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The role of glucose in physiological and pathological heart formation

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Abstract

Cells display distinct metabolic characteristics depending on its differentiation stage. The fuel type of the cells serves not only as a source of energy but also as a driver of differentiation. Glucose, the primary nutrient to the cells, is a critical regulator of rapidly growing embryos. This metabolic change is a consequence as well as a cause of changes in genetic program. Disturbance of fetal glucose metabolism such as diabetic pregnancy is associated with congenital heart disease. *In utero* hyperglycemia impacts the left-right axis establishment, migration of cardiac neural crest cells, conotruncal formation and mesenchymal formation of the cardiac cushion during early embryogenesis and causes cardiac hypertrophy in late fetal stages. In this review, we focus on the role of glucose in cardiogenesis and the molecular mechanisms underlying heart diseases associated with hyperglycemia.

Introduction

Although the study of heart formation has largely been focused on its genetic regulatory mechanisms, the impact of non-genetic factors on heart formation has been known for a long time. For example, mechanical force is an independent regulator of cardiogenesis (deAlmeida et al., 2007). Also, fetal exposure to the teratogens and pathogens causes malformation of organs. Among them, *in utero* hyperglycemia is associated with an increase in the risk of congenital heart disease (CHD) (Centers for Disease, 1990; Lisowski et al., 2010; Liu et al., 2013; Øyen et al., 2016; Simeone et al., 2015; Wren et al., 2003; Yogev &

Visser, 2009). Epidemiological studies have indicated a significant role for environmental risk factors in the etiology of CHD (Gilbert-Barness, 2010; Lage et al., 2012). More recently, two genomic sequencing studies revealed that a genetic diagnosis was made only in 10% of the patients with CHD. Damaging *de novo* mutations in cardiac genes were identified only in 28% of syndromic and 3% of non-syndromic CHD (Jin et al., 2017; Sifrim et al., 2016). These results clearly demonstrate that non-genetic contributors are no less important to cardiogenesis.

Among non-genetic factors influencing the fetal heart growth, environmental glucose is the most fundamental nutrient. In fact, maternal hyperglycemia is the most common medical condition associated with a 2 to 5-fold increase in CHD independent of genetic contributors (Centers for Disease, 1990; Simeone et al., 2015; Yogev & Visser, 2009). Despite the established association between maternal hyperglycemia and CHD, little is known about how glucose impacts cardiomyocyte development and how hyperglycemia affects heart formation in diabetic pregnancy (Gaspar et al., 2014).

In this review, we focus on the progress in the research on the role of glucose on cardiogenesis and the molecular mechanisms underlying CHDs associated with hyperglycemia. We begin with a discussion of the regulation of glucose transport between mother and fetus, glucose uptake by cardiomyocytes, and glucose metabolism in cardiomyocytes. Next, we introduce the process of normal cardiac morphogenesis with an emphasis on early cardiac cushion formation and late cardiomyocyte maturation, two major steps that are affected by diabetic pregnancy. The last part will highlight the current understanding of the molecular pathways underlying maternal hyperglycemia-induced heart diseases.

Glucose metabolism between maternal-fetal circulation and the heart

Placental glucose transport

Glucose is the primary fuel for rapidly growing embryos. It is the major source for both catalytic extraction of energy and anabolic synthesis of building blocks required for normal organogenesis. Glucose enters fetal circulation through placental membrane via facilitative glucose transporters (GLUTs) (Figure 1): fetal glucose concentration is lower than maternal glucose, which drives the net glucose transport toward the fetus (Lager & Powell, 2012). The human placenta expresses seven isoforms of the glucose transporter, GLUT1, 3, 4, 8, 9, 10 and 12 in a variety of locations. Their expression patterns change over the course of gestation (Illsley & Baumann, 2020). GLUT1 is the most abundantly expressed and is currently considered as the primary transporter across the placenta. GLUT1 is asymmetrically distributed between the maternal-side (microvillous-side) and fetal-side (basal-side) of the plasma membranes of trophoblast syncytium. The expression of GLUT1 in the basal plasma membrane is approximately 1/3 of that of the microvillous membrane (Barros et al., 1995; Jansson et al., 2002). Therefore, GLUT1 expression in the basal plasma membrane of the syncytiotrophoblast is a major determinant of the fetal glucose supply, and the current model of transsyncytial glucose transport across the placenta assumes that the basal plasma membrane is the rate-limiting site (Vardhana & Illsley, 2002).

Fetal blood glucose level is primarily determined by the maternal glucose concentration. In experimental models of streptozotocin (STZ)-induced pregestational diabetic pregnancy, the fetal plasma glucose level increases proportionally to the maternal glucose level (Pitkin & Van Orden, 1974; Singh et al., 1997). In human studies, pregestational diabetic pregnancy is associated with 40–100% increase in GLUT1 expression in the basal plasma membrane of syncytiotrophoblast and 40–59% increase in glucose uptake by the placenta. (Gaither et al., 1999; Jansson et al., 1999). However, the results are controversial in gestational diabetes mellitus (GDM); some studies report increase (Gaither et al., 1999; Stanirowski et al., 2017), while others found no difference (Jansson et al., 2001) in GLUT1 expression in the basal plasma membrane of syncytiotrophoblast isolated from the placenta of GDM. The functional contribution of other GLUTs in diabetic pregnancy is still largely unclear, but it is clear that GLUT1 on the basal plasma membrane of the syncytiotrophoblast is the major regulators of fetomaternal glucose transport (Gallo et al., 2017; Illsley & Baumann, 2020).

Intracellular glucose metabolism

Intracellular glucose is first phosphorylated by hexokinase to glucose-6-phosphate (G6P) and is metabolized via glycolysis to pyruvate (Figure 2) (Depre et al., 1999; Kolwicz & Tian, 2011). Pyruvate transported into mitochondria can enter the tricarboxylic acid (TCA) cycle and become carboxylated to generate large amounts of ATP through the process of oxidative phosphorylation (OXPHOS) (Lunt & Vander Heiden, 2011; Owen et al., 2002). While aerobic mitochondrial OXPHOS yields higher net ATP than anaerobic glycolysis, mitochondrial OXPHOS is accompanied with unavoidable by-products of reactive oxygen species (ROS) (Yaribeygi et al., 2019). Imbalance between ROS production and the antioxidative defense system leads to oxidative stress, a key pathogenesis associated with diabetes-induced embryopathy (Maritim et al., 2003; Rochette et al., 2014). In addition to these catabolic pathways, cellular building blocks are generated through the accessory pathways including the glycogen synthesis pathway, the hexosamine biosynthesis pathway, the pentose phosphate pathway (PPP), and the polyol pathway. Figure 1 provides an overview of the various metabolic fates of glucose. (1) G6P can enter intracellular glycogen storage. The glycogen is abundant in cardiomyocytes throughout fetal development occupying 30% of the cell volume in fetal/neonatal cardiomyocytes. It drops to about 2% of the cell volume in adult cardiomyocytes (Depre et al., 1999; Kim et al., 1992; Shao & Tian, 2015). The change in the cardiac glycogen pool is dynamic in response to physiological and pathological conditions (Shao & Tian, 2015). (2) Hexosamine biosynthesis pathway (HBP) produces uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), which serves as a donor for post-translational modification of proteins (Hart, 2019; Issad & Kuo, 2008) (3) In the PPP, the two most important products are nicotinic acid adenine dinucleotide phosphate (NADPH) and ribose-5-phosphate, which are important for redox reaction and nucleotide synthesis, respectively (Kolwicz & Tian, 2011). (4) In addition, excessive glucose is metabolized by aldose reductase through the polyol pathway and converted into sorbitol. This pathway can reduce glucose to polyol by NADPH. Since NADPH is a cofactor required to regenerate reduced glutathione (GSH), a major scavenger of ROS, an excessive flux of the polyol pathway can exacerbate oxidative stress (Giacco & et al., 2010).

High glucose as a teratogen

Diabetic pregnancy is often accompanied by maternal complications including vasculopathy, neuropathy, and insulin resistance, which potentially affect the fetal cardiac formation indirectly. These systemic complications are often subclinical, hindering the dissection of the pathomechanism of CHD in the fetus. However, high levels of glucose are considered to be primary teratogen during the diabetic pregnancy. In animal experiments, exposure to the hyperglycemia causes fetal anomaly typical of diabetic embryopathy even in the absence of maternal diabetes (Garnham et al., 1983; Hughes et al., 1974; Jin et al., 2019). Even a short-term exposure to hyperglycemia at the time of conception results in a higher rate of birth defects in the newborns (Rousseau-Ralliard et al., 2019). These data support the notion that high glucose itself is teratogenic. Increased oxidative stress is central to the teratogenic effect of glucose. High glucose influx also impacts cardiogenesis through its downstream intermediate metabolites, although the precise mechanisms remain elusive (Scholtens et al., 2014).

Glucose and cardiogenesis

A number of clinical studies demonstrated that maternal diabetes is associated with CHD of the fetus (Jin et al., 2017; Sifrim et al., 2016). Type 1 diabetes mellitus (DM) is caused by lower insulin production, while type 2 DM is more commonly characterized by insulin resistance. GDM occurs during the late gestation of pregnancy, and is typically transient and less severe than type 1 and type 2 DM. Although type 1 and type 2 diabetes are more strongly associated with structural defects including heterotaxia (left-right axis defect), and conotruncal defects (double outlets of the right ventricle, transposition of great arteries, persistent truncus arteriosus, single ventricle, etc.) (Depla et al., 2020; Ferencz et al., 1990; Liu et al., 2013; Loffredo et al., 2001; Øyen et al., 2016), all forms of maternal hyperglycemia are associated with the similar spectrum of fetal cardiac anomalies including heterotaxia conotruncal defects, septal defects, and fetal cardiac hypertrophy. In this section, we briefly summarize the morphogenic processes of heart formation as pertinent to the anomalies associated with maternal hyperglycemia.

Morphogenesis

Cardiogenesis is a sequence of morphogenic events that involves multiple cell lineages and cell types. Each step of differentiation and interaction of cellular components is governed by specific gene regulatory networks that together serve to the formation of a functional four-chambered heart (Meilhac & Buckingham, 2018; Moorman & Christoffels, 2003). During early heart development, cardiac progenitors originating from the anterior region of the primitive streak migrate toward the anterior lateral plate mesoderm and coalesce to form the cardiac crescent (Lescroart et al., 2014; Saga, 2000). The bilateral cardiac crescent fuse at the midline to form a linear heart tube, which then undergoes right-ward looping (Ivanovitch et al., 2017). The migration of cardiac progenitors continues even after the completion of looping. While early migrating progenitors, also known as first heart field progenitors, form the initial heart tube, the late migrators, or second heart field progenitors, add additional myocardium to the initial heart tube mainly through the anterior and posterior poles of the heart tube but also through dorsal mesocardium (Meilhac & Buckingham, 2018). The initial

heart tube is the bilayer of the myocardium on the outer layer and the endocardium lining the inner lumen. These layers are separated by an acellular matrix named cardiac jelly. The looped heart tube undergoes the ballooning process to form the cardiac chambers. The heart growth at this stage is achieved largely by proliferation of existing cardiomyocytes as well as by the addition of late migrating progenitors (Dyer & Kirby, 2009; Kelly & Buckingham, 2002).

During the looping and the following stages, cardiac septation and valve formation are initiated to separate the chambers and great arteries. These are critical steps that establish double circulation, both systemic and pulmonary in a unidirectional manner (Gittenberger-De Groot et al., 2014). The proper septation of cardiac chambers and outflow tract (OFT) requires three key components: 1) addition of second heart field progenitors to the arterial and venous poles, 2) transformation of endocardial cells into mesenchymal cells (endocardial-mesenchymal transition; EMT) in the atrioventricular canal (AVC) and OFT cushion (de Lange et al., 2004; Lincoln et al., 2004; Markwald et al., 1977; Markwald et al., 1975), and 3) migration of cardiac neural crest cells (CNCCs) to OFT (Yamagishi, 2020).

Addition of cardiac progenitor cells from SHF is critical for conotruncal heart formation and proper chamber septation (Briggs et al., 2012; Meilhac & Buckingham, 2018; Nakajima, 2010). The SHF contains two primary regions, an anterior (cranial) SHF contributes to the OFT myocardium and SMC (Verzi et al., 2005; Waldo et al., 2005) and the posterior (caudal) SHF contributes to dorsal mesenchymal protrusion that is critical for atrial septation (Snarr et al., 2007). Unsuccessful completion of this process will result in septal defects and malpositioning of the great vessels (Watanabe et al., 2010; Yelbuz et al., 2002).

Endocardial cells of the OFT and AVC segment are distinct from chamber endocardial cells in that they express receptors for EMT-inducing signals from the myocardium (Bernanke 1984, Mjaatvedt1987, Ma 2005 (Nakano et al., 2016). Likewise, the myocardium in the cushion region specifically expresses ligands critical for EMT induction (Ma et al., 2005; Mjaatvedt et al., 1987). Those signaling mechanisms include TGFβ/BMP (Ma et al., 2005; Mercado-Pimentel & Runyan, 2007; Nakajima et al., 2000), Notch (Hofmann et al., 2012; Timmerman et al., 2004), and Wnt (Liebner et al., 2004). The mesenchymal cells transformed from endocardial cells by EMT contributes to valve formation. While atrioventricular valves (mitral and tricuspid valves) are derived entirely from the endocardium, the semilunar valves (aortic and pulmonary valves) consist of cells not only from endocardium but also from the cardiac neural crest and epicardium. Perturbation of the EMT process may cause hypo- or hypercellularized cushions which subsequently could lead to a spectrum of malformations affecting the valves, septae and cardiac chambers (MacGrogan et al., 2014)

Cardiac neural crest cells (CNCCs) are critical cell population contributing to OFT septation from outside of first and second heart field (Kirby et al., 1983). CNCCs are initially specified at the border of the neural plate and migrate to the heart via the third/forth/sixth branchial arches (Miyagawa-Tomita et al., 2016; Schussler et al., 2020). Among several key transcription factors (TF), Pax3, a member of paired box TF, is essential not only for the induction of CNCCs but also for their emigration from the neural crest. The downstream

target genes includes SNAIL, FOXD3, SOX9 or TWIST (Monsoro-Burq, 2015). The migration of CNCCs toward the base of the OFT is partly controlled by SHF as inhibition of Notch signaling within SHF results in abnormal migration of CNCCs into OFT (High et al., 2009). Furthermore, CNCC ablation leads to OFT septation defect along with increased cells from SHF, suggesting that the coordination of these two cell populations is essential for septation.

The interplay among cardiac progenitor cells from SHF, CNCCs, and cushion endocardial cells at this stage is highly coordinated in a temporary and spatially manner and highly sensitive to hyperglycemia.

Cardiac maturation and glucose

During mid-late gestational stages and neonatal stages, cardiomyocytes undergo functional maturation in preparation for the high demand of oxygen and nutrient supply throughout life (Figure 3). In comparison to early cardiogenesis, the maturation process in the heart at cellular and tissue levels has not been studied as thoroughly until recently. Cardiomyocyte maturation occurs concomitantly with the postnatal withdrawal of the cell-cycle and involves several biological processes: sarcomere maturation, electrophysiological maturation, and metabolic maturation. Shortly after birth, cardiomyocytes undergo terminal differentiation and permanently exit from the cell cycle. After the terminal differentiation, cardiomyocytes grow in size but not in number. During the physiological and pathological hypertrophy, cardiomyocytes synthesize DNA without cytokinesis, resulting in mainly tetraploidic cardiomyocytes in humans and binucleic cardiomyocytes in rodents. The details of cardiomyocyte maturation have been reviewed elsewhere (Guo & Pu, 2020; Karbassi et al., 2020; Marchianò et al., 2019).

Cells display distinct metabolic characteristics depending on its differentiation stage (Carey et al., 2015; Tohyama et al., 2016; Wang et al., 2009). The fuel type of the cells serves not only as a source of energy but also as a critical regulator of self-renewal and differentiation of stem/progenitor cells (Oburoglu et al., 2014; Shiraki et al., 2014; Shyh-Chang et al., 2013). Cardiomyocytes shift their energy substrate during late embryonic and neonatal stages (Makinde et al., 1998). Glucose is the major energy source during the early developmental stages. The mitochondrial structure of the embryonic cardiomyocytes rapidly maturates between E10.5 and 14.5 in developing rodent hearts (Mackler et al., 1971). This coincides with the rapid increase in OXPHOS (Cox & Gunberg, 1972). This timing is significantly earlier than the neonatal stage when fatty acid oxidation becomes the predominant source of ATP production (Warshaw & Terry, 1970). This metabolic switch has been explained as an adaptation to the high energy demand of the maturing heart and as a result of the changes in the gene expression of metabolic enzymes and transporters. However, this metabolic change also serves as a driver of the cardiac differentiation program (Nakano et al., 2017).

Glucose is transported to the fetal cardiomyocytes by GLUT1 and GLUT4. GLUT1 is insulin-independent and constitutively active transporter that is dominant in embryonic cardiomyocytes and gradually is downregulated after E10.5. Towards the end of gestation, GLUT4 becomes a predominant transporter. GLUT4 is an insulin-dependent transporter that

translocates upon insulin stimulation (Abel, 2004; Shao & Tian, 2015). Therefore, the glucose transport capacity is significantly lower than GLUT1(Abel, 2004; Shao & Tian, 2015). In *in vitro* experiments using human embryonic/induced pluripotent stem cell derived cardiomyocytes (hESC/iPSC-CMs), glucose deprivation from the media induces the GLUT1 to GLUT4 isoform switch. This GLUT transition restricts the intra-cardiomyocyte glucose level, which further promotes the GLUT1 to GLUT4 switch (Nakano et al., 2017). Thus, this positive feedback mechanism likely facilitates the progressive maturation of cardiomyocytes at late embryonic-early neonatal stages (Figure 3). On the contrary, an increase in the glucose concentration in culture media upregulates GLUT1, further promoting the glucose uptake and inhibiting cardiomyocyte maturation. Disturbance of this feedback loop possibly contributes to the pathomechanism of how hyperglycemia inhibits cardiomyocyte maturation in diabetic pregnancy. GLUT3 is also expressed in the embryonic heart, but its function is not characterized well (Abel, 2004). Less is known as to how glucose restriction promotes cardiac maturation during late embryonic stages. In an *in vitro* study using human pluripotent stem cell-derived cardiomyocytes, restriction of glucose from the culture media induces cardiac maturation at genetic, structural, metabolic, electrophysiological and biomechanical levels (Nakano et al., 2017). Although the mitochondrial respiration has been implicated in the cardiac differentiation, inhibition of glycolysis has no effect on the maturity nor the OXPHOS activity. Rather, gain- and loss-of-function assays suggest that the effect of glucose deprivation was due to the restriction of the pentose phosphate pathway and nucleotide biosynthesis (Nakano et al., 2017).

Molecular mechanisms of fetal cardiac defects associated with maternal diabetes

It is well-recognized that maternal diabetes increases the risk of heart disease. Multiple molecular pathways critical in cardiac development have been shown to be dysregulated in the fetal heart exposed to hyperglycemia (Figure 4).

Oxidative stress

Oxygen is crucial for respiration, and the reduction of oxygen molecules leads reactive oxygen species (ROS) to take the form of free radicals (superoxide anion radical and the hydroxyl radical) or hydrogen peroxide. Cells in the developing embryo are in constant communication and molecular signals are essential for normal embryogenesis. Redox signaling plays important roles in chemical modulation of signaling pathways in development. Regulation of signaling pathways by redox control can take place via control of single enzymatic activity or at the transcriptional level (Basson, 2012; Sies et al., 2017). Imbalances of oxidation-reduction reactions favoring oxidation can disrupt cell fate and impair organogenesis. Hyperglycemia has been shown to induce excess ROS and diminish the level of intracellular antioxidant capacity (Brownlee, 2001; El-Bassiouni et al., 2005; Jain et al., 1989). The major source of ROS is the mitochondrial OXPHOS. In the hyperglycemic condition, the polyol pathway can also contribute to oxidative stress. Hexokinase becomes saturated in the presence of hyperglycemia and the excess glucose can be channeled to the polyol pathway, which oxidizes NADPH to NADP+ during the generation of sorbitol. When ROS are formed, antioxidant enzymes such as superoxide

dismutase (SOD), catalase, and glutathione peroxidase (GPx) are important in scavenging ROS and avoiding toxic effects (Laforgia et al., 2018). Oxidative stress activates a set of proapototic kinase signalings leading to abnormal cell death (Yang et al., 2015). Apoptosis signal-regulating kinase 1(Ask1), a mitogen-activated protein kinase kinase (MAPKK), responds to oxidative stress and induces apoptosis and cell cycle arrest in cardiac cushion via the activation of JNK1/2 phosphorylation in multiple fetal organs including the heart and neural tube in diabetic pregnancy (Wang, Wu, et al., 2015; Yang et al., 2013). Ask1 deletion restored the diabetes-induced fetal heart defects including VSD and PTA (Wang, Wu, et al., 2015). Oxidative stress also impairs the migration of CNCCs (see also Neural crest migration defect section).

It is not readily distinguishable whether oxidative stress is the cause or the consequence of diabetes induced CHD; therefore, whether cardiac anomalies are rescued by antioxidants is important to dissect the mechanism. Administration of antioxidants including Vitamin E given during diabetic pregnancy abolished the effect of hyperglycemia and significantly reduced anomalies of neural tube and cardiac outflow tract defects (Chang et al., 2003; Morgan, Lee, et al., 2008; Simán et al., 2000). In addition, administration of N-Acetylcysteine in drinking water from E0.5 to the end of gestation of diabetic pregnancy rescued cardiac defects and prevented alteration of the expression of Gata4, Gata5 and Vegf, key regulators of cardiac development (Moazzen et al., 2014). Consistent with these results, cardiac phenotypes were rescued when superoxide dismutase 1 (SOD1) was overexpressed in embryonic hearts exposed to maternal diabetes; suggesting that oxidative stress is responsible for heart defects under maternal diabetic conditions (Wang, Fisher, et al., 2015). These results may indicate that ROS can be the therapeutic target to prevent heart disease associated with diabetic pregnancy.

Nitric oxide

Nitric oxide (NO) is produced by NO synthase (NOS). NO interacts with reactive oxygen species (ROS) and generates reactive nitrogen species (RNS). Although an excessive amount of RNS reacts with a wide variety of molecules and causes cell damage, the physiological level of RNS is vital for many biological processes and protects against ROS-mediated pathogenesis of diabetes-induced CHD (Engineer et al., 2018). Endothelial NOS (eNOS/NOS3) is expressed in the fetal endothelium and multiple other cells including cardiac progenitors (Bloch et al., 1999). Knockout (KO) models demonstrate an important role of eNOS during cardiac chamber septation and valve formation. In diabetic pregnancy, NO level and eNOS/NOS3 mRNA are decreased in the fetal heart (Basu et al., 2017; Engineer et al., 2019; Kumar et al., 2008; Moazzen et al., 2015). eNOS KO postnatal mice result in congenital septal defects in accordance with increased cardiomyocyte apoptosis and higher mortality (Feng et al., 2002). Adult eNOS KO mice shows 5 out of 12 had aortic bicuspid valves compared to none of 26 controls (Lee et al., 2000). Several mechanisms may explain how high glucose causes a decrease in NO: chromatin accessibility of the eNOS locus is reduced in the fetal heart from diabetic pregnancy, suggesting that an alteration in the chromatin level is involved (Basu et al., 2017). The level of tetrahydrobiopterin (BH4), a co-factor for the eNOS enzyme, is also lower, possibly contributing to the eNOS uncoupling

(Engineer et al., 2018). Interestingly, supplementation of BH4 decreased the incidence of CHD in diabetic pregnancy possibly by restoring the eNOS coupling.

Notch signaling pathway

Notch signaling is involved in endocardial EMT. Although Notch1 is expressed broadly in endocardial cells, its activity is restricted to the cushion endocardium (de la Pompa et al., 1998). BMP, TGFβ, and Notch signaling all induce the expression of snail family protein, transcriptional repressors that repress VE-cadherin to facilitate endocardial delamination(Niessen et al., 2008; Romano & Runyan, 1999, 2000).

Mutations in *NOTCH1* are associated with cardiovascular defects including conotruncal malformations and semilunar valve anomalies (Freylikhman et al., 2014; Garg et al., 2005; Kelle et al., 2015; Kerstjens-Frederikse et al., 2016; Theis et al., 2015). Murine models revealed that the phenotype of Notch1 mutations are enhanced when the mothers are diabetic, suggesting a gene-environment interaction between Notch signaling and diabetic pregnancy (Basu et al., 2017). Several mechanisms are proposed as to how the diabetic environment enhances the severity of cardiovascular anomalies by Notch mutation. High glucose impedes Notch signaling in the node, thereby hindering the establishment of the left-right axis and causes heterotaxia (Hachisuga et al., 2015). In addition, Notch signaling crosstalks with NO signaling during cardiovascular development. Compound mutants for Notch1 and eNOS demonstrate conotruncal and valvular defects in synergistic manner, suggesting that a genetic interaction exists between these two signaling pathways (Bosse et al., 2013). Notch signaling and NO signaling form a positive feedback loop: Notch promotes NO production via activation of PI3k/Akt that phosphorylates eNOS (Chang et al., 2011), while NO from aortic valve endothelial cells prevents the calcification of valvular interstitial cells by upregulating Notch signaling (Bosse et al., 2013). In the diabetic pregnancy setting, diminished NO production is associated with increased expression of Jarid2, a component of the polycomb repressive complex 2 (PRC2), which epigenetically suppresses the expression of Notch1 (Basu et al., 2017).

Wnt signaling pathway

Wnt signaling regulates cushion cellularization. Overexpression of APC or Dickkopf1, endogenous Wnt inhibitors, inhibits cardiac cushion formation, while their truncated mutants display hyperplastic cushion (Dye & Lincoln, 2020; Gessert & Kühl, 2010; Hurlstone et al., 2003). TGFβ-mediated induction of EMT in the cardiac cushion is affected in the endocardial specific deletion of canonical Wnt signaling, suggesting the crosstalk of TGF and Wnt signaling in cushion formation (Dye & Lincoln, 2020; Liebner et al., 2004).

Both canonical and noncanonical Wnt signaling activities are suppressed in heart defects associated with maternal diabetes. Gene deletions of key Wnt signaling intermediates of both canonical and noncanonical pathways cause heart defects including cardiac outflow tract defects similar to those found in diabetic pregnancies (Hamblet et al., 2002; Yu et al., 2012). Although none of the canonical Wnt ligands are significantly decreased in the embryonic hearts exposed to the STZ-induced diabetic mouse, two Wnt antagonists, sFRP (secreted frizzled-related protein 1) and DKK1 (Dickkopf Wnt signaling pathway inhibitor

1), were upregulated (Wang, Fisher, et al., 2015). Consistent with this finding, maternal diabetes-exposed hearts showed decreased phosphorylation of Dvl2, a downstream effector, and increased activity of GSK3b, a negative regulator of canonical Wnt signaling (Wang, Fisher, et al., 2015). Furthermore, STZ-induced maternal diabetes causes upregulation of endogenous Wnt inhibitors including Wif1 (Wnt inhibitory factor 1), Aes (amino-terminal enhancer of split) and b-catenin destructor, Csnk2a1 (Casein kinase2, a1 polypeptide), in the embryonic hearts (Zhao, 2014), suggesting that canonical Wnt signaling is suppressed in maternal diabetic pregnancy. In addition, Wnt5a, a major noncanonical Wnt ligands in the heart, was significantly downregulated in the embryonic hearts from diabetic dams resulting in an increased level of phosphorylation of NFAT4, suggesting that suppression of noncanonical Wnt signaling is also involved (Wang, Fisher, et al., 2015). Thus, hyperglycemia causes cushion defects possibly via suppression of canonical and noncanonical Wnt signaling.

Hif1 Pathway

Compromised Hif1–1 signaling is implicated as a contributing factor of the teratogenicity of maternal diabetes. Hif-1 is a master regulator of cellular oxygen sensing; however, more recently, it has gained attention to a role in regulating glycolytic genes. Hif1 promotes the metabolic shift from OXPHOS to glycolysis in cancer biology (Ishida et al., 2020; Moldogazieva et al., 2020). Hif1a protein expression is found to be significantly upregulated in embryonic hearts exposed to maternal diabetes (Bohuslavova et al., 2013; Pavlinkova et al., 2009). In line with this, 20 out of 22 Hif1a-target genes are upregulated including enzymes critical in glucose metabolism such as Glut1, Hk2, and Ldha (Pavlinkova et al., 2009). Hif1a+/− embryos at E14.5 displayed increased incidence of cardiac anomalies when they are exposed to maternal hyperglycemia (Bohuslavova et al., 2013). The observed cardiac phenotypes include atrioventricular septal defects, hypoplastic left heart syndrome, and persistent truncus arteriosus as the most frequent types. They also showed that some of the cardiac genes were abnormally upregulated in Hif1a+/− embryonic hearts in diabetic pregnancy: Nkx2.5, Tbx5, Nppa, Cx43, and Mef2c. Another recent publication from the same group reported the long-term effect of the exposure to maternal hyperglycemia on the cardiac function in adult mice (Cerychova & Pavlinkova, 2018). Hif1a+/− offspring from a diabetic mother were normal shortly after birth. However, they developed left ventricular dysfunction after twelve-week-old, suggesting that Hif1 haploinsufficiency and *in utero* exposure to hyperglycemic environment predispose the offspring to cardiac dysfunction later in life. Supporting this idea, RNA-seq analysis of 12-week-old ventricular hearts revealed the differentially expressed genes prominently in the Hif1+/− heart from the diabetic pregnancy compared to all other groups (WT or Hif1+/− heart from WT dam and WT heart of diabetic mother). 53% of these genes are observed or predicted targets of Hif1 including Ldha, Cd36 and Vegfa (Cerychova & Pavlinkova, 2018). Altogether, Hif1 haploinsufficiency contribute to the fetal programming by diabetic pregnancy that has even longer effects on the heart function of offspring from a diabetic mother (Cerychova et al., 2018).

TGFβ signaling pathway

The Transforming growth factor beta (TGFβ) signaling pathway is known to play a critical role in early cardiac development that is suppressed in embryonic hearts from diabetic

pregnancy (Fein et al., 2002; Sanford et al., 1997; Wang, Reece, et al., 2015). Several key processes in cardiogenesis including chamber septation, valvular formation, and coronary vasculogenesis are initiated by EMT of endocardium and epicardium, and TGFβ signaling is key regulator of inducing EMT (Armstrong & Bischoff, 2004; Azhar et al., 2003; Boyer et al., 1999; Combs & Yutzey, 2009; Compton et al., 2007; Mercado-Pimentel & Runyan, 2007). TGFβ ligands (TGFβ1, TGFβ2, and TGFβ3) and main TGFβ receptor proteins (TβRI and TβRII) are expressed in different compartment of the developing heart: TGFβ1 is initially expressed in endocardium while TGFβ2 is expressed both in endocardium and myocardium of OFT and AV regions during cushion formation. TGFβ3 is expressed in the endocardium and mesenchymal cells after the onset of EMT. TGFβ1-null embryos by themselves show no phenotype; however, they exhibit a cardiac phenotype when they are born to TGFβ1-null mothers, suggesting an important role of maternal source of TGF β1 during development (Letterio et al., 1994). TGFβ2-knockout mice exhibit OFT malformation and valvular defects (Bartram et al., 2001; Sanford et al., 1997). *TGFβ3* mutation in human is associated with cardiomyopathy (Chakrabarti et al., 2020). In the context of diabetic pregnancy, two major works demonstrated that the TGF*β* signaling is suppressed in the embryonic hearts exposed to maternal hyperglycemia (Wang, Reece, et al., 2015; Zhao, 2010). Among 84 TGFβ signaling molecules analyzed by real-time RT-PCR between malformed E10.5 hearts exposed to STZ-induced maternal hyperglycemia and normal hearts from the non-diabetic mother, over 70 % of the differentially expressed genes (16 out of 22) were downregulated including TGF*β*3, all TGF*β* receptors and GDF1. In line with this, Wang et al. reported that TGFβ1 and TGFβ3 were downregulated in the embryonic mouse hearts at E12.5 from the STZ-induced diabetic mother as well as the level of phosphorylation of their downstream effectors, TβRII and pSmad2/3. TGFβ-responsive genes, Snai2, CTGF, GDF1, were also down regulated (Wang, Reece, et al., 2015). Interestingly, this group demonstrated overexpression of SOD1 restored the expression of TGFβ ligands in embryos exposed to a diabetic mother. In addition, SOD1 overexpression restored the level of phosphorylation of TβRII and Smad2/3 and the expression of the TGFβ-responsive genes. This indicates the crosstalk of oxidative stress and TGFβ signaling. However, the rescue of cardiac phenotypes by SOD1 overexpression in embryos has not been reported. One report demonstrated slightly increased TGFβ1 expression with no changes in TGFβ2 and 3 but with increased fibronectin in ex vivo mouse embryonic hearts at E9.5 exposed to high glucose (600 mg/dl) (Smoak, 2004). These confounding results of TGFβ ligands expression in the heart exposed to high glucose may be attributed to the experimental setting and the developmental stages.

Neural crest migration defect

Cardiac neural crest cells (CNCCs) are particularly essential for OFT development. Lineage tracing demonstrated that endocardial cells give rise to the majority of mesenchymal cells in AVC with minor contribution of epicardially-derived cells, while CNCCs contribute to the distal part of OFT cushion (de Lange et al., 2004; Jain et al., 2011; Jiang et al., 2002; Li et al., 2000; Verzi et al., 2005; Wessels et al., 2012). In diabetic embryopathy, cushion, septa and outflow structures are commonly affected resulting in persistent truncus arteriosus, pulmonary stenosis, double outlet of right ventricle, and tetralogy of Fallot (Corrigan et al., 2009; Ferencz et al., 1990; Neeb et al., 2013; Zhao, 2014). Despite the importance of CNCC

contribution to OFT formation, little is known about the molecular mechanism affecting CNCCs in diabetic embryopathy. Pregnant mice injected with STZ at E7.5 exhibited hyperglycemia and caused neural crest migration defects in the embryos. Pax3 was downregulated in the neural crest of embryos in diabetic pregnancy, suggesting that hyperglycemia affects migration of CNCCs (Morgan, Relaix, et al., 2008b). This phenotype is attributed to the increase in ROS because injection of Antimycin A, an inhibitor of mitochondrial electron transfer chain complex III, recapitulated the phenotype as a resultant of increased apoptosis mediated by p53 (Morgan, Relaix, et al., 2008a). In addition to Pax3, reduced expression of Msx1 and Bmp4 in the embryonic heart of diabetic model was reported. Both of genes are crucial genes for CNCC induction, chamber septation and valve formation, suggesting that downregulation of these genes may be associated with AV canal and OFT defects in diabetic pregnancy (Kumar et al., 2007).

Fetal cardiac hypertrophy

Maternal diabetes is associated with increased risk for not only structural anomalies including septal defects and transposition of great arteries, but also fetal cardiac hypertrophy. However, their mechanisms seem to be distinct. Recent meta-analysis of 39 clinical studies including 2,276 controls and 1,925 women with diabetic pregnancies showed impaired diastolic function in the fetal hearts from diabetic pregnancy (Depla et al., 2020). The incidence of fetal hypertrophy may depend on the cause of maternal diabetes. The fetal hearts were found to be hypertrophic in 44% of the case if the mothers have type 1 diabetes, 28% with type 2 diabetes, and a few % with GDM, although the maternal blood glucose level is often better controlled in GDM (Ullmo et al., 2007). Fetal cardiac hypertrophy is already present in the second trimester, but most profound in the third trimester (Atiq et al., 2017; Kulkarni et al., 2017). Characteristic to diabetes-induced fetal cardiomyopathy is disproportionate hypertrophy (thickening of interventricular septum), irrespective of pre-gestational DM or GDM. In addition, impaired cardiac function occurred even in well-controlled diabetes (Armstrong & Bischoff, 2004; Balli et al., 2014; Chu et al., 2012; Sanhal et al., 2017). In most cases, the hypertrophy appears to spontaneously regress within the first year of life (Deorari et al., 1989; El-Ganzoury et al., 2012; Narchi & Kulaylat, 2000; Oberhoffer et al., 1997; Reller & Kaplan, 1988; Way et al., 1979). These distinct clinical features suggest a complex pathomechanism of diabetes-induced fetal cardiac hypertrophy.

Unlike the hypertrophy of the adult heart, fetal cardiac enlargement is often “hyperplasia”. Indeed, an animal study suggests that diabetes-induced fetal heart enlargement is associated with smaller cardiomyocyte size (Dowling et al., 2014; Nakano et al., 2017). *In vitro* studies using pluripotent stem cells revealed a direct link between glucose and cardiac hypertrophy (Nakano et al., 2017). Glucose is metabolized in multiple intracellular pathways including anabolic mechanisms (PPP, hexosamine biosynthesis pathway, etc) and catabolic mechanisms (glycolysis and TCA cycle). High glucose promotes the fetal cardiomyocyte proliferation at the expense of cardiomyocyte maturity via the PPP) and nucleotide biosynthesis. Inhibition of the PPP and nucleotide synthesis blocked the over-proliferation and induced maturation and enlargement of cardiomyocyte size even in the presence of high glucose *in vitro*. It is unclear whether the PPP and nucleotide biosynthesis play key roles *in*

vivo and whether inhibition of these mechanisms restore the fetal cardiomyopathy in diabetic pregnancy.

Conclusion

The metabolic environment is not only the consequence of genetic programs but also the driver of genetic regulation. Glucose, the most fundamental of nutrients, plays various roles during embryogenesis. Although it has long been established that hyperglycemia affects the fetal development, we are at the very primitive stage of understanding how glucose impacts heart formation. The study of the pathological role of hyperglycemia has been largely centered around oxidative stress hypothesis. However, the recent studies have highlighted non-ROS mediated mechanism through accessory pathways of glucose metabolism. Further study will elucidate the links between ROS and specific signaling mechanisms. Some metabolites serve as substrates for post-transcriptional chromatin modifications including methylation, acetylation, GlcNAcylation, etc., and the list of chromatin-modifying metabolites keeps increasing. Another possible future direction includes the study of how glucose directly or indirectly modifies chromatin state. Thirdly, the knowledge from developmental metabolism can be applied to the study of regeneration. Accumulating evidence suggests that suppression of OXPHOS is preferred for cardiac regeneration. (Fukuda et al., 2020; Puente et al., 2014; Sauer et al., 2000). Further studies may open a new strategy for cardiac regeneration. Lastly, the *in utero* nutritional environment may have an impact on health and disease in adulthood. Although the concept of the developmental origins of health and disease (DOHaD) is well studied in the context of maternal malnutrition, few studies suggest that offspring of maternal overnutrition also tend to develop cardiovascular diseases later in life. Although the mechanism of this hypothesis remains elusive, the interaction between genetic and non-genetic factors in epigenetic modifications including DNA methylation and histone modification as well as posttranslational modification of cardiac regulatory genes are likely involved. The global prevalence of diabetes among adults over 18 years of age keeps rising. The increase in the proportion of women with pregestational diabetes raises a great concern as it has serious implications for both maternal and fetal welfare. Further advancement of our knowledge of whether and how glucose impacts heart development is critical to tackle this world-wide concern.

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Highlights

- Maternal hyperglycemia increases the risk of congenital heart defects including heterotaxia and conotruncal defects.
- Although diabetic pregnancy is often associated with maternal complications such as hyperinsulinemia, neuropathy, vasculopathy, neuropathy, etc., high glucose is likely the major teratogen for the congenital heart defects in the fetus.
- Hyperglycemia-induced pathology has been largely centered around oxidative stress. Although, recent studies highlighted non-ROS mediated mechanism through accessory pathways of glucose metabolism including pentose phosphate pathway.

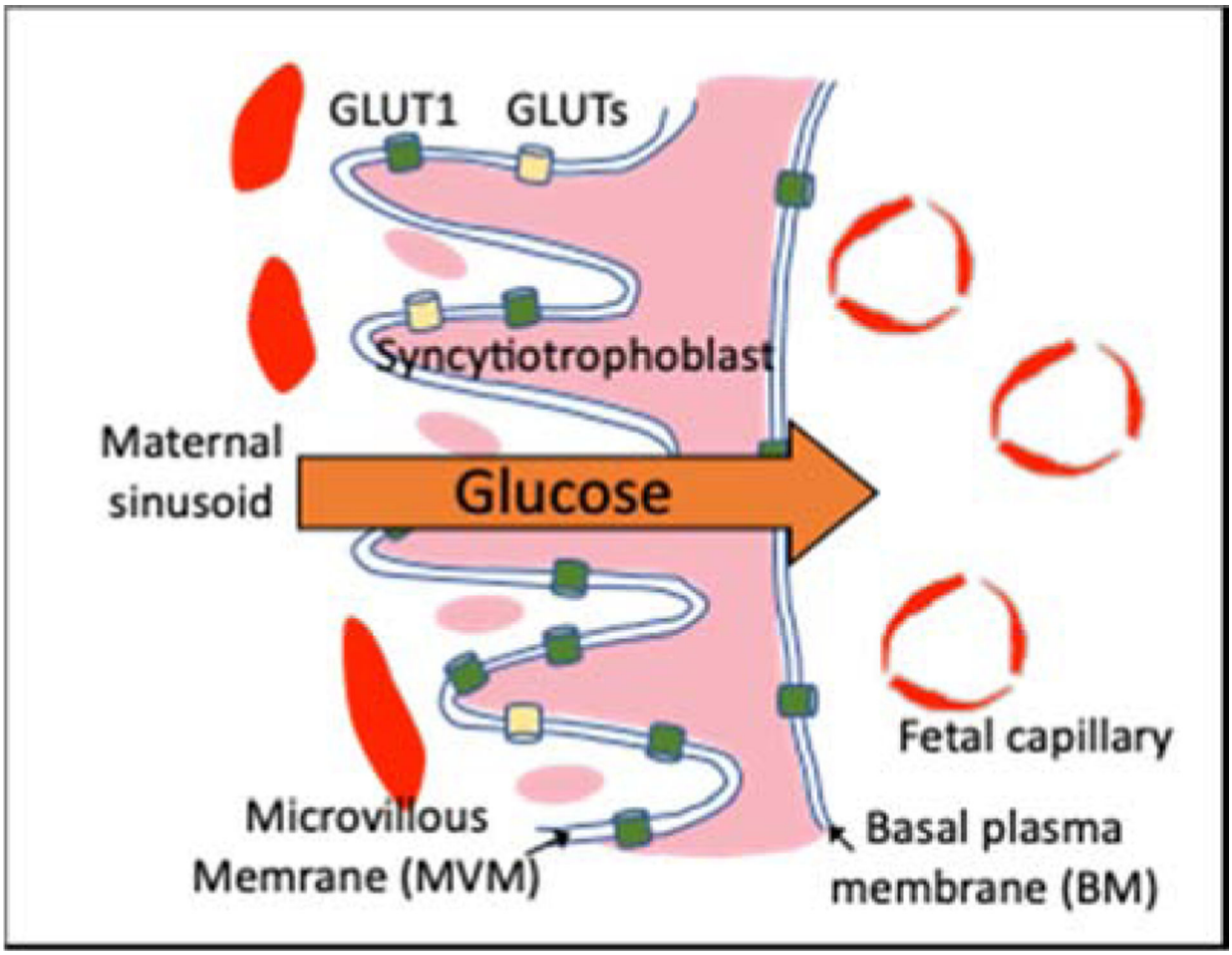

Figure 1.
Placental glucose transport. Glucose transporter isoforms on placental membrane mediate fetal glucose uptake. Excessive glucose causes imbalance of intracellular oxidative stress and its defense system, which then alters signaling pathways.

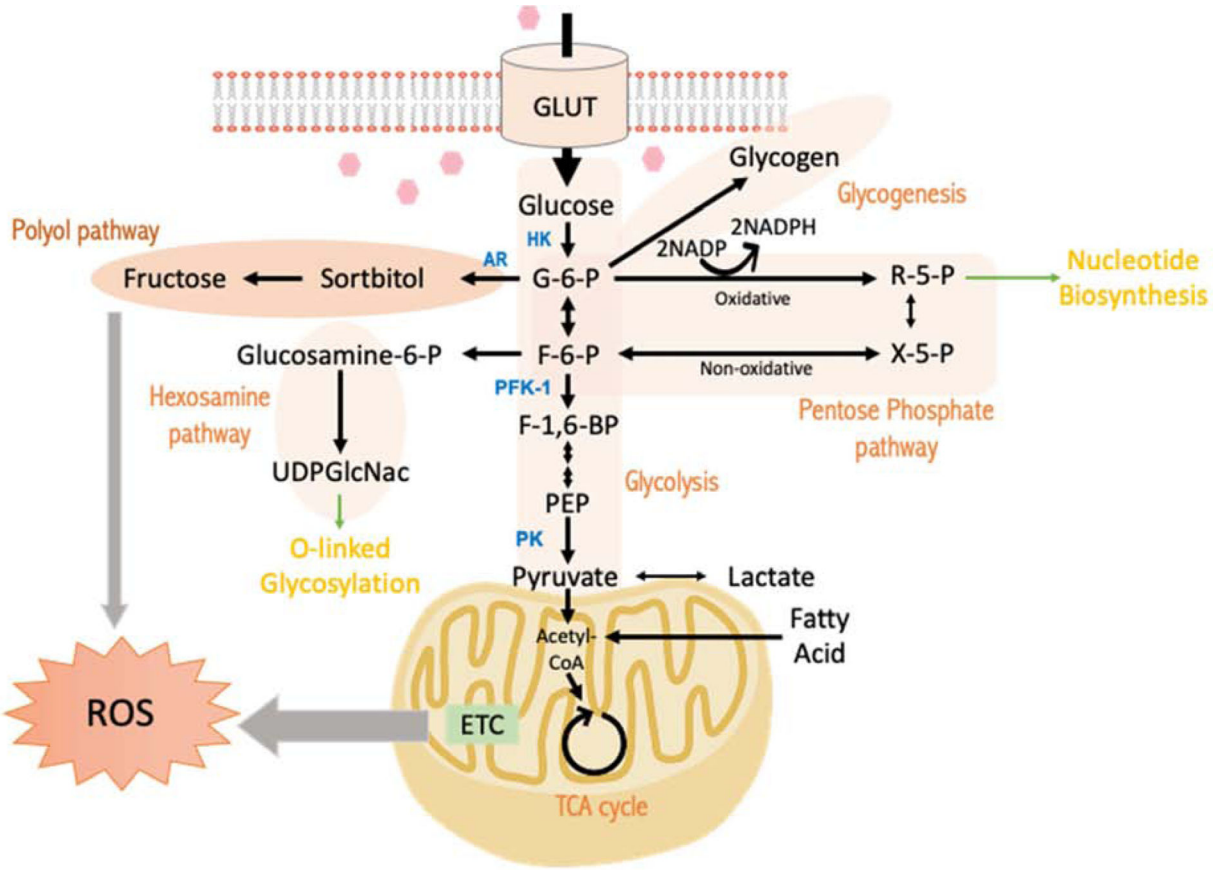

Figure 2.

Intracellular glucose metabolism pathways

Intracellular glucose is phosphorylated by hexokinase to G-6-P, and further metabolized in the glycolytic pathway. G-6-P can be metabolized in several accessory pathways such as PPP, glycogenesis and polyol pathway. In addition, F-6-P can be metabolized in the hexosamine pathway.

AR, aldose reductase; ETC, electron transport chain; GLUT, glucose transporter; HK, hexokinase; PFK-1, phosphofructokinase-1; PK, pyruvate kinase; F-6-P, fructose-6-phosphate; F-1,6-BP, fructose-1, 6-biphosphate; G-6-P, glucose-6-phosphate; NADP, Nicotinamide adenine dinucleotide; NADPH, Nicotinamide adenine dinucleotide phosphate; PEP, phosphoenolpyruvate; R-5-P, ribose-5-phosphate; X-5-P, xylose-5-phosphate

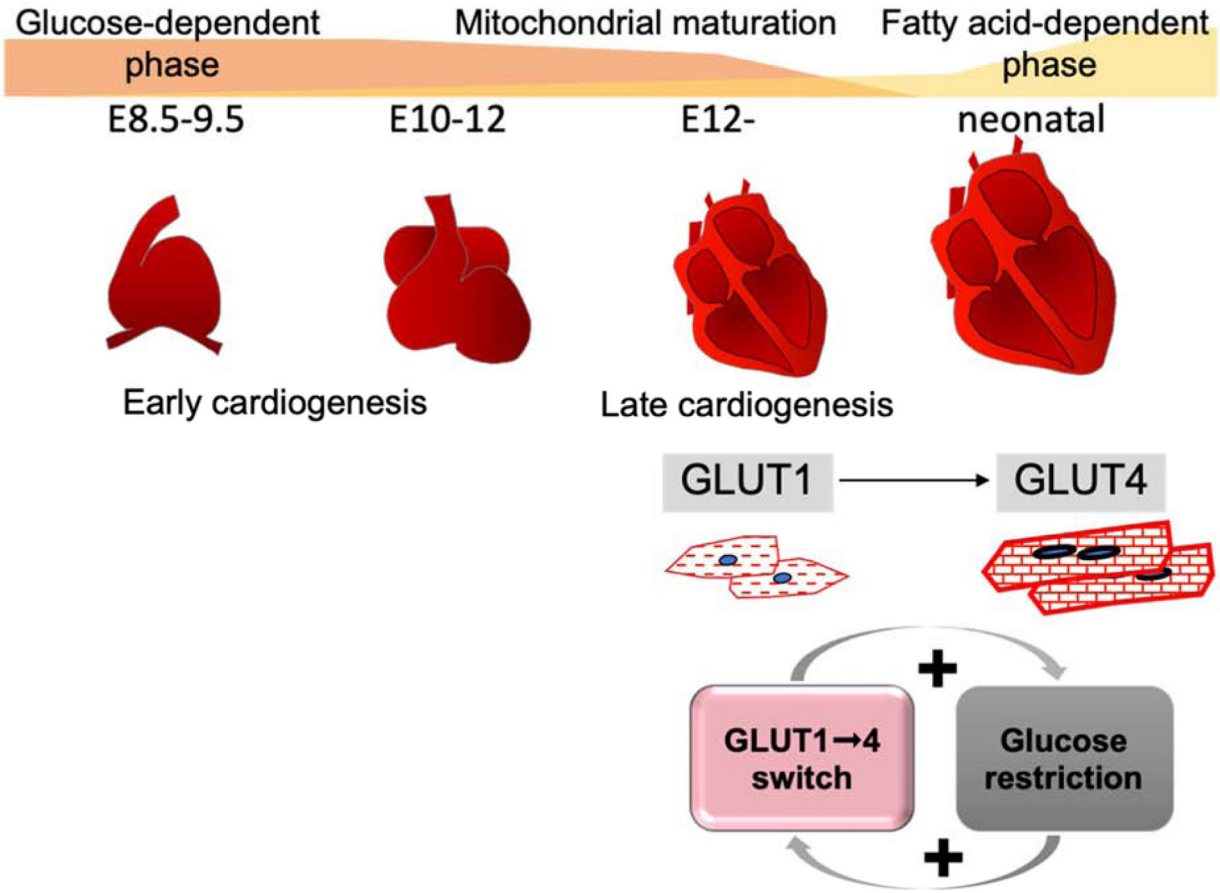

Figure 3.

Metabolic switch during cardiogenesis

During early cardiogenesis, cardiomyocytes are largely dependent on glycolysis. Along with mitochondrial maturation in cardiomyocytes, metabolic substrate switches from glucose to fatty acid. Glucose transporter switches from GLUT1 to GLUT4 during late gestational stages.

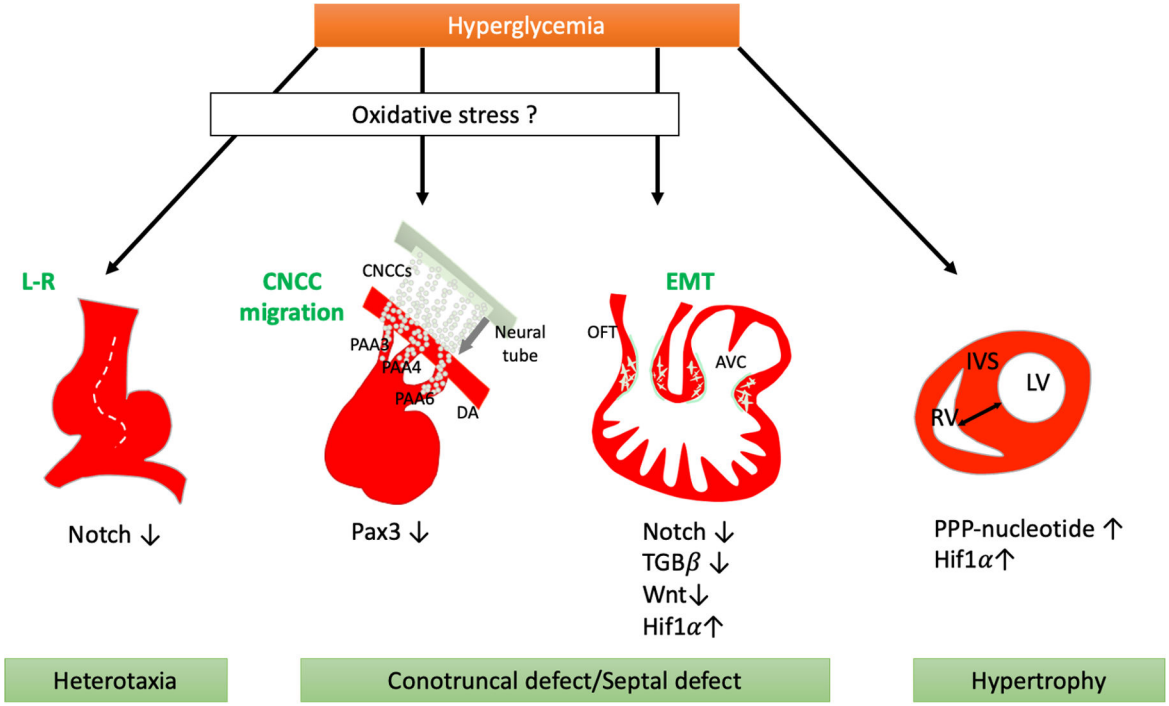

Figure 4.

Maternal glycemia-induced embryopathy

Fetal hyperglycemia alters gene regulatory networks and changes cellular responses such as endocardial epithelial-to-mesenchymal transition that is critical for vascular remodeling, as well as cellular interaction of cardiac progenitor cells from CNCCs (cardiac neural crest cells) in early cardiogenesis. In late cardiogenesis, altered glucose metabolism pathways such as PPP (pentose phosphate pathway) can cause asymmetric cardiac hypertrophy. DA, dorsal aorta; IFT, inflow tract; IVS, interventricular septum; L-R, left-right; LV, left ventricle; OFT, outflow tract; PAA, pharyngeal arch artery; RV, right ventricle.