Comparison of the Size and Number of Nuclei in Single Cardiomyocyte Level in Male and Female Adult Mice

Hongyan Guo[1,2,3,4, a], Binglin Lai[1,2,3,4, b], Yanmei Li[1,2,3,4, c], Shanshan Feng[1,2,3,4, d, *]

[1]Key Laboratory of Regenerative Medicine, Ministry of Education, Jinan University, Guangzhou 510632, China.

[2]Joint Laboratory for Regenerative Medicine, Chinese University of Hong Kong-JinanUniversity, Guangzhou 510632, China.

[3]International Base of Collaboration for Science and Technology (JNU), Ministry of Science and Technology, Guangdong Province, Guangzhou 510632, China.

[4]Department of Developmental and Regenerative Biology, Jinan University, Guangzhou 510632, China.

[a]1360644432@qq.com, [b]1277268544@qq.com, [c]liyanmei010911@163.com,

[d, *] fengss2000@126.com

Abstract

In this study, single cardiomyocyte of male and female mice was isolated. The nuclei of the cardiomyocytes were stained with Hochest-33342. The cardiomyocytes size, nucleus number and area in male and female mice were analyzed in single cardiomyocyte level. We have found that adult male and female cardiomyocytes are mainly binuclear, but the average nucleus number of male cardiomyocytes is smaller than that of female cardiomyocytes. The nucleus and cardiomyocyte area of male cardiomyocytes are larger than that of female cardiomyocytes. In addition, the ratio of cardiomyocytes area/ cardiomyocytes nucleus area in male mice is smaller than that in female mice. It is believed that the results of this study provide more accurate data in the single cardiomyocyte level.

Keywords

Cardiomyocyte; Cell area; Nucleus number.

1. Introduction

Research shows that the adult cardiomyocytes of rodents and humans can be mononuclear, binuclear, and multinuclear cells with more than three nuclei [1-2]. Although the 70% volume of heart is the cardiomyocytes, but only 20-30% nuclei of heart belong to the cardiomyocytes, and another 70% nuclei of heart belong to non-cardiomyocytes. Generally, histological section combing with nucleus staining is applied to analyze and measure the number of nucleus in myocardium and cardiomyocytes [3]. Although this method can show the total number of nucleus in myocardium or in the cardiomyocyte, however, due to the reason that it is difficult to find out the exact boundary of cardiomyocytes in tissue sections, therefore, it limits us to count the exact number of the nucleus in each cardiomyocyte accurately. Furthermore, it makes us difficult to analyze the ratio of cardiomyocytes area/cardiomyocytes nucleus area accurately under physiopathological conditions.

Recent studies have reported that there is a technical limitation in the method of conventional histological section and staining for counting cardiomyocyte nucleus number accurately. Using transgene technology, the myocardial cell nuclei can be labeled with the specific alpha myosin heavy

chain promoter, combined with the method of labeling the membrane of the cardiomyocytes, which is more accurate comparing with the method of labeling the nuclei with Hochest-33342 to identify the nuclei of cardiomyocytes in heart section. It was demonstrated that conventional histological section with Hochest-33342 to count cardiomyocyte nucleus strategy will easy incur misjudgment for the nucleus at boundary of cardiomyocyte as non-cardiomyocyte nucleus [4]. Accordingly, in this study, single male and female cardiomyocyte was isolated. The nuclei of the cardiomyocytes are stained with Hochest-33342. The cardiomyocytes size, nucleus number and area as well as the ratio of cardiomyocytes area/cardiomyocytes nucleus area in male and female mice were analyzed in single cardiomyocyte level.

2. Methods

2.1 Animals

The SPF grade C57 mice (2 month old) were provided by Guangdong animal experimental center. Animal care and handling procedures in this study were performed in accordance with the rules of The Ministry of Science and Technology of the People's Republic of China ([2006]398).

2.2 Isolation of Cardiomyocytes

2-month-old male and female C57 mice (3 in each group) are injected with 100 μL Heparin (125 U/mouse) via orbital injection. After 15 min, the abdominal cavity of the mouse is injected with 200 μL 20% ethylurethanm (40 mg/mouse), then the cardiomyocytes are isolated by the Langendorff perfusion method. Briefly, After disinfecting the mice with 70% alcohol, the heart is quickly taken out at a sterile hood and put into the pre-cold perfusion buffer (NaCl 135 mM; KCl 14.7 mM; $MgCl_2$ 1 mM; HEPES 10 mM; NaH_2PO_4 0.33 mM; BDM 30 mM; D-Glucose 10 mM; Taurine 30 mM; Creatine 5 mM; Sodium pyruvate 2 mM; $NaHCO_3$ 25 mM; L-Glutamine 2mM; KH_2PO_4 0.6 mM; adenosine 5 mM.). After removing the non-cardiac tissues, the aorta of the isolated heart is inserted into the Langendorff perfusion system and fixed with fine wire, and perfused at the velocity of 3 mL/min, instilling 3 min with perfusion buffer. Then the heart is digested for 5-10min with digestion buffer (0.01% collagenase Ⅱ+0.01% collagenase Ⅳ). When the myocardial tissue becomes loose, soft and pink, which indicates the digestion is sufficient. The isolated heart is put into the termination buffer and torn into small peices, followed by blowing with pipette to isolate single cardiomyocytes. After the isolated cardiomyocytes suspension is passed through 250 μm filter to remove the undigested tissue blocks and then the gradient centrifugation (50×g, 3 min; 30×g, 2 min; 20×g, 2 min) is used to remove the cell fragments, blood cells and endothelial cells [5-9], the isolated cardiomyocytes are suspended by terminated buffer and used for nucleus staining and analysis.

2.3 Staining of Cardiomyocytes Nucleus

2 μL Hochest-33342 (5 mg/mL) is added to the diluted cardiomyocytes suspension (2.5×10^4) of 1 mL, and incubated at room temperature for 15 min. After three washings with terminated buffer, the suspension was placed on the inverted fluorescence microscope, and captured under UV light.

2.4 Measurement of Cardiomyocytes Size, Nucleus Number and Nucleus Area

The Image-Pro Plus 6 image processing software was used to measure and count the length, width, area, nucleus area and nucleus number of cardiomyocytes. The cardiomyocytes (female: 1004 cardiaomyocytes; male: 1007 cardiaomyocytes) were collected from 3 male mice and 3 female mice.

2.5 Statistics

The SPASS19 software is used for statistical analysis, and all measured data are presented as the means±standard errors of mean (SEM). Two-tailed student's *t*-test is used to calculate the statistical significance between two groups, $P<0.05$ was considered statistically significant. And the frequency

distribution histogram of each index is plotted with Origin 9.4 software and fitted by Gauss fitting algorithm.

3. Results

3.1 The Number and Distribution of the Nuclei in Adult Mice Cardiomyocytes Have Gender Differences.

In this study, the nuclei of the isolated cardiomyocytes (female: 1004 cardiaomyocytes; male: 1007 cardiaomyocytes) are stained with Hochest-33342. It was found that the number of cardiomyocyte with single nucleus, double nuclei, three nuclei, or four and more than four nuclei was 40 (4%), 728 (72.8%), 130 (13%), 96 (9.6%), or 10 (0.8%) respectively in female mice, while it was 78 (7.7%), 810 (80%), 98 (9.7%), 21 (2.1%), or 0 (0%) respectively in male mice. The average nucleus number of each cardiomyocyte was 2.31±0.02 in female, which was significantly higher than that of male (2.06±0.02) (Table 1; $p<0.001$). In addition, the distribution of nucleus number was analyzed. It was found that most cardiomyocytes were binuclear in both female cardiomyocyte (73%) and male cardiomyocyte (80%). The number of mononuclear and binuclear cardiomyocytes in female cardiomyocytes were less than that in male cardiomyocytes, while the number of multinuclear (three nuclei or four and more than four) cardiomyocytes in female was higher than that in male (Fig 1).

Table 1. The measurement of parameters in adult male and female cardiomyocytes

	Female	Male
Nucleus number/ cardiomyocyte	2.31±0.02	2.06±0.02***
Nucleus area	146.50±2.08	158.60±1.99***
Cardiomyocyte area	2552±24.04	2747±27.08***
Cardiomyocyte length	105.30±0.83	103.40±0.71
Cardiomyocyte width	33.67±0.21	36.54±0.24***
Cardiaomyocytes area/cardiomyocytes nucleus area	20.76±0.37	19.06±0.24**
Counted cardiomyocyte number	1004	1007

: compared with female mice p<0.01; *: compared with female mice p<0.001;

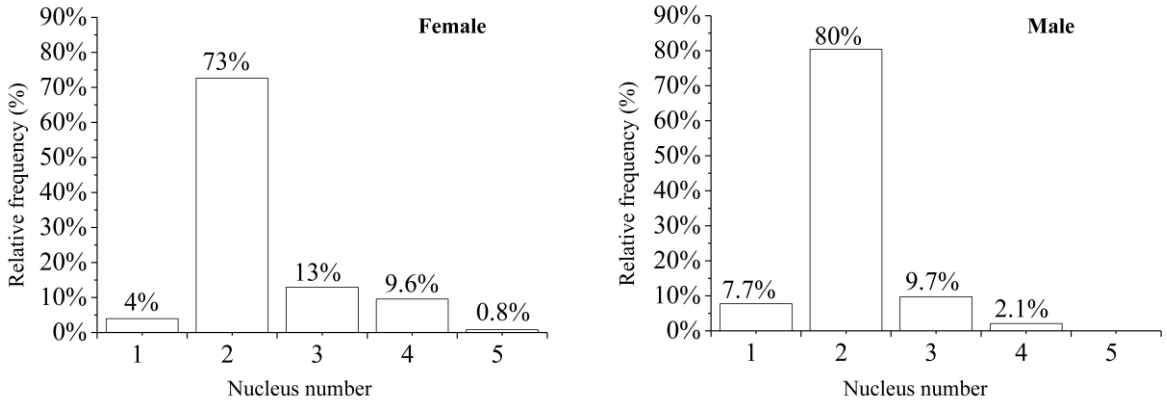

Fig 1. The distribution of the nucleus number in adult male and female cardiomyocyte.

3.2 The Nucleus Area of Male Cardiomyocytes is Significantly Larger than that of Female Cardiomyocytes.

It was found that the average area of nucleus in male cardiomyocytes (158.60±1.99 μm^2) was significantly larger than that in female cardiomyocytes (146.50±2.08 μm^2) (Table 1; p<0.001). In addition, the distribution of nucleus area was analyzed. The 93% nucleus area of female cardiomyocytes was 50-250 μm^2, while, the 90.1% nucleus area of male cardiomyocytes was 50-250 μm^2. For the distribution which ratio was equal or larger than 10%, it was found that: 1) The nucleus area of female cardiomyocytes was distributed 50-250 μm^2 (93%), while it was 50-200 μm^2 (81%) for male cardiomyocytes; 2) The maximal nucleus area of male and female cardiomyocytes (ratio equal or more than 1%) was 350 μm^2 (Fig 2). The results suggested that around 80% nucleus area in male and female cardiomyocytes was roughly the same.

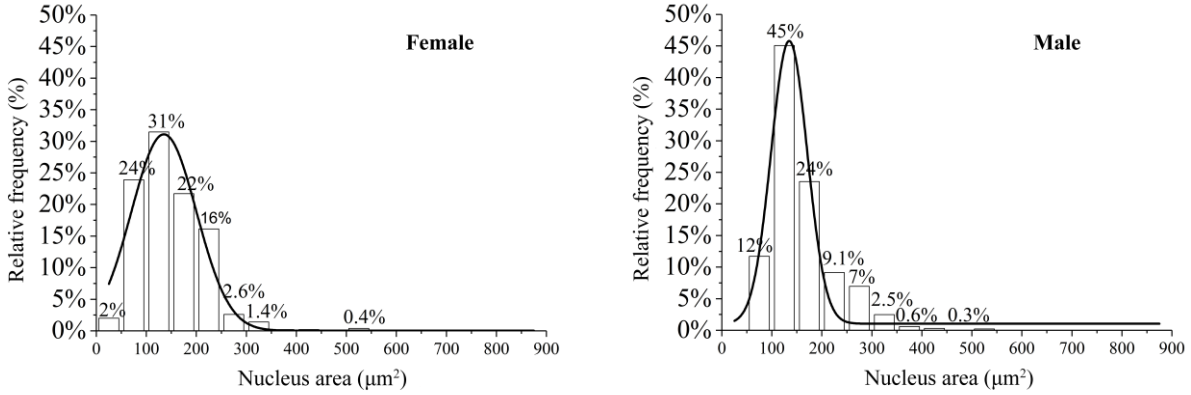

Fig 2. The distribution of cardiomyocytes nucleus area in adult male and female mice.

3.3 The Male Cardiomyocytes Area is Significantly Larger than that of Female Cardiomyocytes.

The measurement of isolated cardiomyocytes showed that the average length of female cardiomyocytes (105.30±0.83 μm) was larger than that of male cardiomyocytes (103.4±0.71 μm), however, the difference was not statistically significant (Table 1; p>0.05). The analysis for distribution of cardiomyocyte length revealed that the female and male cardiomyocyte length was mainly in the range of 60-160 μm and top listed distribution was 80-100 μm, accounted for 35%. For the distribution which ratio was equal or larger than 10%, it was showed that: 1) The length of female cardiomyocytes was ranged 60-140 μm (91%), while it was ranged 60-140 μm (93%) in male cardiomyocytes; 2) The maximal length of female cardiomyocytes (ratio is equal or larger than 1%) was 180 μm, while it was 160 μm in male cardiomyocytes (Fig 3). The results suggested that more than 90% male and female cardiomyocyte length were roughly the same and ranged 60-140 μm. In addition, the percentage of maximum length of female cardiomyocytes (ratio is equal or larger than 1%) was larger than that of the male cardiomyocytes.

For the width of cardiomyocytes, it was showed that the average width of female cardiomyocytes was 33.67±0.21 μm, which was significantly smaller than that of male cardiomyocytes (36.54±0.24 μm) (Table 1; p<0.001). And the widths of female cardiomyocytes ranged 20-60 μm and top listed ratio was 30-35 μm (30%), while the male cardiomyocytes widths ranged 15-60 μm and top listed ratio was more than 40 μm (30.6%). For the distribution which ratio was equal or larger than 10%, it was showed that: 1) The width ranged 25-45 μm was 90% in female cardiomyocytes, while it was 85% in male cardiomyocytes; 2) The maximal width of female cardiomyocytes (ratio is equal or larger than 1%) was 60 μm, while it was 55 μm in male cardiomyocytes (Fig 4). The results suggested that 85% male and female cardiomyocytes widths range 25-45 μm, the precentage of maximum width of female cardiomyocytes (ratio is equal or larger than 1%) was larger than that of the male cardiomyocytes.

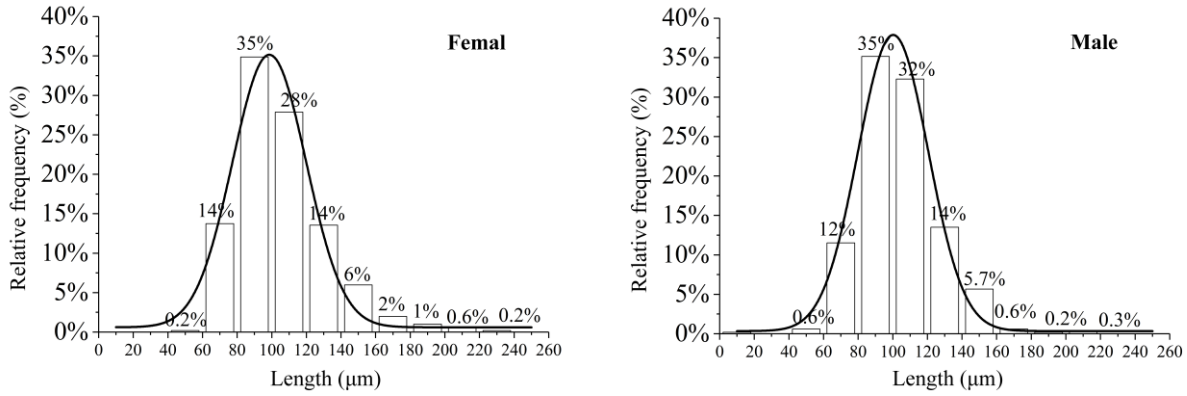

Fig 3. The distribution of cardiomyocytes length in adult male and female mice.

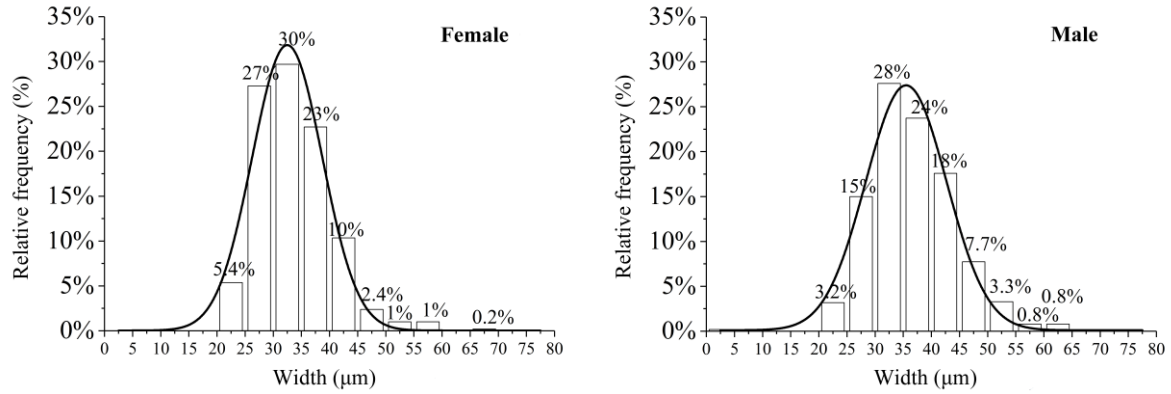

Fig 4. The distribution of cardiomyocytes width in adult male and female mice.

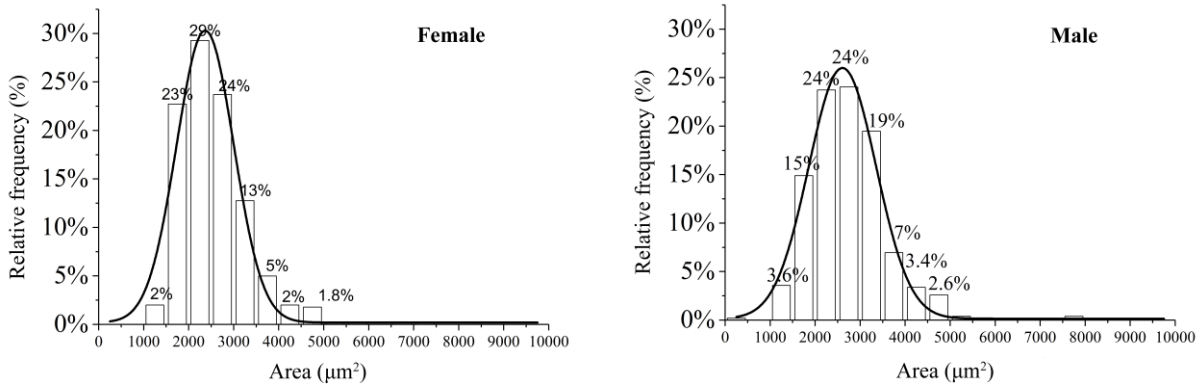

Fig 5. The distribution of cardiomyocytes area in adult male and female mice.

For the measurement of isolated cardiomyocytes area, It was found that the average area of male cardiomyocytes (2747±27.08 μm^2) was significantly larger than that of female cardiomyocytes (2552±24.04 μm^2) (Table 1; $p<0.001$). The male and female cardiomyocytes area were mainly in the range of 1000-5000 μm^2, and top listed distribution was 2000-3000 μm^2, accounted for 48% in male and 53% in female. For the distribution which ratio was equal or larger than 10%, it was showed that: 1) 89% of female cardiomyocytes area ranged 1500-3500 μm^2, while it was 82% in male cardiomyocytes. 2) The maximal area of male and female mice cardiomyocyte (ratio is equal or larger than 1%) was 5000 μm^2 (Fig 5). The results suggested that more than 80% male and female

cardiomyocytes area ranged 1500-3500 μm^2 and the maximum area of cardiomyocytes (5000 μm^2) (ratio is equal or larger than 1%) was the same in male and female cardiomyocytes. However, the percentage of male cardiomyocytes area in the range of 3000-5000 μm^2 (32%) was greater than that of female mice cardiomyocytes (21.3%).

3.4 The Ratio of Cardiomyocytes Area/Cardiomyocytes Nucleus Area in Male Mice is Significantly Smaller Than that in Female Mice.

It was found that the ratio of male cardiomyocytes area/cardiomyocytes nucleus area (19.06±0.24) was significantly smaller than that of female cardiomyocytes area/cardiomyocytes nucleus area (20.76±0.37) (Table 1, Fig 6; $p<0.01$).

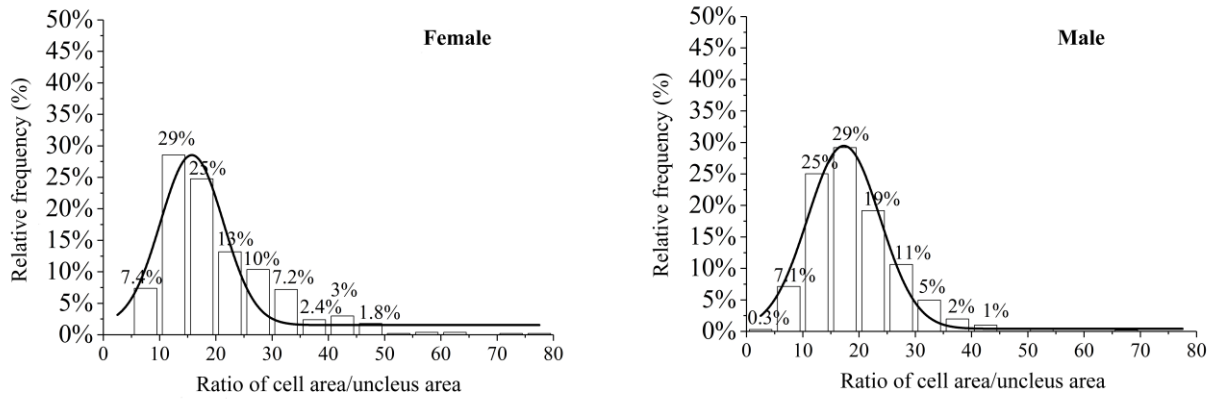

Fig 6. The distribution of cardiaomyocytes area/cardiomyocytes nucleus area ratio in adult male and female mice.

4. Discussion

Cardiomyocytes are the key functional cells in heart to maintain systolic function. It is well identified that cardiomyocyte can be mononucleated or multinucleated cell. However, the conventional histological section combing with nucleus staining methods to analyze the nucleus of the cardiomyocytes are difficult to accurately distinguish nuclei located in the boundary of cardiomyocytes on the heart section. In this study, the cardiomyocytes size, nucleus number and area in male and female mice were analyzed in single cardiomyocyte level. The results of present study showed that although the nuclear number of cardiomyocytes in both male and female mice was mainly two, the percentage of multinuclear (more than two) cardiomyocytes in male cardiomyocytes is lower than that in female cardiomyocytes, while the number of mononuclear and binuclear cardiomyocytes in male is higher than in female.

The comparison of nucleus area showed that, around 80% nucleus area of male and female cardiomyocytes was roughly the same. However, the average of nucleus area of male cardiomyocytes was significantly larger than that of the female cardiomyocytes. The distribution analysis further showed that the difference between male and female was mainly due to the increase in the proportion which is ranged larger than 100 μm^2. The comparison of the cardiomyocytes size showed that more than 90% male and female cardiomyocyte length was the same, and 85% male, and female cardiomyocytes width was the same, however, the average of width of male cardiomyocytes was significantly larger than that of the female cardiomyocytes. In addition, the average of area of male cardiomyocytes was significantly larger than that of female cardiomyocytes. Furthermore, the comparison of the cardiomyocytes area/cardiomyocytes nucleus area in male and female mice showed that, the ratio of cardiomyocyte area/cardiomyocyte nucleus area of male cardiomyocytes was significantly smaller than that of the female cardiomyocytes.

The results of present study suggested that cardiomyocyte size, nucleus number, nucleus area and their distribution in adult mice heart exist gender differences. In addition, it revealed that even though the nucleus area and area of cardiomyocytes in male cardiomyocytes were both singifcantly larger than those of in the female cardiomyocytes, however, due to the reason of decrease of nucleus number per male cardiomyocytes compared with female cardiomyocytes, therefore, the cell area corresponded by per unit nuclear area in male cardiomyocytes was larger than that of the female cardiomyocytes, which suggested the important structural difference between male and female cardiomyocytes in mice.

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