations were measured using a protein assay kit (Bio-Rad, Hercules, CA). Samples with equal amounts of protein were loaded onto 10% polyacrylamide gel with 0.1% SDS and separated by electrophoresis at 100 V for 90 min. Proteins were then transferred onto nitrocellulose membranes. Nonspecific binding sites was blocked for 1 h at room temperature in a Tris-buffered saline solution containing 5% dry-milk. The membranes were then probed with primary antibodies against collagen I, III, MMP-2 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:300 dilution), MMP-1 (Calbiochem, San Diego, CA; 1:1000 dilution), MMP-9, TIMP-1, -2, -3 (Millipore, Temecular, CA; 1:1000 dilution) and TIMP-4

(Abcam Inc, Cambridge, MA). To assure equal loading, band intensities were normalized to beta2-microglobulin (B2M) determined by its antibody (Abcam Inc, Cambridge, MA). After washing, membranes were incubated with secondary horseradish peroxidase-conjugated antibodies. Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. The results were analyzed with the Kodak ID image analysis software.

Gelatin Zymography

Hearts were homogenized in a lysis buffer containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% nonidet P-40, 0.1% SDS, 0.1% deoxycholic acid, 1% protease inhibitor, 0.5% PMSF. Homogenates were then centrifuged at 4 °C for 20 min at 15,000 ×g, and supernatants collected. Protein concentrations were measured using a protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein (60 μg) were loaded and separated by 10% Tris-glycine gel with 0.1% gelatin as substrate. The gel was renatured by renaturation buffer (Bio-Rad, Hercules, CA) for 1 h and then incubated with a development buffer (Bio-Rad, Hercules, CA) at 37 °C for 48 h. The gel was stained with 0.5% Coomassie blue R-250 (Bio-Rad, Hercules, CA) for 1 h and then destained with destaining buffer (Bio-Rad, Hercules, CA) till the bands became clear. Data were analyzed with the Kodak ID image analysis software. Gelatinolytic activity was determined as clear zones or bands at the appropriate molecular weights. Mouse active MMP-9 (Chemicon, Temecular, CA) was used as a standard.

Statistical Analysis

Data are expressed as mean ± SEM. Experimental number (n) represents the hearts of fetuses or neonates from different dams. Statistical significance ($p < 0.05$) was determined by analysis of variance (ANOVA) or Student's *t* test, where appropriate.

Results

Effect of Hypoxia on Fetal and Neonatal Heart Weight and Ventricular Wall Thickness

Maternal hypoxia significantly decreased fetal (3.9 ± 0.1 g *vs.* 3.2 ± 0.1 g, $p < 0.05$) and neonatal (15.0 ± 0.3 g *vs.* 9.2 ± 0.6 g, $p < 0.05$) body weight. There was no significant difference in fetal heart weight between the control and hypoxic animals (23.9 ± 0.9 mg *vs.* 22.6 ± 0.7 mg, $p > 0.05$). However, hypoxia significantly increased the heart to body weight ratio in fetal rats (6.1 ± 0.1 mg/g *vs.* 7.0 ± 0.2 mg/g, $p < 0.05$). Neonatal heart weight was significantly decreased in hypoxic animals (119.5 ± 3.6 mg *vs.* 75.8 ± 6.0 mg, $p < 0.05$), while the heart to body weight ratio in neonatal rats was not changed by hypoxia (8.0 ± 0.2 mg/g *vs.* 8.2 ± 0.5 mg/g, $p > 0.05$). To determine the effect of hypoxia on left ventricular wall thickness, hematoxylin and eosin-stained tissue slides were examined. As shown in Fig. 9, the thickness of anterior wall, septal wall and free wall was decreased in the fetal heart by hypoxia. As expected, the wall thickness of left ventricle was significantly increased in the neonatal heart, as compared with that of fetal heart. However, fetal hypoxia resulted in significant decreases in the thickness of anterior wall, posterior wall, septal wall and free wall of left ventricle in the neonatal heart (Fig.

9). Additionally, the epicardial detachment from underling myocardium was seen in all samples of fetal hearts treated with maternal hypoxia (Fig. 9).

Effect of Hypoxia on Fibrillar Collagen Structure and Composition in Fetal and Neonatal Hearts

Fibrillar collagen structure and composition were assessed by scanning electron microscope in the heart from fetal and neonatal rats. As shown in Fig. 10, fibrillar collagen fibers were cross-linked randomly to form a complicated matrix network in both fetal and neonatal hearts. Maternal hypoxia did not change collagen matrix in the fetal heart, but increased fibrillar collagen weave matrix in the neonatal heart (Fig. 10). The collagen content and distribution in the left ventricle were examined further by the collagen staining using picrosirius red. As shown in Fig. 11A, collagen forms the fiber bundles in the interstitial space. Maternal hypoxia did not change the total collagen content in the fetal heart but increased it in the neonatal heart (Fig. 11A). The soluble collagen content in the heart was determined using a collagen assay kit. Fig. 11B shows that maternal hypoxia had no significant effect on soluble collagen content in the fetal heart. The collagen content in the heart showed a development-dependent increase from the fetus to the neonate, and maternal hypoxia resulted in a significantly greater increase in fibrillar collagens in the neonatal heart (Fig. 11B). The expression of major types of collagens in the heart, collagen I and collagen III, were determined by Western blots (Fig. 11C). Maternal hypoxia caused a reduction in collagen I in the fetal heart but a significant increase in collagen I in the neonatal heart. In contrast, collagen III levels were not significantly altered in either fetal or neonatal hearts.

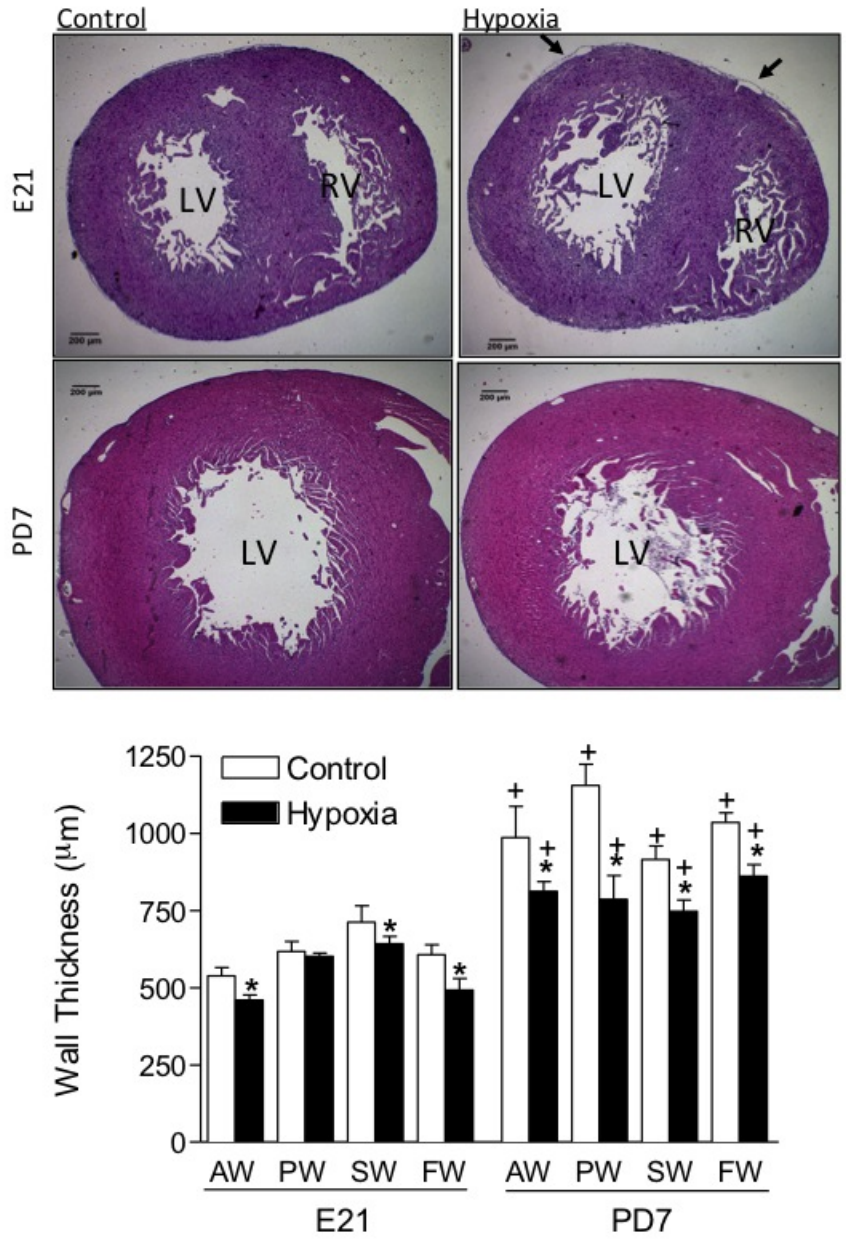

Figure 9. The effect of maternal hypoxia on ventricular morphology. Hearts were isolated from 21-day fetal (E21) and postnatal day 7 (PD7) rats in the control and hypoxic groups. Arrows show the detachment of epicardium in the hypoxic fetal heart. The left ventricular wall thickness was determined at anterior wall (AW), posterior wall (PW), septal wall (SW) and free wall (FW). LV: left ventricle; RV: right ventricle. Data are means ± SEM. Data were analyzed by two-way ANOVA. * $P < 0.05$, hypoxia *vs.* control; † $P < 0.05$, PD7 *vs.* E21. n = 3-5 per group.

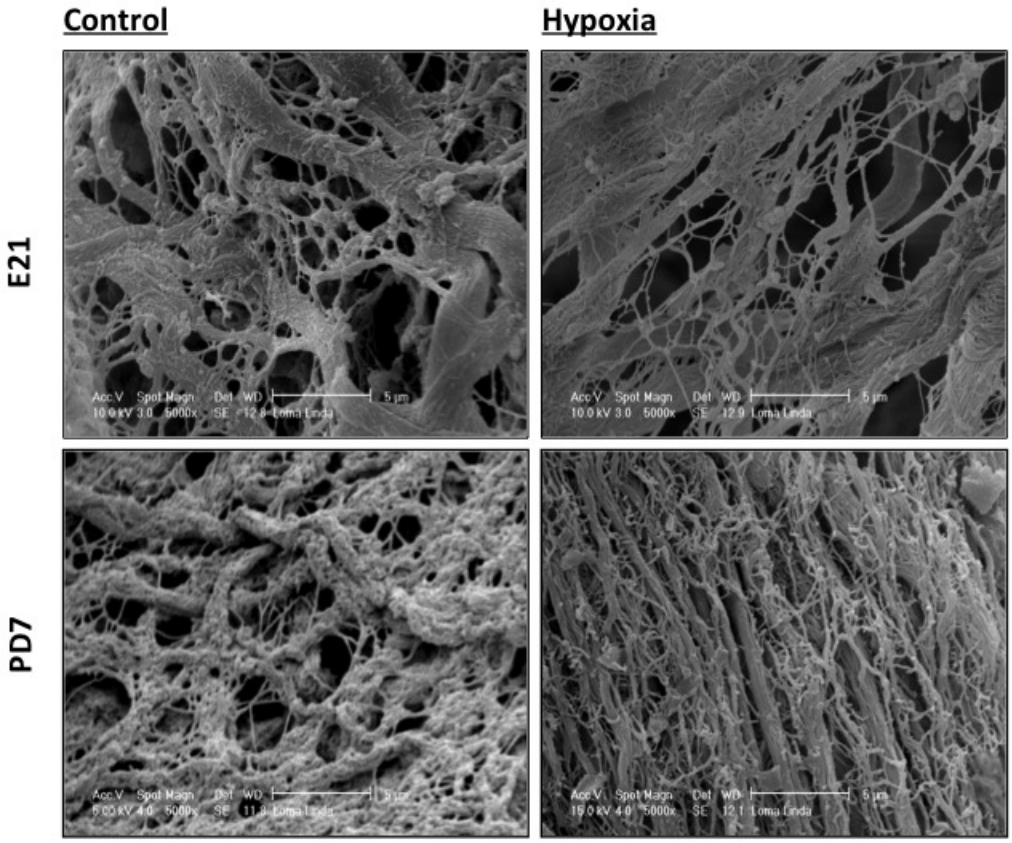

Figure 10. The effect of maternal hypoxia on fibrillar collagen structure and composition. Hearts were isolated from 21-day fetal (E21) and postnatal day 7 (PD7) rats in the control and hypoxic groups. Fibrillar collagen structure and composition were examined by scanning electron microscope. Hypoxia increased the collagen matrix in PD7 but not E21 hearts. The images were representatives of the heart samples from 4 control E21, 4 hypoxic E21, 5 control PD7, and 6 hypoxic PD7 rats.

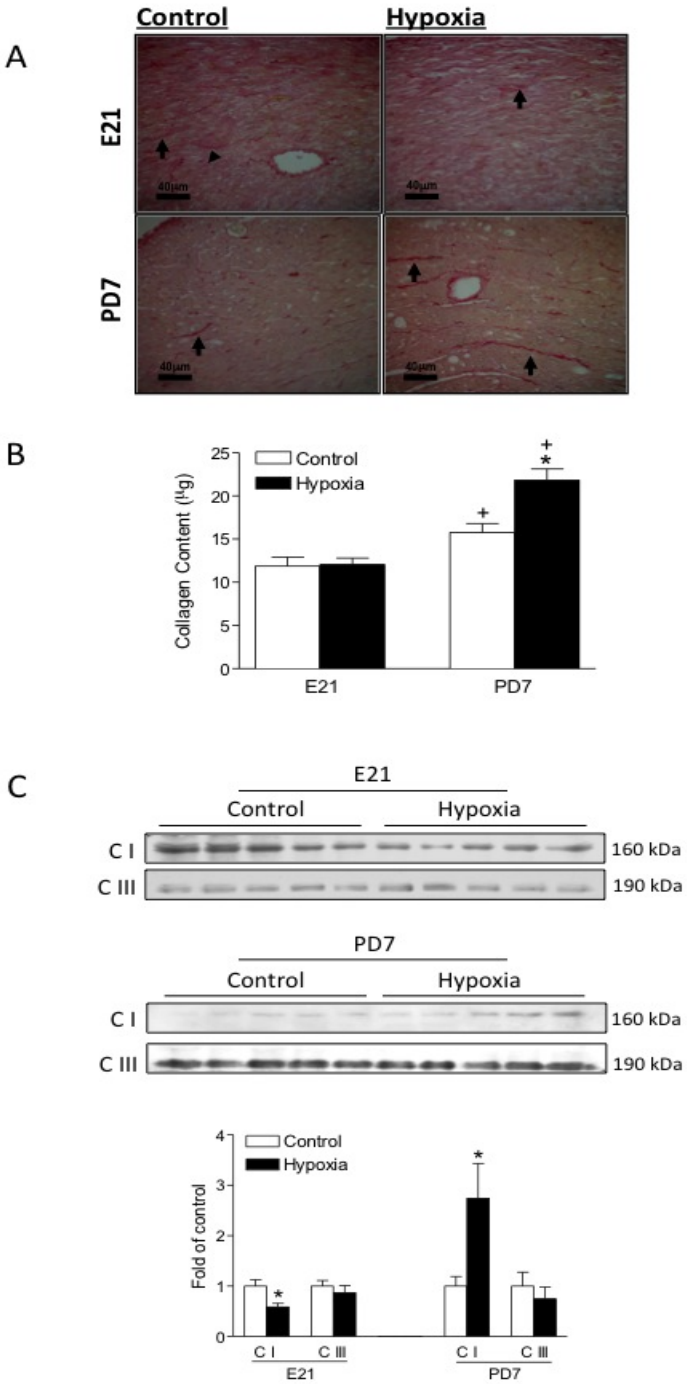

Figure 11. The effect of maternal hypoxia on the fibrillar collagen content. Hearts were isolated from 21-day fetal (E21) and postnatal day 7 (PD7) rats in the control (C) and hypoxic (H) groups. The collagen content in tissue sections (**A**) and the soluble fraction of collagen content in the heart (**B**) were determined by picrosirius red staining. Arrows show the collagen deposition in the anterior wall of left ventricle. Protein levels of collagen I (C I) and collagen III (C III) were determined by Western blots (**C**). Data are means ± SEM. Data in panel **B** were analyzed by two-way ANOVA. Data in panels **C** were analyzed by *t*-test. * $P < 0.05$, hypoxia *vs*. control; † $P < 0.05$, PD7 *vs*. E21. n = 5 per group.

Effect of Hypoxia on Cell Proliferation in Fetal and Neonatal Hearts

Cardiomyocyte proliferation in the fetal and neonatal hearts was determined by examining the immunostaining of the nuclear protein, Ki-67. The Ki-67 expression occurs throughout all phases of the cell cycle, except for the G0 phase. As shown in Fig. 12, the immunostaining of Ki-67 revealed the dark brown dots within the cells. Maternal hypoxia significantly decreased Ki-67 positive nuclei in both the fetal and neonatal hearts, suggesting a reduced proliferative activity of cardiomyocytes (Fig. 12).

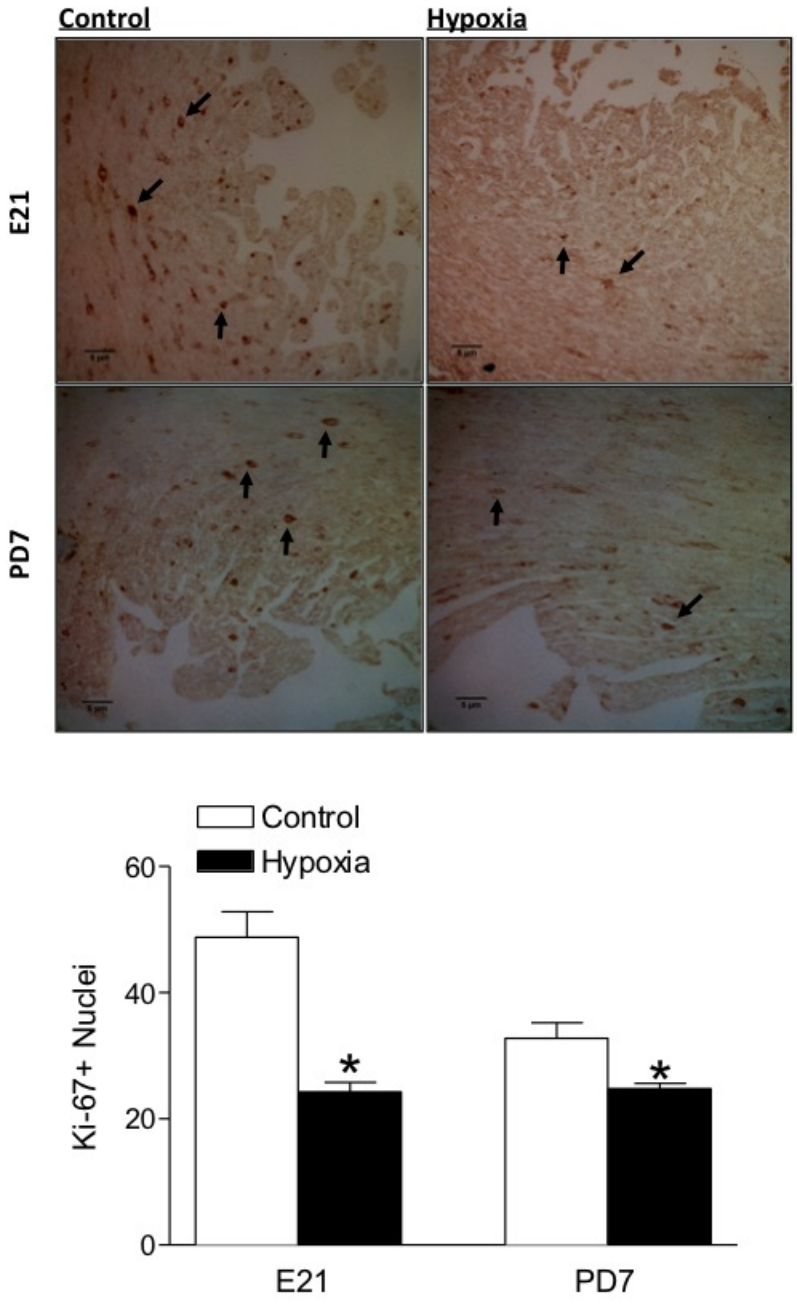

Figure 12. The effect of maternal hypoxia on cardiomyocyte proliferation. Hearts were isolated from 21-day fetal (E21) and postnatal day 7 (PD7) rats in the control and hypoxic groups. Cell proliferation was examined by the Ki-67 staining. Arrows show Ki-67 positive nuclei. Data are means ± SEM. Data were analyzed by *t*-test. * $P < 0.05$, hypoxia *vs*. control. n = 4 per group.

Effect of Hypoxia on MMPs and TIMPs in Fetal and Neonatal Hearts

To elucidate the potential mechanisms underlying the hypoxia-induced cardiac remodeling, we determined the effect of maternal hypoxia on the expression of active MMP-1, -2, -9, -13, MT1-MMP and the expression of TIMP-1, -2, -3, -4 in fetal and neonatal hearts. As shown in Fig. 13, maternal hypoxia resulted in differential expression patterns of MMPs in the fetal and neonatal hearts. The MMP-1 expression was decreased in the fetal heart, but was increased in the neonatal heart. Although it may be debating whether rodents express MMP-1, previous studies clearly demonstrated the expression of MMP-1 in rat hearts (Chen et al., 2004; Kwak et al., 2011). MMP-13 was increased in both the fetal and neonatal hearts. MT1-MMP was increased only in the fetal heart. In contrast, the expression of active MMP-2 and MMP-9 were not altered in either fetal or neonatal hearts. Additionally, the proteolytic activities of MMP-2 & -9 were measured with gelatin zymography. As shown in Fig. 14, there was a lack of activity of active MMP-9 at 82 kDa in fetal or neonatal hearts. MMP-2 activities at 62 kDa and 72 kDa were not significantly altered by maternal hypoxia in either fetal or neonatal hearts. Although maternal hypoxia had no significant effect on TIMP-1 and TIMP-2 expressions, it significantly increased TIMP-3 and TIMP-4 levels in both the fetal and neonatal hearts (Fig. 15). Table 1 summarizes the relative changes of MMPs, TIMPs and collagens in fetal and neonatal hearts in response to maternal hypoxia.

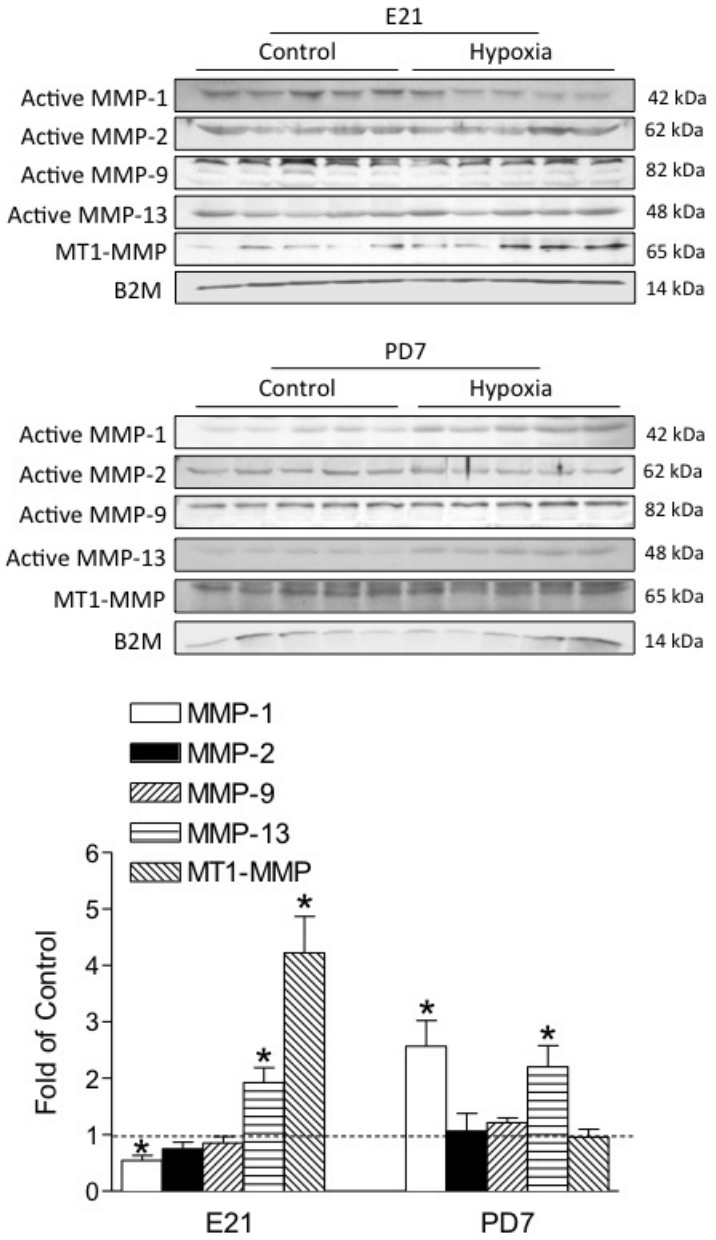

Figure 13. The effect of maternal hypoxia on MMPs expression. Hearts were isolated from 21-day fetal (E21) and postnatal day 7 (PD7) rats in the control and hypoxic groups. Active MMPs protein levels were determined by Western blots. Data are means ± SEM. Data were analyzed by *t*-test. * $P < 0.05$, hypoxia *vs*. control. n = 5 per group.

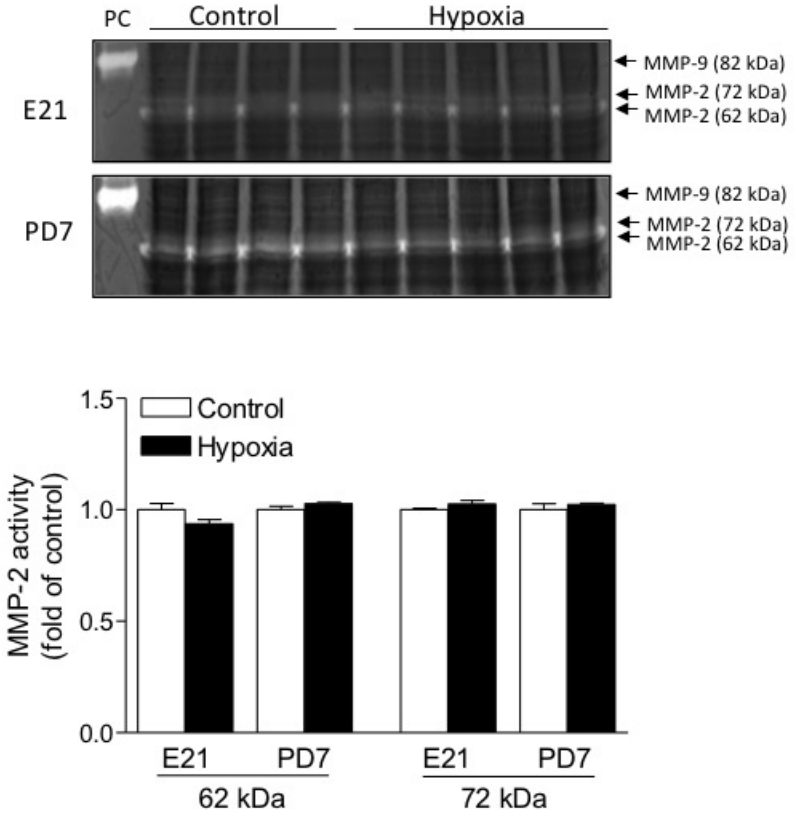

Figure 14. The effect of maternal hypoxia on the activity of MMP-2 & -9. Hearts were isolated from 21-day fetal (E21) and postnatal day 7 (PD7) rats in the control and hypoxic groups. The activities of MMP-2 & -9 were determined by gelatin zymography. Clear bands indicate positive MMP activity. MMP-2 activities are indicated by clear bands associated with molecular weights of 62 kDa and 72 kDa. No clear bands associated with active MMP-9 at 82 kDa were present in the hearts. PC: positive control of active MMP-9 at 82 kDa. The bottom graph illustrates the mean values of densitometric analysis of MMP-2 activities in fetal and neonatal hearts. Data are means ± SEM. Data were analyzed by *t*-test. n = 4-5 per group.

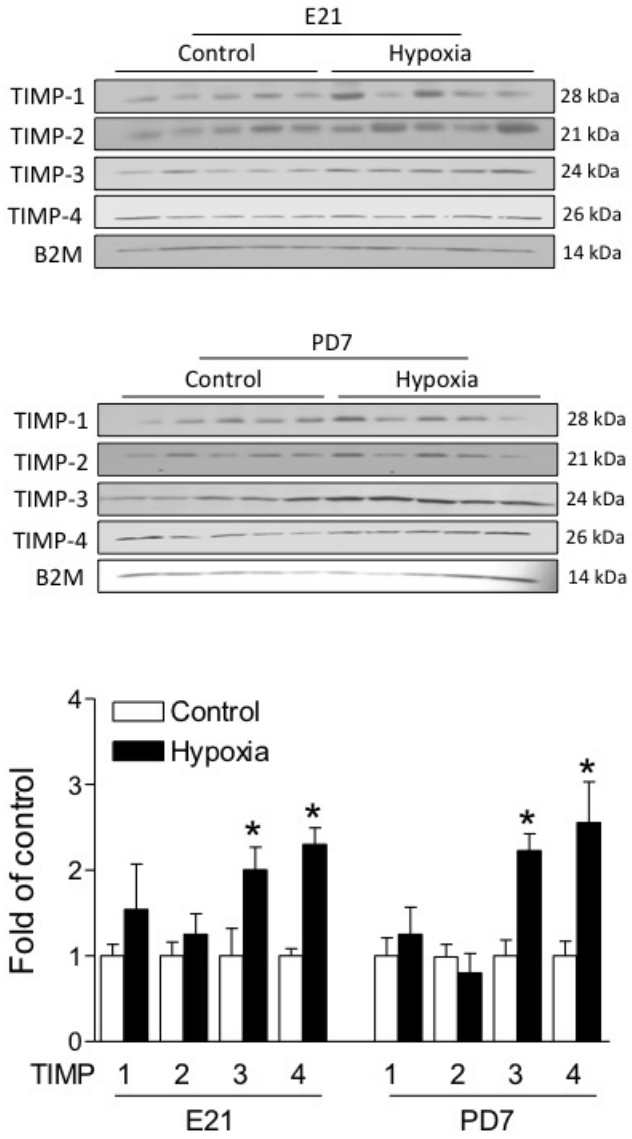

Figure 15. The effect of maternal hypoxia on TIMPs levels. Hearts were isolated from 21-day fetal (E21) and postnatal day 7 (PD7) rats in the control and hypoxic groups. TIMPs protein levels were determined by Western blots. Data are means ± SEM. Data were analyzed by *t*-test. * $P < 0.05$, hypoxia *vs*. control. n = 5 per group.

Table1: Maternal hypoxia-induced changes in the expression of MMPs, TIMPs and collagens. Hearts were isolated from 21-day fetal (E21) and postnatal day 7 (PD7) rats in the control and hypoxic groups, and expression levels of MMPs, TIMPs and collagens were determined by Western blot. ↑: increase by hypoxia; ↓: decrease by hypoxia; —: no change by hypoxia.

	MMPs					TIMPs				Collagens	
	1	2	9	13	MT1	1	2	3	4	I	III
E21	↓	—	—	↑	↑	—	—	↑	↑	↓	—
PD7	↑	—	—	↑	—	—	—	↑	↑	↑	—

Discussion

The present study demonstrates in a rat model that maternal chronic hypoxia causes remodeling of the developing heart in the fetus and neonate by decreasing cardiomyocyte proliferation and increasing the collagen deposition. Previous studies demonstrated that maternal hypoxia increased hypoxia-inducible factor 1α (HIF-1α) protein levels in rodent fetal hearts (Bae et al., 2003; Ream et al., 2008), indicating tissue hypoxia of the fetal heart in response to maternal hypoxia. In the present study, the finding that maternal hypoxia significantly decreased immunostaining of the proliferation marker Ki-67 in fetal and neonatal hearts is intriguing. Although Ki-67 is a nonspecific cell proliferation marker and may stain both cardiomyocytes and cardiac fibroblasts, the previous finding that hypoxia promoted cardiac fibroblast proliferation in rodents and humans (Agocha et al., 1997; Kacimi et al., 2007) suggests that the maternal hypoxia-mediated decrease in the Ki-67 staining in fetal and neonatal hearts in the present study is primarily due to the reduced cardiomyocyte proliferation. In rat heart development, the transition of proliferative and hyperplasic growth of mononucleated cells to hypertrophic growth of binucleated cells and terminal differentiation of cardiomyocytes take place within the first two weeks after birth (Louey and Thornburg, 2005). Consistent with the present finding, previous studies demonstrated an increase in the percentage and cell size of binuleated myocytes in fetal rat heart in response to maternal hypoxia (Bae et al., 2003), an early morphologic indicator of cardiomyocyte hypertrophy (Corstius et al., 2005). Taken together, these studies suggest that fetal hypoxia causes a premature exit of the cell cycle in the fetal heart leading to a fewer but larger cardiomyocytes in offspring. Indeed, enlarged myocytes have been demonstrated in the heart of adult offspring rats

that had been exposed to hypoxia before birth (Li et al., 2004; Xu et al., 2006). The finding of maternal hypoxia-mediated remodeling of the heart during its critical developmental stages of the fetus and neonate suggests programming of aberrant heart function. Indeed, the functional impact of remodeling on postnatal development in the adult heart has been demonstrated in an essentially same animal model of maternal hypoxia, in which reduced MMP-2 and enhanced collagen accumulation were found along with left ventricular hypertrophy and stiffening, diastolic dysfunction, and increased ischemic injury in 4 and 7 month old offspring (Xu et al., 2006). Similar findings that maternal hypoxia causes fetal programming of ischemia-sensitive phenotype in the adult heart have been demonstrated in our previous studies (Li et al., 2004; Li et al., 2003; Patterson et al., 2010; Xue et al., 2011; Xue and Zhang, 2009).

Whereas the mechanisms underlying the hypoxia-mediated reduction of myocyte proliferation in the fetal heart remain elusive, the present finding of the epicardial detachment in the hypoxic fetus provides a possible mechanism by which hypoxia inhibits proliferative and hyperplasic growth of the fetal heart. During the fetal development, the epicardium provides the precursor cells that give rise to various cell types in the heart, and it also supplies multiple growth factors to stimulate cardiac myocyte proliferation, including fibroblast growth factor (FGF)-2, Wingless-type MMTV integration site (Wnt)-9b, FGF-9 and other epicardially-derived factors (34, 39). The detachment of epicardium from myocardium is likely to reduce the availability of mitogenic factors and growth factors to the myocardium and subsequently to inhibit myocardial proliferation (Ream et al., 2008). Additionally, the present study demonstrates that fetal hypoxia increases TIMP-3 and TIMP-4 expression levels in both

fetal and neonatal hearts, suggesting another possible mechanism in the hypoxia-mediated down-regulation of myocyte proliferation. In addition to their roles in modulating MMPs, it has been demonstrated that both TIMP-3 and TIMP-4 play a key role in inhibiting cardiomyocyte proliferation in rat hearts possibly in an MMP-independent and receptor-mediated manner (Fedak et al., 2004; Hammoud et al., 2009; Hammoud et al., 2007; Vanhoutte and Heymans, 2010).

In the present study, the finding that maternal hypoxia causes an increased fibrillar collagen content in neonatal, but not fetal, hearts is intriguing, and suggests a critical window in compensatory remodeling of the collagen matrix in the neonatal heart resulting from hypoxia-mediated premature exit of cell cycle in the fetal heart. In the heart, collagens are the primary extracellular proteins supporting the myocardium and determining the tissue stiffness. The present study demonstrates that hypoxia differentially regulates collagen I and collagen III expression in the developing heart. The increased collagen content found in the neonatal heart is mainly due to an increase in collagen I. During the postnatal development, collagen I represents more than 85% of the total collagen in the heart (Carver et al., 1993; Eghbali and Weber, 1990). Unlike the finding in the neonatal heart, collagen I was decreased in the fetal heart by hypoxia. Although it remains unclear what is the major type of collagens in the fetal heart, collagen III is suggested to be one of the most important fibrillar collagens in the heart during the fetal development (Jackson et al., 1993). The finding that hypoxia did not significantly affect collagen III levels in the fetal heart consists with the lack of apparent changes in the total fibrillar collagen content in the fetal heart. Although the lack of changes in collagen III was also demonstrated in neonatal hearts in the present study, a

previous study showed that maternal hypoxia increased the deposition of both collagen I and III in the heart of adult offspring (Xu et al., 2006), suggesting a continuous remodeling process in the heart during the postnatal development.

The present findings of decreased MMP-1 in the fetal heart and increased MMP-1 in the neonatal heart are somewhat surprising, given that MMP-1 as collagenase 1 is a primary enzyme that digests collagen I. This suggests a complex pattern of the interaction between collagens and MMPs in the developing heart. It is possible that fetal hypoxia caused an imbalance of collagen synthesis and degradation in the developing heart with the effect of synthesis predominant. Multiple pathways have been identified in the hypoxia-enhanced synthesis of collagen I, including reactive oxygen species, mitogen-activated protein kinase, and transforming growth factor-beta 1 (Agocha et al., 1997; Higgins et al., 2008; Hu et al., 2007). Although it is not known at present, the possibility that the collagen I and III genes have different HIF-1 promoter sites that may regulate differentially expressions of collagen I and III in the heart remains an intriguing area for the further investigation. The changes in MMP-1 levels in the same direction of those in collagen I observed in the hypoxic hearts may reflect a compensatory response to the synthesis of collagen I. On the other hand, hypoxia-mediated changes in MMP-1 expression levels may in turn lead to a compensatory response of collagen I synthesis that exceeds its degradation. Similar findings of the apparent paradoxical changes of collagens and MMPs in the same direction resulting from the hypoxia treatment were also obtained in mice (Hu et al., 2007), supporting the notion of feedback regulation of collagens on MMPs levels. It has been shown that the increased collagens can activate the discoidin domain receptor (DDR) and therefore up-regulate MMPs (Vogel et al.,

1997; Xu et al., 2005). In the heart, DDR 2 is primarily expressed in fibroblasts that are the major source of ECM components and MMPs (Goldsmith et al., 2004; Kania et al., 2009). Similarly, MMP-13 (collagenase 3) was significantly increased by hypoxia in both fetal and neonatal hearts, whereas collagen III remained unchanged. This further supports the notion that fibrillar collagens in the developing heart are regulated primarily through their synthesis rather than their degradation via collagenases. In addition to collagenases, MT1-MMP is another primary enzyme that digests fibrillar collagens in the heart (D'Armiento, 2002; Ohuchi et al., 1997). The increased MT1-MMP levels in the hypoxic fetal heart may contribute to the decreased collagen I, and the lack of MT1-MMP increment in the neonatal heart may enhance the accumulation of collagen I synthesis resulting from fetal hypoxia. While collagenases (MMP-1 and MMP-13) possess high substrate specificity for fibrillar collagens, MT1-MMP has been shown to degrade nonmatrix substrates including cytokines, bioactive peptides, and growth factors in the myocardium (Spinale, 2007). It is possible that the elevated MT1-MMP also contributes to the reduced cardiomyocyte proliferation observed in the hypoxic fetal heart. The lack of effect of fetal hypoxia on MMP-2 and MMP-9 expression levels and activities and their endogenous inhibitors TIMP-2 and TIMP-1 in the heart indicates the minimal role of the gelatinases in hypoxia-mediated remodeling of fetal and neonatal hearts. Nonetheless, the active MMP-2 levels were found decreased in the heart of adult rats that had been exposed to hypoxia before birth (Xu et al., 2006), suggesting a continuous programming of MMPs expression patterns in the heart during the postnatal development.

The present study provides new insights in the maternal hypoxia-mediated heart remodeling during its critical developmental stages of the fetus and neonate, and suggests a role of altered MMP-TIMP expressing patterns in the developing heart. Although it is difficult to demonstrate a true cause-effect relation of MMPs-TIMPs and cardiac remodeling in living animals particularly in a pregnant animal model at present due to the lack of selective inhibitors and the difficulty of their use in a pregnant animal model, as well as the difficulty of transgenic approach in a rat model, the present findings provide important physiological information and basis for further more mechanistic investigation possibly using an approach of siRNA in cultured organ/heart or cardiomyocytes. Given that hypoxia is one of the most important and clinically relevant stresses to the fetus, and that large epidemiological studies indicate a link between *in utero* adverse stimuli during gestation and an increased risk of heart disease in the adulthood, the possibility that fetal hypoxia may result in programming of a heightened vulnerability of remodeling and failing heart later in life provides a mechanistic understanding worthy of investigation in humans. Indeed, it has been shown in rats that antenatal hypoxia enhances collagen accumulation and left ventricular hypertrophy along with stiffening, diastolic dysfunction, and the increased heart susceptibility to ischemia and reperfusion injury in adult offspring (Li et al., 2004; Li et al., 2003; Patterson et al., 2010; Xu et al., 2006; Xue et al., 2011; Xue and Zhang, 2009).

Acknowledgements

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CHAPTER FOUR

DIRECT INHIBITORY EFFECT OF HYPOXIA ON CARDIOMYOCYTE PROLIFERATION IN FETAL RAT HEARTS

by

Wenni Tong, Fuxia Xiong, Yong Li and Lubo Zhang

Abstract

Maternal hypoxia inhibits cell proliferation in the heart of fetal and neonatal rats. This study determined the direct effect of hypoxia on cardiomyocyte proliferation in fetal rat hearts. Isolated hearts of day 17 fetal rats and rat embryonic ventricular myocyte H9c2 cells were treated ex vivo with 21% or 1% O2 for 48 or 24 h. Bromodeoxyuridine (BrdU) and Ki-67 were examined by immunohistochemistry and immunofluorescence, respectively. The expression of tissue inhibitors of metalloproteinases (TIMP) -3 & -4, cell division marker Cyclin D2 and cell division inhibitor p27 was measured by Western blots. IHC staining indicated decreased numbers of BrdU positive nuclei in hypoxic-treated fetal hearts. Analysis of double immunofluorescence revealed a predominant distribution of Ki-67 in cardiomyocytes in fetal hearts, and hypoxia caused a reduction of Ki-67 colocalization with cardiomytocytes. In accordance, decreased Ki-67 positive nuclei were demonstrated in H9c2 cells under the hypoxic condition. Cyclin D2 was decreased in hypoxic fetal hearts and H9c2 cells. In contrast, hypoxia enhanced the expression of p27, TIMP-3 and TIMP-4 in fetal hearts and H9c2 cells. Additionally, the presence of TIMP-4 siRNA restores the cell proliferation in the H9c2 cells following hypoxia. The results suggest that hypoxia has direct inhibitory effect on cardiomyocyte proliferation in the developing hearts, which can be mediated by the upregulation of TIMP-4.

Introduction

Large epidemiological studies and animal studies have indicated a clear association of adverse intrauterine environment with an increased incidence of low birth weight, perinatal mortality, and cardiovascular disease in later adult life (Barker and Osmond, 1986; Bateson et al., 2004; Gluckman et al., 2008; McMillen and Robinson, 2005). In humans, one of the common stresses in utero is hypoxia, which may occur under many conditions, including pregnancy at high altitude, pregnancy with cigarette smoke, drug abuse, anemia, pulmonary disease and hypertension (Zhang, 2005). Previous studies in rats have demonstrated that maternal hypoxia increased apoptotic cell death and inhibited cell proliferation in the fetal heart, both of which contribute to a fewer but larger cardiomyocytes in offspring (Bae et al., 2003; Tong et al., 2011). In addition, chronic maternal hypoxia alters the expression of matrix metalloproteinases (MMPs) and their endogenous inhibitors - tissue inhibitors of metalloproteinase (TIMPs), and enhances the collagen accumulation and left ventricular hypertrophy along with stiffening, diastolic dysfunction and the increased heart susceptibility to ischemia-reperfusion injury in adult offspring (Li et al., 2004; Li et al., 2003; Patterson et al., 2010; Tong et al., 2011; Xu et al., 2006; Xue et al., 2011; Xue and Zhang, 2009).

It is known that growth of the fetal heart normally occurs by myocyte division and enlargement along with proportional growth of the other types of cardiac tissue (Jonker et al., 2010). Inappropriate prenatal loss of cardiomyocytes through reduced proliferation appears to play a role in a variety of cardiac dysfunctions in infants and adults. Nonetheless, it remains unclear whether hypoxia induced down-regulation of cardiomyocyte proliferation is a result of hypoxia acting directly on the fetal heart or a result of secondary effects induced by maternal hypoxic insult. For instance, maternal

hypoxia may increase circulating glucocortiods level that is associated with several adverse effects including lower birth weight and apoptosis (Gardner et al., 2001; Larsen et al., 1997; Levitt et al., 1996; Zuloaga et al., 2011). The present study tested the hypothesis that hypoxia has direct effect on myocyte proliferation in the fetal rat hearts and rat embryonic ventricular H9c2 cells.

In addition, the mechanisms whereby prenatal hypoxia inhibits cell proliferation in the fetal hearts are poorly understood. Our recent data have demonstrated fetal hypoxia significantly increased TIMP-3 and TIMP-4 expression levels in both fetal and neonatal hearts (Tong et al., 2011), suggesting a possible mechanism in the hypoxia-mediated downregulation of myocyte proliferation. In addition to the roles in modulating MMPs, it has been shown that both TIMP-3 and TIMP-4 play a key role in inhibiting cardiomyocyte proliferation in rat hearts possibly in an MMP-independent and receptor-mediated manner (Hammound et al., 2009; Hammound et al., 2007; Vanhoutte and Heymans, 2010). Herein, we present evidence that 1) hypoxia has direct inhibitory effect on cardiomyocyte proliferation in the developing heart; 2) the knockdown of TIMP-3 or TIMP-4 abrogates hypoxia-inhibited cell division in H9c2 cells.

Methods

Experimental Animals

Time-dated pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI). Isolated fetal hearts were studied. All procedures and protocols used in the present study were approved by the Institutional Animal Care and Use Committee of Loma Linda University, and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Intact Heart Culture and Cell Culture

Hearts were isolated from gestational day 17 fetal rats and were cultured in M199 media (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 1% (10000 U/ml penicillin and 10000μg/ml streptomycin) at 37°C in 95% air/5% CO_2. Hearts were given 24h to recover before the treatment. To study the direct effect of hypoxia on the fetal heart, hearts were incubated at 37°C in 21% O_2 or 1% O_2 for 48 hours, as reported previously (Patterson et al 2010). Hearts were observed to spontaneously beat after the hypoxic treatment. For the in vitro study, an embryonic rat heart cell line H9c2 obtained from ATCC (Rockville, MD) were grown and subcultured. Experiments were performed at 70% to 80% confluent. For hypoxic studies, cells were treated with 1% O_2 for 24 hours.

Immunofluorescence Staining

The expression of Ki-67 and α-sarcomeric actinin (cardiomyocyte marker) were assessed in the fetal hearts and H9c2 cells by double immunofluorescence staining. For the fetal hearts ex vivo model, the samples were first dehydrated for paraffin embedding and were cut at 5μm thicknesses. The transverse slices then deparaffinized in xylene and rehydrated with a series of decreased concentrations of alcohol (100%, 95%, 90%, 75%). To block the endogenous peroxidase activity, the slices were incubated with 0.3% H_2O_2 for 10min. The antigen retrieval was done by microwaving sections in a citrate buffer for 10 min before the immunofluorescence staining procedure. For the H9c2 cells in vitro model, H9c2 cells were first grown on glass coverslips, and then were fixed in acetone for 10 min. Similarly, the slices of the cells were treated with 0.3% H_2O_2 to block the

endogenous peroxidase activity before the immunofluorescence staining procedure. After blocking the tissue or cell slices with 1% bovine serum albumin (BSA) (Fisher, Tustin, CA) for 1 h at room temperature, the sections were incubated with the following primary antibodies: rabbit anti Ki-67 (Abcam, Cambridge, MA) and mouse anti α-sarcomeric actinin (Sigma, St.Louis, MO) antibody (1:100) at 4°C, overnight. The sections were then incubated with the secondary antibodies: anti-mouse FITC-conjugated and anti-rabbit Texas Red-conjugated antibodies (1:200) at room temperature for 1h. After three washes, the slides were stained with Hoechst 33258 (5ug/ml) (Sigma) for 1 min. The sections then were cover slipped with Permount reagent (Fisher) and visualized using the fluorescent laser scanning confocal microscope (LSM 710 laser scanning microscope; Zeiss). The quantitative analysis of colocalization was carried out by Image J software (version 1.45).

Bromodeoxyuridine (BrdU) Staining

As described previously (Horie et al., 2008; Zhao et al., 2012), the DNA synthesis of fetal heart ex vivo were examined by BrdU staining. After 48 hours of incubation in the normoxic or hypoxic chamber, fetal hearts were then incubated in the M199 media supplemented with BrdU labeling reagent (Invitrogen, Camarillo, CA) at 37°C for additional 6 hours in the normoxic or hypoxic chamber, respectively. The hearts were washed in phosphate buffered saline (PBS) (Bio-Rad, Hercules, CA) and then post fixed in formalin at 4°C. Transverse sections of 5□m were prepared from the middle portion of each heart. Immunohistochemical detection of BrdU in fetal hearts was performed using BrdU staining kit (Invitrogen) according to the instructions of the manufacturer. In brief,

transverse slices of hearts were first deparaffinized in xylene and rehydrated with a series of decreased concentrations of alcohol (100%, 95%, 90%, 75%). To block the endogenous peroxidase activity, the slices were incubated with 0.3% H_2O_2 for 10min. Nonspecific binding sites were blocked in the blocking solution for 10min. The slices were then incubated with biotinylated mouse anti-BrdU for 1h at room temperature. After rinsed three times in PBS for 5min each time, the samples were then exposed to strptavidin-peroxidase and reacted with diaminobenzidine substrate solution according to the manufacturer's recommendations. The slices were viewed with a Zeiss microscope, and images were captured with an attached SPOT digital camera imaging system.

Western Blot Analysis

Fetal hearts and H9c2 cells were homgenized in a lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, 10 mM EDTA, 0.1% Tween-20, 1% Triton, 0.1% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin (pH 7.4) and allowed to incubate for 1 h on ice. Homogenates were then centrifuged at 4°C for 10 min at 10000g, and supernatants were collected. Protein concentration was measured in the supernatant using a protein assay kit (Bio-Rad). Samples with equal amounts of proteins were loaded onto 12% polyacrylamide gel with 0.1% sodium dodecyl sulfate and separated by electrophoresis at 100V for 1.5h. Proteins were then transferred onto nitrocellulose membranes. Nonspecific binding sites were blocked for 1h at room temperature in a Tris-buffered saline solution containing 5% dry milk. The membranes were then probed with primary antibodies against Cyclin D2, p27, TIMP-4 (Abcam; 1:1000 dilution), TIMP-3 (Millipore, Temecular, CA; 1:1000 dilution).

To assure equal loading, band intensities were normalized to β2-microglobulin (B2M) (Abcam; 1:10000 dilution) and actin (Sigma; 1:5000 dilution). After washing, membranes were incubated with secondary horseradish peroxidase-conjugatedd antibodies. Proteins were visulized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. The results were analyzed with the Kodak ID image analysis software.

siRNA Transfection

The Silencer-Select Pre-designed siRNAs against rat TIMP-3 and TIMP-4 genes were obtained from Invitrogen (Camarillo, CA). Nontargeting siRNAs (NC) were used as negative control for the gene specific siRNAs. The siRNAs were dissolved in nuclease-free water and transfected into H9c2 cells with the siPORTTM NeoFXTMagent (Invitrogen), following the manufacturer's instructions. Briefly, H9c2 cells were trypsinized and diluted in normal growth medium and set aside at 37°C. siPORTTM NeoFXTMagent was diluted in OPTI-MEM I medium (Invitrogen) and incubated for 10 minutes at room temperature. siRNAs were diluted in OPTI-MEM I medium, and then mixed with the diluted siPORTTM NeoFXTMagent. The mixture was incubated 10 minutes at room temperature, and dispensed with H9c2 cells into 6-well plates, and incubated for 24 hours before starting the treatment. To study the role of TIMP-3 & -4 in hypoxia-reduced cell proliferation, the H9c2 cells were then placed in the hypoxic chamber (1% oxygen) for 24 hours.

Statistical Analysis

Data are expressed as means± SEM. Statistical significance ($p < 0.05$) was determined by analysis of variance (ANOVA) or Student's *t* test, where appropriate.

Results

Direct Hypoxic Exposure Decreases Cardiomyocyte Proliferation and DNA Synthesis in the Intact Fetal Hearts

Cardiomyocyte proliferation in the fetal hearts was determined by examining the double immunofluorescence staining of the nuclear protein Ki-67 and cardiomyocyte marker α- sarcomeric actinin. The Ki-67 expression occurs throughout all phases of the cell cycle, except for the G0 phase (Gerdes et al., 1984). As shown in Fig.16, double immunofluorescence staining of the fetal heart sections revealed a predominant distribution of Ki-67 in the cardiomyocytes. Quantification colocalization analysis for Ki-67 and □-sarcomeric actinin demonstrated hypoxia significantly decreased the percentage of cardiomyocytes that expressed Ki-67 compared with normoxic condition, which suggested less number of myocytes underwent cell division and a reduced proliferative activity in the fetal hearts (Fig. 16). In addition, the direct effect of hypoxia on DNA synthesis in the fetal heart was measured by immunostaining of BrdU. As shown in Fig.17, the immunostaining of BrdU demonstrated the dark brown dots within the cells that located at the outer layer of the fetal hearts. Following hypoxia a tremendous reduce in number of BrdU positive cells was observed and those cells formed a very thin layer that located at the edge of the fetal hearts (Fig.17). From these data and the immunofluorescence of Ki-67, we conclude that hypoxia directly decreased cardiomyocyte proliferation and DNA synthesis in the fetal hearts. To further verify this

conclusion, we performed Western blot analysis to determine the expression of two cell cycle related proteins: Cyclin D2 and p27. Cyclin D2, a known proliferative marker (Novoyatleva et al., 2010), was significantly decreased in hypoxic fetal hearts, while hypoxia resulted in the upregulation of p27, a cell cycle inhibitor (Novoyatleva et al., 2010), in the fetal hearts (Fig.18), which collectively demonstrated the cardiomyocyte proliferation is directly inhibited by hypoxic exposure in fetal rat hearts ex vivo.

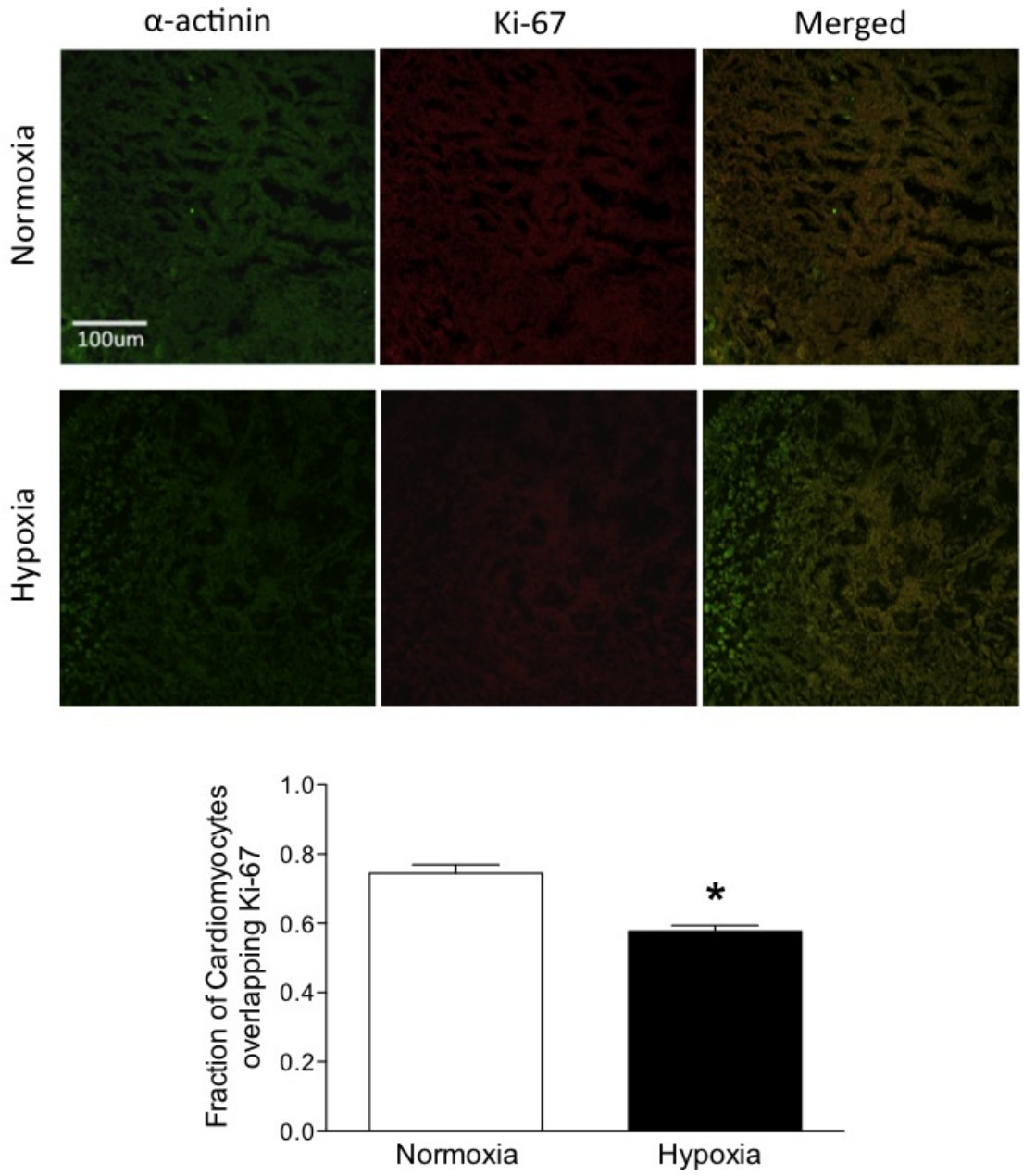


Figure 16. The effect of hypoxia on cardiomyocyte proliferation in the intact fetal hearts. Isolated fetal hearts were treated with 1% O_2 (hypoxia) and 21% O_2 (normoxia) for 48 hours. Cell proliferation was examined by the Ki-67 immunofluorescence. Representative example of cardiomyocytes is stained for □-sarcomeric actinin (green) and Ki-67 (red). The images were representatives of the heart samples from 6 normoxic and 6 hypoxic rats Data are means ± SEM. Data were analyzed by t-test. *p<0.05, hypoxia vs. normoxia, n=5-6 per group.

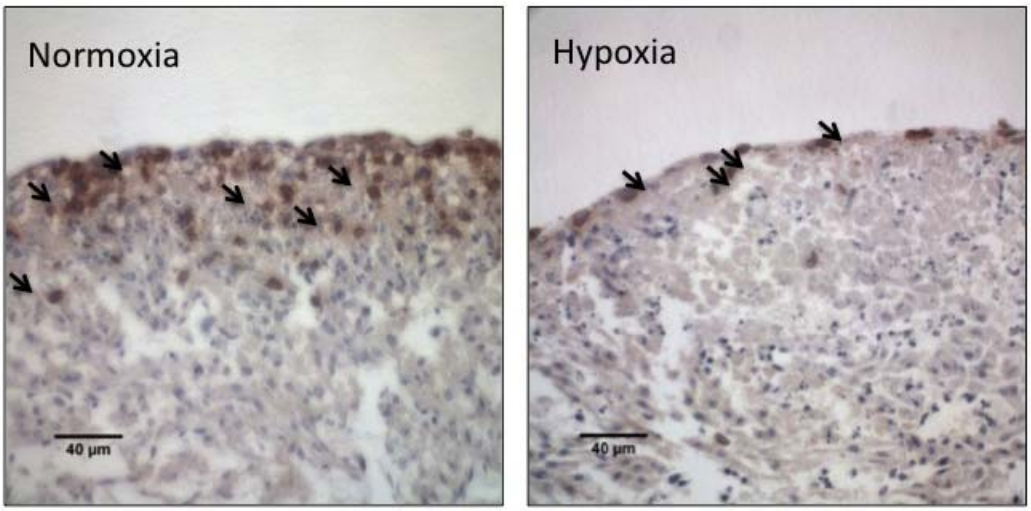


Figure 17. The effect of hypoxia on DNA synthesis in the intact fetal hearts. Isolated fetal hearts were treated with 1% O_2 (hypoxia) and 21% O_2 (normoxia) for 48 hours. DNA synthesis was evaluated by bromodeoxyuridine (BrdU) immunostaining. Arrows show BrdU positive nuclei. The images were representatives of the heart samples from 4 normoxic and 5 hypoxic rats.

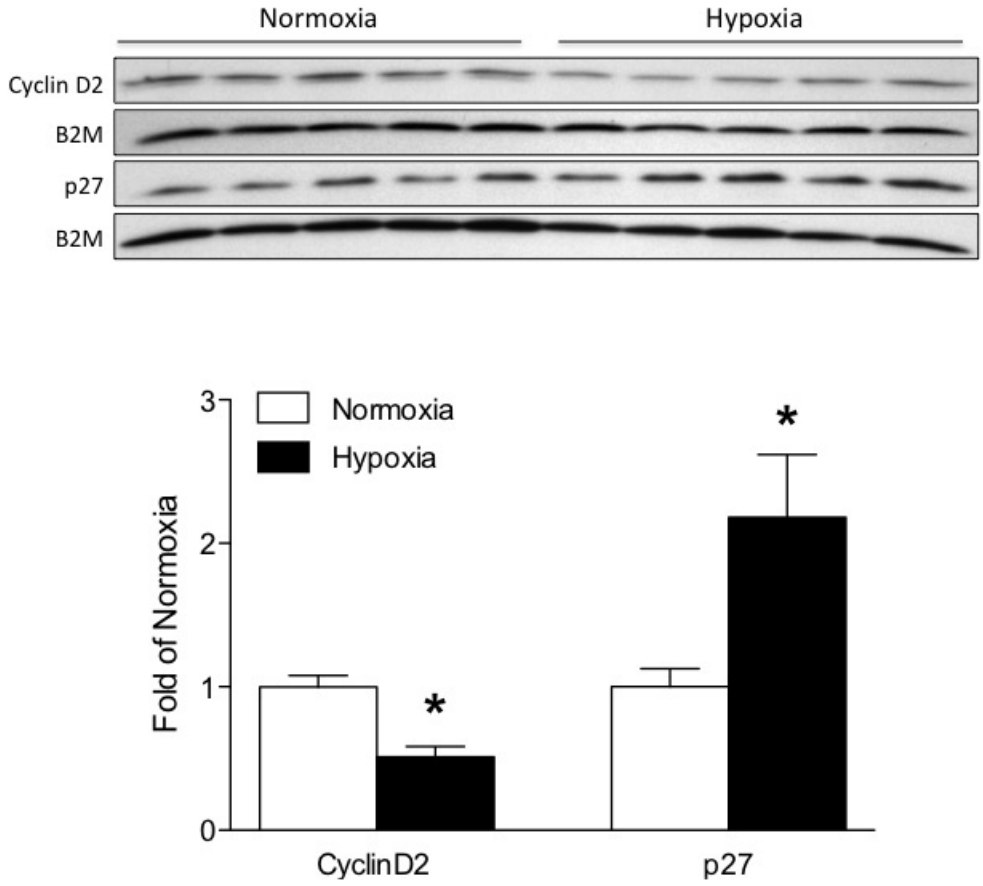


Figure 18. The effect of hypoxia on the expression of cell cycle related proteins in the intact fetal hearts. Isolated fetal hearts were treated with 1% O_2 (hypoxia) and 21% O_2 (normoxia) for 48 hours. Cyclin D2 and p27 protein levels were determined by Western blots. B2M, □2-microglobulin. Data are means ± SEM. Data were analyzed by t-test. *p<0.05, hypoxia vs. normoxia, n=5 per group.

Direct Hypoxic Exposure Inhibited Proliferation in the H9c2 Cells

To elucidate the potential mechanisms underlying the hypoxia-reduced cardiomyocyte proliferation, we first determined the direct effect of hypoxia on cell proliferation in vitro. The double immunofluorescence staining clearly demonstrated that Ki-67 was mainly localized within the cardiomyocyte nuclei (Fig.19). As expected, the ratio of the number of Ki-67 positive nuclei to the total number of nuclei was significantly reduced following 24 h of hypoxia (Fig.19), suggesting hypoxic cells underwent less proliferation compared to the normoxic cells. These results were further confirmed with the expression of Cyclin D2 and p27. Consistent with the finding in the isolated fetal hearts ex vivo, hypoxia resulted in a significantly downregulation in Cyclin D2 expression but an upregulation in p27 expression in the H9c2 cells (Fig.20).

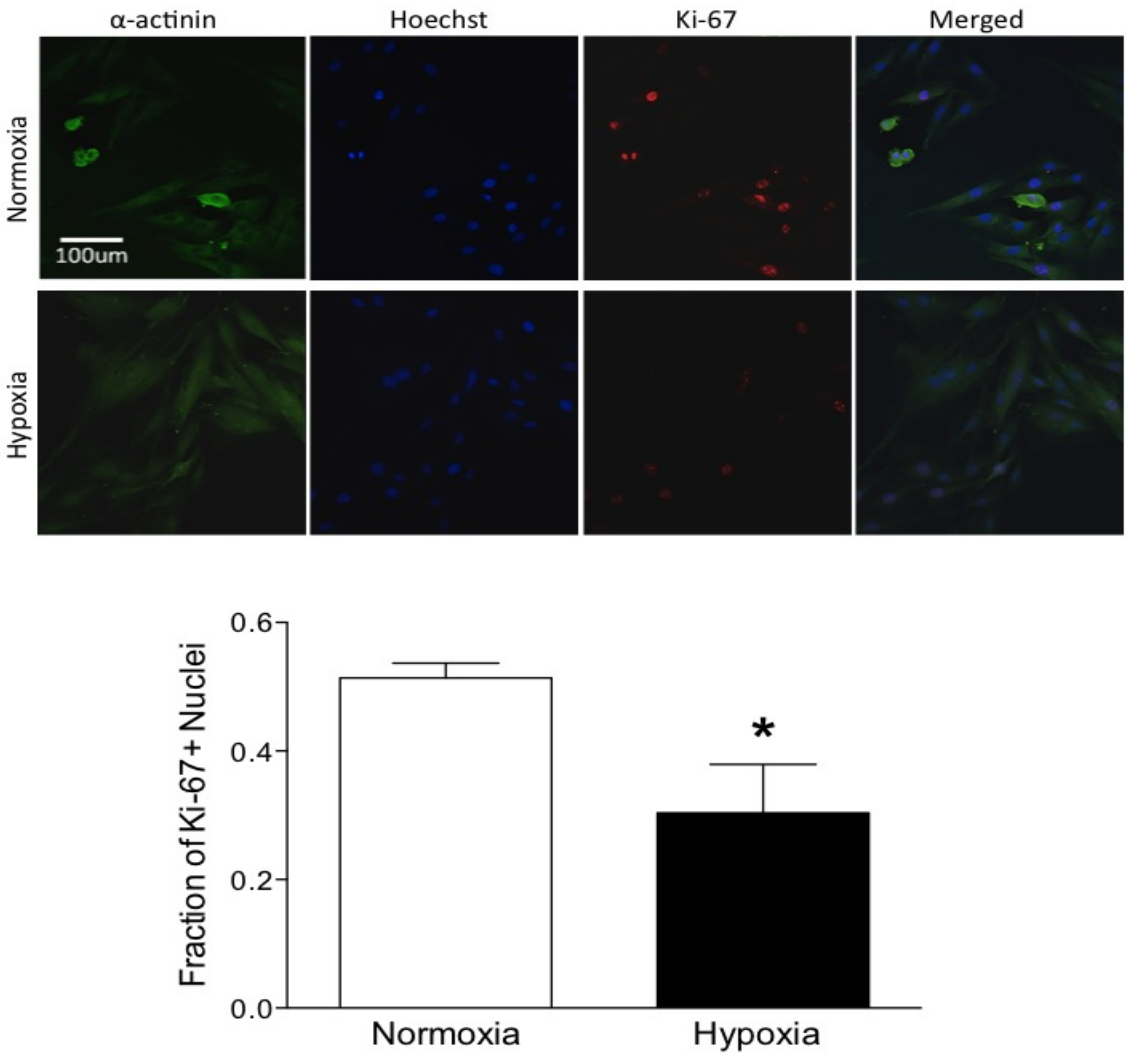


Figure 19. The effect of hypoxia on cardiomyocyte proliferation in the H9c2 cells. Cell proliferation was assessed by the Ki-67 immunofluorescence in the H9c2 cells treated with 1% O_2 versus 21% O_2 for 24 hours. Representative example of cardiomyocytes is stained for □-sarcomeric actinin (green) and Ki-67 (red). The nuclei were visualized using Hoechst 33342 (blue). Data are means ± SEM. Data were analyzed by t-test. *p<0.05, hypoxia vs. normoxia, n=6 per group.

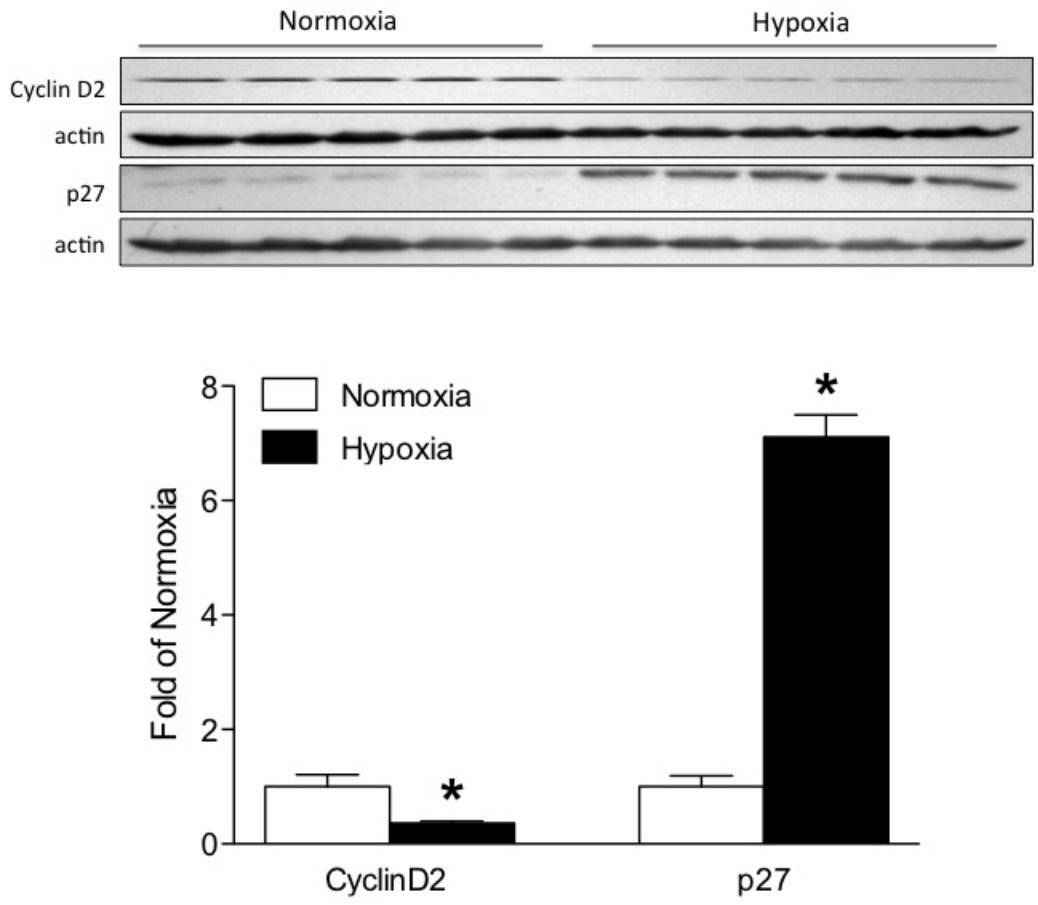


Figure 20. The effect of hypoxia on the expression of cell cycle related proteins in the H9c2 cells. H9c2 cells treated with 1% O_2 versus 21% O_2 for 24 hours. Cyclin D2 and p27 protein levels were determined by Western blots. B2M, □2-microglobulin. Data are means ± SEM. Data were analyzed by t-test. *$p<0.05$, hypoxia vs. normoxia, n=5 per group.

Effect of Hypoxia on the Expression of TIMP-3 & -4 in the Fetal Hearts and H9c2 Cells

To further assess whether potential mediators TIMP-3 & -4 is involved in hypoxia-inhibited cardiomyocyte proliferation, the expression of TIMP-3 & -4 were measured by Western blot analyses. Consistent with the finding in the fetal rat hearts in vivo, hypoxia caused similar expression patterns of TIMPs in ex vivo and in vitro models. As shown in Fig.21A, the expression of both TIMP-3 & -4 was significantly increased in hypoxic fetal hearts compared to the normoxic condition (Fig.21A). As expected, both of TIMPs expressions were significantly upregulated in the H9c2 cells in response to hypoxic exposure (Fig.21B).

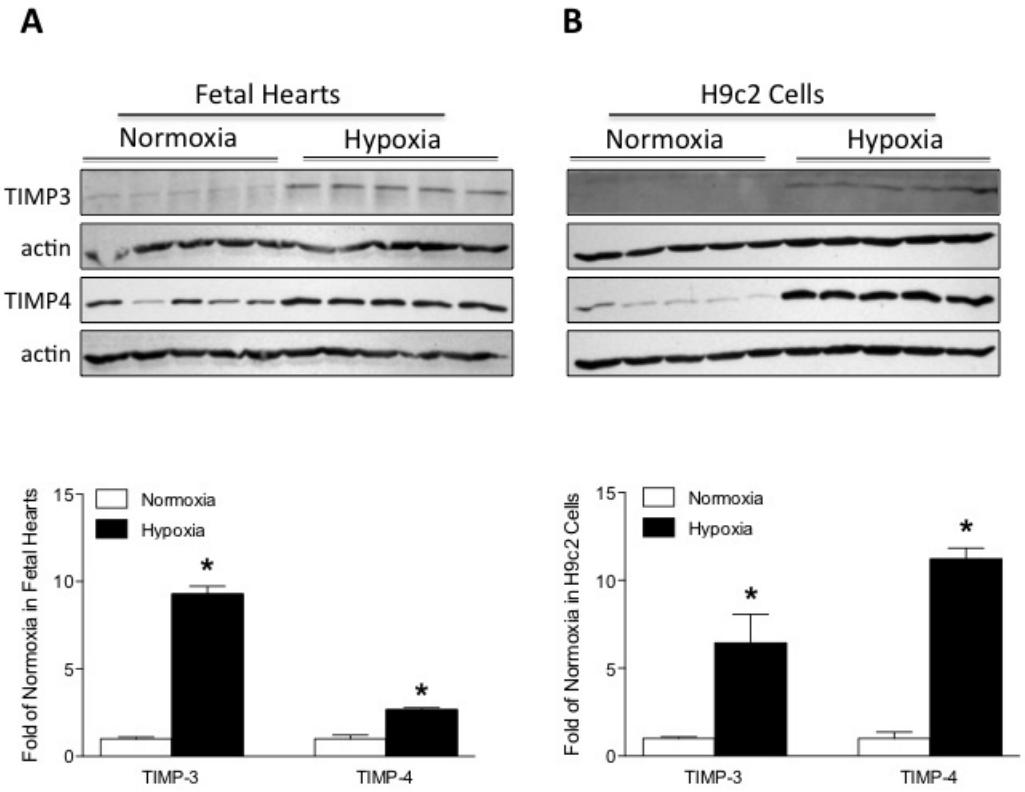


Figure 21. The effect of hypoxia on TIMP-3 & -4 expression in the intact fetal herats and H9c2 cells. The expression pattern of tissue inhibitor of metalloproteinase (TIMP) -3 & -4 were examined by Western blots in isolated fetal hearts and H9c2 cells treated with 1%O_2 versus 21%O_2 for 48 hours or 24 hours, respectively. B2M, □2-microglobulin. Data are means ± SEM. Data were analyzed by *t*-test. *p<0.05, hypoxia vs. normoxia, n=5 per group.

Knockdown of TIMPs Reverses the Inhibitory Effect on Proliferation by Hypoxia

We further determined the mechanism of how hypoxia mediated inhibitory effect in cardiomyocyte proliferation in the H9c2 cells in vitro. As shown in Fig.22A, the treatment of scramble siRNA (NC) had no significant effect in decreasing the expression of TIMP-3 or TIMP-4 after hypoxia, while the treatment of TIMP-3 or -4 siRNA effectively suppressed the expression of TIMP-3 or -4 following hypoxia, respectively (Fig. 22A). In the presence of TIMP-3 siRNA, hypoxia led to downregulation of Cyclin D2 and upregulation of p27 (Fig.22B). Consistently, the ratio of Ki-67 positive nuclei was also decreased with TIMP-3 siRNA treatment under the hypoxic condition (Fig.23). In addition, TIMP-3 siRNA significantly enhanced Cyclin D2 expression level and the ratio of Ki-67 positive nuclei in the normoxic cells compared to the negative controls (Fig.22B & Fig.23). Unlike TIMP-3 siRNA, hypoxia had no significant effect on altering the expression of Cyclin D2 and p27 after the treatment of TIMP-4 siRNA (Fig.22B). Similarly, hypoxia had no effect in reducing the ratio of Ki-67 positive nuclei in the presence of TIMP-4 siRNA (Fig.23). Taken together, these data indicate the treatment of TIMP-4 siRNA alone restore the cardiomyocyte proliferation in H9c2 after hypoxia.

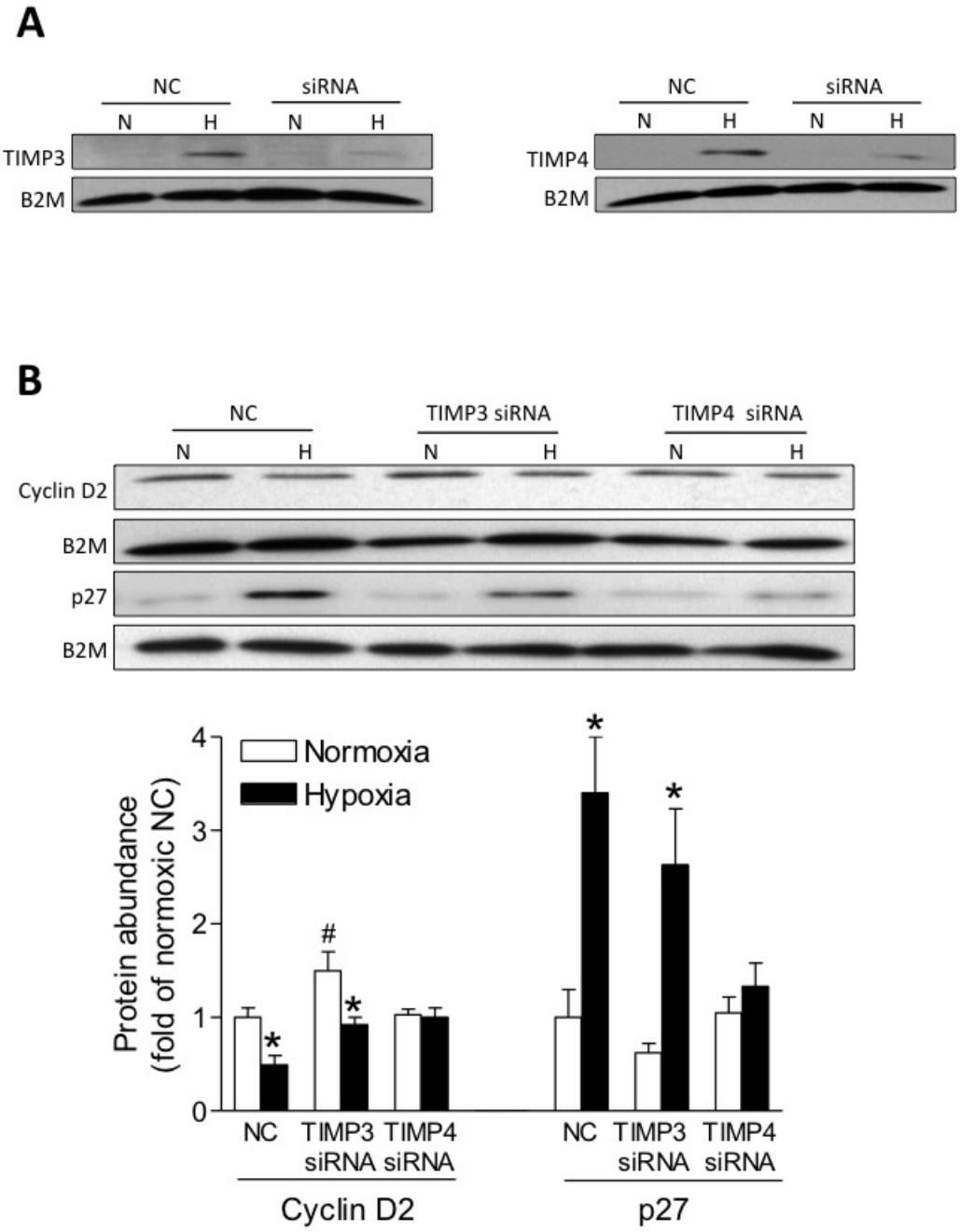


Figure 22. The effect of TIMP-3/ -4 siRNA on the expression of Cyclin D2 and p27 in the H9c2 cells. H9c2 cells were treated with 1% O_2(H) and 21% O_2(N) for 24 hours in the presence of TIMP-3/-4 siRNA or nontargeting siRNA (NC). Data are means ± SEM. Data were analyzed by ANOVA. *$p<0.05$, hypoxia vs. normoxia, #$p<0.05$, siRNA vs. negative control (NC), n=4-5 per group.

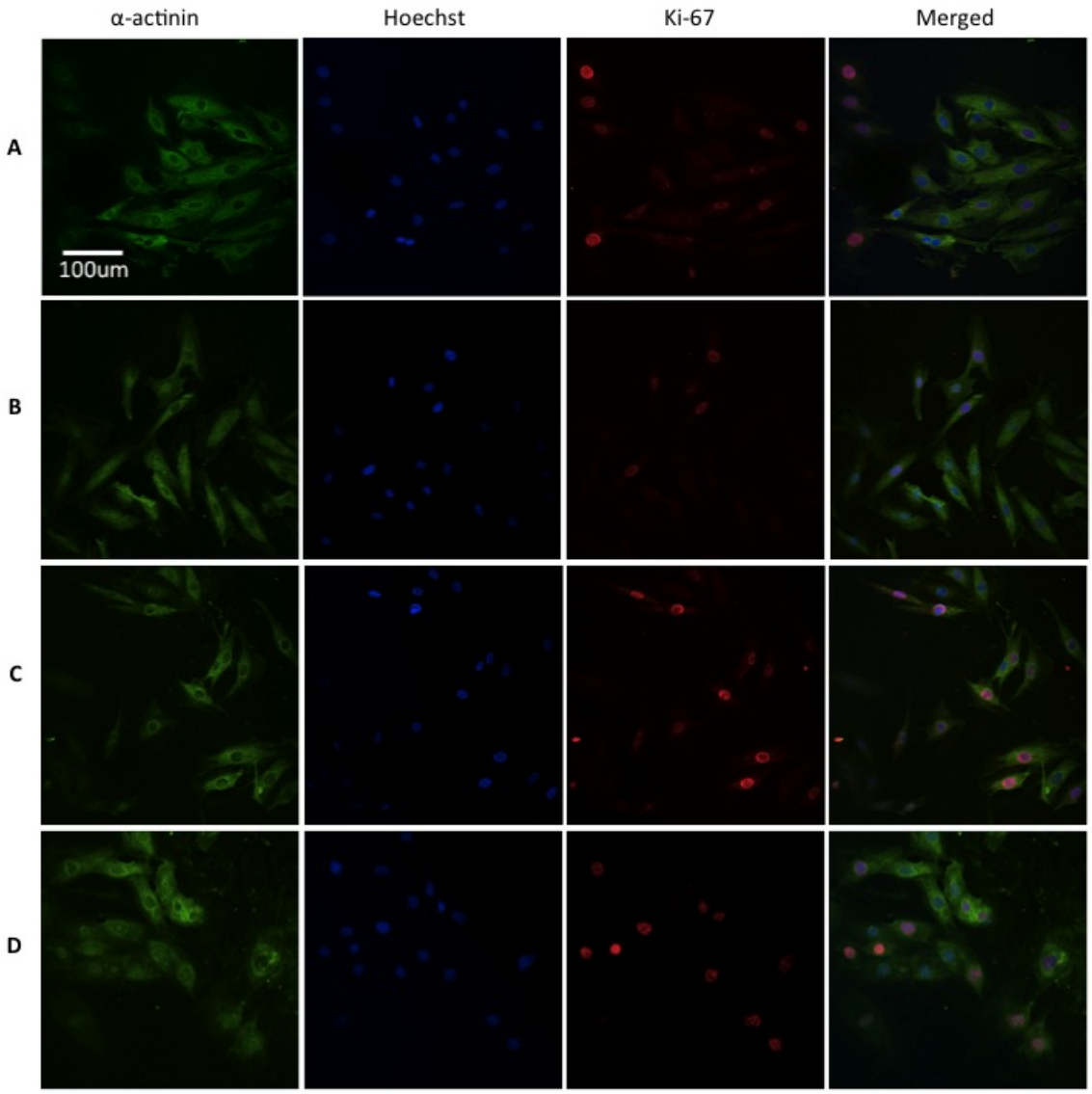
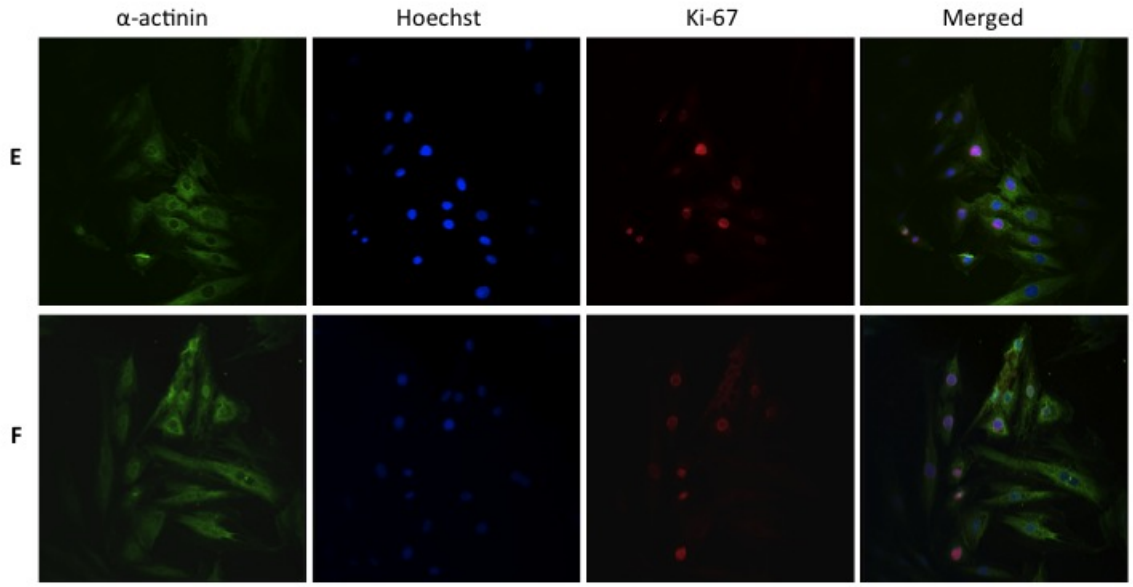


Figure 23. The effect of TIMP-3/ -4 siRNA on cardiomyocyte proliferation in H9c2 cells. Cell proliferation was determined by the Ki-67 immunofluorescence in the H9c2 cells treated with 1% O2 versus 21% O2 for 24 hours in the presence of TIMP-3/-4 siRNA or nontargeting siRNA. A. normoxia+negative control, B. hypoxia+negative control, C. normoxia+TIMP-3 siRNA, D. hypoxia+TIMP-3 siRNA.



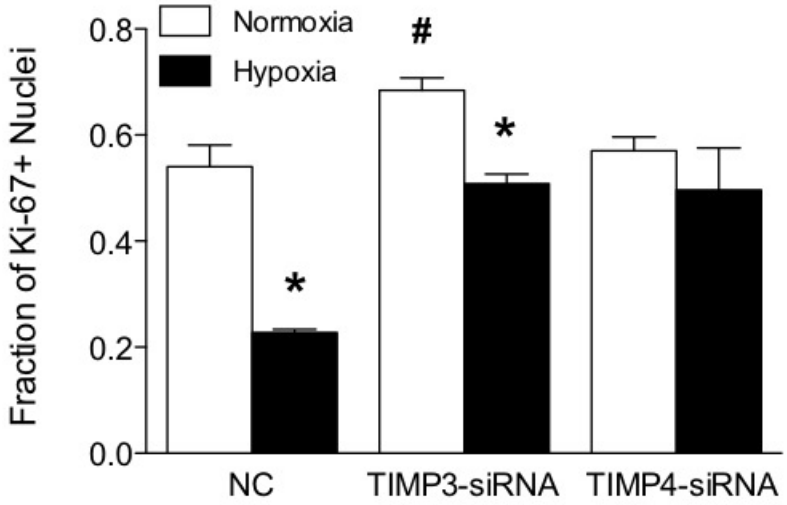


Fig.23 (Continued) E. normoxia+TIMP-4 siRNA, F. hypoxia+TIMP-4 siRNA. Data are means ± SEM. Data were analyzed by ANOVA. *p<0.05, hypoxia vs.normoxic, #p<0.05, siRNA vs. negative control (NC), n=3-6 per group.

Discussion

The findings of the present study show that hypoxia directly impacts the fetal heart to reduce myocyte proliferation, which is correlated with the increased expression of TIMP-3 and TIMP-4. The causal effect of TIMP-4 in the hypoxia-reduced cell division is demonstrated through the use of TIMP-4 siRNA, which can effectively knock down the expression of TIMP-4 and restore cardiomyocyte proliferation after hypoxia.

The present study follows up our previous work that has shown the upregulation of TIMP-3 & -4 and downregulation of myocyte proliferation in the fetal and neonatal rats in response to maternal hypoxia by investigating the direct effects of hypoxia on the fetal heart. The animal models used in our previous studies induce hypoxia by exposing pregnant rats to 10.5% atmosphere O_2. Although this is sufficient to cause oxygen insufficiency in utero (Bae et al., 2003), there are secondary effects that may influence normal heart growth. In the ovine model, chronic hypoxia significantly reduces fetal cardiac output and causes the redistribution of blood flow in favor of the vital organs (heart and brain) and away from other non-essential organs (Kamitomo et al., 1993). The redistribution of fetal blood flow can be accomplished by reflex (adrenergic), local (nitric oxide) and humoral (angiotensin, vasopressin, catecholamine etc.) mechanisms (Martin, 2008). Recent evidence indicates that adaptation to hypoxia is affected by the activity of hypothalamic-pituitary-adrenal axis. For example, blood levels of glucocorticoid are increased under intrauterine hypoxia and associate with several adverse effects including lower birth weight (Gardner et al., 2001; Larsen et al., 1997; Torres et al., 1997; Levitt et al., 1996). Whereas hypoxia also induces apoptosis in cultured neonatal rat cardiomyocytes, therefore, it is likely that there is also a direct connection between hypoxia and cardiac tissue remodeling in the fetal heart (Zhang et al., 2005). In the

present study, we have used both ex vivo model of intact fetal heart and in vitro model of H9c2 cell line and exposed them to hypoxia directly. This eliminates most of the indirect effects of maternal hypoxia such as systemic response to the hypoxic stress and allows the direct addition of pharmacologic interventions.

H9c2 cell line has similar structure to immature embryonic heart cells and keeps the electrical and hormone phenotype consistent with cardiomyocytes (Patterson and Zhang, 2010). It has been widely used to study various mechanisms in heart development including differentiation, hypertrophy and apoptosis (Pereira et al., 2011, Watkins et al., 2011; Shin et al., 2009). In the present study, this cell line was used in conjunction with data gathered from an intact fetal heart ex vivo model. It has been shown that intact fetal hearts can live and beat for at least 6 days in H199 media and can be extended to 2-3 weeks with the media supplemented with insulin and serum (Wildenthal, 1970). This model has been employed to provide many valuable insights in investigating cardiac development. By using this ex vivo model to administer drugs directly to the heart, our laboratory has demonstrated that cocaine directly affects the fetal heart to increase DNA methylation at specific transcription factor binding sites in the PKC□ promoter region, causing a decrease in PKC□ expression (Meyer et al., 2009). More recently, Xue et al has shown a novel mechanism of glucocorticoids in regulating the ontogeny of type 2 angiotensin II receptor (AT2R) in the intact fetal rat heart (Xue et al., 2011). These hearts in organ culture have shown to maintain spontaneous contraction and beat throughout the study period (Meyer et al., 2009), allowing us to determine the direct effect of hypoxia on cardiac remodeling. Previously, hypoxia enhanced the expression of TIMP-3 and TIMP-4 in fetal hearts in vivo. In the present study, hypoxia-induced increase in TIMP-3 and

TIMP-4 expression is observed in fetal cardiomyocytes in vitro and ex vivo, which collectively suggests a congruent underlying mechanism for each model and provides a comparable model of H9c2 cells in the mechanistic study of TIMP-3 & -4 mediated cardiomyocyte proliferation after hypoxia.

The finding of the decreased cardiomyocyte proliferation in response to hypoxic exposure in the intact fetal heart and H9c2 cell line are consistent with the impact of maternal hypoxia treatment on the heart in the fetus in vivo (Tong et al., 2011), demonstrating that the effect of hypoxia on myocyte proliferation in the fetus is primarily due to a direct action of hypoxia on the heart. This finding is the first step to understanding the process of fetal heart development under the hypoxic condition and gives us the ideal models to further investigate both the effect of hypoxia on the heart and the mechanism responsible for these effects. In present study, the immunostaining of the proliferation marker ki-67 has shown the number of proliferative cells in fetal heart and H9c2 cells is significantly reduced in response to hypoxia, additionally, hypoxia results in the downregulation of Cyclin D2, a protein known to be necessary for cardiomyocyte proliferation (Lafontant and Field, 2006), and induces the suppression of the cell cycle inhibitor p27 (Besson et al., 2008). The decreased number of BrdU-positive nuclei is also observed in the hypoxic fetal heart, which indicates less DNA synthesis of the cardiomyocyte. Given that cardiomyocyte DNA synthesis is associated with cell proliferation during fetal life (Zhang et al., 2007), these data suggest an inhibitory cell growth after hypoxic exposure. Interestingly, previous in vitro study has shown short time exposure to hypoxia inhibited cell death and downregulated p27, promoting cardiomyocyte growth (Takahashi et al., 2006). This suggests differential regulations of

short-and long-term hypoxia on the proliferative activity in the heart. Whereas the mechanisms underlying the hypoxia-mediated reduction of myocyte proliferation in the fetal heart remain elusive, the previous finding of enhanced expression of TIMP-3 or/and TIMP-4 implies a possible mechanism in the inhibitory effect on cell division after hypoxia (Tong et al., 2011).

In the present study, the potential causal effect of TIMP-3 or -4 in hypoxia-reduced cardiomyocyte proliferation is further demonstrated by siRNA transfection experiments in H9c2 cells. Our data reveals knockdown of TIMP-4 alone restores the expression of Cyclin D2, p27 and the number of Ki-67 positive cells, and restores the cardiomyocyte proliferation under the hypoxic condition, which confirms the involvement of TIMP-4 in regulating the cell division after hypoxia. Whereas knockdown of TIMP-3 has no significant effect on hypoxia-reduced cell proliferation, suggesting that TIMP-3 does not play a significant role in affecting the myocyte proliferation following hypoxia. It is well documented that TIMPs are linked to MMP-independent mechanisms, such as cell proliferation and apoptosis, in addition to their inhibitory effect on specific MMPs (Vanhoutte and Heymans, 2010). Previous data have shown that neonatal cardiomyocytes proliferation was enhanced in TIMP-3 deficient mouse hearts (Fedak et al., 2004), TIMP-3 inhibited neonatal mouse cardiomyocyte proliferation via epidermal growth factor/c-Jun NH2-terminal kinase/sp1 pathway (Hammoud et al., 2009). Consistent with the previous findings, our present study has demonstrated TIMP-3 siRNA-treated cells exhibit significantly higher expression of Cyclin D2 and number of Ki-67 positive nuclei compared to scramble siRNA (nontargeting siRNA) treated cells under the normoxic condition, indicating knockdown of TIMP-3 plays a positive role in

cell proliferation under the normoxic condition. Additionally, our data provide the evidence for the first time that TIMP-3 is not involved in regulating the proliferation in the hypoxic fetal hearts, albeit it shows inhibitory effect on normoxic cardiomyocyte growth. It is possible that the normoxic activity of TIMP-3 is relatively high and reaches its maximal inhibitory effect on cell division. The further upregulation of TIMP-3 by hypoxia may not able to alter its inhibitory effect on the cardiomyocyte proliferation. On the other hand, TIMP-4 appears to play dual roles in regulating cell proliferation, and its effect is tissue-specific (Melendez-Zajgla et al., 2008). Our data have shown TIMP-4 siRNA abolish hypoxia-reduced cell proliferation, suggesting its pro-proliferative role in the fetal heart cells. Unlike TIMP-3, the promoter region of TIMP-4 does not contain (activator protein) AP1 site that is related to basal expression of TIMP-3 (Young et al., 2002). Therefore, the basal level of TIMP-4 may be relatively low, and its inhibitory effect on cell proliferation could be significantly induced by hypoxia. The precise mechanisms of how hypoxia enhances the TIMP-3 & -4 expression is not fully understood yet, the epigenetic modification is likely to be involved in the reprogramming of TIMP-3 & -4 (Tong and Zhang, 2012).

The present study provides insight into the direct effect of hypoxia on cardiomyocyte proliferation in the fetal heart. Our collective observations indicate an inhibitory effect on cell proliferation in the developing hearts following hypoxia, which can be mediated by upregulation of TIMP-4. While this study focused exclusively on whether TIMP-3 & -4 are involved in modulating cell division under hypoxic condition, further study is needed to determine the mechanisms of how TIMP-4 inhibits

cardiomyocyte proliferation after hypoxia and of how hypoxia upregulates TIMP-4 in the fetal heart.

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CHAPTER FIVE

GENERAL DISCUSSION

Hypoxia is one of the most common stresses during the pregnancy, which can adversely impact prenatal and postnatal development in the offspring. During the critical developmental stages of the fetus and neonate, however, little is known about cerebral and cardiac remodeling and related genes expression patterns in response to fetal hypoxia. It has been demonstrated that the timely breakdown and restructure of ECM are critical for the normal fetal organ development (Nagase and Woessner, 1999), and that MMPs are the one of the most significant mediators in the ECM turnover. MMPs have been implicated in a variety of physiological and pathological processes in the cardiovascular and central nervous systems, including modulating fibrillar collagen structure and deposition (Kandasamy et al., 2010), regulating cell proliferation and cell death (Lee and Lo, 2004), etc. Previous studies have demonstrated MMPs can trigger endothelial cell death after hypoxia-reoxygenation in the in vitro model (Lee and Lo, 2004) and impair the blood brain barrier integrity after hypoxia-ischemia insult in neonatal rats (Chen et al., 2009). My research project has provided the evidence in a rat model that maternal hypoxia during gestation alters MMPs and TIMPs expression patterns in the developing brain and heart and results in the abnormal cerebral and cardiac growth pattern, such as increased neuronal cell death, reduced cardiomyocyte proliferation with the aberrant content of fibrillar collagen network. Additionally, this

project has also revealed an important role of TIMP-4 in the inhibitory effect on cardiomyocyte proliferation in the presence of hypoxia.

Hypoxia in the Regulation of MMP Protein Expression Patterns

Our studies have demonstrated prenatal hypoxia induces abnormal tissue growth in the developing brain and heart, and the alteration of MMPs has a crucial role in tissue remodeling in both the heart and brain. In chapter 2, we found that fetal hypoxia increased the MMP-2 and MMP-9 activity in the brain on postnatal days 0 and 4 neonatal rats, whereas no differences were seen afterwards. In chapter 3, we found hypoxia enhanced the expression of MMP-13 and MT1-MMP in the fetal heart and increased the expression of MMP-1 and MMP-13 in the neonatal heart.

Although the mechanisms of MMP upregulation by chronic prenatal hypoxia are not fully understood, it is known that MMPs can be regulated by transcription, pro-enzymatic activation and endogenous inhibition. MMPs are mainly regulated at the transcriptional level with relatively low basal levels during normal development (Fanjul-Fernández et al., 2010). Hypoxia might activate several transcriptional factors that subsequently bind to some of the key transcriptional binding sites, enhancing or repressing MMP gene expression. The activating protein 1 (AP-1) site, nuclear factor kappa B (NF-κB) site and STAT site are involved primarily in the regulation of MMP genes (Fanjul-Fernández et al., 2010). Increases in MMP-2 synthesis were found in a culture of rat cardiac fibroblasts exposed to 1% oxygen for 24 h, and a functional AP-1 site mediated MMP-2 transcription through the binding of distinctive Fra1-JunB and FosB-JunB heterodimers (Bergman et al., 2003). In vitro studies have shown a rapid

activation in the binding of DNA to AP-1 during hypoxia, and AP-1 synthesis was enhanced to activate many AP-1 and AP-1 family-controlled genes (Rupec and Baeuerle, 1995) that possibly include various MMP genes. The study also suggested that the activation of NF-κB by hypoxia reoxygenation is slow, which implies that NF-κB is indirectly activated and probably by other gene products newly induced by HIFs (Rupec and Baeuerle, 1995). Our previous data demonstrated that maternal hypoxia increased HIF-1□ protein levels in rodent fetal hearts. Collectively, these data implied NF-κB may one of the mediators that responsible for fetal hypoxia-upregulated MMPs levels in the present study. Chen et al. reported that the expression of MMP-1 in cardiac fibroblast is increased, accompanied by elevated ROS and NF-κB after the onset of hypoxia, and continues to be upregulated during prolonged hypoxia (Chen et al., 2004). Given that the generation of ROS from hypoxia is believed to activate a range of intracellular signaling pathways, including NF-κB (Nanduri et al., 2008), this finding implies that oxidative stress is associated with hypoxia-triggered upregulation of MMP, potentially through the NF-κB binding site. Although hypoxia might activate STAT protein by various pathways (Joung et al., 2003), it is unclear whether STAT proteins can bind directly to the transcriptional regulatory region of MMP genes (Vincenti and Brinckerhoff ,2007); thus, the role of the STAT pathway in the upregulation of MMP transcription induced by hypoxia remains elusive.

MMPs are also regulated by Smad family proteins that repress or enhance TGF-β-mediated gene expression, implicating a dual role for TGF-β in modulating MMP genes in tissue remodeling and cancer (Fanjul-Fernández et al., 2010). It has been demonstrated in an in vitro study that hypoxia increased TGF-β expression (Hu et al., 2007), and TGF-

β has been shown to inhibit MMP-1 after ischemic reperfusion in the heart (Chen et al., 2003). Although TGF-β is implicated in regulating MMP expression, whether hypoxia-mediated TGF-β regulates MMPs at the transcriptional level remains to be elucidated.

Imbalance of MMPs and TIMPs and Aberrant Tissue Remodeling

In chapter 2, our finding indicated that not only the alteration of MMPs alone affected the tissue growth and remodeling in the hypoxic condition, but the dynamic changes of MMPs and TIMPs together also contribute to aberrant remodeling during the development following maternal hypoxia. Given that a wide range of neurovascular matrix and cell-surface proteins are the substrates for MMP-2 and -9, they have been extensively investigated together with their endogenous inhibitors (TIMP-2 and -1) in the brain (Tian et al., 2007; Sulik and Chyczewski, 2008). MMP-2 and -9 are released mainly from astrocytes (Vaillant et al., 1999). Our present studies demonstrated that chronic maternal hypoxia alters the TIMP-1: MMP-9 and TIMP-2: MMP-2 ratios by suppressing TIMP expression, resulting in reduced cell proliferation and increased cell death in the neonatal brain. Additionally, the finding of the worsened performance in the wire-hanging test in the hypoxic animals has confirmed that the region of cortex is sensitive to hypoxic insult and fetal hypoxia significantly leads to abnormal cortex remodeling. It is known that the central nervous system begins to form as the neuropithelium at E11 (Dwyer et al., 2009). The rostral section of the neuropithelium generates the forebrain and a portion of these cells migrates to form the cortical plate later on (Dwyer et al., 2009). It is possible that exposure to hypoxia during E15 to 21 interferes cortex remodeling in its critical developmental stage, leading to restricted locomotors function

in the neonatal offspring. Studies in humans reported that serum levels of MMP-9 on the day of birth were significantly increased in perinatal asphyxiated neonates followed by a subsequent increase in serum TIMP-1 levels and a decrease in serum MMP-9 on the day after birth, suggesting that TIMP-1 increases in response to MMP-9 and it, in turn, suppresses MMP-9 (Sunagawa et al., 2009). The study also showed that the serum MMP-9: TIMP-1 ratio in asphyxiated neonates with neurological sequelae is significantly higher than in those without sequelae (Sunagawa et al., 2009). Taken together, these data indicated that the imbalance of MMP-9 and TIMP-1 is linked to prenatal hypoxia-induced neurological deficits during brain development. Ryu et al. explored the influence of chronic hypoxia on the developmental expression profile of additional MMPs and TIMPs in the mouse lung (Ryu et al., 2005). Prolonged neonatal hypoxia resulted in an arrest in alveolarization, elevated MMP-2 and lowered TIMP-2 levels, whereas no significant changes were found in MMP-9, MT1-MMP, TIMP-1 and TIMP-3 (Ryu et al., 2005). This study confirmed that chronic hypoxia breaks the delicate balance of proteolytic and anti-proteolytic forces during lung development, which accounts for several pulmonary pathologies, including pulmonary fibrosis. The TIMP-1: MMP-1 ratio was also remarkably increased in patients with severe left ventricular hypertrophy, showing that the interaction of TIMP-1 with another MMP besides MMP-9 also has a significant role in the pathophysiological remodeling (Deschamps and Spinale, 2005). The disruption of fine dynamics of MMP–TIMPs by acute hypoxia is also supported by studies using a neonatal hypoxic ischemia (HI) model. Activation of MMP-9 was followed by the delayed elevation of TIMP-1, and the mismatch of MMP-9–TIMP-1 elevation and the increased MMP-9: TIMP-1 ratio might be responsible for the blood

brain barrier (BBB) breakdown that occurs after hypoxic injury in developing brain (Chen et al., 2009). The temporal profiles of MMP-2–TIMP-2 were not altered dramatically after HI, distinct to our present findings in the brain after chronic hypoxia. This discrepancy implies that MMP-2 and TIMP-2 are not fully responsible for hypoxia-induced brain remodeling. In addition, changes in the MMP: TIMP ratio might have different roles in acute and prolonged tissue remodeling after hypoxia. In the rapid response to hypoxia, MMPs are released by early gene expression and cause the BBB breakdown and cell death, whereas in the prolonged phase after hypoxia, MMPs and TIMPs might participate in neurogenesis and angiogenesis to recover from the brain injury by different pathways.

Role of MMPs in Fibrillar Collagen Deposition

The primary effect of MMPs, which was first discovered by Gross and colleagues in 1962, is to digest a wide range of collagens (Gross, 2004); therefore, MMPs are key regulators of ECM homeostasis. Collagen degradation is a major step in tissue remodeling, thus in the present study we investigated the effect of fetal hypoxia on the primary enzymes (MMP-1, -13 and MT1-MMP) that digest fibrillar collagen (Ohuchi et al., 1997), as well as the collagen deposition in the developing hearts after fetal hypoxia. Generally, MMPs cleave the native triple helix of collagen at a Gly-Leu or Gly-Ile site, leading to an unstable conformation at body temperature and further degradation by nonspecific proteases (D'Armiento, 2002). The enhanced cardiac collagen accumulation in our present finding may attribute to excessive synthesis of collagen and reduced degradation of collagens, causing ventricular stiffening and impaired diastolic filling. The reduced digesting of collagens does not necessarily result from the low levels of MMPs;

in fact, increased collagens can activate its discoidin domain receptor (DDR) and therefore upregulate MMP-1 expression (Vogel et al., 1997). In the heart, DDR2 is primarily expressed on cardiac fibroblasts, which appear to be the major source of ECM components and MMPs (Kania et al., 2009; Goldsmith et al., 2004). The role of DDR2 in fibroblast proliferation and migration has been demonstrated by primary cell culture from DDR2-knockout mice, and the reduced growth of fibroblast from DDR2-null mice is probably the result of the reduced expression of MMP-2 (Olaso et al., 2002). An in vitro study of DDR1-null smooth muscle cells also indicated a decreased proliferative and migratory response and the involvement of both MMP-2 and MMP-9 expression (Hou et al., 2002). It has been reported that DDR2 expression is observed during early cardiac development under normal conditions, whereas hypoxia, as well as other pathologies, might regulate DDR1 and DDR2 expression by a variety of pathways (Chen et al., 2008; Ferri et al., 2004; Goldsmith et al., 2010). In agreement with our present finding in chapter 3, Hu and colleagues suggested that hypoxia–reoxygenation increases the signals for collagen I and MMPs, and the elevated levels of MMPs are the result of an autoregulatory response to collagen I signal in cardiac fibroblasts (Hu et al., 2007). However, how MMPs are autoregulated by collagen I was not determined in the study. Taken together, in addition to the inadequate inhibition by TIMPs (Polyakova et al., 2004), collagen receptors DDR1 and DDR2 might have an essential role in hypoxia-induced adverse tissue remodeling and might serve as an adaptive mechanism for the upregulation of MMPs by collagens after chronic hypoxia.

In brain tissue, collagens are generally rare and exist principally at the meninges, the basement membranes and the sensory end organs (Hubert et al., 2009). Increasing

amounts of data have shown that collagens have an active role during the development of the nervous system, including axon guidance and synaptogenesis in the establishment of the architecture of the brain (Hubert et al., 2009). DDR1 was found to be expressed prenatally in neurons of the proliferative areas, and yet was not detectable postnatally during brain development in mouse (Roig et al., 2010). Whether MMPs can be regulated by collagens and whether DDR participates in the regulation of MMPs in the fetal brain are not fully understood at present; it is also necessary to determine further the role of DDR in fetal hypoxia-promoted brain remodeling.

Role of TIMPs in Cell Proliferation and Cell Death

TIMPs are linked to MMP-independent mechanisms, such as cell proliferation and apoptosis, in addition to their inhibitory effect on specific MMPs in hypoxia-induced tissue development. In some non-expressing MMP cell lines, TIMP-1 promotes cell proliferation, as measured by DNA content (Hayakawa et al., 1992). The reductive alkylated TIMP-1 and -2 have no MMP inhibitory activity, but both significantly enhance cell proliferation. Moreover, the activity is not seen with the complex of proMMP2–TIMP-2 or proMMP9–TIMP-1 (Hayakawa et al., 1994). Taken together, our present data in the brain suggested maternal hypoxia altered the ratio TIMPs: MMPs by suppressing the TIMPs expression, which is likely to contribute to the reduced cell growth and proliferation and the increased neuronal death in the developing neonatal brains. These studies demonstrate that the cell-proliferating activity of TIMPs is independent of their inhibition of MMP activity. Previous study indicated the MAPK pathway and cAMP–protein kinase A (PKA) pathway might be related to TIMP growth-promoting activity (Stetler-Stevenson, 2008). The cell-proliferation activity might be the result of a TIMP-1

sequence that is homologous with human granulocyte-macrophage colony-stimulating factor, whereas TIMP-2 seems to be devoid of such a sequence (Hayakawa et al., 1992). However, subsequent studies demonstrated that the cell surface receptor might be present for TIMP-2 for its cell-proliferating action (Hayakawa et al., 1994). On the other hand, TIMP-3 and -4 have been shown to inhibit several MMPs and to be expressed at high levels in cardiac tissue. TIMP-3 is unique within in the TIMP family, because it binds firmly to ECM and has pro-apoptotic and anti-proliferative effects (Woessner, 2001). The direct binding to ECM might stabilize the MMP–TIMP complex within the interstitial space (Spinale, 2007). The gene encoding TIMP-3 has no TATA sequence, but contains many Sp1 binding sites in its promoter region, which suggests a potential epigenetic modification through DNA methylation. TIMP-3 can inhibit neonatal cardiomyocyte proliferation possibly by the epidermal growth factor receptor (EGFR)/c-Jun NH2-terminal kinase (JNK)–SP-1–p27 signaling pathway (Hammoud et al., 2009). Consistent with the previous finding, our present study confirmed the knockdown of TIMP-3, not TIMP-4, enhanced cardiomyocyte proliferation under the normoxic condition. Furthermore, in the presence of hypoxia, only knockdown of TIMP-4 could restore the cell proliferation. These data suggested that TIMP-4 appeared to have a detrimental effect on cardiac remodeling after hypoxic insults. Interestingly, previous data have indicated genetic manipulation to deplete some TIMPs could have dramatic actions on cardiac phenotype. TIMP-3 knockout animals have been shown dilated cardiomyopathy and compromised cardiac contractile performance (Fedak et al., 2004), which indicated TIMP-3 might play a critical role in maintaining normal cardiac function. Although TIMP-4 knockout mice have been generated, there is no information available yet

investigating its effect on cardiac phenotype (Schulz, 2007). These data collectively suggested TIMPs might comprehensively modulate cardiac or cerebral tissue remodeling in response to fetal hypoxia. More studies demonstrating whether or how knockdown of those TIMPs affected cardiac function in the offspring that exposed to fetal hypoxia are warranted.

Conclusion

To date, the fundamental mechanisms of prenatal hypoxia in cardiac and cerebral development are still not fully understood. Our study has demonstrated the reprogramming of the expression patterns of MMPs and TIMPs is linked to fetal hypoxia and has a central role in normal growth and tissue remodeling in the immature heart and brain. Present data have also suggested collagens are a major type of protein in the ECM, and their synthesis can be enhanced by fetal hypoxia. Meanwhile, the alteration of MMPs and TIMPs might be initiated to compensate for the accumulation and deposition of collagens; however, the interruption of the fine balance between MMPs and TIMPs after hypoxia might eventually decompensate and impair the fetal heart and brain morphology and function. In addition, we have demonstrated TIMPs might have an important role in MMP-independent pathways, such as cell proliferation and cell death, suggesting that TIMPs per se regulate fetal development. Given that fetal programming of certain genes is believed to contribute to the heightened susceptibility to challenges later in life, the reprogramming of MMPs and TIMPs might predispose offspring to heart disease and brain injury. Understanding the precise mechanisms by which hypoxia modifies these gene expression patterns is important and deserves further attention. Given that ECM and related proteins are the key mediators in tissue remodeling after fetal hypoxia, possible

interventions might be to target MMPs or TIMPs to restore normal morphology and function. Furthermore, whether, and to what extent, the alteration of the expression patterns of MMPs and TIMPs in the fetus persist long term into adulthood require further investigation, and identifying the underlying mechanisms could provide useful insights into future clinical therapeutic approaches.

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