European Cells and Materials Vol. 24 2012 (pages 1-17) DOI: 10.22203/eCM.v024a01 ISSN 1473-2262

IN SEARCH OF AN OSTEOBLAST CELL MODEL FOR *IN VITRO* RESEARCH

E.M. Czekanska[1,2], M.J. Stoddart[1], R.G. Richards[1,2] and J.S. Hayes[3,*,§]

[1]AO Research Institute, Davos, Switzerland
[2]Cardiff University, Cardiff, Wales
[3]REMEDI, National Centre for Biomedical Engineering Science, National University of Ireland, Galway, Ireland

[§]The authors are not compensated and there are no other institutional subsidies, corporate affiliations, or funding sources supporting this work unless clearly documented and disclosed.

Abstract

The process of bone formation, remodelling and healing involves a coordinated action of various cell types. Advances in understanding the biology of osteoblast cells during these processes have been enabled through the use of various *in vitro* culture models from different origins. In an era of intensive bone tissue engineering research, these cell models are more and more often applied due to limited availability of primary human osteoblast cells. While they are a helpful tool in developing novel therapies or biomaterials; concerns arise regarding their phenotypic state and differences in relation to primary human osteoblast cells. In this review we discuss the osteoblastic development of some of the available cell models; such as primary human, rat, mouse, bovine, ovine and rabbit osteoblast cells; as well as MC3T3-E1, MG-63 and SaOs-2 cell lines, together with their advantages and disadvantages. Through this, we provide suggestions on the selection of the appropriate and most relevant osteoblast model for *in vitro* studies, with specific emphasis on cell-material based studies.

Keywords: Osteoblast; differentiation; primary cells; cell lines; *in vitro* cell models.

*Address for correspondence:
Jessica Hayes
Regenerative Medicine Institute
National Centre for Biomedical Engineering Science
National University of Ireland, Galway
E-mail: jessica.hayes@nuigalway.ie

Introduction

Bone is a complex and dynamic vascular mineralised tissue with various functions. It serves as an attachment site for muscles and tendons, protects and supports internal organs, and acts as a mineral reservoir. In the context of tissue engineering, bone also encompasses the important function of housing bone marrow, the principal source of multipotent cells and the centre of haematopoiesis.

Since the first attempt of isolation and *in vitro* culture of osteoblast cells from adult human bone (Bard *et al*., 1972), a great improvement in the knowledge of bone biology and, in particular, osteoblastic cells have been made. Stabilised osteoblastic cell lines have been developed as models for *in vitro* investigation of the regulation of cell differentiation, cytokine and hormonal regulation, synthesis and secretion of matrix proteins, molecular mechanisms of bone diseases, and drug pharmacokinetics. In more recent years osteoblast cell culture has also evolved to include cytocompatibility and osteogenicity evaluation of novel biomaterials.

Various cell culture models have been employed for studying osteoblast cell biology, including primary cells from different species, induced osteoblasts from pluripotent stem cells, immortalised and malignant cell lines. The use of these osteoblast models, as well as any *in vitro* cell model, presents advantages and disadvantages which need to be considered before embarking on any notable evaluation (Table 1). For instance, osteosarcoma cell lines are available in unlimited number without the need for time consuming isolation or ethical approval, with the advantage of more reliable reproducibility. Primary cells have obvious attractiveness in terms of encompassing cell behaviour more reflective of the *in vivo* niche, and therefore, have more preclinical and clinical applicability. Nevertheless, existing species differences complicate extrapolation to human clinical disease and treatment outcomes. Hence, this review presents and discusses factors that influence the phenotype of osteoblast cells derived from different sources. Along with this, it highlights the advantages and disadvantages of these cell types. Consequently, it aims to help with the selection of the appropriate and most relevant osteoblast model for *in vitro* studies, with specific emphasis on cell-material interaction studies.

Table 1. Advantages and disadvantages of primary osteoblast cells and osteoblastic cell line models.

Cell type	Advantages	Disadvantages	References
Primary human cells	• no interspecies differences • relevant for clinical studies	• heterogeneous phenotype • long isolation procedure • limited accessibility • cell phenotype sensitive to donor-related factors	Kasperk *et al*., 1995 Martinez *et al*., 1999 Evans *et al*., 1990 Voegele *et al*., 2000 Gallager, 2003 Jonsson *et al*., 1999 Fedarko *et al*., 1992 Battmann *et al*., 1997 Siggelkow *et al*., 1999
Primary mouse/rat cells	• easily available • possibility to control the selection of donor-animals • cell extraction from all bones in the skeleton	• interspecies differences • genomic differences • cell phenotype sensitive to age and site of isolation factors	Bakker & Klein-Nulend, 2003 Soejima *et al*., 2001 Carpenter *et al*., 1998 Declercq *et al*., 2004 Lian and Stein, 1992 Manduca *et al*., 1997 Stringa *et al*., 1995
Primary bovine/ ovine/ rabbit cells	• potential for improved in vitro extrapolation of bone remodelling and healing process to current in vivo models. • potential formation of trabecular structures (bovine osteoblasts)	• inconsistent results regarding cell mineralisation • need for optimising culture conditions • lack of extensive characterisation of cells • limitations for molecular biology methods	Ibaraki *et al*., 1992 Newman *et al*., 1995 Neyt *et al*., 1998 Cao *et al*., 2006 Whitson *et al*., 1992
SaOs-2	• no interspecies differences • unlimited number of cells • homogenous • cytokine and growth factor expression profile similar to human Ob cells • sensitive to hormonal administration • matrix mineralisation	• do not mirror the whole range of osteoblast phenotypic changes • sensitive to Pi substrates	Masuda *et al*., 1987 Murray *et al*., 1987 Bilbe *et al*., 1996 Rodan *et al*., 1987 Fernandes *et al*., 2007 Rao *et al.,* 1996
MG-63	• no interspecies differences • unlimited number of cells • hormonal administration response similar to human Ob cells • similar to human integrin subunits profile	• arrested in pre-osteoblast state • inconsistent regarding cell mineralisation	Heremans *et al*., 1978 Clover *et al*., 1992 Olivares-Navarrete *et al*., 2008 Kumarasuriyar *et al*., 2009 Saldana *et al*., 2011 Pierschbacher *et al*., 1988
MC3T3-E1	• unlimited number of cells • homogenous character • phenotypic differentiation from pre-osteoblasts to mature osteoblasts	• interspecies differences • some signs of cellular replicative senescence	Wang *et al*., 1999 Sudo *et al*., 1983 Quarles *et al*., 1992 Grigoriadis *et al*., 1985

Osteoblast cell models in *in vitro* research

Human isolated cells

The main advantage to using primary human cells is their clinical applicability and the reduced need for accounting for interspecies differences, as is the case when other animal cell sources or cell lines are used. An example of such distinctions is exhibited in Fig. 1 where differences in spatial staining for alkaline phosphatase (ALP) and Alizarin Red are evident between different cell sources. Furthermore, primary human osteoblasts tend to retain their differentiated phenotype *in vitro*. On the other hand, human isolated cells represent a heterogeneous cell population, and therefore exhibit phenotypic differences relating to the skeletal location from which they were isolated (Kasperk *et al*., 1995; Martinez *et al*., 1999).

Several primary human osteoblast isolation methods have been developed, including enzymatic digestion and spontaneous outgrowth cultures (Gotoh *et al.*, 1990; Gallager, 2003). Several studies have identified an effect of the isolation technique on subsequent *in vitro* performance (Jonsson *et al*., 1999; Voegele *et al*., 2000)**.** Specifically, cells obtained through enzymatic isolation proliferate faster than cells from outgrowth cultures, but ALP activity is comparable in cells from both isolation types (Voegele *et al.,* 2000). Contradictory results in terms of ALP activity were found in another study where the enzyme level was higher in cells obtained from outgrowth cultures (Jonsson *et al.*, 1999). This can be further influenced by donor age. Cells from donors younger than 65 years old were shown to have shorter population doubling times than osteoblast cells from older donors (Voegele *et al.*, 2000). In agreement with this, a decreased rate of proliferation in trabecular bone cell cultures of donors over 60 years of has been reported elsewhere (Evans *et al.*, 1990), with the most significant decline being attributed to the first three decades of life (Fedarko *et al.*, 1992; Shigeno and Ashton, 1995). Moreover, in cells isolated via outgrowth cultures mineral deposition was more pronounced 4 weeks after the osteogenic induction than in cells from enzymatically digested bone specimens (Jonsson *et al*., 1997).

In correlation with the proliferation potential of cells, the expression of genes and protein synthesis associated with osteoblast phenotype is also influenced by donor age. Synthesis of type I collagen and osteonectin were at the highest level in cells isolated from donors from foetal bones to 20 year old donors. A decrease of approx. 65 % in collagen levels was observed in cells from donors above the age of 20 years old (Fedarko *et al*., 1992). Additionally, after induction with $1,25(OH)_2D_3$ the level

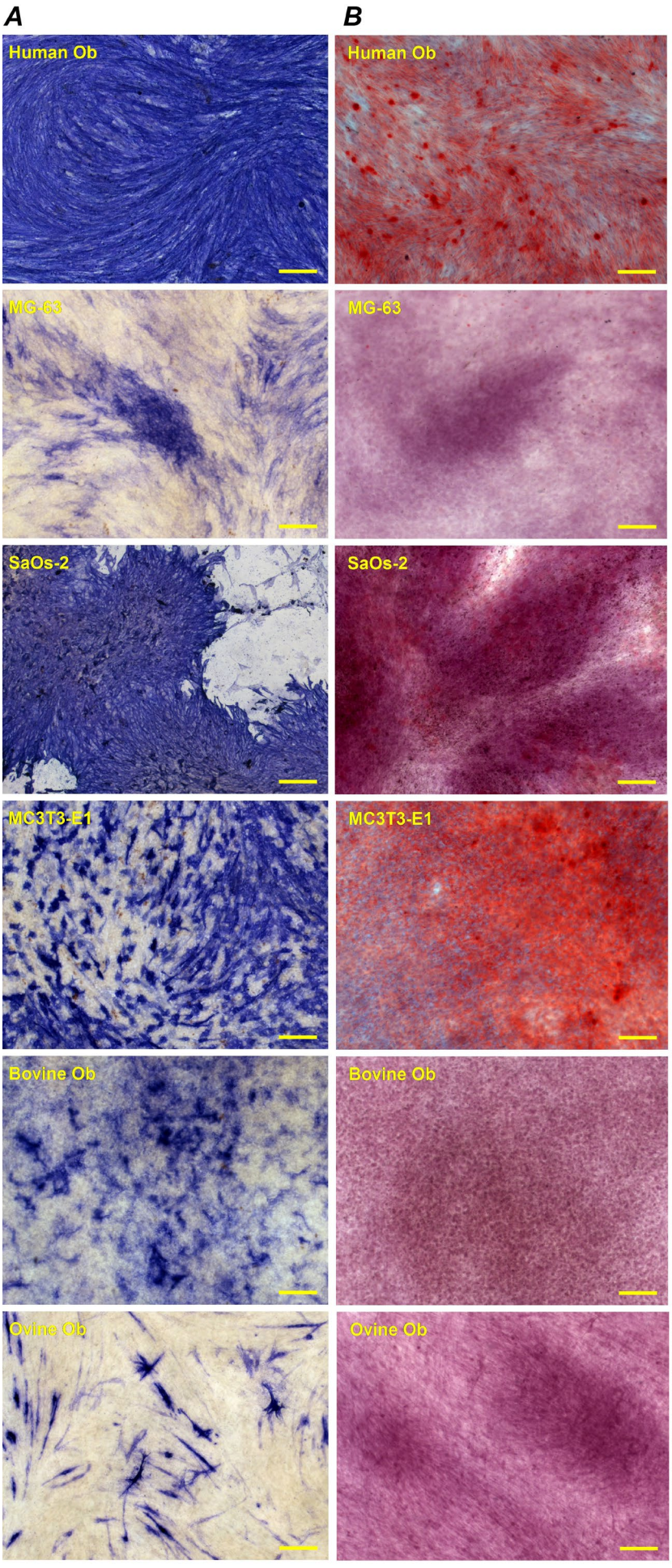

Fig. 1. Light microscopy image representing differences in **(A)** ALP activity at day 21 and (**B**) mineral deposition at day 28 in primary human osteoblast cells, MG-63, SaOs-2, MC3T3-E1 and primary ovine and bovine osteoblast cells. Cells were plated at the density 5000 cells/cm^2 in 6 well plates and cultured in presence of ascorbic acid 2-phosphate and bGP (except SaOs-2, which were supplemented only with ascorbic acid 2-phospate) for 28 days. Note the differences in staining intensity; the spatial differences in positively stained cells and the morphology of the cells. Scale bar 200 µm. Authors' unpublished images.

of the osteocalcin secreted by cells isolated from foetal cortical femoral bone was less than cells from postnatal (age 4-12 years) and adult donors (20-30 years) (Morike *et al.,* 1993; Morike *et al.*, 1995). In cells from donors in the third decade of life, the protein level was 10-fold higher than in foetal bone cells (Morike *et al*., 1995). Furthermore, osteocalcin expression became higher in foetal cells after 10 days of culture with osteogenic supplements and $1,25(OH)_2D_3$ (Montjovent *et al*., 2004).

Considering the changes in osteoblast phenotype relating to donor age, it is important to emphasise that bone aging, determined as changes in the degree and distribution of mineralisation, occurs differently in cortical and cancellous bone (Bonjour *et al.*, 1991; Bergot *et al.*, 2009). This is reflected in osteoblast cell physiology with slower proliferation and increased secretion of ALP and osteocalcin in cells derived from femoral head compared to upper tibia and lower femur (Martinez *et al.*, 1999). Kasperk and colleagues (1995) conducted within-patient comparisons and demonstrated that mandibular derived osteoblast cells had a higher proliferative rate associated with decreased ALP than cells isolated from the iliac crest. Furthermore, decreased expression of FGFβ and IGFII genes was also observed in mandibular-derived osteoblast cells (Kasperk *et al.*, 1995). With the inclusion of age-defined differences it was shown that cells from the knee had higher ALP activity in patients over 70 years old than in those between 50 and 70. The opposite was seen in osteoblasts derived from the femoral head (Martinez *et al.*, 1999).

Age-related changes occurring in bone composition occur mostly in bones in lumbar and hip regions in postmenopausal women due to the decline in systemic oestrogen levels (Davis *et al*., 1994). Indeed, Zhang and colleagues (2004) showed that the expression of ALP, bone sialoprotein (BSP) and type I collagen was significantly increased in cells isolated from female patients below 15 years old, which was subsequently observed to decline with increasing age. Furthermore, a 3.8-fold difference in ALP activity was noted between young and postmenopausal women while the osteocalcin level was shown to be higher in cells from premenopausal women below 50 years old (Zhang *et al*., 2004). In contrast, osteoblasts derived from male femoral head and long bones did not show age related differences in terms of ALP and osteocalcin level (Katzburg *et al*., 1999). Conversely, Battmann and co-workers (1997) reported that osteocalcin production by cells from femoral head or iliac crest changed markedly with age, but were not correlated with gender differences (Battmann *et al*., 1997).

As presented, many factors influence primary human osteoblast cell behaviour; age, site of isolation and gender differences are the most debatable. Thus, the time frames for phenotypic changes *in vitro* differ for cells isolated from different origins. Some studies indicate that nodule formation in mineralisation of human bone cell cultures may not occur (Siggelkow *et al.*, 1999). Furthermore, the question arises, which factors are of utmost importance and should be included for screening cells (or donors) before applying them for research purposes? The answer is not simple and the selection of cells should be controlled and judged on the perspective of addressed subject and aim of study.

Animal primary cells

Due to limited accessibility of human osteoblast cells and their phenotypic heterogeneity, cells isolated from other species can provide an alternative *in vitro* research model. A distinct advantage is their ease of attainability compared to human cell sources. For humans, the location of bone specimens for cell isolation are limited mainly to long bones, mandible and the iliac crest, whereas they can be obtained more easily from additional sites from animals, such as the calvaria. The selection of donor animals can be more controlled in relation to age, weight and sex. Unlike human donors, isolation from animals is not only restricted to pathological subjects. On the other hand, the distinct disadvantage of animal cells is the interspecies differences making extrapolation problematic. Furthermore, the biology and structure of bone is different among animals. Here, the sizes of the animal and weight influence bone composition, quality, remodelling, healing and signalling pathways driving these processes (Pearce *et al*., 2007).

Rat cells

Rat osteoblast cells serve as a model in *in vivo* and *in vitro* research. In 1994 the FDA presented guidelines for preclinical and clinical evaluation of agents used in the treatment or prevention of postmenopausal osteoporosis, with the recommendation of using two animal models, rat and sheep, for this evaluation (Thompson *et al*., 1995). Due to the wide availability of bone samples and known genome, rat osteoblast cells are an attractive model for research focused on hormonal regulation of cell phenotype (Aronow *et al.*, 1990; Abe *et al*., 2000), as well as the osteoinductivity and cytotoxicity of polymer or implant biomaterials (Liao *et al*., 2003; Wirth *et al.*, 2008; Hayes *et al*., 2010; Washington *et al.*, 2011).

Rat osteoblast cells isolated from foetal, neonatal/adult calvaria or long bones retain their osteoblastic phenotype in culture (Manduca *et al*., 1997; Yamamoto *et al*., 2002). Osteoblast isolation from rat tissue is performed mainly through repeated enzymatic digestion and less often through the spontaneous migration from explant specimens. The use of enzymatic treatment was shown to be an effective isolation method of cells from calvarial bones of foetal or neonatal animals (Declercq *et al*., 2004). As a result of enzymatic isolation, cells derived from individual fractions of digestion exhibit different osteoblastic differentiation ability and response to hormones (Lian and Stein, 1992). When calvarial cells from five fractions obtained from rat foetuses were compared, the first 3 fractions responded positively to dexamethasone administration with increased ALP activity and calcium deposition, whereas cells from last two fractions did not show this response. Moreover, based on the number of nodules formed and the assumption that nodule formation is correlated to the number of osteoprogenitor cells, fraction three contained the highest number of osteoprogenitor cells (Yamamoto *et al*., 2002).

Despite the fact that the rat 'donor' selection can be more controlled than for humans, the cell phenotype is similarly affected by age and sex of the animals, as well

as the tissue origin. A comparative study by Declercq and colleagues (2004), evaluating osteoblastic cells derived from adult and foetal rat via explant culture or enzymatic digestion showed that osteogenic differentiation was enhanced in cells isolated by proteolytic treatment, as determined by matrix mineralisation and nodule formation. Additionally, for cells originating from explant cultures, both calvarial and long bone (tibia) samples had lower ALP activity and a more diffuse mineralisation pattern compared to foetal cells from enzymatically digested tissue. Moreover, the temporal pattern of mineralisation differed between types of bone (Declercq *et al*., 2004). Other studies have shown that cells from the mandible of rat foetus (20 day old) demonstrated β-glycerophosphate (bGP)-independent matrix mineralisation, whereas bGP was required for nodule formation and mineralisation of calvarial cells obtained from the same animals (Bellows *et al*., 1987; Bellows *et al*., 1992; Abe *et al*., 2000).

Mineralisation has also been noted to be influenced by donor age differences. For instance, cells isolated from 7 day old rat tibia formed mineralised nodules after 41 days in culture compared to positive osteocalcin detection after just 21 days for foetal mandibular cells (Stringa *et al*., 1995). A more recent study has also revealed gender differences in osteoblast cells isolated from calvaria. Specifically, ALP activity in osteoblasts cells from both genders was positively regulated by $1,25(OH)_2D_3$ in a similar manner, but cells derived from 6-week old male rats had a higher initial ALP activity compared to aged matched female counterparts. The dose-dependent response to $1,25(OH)_2D_3$ was evident regarding cell proliferation and osteocalcin production, with decreased cell number and increased protein synthesis in osteoblast cells isolated from male donors (Olivares-Navarrete *et al*., 2010).

Mouse cells

Osteoblast cell populations can be obtained from various skeletal locations from mice, including calvaria, long bone and vertebrae. Ecarot-Chartier and co-workers (1983) initially isolated primary mouse osteoblasts from new-born mouse calvaria via out-migration from explant cultures. Similarly, out-migration has been used to isolate cells from trabecular bone from mouse caudal vertebrae (Lomri *et al*., 1988). Additionally, out-migration proceeded with collagenase is generally applied for isolation of cells from the diaphyses of long bones of adult mice (Soejima *et al*., 2001; Bakker and Klein-Nulend, 2003). Murine osteoblast cells from outgrowth cultures, from both calvaria and caudal vertebrae, reportedly synthesise ECM which undergoes calcification in the presence of phosphate substrate (Ecarot-Charrier *et al*., 1988; Lomri *et al*., 1988). This process produced a tissue resembling woven bone ultrastructure (Ecarot-Charrier *et al*., 1983; Ecarot-Charrier *et al*., 1988).

The isolation from outgrowth cultures described above reportedly yielded approximately 90 % pure osteoblast cell population with less than 10 % accounting for contaminating cells (subsequently identified as granulocytic eosinophils and neutrophils; Lomri *et al*., 1988). In contrast, genome array analyses of mouse cells isolated by sequential digestion (second fraction) displayed adipogenic and muscle specific genes expression, indicating an impure population of isolated cells believed to be of bone or marrow origin (Garcia *et al*., 2002). According to Bekker and Klein-Nulend only subsequent third and fourth populations of enzymatically isolated cells are enriched for differentiated osteoblasts (Bakker and Klein-Nulend, 2003). Unfortunately, this was not further elucidated and the osteoblastic phenotype of the isolated cells was not confirmed by functional analysis.

Murine osteoblast cell phenotype is affected by age. Differences in osteocalcin synthesis are manifested in donors of different ages. Osteocalcin synthesis was found to be 6-fold higher after 3 weeks in culture in cells isolated form mouse calvaria isolated 30-36 h after birth compared to 78-84 h after birth (Carpenter *et al*., 1998). Moreover, the age and site of isolation affect ALP enzyme activity. This was reported at higher levels in cells from mouse adult long bone than adult and neonatal calvarial cells (from 3-4 day old animals). Interestingly, the stimulation with $1,25(OH)_2D_3$ induced a 3-fold increase in ALP activity in neonatal osteoblast cells compared to osteoblasts isolated from long bone and calvaria of adult animals (Soejima *et a*l., 2001). It has also been reported that cells isolated from mouse calvarial have higher basal osteocalcin production compared to rat calvarial osteoblast cells. Moreover, in mouse osteoblasts cultures this production is negatively affected by both acute and chronic addition of $1,25(OH)_2D_3$. Contrarily, $1,25(OH)_2D_3$ administration in both human and rat primary osteoblast cultures induces osteocalcin levels (Morike *et al*., 1993; Morike *et al*., 1995; Carpenter *et al*., 1998; Montjovent *et al*., 2004).

Bovine cells

Bovine and ovine derived osteoblast cells are less often used as osteoblast cell models compared to rodents. This is surprising, considering that these animals are frequently used as large animal *in vivo* models, and other skeletal cells from these animals serve as established *in vitro* models. For instance, chondrocytes or intervertebral disc cells from bovine specimens are widely used in tissue engineering research and for investigating basic biological mechanisms (Dowthwaite *et al*., 2004; Sakai *et al.,* 2009; Collin *et al*., 2011; Hoshiba *et al*., 2011; Peroglio *et al*., 2011). However, studies focused on bovine osteoblast cells are limited. The first attempts to explore *in vitro* bovine osteoblast culture system involved cells isolated from the hind-limbs of 3 to 6 month old foetal calves (Whitson *et al.,* 1992). This resulted in the identification of functional osteoblast cells that secrete ALP, type I collagen, BSP, osteonectin and bone proteoglycans. Moreover, investigations showed 4.5-6 month old foetuses to be optimal for osteoblast cell isolation. The enzymatic extraction of osteoblast cells from foetuses at this stage resulted in a higher number of viable cells and superior proliferation compared to older foetuses. Additionally, cells from younger foetuses had a slower mineralisation potential when compared to those from 4.5-6 months old foetuses. In bovine cell cultures after stimulation, ALP activity increased in cultures by day 6, which was followed by an increase in mRNA expression of type I collagen, osteonectin and osteopontin (Whitson *et al.,* 1992). In this system the deposition of minerals and

nodule formation were initiated 6 days after osteogenic induction with β-glycerophosphate and ascorbic acid (Ibaraki *et al*., 1992).

The centres of mineralisation appeared and expanded in the matrix located between the single cell layer adhering to the culture surface/dish and continuous multi-cellular layer located above the mineralising matrix (Whitson *et al*., 1984). Interestingly, these cells also formed independent and interconnected mineralised trabecular structures (Whitson *et al.,* 1992). Interestingly, we have observed that explant cultured cells isolated from 6 month old bovine specimens do not have the ability to form nodules or mineralised matrix. Furthermore, ALP activity remained at basal levels over a 21-day period but then increased rapidly by day 28 (unpublished observations).

Ovine cells

In the majority of the orthopaedic research studies, sheep is considered an established *in vivo* model for the testing of novel implant systems in fracture healing. This is due to the advantage of adult sheep having similar body weight to humans and the dimensions of long bones being appropriate for the implantation of human implants and prostheses (Newman *et al*., 1995). Thus, the inclusion of ovine osteoblast cells as an *in vitro* model holds several clear advantages. Unfortunately, there are few *in vitro* studies using ovine osteoblast cells. The main reason for this is the lack of a fully sequenced genome, which limits the application of many molecular biology methods. Currently, institutions such as the International Sheep Genomic Consortium are attempting to address this issue.

The available literature focusing on osteoblastic activities of ovine cells *in vitro* indicate these cells are capable of mineralisation (Collignon *et al*., 1997). Foetal ovine osteoblast cells stimulated post-proliferatively with ascorbic acid, β-glycerophosphate and $CaCl_2$ exhibited the highest ALP activity after 6 days culturing and formation of mineralisation centres from day 12. Culture medium analysis demonstrated a rapid increase in ALP concomitant with initiation of mineralisation. Furthermore, ovine osteoblast cells produced osteocalcin in a time-dependent manner from day 6 (Collignon *et al.*, 1997). Interestingly, a more recent study has shown that trabecular bone from ovine specimens classed as middle-aged (3-5 year old) behaved similarly to human cells isolated from the femoral condyle (donor age not known). Ovine cells responded to $1,25(OH)_2D_3$ stimulation similarly to human osteoblast cells, which resulted in an increase in ALP, osteocalcin and transforming growth factor (TGF)β1 (Torricelli *et al*., 2003). However, ovine osteoblast cells isolated from trabecular bone from 2 year old animals, used in our laboratory failed to mineralise. It is not known how common this phenomenon is since, to our knowledge, we are the only group to demonstrate the lack of mineralisation in ovine and bovine cells of this age under a variety of established culture conditions (unpublished observation).

Rabbit cells

Rabbits are animals widely used as *in vivo* models for various biomedical research applications, such as musculoskeletal research (Neyt *et al*., 1998); including implant biomaterials testing, infection or gene therapy studies. Differences in bone structure, composition and remodelling between rabbit bone and human bone has been discussed and reviewed recently (Pearce *et al*., 2007) yet, not much is known about the biology of rabbit osteoblastic cells *in vitro*. Similarly to bovine and ovine osteoblast cells, the available literature is limited and gives poor insight regarding phenotype and behaviour. Early reports established an isolation protocol of enzymatically digesting rabbit bones with 0.1 % crude collagenase (Yee, 1983; Shaw *et al*., 1989). Later, explant cultures from cervical vertebrae were used for isolating cells. The morphological and functional evaluation of cells isolated from cortical endosteal bone surface of adult rabbits identified that the almost all populations of cells had osteoblast typical polygonal-shaped cells, whereas cells migrating out from the cervical bone had more spindle or triangular shapes (Cao *et al*., 2006). The former were positive for ALP activity and were capable of mineralisation. Stimulation with parathyroid hormone (PTH) positively influenced cAMP production (Shaw *et al*., 1989). Extracellular matrix mineralisation and nodule formation was reported after 3 weeks of culturing rabbit osteoblast cells with ascorbic acid, dexamethasone and BGP (Cao *et al*., 2006). However, current knowledge regarding rabbit osteoblast cells is lacking and further studies are needed to evaluate rabbit osteoblast physiology and its relation to human osteoblast cells. Results from a recent study focusing on rabbit bone marrow stromal cells intensifies that need as the authors show that rabbit cells can undergo spontaneous immortalisation after prolonged culture and lose their potential to differentiate into chondrocytes and adipocytes (Ahmadbeigi *et al*., 2011).

Cell lines

The list of advantages of immortalised cell lines is long, including ease of maintenance, unlimited number of cells without the need for isolation and relative phenotypic stability. On the other hand, some reports present evidence of progressing phenotypic heterogeneity among cell lines, which is correlated with prolonged passaging of cells (Grigoriadis *et al*., 1985; Leis *et al.*, 1997; Wang *et al.*, 1999). Additionally, both transformed and non-transformed cell lines, as they are stage arrested, do not reflect the whole range of phenotypic features of normal osteoblast cells. Specifically, in the case of malignant cell lines, proliferation is non-physiological since the mechanism of contact inhibition amongst several other cell division related processed are disturbed. Despite these disadvantages, the use of osteoblast cell lines is prevalent. The most commonly used cell lines are MC3T3-E1(Quarles *et al*., 1992), hFOB (Harris *et al.*, 1995), MG-63 (Billiau *et al*., 1977), SaOs-2 (Rodan *et al.*, 1987) and U2OS (Ponten and Saksela, 1967). The reader is directed to the excellent review of Kartsogiannis and Ng (2004) which discusses the aforementioned cell lines, in addition to many not covered here.

Human cell lines

Rodan and colleagues (Rodan *et al*., 1987) characterised osteoblastic properties of the human osteosarcoma cell

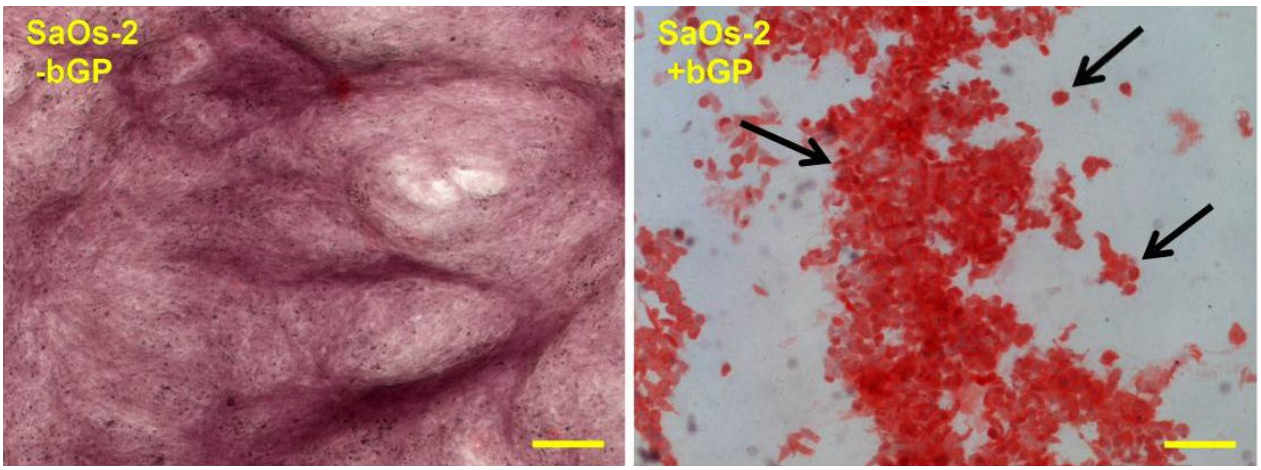

Fig. 2. SaOs-2 cells maintained in culture medium with ascorbic acid (50 μg/mL) and in the presence (5 mM) or absence of glycerol-2-phosphate (bGP). In the absence of bGP cells form multilayers with areas developing into nodules. In the presence of bGP, cells aggregated into groups throughout the culture dish and with time cells began detaching from the surface (black arrows). The strong staining of cells indicates the accumulation of calcium on the cell surface membrane. Images represent cell culture at day 14 (scale bar: 200 μm). Authors' unpublished images.

line SaOs-2, which was isolated from an 11-year old Caucasian female in 1975. These cells have a mature osteoblast phenotype with high levels of ALP activity. This marker was reported at much higher level than in other osteosarcoma cell lines, such as MG-63 and SaOs-1 (Murray *et al*., 1987). Additionally, when compared to human primary osteoblast cells, the ALP activity was similar at the early time points, but 120-fold higher after 14 days of culturing cells under the same conditions (Saldana *et al*., 2011). Interestingly, SaOs-2 cells have been reported to form a calcified matrix typical of woven bone (Rodan *et al.*, 1987). Detailed analysis of the collagen structure synthesised by SaOs-2 revealed that it is similar to collagen formed by primary human osteoblast cells but with higher level of lysyl hydroxylation in SaOs-2 cells (Fernandes *et al*., 2007).

ALP in SaOs-2 cells can be further stimulated by dexamethasone (Rao *et al.*, 1996) and also phosphate substrates (Muller *et al.*, 2011). Continuous culturing in medium with dexamethasone has been reported to increase ALP activity and produce a more differentiated phenotype (Rao *et al*., 1996). Due to the much higher ALP activity level in SaOs-2 cells compared to primary cells in basal conditions, the necessity of the additional administration with dexamethasone should be balanced. Moreover, phosphate substrate supplementation of SaOs-2 cells should be introduced at much lower levels (if at all) compared to other cell types and specifically at the post-proliferative stage when a layer of matrix has been produced, to avoid deleterious effects on viability (Fig. 2). Finally, cytokine and growth factor expression of SaOs-2 cells have been shown to be similar to primary normal human osteoblast cells (Bilbe *et al.*, 1996). Specifically, SaOs-2 cells have been shown to express receptors for parathyroid hormone (PTH) and $1,25(OH)_2D_3$ similar to those expressed on osteoblasts *in vitro* and *in vivo*. Dexamethasone administration of SaOs-2 cells increases their sensitivity to PTH, $1,25(OH)_2D_3$ and 17-b oestradiol (Rao *et al*., 1996).

The MG-63 cell line derives from a juxtacortical osteosarcoma diagnosed in the distal diaphysis of the left femur of a 14 year old male (Billiau *et al*., 1977). These cells display rapid cell growth without exhibiting contact inhibition, resulting in the formation of aggregates (Heremans *et al.*, 1978). This cell type attracted much attention as it produces high yields of interferon (Billiau *et al.*, 1977). Further research identified the responsiveness of MG63 cells to $1,25(OH)_2D_3$ as affecting cellular morphology and phenotype by increasing ALP activity (Franceschi *et al.*, 1985). MG-63 cells represent an immature osteoblast phenotype and undergo temporal development in long term culture. However, inconsistencies exist in the literature regarding mineralisation capabilities of monolayer MG63 cells. In a recent study, Kumarasuriyar and colleagues (2009) reported changes in MG63 phenotype that replicate the differentiation model proposed by Lian and Stein (Lian and Stein, 1992). Briefly, ALP activity increased by day 15 and then declined to basal levels, whereas type I collagen expression increased during the second week of the culture. Osteocalcin and osteonectin expression were observed at day 15 and 29, whereas Runx2/Cfba1, BSP and osteopontin were not detected while calcium accumulation was initiated after day 28 (Kumarasuriyar *et al.,* 2009). Moreover, other reports showed that MG-63 cells have low ALP enzyme activity and did not mineralise (Pierschbacher *et al.*, 1988; Saldana *et al*., 2011).

The response of MG-63 cells to $1,25(OH)_2D_3$ administration has been shown to be similar to normal human osteoblast cells (Clover and Gowen, 1994). Additionally, only after $1,25(OH)_2D_3$ treatment was osteocalcin detected in the supernatants of the cells, with increases reported from 9- to 20-fold (Franceschi *et al.*, 1988; Clover *et al*., 1992). The time and dose dependent stimulation of protein secretion by $1,25(OH)_2D_3$ was reported in both isolated and MG-63 cells. The $1,25(OH)_2D_3$ stimulates the synthesis of a1(I) and a2(I) chains of type I collagen together with type III collagen and fibronectin synthesis (Franceschi *et al.*, 1988). Additionally, both

human and MG63 cells produce cAMP in response to PTH treatment (Lajeunesse *et al.*, 1990). Johansen and colleagues (1992) showed differences in matrix protein secretion by MG-63 cells and normal human osteoblast cells isolated from foetal and adult femoral head (Johansen *et al*., 1992). Furthermore, MG-63 cells secreted gelatinase, YKL-40 protein, tissue inhibitor of metalloproteinase 1 (TIMP-1) and 2 (TIMP-2) and β-microglobulin but no C-terminal propeptides of the a1 and a2 chains of type I collagen. Moreover, normal human osteoblasts from foetal trabecular bone secrete primarily matrix constituent proteins such as C-terminal propeptides of α1 and α2 chains of type I collagen together with osteonectin, while cells from adult donors express in addition to these gelatinase, TIMP-1 and β-microglobulin (Pierschbacher *et al*., 1988).

Collectively these results indicate the similarity of MG-63 and normal osteoblasts in their response to $1,25(OH)_2D_3$ and PTH administration which makes them attractive model for hormonal regulation of phenotypic change studies. However, the inconsistency in the expression of matrix proteins resulting from their clonal heterogeneity limits their use as a model for osteoblast phenotype development and matrix mineralisation.

Mouse cell lines

MC3T3-E1 represents a popular osteoblast cell line representative of a pre-osteoblastic phenotype. Several sub-clones have been established from this new-born mouse calvaria clonal cell line. Five of them, sub-clone 4, 8, 11, 14 and 26, demonstrate mineralisation with the addition of ascorbic acid and inorganic phosphate (Wang *et al*., 1999). Mineralising sub-clones express high levels of mRNA for BSP, osteocalcin, and the PTH/PTHrP receptor. From these clones only sub-clone 4 and 14 have been shown to mineralise the collagenous extracellular matrix. Sub-clone 4 was shown to undergo temporal changes from proliferation to nodule formation and mineralisation in a similar manner to intramembranous osteogenesis *in vivo* (Sudo *et al.,* 1983). Cells proliferated actively *in vitro* and synthesised type I collagen from day 3 in culture. Simultaneously, ALP enzyme activity increased from day 3 to day 21 and the mineral deposition was reported early at day 14 (Quarles *et al*., 1992).

Ascorbic acid and bGP supplementation is a prerequisite for matrix mineralisation in MC3T3-E1 cells. In the absence of these stimulators, ALP activity remains low and fails to result in mineralisation (Hong *et al*., 2010). Differentiation of MC3T3-E1 cells is influenced also by the type of serum used in culture medium, and serum derived cytokines and growth factors (Yohay *et al*., 1994). With the addition of complete foetal bovine serum, cells have increased proliferative capabilities during early differentiation. Furthermore, ALP activity, protein and mRNA expression is increased in the post-proliferative stage compared to cells maintained in medium with resin/charcoal-stripped serum (Yohay *et al.*, 1994). Although being a cell line, MC3T3-E1 at high passages (above 36) have reportedly decreased proliferation. At very high passages (above 60) they exhibit inconsistent cell cycling, indicative of replicative senescence (Grigoriadis *et al*., 1985) similar to human cells. This makes them an attractive tool for *in vitro* investigations relating to bone remodelling and formation.

Considerations on media supplementation

For osteoblastic cells a variety of basic medium types can be used including, but not limited to, DMEM, DMEM/F12 or α-MEM. Due to different components and concentration of additives, results can vary depending on the type of medium used (Coelho *et al.*, 2000) and its pH (Arnett *et al.*, 1994; Arnett, 2003). Commonly applied additives, namely dexamethasone, $1,25(OH)_2D_3$, β-glycerophosphate and ascorbic acid have been shown to direct osteoblast function (Table 2; Arnett, 1990; Jorgensen *et al*., 2004). Supplementation of primary human and rat osteoblast cells medium with physiological concentrations (10^{-7} to 10^{-9} M) of dexamethasone supports a differentiated phenotype as well as increased nodule formation (Bellows *et al*., 1987; Yang *et al*., 2003). In this concentration range, dexamethasone treatment has also been reported to significantly increase ALP activity, osteocalcin synthesis and PTH-stimulated cyclic AMP accumulation (Wong *et al*., 1990; Sutherland *et al*., 1995; Yang *et al*., 2003). However, chronic exposure of mouse osteoblast cells to dexamethasone during proliferation has been reported to block differentiation, suggesting a differential species-specific mechanism (Lian *et al*., 1997; Chen and Fry, 1999).

Detailed information about the action and molecular effects of $1,25(OH)_2D_3$ are still poorly defined. Recently, it has been demonstrated that short (24 h) stimulation of confluent primary human osteoblast cells with $1,25(OH)_2D_3$ modulates the expression of genes associated with not only osteoblast phenotype and vitamin D_3 pathway, but also related to immunological function (Tarroni *et al*., 2012). Moreover, it has been known that the differential effect on osteoblast phenotype depends on the timing of the administration and its duration (Siggelkow *et al*., 1999; Owen *et al*., 1991). Chronic addition of $1,25(OH)_2D_3$ to proliferating rat osteoblasts resulted in inhibition of osteocalcin and nodule development, whereas acute addition of $1,25(OH)_2D_3$ post-proliferatively stimulated ALP, type I collagen and osteocalcin synthesis (Owen *et al*., 1991). Additionally, $1,25(OH)_2D_3$ reportedly promotes mineralisation via increased production of matrix vesicles (Woeckel *et al*., 2010).

Supplementation with ascorbic acid cofactor for the hydroxylation of proline and lysine residues in collagen, to the cell culture media is essential for the proper collagenous extracellular matrix synthesis (Franceschi *et al*., 1994; Hulmes, 2008). Since the ascorbic acid is unstable under cell culture conditions, its long-acting derivative ascorbic acid 2-phosphate is used (Hata and Senoo, 1989). Supplementation with this compound of osteoblast cell culture increases cell proliferation, collagen and ALP gene expression and protein synthesis (Takamizawa *et al*., 2004). The concentration of ascorbic acid 2-phosphate at a level of 50 μg/mL is sufficient for osteoblast cell cultures (Table 2).

Table 2. Culture conditions of popular osteoblast culture models. The mature phenotype refers to the capability of cells to deposit calcium in matrix. Dex – dexamethasone; bGP - glycerol-2-phosphate; Ca – calcium; a.a. - ascorbic acid; ITS - insulin, transferrin, selenium; * related to manuscripts cited within this review. Firstly, papers were sorted based on the method of isolation used. Then, how isolation influences cell behaviour and phenotype, with specific emphasis on the influence of isolation on calcium deposition/mineralisation.

Cell type	Phenotype	Favoured isolation method*	Culture conditions for matrix mineralisation	References
Primary Human Osteoblast cells	Pre- to mature osteoblast	Outgrowth	50 µg/mL a.a.; 5-10 mM bGP, 10-100 nM dex	Gallager, 2003 Gotoh *et al*., 1990 Toesca *et al*., 2001 Siggelkow *et al*., 1999
Primary Mouse/Rat Osteoblast cells	Pre- to mature osteoblast	Outgrowth (Mouse Obs) Enzymatic digestion (Rat Obs)	50 µg/mL a.a.; 5-10 mM bGP	Ecarot-Charrier *et al*., 1983 Ecarot-Charrier *et al*., 1988 Lomri *et al*., 1988 Garcia *et al*., 2002 Roman-Roman *et al*., 2003 Bhargava *et al*., 1988 Siggelkow *et al*., 1999 Lynch *et al*., 1995 Yamamoto *et al*., 2002 Bellows *et al*., 1986
Primary Bovine/Ovine Osteoblast cells	Immature to mature osteoblast	Enzymatic digestion (foetal tissue); outgrowth culture (adult animal)	50 µg/mL a.a., 10 mM bGP, 0.6 mM Ca; ITS	Whitson *et al*., 1992 Ibaraki *et al*., 1992 Whitson *et al*., 1984 Collignon *et al*., 1997
Primary Rabbit Osteoblast cells	Mature osteoblasts	Enzymatic digestion (0.1 % crude collagenase)	Not clearly defined	Yee, 1983 Shaw *et al*., 1989 Cao *et al*., 2006
MG-63	Immature osteoblast	Not applicable	50 µg/mL a.a.; 5-10 mM bGP	Franceschi *et al*., 1988
SaOs-2	Mature osteoblast	Not applicable	50 µg/mL a.a.; 0-10 mM bGP (only post-confluent)	Ahmad *et al*., 1999 Saldana *et al*., 2011 Hausser and Brenner, 2005 Rodan *et al*., 1987 Rao *et al*., 1996 Muller *et al*., 2011
MC3T3-E1	Mature osteoblast	Not applicable	25-50 µg/mL a.a.; 5-10 mM bGP	Hong *et al*., 2010 Quarles *et al*., 1992 Yohay *et al*., 1994

The use of phosphate substrates for matrix mineralisation has been widely debated. Supplementation of certain cultures with 10 mM β-glycerophosphate results in spontaneous mineral deposition and formation of ectopic mineralisation (Chung *et al*., 1992). Furthermore, an excessively high extracellular concentration of β-glycerophosphate or Pi affects cell viability by increasing lactate dehydrogenase production (Roach, 1992). A detailed explanation of the nature of minerals formed in *in vitro* systems as well as the influence of seeding density of cells has been recently covered (Boskey, 2008).

The *in vitro* use and limitations of cell models in biomedical research

Keeping in mind the advantages and disadvantages of different osteoblastic cells as well as their behaviour, the selection of an appropriate model for the study appears crucial. Primary osteoblast cells from different origins and various cell line models have been used not only for answering basic biological questions, but have widespread applicability in bone tissue engineering and the orthopaedic research field. These include the development of novel biomaterials and new therapeutic strategies against acquired or genetic bone diseases. Here, we focus on the former.

Osteoblast models and initial cell-surface interaction

For the development of novel biomaterials to improve their performance when they are implanted into the body, the exploration of the bone cell response to and interaction with these materials is necessary. Recently, in their review dedicated to the subject of focal adhesion formation and cell/biomaterial interaction, Biggs and Dalby emphasised the role of integrins in mediating various cellular functions in osteogenesis (Biggs and Dalby, 2010). The interaction between cells and extracellular environment involves integrins, transmembrane proteins interacting with the extracellular matrix (ECM) and the cytoskeleton. These interactions are crucial for cell behaviour proliferation, movement and ultimately differentiation since topography of ECM and applied stress signals are transmitted to the nucleus through expansion of the laminar network (Maniotis *et al*., 1997; Dahl *et al*., 2004; Dalby *et al*., 2007). Furthermore, integrins can act synergistically with growth factor receptors (Alam *et al*., 2007).

Many studies exploring cell-material interactions utilise various cell types, including MG-63, SaOs-2 cell lines and primary rat osteoblast cells. From both a biological

and tissue engineering point of view, it is important to track and understand the existing differences among integrin-mediated cell-surface interactions in various cell types since this may result in differential responses and activation of specific distinct signalling pathways. It has been previously shown that primary human osteoblast cells express various integrin subunits, including α_4, α_5, α_V, β_1, α_2, α_4, and β_3 (Clover *et al*., 1992; Sinha and Tuan, 1996). Similar to primary cells, the subunits α_2, α_5, α_V, β_1, β_3 have been reported for MG63. However, the distinctive presence of α_4 and α_6 subunits was also reported for this cell line and not for primary human osteoblast cells (Olivares-Navarrete *et al*., 2008). Another important consideration is the relative concentration of expressed subunits, with α_1, $\alpha 2$ and α_5 reported at higher levels in primary human osteoblast compared toMG-63 cell line (Clover and Gowen, 1994).

The β_1 and α_V subunits have been shown to be necessary for SaOs-2 cell adhesion and spreading (Degasne *et al*., 1999). A recent study revealed that the mode of cell binding to the surface differs among cell types. For instance, MG-63 and SaOs-2 cells interacted stronger with a fibronectin coated surface which was mediated by both α_5 and α_V integrin containing heterodimers (Vohra *et al*., 2008). In addition, this contact was mediated via the α_3 subunit in MG63 cells (Pegueroles *et al*., 2011) and primary human osteoblast cells (Sinha and Tuan, 1996). Moreover, primary human osteoblast cells express the α_5, but not the α_v subunit upon interaction with fibronectin coated surface (Sinha and Tuan, 1996). Extensive studies of rat integrin subunit expression revealed a constitutive expression of wide range of integrin subunits. Strongly expressed integrin subunits included α_1, α_2, α_4, α_V and α_6, while β_1 and β_3 were reported but to a lesser extent (Castoldi *et al*., 1997). Furthermore, the interaction of osteoblast cells with fibronectin is mediated in rat and human osteoblasts in similar manner via $\alpha_5\beta_1$ integrin (Pistone *et al*., 1996; Castoldi *et al*., 1997).

Osteoblast model phenotype and biomaterials

In long-term studies assessing the effects of biomaterials on cell behaviour, MG-63 cells have been used regardless of their limitations (Lincks *et al*., 1998). Recently, a systematic study reported the responses of SaOs-2, MG-63 and U-2 OS cell lines on Ti-6 Al-4 V (TAV) surfaces, including their interaction with Ti particles, as compared with the responses of primary human osteoblasts (Saldana *et al*., 2011). The expression of Runx2 and Sp7 genes were not detected in MG63, whereas high levels were reported for primary human cells. ALP activity was similar on tissue culture polystyrene and TAV, for MG-63, but primary human osteoblast cells demonstrated increased actively when cultured on TAV. Thus, under these parameters MG-63 cells were compared unfavourably to human primary osteoblast on both tissue culture plastic and a clinically relevant implant material. One positive outcome was that MG63 cells were identified as expressing a similar integrin subunit profile to HOb cells (Clover *et al*., 1992). Therefore, MG63 may provide a good alternative for studies interested in initial attachment to various materials.

On the other hand, SaOs-2 cells manifested responses more closely resembling those of HOb cells, including the expression of osteoblastic factors and reaction to Ti particles. This favours them as a model for studying some osteoblast functions, such as synthesis of some osteoblast-specific proteins. It has to be emphasised that since the SaOs-2 cells has a mature osteoblastic phenotype and possess a high matrix mineralisation capacity they should not be avoided as a cell type for assessing material osteoconductivity.

A more appropriate model would be the use of primary rat cells or MC3T3-E1 cells, that have additional advantage of similar growth rate to the human osteoblast cells. Additionally, due to the similarity in the molecular regulation of gene expression, such as in C/EBPβ - P1 promoter - Runx2/p57 axis (Henriquez *et al*., 2011), these cells are suitable for investigating the expression of certain genes and their regulation. Many studies have utilised them for biomaterial evaluation and studying signalling pathways involved in regulation of cell phenotype. In fact, MC3T3-E1 appears to be favoured more regularly than primary murine osteoblast cells in cell-material interaction studies. More often, primary mouse cells are used in transgenic studies to elucidate the effects of mutations of particular genes relating to osteogenesis and bone development (Bouvard *et al*., 2001; Malaval *et al*., 2009; Monfoulet *et al*., 2010; Watabe *et al*., 2011). Nevertheless, more recently primary mouse osteoprogenitors transduced with green fluorescent protein have been employed for tracking osteoblast cell *in vitro* and *in vivo* differentiation on biomaterials, including hydroxyapatite (Kuhn *et al*., 2010) and various hydrogels and calcium alginate beads (Roman *et al*., 2007).

Human and rat osteoblasts show similar profiles of ALP activity and procollagen I production; however, the former reached the maximum levels earlier (Siggelkow *et al*., 1999). Furthermore, the nature of the minerals formed by foetal calvaria, periosteum or bone marrow cells is restricted to the nodules. Mineralisation of ECM formed by foetal and adult long bone cells was diffuse and characterised by poorly crystalline apatite (Declercq *et al*., 2004). While MC3T3-E1 cells were shown to mineralise their matrix (Sudo *et al*., 1983; Franceschi and Iyer, 1992) similarly to foetal rat calvaria osteoblasts (Declercq *et al*., 2004), detailed Fourier transform infrared spectroscopy (FTIR) analysis of the deposits demonstrated a lack of calcium phosphate mineralisation, as well as the presence of dystrophic mineralisation of unknown origin (Bonewald *et al*., 2003). On the other hand, the formation of mineral deposits by foetal rat osteoblast cells were characterised by bone-like apatite structures (Boyan *et al*., 2002), similar to that observed for primary human osteoblast cells isolated from the femoral head (Gough *et al*., 2004).

At this point it has to be emphasised that the surface properties of a biomaterial will directly dictate the initial cell-material interaction and subsequent cell behaviour. Moreover, the maturation stage of the cells determines their differential response to the different surface roughness of surface material. Lohmann and colleagues (2000) compared cellular response of osteoblast cells at different maturation stages on materials with different surface roughnesses (0.5, 4.1 and 4.9 μm) and concluded that more phenotypically immature osteoblast cells are more sensitive to the substrate pertaining to phenotypic

progression and $1,25(OH)_2D_3$ responsiveness (Lohmann *et al*., 2000). Indeed, rat calvaria osteoblast cells isolated from neonatal animals displayed differential mRNA expression in response to different surface topographies (Hayes *et al.*, 2010). Moreover, foetal rat calvaria osteoblast cells have been shown to have similar sensitivity to that of primary human osteoblasts to nickel chloride (Macnair *et al.*, 1997). Hence, for osteoconductivity assessment of biomaterials, rat calvarial osteoblast cells appear to be one of the more appropriate and reliable models.

Due to the minimal literature and insufficient information regarding biological performance and limited genomic data, bovine, ovine and rabbit osteoblasts are not a popular choice in applied musculoskeletal research. One study assessing the behaviour of primary foetal bovine mandibular cells on commercially pure titanium and plasma-sprayed hydroxyapatite revealed these cells form a complex multilayer. In these cultures, the presence of the osteoblastic markers was spatially distributed, with the ALP being dominant in cells adjacent to the culture surfaces, BSP in the layer above ALP-positive cells and osteocalcin in the central and upper regions of the culture (Yliheikkila *et al*., 1996). A more recent study compared the behaviour of 18-24 month old bovine osteoblast cells isolated from animals on Zr and Ti implant material surfaces. Immunohistochemical analysis indicated the expression of type I collagen, osteocalcin and osteonectin on both control and implant surfaces at day 7, with subsequent increase in type I collagen levels on Ti surfaces (Depprich *et al*., 2008). Additional evaluation of bovine, ovine and rabbit osteoblast cells is required before their widespread acceptance and application to bone tissue research becomes reality. This would be advantageous to the understanding of interspecies differences and the elucidation of the results obtained from *in vitro* as well as *in vivo* studies.

Conclusions

The correct *in vitro* experimental design is of utmost importance to answer specific research questions. To this end, the selection of an appropriate cell model is crucial as it can contribute to a better understanding of physiological, pathological and regenerative processes involved. In this review, the major *in vitro* differences between osteoblast cells from different sources were emphasised. Due to the considerable phenotypic differences shown, isolated human osteoblast cells should be the cells of choice to be used in all areas of *in vitro* bone biology research. It is not possible to fully standardise the osteoblast cells donor selection. Since the factors related to donors greatly influence the phenotype of the isolated cells, they should be controlled before subjecting cells to an in *in vitro* study. Between the fourth and sixth decade of life the differences in cell behaviour between male and female are more pronounced. Therefore, the selection of donors should be highly controlled at this time, taking great care to screen for bone diseases or health states that might influence bone structure. Nevertheless, for some areas of medical research, such as when searching for therapeutic agents for pathological conditions that occur at a certain time of life (e.g., osteoporosis) or certain molecular mechanisms involved in that state, studying cells derived from donors with diagnosed illness are beneficial. Additionally, the use of human osteoblast cells gives more relevant results and brings the clinical benefit in the translational process.

Other primary cells, especially rat cells, are useful for studying the processes of bone regeneration and remodelling. The main advantage of these cells is that they address the shortcomings of human cells, including the problems regarding donor age and sex differences. It is also possible to obtain a large number of cells from animals at the same stage of life, from the same gender and from the same skeletal site. It is also possible to recapitulate bone formation, remodelling and repair by using the rat model, both *in vitro* and *in vivo*.

Cell-line osteoblasts provide a more homogenous population of cells and allow the study of particular stages of osteoblast phenotype. They are very useful at the early stages of assessing the therapeutic agents or for cytocompatibility testing; however, they do not fully reflect the behaviour of primary cells. From the three cell lines described in this review, MC3T3-E1 represents a reliable alternative to the primary human osteoblast *in vitro* cell model for various research areas. Yet, species-related differences pose the most concerns regarding translation of results from animal studies to a clinical setting. Therefore, results should be extrapolated with care and limitations should be highlighted and conclusions drawn based only upon the parameters of the specific study.

Finally, it has to be pointed out that other factors can influence cell behaviour *in vitro,* which can result in different outcomes. For instance, the medium and its additives used for cell culturing, the cell seeding density as well as the culture system itself. For a long time, cell monolayer culture has been a standard method for maintaining osteoblast cells *in vitro*. Since this does not reflect natural conditions and mineralisation is not always achieved, more interest is paid to establishing a cell system that mimics more closely the natural environment of cells. It has been revealed that cells maintained in 3D systems, such as spheroids (Kale *et al.*, 2000), micromass (Gerber and ap Gwynn, 2002) and pellets (Jahn *et al*., 2010), promote osteogenic progression leading to osteoblast cell maturation. These systems were utilised for culturing primary human or rat osteoblast cells and revealed considerable differences compared to standard monolayer conditions. Further exploration of osteoblast cells from different sources should be performed to obtain more details regarding differences in osteoblast cell maturation.

References

Abe Y, Aida Y, Abe T, Hirofuji T, Anan H, Maeda K (2000) Development of mineralized nodules in fetal rat mandibular osteogenic precursor cells: requirement for dexamethasone but not for beta-glycerophosphate. Calcif Tissue Int **66**: 66-69.

Ahmad M, McCarthy MB, Gronowicz G (1999) An *in vitro* model for mineralization of human osteoblast-like cells on implant materials. Biomaterials **20**: 211-220.

Ahmadbeigi N, Shafiee A, Seyedjafari E, Gheisari Y, Vassei M, Amanpour S, Amini S, Bagherizadeh I, Soleimani M (2011) Early spontaneous immortalization and loss of plasticity of rabbit bone marrow mesenchymal stem cells. Cell Prolif **44**: 67-74.

Alam N, Goel HL, Zarif MJ, Butterfield JE, Perkins HM, Sansoucy BG, Sawyer TK, Languino LR (2007) The integrin-growth factor receptor duet. J Cell Physiol **213**: 649-653.

Anderson HC (2003) Matrix vesicles and calcification. Curr Rheumatol Rep **5**: 222-226.

Arnett T (2003) Regulation of bone cell function by acid-base balance. Proc Nutr Soc **62**: 511-520.

Arnett TR (1990) Update on bone cell biology. Eur J Orthod **12**: 81-90.

Arnett TR, Boyde A, Jones SJ, Taylor ML (1994) Effects of medium acidification by alteration of carbon dioxide or bicarbonate concentrations on the resorptive activity of rat osteoclasts. J Bone Miner Res **9**: 375-379.

Aronow MA, Gerstenfeld LC, Owen TA, Tassinari MS, Stein GS, Lian JB (1990) Factors that promote progressive development of the osteoblast phenotype in cultured fetal rat calvaria cells. J Cell Physiol **143**: 213-221.

Bakker A, Klein-Nulend J (2003) Osteoblast isolation from murine calvariae and long bones. In: Bone Research Protocols (Helfrich MH, Ralston SH, eds). Humana Press, Totowa, NJ, pp 19-28.

Bard DR, Dickens MJ, Smith AU, Zarek JM (1972) Isolation of living cells from mature mammalian bone. Nature **236**: 314-315.

Battmann A, Battmann A, Jundt G, Schulz A (1997) Endosteal human bone cells (EBC) show age-related activity *in vitro*. Exp Clin Endocrinol Diabetes **105**: 98-102.

Bellows CG, Aubin JE, Heersche JN (1987) Physiological concentrations of glucocorticoids stimulate formation of bone nodules from isolated rat calvaria cells *in vitro*. Endocrinology **121**: 1985-1992.

Bellows CG, Heersche JN, Aubin JE (1992) Inorganic phosphate added exogenously or released from beta-glycerophosphate initiates mineralization of osteoid nodules *in vitro*. Bone Miner **17**: 15-29.

Bergot C, Wu Y, Jolivet E, Zhou LQ, Laredo JD, Bousson V (2009) The degree and distribution of cortical bone mineralization in the human femoral shaft change with age and sex in a microradiographic study. Bone **45**: 435-442.

Bhargava U, Bar-Lev M, Bellows CG, Aubin JE (1988) Ultrastructural analysis of bone nodules formed *in vitro* by isolated fetal rat calvaria cells. Bone **9**: 155-163.

Biggs MJ, Dalby MJ (2010) Focal adhesions in osteoneogenesis. Proc Inst Mech Eng H **224**: 1441-1453.

Bilbe G, Roberts E, Birch M, Evans DB (1996) PCR phenotyping of cytokines, growth factors and their receptors and bone matrix proteins in human osteoblast-like cell lines. Bone **19**: 437-445.

Billiau A, Edy VG, Heremans H, Van DJ, Desmyter J, Georgiades JA, De SP (1977) Human interferon: mass production in a newly established cell line, MG-63. Antimicrob Agents Chemother **12**: 11-15.

Bonewald LF, Harris SE, Rosser J, Dallas MR, Dallas SL, Camacho NP, Boyan B, Boskey A (2003) Von Kossa staining alone is not sufficient to confirm that mineralization *in vitro* represents bone formation. Calcif Tissue Int **72**: 537-547.

Bonjour JP, Theintz G, Buchs B, Slosman D, Rizzoli R (1991) Critical years and stages of puberty for spinal and femoral bone mass accumulation during adolescence. J Clin Endocrinol Metab **73**: 555-563.

Boskey AL (2008) Cell culture systems for studies of bone and tooth mineralization. **108**: 4716-4733.

Bouvard D, Brakebusch C, Gustafsson E, Aszodi A, Bengtsson T, Berna A, Fassler R (2001) Functional consequences of integrin gene mutations in mice. Circ Res **89**: 211-223.

Boyan BD, Bonewald LF, Paschalis EP, Lohmann CH, Rosser J, Cochran DL, Dean DD, Schwartz Z, Boskey AL (2002) Osteoblast-mediated mineral deposition in culture is dependent on surface microtopography. Calcif Tissue Int **71**: 519-529.

Cao XY, Yin MZ, Zhang LN, Li SP, Cao Y (2006) Establishment of a new model for culturing rabbit osteoblasts *in vitro*. Biomed Mater **1**: L16-L19.

Carpenter TO, Moltz KC, Ellis B, Andreoli M, McCarthy TL, Centrella M, Bryan D, Gundberg CM (1998) Osteocalcin production in primary osteoblast cultures derived from normal and Hyp mice. Endocrinology **139**: 35-43.

Castoldi M, Pistone M, Caruso C, Puddu A, Filanti C, Piccini D, Tacchetti C, Manduca P (1997) Osteoblastic cells from rat long bone. II: Adhesion to substrata and integrin expression in primary and propagated cultures. Cell Biol Int **21**: 7-16.

Chen TL, Fry D (1999) Hormonal regulation of the osteoblastic phenotype expression in neonatal murine calvarial cells. Calcif Tissue Int **64**: 304-309.

Chung CH, Golub EE, Forbes E, Tokuoka T, Shapiro IM (1992) Mechanism of action of beta-glycerophosphate on bone cell mineralization. Calcif Tissue Int **51**: 305-311.

Clover J, Gowen M (1994) Are MG-63 and HOS TE85 human osteosarcoma cell lines representative models of the osteoblastic phenotype? Bone **15**: 585-591.

Clover J, Dodds RA, Gowen M (1992) Integrin subunit expression by human osteoblasts and osteoclasts in situ and in culture. J Cell Sci **103**: 267-271.

Coelho MJ, Cabral AT, Fernande MH (2000) Human bone cell cultures in biocompatibility testing. Part I: osteoblastic differentiation of serially passaged human bone marrow cells cultured in alpha-MEM and in DMEM. Biomaterials **21**: 1087-1094.

Collignon H, Davicco MJ, Barlet JP (1997) Isolation of cells from ovine fetal long bone and characterization of their osteoblastic activities during *in vitro* mineralization. Arch Physiol Biochem **105**: 158-166.

Collin EC, Grad S, Zeugolis DI, Vinatier CS, Clouet JR, Guicheux JJ, Weiss P, Alini M, Pandit AS (2011) An injectable vehicle for nucleus pulposus cell-based therapy. Biomaterials **32**: 2862-2870.

Dahl KN, Kahn SM, Wilson KL, Discher DE (2004) The nuclear envelope lamina network has elasticity and a compressibility limit suggestive of a molecular shock absorber. J Cell Sci **117**: 4779-4786.

Dalby MJ, Biggs MJ, Gadegaard N, Kalna G, Wilkinson CD, Curtis AS (2007) Nanotopographical stimulation of mechanotransduction and changes in interphase centromere positioning. J Cell Biochem **100**: 326-338.

Davis JW, Ross PD, Wasnich RD (1994) Evidence for both generalized and regional low bone mass among elderly women. J Bone Miner Res **9**: 305-309.

Declercq H, Van den Vreeken N, De Maeyer E, Verbeeck R, Schacht E, De Ridder L, Cornelissen M (2004) Isolation, proliferation and differentiation of osteoblastic cells to study cell/biomaterial interactions: comparison of different isolation techniques and source. Biomaterials **25**: 757-768.

Degasne I, Basle MF, Demais V, Hure G, Lesourd M, Grolleau B, Mercier L, Chappard D (1999) Effects of roughness, fibronectin and vitronectin on attachment, spreading, and proliferation of human osteoblast-like cells (Saos-2) on titanium surfaces. Calcif Tissue Int **64**: 499-507.

Depprich R, Ommerborn M, Zipprich H, Naujoks C, Handschel J, Wiesmann HP, Kubler NR, Meyer U (2008) Behavior of osteoblastic cells cultured on titanium and structured zirconia surfaces. Head Face Med **4**: 29.

Dowthwaite GP, Bishop JC, Redman SN, Khan IM, Rooney P, Evans DJ, Haughton L, Bayram Z, Boyer S, Thomson B, Wolfe MS, Archer CW (2004) The surface of articular cartilage contains a progenitor cell population. J Cell Sci **117**: 889-897.

Ecarot-Charrier B, Glorieux FH, van der Rest M, Pereira G (1983) Osteoblasts isolated from mouse calvaria initiate matrix mineralization in culture. J Cell Biol **96**: 639-643.

Ecarot-Charrier B, Shepard N, Charette G, Grynpas M, Glorieux FH (1988) Mineralization in osteoblast cultures: a light and electron microscopic study. Bone **9**: 147-154.

Evans CE, Galasko CS, Ward C (1990) Effect of donor age on the growth *in vitro* of cells obtained from human trabecular bone. J Orthop Res **8**: 234-237.

Fedarko NS, Vetter UK, Weinstein S, Robey PG (1992) Age-related changes in hyaluronan, proteoglycan, collagen, and osteonectin synthesis by human bone cells. J Cell Physiol **151**: 215-227.

Fernandes RJ, Harkey MA, Weis M, Askew JW, Eyre DR (2007) The post-translational phenotype of collagen synthesized by SAOS-2 osteosarcoma cells. Bone **40**: 1343-1351.

Franceschi RT, Iyer BS (1992) Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. J Bone Miner Res **7**: 235-246.

Franceschi RT, James WM, Zerlauth G (1985) 1 alpha, 25-dihydroxyvitamin D3 specific regulation of growth, morphology, and fibronectin in a human osteosarcoma cell line. J Cell Physiol **123**: 401-409.

Franceschi RT, Romano PR, Park KY (1988) Regulation of type I collagen synthesis by 1,25-dihydroxyvitamin D3 in human osteosarcoma cells. J Biol Chem **263**: 18938-18945.

Franceschi RT, Iyer BS, Cui Y (1994) Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. J Bone Miner Res **9**: 843-854.

Gallager JA (2003) Human osteoblast culture. In: Bone Research Protocols (Helfrich MH, Ralston SH, eds). Humana Press, New Jersey, pp 3-18.

Garcia T, Roman-Roman S, Jackson A, Theilhaber J, Connolly T, Spinella-Jaegle S, Kawai S, Courtois B, Bushnell S, Auberval M, Call K, Baron R (2002) Behavior of osteoblast, adipocyte, and myoblast markers in genome-wide expression analysis of mouse calvaria primary osteoblasts *in vitro*. Bone **31**: 205-211.

Gerber I, ap Gwynn I (2002) Differentiation of rat osteoblast-like cells in monolayer and micromass cultures. Eur Cell Mater **3**: 19-30.

Gotoh Y, Hiraiwa K, Nagayama M (1990) *In vitro* mineralization of osteoblastic cells derived from human bone. Bone Miner **8**: 239-250.

Gough JE, Notingher I, Hench LL (2004) Osteoblast attachment and mineralized nodule formation on rough and smooth 45S5 bioactive glass monoliths. J Biomed Mater Res A **68**: 640-650.

Grigoriadis AE, Petkovich PM, Ber R, Aubin JE, Heersche JN (1985) Subclone heterogeneity in a clonally-derived osteoblast-like cell line. Bone **6**: 249-256.

Harris SA, Enger RJ, Riggs BL, Spelsberg TC (1995) Development and characterization of a conditionally immortalized human fetal osteoblastic cell line. J Bone Miner Res **10**: 178-186.

Hata R, Senoo H (1989) L-ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation, and formation of a three-dimensional tissuelike substance by skin fibroblasts. J Cell Physiol **138**: 8-16.

Hausser HJ, Brenner RE (2005) Phenotypic instability of Saos-2 cells in long-term culture. Biochem Biophys Res Commun **333**: 216-222.

Hayes JS, Khan IM, Archer CW, Richards RG (2010) The role of surface microtopography in the modulation of osteoblast differentiation. Eur Cell Mater **20**: 98-108.

Henriquez B, Hepp M, Merino P, Sepulveda H, van Wijnen AJ, Lian JB, Stein GS, Stein JL, Montecino M (2011) C/EBPbeta binds the P1 promoter of the Runx2 gene and up-regulates Runx2 transcription in osteoblastic cells. J Cell Physiol **226**: 3043-3052.

Heremans H, Billiau A, Cassiman JJ, Mulier JC, de Somer P (1978) *In vitro* cultivation of human tumor tissues. II. Morphological and virological characterization of three cell lines. Oncology **35**: 246-252.

Hong D, Chen HX, Yu HQ, Liang Y, Wang C, Lian QQ, Deng HT, Ge RS (2010) Morphological and proteomic analysis of early stage of osteoblast differentiation in osteoblastic progenitor cells. Exp Cell Res **316**: 2291-2300.

Hoshiba T, Yamada T, Lu H, Kawazoe N, Chen G (2011) Maintenance of cartilaginous gene expression on extracellular matrix derived from serially passaged chondrocytes during *in vitro* chondrocyte expansion. J Biomed Mater Res A **100**: 694-702.

Hulmes DJS (2008) Collagen diversity, synthesis and assembly. In: Collagen structure and mechanics (Fratzl, P., ed). Springer, New York, NY, 15-48.

Ibaraki K, Termine JD, Whitson SW, Young MF (1992) Bone matrix mRNA expression in differentiating fetal bovine osteoblasts. J Bone Miner Res **7**: 743-754.

Jahn K, Richards RG, Archer CW, Stoddart MJ (2010) Pellet culture model for human primary osteoblasts. Eur Cell Mater **20**: 149-161.

Johansen JS, Williamson MK, Rice JS, Price PA (1992) Identification of proteins secreted by human osteoblastic cells in culture. J Bone Miner Res **7**: 501-512.

Jonsson KB, Frost A, Larsson R, Ljunghall S, Ljunggren O (1997) A new fluorometric assay for determination of osteoblastic proliferation: effects of glucocorticoids and insulin-like growth factor-I. Calcif Tissue Int **60**: 30-36.

Jonsson KB, Frost A, Nilsson O, Ljunghall S, Ljunggren O (1999) Three isolation techniques for primary culture of human osteoblast-like cells: a comparison. Acta Orthop Scand **70**: 365-373.

Jorgensen NR, Henriksen Z, Sorensen OH, Civitelli R (2004) Dexamethasone, BMP-2, and 1,25-dihydroxyvitamin D enhance a more differentiated osteoblast phenotype: validation of an *in vitro* model for human bone marrow-derived primary osteoblasts. Steroids **69**: 219-226.

Kale S, Biermann S, Edwards C, Tarnowski C, Morris M, Long MW (2000) Three-dimensional cellular development is essential for *ex vivo* formation of human bone. Nat Biotechnol **18**: 954-958.

Kartsogiannia V, Ng KW (2004) Cell lines and primary cell cultures in the study of bone cell biology. Mol Cell Endocrinol **228**: 79-102

Kasperk C, Wergedal J, Strong D, Farley J, Wangerin K, Gropp H, Ziegler R, Baylink DJ (1995) Human bone cell phenotypes differ depending on their skeletal site of origin. J Clin Endocrinol Metab **80**: 2511-2517.

Katzburg S, Lieberherr M, Ornoy A, Klein BY, Hendel D, Somjen D (1999) Isolation and hormonal responsiveness of primary cultures of human bone-derived cells: gender and age differences. Bone **25**: 667-673.

Kuhn LT, Liu Y, Advincula M, Wang YH, Maye P, Goldberg AJ (2010) A nondestructive method for evaluating *in vitro* osteoblast differentiation on biomaterials using osteoblast-specific fluorescence. Tissue Eng Part C Methods **16**: 1357-1366.

Kumarasuriyar A, Murali S, Nurcombe V, Cool SM (2009) Glycosaminoglycan composition changes with MG-63 osteosarcoma osteogenesis *in vitro* and induces human mesenchymal stem cell aggregation. J Cell Physiol **218**: 501-511.

Lajeunesse D, Frondoza C, Schoffield B, Sacktor B (1990) Osteocalcin secretion by the human osteosarcoma cell line MG-63. J Bone Miner Res **5**: 915-922.

Leis HJ, Hulla W, Gruber R, Huber E, Zach D, Gleispach H, Windischhofer W (1997) Phenotypic heterogeneity of osteoblast-like MC3T3-E1 cells: changes of bradykinin-induced prostaglandin E2 production during osteoblast maturation. J Bone Miner Res **12**: 541-551.

Lian JB, Stein GS (1992) Concepts of osteoblast growth and differentiation: basis for modulation of bone cell development and tissue formation. Crit Rev Oral Biol Med **3**: 269-305.

Lian JB, Shalhoub V, Aslam F, Frenkel B, Green J, Hamrah M, Stein GS, Stein JL (1997) Species-specific glucocorticoid and 1,25-dihydroxyvitamin D responsiveness in mouse MC3T3-E1 osteoblasts: dexamethasone inhibits osteoblast differentiation and vitamin D down-regulates osteocalcin gene expression. Endocrinology **138**: 2117-2127.

Liao H, Andersson AS, Sutherland D, Petronis S, Kasemo B, Thomsen P (2003) Response of rat osteoblast-like cells to microstructured model surfaces *in vitro*. Biomaterials **24**: 649-654.

Lincks J, Boyan BD, Blanchard CR, Lohmann CH, Liu Y, Cochran DL, Dean DD, Schwartz Z (1998) Response of MG63 osteoblast-like cells to titanium and titanium alloy is dependent on surface roughness and composition. Biomaterials **19**: 2219-2232.

Lohmann CH, Bonewald LF, Sisk MA, Sylvia VL, Cochran DL, Dean DD, Boyan BD, Schwartz Z (2000) Maturation state determines the response of osteogenic cells to surface roughness and 1,25-dihydroxyvitamin D3. J Bone Miner Res **15**: 1169-1180.

Lomri A, Marie PJ, Tran PV, Hott M (1988) Characterization of endosteal osteoblastic cells isolated from mouse caudal vertebrae. Bone **9**: 165-175.

Lynch MP, Stein JL, Stein GS, Lian JB (1995) The influence of type I collagen on the development and maintenance of the osteoblast phenotype in primary and passaged rat calvarial osteoblasts: modification of expression of genes supporting cell growth, adhesion, and extracellular matrix mineralization. Exp Cell Res **216**: 35-45.

Macnair R, Rodgers EH, Macdonald C, Wykman A, Goldie I, Grant MH (1997) The response of primary rat and human osteoblasts and an immortalized rat osteoblast cell line to orthopaedic materials: comparative sensitivity of several toxicity indices. J Mater Sci Mater Med **8**: 105-111.

Malaval L, Monfoulet L, Fabre T, Pothuaud L, Bareille R, Miraux S, Thiaudiere E, Raffard G, Franconi JM, Lafage-Proust MH, Aubin JE, Vico L, Amedee J (2009) Absence of bone sialoprotein (BSP) impairs cortical defect repair in mouse long bone. Bone **45**: 853-861.

Manduca P, Palermo C, Caruso C, Brizzolara A, Sanguineti C, Filanti C, Zicca A (1997) Rat tibial osteoblasts III: propagation *in vitro* is accompanied by enhancement of osteoblast phenotype. Bone **21**: 31-39.

Maniotis AJ, Chen CS, Ingber DE (1997) Demonstration of mechanical connections between integrins, cytoskeletal filaments, and nucleoplasm that stabilize nuclear structure. Proc Natl Acad Sci USA **94**: 849-854.

Martinez ME, Del Campo MT, Medina S, Sanchez M, Sanchez-Cabezudo MJ, Esbrit P, Martinez P, Moreno I, Rodrigo A, Garces MV, Munuera L (1999) Influence of skeletal site of origin and donor age on osteoblastic cell growth and differentiation. Calcif Tissue Int **64**: 280-286.

Masuda H, Miller C, Koeffler HP, Battifora H, Cline MJ (1987) Rearrangement of the p53 gene in human osteogenic sarcomas. Proc Natl Acad Sci USA **84**: 7716-7719.

Monfoulet L, Malaval L, Aubin JE, Rittling SR, Gadeau AP, Fricain JC, Chassande O (2010) Bone sialoprotein, but not osteopontin, deficiency impairs the mineralization of regenerating bone during cortical defect healing. Bone **46**: 447-452.

Montjovent MO, Burri N, Mark S, Federici E, Scaletta C, Zambelli PY, Hohlfeld P, Leyvraz PF, Applegate LL, Pioletti DP (2004) Fetal bone cells for tissue engineering. Bone **35**: 1323-1333.

Morike M, Schulz M, Brenner RE, Bushart GB, Teller WM, Vetter U (1993) *In vitro* expression of osteoblastic markers in cells isolated from normal fetal and postnatal human bone and from bone of patients with osteogenesis imperfecta. J Cell Physiol **157**: 439-444.

Morike M, Schulz M, Nerlich A, Koschnik M, Teller WM, Vetter U, Brenner RE (1995) Expression of osteoblastic markers in cultured human bone and fracture callus cells. J Mol Med **73**: 571-575.

Muller WE, Wang X, Diehl-Seifert B, Kropf K, Schlossmacher U, Lieberwirth I, Glasser G, Wiens M, Schroder HC (2011) Inorganic polymeric phosphate/polyphosphate as an inducer of alkaline phosphatase and a modulator of intracellular Ca^{2+} level in osteoblasts (SaOS-2 cells) *in vitro*. Acta Biomater **7**: 2661-2671.

Murray E, Provvedini D, Curran D, Catherwood B, Sussman H, Manolagas S (1987) Characterization of a human osteoblastic osteosarcoma cell line (SAOS-2) with high bone alkaline phosphatase activity. J Bone Miner Res **2**: 231-238.

Newman E, Turner AS, Wark JD (1995) The potential of sheep for the study of osteopenia: current status and comparison with other animal models. Bone **16**: 277S-284S.

Neyt JG, Buckwalter JA, Carroll NC (1998) Use of animal models in musculoskeletal research. Iowa Orthop J **18**: 118-123.

Olivares-Navarrete R, Raz P, Zhao G, Chen J, Wieland M, Cochran DL, Chaudhri RA, Ornoy A, Boyan BD, Schwartz Z (2008) Integrin alpha2beta1 plays a critical role in osteoblast response to micron-scale surface structure and surface energy of titanium substrates. Proc Natl Acad Sci USA **105**: 15767-15772.

Olivares-Navarrete R, Hyzy SL, Chaudhri RA, Zhao G, Boyan BD, Schwartz Z (2010) Sex dependent regulation of osteoblast response to implant surface properties by systemic hormones. Biol Sex Differ **1**: 4.

Owen TA, Aronow MS, Barone LM, Bettencourt B, Stein GS, Lian JB (1991) Pleiotropic effects of vitamin D on osteoblast gene expression are related to the proliferative and differentiated state of the bone cell phenotype: dependency upon basal levels of gene expression, duration of exposure, and bone matrix competency in normal rat osteoblast cultures. Endocrinology **128**: 1496-1504.

Pearce AI, Richards RG, Milz S, Schneider E, Pearce SG (2007) Animal models for implant biomaterial research in bone: a review. Eur Cell Mater **13**: 1-10.

Pegueroles M, Aguirre A, Engel E, Pavon G, Gil FJ, Planell JA, Migonney V, Aparicio C (2011) Effect of blasting treatment and Fn coating on MG63 adhesion and differentiation on titanium: a gene expression study using real-time RT-PCR. J Mater Sci Mater Med **22**: 617-627.

Peroglio M, Grad S, Mortisen D, Sprecher CM, Illien-Junger S, Alini M, Eglin D (2011) Injectable thermoreversible hyaluronan-based hydrogels for nucleus pulposus cell encapsulation. Eur Spine J, in press.

Pierschbacher MD, Dedhar S, Ruoslahti E, Argraves S, Suzuki S (1988) An adhesion variant of the MG-63 osteosarcoma cell line displays an osteoblast-like phenotype. Ciba Found Symp **136**: 131-141.

Pistone M, Sanguineti C, Federici A, Sanguineti F, Defilippi P, Santolini F, Querze G, Marchisio PC, Manduca P (1996) Integrin synthesis and utilization in cultured human osteoblasts. Cell Biol Int **20**: 471-479.

Ponten J, Saksela E (1967) Two established *in vitro* cell lines from human mesenchymal tumours. Int J Cancer **2**: 434-447.

Quarles LD, Yohay DA, Lever LW, Caton R, Wenstrup RJ (1992) Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an *in vitro* model of osteoblast development. J Bone Miner Res **7**: 683-692.

Rao LG, Sutherland MK, Reddy GS, Siu-Caldera ML, Uskokovic MR, Murray TM (1996) Effects of 1alpha,25-dihydroxy-16ene, 23yne-vitamin D3 on osteoblastic function in human osteosarcoma SaOS-2 cells: differentiation-stage dependence and modulation by 17-beta estradiol. Bone **19**: 621-627.

Roach HI (1992) Induction of normal and dystrophic mineralization by glycerophosphates in long-term bone organ culture. Calcif Tissue Int **50**: 553-563.

Rodan SB, Imai Y, Thiede MA, Wesolowski G, Thompson D, Bar-Shavit Z, Shull S, Mann K, Rodan GA (1987) Characterization of a human osteosarcoma cell line (Saos-2) with osteoblastic properties. Cancer Res **47**: 4961-4966.

Roman I, Vilalta M, Rodriguez J, Matthies AM, Srouji S, Livne E, Hubbell JA, Rubio N, Blanco J (2007) Analysis of progenitor cell-scaffold combinations by *in vivo* non-invasive photonic imaging. Biomaterials **28**: 2718-2728.

Roman-Roman S, Garcia T, Jackson A, Theilhaber J, Rawadi G, Connolly T, Spinella-Jaegle S, Kawai S, Courtois B, Bushnell S, Auberval M, Call K, Baron R (2003) Identification of genes regulated during osteoblastic differentiation by genome-wide expression analysis of mouse calvaria primary osteoblasts *in vitro*. Bone **32**: 474-482.

Sakai D, Nakai T, Mochida J, Alini M, Grad S (2009) Differential phenotype of intervertebral disc cells: microarray and immunohistochemical analysis of canine nucleus pulposus and anulus fibrosus. Spine (Phila Pa 1976) **34**: 1448-1456.

Saldana L, Bensiamar F, Bore A, Vilaboa N (2011) In search of representative models of human bone-forming cells for cytocompatibility studies. Acta Biomater **7**: 4210-4221.

Shaw KY, Tang JS, Chao CF (1989) Functional evaluation of cultured rabbit osteoblast-like cells. Proc Natl Sci Counc Repub China B **13**: 192-200.

Shigeno Y, Ashton BA (1995) Human bone-cell proliferation *in vitro* decreases with human donor age. J Bone Joint Surg Br **77**: 139-142.

Siggelkow H, Rebenstorff K, Kurre W, Niedhart C, Engel I, Schulz H, Atkinson MJ, Hufner M (1999) Development of the osteoblast phenotype in primary human osteoblasts in culture: comparison with rat calvarial cells in osteoblast differentiation. J Cell Biochem **75**: 22-35.

Sinha RK, Tuan RS (1996) Regulation of human osteoblast integrin expression by orthopedic implant materials. Bone **18**: 451-457.

Soejima K, Klein-Nulend J, Semeins CM, Burger EH (2001) Different responsiveness of cells from adult and neonatal mouse bone to mechanical and biochemical challenge. J Cell Physiol **186**: 366-370.

Stringa E, Filanti C, Giunciuglio D, Albini A, Manduca P (1995) Osteoblastic cells from rat long bone. I. Characterization of their differentiation in culture. Bone **16**: 663-670.

Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S (1983) *In vitro* differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol **96**: 191-198.

Sutherland MS, Rao LG, Muzaffar SA, Wylie JN, Wong MM, McBroom RJ, Murray TM (1995) Age-dependent expression of osteoblastic phenotypic markers in normal human osteoblasts cultured long-term in the presence of dexamethasone. Osteoporos Int **5**: 335-343.

Takamizawa S, Maehata Y, Imai K, Senoo H, Sato S, Hata R (2004) Effects of ascorbic acid and ascorbic acid 2-phosphate, a long-acting vitamin C derivative, on the proliferation and differentiation of human osteoblast-like cells. Cell Biol Int **28**: 255-265

Tarroni P, Villa I, Mrak E, Zolezzi F, Mattioli M, Gattuso C, Rubinacci A (2012) Microarray analysis of $1,25OH_2D_3$ regulated gene expression in human primary osteoblasts. J Cell Biochem **113**: 640-649.

Thompson DD, Simmons HA, Pirie CM, Ke HZ (1995) FDA Guidelines and animal models for osteoporosis. Bone **17**: 125S-133S.

Toesca A, Pagnotta A, Specchia N (2001) Characterization of human bone cells in culture. Ital J Anat Embryol **106**: 13-26.

Torricelli P, Fini M, Giavaresi G, Borsari V, Carpi A, Nicolini A, Giardino R (2003) Comparative interspecies investigation on osteoblast cultures: data on cell viability and synthetic activity. Biomed Pharmacother **57**: 57-62.

Voegele TJ, Voegele-Kadletz M, Esposito V, Macfelda K, Oberndorfer U, Vecsei V, Schabus R (2000) The effect of different isolation techniques on human osteoblast-like cell growth. Anticancer Res **20**: 3575-3581.

Vohra S, Hennessy KM, Sawyer AA, Zhuo Y, Bellis SL (2008) Comparison of mesenchymal stem cell and osteosarcoma cell adhesion to hydroxyapatite. J Mater Sci Mater Med **19**: 3567-3574.

Wang D, Christensen K, Chawla K, Xiao G, Krebsbach PH, Franceschi RT (1999) Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct *in vitro* and *in vivo* differentiation/mineralization potential. J Bone Miner Res **14**: 893-903.

Washington JT, Schneiderman E, Spears R, Fernandez CR, He J, Opperman LA (2011) Biocompatibility and osteogenic potential of new generation endodontic materials established by using primary osteoblasts. J Endod **37**: 1166-1170.

Watabe H, Furuhama T, Tani-Ishii N, Mikuni-Takagaki Y (2011) Mechanotransduction activates alphabeta integrin and PI3K/Akt signaling pathways in mandibular osteoblasts. Exp Cell Res **317**: 2642-2649.

Whitson SW, Harrison W, Dunlap MK, Bowers DE Jr, Fisher LW, Robey PG, Termine JD (1984) Fetal bovine bone cells synthesize bone-specific matrix proteins. J Cell Biol **99**: 607-614.

Whitson SW, Whitson MA, Bowers DE Jr, Falk MC (1992) Factors influencing synthesis and mineralization of bone matrix from fetal bovine bone cells grown *in vitro*. J Bone Miner Res **7**: 727-741.

Wirth C, Grosgogeat B, Lagneau C, Jaffrezic-Renault N, Ponsonnet L (2008) Biomaterial surface properties modulate *in vitro* rat calvaria osteoblasts response: Roughness and or chemistry? Mat Sci Eng C **28**: 990-1001.

Woeckel VJ, Alves RD, Swagemakers SM, Eijken M, Chiba H, van der Eerden BC, van Leeuwen JP (2010) 1Alpha,25-(OH)2D3 acts in the early phase of osteoblast differentiation to enhance mineralization via accelerated production of mature matrix vesicles. J Cell Physiol **225**: 593-600.

Wong MM, Rao LG, Ly H, Hamilton L, Tong J, Sturtridge W, McBroom R, Aubin JE, Murray TM (1990) Long-term effects of physiologic concentrations of dexamethasone on human bone-derived cells. J Bone Miner Res **5**: 803-813.

Yamamoto N, Furuya K, Hanada K (2002) Progressive development of the osteoblast phenotype during differentiation of osteoprogenitor cells derived from fetal rat calvaria: model for *in vitro* bone formation. Biol Pharm Bull **25**: 509-515.

Yang L, Tao T, Wang X, Du N, Chen W, Tao S, Wang Z, Wu L (2003) Effects of dexamethasone on proliferation, differentiation and apoptosis of adult human osteoblasts *in vitro*. Chin Med J (Engl) **116**: 1357-1360.

Yee JA (1983) Properties of osteoblast-like cells isolated from the cortical endosteal bone surface of adult rabbits. Calcif Tissue Int **35**: 571-577.

Yliheikkila PK, Masuda T, Ambrose WW, Suggs CA, Felton DA, Cooper LF (1996) Preliminary comparison of mineralizing multilayer cultures formed by primary fetal bovine mandibular osteoblasts grown on titanium, hydroxyapatite, and glass substrates. Int J Oral Maxillofac Implants **11**: 456-465.

Yohay DA, Zhang J, Thrailkill KM, Arthur JM, Quarles LD (1994) Role of serum in the developmental expression of alkaline phosphatase in MC3T3-E1 osteoblasts. J Cell Physiol **158**: 467-475.

Zhang H, Lewis CG, Aronow MS, Gronowicz GA (2004) The effects of patient age on human osteoblasts' response to Ti-6Al-4V implants *in vitro*. J Orthop Res **22**: 30-38.

Discussion with Reviewer

Reviewer I: The differences among osteoblast cell models relating to the ALP activity and calcium deposition, as presented in Fig. 2, are not clear. It is known that after osteoblastic *in vitro* differentiation non-homogenous cell distribution could be observed in the culture dishes. Therefore, any conclusions about differences between

species or cell lines (e.g., between human and ovine or bovine osteoblasts) cannot be drawn from single culture images as presented in this figure.

Authors: Fig 2 was included as a visual presentation of the differences in distribution and intensity of the parameters mentioned between species. We believe this helps highlight our general point relating to the fact that researchers need to really consider the research question in hand and whether the model they are using is appropriate given the noted differences highlighted between species.