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# Relation between *in vitro* and *in vivo* osteogenic potential of cultured human bone marrow stromal cells

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The use of cell therapies in bone reconstruction has been the subject of extensive research. It is known that human bone marrow stromal cell (HBMSC) cultures contain a population of progenitor cells capable of differentiation towards the osteogenic lineage. In the present study, the correlation between the *in vitro* osteogenic potential of HBMSC cultures and their capacity to form bone *in vivo* was investigated. HBMSC cultures were established from 14 different donors. Fourth passage cells were examined for the expression of alkaline phosphatase (ALP), procollagen I (PCI) and osteopontin (OP), through flow cytometry and the effect of the osteogenic differentiation factor dexamethasone (Dex) on this expression was evaluated. In addition, the capacity of the cultures to induce *in vivo* bone formation was analysed by culturing the cells on porous hydroxyapatite (HA) scaffolds followed by subcutaneous implantation of these constructs in nude mice. Results showed expression of PCI, OP and ALP in all cultures, irrespective of the presence of Dex in the culture medium. Dex failed to have a significant effect on the expression of PCI and OP but it induced a consistent increase in the relative amount of cells expressing ALP. Nevertheless, although *in vitro* testing clearly indicated osteogenic potential in all cultures, HBMSC from six of the 14 tested donors did not form bone *in vivo*. The results, therefore, demonstrate that neither the expression of PCI, OP and ALP nor the absolute increase in Dex-stimulated ALP expression can as yet be used as predictive markers for *in vivo* bone formation by HBMSC. However, preliminary data indicate that not the absolute, but the relative increase in the percentage of ALP expressing cells caused by Dex stimulation may be related to the ability of the HBMSC to form bone.
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## Introduction

The increasing demands for organ and tissue transplants has motivated many scientists to perform research in the field of tissue engineering. At present, numerous investigators have proposed the use of autologous cultured tissue approaches as an alternative to the traditional bone grafting therapies [1–10]. The engineering of bone tissue is based on the idea of seeding a suitable implant material with patient own cells that, during *in vitro* culture and prior to transplantation into the defect site, will form a bone tissue coating over the material surface [10, 11].

The bone marrow stromal cell population is known to contain progenitors capable of differentiation into mesenchymal lineages such as bone, cartilage, fat and other connective tissues [12–15]. Therefore, they constitute an interesting population of cells for use in cell therapies. Furthermore, bone marrow stromal cells can be easily isolated, extensively expanded and induced to further differentiate into the relevant lineage [15–18]. The *in vitro* and *in vivo* osteogenic potential of adult human bone marrow stromal cells (HBMSC) cultured on porous calcium phosphate scaffolds has already been reported [7, 10, 19–24]. However, in several of these studies, *in vivo* bone formation by HBMSC did not occur in all of the assessed cultures [10, 19, 21, 23]. Moreover, osteogenic potential of the cultures was found to decrease with patient age [19, 25]. Therefore, the development of

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an analysis method that will allow predicting *in vitro* the performance of the tissue-engineered constructs after implantation is of extreme importance. Bone tissue contains high levels of type I collagen and several non-collagenous proteins (such as osteopontin, bone sialoprotein and osteocalcin) that distinguish it from other types of tissues [26–28]. However, alkaline phosphatase (ALP) is the most widely recognised marker for osteoblast activity [16, 22, 25, 26, 29–32]. In bone, high levels of ALP are present in pre-osteoblasts and, in culture, osteogenic cells are also known to express high levels of this enzyme [26]. The synthetic glucocorticoid, dexamethasone, has been extensively reported to induce cultures of bone marrow cells to differentiate along the osteogenic lineage [7, 8, 10, 16, 19, 29, 31–34]. Signs of differentiation induced by dexamethasone (Dex) include morphological changes from an elongated to a more cuboidal cell shape and an increase in the expression of osteoblast markers such as ALP [32–35], osteopontin and osteocalcin [36]. The effects of this glucocorticoid on collagen I expression are dependent on the culture conditions and period [33, 35].

The aim of this study was to find a correlation between *in vitro* and *in vivo* osteogenic potential of HBMSC cultures by evaluating both the expression of osteogenic markers and their capacity to form bone. HBMSC were screened for ALP, pro collagen I (PCI) and osteopontin (OP) expression during culture. The degree of cell stimulation caused by the presence of dexamethasone in the medium was measured through the effect of this differentiation factor on the expression of the bone cell markers. Finally, the *in vitro* results were related to the ability of the cells to form bone after subcutaneous implantation in a nude mice model.

## Materials and methods

### HBMSC harvest and culture

Bone marrow aspirates (10–30 ml) were obtained from 14 patients that had given written informed consent. Donor information is summarised in Table I. The bone marrow specimens were collected in heparinised tubes, transported at room temperature and expanded as previously reported [10, 19]. Cells were re-suspended with a 20 G needle, plated at a density of 500 000 nucleated cells/cm$^2$ and cultured in minimum essential medium (α–MEM, Life Technologies, The Netherlands) containing 10% of a selected batch of foetal bovine serum (FBS, Life Technologies, The Netherlands), antibiotics (AB), 0.2 mM L-ascorbic acid 2-phosphate (AsAP, Life Technologies, The Netherlands) and 1 ng/ml basic fibroblast growth factor (bFGF, Instruchemie, The Netherlands). Cells were grown at 37 °C and in a humid atmosphere with 5% $CO_2$. The culture medium was refreshed twice a week and, at near confluence, the adherent cells were washed with phosphate buffered saline solution (PBS; Life Technologies, The Netherlands) and enzymatically released by means of a 0.25% trypsin – EDTA solution (Sigma, The Netherlands). Cells were plated at a density of 5000 cells per cm$^2$ and subsequent passages were performed when cells were near confluence (80–90%).

TABLE I HBMSC donor information

| Donor | Source of bone marrow | Gender | Age |
|---|---|---|---|
| 1 | Iliac crest | M | 75 |
| 2 | Acetabular fossa | M | 86 |
| 3 | Iliac crest | M | 74 |
| 4 | Iliac crest | M | 45 |
| 5 | Iliac crest | F | 39 |
| 6 | Acetabular fossa | F | 54 |
| 7 | Spine | M | 44 |
| 8 | Iliac crest | F | 69 |
| 9 | Iliac crest | M | 74 |
| 10 | Acetabular fossa | F | 72 |
| 11 | Iliac crest | F | 70 |
| 12 | Iliac crest | F | 74 |
| 13 | Acetabular fossa | F | 67 |
| 14 | Spine | M | 44 |

F = female; M = male.

### Scaffold material

Porous granules of coralline hydroxyapatite (HA, Pro-Osteon 500, Interpore) with an average surface area of 0.2–0.3 cm$^2$ were used as scaffold material. The interconnected pores had a median diameter of 435 μm and the size of the particles was approximately 3 × 2 × 2 mm.

### Antibodies

The purified anti-ALP (hybridoma B4-78), anti-PCI (M-38) and anti-OP (MPIIIB10) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, USA). The control mouse immunoglobulin G (IgG2a) monoclonal antibody and the secondary antibody goat anti-mouse IgG γ-chain-specific-FITC were purchased from Dako (Denmark).

### Expression of PCI and OP

Fourth passage HBMSC (donors 1–7) were plated at a density of 5000 cells per cm$^2$ and cultured until confluency in two different types of media: (i) α–MEM containing 10% FBS, AB, 0.2 mM AsAP and 0.01 M β-glycerophosphate (βGP, Sigma, The Netherlands) (control medium) and (ii) the same medium with the addition of $10^{-8}$ M dexamethasone (Dex, Sigma, The Netherlands) (+ Dex medium). The expression of PCI and OP was evaluated by flow cytometry. Briefly, after trypsinisation, cells were washed twice at 4 °C in PBS containing 1% bovine serum albumin and 0.1% $NaN_3$ (wash buffer). Before antibody labelling, and to block potential non-specific binding, cells were resuspended in PBS containing 5% BSA and 10% human serum and incubated for 30 min on ice. Cells (approx. 0.1–0.3 E6/staining) were then resuspended in fixative solution (Fix and Perm kit, Caltag Lab., Burlingame, CA) for 15 min, at room temperature, and then washed twice. Afterwards, the cell were resuspended in permeabilisation medium (Fix and Perm kit, Caltag Lab., Burlingame, CA) and blocking buffer containing: (a) control mouse anti-human IgG2a (1 : 5 dilution); (b) anti-PCI (1 : 5 dilution) and (c) anti-OP (1 : 5 dilution). Cells were incubated at room temperature, for 15 min, and then washed twice.

Antibody reactivity was detected by suspending the cells with blocking buffer containing goat anti-mouse IgG γ-chain-specific-FITC (1 : 5 dilution). Cells were incubated on ice and in the dark for 30 min. After washing, the cells were resuspended in 200 µl of FACS-flow/staining and analysed using a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry systems). For each measurement 10 000 events were collected.

### Temporal expression of ALP

Fourth passage HBMSC (donors 1–14) were plated at a density of 5000 cells per $cm^2$ and cultured up to nine days both in control and (+) Dex medium. The expression of ALP was evaluated by flow cytometry at several culture periods (three to four measurements were performed for each culture). Briefly, after trypsinisation, cells were washed twice in wash buffer and blocked against non-specific binding (see above). Cells (approx. 0.1–0.3 E6/staining) were then resuspended in blocking buffer containing: (a) control mouse anti-human IgG2a (1 : 5 dilution) and (b) ALP monoclonal antibody (1 : 10 dilution). After incubation on ice for 45 min and washing, antibody reactivity and measurements were performed as described above for PCI and OP.

### *In vivo* osteogenic potential of HBMSC

HBMSC ( passage 4, donor 1–14) were seeded on porous HA granules, at a density of 200 000 cells/particle and cultured for one week in (+) Dex medium. Following this period, and prior to implantation, the tissue engineered samples were soaked in serum free medium and washed in phosphate buffered solution pre-warmed to 37 °C. Samples ($n = 6$ per donor) were then implanted into subcutaneous pockets created in the back of immune-deficient mice (HsdCpb:NMRI-nu, Harlan, The Netherlands). Samples of each culture were divided at least over two animals. At the end of the six-week survival period, the implants were removed and fixed in 1.5% glutaraldehyde in 0.14 M cacodylic acid buffer, pH 7.3. The fixed samples were dehydrated and embedded in methyl methacrylate. Approximately 10 µm thick sections were processed undecalcified on a histological diamond saw (Leica SP1600, Leica, Germany) and then stained with basic fuchsin and methylene blue in order to visualise bone formation.

### Statistics

Statistical analysis was performed using both *t*-student tests and Mann-Whitney *U*-tests assuming non-equal variances. Statistical significance was defined as $p < 0.05$.

## Results

### Expression of PCI and OP

Expression of intracellular type I collagen was detected in all HBMSC cultures, irrespective of the presence of Dex in the culture medium. The proportion of cells that stained for PCI was consistently high, comprising 81.8 ± 21.4% of the total cell population. A high donor variation was found in the values expressed by each individual culture, which ranged from 45.3 to 99.1% of the total cell amount. The addition of Dex to the culture medium did not induce significant changes in the relative proportion of cells expressing intracellular collagen I. With regard to osteopontin expression, positive cells were detected in all confluent cultures, comprising in average 20% of the total cell population. However, the range of individual values was extremely wide (3.2–58.9%), indicating that the exact proportion of OP positive cells was strongly donor dependent. In addition, in the majority of the donors tested, the cells that stained positively for OP generated fluorescence signals that were only marginally above control values (data not shown), indicating a low intracellular content of this protein on the positive cells. Dex treatment of the cultures had no stimulatory effect on the relative amount of OP positive cells or on the intensity of their fluorescence signal.

### Temporal expression of ALP

In HBMSC cultures from each donor, the pattern of expression of ALP positive cells during time was similar in both culture conditions. However, in cultures treated with Dex, the fraction of ALP positive cells was consistently higher as compared to control cultures (Fig. 1(a)). Statistical analysis revealed that after the first two days of culture, the proportion of ALP positive cells in the (+) Dex condition was significantly higher as compared to the control ($p < 0.05$), suggesting that Dex stimulation induced an increase in the fraction of committed osteoprogenitor cells. In the majority of the donors tested (12 of 14), the relative amount of ALP positive cells increased during culture period reaching a maximum value and decreased thereafter. The time period required to achieve the maximum of ALP expression, as well as the value of the maximal fraction of ALP positive cells, was affected by the culture

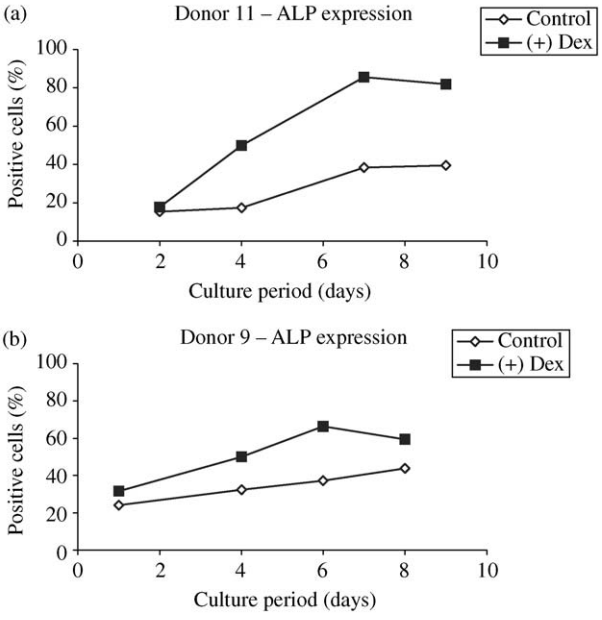


*Figure 1* Temporal expression of ALP in HBMSC cultures: Effect of dexamethasone treatment and variance between donors. (a) Donor 11 and (b) Donor 9.

conditions and markedly donor dependent (Fig. 1(a) and (b)). In HBMSC cultures from two of the 14 patients, the percentage of cells expressing ALP was above 80% in the beginning of the culture and decreased thereafter (data not shown).

## Quantification of osteoprogenitor cells in culture

An approach that may allow for the indirect quantification of early osteoprogenitor cells is the degree of culture stimulation by Dex with regard to the fraction of ALP positive cells. That is, cultures exhibiting a high increase in the amount of cells expressing ALP due to Dex treatment most likely contain a higher proportion of osteoprogenitor cells as compared to cultures in which stimulation by Dex induces a lower increase in ALP expression. Therefore, for each donor and culture period, the degree of stimulation by Dex was measured through the ratio between the fraction of ALP positive cells in the (+) Dex and control conditions. Both *t*-student and Mann–Whitney *U*-tests indicated that, after the first two days in culture, this ratio was time independent, revealing that the optimal cell response to Dex treatment occurred after the first 48 h. To verify whether the degree of culture response to Dex was correlated to the *in vivo* bone formation ability of the cultures, for each donor the average ratio was determined using the measurements performed from day 3 to day 9 (Table II). This ratio, taken as an indirect measure for the proportion of early osteoprogenitor cells, was then compared to the *in vivo* osteogenic potential of the cultures.

## *In vivo* osteogenic potential of HBMSC

Six weeks post implantation, *de novo* formed bone was found in all the samples from eight of the 14 assessed donors (1–2, 4–5, 8, 11–12, 14). The extent of bone formation varied from donor to donor ranging from a small quantity (up to 10% of the available pore area) to a significant amount of this tissue (up to 50% of the available pore area). Fig. 2 illustrates a representative section from the histological analysis. Mineralised bone tissue was observed in direct contact with the ceramic material, indicating that the implanted cells survived and further differentiated into osteoblasts. The bone matrix displayed embedded osteocytes and blood vessels were often observed close to the newly deposited bone. The HBMSC cultures from these donors revealed a good agreement between the *in vivo* and *in vitro* data, in which the osteogenic character of the cultures was demonstrated by the expression of PCI, OP (donor 1, 2, 4, 5) and by an increase in ALP expression after treatment with Dex. However, HBMSC cultures from donors 3, 6, 7, 9, 10 and 13 failed to induce *in vivo* osteogenesis despite the fact that *in vitro* testing also indicated expression of PCI, OP (donor 3, 6, 7) and an increase in ALP expression after treatment with Dex.

TABLE II Degree of Dex stimulation measured as the ratio between the fraction of ALP positive cells in the (+) Dex and control conditions

| Donor | Ratio | Log (ratio) | *In vivo* result |
|---|---|---|---|
| 1 | 1.53 | 0.18 | + |
| 2 | 1.53 | 0.18 | + |
| 3 | 1.40 | 0.15 | − |
| 4 | 2.71 | 0.43 | + |
| 5 | 2.53 | 0.40 | + |
| 6 | 1.52 | 0.18 | − |
| 7 | 1.25 | 0.10 | − |
| 8 | 3.72 | 0.57 | + |
| 9 | 1.56 | 0.19 | − |
| 10 | 2.12 | 0.33 | − |
| 11 | 2.40 | 0.38 | + |
| 12 | 2.56 | 0.41 | + |
| 13 | 1.55 | 0.19 | − |
| 14 | 1.80 | 0.26 | + |

+, Bone formation; −, Lack of bone formation.

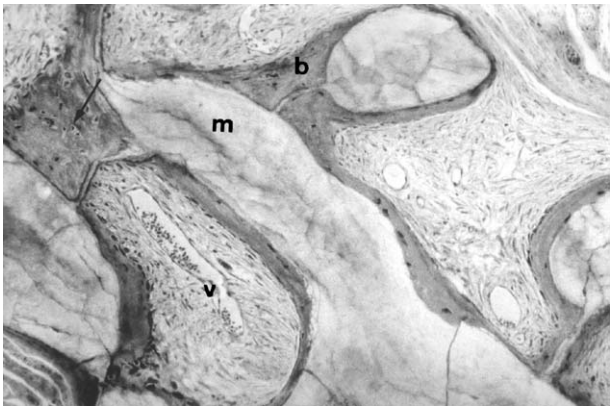


*Figure 2* Light micrograph illustrating a representative histological section of the samples after six weeks of subcutaneous implantation in nude mice. Note mineralised bone matrix (b) with embedded osteocytes (arrow), formed in direct apposition to the scaffold material (m). Blood vessels (v) were present in the vicinity of the newly formed bone, 100× .

## *In vivo* osteogenic potential versus degree of stimulation by Dex with regard to ALP expression

The *in vivo* bone formation capacity of HBMSC could not be related to their *in vitro* expression of PCI, OP and ALP nor to an increase in the percentage of ALP positive cells after Dex stimulation. However, our data indicated that the relative increase in the proportion of ALP positive cells following Dex treatment may be related to the *in vivo* bone formation capacity of the cultures. This relative increase, expressed by the ratio between the fraction of ALP positive cells in (+) Dex and control conditions, was in average higher in bone forming cultures as compared to cultures that failed to induce osteogenesis (Fig. 3 and Table II). Both *t*-student and Mann–Whitney *U*-tests revealed a statistically significant difference between bone forming and non bone forming cultures with regard to the increase on ALP expression after Dex treatment ($p = 0.021$, *t*-student test; $p = 0.029$, Mann–Whitney *U*-test).

## Discussion

Our results have demonstrated that all HBMSC cultures established from 14 different donors contained a fraction of cells expressing markers of the osteoblast phenotype,

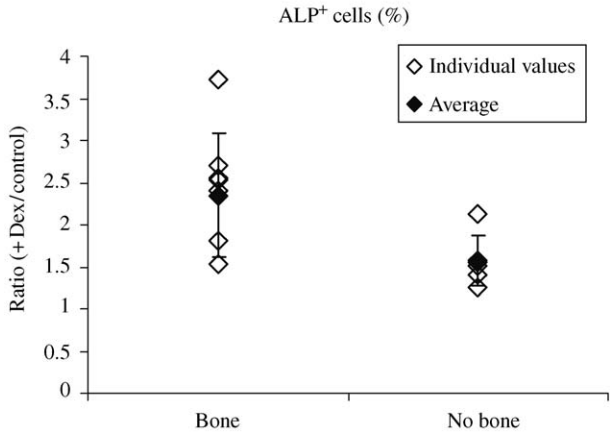


*Figure 3* Relative increase in the fraction of $ALP^+$ cells in bone-forming and non-bone-forming cultures, after Dex treatment. (◇) Individual values of 14 donors; (◆) Average of each population; (*) Statistical significance was observed: $p = 0.021$ in *t*-test and $p = 0.029$ in Mann–Whitney *U*-test.

such as, PCI, OP and ALP, indicating that each culture contained a population of cells committed to differentiate along the osteogenic pathway. Since a wide donor variability was observed in the expression of the assessed markers, and reactivity with both the early (ALP) and later (OP) osteogenic proteins [37, 38] was detected, these data further supports that the HBMSC cultures are not a uniform population of mesenchymal stem cells, but are composed of an heterogeneous mixture of cells at various stages of differentiation and with distinct osteogenic properties. These observations are consistent with a report by Kuznetsov *et al*. [24], in which it was demonstrated that only 59% of clonally derived human marrow stromal fibroblasts, established from different donors, were able to form bone when implanted in immune-deficient mice. In our study, the strong donor dependency observed with regard to the fraction of cells expressing PCI, OP and ALP, is in agreement with studies by Jaiswal *et al*. [16], Stewart *et al*. [29] and Phinney *et al*. [39] which also reported a large variability in ALP expression by cultures derived from human bone marrow of different donors. Differences in the physiological status of the donor, as well as the aspiration site and procedure can account for these variations. With regard to the aspiration site, Phinney *et al*. [39] detected a large variation in the expression of ALP enzyme activity in HBMSC cultures from different donors despite the fact that all aspirates were obtained from the iliac crest. Furthermore, they observed clear differences in ALP activity of cultures established from the same donor over a six month period, which indicated that the method of bone marrow harvest plays a major role in producing cellular heterogeneity.

The differentiation of osteogenic cells from their precursors is known to be enhanced by Dex. Therefore, the effect of this glucocorticoid on PCI, OP and ALP expression was determined. Our data revealed that Dex treatment had no stimulatory effect on the relative proportion of cells expressing PCI or OP. With regard to pro-collagen I reactivity, conflicting results have been published in literature [33, 35], in which Dex was reported to have either an inhibitory or no effect. This discrepancy of results is most likely due to two main factors: the culture conditions and the culture period at which the analysis was performed. With respect to OP expression, the absence of stimulation by Dex can be related to the differentiation stage of the cells. Since Dex would mainly act on early progenitors, it is possible that the stimulated cells, at the time of the measurement, were still in an earlier differentiation stage, not yet expressing OP. This hypothesis is consistent with the fact that Dex invariably increased the proportion of cells expressing ALP, an early osteogenic cell marker [29, 35, 40]. In addition, the observed effect of Dex over the HBMSC populations is in agreement with numerous studies [16, 22, 25, 29, 32–35, 39] and indicates that this glucocorticoid induces progenitors cells to start the process of osteogenic differentiation.

Although we have observed that the addition of ascorbic acid and basic fibroblast growth factor enhance HBMSC cell growth, and that increased cell passage is directly related to decreased osteogenic potency (*in vitro* and *in vivo*; unpublished results), we decided to keep these variables constant in this study so that only the influence of Dex could be evaluated.

Although several reports have demonstrated the therapeutic potential of HBMSC cultures in bone repair [7, 10, 19–24], *in vivo* bone induction by these cultures depends on the presence of a sufficient number of early osteoprogenitors on the implant that can proliferate and further differentiate into osteoblasts. Therefore, the quantification of the osteoprogenitor cell content in the implanted population is of extreme importance. Due to the lack of procedures to isolate early osteoprogenitor cells, we attempted an indirect quantification method based on the hypothesis that after Dex stimulation, the increase on the proportion of cells expressing ALP would provide a measurement for the amount of early (and therefore inducible) osteoprogenitor cells in culture. After calculating the degree of stimulation by Dex displayed by each culture, the results were compared to their ability to induce bone formation in an *in vivo* situation, using a nude mice model. The preliminary data revealed that the average degree of stimulation, with regard to ALP expression, was higher in bone forming cultures as compared to the non-bone forming ones. These results suggested that the ratio between the proportion of cells positive for ALP in the (+) Dex and control conditions may provide a method to assess the early osteoprogenitor cell content of a given population. Nevertheless, it should be noted that the present method does not take into account osteogenic cells that, prior to Dex treatment, had started the process of osteogenic differentiation and this may account for the partial overlapping between the two groups observed in Fig. 3 (bone forming versus non-bone forming). Although these cells may also contribute to the *in vivo* osteogenic potential of the total population, the relation established herein between *in vitro* and *in vivo* data, was based on the measurement of early osteoprogenitors in culture. In future, analysis of a wider donor population will be required to further clarify the observed trend. With respect to the nude mice model used to assess *in vivo* osteogenic potential of HBMSC cultures, it is worth noting that for each donor six tissue engineered samples

were implanted divided over at least two animals, and the presence or absence of newly formed bone in the samples was not affected by the animal in question. However, the use of more than one animal per donor is advisable since previous studies in our group showed that animals can have an influence in the occurrence of bone formation (data not shown). In addition, the nude mouse model presents some drawbacks as it is difficult to extrapolate results obtained in an ectopic site of a small animal to a clinical relevant situation.

## Conclusions

In conclusion, our findings show that although PCI, OP and ALP are widely recognised markers of osteogenic activity, their absolute expression in HBMSC cultures cannot be taken as predictive marker for *in vivo* bone formation by HBMSC. In addition, our data suggests that the proportion of bone forming cells may be directly related to the fold increase in the fraction of cells expressing ALP after Dex treatment.

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