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PELLET CULTURE MODEL FOR HUMAN PRIMARY OSTEOBLASTS

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Abstract

In vitro monolayer culture of human primary osteoblasts (hOBs) often shows unsatisfactory results for extracellular matrix deposition, maturation and calcification. Nevertheless, monolayer culture is still the method of choice for *in vitro* differentiation of primary osteoblasts. We believe that the delay in mature ECM production by the monolayer cultured osteoblasts is determined by their state of cell maturation. A functional relationship between the inhibition of osteoblast proliferation and the induction of genes associated with matrix maturation was suggested within a monolayer culture model for rat calvarial osteoblasts. We hypothesize, that a pellet culture model could be utilized to decrease initial proliferation and increase the transformation of osteoblasts into a more mature phenotype. We performed pellet cultures using hOBs and compared their differentiation potential to 2D monolayer cultures. Using the pellet culture model, we were able to generate a population of cuboidal shaped central osteoblastic cells. Increased proliferation, as seen during low-density monolayer culture, was absent in pellet cultures and monolayers seeded at 40,000 cells/cm^2. Moreover, the expression pattern of phenotypic markers Runx2, osterix, osteocalcin, col I and E11 mRNA was significantly different depending on whether the cells were cultured in low density monolayer, high density monolayer or pellet culture. We conclude that the transformation of the osteoblast phenotype *in vitro* to a more mature stage can be achieved more rapidly in 3D culture. Moreover, that dense monolayer leads to the formation of more mature osteoblasts than low-density seeded monolayer, while hOB cells in pellets seem to have transformed even further along the osteoblast phenotype.

Keywords: Osteoblast, differentiation, pellet culture, 3D, osteocyte.

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Introduction

The pellet culture model is commonly applied to enhance *in vitro* chondrogenesis of primary chondrocytes or bone marrow derived progenitor cells (Johnstone *et al.*, 1998). For chondrocyte differentiation, the pellet culture model simulates the early condensation of mesenchymal stem cells during embryogenesis prior to the onset of chondrogenesis and the production of the extracellular matrix (ECM) by chondrocytes. Therefore, differentiation of the round-shaped cells inside the pellet is increased and apposition of ECM is significantly induced.

Whereas, for chondrocyte differentiation the standard culture model is 3D, the *in vitro* differentiation of osteoblasts is mainly described in 2D culture (Di Silvio and Gurav, 2001; Majeska and Gronowicz, 2002). Monolayer culture of osteoblasts is the most frequently performed method to investigate the effects of growth factors or hormones on the behavior of osteoblast-lineage cells *in vitro*. Lian and Stein (1992) described the processes of *in vitro* differentiation of at low-density seeded monolayer-cultured rat calvaria-derived osteoblasts in detail (Lian and Stein, 1992). Osteoblast differentiation goes through 3 phases – proliferation, matrix maturation and matrix mineralization. Briefly, at the onset of *in vitro* differentiation, spindle-shaped osteoblasts cultured in low-density monolayer proliferate to form a dense multilayer culture. During this stage, the cells undergo morphological changes and express high levels of type I collagen, the most abundant protein in the extracellular matrix of bone. The start of the second phase – matrix maturation – is characterized by an up-regulation of alkaline phosphatase activity (Lian and Stein, 1992). Reaching a constant cell number, characterized by a balance between cell proliferation and cell death, osteoblasts start to produce non-collagenous extracellular matrix proteins, such as osteopontin (Oldberg *et al.*, 1986) and osteocalcin (Hauschka *et al.*, 1989). The maturation of the synthesized ECM is finalized with the incorporation of hydroxyapatite crystals within the matrix. This step can be visualized by the formation of mineralized nodules *in vitro*. The progression of osteoblast maturity is further complicated by the potential of the cells to become embedded with the matrix and further differentiate into osteocytes.

The level of *in vitro* osteoblast maturity in low-density monolayer, with flat and spindle-shaped cells, reflects from a morphological point of view the *in vivo* situation of resting or non-active bone lining cells. These flat cells would have to be activated *in vivo* to become cuboidal-shaped osteoblasts that actively modify bone surfaces and lay down the osteoid, in which they then can be entrapped and be transformed into early osteocytes characterized

by irregular cell morphology and an increased presence of cell processes (Franz-Odendaal *et al.*, 2006). The trigger for this transformation is currently unknown.

Moreover, the potential of *in vitro* osteoblasts to maintain their phenotype, and their level of activity, can vary dramatically, and is undoubtedly dependent on the cell type used and substrate characteristics (Hayes *et al.*, 2010). A popular choice for osteoblast culture studies are immortalized cell lines, i.e. MG-63 or SaOS-2 (Kartsogiannis and Ng, 2004; Pautke *et al.*, 2004). There are many advantages working with a uniform cell population, which are defined by specific characteristics and the known differentiation state. Immortalized cell lines inherit these cell characteristics over a large amount of cell cycles *in vitro*, resulting in a virtually 'never ending' potential use of one cell batch. However, no immortalized cell line truly recapitulates the phenotype of a primary osteoblast and for this reason it is favorable to have a model based on primary cells.

Primary osteoblasts can be harvested from a variety of origins and donors. The isolation methods for osteoblasts are well established and range from enzymatic digestion methods using collagenase type II, to mechanical isolation methods with the intention to collect 'out migrating' osteoblasts (Di Silvio and Gurav, 2001). Primary human osteoblasts can be isolated from i.e. femoral heads of hip replacement patients. One of the main problems using adult human osteoblasts, even at low passage number, is their inadequate ability to maintain a mature phenotype in monolayer culture. To induce *in vitro* activation of primary osteoblasts in monolayer culture, confluence is required and medium additives such as β-glycerolphosphate, dexamethasone or specific nutrient enrichment are commonly used (Di Silvio and Gurav, 2001; Gallagher, 2003). Yet, ECM deposition and calcification by primary osteoblasts in monolayer is limited and can take up to 30 days *in vitro* (Di Silvio and Gurav, 2001), resulting in a prolonged experimental time to achieve the desired osteoblast phenotype. For the *in vitro* monolayer culture of primary osteoblasts, the results of Owen *et al.* (1990) suggested a functional relationship between the inhibition of proliferation and the induction of genes associated with cell differentiation and matrix maturation (Owen *et al.*, 1990). Using fetal rat calvaria cells, Owen *et al.* showed that the inhibition of proliferation by hydroxyurea resulted in a subsequent up-regulation of alkaline phosphatase followed by up-regulation of osteopontin expression and, therefore, activation of osteoblasts *in vitro*. Moreover, it is well known that the formation of nodules, which simulate a micromass, during osteoblast culture results in an increase in osteoblast maturation (Bellows *et al.*, 1986).

Previous attempts have been performed to culture osteoblasts in 3D aggregates. Kale *et al.* (2000) demonstrated that loosely seeded osteoblast suspensions can progress in the presence of $TGF\beta_1$ to form spheroids of variable sizes within 24 h to 48 h of culture (Kale *et al.*, 2000). Very elegantly, a more recent study showed the possibility of folding an existing monolayer culture of hOBs in the presence of mineralization medium lead to the formation of irregularly-shaped 3D aggregates (Ferrera *et al.*, 2002).

Within this study, we aimed to evaluate a rapid method to reproducibly generate cell pellets from human primary osteoblasts, to characterize these pellets and compare them to the conventionally used monolayer culture. We hypothesize, that the pellet culture could serve as a 3D culture model for human primary osteoblasts to accelerate and increase the transformation of osteoblasts *in vitro* to create a more mature osteoblastic cell phenotype within a short culture period in comparison to monolayer culture.

Materials and Methods

Materials

Tissue culture medium (DMEM, powder), Penicillin-Streptomycin (10 kU Penicillin: 10 mg Streptomycin) was purchased from Gibco (Basel, Switzerland), fetal calf serum (FCS) was from Biowest (Nuaillé, France). Sodium bicarbonate was purchased from Merck (Whitehouse Station, NJ, USA), and the L-ascorbic acid phosphate magnesium salt n-hydrate from Wako Chemicals (Neuss, Germany). Bovine serum albumin (BSA), chemically defined lipids and insulin-transferrin-selenium (ITS) were from Gibco. Glycyl-glycine, Polypep, L(+)-Lactic acid, β-Nicotinamide adenine dinucleotice, and Nitrolue tetrazolium (tablets) were purchased from Sigma Aldrich (Buchs, Switzerland). DeadEnd™ Fluorometric TUNEL System was from Promega (Zürich, Switzerland). The anti-C-terminal propeptide of type I collagen (M38) was purchased from Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA, USA), anti-osteocalcin (OC4-30) was purchased from ABCAM (Cambridge, UK), phalloidin (Alexa 488) was from Invitrogen (Basel, Switzerland), and anti-connexin-43 (MAB3068) was from Millipore (Zug, Switzerland). The Vectastain ABC Mouse Elite IgG Kit was from Vector Labs (Peterborough, UK), the alkaline phosphate kit was from Sigma Aldrich. TRI reagent and PolyAcrylCarrier were purchased from Molecular Research Center (Cincinnati, OH, USA). RT and qPCR reagents were purchased from Applied Biosystems (Foster City, CA, USA).

Cell Culture

Human primary osteoblasts (hOBs) were isolated from 3 different donors (2 male and 1 female; average age of 53 years; female 52 years, male 49 years, male 59 years) undergoing hip replacement operations (approved by Ethics Committee Graubünden 18/02) to perform 4 separate experiments comparing low-density monolayer with pellet culture. The osteoblast isolation was performed as previously described (Di Silvio and Gurav, 2001). Primary outgrowing cells were used at passage 6 for pellet and monolayer culture. Each experiment was carried out on a single donor and the data combined. Initially, hOB cells for pellet culture were seeded into 96-V-shaped-non-tissue-culture coated well plates at 36000 cells per well. Pellet formation was achieved by centrifugation at 500x G for 10 min. Loosely formed cell pellets were transferred individually into 1.5 ml Eppendorf (Basel, Switzerland) tubes and cultured overnight under continuous orbital shaking at 40 rpm in 750 µl serum-free (SF) DMEM

containing 5 mg/l L-ascorbic acid-2-phosphate magnesium salt n-hydrate, 1x ITS, 1x chemically defined lipids and 100 mg/l BSA per pellet. Media was replaced on day 1 and then every 48 h with DMEM + 10% FCS + L-ascorbic acid-2-phosphate. Comparative low-density monolayer cultures were performed in 12-well plates with 36000 cells per well. Three additional experiments using cells from 3 different donors (2 male and 1 female; average age of 53 years; female 52 years, male 49 years, male 59 years) to investigate the effect of contact inhibition on the osteoblast phenotype during monolayer culture in comparison to low-density seeded monolayer culture (10000 cells/cm^2) and pellet culture (36000 cells/pellet). Cell densities ranging from a: 18000 cells/cm^2, b: 27000 cells/cm^2, c: 40000 cells/cm^2 were seeded in monolayer, and cultured for 7 days as previous experiments. All cultures were performed in the absence of β-glycerol phosphate and dexamethasone.

Cell number and viability

Cell number and viability were determined on day 1, 3, 5 and 7. Relative estimation of cell number was achieved by DNA quantification using the 'Hoechst' method. For this method, pellets were pooled (3 pellets per sample). Viability was determined using the lactate dehydrogenase (LDH) assay (Wong *et al*., 1982). Therefore, unfixed cryo-sections (12 µm) from pellet centers were prepared. The LDH assay on unfixed cryo-sections was performed as previously described (Stoddart *et al*., 2006). Cell death was assessed qualitatively using the TUNEL (Terminal desoxy-nucleotidyl transferase dUTP-nick-end-labelling) assay. Overview images of 1-7 days cultured pellets were taken.

Immunocytochemical labeling

Vectastain ABC Mouse Elite IgG Kit was used to detect the antibody-antigen reaction. The M38 antibody (DSHB) was utilized to detect the unstable C-terminal propeptide of type I collagen, which is released during type I collagen synthesis through cells, while the OC4-30 antibody (ABCAM) was used to detect osteocalcin production by cells. Immunocytochemical labeling was performed on 12 µm cryo-sections from fixed (15 min in 4°C-pre-cooled 4% neutrally phosphate-buffered formalin) pellet and low-density monolayer cultures from day 1, 3, 5 and 7. Fluorescence labeling of β-actin (phalloidin), together with connexin 43 and DAPI was performed on fixed cryo-sections. The activity of the tissue non-specific alkaline phosphatase (ALP; Alkaline phosphatase kit) was determined on whole pellets and low-density monolayers according to manufactures instructions with a labeling time of 1 h. Cryo-sections of ALP activity labeled pellets were prepared. Images of the different staining were taken using an Axioplan microscope (Zeiss, Oberkochen, Germany).

Alizarin red S (ARS) staining

Fixed monolayer and pellet cultures from day 1, 3, 5 and 7 were stained 40 mM ARS solution (pH = 4.2). The bound ARS was dissolved in 10% w/v cetylpyridinium chloride monohydrate (CPC) solution (pH = 7). Absorbance was measured at 545 nm using a VICTOR^3 ™ plate reader (Perkin Elmer, Waltham, MA, USA).

Table 1. RT-qPCR of Runx2, osterix, type I collagen, osteocalcin and E11/podoplanin.

Gene	Forward primer	Reverse primer	Probe
Runx2	AAGCAGTATTT ACAACAGAGG GTACAAG	GGTGCTCGGAT CCCAAAA	CATCAAACAGC CTCTTCAGCAC AGTGACAC
Osterix	CCTGCTTGAGG AGGAAGTTCA	GGCTAGAGCC ACCAAATTTGC	TCCCCTGGCCA TGCTGACGG
Type I collagen	CCC TGG AAA GAA TGG AGA TGA T	ACT GAA ACC TCT GTG TCC CTT CA	CGG GCA ATC CTC GAG CAC CCT
Osteocalcin	AAG AGA CCC AGG CGC TAC CT	AAC TCG TCA CAG TCC GGA TTG	ATG GCT GGG AGC CCC AGT CCC

RT-qPCR was performed to investigate relative expression levels of Runx2, osterix, type I collagen, osteocalcin and E11/podoplanin. The table shows the primer and probes sequences used.

RT-qPCR

RNA samples from pellet and monolayer cultures were taken every 48 h, starting on day 1. Pellets were pooled (3 pellets per sample). Cells were lysed using TRI reagent and RNA was isolated according to manufactures instructions. Reverse transcription (RT) was performed using 0.5 µg RNA. Quantitative polymerase chain reaction (qPCR) was performed using TaqMan© probes. The dCt was calculated using 18S-rRNA as a house-keeping gene. Expression levels of osteoblastic marker genes (Table 1) were made relative to low-density monolayer culture on day 1.The relative gene expressions were analyzed from the dCT values using 18SrRNA as house-keeping gene. Data visualization was performed using ddCT values, which were determined by normalization to low-density monolayer culture on day 1.

Statistical analysis

Data collected in the performance of DNA analysis and RT-qPCR (ddCt) underwent statistical analysis using SPSS 16.0 (Chicago, IL, USA) and OpenStat (http: statpages.org) software packages. Data sets gained from the cells of different human donors were not normally distributed. Therefore, the Mann-Whitney-U test and Bonferroni correction were used as statistical analyses. Statistical significance was determined as $p \leq 0.05$.

Results

Data obtained from the different donors showed consistent results; therefore the histological images presented are representative. Cell viability within pellets was visualized using an LDH assay. An equal distribution of viable cells throughout the whole cultured pellet was detected until culture day 7 (Fig. 1A-C). Prolonged pellet culture of hOB cells resulted in central areas of cell death. Furthermore, the formation of a surface fibrous-tissue-like cell layer surrounding the inner cells of the pellets was detected by

day 19 (Fig. 1D) As the pellet culture model was changing its morphology so dramatically from day 7 to day 19, further experiments were performed only till day 7. Cell death was determined during the 7-day culture period using the TUNEL assay. By day 1 only a small number of hOB cells inside the pellet were labeled positive for TUNEL (Fig. 2A green cells). However, during the culture period the amount of positively labeled cells increased. By day 7, more cells were labeled positive for TUNEL than on day 1 (Fig. 2B). The quantification of relative cell number was performed during all 7-day culture experiments comparing low-density monolayer culture of hOB cells with pellet culture of the same cell type. Low-density monolayer culture of hOB cells resulted in a steady increase in total DNA amount (p=0.0004) (Fig. 3B light grey data). Contrary, the amount of total DNA in pellet cultures did not increase over time and was significantly lower compared to the amount in low-density monolayer cultures over the whole culture period ($p \leq 0.00001$) (Fig. 3A black data). Moreover, a slight non-significant decrease in the amount of DNA in pellets was determined over 7 culture days. Cells seeded in monolayer at 18,000 and 27,000 cells/cm^2 demonstrated either a modest increase in total DNA by day 7 ($p \leq 0.00001$), while 40,000 cells/cm^2 resulted in a constant amount of DNA over time, similar to the pellet culture (Fig. 3B).

In low-density-seeded monolayer culture hOB cells are known to display a flat and fibroblast-like morphology (Lian and Stein, 1992). *In vivo*, such fibroblast-cell morphology for osteoblastic-lineage cells is only found if osteoblasts are resting and covering inactive bone surfaces as bone-lining cells. During activation and differentiation of pre-osteoblasts to mature osteoblasts, early osteocytes and terminally embedded osteocytes, cell morphological changes are apparent. Active cuboidal osteoblasts that secrete type I collagen can become embedded in ECM and can be transformed into star-shaped osteocytes (Aubin and Liu, 1996). Cell morphology of hOB cells within pellet cultures was determined to investigate, whether cell shape of 3D cultured osteoblasts differs from 2D cultured cells. The morphological analysis demonstrated that hOBs show morphological differences if cultured in low-density monolayer, in dense monolayer / multilayer, or in pellet cultures. The majority of the central pellet cell population presented a cuboidal cell shape (Fig. 4A, C), which was completely different to a fibroblast-like cell morphology. The most outer cell layer of the cultured hOB pellets seemed to have maintained a flat and rather spindle-shaped cell morphology (Fig. 4B, D). Moreover, the size of cultured cells in pellets was with an average of 1000 µm^3 over 10-times smaller than hOB cells in monolayer cultures (12000 µm^3), a typical effect seen during the transformation of osteoblasts into osteocytes (Franz-Odendaal *et al.*, 2006).

While cells in low-density monolayer are characterized by a fibroblast-like morphology (Fig. 4E) the culture in a dense monolayer increases the chance of detecting cuboidal, irregularly-shaped osteoblasts with the presence of more than 2 cell processes connecting to neighboring cells (Fig. 4F). Higher magnification images of LDH stained or actin-connexin 43-double labeled hOB cell pellets showed at least 2 different cell morphologies.

To determine the location of ALP activity during pellet culture of hOB cells, qualitative analysis of the enzyme activity was performed. The low-density monolayer cultures demonstrated an even cell staining of the flat spindle-shaped cells, which increased with increasing cell number from day 3 to day 7 (Fig. 5A, B). During the early stage of pellet culture – day 1 until 3 – ALP activity was found almost exclusively at the outer surface of the hOB cell layer surrounding the central cell population of osteoblasts (Fig. 5C). This distinct pattern of ALP activity location shifted during pellet culture. By day 7 (Fig. 5D), ALP activity was found further into the pellet, with only the most central area being negative. The surface cell layer, which showed activity during the early stage of pellet culture, seemed to be less active in ALP by day 7, suggesting that the spindle-shaped outer cells of the pellet culture progress through a similar but rapid *in vitro* transformation as hOB cells seeded in low-density monolayer.

Type I collagen is the main protein produced by osteoblasts at an early stage of maturation. It accumulates in the ECM and serves as a scaffold for the developing matrix. The C-terminal propeptide of type I collagen (Calvo *et al.*, 1996) was detected during low-density monolayer and pellet culture of hOB cells (Fig. 5E, G). Low-density monolayer cultured cells revealed an even expression by culture day 7, while the labeling for the C-terminal propeptide of type I collagen in pellets was found to be more intense around the surface layer of cultured pellets by day 7 (Fig. 5G). Immuno-labeling for the ECM protein osteocalcin during low-density monolayer culture of hOB cells showed a very slight cellular labeling on day 7 (Fig. 5F). Cells cultured in pellets demonstrated a strong cellular labeling by day 7 and a slight ECM deposition of osteocalcin within the pellets (Fig. 5H).

The quantification of mineral deposition during the pellet and low-density monolayer culture of hOB cells showed significant differences between both cultures (p=0.04) (Fig. 6A). While ARS per DNA in low-density monolayer cultures on day 7 was a level similar to day 1 (Fig. 6B light grey data), ARS per DNA in pellets increased from day 1 to day 7 (Fig. 6B black data). This demonstrates that, contrary to low-density monolayer, mineral deposition per cell increased in pellets with time. The trend with higher density monolayer was less constant as the quantified ARS fluctuated slightly, however by day 7 all three higher density cultures had higher ARS staining than on day 1. For 18,000 cells/cm^2 this trend did not reach significance (p=0.0829). In samples seeded at 27,000 and 40,000 cells/cm^2 the increase in ARS staining was significant ($p \leq 0.0001$; $p \leq 0.0001$, respectively) (Fig. 6B).

To assess osteoblast phenotype, the expression of osteoblast differentiation markers, namely type I collagen, Runx2, osterix, E11/podoplanin and osteocalcin, was investigated. Gene expression was evaluated within the 'standard' low-density monolayer culture and the pellet culture, were compared to three additional monolayer cultures that represented seeding at confluence (a), seeding

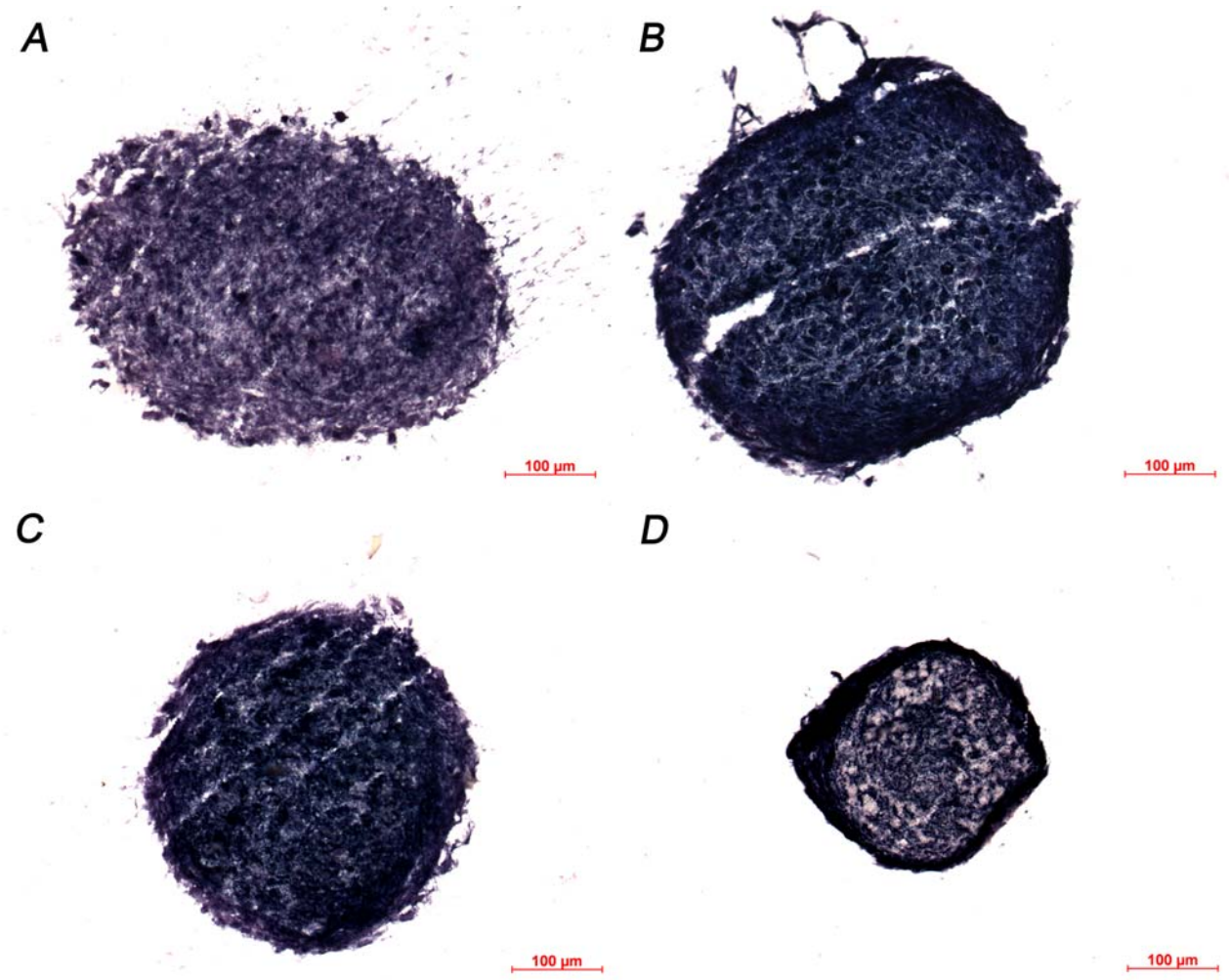

Fig. 1: Representative micrographs of LDH viability labeling were taken from human primary osteoblast cell pellets (male 62 years) cultured for 1 day (**A**), 3 days (**B**), 7 days (**C**) or 19 days (**D**). Evenly distributed LDH activity throughout the pellets was presented till day 7. The pellets on day 19 were much smaller and dominated by a huge fibrous capsule and central areas of cell death.

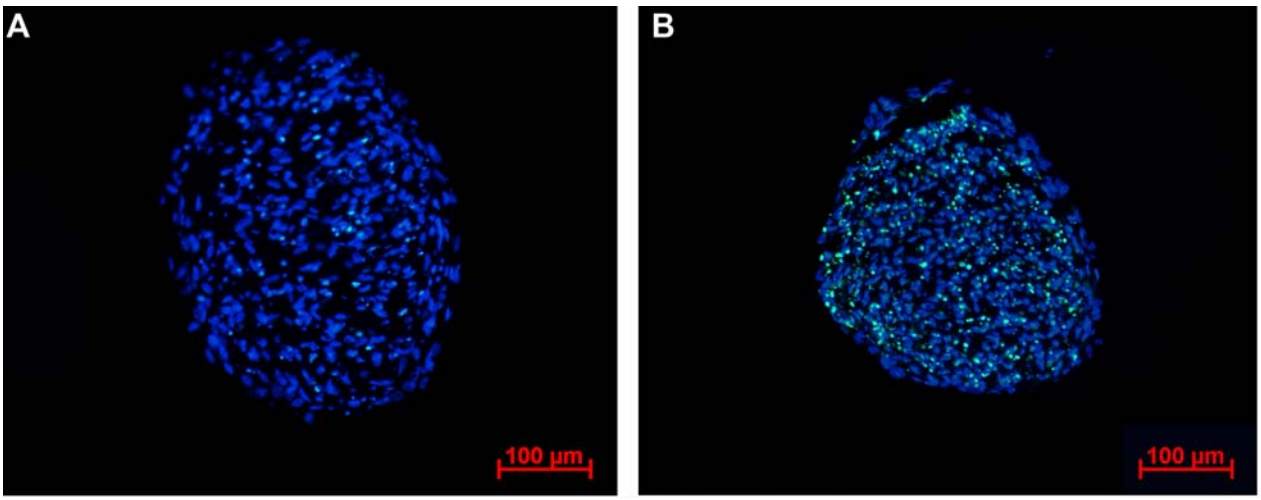

Fig. 2: Representative fluorescence micrographs taken from 1 day (**A**) and 7 days (**B**) cultured osteoblast pellets (female 73 years) stained with DAPI (blue) and analyzed with TUNEL assay. The amount of TUNEL positive cells (green) did increase over culture time.

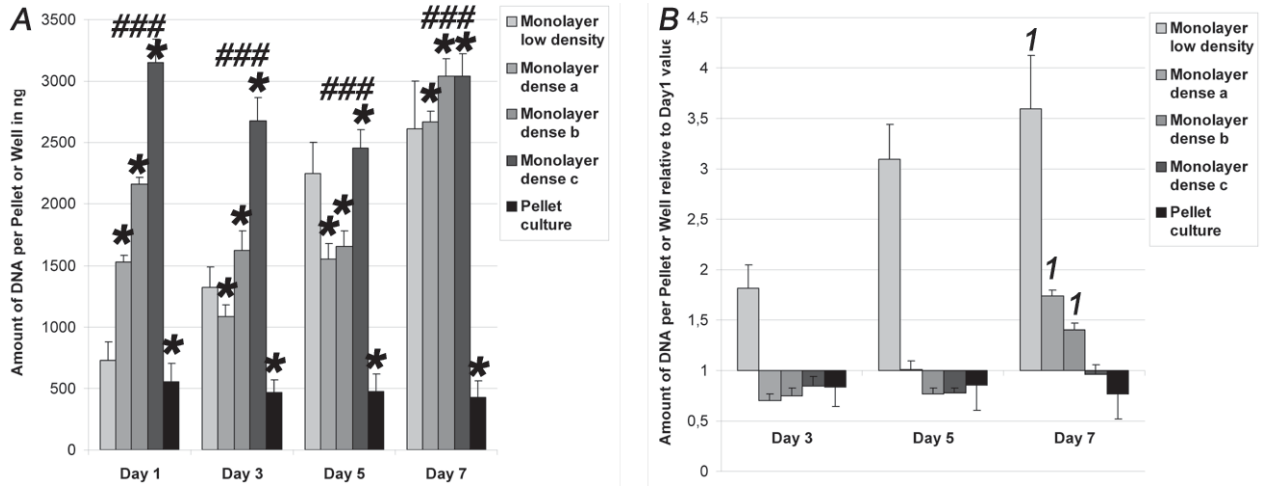

Fig. 3: The diagram **A** shows the amount of mean DNA and the standard error of the mean quantified by Hoechst assay during culture of human primary osteoblasts in low-density monlayer, in dense monolayer (**a**: 18,000 cells/cm² seeded; **b**: 27000 cells/cm² seeded; c: 40000 cells/cm² seeded) and pellet culture (female 52 years, male 49 years, male 59 years; n≤10). Diagram **B** shows the same data but relative to day 1 data values. Data were not normally distributed: Kruskal-Walis and Mann-Whitney-U test with Bonferroni correction were used as statistical analyses with statistical significance at $p \leq 0.05$.
*: significantly different compared to low-density monolayer culture;
#: significantly different compared to pellet culture.
[1]: significantly different from the same group on day 1.
An increase in DNA amount was detected during low-density monolayer culture and denser monolayers **a** and **b**, osteoblasts cultured in multilayers (**c**) and pellets showed no change in DNA over culture time. The amount of DNA in low-density monolayer culture was significantly different to all others (*: $p \leq 0.0003$), so was the DNA amount in pellet culture (#: $p \leq 0.00001$).

a highly packed, overconfluent monolayer (b), and seeding a multilayer (c).

The mRNA expression of early marker type I collagen was significantly increased in denser monolayer cultures compared to the low-density monolayer and pellet culture over all time points ($p \leq 0.00001$) (Fig. 7A). Contrary, the level of type I collagen expression in pellet cultures was significantly lower compared to all monolayer cultures over the whole culture period ($p \leq 0.00001$) (Fig. 7A).

The expression of the early osteoblastic transcription factor Runx2 was up-regulated in denser monolayer cultures compared to the low-density monolayer and pellet culture over all time points ($p \leq 0.00001$) (Fig. 7B). Runx2 expression in pellet cultures was significantly higher compared to low-density monolayer over the whole culture period investigated ($p=0.0021$) (Fig. 7B). The highest expression of Runx2 transcription factor was found during 'multilayer' culture (c) on day 3, with a maximal expression of 108.9-fold change +/- 155.2. ($p \leq 0.00001$).

A very similar expression pattern as for Runx2, was seen for osteocalcin mRNA expression during monolayer and pellet culture (Fig. 7D). The expression of the late osteoblast marker was significantly up-regulated in pellet compared to low-density monolayer culture over the whole culture period ($p=0.0011$). Whereas, osteocalcin expression in the three denser monolayer cultures was significantly higher compared to low-density monolayer and pellet culture over all days ($p \leq 0.00001$) (Fig. 7D).

The expression of the second major transcription factor during osteoblast differentiation, osterix, showed that denser monolayer culture up-regulated mRNA expression compared to low-density monolayer culture over all culture days ($p \leq 0.00001$) (Fig. 7C). However, osterix expression in pellet culture was non-significantly different to denser monolayer cultures on day 3. On day 5 and 7 the highest osterix expression was found within pellet cultures compared to all monolayer cultures ($p=0.0396$).

The expression of the early osteocyte marker E11 was increased in all the denser monolayers as wells as in pellets, compared to low-density monolayer over the whole culture period ($p \leq 0.00001$) (Fig. 7E). By day 7 the expression of E11 was comparable for all the three higher density monolayer cultures and the pellet culture.

Discussion

To determine the optimal culture period of a pellet culture model for human primary osteoblasts, an initial experiment was performed investigating the viability of the cultured cells in 3D. As the ECM deposition of hOB cells in low-density seeded monolayer culture can take up to 3 weeks, the experiment was performed for 27 days in total. Using an LDH assay, we demonstrated that human primary osteoblasts could be kept viable throughout a 7-day culture period. We showed by qualitative TUNEL assay, that cell death slightly increased during the 7-day culture. Nineteen days of culture resulted in areas of cell death in the centre of the pellets. Previous reports demonstrated that 3D cultures of osteoblastic cells will start to produce mature

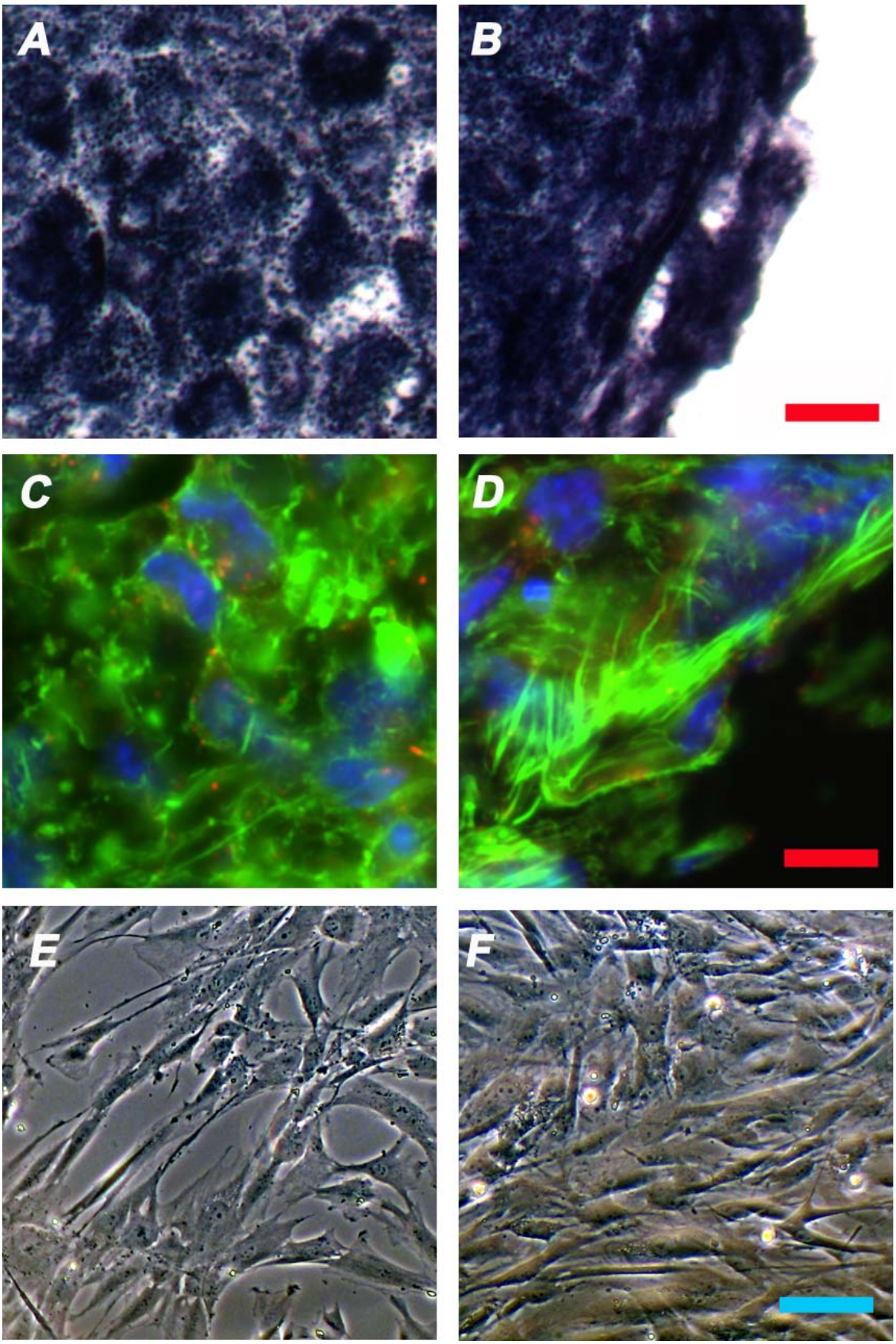

Fig. 4: Representative micrographs A and B represent LDH viability labeling of an osteoblast cell pellet (male 62 years) cultured for 3 days. Central cells demonstrate cuboidal cell morphology (**A**), while surface cells are rather flat and spindle shaped (**B**). These observations on the 2 different cell morphologies were supported by the micrographs **C** and **D** taken from a β-actin (Alexa 488, green) and connexin 43 (Alexa 598, red) immuno-double labeled 5 days cultured osteoblast pellet. Representative brightfield micrographs E and F demonstrate low-density monolayer and multilayer (c) after 2 days culture.

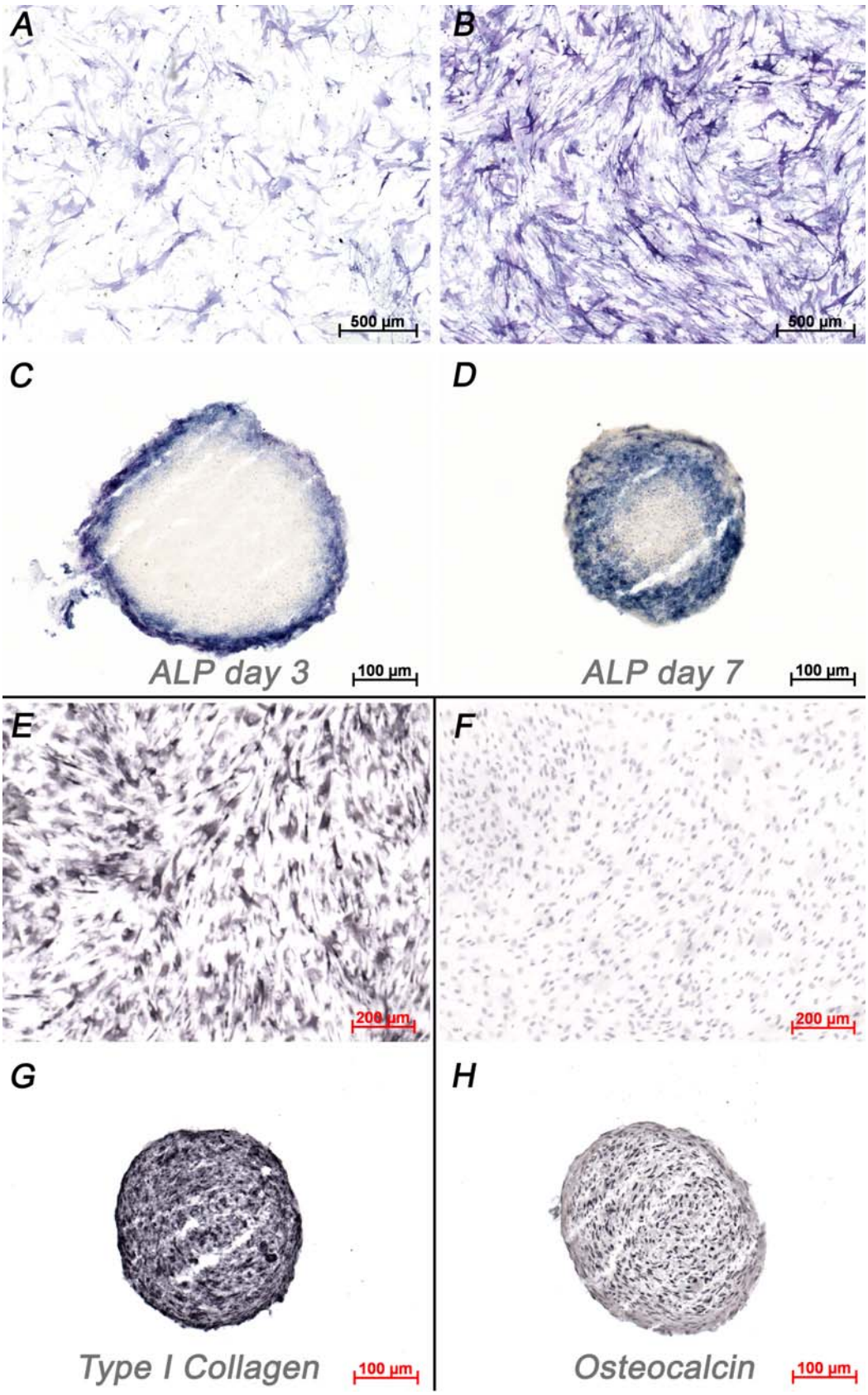

Fig. 5: Representative micrographs A-D represent ALP activity labeling of human primary osteoblasts (female 73 years) cultured in low-density monolayer (**A, B**), or pellets (**C, D**), cultured for 3 days (**A, C**) or 7 days (**B, D**). Low-density monolayer culture showed even activity labeling, while pellets demonstrated concentrated areas of ALP activity predominantly on adjacent surface cells by day 3. On day 7, ALP activity penetrated into deeper zones of the pellet. Micrographs E and G show immuno-labelling of the C-terminal propeptide of type I collagen, detected during monolayer (**E**) and pellet culture (**G**) of human primary osteoblasts (male 59 years) at culture day 7. Micrographs F and H show immuno-labelling of osteocalcin, detected during monolayer (**F**) and pellet culture (**H**) of human primary osteoblasts (male 59 years) at culture day 7.

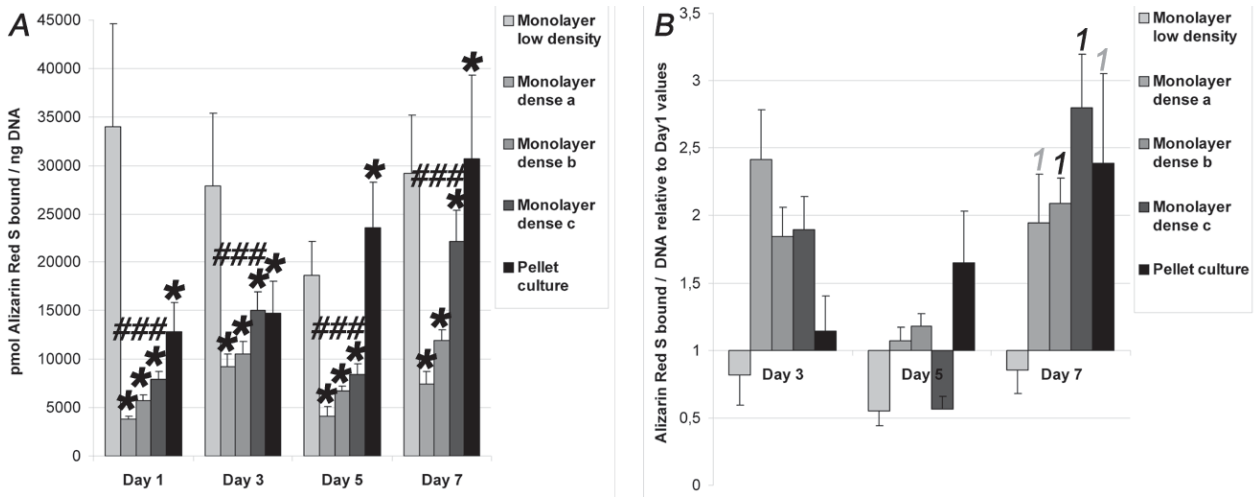

Fig. 6: Human primary osteoblasts were cultured in low-density monolayer, dense monolayers (a, b, c) or pellets (female 52 years, male 49 years, male 59 years; n≤10). Diagram **A** shows the amount of mean ARS staining per total amount of DNA together with the standard error of the mean. Diagram **B** shows the same data but relative to day 1 data values. Data were not normally distributed: Kruskal-Walis and Mann-Whitney-U test with Bonferroni correction were used as statistical analyses with statistical significance at $p \leq 0.05$.

*: significantly different compared to low-density monolayer culture;

#: significantly different compared to pellet culture.

[1]: significantly different from the same group on day 1.

While ARS / DNA in pellet and denser monolayer cultures increased with culture time ($p \leq 0.0829$), the amount of ARS / DNA in low-density seeded monolayers decreased from day 1 to 5 and by day 7 was comparable to day 1 again.

ECM and mineralize this matrix over a prolonged time of culture and the right medium supplementation (Kale *et al.*, 2000; Ferrera *et al.*, 2002). Kale *et al.* (2000) have further demonstrated that the addition of $TGF\beta_1$ is beneficial to the formation of 3D spheroids (Kale *et al.*, 2000). In recent studies, we were able to demonstrate that in multilayer cultured hOB cells in the presence of $TGF\beta_3$ roll-up within 4-6 days of culture to form irregularly-shaped 3D aggregates (unpublished data). Within this evaluation study, we concentrated on the rapid induction of a later osteoblastic / early osteocytic phenotype within pellet culture without any further differentiation stimuli (apart from the addition of ascorbic acid).

With the pellet culture model, we concentrated on limiting the size of the cultured pellets in order to maintain suitable oxygen availability in the center of the pellets. Initial experiments performed (data not shown) were used to determine the diameter of the potential 3D pellet in relation to the seeded cell number. According to previous reports on cell pellet cultures (Carpenedo *et al.*, 2007; Johnstone *et al.*, 1998), we aimed to achieve osteoblast cell pellets of 200-800 µm diameter. In our hands, using 36,000 primary human osteoblasts per pellet resulted in the intended pellet size.

For initial comparison we cultured human primary osteoblasts in a low-density monolayer of 10,000 cells/cm^2, a density commonly used for osteoblastic induction (Gallagher, 2003). When cultured in a 12 well plate this corresponds to 36, 000 cells making it comparable in cell number to the pellets. Under these monolayer conditions there was the expected increase in DNA amount over time. In contrary, the total amount of DNA in pellets did not change significantly over time, demonstrating that increased initial proliferation in cell pellets was absent and this is likely due to contact inhibition. Resulting from *in vitro* osteoblast differentiation studies (Gallagher, 2003; Kartsogiannis and Ng, 2004; Lian and Stein, 1992), we predicted that a reduction in cell proliferation, as seen during osteoblast pellet culture, would result in immensely increased osteoblast differentiation. To test this hypothesis we first investigated the activity of the tissue-nonspecific alkaline phosphatase (ALP) an early marker for the osteoblastic phenotype, indicating the transition of proliferating osteoblasts to mature-ECM-producing cells (Lian and Stein, 1992; Ali *et al.*, 1970). Within the pellet culture system, the activity of ALP was found predominantly in the surface layer of flat-spindle shaped osteoblasts leaving the most central cells ALP-negative. As it is known that early osteoblasts demonstrate high ALP activity, while later more mature osteoblasts, as well as osteocytes, are characterized by no or weak ALP activity (Lian and Stein, 1992; Aubin and Liu 1996), we believe that the central population of hOB cells within pellets was of later osteoblast / early osteocyte differentiation stage, while the surface osteoblasts are characterized by a much earlier phenotype. It is hard to speculate what the reason for the detected shift in ALP activity over culture time in pellets was. We hypothesize that during the early culture of osteoblast pellets (day 1- 3) the surface population of cells received more nutrients in comparison to the central

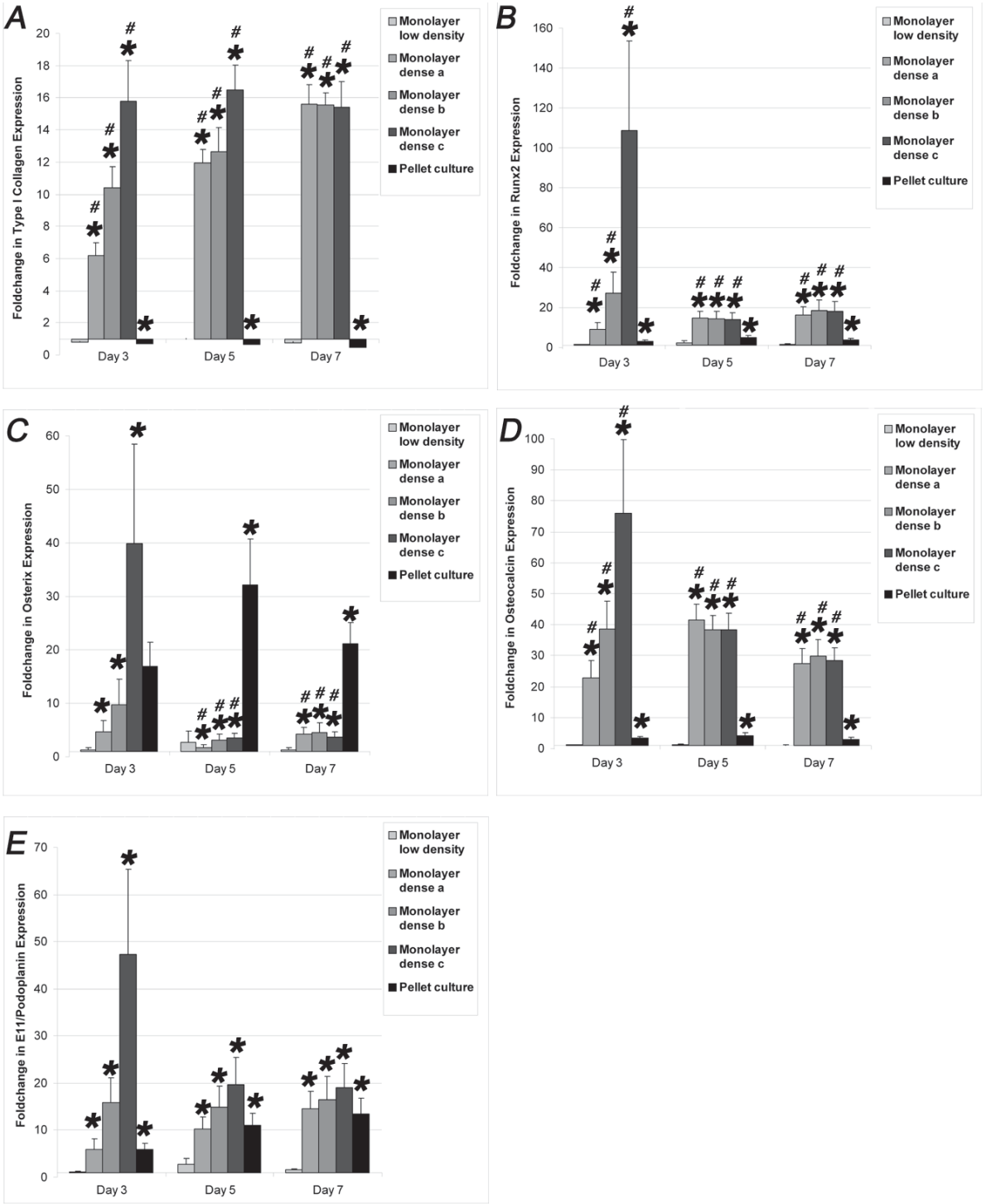

Fig. 7: Comparative qPCR of type I collagen (A), Runx2 (**B**), osterix (**C**), osteocalcin (**D**) and E11 (**E**) was performed during the 7 day culture period of osteoblasts within low-density monolayer, dense monolayer cultures (a: 18,000 cells/cm² seeded; b: 27000 cells/cm² seeded; c: 40000 cells/cm² seeded), or pellet culture (female 52 years, male 49 years, male 59 years); n=12). Diagrams show the mean fold-change in relative gene expression and the standard error of the mean. Gene expression levels were normalised to 18SrRNA, and were made relative to gene expression levels in low-density monolayer at culture day 1. The dCT data was used for statistical analysis data was not normally distributed and analysed with Mann-Whitney test and Bonferroni correction. Statistical significance was determined as $p \leq 0.05$;

*: significantly different from low-density monolayer;

#: significantly different from pellet culture.

The expression of all genes investigated was increased by culturing hOB cells in denser monolayers compared to low-density monolayer. Pellet cultures showed increased Runx2, osterix, osteocalcin and E11 expression compared to low-density monolayer ($p<0.0396$), but Runx2 and osteocalcin expression were lower compared to dense monolayer culture over the whole culture period ($p \leq 0.00001$). The lowest type I collagen expression was found in pellet cultures ($p \leq 0.00001$).

population, this may result in a later onset of ALP activity in deeper zones of the pellet. By culture day 7 ALP activity was also present in deeper zones, but not the central zone, of hOB pellets. ALP activity in these zones might be needed for the onset of mineralization.

It is known that in 2D culture osteoblasts produce connexin 43 gap junctions (Sharrow *et al*., 2008). Within our study we have also demonstrated that such junctions form within pellet cultures.

The potential of the hOB pellets for following mineralization studies was further highlighted by the ARS data investigating the calcium incorporation into the ECM within the short period of pellet culture (7 days). ARS per DNA in pellet culture demonstrated a significant increase during culture time, demonstrating increased cellular activity in the form of mineral deposition in pellets compared to monolayer culture. Therefore, the pellet culture of primary osteoblasts showed a more rapid mineral deposition increase than monolayer culture even within 7 culture days and without any additional stimulus.

To further investigate the role of cell density we investigated the expression of a number of osteoblast markers after 7 days of culture, comparing a number of different seeding densities from 10,000-40, 000 cells/cm^2 and the previously mentioned pellet culture (36,000 cells).

Due to the still existing lack of defined osteocyte markers, Franz-Odendaal *et al.* (2006) highlighted that osteocytes could potentially be distinguished by reduced expression of type I collagen, Runx2 and osteocalcin compared to osteoblasts (Franz-Odendaal *et al.,* 2006). E11 is not exclusive to osteocytes but its expression is greatly increased during osteoblast differentiation and can be correlated to the formation of dendrites (Zhang *et al*., 2006). Due to its important role in osteocyte morphology and the maintenance of the osteocyte network, E11 is thought to play a crucial role in osteocyte development and viability. The mRNA relative expression levels of E11 were quantified in monolayer or in pellet culture. Both dense monolayer and pellet culture showed significantly increased E11 expression compared to low-density monolayer, further underlining the proposed role of E11 in the transformation of osteoblasts into a later phenotype. It is interesting to note that under the conditions applied all culture conditions had a comparable E11 expression except the cells seeded at 10,000 cell/cm^2 where the expression remained unexpectedly low.

The evaluations on the amount of osteoblastic marker genes expressed in the different cultures revealed significant changes. While type I collagen expression was significantly increased by culturing hOB cells in monolayer in the presence of contact inhibition (dense monolayer) rather than low-density seeded cells, the relative gene expression of type I collagen in pellets was significantly decreased. Type I collagen is the main ECM protein found in bone and it serves as a scaffold for the forming bone matrix, with its peak expression prior to ECM maturation and mineralization (Lian and Stein, 1992). Therefore, we compared the expression pattern with the different osteoblast phenotypes during transformation (Lian and Stein, 1992; Franz-Odendaal *et al.,* 2006). We hypothesize, that in low-density monolayer cultured hOB cells compare to less mature, less active osteoblasts, as they are characterized by spindle-shaped morphology, secreting a low amount of type I collagen. Osteoblasts cultured in denser monolayers on the other hand seem to resemble polarized, active osteoblasts that are characterized by large type I collagen expression, as seen prior to the onset of ECM maturation and also prior to the formation of early osteocytes. Therefore, we hypothesize that osteoblasts embedded in pellets are comparable to early osteocytes with a lower expression of type I collagen compared to dense monolayer culture. This conclusion is further supported by the lower expression of Runx2 and osteocalcin by pellet cultures compared to dense monolayer, while the E11 and osterix expression is comparable. Osteocalcin is a small calcium-binding protein found mainly in the ECM of bone, but a small amount enters the blood (Calvo *et al*., 1996; Hauschka *et al*., 1989). Even though its function is still not completely understood, it is expressed by fully differentiated osteoblasts (Hauschka, 1986; Aarden *et al*., 1996). The role of Runx2 during osteoblast differentiation and maturation is of crucial importance. Runx2 was the first transcription factor, identified through its binding site (OSE2) within the osteocalcin promoter (Ducy, 2000). Its expression is up-regulated during mesenchymal condensation leading to the formation of pre-osteoblasts (Ducy *et al*., 1997). Therefore, Runx2 has been described as an early osteoblast differentiation marker.

The role of osterix within the pellet culture model, and its significantly higher expression level compared to all other cultures, is currently unknown. However, even though the importance of osterix for initial bone formation has been demonstrated by Nakashima *et al.* (2002), its role during the later stage of osteoblast transformation and life cycle is to our knowledge still to be determined. Nakashima *et al.* (2002) demonstrated that osterix -/- mice do not show signs of intramembranous or endochondral bone formation with the absence of bone markers such as osteocalcin, osteopontin, or osteonectin (Nakashima *et al*., 2002). These osterix -/- mice expressed Runx2, suggesting osterix acting downstream of Runx2. Yet, other Runx2-independent roles for osterix during osteoblast differentiation have been suggested (Matsubara *et al.,* 2008).

The overall expression pattern in pellet of high E11 and osterix combined with low Runx2, Col I and osteocalcin would suggest the cells are maturing into an early osteocyte phenotype. Whereas with high- density monolayer the high Runx2, Col I, E11 and osteocalcin combined with a lower osterix would suggest a more active, mature osteoblastic phenotype. As expected within monolayer the gene expression of the markers was density dependent, starting relatively high at the higher densities, while increasing with time in culture at lower densities as the cell confluency increased. The exception to this was the cells seeded at the lowest density (10,000 cells/cm^2) where the expression of all markers investigated remained comparably low.

The trigger for a mature osteoblast to become an osteocyte is currently unknown. The different expression

patterns seen between highly dense monolayers (which would contain polarized cells) and pellet cultured cells (which are in 3D culture) suggest that the progression to a more osteocyte phenotype is induced by being presented with a 3D environment and not due to cues from a mineralized matrix. This would need more studies to confirm.

Taken together, we demonstrated that pellet-cultured human primary osteoblasts show faster transformation into a later osteoblast phenotype, possibly even early osteocyte phenotype when compared to low or high density monolayer culture. We believe the pellet culture model for human primary osteoblasts offers the potential further studies aiming to use highly differentiated primary osteoblasts / osteocyte-like cells.

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Discussion with Reviewer

Reviewer I: Although the authors repeatedly use the word "differentiation" with regard to their cultures, it is not appropriate unless these cells de-differentiated during expansion. Since the cells used were primary osteoblasts, they are already differentiated. Word choice should be reconsidered.

Authors: This is a very valid point which is complicated by the fact that "differentiation" is commonly used within the literature even when primary osteoblasts are the cell used. We have adopted the following nomenclature- when the data is being discussed we describe the state of maturity, from proliferating to more active, to potentially late stage osteoblast/early osteocyte. When referring to published manuscripts we have maintained the terminology used in that paper e.g. Lian and Stein (1992) where they refer to differentiation of osteoblasts.

Reviewer I: The pellet culture system is used to induce chondrogenesis of mesenchymal stem cells (MSC) (referenced in the first paragraph) because it is necessary to maintain these cells in a rounded shape for successful chondrogenesis. This is not true of MSC osteogenesis. This rounded morphology is also key to maintaining the phenotype of primary chondrocytes. Given these differences in cell type, how does this paragraph motivate your study?

Authors: An increasing number of researchers believe that the 2D monolayer system does not adequately reproduce the full range of osteogenic maturity. Some have even suggested that most (if not all) mineralization seen in 2D culture is artefact (See for example the work of Tim Arnetts group). This has already led to investigations into 3D culture of osteogenic cells (Kale *et al*., 2000; Ferrara *et al*., 2002, text references). More recently it has been suggested by Cancedda that a transition to 3D cultures should be more robustly investigated (Tortelli and Cancedda, 2009). Taken together this would indicate that in some incidences 2D culture on tissue culture plastic may not be the optimal model and other systems should be investigated. The different mRNA expression profiles obtained in this study between high density monolayer and pellet culture also suggests there are some fundamental differences between the two culture models. We suggest that the high density monolayer has a polarized cell layer similar to that found in osteoid, whereas the 3D pellet is a later, less active stage that would be more associated with an embedded cell.

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