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Generating double knockout mice to model genetic intervention for diabetic cardiomyopathy in Humans

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Summary

Diabetes is a rapidly increasing disease that enhances the chances of heart failure 2–4 folds (as compared to age and sex matched non-diabetics) and becomes a leading cause of morbidity and mortality. There are two broad classifications of diabetes: type1 diabetes (T1D) and type2 diabetes (T2D). There are several mice models that mimic both T1D and T2D in humans. However, the genetic intervention to ameliorate diabetic cardiomyopathy in these mice often requires creating double knockout (DKO). In order to assess the therapeutic potential of a gene, that gene is either overexpressed (transgenic expression) or abrogated (knockout) in the diabetic mice. If the genetic mice model for diabetes is used, it is necessary to create DKO with transgenic / knockout of the target gene to investigate the specific role of that gene in pathological cardiac remodeling in diabetics. One of the important genes involved in extracellular matrix (ECM) remodeling in diabetes is matrix metalloproteinase-9 (Mmp9). Mmp9 is a collagenase that remains latent in healthy hearts but induced in diabetic hearts. Activated Mmp9 degrades extracellular matrix (ECM) and increases matrix turnover causing cardiac fibrosis that leads to heart failure. Insulin2 mutant (Ins2+/-) Akita is a genetic model for T1D that becomes diabetic spontaneously at the age of 3–4 weeks and show robust hyperglycemia at the age of 10–12 weeks. It is a chronic model of T1D. In Ins2+/- Akita, Mmp9 is induced. To investigate the specific role of Mmp9 in diabetic hearts, it is necessary to create diabetic mice where Mmp9 gene is deleted. Here, we describe the method to generate Ins2+/- /Mmp9-/- (DKO) mice to determine whether the abrogation of Mmp9 ameliorates diabetic cardiomyopathy.

Keywords

Ins2+/- Akita; Mmp9; diabetes; heart failure; cardiomyopathy

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1. Introduction

The alarming rate of diabetics [1] and its impact on cardiovascular diseases [2–8] make it a greatest challenge for medical sciences. Diabetes is a global menace [9, 10] that requires global attention. T1D are typically present in the young (therefore also called juvenile diabetes). It is caused either due to mutation of insulin gene that impairs glucose metabolism [11] or developed as an autoimmune disease, where pancreatic beta cells are targeted and destroyed by immune cells [12, 13]. The beta cells are responsible for producing insulin that regulate glucose metabolism. After food intake, blood glucose level increases that triggers signals to release insulin from pancreatic beta cells. Insulin activates Insulin receptor signaling that removes excess glucose from the blood into different cells in body using glucose transporter (GLUT4). It also helps in glucose metabolism (Figure 1). When beta cells are less or absent due to either genetic mutation or autoimmune destruction, the insulin released in response to high blood glucose level is either too less or absolutely absent. This causes low levels of insulin and high levels of glucose in the blood, a signature of T1D (Figure 1). T1D is less prevalent (~5%) in the USA population [14, 15]. Insulin injection before meal is the only clinical treatment practiced for regulating hyperglycemia in T1D patients [12]. Insulin injection sometimes causes hypoglycemia and fluctuation of hyper-to-hypo-glycaemia has devastating impact on different organs including the heart. Heart is a sophisticated but vulnerable organ and diabetes increases the incidence of heart failure [5, 16, 17]. Therefore, investigating an intervention tool to mitigate diabetic cardiomyopathy is indispensable.

T2D results due to insulin insensitivity or resistance and is more prevalent (~90%) [18–21]. In T2D, although beta cells release insulin in response to elevated blood glucose level, insulin is unable to trigger GLUT4 due to defective insulin receptor signaling that results into increased level of glucose in the blood (Figure1). High level of blood glucose triggers beta cells to constantly release insulin; the insulin level becomes high in blood. Therefore, T2D is diagnosed by increased levels of both glucose and insulin. Chronic T2D puts extensive workload on beta cells to secrete insulin that ultimately causes their death. Therefore, long-term T2D leads to T1D.

We have reported that diabetes induces Mmp9 [22, 23] that in turn impairs contractility of cardiomyocytes [24], induces cardiac fibrosis [23, 25] and differentially express several miRNAs [24] that regulates cardiac functions. Recently, we have demonstrated that Mmp9 is also involved in cardiac stem cell survival and may be involved in their differentiation into cardiomyocytes [25, 26]. We also found that ablation of Mmp9 mitigates cardiac fibrosis [25]. Clinical study demonstrates that MMP9 (human matrix metalloproteinase-9) decreases survival probability and exacerbates mortality in heart failure patients with diastolic dysfunction [27]. Mmp9 is also implicated in hypertension and left ventricle remodeling [28–31]. Ablation of Mmp9 decreases the infarct size in ischemia-reperfusion injury [32].

Since Mmp9 is up regulated in diabetic hearts [23], we created DKO (Ins2+/- /Mmp9-/-) mice, where Insulin gene is mutated (T1D) and Mmp9 gene is deleted to assess the specific role of Mmp9 in diabetic cardiomyopathy. The process of generating DKO can be used for combination of other genes. After validating the expression of Mmp9 by genotyping, in-gel-

gelatin zymography and immunohistochemistry; cardiac fibrosis was determined in the three groups: WT, Ins2+/- and Ins2+/- MMP9-/- . Interestingly, fibrosis was significantly decreased in Ins2+/- /Mmp9-/- as compared to the Ins2+/- hearts [25]. We also determined the mitigation of cardiac dysfunction in DKO mice by measuring the levels of atrial natriuretic peptide (a marker of cardiac hypertrophy) (Figure 4). The hypertrophy can also be observed by staining the cell membranes with wheat germ agglutinin (Invitrogen) (Figure 5).

2. Materials

Mice should be ordered from authorized vendor such as Jackson Laboratory.

1. Mice: Ins2+/- Akita The Jackson Lab stock number is 003548 and the genetic background is C57BL/6J) and Mmp9-/- (The Jackson Lab stock number is 007084 (B6.FVB (Cg)-Mmp9^{tm1Tvu}/J).
2. Glucose measurement: Glucometer, glucose strips.
3. Genotyping: Scissors, Ice cold ethanol (see Note 1), DNA extraction kit (Qiagen, USA).
4. Nanodrop for measuring quality and quantity of DNA, PCR instrument, PCR master mix, primers for Insulin and Mmp9 gene, nuclease free water, Agarose powder (Sigma,USA) TAE buffer (BioRad, USA), Ethidium bromide (Invitrogen, USA), Gel apparatus, BioRad ChemiDoc instrument (Note 2).
5. 0.1% gelatin (Sigma, USA)

3. Methods

3.1. Cross –breeding of Ins2+/- and Mmp9-/- to create Ins2+/- /Mmp9-/-

In mouse, there are two Insulin genes (Insulin 1 and Insulin 2) but in human, only one Insulin gene is present. Insulin2 gene of mouse is orthologous to human Insulin and mutation of this gene causes T1D. Insulin gene is located on chromosome 7 whereas Mmp9 gene is located on chromosome 2. After genotyping mice, Ins2+/- females are crossbred with Mmp9-/- male (female: male= 2:1). Since Ins2+/- is heterozygous, the two alleles are different (+ and -). In F1 generation, the two alleles of Ins2+/- are hybridized with one allele (+) of Mmp9 to make two types of genotypes (+/+) and (+/-). For Mmp9 gene there is only “+” allele in Ins2+/- Akita and “-” allele in Mmp9-/- mice and in F1 hybrids only one genotype “+/-” are present. There are two genotypes for F1 hybrids: Ins2+/+ / Mmp9+/- and Ins2+/- /Mmp9+/- . The Ins2+/- /Mmp9+/- heterozygous females are selected for backcrossing with Mmp9-/- males. There are “+” and “-”alleles for both Ins2 and Mmp9 genes in the Ins2+/- /Mmp9+/- hybrids. In F2 generation, the genotypes are “Ins2+/+ / Mmp9+/-”, “Ins2+/- / Mmp9+/-”, “Ins2+/- / Mmp9-/- (DKO)”. The scheme of generating DKO mice is given in Figure 2. The DKO are validated by genotyping as well as expression assay. For MMP9 activity, gelatin zymography are performed. Mmp9 expression is also validated at immunohistochemistry level by measuring fluorescence intensity [25].

3.2. Genotyping of *Ins2*^{+/-}, *Mmp9*^{-/-} and *Ins2*^{+/-} /*Mmp9*^{-/-} mice

3.2.1 DNA extraction—DNA is extracted from the tail of WT, Akita, and DKO mice following the manufacturer's protocol for the kit

Cut 0.4–0.6 cm length of mouse tail and store it in –20°C until DNA extraction

1. Mix 180 µL buffer ATL and 20 µL of proteinase K, which are supplied with the kit, and incubate at 56 °C under rotation for overnight or until completely lysed.
2. For each tail, add 410 µL of buffer AL – ethanol (205 µL AL + 205 µL ethanol) and mix by vortexing.
3. Transfer the mixture to a DNeasy Mini spin column in a 2 mL collection tube and centrifuge at 3300g for 10 min.
4. Discard the collection tube and the flow-through, and place the column in a new 2 mL collection tube.
5. Add a volume of 500 µL AW1 buffer to the spin column and centrifuge at 3300g for 5 min.
6. Discard the flow through and the collection tube, and the column is placed into a new collection tube.
7. Add the same volume (500 µL) of AW2 buffer to the column and centrifuged at 3300g for 15 min.
8. After discarding the flow through and the collection tube, place the column into another new collection tube, and add 200 µL of AE buffer for elution. Incubate it for 1 min at room temperature and then centrifuge for 2 min at 3300g.

The flow through (DNA) was collected and stored at –20 °C for polymerase chain reaction (PCR).

3.2.2. Polymerase chain reaction (PCR)

1. For genotyping the *Insulin2* and *Mmp9* mutants, follow the PCR primers and programs from the Jackson Laboratory protocols.
2. For *Insulin 2* gene, the following primers can be used: forward, 5'-TGC TGA TGC CCT GGC CTG CT-3'; and reverse, 5'-TGG TCC CAC ATA TGC ACA TG- 3'.
3. The PCR program is : 94 °C, 3 min; 94 °C, 20 s; 64 °C, 30 s; decrease in temperature of –5 °C per cycle; 72 °C, 35 s; repeat steps for 12 cycles, 94 °C, 20 s; 58 °C, 30 s; 72 °C, 35 s; repeat steps for 25 cycles; 72 °C, 2 min; 10 °C, hold.
4. For *Mmp9* gene, 4 set of primers: 2 sets for the mutant, and 2 sets for wild type gene. The primers for the mutant gene as follows: forward, 5'- CTG AAT GAA CTG CAG GAC GA-3'; and reverse, 5'-ATA CTT TCT CGG CAG GAG CA-3'.

5. For wild type gene of Mmp9, the primers are: forward, 5'-GTGGGACCA TCA TAA CAT CAC A-3'; and reverse, 5'-CTC GCG GCA AGT CTT CAG AGT A-3'. The cycling conditions are as follows: 94 °C, 3 min; 94 °C, 30 s; 66 °C, 1 min; 72 °C, 1 min; repeat steps for 35 cycles; 72 °C, 2 min; 10 °C, hold.

3.2.3. Agarose gel electrophoresis

1. Separate the PCR product by electrophoresis using 1.5% Agarose gel, and ethidium bromide (0.008%) and visualize the bands under UV light.
2. For 100ml of 1.5% Agarose gel, weigh 1.5 g of Agarose and dissolve in 1X TAE buffer and boil it for 2 min.
3. Allow it to cool down to ~ 60°-70°C and then add 8µl of ethidium bromide.
4. Pour the solution into the Agarose gel cast and allow it to solidify.
5. Perform gel electrophoresis in 1X TAE buffer at 70V.

3.2.4. Analyses of bands

1. In Akita mice, Insulin2 gene is heterozygous. The Insulin2 gene amplification product will show 2 bands of 140 and 280 base pairs.
2. The WT mice will have only 140 bp band.
3. In DKO (Ins2+/- /Mmp9-/-) mice, the PCR product for Mmp9 gene primer will show single band at 172 bp.
4. In WT mice, there will be single Mmp9 band of 277 bp, whereas in heterozygous mice there will be 2 bands of 172 and 277 bp [25] (Figure 3).

3.3. In-gel-gelatin zymography

1. Prepare SDS –substrate PAGE with gels with 0.1% gelatin in both the stacking and resolving gels.
2. To prepare the 0.1% gelatin solution, weigh 0.1g of gelatin and dissolve in 100ml of distilled water. Gelatin will not be dissolve at room temperature. Heat the solution to 50–60°C until the gelatin is completely dissolved (Note 3).
3. Cool down the solution to room temperature and prepare the SDS PAGE gel with stacking (4–5%) and resolving (7.5–10%) gels, in place of water use this 0.1% gelatin solution.
4. For sample preparation, take the desired concentration of protein and add 2x sample buffer (0.5M Tris HCl, pH 6.8– 2.5 ml; Glycerol- 2.0ml ; 10% SDS –4ml, 0.1% bromophenol blue- 0.5ml and make up the final volume to 10 ml with distilled water).
5. Mix the protein and 2X sample buffer and keep it at room temperature for 10 minutes (Note 4).

6. Load 10–30 μ l protein samples and perform gel electrophoresis in 1X Tris-Glycine-SDS running buffer at 70 V.
7. Once the tracking dye passes through the bottom of gel, remove the resolving gel and wash it in distilled water.
8. Incubate the resolving gel in 1X re-naturing buffer (Triton $\times$ 100 – 2.5% v/v in distilled water) with gentle shaking for 30 minutes. Repeat this step two times, and each time use fresh re-naturing buffer.
9. After this, keep the gels in 1X developing buffer (Tris base 1.21 g, Tris HCl-6.3g, NaCl –11.7g, CaCl₂ –0.74, Brij 35–0.02% in 1 L of distilled water) at 37°C at slow shaking for 48 hours.
10. Wash the gels with distilled water and stain the gels with Coomassie blue R-250 (0.5% w/V in methanol) for 30 minutes.
11. To see the clear bands wash the gels with distilled water.
12. De-staining solution (30% methanol and 10% v/v acetic acid) can also be used to remove the excess stain of the gel.
13. Gelatinase activity could be seen in any Gel Doc instrument with UV light as cleared (unstained) regions on the blue background.

3. Notes

1. When genotyping is done in 10–21 days old mice, ice cold ethanol is used as local anesthesia before clipping 1–2 mm tail.
2. Any Gel Doc instrument with UV light will serve the purpose.
3. Excessive heat or boiling of sample may degrade gelatin. Since gelatin is the substrate for Mmp9, it is necessary to dissolve the gelatin without boiling it.
4. The samples should not be boiled as it alters the quaternary structure of protein and impairs the enzymatic activity of Mmp9.

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Figure 1:
Schematics showing the process of glucose metabolism and their dysfunction in T1D and T2D.

Figure 2:
Scheme for generating Ins2^{+/-} /Mmp9^{-/-} (DKO) from Ins2^{+/-} and Mmp9^{-/-} mice.

Figure 3:

The genotypes of WT, Akita, Mmp9^{+/-} and Mmp9^{-/-} mice.

A) PCR amplification of Insulin2 gene showing two bands in WT (Ins2^{+/+}) and one band in Ins2^{+/-} mice.

B) PCR amplification of Mmp9 gene showing genotypes for Mmp9 knock out (Mmp9KO), WT (Mmp9^{+/+}), double knockout homozygous (DKO) (Ins2^{+/-} /Mmp9^{-/-}) and DKO heterozygous (Ins2^{+/-} /Mmp9^{+/-}HT) mice.

Figure 4.

The expression of Atrial Natriuretic Peptide (ANP) in the hearts of WT, Akita, and double knockout (DKO) mice. A) Representative bands (Western blot) for the expression of ANP and Actin (loading control). B) Bar graph showing the relative expression of ANP in the three groups. N=3, *p<0.05.

Figure 5. A.

Fluorescent wheat germ agglutinin (WGA, green color) is used to demarcate cell boundaries as it stains cell membrane. DAPI (blue) is used to stain cell nuclei. The transverse sections of hearts of WT, Akita and DKO mice are used. **B.** Bar graph showing diameter of the cardiomyocytes, which is used to measure cardiomyocyte hypertrophy, *, $p < 0.05$.