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Use of an alpha-smooth muscle actin (SMAA) GFP reporter to identify an osteoprogenitor population

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Abstract

Identification of a reliable marker of skeletal precursor cells within calcified and soft tissues remains a major challenge for the field. To address this, we used a transgenic model in which osteoblasts can be eliminated by pharmacological treatment. Following osteoblast ablation a dramatic increase in a population of α -smooth muscle actin (α -SMA) positive cells was observed. During early recovery phase from ablation we have detected cells with the simultaneous expression of SMAA and a preosteoblastic 3.6GFP marker, indicating the potential for transition of α -SMA⁺ cells towards osteoprogenitor lineage. Utilizing α -SMAGFP transgene, α -SMAGFP⁺ positive cells were detected in the microvasculature and in the osteoprogenitor population within bone marrow stromal cells. Osteogenic and adipogenic induction stimulated expression of bone and fat markers in the α -SMAGFP⁺ population derived from bone marrow or adipose tissue. In adipose tissue, α -SMA⁺ cells were localized within the smooth muscle cell layer and in pericytes. After in vitro expansion, α -SMA⁺/CD45⁻/Sca1⁺ progenitors were highly enriched. Following cell sorting and transplantation of expanded pericyte/myofibroblast populations, donor-derived differentiated osteoblasts and new bone formation was detected. Our results show that cells with a pericyte/myofibroblast phenotype have the potential to differentiate into functional osteoblasts.

Keywords

myofibroblast; pericyte; osteoblast; adult progenitor cell; GFP

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INTRODUCTION

Mature osteoblasts are responsible for the new bone formation and are major players in achieving an adequate balance between bone resorption and formation. Therefore, the burden of maintenance of bone mass depends not only on the function of mature osteoblasts but also on the ability of mesenchymal progenitor cells to generate sufficient numbers of osteoblasts as well. Cells with the properties of mesenchymal stem cell may be present within the bone marrow and other tissues, but it is not yet possible to identify and isolate this population of progenitor cells.

The osteoprogenitor lineage is one of the cell populations that form by the process of maturation from mesenchymal stem cells [1,2]. Currently, identification of cells at different stages of osteoblast maturation is defined by the temporal expression of various bone markers. Commitment to pre-osteoblasts can be detected by expression of alkaline phosphatase (ALP) and osteopontin, while more mature and bone matrix producing osteoblasts express bone sialoprotein and osteocalcin (OC) [3]. A number of cell surface epitopes have been proposed as markers to identify and isolate progenitor cells within the lineage. To define each stage of progenitor cell differentiation a number of markers have been identified. However, these markers vary significantly between species, and are likely to identify partially overlapping populations of progenitors at different levels of maturation. In human bone marrow stromal cells the most characterized has been a cell surface marker STRO1 [4,5], shown to precede the expression of preosteoblastic and osteoblastic alkaline phosphatase [6]. Other markers identifying a population of progenitors in human bone marrow stromal cells are Alcam/CD166 (SB-10) [7,8], a member of the tetraspan family of cell surface glycoproteins CD63 (HOP-26) [9,10]. A more recent study showed that a progenitor population exhibits simultaneous expression of HOP-26, CD166 and CD49a and has the ability to form CFU-F colonies [11, 12]. In addition a population of cells localized to subendothelial areas within the bone marrow has been shown to have osteoprogenitor phenotype. These cells are marked by high expression of melanoma-associated cell adhesion molecule MCAM/CD146 and exhibit potential for self-renewal and osteogenic differentiation [13].

The applicability of markers identified in human cells to murine stromal cells has been limited, so additional markers have been acquired to identify murine mesenchymal stem cells. Besides Alcam, [14] of particular interest is stem cell antigen 1, Ly6 (Sca1), a cell surface glycoprotein that appears to be an active participant in the stem cell renewal process [15]. However, availability and usefulness of cell surface markers in mesenchymal stem cell biology has never approached the level of utility that has been developed in the hematopoietic field. This lack of optimal markers has slowed the studies determining which mesenchymal lineages are formed in vivo from isolated and defined mesenchymal progenitor cells.

In this study we show that a population of cells present within the bone marrow stromal cells and adipose tissue expresses other markers characteristic of mesenchymal progenitor cells. This population with myofibroblast/pericyte phenotype has ability to expand in vitro and generate functional and mature osteoblast lineage cells after transplantation into an osteogenic in vivo environment.

MATERIALS AND METHODS

Experimental mouse models

Osteoblast ablation model—The Col2.3 Δ tk mouse harbors a truncated version of the tk gene under the control of a 2.3 kb segment of the rat collagen type I promoter gene [16]. Utilizing this transgenic mouse we can selectively eliminate mature cells within osteoprogenitor lineage. To achieve this, 6–7 week old mice were injected intraperitoneally

with 8 mg/kg/d of gancyclovir (Cytovene-IV; Roche Pharmaceutical, Nutley, NJ, USA) twice daily for 16-days. After the discontinuation of treatment, experimental mice were allowed to recover. During the entire experimental procedure, all mice were provided with crushed food and sacrificed at time point of 0, 4, 7, 12, 17, 21 and 28 days of recovery.

Osteoblast lineage directed markers—To define cells as mature osteoblasts we utilized a transgenic mouse in which a 2.3 kb collagen type I promoter directs the expression of cyan or emerald variants of GFP to mature osteoblast lineage cells (Col2.3GFP(green) or Col2.3CFP (blue) [17,18]. Col3.6GFPTpz directs expression to cells at the stage of a preosteoblast in vitro, and to periosteal fibroblasts. Following osteogenic induction, Col3.6GFP cells have the potential to differentiate into mature osteoblasts. We crossed homozygous Col2.3 Δ tk mice with either homozygous Col3.6GFP or homozygous Col2.3GFP mice. Animals heterozygous for both transgenes (tk and GFP) were used.

Pericyte directed GFP expression: To identify the pericyte population we utilized a smooth muscle alpha actin promoter (termed SMP8) to direct the expression of enhanced GFP, a transgenic model developed by Dr. Jen-Yue Tsai [19,20].

Histological and μ -CT analysis of bone

Paraffin histological methods—Femurs were fixed in 4% paraformaldehyde at +4°C for 7 days. Following fixation, they were decalcified in 15% ethylene-diamine tetraacetic acid (EDTA) for 3–4 days at room temperature, dehydrated with increasing concentrations of ethanol and cleared in xylene. Samples were embedded in paraffin (paraplast X-TRA tissue embedding medium, Fisher, Pittsburg) at 56°C and 5 μ m thick sections were deparaffinized in xylene, hydrated in descending concentrations of ethanol and 2 mM MgCl₂ in PBS. Glycerol/PBS mounted slides were examined by fluorescent microscopy using dual FITC/Texas red filter cube of the Zeiss Axioplan 200 microscope [21]. The same sections were subsequently stained with hematoxylin and eosin.

μ -CT analysis—Analysis of bone geometry is performed at the University of Connecticut Health Center μ -CT facility. Mouse femur morphometry was assessed using conebeam micro-focus X-ray computed tomography (μ CT40, Scanco Medical AG, Bassersdorf, Switzerland). Serial tomographic images were acquired at 55 kV and 145 μ A, collecting 1000 projections per rotation at 300 msec integration time. Three-dimensional 16-bit grayscale images were reconstructed using standard convolution back-projection algorithms with Shepp and Logan filtering, and rendered within a 12.3 mm field of view at a discrete density of 578,704 voxels/mm³ (isometric 12- μ m voxels). Segmentation of bone from marrow and soft tissue was performed in conjunction with a constrained Gaussian filter to reduce noise, applying a hydroxyapatite-equivalent density threshold of 470 mg/cm³. Three mice were used per time period.

RNA extraction and Northern blot analysis

Animals were sacrificed with CO₂ asphyxiation and epiphyseal portions of the long bones (tibia and femur) were cleaned of attached muscle prior to freezing. Total RNA was prepared using TRI Reagent according to the manufacturer's instructions, using a previously described modification [17]. RNA was separated on a 2.2 M formaldehyde/1% agarose gel and transferred onto a nylon membrane (Maximum Strength Nytran Plus, Schleicher & Schuell). Membranes were probed with the 900 bp PstI fragment of rat Col1a1 (p α 1R2)[22], a 440 bp mouse OC fragment (p923)[23], and 1000 bp mouse BSP and osteopontin (OP) fragments [24]. Probes were radiolabeled by the random primer method using (α -³²P) dCTP (New England Nuclear, 3000 Ci/mmol) to obtain probes with specific activities of approximately 1 \times 10⁹ cpm/ μ g. Filters were hybridized with 3 \times 10⁶ cpm/ml ³²P-labeled probe at 42°C in 50%

formamide, 5×SSPE (1×SSPE = 0.149M NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, pH 7.4), 1.2 × Denhardt's and 0.5% sodium dodecyl sulfate and washed according to published procedures [25].

Histological evaluation of GFP expression, immunofluorescence and histochemical stainings

Preparation of cryosections—Bones were fixed for 3–4 days in 4% paraformaldehyde/PBS (pH 7.4) at 4°C and decalcified 3 days as described above. Thereafter they were placed in 30% sucrose/PBS overnight and embedded in frozen specimen embedding media (Cryomatrix, ThermoShandon, Pittsburgh, USA) at –60°C. Adipose tissues were fixed overnight and placed in sucrose overnight and then embedded and cryosectioned. Bones and soft tissues were cryosectioned in 5µm sections using a CryoJane tape transfer system (Instrumedics, NJ, USA). After rehydration in 1 mM MgCl₂/physiological saline, GFP expression was observed and photographed using a Zeiss Axiovert 200M microscope and an Axiocam digital camera. A GFP-variant specific filters were utilized; GFP/Texas Red dual filter cube for visualization of green fluorescent protein, and Cyan/Texas red dual cube for blue fluorescent protein as described [21]. The filters were obtained from Chroma (Rockingham, VT). The dual bandpass design is required to distinguish the color of the GFP signal from the autofluorescence of the bone and bone marrow.

Immunostaining and Hematoxyllin Eosin (HE) staining—Following detection and imaging of GFP expression, slides were soaked in PBS for 30 min. to remove the coverslides. Sections were blocked with 1% BSA in PBS for 30 min. at room temperature. Sections were stained with Cy3-conjugated mouse monoclonal anti- α -smooth muscle actin (clone 1A, SIGMA, St. Louis, MO, USA) antibody for 1 hr at 1:800 dilution. We utilized monochrome filters to detect transgene driven GFP expression (Col3.6 and Col2.3GFP) in relation to α -SMA immunostaining (FITC filter, excitation 500/20, emission 535/30; TRITC filter, excitation 545/30, emission 620/60). Images were pseudocolored and composite images were generated using Photoshop. The number of single GFP expressing cells (α -SMA⁺ or Col3.6GFP⁺) or dual expressing cells were counted. Expression was evaluated in an area that is 2mm distal from growth plate and in proximity to the endocortical surface (selected area was 0.4mm²).

To detect endothelial cells we utilized immunohistochemistry for CD31 (PECAM). Briefly, sections were washed in PBS and then placed in 3% H₂O₂ for 30 min, followed by a blocking procedure at room temp for 20min. Sections were incubated with rat anti-mouse CD31 primary antibody at 1:100 dilution (BD Biosciences, San Jose, CA, USA) at 4°C overnight. Rabbit biotinylated anti-rat secondary antibody (Vector laboratories, Burlingame, CA, USA) was used at 1:200 dilution. Detection was achieved using a Vectastain ABC kit followed by DAB reaction according to manufacturer instructions (Vector laboratories). After immunostaining sections were washed, mounted with 50% glycerol and imaged. Then HE staining was done to indicate localization of GFP positive cells.

Preparation of primary cell cultures

Bone marrow stromal cell cultures (BMSC)—Four-to 6-week old mice were sacrificed by CO₂ asphyxiation. Femurs and tibiae were dissected from surrounding tissues. The epiphyseal growth plates were removed and the marrow was collected by flushing with α MEM culture medium containing 100 U/ml Penicillin, 100 µg/ml streptomycin and 10% FCS, Hyclone (serum lot was previously tested for optimal osteogenic differentiation) with a 25 gauge needle. Single cell suspensions were prepared by passing the cell clumps through an 18-gauge needle followed by filtration through a 70-µm strainer and cells were plated at a density 1×10^6 cells/cm².

Adipose derived stromal cell cultures (ADSC)—Adipose tissue was dissected from inguinal fat pads of 2–3 month old mice. Briefly, after removal, tissue was subjected to sequential 75 min digestions in an enzyme mixture containing Collagenase A (Roche) at a concentration of 0.15%. Cell digestion was resuspended with syringe and 18G needle, filtered using 75µm cell strainer, centrifuged, and resuspended in DMEM containing 10% FCS (serum lot was previously tested for optimal osteogenic differentiation). Cells were plated in 100mm dishes ($8\text{--}10 \times 10^6$ /per plate) in DMEM containing 10% FCS.

Induction of osteogenesis and adipogenesis—Osteogenesis was induced by addition of 10^{-8} M dexamethasone, 50µg/ml ascorbic acid and 8mM β -glycerol phosphate on day 7 of culture and every 2 days for a period of 2 weeks. Adipogenic induction was obtained by adding a cocktail of 0.5µM rosiglitazone and 1µM insulin for a period of 7 days.

Histochemical analysis of cell cultures—Histochemical staining for ALP activity was performed using a commercially available kit (86-R Alkaline Phosphatase, Sigma Diagnostics, Inc. ST. Louis, MO, U.S.A.) according to the manufacturer's instructions. Mineralization was assessed using a modified von Kossa silver nitrate staining method. Detection of lipid vacuoles was done using an Oil-Red-O (Sigma, USA). Stock solution was prepared as 0.5% in 100% isopropanol, and then fresh diluted to 0.3% before use. Cells were fixed in neutral buffer formalin, and stained for 1 hr. at room temperature. Images were acquired using a flat scanner and processed into a composite image using Adobe Photoshop.

Fluorescent microscopy of cultured cells—GFP expression in cell culture was visualized using an Olympus IX50 inverted system microscope equipped with IX-FLA inverted reflected light fluorescence (Olympus America Inc., Melville, NY). A specific excitation wavelength was obtained using FITC filter (exciter: 470/40; emitter: D525/50) to evaluate the expression of the α -SMAGFP. Cultures derived from dual α -SMAGFP/Col2.3GFPcyan mice were imaged using following filters: (GFP; excitation 500/20, emission 535/30) and (CFP; excitation 436/20, emission 480/40) to separate GFP from CFP. Images were recorded with a SPOT-camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Phenotyping of ADSC populations

Preparation of cell suspension—Freshly isolated adipose derived cells derived from α -SMAGFP mice were prepared as described in preparation of ADSC cultures. In addition, cells were washed one time in PBS and filtered into a new tube using a 75µm cell strainer to remove adipose tissue residues. Samples of ADSC derived from α -SMAGFP mice were obtained from 6-day-old culture by digesting the cells with 0.2% collagenase A/0.2% hyaluronidase for 20 min. Cells were centrifuged and washed in PBS. After washing, red blood cells were lysed using ammonium chloride.

Antibodies—The expression of α -SMAGFP was observed in context of the following combinations; a) α -SMAGFP⁺, anti-mouse CD45R-APC (clone RA3-6B2) and anti-mouse CD11b-PE (clone M1/70); b) α -SMAGFP, anti-mouse CD45R-APC, anti-mouse CD117-PE (clone 2B8); c) α -SMAGFP, anti-mouse CD45R-APC, anti mouse Sca1-PE or d) α -SMAGFP, anti-mouse CD45R-APC and anti mouse CD90-PE. All antibodies reagents were purchased from commercial sources (Pharmingen and eBioscience). Flow cytometry was done on a BD FACS Cibur cytometer and data were processed using Cell Quest software.

Cell sorting and transplantation procedure

Cell sorting—Offspring of dual transgenic mice were established by breeding the α -SMAGFP(green) with Col2.3GFPcyan (blue) to have the ability to identify populations of progenitor cells by expressing only SMAA-GFP and to follow their differentiation into

osteoblast lineage by activation of Col2.3GFPcyan. ADSC from dual transgenic mice were cultured for 7 days to expand a population of progenitors and then were prepared for cell sorting to selectively purify α -SMAGFP⁺/Col2.3cyan⁻ populations. The population of interest was sorted by FACS using a BD Vantage SE with FACSDiva option (BD Biosciences). After sorting, purity of the isolated population was assessed.

Preparation of recipient mice—Wild type recipient mice received a lethal dose of 900 cGy total body gamma irradiation (TBI) using a ¹³⁷Cs source (Nordion Gammacell 40 Irradiator; Atomic Energy of Canada, Ottawa, Canada), with a dose rate of 66.5 cGy/min. Rescue of the hematopoietic system of irradiated mice was performed by systemic infusion of $2.5 \times 10^6/100\mu\text{l}$ of donor total bone marrow cells derived from GFP negative (TBMc) into the retro-orbital sinus of anesthetized animals.

Transplantation procedure—Local injection into the medullary space of the tibia or femur was performed in ketamine/xylazine-anesthetized recipient mice. The knee was flexed to 90° and a 26-gauge needle was inserted into the joint surface of the femur or tibia through the patellar tendon and then inserted into the medullary space. The needle was removed and another 26-gauge needle attached to a 1-mL syringe was inserted into the needle tract and extended into the bone marrow space. Donor cells (α -SMAGFP⁺), $5 \times 10^5/20\mu\text{L}$ were slowly injected into the marrow cavity as the needle was being withdrawn from the intramedullary space [26]. Four wild type mice were used to evaluate the osteogenic potential of α -SMAGFP⁺ cells. To detect new bone formation in relation to the specific donor cell population, mice received a 100 μL intraperitoneal injection of freshly prepared xylenol orange (XO; 30 mg/ml, 90 mg/kg). Images were obtained using FITC/Texas Red and Cyan/Texas red dual cube as previously described [21].

RESULTS

Stimulating expansion of osteoprogenitor cells

We utilized a previously developed transgenic mouse model of osteoblast ablation and recovery to stimulate a defined wave of new bone formation. Specificity of the ablation was achieved by directing the HSV thymidine kinase gene to mature osteoblast lineage cells by using a 2.3kb fragment of type I collagen gene (Col2.3 Δ tk) [16]. Following 16 days of ganciclovir (GCV) treatment the osteoblast lineage was completely depleted. In this study we assessed the recovery process and examined the origin of cells and their progression toward a mature osteoblast. As shown on figure 1a, (day 0-day 21), the process of osteogenic regeneration is extensive and three weeks into recovery numerous trabeculae are observed extending into diaphyseal bone. Volumetric imaging by X-ray CT (μCT) analysis reveals process of bone regeneration indicating the proximity of the endosteal surface as an area where new centers of bone formation have occurred (figure 1b). At day 12 into recovery an indication of new bone formation was detected as budding of mineralized bone on the endocortical surface (suppl. figure 1). To examine the activation of osteoblast lineage during regeneration we analyzed the gene expression of markers known to be active at certain stages of osteogenic maturation. Following GCV treatment we detected almost a complete disappearance of all markers of mature osteoblast lineage (Col1a1, BSP, OP and OC). During an early phase of recovery (day 7) increases in the expression of Col1a1 and BSP were detected that peaked at day 14, while the expression of osteocalcin was detected at a later stage (day 14) and peaked at day 21. This response is indicative of the process of bone repair and stage-related lineage progression (figure 1c). In addition following ablation of the osteoblast lineage we detected the presence of numerous α -SMA⁺ cells in the treated bones. In comparison with GCV untreated bone (suppl. figure 2a) in which α -SMA is detected only at the capillary walls, large populations of α -SMA⁺ cells were present following termination of the treatment and at seven days of recovery

(suppl. figure 2b–c). As recovery progressed areas previously abundant with α -SMA⁺ cells were replaced with matrix-producing osteoblasts (suppl. figure d–e, arrows) and woven bone (suppl. figure 2j–k). After four weeks into the recovery period, expression of α -SMA receded and areas previously populated with α -SMA⁺ cells were fully replaced with new bone (suppl. figure 2f,l).

Activation of osteoblast lineage GFP markers during early phase of lineage reactivation

The sequential activation of osteogenesis provides an opportunity to assess the lineage progression utilizing visual markers of osteogenic differentiation. We have previously characterized the expression pattern of pOBCol3.6GFP (Col3.6GFP) as a preosteoblastic marker, while pOBCol2.3GFP (Col2.3GFP) selectively identifies mature osteoblasts and osteocytes [17,27]. We have generated crosses of Col2.3 Δ tk transgenic mice with the Col3.6GFP or Col2.3GFP as indicated in figure 2a, and followed the activation of GFP transgenes during the recovery process. Following termination of GCV treatment, osteoblasts on the endocortical surface that were Col3.6GFP⁺ were completely absent (figure 2b–c, indicated by arrow). Similarly, Col2.3GFP⁺ osteoblasts were completely ablated by the treatment (figure 2d–e, indicated by arrows). In analysis of early phase of the recovery process we evaluated the appearance of the GFP transgenes at day 7 using paraffin embedded samples. The appearance of the preosteoblastic marker Col3.6GFP was detected within the bone marrow in areas close to the endocortical and trabecular surfaces. Localization of Col3.6GFP cells in vicinity to the blood vessels was also observed (figure 2f–i, arrows). Col2.3GFP expression was restricted to areas on the bone surface associated with more mature osteoblasts (figure 2j–k, arrows).

Activation of osteoblast lineage transgenes Col3.6GFP and Col2.3GFP

Validity of the concept that α -SMA⁺ cells are potential osteoprogenitor cells was evaluated in two dual transgenic mouse models; Col3.6GFP/Col2.3 Δ tk and Col2.3GFP/Col2.3 Δ tk by correlating α -SMA expression with preosteoblastic Col3.6GFP or osteoblastic Col2.3GFP activity. Following termination of treatment and into the first week of recovery we detected co-expression of α -SMA immunostaining and Col3.6GFP fluorescence, predominantly localized within the bone marrow space (figure 3a–d, indicated by arrows). A population of 44 \pm 13% (n=3) α -SMA⁺ cells also expressed Col3.6GFP (dual α -SMA/Col3.6GFP). In addition majority of Col3.6GFP⁺ cells were also α -SMA⁺ (91 \pm 2%). This observation is indicative of commitment towards the early stage of an osteogenic pathway. In contrast, at 7 days after treatment, isolated areas of new matrix deposition and Col2.3GFP signal were observed (figure 3e–h). These areas contain osteoblasts that are negative for α -SMA. Only an occasional cell showed dual expression for Col2.3GFP and α -SMA in the areas of rapid bone formation (data not shown). Collectively, these data indicate that as preosteoblast differentiation progresses into more mature stages of the lineage, SMA expression decreases.

Evaluation of SMAA directed transgene expression

We have acquired transgenic mice in which a 1.1 kb of α -SMA promoter drives a GFP transgene [19,20,28]. A detailed analysis of α -SMAGFP expression was completed in osseous (supplementary figure 3) and non-osseous tissues (supplementary figure 4) in relation to the endogenous α -SMA expression. Expression was observed in cells associated with blood vessels and capillaries and in the myofibroblastic cells located in areas containing osteoprogenitor cells such as cranial sutures and periosteum. A close correlation in expression of α -SMAGFP with endogenous α -SMA expression was observed in all tested tissues. At six weeks of age, few α -SMAGFP⁺ cells are detected in the periosteum of long bone in association with blood vessels (supplementary figure 5).

In vitro analysis of differentiation potential of α -SMAGFP⁺ cells

The differentiation potential of α -SMAGFP⁺ cells was evaluated using primary tissue culture system. Bone marrow stromal cell cultures and adipose derived stromal cell were prepared from α -SMAGFP transgenic mice. Following expansion and separation of cell populations by FACS with purity >99%, cells were induced to osteogenesis and adipogenesis (figure 4a). Sorted α -SMAGFP⁺ cells continued to proliferate and differentiate into osteogenic and adipogenic lineages with strong expression of ALP, mineralization or lipid vacuoles. The α -SMAGFP⁻ population remained ALP negative, with no detectable mineralization (figure 4a–b). α -SMAGFP⁺ cells derived from adipose tissue reached confluence and showed evidence of osteogenic differentiation (figure 4c–d). A large proportion of α -SMAGFP⁻ population was CD31⁺, indicating the presence of endothelial cells (data not shown).

Defining the α -SMAGFP⁺ population derived from adipose tissue

The ability of the α -SMAGFP⁺ population derived from adipose tissue to generate osteoblasts in vitro directed us to more clearly define the in vivo origin of these cells and their characteristics. To localize the α -SMAGFP⁺ cells, inguinal fat pads were frozen-embedded and cryosectioned. Immunostaining for endothelial marker CD31 indicated a close proximity of α -SMAGFP⁺ cells to the endothelial cells associated with the microvasculature in the adipose tissues (figure 5a). No GFP signal and no CD31 staining were detected in adipocytes. Collagenase digested adipose tissue yielded between 1–3% of cells expressing α -SMAGFP marker, of which a high proportion (>50%) were Sca1⁺ and Thy1⁺ (data not shown). In vitro expanded populations of GFP positive cells were analyzed at an early stage of the primary culture. To obtain a better definition of α -SMAGFP⁺ cells, flow cytometry analysis of 6-day old cultures was done. Detection of GFP transgene was combined with immunolabeling for CD45, Sca-1, CD90, CD117, and CD11b. The α -SMAGFP⁺ cells accounted for 79±6% of the cell culture (figure 5b), were CD45⁻ and did not express CD117 or CD11b (GFP⁺/CD45⁻/CD117⁻/CD11b⁻). Large proportions of α -SMAGFP⁺ cells were Sca1⁺ and Thy1⁺, indicative of a presence of the mesenchymal progenitor population within the α -SMAGFP⁺ cells (figure 5c). Following evidence that adipose-derived α -SMAGFP⁺ cells have the ability to differentiate into osteoblast lineage in vitro, our goal was to utilize the promoter-GFP transgenic approach that would confirm these observations using an in vivo transplantation model. Dual GFP transgenic mice harboring α -SMAGFP(green) expression and bone-specific Col2.3CFP(blue) directed expression were utilized in this analysis. Seven day old primary adipose stromal cell culture expressing α -SMAGFP(green) was separated from both GFP⁻ and dual GFP expressing cells. A high degree of purity of isolated α -SMAGFP⁺ cells (>99%) was obtained by FACS and in vitro evaluation of GFP expression. (figure 6a–c). Cells were placed in culture to recover from the sorting procedure, and after 48 hours α -SMAGFP⁺ cells were transplanted into the bone marrow cavity of irradiated wild type mice. We find this recovery period necessary, as a high proportion of cells can be damaged during the sorting procedure and this can subsequently affect osteogenic ability after in vivo transplantation. Following transplantation of α -SMAGFP⁺ cells, new bone formation was detected after 4 weeks as indicated by freshly deposited xylenol orange (red) label that was injected 24 hours prior to sacrificing the animals. We can clearly identify a Col2.3CFP (blue) positive cell population lining the newly formed bone matrix (figure 6d–e). Although adipose tissue has already been utilized as a valuable source of osteoprogenitor cells, these results provide a more clearly delineated definition of the pericyte/myofibroblasts within adipose tissue as a population of cells capable of differentiating into functional osteoblasts.

DISCUSSION

During the last decade the term adult-derived stem cells has become very popular in regenerative biology. Cells with various differentiation properties can be obtained from a

variety of tissue sources including bone marrow [29], dental pulp [30], muscle [31], adipose tissue [32], tendon [33,34] and hair follicles [35]. Furthermore, it has been shown that cells residing within subcutaneous tissue or muscle can differentiate into mature osteoblasts following implantation of BMP in collagen gels or in blood clot as a carrier [36,37]. It is intriguing that there is a wide tissue distribution of cells with mesenchymal progenitor properties, indicative of a common progenitor population residing in these tissues. These adult tissue-derived mesenchymal progenitors exhibit morphology, immunophenotype, and growth properties similar to bone marrow derived mesenchymal stem cells [38]. A perivascular niche has been implicated as a source of the mesenchymal progenitor by a number of laboratories [30,39,40]. Studies have shown that when placed in vitro, retinal pericytes can generate mineralizing nodules [41], chondrocytes and adipocytes [42].

In this study we have defined a population of α -SMA expressing cells as osteogenic precursor cells. The α -SMA cells were localized to a perivascular niche, periosteum, and suture; areas reported to contain osteoprogenitor cells. The α -SMAGFP⁺ cells derived from primary bone marrow stromal and adipose-derived stromal cells exhibit both osteogenic and adipogenic potential in vitro. However, the expression of α -SMA is not specific to pericytes; it is also present in vascular smooth muscle cells of larger blood vessels. This finding increases the complexity in studying this population of cells, and indicates the importance of the generation of new markers that would be pericyte restricted. A potentially successful approach would require generation and characterization of monoclonal antibodies against isolated α -SMAGFP⁺ cells. This could lead to the identification of cell surface markers that, in combination with known markers, would permit better identification and characterization of the pericytes.

The results of our study indicate that the expansion of α -SMA positive cells within the bone marrow itself is detected following ablation of the osteoblast lineage. Localization of the α -SMA⁺ cells is first observed in proximity to the endocortical and trabecular surfaces, areas of bone that are rich in capillary network [43,44]. The activation of Col3.6GFP transgene in a portion of the α -SMA⁺ cells was observed by dual fluorescent imaging. Supporting in vitro data obtained from marrow stromal cells of dual transgenic mice expressing α -SMA and Col3.6GFP revealed that activation of Col3.6GFP in α -SMA is an indicator of preosteoblastic differentiation [45]. In addition a microarray study obtained from primary calvarial osteoblast cultures indicated that the transition from Col3.6GFP⁻ to Col3.6GFP⁺ population is characterized by a decrease in expression of a number of genes associated with the myofibroblastic phenotype [27,45]. Furthermore, we can occasionally detect a rare population of dual expressing α -SMAGFP⁺/Col2.3GFP⁺ cells as a transitional stage to mature osteoblasts. This finding is observed in primary bone marrow stromal cell cultures and may be explained by the long half life of GFP that allows for the detection of Col2.3GFP while the α -SMAGFP signal becomes weaker (data not shown).

Collectively, our study indicates that expanded α -SMA⁺ cells can actively participate in new bone formation following an induced injury to the osteoblast lineage. Other studies have reported that α -SMA⁺ myofibroblasts can participate in fibrotic changes of various tissues following different kind of injuries [46]. Myofibroblasts responsible for lung fibrosis are potentially derived from perivascular cells [46,47] and fibrotic changes in liver as a wound healing response to various injuries are also based on myofibroblastic response [48]. Similarities of these populations exist in localization to the perivascular region and expression of the α -SMA gene (supplementary figure 3–5) [49]. The physiological relevance of α -SMA⁺ cells as osteoprogenitors must be further studied. A Col2.3 Δ tk model represents a specific injury to the lineage, and a response of α -SMA⁺ cells in the form of osteogenic differentiation could be a characteristic of this specific tissue damage. The use of dual transgenic mice will allow the separation of cells at a point of activation of the preosteoblastic

Col3.6GFP transgene, enabling analysis of the regulation of osteogenic gene profiles toward elucidation of the pathways regulating the mechanism of osteoprogenitor induction. A recent publication by Sacchetti et al. defined a cell population of osteoprogenitors as MCAM/CD146⁺ subendothelial cells in human bone marrow stroma [13,50]. The approach utilized in their study adds validity to observations that perivascular cells exhibit the osteoprogenitor ability supportive of hematopoiesis [13].

Tissues other than bone marrow contain perivascular progenitor cells. From a tissue regeneration standpoint, adipose tissue represents an attractive, easily accessible, and abundant source of pericytes.

Recent study by Zannettino et al. presented data describing a presence of multipotential cell population within adult human adipose tissue, which appears to be intimately associated with perivascular cells [51]. We have characterized a population of α -SMAGFP⁺ cells residing in adipose tissue by cell surface markers currently used as characteristic epitopes of mesenchymal progenitor cells. A high proportion of α -SMAGFP⁺ cells isolated from freshly digested adipose tissue were positive for the stem cell markers [52–54], stem cell antigen 1 (SCA-1⁺) and CD90⁺, while they did not express CD117 (c-kit) or CD11b (Mac1) (data not shown). Following a brief culturing period, population of α -SMAGFP⁺/Sca1⁺ and α -SMAGFP⁺/CD90⁺ cells were expanded and represented 75±12% and 59±13% of cells respectively. To substantiate our observation that the α -SMAGFP⁺ cells are osteoprogenitors, we completed an in vivo study that showed evidence that adipose derived α -SMAGFP⁺ cells were able to differentiate into functional osteoblasts. This finding is consistent with previously published reports in which the osteogenic potential of unseparated adipose derived stromal cells was detected [32,55]. Our study defined the pericyte as a population of cells that resides in adipose tissue in close proximity to endothelial cells and exhibits in vitro and in vivo ability to generate osteoprogenitor lineage. These characteristics, indicate that adipose tissue derived α -SMAGFP cells have the potential to be utilized for bone tissue regeneration. Future studies will determine if the population of myofibroblast/pericytes is responsible for the ectopic calcification occurring in tendon and muscle in patients with fibrodysplasia ossificans progressiva[56] or for the other forms of tissue calcifications [57–59].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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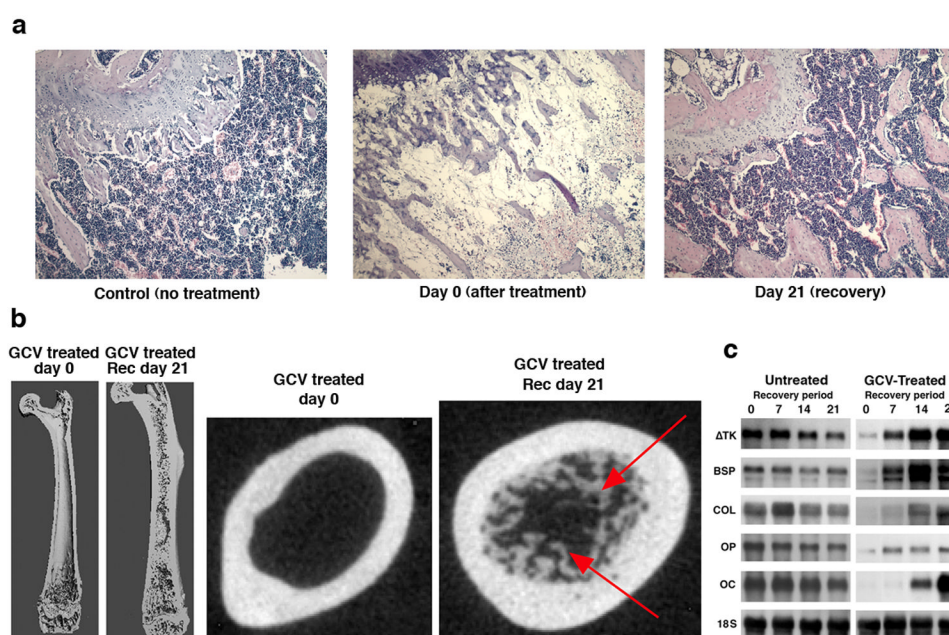


Figure 1. Using a transgenic model Col2.3Δtk for expansion of osteoprogenitor cells

(a) Hematoxylin eosin staining of sections of femurs derived from Col2.3Δtk transgenic mice (control, no treatment), following osteoblast ablation (day 0, after treatment), and after three weeks of recovery from the treatment (day 21, recovery). (b) microCT images of whole femur and diaphyseal cross-sections at day 0 and 21 days after the termination of GCV treatment, (arrows indicate new bone formation arising from endocortical surface of the bone). (c) Northern blot analysis of osteoblast lineage differentiation associated genes. Untreated, or GCV treated mice were evaluated at time points after the termination of treatment (day of recovery from treatment 0, 7, 14, 21) which corresponds to the age of 8–11 weeks. During this period, rapid growth begins to slow, a process that is represented by decreases in expression of Col1a1, osteocalcin (OC), bone sialoprotein (BSP), and transgene Col2.3Δtk as well. Treatment with GCV completely abolished the expression of bone markers, while during the process of recovery an increase in Col1a1 was observed, followed by an increase in BSP and then OC. This activation of osteoblast lineage markers indicates gradual differentiation of osteoprogenitor cells into mature osteoblasts.

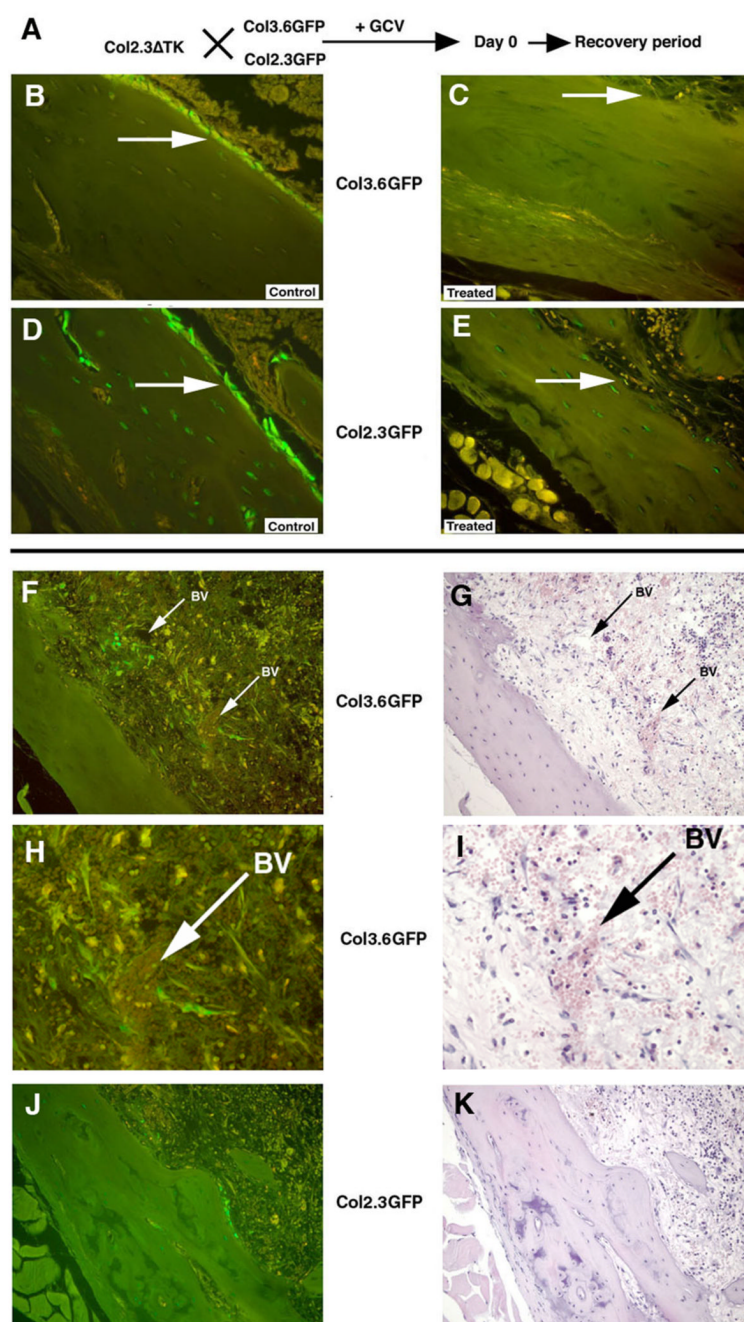


Figure 2. Use of osteoblast lineage GFP transgenes to trace osteoprogenitor lineage activation
 Upper panel. (a) Breeding procedure required crossing Col2.3Δtk with Col3.6GFP or Col2.3GFP transgenic mice. (b–c) Expression of Col3.6GFP transgene as untreated (b) and after GCV treatment (c); arrows indicate a layer of osteoblasts lining endocortical surface (b) or their complete absence after GCV treatment (c). (d–e) Expression of Col2.3GFP is localized to osteoblasts and osteocytes without treatment (d) or absent in osteoblasts after the treatment, indicated by the arrows (e).
 Lower panel. (f–k) GFP expression at 7 days after the termination of treatment. (f–g) Col3.6GFP expression localizes within bone marrow in the proximity to the endocortical surface and in association to the blood vessels (bv, indicated by arrows). (h–i) higher

magnification of Col3.6GFP expression. (j–k) Col2.3GFP is detected in pockets lining endocortex, but it was not detected within the bone marrow.

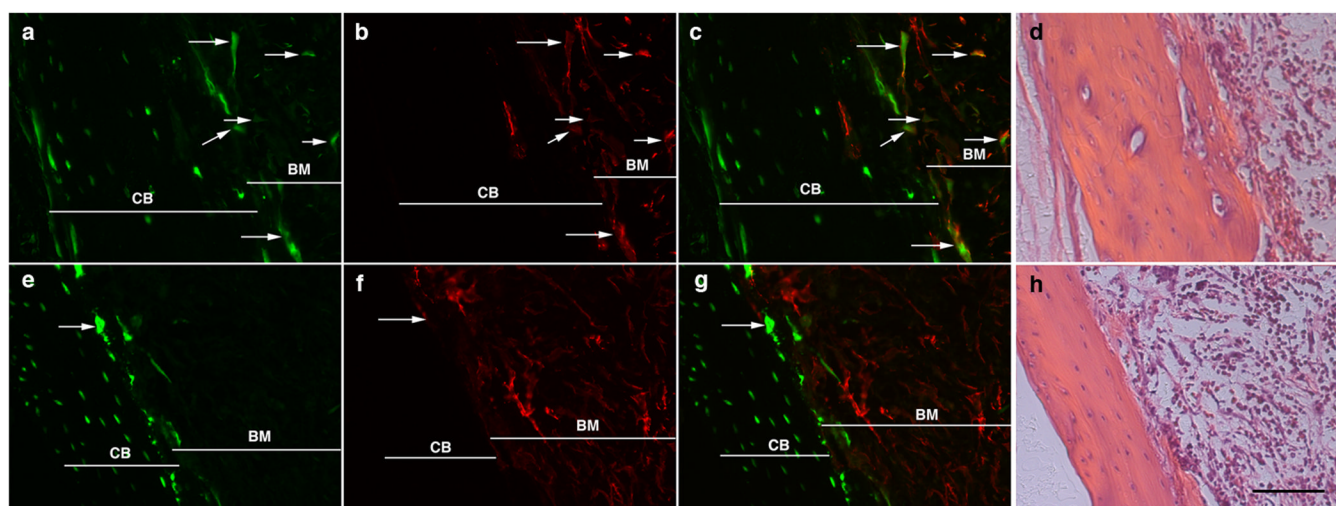


Figure 3. Colocalization of α -SMA with Col3.6GFP transgenes

During the phase of recovery, activation of Col3.6 and Col2.3GFP was observed. To indicate the activation of Col3.6GFP transgene in cells with the expression of α -SMA we utilized dual transgenic mice (Col2.3tk/Col3.6GFP, a–d) or (Col2.3tk/Col2.3GFP, e–h) in combination with immunostaining for α -SMA and HE staining (d,h). Col3.6GFP activates in the bone marrow (BM) in cells that express Col3.6GFP (a–c; indicated by the arrows). The expression of Col2.3GFP was restricted to the osteocytes inside the cortical bone (CB), and it does not colocalize with the α -SMA expressing cells (e–g, see arrows). Bar = 200 μ m.

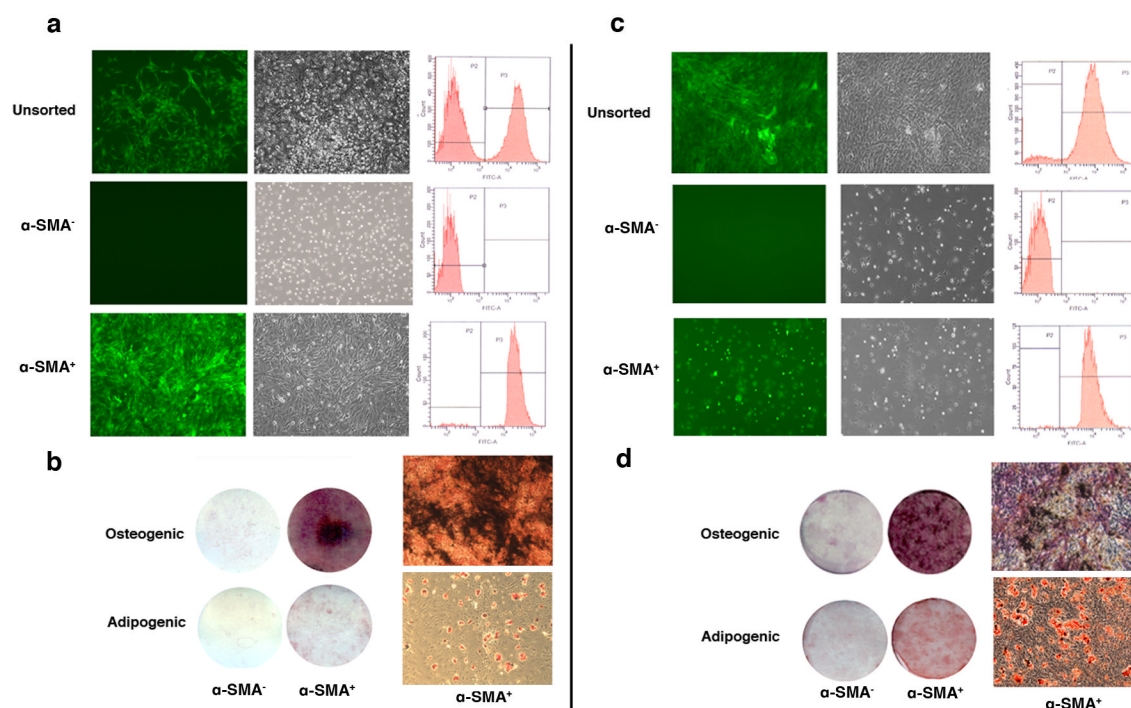


Figure 4. In vitro analysis of differentiation potential of α -SMA-GFP⁺ cells

(a) primary bone marrow stromal cells cultured for 7 days before FACS sorting (unsorted), and α -SMA-GFP⁺ and GFP⁻ population after sorting and replating. Flow cytometry diagrams show reanalysis of purity of populations. From 50% of α -SMAGFP⁺ cells, highly pure populations of α -SMAGFP⁺ and GFP⁻ cells were isolated. (b) Following separation, cells were replated and placed under osteogenic or adipogenic inductive conditions. Cultures were stained for alkaline phosphatase, and for mineralization by the von Kossa silver nitrate method to detect osteogenesis, and by oil-red-0 staining for presence of lipid vacuoles. Osteogenic and adipogenic differentiation was present in α -SMAGFP⁺ cells while α -SMAGFP⁻ cells did not progress towards differentiated phenotypes. (c) The same populations were separated from primary adipose derived stromal cells cultures. (d) Similar to BMSC adipose derived stromal α -SMAGFP⁺ cells exhibited the ability to differentiate into osteoblasts (ALP⁺ and mineralized nodules) and adipocytes (oil red).

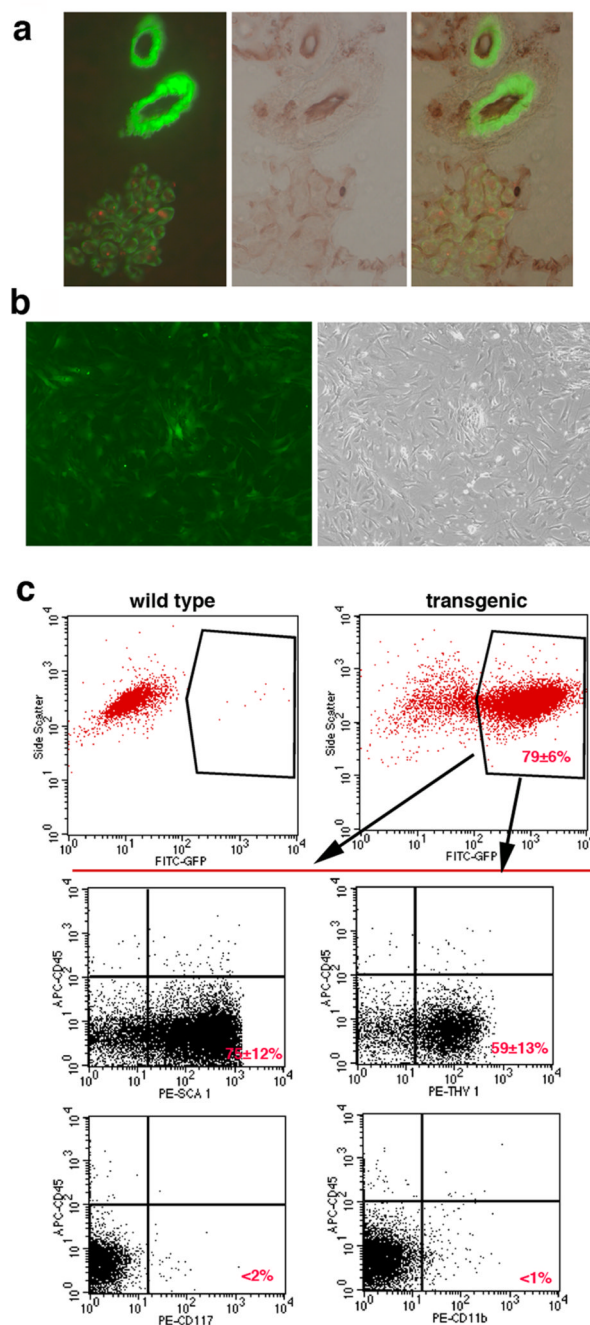


Figure 5. Phenotype of α -SMAGFP⁺ cells residing in adipose tissue

(a) Localization of α -SMAGFP⁺ cells (left panel) in relation to the CD31⁺ cells (immunohistochemistry, middle panel) and composite image (right panel) indicates close proximity of α -SMAGFP⁺ cells to the endothelial cells. (b) Epifluorescence and brightfield images of 7 day old primary cultures derived from adipose tissue indicates high proportions of cells expressing GFP. (c) Flow cytometry analysis of α -SMAGFP⁺ cells derived from adipose tissue cultures. An average of 79±6% of cells are α -SMAGFP⁺ (non-GFP expressing cells were used as negative control). Profiling of adipose derived cells was done using antibodies for CD45, Sca-1, CD90, CD117 and CD11b. α -SMAGFP⁺ cells were CD45⁺,

CD117⁺ and CD11b⁺ and positive for Sca-1⁺ (75±12%), CD90⁺ (59±13%). Representative plots derived from seven individual cultures are shown.

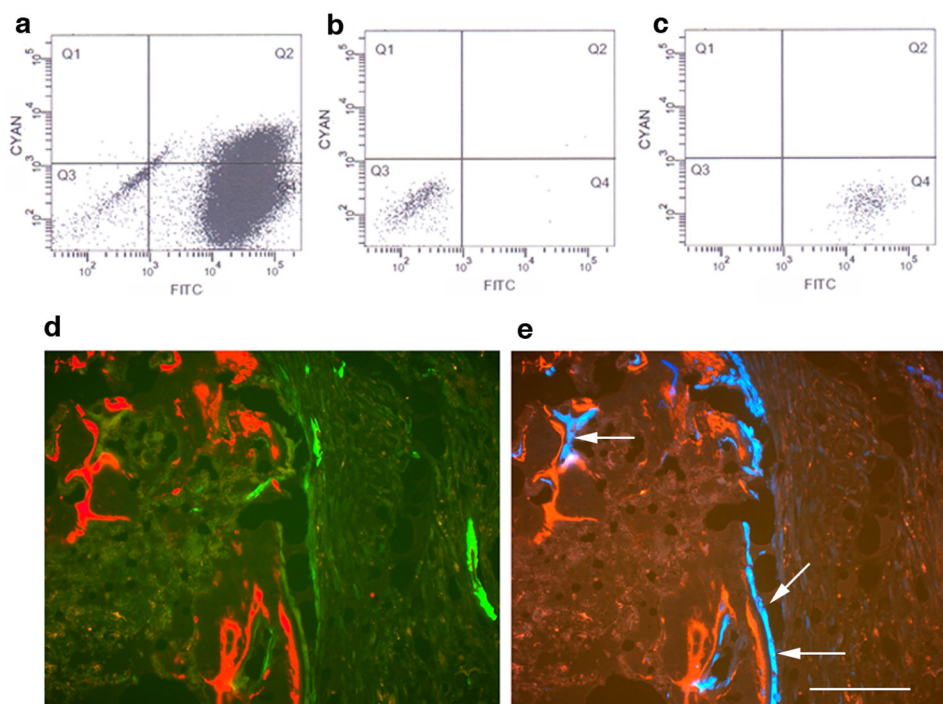


Figure 6. In vivo analysis of osteogenic differentiation of adipose derived α -SMAGFP⁺ cells (a–c) Isolation of α -SMAGFP⁺ cells from adipose derived stromal cells cultures from dual transgenic mice (α -SMAGFPgreen/Col2.3GFPcyan); (a) analysis prior to cell separation, (b) reanalysis of α -SMAGFP[−] and (c) α -SMAGFP⁺ population for post sorting purity. (d–e) Histological sections derived from bones transplanted with sorted α -SMAGFP⁺ cells. Weak expression of α -SMAGFP (green) was observed in fibroblastic cells (d) while a strong GFPcyan (blue) was detected in newly formed bone (e, see arrows) localized along the deposition of xylene orange (red color). As sorted cells were only α -SMAGFP⁺, this provides supporting evidence that this population of cells contains cells with osteogenic potential. Bar = 100 μ m.