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Rac1-PAK2 pathway is essential for zebrafish heart regeneration

Xiangwen Peng [a, 1], Quanze He [b, 1], Guobao Li [a], Jinmin Ma [a], Tao P. Zhong [a, c, *]

[a] State Key Laboratory of Genetic Engineering, Department of Genetics, School of Life Sciences, Fudan University, Shanghai 201203, China
[b] Center for Reproduction and Genetics, Suzhou Municipal Hospital, Jiangsu 215002, China
[c] Department of Medicine, Vanderbilt University School of Medicine, TN 37232, USA

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ABSTRACT

P-21 activated kinases, or PAKs, are serine–threonine kinases that play important roles in diverse heart functions include heart development, cardiovascular development and function in a range of models; however, the mechanisms by which PAKs mediate heart regeneration are unknown. Here, we demonstrate that PAK2 and PAK4 expression is induced in cardiomyocytes and vessels, respectively, following zebrafish heart injury. Inhibition of PAK2 and PAK4 using a specific small molecule inhibitor impedes cardiomyocyte proliferation/dedifferentiation and cardiovascular regeneration, respectively. Cdc42 is specifically expressed in the ventricle and may function upstream of PAK2 but not PAK4 under normal conditions and that cardiomyocyte proliferentation during heart regeneration relies on Rac1-mediated activation of Pak2. Our results indicate that PAKs play a key role in heart regeneration.

1. Introduction

Heart failure remains the leading cause of mortality worldwide. The loss of cardiomyocytes due to heart failure results in fibrosis [1,2], and the low proliferation rate of human myocytes can ultimately lead to heart failure and death [3,4]. Adult zebrafish are an ideal model for heart regeneration as the adult zebrafish heart can fully regenerate even after losing 20% of cardiac muscle tissue [5]. An important mechanistic feature of zebrafish heart regeneration was revealed in genetic fate-mapping experiments demonstrating that new cardiomyocytes are derived from existing cardiomyocytes that facilitate dedifferentiation and proliferation [6,7]. Although a number of studies have revealed several signaling pathways associated with heart regeneration, including the Hippo [8–10], IGF [11], Jak1/Stat3[12] and retinoic acid (RA) signaling pathways [13], the mechanism of heart regeneration is not fully understood. Further investigation into the distinct mechanisms mediating cardiac repair and regeneration is required for the development of regenerative therapies for patients suffering from heart failure.

P21-activated kinases (PAKs) are a family of serine/threonine protein kinases comprising six members that are classified as group I (isoforms 1, 2 and 3) or group II (isoforms 4, 5, 6) according to the similarity of their catalytic domains and regulatory functions [14,15]. PAKs are evolutionarily conserved molecules involved in diverse biological processes such as cytoskeletal rearrangements [16–18], cardiovascular development [14,15], cardiac physiology, heart development and inflammation [19]. Heart regeneration is a complex process that is associated with inflammation [20], cardiovascular regeneration, terminally differentiated cell dedifferentiation and proliferation [7,9,14,21]. A recent study found that cardiomyocyte sarcomeres disassemble during regeneration [22]. Pak1 plays an important role in cardiovascular signaling [23,24]. In zebrafish, a loss-of-function mutation in the Pak2a gene is associated with cerebral hemorrhage without any other detectable phenotype [25]. The deletion of Pak4 in mice is associated with embryonic lethality at approximately embryonic day 11.5 (E11.5). By E9.5, Pak4-deficient embryos exhibit a significantly smaller outflow tract, thinning of the ventricular myocardium, and a decreased and irregular heart rate [26]. Together, these findings indicate that PAKs may play an important role in heart regeneration. Consistent with this hypothesis, we found that PAKs induced heart regeneration and were required for cardiomyocyte dedifferentiation and vascular regeneration. The results of our study indicate that PAKS are a potential therapeutic target for the treatment of heart disease.

* Corresponding author. State Key Laboratory of Genetic Engineering, Department of Genetics, School of Life Sciences, Fudan University, Shanghai 201203, China. Tel.: +86 021 51630546.
E-mail address: taozhongfudan@yahoo.com (T.P. Zhong).
[1] These authors contributed equally to this work.

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2. Results

2.1. PAK activity is induced during zebrafish heart regeneration

To identify pathways related to heart regeneration, we used mass spectrometry and label-free phosphoproteomic quantification to analyze global protein phosphorylation profiles during cardiac regeneration in neonatal mice. We found that phosphorylated Pak2 levels increased at 7 days post amputation (dpa) in injured hearts compared with uninjured hearts (data not show). This finding indicates that the PAKs family of proteins may play an important role in heart regeneration. PAKs are downstream effectors of the Rho GTPases Rac and Cdc42. The PAK family consists of six members that are segregated into two subgroups (Group I and Group II) based on sequence homology. To determine if the expression of PAK genes increases during zebrafish heart regeneration, we performed RT-PCR to evaluate the expression of all PAK genes in zebrafish hearts at 7 dpa and in uninjured hearts (data not show). We found that the expression of 3 PAK genes, pak2a, pak2b and pak4, increased after heart injury; however, no increase in the expression of pak6, pak6b or pak7 was detected (data not show). We confirmed that an increase in PAK activity is induced following injury using immunofluorescence to examine Pak2 and Pak4 expression in regenerating hearts (Fig. 1 A–L). We found that Pak4 was expressed in the epicardium and the vasculature in the compact muscle layer in normal hearts (Fig. 1 A) and was expressed throughout the vasculature and epicardium at 3 and 7 dpa (Fig. 1 B–D). In addition, colocalization studies with Tg(*tcf21:nEGFP*) transgenic fish revealed that Pak4 localized to the epicardium in injured and uninjured hearts (Fig. 1 E–H). Another PAK gene, pak2, was expressed in cardiomyocytes but not in the compact muscle layer in uninjured hearts (Fig. 1 I, J). The expression of Pak2 gradually increased in cardiomyocytes during regeneration (Fig. 1 K, L). Colocalization studies revealed that Pak2 and Pak4 are activated in cardiomyocytes and cardiovascular tissue, respectively, with particularly pronounced Pak2 activation in regenerating muscle.

2.2. Cdc42 and Rac1 act upstream of Pak2 in uninjured and regenerating heart, respectively

The p-21-activated kinases (PAKs) are downstream effectors of the Rho GTPases Rac and Cdc42, and Cdc42 and Rac1 can directly interact with PAKs [17,27,28]. Pak2 and Pak4 are activated in muscle and cardiovascular tissue, respectively. To determine if Cdc42 and Rac1 activate Pak2 and Pak4, respectively, we analyzed the expression of Cdc42 and Rac1 during heart regeneration. Interesting, we found that Cdc42 was specifically expressed in ventricular muscle (Fig. 2 A) and that its expression was unaffected by cardiac injury and regeneration (Fig. 2 B, C). The PAK activator Rac1 was expressed in muscle adjacent to the site of injury in injured

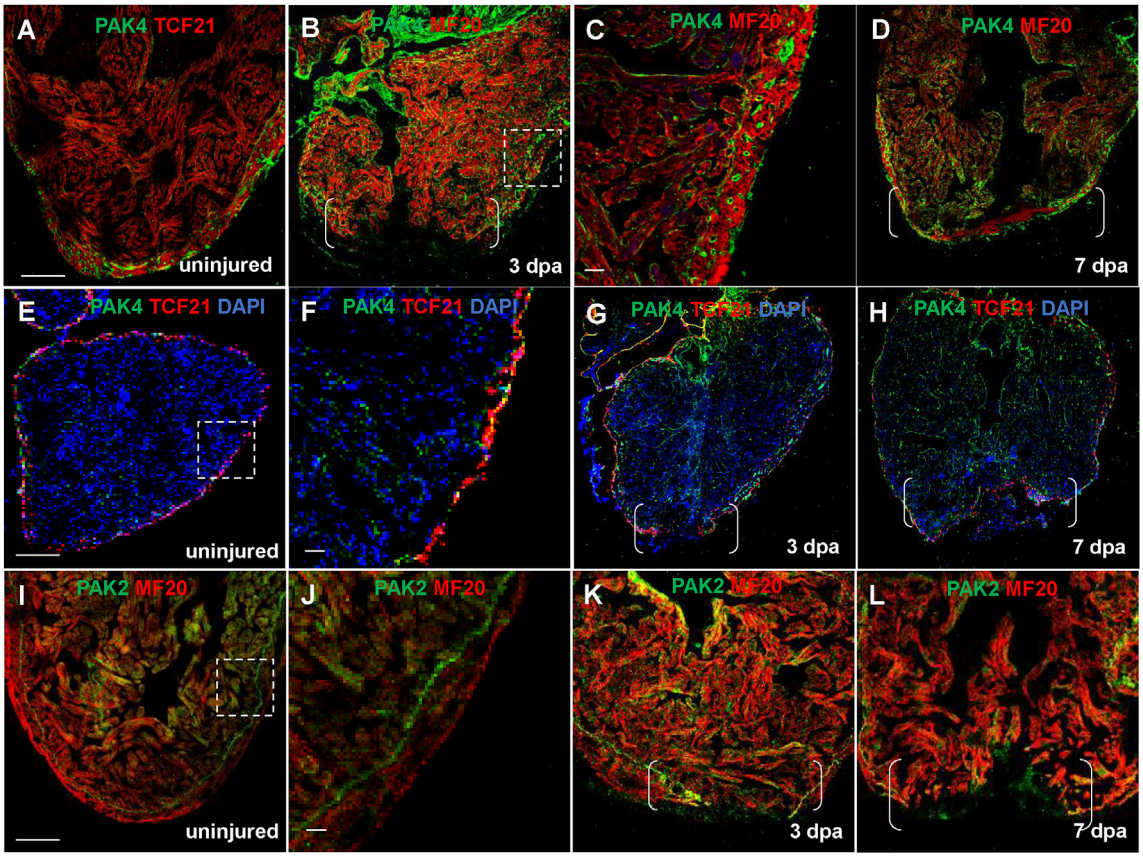

Fig. 1. *pak4* and *pak2* are activated in epicardial cells and cardiomyocytes, respectively, during heart regeneration. (**A**–**D**) Confocal images demonstrating *pak4* expression during regeneration. Pak4 is expressed in the epicardium and vasculature in the compact muscle layer in uninjured hearts, and epicardium Pak4 expression is expanded to all cardiovascular tissue at 3 dpa and 7 dpa. B represents a high-magnification image of the box in A. Green, Pak4; Red, MF20. (**E**–**H**) Colocalization of Pak4 and Tcf21 in tg(tcf21:EGFP) fish. Pak4 expression was observed in the epicardium of tcf21:EGFP^{+} uninjured hearts. At 3 dpa and 7 dpa, Pak4 expression expands throughout the entire cardiovascular tissue. F represents a high-magnification image of the box in E. Green, Pak4; Red, anti-EGFP. (**I**–**L**) Confocal images displaying Pak2 expression during regeneration in injured and uninjured ventricles. Pak2 was expressed in the cardiomyocytes but not in the compact muscle. Pak2 expression increased in cardiomyocytes at the apical edge of the wound at 3 dpa and 7 dpa. J represents a high-magnification image of the box in I. Green, Pak2; Red, MF20. Brackets indicate amputation plane (B-D; G–H; K-L). Scale bar, 100 μM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)