Electronic Supplementary Material (ESI) for Chemical Communications

Creation of Novel Signalling Modulators from Existing Cytokine using Scanning Motif-Programming

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Supporting Information

A. Materials and Methods

Materials. Human recombinant BMP-4 (hBMP-4) was purchased from R&D Systems (Minneapolis, MN). Two selective inhibitors of activin receptor-like kinase, SB431542 and dorsomorphin, were from Sigma-Aldrich. Slide-A-Lyzer Dialysis Cassettes (MWCO 3,500) and BCA Protein Assay Reagent were from PIERCE (Rockford, IL). Firefly luciferase reporter assay reagent was from Promega (Madison, WI). FuGENE 6 transfection reagent was from Roche (Mannheim, Germany). Fetal bovine serum (FBS) was from JRH Bioscience (Lenexa, KS). The mammalian expression vector pcDNA3, Dulbecco's modified Eagle's medium (DMEM), Dulbecco's PBS, Geneticin (G418), and trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA-4Na) were all from Invitrogen (Carlsbad, CA). Cell culture dishes were from BD Bioscience (San Jose, CA). Eagle MEM was from Nissui Pharmaceutical (Tokyo, Japan).

Vent DNA polymerase, restriction enzymes, T4 DNA ligase, and DH5α were purchased from New England Biolabs (Beverly, MA). Bacto™ Trypton and Yeast extract were from BD (Franklin Lakes, NJ). Isopropyl β-thiogalactopuranoside and carbenicillin sodium salt were from Wako (Osaka, Japan).

Construction and purification of artificial proteins. We initially selected four regions, 20 or 21 amino acid residues in length, from human (h)BMP-2 (Fig. 1). Four microgenes (MGs) (Fig. 1b) encoding the corresponding regions were designed so that one of the reading frames would encode the BMP-2 sequence, and one of the other two

reading frames would encode a polypeptide with a propensity to form β-strands.[1] Then, each MG was polymerized using MPR.[2] The resultant microgene polymers were ligated into the *Sma*I site of a bacterial cloning vector, pTZ19R, and sequenced. *Bam*HI-*Asp*718 fragments from pTZ19R containing the microgene polymers were cloned into the *Bgl*II-*Asp*718 sites of vectors pKS600-605, each of which could translate one of the six coding frames of the microgene polymers as an N-terminal His_6-tagged fusion protein.[3]

The artificial proteins were expressed in *E. coli* XL1Blue (Stratagene, La Jolla) using the manufacture's protocol, after which the cells were pelleted by centrifugation at 7,000 *g* for 15 min at 4˚C. The pellet was then frozen at -80˚C and lysed in buffer containing 6 M guanidine hydrochloride, 50 mM sodium phosphate, 10 mM Tris-HCl (pH 8), 100 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. The lysate was cleared by centrifugation at 7,000 *g* for 30 min at 4˚C, and the supernatant was passed over a TALON cobalt affinity column (Clontech, Palo Alto). After washing with buffer containing 8 M urea (pH 7), 50 mM sodium phosphate, 100 mM NaCl, and 15 mM imidazole, the artificial proteins were eluted with buffer containing 8 M urea (pH 5), 50 mM sodium phosphate, 20 mM 2-(*N*-morpholino)ethanesulfonic acid, 100 mM NaCl, and 250 mM imidazole. Purified proteins were dialyzed against 4 mM HCl to be stored at -80˚C.

Cell Culture. C2C12 mouse myoblast cells and C3H10T1/2 mouse fibroblast cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 15% FBS, 100 U/ml penicillin and 100 μ/gml

streptomycin, or in Eagle MEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μ/gml streptomycin, respectively. The cells were incubated at 37˚C under a humidified atmosphere of 5% CO_2 and 95% air, and passaged every 3-4 days.

Screening system. C2C12 cells were seeded into 6-well plates and cultured for 1 day. Then using FuGENE6 they were cotransfected with 1 μg of the BMP-responsive element (BRE)-driven reporter construct BRE-luc containing the Id-1 promoter[4] and 1 μg of pcDNA3. Stable transfectants were first selected by culture in 0.6 mg/ml G418. Thereafter a highly BMP-2 (-4)-sensitive clone was obtained using a limited dilution technique followed by a firefly luciferase reporter assay (Promega). The luminescence was read using a Mithras LB940 microplate reader (Berthold Technologies, Bad Wildbad, Germany).

Each artificial protein solution was added to separate wells of 96-well plates to a final concentration of 15 μg/cm^2 (Nalge Nunc International, Rochester, NY). The plates were then air-dried for 2 days. C2C12-BREluc cells were seeded into the plates to 1.0 × 10^4 cells/well and then cultured for 1 day. In some cases, SB431542[5] (1 μM), dorsomorphin[6] (1 μM), or a dominant-negative hBMP-2 mutant[7] (A34D; 10 μg/mL) was added to the culture medium. The cells were subsequently lysed with 40 μl of Cell Culture Lysis Reagent (CCLR, Promega), after which 20-μl samples of the lysate were subjected to the luciferase assay. The protein concentration in the rest of the lysate (5-μl samples) was measured using a BCA protein assay (PIERCE). The luciferase activity was normalized to the protein concentration.

Alkaline phosphatase (ALP) assay. C2C12 or C3H10T1/2 cells were seeded into 96-well plates on which an artificial protein was immobilized. One hour after seeding, hBMP-2 was added to the culture medium. C2C12 cells were then cultured for an additional 3 days in DMEM supplemented with 15% FBS (Fig. 3a) or with 2% FBS, 50 μM ascorbic acid, and 10 mM β-glycerophosphate (Fig. 3b).[8] After the addition of hBMP-4, C3H10T1/2 cells were similarly cultured for 3 days in Eagle MEM supplemented with 10% FBS. The ALP activity induced in the cells was assayed using 4-methylumbelliferyl phosphate (Sigma) according to the manufacture's protocol and normalized to the protein concentration.

B. Supplementary Figures

Fig. S1 The primary structures of artificial proteins coded by polymerized MGs. Among them, 8 clones (tmw42, tmw48, tmw51, tmw54, tmw57, tmw61, tmw66, and tmw73) were efficiently produced in *E. coli*.

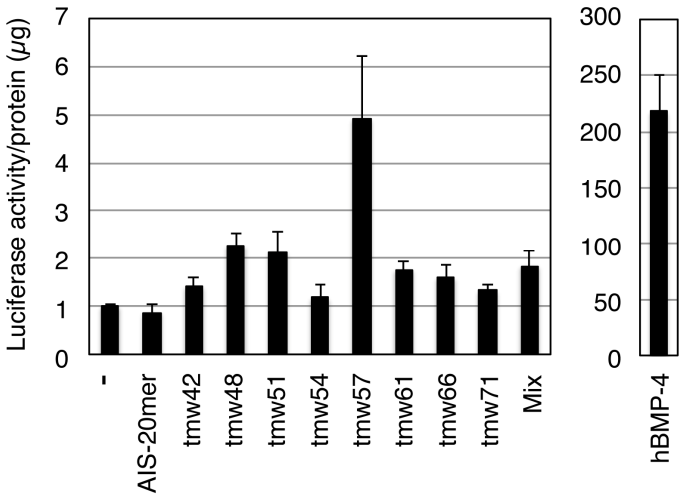

Fig. S2 Screening artificial proteins using BRE-luc reporter assays. C2C12-BREluc cells were cultured for 1 day in plates on which the indicated artificial protein was immobilized (5 μg/well) or in the presence of soluble human BMP-4 (50 μg/mL). AIS-20mer corresponds to tmw5 motif peptide and was immobilized on a plate in the same manner as the other proteins. The "Mix" sample was prepared by mixing equal amounts all 8 artificial proteins so that the total amount was 5 μg/well. Bars represent standard deviations (n=3).

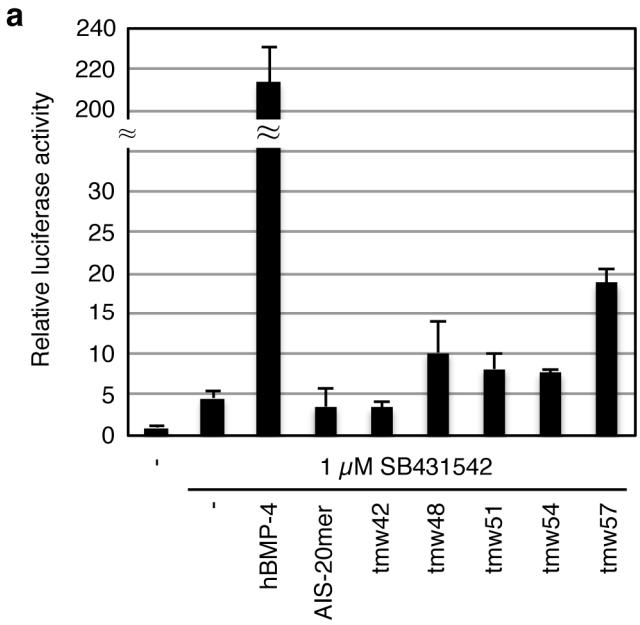

Fig. S3a Effects of inhibition of TGB-β signalling by 1 μM SB431542 on the luciferase activity induced by immobilized tmw57 in C2C12-BREluc cells. TGF-β signalling is known to suppress BMP-2 signalling. The activity of tmw57 increased by approximately four fold, as compared with that in Fig. S2. Bars represent standard deviations (n=3).

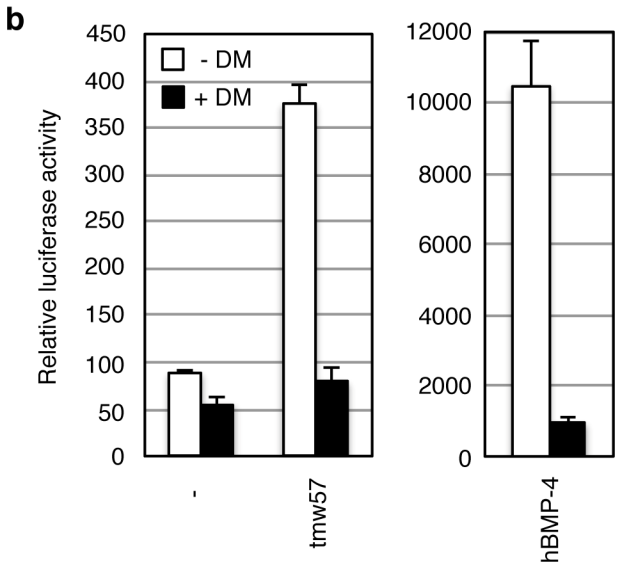

Fig. S3b Effects of blocking BMP receptors using 1 μM dorsomorphin (DM) on the luciferase activity induced by immobilized tmw57 in C2C12-BREluc cells. Dorsomorphin acts intracellularly to inhibit the kinase activity of BMP type I receptors. The activity induced by immobilized tmw57 was almost completely blocked. Bars represent standard deviations (n=3).

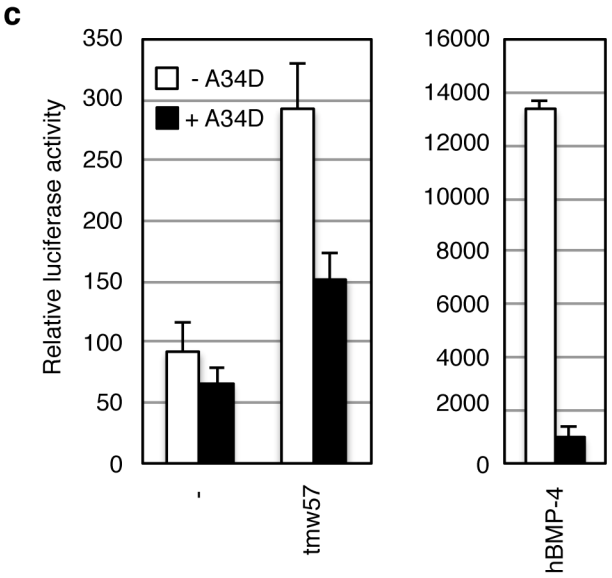

Fig. S3c Effects of addition of a dominant negative hBMP-2 mutant (A34D; 10 μg/mL) on the luciferase activity induced by immobilized tmw57 in C2C12-BREluc cells. The A34D mutant induced a slight reduction in luciferase activity in the cells on untreated plates, which suggests inhibition of endogenous bovine BMPs present in the culture medium. Under these conditions, the activity induced by immobilized tmw57 was partially inhibited. Bars represent standard deviations (n=3).

C. References

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