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An Adult Myometrial Pluripotential Precursor that Promotes Healing of Damaged Muscular Tissues

BEATRIZ G. GÁLVEZ[1], NURIA SAN MARTÍN[1], PATRICIA SALAMA-COHEN[1], JUAN JOSÉ LAZCANO[1], MARÍA JOSÉ CORONADO[1], MARÍA L. LAMELAS[2], ALBERTO ÁLVAREZ-BARRIENTES[1], NOEMÍ EIRÓ[1], FRANCISCO VIZOSO[2] and CARLOS RODRÍGUEZ[1]

[1]*Projech, Science to Technology, Gijón, Spain;*
[2]*Department of Gynaecology and Investigation, Hospital de Jove, Gijón, Spain*

Abstract. *The use of adult stem cells for tissue and organ regeneration constitutes a promising alternative therapy in many human diseases that are currently not treatable. We have isolated a new cell type from mouse adult uterine biopsies (murine adult myometrial precursors or mAMPs) by means of using a simple and non-invasive approach. These cells have been characterized by surface markers, being positive for CD31, CD34, CD44, CD117, Stro-1 and Sca-1. A similar cell population (hAMPs) was isolated from human biopsies. AMPs can differentiate in vitro into a number of mesodermal (smooth and skeletal muscle, osteoblasts and adipocytes) as well as epidermal lineages (all neural lineages). AMPs are unusual adult stem cells as they still express some embryonic antigens and remain undifferentiated through a high number of passages before entering senescence. Importantly, when injected into animal models of muscular disease, AMPs can regenerate new muscle fibers, and promote functional muscular recovery. Moreover, these cells can regenerate the uterine lining after wound healing, reconstructing the uterine muscular architecture. In addition, these cells can form new vessels both in vitro and in vivo. We believe that these cells have superior features to other known adult stem cells and, consequently, their use holds great promise for regenerative medicine, drug development and basic research.*

Stem cells are defined by two main features: i) they are able to self maintain a stable undifferentiated population and ii) they are pluripotential to variable degrees. The term stem cell has been applied to a growing number of cells isolates both from embryonic and adult (*i.e.* post-partum) tissues (1).

Correspondence to: Carlos Rodríguez, MD, Ph.D., Projech, Science to Technology, Avenida Eduardo Castro s/n 33290 – Gijón, Spain. Tel: +34 650311449, e-mail: carlosr@projech.com, jose.schneider@urjc.es

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In the last decade, the choice of using adult stem cells over embryonic stem (ES) cells in regenerative medicine has gained support. A number of reasons account for this ever-growing trend among the regenerative medicine community: it is complicated to control the culture conditions of ES cells without inducing their differentiation; ES cells must go through several intermediate stages before reaching terminal differentiation; the potential therapeutic use of ES has been hindered by safety issues and ethical scrutiny; and finally, cells derived from ES cells are usually rejected by the immunological system since they are allergenic to the recipient. On the contrary, adult stem cells are not rejected by the immune system (2). Furthermore, the fact that they are partially compromised in their differentiation pathway reduces the number of differentiation stages necessary to generate specialised cells. In addition, the use of this type of cells is not associated with any type of legal or ethical controversy. Moreover, although they possess less differentiation potential than ES cells, most of them are really multipotent and can even express some embryonic genes. Indeed, the transcription factor Octamer-4 (Oct-4), important for the maintenance of pluripotency in ES cells, has been shown to be expressed by a variety of adult stem cells (3).

It is widely held that stem cells are slowly cycling but highly clonogenic. Indeed, in many tissues, stem cells divide less frequently than their progeny (4). Stem cells are also defined by their ability to give rise to more stem cells, as well as to cells that differentiate. This is thought to be achieved in a single step through asymmetrical division (5). Symetrical division, instead, provides a mechanism to increase the stem cell population (6).

Different stem cells have already been described in the endometrial layer of the uterus (7-10). These cells are multipotent and can give rise to some of the adult tissues, in particular, to hematopoietic lineages (11), and, in some cases, to the development of uterine tumours and endometriosis (12-14). In a recent paper, a cell population from human myometrium has been reported to be a good candidate for a true myometrial stem cell. These cells can be

isolated owing to their ability to expel the Hoechst 33342 dye (side population) and can reconstitute the myometrial wall in overiectomized mice (15).

Between the diseases to be treated by stem cells therapies, muscular dystrophies primarily affect skeletal muscle, causing fibre degeneration and progressive paralysis(16). Heterologous transplantation of adult mesenchymal stem cells has been shown to induce dystrophin synthesis and partial rescue of the pathology in dystrophic mice (17,18). Bone marrow (BM)-derived cells (BMCs) have also demonstrated a limited myogenic tissue remodelling capacity (19). It is therefore in the greatest interest of patients to assess any possible new human cell source that could represent a candidate for treating muscular diseases.

In this paper, we describe a new stem cell type from normal mouse and human myometrium. We report the broad differentiation potential of these cells and, particularly, their ability to restore muscle anatomy and function.

Materials and Methods

Ethics statement. C57 mice were obtained from Charles and River, Co. and maintained and used in accordance with the National Institutes of Health Animal Care and Use Committee. Patients were treated according to the guidelines used in the Fundación Hospital de Jove. The study adhered to national regulations and was approved by this institution Ethics and Investigation Committee. All patients included in the study have signed a written informed consent.

Uterine explants. Myometrial explants were taken from the lower uterine segment by uterine exfoliation, in mouse and in healthy patients. Both, human and mouse, adult myometrial precursors (AMPs) were obtained from those uterine explants. Briefly, myometrial tissue pieces (10-30 mg) obtained from 4-month-old C57 or green fluorescent protein (GFP)-C57 mice or from human myometrial tissue samples, similar to those obtained for a cervical cytology, were kept in DMEM without fetal calf serum (FCS), with antibiotics. Each sample was then rinsed in phosphate-buffered saline (PBS) with Ca^{++}/Mg^{++} and trimmed of endometrial, serosal, fat and fibrous tissue. Fragments containing small vessels were transferred to a Petri dish coated with 1% gelatin in the presence of 10% FBS-DMEM plus antibiotics. These fragments were cultured for 7 to 15 days and after the initial outgrowth of fibroblast-like cells, small, round, refractile cells appeared. This cell population was easily collected by gently pipetting of the original culture, and was counted and cloned by limited dilution on 1% gelatin-coated 96 well plate. Different clones were selected by phase-contrast morphology and then characterized by the expression of surface markers.

Analysis of cell proliferation. Cells (mAMPs and hAMPs) were plated initially at a density of 1×10^4 cells/cm^2 in complete DMEM and passed on average every three days. At each passage, the number of cells was counted in triplicate in a hemocytometer.

Differentiation assays. Cultures were transferred to differentiation medium (DMEM supplemented with 2% horse serum). Differentiation into smooth muscle cells and osteoblasts was induced by treatment with transforming growth factor-beta 1 (TGFβ1) and bone morphogenetic protein 2 (BMP2) respectively, as previously described (20). Differentiation into skeletal muscle cells was induced by co-culturing mAMPs with myoblasts.

Differentiation into neural cells consist on changing the culture medium to a neural stem cell proliferation medium: DMEM:F12 medium (Sigma, Saint Louis, MO, USA) supplemented with 4.5 mg/ml D-Glucose (Sigma), N2 Supplement, B27 Supplement (both from Gibco-Invitrogen, Grand Island, NY, USA), 20 μg/ml insulin, 2 μg/ml heparin, 20 ng/ml basic fibroblast growth factor (bFGF), 10 ng/ml epidermal growth factor (EGF) (all from Sigma). Further, cells were grown for another week in neural stem cell differentiation medium (DMEM:F12, supplemented with 4.5 mg/ml D-Glucose, N2 Supplement, B27 Supplement, 2 μg/ml heparin and 1% FBS and for another week in specific medium for neuronal culture (Neurobasal-A (Gibco-Invitrogen,), B27 Supplement, Glutamax-I (Gibco-Invitrogen), penicillin-streptomycin solution (Sigma) and oligodendrocyte differentiation (DMEM, 4.5 mg/ml D-glucose, 100 μg/ml bovine serum albumin (BSA), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 60 μg/ml *N*-acetyl-L-cysteine (all from Sigma), N2 Supplement, 20 ng/ml bFGF and 10 ng/ml platelet derived growth factor-AA (PDGF-AA) (PeproTech, Rocky Hill, NJ, USA). After this time, the cells were processed for immunocytochemistry.

In all cases, cells were photomicrographed under a Nikon Elipse TI fluorescence microscope. Identification of nuclei was confirmed by Hoechst. Percentage of differentiation was calculated by counting the number of differentiated mAMPs, with respect to the total cell number.

Immunofluorescence. Cells were grown on gelatin-coated glass coverslips, washed with PBS and fixed with 4% paraformaldehyde for 10 minutes. Samples were frozen in liquid nitrogen-cooled isopentane and serial 8 μm-thick sections were cut with a Leyca cryostat. Cells were permeabilized with 0.2% Triton X-100, 1% BSA in PBS for 30 minutes at room temperature (RT), while tissue sections were incubated without detergent. Cells and tissue sections were incubated with 10% donkey serum for 30 minutes a RT, and incubated overnight at 4˚C with primary antibodies at the appropriate dilution. After incubation, samples were washed twice with the permeabilization buffer and then incubated with the appropriate FITC or Alexa 488 conjugated anti-mouse or anti-rabbit IgG and Hoechst 33342 for 45 minutes at RT. After three final washes, the coverslips were mounted on glass slides using mowiol (Polysciences, Inc., Eppelheim, Germany) in PBS and analyzed under a fluorescent microscope (Nikon).

Antibodies. The following antibodies were used: anti-laminin monoclonal or polyclonal antibodies at 1:100 dilution; MF20 antibody at 1:5 dilution; anti smooth alpha actin 1:300; microtubule-associated protein 2 (MAP2) as neuronal marker; and glial fibrillary acidic protein (GFAP) as astrocyte marker (all from Sigma); polyclonal anti-nestin antibody, beta-III-tubulin, anti-TUJ1 antibody and anti-doublecortin (anti-Dcx) antibody (all from Abcam, Cambridge, UK); anti-receptor interacting protein (RIP) (Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA) as oligodendrocyte marker.

For flow cytometric analysis, a CyAn ADP (Beckman Coulter, Inc. Fullerton, CA, USA) was used with the following antibodies: CD44, CD34, CD45, CD73, CD117, CD133 from BD Biosciences (San Jose, CA, USA) CD31, CD13, from ID labs Inc (London,

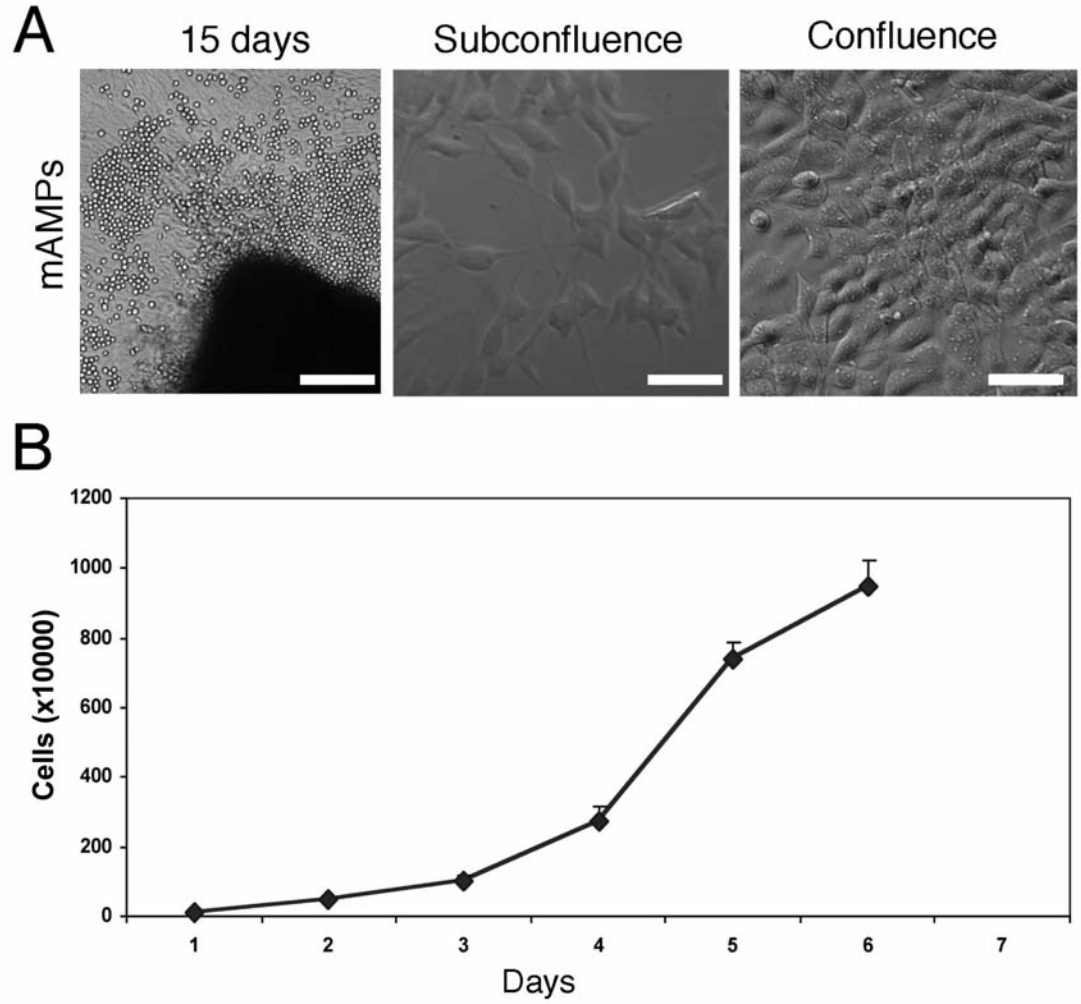


Figure 1. *Characterization of mouse adult myometrial precursors. A: Phase-contrast morphology of mAMPs from the explants 15 days after plating. Bar=25 μm. B: Representative growth curve for the different myometrial precursors. Bars represent standard deviation of the mean for each time point.*

Ontario, USA), CD146 from BioCytex (Marseille, France), CD80, CD90, stage-specific embrionic antigen 4 (SSEA-4), stem cell antigen-1 (Sca-1), Stro-1, TRA1-60 and TRA1-81 (tumor rejection antigen 1) from Miltenyi Biotec (Bergisch-Gladbach, Germany).

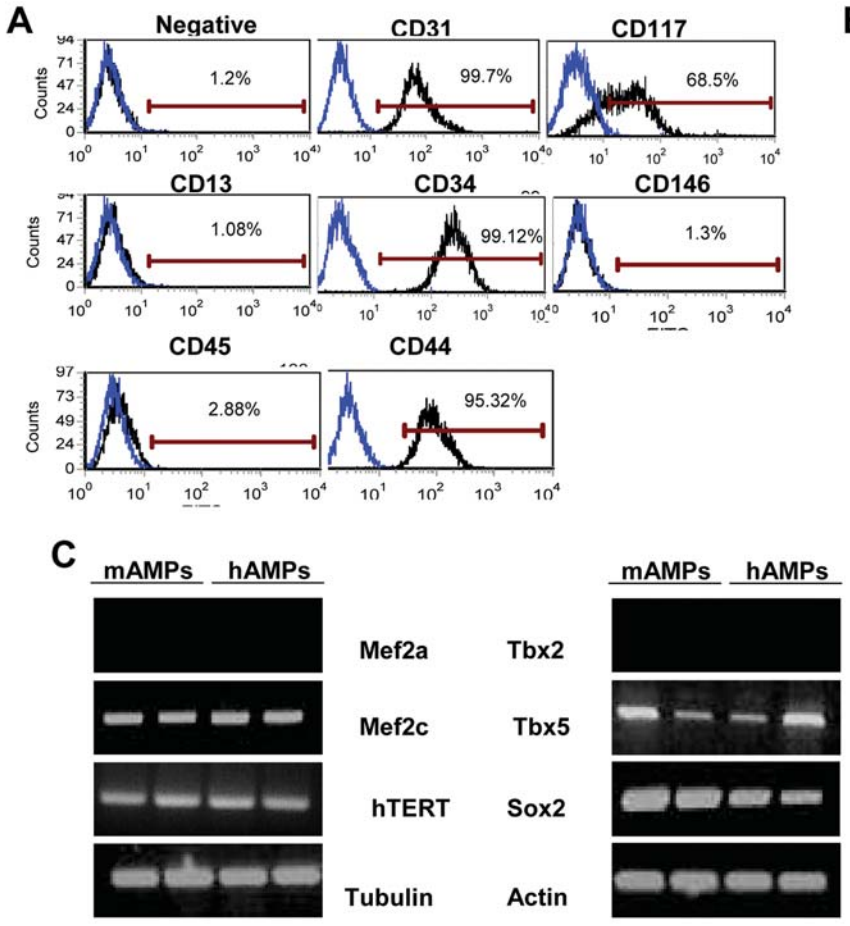
An alkaline phosphatase (ALP) detection kit (Millipore Co., Billerica, MA, USA) was used to test the expression of ALP according to the manufacturer's protocol. mAMPs were grown for 5 days in 24-well plates, fixed with 4% paraformaldehyde and 90% methanol/10% formaldehyde for 2 minutes, and washed with rinse buffer (20 mmol/l Tris-HCl, pH 7.4, 0.15 mmol/l NaCl, 0.05% Tween-20 [TBST]). The substrate solution was prepared by adding fast red violet (FRV) with naphthol AS-BI phosphate solution and water in a 2:1:1 ratio (FRV:naphthol:water). The substrate solution was added to each well and incubated in dark at room temperature for 15 minutes. Following this, the plates were washed with TBST and the number of violet cells was counted under microscope.

Gene expression analysis. RNA was extracted from the different mAMP clone cells while growing. Reverse-transcriptase PCR (RT-PCR) was performed for analysing the expression of different genes involved in development or differentiation previously described by other groups (myocyte enhancer factor 2C (*Mef2c*), SRY (sex determining region Y)-box 2 (*Sox2*), T-box 5 (*Tbx5*), human telomerase reverse transcriptase catalytic subunit (*hTERT*), myocyte enhancer factor 2A (*Mef2a*) and T-box 2 (*Tbx2*)).

The conditions for the PCR were general for all primers: 94˚C for 4 minutes, 30 cycles of 94˚C for 45 s; 55˚C for 45 s; 72˚C for 45 s and a final step of 72˚C for 10 min. The sequences of the primers are listed in Table I. *mTERT*: human/mouse *TERT* primer pair (R&D systems).

RNA was extracted from the different mAMP clone while growing or after differentiation with different media. RT-PCR was performed for analyzing the expression of different genes involved in differentiation. The sequences of the used primers are also listed in Table I.

Angiogenesis. The surface of 24-well plates was coated with the basement membrane Matrigel (400 μl/well; 10 mg/ml). Matrigel was then allowed to polymerize at 37˚C for 30 min. Both mAMPs



Marker	%Positive cells
CD13	<1
CD31	99.7
CD34	99.12
CD44	95.32
CD45	<1
CD80	2.88
CD90	11,76
CD117	68.5
CD133	<1
CD146	<1
HLA-DR	28.91
TRA1-60	<1
TRA1-81	<1
SSEA-4	58.59

Figure 2. *Analysis of surface marker expression on mAMPs. A and B: Surface markers expression analyzed by flow cytometry. Flow cytometric analysis using a panel of antibodies: CD13, CD31, CD34, CD44, CD45, CD73 CD80, CD90, CD117, CD133, CD146, PAL, HLA-DR, TRA1-60, TRA1-81, SSEA-4, Stro-1 and Sca-1. C: Gene expression of mAMPs and hAMPs as analyzed by PCR.*

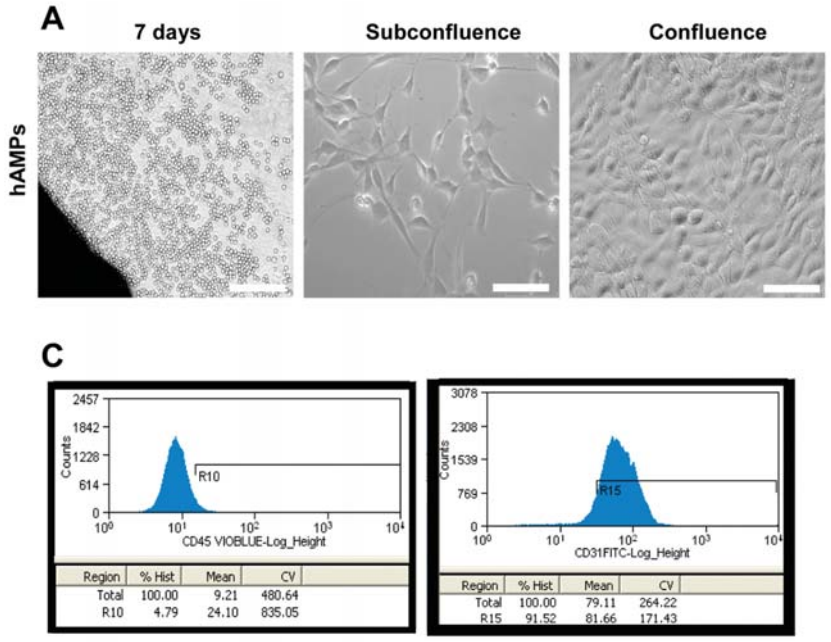
and hAMPs were seeded, separately, on Matrigel-coated plates in the presence of complete DMEM (10×10^3 /well). After a 6 h incubation at 37˚C in a 5% CO_2 incubator, the wells were photographed and the number of vessels was counted under an inverted microscope. *In vivo* angiogenesis assays consist of injection of 25×10^3 GFP-mAMPs into the myometrium wall of C57 female mouse. Uterine tissue was collected one week later and immunohistology was performed on those samples.

In vivo regeneration model for damaged skeletal muscle. All mice were on a C57 background. The age range of experimental animals was between 2 months and 6 months. Control and disease animals were matched by age and gender. Muscle injury was performed in mice anesthetized by metofane inhalation. Tibialis muscle was injected with 50 ng/ml cardiotoxin (Sigma), for 48 h and afterwards, 5×10^5 allogeneic GFP-mAMPs were injected intramusculary. Muscles were collected after one month and analyzed for the presence of GFP by RT-PCR and the differentiation potential was analyzed by immunofluorescence. Functional recovery was analyzed by the running trail, we compared the mobility and running speed between mAMPs-injected and non-injected mice.

Wound healing. Uterine wall damage was performed with scrapers in ten 2- to 6-month-old C57mice; 48 h after the wound, mice were sacrified. The uterine wall was sectioned and stained with myosin peroxidase antibody in order to localize the myometrial muscle. The presence of myometrial precursors in the wound healing was then determined with the detection of alkaline phosphatase positive cells (ALP^+ cells). Images were taken under an inverted microscope (Nikon).

Results

Isolation and *in vitro* expansion of cells from primary uterine biopsies. As mentioned above, biopsy samples of mouse uterus were dissected under the microscope; fragments of vessels and surrounding mesenchymal tissue were dissected and plated on gelatin-coated dishes as previously described for other cell types (18, 20, 21). These samples were cultured for 15 days and after the initial outgrowth of fibroblast-like cells, small round and refractile cells appeared (Figure 1A, left panel). These cells (mAMPs) adhered poorly to the substratum and



Marker	% Positive cells
CD45	< 5
CD34	< 1
CD31	>90%
CD44	>90%
CD90	>70%
CD105	>50%
CD133	< 1
CD117	< 1
TRA1/60	< 1
TRA1/61	< 1
SSA4	>90%

Figure 3. *Isolation of hAMPs. A: Phase-contrast morphology of human myometrial precursors from the explant after 7 days. Bar=5 μm. B and C: Expression of surface protein markers.*

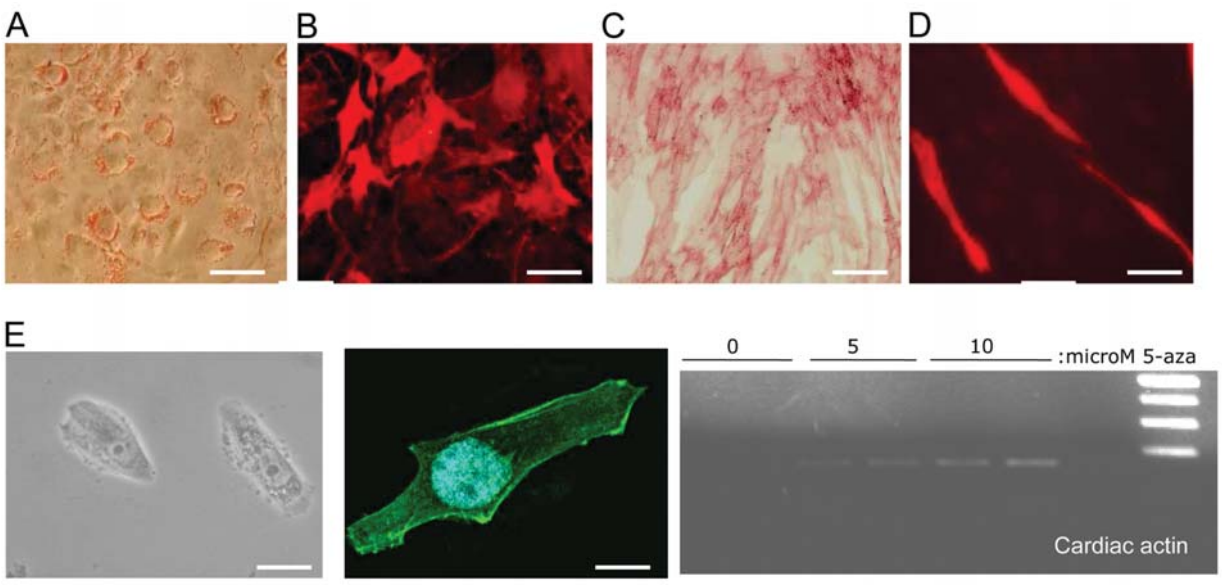


Figure 4. *In vitro differentiation of mAMPs. mAMPs differentiated into: A: Adipocytes, stained with oil red, bar=25 μm. B: Smooth muscle stained with alpha-smooth actin antibody (red), bar=50 μm; Nuclei were stained in blue with Hoechst. C: Osteocytes stained with alizarin red, bar=100 μm. D: Skeletal muscle stained with myosin (red), bar 50 μm; Nuclei were stained in blue with Hoechst. E: Cardiomyocytes observed by light microscope (left panel) or stained with cardiac actin (green, central panel), bars=10 μm and 5 μm, respectively. Right panel: RT-PR for cardiac actin of mAMPs cultivated for 48 h with 5 or 10 μM 5-azacytidine.*

were thus collected by gentle pipetting. Floating cells were either grown as a polyclonal population or, in some cases, cloned by limited dilution. The large majority of the cells in the population acquired a triangular shape (Figure 1A, central and right panel) and maintained a high proliferation rate for approximately 40 passages with a doubling time of approximately 36 hours in a culture medium consisting of DMEM+10% FBS+5 mM glutamine with antibiotics (Figure

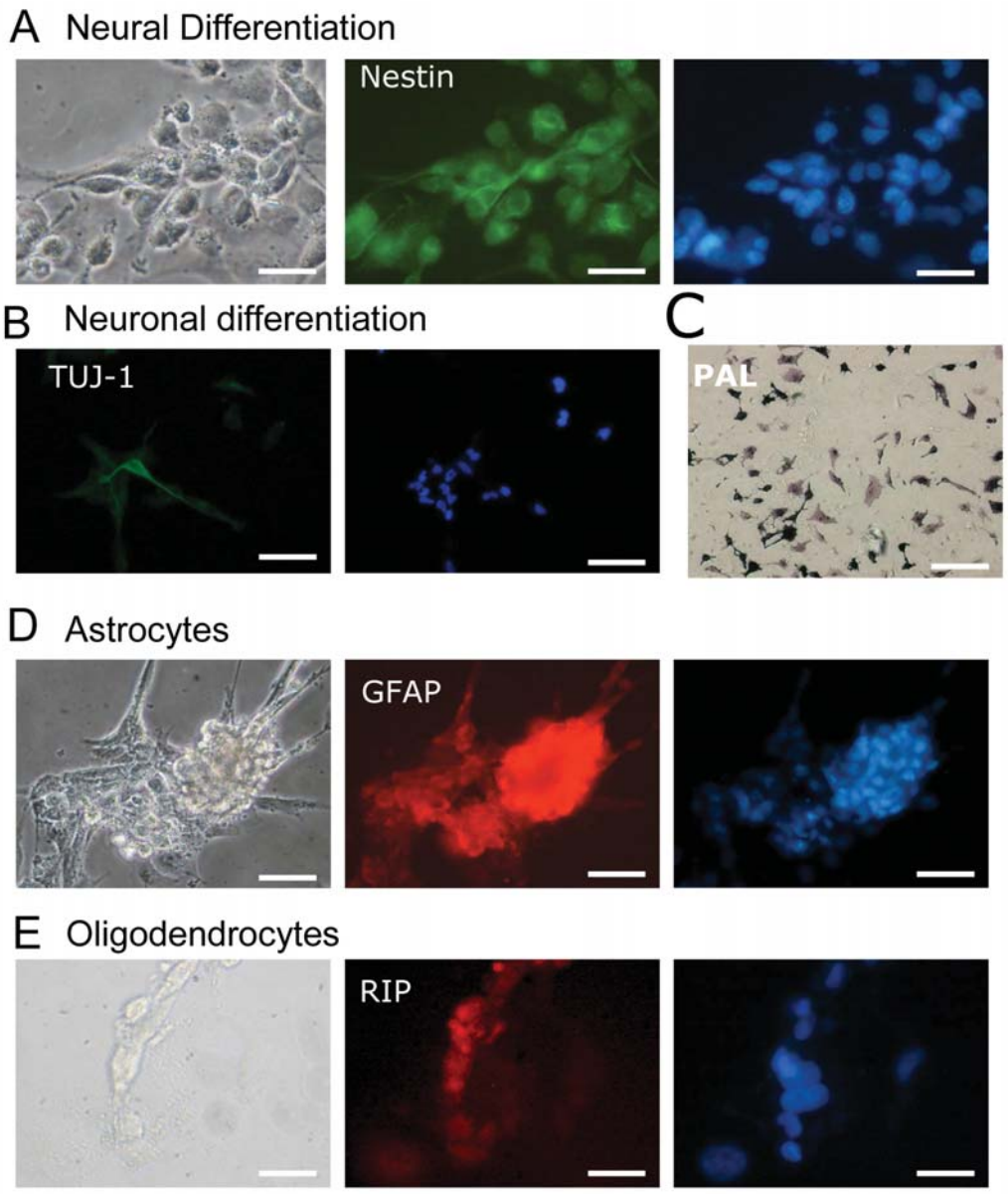


Figure 5. *Neural differentiation of mAMPs. A: mAMP immunostaining for nestin after one week in culture in neural stem cell proliferation medium. Bar=50 μm. B, D and E: Markers of mAMPs after culture in neuronal proliferation medium. Phase-contrast (left panel), primary antibody (middle panel) and nuclei (right panel). Bar=50 μm, 100 μm and 50 μm, respectively. C: Alkaline phosphatase reaction on mAMP monolayer. Bar=100 μm.*

1B). The cell proliferation rate was largely independent of mouse age (ranging from 2 to 6 months) and, on average, led to a final number of approximately 3×10^9 cells per mouse. This number of cells would be suitable for injections. Interestingly, both mAMPs and hAMPs have symmetrical division which allow them to proliferate quickly.

To test for tumorigenicity, 10^7 mAMPs were injected subcutaneously into SCID/beige mice. Ten injected mice were maintained up to 6 months after the injection and none of them developed any visible tumour that could be detected macroscopically at autopsy (data not shown).

Phenotype of mAMPs. mAMPs were further characterized by flow cytometry and PCR gene expression, as well as by their ability to differentiate into different cell types.

mAMP clones were analyzed by flow cytometry for cell surface expression of stem cell markers. All clones were positive for CD31 (endothelial lineage marker), CD34, CD44 and Stro-1

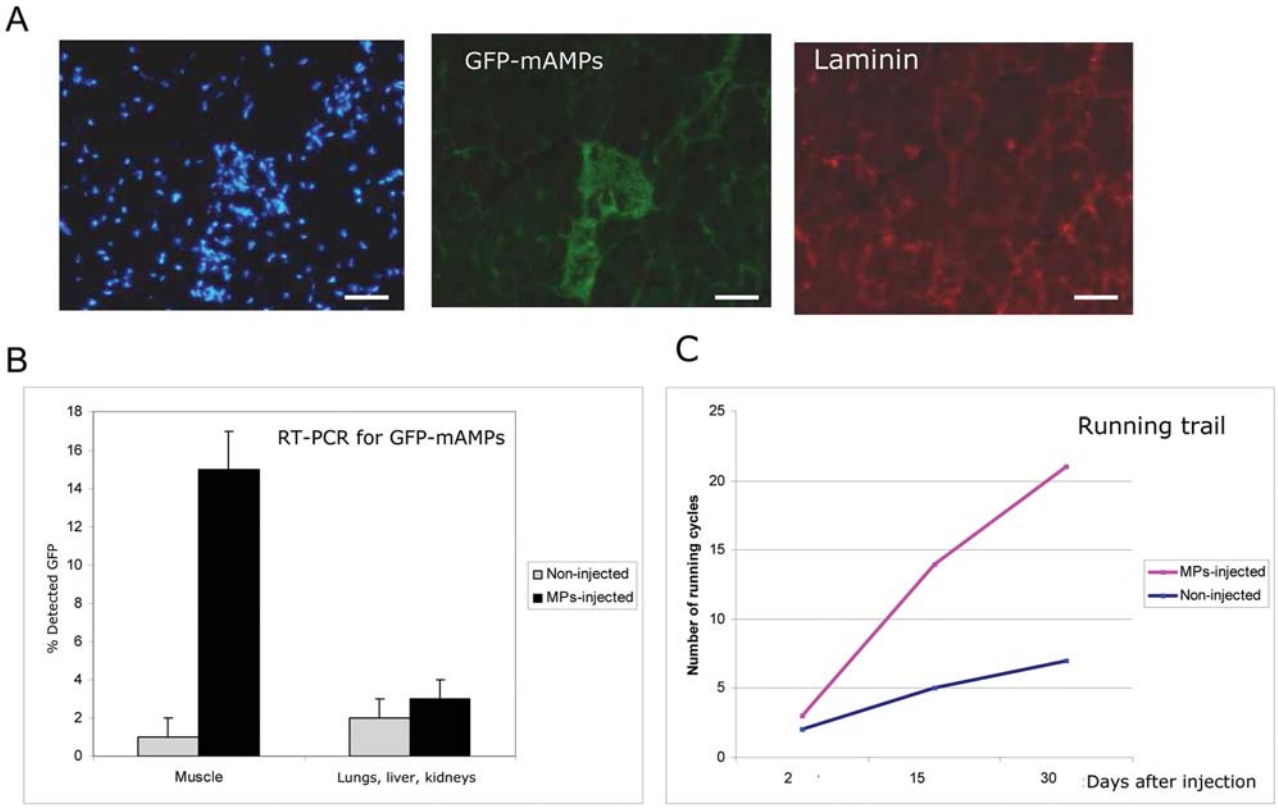


Figure 6. *In vivo muscle regeneration. A: Immunofluorescence in situ showing the localization and differentiation (laminin staining in red) of GFP-mAMPs injected into muscle fibers. Bar=200 μm. B: Graph showing the percentage of GFP-mAMPs found inside the damaged muscles. C: Functional recovery of atrophic muscle in mice after mAMP injection.*

(mesenchymal stem cell markers), SSEA-4 (embryonic stem cell marker), CD117 and Sca-1 (broad stem cell markers) and HLA-DR (inflammatory cell marker) and negative for CD13, CD45, CD73, CD80, CD133 and CD146 (all of which are haematopoietic cell or lineage markers), TRA1-60 and TRA1-81 (tumor rejection antigen 1; embryonic stem cell and tumorogenic cell markers) (Figure 2A and 2B). Therefore, AMPs are clearly stem cells of mesenchymal lineage that still maintain some embryonic and endothelial makers.

An RNA expression signature (RES) was defined after RT-PCR analysis of the expression of different genes known for their involvement in development or differentiation, and previously described by other groups. mAMPs were positive for some embryonic genes such as, *Mef2c, Sox2, Tbx5* and *TERT* genes and negative for *Mef2a* and *Tbx2* genes (Figure 2C, two left lines of the gels).

Isolation and in vitro expansion of cells from primary human uterine biopsies. We then looked to apply the same cell isolation protocol to other mammals. We demonstrated that these cells can be obtained by conventional means from human myometrial tissue (Figure 3A). In humans, myometrial tissue samples were obtained from the lower uterine segment by uterine exfoliation, during routine gynaecological sampling for uterine cytology. Most of the samples were obtained from non-pathological post-natal mammalian myometrial tissue. As shown in Figure 3, hAMPs are phenotypically similar to mAMPs, and can be easily isolated following a protocol that is essentially the same that used for mAMP isolation. Interestingly, compared with their mouse counterparts, the time required for the initial isolation is shorter for hAMPs. Indeed, after only seven days, refractile rounded cells were regularly found from the human explants. The hAMP population was then collected and a growth curve estimated (data not shown). Ten out of 99 patients did not yield any hAMPs using this same isolation protocol; interestingly, these samples corresponded to patients who were 50 years of age or more (data not shown). We hypothesize that post-menopausal women may undergo starvation of the pool of hAMPs that are conspicuously present in the myometrium layer in pre-menopausal women.

The pattern of cell surface marker expression was analyzed by flow cytometry and was consistently reproduced in all cases of isolated hAMPs (Figure 3B and 3C). hAMPs were positive for CD31, CD44, CD90, CD105, SSEA-4,

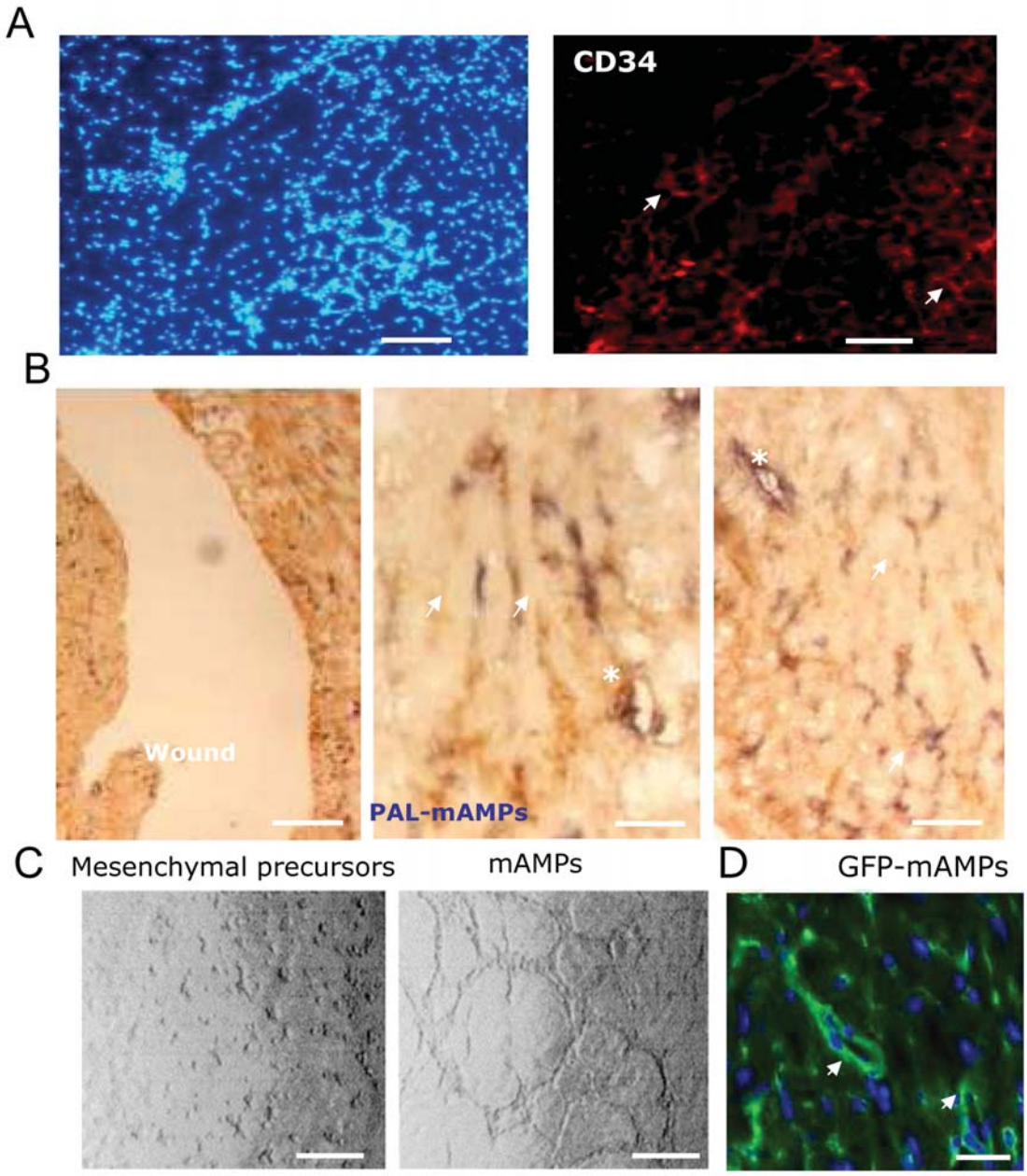


Figure 7. *Niche of AMPs. A: mAMPs (CD34-positive cells) localized around the normal myometrial tissue, in close proximity to vessels (see arrows). Bar=200 μm. B: mAMPs regenerate uterine tissue in the wound healing assay. mAMPs are stained in violet as PAL-positive cells (see arrows for muscle fibers, asterisks for vessels). Bar=150 μm. C: mAMPs are able to generate new vessels. mAMPs were seeded onto matrigel coated-plates and the number of new capillaries was counted after 6 h. Bar=150 μm. D: GFP-mAMPs were localized inside the myometrium, forming new vessels in vivo (see arrows). Bar=150 μm.*

Stro-1 and Sca-1, while they were negative for CD45, CD34, CD73, CD133, CD146, CD117, TRA1/60 and 1/61.

The RT-PCR for hAMPs reproduced the same results as for mAMPs, notably including the expression of *hTERT*, the catalytic unit of human telomerase (Figure 2C, two right lines of gels).

Differentiation potency of mAMPs. To complete the *in vitro* characterization of mAMPs, their potency to undergo terminal differentiation into mesoderm cell lineages was tested. As shown in Figure 4A, mAMPs readily differentiated into adipocytes (with a 60% of red oil-positive cells) and expressed LPL and PPAR-gamma when treated with insulin-

Table I. *Oligonucleotide primer sequences used for RT-PCR analyses in mAMPs.*

Name	Forward primer (5'-3')	Reverse primer (5'-3')
Myocyte enhancer factor 2A (*Mef2a*),	TTGAGGCTCTGAACAAGAAGG	GCATTGCCAGTACTTGGTGG
Myocyte enhancer factor 2C (*Mef2c*),	AACACGGGGACTATGGGGAGAAA	TATGGCTGGACACTGGGATGGTA
T-box 2 (*Tbx2*)	GGTGCAGACAGACAGTGCGT	AGGCCAGTAGGTGACCCATG
T-box 5 (*Tbx5*)	CCAGCTCGGCGAAGGGATGTTT	CCGACGCCGTGTACCGAGTGAT
SRY (sex determining region Y)-box 2 (*Sox2*)	GGCAGCTACAGCATGATGCAGGAGC	CTGGTCATGGAGTTGTACTGCAGG
Lipoprotein lipase (*LPL*)	CTGCTGGCGTAGCAGGAAGT	GCTGGAAAGTGCCTCCATTG
Peroxisome proliferator-activated receptor gamma (*PPAR-γ*)	TCTGGCCCACCAACTTTGGG	CTTCACAAGCATGAACTCCA
Calponin (*Calp*)	GATACGAATTCAGAGGGTGCAGACGGAGGCTC	GATACAAGCTTTCAATCCACTCTCTCAGCTCC
Smoothelin (*Smtn*)	ACGTTGCTGAACCGGCCTGGGCTCT	AGGGGGCAGTATGAAGACTAC
α-Smooth actin (*α-Sma*)	GCATCCACGAAACCACCTA	CACGAGTAACAAATCAAAGC
Osteocalcin (*Osc*)	CAAGTCCCACACAGCAGCTT	AAAGCCGAGCTGCCAGAGTT
Collagen type-I (*Col-I*)	CCAAGCAGTCATGCCTGAT	GACACGGGCATACCTGTTACC
Myosin heavy chain type 2b (*MyHC-Iib*)	TCTGTCACTCGGTGCT TCC	AGGGTTTTTGGAGGCTGTTT

dexamethazone (D). mAMPs were also differentiated into smooth muscle cells (with 90% of α-Sma-positive cells by immunofluorescence) and expressed Calp and higher amounts of α-Sma, when treated with TGFβ; no expression of *Smtn* was detected by RT-PCR (Figure 4B). mAMPs expressed Osc and increased amounts of Col-I after treatment with BMP2; more than 90% of mAMPs were differentiated into osteoblasts and detected as alizarin red-positive cells (Figure 4C). Skeletal muscle differentiation was induced by co-culturing mAMPs with myogenic cells, resulting in a high percentage (more than 25%) of mAMPs fused into hybrid myotubes (Figure 4D); mAMPs differentiated into skeletal muscle (D) also expressed MyHC-IIb by RT-PCR (Figure 4D).

Finally, mAMPs were also able to differentiate into neural tissue after changing to neural stem cell proliferation medium (see Material and Methods). With the neural stem cell proliferation medium around 90% of mAMPs were positive for nestin (Figure 5A). Furthermore, as shown in Figure 5B, D and E, respectively, the cells exhibited positive staining (30%) for neuronal marker Tuj-1, (70%) for the astrocyte marker GFAP, as well as positive staining (30%) for the oligodendrocyte marker RIP. On an additional note, mAMPs were naturally positive for ALP (Figure 5C), a marker usually associated with undifferentiated pluripotent stem cells.

In vivo differentiation and regeneration. GFP-mAMPs were injected intramuscularly into cardiotoxin-damaged muscle (tibialis) of C57 mice. We observed around 15% of muscle fibers formed by the injected GFP-mAMPs (Figure 6A). When muscles were analyzed by immunohistology, mAMPs were found inside the muscle fibers, regenerating new muscle tissue (Figure 6A). After 1 month, mAMPs were detected in all injected muscles by quantative RT-PCR against GFP (Figure 6B). Non mAMPs were detected in liver, lung and kidney. We measured the functional recovery of these mice by the running trail method. mAMP-injected mice had improved their mobility and running speed (Figure 6C).

Niche of MPs. AMPs seemed to be localized around the muscle layer of the myometrium, usually close to the vessels. As shown Figure 7A, AMPs were be localized as CD34-positive cells around capillaries in the uterus. The number of cells was low and distributed throughout the layer under normal conditions.

AMPs can also regenerate uterine tissue *in vivo*. After damaging the uterine wall with scrapers (*in vivo* wound healing assay), PAL-positive myometrial precursors increased in number and were found distributed around the myometrial layer (Figure 7B). As evident in the same figure, myometrial precursors tended to form new muscle fibers and new vessels within the myometrial layer (see arrows and asterisks). Figure 7C provides additional evidence of the angiogenic potential of mAMPs: myometrial precursors, in contrast to other mesenchymal precursors, generated new capillaries *in vitro* when cultured in the presence of an artificial matrix (Matrigel) for 6 h, and *in vivo*, since intra-myometrial injection of GFP-mAMPs generated new vessels *in situ* and contributed to the uterine capillary network (Figure 7D).

Discussion

This work shows the isolation of myometrial precursors from mouse and human adult uterine tissue (mAMPs and hAMPs, respectively). AMPs are unusual adult stem cell precursors in several aspects: i) In contrast with other adult stem cells,

AMPs express some distinctively embryonic antigens in culture and they do not give rise to immortal clones after long-term culture; ii) Cell cultures of AMPs are able to undergo a high number of passages before entering senescence; iii) AMPs are capable of differentiation into different types of mesodermal as well as epidermal tissues; and iv) AMPs divide symmetrically under standard culture conditions.

The latter point is especially interesting since it is well accepted that expansion of stem cells, whether *in vivo* or *in vitro*, requires symmetrical self-renewal. However, with the exception of embryonic stem cells, it has proven extremely difficult to propagate uniform cultures of stem cells *ex vivo*. Recently, however, researchers have had success in the derivation and continuous expansion by symmetrical division of pure cultures of neural stem cells (22). This finding provides an interesting link with our results, particularly considering the fact that AMPs can easily differentiate into neural stem cells.

AMPs constitute a new source of adult stem cells. In recent years, a variety of different types of mesoderm stem cells has been isolated from both mouse and human tissues and characterized to different extents. This includes endothelial progenitor cells (EPCs), multipotent adult progenitor cells (MAPCs), side population cells (SPs), and stem cells from muscle, sinovia, dermis, and adipose tissue. Different experimental procedures, different sources and partial characterization still prevent a complete understanding of the heterogeneity of these cells. Some cell types grow extensively *in vitro* but others such as EPCs and SPs do not; on the other hand, EPCs and SPs can circulate, whereas systemic delivery has not been tested for most of the other cell types. Stem cells isolated from adipose tissue can be grown extensively *in vitro* and differentiate into several tissues. The myoSP population, recently described by Ono and co-workers as Hoechst-selected population in the myometrium (15), expresses CD31 and CD34 markers and can differentiate into mesenchymal cell lineages. In contrast, AMPs (also derived from the myometrial layer) express not only CD31 and CD34 but also other important stem cells markers (such as Sca-1 and Stro-1) and, more importantly, they can differentiate into distinct cell types of the three lineages (endoderm, ectoderm and mesoderm), not only along the osteoblast or the adipocyte lineage (much like myoSPs do), but also into skeletal muscle cells or, especially, into neuron-type cells. We hypothesize that AMPs could therefore represent an earlier stage of myometrial development in comparison with myoSP, a cell type that is already more committed to the mesenchymal lineage. Interestingly, we have proven the potential of mAMPs to generate neural stem cells, as well as cells of the different neural lineages: neurons, astrocytes and oligodendrocytes. Ongoing experiments are addressing their engraftment after transplantation into the adult mouse brain. Whilst long-term stability and functional integration *in vivo* will have to be established in future studies, preliminary data on hAMP cells provide encouragement for this approach.

Gene or cell therapy approaches for muscular dystrophies have no far produced negative or modestly positive results (23). We show here that it is possible to transplant mAMPs into dystrophic animals and obtain extensive reconstitution of fibers expressing dystrophin, improvement in the contraction force and, in many cases, preservation of walking ability. Previous work in the mouse showed that some stem cells can eventually fuse with muscle fibers and contribute to regenerating muscle fibers (24, 25). Extrapolation to humans of these results holds hope that hAMP transplantation would permit muscle regeneration and functional recovery of muscular dystrophy patients. Thus, the work reported here sets the premise for the start of clinical experimentation that may lead to an efficacious therapy for Duchenne muscular dystrophy as well as for other muscular pathologies.

Perspectives for clinical trials. In order to succeed in a cell-based therapeutic strategy, stem cells should comply with a few important criteria: i) Cell expansion may be essential for cell supply. In this regard, AMPs have an intrinsic symmetrical division and, therefore, provide an excellent source of human adult stem cells for therapies involving cell transplantation. ii) In future clinical protocols, systemic delivery appears to be an obligate choice. The myometrial precursors used here express some of the proteins that leukocytes use to adhere to and cross the endothelium. iii) Stem cells should not show signs of immortalization *in vitro* or tumorigenicity *in vivo*. The data presented here demonstrate that these cells can be grown extensively but not indefinitely *in vitro*. These cells maintain a diploid karyotype, are not tumorigenic in immune-deficient mice and undergo senescence after approximately 40 passages *in vitro*.

Apart from the potential of AMPs to regenerate in other tissues, our results strongly suggest that these cells have their own natural role in uterine homeostasis. Indeed, their potential for generating new vessels *in vitro* and *in vivo* has been unambiguously demonstrated. Our data also show that AMPs help reconstitute the uterine wall after wound healing, through the generation of both new myometrial muscle fibers, as well as new vessels. It remains an intriguing possibility that these cells could influence the recovery of the uterine lining after delivery or after the menstrual cycle. Whether these cells have a physiological role in the natural healing and regeneration of uterine tissue is a question of the greatest interest to us and deserves further investigation.

In summary, myometrial precursors can easily be isolated from mouse and human explants. These cells can grow naturally, in sufficient quantity for injection purposes and have the potential to differentiate into different tissues. Their

use for regenerative medicine is promising. At the same time, their niche and *in situ* role highlight these cells as being important for *in situ* regeneration.

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Conflict of Interest

The Authors declare no conflict of interest.

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