# Bmi1 and BRG1 drive myocardial repair by regulating cardiac stem cell function in acute rheumatic heart disease

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**Abstract.** Rheumatic heart disease (RHD) occurs due to the accumulation of complications associated with rheumatic fever, and it results in high morbidity and mortality. The majority of cases of RHD are diagnosed in the chronic stages, when treatment options are limited. A small reservoir of cardiac stem cells is responsible for maintaining cardiac homeostasis and repairing tissue damage. Understanding the role of cardiac stem cells and the various proteins responsible for their functions in different pathological stages of RHD is an important area of investigation. Polycomb complex protein BMI-1 (Bmi1) and transcription activator BRG1 (BRG1) are associated with the maintenance of stemness in various types of stem cells. The present study investigated the role served by Bmi1 and BRG1 in cardiac stem cells during various pathological stages of RHD through immunohistochemistry and western blotting. A rat model of RHD was established via immunization with the Group A Streptococcus M5 protein. The rat was demonstrated to develop acute RHD 2 months after the final immunization, characterized by cardiac inflammation and tissue damage. Chronic RHD was identified 4 months after the final immunization, revealed by cardiac tissue compression and shrinkage. Expression of the cardiac stem cell marker mast/stem cell growth factor receptor kit was identified to be elevated during acute RHD, but downregulated in the chronic stages of RHD. A similar pattern of expression was revealed for Bmi1 and BRG1, indicating that they serve a role in regulating cardiac stem cell proliferation during acute RHD. These results suggest that cardiac stem cells serve a supportive role in the acute, but not chronic, stages of RHD via expression of Bmi1 and BRG1.

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*Key words:* polycomb complex protein BMI-1, transcription activator BRG1, rheumatic heart disease, group A streptococcal M5 protein

## Introduction

Rheumatic heart disease (RHD) is a major health issue worldwide, which primarily results in cardiovascular disease and associated morbidities (1,2). RHD most frequently affects children between 5 and 14 years old (3). The diagnosis of RHD has gradually increased, particularly in the latent stage of the disease (4,5). A previous pathological study demonstrated that valvular inflammation occurs as the disease progresses, which is due to the presence of immunodominant epitopes following infection (6). There are numerous limitations including the asymptomatic nature of the disease and a lack of trained practitioners for screening RHD in the latent stage of the disease (7). In certain cases of chronic RHD, valve replacement is the only treatment option (8). The formation of rheumatic lesions and Aschoff bodies are indicative of the active form of RHD (9-11).

Cardiac stem cells have the ability to convert fibrotic scars, which are formed through injury or inflammation, into nascent cardiomyocytes (12). Cardiomyocytes have a low turnover, which reflects the small reservoir of cardiac stem cells (13). The underlying molecular mechanisms of the self-renewal, potency and survival abilities of cardiac stem cells remain unclear and require further study (14). A number of biomarkers are used to define the stem cell response to injury or inflammation; for example, the expression of mast/stem cell growth factor receptor kit (c-kit) and interleukin (IL)-6 family receptors indicates the proliferative and survival ability of stem cells (15,16). Polycomb complex protein BMI-1 (Bmi1) is a transcriptional factor that is associated with numerous important biological functions, including adult stem cell differentiation, embryonic development, organ development and tumorigenesis (17). The results of a recent study suggest that Bmi1-positive cardiac cells serve a major role in myocardial renewal (18). Transcription activator BRG1 (BRG1) is associated with chromatin remodeling, and serves a regulatory role in cardiac growth and differentiation (19). The present study aimed to elucidate the role served by Bmi1 and BRG1 in cardiac stem cells following RHD.

## Materials and methods

*Rat model of RHD.* A rat model of RHD was developed using 18 8-week-old female Lewis rats (strain code, 004) purchased from Charles River Laboratories, Inc. (Wilmington, MA, USA). Rats were maintained at 26±2˚C and 50-60% humidity with a 12 h light/dark cycle. Rats were provided with free access to food and water. The rats were immunized intraperitoneally with 0.5 mg Group A Streptococcus (GAS) M5 protein along with complete Freund's adjuvant (both Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in a 1:1 ratio with a total volume of 200 $\mu$l, as previously described (20). After the initial dose the rats were carefully monitored and provided with a protein rich pellet form of commercial diet. Booster doses of the immunogen were given on days 7 and 21. The rats subsequently developed a condition mimicking acute RHD 2 months after the final booster dose. Furthermore, a condition mimicking the chronic form of RHD developed 4 months after the final booster dose. The animal protocols used in the present study were approved by the Animal Ethics Committee of Nanjing Medical University (Nanjing, China).

*Histology and immunohistochemistry.* The rats were anesthetized using 2.5% of ether solution for 5 min, and after inactivation cardiac samples (from the left atria) were carefully dissected from control rats (treated with saline) and rats with RHD. The samples were fixed with formalin and embedded in paraffin to make a tissue block, as previously described (21). The tissues were sectioned (6-$\mu$m-thick) using a microtome and dewaxed with xylene. Endogenous peroxidase activity was blocked by treating with 10% $H_2O_2$ for 30 min at room temperature. For histology the processed sections in the slide were stained with hematoxylin (5-7 min) and eosin (30-40 sec) at room temperature for clear visualization of the cells in the tissue. Non-specific sites were blocked using 4% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) solution at room temperature for 1 h. Subsequently, the sections were treated with primary antibodies (1:400) directed against c-kit (cat. no. ab32363), Bmi1 (cat. no. ab38295) or BRG1 (cat. no. ab70558; all Abcam, Cambridge, UK) overnight at 4˚C. The sections were washed with 1X PBS and then incubated with Goat Anti-Rabbit IgG conjugated to horseradish peroxidase (Abcam; cat. no. 6721; 1:4,000) for 2 h at room temperature. The sections were washed again with 1X PBS and antibody signals were visualized with 3,3'-diaminobenzidine solution. The obtained results are documented using a light microscope and software (NIS-Elements Viewer 4.3; Nikon Corporation, Tokyo, Japan).

*Western blotting.* Dissected heart tissues were homogenized, and the total protein was extracted and resolved on a 10% gel via SDS-PAGE (70 $\mu$g per lane), as described previously (22). The separated proteins were transferred onto a polyvinylidene difluoride membrane. The membrane was blocked using 4% BSA solution at room temperature for 1 h. The membrane was treated with anti-c-kit (cat. no. ab32363; Abcam), anti-Bmi1 (cat. no. ab38295; Abcam), anti-BRG1 (cat. no. ab70558; Abcam) primary antibodies at 1:400 and anti-tubulin antibody (cat. no. ab4074; Abcam; 1:200) overnight at 4˚C. The membrane was washed with 1X Tris-buffered saline-Tween-20 buffer and then incubated with secondary antibodies (Goat-Anti-Rabbit IgG conjugated with Alkaline Phosphatase cat. no. A3687; Sigma-Aldrich; Merck KGaA) at 1:8,000 for 2 h at room temperature. Protein bands were visualized by developing the signal using BCIP/NBT (Sigma Aldrich; Merck KGaA).

## Results

*Histology of heart tissue samples from the rat model of RHD.* Lewis rats were immunized with the GAS M5 protein to produce a condition similar to human RHD. Over the course of incubation, the rats developed acute and chronic RHD. The acute and chronic stages were observed 2 and 4 months after the final booster dose, respectively. Histology was performed on heart tissue samples in these stages of RHD (Fig. 1). Normal heart tissue from the control rats exhibited cardiac muscles with interrupted striation that were arranged in a regular fashion (Fig. 1A). Heart tissue samples taken 2 months after the final immunization exhibited inflammation and tissue damage (Fig. 1B). Samples taken 4 months after the final booster dose exhibited tissue compression and abundant tissue damage (Fig. 1C), which is evident in chronic RHD and inhibits normal cardiac function.

*Cardiac stem cell response to acute and chronic RHD.* Cardiac stem cells serve a key role in responding to cardiac damage and maintaining homeostasis of the heart (23). Cardiac stem cell behavior in different pathological stages of RHD was assessed in the current study using immunohistochemistry (Fig. 2). Cardiac stem cells were identified via expression of c-kit, which is a marker of self-renewal (24). Normal heart tissue from the control rats exhibited optimal expression of c-kit (Fig. 2A), which is essential for the maintenance of cardiac function during injury. Elevated expression of c-kit was observed in cardiac samples from acute RHD (Fig. 2B). However, c-kit expression decreased markedly in the chronic stage of RHD (Fig. 2C), indicating a reduction in the amount of cardiac stem cells.

*Role of Bmi1 and BRG1 in regulating cardiac stem cell function in RHD.* Bmi1 serves a role in stem cell maintenance by repressing expression of genes associated with cell aging and senescence (25). Similarly, BRG1 is required to maintain pluripotency in stem cells, including embryonic (26), neural (27) and intestinal (28) stem cells. The expression of Bmi1 and BRG1 in different pathological stages of RHD was examined in the present study (Fig. 3). Bmi1 (Fig. 3A) and BRG1 (Fig. 3B) were expressed in a similar pattern in the cardiac tissue of the control rats. Acute RHD tissue exhibited upregulated expression of Bmi1 (Fig. 3C) and BRG1 (Fig. 3D), which indicates that Bmi1 and BRG1 serve a role in the tissue repair process performed by cardiac stem cells in acute RHD. However, the expression of Bmi1 (Fig. 3E) and BRG1 (Fig. 3F) was downregulated in the chronic stage of RHD. These results are similar to those observed for c-kit.

*Expression of c-kit, Bmi1 and BRG1 in acute and chronic RHD.* The results obtained from immunohistochemistry were validated via western blotting. Protein samples were prepared from control, acute RHD and chronic RHD heart tissue, and the expression of c-kit, Bmi1 and BRG1 was analyzed using

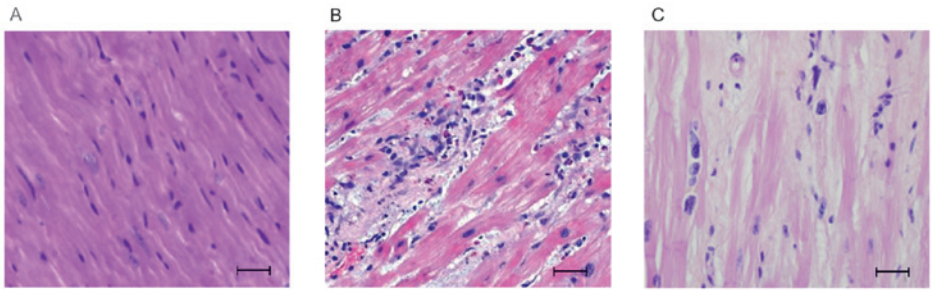


Figure 1. Histological observation of heart tissue samples from different pathological stages of RHD. (A) Control heart tissue with undisturbed layers of tissue. (B) Acute RHD heart tissue exhibiting inflammation and tissue damage. (C) Chronic RHD heart tissue with compression of the soft tissue. Scale bar, 50 $\mu$m. RHD, rheumatic heart disease.

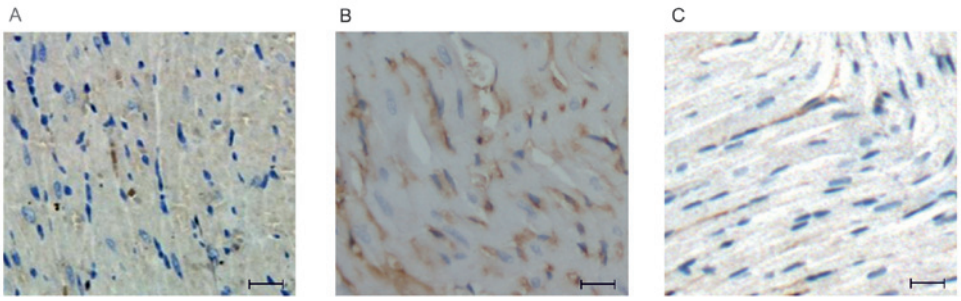


Figure 2. Immunohistochemistry of cardiac stem cells at different pathological stages of RHD. (A) Optimum expression of c-kit protein, a cardiac stem cell marker, was observed in heart tissue from the control rats. (B) High expression of c-kit was observed in the acute RHD tissue section. (C) Low expression of c-kit was identified in the chronic RHD tissue section. Scale bar, 50 $\mu$m. RHD, rheumatic heart disease.

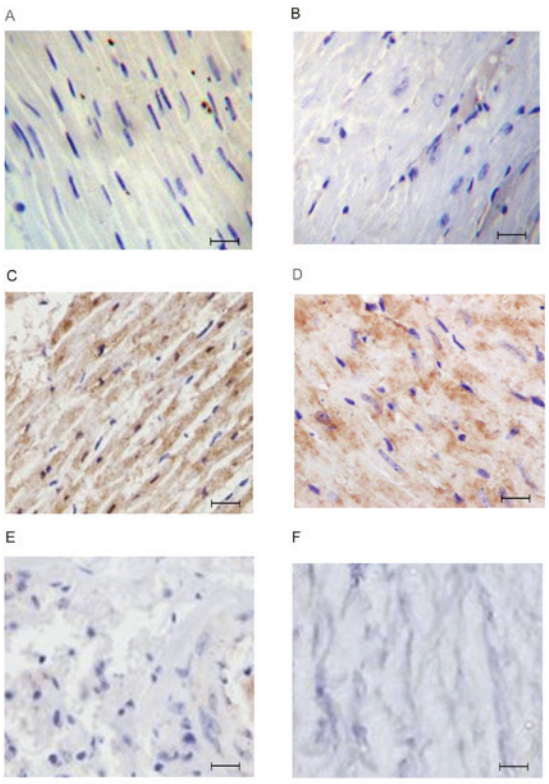


Figure 3. Bmi1 and BRG1 expression at different pathological stages of RHD. Moderate expression of (A) Bmi1 and (B) BRG1 was observed in heart tissue from the control rats. Upregulated expression of (C) Bmi1 and (D) BRG1 was identified in the acute RHD tissue sections. Downregulated expression of (E) Bmi1 and (F) BRG1 was demonstrated in chronic RHD tissue sections. Scale bar, 50 $\mu$m. RHD, rheumatic heart disease; Bmi1, polycomb complex protein BMI-1; BRG1, transcription activator BRG1.

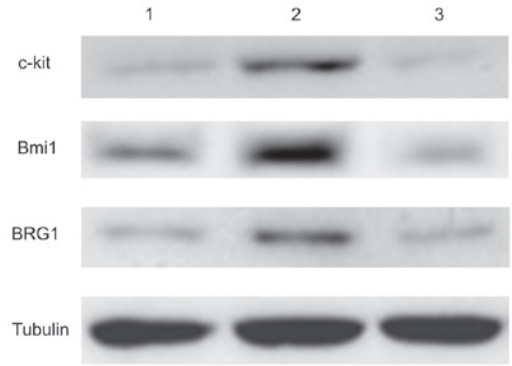


Figure 4. Western blot analysis for the expression of c-kit, Bmi1 and BRG1 at different pathological stages of RHD. Lane 1, control sample; lane 2, acute RHD sample; lane 3, chronic RHD sample. RHD, rheumatic heart disease; c-kit, mast/stem cell growth factor receptor kit; Bmi1, polycomb complex protein BMI-1; BRG1, transcription activator BRG1.

specific antibodies. This revealed a similar expression profile to that observed through immunohistochemistry (Fig. 4). c-kit expression was elevated in acute RHD when compared with normal heart tissue, but its expression decreased in chronic RHD. A similar expression pattern was observed for Bmi1 and BRG1.

## Discussion

The early detection and treatment of RHD is essential, as in its advanced stages RHD may result in congestive heart failure and mortality (29). Cardiac stem cells serve an essential role

in repairing cardiac damage (23). Various proteins are responsible for the characteristics of stem cells, including Bmi1 and BRG1. However, the role served by Bmi1 and BRG1 in cardiac stem cells remains unclear. It is difficult to create animal models of RHD and studying the different pathological stages of RHD remains a challenge (30).

In the present study, an animal model of RHD that mimicked the pathological characteristics observed in humans with RHD was successfully developed. Immunohistochemistry of cardiac samples from this model revealed similar features to the acute and chronic forms of RHD. The acute and chronic forms of RHD exhibited inflammation, tissue damage, and cardiac tissue compression and shrinkage, which are primarily due to an autoimmune response (31).

A small reservoir of cardiac stem cells responds to various pathological and physiological heart conditions (32). The response of cardiac stem cells in different pathological stages of RHD was assessed in the present study via measuring the expression of c-kit. The results of the present study suggest that cardiac stem cells proliferate more in the acute stage of RHD compared with the chronic stage. These results indicate that cardiac stem cells proliferate in order to maintain homeostasis during acute RHD, but serve no role in the chronic form of RHD.

Bmi1 and BRG1 are associated with the maintenance of stemness in different types of stem cells (25-28). The current study investigated the role of Bmi1 and BRG1 in cardiac stem cells. Similarly to the results for c-kit, this revealed that the expression of Bmi1 and BRG1 increased in acute RHD, but decreased in the chronic stage of RHD.

In conclusion, the present study successfully established an animal model of RHD. In addition, experiments using this model indicate that cardiac stem cells serve supportive role in the acute stage of RHD, but not in the chronic stage of RHD. The results of the present study also aid in understanding the association between Bmi1, BRG1 and cardiac stem cells during different stages of RHD. Further studies are required to understand the therapeutic link of Bmi1 and BRG1 associated with cardiac stem cells.

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