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Intrauterine Programming of Diabetes Induced Cardiac Embryopathy

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Abstract

Background: Maternal hyperglycemia is a well-recognized risk factor for fetal congenital heart disease. However, the underlying cellular and molecular mechanisms are not well characterized. We hypothesize that maternal hyperglycemia leading to congenital heart are linked to abnormal DNA methylation and mRNA expression at cardiac specific loci.

Methods: Hyperglycemia was induced in normal 8-week old CD-1 female mice with a one-time intraperitoneal injection of 150 mg/kg of streptozotocin (STZ) 2 weeks prior to mating. Histological analysis of fetal cardiac morphology was evaluated for malformations on embryonic day (E) 16.5 of control pups and pups exposed to maternal hyperglycemia. We used a massively-parallel sequencing-based methylation sensitive restriction based assay to examine genome-wide cytosine methylation levels at >1.65 million loci in neonatal hearts on post-natal (P) day 0. Functional validation was performed with real time quantitative polymerase chain reaction (RT-qPCR).

Results: Cardiac structural defects occurred in 28% of the pups (n=12/45) of hyperglycemic dams versus 7% (n=4/61) of controls. Notable phenotypes were hypoplastic left or right ventricle, double outlet right ventricle, ventricular septal defect, and left ventricular outflow tract obstruction. A 10-fold increase in DNA methylation of gene promoter regions was seen in many cardiac important genes in the experimental versus control P0 neonates and have corresponding decreases in gene expression in 21/32 genes functionally validated.

Conclusion: Maternal hyperglycemia alters DNA methylation and mRNA expression of some cardiac genes during heart development. Quantitative, genome-wide assessment of cytosine methylation can be used as a discovery platform to gain insight into the mechanisms of hyperglycemia-induced cardiac anomalies.

Keywords: Cardiac Embryopathy; Congenital Heart Defects; DNA Methylation; Euglycemic and Hyperglycemic

Introduction

Women with diabetes at the time of conception are five times more likely to have infants with congenital heart defects (CHD) [1-3]. In the United States, three million pregnancies are affected with pre-gestational diabetes mellitus [4]. The obesity epidemic has led to the increase in Type 2 diabetes mellitus (T2DM) in the United States and consequently the increase in incidence of diabetes-related heart defects in the offspring [5,6]. Although CHD affects roughly one percent of the population, it disproportionately accounts for 25% mortality in infants [7]. CHD can result in both short and long-term mortality. Its survivors experience long term sequelae such as cardiac arrhythmia, valvular disease (predisposing patients to endocarditis), cognitive and developmental delay [7]. CHD also confer significant economic burden. The cost associated with an atrial septal defect to complex arterial switch procedures ranges from just under $4,000 (benign disease) to over $100,000 (cyanotic heart lesions) [7,8].

Diabetic embyopathy is directly and positively correlated to first trimester hemoglobin A1C during the time of embryogenesis and sharply rises with A1C % >12 corresponding to severe hyperglycemia regardless of Type 1 or Type 2 DM status [9]. Streptozotocin (STZ) is a chemical that selectively destroys the insulin producing beta-islet cells of the pancreas. We used the STZ induced diabetes mouse model because it is a well-established and highly effective way to induce severe maternal hyperglycemia in mice and other mammals. Severe hyperglycemia is the driving factor in the development of CHD [10].

Maternal diabetes is teratogenic to several organ systems particularly the cardiovascular and central nervous system [3]. Hyperglycemia alters gene expression at various stages of heart development including cardiac neural crest cell migration, outflow tract formation and inflow tract formation [11,12]. Despite these observations, a clear mechanism of gene expression alternations is still elusive. However, we hypothesized that aberrant DNA methylation in the setting of maternal diabetes alters gene expression is cardiac important genes.

Epigenetic modifications are reversible modifications regulate gene expression without altering the DNA sequence [13]. They play a significant role in terms of regulation of gene expression of cellular processes of differentiation and development [14,15]. DNA methylation occurs when a methyl group attaches to CG sites often located upstream from the promoter region and is generally associated with gene silencing [16]. DNA methylation has recently been proposed to be an important regulator of cardiac gene expression required for normal cardiac formation [17]. Conditional DNA methyl transferase 3-B (DMT3B) knock-out is associated with such phenotypes such as ventricular septal defects and endocardial cushion defects [17]. A link exists between maternal diabetes and changes in DNA methylation [18,19]. Furthermore, DNA methylation is important in the normal development of the heart. In case controlled human studies, CHD has been associated with abnormal DNA methylation [18,20,21]. Our objective was to examine the hypothesis that epigenetic modifications such as abnormal DNA methylation disrupt cardiac gene expression setting the stage for the phenotypes we observed in mice.

Materials and Methods

Animal Husbandry

Animals were housed in micro isolation colorless cages and given food and water ad libitum when not fasting for blood glucose measurements. Housing rooms were temperature controlled with 12 hours alternating light and dark cycle. All mouse experiments were performed according to the guidelines of the National Institute of Health and the protocol approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine.

Induction of Maternal Diabetes

Eight week old virgin female CD-1 (n=4) wild type mice bred in house were administered a single dose of 150mg/kg of streptozotocin (STZ) via intraperitoneal injection. Control females (n=6) were given an equal volume, intraperitoneal injection of Sodium Citrate ($NaCO_3$)-the buffer that the STZ was dissolved in. Both groups of animals were injected prior to mating. After fasting animals for 6 hours, basal glucose levels were measured in both groups using a One Touch commercial glucometer in weekly intervals for 2 weeks before mating and on the day of cesarean/sacrificing the dams. The glucose values (mg/dL) reported represent an average of the glucose values for each mouse. Hyperglycemia was defined as a fasting glucose value >200 mg/dL.

Timed Mating

At 10 weeks of age, both groups of females mated with normal male CD-1 mice for timed pregnancies. Noon on

the day of observing vaginal plugs was designated as embryonic day (E) 0.5. The day the female gave birth was designated as postnatal day (P) 0.

Histological Analysis

The pregnant dams were euthanized on E 16.5 and embryonic hearts were collected from hyperglycemic and euglycemic females, respectively. We performed histology on embryonic day E16.5. Gross morphological analysis was done at the time of dissection. The hearts were isolated from the fetal thorax, formalin fixed and paraffin embedded and processed for histology. We deparaffinized slides with xylene prior to staining with hematoxylin and eosin (H&E) and imaged using Olympus DXS-high-resolution microscope at 5x, 10x and 20-x magnification to study morphology. The morphology of each heart was determined adequate if the following structures were identified left ventricle, right ventricle, outflow tracts, mitral valve, tricuspid valves and interventricular septum. A single blinded examiner performed the interpretation of morphology and a second blinded examiner, a co-investigator, determined any uncertainty with diagnosis.

DNA Extraction

DNA was extracted from a litter of neonate hearts in both groups using the high molecular weight genomic DNA extraction protocol described by Khulan [22]. The protocol for DNA isolation and library preparation is available on the Einstein's Center for Epigenomics website (http://wasp.einstein.yu.edu/index.php/Protocol:DNA_extraction). Essentially, hearts were homogenized, incubated with proteinase K, and purified using phenol and chloroform extractions. The DNA was washed in diluted SSC buffer for 24 hours and concentrated with polyethylene glycol while in dialysis tubing. Concentration of DNA was determined with the Nano-drop spectrophotometer and stored at -20 degrees C.

Methyl Sensitive Tiny Fragment Enrichment/Massively Parallel Sequencing (MSFE/MPS)

Neonatal hearts were collected at P0 from offspring of hyperglycemic and euglycemic females. Genomic DNA was extracted using the high molecular weight genomic DNA extraction protocol described previously [22]. A total of 5 μg extracted DNA from P0 hearts was used for the MSFE/MPS assay as described in Suzuki et al. [23]. We modified the original assay by replacing HpaII with HPYCH4IV; the restriction enzyme recognizes 'ACGT' sites and is sensitive to methylation at CpG-sites [17]. Methylation sensitive restriction enzymes, such as HPYCH4IV, are affected by the presence of a methyl group at CpG loci within its recognition sequence, when a methyl group is present the restriction enzyme is not able to cut, conversely cutting occurs at sites where no methyl group is present. We chose to assay methylation at 'ACGT' sites, as it is within the core-binding site for the transcription factor Hif1-α, a key transcription factor for heart morphogenesis ad post-natal vascular morphogenesis. This modification allows us to study directly how DNA methylation may alter the HIF1-α regulatory network in the future.

After HPYCH4IV digestion, the sequencing libraries were generated using Ligation Mediated PCR (LM-PCR) [23,24]. To prevent contamination by single adapter fragments an adapter containing an EcoPI5I recognition site and a T7 promoter sequence was ligated to our fragments, this adapter also served as a measure of quality. We then ligated an Illumina sequencing adapter, reverse transcribed and amplified the ligated DNA fragments.

The generated libraries were submitted to the Epigenomics Shared Facility at the Albert Einstein College of Medicine for massively parallel sequencing using the Illumina Hi-Seq sequencer, (Illumina Inc., San Diego, CA). Sequencing was performed on individual libraries prepared from the hyperglycemic and euglycemic samples. The quality of the sequencing results was determined by a quality control (QC) software using the parameters of length and peak value of sequence reads developed at the Einstein Epigenetics Core.

Bioinformatics Analysis to Profile Genome-Wide DNA Methylation of ACGT Sites

The sequencing reads were aligned to the mouse genome (mm9) and the number of mapped reads with their 5' ends starting at each 'ACGT' site were recorded using the automated data analysis pipeline created by the Epigenomics Center and the Computational and Statistical Epigenomics Group at Albert Einstein College of Medicine [23]. The read counts are inversely proportionate to the percentage of methylation [16]. The read counts at individual 'ACGT' sites from hyperglycemic and euglycemic samples were compared and EdgeR determined sites with significantly different counts, a Bioconductor package designed for analysis of count based genome-wide sequencing data [25]. Differentially methylated loci were defined by >50% difference

between the experimental hearts and control hearts. Loci that had greater than three positions in the promoter regions that were differentially methylated were included in our ontological analysis. The resultant sites were associated to genes if they were located in promoters, gene bodies, or within 50 kb of genes. We focused our attention on gene promoters, as hypermethylation of gene promoters is generally associated with gene silencing [16].

Gene Ontology

Promoter regions (defined as <5000 bp from the transcription start site) were included in computational analysis. In order to compile a manageable list of candidate genes, loci that had three or more positions that were differentially methylated in the promoter region were included in the ontological analysis. Each of the 655 genes was input into GENEMANIA. org, an ontological website that gives information regarding gene function and the tissue in which a particular gene is expressed. The genes were then categorized based on tissue expressed and all cardiac genes (101 genes) were further categorized into the following classes: cardiac morphology, autonomics, cardiac function and vasculogenesis.

Gene Expression Analysis

Gene expression was analyzed by quantitative polymerase chain reaction (qPCR) for 32 selected gene candidates known to be previously published in cardiac function. Primers for candidate sites were designed between exons to provide an internal control. Reactions were run using SYBR green and submitted to the genomics core facility where samples were run on the Real Time qPCR (ABI 7900) 384 well instruments. ΔCt values were calculated by normalizing to an endogenous control (Gapdh), and relative expression change was calculated using the 2-ΔΔCt method [26].

Statistical Analysis

Diabetic model

Microsoft Excel and the data were presented as mean ± standard error (SE). Student's t-Test was used for comparison between euglycemic and hyperglycemic dams. A p-value < 0.05 was considered as significant. Pearson correlation was used to evaluate the incidence of cardiac defects in each litter with respect to the maternal blood glucose concentration.

Modified HELP- assay

Pearson correlation was used to evaluate the overall similarity of MSFE/MPS tag counts between groups. Two-sided t-test was used to evaluate the difference of tag counts at 'ACGT' sites located to different genomic contents, while the hypergeometric test was used to evaluate the enrichment of DM sites in promoters.

Gene expression

The statistical analysis of differential gene expression was performed using Bonferroni's correction was applied to account for multiple testing in gene expression analysis.

Results

The Diabetic Animal Model

The average litter size of the hyperglycemic and control dams were 12+/- 2.5 and 14 +/- 1.17; p=0.17 respectively. Maximum hyperglycemia was attained at 2 weeks after administration of streptotozocin and persisted throughout gestation up to the day of euthanizing the dams. Hyperglycemia was defined as >200mg/dL. The glucose concentration for pregnant dams was 135 +/- (18 mg/dL) in the control dams and 428+/- (146 mg/dL) in the hyperglycemic dams (p=0.0005).

Histological Analysis

We performed histological analysis on E16.5 which is one day after cardiac morphogenesis is expected to be complete [27]. Maternal hyperglycemia was associated with fetal cardiac defects as demonstrated in **(Figure 1-A).** Cardiac structure defects occurred in 28% of the pups (n=12/45) of hyperglycemic dams versus only 7% of the control pups (n=4/61; p=0.003). The distribution of phenotypes noted were hypoplastic left or right ventricle (7/12), double outlet right ventricle (2/12), and ventricular septal defect (1/12), endocardial cushion defect 1/12), and left ventricular outflow tract obstruction (1/12) **(Figure 1-B).** Moreover, the incidence of cardiac defects among individual litters increase in relationship to the level of hyperglycemia with the greatest incidence of 50% in a litter with the maternal blood glucose concentration of 565 mg/dL as shown in **Figure 1-C**. There was a heterogeneous distribution of cardiac phenotypes irrespective of maternal glucose levels as shown in **Table 1.**

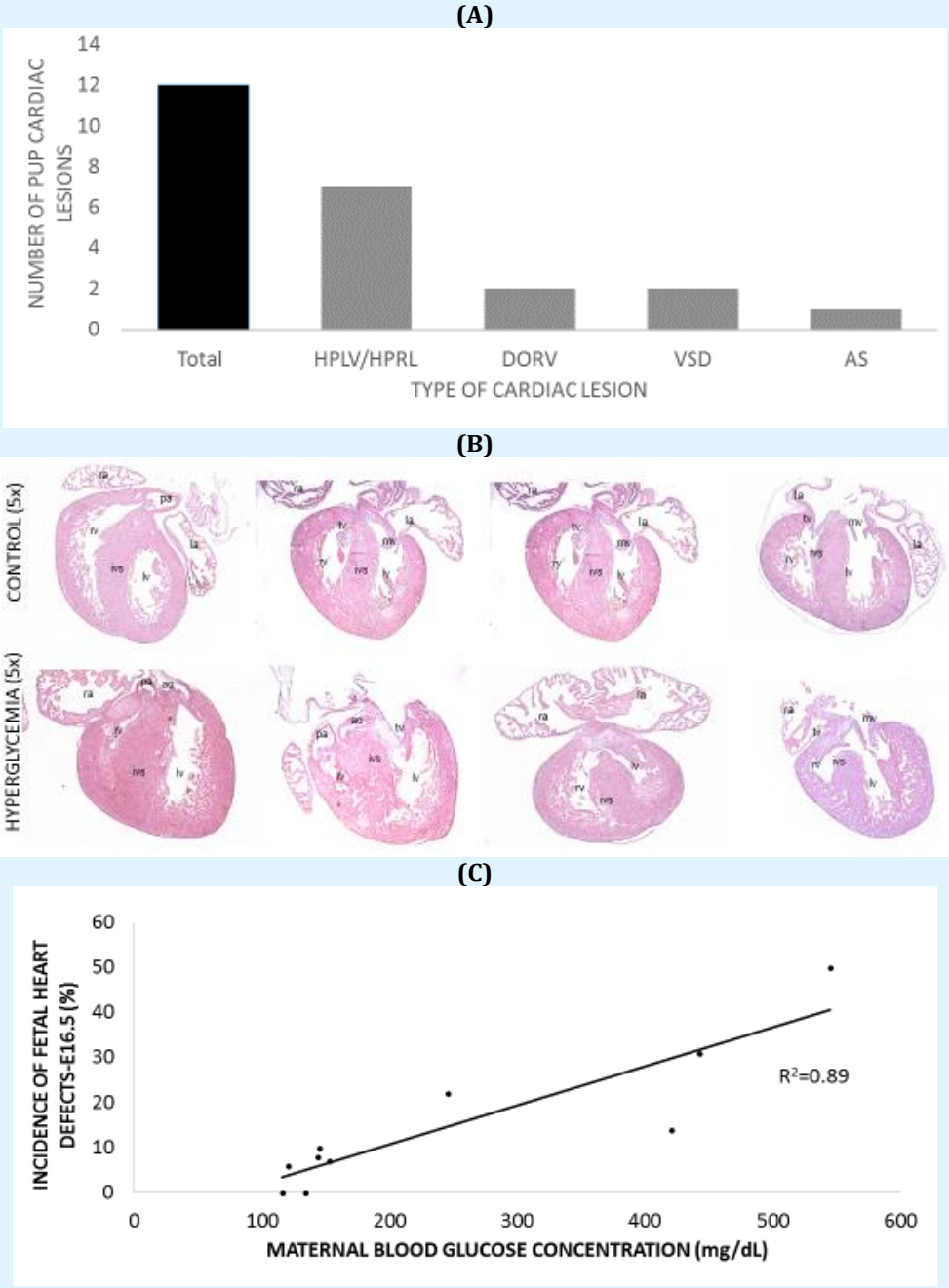


Figure 1: Mouse Diabetic Model Of Cardiac Embryopathy**.**

A. The distribution of cardiac defects in the pups exposed to hyperglycemia at E16.5: On the X-axis is the specific heart lesion. On the Y-axis is the number of fetal heart defects. HPLV/RV 7/12, DORV 2/12, VSD 2/12, AS 1/12.**B.** Abnormal histology examples from hyperglycemia exposed fetal pup hearts. (From left to right) Hypoplastic right heart, double outlet right ventricle, ventricular septal defect, endocardial cushion (Hematoxylin & Eosin staining). Abbreviations: Pulmonary artery(Pa), aorta(ao), aortic valve(av), mitral valve(mv), pulmonary valve (pv), right ventricle(rv), left ventricle (lv). **C**. There is a positive correlation between maternal blood glucose concentration (mg/dL) in the X-axis and the incidence of fetal cardiac defects in their respective litter in the Y-axis.

Maternal blood glucose concentration (mg/dL)	Euglycemia or Hyperglycemia	Number of pups in each litter	Type AND NUMBER of congenital heart defect (CHD)/litter
116	Euglycemia	6	None
121	Euglycemia	16	DORV
134	Euglycemia	3	None
144	Euglycemia	13	VSD
145	Euglycemia	10	Aortic valve duplication
153	Euglycemia	14	DORV
246	Hyperglycemia	9	DORV, HPRV
421	Hyperglycemia	14	HPRV, HPLV
443	Hyperglycemia	14	HPRV (2), DORV, AV Canal defect
545	Hyperglycemia	10	LVOT stenosis, VSD (2), HPRV

Table 1: The type of cardiac defect in the pups of exposed to maternal hyperglycemia were different from one another within the same litter regardless of the degree of hyperglycemia.

Genome wide methylation

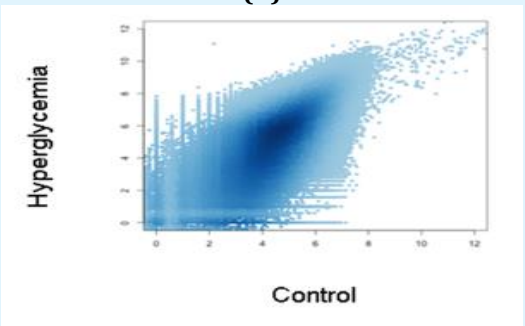
Increased levels of DNA methylation were documented at the promoter sites of the hearts exposed to hyperglycemia. Loci with differential methylation >10 fold increase in methylation are shown in the scatter plot in **Figure 2-A.** The number of reads inversely correlates with the degree of methylation. The number of reads at each differentially methylated loci was averaged and compared between control and diabetic exposed hearts respectively. In each of the differentially methylated gene promoters, a decreased number of tag counts (i.e reads) in the hyperglycemic exposed hearts corresponding to increased methylation as compared to the euglycemic exposed hearts. For these gene promoters, a higher degree of methylation is observed in the hyperglycemia exposed hearts shown in **Figure 2-B.** Embryonic hearts from pregnant dams with STZ-induced hyperglycemia have measurable alterations in DNA methylation associated with genes critical for cardiac development.

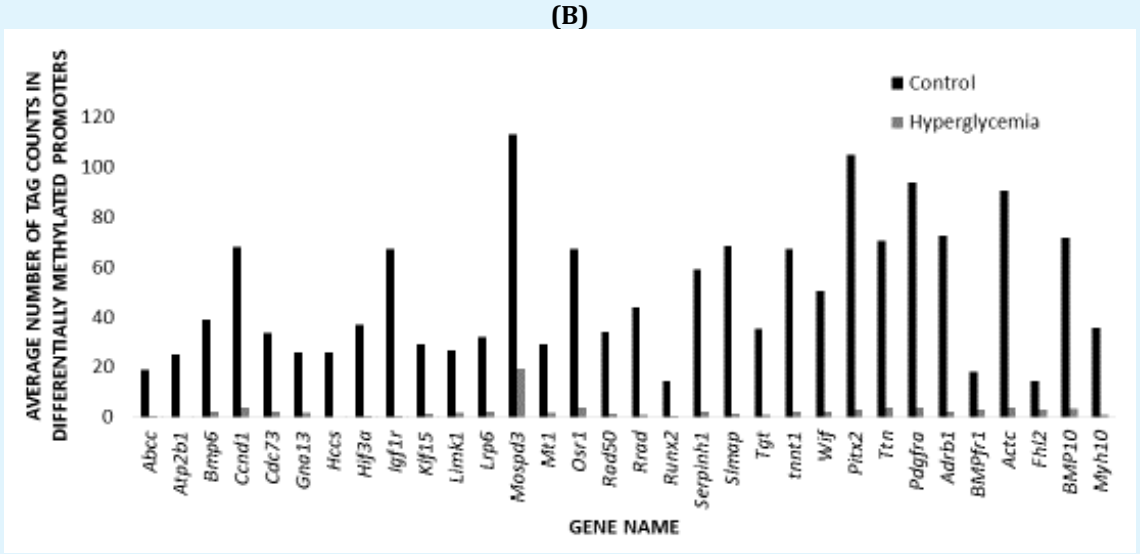
Functional Validation (Expression data)/Ontology

Of the genes whose promoters were hypermethylated, there was a correlative change in mRNA expression of 22/32 of the genes. Among the 32 genes that were tested, 21/32 demonstrated decreased mRNA expression. These genes were *Abcc1, Atp2b1, Bmp6, Cdc 73, Gna13, Hccs, Hif3a, Igf1r, Klf15, Limk1, Lrp6, Mospd3, Mt1, Osr1, Rad50, Rrad, Runx2, Serpinh1, Slmap, Tgt, tnnt1, Wif1, Pitx2, Pdgfra, Adrb1, Fhl2, Bmp10, Myh10* shown in **Figure 2-C**. One of the genes tested, Actin alpha cardiac muscle 1 (*actc 1*), showed an increase in mRNA expression. Six-hundred seventy eight genes had three or more positions that were differentially methylated and were included in the ontological analysis**.** Of the 678 genes analyzed, 101 or 19% were noted to be related to cardiac development or function **(Figure 3-A,B).**

The remainder of the genes were primarily notable in cell cycle (47%), immunologic system (7%), nervous system (11%), other (10%), and no published function (7%). Based on PubMed data mining and GENEmania.org, we categorized the functions of the 101 genes into morphology (43 genes), cardiac function (34 genes), angiogenesis (20 genes), and autonomic function (3 genes), **(Figure 3-A, B).**

(A)





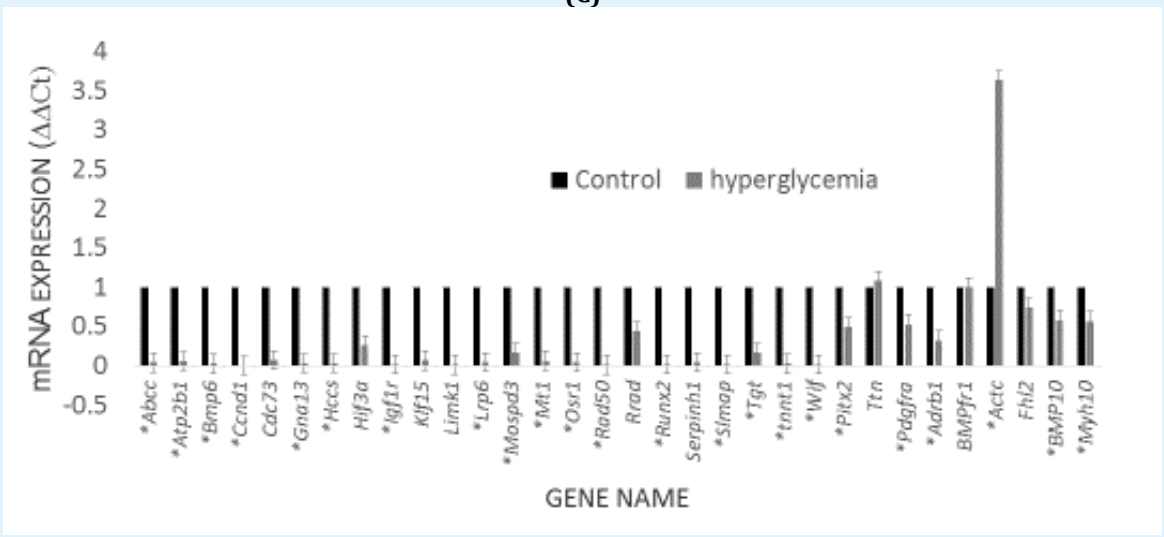


Figure 2: Genome Wide Methylation Assay And Corresponding Gene Expression.

A. Using a threshold of >50% differential methylation, comparison of tag counts for hyperglycemic hearts at Post-natal day 0 (n=3) (Y-Axis, NE tag count) and euglycemic hearts (X-Axis, Ctrl Tag Count) reveals, while sites with similar levels of methylation exist (dark blue) the majority of sites experience changes in DNA methylation. A 10-fold increase in methylation was detected between hyperglycemic and control hearts. **B.** The number of reads inversely correlates with the percent of methylation. For these genes, a higher level of methylation was observed in the promoter regions of hyperglycemia exposed hearts. **C.** *There was a correlative decrease in gene expression in 21/32 selected cardiac genes that corresponded with hypermethylation in promoter regions of hyperglycemia exposed hearts versus controls. *P<0.05.*

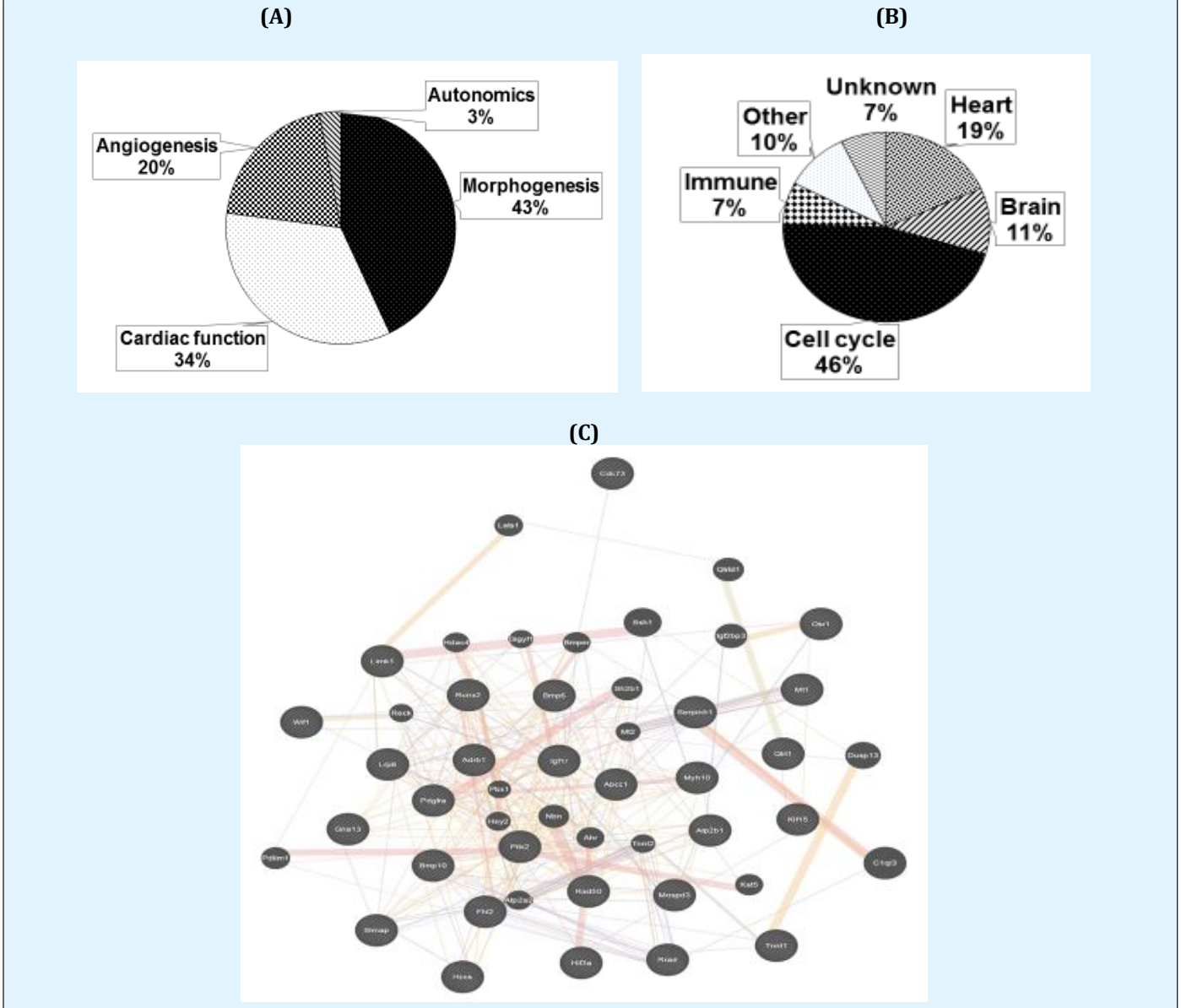


Figure 3: Ontological Analysis.

A. Of the 655 genes analyzed with GENE mania, 46% were related to cell cycle and 19% were related to cardiac development or function. The other genes are related to brain development or neurulation (11%), other (10%) immune function (7%) or unknown (7%). **B.** When analyzing the 101 cardiac genes, we found that 43 were identified to be related to cardiac morphogenesis, 34 were related to cardiac function, 20 were related to angiogenesis or vascular function and 3 were related to autonomics. **C.** The 21/32 genes that were differentially expressed were input into gene mania to identify interactions between the different genes in the pathway analysis. The gene mania report describes the various other co-factors that regulate the cardiac genes that were hypermethylated in the promoters and corresponding decreased mRNA expression.

Discussion

In summary, we conclude that maternal hyperglycemia increases DNA methylation in several cardiac gene promoters and corresponds to differential expression. Our model of streptozotocin-induced diabetes is a sufficient model that yielded a significant increase in cardiac defects in the offspring. The incidence of cardiac defects was proportional to the degree of hyperglycemia of the mother. Interestingly, the incidence of cardiac defects in hyperglycemia exposed pups was four times that of euglycemia exposed hearts which is consistant with epidemiologic literature [9].

We believe that this finding speaks to one of the potential associations in diabetes associated cardiac heart defects. Cardiac dysmorphology is a complex interplay of maternal environment, genetic predisposition and gene regulation. Looking at the gene regulatory mechanisms in the setting of maternal hyperglycemia via a genome wide approach may be used as a discovery platform to identify differentially methylated genes as well as to identify novel genes, not previously known to be implicated in the normal cardiac development. For example, Bmp 10 is important in the morphology of the right ventricle and aberrant expression of this gene can lead to hypoplastic right ventricular syndrome [28]. Myh10 has been previously published to be implicated with outflow tract formation and body wall formation. Abnormal or decreased expression of this gene produces such phenotypes as we have seen such as double outlet right ventricle [29]. PitX2 is important in endocardial cushion formation and the knockout of this gene has produced phenotypes such as atrial septal defects [30].

The clinical significance of our findings are related to the fact that the incidence of CHD rises sharply with first trimester A1C >12 corresponding to mean glucose values of 300 mg/dL [31]. Up to 20% of individuals with diabetes do not know they have the disease [32]. In addition, one half of all pregnancies are unintended or unplanned [33]. This combination of phenomena is potentially a set-up for uncontrolled diabetes during the critical period of embryonic development. Thus, when we embark upon studying the role of hyperglycemia and the development of congenital heart defects, we are intentionally selecting out a subset of very poorly controlled diabetics during organogenesis. Using a rodent model allows us to see the natural course of severe untreated hyperglycemia and its role in creating perturbations in the gene regulatory networks that ultimately are linked with congenital heart defects. Although the upper limits of hyperglycemia approach >500 mg/dL, even at lower levels of hyperglycemia, we identified increased incidence of abnormal phenotypes. We have confirmed that maternal diabetes is associated with a high incidence of cardiac defects. With the described model, we set forth a goal to shed light on the underlying mechanism of the observed phenotype.

Gene Name	Gene Function	Publication(s)related to cardiovascular function	Reference
Abcc	Multidrug resistance. Defense against toxins	Cardiac remodeling after myocardial infarction	[34,35]
Atp2b1	ATPase Ca++ transporter	Vasoconstriction and hypertension	[36]
BMP6	Mesoderm morphogenesis	Developing in endocardial cushion	[37]
Ccnd1	Cell cycle maintenance	Adult cardiomyocytes reentrance into cell cycle	[38]
Cdc73	Regulation of mRNA	Heart morphogenesis	[39]
Gna13	G-protein receptor signal	Vasculogenesis	[40]
Hccs	Activation of respiratory cytochromes	Mitochondrial respiration	[41]
Hif3α	Hypoxia dependent Transcription regulation	Angiogenesis/outflow tract development	[42]
Igf1r	Cellular response to insulin stimulus	Cardiac hypertrophy	[43]
Kif15	Microtubule cytoskeleton organization	None reported	[44]
Limk1	Actin polymerization	Reduced sarcomere structure and contractility	[45]
Lrp6	Wnt signaling pathway	Outflow tracts and valve formation	[46]
Mospd3	Cardiac muscle tissue development	Right Ventricle morphogenesis	[47]
Mt1	Metal ion homeostasis	Pressure overload and myocardial dysfunction	[48]
Osr1	Connective tissue development	Atria and septal development	[49]
Rad50	DNA repair	Expressed in adult myocardium	[50]

Rrad	Cardiac muscle	Congestive heart failure	[51]
RunX2	Regulation of ossification	Interstitial cells in aortic valve stenosis	[51]
Serping1	Protein activation cascade	Reperfusion after ischemia of heart	[52]
Slmap	Lymphocyte proliferation	Excitation-contraction coupling	[53]
Tgtp2	T cell activation	Unknown	[54]
Tnnt1	Contractile fiber development	Ventricular muscle development	[55]
Wif1	Wnt inhibitory signaling	Cardiomyocyte differentiation	[56]
Pitx2	Embryonic organ morphogenesis	Cardiac outflow tract remodeling	[57]
Ttn1	Contractile fiber	Myocardial contractility	[58]
Pdgfra	Platelet growth factor	Left ventricular thickening and thrombus	[59]
Adrb 1	Adrenergic receptor signaling	Cardiac remodeling	[60]
BMPr1b	Limb morphogenesis	myocardial intracellular calcium regulation	[61]
Actc 1	Contractile fiber	Heart contraction	[62]
Fhl2	Development and maintenance of cardiovascular and skeletal systems	Regulation of angiogenesis	[63]
Bmp10	Cardiac muscle tissue development	Cardiac contractility	[64]
Myh10	Voltage-gated channel activity	Heart development and body formation	[29]

Table 2: Using gene mania and PubMed data mining, we identified genes that are related to heart function. The overall function of the gene is displayed and that which is related to either cardiac development or function.

Our study has strengths and limitations. Firstly, we have created an animal model of streptozotocin induced pregestational maternal diabetes that produces significant increases in abnormal cardiac phenotypes. This aligns with the observed clinical literature that pregestational diabetes is associated with an increase of fetal cardiac defects in a dose responsive manner. We have a large cohort of fetal pup hearts that were evaluated in a blinded fashion reducing interpretation bias. Secondly, we have proposed a novel mechanism to shed light on the underlying mechanism for diabetes associated cardiac embryopathy. Utilizing a methylation sensitive restriction enzyme digestion coupled with genome wide cytosine methylation profiling serves as a non-biased approach to interrogate the sites that are differentially methylated between our control and experimental groups and although we started looking at the promoter regions that were differentially methylated, this technology can give a wealth of information about methylation of the gene body and intergenic regions as well. We focused our attention on genes that have been previously published to be implicated in cardiac development or function, however, this non-biased approach will enable us to potentially identify genes that had not previously been known to be implicated in cardiac development and thus may serve as a discovery platform for future studies and applications.

We acknowledge that extracted DNA and RNA from pooled whole hearts from a litter limits our ability to characterize differences in methylation and corresponding mRNA expression. Changes in methylation and gene expression may be tissue specific and homogenizing whole hearts does not allow us to interrogate the changes of methylation that may vary from one tissue type to another within the same organ. Furthermore, if there are individual differences in DNA methylation from one heart to another within the same litter, we would not be able to distinguish this with our design of using a pooled litter of hearts in each group. Nonetheless, collecting a pooled sample allows us to see the global shift of methylation patterns of pup hearts exposed to maternal hyperglycemia versus control. With the heterogeneity of cardiac phenotypes even in the same litter, it may be postulated that the differences in methylation would be more pronounced in hearts with severe defects versus normal or less severe defects. Therefore, pooling the hearts enabled us to see an average of the methylation profile encompassing all hearts whether normal or abnormal in that litter.

Our validation included functional validation by quantifying mRNA expression of selected gene candidates. Moreover, mRNA expression was expectantly decreased in the majority of gene whose gene promoters were hypermethylated correlating with gene silencing. However, we were not successful in technically validating DNA methylation at the exact position of loci differentially methylated from our genome wide dataset. This may have been due to regions in the genomic DNA that were unassayable in our primer design or difficulty amplifying targeted regions of bisulphite converted DNA.

We acknowledge that our wide range of hyperglycemia especially at extreme levels of >400 mg/dL does not represent the majority of human patients with Type 1 diabetes. If they are in the care of a physician, they will be treated with insulin or oral hypoglycemic medications for patients with Type 2DM.

Although the presence of hyperglycemia is associated with cardiac defects the underlying reason for the heterogeneous nature of the defects is still a question. For example, the types of heart defects range from ventricle septal defects to severe lesions such as double outlet right ventricle and hypoplastic left and right heart syndrome. It is not well understood why littermates exposed to the same hyperglycemic environment experience different types of cardiac lesions ranging from normal to complex cardiac lesions. It is also not known whether DNA methylation differs from one littermate to another. It is a question as to what biological components ultimately determine whether a congenital heart will develop or not. Future studies will link the specific lesion with DNA methylation and mRNA expression to give further insight to this question.

Conclusion

Maternal hyperglycemia is a known entity that is associated with congenital heart defects in her progeny. To date although we have long observed this association, the mechanisms that underlie these cardiac defects are elusive. We feel that we have opened the conversation on the possible pathogenesis of diabetes associated cardiac defects and have employed a novel method in profiling the entire genome using it as a discovery platform in which to identify differential methylation in our experimental and control groups. Genome-wide assessment of cytosine methylation may be used as a discovery platform to gain insight into the mechanisms of diabetes associated cardiac embryopathy.

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