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Retinoic acid inhibits BMP4-induced C3H10T1/2 stem cell commitment to adipocyte via downregulating Smad/p38MAPK signaling

Jeong Soon Lee [a], Jin-Hee Park [a], Il Keun Kwon [b], Jung Yul Lim [a,b,*]

[a] Department of Engineering Mechanics, College of Engineering, University of Nebraska-Lincoln, Lincoln, NE 68588, USA
[b] The Graduate School of Dentistry, Kyung Hee University, Seoul, South Korea

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ABSTRACT

Increased adipocyte formation from mesenchymal stem cells (MSCs) is typical for obesity. It is recently observed that bone morphogenetic proteins (BMPs) provide instructive signals for the commitment of MSCs to adipocytes. We examined potential role of retinoic acid (RA) in inhibiting the BMP4 induction of MSC commitment toward adipocyte. BMP4-treated C3H10T1/2 MSCs, when further exposed to adipogenic differentiation media, displayed distinct adipocytic commitment and differentiation. This could be inhibited by RA exposure during the BMP4 treatment stage (commitment stage before adipogenic hormonal inducers were given), as was observed by reductions in key adipogenic genes/transcription factors (C/EBPα, PPARγ, aP2), lipogenic genes (LPL, FAS, GLUT4), and lipid accumulation. Among RA receptors (RARs) screened, RARβ was mainly upregulated under RA exposure. BMP4 signaled through both Smad1/5/8 and p38 mitogen-activated protein kinase (MAPK) and RA significantly suppressed the BMP4-triggered phosphorylation of both Smad1/5/8 and p38MAPK. These data suggest that RA has inhibitory effects on the BMP4 induction of C3H10T1/2 adipocytic commitment via downregulating Smad/p38MAPK signaling. How to inhibit MSC adipocytic commitment, as partly revealed in this study, will have a significant impact on treating obesity and related diseases.

1. Introduction

Obesity is a critical risk factor for various metabolic diseases, including cardiovascular dysfunction, type 2 diabetes, liver steatosis, and even Alzheimer's disease and cancers [1–3]. Improved understanding of the molecular mechanism of obesity is in urgent need to advance comprehensive strategies to prevent and treat obesity-related diseases. The excessive growth, differentiation, and hypertrophy of adipocytes are the fundamental processes of obesity. The increase in adipocyte number is mainly achieved by the recruitment of new preadipocytes from mesenchymal stem cells (MSCs) [4] and by the mitotic clonal expansion of such preadipocytes [5]. Since MSCs have plasticity to commit to various lineages, e.g., adipocytes, osteocytes, myocytes, or chondrocytes, it is critical to understand what factors regulate MSCs to commit toward the adipocytic lineage for obesity study.

Bone morphogenetic proteins (BMPs), members of the transforming growth factor β family, are involved in a variety of developmental processes including heart, central nervous system, cartilage, and post-natal bone [6]. BMPs were initially named for their ability to induce bone formation, and BMP2/4/7 was shown to play crucial roles in osteoblast differentiation and bone formation [7]. Interestingly, it was recently revealed that BMP4 has the potential to induce MSC commitment to preadipocyte [8–11]. Exposure of proliferating C3H10T1/2 MSCs to BMP4 induced their commitment to preadipocytes, and these cells underwent adipogenesis when further exposed to adipogenic differentiation media. If BMP4 signaling is blocked by Noggin, a BMP antagonist, the adipogenesis of C3H10T1/2 MSCs was not observed [8]. BMP2, similar to BMP4, showed the potential to induce MSC adipocytic commitment but at a higher concentration [12]. It is now recognized that BMPs provide instructive signals for MSC commitment to preadipocyte, but relevant mechanisms are less fully understood.

Retinoic acid (RA), biologically active form of vitamin A, plays an important role in diverse cell processes including embryogenesis and differentiation [13]. RA regulation of adipogenesis has been highlighted, but it is still a controversial topic. Several studies showed evidence for RA-induced adipogenesis inhibition. RA inhibited the adipogenic differentiation of 3T3-L1 preadipocytic cells by blocking CCAAT/enhancer binding protein (C/EBP) β-mediated transcription [14]. Also, RA inhibited the adipogenesis of 3T3-L1 cells via RA receptor (RAR) signaling [15]. Another study demonstrated that RA stimulates the nuclear accumulation and transcriptional activity of Smad3, which acts as an adipogenesis inhibitor [16]. Some studies, on the other hand, showed contrary

* Corresponding author at: Department of Engineering Mechanics, College of Engineering, University of Nebraska-Lincoln, W303 Nebraska Hall, Lincoln, NE 68588, USA. Fax: +1 402 472 8292.
E-mail address: jlim4@unl.edu (J.Y. Lim).

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RA effects on adipogenesis especially for embryonic stem cells (ES). RA activated extracellular signal-regulated kinase (ERK) in ES cells, which was required for ES commitment to adipocyte [17]. RA also directed mouse ES fate to adipocyte via signaling through RARβ [18]. Together, RA regulation of adipogenesis is yet to be concluded and little is known for MSCs especially when BMPs direct MSC fate.

In this study, we demonstrated the potential inhibitory effects of RA on the BMP4 induction of MSC adipogenic commitment and differentiation. To reveal the RA interference of BMP4 function at the commitment stage, RA was co-treated during the BMP4 treatment stage before adipogenic induction media were given. As a potential mechanism, we observed BMP4-triggered phosphorylation of Smad1/5/8 and p38 mitogen-activated protein kinase (MAPK) was substantially attenuated under RA.

2. Materials and methods

2.1. Cell culture and BMP4 induction of MSC adipogenesis

C3H10T1/2 MSCs (ATCC, CCL-226) were maintained using growth media composed of Dulbecco's Modified Eagle's medium, 10% fetal bovine serum, and 1% antibiotics. Cells were differentiated to adipocytes following the reported [19] and our [20] protocols (Fig. 1A). Cells were grown using the growth media for 4 days, stimulated for 2 days by adipogenic induction media (10 μg/ml insulin, 1 μM dexamethasone, 0.5 mM methylisobutylxanthine, IBMX), and kept with adipogenic maintenance media (10 μg/ml insulin) for additional 6 days with media changed every 2 days. The BMP4 induction of MSC commitment to preadipocyte was performed similar to the published protocol [8]. During the 4 days of proliferating period, cells were exposed to 50 ng/ml of recombinant BMP4 (R&D Systems, 5020-BP-010). From day 0, cells were exposed to adipogenic induction and maintenance media the same as above. For BMP4 plus RA treatment, all-trans-RA (Sigma, R2625) was further added at 1 μM. Additionally, to check if RA pretreatment alone affects MSC adipogenesis, cells were exposed to 1 μM RA. Four pretreatment conditions (control, BMP4, BMP4 plus RA, RA) were thus tested. Note that cells reached confluence on day 0 for all four pretreatment conditions before cells were exposed to adipogenic inducers (see Supplementary data).

RT-PCR. Quantitative RT-PCR was performed following our published protocol [20] using PCR primer pairs as below. C/EBPα: sense GGG TGA GTT CAT GGA GAA TGG, anti-sense CAG TTT GGC AAG AAT CAG AGC A; peroxisome proliferator-activated receptor (PPAR) γ: sense AGG CCG AGA AGG AGA AGC TGT TG, anti-sense TGG CCA CCT CTT TGC TCT GCT G; adipocyte protein 2 (aP2): sense TCT CAC CTG GAA GAC AGC TCC TCG, anti-sense TTC CAT CCA GGC CTC TTC CTT TGG CTC; lipoprotein lipase (LPL): sense AGG GCT CTG CCT GAG TT, anti-sense AGA AAT CTC GAA GGC CTG GT; fatty acid synthase (FAS): sense AGC CAC GAG TGA GTG TAC GGG AG, anti-sense GGG ACA GGA CAA GAC AAA AAG GG; glucose transporter type 4 (GLUT4): sense GCT TTG TGG CCT TCT TTG AG, anti-sense CAG GAG GAC GGC AAA TAG AA; RARα: sense TCA GTG AAC TCT CCA CCA AG, anti-sense TCC AGT CTC AGC ATC GTC C; RARβ: sense GTT CAC CTT TGC CAA CCA G, anti-sense TGA GAG GTG GCA TTG ATC C; RARγ: sense CTT CCC AGG TGC ACT CAG AG, anti-sense CGA TTT CTG GTG ACC TTG TTG; GAPDH: sense CAT GTT CCA GTA TGA CTC CAC TC, anti-sense GGC CTC ACC CCA TTT GAT GT.

2.2. Immunoblotting of Smad and p38 phosphorylation

BMP4 and RA dependent Smad and p38 phosphorylation was quantified. Cells were exposed to 50 ng/ml BMP4, with or without 1 μM RA, for various time periods (15, 30, 60, and 120 min). Western immunoblotting was performed following our published protocol [21]. Primary antibodies specific to Smad1/5/8, phosphorylated Smad1/5/8, p38MAPK, and phosphorylated p38MAPK (Cell Signaling) were used at 1:1000. Immunopositive bands were visualized using enhanced chemiluminescence and band intensity was quantified by using ImageJ software.

2.3. Oil Red O staining and lipid quantification

On day eight cells were fixed with 10% formalin for 60 min and stained with oil Red O solution. The stained cells were photographed using an optical microscope. The stained oil Red O was extracted with isopropanol and the amount was quantified by measuring the optical absorbance at 570 nm using a BioTek spectrophotometer.

2.4. Statistics

Three assays, each in triplicate, were performed. One-way analysis of variance (ANOVA) followed by Student–Newman-Keuls post hoc tests was performed. Mean ± standard deviation is shown in the figures. Comparison with the control is shown with *, ** and comparison with BMP4-treated cells with $^{\#}$, $^{\#\#}$ at a significance level of $p < 0.05$ (*, $^{\#}$) and $p < 0.01$ (**, $^{\#\#}$).

3. Results

3.1. RA inhibits BMP4 induction of C3H10T1/2 MSC adipogenic commitment and differentiation

Proliferating C3H10T1/2 MSCs were treated with BMP4 and/or RA for 4 days, after which cells were induced to differentiate to adipocytes for 8 days. BMP4 significantly enhanced the commitment of C3H10T1/2 MSCs to preadipocytes that have the capacity to differentiate to adipocytes when further treated with adipogenic hormonal inducers. This was confirmed by the significant upregulation in key adipogenic genes/transcription factors (C/EBPα, PPARγ, aP2) on day 1 for BMP4-treated MSCs relative to the control (Fig. 1B). Such increase in commitment genes by BMP4 could be completely blocked by RA co-pretreatment. Lipogenic gene expression (LPL, FAS, GLUT4), a marker of terminal adipogenesis, showed on day 8 the same trend with respect to BMP4 and RA pretreatment (Fig. 1B). BMP4-pretreated cells showed distinct lipid accumulation on day 8, as seen in oil Red O staining and spectrophotometer quantification (Fig. 1C). Control cells, stimulated for 8 days with adipogenic hormonal inducers the same but without BMP4 pretreatment, did not secrete any lipids for the same time period. Such BMP4-induced lipid synthesis was significantly suppressed when RA was co-pretreated. Cells pretreated with RA alone did not show noticeable changes in adipogenic and lipogenic genes and lipid synthesis relative to the control. Note that two of the RA-treated cells (BMP4 plus RA, RA) displayed elongated cell morphology relative to RA-untreated cells (control, BMP4). See Supplementary data for cell morphology on day 0 and 1.

3.2. RARβ was upregulated when MSCs were exposed to RA

RA binds to RAR/retinoid X receptor (RXR) heterodimer to mediate downstream gene transcription [22]. To reveal which RAR is mainly involved in RA-BMP4 co-control of MSC fate, we examined RAR isotypes. Cells were treated with BMP4 and/or RA for 4 days and with adipogenic induction media for 24 h. RARα mRNA expression did not change with RA exposure, with or without BMP4 (Fig. 2). On the other hand, RARβ was significantly increased in

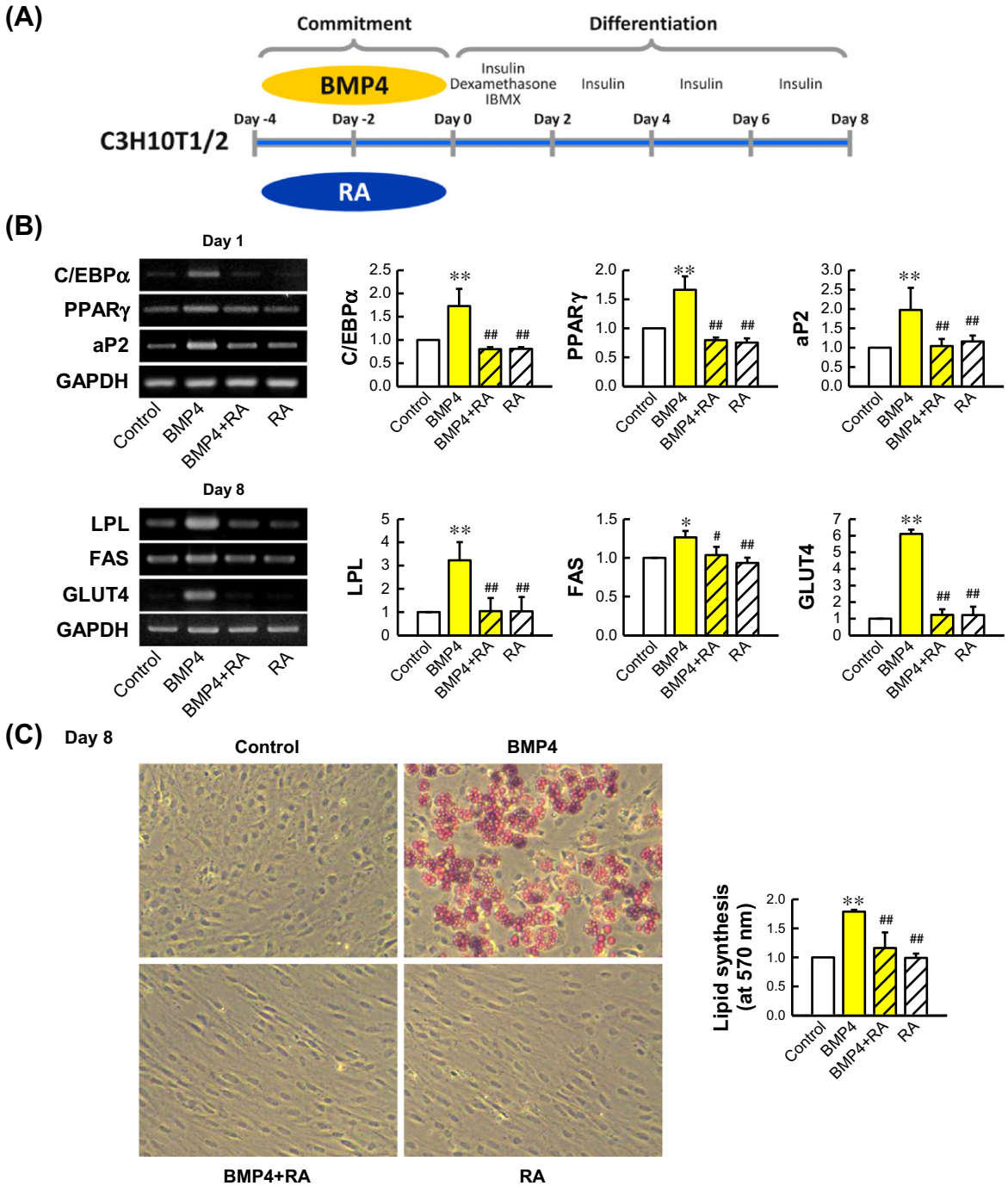

Fig. 1. BMP4 induction of C3H10T1/2 MSC adipogenic commitment and differentiation is inhibited by RA. (A) C3H10T1/2 MSCs were treated for 4 days with growth media or growth media further supplemented with BMP4, BMP4 plus RA, or RA. Cells were then induced for 8 days to differentiate to adipocytes using adipogenic induction and maintenance media. (B) Adipogenic (C/EBPα, PPARγ, aP2) and lipogenic (LPL, FAS, GLUT4) mRNA expressions, assessed on day 1 and 8 respectively, showed significant increases in BMP4-pretreated MSCs. Such upregulation was eliminated when RA was co-pretreated with BMP4. Quantitative RT-PCR data were shown with the control sample data as 1. (C) BMP4-induced lipid accumulation was significantly blocked with RA co-pretreatment. Oil Red O stained images and oil Red O amount quantified by spectrophotometer. RA pretreatment alone did not induce changes in adipogenic and lipogenic gene expression and lipid synthesis relative to the control. Comparison with the control (*, **) and with BMP4 ($^{\#}$, $^{\#\#}$) shown at a significance of $p < 0.05$ (*, $^{\#}$) and $p < 0.01$ (**, $^{\#\#}$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

two RA-treated cells (BMP4 plus RA, RA) relative to RA-untreated cells (control, BMP4). While RARγ did not change under RA relative to the control, RARγ expression in BMP4 plus RA was slightly higher than that of the BMP4. The trend in RARγ expression was less systematic than that of RARβ.

3.3. BMP4-triggered Smad1/5/8 and p38MAPK phosphorylation is suppressed by RA

To reveal the potential mechanism of RA blockage of BMP4 direction of MSC adipogenesis, we examined Smad and p38

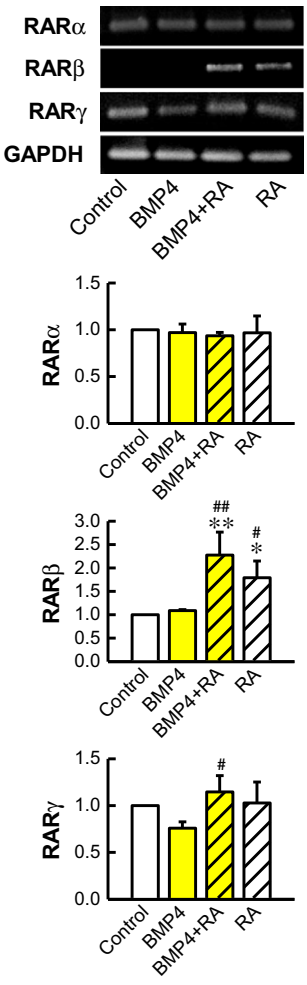

Fig. 2. RARβ is systematically upregulated in C3H10T1/2 MSCs under RA exposure. Among RARs screened, RARβ mRNA expression showed upregulation for two RA-treated cells (BMP4 + RA, RA) relative to RA-untreated cells (control, BMP4). Quantitative RT-PCR data were shown with the control sample data as 1. See Fig. 1 legend for statistics.

phosphorylation. Both pathways are reported to act downstream of BMP type I and II receptor signaling [6]. Cells were treated with BMP4 at varying time periods in the presence or absence of RA, and the phosphorylation of Smad1/5/8 and p38MAPK was quantified by normalizing the immunoblot band intensity of phosphorylated (p−) protein with that of the corresponding total protein (Fig. 3). Obtained ratio data were shown with BMP4-untreated control (Un) as 1. Phosphorylation of both Smad1/5/8 and p38MAPK was significantly increased at 15 and 30 min of BMP4 exposure relative to the untreated control (*, **). Such BMP4-triggered activation in Smad1/5/8 and p38MAPK was significantly suppressed in the presence of RA at the same time points (#, ##), suggesting that RA downregulates BMP4-Smad/p38MAPK signaling. RA treatment alone did not induce the phosphorylation of Smad or p38MAPK (data not shown).

4. Discussion

The role of RA on adipogenesis is yet to be determined, since previous reports have shown contradictory data depending on the adipose precursor cell type, e.g., RA inhibition of 3T3-L1 preadipocytic cell adipogenesis [14–16] or RA regulation of ES cell adipogenesis [17,18]. We demonstrated for MSCs that RA has inhibitory effects on the BMP4 induction of adipogenic commitment and differentiation. Furthermore, specific to this study, we showed that MSC adipogenesis could be blocked via exposing cells to RA during the BMP4 treatment stage (4 days of proliferation period until cell confluence was reached on day 0, which is before adipogenic induction was attempted). Our data are thus in contrast to those of other studies, in which the inhibition of adipogenesis was achieved by applying RA while cells were exposed to adipogenic differentiation media [14–16]. The observation that BMP4 triggering of Smad/p38MAPK phosphorylation was significantly suppressed in the presence of RA suggests potential regulatory mechanism.

C3H10T1/2 MSCs have been focused as a cell model for adipocytic commitment, since they do not usually respond to adipogenic hormonal inducers [23]. BMPs, other than the role in bone formation, have shown the potential to drive C3H10T1/2 MSCs to commit to preadipocytes and respond to adipogenic hormonal inducers [8–12]. Our data are consistent with these studies. BMP4-treated C3H10T1/2 MSCs displayed accelerated adipogenesis as it required only 8 days for producing mature lipid-laden adipocytes (Fig. 1C). Without BMP4 pretreatment C3H10T1/2 adipogenesis usually takes about 14 days or more, as in our previous study [20]. RA inhibition of the BMP4 induction of adipocytic commitment was confirmed by significantly downregulated

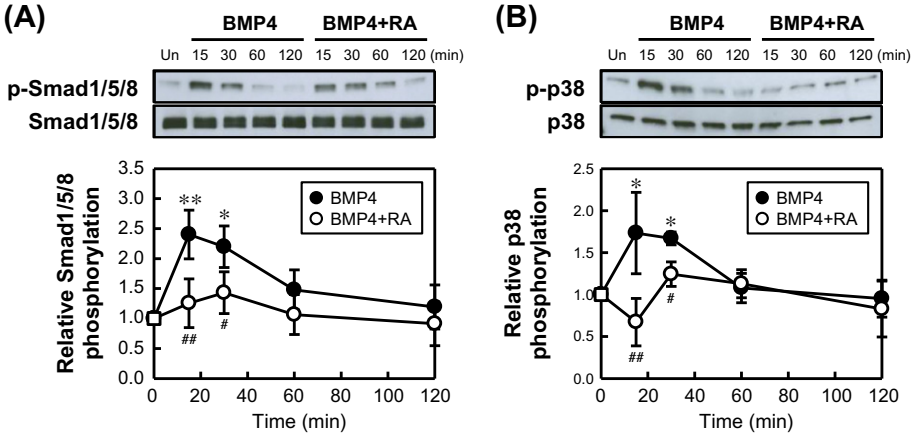

Fig. 3. RA inhibits BMP4-triggered Smad1/5/8 and p38MAPK phosphorylation in C3H10T1/2 MSCs. Immunoblotting showed that BMP4 treatment induced the phosphorylation of Smad1/5/8 and p38MAPK, e.g., increased p-Smad1/5/8 and p-p38 at 15 and 30 min of BMP4 exposure relative to BMP4-untreated control (Un, empty square). These increases were significantly reduced with RA co-exposure. Comparison with untreated control is shown with (*, **) and comparison with BMP4 sample at the same time points is noted with (#, ##) at a significance of $p < 0.05$ (*, #) and $p < 0.01$ (**, ##).

adipogenic transcription factors, e.g., C/EBPα and PPARγ (Fig. 1B). Activation of defined transcription factors is the essential step for stem cell lineage commitment. Differentiation to preadipocytes is initiated by C/EBPβ, which activates PPARγ [24,25]. PPARγ and C/EBPα form positive transcriptional feedback loop orchestrating downstream adipocyte biology [26]. Similar to the trend of commitment genes, BMP4-induced upregulation in terminal adipogenesis markers (lipogenic genes, lipid synthesis) showed significant reduction by RA (Fig. 1B and C).

BMPs typically signal through Smad pathways [6]. BMPs activate BMP type II receptor, which phosphorylates BMP type I receptor. This activates Smad signaling via phosphorylation of Smad1/5/8 and their association with co-Smad (Smad4). The heteromeric Smad1/5/8-Smad4 complex translocates to the nucleus and controls the transcriptional activity of the target genes, regulating cell commitment and differentiation. For example, it has been proposed that BMP signaling through Smads controls the transcriptional activities of C/EBP family and PPARγ in the case of BMP2/4 induction of C3H10T1/2 MSC adipogenesis [12]. BMPs also signal through non-Smad pathways such as MAPKs, including p38, ERK, and c-Jun N-terminal kinase (JNK) [6]. These MAPK pathways also act as downstream of BMP type I and II receptor signaling. Huang et al. [12] recently proposed that, while both Smad and p38 are involved in the BMP2/4 induction of MSC adipocytic commitment, Smad1/5/8 may have a dominant role since the knockdown of p38 has relatively little effect on BMP-induced adipogenesis. On the other hand, other studies observed BMP signaling mainly through MAPKs, e.g., BMP4 activation of p38 in myocytes [27] and BMP2 phosphorylation of ERK1/2 during the osteoblastic differentiation of C3H10T1/2 MSCs [28]. We screened two major BMP signaling pathways, Smad and p38, as a potential mechanism of BMP4-RA co-control of MSC adipogenesis. BMP4 phosphorylated both Smad1/5/8 and p38MAPK and both activations were significantly suppressed in the presence of RA (Fig. 3). This suggests that BMP4 signaling through both Smad1/5/8 and p38MAPK may play a certain role in BMP4-induced MSC adipogenic activities and that the RA inhibition of BMP4 functioning is achieved by RA-induced downregulation of BMP4-Smad/p38MAPK signaling.

It has been proposed that RARs are involved in the adipogenesis. The repertoire of RAR and its change with adipogenic differentiation are, however, not consistent depending on the cell type, in vitro or in vivo condition, etc. For example, RARα and -γ were abundant but RARβ showed only a trace in 3T3-L1 preadipocytes [29]. During the adipogenesis of 3T3-L1 cells, RARα remained constant while RARγ mRNA and protein levels dramatically decreased [30]. In a recent study by Berry and Noy [15], 3T3-L1 cells displayed RARα and -γ and both of them were downregulated during the adipogenic differentiation. In the same study, on the other hand, lean and obese mouse models exhibited all three RARs (-α, -β, -γ), and when obese mice were treated with RA the mice displayed weight loss and a significant upregulation only in RARβ [15]. Overall, for 3T3-L1 preadipocytes and animal model is reported the antagonistic correlation between RAR level and adipogenesis/obesity phenotype. On the contrary, RAR signaling, such as RARβ, was required for ES cell adipogenic differentiation [18]. For MSC adipogenesis little is known about RAR repertoire. In our study, all RARs (-α, -β, -γ) were detected in C3H10T1/2 MSCs and among them RARβ showed the most notable and systematic changes such that only RARβ mRNA was significantly upregulated under RA exposure (Fig. 2). Our data suggest a mechanism similar to that of animal model and 3T3-L1 cells [15,29,30], i.e., antagonistic correlation between RAR and adipogenesis/obesity. The difference though exists in the RAR repertoire in that RARα and -γ were the main RARs for 3T3-L1 preadipocytes while we observed major changes in RARβ for C3H10T1/2 MSCs.

It is premature to conclude a direct regulatory mechanism between RARβ and BMP4 signaling. However, based on the observed data on RAR expression under RA (Fig. 2) and RA inhibition of BMP4-mediated signaling (Fig. 3) and commitment genes (Fig. 1B), tentative conclusion may be drawn. RA may interfere with BMP4-induced Smad/p38MAPK regulation of the transcriptional activities of adipogenic commitment genes (C/EBPα, PPARγ) and this is achieved potentially via upregulated RA-RARβ signaling. Further studies are required on how RAR may directly regulate BMP4 activities during the MSC adipocytic commitment.

Potential competitive role of RA and BMP in directing cell fate is recently highlighted. Kennedy et al. [31] reported that RA enhances skeletal muscle progenitor formation and during this process the inhibition by BMP4 could be bypassed by RA. They showed that during the embryonal carcinoma cell differentiation RA overwhelmed the effect of BMP4 by upregulating BMP4 inhibitor, Tob1, and enhanced myogenesis. This may suggest an analogous mechanism such that RA may act as an antagonist for BMP4 when BMP4 directs MSC fate toward adipogenesis.

The data by Kennedy et al. [31], RA stimulation of myogenesis overcoming BMP4, may suggest another regulatory mechanism, i.e., cell shape control of stem cell fate. We observed elongated cell morphology when MSCs were treated with RA (Supplementary data, Fig. 1C). Similar cell shape was seen for RA-treated C3H10T1/2 [16]. Elongated, spindle-shaped cell morphology is one of the distinct markers of stem cell lineage commitment toward myogenesis. For instance, during the extracellular milieu-controlled MSC fate direction cell morphology could clearly represent the MSC lineage commitment, e.g., spindle cell shape for myogenesis [32]. Moreover, it is well-established that RA has a strong stimulatory role in myogenesis [33]. Combined, it is speculated the RA inhibition of BMP4-induced MSC adipocytic commitment observed in this study may be partly because RA tends to direct MSCs to differentiate toward the other lineage, e.g., myogenesis. However, since RA is also involved in many other differentiation lineages including osteogenesis, neurogenesis, etc. [34,35], systematic differentiation studies for various fates are required to assess whether RA, in the presence or absence of BMP4, directs MSCs to choose lineages other than adipogenesis.

In conclusion, we showed that BMP4-induced upregulation in adipogenic/lipogenic genes and lipid secretion in C3H10T1/2 MSCs was significantly inhibited with RA co-exposure during the BMP4 treatment (commitment) stage. Among RA receptors, RARβ appeared to be mainly involved in this process. BMP4 activated Smad1/5/8 and p38MAPK phosphorylation and this was significantly suppressed in the presence of RA, suggesting potential mechanism. Our data may further suggest an intriguing implication on stem cell fate switching such that RA inhibition of BMP4-mediated MSC adipogenic commitment may potentially be reflected into enhanced MSC commitment toward the other lineages.

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Appendix A. Supplementary data

Supporting data are provided to show cell morphology on day 0 and 1. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.05.042.

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