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Effects of Age and Gender on WNT Gene Expression in Human Bone Marrow Stromal Cells

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ABSTRACT

WNT signaling pathways play important roles in the behavior of human bone marrow stromal cells. Although WNT expression has been examined in human bone marrow stromal cells (hMSCs) with limited numbers of subjects or from commercial sources, there are conflicting results on WNT gene expression in hMSCs. Furthermore, the effects of age and gender on WNT expression in hMSCs are largely unknown. In this study, we evaluated RNA expression of all the WNT genes in hMSCs from 19 subjects, 12 women and 7 men, aged from 36 to 85 years. Analysis of WNT gene expression in young and old groups indicated that WNT7B and 14 were expressed significantly higher in the young group. WNT2 and WNT13 showed a trend of higher expression in young group. WNT7B, 13, and 14 were inversely correlated with age. Further analysis for gender-specific difference indicated that WNT16 was expressed significantly higher in men than in women. WNT11 showed a trend of higher expression in hMSCs from women. For the hMSCs from women, WNT13 was inversely correlated with age and WNT4 was positively correlated with age. For the hMSCs from men, WNT7B and WNT14 were inversely correlated with age. These data indicated that most of the age-related WNT genes belong to the canonical WNT signaling pathway. Further, there are gender-specific differences in the expression of WNT4, 7B, 13, 14, and 16 in hMSCs. Age and gender account for many of the sample-to-sample variations in WNT gene expression in human marrow stromal cells. J. Cell. Biochem. 106: 337–343, 2009.

KEY WORDS: WNT; HUMAN MARROW STROMAL CELLS; AGE; GENDER; EXPRESSION PROFILING

Human bone marrow stromal cells (hMSCs), also known as bone marrow-derived mesenchymal stem cells, have the potential to differentiate into various types of connective tissue cells, including osteoblasts, chondrocytes, and adipocytes [Imabayashi et al., 2003; Sekiya et al., 2004; Titorencu et al., 2007]. Wnt proteins constitute a family of secreted proteins that regulate many aspects of gene expression, cell proliferation, and cell differentiation [Gregory et al., 2003; Nusse, 2008]. Evidence shows that Wnt signaling pathways play important roles in cell behavior of MSCs from various species. The canonical WNT signaling is critically involved in the regulation of the proliferation, as well as of the migration/invasion capacity of hMSCs [Neth et al., 2006]. There is evidence that the canonical Wnt signaling pathway is essential for bone formation [Piters et al., 2008]. Further, bone morphogenic protein 2 stimulation of osteogensis by hMSCs in vitro is sustained by WNT signaling [Gregory et al., 2005]. It has also been reported that activation of the canonical Wnt/β-cantenin signaling pathway promotes chondrocyte differentiaton in the murine cell line C3H10T1/2 [Yano et al., 2005]. Sustained WNT protein expression was detected in chondral constructs generated with hMSCs [Nishioka et al., 2005]. Due to the importance of WNT signaling during normal development or pathological progress of disease, WNT expression has been examined in rat hepatic stellate cells [Jiang et al., 2006], human brain tumors [Howng et al., 2002], and chondroinduced human dermal fibroblasts [Yates, 2004].

Previously, we examined WNT signaling pathway in KM101 cells, a line of immortalized hMSCs; 12 WNT genes were constitutively expressed and many were regulated in those cells [Zhou et al., 2004]. Recently, WNT gene expression has been studied in normal hMSCs by several groups. Boland et al. [2004] reported that 7 of the 19 WNT family members were expressed in one sample of hMSCs. Etheridge et al. [2004] described expression of 5 WNT genes in hMSCs isolated from three donors and a commercial source. Okoye et al. [2008] reported that 13 WNT genes were expressed in hMSCs that were acquired from a commercial source. Data from those reports showed some agreement and some differences. WNT5A was the only WNT

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expressed in hMSCs in all the studies and all found that there was no expression of WNT8A, 8B, and 9B. Only the work with the immortalized line showed expression of WNT10A [Zhou et al., 2004].

We speculated that age and gender and other clinical variables may account for the discrepancies regarding WNT expression in hMSCs in the literature. Our previous studies indicated an effect of age on many properties of hMSCs [Cheleuitte et al., 1998; Mueller and Glowacki, 2001; Zhou et al., 2008]. It is known that Wnt signaling pathways are dysregulated in the Klotho aging mouse [Liu et al., 2007]. In addition, Wnt is required for sex differentiation [Kozopas et al., 1998; Vainio et al., 1999] and is expressed with gender-specificity in neurons from women and men with Parkinson's Disease [Cantuti-Castelvetri et al., 2007].

In this study, we evaluated constitutive expression of the 19 known WNT genes in hMSCs from young and old women and men.

MATERIALS AND METHODS

SUBJECTS

Bone marrow samples were obtained with IRB approval from femoral tissue discarded during hip replacement surgery for advanced non-inflammatory osteoarthritis. Exclusion criteria were cancer, rheumatoid arthritis, and other comorbid conditions that may influence skeletal metabolism, such as renal insufficiency, alcoholism, active liver disease, malabsorption, hyperthyroidism, ankylosing spondylitis, aseptic necrosis, hyperparathyroidism, morbid obesity, and diabetes. Also excluded were patients who were taking medications that may influence skeletal metabolism (e.g., thyroid hormone, glucocorticoids, NSAIDs, and bisphosphonates). A total of 19 subjects, 12 women and 7 men, age ranging from 36 to 85 years old, were included in this study. Of them, 9 subjects were classified as young ($\leq$50-years, mean 42.0 ± 4.6 years) and 10 were classified as old ($\geq$55-years, mean 69.3 ± 9.5 years). There were 12 samples from women (37- to 85-years, mean 58.6 ± 16.0 years) and 7 from men (36- to 82-years, mean 52.6 ± 16.0 years).

PREPARATION OF HUMAN BONE MARROW-DERIVED STROMAL CELLS

Human bone marrow stromal cells (hMSCs) were prepared from femoral bone marrow that was obtained as discarded material from patients undergoing hip replacement. Low-density mononuclear cells were isolated by density centrifugation on Ficoll/Histopaque 1077 (Sigma, MO). This procedure enriches for undifferentiated cells and includes a fraction of non-adherent hematopoietic cells and a fraction capable of adherence and differentiation into musculoskeletal cells. The low density cells were placed into tissue culture dishes and were cultured in phenol red-free α-MEM medium, 10% Fetal Bovine Serum–Heat Inactivated (FBS-HI), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a humid atmosphere with 5% CO_2. After 24 h, non-adherent cells were removed, and adherent hMSCs were expanded. Medium was changed twice a week. On reaching confluence, the cells were subcultivated at 1:5 ratio. Passage 2 or 4 cells were used.

In each experiment, standardized conditions were used for all samples, including the same cell passage, identical medium, serum, and reagents. For RNA isolation, cells were cultured, harvested, and stored in TRIZOL reagent (Invitrogen) at −80°C for analysis at the same time to avoid technical differences between assays.

RNA ISOLATION AND RT-PCR

Total RNA was isolated from hMSCs with TRIZOL reagent when they were at 80% confluence. For RT-PCR, 2 μg of total RNA was reverse-transcribed into cDNA with M-MLV (Promega), following the manufacturer's instructions. One-twentieth of the cDNA was used in each 50 μl PCR reaction (30–40 cycles of 94°C for 1 min, 55–60°C for 1 min, and 72°C for 2 min) as described [Zhou et al., 2004]. The gene-specific primers for human WNT genes were previously described [Yates, 2004]. PCR products were measured by densitometry of captured digitized images with KODAK Gel Logic 200 Imaging System and KODAK Molecular Imaging Software, following the manufacturer's instructions (KODAK, Molecular Imaging Systems, New Haven, CT). Gene expression levels were measured by semi-quantitative RT-PCR relative to the level of expression of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

STATISTICAL ANALYSES

All experiments were performed at least in triplicate. Group data were presented as mean value $\pm$ standard deviation (SD). Datum that was more than $\pm 5 \times$ SD from the mean of the rest of the samples was excluded as an outlier; this occurred in analyses for WNT2, 4, 7B, and 10B. Data were analyzed with unpaired *t*-test or non-parametric tools, either the Mann–Whitney test for group comparisons or Spearman correlation test, when appropriate. A value of $P < 0.05$ was considered significant; if the P value was higher than 0.05 but less than 0.1, it was reported as a trend.

RESULTS

EFFECTS OF PASSAGE ON EXPRESSION OF WNT GENES IN hMSCs

In a pilot study we tested whether WNT expression was affected by cell passaging. A comparison of gene expression of WNT2, 5A, and 10B was performed, with second passage (P2) and fourth passage (P4) cells from the same subjects. Differences in WNT gene expression were found: WNT 2 increased (2.0-fold) and WNT10B decreased to 40% of P2, WNT5 was unchanged with passaging (Fig. 1). Subsequent experiments used P2 cells.

TWELVE WNT GENES WERE EXPRESSED IN HUMAN BONE MARROW STROMAL CELLS

We surveyed WNT gene expression in hMSCs from 19 subjects. Expression of all known 19 human WNT genes including both canonical and non-canonical WNT genes was analyzed by RT-PCR in hMSCs cultured in basal medium. Twelve of the 19 WNT genes, WNT2, 3, 4, 5A, 5B, 6, 7B, 10B, 11, 13, 14, and16B were expressed in hMSC cultures, although many showed a wide range of expression in this series of specimens (Fig. 2). Expression of WNT1, 3A, 7A, 8A,

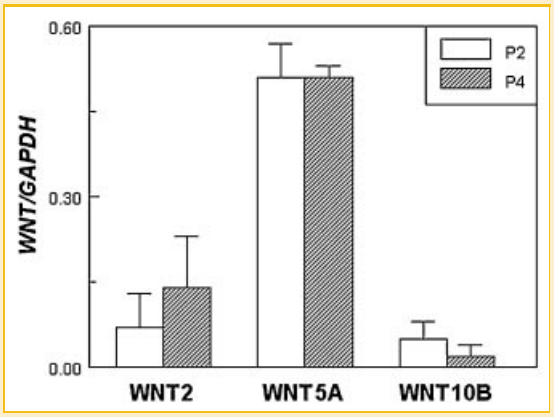

Fig. 1. Effect of passage on the expression of WNT genes in hMSCs. Expression of WNT2, WNT5A, and WNT10B was measured in passage 2 (P2) and passage 4 (P4) for five samples of hMSCs. Data are shown as the mean ± standard deviation (SD) for each gene relative to expression of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

8B, 10A, and 15 was not detectable in any of the 19 samples hMSCs with this method (data not shown). The expression levels of WNT5A and WNT5B appeared similar in hMSCs from the 19 subjects. Dramatic differences were seen for the other WNT genes. Some specimens showed absence of WNT genes that were strongly expressed in others.

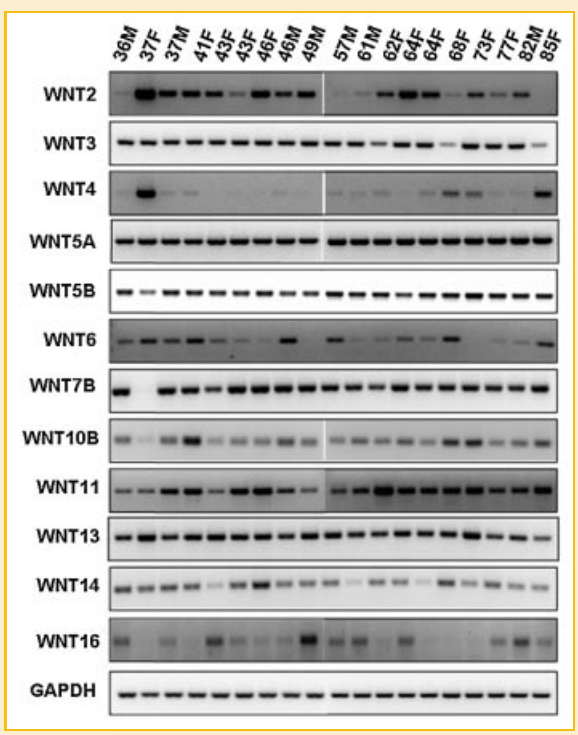

Fig. 2. Expression of WNT genes in hMSCs. RNA was extracted from hMSCs from 19 subjects, age 36 to 85 years, including women (F) and men (M). RT-PCR products were separated by Gel electrophrosis, and the bands were captured digitally with KODAK Gel Logic 200 Imaging System.

EFFECTS OF AGE ON THE EXPRESSION OF WNT GENES IN hMSCs

We tested whether there is an effect of age on the expression of WNT genes in hMSCs. We compared expression level of every detected WNT gene in hMSCs obtained from a group of young ($n = 9$, <50 years old), and a group of old ($n = 10$, >55 years old) subjects (Fig. 3, upper). There was significantly greater expression of WNT7B and WNT14 (1.3-fold, $P = 0.011$; 1.6-fold, $P = 0.020$ respectively) in hMSCs obtained from the young group compared with the old group. In addition, WNT2 and WNT13 showed trends of higher expression in the young group compared with the older group (2.2-fold, $P = 0.088$; 1.3-fold, $P = 0.077$ respectively). The expression of many of the other WNT genes ranged from 1.1- to 3.8-fold higher in the young group, compared with the older group. Expression of WNT4 appeared higher in the older group (4-fold), but this difference was not statistically significant. With the numbers available, the other WNT genes did not show difference between the two age groups.

Analysis of correlations between the expression of WNT genes and age showed that there were inverse relations with age for WNT7B expression (Spearman $r = -0.477$, $P = 0.045$), WNT13 expression (Spearman $r = -0.538$, $P = 0.018$), and in WNT14 expression (Spearman $r = -0.541$, $P = 0.017$) (Fig. 4). None of the other WNT genes showed a significant relationship with age.

EFFECTS OF GENDER ON THE EXPRESSION OF WNT GENES IN hMSCs

We tested whether there were gender differences in WNT gene expression in hMSCs (Fig. 3, lower). There were 12 samples from women (37- to 85-year, mean 58.6 ± 16.0 years) and 7 from men (36- to 82-year, mean 52.6 ± 16.0 years). There was significantly greater expression of WNT16B (threefold, $P < 0.01$) in hMSCs obtained from men, compared with women. WNT11 showed a trend of higher expression in the hMSCs from women, compared with men (1.74-fold, $P = 0.058$). WNT2 was expressed higher in hMSCs from women, compared with men (1.38-fold), and WNT13 expression levels were higher in hMSCs from men than women (1.1-fold), but neither of these was statistically significant. The expression of many of the other WNT genes including WNT 3, 5A, 5B, 6, 7B, 10B, and 14 was slightly higher in hMSCs from men, compared with women (1.02- to 1.22-fold), but they were not statistically significant with the numbers available.

GENDER-SPECIFIC EFFECTS OF AGE ON THE EXPRESSION OF WNT GENES IN hMSCs

Subanalysis of WNT gene expression by age in women and men revealed gender-specific relationships (Fig. 5). WNT5A expression in hMSCs was slightly but significantly higher in hMSCs from 5 young (37- to 46-year, mean 42.0 ± 3.3) women (1.16-fold, $P = 0.040$) compared with the group of 7 old women (62- to 85-year, mean 70.4 ± 8.4). WNT13 showed a trend for higher expression in hMSCs obtained from young women (1.4-fold, $P = 0.070$) than hMSCs from old women. There were no significant differences in expression of the other 10 WNT genes in the hMSCs from groups of young and old women. Analysis for correlation with age showed an inverse relationship for WNT13 expression in women ($r = -0.608$,

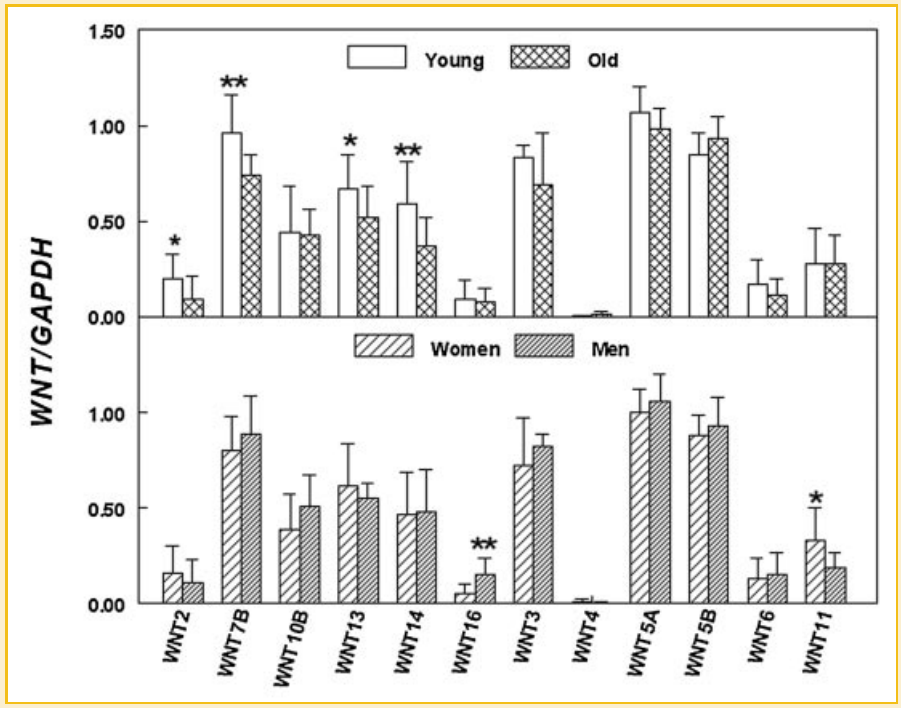

Fig. 3. Effects of age and gender on the expression of WNT genes in hMSCs. Upper graph: effects of age on the expression of WNT Genes in hMSCs. Expression of each WNT gene was compared in 9 young and 10 old subjects. Data are shown as the mean ± standard deviation (SD) for each gene relative to expression of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Bottom graph: effects of gender on the expression of WNT genes in hMSCs from 12 women and 7 men. Data are shown as the mean ± SD for each gene relative to expression of GAPDH ($^{**}P < 0.05$, $^{*}0.05 < P < 0.1$).

$P = 0.034$) and a positive correlation for WNT4 ($r = 0.640$, $P = 0.034$) (Fig. 4), but WNT4 expression showed no significant relation with age when hMSCs from both genders were included. WNT5A expression showed a small trend for an inverse correlation with age in women (data not shown).

Subanalysis of WNT expression in hMSCs from men showed trends for higher expression of WNT7B (1.5-fold, $P = 0.057$), WNT10B (1.6-fold, $P = 0.057$), and WNT14 (1.9-fold, $P = 0.076$) in the hMCSs from 4 young men (36- to 49-year, mean 42.0 ± 6.5 years), compared with the 3 older men (57- to 82-year, mean

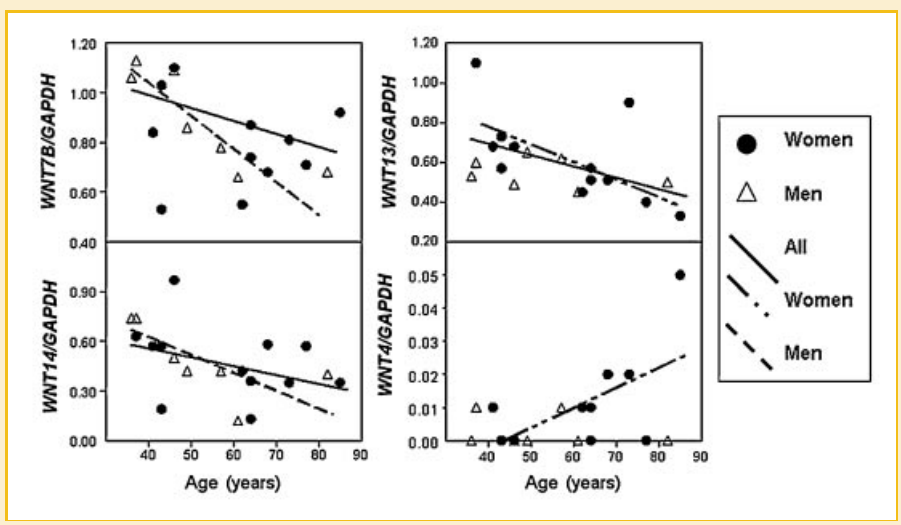

Fig. 4. Correlation of WNT gene expression in 19 hMSCs with age and subcorrelation of WNT gene expression in hMSCs with age and gender. Solid lines represent significant correlations for each WNT gene with age of all subjects. Significant age-related correlations are shown for subgroups of men or women.

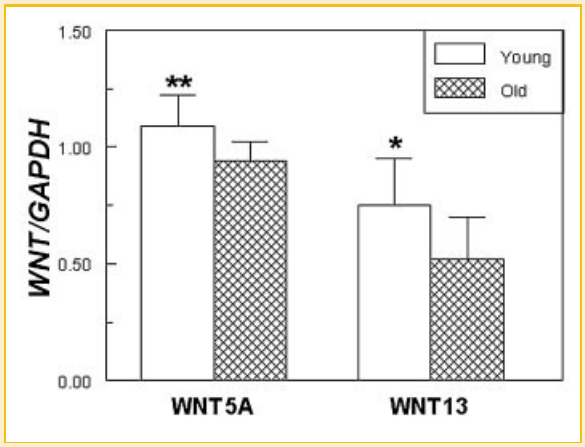

Fig. 5. Effects of age on the expression of WNT genes in hMSCs from women. Expression of WNT5A and WNT13 in hMSCs from a group of 5 young women was compared with a group of 7 old women. Data are shown as the mean ± standard deviation (SD) for each gene relative to expression of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ($^{**}P = 0.040$; $^{*}P = 0.070$).

66.7 ± 13.4 years) (data not shown). Correlation analysis of data for men showed inverse relationships with age for WNT7B (Spearman $r = -0.861$, $P = 0.013$) and for WNT14 (Spearman $r = -0.675$, $P = 0.097$) (Fig. 4). The age dependent correlations for WNT7B and 14 were not seen for women.

DISCUSSION

These studies show variances in constitutive expression of many WNT genes in 19 samples of freshly isolated human bone marrow stromal cells. Further, they indicate effects of age and gender on expression of some WNT genes. These data resolve some of the discrepancies in the literature from studies that used one or few samples of hMSCs without information about gender, age, or passage.

We used RT-PCR to survey the expression profile of WNT genes in hMSCs. A pilot study revealed that WNT2 and 10B, but not WNT5 showed differences in expression between P2 and P4 respectively. Because passage of hMSCs may confound interpretation of expression profiling, subsequent experiments were done with P2 samples.

The human WNT gene family consists of 19 members, clustered in different human chromosomes [Katoh, 2002]. There are reports on the expression of WNT genes in a number of pathological cells and tissues [Howng et al., 2002; O'Gorman et al., 2006; Memarian et al., 2007; Pilarsky et al., 2008], but more information is needed about subject-to-subject variances in normal tissues. This evaluation of WNT expression in hMSCs from a series of young and old women and men emphasizes the need to use multiple samples and to characterize the cell source before drawing generalized conclusions about these genes. In this study of 19 samples, 12 of the 19 known WNT genes were expressed in hMSCs, although some of the genes were not expressed in every subject, or showed variable ranges of difference between subjects. All samples showed similar expression of WNT5A and WNT5B. None showed expression of WNT1, 3A, 7A, 8A, 8B, 10A, or 15. The greatest variability was seen in the other WNT genes. Previous research with hMSCs from limited numbers of subjects or from commercial sources drew different conclusions about WNT gene expression. Boland et al. [2004] reported that expression of WNT1, 3, 5A, 10B, 11, 13, and 14 was detected in hMSCs. Not consistent with those results, Etheridge et al. [2004] reported that expression of WNT2, 4, 5A, 11, and 16 was detected in hMSCs. In a previous study from our group with a line of hMSCs, KM101, we found that they expressed WNT 2, 3, 4, 5A, 5B, 7A, 7B, 10A, 10B, 13, 14, and 16 [Zhou et al., 2004]. A recent report from Okoye et al. [2008] indicated that WNT2, 3, 3A, 4, 5A, 5B, 6, 7A, 7B, 10B, 13, 14, and 16 were expressed in hMSCs from a commercial source. To our knowledge, this current study represents the first comprehensive evaluation of the expression profile of WNT genes in hMSCs from a variety of women and men with a wide range of age. Since Wnt signaling is critical for regulating stem cell renewal [Reya and Clevers, 2005], and effects of gender on WNT gene expression have been reported [Cantuti-Castelvetri et al., 2007], it would seem prudent to identify the age and gender of hMSCs and to use replicates in order to draw meaningful conclusions about WNT function and regulation.

We previously reported other influences of subject age and gender on hMSCs functions. We reported striking age-dependent decreases in proliferation and osteoblast differentiation and increases in SA-β-gal activity and apoptosis with age in hMSCs [Mueller and Glowacki, 2001; Zhou et al., 2008]. We speculated that WNT signaling pathway genes change with age in hMSCs. Expression of both WNT7B and 14 was significantly higher in the young group than in the older group. Other genes, WNT2 and 13, showed trends for an age effect. Expression of WNT7B, 13, and 14 was inversely correlated with age. A recent review concluded that activation of canonical Wnt signaling delays the onset of cellular senescence by β-catenin/TCF/LEF interactions [DeCarolis et al., 2008]. Our data showed effects of age on canonical WNT genes, and WNT7B and WNT14 were expressed higher in the young group than in the old group. Expression of WNT2 ligand and downstream canonical WNT signals were reported to be repressed in senescent human cells [Ye et al., 2007], but our data suggested WNT2 expression was not strongly correlated with in vivo age. WNT13 functions as the stem cell factor for neural or retinal progenitor cells during embryogenesis [Katoh, 2005] and knocking down WNT14 mRNA levels increased the proliferation of human breast cell Line MCF-7 [Xiang et al., 2008], but the function of these two WNT genes in hMSCs still remains unknown.

Our results also showed that WNT16 was expressed significantly higher in men than in women. There are two isoforms (A and B) from the WNT16 locus [Fear et al., 2000], but only WNT16B expression was detected with our technique. There is little known about WNT16. Dell'Accio et al. [2008] reported that in osteoarthritis (OA), WNT16 was barely detectable in preserved cartilage areas, but was dramatically up-regulated in areas of the same articular cartilage with moderate to severe OA damage. This study showed a trend for higher expression of WNT11 in women, compared with men. It has been shown that Wnt11 plays an important role in cardiac development by embryoid bodies, and may be a key regulator of

cardiac muscle cell proliferation and differentiation during heart development [Terami et al., 2004]. Further identification of the critical effects of the WNT16 and 11 signaling pathway in hMSCs may help in understanding their gender-related differences.

We also tested for gender-specific effects of age on the expression of WNT genes in hMSCs from females and males separately. Correlation analysis showed that the effect of age on WNT7B and 14 was due to an inverse age effect with samples from men and not from women. In contrast, the age-related decline in WNT13 was attributable to changes in women not men. It is notable that WNT4 expression was the only one that was greater in samples from older subjects; correlation analysis showed that this was true for only the women. Wnt4 is necessary for the initiation of female sex determination in mice [Bernard and Harley, 2007], and deficiency of Wnt4 and 5a results in sex reversal, infertility, and/or malformation of the internal and external genitals in mice [Heikkila et al., 2001]. This evidence indicates that WNT genes related to sexual development may be relevant for hMSCs. In other studies with hMSCs, we reported gender-specific differences, including cytokine secretion [Cheleuitte et al., 1998] and responsiveness to sex hormones [Gordon et al., 2001]. Taken together with the new data reported herein, growing evidence shows the importance of age, gender, and passage on in vitro activities of hMSCs.

In summary, we evaluated the expression of WNT genes in hMSCs from young and old women and men. Our results indicated that most of the age-related WNT genes belong to the canonical WNT signaling pathway. Further, there are gender-specific differences in the expression of WNT4, 7B, 13, 14, and 16 in hMSCs. Age and gender account for many of the sample-to-sample variations in WNT gene expression.

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