# WNT16 induces proliferation and osteogenic differentiation of human perivascular stem cells

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ABSTRACT

Perivascular stem cells (PSC) are a progenitor population defined by their perivascular residence. Recent studies have examined the relative difference in Wnt ligands to induce PSC differentiation, including Wnt16. Here, we examine the role of Wnt16 in the proliferation and osteogenic differentiation of human PSC. Treatment of PSC with WNT16 significantly increased cell proliferation to a greater extent than did WNT3A. In addition, WNT16 showed a significant increase in osteogenic gene expression among PSC. These data demonstrate that WNT16 represents a combined mitogenic/pro-osteogenic stimulus that may play a functional role in human mesenchymal stem cell mediated bone repair.

## 1. Introduction

Mesenchymal stromal cells (MSC) are a multipotent stromal cell population capable of mesenchymal differentiation into multiple cell types, including: adipogenic, chondrogenic, and osteogenic cell fates.[1] Multiple applications exist for MSC in tissue regeneration, which primarily lie in MSC ability to function as paracrine regulators of tissue repair.[2] Adipose tissue (AT) is an appealing cell source as it is readily available, accessible and dispensable by routine liposuction procedures. The stromal vascular fraction (SVF) has been previously used for bone repair, but forms bone tissue unreliably[3] or with a low efficacy.[4]

To improve upon existing AT stromal therapies, we previously purified a population of MSC termed perivascular stem/stromal cells (PSC) from the SVF of human subcutaneous white adipose tissue.[5] PSC are purified by fluorescence activated cell sorting (FACS) and represent a comparatively homogenous MSC population for regenerative medicine applications.[6,7] PSC are abundant in human white adipose tissue, and are present in clinically relevant numbers for efforts in tissue engineering (∼40% of viable mononuclear SVF).[7] PSC originate in the vessel wall, which represents a well-established source of mesenchymal progenitor cells.[8,9] PSC are composed of two distinct yet related cell populations, including pericytes (CD34-CD146 + CD45-) and adventitial progenitor cells (CD34 + CD146-CD45-).[9,10] PSC have been shown to promote *in vivo* bone regeneration across animal models, including a rat spinal fusion model[6,11] and a calvarial defect model.[12]

The commitment of MSC to an osteogenic cell fate relies on many signaling, including both β-Catenin dependent canonical Wnt signaling, and β-catenin independent noncanonical Wnt signaling.[13–15] See[16] for a review of canonical and non-canonical Wnt signal transduction. WNT16, a mixed canonical and non-canonical Wnt signaling ligand, was previously observed to be enriched within the transcriptome of human PSC. In our recent observations,[17] we found that sustained treatment with rWNT16 increased osteogenic differentiation in a c-Jun N-terminal kinase (JNK) pathway dependent fashion. In contrast, sustained rWNT3A treatment significantly decreased PSC osteogenic differentiation. Conversely, *WNT16* knockdown significantly diminished PSC osteogenic differentiation. These data suggested that WNT16 plays a functional and necessary role in PSC osteogenesis. Here, we examine in more detail the role of recombinant Wnt16 in the proliferation and osteogenic differentiation of human PSC *in vitro*.

## 2. Methods

### 2.1. Perivascular stem/stromal (PSC) cell isolation

PSC were isolated from human lipoaspirate via fluorescence activated cell sorting (FACS). The stromal vascular fraction (SVF) was obtained by collagenase digestion. Briefly, lipoaspirate was diluted with an equal volume of phosphate-buffered saline (PBS) before digestion with Dulbecco's modified Eagle's medium (DMEM) containing 3.5%

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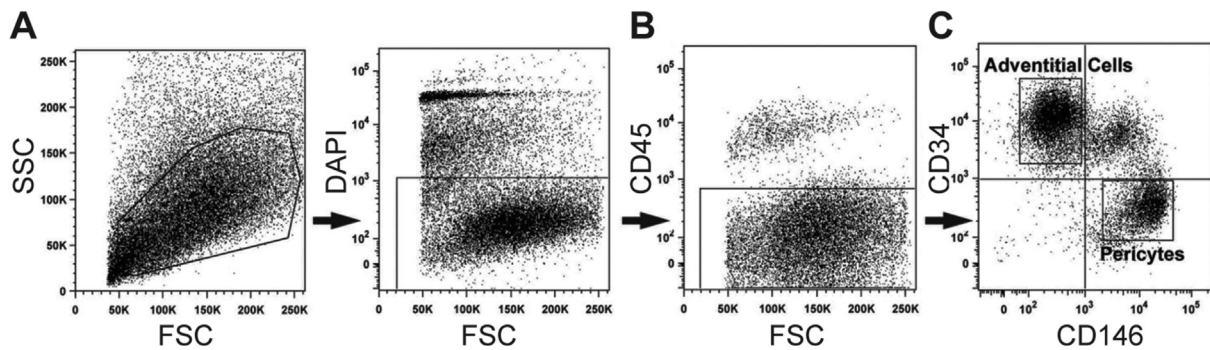
**Fig. 1.** Human perivascular stem/stromal cell (hPSC) isolation and osteogenic differentiation. (A–C) Fluorescence-activated cell sorting (FACS) isolation method for hPSC. (A) DAPI + non-viable cells and (B) CD45 $^+$ hematopoietic cells were excluded from the stromal vascular fraction of human lipoaspirate. (C) Purified hPSC consist of CD146 + CD34-pericytes and CD34 + CD146-adventitial cells. Reproduced with permission from Askarinam & James et al., *Tissue Eng Part A*, 2013, PMCID: PMC3638559.

bovine serum albumin (Sigma-Aldrich, St. Louis) and 1 mg/ml type II collagenase for 70 min under agitation at 37 °C. Next, adipocytes were separated and removed by centrifugation. The pellet was then re-suspended in red-cell lysis buffer (155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA) and incubated for 10 min at room temperature. After centrifugation, pellets were resuspended in PBS and filtered at 70 μm. The resulting SVF was incubated with a mixture of the following di-rectly conjugated antibodies: anti-CD34-phycoerythrin (1:100; Dako, Glostrup, Denmark), anti-CD45-allophycocyanin (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA), and anti-CD146-fluorescein iso-thiocyanate (1:100; AbD Serotec, Raleigh, NC). All incubations were performed at 4 °C for 15 min in the dark. Before sorting, 4′,6-diamidino-2-phenylindole (DAPI; 1:1000; Invitrogen, Carlsbad, CA) was added for dead cell exclusion; the solution was then passed through a 70-μm cell filter and then run on a FACSAria cell sorter (BD Biosciences, San Diego, CA). Sorted cells were plated for *in vitro* studies. In this manner, peri-cytes (CD34−CD146 + CD45−) and adventitial cells (CD34 + CD146−CD45−) were isolated and combined to constitute the PSC population. Cells were cultured at 37 °C in a humidified at-mosphere containing 95% air and 5% $CO_2$. The expansion of cells was performed in DMEM, 20% fetal bovine serum (FBS), 1% penicillin/streptomycin. Medium was changed every 3 d unless otherwise noted.

### 2.2. Cell proliferation

PSC were seeded in 96 well plates at a density of 1000 cells per well and allowed to adhere overnight. Cells were cultured in DMEM + 20% FBS + 1% Pen Strep and treated with rWNT16 (80 ng/mL), rWNT3A (50 ng/mL), or rDKK1 (50 ng/mL) for 3 d followed by MTS assay per the manufacturer's instructions (Promega, Madison, WI).

### 2.3. Osteogenic differentiation

Assays for PSC differentiation are adapted from our prior publica-tions.[18,19] The osteogenic differentiation medium (ODM) included 10 mM β-glycerophosphate and 50 μM ascorbic acid in DMEM + 20% FBS. ODM with recombinant proteins was changed every third d. RNA isolation for specific gene expression was performed on 0, 3, 6, and 9 d of differentiation.

### 2.4. Ribonucleic acid (RNA) isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Gene expression was assayed by quantitative RT-PCR, based on our previous methods.[19,20] Primers are in Supplemental Table 1. Time-points for specific gene expression include 0, 3, 6, and 9 d of differ-entiation. Briefly, total RNA was extracted using RNEasy Kit (Qiagen, Santa Clarita, CA). 1 μg of total RNA from each sample was subjected to

first-strand complementary deoxyribonucleic acid (cDNA) synthesis using the SuperScript III Reverse-Transcriptase Kit (Life Technologies) to a final volume of 20 μL The reverse transcription reaction was per-formed at 65 °C for 5 min, followed by 50 °C for 50 min and 85 °C for 5 min. For qRT-PCR, the reaction was performed using 2 × SYBR green RT-PCR master mix and an ABI PRISM 7300 qRT-PCR system instru-ment (Applied Biosystems, Foster City, CA). qRT-PCR was performed using 96 well optical plates at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and at 60 °C for 60 s. The relative quantification of gene expression was performed using a Comparative CT method according to the manufacturer's protocol and was normalized to the expression levels of the housekeeping gene, *ACTB,* in each sample.

### 2.5. Statistical analysis

All results were expressed as mean ± standard deviation (SD). Statistical analyses were performed using the SPSS16.0 software. All data were normally distributed. Student's *t*-test was used for two-group comparisons, and one-way ANOVA test was used for comparisons of 3 or more groups, followed by Tukey's *post hoc* test. Differences were considered significant when $P < 0.05$.

### 3. Results

### 3.1. Perivascular stem/stromal derivation

First, perivascular stem/stromal cells (PSC) were purified from human lipoaspirate using FACS to detect a population of pericytes and adventitial progenitor cells based on expression of CD146 and CD34 (Fig. 1). Briefly, using previously established protocols, the stromal vascular fraction (SVF) of lipoaspirate was processed so as to remove DAPI + non-viable cells (Fig. 1A), as well as CD45 $^+$ hematopoietic cells (Fig. 1B). Next, pericytes were defined as a CD146 + CD34-CD45-cell population while adventitial progenitor cells are CD34 + CD146-CD45-cell population (Fig. 1C). When combined, this bipartite popu-lation is termed PSC.[9,10] Prior studies have confirmed that PSC have multilineage differentiation potential, including an ability to differ-entiate down osteogenic, adipogenic and chondrogenic lineages.[9,10]

### 3.2. WNT16 induces perivascular stem cell proliferation

Our prior examination identified an enrichment of *WNT16* trans-cripts among human PSC, and that WNT16 represented a context de-pendent stimulator of osteogenic and adipogenic differentiation.[17] As well, *WNT16* gene expression was observed to peak early in the process of PSC osteogenic differentiation (day 3), suggesting a role in osteo-progenitor cell expansion or early commitment.[17] We next sought to examine the effects of WNT16 on cellular proliferation in more detail