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In vitro induction of alkaline phosphatase levels predicts *in vivo* bone forming capacity of human bone marrow stromal cells☆,☆☆

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Abstract One of the applications of bone marrow stromal cells (BMSCs) that are produced by *ex vivo* expansion is for use in *in vivo* bone tissue engineering. Cultured stromal cells are a mixture of cells at different stages of commitment and expansion capability, leading to a heterogeneous cell population that each time can differ in the potential to form *in vivo* bone. A parameter that predicts for *in vivo* bone forming capacity is thus far lacking. We employed single colony-derived BMSC cultures to identify such predictive parameters. Using limiting dilution, we have produced sixteen single CFU-F derived BMSC cultures from human bone marrow and found that only five of these formed bone *in vivo*. The single colony-derived BMSC strains were tested for proliferation, osteogenic-, adipogenic- and chondrogenic differentiation capacity and the expression of a variety of associated markers. The only robust predictors of *in vivo* bone forming capacity were the induction of alkaline phosphatase,

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(ALP) mRNA levels and ALP activity during *in vitro* osteogenic differentiation. The predictive value of *in vitro* ALP induction was confirmed by analyzing "bulk-cultured" BMSCs from various bone marrow biopsies. Our findings show that in BMSCs, the additional increase in ALP levels over basal levels during *in vitro* osteogenic differentiation is predictive of *in vivo* performance.

Introduction

Stem cells within the bone marrow stromal cell (BMSC) population have the capacity to form bone, cartilage, stroma and fat, and are present at frequency between 0.01% and 0.001%. These non-hematopoietic stem cells in the BM were first described by Friedenstein as colony-forming-units-fibroblasts (CFU-Fs) and its progeny of plastic adherent multipotent cells as BMSCs (Friedenstein et al., 1968, 1974, 1976). Not only the multipotency, but also the heterogeneity of BMSC clones was shown in 1997 by Kuznetsov et al. (1997). The lineages of differentiation of BMSCs and the methods to expand BMSCs *ex vivo*, including clinically approved expansion protocols, have increased in the last four decades (Pittenger et al., 1999) (Jiang et al., 2002) (Kolf et al., 2007) (Phinney and Prockop, 2007) (Seshi et al., 2000) (Liu et al., 2005) (Quirici et al., 2002) (Gentry et al., 2007) (Leyva-Leyva et al., 2013) (Buhring et al., 2007) (Chung et al., 2013) (Gang et al., 2007) (Simmons and Torok-Storb, 1991). The International Society for Cellular Therapy has described minimal criteria to define multipotent BMSCs in an attempt to foster a more uniform characterization (Dominici et al., 2006). However, there is compelling evidence that BMSCs are a mixture of cells that can differ not only in size and morphology but also in proliferation rates and *in vitro* differentiation capacity (Kuznetsov et al., 1997; Satomura et al., 1998). Although a large number of cell surface markers show consistent and homogeneous expression on all BMSCs in culture, large variation is observed in the expression of newly identified markers and the question arises as to whether this relates to donor-to-donor variation or heterogeneity in the expanded BMSC cultures (Dominici et al., 2009) (Sarugaser et al., 2009) (Lee et al., 2010) (Russell et al., 2010). Observations concerning heterogeneity have mostly been reported for *in vitro* studies, but little is known about how this translates into differences in outcome when BMSCs are used for *in vivo* applications.

BMSCs hold great promise for use in cell-based bone tissue engineering. In a previous study, we showed that by substituting human platelet lysate (PL) for fetal bovine serum (FBS), we could generate bone from 9 out of 9 for PL and in 6 out of 9 for FBS cultured donors in an *in vivo* ectopic murine bone formation model. However, the amount of bone that was produced by the various BMSC preparations demonstrated a large variation (Prins et al., 2009), as was consistent with the observations of other groups (Kuznetsov et al., 1997) (Digirolamo et al., 1999) (Mendes et al., 2002) (Siddappa et al., 2007) (Siddappa et al., 2008) (Agata et al., 2010). Although density dependent cells are present in initial BM cultures, total BMSC cultures are mainly the result of expansion of cells that formed the initial colony that was started from the adherent cells with clonogenic potential also referred to as colony forming unit-fibroblast (CFU-F). After passaging of the initial cultures, the cultures will mainly consist of cells with the highest replating potential. The final total culture will only result in balanced cell product if the original CFU-Fs have similar rates of proliferation and similar differentiation potential. Heterogeneity in these characteristics will result in great heterogeneity in the end product. This requires the definition of predictive parameters to determine whether the cultured BMSCs meet the requirements for their intended use.

The aim of the current study was to obtain greater insight in the heterogeneity of clonal populations that contributes to a BMSC culture with the goal of correlating *in vivo* bone forming capacity with *in vitro* predictors. We therefore expanded single CFU-F-derived strains using PL and determined the *in vivo* bone forming capacity of the subsequent clonal BMSC cultures and correlated this to the cumulative total cell production, to the *in vitro* differentiation capacity towards adipocytes, chondrocytes, osteoblasts, and to the cell surface marker expression using a large panel of monoclonal antibodies (mAbs). Our data demonstrate that clonal BMSC cultures differ considerably in these characteristics which are reflected in the heterogeneity within the total BMSC population. We could demonstrate that *in vivo* bone forming capacity of the clonal BMSC strains can be predicted by the magnitude of induction of alkaline phosphatase (ALP) during the *in vitro* osteogenic differentiation process. This was confirmed in non-clonal total BMSC cultures derived from BM biopsies from various donors. This finding could be beneficial for the selection of BMSCs with bone forming capacity.

Materials and methods

Bone marrow stromal cell expansion

Human BM aspirates were obtained from the iliac crest during standard hip operations or BM stem cell harvest procedures from donors that had given written informed consent. The single cell-derived BMSC strains were generated from a BM aspirate collected from an 11-year old patient for routine diagnostic purposes, as approved by the institutional medical ethics committee. The BM aspirate was filtered through a 70 μm nylon cell strainer to remove cell aggregates and fragments of bone. Mononuclear cells (MNCs) were isolated by density gradient centrifugation (Ficoll 1.077 g/cm^3). To obtain single cell-derived BMSC strains seven 96-wells plates were seeded with 10,000 MNCs per well. Cells were cultured in α-minimal essential medium supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies), 10 U/ml heparin. For growth stimulation we added 5% human platelet lysate (PL), which was refreshed every three or four days as previously described (Prins et al., 2009) and maintained at 37 °C and 5% CO_2. After 2 weeks, fifty single CFU-F-derived colonies were expanded using PL supplemented medium and tested for their expansion capacity by measuring the

cumulative cell counts as well as the production of colonies weekly (secondary colony forming efficiency).

Colony forming efficiency assay

Cells were seeded in a colony forming efficiency (CFE) assay as described previously (Prins et al., 2009). In brief, BMSCs from primary cultures were trypsinized and seeded in 6-wells plates at 3000; 1000 and 333 cells, per well in duplicate to generate secondary colonies. Secondary colonies were trypsinized and seeded to generate tertiary colonies. Ten days after seeding, colonies were fixed with ice-cold methanol for 15 min and stained with 1:8 in distilled H_2O diluted Giemsa stain (Sigma-Aldrich) for 15 min at room temperature (RT). The number of colonies, consisting of more than 50 cells, was scored using an inverted microscope (Zeiss). The calculated cumulative colony production was determined by multiplying the cumulative cell number with the observed colony frequency.

In vivo bone formation in a murine ectopic bone formation model

To evaluate the *in vivo* bone forming capacity of each clonal strain that we generated, we used an ectopic bone-forming mouse model as previously described (Alves et al., 2013; Groen et al., 2012; Prins et al., 2009; Siddappa et al., 2007, 2008) with the only modification that for this study, eighteen immunodeficient RAG-$2^{-/-}$ $\gamma c^{-/-}$ Balb/c mice were used instead of nude mice. For each clonal strain six hybrid constructs, consisting of 2×10^5 BMSCs seeded onto three 2–3 mm biphasic calcium phosphate (BCP) particles, were implanted in six individual mice. BCP particles were obtained from Xpand Biotechnology and consist of biphasic 60% hydroxyapatite/40% β-tricalcium phosphate (Yuan et al., 2002). Experiments were conducted after acquiring permission of the local Ethical Committee for Animal Experimentation and in accordance with the Dutch law on animal experimentation. After six weeks, the mice were euthanized, the constructs were retrieved from the mice and evaluated for bone formation by determining the percentage of bone area in the available pore space as previously described (Prins et al., 2009).

Osteogenic differentiation and alkaline phosphatase measurement

To obtain quantitative ALP activity measurements, the BMSCs were seeded in triplicate at 2×10^4 cells per well in a 24-wells plate (Nunc). After 24 h, medium was replaced by NH OsteoDiff medium (Miltenyi Biotec) and cultured for 7 and 11 days, respectively. For a reference value one plate was fixed at day 1. At the end of the culture period, cells were fixed with 4% formalin for 15 min at RT, and washed twice with PBS. Osteogenic commitment was quantified by staining the cells for ALP using a fluorescent ALP substrate kit (Vector Laboratories), according to the manufacturer's protocol. Briefly, the cells were stained with Vector® Red substrate working solution for 1 h at RT in the dark and washed with PBS. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1:10,000 in PBS) for 15 min at RT. After another wash with PBS, plates were scanned using the Thermo Scientific Cellomics® ArrayScan® VTI. Per well, a maximum of 20 scanned fields or a minimum of 10,000 objects, using a 10× objective, were analyzed. For the analysis vHCS™:View software (both from Thermo Fisher) was used to determine the average ALP intensity per DAPI positive cell. ALP measurements were normalized to the reference plate of day 1 and expressed as the ALP fold induction.

qRT-PCR for osteogenic gene expression

mRNA levels of osteogenic markers (Table 1) were determined using quantitative real time polymerase chain reactions (qPCR) during osteogenic differentiation. BMSCs were seeded at 1×10^5 cells per well in a 6-wells plate and cultured in NH OsteoDiff medium (Miltenyi Biotec) for 7 and 11 days, respectively. Reference samples were collected at day 1. RNA was isolated from cell lysates by using TRIzol according to manufacturer's protocol (Life Technologies). RNA was DNase I treated and prepared by using QIAGEN RNAse-free DNA Set and RNeasy kit (QIAGEN Corp.). The RNA isolates were verified at 1:50 dilution for purity and quantity by spectrophotometry at 260 nm and 280 nm, followed immediately by cDNA synthesis using 0.5 μg of RNA and Bio-Rad iScript according to manufacturer's protocol (Bio-Rad Laboratories). A 1:50 dilution of cDNA was used for amplification. qPCR was performed on a Light Cycler real time PCR machine (Bio-Rad) using SYBR green I master mix (Bio-Rad). Gene expression was calculated relative to GAPDH levels by the comparative ΔCT values method and fold induction was calculated by normalizing the data to the values at day 1. The following primers were used: GAPDH (NM_002046.4) 5′-aaatcccatcaccatcttccaggagc-3′ and 5′-catggttcacacccatgacgaaca-3′; Osteocalcin (NM_199173.4) 5′-ggcagcgaggtagtgaagag-3′ and 5′-gatgtggtcagccaactcgt-3′; Osteopontin (NM_001040058.1) 5′-ccaagtaagtccaacgaaagc-3′ and 5′-gcatcagggtactggatgtc-3′; ALP (NM_00478.4) 5′-acaagcactcccacttcatctgga-3′ and 5′-tcacgttgttcctgttcagctcgt-3′;

Table 1 mRNA levels of osteogenic markers. The changes of mRNA levels of multiple genes that are considered as markers for osteogenic differentiation were analyzed by quantitative polymerase chain reaction. We determined the utility of the increase in expression of these marker genes as a prognostic indicator for *in vivo* bone forming capacity by a Receiver Operating Characteristic (ROC) curve. Using the ROC analysis we calculated the area under the curve (AUC). The *p* value indicates the statistical significance to distinguish between clones with *in vivo* bone forming capacity and clones without *in vivo* bone forming capacity.

Marker	AUC	*p* value
Alkaline phosphatase	0.95	0.005
Runx2	0.80	0.06
Bone sialoprotein	0.78	0.08
Osteocalcin	0.76	0.10
Collagen type Iα1	0.71	0.19
Osteopontin	0.69	0.23
Osteonectin	0.60	0.53

Col1a1(NM_000088.3) 5′-agggccaagacgaagacatc-3′ and 5′-agatcacgtcatcgcacaaca-3′; Bone sialoprotein (NM_004967.3) 5′-tccagttcagggcagtagtg-3′ and 5′-accctgtgccaggcgtgg-3′; Runx2 (NM_001024630.3) 5′ tccggaatgcctctgctgttatga 3′ and 5′-aaggtgaaactcttgcctcgtcca-3′; Osteonectin (NM_003118.3) 5′ cagcaccccattgacgggt 3′ and 5′-gagagaatccggtactgtgg-3′.

Adipogenic differentiation

Differentiation of the cells towards the adipogenic lineage was induced by incubating the cells in adipogenic differentiation medium as previously described (Pittenger et al., 1999). In short, cells were seeded in triplicate into a 24-wells plates (Nalgene Nunc) at a concentration of 2×10^4 cells per well. After 24 h, the medium was replaced by adipogenic induction medium (MDI). The medium contained high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen), 10% Hyclone fetal bovine serum (FBS), 0.1 μM dexamethasone, 0.5 μM isobutyl methyl xanthine, 10 μg/ml insulin and 100 μM indomethacin (all from Sigma-Aldrich). After 2 days, the medium was changed to adipogenic maintenance medium (AM), consisting of DMEM, 10% FBS, and 10 μg/ml insulin and incubated for 24 h, subsequently incubated in MDI medium and medium was changed every 2 days for 4 days, and finally for 1 week in AM medium. Adipogenic differentiation was evaluated qualitatively on the basis of the morphological appearance of stained Oil Red O lipid droplets as previously described (Prins et al., 2009). To quantify the amount of lipid droplets formed, cells were fixed with 10% paraformaldehyde, and stained using fluorescent Nile Red O (Sigma-Aldrich). Cells were washed twice with PBS and incubated for 5 min with a solution containing DAPI and 0.5 μg/ml Nile-Red-O at RT and in the dark. After washing 2 times with PBS, cells were scanned using the Thermo Scientific Cellomics® ArrayScan® VTI. vHCS™:View software was used to determine the average Nile Red O intensity per DAPI-positive cell. Images were taken using an EVOS *fl* fluorescent microscope (Advanced Microscope Group, Mill Creek, WA, USA).

Chondrogenic differentiation

Pellets of 250,000 BMSCs were formed by centrifugation of the cells produced by the individual clonal strains for 5 min at 1500 rpm in round bottom ultra-low attachment 96-wells plates (Corning, NY, USA). The pellets were maintained in chondrogenic differentiation medium at 37 °C and 5% CO_2. The medium contained high glucose DMEM (Invitrogen) supplemented with 1% ITS + premix (BD Biosciences, Belgium), 10^{-7} M dexamethasone (Sigma), 0.2 mM L-ascorbic acid 2-phosphate (Sigma), 100 units/ml penicillin and 100 mg/ml streptomycin (Invitrogen), and 10 ng/ml TGF-β2 (R&D Systems). The medium was refreshed twice each week. After a period of 4 weeks the cell pellets were processed for histology (n = 2) and for quantitative glycosaminoglycan (GAG) analysis (n = 3).

Quantitative analysis of glycosaminoglycans

Quantification of sulfated GAG in the pellets was performed using the 1,9-dimethyl-methylene blue (DMMB; Sigma) assay as described previously (Farndale et al., 1986). Briefly, pellets were digested overnight at 60 °C in 250 μg/ml papain buffer (Sigma) with 0.2 M NaH_2PO_4 (Merck), and 0.01 M EDTA (Sigma). Reference samples of known concentrations of chondroitin-6-sulfate were prepared. DMMB staining solution (46 μM DMMB, 0.04 M NaCl, 0.04 M glycine; all from Sigma) was then added to the samples and standards. Absorbance was measured at 540 and 595 nm on a 96-wells plate reader (Bio-Rad). Concentrations of GAG were calculated by taking the ratio of absorbances at 540/595 nm and based on a second degree polynomial fit of the standard curve. The amount of GAG per pellet was normalized to the total amount of DNA per pellet. Total DNA was assessed in digested samples using a Quant-iT PicoGreen dsDNA kit according to manufacturer's instructions (Molecular Probes, Invitrogen).

(Immuno)histochemical staining

Pellets were fixed in formalin, embedded in paraffin and 5 μm sections were cut. After deparaffinization and rehydration safranin-O staining and collagen type II immunohistochemistry were performed on three sections per pellet. Sections were first stained in Weigert's hematoxylin (Klinipath BV) to detect cell nuclei and subsequently in 0.4% fast green (Merck). Next, proteoglycans were detected with 0.125% safranin-O (Merck) before dehydration and mounting in DePex. For detection of collagen type II, antigen retrieval was carried out by incubating with 1 mg/ml pronase and 10 mg/ml hyaluronidase (both from Sigma). Subsequently, tissue sections were blocked in 0.3% H_2O_2 and in 5% bovine serum albumin in PBS, followed by incubation with the primary antibody recognizing specific collagen type II (1:100, II-II6B3, Developmental Studies Hybridoma Bank) overnight at 4 °C. A secondary goat anti-mouse antibody, conjugated with horse radish peroxidase (Dako, Denmark), was applied at RT for 1 h in a 1:200 dilution and antibody binding was detected by incubation with 3,3′-diaminobenzidine tetrahydrochloride hydrate (DAB; Sigma) for 10 min. Finally, cell nuclei were counterstained with hematoxylin and sections were mounted. Isotype control staining was performed by replacement of the primary antibody with mouse isotype IgG_1 monoclonal antibody (Friedenstein et al.) at matching concentrations.

Cell surface marker expression of single colony-derived BMSC cultures

Passage 3 BMSCs were analyzed for antigen expression profile using monoclonal antibodies (mAbs) against human cell surface markers. For direct conjugated antigen expression studies 3×10^4 cells were washed with PBS and stained with specific monoclonal antibody or isotype controls for 30 min on ice in the dark. For indirect immunofluorescence, cells were washed twice after specific first labeling and subsequently incubated for 30 min with mouse secondary antibodies on ice and in the dark. Samples were washed and analyzed in a FACSCanto flow cytometer (Abdallah & Kassem, 2008) with BD FACSDiva™ software. The mAbs used for immunophenotyping were as shown in Supplemental Table 1.

Statistical analysis

Data were presented as mean ± standard deviations. Spearman's rank correlations were used to determine correlations between the cumulative CFU-F, cell numbers

and *in vivo* bone formation. To determine the ability to distinguish between clones with and those without bone forming capacity, a Receiver Operating Characteristic (ROC) curve was made using GraphPad Prism 4, and the area under the curve (AUC) was calculated. ROC curve is a widely used standard method to compare the accuracy of a diagnostic test. It represents the tradeoff between false negative and false positive for every possible cutoff. A higher area under the curve (AUC) for a given parameter indicates a higher probability of this parameter to predict for *in vivo* bone forming capacity. A test was considered statistically significant when the *p*-value was less than 1%. Correlations between variables were assessed by Spearman's rank correlation coefficient. Significant differences between clones for GAG/DNA data were analyzed by ANOVA and a Bonferroni correction in SPSS 15.0.

Results

Heterogeneity in proliferation of the clonal BMSC cultures

To investigate the heterogeneity within BMSC cultures, we isolated clonal BMSC strains from human BM by seeding 10,000 mononuclear BM cells per well in seven 96-wells plates. Outgrowth of single BMSC colonies from the adherent cell fraction was observed in 50 out of 672 seeded wells, indicating a frequency of one colony per 134,400 cells. The low frequency of 7% of the wells with cell growth is in agreement with the assumption that only one clonogenic cell was present in these wells. Moreover, we did not observe outgrowth of more than one CFU-F per well. A total of sixteen of the 50 clonal BMSC cultures could be expanded through passage three to produce sufficient cells for further characterization. We calculated the cumulative colony forming efficiency and total cell production per clone after passage four as a percentage of the total cumulative CFE and total cell number of the sixteen selected clones. These differed approximately 100-fold between clone 38 which had the lowest proliferative capacity of 2.3×10^7 and clone 32 which had the highest proliferative capacity of 2.0×10^9 cells. The cumulative number of colonies that could be generated from these sixteen clones differed more than 7000-fold, ranging from 2.3×10^4 colonies for clone 12 to 1.7×10^8 colonies for clone 32. The relative contribution of each individual clone was calculated and depicted as the percentage of the total cell production (Fig. 1A) and generated colonies (Fig. 1B). The percentages are shown only for the clones with *in vivo* bone forming capacity. Spearman's rank correlations showed a statistically significant correlation between the cumulative number of colonies and the cumulative number of cells (Spearman $r = 0.77$; $p < 0.01$). These data demonstrate the heterogeneity in proliferation capacity in primary BMSC clones derived from the same donor.

Heterogeneity of *in vivo* bone formation of the clonal BMSC cultures

Using an *in vivo* model for bone formation (Groen et al., 2012; Prins et al., 2009; Siddappa et al., 2007, 2008), bone forming capacity was demonstrated for five of the sixteen clones. Of these, clone 8 showed the most extensive bone forming capacity (13.1%) while clone 42 only showed minimal bone formation (Figs. 2A, B), and eleven of the sixteen clones did not show any *in vivo* bone forming capacity (Fig. 2A). The bone that is deposited on the scaffold material contained embedded osteocytes as well as a prominent layer of osteoblast (Fig. 2B). We did not find a correlation between the percentage of bone formation and the cumulative colony numbers, (Spearman $r = 0.36$; $p = 0.16$), indicating that the formation of secondary and tertiary colonies is not a predictor for *in vivo* bone formation.

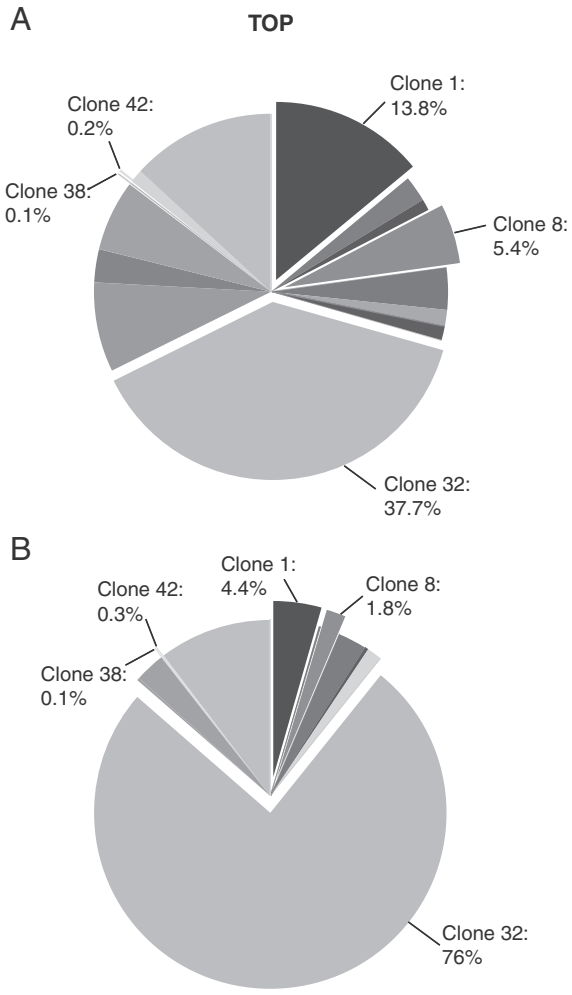


Figure 1 Cumulative cell counts and colony numbers. The theoretical cumulative total cell production and number of generated colonies for each individual clone were calculated and the relative contribution of each individual clone depicted as the percentage of the total cell production (A) and generated colonies (B). The percentages are shown only for the clones with *in vivo* bone forming capacity.

Cell surface marker expression

To investigate if a cell surface marker could predict *in vivo* bone formation, we determined the immunophenotype of the cultured BMSCs using mAb staining and flow cytometry analysis. More than 90% of the cells of all clonal strains

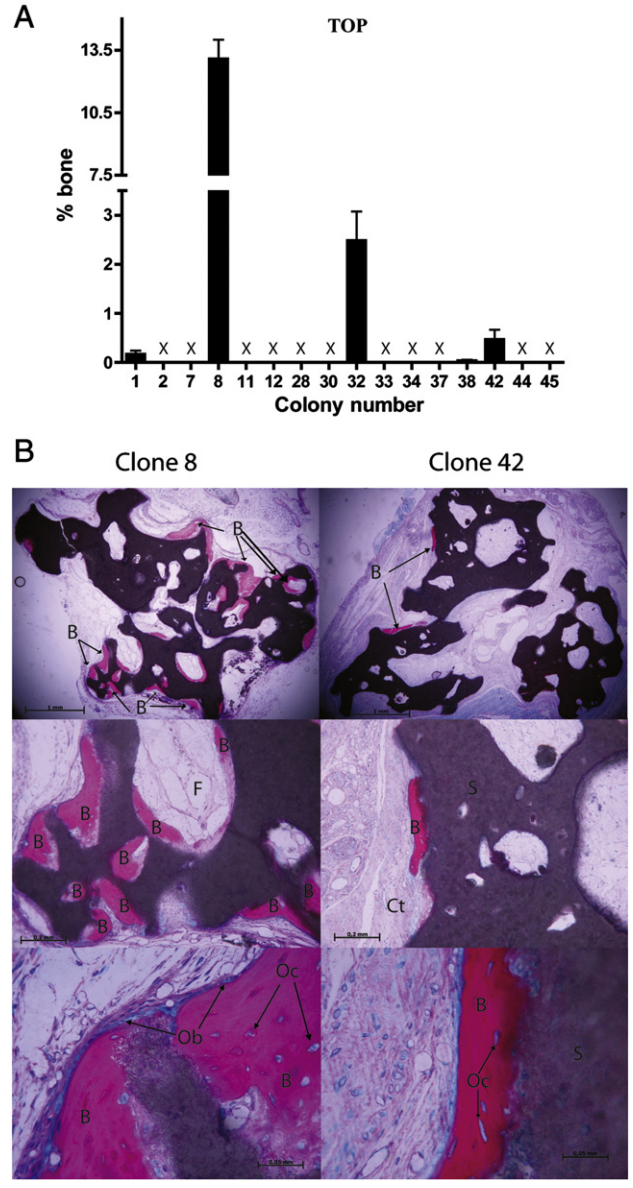


Figure 2 *In vivo* bone formation. *In vivo* bone formation was tested in an ectopic murine immune-deficient model. (A) The average percentage of bone per pore available space for each individual clone. X indicates no *in vivo* bone formation. (B) Representative histological sections of bone formation by BMSCs from clone 8 (left) and clone 42 are shown at increasing microscopic magnifications (original magnification 25×, 100×; 400× from top to bottom). Scaffold material is shown in gray/black (S), newly formed bone is basic fuchsin-stained pink. The bone lining the scaffold contained embedded osteocytes (Oc) and a prominent osteoblastic (Ob) layer on the luminal surfaces. Connective tissue (Ct) was present between and around the scaffolds.

expressed the recognized BMSC markers CD73, CD90, CD13, CD105, CD166, CD63, CD29, CD49e, CD44, CD71, CD146, HLA-ABC, and β2-microglobulin and lacked expression (<1%) of HLA-DR and hematopoietic markers, including CD3, CD19, CD34, CD45, CD117 (Supplemental Table 1). The markers identifying BMSC subpopulations with variable expression between the clones (1–90%) were considered to be the most promising to identify cells with bone forming capacity. These include the monocyte marker CD14, the endothelial marker CD31 (PECAM-1), the decay-accelerating factor CD55, the erythroid marker glycophorin A (CD235a), the low-affinity nerve growth factor receptor (CD271), the neural ganglioside GD2 (NG-GD2), the stromal cell precursor marker in human bone marrow Stro-1, the stage-specific embryonic antigen 4 (SSEA-4), a mAb recognizing subpopulations of BMSC and neural progenitor cells (W4A5) and tissue non-specific alkaline phosphatase (TNAP/MSCA-1)(Supplemental Table 1). The results of the membrane marker expression analysis once more showed that there is substantially more heterogeneity in BMSC cultures than is generally recognized. None of the markers however, correlated with or were predictive of *in vivo* bone formation.

Induction of ALP predicts *in vivo* bone formation

Third passage clonal BMSC strains were differentiated towards the osteogenic lineage and at day one, and after seven and 11 days of differentiation, ALP enzymatic activity was analyzed and quantified as mean ALP activity per cell using the Thermo Cellomics® ArrayScan® VTi. Remarkable differences were observed in the absolute amounts of ALP activity per cell for the clones at day one (Fig. 3A). Some of the clones showed an increase of ALP activity at day seven and/or day 11 upon differentiating the cells towards the osteogenic lineage, whereas other clones did not (Fig. 3B). Following normalization of ALP levels to day one, ALP activity fold-induction ranged from a 1.1-fold increase to a 5.5-fold increase at day 11 (Fig. 3B). We performed ROC analysis to determine whether ALP activity could serve as a marker to identify clones with *in vivo* bone forming capacity. This analysis is a widely used standard method to compare the accuracy of a diagnostic test (Mentink et al., 2013). It represents the tradeoff between false negative and false positive for every possible cutoff. A higher area under the curve (AUC) for a given parameter indicates a higher probability that this parameter will predict for *in vivo* bone forming capacity. A test was considered statistically significant when the *p*-value was less than 1%. A diagnostic test always has false positive and negative results. The AUC of >0.93 that we observed, indicates a very high probability of ALP induction to predict for *in vivo* bone formation and implicates that the level of induction of ALP activity during *in vitro* osteogenic differentiation has a high probability to predict for *in vivo* bone formation ($p < 0.01$) (Fig. 3C).

To extend our observation that induction of ALP activity could serve as a predictive parameter for *in vivo* bone formation, we analyzed ALP mRNA levels using qRT-PCR (Fig. 3D). For comparison, we also analyzed the mRNA expression of other known osteogenic markers including collagen type Iα1 (Col-Iα1), non-collagenous proteins of the extracellular matrix such as osteocalcin (OC), osteonectin (ON), osteopontin (OP), and bone sialoprotein (BSP), and the bone-related transcription factor Runx2. mRNA expression levels were determined during osteogenic differentiation at days seven and 11. Table 1 shows the AUC for ROC curves and associated *p* value for the markers tested. We typically observed that OC, Runx2 and BSP mRNA levels increased progressively during osteogenic differentiation for the clones

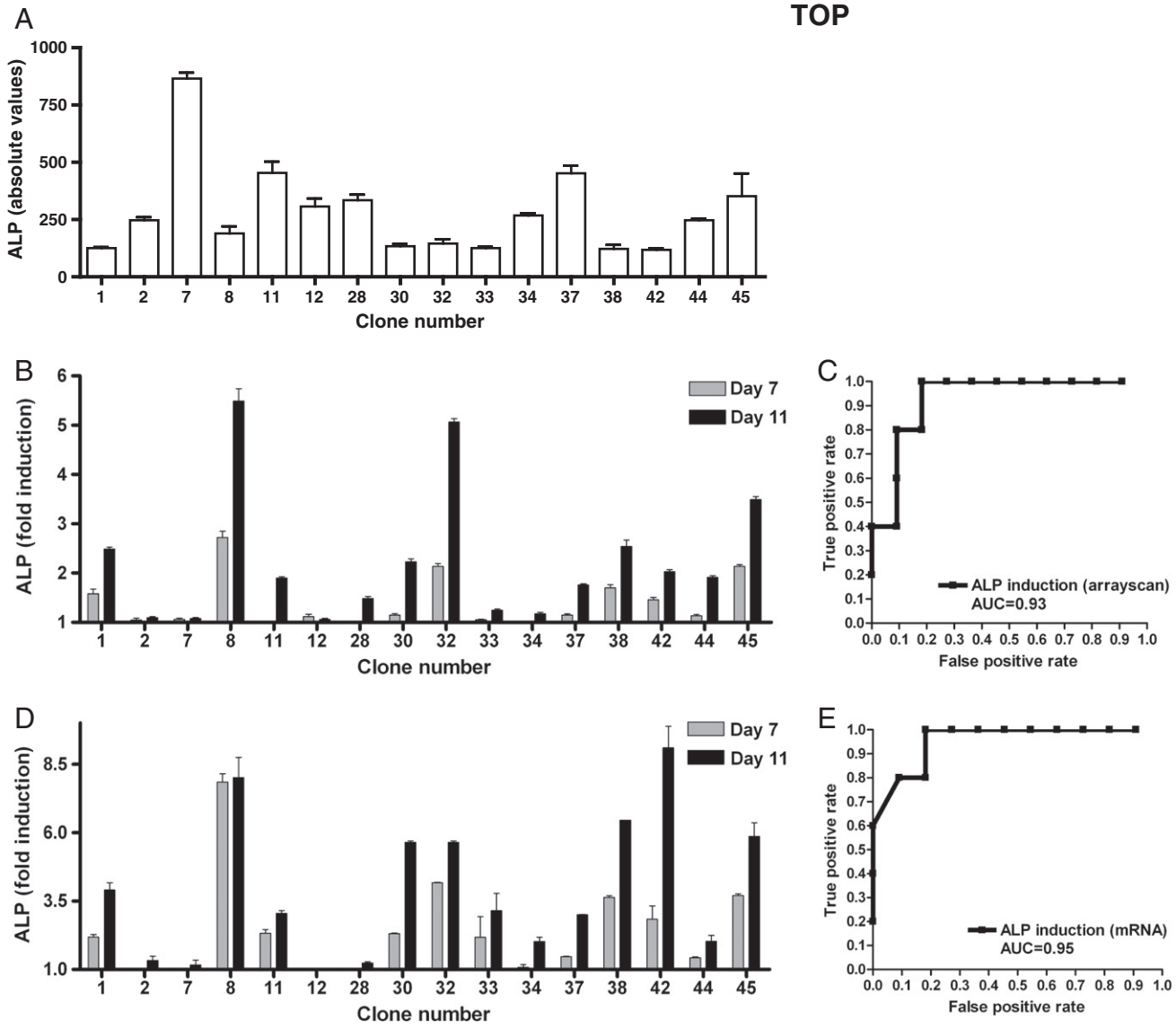


Figure 3 Alkaline phosphatase expression and induction in clonal BMSC strains. The ALP activity was determined using a fluorescent ALP substrate. Fluorescence was measured on a Cellomics ArrayScan VTI High Content Screening (HCS) platform and calculated per DAPI-positive stained cell. Absolute values of alkaline phosphatase (ALP) activity are shown for each individual clone (A). ALP activity was normalized to day 1 and depicted as ALP-fold induction after induction of osteogenic differentiation at day 7 and day 11 (B). The ALP activity determined at mRNA level and the calculated fold-induction is depicted in (D). The probability of ALP induction as a predictive marker for *in vivo* bone forming capacity was evaluated using a Receiver Operating Characteristic (ROC) curve for the ALP activity level (C) and for the mRNA level (E). A completely random predictor would be given by a diagonal 'line of no discrimination' (dotted line).

that formed bone *in vivo*, whereas less increase was observed in the clones that did not result in *in vivo* bone formation. However, there was no statistical significance ($p = 0.10$; $p = 0.06$; $p = 0.08$, respectively), indicating that these markers do not have a high probability of predicting *in vivo* bone formation. Expression of ON, OP, and Col-1 resulted in values reflecting more random predictions for *in vivo* bone forming capacity ($p = 0.53$; $p = 0.23$; $p = 0.19$, respectively) implicating that there is no correlation between *in vitro* differentiation and expression of ON, OP and Col-1. ALP mRNA induction showed the highest AUC (0.95) and a *p*-value of <0.01 (Fig. 3E). This supports the data from the ALP activity measurements, and suggests that for BMSCs *in vitro* changes in ALP expression are highly predictive of *in vivo* bone forming capacity.

In vitro differentiation capacity of the clonal BMSC strains

To evaluate the multi-potency of clonal BMSC cultures, cells were also differentiated towards adipocytes and chondrocytes. Cells from all 16 clones were differentiated towards adipocytes for 14 days in specific differentiation media. Adipocyte differentiation was defined by the appearance of Oil Red O-positive stained lipid droplets after 14 days (Fig. 4A; Clone

28 is shown as an example). Quantification of the induction of Nile Red-positive lipid containing droplets per DAPI-positive cell (Fig. 4B) revealed an increase of the fluorescence for 11 from the 16 clones from 3.9 ± 0.5 to 1.1 ± 0.2 fold (Fig. 4C). No Nile Red O-positive cells were detected in the uninduced control cells. Cells from 14 of the 16 clones were also analyzed for chondrogenic differentiation. Clones 12 and 34 could not be included due to insufficient numbers of cells at passage three. *In vitro* chondrogenesis was evaluated by safranin-O and toluidine blue staining to detect GAGs, and staining for cartilage-specific collagen type II. Positive staining for all markers was only observed in clones 32 and 42 (Fig. 4D; E, Supplemental Fig. Fig. 1) indicating the ability to differentiate into chondrocytes. Quantification of the amount of sulfated GAGs in the pellets revealed that only those from clones 32 and 42 produced significantly more GAG/DNA (>10) compared to those of all other clones (Fig. 4F), which confirmed the histological results. Lacunae resembling those surrounding chondrocytes were evident in histological sections from clones 32 and 42 (Fig. 4E, Supplemental Fig. 1).

The differences in the capacity to differentiate towards adipocytes and chondrocytes demonstrate the heterogeneity in multi-potency among the clones. The capacity to differentiate to adipocytes or chondrocytes was not predictive of *in vivo* bone formation.

The magnitude of induction of ALP is predictive of *in vivo* bone forming capacity of non-clonal BMSC cultures

To extend and confirm our initial observation that induction of ALP levels during *in vitro* osteogenic differentiation in clonal BMSCs predicts *in vivo* bone forming capacity, we analyzed the induction of ALP during *in vitro* differentiation of total BMSC cultures derived from different donors and correlated this to the *in vivo* bone forming capacity of each non-clonal BMSC culture. Previously we characterized and reported the *in vivo* bone forming capacity of several BMSC cultures from different donors (Prins et al., 2009), and we now measured induction of ALP activity during *in vitro* differentiation. BMSCs that showed a low magnitude of induction (<5.2 fold) of ALP activity at day 11 of *in vitro* osteogenic differentiation did not form bone *in vivo* (Fig. 5). However, BMSCs that showed a high magnitude of induction (>8.2 fold) of ALP activity at day 11 of *in vitro* osteogenic differentiation were successful in generating bone *in vivo* (Fig. 5). These data extend and confirm the data obtained with the BMSC clones and show that ALP induction in non-clonal BMSC strains is also predictive of *in vivo* bone-forming capacity.

Discussion

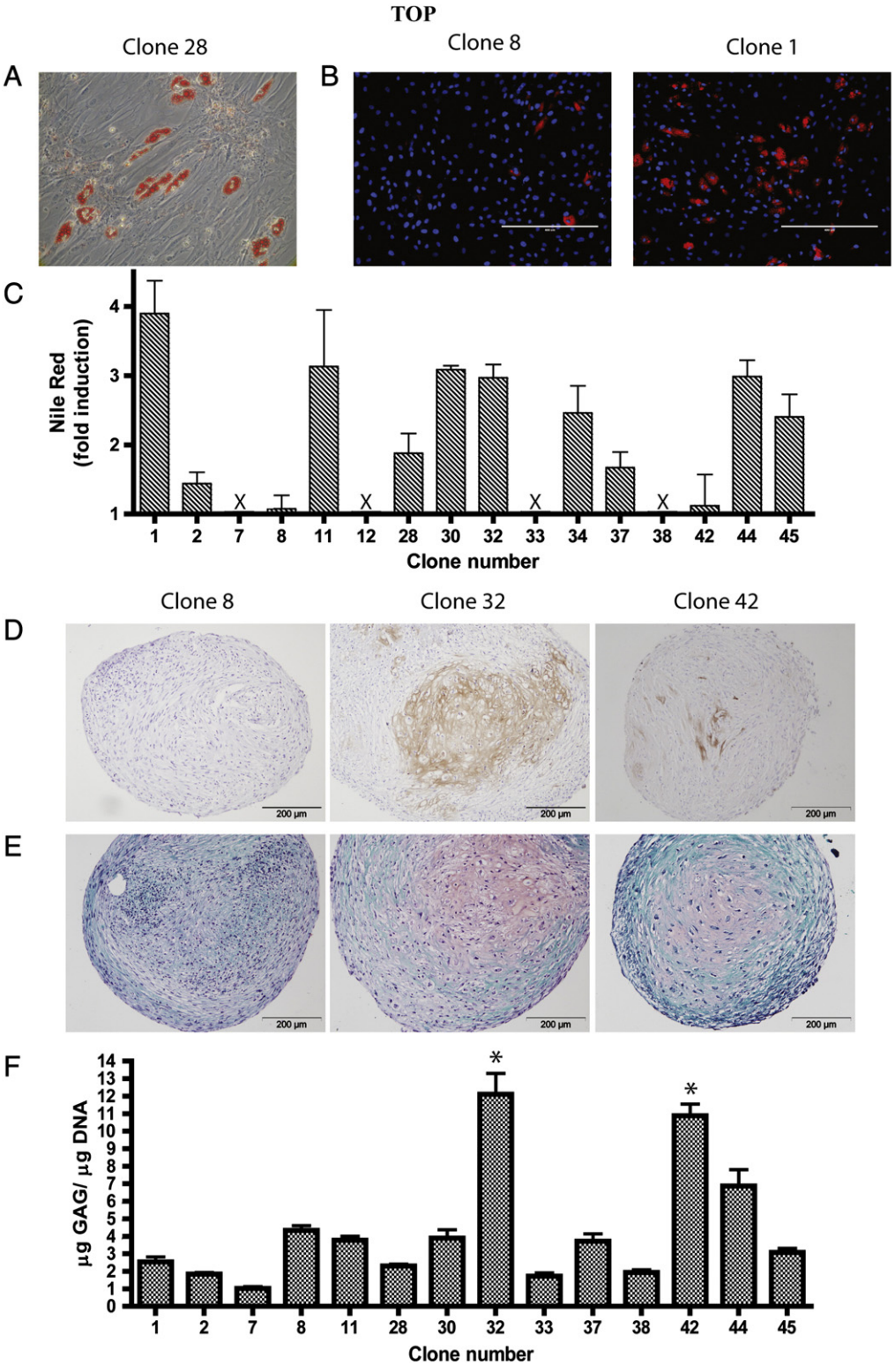
Our study on the characteristics of clonally expanded BMSC cultures strongly supports the postulate that there is a large degree of heterogeneity in BMSC cultures, not only on the basis of *in vitro* parameters for characterization, but also when using the *in vivo* test for functional bone forming capacity. Previous *in vitro* studies have shown heterogeneity in BMSC cultures (Digirolamo et al., 1999) (Colter et al., 2000; Colter et al., 2001; Sekiya et al., 2002; Smith et al., 2004), however, until now, a specific marker profile for cells with specific differentiation properties has not been described. Our study shows that single colony-derived clonal BMSC strains derived from the same donor exhibit a broad *in vitro* proliferation and differentiation profile and this was extended to *in vivo* bone formation. The most rapidly proliferating single colony-derived stromal cell culture in our study (clone 32; Fig. 1) is composed of cells with the highest differentiation capacity. The cells showed potential to differentiate into the osteogenic, adipogenic and chondrogenic lineages as well as to form bone *in vivo* in substantial amounts (Figs. 2, 4C, F; Table 2).

Only a limited number (31%) of single colony cultures exhibited *in vivo* bone forming capacity. These observations are in line with a previous report by Kuznetsov et al., who showed that a similar proportion, (33.3%), of clonally derived human marrow stromal fibroblasts (which he defined as "strains"), established from different donors, exhibited *in vivo* bone forming potential (Kuznetsov et al., 1997). The accurate selection or identification of clones with *in vivo* osteogenic capacity would benefit patient treatment for osteogenic defects. Therefore, we investigated several parameters that might be utilized to predict *in vivo* osteogenic capacity. Neither the number of cells produced per initial CFU-F nor the colony producing capacity was useful for predicting *in vivo* bone forming capacity.

Our data indicate that the large variability between donors can be explained by the composition and properties of a given clonal mixture from the primary isolated BMSCs. This clonal mixture consists of several populations among which are multi-potent stem cells, committed progenitors, lineage restricted cells, and senescent cells. We showed that 2 of 16 clones were multi-potent and able to differentiate into osteoblasts as well as chondrocytes and adipocytes; 4 of 16 clones were bi-potent and able to differentiate into osteoblasts as well as into adipocytes; 7 of 16 clones were uni-potent and their differentiation potential was restricted to either the adipocytic or osteogenic lineage; 3 of 16 clones were not able to differentiate into any of the three lineages. A most striking observation was that the chondrocytic differentiation potential was only observed in combination with adipocyte and osteoblast potential, suggesting a hierarchy in differentiation potential (Pittenger et al., 1999; Russell et al., 2010). From the seven clones with *in vitro* osteogenic potential, only five also exhibited *in vivo* bone forming potential (Table 2). These phenomena might explain why the first attempts to generate osteogenic grafts with human BMSCs were unsuccessful in standard clinical application (Goldstein et al., 1999) (Meijer et al., 2007; Quarto et al., 2001; Schimming and Schmelzeisen, 2004; Schultz et al., 2000; Ueda et al., 2008). In an attempt to identify a surface marker that could distinguish between cells with and without *in vivo* bone forming capacity, we evaluated our clonal BMSC strains for several previously reported cell surface markers using flow cytometry. Some of these markers were included as they are known to react with specific subpopulations within the CFU-F fraction in freshly collected BM (Aslan et al., 2006; Baksh et al., 2007; Battula et al., 2008; Deschaseaux et al., 2003; Gang et al., 2007; Groen et al., 2011; Majumdar et al., 2000; Martinez et al., 2007; Rider et al., 2007; Rozemuller et al., 2010; Sacchetti et al., 2007; Simmons and Torok-Storb, 1991; Sobiesiak et al., 2010; Sorrentino et al., 2008; Stewart et al., 2003; Tormin et al., 2009; Vogel et al., 2003). We

observed that BMSCs derived from single clones exhibit a variable expression of several markers (Supplemental Table 1). However, we could not find a single marker with a sufficiently strong positive or negative correlation to identify the clones with *in vivo* bone forming capacity. The heterogeneity in the reactivity profiles of the mAbs that we used lends support to the concept that subpopulations in the BM as well as cultured BMSCs exist and it opens the possibility to use multiparameter flow sorting to identify a specific BMSC subfraction with *in vivo* bone forming potential.

All the potential markers and parameters discussed above failed to correlate with the capacity of the clonal derived BMSCs to form bone *in vivo*. However, in our analysis of clonally-derived BMSC strains, we identified a novel



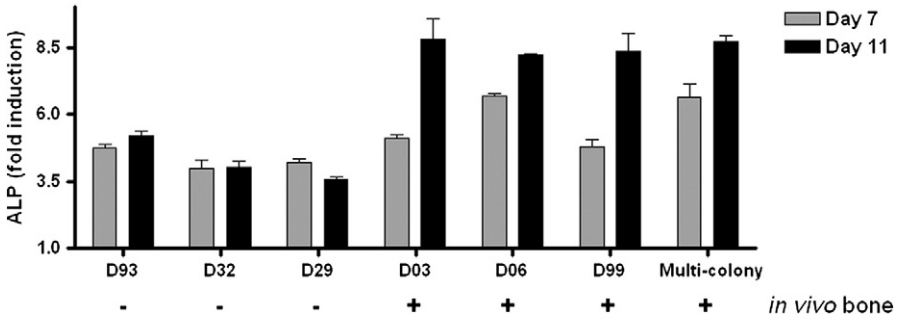


Figure 5 Fold ALP induction of *in vitro* osteogenic differentiation and *in vivo* osteogenic differentiation of BMSCs. Fold induction of ALP levels during *in vitro* osteogenic differentiation at days 7 and 11 of total BMSC populations of different donors compared to *in vivo* osteogenic differentiation capacity (+ or −) of the same donors. D29, D32, D93, D03, D06, D99 and "multi-colony" are the different donors and "multi-colony" is the same donor the clonal MSC strains were derived from.

parameter that can predict the *in vivo* differentiation capacity of BMSCs: induction of ALP mRNA and activity levels. We showed that induction of ALP levels (Figs. 3B, C) but not the initial levels of ALP (Fig. 3A) is highly predictive for the *in vivo* osteogenic differentiation capacity of these clones (Fig. 2A). Moreover, we confirmed our observations by using total BMSC preparations derived from different donors. Analysis of these strains and the original clonal strains showed a 100% correlation between *in vivo* bone forming capacity and ALP induction during *in vitro* osteogenic differentiation (Fig. 5).

ALP enzyme activity is increased in the early stages of osteoblast commitment and upregulation of ALP activity during osteogenic differentiation is assumed to reflect the number of osteogenic committed progenitor cells in a cell population. High variations in ALP levels and in *in vitro* induction kinetics that differentiate between human BMSC donor populations have previously been reported (Phinney et al., 1999) (Jaiswal et al., 1997). We found a novel correlation between the degree to which ALP activity of a given clone could be induced and the *in vivo* bone forming capacity, both at the mRNA level as well as in enzyme activity (Figs. 3C, E). We showed that, upon differentiation of the clonally-derived BMSCs towards the osteogenic lineage, ALP induction of more than two-fold and mRNA induction of more than four-fold after 11 days of differentiation predicts the capacity for *in vivo* bone forming capacity in 14 of 16 clones. The clonal BMSCs with *in vivo* bone forming capacity also displayed the lowest ALP levels at the onset of the differentiation assay as compared to those without *in vivo* bone forming capacity, indicating that high ALP levels in BMSC cultures, while suggesting *in vivo* bone forming capacity, in fact anticorrelate with *in vivo* bone forming capacity. Moreover, we extended our observations to non-clonal BMSC populations and showed that from seven donors, four donors showed a high induction of ALP during *in vitro* differentiation (>8.2 fold, 11 days), which correlated fully with the *in vivo* bone forming capacity of these donors. Three of the seven donors did not show a high magnitude of induction of ALP during *in vitro* differentiation and here we could not detect any *in vivo* bone formation. A possible relationship between *in vitro* ALP induction and *in vivo* bone forming capacity has been previously suggested (Mendes et al., 2004; Siddappa et al., 2007). The discrepancies between the thresholds of ALP induction during *in vitro* differentiation of the clonal BMSC strains and the non-clonal BMSC populations and *in vivo* bone forming capacity could perhaps be explained by the higher number of population doublings of the clonal BMSC strains and their gradual subsequent loss of response to *in vitro* osteogenic stimuli (Siddappa et al., 2007). Inclusion of control cells during *in vitro* osteogenic differentiation would allow controlling for differences between experiments and would be beneficial in determining a threshold that could be used for the selection of BMSCs with *in vivo* bone forming capacity.

Although the same scaffolds were used throughout our study to determine the *in vivo* bone forming capacity, the type of material and composition of the scaffold do affect some of the *in vivo* properties of BMSCs (Yuan et al., 2010). The formation of bone tissue and bone marrow like structures, blood vessels and hematopoietic cells therein can vary depending on the scaffold used. The use of a different substrate or scaffold could induce the cells into a different lineage (Engler et al., 2006) (Dupont et al., 2011).

The generation of osteogenic grafts with human BMSCs for clinical application requires cells with *in vivo* osteogenic potential. Since prolonged culturing of cells reduces the osteogenic potential (Siddappa et al., 2007) of BMSCs, an

Figure 4 Adipogenic and chondrogenic differentiation. Differentiation to adipocytes was verified by staining the accumulation of lipid-rich vacuoles within the differentiated cells. Lipid vacuoles were stained using Oil Red O for visual inspection (A; original magnification 200×) and quantification was performed after fluorescent Nile Red O staining; nuclei were counter-stained using DAPI (B; scale bar = 400 μm). Nile Red O staining at day 14. X indicates no adipogenic differentiation (C). For analyses of chondrogenesis, pellets were stained for cartilage-specific collagen type II by immunohistochemistry using a specific antibody (D) showing the type II collagen expression in the extensive extracellular matrix. Pellets were stained for glycosaminoglycans by safranin-O staining (n = 2; E), Chondrocyte-like lacunae are evident in histological sections from clones 32 and 42 (E). Pellets were harvested for quantitative sulfated glycosaminoglycans (GAGs) analysis (n = 3). The GAG content of the pellets is expressed as GAG per DNA (F). Differences between clones for GAG/DNA are considered to be significant when $p < 0.01$ and are marked with an asterisk (*). Sections were counterstained with hematoxylin, staining the nuclei blue (E).

Table 2 Multi-differentiation potential. The clones that displayed the capability to be differentiated towards adipocytes (A), osteoblasts (O), chondrocytes (C) and/or form *in vivo* bone (B), are marked with X. NA = not analyzed.

Clone	A	O	C	B
1	X	X		X
2	X			
7				
8	X	X		X
11	X			
12			NA	
28	X			
30	X	X		
32	X	X	X	X
33				
34	X		NA	
37	X			
38		X		X
42	X	X	X	X
44	X			
45	X	X		

alternative procedure would be to directly load BMSCs on scaffolds such as has been proposed by the group of Martin (Di Maggio et al., 2011). They showed that prolonged perfusion of, in this case total bone marrow, and immediate implantation resulted in *in vivo* bone formation. However, for large scale scaffold loading it might be more efficient to increase the frequency and thereby increase the total number of BMSC with bone forming capacity by concentrating the BMSC based on their CD marker profile.

So far, prospective markers to isolate or identify the BMSC population with *in vivo* osteogenic potential have been scarce and with limitations, but it has been described that sorting for CD271, CD56, MSCA-1 and MSCA-2 (Battula et al., 2008) (Rozemuller et al., 2010), CD146 (Sacchetti et al., 2007), CD90 (Chung et al., 2013) and ALDH (Gentry et al., 2007) results in enrichment of BMSC with multipotent capacity, including *in vivo* bone formation. Recently, Larsen et al. identified decorin, lysyl oxidase-like 4, natriuretic peptide receptor C, and tetranectin as being upregulated in cells that are osteogenic *in vivo* by comparing low *versus* high bone forming hMSC-TERT cell populations (Larsen et al., 2010). However, in this study the identified molecular phenotype was not tested in total BMSC cultures from primary bone marrow cells from different donors. The results from our study could explain the fact that in their study a positive correlation for ALP levels was not found, since we demonstrated that induction of ALP levels (and not necessarily the actual level of ALP) is predictive for the *in vivo* osteogenic differentiation capacity.

Finally, our data show that an *in vitro* osteogenic differentiation capacity parameter can be used to classify produced clinical BMSC batches for the best *in vivo* osteogenic potential within twelve days. Despite the heterogeneity within BMSC cultures and between cultures from different donors, the magnitude of ALP induction during *in vitro* osteogenic differentiation predicts the *in vivo* osteogenic differentiation capacity for most of the cases that we studied and will aid in the selection of the optimal BMSC population for use in patients. Our findings should be taken into consideration when developing clinical protocols that aim at generating BMSCs for the engineering of human bone.

Author disclosure statement

No competing financial interests exist.

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