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Maternal hyperglycemia and fetal cardiac development: clinical impact and underlying mechanisms

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Abstract

Congenital heart disease (CHD) is the most common type of birth defect and is both a significant pediatric and adult health problem, in light of a growing population of survivors. The etiology of CHD has been considered to be multifactorial with genetic and environmental factors playing important roles. The combination of advances in cardiac developmental biology, which have resulted in the elucidation of molecular pathways regulating normal cardiac morphogenesis, and genome sequencing technology have allowed the discovery of numerous genetic contributors of CHD ranging from chromosomal abnormalities to single gene variants. On the other hand, mechanistic details of the contribution of environmental factors to CHD remain unknown. Maternal diabetes mellitus (matDM) is a well-established and increasingly prevalent environmental risk factor for CHD, but the underlying etiologic mechanisms by which pre-gestational matDM increases the vulnerability of embryos to cardiac malformations remains largely elusive. Here, we will briefly discuss the multifactorial etiology of CHD with a focus on the epidemiologic link between matDM and CHD. We will describe the animal models used to study the underlying mechanisms between matDM and CHD and review the numerous cellular and molecular pathways affected by maternal hyperglycemia in the developing heart. Lastly, we discuss how this increased understanding may open the door for the development of novel prevention strategies to reduce the incidence of CHD in this high-risk population.

Keywords

Maternal diabetes; hyperglycemia; gene-environment interaction; cardiovascular development; congenital heart defects

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Conflict of Interest Statement

The authors have declared that no conflict of interest exists.

Introduction

Congenital heart disease (CHD) is the most common type of birth defect with an incidence of ~1% and an estimated prevalence of ~2% when including bicuspid aortic valve (Hoffman & Kaplan, 2002). CHD remains the leading non-infectious cause of death in infants even with the tremendous improvements in medical and surgical therapies. These advances have resulted in an increasing number of adult survivors of CHD. Unfortunately, a significant subset suffer long-term cardiovascular morbidities and shortened life expectancies. Accordingly, it remains critical to define the etiologic origins of CHD to devise novel preventative therapies and better understand the contributors to adult-onset complications.

Simply defined, CHD is a congenital cardiac malformation that is the result of abnormal heart development. Cardiac development is a complex process that involves the interplay of several cell lineages and cellular processes, all tightly controlled by their corresponding genetic programs (Schleich et al., 2013). This developmental process has been carefully documented in several animal models and involves a combination of hemodynamic forces and morphogenetic events. Many studies have demonstrated the importance of several cellular contributions from the first and second heart field (FHF, SHF) lineages, the migrating neural crest cells (NCC) and epicardial cells for proper cardiac morphogenesis (Figure 1). Briefly, in humans cardiogenesis starts from specification of cardiac progenitor cells (CPCs) at day 15 (corresponds to E7.5 in mice) where the anterior and pharyngeal mesoderm give rise to FHF and SHF cell populations that are organized in the shape of a crescent (Figure 1a). The progenitor cells in the FHF primarily contribute to the left ventricle (LV) and parts of the atria, while the SHF cell population forms the major portions of the right ventricle (RV), outflow tract (OFT) along with some contribution to the atria. (Kelly et al., 2014; Yashiro & Suzuki, 2016). Following specification, around the third week of gestation in the human (E8.0 in mice), these bilaterally symmetric heart primordial cells migrate to the midline and fuse to form a single linear heart tube composed of an inner endothelial lining surrounded by an outer myocardial cell layer that is separated by an extracellular matrix (ECM) (Figure 1b). The primitive tubular heart then undergoes rightward looping, to acquire left–right asymmetry at four weeks of gestation (E8.5–E9.0 in mice). The looped heart then begins to segment into the right and left atria and ventricles, while the future atrioventricular (AV) canal and proximal outflow tract (OFT) cushions expand. Upon endothelial-to-mesenchymal transition (EMT), mesenchymal cells start to proliferate and migrate to fill the AV and OFT cushions around five weeks of gestation (E9.5–E10.5) (Figure 1c). Subsequently, mesenchymal cells of NCC origin populate the truncal cushions of distal OFT, during 6th–7th weeks of human gestation, the common OFT (truncus arteriosus) is septated into the aorta and the pulmonary artery. In addition, the heart completes septation into four distinct chambers and the heart valves undergo remodeling (Figure 1d) (Buckingham et al., 2005; Garg, 2006; Epstein, 2010). The different CPCs contribute to distinct regions of the heart and are controlled by specific gene-regulatory network and signaling pathways, which determine their behavior (Xin et al., 2013; Gelb & Chung, 2014). In the past two decades, much knowledge has been gained about the molecular pathways that regulate each of these morphogenetic processes and supported the discovery of novel CHD associated variants in genes important for heart development.

Multifactorial etiology of congenital heart defects

With advancements in genetic technologies and the increased availability of genetic testing, numerous genetic contributors have been identified to be associated with CHD ranging from chromosomal abnormalities (e.g. Trisomy 21 and 22q11 deletion) to single gene defects in syndromic and non-syndromic CHDs (Pierpont et al., 2007; Richards & Garg, 2010; Andersen et al., 2014). The initial discoveries that identified CHD-causing genes were often performed by studying large families with autosomal dominant forms of syndromic and non-syndromic CHD or by screening candidate genes in affected populations. The identified disease genes primarily encoded for transcription factors (e.g. *GATA4*, *TBX5*, *NKX2.5*), ligands and receptors of cell signaling pathways (e.g. *JAG1*, *NOTCH1*, *NOTCH2*), structural proteins (e.g. *ACTC1*, *MYH6*, *MYH7*, *MYH11*), and laterality pathways (e.g. *NODAL*, *LEFTY*, *CITED2*). These CHD-causing genes along with many others have been studied using animal models, and further contributed to the refinement of the molecular pathways regulating different aspects of heart formation (Garg, 2006; Fahed et al., 2013). While this knowledge contributed to our understanding of the genetics of CHD, recent advances in genomic technologies have uncovered a surprising complex genetic architecture underlying isolated and apparently non-syndromic cases of CHD.

Recent large scale genomic sequencing initiatives in non-familial CHD led by the Pediatric Cardiac Genomics Consortium (PCGC) and others have identified an increased frequency of *de novo* damaging mutations in genes which are expressed in the developing heart. In particular, there was a preponderance of potentially pathologic sequence variants in genes encoding for histone modifying enzymes (e.g. *EP300*, *KAT6A*, *KDM6B*, *CHD7*, *KMT2D*, *WDR5*, *KDM5A*, *KDM5B*, *UBE2B*, *RNF20*, *USP44*) suggesting the regulation of chromatin through histone modifications plays a key role in human CHDs (Zaidi et al., 2013; Homsy et al., 2015). In 2016 and 2017, two important studies using large CHD populations provided important insights into the genetic architecture underlying CHD (Sifrim et al., 2016; Jin et al., 2017). The PCGC published genomic sequencing data from the exome sequencing of nearly 3000 affected children. The findings of this work supported earlier studies by them, which demonstrated an increased frequency (28%) of damaging *de novo* mutations in genes expressed in the developing heart in children with CHD and neurodevelopmental as well as extra-cardiac anomalies (Sifrim et al., 2016; Jin et al., 2017). Within the isolated CHD cases, only 3% had damaging *de novo* mutations. Work from the worldwide collaboration with the Deciphering Developmental Disorders study reported similar findings in that there was enrichment in *de novo* protein truncating variants in children with syndromic CHD. Interestingly, the investigators of this study also identified a significantly increased frequency of inherited protein-truncating variants in children with non-syndromic CHD. In combination, these large-scale studies demonstrate the importance of *de novo* genetic contributors especially in the case of CHDs associated with neurodevelopmental disorders. For isolated, non-syndromic CHD, it has been suggested that inherited variants in cardiac developmental genes are also contributing to disease, potentially in an oligogenic manner where other genetic contributors such as common variants, structural variation, or non-coding variation function together to cause disease.

While technological improvements have accelerated the discovery of genetic causes of CHD, epidemiological studies have indicated a significant role for environmental risk factors in the etiology of CHD (Nora, 1968; Hinton, 2013; Gelb & Chung, 2014). It is estimated that a significant fraction of CHD results due to the adverse effect of environmental factors on prenatal development (Gilbert-Barness, 2010; Lage et al., 2012). These non-inherited modifiable risk factors include: (a) maternal illnesses and disease conditions, including type 1 and 2 diabetes mellitus (DM), obesity and hypertension, (b) maternal infections including rubella and influenza, (c) exposure to toxic metals and chemicals such as lead, mercury, lithium, toluene, (d) exposure to ethanol, tobacco, folic acid antagonists and therapeutic/non-therapeutic drugs (Gilbert-Barness, 2010). Many of these teratogens are associated with a spectrum of CHD including laterality and looping defects, conotruncal malformations and septal defects (Jenkins et al., 2007). Accordingly, the large-scale genetic studies are consistent with a potential role for prenatal/environmental risk factors that interact with this genetic susceptibility to disrupt heart development in non-syndromic cases of CHD (Fung et al., 2013). While environmental risk factors have long been recognized, the mechanisms by which environmental factors disrupt molecular pathways during cardiac development to cause CHD are not well defined (Nora, 1968; Hinton, 2013).

Diabetes mellitus: a non-genetic risk factor for congenital heart disease

A classic example of a perturbed maternal environment that is strongly associated with CHD is pre-gestational type 1 and type 2 DM (Loffredo et al., 2001; Wren et al., 2003; Lisowski et al., 2010; Liu et al., 2013). The incidence of DM has been increasing over recent decades. It is estimated that 7.3 billion adults are affected with DM worldwide, and this will reach to 9.0 billion by 2040 (WHO, 2016). Among this, 129.4 million women aged 20–49 years are diagnosed with DM and the global prevalence of live births affected by matDM is approximately 20.9 million (Ogurtsova et al., 2017). The primary teratogen in all human diabetic pregnancies is hyperglycemia (Negrato et al., 2012). DM is broadly classified into the following three types: (i) pre-existing type 1, characterized by deficiency in insulin production, (ii) type 2, results from body's ineffective use of insulin and often associated with obesity and (iii) gestational diabetes (GDM), a transient condition that arises during pregnancy and is most commonly diagnosed in the second or third trimester. Increasing obesity rates indicate that the proportion of pregnancies complicated by both type 2 DM and GDM is likely to continue to rise (Marco et al., 2012). Diabetes mellitus with its hyperglycemic milieu before conception and during the first trimester is associated with diabetic embryopathy in the developing fetus, which affects the heart, great vessels and neural tube (Zhao & Reece, 2013). In contrast, GDM develops during the latter half of pregnancy and is associated with fetal macrosomia, cardiomyopathy and increased incidence of perinatal complications and mortality (Kc et al., 2015).

Association between maternal DM and CHD

Numerous epidemiological studies have demonstrated a strong correlation between matDM and significantly elevated risk of CHD in the offspring of affected mothers. From initial work decades ago performed in small populations of infants of diabetic mothers to recent studies using a population of nearly 2.3 million infants born in Canada between 2002–2010,

investigators have confirmed the association of type 1 and type 2 matDM with CHD (Rowland et al., 1973; Liu et al., 2013). In most cases, the risk was 4-5% vs. 1% in control offspring (Liu et al., 2013). In addition, there is a suggestion that exposure to the matDM environment increases the risk of certain CHD subtypes at different levels. The Baltimore-Washington Infant Study was a population based case-control study performed between 1981-1989 whose goal was to identify risk factors for CHD. It described an increased risk of laterality and cardiac looping defects (or heterotaxy), cardiac outflow tract anomalies, atrioventricular and membranous ventricular septal defects but no increased risk of left and right-sided obstructive defects, muscular ventricular septal defects, atrial septal defects and persistent ductus arteriosus (Ferencz et al., 1997). There was an increased risk of hypertrophic cardiomyopathy related to exposure to matDM during late gestation (Loffredo et al., 2001).

The above mentioned Canadian study with its large population size also investigated the link between type 1 and type 2 matDM with specific CHD subtypes. The investigators found that type 2 matDM was associated with the highest risk of heterotaxy and left ventricular outflow tract obstructive malformations, while type 1 matDM had the highest risk for conotruncal malformations and atrioventricular septal defects (Liu et al., 2013). Of note, type 1 and type 2 matDM raised the risk of other types of CHD examined (right ventricular outflow tract obstructive malformations and atrial and ventricular septal defects) albeit to lower levels. Other population-based studies have consistently indicated 3-5-fold higher prevalence of CHD among offspring when exposed to pre-existing matDM (Janssen et al., 1996; Eidem et al., 2010; Simeone et al., 2015; Øyen et al., 2016; Hoang et al., 2017).

Maternal hyperglycemia as the primary teratogen

The pathophysiology of matDM-mediated birth defects is complex as the abnormal metabolic disease state has pleiotropic effects. MatDM is characterized by abnormal homeostasis of carbohydrate metabolism, inadequate or complete absence of insulin secretion/function, derangements in metabolism of inositol, arachidonic acid and prostaglandins leading to an overall metabolic syndrome (Baker et al., 1990; American Diabetes Association, 2009; Zabihi & Loeken, 2010). Despite having multiple irregularities in the processing of other metabolic molecules, all forms of DM point to an increase in maternal glucose levels (hyperglycemia) as the primary teratogen. The underlying molecular mechanisms by which hyperglycemia exerts its teratogenic effects and how those contribute to birth defects in susceptible infants during development are actively being investigated.

Previous studies have shown that during embryogenesis, maternal hyperglycemia is the major energy substrate and is transported across the placenta by facilitated diffusion (primarily through GLUT1 receptor) to the fetus in an insulin-independent manner; therefore it is extremely critical to maintain glucose flux at the time of organogenesis (Hay, 2006; Zhang et al., 2015). Along these lines, it was recently demonstrated that even mild elevations in maternal serum glucose were associated with Tetralogy of Fallot in offspring from diabetic mothers. This was not observed in controls or in mothers carrying infants with transposition of the great arteries (Priest et al., 2015). This suggested that maternal glucose levels could serve as a risk for CHD and this was further supported by large retrospective

study of over 19,000 mother-child dyads where elevated glucose levels in the first trimester were associated with an increased risk of CHD in the offspring (Helle et al., 2018).

Rodent models to study mechanisms associated with matDM

Studies have demonstrated that exposing developing embryos to a hyperglycemic environment *in utero* is sufficient to cause cellular damage and recapitulate the cardiac anomalies seen in human diabetic pregnancies (Rees & Alcolado, 2005; Cefalu, 2006; Kiss et al., 2009). The animal models range from chemically induced, inbred mouse models, naturally occurring mutations, genetically engineered mouse models, global and tissue-specific knockouts and transgenic models (Rees & Alcolado, 2005; Neubauer & Kulkarni, 2006). Among chemically induced animal models, intraperitoneal administration of toxins such as, Streptozotocin (STZ) at multiple or single low and high doses, is the most commonly used to induce mild and severe diabetes in rats and mice to achieve total destruction of the pancreatic β -cells. This results in a phenotype resembling insulin-dependent type 1 DM such that hyperglycemia is present (Table 1). In parallel, animal models exist that do not need external intervention to induce DM. For example, the non-obese diabetic (NOD) mouse, which develops diabetes spontaneously, is another established model of type 1 DM and the embryos of diabetic NOD mice demonstrate a higher rate of neural tube and cardiac defects (Otani et al., 1991; Morishima et al., 1996; Rees & Alcolado, 2005; Anderson & Bluestone, 2005; Salbaum et al., 2015). Dietary changes could also result in DM-like phenotype. High-fat diet (HFD) induced murine type 2 DM model exhibited modest hyperglycemia, glucose intolerance, insulin resistance and hyperinsulinemia, all of which are characteristics of human type 2 diabetes (Winzell & Ahrén, 2004; Wu et al., 2015;). Similar to type 1 DM murine models, the HFD-induced type 2 matDM model has adverse effects on the embryonic heart and leads to CHD, including ventricular septal defects and persistent truncus arteriosus (Wu et al., 2016). Of note, a similar association with CHD was not seen in a different genetic background when exposed to HFD for a shorter period of time (Schulkey et al., 2015). Studies using STZ, HFD and high-fat high-sucrose diet induced rodent models have also been carried out to examine maternal pathologies during late onset of DM during pregnancy or GDM that may affect fetal outcomes (Pasek & Gannon, 2013; Pennington et al., 2017). Additionally, the influence of specific genetic background on the propensity to develop each types of diabetes is also being recognized in mouse models (Wolf et al., 1984; Leiter, 1989).

Molecular pathways dysregulated in the heart with exposure to matDM

The fetal response to maternal hyperglycemia is dependent on multiple factors including the severity and timing of exposure, other metabolic derangements and genetic susceptibility. These responses result in transcriptional and epigenetic changes during critical periods of heart development (Figure 2). The mechanisms linking impaired molecular signaling to heart defects in offspring of diabetic mothers are now being increasingly described and are discussed below:

1. Oxidative stress

The “oxidative stress hypothesis” posits that excessive stress is the key mechanism underlying the formation of diabetic embryopathy, which includes the phenotypes of growth retardation and cardiac and neural tube defects found in rodent embryos (Brownlee, 2001; Zabihi & Loeken, 2010). It appears that reactive oxygen species (ROS)-mediated oxidative stress is the initial responder to maternal hyperglycemia, which then regulates multiple molecular pathways required for cardiac development. The antioxidant capacity of the developing embryo is known to be limited, and ROS production is exacerbated as the expression and activities of major ROS scavenging enzymes including superoxide dismutase (*Sod1*) and glutathione peroxidase (*Gpx*) are decreased during matDM (el-Hage & Singh, 1990; Ishibashi et al., 1997; Sivan et al., 1997; Wentzel et al., 2008). CHD associated with matDM was shown to be completely rescued by reducing glutathione (GSH) and ROS levels by N-acetyl cysteine (NAC) treatment, and consistent with this, there was restoration of *Gata4*, *Gata5* and *Vegfa* expression in the fetal heart of matDM offspring (Moazzen et al., 2014). However, conflicting results exist that show only a partial rescue with NAC treatment and that NAC can increase apoptosis and induce ~10% septal defects in control embryos suggesting that alternative mechanisms disrupting cardiac developmental pathways may also be present (Moazzen et al., 2014; Basu et al., 2017). Increased production of mitochondrial ROS has also been shown to disrupt endogenous antioxidant activities, trigger caspase3/8-dependent apoptosis and reduce cell proliferation in embryonic hearts exposed to matDM (Sivan et al., 1997; Morgan et al., 2008; Wentzel et al., 2008; Li et al., 2011; Wang et al., 2015). The enrichment of apoptotic cells was found in the AV and OFT endocardial cushions and also in the myocardium that may contribute to the development of ventricular septal defect and persistent truncus arteriosus in response to matDM. Consistent with these hypotheses, rescue of cardiac phenotypes has been demonstrated by either overexpression of superoxide dismutase 1 transgene (SOD1-OE) *in vivo* and by administration of SOD1-mimetic, tempol *in vitro* (Wang et al., 2015).

2. Wnt signaling

Previous studies have demonstrated that Wnt signaling is crucial for embryogenesis and targeted deletion of key components of canonical and non-canonical Wnt-pathway results in CHD similar to those observed in human diabetic pregnancies (Schleifarth et al., 2007; Lin et al., 2007; Yu et al., 2012). The STZ-induced mouse model of matDM is shown to increase the expression of Wnt antagonists, *sFRP1* and *Dkk1* in embryonic hearts, along with downregulation of Dvl2 and Gsk3b phosphorylation levels, suggesting inactivation of canonical Wnt pathway (Wang et al., 2015). In contrast, the expression of non-canonical *Wnt5a*, which is predominantly localized to the cardiac OFT, was shown to be downregulated and phosphorylation of NFAT4 was increased in matDM-exposed embryos compared to normoglycemic control embryos. The effect of SOD1-OE, which abrogated oxidative stress in developing fetal heart and significantly rescued defects in ventricular septation and outflow tract development, was demonstrated to occur by specifically inhibiting the canonical Wnt pathway antagonists, *sFRP1* and *Dkk1*, and activating downstream targets such as *Islet1*, *Gja1* and *Versican* as well as by restoring matDM-suppressed Wnt5a-Ca²⁺/NFAT pathway components in the developing heart (de la Pompa et al., 1998; Schleifarth et al., 2007; Wang et al., 2015).

3. Nitric oxide and Notch signaling

Excess production of ROS is the hallmark of diabetes, but reactive nitrogen species has also been extensively shown to contribute to endothelial dysfunction and reduce endothelial nitric oxide synthase (eNOS) expression, a major source of nitric oxide (NO) production in endothelial cells. NO serves as an important cardiovascular signaling molecule and is regulated by multiple transcriptional, posttranscriptional, and posttranslational mechanisms (Tai et al., 2004; Matouk & Marsden, 2008; Krause et al., 2013). Hyperglycemia alters *eNOS* and *Vegf* gene expression in embryos from matDM mice and both eNOS and Vegf play roles in the regulation of cell growth and vasculogenesis (Srinivasan et al., 2004; Kumar et al., 2008). In contrast, we and others have postulated parallel mechanisms for eNOS downregulation in response to hyperglycemia. Specifically, we demonstrated relative chromatin inaccessibility at *eNOS* loci by ATAC-sequencing and others have shown decreased phosphorylation at Ser1177 to reduce NO bioavailability under hyperglycemic stress (Chen et al., 2007; Basu et al., 2017). Supplementation with exogenous NO donor rather than antioxidants (such as NAC) was able to restore *Jarid2* mediated *Notch1* suppression in murine embryonic AV cushion mesenchymal (AVM) cell line and rescued hyperglycemia-mediated senescence, impaired migration and tube formation of late endothelial progenitor cells, suggesting an oxidative stress independent teratogenicity of hyperglycemia (Chen et al., 2007; Basu et al., 2017). Previous studies have shown that global deletion of *eNOS* in mice results in CHD and *eNOS*^{-/-}; *Notch1*^{+/-} compound mutant mice exhibit a range of conotruncal and semilunar valve malformations, demonstrating the requirement of endothelial NO for proper heart development (Lee et al., 2000; Feng et al., 2002; Bosse et al., 2013). Apart from this genetic interaction between NO and Notch1, NO has been shown to regulate active Notch1 signaling in aortic valve cells and Notch alters NO production by activating a PI3 kinase/Akt pathway to phosphorylate eNOS as part of an autocrine loop necessary to drive EMT in the developing AV canal (Chang et al., 2011; Bosse et al., 2013). Recent findings in STZ-treated mouse and *Drosophila* matDM models revealed a “gene-environment interaction”, where haploinsufficiency of *Notch1* in developing embryos together with hyperglycemic exposure resulted in increased incidence of ventricular septal defects or developmental mortality. Evidence supported that this interaction was epigenetically regulated by a polycomb repressive complex 2 (PRC2) component, *Jarid2* (Basu et al., 2017). Further evidence suggesting a link between matDM and Notch signaling was shown, where mouse embryos exposed to the matDM environment exhibited defects in left-right axis formation via a reduced Notch1 signaling mechanism (Hachisuga et al., 2015).

4. Hif1α pathway

Global gene expression studies on E10.5 embryos exposed to matDM revealed increased expression 20 genes regulated by Hif1α, possibly reflecting embryonic response to the matDM environment is a consequence of increased oxidative stress and hypoxia (Pavlinkova et al., 2009). Haploinsufficiency of *Hif1α* in embryos from diabetic pregnancies demonstrated increased incidence of cardiovascular anomalies compared to *Hif1α*^{+/-} embryos from non-diabetic dams, but was not statistically significant when compared to wildtype control littermates exposed to matDM (Bohuslavova et al., 2013). In addition, there was significant elevation of *Nkx2.5*, *Tbx5*, *Nppa*, *Connexin 43* and *Mef2C* expression in

matDM-exposed *Hif1a*^{+/-} hearts when compared to either diabetic wildtype or non-diabetic *Hif1a*^{+/-} embryonic hearts (Bohuslavova et al., 2013). Another recent study demonstrated that *Hif1a*^{+/-} offspring from a diabetic pregnancy developed left ventricular dysfunction at 12 weeks of age, as manifested by decreased fractional shortening and structural remodeling of the myocardium (Cerychova et al., 2018). Transcriptional profiling by RNA-sequencing revealed ~53% of differentially expressed genes were direct or predicted targets of *Hif1a* and included the categories of heart development, response to stress, ECM organization, apoptosis, cell proliferation and angiogenesis (Cerychova et al., 2018). Taken together, these results demonstrate that *Hif1a* haploinsufficiency in mice increases the predisposition of offspring exposed to matDM to a higher risk of CHD.

5. Tgfβ pathway

Tgfβ is known to play a critical role in early stages of cardiac development and mice harboring null-alleles for components of the Tgfβ pathway are known to cause defects in the cardiac outflow tract and ventricular septum (Sanford et al., 1997; Compton et al., 2007). MatDM is shown to suppress Tgfβ signaling in the exposed embryonic hearts by downregulating the expression of its ligands (*Tgfβ1* and *Tgfβ3*) and phosphorylation levels of downstream effectors (TβRII, Smad2/3), which may contribute to the development of hyperglycemia-induced cardiac malformations (Wang et al., 2015). SOD1-OE and *in vitro* treatment with either tempol or recombinant TGFβ protein were able to restore HG-suppressed expression of Tgfβ target genes (*Snai2*, *Ctgf* and *Gdf1*), which have previously been implicated as essential regulators of cardiac cushion morphogenesis, cell adhesion, migration and proliferation and left-right patterning (Niessen et al., 2008; Zhao, 2010; Jun & Lau, 2011; Karkera et al., 2007; Wang et al., 2015).

6. Other cardiac molecular pathways

Hyperglycemia and associated oxidative stress have been shown to impact an array of transcriptional and signaling pathways and there are varying levels of evidence supporting these potential roles. For example, deletion of apoptosis signal-regulating kinase 1 (*Ask1*), an oxidative stress-responsive gene, have been demonstrated to prevent ventricular septal and outflow tract defects by diminishing diabetes-induced JNK1/2 phosphorylation and significantly restoring the levels of endoplasmic reticulum (ER) stress markers in embryonic hearts from matDM dams compared to non-diabetic controls (Saitoh et al., 1998; Wang et al., 2015). STZ treated *Ask1*^{-/-} mice were shown to rescue the expression of cardiac transcription factors, *Bmp4*, *Nkx2.5*, and *Gata5* and Smad1/5/8 phosphorylation along with deficits in cellular proliferation (Wang et al., 2015). Previous findings suggested a negative feedback loop between *Ask1* activation and *Hif1a*, essential for proper cardiac looping and modulation of neural crest cell migration and survival (Kotch et al., 1999; Compennolle et al., 2003; Zhou et al., 2004; Yang et al., 2013). Using experimental animal models, hyperglycemia is sufficient to cause dysmorphogenesis of embryos in glucose-injected dams or in *ex vivo* culture affecting endocardial cushion and cardiac neural crest cells (Wentzel et al., 2001; Kumar et al., 2007). Downregulation of *Bmp4*, *Msx1* and *Pax3* gene expression in diabetic embryonic heart is shown to impair EMT during cardiogenesis, affect myocardial proliferation, disorganize the conotruncal structure and affect development and/or migration of cardiac NCCs leading to atrioventricular septation and cardiac outflow tract defects

(Conway et al., 1997; Phelan et al., 1997; Jiao et al., 2003; Stottmann et al., 2004; Ishii et al., 2005; Kumar et al., 2007; Morgan et al., 2008). Global gene-expression studies on different developmental stages of the murine heart revealed that dysregulated genes and their molecular functional classification mainly include transcription factors (e.g. *Hif1a*, *Klf9*, *Cited4*), chromatin/DNA binding (e.g. *Atrx*, *Hmga1*, *Setdb1*), signal transduction components (e.g. *Mapk10*, *Arid4b*, *Pik3c2a*), cell surface receptors (e.g. *Efnb2*, *Pdgfra*, *Sema3a*, *Tgfbr1*), extracellular matrix/adhesion molecules (e.g. *Adam10*, *Twsg1*), cytoskeletal assembly factors (e.g. *Dcx*, *Vcl*, *Tubb2b/2c*) and genes regulating RNA-binding, transport, metabolism, metal-ion homeostasis and cell cycle/apoptosis (Pavlinkova et al., 2009; Vijaya et al., 2013). However, available single cell transcriptomic analysis studies performed on early and late embryonic hearts is yet to decipher the cell-lineage contribution of these genes and how they are sensitized during diabetic embryopathy (DeLaughter et al., 2016; Mohammed et al., 2017).

7. Pentose phosphate pathway

Glucose serves as a major energy source during early developmental stages and critical regulator of self-renewal and differentiation of progenitor cells, however little is known about how metabolic changes regulate the cardiac differentiation program (Harris et al., 2013; Oburoglu et al., 2014; Gaspar et al., 2014). Recently, Nakano et al. (2017) have demonstrated that hyperglycemia boosts the pentose phosphate pathway, an anabolic reaction to metabolize glucose. This not only induces cardiomyocyte proliferation but also inhibits cardiomyocyte maturation in both *in vitro* human embryonic stem cell-derived cardiomyocytes (hESC-CMs) and *in vivo* murine diabetic model. hESC-CMs when exposed with high (25mM) glucose were shown to reduce the expression of cardiac markers, *TNNT2*, *NKX2.5* and mitochondrial marker, *PPARGC1A* (Nakano et al., 2017). Gene expression analysis using RNA-seq revealed that genes related to cardiac muscle and function are enriched in hESC-CMs exposed to normal glucose (5.5mM), and genes associated with mitosis and cell cycle are enriched in the hyperglycemic (25mM) group (Nakano et al., 2017). Another finding revealed hyperglycemia inhibited ESC differentiation to cardiomyocytes by suppressing potassium channels *HCN1* and *KCN1* essential for cardiomyocyte contraction and by reducing the maturation markers, *TNNT2* and *MEF2C* (Yang et al., 2016). Therefore, using embryonic stem cells can provide an excellent approach to examine the adverse effects of hyperglycemic dose on cardiac lineage specification during their differentiation into cardiomyocytes, smooth muscle cells and endothelial cells and lead to a better understanding of the mechanisms associated with CHD in matDM.

Cardiac hypertrophy and hyperglycemia during second half of pregnancy

Cardiac hypertrophy is the most common heart abnormality found with matDM reportedly affecting 50% of infants of mothers with type 1 DM and 25% of type 2 DM in one study (Ullmo et al., 2007). However, it is the only cardiac abnormality found with GDM even though the incidence is low. Routine echocardiographic analyses on infants of diabetic mothers have shown prevalence of hypertrophy, with increased left ventricular mass and contractility, left ventricular outflow tract obstruction with apposition of anterior leaflet of the mitral valve to interventricular septum during systole. The cardiac output is significantly

reduced directly related to disproportionate septal enlargement (Walther et al., 1985). This cardiomyopathy phenotype can be mild in nature or can lead to much severe congestive heart failure (Reller & Kaplan, 1988). In an experimental GDM model, where glucose was infused directly into the left uterine artery late in gestation, there was significantly higher septal width and increased myocardial proliferation in fetuses exposed to HG compared to untreated right-side control littermates suggesting that even transient hyperglycemic exposure induces septal overgrowth/hypertrophy (Gordon et al., 2015). The underlying mechanism for cardiac hypertrophy is not related to a disruption of cardiac developmental pathways but is proposed to be related to fetal hyperinsulinemia and increased expression of insulin receptors which leads to proliferation and hypertrophy of cardiomyocytes (Breitwieser et al., 1980; Buchanan & Kitzmiller, 1994; Hornberger, 2006). Activation of growth/stress signaling kinases including PI3-Akt and MAP-kinases JNK, ERK, and p38 are proposed to cause diabetes induced cardiac overgrowth (Bueno et al., 2000; Chang et al., 2010; Shen et al., 2011; Chung & Leinwand, 2014; Gordon et al., 2015). While the clinical presentation is varied, the cardiac hypertrophy in the infants of diabetic mothers is reported to be benign in the majority of infants and spontaneously resolves within the first year of life (Way et al., 1979; Narchi & Kulaylat, 2000).

Concluding remarks

In summary, the mechanistic etiologies behind the variability in matDM-associated CHD phenotypes are manifold. Current evidence points to (i) ROS-mediated direct and indirect effects on cardiac morphogenesis that affects cell proliferation, apoptosis, migration and differentiation pathways, (ii) alterations of cardiac signaling pathways including Wnt, Notch, Tgf β , Hif1 α , at the transcriptional level in response to maternal hyperglycemia and associated oxidative stress and (iii) potential changes in the epigenetic landscape with maternal diabetic milieu that in turn affects expression of cardiac developmental genes in a spatiotemporal manner (Figure 2). With the utilization of newer high throughput genomic technologies, we can tease apart the cellular and molecular changes in the developing heart to hyperglycemic conditions during pregnancy at a single cell level and further mechanistically define these perturbations.

Successful elucidation of the mechanisms by which maternal hyperglycemia disrupt cardiac developmental pathways may open the door to investigate gene-environment interactions. Why a subset of infants is susceptible to CHD with exposure to an environmental risk factor, such as matDM, is a multifaceted question, but may involve the genetic variations that increase sensitivity to environmental conditions (Nora, 1968; Jenkins et al., 2007). This hypothesis may begin to explain the etiology of a significant fraction of CHD that still remains unexplained even with genome sequencing. Additionally, there is speculation that maternal vascular complications in hyperglycemic milieu alters maternal-placental as well as fetoplacental blood-flow which may trigger the molecular responses leading to CHD (Salafia et al., 2010). Placental dysfunction can also lead to inflammation and oxidative stress to impede signaling cascades that affect different aspects of embryonic development including heart development (Maslen, 2018; Courtney et al., 2018). Lastly, other modifiable risk factors like maternal age and exercise have complex effects on physiology and metabolism and therefore could also play a functional role in mitigating the risk of CHD in offspring.

carrying a pathogenic sequence variant (Ramírez-Vélez et al., 2009; Schulkey et al., 2015; Jay et al., 2016).

Ultimately, the hope is to translate these findings to humans by screening mothers with environmental risk factors and their children for genetic variants in cardiac regulatory genes. We predict that these genetic variants will serve as risk factors for the development of CHD in high-risk populations, such as those with matDM. These studies may allow for targeted approaches in high-risk populations to reduce the incidence of CHD. While beyond the scope of this review, it also raises the possibility that exposure to these environmental risk factors during cardiac development may result in epigenetic alterations in cardiac genes that predispose to the development of adult cardiovascular disease in CHD survivors.

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Figure 1: Stages of cardiac morphogenesis
Summary of the key stages of heart development in human and mouse. (a) Specification of cardiac progenitor cells (CPCs) into first (red) and second (blue) heart field (FHF/SHF) cells form a crescent like structure; day 15 of gestation (E7.5). (b) CPCs migrate to midline and fuse to form linear heart tube; day 21 (E8.0). (c) Heart tube undergoes rightward looping along the anterior–posterior axis, which segments the heart into atria, ventricles, atrioventricular (AV) and outflow tract (OFT) endocardial cushions. Following looping, AV and OFT cushions are populated by mesenchymal cells after endothelial to mesenchymal transition and cardiac neural crest cells (green) migrate via the pharyngeal arches arteries (III, IV, VI) to OFT; day 28 (E10.5). (d) Mature four chambered heart forms as a result of cushion remodeling and valve formation, complete septation of OFT resulting in pulmonary and systemic circulations; at birth. RA= right atria, LA= left atria, RV= right ventricle, LV= left ventricle, Ao= aorta, Pu= pulmonary. AVC= atrioventricular cushion, IVS= interventricular septum.

Figure 2: Fetal molecular responses to maternal hyperglycemia

Fetal molecular responses with type 1 and 2 matDM are mediated by hyperglycemia, the major teratogen. The underlying mechanism is complex and multifactorial in nature and includes: (a) increase in reactive oxygen species (ROS) induced oxidative stress during embryogenesis that primarily induces DNA damage, protein/lipid oxidation and has multiple indirect effects on cell apoptosis, proliferation, differentiation and inflammation via protein kinase C (PKC) mediated changes in expression of vascular endothelial growth factor (VEGF), nuclear factor-kappa beta (NF-κB), transforming growth factor-beta (TGFβ), endothelial nitric oxide synthase (eNOS). In addition to ROS, reduced nitric oxide (NO) bioavailability leads to endothelial dysfunction. This is observed due to several parallel or overlapping mechanisms such as increased ROS activity, eNOS uncoupling and/or reduced chromatin accessibility at eNOS locus in hyperglycemic milieu; (b) changes in gene expression of antioxidant enzymes (e.g. *Sod1, Gpx*), cardiac developmental genes/pathways (e.g. *Wnt, Notch1, Tgfb, Hif1a*) and chromatin modifiers (e.g. *Jarid2, Setdb1, Smyd1*) and (c) epigenetic modifications include active and repressive marks (e.g. histone/DNA methylation, acetylation, microRNAs, non-coding RNA and post-translational modifications) that result in transcriptional changes of target genes and lead to maternal hyperglycemia associated CHD.

Table 1:

Dysregulated genes in maternal hyperglycemia associated congenital heart malformations

Rodent strain of matDM	Altered fetal cardiac gene expression/Genome-wide expression changes (NCBI GEO accession #)	Reference(s)
(A) Streptozotocin (STZ)-induced diabetes		
Swiss Albino mice	<i>Bmp4, Msx1, Pax3</i>	Kumar et al., 2007
	Affymetrix Mouse Genome 430 2.0 microarrays (GSE32078)	Vijaya et al., 2013 Lin et al., 2018
FVB mice	<i>Pax3</i>	Morgan et al., 2008
	<i>Hif1a, Nkx2.5, Tbx5, Mef2C, α-SMA Cx43, Nppa</i>	Bohuslavova et al., 2013
	Affymetrix Mouse 430 2.0 arrays (GSE9675)	Pavlinkova et al., 2009
	SOLiD SAGE mRNA deep sequencing (PRJNA275285)	Zhao et al., 2016
	RNA-seq on diabetes exposed <i>Hif1a</i> ^{+/-} offspring (GSE109633)	Cerychova et al., 2018
C57BL/6J mice	<i>Notch1, Hey2, Bmp10, EfnB2, Nrg1 Jarid2, eNOS</i>	Basu et al., 2017
	<i>sFRP1, Dkk1, β-catenin, Islet1, Gja1, Versican Wnt5a, NFAT2/4, Mrtf-b, Tpm1, Rcan1</i>	Wang et al., 2015
	<i>Ask1, TRADD, Caspase8, Foxo3a</i>	Yang et al, 2013
	<i>Gata4, Gata5, Vegfa</i>	Moazzen et al., 2014
	<i>TGFβ1, TGFβ3, TβRII, Smad2/3, Snai2, CTGF, GDF1</i>	Wang et al., 2015
	<i>Nppa, Nppb, Myh2, Myh3, Atp2a2, Kcnip2, Ucp2/3, Slc2a4, EglN3, Tnfrsf12a</i>	Lehtoranta et al., 2013
Sprague-Dawley rats	<i>CuZnSOD, MnSOD, and Gpx-1</i>	Wentzel et al., 2008
(B) High fat diet (HFD) induced diabetes		
C57BL/6J	<i>CHOP, BiP, Calnexin, PDIA, GRP94</i> p-IRE1α, p-eIF2α, p-PERK, XBP1 cleaved Caspase3/8	Wu et al., 2016
(C) High glucose during late gestation		
Sprague-Dawley rats	<i>PI3-Akt, MAP-kinase, JNK, ERK, p38</i>	Gordon et al., 2015