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Inhibitory effect of progesterone during early embryonic development: Suppression of myocardial differentiation and calcium-related transcriptome by progesterone in mESCs Progesterone disturb cardiac differentiation of mESCs through lower cytosolic Ca^{2+}

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ABSTRACT

Progesterone (PG) and its derivates are used in prevention of spontaneous miscarriage. However, some studies have reported that exposure to PG and its derivates during pregnancy can cause malformations and affect both blood pressure and the cardiovascular system. The effect of PG on cardiomyogenesis of mouse embryonic stem cells (mESCs) is not well known. Expression of *Pgr* mRNA showed an opposite pattern of beating-ratio during differentiation. PG treatment resulted in reduction of the beating ratio to $60.45 \pm 1.54\%$ from $92.17 \pm 2.98\%$ in normal differentiation, reduced transcripts of heart morphogenesis and Ca^{2+} binding-related genes in the next generation sequencing data and significantly decreased expression levels of Ca^{2+}/contraction-related genes including *Ryr2, Calm2, Trpv2,* and *Mylk3*, the intracellular Ca^{2+} level, and the beating frequency. These results suggest that PG exerts inhibitory effects on differentiation of mESCs into functional cardiomyocytes.

1. Introduction

Progesterone (PG), a steroid hormone consisting of 21 carbons, regulates the female menstrual cycle and supports gestation and embryogenesis by inducing secretory changes essential for successful implantation of a fertilized egg [1]. PG prepares the uterus to receive and support the fertilized egg [2]. Derivates of PG, including dydrogesteronein and medroxyprogesterone acetate (MPA), are administered for hormone therapy for pregnant women undergoing IVF and embryo transfer treatment with PG [2]. PG and its derivates reduce the risk of spontaneous pregnancy loss in women with habitual abortion or recurrent miscarriage [3]. Treatment is usually administered in the first trimester of pregnancy in an effort to prevent spontaneous miscarriage. However, some studies have reported that PG-derivates such as MPA can cause fetal deformities and are embryotoxic [3,4]. Thus, study on the safety of PG treatment during embryonic development is required.

Mouse embryonic stem cells (mESCs) are an attractive *in vitro* model system for study of gene regulation and estimation of impaired differentiation and function by various agents during early embryonic development [5–7]. ESCs have two distinct properties from other cells, self-renewal and pluripotency. Pluripotent ESCs can differentiate into any of the three germ layers through the formation of three-dimensional multicellular aggregates known as embryoid bodies (EBs) [8]. EBs with embryo-like structures act as the onset point of *in vitro* differentiation, and are therefore useful for *in vitro* model systems for study of embryogenesis [5].

mESCs can differentiate into cardiomyocytes. Differentiated mESCs contract like cardiomyocytes and express a number of cardiac-specific markers, including T-box 20 (*Tbx20*) and troponin I, cardiac 3 (*Tnni3* or *Ctn1*), suggesting that this system can serve as a model for study of the early aspects of cardiogenesis [9]. In addition, they express not only cardiac-specific marker proteins, but also Ca^{2+}-handling proteins including ryanodine receptor (RyR), Na^{+}/Ca^{2+} exchanger (NCX), and sarcoplasmic reticulum

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Ca^{2+}-ATPase (SERCA) [10]. At the early stage, contractions of cardiomyocytes are accompanied by Ca^{2+}-dependent pacemaker action potentials [11]. Intracellular calcium is a key regulator of cardiac function. Calcium (Ca^{2+}) acts as a secondary messenger, playing an important role in regulation of cardiac physiology and pathophysiology [12]. Maintenance of intracellular Ca^{2+} homeostasis in cardiomyocytes is important for many aspects of cardiac function, including cardiac excitation-contraction coupling and relaxation.

Cardiac excitation-contraction coupling (ECC) is closely associated with Ca^{2+}-induced release of Ca^{2+} [13]. Movement of Ca^{2+} occurs through transmembrane and intracellular calcium channels and transporters. Cardiac contraction is initiated by activation of voltage-gated L-type Ca^{2+} channels and a subsequent increase in intracellular Ca^{2+} concentration because of the influx of extracellular Ca^{2+} through the transient receptor potential (TRP) channel, including transient receptor potential cation channel subfamily V member 1 (TRPV1) and transient receptor potential cation channel subfamily V member2 (TRPV2) [14]. Ca^{2+} influx causes a substantial release of Ca^{2+} through RyR channels from the sarcoplasmic reticulum (SR) with connection of a subsequent Ca^{2+} to Ca^{2+}-sensitive proteins, including myosin, troponin, and actin, leading to initiation of cardiac contraction. Following cardiac contraction, Ca^{2+} is released from the filaments and returns to the SR through the SERCA pump, initiating cardiac relaxation. Ca^{2+} is then transported to the extracellular location through the NCX [15].

Sex steroid hormones have been defined by their traditional role in normal reproductive function. However, steroid hormones are also produced locally by peripheral conversion in target tissues including fat and the liver [16]. These hormones may act in a paracrine manner or circulate to act on target tissues in an endocrine fashion. Researchers have recently challenged the classic dogma regarding the function of sex hormones [16]. Information concerning their variability in ligand availability, newly recognized alternative forms of sex steroid receptors, previously unrecognized targets of steroid hormones, and different modes of genomic and non-genomic actions has altered our knowledge of normal physiology [17].

PG signaling affects embryogenesis, including cardiac differentiation. However, the function of PG signaling in mESCs is unknown, and no current research regarding the effects of PG on differentiation of mESCs into functional cardiomyocytes during the early differentiation stage has been reported. In this study, to examine the effect of PG on early differentiation into cardiomyocytes in mESCs, changes to the transcriptome by PG were examined by next generation sequencing (NGS) and the expression of a number of cardiac genes and Ca^{2+}-handling genes was observed using quantitative real-time PCR and Western blotting. In addition, cells were evaluated by immunocytochemistry and confocal laser scanning microscope analysis.

2. Materials and methods

2.1. mESCs cell culture and differentiation into cardiomyocytes

2.1.1. Maintenance of pluripotent mESCs

mESCs were purchased from the American Type Culture Collection (ES-E14TG2a cell line). Pluripotent mESCs were cultured in basal medium with leukemia inhibitory factor (LIF, 10 ng/ml; Chemicon) and grown on mitomycin C-treated mouse embryonic fibroblasts in a 60 mm plate (Falcon) at 37 °C in a 5% CO_2 humidified tissue culture incubator (Sanyo). Basal medium consisted of DMEM/F-12 (Gibco) supplemented with non-essential amino acids (NEAA, 1X), 10% heat inactivated and certified fetal bovine serum (FBS; GIBCO), 2-mercaptoethanol (10^{-4} M), penicillin (100 U/ml), and streptomycin (100 μg/ml).

2.1.2. Differentiation protocol of mESCs

To induce differentiation, mESCs were suspended in differentiation medium containing 15% FBS without LIF based on basal medium. For formation of mouse embryoid bodies (mEBs), a cell suspension of 3.2×10^4 cells/ml was prepared and 25 μl X 90 drops were plated on the lid of a 90 mm Petri dish (SPL, South Korea) and cultured as hanging drops after turning over the lids; 6 ml PBS was added to the bottom plate to prevent drying of the drops. Three days later, the mEBs formed on the lid of 2 plates were transferred into a non-coated Petri dish with 6 ml differentiation medium without LIF. After suspension culture for one day, the mEBs were transferred into and attached on a 6-well plate in 2 ml differentiation medium (seven mEBs per well) and were grown for six or ten days.

2.1.3. Treatment of PG during differentiation of mESCs

To examine the effect of PG on cardiac differentiation of mESCs, pluripotent/undifferentiated mESCs were transferred to basal medium without phenol-red and with 10% charcoal-dextran treated certified FBS (CD-FBS) instead of general FBS for two days. Hanging drops for four days and suspension culture for two days were performed using the above mentioned protocol using differentiation medium without phenol-red and with 15% CD-FBS. Attached cells were grown for 12 or 20 days. On day 2 after attachment, mESCs were treated with 10^{-8} M PG for10 days. Some of the 10^{-8} M PG-treated group were treated with 10^{-7}, 10^{-6}, and 10^{-5} M RU486, an antagonist of PG, on day 11 after attachment of mEBs and cells were exposed for one day. All experiments were performed four or more times in triplicate.

2.2. Methods used in measuring the effect of PG

2.2.1. Assessment of cardiomyocyte differentiation using beating ratio and frequency

Spontaneous contraction of differentiated mESCs was observed manually using a phase-contrast microscope (Olympus, IX71), and beating ratio was determined by the number of contracting cell populations to the number of attached mEBs. One cell population means differentiated cells from one mEB. To measure beating frequency, Ten beating populations per treatment group were recorded on video for 2 min by Olympus DP Controller software, and then the number of excitation-contractions was counted manually.

2.2.2. Total RNA extraction, reverse transcription, and quantitative PCR

Total RNA was extracted using Trizol reagent (Ambion) according to the manufacturer's instructions and the concentration of total RNA was determined by measuring the absorbance at 260 nm using an Epoch microplate spectrophotometer (BioTek Instruments, Inc.). Reverse transcription of 1 μg of total RNA was performed using MMLV reverse transcriptase (Invitrogen) and random primers (9-mers; TaKaRa Bio Inc.). Quantitative real-time PCR was performed in a 12.5 μl mixture of 6.25 μl of 2 × SYBR Premix Ex Taq (Genet Bio Inc.), 0.25 μl Rox dye (Genet Bio Inc.), 0.25 μl each of forward and reverse primers (Table 1), and 4.5 μl distilled water using an ABI Prism 7300 Sequence 10 detection system (Life Technologies) equipped with a 96-well optical reaction plate under the following conditions: denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. Fluorescence intensity was measured at the end of the extension phase of each cycle. The reaction cycle at which PCR products exceeded the fluorescence intensity threshold in the exponential phase of PCR amplification was considered the threshold cycle (CT). The amount of transcript showed inverse relation to the observed CT and CT was expected