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Nanog expression in heart tissues induced by acute myocardial infarction

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Summary. Nanog is a potential stem cell marker and is considered a as regeneration factor during tissue repair. In the present study, we investigated expression patterns of nanog in the rat heart after acute myocardial infarction by semi-quantitative RT-PCR, immunohistochemistry and Western blot analyses. Our results show that nanog at both mRNA and protein levels is positively expressed in myocardial cells, fibroblasts and small round cells in different myocardial zones at different stages after myocardial infarction, showing a spatio-temporal and dynamic change. After myocardial infarction, the nanog expression in fibroblasts and small round cells in the infarcted zone (IZ) is much stronger than that in the margin zone (MZ) and remote infarcted zone (RIZ). From 7-day 7 after myocardial infarction, the fibroblasts and small cells strongly expressed nanog protein in the IZ, and a few myocardial cells in the MZ and the RIZ and the numbers of nanog-positive fibroblasts and small cells reached the highest peak at 21 -days after myocardial infarction, but in this period, the number of nanog-positive myocardial cells decreased gradually. At 28 -days after myocardial infarction, the numbers of all nanog-positive cells decreased and maintained into a low level. Therefore, our data suggest that all myocardial cells, fibroblasts and small round cells are involved in myocardial reconstruction after cardiac infarction. The nanog-positive myocardial cells may respond to the early myocardial repair, and the nanog-positive fibroblasts and small round cells are the main source for myocardial reconstruction after cardiac infarction.

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Introduction

Acute myocardiac ischemia, which accounts for more than 50% of the mortality in cardiovascular disease, is one of the main factorsreason leading to human death (Cohn et al., 1997). After myocardium damage, the impaired myocardiac cells are usually replaced by fibrous tissue during the myocardiac reconstitution, forming a the permanent scar, which severely impacts on myocardial function. Recently, many reports have shown that stem cells in the heart are involved in the physiologically myocardial reconstitution. They are generated quickly and participate in myocardiac repairing after ischemia, although their origin and functional role are still in debate, e.g., whether they are derived directly from the myocardial tissue or from the circulating blood, after myocardiac ischemia (Wojakowski et al., 2011). Nanog is a transcription factor expresseding in blastula inner mass, archaeocyte and embryonic stem cells (ESCs) (Chambers et al., 2003; Mitsui et al., 2003). Nanog is involved in the self-renewal of ESCs, maintains cells in un-differentiation phase, and promotes cell proliferation (Zhang et al., 2005). As a regeneration factor, nanog can reprogram somatic cells into embryonic cells, which can develop into a new type of cell or tissue to replace the destroyed failure tissue or organs (Cavaleri and Scholer, 2003). Thus, the expression of nanog plays an important role in cell division and proliferation. Therefore, the expression and functional role of nanog during pathological processes, e.g., in myocardiac ischemia, is

interestinged. The investigation of nanog expression in the infarcted myocardium may give cluesa hint about stem cell proliferation and differentiation and help us understand the mechanism of myocardium reconstruction. In the present study, we established an acute myocardiac infarction (AMI) model in the rat and spatio-temporally investigated the expression pattern of nanog in different regions during myocardiac reconstruction.

Materials and methods

Reagents and instruments

Primary rabbit anti-mouse nanog antibody (abcam, U.S.) and mouse monoclonal ß-tubulin IgG (Shanghai Abmart company, China) were used. The appropriate kits, including secondary antibodies, anti-reagent box (Beyotime company), DAB color kit, Peroxidase-Conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) and Peroxidase-Conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (Zhongshan Golden Bridge Company, China) were used. Western blotting kit was purchased from Beyotime company and the mouse total RNA extraction kit from Shanghai Sangon Biological Engineering Company, China; RT-PCR kit from TaKaRa company, China; The gel imaging system from SIM, U.S;A and animal breathingmachine, ECG, and small vertical electrophoresis apparatus from Bio-Rad-U.S. The staining was imaged with an Olympus imaging microscope system.

Preparation of acute myocardial infarction

Forty adult healthy SD rats (weight 220±11 g) were randomly divided into 4 groups for different time points (each n=10). After 10% hydrated chloral (3 ml/kg) was injected intraperitoneally, the ECG was monitored by limb-lead connection. When the rat breathed smoothly, the tracheal intubation was used to for assisting breathing with a respiratory rate of 100 beats / min, tidal volume of 6 ml and breathing ratio of 1:2. Then, the left pectoralis major was separated along the chest median line to expose the third intercostal, and the intercostal muscles wereas incised to expose the heart. Subsequently, ramus descendens anterior arteriae coronariae sinistrae was ligated atin the location of 1-2 mm below circumflex bifurcation using ophthalmic suture needles. After ligation, the left ventricular anterior wall changed into dark red or pale, heart volume became larger, heart rate slowed down, and ST-segment in the ECG elevated, demonstrating that an acute ischemic model was successfully established. After suturinged the major muscle and the skin, the breathing machine was removed from the rat traachea when spontaneous breathing resumed.

Heart tissues were used for RT-PCR, Werstern blot, and immunohistochemistry analyses were taken from the rats, which were of each grouped according to in the time points at 7-day 7, 14-day 14, 21-day 21, and 28-dday 28 after myocardial infarction for RT-PCR, Werstern blot, and immunohistochemistry. For Western blot and RT-PCR detection, distinct parts of the infarcted region wereas estimated under the stereomicroscope as three sub-regions: an infarcted zone (IZ), a margin zone (MZ) surrounding the IZ and a remote infarcted zone (RIZ) around the MZ. They were separately collected, immediately frozen in liquid nitrogen, and then stored at -80°C temperature. refrigerator; For immunohisto-chemistry, the whole heart was fixed in 4% paraformaldehyde at 4°C for 24h. The experimental protocol of myocardial infarction in the rat was approved by the ethics committee of Xinxiang Medical University (52346178XXX).

Masson's trichrome staining

The infarcted hearts were fixed in 4% formaldehyde at 4°C for 24h, and then dehydrated and embedded inwith paraffin. After cutting into a thickness of 8Ìm, the slices were used for regular Masson's trichrome staining using the Masson's trichrome stain kit according to the manufactureory's protocolguidance (Shanghai Bogoo Bio-technology Company Limited from China).

Western blot analysis

The sample was added towith RIPA lysis buffer (10 ml/g) and homogenized by an electric homogenizer in ice for 2 min. After the protein concentration was detected by BCA method, 5x loading buffer with 1:5 volume ratio was added and cooked in boiling water for 5 min. After running in a SDS-polyacrylamide gel, the protein was transferred to a PVDF membrane, followed by blockinglocked with 5% dried milk TBST buffer for 1 hour at room temperature, and the rabbit anti-mouse nanog (1:800) and mouse monoclonal ß-tubulin (1:1000) primary antibodies were added for incubation at 4°C overnight. After washinged with TBST, the secondary antibody of goat anti-rabbit IgG (H+L; 1:500) and goat anti-mouse IgG (H+L; 1:1000) in blocking solution was added at room temperature for 2 h. Finally, the protein band was detected in the film by ECL method. The protein level was measured by semi-quantitative analysis with Motic Images Advanced 3.2 software (Moticsoft Technology Company Limited from Beijing, China). The amount of nanog was normalized to ,-tubulin.

RT-PCR

Total RNA was extracted with total RNA extraction kit from Sigma (USA) according to the manufactureory's protocolguidance. After the first strain cDNA was synthesized, PCR was performed under the condition with a denaturation at 94°C for 20 min, then 35 cycles with 94°C for 30 sec, 58°C for 30 sec, 72°C for 45 sec, and an extra extension at 72°C for 7 min to gget a 266 bp band for nanog with the primers: 5'-

cacagtctgcctagttgt gagg-3' (forward) and 5'-ttggtga ggaccttgttctctt-3' (reverse); and a 595 bp band for GAPDH with the primers: 5'-cagtgccagcctcgtctcat-3' (forward) and 5'-aggggccatccacagtcttc-3' (reverse). After PCR products were electrophoresed with 1.2% agarose gel, mRNA bands were semi-quantified using SIM gel imaging system (Gene Company Limited from Hong Kang, China). The nanog mRNA amount was normalized to GAPDH loading control.

Immunohistochemistry

Paraffin-embedded heart samples were cut into slices with a thickness of 8 μm and antigen retrieval was performed in 0.01 mol/L citrate buffer using a microwave for 15 minutes. The endogenous peroxidase was blocked by 3% hydrogen peroxide for 10 min. After the slice was blocked by 5% normal goat serum in the PBS, the primary mouse antibody against nanog was applied (1:100) overnight at 4°C. Subsequently, peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG was added and the immuno-interaction was visualized by adding the emzymatic substrate solution in DAB kit. The nucleus was counterstained with hematoxylin. The negative control experiment was performed using only the secondary antibody. After immunohistochemical staining, 10 visual fields of IZ, MZ and RIZ from each section were imaged (total 10 sections from each group) and then 60 imagesvisions in each group of each region were randomly selected for the number count of nanog-positive cells.

Statistical analysis

All data were represented as means ± SD (standard deviation) and evaluated by one-way ANOVA by SPSS software 18.0 (SPSS company, U.S.A). Q test was further used for analysis in multiple groups when the difference was significant (p value is less than 0.05).

Results

Pathological change of infarcted myocardium

We first investigated the myocardium at 7-days after myocardial infarction by Masson's trichrome staining to identify the pathological change of the infarcted myocardium. The results showed that the anterior wall of the left ventricle in the infarcted zone became thinner and the myocardial cells were replaced by fibrous tissue forming a scar, as indicated by the blue-labeled collagen without any myocardial cells (red) inside the IZ (Fig. 1). The different regions, such as the IZ, the MZ and the RIZ, were also clear to be recognizedshown (Fig. 1).

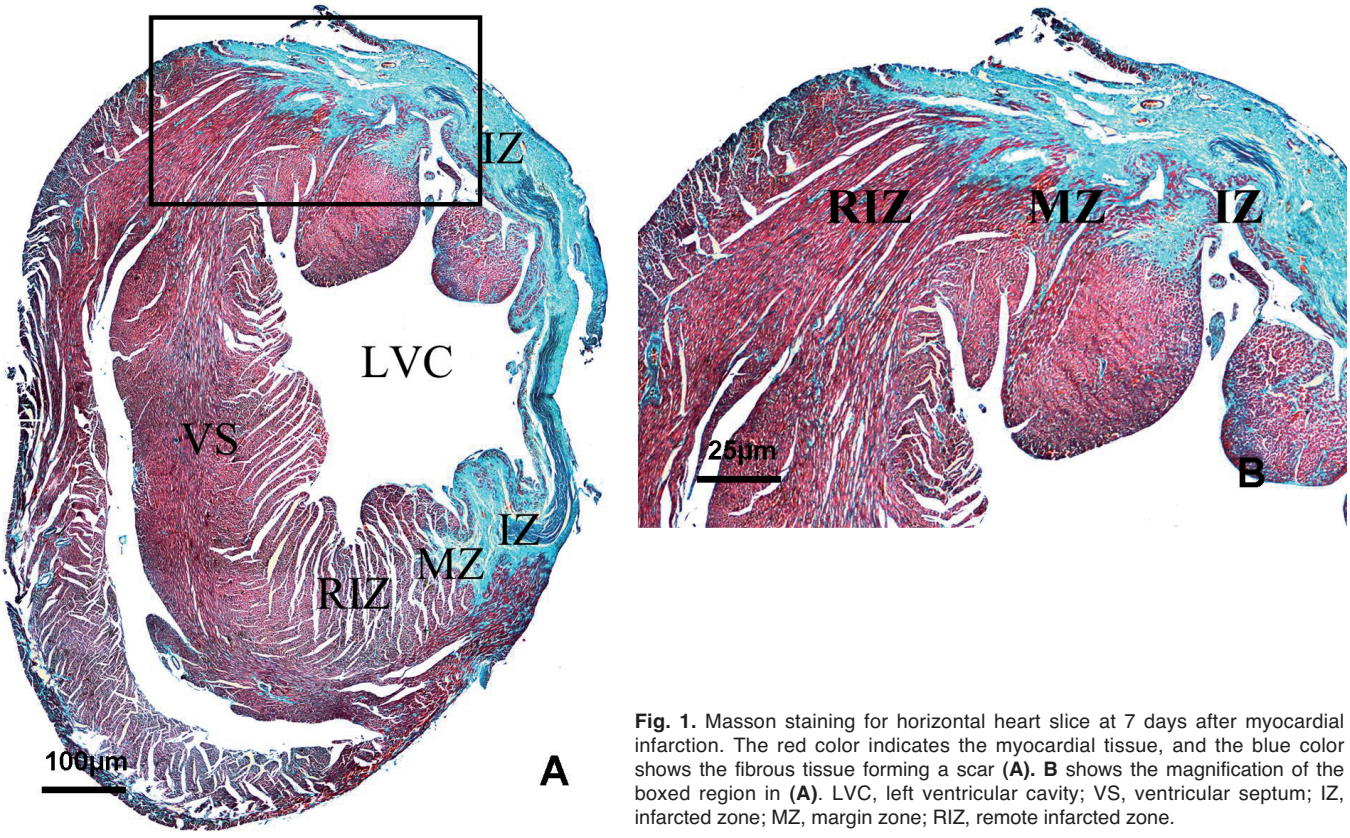

Fig. 1. Masson staining for horizontal heart slice at 7 days after myocardial infarction. The red color indicates the myocardial tissue, and the blue color shows the fibrous tissue forming a scar **(A). B** shows the magnification of the boxed region in **(A)**. LVC, left ventricular cavity; VS, ventricular septum; IZ, infarcted zone; MZ, margin zone; RIZ, remote infarcted zone.

Nanog mRNA expression analyzed by RT-PCR

To monitor the mRNA expression of nanog at different time points in different regions, RT-PCR was performed. The results demonstrated that the expression of nanog mRNA was very low at 7- and 14-days after infarction (Fig. 2A, lane 1-6), and increased to the highest level at 21-days (Fig. 2A, lane 7-9), then decreased gradually and maintained stably at 28-days (Fig. 2A, lane 10-12), suggesting that nanog was induced to be expressed after myocardial infarction. Furthermore, the mRNA level in different regions at the same time point was also different. The nanog mRNA in the IZ was weaker than that of the RIZ and MZ at the 7- and 14-days after infarction, but increased gradually at the 21- and 28- days compared to the RIZ and MZ (Fig. 2B), suggesting that the response of the cells in the different regions to the infarction was different.

Nanog protein expression analyzed by Western blot

The expression patterns of nanog protein at different time points were consistent with the results from mRNA expression, i.e., at the 7-, 14- and 28-days after infarction, the nanog protein level in the different regions was relatively lower than that at 21-days (Figs. 3A,B), and the protein in the IZ reached at the highest level at 21-days after infarction compared to other time points (Figs. 3A,B), suggesting that nanog protein was induced to be synthesizeds after myocardial infarction.

Nanog expression revealed by immunohistochemistry

Nanog expression at both mRNA and protein levels were induced atin different time points and distinct regions after myocardial infarction. Therefore, we continued to investigate in which types of cells nanog was induced using the immunohistochemistry method. The results showed that nanog was positively expressed by myocardial cells, fibroblasts and some small round cells located in IZ, MZ and RIZ (Fig. 4). The number of nanog positive cells in IZ was significantly higher than those in MZ and RIZ ($P<0.05$; Fig. 4Q). The expression of nanog was dynamically changed in different regions at different stages after myocardial infarction. For example, at 7-days after infarction, only a few nanog-positive myocardial cells were found in MZ and RIZ (Fig. 4A-D), but manya lot in IZ, where they located as a small groups surrounding the perivascular or connective

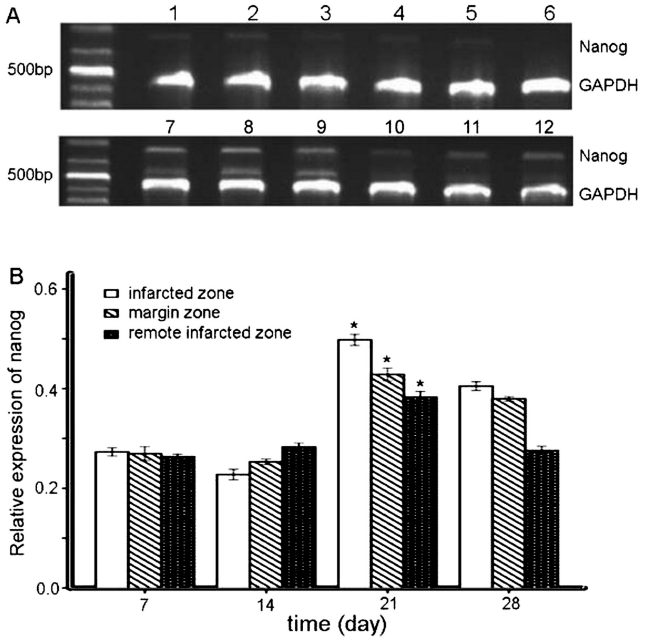

Fig. 2. The mRNA expression of nanog in differentce zones at different stages after myocardial infarction by RT-PCR **(A)** and related semi-quantification **(B)**. Lanes 1-3 indicate nanog mRNA levels in the infracted zone (IZ), margin zone (MZ) and remote infracted zone (RIZ) at 7-days after myocardial infarction; Lanes 4-6 in the IZ, MZ and RIZ at 14-days; Lanes 7-9 in the IZ, MZ and RIZ at 21-days; and lanes 10-12 in the IZ, MZ and RIZ at 28- days after myocardial infarction. * $P<0.01$ compared to the other three groups.

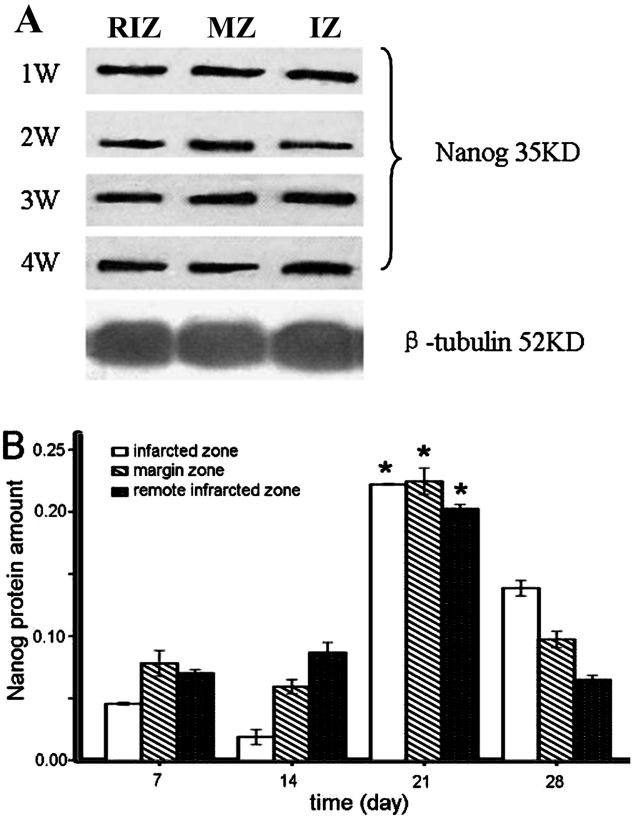

Fig. 3. The expression of nanog protein investigated in different zones at different stages after myocardialinfarction by Western blotting **(A)** and related semi-quantification **(B)**. IZ, infarcted zone; MZ, margin zone; RIZ, remote. * $P<0.05$ compared to other three groups.

tissue. Fibrocytes and fibroblasts in the connective tissue also strongly expressed nanog protein. At 14-days, the numbers of the nanog-positive cells in different regions maintained a in the similar level as at 7-days (Fig. 4E-H,Q). At 21-days,, although the number of nanog-positive myocardial cells decreased, but the number of nanog-positive small cells in different regions significantly increased and reached the highest peak (Fig. 4I-L,Q). At 28-day, the numbers of nanog-positive cells in each region decreased, but the number of nanog-

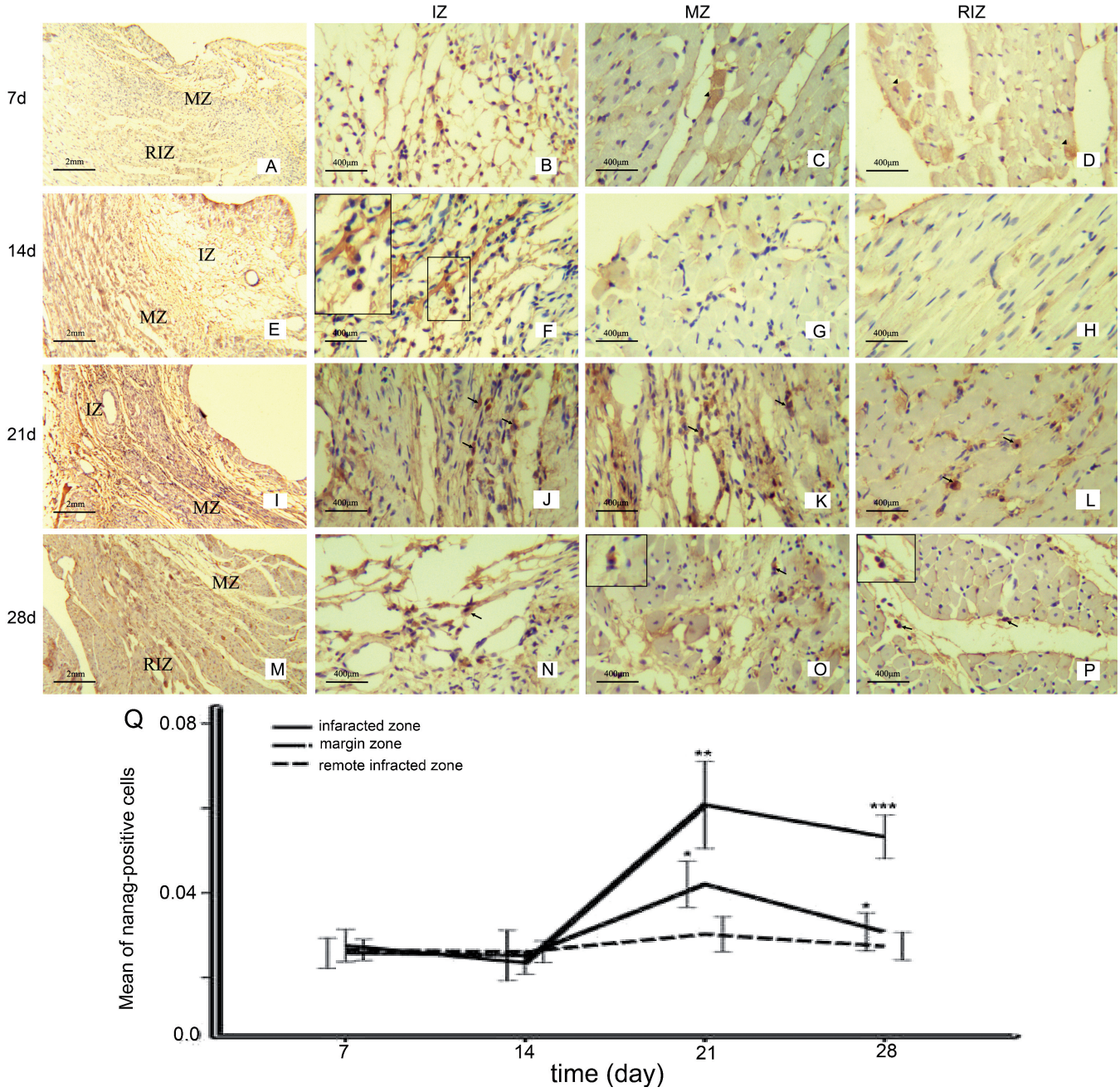

Fig. 4. The expressions of nanog protein in different zones and at different times after myocardial infarction by immunohistochemical staining **(A-P)** and quantification of nanog-positive cells based on the immunostaining **(Q)**. Arrowheads indicate nanog-positive myocardial cells and arrows show nanog-positive small cells cells. IZ, infarcted zone; MZ, margin zone; RIZ, remote. *P<0.05, **P<0.01 and ***P<0.001 when compared to other three groups. arrows show nanog-positive small cells cells. Scale bar: A, E, I, M, 2 mm; B-D, F-H, J-L, N-P, 400 μm.

positive cells in IZ was still significantly higher than that in MZ and RIZ (Fig. 4M-P,Q). All these data suggested that nanog expression was induced by infarction in with a spatio-temperatelyly dynamic change.

Discussion

Myocardial infarction is one of the main reasons reasons for death in heart diseases. TheIt is one of the most interestinged points for myocardial regeneration is that which type of cells participatinge in myocardial tissue regeneparation processes after myocardial infarct. It has been shown from most clinical and animal experiments that cells around the infarcted region can respond to infarction damage, differentiating into myocardial cells and substituting the scar tissue (Wojakowski et al., 2010), but the precise cell type with differentiation potential and their distribution region are still open to question, maybe coming from stem cells, fibroblasts or other cells (Rohr, 2011; Takamiya et al., 2011). The breakthrough discovery of somatic cell reprogramming is a promising approach for the generation of autologous, pluripotent stem cells for use in regenerative medicine. Nevertheless, a few studies have focused on myocardial reparability of iPSC (Ahmed et al., 2011; Nelson et al., 2009). Moreover, these studies have published conflicting results in terms of the teratogenicity of the transplanted cells. Therefore, both the safety and efficacy of iPSC warrant in-depth and systematic assessment in experimental animal models. However, endogenous the autoalergicadult stem tissue cells, instead of transplanted cell, could be filled up above both insufficiency.

Nanog is one of the molecule markers characterized by stem cells and is a key pluripotent transcription factor, involveding not only in formation of embryonic stem cells, but also in regulating the pluripotent function of somatic cells. AsDuring embryo developsnic development, the expression of nanog protein in myocardial cells decreases gradually, but it can be expressed in in endothelial cells oflifespan in the heart (Wu et al., 2008). It has been reported that nanog protein is related to the growth of myocardial cells (Chang et al., 2007). Therefore, in the present study, we investigated the nanog expression and tried to discoverfind out the which role of nanog protein in the myocardial reparation after myocardial infarction. The results showed that atfrom both mRNA and protein levels, nanog is expressed by small cells in IZ and by myocardial cells in MZ and RIZ at 7-days after myocardial infarction (Figs. 2-4). As time passes-develops, the number of nanog-positive myocardial cells is gradually reduced in IZ and almost disappeared at 21-days after infarction in RIZ and MZ, but the number of nanog-positive small cells in the three zones increases gradually and reaches a peak at the 21-days. At 28-days after infarction, the total number of nanog-positive cells in three zones decreases again. Furthermore, the number of nonag-positive fibroblasts reaches the highest peak at 21-days after infarction and then decreases at the 28-days (Fig. 4). Our data suggest that the cells in the heart are responded to myocardial infarction and showing a spatio-temporal and dynamic change, i.e., in the early period of myocardial infarction at the first week, myocardial cells are responsivee to be proliferated, then the small cells and fibroblasts. Specificaally, at 21-days after infarction, the nanog-positive small cells and fibroblasts play an important role in the morphological and functional recovery in different infarcted zones. It should be noted that the amount of nanog mRNA and protein at 7- and 14-days in the IZ is slightly less than that in the MZ and RIZ, because the number of living cells in the IZ is less due to based onthe death of cells the infraction, although they respond to the infraction stimulation first (Figs. 2, 3). Furthermore, as time passedevelops, the amount of nonag mRNA and protein also increases also in the RIZ as well as in besides the IZ and MZ, suggesting a response of the cells in the RIZ and/or migrating cells that respond to the infraction stimulation, as reported in athe previous study (Morales et al., 2001).

Kucia and his colleagues have isolated a type of very small embryonic-like stem cells (VSEL-SCs) from the mouse bone marrow and the human cord blood (Kucia et al., 2006; Zuba-Surma et al., 2008a). Furthermore, Shin and his colleagues have reported that the morphological features and cell markers of VSEL-SCs are similar to those of embryonic stem cells, having a pluripotent characteristic and expressing nanog, but not CD45, suggesting that these VSEL-SCs belong to a type of non-hematopoietic stem cells (Shin et al., 2009). Our previous study has shown that the small cells in myocardial tissue express not only nanog, but also $Ki67^{+}$ and possess the feature of stem cells (Zuba-Surma et al., 2008b). Moreover, the morphological features of nanog-positive small cells and nanog biological behavior investigated in the present study are similar to the small cells in our previous study, therefore, we suppose the small cells studied in this work might be one kind of myocardial stem cells, which can regenerate myocardial tissue and improve cardiac function after acute myocardial infarction (Wojakowski et al., 2009; Zuba-Surma et al., 2011). Further study should be performed to investigate the which origins of these small cells come from.

Page et al. (2009) have found that isolated adult fibroblasts express Oct4, Sox2 and nanog in vitro, indicating that fibroblasts possess the potential characteristics of stem cells. In 2011 a report has been showedn that isolated Ki67-$^{+}$and CD45-negative fibroblasts express nanog, TERT and CD34 from the rat infarcted myocardium and the shape of these cells, with a large nucleusar and less cytoplasm, is smaller than those of bone marrow-derived stem cells (Carlson et al., 2011). From the present study, we have found that the fibroblasts in myocardial tissue expressing nanog after acute myocardial infarction, suggesting that 1) the fibroblasts are involved in the repair process of

myocardial scar; 2) the fibroblasts possesses a characteristic of stem cell. Therefore, it is important to know whether the nanog-positive fibroblasts in the heart can be transformed into myocardial cells or not bywith the further investigation.

Together, the fibroblasts and the small cells express nanog after myocardial infarction with variations in different stages and regions, suggesting that these cells play an important role in reconstruction of the heart after myocardial infarction.

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