

# Functional role of NKX2-5 and Smad6 expression in developing rheumatic heart disease

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**Abstract. – OBJECTIVE:** Rheumatic heart disease (RHD) results due to the cross reaction of the host immune system when it develops immunity against group A streptococcal infection. This autoimmune disease progress with different pathological conditions and the genes associated with it are still less understood.

**MATERIALS AND METHODS:** To understand the role of NKX2-5 and Smad-6 in developing an RHD, we successfully developed RHD model using BALB/c mice and we evaluate the expression of NKX2-5 and Smad-6 in different conditions.

**RESULTS:** The disease conditions are confirmed through histological sectioning of RHD heart tissue with its associated Aschoff bodies. The histological of control heart tissue in the absence of NKX2-5 looks abnormal with an enlarged nucleus and in the absence of Smad-6 the solid nature of heart tissue loosens. The mice developed a complex form of acute RHD with tissue hardening in the absence of either NKX2-5 or Smad-6 which are confirmed in NKX2-5 or Smad-6 null mice. Immunohistochemical studies reveal that the NKX2-5 and Smad-6 expression get down regulated on developing with RHD. Through experiments, we detected that both NKX2-5 and Smad-6 are both inter-dependable and it negatively regulated each other by inhibiting them. In the absence of NKX2-5 or Smad-6, a severe form of RHD is observed together with down-regulation of either NKX2-5 or Smad-6.

**CONCLUSIONS:** The present investigation of NKX2-5 and Smad-6 in RHD provides a new insight of data that helps to understand the disease pathogenesis.

*Key Words:*

Rheumatic heart disease, NKX2-5, Smad-6, Aschoff bodies, BALB/c mice.

## Introduction

Rheumatic Heart Disease (RHD) is still a major health problem in developing countries that results with heart valve damage<sup>1</sup> as well as valve deformation<sup>2</sup>. Each year it was calculated that

around 500,000 individuals are died due to Rheumatic heart disease<sup>3,4</sup>. The reason for developing RHD was due to the cross reactions that are associated with *Streptococcus pyogenes*<sup>5</sup>. All RHD occur as a result of rheumatic fever and it only developed into RHD at the adverse stage of rheumatic fever<sup>6</sup>. Understanding the risk factors that result into RHD is more critical in preventing the disease. The genes associated with the pathogenesis of RHD are more complex to understand and up to now little is known related to RHD<sup>7</sup>.

The most of the genes that are identified as a primary cause of rheumatic fever are related to immune response but latter pathological changes associated with RHD are results from cellular genes<sup>8,9</sup>. Among the different genes that are related to heart valve the role of NKX2-5 and Smad6 are important because any mutation in this gene results with deformities in heart valve and impairment in their development<sup>10,11</sup>. NKX2-5 is a vertebrate homeobox gene that regulates many transcriptional factors that are involved in cardiac development and it also helps to maintain the homeostasis of an adult heart<sup>12</sup>. Another important role of NKX2-5 is that it is essential for the differentiation of the cardiac cells and its absence has a direct effect on cardiac development<sup>13,14</sup>. Similarly, Smad6 is also more important in maintaining the cardiac homeostasis because its suppression could result into hyperplasia and metaplasia<sup>11</sup>.

The T cells are the main reason for developing valve damage<sup>15</sup>, but still the molecular level of a series of reactions that contribute to valve damage are not still clear<sup>16</sup>. The role of NKX2-5 and Smad6 in cardiac development and maintaining its homeostasis are still gaining new improvement in understanding to define its function. But it cannot so far analyze in the context of RHD. In this manuscript we used an RHD mouse model to identify the role of NKX2-5 and Smad6 in developing RHD.

## Materials and Methods

### *Experimental Animals*

To initiate the animal model with RHD, the BALB/c mice were developed with myocardial lesions which mimic condition similar to RHD. To initiate this BALB/c female mice were subcutaneously injected with 500 µg of group A streptococcal recombinant M5 protein. Following them, the mice were intraperitoneally injected with 0.3 µg of pertussis toxin on 1<sup>st</sup> and 3<sup>rd</sup> day after initial injection immunization following them booster dose of 500 µg of group A streptococcal recombinant M5 protein was given on the 7<sup>th</sup> day as described previously<sup>17</sup>. The mice were undisturbed for till 4<sup>th</sup> weeks after that the mice were analyzed and examined for RHD. The control mice were injected with 1X PBS. NKX2-5 and Smad-6 null mice were also used in these experiments. The NKX2-5 null mice obtained from an experimental animal resource unit of the University of Michigan Medical Center, USA, and Smad-6 null mice were obtained from Millennium Pharmaceuticals. The mice that are undergoes this present study were approved by the host institutional ethical committee.

### *Immunohistochemistry*

The heart tissue dissected from control and RHD heart was formalin fixed by keeping them in 37° C for 24 hrs. The tissues were then washed with water and subjected to dehydration using gradient increase in ethanol concentration. After clearing step by using xylene, the tissue was paraffin embedded. The tissues were then sliced using fine blades using a microtome to a thin size of 6 µm. The ribbons of sections were placed in a glass slide and they were de-paraffinized using xylene. The endogenous peroxidase activity was subsequently blocked by dipping them in freshly prepared 3% H<sub>2</sub>O<sub>2</sub> in methanol for 20 mins. It is followed by heat-induced antigen retrieval in 10 mmol/L citrate buffer (pH 6.0). Nonspecific sites in sections were blocked by incubating them in 10 % serum in PBS for 1 hour. Following them the primary antibody, anti-NKX2-5 antibody [2E1], Abcam, (ab91196) or anti-Smad-6 antibody [4F3] Abcam, (ab110156) was placed over the sections and incubating for 1 hour at room temperature. The sections were then washed and allowed to react with the suitable secondary antibody. After washing, DAB (diaminobenzidine) staining was performed to identify the specific protein and the results obtained are documented.

### *Western Blot Analysis*

The tissue samples from normal and RHD heart tissue were dissected to prepare the protein cell lysate. The resulting cellular proteins were subjected to 12% SDS-PAGE gel by following the protocol as previously described<sup>18</sup>. After that, the membrane was blocked with 4% bovine serum albumin (BSA) solution, then the membrane was incubated in 4° C for 6 hours with primary antibody, anti-NKX2-5 antibody [2E1], Abcam, (ab91196) or anti-Smad-6 antibody [4F3] Abcam, (ab110156) in the dilution ratio of 1:500. After washing the non-specific binding of primary antibody, the membranes were incubated further with the suitable secondary antibody of dilution concentration (1:5000). After washing, the membrane was developed to obtain the specific signal.

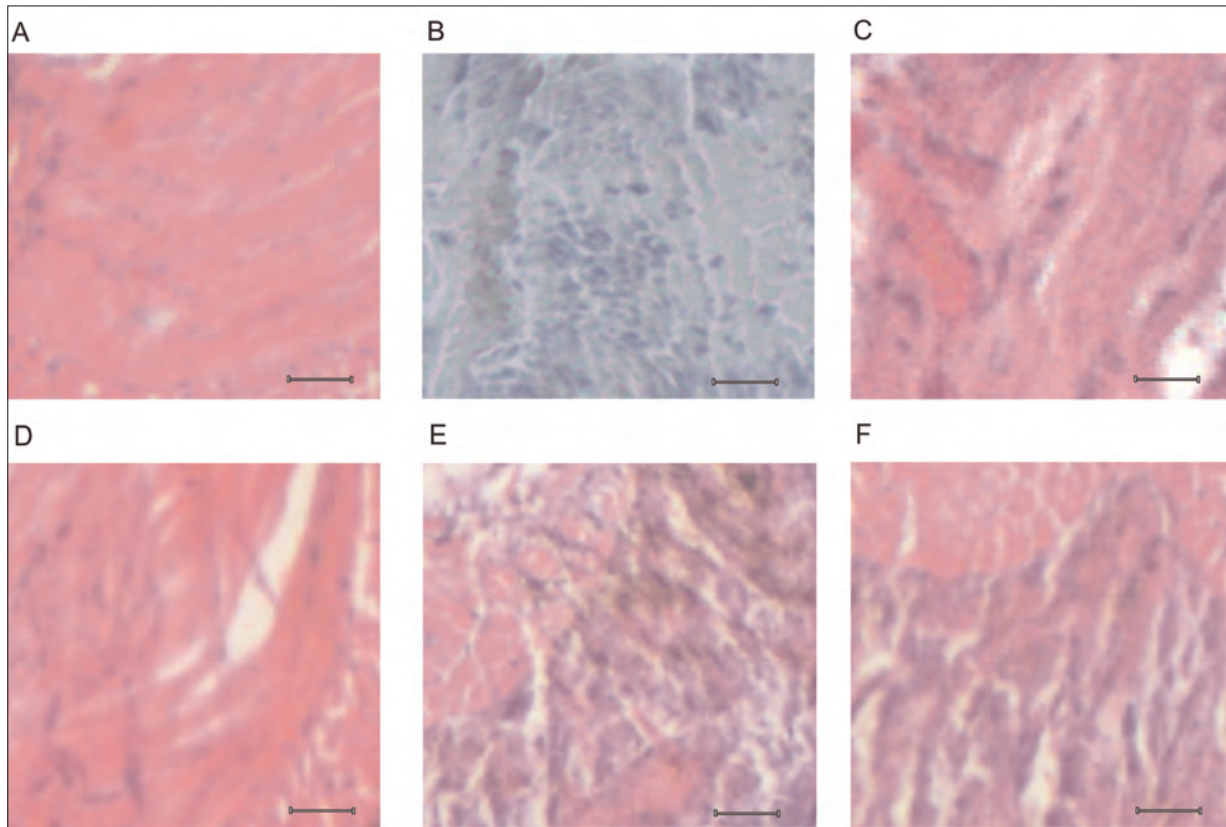
## Results

### *Histopathological Variations Between Normal and RHD Heart Tissue*

To analyze the histopathological changes associated with RHD in the context of NKX2-5 and Smad-6. The normal mice heart tissue, RHD mice heart tissue, normal and RHD mice, which are null for NKX2-5 and Smad-6 were subjected to histological analysis and their results are documented as shown in Figure 1. The normal mice heart tissues are solid in nature and it is also having striations with equal sized cell nucleus (Figure 1A). In case of RHD, the heart tissues were developed with cardiac lesions (Figure 1B). The lesions were primarily developed due to the formation of Aschoff bodies, which are resultant with the accumulation of activated T cells.

### *Nkx2-5 and Smad-6 Null Mice Shows Adverse RHD*

The heart tissue lacking NKX2-5 expression shows abnormal cells with elongated nucleus, additionally, the regular solid pattern of tissue get disturbed (Figure 1C). Similarly, the heart tissue lacking Smad-6 shows mild histological variations; especially we observed the solid packing of the heart tissue is disturbed (Figure 1D). The NKX2-5 and Smad-6 Null mice show more adverse histopathological variations when they were developed with RHD. The NKX2-5 null mouse with RHD shows more acute inflammation with advanced development of Aschoff bodies together with hardening of tissue (Figure



**Figure 1.** Histopathological variations observed between normal and RHD heart tissue. **A**, Histological section of normal heart tissue of mice, which shows striations with equal sized nucleus. **B**, Histology of RHD heart tissue with Aschoff bodies associated lesions. **C**, Cardiac tissue deficient in NKX2-5 shows enlarged nucleus. **D**, Smad-6 null mice with unpacked cardiac tissue. Scale: 50  $\mu$ m.

1E). Like that, the Smad-6 null mouse with RHD was observed with thickening of the heart tissue along with incompact tissue pattern (Figure 1F).

#### ***Nkx2-5 and Smad-6 Expressions Are Down-regulated in RHD***

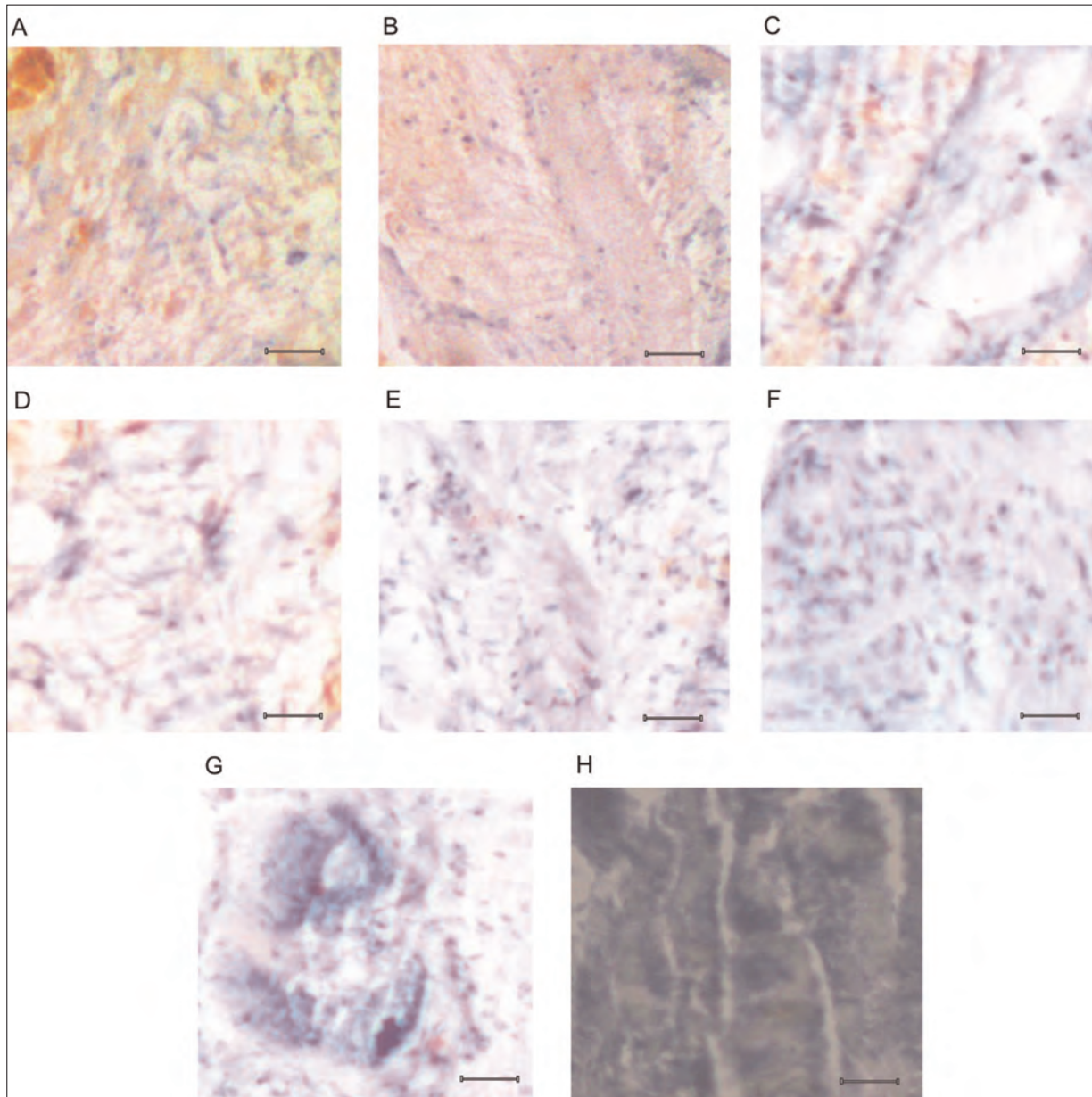
NKX2-5 and Smad-6 expressions are vital for maintaining cardiac homeostasis and their absence affects the normal physiology of the heart tissue. In order to assess the role of NKX2-5 and Smad-6 expression in RHD, Immunohistochemistry was performed against NKX2-5 and Smad-6 proteins and their results are shown as in Figure 2. The normal heart tissues were observed with an intensive expression of NKX2-5 (Figure 2A) and we also observed uniform expression pattern of Smad-6 expression with less intensive staining when compared with NKX2-5 was observed in control heart tissue (Figure 2B). But after developing with RHD, the NKX2-5 (Figure 2C) and Smad-6 (Figure 2D) expressions are down-regulated along with destructive changes in normal tissue of heart architecture.

#### ***Nkx2-5 and Smad-6 are Negatively Regulated by Inhibiting Each Other and Both of Them Produce Interdependent Effects on Developing RHD***

The normal mice heart tissue with null Smad-6 are analyzed for the expression of NKX2-5 and we observed there is only a mild expression of NKX2-5 (Figure 2E). Interestingly the NKX2-5 null mice show very lower expression of Smad-6 in their heart tissue with enlarged nucleus (Figure 2F). In the absence of Smad-6, the expression of NKX2-5 is severely affected after developed with RHD together with infiltration of immune cells (Figure 2G) the condition is still worsened in NKX2-5 null mice, which also shows mild expression of Smad-6 along with hardening of heart tissue (Figure 2H).

#### ***Western Blotting Analysis***

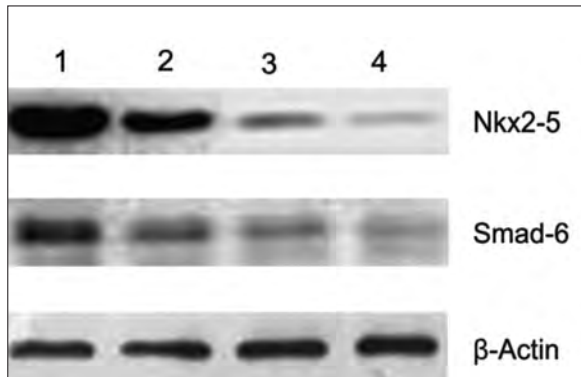
To analyze and confirm the results further that are obtained in immunohistochemistry, we performed Western blotting and their results are shown as in Figure 3. The lane 1 represents normal



**Figure 2.** Immunohistological variation associated with Nkx2-5 and Smad-6 Null mice shows adverse RHD. **A**, Immunohistological section of normal heart tissue, which shows intensive expression of Nkx2-5. **B**, Immunohistological section of normal heart tissue, which shows intensive expression of Smad-6. **C**, Mice developed with RHD shows down regulated expression of Nkx2-5. **D**, Mice developed with RHD shows down regulated expression of Smad-6. **E**, Normal heart tissue showing down regulated expression of Nkx2-5 in the absence of Smad-6. **F**, Normal heart tissue showing down regulated expression of Smad-6 in the absence of Nkx2-5. **G**, RHD heart tissue showing down regulated expression of Smad-6 in the absence of Nkx2-5 with tissue hardening. **H**, RHD heart tissue showing down regulated expression of Nkx2-5 in the absence of Smad-6 with acute tissue hardening. Scale: 50  $\mu$ m.

heart tissue, lane 2 with samples from RHD, lane 3 represent normal heart tissue samples from null mice NKX2-5 and Smad-6, respectively, and lastly lane 4 represents RHD heart tissue samples from null mice NKX2-5 and Smad-6, respectively. The expression of NKX2-5 and Smad-6 is down-regu-

lated in RHD conditions and the condition get worse with intense down-regulated signals, in the absence of either NKX2-5 or Smad-6. The results obtained through Western blotting are likely similar to the results obtained through immunohistochemistry.



**Figure 3.** Validation of NKX2-5 and Smad-6 protein expression by western blotting. Upper Lane. Lane 1-4 represents the NKX2-5 protein expression in normal, RHD, Smad-6 negative control heart and Smad-6 negative RHD heart tissue samples. Middle Lane. Lane 1-4 represents the Smad-6 protein expression in normal, RHD, NKX2-5 negative control heart and NKX2-5 negative RHD heart tissue samples. For control purposes  $\beta$ -actin was used as a loading control.

## Discussion

Animal models are not the ultimate substitute for human disease, but they help to understand the pathological conditions and they facilitate to reveal the mechanism behind RHD<sup>17</sup>. The experiments here are followed with BALB/c mice and it develops similar pathological condition resembles to human<sup>19</sup>. The role of NKX2-5 was identified to be important for heart development<sup>13</sup> and it also aids in maintaining the normal physiology of the heart<sup>12</sup>. The mutation in NKX2-5 gene results into development of congenital heart disease and their different pathological conditions are studied in animal models which mimic similar conditions<sup>20</sup>. The functional role of Smad-6 was also well studied, and it was found that it regulates cardiogenesis through the inhibitory action of the BMP pathway<sup>11,21</sup>. Yet the functional role of NKX2-5 and Smad-6 in RHD is not documented so far.

We are the first to demonstrate that the condition of RHD has a deteriorate effects when it has coupled with either absence of NKX2-5 (Figure 1E) or Smad-6 (Figure 1F). When compared with the normal development of RHD (Figure 1B) the NKX2-5 or Smad-6 null mice shows complex forms of Aschoff bodies with hardened tissue (Figure 1E-F). The results imply that the synergy of pathological lesions are adverse either in the absence of NKX2-5 or Smad-6. The formation of

Aschoff bodies is due to the accumulation of T cells that result with different pathological conditions<sup>22</sup>. From the results, we are pointing out that either NKX2-5 or Smad-6 deficiency have correlated effects on T cell infiltration and its interrelated lesions.

Additionally, the results with the immunohistochemistry help to understand that the development of RHD is associated with the down-regulation of NKX2-5 (Figure 2C) and Smad-6 (Figure 2D). The Smad-6 deficient mice are showing inhibitory expression of Nkx2-5 (Figure 2E) and vice versa the Nkx2-5 deficient mice are showing inhibitory expression of Smad-6 (Figure 2F). The result reveals that there is a tight coordination between NKX2-5 and Smad-6 that oppositely regulate each other.

Another interesting fact we investigate here is that the smad-6 and NKX2-5 null mice develop an acute form of RHD with tissue hardening (Figure 2G and 2H), and it implies either the NKX2-5 or Smad-6 deficiency has a severe effect on heart tissue architecture. The results obtained in immunohistochemistry are cross-checked with Western blotting (Figure 3). The Smad-6 null mice failed to negatively control the bone morphogenetic protein (BMP) signaling as like in results obtained in cell culture<sup>23</sup>. The development of RHD associated with the absence of either NKX2-5 or Smad-6 has a synergic effect that results with more acute RHD.

## Conclusions

From our results, we are able to conclude that in the absence of smad-6 or NKX2-5 the development of RHD is more complex in nature. The smad-6 or NKX2-5 in their absence negatively regulates each other, and we found out that, there is a tight correlation between each other. The mouse developed with RHD in the absence of NKX2-5 has severely resulted with tissue hardening. Overall, the present studies add valuable findings in disease progression and help to understand the key mechanism that attributes to RHD.

## Acknowledgements

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### Conflict of Interest

The Authors declare that there are no conflicts of interest.

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