

**Alteration in the expression of the renin-angiotensin system in the myocardium of mice
conceived by in vitro fertilization**

Running title: Effects of *in vitro* fertilization on myocardium

Summary sentence: Altered expression of the renin-angiotensin system in myocardial tissue might contribute to an increased risk of cardiovascular malformations and dysfunction in IVF offspring, and is involved in abnormal regulations of DNA methylation and microRNAs.

Key words: DNA methylation; *in vitro* fertilization; mouse; myocardium; renin-angiotensin system; microRNA.

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ABSTRACT

Epidemiological studies have revealed that offspring conceived by *in vitro* fertilization (IVF) have an elevated risk of cardiovascular malformations at birth, and are more predisposed to cardiovascular diseases in late childhood. Unfortunately, the underlying mechanisms influencing compromised cardiovascular function in IVF-conceived children remain unclear. Moreover, the continuing implications for the cardiovascular health of these children as they move into adulthood and old age, have yet to be determined. The renin-angiotensin system (RAS) plays an essential role in both the pathogenesis of congenital heart disease in fetuses and cardiovascular

dysfunction in adults. This study aimed to assess the relative expression levels of genes in the RAS pathway in mice conceived using IVF, compared to natural mating with superovulation. In addition, the relative expression of candidate genes expressed in the myocardial tissue of newborn, adult and old mice was determined between the IVF and naturally mated populations. Results demonstrated that expression of the angiotensin II receptor type 1 (AGTR1), connective tissue growth factor (CTGF), and collagen 3 (COL3), in the myocardial tissue of IVF-conceived mice, was elevated at 3 weeks, 10 weeks, and 1.5 years of age, when compared to their non-IVF counterparts. These data were supported by microRNA microarray analysis of the myocardial tissue of aged IVF-conceived mice, where *miR-100*, *miR-297*, and *miR-758*, which interact with COL3, AGTR1 and COL1 respectively, were upregulated when compared to naturally mated mice of the same age. Interestingly, bisulfite sequencing data indicated that IVF-conceived mice exhibited decreased methylation of CpG sites in *Col1*. In support of our *in vivo* investigations, *miR-297* overexpression was shown to upregulate AGTR1 and CTGF, and increased cell proliferation in cultured H9c2 cardiomyocytes. These findings indicate that the altered expression of RAS in myocardial tissue might contribute to cardiovascular malformation and/or dysfunction in IVF-conceived offspring. Furthermore, these cardiovascular abnormalities might be the result of altered DNA methylation and abnormal regulation of microRNAs.

INTRODUCTION

Since the birth of Louise Brown, the first baby conceived via *in vitro* fertilization and embryo transfer (IVF-ET) in 1978, more than 5 million newborns have been conceived through assisted reproductive technology (ART) [1, 2]. ART includes controlled ovarian hyperstimulation,

IVF-ET, intracytoplasmic sperm injection (ICSI), preimplantation genetic diagnosis, and gamete and embryo cryopreservation. The clinical implementation of ART has made a significant contribution to the treatment of infertility [3]. However, ART intervenes several key points during the process of human conception and early development, specifically gametogenesis, fertilization, and early embryonic maturation. Data has shown that during the periconceptional period, the oocyte and embryo are highly sensitive to signals from their surrounding environment and as such, are particularly susceptible to damage which might manifest in an altered adult phenotype [4]. Follow-up studies on the growth and development of ART-conceived human offspring have aroused widespread concern as their findings indicate that, when compared with naturally conceived newborns, those conceived by IVF are at greater risk of low birth weight (LBW), increasing their susceptibility in adulthood to cardiovascular disease and diabetes [5-7]. These data are of particular concern given the extensive clinical application of ART and a large number of resultant ART-conceived offspring [8]. Multicenter epidemiological studies have shown that the incidence of cardiovascular malformations in ART babies is markedly increased compared with that of naturally conceived offspring [9-11]. Furthermore, follow-up surveys of ART-conceived offspring have shown an increased risk of dysfunction [12] and remodelling in the cardiovascular system [13], including higher arterial blood pressure, altered vascular intima-media thickness, and compromised myocardial regulatory function [14]. Investigations using mouse models have further illustrated that ART-conceived offspring have abnormal vascular endothelial function and changes in lipid metabolism [15, 16]. While it is widely accepted that IVF-conceived offspring have an increased risk of poor cardiovascular health, the

precise mechanisms underlying cardiovascular malformation and dysfunction remain unclear.

The renin-angiotensin system (RAS) is a major physiological system that regulates blood pressure and the balance of fluids and electrolytes within the body. In addition, the RAS is known to play an essential role in the pathogenesis of several types of cardiovascular disease as well as in cardiac remodelling [17, 18]. Growing evidence reveals that in addition to the classic systemic RAS, a local RAS operates, in organs such as the heart, large arteries, kidneys, and even in single cells to further modulate cardiovascular regulation [19, 20]. In the RAS, the precursor protein angiotensinogen (AGT) is first converted into angiotensin I (Ang I) by renin (Ren). Then, Ang I is hydrolyzed to Ang II-IX by angiotensin-converting enzymes (ACE). Ang II is a biological key hormone, and its function is mediated through the receptors of Ang II type 1 (AGTR1) or Ang II type 2 (AGTR2) [21]. Excessive activation of AGTR1 results in increased expression levels of the extracellular matrix components, such as collagen 1 (COL1) and collagen 3 (COL3) [22] which are distributed in several layers of the heart wall to provide rigidity and elasticity [23]. During the embryonic period, abnormal expression of COL1 and COL3 can affect the normal development of the cardiovascular structures [24, 25]. In adulthood, overstimulation of the RAS can contribute to the pathogenesis of a number of cardiovascular diseases, affecting the normal function of cardiovascular tissue cells and increasing the risk of hypertension, heart arrhythmias, and cardiac hypertrophy [26]. Connective tissue growth factor (CTGF) promotes fibrosis therefore playing a further key role in AT1-mediated intracellular signal transmission that results in myocardial remodelling and vascular fibrosis [27].

During gametogenesis and early embryogenesis, parental genomes undergo demethylation

and remethylation for epigenetic reprogramming. The first round of genome reprogramming occurs in the germline, while the second round of genome reprogramming occurs after fertilization when somatic methylation patterns are generated [28]. Evidence suggests that the early embryo, and later the fetus, is highly sensitive to its *in utero* environment, and that a suboptimal uterine milieu, such as is experienced by the fetus during maternal nutritional restriction, could increase the incidence of chronic diseases in adulthood via epigenetic modifications in the embryo or fetus [4, 29, 30]. Since IVF-ET overlaps with the epigenetic reprogramming period of the embryo, the risk of imprinting defects that cause imprinted disorders such as Prader-Willi syndrome (PWS) and Angelman syndrome (AS) may increase when fertilization and early embryonic development occur *in vitro* [31]. Recent experiments in humans and mice have revealed differences in the methylation levels of CpG islands in imprinting genes and developmentally important genes between ART-conceived offspring and control groups [32, 33]. A previous study from our research group showed alterations in *Igf2/H19* expression and DNA methylation in both the liver and the skeletal muscle of IVF mice [34]. Interestingly, ICSI-conceived fetuses also exhibit a decreased rate of CpG island methylation, accompanied by increased expression of Insulin-induced gene 1 (INSIG1) and resulting in significantly increased umbilical cord blood lipid levels [35]. In addition to ART-associated DNA methylation and chromatin acetylation modifications, the role of noncoding RNAs in regulating gene expression has become a concern in IVF-ET offspring [36]. It is known that multiple microRNAs (miRNAs) are involved in the regulation of hypertension, coronary heart disease, and glycolipid metabolic disorders [37, 38]. However, few studies have focused on whether ART

affects the expression of miRNAs. Therefore, a miRNA array was performed in this present study to identify associations between miRNA expression and the use of IVF-ET, and also to explore possible miRNA mediated mechanisms influencing the increased incidence of cardiovascular disease in ART offspring.

Variations in the health status diseases, fertility, genetic background, and living environment of human patients make it difficult to clarify a causal link between the use of ART and the development of cardiovascular aberrations in offspring. In addition, it is currently almost impossible to assess the long-term health of a population conceived through ART, since most IVF-conceived offspring are less than 30 years old. Therefore, a mouse model was used in this study to evaluate the expression levels of RAS components in the myocardial tissue of IVF and naturally conceived mice, from 3 weeks to 1.5 years. In addition, corresponding DNA methylation levels in the RAS, and miRNA expression profiles were evaluated, with the view to investigate the presence of underlying epigenetic regulatory mechanisms that might influence cardiovascular dysfunction in ART-conceived offspring.

MATERIALS AND METHODS

Experimental animals

All treatment protocols were approved by the Animal Care Ethics Committee of Zhejiang University (No. ZJU2009101007Y). The methods used in these investigations were performed in accordance with the approved guidelines. C57BL/6J female mice (6-8 weeks old) and male mice (10-12 weeks old) were used in this study. All animals were provided with nesting materials and housed with a constant 12-hour light/12-hour dark cycle at $25^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ with 50%-60%

humidity. Mice were fed standard chow and water.

Experimental design

All female mice were superovulated and randomly divided into the IVF group and the *in vivo* naturally mated group.

Superovulation

C57BL/6J female mice were injected intraperitoneally with 7.5 IU (0.15 ml) of pregnant mare serum gonadotropin (PMSG) (GEN's, Hangzhou, China). After 48 hours, the female mice were injected with 7.5 IU (0.15 ml) of human chorionic gonadotropin (hCG) (GEN's).

In vitro fertilization

Human tubal fluid (HTF) (Irvine Scientific, CA, USA) medium containing 10% synthetic serum substitute (SSS; Irvin Scientific) was equilibrated at 37°C with 5% CO₂ and 95% humidity overnight before IVF. In the IVF group, sperms were collected from the cauda epididymis of C57BL/6J male mice in warmed modified HTF (mHTF) (containing 10% SSS) (Irvine Scientific) and then transferred to freshly warmed HTF for capacitation. Capacitation of the spermatozoa was performed in a 37°C environment with 5% CO₂ and 95% humidity for 1-2 hours. C57BL/6J female mice were euthanized by cervical dislocation at 15 hours after hCG injection. The oviducts were separated and placed in warmed mHTF, and the ampulla of the fallopian tube was punctured. Cumulus-oocyte complexes were washed twice in mHTF and then transferred to HTF droplets. The capacitated sperm suspension was gently added to the HTF medium with metaphase II oocytes in a final sperm concentration of $1-2 \times 10^6$ /ml. Sperm and oocytes were co-cultured for 6 hours in the insemination medium. Then, the presumptive

embryos were gently washed twice for further culture in fresh HTF. The two-cell-stage embryos of the IVF group were obtained 24 hours later.

In the *in vivo* group, C57BL/6J female mice were caged with male mice at a ratio of 1:1 after hCG injection. The next day, female mice with a vaginal plug were separated from the males. After 44 hours of hCG injection, the female mice were euthanized by cervical dislocation and two-cell embryos were obtained from the oviducts. The operating conditions were the same as those of the IVF group.

Embryo transfer

Female mice at least 8 weeks of age from the Institute of Cancer Research (ICR) were used as pseudopregnant recipients. Vaginal plugs were detected the morning after mating with vasectomized males from the Institute of Cancer Research and the animals were considered to be at day 0.5 of pseudopregnancy. Twelve morphologically normal two-cell-stage embryos in each group were transferred to the oviducts of pseudopregnant recipients.

Postnatal growth

The birth outcomes of mice were described previously [34]. Fifty-one mice were born in each of the IVF and *in vivo* groups. No significant differences were observed in pregnancy rate, birth rate, mortality, or sex ratio. The wet weights of mice were recorded weekly from birth to 10 weeks of age, and biweekly from 11 weeks to 1.5 years of age. The animals were euthanized, and the myocardial tissue was dissected. A part of the tissue was snap frozen in liquid nitrogen and stored at 80°C for RNA, DNA, and protein detection. The remaining tissue was fixed overnight with 4% paraformaldehyde in phosphate-buffered saline (PBS) for immunohistochemistry.

Cell culture

The H9c2 (2-1) cell line was purchased from American Type Culture Collection (ATCC, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Sciences, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, NY, USA) and 1% penicillin/streptomycin solution (Gibco) in 100-cm² flasks at 37°C in a humidified atmosphere of 5% CO₂.

MicroRNA transfection

The H9c2 cells in the exponential phase of growth (2×10^5 /well) were cultured overnight in six-well plates to overexpress *miR-297*. The cells were transfected with a *miR-297* mimic (100nM) or negative control RNA (100nM) using the riboFECT CP reagent and buffer (RiboBio) according to the manufacturer's protocol. First, 10 µl of miRNAs was diluted with 120 µl of riboFECT CP buffer for 10 min at room temperature. This diluent was mixed with 12 µl of riboFECT CP reagent and incubated for 10 min at room temperature. Finally, the total mixture was added to each well of the plate together with 1.8 ml of DMEM and incubated at 37°C. RNA and protein was extracted from the H9c2 cells after 24 hours and 48 hours, respectively.

RNA extraction

Total RNA was extracted from the myocardial tissue of mice and H9c2 cells after transfection according to the manufacturer's protocol using the RNAiso Plus kit (TaKaRa, Tokyo, Japan).

Reverse transcription

For mRNAs (*Ren1*, *Ace*, *Ace2*, *Agt*, *Agtr1a*, *Agtr1b*, *Agtr2a*, *Colla1*, *Colla2*, *Col3*, *Ctgf*), 0.5- μ g RNA samples were reverse-transcribed with PrimeScript RT Reagent Kit (TaKaRa). For miRNAs (*miR-100*, *miR-297*, *miR-758*), reverse transcription was performed with Mir-X microRNA FirstStrand Synthesis Kit (TaKaRa). U6 snRNA controls served as a reference gene for analyzing microRNA expression levels.

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (PCR) analysis was performed using SYBR Premix Ex Taq (TaKaRa) for mRNAs and SYBR Premix Ex Taq II for miRNAs in an ABI 7900 thermocycler (Applied Biosystems, CA, USA). *Gapdh* served as a reference gene for analyzing gene expression levels. The real-time PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method. The primers were synthesized by Sangon (Shanghai, China). The primer sequences for real-time PCR are shown in Supplementary Tables 1 and 2.

Western blotting

Myocardial tissues and H9c2 cells were homogenized in radioimmunoprecipitation assay lysis buffer (CW BIO, Beijing, China), incubated on ice for 20 min, and centrifuged at 14,000g for 10 min at 4°C. The supernatant was transferred to a tube, and concentrations were detected

using the Bradford assay (Bio-Rad Laboratories, CA, USA). Then, 50 µg of protein was transferred to each lane on a 10% SDS-polyacrylamide gel. These samples were transferred to polyvinylidene fluoride membranes (Millipore, MA, USA) and incubated with the following primary antibodies (Supplementary Table 3): anti-renin antibody (sc-137252; Santa Cruz Biotechnology, California, USA); anti-angiotensin converting enzyme 1 antibody (ab11734; Abcam, Cambridge, UK); anti-angiotensin II type 1 receptor antibody (ab124505; Abcam, Cambridge, UK); anti-angiotensin II type 2 receptor antibody (ab92445; Abcam); anti-collagen 3 antibody (ab7778; Abcam); anti-CTGF antibody (ab6992; Abcam); and anti-glyceraldehyde 3 phosphate dehydrogenase (GAPDH) antibody (ab181602; Abcam) overnight at 4°C. They were incubated with fluorescent secondary antibodies (680 and 790 nm; Jackson, PA, USA) for 2 hours during RT. The protein bands were visualized using the Odyssey Imager (Li-Cor Biosciences, NE, USA). The GAPDH protein levels were used as the control protein.

DNA methylation analysis

Targeted bisulfite sequencing

The process of targeted bisulfite sequencing was performed to analyze DNA methylation, as described in a previous study [39]. The genomic DNA of myocardial tissue was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Genome bisulfite modification was performed using the EpiTect Bisulfite Kit (Qiagen) according to the operation manual, and its products were used for subsequent PCR amplification. Targeted sequences of primers are shown in Supplementary Table 4. Furthermore, a 1-µl bisulfite-converted DNA template was performed using the PCR program. The PCR conditions were as follows: 3 min at 94°C, 5 cycles

of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C (decrease of 0.5°C in each cycle); then, 30 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C; and finally 72°C for 5 min. The PCR products were performed with end-repair, A-tailing, adaptor ligation, and clean-up according to the recommendations of KAPA Biosystems (MA, USA). A PCR program using Kapa Hifi DNA Polymerase (KAPA Biosystems) was performed as follows: 3 min at 94°C, 9 cycles of 30 s at 94°C, 30 s at 62°C, and 30 s at 72°C. The sequencing of samples was performed on the MiSeq System (Illumina, CA, USA) using 100-bp paired-end reads.

The methylation levels of each CpG site were calculated using the Bismark's methylation extractor tool [40] and shown using R scripts. Average methylation levels of the target regions and other information were generated with the local Perl, Python, and Shell scripts to compile counts of methylated and unmethylated reads at each CpG site.

MicroRNA microarray and bioinformatic analysis

MicroRNA profiling was performed by RiboBio. The quality and quantity of RNA were assessed using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). $A_{260}/A_{280} \geq 1.5$ and $A_{260}/A_{230} \geq 1$ were acceptable RNA purity levels, whereas an acceptable RNA integrity number (RIN) value was ≥ 5 using the Agilent 2200 RNA assay (Agilent Technologies, Santa Clara, CA, USA). gDNA contamination was detected using gel electrophoresis; 1 μ g of total RNA was labeled with cy3 using the (Universal Linkage System) ULS method and hybridization was performed using RiboArray miDETECT mouse array (RiboBio). After the data were acquired, microRNA targets were predicted using the following databases: TargetScan (<http://www.targetscan.org>), microRNA.org (<http://www.microrna.org>),

miRDB (<http://www.mirdb.org/>), and CLIP (<http://lulab.life.tsinghua.edu.cn/clipdb/>).

Luciferase assay

The AGTR1 3'-UTR or AGTR1 3'-UTR mutant fragment was amplified by PCR and inserted into the pmiR-RB-Report vector (Guangzhou Ruibo Biotech Company, China) to generate a luciferase reporter vector. Co-transfection of 293T cells with the recombinant vectors alongside miR-297a-3p mimic or negative control (NC) was performed with lipofectamine 2000 transfection reagent (Invitrogen, USA), according to the manufacturer's instructions. The relative luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega, USA). The reporter vector used was hRluc, with hluc as the internal reference corrected fluorescence. All transfection assays were performed in three independent biological replicates.

Cell proliferation assay

H9c2 cells were transfected with miRNA mimics in six-well plates. Forty-eight hours after cell transfection, 5-ethynyl-20-deoxyuridine (EdU) (10 μ M) was added to the cultures and cells were grown for an additional 2 hours.

For imaging analysis of cell proliferation, the cells were fixed with 4% formaldehyde in PBS for 30 minutes. After labelling, H9c2 cells were assayed using the Cell-Light EdU Apollo 488 *in vitro* imaging kit (RiboBio) according to the manufacturer's protocol. The proliferation rate was calculated after normalizing the number of EdU-positive cells to that of DAPI-stained cells in five randomly selected high power fields. Images were acquired under an FV1200 laser confocal microscopy (Olympus, PA, USA).

For flow cytometry analysis of cell proliferation, EdU (10 μ M) (Cell Light EdU Apollo 488

in vitro flow cytometry kit; RiboBio) was added and the cells were cultured for an additional 2 hours according to the manufacturer's protocol. H9c2 cells were resuspended in PBS and centrifuged at 15,000 rpm for 5 minutes. H9c2 cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. H9c2 cells were resuspended in PBS cells. Then, they were immunocytochemically stained with antibodies. The following tests were performed using ACEA NovoCyte™ flow cytometry (ACEA, San Diego, CA, USA).

Statistical analysis

The experimental data were compared using the Student's *t*-test (version 19.0; SPSS, NY, USA) to perform statistical analyses of the results of the two groups. The results were presented as mean \pm standard error of the mean. For all results, statistically significant differences were defined as $P < 0.05$ and $P < 0.01$.

RESULTS

IVF altered the expression of genes in the RAS components and those related to myocardial remodelling in myocardial tissue

The expression levels of genes in the RAS components and corresponding effect genes were detected using quantitative RT-PCR to investigate the effect of IVF on the expression of essential genes related to the health of the cardiovascular system (Fig. 1). The genes in the RAS components examined in this study included *Ren1*, *Ace*, *Ace2*, *Agt*, *Agtr1a*, *Agtr1b*, and *Agtr2*. The corresponding effect genes of the RAS included *Colla1*, *Colla2*, *Col3*, and *Ctgf*. At 3 weeks of age, the expression levels of *Ren1*, *Ace*, *Agtr1a*, *Col3*, and *Ctgf* were found to be

significantly increased in the IVF group compared with those in the *in vivo* group (1.14-fold, $P < 0.05$; 2.15-fold, $P < 0.05$; 2.01-fold, $P < 0.05$; 1.62-fold, $P < 0.05$; and 2.13-fold, $P < 0.05$, respectively) (Fig. 1A). No statistically significant differences were observed in the expression levels of *Ace2*, *Agt*, *Agtr1b*, *Agtr2*, *Colla1* and *Colla2* ($P > 0.05$) between the *in vivo* group and the IVF group (Fig. 1A). At 10 weeks of age, the expression levels of *Ace*, *Agt*, *Agtr1b*, *Agtr2*, *Colla1*, and *Col3* were also found to be significantly increased in the IVF group compared with those in the *in vivo* group (1.74-fold, $P < 0.05$; 2.04-fold, $P < 0.05$; 1.79-fold, $P < 0.05$; 2.08-fold, $P < 0.05$; and 1.62-fold, $P < 0.05$, respectively) (Fig. 1B). But no statistically significant differences were found in the expression levels of *Ren1*, *Ace2*, *Agtr1a*, *Colla2* and *Ctgf* ($P > 0.05$) between the *in vivo* group and the IVF group (Fig. 1B). When the mice reached the age of 1.5 years, the expression levels of *Ren1*, *Ace*, *Agtr1b*, *Col3*, and *Ctgf* were still increased in the IVF group compared with those in the *in vivo* group (2.37-fold, $P < 0.01$; 2.38-fold, $P < 0.05$; 1.68-fold, $P < 0.05$; 1.86-fold, $P < 0.05$; and 1.81-fold, $P < 0.05$, respectively) (Fig. 1C). However, no significant differences were found in the expression levels of *Ace2*, *Agt*, *Agtr1a*, *Agtr2*, *Colla1* and *Colla2* ($P > 0.05$) between the *in vivo* group and the IVF group (Fig. 1C).

The protein expression levels of genes in the RAS components and corresponding effect genes were further assessed using quantitative western blotting (Fig. 2). At 3 weeks of age, the expression levels of REN1, ACE, AGTR1 and CTGF were significantly increased in the IVF group compared with the *in vivo* group (1.11-fold, $P < 0.05$; 1.26-fold, $P < 0.05$; 1.79-fold, $P < 0.05$; 1.85-fold, $P < 0.05$, respectively) (Fig. 2A). No significant differences were found in

expression levels of AGTR2 and COL3 ($P > 0.05$) (Fig. 2A). At 10 weeks of age, the expression levels of ACE were significantly increased in the IVF group compared with the *in vivo* group (1.14-fold, $P < 0.05$) (Fig. 2B). The protein expression levels of AGTR1, AGTR2, and CTGF were not statistically different between the two groups at 10 weeks of age ($P > 0.05$) (Fig. 2B). In old age, the protein expression of ACE and AGTR1 was upregulated in the IVF group (1.13-fold, $P < 0.05$; 1.40-fold, $P < 0.05$, respectively) (Fig. 2C), but the protein expression levels of REN1, AGTR2, CTGF and COL3 were not significantly different ($P > 0.05$) (Fig. 2C).

The altered methylation levels of CpG island regions of Colla1 in IVF-conceived mice

Among the differentially expressed genes, there are CpG islands near the promoters regions of and *Colla1*. Thus, *Colla1* were selected to explore whether the methylation levels of CpG island regions of *Colla1* would change in IVF-conceived mice (Fig. 3). Targeted bisulfite sequencing analysis was used to detect the methylation level of each CpG site in the targeted gene. Using this method, we measured methylation levels of 14 CpG sites in *Colla1* target regions. The schematic overview of the CpG island regions of the *Colla1* is shown in Figure 3A. No statistically significant differences were observed in the mean DNA methylation level of *Colla1* between the IVF and *in vivo* groups (Fig. 3B). At 3 weeks of age, the methylation level of the CpG site 4 of *Colla1* was significantly reduced in the IVF group ($P < 0.05$). The methylation levels of CpG sites 6 and 8 of *Colla1* at 10 weeks and of CpG sites 1, 2, and 3 of *Colla1* at 1.5 years of age were significantly reduced in the IVF group ($P < 0.05$). Only the

methylation level of CpG site 7 at 3 weeks of age in the IVF group was increased ($P < 0.05$) (Fig. 3B).

MiRNAs were involved in the regulation of IVF-induced abnormal cardiac gene expression

A microarray analysis was first used to detect the expression profile of miRNA in myocardial tissue in the IVF-conceived and *in vivo* groups at 1.5 years (Fig. 4A). While some upregulated miRNAs were observed, the present study focused on significantly upregulated *miR-100*, *miR-297*, and *miR-758*. The *miR-100*, *miR-297* and *miR-758* were predicted to interact with *Col3a1*, *Agtr1a*, and *Col1a2*, respectively. Next, quantitative RT-PCR was used to detect the expression of *miR-100*, *miR-297* and *miR-758* in myocardium from the IVF-conceived and *in vivo* groups at 3 weeks, 10 weeks and 1.5 years of age. At 3 weeks, the expression levels of *miR-100*, *miR-297* and *miR-758* were not found to be significantly altered between the two groups ($P > 0.05$) (Fig. 4B), whereas at 10 weeks, a significant increase in *miR-100* and *miR-758* was observed in the IVF group compared with the *in vivo* group (4.24-fold, $P < 0.05$ and 4.03-fold, $P < 0.01$, respectively) (Fig. 4C). A similar trend was found during old age; the expression levels of *miR-100*, *miR-297* and *miR-758* were found to be significantly increased in the IVF group (1.76-fold, $P < 0.05$; 2.04-fold, $P < 0.05$; and 2.52-fold, $P < 0.05$) (Fig. 4D). To further verify the interaction between *Agtr1* and *miR-297*, this study performed luciferase activity assay to confirm the prediction (Fig. 4E and 4F). The results of the luciferase activity assay showed that *miR-297a-3p* mimic significantly modulated the luciferase activity of the pmiR-AGTR1-WT vector. However, the luciferase activity of the pmiR-AGTR1-Mut vector was

not affected by the miR-297a-3p mimics. This evidence indicated that miR-297a-3p directly targeted AGTR1 and regulated its expression (Fig. 4E and 4F).

Proliferation of H9c2 was promoted by miR-297 probably through regulating the expression of AGTR1 and CTGF

Because *miR-297* was found to have an upregulation tendency in the myocardial tissue of IVF-conceived mice compared with the *in vivo* group, H9c2 cardiomyocytes were transfected with 100nM *miR-297* mimic to increase their expression level of *miR-297* (Fig. 5A). The expression of AGTR1 and CTGF did not change significantly after 24 hours of overexpression of *miR-297*. However, the protein expression of AGTR1 and CTGF in the *miR-297* overexpression group significantly increased after 48 hours, indicating that *miR-297* could increase the expression of AGTR1 and further promote the expression of CTGF protein.

To examine whether *miR-297* affects cell proliferation in H9c2 cells, the H9c2 cells were treated with 100nM *miR-297* mimic for 48 hours. Cell proliferation was examined to detect EdU-positive cells using flow cytometry and fluorescence microscopy. The flow cytometry results showed that the overexpression of *miR-297* was able to promote cell proliferation ($23.57\% \pm 0.30\%$) compared with the control group ($13.23\% \pm 1.95\%$; Fig. 5B). Significantly more EdU-positive cells were observed in the *miR-297* mimic group ($25.47\% \pm 2.74\%$) compared with the control group ($15.79\% \pm 1.43\%$; Fig. 5C) using fluorescence microscope imaging.

DISCUSSION

Since it was initially proposed that conception via ART might be linked to an increased risk of developing long-term cardiovascular health issues, several studies have confirmed an altered cardiovascular phenotype and elevated metabolic risk in children born following IVF [13, 14]. For example, investigations have shown that IVF-conceived children have significantly higher blood pressure compared with spontaneously conceived children [41-44], something that has recently been supported by a meta-analysis of 2112 children conceived by ART, where data showed that there was a slight, yet statistically significant, increase in blood pressure compared with naturally conceived offspring [45]. Furthermore, Brenda *et al.* reported that children conceived through ART showed cardiac and vascular remodelling during the fetal period that persisted in postnatal life [13]. Another meta-analysis of 25,856 children conceived using IVF/ICSI techniques showed an increased risk of developing congenital heart defects compared with those conceived spontaneously [46]. Using an IVF mouse model, this study goes some significant way to addressing the urgent need for us to better understand the underlying mechanisms influencing ART-induced cardiovascular dysfunction (Figure 6). Furthermore, we have clarified the long-term health effects of an ART-induced compromised cardiovascular system in mice from aged 3 weeks to 1.5 years.

Several studies have shown that structural and functional alterations of the heart are mediated through stimulation of a local cardiac RAS system in the cardiac tissue and large arteries. Data suggests that the cardiac RAS may act in an autocrine or paracrine manner by binding to AGTR1 [47, 48], an idea supported by evidence which suggests that the cardiac RAS is activated at

advanced stages of heart failure in humans [47, 48]. Besides that, AGTR1 activation has an important role in the development of load-induced cardiac hypertrophy, even without Ang II involvement [49, 50]. In this present study, the mRNA expression level of *Ace*, *Agtr1* and *Col3* was significantly increased in the myocardial tissue of IVF-conceived mice at 3 weeks and 1.5 years of age when compared to their naturally mated counterparts. These findings were reflected at the protein level, where AGTR1 was elevated in the myocardium of IVF-conceived mice at both 3 weeks and 1.5 years of age. These results might be applied to human physiology, providing a potential explanation for the cardiovascular alterations observed in ART-conceived children.

Based on the hypothesis of ‘developmental origins of health and disease’ put forward by Barker, adverse events during the fetal development period can increase the risks of some adult diseases, including cardiovascular disorders [4]. Thus it is not unreasonable to conclude that ART intervention during the periconception period might induce a health risk in the resulting offspring. During ART, specifically in vitro gamete manipulation and embryo system, spermatozoa, oocytes and embryos are exposed to various nonphysiological factors, such as culture media, altered pH [51], light [52, 53], higher oxygen pressure [54], increased concentration of reproductive hormones [55] and mechanical injection or biopsy, all of which contribute to an altered periconception environment [56]. In addition to *in vitro* ART, a sub-optimal uterine environment following embryo transfer, is thought to further influence the long-term viability of offspring. Interestingly, factors that create an adverse interuterine environment, including maternal high-salt diet, inflammation and dietary restriction have also been shown to alter the fetal and

offspring RAS [57-60].

CTGF, an immediate response gene of the CCN (*Cyr61*, *Ctgf*, *Nov*) family, is a critical cytokine in the pathophysiology of fibrosis that upregulates COL1 and COL3 in cardiomyocytes [61]. CTGF is essential in AGTR1-mediated intracellular signalling and can activate cytokines, such as transforming growth factor- β , resulting in myocardial remodelling, vascular fibrosis, endothelial dysfunction, and atherosclerosis progress [62]. Increased plasma levels of CTGF may be a marker of pulmonary arterial hypertension associated with congenital heart disease [63]. An abnormal expression of COL1 and COL3 in the embryonic period may affect the heart's structural development [25]. Sustained changes in the expression of COL1 and COL3 in adulthood would further increase the risk of cardiovascular diseases, such as hypertension, arrhythmia, and cardiac hypertrophy, and enhance the process of cardiovascular fibrosis and remodelling [26]. The effects of ART on genome-wide changes in DNA methylation levels have been confirmed by several studies [64-66]. For instance, ART-conceived mice showed arterial tissue endothelial dysfunction and increased DNA methylation of the promoter of the *eNOS* gene [15]. CpG islands are located in or near the promoter region of genes and hence, prevent transcription factors from binding, thus preventing transcription [67]. At 3 weeks and 1.5 years of age, the methylation levels of some CpG sites in *Colla1* were significantly decreased in the IVF-conceived group, which might contribute to the changes in genes expression of *Colla1*.

In addition to DNA methylation and chromatin histone acetylation modification, accumulating studies have also shown noncoding RNA to be involved in the epigenetic modification of growth and development [68]. MiRNAs are small noncoding RNA molecules of

approximately 22-24 nucleotides that participate in the transcriptional and posttranscriptional regulation of gene expression [69]. A variety of miRNAs have been shown to regulate hypertension, coronary heart disease, and glucose and lipid metabolism disorders [70, 71]. In this study, *miR-100*, *miR-297* and *miR-758* were chosen as candidate genes after miRNA database prediction indicated that they interact with *Col3a1*, *Agtr1a*, and *Colla2*, respectively. Previous studies have shown that plasma *miR-100* is positively associated with coronary plaque vulnerability [72], can inhibit angiogenesis [73] and is involved in the development and growth of pancreatic cancer [74], while *miR-297* is involved in septic shock [75]. In this study, the luciferase assay was used to demonstrate that *miR-297* targets AGTR1 and promotes the proliferation of cardiomyocytes by regulating the expression of AGTR1 and CTGF. Moreover, the expression of *miR-297* was shown to promote the proliferation of H9c2 cells in culture, which might also indicate a role for *miR-297* in cardiac structural and functional abnormalities. In accordance with the present results, previous studies have also demonstrated that up-regulation of *miR-297* promotes cardiomyocyte hypertrophy induced by Ang II [76]. The functions of the miR might be extensive due to its ability to bind multiple molecules [77]. The *miR-297* could directly inhibit gene expression by targeting on the 3'UTR region of mRNA [76]. In addition, miRs also indirectly affect the gene expression by regulating transcription factors, nuclear factor and binding proteins which are closely related to gene expression [77]. The miRs might also target important molecules acting on cellular signalling, indirectly affecting gene expression [78]. Therefore, we speculated that *miR-297* may also interact with other regulatory factors in the signalling pathway regulated by AGTR1, and other mechanisms need to be

explored in future studies.

Evidence presented in this manuscript indicates that the expressions of RAS associated gene and protein expression in myocardial tissue have been changed in the myocardium of IVF-conceived mice. Furthermore, modifications to DNA methylation and abnormal patterns of expression of miRNAs may be involved in the regulations, and thus the cardiovascular health of IVF-conceived fetuses and offspring. The application of an IVF mouse model has enabled us to investigate the long-term health of ART offspring. Given that ART is now widely utilized, it is important to further explore the exact signaling pathways and genes involved in embryonic defects, associated with environmental factors, which manifest as clinical disorders in the adult. These findings provide new and important insights into the mechanisms which control the development of cardiovascular disease and will allow us to develop tools to minimize ART-induced cardiovascular dysfunction at the point of fetal origin and also as the offspring progress into adulthood [79].

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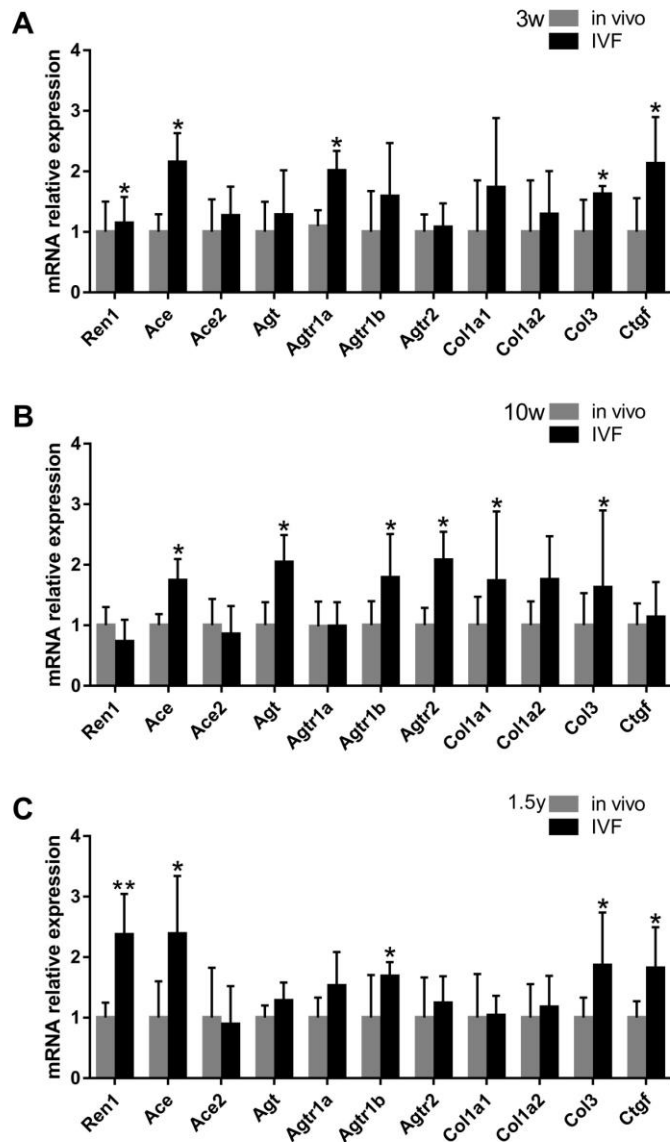


FIG. 1. The mRNA relative expression of genes in the RAS components and those related to myocardial remodelling in myocardial tissue in the *in vivo* and IVF groups at (A) 3 weeks, (B) 10 weeks, and (C) 1.5 years. *Ren1*, *Ace*, *Ace2*, *Agt*, *Agtr1a*, *Agtr1b*, and *Agtr2* were critical elements in the RAS. *Col1a1*, *Col1a2*, and *Col3* were the major components of the extracellular matrix of the heart and also crucial in the development of heart and myocardial remodelling. *Ctgf*

promotes the pathological process of fibrosis. The data are presented as mean \pm standard error of the mean. The relative levels were calculated using the $2^{-\Delta\Delta CT}$ method. * $P < 0.05$ and ** $P < 0.01$, compared with the *in vivo* group; Student's *t*-test (*N*, *in vivo* = 6, IVF = 6 in 3-week-old mice; *N*, *in vivo* = 6, IVF = 6 in 10-week-old mice; and *N*, *in vivo* = 6, IVF = 6 in 1.5-year-old mice).

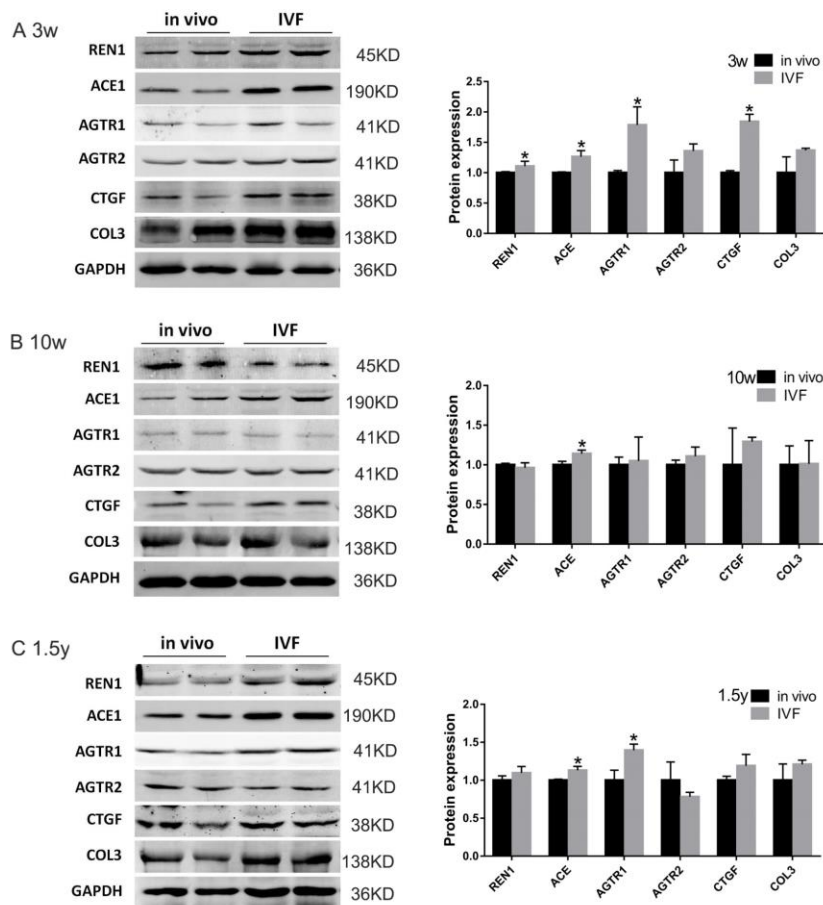


FIG. 2. Western blot analysis of REN1, ACE1, AGTR1, AGTR2, CTGF, and COL3 in myocardial tissue in the *in vivo* and IVF groups at (A) 3 weeks, (B) 10 weeks, and (C) 1.5 years of age. GAPDH was used as the internal control. Data are presented as mean \pm standard error of the mean. * $P < 0.05$ and ** $P < 0.01$ compared with the *in vivo* group; Student's *t*-test (*N*, *in vivo* =

4; IVF = 4 in 3-week-old mice; *N*, *in vivo* = 4; IVF = 4 in 10-week-old mice; and *N*, *in vivo* = 4; IVF = 4 in 1.5-year-old mice).

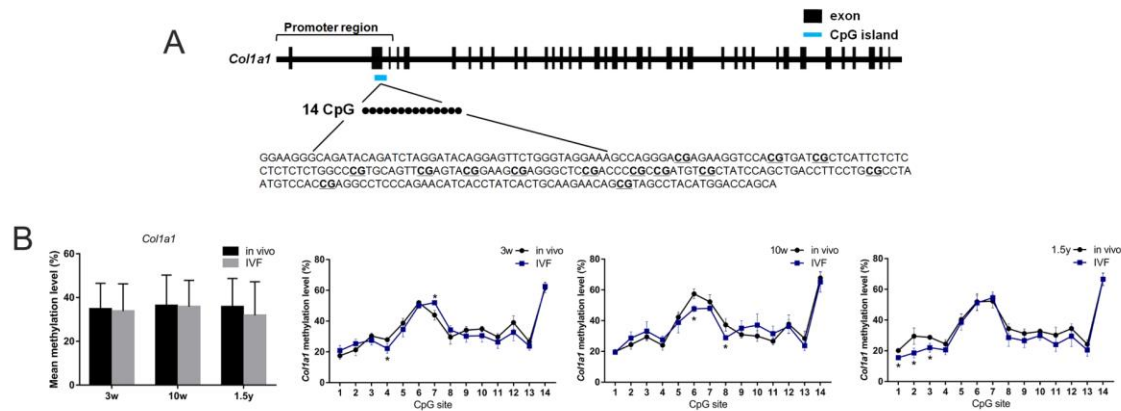


FIG. 3.

Schematic overviews of the CpG island regions of *Colla1* (A). The target sequences examined by targeted bisulfite sequencing were shown, and CpG sites were underlined. Average DNA methylation levels for the whole regions of CpG islands and the mean methylation levels for the individual CpG site of *Colla1* (B) in the *in vivo* and IVF groups at 3 weeks, 10 weeks, and 1.5 years. Data are presented as mean \pm standard error of the mean. * $P < 0.05$ and ** $P < 0.01$ compared with the *in vivo* group; Student's *t*-test (*N*, *in vivo* = 6; IVF = 6 in 3-week-old mice; *N*, *in vivo* = 6; IVF = 6 in 10-week-old mice; and *N*, *in vivo* = 6; IVF = 6 in 1.5-year-old mice).

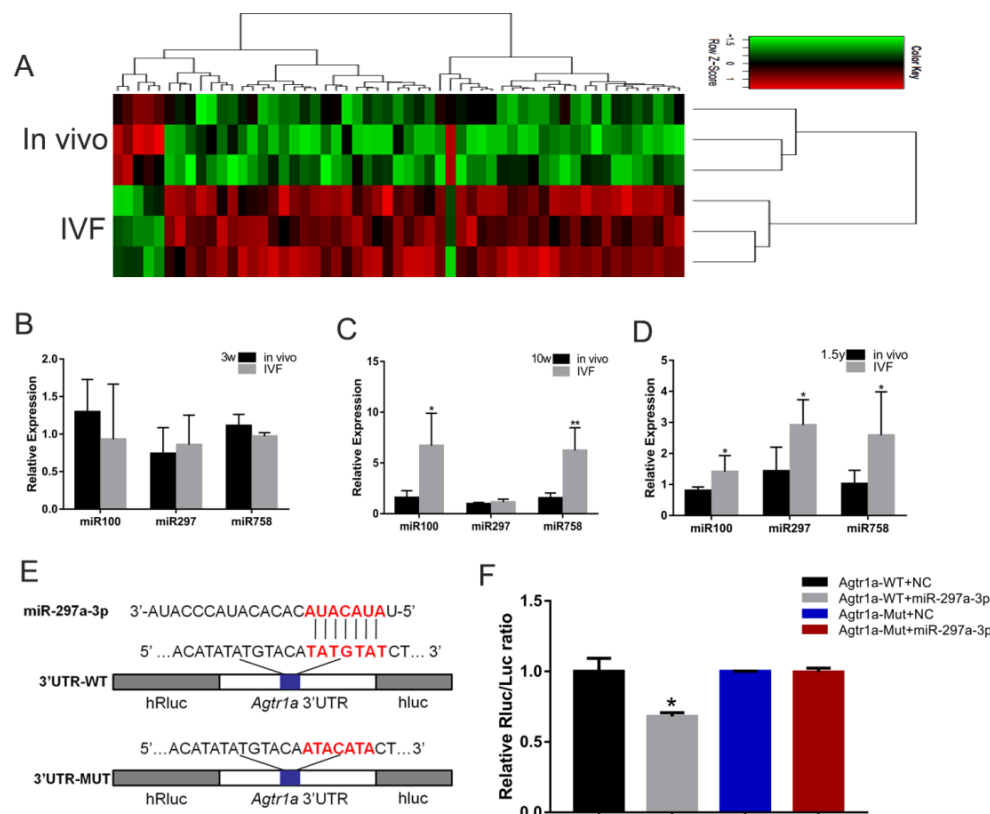


FIG. 4.

(A) Heat map showing the hierarchical clustering of miRNAs in the *in vivo* and IVF groups at 1.5 years. (B-D) Expression of *miR-100*, *miR-297* and *miR-758* was altered in the myocardial tissue of the IVF-conceived mice. The gene expression of *miR-100*, *miR-297* and *miR-758* at (B) 3 weeks, (C) 10 weeks, and (D) 1.5 years. (E and F) Correlation between AGTR1 and miR-297a-3p detected using the luciferase reporter gene assay. (E) The pmiR-AGTR1-WT plasmid was mutated in the miRNA-predicted target site and designated as pmiR-AGTR1-Mut. (F) Luciferase activity of AGTR1 3'UTR luciferase reporters was detected after transfecting the recombinant vectors along with miR-297a-3p mimics or NC. Data concerning the relative

concentrations of *miR-100*, *miR-297* and *miR-758* were normalized to U6 snRNA; the relative levels were calculated using the $2^{-\Delta\Delta CT}$ method. The data are presented as mean \pm standard error of the mean. * $P < 0.05$ and ** $P < 0.01$, compared with the *in vivo* group; Student's *t*-test (*N*, *in vivo* = 6, IVF = 6 in 3-week-old mice; *N*, *in vivo* = 6, IVF = 6 in 10-week-old mice; and *N*, *in vivo* = 6, IVF = 6 in 1.5-year-old mice). The data of luciferase activity are presented as mean \pm standard error of triplicate cell cultures. * $P < 0.05$ and ** $P < 0.01$.

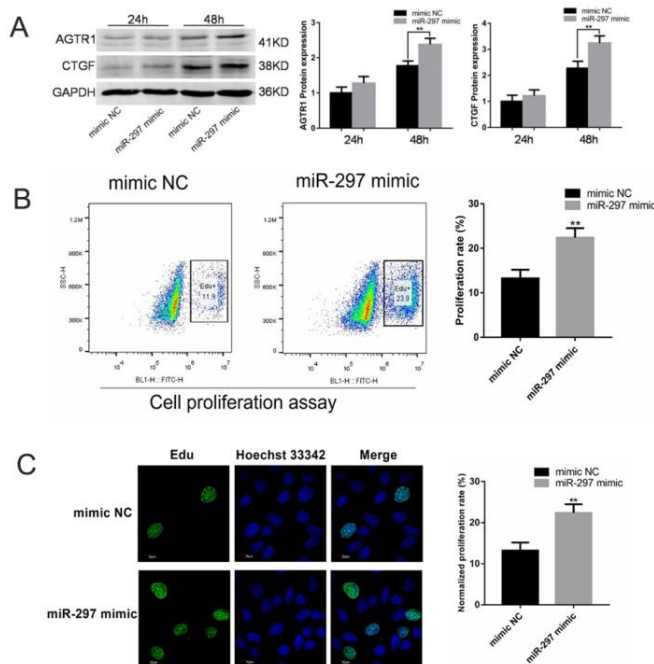


FIG. 5.

(A) The *miR-297* promoted the expression of AGTR1 and CTGF in the proliferation of H9c2 cardiomyocytes. The H9c2 cells were transfected with negative control (NC) or *miR-297* mimic for 24 or 48 h. The Western blot analysis was used to analyze the expression of AGTR1 and CTGF proteins. GAPDH was the internal control. (B and C) *miR-297* was involved in the

regulation of the proliferation of cardiac H9c2 cells. The H9c2 cell proliferation assay using flow cytometry (B) and laser confocal microscopy (C). The cells were transfected with *miR-297* and negative control (NC) after 48 h. The data are presented as mean \pm standard error of the mean. * $P < 0.05$ and ** $P < 0.01$, compared with the *in vivo* group; Student's *t*-test.

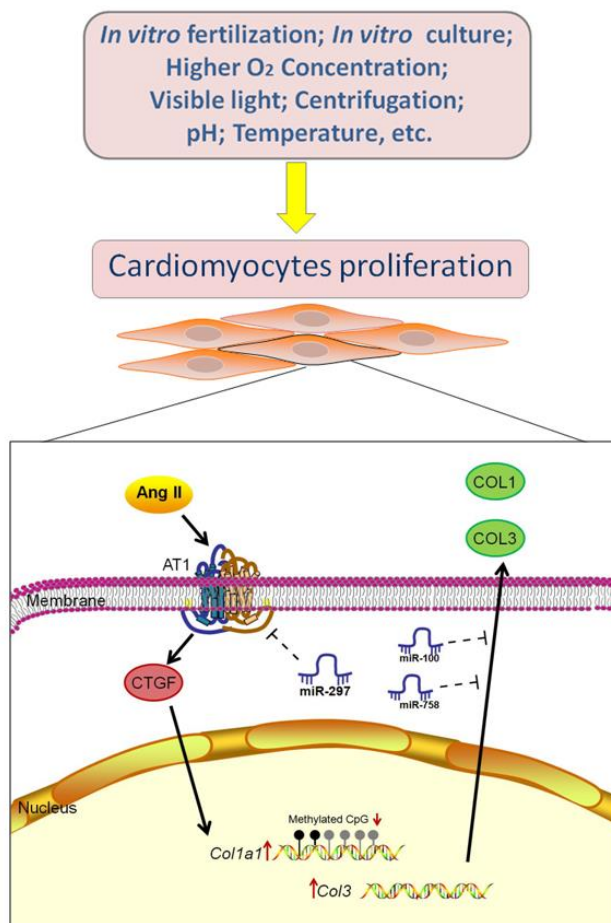


FIG. 6.

ART procedures affected the expression of RAS and epigenetic modifications in the myocardium. Oocytes and embryos were exposed to various non-physiological factors during IVF, such as culture media, altered pH, visible light, higher oxygen pressure, and reproductive hormones.

These factors might result in the increased expression of *Agt*, *Agtr1*, and other genes in the RAS. CTGF is crucial in AT1-mediated intracellular signalling and can promote the expression of COL1 and COL3. Moreover, it was suggested that the changes in the expression of *Colla1* genes in IVF-conceived mice might have been caused by the decreased methylation of CpG sites in these genes. *MiR-100*, *miR-297* and *miR-758* might be involved in the regulation of the expression of *Col3a1*, *Agtr1a*, and *Colla2*. In particular, *miR-297* could promote the proliferation of cardiomyocytes by interacting with AGTR1.