has the ability to bind to collagen and promote hydroxyapatite formation *in vitro* (Termine et al. 1981). Bone sialoprotein (Franzen & Heinegard 1985) is characterized by its ability to mediate initial formation of hydoxyapatite crystals (Oldberg et al. 1988) and is transiently expressed very early (Malavel et al. 1999, Aubin 2000, Sodek & Cheifitz 2000) and then upregulated again in differentiated osteoblasts actively involved in mineralization (Chen et al. 1992, Malavel et al. 1999, Aubin 2000, Sodek & Cheifitz 2000). These bone-matrix proteins have proven to be particularly useful osteogenic markers (Sodek & Cheifitz 2000). Consequently, because there is no specific single marker for osteoblasts, relevance has to be placed upon the cellular expression of a range of non-collagenous and collagenous bone-related proteins as well as alkaline phosphatase, when examining cellular differentiation.

These studies investigate the effect of a series of novel rapidly resorbable calcium phosphates, glass ceramics and a calcium phosphate bone cement as compared to α-TCP on the expression of bone-related genes and proteins by human bone-derived cells (HBDC). Thereby the effect of these novel bone substitute materials on osteoblastic cell differentiation is evaluated.

I/2 Materials and Methods

I/2.1 Test Materials

I/2.1.1 Test Materials Study A

In study A, four calcium phosphate materials which were created from β-Rhenanite ($CaNaPO_4$) and its derivatives were tested and compared to α-TCP ($Ca_3(PO_4)_2$): β-Rhenanite ($CaNaPO_4$) was denominated R1. Other materials resulted from modification of $CaNaPO_4$ using magnesium or potassium phosphate or silicate.

These materials were made from $CaNaPO_4$ and 20mol% Mg_2SiO_4 (material denominated R1/M2), $CaNaPO_4$ and 9% SiO_2 (wt%) (material denominated R1+SiO_2), or $CaKPO_4$ and $CaNaPO_4$ which react to form $Ca_2KNa(PO_4)_2$ (material denominated R17) (Schneider et al. 1994, Berger et al. 1995a, 1995b). Compounds were melted from carbonates and phosphates (Schneider et al. 1994, Berger et al. 1995a, 1995b). All specimens were prepared by compressing powder (powder size 40 µm) followed by sintering to form 10-mm diameter discs. The surface roughness of the specimens was characterized by profilometry using a Hommel T 8000 Surface Profile Measuring System with a diamond stylus (Hommel Inc., Germany). Parameters used to quantify surface roughness were: R_a (the arithmetic mean of departures of the roughness profile from the mean line) and R_z (the average of five consecutive values of roughness height which is defined as the distance between the top of the highest peak and the bottom of the deepest valley). Table 2 lists the results of the surface roughness assessment. The ceramic specimens were sterilized at 300°C for 3 h. Phase transformations do not occur below 600°C. Tissue culture polystyrene served as an internal control (Co).

Table 2 Surface roughness of the different bioceramics examined in study A

Biomaterial	**Surface roughness**	
	R_z (mean) [µm]	R_a (mean) [µm]
TCP	15.43	2.22
R1	13.12	2.13
R1/M2	13.37	2.21
R1+SiO_2	13.24	2.18
R17	13.86	2.23

I/2.1.2 Test Materials Study B

In study B, three calcium phosphates were tested and compared to α-TCP: two rapidly resorbable glassy crystalline materials resulting from modifying $Ca_2KNa(PO_4)_2$ by using either magnesium potassium phosphate or silica phosphate. These materials are based on calcium orthophosphates and have been developed by Berger et al. (1995b) to crystallize spontaneously and directly form the melt. The main crystalline phase consists of the newly synthezised chemical $Ca_2KNa(PO_4)_2$. These materials have a higher solubility than tricalcium phosphate. The dissolution rate is controlable by the amount of added ions, such as Na, K, or Mg. The materials examined in study B were two sintered calcium alkali orthophosphates with the main crystalline phase $Ca_2KNa(PO_4)_2$ and a small amorphous portion containing either magnesium potassium phosphate (material denominated GB14) or silica phosphate (material denominated GB9) (Berger et al. 1995a, 1995b). The compositions (wt%) of these ceramics are listed in Table 3. Both compositions are meltable and crystallize spontaneously from the melt. Thus, they can easily be fabricated. GB9 samples have previously been referred to as GB9/1 (Berger et al. 1995b). The dissolution rate was measured *in vitro* (0.2M Tris-HCL) and yielded rates of 460 ± 40 and 430 ± 40 mg/l for GB9 and GB14, respectively, compared to 30.6 ± 10 mg/l for TCP discs (Berger et al. 1995a). α-TCP, GB14 and GB9 specimens were prepared by compressing powder (powder size 40 µm) followed by sintering to form 10-mm diameter discs.

The third material investigated was a calcium phosphate cement (material denominated Biocement D). These cements are blends of amorphous and crystalline calcium phosphate compounds and are set to produce either calcium phosphate dihydrate, calcium deficient hydroxyapatite (CDHA), or amorphous calcium phosphate. Depending on the type of calcium phosphate formed during setting,

different groups of calcium phosphate cements are known. Biocement D belongs to the group of cements which form calcium deficient hydroxyapatite (Khairoun et al. 1997). Biocement D (Bioc) is made by mixing a powder (powder size 2-5 µm) consisting of 58% α-TCP, 25% $CaHPO_4$, 8.5% precipitated hydroxyapatite and 8.5% calciumcarbonate (wt%) with an aqueous solution containing 4% Na_2HPO_4 (0.32 ml fluid per g powder). After setting Biocement D (Bioc) is composed of carbonated apatite, $CaHPO_4$ and 3% $CaCO_3$ (Khairoun et al. 1999). Discs (10 mm in diameter) were made from Biocement D. The surface roughness of the specimens was determined by profilometry using a Hommel T 8000 Surface Profile Measuring System with a diamond stylus (Hommel Inc., Germany). Table 4 lists the results of the surface roughness measurements. Due to the difference in powder size and fabrication process the TCP, GB9 and GB14 specimens exhibited a significantly higher surface roughness than the Biocement D specimens (Table 4). Tissue culture polystyrene served as an internal control (Co). The ceramic specimens were sterilized at 300°C for 3 h. Phase transformations do not occur below 600°C.

Table 3 Compositions of calcium alkali orthophosphates examined in study B

Material Code	**Composition (wt%)**						
	CaO	P_2O_5	Na_2O	K_2O	MgO	SiO_2	TCP
α-TCP $Ca_3(PO_4)_2$							100
GB9 $Ca_2KNa(PO_4)_2$ + Mg_2SiO_4	32.25	40.81	8.91	13.54	2.57	1.92	
GB14 $Ca_{1,8}KMg_{0,4}Na(PO_4)_2$	30.67	43.14	9.42	14.32	2.45		

Table 4 Surface roughness of the different bioceramics examined in study B

Biomaterial	**Surface roughness**	
	R_z (mean) [µm]	R_a (mean) [µm]
TCP	15.43	2.22
GB9	13.03	2.17
GB14	17.89	2.90
Bioc	8.28	1.33

I/2.1.3 Test Materials Study C

In study C, three glass ceramics were examined and compared to α-TCP: First, a surface-treated rapidly resorbable sintered material with crystalline phase $Ca_2KNa(PO_4)_2$ (Schneider et al. 1994, Berger et al. 1995a, 1995b), and a small amount of silica phosphate (material denominated GB9N). The surface treatment partially removed the rapidly soluble alkali phosphates from the surface of the material thus leading to a layer of decreased reactivity. This way, the surface treatment chemically modified the material surface and influenced the release kinetics and pH level during degradation as described previously (Reif et al. 1998). In brief, this treatment resulted in a change of pH of the biological fluid after immersion of this material from a more alkaline to a more neutral pH (Reif et al. 1998, Ignatius et al. 2001). Second, a rapidly resorbable glass ceramic (material denominated Mg5) (Reif et al. 1998) and, third, a bioactive glass ceramic based on crystalline phases of apatite and wollastonite (material denominated AP40) (Knabe et al. 1998). The compositions (wt%) of the ceramics examined are listed in Table 5. TCP, GB9N and Mg5 specimens were prepared by compressing powder (powder size 40 µm) followed by sintering to form 10-mm diameter discs. AP40 specimens were prepared

by sectioning discs from cast cylinders. The cut surface was subsequently temperature treated. The surface roughness of the specimens was measured by profilometry as described above. Table 6 lists the surface roughness data for the bioceramics examined in study C. Tissue culture polystyrene (Primaria®, Becton Dickinson, Franklin Lakes, NJ, USA) served as an internal control (Co). The ceramic specimens were sterilized at 300°C for 3 h. Phase transformations do not occur below 600°C.

Table 5 Compositions of bioactive ceramics examined

Material Code	**Composition (wt%)**							
	CaO	P_2O_5	Na_2O	K_2O	MgO	SiO_2	TCP	CaF_2
α-TCP $Ca_3(PO_4)_2$							100	
AP40	16-19		3-5	0.1-0.3		42-45	23-25	3.0-5.5
GB9N	35.9	43.2	7.5	10.7	1.1	1.1		
Mg5	20.6	58.5	14.4		4.1			2.4

Table 6 Surface roughness of the different bioceramics examined in study C

Biomaterial	**Surface roughness**	
	R_z (mean) [μm]	R_a (mean) [μm]
TCP	38.4	2.22
GB9N	37.1	5.78
Mg5	32.3	5.03
AP40	4.02	0.43

I/2.2 Cell isolation and cultures

Human bone-derived cells (HBDC) were grown from small pieces of vertebral bone harvested from healthy female patients under 15 years of age using a modified enzymatic digestion technique (Gehron-Robey 1989, Bellows et al. 1996, Zreiqat et al. 1996, Zreiqat & Howlett 1999). The selected patients did not suffer from any conditions known to affect the healing or metabolism of bone. As-received bone was trimmed of fat and soft tissue and then cut into about 1 mm^3 pieces, washed several times in 0.02% (w/v) trypsin (Sigma, USA) in calcium-free and magnesium-free phosphate buffered saline (PBS) (Appendix B1) and then allowed to digest in this solution for 90 min. Digested bone chips were washed several times in α-Minimal Essential Medium (α-MEM, Gibco Laboratories, USA) and then placed in 25 cm^2 tissue culture flasks (Nunc, # 3013, Denmark) containing α-Minimal Essential Medium (α-MEM, Gibco Laboratories, USA), 10% (v/v) fetal calf serum (FCS, Gibco Laboratories, USA), 2mM L-glutamine (Gibco Laboratories, USA), 25mM Hepes Buffer (Gibco Laboratories, USA), 30 μg/ml penicillin, 100 μg/ml streptomycin (Gibco Laboratories, USA) and 0.1M L-ascorbic acid phosphate magnesium salt (Wako Pure Chemicals, Osaka, Japan) in which the phosphate compound is more stable than the natural form (Hata & Seno 1989) (Appendix B7). HBDC obtained in that manner were osteoblast-like cells grown from explanted bone chips. HBDC were cultured in α-Minimal Essential Medium (α-MEM, Gibco Laboratories, USA), 10% (v/v) fetal calf serum (FCS, Gibco Laboratories), 2mM L-glutamine (Gibco Laboratories), 25mM Hepes Buffer (Gibco Laboratories), 30 μg/ml penicillin, 100 μg/ml streptomycin (Gibco Laboratories) and 0.1M L-ascorbic acid phosphate magnesium salt (Wako Pure Chemicals, Osaka, Japan) (Appendix A1, Appendix B7). Permission to use discarded human tissue was granted by the Human Ethics Committee of the University of New South Wales (Ethic clearance CEPIHS 97043).

All cultures were incubated at 37°C without CO_2 and the medium was changed three times per week. HBDC were harvested at confluency and passaged using 0.1% (w/v) trypsin (Sigma Bio-Scientific, T-8253, USA) with 0.2mM EDTA (Boehringer-Mannheim, # 200-0081, Germany) in calcium-free and magnesium free PBS. To harvest cultures, spent culture medium was removed, and flasks rinsed with a small quantitity of harvesting solution. Approximately 2 ml of fresh harvesting solution was added to each 25 cm^2 culture flask (Nunc # 3013, Denmark), and flasks incubated at 37°C for 2 minutes when most of the cells could be freed from the culture surface by gentle tapping of the flask. The cell suspension was collected in a test-tube and immediately centrifuged at 3,000 rpm for 3 minutes at room temperature. The resulting pellet of cells was resuspended and washed in serum free medium. For sub-culturing, the cell suspension was centrifuged again as before, and the cell pellet resuspended in 1 ml of culture medium and seeded into a fresh 75 cm^2 flask (Falcon, # 3024, USA) (cells harvested from one 25 cm^2 flask were transferred to one 75 cm^2 flask). For use in experimental assays the cell suspension was centrifuged again as before, and the cell pellet resuspended in 1 ml of serum-free culture medium. For experimental assays 50 µl of this cell suspension were diluted 1/10 in 0.5% (w/v) trypan blue (50 µl of cell suspension and 450 µl of trypan blue) and counted using a hemacytometer. For cell seeding the cell concentration was then adjusted to 5 $x10^4$ cells per ml. Test materials with a diameter of 10 mm were placed into 48-well tissue culture polystyrene plates (Nunc, Denmark # 150687) and preincubated in 500 µl of culture medium for 24 hours without cells, and the 24-h wash was then discarded. HBDC were seeded in the culture medium described above at a density of $2.83x10^4$ $cells/cm^2$ on the different substrates. On every occasion 6 wells were seeded for each substratum at each time point. Subsequently, plates were incubated at 37°C for the predetermined experimental time-period.

The phenotype and function of HBDC cultured in this manner were characterized by their expression of a number of parameters, including intracellular alkaline phosphatase (ALP), increased production of cAMP (cyclic adenosine monophosphate) in response to parathyroid hormone, synthesis of type I collagen, and deposition of calcium phosphate aggregates when the medium contained 4 mM β-glycerophosphate, and mineral deposition in the presence of an organic source of calcium phosphate (Zreiqat et al. 1996, Zreiqat 1997). The formation of mineralized extracellular matrix is an essential phenotypic marker of any bone-like culture system (Bellows et al. 1996) and cells isolated from human bone chips in the described manner have this functional characteristic of osteoblasts (Zreiqat 1997). HBDC cultured in this manner with L-ascorbic acid phosphate magnesium salt and serum containing medium form calcifying nodules and thus produce viable mineralized tissue-like extracellular matrix when maintained under defined conditions as described above (Zreiqat 1997). Addition of L-ascorbic acid phosphate magnesium salt and serum to the culture medium are a prerequisite for matrix mineralization in the present culture system. X-ray diffraction analysis of this matrix revealed a crystalline hydroxyapatite, with a correct composition of Ca/P ratio of 1.6. This ratio is consistent with the Ca/P ratio of hydroxyapatite crystals *in vivo* (Junqueira et al. 1989) and the control bone run in parallel to the calcifying nodule (Zreiqat 1997). Not only did this mineralized matrix exhibit Ca and P peaks, but also a strong sulphur signal (Zreiqat 1997) suggesting the presence of sulphated glycosaminoglycans in the osseous matrix (Davies et al. 1991).

I/2.3 Cellular quantitative *in situ* hybridization assay (QISH)

The non-isotopic biotin labeling quantitative *in situ* hybridization assay enabling hybridization to be carried out directly on cells centrifuged in 96-well microtiter plates was originally adapted from a methodology developed by Markovic et al. (1994).

All test materials were preincubated in 500 µl of culture medium for 24 hours without cells, and the 24-h wash was then discarded. HBDC were seeded in the culture medium described above at a density of 2.83×10^4 cells/cm^2 on the different substrates and then cultured for 3, (5 in study A and C), 7, 14 and 21 days. At these predetermined time points, the medium was removed from each well and cells were washed twice with warm α-MEM (Gibco Laboratories, USA) to remove any serum. The final α-MEM wash was aspirated and HBDC were harvested from the surface of the substratum by adding 1 ml of 1% (w/v) trypsin (Sigma Bio-Scientific, USA) with 0.2 mM EDTA (Boehringer-Mannheim, Germany) in PBS to each well and incubating at 37°C for 2 min to allow attached cells to detach from the culture surface. Disks were agitated to yield a free cellular suspension, in this way 99% of cells from the different biomaterial substrata were isolated. Harvested cells were washed once in full culture medium by centrifugation for 3 minutes at 4,000 rpm and resuspended in warm PBS. This was followed by two additional washes in PBS. Cells were again resuspended in PBS and cellular viability was determined using the trypan blue exclusion method as described above. Cellular viability was found to be not less than 98%, and cells were counted with a hemocytometer. Subsequently the cellular concentration was adjusted to 1×10^5 cells per ml PBS and 100 µl of this suspension was pipetted into each well of tissue culture polystyrene 96-well microtiter plates (Costar # 3596, USA). Plates were centrifuged at 1,200 rpm for 10 minutes. The supernatant form each well was vacuum aspirated using a fine needle, and cells were dried to the plates for 30 min in a fan forced incubator at 37°C (Appendix A6.1). Plates thus dried were wrapped in plastic film and stored at room temperature ready to be used for quantitative *in situ* hybridization (QISH) and/or immunocytochemistry detection. Previous research has shown that the mRNA was preserved for several

months without any major decreases in absorbance values obtained using the QISH assay (Markovic et al. 1994, Zreiqat et al. 1996).

Fixation and Pretreatment

Cells dried to the plates were fixed by immersing the entire plates in 10% (v/v) formalin (analytical grade, Ajax Chemicals, Australia) buffered in PBS. Cellular permeabilization (to allow probe penetration) was achieved by immersing these plates in two changes of PBS containing 0.25% (v/v) Triton X-100 (ICN, USA) and 0.25% (v/v) Nonidet P40 (Sigma, USA). Following additional washings (Appendix A6.2), the cells were dried in an incubator at 42°C. Visual assessment of plates prior to, after fixation, and on completion of the detection protocol showed minimal loss of cells from wells at each of these time points.

cDNA Probes and Labeling

cDNA (complimentary deoxyribonucleic acid) probes for type I collagen α 2 (col Iα2), ALP, osteocalcin (OC), osteopontin (OP) and osteonectin (ON) were used as whole plasmids (Dracapoli et al. 1991, Villarreal et al. 1989, Kiefer et al. 1989, Myers et al. 1981) (Appendix A3, Table A1) and were isolated using the Wizard™ Maxipreps DNA Purification System (Promega, USA) (Appendix A4). cDNA probes were checked for purity using restriction enzyme digests of the cDNA probes and were found to have no evidence of DNA contamination. Respective plasmid vectors were used as negative controls, while the detection of β-actin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA were used as an internal positive control (Erba et al. 1986). Each cDNA probe was adjusted to reach a final concentration of 1 μg/μl in sterile water and 20μg lots were labeled at one time using the Photobiotin labeling method (Bresatec, Australia) (Appendix A5).

All the cDNA probes were checked against the Genebank Database (Los Alamos, NM) to ascertain that sequences were as unique as possible to the target of

interest, using the Blast-Align program (Altschul et al. 1990) located at the internet site http://www.ncbi.nlm.nih.gov/BLAST/ and highest homology was found to be only 44%.

Hybridization Conditions

Fifty microliters of hybridization solution, composed of 5 µg heat dissociated photobiotin labeled cDNA probe per 1 ml hybridization buffer (Appendix B4), was added to each well. The plates were sealed and placed in a humidified chamber for 22h at 42°C to hybridize (Appendix A6).

Posthybridization and Detection

The plates were decanted of probes and hybridization buffer. Excess unbound probe and mismatched bound probe were removed by copious post-hybridization washings initially with 2X sodium citrate chloride (SSC) and sodium chloride solution (Appendix B5) in 50% (v/v) formamide for 15 min at 42°C; then 2X SSC-0.1% (w/V) sodium dedocyl sulphate (SDS) and 0.4XSSC-0.1SDS at 42°C, for 15 minutes each. The plates were then washed four times with 0.4XSSC-Milli-Q-water at room temperature to remove the SDS, and then placed in 2X PBS and then Tris buffered saline 1 (pH 7.6) (Appendix B5) for 5 minutes each. Plates were then decanted. The biotin on the hybridized probe was detected using an *in situ* hybridization kit (Dako, Denmark, # K0600) with 10-min incubation steps of streptavidin 1:100 dilution, biotinylated ALP 1:100 dilution, streptavidin 1:500 dilution and biotinylated ALP 1:100 dilution (Appendix A6.3). Plates were washed 4 times between each step in Tris buffered saline 1 (pH 7.6) (Appendix B5). After the last application of biotinylated ALP; plates were washed again four times in Tris buffered saline 1 (pH 7.6), then incubated in Tris buffered saline 2 (pH 9.5) (Appendix B5) for 5 minutes and then decanted. Quickly, 100 µl of p-nitrophenyl phosphate (p-NPP) substrate in 5 ml diethanolamine, preheated to 37°C, was added to each well. The plates were then

incubated in a humidified chamber in the dark at 37°C for 20 min. The colour developed by the colourimetric conversion of p-nitrophenyl phosphate (p-NPP) to p-nitrophenyl (p-NP) was yellow and the reaction was stopped by adding 100 µl of 0.5 M NaOH to each well (Appendix A6.3, Figure A3). The optical density of the yellow colour (p-NP) was read by a TIM-200 plate reader (InterMed, Denmark) at 405 nm. The results were normalized to the internal control β-actin or GAPDH mRNA.

The present technique offers many advantages over Northern or dot blot methods for quantification of mRNA (Markovic et al. 1994. Zreiqat et al. 1998). Previously, it was shown that the quantitative ISH technique had the same sensitivity as that of the RNase protection assay (Markovic et al. 1994). Furthermore, the quantitative aspects of this technique were investigated previously and a log-linear relationship between absorbance and mRNA abundance was found (Zreiqat et al. 1998). The ability to use a 96-well microtiter plate together with progress in new sensitive avidin-biotin methods (Pringle et al. 1987, Wilchek & Bayer 1988) allows the determination of mRNA for collagenous and non-collagenous bone-related genes directly from isolated human bone-derived cells cultured on biomaterials, with minimal cell manipulation (Markovic et al. 1994). Furthermore, the ISH technique is quantitative and requires substantially fewer cells than conventional Northern blot analyses. The latter is of importance when characterizing new biomaterial surfaces. Thus, within one experiment the same cells can be used to study gene expression and protein production, an important feature because it is paramount to ensure that an increase in mRNA is translated to proteins.

I/2.4 Cellular quantitative immunocytochemistry assay

An indirect immunocytochemical method was used utilising rabbit anti-mouse antibodies labeled with biotin, enabling detection with avidin-alkaline phosphatase

and visualization using the detection method described above for the QISH assay. The expressed bone-related intracellular proteins were detected using monoclonal antibodies for alkaline phosphatase (ALP) (Sigma, USA), type I collagen (Col I) (Amersham, Australia), β-actin (Sigma, USA), as well as polyclonal antibodies for osteopontin (OP) (LF-123), osteonectin (ON) (BON-I), osteocalcin (OC) (LF-32) and bone sialoprotein (BSP) (LF-83). The polyclonal antibodies used in these studies were generously provided by Dr. Larry Fisher (NIDCR; Bethesda, Maryland). The specificity of all the primary antibodies used in these studies was checked by the providers of these antibodies.

All test biomaterials were preincubated in 500 µl of culture medium for 24 hours without cells, as descibed above. HBDC were seeded at a density of 2.83×10^4 cells/cm^2 on the different substrates and then cultured for 3, (5 in study A and C), 7, 14 and 21 days. At these predetermined time points, cells were harvested and centrifuged onto the 96-well-plates as described above and then fixed for 1 min with methanol:acetone (9:1) at room temperature. Subsequently cells were air-dried and then washed in two changes of PBS. This was followed by incubation in 0.25% Triton 100-X (ICN, USA), 0.25% Nonidet NP40 (Sigma, USA) in PBS, for 5 minutes to permeabilize the cell membrane. This solution was decanted and cells were washed in two changes of 0.05% (v/v) Triton 100-X in PBS. In order to reduce non-specific binding 50 µl of the blocking solution consisting of 2% (w/v) bovine serum albumin (BSA), heat inactivated (Sigma Aldrich, USA) in Hanks' balanced salt solution (HBSS) (Sigma Aldrich, USA, # H 9269) was added to each well, and plates were incubated for 20 min at room temperature in a humidified chamber. The blocking solution was then decanted and the wells drained, before the monoclonal (mABs) or polyclonal antibodies (pAbs) listed above were added (Appendix 7.1, Table A2). mAbs were used at a 1/100 dilution, and pAbs at a 1/200 dilution. Primary

antibodies were diluted in HBSS containing 1% (w/v) BSA, added to the cells and incubated at room temperature for 30 min in a humidified chamber, 1% (w/v) BSA in HBSS only was used as negative control. After the primary incubation, plates were washed in four changes of 0.05% (v/v) Triton 100-X in PBS: mAbs were detected with a biotin labeled $F(ab)_2$ fragment of rabbit anti-mouse antiserum (Dako, Denmark, # E0413), while the pAbs were detected using a Multi-link antibody which is labeled with biotin (Dako, Denmark, # E0453). The secondary antibodies (Appendix A7.1, Table A3), were diluted 1:200 with HBSS-1%BSA and 50 µl applied to each well. The plates were again placed in a humidified chamber and incubated at room temperature for 30 min. After the secondary incubation, plates were again washed in four changes of 0.05% (v/v) Triton 100-X in PBS and then placed in two changes of Tris buffered saline 1 (pH 7.6) (Appendix B5) for 5 minutes each. The biotin of the secondary antibody was detected using the *in situ* hybridization kit (Dako, Denmark, # K0600) described above with 10-min incubation steps of 100 µl of streptavidin 1:100 dilution and 100 µl of biotinylated ALP 1:100 dilution (Appendix A7.1). Plates were washed 4 times between each application in Tris buffered saline 1 (pH 7.6) (Appendix B5). After the application of the biotinylated ALP, plates were rinsed once in Tris buffered saline 1 (pH 7.6), and then incubated in two changes of Tris buffered saline 2 (pH 9.5) (Appendix B5) for 5 minutes and then decanted. The alkaline phosphatase of the detection process was vizualized using the p-NPP substrate described above for the *in situ* hybridization detection procedure. In addition, an amount of 5mM of levamizole was added to inhibit endogenous ALP activity. The measurement and quantitation was performed as described above for the *in situ* hybridization detection (I/2.3 and Appendix A6.3), and results were normalized to the internal control β-actin protein.

This methodology is very useful as it allows within on experiment the same cells to be used to study gene and protein expression. Thus ensuring that the mRNA is translated to proteins.

Previously, it was shown that there was a linear relationship for the majority of the probes and antibodies over the range of 10,000-60,000 cells/well for the detection of probes and 10,000-80,000 cells/well for the detection of the antibodies (Zreiqat 1997, Zreiqat et al. 1998). This clearly demonstrated that there was a direct linear relationship between the absorbance obtained from a probe or antibody and the concentration of cells using the QISH and or/or immunocytochemistry techniques. This linearity was maintained up to a point near confluence of cells of the microtiter well surface.

I/2.5 Statistical analysis

Three separate studies were performed and assays were run in triplicate for each material and each time point. First it was determined that all data showed a normal distribution. This was followed by analysis of the data using student’s t-test, and significance was considered achieved at $p<0.05$. Student's t-test was used to determine whether the average mean mRNA or protein expression generated on biomaterial A for a given osteogenic marker at a given time point was significantly different compared to the expression generated on biomaterial B.

I/3 Results

I/3.1 Results Study A

Cellular Proliferation

All substrates supported continuous cellular growth for 21 days (Fig. 2). At day 14, all test surfaces displayed higher cell numbers than the control (Fig. 2), and by