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microRNA expression profiling and functional annotation analysis of their targets modulated by oxidative stress during embryonic heart development in diabetic mice

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ABSTRACT

Maternal pregestational diabetes mellitus (PGDM) induces congenital heart defects (CHDs). The molecular mechanism underlying PGDM-induced CHDs is unknown. microRNAs (miRNAs), small non-coding RNAs, repress gene expression at the posttranscriptional level and play important roles in heart development. We performed a global miRNA profiling study to assist in revealing potential miRNAs modulated by PGDM and possible developmental pathways regulated by miRNAs during heart development. A total of 149 mapped miRNAs in the developing heart were significantly altered by PGDM. Bioinformatics analysis showed that the majority of the 2111 potential miRNA target genes were associated with cardiac development-related pathways including STAT3 and IGF-1 and transcription factors (Cited2, Zeb2, Mef2c, Smad4 and Ets1). Overexpression of the antioxidant enzyme, superoxide dismutase 1, reversed PGDM-altered miRNAs, suggesting that oxidative stress is responsible for dysregulation of miRNAs. Thus, our study provides the foundation for further investigation of a miRNA-dependent mechanism underlying PGDM-induced CHDs.

1. Introduction

Congenital heart defects (CHDs) are the most common structural birth defects, and the causes of CHDs are still largely unknown [20,40]. Pregestational maternal diabetes mellitus (PGDM) increases the risk of CHDs in the offspring five-fold, compared with the general population [32,44,56,59]. The major heart defects associated with PGDM are outflow tract (OFT) defects and ventricular septal defects (VSDs) [32,56]. PGDM-induced CHDs have become a significant public health concern because the number of women of reproductive age with diabetes is rising rapidly [10,26,44]. Previous studies have demonstrated that PGDM alters developmental pathways and gene expression essential for heart development [35,43,48,50,52], whereas the mechanism underlying PGDM-induced gene dysregulation is unclear.

MicroRNAs (miRNAs) are small, non-coding RNAs that negatively regulate gene expression at the post-transcription level [4]. By an imperfect sequence complementation, miRNAs recognize and bind to the 3′-untranslated regions (3′UTR) of target mRNAs, thereby inhibiting mRNA function through degradation, repression of translation, or both [27]. Approximately 50% of all mammalian protein-coding genes are post-transcriptionally controlled by miRNAs [24]. Currently, 2588 mature miRNAs have been described in humans, and 1915 mature miRNAs have been described in the mouse (miRbase release 21, June 2014, http://www.mirbase.org). Our recent studies have demonstrated that PGDM affects the expression of several miRNAs in the developing embryo [12,18,19], suggesting the importance of miRNA changes in PGDM-induced CHD formation.

miRNAs play important roles in many biological processes, including development, differentiation, proliferation and apoptosis [3]. Since the discovery of miRNAs, numerous studies have deter-

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mined that miRNAs are differentially expressed in many diseases such as cancer, stroke, Alzheimer's disease, diabetes, and nephropathy [2,29]. In addition, there is ample evidence that miRNAs are associated with cardiovascular diseases, including cardiac hypertrophy, myocardial infarction, cardiac fibrosis, arrhythmia, heart failure and vascular disease [38,42]. However, although the function of miRNAs in heart development has been previously described [31], only a few studies have attempted to elucidate the role of miRNAs in the pathogenesis of CHD formation. A comprehensive miRNA profile in embryonic hearts from diabetic pregnancies has not been previously performed.

PGDM exerts harmful effects on the developing embryo because uncontrolled hyperglycemia resulting from PGDM in early pregnancy (2–8 weeks' gestation) can significantly influence development of the primary vital organs, including the heart [16,49,62]. We have developed a murine type 1 diabetes mellitus model that can successfully and consistently recapitulate the negative effects of human maternal diabetes on embryonic development, and have previously determined that embryonic day (E) 12.5 in the mouse corresponds approximately to the critical period of cardiogenesis in humans [48,52]. Hence, in the present study, we investigated the miRNA expression profiles of E12.5 hearts of embryos from nondiabetic and diabetic dams. We identified a set of potential miRNAs which may be associated with PGDM-induced CHDs. In addition, we explored possible target genes of those miRNAs modulated by PGDM.

2. Materials and methods

2.1. Mice and reagents

Wild-type (WT) C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The superoxide dismutase 1 transgenic (SOD1-Tg) mice in a C57BL/6J background were revived from frozen embryos by the Jackson Laboratory (Stock No.002298). Streptozotocin (STZ) from Sigma (St. Louis, MO) was dissolved in sterile 0.1 M citrate buffer (pH4.5).

2.2. Mouse models of diabetic embryopathy

All procedures for animal use were approved by the Institutional Animal Care and Use Committee of University of Maryland School of Medicine. The mouse model of PGDM-induced embryopathy was previously described [48,50,52]. Briefly, ten-week old WT female mice were intravenously injected daily with 75 mg/kg STZ for two days to induce diabetes. Diabetes was defined as a 12-h fasting blood glucose level of $\geq$16.7 mM. Male (WT or SOD1-Tg) and WT female mice were paired at 3:00 P.M., and day 0.5 (E0.5) of pregnancy was established at noon of the day when a vaginal plug was present. Embryonic hearts were harvested for miRNA microarray analysis on E12.5. The embryonic hearts from one litter were pooled as one sample.

2.3. Total RNA preparation and quality control

Total RNA was extracted from embryonic hearts using the miRNeasy Mini Kit (Qiagen, Valencia, CA). The RNA integrity level was measured for each RNA sample using the Agilent Bioanalyzer 2100 in order to obtain a RIN value (RIN = RNA integrity number). All samples were found to have RIN values higher than 7, indicating RNA of suitable quality for microarray analysis.

2.4. miRNA array profiling

The miRNA array was conducted at Exiqon Services, Denmark. Total RNA (750 ng) from both sample and reference was labeled with Hy3™ and Hy5™ fluorescent label, respectively. The Hy3™-labeled samples and a Hy5™-labeled reference RNA sample were mixed pair-wise and hybridized to the mercury LNA™ microRNA Array 7th Gen (Exiqon, Denmark), which contains capture probes targeting all murine miRNAs in the miRBASE 19.0. The hybridization was performed according to the miRCURY LNA™ microRNA Array Instruction manual using a Tecan HS4800™ hybridization station (Tecan, Austria). After hybridization the microarray slides were scanned and stored in an ozone free environment (ozone level below 2.0 ppb) in order to prevent potential bleaching of the fluorescent dyes. The miRCURY LNA™ microRNA Array slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc., USA) and the image analysis was carried out using the ImaGene® 9 (miRCURY LNA™ microRNA Array Analysis Software, Exiqon, Denmark). The quantified signals were background corrected (Normexp with offset value 10) and normalized using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm.

2.5. miRNA expression profiling data analysis

Hierarchical clustering is used for grouping similar objects into "clusters". Hierarchical clustering produces a tree (also called a dendogram) that shows the hierarchy of the clusters. This allows for exploratory analysis to determine the proximity of the samples inside an experimental based on similarity of features (miRNA expression levels). Principal components analysis (PCA) is a procedure for identifying a small number of uncorrelated variables, called "principal components,from a large dataset. The aim of PCA is to explain the maximum amount of variance with the fewest number of principal components. The hierarchical clustering and PCA analysis based on the entire miRNA expression dataset were used for comparing their similarity within and between the two experimental groups (nondiabetic and diabetic). To identify PGDM-regulated miRNAs between the two groups, a random variance *t* test was used to improve estimates of miRNA-specific variances without assuming that all miRNAs have the same variance [45]. The criteria for inclusion of a miRNA in the PGDM-regulated miRNA list were set to $P < 0.05$. A hierarchical clustering analysis (heat map) was used for visualizing the normalized expression values of the PGDM-regulated miRNAs

2.6. miRNA target gene prediction

A two-step bioinformatics approach was used to generate a high-confidence candidate miRNA target list. First, to increase the accuracy of in-silico miRNA target prediction, we generated a computationally based candidate miRNA target list by taking advantage of the following five in-silico computational miRNA target prediction tools: targetScan [1], picTar [23], RNA22 [34], PITA [22], and miRanda [6]. For each miRNA, one mRNA was considered its high-confidence predicted target gene if this regulation was predicted by at least two out of the above five prediction tools. Second, we identified miRNA-mRNA regulation relationships from public literature collected by the Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com/) software.

2.7. Functional annotation analysis of the predicted targets

The high-confidence miRNA target gene list was further annotated with the Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com/) platform. We queried IPA with this list aiming to map and generate putative biological processes/functions, networks and pathways based on the manually curated knowledge database of molecular interactions extracted from the public literature. Molecular and cellular functional analysis, physiological