CHAPTER V

DISCUSSION AND CONCLUSION

This study has shown that cells derived from human alveolar bone express osteoblastic markers and have ability to form mineralized nodules, when cultured in media supplemented with AA and β-GP. We collected bone samples from 15 donors, but only 8 samples were successfully established. Delayed transfer of samples to medium, or delayed processing of bone to culture condition may affect the viability of cells in bone samples. The cultured cells have morphology very similar to fibroblasts. Accordingly, we used ALP activity at 14 days in culture with inducing agent as an inclusion criterion to examine whether these cells were of ostoblastic lineage. All 3 samples that we used in this study achieved the ability to form mineralized nodules, therefore the inclusion criterion used is appropriate for establishment of human primary osteoblast cell lines in culture.

COLI is a major component in bone matrix, and the most abundant extracellular matrix protein in vertebrates. It is synthesized by osteoblasts, fibroblasts, and odontoblasts. In this study, COLIA2 mRNA expressed in the human primary osteoblasts at all time points (days 3, 7, 14, 21 and 28). Our results agree with many previous studies of osteoblasts. Collagen protein synthesis of mouse calvariae bone cells retained up to 14 days in β-GP cultured medium (Ecarot-Charrier et al., 1983). Review literature of osteoblast differentiation reported high expression of COLI from osteoprogenitors to mature osteoblasts (Aubin et al., 1995). Study by Southern blots in rat calvaria, COLI was uniformly expressed in osteoprogenitors, pre-osteoblasts,

osteoblasts and mature osteoblasts (Liu et al., 2003). In contrast to our study, Stein and Lian reported the down-regulation of COLI after 14 days in mouse calvaria osteoblasts (Stein and Lian, 1993). In our study, cells in the inducing condition were steadily expressed COLIA2. Cells grown in medium without inducing agent also expressed COLIA2.

One study in stromal cell line demonstrated that ascorbic acid induces the osteoblastic differentiation, which requires the formation of a collagen-containing extracellular matrix in response to ascorbic acid (Otsuka et al., 1999). Ascorbic acid is important for collagen synthesis due to its role as a cofactor for proline hydroxylase and lysine hydroxylaes (Kivirikko and Prockop, 1972, Hausmann, 1967), which are important in the hydroxylation of collagen. Integrins, implicated in cell attachment to the extracellular matrix, were up-regulated in a dose-dependent manner of ascorbic acid. Moreover, collagen was significantly underhydroxylated in bones treated with 0, 1, and 10 μg/ml ascorbate compared with control bone with 100 μg/ml ascorbate (Ganta et al., 1997). Ascorbic acid increased the rate of procollagen secretion from cell layers to culture medium (Franceschi et al., 1994). At day 28, cells in the inducing condition were more difficult to trysinized from the culture plate, compared to cells without AA and β-GP condition in the same culture period. This may be due to explain the difference in the quality of collagens secreted in cells grown in medium AA.

In this study, ALP mRNA expression was detected at all time points for all primary lines. In two of the lines, the ALP expression reached the highest level at day 14, then gradually decreased. It was reported that in heavily mineralized condition, cellular levels of ALP mRNA declined (Stein and Lian,

1993). This agrees with our results. One of the primary line, HOB3, had ALP peak level in day 7, corresponding with its onset of nodule formation, which was 1 week earlier than the others. However, some other studies showed slightly different expression pattern. Cells derived from rat calvaria demonstrated the peak level of ALP expression at day 21 in culture with AA and β-GP, and gradually declined two weeks after (Stein and Lian, 1993). Perinpanayagam and colleagues showed that ALP gene expression in human alveolar bone osteoblats was the highest expression at 7 days in culture with Dex, AA and β-GP, and slowly declined after that (Perinpanayagam et al., 2006). However, we could not compare our results at days 21 and 28 to the study, since their experimental period is 14 days.

The ALP activity of our primary cells started from day 14. HOB1 distinctly reached a peak at day 21 and gradually decreased, confirm a previous study of human marrow cells. ALP activity of human bone marrow cells grown in AA and β-GP showed the highest level at day 21 (Coelho and Fernandes, 2000). HOB2 had upregulated at day 14 and remained until day 28. The latter might be mixed cells in various stages of differentiation, so less differentiating cells showed late ALP activity. ALP activity of HOB3 had peak at day 14 slightly declined in day 21. This result is similar to the study in serially passaged human alveolar bone cells revealed the peak level of ALP activity at day 14, after cultured in medium with AA, β-GP and Dex (Fernandes et al., 1997). In contrast, Perinpanayagam et al., 2006, showed the highest ALP activity of human alveolar bone grown in medium with AA, β-GP and Dex at day 7. Human marrow cells demonstrated that cultures grown in the presence of Dex revealed a significant induction in ALP activity, comparing to those grown in standard medium or standard medium with AA and β-GP (Coelho

and Fernandes, 2000). Although we did not add Dex in our experiment, HOB3 showed relatively high mRNA expression of ALP earlier than the others. From our observation, HOB3 had swift proliferation stage, early mRNA expression of ALP, and also early mineralization, 1 week prior to the other cell lines. We believe that these cells could be at more mature stage than HOB1 and HOB2.

Mineralization stage of rat calvarial cells occurs from 28 to 35 days after cultured in medium with AA and β-GP (Stein and Lian, 1993). In this study, mineralized nodules were detected in all primary lines at day 28. Our result confirmed the study of Coelho and Fernandes, which showed mineralized nodules after 28 days in culture of marrow cells in AA and β-GP. For HOB2, nodule formation started from day 21, and strongly detected at day 28. HOB3 strongly stained Alizarin red S at day 21, and more densely in day 28. This corresponded to the early ALP mRNA expression at day 7.

In present study, cells grown in condition without AA and β-GP did not show nodule formation. β-GP is almost completely degraded to inorganic phosphate (Morais et al., 1999), is a source of substance for mineralization. Cells grown in condition without AA and β-GP may not have source of inorganic phosphate. Besides, this condition did not have ascorbic acid, which induces the osteoblastic differentiation (Otsuka et al., 1999).

BSP2 constitutes from 8 to 12% of the total non-collagenous proteins in bone and cementum (Fisher et al., 1983, Fisher et al., 1987). It is important in hydroxyapatite nucleation (Hunter and Goldberg, 1993), calcium binding (Chen et al., 1992), and mediate cell attachment (Oldberg et al., 1988). Its expression was reported in mature osteoblasts (Aubin et al., 1995). In present

study, we found different patterns of BSP2 mRNA expression in the three primary cell lines. HOB1 mRNA expression was first weakly detected at day 7 and more intense in day 28. This result agrees with previous studies (Aubin et al., 1995). Aubin and coworkers reviewed BSP2 expression in mature stage of osteoblasts by Southern blots obtained from poly(A)-PCR amplified samples. Perinpanayagam et al. detected the up-regulation of BSP2 expression in human alveolar bone cells cultured in medium with Dex, AA and β-GP for 14 days. This is similar to HOB2 which expressed BSP at days 7 and 14. We found a decline of BSP2 expression at day 21, and then an increase again at day 28. Previous study in rat calvarial cells using Sourthern blots analysis showed early and transient BSP2 expression in stage of osteoprogenitor cells, prior expression declined, then increased again in stage of osteoblasts (Liu et al., 2003). This may explain the pattern of BSP2 expression in HOB2. Albeit, BSP2 is important in hydroxyapatite nucleation (Hunter and Goldberg, 1993), even so we could not detect it in one of our cell lines, HOB3. The time points that we used in this study may not be an appropriative interval for HOB3. The cells may had intermittent expression of BSP2. I ts expression may down-regulated at time points that we analyzed, so we could not detect BSP2 expression in HOB3.

Many researchers reported that OPN could both promote (Giachelli and Steitz, 2000, Morinobu et al., 2003) and inhibit hydroxyapatite deposition (Boskey et al., 1993). OPN is expressed in two periods in rat calvaria cell culture, a low level of expression in active proliferation and a peak expression in mineralization (Stein and Lian, 1993). It has been immunolocalized in osteoblasts, osteocytes and pre-osteoblasts in the woven bone of neonatal rats (Mark et al., 1987). But in present study, we found OPN expression of

primary human osteoblasts only in mineralization period. We may have missed the first expression in active proliferation because the time point of expression period and RNA extraction was not frequent enough to detect the narrow expression period.

OCN, an indicator of bone matrix metabolism or turnover (Kleerekoper and Edelson, 1996). It is produced by osteoblasts during the mineralization stage (Power and Fottrell, 1991). OCN expression in the primary human osteoblasts was different from those reported previously. In this study, we found early expression of OCN mRNA before mineralization period and also in mineralization period. In a pilot study, we used primary cells derived from torus mandibularis. These cells showed OCN expression from day 3 to day 28 at a constantly level (data not shown). OCN is involved in the formation and remodeling bone (Ducy et al., 1996), however, OCN has no demonstratable nucleating activity (Hauschka, 1985). This may explain why there was no nodule formation at the earlier culturing period, although OCN expression was found. Studies in rat calvaria (Stein and Lian, 1993, Liu et al., 2003) showed different OCN expression, which was demonstrated in matrix maturation and mineralization stage. We suggest that primary cells derived from human alveolar bone may behave slightly differently from those derived from rat calvaria.

In present study, even all donors were the same age (21 years old), they had slightly different gene expression and nodule formation patterns, especially in HOB3. HOB3 was derived from a male donor, whereas HOB1 and HOB2 were from females. HOB3 was obtained during routine bilateral sagittal split osteotomy, while HOB1 and HOB2 were obtained from routine

extraction of upper third molars. Eventhough the sample size in this study is small, we suspect that using bone samples obtained from different areas or from different genders, may result in acquiring cells at different stages of differentiation.

We used SaOS2 at day 7 in culture condition as a positive control for all bone markers. These cells could not grow in muticellular layer, and did not survive after day 14 without subculturing. Therefore, we could not collect RNA at later time points for SaOS2. That is the reason why we did not compare SaOS2 mRNA expressions to our primary cells at all time points.

In conclusion, we demonstrated that cells derived from human alveolar bone showed osteoblast phenotype *in vitro*, under the inclusion criteria of ALP expression at 14 days. These cells expressed specific bone markers, COLIA2, ALP, BSP2, OPN and OCN. All cell lines had ability to create mineralized nodules, in medium with AA and β-GP. From this study, alveolar bone specimens provided available source of human osteoblastic cells for culture. These primary cells have potential to be a clinically relevant *in vitro* models of human osteoblasts from alveolar bone, for further studies in alveolar bone biology.