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Chapter 6

Exon Skipping in Activin-receptor-like Kinase 2: Towards a Treatment for Fibrodysplasia Ossificans Progressiva

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Progress report
Abstract

Bone morphogenetic proteins (BMPs) are potent stimulators of osteoblast differentiation and bone formation. Fibrodysplasia Ossificans Progressiva (FOP) is a rare heritable disease in which connective tissues turn into bone tissue. Mutations in the gene encoding activin receptor-like kinase2 (ALK2), a BMP type I receptor, have been linked to FOP. FOP patients with classical clinical features have a mutation that results in a R206H amino acid change in ALK2. ALK2 R206H mutant has constitutive activity and sensitizes the cells to BMP signalling. In this study, the aim is to cause the knockdown ALK2 by means of exon skipping using antisense oligonucleotides (AONs), which could potentially be used to normalize the excessive ALK2 activity in FOP patients. The ALK2 exon harbouring the FOP mutant allele was targeted in different cell types using antisense oligonucleotides (AON) in different cell types. The exon skipping and expression of ALK2 as well as the BMP6-induced responses were assessed. ALK2 AONs induce skipping of the exon that harbours the mutant ALK2 allele in cell cultures, and was accompanied by a decrease in ALK2 mRNA. In addition, the ALK2 AONs potentiate muscle differentiation. Furthermore, ALK2 AONs repressed BMP6-induced osteoblast differentiation. We conclude that ALK2 AONs can be used to attenuate ALK2 function in cell culture and thereby inhibit BMP6-induced responses. It will be interesting to test ALK2 AONs in vivo to inhibit heterotopic bone formation in ALK2 R206H knock in mice.

Key words: Antisense oligonucleotide (AON), ALK2, BMP signalling, Fibrodysplasia Ossificans Progressiva
Introduction

Bone Morphogenetic Proteins (BMPs) are cytokines that belong to the transforming growth factor (TGF)β family [1]. Like other TGFβ family members, BMPs signal across the plasma membrane via the formation of a heteromeric complex comprised of BMP type I and type II serine/threonine kinase receptors. Upon complex formation, the type II receptor phosphorylates specific serine and threonine residues of the type I receptor that are located in the Glycine-Serine (GS) rich juxtamembrane region [1]. The activated BMP type I receptor initiates intracellular signalling by phosphorylating receptor regulates Smads (R-Smads), which are Smad1, -5 and -8. The phosphorylated R-Smads can form heteromeric complexes with a common Smad (Co-Smad), Smad4. Subsequently the Smad complex accumulates in the nucleus to initiate the transcription of target genes in collaboration with other transcription factors, co-activators and co-repressors [1, 2]. In mammals, BMPs mainly utilize BMPR-II, ActR-II and ActR-IIB, as BMP type II receptors and ALK1, -2, -3 and -6 serve as their type I receptors, which are expressed in multiple tissues [1].

The GS domain is an important regulatory domain for the activity of type I receptors. Notably, type II receptors can form oligomeric complexes with type I receptors even in the absence of BMP ligands [1, 3]. To prevent type I receptors from activation by type II receptors, the negative regulator FKBP12 binds to the GS domain in the absence of BMP ligands [4, 5]. Upon stimulation by BMPs, FKBP12 will dissociate from the type I receptor, thereby exposing the phosphorylation site on the GS domain to the type II receptor [5, 6]. Subsequently, the type II receptor can phosphorylate the type I receptor and initiate intracellular signal transduction.

BMPs are multi-functional growth factors that play vital roles in bone formation, and heart and liver development [7-10]. The activity of the BMP pathway is precisely regulated to elicit its function in different cellular contexts. Perturbation of BMP pathways can lead to multiple diseases, such as Fibrodysplasia Ossificans Progressiva (FOP), a genetic disease caused by overactive BMP receptor signalling [11-15].

FOP is a rare disease in which acute inflammation results in progressive ossified fibrous tissue. Minor traumas such as intramuscular immunization, muscle fatigue or muscle trauma from bumps or bruises can initiate the painful formation of new bones in the soft tissue [16]. Therefore the traditional surgical treatment to remove ectopic bone is not applicable in FOP patients as the surgical trauma can quickly induce more bone formation. In the past decade, a variety of gene mutations in the ALK2 gene were found in most FOP patients [11, 17-20]. The classic FOP mutation, a nucleotide change of guanine (G) into adenine (A) in the ALK2 gene, leads to a substitution of arginine with histidine (R206H) in its GS domain [11]. The FOP ALK2 shows a lower binding affinity for FKBP12, which results in elevated BMP signalling in cells, either in the presence or absence of exogenous BMP ligands [14, 15, 21].

Recently, Medici and colleagues suggested that activated ALK2 can mediate transition of mature endothelial cells into mesenchymal stem cells-like cells, which could be further differentiated into osteoblasts under osteogenic conditions [22]. In
addition, the FOP ALK2 can sensitize cells to BMP-induced osteoblast differentiation [14]. Therefore a putative therapy for FOP patients could be the correction of the excessive ALK2 activity in these patients.

Small molecules have been designed as BMP inhibitors that repress the activity of the BMP type I receptors, such as Dorsomorphin and LDN-193189 (LDN) [12, 23]. However, Dorsomorphin and LDN can inhibit both ALK2 and ALK3 activity [12, 24]. Other studies have suggested that the inhibitors are not specific for BMP activity, as at a certain concentration, the inhibitors could even block TGFβ activity [25]. The ideal BMP inhibitor for FOP patients would be one that normalizes excessive ALK2 activity without affecting the functions of other kinases.

In this manuscript we address the possibility of applying antisense oligo nucleotides (AONs) to specifically target mutant ALK2 gene. AONs are already widely used in both fundamental, preclinical and clinical experiments to block or manipulate specific gene expression [26]. Here, the AONs were designed to selectively modify the pre-mRNA splicing of ALK2 in order to inhibit ALK2 expression and thereby antagonize ALK2-induced responses.

**Materials and methods**

**AONs**

The AONs were specially designed to target exon 7 of both wild type and FOP ALK2, and exon 8 of wildtype mouse ALK2; h-wt: 5’ gug uaa ucu ggc gag cca cug uuc u 3’, h-fop: 5’ gug uaa ucu ggu gag cca cug uuc u 3’, mouse: 5’ ggg uua ucu ggc gag cca cca cgg uuc u 3’

The AON we used for control (con-AON) is: 5’ uca agg aag aug gca uuu cu 3’

All AONs contain full length phosphorothioate backbones, and 2’-O-methly-modified ribose molecules. The WT AON and FOP AON that target human ALK2 were purchased from Eurogentec (Belgium), the mouse ALK2 AON targeting mouse ALK2 was obtained from Prosensa Therapeutics B.V (the Netherlands).

**Ligands**

Recombinant human BMP6 was obtained from R&D systems (Minneapolis, MN, USA). Recombinant human TGFβ3 was kindly provided by Dr K Iwata (OSI Pharmaceuticals, Melville, NY, USA)

**Cell culture**

The human breast cancer cell lines MDA-MB231, mouse C2C12 myoblast cell line and mouse embryonic endothelial cells (MEECs) were maintained in DMEM medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), and penicillin/streptomycin (Invitrogen, Carlsbad, CA). For myogenic differentiation, C2C12 cells were cultured in DMEM (Gibco, Carlsbad, CA) supplemented with 2% FBS (Invitrogen, Carlsbad, CA). Mouse osteoprogenitor cells KS483 were maintained in α-MEM medium (Gibco, Carlsbad, CA) supplemented with 10% FBS, and penicillin/streptomycin. All of the cells were grown at 37 °C in a humidified incubator with 5% CO₂.
AON transfection

ExoGen 500, the linear 22 KDa form of polyethylenemine (PEI, MBI, Fermentas, St.Leon-Rot, Germany), was used as a transfection reagent for KS483, MEECs and MDA-MB231 cells, and dharmaFECT duo (Thermo Scientific, USA) was used as transfection reagent for C2C12 cells. Transfection was performed according to the manufacturer’s instructions.

RNA isolation and analysis

Total RNA was isolated using the RNAII isolation kit (Machery Nagel, Düren, Germany) according to the manufacturer’s protocol. Subsequently, cDNA was reverse transcribed from using the Revert Aid protocol (Fermentas, St.Leon-Rot, Germany) with oligo dT primers. Quantitative Real-Time PCR (qPCR) analysis was performed using the Roche LightCycler 480 and the relative expression level of the genes of interest were determined in triplicate for each sample using the $2^{-\Delta\Delta CT}$ method. Values were normalized to GAPDH expression. Primers sequences to detect skipped products are as follows:

- human-E6-FW: 5`GTATGGCACTATCGAAGGG 3`;
- human-E8-RV: 5`CATCACGGGAGGAGAAGA 3`;  
- mouse-E7-FW: 5`AAGTTGGCCTTATCATCC 3`;  
- mouse-E9-RV: 5`GTACAATTCCGTCTCCCT 3`;  

Primer sequences for qPCR are as follows:

- human-q-FW: 5`GTGGCTCGCCAGATTACA 3`;  
- human-Q-RV: 5`CATCACGGGAGGAGAAGA 3`;  
- mouse-Q-FW: 5`TGGCTCCGGTCTTCCTTT 3`;  
- mouse-Q-RV: 5`AGCGACATTTTCGCTTG 3`

Alkaline Phosphatase (ALP) Assays

For the ALP activity assay in KS483 cells, cells were seeded in a 96-well plate with 5 x 10³ cells per well. 2 days after AON transfection, cells were stimulated with 100 ng/ml BMP6 for a 2 subsequent days. Cells were then washed with phosphate buffered saline (PBS), and lysed for use in the ALP activity assay.

For the ALP activity assay in MEECs, 2 x 10⁴ cells were seeded in a 48-well plate, and, 1 day after AON transfection, cells were stimulated with TGFβ for 2 days. Then, cells were stimulated with BMP6 in proliferation medium for another 2 days before the ALP activity assay was performed. Histochemical examination of ALP activity was performed using naphtol AS-MX phosphate(Sigma) and fast blue RR salt (Sigma, St Louis, MO, USA), as described previously [14].

Mineralization Assay

Confluent KS483 cells were transfected with AON for 2 days, then stimulated with 100 ng/ml BMP6 for 4 days in growth medium. Then cells were switched into an osteogenic medium, which is comprised of α-MEM supplemented with 5% FBS, 0.2 mM of ascorbic acid and 10mM of β-glycerol phosphate (Sigma, St Louis, MO, USA), for the subsequent 14 days. Medium refreshment was performed every 3-4
days. MEECs were transfected with AON for 1 day, then stimulated with 5 ng/ml of TGFβ for 2 days in growth medium. After that cells were cultured in osteogenic medium containing 100 ng/ml of BMP6 for another 4 days. To visualize mineralization, cells were stained with 2% Alizarin Red S solution.

**Immunofluorescence**

Antibodies used for immunoblotting were Desmin (SantaCruz) and Myosin heavy chain (MF20; Developmental Studies Hybridoma Bank, USA). The immunofluorescent procedure was performed as described previously [25].

**Western Blotting**

Western blotting was performed as previously described using standard techniques [25]. The antibodies used for immunoblotting were Smad1/5/8 phosphorylation (Cell signaling, USA) and GAPDH (Sigma, St Louis, MO, USA). GAPDH was analyzed as the loading control.

**Results**

**Specificity of ALK2 AON**

The classic FOP mutation, a G-A substitution located in the exon 7 of the human ALK2 gene, leads to a R206H mutation in the GS domain of ALK2 protein, which confers elevated BMP signalling in FOP patients [11, 14]. In an attempt to specifically target the mutated FOP ALK2, AONs were designed that covered the mutated nucleotide in exon 7 in ALK2 to induce exon skipping (Fig. 1A). Upon transfection and entry of the ALK2 AON into the cell nucleus, the AON may mask the targeted exon and modulate the pre-mRNA splicing. This may then result in skipping of the exon 7 or exon 8 in the human or mouse ALK2 respectively, upon which the reading frame is changed and a premature stop codon is encountered (Fig. 1A). The truncated ALK2 mRNA without exon 7, or mouse ALK2 mRNA without exon 8, may be degraded via nonsense-mediated decay. The locations of primers to detect the skipped exons are indicated as black arrows in Fig. 1A.

AONs were designed that either targeted wild-type ALK2 or FOP ALK2. Since there is only one mismatched nucleotide between the WT and FOP AON, the study aimed to determine whether one mismatch is sufficient to confer the AON specificity. The human ALK2 AON target sequence is highly similar to the mouse ALK2 mRNA. There are 3 mismatches (labelled in grey color in the mouse sequence) between human and mouse sequence in the human AON target sequence (Fig. 1A). Therefore an AON was also designed that was completely complementary to the target sequence in mouse.

To test the specificity of these AONs, the effect of WT AON and FOP AONs on ALK2 expression in the human breast cancer cell lines MDA-MB231 was tested. The transfection of fluorescent AONs into MDA-MB231 indicated the transfection efficiency was very high (>95%, Fig. 1B). Using the same method, MDA-MB231 cells were transfected with WT AON, FOP AON or a non-fluorescent control AON. Both WT AON and FOP AON were able to induce exon skipping, as visualized by a
shorter fragment obtained after RT-PCR analysis (Fig. 1B). Notably, the WT and FOP AONs are equally efficient with regards to decreasing the expression of full length ALK2 (Fig. 1B), suggesting that one mismatch in a 25-mer AON may not always ensure AON specificity.

To further assess AON specificity, both WT AON and mouse AONs were used to transfect differentiated mouse C2C12 cells. AONs were found to be equally effective at inducing skipping and decreasing full-length ALK2 expression in mouse C2C12 cells (Fig. 1C). Therefore our results suggested that even 3 mismatches were not sufficient to ensure ALK2 AON specificity.

Fig1: Schematic overview of exon skipping and a specificity test of ALK2 AONs. (A) The AONs are indicated by purple bars. The primers for detecting of the skipped bands are indicated by black arrows. The full sequence of AONs used in the article are shown and compared. The red position marked the mutated nucleotide in FOP patients. The grey nucleic acids indicate the different nucleotides in the mouse sequence. (B) The human breast cancer cell MDA-MB231 was transfected with 100 nM of fluorescent AON to visualize the transfection efficiency of AON in the cells. In the meantime, the cells were transfected with either CON AON, or WT AON or FOP AON at different concentrations, with the control of the samples without transfection. Two days after transfection, RNA was isolated and cDNA was synthesized. Then PCR was performed to
visualize the full length ALK2 composed of exon6, 7 and 8, and the skipped band composed of only exon6 and exon8. Q-PCR was performed to quantify the full length ALK2 expression. Gene expression was normalized to GAPDH. (C) C2C12 cells were cultured in differentiation medium for 7 days when fully differentiated myotubes were available. Cells were then transfected with either WT AON or mouse AON. Two days after transfection, RNA was isolated and cDNA was synthesized. PCR was performed to visualize the full length mouse ALK2 composed of exon7, 8 and 9; and skipped band composed of exon7 and exon9. Q-PCR was performed to quantify the relative full length ALK2 expression in C2C12 cells. The CON samples are not transfected with AONs. Gene expression was normalized to GAPDH.

**ALK2 AON potentiates muscle differentiation**

As shown, the WT AON can induce skipping and reduce full length ALK2 expression. The question of whether the ALK2 AON can also decrease BMP signalling was also investigated. BMP signalling is known to repress myogenic differentiation, and BMP inhibitors have been proven to potentiate the differentiation of myoblast into myotubes [25, 27]. Therefore the next step was to test whether ALK2 AONs can function as BMP inhibitors to potentiate myogenic differentiation from myoblasts.

Undifferentiated C2C12 myoblasts could be transfected with AON with high efficiency (>90%) as visualized by fluorescent AON (Fig. 2A). C2C12 myoblasts were transfected with ALK2 AON, and a skipped band could be detected. Q-PCR analysis showed that full-length ALK2 expression was decreased in cells treated with ALK2 AON, while the ALK3 expression was not affected (Fig. 2B). Seven days after transfection, cells were fixed and stained for myosin with immuno-fluorescence to visualize the differentiated myotubes. The differentiation of myoblasts into myotubes was measured by two indices: the differentiation index and the fusion index. The differentiation index is calculated as the percentage of myosin-positive cells out of all myogenic (desmin-positive) cells. The fusion index is calculated as the average number of nuclei in the differentiated myotubes. ALK2 AON was found to enhance both the differentiation index and fusion index in C2C12 cells (Fig. 2C), suggesting ALK2 AON potentiates muscle differentiation in these cells and implying that the endogenous BMP signalling in C2C12 cells is repressed by the ALK2 AONs.
Fig2: ALK2 AON enhanced myogenic differentiation in C2C12 cells. (A) C2C12 cells were transfected with 500 nM fluorescent AON in differentiation medium, two days after transfection, fluorescent signal was checked and pictures were taken. (B) QPCR to analyze the relative full length ALK2 and ALK3 expression in C2C12 cells transfected with 200 nM of CON AON or 200 nM of mouse ALK2 AON for 2 days in differentiation medium. GAPDH was used to normalize gene expression. (C) C2C12 cells were transfected with either 200 nM of CON AON or 200 nM of mouse ALK2 AON in differentiation medium for 7 days. Then cells were stained with myosin and desmin by immunofluorescence. Myosin was used to label the differentiated cells while desmin was used to label myogenic cells. Error bars represent the standard deviation of triplicate measurements.

ALK2 AON decreased BMP6-induced osteoblast differentiation in KS483 cells

After demonstrating the ALK2 AON can promote myogenic differentiation in
C2C12 cells, we further sought to examine whether the ALK2 AON can repress BMP-induced osteoblast differentiation, to provide further evidence on the therapeutic potential of the ALK2 AON for FOP patients [11]. KS483 osteoprogenitor cells are a frequently used model system for BMP-induced osteoblast differentiation. Transfection with the fluorescent AONs indicated that AONs can be easily delivered into KS483 cells (Fig. 3A). ALK2 AONs transfection resulted in exon skipping and reduced expression of the full-length ALK2 in KS483 cells (Fig. 3B, C). Further results revealed that the ALK2 AON efficiently repressed BMP6-induced osteoblast differentiation in KS483 cells, as visualized by the ALP activity (Fig 3E) and the mineralization assays (Fig 3F). Our results showed that ALK2 AON reduced the BMP6-induced osteoblast differentiation though it had no effect on Smad1/5/8 phosphorylation (Fig 3D).

Fig3: ALK2 AON reduced osteogenic differentiation in KS483 cells. (A) KS483 cells were transfected with 200 nM of fluorescent AON, two days after transfection, fluorescent signal was checked and photos were taken. (B) KS483 cells were transfected with 200 nM of mouse ALK2 AON, 500 nM of mouse ALK2 AON, 200 nM of CON AON or 500 nM of CON AON. The transfection was performed in proliferation medium.
Two days after transfection, RNA was isolated, and cDNA was synthesized. PCR was performed to visualize the skipped band. (C) KS483 cells were transfected with 200 nM of CON AON, or 200 nM of mouse ALK2 AON in proliferation medium. CON indicates samples without AON transfection. 2 days after transfection, RNA was isolated. Full length ALK2 expression was quantified by q-PCR. GAPDH was used to normalize gene expression. The error bars represent the mean of ± S.D. of triplicate experiments. (D) KS483 cells were transfected with 200 nM of CON AON, 200 nM of ALK5 AON and 200 nM of mouse ALK2 AON in proliferation medium. The CON indicates samples without AON transfection. Two days after transfection, cells were starved in serum free medium over night and then stimulated with 100 ng/ml of BMP6 for 1 hour. Proteins were isolated and western blot was performed. Smad1/5/8 phosphorylation was examined; GAPDH was used as loading control. (E) Confluent KS483 cells were transfected with 200 nM of CON AON, or 200 nM of mouse ALK2 AON for 2 days in proliferation medium. Then cells were stimulated with 100 ng/ml of BMP6 for another 2 days in proliferation medium. Cells lysates were harvested. The ALP activity normalized by protein concentration were shown as the mean ± S.D. of three independent experiments. (F) Confluent KS483 cells were transfected with 200 nM of CON AON, or 200 nM of mouse ALK2 AON for 2 days in proliferation medium. The cells were then stimulated with 100 ng/ml of BMP6 in proliferation medium for 4 days. Then cells were switched into osteogenic medium for another 10 days. Medium refreshment was done every 3-4 days. The cells were finally stained with alizarin red S solution to visualize the mineralized area in KS483 cells.

**ALK2 AON decreased ALP activity and mineralization in MEECs**

ALK2 AONs were shown to be able to repress BMP6-induced osteoblast differentiation in KS483 cells. Recently, endothelial cells were reported as potential osteo-precuror cells for ectopic bone formation in FOP patients [22, 28]. Therefore the next step was to use endothelial cells as a cell model to test whether ALK2 AON can repress BMP-induced osteoblast differentiation in endothelial cells.

High transfection efficiency was observed upon fluorescent AON transfection in MEECs (Fig 4A). Consistent with the previous cell lines, ALK2 AON can induce skipping of exon8, as shown by the skipped band composed of exon7 and exon9(Fig 4B), and decreased full-length ALK2 expression in MEECs (Fig. 4D). MEECs were then cultured in osteogenic conditions to induce the differentiation towards the osteoblast lineage. The results showed that the ALP activity (Fig. 4C), and mineralization (Fig 4D) was repressed in the samples treated with ALK2 AON. The results also revealed that expression of the BMP direct target gene, Id3, and other osteogenic genes, including RunX2, OSC and BSP, were decreased by ALK2 AON (Fig. 4E), suggesting that ALK2 AON decreased BMP-induced osteoblast differentiation.
Fig 4: ALK2 AON reduces transdifferentiation of MEECs. (A) MEECs were transfected with 200 nM of Fluorescent AON. One day after transfection, fluorescent signal was examined and photos were taken. (B) MEECs were transfected with CON AON or ALK2 AON, one day after transfection, RNA was isolated and cDNA was synthesized. PCR was performed to visualize the skipped band composed of exon 7 and exon 9. (C) MEECs were transfected with 200 nM of CON AON or 200 nM of ALK2 AON. The LDN sample indicated 120 nM LDN was present during the whole experiment. ALP activity was measured and visualized by ALP staining. The ALP activity normalized by protein concentration was shown as the mean ± S.D. of three independent experiments. (D) MEECs were transfected with either 100 nM of CON AON or 100 nM of ALK2 AON in growth medium. The mineralization was visualized by alizarin red staining. (E) MEECs were transfected with 200 nM of CON AON or 200 nM of ALK2 AON. After mineralization was induced, the RNA was isolated and qPCR was performed to check
the relative full length ALK2 expression, and the other osteogenic gene Runx2, Osc and Bsp expression. The BMP target gene Id3 was also checked. All of the gene expression is normalized by Gapdh.

Discussion
Relying on the rule of base-pairing of nucleic acid, it is simple to design AONs to block specific gene expression. Currently, a notable application of AONs is to use RNase-H resistant AONs to modify the pre-mRNA splicing, in which AONs were used to block the splicing signal [26]. In this case, AONs were used either to restore cryptic splicing site, or to change the levels of alternative splicing. Through modulation of gene splicing, we can either decrease specific gene expression or restore a certain gene expression. The technique has already been successfully applied to restore the functional Dystrophin protein in the skeletal muscle of DMD patients without causing severe adverse effect on other non-target tissues or organs [29, 30]. Therefore to use the same technique to modulate mutant gene expression might be an attractive therapeutic option for genetic disease.

In this study, using the same design strategy, we have obtained an AON that could specifically target ALK2 and decreased ALK2 expression in various types of cells. In the meanwhile, the in vitro experiments showed that the endogenous BMP signalling and BMP induced osteoblast differentiation was impaired after cells were treated with ALK2 AON, suggesting ALK2 AON is an efficient genetic tool to modulate endogenous ALK2 expression and BMP activity. The next step would be to test whether the ALK2 AON could be efficient in vivo to decrease ALK2 expression and ALK2 mediated BMP signalling. In this respect, it may be of great interest to test whether ALK2 AON can inhibit the heterotopic ossification in ALK2 R206H knock in mice that have recently been developed [31].

Using the allele specific siRNA technique, Kaplan and colleagues have successfully obtained siRNAs that target the disease causing ALK2, without affecting normal ALK2 expression [32]. The siRNAs were used in FOP mesenchymal progenitor cells to restore normal BMP activity and osteogenic differentiation [32]. Our AON is not so specific, as it decreased both WT ALK2 and FOP ALK2 expression. However, the ALK2 AON mediated decrease in ALK2 expression may also lead to restoration in normal BMP activity in FOP cells. Therefore in future, we could also apply ALK2 AON in FOP cells to check whether ALK2 AON could be as efficient as siRNA to restore BMP activity in those cells.

Reference
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