1. Introduction

Diabetic pregnancy is associated with an increased incidence of congenital malformations compared with non-diabetic pregnancy [1,2]. Diabetic embryopathy can affect any developing organ system, although congenital heart defects are the most frequent malformations in the offspring of diabetic women [1]. Atrioventricular septal (AVS) defects, hypoplastic left heart syndrome, and persistent truncus arteriosus are the most frequent cardiac malformations detected in clinical studies [1,3–5]. The same types of heart malformations that are associated with diabetic pregnancies in humans have been reported in animal models [4,6–8]. Animal studies have indicated that hyperglycemia, hypoxia, fetal acidosis, and abnormal maternal/fetal fuel metabolism are responsible for changes in embryonic development [9–15]. Recent studies suggest that a major teratogenic effect of the diabetic-hyperglycemic milieu is mediated by increased oxidative stress and that administration of anti-oxidants reduces the occurrence of developmental defects [13–16]. However, the molecular mechanisms by which hyperglycemia or oxidative stress lead to the dysregulated gene expression that ultimately results in malformations have not yet been elucidated.

Using global gene expression profiling, we previously showed that maternal diabetes alters embryonic gene expression [17,18]. In particular, twenty genes regulated by hypoxia-inducible factor 1 (HIF-1) exhibited increased expression in diabetes-exposed embryos at E10.5, possibly reflecting an adaptive embryonic response to the diabetic environment of increased oxidative stress and hypoxia [17]. HIF-1 activates over 800 target genes that are involved in cell proliferation, angiogenesis, erythropoiesis, metabolism, and apoptosis [19]. Like other essential regulatory proteins, the expression levels of HIF-1 are highly controlled through the combinations of transcriptional, post-transcriptional, and post-translational mechanisms, and further influenced by protein stability and transactivation processes [19,42]. Oxygen tension plays a key role in the regulation of HIF-1α expression, stabilization, and activation [19]. The amplitude of this response is also modulated by growth factor and cytokine-dependent signaling pathways [20,21]. Furthermore, emerging evidence indicates that mitochondrial reactive oxygen species (ROS) are both necessary and sufficient to initiate the...
stabilization and activation of HIF-1α, and that treatment with antioxidants prevents HIF-1α protein stabilization [22].

The critical role of HIF-1 in development is demonstrated by the consequences of homozygosity for a null allele at the Hif1a locus encoding the HIF–1α subunit. Hif1a+/− knockout mouse embryos die at mid-gestation due to cardiovascular and neural tube defects [23,24]. HIF-1α is essential for proper cardiac looping and the modulation of neural crest cell (NCC) migration and survival [24,25]. Interestingly, exposure of mouse embryos to increased ambient O2 concentrations partially rescues development of the Hif1a+/− embryonic heart, specifically chamber formation. However, hypoxia fails to rescue pharyngeal arch development and NCC migration. Furthermore, mice with global heterozygous deletion of HIF-1α concomitant with a cardiac specific homozygous deletion of HIF-1α in ventricular cardiomyocytes exhibit embryonic lethality due to abnormal cardiac development between E8.5 and E10.0 [26]. The global nature of the HIF-1α deletion in these studies does not allow a definitive attribution of the noted effects to a single cell type. Not only HIF-1α loss-of-function, but also excessive HIF-1 activity, may result in birth defects. CITEd2 is a negative regulator of HIF-1 transcriptional activity and Cited2−/− mouse embryos manifest overexpression of HIF-1 target genes, such as Vegfa, Glut1, and Psgk1, as well as neural tube and cardiovascular defects [27]. Cardiac defects were partially rescued in Cited2−/−/Hif1a−/− embryos [28]. Localized adenosine overexpression of HIF-1α in the chick heart was sufficient to cause coronary artery anomalies [29]. Thus, increased or decreased HIF-1 activity results in neural and cardiovascular defects, which are the most frequent developmental defects associated with diabetic embryopathy.

Based on our finding that the expression of HIF-1 target genes is induced by maternal diabetes [17], and the knowledge that Hif1a+/− embryos have major defects in cardiovascular development [23-24], we hypothesized that HIF-1 transcriptional activity represents a protective response of the embryo to maternal diabetes and that loss of HIF-1 activity increases susceptibility to heart defects observed in diabetic embryopathy. Hif1a+/− heterozygous-null mice develop normally but demonstrate impaired responses when challenged with hypoxia after birth [30,31]. We hypothesized that limiting levels of HIF-1α may compromise embryonic development under conditions of aggravated hypoxia, which may occur in the context of maternal diabetes. We tested this hypothesis by exposing Hif1a+/− embryos to maternal diabetes. We analyzed the frequency and morphology of heart defects in diabetes-exposed Hif1a+/− and wild type (Wt) littermate embryos. Our results suggest an important role for HIF-1 in embryonic responses to the diabetic environment, including regulation of the key cardiac transcription factors Nkx2.5, Tbx5, and Mej2C.

2. Methods

2.1. Experimental animals

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). Diabetes was induced in female inbred FVB mouse strain (strain code 207, Charles River), aged 7–9 weeks, by 2 intraperitoneal injections of 100 mg/kg body weight of streptozotocin (STZ; Sigma, St. Louis, MO), as described [17]. Blood glucose levels were measured in fasted animals by glucometer (COUNTOUR TS, Bayer, Switzerland). We analyzed embryos from 52 diabetic dams that maintained blood glucose levels above 13.9 mmol/L (classified as diabetic) with blood glucose levels (mean ± SD) of 10.0 ± 1.2; 18.3 ± 4.3; and 28.7 ± 6.36 mmol/L before STZ treatment, on the mating day, and at embryo harvest, respectively. Mouse embryos were isolated from diabetic or control dams between E10.5 and E18.5. Noon of the day on which the vaginal plug was found was designated E0.5. Diabetic FVB Wt females were mated to Hif1a+/− males (with the Hif1a+tm/jhu knockout allele [23] on an FVB background) to generate Hif1a+/− and Wt (Hif1a+/+) littermate embryos. Hif1a+/− mice are defective in the induction of HIF-1α protein in response to hypoxia or ischemia [32,33]. Offspring of Wt × Hif1a+/− matings were genotyped by PCR [23,34]. Non-diabetic embryos were generated from crosses between mock-induced Wt females (i.e. no STZ treatment) to Hif1a+/− males. This breeding scheme minimized the potential influence of maternal genotype since the mutant allele was paternally contributed. The developmental stage was classified for each embryo by morphological criteria, including somite number as well as central nervous system, limb, and eye development. Embryonic and cardiac morphology was assessed using a Nikon SMZ dissection microscope. Digital images of whole embryos were captured with a Nikon DS-Fi1 camera. Measurements of the crown-rump length and relative heart area were obtained ex vivo by light microscopy using the NIS-elements software program (Nikon). We analyzed embryos that were comparable in their developmental progression.

2.2. Morphological analysis and immunostaining

Dissected thoraces from E14.5 embryos were fixed with 4% paraformaldehyde in PBS (pH 7.4) at 4 °C overnight, dehydrated, and embedded in paraffin. Quantification of myocardial tissue area (ventricular compact myocardium and trabeculae) and the area of the ventricular lumens was performed in Wt and Hif1a+/− diabetes-exposed and control hearts (n = 5 each) using the threshold tool in the NIH ImageJ program (http://imagej.nih.gov/ij/download.html), as described [35]. We analyzed 3 consecutive sections of 7-μm thickness running through the atrioventricular (AV) junction with a four-chamber view. Immunohistochemistry was performed with anti-VEGF-A antibody at 1:50 dilution (#sc-7269, Santa Cruz Biotechnology, CA, USA), anti-α-smooth muscle actin (α-SMA) antibody at 1:500 dilution (#A2547, Sigma-Aldrich), and anti-phospho-histone H3 (pH3) antibody (#06-570; Merck Millipore, Germany) at dilution 1:100; each analysis was repeated a minimum of 3 times on an average of 3 embryos per genotype and included appropriate controls. The sections were analyzed under a Nikon Eclipse 50i microscope with a 20 × magnification objective using the NIS-element program. α-SMA and pH3 immunostaining was analyzed with a two-photon microscope (Zeiss MP7). VEGF-A expression and α-SMA expression were quantified using ImageJ software. Immunopositive pH3+ nuclei in compact myocardium were counted using Adobe Photoshop CS5.11.

2.3. TUNEL assay

For diabetes-exposed and control Wt and Hif1a+/− embryos, we analyzed 3 cardiac sections running through the AV junction with a four chamber view from 3 embryos of different litters for each genotype. Tissue sections (7 μm) of dissected E14.5 thoraxes were treated with 20 μg/ml proteinase K for 20 min at room temperature. The sections were incubated with the TUNEL labeling kit (Roche) for 1 h at 37 °C and Hoechst 33342 was used as a nuclear counterstain. The sections were analyzed under a Nikon Eclipse E400 fluorescent microscope.

2.4. Real-time reverse-transcription quantitative PCR

Total RNA was isolated from hearts at E14.5 from experimental samples (EX; diabetes-exposed Wt, diabetes-exposed Hif1a+/−, and non-diabetic Hif1a+/−) and from non-diabetic Wt (control). Following reverse transcription (RT), quantitative real-time PCR (qPCR) was performed with the initial AmpliTaq activation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s, as described [34]. The Hprt1 gene was selected as the best reference gene for our analyses from a panel of 12 control genes (TATAA Biocenter AB, Sweden). The relative expression of a target gene was calculated, based on qPCR efficiencies (E) and the crossing point (Cp) difference (Δ) of an experimental sample versus control (ratio = (Etarget/EX)Cp Gene(Mean control − Mean EXP)).
3. Results

3.1. Effects of diabetes-exposure on litter size, embryonic growth, and external morphology of Hif1a+/− and Wt mice

Wt (n = 195) and Hif1a+/− (n = 163) embryos from 52 diabetic pregnancies, and Wt (n = 120) and Hif1a+/− (n = 117) embryos from 29 non-diabetic pregnancies were collected between E10.5 and E18.5 (Supplemental Table S2). The number of resorbed embryos was significantly increased in diabetic pregnancies with severe hyperglycemia (blood glucose level > 30 mmol/L; 24% of embryos) compared to non-diabetic pregnancies (2% of embryos) or diabetic pregnancies with blood glucose levels <30 mmol/L (3% of embryos). The average number of Wt embryos per litter was not significantly different in diabetic pregnancies with glucose levels >30 mmol/L (severe hyperglycemia; 3.97 ± 0.4) and in diabetic pregnancies with blood glucose levels between 13.9 mmol/L and 30 mmol/L (moderate hyperglycemia; n = 34 litters). The number of Hif1a+/− embryos/litter was significantly decreased in diabetic pregnancies compared to non-diabetic pregnancies. Statistical significance was assessed by one-way ANOVA (brackets at top) followed by Dunnett’s post-test (all groups vs. non-diabetic Wt group). The values represent means ± SEM. *, P < 0.05; **, P < 0.001; #, P < 0.037, t-test.

We detected a significant decrease in the average crown-rump length of Wt and Hif1a+/− diabetic embryos at E18.5 (P = 0.0001, one-way ANOVA; Supplemental Fig. S1A). The heart size of embryos from diabetic pregnancies was smaller than the sizes of embryonic hearts from non-diabetic pregnancies at E18.5 (P = 0.0002; Supplemental Fig. S1B).

Dissected embryos were examined for defects in gross morphology (Table 1). Diabetes-exposed embryos displayed a variety of external developmental defects, including neural tube defects and cardiovascular anomalies (Figs. 2A–J). Cardiovascular anomalies were the most frequent and were manifested by hemorrhage, edema, and in some cases, anemia. The evaluation of external phenotype of diabetes-exposed embryos demonstrated that Hif1a+/− embryos from diabetic pregnancies had an increased incidence of developmental defects of 22% (36 of 163) compared to 13% (26 of 195) of Wt littermate embryos (Fig. 2K; P = 0.03, Fisher’s exact test). No significant effect of genotype on the frequency of developmental defects was detected in pregnancies with glucose levels >30 mmol/L (severe hyperglycemia) with frequencies of 26% (15 of 58) and 28% (18 of 64) for Hif1a+/− and Wt embryos, respectively (P = 0.8, Fisher’s exact test). However, a significant effect of genotype on the incidence of developmental defects was observed

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Changes in gross morphology of diabetes-exposed embryos.</th>
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<tr>
<td><strong>External developmental defects</strong></td>
<td><strong>Wt (n = 195)</strong></td>
</tr>
<tr>
<td>NTDa</td>
<td>2</td>
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<tr>
<td>NTD in the brain region/exencephaly</td>
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<tr>
<td>NTD in the posterior region/spina bifida</td>
<td>2</td>
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<tr>
<td>Zig-zag closure</td>
<td>1</td>
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<tr>
<td>Combination of NTD and cardiovascular anomaliesb</td>
<td>7</td>
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<tr>
<td>NTD in the brain region/exencephaly</td>
<td>5</td>
</tr>
<tr>
<td>Anencephaly</td>
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<tr>
<td>NTD in the posterior region/spina bifida</td>
<td>2</td>
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<tr>
<td>Craniorachischisis</td>
<td>1</td>
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<tr>
<td>Caudal regression</td>
<td>1</td>
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<tr>
<td>Cardiovascular anomaliesb</td>
<td>10</td>
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<tr>
<td>Developmental arrest</td>
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a NTD, neural tube defects.
b Cardiovascular anomalies were mostly manifested by hemorrhages and edema.
in diabetic pregnancies with blood glucose levels of moderate hyperglycemia, in which 20% (21 of 105) of Hif1a+/− embryos, compared to only 6% (8 of 131) of Wt embryos, demonstrated developmental defects (P = 0.002, Fisher’s exact test). Since we detected a significant effect of genotype on the incidence of developmental defects in embryos from diabetic pregnancies with moderate hyperglycemia, we used only embryos from pregnancies with moderate hyperglycemia for our subsequent analyses.

3.2. The frequency and morphology of cardiovascular defects

To assess the morphology and frequency of cardiovascular defects, we examined heart development and heart morphology by histological analysis of thorax sections of E14.5 embryos (Figs. 3A–D). Since HIF-1α modulates NCC survival and migration [25], we have carefully analyzed morphology of the outflow tract and pharyngeal arch arteries, because NCCs contribute to their formation. We analyzed 6 litters from diabetic pregnancies and 4 litters from non-diabetic (A, F) and diabetic pregnancies (blood glucose levels > 13.9 mmol/L, severe hyperglycemia) with frequencies of 26% (n = 1 of 117; P = 0.0001, Fisher’s exact test) and to 1% of non-diabetic Hif1a+/− embryos (n = 1 of 117; P = 0.0001, Fisher’s exact test). No significant effect of genotype on the incidence of congenital defects was detected in diabetic pregnancies with glucose levels > 13.9 mmol/L (severe hyperglycemia) with frequencies of 26% (n = 15 of 195; P = 0.002, Fisher’s exact test) and to 1% of non-diabetic Hif1a+/− embryos (n = 1 of 117; P = 0.0001, Fisher’s exact test). No significant effect of genotype on the incidence of congenital defects was detected in diabetic pregnancies with glucose levels > 13.9 mmol/L and 30 mmol/L (moderate hyperglycemia), in which 20% of Hif1a+/− embryos (n = 21 of 105) compared to 6% of Wt embryos (n = 8 of 131, P = 0.002, Fisher’s exact test) demonstrated congenital defects. *, P < 0.03, Fisher’s exact test.

Fig. 2. External morphological changes in diabetes-exposed embryos at E14.5. The external appearances of Wt and Hif1a+/− embryos from non-diabetic (A, F) and diabetic pregnancies are compared at E14.5 (B–E, G–J). The most frequent defects associated with diabetic pregnancies were cardiovascular anomalies and neural tube defects. Neural tube defects were displayed mainly as neural tube closure defects (arrow in j and l) and in some cases as a phenotype of zig-zag closure line of neural tube (E). Cardiovascular anomalies were manifested by hemorrhages (B, C, G, I), edema (C, D, G, arrow head), and anoxic phenotype in live embryos (H). Scale bar: 5 mm. (K): Incidence of congenital defects in embryos between E10.5 and E18.5 was affected by maternal diabetes. Hif1a+/− embryos from diabetic pregnancies (blood glucose levels > 13.9 mmol/L) showed an increased rate of congenital malformations of 22% (n = 36 of 163) compared to 13% of diabetes-exposed Wt (n = 26 of 195; P = 0.03, Fisher’s exact test) and to 1% of non-diabetic Hif1a+/− embryos (n = 1 of 117; P = 0.0001, Fisher’s exact test). Since we detected a significant effect of genotype on the incidence of congenital defects was detected in diabetic pregnancies with glucose levels > 13.9 mmol/L (severe hyperglycemia) with frequencies of 26% (n = 15 of 58) and 28% (n = 18 of 64; P = 0.8, Fisher’s exact test) for Hif1a+/− and Wt embryos, respectively. However, the significant effect of genotype on the incidence of congenital defects was found in diabetic pregnancies with blood glucose levels between 13.9 mmol/L and 30 mmol/L (moderate hyperglycemia), in which 20% of Hif1a+/− embryos (n = 21 of 105) compared to 6% of Wt embryos (n = 8 of 131, P = 0.002, Fisher’s exact test) demonstrated congenital defects. *, P < 0.03, Fisher’s exact test.

The frequency and morphology of cardiovascular defects

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We closely observed the cardiovascular malformations involving cardiac outflow tract defects. In 35 analyzed Hif1a+/− and Wt embryos from diabetic pregnancies, we identified one case of persistent truncus arteriosus in a Hif1a+/− embryo. We did not observe any cases of transposition of the great vessels or double outlet right ventricle. However, histological analysis showed that ventricular myocardial mass was profoundly reduced and the compact ventricular myocardial walls were thinner in all diabetes-exposed embryos at E14.5 (Figs. 3A–D, E; one-way ANOVA, P < 0.0001). Additionally, in diabetic pregnancies, the myocardial volume of the left ventricle (LV) of Hif1a+/− embryos was significantly decreased compared to Wt littermates (Fig. 3E). Interestingly, a sponge-like layer of myocardium (trabeculae) was increased in both diabetes-exposed Wt and Hif1a+/− embryos compared to non-diabetic Wt embryos (Fig. 3F). Since the ventricular trabeculae
are thought to increase surface area to maximize oxygen uptake by the myocardium, our results are consistent with the hypothesis that the diabetic embryonic environment impairs tissue oxygen availability and may activate compensatory mechanisms to satisfy oxygen demands. To investigate the mechanistic bases for the observed thin-walled myocardium, we quantified proliferation and apoptosis in the compact myocardium in both ventricles at E14.5. Isolated TUNEL-positive cells were seen in the endocardium, mesenchymal tissues of the forming cardiac skeleton, remodeling AV and outflow tract valves, and the interventricular septum of control embryos. A higher frequency of apoptotic cells was detected, mainly in the endocardial cushions and AV septum, of diabetes-exposed embryos. Since the majority of heart defects observed in diabetes-exposed embryos were AVS defects, we also analyzed apoptosis specifically in hearts with AVS defects. We detected a significant increase in apoptotic cells in Hif1α−/− diabetes-exposed embryos with AVS defects compared to Hif1α−/− embryos from non-diabetic pregnancies (Fig. 4; one-way ANOVA, P < 0.02).

Since the LV myocardial volume of diabetes-exposed Hif1α−/− embryos was significantly decreased compared to Wt littermates, we quantified cell proliferation by immunostaining for pH3 at E10.5, and from E12.5 to E14.5 (Fig. 5). Mitotic activity was significantly decreased in the compact myocardium of diabetes-exposed Hif1α−/− hearts relative to the non-diabetic Wt hearts at E10.5 and E12.5 (Figs. 5E–F). These data are consistent with previously published results [6,7] and suggest that the

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**Fig. 3.** Morphological changes in embryos exposed to maternal diabetes. H&E staining of mouse embryonic transverse sections of E14.5 thorax demonstrated an increased rate of cardiovascular defects in diabetes-exposed embryos (A–D). In non-diabetic Wt (A) and Hif1α−/− (C) embryos from non-diabetic pregnancies, the right and left ventricles were separated by the interventricular septum (IVS). In diabetes-exposed embryos (B, D), the 65% of detected cardiovascular defects were ventricular septal defects (VSD). Ventricular myocardial walls were thinner in all diabetes-exposed compared to non-diabetic embryos at E14.5. Scale bar: 0.5 mm. RV, right ventricle; LV, left ventricle; IVS, interventricular septum; VSD, ventricular septal defect. (E) The relative myocardial volumes (μm³) of compact layer of diabetes-exposed RV of Wt (P < 0.003), RV of Hif1α−/− (P < 0.01), and LV of Hif1α−/− (P < 0.001) were significantly smaller compared to non-diabetic Wt and Hif1α−/− hearts. The compact myocardium of diabetes-exposed Hif1α−/− LV was significantly more affected than diabetes-exposed Wt LV (P < 0.01). The relative myocardial volume of compact layer (μm³) was estimated from the areas of LV with length of 0.2 mm and RV with the length of 0.4 mm (μm²) multiplied by the thickness of sections (7 μm). (F) Thickness of trabecular myocardium at E14.5. The average area of trabecular myocardium of both the LV (P < 0.0004) and RV (P < 0.0076) was significantly increased in the diabetes-exposed hearts of Wt and Hif1α−/− embryos compared to non-diabetic Wt. Ventricular trabecular area was quantified using ImageJ software. We analyzed compact and trabecular myocardium in 3 subsequent sections of E14.5 thorax running through the AV junction with a four chamber view from 5 embryos from 3 litters/each group. The values represent means ± STDEV. *, P < 0.05, **, P < 0.01; one-way ANOVA (brackets at top) with Bonferroni’s multiple comparison post-test.
formation of a thin myocardium in diabetes-exposed hearts is the result of decreased cell proliferation. Importantly, our analysis revealed a marked decrease in mitotic activity in the LV compact myocardium of Hif1a−/−/diabetes-exposed hearts compared to Wt littermates at E12.5, E13.5, and E14.5 (Figs. 5F–H). Thus, cell proliferation in the LV compact myocardium of diabetes-exposed Hif1a−/− embryos is considerably reduced, which represents a cellular mechanism for the decreased volume of the LV compact myocardium in diabetic Hif1a+/− embryos.

3.3. Analysis of HIF-1α protein levels

To understand the basis for the escalation of congenital defects in Hif1a+/−/diabetic embryos, we analyzed HIF-1α protein levels. Protein lysates were prepared from Wt and Hif1a+/− whole embryos and hearts at E10.5 (Fig. 6). Quantitative Western blot analysis showed significantly decreased HIF-1α levels in whole Hif1a+/−/embryos by 1.9 fold relative to Wt embryos. In contrast, HIF-1α protein levels in the hearts of Wt and Hif1a+/−/littermates from non-diabetic pregnancies were similar. HIF-1α levels were increased 2.9-fold in diabetes-exposed Wt hearts but only 1.8 fold in Hif1a+/− hearts, compared to non-diabetic Wt hearts.

3.4. Analysis of cardiac VEGF-A and α-SMA expression

Next, we focused on cardiac expression of VEGF-A, a key HIF-1 target gene product. VEGF-A is an essential modulator of cardiovascular development and modest increases or decreases in VEGF-A levels lead to embryonic lethality [37,38]. We analyzed histological sections of E14.5 hearts to establish the spatial expression of VEGF-A using immunohistochemistry. VEGF-A was detected in the interventricular septum, myocardial cells lining the endocardial cushions of both the inflow tract and the outflow tract, and in the ventricular myocardium (Figs. 7A–F). Diabetes significantly increased VEGF-A protein levels in Wt hearts but not in Hif1a+/− hearts (Fig. 7G). In diabetes-exposed Hif1a+/−/hearts, the expression of VEGF-A was increased in three of the four analyzed embryos. We also used RT-qPCR to analyze Vegfa mRNA levels in the embryonic hearts at E14.5 (Fig. 7H). We detected a marked variability in Vegfa mRNA expression levels in the diabetes-exposed hearts. However, mean Vegfa mRNA levels in the hearts of diabetes-exposed Hif1a+/−/embryos were markedly reduced compared to Wt littermates.

To further elucidate the roles of HIF-1α in cardiac responses to the diabetic environment, we analyzed spatial expression of α-SMA, a marker of immature cardiomyocytes. The expression of α-SMA in early cardiomyocytes is more prominent before E12.5 and its expression is decreased with cardiomyocyte maturation at E14.5 [39]. We observed a reduced number of α-SMA+ cardiomyocytes in both the LV and RV of diabetic Hif1a+/−/embryos compared to non-diabetic embryos at E14.5 (Figs. 8A–B). Although the RV myocardial volume in diabetes-exposed Wt embryos was significantly decreased compared to non-diabetic Wt embryos (Fig. 3), the expression of α-SMA was similar in diabetic and non-diabetic RVs. Since mature cardiomyocytes lose α-SMA expression, the differences in the number of α-SMA+ cardiomyocytes may indicate dysregulated differentiation (maturation) of the ventricular cardiomyocytes in diabetic embryos.

3.5. Analysis of cardiac gene transcription

RT-qPCR was performed to analyze the expression of genes encoding molecules important for myofibrillogenesis, transcriptional regulation, differentiation, and proliferation, including T-box 5 (Tbx5), NK class homeodomain protein (Nkx2.5), atrial natriuretic peptide (Nppa), gap-junction gene connexin 43 (Cx43), myocyte enhancer factor 2C (Me2cC), skeletal α-actin 1 (Acta1), myosin light chain isofrom (Mlc2v), and cardiac troponin I (Tnni3). Whereas expression of Tbx5 was significantly decreased in non-diabetic Hif1a+/−/compared to Wt hearts, it was markedly increased in diabetes-exposed Hif1a+/−/hearts compared to non-diabetic and diabetic Wt hearts (Fig. 9). Expression of the cardiogenic factor Nkx2.5 mRNA expression was also significantly increased in diabetes-exposed Hif1a+/−/hearts. Tbx5 and Nkx2.5 can synergistically activate the Nppa gene promoter [40,41]. Expression levels of Nppa were significantly increased in diabetes-exposed Hif1a+/−/embryos in accordance with the expression patterns of Tbx5 and Nkx2.5. Next, we analyzed the expression of Mef2c, which encodes another cardiac-specific transcription factor. Mef2c mRNA levels were also increased in diabetes-exposed Hif1a+/−/hearts but not in diabetes-exposed Wt hearts at E14.5. In contrast, maternal diabetes modestly increased the expression of a marker of myocardial cell differentiation, Cx43, in both Wt and Hif1a+/−/hearts. The expression of Acta1 was modestly decreased in both the Wt and Hif1a+/−/diabetes-exposed hearts compared to non-diabetic hearts. The expression of myofilament genes, Tnni3 and ventricle-specific Mlc2v, was not significantly affected by Hif1a genotype or the diabetic environment. Taken together, our data are consistent with the hypothesis that dysregulated gene expression in the hearts of Hif1a+/−/embryos subjected to the diabetic environment underlies the increased incidence of congenital cardiac defects in these embryos.

4. Discussion

The teratogenic process in diabetic pregnancy is multifactorial. It is associated with numerous disturbances in embryonic development and growth and with compromised placental function. In this study, we have analyzed the effects of global heterozygous deletion of Hif1a on the embryonic response to maternal diabetes. Our study revealed that compared to Wt littermates, mouse embryos heterozygous for a knockout allele at the Hif1a locus have a decreased number of embryos per litter and increased incidence of malformations in the teratogenic environment of maternal diabetes. Previous studies have documented that embryos with the global knockout of Hif1a die by E10.5 displaying severe cardiovascular and neural tube defects [23,24]. In contrast, a cardiac myocyte-specific deletion of Hif1a is not lethal and does not cause an increased incidence of developmental cardiac defects [42]. Although in this study, we observed that cardiovascular defects were the most frequent defects in Hif1a+/−/embryos from diabetic pregnancies, we cannot determine which cell type or which combinations of cell types are contributing to the increased susceptibility of Hif1a+/−/mice to diabetic embryopathy due to the global nature of the Hif1a deletion. At the same time, the global deletion of Hif1a may additionally affect gene regulation in the embryonic
compartments of the diabetic placenta. It can also contribute to placental dysfunction and to an overt pathological response in diabetic pregnancy.

In the present study, we have demonstrated increased HIF-1α levels in diabetes-exposed hearts compared to non-diabetic hearts at E10.5. Additionally, cardiac HIF-1α levels were reduced by 40% in diabetes-exposed hearts from Hif1a+/− embryos compared to Wt littermates, reflecting Hif1a haploinsufficiency. These differences in cardiac HIF-1α levels are evidence of differential responses between Wt and Hif1a+/− diabetes-exposed hearts. Under normoglycemic conditions, compensation for heterozygosity for the Hif1a knockout allele occurs, presumably by changes in the rate of synthesis or degradation of HIF-1α mRNA or protein. As a result, HIF-1α protein levels are not significantly decreased in the hearts of Hif1a+/− embryos and development proceeds normally. However, under the stress of maternal hyperglycemia, the adaptive increase in HIF-1α protein levels that is observed in Wt embryos cannot be achieved in Hif1a+/− embryos, thereby increasing their risk for congenital heart defects.

Fig. 5. Quantification of cellular proliferation in compact myocardium of the left ventricle of the diabetes-exposed and non-diabetic hearts of Wt and Hif1a+/− embryos. Heart sections (7 μm) were immunostained for phospho-histone H3 (pH3) to detect mitotic cells in the compact myocardium at E10.5, E12.5, E13.5, and E14.5 (3 embryos per each group and 2–3 slides per embryo). Confocal imaging of transverse sections of the embryonic heart of stage E14.5 (green autofluorescence) stained with anti-pH3 antibody (red) showed mitotic cells in the left ventricle of non-diabetic Wt (A) and Hif1a+/− (C), and diabetes-exposed Wt (B) and Hif1a+/− (D) embryos. Hoechst 33342 (blue) was used as a nuclear counter-stain. Images are stacked Z-plane sections from confocal microscopy. Scale bar: 0.1 mm. (E–H) Quantification of pH3+ cells was determined as an average pH3+ nuclei per total tissue area of the left ventricular compact myocardium of the field using ImageJ software. The values represent means of number pH3+ nuclei/μm² of left ventricular compact layer ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001; one-way ANOVA (brackets at top) followed by post-t-test. LV, left ventricle.
Interestingly, we found that the LV myocardial volume in diabetes-exposed \textit{Hif1a}^{+/-} hearts was significantly reduced compared to WT littermates at E14.5. We detected significantly increased expression of \textit{Tbx5} in diabetes-exposed \textit{Hif1a}^{+/-} hearts compared to WT littermates that may play a major role in the pathogenesis of reduced LV myocardial volume. \textit{Tbx5} is one of the important cardiac factors with distinct asymmetric expression, specifying the identity of the LV [43,44]. Existing evidence supports the role of \textit{Tbx5} as a regulator of myocardial cell proliferation. Overexpression of \textit{Tbx5} in embryonic chick heart inhibits myocardial cell proliferation [45]. Similarly, ubiquitous dysregulated expression of \textit{Tbx5} in the embryonic heart is associated with a thinner ventricular wall compared to the normal heart [46]. \textit{Tbx5} activates the \textit{Nkx2.5} gene [40,41,46]. Additionally, \textit{Tbx5} and \textit{Nkx2.5} synergistically activate the \textit{Nppa} gene [40]. Accordingly, we detected an increased expression of \textit{Nkx2.5} and \textit{Nppa} in the hearts of diabetes-exposed \textit{Hif1a}^{+/-} embryos but not WT embryos at E14.5. \textit{Nppa} is also specifically expressed in the LV but not in the RV in mid-gestation similar to \textit{Tbx5} [57]. Existing evidence strongly suggests that the levels of \textit{Tbx5} expression in the developing heart are crucial. Overexpression of \textit{Tbx5} in the heart is associated with abnormalities of early chamber development, hypoplasia, loss of ventricular-specific gene expression, and embryonic lethality [45,58]. Similarly, homozygous \textit{Tbx5}^{+/-} mutants demonstrate the arrest in heart development at E9.5. Heterozygous \textit{Tbx5}^{+/-} mice show the congenital defects, including cardiac hypoplasia, atrial and ventricular septal defects, of Holt–Oram syndrome mutations in humans [40]. \textit{Nkx2.5} mutations in humans are also associated with congenital heart defects similar to Holt–Oram syndrome [59]. Based on our results, we propose that the diabetic environment affects \textit{Hif1a} regulation in the developing heart and that haploinsufficiency of \textit{Hif1a} alters compensatory mechanisms involving key transcriptional factors that regulate cardiac differentiation, morphogenesis, and growth.

Although hypoplasia of the heart ventricles was observed in all diabetes-exposed embryos, we did not detect a significant increase in apoptotic cells at E14.5. However, a statistically significant increase in the number of apoptotic cells was detected in \textit{Hif1a}^{+/-} hearts with AVS defects. Increased apoptosis in the cardiac cushions in pre-septation stages was associated with subsequent defects of cardiac septation in TGF-\textit{fi}2-deficient mice [47]. Our findings of increased apoptosis at the stage where the defects are already established may reflect either a persistence of the apoptotic stimulus or abnormal hemodynamic conditions in the malformed heart, which can also lead to apoptosis [48]. However, the majority of apoptotic cells in both malformed and normal hearts were found in physiological zones of programmed cell death associated with heart morphogenesis [49], specifically the mesenchymal cushions, valves, and interventricular septum, rather than in the myocardium of the free ventricular wall. These observations suggest that ventricular wall mass was not reduced because of increased apoptosis and are consistent with other studies, which have implicated decreased cell proliferation in the ventricular myocardium as the cause of hypoplastic myocardium in diabetes-exposed hearts [6,7]. The proliferation of cardiomyocytes is necessary to support the increasing hemodynamic load at mid-gestation [50]. During normal heart development, the number of cardiomyocytes in the compact layer of the ventricles increases 5-fold between E12.5 and E14.5 [51]. Previous work has associated decreased proliferation with the formation of a thin myocardium in diabetes-exposed hearts [6,7,52]. Consistent with these results, we observed significantly decreased mitotic activity in the compact myocardium of E10.5 and E12.5 diabetes-exposed embryos compared to non-diabetic embryos. Interestingly, we detected substantially reduced proliferation in the compact myocardium of the left ventricle in diabetes-exposed \textit{Hif1a}^{+/-} embryos compared to WT littermates (Fig. 5), which corresponded to the more profound phenotype of thin ventricular compact myocardium and to the increased expression of \textit{Tbx5}, an inhibitor of cardiomyocyte proliferation [45]. Moderately elevated mitotic activity in the compact myocardium of E14.5 diabetes-exposed WT embryos suggests compensatory responses to the teratogenic diabetic environment. These different cellular responses between diabetes-exposed WT and \textit{Hif1a}^{+/-} embryos may contribute to increase susceptibility to diabetic embryopathy in \textit{Hif1a}^{+/-} embryos. In contrast to morphological changes in the ventricular myocardium, the epicardial derivatives of diabetes-exposed hearts was characteristically spread over the myocardium, without any signs of detachment or blebbing. Since epicardial–myocardial interactions regulate differentiation and proliferation of the ventricular wall, the possibility that epicardium-mediated signals are altered in diabetes-exposed embryos remains to be determined.

Our data demonstrate that, compared to WT embryos, \textit{Hif1a}^{+/-} littermates from diabetic pregnancies with moderate hyperglycemia are more susceptible to diabetic embryopathy. To further evaluate the role of \textit{Hif-1}–regulated pathways in diabetic embryopathy, we analyzed the expression of a key \textit{Hif-1} target gene, \textit{Vegfa}, which is tightly regulated during normal embryonic development. Heterozygous \textit{Vegfa}^{+/–} mutant embryos die at E10.5, displaying abnormal vascularization. Transgenic embryos overexpressing \textit{Vegf-A} also die with severe cardiac abnormalities at E12.5–E14 [37,38,53]. Our previous research has demonstrated that the expression of \textit{Vegf} is altered by the diabetic environment at E10.5 [17]. Cultured mouse embryos exposed to high glucose from E7.5 until E9.5 showed a reduction in \textit{Vegf-A} levels and significant vascular abnormalities [54]. A decrease in \textit{Vegfa} mRNA was observed in the hearts of E14 rat embryos (corresponding to E12.5 in the mouse) from diabetic pregnancies, although 25% of the analyzed embryos were not affected by the diabetic environment [55]. In contrast, an increased expression of \textit{Vegfa} mRNA and \textit{Vegf-A} protein was detected in the hearts of E13.5 mouse embryos from diabetic pregnancies [56]. In the present study, \textit{Vegf-A} levels were on average increased in WT but not \textit{Hif1a}^{+/-} embryos at E14.5. Our RT-qPCR analysis showed a high variability in \textit{Vegfa} mRNA levels with a prevailing trend of decreased \textit{Vegfa} expression in \textit{Hif1a}^{+/-} diabetes-exposed embryos at E14.5, supporting the idea of altered \textit{Hif-1} regulation that may increase the risk of cardiac malformations in \textit{Hif1a}^{+/-} embryos.

Maternal-diabetes–induced specific morphogenetic defects represent a phenotype of incomplete penetrance. A recently proposed explanation of the etiology of diabetic embryopathy based on gene expression highlighted two components, the deregulation of gene expression and increased variability of gene expression [60]. In our case, \textit{Hif1a}^{+/-} genotype and an increased variability of gene expression produce discrete differences among embryos, which can trigger pathogenic events resulting in congenital defects.

![Figure 6: Protein levels of HIF-1α.](image-url)
Studies in adult mice have demonstrated that HIF-1-dependent vascularization following femoral artery ligation or cutaneous wounding is impaired in diabetic mice, which can be rescued by experimental manipulations that increase HIF-1α expression [61–63]. Loss of HIF-1 activity appears to play a role in the pathogenesis of type 2 diabetes [64,65]. Thus, environmental (maternal diabetes) and genetic (Hif1a mutation)
factors may each reduce HIF-1 activity in embryos and result in congenital malformations if either abnormality is severe enough (i.e. maternal blood glucose > 30 mmol/L or Hif1a\(^{-/-}\) genotype) or when more modest abnormalities are present in combination (i.e. maternal blood glucose between 13.9 and 30 mmol/L and Hif1a\(^{+/+}\) genotype). Furthermore, clinical studies have associated a loss of gene copy of HIF1A with ventricular septal defects [66]. Taken together with the results presented here, these data raise the possibility that genetic variation at the HIF1A locus may influence malformation risk for infants of diabetic mothers.

5. Conclusions

We used a genetic mouse model of partial global HIF-1\(\alpha\) deficiency to test our hypothesis that induction of HIF-1\(\alpha\) represents one of the
adaptive responses to maternal diabetes and that a failure to ade-
quately induce expression of HIF-1α increases susceptibility to diabetic
embryopathy. HIF-1α heterozygous-null and Wt littermate embryos
were exposed to the intrauterine environment of a diabetic mother
and the frequency and morphology of heart defects were analyzed.
We found that the global reduction in functional HIF-1α gene dosage
decreased the number of embryos per litter and increased the incidence
of heart malformations, mainly AVS defects and reduced ventricular
myocardial mass, in diabetes-exposed HIF-1α−/− compared to Wt
littermates. We also detected significant changes in the expression of Vegfa
and key cardiogenic transcription factors Tbx5, Nkx2.5, and Mef2c in
diabetes-exposed HIF-1α−/− compared to Wt embryonic hearts. These
changes provide a molecular mechanism by which HIF-1α loss-of-function may increase the risk of congenital malformations.
Taken together, these results are compelling evidence that impairment
of HIF-1α-controlled hypoxia-response pathways may play a function-
ally causative role in diabetic embryopathy.

Supplementary data to this article can be found online at http://
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Fig. 9. Gene expression changes in Wt and Hif1a−/− hearts exposed to maternal diabetes at E14.5. The expression of selected genes was analyzed using RT-qPCR. The relative
expression levels were quantified using ΔΔCT method. The data represent an expression of mRNA relative to non-diabetic Wt expression of mRNA, normalized by the housekeeping
mRNA of Hprt1. The values represent means ± SEM (each experiment in duplicate; n = 8 per groups of diabetic Wt and Hif1a−/−, non-diabetic Hif1a−/−; n = 6 per non-diabetic Wt).
Differences in normalized Ct values were tested for statistical signi-
ficance by one-way ANOVA (brackets at top) followed by Bonferroni’s multiple comparison post-test. *, P < 0.05,
**, P < 0.01, *** P < 0.001.

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Disclosure statement

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