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Research Article

Involvement of WNT Signaling in the Regulation of Gestational Age-Dependent Umbilical Cord-Derived Mesenchymal Stem Cell Proliferation

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Mesenchymal stem cells (MSCs) are a heterogeneous cell population that is isolated initially from the bone marrow (BM) and subsequently almost all tissues including umbilical cord (UC). UC-derived MSCs (UC-MSCs) have attracted an increasing attention as a source for cell therapy against various degenerative diseases due to their vigorous proliferation and differentiation. Although the cell proliferation and differentiation of BM-derived MSCs is known to decline with age, the functional difference between preterm and term UC-MSCs is poorly characterized. In the present study, we isolated UC-MSCs from 23 infants delivered at 22–40 weeks of gestation and analyzed their gene expression and cell proliferation. Microarray analysis revealed that global gene expression in preterm UC-MSCs was distinct from term UC-MSCs. WNT signaling impacts on a variety of tissue stem cell proliferation and differentiation, and its pathway genes were enriched in differentially expressed genes between preterm and term UC-MSCs. Cell proliferation of preterm UC-MSCs was significantly enhanced compared to term UC-MSCs and counteracted by WNT signaling inhibitor XAV939. Furthermore, WNT2B expression in UC-MSCs showed a significant negative correlation with gestational age (GA). These results suggest that WNT signaling is involved in the regulation of GA-dependent UC-MSC proliferation.

1. Introduction

Mesenchymal stem cells (MSCs) are a heterogeneous cell population that has a potential to proliferate and differentiate into trilineage mesenchymal cells: adipocytes, osteocytes, and chondrocytes. MSCs were initially isolated and characterized from the bone marrow (BM) [1, 2] and subsequently derived from almost all tissues including adipose tissue (AT), synovium, skin, dental pulp, umbilical cord blood (UCB), placenta, and umbilical cord (UC) [3]. Due to the ability to home to sites of injury, undergo differentiation, suppress immune responses, and modulate angiogenesis, MSCs are paid an increasing attention as a source for cell therapy against various degenerative diseases. Currently, MSCs from different sources have been tested in clinical studies for treatment of graft-versus-host disease, myocardial infarction, cerebral infarction, and so on [4, 5].

Although BM is the most well-characterized source of MSCs, it has certain limitations with the invasive BM aspiration and the decline in MSC proliferation and
2. Materials and Methods

2.1. Patients and Samples. Human UCs were obtained from 23 infants delivered at 22–40 weeks of gestation with parental written consent. This study was approved by the Ethics Committee at Kobe University Graduate School of Medicine (approval number 1370) and Hyogo Prefectural Kobe Children’s Hospital (approval numbers 24-25) and conducted in accordance with the approved guidelines.

2.2. Preparation of UC-MSC. The umbilical cord (2-3 g weight) was collected, cut into 2-3 mm pieces, enzymatically dissociated with Liberase DH Research Grade (Roche, Mannheim, Germany) in PBS for 45–60 min at 37°C followed by the addition of 10% fetal bovine serum (FBS; Sigma, St. Louis, MO) to inhibit enzyme activity, and filtered through a 100 μm cell strainer (BD Bioscience, Bedford, MA). The resulting cells derived from all compartments of the umbilical cord (whole UC) were cultured at 37°C (5% CO2 and 95% air) in MEM-α (Wako Pure Chemical, Osaka, Japan) containing 10% FBS and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA) until confluent primary cultures were established. The cells were then dissociated with trypsin-EDTA (Wako Pure Chemical), and the trypsinized cells were seeded into fresh dishes and passaged to confluence. Serial passaging was carried out until the tenth passage. The cells at fifth to eighth passages were used in the present experiments.

2.3. Cell Surface Marker Analysis. UC-MSCs were dissociated with 0.25% trypsin-EDTA for 10 minutes, washed with PBS and suspended at ~1 × 10^6 cells/ml in FCM buffer containing 1 × PBS, 2 mM EDTA, and 10% Block Ace (Dainippon Pharmaceutical, Osaka, Japan). The cells were incubated with phycoeryhrin-(PE-) conjugated mouse primary antibodies against CD14, CD19, CD34, CD45, CD73, CD90, CD105, or HLA-DR (BD Bioscience, Franklin Lakes, NJ) for 45 min on ice, washed with PBS, incubated with Fixable Viability Stain 450 (BD Bioscience) for 15 min at room temperature, washed with PBS, and filtered through a 70 μm cell strainer (BD Bioscience). PE-conjugated mouse IgG1 k, IgG2a k, or IgG2b k isotype control (BD Bioscience) was used as a negative control for each primary antibody. Flow cytometric analysis was performed using FACSARia III carrying a triple laser (BD Bioscience) and FACSDiva software (BD Bioscience).

2.4. Cell Differentiation. To verify the multipotency of UC-MSCs, the cells were induced to differentiate into the adipogenic, osteogenic, and chondrogenic lineages. Adipogenic differentiation was induced in STEMPRO adipogenesis differentiation medium (Invitrogen) for 2-3 weeks and stained, and the differentiation was investigated by staining lipid vesicles with Oil Red O (Sigma). Osteogenic differentiation was induced in STEMPRO osteogenesis differentiation medium (Invitrogen) or STK-3 (DS Pharma Biomedical, Osaka, Japan) for 1-2 weeks, and the differentiation was examined by staining with Arizarin Red S (Sigma) reacting to calcium cation. Chondrogenic differentiation was induced by forming cell aggregates in micromass culture in STEMPRO chondrogenesis differentiation medium (Invitrogen) for...
1 week, and the differentiation was assessed by staining anionic glycoconjugates with Toluidine Blue (Sigma). Cell images were acquired using a BZ-X700 microscope (Keyence, Osaka, Japan).

2.5. RNA Extraction. Total RNA from UC-MSCs and fibroblasts was extracted with a TRIZOL Plus RNA purification kit (Life Technologies) according to the manufacturer’s instructions. RNA integrity was evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using RNA 6000 nanokit (Agilent Technologies) according to the manufacturer’s instructions.

2.6. Gene Expression Microarray Analysis. Total RNA from three term and five preterm UC-MSCs (Table 1) was subjected to global gene expression analysis using the Low Input Quick Amp Labeling Kit One-Color (Agilent Technologies) and SurePrint G3 Human Gene Expression v3 8 × 60K Microarray Kit (Agilent Technologies) according to the manufacturer’s instruction. Briefly, double-stranded cDNA was synthesized from 100 ng of total RNA by AffinityScript-RT using T7 promoter-incorporated Oligo-dT primer. Cyanine 3- (Cy3-) CTP-incorporated RNA (cRNA) was generated using the second strand cDNA as a template via an in vitro transcription reaction. The amplified cRNA was purified with the RNeasy mini kit (Qiagen, Valencia, CA) and quantified cRNA by the NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA). 600 ng of Cy3-labeled cRNA was hybridized to the microarray slides at 65°C for 17 hr with rotation at 10 rpm. After hybridization, the slides were washed and scanned by the SureScan (Agilent Technologies), the images were subsequently extracted using the Feature Extraction Software (Agilent Technologies). Extracted data with good QC metrics were normalized (percentile shift to the 75th percentile) and filtered by gene expression (20.0–100.0 percentile), flags for signals and error for CV in the GeneSpring GX (v 14.5) (Agilent Technologies). The processed data were subjected to statistical analysis (moderated T-test with Benjamini-Hochberg FDR), and the corrected p value <0.05 was determined to be significant (n = 3–5). The following analyses were performed for further data interpretation: principal component analysis (PCA), clustering analysis, GO (gene ontology) analysis, and pathway analysis with curated datasets of WikiPathways (413 pathways) and KEGG (10 pathways). A gene-set list associated with human WNT signaling pathway (150 genes, 40310 from KEGG pathways) was obtained from a public database (https://www.stemformatics.org/).

2.7. Quantitative RT-PCR (RT-qPCR). cDNA was synthesized from 1 μg of total RNA from UC-MSCs by using a QuantiTect reverse transcription kit (Qiagen). Real-time PCR analysis was performed with an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA) using FastStart Universal SYBR Green master mix (Roche) with 0.5 μM sense and antisense primers and cDNA (corresponding to 12.5 ng total RNA) according to the manufacturer’s instructions. Each cDNA was amplified with a preincubation hold at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 60 sec, and one cycle at 95°C for 15 sec, 60°C for 60 sec, 95°C for 15 sec, and 60°C for 15 sec. Relative expression of each transcript was calculated based on the ΔΔCt method using β-actin (ACTB) as an endogenous reference for normalization. Primer sequences for WNT2, WNT2B, WNT3A, WNT4, WNT5B, WNT6, SFRP1, and ACTB were shown in Table 2. All sample measurements were repeated at least three times, and the results were expressed as the mean ± SE.

2.8. Ki-67 Staining. Cell suspensions of UC-MSCs were centrifuged at 3000 rpm for 5 min, and two smears were immediately prepared. Slides were fixed in 95% ethanol for immunostaining or fixed in 20% formalin and 80% methanol and stained with hematoxylin and eosin (H&E), respectively. Immunostaining was performed with antibody against Ki-67 (Clone MIB-1, Dako, Santa Clara, CA) using Leica BondMax automation and Bond Polymer Refine detection kit (Leica Biosystems, Nussloch, Germany) according to manufacture’s instructions. IHC cytology protocol included primary antibody incubation for 15 min, post primary for 5 min, polymer for 8 min, peroxide block for 5 min, mixed DAB reine for 10 min, and followed by 5 min hematoxylin counterstaining.

2.9. MTS Assay. UC-MSCs were seeded at the density of 12,000 cells/well in a 12-well plate, incubated in 1 ml of MEM-α with 10% FBS in the presence or absence of 10 μM XAV939 (Selleck Chemicals, Houston, TX) at 37°C (5% CO2 and 95% air) for 24, 48, or 72 h. Cell proliferation was then determined by the CellTiter 96H AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) according to the manufacturer’s instruction. Briefly, 200 μl of MTS reagent (a tetrazolium compound) was added into each well and incubated at 37°C (5% CO2 and 95% air) for 4h. The absorbance at 490 nm was measured using an EnSpire Microplate Reader (Perkin Elmer, Poland, OR). All experiments were repeated at least three times, and the results were expressed as the mean ± SE.

2.10. Statistical Analysis. Pearson’s correlation coefficients were determined, and the Mann–Whitney U test was used to compare two independent datasets, using Excel software (Microsoft, Redmond, WA) and Excel Statistics (Statcel 3; Social Survey Research Information, Tokyo, Japan). Differences were considered statistically significant for p < 0.05.

3. Results

3.1. UC-MSCs Isolated from Infants Delivered at 22–40 Weeks of Gestation. We first obtained UCs from infants delivered at 22–40 weeks of gestation and then isolated the plastic-adherent cells from these UCs (Table 1). The cells exhibited a spindle-like shape (Figure 1(a)). Their cell surface markers were positive for MSC signature markers CD73, CD90, and CD105 but negative for hematopoietic, macrophage, and endothelial markers CD14, CD19, CD34, CD45, and HLA-DR by flow cytometric analysis (Figure 1(b)). There were no statistically significant differences in the percentages of MSC signature marker-positive cells (CD73: 99.9 ± 0.1%
| Sample | Gestational age (weeks) | Birth weight (g) | Sex | Apgar score 1 min | Apgar score 5 min | Paternal age (years) | Maternal age (years) | Gravidity | Parity | Perinatal history | Maternal complication | Usage in the present study |
|--------|------------------------|------------------|-----|-------------------|------------------|---------------------|----------------------|-----------|-------|------------------|------------------------|--------------------------|
| Pre-1  | 24                     | 530              | Female | 1                 | 3                | 42                  | 38                   | 2         | 2     | Cesarean section due to non-reassuring fetal status involved with abruption of placenta | Pregnancy-induced hypertension | Figures 2, 3, 5, and 6, S |
| Pre-2  | 25                     | 656              | Male  | 4                 | 8                | 26                  | 31                   | 0         | 0     | Cesarean section due to active premature labor | Cesarean section due to active premature labor | Figures 2, 3, 5, and 6, S |
| Pre-3  | 26                     | 338              | Male  | 2                 | 2                | 37                  | 36                   | 1         | 1     | Cesarean section due to active premature labor involved with placental hematoma | Cesarean section due to active premature labor | Figures 2, 3, 4, 5, and 6, S |
| Pre-4  | 22                     | 550              | Male  | 1                 | 4                | 26                  | 25                   | 1         | 1     | Cesarean section due to active premature labor | Cesarean section due to active premature labor | Figures 2, 3, 5, and 6, S |
| Pre-5  | 23                     | 530              | Female | 2                 | 4                | 32                  | 31                   | 1         | 1     | Vaginal delivery due to active premature labor | Cesarean section due to active premature labor | Figures 2, 3, 5, and 6, S |
| Pre-6  | 23                     | 478              | Female | 1                 | 5                | 32                  | 32                   | 2         | 1     | Cesarean section due to active premature labor | Cesarean section due to active premature labor | Figures 5 and 6, S |
| Pre-7  | 24                     | 642              | Male  | 1                 | 3                | 16                  | 17                   | 0         | 0     | Cesarean section due to active premature labor | Cesarean section due to active premature labor | Figures 1, 5, and 6, S |
| Pre-8  | 26                     | 750              | Female | 5                 | 9                | 39                  | 38                   | 2         | 1     | Cesarean section due to advancing pregnancy-induced hypertension | Cesarean section due to advancing pregnancy-induced hypertension | Figures 4, 5, and 6, S |
| Pre-9  | 26                     | 568              | Female | 4                 | 7                | 38                  | 36                   | 2         | 1     | Cesarean section due to non-reassuring fetal status | Cesarean section due to non-reassuring fetal status | Figure 4 |
| Int-1  | 30                     | 1546             | Female | 7                 | 8                | 44                  | 42                   | 0         | 0     | Cesarean section due to non-reassuring fetal status | Cesarean section due to non-reassuring fetal status | Figure 6, S |
| Int-2  | 31                     | 1170             | Male  | 4                 | 7                | 27                  | 28                   | 1         | 1     | Cesarean section due to non-reassuring fetal status | Cesarean section due to non-reassuring fetal status | Figure 6, S |
| Int-3  | 34                     | 2062             | Female | 7                 | 9                | 48                  | 42                   | 3         | 2     | Cesarean section due to non-reassuring fetal status | Cesarean section due to non-reassuring fetal status | Figure 6, S |
| Term-1 | 38                     | 2550             | Male  | 8                 | 8                | 37                  | 34                   | 0         | 0     | Cesarean section due to placental previa | Cesarean section due to placental previa | Figures 2, 3, and 6, S |
| Term-2 | 40                     | 2546             | Male  | 2                 | 6                | 40                  | 44                   | 1         | 1     | Cesarean section due to non-reassuring fetal status | Cesarean section due to non-reassuring fetal status | Figures 2, 3, and 6, S |
| Term-3 | 38                     | 3314             | Male  | 8                 | 8                | 40                  | 33                   | 0         | 0     | Cesarean section due to breach presentation | Cesarean section due to breach presentation | Figures 5 and 6, S |
| Term-4 | 37                     | 2750             | Female | 8                 | 9                | 36                  | 33                   | 0         | 0     | Cesarean section due to placental previa | Cesarean section due to placental previa | Figures 5 and 6, S |
| Term-5 | 37                     | 3062             | Male  | 9                 | 10               | 35                  | 35                   | 1         | 1     | Repeated cesarean section | Repeated cesarean section | Figure 5 |
| Sample | Gestational age (weeks) | Birth weight (g) | Sex | Apgar score 1 min | Apgar score 5 min | Paternal age (years) | Maternal age (years) | Gravidity | Parity | Perinatal history | Maternal complication | Usage in the present study |
|--------|------------------------|------------------|-----|------------------|------------------|---------------------|---------------------|-----------|-------|-----------------|------------------------|--------------------------|
| Term-6 | 37                     | 2776             | Female | 8 | 9 | 42 | 45 | 2 | 1 | Repeated cesarean section |                       | Figures 5 and 6, S |
| Term-7 | 38                     | 3390             | Male | 9 | 10 | 32 | 26 | 1 | 1 | Normal vaginal delivery |                       | Figures 5 and 6, S |
| Term-8 | 38                     | 2960             | Male | 8 | 9 | 38 | 39 | 1 | 1 | Repeated cesarean section |                       | Figures 1, 5, and 6, S |
| Term-9 | 38                     | 3144             | Female | 8 | 8 | 39 | 39 | 1 | 1 | Repeated cesarean section |                       | Figure 4 |
| Term-10| 38                     | 2666             | Female | 8 | 9 | 28 | 28 | 0 | 0 | Cesarean section due to breech presentation |                       | Figure 4 |
| Term-11| 39                     | 2892             | Female | 9 | 9 | 37 | 31 | 1 | 0 | Cesarean section due to breech presentation |                       | Figure 4 |
and 99.6 ± 0.4%, CD90: 99.9 ± 0.1% and 99.5 ± 0.5%, and CD105: 99.7 ± 0.4%) between preterm and term UCs.

Under standard in vitro differentiation conditions, both preterm and term UC-MSCs were induced to differentiate into osteocytes, adipocytes, and chondrocytes (Figure 1(c)). Preterm UC-MSCs did not qualitatively differ from term UC-MSCs in their capacity to differentiate into trilineage mesenchymal cells. Taken together, the resulting cells fulfilled the criteria defined by the ISCT position paper [22] and were defined as UC-MSCs.

### 3.2. Differentially Expressed Genes between Preterm and Term UC-MSCs.

To get an insight into the functional difference between preterm and term UC-MSCs, we extracted total RNA from five preterm and three term UC-MSCs (Table 1) and performed microarray analysis. Principal component analysis (PCA) for global gene expression revealed that preterm UC-MSC samples were clustered together and were separated from term UC-MSC samples (Figure 2(a)). In total, 5578 unique genes (4272 upregulated and 1306 downregulated) showed greater than twofold-expression changes between preterm and term UC-MSCs with a corrected p value less than 0.05 (Figure 2(b), Supplementary Table S1 available online at https://doi.org/10.1155/2017/8749751).

The pathway analysis of all differentially expressed genes identified significant enrichment of signaling pathways for immune/inflammatory reactions, cell-cell/cell-extracellular matrix interactions, glucose/lipid metabolism, and cell proliferation and differentiation (Table 3). Among these signaling pathways, we focused WNT signaling pathway that was previously implicated in the regulation of MSC proliferation and differentiation. Noticeably, 32/150 of WNT signaling pathway genes were overlapped with differentially expressed genes between preterm and term UC-MSCs (Figures 2(c) and 2(d)).

We then confirmed a subset of these WNT signaling pathway genes by RT-qPCR using cDNA from the same five preterm and three term UC-MSCs as a template. A subset included secreted WNT ligands and modulators: WNT2, WNT2B, WNT3A, WNT4, WNT5B, WNT6, and SFRP1. Consistent with microarray analysis, upregulated WNT2, WNT2B, WNT3A, WNT4, and WNT6 showed increased expression in preterm UC-MSCs compared to term UC-MSCs by RT-qPCR (Table 4, Figure 3). Decreased expression of downregulated WNT5B and SFRP1 was also detected by RT-qPCR (Table 4, Figure 3). Collectively, these results suggested that WNT signaling pathway gene expression in preterm UC-MSCs was distinct from term UC-MSCs.

### 3.3. Cell Proliferation of Preterm and Term UC-MSCs.

To examine the function of WNT signaling pathway genes in preterm and term UC-MSCs, we isolated UC-MSCs from nine preterm (22–26 weeks of gestation) and nine term (37–39 weeks of gestation) infants (Table 1) and analyzed their cell proliferation. We first evaluated the expression of Ki-67, a marker of proliferating cells expressed in all active phases of the cell cycle (G1, S, G2, and M), by immunocytochemistry [23]. The percentages of Ki-67-positive cells were markedly increased in preterm UC-MSCs as compared to term UC-MSCs, albeit not statistically significant (Figure 4).

We then analyzed cell proliferation of preterm and term UC-MSCs by MTS assay. Although both preterm and term UC-MSCs showed vigorous proliferation, the proliferation rate of preterm UC-MSCs measured at 72 h was significantly faster than term UC-MSCs (Figure 5(a)). Next, we examined the effect of WNT signaling inhibition on the growth of preterm and term UC-MSCs using a small molecule XAV939. XAV939 is a potent inhibitor of Tankyrase1 and Tankyrase2, and this inhibition stabilizes Axin1 and Axin2, the concentration-limiting component of the WNT pathway transcription factor β-catenin destruction complex. Increased levels of Axin1 and Axin2 stimulate β-catenin degradation and thereby inhibit β-catenin-mediated transcription [24]. Treatment of preterm UC-MSCs with 10 μM XAV939 resulted in significant inhibition of cell proliferation (Figure 5(b)). Term UC-MSC proliferation was also reduced by 10 μM XAV939, but there was no statistical significance (Figure 5(c)). These results suggest that WNT signaling is involved in the enhanced cell proliferation of preterm UC-MSCs compared to term UC-MSCs.

### 3.4. Gestational Age-Dependent Expression of WNT Signaling Pathway Genes.

We further analyzed WNT2, WNT2B, WNT3A, WNT4, WNT5B, WNT6, and SFRP1 expressions in UC-MSCs isolated from other 10 infants delivered at 22–40 weeks of gestation by RT-qPCR. Expression of these WNT signaling pathway genes tended to decrease or increase with gestational age. Among them, WNT2B expression showed a statistically significant negative correlation with gestational age (Figure 6, Supplementary Figure S1).

### 4. Discussion

In the present study, we isolated UC-MSCs from 23 infants delivered at 22–40 weeks of gestation and obtained the following findings. (1) Global gene expression in preterm UC-MSCs was distinct from term UC-MSCs. (2) WNT signaling pathway genes were enriched in differentially expressed genes between preterm and term UC-MSCs. (3) Preterm UC-MSC proliferation was faster than term UC-MSCs. (4) WNT signaling inhibitor XAV939 significantly inhibited the cell proliferation of preterm but not term UC-MSCs. (5) WNT signaling inhibition significantly increased cell proliferation of preterm UC-MSCs more than term UC-MSCs. We further analyzed WNT2, WNT2B, WNT3A, WNT4, WNT5B, WNT6, and SFRP1 expressions in UC-MSCs isolated from other 10 infants delivered at 22–40 weeks of gestation by RT-qPCR. Expression of these WNT signaling pathway genes tended to decrease or increase with gestational age. Among them, WNT2B expression showed a statistically significant negative correlation with gestational age (Figure 6, Supplementary Figure S1).
Figure 1: Characterization of UC-MSCs from term and preterm infants. (a) UC-MSCs from preterm (24 weeks of gestation, preterm UC-MSCs) and term (38 weeks of gestation, term UC-MSCs) newborns at passage numbers 6 to 7 were examined by phase-contrast microscopy. The images shown are representative of three independent experiments. Scale bars show 100 μm. (b) Preterm and term UC-MSCs were analyzed by flow cytometer using antibodies against MSC markers (CD14, CD19, CD34, CD45, CD73, CD90, CD105, and HLA-DR) defined by ISCT [22]. The histograms shown are representative of three independent experiments. (c) Preterm and term UC-MSCs were differentiated into adipocyte as visualized by Oil Red O and into osteocyte as visualized by Alizarin Red S and chondrocyte as visualized by Toluidine Blue. The images shown are representative of three independent experiments. Scale bars represent 50 μm.
Figure 2: Gene expression microarray analysis of preterm and term UC-MSCs. (a) PCA mapping of gene expression profile for preterm (pre-1-5) and term (term-1-3) UC-MSCs. (b) Heat map of 5578 differentially expressed gene with greater than twofold changes in preterm UC-MSCs as compared to term UC-MSCs at a corrected p value less than 0.05. Green color refers to low levels of gene expression and red color to high levels. (c) Pie chart of altered expression genes and WNT signaling pathway genes. The list shows overlapped 32 genes. (d) Heat map of 32 genes extracted from (c). Green color refers to low levels of gene expression and red color to high levels.
Table 3: Pathway analysis of differentially expressed genes between preterm and term UC-MSCs.

| Pathway                                                                 | p value     | Matched entities | Pathway entities |
|------------------------------------------------------------------------|-------------|------------------|------------------|
| Hs_Interferon_alpha-beta_signaling_WP1835_83224                        | 1.91E-09    | 29               | 122              |
| Hs_GPCR_downstream_signaling_WP1824_83301                              | 1.85E-07    | 171              | 919              |
| Hs_Interferon_gamma_signaling_WP1836_83234                             | 8.41E-06    | 32               | 170              |
| Hs_NRF2_pathway_WP2884_83041                                           | 3.20E-05    | 36               | 143              |
| Hs_Immunoregulatory_interactions_between_a_Lymphoid_and_a_non-Lymphoid_cell_WP1829_83164 | 5.33E-05    | 32               | 332              |
| Hs_Gastrin-CREB_signalling_pathway_via_PKC_and_MAPK_WP2664_83266        | 1.55E-04    | 42               | 180              |
| Hs_Focal_Adhesion_WP306_80308                                           | 2.25E-04    | 45               | 191              |
| Hs_GPCR_ligand_binding_WP1825_83346                                    | 2.99E-04    | 85               | 438              |
| Hs_Immunoregulatory_interactions_between_a_Lymphoid_and_a_non-Lymphoid_cell_WP1829_83164 | 5.33E-05    | 32               | 332              |
| Hs_Glycerophospholipid_biosynthesis_WP2740_83341                        | 4.59E-04    | 26               | 96               |
| Hs_Allograft_Rejection_WP2328_78554                                     | 5.49E-04    | 24               | 100              |
| Hs_Immunoregulatory_interactions_between_a_Lymphoid_and_a_non-Lymphoid_cell_WP1829_83164 | 5.33E-05    | 32               | 332              |
| Hs_Extracellular_matrix_organization_WP2703_83106                       | 6.47E-04    | 22               | 78               |
| Hs_Selenium_Micronutrient_Network_WP15_82705                            | 6.56E-04    | 23               | 84               |
| MAPK signaling pathway                                                  | 7.53E-04    | 55               | 257              |
| Hs_MicroRNAs_in_cardiomyocyte_hypertrophy_WP1544_75258                 | 7.89E-04    | 23               | 104              |
| Hs_DNA_Damage_Response_(only_ATM_dependent)_WP710_79974                 | 8.29E-04    | 29               | 114              |
| Hs_Parkin-Ubiquitin_Proteasomal_System_pathway_WP2359_72121             | 9.16E-04    | 20               | 73               |
| Hs_Cell_surface_interactions_at_the_vascular_wall_WP1794_83824          | 0.0010894155| 25               | 99               |
| Hs_BDNF_signaling_pathway_WP2380_79953                                  | 0.0012996251| 34               | 144              |
| Hs_Nuclear_Receptors_Meta-Pathway_WP2882_83040                          | 0.0013138579| 62               | 318              |
| Hs_Wnt_Signaling_Pathway_WP428_79854                                    | 0.0013306512| 19               | 67               |
| Hs_B_Cell_Receptor_Signaling_Pathway_WP23_79985                         | 0.0014992601| 25               | 98               |
| Hs_MAPK_Signaling_PATHWAY_WP382_79951                                   | 0.0016350249| 38               | 168              |
| Hs_SIIDS_Susceptibility_Pathways_WP706_80056                            | 0.0017464098| 37               | 166              |
| Hs_O-linked_glycosylation_WP3315_83262                                  | 0.0020349505| 25               | 104              |
| Hs_NG_F_signalling_via_TRKA_from_the_plasma_membrane_WP1873_83147       | 0.0023317037| 20               | 77               |
| Hs_Arachidonic_acid_metabolism_WP2650_83044                             | 0.0026565776| 15               | 53               |
| Hs_Vitamin_B12_Metabolism_WP1533_82707                                  | 0.0026565776| 15               | 53               |
| Hs_Folate_Metabolism_WP176_82704                                       | 0.0028243104| 18               | 67               |
| Hs_NGC signalling_via_TRKA_from_the_plasma_membrane_WP1873_83147       | 0.003263897 | 10               | 28               |
| Hs_Muscle_contraction_WP1864_83290                                      | 0.0033954314| 17               | 63               |
| Hs_NCAM_signalling_for_neurite_out-growth_WP1866_83314                 | 0.003398262 | 12               | 40               |
| Hs_Corticotropin-releasing_hormone_WP2355_79973                         | 0.0034443145| 23               | 92               |
| Hs_Cori_Cycle_WP1946_79691                                             | 0.0037244426| 7                | 23               |
| Pathway                                                                 | \( p \) value    | Matched entities | Pathway entities |
|------------------------------------------------------------------------|------------------|------------------|------------------|
| Hs_Eicosanoid_Synthesis_WP167_82702                                    | 0.0037961914     | 8                | 25               |
| Hs_Serotonin_Receptor_2_and_ELK-SRF-GATA4_signaling_WP732_80010        | 0.0037961914     | 8                | 20               |
| Hs_Semaphorin_interactions_WP1907_83271                                | 0.004035725      | 18               | 67               |
| Hs_Histidine_lysine_phenylalanine_tyrosine_proline_and_trypotoan_catabolism_WP3573_83463 | 0.005489827      | 12               | 39               |
| Hs_ACE_Inhibitor_Pathway_WP554_77712                                   | 0.0055746404     | 7                | 17               |
| Hs_Integrin-mediated_Cell_Adhesion_WP185_80036                         | 0.005693194      | 24               | 101              |
| Hs_Prostaglandin_Synthesis_and_Regulation_WP98_72088                   | 0.0058502043     | 10               | 31               |
| Hs_GPCR_s.Class_A_Rhodopsin-like_WP455_81793                            | 0.0062826765     | 50               | 262              |
| Hs_Phase_I_functionalization_of_compounds_WP1879_83057                 | 0.006725243      | 21               | 88               |
| Hs_Complement_and_Coagulation_Cascades_WP558_79680                      | 0.00709079       | 16               | 61               |
| Hs_Lipid_digestion_mobilization_and_transport_WP2764_83187             | 0.0071218973     | 15               | 61               |
| Hs_Type_II_diabetes_mellitus_WP1584_81779                               | 0.0074452385     | 8                | 22               |
| Hs_miRNA_targets_in_ECM_and_membrane_receptors_WP2911_83020             | 0.0074452385     | 8                | 45               |
| Hs_Insulin_Signaling_WP481_82731                                       | 0.008477005      | 34               | 161              |
| Hs_L1CAM_interactions_WP1843_83082                                     | 0.008832967      | 21               | 92               |
| Hs_Calcium_Regulation_in_the_Cardiac_Cell_WP536_80211                   | 0.008975188      | 32               | 150              |
| Hs_Cell_junction_organization_WP1793_83402                              | 0.00935909       | 20               | 85               |
| Hs_XBP1(S)_activates_chaperone_genomes_WP3472_83243                     | 0.01072612       | 22               | 98               |
| Hs_G1_to_S_cell_cycle_control_WP45_80001                                | 0.010931775      | 17               | 68               |
| Hs_Prostate_Cancer_WP2263_80439                                        | 0.010931775      | 25               | 117              |
| Hs_Wnt_Signaling_Pathway_and_Pluripotency_WP399_79474                    | 0.011321301      | 23               | 101              |
| Hs_Human_Complement_System_WP2806_83005                                  | 0.011321301      | 23               | 136              |
| Hs_Assembly_of_collagen_fibrils_and_other_multimeric_structures_WP2798_83231 | 0.0113662705     | 9                | 29               |
| Hs_DSCAM_interactions_WP1808_83372                                      | 0.0118092755     | 5                | 11               |
| Hs_Arrhythmogenic_Right_Ventricular_Cardiomyopathy_WP2118_71265          | 0.012062866      | 18               | 78               |
| Hs_Endothelin_Pathways_WP2197_74852                                     | 0.0121577475     | 10               | 33               |
| Hs_Nuclear_Receptors_WP170_71083                                       | 0.012546546      | 11               | 38               |
| Hs_Metapathway_biotransformation_WP702_73516                             | 0.013431928      | 34               | 188              |
| Hs_Potassium_Channels_WP2669_83272                                     | 0.0139101725     | 15               | 59               |
| Hs_Myometrial_Relaxation_and_Contraction_Pathways_WP289_81078           | 0.014395579      | 32               | 156              |
| Hs_Collagen_biosynthesis_and_modifying_enzymes_WP2725_83130             | 0.014458477      | 14               | 54               |
| Hs_Cardiac_Hypertrophic_Response_WP2795_78544                            | 0.014458477      | 14               | 54               |
| Hs_IL-3_Signaling_Pathway_WP286_78583                                   | 0.014911717      | 13               | 49               |
| Hs_Hematopoietic_Sem_Cell_Differentiation_WP2849_83039                  | 0.015318828      | 11               | 98               |
| Pathway                                                                 | p value     | Matched entities | Pathway entities |
|------------------------------------------------------------------------|-------------|------------------|------------------|
| Hs_Telomere_Maintenance_WP1928_83097                                   | 0.016210536| 15               | 61               |
| Hs_Signaling_Pathways_in_Glioblastoma_WP2261_81197                     | 0.016799    | 19               | 83               |
| Glycolysis/Gluconeogenesis                                              | 0.017775405| 16               | 66               |
| Hs_Reversible_hydration_of_carbon_dioxide_WP2770_83176                 | 0.01794276  | 5                | 12               |
| Hs_Transport_of_inorganic_cations-anions_and_amino_acids-oligopeptides_WP1936_83267 | 0.01851932  | 21               | 97               |
| Hs_Neural_Crest_Differentiation_WP2064_79263                           | 0.0192032   | 22               | 101              |
| Hs_Signaling_by_the_B_Cell_Receptor_(BCR)_WP2746_83158                 | 0.020346014 | 24               | 247              |
| Hs_Adipogenesis_WP236_80209                                             | 0.02145827  | 27               | 131              |
| Hs_Secretion_of_Hydrochloric_Acid_in_Parietal_Cells_WP2597_78485       | 0.021901488 | 3                | 5                |
| Pathways in cancer                                                      | 0.023056423 | 59               | 327              |
| Hs_NLR_Proteins_WP288_80026                                             | 0.02675793  | 4                | 10               |
| Hs_Neurotransmitter_Release_Cycle_WP1871_83254                          | 0.02710882  | 10               | 39               |
| Hs_Transport_of_vitamins_nucleosides_and_related_molecules_WP1937_83207 | 0.02710882  | 10               | 38               |
| Hs_S_Phase_WP2772_83395                                                 | 0.02769413  | 25               | 123              |
| Hs_Integrin_cell_surface_interactions_WP1833_83181                     | 0.028421601 | 15               | 66               |
| Hs_GABA_synthesis_release_reuptake_and_degradation_WP2685_83090         | 0.03075299  | 6                | 19               |
| Hs_Transport_of_glucose_and_other_sugars_bile_salts_and_organic_acids_and_amine_compounds_WP1935_83132 | 0.0316663   | 21               | 100              |
| Hs_Class_I_MHC_mediating_antigen_processing_presentation_WP3577_83467   | 0.032386538 | 49               | 330              |
| Hs_Formation_of_Fibrin_Clot_(Clotting_Cascade)_WP1818_83143             | 0.032393858 | 10               | 39               |
| Hs_Parkinsons_Disease_Pathway_WP2371_79766                              | 0.032393858 | 10               | 71               |
| Hs_ErbB_Signaling_Pathway_WP673_80202                                   | 0.032395784 | 13               | 55               |
| Hs_Interferon_type_1_signaling_pathways_WP585_80201                     | 0.032395784 | 13               | 54               |
| Hs_Metabolism_of_water-soluble_vitamins_and_cofactors_WP1857_83083     | 0.03357853  | 19               | 93               |
| Hs_Primary_Focal_Segmental_Glomerulosclerosis_FSGS_WP2572_79947         | 0.033947315 | 16               | 74               |
| Hs_Glycolysis_and_Gluconeogenesis_WP534_78585                           | 0.034427498 | 12               | 49               |
| Hs_Interleukin-11_Signaling_Pathway_WP2332_79525                         | 0.034641972 | 11               | 44               |
| Hs_Sleep_regulation_WP3591_83861                                        | 0.03482186  | 10               | 39               |
| Hs_Regulation_of_toll-like_receptor_signaling_pathway_WP1449_81172      | 0.03940498  | 27               | 149              |
| Hs_PI_Metabolism_WP2747_83160                                           | 0.03972961  | 12               | 51               |
| Hs_Signal_regulatory_protein_(SIRP)_family_interactions_WP1909_83190   | 0.0397491   | 4                | 11               |
| Hs_Neurotoxicity_of_clostridium_toxins_WP2665_83321                     | 0.0397491   | 4                | 22               |
| Hs_Overview_of_nanoparticle_effects_WP3287_82926                        | 0.039779507 | 6                | 22               |
| Hs_Protein_folding_WP1892_83103                                         | 0.039869573 | 9                | 34               |
| Hs_Platelet_homeostasis_WP1885_83192                                    | 0.041275427 | 15               | 70               |
| Pathway                                                                 | p value      | Matched entities | Pathway entities |
|------------------------------------------------------------------------|--------------|------------------|------------------|
| Hs_Synthesis_of_DNA_WP1925_83144                                       | 0.04189585   | 20               | 98               |
| Hs_Pathogenic_Escherichiacoli_infection_WP2272_78594                   | 0.042428713  | 13               | 64               |
| Hs_Mitotic_G1-G1-S_phases_WP1858_83315                                  | 0.042929105  | 25               | 128              |
| Hs_Synaptic_Vesicle_PATHWAY_WP2267_78595                                | 0.04557775   | 12               | 51               |
| Hs_Spinal_Cord_Injury_WP2431_80343                                      | 0.046727844  | 23               | 117              |
| Hs_Gamma_carboxylation_hypusine_formation_and_arylsulfatase_activation_WP2762_83388 | 0.04724196   | 9                | 36               |
| Hs_RAF-MAP_kinase_cascade_WP2735_83142                                   | 0.047904383  | 36               | 216              |
| Hs_Binding_and_Uptake_of_Ligands_by_Scavenger_Receptors_WP2784_83217     | 0.048846867  | 11               | 195              |
| Hs_Extracellular_vesicle-mediated_signaling_in_recipient_cells_WP2870_79555 | 0.04923168   | 8                | 30               |
UC-MSCs. (5) WNT2B expression in UC-MSCs showed a significant negative correlation with GA.

MSCs are isolated from a variety of tissues and result in so heterogeneous population of cells, and not all of them express the same phenotypic markers. In the case of BM-MSCs, younger donor-derived BM-MSCs showed greater proliferative and differentiative potential than older counterparts and may have more potential for cell therapy [25, 26]. Although fetal MSCs could be isolated from newborns delivered at a wide range of GA as a result of preterm, term, and postterm delivery, their GA-dependent function remained poorly characterized [8, 9]. With regard to UCB-MSCs, the

Table 4: Differentially expressed WNT pathway genes between preterm and term UC-MSCs.

| Gene       | FC (pre versus term) | p (Corr)  |
|------------|----------------------|-----------|
| **Ligands**|                      |           |
| WNT2       | 4.07917              | 0.01631   |
| WNT2B      | 2.64791              | 0.02698   |
| WNT3A      | 2.56017              | 0.02584   |
| WNT6       | 2.35531              | 0.00938   |
| WNT4       | 2.11623              | 0.00510   |
| WNT5B      | −3.93533             | 0.01317   |
| **Receptors** |                   |           |
| FZD9       | 3.47820              | 0.04035   |
| TCF7L2     | 2.29222              | 0.01187   |
| **Extracellular modulators** | | |
| DKK4       | 3.79029              | 0.01143   |
| DKK2       | −2.65327             | 0.01720   |
| SFRP1      | −3.13336             | 0.00621   |
| **Intracellular signaling molecules** | | |
| CCND2      | 3.49892              | 0.02933   |
| DAAM2      | 2.71289              | 0.00467   |
| CER1       | 2.40160              | 0.03446   |
| MAPK8      | 2.36091              | 0.00501   |
| NFATC4     | 2.25687              | 0.01089   |
| APC2       | 2.21774              | 0.04864   |
| PPP2R5B    | 2.20956              | 0.00501   |
| PRKCB      | 2.11780              | 0.00504   |
| CAMK2A     | 2.03443              | 0.02825   |
| APC        | −2.01861             | 0.00578   |
| FOSL1      | −2.06872             | 0.03346   |
| PRKACA     | −2.16403             | 0.02705   |
| PPP2R1A    | −2.19719             | 0.04119   |
| CCND3      | −2.20727             | 0.01748   |
| RUVBL1     | −2.24427             | 0.02646   |
| AXIN1      | −2.47832             | 0.02670   |
| RAC2       | −2.51880             | 0.00902   |
| TBL1X      | −2.73244             | 0.01261   |
| DVL1       | −2.77299             | 0.00726   |
| NFATC3     | −2.82561             | 0.02238   |
| CCND1      | −3.28580             | 0.03436   |

Figure 3: WNT signaling pathway gene expression in preterm and term UC-MSCs. The relative expression of WNT2, WNT2B, WNT3A, WNT4, WNT6, WNT5B, and SFRP1 mRNA in preterm \((n = 3)\) and term \((n = 5)\) UC-MSCs was analyzed by RT-qPCR. The mean of term UC-MSCs was set as 1. The results shown are the mean ± SE.

Figure 4: Ki-67 staining of preterm and term UC-MSCs. (a) Smears of preterm \((n = 3)\) and term \((n = 3)\) UC-MSCs were prepared, immunostained with anti-Ki-67 antibody, and counterstained with hematoxylin. The images shown are representative of three independent experiments. (b) The percentage of Ki-67 positive was determined by manually counting 1000 cells and expressed as the mean ± SE.
MSC population in UCB was significantly higher in preterm newborn compared to term newborn [27, 28]. In the case of UC-MSCs, MSCs were isolated from different UC compartments including cord lining, perivascular region (PV), Wharton’s jelly (WJ), and whole UC [29–31]. Preterm UCs were shown to contain more perivascular cells (PVCs), identical to MSCs, than term UCs [32]. Preterm PVCs/UC-MSCs isolated from fetuses aborted at 8–12 weeks of gestation were reported to exhibit a greater proliferative potential, a more efficient differentiation into chondrogenic and adipogenic cell lineages, and a differential gene expression profile compared to term PVCs/UC-MSCs isolated from newborns delivered at 37–40 weeks of gestation [33]. Although we isolated UC-MSCs from the whole UC and preterm newborns delivered at 22–26 weeks of gestation, the present study and others supported that proliferative capacity of UC-MSCs declined with GA.

Global gene expression analysis identified 5578 differentially expressed genes between preterm and term UC-MSCs (Figure 2(a), Table S1). The pathway analysis revealed significant enrichment of 111 signaling pathways (Table 3). Immune/inflammatory reaction-associated signaling pathways were top-ranked among the list (Table 3). The upregulation of interferon (IFN) signaling pathways in preterm UC-MSCs may be interpreted as the consequence of

Figure 5: Cell proliferation of preterm and term UC-MSCs. Preterm (n = 8) and term (n = 6) UC-MSCs were cultured in the absence or presence of XAV939 for 24, 48, and 72 h. Their cell proliferation was determined by MTS assay and expressed as the percent increase. The results shown are the mean ± SE of (a) preterm and term UC-MSCs, (b) preterm UC-MSCs ± XAV939, and (c) term UC-MSCs ± XAV939.

Figure 6: Gestational age-dependent expression of WNT signaling pathway genes. The relative expression of WNT2B mRNA in UC-MSCs isolated from 18 infants delivered at 22–40 weeks of gestation was analyzed by RT-qPCR. The mean of all UC-MSCs was defined as 1.
preterm delivery that has inherent fetal and/or maternal indications (Table 1, Table 3). Although cell cycle and senescence-associated secretory phenotype pathways were also expected to affect the growth rate and GA-dependent changes of UC-MSCs, these pathways were not included in the list (Table 3).

WNT signaling is a key regulator of stem cell functions in development, renewal, and regeneration of multiple tissues [13–15]. In the case of MSCs, mRNA expression of a subset of WNT signaling pathway genes including WNT2, WNT4, WNT5A, WNT11, WNT16, SFRP2, SFRP3, and SFRP4 was detected in BM-MSCs [34]. WNT2, WNT2B, WNT4, WNT5A, WNT5B, SFRP1, and SFRP4 were also highly expressed in AT-MSCs under hypoxic stress conditions [35]. Comparison of BM-MSCs with UC-MSCs revealed lower differentiation capacity toward osteocytes and adipocytes along with the downregulation of WNT3A, WNT5A, WNT5B, WNT7B, WNT8A, SFRP1, and SFRP4 in UC-MSCs compared to BM-MSCs [36]. Consistent with these observations, the present study revealed a significant enrichment of WNT2, WNT2B, WNT3A, WNT4, WNT5B, WNT6, and SFRP1 in differentially expressed genes between preterm and term UC-MSCs (Figure 2(c)). Noticeably, WNT2, WNT2B, WNT4, WNT5B, WNT6, and SFRP1 were associated with a noncanonical WNT pathway, as opposed to only WNT3A with a canonical WNT pathway among these WNT ligands and modulators in UC-MSCs [12]. In contrast, the enhanced cell proliferation of preterm UC-MSCs was abolished by XAV939, which selectively decreased β-catenin expression through Tankyrase1 and Tankyrase2 inhibition and increased Axin1 and Axin2 expression (Figure 5) [24]. Accumulating evidence indicates that noncanonical WNT signaling can inhibit canonical WNT signaling [37, 38] and that activation of either canonical or noncanonical WNT signaling is highly dependent on the cell type and on specific receptors expressed by the cells [39, 40]. Further understanding of how WNT signaling pathway controls the GA-dependent proliferation of UC-MSC will be crucial to develop UC-MSC-based cell therapy.

In summary, preterm UC-MSC proliferation is significantly faster than term UC-MSCs, and WNT signaling is involved in the regulation of this GA-dependent proliferation of UC-MSCs.

**Conflicts of Interest**

The authors declare that there is no conflict of interest regarding the publication of this paper.

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