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

day 21 all novel calcium phosphates had as many or more cells than TCP; surfaces of R1+SiO_2 and R17 had the highest number of HBDC (Fig. 2).

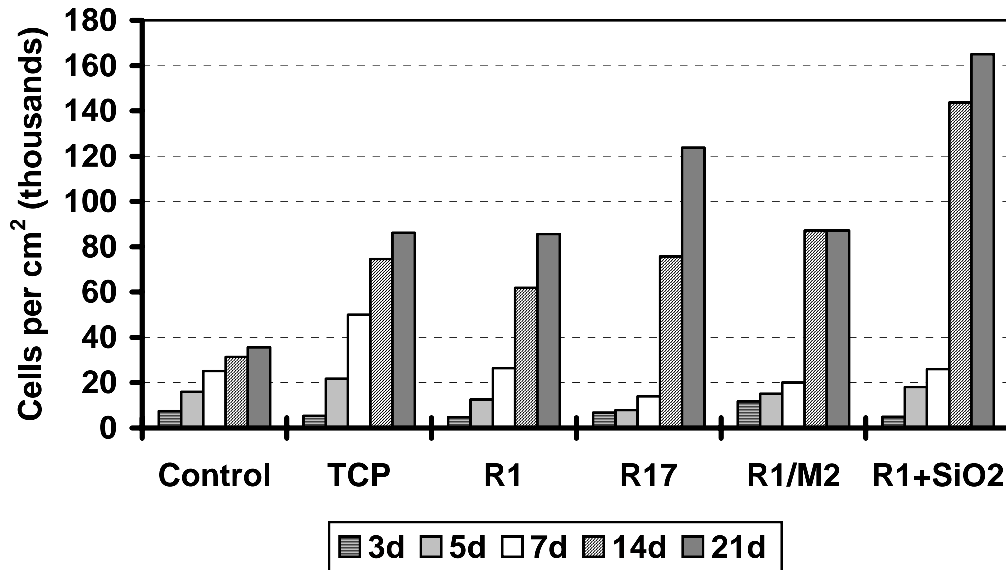

Figure 2. Number of HBDC cultured over 21 days on different bone substitute materials in study A.

Cellular Differentiation

At day 3, HBDC cultured on R1 expressed significantly higher mRNA levels for Col Iα2 compared to cells grown on Co and R17 ($p<0.002$) (Fig. 3(a)). Also for ALP and ON mRNA significantly higher levels were expressed by HBDC cultured on R1 compared to cells grown on Co, TCP, R17 and R1/M2 ($p<0.02$). Cells cultured on R1+SiO_2 had significantly higher levels of Col Iα2, ALP and ON mRNA compared to α-TCP, R17, R1/M2 and the control ($p<0.05$) (Fig. 3(a)). Protein production by HBDC for Col I, ALP, OP, OC, ON, and BSP was higher, when these cells were cultured on R1 compared to all other surfaces tested ($p<0.05$) (Fig. 4(a)).

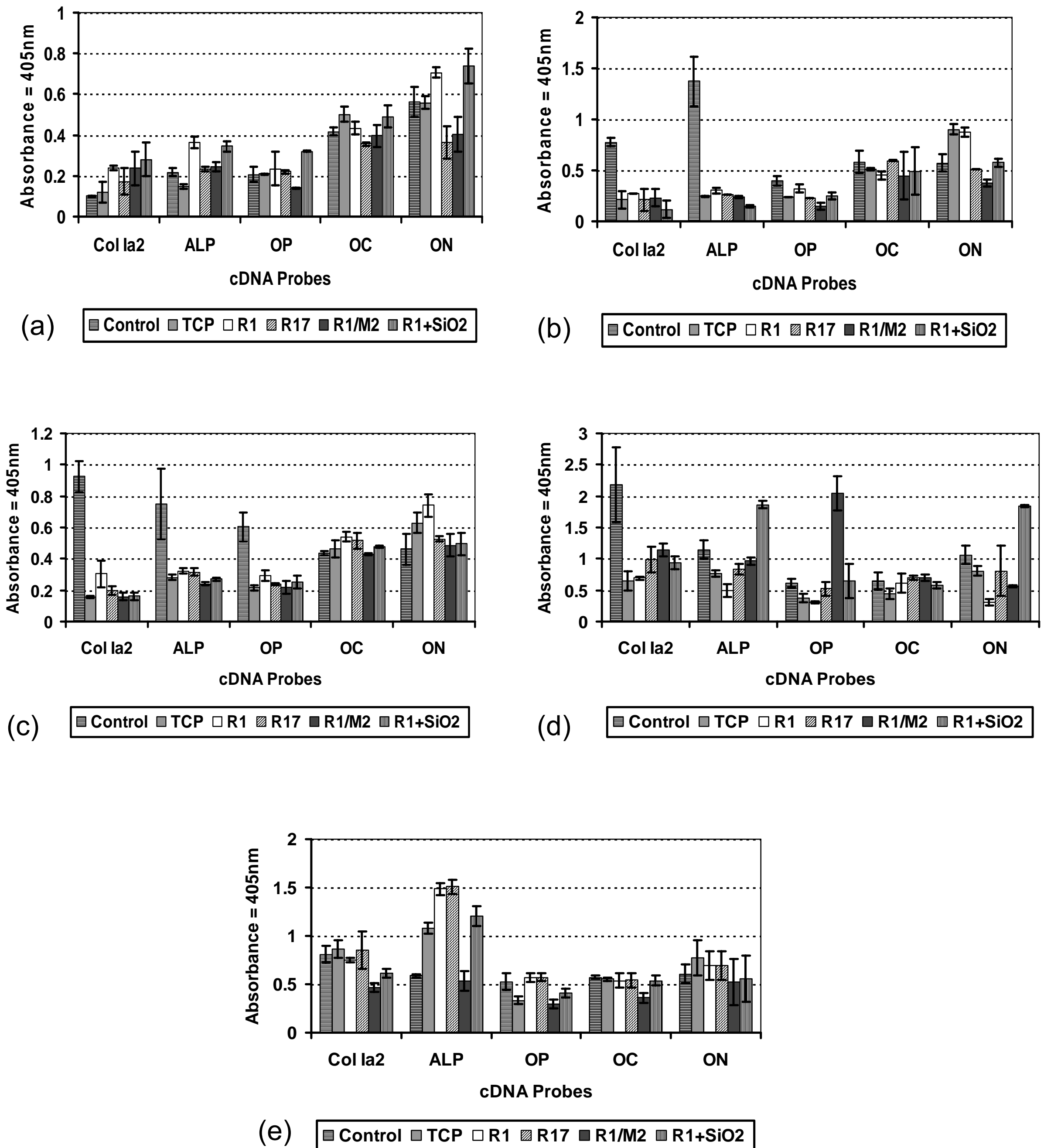

Figure 3. The temporal expression of osteogenic mRNA by HBDC cultured on different calcium phosphate ceramics for 3 weeks in study A. (a) Day 3, (b) day 5, (c) day 7, (d) day 14, (e) day 21. Cellular mRNA expression by HBDC is at (a) 3, (b) 5, (c) 7, (d) 14, and (e) 21 days of culture on the polystyrene control, TCP, R1, R17, R1/M2 and R1+SiO_2. Results are normalized to the internal control β-actin mRNA for each time point and each substratum. Three runs of experiments were performed in which there were three replicates. All values are mean ± standard deviation. Col Iα2, procollagen Iα2; ALP, alkaline phosphatase; OP, osteopontin; OC, osteocalcin; and ON, osteonectin.

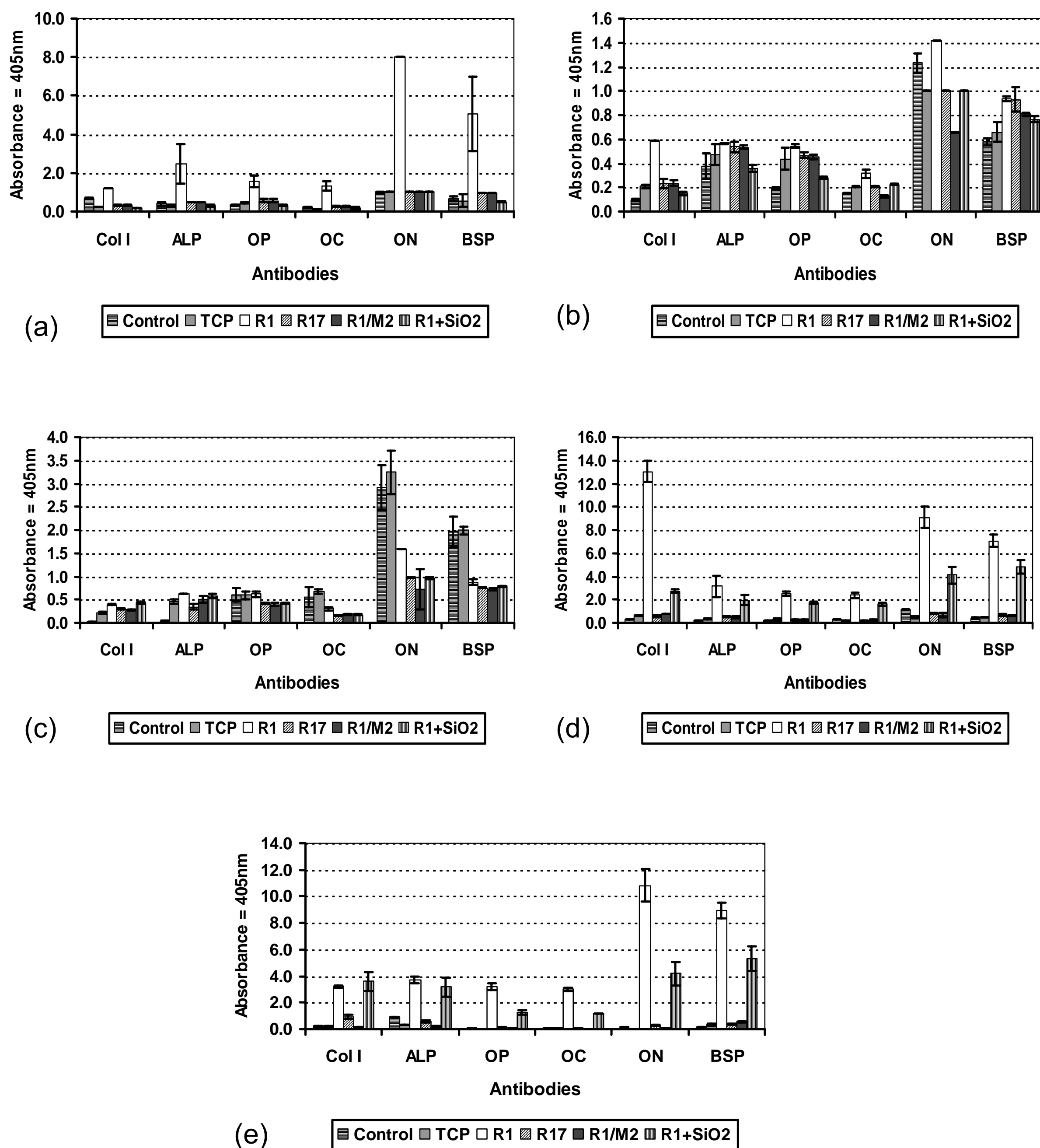

Figure 4. The temporal expression of bone-related proteins by HBDC cultured on different biomaterials for 3 weeks in study A. (a) Day 3, (b) day 5, (c) day 7, (d) day 14, (e) day 21. Intracellular protein expression by HBDC is at (a) 3, (b) 5, (c) 7, (d) 14, and (e) 21 days of culture on the polystyrene control, TCP, R1, R17, R1/M2 and R1+SiO_2. Results are normalized to the internal control β-actin protein for each time point and each substratum. Three runs of experiments were performed in which there were three replicates. All values are mean ± standard deviation. Col I, type I collagen; ALP, alkaline phosphatase; OP, osteopontin; OC, osteocalcin; ON, osteonectin; and BSP, bone sialoprotein.

At day 5, mRNA levels for Col Iα2, ALP, OP, OC and ON were similar for all ceramic substrata tested (Fig. 3(b)), whereas a different pattern was observed at the protein level: HBDC cultured on R1 expressed significantly higher protein levels for Col I, OP and ON compared to all other surfaces ($p<0.04$) (Fig. 4(b)). The same was true when comparing OC protein levels on R1 to these on Co, TCP, R17 and R1/M2 ($p<0.04$), and when comparing BSP protein levels on R1 to these on the Co, TCP, R1/M2 and R1+SiO_2 specimens ($p<0.04$) (Fig. 4(b)).

At day 7, cells grown on R1 expressed significantly higher mRNA levels for Col Iα2, ALP and OP compared to identical cells grown on TCP (Fig. 3(c)). Also mRNA levels for Col Iα2, ALP, OC and ON on R1 were significantly higher compared to cells on R1/M2 ($p<0.05$). Furthermore, HBDC on R1 had more Col Iα2, ALP and ON mRNA then cells on R1+SiO_2 ($p<0.05$) (Fig. 3(c)) and more OP and ON mRNA than cells on R17 ($p<0.04$). At the protein level, HBDC cultured on R1 expressed significantly higher protein levels for Col I and ALP compared to Co, TCP, R17 and R1/M2 ($p<0.04$) (Fig. 4(c)). The same was true when comparing OP protein expression on R1 to that on TCP, R17, R1/M2 and R1+SiO_2 ($p<0.004$). Moreover, cells cultured on R1+SiO_2 expressed significantly higher protein levels for Col I compared to the control, TCP, R17 and R1/M2 surfaces ($p<0.003$) (Fig. 4(c)). Protein production for ON and BSP, however, was highest on the control and TCP specimens ($p<0.02$) (Fig. 4(c)).

At day 14, HBDC grown on R1/M2 and R1+SiO_2 expressed significantly higher levels of Col Iα2, ALP and OP mRNAs than cells cultured on TCP ($p<0.04$) (Fig. 3(d)). The same was true when comparing ON mRNA levels on R1+SiO_2 to those on TCP and all other surfaces ($p<0.05$). Moreover, cells cultured on R17 and R1/M2 had more OC mRNA than identical cells grown on TCP ($p<0.02$) (Fig. 3(d)).

Col I, OP, OC, ON and BSP protein levels were significantly higher for R1 compared to all other surfaces ($p<0.008$) (Fig. 4(d)). The same was true when comparing ALP protein levels on R1 to these on Co, TCP, R1/M2 and R17 surfaces ($p<0.008$). Cell numbers, however, were lower on R1 compared to those on TCP, R17, R1/M2 and R1+SiO_2 (Fig. 2). At day 14, significantly higher protein levels for Col I, ALP, OP, OC, ON and BSP were expressed by cells grown on R1+SiO_2 compared to Co, TCP, R17, and R1/M2 specimens ($p<0.005$) (Fig. 4(d)).

At day 21, HBDC cultured on R1 and R17 expressed significantly higher levels of ALP, and OP mRNAs (Fig. 3(e)) than cells on TCP ($p<0.003$). On R1+SiO_2 surfaces, more ALP and OP mRNA was noted compared to TCP, however, this was not statistically significant. mRNA expression for Col Iα2, OC and ON was similar for all substrata tested (Fig. 3(e)). At the protein level the pattern observed at day 14 was also maintained at day 21. In detail, protein expression by HBDC cultured on R1 was significantly higher for OP, OC, ON and BSP than in cells on all other substrata ($p<0.01$) (Fig. 4(e)). Protein production for Col I and ALP was significantly higher in cells grown on R1 and R1+SiO_2 than in cells on the Co, TCP, R17 and R1/M2 specimens ($p<0.005$), and cells on R17 expressed more Col I and ALP protein than cells on TCP ($p<0.02$) (Fig. 4(e)). The same was true when comparing protein expression for OP, OC, ON and BSP by HBDC grown on R1+SiO_2 to that in cells cultured on Co, TCP, R1, R17 and R1/M2 surfaces ($p<0.05$) (Fig. 4(e)). Moreover, R1+SiO_2 had the highest cell numbers at the end of the incubation period (Fig. 2).

I/3.2 Results Study B

Cellular Proliferation

All substrates supported continuous cellular growth for 21 days (Fig. 5). By day 21, the number of cells on GB9 was higher than on TCP, GB14 and the control.

GB14 displayed higher cell numbers than the control. Biocement D had less cells than the control.

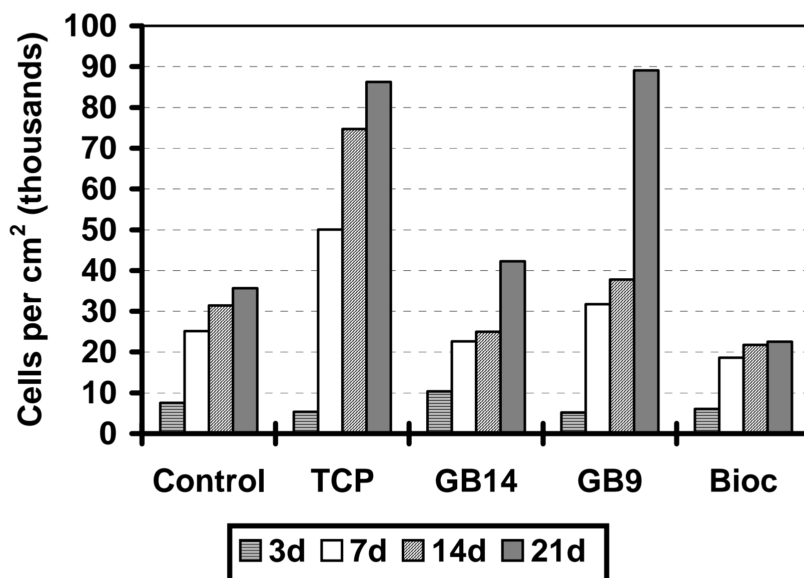

Figure 5. Number of HBDC cultured over 21 days on different bioceramics in study B.

Cellular Differentiation

At day 3, HBDC cultured on GB9 expressed significant higher mRNA levels for ColIα2, ALP, OP, OC and ON compared to the other surfaces ($p<0.05$) (Fig. 6(a)). Cells grown on the novel biomaterial GB14 had significantly higher levels of Col Iα2 and ALP mRNA compared to α-TCP and the control ($p<0.05$) (Fig. 6(a)). Protein production by HBDC for ALP, OC and BSP was higher, when these cells were cultured on GB14, Biocement D and GB9 than for the same cells grown on TCP (Fig. 7(a)). These differences were not statistically significant, however.

At day 7, cells grown on Bioc expressed significantly higher levels of all osteogenic mRNAs than HBDC grown on all other calcium phosphates ($p<0.002$)

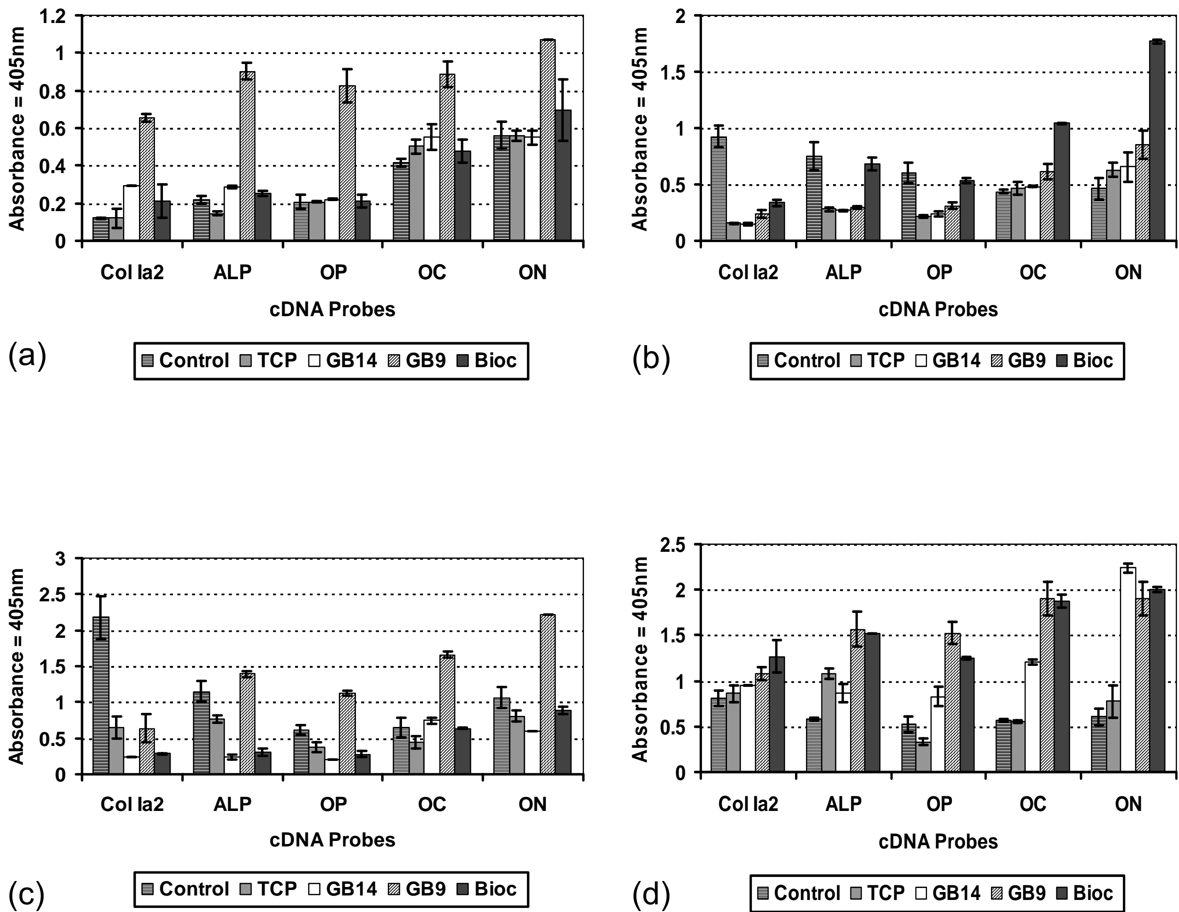

Figure 6. The temporal expression of osteogenic mRNA by HBDC cultured on different calcium phosphates for 3 weeks in study B. (a) Day 3, (b) day 7, (c) day 14, (d) day 21. Cellular mRNA expression by HBDC is at (a) 3, (b) 7, (c) 14, and (d) 21 days of culture on the polystyrene control, TCP, GB14, GB9 and Biocement D (Bioc). Results are normalized to the internal control β-actin mRNA for each time point and each substratum. Three runs of experiments were performed in which there were three replicates. All values are mean ± standard deviation. Col Ia2, procollagen Iα2; ALP, alkaline phosphatase; OP; osteopontin; OC, osteocalcin; and ON, osteonectin.

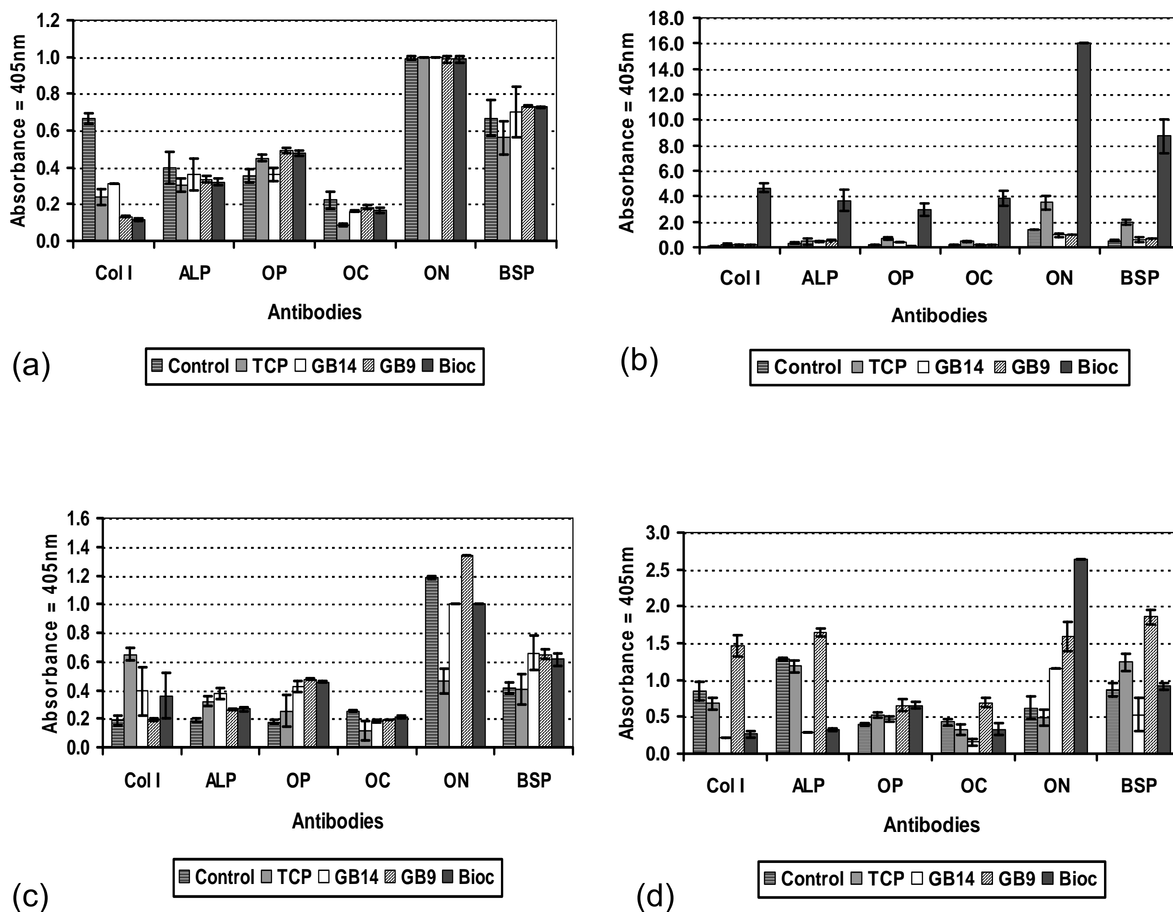

Figure 7. The temporal expression of bone-related proteins by HBDC cultured on different biomaterials for 3 weeks in study B. (a) Day 3, (b) day 7, (c) day 14, (d) day 21. Intracellular protein expression by HBDC is at (a) 3, (b) 7, (c) 14, and (d) 21 days of culture on the polystyrene control, TCP, GB14, GB9 and Biocement D (Bioc). Results are normalized to the internal control β-actin protein for each time point and each substratum. Three runs of experiments were performed in which there were three replicates. All values are mean ± standard deviation. Col I, type I collagen; ALP, alkaline phosphatase; OP, osteopontin; OC, osteocalcin; ON, osteonectin; and BSP, bone sialoprotein.

(Fig. 6(b)). Moreover, cells cultured on Bioc also expressed significantly higher levels of all bone-related proteins than HBDC grown on all other materials ($p<0.003$) (Fig. 7(b)). Cell growth, however, was the lowest on Bioc specimens (Fig. 5).

At day 14, HBDC grown on GB9 expressed significantly higher levels of ALP, OP, OC and ON mRNAs than cells cultured on all other surfaces ($p<0.003$) (Fig. 6(c)). Furthermore, protein production by HBDC for OP, OC, ON and BSP was higher when these cells were cultured on GB 14, Bioc and GB9 than for the same cells grown on TCP (Fig. 7(c)), while cell numbers were lower (Fig. 5). However, these differences in protein production were only statistically significant for ON ($p<0.004$).

At day 21, HBDC cultured on Bioc expressed significantly higher levels of the ALP, OC, OP and ON mRNAs (Fig. 6(d)) than cells on TCP ($p<0.003$), while cell numbers were lower (Fig. 5). On the protein level this tendency was only present for OP and ON ($p<0.04$). With GB14 specimens mRNA expression for OP, OC and ON ($p<0.005$) and protein formation for ON ($p<0.004$) by HBDC was higher than in cells cultured on TCP. GB9 had the highest cell numbers (Fig. 5) expressing significantly higher levels of ALP, OP and OC mRNA ($p<0.05$) than cells grown on the control, TCP and GB14 specimens (Fig. 6(d)). Protein formation in HBDC cultured on GB9 was significantly higher for Col I, ALP, OP, OC and BSP than in cells on the control and GB14 specimens ($p<0.05$) (Fig. 7(d)). The same was true when comparing protein expression for Col I, ALP, OC and BSP to that in cells cultured on Bioc specimens ($p<0.02$). Moreover, HBDC cultured on GB9 displayed higher protein levels of all bone-related proteins compared to those in cells grown on TCP specimens (Fig. 7(d)). These differences, however, were only statistically significant for ON and BSP ($p<0.003$).

I/3.3 Results Study C

Cellular Proliferation

All substrates supported continuous cellular growth for 21 days (Fig. 8). The number of cells on GB9N was higher than on TCP, AP40 and the tissue culture polystyrene surfaces (Co) at day 21. TCP and AP40 displayed higher cell numbers than Co (Fig. 8).

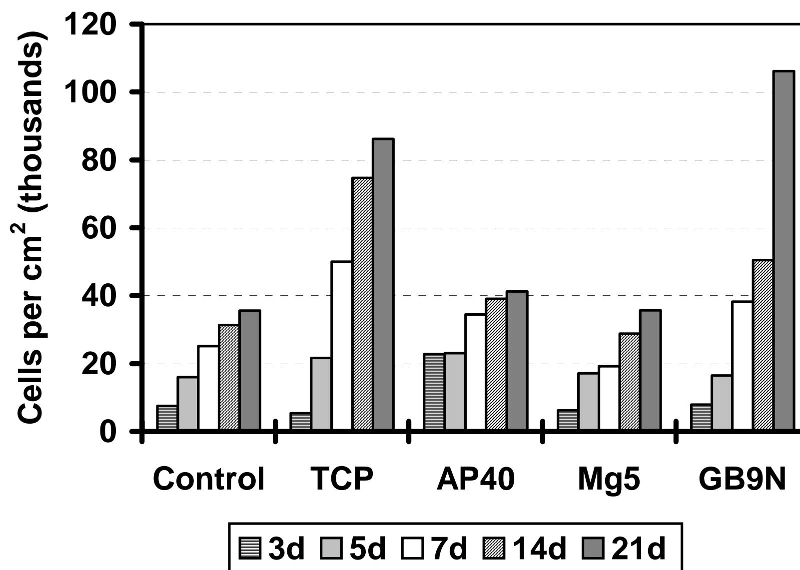

Figure 8. Number of HBDC cultured over 21 days on different bone substitute materials in study C.

Cellular Differentiation

At day 3, HBDC cultured on GB9N expressed significantly higher mRNA levels for Col Iα2, ALP, OP and OC compared to cells grown on Co, TCP, AP40 and Mg5 ($p<0.03$), and significantly more ON mRNA compared to HBDC grown on Co, TCP, and AP40 ($p<0.01$) (Fig. 9(a)). Cells cultured on the novel biomaterial Mg5 had significantly higher levels of ALP mRNA compared to α-TCP, AP40, and Co ($p<0.03$) (Fig. 9(a)). mRNA levels for Col Iα2, OC, OP and ON expressed by HBDC grown on

the glass ceramics Mg5 and AP40 were similar to those on TCP and Co. Protein production by HBDC for Col I and OP was higher, when these cells were cultured on GB9N than for the same cells grown on TCP ($p<0.05$) (Fig. 10(a)). The same was true when comparing protein levels for Col I on AP40 to those on TCP ($p<0.003$). Moreover, cells grown on Mg5 expressed significantly higher levels of Col I protein compared to HBDC cultured on TCP ($p<0.004$) and more Col I and OP protein than cells on Co ($p<0.004$) (Fig. 10(a)).

At day 5, cells grown on Mg5 expressed significantly more OC mRNA than cells cultured on all other substrata ($p<0.03$) and more ALP mRNA than cells on AP40 ($p<0.004$) (Fig. 9(b)). mRNA levels for Col Iα2, OP and ON were similar for all ceramic substrata tested. mRNA expression for Col Iα2, ALP and OP, however, was highest on the Co specimens ($p<0.02$) (Fig. 9(b)). At the protein level, HBDC cultured on GB9N expressed significantly more Col I protein compared to all other surfaces ($p<0.02$) (Fig. 10(b)). The same was true when comparing OC protein levels on Mg5 to these on Co, TCP and GB9N ($p<0.003$), and when comparing BSP protein levels on AP40, Mg5 and GB9N to these on the Co and TCP specimens ($p<0.008$) (Fig. 10(b)).

At day 7, cells grown on GB9N expressed significantly higher mRNA levels for Col Iα2, ALP, OP, OC and ON compared to identical cells grown on all other ceramic substrata ($p<0.004$) (Fig. 9(c)). Furthermore, HBDC on Mg5 had more Col Iα2 and OP mRNA than cells on TCP ($p<0.02$) and more Col Iα2, ALP, OP and ON mRNA than cells on AP40 ($p<0.005$) (Fig. 9(c)). At the protein level, HBDC cultured on GB9N expressed significantly higher protein levels for Col I, ALP, OP, OC and ON and than HBDC grown on all other materials ($p<0.004$) (Fig. 10(c)). The same was true when comparing BSP protein expression on GB9N to that on AP40, Mg5 and Co ($p<0.002$) (Fig. 10(c)).

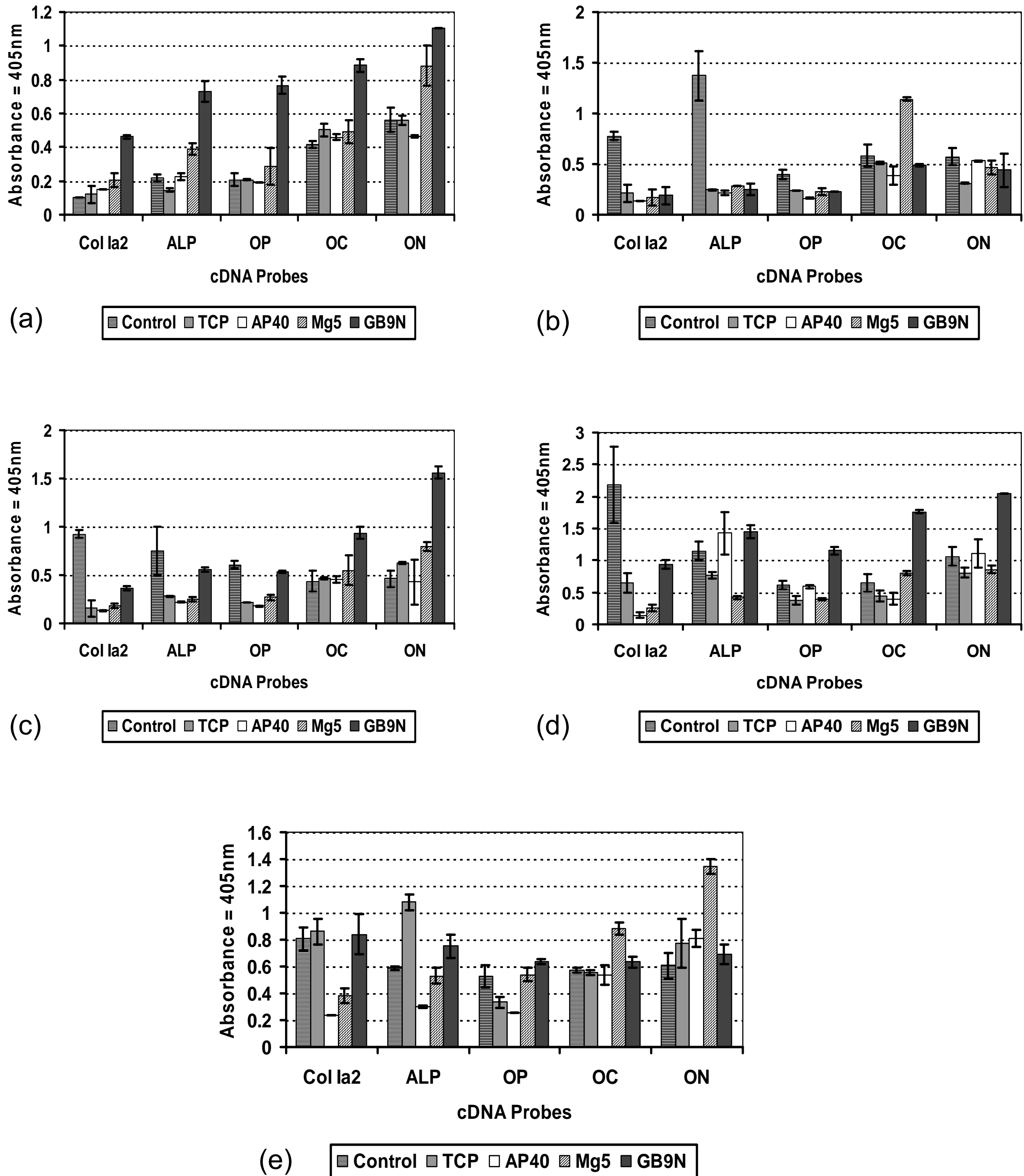

Figure 9. The temporal expression of osteogenic mRNA by HBDC cultured on different bioactive ceramics for 3 weeks in study C. (a) Day 3, (b) day 5, (c) day 7, (d) day 14, (e) day 21. Cellular mRNA expression by HBDC is at (a) 3, (b) 5, (c) 7, (d) 14, and (e) 21 days of culture on tissue culture polystyrene (Co) and TCP, AP40, Mg5 and GB9N specimens. Results are normalized to the internal control β-actin mRNA for each time point and each substratum. Three runs of experiments were performed in which there were three replicates. All values are mean ± standard deviation. Col Ia2, procollagen Iα2; ALP, alkaline phosphatase; OP; osteopontin, OC, osteocalcin; and ON, osteonectin.

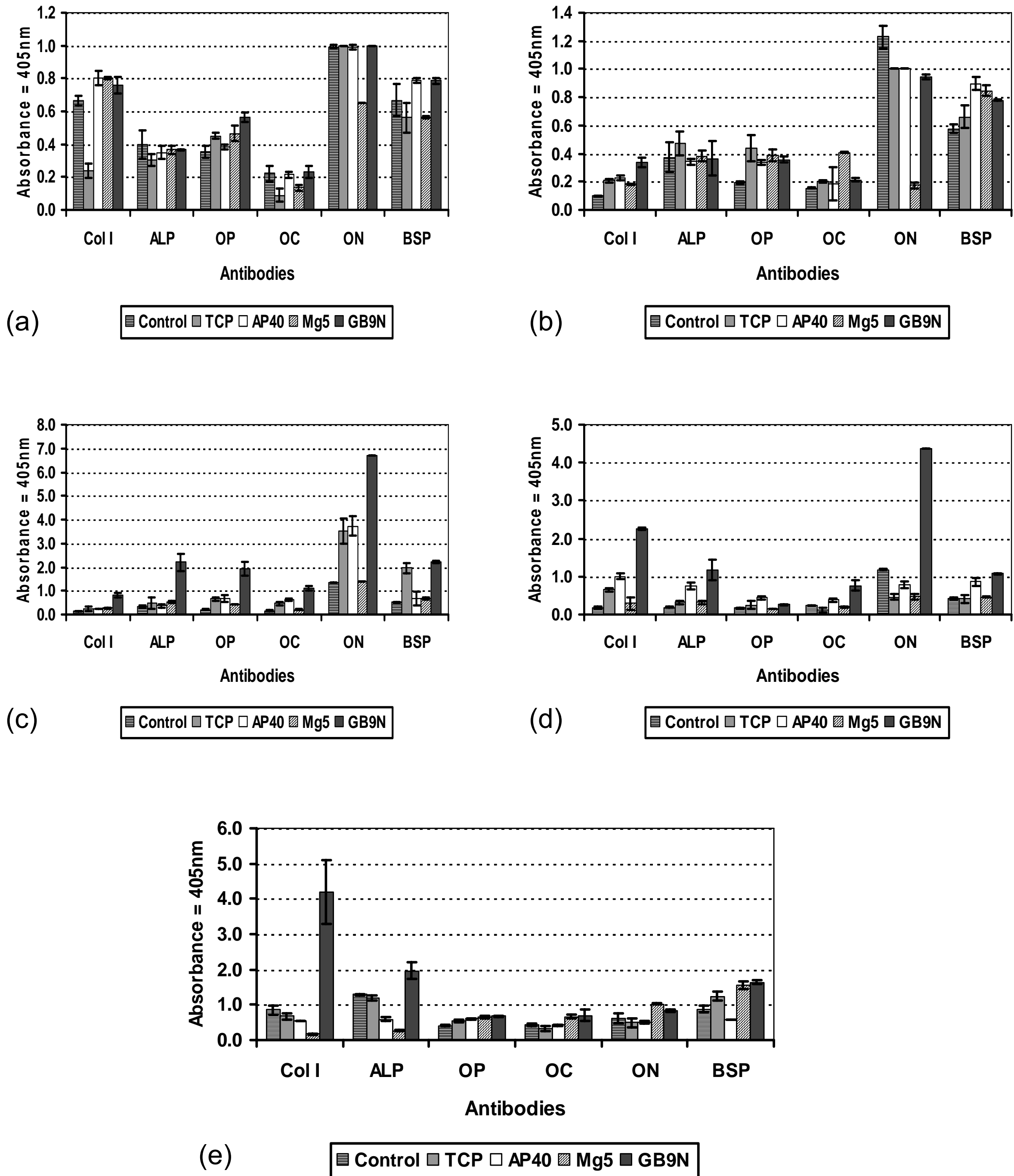

Figure 10. The temporal expression of bone-related proteins by HBDC cultured on different biomaterials for 3 weeks in study C. (a) Day 3, (b) day 5, (c) day 7, (d) day 14, (e) day 21. Intracellular protein expression by HBDC is at (a) 3, (b) 5, (c) 7, (d) 14, and (e) 21 days of culture on the tissue culture polystyrene (Co) and TCP, AP40, Mg5 and GB9N ceramic specimens. Results are normalized to the internal control β-actin protein for each time point and each substratum. Three runs of experiments were performed in which there were three replicates. All values are mean ± standard deviation. Col I, type I collagen; ALP, alkaline phosphatase; OP, osteopontin; OC, osteocalcin; ON, osteonectin; and BSP, bone sialoprotein.

At day 14, HBDC grown on GB9N expressed significantly higher levels of Col Iα2, OP, OC and ON mRNAs than cells cultured on all other ceramic substrata ($p<0.05$) (Fig. 9(d)) and more ALP than cells on Co, TCP and Mg5 ($p<0.03$). The same was true when comparing ALP and OP mRNA levels on AP40 to those on TCP ($p<0.03$). Moreover, cells cultured on Mg5 had more OC mRNA than identical cells grown on TCP ($p<0.005$) (Fig. 9(d)). Col Iα2 mRNA levels were significantly ($p<0.005$) reduced on AP40 and Mg5 compared to those expressed on TCP and Co (Fig. 9(d)). The same was true for ALP mRNA levels expressed on Mg5 ($p<0.0005$) (Fig. 9(d)). Protein production by HBDC for Col I, ALP, OC, ON and BSP was significantly higher when these cells were cultured on GB9N than for the same cells grown on all other surfaces ($p<0.002$) (Fig. 10(d)), while cell numbers were lower for GB9N than on TCP (Fig. 8). The same was true when comparing OP protein levels on GB9N to these on Co and Mg5 surfaces ($p<0.008$). Cells grown on AP40 expressed significantly higher protein levels for ALP, OP, OC and BSP at day 14 than HBDC cultured on the Co, TCP and Mg5 specimens ($p<0.005$). Furthermore, HBDC on AP40 produced more ON protein than cells on TCP and Mg5 ($p<0.002$) and more Col I protein than HBDC on Mg5 and Co surfaces ($p<0.02$) (Fig. 10(d)).

At day 21, HBDC cultured on GB9N expressed significantly higher levels of OP mRNA (Fig. 9(e)) compared to all other ceramics ($p<0.009$). The same was true when comparing OC mRNA levels on GB9N to these on the TCP and Co surfaces ($p<0.04$). Furthermore, cells on GB9N expressed more Col Iα2 and ALP mRNA compared to cells on AP40 and Mg5 ($p<0.007$) (Fig. 9(e)). HBDC cultured on Mg5 expressed significantly higher mRNA levels for OC ($p<0.02$) and ON ($p<0.05$) compared to all other surfaces tested. The same was true when comparing OP mRNA levels on Mg5 to those on TCP and AP40 ($p<0.003$) and Col Iα2 and ALP mRNA levels on Mg5 to these on AP40 ($p<0.009$) (Fig. 9(e)). At day 21, protein

formation in HBDC cultured on GB9N was significantly higher for Col I and ALP than in cells on all other substrata ($p<0.02$) (Fig. 10(e)). The same was true when comparing OC ($p<0.05$), BSP ($p<0.05$) and ON ($p<0.01$) protein production on GB9N and Mg5 to that on TCP, Co and AP40, and OP protein levels on GB9N to these on TCP and Co surfaces ($p<0.05$) (Fig. 10(e)). GB9N had the highest cell numbers at the end of the incubation period (Fig. 8).

I/4 Discussion

I/4.1 General Discussion Part I

Cell and tissue response to implant materials is one of the most important themes in the field of biomaterials (Jarcho 1981, Klein et al. 1983, LeGeros & Daculsi 1990, Gross et al. 1991, Kotani et al. 1991, Ducheyne & Cuckler 1992, Neo et al. 1992, Davies 1996, Hollinger et al. 1996, Yaszemski et al. 1996, Hench 1998, Ducheyne & Qui 1999, Oonishi et al. 1999). Implanted calcium phosphate ceramics (Jarcho 1981, Klein et al. 1983, LeGeros & Daculsi 1990, Ohgushi et al. 1990, Gross et al. 1991, Kotani et al. 1991, de Bruijn et al. 1994, Hollinger et al. 1996, Ducheyne 1998) and glass ceramics (Hench & Paschall 1973, Gross et al. 1991, Schepers et al. 1991, 1993, 1998, Schepers & Ducheyne 1997, Hench 1998, Ducheyne & Qui 1999) are known to bond directly to bone. However, differences in these materials are reflected in the rate of bone formation on their surfaces (Ohgushi et al. 1990, Gross et al. 1991, Neo et al. 1992, Schepers & Ducheyne 1997, Hench 1998, Ducheyne & Qui 1999). Ideally bioactive calcium phosphate ceramics for use in bone regeneration should possess the ability to activate bone formation (Hench & Paschall 1973, Ohgushi et al. 1990, Davies 1996, Hench 1998, Ducheyne & Qui 1999). This in turn requires the ability to differentiate stromal bone marrow cells into osteoblasts on