



# Purification and functional assessment of smooth muscle cells derived from mouse embryonic stem cells

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## Abstract

**Objective** To obtain a pure population of smooth muscle cells (SMC) derived from mouse embryonic stem cells (ESC) and further assess their functions. **Methods** A vector, expressing both puromycin resistance gene (*puro*<sup>r</sup>) and enhanced green fluorescent protein (EGFP) gene driven by smooth muscle 22 $\alpha$  (SM22 $\alpha$ ) promoter, named pSM22 $\alpha$ -*puro*<sup>r</sup>-IRES2-EGFP was constructed and used to transfect ESC. Transgenic ESC (Tg-ESC) clones were selected by G418 and identified by PCR amplification of *puro*<sup>r</sup> gene. The characteristics of Tg-ESC were detected by alkaline phosphatase (ALP) staining, SSEA-1 immunofluorescence and teratoma formation test *in vivo*. After induction of SMC differentiation by all-trans retinoic acid, differentiated Tg-ESC were treated with 10  $\mu$ g/mL puromycin for three days to obtain purified SMC (P-SMC). Percentage of EGFP<sup>+</sup> cells in P-SMC was assessed by flow cytometer. Expressions of smooth muscle specific markers were detected by immunostaining and Western blotting. Proliferation, migration and contractility of P-SMC were analyzed by growth curve, trans-well migration assay, and carbachol treatment, respectively. Finally, both P-SMC and unpurified SMC (unP-SMC) were injected into syngeneic mouse to see teratoma development. **Results** Tg-ESC clone was successfully established and confirmed by PCR detection of *puro*<sup>r</sup> gene in its genomic DNA. The Tg-ESC was positive for ALP staining, SSEA-1 staining and formed teratoma containing tissues derived from three germ layers. After retinoic acid induction, large amount of EGFP positive cells outgrew from differentiated Tg-ESC. Three days of puromycin treatment produced a population of P-SMC with an EGFP<sup>+</sup> percentage as high as 98.2% in contrast to 29.47% of unP-SMC. Compared with primary mouse vascular smooth muscle cells (VSMC), P-SMC displayed positive, but lowered expression of SMC-specific markers including SM  $\alpha$ -actin and myosin heavy chain (SM-MHC) detected either, by immunostaining, or immunoblotting, accelerated proliferation, improved migration ( $99.33 \pm 2.04$  vs.  $44.00 \pm 2.08$  migrated cells/field,  $P < 0.05$ ), and decreased contractility in response to carbachol ( $7.75 \pm 1.19$  % vs.  $16.50 \pm 3.76$  % in cell area reduction,  $P < 0.05$ ). *In vivo* injection of unP-SMC developed apparent teratoma while P-SMC did not. **Conclusions** We obtained a pure population of ESC derived SMC with less mature (differentiated) phenotypes, which will be of great use in research of vascular diseases and in bio-engineered vascular grafts for regenerative medicine.

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**Keywords:** Smooth muscle cell; Embryonic stem cell; Differentiation; SM22 $\alpha$ ; Phenotype

## 1 Introduction

Cardio and cerebrovascular diseases are the two leading causes of disability and mortality in elderly people worldwide.<sup>[1]</sup> As a major cellular component of blood vessels, smooth muscle cells (SMC) play an important role in these diseases, including atherosclerosis, hypertension, stroke,

restenosis, *etc.* Unfortunately, the mechanism underlying the roles of SMC involved in pathogenesis and development of vascular disease is far from being fully elucidated. One of the obstacles is the lack of suitable *in vitro* model recapitulating developmental process of SMC *in vivo*. Although several lines of multipotential cells including 10T 1/2,<sup>[2]</sup> MONC-1<sup>[3]</sup> and P10 embryonal carcinoma cells<sup>[4]</sup> have been successfully induced to produce SMC like cells expressing one or more SMC specific markers, but these cells fail to exhibit the characteristic contractile function of SMC. Consequently, SMC like cells derived from the above cell sources may not be the best choice for SMC developmental study. Moreover, it's also questionable whether these multipotential cells are true origins of SMC *in vivo*. Therefore, it is essential to obtain functional SMC from an *in vitro* sys-

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tem where true SMC development and maturation are recapitulated as *in vivo* from a definite origin.

In addition, SMC also serve as indispensable seeds for engineered vascular grafts, but lack of ideal cell sources for SMC has greatly impeded progress in this field. The widely used SMC isolated from adult blood vessels have limited proliferative ability and will lose contractility during long term culture *in vitro*.<sup>[5]</sup> SMC from other sources like mesenchymal stem cells,<sup>[6]</sup> adipose tissues<sup>[7]</sup> and hair follicles<sup>[8]</sup> also displayed limited proliferation, and required frequent traumatic procedures to harvest a sufficient quantity of cells. Moreover, the above source tissues isolated from patients are usually senile or diseased, thus leading to production of unqualified SMC for therapeutic regeneration due to their impaired functions.

Embryonic stem cells (ESC) have been previously established from the inner cell mass of blastocysts. These cells have ability of indefinite self-renewal and pluripotency to generate almost over 200 known cell types *in vivo* and *in vitro*,<sup>[9,10]</sup> making it an ideal cell source for SMC. However, ESC pluripotency will result in “contamination” of SMC by other cell types during differentiation, which calls for the necessary isolation of pure SMC from the cell mixture differentiated from ESC. So in the present study, we aimed to obtain a pure population of SMC derived from ESC, and further assess their functions.

## 2 Methods

### 2.1 Construction of vector pSM22 $\alpha$ -*puro*<sup>r</sup>-IRES2-EGFP

Procedures performed in the mouse conformed to the “Position of the American Heart Association (AHA) on research animal use” and was approved by Ethics Committee of our institution. Mouse genomic DNA was extracted from 129/Sv mouse tail by using DNA extraction kit (Promega, Madison, WI, USA) according to the instructions of the manufacturer. The 541bp SM22 $\alpha$  promoter (NC\_000075) was amplified from mouse genomic DNA using high fidelity PCR with the following primers: forward AGTTATATTAATTTTGCATAGTGCCTGGTTG, and reverse GCGCTAGCTACAAGGCTTGGTCGTTT (Takara, Dalian, China). The puromycin resistance (*puro*<sup>r</sup>) gene was excised from pSM2C (Invitrogen, Carlsbad, CA, USA) with HindIII/ClaI (Takara). The SM22 $\alpha$  promoter and *puro*<sup>r</sup> gene were subcloned into pIRES2-EGFP vector (Clontech, Mountain View, CA, USA) with AseI/NheI (NEB, Ipswich, MA, USA) and BglIII/AccI (Takara), respectively, to construct pSM22 $\alpha$ -*puro*<sup>r</sup>-IRES2-EGFP (Figure 1A), which encoded *puro*<sup>r</sup> and EGFP under control of the SM22 $\alpha$  promoter. All the constructed vectors were finally confirmed by DNA sequencing.

### 2.2 ESC culture and generation of transgenic ESC (Tg-ESC)

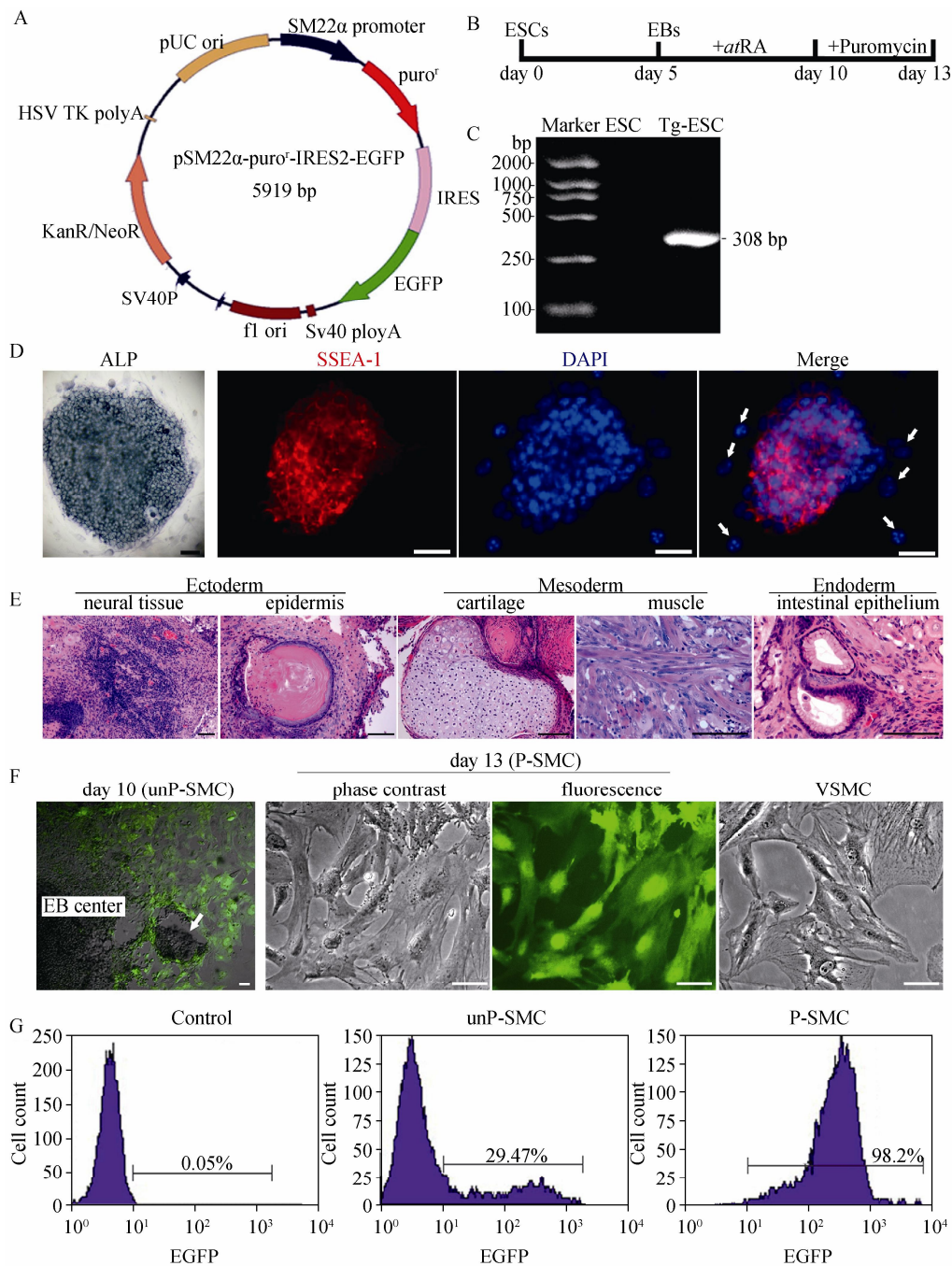
Mouse ESC R1 (ATCC, Manassas, VA, USA) were routinely cultured on mitotically inactivated STO feeder layers in ESC medium containing high glucose DMEM (Hyclone, Logan, UT, USA) in the presence of 15% ESC qualified fetal bovine serum (Gibco, Carlsbad, CA, USA), 0.1 mmol/L 2-mercaptoethanol, 1 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 0.1 mmol/L non-essential amino acids, 100 U/mL penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and 1000 U/mL leukemia inhibitory factor (LIF, Millipore, Billerica, MA, USA). Cells were fed every day and passaged when reaching 80% confluence. Before transfection, ESC trypsinized to single cells were cultured on 6-well plate coated with 0.1% gelatin (Sigma-Aldrich) without feeder layer. Then, ESC were transfected with 4  $\mu$ g pSM22 $\alpha$ -*puro*<sup>r</sup>-IRES2-EGFP plasmid DNA using Lipofectamine 2000 (Invitrogen) per the instructions of the manufacturer. Cells were incubated overnight and replated to a 10 cm plate coated with 0.1 % gelatin. Twenty four hours later, 500  $\mu$ g/mL G418 was used to select transgenic ESC clones. Colonies that survived two weeks of G418 selection were amplified. One half of the survived ESC clones were cryopreserved, and the other half was screened for the presence of the *puro*<sup>r</sup> gene in genome by PCR. The forward primer for PCR reaction 5'GCAACCTCCCTTCTACGAGC3'; and reverse primer, 3'CCTCACATTGCCAAAAGACG5'. The expected length of PCR product is 308 bp.

### 2.3 Primary mouse vascular smooth muscle cells (VSMC) culture

All procedures involving animals in our study were approved by the Animal Ethics Committee of our hospital. The aortas of 10 three-months old male Sv/129 mice were removed from the aortic arch to the femoral bifurcation stripped of adventitia and minced. The minced pieces are spread evenly on the 60-mm dishes under the humidified conditions. After these pieces adhere firmly to plates, fresh, pre-warmed media is added and the plate is placed in a humidified incubator containing 5% CO<sub>2</sub>. At the end of the 2<sup>nd</sup> week, explants are carefully removed without disturbing the SMC outgrowths. The culture medium is replaced and proliferative cells are allowed to grow for further 2–3 weeks to form monolayer. Cells were used between passage 2 and 5.

### 2.4 *In vitro* pluripotency characterization of Tg-ESC

Alkaline phosphatase (ALP) staining, SSEA-1 immuno-



**Figure 1. Production of pure population of SMC by puromycin selection.** (A): Schematic of vector pSM22 $\alpha$ -puro<sup>R</sup>-IRES2-EGFP. (B): Protocol for induction and purification of SMC. (C): Tg-ESC was confirmed by detection of 308 bp fragment of *puro<sup>R</sup>* gene by PCR assay. Wild type ESC was used as negative control. (D) Tg-ESC were positive for ALP staining and SSEA-1 staining. Feeder cells (indicated by arrows) showed no SSEA-1 staining. Scale bars, 50  $\mu$ m. (E) Tg-ESC were capable of forming teratoma containing structures from three germ layers, including ectoderm (neural tissue and epidermis), mesoderm (cartilage and muscle) and endoderm (intestinal epithelium). Scale bars, 100  $\mu$ m. (F): Day 10 (unP-SMC): EGFP positive SMC mainly located peripherally to the EB center before puromycin selection. Cell colony with morphology of undifferentiated ESC could also be seen (indicated by arrow) within SMC population; day 13 (P-SMC): Pure population of P-SMC showed spindle-like shape under both phase contrast and fluorescence microscope after puromycin selection; VSMC: morphology of primary mouse VSMC under phase contrast microscope. Scale bars, 100  $\mu$ m. (G): Flow cytometry analysis showed that P-SMC had 98.20% EGFP<sup>+</sup> cells, which is much higher than 29.47% of unP-SMC without puromycin selection. Cells derived from wild type ESC without EGFP expression were used as negative control. EGFP: enhanced green fluorescent protein; SMC: smooth muscle cell; Tg-ESC: transgenic embryonic stem cell; unP-SMC: unpurified SMC; EB: embryoid body; P-SMC: purified SMC; VSMC: vascular SMC.

fluorescence and *in vivo* teratoma formation were used to assess pluripotency of Tg-ESC.

For ALP staining, Tg-ESC were cultured in 6-well plates until 70% confluent. Then cells were fixed by 4% paraformaldehyde (PFA, Sigma-Aldrich) for one min at room temperature (RT). Remove PFA and wash cells 3 times with 2 mL of 0.05 % Tween-20 in PBS. Prepare 2 mL staining solution according to the instructions of the manufacturer of the ALP kit (Sidansai Biotechnology, Shanghai, China) and incubate Tg-ESC in the dark for 15 min at RT. Positive staining will appear as blue.

For SSEA-1 immunofluorescence, Tg-ESC grown on coverslips were fixed with 4% PFA for 5 min, permeabilized with 0.2% Triton X-100 for 10 min and blocked with 5% goat serum for 1 h. Cells were then incubated with mouse monoclonal antibody SSEA-1 (1: 100, Cell Signaling, Danvers, MA, USA) at 4°C overnight. On the next day, cells were incubated with anti-mouse IgM antibody (1: 100, Cell signaling) for 1 h at RT. Cells were washed 3 times with PBS for 5 min between each procedure. After being mounted with DABCO glycerol anti-fading media (Sigma-Aldrich), cover-slips were sealed and examined and images were taken by Olympus IX70 inverted fluorescence microscope (Olympus, Tokyo, Japan).

For *in-vivo* teratoma formation test,  $1 \times 10^6$  Tg-ESC in a 0.1 mL 50% matrigel suspension was subcutaneously injected into the hind limb of syngeneic Sv/129 hosts ( $n = 6$ ). After 4 weeks of incubation, the animals were euthanized, and the teratoma was dissected out. Teratoma was fixed, paraffin embedded, microtome sectioned, and histologically stained with routine hematoxylin and eosin (HE) staining. Structures from their germ layers were identified under the microscope.

## 2.5 Induction of SMC differentiation and SMC purification

Induction protocol was displayed as Figure 1B. In detail, wild type ESC and Tg-ESC were trypsinized to single cells and cultured in Petri dish with ESC medium without LIF to form suspended cell aggregates called embryoid body (EB). On day 6, suspended EB were replated onto a 0.1% gelatin-coated 10 cm plate in the presence of 10 nmol/L of all-trans retinoic acid (*atRA*). The culture was continued for five days with a daily change of fresh *atRA*-containing medium. On day 11, a series of puromycin concentrations, i.e., 1, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20  $\mu\text{g}/\text{mL}$ , were used for 3 days to differentiate wild type ESC and Tg-ESC. The lowest concentration that kills all the cells in wild type ESC group and leaving least non-EGFP<sup>+</sup> cells in Tg-ESC group was determined as the final concentration for puromycin

selection. In our study, this concentration was 10  $\mu\text{g}/\text{mL}$  and was adopted in all the later purification procedures. After 3 days of puromycin selection, purified SMCs (P-SMCs) were obtained and expanded. Cells between passage 2 and 5 were used for further assessment.

## 2.6 Flow cytometry analysis

Unpurified SMC (unP-SMC) and P-SMC derived from Tg-ESC were harvested by trypsin digestion and cell suspension was fixed by ice cold 70% ethanol for 30 min at 4°C. Acquisition of 10 000 events was made with FACScan (BD Biosciences, Rockville, MD, USA), and EGFP positive SMC were analyzed with CellQuest software (BD). Cells derived from wild type ESC without EGFP expression were used as negative controls.

## 2.7 Immunofluorescence

Mouse VSMC and P-SMC were plated onto 0.1 % gelatin coated cover slips. One day after plating, cells were fixed with 4 % PFA in PBS for 5 min, permeabilized with 0.2 % Triton X-100 (Sigma-Aldrich) in PBS for 15 min, and blocked with 5% goat serum (Gibco) in PBS for one h at room temperature. Cells were incubated with primary antibodies overnight at 4°C. Primary antibodies were mouse anti-SM  $\alpha$ -actin (1: 100), Abcam, Cambridge, MA, USA) and rabbit anti-SM myosin heavy chain (SM-MHC, 1: 100, BTI, Stoughton, MA, USA). The cells were then incubated with TRITC-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. Cover-slips were sealed and examined by Olympus IX70 inverted fluorescence microscope (Olympus).

## 2.8 Western blot analysis

Protein samples of VSMC, P-SMC were extracted by RIPA buffer, supplemented with protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA). Equal amounts of 50  $\mu\text{g}$  proteins were run on 8%–12% SDS gel and transferred to PVDF membranes. Nonspecific binding was blocked by incubation for one h with 5% nonfat dry milk in TBS-T (25 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 0.1% Tween 20). Primary antibody against SM22 $\alpha$  (1: 1000, Abcam), SM  $\alpha$ -actin (1: 1000, Abcam), SM-MHC (1: 500, BTI),  $\beta$ -actin (1: 2000, Abcam) and secondary anti-mouse- HRP (1: 2000) and anti-rabbit-HRP (1: 2000) antibodies (Jackson ImmunoResearch) were used to blot the membrane. Detection of protein was performed by using ECL system (Thermo Scientific) according to the manufacturer's instruction. Bands were scanned and quantified by Gel-pro Analyzer 4.0 software (Media Cybernetics, Rockville, MD, USA).

## 2.9 Proliferation assay

Proliferation of VSMC and P-SMC was assessed by counting cell numbers to plot growth curve. Cells were seeded onto 24-well plates at the concentration of 10000 cells/well in 10% FBS-DMEM (day 0) and the medium replaced every other day. Cells were collected every day and cell numbers were determined by hemacytometer up to seven days.

## 2.10 Migration assay

Migration of VSMC and P-SMC were assayed by Trans-well (Corning, Tewksbury, MA, USA) 24-well tissue-culture plates with 3  $\mu\text{m}$  pores. Cells were seeded at a density of  $4 \times 10^4$  cells/well on the inner chamber. After 12 h incubation, cells were fixed and stained with Giemsa stain (Sigma-Aldrich). The number of migrated cells was counted in randomly chosen fields of triplicate chambers by microscope at  $100 \times$  magnification for each sample.

## 2.11 Contractility assay

VSMC and P-SMC were replated onto 0.1% gelatin-coated cover slips. After 12 h, carbachol (Sigma-Aldrich) was added into culture medium at a final concentration of 10  $\mu\text{mol/L}$ . Pictures were acquired at 10 min after treatment. Cell surface area was measured by Image-pro Plus 6.0 software (Media Cybernetics) and the average cell area reduction was calculated.

## 2.12 *In-vivo* teratoma formation

$1 \times 10^6$  unP-SMC in a 0.1 mL 50 % matrigel suspension was subcutaneously injected into left hind limb of syngeneic Sv/129 host, and the other  $1 \times 10^6$  P-SMC into right hind limb ( $n = 6$ ). After 4 weeks of incubation, teratoma formation was recognized with the naked eye.

## 2.13 Statistical analysis

Data are presented as mean  $\pm$  SD of three independent experiments unless otherwise stated. Data analyses were performed using SPSS 18.0 software. Differences between two groups were analyzed by two-tailed Student's *t*-test and significant differences are indicated as  $P < 0.05$ .

# 3 Results

## 3.1 Production of high purity SMC by puromycin selection

The vector pSM22 $\alpha$ -puro<sup>r</sup>-IRES2-EGFP was successfully constructed and confirmed by DNA sequencing (Fig-

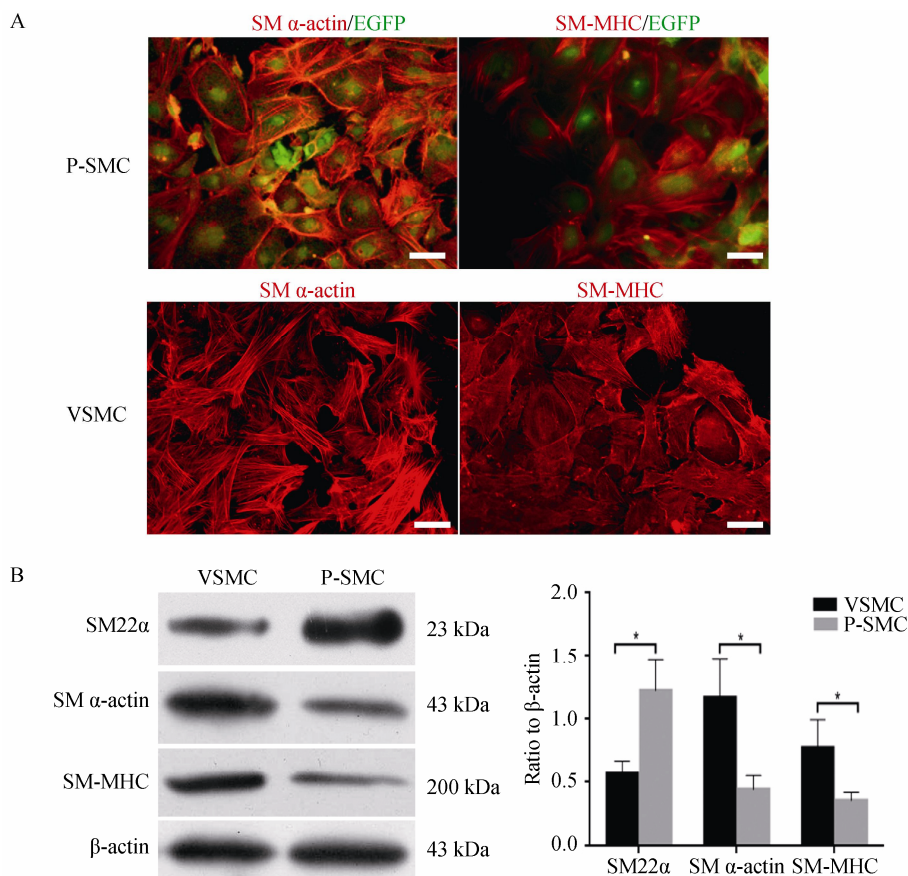
ure 1A, data not shown). The Tg-ESC line, expressing puro<sup>r</sup> gene and EGFP driven by SM22 $\alpha$  promoter, was also established by G418 selection for two weeks, and identified by detection of 308 bp puro<sup>r</sup> gene fragment (Figure 1C) in its genome. These Tg-ESC were positive for ALP staining, SSEA-1 staining (Figure 1D), and were capable of forming teratoma containing structures from three germ layers, including ectoderm (neural tissue and epidermis), mesoderm (cartilage and muscle) and endoderm (intestinal epithelium) (Figure 1E). Following the *atRA* induction protocol (Figure 1B), considerable amounts of EGFP positive SMC were induced and outgrew from the center of the attached EB (Figure 1D, day 10). Although most of these SMC located peripherally to the EB center, some non-EGFP expressing colonies with typical morphology of undifferentiated ESC could still be seen among them (Figure 1F, day 10, indicated by arrow). After additional three days of puromycin selection, EGFP positive P-SMC (Figure 1F, day 13 (P-SMC)) with similar morphology to VSMC (Figure 1F, VSMC) were obtained. Flow cytometry analysis showed that P-SMC had 98.20% EGFP<sup>+</sup> cells, which is much higher than 29.47 % of unP-SMC without puromycin selection (Figure 1G).

## 3.2 P-SMC expressed smooth muscle specific markers

To characterize the pure population of P-SMC in detail, we further underwent immunofluorescence and immunoblotting to detect the expression of smooth muscle specific markers. The immunofluorescence study showed that, in addition to EGFP expression, P-SMC displayed similar staining pattern of SM  $\alpha$ -actin and SM-MHC to that of VSMC. But compared to VSMC, staining of P-SMC was less intense, especially for SM-MHC (Figure 2A). Immunoblotting showed that SM22 $\alpha$  expression of P-SMC was significantly higher than that of VSMC (Figure 2B). This was not surprising because P-SMC was purified relying on puro<sup>r</sup> gene expression driven exclusively by SM22 $\alpha$  promoter. So, all P-SMC were supposed to be SM22 $\alpha$  positive, while VSMC were not. Expression of SM  $\alpha$ -actin and SM-MHC of P-SMC were significantly lower than those of VSMC (Figure 2B).

## 3.3 P-SMC displayed improved proliferation, accelerated migration, decreased contractility and no tumorigenic potential

Functions of P-SMC including proliferation, migration and contraction were also analyzed. Growth curve assay showed that P-SMC had stronger proliferative ability than VSMC. There was no difference between P-SMC and



**Figure 2. P-SMC expressed smooth muscle specific markers.** (A): P-SMC immunostained positively for SM  $\alpha$ -actin (red) and SM-MHC (red) in addition to EGFP expression (green). But the staining of the two markers is less intensive compared with VSMC. (B): Immunoblotting showed P-SMC expressed significantly higher level of SM22 $\alpha$ , but lower level of SM  $\alpha$ -actin and SM-MHC than VSMC. Scale bars, 100  $\mu$ m. \* $P < 0.05$ . P-SMC: purified SMC; SM-MHC: smooth muscle myosin heavy chain; VSMC: vascular SMC.

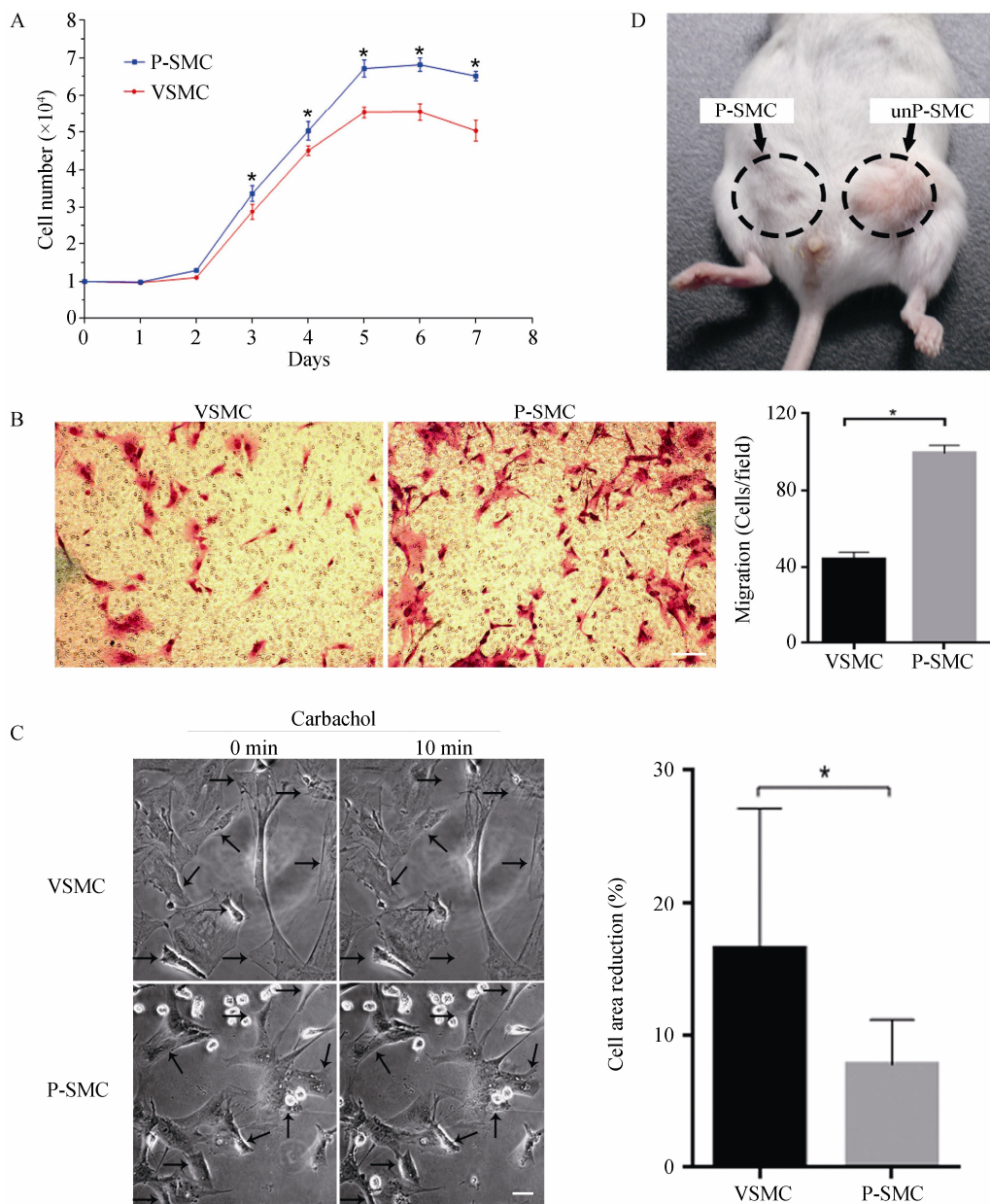
VSMC in cell numbers in the first two days (Figure 3A). But from day 3 to day 7, cell numbers of P-SMC was much higher than that of SMC ( $P < 0.05$ ). Also, P-SMC presented with an attenuated declining trend of growth rate after day 5 (Figure 3A). Transwell migration assay showed that P-SMC had improved migration activity compared with VSMC (Figure 3B,  $99.33 \pm 2.04$  vs.  $44.00 \pm 2.08$  migrated cells/field,  $P < 0.05$ ). For contractility assessment, P-SMC contracted less rigorously than VSMC in response to cholinergic agonist, carbachol (Figure 3C,  $7.75\% \pm 1.19\%$  vs.  $16.50\% \pm 3.76\%$  in cell area reduction,  $P < 0.05$ ).

In addition, subcutaneous injection of unP-SMC resulted in large teratoma *in vivo*, while injection of P-SMC showed no teratoma development.

## 4 Discussion

Differentiation of diverse cell types together with SMC in the ESC system makes it difficult to analyze mechanisms responsible for SMC differentiation, or to use them in vas-

cular regeneration. For this reason, it is crucial to obtain pure SMC populations. Three strategies have been frequently used to purify target cells from ESC derivatives: (1) fluorescence-activated cell sorting (FACS) based on surface marker labeling, or tissue-specific, promoter-controlled, fluorescence protein expression;<sup>[11]</sup> (2) magnetic activated cell sorting (MACS) based on surface marker labeling;<sup>[12]</sup> and (3) drug selection based on tissue-specific, promoter-controlled, antibiotic resistance gene expression.<sup>[13]</sup> FACS sorting has the advantage of high-purity sorting of ESC derived target cells. However, the cells suffered great injury during long period of sorting. MACS have recently been shown to be insufficient to meet the purification needs for cell therapy.<sup>[12]</sup> In our study, we developed a purification method by use of puromycin selection. Tg-ESC expressed the puromycin resistance gene and EGFP under the control of SM22 $\alpha$  promoter. Through three days of selection, we obtained high purity SMC confirmed by flow cytometry. This purification strategy was proven to be easy and efficient in our study. Furthermore, the co-expressed EGFP in



**Figure 3. P-SMC showed increased proliferation, migration and decreased contractility.** (A): Growth curve showed that P-SMC had increased proliferative ability than VSMC. (B): Transwell assay showed that P-SMC migrated more than VSMC per 100 $\times$  high magnification field ( $99.33 \pm 2.04$  vs.  $44.00 \pm 2.08$ ,  $P < 0.05$ ). (C): P-SMC displayed contractile ability in response to carbachol treatment. P-SMC contracted less than VSMC measured by cell surface area reduction ( $7.75\% \pm 1.19\%$  vs.  $16.50\% \pm 3.76\%$ ,  $P < 0.05$ ). (D): Subcutaneous injection of unP-SMC into left hind limb of syngeneic mouse resulted in large teratoma, while P-SMC into right side showed nothing. Scale bar, 20  $\mu$ m. \* $P < 0.05$ . P-SMC: purified SMC; unP-SMC: unpurified SMC; VSMC: vascular SMC.

P-SMC can serve as a tracing marker when being utilized *in vitro*, or *in vivo* for future studies.

Purification of ESC derived SMC driven by SM  $\alpha$ -actin and SM-MHC promoters have been reported.<sup>[13,14]</sup> However, it is well known that SM  $\alpha$ -actin is not a definitive SMC lineage marker as it also being expressed in a wide variety of non-SMC cell types, including skeletal and cardiac mus-

cle during normal development,<sup>[15]</sup> fibroblasts,<sup>[16]</sup> endothelial cells,<sup>[17]</sup> and various tumor cells.<sup>[18]</sup> In this study, we employed another SMC specific SM22 $\alpha$  promoter to purify SMC populations from mixed cell types derived from ESC. SM22 $\alpha$  is a marker exclusively expressed in visceral and vascular SMCs.<sup>[19]</sup> Compared to other SMC-specific proteins such as SM  $\alpha$ -actin and SM-MHC, SM22 $\alpha$  offers ad-

ditional advantages as a differentiation marker due to its high specificity, small size, and high efficiency.<sup>[20]</sup> Using a series of promoter deletions, a 441 bp<sup>[19]</sup> and 445 bp<sup>[21, 22]</sup> region, encoding a 59-flanking sequence in the murine SM22 $\alpha$  promoter, has been shown to be necessary and sufficient to drive high-level transgene expression in SMC lineages. In the present study, a SM22 $\alpha$  promoter with 541 bp was cloned from mouse genome DNA and proved to be effective, as indicated by the high purity of EGFP expressing SMC.

Contractile (differentiated) and synthetic (de-differentiated) SMC, representing the two ends of a spectrum of SMC with intermediate phenotypes, clearly show different morphology and functions.<sup>[23]</sup> The P-SMC derived from ESC in our study seemed to have the contractile phenotype, as they displayed spindle-shaped morphology, expressed lineage specific proteins including SM22 $\alpha$ , SM  $\alpha$ -actin and SM-MHC, and contracted in response to carbachol exposure. But compared to VSMC from adult mice, P-SMC also seemed to be less mature due to their improved proliferation, increased migration, decreased expression level of lineage specific markers, and impaired contractility. This less mature phenotype of P-SMC population is of importance in clarifying the mechanism underlying SMC development, as it could model SMC maturation process. The improved proliferative activity is also beneficial in generating more seeds for engineered vessel grafts.<sup>[24]</sup> Recently, Tang *et al.*<sup>[25]</sup> reported that differentiated VSMC are incapable of switching to de-differentiated phenotype either *in vivo*, or *in vitro*. It is activation and differentiation of multi-potential vascular stem cells, instead of SMC de-differentiation, that results in the proliferative and synthetic cells in the vascular wall. Their findings challenge the widely accepted dogma of “phenotypic modulation” existing for almost 50 years.<sup>[26]</sup> P-SMC developed in our study showed accelerated proliferation, indicating the possibility that they might still have some stem cell characteristics. Detailed lineage tracing studies by combining use of multiple lineage specific markers to accurately define SMC population are badly needed in the future investigation. Moreover, P-SMC showed no tumorigenic potential compared to unP-SMC, which developed large teratoma *in vivo*. This result highlighted the necessity of obtaining pure population of cells originated from stem cells, and implied that p-SMC in our study might be more safer and appropriate for tissue engineering.

In conclusion, we successfully developed an easy and efficient method to obtain pure population of SM22 $\alpha$  positive SMC derived from ESC. The P-SMC displayed less mature (differentiated) phenotypes, which will be of great use in disclosing mechanisms underlying SMC develop-

ment and SMC related diseases, and in application of bio-engineered vascular grafts for regenerative medicine.

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