SUMMARY

MSCs are fundamental to bone regeneration and healing. The ease of isolation, the capacity for in vitro proliferation and osteoblast differentiation makes them the most explored cell source for bone regenerative therapies. In the in vivo scenario, the ECM surrounding the cells regulates the cell properties, behavior and functions. In this research project we studied in detail the ECM mediated regulation of hMSC adhesion, proliferation and differentiation. This knowledge led to the development of novel biomimetic scaffolds for bone tissue engineering applications.

Our studies revealed that ECM proteins, such as collagen type I and fibronectin and laminin are ideal for improving adhesion and proliferation of hMSCs during in vitro culture. On the other hand, ECM-GAGs, such as hyaluronic acid, heparin and chondroitin-6-sulphates, are ideal for enhancing osteoblast differentiation and mineralization of hMSCs. Our attempt to understand the mechanism by which the ECM proteins and proteoglycans enhanced osteoblast differentiation gave us interesting results.

The mechanism of osteogenesis by hMSCs is a complex process and is not well understood. Differentiation of hMSCs to mineralizing osteoblasts can be confirmed by demonstrating calcium deposits by simple staining methods like von Kossa or Alizarin Red S. However, it is not possible to evaluate the progress of differentiation and the degree of mineralization by a single test. Therefore, we have combined a series of techniques to study and compare the osteoblast differentiation on various ECM protein and GAG treated plates with respect to the untreated tissue culture plate.
Alkaline phosphatase (ALP) is the most widely recognized biochemical marker of the osteoblast differentiation. Staining for ALP on day 7 of osteogenic induction gave the first visuals and preliminary comparison of the commitment of hMSCs to osteoblast lineage. ALP assay that measured the ALP enzymatic activity was very useful in assessing the initiation and progress of mineralization at different stages of osteoblast differentiation. Human MSCs maintained in osteogenic induction media showed significant increase in the ALP activity with respect to the hMSCs maintained in non-osteogenic media, irrespective of the culture plate treatment. ALP activity, generally followed the trend of lower ALP activity during early stage (day 7) of osteogenic differentiation, which then showed a significant peak in expression during mid stage (day 14) and then a reduction in activity towards the late stage (day 21). The peak in ALP expression normally coincided with the initiation of mineralization. Although most of the plates followed this trend, a significant difference in the level of ALP activity was observed among different sets of experiments. We also noted that, there was discrepancy between the ALP expression at the gene level and protein level (ALP activity). In addition, higher ALP activity was not always an indication of higher mineralization.

Alizarin Red S on day 14 of osteogenic induction helped in visualizing and comparing the initiation of mineralization. Von Kossa staining was useful in confirming mineralization and comparing the secreted bone matrix at the end of differentiation. Von Kossa staining, not only indicated the amount of calcium containing mineral matrix but also reflected the amount of organic bone matrix present. Thus, high intensity of staining suggested high amounts of bone matrix that included both organic and inorganic matrix.

The amount of calcium in the secreted bone matrix determined the strength of the newly formed bone. ECM proteins like collagen type I and fibronectin showed high amount of bone matrix, but the amount of calcium was less compared to vitronectin.
Two-fold higher amount of calcium was found on vitronectin treated plate when compared to untreated and other ECM protein treated plates. ECM proteins interact with specific integrins present on the cell surface and regulate osteoblast differentiation mainly through integrin mediated signal transduction by ERK/ MAPK pathway. The difference in the mineralization observed among the different ECM protein treated plates could be due to the difference in the integrin binding and subsequent signal transduction.

When compared to ECM proteins, all the ECM-GAGs except chondroitin-4-sulphate, showed significant enhancements of mineralization with four fold higher calcium deposition. C4S and C6S differed only in the position of the sulphate group showed significance difference in mineralization. We could not explain how the position of sulphate group influenced mineralization. Although, hyaluronic acid, heparin, C6S and DS showed similar amount of calcium, there was notable difference in the secreted bone matrix. This was evident from the difference in the intensity of von Kossa staining observed on these plates.

The difference in the mineralization observed among ECM proteins and GAG is again due to the difference in the regulatory mechanisms of these components. During the in vitro differentiation of hMSCs to osteoblasts, ALP gene (ALPL) get up-regulated in the early phase of differentiation and get subsequently down-regulated in the later phase. ALP plays a significant role in initiating mineralization by hydrolysing phosphate substrate releasing Pi and there by acting as the nucleation point for deposition of the bone mineral, calcium phosphate. ECM proteins, which enhanced mineralization such as collagen type I, fibronectin, and vitronectin respectively, showed 50 fold, 25 fold and 10 fold higher ALPL expressions during the early phase of differentiation; whereas, the GAGs did not show any significant increase in ALPL expression associated with enhanced mineralization. Osterix (OSX), which is downstream of RUNX2, is identified
as a key transcription factor essential for the differentiation of pre-osteoblasts to mature osteoblasts. GAGs like hyaluronic acid and heparin which showed higher bone matrix with significant amount of calcium, showed a 150 fold higher expression of OSX. Other ECM components like C6S and fibronectin, which enhanced mineralization, also showed four-fold increases in OSX expression. Other major osteoblast differentiation associated genes which showed relative up-regulation associated with enhanced mineralization include, OCN on collagen type I, fibronectin, hyaluronic acid and heparin; OPN on collagen type I and vitronectin; IBSP on hyaluronic acid, collagen type I and vitronectin; and ON on hyaluronic acid.

Chitosan, which usually has a hostile surface for cell adhesion, supported cell adhesion at lower plate coating densities. In this study, we had proved the osteogenic potential of chitosan by demonstrating enhanced osteoblast differentiation and mineralization on chitosan coated plate when compared to untreated plate. Chitosan showed significant up-regulation of ALPL, COL1A1, OPN, ON and OCN genes.

Our ECM-based biomimetic approach for improving osteoblast differentiation and mineralization showed promising results. The tripolymer composite coating consisting of chitosan, collagens type I and hyaluronic acid showed significant enhancement of mineralization and calcium deposits with respect to their individual components. Chitosan-collagen (1:1) plate surface modified with hyaluronic acid showed the highest amount of calcium in the bone matrix. Although, collagen type I alone showed comparatively less amount of calcium, it was found to play significant role in enhancing calcium deposition in the composite coatings.

We also developed, stable biomimetic chitosan-collagen type I-hyaluronic acid based scaffolds, which supported and promoted hMSC adhesion and osteoblast
differentiation. It was noted that hMSCs grew as cell aggregates on chitosan scaffold since a higher concentration of chitosan was used for preparing strong and stable scaffolds. The addition of collagen and hyaluronic acid made the chitosan scaffolds cell adhesive. The tested pure chitosan and tripolymer scaffolds were non-toxic, suitable for hMSC culture and delivery; and were found to be osteoconductive. The scaffolds were porous and thus had large surface area making it possible to seed large number of cells. There was enhanced cell proliferation and the entire scaffold surface got covered with layers of cells in short period. When placed in appropriate environment, the cells were able to migrate from the scaffolds to the surrounding media. This ability of the scaffolds to support cell migration signifies the use of scaffolds seeded with undifferentiated or differentiated hMSCs for various cell based therapeutic applications. Pure chitosan scaffolds, which supported the proliferation and osteoblast differentiation of hMSCs as aggregates, also hold good promise for various tissue engineering applications.

To conclude, the ECM components indeed modified the adhesion, proliferation and osteoblast differentiation of hMSCs in vitro. The difference in the osteoblast differentiation and associated gene expression observed on various ECM component treated plates indicates the difference in the regulatory mechanisms. We have successfully developed ECM based, tripolymer composites consisting of chitosan, collagen type I and hyaluronic acid which holds great promise as a biomimetic coating or scaffold for bone tissue engineering and bone regenerative therapies.