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Utilization of transgenic models in evaluation of osteogenic differentiation of embryonic stem cells

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Abstract

Previous studies reported that embryonic stem cells (ESCs) can be induced to differentiate into cells showing a mature osteoblastic phenotype by culturing them under osteo-inductive conditions. It is probable that osteogenic differentiation requires that ESCs undergo differentiation through an intermediary step involving a mesenchymal lineage precursor. Based on our previous studies indicating that adult mesenchymal progenitor cells express αSMA, we have generated ESCs from transgenic mice in which an αSMA promoter directs the expression of red fluorescent protein (RFP) to mesenchymal progenitor cells. To track the transition of ESC-derived MSCs into mature osteoblasts, we have utilized a bone-specific fragment of rat type I collagen promoter driving green fluorescent protein (Col2.3GFP).

Following osteogenic induction in ESCs, we have observed expression of alkaline phosphatase and subsequent mineralization as detected by von Kossa staining. After one week of osteogenic induction, ESCs begin to express αSMARFP. This expression was localized to the peripheral area encircling a typical ESC colony. Nevertheless, these αSMARFP positive cells did not show activation of the Col2.3GFP promoter, even after 7 weeks of osteogenic differentiation *in vitro*. In contrast, Col2.3GFP expression was detected *in vivo*, in mineralized areas following teratoma formation.

Our results indicate that detection of alkaline phosphatase activity and mineralization of ESCs cultured under osteogenic conditions is not sufficient to demonstrate osteogenic maturation. Our study indicates the utility of the promoter-visual transgene approach to assess the commitment and differentiation of ESCs into the osteoblast lineage.

Keywords

Embryonic stem cells; mesenchymal stem cells; differentiation; osteogenesis; GFP; teratoma

Introduction

Embryonic stem cells (ECSs) are capable of differentiating into cells and tissue belonging to all three germ layers. When injected into adult mice, ESCs produce teratomas containing a

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wide variety of mature cell types. Although this differentiation ability provides enormous regenerative potential, it also poses a major challenge to understand the steps and controlling mechanisms that can direct ESCs towards specific lineages [1].

In contrast to embryonic stem cells, the ability of adult mesenchymal stem cells (MSCs) to generate bone is well characterized. The availability of adult-derived stem cells, and reports of their successful use in transplantation approaches have stimulated the recent expansion in the tissue regeneration field [2–4]. Protocols in regenerative medicine utilize different sources of progenitor cells, including adipose-derived stem cells (ADSC) or bone marrow-derived stem cells (BMSC), in conjunction with osteoinductive factors and different carriers that provide three-dimensional scaffolding [5–8]. Approaches utilizing ADSC or BMSC represent a major new therapeutic direction for treatment of musculoskeletal diseases [9–12]. However, despite the advantages of adult stem cells, therapeutic approaches using these cells also face significant obstructions, due to the high cost of cell isolation, the limited proliferation potential of these cells, and regulatory requirements for harvesting, and *in vitro* expansion procedures. In contrast, once appropriate differe6ntiation protocols have been developed, ESCs have the potential for generating large numbers of skeletal progenitor cells. It has been proposed that organized banks of ESCs can be established that contain cell lines with the majority of possible HLA types to serve as a source of donor cells to treat the majority of patients.

Recent studies have reported the ability to differentiate mouse or human ESCs into osteoblasts [13–17]. However, when osteogenic differentiation of ESCs is contrasted with BMSC, limited osteogenecity of ESCs is observed. This is clearly evident in the *in vivo* studies in which transplanted MSCs showed bone forming potential, while ESCs did not [18]. Given the potential advantages of ESCs for bone regeneration applications, it is critical to carefully characterize their osteoblast lineage differentiation potential. A long term goal of our studies has been to identify markers of different stages of osteogenic differentiation from MSCs, and to develop a system in which different stages of differentiation can be identified and isolated using visual promoter-transgene reporters. To identify cells within the osteoblast lineage, we have utilized distinct promoter fragments of the rat Col1a1 gene to drive GFP transgenes. These include Col3.6GFP, which becomes active at the preosteoblast stage, and Col2.3GFP, which activates at the mature osteoblast stage [19]. To apply our experience in utilizing visual markers that can identify MSCs, and reflect different stages of osteogenic lineage differentiation to analyze osteoblastic differentiation of ESCs, we have generated ESCs derived from transgenic mice in which an α-smooth muscle actin (αSMA) promoter directs the expression of red fluorescent protein (RFP) to adult mesenchymal progenitor cells [8, 20]. To track the transition of ESC-derived MSC into mature osteoblast lineage cells we have used Col2.3GFP [19]. Osteogenic differentiation of the adult MSCs and the ESCs derived from dual transgenic mice was evaluated *in vitro* and using *in vivo* functional assay of teratoma formation from ESCs.

Materials and Methods

Transgenic mice

Mice transgenic for αSMA promoter driving RFP-cherry reporter gene (αSMARFP) and pOBCol2.3GFP mice have been previously developed and characterized [19, 20]. Teratoma formation assays were performed using 3–4 month old NOD/SCID/interleukin 2 receptor [IL2r] gamma (null) (NSG) mice as recipients [21].

Generation and culture of ESCs

Murine ESC lines were derived from αSMARFP/Col2.3GFP transgenic mice. Briefly, following mating, and detection of the plugs, pregnant mice were sacrificed. Blastocysts were dissected and embryonal stem cell lines were prepared by the UCHC-Gene targeting and transgenic facility [22]. Genotyping of ESCs was done using eGFP (5'-TCATCTGCACCACCGGCAAGC; 5'-AGCAGGACCATGTGATCGCGC) and RFP (5'-CCATCATCAAGGAGTTCATGC; 5'-TCTTGACCTCAGCGTCGTAGT) primers sets. ESCs derived from C57Bl6 non-transgenic mice were purchased (Life Technologies, Carlsbad, CA). ESCs were cultured on feeder cells (Mouse Embryonic Fibroblasts, mitotically inactivated using gamma-rays) in ESC medium: Dulbecco's modified Eagle's medium (DMEM, embryomax), containing 15% fetal bovine serum (FBS, HyClone, Logan, UT, USA), 1% penicillin-streptomycin (penicillin at 100 U/mL and streptomycin at 100 μg/mL, PS), leukemia inhibitory factor (LIF), sodium pyruvate, β-mercaptoethanol, non-essential amino acids and L-glutamine.

In vitro assay of osteogenic differentiation

Osteogenic differentiation using monolayer culture—ESCs were trypsinized (0.05% Trypsin/EDTA) and passaged onto 0.1% gelatin-coated plates for 4 days in DMEM High Glucose media supplemented with 20% FBS and 1% PS. Then cells were trypsinized and plated on gelatin-coated 6-well dishes (100,000 cells/well) and differentiated for 21 days in osteogenic media: αMEM medium supplemented with 10% FBS, ascorbic acid (50 μg/mL) and β-glycerophosphate (BGP) (8 mM) [13, 14].

Osteogenic differentiation of ESCs through embryoid body (EB) step—EBs were prepared using the “suspension culture in bacterial-grade dishes” method. ESCs were trypsinized (0.05% Trypsin/EDTA) and passaged onto 0.1% gelatin-coated plates for 2 days followed by replating in bacteriological-grade petri dishes for 6 days in ESC medium. EBs were dispersed with 0.25% trypsin–EDTA solution and the cells (100,000 cells/well) were transferred to 0.1% gelatin-coated 6-well tissue culture plates. Cells were then cultured for 4 days in DMEM 20% FCS, 1% PS followed by osteogenic induction (EB-D), or directly under osteogenic media (EB-O) [13, 23, 24].

Primary bone marrow stromal cells (BMSC)

Six- to eight-week-old mice were killed by CO_2 asphyxiation. Femurs and tibias were dissected from surrounding tissues. The epiphyseal growth plates were removed and bone marrow was collected by flushing bones with αMEM 10% FBS 1% PS with a 25-G needle. Single cell suspensions were prepared by passing the cells through an 18-G needle followed by filtration through a 70-μm cell strainer. Cells were plated in 100-mm culture plates at a density $10^6/cm^2$. On day 4, half of the medium containing non-adherent cells was replaced. On day 7 plates for cell sorting were washed, incubated for 15 min with 2.5% trypsin (Gibco, Life Technologies). Cells were sorted using BD Aria and replated at $5\times10^4/cm^2$ and induced to osteogenesis as previously described [8], or were plated as spot cultures of 1×10^5 cells per 100μl of media [25]. Following one week in basal conditions, osteogenic differentiation is induced by addition of ascorbic acid (50 μg/mL) and BGP (8 mM). Primary BMSCs were also grown under osteogenic conditions from day 7 to day 21 and RNA was harvested for analysis of ostegenic differentiation markers.

In vivo evaluation of osteogenic potential

Teratoma formation assay—ESCs derived from αSMARFP/Col2.3GFP mice were grown as a monolayer culture on gelatin. Following the second passage, 10^6 cells were injected into femoral muscle of NSG mice [26]. Formation of teratomas was analyzed six

weeks later. Following x-ray imaging, tissues were collected, dissected, fixed in 4%PFA, placed in 30% sucrose and embedded. Cryosections were obtained from tissues dissected from teratoma, and evaluated for expression of Col2.3GFP and for the presence of mineralized matrix.

Detection of epifluorescence

In vitro—GFP expression was visualized using an Olympus IX50 inverted system microscope equipped with IX-FLA inverted reflected light fluorescence (Olympus America Inc., Melville, NY). Cultures derived from dual αSMARFP/Col2.3GFP mice were imaged using following filters: GFP (excitation 500/20, emission 535/30) and RFP (excitation 560/40, emission 630/75) to separate GFP from RFP. Images were recorded with a SPOT-camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

In vivo—Tissues were fixed overnight and after 24 hours in sucrose they were embedded and sectioned. Sections of 5 μm were obtained using a Leica cryostat and tape transfer system. Images were obtained by appropriate filter cubes optimized for GFP variants (Chroma) using a Zeiss Observer. Z1 microscope. Images were obtained in grey scale, pseudocolored and composite images were assembled [20]. To obtain a full size image, tissues were scanned at high power and then stitched into a composite. Following fluorescent imaging sections were stained with von Kossa and reimaged.

Histochemical analysis of cell cultures

Histochemical staining for alkaline phosphatase (ALP) activity was performed using a commercially available kit (86-R Alkaline Phosphatase, Sigma Diagnostics, Inc. Saint Louis, MO, USA) according to the manufacturer's instructions. Mineralization was assessed using a von Kossa silver nitrate staining method and by Alizarin red staining. Images were acquired using a flat bed scanner and processed into a composite image using Adobe Photoshop [19].

RNA isolation and analysis of gene expression

We isolated total RNA, using the Trizol method according to the manufacturer's protocol (Invitrogen). The quality and quantity of RNA was analyzed by gel electrophoresis and spectrophotometry. RNA (1 μg) was used for first-strand cDNA synthesis, using Superscript III (Invitrogen) according to the manufacturer's protocol. For real-time PCR, 1 μl of 100× diluted cDNA was used for GAPDH amplification (Mm99999915_g1) and 1 μl undiluted cDNA was used for Taqman assays amplification for osteocalcin (Oc, Mm03413826_mH), bone sialoprotein (Bsp, Mm00492555_m1) Osterix (Osx, Mm00504574_m1), Runx2 (Mm00501584_m1), Type I collagen (Col1a2, Mm00483888_m1).

Results

Activation of promoter-transgene constructs in primary BMSC

To identify a population of cells that has mesenchymal progenitor potential, we have used a previously described trangenic mouse in which the αSMA promoter directs the expression of RFP. This transgene is active within a population of cells that have the ability to differentiate into mature osteoblast lineage cells *in vitro* and *in vivo*. To assess this capability we have used Col2.3GFP mice that have been shown to express the transgene within osteoblasts and osteocytes. The transition of mesenchymal progenitors into osteoblasts was evaluated in primary BMSCs. Cultures were established from αSMARFP/Col2.3GFP dual transgenic mice (Figure 1A) and grown under basal conditions for one week. During this period we observed a population of αSMARFP expressing cells (Figure 1B–C). Following

cell sorting, replated αSMARFP+ cells (20–30%) were cultured for 1 week under basal conditions (Figure 1D). Cells were induced to osteogenesis, and activation of osteoblast specific transgene Col2.3GFP confirmed osteogenic ability of αSMARFP+ cells. Strong expression of Col2.3GFP was observed when sorted αSMARFP expressing cells were replated as spot cultures and induced to osteogenesis (Figure 1E). Osteogenic differentiation was confirmed by detection of mineralization with von Kossa method (Figure 1F) and by up-regulation of markers of mature osteoblast lineage cells; osteocalcin and bone sialoprotein (Figure 1G).

Generation of ESCs from dual transgenic mice

We have generated ESCs from dual transgenic mice. Briefly, blastocysts were utilized to obtain inner mass cells and establish ESCs. Cells were grown on a fibroblast feeder layer in ESC media containing LIF. Due to the breeding of heterozygous αSMARFP to homozygous Col2.3GFP, we have genotyped ESCs using GFP and RFP specific primers. As indicated on Figure 2A, three different clones harboring both transgenes were obtained. Following cultivation without feeder cells or LIF-containing media, ESCs generate fibroblastic shaped cells that express αSMARFP. The αSMARFP signal was not detected within the ESC colony, but was very strong in the fibroblastic shape cells surrounding the ESC clone (Figure 2B).

Use of standard methods to detect osteogenesis by ESCs

Two protocols have been utilized to expand ESCs and to prepare them for osteogenic induction: a monolayer culture (Figure 3) and cultures that involve EB formation (Figure 4). We have followed previously published studies that report induction of osteogenesis of ESCs by addition of ascorbic acid and BGP. To assess osteogenic differentiation, conventional endpoints such as expression of alkaline phosphatase (ALP staining) and formation of mineralized matrix (von Kossa and Alizarin red staining) were evaluated. Seven-day old ESCs show ALP activity which becomes more robust by day 14 and day 21 (Figure 3B and Figure 4B). On day 21, mineral deposition can be observed by von Kossa staining and Alizarin red in a high proportion of ESC derived colonies (Figure 3B, 4B, Supplementary figure 1. In addition, osteogenic commitment and differentiation was assessed by the expression of a number of marker genes (Figure 5). Cells undergoing commitment to the osteogenic lineage would be expected to show up-regulation of master transcription factors Runx2 and Osterix, followed by increases in expression of bone matrix proteins and marker genes such as type I collagen (Col1a2), Bsp, and Oc as they differentiated into osteoblasts [19]. None of these genes were expressed in undifferentiated ESCs or at 7 days after induction of monolayer cultures of ESCs. By day 14 we can detect an increase in Bsp and Col1a2 expression. Runx2 was detected only at day 42 of culture. The only gene that showed increased expression during osteogenic induction was Bsp. The expression of Osterix and the mature osteoblast marker Oc was very low or not detected at any stage. To confirm that the levels of the bone marker genes showed only minimal increases, we evaluated the expression of these genes in the BMSCs at day 21 of culture under osteogenic conditions.

In another set of experiments we have evaluated differentiation of cells derived from digestion of the EBs. Cells were expanded for four days in DMEM medium (non-osteogenic conditions) prior to osteogenic induction (EB-D) or placed directly under osteogenic conditions (EB-O). Similar to the monolayer-derived cultures, only low levels of Col1a2 and Bsp were detected. All other markers show no or very low expression in contrast to the differentiated BMSCs.

Use of promoter-transgene to detect osteogenesis by ESCs

In osteogenic ESC cultures, expression of mesenchymal marker αSMARFP was observed in cells around ESC colonies, a population that expanded from day 7 to day 14 in both culture models (monolayer and from EB derived population) (Figure 3C and Figure 4C). To detect differentiation into mature osteoblasts we have used Col2.3GFP transgene. Despite the presence of mineralized colonies we did not detect expression of Col2.3GFP in cells derived from monolayer ESCs (Figure 3C). The expression of very few Col2.3GFP expressing cells in EB derived cultures was observed only in one of the four biological replicate cultures (Figure 4C). In order to confirm that the GFP transgenes did not affect differentiation of ESCs we have evaluated osteogenic differentiation of wild type (non-transgenic) ESCs using the monolayer method. These cultures show similar expression of ALP, and mineralization and also lack induction of osteogenic marker genes (Supplementary Figure 2).

To confirm that Col2.3GFP activation in this ESC line occurs, we have completed an *in vivo* teratoma assay. ESCs from dual transgenic mice were expanded and transplanted into the femoral muscle of immunodeficient mice. Presence of mineralized tissue was detected 6 weeks after implantation. Histological sections revealed a strong GFP signal in the areas that stain positive for mineralization by von Kossa (Figure 6). These data confirm that Col2.3GFP expressing cells can be generated from ESCs in the functional *in vivo* assay, and that this transgene can be utilized to confirm their osteogenic differentiation.

Discussion

To identify adult MSCs, a number of markers have been characterized. Most commonly used for human cells are CD146 [27] and STRO1 [28], while nestin [29], αSMA [20] and osterix [30] have been postulated to identify populations of murine MSCs within bone marrow. MSC commitment to the osteogenic lineage and stages of differentiation are well defined. Genes with master regulatory roles have been identified, including Runx2 and osterix, which are crucial for the regulation of osteogenic gene expression, and are markers of the osteoprogenitor stage. The commitment to the preosteoblast stage is characterized by expression of ALP, osteopontin, and Bsp, while terminal osteogenic differentiation can be evaluated by expression of Oc and dentin matrix protein-1. We have successfully utilized visual markers to identify different stages of the osteogenic lineage. Our previous results indicate that αSMA expression is characteristic of mesenchymal progenitor cells, while expression of the Col2.3 promoter is a marker for mature osteoblast lineage cells [20]. Utilizing dual transgenic mice and cell sorting we have shown that MSCs expressing αSMA, can differentiate and activate Col2.3GFP expression. To achieve osteogenic differentiation of ESCs, some procedures utilize generation of EBs, while others use a direct differentiation protocol [31, 32]. Osteogenic differentiation requires that ESCs undergo differentiation through a mesenchymal lineage precursor. These cells are known as ESC-derived MSCs, and they can be identified by functional and morphological criteria [17]. They can be grown on tissue culture plastic, and are defined by the expression of characteristic MSC surface markers, and their ability to undergo multi-lineage differentiation [33].

In the present study we aimed to evaluate the ability of ESCs to differentiate into cells of the osteogenic lineage. We have utilized conventional methods (ALP activity, mineralization and gene expression analysis), along with the use of visual markers of MSCs (αSMARFP) and osteoblasts (Col2.3GFP) [19]. Osteogenic differentiation of the adult MSCs and the ESCs derived from dual transgenic mice was evaluated *in vitro*, and *in vivo* by evaluating teratoma formation from ESCs. Following initial expansion of the ESCs, we have induced osteogenic differentiation and evaluated osteogenesis 1, 2 and 3 weeks later. Over this time course, BMSC cultures show a gradual increase in expression of bone markers [19]. In

addition, by week one, both cultures show ALP activity, and by 3 weeks both BMSCs and ESCs show evidence of mineral deposition. In addition, we have evaluated a later time point (day 42) to ensure that ESCs did not differentiate more slowly than BMSCs.

Although evidence for osteogenic differentiation of ESCs (mineralization and expression of osteogenic genes) has been shown by a number of research groups [13, 16, 32, 34, 35], a recent study by Both et al. indicated that both human and murine ESCs have impaired osteogenic response following *in vivo* implantation [18]. In line with these findings are our *in vitro* results, indicating that osteogenic differentiation of ESCs has to be carefully evaluated, as expression of ALP and the presence of mineralization are not sufficient criteria for osteogenecity of the ESCs. Osteogenic growth media are commonly supplemented with BGP, which undergoes hydrolysis by ALP, generating the phosphate required for bone mineralization. Standard differentiation protocols utilize high concentrations of exogenous phosphate (BGP), which has been reported to cause dystrophic deposition of mineral [36, 37]. In addition, we detected calcium deposition in ESC cultures by alizarin red staining confirming that detection of mineralization should be utilized only in conjunction with the expression of bone matrix specific genes such as osteocalcin. It may also be appropriate to confirm osteogenicity of cells using *in vivo* models such as ectopic bone formation or critical defect healing. A potential explanation for mineral deposition in cultures lacking osteoblasts could be a strong correlation between mineralization and cell death, as pathological mineralization has often been associated with apoptotic or necrotic processes *in vivo* [38]. As ALP is critical for this process, it is important to note that ESCs *in vitro* express ALP even prior to induction of osteogenic differentiation. This observation adds to the complexity of interpretation of the presence of the mineralization in cultures derived from ESCs.

In addition to ALP and mineralization, the expression of bone related genes has been utilized as a method to assess osteogenic differentiation of ESCs. Most studies utilize the expression of Runx2, osterix, type I collagen, Bsp and Oc, as molecular markers characteristic of osteoblasts. However, detecting a few fold increase in the magnitude of expression of these genes over the undifferentiated cultures that generally show low or undetectable levels of bone marker genes is not sufficient to demonstrate osteogenic differentiation. We have therefore contrasted the increase in differentiation markers by ESC cultures with the levels present in differentiated BMSCs. Although we can observe increases in expression in some of the genes analyzed (Col1a2, Bsp), most of them appeared minimal compared to the levels expressed by the BMSCs, even after seven weeks of osteogenic induction (Figure 5). In addition, we would expect stem cells undergoing osteogenic differentiation to initially up-regulate expression of transcription factors like Runx2 and osterix that are critical for lineage commitment, followed by increases in mature lineage markers, and this pattern was not observed in our ESC cultures. It is intriguing to observe the stronger increase in Bsp expression, which could be due to cells other than osteoblasts, including tissues that are derived from the embryo and participate in formation of the placenta [39].

In this study we have utilized a visual transgene system to assess osteogenesis. In previous work we have shown that the Col2.3 promoter is expressed in cells that show formation of mineral (osteoblasts) and cells embedded within bone matrix (osteocytes) [19, 40]. Although numerous other cells types express type I collagen, this 2.3kb fragment of rat type I collagen promoter has been shown to be preferentially active in the mature osteoblast lineage cells. By generating a visual reporter, cell differentiation can be visualized in real time without the need to terminate the cultures. Our evidence indicates that, in the dual transgenic ESC line that we have developed, bona fide induction of osteoblast differentiation would be demonstrated by induction of the Col2.3GFP marker.

The ESCs were expanded as monolayered culture. Following three weeks in differentiation media, we have not observed expression of Col2.3GFP when using monolayer cultures of ESCs, although we have detected expression of ALP and deposition of mineral. However, in one out of four experiments in which cells were derived from EBs, we detected a small number of cells expressing Col2.3GFP. Following detection of no or minimal Col2.3GFP expression *in vitro*, we were able to confirm that this transgene can activate in bone tissue derived from ESCs. During teratoma formation, bone formation was detected, with a strong activity of Col2.3GFP in areas that represent bone tissue. This confirmed the validity of the use of Col2.3GFP as a marker of osteoblasts when assessing differentiation of ESCs.

The results of our study underline the importance of using a cautious approach to assess osteogenic differentiation of ESCs. The detection of ALP staining and mineralization are not sufficient to establish the presence of mature osteoblasts. Robust osteogenesis is characterized by a tremendous increase in the expression of osteocalcin in particular, as seen in primary BMSCs [19]. The ESCs derived from αSMARFP/Col2.3GFP mice represent a model that can be used to assess osteogenesis of ESCs *in vitro* and *in vivo*. Further studies will have to develop more detailed protocols that will direct the osteogenic differentiation of ESCs, while using clearer criteria to evaluate the commitment to the osteogenic lineage and maturation to matrix-producing functional osteoblasts. To our knowledge, this study is the first to utilize the expression of Col2.3GFP transgene in the ESCs derived from transgenic mice. The use of ESCs that harbor Col2.3GFP transgene are an excellent model by which we can evaluate the osteogenic differentiation of ESCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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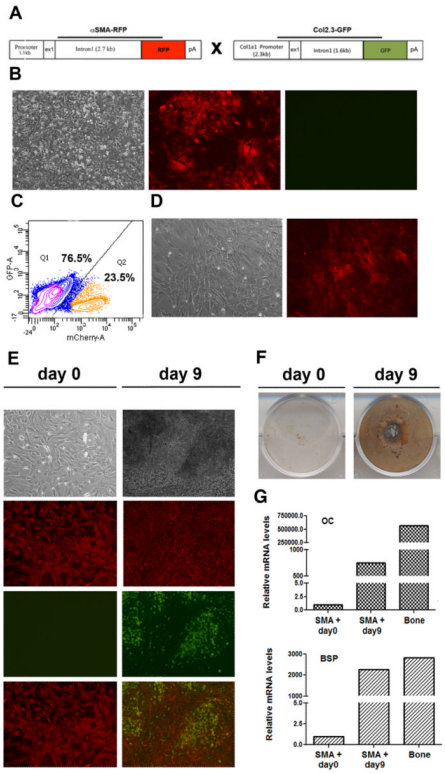

Figure 1. Osteogenic potential of primary bone marrow stromal cells

(A) Diagram of αSMARFP and Col2.3GFP transgenic constructs. (B) Images of day 7 primary BMSC cultures derived from dual αSMARFP/Col2.3GFP transgenic mice (brightfield, left panel; RFP, middle panel; GFP, right panel). C) FACS analysis confirming the activity of αSMARFP and absence of Col2.3GFP before sorting. D) Images obtained one week after sorting, prior to osteogenic induction, and E) nine days after osteogenesis was induced (brightfield images red (αSMARFP) and green fluorescence (Col2.3GFP) and overlayed images are shown). Mineralization was assessed with von Kossa method (F) and expression levels of mature osteoblast markers osteocalcin and bone sialoprotein were evaluated by real-time PCR (Figure 1G). Transgene activation and osteogenic differentiation was evaluated in three independent biological experiments and results from a representative experiment are presented.

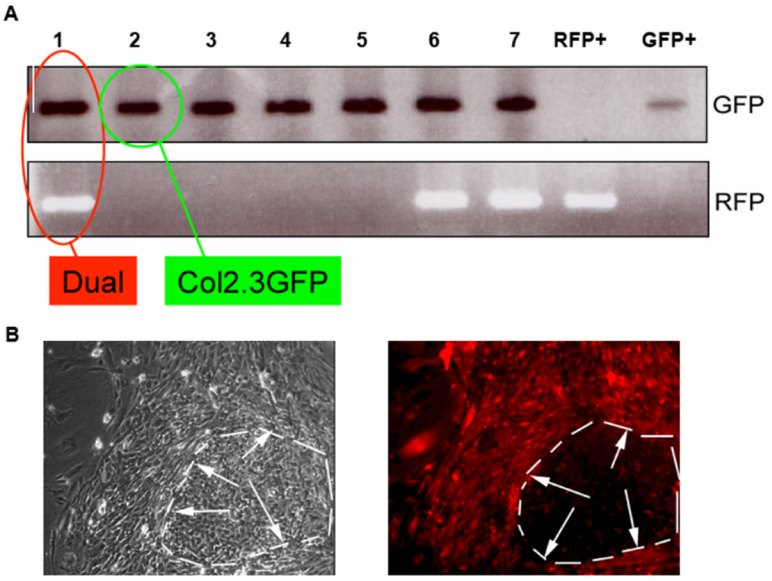

Figure 2. Generation of murine ESCs lines

A) By crossing αSMARFP × Col2.3GFP mice, we have generated dual transgenic ESC lines. Genotyping was done using a set of primers that distinguishes GFP from RFP sequences. B) Following induction of differentiation the αSMA expression was detected in cells surrounding ESC colonies, with no expression of αSMARFP within ESC colony. Morphologically αSMARFP+ cells exhibited fibroblastic shape. Edges between the two distinct populations are indicated using arrows.

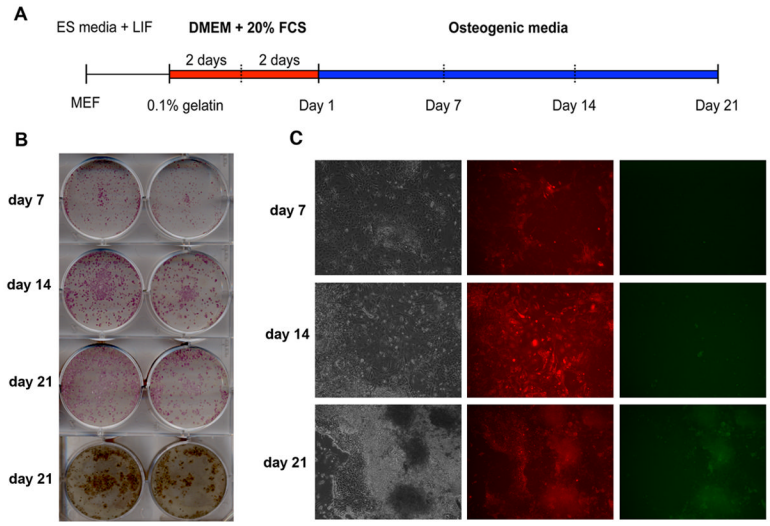

Figure 3. Evaluation of osteogenic differentiation of ESCs using monolayer conditions

(A) Description of the cell culture protocol and timeline for analysis. (B) Histochemical detection of alkaline phosphatase. Mineralization was detected using von Kossa method on day 21. (C) Images of mouse ESCs following osteogenic induction. Phase contrast, left panel; RFP, middle panel; GFP, right panel. Epifluorescence imaging for GFP shows only a background signal from mineralized tissue. Experiments were completed as three independent biological replicates.

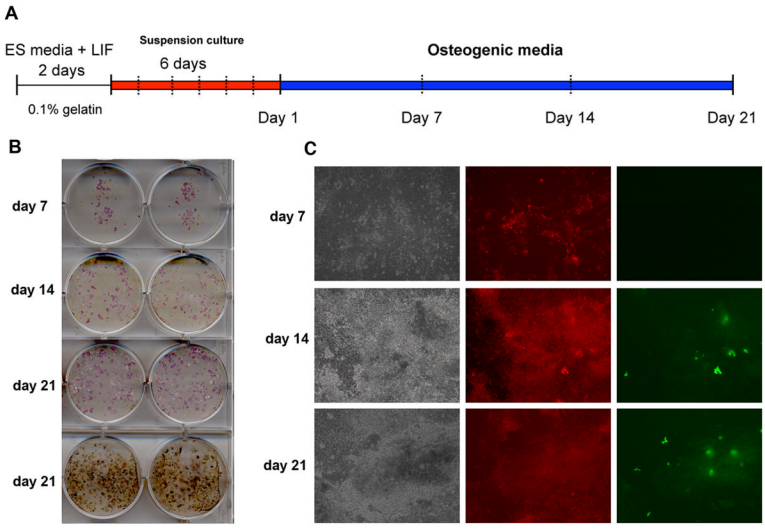

Figure 4. Evaluation of osteogenic differentiation of ESCs derived through EB step

(A) Description of the cell culture protocol and timeline for analysis. (B) Detection of ALP on day 7–21 and mineralization on day 21. (C) Phase contrast image of mouse ESCs after osteogenic induction (left panel). αSMARFP expression is shown in middle panel, while expression of Col2.3GFP is shown on right panel. Experiments were completed as four independent biological replicates.

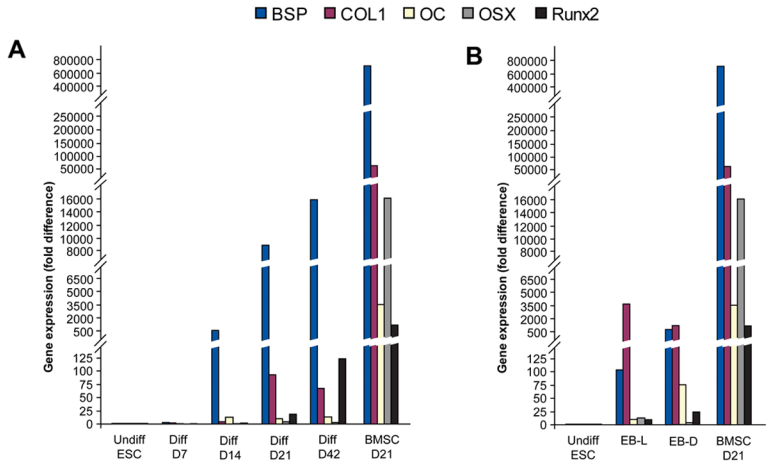

Figure 5. Analysis of gene expression during osteogenic induction of ESCs

Time course of bone marker expression in ESCs differentiated as monolayered cultures (A) or through formation of EBs (B). RNA was extracted at various time points during osteogenic differentiation and assayed for Runx2, Osterix, Col1a2, Bsp and Oc. Undifferentiated ESCs were used to normalize the expression, and RNA from day 21 BMSCs was used as a positive control. Results presented are from one of three independent experiments.

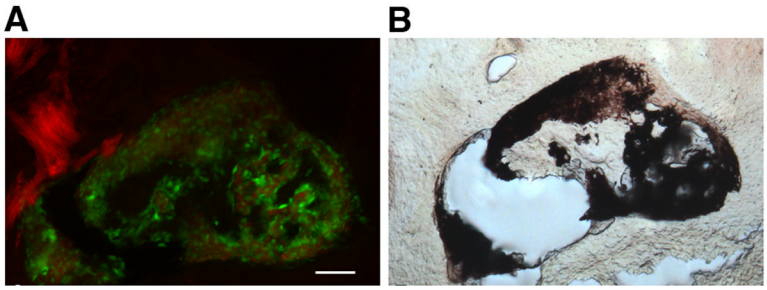

Figure 6. ***In vivo*** **analysis of Col2.3GFP expression using teratoma formation assay**

ESCs from αSMARFP/Col2.3GFP transgenic mice were transplanted into the femoral muscle of immunodeficient mice. (A) Epifluorescence and (B) von Kossa staining of the corresponding section confirmed the presence of mineralized tissues in the areas in which Col2.3GFP activates. Four mice were utilized and ESCs were injected in femoral muscle on both sides. (Bar= 100μm).