CHAPTER 6

Study the effect of various glycosaminoglycans on adhesion, proliferation and osteoblast differentiation of bone marrow derived human mesenchymal stem cells

6.1 Introduction

Extracellular matrix (ECM) is an important part of the cellular microenvironment, which along with various growth factors play a significant role in regulating the differentiation and development (Adams and Watt, 1993; Flaumenhaft and Rifkin, 1992). ECM is referred to as the part of the “language” of the intercellular communications where cytokines are viewed as the symbols (Nathan and Sporn, 1991). ECM can bind and regulate the local concentration and availability of various growth factors. The ECM-cell interaction takes place through ligand-receptor binding, which activates specific sets of genes that regulates various signalling pathways (Flaumenhaft and Rifkin, 1992; Kim et al, 2011). The ECM of animal tissue mainly consists of proteins and polysaccharides. Most of the ECM proteins are associated with structural and adhesive functions. Major part of ECM is constituted by polysaccharides which have long chains of glycosaminoglycans (GAGs) usually linked to proteins forming proteoglycans. The GAGs interact with various secreted growth factors, chemokines and proteins that regulate cell signalling and differentiation (Wight et al., 1992). The current focus of the research is in understanding the diversity of ECM components and elucidating the complex mechanism of signal transduction.
The major GAGs of fibroblast ECM include hyaluronic acid (HA), heparin (HEP), heparan sulphate (HS), chondroitin sulphate (CS), dermatan sulphate (DS) and keratan sulphate (KS) (Ashhurst et al., 1990; Gallagher et al., 1983; Siczkowski et al., 1993). The major part of the inorganic bone matrix is made up of the hydroxyapatite and the organic matrix mainly consists of collagens and GAGs, chondroitin-4-sulphate (C4S), DS chondroitin-6-sulphate (C6S) and KS (Kazama et al., 1992). HA, the only non- sulphated GAG, can bind to cell surface directly through the CD44 receptor and mediate tissue regeneration and repair. The sulphated GAGs, which are rich in negatively charged sulphate groups, can bind to the positively charged amino group of various proteins and growth factors, thus increasing their local availability (Taipele and Keski-Oja, 1997).

Sulphated GAGs are important for bone formation as they bind to most of the growth factors like fibroblast growth factor (FGF), bone morphogenic proteins (BMPs), transforming growth factor-β1 (TGF-β1) and Insulin like growth factor II (IGF-II) involved in regulating the cells of osteoblast lineage (Benoit and Anseth, 2005). It has been shown that maintaining aged mesenchymal stem cells (MSCs) in new ECM restored its self-renewal and osteoblast differentiation in mice (Sun et al., 2011). Similarly, artificial biomimetic scaffold containing CS has been demonstrated to promote osteoblast differentiation of human MSCs (hMSCs) more specifically (Wollenweber et al., 2006).

To adopt the cell mimetic approach for bone regeneration, one should have an ideal cell source with well-established osteoblast differentiation protocols and a biodegradable scaffold providing mechanical support and cues for the intended differentiation. In this study, we have selected five GAGs; C4S, DS, C6S, HEP and HA present in the ECM of hMSCs or /and bone matrix to elucidate their regulatory role in the adhesive properties and osteogenic potential of human bone marrow derived MSCs. The aim of this study is
to identify various ECM-GAGs that promote and enhanced hMSCs proliferation and osteoblast differentiation in order to provide insights into their regulatory roles. This knowledge will help in developing a cell mimetic niche for directing hMSCs to achieve a functional bone construct for orthopaedic applications.

6.2 Materials and methods

6.2.1 Preparation of glycosaminoglycan treated culture plates

The ECM glycosaminoglycans (GAGs) such as hyaluronic acid (HA) from human umbilical cord, heparin (HEP) from porcine intestinal mucosa, chondroitin sulphate A or C4S from bovine articular cartilage, dermatan sulphate (DS) from bovine intestinal mucosa, and chondroitin sulphate C or C6S from shark cartilage, were purchased from Sigma Aldrich (St. Louis, MO, USA). One percentage solution of each GAG was prepared in HEPES buffered saline (pH 7.4) in a laminar flow hood under sterile conditions. Tissue culture plates were coated with the GAG solutions at an optimised coating density of 50µg/cm². The solution was allowed to dry completely in a laminar flow hood and the plates were washed once with distilled water before using for cell culture. These plates will be referred to as GAG treated plates henceforth. Normal tissue culture plate without any treatment will be referred to as untreated plates henceforth.

6.2.2 Human MSC culture on GAG treated plates

Mesenchymal stem cells were isolated and characterized from the bone marrow of adult human volunteers by the method mentioned under section 4.2.5. The cells were isolated and propagated in hMSC culture media (Table 4.2). Human MSCs were seeded at a density of 5000 cells per sq. cm on each of the ECM-GAG treated plates. Untreated tissue culture plates were used as the control for these experiments. The plates were
placed in a humidified incubator maintained at 37 °C with 5% CO₂ in the air. After 24h of incubation, they were observed under an inverted phase contrast microscope (Nikon Eclipse TE2000-S, Nikon Instruments Inc, Melville, NY, USA) for cell morphology and adherence. The viability of hMSCs (Section 4.2.7) and the doubling time (DT) on each GAG treated plate were also calculated (Section 5.2.2.1).

6.2.3 Osteoblast differentiation studies on the ECM-GAG treated plates

Human MSCs grown on ECM-GAG treated plates were directed to osteoblast lineage by growing them in osteogenic induction media (table 4.3). The osteogenic potential of the ECM-GAG treated plates were evaluated at different time points by methods like alkaline phosphate (ALP) staining (Section 5.2.3.1), ALP assay (Section 5.2.3.2), histochemical staining for secreted mineral matrix (Alizarin Red S, Section 4.2.9.3.1 and von Kossa staining, Section 4.2.9.3.2), calcium assay (Section 5.2.3.4) and osteoblast differentiation associated gene expression profile (Section 5.2.3.5).

6.2.4 Statistical analysis

The experiments were repeated with three different donor samples. Measures for each sample were performed in triplicates or duplicates and the results were expressed as mean ± standard deviation (SD). The statistical analysis was done by the two tailed, paired Student’s t test. In all the analysis a p-value less than 0.05 was considered significant and asterisks were given accordingly to indicate the level of significance as *p<0.05, **p<0.01 and ***p<0.001.
6.3 Results

6.3.1. hMSC culture on GAG treated plates

Phase contrast microscopy revealed no change in the morphology and adhesive property of hMSCs on GAG treated plates when compared to the untreated plate. Both the untreated and GAG treated plates showed adherent, fibroblast like cells (Fig. 6.1).

Cell viability assay by trypan blue exclusion test showed good cell viability (>87%) on all the GAG treated plates (Table 6.1). Cell viability decreased slightly when the cells were grown on the GAG treated plates when compared to the untreated plate. Among the GAGs, HA treated plates showed slightly lower cell viability (87.2 ± 5.3) than the rest of the plates (>91%).

Figure 6.1 Human MSCs on GAG treated plates. Phase contrast micrographs showing hMSC adhesion on GAG treated plates after 24h of incubation. All the GAG treated plates supported hMSCs adhesion and there was no significant morphological change. Scale bar - 50µm.
The hMSCs used in this experiment showed a doubling time of $37.1 \pm 2.9$ h on normal tissue culture plates at optimal culture conditions (Table 6.2). Tissue culture plate treated with various ECM-GAGs such as DS ($38.7 \pm 2.5$ h), C6S ($37.8 \pm 6.4$ h) and HEP ($34.2 \pm 2.6$ h) did not show any significant change in the doubling time when compared to the untreated plate ($37.1\pm 2.9$ h). However, a significant increase in doubling time was noticed on C4S ($56.4 \pm 3.1$ h) and HA ($51.6 \pm 5.6$ h) plates.
6.3.2 Osteoblast differentiation and mineralization on the GAG treated plates

Phase contrast microscopy showed early mineralization on HA treated plates, as mineral deposits were visible on day 7 of osteogenic induction (Fig. 6.2). All other plates showed visible mineralization in the second week of osteogenic induction. Mineral deposits were observed on all the tested plate on day 14 of osteogenic induction with HA and HEP treated plate showing higher mineralization (Fig. 6.2). Day 21 of osteogenic induction showed highest mineralization on HA followed by HEP, C6S and DS treated plates. The mineralization was least on C4S treated plate (Fig. 6.2).

6.3.2.1 ALP staining and assay

ALP staining performed on day 7 of osteogenic induction showed ALP positive cells on all the tested plates (Fig. 6.3). More number of ALP positive cells was generally present in the periphery of the culture plates.

Figure 6.2 Osteoblast differentiation of hMSCs on GAG treated plates. Phase contrast microphotographs depicting the progress of osteoblast differentiation and mineralization. Initiation of mineralization was appreciated only on HA treated plate on day 7 (L, yellow arrow, inset shows the area marked within the red squire at higher magnification). Day 14 showed mineral deposits on all the tested plates with HA and HEP treated plates showing higher mineralization (R, Q). There was increase in the mineral deposits by day 21 of differentiation. HA and HEP treated plates showed remarkably higher mineral deposits on day 21 of differentiation (X, W). Scale bar - 10µm.
There was marked difference in the number of positive cells on the untreated plate and the various GAG treated plates. The entire GAG treated plates showed more ALP positive cells than the untreated plate. Among the GAGs HEP and HA treated showed the maximum number of ALP positive cells followed by DS and C6S treated plates. Whereas C4S treated plates showed the lowest number of ALP positive cells.

**Figure 6.3 ALP staining by BCIP/NBT method.** Phase contrast micrographs showing hMSCs undergoing osteoblast differentiation on various GAG treated plates, stained for ALP on day 7 of osteogenic induction. ALP positive cells (bluish-black) were present on all the tested plates. More number of ALP positive cells were found on GAG treated plates (B-F) when compared to the untreated plate (A). Among the GAG treated plates, HEP (E) and HA (F) treated plates had more number of ALP positive cells and C4S (B) treated plates showed the least number of ALP positive cells. Scale bar - 10µm.

ALP assay showed a significant increase (as high as 80 fold) in the ALP level during the mid-stage of osteoblast differentiation when compared to the ALP level in the undifferentiated population (Fig. 6.4).

There was significant increase in the ALP activity in the mid-stage (day 14) of osteoblast differentiation on the untreated plate. Similar results were observed in HEP
and HA treated plates. The ALP activity was reduced at late stage of differentiation (21 days) on these plates. C4S, DS and C6S treated plates however, showed significantly lower ALP activity when compared to that of untreated, HEP and HA treated plates. Though there was difference in the level of ALP activity, all the tested plates except C6S, showed similar pattern of expression showing a peak in the activity in the mid-stage (day 14) of differentiation.

Figure 6.4 ALP assay. The dotted line shows the level of ALP in the initial undifferentiated (UD) population and the bars represent the ALP levels of cells undergoing osteoblast differentiation. There was significant increase in the ALP activity during osteoblast differentiation. Human MSCs on the entire GAG treated plates except C6S, showed similar pattern of expression with a peak in the expression in the second week of differentiation, which coincided, with the initiation of mineralization. Significance (p-value) was calculated with respect to the untreated plate at different time points and asterisks were given accordingly to indicate the level of significance as *p<0.05, **p<0.01 and ***p<0.001.
6.3.2.2 Alizarin red S and von Kossa staining for calcium

Alizarin staining performed on day 7 showed an early onset of mineralization as mineral deposits were appreciated on these plates as early as 7 days of osteogenic induction (Fig. 6.5).

![Figure 6.5 Alizarin Red S staining for calcium.](image)

Significantly, high amount of calcium was observed on DS (O), C6S (P), HEP (Q) and HA (R) treated plates on day 14 of osteoblast differentiation. No significant enhancement of mineralization was observed on C4S treated plate (N). Scale bar-10µm.

![Figure 6.6 Von Kossa staining for calcium.](image)

HA treated plate (F, L) showed the highest amount of bone matrix deposition followed by HEP (E, K). C6S (D, J) showed more mineralization than C4S (B, H) and DS (C, I) treated plates. Scale bar-10µm.
Alizarin Red S staining showed high amount of calcium deposition on HA treated plates. Positive staining was observed on HEP and C6S treated plates. As Alizarin Red S stain binds to the intra-cellular calcium, calcium binding proteins and proteoglycans also, there was intense staining on the plates after 14 days of osteogenic induction, making it difficult to assess the difference in the mineralization by the macroscopic view (Fig. 6.5). However, the phase contrast micrographs showed a comparatively high staining on HA, HEP and C6S, and a low stain on C4S and untreated plates.

Von Kossa staining performed at the end of differentiation showed significant difference in the staining intensity between the untreated and GAG treated plates. HA showed the highest staining followed by HEP and C6S (Fig. 6.6A, B). DS, C4S and untreated plates showed low staining.

6.3.2.3 Calcium assay

Calcium formed a purple-coloured complex with O-cresolphthalein complexone in an alkaline medium. The presence of calcium was detected only in the plates with cells growing in osteogenic induction media. They showed detectable amount of calcium at late stage of differentiation. All GAG treated plates showed significant increase for calcium when compared to the untreated tissue culture plate (Fig. 6.7). There was 4-5 fold higher calcium deposition in HA treated plates followed by HEP, C6S and DS treated plates. HA treated plate showed the highest amount of calcium deposition (3.5mg/mg of total protein) among all the GAGs and C4S, the lowest (1.2mg/mg of total protein).
Figure 6.7 Calcium assay. There was significant increase for the calcium present in the secreted bone matrix on all the GAG treated plates. The quantity of calcium on DS, C6S, HEP and HA treated plates was four times higher than that on the UT plates and HA showed the highest amount. C4S, C6S, which differed only in the location of their sulphate group, showed significant difference in calcium deposition. C4S did not enhance mineralization whereas C6S promoted mineralization showing almost three times higher amount of calcium deposition compared to C4S. The data presented is of a representative experiment done in duplicates. (*p<0.05, **p<0.01 and ***p<0.001).

6.3.2.4 Osteoblast differentiation associated gene expression profile on the GAG treated plates

The fold difference value indicated the fold difference in the expression of a particular gene with respect to the undifferentiated population on the GAG treated plate compared to the untreated plates. This value indicated the up regulation or down regulation of a particular gene caused by a particular GAG. Human MSCs undergoing differentiation on the GAGs treated plate has revealed significant difference in the relative gene expression profile, when compared to the untreated tissue culture plate (Fig. 6.8). In addition, there was relevant difference in the fold change in gene expression on various GAG treated plates. HA treated plates maintained a high level of OSX expression in all the stages of osteoblast differentiation. OSX level was 76 fold higher at early stage,
which later up-regulated to 168 fold (p< 0.01) at mid-stage differentiation and then got down-regulated to 12 fold at late stage of differentiation. Beside OSX gene expression, HA also showed higher expression of IBSP (10 fold, p< 0.05) at mid-stage; ON (8 fold, p< 0.05) and OCN (6 fold, p< 0.05) at late stage of osteoblast differentiation. HA however, did not stimulate RUNX2, COL1A1, OPN and ALPL gene expression. HA and HEP treated plate which had similar ALP activity as that of the untreated plate showed similar expression in ALPL gene. Results showed that HA treated plates expressed both early stage markers IBSP and ON; and late stage markers OSX and OCN that is necessary for optimum osteoblast differentiation.

HEP treated plates also showed similar up regulation of OSX expression as in HA treated plates. Compared to the untreated plates, the cells on HEP treated plates showed 46 (p< 0.05), 172 and 4 fold higher OSX expressions at the early, mid and late stage of differentiation respectively. Two final phase osteoblast differentiation associated gene ON and OCN showed around 3 times higher fold difference in expression towards the final stage of differentiation. Other genes like RUNX2, COL1A1, OPN, ALPL and IBSP did not show significant up regulation on HEP treated plates.

C6S, DS and C4S coated plates showed relatively lower expression of all the tested genes in this experiment compared to that of HA and HEP. In C6S coated plates only gene that showed marginally higher expression is OSX (4 fold, p< 0.05). None of the other genes showed higher expression than that of untreated control. Similarly in DS coated plate, there was marginal increase in the expression genes such as RUNX2, ALPL and IBSP, which are early stage genes of osteoblast differentiation. Expression level of late stage differentiation genes like OSX and OCN in DS coated plates were unchanged or similar to that of the untreated plates.
Figure 6.8 Osteoblast differentiation associated gene expression profile. The real time PCR reaction was carried out in triplicates and the mean fold difference with standard deviation is represented in the graph. The dotted line represented the level of expression on the untreated (UT) plates taken as one. There was significant difference in the gene expression profile on all the GAG treated plates with respect to the UT plates. OSX showed around 160 fold increase in the expression on HA and HEP treated plates. OSX also was significantly up regulated on C6S treated plates. (*p<0.05, **p < 0.01 and ***p< 0.001).

Similar results were also observed in C4S coated plates where early differentiation genes were shown marginally higher expression such as COL1A1 (2 fold, p< 0.05) IBSP (6 fold) ON (4 fold, p< 0.01). Late stage genes like OSX and OCN were however unchanged. Due to low level of osteogenic differentiation associated genes in
C4S, DS and C6S, we observed low differentiation of osteoblast and ALP activity compared to that of HA and HEP.

6.4 Discussion

For the development of a functional tissue construct, it is important to understand the natural microenvironment of the source cell and the innate mechanisms of cell differentiation and tissue regeneration. This knowledge will form the back bone of the biomimetic approach for cell differentiation and tissue regeneration. This is the first study reporting the effect of ECM-GAG treated plates on the gene expression profile and bone matrix deposition and mineralization of human mesenchymal stem cells undergoing osteoblast differentiation.

Good viability of hMSCs on all the GAG treated plates indicated the suitability of using GAGs in cell culture and tissue engineering applications. The adhesive functions of ECM are mediated mainly through its protein components like fibronectin (Ode et al., 2010; Pankov et al., 2002). This data showed no significant enhancement of hMSC adhesion and proliferation on GAG treated plates. Uygun et al (2009), in their study using GAGs and rat bone marrow MSCs, showed that the MSC proliferation rates on heparin, heparan sulphate, DS, and C6S exhibited nonlinear increases with the level of fibronectin binding on these surfaces. In contrast, MSC proliferation on hyaluronic acid and C4S was found to be independent of fibronectin or vitronectin binding on the surfaces. Since both HA and C4S can bind with CD44 (Peach et al., 1993) they suggested the possibility of a specific CD44–GAG interactions responsible for the initial cell attachment, spreading and subsequent growth on HA- and C4S-immobilized membranes. This difference in the regulatory mechanism of cell proliferation was evident from our results also, as HEP, DS and C6S had comparatively similar DT with heparin showing the least DT; whereas C4S and HA had similar DT which was significantly higher than the untreated plate.
Therefore, among the GAG heparin provided the most favourable surface for the hMSC adhesion and proliferation. It has been reported that heparin modified gels promoted hMSC adhesion and proliferation (Benoit et al., 2005). They suggested that heparin act as sequestering and / or stabilizing vehicle increasing the surface concentration of proteins stimulating cell proliferation. Another interesting observation we made from the cell adhesion and proliferation experiment is that C4S and C6S, differing only in the location of sulphate group in the disaccharide unit, showed considerable difference in cell attachment as well as proliferation. C6S was found to be superior with better cell adhesion and population doubling rate than C4S.

Osteoblast differentiation is marked by an initial proliferative phase followed by an extracellular matrix synthesis phase and a final phase of mineralization (Lian et al., 2003; Lian et al., 1995). The osteoblast differentiation experiments with the GAGs provided new insights into the role of ECM GAGs in osteogenesis and its regulation. Among the GAGs, HA was proved to have the best osteogenic potential followed by heparin, C6S, and DS. The overall histochemical staining for calcium and bone matrix demonstrated the HA surface to be the most preferable condition for osteoblast differentiation and mineralization. There was early onset of mineralization and significantly large amount of mineral deposition with high calcium content on HA treated plates. HA based hydrogels with BMP-2 and hMSCs had the highest expression of osteocalcin and mature bone formation with vascular markers (Kim et al., 2007). In addition, there are studies reporting enhanced mineralization, with increased ALP activity and OPN and COL1 expression by human mesenchymal stromal cells on a biomimetic HA based scaffolds (Manferdini et al., 2010). Though there are many studies (Huang et al., 2003; Kawano et al., 2011) proving the osteogenic potential of hyaluronic acid, only a few researchers report the possible mechanism of the osteogenesis by HA. A recent
A study demonstrating the differentiation of hMSCs to pre-osteoblasts in the absence of osteogenic supplements in a HA based gel suggested the integrin mediated hMSC adhesion and subsequent sequestration of native ECM components and soluble cytokines with osteoconductive properties (Jha et al., 2011). This shows the innate osteogenic potential of HA. Another school of thought is that as MSCs possess CD44 on its surface, the CD44-HA interaction is responsible for facilitating the osteogenic differentiation. The CD44 receptor serve as the primary receptor for the ECM hyaluronic acid and regulate both cell-cell and cell-ECM interactions (Underhill, 1992). CD44-HA binding increases the cellular response to BMP7, which activates the Smad-1 signalling pathway inducing osteoblast differentiation through Hoxc-8 (Peterson et al., 2004).

Heparin was another GAG that showed high osteogenic potential. Though the onset of mineralization was later compared to HA, heparin showed equally good amount of calcium in the mineral deposits. There are studies reporting the osteogenic potential of heparin (Ling et al., 2010, Hausser et al., 2004) and also the inhibitory effect of heparin on osteoblast differentiation and mineralization (Kock et al., 2002; Yang et al., 2005). Therefore, the exact role of heparin in bone biology is still not very clear. There was significant difference in the osteogenic potential of C4S, C6S and DS, though they differed only in the position of sulphate group and the presence of iduronic acid in the case of DS. C6S provided a highly favourable condition for osteoblast differentiation and mineralization, followed by DS. C4S provided the least favourable condition. The amount of calcium deposited on C6S and DS was two times higher than that of C4S. There was difference in the ALP expression level also. When the fold difference in the osteoblast differentiation associated gene expression relative to the untreated plate was calculated, there was remarkable difference between the expression profile on C4S, C6S and DS. Though this result clearly indicates the stringent binding specificity of these GAGs to
growth factors or other regulatory molecules, we were not able to establish the relation
between the position of sulphate group and osteogenic potential or elucidate the exact
mechanism of regulation of osteogenesis by these ECM components. We found similar
reports stating the difference in the functional properties of these ECM components in a
hematopoietic cell culture experiment (Madihally et al., 1999). They observed a
difference in the response to C4S and C6S by hematopoietic cell population. C6S showed
a high cell proliferation rate whereas C4S had a toxic effect on the cell culture. DS also
differed in cell culture preference and CD 34+ cell enrichment. They suggested that the
difference in the biological properties of these GAGs is most likely to be due to their
unique binding characteristics and highly specific interaction modes.

The calcium quantification showed significantly high amount of calcium on DS,
C6S, HEP and HA plates with HA showing the highest amount. Whereas von Kossa
staining showed very intense staining on HA plate followed by HEP and lower intensities
for C6S and DS. This difference between the calcium quantification and staining for
calcium clearly indicates the presence of large amount of organic components in the bone
matrix of osteoblasts on HA and HEP treated plates. Calcium assay confirmed that all the
GAG treated plates except C4S have significant osteogenic potential and formed
mineralized bone matrix with good amount of calcium irrespective of the organic
components in the extra-cellular deposits, indicating their therapeutic potential in bone
fracture healing.

The most important findings of this study were made in the osteoblast
differentiation associated gene expression profile of different GAG treated plates. Among
the eight genes studied, OSX was found to have the most significant role in regulating the
GAG mediated osteoblast differentiation. OSX is a zinc finger-containing transcription
factor, which was recently been found to have critical role in osteoblast differentiation
and bone formation (Nakashima et al., 2002). OSX is the downstream effector for the osteoblast differentiation master-switch, RUNX2. RUNX2 is expressed by the multi-potential MSC population and it acts as the key factor regulating their differentiation to multiple lineages. RUNX2, responsible for the osteoblast lineage commitment, enhances osteoblast differentiation at an early stage and inhibits the later stage of osteoblast maturation (Komori et al., 1997). Now, OSX is identified as a key transcription factor essential for the differentiation of pre-osteoblasts to mature osteoblasts (Zhang et al., 2010). No bone formation occurs in OSX-null mice and their skeleton is composed entirely of cartilage with no osteoblasts and mineral bone matrix. OSX is not expressed in RUNX2 null mice while RUNX2 is expressed in OSX-null mice. These findings suggested that OSX is essential for osteoblast differentiation and bone matrix deposition and it acts genetically downstream of RUNX2 (Deng et al., 2008). When MSCs were transfected with OSX gene, the over expression of OSX increased MSC proliferation, ALP activity and mineralization in vitro (Tu et al., 2006). They also report that over expression of OSX in MSCs did not favour chondrogenic or adipogenic differentiation. In another experiment they demonstrated that OSX over expressing MSCs could effectively stimulate healing of critical sized defects in murine calvarial bone by promoting bone regeneration (Tu et al., 2007). As all these data suggest the crucial role of OSX in osteoblast differentiation, the high level of OSX expression observed on HEP and HA treated surface in this experiment holds significant relevance. Higher the OSX expression level more was the bone matrix deposition, which included the inorganic calcium as well as the organic matrix. The average OSX expression level was highest on HA treated plate that showed the highest bone matrix deposition, which was followed by HEP in both OSX expression, and matrix deposition. HA treated plate showed an increase in the expression of COL1A1 and IBSP, the early markers; and ON and OCN, the late markers
of osteoblast differentiation. HEP also showed an increase in the expression of ON and OCN. There are reports showing the increased expression of OCN, ALP, IBSP and OPN caused by the over expression of OSX (Tu et al., 2006). In C6S treated plate also OSX played the key role in increasing the mineralization. Thus from these observations we conclude that OSX is responsible for the enhancement of osteoblast differentiation and bone matrix deposition by hMSCs on ECM-GAG treated plates.

To summarise, the GAGs did not enhance hMSC adhesion and proliferation significantly but played a major role in regulating hMSC differentiation to the osteoblast lineage. Enhancement of mineralization with significant calcium deposition was observed in hMSCs undergoing osteoblast differentiation on hyaluronic acid, heparin, chondroitin-6-sulphate and dermanate sulphate treated tissue culture plates. Hyaluronic acid showed superior osteogenic potential by up regulating osteoblast differentiation associated genes and thus stimulating higher amount of bone matrix deposition with significant amount of calcium. We identified OSX as the key transcription factor responsible for the enhancement of bone matrix deposition. Identification of the right kind of glycosaminoglycans promoting osteogenesis would enable us to fabricate osteoconductive biomimetic scaffolds for developing human mesenchymal stem cell based bone graft for clinical applications. This data advocates the use of hyaluronic acid for bone healing applications.