SUPPLMENTAL MATERIAL

## METHODS

### *Mice and reagents*

C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). *SOD1*-Tg mice in a C57BL/6J background were revived from frozen embryos by the Jackson Laboratory (stock no. 002298). *Wnt5a*$^{+/-}$ mice were provided by Dr. Terry Yamaguchi at the Center for Cancer Research, NCI-Frederick, NIH, as previously described[1]. *SOD1*-Tg male mice were mated with nondiabetic or diabetic female WT mice to generate SOD1 overexpressing and WT embryos. *SOD1*-Tg male mice mated with *Wnt5a*$^{+/}$- female mice to generate *SOD1; Wnt5a*$^{+/-}$ male mice. *SOD1; Wnt5a*$^{+/-}$ male mice mated with *Wnt5a*$^{+/-}$ female mice to Wnt5a null embryos with or without SOD1 overexpression. Streptozotocin (STZ) from sigma (St. Louis, MO) was dissolved in sterile 0.1M citrate buffer (pH 4.5). Sustained-release insulin pellets were purchased from Linplant (Linshin, CA).

### *Mouse models of diabetic embryopathy*

Twelve-week old wild-type (WT) female mice were intravenously injected with 75 mg/kg streptozotocin (STZ) on consecutive days. Diabetes was defined as 12 hours fasting blood glucose levels ≥ 250 mg/dL. Insulin pellets were subcutaneously implanted in these diabetic mice to restore euglycemia prior to mating. Insulin implantation during early pregnancy stages is essential for successful embryo implantation[2, 3]. Diabetic wild-type female (DM-WT) mice were then mated with SOD1 transgenic or wild-type male mice at 3:00 P.M to produce wild-type and SOD1 overexpressing embryos. Pregnancy was established by the presence of the vaginal plug the next morning and noon of that day was designated as Embryonic day 0.5 (E0.5). On E5.5,

insulin pellets were removed to ensure that the developing embryos would be exposed to hyperglycemia during the critical period of cardiogenesis (E8.5-E13.5). Nondiabetic WT (ND-WT) female mice with vehicle injection and sham operation of insulin pellet implantation/removal served as nondiabetic controls. On E11.5, E12.5 and 17.5, embryonic hearts were harvested for analyses. The findings in E11.5 hearts in molecular and biochemical analyses were identical or similar to those of E12.5 hearts. To avoid redundancy, data in E11.5 hearts was not included.

*India ink injection and Hematoxylin-eosin staining*

E17.5 hearts were collected for morphological examination. For India ink injections, mouse embryos were collected at E17.5 and diluted (1:100) India ink was injected into the left ventricle and perfused through the vascular system using μTIP (TIP10TW1-L, world precision instrument, Inc., Sarasota, FL). Hearts were then fixed in methacarn (methanol, 60%; chloroform, 30%; glacial acetic acid, 10%), embedded in paraffin, and cut into 8-μm sections. After deparaffinization and rehydration, all specimens then underwent hematoxylin and eosin (H&E) staining in a standard procedure. All heart sections were photographed and examined for heart defects.

*LPO (Lipid Hydroperoxide) Assay*

The degree of lipidperoxidation in the developing heart was assessed by the LPO assay using the Calbiochem Lipid Hydroperoxide Assay Kit (Millipore, Bedford, MA) as per the manufacturer's instructions. Briefly, E12.5 hearts were homogenized in HPLC-grade water. The lipid hydroperoxides of each heart were extracted by deoxygenated chloroform, and reacted with chromogen. The optical density was then measured at the absorbance of 500 nm. The results

were expressed as μM lipid hydroperoxides per gram protein. Protein concentrations were determined by the BioRad DC protein assay kit (BioRad, Hercules, CA).

*Ex vivo embryonic heart culture*

*Ex vivo* embryonic heart culture was performed as described by Hisayuki Hashimoto *et al*[4]. Briefly, E11.5 embryonic hearts were quickly explanted from nondiabetic WT (ND-WT) dams and placed in a 24-well plate casted with collagen gel (A10483-01, BD Gibco). The collagen gel was prepared in 5mM (low glucose, LG) in M199 culture media (M4530, Sigma) and then hydrated by warmed Opti-MEM media plus 1% fetal bovine serum (FBS, Gibco) and insulin-transferrin-selenium (ITS,, Corning). After incubated overnight at 37°C in a humidified atmosphere of 5% $CO_2$, hearts were culturedunder 5 mM, or 25 mM D-glucose (high glucose, HG) conditions with or without 5 mM Tempol, (Enzo Life Science) 1μM sodium peoxynitrite (CAS14042-01-4, Cayman Chemical Company Inc.), or 100μM $H_2O_2$ (Sigma) for 24 hours.

*Western blotting*

Western blotting was performed as previously described[5-7]. Briefly, E12.5 embryonic hearts were sonicated in ice-cold RIPA lysis buffer (Millipore, Bedford, MA) containing a protease inhibitor cocktail (Sigma, St Louis, MO). Nuclear protein extraction was prepared by using the EpiQuik Nuclear Extraction Kit (Epeigentek Group Inc, Farmingdale, NY). Proteins from different experimental groups were separated by 6%-12% SDS-PAGE and immunoblotted using primary antibodies at 1:1000 to 1:2000 dilutions in 5% nonfat milk. Antibodies of Dvl2, β-catenin, phosphor-(p-)GSK3β, p-CaMKII, NFAT2, NFAT4 and SOD1 were from Cell Signaling Technology (Boston, MA). Anti-caspase3 and anti-caspase8 were from Millipore (Bedford, MA). The antibody of Wnt5a was from R&D system (Minneapolis, MN). The intensity of the protein

bands were determined by densitometry and normalized by the densities of β-actin (Abcam, Cambridge, MA), or Histone H3 (Cell Signaling Technology, Boston, MA) for nuclear proteins or corresponding total proteins for phosphorylated proteins in the same preparation. Signals were detected using SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific, Rockford, IL). All experiments were repeated three times with the use of independently prepared tissue lysates.

*Real-time PCR*

Total RNA was isolated from embryos using an RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed by using the high-capacity cDNA archive kit (Applied Biosystem, Grand Island, NY). RT-PCR for Wnt ligands (Wnt1, 2a, 3a, 5a, 7b, 8a), Wnt antagonists ( WIF1 (wnt inhibitory factor 1), sFRP1 (secreted frizzled-related protein 1), DKK1 (dickkopf 1), Wnt target genes (*Islet1*, *GJA1* (gap junction alpha 1), *Versican*, *Mrtf-b* (myocardin related transcription factor B), *Tpm1* (alpha tropomyosin 1), *Rcan1* (regulator of calcineurin)) and *β-actin* were performed using Maxima SYBR Green/ROX qPCR Master Mix assay (Thermo Scientific, Rockford, IL). RT-PCR and subsequent calculations were performed by using the StepOnePlus real-time PCR system (Applied Biosystem, Grand Island, NY).

*Immunofluorescence and DHE staining*

E12.5 hearts were fixed in 4% paraformaldehyde overnight followed by embedding in OCT (optimal cutting temperature, Sakura finetek, Torrance, CA) compound. 10-μm cryosections of heart tissues were antigen-unmasked using citrate buffer and blocked in 5% bovine serum albumin in PBST (0.1% Triton X-100 in PBS) for 1 hour. The following antibodies were used as primary antibodies: β-catenin (1:200) (Cell Signaling Technology,

Boston, MA), Wnt5a (1:50) (ThermoFisher Scientific, Rockville, MD) and p-Histone H3 (1:100) (Millipore, Bedford, MA). Normal rabbit or mouse IgG at the same dilutions as those for antibodies was used as controls. Sections were counterstained with DAPI and mounted with aqueous mounting medium (Sigma, St Louis, MO). Images were captured using an inverted microscope (Nikon Eclipse E1000M). For fluorescence detection of superoxide, the frozen heart sections were incubated with 1.5μM DHE (dihydroethidium) for 5 min at room temperature and then washed for 3 times with PBS, 5 min each time. Sections were counterstained with DAPI and mounted with aqueous mounting medium (Sigma, St Louis, MO). The DHE staining has been successfully used in assessing superoxide levels in tissue sections such as kidney sections[8]. However, the DHE → E reaction may detect ROS broadly including hydrogen peroxide and hydroxyl, in addition to superoxide. For the evaluation of cell proliferation, p-Histone H3 positive cells were counted on three heart sections from three different dams per group.

*TUNEL Assay*

The TUNEL assay was performed as previously described by using the In Situ Cell Death Detection Kit (Millipore, Billerica, MA)[2, 7]. 10-μm serial heart frozen sections were fixed with 4% paraformaldehyde in PBS and incubated with TUNEL reagent. Sections were counterstained with DAPI and mounted with aqueous mounting medium (Sigma, St Louis, MO). TUNEL-positive cells in the heart of each section were counted. Heart sections from three embryos of different dams per group and three sections per heart were analyzed. The percentage of apoptotic cells was calculated as number of apoptotic cells divided by 200 cells in a selected area.

## References


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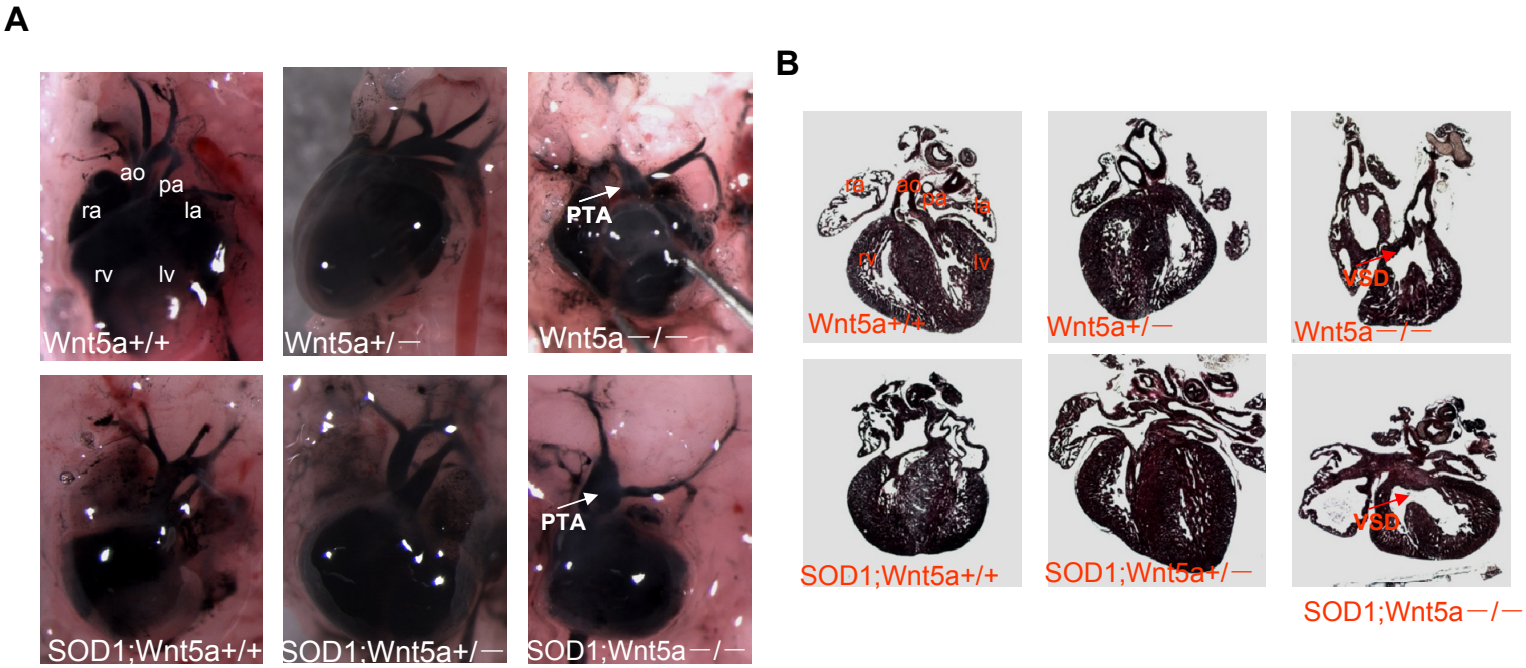
**Supplementary Table 1.** Effect of SOD1 overexpression on heart defect incidence in Wnt5a null embryos

| | Experimental group | Blood glucose levels (mg/dl) | Embryo Genotype | Number of embryos | Number with heart defects |
|---|---|---|---|---|---|
| ND | SOD1; $Wnt5a^{+/-}$ ♂ x $Wnt5a^{+/-}$ ♀ (9 litters) | 103.2±12.6 | $Wnt5a^{+/+}$ | 11 | 0 |
| | | | $Wnt5a^{+/-}$ | 15 | 0 |
| | | | $Wnt5a^{-/-}$ | 5 | 5 |
| | | | SOD1;$Wnt5a^{+/+}$ | 7 | 0 |
| | | | SOD1;$Wnt5a^{+/-}$ | 12 | 0 |
| | | | SOD1;$Wnt5a^{-/-}$ | 5 | 5 |

ND: nodiabetic; +/+: wild-type; +/-: heterozygous; -/-: Wnt5a null.




**Supplementary Figure 1.** Heart defects in Wnt5a null embryos with or without SOD1 overexpression. A, Representative images of India ink injections revealed persistent truncus arterious (PTA) in Wnt5a$^{-/-}$ (null) embryos with or without SOD1 overexpression. B, Representative images of serial heart sections showed ventricular septum defect (VSD) in Wnt5a null embryos. AO: Aorta; PA: Pulmonary Artery; LA: Left Atrium; RA: Right Atrium; LV: left ventricle; RV: right ventricle.