

Research Paper

Endothelin-1 Promotes Cardiomyocyte Terminal Differentiation in the Developing Heart via Heightened DNA Methylation

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Abstract

Aims: Hypoxia is a major stress on fetal development and leads to induction of endothelin-1 (ET-1) expression. We tested the hypothesis that ET-1 stimulates the terminal differentiation of cardiomyocytes from mononucleate to binucleate in the developing heart.

Methods and results: Hypoxia (10.5% O₂) treatment of pregnant rats from day 15 to day 21 resulted in a significant increase in prepro-ET-1 mRNA expression in fetal hearts. ET-1 *ex vivo* treatment of fetal rat cardiomyocytes increased percent binucleate cells and decreased Ki-67 expression, a marker for proliferation, under both control and hypoxic conditions. Hypoxia alone decreased Ki-67 expression and in conjunction with ET-1 treatment decreased cardiomyocyte size. PD145065, a non-selective ET-receptor antagonist, blocked the changes in binucleation and proliferation caused by ET-1. DNA methylation in fetal cardiomyocytes was significantly increased with ET-1 treatment, which was blocked by 5-aza-2'-deoxycytidine, a DNA methylation inhibitor. In addition, 5-aza-2'-deoxycytidine treatment abrogated the increase in binucleation and decrease in proliferation induced by ET-1.

Conclusions: Hypoxic stress and synthesis of ET-1 increases DNA methylation and promotes terminal differentiation of cardiomyocytes in the developing heart. This premature exit of the cell cycle may lead to a reduced cardiomyocyte endowment in the heart and have a negative impact on cardiac function.

Key words: Endothelin-1, Hypoxia, Heart, Fetal development, Epigenetic

Introduction

Heart disease is the leading cause of death in the United States. It has been well established that an adverse intrauterine environment increases vulnerability to cardiovascular disease later in life [1, 2]. Environmental factors during the critical period of fetal development can influence the maturation of organs, such as the heart. Involved in this maturation is a transition of cardiomyocytes from a mononucleate to a binucleate phenotype. This normal transition occurs during fetal and early postnatal life, and is attributed

to the uncoupling of cytokinesis from karyokinesis [3]. Mononucleate cardiomyocytes retain the ability to proliferate whereas the binucleate cells do not, and this is because they have exited the cell cycle and become terminally differentiated [4]. Alterations to the timing of this critical transition may have long-term consequences on heart development and function throughout life.

Hypoxia is a major stress to the fetal development. Our previous studies have shown that an *in vivo*

rat model of maternal hypoxia results in fetal cardiomyocytes prematurely exiting the cell cycle [5-7]. This early-onset transition leads to fewer but larger cardiomyocytes as a result of increased binucleation and hypertrophy, and decreased proliferation of the cells. The timing of this transition is critical in determining the number of cardiomyocytes endowed in the heart for a lifetime. Whereas these *in vivo* studies showed the effect of hypoxia on fetal heart development, the mechanisms remain unknown.

Hypoxia is a known inducer of endothelin-1 (ET-1) expression [8-11]. ET-1 plays an important role in regulating cell cycle, and the cardiomyocyte is both a site of synthesis and action of ET-1 [12, 13], suggesting a localized role for hypoxia-induced ET-1 action in the heart. Thus, the present study tested the hypothesis that ET-1 induces a premature cardiomyocyte transition in the developing heart. Given a recent finding that the terminal differentiation of cardiomyocytes is characterized by a hypermethylated genome and compact chromatin [14], we further tested the hypothesis that ET-1 promotes cardiomyocyte terminal differentiation by an increase in DNA methylation. Herein, we present evidence that ET-1 *via* action of ET-1 receptors stimulates the premature transition of fetal cardiomyocytes, characterized by increased binucleation and decreased proliferation. DNA methylation of fetal cardiomyocytes is increased with ET-1 treatment, and the ET-1-induced changes in binucleation and proliferation are blocked by a DNA methylation inhibitor 5-aza-2'-deoxycytidine. Altogether the results suggest that epigenetic regulation *via* DNA methylation is involved in the cardiomyocyte transition stimulated by increased synthesis of ET-1.

Methods

Experimental animals.

Time-dated pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI) and divided into two groups: (i) normoxic control and (ii) 10.5% O₂ hypoxia treatment from gestational day 15 to 21, as previously described [15, 16]. Hearts were isolated from day 21 fetuses. To isolate hearts, pregnant rats were anesthetized with isoflurane, and adequate anesthesia was determined by loss of pedal withdrawal reflex. Fetuses were removed and pregnant rats killed by removing the hearts. Fetal hearts were isolated for the studies. All procedures and protocols were approved by the Institutional Animal Care and Use Committee and followed the guidelines by US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Primary cardiomyocyte culture and treatment.

Cardiomyocytes were isolated from normoxic day 21 fetal rats as previously described [17]. Cells were cultured in Hyclone Medium 199 (Thermo Scientific) supplemented with 10% fetal bovine serum (Gemini Bio-Products) and 1% antibiotics (10,000 I.U./mL penicillin, 10,000 µg/mL streptomycin) at 37°C in 95% air/5% CO₂. BrdU (0.1mM) was added to the medium to prevent fibroblast proliferation. Within three days of culture, the cells formed a monolayer with synchronized beating, characteristic of viable cardiomyocytes. Experiments were performed at 70-80% confluency. Cells were treated under normoxia (21% O₂) or hypoxia (1% O₂) for 24 hours, in the absence or presence of ET-1 (Sigma; 10 nM), PD145065 (Calbiochem; 10 nM), or 5-aza-2'-deoxycytidine (Sigma; 10 µM).

Quantitative real-time PCR.

RNA was isolated from the fetal hearts and pre-pro-ET-1 mRNA abundance was determined by real-time RT-PCR using Icyler Thermal cycler (Bio-Rad), as described previously [16]. Reverse transcription and cDNA synthesis was performed using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). The primers are 5'-CTAGGTCTAAGCGATCCTTGAA-3' (forward) and 5'-CTTGATGCTGTTGCTGATGG-3' (reverse). PCR was performed in triplicate, and threshold cycle numbers were averaged.

Immunocytochemistry.

Primary cardiomyocytes were double stained with alpha-actinin, a cardiomyocyte marker, and Ki-67, a proliferation marker as described previously [5]. Cardiomyocytes were plated on coverslips and fixed with acetone for 10 minutes. The cells were blocked with 1% bovine serum albumin for 1 hour at room temperature before incubation with the primary antibodies: mouse anti- α -sarcomeric actinin (Sigma, St.Louis, MO) (1:200) and rabbit anti-Ki-67 (Abcam, Cambridge, MA) (1:100) in 4°C overnight. The samples were incubated with the secondary antibodies: anti-mouse FITC-conjugated and anti-rabbit Texas Red-conjugated antibodies for 1 hour at room temperature. Nuclei were stained with Hoescht (Sigma) for 1 minute. The immunofluorescence staining was assessed using a Zeiss Axio Imager.A1 microscope and quantitative analysis was carried out using Image J software. Percent binucleation, Ki-67 expression, and cell size were measured.

5-mC DNA ELISA.

DNA methylation in primary fetal cardiomyo-

cytes was determined by measuring 5-methylcytosine (5-mC) using a 5-mC DNA ELISA kit (Zymo Research). The kit features a unique anti-5-mC monoclonal antibody that is both sensitive and specific for 5-mC. The protocol for measurement of 5-mC level is described in the manufacturer's instruction. Briefly, 100 ng of genomic DNA from cardiomyocytes and standard controls provided by the kit was denatured and used to coat the plate wells with 5-mC coating buffer. After incubation at 37°C for 1 hour, the wells were washed with 5-mC ELISA buffer and then an antibody mix consisting of anti-5-mC and a secondary antibody was added to each well. The plate was covered with foil and incubated at 37°C for 1 hour. After the antibody mix was washed out from the wells with the 5-mC ELISA buffer, a HRP developer was added to each well and incubated at room temperature for 1 hour. The absorbance at 405 nm was measured using an ELISA plate reader. The percent 5-mC was calculated using the second-order regression equation of the standard curve that was constructed with negative control and positive controls in the same experiment.

Statistical Analysis.

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

Results

Maternal hypoxia increased prepro-ET-1 mRNA in fetal hearts.

Animals were exposed to maternal hypoxia from gestational day 15-21; at the end of treatment hearts were isolated from day 21 fetal rats. Figure 1 demonstrated a significant increase in prepro-ET-1 mRNA abundance in fetal hearts exposed to 10.5% O₂, as compared to the normoxic control (21% O₂).

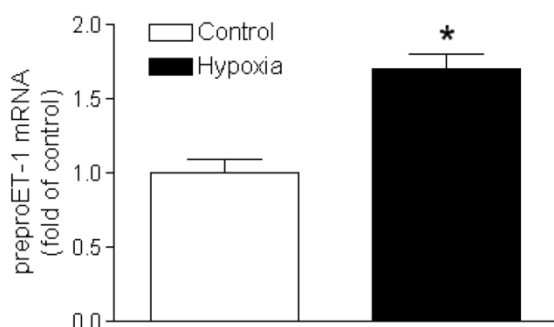
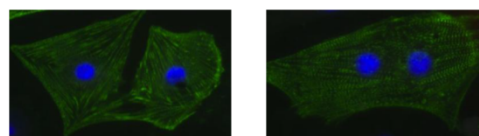


Figure 1. Effect of hypoxia on prepro-ET1 mRNA in the fetal heart. Hearts were isolated from near-term fetuses of pregnant rats treated with control or hypoxia. mRNA abundance of prepro-ET-1 was determined by real-time RT-PCR. Data are means \pm SEM. * $P < 0.05$, hypoxia vs. control. $n = 7-8$

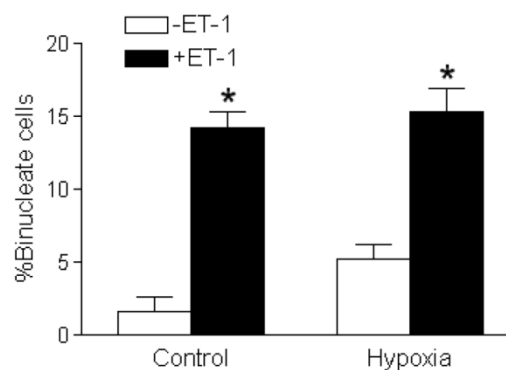
ET-1 stimulated binucleation and inhibited proliferation of fetal cardiomyocytes.

The morphology of mononucleate cells and binucleate cells of primary fetal cardiomyocytes are shown in Figure 2A. Whereas the basal levels of binucleate cardiomyocytes in fetal hearts were low, the treatment of cardiomyocytes with ET-1 resulted in a significant increase in percent binucleation, as compared to the control in both normoxic and hypoxic conditions (Figure 2A). Hypoxia alone in the absence of ET-1 had a slight increase in percent binucleation but it did not reach a significant level, as compared to the normoxic control. Percent Ki-67 positive cells, indicating proliferation, in ET-1 treated cardiomyocytes were significantly decreased in both normoxia and hypoxia (Figure 2B). Unlike the effect of binucleation, hypoxia alone in the absence of ET-1 significantly decreased percent of Ki-67 positive cells (Figure 2B).

A



B



C

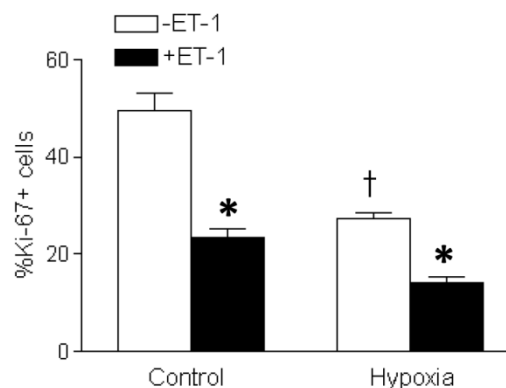


Figure 2. Effect of ET-1 on binucleation and proliferation of fetal cardiomyocytes. Cardiomyocytes isolated from fetal hearts were treated with ET-1 (10 nM) under normoxic control (21% O₂) or hypoxic (1% O₂) conditions for 24 h. **A.** Morphology of mononucleate and binucleate fetal cardiomyocytes. **B.** Binucleation result. **C.** Proliferation result.

Data are means \pm SEM. * $P < 0.05$, +ET-1 vs. -ET-1; † $P < 0.05$, hypoxia vs. control. $n = 5$

Interaction of ET-1 and hypoxia decreased cardiomyocyte size.

Neither ET-1 nor hypoxia alone had a significant effect on cardiomyocyte size (Figure 3). However, cardiomyocyte size was significantly decreased with the ET-1 treatment under the hypoxic condition (Figure 3).

PD145065 inhibited the effects of ET-1.

PD145065, a non-selective ET-receptor antagonist, blocked the effects of ET-1 on percent binucleation (Figure 4A) and Ki-67 expression (Figure 4B) in fetal cardiomyocytes. PD145065 in the absence of ET-1 had no significant effect on either binucleation or proliferation of cardiomyocytes.

ET-1 increased DNA methylation in fetal cardiomyocytes.

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

5-Aza-2'-deoxycytidine abrogated the effects of ET-1 on binucleation and proliferation in fetal cardiomyocytes.

In the presence of 5-aza-2'-deoxycytidine, ET-1-induced stimulation of binucleation (Figure 6A) and inhibition of Ki-67 expression (Figure 6B) in fetal cardiomyocytes were blocked. Whereas 5-aza-2'-deoxycytidine alone in the absence of ET-1 had a tendency to increase cardiomyocyte binucleation, this effect did not reach the significant level (Figure 6A).

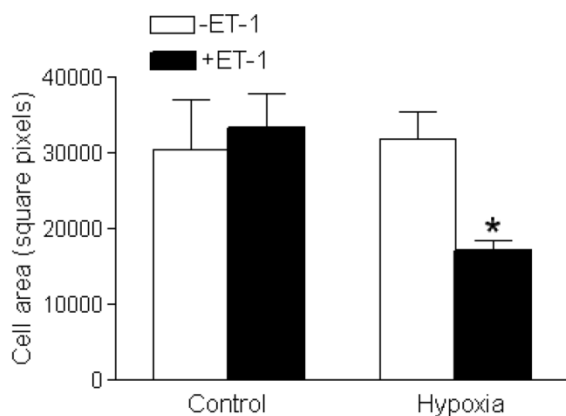


Figure 3. Effect of ET-1 on fetal cardiomyocyte size. Cardiomyocytes isolated from fetal hearts were treated with ET-1 (10 nM) under normoxic control (21% O₂) or hypoxic (1% O₂) conditions for 24 h. Data are means ± SEM. * P < 0.05, +ET-1 vs. -ET-1. n = 7-10

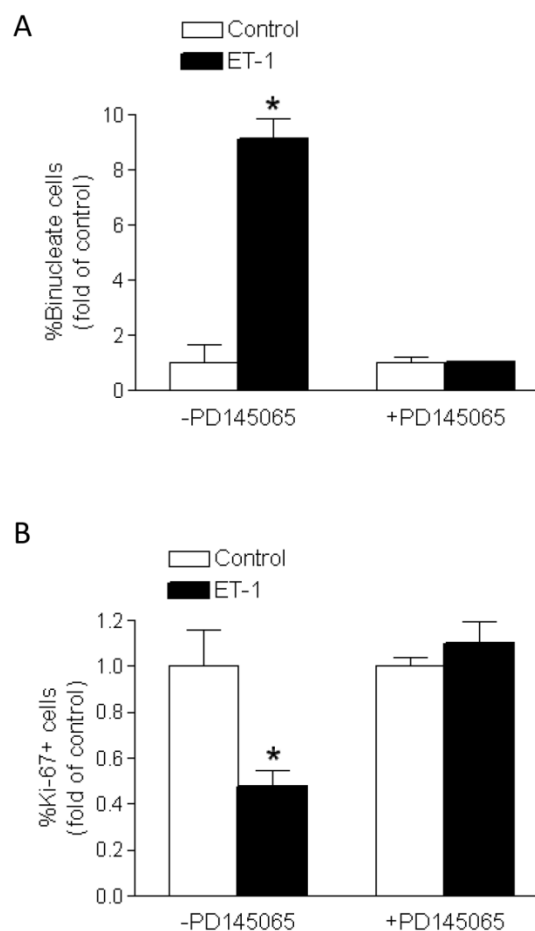


Figure 4. PD145065 abrogates ET-1-mediated effects on binucleation and proliferation of fetal cardiomyocytes. Cardiomyocytes isolated from fetal hearts were treated with ET-1 (10 nM) for 24 h in the absence or presence of PD145065 (10 nM). **A.** Binucleation result. **B.** Proliferation result. * P < 0.05, ET-1 vs. control. n = 5

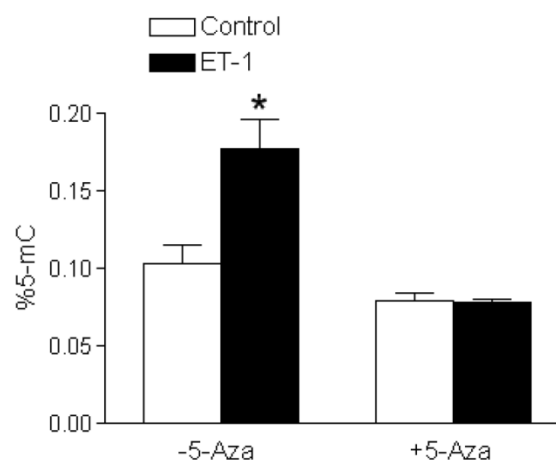


Figure 5. 5-Aza-2'-deoxycytidine blocks ET-1-increased DNA methylation in fetal cardiomyocytes. Cardiomyocytes isolated from fetal hearts were treated with ET-1 (10 nM) for 24 h in the absence or presence of 5-aza-2'-deoxycytidine (5-Aza, 10 μM). * P < 0.05, ET-1 vs. control. n = 5

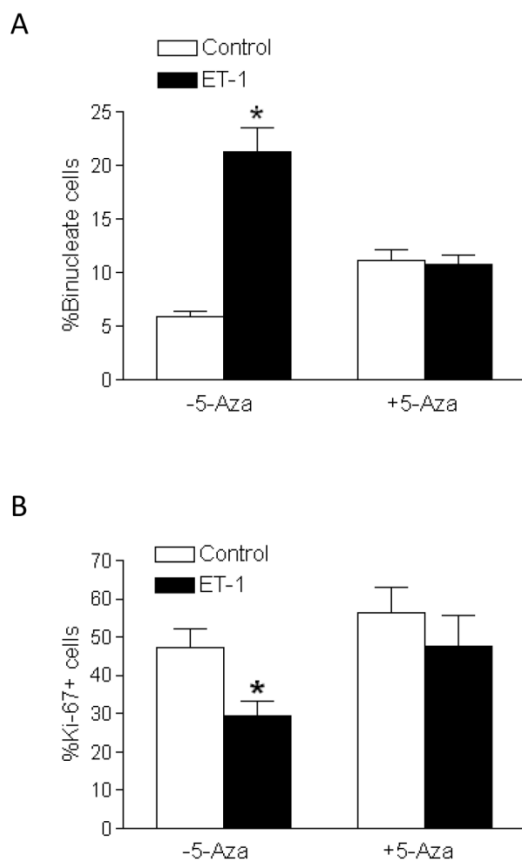


Figure 6. 5-Aza-2'-deoxycytidine abrogates ET-1-mediated effects on binucleation and proliferation of fetal cardiomyocytes. Cardiomyocytes isolated from fetal hearts were treated with ET-1 (10 nM) for 24 h in the absence or presence of 5-aza-2'-deoxycytidine (5-Aza, 10 μ M). **A.** Binucleation result. **B.** Proliferation result. * $P < 0.05$, ET-1 vs. control. $n = 5$

Discussion

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

The ET-1 synthesis pathway begins with the transcription of prepro-ET-1 mRNA, which is translated into prepro-ET-1. A series of enzymatic cleavages produce Big-ET-1 and the matured ET-1, a 21-amino acid peptide [18]. Several studies have reported the regulation of ET-1 synthesis by hypoxia, including the identification of a HIF-1 binding site on the prepro-ET-1 gene promoter [19, 20]. Specifically in

cardiomyocytes, a HIF-1 α binding site has been identified on the 5'-promoter region of the prepro-ET-1 gene [21]. Additionally, previous studies demonstrated a negative correlation between pO₂ and plasma ET-1 levels in fetal goats [11] as well as pO₂ and ET-1 levels in human amniotic fluid [10]. ET-1 mRNA levels were significantly increased in rat lung [22] and placentas [23] exposed to hypoxia. In agreement with these findings, the present study demonstrated a significant increase in prepro-ET-1 mRNA in the fetal rat heart resulting from *in utero* hypoxia, suggesting a local paracrine action of ET-1 in hypoxia-mediated effect on the fetal heart.

Physiological circulating levels of ET-1 are in the low picomolar range [12, 24] and may be significantly upregulated in pathophysiological conditions, such as hypoxia, heart failure, hypertension, and preeclampsia [25-28]. ET-1 acts in a paracrine and/or autocrine fashion and therefore tissue concentrations are significantly higher than those in the circulation [29]. The concentration of ET-1 (10 nM) was chosen based on other studies [30-33] and the rationale that ET-1 levels in the local tissue are much greater than in circulation. In the present study, we found that fetal rat cardiomyocytes exposed to elevated levels of ET-1 exhibited both increased binucleation and decreased proliferation. The binucleate cells are unable to proliferate and thus become terminally differentiated. In the rat heart, normal transition of cardiomyocytes to the binucleate form starts at birth and lasts during the first two weeks of postnatal life [3]. Therefore, the accelerated transition in the fetal heart due to increased levels of ET-1 has long-term implications. A premature transition of terminal differentiation may result in a reduced number of cardiomyocytes and altered cardiac growth after birth. As previous work has shown, hypoxia causes a premature exit of cell cycle in fetal cardiomyocytes [7], but the downstream regulators are not known.

The present study demonstrated that ET-1 increased the percent of binucleate cells independent of hypoxia, and hypoxia alone had no significant effect on the binucleation of cardiomyocytes. These findings suggest a lack of direct effect of hypoxia on the cardiomyocyte transition and provide evidence that ET-1 is a key downstream regulator of the premature exit of cell cycle in the fetal heart, observed *in vivo* in fetal hypoxia [7]. It is important to note that while cardiomyocytes have the ability to produce ET-1, endothelial cells contribute a large portion of its production. The isolated cardiomyocytes may not provide the full mechanism for hypoxia to produce a sufficient ET-1 response and the subsequent effects on binucleation and cell size. The finding that PD145065 blocked the ET-1-induced increase in binucleation and decrease in

proliferation of fetal cardiomyocytes indicates the ET-1 receptor-mediated effects. PD145065 is a non-selective ET_A- and ET_B-receptor antagonist and has been shown to block the effect of ET-1 *via* the ET-receptors [34-36].

The finding that hypoxia decreased proliferation of fetal cardiomyocytes is in agreement with previous reports [5-7]. Prior studies have shown that hypoxia promotes HIF-1 α association with HIF-1 β and enhances the expression of cyclin-dependent kinase inhibitors (CKIs), which in turn inhibits cell cycle proteins and decreases cell proliferation [37]. It is also suggested that HIF-1 regulates the G1/S phase transition by regulating the expression of cyclin E, a required factor for the transition [38]. Hypoxia has also been shown to induce expression of metalloproteinase inhibitors (TIMPs) that may have an inhibitory or stimulatory effect on cellular proliferation depending on the subtype and tissue involved [39]. In the heart, TIMP-3 is highly expressed and shown to inhibit proliferation in neonatal mouse cardiomyocytes [40]; a result of up-regulated p27 expression *via* the EGFR-JNK-SP-1 mediated pathway [41]. Although TIMP-3 and -4 are upregulated by hypoxia, their promoters do not contain HIF-responsive elements. Thus the regulation of these inhibitors and the subsequent effects on proliferation appear to be mediated by other genes that contain HIF-responsive elements.

ET-1 is one possible candidate considering it both contains a HIF-response element in its promoter [19, 20] and has been shown to regulate proliferation [5-7, 42]. The finding that, unlike ET-1, hypoxia had no significant effect on binucleation suggests that hypoxia-induced effect on proliferation was not mediated by ET-1, but rather by an independent and direct effect of hypoxia. The synergistic effect of hypoxia and ET-1 more closely mimics the physiological system as a whole. Ki-67 expression and cell size were significantly decreased by hypoxia and ET-1 treatment together. These results agree with previous reports, from our lab and others, that hypoxia alone had a direct effect in decreasing proliferation of cardiomyocytes [5-7, 42].

A change in cell size was only observed with the addition of both ET-1 and hypoxia. Both ET-1 and hypoxia are known hypertrophic factors [43-45]. In neonatal rat cardiomyocytes, mild hypoxia (10% O₂) has been found to induce hypertrophy [45, 46]. However more severe hypoxia (1% O₂), as was done in our study, appears to elicit the opposite effect leading to a reduction in cell size. Thus the severity at which hypoxia is induced likely has a differential effect on changes in cellular size. ET-1 has also been shown to stimulate cardiomyocytes to proliferate, and in the case of terminally differentiated cells it leads to

hypertrophy [32, 47]. Furthermore previous studies have found that hypertrophic growth is initially observed in the first week of postnatal life [3, 48]. Given that fetal and neonatal hearts are at very different developmental stages and experience very different oxygen tensions, it is possible that cardiomyocytes of the fetal heart respond differently to environmental cues such as hypoxia as that seen in the neonatal cardiomyocytes.

The finding that 5-aza-2'-deoxycytidine blocked the ET-1-induced increase in binucleation and decrease in proliferation is intriguing and suggests that ET-1 induces methylation of DNA as a means of involvement in cardiomyocyte terminal differentiation and suppression of proliferation. Whereas the present study focused on the downstream mechanisms of ET-1 in regulating terminal differentiation of cardiomyocytes, whether DNA methylation plays a role in the hypoxia-mediated direct effect on proliferation remains to be determined. 5-Aza-2'-deoxycytidine has been widely used as a DNA methylation inhibitor, and in the concentration range of 1 to 30 μ M it inhibits DNA methylation both globally and at specific sites of DNA [14, 49-52]. In the present study, we found that ET-1 significantly increased global DNA methylation in cardiomyocytes and this was blocked by 5-aza-2'-deoxycytidine. Epigenetic mechanism of DNA methylation acts to silence gene transcription, typically at cytosine residues within CpG dinucleotides. A previous study showed that methylation gradually increases over the course of development in neonatal cardiomyocytes [14], the same time frame for which binucleation occurs. Furthermore, expression of DNA methyltransferases involved in *de novo* DNA methylation (DNMT3a and DNMT3b) was significantly increased during the first 90 days of postnatal life. Inhibition of methylation with 5-aza-2'-deoxycytidine during neonatal day 7 and 10 resulted in a marked increase in DNA synthesis and delayed maturation [14]. It is well known that environmental cues during fetal development can profoundly alter the structure and function of an organ *via* epigenetic regulation. Particularly in the heart, cardiac function is dependent in part on cardiomyocyte number. Thus hypoxia-mediated ET-1 may signal through epigenetic mechanisms to negatively impact cardiomyocyte development. The present study suggests that DNA methylation is an epigenetic mechanism through which ET-1 stimulates cardiomyocyte transition of terminal differentiation. Ultimately, this may lead to reduced total cardiomyocyte number in the heart. Many studies have demonstrated that genes associated with the cell cycle and cytokinesis are involved in this transition process [53-57]. Adult cardiomyocytes from knockout mice lacking Rb and p130 show a de-

crease in heterochromatin and an increase in proliferation associated with derepression of cell cycle genes [57]. These genes may be differentially regulated by changes in methylation patterns thus altering the cell cycle and cytokinesis. Future studies will have to elucidate the methylation status of specific genes during this transition phase.

The present study identifies a novel mechanism of ET-1-induced hypermethylation as a downstream regulator of hypoxia-mediated cardiomyocyte transition from mononucleate to binucleate cells in the developing heart. Cardiomyocyte endowment is determined during fetal and early postnatal development, when most cardiomyocytes become binucleate and cease to proliferate [58, 59]. Given that hypoxia is one of the most important and clinically relevant stresses to the fetal development, and that fetal hypoxia results in fewer but larger cardiomyocytes and increases the susceptibility of the heart to ischemic injury in offspring [7, 60-62], the present study provides a mechanistic understanding worthy of further investigation in humans.

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Competing Interests

The authors have declared that no competing interest exists.

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