IN VITRO HYPERGLYCEMIA OR A DIABETIC INTRAUTERINE 
ENVIRONMENT REDUCES NEONATAL ENDOTHELIAL COLONY 
FORMING CELL NUMBERS AND FUNCTION

David A. Ingram, Izlin Z. Lien, Laura E. Mead, Myka Estes, Daniel N. Prater, 
Ethel Derr-Yellin, Linda DiMeglio, and Laura S. Haneline

Department of Pediatrics, Herman B Wells Center for Pediatric Research, 
Department of Biochemistry and Molecular Biology, 
Department of Microbiology and Immunology, Indiana University School of Medicine, 
Indianapolis, 46202

Running title: Maternal diabetes reduces neonatal EPC function

Corresponding Author: 
Laura S. Haneline 
Herman B Wells Center for Pediatric Research 
1044 W. Walnut St. R4-476 
Indianapolis, IN 46202 
lhanelin@iupui.edu

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ABSTRACT

Objective: Emerging data demonstrate that maternal diabetes mellitus (DM) has long-term health consequences for her offspring including the development of hypertension. In adults, circulating endothelial progenitor cells (EPCs) participate in vascular repair, and EPC numbers and function inversely correlate with the risk of developing vascular disease. Therefore, our objectives were to determine whether hyperglycemia (HG) or exposure to a diabetic intrauterine environment alters EPC function.

Research Design and Methods: We utilized well established clonogenic endothelial colony forming cell (ECFC) assays and murine transplantation experiments to examine human vasculogenesis.

Results: Both in vitro HG and a diabetic intrauterine environment reduced ECFC colony formation, self-renewal capacity, and capillary-like tube formation in matrigel. This cellular phenotype was linked to premature senescence and reduced proliferation. Further, cord blood ECFCs from diabetic pregnancies formed fewer chimeric vessels de novo after transplantation into immunodeficient mice compared to neonatal ECFCs harvested from uncomplicated pregnancies.

Conclusions: Collectively, these data demonstrate that HG or exposure to a diabetic intrauterine environment diminishes neonatal ECFC function both in vitro and in vivo, providing potential mechanistic insights into the long-term cardiovascular complications observed in newborns of diabetic pregnancies.


KEY WORDS. diabetes, hyperglycemia, endothelial cells, senescence, fetal programming
The incidence of diabetes mellitus (DM) is increasing exponentially. According to the U.S. Department of Health and Human Services, ~10% of the population over age 20 have DM (type 1 + type 2) while another 26% have prediabetes (defined as impaired fasting glucose levels); many of whom are unaware of their diagnosis. Vascular diseases associated with DM contribute significantly to the morbidities and mortality of this chronic disease. In fact, 73% of adults with DM are hypertensive and > 65% die from cardiovascular disease or stroke. Intensive treatment of type 1 DM patients decreases cardiovascular and microvascular complications, suggesting that the degree of hyperglycemia (HG) correlates directly with the development of vascular morbidities and is a key pathogenic factor in the development of endothelial dysfunction.

The burden of DM in pregnancy (type 1, type 2, and gestational) is also increasing; currently affecting ~6-10% of all pregnant women. It has long been recognized that maternal DM is associated with numerous fetal and neonatal morbidities including congenital anomalies, premature delivery, fetal macrosomia, neonatal respiratory distress, and neonatal hypoglycemia. However, emerging data suggest that maternal DM also has long-term health consequences for her offspring. The most compelling data are studies in Pima Indians where offspring of women with either gestational DM or type 2 DM have an increased risk of developing the metabolic syndrome, hypertension, type 2 DM, and obesity, and in many cases, elevated blood pressure, insulin resistance, and increased body mass index occur in childhood. The concept that alterations in the intrauterine environment results in fetal adaptations that increase the risk for subsequent disease is well described. However, the current challenge is to understand the underlying mechanisms responsible for these effects in order to design preventative strategies for individuals. Given that hypertension is prevalent in offspring of diabetic mothers, it is critical to address how the diabetic intrauterine environment affects the developing vascular system.

Multiple factors contribute to the maintenance of vascular integrity, thereby protecting an individual from vascular disease. A critical component of vascular health is efficient repair of damaged endothelium and the ability to form new blood vessels. Endothelial progenitor cells (EPCs) participate in both of these processes. While significant debate remains over the optimal method for defining EPCs, numerous studies demonstrate an indirect correlation between circulating EPC numbers and vascular disease risk. Initial studies reported a reduction in circulating EPCs in individuals with coronary artery disease and in those adults with a high cardiovascular disease risk predicted by a Framingham risk factor score. Interestingly, this paradigm has held for multiple disease states in adults including DM, peripheral vascular disease, rheumatoid arthritis, and pre-eclampsia. However, no studies have been reported in infants or children.

Previously, we characterized circulating EPCs as endothelial colony forming cells (ECFCs) using functional parameters routinely used to define progenitor cells such as proliferative potential and self-renewal capacity. ECFCs circulate in the blood (cord blood
and adult peripheral blood) and reside in the endothelium of vessel walls. In addition, ECFCs are highly proliferative, capable of 100 population doublings for cord blood, exhibit self-renewal capacity demonstrated by replating ability, and are capable of de novo vessel formation in vivo. Given that reduced circulating EPC numbers in adults are associated with increased vascular disease risk together with the observation that offspring of diabetic mothers have an increased incidence of hypertension, we questioned whether fetal exposure to a diabetic intrauterine environment would result in reduced cord blood ECFC numbers and function.

**RESEARCH DESIGN AND METHODS**

**Umbilical cord blood samples and subject characteristics.** Human umbilical cord blood (UCB) samples (40-60ml) from healthy newborns and infants of diabetic mothers (gestational age 38-42 weeks) were collected in heparinized solution. At Indiana University School of Medicine, screening oral glucose tolerance tests are performed as part of routine care on all non-diabetic pregnant women between 24 and 28 weeks gestation. All control mothers had a normal screening 50 gram oral glucose challenge, defined as a 1 hour glucose <140, and normal blood pressure throughout pregnancy. All diabetic mothers had an established diagnosis of diabetes prior to pregnancy and were on insulin at delivery. Diabetic mothers had normal blood pressure and renal function throughout pregnancy documented by serum creatinine, serum blood urea nitrogen, and urinalysis. In addition, none of the women had evidence of retinopathy prior to pregnancy and fundoscopic exams in 3 of the 4 women during pregnancy (16-32 weeks gestation) were normal. The fourth diabetic woman did not have an ophthalmologic exam during pregnancy. Women with illnesses known to affect glucose metabolism (i.e. polycystic ovarian syndrome, Cushing syndrome) and taking medications known to affect glucose metabolism (i.e. glucocorticoids) were excluded from the study. The Institutional Review Board at the Indiana University School of Medicine approved all protocols, and informed consent was obtained from all pregnant women.

**Culture of Endothelial Colony Forming Cells (ECFCs).** Immediately after collection of UCB samples, human mononuclear cells (MNCs) were obtained by diluting blood with PBS 1:1 which was underlaid with an equivalent volume of Ficoll-Paque PLUS™ (GE Healthcare, Piscataway, NJ). Cells were centrifuged for 30 minutes at room temperature at 740g. MNCs were isolated and washed with EBM-2 medium (Cambrex, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2% penicillin/streptomycin and 0.25µg/mL amphotericin (Invitrogen, Grand Island, NY) (complete EBM-2 medium).

MNCs were resuspended in complete EGM-2 medium (complete EBM-2 supplemented with EGM-2 bullet kit, Cambrex). Six-well tissue culture plates pre-coated with type 1 rat tail collagen (BD Biosciences, Bedford, MA) were seeded with 5 x 10^7 cells/well and cultured in a humidified incubator. After 24 hours of culture, adherent cells were washed with complete EGM-2 and complete EGM-2 medium was added to each well. Medium was changed daily for 7 days and then every other day until the first passage.

Colonies of endothelial cells appeared between 5-8 days of culture and were
Maternal diabetes reduces neonatal EPC function

identified as circumscribed monolayers of cobblestone-appearing cells. Upon confluency, endothelial cells were detached with 0.25% trypsin-EDTA (Invitrogen), resuspended in complete EGM-2 media, and plated tissue culture flasks coated with type 1 rat tail collagen.

For this study, HG treatment included a 7 day exposure of early passage (P3-4) ECFCs of healthy newborns with D-glucose (Invitrogen) added to complete EGM-2 media. Complete EGM-2 media is considered euglycemic with a basal concentration of 5mM dextrose.

Colony forming assays. For limiting dilution assays, ECFCs were seeded in 6-well plates precoated with type 1 rat tail collagen (200 cells/well). Each condition was plated in triplicate wells. On day 7 after initial plating, wells were scored for colony formation by visual inspection with an inverted microscope (Olympus, Lake Success, NY) under 40x magnification.

ECFC growth kinetics. Cord blood ECFCs were passaged upon reaching 90% confluency. At each passage, ECFCs were counted by trypan blue exclusion for calculation of growth kinetic curve and cumulative population doubling levels (CPDLs). The number of population doublings (PDs) occurring between passages was calculated according to the equation PD = log₂ (C_H/C_S) where C_H is the number of viable cells at harvest and C_S is the number of cells seeded. The sum of all previous PDs determined the CPDL at each passage.

Senescence associated-β-galactosidase (SA-β-gal) staining. ECFCs were cultured in 6 well plates for 7 days prior to β-galactosidase staining to assess for senescence. Wells were washed, fixed with 2% formaldehyde/0.2% gluteraldehyde, and stained for 24 hours with β-galactosidase solution (Acros, NJ) 150mM, MgCl₂ (Fisher, Fair Lawn, NJ) 2mM, Trisodium Citrate (Sigma-Aldrich, St Louis, MO) 40mM, Potassium Ferricyanide (Sigma-Aldrich) 5mM and Potassium Ferrocyanide (Sigma-Aldrich) 5mM; adjusted to pH 6 containing X-Gal (5-bromo-4-chloro-3-indoyl-beta-D-galactopyranoside) (Invitrogen). Senescence was examined by visual inspection of deep blue stained cells with an inverted microscope under 40x magnification. All conditions were conducted in triplicate and at least 100 cells were scored per replicate.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) Assay. ECFCs that were either untreated or treated with D-glucose for 1-7 days were examined for apoptosis using a TUNEL assay per manufacturer’s recommendation (Roche, Indianapolis, IN). All conditions were conducted in triplicate and at least 100 cells were scored per replicate.

H³ Thymidine Proliferation Assay. ECFCs were starved for 24 hours without growth factors in EBM-2 media supplemented with 5% FBS. Cells were plated at 50,000 cells/well in a 6-well collagen coated plate in triplicate and starved overnight in EBM-2 supplemented with 1% FBS. ECFCs were then cultured in EBM-2 without serum for eight hours to achieve quiescence. Media was then changed to EBM-2 supplemented with 10% FBS, and cells were either untreated, stimulated with 25 ng/ml vascular endothelial growth factor (VEGF), or stimulated with 25 ng/ml basic fibroblast growth factor (bFGF) (Peprotech, Rocky Hill, NJ) for 16 hours. Cells were pulse-labeled with 1 µCi (0.037 MBq) tritiated thymidine (Perkin Elmer Life Sciences Products, Boston, MA) for 5 hours and lysed in
Maternal diabetes reduces neonatal EPC function

0.1M NaOH for one hour before counting in a liquid scintillation counter (Beckman Coulter).

Matrigel Assay. ECFCs were cultured at 8000 cells/well in 96 well tissue culture plates coated with 50µl Matrigel (BD Biosciences). At 24 hours, images were collected using a Zeiss Axiovert 1 inverted microscope with a 5x CP-AChROMAT/0.12 NA objective. Images were acquired using a SPOT RT color camera (Diagnostic Instruments, Sterling Heights, MI) with the manufacturer’s software. Each experiment was done in triplicate.

Xenograft Transplant. Cellularized gel implants were cast as previously described.28,29 Cord blood ECFCs were suspended (2x10^6 cells/ml) in complete EGM-2 supplemented with 1.5 mg/ml rat-tail collagen I, 100 ng/ml human fibronectin (Chemicon), 1.5 mg/ml sodium bicarbonate (Sigma), 25 mM HEPES (Cambrex), and 10% FBS. Pericytes isolated from human adipose tissue were added to the gels to provide mural support to newly forming vessels as previously described.30 Cell suspensions were placed in a 12-well tissue culture dish for 30 minutes at 37°C for polymerization. The gels were then covered with complete EGM-2 for overnight incubation. Gels were implanted the following day into 3 anesthetized NOD/SCID mice. The right flank of each mouse was implanted with control ECFCs, and the left flank was implanted with ECFCs from diabetic pregnancies. Fourteen days after transplantation, grafts were excised and analyzed for vessel formation by immunohistochemistry. All experiments were approved by the Indiana University Laboratory Animal Research Center.

Immunohistochemistry. All reagents for immunohistochemistry studies were purchased from DAKO (Carpenteria, CA) unless otherwise specified. For anti-human CD31 staining formalin-fixed, paraffin-embedded tissue sections were deparaffinized and immersed in a retrieval solution for 20 minutes at 95-99°C. Slides were incubated at room temperature with anti-human CD31 (clone JC70A) for 30 minutes followed by 10 minute incubations with LSAB2 link-biotin and streptavidin-HRP, then developed with DAB solution for 5 minutes. Slides were dehydrated and mounted using Cytoseal (Richard-Allen Scientific, Kalamazoo, MO). Enumeration of perfused vessels staining positively for anti-human CD31 was performed by visual inspection under 100 X magnification.

RESULTS

HG reduces cord blood ECFC clonogenic growth via premature senescence. To test whether neonatal ECFCs are sensitive to HG stress, cord blood ECFCs from uncomplicated pregnancies were cultured for 7 days with increasing dextrose concentrations in clonogenic progenitor assays as described.29 A range of dextrose concentrations were tested including levels of HG observed in pregnant women with DM. ECFC colony formation was reduced at 10, 15, and 30 mM dextrose concentrations compared to normoglycemic controls (5mM dextrose) (Figure 1A). Interestingly, the most profound reduction was between the normoglycemic control and the lowest dextrose concentration tested (10 mM or 180 mg/dL). To interrogate the mechanism responsible, TUNEL assays and SA-β-gal staining were conducted to examine for increased apoptosis and/or
Maternal diabetes reduces neonatal EPC function

...senescence, respectively. No increase in apoptosis was observed in ECFCs treated with 10-15 mM dextrose for 1, 3, 5, or 7 days (data not shown). However, cord blood ECFCs exhibited a 2-fold increase in SA-β-gal positive cells after treatment with 10 and 15 mM dextrose (Figure 1B). Together these data suggest that enhanced senescence, but not apoptosis, contributes to the HG induced reduction of cord blood ECFC colony formation.

Given our observation that HG induces enhanced senescence of cord blood ECFCs, we tested whether HG diminishes tube formation of cord blood ECFCs using matrigel assays as described. Figure 2A shows representative photomicrographs of cord blood ECFCs cultured under normoglycemia and HG conditions. Quantitative assessment of tube formation was conducted by scoring the number of closed capillary tube networks per well (Figure 2B). A 30% reduction in tube formation was observed in cord blood ECFCs cultured under HG conditions compared to control ECFCs. Collectively, these data demonstrate that exposure to levels of HG seen routinely in pregnant diabetic women is sufficient to impair cord blood ECFC tube forming ability.

ECFCs exposed to a diabetic environment in utero exhibit premature senescence and reduced proliferation. Emerging evidence suggests that maternal DM increases the risk for offspring to develop cardiovascular disease; however the underlying cellular mechanisms responsible are unknown. Given our data demonstrating that HG induces significant functional deficits in cord blood ECFCs harvested from uncomplicated pregnancies, we hypothesized that maternal DM would alter fetal ECFCs in utero resulting in reduced circulating cord blood ECFC numbers and function. To test this hypothesis, ECFC colonies were enumerated and cell lines established from 9 pregnancies (4 DM and 5 controls). Tables 1 and 2 include clinical data from the women and infants, respectively, in this study. Pregnant subjects with DM (two type 1 and two type 2) were considered well-controlled with HgbA1C’s below 6%, and none of the women had evidence of hypertension, nephropathy, retinopathy, or coronary artery disease. Two of the infants born to diabetic mothers had increased ponderal indices with weight/height ratios above the 90th percentile. None of the infants born to the diabetic mothers developed hypoglycemia after birth. One infant born to a diabetic mother was diagnosed with respiratory distress secondary to hyaline membrane disease versus pneumonia and was treated with oxygen and antibiotics in the neonatal intensive care unit.

To evaluate the effect of maternal DM on fetal ECFCs, the total number of ECFC colonies harvested from cord blood MNCs were enumerated. No differences were observed in the number of MNCs obtained from either experimental group (data not shown). However, a significant reduction in the number of cord blood ECFC colonies/10^8 MNCs was observed in samples from diabetic pregnancies compared to uncomplicated pregnancies (13.2±2.6 vs. 40.1±6.9, p<0.01). Data from control ECFCs are similar to our previously published data. To examine whether in utero exposure to a diabetic environment alters the function of neonatal ECFCs, the primary cell progeny from all samples were expanded in culture, and functional assays were conducted. In addition to an overall decrease in the total number of ECFCs...
Maternal diabetes reduces neonatal EPC function

harvested from cord blood samples from diabetic pregnancies, the frequency of clonogenic precursors from these samples was significantly reduced compared to cord blood ECFCs from uncomplicated pregnancies (Figure 3).

Since in vitro exposure of cord blood ECFCs to HG increased senescence, we tested whether ECFCs from diabetic pregnancies also exhibited enhanced senescence, contributing to the reduction in clonogenic capacity. SA-β-gal assays demonstrated a 2 fold increase in senescent ECFCs from diabetic pregnancies compared to controls (Figure 4A-B). Given that cord blood ECFCs are highly proliferative with self-renewal ability, we hypothesized that increased senescence would result in diminished proliferative capacity of ECFCs from diabetic pregnancies. To test this hypothesis, population doubling assays were conducted as described. These studies revealed that ECFCs from diabetic pregnancies exhibited a marked reduction in cumulative population doubling levels (CPDLs) (Figure 4C). To assess growth factor induced proliferative responses, ECFCs from control and diabetic pregnancies were starved of serum and growth factors before stimulating with either bFGF or VEGF, two cytokines important for endothelial cell proliferation. ECFCs from diabetic pregnancies exhibited a significant reduction in baseline, bFGF induced, and VEGF induced proliferation compared to controls (Figure 4D). Collectively, these data suggest that maternal DM results in an overall decline in proliferative capacity of cord blood ECFCs due to premature senescence.

**ECFCs exposed to a diabetic environment in utero exhibit profound deficits in vascular regeneration.** Impaired vascular regeneration at multiple anatomic sites leads to a variety of vasculopathies in diabetic patients. Given the effects of the diabetic environment on cord blood ECFCs outlined above, we next tested whether ECFCs from diabetic pregnancies exhibited decreased capillary forming ability in vitro and diminished vasculogenesis in vivo. Figure 5A shows representative photomicrographs of capillary formation from ECFCs isolated from uncomplicated or diabetic pregnancies. Quantitative assessment of tube formation was conducted by scoring the number of closed capillary tube networks per well (Figure 5B). A 66% reduction in tube formation was observed in cord blood ECFCs from diabetic pregnancies compared to control ECFCs (Figure 5B).

A characteristic of stem and progenitor cells, including ECFCs, is their ability to be adoptively transplanted into hosts and repopulate the mature cells of the intended lineage. Therefore, we compared the ability of cord blood ECFCs isolated from either diabetic or uncomplicated pregnancies to form vascular structures de novo utilizing an established methodology for subcutaneous transplantation of cellularized gel implants into immunodeficient mice. Early passage cord blood ECFCs from diabetic or control pregnancies were generated from three different cord bloods respectively, suspended in collagen/fibronectin gels, and transplanted into immunodeficient mice. At 14 days, mice were sacrificed and the grafts analyzed for chimeric vessel formation. Photomicrographs of cellularized gels and enumeration of vessels from diabetic or control ECFCs are shown (Figure 5C-D). In 3 independent experiments, cord blood ECFCs from diabetic pregnancies had a two fold reduction in the number of
chimeric vessels perfused with mouse red blood cells in the transplanted graft compared to control ECFCs. Collectively, these data demonstrate that cord blood ECFCs from diabetic pregnancies exhibit diminished functional capacity both \textit{in vitro} and \textit{in vivo}.

\textbf{DISCUSSION}

Barker et al provided the initial epidemiologic evidence that infants born small for gestational age are at increased risk to develop a spectrum of adult diseases including the metabolic syndrome, cardiovascular disease, type 2 DM, and obesity\textsuperscript{31}. These seminal observations were the first to suggest that an individual's long-term risk for disease could be impacted by prenatal events. Since these early reports, numerous studies in humans and animals also demonstrate that intrauterine exposure to an energy rich environment, such as a high calorie diet, maternal DM, or maternal obesity, increase the risk for offspring to develop the metabolic syndrome, cardiovascular dysfunction, type 2 DM, and obesity\textsuperscript{5,13,32,33}. While clear evidence exists to show that alterations in the intrauterine environment increases disease risk in offspring, the underlying pathologic processes remain unknown.

Given the importance of EPCs in the maintenance and repair of the vascular system in adults together with the observed decrease in circulating EPCs in numerous adult disease states including DM, we speculated that \textit{in utero} exposure to maternal DM during rapid growth of the fetal vasculature would diminish the numbers and function of circulating fetal EPCs. Previous studies by our group classified ECFCs as EPCs that circulate in cord blood and peripheral blood, are resident in blood vessel walls, and exhibit essential progenitor characteristics; high proliferation potential, self-renewal capacity, and \textit{de novo} blood vessel formation \textit{in vivo}\textsuperscript{26,27,29}. Since maternal glucose freely diffuses across the placenta into the fetal circulation, initial studies examined whether HG directly impairs fetal ECFC function. After HG treatment, neonatal ECFCs from uncomplicated pregnancies exhibited significant functional deficits including a reduction in colony formation, enhanced senescence, and impaired tube forming ability. The magnitude of the functional deficits observed at the lowest dextrose dose (10 mM) was unexpected given that this concentration correlates with modest HG in a diabetic pregnant women (180 mg/dL) and can be seen routinely after a carbohydrate-rich meal or during an illness.

The majority of previous studies assessing the direct effect of HG on EPCs were conducted on cell populations that express both hematopoietic and endothelial markers and have since been determined to be angiogenic macrophage precursors\textsuperscript{34}. Recently however, Chen et al demonstrated that adult peripheral blood ECFCs treated with HG exhibited enhanced senescence and reduced tube forming ability \textit{in vitro}\textsuperscript{35}, though the dextrose concentrations required to induce these phenotypes were much higher (20-30 mM or 360-540 mg/dL) compared to our studies. The distinction between the degree of HG required to induce functional changes in neonatal versus adult ECFCs may reflect important developmental differences that deserve further investigation.

To examine whether circulating ECFCs from diabetic pregnancies displayed similar functional deficits as HG treated ECFCs, cord blood ECFCs were harvested from uncomplicated and
diabetic full-term pregnancies. Interestingly, cord blood from diabetic pregnancies had only one-third the number of circulating ECFCs compared to control cord blood samples. Our data support diminished growth factor dependent proliferation and premature senescence of neonatal ECFCs from diabetic pregnancies as contributing factors involved in reduced ECFC colony forming ability. To rigorously examine whether the functional capacity of neonatal ECFCs was impaired after exposure to a diabetic intrauterine environment, ECFCs were transplanted into NOD/SCID mice using a well-established in vivo vasculogenesis assay\(^\text{28}\). Remarkably, ECFCs from diabetic pregnancies formed half as many human-murine chimeric vessels compared to controls. Collectively, these data suggest that in utero exposure to a diabetic environment severely diminishes the functional capacity of fetal circulating ECFCs.

Optimal glycemic control is the mainstay of therapy in diabetic pregnancies and is evaluated by serial blood glucose measurements and HgbA1C levels. Given these criteria, the maternal subjects in our study were considered well-controlled by their obstetricians with HgbA1C levels <6%. Therefore, the magnitude of cord blood ECFC functional deficits observed in the offspring of these women may initially appear surprising. However, intermittent episodes of mild-moderate HG do not increase HgbA1c levels appreciably. Our data showing that a short-term treatment with modest HG (10 mM dextrose or 180 mg/dL) significantly reduces neonatal ECFC function supports the idea that elevations in HgbA1C levels may not be required for impairments in fetal ECFC function. Consistent with this notion, some investigators argue that targeting postprandial glucose levels, which are generally the daily peak HG periods, should be routinely practiced in diabetic individuals to reduce their cardiovascular disease risk and improve neonatal outcomes (reviewed in \(^\text{36}\)). Furthermore, while HG is a major metabolic disturbance in DM, numerous metabolic, cellular, and biochemical derangements may be observed in DM; each of which could independently contribute to diminished fetal ECFC function. In support of this idea, evidence for fetal effects of an intrauterine diabetic environment was present in our study infants even though the mothers had normal HgbA1C values. For example, two of the infants had increased ponderal indices (weight/height > 90%ile), and a third infant (4.01 kg and weight/height ratio 75-90%ile) experienced respiratory distress after delivery at full-term. Collectively, these observations suggest that HG directly impairs fetal ECFC function; however additional factors likely contribute to fetal ECFC dysfunction in vivo given the complexity of the intrauterine diabetic environment. A limitation of this study was the number of cord blood ECFCs analyzed. However, the consistency of the data in all ECFC cell lines derived from diabetic pregnancies compared to five different control ECFC cell lines strongly supports the contention that fetal exposure to maternal diabetes has detrimental effects on neonatal circulating ECFCs. Future studies to explore additional factors involved in ECFC dysfunction are planned.

Our data demonstrating enhanced senescence in cord blood ECFCs from diabetic pregnancies are intriguing given previous studies supporting a role for enhanced endothelial cell senescence in
Maternal diabetes reduces neonatal EPC function

the pathogenesis of vasculopathies (reviewed in 37). Senescent cells are active participants in vascular disease progression via the induction of vascular structural changes, inflammatory cytokine production, and loss of vascular reparative capacity. Typically, endothelial cell senescence is observed during the process of aging or after damage from a chronic disease such as DM. Therefore, our data showing that neonatal ECFCs from diabetic pregnancies prematurely senesce are disconcerting given the tremendous vascular growth that occurs from infancy through adolescence. Collectively, our data together with previous studies correlating reduced circulating EPC numbers with cardiovascular disease risk in adults suggest that reduced circulating cord blood ECFC numbers and premature ECFC senescence may predispose infants born to diabetic mothers to develop endothelial dysfunction and ultimately cardiovascular disease.

ACKNOWLEDGEMENTS

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REFERENCES

**TABLE 1.** Clinical data for maternal subjects.

<table>
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<th>Maternal subjects</th>
<th>Maternal age</th>
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<th>Length of DM (years)</th>
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<th>Maternal pre-pregnancy BMI</th>
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TABLE 2. Clinical data for infant subjects

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Maternal diabetes reduces neonatal EPC function

FIGURE LEGENDS

Figure 1A-B. Hyperglycemia reduces cord blood ECFC clonogenic capacity via senescence. A) Number of ECFC forming colonies 7 days after dextrose treatment. Results represent the mean ± SEM, n=4, *P< 0.03 for dextrose treated vs. the untreated control (C) by Student t test. B) Percentage of ECFC, which stained positively for SA β-gal 7 days after dextrose treatment. Results represent the mean of 3 independent experiments ± SEM, *P< 0.05 for dextrose treated vs. control by Student t test.

Figure 2A-B. Hyperglycemia reduces in vitro capillary formation. A) Representative photomicrographs of ECFCs after plating on Matrigel. Results are representative of 3 independent experiments. Scale bar represents 200 µm. B) Quantitation of capillary vessel density of control ECFCs (C) or ECFCs treated with 15 mM dextrose (Dex). Data shown are the mean ± SEM, n=3. *P< 0.05 by Student t test.

Figure 3. In utero exposure to a diabetic environment reduces ECFC clonogenic capacity. Number of ECFCs derived from cord blood from uncomplicated (C) or diabetic (IDM) pregnancies, which formed colonies 7 days after plating at limiting dilution. Results represent the mean ± SEM, n=4, *P< 0.003.

Figure 4A-D. Enhanced senescence of ECFCs exposed to a diabetic intrauterine environment correlates with reduced proliferative capacity. A) SA-β-gal staining of ECFCs derived from uncomplicated (C) or diabetic (IDM) pregnancies. Results are representative of four independent experiments. Scale bars represent 100 µm. B) Percentage of ECFCs derived from uncomplicated or diabetic pregnancies, which stained positively for SA β-gal. Results represent the mean ± SEM, n=4, *P< 0.002 by Student t test. C) Cumulative population doubling level (CPDL) of ECFCs derived from uncomplicated or diabetic pregnancies after 50 days of culture. Results represent the mean ± SEM, n=4, *P< 0.002 by Student t test. D) Proliferation of ECFCs derived from cord blood from uncomplicated (closed bars) or diabetic (open bars) pregnancies in response to 25 ng/ml VEGF or 50 ng/ml bFGF stimulation. Results represent mean radiation counts per minute performed in triplicate from a representative experiment, *P< 0.001 compared to control, **P< 0.002 compared to untreated (UT) IDM condition, +P<0.002 compared to UT control condition by Student t test. Similar results were seen in two additional independent experiments.

Figure 5A-D. In vitro and in vivo capillary formation of ECFCs exposed to a diabetic intrauterine environment is impaired. A) Representative photomicrographs of ECFCs derived from uncomplicated (C) and diabetic (IDM) pregnancies 24 hours after plating on Matrigel. Results representative of 3 independent experiments. Scale bar represents 30 µm. B) Quantitation of capillary vessel density 24 hours after plating on Matrigel. Data shown are the mean ± SEM, n=4. *P< 0.002 by Student t test. C) Representative photomicrographs (100x magnification) of cellularized grafts and surrounding murine tissue 14 days after implantation into NOD/SCID mice stained with anti-human CD31 (brown) to identify human blood vessels. Grafts contained ECFCs from either an uncomplicated or diabetic pregnancy. Arrows indicate anti-human CD31.
positive vessels and capillaries in the ECFC grafts, which were perfused with murine red blood cells. Results shown are representative of 3 independent experiments utilizing cells from different donors. D) Quantitation of capillary density within cellularized grafts containing ECFCs from uncomplicated or diabetic pregnancies 14 days after implantation. Results shown are the average number of capillaries containing murine red blood cells/mm² of graft tissue ± SEM, n=3, *P<0.001 by Student’s t test.
Maternal diabetes reduces neonatal EPC function

Figure 1 A - B
Maternal diabetes reduces neonatal EPC function

Figure 2 A - B

A

C

15mM Dextrose

B

Number of closed network units/well

C

Dex

*
Maternal diabetes reduces neonatal EPC function

Figure 3

Number of colonies

C  IDM

*
Maternal diabetes reduces neonatal EPC function

Figure 4 A-D
Maternal diabetes reduces neonatal EPC function

Figure 5 A - D