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Activation of non-canonical Wnt/JNK pathway by Wnt3a is associated with differentiation fate determination of human bone marrow stromal (mesenchymal) stem cells

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ABSTRACT

The canonical Wnt signaling pathway can determine human bone marrow stromal (mesenchymal) stem cell (hMSC) differentiation fate into osteoblast or adipocyte lineages. However, its downstream targets in MSC are not well characterized. Thus, using DNA microarrays, we compared global gene expression patterns induced by Wnt3a treatment in two hMSC lines: hMSC-LRP5^{T253} and hMSC-LRP5^{T244} cells carrying known mutations of Wnt co-receptor LRP5 (T253I or T244M) that either enhances or represses canonical Wnt signaling, respectively. Wnt3a treatment of hMSC activated not only canonical Wnt signaling, but also the non-canonical Wnt/JNK pathway through upregulation of several non-canonical Wnt components e.g. naked cuticle 1 homolog (NKD1) and WNT11. Activation of the non-canonical Wnt/JNK pathway by anisomycin enhanced osteoblast differentiation whereas its inhibition by SP600125 enhanced adipocyte differentiation of hMSC. In conclusion, canonical and non-canonical Wnt signaling cooperate in determining MSC differentiation fate.

1. Introduction

Canonical and non-canonical Wnt signaling pathways play important roles in a variety of cellular activities, including cell fate determination, proliferation, migration, polarity and gene expression [1]. The canonical Wnt pathway i.e. β-catenin dependent pathway, is initiated by the binding of Wnt ligands, for example Wnt3a to Frizzled receptor and low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6), leading to the stabilization of cytosolic β-catenin which translocates to the nucleus and binds to T-cell factor/lymphoid enhancer binding factor (TCF/LEF) binding sites in promoters of target genes [2]. A large body of evidence based on *ex vivo* and *in vivo* studies has demonstrated that the canonical Wnt pathway is important for bone mass accrual and maintenance [3–5].

The non-canonical Wnt signaling pathway is initiated by non-canonical Wnt ligands, e.g. Wnt5 or Wnt11, and stimulates a number of signaling pathways e.g. calcium/calmodulin-dependent kinase (CaMKII), protein kinase C (PKC) (Wnt/Ca^{2+} pathway), or through small GTPases, the heterotrimeric G proteins [6]. An increasing number of recent studies have suggested a role for non-canonical Wnt signaling in osteoblast differentiation and bone formation. Takada et al. demonstrated that the non-canonical Wnt5A ligand induces CaMKII-TAK1-TAB2-NLK signaling that transcriptionally represses PPARγ transactivation function through chromatin inactivation and induces Runx2 expression, thus inhibiting adipogenesis while promoting osteoblastogenesis in the bipotential murine stromal cell line ST2 [7]. A second study employing the same ST2 cell line reported that Wnt3a and Wnt7b, which are known canonical Wnt ligands, enhance osteoblast differentiation through activation of G-proteins, composed of the Gq subfamily and PKCδ, and not through the canonical β-catenin dependent mechanism [8]. In human MSC, the non-canonical Wnt ligand Wnt4 enhanced *in vitro* osteogenic differentiation of MSCs isolated from human adult craniofacial tissues, and genetically modified MSC overexpressing Wnt4 exhibited improved repair capacity of craniofacial defects *in vivo* by activation of p38 MAPK pathway [9]. Interestingly, parathyroid hormone (PTH) has been demonstrated to stimulate Wnt4 through the protein kinase A pathway thus linking PTH anabolic effects on osteoblastic cells and the stimulation of the non-canonical Wnt pathway [10].

c-Jun N-terminal kinase (JNK) pathway is a target pathway for non-canonical Wnt signaling named Wnt/PCP pathway [6]. Recently, the JNK signaling pathway has been implicated in promoting osteoblastic differentiation and repressing adipocytic differentiation in both human and rat MSC [11,12]. Additionally, Wu et al. demonstrated that nuclear accumulation of β-catenin,

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in response to canonical Wnt activation, involves non-canonical Wnt signaling components including Rac1 and JNK2 thus suggesting a possible link between canonical Wnt and Wnt/JNK pathways [13]. The implication of the link between canonical and non-canonical Wnt signaling on hMSC differentiation fate are unknown.

We have recently demonstrated that canonical Wnt signaling plays an important role in lineage fate determination of hMSC into osteoblasts versus adipocytes through activation of canonical Wnt signaling [14,15]. In the present study, we examined the interaction between canonical and non-canonical Wnt signaling in mediating the biological effects on hMSC. We report that Wnt3a, a canonical Wnt ligand, not only activates canonical Wnt signaling but also stimulates the expression of non-canonical Wnt components such as naked cuticle 1 homolog (NKD1) and non-canonical Wnt ligand e.g. WNT11 and activates Wnt/JNK pathway. Furthermore, modulation of Wnt/JNK activity using chemical biology approaches was associated with changes in hMSC differentiation towards the osteoblast or adipocyte lineage.

2. Materials and methods

2.1. Cell culture and condition medium

The creation, characterization and culture of hMSC-LRP5^{T253} and hMSC-LRP5^{T244} have been reported previously [14,16]. Wnt3a conditioned medium (W3A) as well as control conditioned medium (CCM) were prepared as described [14,16].

2.2. Chemicals and antibodies

Canonical Wnt activators lithium chloride (LiCl, Sigma) and (2′Z, 3′E)-6-Bromoindirubin-3′-oxime (BIO, Calbiochem) as well as JNK inhibitor SP600125 (Sigma) and JNK activator anisomycin (Sigma) were used. Human β-catenin (#9581), c-Jun (#9165) and phospho-c-Jun (Ser63) II (#9261) antibodies were purchased from cell signaling and actin antibody (A3853) was purchased from Sigma. Alex 488 conjugated donkey anti-Rb IgG (A-21206) and DAPI solution were purchased from Molecular probe and Vector laboratories, respectively.

2.3. Real-time qRT-PCR

Real-time qRT-PCR was performed using fast SYBR® green master mix (Applied Biosystem) on StepOnePlus™ system (Applied Biosystem) according to the manufacturer's protocol. RNA isolation, cDNA synthesis, and data analysis were carried out as described previously [14,15]. Primers used in this study are: NKD1 forward primer 5′-gatggagagagtgagcgaacc-3′ and reverse primer 5′-catagatggtgtgcagcaagc-3′, WNT11 forward primer 5′-acaacctcagctacg ggctcct-3′ and reverse primer 5′-cccaccttctcattcttcatgc-3′, WNT2B forward primer 5′-tcatgctcagaagtagccgaga-3′ and reverse primer 5′-tggcacttacactccagcttca-3′ , WNT5A forward primer 5′-ttttttctccttc gcccaggttgt-3′ and reverse primer 5′-ggctcatggcgttcaccac-3′ , and WNT5B forward primer 5′-cagcttctgacagacgccaact-3′ and reverse primer 5′-gcctatctgcatgactctccca-3′. Primer sequence for adipogenic markers were described as previous [14]. GAPDH forward primer 5′-ggcgatgctggcgctgagtac-3′ and reverse primer 5′-tggttcacacccatgacga-3′ was used as control.

2.4. Immunostaining and Western blotting

For immunostaining, cells were treated with 50% CCM or W3A for 2 h and fixed in PBS buffered formaldehyde (pH 7.0) for 10 min followed by incubation with TBS buffered Triton X-100 (pH 7.4) for 10 min. After blocking with PBS containing 10% donkey serum for 30 min, cells were incubated with rabbit anti-β-catenin antibody (1:100 dilution) for 1 h and then incubated with Alex 488 conjugated donkey anti-Rb IgG (1:500 dilution) for 1 h. Nuclei were counterstained with DAPI solution and images were taken by Leica fluorescent microscope.

For Western blot analysis, cells were treated with normal culture medium, 50% CCM or 50% W3A for 24 h and then 30 μg total proteins were used as described [14]. Semi-quantitation of the bands was performed by ImageJ software according to the software's instruction.

2.5. Cytotoxicity MTS assay

Cells were seeded at 6000 cells/well of 96-well plate and treated with 50% W3A plus JNK activator anisomycin (0–0.2 μg/ml) or inhibitor SP600125 (0–2 μM) for 2 days. MTS assay was performed by using CellTiter 96® AQ_{ueous} one solution reagent containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and an electron coupling reagent phenazine ethosulfate (Promega) following the manufacturer's protocol.

2.6. hMSC differentiation

Osteoblast differentiation: cells were treated with 50% W3A containing anisomycin (0–0.1 μg/ml) or SP600125 (0–2 μM) for 7 days. Osteoblastic differentiation marker alkaline phosphatase (ALP) activity was measured by using p-nitrophenylphosphate (Fluka) as substrate and normalized against cell number determined by CellTiter-Blue reagent (Promega) as described [14,15].

Adipocyte differentiation: cells were treated with adipogenic induction medium [14] supplemented with anisomycin (0.2 μg/ml) or SP600125 (2 μM) for 7 days. Expression of adipocytic differentiation markers were analyzed as described [14,15].

2.7. Statistical analysis

Differences among groups were determined by Student's *t*-test and $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Wnt3a stimulates the expression of NKD1 and WNT11

We have previously established two hMSC lines carrying known mutations of Wnt co-receptor LRP5 i.e. T253I (hMSC-LRP5^{T253}) or T244M (hMSC-LRP5^{T244}) that either cause high bone mass phenotype (HBM) or osteoporosis pseudoglima syndrome (OPPG). We have also reported that hMSC-LRP5^{T253} transduces higher canonical Wnt signaling, exhibits enhanced osteoblast differentiation in *ex vivo* cultures and higher bone formation *in vivo* as well as reduced adipocytic differentiation capacity compared to hMSC-LRP5^{T244} [14]. To identify downstream targets responsible for phenotypic differences, we compared gene expression profiles of hMSC-LRP5^{T253} and hMSC-LRP5^{T244} in response to Wnt3a-treatment by Illumina® microarray system [15].

Bioinformatic analysis revealed that several known Wnt signaling components including AXIN2, DKK2, FZD1, NKD1, PLCB1, PRICKLE1 and SFRP2 were upregulated by Wnt3a treatment (Fig. 1A). Among these genes, NKD1 was one of the most differentially upregulated genes: 3- and 9-fold upregulation in hMSC-LRP5^{T244} and hMSC-LRP5^{T253}, respectively. Real-time qRT-PCR confirmed that NKD1 upregulation was higher in hMSC-LRP5^{T253} compared to hMSC-LRP5^{T244} cells and the upregulation was time-dependent (Fig. 1B and C). Both LiCl and BIO, which are known inhibitors of

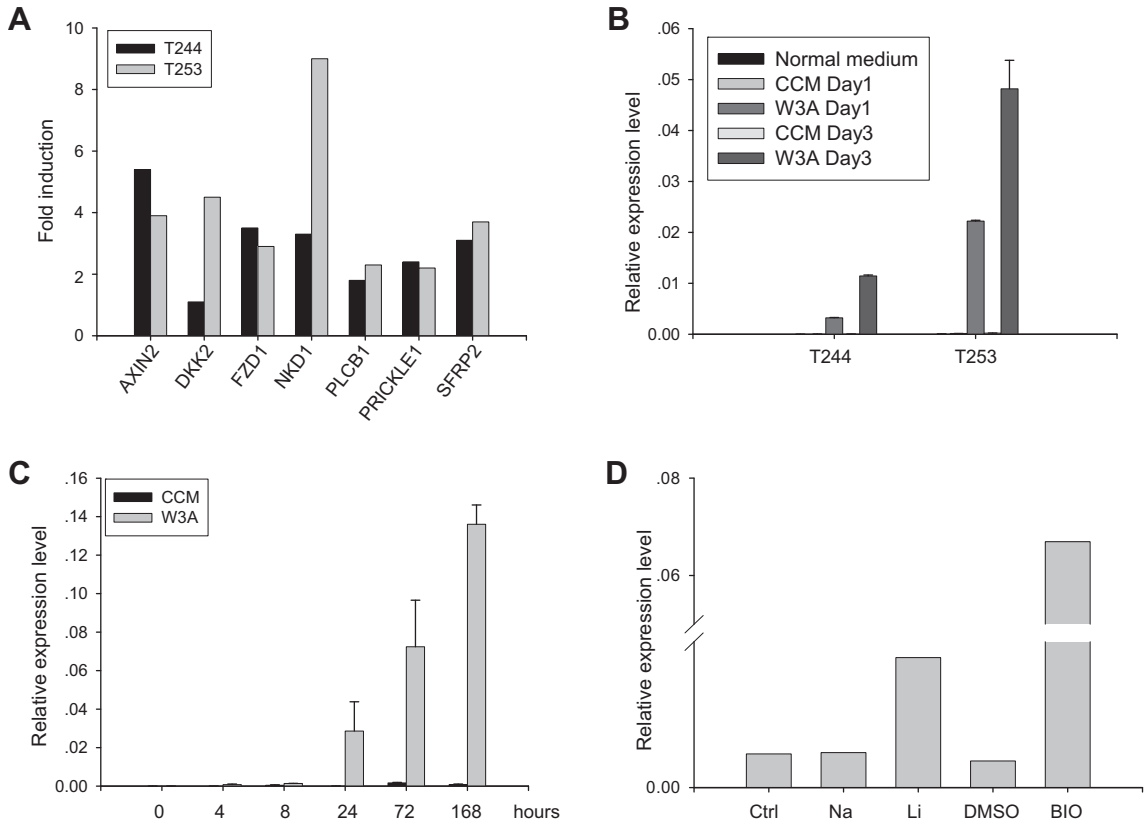

Fig. 1. Wnt3a activates the expression of NKD1. (A) hMSC-LRP5^{T244} (T244) and hMSC-LRP5^{T253} (T253) cells were treated with 50% control condition medium (CCM) or Wnt3a condition medium (W3A) for 24 h and their gene expression profiles were analyzed by Illumina microarray system. Several canonical and non-canonical Wnt components were upregulated by W3A compared with CCM and fold induction was presented. (B–D) NKD1 expression was analyzed by real-time qRT-PCR. T244 and T253 cells were treated with normal medium, CCM or W3A for 1 or 3 days (B). T253 cells were treated with CCM or W3A for indicated hours (C). T244 cells were treated with normal medium plus vehicle (Ctrl), 20 mM NaCl (Na), 20 mM LiCl (Li), DMSO or 5 μM (2′Z, 3′E)-6-Bromoindirubin-3′-oxime (BIO) for 24 h (D). NKD1 expression was normalized against GAPDH.

GSK3β [17,18] leading to stabilization of β-catenin, increased the expression of NKD1 dramatically (Fig. 1D) supporting the view that NKD1 is a downstream target of canonical Wnt signaling.

NKD1 is known to block canonical Wnt signaling and to activate Wnt/JNK pathway [19–21]. hMSC lines also express several Wnt ligands including non-canonical WNT5A, WNT5B, WNT11 and canonical WNT2B [14] but they were not detected as significant differentially regulated genes by microarray. So we measured their expression upon Wnt3a treatment up to 3 days by real-time qRT-PCR. As a result, Wnt3a upregulated WNT11 on day 1 only in hMSC-LRP5^{T253} and both WNT11 and WNT5A were upregulated on day 3 in hMSC-LRP5^{T244} and hMSC-LRP5^{T253}. Another non-canonical Wnt ligand WNT5B did not exhibit significant changes (Fig. 2). On the other hand, canonical Wnt ligand WNT2B expression decreased in hMSC-LRP5^{T253} cells after 3 days treatment with Wnt3a comparing with control conditioned medium. Several Wnt signaling components have been reported as Wnt target genes but few of them are Wnt ligands [22]. We determined whether WNT2B, WNT5A, WNT5B and WNT11 expressed in our hMSC cells are regulated directly by Wnt3a by performing promoter analysis of all their transcriptional variants as described previously [15]. We identified several TCF/LEF binding sites in these genes but most of these sites were located 2 kb upstream of the transcription start site (Supplementary Table) suggesting that these Wnt ligands may respond weakly to canonical Wnt activation. In consistent with bioinformatic analysis, we observed the significant upregulation of WNT11 and WNT5A following Wnt3a treatment for 3 days by real-time qRT-PCR suggesting that they may be novel Wnt target genes. The observed significant upregulation of WNT11 by Wnt3a may be due to the proximal TCF/LEF site (−44 to −38) in its promoter (Supplementary Table). However, the expression of these Wnt ligands was not upregulated by LiCl and BIO possibly due to the short treatment period (24 h) while longer treatment induces toxicity to the cells (Supplementary Fig. 1).

Steady state gene expression revealed by microarray analysis and real-time qRT-PCR suggested that Wnt3a treatment may trigger a negative feedback mechanism to regulate canonical Wnt pathway evidenced by the upregulation of canonical Wnt antagonists including AXIN2, DKK2 and SFRP2 as well as downregulation of canonical Wnt ligand WNT2B [2]. More importantly, we revealed that Wnt3a also activated key components of Wnt/PCP pathway such as WNT5A, WNT11, NKD1 and PRICKLE1 suggesting a possible relationship between canonical and non-canonical Wnt pathways.

3.2. Wnt3a activates both β-catenin and JNK pathway

Wnt3a has been reported to function via non-canonical Wnt signaling pathway in addition to its role as a canonical Wnt ligand [8]. To further confirm that Wnt3a triggers canonical Wnt/β-catenin pathway in hMSC, we examined the translocation of β-catenin by immunostaining. As shown in Fig. 3A, nuclear β-catenin could be observed as early as 2 h after treatment with Wnt3a and these effects corroborate our previous results that Wnt3a stimulated canonical Wnt activity as evidenced by TCF-luciferase reporter assay and this effects was blocked by canonical Wnt antagonist DKK1

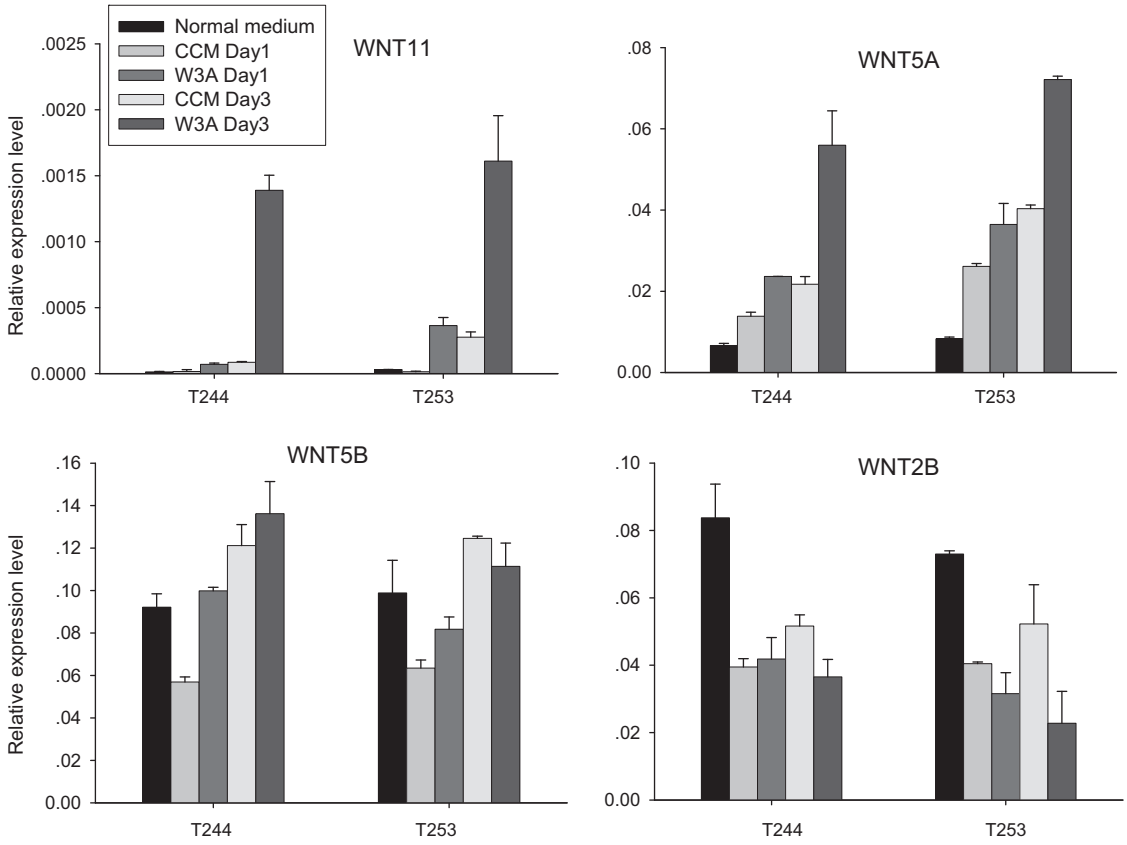

Fig. 2. Wnt3a activates the expression of non-canonical Wnt ligands. hMSC-LRP5^{T244} (T244) and hMSC-LRP5^{T253} (T253) cells were treated with normal medium, 50% control condition medium (CCM) or Wnt3a condition medium (W3A) for 1 or 3 days and gene expression was analyzed by real-time qRT-PCR and normalized against GAPDH.

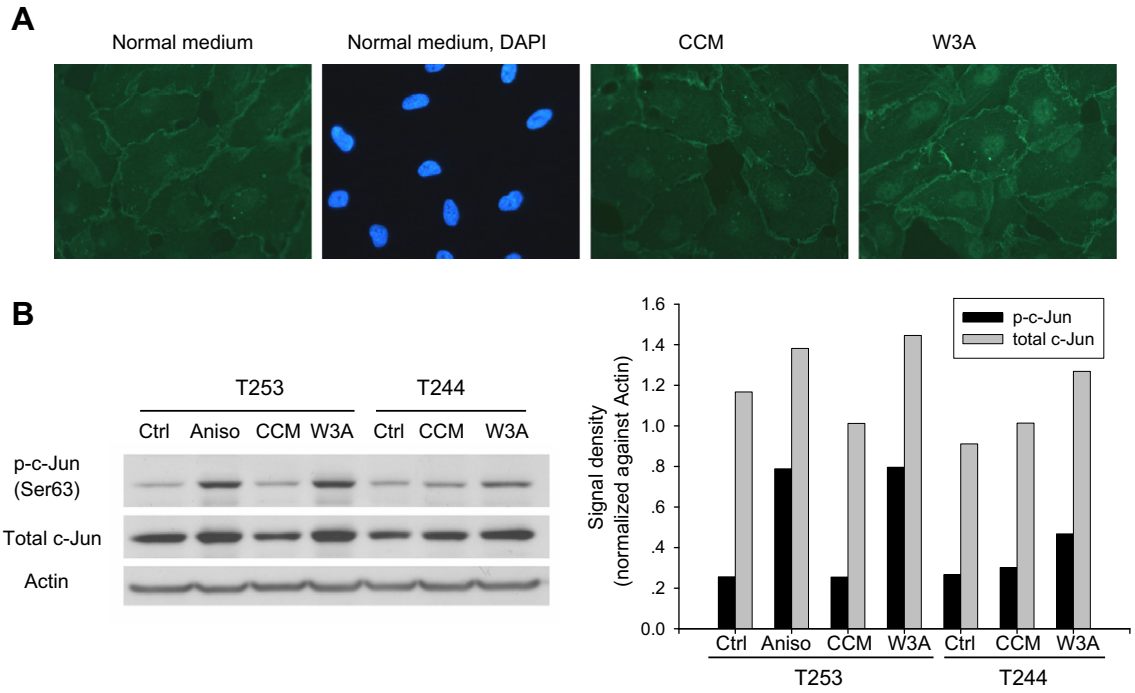

Fig. 3. Wnt3a activates both canonical and non-canonical Wnt signaling pathways. (A) hMSC-LRP5^{T253} (T253) cells were treated with normal medium, 50% control condition medium (CCM) or Wnt3a condition medium (W3A) for 2 h and stained with β-catenin antibody. Nuclei were counterstained with DAPI and images were taken by Leica fluorescence microscopy (Magnification, 200×). (B) T253 and hMSC-LRP5^{T244} (T244) cells were treated with normal medium plus water as negative control (Ctrl), 0.1 μg/ml anisomycin (Aniso) for 1 h as a positive control, 50% CCM or W3A for 24 h and analyzed by Western blotting for total c-Jun and phosphor-c-Jun (p-c-Jun). The signal density was semi-quantitated by ImageJ software.

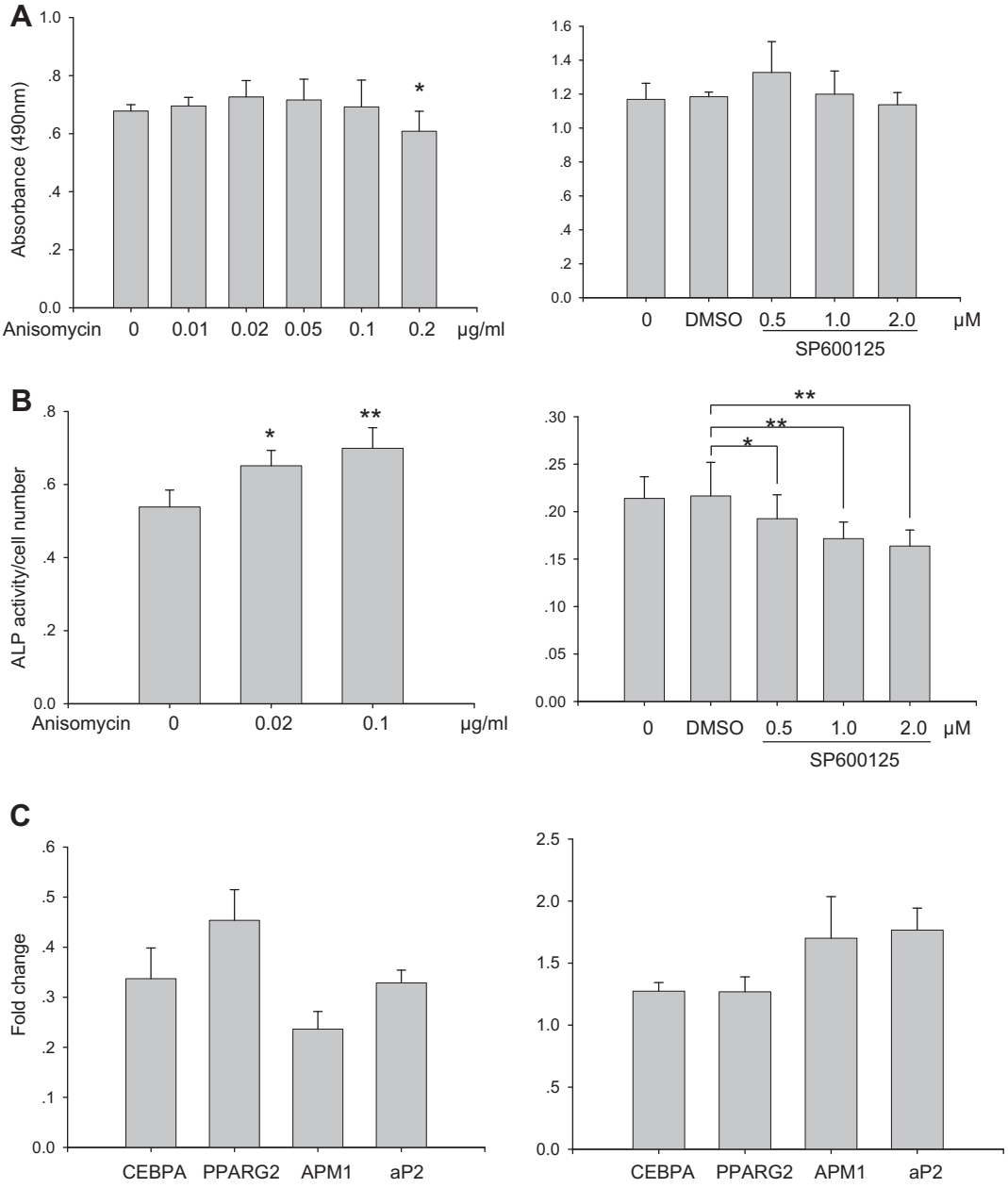

Fig. 4. Wnt/JNK pathway is involved in determination of human bone marrow stromal (mesenchymal) stem cell (hMSC) differentiation fate. (A) hMSC-LRP5^{T244} (T244, left) and hMSC-LRP5^{T253} (T253, right) cells were treated with anisomycin or SP600125, respectively for 2 days and cell viability was measured by MTS assay. Results shown are mean + SD of eight replicates. (B) hMSC-LRP5^{T244} (T244, left) and hMSC-LRP5^{T253} (T253, right) cells were treated with 50% Wnt3a conditioned medium plus anisomycin or SP600125, respectively for 7 days. ALP activity was measured by using P-nitrophenylphosphate (Fluka) as substrate and normalized against cell number determined by CellTiter-Blue reagent. Results shown are mean + SD of six to eight replicates. (C) hMSC-LRP5^{T244} (T244) cells were treated with adipogenic induction medium (AIM) plus 0.2 μg/ml anisomycin or equal amount of vehicle (water) as control (left), or 2 μM SP600125 or DMSO as control (right) for 7 days. Steady state gene expression of adipocytic differentiation markers was analyzed by real-time qRT-PCR and gene expression level in cells treated with AIM plus vehicle (water or DMSO) was set to one. Fold change below one indicated down-regulation by anisomycin (left) and above one indicated upregulation by SP600125 (right). Results shown are mean + SD of three to four replicates. $^{*}P < 0.05$; $^{**}P < 0.01$.

[14]. Thus, Wnt3a functions as a canonical Wnt ligand in hMSC. Next, we tested whether Wnt3a treatment induces non-canonical Wnt/JNK signaling in hMSC by Western blotting using antibody against phosphor-c-Jun which is phosphorlyated by activated JNK at Ser63 [23]. In hMSC-LRP5^{T253} cells, phosphor-c-Jun increased significantly by Wnt3a treatment for 24 h to comparable levels induced by JNK activator anisomycin and almost 3-fold higher than cells treated with control conditioned medium. We found that phosphor-c-Jun did not changed by Wnt3a treatment in hMSC-LRP5^{T244} cells which transduce lower canonical Wnt activity and thus lower expression of non-canonical Wnt components (i.e. NKD1, WNT11 and WNT5A on day 1). Besides, the difference in expression of total c-Jun was not as significant as phosphor-c-Jun between hMSC-LRP5^{T253} and hMSC-LRP5^{T244} upon treatment with Wnt3a by both Western blot (Fig. 3B) and microarray (Supplementary Fig. 2) although c-Jun was reported to be a direct target of canonical Wnt pathway [24]. These results indicated that c-Jun phosphorylation reflects the activation of Wnt/JNK signaling and this is not due to increased expression of total c-Jun by Wnt/β-catenin signaling.

Unlike observations in ST2 cells that Wnt3a activates PKCδ but not β-catenin [8], in hMSC Wnt3a functions as canonical Wnt ligand to activate downstream target for example TNFRSF19 that regulates hMSC differentiation [15]. On the other hand, activated canonical Wnt pathway increased the expression of several non-canonical Wnt components directly or indirectly to activate Wnt/JNK pathway. It is plausible that such activation represents either a feedback mechanism for canonical Wnt signaling regulation or a signaling cascade downstream Wnt/β-catenin that induces osteoblast differentiation.

3.3. Wnt/JNK activity is involved in hMSC differentiation into osteoblast or adipocyte lineage

hMSC are clonogenic, non-haematopoietic stem cells present in the bone marrow stroma and are able to differentiate into multiple mesoderm-type cells e.g. osteoblasts, adipocytes [25]. *Ex vivo* and *in vivo* studies give support to the hypothesis of the presence of an inverse relationship between the commitment of MSC to the osteoblast and adipoctye lineage pathways [26] and this balance between osteoblast and adipocyte differentiation of hMSC may play a role in bone mass regulation during aging and in osteoporosis [27]. Identifying signaling pathways that control hMSC lineage commitment has clinical relevance in order to develop therapeutics to increase bone mass by changing the balance of osteoblast versus adipocyte differentiation of hMSC. Studies from our group and other groups have demonstrated that canonical Wnt signaling controls hMSC differentiation into osteoblasts and adipocytes [14,15,28,29]. However, the role of non-canonical Wnt signaling in regulation of MSC differentiation is still not clear.

In order to study the role of non-canonical Wnt signaling on hMSC differentiation, we treated hMSC lines with JNK activator anisomycin [30,31] or specific inhibitor SP600125 [32]. First, cytotoxicity of the chemicals was determined by MTS assay. We observed that cell viability was not significantly affected by using anisomycin within 0.1 μg/ml and SP600125 within 2 μM (Fig. 4A). Second, adding anisomycin or SP600125 to Wnt3a conditioned medium (50%) enhanced and inhibited ALP activity in a dose dependent manner, respectively (Fig. 4B). Furthermore, inducing hMSC lines to adipocytes in the presence of anisomycin in adipogenic induction medium resulted in inhibition of adipocyte specific gene expression (CEBPA, PPARG2, APM1 and aP2) to 20–45% comparing to adipogenic induction medium without anisomycin (Fig. 4C, left). On the other hand, inhibition of JNK using SP600125 increased the expression of adipocytic gene expression from 25% to 75% (Fig. 4C, right). Therefore, in contrast to the upregulation of antagonists of canonical Wnt pathway e.g. AXIN2, DKK2 and SFRP2 which could be regarded as a negative feedback mechanism for canonical Wnt signaling, increased expression of non-canonical components and activated Wnt/JNK pathway may represent a non-canonical Wnt signaling cascade with potential role in control of MSC differentiation.

Although activation of Wnt/JNK increased ALP activity in the presence of Wnt3a, cells treated with anisomycin alone have lower ALP activity than cells treated with Wnt3a alone (Supplementary Fig. 3). Besides, anisomycin inhibited the expression of adipogenic markers in hMSC-LRP5T244 by 20–45% (Fig. 4C, left) but Wnt3a inhibited those gene expression more than 50% [14]. Therefore, it is plausible that regulation of hMSC differentiation by canonical Wnt pathway involves multiple downstream molecules and/or signaling pathways.

In conclusion, we revealed a novel interaction between canonical and non-canonical Wnt pathways in hMSC evidenced by upregulation of non-canonical Wnt components and increased phosphor-c-Jun downstream of activated Wnt/β-catenin pathway. Such interaction results in regulation of hMSC differentiation which may be relevant mechanism for control of bone formation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.08.061.

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