

Isolation of an adult blood-derived progenitor cell population capable of differentiation into angiogenic, myocardial and neural lineages

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Stem cells for therapeutic use can be obtained from embryonic tissue, umbilical cord blood and adult tissues (Passier & Mummery, 2003; Rogers & Casper, 2004; Sylvester & Longaker, 2004). Adult stem cells, identified in bone marrow (BM) and in other tissues, have the ability to differentiate into a variety of cell types, moreover, these cells can be used autologously, thereby eliminating risks of rejection or graft versus host diseases (Morrison *et al*, 1997; Fuchs & Segre, 2000; Forbes *et al*, 2002). As demonstrated in numerous studies, stem cells can be administered therapeutically to repair and regenerate damaged tissue (Gussoni *et al*, 1999; Kalka *et al*, 2000; Lagasse *et al*, 2000, 2001; Bianco & Robey, 2001; Forbes *et al*, 2002; Badorff *et al*, 2003; Grove *et al*, 2004; Guo *et al*, 2004; Petite *et al*, 2000; Ramiya *et al*, 2000; Stock &

Summary

Blood-derived adult stem cells were previously considered impractical for therapeutic use because of their small numbers. This report describes the isolation of a novel human cell population derived from the peripheral blood, termed synergetic cell population (SCP), and defined by the expression of CD31^{Bright}, CD34⁺, CD45^{-/Dim} and CD34^{Bright}, but not lineage-specific features. The SCP was capable of differentiating into a variety of cell lineages upon exposure to defined culture conditions. The resulting cells exhibited morphological, immunocytochemical and functional characteristics of angiogenic, neural or myocardial lineages. Angiogenic cell precursors (ACPs) expressed CD34, CD133, KDR, Tie-2, CD144, von Willebrand factor, CD31^{Bright}, concomitant binding of Ulex-Lectin and uptake of acetylated low density lipoprotein (Ac-LDL), secreted interleukin-8, vascular endothelial growth factor and angiogenin and formed tube-like structures *in vitro*. The majority of CD31^{Bright} ACP cells demonstrated Ac-LDL uptake. Neural cell precursors (NCPs) expressed the neuronal markers Nestin, β III-Tubulin, and Neu-N, the glial markers GFAP and O4, and responded to neurotransmitter stimulation. Myocardial cell precursors (MCPs) expressed Desmin, cardiac Troponin and Connexin 43. In conclusion, the simple and rapid method of SCP generation and the resulting considerable quantities of lineage-specific precursor cells makes it a potential source of autologous treatment for a variety of diseases.

Keywords: stem cells, progenitor cells, cell culture, cell therapy, differentiation.

Vacanti, 2001; Rafii & Lyden, 2003; Losordo & Dimmeler, 2004). To date, for therapeutic purposes the most extensively used stem cells are BM and mobilised BM cells.

The more accessible, blood-derived adult stem cells are now being evaluated as a potential source for different cell lineages (Assmus *et al*, 2002; Abuljadayel, 2003; Rehman *et al*, 2003; Zhao *et al*, 2003; Dobert *et al*, 2004; Romagnani *et al*, 2005). Endothelial progenitor cells (EPCs), similar to the progenitor cells first reported by Asahara *et al* (1997), and to the angiogenic cell precursors (ACPs) reported in this study, have been utilised in most of the therapeutic angiogenesis trials involving blood-derived adult stem cells (Kalka *et al*, 2000; Assmus *et al*, 2002). Several groups have recently studied the phenotype, function and therapeutic potential of these cells

(Rehman *et al*, 2003, 2004,) focusing on CD34, CD14 and CD31 as defining markers for a multipotent cell population. (Gulati *et al*, 2003; Kanayasu-Toyoda *et al*, 2003; Kawamoto *et al*, 2003; Romagnani *et al*, 2005; Yoon *et al*, 2005).

The present study describes a method for the isolation of a multipotent progenitor cell population, designated a synergetic cell population (SCP), from adult peripheral blood. The SCP is a heterogeneous population, rich in CD45, CD31^{Bright}, CD34⁺CD45^{-/Dim} and CD34^{Bright} cells, composed of multipotent progenitor cells supported by other cellular elements that can give rise to a variety of lineages.

Materials and methods

SCP isolation

Individual blood samples from healthy adults were obtained from the Israeli Blood Bank. Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep Ficoll gradient (Axis-Shield PoC AS, Oslo, Norway). Cells were centrifuged on a Ficoll gradient for 20 min at 2050 g, 21°C without brake. After washing with phosphate-buffered saline (PBS), cells were re-suspended in a small volume (1.5–3.0 ml) of X-vivo 15 serum-free medium (Cambrex, East Rutherford, NJ, USA) and subjected to a second density-based cell enrichment step using either OptiPrep (Axis-Shield PoC AS) or Percoll (GE Healthcare, Amersham Biosciences, Uppsala, Sweden). Cells subjected to OptiPrep gradient were centrifuged for 30 min at 700 g, 21°C without brake; Cells subjected to Percoll were centrifuged for 30 min at 1260 g, 13°C without brake. Layers of cells having a density of less than 1.072 g/ml were collected to a 50 ml tube pre-filled with PBS, washed twice with PBS and cultured *in vitro*. Both Optiprep and Percoll gradients were equally effective.

Cell Culture

Synergetic cell population cells, seeded at a concentration of $1.5\text{--}3 \times 10^6$ cells/ml in X-vivo 15 medium supplemented with 10% autologous serum, were cultured on 25 µg/ml fibronectin (Chemicon, Temecula, CA, USA) or autologous plasma coated dishes (Corning, Corning, NY, USA). Further differentiation was achieved by growing the SCP under culture conditions specific for each lineage. Upon termination of the culture, non-adherent cells were collected and combined with the mechanically detached adherent cells. To generate ACPs, SCP cells were cultured at a concentration of $1.5\text{--}3.0 \times 10^6$ cells/ml as described above and further supplemented with 1–10 ng/ml vascular endothelial growth factor (VEGF, R&D Systems, Minneapolis, MN, USA) and 5 IU/ml heparin (Kamada, Beit-Kama, Israel). To generate neural cell precursors (NCPs), $1.5\text{--}2.5 \times 10^6$ SCP cells/ml were supplemented with 10 ng/ml basic fibroblast growth factor (bFGF, R&D Systems), 25 ng/ml brain-derived neurotrophic factor (BDNF, PeproTech, Rocky Hill, NJ, USA), 50 ng/ml nerve growth factor (NGF, Pepro-

Tech), and 5 IU/ml heparin. After 8 d, cells were washed and incubated in X-vivo 15 medium containing 33% F12, 2% B27 (Sigma-Aldrich, St Louis, MO, USA), 10 ng/ml bFGF, 25 ng/ml BDNF, 50 ng/ml NGF, 20 ng/ml epidermal growth factor (EGF, PeproTech), and 5 IU/ml heparin. To generate myocardial cell precursors (MCPs), $2.0\text{--}3.0 \times 10^6$ SCP cells/ml were cultured as described above and further supplemented with 10 ng/ml bFGF and 5 IU heparin. Ten days after culture onset, 3 µM 5-azacytidine (Sigma-Aldrich) was added for 24 h.

Tube formation assay

Tube formation was tested using an *in vitro* angiogenesis assay kit (Chemicon). Briefly, harvested ACPs ($0.1\text{--}0.4 \times 10^6$ cells/ml) or acetylated low density lipoprotein (Ac-LDL)-DiO (BTI, Stoughton, MA, USA) pre-loaded ACPs, were cultured overnight in a 96-well plate using M199 medium (Sigma-Aldrich) containing 10% autologous serum, 10 ng/ml VEGF, 10 ng/ml bFGF, 5 IU/ml heparin, and 25 µg/ml endothelial cell growth supplement (ECGS; BTI) on extra cellular matrix (ECM) gel. Tube formation was assessed visually using an inverted light microscope (Nikon ECLIPSE TS-100; Nikon, Melville, NY, USA). Angiogenic pattern and vascular tube formation were scored as previously described (Kayisli *et al*, 2004): grade 0 – scattered individual cells; grade 1 – cells beginning to align with each other; grade 2 – organization into visible capillary-like structures; grade 3 – sprouting of secondary capillary tubes; grade 4 – closed polygons of capillaries beginning to form and grade 5 – complex mesh-like capillary structures.

Immunocytochemistry

Cells were grown on Permanox (Nunc, Rochester, NY, USA) slides or loaded on slides after harvesting and fixed in 3% paraformaldehyde (PFA, Sigma-Aldrich) for 15 min at room temperature. Following a 30 min non-specific stain blocking step (4% normal serum, 1% bovine serum albumin (BSA), and 0.1% Triton X-100; Sigma-Aldrich), cells were incubated overnight at 4°C in the dark with specific anti-human antibodies or matched non-specific isotype controls. The various cell lineages were stained using the following: ACPs – CD31-phycoerythrin (PE) or CD31-fluorescein isothiocyanate (FITC) (eBioscience, San Diego, CA, USA) and FITC-labelled Lectin from *Ulex europaeus* (Ulex-Lectin, Sigma-Aldrich); NCPs – Neu-N-Alexa 488 (Chemicon), Glial fibrillary acidic protein (GFAP, DakoCytomation, Glostrup, Denmark), Nestin, βIII-Tubulin, and Oligodendrocyte (O4, R&D Systems); MCPs – cardiac Troponin T, Desmin, and Connexin 43 (Chemicon). Goat anti-mouse (GaM) IgG-FITC, GaM IgG PE (Chemicon) were used as isotype controls. The primary antibodies were visualised by GaM IgG-FITC, GaM IgG-PE (Chemicon) or Rabbit anti-mouse IgG-Cy3 (Jackson ImmunoResearch, West Grove, PA, USA). For Ac-LDL uptake, cells were incubated in the presence of 0.8 µg/ml Ac-LDL (Alexa

Fluor488 AcLDL - Invitrogen, Carlsbad, CA, USA or Ac-LDL-DiI – Biomedical Technologies, Inc., Stoughton, MA, USA) for 15 min at 37°C, after which they were washed, fixed in 3% PFA and stained with CD31-FITC, CD31-PE (eBioscience) or FITC-labelled Ulex-Lectin (Sigma-Aldrich). Slides were mounted with a fluorescent mounting solution containing the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) (Vector, Burlingame, CA, USA) and examined on either an Olympus BX-50 or a Nikon E400 microscope equipped with appropriate excitation and barrier filters. Slides stained with hematoxylin and eosin (H&E) were examined on a Nikon E200 light microscope.

Flow cytometry

Harvested cells were washed in PBS and cell pellets were re-suspended in 100 µl PBS, stained with specific fluorochrome-conjugated or non-conjugated primary anti-human antibodies or isotype-matched non-specific controls, incubated in the dark for 30 min on ice; in case of non-conjugated primary antibody it was followed by fluorochrome-labelled secondary antibody. SCPs were stained using the following antibodies: CD31-FITC, CD45-PE (eBioscience) and CD34-APC. ACPs were stained using CD14-FITC, CD31-PE or CD31-FITC, CD34-APC, CD117-APC (DakoCytomation), CD133-PE, CD144-FITC, KDR-PE, Tie-2-PE (R&D Systems), VWF-FITC (Chemicon) and Ulex-Lectin-FITC. NCPs were stained using Nestin and βIII-Tubulin. MCPs were stained using Desmin and cardiac Troponin T. GaM IgG-FITC and GaM IgG-PE were used as secondary antibodies. In the case of CD31 and CD34 the results represent the percentage of cells with bright intensity (CD31^{Bright} and CD34^{Bright} respectively); cell staining was considered bright if staining intensity was at least 50 times higher than the intensity of the corresponding isotype control staining. For Ac-LDL uptake, cells were incubated in the presence of 0.8 µg/ml Ac-LDL (Alexa Fluor488 AcLDL or Ac-LDL-DiI) for 15 min at 37°C, after which they were washed and stained with FITC- or PE- conjugated CD31. Exclusion of dead cells was performed using 7-aminoactinomycin D (7-AAD; eBioscience) staining. Intracellular staining was carried out on cells fixed in 3% PFA and permeabilized by 0.1% Triton X-100. Five hundred thousand cells per sample were stained; at least 10 000 cellular events per sample were assessed by flow cytometry (FACScalibur, Becton Dickinson, Rockville, MD, USA) and analysed by CELLQUEST PRO software (Becton Dickinson). The results are expressed as mean ± standard error (SE) of the percentage of stained cells.

Analysis of cytokine secretion

Harvested cells were washed in PBS, cell pellets were re-suspended to 1×10^6 cells in 1 ml X-vivo 15 and grown for 24 h in 24-well plates. Cytokine secretion to the super-

natant was tested using flow cytometry, applying the BDTM CBA Human Angiogenesis Kit (Becton Dickinson).

Calcium uptake assay

Ca²⁺ influx through voltage-gated calcium channels in response to neurotransmitter stimulation with 100 µmol/l glutamate and 100 µmol/l GABA (Sigma-Aldrich), was performed as previously described (Hershinkel *et al*, 2001). Briefly, harvested cells were cultured overnight on 33 mm glass slides coated with poly-L-lysine. Cells were incubated for 30 min with 5 µmol/l Fura-2 acetoxymethyl ester (AM; TEF-Lab, Austin, TX, USA) in 0.1% BSA in NaCl Ringer's solution. After dye loading, the cells were washed in Ringer's solution, and the cover slides were mounted in a chamber that allowed the superfusion of cells. Free cellular Ca²⁺ level measured by Fura-2 that was excited at 340 nm and 380 nm and imaged with a 510 nm long-pass filter. The imaging system consisted of an Axiovert 100 inverted microscope (Zeiss, Göttingen, Germany), Polychrome II monochromator (TILL Photonics, Planegg, Germany), and a SensiCam cooled charge-coupled device (PCO). Fluorescent imaging measurements were acquired with Imaging Workbench 2 (Axon Instruments, Foster City, CA, USA).

Statistical methods

The results are presented as mean ± SE of independent experiments. Statistical analyses was performed using two-tailed Student's *t*-test; $P \leq 0.05$ was considered a significant difference. The correlation graph was analysed by a nonparametric two-tailed analysis (GRAPHPAD PRISM software; GraphPad Software, San Diego, CA, USA). $P \leq 0.05$ was considered a significant difference.

Results

Characterisation of SCP

Peripheral blood mononuclear cells obtained from individual normal blood donations were used in independent experiments to isolate the SCP. CD45 cells comprised more than 85%, in both PBMC and enriched SCP (data not shown). As can be seen in Fig 1A1 and A2, the percentage of CD34^{Bright} cells in the SCP was 3.5-fold higher than in the PBMC population. Furthermore, the percentages of CD31^{Bright}, CD34⁺CD45^{-Dim} and CD34^{Bright} cells in the SCP and the PBMC populations were $67.2 \pm 3.5\%$, $3.12 \pm 0.58\%$ and $0.36 \pm 0.07\%$ vs. $18.2 \pm 1.5\%$, $1.04 \pm 0.18\%$ and $0.09 \pm 0.02\%$ respectively (Fig 1B). Cultured SCP cells adhered to the culture dish surface and, after 4 d of culture, two main types of cell morphologies, mitotic and multinucleated, were observed (Fig 1C). The morphology of the multinucleated cells and the expression of CD31 on both SCP and ACPs suggested that some of them may be osteoclasts or megakaryocytes, both

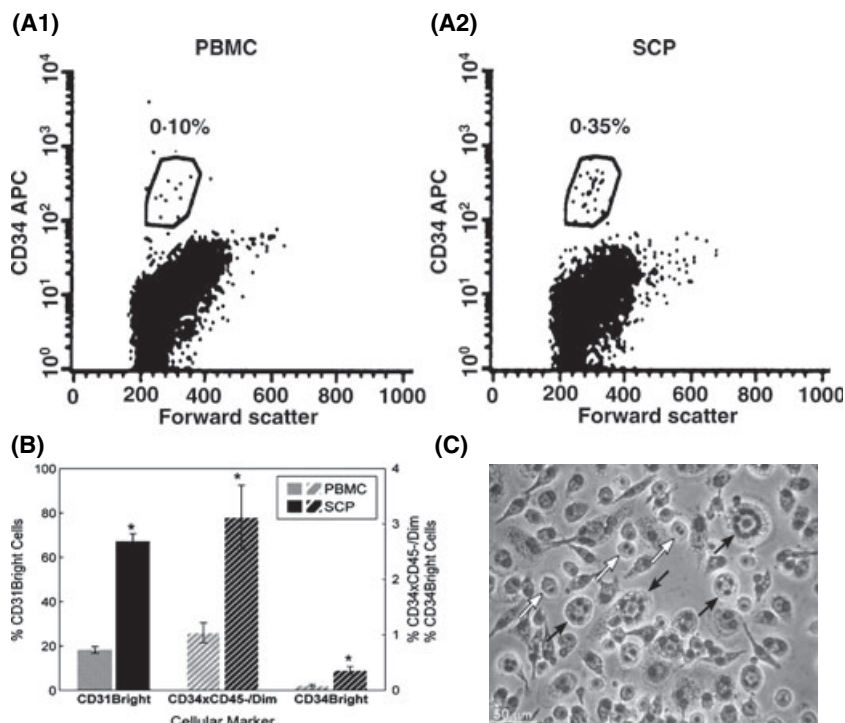


Fig 1. Characterisation of the synergistic cell population (SCP). (A) Flow cytometry analysis of expression of multipotent haematopoietic cellular marker CD34, detected using anti-CD34-APC on freshly prepared peripheral blood mononuclear cells (PBMC) (A1) and on SCP (A2). (B) Flow cytometry analysis of PBMC and SCP stained with anti-CD31-fluorescein isothiocyanate (FITC) (left y-axis; grey histograms represent PBMC; black histograms represent SCP), anti-CD45-PE and anti-CD34-APC (right y-axis; grey striped histogram represents PBMC; black striped histogram represents SCP). The percentage of cells expressing the markers is presented as mean \pm SE (CD31^{Bright}, $n = 16$; CD34⁺CD45^{-/Dim}, $n = 40$ and CD34^{Bright}, $n = 18$), statistically significant ($P < 0.01$) differences are marked by asterisks. Matched isotype control antibody staining results are deducted from specific antibody results. (C) Representative morphological overview of SCP cells after 4-d culture. Cell nuclei are stained with haematoxylin; mitotic cells indicated by white arrows; multinuclear cells indicated by black arrows.

characterised by CD51/CD61, receptor activator of nuclear factor kappaB ligand (RANKL) and its down stream indicator tartrate-resistant acid phosphatase (TRAP). However, their specific nature and biological activity were not determined during the culture period and will be addressed in future studies.

These results demonstrated the preferential expression of CD31^{Bright}, CD34⁺CD45^{-/Dim} and CD34^{Bright} in the SCP that can, subsequent to culturing on fibronectin or plasma, give rise to cells that exhibit a pronounced differentiation potential.

Characterisation of ACPs

The SCP seeding efficiency was $38.4\% \pm 2.4\%$ ($n = 14$). When grown for 5 d in a medium containing autologous serum, heparin and VEGF, SCP differentiated into ACPs exhibiting the characteristic elongated, spindle-shaped morphology (Fig 2A). Despite losing this morphology following harvesting, ACPs retained the ability to renew fully differentiated cultures of elongated and spindle-shaped cells when replated on a fibronectin surface for 24 h (Fig 2B). The function of differentiated ACPs was tested *in vitro*: angiogenic potency was assessed by microscopic examination of vascular

tube formation pattern 18–48 h after cell seeding on ECM. Semi-closed and closed polygons of capillaries and complex mesh-like capillary structures were observed and scored as grade 4–5 (Fig 2C). These tube-forming cells originated mainly from ACPs capable of Ac-LDL uptake (data not shown). Supportive cytokine secretion by 10^6 ACP cells cultured for 24 h in serum-free medium (X-vivo 15) was assessed using the flow cytometry-based CBA kit (BD Biosciences). Results show that, when compared to X-vivo 15 control, ACPs secreted interleukin (IL)-8 ($10\,107 \pm 1108$ pg/ml), VEGF (165 ± 6 pg/ml) and angiogenin (615 ± 62 pg/ml (Fig 2D) but not tumour necrosis factor (TNF) and b-FGF (data not shown). Immunostaining of cells harvested and fixed on slides showed typical angiogenic characteristics of concomitant binding of Ulex-Lectin and uptake of Ac-LDL (Fig 3A1, A2 and B). Flow cytometry assessment of ACPs showed expression of the stem cell markers CD34 ($23.6 \pm 3.6\%$ of the cells), CD133 ($10.1 \pm 2.1\%$) and CD117 ($7.0 \pm 1.9\%$) and endothelial/angiogenic markers KDR ($10.2 \pm 4.8\%$), Tie-2 ($31.8 \pm 4.2\%$), CD144 ($24.4 \pm 5.3\%$), von Willebrand factor (VWF; $30.1 \pm 8.1\%$) and CD31^{Bright} ($67.9 \pm 4.5\%$). Additionally, $68.2 \pm 7.6\%$ of ACPs showed concomitant binding of Ulex-

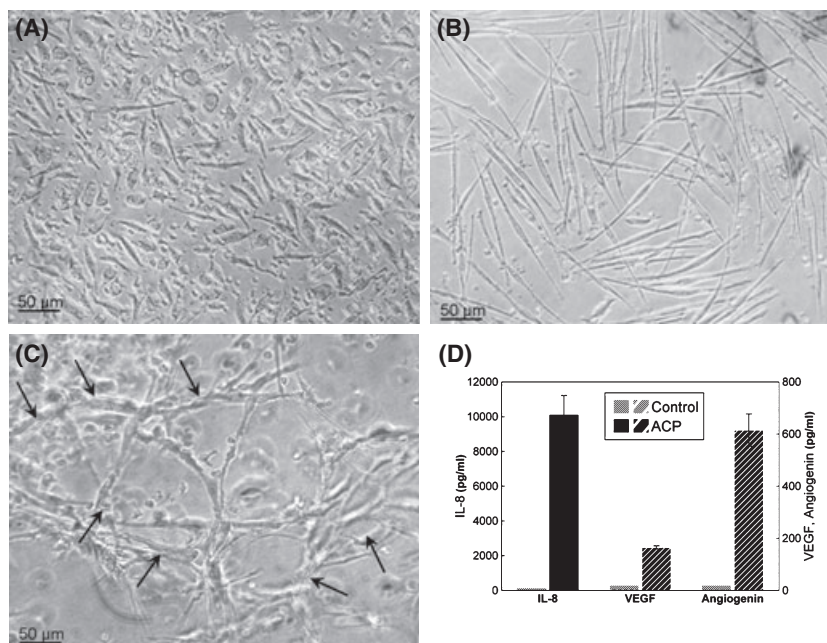


Fig 2. Characterisation of angiogenic cell precursors (ACPs). Morphology, immunostaining and functional examination. Microscopic morphology illustrating: (A) Typical elongated, spindle-shaped cells, (B) Renewal of ACP culture morphology. Harvested ACPs were replated for 24 h on fibronectin-coated 24-well plates. (C) Tube formation assay: arrows indicate cell organisation into tube-like structures. (D) Cytokine secretion by 10^6 ACP cells cultured for 24 h in serum-free medium was assessed using the flow cytometry-based CBA kit. Medium with no cultured cells served as control. Secretion of interleukin-8 (left y-axis; black histogram represents secretion by ACPs; grey histogram represents medium control); VEGF and Angiogenin (right y-axis; black striped histograms represent ACP secretion; grey striped histogram represents medium control).

Lectin and uptake of Ac-LDL and $58.8 \pm 4.3\%$ of the ACPs both expressed CD31^{Bright} and displayed uptake of Ac-LDL (Fig 3C and D). Moreover, the majority of CD31^{Bright} cells showed both binding of Ulex-Lectin ($92.9 \pm 5.2\%$; Fig 4A1 and A2) and uptake of Ac-LDL ($86.9 \pm 2.9\%$; Fig 4 B1, B2 and D). Concurrent expression of CD31^{Bright} and uptake of Ac-LDL were consequently used to define the differentiated ACP cells, was limited to SCP cells (Fig 4C and D). Similar characterisation results were obtained when cells were cultured in plates coated with either fibronectin or autologous plasma that can be safely used for the development of therapeutic cellular products. An average of $25.1 \pm 3.7 \times 10^6$ CD31^{Bright}-xAc-LDL cells was generated from 450 ml blood ($n = 14$). Interestingly, a non-parametric two-tailed analysis of 11 individual blood donations confirmed a significant negative correlation ($r = -0.74$, $P < 0.01$) between percentages of cells expressing the multipotent haematopoietic stem cell marker CD34 and the percentage of cells exhibiting the angiogenic differentiation phenotype of CD31^{Bright}-xAc-LDL (Fig 4E).

Characterisation of NCPs

Synergetic cell population cultures were induced to differentiate into NCPs and an average of 13.5×10^6 ($n = 5$) NCPs were generated from 450 ml blood. These cells developed irregular perikarya, from which filamentous extensions spread and contacted neighboring cells, forming a net-like organisa-

tion (Fig 5A). NCPs expressed the neural progenitor markers Nestin and β III-Tubulin, typical of newly differentiated neurons (Fig 5B and C), and Neu-N, a nuclear protein present in neurons (Fig 5D). Other cells from these cultures expressed O4 and GFAP, oligodendrocyte and astrocyte markers, (Fig 5E and F). Flow cytometry analysis showed that $49.4 \pm 6.3\%$ and $34.0 \pm 5.9\%$ of NCPs expressed Nestin and β III-Tubulin respectively (Fig 5G). In addition to demonstrating neural lineage, the differentiated NCPs responded to the neurotransmitters glutamate and GABA, as detected by calcium influx through voltage-gated calcium channels (Fig 5H).

Characterisation of MCPs

In preliminary experiments ($n = 3$) SCP cultures were induced to differentiate into MCPs. Morphologically, MCPs appeared elongated with dark cytoplasm, possibly indicating high protein content (Fig 6A). Furthermore, the cells expressed the myocardial markers cardiac Troponin T (Fig 6B) and the gap junction marker Connexin 43 (Fig 6C). Flow cytometry analysis showed the expression of Desmin and cardiac Troponin T (on 19.7% and 52.3% of cells respectively) (Fig 6D and E).

Lineage-specific differentiation

The specificity of the differentiation processes is summarised in Table I. In contrast to differentiated, lineage-specific

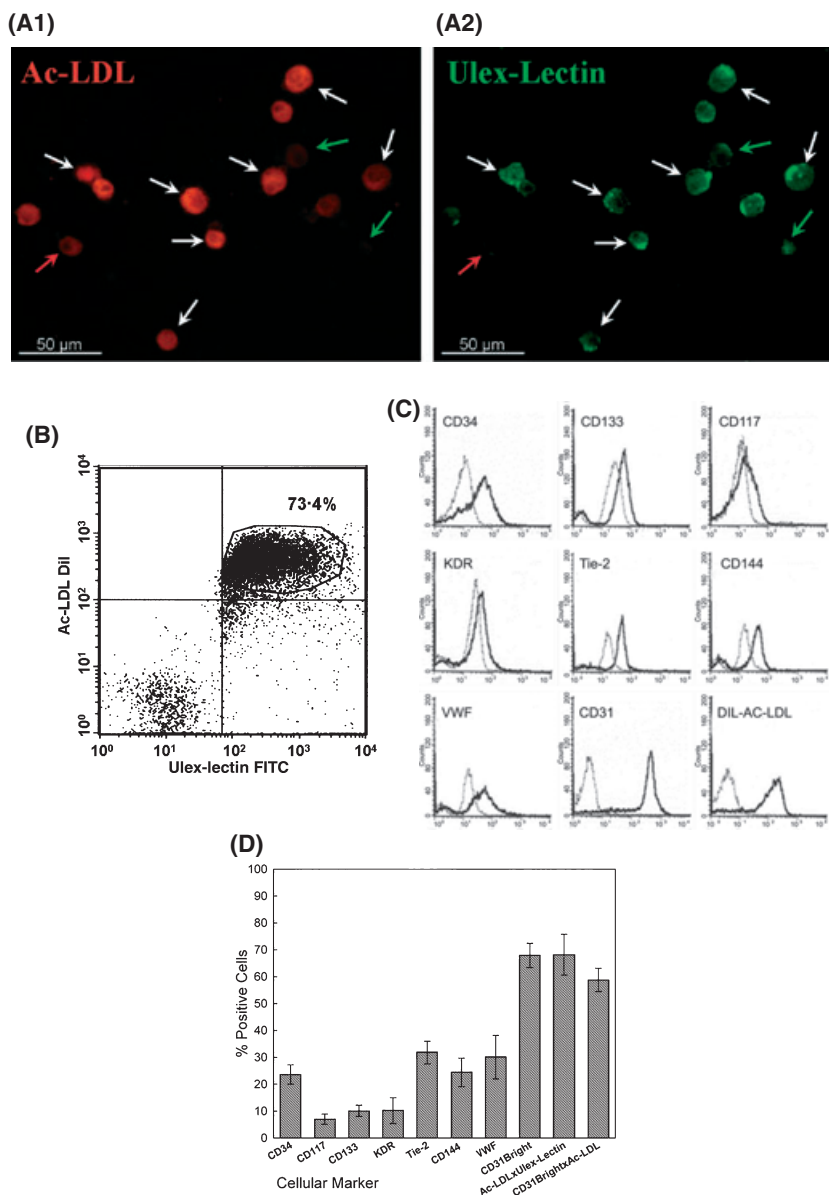


Fig 3. Characterisation of ACPs. A representative field of harvested, slide-fixed, specifically labelled ACPs was imaged using two wavelength filters: (A1) acetylated low density lipoprotein (Ac-LDL)-Dil imaged at 565 nm. (A2) Ulex-Lectin-fluorescein isothiocyanate (FITC) imaged at 505 nm. Cells that showed only Ac-LDL uptake are indicated by red arrows; cells stained solely by Ulex-Lectin-FITC are indicated by green arrows; and cells that show concomitant expression of both Ac-LDL uptake and binding of Ulex-Lectin are indicated by white arrows. Flow cytometry of harvested ACPs stained with: (B) Ulex-Lectin-FITC and Ac-LDL-Dil; (C) anti-CD34-APC, CD133-PE, CD117-APC, KDR-PE, Tie-2-PE, CD144-FITC, VWF-FITC, CD31-FITC and Ac-LDL-Dil. (D) The percentage of cells expressing the markers is presented as mean \pm SE (CD34, $n = 33$; CD117, $n = 29$; CD133, $n = 5$; KDR, $n = 10$; Tie-2, $n = 21$; CD144, $n = 11$; von Willebrand factor (VWF), $n = 9$; CD31^{Bright}, $n = 24$; and CD31^{Bright}xAcLDL, $n = 14$). Matched isotype control antibody staining results are presented in Fig S1 and deduced from specific antibody results.

precursors, freshly isolated SCP cells failed to express ACP, NCP or MCP-specific markers. They did not generate tube-like structures and less than 1% of SCP cells showed concomitant expression of CD31^{Bright} and Ac-LDL uptake (Fig 4C), characteristics typical of ACPs. Furthermore, they expressed neither the NCP markers β III-Tubulin and GFAP nor the MCP markers Connexin 43 and cardiac Troponin T. Differentiated cells, on the other hand, expressed only their lineage-specific markers but not those typical of the other lineages: ACPs that

expressed specific lineage characteristics, such as CD31, KDR and Tie-2, significant Ac-LDL uptake and binding of Ulex-Lectin did not express the NCP-specific markers β III-Tubulin and GFAP or the MCP-specific markers Connexin 43 and cardiac Troponin T; differentiated NCPs that expressed NeuN, β III-Tubulin, and GFAP did not express the MCP markers cardiac Troponin T and Actin; and MCPs that expressed cardiac Troponin T, Desmin and Connexin 43 did not express β III-Tubulin and GFAP, markers of NCPs.

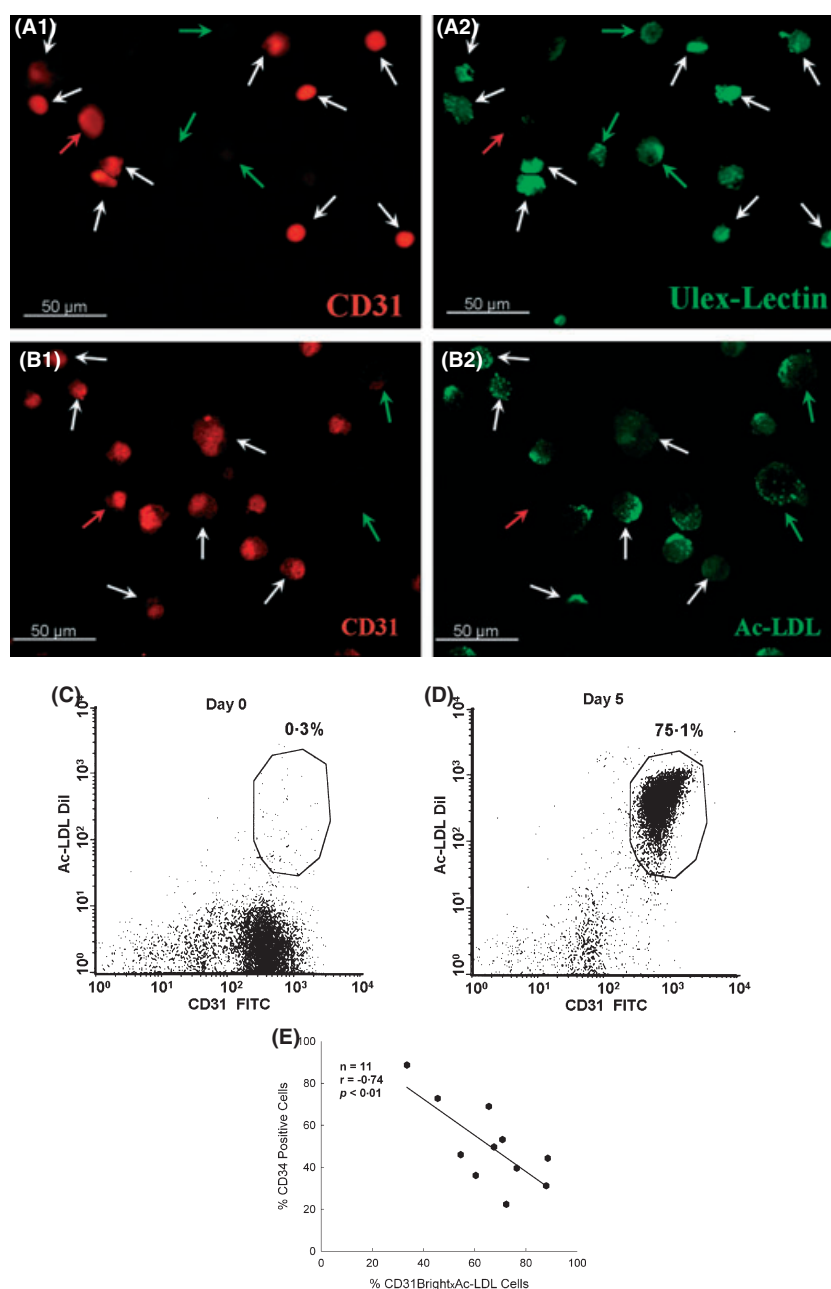


Fig 4. Characterisation of CD31^{Bright} ACPs. A single field of harvested, slide-fixed, specifically labelled ACPs was imaged using two wavelength filters: (A1) anti-CD31-PE imaged at 565 nm. (A2) Ulex-Lectin-FITC imaged at 505 nm. Cells stained solely by anti-CD31 are indicated by red arrows; cells stained solely by Ulex-Lectin are indicated by green arrows; and cells that show concomitant expression of both anti-CD31 and Ulex-Lectin are indicated by white arrows. (B1) Anti-CD31-PE imaged at 565 nm. (B2) Uptake of Ac-LDL-Alexa488 imaged at 505 nm. Cells stained solely by anti-CD31 are indicated by red arrows; cells that showed only Ac-LDL uptake are indicated by green arrows; and cells that show concomitant expression of both anti-CD31 and Ac-LDL uptake are indicated by white arrows. Flow cytometry analysis of concomitant expression of anti-CD31-FITC and uptake of Ac-LDL-Dil. (C) SCP (day 0 of culture) and (D) ACP (day 5 of culture). (E) Negative correlation between the expression of CD34 and the concomitant expression of CD31 and uptake of Ac-LDL-Dil on ACPs. The correlation, generated from 11 individual blood donations, resulted from a nonparametric two-tailed analysis using GRAPHPAD PRISM software.

Discussion

Most attempts to develop stem cell therapy have focused on the direct isolation or mobilisation of BM cells (Gussoni *et al*, 1999; Fuchs & Segre, 2000; Kalka *et al*, 2000; Lagasse *et al*, 2000, 2001; Bianco & Robey, 2001; Forbes *et al*, 2002;

Badorff *et al*, 2003; Grove *et al*, 2004; Guo *et al*, 2004, Morrison *et al*, 1997; Petite *et al*, 2000; Ramiya *et al*, 2000; Stock & Vacanti, 2001; Rehman *et al*, 2003; Losordo & Dimmeler, 2004; Matsubara, 2004). Blood-derived adult stem cells are currently being evaluated as a potential source of different cell lineages. Recent reports have described

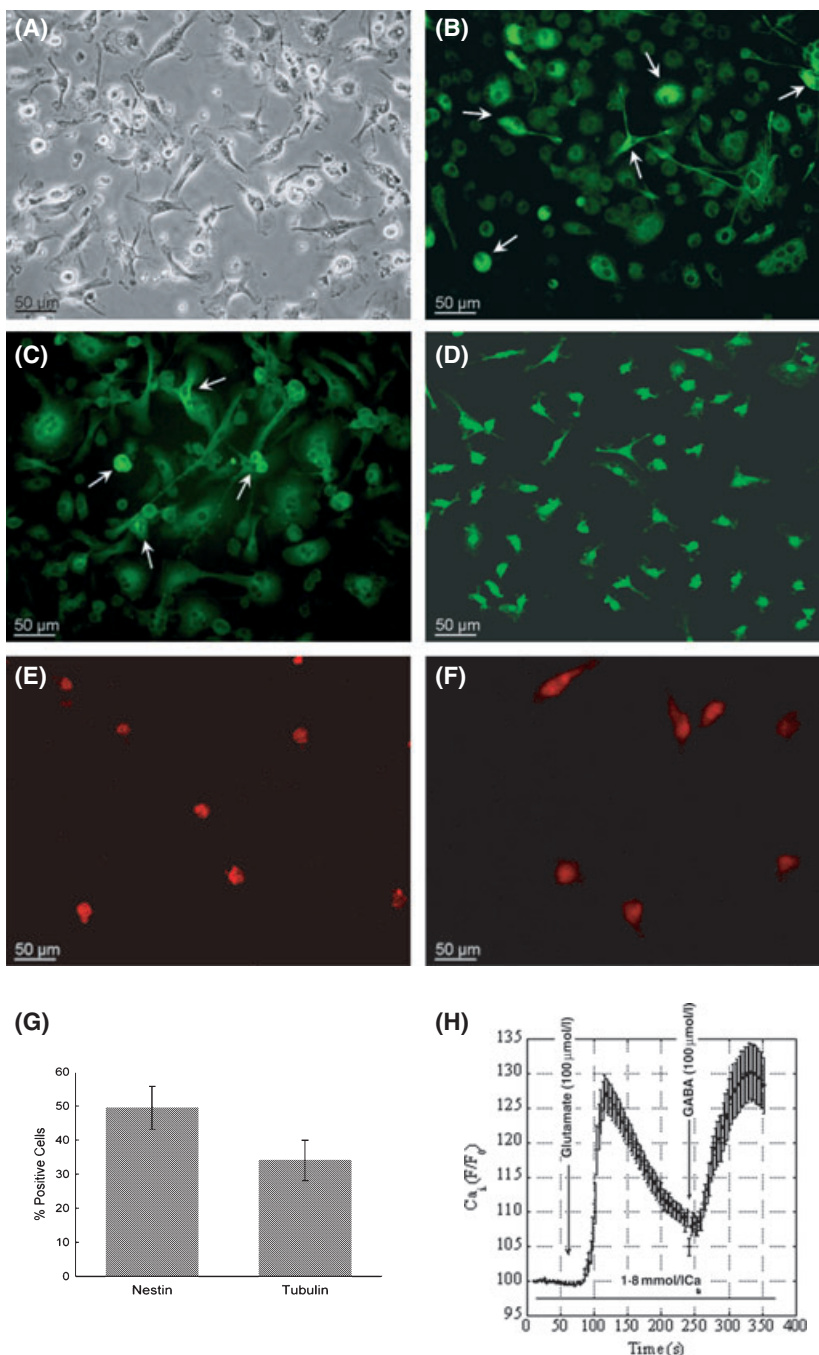


Fig 5. Characterisation of the neural cell precursors (NCPs). Morphology, immunostaining and functional examination of neural progenitors. (A) Microscopic examination of neural progenitor cells morphology shows irregular cell bodies from which filamentous extensions spread and create connections, forming a net-like structure. Slide-fixed neural progenitor cells stained with: (B) anti-Nestin detected by goat anti-mouse (GaM) immunoglobulin G (IgG)-fluorescein isothiocyanate (FITC); (C) anti-βIII-Tubulin detected by GaM IgG-FITC (positive cells marked by arrows); (D) anti-Neu-N-Alexa 488; (E) anti-O4 detected by GaM IgG-Cy3; (F) anti-GFAP detected by anti-mouse IgG-Cy3. (G) Flow cytometry analysis results, presented as the percentage mean ± SE of harvested fixed neuronal progenitor cells stained with anti-βIII-Tubulin and anti-Nestin detected by GaM IgG-FITC. Matched isotype control antibody staining results are presented in Fig S2 and deducted from specific antibody results. (H) Results of Ca²⁺ release test following activation of neural progenitor cells with glutamate and GABA.

procedures for the generation of progenitor cells from peripheral blood. Assmus *et al* (2002) used purified, cultured cells from peripheral blood in a clinical study (TOPCARE-AMI) but did not demonstrate multiple lineage potential for

these cells, while other reports demonstrated differentiation into multiple lineages, but on a small scale (Abuljadayel, 2003; Rehman *et al*, 2003; Zhao *et al*, 2003; Dobert *et al*, 2004; Romagnani *et al*, 2005).

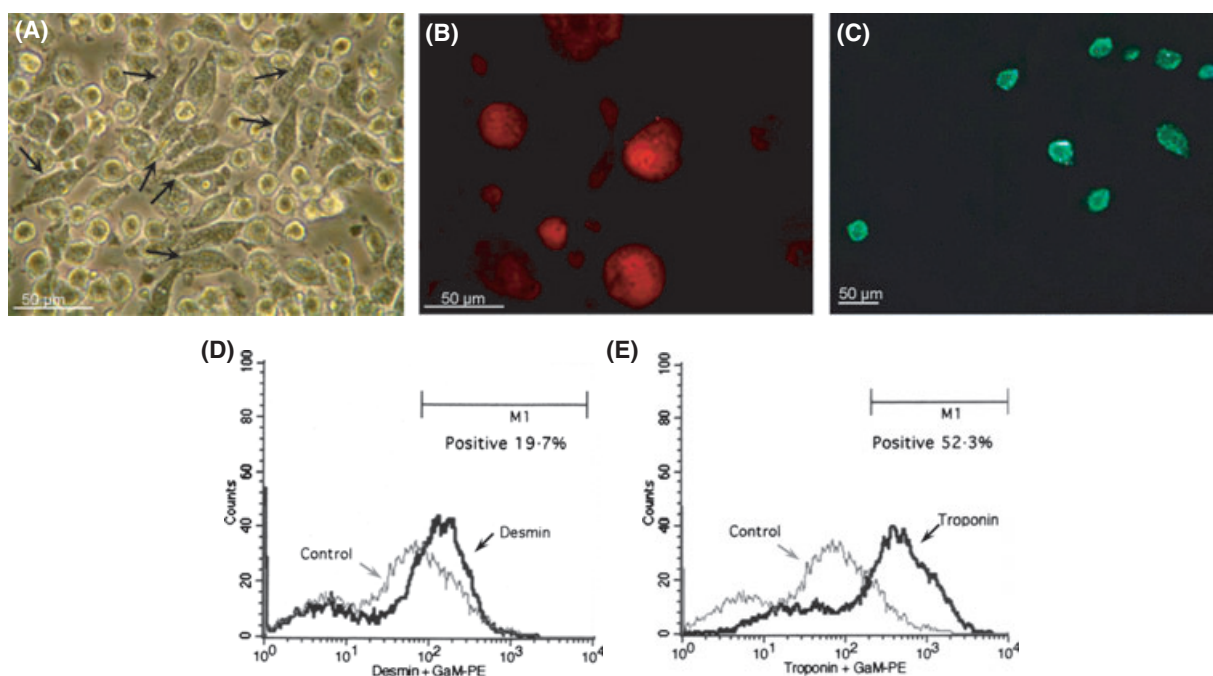


Fig 6. Characterisation of the myocardial cell precursors (MCP). Morphology and immunostaining of MCPs. (A) Microscopic examination of morphology shows elongated cells with dark cytoplasm (marked by arrows). Harvested slide-fixed MCPs stained with: (B) anti-cardiac Troponin T detected by goat anti-mouse (GaM) immunoglobulin G (IgG)-Cy3 and (C) anti-mouse Connexin 43 detected by GaM IgG-FITC. Flow cytometry analysis of cardiomyocyte progenitors stained with: (D) anti-cardiac Troponin T detected by GaM IgG-phycoerythrin (PE) and (E) anti-Desmin detected by GaM IgG-PE. Matched isotype control antibody staining results are presented in histograms 6D and 6E and in Fig S3.

Table I. Summary of lineage characteristics expression by freshly isolated SCP cells and by specific angiogenic, neural and myocardial lineage precursor cells.

Cell type	SCP	ACP	NCP	MCP
ACP characteristics:				
LDLxCD31 ^{Bright} (%)	<1%	58.8% ± 4.3%	NT	NT
Tube formation (0-5)	0	5	NT	NT
NCP characteristics:				
βIII-Tubulin (+/-)	-	-	+	-
GFAP (+/-)	-	-	+	-
MCP characteristics:				
Cardiac troponin T (+/-)	-	-	-	+
Connexin 43 (+/-)	-	-	-	+

SCP, synergetic cell population; ACP, angiogenic cell precursors; NCP, neural cell precursors; MCP, myocardial cell precursors; LDL, low density lipoprotein; GFAP, glial fibrillary acidic protein; NT, not tested.

The synergetic cell population, described here for the first time, contains increased numbers of CD34⁺CD45^{-/Dim} and CD31^{Bright} multipotent cells but not cells expressing mature lineage markers. The SCP can be induced to lineage-specific differentiation. Our approach provides the means to simply and reliably obtain more than 10⁷ differentiated precursor cells from 450 ml of blood. For example, a mean of 25.1 × 10⁶ ACPs obtained from blood samples is comparable with (or

even higher than) the amount of specific progenitor cells obtained from 10⁹ BM cells (Dobert *et al*, 2004; Schachinger *et al*, 2004; Pompilio *et al*, 2005; Strauer *et al*, 2005).

The SCP, a multipotent cell population, is purified based on cell density and is therefore more affluent than PBMCs in progenitor cells, as demonstrated by the levels of CD34^{Bright}, CD34⁺CD45^{-/Dim} and CD31^{Bright} cells. Under specific culture conditions, cells of the SCP can differentiate into angiogenic, myocardial and neural lineages. Further studies are needed to explore culture conditions under which the SCP may differentiate to other cell lineages.

Angiogenic cell precursors generated from the SCP expressed CD34, CD117 and CD133, typical of multipotent haematopoietic stem cells as well as KDR, Tie-2, CD144, VWF, CD31^{Bright} and displayed both Ulex-Lectin and Ac-LDL uptake, which is typical of angiogenic/endothelial cells.

CD31/PECAM-1 is expressed on hematopoietic progenitor cells and is a major constituent of the endothelial cell intercellular junction, where up to 10⁶ molecules are concentrated (resulting in CD31^{Bright} cells) (Sheibani *et al*, 1999). CD31 is not present on fibroblasts, epithelium, muscle, or other nonvascular cells (Newman, 1997); thus evaluation of CD31⁺ cell involvement in the angiogenic processes is of particular interest. Previous studies reported that CD31⁺ cells demonstrate the ability to differentiate into endothelial cells and significantly improve symptoms in models of myocardial infarction (Kanayasu-Toyoda *et al*, 2003; Kawamoto *et al*, 2003). Our data support the importance of CD31 as a marker

for multipotent progenitor cells that can differentiate into a variety of lineages including the ACP lineage. Upon differentiation into ACPs, CD31^{Bright} cells acquire endothelial cell-specific characteristics, such as the uptake of Ac-LDL. We found that the majority of CD31^{Bright} cells in the ACP population, but not in the source SCP, demonstrated Ac-LDL uptake and binding of Ulex-Lectin. These CD31^{Bright}Ac-LDL positive cells showed typical morphology of elongated, spindle-shaped cells that not only expressed ACP markers but also showed specific angiogenic biological activity: secretion of tissue regeneration factors, such as IL-8 (also known as the chemokine CXCL8), VEGF and angiogenin (Han *et al*, 1997; Wiedlocha, 1999; Rivera *et al*, 2001; Pruijt *et al*, 2002; Li *et al*, 2003) and formation of tube-like structures (Kayisli *et al*, 2004).

Thus, we describe here a methodology for characterising the angiogenic/endothelial lineage based on concomitant expression of CD31^{Bright} and uptake of Ac-LDL. Using this approach, we observed a negative correlation ($r = -0.74$, $P < 0.01$) between the percentages of differentiated angiogenic cells (CD31^{Bright}Ac-LDL) and undifferentiated, multipotent, haematopoietic CD34⁺ cells that could indicate the differentiation/multipotential status of the angiogenic populations.

Synergetic cell population-derived NCPs expressed the neuronal and glial markers Nestin, β III-Tubulin, Neu-N, GFAP and O4 (Steindler & Pincus, 2002; Goolsby *et al*, 2003) and responded to neurotransmitter stimulation, whereas SCP-derived MCPs expressed Desmin, cardiac Troponin T and the gap junction marker Connexin 43 (Grounds *et al*, 2002; Nygren *et al*, 2004).

The specificity of differentiation processes can be demonstrated by the fact that the SCP does not express lineage-specific markers and that the differentiated cells strictly express high levels of specific characteristics, but not those of other lineages.

The isolation of multipotent cells, the nature of the interactions between the cellular elements of the SCP, and the methods required to facilitate production of additional lineage-specific progenitors will be the subject of future studies. The vitality and plasticity shown by the SCP and the committed precursor cells generated thereof can potentially form the basis for safe and effective autologous cell therapies applicable to a wide range of clinical disorders. However, the biological activity of the lineage specific precursors should be addressed *in vivo* in order to evaluate their therapeutic potential.

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Supplementary material

The following supplementary material is available for this article online:

Fig S1. Supplemental data for Fig 3 and 4 – ACP Isotype control.

Fig S2. Supplemental data for Fig 5 – NCP Isotype control.

Fig S3. Supplemental data for Fig 6 – MCP Isotype control.

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