CHAPTER IV

RESULTS

4.1 Primary cells derived from human alveolar bone

Approximately 2 weeks after plating, cells started to migrate from bone samples, and reached confluency at 4 weeks. In general, it takes around 2 months from the time of explantation to culture these cells for all experiments. Cells at the 3rd or 4th passage were used in this study. We received bone samples from 15 individuals, but we could grow cells from 8 samples. After cultured in the inducing condition for 14 days, three of the primary human osteoblast cell lines, HOB1, HOB2, HOB3, that positive stained to alkaline phosphatase substrate were used in our experiments. HOB1 and HOB2 were collected from 21-year-old females, and HOB3 was collected from a 21-year-old male. These cells were spindle-shaped and grown in multilayer after 14 days culture. Cells from different patients grew at slightly different rates. HOB2 grew a little faster than the others. At day 21, we observed scatter foci on culture plates consistent with the mineralization nodules.

4.2 RT-PCR analysis

RT-PCR analysis of RNA extracts demonstrated that these primary cells from human alveolar bone expressed osteogenic markers, COLIA2, ALP, BSP2, OPN and OCN. COLIA2 was expressed at all time points and also expressed in cells grown in medium without AA and β-GP in all primary lines (figure 5). For HOB1 and HOB2, the relative steady state levels of COLIA2 mRNA remained throughout the 28-day culture period. HOB3 COLIA2 expressed at relatively higher levels at days 7 and 21 (figure 6).

For HOB1 and HOB2, ALP were slightly expressed at day 3, and increased at day 7 to the highest level at day 14. Its expression declined after day 14. However, the highest level of HOB2 was about 1.3-fold increase, while that of HOB1 was about 10-fold increase at day 14 (figure 8). Only HOB3 showed peak 6.7-fold increase of ALP expression in day 7 (figure 8). Cells grown in medium without AA and β-GP, also expressed ALP (figure 7). Semi-quantitative RT-PCR analysis of ALP gene expression of HOB1, HOB2 and HOB3 was presented in figure 8.

BSP2 mRNA expression of HOB1 was first detected at day 7 and gradually increased 12-fold at day 28 (figure 9 and 10). HOB2 expressed BSP2 at days 7 and 14. The expression declined at day 21, and then upregulated again at day 28. It was weakly expressed in condition without inducing agent (figure 9). Surprisingly, BSP2 expression could not be detected in HOB3. Semi-quantitative PCR analysis of BSP gene expression of HOB1 and HOB2 was presented in figure 10.

RT-PCR analysis did not show OPN expression until the third week of culture in all three primary lines. Its expression upregulated at day 28. OPN gene expression could not be detected in HOB1 and HOB2 grown in medium without AA and β-GP at day 28, however it could be detected in HOB3 (figure 11). Semi-quantitative RT-PCR analysis of OPN gene expression was presented in figure 12.

We detected OCN expression at day 3 in HOB1 and HOB2. The expression declined at day 7 to the lowest level at day 14, then substantially increased at day 28 (figure 13). However, for HOB3, the relative intensity of HOB3 demonstrated the different expression pattern. OCN expression of HOB3 was detected at day 3, up-regulated at day 7 and then down-regulated at day 14. Its expression increased again at day 21, then declined at day 28 (figure 14). HOB2 and HOB3 weakly expressed OCN in condition without AA and β-GP (figure 13). Semi-quantitative RT-PCR analysis of OPN gene of HOB1, HOB2 and HOB3 were presented in figure 14.

Semi-quantitative RT-PCR of bone marker gene expressions of HOB1 were demonstrated in figure 15. SaOS2, a positive control cell line expressed all of bone marker genes. PBMC, a negative control for bone marker preimers, did not express any of bone markers (data not shown).

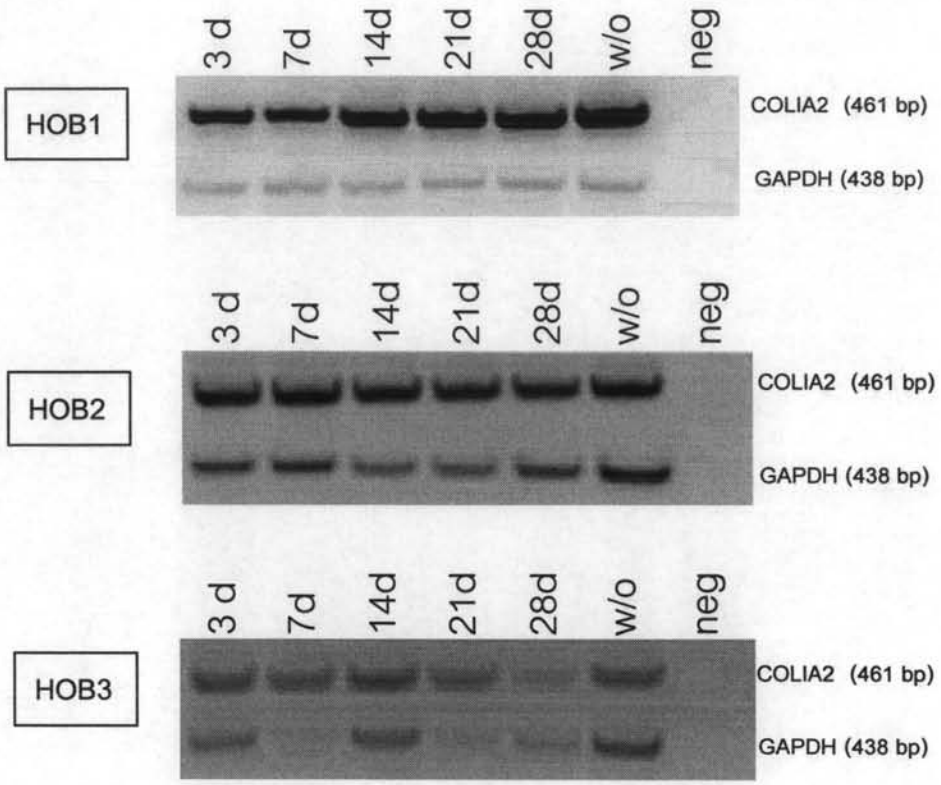

Figure 5. COLIA2 mRNA expression. Cells were plated in 60-mm culture dishes, in cultured with 50 μg/ml of AA and 10 mM of β-GP. RNA was extracted at days 3, 7, 14, 21 and 28 as detailed in Materials and Methods. Ethidium bromide-stained PCR products were photographed, and then the images were analyzed using Gel Imaging Analysis. w/o = cells grown in medium without AA and β-GP for 28 days. Neg = No cDNA templates.

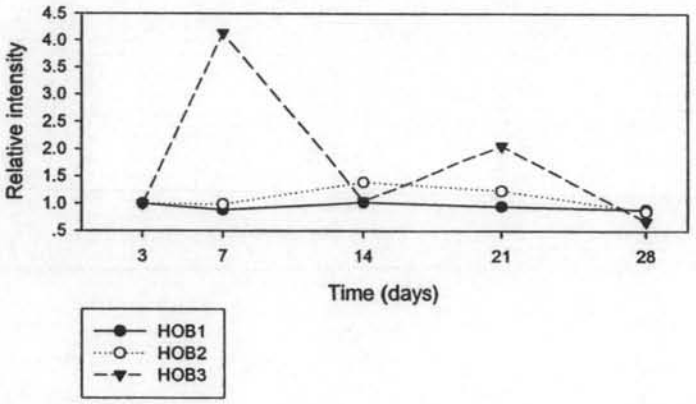

Figure 6. Semi-quantitative RT-PCR analysis of COLIA2 gene expression. Cells were plated in 60-mm culture dishes. RNA was extracted at days 3, 7, 14, 21 and 28. The band intensities of the RT-PCR products for ALP were measured and normalized to GAPDH as the internal standard. The relative intensity was calculated as ratio of the intensity of each time point to the lowest detected intensity.

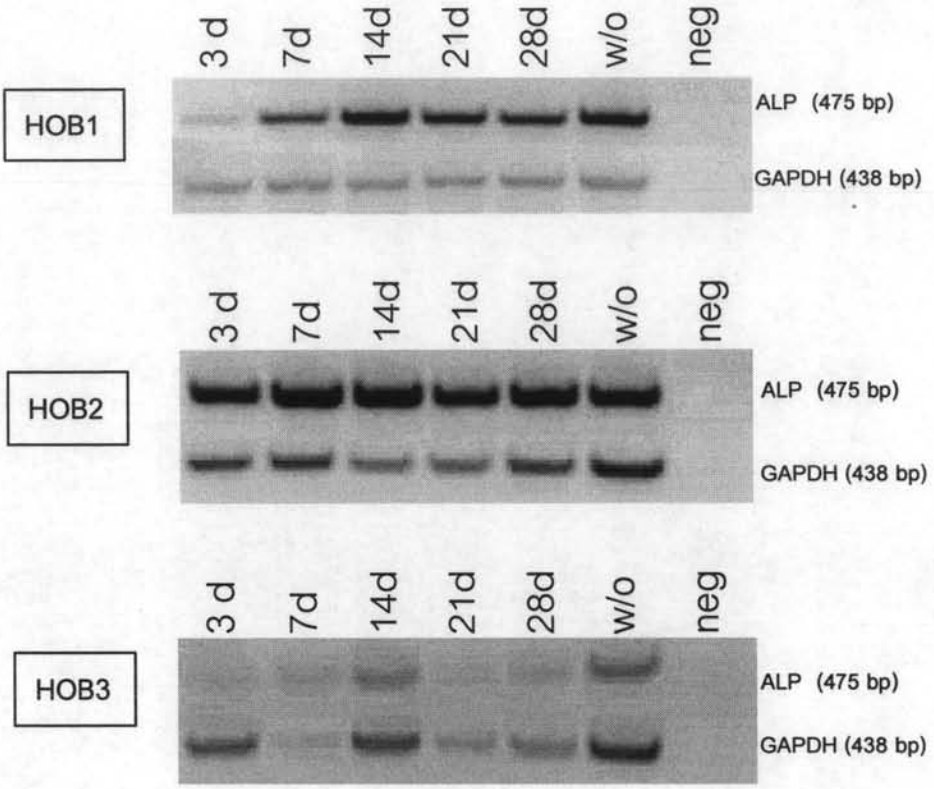

Figure 7. ALP mRNA expression. Cells were plated in 60-mm culture dishes, in cultured with 50 μg/ml of AA and 10 mM of β-GP. RNA was extracted at days 3, 7, 14, 21 and 28 as detailed in Materials and Methods. Ethidium bromide-stained PCR products were photographed, and then the images were analyzed using Gel Imaging Analysis. w/o = cells grown in medium without AA and β-GP for 28 days. Neg = No cDNA templates.

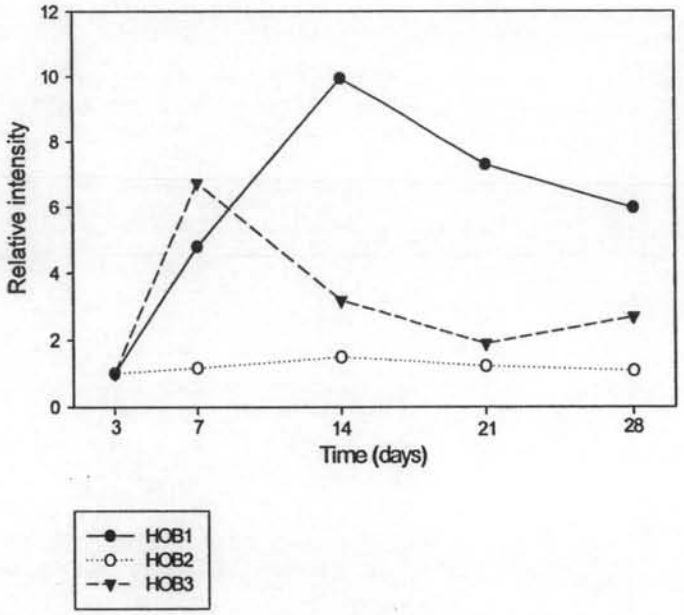

Figure 8. Semi-quantitative RT-PCR analysis of ALP gene expression. Cells were plated in 60-mm culture dishes. RNA was extracted at days 3, 7, 14, 21 and 28. The band intensities of the RT-PCR products for ALP were measured and normalized to GAPDH as the internal standard. The relative intensity was calculated as ratio of the intensity of each time point to the lowest detected intensity or.

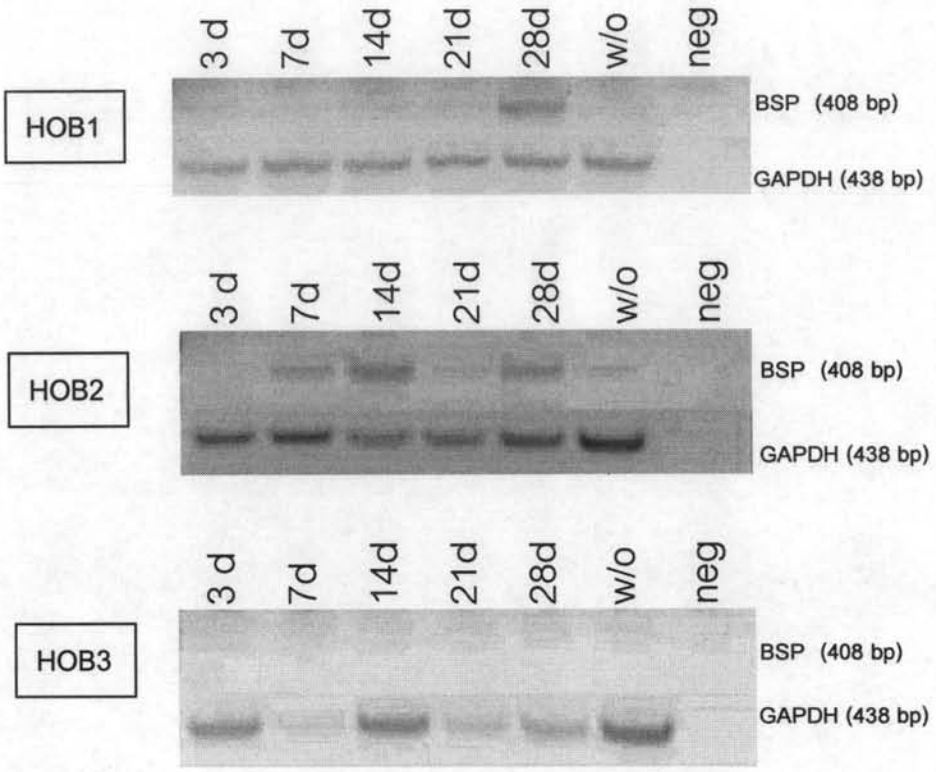

Figure 9. BSP2 mRNA expression. Cells were plated in 60-mm culture dishes, in cultured with 50 μg/ml of AA and 10 mM of β-GP. RNA was extracted at days 3, 7, 14, 21 and 28 as detailed in Materials and Methods. Ethidium bromide-stained PCR products were photographed, and then the images were analyzed using Gel Imaging Analysis. w/o = cells grown in medium without AA and β-GP for 28 days. Neg = No cDNA templates.

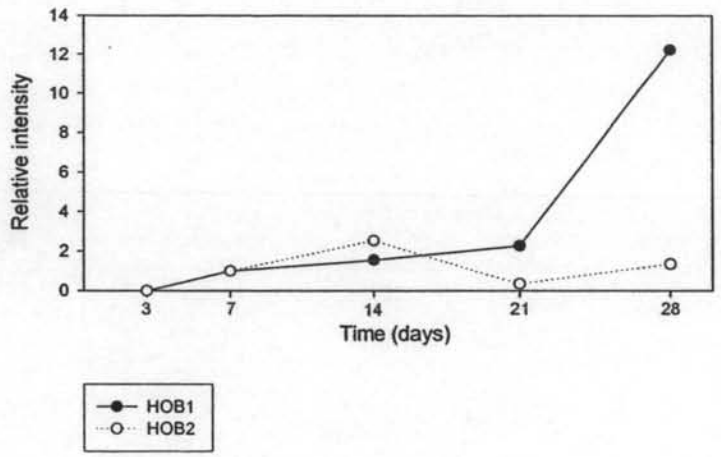

Figure 10. Semi-quantitative RT-PCR analysis of BSP2 gene expression. Cells were plated in 60-mm culture dishes. RNA was extracted at days 3, 7, 14, 21 and 28. The band intensities of the RT-PCR products for BSP were measured and normalized to GAPDH as the internal standard. The relative intensity was calculated as ratio of the intensity of each time point to the lowest detected intensity.

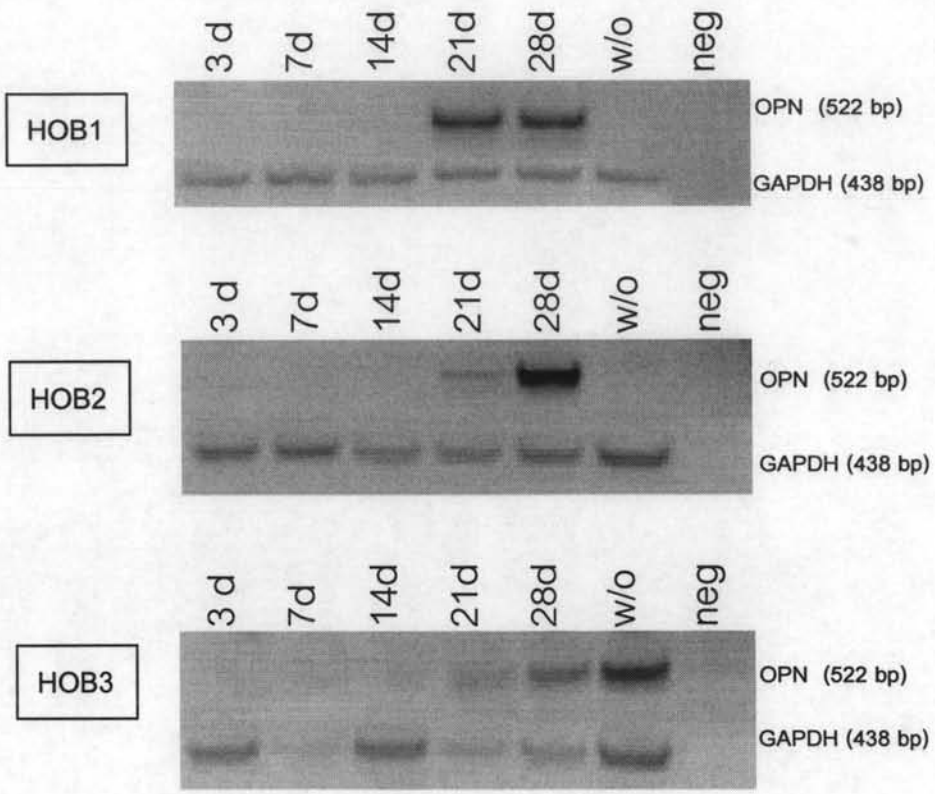

Figure 11. OPN mRNA expression. Cells were plated in 60-mm culture dishes, in cultured with 50 μg/ml of AA and 10 mM of β-GP. RNA was extracted at days 3, 7, 14, 21 and 28 as detailed in Materials and Methods. Ethidium bromide-stained PCR products were photographed, and then the images were analyzed using Gel Imaging Analysis. w/o = cells grown in medium without AA and β-GP for 28 days. Neg = No cDNA templates.

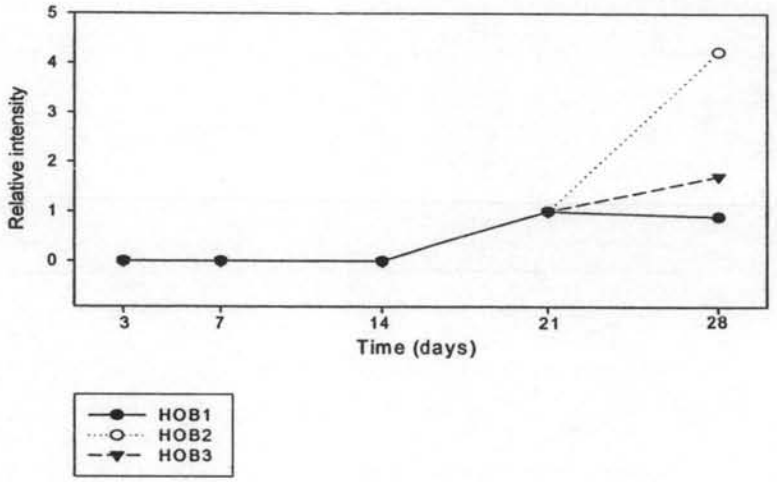

Figure 12. Semi-quantitative RT-PCR analysis of OPN gene expression. Cells were plated in 60-mm culture dishes. RNA was extracted at days 3, 7, 14, 21 and 28. The band intensities of the RT-PCR products for OPN were measured and normalized to GAPDH as the internal standard. The relative intensity was calculated as ratio of the intensity of each time point to the lowest detected intensity.

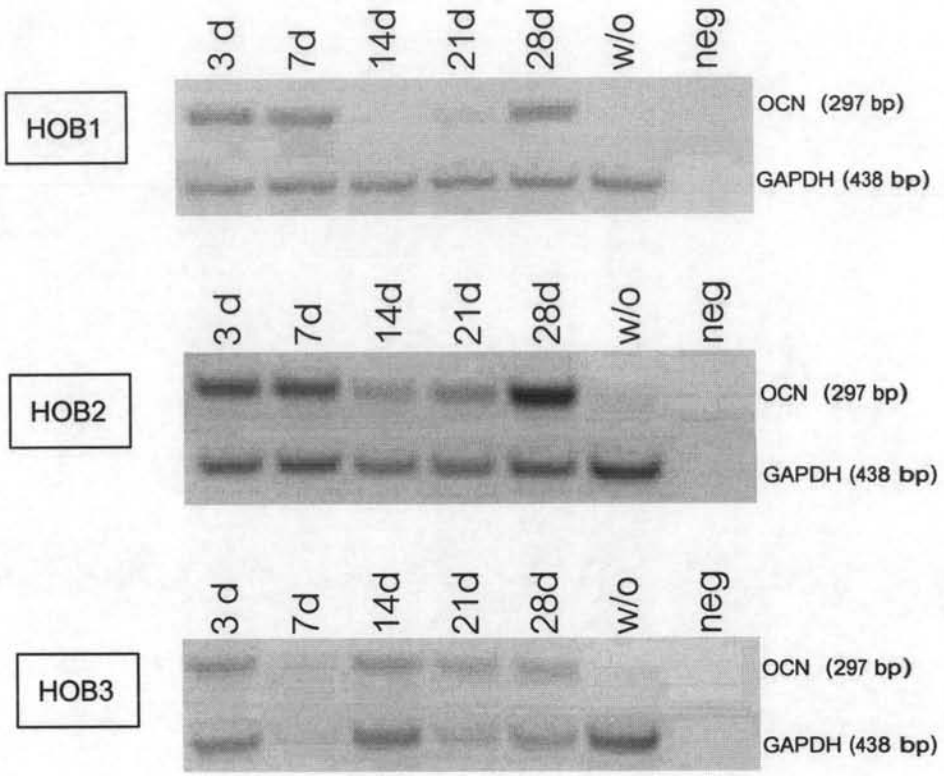

Figure 13. OCN mRNA expression. Cells were plated in 60-mm culture dishes, in cultured with 50 μg/ml of AA and 10 mM of β-GP. RNA was extracted at days 3, 7, 14, 21 and 28 as detailed in Materials and Methods. Ethidium bromide-stained PCR products were photographed, and then the images were analyzed using Gel Imaging Analysis. w/o = cells grown in medium without AA and β-GP for 28 days. Neg = No cDNA templates.

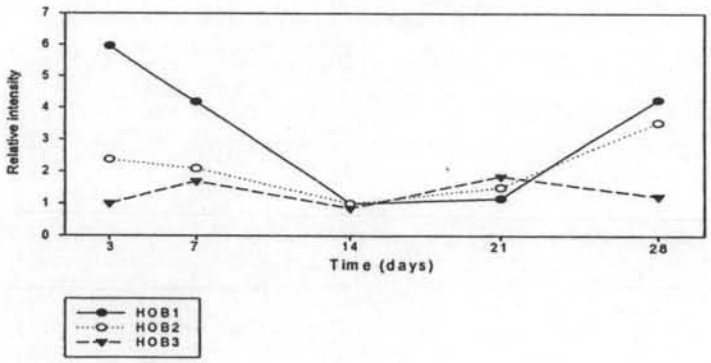

Figure 14. Semi-quantitative RT-PCR analysis of OCN gene expression. Cells were plated in 60-mm culture dishes. RNA was extracted at days 3, 7, 14, 21 and 28. The band intensities of the RT-PCR products for OCN were measured and normalized to GAPDH as the internal standard. The relative intensity was calculated as ratio of the intensity of each time point to the lowest detected intensity.

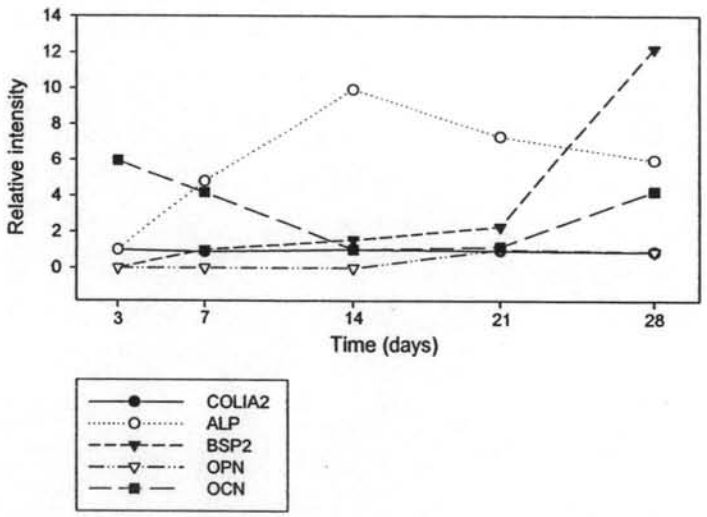

Figure 15. Semi-quantitative RT-PCR of bone markers in HOB1. Cells were plated in 60-mm culture dishes. RNA was extracted at days 3, 7, 14, 21 and 28. The band intensities of the RT-PCR products for BSP were measured and normalized to GAPDH as the internal standard. The relative intensity was calculated as ratio of the intensity of each time point to the lowest detected intensity.

4.3 Alkaline phosphatase activity assay

Positive stained could be detected from day 14 to day 28. In HOB1, the highest activity was observed at day 21. HOB2 were stained at the same intensity at days 14, 21 and 28 (figure 16). For HOB3, we observed the strong intensity at day 28 by naked eyes. However, under microscope, these were stained scatter foci, which corresponded with as mineralized nodules. The highest ALP activity of HOB3 was presented at day 14 under microscope observation (figure 18). At day 28, primary human osteoblasts without AA and β-GP were also positive stained (figure 16). In the positive wells, we could observe all of cells stained under light microscope (figure 17).

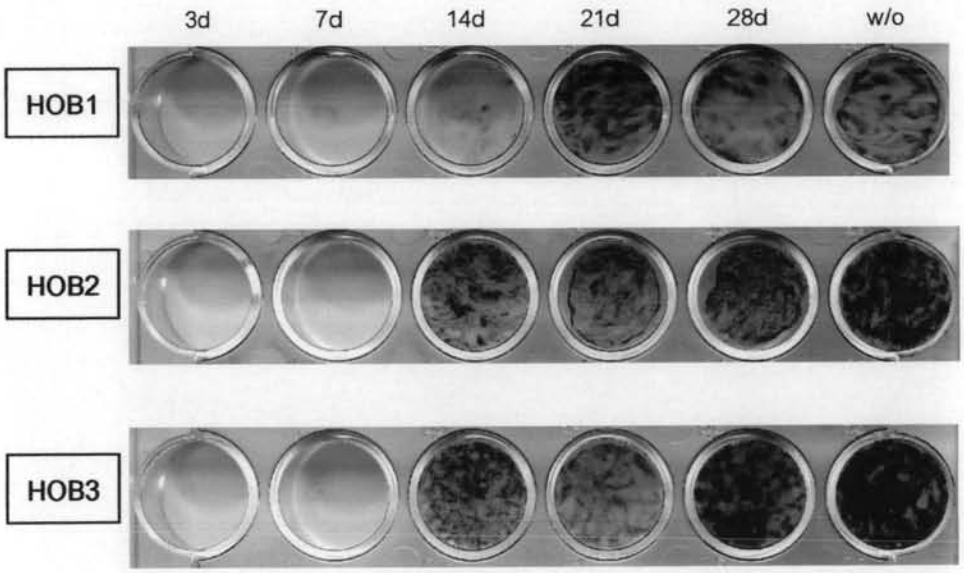

Figure 16. ALP activity. Cells were cultured in the medium containing 50 μg/ml of AA and 10 mM of β-GP at days 3, 7, 14, 21 and 28 as described in Materials and Methods. w/o = cells grown in medium without AA and β-GP for 28 days.

Figure 17. ALP activity of HOB2 in cultured with 50 μg/ml of AA and 10 mM of β-GP for 14 days (40x magnification).

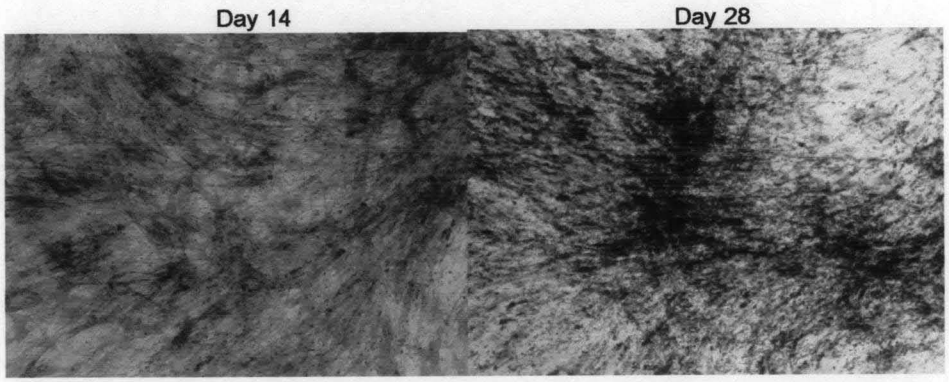

Figure 18. ALP activity of HOB3 in cultured with 50 μg/ml of AA and 10 mM of β-GP for 14 days. At day 14 (left), cells were stained in highest level. At day 28 (right), scatter foci of mineralized nodules were stained. (40x magnification).

4.4 Alizarin red S staining of mineralized nodules

The mineralized nodules could be detected in day 28 in all primary cell lines. However for HOB2 and HOB3, they started to form mineralized nodules at day 21 (figure 19). We did not find any mineralization in cells grown in medium without AA and β-GP. Mineralization nodules of HOB2 at day 28 shown in figure 20.

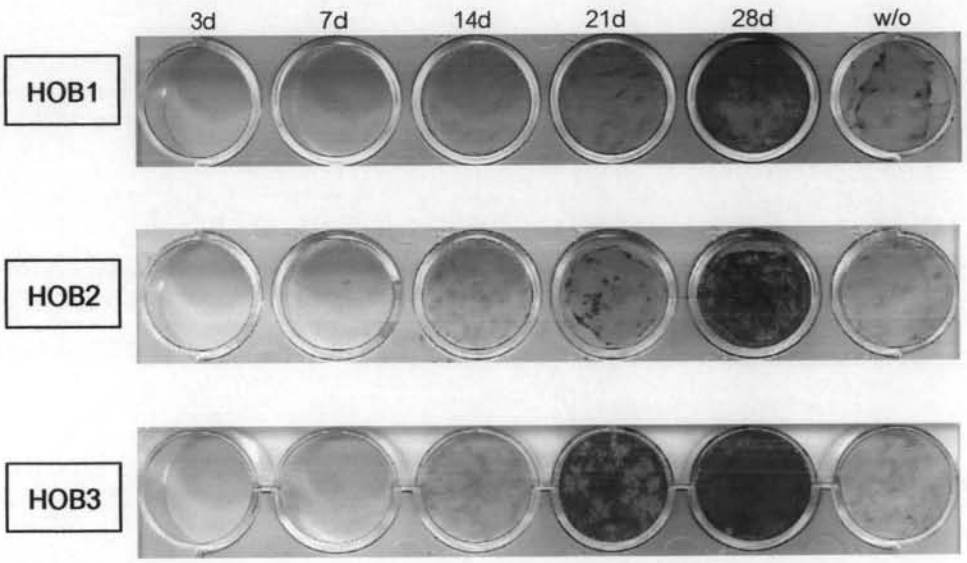

Figure 19. Alizarin red S staining. Cells were cultured in medium with 50 μg/ml of AA and 10 mM of β-GP for 3, 7, 14, 21 and 28 days. In HOB2 and HOB3, mineralized nodules were first detected at day 21, and heavily stained at day 28. w/o = cells grown in medium without AA and β-GP for 28 days.

Figure 20. Mineralization nodules of HOB2. HOB2 after 28 days cultured in medium with 50 μg/ml of AA and 10 mM of β-GP, were stained with Alizarin Red S. Mineral deposits were stained red (40x magnification).