

## Original Article

# All-trans retinoic acid in combination with collagen IV induces the differentiation of mouse embryonic stem cells into smooth muscle cells

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**Abstract:** All-trans retinoic acid (atRA) and collagen IV (ColIV) have been reported to play key roles in embryogenesis. Differentiation of smooth muscle cells (SMC) plays an important role in human diseases including atherosclerosis, hypertension and neoplasms. Here, atRA and ColIV were applied separately and in combination to investigate the differentiation of mouse embryonic stem cells (ESC) into SMC. The totipotency of ESC was examined by ALP (alkaline phosphatase) staining, SSEA-1 (stage-specific embryonic antigen-1) staining and teratoma formation test in vivo. Real-time Quantitative PCR (qRT-PCR), Western blot and immunofluorescence staining were used to detect  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and smooth muscle myosin heavy chain (SM-MHC) expression in differentiation of ESC. We observed that expression of ALP and SSEA-1 was positively localized in ESC. The histochemical staining (HE) showed that ESC had the capacities of totipotency and could differentiate embryoid body. Interesting, we found that pretreatment of ESC with either atRA or ColIV could increase mRNA and protein expression levels of  $\alpha$ -SMA and SM-MHC in differentiation of ESC at 7 and 14 days. More important, combined treatment with atRA and ColIV resulted in a significant promotion of  $\alpha$ -SMA and SM-MHC expression compared with atRA or ColIV treated ESC at 7 and 14 days. In conclusion, ESC has the potential to differentiate to SMC. AtRA in combination with ColIV can efficaciously induce the differentiation of mouse ESC into SMC.

**Keywords:** AtRA, ColIV, ESC, SMC, differentiation

## Introduction

Embryonic stem cells (ESC) derived from the inner cell mass (ICM), which have unlimited self-renewal potential in vitro and can give rise to all three germ layers of embryo [1, 2]. Cellular differentiation involves initial commitment of ESC to a specific cellular lineage and subsequently differentiation of committed cells. Cellular differentiation is characterized by coordinate induction of a repertoire of cell-specific proteins necessary for specialized functions [3].

Differentiation and phenotypic plasticity of smooth muscle cells (SMC) act important roles in vasculogenesis and many human diseases [4, 5]. SMC are heterogeneous cells with a wide range of different phenotypes at different developmental stages [6, 7]. In the last few

years, several studies have demonstrated that ESC can develop into SMC in vitro [8]. For example, Xiao et al. showed that Pla2g7 (phospholipase A2, group 7) plays a crucial physiological role in SMC differentiation from ESC [9]. Yamaguchi et al. demonstrated that miR-145 can regulate the fate and phenotype of human ESC-pre-SMC [10]. Xiao et al. found that Cbx3 (chromobox protein homolog 3) is essential for ESC differentiation to SMC in vitro [11]. In addition, Simpson et al. reported that Hyaluronan is crucial for ESC differentiation into SMC [12].

The all-trans retinoic acid (atRA) is a potent regulator of cell proliferation, differentiation, and matrix formation of various cell types and plays a key role in embryogenesis [13]. Collagen IV (ColIV) has been reported to direct ESC differentiation to mesodermal lineages in both mouse and human [14]. However, the signifi-

**Table 1.** The information of primers for qRT-PCR

Gene	Forward (5'-3')	Reverse (5'-3')
$\alpha$ -SMA	ACTGCCGAGCGTGAGATT	TCCAGGGAGGAAGAGGAG
SM-MHC	AAGTCTAGGGCTATTCG	ATGGCTTCCAGTGTCTCC
$\beta$ -Actin	AATCGTGCGTGACATCAA	AGAAGGAAGGCTGGAA

cance and exact function of atRA and ColIV in SMC differentiation remain to be elucidated. In this study, we demonstrated that both atRA and ColIV could enhance mouse ESC differentiation from ESC. More important, AtRA in combination with ColIV could efficaciously induce the differentiation of mouse ESC into SMC.

## Materials and methods

### ESC culture

Mouse ESC was obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA). The ESC was cultured on  $\gamma$ -irradiated mouse embryonic fibroblasts (MEFs) at 37°C and 5% CO<sub>2</sub> with daily change of the medium. The medium was composed of Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA), 15% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 1% of 100 U/ml penicillin, 1% of 100 mg/ml streptomycin sulfates, 0.1 mM mercaptoethanol, 2 mM glutamine, 0.1 mM nonessential amino acids, and 1,000 U/ml recombinant leukemia inhibitory factor.

### SMC differentiation

The resuspended ESC was cultivated on 12-well cell culture plates at a density of approximately 3×10<sup>5</sup>/well at 37°C with 5% CO<sub>2</sub> in 2 ml of differentiation medium with the presence of 20  $\mu$ M atRA or 10  $\mu$ M ColIV or 10  $\mu$ M atRA and 5  $\mu$ M ColIV. The differentiation medium was made of DMEM, 15% fetal bovine serum, 2 mM L-glutamine, 1 mM MTG, 0.1 mM nonessential amino acids, 1% of 100 U/ml penicillin and 1% of 100 mg/ml streptomycin sulfates. The culture was continued for 10 days with daily change of fresh media.

Starting from the 11th day, the differentiation medium was replaced by the serum-free culture medium, which was composed of DMEM, 15% knock-out serum replacement, 2 mM L-glutamine, 1 mM MTG, 0.1 mM nonessential amino acids, 1% of 100 U/ml penicillin and 1%

of 100 mg/ml streptomycin sulfates. The cultures were continued for another 10 days with daily change of the serum-free medium.

### Teratoma formation test

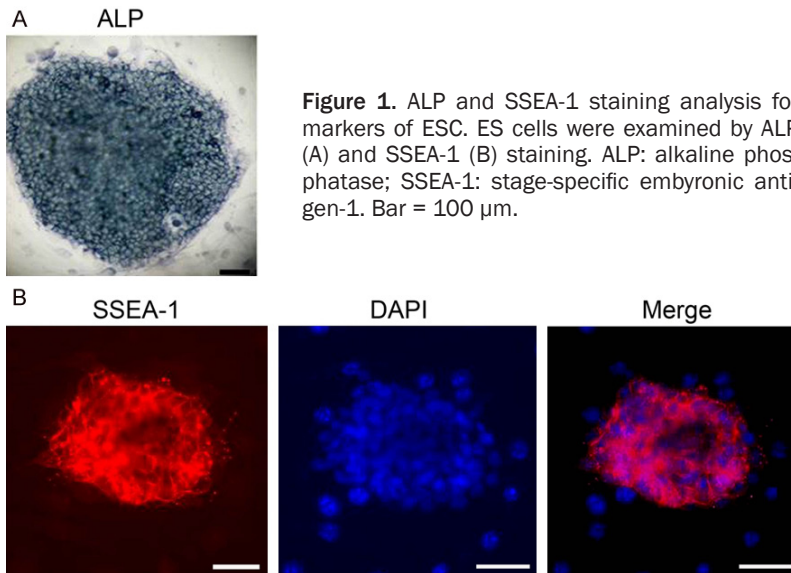
Pluripotency *in vivo* was assessed by teratoma formation in immunodeficient nude mice (BALB/cAJcl-nu/nu; CLEA Japan Inc. Tokyo, Japan). A 60 mm plate of undifferentiated ESC was washed with phosphate-buffered saline (PBS) and the cells were harvested with a cell scraper. The cell suspension was collected into a 15-ml conical tube and spun down at 1000 rpm for 4 min. The cell pellet was resuspended by addition of a 1:1 mixture of ESC culture medium and Matrigel (BD Biosciences, NJ, USA) to a final total volume of 400  $\mu$ l. Approximately 2~5×10<sup>6</sup> cells in 200  $\mu$ l/injection site were injected in the dorsolateral area into the subcutaneous space on both sides. Tumors were excised surgically. All animals were treated according to the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources for the National Research Council. The study was approved by The Ethics Committee of the Affiliated Hospital of Guizhou Medical University. All experiments with mice were subject to the 3 R consideration (refine, reduce replace) and all efforts were made to minimize animal suffering, and to reduce the number of animals used.

### Total RNA isolation and real-time quantitative PCR (qRT-PCR)

Total RNAs were isolated from tissues and cells by TRIzol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocols. The RNA was immediately frozen stored at -80°C until further experiments.

For detect  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and smooth muscle myosin heavy chain (SM-MHC) expression, reverse transcription was performed by using a Reverse Transcriptase M-MLV (Takara, Dalian, China). qRT-PCR reaction was performed by using a SYBR® Premix EX Taq™ IIPCR Kit (Takara, Japan) on ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).  $\beta$ -actin was used as internal control. The primers sequences were shown in **Table 1**. The  $\alpha$ -SMA and

## AtRA and colIV induce differentiation



**Figure 1.** ALP and SSEA-1 staining analysis for markers of ESC. ES cells were examined by ALP (A) and SSEA-1 (B) staining. ALP: alkaline phosphatase; SSEA-1: stage-specific embryonic antigen-1. Bar = 100  $\mu$ m.

Large tumors were cut in half before fixation, and each of the two halves were placed in the paraffin block so that the central parts of tumor (with the larger surface area) were face up. To enable analysis of the different parts of large tumors, the sectioning was performed from the upper part of the paraffin block, containing the central parts of both halves of the tumor, towards the tumor's edges. The results were observed by a Laser Scanning Confocal Microscopy (Zeiss, Germany).

SM-MHC expression levels were normalized to those of  $\beta$ -Actin by using the  $2^{-\Delta\Delta Ct}$  method [15].

### Western blot

Total protein was isolated by ice-cold RIPA buffer (Invitrogen, Carlsbad, CA, USA). Protein was quantitated by a BCA kit (Millipore, MA, USA) according to the manufacturer's protocols. The protein samples were separated in 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (PVDF; Millipore, Billerica, MA, USA). After that, the membrane was blocked in 5% non-fat milk overnight and incubated with the mouse monoclonal against  $\alpha$ -SMA antibody (#ab7817; 1:500; Abcam, Cambridge, USA), SM-MHC antibody (#ab683; 1:1000; Abcam, Cambridge, USA) and  $\beta$ -actin antibody (#ab-8226; 1:1000; Abcam, Cambridge, USA) at 37°C for 2 h. After washing with TBST buffer, the membrane was incubated with the HRP-conjugated goat anti-mouse secondary antibody (#ab6789; 1:2000; Abcam, Cambridge, USA) for 1 h at 37°C. The protein bands were detected by using an enhanced chemiluminescence (ECL)-based detection system (Millipore, MA, USA).

### Histological analysis

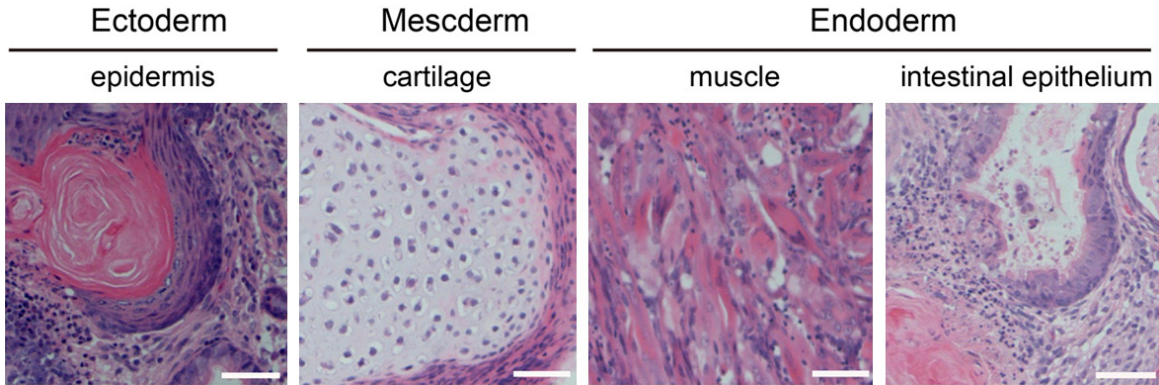
The tumor-containing tissues were fixed in 4% paraformaldehyde (PFA), embedded in paraffin and serially sectioned into 5 micron sections. Various parts of the tumor were stained with hematoxylin and eosin (HE), and subjected to histological analysis by certified pathologists.

### ALP (alkaline phosphatase) staining

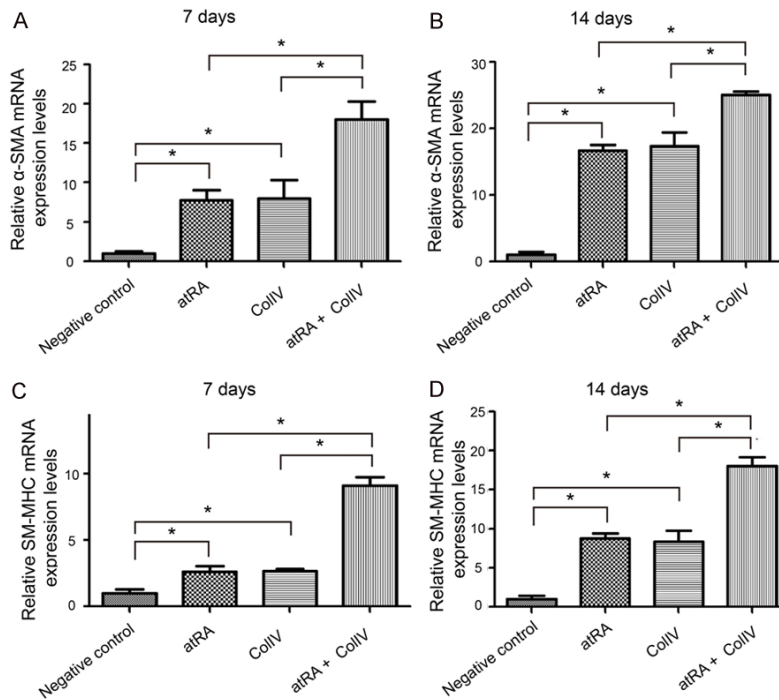
ESC was subjected to alkaline phosphatase staining on day 5 of culture. In detail, ESC was freed from culture media and fixed in 4% paraformaldehyde for 10 min, fixed cells were washed with DPBS and incubated in AP staining solution containing of 25 mM Tris-HCl, 150 mM NaCl, 8 mM  $MgCl_2$ , 0.4 mg/ml Naphthol AS-MX Phosphate and 1 mg/ml FastRed TR salt for 30 min at 37°C.

### Immunofluorescence staining

Prior to immunofluorescence staining, all cells were washed with 1 $\times$ PBS, fixed for 10 minutes in 4% paraformaldehyde (Sigma), and rinsed twice in PBS. Cells were then permeabilized using 1% Triton X-100 (Sigma, USA) for 30 minutes and subsequently incubated in 10% goat serum (Sigma, USA). After rinsing with 0.05% Tween 20, cells were incubated with the SSEA-1 (stage-specific embryonic antigen-1) anti- $\alpha$ -SMA and SM-MHC primary antibody overnight at 4°C. For secondary antibody detection, the appropriate Alexa Fluor-conjugated antibodies were incubated at a 1:250 dilution in PBS for 30 minutes at room temperature in the dark. After secondary antibody incubation, the cells were washed with PBS and incubated in a DAPI solution (Invitrogen, Carlsbad, CA, USA) followed by washes with PBS. In a final step, the cell culture chambers were removed from the slides and slides and coverslips were mounted using ProLong Gold antifade mounting medium (Invitrogen, Carlsbad, CA, USA). The slides were



**Figure 2.** Morphometric analysis of teratomas. The totipotency of ESC in vivo teratoma formation was detected by hematoxylin and eosin (HE) staining. Bar = 100  $\mu$ m.



**Figure 3.** The mRNA expression of  $\alpha$ -SMA and SM-MHC in differentiation of ESC at different times. Real-time Quantitative PCR (qRT-PCR) was performed to detect  $\alpha$ -SMA (A and B) and SM-MHC (C and D) expression in differentiation of ESC at 7 and 14 days.  $\beta$ -Actin was used as internal control.  $\alpha$ -smooth muscle actin:  $\alpha$ -SMA; smooth muscle myosin heavy chain: SM-MHC. \* $P < 0.05$ .

then stored in the dark overnight at 4°C prior to imaging. Images were acquired using a Laser Scanning Confocal Microscopy (Zeiss, Germany).

#### Statistical analysis

The SPSS 18.0 software (SPSS Inc; Chicago, IL, USA) was used to perform statistical analysis. All data were shown as the mean  $\pm$  standard

deviation (SD) from at least three times independently experiments. Student's t-test or ANOVA was used to determine statistical significance. A value of  $P < 0.05$  was considered to be statistically significant.

#### Results

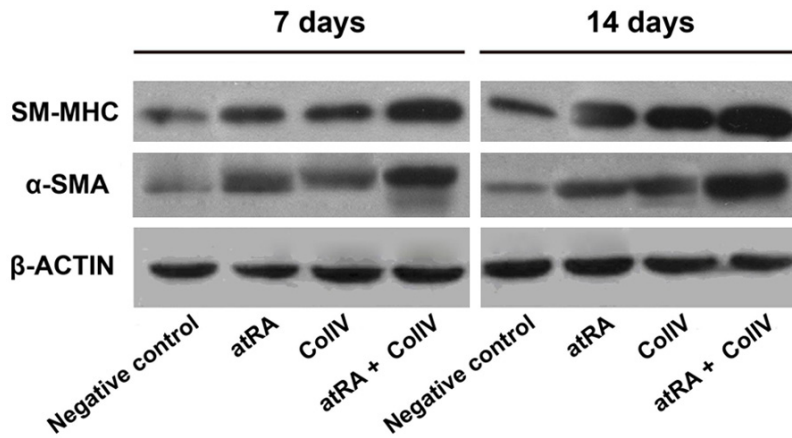
##### ALP and SSEA-1 staining analysis for markers of ESC

Expression of pluripotency related marker genes like ALP and SSEA-1 were positively localized in ESC at 5 days of culture. As shown in **Figure 1**, localization of these markers was cytoplasmic and distributed homogeneously all over the ESC.

##### Morphometric analysis of teratomas

To further investigate the predisposition of ESC to differentiate in vivo, we measured the areas of histological components including ectoderm, mesoderm and endoderm. The results showed that cartilage, epidermis, muscle and intestinal epithelium were successful generated by ESC, suggesting ESC has the capacities of totipotency and can differentiate embryoid body (**Figure 2**).





**Figure 4.** The protein expression of  $\alpha$ -SMA and SM-MHC in differentiation of ESC at different times. Western blot was performed to detect  $\alpha$ -SMA and SM-MHC expression in differentiation of ESC at 7 and 14 days.

higher than atRA or CollIV treated group (Figure 3,  $P < 0.05$ ). Meanwhile, we also observed that the protein levels of  $\alpha$ -SMA and SM-MHC in atRA and CollIV treated ESC were also higher than atRA or CollIV treated group (Figures 4 and 5). All of these data demonstrate that both atRA and CollIV enhance mouse ESC differentiation from ESC. More important, AtRA in combination with CollIV can efficaciously induce the differentiation of mouse ESC into SMC.

*All-trans retinoic acid (atRA) and collagen IV (CollIV) enhance mouse ESC differentiation from ESC*

ESC is characterized by unlimited self-renewal and the potential to differentiate into SMCs in vitro SMC [16, 17]. SMC can express specific cell markers such as  $\alpha$ -SMA and SM-MHC [18, 19]. After pretreatment with atRA or CollIV, the mRNA expression of  $\alpha$ -SMA and SM-MHC in differentiation of ESC at different times was determined by qRT-PCR. As shown in Figure 3, the expression levels of  $\alpha$ -SMA and SM-MHC in atRA or CollIV or atRA and CollIV treated ESC at 7 and 14 days were significantly increased compared with negative control group ( $P < 0.05$ ). The expression levels of  $\alpha$ -SMA and SM-MHC in atRA and CollIV treated ESC have not statistically significant.

In addition, Western blot and immunofluorescence staining was also applied to detected protein levels of  $\alpha$ -SMA and SM-MHC in ESC. Interesting, as shown in Figures 4 and 5, we found that  $\alpha$ -SMA and SM-MHC protein expression in atRA or CollIV treated ESC at 7 and 14 days was also up-regulated compared with negative control group. The protein levels of  $\alpha$ -SMA and SM-MHC in atRA and CollIV treated ESC have not statistically significant.

*AtRA in combination with CollIV efficaciously induce the differentiation of mouse ESC into SMC*

Pretreatment with atRA and CollIV in ESC,  $\alpha$ -SMA and SM-MHC mRNA expression was

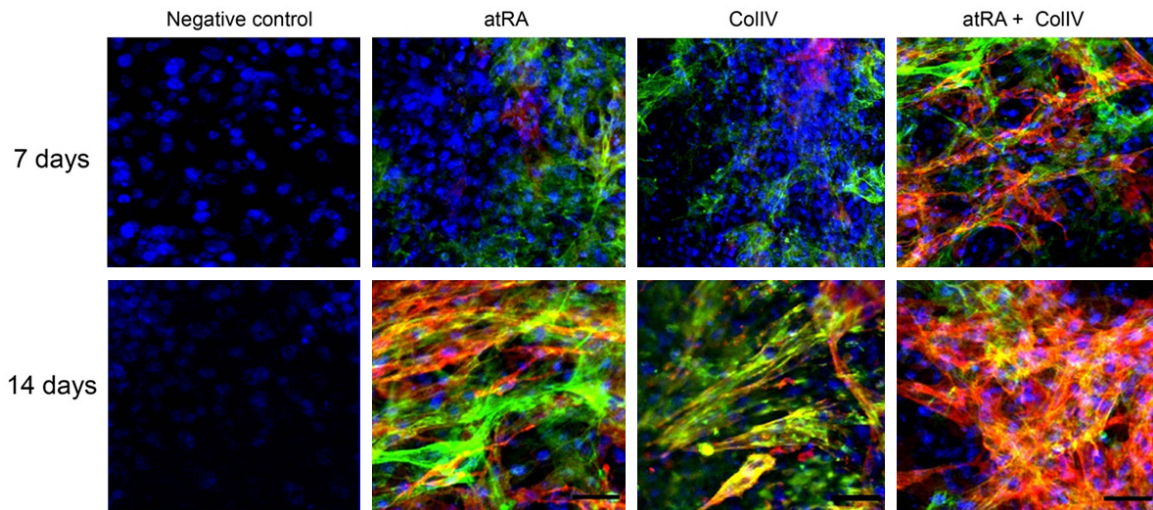
**Discussion**

SMC is a key component of healthy and tissue engineered vessels and play a crucial role in vascular development and the pathogenic events of vascular remodeling [4, 5]. The overall process of SMC differentiation is complex and involves the co-operative interaction of numerous factors. Myocardin, the transcriptional co-factor of serum response factor (SRF), is found to be required for the expression of many SMC differentiation markers [20]. It is crucial in the initial differentiation of SMC during development [21, 22]. Over-expression of myocardin induces ESC to express multiple SMC genes including  $\alpha$ -SMA and SM-MHC [23].

ESC has the remarkable capability to differentiate into vascular SMC in response to specific stimuli, which provides a useful model for studying SMC differentiation [24, 25]. AtRA, a vitamin A derivative, exerts a wide range of biological effects. Previous studies suggested that AtRA is involved in the control of cellular differentiation and proliferation [13]. Collagen IV (CollIV) has been reported to direct ESC differentiation to mesodermal lineages in both mouse and human [14]. However, the significance and exact function of atRA and CollIV on SMC differentiation remain to be elucidated.

In this study, we found expression of pluripotency related marker genes like ALP and SSEA-1 were positively localized in ESC at 5 days of culture. The cartilage, epidermis, muscle and intestinal epithelium were successful generated by ESC. These results suggested that ESC

## AtRA and ColIV induce differentiation



**Figure 5.** Immunofluorescence staining analysis of  $\alpha$ -SMA and SM-MHC expression in differentiation of ESC at different times.  $\alpha$ -SMA (green) and SM-MHC (red) staining were captured by using a Laser Scanning Confocal Microscopy and representative photographs were shown. Bar = 100  $\mu$ m.

had the capacities of totipotency and could differentiate embryoid body. Next, we detected the effects of AtRA and ColIV on SMC differentiation. Our results revealed that the expression levels of  $\alpha$ -SMA and SM-MHC in atRA or ColIV treated ESC at 7 and 14 days were significantly increased compared with negative control group, while  $\alpha$ -SMA and SM-MHC expression in atRA or ColIV treated ESC have not statistically significant. More important, Pretreatment with atRA and ColIV in ESC,  $\alpha$ -SMA and SM-MHC expression was higher than atRA or ColIV treated group. All of these data demonstrated that both atRA and ColIV could enhance mouse ESC differentiation from ESC. More important, atRA in combination with ColIV could efficaciously induce the differentiation of mouse ESC into SMC.

In summary, we report that ESC has the potential to differentiate to SMC. AtRA in combination with ColIV can efficaciously induce the differentiation of mouse ESC into SMC. These findings significantly increase our understanding of the molecular mechanisms in SMC differentiation and will benefit future applications in regenerative medicine.

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### Disclosure of conflict of interest

None.

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