Hyperglycemia impairs left–right axis formation and thereby disturbs heart morphogenesis in mouse embryos

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Congenital heart defects with heterotaxia are associated with pregestational diabetes mellitus. To provide insight into the mechanisms underlying such diabetes-related heart defects, we examined the effects of high-glucose concentrations on formation of the left–right axis in mouse embryos. Expression of Pitx2, which plays a key role in left–right asymmetric morphogenesis and cardiac development, was lost in the left lateral plate mesoderm of embryos of diabetic dams. Embryos exposed to high-glucose concentrations in culture also failed to express Nodal and Pitx2 in the left lateral plate mesoderm. The distribution of phosphorylated Smad2 revealed that Nodal activity in the node was attenuated, accounting for the failure of left–right axis formation. Consistent with this notion, Notch signal-dependent expression of Nodal-related genes in the node was also down-regulated in association with a reduced level of Notch signaling, suggesting that high-glucose concentrations impede Notch signaling and thereby hinder establishment of the left–right axis required for heart morphogenesis.

Significance

Epidemiological studies have revealed that pregestational diabetes mellitus increases the risk for congenital anomalies, including congenital heart defects (CHDs). Despite the importance of preventing diabetes-related congenital malformations, however, the underlying pathogenic mechanisms have remained largely unknown. Pregestational diabetes mellitus is associated specifically with CHDs accompanied by heterotaxia. We have now examined left–right (L–R) axis formation in embryos of diabetic female mice as well as in mouse embryos exposed to high-glucose concentrations in culture. We found that high-glucose levels prevent establishment of the L–R axis required for heart morphogenesis and the L–R asymmetry of visceral organs. Such a mechanism may thus explain, at least in part, the CHDs and accompanying heterotaxia in the offspring of diabetic mothers.

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activates its own expression in the left LPM (27, 30, 33, 37). The localized expression of Nodal in the left LPM then expands to encompass the entire LPM as a result of the operation of the positive loop (27, 38). At the anterior end of the left LPM, Nodal signals in the left side of the heart primordium, which determines the direction of heart looping (19, 24). Nodal signaling in the left LPM and heart primordium induces the expression of the homeobox gene Pitx2 (39, 40), as a result of which visceral organs acquire their L–R asymmetric morphology. In the heart, the asymmetric expression of Pitx2 is not involved in looping but is required for proper morphogenesis, with Pitx2 mutant mice developing CHDs (21–23, 25, 26).

To understand the molecular etiology of CHDs and heterotaxia associated with maternal diabetes, we examined formation of the L–R axis in embryos derived from female mice with streptozotocin-induced diabetes, as well as in mouse embryos cultured in high-glucose medium. We found that high glucose attenuates Notch signaling in the node, which is followed by down-regulation of Nodal activity at the initial step of L–R axis formation. The reduction in Nodal activity in the node thus prevents the establishment of Nodal expression in the left LPM and consequent Pitx2 expression in the left LPM and heart primordium. Human CHDs accompanied by right isomerism might be explained by this mechanism.

Results

The L–R Axis Is Affected in Embryos of Diabetic Female Mice. To provide insight into the pathogenesis of heterotaxia in offspring of mothers with pregestational DM, we first examined embryos of female mice with streptozotocin-induced diabetes as an animal model. All E8.7 embryos (n = 126) derived from diabetic females (n = 10) with a blood glucose concentration of 300–490 mg/dL manifested normal looping of the heart tube to the right side (d-loop), as did control embryos of nondiabetic dams (Fig. 1A and H). In contrast, 6 of 10 litters from dams with a blood glucose level of >490 mg/dL included embryos with abnormal heart looping. In the affected six litters, 11 of 66 evaluable embryos manifested reversed heart looping (l-loop) (Fig. 1B, C, and H), whereas 10 embryos had the heart tube at the midline without apparent L–R asymmetry (Fig. 1D). Given that the embryos without asymmetric heart looping were at around the seven- to eight-somite stage, when the heart tube begins to elongate, the looping process may have been delayed in these embryos. Embryonic turning is another indicator of the L–R axis, with normal embryos showing clockwise rotation along the long axis as viewed from the cranial to caudal portion. Seven of 52 evaluable embryos in the six litters showing abnormal heart looping also manifested reversed embryonic turning (Fig. 1H). Taken together, these observations suggested that L–R asymmetric morphogenesis might be disturbed as a result of aberrant L–R axis formation in the offspring of female mice with streptozotocin-induced diabetes.

To examine whether L–R axis formation was indeed affected in such embryos, we performed in situ hybridization analysis of Pitx2 expression. Pitx2 is L–R asymmetrically expressed, with its expression being localized predominantly to the left LPM and heart primordium, as well as the head mesenchyme at E8.7 (Fig. 1.4 and E). The expression of Pitx2 was altered in the litters that included embryos with abnormal heart looping or turning, being lost either in both the left LPM and heart primordium (8 of 66 embryos) (Fig. 1B and F) or in only the left outflow tract (8 of 66 embryos) (Fig. 1D), whereas the expression in head mesenchyme was not affected. Of note, four embryos with Pitx2 expression in the left LPM and heart primordium showed an l-loop of the heart tube (Fig. 1C and G). Given that the asymmetric expression of Pitx2 is required for heart morphogenesis and L–R asymmetric organogenesis (20–23, 25, 26, 41), CHDs accompanied by heterotaxia in diabetic embryopathy are likely explained by the loss of Pitx2 expression in the left LPM and heart primordium (Discussion).

High Glucose Affects L–R Axis Formation in Mouse Embryos. The loss of Pitx2 expression indicated that the L–R axis is affected in embryos of diabetic female mice. To understand the mechanism by which the L–R axis is affected by hyperglycemia, we examined embryos exposed to a high-glucose concentration in whole-embryo culture (hereafter referred to as high-glucose embryos). We first determined the glucose concentration at which Pitx2 expression in the LPM is lost. Embryos at the head-fold stage (E7.75), before the onset of L–R axis formation, were cultured for 16 h in control (140 mg/dL) or high-glucose (390, 530, or 720 mg/dL) medium, during which time the embryos developed to the six- to seven-somite stage. Whereas more (21 of 23) control embryos expressed Pitx2 in the left LPM (Fig. 2A), the expression was lost in high-glucose embryos in a concentration-dependent manner [390 mg/dL, 7 of 16; 530 mg/dL, 12 of 16 (P < 0.0001); 720 mg/dL, 21 of 21 (P < 0.0001)] (Fig. 2B). To confirm that the change in osmolarity of the medium caused by glucose addition did not affect L–R axis formation, we added t-glucose at 580 mg/dL.
Fig. 2. Loss of Nodal expression in the LPM of embryos exposed to high glucose in culture. (A–G) Whole-mount in situ hybridization analysis of Pits2 (A–C), Nodal (D, E), and Lefty1 & Lefty2 (F, G) expression in embryos cultured in control (A, D, F), high–d-glucose (B, E, G), or high–c–glucose (C) medium from the 16h to 12h time points. The expression of Uncx was simultaneously examined as an indicator for somite number in D–G. Anterior views (A–C) or ventral views with anterior to the top (D–F) are shown. All isoforms of Pits2 mRNA are detected (A–C). Lefty1 and Lefty2 expression is simultaneously detected by corresponding specific probes (F, G). Black arrowheads indicate expression of Pits2, Nodal, or Lefty2 in the left LPM. White arrowheads indicate Lefty1 expression. Asterisk marks head mesenchyme. (Scale bar, 200 μm.) (H–K) Embryos cultured for 48 h from the head-fold stage in control (H, J) or high–c–glucose (I, K) medium. The placenta and yolk sac of the embryos in (H) and (J) were removed to show the ventral view of the heart in (J) and (K), respectively. White dashed lines show the direction of heart looping. (Scale bars in H and J, 400 μm.) (L) Summary of the numbers of embryos examined for the direction of heart looping and embryonic turning after culture for 48 h. Numbers in parentheses indicate embryonic stages. As well as the control culture, high–d–glucose medium showed normal expression of Pits2 (Fig. 2C), suggesting that d-glucose itself was responsible for the observed disturbance of the L–R axis formation.

Pits2 expression is observed in the left LPM (Fig. 2F) and larval yolk sac of the embryos in (H) and (J) were removed to show the ventral view of the heart in (J) and (K), respectively. White dashed lines show the direction of heart looping. (Scale bars in H and J, 400 μm.) (L) Summary of the numbers of embryos examined for the direction of heart looping and embryonic turning after culture for 48 h. Numbers in parentheses indicate embryonic stages. As well as the control culture, high–d–glucose medium showed normal expression of Pits2 (Fig. 2C), suggesting that d-glucose itself was responsible for the observed disturbance of the L–R axis formation.

To reproduce the L–R morphological defects observed in the offspring of female mice with streptozotocin-induced diabetes, we subsequently analyzed embryos cultured in high-glucose medium containing glucose at 720 mg/dL. Despite the presence of this high concentration of glucose, embryos at the head-fold stage achieved the stage corresponding to E9.25 after culture for 48 h, although the high-glucose embryos were smaller than control embryos (Fig. 2 H and J). With regard to the L–R axisymmetric morphogenesis, heart looping occurred normally in control embryos (20 of 20), whereas reversed heart looping was observed in 9 of 14 high-glucose embryos (P < 0.001) (Fig. 2 J–L). As a consequence of normal embryonic turning, the placenta becomes positioned on the right side of the embryo. For control embryos (19 of 20), the placenta was normally positioned on the right side, whereas the placenta was found on the left side for half (7 of 14; P < 0.01) of high-glucose embryos (Fig. 2 H, I, and L), suggesting that the turning process was randomized in these latter embryos. To further confirm that high glucose affects the process of L–R axis formation, we cultured embryos in high-glucose medium for the initial 14 h, during which time the L–R axis is established in control embryos, and in normal medium for the subsequent 34 h. The embryos still manifested reversed heart looping (6 of 12; P < 0.01) with the placenta positioned on the left side (5 of 12; P < 0.01) (Fig. 2L), suggesting that transient elevation of the glucose level at this initial stage can induce heterotaxia.

We next explored why Pits2 expression in the left LPM is lost in high-glucose embryos. Embryos at the four- to five-somite stage were cultured for 12 h to 14 somite stages. Transient expression of Nodal in the left LPM not only determines the direction of heart looping and embryonic turning, but also induces the expression of Pits2 (19, 24, 39). In most (16 of 20) control embryos at the four- to five-somite stage, Nodal was normally expressed in the left LPM (Fig. 2D). However, Nodal expression was not detected in any of the high-glucose embryos examined (Fig. 2E). As consequence of normal embryonic turning and Nodal expression domain to the left side (24, 27, 36, 42, 43). Lefty1 and Lefty2 were normally expressed in most control embryos at the four- to five-somite stage (9 of 14 for both Lefty1 and Lefty2) (2). In contrast, Lefty2 expression was lost (14 of 14; P < 0.001) and Lefty1 expression was detected in only a few cells of the node (11 of 14) in high-glucose embryos (Fig. 2G). The loss of Lefty expression in high-glucose embryos was likely because of the absence of Nodal expression in the left LPM. Taken together, these results thus indicated that high glucose inhibits Nodal expression in the left LPM, which in turn results in the loss of Pits2 expression and randomization of heart looping and embryonic turning.

LPM is Competent to Express Nodal in High-Glucose Embryos. Establishment of concordant L–R asymmetry of visceral organs requires expansion of Nodal expression in the entire LPM. Nodal expression in LPM is first induced locally by a signal from the node and then expands along the anteroposterior axis through the action of the Nodal positive loop (27–29, 36). Cryptic, which encodes a cofactor of Nodal signaling, is expressed in the LPM, floor plate, and node, and its expression is required for Nodal expression in the LPM (24). We found that Cryptic was normally expressed in these tissues in both control (n = 7) and high-glucose (n = 10) embryos (Fig. 3A and B). To examine whether the LPM is competent to express Nodal in high-glucose embryos, we subjected embryos at the four-fold stage to local transfection with Nodal and GFP expression vectors in the right posterior LPM, which time the L–R axis is established in control embryos, and in normal medium for the subsequent 34 h. The embryos still manifested reversed heart looping (6 of 12; P < 0.01) with the placenta positioned on the left side (5 of 12; P < 0.01) (Fig. 2L), suggesting that transient elevation of the glucose level at this initial stage can induce heterotaxia.
Smad2 in mouse embryonic stem (ES) cells exposed to high glucose. Binding of Nodal to Activin receptors induces the phosphorylation of Smad2 and its translocation to the nucleus, where it exerts its transactivation activity. Smad2 is phosphorylated in mouse ES cells as a result of the autonomous production of Nodal and Activin (44). As expected, pSmad2 was detected in the nuclei of ES cells cultured in standard medium (Fig. 3F). Whereas SB431542, a potent inhibitor of type I Activin receptor-like kinase receptors, attenuated Smad2 phosphorylation in ES cells, culture of the cells in high-glucose medium (containing high glucose at 850 mg/dL) for 12 h had no such effect (Fig. 3G and H), indicating that high glucose does not interfere with Nodal-Activin signaling in ES cells. Taken together, these results suggested that high glucose impairs left-right axis formation before the induction of Nodal expression in LPM rather than at the amplification stage.

**High Glucose Reduces Nodal Activity in the Node.** Nodal produced in the crown cells of the node is required for the subsequent expression of Nodal in the left LPM (30, 32, 37). We therefore next examined whether the expression of Nodal-related genes, including Nodal, Gdf1, and Cerl2, was affected in the node of high-glucose embryos. In cultured control embryos, Nodal expression was normally observed in crown cells at the three- to four-somite stage (n = 8) (Fig. 4A). In high-glucose embryos, however, the Nodal expression domain was found to be narrowed and slightly shortened and to have lost its crescent shape (n = 8; P < 0.001) (Fig. 4B). The product of Gdf1 expressed in crown cells and the LPM enhances the activity of Nodal through formation of a Nodal-GDF1 heterodimer (31). Gdf1 expression in the node appeared normal in control embryos (n = 10), whereas its domain in high-glucose embryos was also narrowed (7 of 12) and showed various degrees of shortening (n = 12) (Fig. 4C-D, and D′). These results thus suggested that the amount of Nodal-GDF1 produced in the node is reduced in the high-glucose condition. Expression of Nodal in the left LPM requires the activity of Nodal in crown cells to be regulated in an L-R asymmetric manner, with Cerl2 playing an essential role in such regulation through suppression of Nodal activity (32, 33, 35). Cerl2 is expressed in crown cells, with the level of expression being higher on the right side. In control embryos (13 of 14), the depth of staining for Cerl2 expression became symmetric as a result of culture, whereas the expression domain was larger on the right side than on the left side. In high-glucose embryos, however, Cerl2 expression was down-regulated, although the extent of this change was not as great as that for Nodal or Gdf1 (n = 15; P < 0.001) (Fig. 4E and F). The right-sided dominance of the Cerl2 expression domain remained apparent in most high-glucose embryos (12 of 15). Collectively, these results indicated that the expression of genes that determine the activity of Nodal in the node is attenuated in high-glucose embryos.

We next examined Nodal activity by detecting the distribution of pSmad2 in high-glucose embryos by immunohistochemistry. The phosphorylation of Smad2 at early somite stages depends on Nodal signaling, which is thought to occur first in the node and then to spread to the left LPM before the onset of Nodal expression in LPM (33). In cultured control embryos, pSmad2 was detected in crown cells and the left LPM at the four-somite stage (n = 17) (Fig. 4G and I). In high-glucose embryos, however, pSmad2 was not detected in either crown cells or the left LPM at the four- to five-somite stage (n = 23) (Fig. 4H and J). Instead, pSmad2 was detected only in pit cells of the posterior node (16 of 23 embryos; P < 0.001). These results suggested that the activity of Nodal produced in the node of high-glucose embryos is markedly reduced, resulting in the failure to express Nodal in the LPM.

**Notch Signaling Is Attenuated in the High-Glucose Condition.** We next explored why the expression of Nodal-related genes is attenuated in control embryos. To rule out the possibility that formation of the node is impaired in these embryos, we examined the expression of sonic hedgehog (Shh) and Foxa2, representative marker genes for the node and floor plate. Both Shh and Foxa2 were normally expressed in the node of high-glucose embryos (n = 9 and 11, respectively) at the three- to four-somite stage at levels similar to those observed in control embryos (n = 10 and 10) (Fig. 5A–D). Expression of FoxJ1 in the node is required for cilogenesis and the function of monocilia in the node (45). Expression of this gene appeared normal in the node of both control (n = 11) and high-glucose (n = 12) embryos (Fig. 5E and F). We next examined the morphology of the node in cultured embryos at the three- to four-somite stage by scanning electron microscopy. In some cultured embryos, large cells lacking a cilium were apparent among the pit cells (4 of 11 for control; 5 of 9 for high glucose) (Fig. SI B and C). Although most high-glucose embryos (7 of 9) manifested ciliated pit cells similar to those of control embryos (n = 11) (Fig. SI A–C), the pit cells lacking a cilium occupied a large area of the node in two high-glucose embryos (Fig. SI D). Leftward nodal flow was observed in high-glucose embryos as well as in control embryos (n = 11 for control; 7 of 9 for high glucose) (Movies S1 and S2). Even in the two high-glucose embryos with defective pit cells, a weak nodal flow was observed in the ciliated region (Movie S3). Taken
together, these results suggested that node formation was not severely impaired in most embryos exposed to high glucose.

Expression of Nodal in the node is induced by Notch signaling (46-48). We have also previously shown that Gdf1 and Cer12 expression in crown cells is extinguished by a Notch signaling inhibitor (48). We examined whether Notch signaling is affected in high-glucose embryos at early somite stages. Delta-like 1 (Dll1) induces Nodal expression in crown cells through interaction with its receptors Notch1 and Notch2 (46, 47). There was no apparent difference in the expression of Dll1 around the node (n = 12 and 11, respectively), in that of Notch1 in crown cells and paraxial mesoderm (n = 12 and 12), and in that of Notch2 in the node and paraxial mesoderm (n = 9 and 9) between control and high-glucose embryos (Fig. 5 G-L). We also examined the distribution of Notch1 in control and high-glucose embryos by immunofluorescence analysis. Notch1 was detected predominantly around the node including the crown cells, in the nascent mesoderm at the anterior primitive streak, and in newly generated somites in control embryos (n = 5) (Fig. 6 A-E). In high-glucose embryos, although the staining in the nascent mesoderm was irregular, we did not detect an apparent change in Notch1 distribution in crown cells (n = 6) (Fig. 6 B and E). We next examined whether Notch signaling might be affected in high-glucose embryos by immunostaining for the cleaved Notch1 intracellular domain (cNICD1) (49). We found that the amount of cNICD1 was significantly reduced in crown cells of the cleaved Notch1 intracellular domain (cNICD1) (49). We found that the amount of cNICD1 was significantly reduced in crown cells of high-glucose embryos (n = 13; P < 0.001) at early somite stages compared with that in control embryos (n =14) (Fig. 6 C-E). These results thus suggested that high glucose impairs Notch signaling in the node at the level or upstream of cleavage of the Notch intracellular domain, possibly accounting for the down-regulation of Nodal-related gene expression in crown cells.

In summary, high glucose was found to directly affect L-R axis formation in mouse embryos. Attenuation of Notch signaling in crown cells of the node may prevent the establishment of a sufficient level of Nodal and Gdf1 expression in the node of high-glucose embryos. Expression of Nodal is therefore not initiated in the left LPM of these embryos, leading to failure of Pitx2 expression. Thus, the loss of Nodal and Pitx2 expression likely accounts for CHDs and abnormal L-R asymmetric morphogenesis apparent in the offspring of diabetic mothers.

**Discussion**

Although case-control studies have shown that CHDs and accompanying laterality defects are associated with pregestational DM (1, 4, 15, 16), the molecular etiology of these defects has remained largely unknown. We have now shown that maternal...
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Inhibition of Notch signaling in the node by high glucose. Embryos
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Pitx2 in the node of high-glucose embryos, which probably accounts for the loss of Nodal expression in the LPM. Although the mechanism by which Notch signaling is suppressed by high glucose remains to be determined, our findings indicate that high glucose affects the initial stage of L–R axis formation. In addition, these findings suggest that a transient deviation of glycemic control at this stage, rather than continuous hyperglycemia at later stages of L–R axis formation, may give rise to CHDs in human patients.
Reversed heart looping or embryonic turning frequently occurred in litters of dams with a blood glucose concentration of >490 mg/dL. Our finding that the L–R axis is affected only at such high-glucose levels might explain the rarity of heterotaxia associated with pre-gestational diabetes in humans. It does not necessarily suggest, however, that heterotaxia does not occur at less-pronounced glucose levels in humans. Somitogenesis progresses faster in mice than in human embryos, suggesting that the period of L–R axis formation is also longer in humans. If the sum of excess glucose is the key determinant at this stage, then longer exposure to lower glucose levels may affect L–R axis formation in human embryos.
In mice, the failure to acquire Nodal expression in the LPM results in right isomerism of viscera and specific types of CHD. For example, in Cryptic−/− mutant mice, which do not establish Nodal expression in the LPM, the position of the stomach is L–R randomized in association with asplenia or hyposplenia in the abdomen, whereas right isomerism of the lung and atrium is apparent in the thorax in association in about half of neonates with dextrocardia or mesocardia (24). Of note, Cryptic neonates manifest CHDs, including transposition of the great arteries (TGA) and atrial septal defect (ASD). TGA associated with right isomerism has also been described in Nodal mutants (19, 23, 25, 26). Nodal is induced by Nodal signaling, a fundamental role in heart morphogenesis. Pitx2 mutant mice thus develop double-outlet right ventricle (DORV), complete atrioventricular canal defect (CAVC), ASD, and ventricular septal defect (20–23, 25, 26). Unfortunately, given that the culture period that can support embryonic development is limited, it was not possible to determine which type of CHDs would become manifest in embryos cultured in high-glucose medium. The loss of Nodal expression in the LPM of these embryos, however, would be expected to result in TGA and DORV associated with heterotaxia based on right isomerism. In support of this notion, DORV, TGA, and CAVC associated with right isomerism are often apparent in fetuses of female nonobese diabetic mice (17). On the other hand, cases of heterotaxia in offspring of human diabetic mothers include left isomerism (50). The mechanism underlying the development of such left isomerism remains unknown.
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expression in the LPM (37). Second, the amount of cNICD1 in crown cells was thus significantly reduced in high-glucose embryos. Despite its small size, the node plays a fundamental role in L–R axis formation. First, Nodal produced by crown cells induces Nodal expression in the LPM (37). Second, the L–R asymmetric activity of Nodal in the node determines the side on which Nodal is expressed in the LPM (32, 33, 35). Notch signaling is required for Nodal expression in the node (46, 47). In addition, the expression of Gdf1, which encodes a heterodimerization partner of Nodal, is also likely induced by Notch signaling (48). The attenuated expression of Nodal and Gdf1 in the node of high-glucose embryos is thus likely attributable to the down-regulation of Notch signaling. The activity of Nodal was virtually undetectable in the node of high-glucose embryos, which probably accounts for the loss of Nodal expression in the LPM. Although the mechanism by which Notch signaling is suppressed by high glucose remains to be determined, our findings indicate that high glucose affects the initial stage of L–R axis formation. In addition, these findings suggest that a transient deviation of glycemic control at this stage, rather than continuous hyperglycemia at later stages of L–R axis formation, may give rise to CHDs in human patients.
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In mice, the failure to acquire Nodal expression in the LPM results in right isomerism of viscera and specific types of CHD. For example, in Cryptic−/− mutant mice, which do not establish Nodal expression in the LPM, the position of the stomach is L–R randomized in association with asplenia or hyposplenia in the abdomen, whereas right isomerism of the lung and atrium is apparent in the thorax in association in about half of neonates with dextrocardia or mesocardia (24). Of note, Cryptic neonates manifest CHDs, including transposition of the great arteries (TGA) and atrial septal defect (ASD). TGA associated with right isomerism has also been described in Nodal mutants (19, 23, 25, 26). Nodal is induced by Nodal signaling, a fundamental role in heart morphogenesis. Pitx2 mutant mice thus develop double-outlet right ventricle (DORV), complete atrioventricular canal defect (CAVC), ASD, and ventricular septal defect (20–23, 25, 26). Unfortunately, given that the culture period that can support embryonic development is limited, it was not possible to determine which type of CHDs would become manifest in embryos cultured in high-glucose medium. The loss of Nodal expression in the LPM of these embryos, however, would be expected to result in TGA and DORV associated with heterotaxia based on right isomerism. In support of this notion, DORV, TGA, and CAVC associated with right isomerism are often apparent in fetuses of female nonobese diabetic mice (17). On the other hand, cases of heterotaxia in offspring of human diabetic mothers include left isomerism (50). The mechanism underlying the development of such left isomerism remains unknown.
Nodal expression spreads in the left LPM to provide L–R axis information to precursor cells of visceral organs that are constructed along the anteroposterior axis of embryos. The heart primordium at the anterior end of the LPM is the cell population that receives the Nodal signal last. Nodal expression expands in the LPM at the four-somite stage, when the heart primordium is crescent-shaped. Its morphology subsequently undergoes a marked change associated with the formation of the heart tube and loops. The time course of Nodal expression indicates that the heart primordium receives the Nodal signal at a specific stage of heart development. Given that high glucose reduces Nodal...
activity in the node, hyperglycemia at a threshold level may delay Nodal expression in LPM rather than completely abolish it. Although it is not known whether such a delay in Nodal expression in the LPM would affect heart morphogenesis, the precise area of Pitx2c expression might not be established in the heart primordium at more advanced stages. This scenario would be expected to give rise to CHDs without laterality defects of visceral organs. In support of this notion, four embryos with 0-glucose was added to the basal medium to a final concentration of 10 μM to suppress Nodal-Activin signaling. We have now shown that Notch signaling is affected by high glucose, suggesting that the multiple defects associated with diabetic pregnancy may be explained by impairment of separate developmental processes and different signaling cascades. Notch signaling plays a key role in various processes of development, and its disturbance causes human congenital defects, including CHDs and skeletal abnormalities (51). It will thus be important to investigate whether Notch signaling is affected in developmental processes other than L–R axis formation in diabetic embryopathy. Of note, we also found that the somites of high-glucose embryos are smaller along the anteroposterior axis compared with those in control embryos. The smaller somites observed in high-glucose embryos might possibly be related to the observation that the risk for costovertebral defects and VACTERL (vertebral defects, anal atresia, cardiac defects, tracheo-esophageal fistula, renal anomalies, and limb abnormalities) is increased in human diabetic embryopathy (52). Somitogenesis is regulated by oscillation of Notch signaling in presomitic mesoderm (49). Given that the expression pattern for cNICD1 itself appeared normal in presomitic mesoderm of high-glucose embryos at the resolution level of our experiments, the reason why somitogenesis is affected is unclear. A Notch reporter assay performed in HEK293T cells failed to reveal a suppressive effect of high glucose on reporter activity (Fig. S2), suggesting that the suppression of Notch signaling by high glucose might depend on cell type.

Materials and Methods

Mice. Imprinting control region mice were mated to obtain embryos. Noon of the day on which a vaginal plug was detected was designated as E0.5. Dams at E3.5 were injected intraperitoneally with streptozotocin (200 μg per gram of body weight) (Wako) to induce type 1 DM. Food (CR-LPF, Oriental Yeast) intake was limited to 5 g/d beginning 3 d after the injection, and 5% (wt/vol) glucose solution was given as drinking water from E7.5. Control female mice were fed ad libitum until E6.5 and at 5 g/d thereafter. The pregnant female mice were killed by cervical dislocation to collect embryos at the indicated stages. The blood glucose concentration of streptozotocin-treated mice was measured with a glucometer (Glucose Pilot, Aventir Biotech). The study was approved by the Animal Care and Use Committee of Kyushu University.

Whole-Embryo Culture. Embryos were removed from the decidua in DMEM supplemented with 10% (vol/vol) FBS. After removal of Reichert’s membrane, the developmental stage was determined on the basis of morphological criteria. The head-fold stage embryos at the early to late heart formation stage were transferred to a 50-ml conical tube containing 1 mL of DMEM with a 0-glucose concentration of 100 mg/dL and supplemented with 75% (vol/vol) rat serum. The embryos were then subjected to rotation culture at 37 °C in a humidified incubator containing 5% CO2. The glucose concentration of the culture medium as measured with a glucometer (GF501, TANITA) was 144 ± 16.5 mg/dL, depending on the lot of rat serum (mean ± SD for 32 lots). For high-glucose culture, 0-glucose was added to the medium up to the maximum measured concentration of 720 mg/dL. For long-term culture (48 h), the medium was replaced with fresh every 12 h after the initial 14 h. Where indicated, some embryos were returned to control medium after the initial 14 h of high-glucose culture. High-glucose culture was always performed simultaneously with control culture.

ES Cell Culture. Mouse ES cells (G4) were cultured in a gelatin-coated dish with 21 + LIF culture medium (53). The medium was based on DMEM containing 0-glucose at 450 mg/dL. For high-glucose culture, ES cells were incubated for 12 h in medium containing 0-glucose at 850 mg/dL (Sigma-Aldrich) was added to the basal medium to a final concentration of 10 μM to suppress Nodal-Activin signaling.

Local Transfection of Embryos. Lipofection solution (Lipofectamine2000, Invitrogen) containing Nodal and GFP expression vectors was transfected at the right posterior mesoderm with the use of a glass capillary connected to an injector (Narishige) (27). Injected embryos were then developed by whole-embryo culture.

Whole-Mount In Situ Hybridization. Embryos were fixed overnight with 4% (wt/vol) paraformaldehyde in PBS and then subjected to whole-mount in situ hybridization according to standard protocols. In brief, fixed embryos were incubated with PBS containing 0.1% Tween 20 (PBST) and dehydrated with increasing concentrations of methanol. After exposure to 6% (vol/vol) H2O2 in methanol, the embryos were rehydrated with PBST, treated first with proteinase K (10 μg/mL) in PBST and then with glycine (2 mg/mL) in PBST, and fixed again with 4% paraformaldehyde and 0.2% glutaraldehyde in PBST. After prehybridization, the embryos were incubated overnight with hybridization solution containing various digoxigenin-labeled probes, extensively treated with RNase A (50 μg/mL) and washed again. They were then exposed to 10% (vol/vol) sheep serum, incubated overnight with alkaline phosphatase-conjugated antibodies to digoxigenin (Roche), and washed extensively, after which the color was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. Probes included those for Pitx2, Nodal, Lefty1, Lefty2, Uncx, Gad1, Cryptic, Ceri2, Shh, Foxa2, Fox1, Notch1, and Notch2.

Immunostaining of Whole Embryos. Embryos were fixed for 20 min at 4 °C with 4% paraformaldehyde in PBS, washed with Tris-buffered saline containing 0.1% Tween 20 (TBST), treated with 100% methanol, and rehydrated with TBST. For the immunodetection of pSmad2, the embryos were bleached with 10% H2O2 in methanol for 20 min after the treatment with 100% methanol. Nonspecific sites were blocked by incubation for 30 min with 0.5% TSA Blocking Reagent (Perkin-Elmer). The embryos were then incubated with rabbit primary antibodies to cNICD1 (#2421, Cell Signaling), to the COOH terminus of Nodal (sc-6014, Santa Cruz Biotechnology), or to pSmad2 (#3101, Cell Signaling), all of which were diluted with blocking buffer (1:50). After washing with TBST, the embryos were incubated with Alexa Fluor 488-conjugated goat antibodies to rabbit IgG (Molecular Probes) and with TO-PRO3 (Molecular Probes) to detect nuclei. Immunostaining of control and high-glucose embryos was always performed simultaneously. Confocal images were acquired with a Leica TCS SPS system, and fluorescence of Alexa Fluor 488 and TO-PRO3 was converted to red and blue colors, respectively.

Quantitation of Immunofluorescence. Crown cells of the node were identified by 3D projection of TO-PRO3 confocal images (Leica LAS AF). Crown cells show...
The function of ImageJ. The pixel intensities of crown cells on the lateral plate of developing mouse embryos were measured with the "Analyze Particles" function of ImageJ. The pixel intensities of crown cells on both sides of the node were summed and averaged. The averaged value of Alexa Fluor 488 was then divided by that of TOPRO3 to correct for the bias because of the distance between the cleftorip and the specimen.

Statistical Analysis. The Wilcoxon rank sum test (R package) for comparison of immunofluorescence intensity of two-tailed Fisher's exact probability test for the other experiments was performed according to the null hypothesis of there being no differences between control and high-glucose embryos. A P value < 0.05 was considered statistically significant.

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