Designer Nodal/BMP2 Chimeras Mimic Nodal Signaling, Promote Chondrogenesis, and Reveal a BMP2-like Structure

Received for publication, October 22, 2013, and in revised form, November 20, 2013
Published, JBC Papers in Press, December 5, 2013
DOI 10.1074/jbc.M113.529180

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Nodal, a member of the TGF-β superfamily, plays an important role in vertebrate and invertebrate early development. The biochemical study of Nodal and its signaling pathway has been a challenge, mainly because of difficulties in producing the protein in sufficient quantities. We have developed a library of stable, chemically refoldable Nodal/BMP2 chimeric ligands (NB2 library). Three chimeras, named NB250, NB260, and NB264, show Nodal-like signaling properties including dependence on the co-receptor Cripto and activation of the Smad2 pathway. NB250, like Nodal, alters heart looping during the establishment of embryonic left-right asymmetry, and both NB250 and NB260, as well as Nodal, induce chondrogenic differentiation of human adipose-derived stem cells. This Nodal-induced differentiation is shown to be more efficient than BMP2-induced differentiation. Interestingly, the crystal structure of NB250 shows a backbone scaffold similar to that of BMP2. Our results show that these chimeric ligands may have therapeutic implications in cartilage injuries.

The TGF-β superfamily of proteins plays crucial roles during development and homeostasis of both vertebrate and invertebrate organisms. Nodal is a TGF-β superfamily member that controls mesoderm formation (1), establishes left-right asymmetry in vertebrates (2), and maintains pluripotency of human embryonic stem cells (3), among other roles. Activins, which comprise another TGF-β subfamily, also regulate wide ranging biological processes including erythropoiesis (4), follicle-stimulating hormone secretion (5), and differentiation of macrophages (6).

Remarkably, despite the fact that they have very different biological roles, Activins and Nodal signal via the same type II receptor (ActRII/IIb) and type I (ALK4) serine/threonine kinase receptors and activation of cytoplasmic Smad2/3 proteins. Unlike Activin, however, Nodal signaling requires a co-receptor from the epidermal growth factor-Cripto/FRL1/Cryptic protein family such as Cripto. Cripto interacts with Nodal through its EGF-like domain and with ALK4 through its CFC domain (7). Cripto is not required for Activin signaling but rather binds to the Activin-receptor complex to reduce Activin signaling capacity (8). It is intriguing and remains unclear from a structural and mechanistic standpoint how Cripto is required for Nodal signaling but inhibits Activin signaling via the same signaling receptors.

Bone morphogenetic proteins (BMPs), the major subfamily of the TGF-β superfamily, control development of the skeletal system, as well as cartilage (9) and tendon formation (10). Even though BMPs can utilize the same type II receptor as Activin and Nodal, their signaling is different, being propagated through BMP-specific type I receptors and activation of Smad 1/5/8 proteins. Nonetheless, studies have shown the existence of cross-talk between the Activin and BMP signaling pathways during chondrogenesis (11, 12).

The comparative biochemical and biophysical studies of Nodal versus Activin signaling have been hampered by the
inability to produce these proteins in quantities suitable for structural and functional studies. A chemical refolding protocol that has been successful for many ligands (13) has failed for both Nodal and Activin. A strategy known as random assembly of segmental chimera and heteromers (RASCH) has been devised to combine sections of Activin and BMP2 to produce Activin/BMP2 chimeras that we termed the AB2 library (14). From the AB2 library, we found a chimera, AB208, which achieved the refolding efficiency of BMP2 while exhibiting the signaling properties of Activin (14). Additionally, the RASCH strategy has yielded other chimeras such as AB204 and AB215 with enhanced SMAD 1/5/8 signaling capabilities compared with wild type BMP2. Thus, this strategy enabled us not only to produce properly refolded chimeras with the properties of their parental ligands (e.g., AB208), but also to produce chimeras that exhibit novel signaling characteristics.

In this study, we apply the RASCH strategy to combine Nodal and BMP2 and create Nodal/BMP2 chimeras (NB2 library). We identify three chimeras (NB250, NB260, and NB264) with the refolding capabilities of BMP2 and the Cripto-dependent signaling properties of Nodal. Additionally, we find that NB250, like Nodal, alters heart looping during the establishment of embryonic left-right asymmetry. Interestingly, we observe that NB250 and NB260 strongly induce chondrogenic differentiation of human adipose-derived stem cells (hASCs) in a manner similar to Nodal and more efficiently than BMP2 or Activin A. Finally, we solve the crystal structure of NB250 and demonstrate that its fundamental scaffold remains identical to that of BMP2 except for small local conformational changes in the receptor-binding regions. This suggests that Nodal itself likely adopts a BMP2-like fold. Together, our results show that these Nodal-like chimeric ligands will be useful for additional structural studies and may have therapeutic implications for the treatment of cartilage injuries and other disorders.

**EXPERIMENTAL PROCEDURES**

**NB2 Chimera Expression and Purification**—To create the NB2 chimera library, the mature domains of human Nodal and human BMP2 were divided into six segments. Twelve Nodal/BMP2 primers corresponding to each segment (Integrated DNA Technologies, Coralville, IA) were combined by an overlapping PCR strategy to produce a full-length PCR fragment of each chimera. To create overlapping regions for PCR, we substituted residues Ala77 and Leu95 in Nodal to valine. The constructs were cloned into pET21A vector (EMD Biosciences, Darmstadt, Germany) and propagated in Nova Blue cells (EMD Biosciences). Final expression constructs were confirmed by DNA sequencing.

All NB2 chimeras were expressed in Escherichia coli as inclusion bodies and isolated, purified, and refolded using a modified protocol (13). The refolded ligands were initially purified using Hi-trap heparin sulfate (GE Healthcare) and a sodium chloride gradient. Fractions containing the refolded ligand were submitted to a second round of purification using a C4 reverse phase column (GraceVydac). The protein was eluted using an acetonitrile gradient. Nodal was purchased from R&D Systems.

**NB250 Crystallization**—Lyophilized NB250 was resuspended in water at 10 mg/ml, and crystallization trials with several commercial screening kits, Crystal Screen, Crystal Screen 2 (Hampton Research), Wizard Screens I and II (Emerald Biosstructures), PEG/ION, and Nextal Classics suite (Qiagen) were conducted using the Mosquito crystallization robot (TTP Labtech, Cambridge, MA). The trials yielded several crystallization hits, which were subsequently optimized. The final crystal was obtained using the hanging-drop vapor diffusion method, in which 1 μl of 10 mg/ml protein was mixed with 1 μl of a reservoir solution of 0.2 M NaCl, 0.1 M sodium acetate, pH 4.6, 30% 2-methyl-2,4-pentanediol. Crystals appeared after 3 weeks at 15 °C and were frozen in liquid N2 using a cryoprotectant comprised of the original reservoir solution supplemented with 15% (v/v) glycerol. Diffraction data were collected at the Stanford Synchrotron Radiation Laboratory using Beamline 11-1 at the resolution of 1.9 Å. The space group of NB250 was found to be R32:H with the following cell dimensions: a, b = 95.57; c = 97.49; α, β = 90; γ = 120. Data were processed using XDS software (15), and the structure was solved using molecular replacement with BMP2 as a model. Model building...
was done in COOT version 0.6.2 (16). Refinement of the structure was completed using PHENIX (17) to R and Rfree, of 0.175 and 0.203, respectively. The final data processing and refinement statistics are listed in Table 2. The root mean square deviations in bond lengths and bond angles are 0.006 Å and 1.14°, respectively. All structure figures were generated using PyMOL (version 1.3; Schrödinger) and Molscript version 2.1.2 (19).

**Luciferase, Phospho-SMAD2 Assays, and ASCs Cell Culture—** Luciferase and phospho-Smad2 assays were performed as described previously (8). Human mesenchymal stem cells were derived from the adipose tissue of the subcutaneous abdomen of a 37-year-old Caucasian female (lot number 9061601.12; Promocell, Heidelberg, Germany). Cells were cultured in growing medium (high glucose DMEM (Invitrogen) with 10% fetal bovine serum and 1% Penicillin (Invitrogen)).

### TABLE 1
List of NB2 chimeras

| Name     | Sequence   | RE  |
|----------|------------|-----|
| NB201    | NNNNNN     | −   |
| NB203    | NRBNNB     | −   |
| NB205    | NNNNNB     | −   |
| NB206    | NNNNNB     | −   |
| NB207    | NNNNNB     | −   |
| NB208    | NNNNNB     | −   |
| NB209    | NNNNNB     | −   |
| NB210    | NRBNNB     | ++  |
| NB211    | NRBNNB     | −   |
| NB212    | NRBNNB     | −   |
| NB213    | NNNNNB     | −   |
| NB214    | NNNNNB     | −   |
| NB215    | NNBNNN     | −   |
| NB216    | NNBNNN     | −   |
| NB217    | NNBNNN     | −   |
| NB218    | NNBNNN     | −   |
| NB219    | NBBNNN     | −   |
| NB220    | NNNNNB     | −   |
| NB221    | NNNNNB     | −   |
| NB222    | NNNNNB     | −   |
| NB223    | NNNNNB     | −   |
| NB224    | NNNNNB     | −   |
| NB225    | NNNNNB     | −   |
| NB226    | NNNNNB     | −   |
| NB227    | NNNNNB     | −   |
| NB228    | NNNNNB     | −   |
| NB229    | NNNNNB     | −   |
| NB230    | NNNNNB     | −   |
| NB231    | NNNNNB     | −   |
| NB232    | NNNNNB     | −   |
| NB233    | NNNNNB     | −   |
| NB234    | NNNNNB     | −   |
| NB235    | NNNNNB     | −   |
| NB236    | NNNNNB     | −   |
| NB237    | NNNNNB     | −   |
| NB238    | NNNNNB     | −   |
| NB239    | NNNNNB     | −   |
| NB240    | NNNNNB     | −   |
| NB241    | NNNNNB     | −   |
| NB242    | NNNNNB     | −   |
| NB243    | NNNNNB     | −   |
| NB244    | NNNNNB     | −   |
| NB245    | NNNNNB     | −   |
| NB246    | NNNNNB     | −   |
| NB247    | NNNNNB     | −   |
| NB248    | NNNNNB     | −   |
| NB249    | NNNNNB     | −   |
| NB250    | NNNNNB     | −   |
| NB251    | NNNNNB     | −   |
| NB252    | NNNNNB     | −   |
| NB253    | NNNNNB     | −   |
| NB254    | NNNNNB     | −   |
| NB255    | NNNNNB     | −   |
| NB256    | NNNNNB     | −   |
| NB257    | NNNNNB     | −   |
| NB258    | NNNNNB     | −   |
| NB259    | NNNNNB     | −   |
| NB260    | NNNNNN     | +   |
| NB261    | NNNNNN     | +   |
| NB262    | NNNNNN     | +   |
| NB263    | NNNNNN     | +   |
| NB264    | NNNNNN     | +   |
| 5'-AGGATGGCTTCCACCACTGAC-3’ and 5'-TCAGGGATGACCCTTGGCCACAG-3' for GAPDH; 5'-AGGATGGCTTCCACCACTGAC-3’ and 5'-TCAGGGATGACCCTTGGCCACAG-3' for GAPDH;
values and are shown as fold change relative to the value of the control sample. All the samples were done in triplicate.

Cell Monolayer and Cell Pellet Processing—For histological and immunocytochemistry analysis, culture pellets were fixed with 4% paraformaldehyde for 20 min at room temperature and embedded in 2.3 M sucrose for 1 h. Cell pellets were embedded in tissue freezing medium, Blue (Electron Microscopy Sciences) and frozen on dry ice. Sections of 2–6 μm in thickness were cut with a microtome and placed in the center of a coated slide. Sections were washed with PBS in a humid chamber, until excess sucrose was washed away.

Toluidine Blue Staining—Briefly, 0.1 g of toluidine blue (Sigma) was dissolved in 100 ml of distilled H2O. Pellet sections were stained in toluidine blue solution for 1–5 min at room temperature and rinsed with distilled H2O until excess stain was washed away.

In Vivo Assays—Chick embryos were explanted and grown in vitro as described (21). RCAS-Nodal retroviral stocks were produced as described (21) and used to infect, by air pressure, the right side of Hensen’s node in developing chick embryos. Beads were soaked in Nodal-like NB250 compound and similarly applied locally on the right side of Hensen’s node. Embryos were processed for whole mount Pitx2 in situ hybridization as described previously (21) or fixed in paraformaldehyde for heart looping visualization.

Fluorescence Microscopy—Briefly, after fixation, pellet and sections were blocked and permeabilized for 1 h at 37 °C with 5% BSA, 5% appropriate serum, 1× PBS with 0.1% Triton X-100. Subsequently, cells and sections were incubated with the indicated primary antibody overnight at 4 °C. Pellet sections were then washed thrice with 1× PBS and incubated for 2 h at 37 °C with the respective secondary antibody. Pellet sections were then washed thrice with 1× PBS; DAPI (0.5 μg/ml in PBS) was added to the last wash. These sections were mounted with aqueous mounting medium before analysis.

The primary antibodies used were anti-type I Collagen antibody (rabbit polyclonal antibody (ab292); Abcam, Cambridge, MA), anti-type II Collagen antibody (mouse monoclonal antibody (ab3092); Abcam), and anti-Sox9 antibody (rabbit polyclonal antibody (AB5535); Chemicon). Alexa Fluor 568 or 488 (Neomarkers) were used as secondary antibodies. The localizations of the proteins were observed with a Leica TCS SP2 AOBS confocal or Nikon E-800 microscope.

Surface Plasmon Resonance (BIAcore) Affinity Studies—The affinity of NB250 to ActRII-ECD was measured using a Biacore 3000 (GE Healthcare). Using primary amine coupling, the...
receptor ECD was immobilized on a CM5 chip independently using flow cell 2. No protein was immobilized on flow cell 1 as a negative control. For kinetic analysis, all tests were performed in duplicate using a minimum of five concentrations, plus a zero concentration. Binding data were analyzed with BIAevaluation software ver. 4.1 (GE Healthcare) and fit using a global 1:1 Langmuir binding with mass transfer model.

RESULTS

NB2 Chimera Production—NB2 chimeras were designed using the RASCH strategy (14). We exploited the sequence similarity between BMP2 and Nodal to enable proper chemical refolding under in vitro conditions to develop NB2 chimeras. Nodal and BMP2 were each divided into six segments based on the alignment of their sequences, thereby minimizing disruption of secondary structural motifs (Fig. 1). These segments were recombined using the overlapping PCR method to produce $2^6 = 64$ constructs and were given an identification number, NB2XX, where N (Nodal) and B2 (BMP2) are followed by the serial numbers ranging from 1 to 64 for XX. The collection of these 64 chimeras is referred to as the NB2 library. NB2 chimeras can also be identified using a code comprised of a six-letter sequence of N and B that indicates their segmental makeup. For instance, NB250 can be referred to as BNNNBB, because segments 1, 5, and 6 are derived from BMP2, and segments 2, 3, and 4 are derived from Nodal.

In short, these 64 NB2 library chimeras were purified from E. coli inclusion bodies, denatured, chemically refolded, and purified using affinity chromatography and reverse phase chromatography. Their production efficiency is reported in Table 1. Interestingly, chimeras beginning with segment 1 derived from Nodal did not produce a stable dimer during refolding, with the exception of two ligands that refolded correctly with noticeable efficiency. On the other hand, 21 of 32 chimeras with BMP2 segment 1 can be refolded and purified. As a result, we obtained a total of 23 properly refolded NB2 ligands of 64 possible. Based on these results, it appears that BMP2 segment 1 significantly improves proper chemical refolding of the NB2 chimera sequences.

NB250, NB260, and NB264 Signal Like Nodal in a Cripto-dependent Manner—After screening several chimeras, we found that NB250 (BNNNBB), NB260 (BNNNBN), and NB264 (BNNNNN) have Cripto-dependent activation of a Smad2-responsive luciferase reporter (Fig. 2A). These three chimeras were also similar to Nodal in their ability to induce Cripto-dependent Smad2 phosphorylation (Fig. 2B). Additionally,
NB250, the most divergent from Nodal of these three chimeras, shows a dose dependence similar to that of Nodal. Our results indicate that Cripto-dependent activation of Smad2 by NB250 closely follows that of Nodal in the concentration range of 0.01–30 nM (Fig. 2C). Moreover, like Nodal, NB250 does not activate a BMP-specific luciferase reporter that is activated by BMP2 (Fig. 2D).

**NB250 Alters Heart Looping in a Manner Very Similar to That of Nodal**—We compared the activity of NB250 and Nodal in vivo and found that both ligands induce ectopic expression of Pitx2 and alter heart looping during the establishment of vertebrate embryonic left-right asymmetry in a very similar manner. During normal development, Pitx2 transcripts are detected asymmetrically along the entire left side of the lateral plate mesoderm in developing chick embryos (Fig. 3A). We discovered that NB250 induces ectopic expression of Pitx2 and alters heart looping during the establishment of vertebrate embryonic left-right asymmetry. This activity is very similar to that of Nodal. Ectopic expression of Nodal on the right side of chick embryos leads to induction of Pitx2 transcripts on the right side of the lateral plate mesoderm (Fig. 3B, red arrow). Similarly, application of a bead soaked in NB250 induces ectopic Pitx2 transcripts along the right lateral plate mesoderm (Fig. 3C, red arrow). The wild type chick embryo develops with a rightward heart looping (Fig. 3D). However, Nodal and the NB250 chimera are each able to reverse heart looping in a similar leftward orientation (Fig. 3E and F), indicating that the in vivo action by NB250 mimics that of Nodal.

**NB250 and NB260 Induce Chondrogenic Differentiation of Human Adipose-derived Stem Cells**—BMP2 (22) and Activin (23) have each been observed to play a role in promoting chondrogenesis, whereas a role for Nodal in this process has not yet been established. However, because Nodal shares the same signaling pathway with Activin, we inferred that it might also induce chondrogenesis. We treated monolayers of expanded hASCs with 10 ng/ml of Nodal, NB250, NB260, or BMP2 for 2 weeks and compared the ability of each of these ligands to induce expression of chondrogenic genes using real time PCR. The results show that each of these ligands induced the expression of Col II, Sox9, and Aggrecan in a very similar manner (Fig. 4).

To further evaluate the chondrogenic potential of the chimeras, we used a cell pellet culture model to mimic the cellular condensation process occurring in normal limb development (24). Representative images of two independent experiments, using duplicates for each, are shown in Fig. 5. After 6 weeks of culture, pellets supplemented with BMP2, NB250, or NB260 achieved a cartilage-like appearance with a white shiny look resembling cartilage tissue and showed a marked increase in size compared with cell pellets grown in complete chondrogenic medium (control) (Fig. 5A).

We explored the formation of cartilage-like tissue by staining sections of the pellets with toluidine blue. Active matrix production and cartilage-like tissue formation was confirmed in BMP2, NB250, and NB260 supplemented pellets (Fig. 5B). Purple-dark blue metachromasia is even more apparent in NB2 chimera-treated pellet than in BMP2-treated pellet, suggesting higher deposition of glycosaminoglycans and formation of denser extracellular matrix. On the other hand, the control cells did not display tissue-like organization and rather present the appearance of aggregated cells with no clear organization. Additionally, the degree of maturation after chondrogenic differentiation was evaluated by immunohistochemical techniques using cells derived from a pellet culture system. Immunostaining of Col II and Col I in cell pellet sections showed a substantial number of Col II-positive cells, and a dense filamentous network of Col II connecting the ligand-treated cells (Fig. 5C). Interestingly, we did not detect co-localization of Collagen I and II in cell pellet sections showed a substantial number of Collagen II-positive cells, and a dense filamentous network of Collagen II-positive cells, indicating the resemblance of a stratified structure similar to that found in cartilage tissue (Fig. 5E). Expression of the chondrogenic transcription factor Sox9 was highly enriched in ligand-treated cells when compared with control cells (Fig. 5F). The detailed picture of Sox 9 nuclear localization is created by a Z-stack of the NB260-treated sections and shown in the upper left corner of the far right panel of Fig. 5F. Altogether, these data demonstrate that NB250 and NB260 can cause hASCs to acquire a mature chondrocyte-like phenotype in a more efficient manner than BMP2.

**The Structure of NB250 Closely Resembles That of BMP2**—We determined the crystal structure of NB250 at 1.9 Å resolution by the molecular replacement method using the known
BMP2 structure. Processing and refinement statistics are presented in Table 2. Similar to BMP2, the NB250 monomer has seven cysteine residues. These residues allow the monomer to form three intradisulfide bonds (Cys14/Cys80, Cys43/Cys112, and Cys47/Cys114) and one interdisulfide bond, which is formed between the Cys79 of each monomer. These disulfide bonds, along with α helix α1 formed by the region between residues Asn59 and Tyr70, comprise the core of the protein. The overall architecture of the dimer is dictated by these disulfide bonds (cysteine knot motif) coupled with four anti-parallel β-strands extending outward from the core, giving it the appearance of the spread wing shape of a butterfly, similar to that of BMP2 (Fig. 6A). The pre-helix loop, helix α1, and β sheet β2 consist of segments derived from Nodal and are depicted in yellow in Fig. 6A, whereas β sheets β1, β3, and β4 are BMP2 segments and are depicted in purple. When the structures are superimposed based on their shared sections (i.e., 1B, 5B, and 6B, which are NB250 segments derived from BMP2), the Cα root mean

**FIGURE 5.** NB250 and NB260 induce the formation of cartilage-like tissue. A, hASCs cultured in a pellet system treated with Nodal-like ligands (NB250 and NB260) or BMP2 or grown in incomplete chondrogenic medium as negative control (CTL). Scale bar, 1 mm. B, toluidine blue stain of sections of hASC pellets grown in incomplete chondrogenic medium or supplemented with BMP2, NB250, or NB260. Scale bar, 350 μm. C–E, immunochemistry assays of monolayer cells treated with BMP2, NB50, or NB260 for 4 weeks. Type II collagen is stained green, type I collagen is stained red, and DAPI is stained blue. Scale bar, 65 μm. F, expression of chondrogenic transcription factor Sox9 in culture pellet sections treated with BMP2, NB250, or NB260. Sox9 is shown in green, and cell nuclei are in blue. Ortho view of Z-stack, a representative cross-sectional image of x-y (box a), y-z (box b), and x-z (box c) coordinates is shown in the far right panel. Scale bar, 4 μm. The data are representative of two independent experiments performed in duplicate.
square deviation is 0.540 Å. As expected, the regions of highest structural discrepancy lie in the 2N3N4N region of NB250.

We superimposed NB250 onto BMP2 of the BMP2-BMPRIa-ActRII ternary complex to identify residues responsible for potential differences between BMP2 and NB250 in type II receptor and type I receptor binding. Previous studies have identified Ala34, Pro35, Ser88, Met89, and Leu90 in BMP2 as required for ActRII binding via its hydrophobic residues Phe83, Phe49, and Trp60 in BMPRIa (25). NB250 shares these residues with BMP2 except for Ala343, which is substituted by Tyr34 in NB250. After aligning the NB250 structure with BMP2 of the BMP2-BMPRIa-ActRII ternary complex (Fig. 6B), it can be seen that the aromatic side chain in tyrosine can form pi-stacking interactions with Phe83 of ActRII providing a basis for enhanced affinity for potential differences between BMP2 and NB250.

To test this possibility directly, we used surface plasmon resonance (Biacore) to measure the affinity of NB250 to ActRII-Alk4 complex, which adopts a BMP2-like fold. Thus, we predict that NB250 will likely adopt a BMP2-like fold. This is surprising but may shed some light on its functional properties. Combining this with the fact that NB250 appears to be indistinguishable from Nodal in its mode of signaling and effects on left-right heart looping in chick embryos and chondrogenesis, it can be concluded that Nodal itself retains Nodal functionality the minimal required Nodal segment sequence is 2N3N4N, with NB250 (BNNNBB) being a “minimalistic” chimaera to carry out Nodal functionality.

Nodal and NB2 ligands display strikingly similar effects in complex bioassays. We demonstrate that NB250 alters heart looping during the establishment of vertebrate embryonic left-right asymmetry in a manner indistinguishable from that of Nodal. Further, we show for the first time the chondrogenic effect of Nodal and that it is mimicked by the NB2 ligands. Previous studies, as well as our own assays, have shown that Activin/Nodal/TGF-β signaling plays a role in chondrogenesis (23). The Activin/BMP2 chimeric ligand AB235 was shown to direct adipose-derived stem cells to chondrogenic differentiation and exhibit a pattern of enhanced gene marker expression similar to that observed for Nodal, BMP2, and the NB250 and NB260 chimeras (27). NB250 and NB260 also induce chondrogenesis of human adipose-derived stem cells with an efficacy that appears to exceed that of BMP2 and AB235. Histological and immunohistochemical studies further demonstrate that NB260 and NB250 induce the growth of cartilage tissue and a dense filamentous network that resembles the stratified structure found in cartilaginous tissue.

Intriguingly, the overall structure of NB250 is very similar to that of BMP2. This is surprising but may shed some light on its functional properties. Combining this with the fact that NB250 appears to be indistinguishable from Nodal in its mode of signaling and effects on left-right heart looping in chick embryos and chondrogenesis, it can be concluded that Nodal itself adopts a BMP2-like fold. Thus, we predict that NB250 will likely be a structural as well as a functional mimic of Nodal. Importantly, its rigidity should facilitate structural studies of relevant complexes including the NB250-Cripto-ActRII-Alk4 complex. The elucidation of this complex, together with the Activin-ActRII-Alk4 complex, will be instrumental to understanding

**DISCUSSION**

Our previous studies of chemical refolding of Activin/BMP2 chimeras (14) revealed that the first BMP2 segment increases the probability of proper chemical refolding and dimer formation. This study reasserts the finding, because it shows that the first BMP2 segment also facilitates proper refolding of NB2 chimeras. It is noteworthy that the first segment of BMP2 has four fewer basic residues than the first segment of Nodal, and this difference may allow this BMP2 segment to promote chimaera refolding.

Our functional studies in vitro show that the NB2 chimeras NB250, NB260, and NB264 signal like Nodal in a Cripto-dependent manner. It appears that the chimeras may require segments 2, 3, and 4 of Nodal to achieve Cripto-dependent receptor activation and Smad2 phosphorylation. Therefore, we infer that these segments, which include residues structurally crucial to the putative type I receptor binding epitope, may play a pivotal role in proper Cripto binding and Cripto-dependent signaling. Indeed, previous modeling studies suggest that sections 2N, 3N, and 4N are relevant for Cripto dependence because they encompass structural elements proposed to play a role in Nodal binding to ALK4 in the presence of Cripto (26). Our activity assays are consistent with this idea. We propose that to retain Nodal functionality the minimal required Nodal segment sequence is 2N3N4N, with NB250 (BNNNBB) being a “minimalistic” chimaera to carry out Nodal functionality.

NB250 and NB260 chimeras (27). NB250 and NB260 also induce chondrogenesis of human adipose-derived stem cells with an efficacy that appears to exceed that of BMP2 and AB235. Histological and immunohistochemical studies further demonstrate that NB260 and NB250 induce the growth of cartilage tissue and a dense filamentous network that resembles the stratified structure found in cartilaginous tissue.

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how Cripto exerts opposing effects on Nodal and Activin signaling (8).

Our studies show that NB250 is functionally indistinguishable from Nodal. However, its sequence is approximately one-half Nodal and one-half BMP2. Interestingly, NB250 does not signal through the canonical BMP pathway, i.e., via Smads 1, 5, and 8. Nodal and BMP2 share the same type II receptor, ActRII, and the type II receptor binding epitope of NB250 largely resembles that of BMP2. Nodal receptor assembly is critically regulated by Cripto, which binds to Nodal via its EGF-like domain and to the type I receptor ALK4 via its CFC domain (18). It is perhaps not surprising, therefore, that replacing the type II receptor binding epitope of Nodal with that of BMP2 in NB250 does not disrupt Nodal function because the Cripto binding site is preserved.

The affinity of NB250/ActRII is two times higher than that of BMP2/ActRII, which may be explained by a single residue change in the NB250 ActRII binding epitope that could slightly enhance the affinity of this chimera for its type II receptor. We determined that an Ala to Tyr change in the NB250 type II binding epitope may establish a novel interaction with the ActRII residues that bind to this pocket.

On the other hand, failure to observe BMP2-like signaling through Smad1/5/8 suggests that NB250 has a substantially lower affinity for its type I receptor.
decreased affinity for the canonical BMP type I receptor, BMPR1a. This may be caused by disruptive residue changes that are the product of the chimeric makeup of NB250, the most significant being a Phe to Asn substitution in the NB250 type I receptor binding pocket. Indeed, a change from a hydrophobic to a polar residue in this region would greatly reduce, if not abolish, the hydrophobic interactions required for BMPR1a to bind to this ligand.

The NB2 chimeras employed in this work are easily produced and will accelerate the development of cost-effective protocols for structural studies and for biological purposes such as deriving chondrocytes in vitro. We have shown that NB250 is a suitable substitute for Nodal and that it is able to mimic Nodal’s heart looping properties as well as Nodal’s newly found chondrogenic property. Furthermore, the chondrocytes obtained by the library chimeras may have important implications for cell replacement therapies in cartilage repair with valuable clinical applications.

REFERENCES

1. Tsuchida, K., Nakatani, M., Uezumi, A., Murakami, T., and Cui, X. (2008) Signal transduction pathway through Activin receptors as a therapeutic target of musculoskeletal diseases and cancer. Endocr. J. 55, 11–21
2. Chea, H. K., Wright, C. V., and Swalla, B. J. (2005) Nodal signaling and the evolution of deuterostome gastrulation. Dev. Dyn. 234, 269–278
3. Vallier, L., Reynolds, D., and Pedersen, R. A. (2004) Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. Dev. Biol. 275, 403–421
4. Shiozaki, M., Sakai, R., Tabuchi, M., Nakamura, T., Sugino, K., Sugino, H., and Eto, Y. (1992) Evidence for the participation of endogenous Activin A/erythroid differentiation factor in the regulation of erythropoiesis. Proc. Natl. Acad. Sci. U.S.A. 89, 1553–1556
5. Rivier, C., and Vale, W. (1991) Effect of recombinant Activin-A on gonadotropin Secretion in the female rat. Endocrinology 129, 2463–2465
6. Shiozaki, M., Sakai, R., Tabuchi, M., Nakamura, T., Sugino, K., Sugino, H., and Eto, Y. (1992) Evidence for the participation of endogenous Activin A/erythroid differentiation factor in the regulation of erythropoiesis. Proc. Natl. Acad. Sci. U.S.A. 89, 1553–1556
7. Rivier, C., and Vale, W. (1991) Effect of recombinant Activin-A on gonadotropin Secretion in the female rat. Endocrinology 129, 2463–2465
8. Shiozaki, M., Sakai, R., Tabuchi, M., Nakamura, T., Sugino, K., Sugino, H., and Eto, Y. (1992) Evidence for the participation of endogenous Activin A/erythroid differentiation factor in the regulation of erythropoiesis. Proc. Natl. Acad. Sci. U.S.A. 89, 1553–1556
9. Rivier, C., and Vale, W. (1991) Effect of recombinant Activin-A on gonadotropin Secretion in the female rat. Endocrinology 129, 2463–2465
10. Shiozaki, M., Sakai, R., Tabuchi, M., Nakamura, T., Sugino, K., Sugino, H., and Eto, Y. (1992) Evidence for the participation of endogenous Activin A/erythroid differentiation factor in the regulation of erythropoiesis. Proc. Natl. Acad. Sci. U.S.A. 89, 1553–1556
11. Montero, J. A., Lorda-Diez, C. I., Gañan, Y., Macias, D., and Hurle, J. M. (2008) Activin/TGFβ and BMP crosstalk determines digt chondrogenesis.