



# Role of Heparin Sodium Salt in the Modulation of Human Umbilical Cord-Derived Mesenchymal Stem Cell Differentiation

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## Abstract

**Introduction:** The present study aims to investigate the role of low molecular weight compound heparin sodium salt (HSS) to control the differentiation of the human umbilical cord (UC) derived mesenchymal stem cells (MSCs) through possible interaction with WWTR1 protein.

**Materials and Methods:** In order to carry out this study, the human UC-derived stem cells were isolated and characterized by stem cell specific markers and the effect of HSS was studied by altering the phenotypes of MSCs. An *In silico* approach was employed to reveal the structural determination of the ligand, the WWTR1 protein binding site and to predict the strength of the interaction. After HSS treatment, WWTR1, Oct4, Nanog, SOX9 gene expressions were studied using real-time polymerase chain reaction (PCR). Cell staining was performed using alizarin red to confirm the formation of osteocytes.

**Results:** Mineralization indicated by osteocytes was confirmed using alizarin red after the treatment of HSS. Post, HSS treatment, OCT4, Nanog, RUNX2, COL1A1 and WWTR1 gene expressions were positively modulated. Heparin treatment of MSCs lead to the up regulation of WWTR1 along with the down regulation of stemness markers Oct4 and Nanog expression. *In silico* studies also predicted the possible interaction of WWTR1 with HS. Results indicated that Amino acid residues ASP57, GLN83, GLN109, THR135, and TYR141 came up as a prominent interaction centre; ASP57, GLN83 and THR135 recorded the highest interaction energy – while ASP57 mostly participated in an electrostatic interaction.

**Conclusions:** To conclude, it can be stated that heparin can possibly interact with WWTR1 along with having the capability to direct cells towards osteogenic lineages.

**Keywords:** Heparin, Mesenchymal Stem Cells, WWTR1, Cell Proliferation, Osteocyte Differentiation

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## Introduction

The ECM is a three-dimensional complex network of macromolecules including collagens, proteoglycans, elastin, and laminins ubiquitously present within all tissues and organs in the body.<sup>1</sup>

Heparin and heparan sulfate are notable extracellular polysaccharides which have recently been investigated for their catalytic role within the extracellular matrix of the cell. Heparin is found to be primarily produced in the specialized secretory cells namely mast cells, as well as stored in the intracellular granules of those cells.<sup>2</sup> The heparin treatment has been proven to be non-toxic for mesenchymal

stem cells (MSCs) which supports and leads them towards differentiation, dedifferentiation and chondrogenesis.<sup>3</sup> They directly bind and trigger the activity of TGF-beta1 and other growth factors, which lead to mitogenic or differentiation effects of the cells.<sup>4</sup> Previous studies have suggested that WWTR1, the hippo pathway regulator co activates RUNX2-dependent transcription are found to direct MSCs towards osteogenic differentiation.<sup>5-7</sup>

However, till now no studies have pointed out to the fact that heparin may be able to modulate WWTR1 expression in MSCs, which possibly leads to the differentiation of osteocytes. Commonly, heparin can repair and allows tissue

regeneration to occur, placing a promising strategy for functional recovery. Also, WWTR1's fate is reversible if not degraded by the cytoplasm through phosphorylation, and is dephosphorylated back to the nucleus, which is a key attribute for its epithelial mesenchymal transition.<sup>8-10</sup> Heparin addition has shown to increase WWTR1 expression and matrix stiffness.<sup>11,12</sup>

It is believed that heparin regulates differentiation by directing cells towards forming osteocytes and chondrocytes when human umbilical MSCs (hUCMSCs) are induced by providing the necessary growth factors. In this study, it has been aimed to investigate the possible interaction of heparin with WWTR1 which may provide additional scope for observing the differentiation potential of MSCs.

## Materials and Methods

### Isolation and Characterization of Mesenchymal Stem Cells

In the present study, after obtaining the patient's consent, fresh umbilical cords (UCs) were retrieved during caesarean deliveries from the Department of Obstetrics and Gynecology of Chettinad Hospital and Research Institute (Chennai, India) and were processed within the optimal period of 12 hours (proposal no.10/IHEC/3-16). The UC sample was collected in a collection medium containing 10 mL PBS (RM7385, Dulbecco-A, HI-MEDIA) solution with 1 mL fetal bovine serum (FBS) and 10 µL antibiotic-antimycotic solution. After transferring samples under a sterile laminar flow cell culture hood, UCs were rinsed twice in phosphate-buffered saline (PBS) containing antibiotic-antimycotic solution (Gibco) until the cord blood was cleared, and cord vessels were removed. The cords were manually minced into pieces of 1–3 mm<sup>3</sup> and were allowed to adhere to flasks for 30 minutes. Then, they were floated in Low Glucose Dulbecco's Modified Eagle's Medium (LG-DMEM) containing 20% FBS, and 1% antibiotic-antimycotic solution. Cord pieces were subsequently incubated at 37°C in humid air with 5% CO<sub>2</sub>. The medium was changed once in every 3 days after initial plating. Fragments of UC were left undisturbed in culture and cells that were released and were further expanded and cultured.<sup>13</sup>

### Immunophenotypic Characterization by FACS

The MSCs specific marker of the isolated umbilical cord mesenchymal stem cells (UC-MSCs) was determined by flow cytometry analysis. The cells were harvested and suspended in PBS and were stained with antibodies: CD73, CD105, CD14, CD34 and CD45 (Table 1). Cells were acquired and

analyzed with the BD FACS ARIA II instrument.

### MTT Assay for the Dose Selection

In order to determine the effect of heparin sodium salt (HSS) on cells in culture, an assay was performed using MTT. In a 96 well plate, 2000 cells/well were seeded and incubated for 48 hours and 72 hours. The control cell did not receive any HSS. After the MTT treatment, the formed intracellular formazan crystals were solubilized with DMSO and the absorbance of the solution was measured at 530 nm using an ELISA reader. The percentage of cell viability was calculated by using the formula; Percentages of Cell Viability = (OD of drug treated sample/ OD of control sample) × 100.

### Treatment of Mesenchymal Stem Cells With Heparin Sodium Salt

An HSS stock solution of 1µM was prepared with serum free-Dulbecco's modified Eagle medium. The cells were exposed to various working concentration of HSS prepared from stock solution. The drug was introduced into the medium every 48 hours and characterized on day 8.

### Colony Forming Unit Assay

The plated cells were briefly fixed with chilled methanol for about 5 minutes and were stained with Giemsa and incubated at room temperature for 10-20 minutes. Colonies were visualized using inverted microscope (Leica Microsystems).

### Cell Differentiation

Induction medium and cell staining techniques was utilized to confirm the formation of osteocytes, chondrocytes and adipocytes. Alizarin Red staining for osteocytes, Toluidine blue for chondrocytes and oil red staining for adipocytes was employed. (HIMEDIA: Catalogue codes: AL521-1X100ML HiAdipoXL TM Adipocyte, AL523-1X100ML HichondroXL TM, AL522-1X100ML HiOsteoXL TM Osteocyte)

### Protein Modeling

The amino acid sequence of WWTR1 were obtained and subjected to the model building using modelling servers namely IntFOLD, I-TASSER, Phyre2 and Raptor X.<sup>14,15</sup> The globular models shaped by I-TASSER and Phyre2 were protonated at physiological pH and ionic strength, subjected to loop refinement, and minimized in a smart minimizer of DS 2.5 to satisfy the root mean square gradient of 0.1 kcal/mol Å. Furthermore, both the direct server output and minimized models were validated using Structural Analysis

**Table 1.** FACS Antibodies

Antibody	Specificity	Conjugate	Catalogue Number and Details
CD 14	Hematopoietic cells (negative marker)	FITC-A	Mouse anti-human CD14 Mouse IgG1, κ FITC BD Biosciences 555397
CD90	Mesenchymal stem cell (positive marker)	FITC-A	Mouse anti-human CD90 Mouse IgG1, κ FITC BD Biosciences 555595
CD34	Hematopoietic cells (negative marker)	PE-A	Mouse anti-human CD34 Mouse IgG1, κ PE BD Biosciences 555822
CD105	Mesenchymal stem cell (positive marker)	PerCP-Cy5-5-A	Mouse anti-human CD105 Mouse IgG1, κ PerCP-Cy <sup>TM</sup> 5.5 BD Biosciences 560819
CD73	Mesenchymal stem cell (positive marker)	APC-A	Mouse anti-human CD73 Mouse IgG1, κ APC BD Biosciences 560847
CD45	Hematopoietic cells (negative marker)	APC-Cy7-A	Mouse anti-human CD45 Mouse IgG1, κ FITC BD Biosciences 555482

and Verification Server (SAVES) (<http://services.mbi.ucla.edu/SAVES/>). The I-TASSER produced the best model on the basis of stereochemistry of the models.<sup>16</sup>

### Protein-Ligand Docking

The structure of Sodium Heparin [PubChem CID: 22833565] was obtained from PubChem<sup>17</sup> and the ligand binding site of WWTR1 was predicted using COACH<sup>18</sup> 3D Ligandsite, CASTp, DoGSiteScorer, FTSite and Sitehound servers. All possible conformers of the ligand were generated and subjected to molecular docking by Genetic Optimization for Ligand Docking (GOLD 5.2) software package on the basis of the detected binding site residues (GLU131 THR135 GLN139 TYR141 LEU143 HIS145 ILE149 TRP152) using ChemPLP and ChemScore scoring function. The best complex was chosen on the basis of ChemPLP Fitness and was minimized in smart minimizer. Also, the binding free energy and interaction energies were calculated in DS 2.5.

### qRT-PCR

The RNA was extracted from the cell sample using TRIzol method. The c-DNA was synthesized using superscript cDNA synthesis kit according to the manufacturer's protocol. The qRT-PCR for the genes (Table 2) was performed using Applied Biosystem 7900 PCR system. The Ct values for target genes were normalized using endogenous control beta actin. The fold difference in the heparin treated cells was calculated by  $2^{-\Delta\Delta Ct}$  with respect to the control.

### Statistical Analysis

Data was presented as mean  $\pm$  SD. Student's *t* test was used to assess differences between the samples. *P* values <0.05 was considered as a significant difference.

## Results

### Isolation

After 10 days of isolation, UC-derived cells were found to migrate and adhered to the surface of the flasks (Figure 1a and 1b). The isolated and expanded UC-MSCs showed spindle-shaped fibroblast morphology.

### Surface Marker Analysis of the Cells

The flow cytometry analysis of the isolated cells displayed a positive expression pattern for CD73, CD105 and lacked

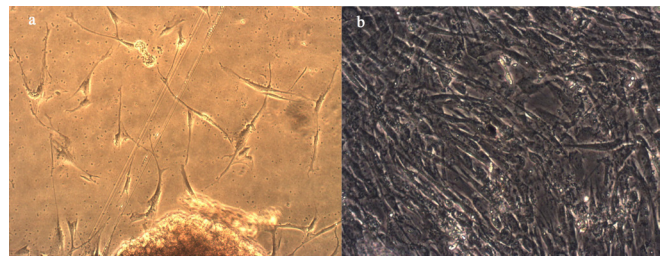


Figure 1. Stem Cells Morphology.

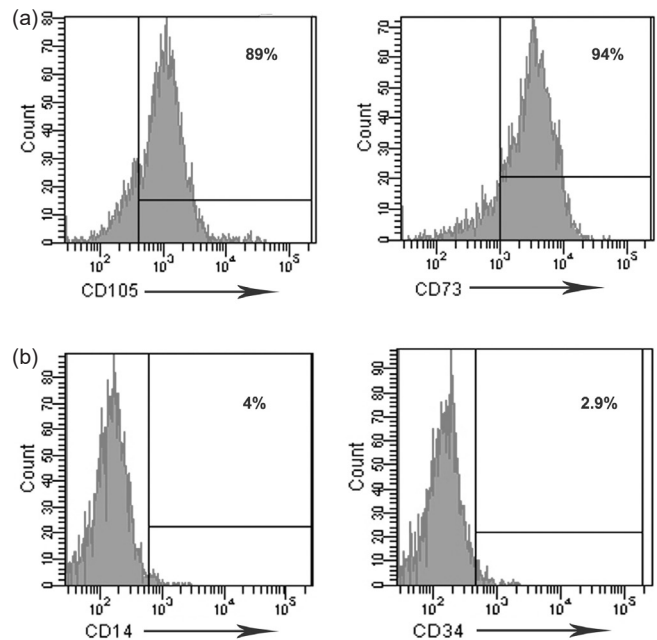


Figure 2. Characterization of MSCs. (a) CD73, CD105, (b) CD14, CD34.

expression for CD14, CD34 (Figure 2) indicating that these cells were MSCs according to the minimal criteria of ISCT.

### Selecting the Dose of Heparin Treatment

The 100 nM concentration was used for this study. Human UC-derived mesenchymal cells are plastic adherent that were expanded in culture in several passages.

### Predictive Interaction of WWTR1- Sodium Heparin

Electrostatic and van der Waal interactions contributed almost equally in the WWTR1-Sodium Heparin interaction profile and was characterized by numerous hydrogen bonds and  $\pi$ -sulfur interaction with considerable binding free energy (Tables 3, 4 and Figure 3), which corroborated the experimental findings (Figure 3 indicating experimental binding study). Amino acid residues ASP57, GLN83, GLN109, THR135, and TYR141 were found to be prominent interaction centers. However, ASP57, GLN83 and THR135 recorded the highest interaction energy. This is while ASP57 mostly participated in electrostatic interaction and the other two made both VDW and electrostatic contacts efficient (Table 4, Figure 3). The implications of heparin binding to WWTR1 and its effect in its functionality is yet to be established. Residues

Table 2. Primer Sequences

Gene	Sequence
WWTR1	F- 5'GTCACGCAGGACCTAGACAC R- 3'CACGAGCTAGGCTTCGGATT
RUNX2	F- CAACAAGACCTGCCCGT. R- TCCCATCTGGTACTCTCCG.
OCT-4	F- 5'CGACCATCTGCCGCTTTGAG R- 3'CCCCCTGTCCCCATTCTA
NANOG	F-5'AGTCCCAAAGGCAACAACCCACTTC R-3'ATCTGCTGGAGGCTGAGGTATTTCTGTCTC
COL1A1	F- GCTACCCAACCTGCCITCATG R- TGCAGTGGTAGGTGATGTTCTGA
B-actin	F: 5' TTCTACAATGAGCTGCGTGTG 3' R: 5' GGGGTGTTGAAGGTCTCAA 3'

**Table 3.** WWTR1 Interactions With Heparin Sodium

Hydrogen Bonds		$\pi$ -sulphur Interaction	Binding Free Energy (kcal/mol)
Conventional	Polarized carbon		
7	16	1	-61.76

**Table 4.** Key Residues of Interactions

WWTR1				
ASP57	HIS84	PRO106	THR135	TYR141
GLY81	VAL85	GLN108	THR137	
ALA82	LEU94	GLN109	GLN139	
GLN83	LEU96	THR133	ARG140	

with interaction energy  $\geq 5$  kcal/mol were reported. Residues involved in  $\pi$ -sulfur interaction were highlighted, amino acids exhibiting interaction energy  $\geq 10$  kcal/mol were labeled bold, amino acids with high electrostatic energies were noted in shaded cells, AAs with prominent van der Waals contribution were in red, AAs participating in conventional HBs were underlined and hydrogen bonds involving polarized carbon were italicized.

#### WWTR1 Expression after HSS Treatment

The expression of WWTR1 was significantly upregulated after heparin treatment which may be significant to the fact that the addition of heparin leading to matrix stiffness is directly proportional to WWTR1 upregulation (Figure 4).

#### Quantitative Real Time PCR Analysis

##### Heparin modulation

The present study has addressed the potential of HSS, to modulate stem cell fate of UC-derived MSCs via regulating the gene expressions of WWTR1, NANOG, OCT4, RUNX2, SOX9 and COL1A1 (Figure 4).

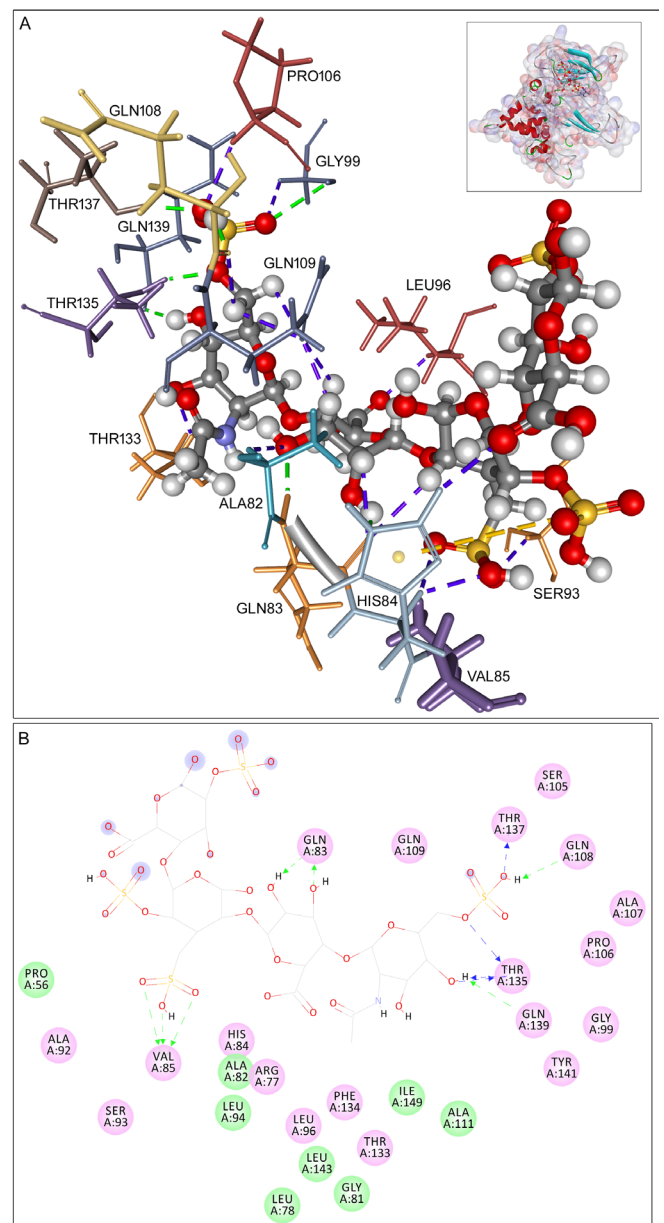
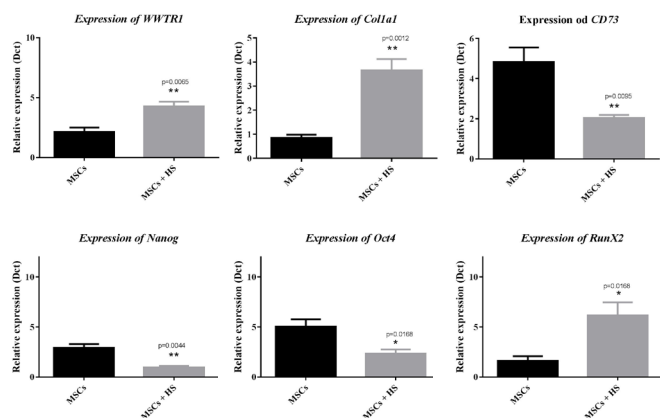
This study also documented the upregulation of RUNX2 gene which is the essential transcription factor for osteoblasts differentiation. Therefore, it is predicted that heparin interacts with WWTR1 thus regulating the proliferation and differentiation of MSCs (Figure 4).

#### Continuous Treatment for 21 Days by Heparin Directs Cells towards Osteogenic Differentiation

Mineralization indicated by osteocytes was confirmed using alizarin red (Figures 5a and 5b). The WWTR1 was seen to be overexpressed along with other traditional stemness markers, such as Oct4 and Nanog that enables to distinguish an undifferentiated MSC from a differentiated one, especially derived from a human UC.

#### Discussion

The primary goal of the present study was to explore the modulatory role of a low molecular weight compound such as HSS in the WWTR1-mediated osteocyte differentiation of MSCs. While the progressive effects of heparin and WWTR1 upon stabilization of cell fate has been explored individually, the novelty of this study was to focus on evaluating the outcomes of the synergistic effect of WWTR1 protein and

**Figure 3.** WWTR1-Heparin Sodium Interaction Profile**Figure 4.** RT-PCR Analysis After HSS Addition. RT-PCR expression of (a) WWTR1, (b) COL1A1, (c) CD73, (d) Nanog, (e) Oct4 and (f) RUNX2

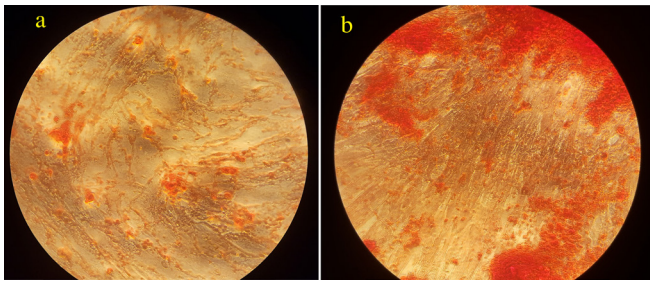


Figure 5. Osteocytes mineralization.

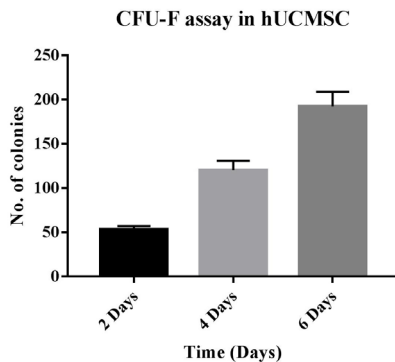


Figure 6. Cell Viability Assay.

heparin compound. Protein-ligand interaction influences the lineage commitment of stem cells as they are largely reliant on specific adhesion signals for the differentiation process. An in-depth understanding of the intricate molecular mechanics occurring in protein-ligands interactions contribute to identifying the ligand binding capacity and affinity states. The molecular functions and biological processes of target proteins are associated with their affinity for the specific ligand. It is interesting to note that osteogenic differentiation is potentially induced in UC-derived MSCs when cultured with various agents including low molecular weight compounds.

A qualitative comparative analysis was performed to view the colony forming units (CFU) (Figure 6). It is worth noting that comparative analysis of control and heparin treated cells of CFU show a higher percentage of cells that confirmed the increased proliferation of stem cells that is triggered by addition of heparin. This is in line with previous experiments where heparin has shown to increase proliferation at low doses such 200 ng.<sup>19,20</sup>

The isolated cells that showed positive for MSC markers CD90, CD73, CD105 and negative for Hematopoietic markers CD14, 34 and 45 denoted that there was no cross contamination with hematopoietic cells that were cultured. This is possibly due to the removal of the 3 vessels carrying cord blood in the human UC which is a gold standard protocol.

The WWTR1 activity increases ECM rigidity that provides the right environment for the anchoring of the osteoblasts. Thus, under the influence of heparin, signals involved in the translocation of WWTR1 into nucleus which may be deduced which can form the nucleosome complex signaling matrix

proteins which have been confirmed by the phenotype of these cells.<sup>21</sup>

With the expression of PPAR $\gamma$ , an adipogenic marker was downregulated in heparin treated cells. This suggests that the direction of adipogenic lineage is suppressed with the use of heparin. Chondrogenic marker SOX9 showed no change in the gene expression after heparin treatment. The RUNX2 genes are specific markers for osteogenic differentiation and RT-PCR analysis showed elevated expression of RUNX2 in heparin treated cells. This confirms that heparin may have an affinity towards forming osteocytes which is important in bone formation and heparin is an important component of bones and cartilages. The COL1A1 ECM marker was upregulated after heparin treatment which is a significant part of this study. The cell treated with heparin substantially lost its stemness marker Oct4 and Nanog expression thus the cells moves towards differentiation. It has been identified that heparin downregulates the concurrent expression of pluripotent integrators such as Nanog and Oct4 via facilitating and initiating several signaling pathways namely the fibroblast growth factor signaling.<sup>22</sup>

Surface marker analysis revealed that MSCs treated with heparin showed a variation in the surface marker expression<sup>2</sup>. Heparin has clearly shown an increase in proliferation that is in line with research on heparin where heparin has shown to increase proliferation at low doses such 200 ng.<sup>23</sup>

Finding have provided new insight into a novel mechanism of the WWTR1 involved in smad-WWTR1 axis as suggested by Park et al. The adipo-osteogenic differentiation of MSCs demonstrate a reciprocal role of WWTR1 as a positive and negative factor in osteogenesis and adipogenesis of MSCs, respectively. The WWTR1 nuclear retention leading to its active transcription regulating hippo pathway eventually leads to hard matrix formation that yields formation of osteocytes.<sup>24</sup> Initial findings of WWTR1 expression was in relation to RUNX2 where this protein was identified to promote osteoblast differentiation and its regulation along with repressing PPAR $\gamma$  and adipogenesis. The findings of this study are in line with Hong et al's study.<sup>8</sup>

Further, we sought to examine the proliferation and trilineage gene expression profile of the MSCs through q-RT PCR. Stemness markers like OCT-4, NANOG, and transcriptional coactivator with PDZ-binding motif (WWTR1) were found to be downregulated in Heparin treated cells. However, the up regulation of the pre-osteoblast marker RUNX2 and major osteocyte protein COL1A1 were observed in MSCs treated with heparin indicating that HSS induces osteogenic differentiation. This result indicates that heparin treatment inhibits the proliferation of MSCs and promotes osteogenesis. Ling et al's study showed that heparin enhances osteogenesis through phosphoinositide 3-kinase/Akt/RUNX2 pathway.<sup>25</sup> The WWTR1 protein interacts with RUNX2 and activates transcription directing MSCs towards osteogenic differentiation.

## Conclusions

This experimental study assessed the effects of heparin on the expression of WWTR1 protein in human UC derived MSCs

and inducing differentiation. Notably, increased WWTR1 expression in MSCs significantly demonstrates WWTR1 as a cofactor for stemness. The *in silico* study highlights the strong binding interaction between WWTR1 and heparin. It is believed that heparin regulates differentiation by directing cells towards forming osteocytes. Despite substantial progress, further studies involving the relationship between WWTR1 and heparin via cell signaling pattern are warranted. The novelty of the undertaken study lies in the *in silico* approach which establishes the rationale involved in the impact of heparin-WWTR1 interaction upon the ECM and stem cell niche. The binding site, affinity and strength of the interaction results in conformational changes and validates the hypothesis. The WWTR1 protein may co-activate the expression of Runx2, a key transcription factor which drives the osteogenic differentiation. The COL1A1 is the principal gene required for providing the primary organic bone material. In this study, the expression of COL1A1 was consistently higher as the cell differentiation process began. The expression of pluripotency regulators such as nanog and oct4 is downregulated under the influence of heparin sodium salt that affects WWTR1 signalling.

#### Authors' Contributions

MV conducted all the experiments, analyzed the results, and wrote the drafts of the manuscript. SKN did the bioinformatics analysis. GJ, SM, RDL and BKM helped in statistical analysis and writing the MS. SP conceived the study and participated in its design, coordination and helped draft the manuscript. All authors finally reviewed the manuscript.

#### Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

#### Ethics Approval

A written informed consent was obtained from all patients and the study protocol was approved by the Institutional Review Board of Chettinad Academy of Research and Education, Chennai.

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#### Availability of Data and Materials

The authors declare that all relevant raw data will be freely available to any scientist wishing to use them for non-commercial purposes.

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