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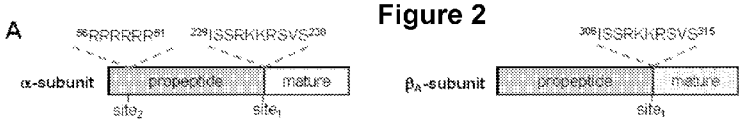
(54) Title: INHIBIN ANALOGS

Figure 2



(57) Abstract: The present invention relates to the recombinant production of inhibin. At least one proprotein convertase cleavage site of the precursor subunits of inhibin is modified through substitution with the more efficiently processed cleavage site ISSRKKRSVSS. Further modifications include mutation of the type I receptor (ALK4) binding epitope of the beta subunit to supress activin bioactivity, and mutation of the homodimerization interface site of the beta subunit to reduce or prevent activin formation. Also disclosed are pharmaceuticals and uses of the inhibin precursor and mature inhibin.

INHIBIN ANALOGS

[0001] This application is associated with and claims priority from Australian Provisional Patent Application No. 2015904898, filed on 26 November, 2015, entitled "Inhibin analogs", the entire contents of which, are incorporated herein by reference, in their entirety. This specification refers to a Sequence Listing. The "ST25.txt" file is in ANSI format. The file is hereby incorporated in its entirety by reference from AU 2015904898 into the subject specification.

FIELD

[0002] The present invention relates to inhibin analogs, their method of production and their use in the treatment and prophylaxis of disease or conditions associated with reduced levels of inhibin and activin-mediated signaling.

BACKGROUND

[0003] Bibliographic details of the publications referred to by author in this specification are collected alphabetically at the end of the description.

[0004] The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgement or admission or any form of suggestion that the prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavor to which this specification relates.

[0005] Follicle stimulating hormone (FSH) regulates spermatogenesis in males and folliculogenesis in females. In response to FSH, testicular Sertoli cells and ovarian granulosa cells produce inhibin A and/or inhibin B, which target the gonadotrope cells of the pituitary to down-regulate the production and secretion of FSH in a cycle-dependent

manner in females and in a tonic pattern in males (Woodruff *et al.* (1996) *Endocrinology 137*:5463-5467). Circulating inhibin levels decrease dramatically across the menopause transition and inversely correlate with increased serum FSH (Robertson *et al.* (2008) *Menopause 15*:1139-1144).

[0006] Inhibin A and B are unique members of the transforming growth factor-β (TGF-β) superfamily as they:(i) are heterodimers composed of α- and β (β_A or β_B)-subunits, whereas most other family members are homodimers; (ii) act as antagonists, rather than agonists, inhibiting signaling of activin-related proteins; and (iii) function in an endocrine rather than autocrine/paracrine manner. These aspects of inhibin biology are crucial for their roles in reproduction, but they also endow these hormones with the potential to regulate additional physiological processes, including bone and muscle growth.

[0007] Analogous to other members of the TGF-β superfamily, inhibin α- and β-subunits are synthesized as large precursor molecules with the N-terminal prodomain mediating the folding and dimerization of the C-terminal mature domains (Walton *et al.* (2009) *J Biol Chem. 284*:9311-9320). Dimeric precursors are cleaved by proprotein convertases and inhibins are secreted from Sertoli and granulosa cells non-covalently associated with their prodomain (pro-mature complexes). Once localized to target tissues, prodomains are displaced, and mature 31 kDa inhibins associate with their cognate receptors.

[0008] It is recognized that inhibin A and B regulate FSH secretion from the anterior pituitary by blocking the stimulatory actions of activins (Wiater and Vale (2003) *J Biol Chem. 278*:7934-7941). Activins are homodimers of inhibin β-subunits:β_A-β_A (activin A), β_B-β_B (activin B). Activins initially bind to type II receptors, ActRIIA or ActRIIB, leading to recruitment, phosphorylation and activation of the type I receptor, ALK4. Activated ALK4 phosphorylates intracellular signaling molecules, Smad2/3, which in turn form a complex with the co-activator, Smad4. The resulting Smad oligomer localizes within the nucleus to regulate target genes (e.g. *Fshb*) in a cell- and context-dependent manner (Massague and Wotton *EMBO J. 19*:1745-1754). Inhibin antagonism of activin-related ligands is dependent upon interactions with betaglycan, a cell-surface proteoglycan that

also acts as a TGF-β co-receptor (Lewis *et al.* (2000) *Nature 404*:411-414). Betaglycan binds inhibin A and B directly and promotes the formation of a stable high affinity complex involving activin type II receptors (Lewis *et al.* (2000) *supra*). Sequestration of type II receptors in this way prevents their interactions with signaling ligands such as activins. In the absence of betaglycan, inhibin cannot block activin-mediated FSH secretion by pituitary gonadotrope cells (Wiater *et al.* (2009) *Mol Endocrinol. 23*:1033-1042).

[0009] Aspects of inhibins' mode of action, including the widespread expression of inhibin receptors and the fact that activins target multiple tissues, presuppose functions beyond the negative regulation of FSH. Indeed, serum inhibin A and B levels correlate inversely with markers of bone formation and bone resorption in women across the menopause transition, and it has been proposed that these decreases in inhibin contribute to the initial bone loss during this period (Perrien *et al.* (2006) *J Clin Endocrinol Metab. 91*:1848-1854). To test whether inhibin A could regulate bone mass *in vivo*, (Perrien *et al.* (2007) *Endorcinology 148*:1654-1665; Perrien *et al.* (2012) *J Orthopaed Res. 30*:288-295) utilized a transgenic model of inducible human inhibin A expression. Inhibin A increased total body bone mineral density (BMD), increased bone volume and improved biomechanical properties at the proximal tibia of intact mice, and also prevented the loss of BMD and bone volume associated with gonadectomy. As activin A, one of the most highly expressed TGF-β proteins in bone, potently suppresses osteoblast differentiation (Lotinun *et al.* (2012) *Curr Mol Pharmacol. 5*:195-204), it is likely that inhibins' anabolic effect is *via* inhibition of this growth factor. Thus, gonadal inhibins are likely components of the normal endocrine repertoire that regulate bone quality, and the loss of inhibins at menopause may play a significant role in osteoporosis progression.

[0010] Definitively characterizing the physiological roles of inhibin A and B, however, has proven difficult for several reasons. First, targeted deletion of the inhibin α-subunit in mice leads to unopposed gonadal expression of activin A and B. As activins stimulate granulosa and Sertoli cell proliferation, inhibin-deficient mice develop sex-cord stromal tumors with 100% penetrance, as early as 4 weeks of age (Matzuk *et al.* (1992) *Nature 360*:313-319).

As tumors progress, serum levels of activin A and B increase up to 500-fold (Li *et al.* (2007) *Mol Human Reprod. 13*:675-683), and mice die from a cachexia-like wasting syndrome between 12-17 weeks (Matzuk *et al.* (1994) *Proc Natl Acad Sci USA 91*:8817-8821). Thus, the phenotype of the inhibin α-subunit knockout mouse does not really reflect the loss of inhibin, but rather the devastating systemic effects of elevated circulating activins. Secondly, i*n vivo* overexpression of inhibins (α/β-subunit heterodimers) is always accompanied by production of activins (β-subunit homodimers), which have pleiotropic effects in multiple organs (Harrison *et al.* (2005) *Trends Endocrinol Metab. 16*:73-78). Finally, recombinant production of inhibins and their separation from contaminating activins is difficult, and the resultant 31 kDa mature inhibin isoforms have short *in vivo* half-lives (Makanji *et al.* (2009) *Endocrinology 150*:4784-4793).

[0011] There is a need to develop an improved system to generate inhibin A and inhibin B for therapeutic use.

SUMMARY

[0012] Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of the sequence identifiers is provided in Table 1. A sequence listing is provided after the claims. Abbreviations used herein are listed in Table 2.

[0013] The present specification teaches a mammalian inhibin analog precursor protein comprising a heterodimer of α-subunit and β-subunit precursors each having at least one proprotein convertase cleavage site wherein at least one proprotein convertase cleavage site in the α-subunit and/or β-subunit is modified by an amino acid substitution mutation to render it more efficiently cleaved by the proprotein convertase. This enables the efficient generation of an inhibin in bioactive form. The term "analog" is used to highlight that the inhibin precursor protein contains an artificially introduced mutation to facilitate more efficient processing. Other terms such as "variant", "mutant" and "modified protein" may also be used to the same effect. The bioactive inhibin and its precursor are useful in the treatment of a range of diseases and conditions such as arising from reduced production of an inhibin or where antagonism of an activin would ameliorate a particular disease or condition. Reduced serum levels of inhibin in post-menopausal women, for example, can exacerbate bone disorders such as osteoporosis.

[0014] In an embodiment, a "primary" proprotein convertase cleavage site in each of the α-subunit and β-subunit precursors is modified by the amino acid substitution mutation. In a further embodiment, the α-subunit protein comprises a secondary proprotein convertase cleavage site, which is modified by an amino acid substitution mutation to render it more efficiently cleaved by the proprotein convertase. In an embodiment, the modified proprotein primary convertase site is defined by the amino acid sequence set forth in SEQ ID NO:18 (ISSRKKRSVSS). The β-subunits may be β_A-subunit leading to an inhibin A analog or a β_B-subunit leading to an inhibin B analog when either subunit forms a heterodimer with the α-subunit. The α- and/or β-subunits may contain additional amino

acid substitutions, additions and/or deletions with the proviso that either or both subunits contain a modified proprotein convertase cleavage site(s) to render convertase processing more efficient compared to cleavage of a wild-type α- and/or β-subunit. An example of another amino acid modification is at the predicted β/β homodimerization interface, required for the formation of an activin. These sites include A347, Y345, F326, V392, P393 and L396 in β_A-subunit (using the wild-type amino acid sequence in SEQ ID NO:2). Sites in β_B-subunit include F308, Y327, G329, I373, P374 and L377 (using the wild-type amino acid sequence in SEQ ID NO:12). Homodimers of β subunits are present in activins ($\beta_A + \beta_A$ in activin A or $\beta_B + \beta_B$ for activin B). A homodimerization interface mutation reduces the ability for homodimers (i.e. activins) to form. The variants of the present invention retain inhibin activity, are generated with improved processing efficiency resulting in higher proportion of active inhibin, exhibit enhanced α/β dimerization and have reduced or silenced activin receptor binding activity. This is especially the case when there is a further mutation such as a mutation at a homodimerization interface site on β_A or β_B to prevent or reduce homodimerization formation.

[0015] The inhibin analog precursor protein may be of human or non-human mammalian origin. Hence, the precursor protein or the inhibin generated therefrom has therapeutic potential in humans and non-human mammals.

[0016] The β-subunit of the inhibin analog precursor protein may further comprise a single mutation within a type I receptor (ALK4) binding epitope of the mature domain leading to inactive activin. Activin is generally co-formed during the synthesis of inhibins and comprises β-subunit homodimers. The mutation in the β-subunit renders inactive any activin formed. A mutation at a homodimerization interface site further facilitates an absence of or reduce amount of active activin.

[0017] For the human β_A-subunit, the mutation includes a M418A (based on numbering in SEQ ID NO:2; equivalent to M432A in SEQ ID NO:4) substitution mutation (numbering from start of prodomain; equivalent to M108A from start of the mature domain). This

means the resident methionine residue is replaced by an alanine residue. For the human β_B-subunit, the mutation includes a M399A substitution mutation (from start of prodomain); numbering from SEQ ID NO:12; equivalent to M410A in SEQ ID NO:14.

[0018] Additional mutation(s) may also be added at the interface site for homodimerization to occur for β_A + β_A dimerization into activin A and β_B + β_B dimerization into activin B. Such mutations reduce or inhibit altogether functional activin molecules being produced. Examples include $A347X_1$, $Y345X_2$, $F326X_3$, $V392X_4$, $P393X_5$ and $L396X_6$ wherein each of X_1, X_2, X_3, X_4, X_5 and X_6 is any amino acid except A, Y, F, V, P and L, respectively (based on numbering in SEQ ID NO:2) in subunit β_A; and $F308X_7$, $Y327X_8$, $G329X_9$, $I373X_{10}$, $P374X_{11}$ and $L377X_{12}$ wherein each of X_7, X_8, X_9, X_{10}, X_{11} and X_{12} is any amino acid except F, Y, G, I, P and L, respectively (using numbering in SEQ ID NO:12). Particular examples including A347H, Y345G and A347H + Y345G in β_A-subunit.

[0019] The inhibin analog precursor protein β_A- or β_B-subunit may further comprise a FLAG tag comprising the amino acid sequence DYKDDDK (SEQ ID NO:16) between amino acids 27 and 28 of β_A or 28 and 29 of β_B. The α-subunit may comprise a poly-histidine tag. These aid in purification of inhibin from conditioned medium by affinity chromatography as well as distinguishing wild-type protein from modified protein.

[0020] The modified proprotein convertase sites improve the efficiency of cleavage of the inhibin analog precursor protein relative to enzymatic cleavage of the wild-type protein. In addition, there is a concomitant reduction in bioactive activin. This is further enhanced by the introduction of the mutation in the ALK4 binding epitope in the β-subunit mature domain and/or mutation at a site required for homodimerization of a β_A-subunit on β_B-subunit.

[0021] Hence, enabled herein is an inhibin α-subunit precursor comprising:

(i) a modified primary proprotein convertase site ("super-cut site1");

(ii) a modified secondary proprotein convertase site ("super-cut site2");

(iii) modified primary and secondary proprotein convertase sites; and optionally together with a poly-histidine tag to assist with purification

.

[0022] Further enabled herein is an inhibin β_A-subunit precursor comprising a modified proprotein convertase site ("super-cut site");

optionally with a mutation disrupting the ALK4 binding epitope in the mature domain and optionally with a FLAG tag to assist in affinity chromatography purification and optionally with a mutation disrupting homodimerization.

[0023] Taught herein is an inhibin β_B-subunit precursor comprising a modified proprotein convertase site ("super-cut site");

optionally with a mutation disrupting the ALK4 binding epitope in the mature domain and optionally with a FLAG tag to assist in affinity chromatography purification and optionally with a mutation disrupting homodimerization.

[0024] Hence, in an embodiment an inhibin analog precursor protein is provided comprising an α-subunit having the amino acid sequence as set forth in SEQ ID NO:8 (α-subunit super-cut site1 + poly-his) or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:8 after optimal alignment with the proviso that it comprises at least one modified proprotein convertase site to enable more efficient cleavage by the convertase.

[0025] Enabled herein is an inhibin analog precursor protein comprising an α-subunit having the amino acid sequence as set forth in SEQ ID NO:10 (α-subunit super-cut sites1 and 2 + poly-his) or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:10 after optimal alignment with the proviso that it comprises at least one modified proprotein convertase site to enable more efficient cleavage by the convertase.

[0026] In an embodiment, an inhibin analog precursor protein is provided comprising an β_A-subunit having the amino acid sequence as set forth in SEQ ID NO:4 (β_A-subunit super-

cut + ALK4 mutation + FLAG tag) or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:4 after optimal alignment with the proviso that it comprises at least one modified proprotein convertase site to enable more efficient cleavage by the convertase.

[0027] Taught herein is an inhibin analog precursor protein comprising an β_A-subunit having the amino acid sequence as set forth in SEQ ID NO:40 (β_A-subunit super-cut + ALK4 mutation + FLAG tag + interface mutation) or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:40 after optimal alignment with the proviso that it comprises at least one modified proprotein convertase site to enable more efficient cleavage by the convertase.

[0028] Enabled herein is an inhibin analog precursor protein comprising an β_A-subunit having the amino acid sequence as set forth in SEQ ID NO:41 (β_A-subunit super-cut + ALK4 mutation + FLAG tag + interface mutation) or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:41 after optimal alignment with the proviso that it comprises at least one modified proprotein convertase site to enable more efficient cleavage by the convertase.

[0029] In an embodiment, an inhibin analog precursor protein is provided comprising an β_A-subunit having the amino acid sequence as set forth in SEQ ID NO:42 (β_A-subunit super-cut + ALK4 mutation + FLAG tap + combined interface mutations) or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:42 after optimal alignment with the proviso that it comprises at least one modified proprotein convertase site to enable more efficient cleavage by the convertase.

[0030] Taught herein is an inhibin analog precursor protein comprising an β_B-subunit having the amino acid sequence as set forth in SEQ ID NO:14 (β_B-subunit super-cut + ALK4 mutation + FLAG tag) or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:14 after optimal alignment with the proviso that it comprises at least one modified proprotein convertase site to enable more efficient cleavage by the

convertase.

[0031] Further enabled herein is a nucleic acid molecule encoding the α-subunit or β_A-subunit or β_B-subunit precursor. The nucleic acid may be operably linked to a promoter and if necessary a terminator sequence or other regulatory sequence. The nucleic acid molecule is used to transfect cells or cell lines to coproduce α- and either β_A- or β_B-subunits.

[0032] In an embodiment, the nucleic acid molecule encoding the α-subunit variant comprises the nucleotide sequence set forth in SEQ ID NO:7 (super-cut site1 + poly-his) or a nucleotide sequence having at least about 80% identity thereto after optimal alignment or a nucleic acid capable of hybridizing to the complement of SEQ ID NO:7 under stringency conditions.

[0033] In another embodiment, the nucleic acid molecule encoding the α-subunit variant comprises the nucleotide sequence set forth in SEQ ID NO:9 (super-cut sites1 and 2 + poly-his) or a nucleotide sequence having at least about 80% identity thereto after optimal alignment or a nucleic acid capable of hybridizing to the complement of SEQ ID NO:9 under stringency conditions.

[0034] A nucleic acid molecule encoding the β_A-subunit includes the nucleotide sequence as set forth in SEQ ID NO:3 (super-cut site + ALK4 mutation + FLAG tag) or a nucleotide sequence having at least about 80% identity thereto after optimal alignment or a nucleic acid capable of hybridizing to the complement of SEQ ID NO:3 under stringency conditions.

[0035] A nucleic acid molecule encoding the β_B-subunit includes the nucleotide sequence as set forth in SEQ ID NO:13 (super-cut site + ALK4 mutation + FLAG tag) or a nucleotide sequence having at least about 80% identity thereto after optimal alignment or a nucleic acid capable of hybridizing to the complement of SEQ ID NO:13 under stringency conditions.

[0036] Further enabled herein is an isolated cell or cell line comprising the nucleic acid encoding the α-subunit and either the β_A-subunit or β_B-subunit variant.

[0037] The above nucleic acid molecules may encode a range of amino acid substitutions, additions and/or deletions provided that they encode a modified proprotein convertase cleavage site which is more efficiently cleaved by the convertase compared to a wild-type α- and/or β-subunit. A cell or cell line transfected with a nucleic acid encoding the α-subunit and β-subunit precursors and cultured in a growth medium generates bioactive inhibin following expression of the nucleic acid to produce inhibin precursor and proprotein convertase cleavage of the inhibin analog precursor protein. Inhibin production is enhanced by modulating the ratio of nucleic acids encoding the α- and β-subunits. Generally, the ratio is α>β, such as but not limited to 3:2, respectively.

[0038] In an embodiment, an equal amount of α-subunit encoding nucleic acid and β-subunit encoding are transfected into the cell or cell line. In another embodiment, the ratio of α-subunit to β-subunit encoding nucleic acid is α>β including the ratio 3:2 (α:β, respectively). In another embodiment, the ratio is α<β including 2:3 (α:β, respectively).

[0039] A method for generating a bioactive inhibin analog is contemplated herein the method comprising co-expressing in a cell or cell line nucleic acid encoding the α-subunit and a β_A-subunit or β_B-subunit as defined herein for a time and under conditions sufficient for an inhibin precursor protein to be produced, cleaved by a proprotein convertase and secreted from the cell or cell line as a bioactive inhibin analog.

[0040] This leads to the generation of inhibin A analog or inhibin B analog.

[0041] In an embodiment, minimal bioactive activin A or activin B is produced.

[0042] Further contemplated herein is a method of treatment of a mammalian subject comprising the administration of an inhibin A or inhibin B analog as defined herein or an

inhibin analog precursor protein thereof.

[0043] In an embodiment, the mammal is a human.

[0044] Taught herein is a pharmaceutical composition comprising an inhibin A or inhibin B analog as defined herein or an inhibin analog precursor protein thereof and one or more pharmaceutically acceptable carriers, diluents and/or excipients.

[0045] Further taught herein is the use of an inhibin A or inhibin B analog as defined herein or an inhibin analog precursor protein thereof in the manufacture of a medicament for the treatment of a mammalian subject in need of therapy. In a related embodiment enabled herein is an inhibin A or inhibin B analog as defined herein or an inhibin analog precursor protein thereof for use in the treatment of a mammalian subject in need of therapy. In an embodiment, the mammal is a human.

[0046] In a further embodiment, the subject has reduced serum levels of inhibin compared to the level in a pre-menopausal healthy subject or has a disease or condition exacerbated by activin-mediated signaling.

[0047] Additional embodiment described herein includes antibodies to the inhibin analogs or their precursors.

[0048] The location of homodimerization interface sites which can or have been mutated are shown in Table 3 (β_A-subunit) and Table 4 (β_B-subunit).

Table 1

Summary of sequence identifiers

SEQUENCE ID NO:	DESCRIPTION
1	Nucleotide sequence encoding wild-type human inhibin β_A-subunit
2	Amino acid sequence of wild-type human inhibin β_A-subunit
3	Nucleotide sequence encoding super-cut variant of human inhibin β_A-subunit (super-cut site + ALK4 mutation + FLAG tag)
4	Amino acid sequence of super-cut variant of inhibin β_A-subunit (super-cut site + ALK4 mutation + FLAG tag)
5	Nucleotide sequence encoding wild-type human inhibin α-subunit
6	Amino acid sequence of wild-type human inhibin α-subunit
7	Nucleotide sequence encoding super-cut variant 1 of human inhibin α-subunit (super-cut site1 + poly-his)
8	Amino acid sequence of super-cut variant 1 of human inhibin α-subunit (super-cut site 1 + poly-his)
9	Nucleotide sequence encoding super-cut variant 2 of human inhibin α-subunit (super-cut sites 1 and 2 + poly-his)
10	Amino acid sequence of super-cut variant 2 of human inhibin α-subunit (super-cut sites 1 and 2 + poly-his)
11	Nucleotide sequence encoding wild-type human inhibin β_B-subunit
12	Amino acid sequence of wild-type human inhibin β_B-subunit
13	Nucleotide sequence encoding super-cut variant of human inhibin β_B-subunit (super-cut + ALK4 mutation + FLAG tag)
14	Amino acid sequence of super-cut variant of human inhibin β_B-subunit (super-cut + ALK4 mutation + FLAG tag)
15	Nucleotide sequence encoding FLAG tag
16	Amino acid sequence of FLAG tag
17	Nucleotide sequence encoding super-cut site1
18	Amino acid sequence of super-cut site1
19	Nucleotide sequence encoding poly-histidine TAG (x9)

SEQUENCE ID NO:	DESCRIPTION
20	Amino acid sequence of poly-histidine TAG (x9)
21	Nucleotide sequence encoding super-cut site 2
22	Amino acid sequence of super-cut site 2
23	Nucleotide sequence of α-NHE1 sense primer
24	Nucleotide sequence of α-ECOR1 antisense primer
25	Nucleotide sequence of α-SCUT (site1) sense primer
26	Nucleotide sequence of α-SCUT (site1) antisense primer
27	Nucleotide sequence of α-SCUT (site2) sense primer
28	Nucleotide sequence of α-SCUT (site2) antisense primer
29	Nucleotide sequence of α-polyH1 Stag sense primer
30	Nucleotide sequence of α-polyH1 Stage antisense primer
31	Nucleotide sequence of βAXBA1 sense primer
32	Nucleotide sequence of βA-NOT1 antisense primer
33	Nucleotide sequence of βA-SCUT (site1) sense primer
34	Nucleotide sequence of βA-SCUT (site1) antisense primer
35	Nucleotide sequence of βA-M418A sense primer
36	Nucleotide sequence of βA-M418A antisense primer
37	Amino acid sequence of human β_A-subunit variant with A347H substitution at interface region for homodimerization.
38	Amino acid sequence of human β_A-subunit variant with Y345G substitution at interface region for homodimerization.
39	Amino acid sequence of human β_A-subunit variant with A347H/Y345G double mutation at interface region for homodimerization.
40	Amino acid sequence of human-β_A-subunit with super-cut + ALK4 mutation FLAG tag + A347H (=A361H) mutation.
41	Amino acid sequence of human-β_A-subunit with super-cut + ALK4 mutation FLAG tag + Y345G (=Y359G) mutation.
42	Amino acid sequence of human-β_A-subunit with super-cut + ALK4 mutation FLAG tag + A347H + Y345G (=A361H + Y359G) mutations.

SEQUENCE ID NO:	DESCRIPTION
43	Nucleotide sequence encoding super-cut variant of human inhibin β_B-subunit (super-cut + ALK4 mutation + FLAG tag): with a modified signal sequence
44	Amino acid sequence of super-cut variant of human inhibin β_B-subunit (super-cut + ALK4 mutation + FLAG tag): with a modified signal sequence

Table 2

Abbreviations

ABBREVIATION	DESCRIPTION
ALK4	Type I receptor activated *via* an activin
BMD	Bone mineral density
E4	Inhibin β_A-subunit monoclonal antibody
FCS	Fetal calf serum
FSH	Follicle stimulating hormone
Interface mutation	Mutation at a site on β_A or β_B which prevents or reduces homodimerization. Also referred to as "dimerization interface mutation" and a "homodimerization interface mutation"
LβT2	Mouse pituitary gonadotrope cell line
PBS	Phosphate buffered saline
R1	Inhibin α-subunit monoclonal antibody
Super-cut site (α- or β-SCUT)	Proprotein convertase cleavage site in α- and β-subunits of an inhibin
Super-cut site1 (α-SCUT1)	Primary proprotein convertase site in α-subunit of inhibin
Super-cut site2 (α-SCUT2)	Secondary proprotein convertase site in α-subunit of inhibin
TGFβ	Transforming growth factor-β
α	α-subunit of inhibin
α- β_A	Inhibin A heterodimer
α–β_B	Inhibin B heterodimer
β_A	β_A-subunit of inhibin
β_A- β_A	Activin A homodimer
β_B	β_B-subunit of inhibin
β_B- β_B	Activin B homodimer

Table 3

Homodimerization interface sites on β_A-subunit

Wild-type β_A-subunit (SEQ ID NO:2)	Variant β_A-subunit (super-cut site + ALK4 mutation + FLAG tag_ (SEQ ID NO:4)	Variant β_A-subunit (super-cut site + ALK4 mutation + FLAG tag + interface mutation (SEQ ID NO:40)	Variant β_A-subunit (supercut site + ALK4 mutation + FLAG tag + interface mutation (SEQ ID NO:41)	Variant β_A-subunit (supercut site + ALK4 mutation + FLAG tag + interface mutation (SEQ ID NO:42)
A347	A361	A361H	A361	A361H
Y345	Y359	Y359	Y359G	Y359G
F326	F340	F340	F340	F340
V392	V406	V406	V406	V406
P393	P407	P407	P407	P407
L396	L410	L410	L410	L410

Table 4

Homodimerization interface sites on β_B-subunit

Wild-type β_B-subunit (SEQ ID NO:12)	Variant β_B-subunit (super-cut site + ALK4 mutation + FLAG tag) (SEQ ID NO:14)[1]
F308	F319
Y327	Y338
G329	G340
I373	I384
P374	P385
L377	L388

[1] This sequence has a Ser Arg His deleted as part of the supercut modification (compared to the wild-type sequence).

BRIEF DESCRIPTION OF THE FIGURES

[0049] Figure 1 is a graphical representation showing the effect of enhanced subunit processing on inhibin/activin production in mammalian cells. (**A**) The native cleavage sites (RXXR) in the inhibin α- and β-subunits were enhanced by site-directed mutagenesis. (**B, C**) Wild-type or mutant inhibin α- and β_A-subunits were co-transfected into HEK293F cells and the resultant inhibin and activin isoforms produced were assessed by Western blot, using antibodies to the α- and β_A-subunits (R1 and E4, respectively). (**D, E**) Specific ELISAs were used to quantitate inhibin and activin produced by HEK293F cells. Results are representative of n=3 experiments.

[0050] Figure 2 is a graphical representation showing enhanced processing within the inhibin α-subunit precursor favors the production of mature inhibins. (**A**) The inhibin α-subunit uniquely contains a second processing site (site2) within its prodomain. This cleavage site was enhanced by targeted mutagenesis, alongside site1, which intervenes the pro- and mature domains. (**B, C**) Wild-type or "super-cut" inhibin α- and β_A-subunits were co-transfected into HEK293F cells and the resultant inhibin and activin isoforms produced were assessed by Western blot (*SCUT-S2* refers to α-subunit with both processing sites enhanced). (**D, E**) Densitometry was used to determine the relative expression of mature (31 kDa) inhibin A and activin A. Results are representative of n=3 experiments.

[0051] Figure 3 is a graphical representation showing inactivation of residual activin A improves inhibin bioactivity. (**A**) An M418A mutation was incorporated into the β_A-subunit to inhibit activin binding to its type I receptor (ALK4). (**B, C**) The effect of the M418A mutation on inhibin and activin Biosynthesis was assessed by Western blot, using antibodies to the α- and β_A-subunits (R1 and E4, respectively). (**D**) The ability of inhibin M418A to suppress activin Bioactivity was determined using an LβT2 gonadotrope FSH bioassay. The LβT2 cells were stimulated with 200 pM activin A and treated with increasing doses of either Inhibin A-SCUT or Inhibin A-SCUT (M418A). (**E**) Activin Bioactivity within the inhibin preparations was assessed using an activin-responsive

luciferase reporter assay (A3-Luc). Transfected HEK293T cells were treated with increasing doses of inhibin preparations, and activity determined by measuring luciferase activity. Results are representative of n=3 experiments.

[0052] Figure 4 is a photographic or graphical representation showing purification of inhibin A-SCUT (M418A) by affinity chromatography. Inhibin A-SCUT (M418A) was purified using immobilized metal ion affinity chromatography (IMAC), and recovery assessed by Western blot (**A**) and ELISA (**B**).

[0053] Figure 5 is a graphical representation showing analysis of Pro-inhibin bioactivity. IMAC purification enabled the isolation of pro-inhibin A complexes carrying the M418A mutation. The ability of Pro-inhibin A (M418A) to suppress activin-induced FSH release was compared to mature inhibin A in both (**A**) rat pituitary cell cultures, and (**B**) mouse gonadotrope LβT2 cells. (**C**) The ability of Pro-inhibin A (M418A) to suppress activin-induced SMAD2 phosphorylation was also assessed in LβT2 cells. Cells were treated with 200 pM activin A in the presence of increasing doses of Pro-inhibin A (M418A) or mature inhibin A. Representative result of n=3 experiments

[0054] Figure 6 is a graphical representation showing the effect of altering the transfection ratio of the α- and β_A-subunits on inhibin production. (**A, B**) The inhibin α- and β_A-subunits with super-cleavage sites (SCUT) were co-transfected into HEK293F cells at varying ratios and the resultant inhibin and activin isoforms produced were assessed by Western blot, using antibodies to the α- and β_A-subunits (R1 and E4, respectively). (**C, D**) Specific ELISAs were used to quantitate inhibin and activin produced by HEK293F cells. Results are representative of n=3 experiments.

[0055] Figure 7 is a representation of human inhibin β_A-subunit super-cut variant + M418A (ALK4) mutation + FLAG tag.

[0056] Figure 8 is a representation of human inhibin α-subunit super-cut 1 variant + poly-his tag.

[0057] Figure 9 is a representation of human inhibin α-subunit super-cut 2 variant (super-cut site1 and 2 + poly-his tag).

[0058] Figure 10 is a representation of human β_B-subunit super-cut variant + M410A (ALK4) mutation + FLAG tag.

[0059] Figure 11 is a photographic representation of purification of inhibin B analog (inhibin β-SCUT M410A) by affinity chromatography. Inhibin β-SCUT (M410A) was purified using IMAC and recovery assessed by Western blot comparing wild-type and mutants of α- and β_B-subunits.

[0060] Figures 12A through C are photographic and graphical representations of (**A**) Western blot analysis of activin expression (E4 detection); (**B**) Western blot analysis of inhibin expression (R1 detection); (**C**) Activity data of super-cut mutant and β_A 347H mutant.

[0061] Figures 13A and B are photographic and graphical representations of (**A**) Western blot analysis of activin expression (E4 detection); and (**B**) Activity data of super-cut mutant and β_A A347H/Y345G double mutants.

[0062] Figure 14 is a representation of human β_B-subunit super-cut variant + M410A (ALK4) mutation + FLAG tag: with a modified signal sequence. This sequence has a Ser Arg His deleted as part of the supercut modification (compared to the wild-type sequence).

DETAILED DESCRIPTION

[0063] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or method step or group of elements or integers or method steps but not the exclusion of any other element or integer or method steps or group of elements or integers or method steps.

[0064] As used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a variant" or "a mutation" includes a single variant or mutation, as well as two or more variants or mutations; reference to "an inhibin" includes a single inhibin, as well as two or more types of inhibins; reference to "the disclosure" includes a single and multiple aspects taught by the disclosure; and so forth. Aspects taught and enabled herein are encompassed by the term "invention". All such aspects are enabled within the width of the present invention. Any variants and derivatives contemplated herein are encompassed by "forms" of the invention.

[0065] Gonadal-derived inhibin A and inhibin B are components of the normal endocrine repertoire that regulated bone quality, and the loss of inhibins at menopause likely contributes to the accompanying decrease in bone mass. Therefore, inhibins have been touted as potential therapeutics for osteoporosis in post-menopausal women as well as the treatment of male and female subjects having a disease or condition exacerbated by low levels of inhibins and/or activin-mediated signaling. However, as heterodimeric proteins of α- and β (β_A or β_B)-subunits, inhibins are difficult to produce recombinantly, they are poorly processed to their mature bioactive forms and their expression is always accompanied by production of activins (β-subunit homodimers).

[0066] The present invention provides a method for generating bioactive inhibin proteins in the form of bioactive inhibin analogs. The precursor form is also proposed herein to be bioactive. The method comprises:

(i) modifying a proprotein convertase cleavage site on one or both of the α-subunit precursor and/or a β-subunit precursor thereby making proprotein convertase cleavage more efficient;

(ii) optionally introducing a mutation to eliminate the type I receptor (ALK4) binding epitope in the β-subunit mature domain thereby rendering inactive any activin formed;

(iii) optionally introducing a mutation to disrupt homodimerization;

(iv) transfecting a cell or cell line with nucleic acid molecules encoding the α-subunit and β-subunit precursors as defined in (i) and optionally (ii) and optionally (iii);;

(v) culturing the transfected cells or cell line for a time and under conditions sufficient for the inhibin precursor protein to be processed by a proprotein convertase and the processed protein released from the cell into conditioned medium; and

(vi) isolating the inhibin or its precursor form from the conditioned medium.

[0067] Both the ALK4 and dimerization interface mutations may be included or one or other or neither. The modified α-subunit precursor and β-subunit precursor may be referred to as a variant, mutant, modified protein or analog or any other term indicating that the amino acid sequence has undergone a modification. In addition to a modified proprotein convertase cleavage site, the amino acid sequence of the α- and/or β-subunit may have one or more other amino acid substitutions, additions and/or deletions. The inhibin is of mammalian origin including a human or non-human primate, a laboratory test animal such as a mouse, rat, rabbit, guinea pig or hamster, a farm animal such as a sheep, cow, pig, horse or deer or a companion animal such as a dog or cat. In an embodiment, the inhibin is of human origin. Reference hereinafter to amino acid positions in a human inhibin includes the equivalent position in a non-human inhibin.

[0068] Reference to an "inhibin" includes inhibin A comprising a heterodimer of an α-subunit and a β_A-subunit and inhibin B comprising a heterodimer of an α-subunit and a β_B-subunit. The modified inhibin precursor protein and the inhibin generated therefrom may be referred to herein as a "inhibin precursor protein analog" and "inhibin analog", respectively. Both are proposed herein to be bioactive. Modified inhibin A is

encompassed by the terms inhibin A variant, mutant, modified protein and analog. Modified inhibin B is encompassed by the terms inhibin B variant, mutant, modified protein and analog. This also applies to a modified inhibin A or inhibin B precursor protein. The inhibin A and/or inhibin B may also contain additional amino acid changes provided at least one proprotein convertase cleavage site on the α- and/or β-subunit precursor is rendered more efficiently cleavable by the convertase compared to wild-type α- and/or β-subunit precursors. Examples of additional mutations include an ALK4 mutation and a homodimerization interface mutation. In relation to homodimerization sites, these sites include A347, Y345, F326, V392, P393 and L396 in β_A-subunit (using the wild-type amino acid sequence in SEQ ID NO:2). Sites in β_B-subunit include F308, Y327, G329, I373, P374 and L377 (using the wild-type amino acid sequence in SEQ ID NO:12).

[0069] The inhibin α-subunit primary protein convertase cleavage site is at ^{229}RARR^{232} in human inhibin A (site1). When modified, a more efficient proprotein cleavage site is substituted comprising ISSRKKRSVSS (SEQ ID NO:18). At site 1 of α-subunit, the amino acid sequence is ^{229}ISSRKKRSVSS^{239}. The substituted cleavage site may further be modified by one or more amino acid substitutions, additions and/or deletions to the amino acid sequence set forth in SEQ ID NO:18. Hence, the substituted proprotein cleavage site may comprise SEQ ID NO:18 or an amino acid sequence having at least 80% similarity to SEQ ID NO:18 after optimal alignment. Reference to "at least 80%" includes 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 and 100%, or a percentage inbetween.

[0070] The α-subunit contains a secondary proprotein convertase site comprising ^{56}RRLPRR^{61} (site2). In an embodiment, the secondary cleavage site is substituted by ^{56}RRRRRR^{61} (SEQ ID NO:22). This amino acid sequence may comprise one or more amino acid substitutions, additions and/or deletions. The modification to change the cleavage sites are also referred to herein as "super-cut" variants. Generally, the primary cleavage site is referred to as super-cut site1 and the modified sequence is SEQ ID NO:18 and the secondary cleavage site is super-cut site2 with SEQ ID NO:22. The present invention may be practised with a wild-type α-subunit precursor, an α-subunit variant

precursor with a modified site1, an α-subunit variant precursor with a modified site2 or an α-subunit variant precursor with a modified site1 and modified site2.

[0071] In an embodiment, the α-subunit variant precursor comprises both modifications at site1 and site 2.

[0072] The α-subunit variant precursor may further comprise a poly-histidine tag comprising the amino acid sequence HHHHHHHHH (SEQ ID NO:20). Such a tag is useful in the purification by affinity chromatography and to distinguish wild-type and modified inhibins.

[0073] Additional mutation(s) may also be added at the interface site for homodimerization to occur for β_A + β_A dimerization into activin A and β_B + β_B dimerization into activin B. Such mutations reduce or inhibit altogether functional activin molecules being produced. Examples include $A347X_1$, $Y345X_2$, $F326X_3$, $V392X_4$, $P393X_5$ and $L396X_6$ wherein each of X_1, X_2, X_3, X_4, X_5 and X_6 is any amino acid except A, Y, F, V, P and L, respectively (based on numbering in SEQ I DNO:2) in subunit β_A; and $F308X_7$, $Y327X_8$, $G329X_9$, $I373X_{10}$, $P374X_{11}$ and $L377X_{12}$ wherein each of X_7, X_8, X_9, X_{10}, X_{11} and X_{12} is any amino acid except F, Y, G, I, P and L, respectively (using numbering in SEQ I DNO:12). Particular examples including A347H, Y345G and A347H + Y345G in β_A-subunit. In an embodiment, the amino acid sequence of β_A-subunit with an interface mutation is set forth in SEQ ID NO:40, 41 and 42 and includes an amino acid sequence having at least 80% similarity to any one of SEQ ID NO:40, 41 or 42 after optimal alignment provided the sequence comprises either a super-cut mutation and/or an interface mutation. The location of the mutations can be found in Tables 3 and 4. As above, reference to "at least 80%" includes 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 and 100% or a percentage inbetween. Any mutation which reduces or inhibits homodimerization may be introduced into β_A or β_B.

[0074] Hence, enabled herein is an inhibin α-subunit precursor comprising:

(i) a modified primary proprotein convertase site ("super-cut site1");

(ii) a modified secondary proprotein convertase site ("super-cut site2");

(iii) modified primary and secondary proprotein convertase sites; and

optionally together with a poly-histidine tag to assist with purification.

[0075] Further enabled herein is an inhibin β_A-subunit precursor comprising a modified proprotein convertase site ("super-cut site");

optionally with a mutation disrupting the ALK4 binding epitope in the mature domain and optionally with a FLAG tag to assist in affinity chromatography purification and optionally with a mutation disrupting or eliminating homodimerization.

[0076] The inhibin β_A-subunit carries a primary proprotein convertase cleavage site at ^{306}RRRRR^{310} in human inhibin A. In an embodiment this is modified to ISSRKKRSVSS (SEQ ID NO:18) which is a more efficient cleavage site. In β_A-subunit, the amino acid sequence is ^{306}ISSRKKRSVSS^{316}. As in the α-subunit, the substituted cleavage site may be further modified by one or more amino acid substitutions, additions and/or deletions to the amino acid sequence set forth in SEQ ID NO:18. Hence, the substituted proprotein cleavage site in β_A-subunit may comprise SEQ ID NO:18 or an amino acid sequence having at least 80% similarity to SEQ ID NO:18 after optimal alignment. As above, reference to "at least 80%" includes 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 and 100% or a percentage inbetween.

[0077] The β_A-subunit variant precursor may further comprise a poly-histidine tag comprising the amino acid sequence HHHHHHHHH (SEQ ID NO:20) such a tag is useful in the purification by affinity chromatography.

[0078] In addition, the β_A-subunit mature domain may further contain a mutation to disrupt the type I receptor (ALK4) binding epitope. This results in inactive activin A (comprising the homodimers β_A-β_A). In an embodiment, the mutation is a single point mutation comprising an M418A substitution (numbering from start of prodomain [see SEQ ID NO:2; equivalent to M432A in SEQ ID NO:4]; equivalent to M108A from start of mature domain; in β_B-subunit, the site is M399A (from start of prodomain [see SEQ ID

NO:12; equivalent to M410A in SEQ ID NO:14]). The effect of more efficient cleavage is an at least a 4 to 9 fold increase in cleavage compared to the wild-type protein. This includes a 4, 5, 6, 7, 8, or 9 fold increase. As indicated above, a mutation eliminating or reducing homodimerization of β_A or β_B may also be introduced (referred to as a dimerization interface mutation).

[0079] Taught herein is an inhibin β_B-subunit precursor comprising a modified proprotein convertase site ("super-cut site");

optionally with a mutation disrupting the ALK4 binding epitope in the mature domain and optionally with a FLAG tag to assist in affinity chromatography purification and optionally with a mutation disrupting or eliminating homodimerization.

[0080] The inhibin β_B-subunit carries a primary proprotein convertase cleavage site at ^{288}RIRKR^{292} in human inhibin B. In an embodiment this is modified to ISSRKKRSVSS (SEQ ID NO:18) which is a more efficient cleavage site. In β_B-subunit, the amino acid sequence is ^{288}ISSRKKRSVSS^{298}. As in the α-subunit, the substituted cleavage site may be further modified by one or more amino acid substitutions, additions and/or deletions to the amino acid sequence set forth in SEQ ID NO:18. Hence, the substituted proprotein cleavage site in β_B-subunit may comprise SEQ ID NO:18 or an amino acid sequence having at least 80% similarity to SEQ ID NO:18 after optimal alignment. Reference to the "at least 80%" is as defined above.

[0081] The β_B-subunit variant precursor may further comprise a poly-histidine tag comprising the amino acid sequence HHHHHHHHH (SEQ ID NO:20) such a tag is useful in the purification by affinity chromatography.

[0082] In addition, the β_B-subunit may further contain a mutation to disrupt the type I receptor (ALK4) binding epitope. This results in inactive Activin A (comprising the homodimers β_B-β_B). In an embodiment, the mutation is a single point mutation comprising an M399A substitution (see numbering in SEQ ID NO:12; equivalent to M410A in SEQ ID NO:14). A dimerization interface mutation may also be introduced to reduce or

eliminate homodimerization. Sites for homodimerization mutations are summarized in Tables 3 and 4.

[0083] The expression "at least 80%" has the same meaning as above.

[0084] Hence, to summarize the modifications, the α-subunit may comprise:

(i) a modified primary proprotein convertase site ("super-cut site 1"); and one or more of;

(ii) a modified secondary proprotein convertase site ("super-cut site 2"); and/or

(iii) a poly-histidine tag to assist with purification;

which α-subunit forms a heterodimer with a β_A- or β_B-subunit wherein the β_A- or β_B-subunit comprises:

(i) a modified proprotein convertase site ("super-cut site"); and one or more of:

(ii) a mutation disrupting the ALK4 binding epitope in the mature domain;

(iii) a FLAG tag to assist in affinity chromatography purification; and/or

(iv) a mutation disrupting or eliminating homodimerization.

[0085] In an embodiment, the α-subunit has (i), (ii) and (iii). In an embodiment, the β_A- or β_B-subunit has (i), (ii), (iii) and (iv). In an embodiment, all mutations are present in the α- and β-subunits together.

[0086] Enabled herein is an inhibin analog precursor protein comprising an α-subunit having the amino acid sequence as set forth in SEQ ID NO:8 (super-cut site1 + poly-his) or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:8 after optimal alignment with the proviso that it comprises at least one modified proprotein convertase site to enable more efficient cleavage by the convertase.

[0087] Enabled herein is an inhibin analog precursor protein comprising an β_A-subunit having the amino acid sequence as set forth in SEQ ID NO:4 (super-cut + ALK4 mutation + FLAG tag) or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:4 after optimal alignment with the proviso that it comprises at least one modified

proprotein convertase site to enable more efficient cleavage by the convertase.

[0088] In an embodiment, an inhibin analog precursor protein is provided comprising an β_A-subunit having the amino acid sequence as set forth in SEQ ID NO:40 (β_A-subunit super-cut + ALK4 mutation + FLAG tag + interface mutation [A347H]) or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:40 after optimal alignment with the proviso that it comprises at least one modified proprotein convertase site to enable more efficient cleavage by the convertase.

[0089] In an embodiment, an inhibin analog precursor protein is provided comprising an β_A-subunit having the amino acid sequence as set forth in SEQ ID NO:41 (β_A-subunit super-cut + ALK4 mutation + FLAG tag + interface mutation [Y345G]) or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:41 after optimal alignment with the proviso that it comprises at least one modified proprotein convertase site to enable more efficient cleavage by the convertase.

[0090] In an embodiment, an inhibin analog precursor protein is provided comprising an β_A-subunit having the amino acid sequence as set forth in SEQ ID NO:42 (β_A-subunit super-cut + ALK4 mutation +FLAG tag + combined interface mutation [A347H/Y345G]) or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:42 after optimal alignment with the proviso that it comprises at least one modified proprotein convertase site to enable more efficient cleavage by the convertase.

[0091] Taught herein is an inhibin analog precursor protein comprising an α-subunit having the amino acid sequence as set forth in SEQ ID NO:10 (super-cut site1 and 2 + poly-his) or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:10 after optimal alignment with the proviso that it comprises at least one modified proprotein convertase site to enable more efficient cleavage by the convertase.

[0092] Further contemplated herein is an inhibin analog precursor protein comprising an β_B-subunit having the amino acid sequence as set forth in SEQ ID NO:14 (super-cut +

ALK4 mutant + FLAG tag) or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:14 after optimal alignment with the proviso that it comprises at least one modified proprotein convertase site to enable more efficient cleavage by the convertase.

[0093] Hence, the α- and/or β-subunit amino acid sequence may have amino acid substitutions, additions and/or deletions in addition to the modified proprotein convertase site. Such amino acid changes are those which would not render the inhibin inactive. The present invention extends to nucleic acid molecules encoding the modified α- and β_A- or β_B-subunits.

[0094] Enabled herein is a nucleic acid molecule encoding an α-subunit of inhibin comprising the nucleotide sequence set forth in SEQ ID NO:7 (super-cut site1 and poly-his) or a nucleotide sequence having at least 80% identity thereto after optimal alignment or a nucleotide sequence capable of hybridizing to the complement of SEQ ID NO:7 under stringency conditions.

[0095] Further enabled here is a nucleic acid molecule encoding an α-subunit of inhibin comprising the nucleotide sequence set forth in SEQ ID NO:9 (super-cut site1 and poly-his) or a nucleotide sequence having at least 80% identity thereto after optimal alignment or a nucleotide sequence capable of hybridizing to the complement of SEQ ID NO:9 under stringency conditions.

[0096] A nucleic acid molecule encoding the β_A-subunit is encompassed by the present invention comprising the nucleotide sequence as set forth in SEQ ID NO:3 (super-cut site + ALK4 mutation + FLAG tag) or a nucleotide sequence having at least about 80% identity thereto after optimal alignment or a nucleic acid capable of hybridizing to the complement of SEQ ID NO:3 under stringency conditions.

[0097] Taught herein is a nucleic acid molecule encoding the β_B-subunit comprising the nucleotide sequence as set forth in SEQ ID NO:13 (super-cut site + ALK4 mutation +

FLAG tag) or a nucleotide sequence having at least about 80% identity thereto after optimal alignment or a nucleic acid capable of hybridizing to the complement of SEQ ID NO:13 under stringency conditions.

[0098] The nucleic acid molecule may be in isolated form or operably linked to a promoter and optionally a terminating sequence or other regulatory sequences.

[0099] A method for generating a bioactive inhibin analog is contemplated herein the method comprising co-expressing in a cell or cell line nucleic acid encoding the α-subunit and a β_A-subunit or β_B-subunit as defined herein for a time and under conditions sufficient for an inhibin precursor protein to be produced, cleaved by a proprotein convertase and secreted from the cell or cell line as a bioactive inhibin analog.

[0100] This leads to the generation of inhibin A analog or inhibin B analog.

[0101] In an embodiment, minimal bioactive activin A or activin B is produced.

[0102] In an embodiment, enabled herein is a process for generating a bioactive inhibin A, the method comprising transfecting a cell or cell line with a nucleic acid encoding an α-subunit precursor comprising the nucleotide sequence as set forth in SEQ ID NO:7 or a nucleic acid having at least 80% identity to SEQ ID NO:7 (super-cut site1 + poly-his) or is capable of hybridizing to the complement of SEQ ID NO:7 with the proviso that the nucleic acid comprises a modified proprotein cleavage site encoded by SEQ ID NO:17; and transfecting the cell or cell line with a nucleic acid encoding a β_A-subunit precursor comprising the nucleotide sequence as set forth in SEQ ID NO:3 (super-cut + ALK4 mutation + FLAG tag) or a nucleic acid having at least 80% identity to SEQ ID NO:3 or is capable of hybridizing to the complement of SEQ ID NO:3 with the proviso that the nucleic acid comprises a modified proprotein convertase cleavage site encoded by SEQ ID NO:17; and culturing the cells for a time and under conditions sufficient for the α-subunit and β_A-subunit variants to be processed by the proprotein convertase and to release inhibin A analog with minimal bioactive activin A.

[0103] In another embodiment, the α-subunit variant comprises the modified primary and secondary proprotein convertase cleavage sites. In an embodiment, enabled herein is a process for generating a bioactive inhibin A with minimal bioactive activin A, the method comprising transfecting a cell or cell line with a nucleic acid encoding an α-subunit precursor comprising the nucleotide sequence as set forth in SEQ ID NO:9 (super-cut site1 and 2 + poly-his) or a nucleic acid having at least 80% identity to SEQ ID NO:9 or is capable of hybridizing to the complement of SEQ ID NO:9 with the proviso that the nucleic acid comprises a modified proprotein cleavage site encoded by SEQ ID NO:17 and SEQ ID NO:21; and transfecting the cell or cell line with a nucleic acid encoding a β_A-subunit precursor comprising the nucleotide sequence as set forth in SEQ ID NO:3 (super-cut site + ALK mutation + FLAG tag) or a nucleic acid having at least 80% identity to SEQ ID NO:3 or is capable of hybridizing to the complement of SEQ ID NO:3 with the proviso that the nucleic acid comprises a modified proprotein convertase cleavage site encoded by SEQ ID NO:17; and culturing the cells for a time and under conditions sufficient for the α-subunit and β_A-subunit variants to be processed by the proprotein convertase and to release inhibin A analog with minimal bioactive activin A.

[0104] In relation to an inhibin B analog, again the α-subunit may contain only the primary proprotein cleavage site modified. Hence, taught herein is a process for generating a bioactive inhibin B with minimal bioactive activin B, the method comprising transfecting a cell or cell line with a nucleic acid encoding an α-subunit precursor comprising the nucleotide sequence as set forth in SEQ ID NO:7 (super-cut site1 + poly-his) or a nucleic acid having at least 80% identity to SEQ ID NO:7 or is capable of hybridizing to the complement of SEQ ID NO:7 with the proviso that the nucleic acid comprises a modified proprotein cleavage site encoded by SEQ ID NO:17; and transfecting the cell or cell line with a nucleic acid encoding a β_B-subunit precursor comprising the nucleotide sequence as set forth in SEQ ID NO:13 (super-cut + ALK mutation + FLAG tag) or a nucleic acid having at least 80% identity to SEQ ID NO:13 or is capable of hybridizing to the complement of SEQ ID NO:13 with the proviso that the nucleic acid comprises a modified proprotein convertase cleavage site encoded by SEQ ID

NO:17; and culturing the cells for a time and under conditions sufficient for the α-subunit and β_B-subunit variants to be processed by the proprotein convertase and to release inhibin B analog with minimal bioactive activin B.

[0105] Still in a further embodiment, the α-subunit comprises both the primary and secondary proprotein convertase cleavage sites modified. The subject specification is hence instructional for a process for generating a bioactive inhibin B with minimal bioactive activin B, the method comprising transfecting a cell or cell line with a nucleic acid encoding an α-subunit precursor comprising the nucleotide sequence as set forth in SEQ ID NO:9 (super-cut sites1 and 2 + poly-his) or a nucleic acid having at least 80% identity to SEQ ID NO:9 or is capable of hybridizing to the complement of SEQ ID NO:9 with the proviso that the nucleic acid comprises a modified proprotein cleavage site encoded by SEQ ID NO:17; and transfecting the cell or cell line with a nucleic acid encoding a β_B-subunit precursor comprising the nucleotide sequence as set forth in SEQ ID NO:13 (super-cut + ALK4 mutation + FLAG tag) or a nucleic acid having at least 80% identity to SEQ ID NO1:3 or is capable of hybridizing to the complement of SEQ ID NO:13 with the proviso that the nucleic acid comprises a modified proprotein convertase cleavage site encoded by SEQ ID NO:17; and culturing the cells for a time and under conditions sufficient for the α-subunit and β_B-subunit variants to be processed by the proprotein convertase and to release inhibin B analog with minimal bioactive activin B.

[0106] As indicated above, the amino acid sequence of α- and/or β-subunit precursor and/or mature form may have one or more amino acid substitutions, additions and/or deletions which do not render the inhibin inactive. Such changes may enhance activity or render the inhibin more stable. It is well understood in the art that some amino acids may be changed to others with broadly similar properties without changing the nature of the activity of the polypeptide. Variant polypeptides encompassed by the present invention are those which are biologically active, that is, they continue to possess the antagonistic biological activity of the propeptide towards an activin or the ability to confer higher affinity binding as described herein. Amino acid modifications are preferably conservative amino acid substitutions although additions and/or deletions may also be made. Examples

include the addition of homodimerization interface mutations.

[0107] Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the polypeptides can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA. 82:*488-492; Kunkel *et al.* (1987) *Methods in Enzymol, 154:*367-382; U.S. Patent No. 4,873,192; Watson *et al.* (1987) "Molecular Biology of the Gene", Fourth Edition, Benjamin/Cummings, Menlo Park, Calif. and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure*, Natl. Biomed. Res. Found., Washington, D.C.

[0108] Variant propeptides may contain conservative amino acid substitutions at various locations along their sequence, as compared to a parent (e.g. naturally-occurring or reference) amino acid sequence. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, which can be generally sub-classified as follows:

[0109] *Acidic*: The residue has a negative charge due to loss of H ion at physiological pH and the residue is attracted by aqueous solution so as to seek the surface positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium at physiological pH. Amino acids having an acidic side chain include glutamic acid and aspartic acid.

[0110] *Basic*: The residue has a positive charge due to association with H ion at physiological pH or within one or two pH units thereof (e.g. histidine) and the residue is attracted by aqueous solution so as to seek the surface positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium at physiological pH. Amino acids having a basic side chain include arginine, lysine and histidine.

[0111] *Charged*: The residues are charged at physiological pH and, therefore, include amino acids having acidic or basic side chains (i.e. glutamic acid, aspartic acid, arginine, lysine and histidine).

[0112] *Hydrophobic*: The residues are not charged at physiological pH and the residue is repelled by aqueous solution so as to seek the inner positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium. Amino acids having a hydrophobic side chain include tyrosine, valine, isoleucine, leucine, methionine, phenylalanine and tryptophan.

[0113] *Neutral/polar*: The residues are not charged at physiological pH, but the residue is not sufficiently repelled by aqueous solutions so that it would seek inner positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium. Amino acids having a neutral/polar side chain include asparagine, glutamine, cysteine, histidine, serine and threonine.

[0114] This description also characterizes certain amino acids as "small" since their side chains are not sufficiently large, even if polar groups are lacking, to confer hydrophobicity. With the exception of proline, "small" amino acids are those with four carbons or less when at least one polar group is on the side chain and three carbons or less when not. Amino acids having a small side chain include glycine, serine, alanine and threonine. The gene-encoded secondary amino acid proline is a special case due to its known effects on the secondary conformation of peptide chains. The structure of proline differs from all the other natural-occurring amino acids in that its side chain is bonded to the nitrogen of the α-amino group, as well as the α-carbon. Several amino acid similarity matrices (e.g. PAM120 matrix and PAM250 matrix as disclosed for example by Dayhoff *et al.* (1978) *supra*, A model of evolutionary change in proteins.

[0115] The degree of attraction or repulsion required for classification as polar or non-polar is arbitrary and, therefore, amino acids specifically contemplated by the invention

have been classified as one or the other. Most amino acids not specifically named can be classified on the basis of known behavior.

[0116] Amino acid residues can be further sub-classified as cyclic or non-cyclic, and aromatic or non-aromatic, self-explanatory classifications with respect to the side-chain substituent groups of the residues, and as small or large. The residue is considered small if it contains a total of four carbon atoms or less, inclusive of the carboxyl carbon, provided an additional polar substituent is present; three or less if not. Small residues are, of course, always non-aromatic. Dependent on their structural properties, amino acid residues may fall in two or more classes.

[0117] Conservative amino acid substitution also includes groupings based on side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. For example, it is reasonable to expect that replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid will not have a major effect on the properties of the resulting variant polypeptide. Whether an amino acid change results in a functional peptide polypeptide can readily be determined by assaying its activity. Amino acid substitutions falling within the scope of the invention, are, in general, accomplished by selecting substitutions that do not differ significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. After the substitutions are introduced, the variants are screened for biological activity as described herein.

[0118] Alternatively, similar amino acids for making conservative substitutions can be

grouped into three categories based on the identity of the side chains. The first group includes glutamic acid, aspartic acid, arginine, lysine, histidine, which all have charged side chains; the second group includes glycine, serine, threonine, cysteine, tyrosine, glutamine, asparagine; and the third group includes leucine, isoleucine, valine, alanine, proline, phenylalanine, tryptophan, methionine, as described in Zubay (1993) *Biochemistry*, third edition, Wm.C. Brown Publishers.

[0119] Thus, a predicted non-essential amino acid residue in a precursor inhibin polypeptide subunit is typically replaced with another amino acid residue from the same side chain family. A "non-essential" amino acid residue is a residue that can be altered from the reference sequence of an embodiment polypeptide without abolishing or substantially altering one or more of its activities. Suitably, the alteration does not substantially alter one of these activities, for example, the activity is at least 60%, 70%, 80%, 90% or 100% of the reference sequence. By contrast, an "essential" amino acid residue is a residue that, when altered from the wild-type sequence of a reference polypeptide, results in abolition of an activity of the parent molecule such that less than 20% of the activity of the reference polypeptide is present. In an embodiment, essential amino acid residues include those that are conserved in inhibin polypeptides across different species.

[0120] The propeptides of the present invention may be prepared by any suitable procedure known to those of skill in the art. Recombinant propeptides can be conveniently prepared using standard protocols as described for example in Sambrook *et al. Molecular Cloning:A Laboratory Manual, 2nd ed.*, Cold Spring Harbor, in particular Sections 13, 16 and 17; Ausubel *et al.* (1994) Current Protocols in Molecular Biology, John Wiley & Sons Inc, in particular Chapters 10 and 16; and Coligan *et al.* (1995-1997) Current Protocols in Protein Science, John Wiley & Sons, Inc., Chapters 1, 5 and 6. Methods of purification include size exclusion, affinity or ion exchange chromatography/separation. The identity and purity of peptides is determined for example by SDS-polyacrylamide electrophoresis or chromatographically such as by high performance liquid chromatography (HPLC). Alternatively, the propeptides or parts of the propeptides may be synthesized by chemical

synthesis, e.g. using solution synthesis or solid phase synthesis as described, for example, in Chapter 9 of Atherton and in Roberge *et al.* (1995) *Science*, 269:202).In some embodiments, the propeptides are prepared by recombinant techniques. For example, the inhibin analogs of the present invention may be prepared by a procedure including the steps of:(a) preparing a construct comprising a nucleic acid sequence that encodes an α- or β-subunit modified as herein described and that is operably linked to a regulatory element; (b) introducing (e.g. transfecting) the construct into a host cell or cell line; (c) culturing the host cell to express the nucleic acid to thereby produce the encoded subunit precursors; and (d) isolating the processed inhibin or its precursor from conditioned medium.

[0121] The invention also contemplates variants of the nucleic acid molecules encoding the subject modified propeptides including the dimerization domain. Nucleic acid variants can be naturally-occurring (native), such as allelic variants (same locus), homologs (different locus), and orthologs (different organism) or can be non naturally-occurring. Naturally-occurring nucleic acid variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as known in the art. Non-naturally occurring polynucleotide variants can be made by mutagenesis techniques, including those applied to polynucleotides, cells, or organisms. The variants can contain nucleotide substitutions, deletions, inversions and insertions. Variation can occur in either or both the coding and non-coding regions. The variations can produce both conservative and non-conservative amino acid substitutions (as compared in the encoded product) and glycosylation variants. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of a reference polypeptide. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis but which still encode a polypeptide. Variants of a particular nucleic acid sequence will have at least about 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs known in the art using default parameters.

[0122] The term "sequence identity" as used herein refers to the extent that sequences are identical on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g. A, T, C, G, I, U) or the identical amino acid residue (e.g. Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e. the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

[0123] A comparison window may comprise additions or deletions (i.e. gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerized implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA) or by inspection and the best alignment (i.e. resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as for example disclosed by Altschul *et al.* (1997) *Nucl. Acids Res. 25*:3389. A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel *et al.* (1994-1998) "Current Protocols in Molecular Biology", John Wiley & Sons Inc, Chapter 15.

[0124] In another aspect, the invention provides a purified nucleic acid molecule that comprises a nucleotide sequence encoding the herein described inhibin subunit precursors including variants having substantial sequence identity or ability to cross hybridise under stringent hybridization conditions, synonymous codon variants and codon optimized variants thereof.

[0125] As known in the art, "stringency" refers to the temperature and ionic strength conditions, and presence or absence of certain organic solvents, during hybridization and washing procedures. The higher the stringency, the higher will be the degree of complementarity between nucleic acid sequences that remain hybridized after washing. The term "*high stringency*" etc. refers to temperature and ionic conditions under which only nucleotide sequences having a high frequency of complementary bases will hybridize. The stringency required is nucleotide sequence dependent and depends upon the various components present during hybridization. The stringency conditions may be high, medium or low.

[0126] The nucleotide sequence may comprise codon substitution with a synonymous codon. The term "synonymous codon" as used herein refers to a codon having a different nucleotide sequence compared to another codon but encoding the same amino acid as that other codon. Codon optimization is standard in the art and is contemplated herein.

[0127] In another aspect the present specification provides nucleic acid constructs encoding a modified propeptide as described herein or a functional fragment thereof. Illustrative nucleic acid sequences characterizing the subject nucleic acid molecules are set out in Table 1 and include SEQ ID NO:3 (β_A-subunit with super-cut site + ALK4 mutation + FLAG tag), SEQ ID NO:13 (β_B-subunit with super-cut site + ALK4 mutation + FLAG tag), SEQ ID NO:7 (α-subunit super-cut site1 + poly-his) and SEQ ID NO:9 (α-subunit super-cut sites1 and 2 + poly-his).

[0128] The invention extends to vectors and other constructs comprising isolated nucleic acid molecules including those capable of expressing (producing) the subject inhibin subunit precursors and to isolated host cells or cell lines comprising same.

[0129] By "vector" is meant a polynucleotide molecule, suitably a DNA molecule derived, for example, from a plasmid, bacteriophage, yeast or virus, into which a nucleic acid molecule can be inserted or cloned. A vector may contain one or more unique restriction sites and can be capable of autonomous replication in a defined host cell including a target

cell or tissue or a progenitor cell or tissue thereof, or be integral with the genome of the defined host such that the cloned sequence is reproducible. Accordingly, the vector can be an autonomously replicating vector, i.e. a vector that exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, e.g. a linear or closed circular plasmid, an extra-chromosomal element, a mini-chromosome, or an artificial chromosome. The vector can contain any means for assuring self-replication. Alternatively, the vector can be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. A vector system can comprise a single vector or plasmid, two or more vectors or plasmids, which together contain the total DNA to be introduced into the genome of the host cell, or a transposon. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. In the present case, the vector is preferably a viral or viral-derived vector, which is operably functional in animal and preferably mammalian cells. Such vector may be derived from a poxvirus, an adenovirus or yeast. The vector can also include a selection marker such as an antibiotic resistance gene that can be used for selection of suitable transformants. Examples of such resistance genes are known to those of skill in the art and include the *nptII* gene that confers resistance to the antibiotics kanamycin and G418 [Geneticin (Registered Trade Mark)] and the *hph* gene that confers resistance to the antibiotic hygromycin B.

[0130] Vectors useful for expressing the subject nucleotide sequence in subject host cells *in vivo* are known to those of skill in the art and are expressly contemplated. They include adenoviral vectors and adeno-associates virus vectors. Illustrative vectors include AAV8 or AAV6 described for example in Qiao *et al.* (2008) *Human Gene Therapy 19:*000-000.

[0131] In another embodiment host cells are provided comprising a nucleic acid construct encoding a modified inhibin subunit precursors as described herein, wherein the host cell or cell line expresses the precursor.

[0132] Host cells are conveniently eukaryotic cells include mammalian, plant, yeast and

insect cells as known in the art. Recombinant proteins are produced by culturing the host cells for a period of time sufficient to allow for expression of the modified polypeptide inhibin subunits in the host cells or, more preferably, secretion of the protein into the culture medium in which the host cells are grown. Suitable mammalian cell lines include, but are not limited to, HEK293T, HEK293, HEK293T-Rex, BHK, VERO, HT1080, RD, COS-7, CHO, Jurkat, HUT, SUPT, C8166, MOLT4/clone8, MT-2, MT-4, H9, PM1, CEM, myeloma cells (e.g. SB20 cells) and CEMX174 are available, for example, from the ATCC. Other host cells include without limitation yeast, e.g. *Pichia pastoris*, or insect cells such as Sf9 cells although such molecules would not ordinarily be glycosylated.

[0133] In another aspect the present invention provides a method of treating or preventing activin-induced conditions, such as muscle wasting, fibrosis or inflammation in a subject, the method comprising administering to the subject an inhibin analog or precursor thereof as described herein or a nucleic acid construct encoding same which provides the modified α- and β-subunits to the subject. In an embodiment, the inhibin analog is inhibin A analog comprising an α-subunit having the amino acid sequence set forth in SEQ ID NO:7 or 9 or an amino acid sequence that is at least 80% identical to SEQ ID NO:7 or 9 and β_A-subunit having the amino acid sequence set forth in SEQ ID NO:3 or an amino acid sequence that is at least 80% identical to SEQ ID NO:3.

[0134] In another aspect the present invention provides a method of treating or preventing activin-induced conditions, such as muscle wasting, fibrosis or inflammation in a subject, the method comprising administering to the subject an inhibin analog or precursor thereof as described herein or a nucleic acid construct encoding same which provides the modified α- and β-subunits to the subject. In an embodiment, the inhibin analog is inhibin B analog or precursor comprising an α-subunit having the amino acid sequence set forth in SEQ ID NO:7 or 9 or an amino acid sequence that is at least 80% identical to SEQ ID NO:7 or 9 and β_A-subunit having the amino acid sequence set forth in SEQ ID NO:9 or an amino acid sequence that is at least 80% identical to SEQ ID NO:9.

[0135] In these embodiments, minimal bioactive activin A or B is generally produced. In

an embodiment, any similar inhibin generally comprises a β-subunit chain with a mutation in the ALK4 binding epitope. In an embodiment, homodimerization interface mutations are included in β_A- or β_B-subunits (for example, refer to Tables 3 and 4).

[0136] "Subjects" contemplated in the present invention are humans or mammals including laboratory or art accepted test or vehicle animals. "Subjects" include human subjects in need of treatment or prophylaxis.

[0137] Usefully, the present invention further provides compositions comprising an inhibin analog or its precursor or nucleic acid encoding inhibin subunits as herein described. The term "compound" includes "medicament", "agent", "therapeutic", "pharmacologically acceptable compound" and "pharmaceutical composition" and the like. In another embodiment, the composition comprises a pharmaceutically or physiologically acceptable carrier or diluent. In an embodiment, the inhibin analog or its precursor are for use in the treatment or prevention of conditions or symptoms of conditions promoted or exacerbated by activin signaling.

[0138] Pharmaceutical compositions are conveniently prepared according to conventional pharmaceutical compounding techniques. See, for example, Remington's Pharmaceutical Sciences (1990) 18th Ed., Mack Publishing, Company. These compositions may comprise, in addition to one of the active substances (inhibin analog or its precursor), a pharmaceutically acceptable excipient, carrier, buffer, stabilizer or other materials well known in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g. intravenous, oral or parenteral.

[0139] The inhibin analogs or their precursors or compositions comprising same are administered in an effective amount. The terms "effective amount" includes "therapeutically effective amount" and "prophylactically effective amount" and mean a sufficient amount of active either in a single dose or as part of a series or slow release system which provides the desired therapeutic, preventative, or physiological effect in

some subjects. Undesirable effects, e.g. side effects, may sometimes manifest along with the desired therapeutic effect; hence, a practitioner balances the potential benefits against the potential risks in determining an appropriate "effective amount". The exact amount of composition required will vary from subject to subject, depending on the species, age and general condition of the subject, mode of administration and the like. Thus, it may not be possible to specify an exact "effective amount". However, an appropriate "effective amount" in any individual case may be determined by one of ordinary skill in the art using routine skills or experimentation. The term "treatment" refers to any measurable or statistically significant amelioration in at least some subjects in one or more symptoms of a condition associated with disregulated or overactive activin signaling in a subject. Prophylactic administration of the compound serves to prevent or attenuate onset of symptoms of a condition associated with disregulated or overactive activin signaling in a subject. In an embodiment, the subject is a post-menopausal female subject with reduced levels of serum inhibin and has or is at risk of developing a bone disorder such as osteoporosis.

[0140] A "pharmacologically acceptable" composition is one tolerated by a recipient patient. A "pharmaceutically acceptable carrier and/or a diluent" is a pharmaceutical vehicle comprised of a material that is not otherwise undesirable i.e. it is unlikely to cause a substantial adverse reaction by itself or with the active composition. Carriers may include all solvents, dispersion media, coatings, antibacterial and antifungal agents, agents for adjusting tonicity, increasing or decreasing absorption or clearance rates, buffers for maintaining pH, chelating agents, membrane or barrier crossing agents. A pharmaceutically acceptable salt is a salt that is not otherwise undesirable. The agent or composition comprising the agent may be administered in the form of pharmaceutically acceptable non-toxic salts, such as acid addition salts or metal complexes.

[0141] For oral administration, the compositions can be formulated into solid or liquid preparations such as capsules, pills, tablets, lozenges, powders, suspensions or emulsions. In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils, alcohols, flavoring agents,

preservatives, coloring agents, suspending agents, and the like in the case of oral liquid preparations (such as, for example, suspensions, elixirs and solutions); or carriers such as starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations (such as, for example, powders, capsules and tablets). Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. Tablets may contain a binder such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate. If desired, tablets may be sugar-coated or enteric-coated by standard techniques. The active composition can be encapsulated to make it stable to passage through the gastrointestinal tract. See for example, International Patent Publication No. WO 96/11698.

[0142] For parenteral administration, the composition may be dissolved in a carrier and administered as a solution or a suspension. For transmucosal or transdermal (including patch) delivery, appropriate penetrants known in the art are used for delivering the composition. For inhalation, delivery uses any convenient system such as dry powder aerosol, liquid delivery systems, air jet nebulizers, propellant systems. For example, the formulation can be administered in the form of an aerosol or mist. The compositions may also be delivered in a sustained delivery or sustained release format. For example, biodegradable microspheres or capsules or other polymer configurations capable of sustained delivery can be included in the formulation. Formulations can be modified to alter pharmacokinetics and biodistribution. For a general discussion of pharmacokinetics, see, e.g., Remington's Pharmaceutical Sciences, 1990 (*supra*). In some embodiments the formulations may be incorporated in lipid monolayers or bilayers such as liposomes or micelles. Targeting therapies known in the art may be used to deliver the agents more specifically to certain types of cells or tissues.

[0143] The present invention further contemplates antibodies to the inhibin analogs, their precursors or their α- or β-subunits. Such antibodies are useful *inter alia* in affinity purification techniques as well as quenching agents to inhibit activity if required.

[0144] Polyclonal antibodies may be generated, however, the use of monoclonal antibodies is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production is derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation (i.e. comprising 35-LM polypeptide) or can be done by techniques which are well known to those who are skilled in the art. (See, for example, Douillard and Hoffman (1981) Basic Facts about Hybridomas, in *Compendium of Immunology* Vol. II, ed. by Schwartz; Kohler and Milstein (1975) *Nature 256:*495-499; Kohler and Milstein (1976) *European Journal of Immunology 6:*511-519). Single chain antibodies or transgenic mice expressing humanized antibodies or other recognition proteins may also be used. Useful proteins in this regard include diabodies, peptide mimetics and antibody fragments such as scFv fragments and Fab fragments.

[0145] Monoclonal antibodies which bind specifically to the inhibin analogs or their precursors or their α- or β-subunits provide a convenient method for detecting and targeting the cells which express the inhibin analogs. The presence of a particular inhibin analog or precursor form or subunit may be accomplished in a number of ways such as by Western blotting and ELISA procedures. A wide range of immunoassay techniques are available as can be seen by reference to U.S. Patent Nos. 4,016,043, 4,424,279 and 4,018,653. These include both single-site and two-site or "sandwich" assays of the non-competitive types, as well as in the traditional competitive binding assays. These assays also include direct binding of a labeled antibody to a target. Monoclonal antibodies may be used as antagonists of inhibin analog activity. They may also be formulated as a composition suitable for administration to an individual in a method of treatment or prophylaxis.

[0146] The antibodies of the present invention are useful in a range of other methodologies including flow cytometry, which typically detects optical parameters. For example, a flow cytometer may be used to determine forward scatter (which is a measure of size of a carrier), side scatter (which is sensitive to refractive index and size of a particle [see

Shapiro (1995) *"Practical flow cytometry"*, 3rd ed. Brisbane, Wiley-Liss]) and fluorescent emission.

EXAMPLES

[0147] Aspects disclosed herein are further described by the following non-limiting Examples. Certain technical details have since the priority date been included in Walton *et al.* (2016) *Endocrinology 157*:2799-2809 the entire contents of which are incorporated herein by reference.

Methods

Generation of mutant inhibins

[0148] The cleavage sites intervening the pro- and mature domains in the inhibin α- and β_A-subunits were modified using site-directed mutagenesis. A pCDNA3.1 (Invitrogen, Carlsbad, CA) vector containing either the full-length wild-type human inhibin α-subunit (sequence reference NM_002191.3), or the β_A-subunit (sequence reference NM_002192.2) served as the templates in these reactions. The native furin cleavage sites (RXXR) were replaced with an ideal theoretical site (ISSRKKRSVSS – SEQ ID NO:18) [Duckert *et al.* (2004) *Protein Eng Des Selec. 17*:107-112] to enhance the processing of pro-inhibin forms. This was achieved by overlap extension PCR, using designed 'super-cut' (SCUT) primers in combination with primers flanking the ORFs (primer details provided in Table 3) to enable cloning into compatible sites of pCDNA3.1. To aid purification, a poly-histidine tag was inserted at a previously determined permissive site (Walton *et al.* (2013) *Mol Cell Endocrinol. 381*:106-114), immediately prior to the pro:mature cleavage site. This was achieved using 'polyHIStag' primers in combination with flanking primers as outlined in Table 5, to enable cloning into pCDNA3.1. All constructs were verified by DNA sequencing.

[0149] Inhibin variants were produced by transient transfection in human embryonic kidney (HEK293T) cells using Lipofectamine 2000 (Invitrogen). In brief, cells were plated at 8×10^5 cells per well in 6-well plates. Wild-type or mutant α-subunit constructs were combined with β_A-subunit variants, and Lipofectamine 2000 was added according to the manufacturer's instructions. After a 20 minute incubation, DNA/Lipofectamine complexes

were added directly to the plated cells and incubated in serum-free Opti-MEM medium (Invitrogen) for a further 48 hours at 37°C in 5% v/v CO_2.

[0150] Western blotting was used to assess inhibin and activin forms in the conditioned medium from transfected HEK293T. At 48 hours after transfection, conditioned medium was combined with 4x LDS loading dye (Invitrogen), and non-reduced samples were separated by 10% w/v SDS-PAGE. After electrophoresis, samples were transferred onto ECL Hybond membranes (GE Healthcare, Giles, Buckinghamshire, UK). Membranes were blocked for a minimum of 1 hour in 1% w/v BSA in Tris-buffered Saline with 0.05% v/v Tween-20 (TBS-T). Inhibin forms were detected using antibodies to the inhibin β_A- (E4, binds residues 401-413 (19)), or α-subunit (R1, binds residues 233-264 [Groome *et al.* (1994) *Clin Endocrinol. 40*:717-723]). The E4 antibody was obtained from Beckman Coulter (Chaska, USA) or Oxford Brookes University (Oxford, UK), and requires an antigen retrieval step during incubation (6% v/v H_2O_2). Antibodies were incubated in 1% w/v BSA/TBS-T for 2 hours and excess antibody removed by multiple washes with TBS-T. Bound R1 and E4 antibodies were then detected by incubation with a mouse secondary antibody conjugated to horseradish peroxidise (mouse IgG-HRP, GE lifesciences). Following multiple washes, chemiluminescence was measured using Lumilight substrates (Roche Applied Sciences) and a Bio-Rad Chemidoc XRS System.

Quantification of inhibin

[0151] Inhibin A levels were determined using recently described inhibin ELISA (Walton *et al.* (2013) *supra*), which employs the β_A-subunit mAb (E4) as a capture antibody, and α-subunit mAb (R1) as the label. For the purposes of this study, all samples were assayed in the low-triton assay format, which favors detection of the mature or 'active' inhibin forms. Samples and standards were first treated with a low-triton assay buffer (0.1% v/v Triton X-100, 1% w/v BSA in TBS), and then oxidized using 1% v/v H_2O_2 (final concentration). Bound inhibin was labelled using biotinylated-R1 antibody diluted in the low-triton assay buffer. Following R1 labelling, plates were treated with Strep-HRP, then washed and developed with TMB substrate (3,3′,5,5′-tetramethylbenzidine, Invitrogen). The ELISA was stopped using 0.1 M H_2SO_4, and absorbance determined at 450 nM on a SpectraMax

plate reader (Molecular devices, CA). Assay sensitivity was 6 pg/ml. Purified 34 kDa inhibin A generated by the laboratory was used as a reference standards (Makanji *et al.* (2007) *Endocrinology 148*:2309-2316).

Quantification of activin

[0152] Activin A was measured using a specific ELISA (Oxford Bioinnovations). In brief, activin A standard and samples (diluted in 5% w/v BSA) in PBS, pH 7.4) were treated with SDS (final concentration 3% w/v) and boiled for 3 minutes. Once cooled, samples were treated with H_2O_2 (final concentration 2% v/v) and incubated for 30 minutes at room temperature. Samples were added to E4 antibody-coated plates and incubated for 1 hour at room temperature. Plates were then probed with biotinylated-E4 antibody and incubated overnight at room temperature. After washing, a streptavidin-horseradish peroxidase (HRP) conjugate was added to the wells and incubated at room temperature for 1 hour. Following further washes, HRP activity was detected with TMB substrate (3,3',5,5'-tetramethylbenzidine; Life Technologies).

Production and purification of inhibins by IMAC

[0153] Pro-inhibin forms were produced by transient transfection in HEK293T cells using lipofectamine 2000. In brief, cells were plated at $11x10^6$ cells per plate on 15 cm plates, and then transfected with an equal ratio of inhibin α- and β-subunit DNA constructs using Lipofectamine 2000 and Opti-MEM media (Invitrogen, according to the manufacturers protocol). Pro-inhibin was then isolated from conditioned media by IMAC immunoaffinity. Conditioned media (100 ml) was first concentrated (twice) using centricon devices with a 5 kDa molecular weight cut-off (Millipore) and resuspended in phosphate buffer (50 mM PO_4, 0.5 M NaCl, pH 8.0). Concentrated media was applied to a Nickel-NTA resin (Invitrogen) and incubated overnight at 4°C. Unbound protein was collected, and the resin washed 4 × with phosphate buffer. Bound inhibins were eluted with 0.5 M imidazole in phosphate buffer (50 mM PO_4, 0.5 M NaCl, pH 8.0). Imidazole was removed by buffer exchange on a PD-10 column (GE Healthcare), and 0.1% w/v BSA was applied to the preparations. IMAC purification was performed twice to enrich inhibins, and deplete contaminating activin forms. The recovery and yield of Pro-inhibin

preparations were determined by Western blot analysis (using R1 MAb), and inhibin ELISA, as described above.

Determination of inhibin bioactivity using FSH in vitro bioassays

[0154] The ability of the inhibin preparations to suppress activin-induced FSH release was examined in cultured rat pituitary cells (Scott *et al.* (1980) *Endocrinology 107*:1536-1542), and in a mouse pituitary gonadotrope cell line (LβT2). For the rat pituitary culture bioassay, the anterior pituitary glands of adult male Sprague Dawley rats (12 weeks) were enzymatically dispersed with trypsin and plated at 50,000 cells/well in 48-well plates in DMEM-Fl2 (Invitrogen) containing 10% v/v fetal calf serum. After incubation at 37°C in 5% v/v CO_2 for 48 hours, cells were washed with 0.1% BSA in DMEM-F12 and incubated for a further 4 hours. Cells were then treated with increasing doses of inhibins (5-800 pM) diluted in 0.1% w/v BSA in DMEM-F12 media. After 48 hours, the cell media were assayed for rat FSH by a specific rat FSH immunofluorometric assay (van Casteren *et al.* (2000) *Biol Reprod. 62*:886-894) using reagents provided from N.V. Organon, Oss, The Netherlands. The sensitivity of the *in vitro* bioassay was 75 pg/well using the highly purified 31-kDa inhibin A preparation (Makanji *et al.* (2007) *supra*).

[0155] For the LβT2 cell *in vitro* bioassay, cells were plated in 48-well plates at a density of 2.5×10^5 cells/well. The cells were allowed to recover for 24 hours in DMEM supplemented with 10% v/v FCS, and then treated with 150 pM activin A in the presence of increasing concentrations of inhibin (0.1-90 nM). Following a 24-hour incubation, the media was collected for FSH assay, as described above.

Smad phosphorylation

[0156] The ability of Pro-inhibin to suppress activin-induced Smad2 phosphorylation, relative to mature inhibin, was determined in LβT2 cells. Cells were seeded at 2×10^6 cells/well in poly-lysine coated six-well plates. The following day, the media was changed to DMEM, 0.2% v/v FCS and 50 mM HEPES, containing 200 pM activin A with increasing concentrations of inhibins (0.3-3 nM). After 30 minutes of treatment, cells were washed with PBS and lysed in 100 µl RIPA buffer (50 mmol/l Tris-base, 1% v/v Nonidet

P-40, 0.5% w/v deoxycholic acid, 0.1% w/v sodium dodecyl sulfate, and 0.9% w/v saline (pH 8.0)), containing protease inhibitor cocktail tablets (Roche Applied Sciences, Penzberg, Germany) and phosphatase inhibitors. Lysates were collected, clarified by centrifugation, and combined with reducing sample buffer (Life Technologies) and analyzed by Western blot. Phospho-Smad2 and Smad2 (Cell Signaling Technologies, Beverly, MA) antibodies were used at 1:2,000 dilutions. Bound primary antibodies were detected using goat anti-rabbit or sheep anti-mouse horseradish peroxidise conjugates (GE Healthcare Life Sciences, Pittsburgh, PA). These studies were undertaken on duplicate cultures.

Introduction of homodimerization interface mutations

[0157] This refers to Example 8. Sites of amino acids involved in homodimerization with β_A-subunit in β_B-subunit are provided in Tables 3 and 4, respectively. Particular examples include A347H and Y345G in β_A-subunit. These amino acid positions are based on the wild-type β_A-subunit sequence (SEQ ID NO:2). The equivalent positions in variant β_A-subunit are A361H and Y359G, respectively.

Inhibin B analogs

[0158] The same approach for generating modified inhibin α- and β_A-subunits, was applied to the generation of β_B-subunits which were then used with modified α-subunits to generate inhibin B analogs.

Table 5

Primer details for the construction of mutant inhibins

Primer reference	Sequence 5'→3'	SEQ ID NO:
α- NHEI –sense	ctaggctagcatggtgctgcacctactgctcttc	23
α- ECORI -antisense	ctaggaattcttagatacaagcacagtgctgcg	24
α-SCUT$_{(site1)}$-sense	cgatgagactgagcgtttctttctcgatgagatctcccctccactg ggtggtctggtccgagtgtg	25
α-SCUT$_{(site1)}$-antisense	atctcatcgagaaagaaacgctcagtctcatcgactcccctgatg tcctggccttggtctccctct-	26
α-SCUT$_{(site2)}$-sense	ccctggagtcaggcggcgacgtcgaagacatgccc	27
α-SCUT$_{(site2)}$-antisense	gggcatgtcttcgacgtcgccgcctgactccaggg	28
α-polyHIStag-sense	atctcatcgagaaagaaacgctcagtctcatcaactcccctgatg tcctggccttggtctcc	29
α-polyHIStag -antisense	tgagactgagcgtttctttctcgatgagatgtgatgatggtggtga tggtgatgatgctccc	30
βA-XBAI -sense	ctagtctagaatgcccttgctttggctgagagg	31
βA-NOTI -antisense	gctagcggccgcctatgagcacccacactcctccacgatc	32
βA-SCUT$_{(site1)}$-sense	atctcatcgagaaagaaacgctcagtctcatcgggcttggagtgt gatggcaaggtcaacatctgc	33
βA-SCUT$_{(site1)}$-antisense	cgatgagactgagcgtttctttctcgatgagatatgagggtggtct tcagactgccgggcctgcag	34
βA-M418A-sense	gacattcagaacgcgatcgtggaggag	35
βA- M418A –antisense	ctcctccacgatcgcgttctgaatgtc	36

EXAMPLE 1

Enhanced processing of the α-subunit favors inhibin production over activin

[0159] The inhibin α- and β-subunits comprise an N-terminal prodomain and a C-terminal mature domain, separated by a proprotein convertase cleavage site (Figure 1A). Following dimerization, the mature inhibin ligand is enzymatically released from its prodomain, enabling bioactivity. However, recombinant production of inhibin A indicates that processing by members of the proprotein convertase family is inefficient as significant amounts of unprocessed pro-inhibin A and full-length 50kDa α-subunit are present in conditioned media, with relatively little mature 31-34kDa inhibin A (Figure 1B, *lane 1*). Further confounding inhibin production/purification are the high levels of mature activin A that are co-produced when HEK293T cells are transfected with wild-type α- and β-subunits (Figure 1C, *lane 1*). To improve enzymatic processing of the α-subunit, the endogenous cleavage site (^{229}RARR^{232}) was replaced with an ideal proprotein convertase cleavage site (^{229}ISSRKKRSVS^{238} – SEQ ID NO:18) [Figure 1A] and this "super-cut" variant was expressed in HEK293F cells. The super-cut form of the inhibin α-subunit resulted in a marked increase in the levels of bioactive inhibin A (Figure 1B, *lane 2*) and a surprising decrease in mature activin A production (Figure 1C, *lane 2*). Specific ELISAs identified similar changes in total inhibin and activin levels (Figure 1D and E).

[0160] Subsequently, the same enhanced cleavage site was introduced into the β_A-subunit and showed that in combination with the wild-type α-subunit this modification also increased mature inhibin A production (Figure 1B, *lane 3*), although mature activin A levels were not altered (Figure 1C, *lane 3*). Combining super-cut versions of both the α- and β_A-subunits led to nearly complete processing of precursor proteins and the greatest proportional ratio of inhibin:activin production (69:1) [Table 6]. These results support the concept that processing of the α-subunit is the limiting step in inhibin production, relative to activin.

Table 6

Ratio of inhibin:activin produced following transient transfection of HECK293F cells with wild-type and super-cut constructs

α-subunit	βA-subunit	Ratio Inhibin:Activin (Mean±SD)
WT	WT	1.5 ± 0.7
SCUT	WT	24.8 ± 0.3
WT	SCUT	2.2 ± 0.3
SCUT	SCUT	68.7 ± 7.3

EXAMPLE 2

Overexpressing the α-subunit greatly increases inhibin production

[0161] In initial experiments, HEK293T cells were transfected with an equal ratio of super-cut α- and β_A-subunit DNA. Varying this ratio (from 4:1 to 1:4) had interesting effects on inhibin and activin production (Figure 6). At a 4:1 or 3:2 ratio of α:β_A-subunit, mature inhibin A levels were relatively high (Figure 6A and C), whereas mature activin A levels were negligible (Figure 6B and D). Interestingly, when the transfection ratio favored β_A-subunit expression (2:3 or 1:4 ratio of α:β_A-subunit) 3-4-fold higher levels of mature inhibin A were actually produced (Figure 6A and C). However, increased β_A-subunit expression was also accompanied by significant activin production (Figure 6B and D). Therefore, a 3:2 ratio of α:β_A-subunit was chosen for all future experiments.

EXAMPLE 3

Modifying the secondary cleavage site in the α-subunit further enhances inhibin A production

[0162] When large-scale production of inhibin A was commenced using the modified α- and β_A-subunit constructs, an increase in free 50kDa α-subunit was noted (Figure 2B) relative to the earlier small-scale transfections (Figure 1B). The α-subunit precursor has a second cleavage site at the N-terminus (Figure 2A), which is critical for inhibin production (Walton *et al.* (2015) *Endocrinology 156*:3047-3057). Here, this secondary cleavage site (^{56}RRLPRR^{61}) was replaced with an improved proprotein convertase cleavage site (^{56}RRRRRR^{61} – SEQ ID NO:22) [Figure 2A] and this super-cut-2 variant was expressed together with the modified β_A-subunit in HEK293T cells. Western analysis showed that these modifications abrogated expression of the free α-subunit and increased mature inhibin A expression (Figure 2B, *lane 3*). Densitometry indicated that improving processing at both sites in the α-subunit increased mature inhibin production 9-fold relative to wild-type and 2-fold relative to the initial super-cut variant (Figure 2D). Mature activin A levels were 12.5-fold lower than wild-type (Figure 2C and E). Thus, improving both processing sites in the α-subunit results in a significant increase in mature inhibin A production and a corresponding decrease in activin A. See also Figures 8 and 9.

EXAMPLE 4

Elimination of contaminating activin bioactivity by a single point mutation in the β_A-subunit

[0163] By improving the processing capacity of the inhibin α-subunit and optimizing the transfection ratio of α:β_A-subunit DNA, inhibin production was greatly favored over that of related activins. However, any residual activin A produced would retain bioactivity and, therefore, have the potential to counteract inhibins' effects. To eliminate contaminating activin Bioactivity, a single point mutation (M418A) [the amino acid position is from the prodomain start site (SEQ ID NO:2), the equivalent site from start to the mature domain is M108A; the site is M432A in SEQ ID NO:4 and Figure 7] was incorporated into the β_A-subunit at the type I receptor (ALK4) binding epitope (Figure 3A) [Harrison *et al.* (2003) *J Biol Chem. 278*:21129-21135]. The M418A mutation did not affect the amounts of inhibin and activin produced by HEK293T cells (Figure 3B and C), however, it resulted in an 8-fold increase in inhibin activity, as measured by the suppression of activin-induced FSH release by LβT2 mouse gonadotrope cells (Figure 3D). To demonstrate that this difference in activity was due to suppression of the residual activin response, HEK293F cells were transfected with an activin-responsive luciferase reporter and treated cells with the inhibin A preparations. This assay system is extremely sensitive to activin stimulation, but does not respond to inhibin due to low betaglycan expression. Inhibin A-SCUT induced a dose-dependent increase in luciferase activity, confirming the presence of active activin A in this preparation, whereas, inhibin A-SCUT (M418A) exhibited a 100-fold lower activin response (Figure 3E). Thus, a single point mutation in the β_A-subunit (M418A) further increases inhibin A activity by inactivating the residual activin A produced. Figure 7 provides the nucleotide and amino acid sequences of the human inhibin β_A-subunit super-cut variant with the ALK4 mutation.

EXAMPLE 5

One-step purification of mature inhibin A

[0164] *S*tudies have shown that mature TGF-β proteins are secreted from cells non-covalently associated with their prodomains (Harrison *et al.* (2011) *Growth Factors 29*:174-186; Sengle *et al.* (2008) *J Mol Biol. 381*:1025-1039; Sengle *et al.* (2011) *J Biol Chem. 286*:5087-5099). To determine if pro-inhibin A was secreted as a non-covalent complex, a HIS-tag was incorporated at the C-terminus of the α-subunit prodomain immediately preceding the super-cut-processing site. Conditioned media from HEK293T cells transfected with HIS-tagged super-cut α-subunit and super-cut β_A-subunit was purified by IMAC affinity chromatography and analyzed by Western blot. More than 90% of mature inhibin A present in the starting material was co-purified with the HIS-tagged prodomain (Figures 4A and B), confirming that the majority of inhibin A remains non-covalently associated with its prodomain upon secretion from the cell.

EXAMPLE 6

Pro-inhibin A is a more potent activin antagonist than mature inhibin A in specific settings

[0165] Using FSH release assays in both primary rat pituitary cells and the LβT2 cell line, the biological activity of IMAC-purified pro-inhibin A and HPLC-purified mature inhibin A (Makanji *et al.* (2007) *supra*) were compared. In primary pituitary cells, pro-inhibin A induced a dose-dependent decrease in FSH release (IC_{50} 75 pM), which was similar to that observed with mature inhibin A (IC_{50} 65 pM) [Figure 5A]. Any possible positive influence of the prodomain in this assay system may have been masked by the very potent activity of inhibin A. Therefore, the comparison of the pro- and mature inhibin isoforms was repeated on FSH release by LβT2 pituitary gonadotrope cells, which are significantly less responsive to inhibin due to low levels of betaglycan expression (Makanji *et al.* (2008) *J Biol Chem. 283*:16743-16751). Remarkably, pro-inhibin A (IC_{50} 45 pM) was 22-fold more potent than mature inhibin A (IC_{50} 1 nM) in this assay system (Figure 5B). These differing activities were mirrored intracellularly where mature inhibin A only partially blocked

activin-induced Smad2 phosphorylation, while pro-inhibin A completely inhibited this response (Figure 5C). Together, these results indicate that in less responsive systems, inhibin A activity is significantly enhanced when non-covalently associated with its prodomain.

EXAMPLE 7

Generation of inhibin B variants

[0166] The same methods were applied to generate modified inhibin β_B-subunits and therefore, in conjunction with a modified α-subunit an inhibin B analog. The same super-cut and M→A substitution (M399A in SEQ ID NO:12 which is equivalent to M410A in SEQ ID NO:14) were included resulting in enhanced inhibin B production with reduced activin B co-production. The results are shown in Figure 11. The amino acid sequence of mutated β_B-subunit is shown in Figure 10. It should be noted that a Ser His Arg sequence was removed just prior to the super-cut site. Figure 14 is the same sequence but with a modified signal nucleotide sequence. This does not result to any change to the amino acid sequence.

EXAMPLE 8

Improvements in the protocol to produce inhibins

[0167] In the previous Examples, a method is described to produce *inter alia* inhibins while limiting contaminating activin formation. Certain aspects have since been published by Walton *et al.* (2016) *supra.* In brief, the Examples showed *inter alia* that by improving processing of the inhibin α-subunits activin co-production can be minimized. Additionally, any residual activin activity is suppressed by modifying the type I receptor binding site on the β_A-subunit (M418A mutation).

[0168] In this Example, the procedure is further advanced using targeted mutagenesis. It is found that mutation of key residues in the β-subunit disrupted activin β/β-dimerization (homodimerization) at the binding interface without impacting inhibin α/β formation. This

finding represents a significant advance for the generation of inhibins, and for progressing their development for clinical applications. Hence, one or more homodimerization interface are introduced to reduce or eliminate homodimerization and therefore active activin production. Sites on β_A-subunit involved in homodimerization are shown in Table 3. Particular examples are A347H and Y345G. Similar sites in β_B-subunit are listed in Table 4. The amino acid position is based on wild-type sequences (SEQ ID NO:2 for β_A-subunit and SEQ ID NO:12 for β_B-subunit).

[0169] Using *in vitro* mutagenesis, residues are mutated in the inhibin β_A-subunit that lie in the predicted β/β homodimerization interface. Mutations were introduced into the inhibin β_A-supercut construct (which has heightened processing [Example 1]). The inhibin β_A-subunit variants were then co-transfected with the inhibin α-subunit (supercut form) into HEK293T mammalian cells, and the resultant inhibin/activin expression examined by Western blot and ELISAs. As seen in previous Examples (and reported in Walton *et al.* (2016) *supra*), enhanced processing of the inhibin subunits (supercut forms), resulted in an increased yield of mature inhibin relative to activin (compare lanes 1 and 2, Figure 12A). Mutation of key residues in the βA-subunit (e.g. A347H) disrupted activin formation – evidenced by decreased dimeric activin (Figure 12A, lanes 3 and 5). The A347H and Y345G mutations appeared to be most disruptive for β/β-dimer formation, but did not limit inhibin synthesis (Figures 12A and B).

[0170] To ascertain that the newly generated inhibin mutants retained biological activity, the A347H mutant was purified following production in mammalian cells (as outlined in Walton *et al.* (2016) *supra*). The activity of this inhibin variant was then tested in a luciferase reporter assay – in which mammalian cells were first transfected with an activin-responsive luciferase reporter, and betaglycan (inhibin co-receptor), and then treated with activin A +/- inhibin A (A347H). The introduced A347H mutation did not hinder inhibin's ability to suppress activin-induced luciferase activity, as this variant had comparable activity to the unmodified inhibin form (Figure 12C).

[0171] Next, the most promising inhibin mutants (A347H/Y345G), which exhibited disrupted activin formation. To this end, a double mutation A347H/Y345G was introduced into the β_A-subunit, and the resultant effect on ligand synthesis and activity examined (as described above). Co-introduction of the A347H/Y345G mutations into the inhibin β_A-subunit essentially abolished the production of activins – as determined using an antibody directed to the activin β_A-subunit (see arrow, Figure 13A, lane 3). The dual mutation had no effect on inhibin production, relative to the supercut variant (compare lanes 1 and 3, Figure 13A). Analysis indicates that Inhibin (A347H/Y345G) has comparable activity to wild type inhibin (Figure 13B).

[0172] Hence, inhibin α- and β_A-subunits have been engineered to allow the production of inhibin in the absence of contaminating activin. Using a systematic approach, the approach has: (1) improved the processing of the inhibin precursor forms, resulting in higher proportions of mature active inhibins; (2) streamlined the purification process to allow the study of inhibins under native conditions; (3) disrupted homodimerization of the β-subunit, and consequently, enhanced inhibin α/β heterodimerization; and (4) inactivated any residual activin by silencing its receptor binding capacity. Together, these steps allow for the targeted production of bioactive inhibins, with yields superior to any process described to date.

[0173] These advances allow the full elucidation of the importance of inhibins in physiological processes, both within and outside the reproductive system, and provide a platform to generate recombinant inhibins for clinical applications.

EXAMPLE 9

Therapeutic potential of inhibins

[0174] Inhibin A and B have the potential to be utilized therapeutically since declining levels of inhibin occur during menopause transition which not only correlated with an increase in FSH, but also a rapid decrease in bone mass (Perrien *et al.* (2006) *supra*; Perrien *et al.* (2007) *supra*). Subsequently, Perrien *et al.* (2007) *supra* used a transgenic approach to show that inhibin A acts as a potent stimulator of bone mass and strength, which could also prevent bone loss associated with gonadectomy. The anabolic effects of inhibin A have been confirmed in a murine model of distraction osteogenesis (Perrien *et al.* (2012) *supra*). The ability of inhibin A to enhance bone repair and regeneration is likely *via* antagonism of activin A, which potently inhibits osteoblast differentiation and matrix mineralization*in vitro* and *in vivo* (Lotinun *et al.* (2012) *supra*). This also applies to inhibin B. In support, blocking activin A signaling, using soluble ActRIIA, increases bone formation and improves skeletal integrity in both normal and ovariectomized mice (Chantry *et al.* (2010) *J Bone Min Res. 25*:2633-2646; Pearsall *et al.* (2008) *Proc Natl Acad Sci USA 105*:7082-7087). Although soluble ActRIIA cannot be used therapeutically because of off-target effects, restoring circulating inhibin levels in post-menopausal women is considered an attractive approach for the treatment of osteoporosis.

[0175] To date, the production and purification of inhibin in therapeutic amounts has proven difficult due to:(i) the inefficient processing of the heterodimeric precursor to the bioactive mature form; (ii) the short *in vivo* half-life of mature inhibin A (Makanji *et al.* (2009) *supra*); and (iii) the effort required to remove contaminating activins (Makanji *et al.* (2007) *supra*). To improve processing, in accordance with the present invention, the focus was on the inhibin α- and β_A-subunit prodomains. It is generally accepted within the TGF-β superfamily that prodomains govern the correct folding of dimeric precursors within the endoplasmic reticulum (Constam (2014) *Stem Cell Dev Biol. 32*:85-97). TGF-β precursors then acquire complex carbohydrate modifications during exocytosis, indicating transit through the trans-Golgi network. Once the correct tertiary structure is achieved, TGF-β

precursors are proteolytically matured by proprotein convertases, either in a late Golgi compartment or extracellularly (Constam (2014) *supra*). For inhibin and activin, PC5/6 and furin have been identified as the processing enzymes (Antenos *et al.* (2011) *PloS one. 6*:e17348; Antenos *et al.* (2008) *J Biol Chem. 283*:33059-33068). However, the high levels of unprocessed inhibin and the low amounts of mature inhibin relative to activin, produced by HEK293T cells, suggested that α-subunit processing is less efficient than that of the β_A-subunit. Therefore, in order to generate inhibin A and B more efficiently the endogenous α-subunit cleavage site (^{229}RARR^{232}) was replaced with an ideal proprotein convertase cleavage site (^{229}ISSRKKRSVS^{238} – SEQ ID NO:18) [Duckert *et al.* (2004) *supra*]. As anticipated, this modification significantly increased the amount of mature inhibin produced by HEK293T cells; however, it also resulted in a dramatic decrease in activin production. As dimerization proceeds processing, this finding indicates that the "super-cut" site introduced into the α-subunit induced a conformational change, which greatly facilitated inhibin A production, relative to activin A.

[0176] Improved inhibin A production was accompanied by a substantial increase in the amount of α-subunit precursor. This 50 kDa species represents free α-subunit that has not dimerized with β_A-subunit and, therefore, has not been processed by proprotein convertases. Interestingly, the inhibin α-subunit is one of a small subset of TGF-β precursors that are cleaved at two distinct proprotein convertase motifs (Shi *et al.* (2011) *Nature 474*:343-349). The second cleavage site (^{56}RRLPRR^{61}) within the α-subunit precursor releases a 43 amino acid fragment that can limit inhibin activity (Walton *et al.* (2015) *supra*). Processing at site2 was absolutely required for the synthesis and secretion of inhibin A and B (Walton *et al.* (2015) *supra*). Incorporating enhanced proprotein convertase cleavage sites at both site1 and site2 of the α-subunit led to a 9-fold increase in mature inhibin A production, ostensibly due to complete dimerization and processing of the α-subunit. Remarkably, enhanced inhibin production was accompanied by a 12.5-fold decrease in activin A levels. Thus, by incorporating improved cleavage sites within the α- and β_A-subunits a regime to produce high levels of bioactive inhibin A has been developed. This also applies to inhibin B.

[0177] Although the modifications had greatly improved the ratio of inhibin A:activin A produced by HEK293F cells, the remaining activin retained biological activity. Therefore, a secondary mutation was incorporated within the β_A-subunit (M418A). Met^{418} resides at the type I receptor (ALK4) interface of activin A and mutating this residue to alanine disrupts activin Activity without affecting binding to ActRIIA/IIB (Harrison *et al.* (2003) *supra*). In the context of inhibin, the M418A β_A-subunit mutation did not affect expression of the super-cut variant, but enhanced activity 8-fold. The improved activity was due to a nearly complete inactivation of residual activin A. Thus, inhibin A and inhibin B can be produced in the virtual absence of contaminating activin Activity, which has long been the major obstacle to the use of inhibin as a therapeutic.

[0178] A further modification incorporated into inhibin A was a HIS-tag at the C-terminus of the α-subunit prodomain. Mature inhibin A (M418A) was co-purified using HIS affinity chromatography, indicating that it is secreted by HEK293F cells in a non-covalent complex with its prodomain (termed pro-inhibin A). This brings the number of TGF-β proteins shown to remain associated with their prodomains extracellularly to 13 (Harrison *et al.* (2011) *supra*; Sengle *et al.* (2008) *supra*; Sengle *et al.* (2011) *supra*; Shi *et al.* (2011) *supra*; Mottershead *et al.* (2015) *J Biol Chem 290*:24007-24020; Robertson *et al.* (2015) *Matrix Biol. 47*:44-53), suggesting this is the default manner in which these proteins are secreted from cells. For most family members, prodomains localized mature growth factors in the vicinity of target cells; however, the affinity of the interaction is not sufficient to suppress biological activity (Harrison *et al.* (2011) *supra*; Sengle *et al.* (2011) *supra*). In contrast, the TGF-β isoforms, myostatin and GDF-11 bind their prodomains with high affinity and are secreted from the cell in a latent form (Shi *et al.* (2011) *supra*). Interestingly, it was found that pro-inhibin A was either equipotent (primary rat pituitary cells) or had enhanced activity (LβT2 pituitary gonadotrope cells) compared to HPLC-purified mature inhibin A. This is likely due to the prodomain facilitating inhibin A binding to its cell surface receptors, betaglycan and ActRIIA/B. In support, the prodomain of cumulin (BMP15:GDF9 heterodimer) was recently shown to be indispensable for this growth factors positive effects on oocyte quality (Mottershead *et al.* (2015) *supra*).

[0179] Yet another mutation disrupts the homodimerization interface site on the β subunits. For example, substitutions at amino acids 347 and 345 of β_A resulted in virtually zero activin A activity. In an embodiment, the individual substitutions are A347H and Y345G. These may be used singularly (e.g. SEQ ID NOs:40 and 41) or in combination (e.g. SEQ ID NO:42). Functionally equivalent mutations can also be made in β_B. Other interface sites are listed in Tables 3 and 4.

[0180] Hence, introduction of a series of mutations into the α- and β_A-/β_B-subunits increase mature inhibin A/B expression, dramatically decreased and inactivated contaminating activin A/B and streamlined the purification process. It is now possible to generate sufficient quantities of recombinant inhibin A and inhibin B to fully explore the considerable therapeutic potential of this molecule on bone and other tissues.

[0181] Those skilled in the art will appreciate that the disclosure described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the disclosure contemplates all such variations and modifications. The disclosure also enables all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of the steps or features or compositions or compounds.

[0182] All patents, applications, publications, test methods, literature, and other materials cited herein are hereby incorporated by reference in their entirety as if physically present in this specification.

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CLAIMS:

1. A mammalian inhibin analog precursor protein comprising a heterodimer of α-subunit and a β-subunit precursors each having proprotein convertase cleavage sites wherein at least one proprotein convertase cleavage site is modified by an amino acid substitution mutation to render it more efficiently cleaved by the proprotein convertase to generate bioactive inhibin wherein the modified proprotein convertase cleavage site is ISSRKKRSVSS (SEQ ID NO:18).

2. The inhibin analog precursor protein of Claim 1 wherein a proprotein convertase cleavage site in each of the α-subunit and a β-subunit precursors is modified by the amino acid substitution mutation.

3. The inhibin analog precursor protein of Claim 1 or 2 wherein the inhibin is of human origin.

4. The inhibin analog precursor protein of Claim 3 wherein the α-subunit protein further comprises a secondary proprotein convertase cleavage site which is modified by an amino acid substitution mutation to render it more efficiently cleaved by the proprotein convertase.

5. The inhibin analog precursor protein of Claim 4 wherein a primary proprotein convertase site in the α-subunit at ^{229}RARR^{232} is replaced by ^{229}ISSRKKRSVSS^{239}.

6. The inhibin analog precursor protein of Claim 4 wherein a secondary site in the α-subunit at ^{56}RRLPRR^{61} is replaced by ^{56}RRRRRR^{61}.

7. The inhibin analog precursor of Claim 5 or 6 further comprising a poly-his tag.

8. The inhibin analog precursor protein of Claim 4 wherein the β-subunit is a β_A-subunit.

9. The inhibin analog precursor protein of Claim 8 wherein the cleavage site ^{306}RRRRR^{310} in the β_A-subunit is replaced with the amino acid sequence ^{306}ISSRKKRSVSS^{316}.

10. The inhibin analog precursor protein of Claim 4 wherein the β-subunit is a β_B-subunit.

11. The inhibin analog precursor protein of Claim 10 wherein the cleavage site ^{288}RIRKR^{292} in the β_B-subunit is replaced by the amino acid sequence ^{288}ISSRKKRSVSS^{298}.

12. The inhibin analog precursor protein of any one of Claims 1 to 11 wherein the β-subunit mature domain further comprises a single point mutation within a type I receptor (ALK4) binding epitope.

13. The inhibin analog precursor protein of Claim 12 wherein the β_A-subunit comprises an M418A substitution mutation.

14. The inhibin analog precursor protein of Claim 12 wherein the β_B-subunit comprises an M410A substitution mutation.

15. The inhibin analog precursor protein of any one of Claims 8 to 14 wherein the β_A- or β_B-subunit further comprises a FLAG tag comprising the amino acid sequence DYKDDDK (SEQ ID NO:16) between amino acids 27 and 28 of β_A or 28 and 29 of β_B.

16. The inhibin analog precursor protein of any one of Claims 1 to 15 further comprising a mutation at a homodimerization interface site on β_A- or β_B-subunit to reduce or eliminate the production of an active activin A or B.

17. The inhibin analog precursor of Claim 16 wherein the mutation on β_A-subunit is a substitution mutation selected from the group consisting of $A347X_1$, $Y345Y_2$, $F326X_3$, $V392X_4$, $P393X_5$ and $L396X_6$ using the numbering system of SEQ ID NO:2 wherein each of X_1, X_2, X_3, X_4, X_5 and X_6 is any amino acid except A, Y, F, V, P and L, respectively.

18. The inhibin analog precursor protein of Claim 17 wherein the substitution mutation is A347H.

19. The inhibin analog precursor protein of Claim 17 wherein the substitution mutation is Y345G.

20. The inhibin analog precursor protein of Claim 17 wherein the substitution mutation is combined A347H and Y345G.

21. The inhibin analog precursor of Claim 16 wherein the mutation in β_B-subunit is a substitution mutation selected from the group consisting of $F308X_7$, $Y327X_8$, $G329X_9$, $I373X_{10}$, $P374X_{11}$ and $L377X_{12}$, using the numbering system of SEQ ID NO:12, wherein each of X_7, X_8, X_9, X_{10}, X_{11} and X_{12} is any amino acid except F, Y, G, I, P and L, respectively.

22. The inhibin analog precursor protein of any one of Claims 1 to 21 comprising an α- and/or β-subunit precursor with one or more amino acid substitutions, additions and/or deletions in addition to the modified proprotein convertase cleavage site.

23. The inhibin analog precursor protein of Claim 1 comprising an α-subunit having the amino acid sequence as set forth in SEQ ID NO:8 or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:8 after optimal alignment with the proviso

that it comprises at least one modified proprotein convertase site to enable more efficient cleavage by the convertase.

24. The inhibin analog precursor protein of Claim 1 comprising an α-subunit having the amino acid sequence as set forth in SEQ ID NO:10 or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:10 after optimal alignment with the proviso that it comprises at least one modified proprotein convertase site to enable more efficient cleavage by the convertase.

25. The inhibin analog precursor protein of Claim 1 comprising a β_A-subunit having the amino acid sequence as set forth in SEQ ID NO:4 or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:4 after optimal alignment with the proviso that it comprises at least one modified proprotein convertase site to enable more efficient cleavage by the convertase.

26. The inhibin analog precursor protein of Claim 1 comprising a β_A subunit amino acid sequence as set forth in SEQ ID NO:37 or 38 or 39 or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:37 or 38 or 39 after optimal alignment with the proviso that the β_A-subunit comprises at least one amino acid mutation to reduce dimerization in the formulation of an activin.

27. The inhibin analog precursor protein of Claim 1 comprising a β_A subunit amino acid sequence as set forth in SEQ ID NO:40 or 41 or 42 or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:40 or 41 or 42 after optimal alignment with the proviso that the β_A-subunit comprises at least one amino acid mutation to reduce dimerization in the formulation of an activin.

28. The inhibin analog precursor protein of Claim 1 comprising a β_B-subunit having the amino acid sequence as set forth in SEQ ID NO:14 or an amino acid sequence having at least 80% sequence similarity to SEQ ID NO:14 after optimal alignment with the

proviso that it comprises at least one modified proprotein convertase site to enable more efficient cleavage by the convertase.

29. A nucleic acid molecule encoding the α-subunit as defined in Claim 23 or 24.

30. The nucleic acid molecule of Claim 29 comprising the nucleotide sequence set forth in SEQ ID NO:7 or a nucleotide sequence having at least about 80% identity thereto after optimal alignment or a nucleic acid capable of hybridizing to the complement of SEQ ID NO:7 under stringency conditions.

31. The nucleic acid molecule of Claim 29 comprising the nucleotide sequence set forth in SEQ ID NO:9 or a nucleotide sequence having at least about 80% identity thereto after optimal alignment or a nucleic acid capable of hybridizing to the complement of SEQ ID NO:9 under medium stringency conditions.

32. A nucleic acid molecule encoding a β_A-subunit of Claim 25.

33. A nucleic acid molecule of Claim 32 comprising the nucleotide sequence as set forth in SEQ ID NO:3 or a nucleotide sequence having at least about 80% identity thereto after optimal alignment or a nucleic acid capable of hybridizing to the complement of SEQ ID NO:3 under medium stringency conditions.

34. A nucleic acid molecule encoding a β_B-subunit of Claim 28.

35. A nucleic acid molecule of Claim 34 comprising the nucleotide sequence as set forth in SEQ ID NO:13 or a nucleotide sequence having at least about 80% identity thereto after optimal alignment or a nucleic acid capable of hybridizing to the complement of SEQ ID NO:13 under medium stringency conditions.

36. An isolated cell or cell line comprising the nucleic acid of any one of Claims 29 to 35.

37. A bioactive inhibin generated following proprotein convertase cleavage of the inhibin analog precursor protein of any one of Claims 1 to 28.

38. A method for generating a bioactive inhibin analog said method comprising co-expressing in a cell or cell line a nucleic acid molecule encoding the α-subunit as defined in Claim 23 or 24 and a β-subunit as defined by any one of Claims 25 to 28 for a time and under conditions sufficient for an inhibin precursor protein to be produced, cleaved by a proprotein convertase and secreted from the cell or cell line as a bioactive inhibin analog.

39. The method of Claim 38 wherein the bioactive inhibin analog is inhibin A analog.

40. The method of Claim 38 wherein the bioactive inhibin analog is inhibin B analog.

41. The method of Claim 31 or 39 or 40 wherein minimal bioactive active A or activin B is produced.

42. A method of treatment of a mammalian subject comprising the administration of an inhibin A or inhibin B analog as defined by Claim 39 or 40 or an inhibin analog precursor protein of any one of Claims 1 to 28.

43. The method of Claim 42 wherein the mammal is a human.

44. The method of Claim 43 wherein the human is a post-menopausal female subject.

45. A pharmaceutical composition comprising an inhibin A or inhibin B analog as defined by Claim 39 or 40 or an inhibin analog precursor protein of any one of Claims 1 to 28 and one or more pharmaceutically acceptable carriers, diluents or excipients.

46. Use of an inhibin A or inhibin B analog as defined by Claim 39 or 40 or an inhibin analog precursor protein of any one of Claims 1 to 28 in the manufacture of a medicament for the treatment of a mammalian subject in need of therapy.

47. Use of Clam 46 wherein the mammal is a human.

48. Use of Claim 47 wherein the human is a female subject who has reduced serum levels of inhibin compared to the level in a pre-menopausal healthy subject.

49. An inhibin A or inhibin B analog as defined by Claim 38 or 39 for use in treating a mammalian subject in need of therapy.

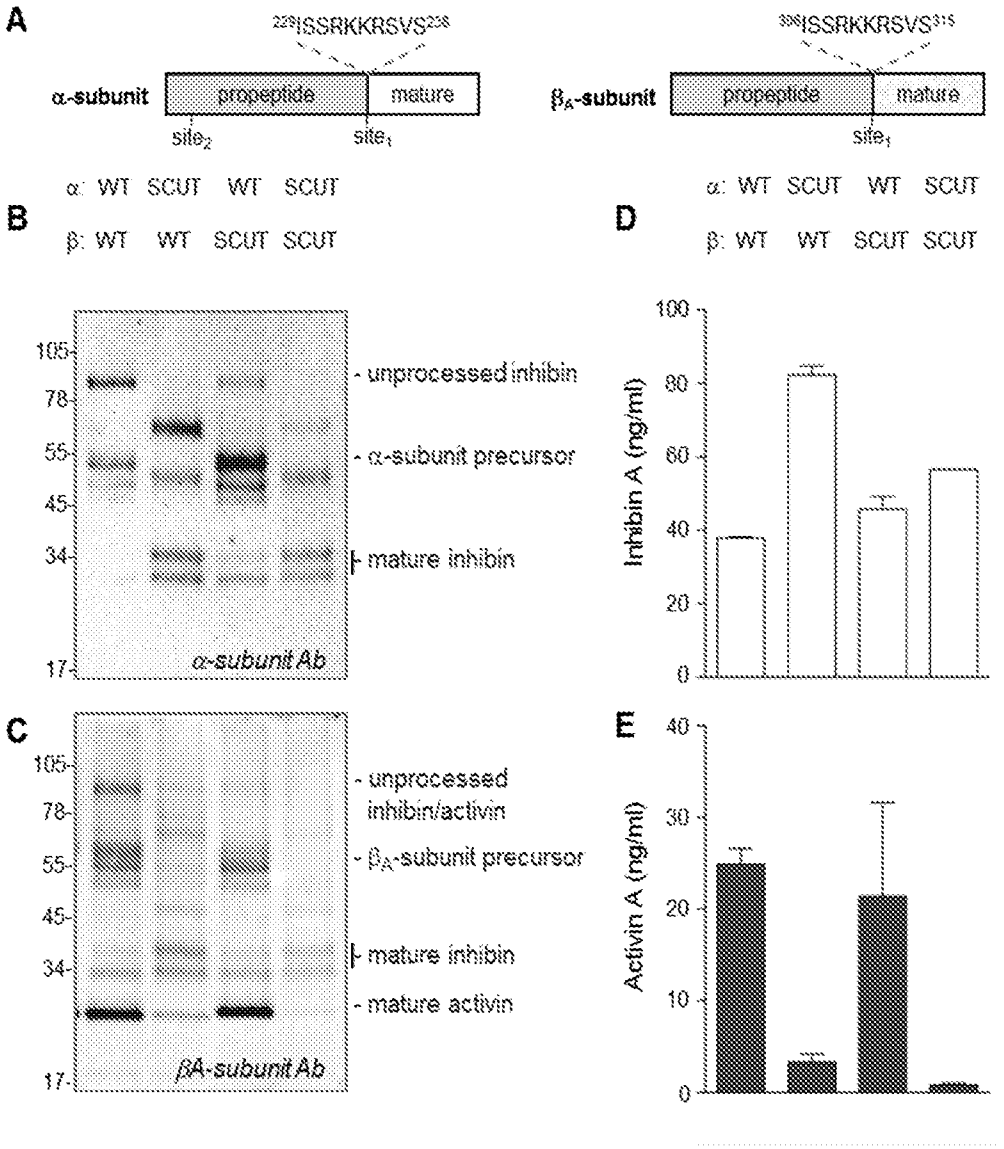
A
229ISSRKKRSVS238
α-subunit
propeptide
mature
site2
site1
339ISSRKKRSVS315
βA-subunit
propeptide
mature
site1
α: WT SCUT WT SCUT
β: WT WT SCUT SCUT
B
105
78
55
45
34
17
- unprocessed inhibin
- α-subunit precursor
- mature inhibin
α-subunit Ab
C
105
78
55
45
34
17
- unprocessed inhibin/activin
- βA-subunit precursor
- mature inhibin
- mature activin
βA-subunit Ab
D
α: WT SCUT WT SCUT
β: WT WT SCUT SCUT
Inhibin A (ng/ml)
100
80
60
40
20
0
E
Activin A (ng/ml)
40
30
20
10
0

Figure 1

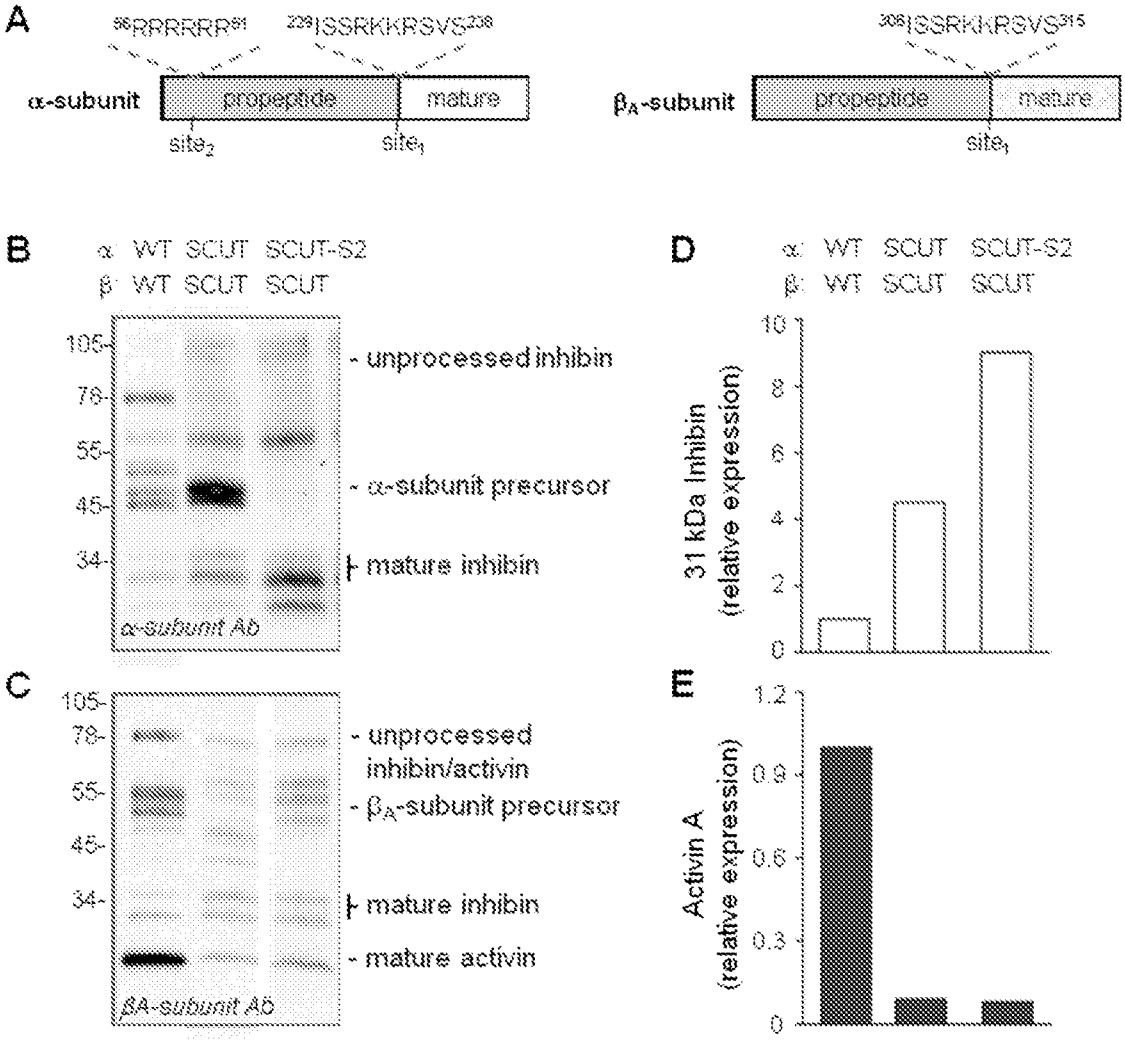
A
86RRRRRR91
229ISSRKKRSVS238
α-subunit
propeptide
mature
site2
site1
308ISSRKKRSVS315
βA-subunit
propeptide
mature
site1
B
α: WT SCUT SCUT-S2
β: WT SCUT SCUT
105-
78-
55-
45-
34-
α-subunit Ab
- unprocessed inhibin
- α-subunit precursor
mature inhibin
C
105-
78-
55-
45-
34-
βA-subunit Ab
- unprocessed inhibin/activin
- βA-subunit precursor
mature inhibin
- mature activin
D
α: WT SCUT SCUT-S2
β: WT SCUT SCUT
31 kDa Inhibin (relative expression)
10
8
6
4
2
0
E
Activin A (relative expression)
1.2
0.9
0.6
0.3
0

Figure 2

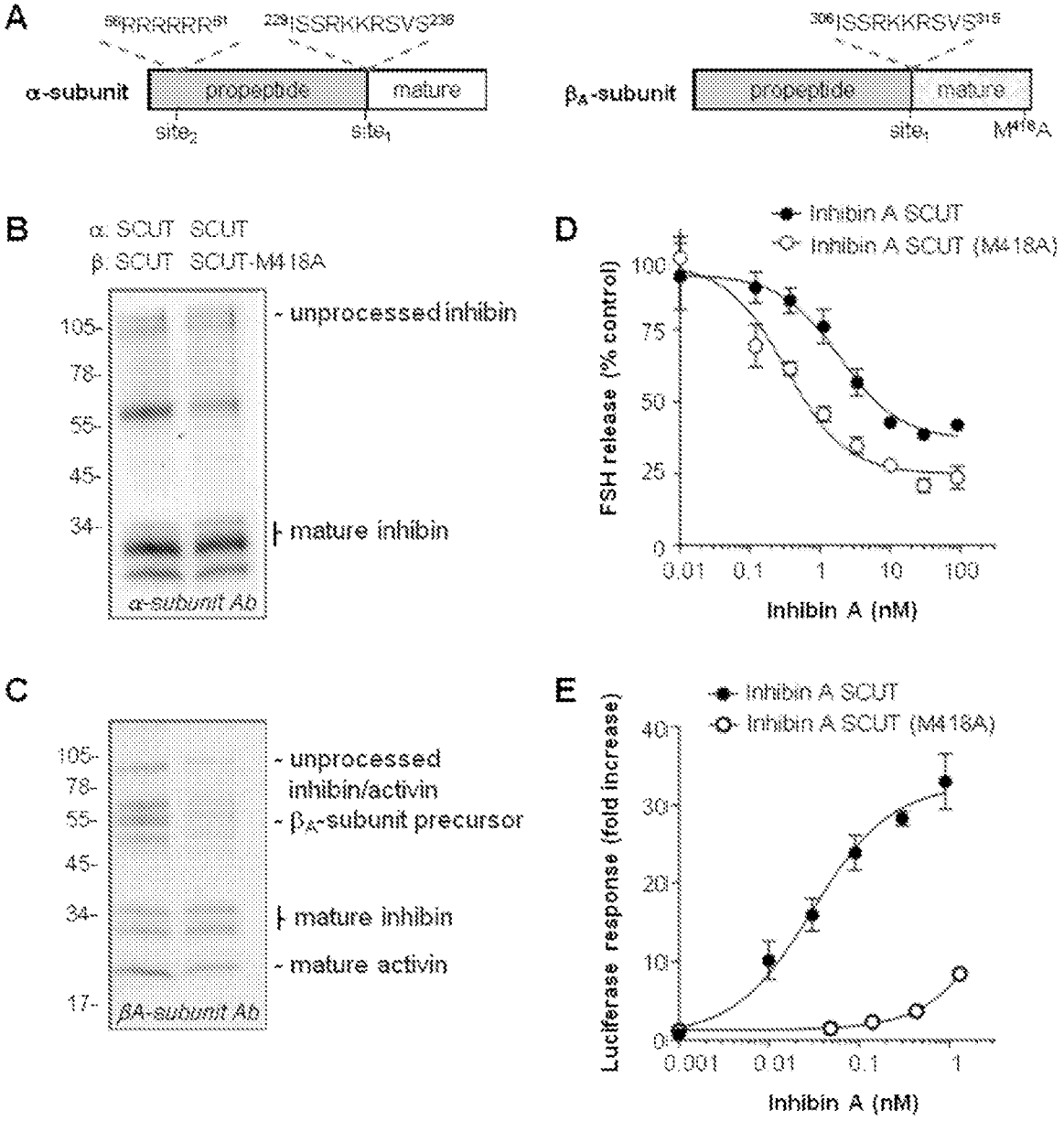
A
88RRRRRR93
229ISSRKKRSVS238
α-subunit
propeptide
mature
site2
site1
βA-subunit
306ISSRKKRSVS315
propeptide
mature
site1
M418A
B
α: SCUT SCUT
β: SCUT SCUT-M418A
105-
78-
55-
45-
34-
~ unprocessed inhibin
mature inhibin
α-subunit Ab
C
105-
78-
55-
45-
34-
17-
~ unprocessed inhibin/activin
~ βA-subunit precursor
mature inhibin
~ mature activin
βA-subunit Ab
D
Inhibin A SCUT
Inhibin A SCUT (M418A)
FSH release (% control)
100
75
50
25
0
0.01
0.1
1
10
100
Inhibin A (nM)
E
Inhibin A SCUT
Inhibin A SCUT (M418A)
Luciferase response (fold increase)
40
30
20
10
0
0.001
0.01
0.1
1
Inhibin A (nM)

Figure 3

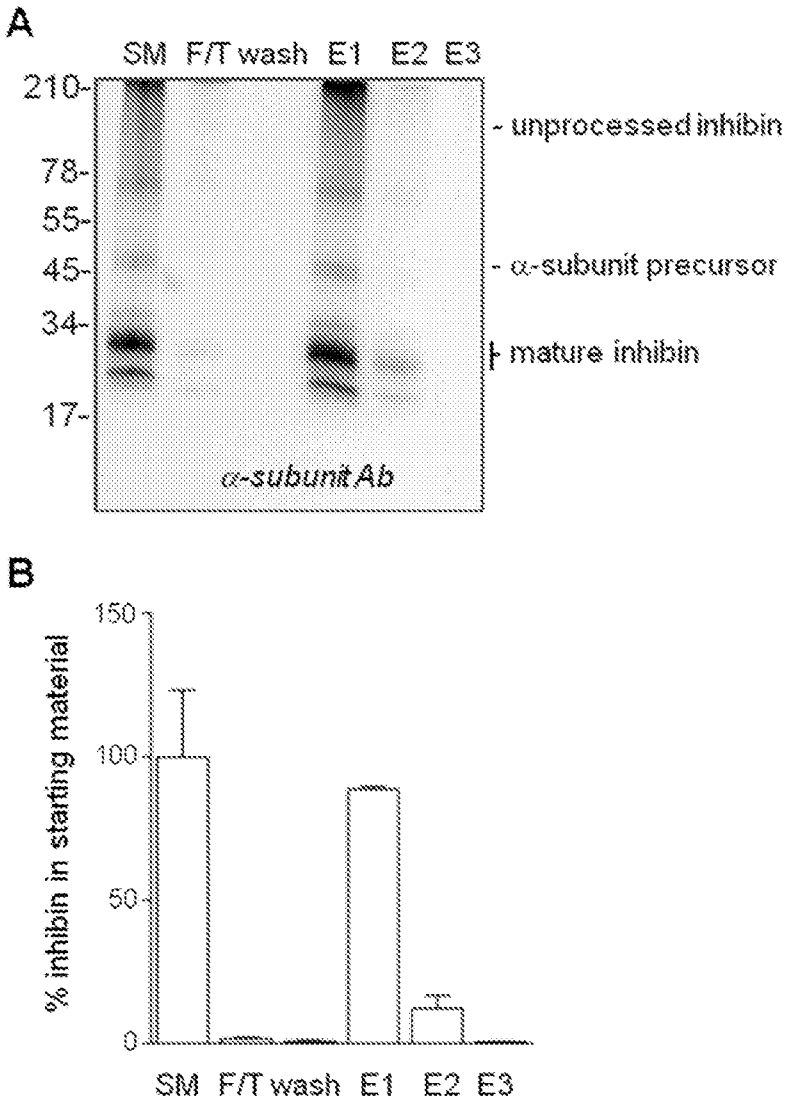
A
SM F/T wash E1 E2 E3
210-
78-
55-
45-
34-
17-
- unprocessed inhibin
- α-subunit precursor
mature inhibin
α-subunit Ab
B
% inhibin in starting material
150
100
50
0
SM F/T wash E1 E2 E3

Figure 4

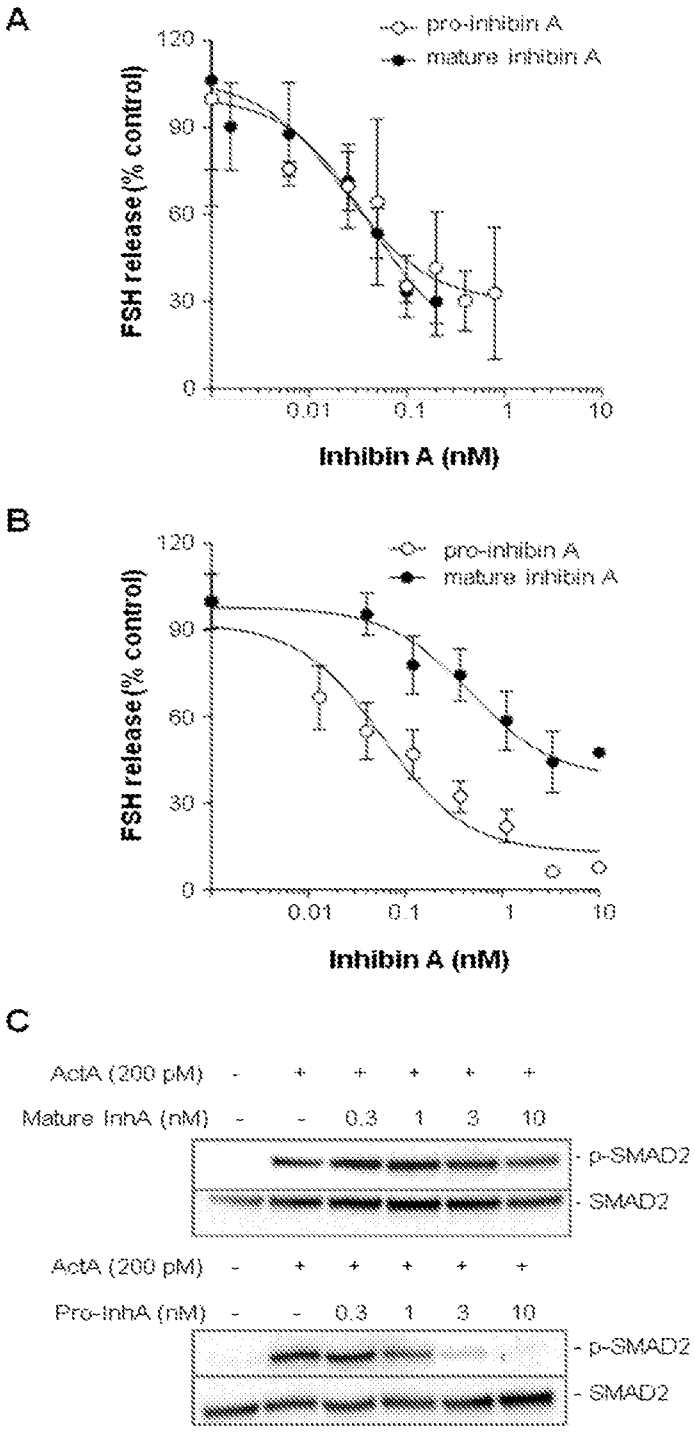
A
pro-inhibin A
mature inhibin A
FSH release (% control)
120
90
60
30
0
0.01
0.1
1
10
Inhibin A (nM)
B
pro-inhibin A
mature inhibin A
FSH release (% control)
120
90
60
30
0
0.01
0.1
1
10
Inhibin A (nM)
C
ActA (200 pM) - + + + + +
Mature InhA (nM) - - 0.3 1 3 10
p-SMAD2
SMAD2
ActA (200 pM) - + + + + +
Pro-InhA (nM) - - 0.3 1 3 10
p-SMAD2
SMAD2

Figure 5

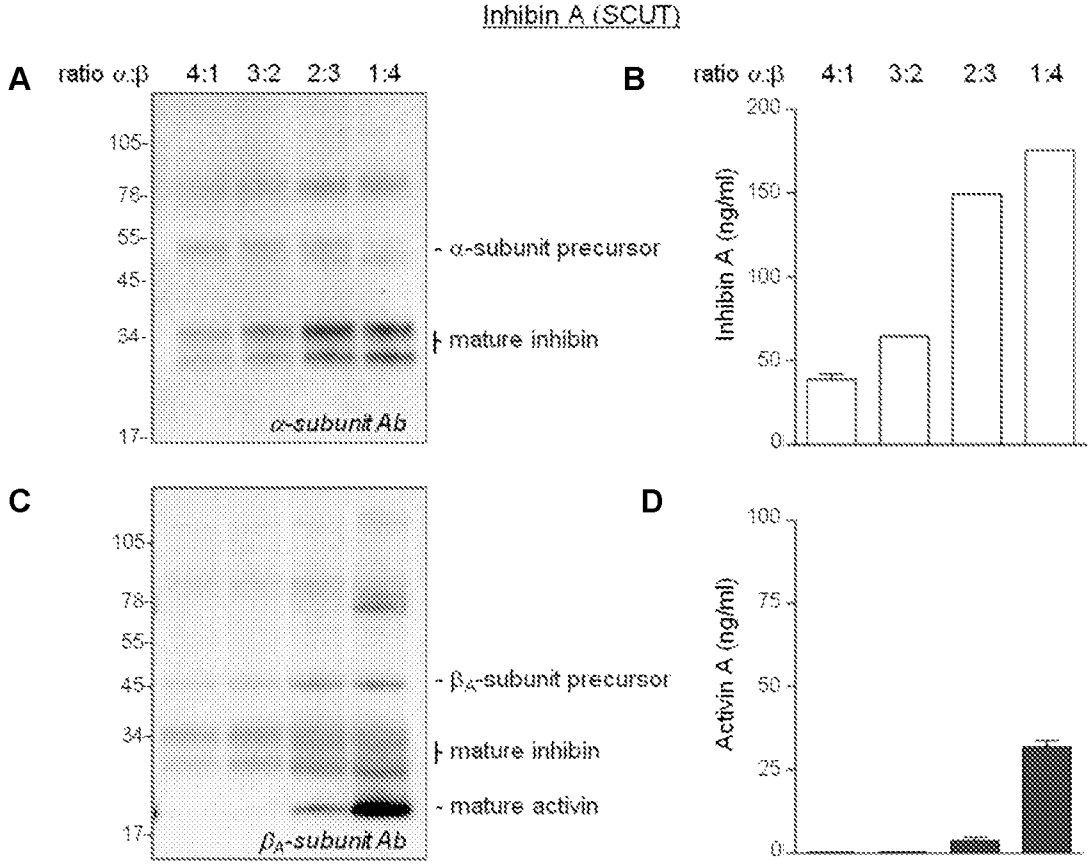
Inhibin A (SCUT)
A
ratio α:β 4:1 3:2 2:3 1:4
105
78
55
45
34
17
α-subunit precursor
mature inhibin
α-subunit Ab
B
ratio α:β 4:1 3:2 2:3 1:4
Inhibin A (ng/ml)
200
150
100
50
0
C
105
78
55
45
34
17
βA-subunit precursor
mature inhibin
mature activin
βA-subunit Ab
D
Activin A (ng/ml)
100
75
50
25
0

Figure 6

Human inhibin βA-subunit super-cut variant + M418A mutation

```
atgcccttgctttggctgagaggatttctgttggcaagttgctggattatagtgaggagt
 M  P  L  L  W  L  R  G  F  L  L  A  S  C  W  I  I  V  R  S
gactacaaagacgacgacgacaaatccccccaccccaggatccgaggggcacagcgcggcc        FLAG tag
 D  Y  K  D  D  D  D  K  S  P  T  P  G  S  E  G  H  S  A  A
cccgactgtccgtcctgtgcgctggccgccctcccaaaggatgtacccaactctcagcca
 P  D  C  P  S  C  A  L  A  A  L  P  K  D  V  P  N  S  Q  P
gagatggtggaggccgtcaagaagcacattttaaacatgctgcacttgaagaagagaccc
 E  M  V  E  A  V  K  K  H  I  L  N  M  L  H  L  K  K  R  P
gatgtcacccagccggtacccaaggcggcgcttctgaacgcgatcagaaagcttcatgtg
 D  V  T  Q  P  V  P  K  A  A  L  L  N  A  I  R  K  L  H  V
ggcaaagtcggggagaacgggtatgtggagatagaggatgacattggaaggagggcagaa
 G  K  V  G  E  N  G  Y  V  E  I  E  D  D  I  G  R  R  A  E
atgaatgaacttatggagcagacctcggagatcatcacgtttgccgagtcaggaacagcc
 M  N  E  L  M  E  Q  T  S  E  I  I  T  F  A  E  S  G  T  A
aggaagacgctgcacttcgagatttccaaggaaggcagtgacctgtcagtggtggagcgt
 R  K  T  L  H  F  E  I  S  K  E  G  S  D  L  S  V  V  E  R
gcagaagtctggctcttcctaaaagtccccaaggccaacaggaccaggaccaaagtcacc
 A  E  V  W  L  F  L  K  V  P  K  A  N  R  T  R  T  K  V  T
atccgcctcttccagcagcagaagcacccgcagggcagcttggacacaggggaagaggcc
 I  R  L  F  Q  Q  Q  K  H  P  Q  G  S  L  D  T  G  E  E  A
gaggaagtgggcttaaaggggagaggagtgaactgttgctctctgaaaaagtagtagac
 E  E  V  G  L  K  G  E  R  S  E  L  L  L  S  E  K  V  V  D
gctcggaagagcacctggcatgtcttccctgtctccagcagcatccagcggttgctggac
 A  R  K  S  T  W  H  V  F  P  V  S  S  S  I  Q  R  L  L  D
cagggcaagagctccctggacgttcggattgcctgtgagcagtgccaggagagtggcgcc
 Q  G  K  S  S  L  D  V  R  I  A  C  E  Q  C  Q  E  S  G  A
agcttggttctcctgggcaagaagaagaagaaagaagaggaggggggaagggaaaaagaag
 S  L  V  L  L  G  K  K  K  K  K  E  E  E  G  E  G  K  K  K
ggcggaggtgaaggtggggcaggagcagatgaggaaaaggagcagtcgcacagacctttc
 G  G  G  E  G  G  A  G  A  D  E  E  K  E  Q  S  H  R  P  F
ctcatgctgcaggcccggcagtctgaagaccaccctcatatctcatcgagaaagaaacgc        Super-cut site
 L  M  L  Q  A  R  Q  S  E  D  H  P  H  I  S  S  R  K  K  R
tcagtctcatcggggcttggagtgtgatggcaaggtcaacatctgctgtaagaaacagttc
 S  V  S  S  G  L  E  C  D  G  K  V  N  I  C  C  K  K  Q  F
tttgtcagtttcaaggacatcggctggaatgactggatcattgctccctctggctatcat
 F  V  S  F  K  D  I  G  W  N  D  W  I  I  A  P  S  G  Y  H
gccaactactgcgagggtgagtgcccgagccatatagcaggcacgtccgggtcctcactg
 A  N  Y  C  E  G  E  C  P  S  H  I  A  G  T  S  G  S  S  L
tccttccactcaacagtcatcaaccactaccgcatgcggggccatagccccttgccaac
 S  F  H  S  T  V  I  N  H  Y  R  M  R  G  H  S  P  F  A  N
ctcaaatcgtgctgtgtgcccaccaagctgagacccatgtccatgttgtactatgatgat
 L  K  S  C  C  V  P  T  K  L  R  P  M  S  M  L  Y  Y  D  D
ggtcaaaacatcatcaaaaaggacattcagaacgcgatcgtggaggagtgtgggtgctca
 G  Q  N  I  I  K  K  D  I  Q  N  A  I  V  E  E  C  G  C  S
tag                                          M418A mutation
 -
```

Figure 7

Human inhibin α-subunit_super-cut 1 variant

```
atggtgctgcacctactgctcttcttgctgctgaccccacagggtgggcacagctgccag
 M  V  L  H  L  L  L  F  L  L  L  T  P  Q  G  G  H  S  C  Q
gggctggagctggcccgggaacttgttctggccaaggtgagggccctgttcttggatgcc
 G  L  E  L  A  R  E  L  V  L  A  K  V  R  A  L  F  L  D  A
ttggggccccccgcggtgaccagggaaggtggggaccctggagtcaggcggctgccccga
 L  G  P  P  A  V  T  R  E  G  G  D  P  G  V  R  R  L  P  R
agacatgccctgggggggcttcacacacaggggctctgagcccgaggaagaggaggatgtc
 R  H  A  L  G  G  F  T  H  R  G  S  E  P  E  E  E  E  D  V
tcccaagccatccttttcccagccacagatgccagctgtgaggacaagtcagctgccaga
 S  Q  A  I  L  F  P  A  T  D  A  S  C  E  D  K  S  A  A  R
gggctggcccaggaggctgaggagggcctcttcagatacatgttccggccatcccagcat
 G  L  A  Q  E  A  E  E  G  L  F  R  Y  M  F  R  P  S  Q  H
acacgcagccgccaggtgacttcagcccagctgtggttccacaccgggctggacaggcag
 T  R  S  R  Q  V  T  S  A  Q  L  W  F  H  T  G  L  D  R  Q
ggcacagcagcctccaatagctctgagcccctgctaggcctgctggcactgtcaccggga
 G  T  A  A  S  N  S  S  E  P  L  L  G  L  L  A  L  S  P  G
ggacccgtggctgtgcccatgtctttgggccatgctccccctcactgggccgtgctgcac
 G  P  V  A  V  P  M  S  L  G  H  A  P  P  H  W  A  V  L  H
ctggccacctctgctctctctctgctgacccaccccgtcctggtgctgctgctgcgctgt
 L  A  T  S  A  L  S  L  L  T  H  P  V  L  V  L  L  L  R  C
cccctctgtacctgctcagcccggcctgaggccacgcccttcctggtggcccacactcgg
 P  L  C  T  C  S  A  R  P  E  A  T  P  F  L  V  A  H  T  R
accagaccacccagtggaggggagcatcatcaccatcaccaccatcatcacatctcatcg
 T  R  P  P  S  G  G  E  H  H  H  H  H  H  H  H  H  I  S  S
agaaagaaacgctcagtctcatcaactcccctgatgtcctggccttggtctccctctgct
 R  K  K  R  S  V  S  S  T  P  L  M  S  W  P  W  S  P  S  A
ctgcgcctgctgcagaggcctccggaggaaccggctgcccatgccaactgccacagagta
 L  R  L  L  Q  R  P  P  E  E  P  A  A  H  A  N  C  H  R  V
gcactgaacatctccttccaggagctgggctgggaacggtggatcgtgtaccctcccagt
 A  L  N  I  S  F  Q  E  L  G  W  E  R  W  I  V  Y  P  P  S
ttcatcttccactactgtcatggtggttgtgggctgcacatcccaccaaacctgtccctt
 F  I  F  H  Y  C  H  G  G  C  G  L  H  I  P  P  N  L  S  L
ccagtccctggggctcctcctaccccagcccagccctactccttgctgccaggggcccag
 P  V  P  G  A  P  P  T  P  A  Q  P  Y  S  L  L  P  G  A  Q
ccctgctgtgctgctctcccagggaccatgaggcccctacatgtccgcaccacctcggat
 P  C  C  A  A  L  P  G  T  M  R  P  L  H  V  R  T  T  S  D
ggaggttactctttcaagtatgagacagtgcccaaccttctcacgcagcactgtgcttgt
 G  G  Y  S  F  K  Y  E  T  V  P  N  L  L  T  Q  H  C  A  C
atctaa
 I  -
```

Poly-histidine TAG (x9)

Super-cut site1

Figure 8

Human inhibin α-subunit_super-cut 2 variant

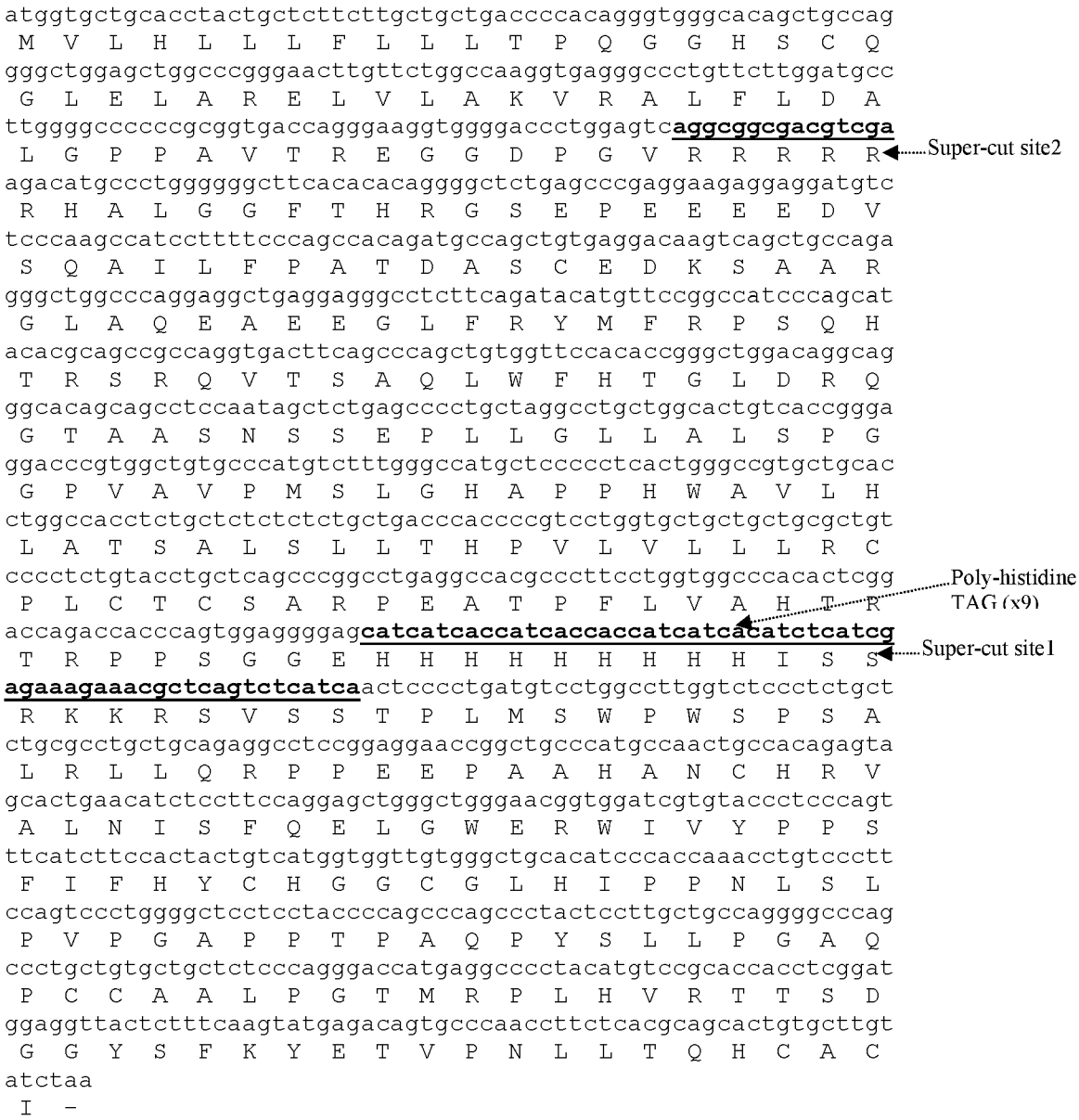

Figure 9

INHBB_super-cut_M410A SEQ (Wt INHBB SEQ)

```
atggacgggctgcccggtcgggcgctgggggccgcctgccttctgctgctggcggccggc
 M  D  G  L  P  G  R  A  L  G  A  A  C  L  L  L  L  A  A  G
tggctggggcctgaggcctggggcgactacaaagacgacgacgacaaatcacccacgccc      FLAG Tag
 W  L  G  P  E  A  W  G  D  Y  K  D  D  D  D  K  S  P  T  P
ccgccgacgcctgccgcgccgccgccacccccgccacccggatccccgggtggctcgcag
 P  P  T  P  A  A  P  P  P  P  P  P  P  G  S  P  G  G  S  Q
gacacctgtacgtcgtgcggcggcttccggcggccagaggagctcggccgagtggacggc
 D  T  C  T  S  C  G  G  F  R  R  P  E  E  L  G  R  V  D  G
gacttcctggaggcggtgaagcggcacatcttgagccgcctgcagatgcggggccggccc
 D  F  L  E  A  V  K  R  H  I  L  S  R  L  Q  M  R  G  R  P
aacatcacgcacgccgtgcctaaggccgccatggtcacggccctgcgcaagctgcacgcg
 N  I  T  H  A  V  P  K  A  A  M  V  T  A  L  R  K  L  H  A
ggcaaggtgcgcgaggacggccgcgtggagatcccgcacctcgacggccacgccagcccg
 G  K  V  R  E  D  G  R  V  E  I  P  H  L  D  G  H  A  S  P
ggcgccgacggccaggagcgcgtttccgaaatcatcagcttcgccgagacagatggcctc
 G  A  D  G  Q  E  R  V  S  E  I  I  S  F  A  E  T  D  G  L
gcctcctcccgggtccgcctatacttcttcatctccaacgaaggcaaccagaacctgttt
 A  S  S  R  V  R  L  Y  F  F  I  S  N  E  G  N  Q  N  L  F
gtggtccaggccagcctgtggctttacctgaaactcctgccctacgtcctggagaagggc
 V  V  Q  A  S  L  W  L  Y  L  K  L  L  P  Y  V  L  E  K  G
agccggcggaaggtgcgggtcaaagtgtacttccaggagcagggccacggtgacaggtgg
 S  R  R  K  V  R  V  K  V  Y  F  Q  E  Q  G  H  G  D  R  W
aacatggtggagaagagggtggacctcaagcgcagcggctggcataccttcccactcacg
 N  M  V  E  K  R  V  D  L  K  R  S  G  W  H  T  F  P  L  T
gaggccatccaggccttgtttgagcggggcgagcggcgactcaacctagacgtgcagtgt
 E  A  I  Q  A  L  F  E  R  G  E  R  R  L  N  L  D  V  Q  C
gacagctgccaggagctggccgtggtgccggtgttcgtggacccaggcgaagagtcgcac
 D  S  C  Q  E  L  A  V  V  P  V  F  V  D  P  G  E  E  S  H
cggccctttgtggtggtgcaggctcggctgggcgacatctcatcgagaaagaaacgctca
 R  P  F  V  V  V  Q  A  R  L  G  D  I  S  S  R  K  K  R  S      Super-cut
gtctcatcggcctggagtgcgatggccggaccaacctctgttgcaggcaacagttcttc
 V  S  S  G  L  E  C  D  G  R  T  N  L  C  C  R  Q  Q  F  F
attgacttccgcctcatcggctggaacgactggatcatagcacccaccggctactacggg
 I  D  F  R  L  I  G  W  N  D  W  I  I  A  P  T  G  Y  Y  G
aactactgtgagggcagctgcccagcctacctggcaggggtccccggctctgcctcctcc
 N  Y  C  E  G  S  C  P  A  Y  L  A  G  V  P  G  S  A  S  S
ttccacacggctgtggtgaaccagtaccgcatgcggggtctgaaccccggcacggtgaac
 F  H  T  A  V  V  N  Q  Y  R  M  R  G  L  N  P  G  T  V  N
tcctgctgcattcccaccaagctgagcaccatgtccatgctgtacttcgatgatgagtac
 S  C  C  I  P  T  K  L  S  T  M  S  M  L  Y  F  D  D  E  Y
aacatcgtcaagcgggacgtgcccaacgcgattgtggaggagtgcggctgcgcctga
 N  I  V  K  R  D  V  P  N  A  I  V  E  E  C  G  C  A  -
                           M410A mutation
```

Figure 10

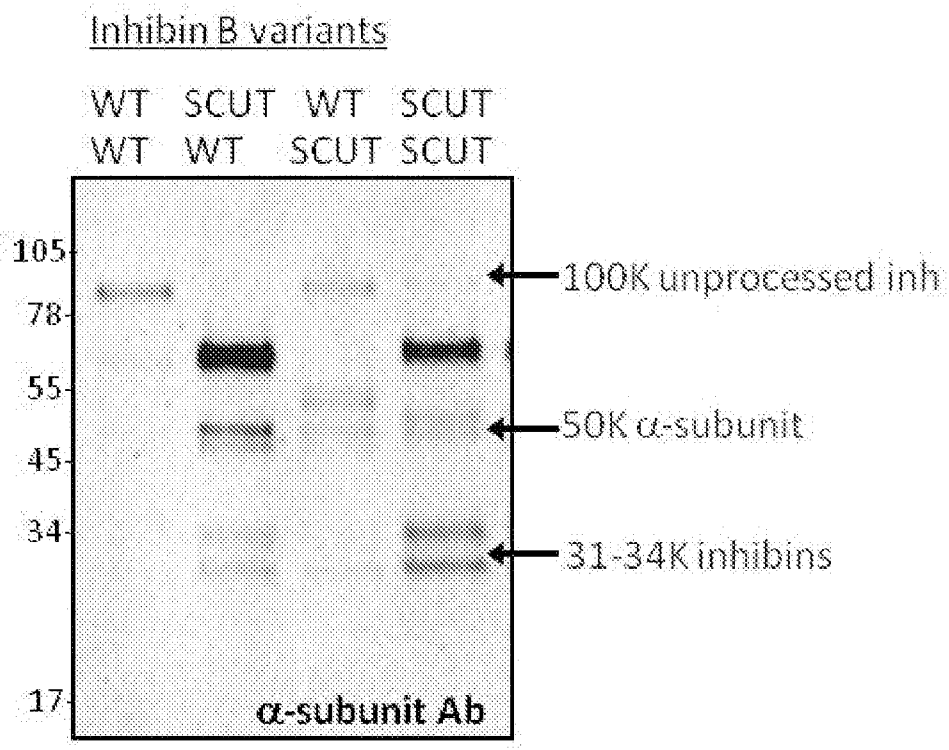
Inhibin B variants
WT SCUT WT SCUT
WT WT SCUT SCUT
105
78
55
45
34
17
100K unprocessed inh
50K α-subunit
31-34K inhibins
α-subunit Ab

Figure 11

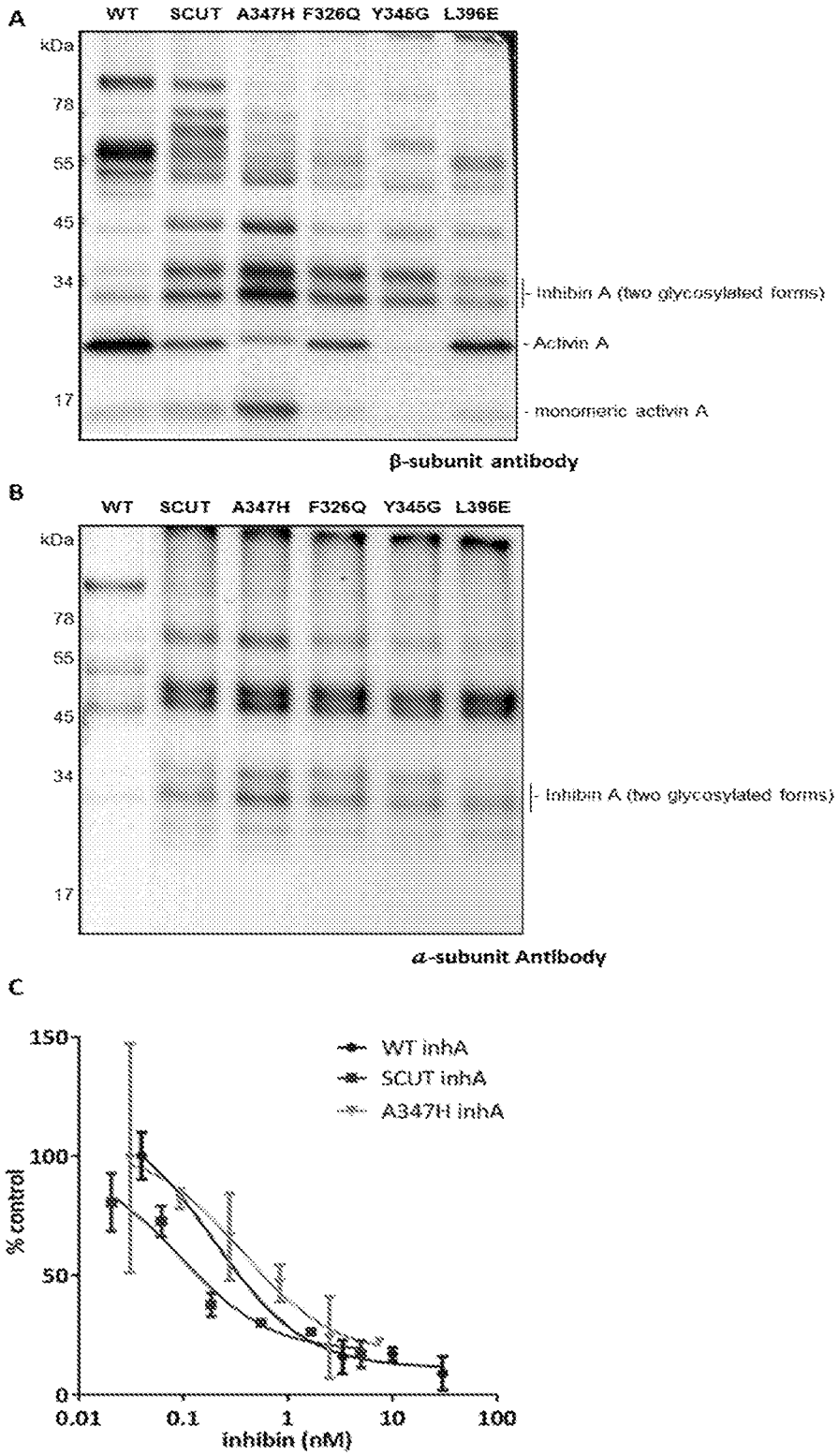
A
WT
SCUT
A347H
F326Q
Y345G
L396E
kDa
78
55
45
34
17
- inhibin A (two glycosylated forms)
- Activin A
- monomeric activin A
β-subunit antibody
B
WT
SCUT
A347H
F326Q
Y345G
L396E
kDa
78
55
45
34
17
- inhibin A (two glycosylated forms)
α-subunit Antibody
C
150
100
50
0
% control
0.01
0.1
1
10
100
inhibin (nM)
WT inhA
SCUT inhA
A347H inhA

Figure 12

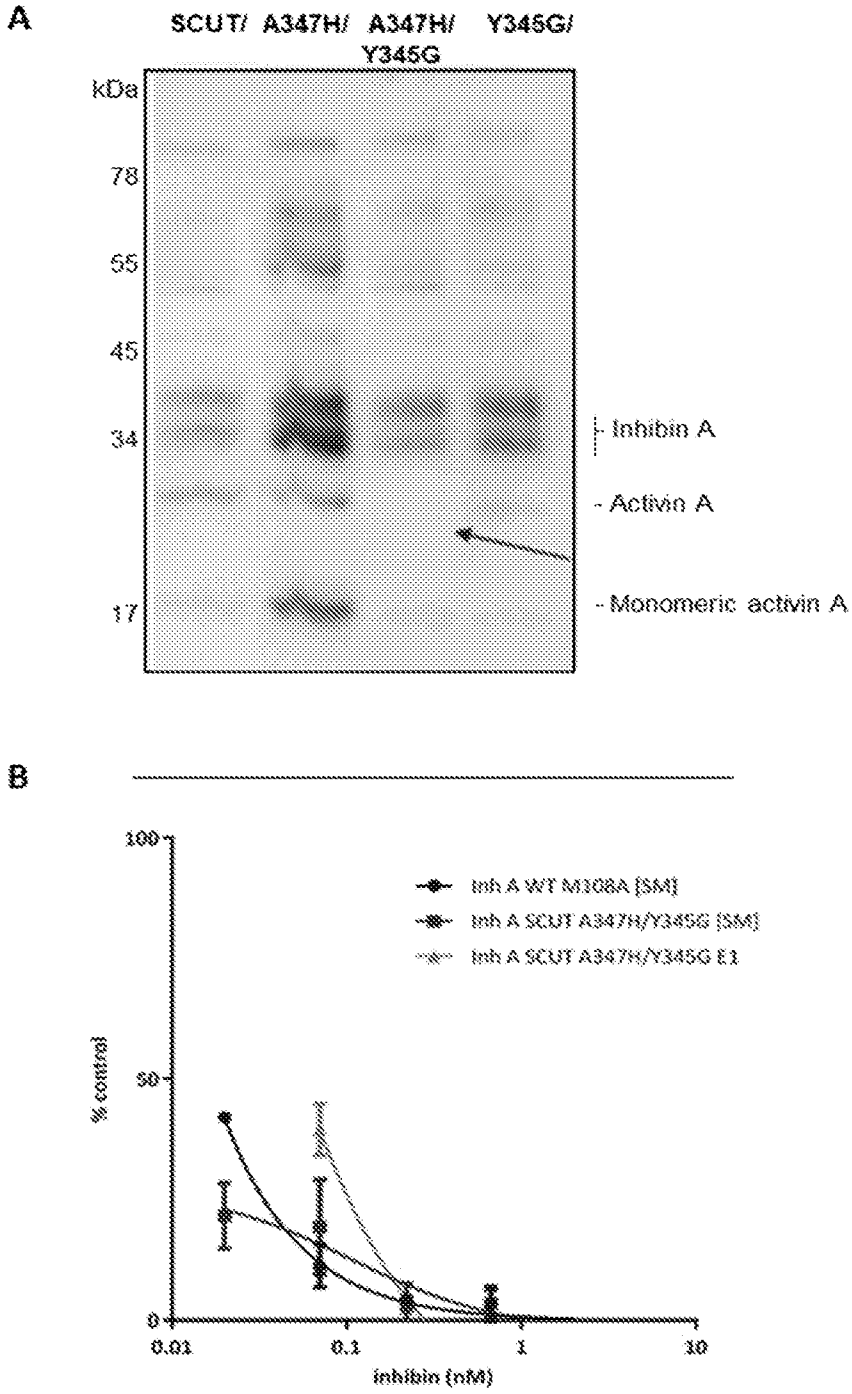
A
SCUT/ A347H/ A347H/ Y345G/
Y345G
kDa
78
55
45
34
17
Inhibin A
Activin A
Monomeric activin A
B
100
50
0
% control
0.01
0.1
1
10
inhibin (nM)
Inh A WT M108A [SM]
Inh A SCUT A347H/Y345G [SM]
Inh A SCUT A347H/Y345G E1

Figure 13

14/14

INHBB_super-cut_M410A SEQ

```
atggatggacttccaggtcgagctctgggggccgcctgccttctgctgctggcggccggc
 M  D  G  L  P  G  R  A  L  G  A  A  C  L  L  L  L  A  A  G
tggctggggcctgaggcctggggc**gactacaaagacgacgacgacaaa**tcacccacgccc
 W  L  G  P  E  A  W  G  **D  Y  K  D  D  D  D  K**  S  P  T  P
ccgccgacgcctgccgcgccgccgccacccccgccacccggatccccgggtggctcgcag
 P  P  T  P  A  A  P  P  P  P  P  P  P  G  S  P  G  G  S  Q
gacacctgtacgtcgtgcggcggcttccggcggccagaggagctcggccgagtggacggc
 D  T  C  T  S  C  G  G  F  R  R  P  E  E  L  G  R  V  D  G
gacttcctggaggcggtgaagcggcacatcttgagccgcctgcagatgcggggccggccc
 D  F  L  E  A  V  K  R  H  I  L  S  R  L  Q  M  R  G  R  P
aacatcacgcacgccgtgcctaaggccgccatggtcacggccctgcgcaagctgcacgcg
 N  I  T  H  A  V  P  K  A  A  M  V  T  A  L  R  K  L  H  A
ggcaaggtgcgcgaggacggccgcgtggagatcccgcacctcgacggccacgccagcccg
 G  K  V  R  E  D  G  R  V  E  I  P  H  L  D  G  H  A  S  P
ggcgccgacggccaggagcgcgtttccgaaatcatcagcttcgccgagacagatggcctc
 G  A  D  G  Q  E  R  V  S  E  I  I  S  F  A  E  T  D  G  L
gcctcctcccgggtccgcctatacttcttcatctccaacgaaggcaaccagaacctgttt
 A  S  S  R  V  R  L  Y  F  F  I  S  N  E  G  N  Q  N  L  F
gtggtccaggccagcctgtggctttacctgaaactcctgccctacgtcctggagaagggc
 V  V  Q  A  S  L  W  L  Y  L  K  L  L  P  Y  V  L  E  K  G
agccggcggaaggtgcgggtcaaagtgtacttccaggagcagggccacggtgacaggtgg
 S  R  R  K  V  R  V  K  V  Y  F  Q  E  Q  G  H  G  D  R  W
aacatggtggagaagagggtggacctcaagcgcagcggctggcataccttcccactcacg
 N  M  V  E  K  R  V  D  L  K  R  S  G  W  H  T  F  P  L  T
gaggccatccaggccttgtttgagcggggcgagcggcgactcaacctagacgtgcagtgt
 E  A  I  Q  A  L  F  E  R  G  E  R  R  L  N  L  D  V  Q  C
gacagctgccaggagctggccgtggtgccggtgttcgtggacccaggcgaagagtcgcac
 D  S  C  Q  E  L  A  V  V  P  V  F  V  D  P  G  E  E  S  H
cggccctttgtggtggtgcaggctcggctgggcgac**atctcatcgagaaagaaacgctca**
 R  P  F  V  V  V  Q  A  R  L  G  D  **I  S  S  R  K  K  R  S**
**gtctcatcg**ggcctggagtgcgatggccggaccaacctctgttgcaggcaacagttcttc
 **V  S  S**  G  L  E  C  D  G  R  T  N  L  C  C  R  Q  Q  F  F
attgacttccgcctcatcggctggaacgactggatcatagcacccaccggctactacggg
 I  D  F  R  L  I  G  W  N  D  W  I  I  A  P  T  G  Y  Y  G
aactactgtgagggcagctgcccagcctacctggcaggggtccccggctctgcctcctcc
 N  Y  C  E  G  S  C  P  A  Y  L  A  G  V  P  G  S  A  S  S
ttccacacggctgtggtgaaccagtaccgcatgcggggtctgaaccccggcacggtgaac
 F  H  T  A  V  V  N  Q  Y  R  M  R  G  L  N  P  G  T  V  N
tcctgctgcattcccaccaagctgagcaccatgtccatgctgtacttcgatgatgagtac
 S  C  C  I  P  T  K  L  S  T  M  S  M  L  Y  F  D  D  E  Y
aacatcgtcaagcgggacgtgcccaac**gcg**attgtggaggagtgcggctgcgcctga
 N  I  V  K  R  D  V  P  N  **A**  I  V  E  E  C  G  C  A  -
```

FLAG tag

Super-cut site

M410A mutation

Figure 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2016/051156

A. CLASSIFICATION OF SUBJECT MATTER

C07K 14/575 (2006.01) A61K 38/00 (2006.01) C12N 15/16 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, WPIAP, TXTE: C07K 14/575 (CPC), C07K 14/- (CPC, IPC), A61K 38/00 (CPC, IPC), inhibin, activin, cleave, dimerize, cleavage site, enhance, modify, mutate, TGF beta, and similar terms.
CAPlus, Medline Biosis: inhibin, activin, cleavage site
Google, PubMed: inhibin, activin, cleavage site, pharmaceutical, administer, menopause and similar terms in various combinations
GenomeQuest: SEQ ID NOs:2, 6, 12, 18; mature beta A and B subunits from SEQ ID NOs: 2 and 12.
An applicant and inventor search was conducted in PatentScope, PubMed, AusPat and internal IP Australa databases.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 March 2017	09 March 2017
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Gareth Cook AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0399359629

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2016/051156

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☒ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

   A statement was furnished confirming that the sequence information in electronic form is identical to the written sequence listing, but there does not appear to be a written sequence listing.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2016/051156

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/AU2016/051156**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Papakonstantinou T *et al*, "Synthesis, purification and bioactivity of recombinant human activin A expressed in the yeast *Pichia pastoris*", *Protein Expression and Purification*, 2009, 64(2):131-138 abstract, pages 132 to 133, figure 2, whole of document	29-36, 38-41
X	Fredericks D *et al*, "Optimization of the expression of recombinant human activin A in the yeast *Pichia pastoris*", *Biotechnology Progress*, 2010, 26(2):372-383 abstract, page 373 to 375, whole of document	29-36, 38-41
X	US 2010/0092463 A1 (ISHIKAWA et al) 15 April 2010 paragraphs [0011] and [0054], whole of document	37, 42-49
X	WO 1997/015321 A1 (THE VICTORIA UNIVERSITY OF MANCHESTER) 01 May 1997 page 2, pages 15 to 21, whole of document	37, 42, 43, 45-47, 49
X	Pangas SA *et al*, "Production and purification of recombinant human inhibin and activin", *The Journal of Endocrinology*, 2002, 172(1):199-210 abstract, figure 2, whole of document	37
X Y	Walton KL *et al*, "A common biosynthetic pathway governs the dimerization and secretion of inhibin and related transforming growth factor beta (TGFbeta) ligands", *Journal of Biological Chemistry*, 2009, 284(14):9311-9320 abstract, figure 1, whole of document figure 7, page 9319, whole of document	37 16
P,X P,Y	Walton KL *et al*, "A Novel, More Efficient Approach to Generate Bioactive Inhibins", *Endocrinology*, July 2016, 157(7):2799-2809 figure 1, figure 2, whole of document whole of document	1-15, 22-25, 28-49 16
T	Bernard DJ, "Disinhibiting an Inhibitor: Genetic Engineering Leads to Improvements in Recombinant Inhibin A Production", *Endocrinology*, July 2016, 157(7):2583-2585 whole of document	1-49
A	Duckert P *et al*, "Prediction of proprotein convertase cleavage sites", *Protein Engineering, Design and Selection: PEDS*, 2004, 17(1):107-112 figure 1, whole of document	
A	WO 2014/182676 A2 (SCHOLAR ROCK, INC.) 13 November 2014 whole of document	
A	WO 1993/011247 A1 (GENENTECH, INC.) 10 June 1993	

Supplemental Box

Continuation of: **Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept. This Authority has found that there are different inventions based on the following features that separate the claims into distinct groups:

•Claims 1 to 28 and 37 (completely) and claims 42 to 49 (partially) are directed to a mammalian inhibin analog precursor wherein at least one proprotein convertase cleavage site has been modified by being replaced with the sequence ISSRKKRSVSS, the processed mature inhibin and uses and composition comprising the inhibin precursor. The feature of one of at least one proprotein convertase cleavage site of the inhibin precursor being replaced by the sequence ISSRKKRSVSS is specific to this group of claims.

•Claims 29 to 31 (completely) and claim 36 (partially) are directed to a nucleic acid molecule encoding an α subunit as defined by claims 23 or 24, having at least one modified proprotein convertase cleavage site to enable more efficient cleavage by the convertase and an isolated cell comprising the nucleic acid molecule. The modified proprotein cleavage site does not have to be that defined in claim 1 or be modified to have the sequence given in claim 1. The nucleic acid encoding α subunit with the modified proprotein convertase cleavage site is specific to this group of claims.

•Claims 32 to 35 (completely) and claim 36 (partially) are directed to a nucleic acid molecule encoding a β subunit is defined by claims 25 or 28, having at least one modified proprotein convertase cleavage site to enable more efficient cleavage by the convertase and an isolated cell comprising the nucleic acid molecule. The modified proprotein convertase cleavage site does not have to be that defined in claim 1 or be modified to have the sequence given in claim 1. The nucleic acid encoding β subunit with the modified proprotein convertase cleavage site is specific to this group of claims.

•Claim 40 (completely) and claims 38, 39 and 41 to 49 (partially) are directed to a recombinant method of producing inhibin using a nucleic acid molecule encoding an α subunit of claim 23 or 24 and a β subunit defined by claims 25 or 28, both subunits having at least one modified proprotein convertase cleavage site to enable more efficient cleavage by the convertase, inhibin produced by the method and uses of the inhibin. Neither proprotein cleavage site has to be the modified proprotein cleavage site of claim 1, or be modified to have the sequence given in claim 1. The method of producing inhibin using a nucleic acid molecule encoding an α subunit of claim 23 or 24 and a β subunit defined by claims 25 or 28 is specific to this group of claims.

•Claims 38, 39 and 41 to 49 (partially) are directed to a recombinant method of producing inhibin using a nucleic acid molecule encoding an α subunit of claim 23 or 24 having a more efficient proprotein convertase cleavage site and a β_A subunit as defined by claims 26 or 27, having at least one amino acid mutation to reduce dimerization in the formulation of activin, inhibin produced by the method and uses thereof. The β_A subunit does not need to have a modified proprotein convertase cleavage site. The method of producing inhibin using a nucleic acid molecule encoding an α subunit of claim 23 or 24 and a β_A subunit defined by claims 26 or 27 is specific to this group of claims

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art. When there is no special technical feature common to all the claimed inventions there is no unity of invention.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. The only feature common to all of the claimed inventions is modification of a proprotein convertase cleavage site of an α or β subunit to enable more efficient cleavage. However, this is known in the prior art for the β subunit from D1 and D2. Therefore this cannot be a common feature to provide unity of invention, *a posteriori*.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2016/051156**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		**Patent Family Member/s**	
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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

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Patent Document/s Cited in Search Report		**Patent Family Member/s**	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
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End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.