Yap Contributes to Cardiomyocyte Proliferation in the Fetal Rat Heart Epicardium with Antenatal Glucocorticoid Administration

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Abstract

**Background**: Our previous reports demonstrated that antenatal glucocorticoid (GC) administration increased cardiomyocyte proliferation in preterm fetuses, leading to the maturation of the heart. Understanding the proliferative mechanisms of fetal cardiomyocytes might be applicable to regenerative therapeutic strategies. In this study, we investigated whether antenatal GC administration affects Yes-associated protein (Yap), a transcriptional activator of cardiomyocyte proliferation.

**Methods and Results**: Eight-week-old pregnant Wistar rats were administered dexamethasone for 2 consecutive days between gestational days 17 and 21 before cesarean section. The production of the proliferation markers, cyclin D1, Ki-67, and Yap in the nuclei of cardiomyocytes was analyzed using immunohistochemistry. To clarify the relationship between upstream factors and Yap, we evaluated the expression of agrin, an extracellular matrix protein, using Western blot analysis. Although cyclin D1-, Ki-67-, and Yap-positive cells gradually decreased from the fetal to neonatal stage, they increased significantly in the epicardium of 19-day fetal groups with antenatal GC administration. Agrin was also significantly increased in fetal hearts in the GC group.

**Conclusions**: These results suggest that antenatal GC administration affects cell proliferation in the fetal epicardium, and the increased agrin and Yap activation may be involved.

**Key Words**

Antenatal glucocorticoid, preterm birth, fetal heart, Yap, cyclin D1

Introduction

Although cardiomyocytes can proliferate in the fetal heart,¹ this ability is lost after birth, when heart growth continues through the expansion in cardiomyocyte volume. It is almost impossible for the adult heart to regenerate after injury due to myocardial infarction, often leading to the development of heart failure, a major cause of death. Understanding the proliferative mechanisms of fetal cardiomyocytes might be applicable to the development of regenerative therapeutic strategies. We reported that antenatal glucocorticoid (GC) administration in animal models leads to heart maturation and demonstrated the mechanisms of cardiomyocyte functions, such as the expression of contraction factors and energy production, etc.²,³ Furthermore, GC enlarges the cross section of the fetal heart as a result of cardiomyocyte proliferation.⁴ One cardiac hypertrophy-related factor, the protein kinase B (Akt) level, was found to be increased in the fetal cardiomyocytes of prenatal rats, and β-catenin and vascular endothelial growth factor

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protein levels were also increased. The transcriptional co-activator Yes-associated protein (Yap) is known to be necessary for cardiomyocyte proliferation during the newborn stage. However, the upstream regulators and signaling pathways that control Yap activity in the heart remain unknown. A study has found that increases in Yap in the nucleus and the cell cycle regulator are associated with cardiomyocyte proliferation in the heart.

The Hippo-Yap pathway plays roles in controlling cell growth, tissue homeostasis, and organ size. Hippo pathway activation, including the sterile-20-like protein kinase (Msts) and phosphorylates large tumor suppressor homologue (Lats) kinases and Salvador (Salv) adaptor, results in phosphorylation and nuclear exclusion of transcriptional cofactors Yap/Taz. On the other hand, inactivation of upstream kinases translocates Yap/Taz in the nucleus, where they consequently interact with various transcription factors. This causes the promotion of transcriptional activity for downstream target genes involved in differentiation, cell proliferation, and survival. We therefore investigated whether antenatal GC administration increases nuclear Yap levels and affects the proliferative potential of cardiomyocytes in fetal rats.

Recently, it has been reported that the maturation of the epicardium is essential for mammalian cardiac development. The epicardium and epicardium-derived factors contribute coronary smooth muscles and fibroblasts to the developing heart and regulate cardiomyocyte proliferation and the enlargement of chamber myocardium. Therefore, in particular, we noted the cell proliferative capacity in cardiomyocytes in the epicardium of preterm rat models.

Methods

Animal Experimental Protocols

All animal procedures were performed according to the Guidelines of the Institute of Experimental Animals, St. Marianna University Graduate School of Medicine for the Care and Use of Experimental Animals (No. 2002008). Eight-week-old pregnant Wistar rats were purchased from CLEA (Tokyo, Japan) and were given dexamethasone (DEX) (Fujifilm Wako Pure Chemical, Osaka, Japan) dissolved in sesame oil. The pregnant rats were randomly divided into 2 groups to receive either DEX or vehicle (sesame oil) as a control at a dose of 0.5, 1.0, or 2.0 mg/kg administered subcutaneously twice daily on gestational days 17 and 18, or 19 and 20, or 20 and 21. The fetuses were delivered by cesarean section on gestational days 19 and 21, the 1-day-old neonates (1N) were euthanized under inhaled isoflurane anesthesia, and the hearts were immediately removed and frozen at -80ºC until use.

Immunohistochemistry

Rat heart tissue sections (5 μm) fixed in 10% formalin were embedded in paraffin, and the samples were deparaffinized in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity in the tissue sections was inactivated with 0.1% hydrogen peroxide. Immunoreactivity in the sections was demonstrated using a Dako catalyzed signal amplification (CSA) system (Carpinteria, CA, USA) according to the manufacturer’s instructions. Microscopic observations were performed with goat IgG antibody used as a negative control. Samples were analyzed by counting 300 cardiomyocytes in 3 different fields for the free walls of the right ventricle (RV) and the left ventricle (LV), and then the mean number of Ki-67-, cyclin D1-, and Yap-positive cells divided by the total number in the nuclei of cardiomyocytes was determined. As shown in Fig. 1A, the positive rate was calculated for each of the 3 compartments. We defined the epicardium as the area within 200 μm from the epicardium edge. Similarly, the endocardium was the area within 200 μm from the endocardial edge, and the myocardium was the area within 100 μm on each side of the midline between the epicardium and endocardium (Fig. 1A) since the wall thickness of the 19-day embryonic group samples were about 600 μm. The anatomical terms endocardium and epicardium are thin membranes, but the endocardium and epicardium defined in this paper are regions. Therefore, the terms endocardium and epicardium in this paper differ from the commonly used anatomical terms. Three samples were examined in each group, except for the 3- and 5-day-old neonate (3N and 5N) groups in which only 1 and 2 samples, respectively, could be obtained.

Western blot analysis

The whole protein of the cardiac tissue was homogenized with lysis solution (CelLytic MT tissue protein extraction solution, Sigma-Aldrich Corp., St. Louis, MO, USA). The proteins in the samples (10 μg) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (ATTO, Tokyo, Japan). The membrane was blocked with 5% skim milk in Tris-buffered saline containing Tween 20 (NaCl 150 mmol, Tris-HCl 100 mmol/L, pH 7.5, Tween
0.5%) overnight at 4°C. Western blot analysis, using anti-agrin (Novus Biologicals, Centennial, CO, USA) and anti-α-tubulin polyclonal antibody (Fujifilm Wako Pure Chemical), was undertaken for 2 h at room temperature. Reactive proteins were viewed under enhanced chemiluminescence (ImmunoStar, Wako, Tokyo). Signal intensities of the detected bands were analyzed using a C-DiGit Chemiluminescent Western Blot Scanner (LI-COR Bioscience, Lincoln, NE, USA). Protein expression was quantified by densitometric analysis. Three samples were used from each group. The expression levels of α-tubulin were confirmed as a control.

Statistical analysis

Data are expressed as mean ± SD. All statistical analyses were performed with the JMP 13 (SAS Institute Inc., Cary NC, USA), and a p value of <0.05 was considered to represent a statistically significant difference. Pearson’s correlation analysis was used to investigate relationships between variables.

Results

Production of cyclin D1 and Ki-67 in rat hearts with growth

We first evaluated cyclin D1 and Ki-67 production in the outer epicardium, middle myocardium, and inner endocardium of fetal to 5N and 8-week adult (8W) rats (Fig. 1). Numerous cyclin D1- and Ki-67-positive cells in the nuclei were histologically observed in the heart tissue of fetal and 1N groups, which gradually decreased from postnatal day 3. Those positive cells were rarely observed in 8W hearts (Fig. 1B). As shown in Fig. 1B, similar frequencies of cyclin D1- and Ki-67-positive cells in the nuclei were observed in the RV and LV. Cyclin D1-positive cells in the endocardium were significantly increased in the 21-day fetal (21F) (73.3 ± 4.7%) compared with the 19-day fetal (19F) (49.6 ± 11.3%) group. On the other hand, Ki-67-positive cells gradually decreased significantly from fetal day 19 (76.4 ± 3.3%) to postnatal day 1 (1N) (64.9 ± 1.9%) (Fig. 1C). The percentage of cyclin D1-positive cells in the myocardium did not change with growth (19F, 46.0 ± 3.4%; 21F, 56.2 ± 10.2%; 1N, 58.5 ± 8.9%), while Ki-67-positive cells significantly increased in the 1N (79.6 ± 4.2%) compared with the 19F (59.0 ± 6.4%) group. Furthermore, cyclin D1- and Ki-67-positive cells in the epicardium markedly increased in the 21F (cyclin D1, 45.6 ± 13.8%; Ki-67, 44.7 ± 5.6%) and 1N (cyclin D1, 47.7 ± 4.9%; Ki-67, 49.7 ± 5.5%) groups compared with the 19F group. The percentages of both types of positive cells in the 3 heart layers in the 8W groups were lower than those in the fetal and 1N groups. There were positive correlations between cyclin D1- and Ki-67-positive cells in the heart of 19F untreated groups ($R^2 = 0.7335, p < 0.01$) (Fig. 1D).

Cyclin D1 and Ki-67 production after antenatal DEX administration

Similar evaluations were carried out in the antenatal DEX administration groups (Fig. 2). Cyclin D1- and Ki-67-positive cells were significantly increased in 19F epicardium after antenatal DEX administration (cyclin D1: untreated [DEX(-)], 22.9 ± 8.9%; 0.5 mg/kg, 24.9 ± 5.1%; 1.0 mg/kg, 37.1 ± 2.7%; 2.0 mg/kg, 42.2 ± 5.0%; Ki-67: DEX (-), 19.6 ± 6.4%; 0.5 mg/kg, 22.0 ± 5.4%; 1.0 mg/kg, 38.7 ± 5.9%; 2.0 mg/kg, 36.8 ± 2.9%). On the other hand, Ki-67-positive cells were significantly decreased in the endocardium (DEX (-), 76.4 ± 3.3%; 0.5 mg/kg, 64.6 ± 2.9%; 1.0 mg/kg, 54.2 ± 3.8%; 2.0 mg/kg, 62.5 ± 6.8%) and myocardium (DEX (-), 59.0 ± 5.5%; 0.5 mg/kg, 47.1 ± 4.4%; 1.0 mg/kg, 45.1 ± 4.9%; 2.0 mg/kg, 57.6 ± 5.4%) in the 19F group. There were no changes in cyclin D1 and Ki-67 production in the 3 heart tissue layers in the 21F group (Fig. 2B).

Interestingly, a significant correlation between cyclin D1 and Ki-67 positivity was seen ($R^2 = 0.5346, p < 0.01$) in the epicardium of the 19F group after antenatal DEX administration (Fig. 2C).

Yap production in rat heart after antenatal DEX administration

Yap-positive cells in the nuclei of fetal and 1N heart tissues increased in the fetal period, decreased after birth, and continued to decrease gradually with age, and thus there were few positive cells in the 8W group (Fig. 3A and 3B). When we investigated Yap-positive cells in each layer, there was a significant decrease in the endocardium in the 1N (43.7 ± 7.3%) and 8W (36.3 ± 11.0%) compared with the fetal groups (19F, 61.3 ± 4.2%; 21F, 77.1 ± 0.7%). However, Yap-positive cells in the epicardium were significantly increased in the 21F (48.5 ± 6.0%) compared with the 19F (33.7 ± 2.8%) group. Yap-positive cells were strongly correlated with cyclin D1- or Ki-67-positive cells in the 19F (cyclin D1, $R^2 = 0.7535, p < 0.01$; Ki-67, $R^2 = 0.8524, p < 0.001$) and 21F (cyclin D1, $R^2 = 0.6403, p < 0.01$; Ki-67, $R^2 = 0.5992, p < 0.05$) untreated groups (Fig. 3C).
Fig. 1. Immunohistochemical analysis of cyclin D1 and Ki-67 in the rat heart with development.

(A) Cross-section of a 19-day-old fetal (19F) rat heart (H&E staining). Original magnification ×40. endo, endocardium; myo, myocardium; epi, epicardium.

(B) Immunohistochemical analysis of cyclin D1- and Ki-67-positive cells in untreated [DEX(-)] rat hearts. Immunostained positive cells appear brown. Data from fetuses, 1-day-old neonates (1N), and 8-week-old adult (8W) rats are representative of 3 independent experiments. Data from 3-day-old (3N) and 5-day-old neonates (5N) are representative of 2 and 1 experiments, respectively. 19F, 19-day-old fetus; 21F, 21-day-old fetus; LV, left ventricle; RV, right ventricle; Original magnification×400.

(C) Cyclin D1- and Ki-67-positive cells in the nuclei as a percentage of the number of total cells. Data are expressed as mean ± SD. Data from fetal, 1N, and 8W rats are representative of 3 independent experiments. ¶, p < 0.01 for each; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

(D) Correlation between cyclin D1- and Ki-67-positive cells in the 3 layers of the heart. Each layer in the 19F and 21F untreated groups is shown in different colors: endocardium, black; myocardium, red; epicardium, blue.
Fig. 2. Immunohistochemical analysis of cyclin D1- and Ki-67-positive cells in rat hearts after antenatal DEX administration.

(A) Immunohistochemical analysis of cyclin D1 and Ki-67 in 19F and 21F hearts after antenatal DEX. Because similar frequencies of cyclin D1- and Ki-67-positive cells in the nuclei were observed in the RV and LV, only LV histograms are shown. Original magnification ×400.

(B) Cyclin D1- and Ki-67-positive cells in the nuclei as a percentage of the total number of cells. Immunostained positive cells appear brown. Data are expressed as mean ± SD. Data from fetal, 1N, and 8W rats are representative of 3 independent experiments.

(C) Correlation between cyclin D1- and Ki-67-positive cells in the 3 heart tissue layers. Each layer in the 19F and 21F untreated groups and after DEX administration groups are shown in different colors: endocardium, black; myocardium, red; epicardium, blue. DEX, dexamethasone.

DEX 0.5 mg/kg (75.8 ± 2.3%) and 1.0 mg/kg (82.4 ± 4.2%) groups showed significant increases in Yap-positive cells compared with the untreated group (61.3 ± 4.2%) in the 19F endocardium after antenatal GC administration. The percentage in the DEX 2.0 mg/kg group (87.2 ± 3.2%) in the 21F endocardium was higher than that in the untreated group (75.4 ± 3.5%) (Fig. 3E).

These results indicate that the localization of cyclin D1, Ki-67, and Yap cells was similar in the 3 layers. Although increases in cyclin D1 and Ki-67 production were strongly correlated in the 19F epicardium with antenatal DEX administration (R² = 0.5364, p < 0.01), the production of Yap was not correlated with that of cyclin D1 (R² = 0.0095) and Ki-67 (R² = 0.0002). Furthermore, there were no correlation between the production of Yap and cyclin D1 nor Ki-67 in cardiomyocytes of myocardium (Fig. 3F and 3G). However, Yap-positive cells were negatively correlated with Ki-67 positive cells in the endocardium of 19F DEX group (R²=0.4008, p < 0.05, Fig 3G). Taken together, these findings suggest that the values of both positive cells in 19F untreated group group were likely affected.

**Production of agrin in rat heart after antenatal DEX administration**

The agrin protein levels in 19F untreated group were approximately equal from 21F to 8W (Fig. 4A and 4C). On the other hand, DEX administration en-
A

| YAP - positive cells in nuclei (%) |
|-----------------------------------|
| 19F  | 21F  | 1N  | 3N  | 5N  | 8W  |
| RV   | LV   |     |     |     |     |
| 100% | 100% |     |     |     |     |

B

| YAP - positive cells in nuclei (%) |
|-----------------------------------|
| 19F  | 21F  | 1N  | 3N  | 5N  | 8W  |
| RV   | LV   |     |     |     |     |
| 100% | 100% |     |     |     |     |

C

19F, DEX (-)

y = 0.748x + 19.298
R² = 0.7535
P < 0.01

21F, DEX (-)

y = 0.6502x + 24.559
R² = 0.6403
P < 0.01

D

| YAP - positive cells in nuclei (%) |
|-----------------------------------|
| 19F  | 21F  | 1N  | 3N  | 5N  | 8W  |
| RV   | LV   |     |     |     |     |
| 100% | 100% |     |     |     |     |
**E**

![Diagram showing YAP-positive cells in nuclei](image)

**19F**

Endocardium

- YAP-positive cells in nuclei (%)
  - DEX (mg/kg): 0, 0.5, 1.0, 2.0
  - **YAP**

Myocardium

- YAP-positive cells in nuclei (%)
  - DEX (mg/kg): 0, 0.5, 1.0, 2.0
  - **Cyclin D1**

Epicardium

- YAP-positive cells in nuclei (%)
  - DEX (mg/kg): 0, 0.5, 1.0, 2.0
  - **Cyclin D1**

**F**

![Diagram showing YAP-positive cells in nuclei](image)

**19F**

Endocardium

- YAP-positive cells in nuclei (%)
  - **YAP**

Myocardium

- YAP-positive cells in nuclei (%)
  - **Cyclin D1**

Epicardium

- YAP-positive cells in nuclei (%)
  - **Cyclin D1**

**21F**

Endocardium

- YAP-positive cells in nuclei (%)
  - **YAP**

Myocardium

- YAP-positive cells in nuclei (%)
  - **Cyclin D1**

Epicardium

- YAP-positive cells in nuclei (%)
  - **Cyclin D1**

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**Graphical Representation**

- **YAP Positive Cells**: Variation with DEX dose across Endocardium, Myocardium, and Epicardium.
- **Cyclin D1 Positive Cells**: Linear regression analysis indicated significant correlations.

**Statistical Analysis**

- **YAP**: Linear correlation with DEX dose.
  - **Equation**: $y = -0.0886x + 85.422$
  - **R²**: 0.0019

- **Cyclin D1**: Linear correlation with DEX dose.
  - **Equation**: $y = 0.0368x + 46.22$
  - **R²**: 0.0095
Fig. 3. Immunohistochemical analysis of Yap in rat hearts after antenatal DEX administration.

(A) Immunohistochemical analysis of Yap in untreated [DEX (-)] rat hearts. Data from fetuses, 1N, and 8W rats are representative of 3 independent experiments. Data from 3N and 5N rats are representative of 2 and 1 experiments, respectively. Original magnification ×400.

(B) Yap-positive cells in the nuclei as a percentage of the number of total cells. Data are expressed as mean ± SD. Data are representative of 3 independent experiments. ¶, p < 0.01 for each; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

(C) Correlation between Yap- and cyclin D1-positive cells. There was a strong correlation between Yap- and cyclin D1-positive cells in 19F and 21F untreated groups. However, Yap-positive cells in the epicardium of 19F and 21F rats were not correlated with cyclin D1-positive cells.

Correlation between Yap- and Ki-67-positive cells. There was a strong correlation between Yap- and Ki-67-positive cells in 19F and 21F untreated groups. However, Yap-positive cells in the epicardium of 19F and 21F rats were not correlated with Ki-67-positive cells.

(D) Immunohistochemical analysis of Yap in 19F and 21F hearts after antenatal DEX administration. Immunostained positive cells appear brown. Data from fetal, 1N, and 8W rates are representative of 3 independent experiments. Data from 3N and 5N rats are representative of 2 and 1 experiments, respectively. Original magnification ×400.

(E) Yap-positive cells in the nuclei as a percentage of the number of total cells. Data are expressed as mean ± SD. Data are representative of 3 independent experiments. *, p < 0.05 vs 19F untreated group; **, p < 0.01 vs 19F untreated group.

(F) Correlation between Yap- and cyclin D1-positive cells in each layer of 19F and 21F hearts after antenatal DEX administration. Yap-positive cells were negatively correlated with Ki-67 positive cells in the endocardium of 19F DEX group.

(G) Correlation between Yap- and Ki-67-positive cells in each layer of 19F and 21F hearts after antenatal DEX administration. However, Yap-positive cells in each layer of 19F and 21F rats were not correlated with Ki-67-positive cells.
hanced the agrin protein levels in 19F and 21F hearts in a dose-dependent manner (Fig. 4B and 4C).

Discussion

Mammalian cardiomyocytes stop proliferating after birth, leading to increases in cell size and physiological hypertrophy with continued heart growth. Although cardiomyocytes proliferate vigorously in embryonic murine fetal hearts, they cease to do so approximately 5 days after birth. In the developing heart, cardiomyocyte proliferation is regulated by several different pathways: the neuregulin/ErbB/ERK pathway is important for primary proliferation in the embryonic heart; PI3K/Akt/GSK3β pathways are required for hyperplastic growth of cardiomyocytes; and the Hippo-Yap pathway is a critical regulator of cardiomyocyte proliferation and organ size throughout development and after birth. Regulatory mechanisms of cardiomyocyte proliferation and the postnatal cell cycle act simultaneously. The expression of cyclin and cyclin-dependent kinase (CDK) decreases and arrests the cell cycle in mammalian cardiomyocytes in the days immediately after birth. Cyclin D synthesized during growth forms a CDK complex by nuclear translocation and promotes the G1-to-S phase transition. Cyclin D1 is a particularly important factor promoting the G1-to-S phase progression, and mitogenic stimulation induces
hypertrophic cell growth and upregulates cyclin D1 expression in postmitotic cardiomyocytes. During the G1 phase, cyclin D1 accumulates in the nucleus of cardiomyocytes, contributing to cardiomyocyte proliferation.18

We previously reported that antenatal DEX administration increases the production of the cell proliferation marker Ki-67 in the fetal rat heart.21 We concluded that the proliferation was attributed to physiological hypertrophy with growth mediated by Akt/Gsk3β/β-catenin activation,49 although it was believed that other mechanisms were also involved. However, the layers of the heart wall consisting of the outer epicardium, middle myocardium, and inner endocardium show different localization of Ki-67 protein and the differential rates of cardiomyocyte maturation and proliferation in the 3 layers remain to be clarified. In this study, we first evaluated cyclin D1 and Ki-67 production in each layer. In immunohistochemical analysis, numerous cyclin D1- and Ki-67-positive cells in the nuclei were observed in rat fetuses and 1-day neonates in all 3 layers, which gradually decreased from postnatal day 3 (Fig 1C). Cyclin D1- and Ki-67-positive cells were rarely observed in 8W rats. That finding is consistent with previous reports.14 Antenatal DEX administration significantly decreased Ki-67-positive cells in the 19F endocardium, while it significantly increased those in the 19F epicardium. The cell proliferative ability in 19F endocardium after antenatal DEX resembles the decrease in cell proliferation with growth. On the other hand, the cell proliferative ability in 19F epicardium was activated by DEX. Although the expression levels of cyclin D1- and Ki-67-positive cells differed in each layer, these factors were positively correlated in 19F and 21F untreated groups (Fig. 1D). Furthermore, cyclin D1-positive cells were positively correlated with Ki-67-positive cells in 19F epicardium after antenatal DEX administration (R² = 0.5346, p < 0.01), suggesting that DEX promotes cardiomyocyte proliferation (Fig 2C). There was no correlation between cyclin D1 and Ki-67 expression in the endocardium and myocardium of 19F hearts after antenatal DEX administration, and there was no change in the 3 layers in the 21F DEX groups.

The proliferation pattern changes markedly before and after birth. Many cardiomyocytes undergo mitosis, resulting in binucleated cardiomyocytes during early postnatal stages, following which the cell cycle arrests irreversibly.19,20 The number of cardiomyocytes increases with cell cycle progression through cytokinesis before birth, however after birth, the number does not change. Furthermore, the expression patterns of cyclins and CDKs and their activities decreased from mid-gestation to postnatal day 5, accompanied by the transition from mono- to binucleated cells after birth.21 With the decrease in cyclin, the arrest of cardiomyocyte proliferation coincides with the onset of polyploidization. Cardiomyocytes in the fetal endocardium and myocardium might be prepared for the transition to binucleation by the arrest of cell cycle progression. Mammalian fetal heart progenitor cells are derived from the mesoderm (endocardium and myocardium), proepicardium (epicardium and coronary vessels), and neural crest (such as heart valves).22 Therefore, differences in the response to antenatal DEX administration occur in the 3 layers.

Antenatal DEX administration affects epicardial cell proliferation in the early fetal stage. The epicardium is a thin mesothelial tissue comprising the outermost layer of the heart. Epicardial maturation was reported to be essential for mammalian heart development.12,13 The epicardium is an active cell population involved in the development of diverse cardiac structures. Epicardial derived progenitors contribute to the differentiation of a variety of cell types, such as coronary smooth muscle cells, interstitial fibroblasts, endothelial cells, and antiventricular cushion mesenchymal cells, comprising the major lineages of the mature heart.11,13,23–25 Federica et al. reported that c-kit- and CD34-positive epicardium-derived cells can be detected in human fetal and adult epicardium, suggesting that the epicardium contains cell populations with proliferative or differentiation ability.26 Fischer et al. demonstrated that cardiac progenitor cells over-expressing Pim-1 (an enhancer of regenerative potential) increased proliferation after DEX exposure.27 Ventricular wall expansion is driven by cardiomyocyte proliferation coupled with morphogenesis that causes wall thickening during fetal development.3,4 Our previous results indicated that enlargement of the fetal heart results from cardiomyocyte proliferation and vascularization,5 suggesting that 19F epicardial cardiomyocytes retain proliferative ability that is promoted by GC. We then investigated the related signaling pathways.

The Yap signaling pathway is also necessary to increase cardiomyocyte numbers and heart size, leading to heart growth. Yap, as a critical downstream effector, is also required for cardiomyocyte proliferation during the newborn stage.28 We observed
numerous Yap-positive cells in all 3 layers of the heart in the fetal to 1N groups, and those numbers tended to decrease with growth. However, Yap production was significantly increased in the endocardium and epicardium in 19F rats after antenatal DEX 0.5 and 1.0 mg/kg administration. Furthermore, Yap-positive cells were increased in 21F rats that received DEX 2.0 mg/kg antenatally. Yap expression in the 3 heart tissue layers was strongly correlated with cyclin D1- and Ki-67-positive cell numbers in fetal untreated groups, while no significant correlation was found in the fetal epicardium after antenatal DEX. Sorrentino et al. reported that GC directly increased Yap activation mediated through GC receptors in breast cancer cells. Yap activation by antenatal DEX administration may contribute to other functions but not to cell proliferation. Singh et al. demonstrated that Hippo pathway-related genes, Lats1/2, and Yap are expressed in the epicardium, and that Yap contributes to coronary vascular development in mice. In addition, mice deficient in epicardial Yap and TAZ, as Hippo pathway effectors, develop profound postmyocardial infarction pericardial inflammation and myocardial fibrosis, resulting in cardiomyopathy and death. Epicardial Yap is required for cardiac maturation, and its administration to preterm neonatal mice lowers the risk of developing chronic heart disease in adulthood. Increased Yap in the epicardium may be involved in various aspects of cardiac development, in addition to cell proliferation. Further studies are needed to clarify the roles of Yap.

Extracellular matrix (ECM) components promote and regulate cardiomyocyte morphogenesis in the mammalian heart. The Hippo signaling pathway is one upstream factor regulating the ECM. After birth, the dystrophin-glycoprotein complex (DGC) component directly binds to Yap, preventing nuclear translocation and inhibiting cardiomyocyte proliferation. Recent studies have reported that epicardium maturation is essential for mammalian heart development, and agrin has attracted attention as a Yap-regulatory factor. Recently, several studies have found that the ECM protein agrin may promote heart regeneration through cardiomyocyte proliferation. The mRNA and protein levels of agrin gradually decrease after birth. However, during heart development, agrin acts as a ligand for several ECM receptors within the transmembrane DGC to destabilize cell interaction and accelerates the nuclear translocation of Yap in cardiomyocytes. In the present study, agrin protein levels were unchanged in fetal and neonatal hearts, and agrin expression was retained from the fetal to 8W stage. Antenatal DEX administration dose-dependently increased agrin protein levels in both the 19F and 21F groups, and no change in agrin localization was observed.

The interaction between cardiomyocytes and the ECM is a critical regulator of cardiac organogenesis and may contribute to the ability to undergo regeneration. The ECM plays irreplaceable cell-autonomous roles in regulating cellular communication, differentiation, and proliferation in endocardial and epicardial cells. Recent studies have reported that the ECM including agrin can affect cardiomyocyte proliferation and/or migration as well as modulate cardiac myocyte contraction. A limitation of this study was that it did not show the direct mechanism by which the agrin-YAP signaling pathway increases cell proliferation. A conclusion has not been reached on the relationship between Yap-induced activation of the epicardium and agrin. Further experiments are therefore necessary to resolve this relationship.

In conclusion, the cell proliferative ability in the epicardium was greater than that in the endocardium and myocardium in the 19F group, and antenatal GC administration promoted cell proliferation in the fetal epicardium. Both cell cycle progression and Yap activation are among the mechanisms involved in fetal cardiomyocyte proliferation in the epicardium. Our results suggest that antenatal GC administration may contribute to cardiac development. Detailed knowledge of the properties of epicardium-based cell proliferation mechanisms will be useful for developing therapeutic strategies to treat cardiovascular disease. The proliferative capacity in cardiomyocytes of the epicardium may contribute to regenerate damaged myocardium.

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Conflicts of Interest

The authors have nothing to disclose.

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