In vitro Assessment of the Bioactivity of rmGDF-9 and rhBMP-15

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ABSTRACT

Growth differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15) are oocyte-derived growth factors that are well known for their important roles in regulating folliculogenesis and female fertility. However, recent publications implicating their involvement in human cancers raises questions about whether GDF-9 and BMP-15 might exert diverse biological functions in non-ovarian tissues. We have purified recombinant mouse GDF-9 and recombinant human BMP-15 from Chinese Hamster Ovary (CHO) cells and evaluated their bioactivities using a range of bioassays. In a functional ELISA, mouse GDF-9 and human BMP-15 bind to the recombinant human/mouse BMP-RII extracellular domain with Kd values of 1.4 nM and 2 nM, respectively. In addition, the two proteins activate Smad 2/3 in P19 cells. Similar to other members of the transforming growth factor-β family, recombinant mouse GDF-9 is able to induce apoptosis in Mv1Lu and DU145 cells. In addition, recombinant human BMP-15, like other BMP family members, is osteogenic and promotes differentiation of MC3T3-E1 cells to osteoblasts. We also found that human BMP-15 is very potent in supporting the survival and proliferation of NIH3T3 cells under nutrient deprived conditions. The differential *in vitro* functions of GDF-9 and BMP-15 provide a basis for new research initiatives and also open a new avenue to explore their functions in cancer biology and reproductive physiology. These studies in turn may lead to an expanded interest into their potential use as therapeutic targets.

INTRODUCTION

GDF-9 and BMP-15 are members of the transforming growth factor-β (TGF-β) superfamily and possess similar structural and functional properties. Both molecules form non-covalently linked homodimers or heterodimers due to the lack of the fourth of seven conserved cysteines present in the majority of the TGF-β family members. GDF-9 and BMP-15 are predominately expressed in the ovary and play important roles in regulating folliculogenesis through several paracrine mechanisms. In GDF-9-deficient mice, expression levels of several ovarian marker genes are reduced and follicular development is arrested at the primary stage. GDF-9 and BMP-15 promote the growth and differentiation of granulosa cells, theca cells, and oocytes. GDF-9 in concert with BMP-15 modulates the cumulus expansion, which is essential for ovulation, fertilization, and implantation.[1] However, the biological significance of GDF-9 and BMP-15 outside the ovary is largely unknown. Recent publications implicating their biological functions in human cancers raised questions of whether GDF-9 and BMP-15 might exert diverse functions in non-ovarian tissues.[2] We have purified the recombinant mouse GDF-9 and human BMP-15 from Chinese Hamster Ovary (CHO) cells and evaluated bioactivity using several bioassays.

Members of the TGF-β superfamily transmit signals to the nucleus by signaling through type II and type I serine-threonine kinase receptors, and intracellular effectors known as Smads. Ligands bind to a type II receptor on the surface of the cell and then recruit a type I receptor, activating it via phosphorylation. This activated complex phosphorylates Smads, which translocate to the nucleus to transduce the signal. Studies indicate GDF-9 and BMP-15 signaling utilizes the TGF-β/BMP pathway, acting through BMP-RII and Smad2/3[3,4], and eliciting cell proliferation, differentiation, and many other cellular processes during folliculogenesis.

RESULTS

FIGURE 1

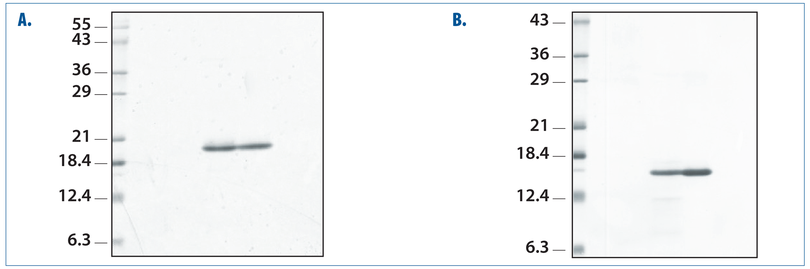

FIGURE 1 Recombinant mouse GDF-9 (rmGDF-9) and recombinant human BMP-15 (rhBMP-15). SDS-PAGE analysis of two lots of purified rmGDF-9 (Gly307-Arg 441), shown in panel A, and two lots of rhBMP-15 (Gln268-Arg392 (Thr266Arg), shown in panel B, under reducing conditions. Samples of rmGDF-9 and rhBMP-15 (1 µg) were loaded onto each lane of 15% SDS-PAGE Gel. The proteins were detected with silver staining.

FIGURE 2

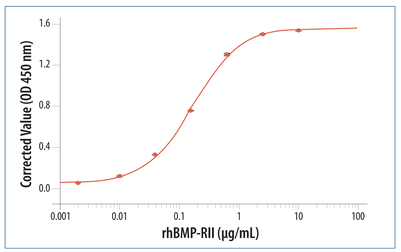

FIGURE 2 rmGDF-9 binds to recombinant h/mBMP-RII/Fc in a functional ELISA. 1 µg/mL of rmGDF-9 was immobilized on an ELISA plate and incubated with various concentrations of rh/rmBMP-RII/Fc as indicated. The amount of rh/rmBMP-RII/Fc bound to rmGDF-9 was detected with a biotinylated goat anti-BMP-RII antibody.

FIGURE 3

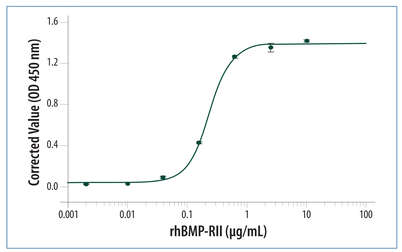

FIGURE 3 rhBMP-15 binds to recombinant h/mBMP-RII/Fc. 2 µg/mL of rhBMP-15 was immobilized on an ELISA plate and incubated with various concentrations of rh/rmBMP-RII/Fc as indicated. The amount of rh/rmBMP-RII/Fc bound to rhBMP-15 was detected with a biotinylated goat anti-BMP-RII antibody.

FIGURE 4

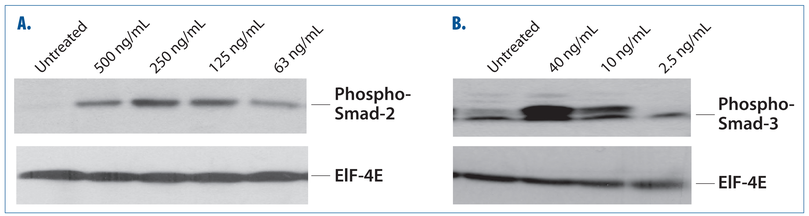

FIGURE 4 Treatment of rmGDF-9 and rhBMP-15 stimulates Smad-2/3 phosphorylation in P19 cells. A. Stimulation of Smad-2 phosphorylation by rmGDF-9. **B.** Stimulation of Smad-3 phosphorylation by rhBMP-15. Immunoblot analysis of Smad-2 and Smad-3 phosphorylation was performed using cell extracts following treatment of P19 cells with rmGDF-9 or rhBMP-15 for 60 minutes. Immunoblotting of EIF-4E was used as the gel loading control.

FIGURE 5

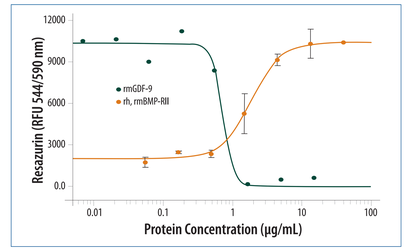

FIGURE 5 rmGDF-9 induces apoptosis of Mv1Lu cells and rh/rmBMP-RII/Fc antagonizes rmGDF-9 induced apoptosis. Mv1Lu mink lung cells were treated with various concentrations of rmGDF-9 as indicated, for 24 hours. The effect of rmGDF-9 on cell viability was measured using resazurin. For the antagonist assay, rh/rmBMP-RII/Fc was incubated with 1 µg/mL of rmGDF-9 for 1 hour before exposure to the cells.

FIGURE 6

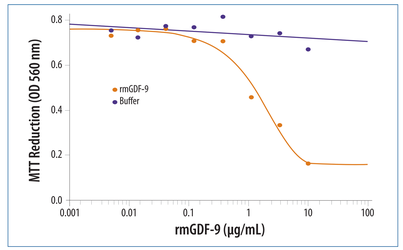

FIGURE 6 rmGDF-9 induces apoptosis of DU145 cells. DU145 human carcinoma cells were incubated with various concentrations of rmGDF-9 as indicated, for 3 days. The effect of rmGDF-9 on the cell viability was measured by MTT.

FIGURE 7

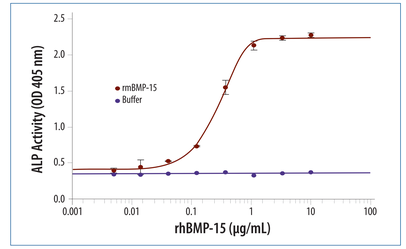

FIGURE 7 rhBMP-15 promotes differentiation of MC3T3-E1 cells. MC3T3-E1 mouse osteoblastic cells were incubated with various concentrations of rhBMP-15 as indicated, for 72 hours. The effect of rhBMP-15 on the cell differentiation was measured by the activity of alkaline phosphatase (ALP).

FIGURE 8

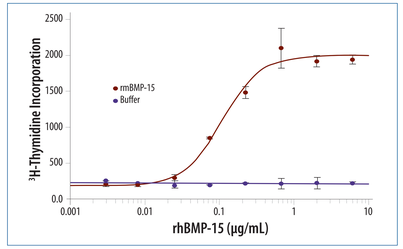

FIGURE 8 rhBMP-15 stimulates proliferation of NIH3T3 cells. NIH3T3 mouse embryonic fibroblast cells were cultured with various concentrations of rhBMP-15 as indicated, for 24 hours. The effect of rhBMP-15 on NIH3T3 cells was measured by the amount of ^{3}H-thymidine incorporated into the DNA.

SUMMARY

- Both rmGDF-9 and rhBMP-15 signal through the BMP-RII receptors and activate Smad2/3.
- rmGDF-9 displays TGF-β like activity *in vitro*, inducing apoptosis of Mv1Lu mink lung and DU145 human carcinoma cells. The activity of rmGDF-9 can be antagonized by soluble BMP-RII.
- rhBMP-15 displays BMP-like activity *in vitro*, promoting the differentiation of MC3T3-E1 mouse osteoblastic cells.
- rhBMP-15 functions as a mitogen and stimulates proliferation of NIH3T3 mouse embryonic fibroblast cells.

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