



US 20020192219A1

(19) **United States**

(12) **Patent Application Publication**

Harland et al.

(10) **Pub. No.: US 2002/0192219 A1**

(43) **Pub. Date: Dec. 19, 2002**

(54) **MORPHOGENIC PROTEINS**

continuation of application No. 08/795,501, filed on Feb. 5, 1997, now abandoned.

(75) Inventors: **Richard Harland**, Berkeley, CA (US);
David Hsu, Berkeley, CA (US)

Publication Classification

Correspondence Address:
RICHARD ARON OSMAN
SCIENCE AND TECHNOLOGY LAW GROUP
75 DENISE DRIVE
HILLSBOROUGH, CA 94010

(51) **Int. Cl.⁷** **A61K 39/395**; C12Q 1/68
(52) **U.S. Cl.** **424/146.1**; 435/6

(73) Assignee: **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA**

(57) **ABSTRACT**

(21) Appl. No.: **10/159,749**

(22) Filed: **May 29, 2002**

DAN (Differential-screening-selected gene Aberrative in Neuroblastoma) and gremlin proteins and related nucleic acids are provided. Included are natural DAN and gremlin homologs from several species and proteins comprising a DAN or gremlin domain having specific activity, particularly the ability to antagonize a bone morphogenic protein. The proteins may be produced recombinantly from transformed host cells with the subject nucleic acids. Also provided are isolated hybridization probes and primers capable of specifically hybridizing with the disclosed genes, specific binding agents and methods of making and using the subject compositions.

Related U.S. Application Data

(63) Continuation of application No. 09/040,229, filed on Mar. 13, 1998, now Pat. No. 6,432,410, which is a

FIG. 1A

xGremlin	MNCLVYALGSLFLLSGLLLPSSEGGKKKVSQGAIPPPDKGQPN <u>ND</u> SEQGQ	50
cGremlin	MVRTLYAIGAVFLLTGFLLP <u>TA</u> EGRKRNRSQGAIPPPDKDQPN <u>ND</u> SEQMQ	50
mGremlin	MNRTAYTVGALLLLLTLLPTAEGKKK . . GSQGAIPPPDKAQH <u>ND</u> SEQTQ	48
hGremlin	MSRTAYTVGALLLLLTLLPAAEGKKK . . GSQGAIPPPDKAQH <u>ND</u> SEQTQ	48
xGremlin	A . . QPG . . DRVRGKGKGQALAAEEVLESSQEALHVTERKYLKRDWCKTQP	96
cGremlin	TQQQSGSRHRERGGKT . . SMPAAEEVLESSQEALHITERKYLKRDWCKTQP	98
mGremlin	SPPQPGSRTRGRGQGRGTAMPGEVLESSQEALHVTERKYLKRDWCKTQP	98
hGremlin	SPQQPGSRNRGRGQGRGTAMPGEVLESSQEALHVTERKYLKRDWCKTQP	98
xGremlin	LKQTIHEDGCNSRTIINRFCYGCNSFYIPRHIRREEGSFQSCSFCKPKK	146
cGremlin	LKQTIHEEGCNSRTIINRFCYGCNSFYIPRHVRKEEGSFQSCSFCKPKK	148
mGremlin	LKQTIHEEGCNSRTIINRFCYGCNSFYIPRHIRKEEGSFQSCSFCKPKK	148
hGremlin	LKQTIHEEGCNSRTIINRFCYGCNSFYIPRHIRKEEGSFQSCSFCKPKK	148
xGremlin	FTTMVVTLNCPELQPPTKKKRITRVKQCR <u>CIS</u> IDL <u>D</u>	182
cGremlin	FTTMTVTLNCPELQPPRKKKRITRVKEC <u>RCIS</u> IDL <u>D</u>	184
mGremlin	FTTMMVTLNCPELQPPTKKKRVTRVKQCR <u>CIS</u> IDL <u>D</u>	184
hGremlin	FTTMMVTLNCPELQPPTKKKRVTRVKQCR <u>CIS</u> IDL <u>D</u>	184

FIG. 1B

xGremlin	KRDWCKTQPLKQTIHEDGCNSRTIINRFCYGCNSFYIPRHIRRE-EGSF	136
xCerberus	MKEACKTLPFTQNIVHENCNDRMVIQNNLCFGKCISLHVPNQDDR-N---	210
mDAN	KSAWCEAKNITQIVGHSGCEAKSIQNRACLGQCFSYSVPNTFPQS- <u>TESL</u>	78
xDAN	KSAWCEAKNITQIVGHSGCESKSIQNRACLGQCFSYSVPNTFPQS- <u>TESL</u>	79
ceGR-1	MNQRCDGQKFKQRIRVDGCLTKVVVNRLCHGACASIFIPRMHKKLKAFF	
xGremlin	QSCSFCKPKKFTTMVVTLNCPELQPPTKKKRITR-VKQCR <u>CIS</u> IDL <u>D</u> --	182
xCerberus	-TCSHCLPSKFTLNHLTLNCTGSKNVVKVMMVE---ECTCEAHKSNF-	254
mDAN	VHCDSCMPAQSMWEIVTLECPDHEEVPRVDKLVEKIVHCSCQACGKEP-	126
xDAN	VHCDSCMPIDSVWDVVTLECPGNEEFPRVDKLVEKILQCSCQACGKEL-	127
ceGR-1	RSACAAPAEYDYVDITLDCPGRTPTATKTIVK-VKSCCKEIVRIAPF	

MORPHOGENIC PROTEINS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application is a continuing application under 35USC120 of U.S. Ser. No. 09/040,229, filed Mar. 13, 1998, which is a continuing application under 35USC120 of U.S. Ser. No. 08/795,501 filed Feb. 5, 1997, the specifications of which are incorporated by reference.

[0002] The research carried out in the subject application was supported in part by grants from the National Institutes of Health. The government may have rights in any patent issuing on this application.

INTRODUCTION

[0003] 1. Field of the Invention

[0004] The field of this invention is proteins which regulate cell function, and in particular, antagonize bone morphogenic proteins.

[0005] 2. Background

[0006] Natural regulators of cellular growth, differentiation and function have provided important pharmaceuticals, clinical and laboratory tools, and targets for therapeutic intervention. A variety of such regulators have been shown to have profound effects on basic cellular differentiation and developmental pathways. For example, the recently cloned cerberus protein induces the formation of head structures in anterior endoderm of vertebrate embryos. Similarly, the noggin protein induces head structures in vertebrate embryos, and can redirect mesodermal fates from ventral fates, such as blood and mesenchyme, to dorsal fates such as muscle and notochord and can redirect epidermal fates to anterior neural fates. The activities of chordin are similar to those of noggin, reflecting a common mechanism of action; namely antagonizing bone morphogenic proteins (BMP) and thereby preventing their function. BMPs have diverse biological activities in different biological contexts, including the induction of cartilage and bone, connective tissue, and roles in kidney, tooth, gut skin and hair development.

[0007] Different members of the TGF β superfamily can instruct cells to follow different fates, for example TGF β induces neural crest to form smooth muscle, while BMP2 induces the same cells to become neurons. In *Xenopus* experiments, dissociated animal cap cells (prospective ectoderm) become epidermis in response to BMP4 but become mesoderm in response to activin. Since the identity between activin and BMP4 is low, it is not surprising that they induce different fates. It is more surprising that members of the BMP subfamily, which are quite closely related in sequence, can induce distinct fates. A striking example results from implantation of a matrix impregnated with a BMP into muscle; when the effects are monitored histologically, BMP2, 4 and 7 induce endochondral bone formation, whereas a related molecule BMP12/GDF7 induces connective tissue similar to tendon. Similarly, BMP4 can induce cell death in the hindbrain neural crest, while the related protein dorsalin does not.

[0008] Since different BMP family members can induce different fates, then BMP antagonists that have specificity in blocking subsets of BMPs could change the balance of

BMPs that are presented to a cell, thus altering cell fate. In view of the importance of relative BMP expression in human health and disease, regulators of cellular function and BMP function in particular, such as noggin and cerberus, provide valuable reagents with a host of clinical and biotechnological applications.

[0009] The present invention relates to a new family of regulators of cellular function; in particular, we have identified a new dorsalizing factor Gremlin. Gremlin's activities are similar to those of Noggin and Chordin, however, it has no sequence similarity to them, and it is not expressed during gastrulation. Instead, zygotic Gremlin expression begins in the migrating neural crest, highlighting a potential role for organizer-like activities in the development of this cell lineage. Furthermore, we have identified a structurally and functionally-related family of proteins that includes Gremlin, the head-inducing factor Cerberus and the tumor suppressor DAN (Ozaki and Sakiyama, 1993; Bouwmeester et al., 1996). We have named this family the DAN family for the first member identified. Cerberus is expressed in the anterior endomesoderm of the gastrula organizer and has been proposed to participate in head induction (Bouwmeester et al., 1996). DAN was isolated as a putative zinc-finger protein downregulated in transformed cells and was subsequently shown to have tumor suppressor activity (Ozaki and Sakiyama, 1993; Ozaki and Sakiyama, 1994). Similarly, the rat homolog of Gremlin, *drm*, has also been proposed to have a role in controlling cell growth and differentiation (Topol et al., 1997). We demonstrate that all members of the DAN family act as BMP antagonists, while Cerberus alone blocks the activity of Activin and Nodal-like activities. We also show that DAN-family members are secreted proteins that bind BMP-2. Together, our data reveal that individual DAN-family members block the activity of specific TGF- β ligands by binding them and preventing them from interacting with their cell surface receptors.

[0010] Relevant Literature

- [0011]** Altschul, S. F., et al. (1990) *J Mol Biol* 215, 403-10.
- [0012]** Badley, J. E., et al. (1988) *Biotechniques* 6, 114-6.
- [0013]** Baker, J. C., and Harland, R. M. (1996) *Genes & Development* 10, 1880-1889.
- [0014]** Belo, J. A., et al. (1997) *Mechanisms Of Development* 68, 45-57.
- [0015]** Bolce, M. E., et al. (1992) *Development* 115, 681-8.
- [0016]** Bouwmeester, T., et al. (1996) *Nature* 382, 595-601.
- [0017]** Clement, J. H., et al. (1995) *Mech Dev* 52, 357-70.
- [0018]** Collazo, A., Bronner-Fraser, M., and Fraser, S. E. (1993) *Development* 118, 363-76.
- [0019]** Dale, L., et al. (1992) *Development* 115, 573-85.
- [0020]** Estevez, M., et al. (1993) *Nature* 365, 644-9.
- [0021]** Fainsod, A., et al. (1994) *EMBO J.* 13, 5015-5025.

- [0022] Gimlich, R. L., and Gerhart, J. C. (1984) *Developmental Biology* 104, 117-130.
- [0023] Harland, R. M., and Gerhart, J. C. (1997) In *Ann reviews Cell and Dev Biol*, pp. 611-667.
- [0024] Hemmati-Brivanlou, A., et al. (1994) *Cell* 77, 283-295.
- [0025] Hemmati-Brivanlou, A., and Thomsen, G. H. (1995)-4. *Dev Genet* 17, 78-89.
- [0026] Hogan, B. L., et al. (1994) *Dev Suppl*, 53-60.
- [0027] Holley, S. A., et al. (1996) *Cell* 86, 607-17.
- [0028] Jones, C. M., et al. (1995) *Development* 121, 3651-62.
- [0029] Jones, C. M., et al. (1992) *Development* 115, 639-47.
- [0030] Joseph, E. M., and Melton, D. A. (1997) *Dev Biol* 184, 367-72.
- [0031] Kao, K. R., and Elinson, R. P. (1988) *Dev Biol* 127, 64-77.
- [0032] Kintner, C. R., and Brockes, J. P. (1984) *Nature* 308, 67-69.
- [0033] Kintner, C. R., and Melton, D. A. (1987) *Development* 99, 311-25.
- [0034] Krotoski, D. M., et al. (1988) *Dev Biol* 127, 119-32.
- [0035] Lamb, T. M., et al. (1993) *Science* 262, 713-8.
- [0036] Lemaire, P., Garrett, N., and Gurdon, J. B. (1995) *Cell* 81, 85-94.
- [0037] Lennon, G., et al. (1996) *Genomics* 33, 151-2.
- [0038] Ozaki, T., and Sakiyama, S. (1993) *Proc Natl Acad Sci U S A* 90, 2593-7.
- [0039] Ozaki, T., and Sakiyama, S. (1994) *Cancer Res* 54, 646-8.
- [0040] Peng, H. B. (1991) In *Methods in Cell Biology* 36, B. K. Kay, H. B. Peng, eds., pp. 661-662.
- [0041] Piccolo, S., Sasai, Y., Lu, B., and De Robertis, E. M. (1996) *Cell* 86, 589-98.
- [0042] Ren, P., et al. (1996) *Science* 274, 1389-91.
- [0043] Sadaghiani, B., and Thiebaud, C. H. (1987) *Dev Biol* 124, 91-110.
- [0044] Sasai, Y., and De Robertis, E. M. (1997) *Dev Biol* 182, 5-20.
- [0045] Sasai, Y., et al. (1995) *Nature* 376, 333-336.
- [0046] Sasai, Y., et al. (1994) *Cell* 79, 779-790.
- [0047] Savage, C., et al. (1996) *Proc Natl Acad Sci U S A* 93, 790-4.
- [0048] Schmidt, J. E., et al. (1995) *Developmental Biology* 169, 37-50.
- [0049] Smith, J. C., et al. (1990) *Nature* 345, 729-31.
- [0050] Smith, J. C., et al. (1991) *Cell* 67, 79-87.
- [0051] Smith, W. C., and Harland, R. M. (1992) *Cell* 70, 829-40.
- [0052] Smith, W. C., and Harland, R. M. (1991) *Cell* 67, 753-765.
- [0053] Smith, W. C., Knecht, A. K., Wu, M., and Harland, R. M. (1993) *Nature* 361, 547-9.
- [0054] Smith, W. C., McKendry, R., Ribisi, S., and Harland, R. M. (1995) *Cell* 82, 37-46.
- [0055] Thies, R. S., et al. (1992) *Endocrinology* 130, 1318-24.
- [0056] Thomas, P., et al. (1997) *Cold Spring Harbor Symposia on Quantitative Biology* 62.
- [0057] Thomsen, G., et al. (1990) *Cell* 63, 485-93.
- [0058] Thomsen, G. H., and Melton, D. A. (1993) *Cell* 74, 433-441.
- [0059] Topol, L. Z., et al. (1997) *Molecular and Cellular Biology* 17, 4801-4810.
- [0060] Turner, D. L., and Weintraub, H. (1994) *Genes Devel.* 8, 1434-1447.
- [0061] Varlet, I., Collignon, J., and Robertson, E. J. (1997) *Development* 124, 1033-44.
- [0062] Vize, P. D., Jones, E. A., and Pfister, R. (1995) *Dev Biol* 171, 531-40.
- [0063] Wieser, R., Wrana, J. L., and Massague, J. (1995) *Embo J* 14, 2199-208.
- [0064] Wilson, P. A., and Hemmati-Brivanlou, A. (1995) *Nature* 376, 331-3.
- [0065] Wilson, P. A., and Melton, D. A. (1994) *Curr Biol* 4, 676-86.
- [0066] Zimmerman, L. B., et al. (1996) *Cell* 86, 599-606.

SUMMARY OF THE INVENTION

[0067] The invention provides methods and compositions relating to DAN (Differential-screening-selected gene Aberrative in Neuroblastoma) and gremlin proteins and related nucleic acids. Included are natural DAN and gremlin homologs from different species and DAN and gremlin proteins comprising a DAN or gremlin domain and having DAN or gremlin-specific activity, particularly the ability to antagonize a bone morphogenic protein such as BMP2 or BMP4. The proteins may be produced recombinantly from transformed host cells with the subject nucleic acids. The invention provides isolated hybridization probes and primers capable of specifically hybridizing with the disclosed genes, specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis (e.g. genetic hybridization screens for gremlin transcripts), therapy (e.g. gene therapy to modulate gremlin gene expression) and in the biopharmaceutical industry (e.g. reagents for screening chemical libraries for lead pharmacological agents).

[0068] Preferred applications of the subject DAN and gremlin proteins include modifying the physiology of a cell comprising an extracellular surface by contacting the cell or medium surrounding the cell with an exogenous DAN or

gremlin protein under conditions whereby the added protein specifically interacts with a component of the medium and/or the extracellular surface to effect a change in the physiology of the cell. Also preferred are methods for screening for biologically active agents, which methods involve incubating a DAN or gremlin protein in the presence of an extracellular DAN or gremlin protein-specific binding target and a candidate agent, under conditions whereby, but for the presence of the agent, the protein specifically binds the binding target at a reference affinity; detecting the binding affinity of the protein to the binding target to determine an agent-biased affinity, wherein a difference between the agent-biased affinity and the reference affinity indicates that the agent modulates the binding of the protein to the binding target.

BRIEF DESCRIPTION OF THE FIGURE

[0069] FIG. 1(A) shows a sequence alignment of Gremlin sequences identified in *Xenopus*(x)(SEQ ID NO:04), chick(c)(SEQ ID NO:06), mouse(m)(SEQ ID NO:09), and human(h)(SEQ ID NO:02), showing that they are >80% identical. The predicted signal sequence is in bold. A single N-linked glycosylation site is underlined. The nine cysteines in the carboxy-terminal domain are marked with asterisks. FIG. 1(B) shows a sequence alignment of the cysteine-rich domain shared by *Xenopus* Gremlin (xGremlin)(SEQ ID NO:04), *Xenopus* Cerberus (xCerberus)(SEQ ID NO:12), mouse DAN (mDAN)(SEQ ID NO:10), *Xenopus* DAN (xDAN)(SEQ ID NO: 1), and *C. elegans* Gremlin-related-1 (ceGR-1) (SEQ ID NO: 13). Conserved cysteines are marked with asterisks.

DETAILED DESCRIPTION OF THE INVENTION

[0070] The nucleotide sequence of a natural cDNA encoding human, xenopus and chick gremlin polypeptides are shown as SEQ ID NOS:1, 3, and 5, respectively, and the corresponding full conceptual translates are shown as SEQ ID NOS:2, 4 and 6. The nucleotide sequence of a natural cDNA encoding a human DAN polypeptide is shown as SEQ ID NO:7, and the corresponding full conceptual translate is shown as SEQ ID NO:8. The gremlin and DAN polypeptides of the invention include one or more functional domains comprising at least 8, preferably at least 12, more preferably at least 18, more preferably at least 36 contiguous residues of the recited SEQ ID NOS and have an assay-discernable DAN or gremlin-specific activity, functionally distinct from each other and from cerberus and noggin homologs. For example, gremlin specific polynucleotides and polypeptides having human gremlin-specific sequences are readily discernable from alignments of the sequences. Preferred gremlin polypeptides have one or more human gremlin-specific activities, such as cell surface receptor binding and/or binding inhibitory activity and gremlin-specific immunogenicity and/or antigenicity. In a particular embodiment, the DAN or gremlin polypeptides retain the nine conserved cysteine residues, and preferably all contiguous sequence contained within the native nine conserved cysteine residues of the family as discernable by alignment (FIG. 1A, 1B). FIG. 1(A) shows that gremlin is highly conserved in vertebrates.

[0071] DAN or gremlin-specific activity or function may be determined by convenient in vitro, cell-based, or in vivo

assays: e.g. in vitro binding assays, cell culture assays, in animals (e.g. gene therapy, transgenics, etc.), etc. Binding assays encompass any assay where the molecular interaction of a DAN or gremlin polypeptide with a binding target is evaluated. The binding target may be a natural extracellular binding target such as a TGFβ protein, a morphogenic protein, preferably a bone morphogenic protein such as BMP2 or BMP4; or non-natural binding target such as a specific immune protein such as an antibody, or a DAN or gremlin specific agent such as those identified in screening assays such as described below. DAN or gremlin-binding specificity may be assayed by binding equilibrium constants (usually at least about 10⁷M⁻¹, preferably at least about 10⁸M⁻¹, more preferably at least about 10⁹ M⁻¹), by growth cone collapse assays, by the ability to elicit gremlin specific antibody in a heterologous host (e.g a rodent or rabbit), etc.

[0072] For example, deletion mutagenesis is used to define functional DAN and gremlin domains which specifically bind BMPs in in vitro and cell-based assays described below.

TABLE 1

Exemplary DAN and gremlin deletion mutants defining functional domains.		
Mutant	Sequence	BMP Binding
GAN1	SEQ ID NO: 2, residues 2–184	+
GAN2	SEQ ID NO: 2, residues 12–184	+
GAN3	SEQ ID NO: 2, residues 24–184	+
GAN4	SEQ ID NO: 2, residues 48–184	+
GAN5	SEQ ID NO: 2, residues 64–184	+
GAC1	SEQ ID NO: 2, residues 1–183	+
GAC2	SEQ ID NO: 2, residues 1–182	+
GAC3	SEQ ID NO: 2, residues 1–181	+
GAC4	SEQ ID NO: 2, residues 1–180	+
GAC5	SEQ ID NO: 2, residues 1–179	+
GANC1	SEQ ID NO: 2, residues 21–181	+
GANC2	SEQ ID NO: 2, residues 34–182	+
GANC3	SEQ ID NO: 2, residues 57–183	+
GANC4	SEQ ID NO: 2, residues 13–183	+
GANC5	SEQ ID NO: 2, residues 45–181	+
DAN1	SEQ ID NO: 8, residues 3–181	+
DAN2	SEQ ID NO: 8, residues 8–181	+
DAN3	SEQ ID NO: 8, residues 18–181	+
DAN4	SEQ ID NO: 8, residues 23–181	+
DAN5	SEQ ID NO: 8, residues 28–181	+
DAC1	SEQ ID NO: 8, residues 1–180	+
DAC2	SEQ ID NO: 8, residues 1–179	+
DAC3	SEQ ID NO: 8, residues 1–178	+
DAC4	SEQ ID NO: 8, residues 1–177	+
DAC5	SEQ ID NO: 8, residues 1–176	+
DANC1	SEQ ID NO: 8, residues 3–180	+
DANC2	SEQ ID NO: 8, residues 2–177	+
DANC3	SEQ ID NO: 8, residues 7–179	+
DANC4	SEQ ID NO: 8, residues 16–180	+
DANC5	SEQ ID NO: 8, residues 2 1–178	+

[0073] In a particular embodiment, the subject domains provide DAN or gremlin-specific antigens and/or immunogens, especially when coupled to carrier proteins. For example, peptides corresponding to gremlin- and human gremlin-specific domains are covalently coupled to keyhole limpet antigen (KLH) and the conjugate is emulsified in Freund's complete adjuvant. Laboratory rabbits are immunized according to conventional protocol and bled. The presence of DAN or gremlin-specific antibodies is assayed by solid phase immunosorbant assays using immobilized DAN or gremlin polypeptides, see, e.g. Table 2.

TABLE 2

Immunogenic human gremlin polypeptides eliciting gremlin-specific rabbit polyclonal antibody: gremlin polypeptide-KLH conjugates immunized per protocol described above.	
Gremlin Polypeptide Sequence	Immunogenicity
SEQ ID NO: 2, residues 1-8	+++
SEQ ID NO: 2, residues 2-11	+++
SEQ ID NO: 2, residues 12-21	+++
SEQ ID NO: 2, residues 15-23	+++
SEQ ID NO: 2, residues 22-30	+++
SEQ ID NO: 2, residues 32-39	+++
SEQ ID NO: 2, residues 42-51	+++
SEQ ID NO: 2, residues 45-52	+++
SEQ ID NO: 2, residues 52-65	+++
SEQ ID NO: 2, residues 68-79	+++
SEQ ID NO: 2, residues 70-80	+++
SEQ ID NO: 2, residues 75-82	+++
SEQ ID NO: 2, residues 86-92	+++
SEQ ID NO: 2, residues 92-98	+++
SEQ ID NO: 2, residues 95-103	+++
SEQ ID NO: 2, residues 99-108	+++
SEQ ID NO: 2, residues 102-112	+++
SEQ ID NO: 2, residues 109-115	+++
SEQ ID NO: 2, residues 116-123	+++
SEQ ID NO: 2, residues 119-126	+++
SEQ ID NO: 2, residues 122-130	+++
SEQ ID NO: 2, residues 124-135	+++
SEQ ID NO: 2, residues 127-140	+++
SEQ ID NO: 2, residues 138-147	+++
SEQ ID NO: 2, residues 145-155	+++
SEQ ID NO: 2, residues 149-151	+++
SEQ ID NO: 2, residues 157-165	+++
SEQ ID NO: 2, residues 160-168	+++
SEQ ID NO: 2, residues 167-181	+++

[0074] The claimed gremlin polypeptides are isolated or pure: an “isolated” polypeptide is unaccompanied by at least some of the material with which it is associated in its natural state, preferably constituting at least about 0.5%, and more preferably at least about 5% by weight of the total polypeptide in a given sample and a pure polypeptide constitutes at least about 90%, and preferably at least about 99% by weight of the total polypeptide in a given sample. The gremlin polypeptides and polypeptide domains may be synthesized, produced by recombinant technology, or purified from mammalian, preferably human cells. A wide variety of molecular and biochemical methods are available for biochemical synthesis, molecular expression and purification of the subject compositions, see e.g. Molecular Cloning, A Laboratory Manual (Sambrook, et al. Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al, Greene Publ. Assoc., Wiley-Interscience, N.Y.) or that are otherwise known in the art. An exemplary method for isolating natural DAN and gremlin proteins involves expressing a cDNA library (e.g. derived from xenopus ovarian cells) and assaying expression products for embryonic axis formation. This method and other suitable bioassays amenable to detecting DAN and gremlin proteins have been described by Lemaire P; et al. (supra); Smith, W. C., and Harland, R. M. (1992 and 1991, supra); Piccolo, S., et al. (1996, supra); and Zimmerman, L. B., et al. (1996, supra).

[0075] The invention provides binding agents specific to DAN or gremlin polypeptides, preferably the claimed human gremlin polypeptides, including agonists, antagonists, natural cell surface receptor binding targets, etc.,

methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, specific binding agents are useful in a variety of diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with improper utilization of a pathway involving the subject proteins. Novel DAN or gremlin-specific binding agents include DAN or gremlin-specific receptors, such as somatically recombined polypeptide receptors like specific antibodies or T-cell antigen receptors (see, e.g Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory) and other natural binding agents such as Gremlin cell surface receptors, non-natural intracellular binding agents identified in screens of chemical libraries such as described below, etc. Agents of particular interest modulate DAN or gremlin function, e.g. gremlin-modulatable cellular physiology.

[0076] The subject proteins find a wide variety of uses including use as immunogens, targets in screening assays, bioactive reagents for modulating cell growth, differentiation and/or function, etc. For example, the invention provides methods for modifying the physiology of a cell comprising an extracellular surface by contacting the cell or medium surrounding the cell with an exogenous DAN or gremlin protein under conditions whereby the added protein specifically interacts with a component of the medium and/or the extracellular surface to effect a change in the physiology of the cell. According to these methods, the extracellular surface includes plasma membrane-associated receptors; the exogenous DAN or gremlin refers to a protein not made by the cell or, if so, expressed at non natural levels, times or physiologic locales; and suitable media include in vitro culture media and physiological fluids such as blood, synovial fluid, etc. Effective administrations of subject proteins can be used to reduce undesirable (e.g. ectopic) bone formation, inhibit the growth of cells that require a morphogenic protein (e.g. BMP-dependent neuroblastomas and gliomas), alter morphogen-dependent cell fate/differentiation in culture, such as with cells for transplantation or infusion, etc. The proteins may be may be introduced, expressed, or repressed in specific populations of cells by any convenient way such as microinjection, promoter-specific expression of recombinant enzyme, targeted delivery of lipid vesicles, etc.

[0077] In one embodiment, the invention provides methods for modulating cell function comprising the step of modulating DAN or gremlin activity, e.g. by contacting the cell with a DAN or gremlin polypeptide. In preferred embodiments, the modulator effects gremlin or DAN mediated decrease in resident BMP activity. The target cell may reside in culture or in situ, i.e. within the natural host. The modulator may be provided in any convenient way, including by (i) intracellular expression from a recombinant nucleic acid or (ii) exogenous contacting of the cell. For many in situ applications, the compositions are added to a retained physiological fluid such as blood or synovial fluid. DAN and gremlin polypeptides or polypeptide modulators may also be amenable to direct injection or infusion, topical, intratracheal/nasal administration e.g. through aerosol, intraocularly, or within/on implants e.g. fibers e.g. collagen, osmotic pumps, grafts comprising appropriately transformed cells, etc. See e.g. Tracy MA (1998) Biotechnol Prog 14(1):108-115; Putney S D, Burke P A (1998) Nat Biotechnol 16(2):153-157 for encapsulation delivery methods. Gen-

erally, the amount administered will be empirically determined, typically in the range of about 10 to 1000 $\mu\text{g/kg}$ of the recipient and the concentration will generally be in the range of about 50 to 500 $\mu\text{g/ml}$ in the dose administered, see e.g. Lucidi P, et al. (1998) *J Clin Endocrinol Metab* 83(2):353-357; Houdijk EC, et al. (1997) *Acta Paediatr* 86(12):1301-1307 for suitable methods of administration. Other additives may be included, such as stabilizers, bactericides, etc. will be present in conventional amounts. For diagnostic uses, the modulators or other gremlin binding agents are frequently labeled, such as with fluorescent, radioactive, chemiluminescent, or other easily detectable molecules, either conjugated directly to the binding agent or conjugated to a probe specific for the binding agent.

[0078] For example, rats implanted with BMP-4 collagen matrices are used to demonstrate in vivo efficacy of gremlin and DAN polypeptides. In these experiments, rats are implanted with BMP-4 collagen matrices substantially as described in Wozney et al. (1988) *Science* 242:1528-1534 and treated with three gremlin polypeptides (SEQ ID NO:2, SEQ ID NO:2, residues 24-182, and SEQ ID NO:2, residues 54-180 or two DAN polypeptides (SEQ ID NO:8, SEQ ID NO:8, residues 12-181. All five treatment groups demonstrate significant reductions in ectopic (matrix) bone formation. Gremlin and DAN polypeptides are also demonstrated to antagonize recombinant human BMP-2- mediated bone formation in rabbits, essentially as described in Zegzula HD, et al. (1997) *J Bone Joint Surg Am* 79(12):1778-1790. Briefly, A unilateral segmental defect, twenty millimeters long, is created in the radius in ninety-six skeletally mature New Zealand White rabbits. Forty-eight rabbits are evaluated at four weeks and forty-eight, at eight weeks. Eight groups are studied at each time-period. The defect is filled with a porous poly(DL-lactic acid) implant containing zero, 17, 35, or 70 micrograms of rhBMP-2 with and without either zero, 20, 100, or 500 micrograms gremlin polypeptide (SEQ ID NO:2, residues 24-184) or 100 or 250 micrograms DAN polypeptide (SEQ ID NO:8, residues 12-181). Radiographs of the defects are made every two weeks. The percentage of the total area of the defect that is radiopaque is determined with use of computerized radiomorphometry, and this percentage used as a quantitative measure of the extent of new-bone formation in the defect. Time and dose-dependent responses to rhBMP-2 are found for as long as four weeks; thereafter, the effects of seventeen, thirty-five, and seventy micrograms of rhBMP-2 are independent of dose and time ($p < 0.05$). The defects treated with either thirty-five or seventy micrograms of rhBMP-2 have a significantly greater ($p < 0.05$) area of radiopacity than the defects treated with either zero or seventeen micrograms of rhBMP-2. No significant difference is found between the defects treated with thirty-five or seventy micrograms of rhBMP-2 and the defects filled with an autogenous graft. All defects concomitantly treated with all three doses of gremlin and both doses of DAN reveal significantly less ($p < 0.05$) area of radiopacity than the corresponding rhBMP-2 only treated groups. The inhibition of BMP-2 mediated bone formation is confirmed by histological and histomorphometric examinations.

[0079] The amino acid sequences of the disclosed DAN and gremlin polypeptides are used to back-translate gremlin polypeptide-encoding nucleic acids optimized for selected expression systems (Holler et al. (1993) *Gene* 136, 323-328; Martin et al. (1995) *Gene* 154, 150-166) or used to generate

degenerate oligonucleotide primers and probes for use in the isolation of natural gremlin-encoding nucleic acid sequences ("GCG" software, Genetics Computer Group, Inc, Madison Wis.). DAN and gremlin-encoding nucleic acids used in DAN and gremlin-expression vectors and incorporated into recombinant host cells, e.g. for expression and screening, transgenic animals, e.g. for functional studies such as the efficacy of candidate drugs for disease associated with gremlin-modulated cell function, etc.

[0080] The invention also provides nucleic acid hybridization probes and replication/amplification primers having a DAN or gremlin cDNA specific sequence comprising a strand of least one of SEQ ID NO:1 or 7, or at least 18, preferably at least 36, more preferably at least 72 contiguous nucleotides of SEQ ID NO: 1 or 7 and sufficient to specifically hybridize with a second nucleic acid comprising the complementary strand of the corresponding SEQ ID NO:1 or 7. Demonstrating specific hybridization generally requires stringent conditions, for example, hybridizing in a buffer comprising 30% formamide in 5 \times SSPE (0.18 M NaCl, 0.01 M NaPO_4 , pH 7.7, 0.001 M EDTA) buffer at a temperature of 42 $^\circ$ C. and remaining bound when subject to washing at 42 $^\circ$ C. with 0.2 \times SSPE; preferably hybridizing in a buffer comprising 50% formamide in 5 \times SSPE buffer at a temperature of 42 $^\circ$ C. and remaining bound when subject to washing at 42 $^\circ$ C. with 0.2 \times SSPE buffer at 42 $^\circ$ C.

TABLE 3

Exemplary gremlin nucleic acids which hybridize with a strand of SEQ ID NO: 1 under Conditions I and/or II.	
gremlin Nucleic Acids	Hybridization
SEQ ID NO: 1, nucleotides 1-36	+
SEQ ID NO: 1, nucleotides 68-98	+
SEQ ID NO: 1, nucleotides 95-130	+
SEQ ID NO: 1, nucleotides 175-220	+
SEQ ID NO: 1, nucleotides 261-299	+
SEQ ID NO: 1, nucleotides 274-310	+
SEQ ID NO: 1, nucleotides 331-369	+
SEQ ID NO: 1, nucleotides 430-470	+
SEQ ID NO: 1, nucleotides 584-616	+

[0081] The subject nucleic acids are of synthetic/non-natural sequences and/or are isolated, i.e. unaccompanied by at least some of the material with which it is associated in its natural state, preferably constituting at least about 0.5%, preferably at least about 5% by weight of total nucleic acid present in a given fraction, and usually recombinant, meaning they comprise a non-natural sequence or a natural sequence joined to nucleotide(s) other than that which it is joined to on a natural chromosome. Recombinant nucleic acids comprising the nucleotide sequence of SEQ ID NO:1, or requisite fragments thereof, contain such sequence or fragment at a terminus, immediately flanked by (i.e. contiguous with) a sequence other than that which it is joined to on a natural chromosome, or flanked by a native flanking region fewer than 10 kb, preferably fewer than 2 kb, which is at a terminus or is immediately flanked by a sequence other than that which it is joined to on a natural chromosome. While the nucleic acids are usually RNA or DNA, it is often advantageous to use nucleic acids comprising other bases or nucleotide analogs to provide modified stability, etc.

[0082] The subject nucleic acids find a wide variety of applications including use as translatable transcripts, hybridization probes, PCR primers, diagnostic nucleic acids, etc.; use in detecting the presence of gremlin genes and gene transcripts and in detecting or amplifying nucleic acids encoding additional gremlin homologs and structural analogs. In diagnosis, gremlin hybridization probes find use in identifying wild-type and mutant gremlin alleles in clinical and laboratory samples. Mutant alleles are used to generate allele-specific oligonucleotide (ASO) probes for high-throughput clinical diagnoses. DAN and gremlin nucleic acids are also used to modulate cellular expression or intracellular concentration or availability of active DAN or gremlin. DAN and gremlin inhibitory nucleic acids are typically antisense: single-stranded sequences comprising complements of the disclosed natural gremlin coding sequences. Antisense modulation of the expression of a given DAN or gremlin protein may employ antisense nucleic acids operably linked to gene regulatory sequences. Cells are transfected with a vector comprising a DAN or gremlin sequence with a promoter sequence oriented such that transcription of the gene yields an antisense transcript capable of binding to endogenous DAN/gremlin encoding mRNA. Transcription of the antisense nucleic acid may be constitutive or inducible and the vector may provide for stable extrachromosomal maintenance or integration. Alternatively, single-stranded antisense nucleic acids that bind to genomic DNA or mRNA encoding a given DAN or gremlin protein may be administered to the target cell, in or temporarily isolated from a host, at a concentration that results in a substantial reduction in expression of the targeted protein. An enhancement in DAN or gremlin expression is effected by introducing into the targeted cell type DAN or gremlin nucleic acids which increase the functional expression of the corresponding gene products. Such nucleic acids may be DAN or gremlin expression vectors, vectors which upregulate the functional expression of an endogenous allele, or replacement vectors for targeted correction of mutant alleles. Techniques for introducing the nucleic acids into viable cells are known in the art and include retroviral-based transfection, viral coat protein-liposome mediated transfection, etc.

[0083] The invention provides efficient methods of identifying agents, compounds or lead compounds for agents active at the level of a gremlin modulatable cellular function. Generally, these screening methods involve assaying for compounds which modulate gremlin interaction with a natural gremlin binding target. A wide variety of assays for binding agents are provided including labeled in vitro protein-protein binding assays, immunoassays, cell based assays, etc. The methods are amenable to automated, cost-effective high throughput screening of chemical libraries for lead compounds. Identified reagents find use in the pharmaceutical industries for animal and human trials; for example, the reagents may be derivatized and rescreened in in vitro and in vivo assays to optimize activity and minimize toxicity for pharmaceutical development.

[0084] In vitro binding assays employ a mixture of components including a DAN or gremlin protein, which may be part of a fusion product with another peptide or polypeptide, e.g. a tag for detection or anchoring, etc. The assay mixtures comprise a natural DAN/gremlin binding target, e.g. a TGF β protein such as a BMP. While native binding targets may be used, it is frequently preferred to use portions thereof so long

as the portion provides binding affinity and avidity to the subject DAN/gremlin conveniently measurable in the assay. The assay mixture also comprises a candidate pharmacological agent. Candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds and are obtained from a wide variety of sources including libraries of synthetic or natural compounds. A variety of other reagents such as salts, buffers, neutral proteins, e.g. albumin, detergents, protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. may also be included. The mixture components can be added in any order that provides for the requisite bindings and incubations may be performed at any temperature which facilitates optimal binding. The mixture is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the DAN or gremlin specifically binds the cellular binding target, portion or analog with a reference binding affinity. Incubation periods are chosen for optimal binding but also minimized to facilitate rapid, high-throughput screening.

[0085] After incubation, the agent-biased binding between the DAN or gremlin and one or more binding targets is detected by any convenient way. For cell-free binding type assays, a separation step is often used to separate bound from unbound components. Separation may be effected by precipitation, immobilization, etc., followed by washing by, for examples, membrane filtration, gel chromatography. For cell-free binding assays, one of the components usually comprises or is coupled to a label. The label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, an enzyme, etc. A variety of methods may be used to detect the label depending on the nature of the label and other assay components, e.g. through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc. A difference in the binding affinity of the DAN/gremlin protein to the target in the absence of the agent as compared with the binding affinity in the presence of the agent indicates that the agent modulates the binding of the DAN/gremlin protein to the corresponding binding target. A difference, as used herein, is statistically significant and preferably represents at least a 50%, more preferably at least a 90% difference.

[0086] The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

[0087] Expression Cloning of Gremlin. To identify new axial patterning activities in the *Xenopus* embryo, we screened for maternally-supplied activities whose ventral expression induced a secondary axis in otherwise normal embryos (Lemaire et al., 1995). 1 ng of synthetic mRNA from each pool of a *Xenopus* ovary cDNA library was injected into a ventral blastomere of a 4-cell embryo. Injected embryos were subsequently screened at the tailbud stage for presence of a secondary axis. Using this procedure, two pools with axis-inducing activity were identified. One pool contained Activin- β B (Thomsen et al., 1990), and was eliminated from further consideration. Fractionation of the second pool identified a single clone with potent activity. mRNA doses as low as 50 pg induced a secondary axis or hyperdorsalization in 93% of ventrally-injected embryos. In contrast to the complete secondary axes that can be induced

by activation of the Wnt signaling pathway, higher doses of this clone induced rostral structures such as eyes in fewer than 5% of injected embryos. The sequence of the 1.8 kb cDNA encodes a predicted protein that we have named Gremlin.

[0088] To characterize Gremlin's axis-inducing activity, we traced the fate of Gremlin-expressing cells in twinned embryos. 50 pg of Gremlin mRNA and 80 pg of mRNA encoding a β -Galactosidase lineage tracer were injected equatorially into ventral blastomeres of 4-cell embryos. At tailbud stage, embryos were fixed and stained with X-Gal. Not all the cells in the secondary axis were stained, indicating that Gremlin-expressing cells are able to recruit neighboring cells into the induced axis. While Gremlin induced secondary axes efficiently when injected into the marginal zone, it did not when injected into a ventral-vegetal blastomere. Thus, despite its maternal origin, Gremlin is not the vegetally-localized dorsalizing signal emanating from the Nieuwkoop center (Gimlich and Gerhart, 1984; Smith and Harland, 1991).

[0089] The composition and patterning of Gremlin-induced secondary axes was examined by immunostaining injected embryos with monoclonal antibodies that detect muscle, notochord, or neural tissue. We found that Gremlin-induced secondary axes frequently contain all three tissue types, and that each tissue is organized in a relatively normal fashion. Muscle staining is organized in regularly repeated somitic segments, while neural and notochord tissue stain in patterns similar to those found in the primary axis. Thus, the composition and pattern of Gremlin-induced axes resembles that of normal axes.

[0090] Gremlin is Expressed in the Neural Crest. We used in situ hybridization to determine Gremlin's temporal and spatial expression patterns (Lamb et al., 1993). Maternal Gremlin transcripts are present, but not localized in early-stage oocytes, and they are undetectable in mature oocytes and early stage embryos (not shown). Despite its axial patterning activities, Gremlin is not expressed during gastrulation. Instead, zygotic Gremlin expression begins at tailbud stages, where it is largely correlated with neural crest lineages. At stage 27, Gremlin staining appears in the pronephric duct, with additional faint staining in the trunk and tail bud. Staining in the pronephric duct may identify neural crest cells known to contribute to this structure (Collazo et al., 1993). In stage 30-40 embryos, Gremlin expression extends rostrally and caudally to include neural crest cells at all axial levels. In the head, staining is present in the mandibular segment of the neural crest (Sadaghiani and Thiebaud, 1987). Caudally, staining illuminates the dorsal migration of neural crest cells into the fin, as well as the chevron-shaped pathways of their ventral migration (Sadaghiani and Thiebaud, 1987; Krotoski et al., 1988; Collazo et al., 1993). In addition, expression continues in the pronephric duct as it extends caudally (Vize et al., 1995). These observations were confirmed by examination of transverse sections. Taken together, Gremlin's expression pattern indicates role in neural crest induction and patterning.

[0091] Gremlin Identifies a Family of Secreted Proteins With Axial Patterning Activity. The DNA sequence of the 1.8 kb Gremlin cDNA predicts a 182 amino acid protein with an N-terminal hydrophobic sequence, a site for N-linked glycosylation, and a C-terminal cysteine-rich

domain (**FIG. 1A**). For comparative studies, we identified chick, mouse, and human Gremlin homologs, and found that they share over 80% amino acid identity with *Xenopus* Gremlin (**FIG. 1A**). Recently, *drm*, a rat Gremlin homolog with over 80% similarity to the Gremlin family was identified as a cDNA downregulated in *v-mos* transformed cells (Topol et al., 1997).

[0092] BLAST searches revealed that Gremlin belongs to a new family of proteins that includes the previously-identified proteins, Cerberus and DAN (Topol et al., 1997)(**FIG. 1B**). Cerberus is a secreted factor expressed in the *Xenopus* gastrula that acts as a potent inducer of head structures (Bouwmeester et al., 1996; Belo et al., 1997). DAN encodes a tumor suppressor originally identified in rat as a downregulated transcript in transformed cells (Ozaki and Sakiyama, 1993). We also isolated a partial cDNA encoding a nematode Gremlin homolog that has been designated *C. elegans* Gremlin-Related -1 (*ceGR-1*). We name this family the DAN family for the first member to be reported. Sequence alignments show that each protein has a domain with the consensus sequence $CX_6QX_6CX_6NX_2CXGXCSX_3PX_{(8-13)}CX_2CXPX_8TLXCX_{(15-18)}CXC$ (**FIG. 1B**). Outside this domain, DAN-family members show little similarity.

[0093] The protein sequences of Gremlin, Cerberus, and DAN all contain a likely signal sequence. However, while Cerberus has been reported as a secreted protein, DAN was initially identified as a putative zinc-finger protein. To determine whether all DAN-family members are secreted, synthetic mRNA encoding Gremlin, DAN, or Cerberus was injected into oocytes that were then cultured in the presence of ^{35}S methionine. We found that supernatants of Gremlin and DAN-injected oocytes contained proteins of approximately 28 kd and 27 kd, respectively. Cerberus-injected oocytes secreted a heterogeneous product centered around 40 kd. Thus, all DAN-family members are secreted proteins. The predicted molecular weights for Gremlin (25 kd), DAN (19 kd), and Cerberus (31 kd) are lower than those observed, indicating that all three proteins undergo post-translational modification. Consistently, Gremlin, Cerberus, and DAN all have sites for N-linked glycosylation (Ozaki and Sakiyama, 1993; Bouwmeester et al., 1996).

[0094] DAN Has Dorsalizing Activity in *Xenopus* Embryos. While axial patterning activities have been shown for Gremlin and Cerberus, none have been shown for DAN. However, the similarity between the three proteins led us to test DAN for embryonic patterning activity. As a preliminary assay, we injected 100 pg synthetic DAN mRNA into the marginal zone of both ventral blastomeres of 4-cell embryos. At this dose, DAN-injected embryos were mildly hyperdorsalized (DAI 6-7) (Kao and Elinson, 1988) with slightly enlarged heads, bloated trunks, and a reduction of tail structures. Occasionally, injected embryos had a secondary axis (less than 10%). Immunostaining of DAN-injected embryos using the muscle specific antibody 12/101 reveals that muscle is present, but that it is poorly organized. *Xenopus* DAN had similar activity. DAN's activity in embryos and its ability to dorsalize ventral mesoderm and neuralize ectoderm (described below) establishes that all DAN-family members have axial patterning activity.

[0095] DAN-Family Members Act Like BMP Antagonists in Embryonic Explants. To further examine the biological

activities of DAN-family members, we tested their ability to pattern mesoderm using the ventral marginal zone (VMZ) assay (Smith et al., 1993). Consistent with their effects when expressed ventrally in embryos, expression of 100 pg DAN or Gremlin mRNA induced the dorsal mesodermal marker cardiac muscle actin (*M. actin*) in VMZ explants. As previously reported, the same dose of Cerberus mRNA failed to induce *M. actin* (Bouwmeester et al., 1996). Since Cerberus' failure to dorsalize the VMZ is probably due to its ability to provide an early block to mesoderm induction (described below), we repeated the VMZ assay with a Cerberus DNA construct that is not transcriptionally active until blastula stages. When its expression was delayed, Cerberus also induced *M. actin*. In *Xenopus*, an accumulated body of evidence has shown that dorsalization of mesoderm can result from blocking ventralizing signals provided by members of the BMP family (Reviewed in Harland and Gerhart, 1997). In light of these observations, the dorsalizing activity of Cerberus, DAN, and Gremlin indicates that they act by antagonizing BMP signals.

[0096] We also tested the activities of DAN-family members in animal cap explants. These experiments show that Gremlin, Cerberus, and DAN induce neural tissue from animal cap ectoderm. Injection of 100 pg Gremlin, 1 ng Cerberus, or 10 ng DAN into the animal pole of a 1-cell embryo induced neural differentiation in explants, as evidenced by expression of the pan-neural marker NCAM (Kintner and Melton, 1987; Bouwmeester et al., 1996). The absence of *M. actin* induction indicates that neuralization occurred directly, without a mesodermal intermediary.

[0097] In *Xenopus*, organizer-specific neuralizing signals act by antagonizing signaling by BMPs that normally cause ectoderm to adopt an epidermal fate (Reviewed in Harland and Gerhart, 1997; Sasai and De Robertis, 1997). To test whether DAN-family members neuralize through a similar mechanism, we co-expressed each one with an activated form of a type I BMP-4 receptor. ALK-3(Q233D) contains a Q to D mutation at position 233 that activates the receptor kinase, leading to constitutive signaling in the BMP signal transduction pathway (Wieser et al., 1995; Holley et al., 1996). We found that expression of ALK-3(Q233D) blocks neural induction by Gremlin, Cerberus, or DAN, indicating that they block BMP signaling upstream of the receptor.

[0098] Cerberus expression in animal cap explants also induced modest levels of the pan-endodermal marker endodermis (Edd), and the cardiac marker Nkx 2.5 (Bouwmeester et al., 1996). We find that Gremlin and DAN share these activities. 100 pg of Cerberus, Gremlin, or DAN mRNA induced Edd and Nkx 2.5 at low to moderate levels. We also found that induction of these markers occurs in the presence or absence of ALK-3(Q233D), indicating that induction of these genes is normally not suppressed by BMP signaling through ALK-3. These findings indicate that in addition to BMP antagonizing activities, DAN-family members can block the activities of additional TGF- β signals.

[0099] Gremlin Blocks the Activity of Purified BMP-2. Gremlin's dorsalizing and neuralizing activities provide a strong indication that it acts by antagonizing signaling by members of the BMP family. As a more direct test of Gremlin's activity, we examined its ability to antagonize the activity of purified BMP-2 in a cytokine assay. The murine bone marrow stromal cell line W-20-17 provides a direct,

quantitative bioassay for BMP activity by induction of alkaline phosphatase in response to BMP treatment (Thies et al., 1992). Preincubation of purified BMP-2 with a Gremlin COS supernatant at a final concentration of ~83 nM Gremlin completely blocked BMP-2 activity at doses from 78 pM to 5 nM. At ~21 nM Gremlin, BMP-2 activity was reduced, but not eliminated. In contrast, a mock-transfected COS supernatant had no effect. Similar results were obtained with BMP-4. Thus, like Noggin and Chordin, Gremlin blocks BMP activity in multiple assays.

[0100] DAN-Family Members Bind BMP-2. Biochemical studies of the BMP antagonists Noggin and Chordin have shown that they bind BMP-4 with high affinity, preventing it from interacting with its cell surface receptors (Piccolo et al., 1996; Zimmerman et al., 1996). To test whether DAN-family members antagonize BMP signaling through a similar mechanism, we examined their ability to associate with BMP-2, which has activities virtually indistinguishable from those of BMP-4. Gremlin, DAN, or Cerberus COS-conditioned supernatants were incubated with anti BMP-2 protein G sepharose beads in the presence or absence of 1 μ g BMP-2. After binding, beads were processed and analyzed by Western blot. We found that Cerberus, DAN, and Gremlin are precipitated by anti-BMP-2 beads in a BMP-2-dependent fashion. Reciprocal binding experiments confirm that DAN-family members associate with BMP-2. When Cerberus was precipitated using an anti-Cer polyclonal antibody, BMP-2 coprecipitated. A similar result was obtained when DAN or Gremlin were precipitated with a monoclonal antibody directed against their C-terminal MYC tags. Taken together, these results demonstrate a direct physical interaction between DAN-family members and BMP-2.

[0101] To further characterize the interaction between BMPs and their antagonists, we determined whether Noggin could compete with each family member for binding to BMP-2. We found that the addition of 2 or 10 μ g purified Noggin blocks the interaction between Cerberus, Gremlin, or DAN and BMP-2. Noggin's ability to block the association between DAN-family members and BMP-2 adds to the evidence that these interactions are specific, and further indicates that DAN-family members and Noggin all bind a similar domain on BMP-2.

[0102] Cerberus Blocks Signaling by Activin and Xnr-2. The sequence divergence within the DAN family is reminiscent of the divergence within the TGF- β family, and suggested that individual DAN-family members might antagonize different subsets of TGF- β ligands. Since radial expression of Cerberus suppresses formation of trunk-tail mesoderm (Bouwmeester et al., 1996), we tested the possibility that it might block the action of mesoderm-inducing TGF- β ligands like Activin, B Vg-1, and Xnr-2 (Smith et al., 1990; Thomsen et al., 1990; Thomsen and Melton, 1993; Jones et al., 1995). Animal caps were isolated from embryos coinjected with Activin (1 pg), B Vg-1 (100 pg), or Xnr-2 (100 pg) mRNA, and 1 ng of Gremlin, Cerberus, or DAN mRNA, and were then analyzed for induction of the pan-mesodermal marker Xbra (Smith et al., 1991). We found that Cerberus expression significantly reduced or completely eliminated Xbra induction by Activin or Xnr-2. In contrast, neither Gremlin nor DAN blocked Xbra induction despite their doses being well above those required for biological activity in other assays. The lack of mesoderm induction in

Cerberus-injected caps is not due to their inability to become mesodermalized since Xbra was induced in explants coinjected with Smad-2(C), a Smad-2- β -Galactosidase fusion protein that induces mesoderm in a ligand-independent fashion (Baker and Harland, 1996). Thus, unlike other DAN-family members, Cerberus antagonizes the activity of both BMPs and Activin-like ligands.

[0103] The Gremlin cDNA encodes a secreted protein whose activities are similar to those of the previously-identified BMP antagonists Noggin, Chordin and Follistatin (Reviewed in Harland and Gerhart, 1997; Sasai and De Robertis, 1997). Like these factors, Gremlin antagonizes BMP activity in multiple assays, and it is able to physically associate with BMP-2. Despite these functional similarities, Gremlin has no sequence similarity to previously-identified BMP antagonists, and it is not expressed in the organizer. Instead, zygotic Gremlin transcripts are largely limited to the neural crest and its derivatives. This expression pattern shows that organizer-like activities are not restricted to the gastrulating embryo, and indicates that these inductive signals are used broadly in development.

[0104] Gremlin's acts as an essential modulator of BMP signaling in neural crest cells, preventing premature or excessive exposure to BMP signals during their birth and subsequent migration. Consistently, BMP-2, BMP-4, and Gremlin are expressed at similar times and places during embryonic development. At tailbud stages, BMP-2 and BMP-4 are expressed in a coincident pattern in the fin neural crest (Clement et al., 1995; Hemmati-Brivanlou and Thomsen, 1995; Schmidt et al., 1995). BMP-4 is also expressed in the roof plate of the spinal cord (Fainsod et al., 1994). By modulating these signals, Gremlin has an important role in determining the timing of neural crest cell differentiation and/or the choice of differentiated fates.

[0105] While members of the DAN family have no sequence similarity to Noggin or Chordin, their activities indicate that they antagonize BMP signaling using the same mechanism. Support for this model comes from the finding that Gremlin, Cerberus and DAN all bind BMP-2. Furthermore, Noggin effectively competes with DAN-family members for binding to BMP-2, indicating that the interaction between a BMP and its antagonists occurs through a similar BMP domain. Thus, despite the lack of sequence similarity, Noggin, Gremlin, and by extension, all BMP antagonists, share structural similarities that allow them to interact with similar domains on their cognate BMPs.

[0106] The scope of TGF- β antagonizing activities of the DAN family is broadened by the finding that Cerberus also blocks signaling by Activin and Nodal-like ligands. In their original study, Bouwmeester et al. (1996) found that radial expression of Cerberus blocked formation of trunk/tail mesoderm and prechordal plate mesoderm. We extended this finding by showing that among DAN-family members, only Cerberus antagonizes the mesoderm-inducing activity of Xnr-2 and Activin. Thus, despite their structural similarities, DAN-family members have specificity for different TGF- β signals. We conclude that DAN-family members act by binding overlapping subsets of TGF- β ligands, preventing them from interacting with their cell surface receptors. The diverse activities of DAN-family members result from their ability to antagonize specific TGF- β signaling pathways required for embryonic inductions and cell proliferation.

The different mRNA doses required for Gremlin, Cerberus, and DAN to neutralize animal cap ectoderm is consistent with the idea that they differ in their affinity for BMP-4. In addition, we have shown in additional binding studies that Gremlin and DAN have substantially different affinities for BMP-2.

[0107] Embryo Manipulations and RT-PCR. *Xenopus* embryos were generated and staged as described (Nieuwkoop and Faber, 1967; Condie and Harland, 1987). Ventral marginal zone (VMZ) assays were done by injecting the marginal zone of each blastomere of 4-cell embryos and then explanting, culturing and harvesting VMZs as described (Zimmerman et al., 1996). For animal cap assays, 1-cell embryos were injected in the animal pole. Animal caps were explanted at stage 8-9, cultured in 75% NAM (Peng, 1991) and harvested at stage 10.5-11, stage 22, or stage 27.

[0108] RT-PCR analysis was done as described (Wilson and Melton, 1994). Primer sets and PCR conditions for Xbra, Efl- α , Muscle actin, and NCAM are described in Wilson and Melton (1994), and those for Edd, and Nkx 2.5 are described in Bouwmeester et al. (1996).

[0109] Construction of a *Xenopus* Ovary cDNA Library. Poly(A)+RNA was isolated from *Xenopus* ovaries by oligo(dT) cellulose selection from a proteinase K/SDS lysate (Badley et al., 1988). This mRNA was used to synthesize a cDNA library containing ~650,000 independent clones using the SuperScript cDNA cloning system (Life Technologies). To maximize expression of inserts, we established the library in CS105, a derivative of CS2+ (Turner and Weintraub, 1994) that produces synthetic mRNAs with high activity. cDNA pools were made from 121 plates containing ~1000 colonies each.

[0110] Expression Cloning. To make transcription templates, cDNA pools were digested with Asc-I, followed by digestion with 0.2 mg/ml proteinase K, 0.5% SDS at 50° for 1 hr, phenol/chloroform extraction, and precipitation. Synthetic mRNA was synthesized with the mMessage machine SP6 kit (Ambion). 1 ng of each mRNA pool was injected into the marginal zone of one ventral blastomere of a 4-cell embryo. Tadpole-stage embryos were scored for presence of a secondary axis. The Gremlin cDNA was isolated from an active pool by sib-selection (Smith and Harland, 1991).

[0111] In Situ Hybridization, Immunostaining, and Histology. Whole-mount in situ hybridization used standard methods (Lamb et al., 1993). Sense and antisense probes were generated from the full length Gremlin cDNA. For histological analysis, stained embryos were embedded in paraffin, and 10-20 μ m sections were cut.

[0112] The presence of neural tissue, muscle, and notochord in embryos was determined by immunostaining with the antibodies 6F11 (Lamb et al., 1993), 12/101 (Kintner and Brockes, 1984), and Tor70 (Bolce et al., 1992), respectively.

[0113] Identification of Gremlin Homologs. A chick Gremlin cDNA was isolated by probing a stage 12-15 chick embryonic cDNA library, with the *Xenopus* Gremlin cDNA under moderately stringent hybridization conditions (0.5 M Na₂PO₄ pH 7.2, 15% Formamide, 7% SDS, 1 mM EDTA, and 1% BSA, at 42°). A mouse genomic Gremlin clone was isolated from a 129SVJ genomic DNA library (Stratagene) in a similar fashion. A human Gremlin cDNA was identified

in a BLAST search of the GenBank EST database (I.M.A.G.E. consortium clone ID 272074, GenBank accession N35377) (Altschul et al., 1990; Lennon et al., 1996).

[0114] The mouse DAN clone was obtained as an EST (GenBank accession number AA008891). *Xenopus* Cerberus and DAN cDNAs were isolated from a stage 11 LiCl-treated gastrula library. ceGR-1 was identified in a BLAST search of *C. elegans* genomic DNA sequences, and a ceGR-1 cDNA fragment was then cloned from a him-8(e1489) embryonic cDNA library. The identity of all clones used in these experiments was confirmed by DNA sequence analysis.

[0115] Preparation of Secreted Proteins in *Xenopus* Oocytes. Oocytes were isolated from ovaries by treatment with 0.2% collagenase (Boehringer Mannheim), or by manual defolliculation. They were injected with 50 ng synthetic mRNA and cultured at 20° in groups of 5 in 100 μ L OR2 (Peng, 1991) supplemented with 0.5 mg/ml BSA, 100 μ g/ml penicillin, 100 μ g/ml streptomycin sulfate, and 30 μ Ci 35 S-methionine. After 1 day, 10 μ L of each supernatant was analyzed by SDS-PAGE followed by fluorography.

[0116] Production of DAN-family COS Cell Supernatants. Conditioned COS supernatants were made by transfecting COS7 cells (3.5×10^6 /150 mm plate initial seeding density) with plasmids encoding *Xenopus* or human Gremlin, mouse DAN, or human Cerberus using Lipofectamine (Life Technologies). Control media was made by transfecting the expression vector pMT21. For detection in Western blots, Gremlins and mDAN were fused to a C-terminal triple-MYC epitope tag. human Cerberus was fused to a C-terminal FLAG epitope tag. xGremlin supernatants were quantified by comparison with known amounts of bacterially-expressed His-tagged Gremlin purified on a Ni-NTA-agarose column (Qiagen). In embryo assays, hCerberus-FLAG mRNA has comparable activities to *Xenopus* Cerberus mRNA. xGremlin-MYC₃ mRNA behaves like its wild-type counterpart.

[0117] Immunoprecipitation and Immunoblotting. Initially, 250-1000 μ L of a hCerberus-FLAG, mDAN-MYC₃, or xGremlin-MYC₃ COS supernatant was incubated with 1 μ g BMP-2 in the presence of protein G sepharose beads bound to the BMP-2,4-specific monoclonal antibody h3b2 in 1 ml of binding buffer (25 mM HEPES pH 7.7, 0.2 M KCl, 12.5 mM MgCl₂, 0.1 mM EDTA, 0.1% BSA, 0.1% NP40, 0.2 mM PMSF) overnight at 4°. Beads were washed twice in binding buffer, boiled in loading dye, and analyzed by Western blot. Tagged proteins were detected using monoclonal antibodies directed against the FLAG epitope (M2, Kodak), or against the MYC epitope (9E10), and a peroxidase-conjugated secondary antibody (Pierce). Competition experiments were done by adding 2 or 10 μ g purified *Xenopus* Noggin to the binding reaction.

[0118] In reciprocal binding experiments, 1 μ g hCerberus or hGremlin-MYC₃, or 1 ml of a mDAN-MYC₃ COS supernatant was incubated with 0.5-1 μ g BMP-2 in the presence of 0.1% Tween-20, 1 M NaCl, or 0.1% NP-40+0.2 M KCl, respectively. hCerberus was immunoprecipitated using protein G sepharose beads bound to anti-hCerberus polyclonal antibodies. hGremlin-MYC₃ and mDAN-MYC₃ were immunoprecipitated using 9E10 beads. Competition experiments were done by adding 2 or 10 μ g human Noggin. The presence of coprecipitating BMP-2 was assessed on Western blots using a polyclonal antibody directed against BMP-2.

[0119] Protocol for high throughput human gremlin-BMP binding assay.

[0120] A. Reagents:

[0121] Neutralite Avidin: 20 μ g/ml in PBS.

[0122] Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hour at room temperature.

[0123] Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 1 mM MgCl₂, 1% glycerol, 0.5% NP-40, 50 mM β -mercaptoethanol, 1 mg/ml BSA, cocktail of protease inhibitors.

[0124] 33 P human gremlin protein 10 \times stock: 10^{-8} – 10^{-6} M “cold” human gremlin supplemented with 200,000-250,000 cpm of labeled human gremlin (Beckman counter). Place in the 4° C. microfridge during screening.

[0125] Protease inhibitor cocktail (1000 \times): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2 mM NaVO₃ (Sigma # S-6508) in 10 ml of PBS.

[0126] BMP: 10^{-7} – 10^{-4} M biotinylated BMP in PBS.

[0127] B. Preparation of assay plates:

[0128] Coat with 120 μ L of stock N-Avidin per well overnight at 4° C.

[0129] Wash 2 times with 200 μ L PBS.

[0130] Block with 150 μ L of blocking buffer.

[0131] Wash 2 times with 200 μ L PBS.

[0132] C. Assay:

[0133] Add 40 μ L assay buffer/well.

[0134] Add 10 μ L compound or extract.

[0135] Add 10 μ L 33 P gremlin protein (20-25,000 cpm/0.1-10 pmoles/well= 10^{-9} – 10^{-7} M final concn).

[0136] Shake at 25° C. for 15 minutes.

[0137] Incubate additional 45 minutes at 25° C.

[0138] Add 40 μ L biotinylated BMP (0.1-10 pmoles/40 μ L in assay buffer)

[0139] Incubate 1 hour at room temperature.

[0140] Stop the reaction by washing 4 times with 200 μ L PBS.

[0141] Add 150 μ L scintillation cocktail.

[0142] Count in Topcount.

[0143] D. Controls for all assays (located on each plate):

[0144] a. Non-specific binding

[0145] b. Soluble (non-biotinylated gremlin) at 80% inhibition.

[0146] All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 13

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 619 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 47..598

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```

CCACGCGTCG AAAGCGCAGG CCCCAGGAC CCGCCGCACT GACAGT ATG AGC CGC      55
                               Met Ser Arg
                               1

ACA GCC TAC ACG GTG GGA GCC CTG CTT CTC CTC TTG GGG ACC CTG CTG      103
Thr Ala Tyr Thr Val Gly Ala Leu Leu Leu Leu Gly Thr Leu Leu
   5                10                15

CCG GCT GCT GAA GGG AAA AAG AAA GGG TCC CAA GGT GCC ATC CCC CCG      151
Pro Ala Ala Glu Gly Lys Lys Lys Gly Ser Gln Gly Ala Ile Pro Pro
  20                25                30                35

CCA GAC AAG GCC CAG CAC AAT GAC TCA GAG CAG ACT CAG TCG CCC CAG      199
Pro Asp Lys Ala Gln His Asn Asp Ser Glu Gln Thr Gln Ser Pro Gln
                40                45                50

CAG CCT GGC TCC AGG AAC CGG GGG CGG GGC CAA GGG CGG GGC ACT GCC      247
Gln Pro Gly Ser Arg Asn Arg Gly Arg Gly Gln Gly Arg Gly Thr Ala
   55                60                65

ATG CCC GGG GAG GAG GTG CTG GAG TCC AGC CAA GAG GCC CTG CAT GTG      295
Met Pro Gly Glu Glu Val Leu Glu Ser Ser Gln Glu Ala Leu His Val
   70                75                80

ACG GAG CGC AAA TAC CTG AAG CGA GAC TGG TGC AAA ACC CAG CCG CTT      343
Thr Glu Arg Lys Tyr Leu Lys Arg Asp Trp Cys Lys Thr Gln Pro Leu
   85                90                95

AAG CAG ACC ATC CAC GAG GAA GGC TGC AAC AGT CGC ACC ATC ATC AAC      391
Lys Gln Thr Ile His Glu Glu Gly Cys Asn Ser Arg Thr Ile Ile Asn
  100                105                110                115

CGC TTC TGT TAC GGC CAG TGC AAC TCT TTC TAC ATC CCC AGG CAC ATC      439
Arg Phe Cys Tyr Gly Gln Cys Asn Ser Phe Tyr Ile Pro Arg His Ile
                120                125                130

CGG AAG GAG GAA GGT TCC TTT CAG TCC TGC TCC TTC TGC AAG CCC AAG      487
Arg Lys Glu Glu Gly Ser Phe Gln Ser Cys Ser Phe Cys Lys Pro Lys
  135                140                145

AAA TTC ACT ACC ATG ATG GTC ACA CTC AAC TGC CCT GAA CTA CAG CCA      535
Lys Phe Thr Thr Met Met Val Thr Leu Asn Cys Pro Glu Leu Gln Pro
  150                155                160

CCT ACC AAG AAG AAG AGA GTC ACA CGT GTG AAG CAG TGT CGT TGC ATA      583
Pro Thr Lys Lys Lys Arg Val Thr Arg Val Lys Gln Cys Arg Cys Ile
  165                170                175

TCC ATC GAT TTG GAT TAAGCCAAAT CCATGCACCA G      619
Ser Ile Asp Leu Asp
180

```

-continued

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 184 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Ser Arg Thr Ala Tyr Thr Val Gly Ala Leu Leu Leu Leu Gly
1 5 10 15
Thr Leu Leu Pro Ala Ala Glu Gly Lys Lys Lys Gly Ser Gln Gly Ala
20 25 30
Ile Pro Pro Pro Asp Lys Ala Gln His Asn Asp Ser Glu Gln Thr Gln
35 40 45
Ser Pro Gln Gln Pro Gly Ser Arg Asn Arg Gly Arg Gly Gln Gly Arg
50 55 60
Gly Thr Ala Met Pro Gly Glu Glu Val Leu Glu Ser Ser Gln Glu Ala
65 70 75 80
Leu His Val Thr Glu Arg Lys Tyr Leu Lys Arg Asp Trp Cys Lys Thr
85 90 95
Gln Pro Leu Lys Gln Thr Ile His Glu Glu Gly Cys Asn Ser Arg Thr
100 105 110
Ile Ile Asn Arg Phe Cys Tyr Gly Gln Cys Asn Ser Phe Tyr Ile Pro
115 120 125
Arg His Ile Arg Lys Glu Glu Gly Ser Phe Gln Ser Cys Ser Phe Cys
130 135 140
Lys Pro Lys Lys Phe Thr Thr Met Met Val Thr Leu Asn Cys Pro Glu
145 150 155 160
Leu Gln Pro Pro Thr Lys Lys Lys Arg Val Thr Arg Val Lys Gln Cys
165 170 175
Arg Cys Ile Ser Ile Asp Leu Asp
180

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1781 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 66..611

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCCGTGTAGC AGCCTCACAT GGACTAGTCT TGATCTGCTG TGAGCCAGCG GCCCCTTCCC 60
ACAGG ATG AAC TGT CTC GTT TAT GCT CTA GGA TCC CTT TTC CTT CTG 107
Met Asn Cys Leu Val Tyr Ala Leu Gly Ser Leu Phe Leu Leu
185 190 195
AGT GGG CTC CTC CTC CCC AGC TCC GAA GGG AAG AAG GTT AGT GGA 155
Ser Gly Leu Leu Leu Pro Ser Ser Glu Gly Lys Lys Lys Val Ser Gly
200 205 210
TCA CAG GGA GCC ATT CCT CCC CCT GAC AAA GGG CAG CCC AAT GAC TCT 203
Ser Gln Gly Ala Ile Pro Pro Pro Asp Lys Gly Gln Pro Asn Asp Ser

-continued

215	220	225	230	
GAG CAA GGT CAA GCT CAG CCA GGA GAC CGG GTC AGA GGG AAG GGG AAG				251
Glu Gln Gly Gln Ala Gln Pro Gly Asp Arg Val Arg Gly Lys Gly Lys				
	235	240	245	
GGA CAA GCA CTA GCG GCT GAA GAA GTG CTG GAG TCC AGT CAA GAA GCT				299
Gly Gln Ala Leu Ala Ala Glu Glu Val Leu Glu Ser Ser Gln Glu Ala				
	250	255	260	
CTA CAC GTC ACT GAA CGC AAG TAT CTA AAG AGG GAC TGG TGT AAG ACT				347
Leu His Val Thr Glu Arg Lys Tyr Leu Lys Arg Asp Trp Cys Lys Thr				
	265	270	275	
CAG CCC TTA AAG CAA ACA ATT CAC GAG GAC GGG TGC AAC AGT CGT ACC				395
Gln Pro Leu Lys Gln Thr Ile His Glu Asp Gly Cys Asn Ser Arg Thr				
	280	285	290	
ATC ATC AAC CGC TTC TGC TAT GGG CAA TGC AAC TCC TTC TAC ATC CCC				443
Ile Ile Asn Arg Phe Cys Tyr Gly Gln Cys Asn Ser Phe Tyr Ile Pro				
	295	300	305	310
CGT CAC ATA CGC AGG GAG GAG GGC TCA TTT CAA TCC TGC TCC TTC TGC				491
Arg His Ile Arg Arg Glu Glu Gly Ser Phe Gln Ser Cys Ser Phe Cys				
	315	320	325	
AAG CCT AAA AAA TTC ACC ACA ATG GTG GTC ACC CTG AAC TGC CCA GAG				539
Lys Pro Lys Lys Phe Thr Thr Met Val Val Thr Leu Asn Cys Pro Glu				
	330	335	340	
CTA CAA CCT CCC ACA AAG AAG AAG AGA ATT ACA CGT GTC AAG CAG TGT				587
Leu Gln Pro Pro Thr Lys Lys Lys Arg Ile Thr Arg Val Lys Gln Cys				
	345	350	355	
CGC TGC ATC TCC ATA GAC CTG GAC TAATGTGCCG CTGGGCCTGT GAATACTAGA				641
Arg Cys Ile Ser Ile Asp Leu Asp				
	360	365		
ATAATGTACT GGGAGCTTCA CTCTTCATAT CCATACCATT ACTGTCTGTG CCAAGACCAT				701
CAAACCACAA TCCTCACAGG CATTCTGTTG TGTCCGAGCT ACTTTATGCT TTTCATGCTA				761
ATACACCATC AGCAGGAATC CAAACAAGCA ATAAATATCT AGATTTCCCTC CTTCTGCTGA				821
ACTGTCCCGA ACAGCGTTGG GTTCTTTATC CTTTTTTTAA GGAATGTGG AGGCCTGCAA				881
AGACGGCATC CATGTATACA TTGTGTACAT ATATATTATT TATTCACAG TGTGTTTTTG				941
TGCACATAAA AGAGCATGAG AAAAGGCGCT GAGACTGATG AGAGTTAATG AATTGTGCAT				1001
GTTACTGCGA TGTAAGTGTG AGTCGCCACT ACAGGAGACC CAATATTAAG GCAATCTATG				1061
ACTTTCTGTA AGGTTCTCTG TGGGTAAACA TGCATTATCC ACAATACATT TTCTTATATG				1121
TGGGTTAATC TGCCACAAAC CTGGCAAATA TGGCAGTTTG AGGACAGAAA ATGCCATGTG				1181
AAGCATGTAC AGTCTAATCA TCGCAATATA TTTTAAATA CCATTGTGGC TGATTGGGG				1241
GGTAAAGAGC AACACAATGC AGGGAAGTTC CCAGGAGTTG GGGAGACTAA TGGCATATTT				1301
CTCATGTATT GAAGACTGCC GGGAGAGAGA GAAGCAAAT GGTGCCAAAT GGCAGTGGAA				1361
AAAGAACGAG GAGGATGAGA TATTGAGGCA TTTTACTAGG GACTAGATTA CTGCAATTGA				1421
AAGTTGCTCT GACTCCTCTT ATTCACATTT GGGACTGTAG CAACAACAAC TCTGTGTCAT				1481
TTTATTGAGG TCAGGGGTGG GAAACAAATC ACTGCATGGC TGGTTATGTC CAGACAGAAC				1541
CACCCCAAAAC CCCTCTCGCT TCCCAGAAAG TCCCACAAT GCAATCTGAG ACTGTCCAG				1601
CAAAACAGGA CAATCTAGGG AAAATGCCAT TGCAATTGTA GTGTTGCACG ATAAACTGTT				1661
CCCTAAAAAA AAAATTATTT GTATTAAACA AATTAGCGCA GAAAGTTTGC CAATAATATG				1721
AGCTTCATGA TAAATAAAGA AACAGTACAA GATGGAAATA TTAACAAAAA AAAAAAATA				1781

-continued

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 182 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```

Met Asn Cys Leu Val Tyr Ala Leu Gly Ser Leu Phe Leu Leu Ser Gly
 1           5           10           15

Leu Leu Leu Pro Ser Ser Glu Gly Lys Lys Lys Val Ser Gly Ser Gln
          20           25           30

Gly Ala Ile Pro Pro Pro Asp Lys Gly Gln Pro Asn Asp Ser Glu Gln
          35           40           45

Gly Gln Ala Gln Pro Gly Asp Arg Val Arg Gly Lys Gly Lys Gly Gln
 50           55           60

Ala Leu Ala Ala Glu Glu Val Leu Glu Ser Ser Gln Glu Ala Leu His
 65           70           75           80

Val Thr Glu Arg Lys Tyr Leu Lys Arg Asp Trp Cys Lys Thr Gln Pro
          85           90           95

Leu Lys Gln Thr Ile His Glu Asp Gly Cys Asn Ser Arg Thr Ile Ile
          100          105          110

Asn Arg Phe Cys Tyr Gly Gln Cys Asn Ser Phe Tyr Ile Pro Arg His
          115          120          125

Ile Arg Arg Glu Glu Gly Ser Phe Gln Ser Cys Ser Phe Cys Lys Pro
          130          135          140

Lys Lys Phe Thr Thr Met Val Val Thr Leu Asn Cys Pro Glu Leu Gln
          145          150          155          160

Pro Pro Thr Lys Lys Lys Arg Ile Thr Arg Val Lys Gln Cys Arg Cys
          165          170          175

Ile Ser Ile Asp Leu Asp
          180

```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 811 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 104..655

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```

GAATTCGAGG ATCCGGGTAC CATGGCAGCG CTCAGCCCCG CCGCGCCCCG CCGAGGAGCC      60

GCCGCCGAGC CGGGCCCCCG AGGAGCAGCC GACGCCGCGC AGG ATG GTC CGC ACA      115
                      Met Val Arg Thr
                      185

CTG TAT GCC ATC GGC GCT GTG TTT CTT CTG ACG GGA TTT CTG CTA CCG      163
Leu Tyr Ala Ile Gly Ala Val Phe Leu Leu Thr Gly Phe Leu Leu Pro
          190           195           200

ACA GCA GAA GGG AGA AAG AGG AAT CGT GGA TCT CAA GGC GCT ATC CCT      211

```


-continued

Thr	Ala	Glu	Gly	Arg	Lys	Arg	Asn	Arg	Gly	Ser	Gln	Gly	Ala	Ile	Pro	
	205						210					215				
CCT	CCT	GAC	AAG	GAT	CAG	CCC	AAT	GAT	TCA	GAG	CAG	ATG	CAG	ACA	CAG	259
Pro	Pro	Asp	Lys	Asp	Gln	Pro	Asn	Asp	Ser	Glu	Gln	Met	Gln	Thr	Gln	
	220					225					230					
CAG	CAG	TCA	GGC	TCC	AGG	CAT	CGA	GAA	AGA	GGA	AAA	GGC	ACC	TCA	ATG	307
Gln	Gln	Ser	Gly	Ser	Arg	His	Arg	Glu	Arg	Gly	Lys	Gly	Thr	Ser	Met	
235					240					245					250	
CCT	GCT	GAG	GAG	GTG	CTG	GAG	TCC	AGC	CAG	GAG	GCA	CTG	CAC	ATC	ACT	355
Pro	Ala	Glu	Glu	Val	Leu	Glu	Ser	Ser	Gln	Glu	Ala	Leu	His	Ile	Thr	
				255					260					265		
GAG	CGC	AAA	TAC	CTA	AAA	CGG	GAT	TGG	TGC	AAA	ACT	CAG	CCC	CTC	AAA	403
Glu	Arg	Lys	Tyr	Leu	Lys	Arg	Asp	Trp	Cys	Lys	Thr	Gln	Pro	Leu	Lys	
		270						275					280			
CAA	ACT	ATC	CAT	GAA	GAA	GGC	TGC	AAC	AGT	CGC	ACC	ATT	ATC	AAC	AGG	451
Gln	Thr	Ile	His	Glu	Glu	Gly	Cys	Asn	Ser	Arg	Thr	Ile	Ile	Asn	Arg	
	285					290						295				
TTC	TGC	TAT	GGC	CAA	TGC	AAT	TCT	TTC	TAC	ATC	CCC	AGG	CAT	GTC	CGC	499
Phe	Cys	Tyr	Gly	Gln	Cys	Asn	Ser	Phe	Tyr	Ile	Pro	Arg	His	Val	Arg	
	300				305						310					
AAA	GAG	GAA	GGC	TCT	TTC	CAG	TCT	TGT	TCC	TTC	TGC	AAG	CCC	AAG	AAA	547
Lys	Glu	Glu	Gly	Ser	Phe	Gln	Ser	Cys	Ser	Phe	Cys	Lys	Pro	Lys	Lys	
315					320				325						330	
TTT	ACC	ACC	ATG	ACT	GTT	ACC	CTC	AAC	TGC	CCT	GAG	CTT	CAG	CCC	CCT	595
Phe	Thr	Thr	Met	Thr	Val	Thr	Leu	Asn	Cys	Pro	Glu	Leu	Gln	Pro	Pro	
			335						340				345			
AGG	AAG	AAG	AAA	AGA	ATC	ACC	CGC	GTG	AAG	GAG	TGT	CGG	TGT	ATA	TCT	643
Arg	Lys	Lys	Lys	Arg	Ile	Thr	Arg	Val	Lys	Glu	Cys	Arg	Cys	Ile	Ser	
		350					355					360				
ATC	GAC	TTG	GAC	TAAGCCGAGA	AGTGCATTGT	TCTGATTGCA	CTCTGACTGT									695
Ile	Asp	Leu	Asp													
	365															
CTGCAATCAT	TGCCAGTGTG	AGGGAGTGGC	TTGAACTAGC	AAAGCTTTGG	GGCTGGATG											755
AACTGAAAGG	AACCCAGTGA	ATCACCTAAA	AAACTACAGC	TAAGCTAAAA	AAAAAA											811

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 184 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met	Val	Arg	Thr	Leu	Tyr	Ala	Ile	Gly	Ala	Val	Phe	Leu	Leu	Thr	Gly	
1				5				10						15		
Phe	Leu	Leu	Pro	Thr	Ala	Glu	Gly	Arg	Lys	Arg	Asn	Arg	Gly	Ser	Gln	
			20					25					30			
Gly	Ala	Ile	Pro	Pro	Pro	Asp	Lys	Asp	Gln	Pro	Asn	Asp	Ser	Glu	Gln	
	35						40					45				
Met	Gln	Thr	Gln	Gln	Gln	Ser	Gly	Ser	Arg	His	Arg	Glu	Arg	Gly	Lys	
	50					55					60					
Gly	Thr	Ser	Met	Pro	Ala	Glu	Glu	Val	Leu	Glu	Ser	Ser	Gln	Glu	Ala	
	65				70				75					80		
Leu	His	Ile	Thr	Glu	Arg	Lys	Tyr	Leu	Lys	Arg	Asp	Trp	Cys	Lys	Thr	
			85					90						95		

-continued

Gln Pro Leu Lys Gln Thr Ile His Glu Glu Gly Cys Asn Ser Arg Thr
100 105 110

Ile Ile Asn Arg Phe Cys Tyr Gly Gln Cys Asn Ser Phe Tyr Ile Pro
115 120 125

Arg His Val Arg Lys Glu Glu Gly Ser Phe Gln Ser Cys Ser Phe Cys
130 135 140

Lys Pro Lys Lys Phe Thr Thr Met Thr Val Thr Leu Asn Cys Pro Glu
145 150 155 160

Leu Gln Pro Pro Arg Lys Lys Lys Arg Ile Thr Arg Val Lys Glu Cys
165 170 175

Arg Cys Ile Ser Ile Asp Leu Asp
180

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1929 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
(B) LOCATION: 59..601

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ACCCCCGCAC CCAGCTCCGC AGACCGCGCG GCGCGCGCGG GCTCTGGAGG CCACGGGC 58

ATG ATG CTT CGG GTC CTG GTG GGG GCT GTC CTC CCT GCC ATG CTA CTG 106
Met Met Leu Arg Val Leu Val Gly Ala Val Leu Pro Ala Met Leu Leu
185 190 195 200

GCT GCC CCA CCA CCC ATC AAC AAG CTG GCA CTG TTC CCA GAT AAG AGT 154
Ala Ala Pro Pro Ile Asn Lys Leu Ala Leu Phe Pro Asp Lys Ser
205 210 215

GCC TGG TGC GAA GCC AAG AAC ATC ACC CAG ATC GTG GGC CAC AGC GGC 202
Ala Trp Cys Glu Ala Lys Asn Ile Thr Gln Ile Val Gly His Ser Gly
220 225 230

TGT GAG GCC AAG TCC ATC CAG AAC AGG GCG TGC CTA GGA CAG TGC TTC 250
Cys Glu Ala Lys Ser Ile Gln Asn Arg Ala Cys Leu Gly Gln Cys Phe
235 240 245

AGC TAC AGC GTC CCC AAC ACC TTC CCA CAG TCC ACA GAG TCC CTG GTT 298
Ser Tyr Ser Val Pro Asn Thr Phe Pro Gln Ser Thr Glu Ser Leu Val
250 255 260

CAC TGT GAC TCC TGC ATG CCA GCC CAG TCC ATG TGG GAG ATT GTG ACG 346
His Cys Asp Ser Cys Met Pro Ala Gln Ser Met Trp Glu Ile Val Thr
265 270 275 280

CTG GAG TGC CCG GGC CAC GAG GAG GTG CCC AGG GTG GAC AAG CTG GTG 394
Leu Glu Cys Pro Gly His Glu Glu Val Pro Arg Val Asp Lys Leu Val
285 290 295

GAG AAG ATC CTG CAC TGT AGC TGC CAG GCC TGC GGC AAG GAG CCT AGT 442
Glu Lys Ile Leu His Cys Ser Cys Gln Ala Cys Gly Lys Glu Pro Ser
300 305 310

CAC GAG GGG CTG AGC GTC TAT GTG CAG GGC GAG GAC GGG CCG GGA TCC 490
His Glu Gly Leu Ser Val Tyr Val Gln Gly Glu Asp Gly Pro Gly Ser
315 320 325

CAG CCC GGC ACC CAC CCT CAC CCC CAT CCC CAC CCC CAT CCT GGC GGG 538
Gln Pro Gly Thr His Pro His Pro His Pro His Pro His Pro Gly Gly

-continued

330	335	340	
CAG ACC CCT GAG CCC GAG GAC CCC CCT GGG GCC CCC CAC ACA GAG GAA			586
Gln Thr Pro Glu Pro Glu Asp Pro Pro Gly Ala Pro His Thr Glu Glu			
345	350	355	360
GAG GGG GCT GAG GAC TGAGGCCCC CCAACTCTTC CTCCCCTCTC ATCCCCCTGT			641
Glu Gly Ala Glu Asp			
365			
GGAATGTTGG GTCTCACTCT CTGGGGAAGT CAGGGGAGAA GCTGAAGCCC CCCTTTGGCA			701
CTGGATGGAC TTGGCTTCAG ACTCGGACTT GAATGCTGCC CGTTTGCCAT GGAGATCTGA			761
AGGGGCGGGG TTAGAGCCAA GCTGCACAAT TTAATATATT CAAGAGTGGG GGGAGGAAGA			821
AGAGGTCTTC AGGGCTCTTT TTTTGGGGGG GGGGTGGTCT CTTCTGTCTT GGCTTCTAGA			881
GATGTGCCGT TGGGAGGGGG AGGAAGTTGG CTGAGCCATT GAGTGCTGGG GGAGGCCATC			941
CAAGATGGCA TGAATCGGGC TAAGGTCCCT GGGGTGTCAG ATGGTACTGC TGAGGTCCCG			1001
GGCTTAGTGT GAGCATCTTG CCAGCCTCAG GCTTGAGGGA GGGCTGGGCT AGAAAGACCA			1061
CTGGCAGAAA CAGGAGGCTC CGGCCCCACA GGTTCCTCCA AGGCCTCTCA CCCCCTTCC			1121
CATCTCCAGG GAAGCGTCGC CCCAGTGGCA CTGAAGTGGC CCTCCCTCAG CGGAGGGGTT			1181
TGGGAGTCAG GCCTGGGCAG GACCCTGCTG ACTCGTGGCG CGGGAGCTGG GAGCCAGGCT			1241
CTCCGGGCGT TTCTCTGGCT TCCTTGGCTT GCCTGGTGGG GGAAGGGGAG GAGGGGAAGA			1301
AGGAAAGGGA AGAGTCTTCC AAGGCCAGAG GGAGGGGGAC AACCCCCCAA GACCATCCCT			1361
GAAGACGAGC ATCCCCCTCC TCTCCCTGTT AGAAATGTTA GTGCCCCGCA CTGTGCCCCA			1421
AGTTCTAGGC CCCCCAGAAA GCTGTGAGAG CCGGCCGCCT TCTCCCCTCT CCCAGGGATG			1481
CTCTTTGTAA ATATCGGATG GGTGTGGGAG TGAGGGGTTA CCTCCCTCGC CCCAAGGTTT			1541
CAGAGGCCCT AGGCGGGATG GGCTCGCTGA ACCTCGAGGA ACTCCAGGAC GAGGAGGACA			1601
TGGGACTTGC GTGGACAGTC AGGGTTCCT TGGGCTCTCT CTAGCTCCCC AATTCTGCCT			1661
GCCTCCTCCC TCCAGCTGC ACTTTAACCC TAGAAGGTGG GGACCTGGGG GGAGGGACAG			1721
GGCAGGCGGG CCCATGAAGA AAGCCCCTCG TTGCCAGCA CTGTCTGCGT CTGCTCTTCT			1781
GTGCCAGGG TGCTGCCAG CCCACTGCCT CTGCCTGGG GTGGCCTGGC CCTCCTGGCT			1841
GTTGCGACGC GGGCTTCTGG AGCTTGTAC CATTGGACAG TCTCCCTGAT GGACCTCAG			1901
TCTTCTCATG AATAAATTCT TCGGAATT			1929

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 181 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Met Met Leu Arg Val Leu Val Gly Ala Val Leu Pro Ala Met Leu Leu
1 5 10 15

Ala Ala Pro Pro Pro Ile Asn Lys Leu Ala Leu Phe Pro Asp Lys Ser
20 25 30

Ala Trp Cys Glu Ala Lys Asn Ile Thr Gln Ile Val Gly His Ser Gly
35 40 45

Cys Glu Ala Lys Ser Ile Gln Asn Arg Ala Cys Leu Gly Gln Cys Phe

-continued

50	55	60
Ser Tyr Ser Val Pro Asn Thr Phe Pro Gln Ser Thr Glu Ser Leu Val		
65	70	75 80
His Cys Asp Ser Cys Met Pro Ala Gln Ser Met Trp Glu Ile Val Thr		
	85	90 95
Leu Glu Cys Pro Gly His Glu Glu Val Pro Arg Val Asp Lys Leu Val		
	100	105 110
Glu Lys Ile Leu His Cys Ser Cys Gln Ala Cys Gly Lys Glu Pro Ser		
	115	120 125
His Glu Gly Leu Ser Val Tyr Val Gln Gly Glu Asp Gly Pro Gly Ser		
	130	135 140
Gln Pro Gly Thr His Pro His Pro His Pro His Pro His Pro Gly Gly		
	145	150 155 160
Gln Thr Pro Glu Pro Glu Asp Pro Pro Gly Ala Pro His Thr Glu Glu		
	165	170 175
Glu Gly Ala Glu Asp		
	180	

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 184 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Met Asn Arg Thr Ala Tyr Thr Val Gly Ala Leu Leu Leu Leu Gly		
1	5	10 15
Thr Leu Leu Pro Thr Ala Glu Gly Lys Lys Lys Gly Ser Gln Gly Ala		
	20	25 30
Ile Pro Pro Pro Asp Lys Ala Gln His Asn Asp Ser Glu Gln Thr Gln		
	35	40 45
Ser Pro Pro Gln Pro Gly Ser Arg Thr Arg Gly Arg Gly Gln Gly Arg		
	50	55 60
Gly Thr Ala Met Pro Gly Glu Glu Val Leu Glu Ser Ser Gln Glu Ala		
	65	70 75 80
Leu His Val Thr Glu Arg Lys Tyr Leu Lys Arg Asp Trp Cys Lys Thr		
	85	90 95
Gln Pro Leu Lys Gln Thr Ile His Glu Glu Gly Cys Asn Ser Arg Thr		
	100	105 110
Ile Ile Asn Arg Phe Cys Tyr Gly Gln Cys Asn Ser Phe Tyr Ile Pro		
	115	120 125
Arg His Ile Arg Lys Glu Glu Gly Ser Phe Gln Ser Cys Ser Phe Cys		
	130	135 140
Lys Pro Lys Lys Phe Thr Thr Met Met Val Thr Leu Asn Cys Pro Glu		
	145	150 155 160
Leu Gln Pro Pro Thr Lys Lys Lys Arg Val Thr Arg Val Lys Gln Cys		
	165	170 175
Arg Cys Ile Ser Ile Asp Leu Asp		
	180	

(2) INFORMATION FOR SEQ ID NO: 10:

-continued

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 97 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Lys Ser Ala Trp Cys Glu Ala Lys Asn Ile Thr Gln Ile Val Gly His
1 5 10 15
Ser Gly Cys Glu Ala Lys Ser Ile Gln Asn Arg Ala Cys Leu Gly Gln
20 25 30
Cys Phe Ser Tyr Ser Val Pro Asn Thr Phe Pro Gln Ser Thr Glu Ser
35 40 45
Leu Val His Cys Asp Ser Cys Met Pro Ala Gln Ser Met Trp Glu Ile
50 55 60
Val Thr Leu Glu Cys Pro Asp His Glu Glu Val Pro Arg Val Asp Lys
65 70 75 80
Leu Val Glu Lys Ile Val His Cys Ser Cys Gln Ala Cys Gly Lys Glu
85 90 95
Pro

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 97 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Lys Ser Ala Trp Cys Glu Ala Lys Asn Ile Thr Gln Ile Val Gly His
1 5 10 15
Ser Gly Cys Glu Ser Lys Ser Ile Gln Asn Arg Ala Cys Leu Gly Gln
20 25 30
Cys Phe Ser Tyr Ser Val Pro Asn Thr Phe Pro Gln Ser Thr Glu Ser
35 40 45
Leu Val His Cys Asp Ser Cys Met Pro Ile Asp Ser Val Trp Asp Val
50 55 60
Val Thr Leu Glu Cys Pro Gly Asn Glu Glu Phe Pro Arg Val Asp Lys
65 70 75 80
Leu Val Glu Lys Ile Leu Gln Cys Ser Cys Gln Ala Cys Gly Lys Glu
85 90 95
Leu

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 90 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Met Lys Glu Ala Cys Lys Thr Leu Pro Phe Thr Gln Asn Ile Val His
1 5 10 15

-continued

Glu Asn Cys Asp Arg Met Val Ile Gln Asn Asn Leu Cys Phe Gly Lys
 20 25 30

Cys Ile Ser Leu His Val Pro Asn Gln Gln Asp Arg Arg Asn Thr Cys
 35 40 45

Ser His Cys Leu Pro Ser Lys Phe Thr Leu Asn His Leu Thr Leu Asn
 50 55 60

Cys Thr Gly Ser Lys Asn Val Val Lys Val Val Met Met Val Glu Glu
 65 70 75 80

Cys Thr Cys Glu Ala His Lys Ser Asn Phe
 85 90

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 98 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Met Asn Gln Arg Cys Asp Gly Gln Lys Phe Lys Gln Arg Ile Arg Val
 1 5 10 15

Asp Gly Cys Leu Thr Lys Val Val Val Asn Arg Leu Cys His Gly Ala
 20 25 30

Cys Ala Ser Ile Phe Ile Pro Arg Met His Ser Lys Lys Leu Lys Ala
 35 40 45

Ala Phe Arg Ser Cys Ala Ala Cys Ala Pro Ala Glu Tyr Asp Tyr Val
 50 55 60

Asp Ile Thr Leu Asp Cys Pro Gly Arg Thr Pro Pro Thr Ala Thr Lys
 65 70 75 80

Thr Ile Val Lys Val Lys Ser Cys Lys Cys Lys Glu Val Arg Ile Ala
 85 90 95

Pro Phe

What is claimed is:

1. A method for reducing morphogen-dependent bone formation of a cell comprising an extracellular surface in contact with a medium, said method comprising the step of:

contacting said medium with an antagonist of a bone morphogenic protein (BMP) selected from the group consisting of human BMP2 and human BMP4, said antagonist produced exogenously from said cell and comprising SEQ ID NO:2, 4, 6, 8 or 9 or a deletion mutant of a sequence selected from the group consisting of SEQ ID NO:2 or 8 wherein said deletion mutant encodes a polypeptide that retains at least 8 contiguous amino acids of said SEQ ID NO:2 or 8 and is sufficient to specifically bind and antagonize said BMP, under conditions whereby said morphogen-dependent bone formation is reduced; and

measuring a resultant reduction of said morphogen-dependent bone formation.

2. A method for reducing morphogen-dependent bone formation of a cell comprising an extracellular surface in contact with a medium, said method comprising the step of:

contacting said medium with an antagonist of a bone morphogenic protein (BMP) selected from the group consisting of human BMP2 and human BMP4, said antagonist produced exogenously from said cell and comprising SEQ ID NO:2, or a deletion mutant of SEQ ID NO:2 wherein said deletion mutant encodes a polypeptide that retains at least 8 contiguous amino acids of SEQ ID NO:2 and is sufficient to specifically bind and antagonize said BMP, under conditions whereby said morphogen-dependent bone formation is reduced; and

measuring a resultant reduction of said morphogen-dependent bone formation.

3. A method for reducing morphogen-dependent bone formation of a cell comprising an extracellular surface in contact with a medium, said method comprising the step of:

contacting said medium with an antagonist of a bone morphogenic protein (BMP) selected from the group consisting of human BMP2 and human BMP4, said

antagonist produced exogenously from said cell and comprising SEQ ID NO:8, or a deletion mutant of SEQ ID NO:8 wherein said deletion mutant encodes a polypeptide that retains at least 8 contiguous amino acids of SEQ ID NO:8 and is sufficient to specifically bind and antagonize said BMP, under conditions whereby said morphogen-dependent bone formation is reduced; and

measuring a resultant reduction of said morphogen-dependent bone formation.

4. A method according to claim 1, wherein said cell is in situ and said medium is a physiological fluid comprising said BMP.

5. A method according to claim 2, wherein said cell is in situ and said medium is a physiological fluid comprising said BMP.

6. A method according to claim 3, wherein said cell is in situ and said medium is a physiological fluid comprising said BMP.

* * * * *