**Wisp1 mediates Bmp3-stimulated mesenchymal stem cell proliferation**

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**Abstract**

Adipose tissue expansion, resulting from adipocyte hyperplasia and/or hypertrophy, is a hallmark of obesity. Adipocytes are derived from mesenchymal stem cells (MSCs) through adipogenesis, a process involving three key steps: proliferation, commitment and differentiation. Although studies have elaborated on the mechanisms regulating adipocyte commitment and differentiation, the factors that control MSC proliferation remain largely unknown. Previously, we demonstrated that bone morphogenetic protein 3 (Bmp3), the expression of which was upregulated in our rat model of hyperplastic visceral adiposity, potently stimulated MSC proliferation. In the present study, we investigate the molecular target of Bmp3. We conducted DNA microarray analysis on MSCs treated with and without Bmp3 and identified WNT1-inducible signaling pathway protein 1 (Wisp1) as a differentially expressed gene, whose expression was upregulated 3.7-fold by Bmp3. Wisp1 is a proliferative agent in various non-adipose cell types and is implicated in adipogenesis. Therefore, we tested the hypothesis that Wisp1 mediates Bmp3 stimulation of MSC proliferation. We showed that Bmp3 increased the expression of Wisp1 as early as 3 h following Bmp3 treatment in MSCs. Importantly, the upregulated Wisp1 expression preceded Bmp3-induced MSC proliferation, as determined by [3H]-thymidine incorporation. Furthermore, treatment of MSCs with recombinant Wisp1 led to a concentration-dependent increase in [3H]-thymidine incorporation with a maximal increase of 300%. In addition, siRNA-mediated knockdown of Wisp1 expression attenuated Bmp3-induced MSC proliferation. Taken together, our present findings reveal Wisp1 as a novel target of Bmp3 and suggest that the Bmp3/Wisp1 signaling pathway play a key role in MSC proliferation, and consequently adipogenesis.

**Introduction**

Obesity has reached epidemic proportions globally and is a major contributor to the global burden of chronic disease and disability (Dixon 2010, WHO 2015). These obesity-associated co-morbidities include insulin resistance, type-2 diabetes, strokes, hypertension, coronary artery disease, various cancers, chronic kidney disease, osteoarthritis, sleep apnea and premature death. At the cellular level, obesity is characterized by excess adipose tissue expansion resulting from an increase in the adipocyte cell number or adipocyte cell size (Hirsch & Batchelor 1976, Shepherd et al. 1993). New adipocytes arise from a pre-existing population of undifferentiated progenitor cells, namely mesenchymal stem cells (MSCs), through adipogenesis, a process that involves three key
steps: proliferation, commitment and terminal differentiation (Moreno-Navarrete & Fernandez-Real 2012).

Adipocyte number is established during early life by various environmental factors (Spalding et al. 2008) as an adaptation to enhance future fat storage in order to face potential adverse environmental conditions in later life (Hales et al. 1997). In particular, low birth weight has been associated with increased adiposity in humans (Osmond & Barker 2000) and animal models (Sarr et al. 2012) during adulthood. We have previously shown that poor nutrition during early life, induced by maternal protein restriction (MPR) during pregnancy and lactation, leads to low birth weight and subsequent development of increased visceral adiposity in adult male rat offspring (Guan et al. 2005). This increased visceral adiposity was characterized by adipocyte hyperplasia. Indeed, we showed that adipocyte precursor cells derived from MPR offspring exhibited an accelerated rate of proliferation, and this persisted days after removal from their in vivo environment (Zhang et al. 2007). Based on these observations, we identified Bmp3 (bone morphogenetic protein 3) as a key gene that was increased twofold in the adipose tissue of our MPR rat model of visceral adiposity (Guan et al. 2005). Bmp3 expression was also upregulated in the adipose tissue of a diet-induced mouse model of obesity (Koza et al. 2006). As a member of the transforming growth factor beta (TGFβ) superfamily, Bmp3 was originally thought to act as a trophic factor for bone growth due to its high expression in bone tissue (Sampath et al. 1987), but subsequent gene-targeting studies revealed that Bmp3 was anti-osteogenic (Bahamonde & Lyons 2001). We showed that Bmp3 promoted MSC and preadipocyte proliferation via the TGFβ/activin-signaling pathway (Stewart et al. 2010). However, the downstream molecular targets through which Bmp3 stimulates MSC proliferation remain unknown. Therefore, the present study was designed to address this important question.

Materials and methods

Cell culture

The C3H10T1/2 MSC line was obtained from the American Type Culture Collection (Manassas, VA, USA). C3H10T1/2 stem cells were cultured in standard growth medium consisting of Minimal Essential Medium (MEM; Sigma–Aldrich Canada Ltd) and 10% fetal bovine serum (FBS; Sigma). All cultures were maintained in a humidified incubator at 37 °C and 5% CO2. Growth medium was replaced every other day.

Genome-wide gene expression profiling: DNA microarray

C3HT10T1/2 MSCs were treated with and without 100 ng/ml recombinant human Bmp3 (R&D Systems, Minneapolis, MN, USA) for 6 h. At the end of treatment, total RNA was extracted from cultured cells using RNasy Mini Kit (QIAGEN, Inc., Mississauga, ON, Canada) coupled with on-column DNase digestion with the RNase-Free DNase Set (QIAGEN) according to the manufacturer’s instructions. A total of four arrays (GeneChip Mouse Genome 430 2.0 Array; Affymetrix, Santa Clara, CA, USA) were conducted using total RNA samples from two control and two Bmp3-treated sets of cells, as previously described (Guan et al. 2005). Briefly, micro-arrays were performed at the London Regional Genomics Centre (London, ON, Canada) following the standard procedures as outlined in the Affymetrix GeneChip Expression Analysis Technical Manual. The complete data set was submitted to the National Center for Biotechnology Information’s Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) database (acc. no. GSE73649). Expression values for the Affymetrix GeneChip data were globally normalized to a preset value and analyzed using GeneSpring version 10.0 Software (Silicon Genetics, Redwood City, CA, USA). Values for the mean expression level for each gene were calculated for the control and Bmp3 treatment microarray data sets. Candidate genes were selected by a combination of statistical analysis (P < 0.05) and comparison analysis (displayed > 1.5-fold increase or decrease from the control to Bmp3 treatment).

Assessment of mRNA levels: real-time quantitative RT-PCR

The relative abundance of various mRNAs was assessed by a two-step real-time quantitative RT-PCR (qRT-PCR), as described previously (Rajakumar et al. 2015). Briefly, 1 μg of total RNA was reverse-transcribed in a total volume of 20 μl using the High Capacity cDNA Archive Kit (Applied Biosystems) following the manufacturer’s instructions. For every RT reaction set, one RNA sample was set up without reverse-transcriptase enzyme to provide a negative control. Gene transcript levels of Gapdh (housekeeping gene), Wisp1 (CCN; human gene nomenclature), Cyr61 (CCN1), Ctgf (CCN2), Nov (CCN3), Wisp2 (CCN5), and Wisp3 (CCN6) were quantified separately by pre-designed and validated TaqMan Gene Expression Assays (Applied Biosystems; Table 1) following the manufacturer’s instructions. Briefly, gene expression assays were performed with the TaqMan Gene Expression Master Mix...
addition of 400 

Cells were then solubilized by the ice-cold PBS, once with 5% trichloroacetic acid (TCA) and Woodbridge, ON, Canada). Cells were washed twice with 75.2 Ci/mmol, PerkinElmer Life and Analytical Sciences, 

The incorporation of [3H]-thymidine into DNA was determined by scintillation counting. Results were expressed as a percentage of control.

Proliferation capacity of C3H10T1/2 MSCs was assessed by measuring [3H]-thymidine incorporation, as described previously (Stewart et al. 2010). Briefly, C3H10T1/2 stem cells were lysed in SDS Sample Buffer (62.5 mM Tris–HCL, pH 6.8, 2% w/v SDS, 10% v/v glycerol, 50 mM DTT and 0.01% w/v bromophenol blue). Equal concentrations of the whole cell lysates were subjected to a standard 10% SDS–PAGE. After electrophoresis, proteins were transferred to PVDF transfer membrane (Amersham Hybond-P, Cat# RPN303F) using a Bio-Rad Mini Transfer Apparatus. Non-specific antibody binding was blocked with 5% w/v milk in TTBS (0.1% v/v Tween-20 in TBS) for 1 h at room temperature. Membranes were then hybridized with primary antibody (WISP1 polyclonal antibody at 1:2000, CAT# PAB7404, Abnova, Taoyuan City, Taiwan; human GAPDH polyclonal antibody at 1:5000, Cat# IMG- 5567, Imgenex Corporation, San Diego, CA, USA) overnight at 4 °C. After three 10-min washes with TTBS, the membrane was incubated with the appropriate HRP-labeled secondary antibody, either anti-Goat (Cat# AP180P, Millipore, Etobicoke, ON, Canada, 1:10 000) or anti-rabbit (R&D Systems, Cat# HAF008, 1:1000) for 1 h at room temperature. Following another three 10-min washes in TTBS, proteins were detected by chemiluminescence (Western Lightning Plus-ECL, PerkinElmer Life and Analytical Sciences, Cat# NEL103001). The membrane was then exposed to X-ray film (Eastman Kodak, Rochester, NY, USA) for 1–60 min.

The siRNA-mediated knockdown of Wisp1 expression

To provide direct evidence for the involvement of Wisp1 in mediating the effects of Bmp3 on MSC proliferation, a siRNA-mediated knockdown approach was utilized (Guan et al. 2013). Briefly, C3H10T1/2 MSCs were plated on 24-well plates and cultured under standard conditions for 48 h. Cells were then transfected 100 nM of the Silencer Select Pre-designed siRNA-targeting mouse Wisp1 (CAT# S76051; Ambion) in Opti-MEM I medium (Invitrogen) containing 2 μl/well of Lipofectamine 2000 (Invitrogen), following the manufacturer’s instructions. Cells were also transfected in an identical manner with 100 nM of the negative control #1 siRNA (Cat# 4624; Ambion) or with the transfection agent alone to serve as controls. At 48 h post-transfection, cells were collected for western blot analysis. Alternatively, 12 h after transfection, cells were treated for 48 h with 100 ng/ml Bmp3. At the end of treatment, proliferation capacity was determined by [3H]-thymidine incorporation, as described above.

Western blot analysis

Western blot analysis was used to determine WISP1 protein levels, as described previously (Stewart et al. 2010). Briefly, C3H10T1/2 stem cells were lysed in SDS Sample Buffer (62.5 mM Tris–HCL, pH 6.8, 2% w/v SD, 10% v/v glycerol, 50 mM DTT and 0.01% w/v bromophenol blue). Equal concentrations of the whole cell lysates were subjected to a standard 10% SDS–PAGE. After electrophoresis, proteins were transferred to PVDF transfer membrane (Amersham Hybond-P, Cat# RPN303F) using a Bio-Rad Mini Transfer Apparatus. Non-specific antibody binding was blocked with 5% w/v milk in TTBS (0.1% v/v Tween-20 in TBS) for 1 h at room temperature. Membranes were then hybridized with primary antibody (WISP1 polyclonal antibody at 1:2000, CAT# PAB7404, Abnova, Taoyuan City, Taiwan; human GAPDH polyclonal antibody at 1:5000, Cat# IMG- 5567, Imgenex Corporation, San Diego, CA, USA) overnight at 4 °C. After three 10-min washes with TTBS, the membrane was incubated with the appropriate HRP-labeled secondary antibody, either anti-Goat (Cat# AP180P, Millipore, Etobicoke, ON, Canada, 1:10 000) or anti-rabbit (R&D Systems, Cat# HAF008, 1:1000) for 1 h at room temperature. Following another three 10-min washes in TTBS, proteins were detected by chemiluminescence (Western Lightning Plus-ECL, PerkinElmer Life and Analytical Sciences, Cat# NEL103001). The membrane was then exposed to X-ray film (Eastman Kodak, Rochester, NY, USA) for 1–60 min.

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**Table 1** TaqMan gene expression assays for various mouse genes.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctgf</td>
<td>Mm01192931_g1</td>
</tr>
<tr>
<td>Cyr61</td>
<td>Mm00487498_m1</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Mm99999915_g1</td>
</tr>
<tr>
<td>Nov</td>
<td>Mm00456855_m1</td>
</tr>
<tr>
<td>Wisp1</td>
<td>Mm00457574_m1</td>
</tr>
<tr>
<td>Wisp2</td>
<td>Mm04974711_m1</td>
</tr>
<tr>
<td>Wisp3</td>
<td>Mm01193670_m1</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Mm99999915_g1</td>
</tr>
</tbody>
</table>
Statistical analysis

Results are presented as mean ± S.E.M. of four independent experiments each performed in triplicate. Data were analyzed using one-way ANOVA followed by Tukey’s post-hoc test, or Student’s t-test as indicated. Significance was set at \( P < 0.05 \).

Results

Effects of \textit{Bmp3} on MSC transcriptome

As a first step in identifying the downstream factors that are responsible for mediating \textit{Bmp3}-induced MSC proliferation, we conducted genome-wide gene expression profiling and identified 134 and 164 genes that were up- and downregulated by \textit{Bmp3} respectively (data not shown). Among the significantly upregulated genes, WNT-inducible secreted protein 1 (\textit{Wisp1/CCN4}), whose expression was upregulated 3.7-fold by \textit{Bmp3} (Fig. 1A), was of particular interest, because it has been previously shown to promote proliferation of cardiac fibroblasts (Venkatachalam et al. 2009), lung epithelial cells (Königshoff et al. 2009) and osteogenic cells (Inkson et al. 2009). Consequently, we selected \textit{Wisp1} as a potential candidate gene and verified the magnitude of the increase in levels of \textit{Wisp1} mRNA following \textit{Bmp3} treatment with qRT-PCR (Fig. 1B; \( P < 0.001 \)). Importantly, we showed that there was a corresponding increase in levels of WISP1 protein (Fig. 1C; \( P < 0.001 \)).

Effects of \textit{Bmp3} on the expression of CCN family genes

\textit{Wisp1} belongs to the CCN family genes, which include \textit{Cyr61 (CCN1)}, \textit{Ctgf (CCN2)}, \textit{Nov (CCN3)}, \textit{Wisp2 (CCN5)}, and \textit{Wisp3 (CCN6)} (mouse and human gene nomenclatures, respectively). To establish whether the effects of \textit{Bmp3} were specific to \textit{Wisp1}, we determined mRNA levels of all six CCN family genes by qRT-PCR. We showed that mRNA levels of \textit{Cry61}, \textit{Ctgf} and \textit{Wisp1} were increased by 1.7-, 6.5- and 4.1-fold following \textit{Bmp3} treatment respectively (Fig. 2). By contrast, the mRNA level of \textit{Nov}, \textit{Wisp2} and \textit{Wisp3} were unchanged following \textit{Bmp3} treatment (Fig. 2).

Effects of \textit{Bmp3} on \textit{Wisp1} expression and MSC proliferation

Given that \textit{Wisp1} was upregulated following \textit{Bmp3} treatment and is a known pro-survival factor in various non-adipose cell types (Inkson et al. 2009, Königshoff et al. 2009, Venkatachalam et al. 2009, Schlegelmilch et al. 2014), coupled with previous findings that human adipocyte differentiation was associated with elevated \textit{Wisp1} expression (Murahovschi et al. 2015), we examined the involvement of \textit{Wisp1} in mediating \textit{Bmp3}-induced MSC proliferation. We first determined if \textit{Bmp3}-induced upregulation of \textit{Wisp1} expression preceded \textit{Bmp3}-stimulated cell proliferation by conducting a time-dependent experiment. We showed that \textit{Bmp3} increased \textit{Wisp1} mRNA as early as 3 h, while an increase in \(^{3}H\)-thymidine incorporation was not observed until 12 h post-treatment (Fig. 3A and B).

Effects of \textit{Wisp1} on MSC proliferation

To determine if \textit{Wisp1} stimulates MSC proliferation, we treated \textit{C3H10T1/2} MSCs with various concentrations of recombinant \textit{Wisp1} and assessed cell proliferation by standard \(^{3}H\)-thymidine incorporation assays. We showed that \textit{Wisp1} increased \(^{3}H\)-thymidine incorporation in a
concentration-dependent manner, with a maximal effect at 1 μg/ml, and to a similar degree as Bmp3 (>3-fold increase; Fig. 4).

Effects of siRNA-mediated knockdown of Wisp1 expression on Bmp3 stimulation of MSC proliferation

To provide direct evidence for the involvement of Wisp1 in mediating Bmp3-induced MSC proliferation, we transfected C3H10T1/2 MSCs with siRNA specific for mouse Wisp1 and examined the effect of Bmp3 on [3H]-thymidine incorporation. As shown in Fig. 5, transfection of the cells with Wisp1-specific siRNA resulted in an 80% reduction in WISP1 protein levels. Furthermore, siRNA-mediated knockdown of Wisp1 expression significantly attenuated the stimulatory effect of Bmp3 on [3H]-thymidine incorporation (from >350 to 150% of control) (Fig. 5C).

Discussion

In the present study, we demonstrate that i) Bmp3 induces dynamic changes in C3H10T1/2 MSC transcriptome, including a significant increase in the expression of Wisp1, a key pro-survival factor previously identified in non-adipose cells (Venkatachalam et al. 2009, Schlegelmilch et al. 2014); ii) Bmp3-induced upregulation of Wisp1 expression proceeds Bmp3-stimulated MSC proliferation; iii) recombinant Wisp1 potently stimulates MSC proliferation; and iv) siRNA-mediated knockdown of Wisp1 expression significantly attenuates the stimulatory effect of Bmp3 on MSC proliferation. Taken together, these findings reveal Wisp1 as a novel target of Bmp3, and demonstrate that the Bmp3/Wisp1 signaling pathway plays an important role in MSC proliferation. However, the physiological significance of these findings in vivo remains to be determined.

Although accelerated adipogenesis is a hallmark of obesity, the precise molecular mechanisms and factors that govern adipogenesis are incompletely understood. Adipogenesis begins with the commitment of MSCs to the adipocyte lineage, followed by terminal differentiation of preadipocytes to lipid-filled mature adipocytes (Rosen & MacDougald 2006, James 2013). Prior to these stages...
however, MSCs undergo proliferation, a process that is critical but poorly understood (Rosen & MacDougald 2006, Moreno-Navarrete & Fernandez-Real 2012). We previously showed that \textit{Bmp3} stimulated MSC proliferation via the TGF-b/activin-signaling pathway (Stewart et al. 2010). In this study, we sought to identify the downstream molecular targets that mediate \textit{Bmp3}-induced MSC proliferation. Using DNA microarray, we first identified genes that were differentially regulated by \textit{Bmp3}. Prominently among the upregulated genes was \textit{Wisp1}, the increased expression of which is known to be associated with human adipocyte differentiation (Murahovschi et al. 2015). \textit{Wisp1} is also a pro-survival factor, because it stimulates proliferation of several non-adipose cell types, including cardiac fibroblasts (Venkatachalam et al. 2009), lung epithelial cells (Königshoff et al. 2009), osteogenic cells (Inkson et al. 2009). Furthermore, reduced expression of \textit{Wisp1} is associated with enhanced apoptosis in cultured human mesenchymal stromal cells (Schlegelmilch et al. 2014). Therefore, we chose \textit{Wisp1} as a candidate gene for this study. We first confirmed the upregulated expression of \textit{Wisp1} by \textit{Bmp3} with qRT-PCR and western blotting. We then determined if the effects of \textit{Bmp3} were specific to \textit{Wisp1} or the entire \textit{CCN} family of genes. Our results indicated that \textit{Bmp3} discretely upregulated the expression of two other \textit{CCN} family genes, \textit{Cyr61} and \textit{Ctgf}, while expression of the remaining members of \textit{CCN} family was unchanged. To the best of our knowledge, this is the first study to identify certain members of the \textit{CCN} family as downstream targets of \textit{Bmp3}. Although \textit{Cyr61} has been shown to play a crucial role in cutaneous wound healing (Jun & Lau 2010), while \textit{Ctgf} is a known mediator of organ fibrosis (Chen & Lau 2009), the functional significance of \textit{Bmp3}-induced upregulation of \textit{Cyr61} and \textit{Ctgf} awaits future exploration.

Our next step was to examine the involvement of \textit{Wisp1} in mediating \textit{Bmp3}-induced MSC proliferation.
By conducting a time-dependent experiment, we determined that Bmp3 upregulated the expression of Wisp1 before stimulating cell proliferation, suggesting the involvement of Wisp1 in mediating BMP3 stimulation of MSC proliferation. To provide direct evidence in support of this contention, we showed that treatment with recombinant Wisp1 led to a concentration-dependent increase in MSC proliferation. This is consistent with the findings of two previous studies showing that Wisp1 was a proliferative factor in human MSCs, aiding in bone development (Thorfve et al. 2014) and fracture repair (French et al. 2004).

To provide additional evidence for the involvement of Wisp1 in mediating Bmp3-induced MSC proliferation, we used a loss-of-gene function approach. We found that siRNA-mediated knockdown of Wisp1 expression significantly attenuated the stimulatory effect of Bmp3 on MSC proliferation, indicating that Wisp1 is a key mediator of Bmp3 stimulation of MSC proliferation. Given that the knockdown of Wisp1 expression did not completely block Bmp3-induced MSC proliferation, it is possible that factors other than Wisp1 may also be involved in mediating the effects of Bmp3. It is also probable that the incomplete knockdown of Wisp1 expression may account for the incomplete blockade of Bmp3-induced MSC proliferation. It is interesting to note that the stimulatory effects of Wisp1 on vascular smooth muscle cell proliferation was attenuated but not prevented by siRNA-mediated knockdown of Wisp1 expression (Liu et al. 2013). Obviously, future studies will be required to explore these possibilities.

In the past several decades, Wisp1 has emerged as a prominent pro-survivor and proliferative factor (Venkatachalam et al. 2009, Maiese 2014). Importantly, aberrant function or dysregulation of the Wisp1 signaling is associated with a variety of disorders, including musculoskeletal, vascular and neurodegenerative (Maiese 2014), as well as various cancers (Gurbuz & Chiquet-Ehrismann 2015), including breast (Chiang et al. 2015), prostate (Ono et al. 2013) and gastrointestinal (White et al. 2012). In the present study, we identify Wisp1 as a novel mediator of Bmp3-induced MSC proliferation, a key but poorly understood component of adipogenesis. We recognize that MSCs can give rise to four distinct cell types, including adipocytes, chondrocytes, myocytes and osteocytes (Rosen & MacDougald 2006). As such, it is possible that increased MSC proliferation may have implications for the commitment and differentiation of these pluripotent MSCs to any of the four diverse cell types. However, we have provided evidence to support the contention that stimulation of MSC proliferation by the Bmp3/Wisp1 signaling pathway is a mechanism important to adipogenesis. We previously demonstrated that Bmp3 was upregulated in the adipose tissue of an experimental rat model of visceral adiposity and promoted proliferation of both MSCs and 3T3-L1 preadipocytes (Stewart et al. 2010). In the present study, we show that Bmp3 potently stimulates the expression of Wisp1, which precedes Bmp3-induced MSC proliferation. We also demonstrate that Wisp1 stimulates MSC proliferation to the same degree as Bmp3. Our contention is also corroborated by a recent study that showed that the expression of Wisp1 was associated with adipocyte differentiation and was elevated in the visceral adipose tissue of obese humans (Murahovschi et al. 2015). However, the question of whether Wisp1 stimulates adipogenic differentiation of MSCs as well as the receptor and the signaling pathway that mediates the effects of Wisp1 remain to be determined.

In conclusion, our present findings reveal that the Wisp1-mediated effects of Bmp3 stimulation of MSC proliferation and may also have important implications for adipogenesis and the pathogenesis of obesity.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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