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Features of Cardiomyocyte Division During Rat Heart Development

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Abstract.- In the present study, the division modalities and regularity of rat cardiomyocytes from different developing stages were investigated with immunohistochemistry, transmission electron microscopy and cell culture *in vitro*. The results showed that the cells with mitotic figure and binuclear cells exist in the normal cardiomyocytes in prenatal and postnatal life. There are two types of binuclear cardiomyocytes with juxtaposed binuclear cells and separated binuclear cells, and both types of cells gradually increase along with developing time. For example, the division index of separated binuclear cells in the cardiomyocytes is 2.54±0.01% in the 20-day rat fetus, 3.86±0.01% in the 6-day postnatal rat, and 6.78±0.03% in the 12-day postnatal, respectively. However, the division index of mitotic cells in the cardiomyocytes is gradually decreased along the developing stages, for example, 1.44±0.02% in the 20-day rat fetus, 1.15±0.04% in 6-day postnatal rat, and 1.00±0.02% in the 12-day rat after birth. These results suggest that both binuclear cells and mitotic cells in rat cardiomyocytes exist and are temporally regulated during prenatal and postnatal life, for instance, mitotic cells are decreased, while binuclear cells are gradually increased during developmental stages. Binuclear cells may come from amitosis which may be a main division pattern during adult stage.

Keywords: Cardiomyocyte, mitotic cells, binuclear cells, amitosis, division index, rat heart.

INTRODUCTION

The cardiomyocytes enter a rapid proliferation phase during the prenatal life but cease to reduplicate in the postnatal period. Cardiac muscle cells have reached a highly differentiated cellular type during postnatal life, which were generally believed to have lost the capability of division growth (Ahuja *et al.,* 2007). Beltrami *et al.* (2001) earlier questioned the notion that cardiomyocytes were not capable of division growth, which made the sensational challenge in cardiovascular science by laser confocal scanning microscopy (Kajstura *et al.,* 1998). Different varieties of modalities have been adopted to confirm their results ever since. Normally, growth signals promote the proliferation of fetal cardiomyocytes, rather than promote adult cardiomyocytes, entering the cell cycle, while they enhance the heart weight by augmenting cellular size or promoting cellular hypertrophy. Therefore, the growth and development of cardiac muscle cells consist of three major formats, namely, proliferation, binucleation and hypertrophy (Ahuja *et al.,* 2007). Nevertheless, some evidences have been accumulated that cardiomyocytes do undergo mitosis (Shperling *et al.,* 1988; Beltrami *et al.,* 2001; Bolshakova, 2008). Furthermore, Baroldi *et al.* (1967) reported that cardiomyocyte amitosis occurs in hearts with an increased frequency in hypertrophic and atrophic hearts. Some researchers presumed that the two adjacent myocardial cell nuclei in the cardiac overloading or in the carcinoma were interpreted as amitosis (Cluzeaud *et al.,* 1984; Chen and Wan, 1986; Schüpbach and Schneider, 1988), although Pfitzer (1980) concluded that the formation of myocardial cells and nuclear rows was the result of mitosis from impeded cellular divisions. All these results about cardiomyocyte binucleation or amitosis came from abnormal cardiomyocytes without sufficient morphological evidence, and cardiomyocyte amitosis is little known in normal heart development.

Together, according to the known knowledge regarding cardiomyocyte division, the following questions remain unanswered at least in the cellular level: Is amitosis present in normal cardiomyocytes? What is the relationship of binuclear cells and mitosis? Is it possible to observe division of cardiomyocytes under light microscope or

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transmission electron microscope? To answer the those questions, multiple staining methods were employed in the present study to analyze the cellular morphology during division at different stages of development using laser confocal scanning microscopy (LSCM), transmission electron microscopy (TEM) etc. The division type of cardiac muscle cells and the relation between cardiomyocyte division and binucleation were investigated. It was found that mitotic cells and binuclear cells are present in the cardiomyocytes both pre- and post-birth, and mitotic cells in the cardiomyocytes are gradually decreased, but binuclear cells are gradually increased along the developing stages. Our present results suggest that mitosis and binucleation or amitosis may both serve for the proliferation and division of rat cardiomyocytes, which enriched the known division patterns of cardiomyocytes and provided the cellular evidence for cardiomyocyte regeneration even at adult stage.

MATERIALS AND METHODS

Animals

Sixty healthy Sprague-Dawley rats were divided into three groups (20 rats for each group), *i.e.* the group of 20-day fetus (Embryonic day 20, E20), the group of 6-day postnatal (P6) rats, and the group of 12-day postnatal (P12) rats, respectively. In each group, 18 rats were used for histological staining and immunohistochemistry staining, and the rest 2 for transmission electron microscopy. After sacrificed by cervical vertebra dislocation, rat hearts were collected and fixed in 4% paraformaldehyde (PFA) in the phosphate buffered saline (PBS) for 1~6 hours according to the size. Then the fixed hearts were embedded by paraffin and sectioned following routine practice (Yin and Guo, 2002). The sections were manipulated to be 6μm in thickness for following use. All experiments were carried out in accordance with national and institutional guidelines on the care of animals in research.

Hematoxylin and Eosin (HE) staining

To demonstrate the cell morphology of rat cardiomyocytes, HE staining were performed using the routine method (Yin and Guo, 2002). Olympus biological photograph microscope (Japan) was employed to observe the cell division and morphology of cardiomyocytes.

Immunohistochemistry staining

The paraffin sections of left ventricle were dewaxed into water and incubated in 3% hydrogen peroxide for 10 min to inhibit endogenous peroxidase. Then the sections were washed in PBS, and kept in 5% citrate sodium solution at 92~98^{o}C for microwave reconditioned antigen for 15min and cooled for 20 min. After blocked with 5% normal sheep serum for 30 min, the sections were incubated with the sheep polyclonal antibody anti rabbit striated muscle actin ZM-0002 (1:200 diluted in PBS; Zymed Co.) at 4^{o}C overnight. After washing in PBS for three times, the sections were incubated with biotin labeled mouse anti sheep IgG at 37^{o}C for 1 hour, following with horse-radish peroxydase labeled streptodornase avidin at 37^{o}C for 30 min. DAB-H_2O_2 was used for coloration. Normal rabbit serum diluted by 1:200 served as a negative control. Mayer's Hematoxylin was performed after DAB staining. Deep buffy grains indicated positive expression of striated muscle actin and the light blue showed cell nuclei. The cleavage and nucleus morphology were observed and microphotographed using Olympus Biological Photograph Microscope.

To view the staining of striated muscle actin in cardiomyocytes under Japanese FU-1000 Laser Confocal Scanning Microscope, the immunofluorescence staining was performed. For the primary antibody incubation, the steps were same as above, then the section was incubated with 1:300 Cy3 labeled anti sheep IgG. DAPI solution was added to stain cell nuclei. Laser Confocal Scanning Microscope was used for observation and photograph.

Isolation and culture of cardiomyocytes

The cardiac muscles from left ventricle of the heart from P6 rats were washed carefully by DMEM in the Petri dish to remove the blood cells. The tissue were slashed into pieces as 0.5-1mm^3 blocks. These blocks were washed again by the medium thoroughly and carefully, then rested for 2min and removed the supernatant (the red blood cells, the

pieces of the broken cells and dead cells are inside the supernatant). The deposition of the cardiac muscle were transferred into one new 10ml tube and digested by the digestion solution (0.1% trypsin, 0.1% type I collagenase and 0.1% hyaluronidase), mixed for 1-3 min and incubated at 37°C for 6min. The digestion step were repeated for 3 times until the digestion was thoroughly. After rested for 10-20s, the supernatant including cardiomyotomes were tranfered into a 15ml tube and stopped the the digestion step by adding the absolute DMEM included 15% fetal bovine serum (FBS), 2mM L-glutamine, 100U/ml ampicilin and 100mg/ml streptomycin). The above solution was filtered, and the cells were collected by 1200r/min centrifuge for 10min, then resuspended the cells with 6ml medium including 15% FBS. The obtained cells were cultured with 5% CO_2 at 37°C for 1, 2, 3, 4, 5 days respectively. The immuofluorescence staining for the cultured cardiomyocytes was same as above.

Ultrathin image acquisition

After vascular perfusion, the left ventricles at P6 and P12 were slashed into 1mm^3 blocks, fixed in 2.5% glutaraldehyde phosphate buffer, and embedded by the methods of routine transmission electron microscopy (Guo, 2007). The cardiomyocyte cleavage and nucleus morphology were observed under Japanese H-7500 transmission electron microscope.

Nuclei counting

The sections with clear view were chosen to demonstrate cardiac tissue under microscope. 10 HE staining sections, 10 immunohistochemistry sections and 2 immunofluorescence sections were randomly selected from each cardiac tissue block of the ventricle. To each section, 2~4 visual fields were counted. The number of cells at different division stages were recorded. It is note that few fibroblasts with negative striated muscle actin expression were not taken into account. To avoid the sources of bias, there were total 3498 visual fields observed and recorded. The following formula was used to calculate the Division Index and Binuclear Cell Index: Division Index (Binuclear Cell Index) = Number of Mitotic Cells (Binuclear Cells) ÷ Total of Cardiomyocytes × 100%. The data were processed by student *t*-test of SPSS analytic package and demonstrated as Mean±Standard Deviation. A value of $p<0.05$ was considered as significant difference.

RESULTS

The mitotic division of cardiomyocytes

Few cardiomyocytes with chromosome aggregations and nuclear membrane disappearing were revealed under immersion objective, which were typical mitotic division (Fig. 1A-C). Some cells were identified in different mitotic phases, such as prophase as chromosomes are aggregated, metaphase as chromosomes are ranged on the equatorial plate (arrows in Fig. 1A and C), and anaphase of cell division as some chromosomes were polarized (arrow in Fig. 1B). Other cells had large, round nucleus with light stain and 3~5 nucleoli. Cell division was most frequently seen in the ventricular cells of E20, less frequently seen in P6 rats, and hardly seen in P12 rats. The Division Index in all groups was shown in Table I in detail.

The characteristics of binuclear cells

A number of cardiac cells from E20 fetal rats had medial line in the nucleus (internuclear line) which longitudinally divided the nucleus into equal halves, i.e. juxtaposed binuclei (arrows in Fig. 1D). During cellular development, the number of the juxtaposed binuclei with medial line is decreased gradually. However, the number of the separated binuclei along the major axis with terminals facing the medial plate (end to end) is increased (arrows and inserted in Fig. 1E). Furthermore, such separated binuclear cells had remarkable difference of distance between the two nuclei, such as the close ones which are connected by filaments on both terminals and the distant ones which are remarkably away (Fig. 1E). The separated binuclear in longitudinal axis of cardiomyocytes were rarely seen before birth, but gradually increased in P6 and P12 rats, and were visible in each vision field in P12 rats. The number of cells with internuclear line and separated binuclei were shown in Table I. The binuclear cells followed regular characteristics: juxtaposed binuclear cells are increased as birth date

Table I.- The nucleus division index and binuclear cells index of rat cardiocytes (Mean±SD).

	All nuclei	Division of mitotic nucleus	Mitotic nucleus index (%)	Number of juxtaposed binuclear cells	Juxtaposed binuclear cells index(%)	Number of separated binuclear cells	Separated binuclear cells index (%)
E20	7850±23.21	113±0.06	1.44±0.02	4±0.01	0.05±0.02	199±0.98	2.54±0.01
P6	7800±18.02	90±0.04	1.15±0.04	16±0.11	0.21±0.03	301±0.86	3.86±0.01
P12	7500±17.03	75±0.03	1.00±0.02	22±0.19	0.29±0.02	508±0.91	6.78±0.03

P<0.05 between any two groups (E20, P6 or P12) of each index.

approached, but decreased after birth; the juxtaposed nuclei gradually moved to the cellular poles and formed separate binuclear cells image along the longitudinal axis. All results are same from different investigated rats. Note that, cell fusion and mitosis without cytokinesis also result in binuclear cells, however, cell fusion is a rare phenomenon, except when at least one of the cells involved is malignant, and mitosis without cytokinesis, which mitosis is prerequisites and the sister nuclei are symmetric and far apart etc., is also rare in cardiomyocytes (Kuhn *et al.,* 1991). While the size of the binuclear cells from our results is same or similar as other cardiomyocytes, and we did not observe malignant cardiomyocytes with two nuclei and binuclear cells possessing the characterizations of the mitosis without cytokinesis, so the binuclear cells from cardiomyocytes may be from amotisis. The Binuclear Cells Index was shown in Table I.

Identification of cell division of cardiomyocytes in vivo

In order to confirm all the mitotic cells and binuclear cells were exclusively cardiomyocytes, we performed immunohistochemistry staining for striated muscle actin which was the specific marker for cardiomyocytes (Cai *et al.,* 2006; Chen *et al.,* 2008; Bye *et al.,* 2008). The results of which indicated that the striated muscle actin was brown positive particles distributed in the form of catenation along the longitudinal axis in the cytoplasma of the cardiomyocytes. Some cardiomyocytes showed clear transverse striation out of positive actin expression, while most cardiomyocytes witnessed well-distributed expression in the cytoplasma with a minority of cardiomyocyte showing strong positive plaque response. The cytoplasma of mitotic cardiomyocytes (arrow in Fig. 1F) and binuclear cells (arrow and the insert in Fig. 1G) were both strong positive (Fig. 1F, G). The fibroblast, vascular endothelial cells and smooth muscle cells all were actin negative.

Under laser confocal scanning microscope, the striated muscle actin was shown with red color, and cellular nuclei were stained with DAPI in blue color. The actin were particles or plaques situated within the cytoplasma. Aggregations of chromosomes were visible in a great number of cardiomyocytes from E20 rats, indicating different stages of mitosis (arrows in Fig. 1H). However, such aggregation are decreased in the cardiomyocytes from P6 rats (arrows in Fig. 1I) and barely seen in the P12 rats. Although mitotic cell was occasionally visible in a few fibroblasts, actin was negative in their cytoplasma (data not shown). The red blood cells appeared to be deep pink.

Identification of cell division of cardiomyocytes in vitro

To obtained experimental evidence to confirm the existence of mitosis and amitosis in cardiomyocytes, which were visible in the culture of cardiomyocytes *in vitro*. The serial observation of cultured cells from 1d to 5d explored that mitosis and amitosis both do exist. Our results showed that binuclear cells were not only existed in the normal cardiac muscle tissue *in vivo*, but also in the cells which were primarily cultured *in vitro* (Fig. 2). Similar to *in vivo* experiments, the cardiomyocytes were recognized by immunostaining with the antibody of the striated muscle actin (red, Fig. 2B, E, H), and the cell nuclei were stained with DAPI (blue, Fig. 2A, D, G). Figure 2C, F, I are the merge

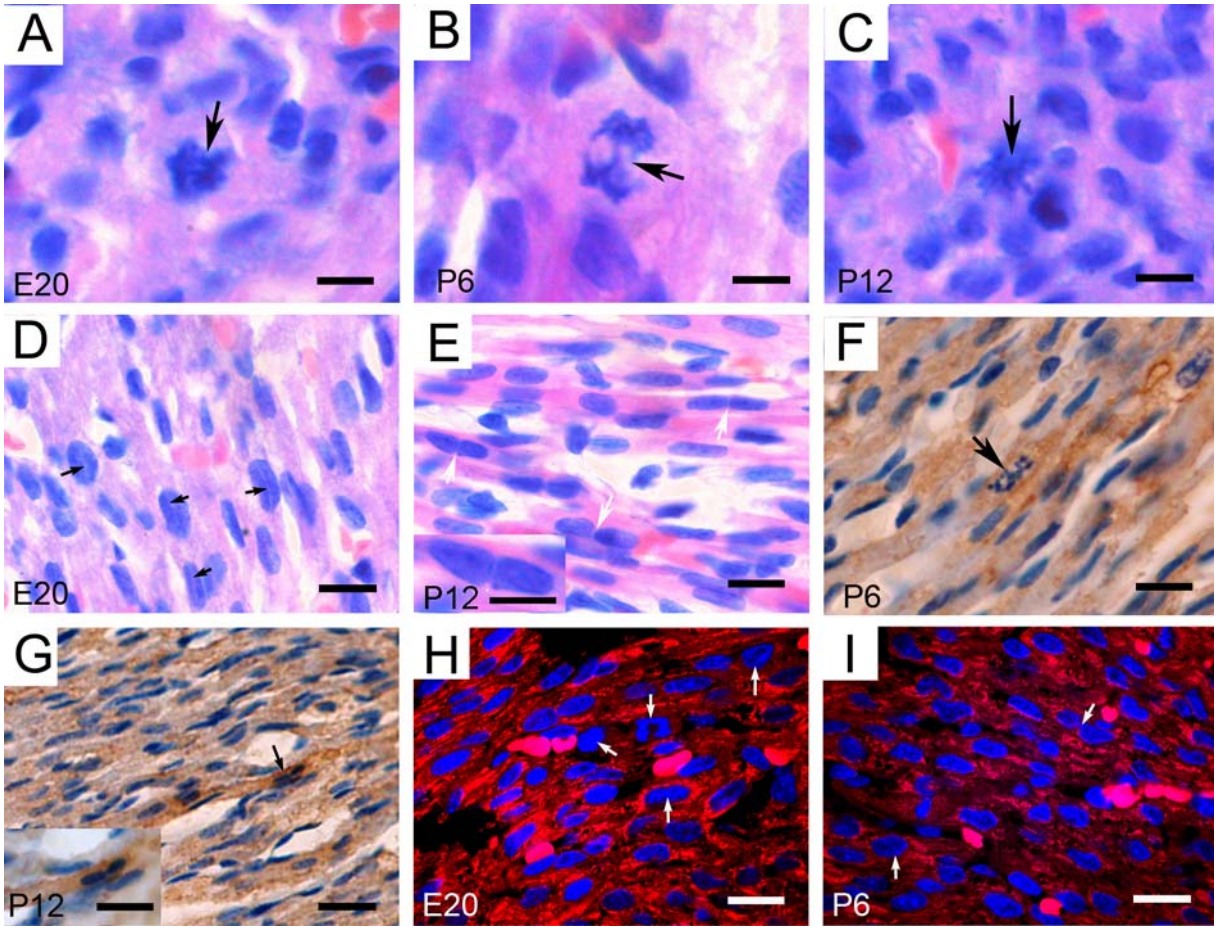

Fig. 1. Mitosis and amitosis of rat cardiomyocytes in different developing stages (marked).
A-E: HE staining. Arrow in A shows the mitosis metaphase of E20 cardiomyocyte; arrow in B shows the mitosis anaphase of P6 cardiomyocyte; arrows in C shows the mid-prophase of P12 cardiomyocyte; arrows in D indicate the juxtaposed binuclear cardiomyocyte with internuclear line; the arrows in E show binuclear cardiomyocyte at P12, and the insert in E is the high magnification of the binuclear cardiomyocyte. **F,G**: Immunohistochemical staining for the striated muscle actin to show cardiomyocytes. Arrow in F shows mitosis of cadiomyocytes at P6; Arrow in G indicates binuclear cardiomyocyte at P12, and the insert in G is the high magnification of the binuclear cardiomyocyte. **H,I:** Immunofluorescence staining for the striated muscle actin to show cardiomyocytes. Arrows in H show the diverse dividing phases of cardiomyocytes at E20; Arrows in I show the juxtaposed binuclear and separate binuclear cardiomyocyte at P12. Scale bar: 10μm in the insert of E; 20μm in A-C and the insert of G; 50μm in D-I.

figures for Fig. 2A, D, G and Fig. 2B, E, H, respectively. At different development stage of the cardiomyocytes, the in vitro cultured cells, which are under division, show two different division types. One is typical mitosis at different division stages (data not shown) while in the other the chromosome aggregations and equatorial plate were not seen which are the typical mitosis characterizations, and malignant cells which is typical characterization for cell fusion. However, the cell nuclei were at different division stages, such as dumbbell-type nuclei which move to two sides of the cell and the middle becomes thin (Fig. 2A-C), splitting nuclei which is close to divide (Fig. 2D-F), and binuclear cells (Fig. 2 G-I). Because the binuclear cells are not same as the cells generated from mitosis and cell fusion (Kuhn *et al.,* 1991), we hereby defined this division type as amitosis. Interestingly, the mitotic cells are more in 1d cultured cardiomyocytes, but decreased gradually

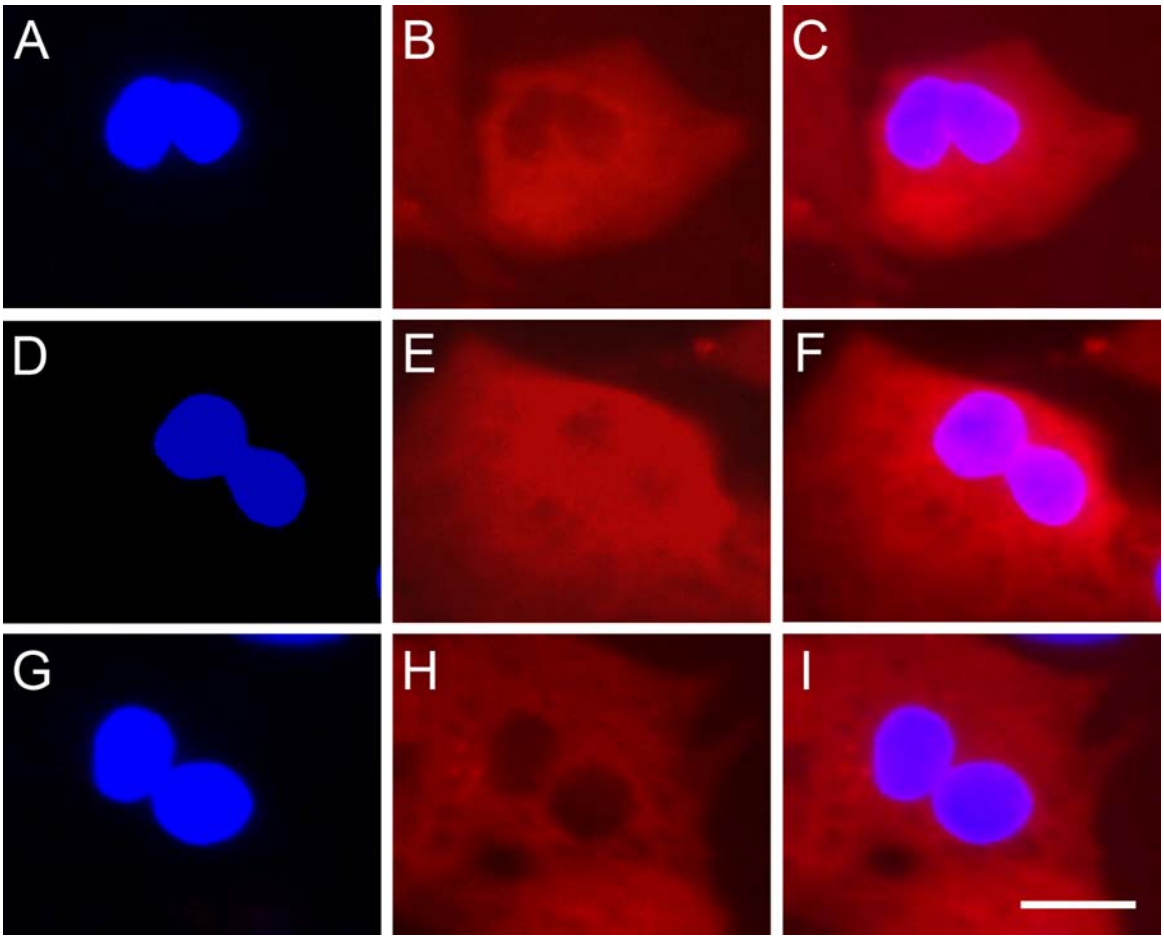

Fig. 2. Binuclear cardiomyocytes at different phases in culturing cardio muscle cells *in vitro*.
A, D, G: The nuclei were shown with DAPI staining (blue). **B, E, H:** Cardiomyocytes were recognized by immunostaining for the striated muscle actin (red). **C, F, I:** The merge for A and B, D and E, and G and H, respectively. Scale bar: 20μm in I for all panels.

according to the culturing time. Contrarily, the binuclear cells were a few in 1d culture, but increased gradually from 2d to 5d. At 5d, about 10 binuclear cells at different division stages can be observed in each vision field under microscope. The results *in vitro* of experiments, further confirm that mitosis or amitosis do exist in cardiomyocytes, which is similar to *in vivo* results (above).

Ultrastructure of rat mitotic cardiomyocyte from transmission electron microscopy

In order to further identify the morphology of the mitotic or amitotic cardiomyocytes, we performed transmission electron microscopy experiments. The fetal rat cardiomyocytes were mostly in the form of stub with the nucleus in the center. However, the cardiomyocytes from P12 rats were in the shape of ellipse, dumbbell, horse-hoof or mushroom, with 1~3 clear nucleoli generally and 5 nucleoli at most (data not shown). The electron microscope revealed the typical image of 4 stages of mitosis of cardiomyocytes. Some cardiomyocytes had aggregated chromosome mottling in nuclei (Fig. 3A), some cardiomyocytes had nuclear chromosome aggregated into plaques during the prophase of cells division (Fig. 3B), some cells had chromosomes arranged on the equatorial plate with nuclear membrane missing (Fig. 3C) or already evolved into two daughter cells but without centriole or spindle fiber in all the stages of cells division. At P12, Some

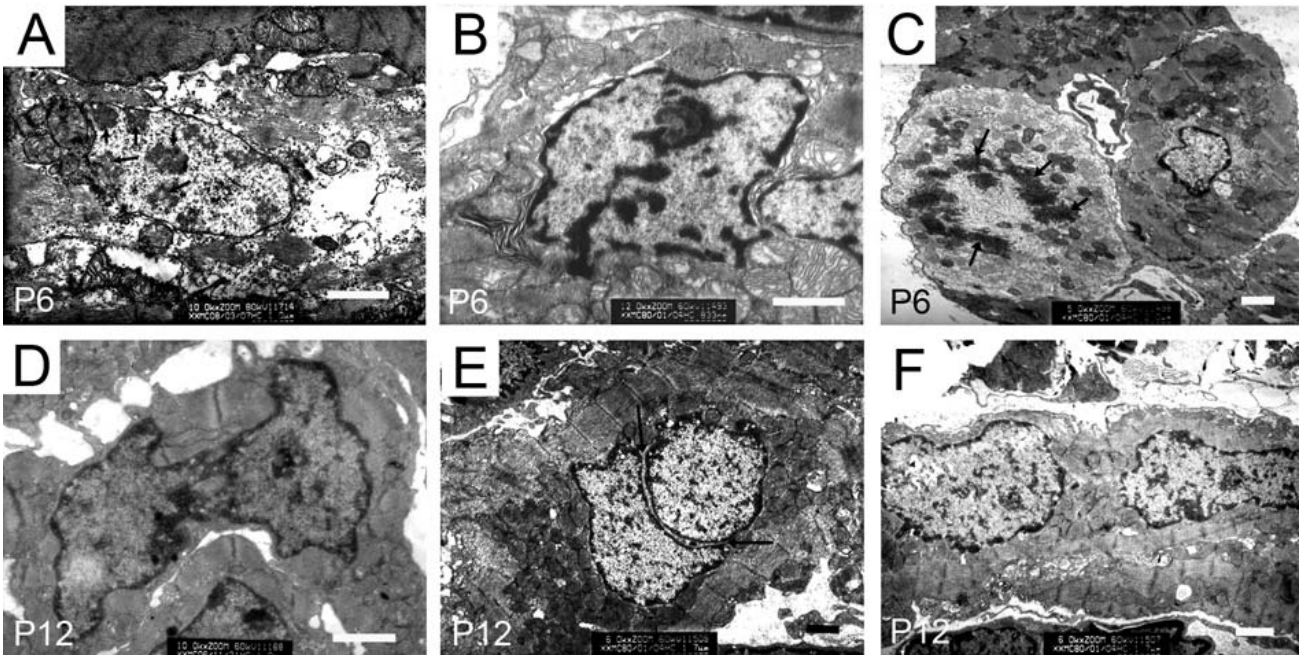

Fig. 3. Ultrastructural mitosis and amitosis of rat cardiomyocytes in different developing stages (marked). All panels are obtained from a transmission electron microscope.
A-C: Mitosis phases of cardiomyocyte at P6. Arrows in A indicate chromatosome aggregation at the mitosis prophase of cardiomyocyte. Arrow in B shows the equatorial plate of the metaphase of cardiomyocyte. Arrows in C indicate chromosomes separated toward bilateralis at the mitotic midanaphase of cardiomyocyte. **D-F:** Binuclear cardiomyocytes at P12. D shows a typical amitotic cardiomyocyte. Arrows in E show a juxtaposed binuclear cardiomyocyte with internuclear line. F displays a separated binuclear cardiomyocyte. Scale bar: 2μm in all panels.

cells had dumbbell shaped nuclei moving towards the cellular poles with the central area gradually thinning and separating, but without the chromatins (Fig. 3D). Most of cells had longitudinal fissure on the nucleus and appeared to be juxtaposed binuclear cells with narrow interstice between the two juxtaposed nuclei (corresponding to the internuclear line under light microscope, Fig. 3E). Cardiomyocytes at P12 possessed a large amount of binuclear cells with the terminals facing each other while a certain distance existed along the longitudinal axis between the two separating nuclei and the cytoplasma between the nuclei were not segregated by intercalated disk (Fig. 3F). In comparison to juxtaposed binuclear cells, we identified them as separate binuclear cells. The binuclear cells and their muscle fibril in the vicinity were well developed. The binuclear cells increased from E20 to P12, which were in consistence with the observation under light microscope.

DISCUSSION

In cardiac tissues, not only cardiomyocytes but also fibroblasts, vascular smooth muscle cells and epithelial cells retained the capability of division. In an effort to confirm the mitotic cells and binuclear cells were cardiomyocytes instead of any other cells, the present study adopted immunological label of striated muscle actin and discrimination of mitotic cells and binuclear cells were performed under both light microscope and laser confocal scanning microscope. The minority of division non-cardiomyocytes including fibroblast, smooth muscle cells etc., which did not express striated muscle actin *in vivo* and *in vitro*, were excluded from the cardiomyocyte division index. Therefore, we could be able to confirm that the mitotic cells and binuclear cells investigated in this study are cardiomyocytes.

It was conventionally held that cardiomyocytes were terminally differentiated cells and were not capable of further differentiation or

proliferation. Linzbach (1960) discovered the increase of absolute cardiomyocyte number in heart failure patients, confirming the ability of proliferation of left ventricular cells. However, their discovery was not paid enough attention to until recently when the discovery was made via laser confocal scanning microscope. Beltrami *et al.* (2001) confirmed that there was myocyte proliferation after myocardial infarction. These results suggested that mitosis was seldom in normal hearts while significantly increased under pathological states (Beltrami *et al.,* 2001; Ahuja *et al.,* 2007; Hosoda *et al.,* 2010). In normal hearts, most cells completed mitosis within 1 hour, which probably diminished the chance of detecting cardiomyocyte mitosis (Anversa and Kajstura, 1998). In addition, classical methods such as morphology of cell proliferation, biochemistry and molecular biological methods were not specific and acute enough for the detection of mitosis in cardiomyocytes. The present study confirmed mitosis in normal heart tissue using both general histological modalities and electron microscopy technologies, which suggested the regularities of mitosis in normal rat muscle cells: the mitotic cells were common in fetal rat hearts, while they decreased in number as the fetus grew older; mitosis at P12 were significantly less frequent, which was in accordance with the results (mitosis ceased 14 days after birth) of Di *et al.* (2007) but disagreed with the conclusion by Bicknell and Brooks (2008), who held that mitosis of cardiomyocytes ceased 3~4 days after birth, with 85% cardiomyocytes in the stasis of G0/G1 phase and the rest in the stasis of G2/M phase.

During the process from fetal cardiac cells till mature cardiac muscles, the volume, quantity and extent of multiplicity of the histological structure of ventricular cells increased continuously (Hirschy *et al.,* 2006). The development of heart and the renovation of cardiomyocytes depended on the balance between cell proliferation and death. As a matter of fact, although mitosis of rat cardiomyocytes ceased soon after birth, this did not affect the multiplication of cardiomyocytes during the development of heart. The reason for this phenomenon was that cardiomyocytes might be capable of another division modality—amitosis. Generally, amitosis was seldom seen in humans, though occasionally found in liver cells, renal tubular epithelial cells, adrenal cortex cells and others. Since long time it was controversial that whether amitosis exists in animals, especially higher animals (Anversa and Kajstura, 1998) or not. Baroldi *et al.* (1967) and Cluzeaud *et al.* (1984) had interpreted binuclear cell as amitosis, however, which was lacking the support of morphological evidence. The present study *in vivo* (heart tissue) or *in vitro* (cultured cardiomyocytes) confirmed the existence of internuclear line, internuclear filament and juxtaposed binuclei under light microscope, and the dumbbell-shaped separate nucleus and interstice between juxtaposed binuclei under electron microscope. These cells did not contain chromosome aggregation, evidence of mitosis, and malignant cells ant cell aggregation with nuclei, the characterization of cell fusion (Kuhn *et al.,* 1991), but formed binuclear cells directly. These phenomena were indicative of amitosis.

The Table I shows that the juxtaposed and separated binuclei were all increased gradually during the development period, the two types of binuclei exist association, which indicate that the separated binuclei may derive from juxtaposed binuclei. According to our present results, therefore, we believed that both mitosis and amitosis were present in rat cardiomyocytes. The serial observation of cultured cells from 1d to 5d further explored that mitosis and amitosis both do also exist in culturing cardiomyocytes (Fig. 2). During the fetal period, cardiac muscle cells were characterized by mitosis, which gradually ceased with time after birth and replaced by amitosis. The nuclei were separated by the internuclear line in amitosis and gradually became two distinctive juxtaposed nuclei, which moved to the cellular poles by the contraction and dilation of muscle fibrils. The nuclei were divided in this process without the segregation of cytoplasma. Normally, nuclei were the indication for the cardiomyocyte counting under light microscope, therefore, the binuclear cells with nucleus far away from each other were easily assessed as two cells, which might undermine the conclusion that the quantity of cells increased relatively, i.e., cellular nucleus were increased but not cardiomyocyte, during growth and development.

Anversa *et al.* (1980) also described excellently the cardiomyocyte binucleation. However, it is also controversial regarding the formation of binuclear cardiomyocytes, their metabolic functions and division capabilities. Some of the scholars hold that binuclear cells come from incomplete mitosis, namely, the cessation of nuclear division without the cleavage of cytoplasma (Pfitzer, 1980). Most of the scholars, however, believe that binuclear cells are nothing but a transitional form during amitosis and widely exist in normal or pathological tissues, for examples, in the cultured mouse trophoblast (Kuhn *et al.,* 1991), in the human adrenal cells (Magalhães *et al.,* 1991), in the liver (David and Uerlings, 1992; Chen and Wang, 1994; Nagata, 2003; Guidotti *et al.,* 2003), in the giant cell tumor of bone (Kusuzaki *et al.,* 2000), and in the chondrocytes of normal cartilage (Kusuzaki *et al.,* 2001). Only a few papers have discussed the mitosis and amitosis of cardiomyocytes (shown in Introduction part). Clubb and Bishop (1984) found that the binucleation of myocardial cells was not due to fusion of mononucleated cells, because there was continued DNA synthesis in the neonatal hearts, and thy conclude that the formation of binucleated myocardial cells is an early indicator of growth hypertrophy in the neonatal rat and a result of mitosis without cytokinesis. Soonpaa *et al.* (1996) considered cardiomyocyte proliferation to have ceased before birth, while the binuclear cells were merely indicative of DNA synthesis, *i.e.*, DNA was synthesized during the process of binuclear cells formation. Though human binuclear cardiac muscle cells gradually increased after birth, they reached a static ratio with mononuclear cardiomyocytes at certain period of time (Soonpaa *et al.,* 1996). Olivetti *et al.* (1996) found that mononuclear cells took 75% of the total cardiomyocytes while binuclear cells 25%; and this ratio did not change regardless of health status, age and gender. Therefore, binuclear cells, which are achieved by amitosis, may be the major form of cardiomyocyte proliferation after birth. Stem cells have been successfully used in myocardial repair and remodeling (Novotny *et al.,* 2008). We also used the cultured rat cardiomyocytes to treat the rabbit acute myocardial infarction through transplantation (unpublished paper). The binuclear cells may show some similarity to the stem cells in the molecular level. It will be of interest to study the relation of the binuclear cells and the embryonic stem cells. As highly differentiated and functional cells, the reason, mechanism and molecular evidence, which amitosis involves in cardiomyocytes proliferation, should be further investigated.

CONCLUSIONS

The present study described the morphological characteristics and development regularity of the division cardiomyocytes in rat hearts at different developmental stages. Our results answered the three questions mentioned in the instruction part and confirmed that the mitosis and binuclear cells from amitosis both exist in rat fetus and the earlier neonatal rats, and the number of mitosis cells decreases but of binuclear cells from amitosis increases gradually during rat heart development.

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