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(54) Title: FOLLISTATIN-RESISTANT ACTIVIN

(57) Abstract: This invention relates to follistatin-resistant variants of activin that are modified to include mutations at residues Asp27 and Gln98. These activin variants are shown to display increased resistance to follistatin inhibition relative to wild-type activin and hence increased signaling potency in cell culture. Modified activin and methods for its production and use are provided.



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Follistatin-Resistant Activin

Field

This invention relates to engineered forms of activin and their use in cell culture.

5 Background

Activin signaling is regulated by follistatin (FST), an important antagonist in the extracellular matrix. Activin induces follistatin expression, generating a feedback inhibitory loop which limits the extent and duration of activin mediated signaling.

- 10 Follistatin inhibits activin by binding directly to the mature growth factor, forming stoichiometric complex with two follistatins interacting with a dimeric activin^{1,2}. This complex is unable to interact with activin receptors and induce signaling and is instead taken into cells through heparin mediated internalization and destroyed.
- 15 An Asp95Ala mutation in activin A has been reported to slightly reduce the inhibitory potency of follistatin on activin A signaling compared to the wild-type activin A (IC_{50} increased from 178 ± 19 pM to 360 ± 49 pM)⁴. However, this modest increase in resistance to follistatin inhibition is insufficient to impact on the signaling potency of activin A in cell culture.
- 20 Reducing or blocking the inhibition of activin by follistatin would be useful in increasing the extent and duration of activin-mediated signaling in cell culture.

Summary

- 25 This invention relates to the unexpected finding that mutations at certain positions in the amino acid sequence of activin dramatically increase the resistance of activin to follistatin inhibition without abolishing its biological signaling activity, thereby increasing its signaling potency in cell culture. Follistatin-resistant activin as described herein may display an extended half-life in cell culture and have biotechnological and therapeutic applications in stem cell research and regenerative medicine.

30

An aspect of the invention provides a modified activin comprising mutations at both Asp27 and Gln98.

- 35 The modified activin may optionally comprise further mutations at one or more of Asp95, Arg87, Lys85 and Lys103. For example, a modified activin may comprise mutations at Asp27, Gln98 and Asp95; mutations at Asp27, Gln98 and Arg87; mutations at Asp27, Gln98 and Lys85; mutations at Asp27, Gln98 and Lys103; mutations at Asp27, Gln98, Arg87 and

Asp95; mutations at Asp27, Gln98, Lys85, and Arg87; and mutations at Asp27, Gln98, Lys85, Arg87 and Asp95.

The modified activin may display increased resistance to inhibition by follistatin relative to unmodified activin.

A preferred modified activin may comprise the amino acid sequence of any one of SEQ ID NOs: 9 to 11 or 13.

Other aspects of the invention provide a nucleic acid encoding a modified activin described herein; a vector comprising a nucleic acid encoding a modified activin described herein; a recombinant cell comprising a nucleic acid or a vector described herein; and a cell culture medium comprising a modified activin described herein.

Another aspect of the invention provides a method of culturing mammalian cells comprising exposing said cells in a cell culture medium comprising modified activin as described herein.

Another aspect of the invention provides the use of modified activin as described herein for the culture of mammalian cells.

Other aspects and embodiments of the invention are described in more detail below.

Brief Description of Figures

Figure 1 shows structural and activity analysis of engineered activin A (Asp27Asn Gln98Arg) (frActA001). Fig1a shows the crystal structures of wild type (light) and engineered activin A (Asp27Asn Gln98Arg) (dark). Fig 1b shows activity analysis of wild type and engineered activin A in cell-based luciferase assay. Data and fit curve are shown for wild-type activin A (ActA; round marks and black curve) and engineered activin A (frActA001; square marks and dotted curve). Error bars are standard deviations from three replicate of data.

Figure 2 shows follistatin inhibition and its interaction with wild-type and engineered activin A (Asp27Asn Gln98Arg) (frActA001). Fig 2a shows analysis of follistatin inhibition of 100 pM wild-type and engineered activin A in cell-based luciferase assay. Data and fit curves are shown for wild-type activin A (100 pM ActA; round marks and black curve) and engineered activin A (100 pM frActA001; square marks and dotted curve). Figs 2b and 2c show BLI analysis of interaction between follistatin and wild-type activin A (ActA) and interaction between follistatin and engineered activin A (frActA001).

Figure 3 shows the analysis of further mutagenesis on the engineered activin A. The mutation sites of each engineered activin A are listed in Table 1. Figs 3a and 3b show the analysis of the signalling activities of activin A variants in the cell-based luciferase assay.

5 Figs 3c and 3d show the analysis of follistatin inhibition of 100 pM activin A variants in the cell-based luciferase assay. Figs 3e and 3f show the analysis of the interaction between follistatin and selected engineered activin A.

Figure 4 shows the concentration of follistatin and activin A in the prolonged culture of iPSCs grown in 10 ng/ml activin A or frActA001 for seven days, as measured by ELISA. The molar concentrations of activin A (ActA) are shown as shaded bars, those of follistatin in activin A-containing culture medium (FST (ActA culture)) are shown as black bars, and those of follistatin in frActA001-containing culture medium (FST (frActA001 culture)) are shown as grey bars. The molar ratios of follistatin to activin A in each culture are shown as black dots with black connecting line (FST:ActA (ActA culture)) and grey dots with grey connecting line (FST:ActA (frActA001 culture)), respectively.

Figure 5 shows the residual activin A signaling activity of the medium samples of iPSC cell culture after extended culturing in the presence of wild type activin A or frActA001. The activities of iPSC culture medium containing activin A (ActA culture) are shown as black round marks with black connecting line and those of iPSC culture medium containing frActA001 (frActA001 culture) are shown as square marks with dotted connecting line. The activin A signalling activities are determined in duplicate using HEK293T cell-based luciferase assay.

25 Figure 6 shows the modelling of residual activin A signaling activity of medium samples of iPSC cell culture in the presence of wild type activin A or frActA001 with the medium being replaced with fresh medium every day. The data for the model is taken from the experimental data in Figure 5.

30 Figure 7 shows the modelling of residual activin A signaling activity of medium samples of iPSC cell culture in the presence of wild type activin A or frActA001 with the medium being replaced with fresh medium every other day. The data for the model is taken from the experimental data in Figure 5.

35

Detailed Description

This invention relates to modified activins which have mutations at Asp27 and Gln98 and

optionally one or more of Asp95, Arg87, Lys85, and Lys103. A modified activin may display increased resistance to follistatin inhibition compared to an unmodified activin which does not have these mutations.

- 5 A modified activin as described herein may have two or more mutations in its amino acid sequence. For example, the modified activin may have two, three, four, five, six or more than six mutations. The residues at both Asp27 and Gln98 are mutated in the modified activin. Mutation of only one of residues Asp27 and Gln98 does not increase resistance to follistatin inhibition. Optionally, in addition to Asp27 and Gln98, one or more of Asp95, Arg87, 10 Lys85 and Lys103 may also be mutated. For example, the modified activin may comprise one of the following combinations of two or more mutations;
- (i) mutations at Asp27 and Gln98; preferably Asp27Asn and Gln98Arg
 - (ii) mutations at Asp27, Gln98 and Asp95; preferably Asp27Asn, Gln98Arg, and Asp95Ala
 - 15 (iii) mutations at Asp27, Gln98 and Arg87; preferably Asp27Asn, Gln98Arg, and Arg87Asn
 - (iv) mutations at Asp27, Gln98, Asp95 and Arg87; preferably Asp27Asn, Gln98Arg, Arg87Asn, and Asp95Ala
 - (v) mutations at Asp27, Gln98 and Lys85; preferably Asp27Asn, Gln98Arg and 20 Lys85X₁,
 - (vi) mutations at Asp27, Gln98, Asp95 and Lys85; preferably Asp27Asn, Gln98Arg, Asp95Ala and Lys85X₁
 - (vii) mutations at Asp27, Gln98, Arg87 and Lys85; preferably Asp27Asn, Gln98Arg, Arg87Asn and Lys85X₁
 - 25 (viii) mutations at Asp27, Gln98, Asp95, Arg87 and Lys85; preferably Asp27Asn, Gln98Arg, Arg87Asn, Asp95Ala and Lys85X₁
 - (ix) mutations at Asp27, Gln98 and Lys 103 preferably Asp27Asn, Gln98Arg and Lys103X₁
 - (x) mutations at Asp27, Gln98, Asp95 and Lys 103; preferably Asp27Asn, Gln98Arg, 30 Asp95Ala and Lys103X₁
 - (xi) mutations at Asp27, Gln98, Arg87 and Lys 103; preferably Asp27Asn, Gln98Arg, Arg87Asn, and Lys103X₁
 - (xii) mutations at Asp27, Gln98, Asp95, Arg87 and Lys 103; preferably Asp27Asn, Gln98Arg, Arg87Asn, Asp95Ala and Lys103X₁
 - 35 (xiii) mutations at Asp27, Gln98, Lys85 and Lys 103; preferably Asp27Asn, Gln98Arg, Lys85X₁ and Lys103X₂

(xiv) mutations at Asp27, Gln98, Asp95, Lys85 and Lys 103; preferably Asp27Asn, Gln98Arg, Asp95Ala, Lys85X₁ and Lys103X₂

(xv) mutations at Asp27, Gln98, Arg87, Lys85 and Lys 103; preferably Asp27Asn, Gln98Arg, Arg87Asn, Lys85X₁ and Lys103X₂

5 (xvi) mutations at Asp27, Gln98, Asp95, Arg87, Lys85 and Lys 103; preferably Asp27Asn, Gln98Arg, Arg87Asn, Asp95Ala, Lys85X₁ and Lys103X₂, or

(xvii) any one of combinations (i) to (xvi) with an additional 4-8 residue N-terminal deletion from the mature activin A or activin B sequence.

10 In (i) to (xvii) above, X₁ and X₂ may be, independently, a non-positively charged amino acid i.e. any amino acid other than Lys, Arg or His.

Activins are dimeric members of the TGF β superfamily. They stimulate SMAD2 and SMAD3 mediated intracellular signaling pathways in mammalian cells and activate the secretion of FSH by the pituitary gland. Activins exert a range of cellular effects via stimulation of the Activin/Nodal pathway and are used for the maintenance of pluripotency in human embryonic stem cells. (Vallier et al., *Cell Science* 118:4495-4509 (2005)). Activins are involved in the development of whisker, palate, and tooth development in mice and play a role in enhancing oligodendrocyte differentiation and wound healing as well. Activins induce differential effects on cells, depending on their concentration, and are used in the differentiation of stem cells. Activins are inhibited by the secreted agonist follistatin, which prevents signalling by binding directly to mature activins and preventing interaction with receptors. Expression of follistatin is induced by activins, causing a negative feedback loop and termination of signalling.

25 Activin as described herein may be vertebrate activin, preferably mammalian activin, most preferably human activin.

Activin as described herein may be activin A or activin B, preferably activin A.

30 Activin A and activin B induce the same signaling pathways but bind to different type 1 receptors (Activin B ALK7 and ALK4; Activin A ALK4). Activin A and activin B have similar biological activities and display similar bioactivity/potency in the luciferase activity assay described below.

35 Activin A (Inhibin β A; INHBA; Gene ID 3624; Uniprot P08476) may have the reference amino acid sequence of NP_002183.1 GI: 4504699 and may be encoded by the reference

nucleotide sequence of NM_002192.2 GI: 62953137. The wild-type activin A precursor may comprise the amino acid sequence of SEQ ID NO: 1 and the mature activin A may comprise the amino acid sequence of SEQ ID NO: 6.

- 5 Activin B (Inhibin β B; INHBB; Gene ID 3625) may have the reference amino acid sequence of NP_002184.2 GI: 154813204 and may be encoded by the reference nucleotide sequence of NM_002193.2 GI: 154813203. The unmodified wild-type activin B precursor may comprise the amino acid sequence of SEQ ID NO: 2 and the mature activin B may comprise the amino acid sequence of SEQ ID NO: 3.

10

The activin residues described herein are numbered according to their position in the mature activin A sequence of SEQ ID NO: 6. Residue 1 corresponds to residue 311 of Uniprot entry P08476 (NP002183.1; SEQ ID NO: 1). The corresponding position in a different activin sequence may be easily identified using standard sequence alignment techniques. For
15 example, Asp27, Arg87, Asp95, and Lys85, and Gln98 of the activin A sequence of SEQ ID NO: 6 correspond to Asp27, Ser86, Asp94, Lys84 and Tyr97 respectively of the activin B sequence of SEQ ID NO: 3.

- The activity of activin may be determined using conventional assays. Suitable assays
20 include cell-based assays using luciferase linked to an activin-responsive promoter, for example a SMAD2/3 response element such as CAGA_n, and assays to measure the release of FSH from a pituitary cell line (e.g. L β T2).

- Follistatin (FST Gene ID 10468) is a single-chain gonadal protein that specifically inhibits
25 follicle-stimulating hormone release. Preferably, follistatin is human follistatin. Follistatin has two isoforms (FST317 and FST344) which result from alternative splicing of the precursor mRNA. Follistatin may be follistatin-317 (also called FST-288) or follistatin-344 (also called FST-315).

- 30 Follistatin-317 precursor may have the reference amino acid sequence of NP_006341.1 GI: 5453652 and may be encoded by the reference nucleotide sequence of NM_006350.3 GI: 331999951. The mature form of Follistatin-317 may have the reference amino acid sequence of residues 30 to 317 of NP_006341.1 GI: 5453652.

- 35 Follistatin-344 precursor may have the reference amino acid sequence of NP_037541.1 GI: 7242222 and may be encoded by the reference nucleotide sequence of NM_013409.2 GI: 331999952. The mature form of Follistatin-344 may have the reference amino acid

sequence of residues 30 to 344 of NP_037541.1 GI: 7242222.

Follistatin binds to activin. The extent or affinity of the binding of follistatin to activin may be determined using conventional techniques, such as isothermal titration calorimetry (ITC),
5 biolayer interferometry (BLI), surface plasmon resonance (SPR) spectroscopy and
fluorescent polarization anisotropy and monitoring binding of radioactive activin to cell
surface receptors. The ability of follistatin to inhibit activin can be measured using cell-based
bioassays by monitoring the reduction in activin signalling outcome. Suitable assays include
production of luciferase enzyme from activin inducible luciferase construct, differentiation of
10 K562 cells and differentiation of embryonic stem cells.

A residue in the amino acid sequence of activin may be mutated by insertion, deletion or
substitution, preferably substitution for a different amino acid residue. Such mutations may
be caused by one or more of addition, insertion, deletion or substitution of one or more
15 nucleotides in the encoding nucleic acid. This may be achieved using standard techniques.

A mutation at a position in the activin sequence may comprise the substitution of the residue
at that position in the unmodified activin sequence for a different naturally-occurring amino
acid, a non-natural amino acid, a modified amino acid or a D-amino acid. The use of such
20 amino acids is well-known to those of skill in the art.

A modified activin of the invention comprises a mutation at Asp27. Preferably, the mutation
is a substitution in which the Asp residue at position 27 is replaced with a non-native residue
(i.e. a residue other than Asp) in the modified activin. Asp27 may substituted for any natural
25 or non-natural amino acid other than Asp. Preferably, Asp27 is substituted for a non-
negatively charged amino acid i.e. an amino acid other than Asp or Glu. Suitable amino acid
residues include a positively charged amino acid, such as Lys, Arg or His, a polar amino
acid, such as Ser, Thr, Cys, Met, Asn, or Gln, an aromatic amino acid, such as Phe, Trp and
Tyr, or a non-polar aliphatic amino acid, such as Gly, Ala, Val, Leu, Ile or Pro. In some
30 embodiments, a non-polar residue, such as Ala or Gly, may be preferred.

In especially preferred embodiments, Asp27 is substituted for Asn (Asp27Asn).

A modified activin of the invention further comprises a mutation at Gln98. Preferably, the
35 mutation is a substitution in which the Gln residue at position 98 is replaced with a non-
native residue (i.e. a residue other than Gln) in the modified activin. Gln98 may substituted
for any natural or non-natural amino acid other than Gln. Suitable amino acid residues

include a positively charged amino acid, such as Lys, Arg or His, a negative amino acid, such as Asp or Glu, an aromatic amino acid, such as Phe, Trp and Tyr, or a non-polar aliphatic amino acid, such as Gly, Ala, Val, Leu, Ile or Pro. In some embodiments, a positively charged amino acid, such as Lys, Arg or His may be preferred.

5

In especially preferred embodiments, Gln98 is substituted for Arg (Gln98Arg).

In some embodiments, the modified activin may comprise a mutation at Asp95 in addition to the mutations at Asp27 and Gln98. Asp95 may be substituted for a different residue.

10 Preferably, the mutation is a substitution in which the Asp residue at position 95 is replaced with a non-native residue (i.e. a residue other than Asp) in the modified activin. Asp95 may be substituted for any natural or non-natural amino acid other than Asp. Preferably, Asp95 is substituted for a non-negatively charged amino acid i.e. an amino acid other than Asp or Glu. Suitable amino acid residues include a positively charged amino acid, such as Lys, Arg
15 or His, a polar amino acid, such as Ser, Thr, Cys, Met, Asn, or Gln, an aromatic amino acid, such as Phe, Trp and Tyr, or a non-polar aliphatic amino acid, such as Gly, Ala, Val, Leu, Ile or Pro.

In especially preferred embodiments, Asp95 is substituted for Ala (Asp95Ala).

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In some embodiments, the modified activin may comprise a mutation at Arg87 in addition to mutations at Asp27 and Gln98. Preferably, the mutation is a substitution in which the Arg residue at position 87 is replaced with a non-native residue (i.e. a residue other than Arg) in the modified activin. Arg87 may be substituted for any natural or non-natural amino acid other
25 than Arg. Preferably, Arg87 is substituted for a non-positively charged amino acid i.e. an amino acid other than Lys, Arg or His. Suitable amino acid residues include a negatively charged amino acid, such as Asp or Glu, a polar amino acid, such as Ser, Thr, Cys, Met, Asn, or Gln, an aromatic amino acid, such as Phe, Trp and Tyr, or a non-polar aliphatic amino acid, such as Gly, Ala, Val, Leu, Ile or Pro. In some embodiments, a small neutral
30 amino acid, such as Gly, Ala or Pro, or a negative amino acid, such as Glu or Asp, may be preferred.

In some preferred embodiments, Arg87 is substituted for Asn (Arg87Asn).

35 In some embodiments, the modified activin may comprise a mutation at Lys85. Lys85 may be substituted for a different residue. Preferably, the mutation is a substitution in which the Lys residue at position 85 is replaced with a non-native residue (i.e. a residue

other than Lys) in the modified activin. Lys85 may substituted for any natural or non-natural amino acid other than Lys. Preferably, Lys85 is substituted for a non-positively charged amino acid i.e. an amino acid other than Lys, Arg or His. Suitable amino acid residues include a negatively charged amino acid, such as Asp or Glu, a polar amino acid, such as Ser, Thr, Cys, Met, Asn, or Gln, an aromatic amino acid, such as Phe, Trp and Tyr, or a non-polar aliphatic amino acid, such as Gly, Ala, Val, Leu, Ile or Pro.

In some preferred embodiments, Lys85 is substituted for Ser.

10 In some embodiments, the modified activin may further comprise a mutation at Lys103. Lys103 may be substituted for a different residue. Preferably, the mutation is a substitution in which the Lys residue at position 103 is replaced with a non-native residue (i.e. a residue other than Lys) in the modified activin. Lys103 may substituted for any natural or non-natural amino acid other than Lys. Preferably, Lys103 is substituted for a non-positively charged amino acid i.e. an amino acid other than Lys, Arg or His. Suitable amino acid residues include a negatively charged amino acid, such as Asp or Glu, a polar amino acid, such as Ser, Thr, Cys, Met, Asn, or Gln, an aromatic amino acid, such as Phe, Trp and Tyr, or a non-polar aliphatic amino acid, such as Gly, Ala, Val, Leu, Ile or Pro.

20 In some preferred embodiments, Lys103 is substituted for Asn.

In some embodiments, in addition to a set of mutations described above, the modified activin may further comprise an N-terminal deletion. For example, the modified activin may lack 1, 2, 3, 4, 5, 6, 7, 8 or more residues at the N terminal, preferably 4-8 residues, for example about 7 residues, relative to unmodified mature activin (for example SEQ ID NO: 3 or SEQ ID NO: 6). When residue 4 (Cys) is removed or mutated, residue 12 may also be mutated to remove the sulfhydryl group of otherwise unpaired half-cysteine. Activin sequences with an N-terminal deletion are shown in SEQ ID NOs: 12 and 13.

30 Other than mutations of residues identified above, a modified activin may have 30 or fewer, or 20 or fewer amino acid residues altered relative to a wild-type activin amino acid sequence (for example the mature activin sequence of SEQ ID NO: 3 or 6), preferably 15 or fewer, 10 or fewer, 5 or fewer or 3 or fewer. For example, a modified activin may comprise the sequence of a wild-type activin with 30 or fewer, 20 or fewer, 15 or fewer, 10 or fewer, 5 or fewer, 3 or fewer, 2 or fewer or 1 or fewer amino acid residues mutated or altered, in addition to Asp27 and Gln98, and optionally Asp95, Arg87, Lys85 and/or Lys103. In some preferred embodiments, a modified activin may comprise the sequence of a wild-type activin

with no amino acid residues mutated or altered other than Asp27 and Gln98, and optionally Asp95, Arg87, Lys85 and/or Lys103.

In some embodiments, the residues in the modified activin that interact with an activin receptor, for example the type I or the type II activin receptor, may not be mutated or altered. Residues in the modified activin that interact with activin receptor include Ser90, Met91, Leu92, Tyr93, Tyr94, Ile100 and Lys102⁴.

The modified activin may share at least 50% sequence identity with the wild-type amino acid sequence of a mature wild-type activin, for example the mature activin B sequence of SEQ ID NO: 3 or the mature activin A sequence of SEQ ID NO: 6, at least 55%, at least 60%, at least 65%, at least 70%, at least about 80%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity.

Sequence identity is commonly defined with reference to the algorithm GAP (Wisconsin GCG package, Accelrys Inc, San Diego USA). GAP uses the Needleman and Wunsch algorithm to align two complete sequences that maximizes the number of matches and minimizes the number of gaps. Generally, default parameters are used, with a gap creation penalty = 12 and gap extension penalty = 4. Use of GAP may be preferred but other algorithms may be used, e.g. EMBOSS Needle (which uses the Needleman and Wunsch algorithm), EMBOSS Stretcher (which uses a modified Needleman and Wunsch algorithm) BLAST (which uses the method of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 405-410), FASTA (which uses the method of Pearson and Lipman (1988) *PNAS USA* 85: 2444-2448), or the Smith-Waterman algorithm (Smith and Waterman (1981) *J. Mol Biol.* 147: 195-197), CLUSTAL ω (EMBL-EBI; Sievers *et al* (2011) *Molecular Systems Biology* 7:539 doi:10.1038/msb.2011.75) or the TBLASTN program, of Altschul *et al.* (1990) *supra*, generally employing default parameters. In particular, the psi-Blast algorithm may be used (Nucl. Acids Res. (1997) 25 3389-3402). Sequence identity and similarity may also be determined using GenomequestTM software (Gene-IT, Worcester MA USA).

Sequence comparisons are preferably made over the full-length of the relevant sequence described herein.

In some preferred embodiments, the modified activin may consist of the amino acid sequence of a wild-type activin with said two or more mutations which increase follistatin resistance as described herein i.e. the only mutations in the sequence of the modified activin are a combination of two or more mutations set out above.

Preferred modified activin sequences may include any one of SEQ ID NOS: 9 to 11 or 14 to 16, most preferably SEQ ID NOs: 9 and 10.

- 5 A modified activin as described herein may be part of a fusion protein, for example a fusion protein which contains the activin prodomain or one or more heterologous amino acid sequences additional to the modified activin sequence. The fusion protein comprising the modified activin may further comprise one or more additional domains which improve the stability, pharmacokinetic, targeting, affinity, purification and production properties of the
10 modified activin.

Modified activins as described herein may be provided using synthetic or recombinant techniques which are standard in the art. In some embodiments, a modified activin may be recombinantly expressed in insoluble inclusion bodies in a prokaryotic expression system
15 such as *E coli*. Following expression, the inclusion bodies may be isolated and solubilised with a denaturant to generate the modified activin re-folded into soluble form.

In other embodiments, a modified activin may be recombinantly expressed in a eukaryotic expression system. The modified activin may be coupled to its own pro-domain and/or a
20 signal leader peptide to direct secretion of the fusion polypeptide from a eukaryotic cell into the culture medium. A range of suitable signal leader peptides are known in the art. The signal leader peptide may be an activin signal sequence or may be heterologous to the modified activin i.e. it may be a non-activin signal sequence. For example, an α -factor secretion signal or BiP signal sequence may be employed. Preferably, the signal peptide
25 and/or pro-domain is removed by post-translational processing after expression of the polypeptide.

In some embodiments, the modified activin may be produced as a fusion protein further comprising an affinity tag, which may, for example, be useful for purification. An affinity tag is
30 a heterologous peptide sequence which forms one member of a specific binding pair. Polypeptides containing the tag may be purified by the binding of the other member of the specific binding pair to the polypeptide, for example in an affinity column. For example, the tag sequence may form an epitope which is bound by an antibody molecule. Suitable affinity tags are well known in the art and are reviewed in Terpe (2003) Appl. Microbiol. Biotechnol.
35 60 523-533. The affinity tag sequence may be separated from the modified activin after purification, for example, using site-specific proteases.

Modified activins as described herein may be isolated, in the sense of being free from contaminants, such as unmodified activins and other polypeptides and/or serum components. Modified activins as described herein are preferably non-naturally occurring proteins.

5

The presence of the two or more mutations reduces the binding affinity of follistatin to the modified activin relative to unmodified activin. For example, the modified activin may bind to follistatin with an affinity that is at least 3 fold, at least 4 fold, at least 5 fold, at least 6 fold, at least 7 fold, at least 8 fold or at least 10 fold lower than the binding affinity of the unmodified
10 activin to follistatin. A suitable modified activin may for example bind to follistatin with a dissociation constant (K_d) of 400 pM or more, 500 pM or more, 600 pM or more, 700 pM or more, 1200 pM or more, or 2000 pM or more.

The modified activin may have lower sensitivity and/or higher resistance than unmodified
15 activin to inhibition by follistatin. For example, the IC_{50} of follistatin for modified activin may be greater than the IC_{50} of follistatin for unmodified activin (252 pM). The IC_{50} of follistatin for modified activin may be at least 2 fold, at least 5 fold, at least 10 fold, at least 50 fold or at least 100 fold higher than the IC_{50} of follistatin for unmodified activin. A suitable modified
20 activin may for example have an IC_{50} of 500 pM or more, 1000 pM or more, 10000pM or more, or 50000pM or more. For example, the IC_{50} of follistatin for modified activin A (Asp27Gln98) is 643 pM; the IC_{50} for modified activin A (Asp27Gln98Arg87) is 1644 pM; and the IC_{50} for modified activin A (Asp27Gln98Asp95) is 53211 pM.

IC_{50} may be measured using any convenient method. Suitable methods include treating
25 HEK293T cells transfected with a plasmid containing a luciferase gene linked to an activin-responsive promoter with a mixture of different concentrations of follistatin and 100pM modified activin and determining the IC_{50} , as described below. Alternatively, the IC_{50} of follistatin to inhibit signalling from 100 pM modified activin in LbT2 cells may be determined⁴.

30 Activin stimulates the Activin/Nodal signaling pathway. Preferably, the presence of the two or more mutations in the activin sequence does not significantly affect the biological activity of the modified activin relative to unmodified activin. For example, the modified activin may have the same activity as unmodified activin; an activity that is greater than unmodified
35 activin, for example up to 20%, up to 50% or more than 50% greater; or an activity that is less than unmodified activin, for example up to 20% or up to 50% less. In some embodiments, activity may be expressed as the EC_{50} (i.e. the concentration required for half maximal effect).

Techniques for measuring activin activity are standard in the art. Suitable techniques include measurement of the activin-induced expression level of luciferase in a transfected mammalian cell (see methods section below); measurement of the secretion level of
5 follistatin from LbT2 induced by activin (see ref 4); detection of the phosphorylation level of SMAD2/3 in the mammalian cells treated with activin; detection of the pluripotency marker/differentiation marker in stem cells treated with activin; and measurement of the inhibition of proliferation of mouse plasmacytoma cell line MPC-11 by activin A.

10 A modified activin may retain at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% of its activity in a five-day culture with mammalian cells, such as iPSCs. In some embodiments, a modified activin may retain at least 30% activity, for example 35% or more activity in a seven-day culture with mammalian cells.

15 Other aspects of the invention provide a nucleic acid encoding a modified activin as described above and a vector comprising such a nucleic acid.

Nucleic acid encoding a modified activin as described herein may be readily prepared by the skilled person using the information and references contained herein and techniques known
20 in the art (for example, see *Molecular Cloning: a Laboratory Manual*: 3rd edition, Sambrook and Russell (2001) Cold Spring Harbor Laboratory Press). For example, nucleic acid may be prepared by conventional solid phase synthesis techniques or may be produced by recombinant means.

25 Nucleic acid encoding a modified activin may be codon optimized for expression in a particular host organism, such as *E. coli*. The nucleic acid encoding the modified activin may display a lower sequence identity to the wild-type activin coding sequence than the sequence identity of the modified activin protein to the wild-type activin protein.

30 The nucleic acid encoding a modified activin may be operably linked to a heterologous regulatory element i.e. a regulatory element that is not naturally associated with the activin coding sequence.

Suitable vectors can be chosen or constructed, containing appropriate regulatory
35 sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Preferably, the vector contains appropriate regulatory sequences to drive the expression of

the nucleic acid in mammalian cells. A vector may also comprise sequences, such as origins of replication, promoter regions and selectable markers, which allow for its selection, expression and replication in bacterial hosts such as *E. coli*.

- 5 Vectors may be plasmids, viral e.g. phage, or phagemid, as appropriate. Preferred vectors for expression in *E. coli* include T7 based vectors, such as the vectors in the pET series (Novagen, USA). For further details see, for example, Molecular Cloning: a Laboratory Manual: 3rd edition, Russell et al., 2001, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of
- 10 nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, are described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds. John Wiley & Sons, 1992.

A nucleic acid or vector as described herein may be introduced into a host cell.

15

Another aspect of the invention provides a recombinant cell comprising a nucleic acid or vector that expresses a polypeptide comprising or consisting of a modified activin as described above.

- 20 A range of host cells suitable for the production of recombinant modified activins are known in the art. Suitable host cells may include prokaryotic cells, in particular bacteria such as *Escherichia coli* and *Lactococcus lactis* and eukaryotic cells, including mammalian cells such as CHO and CHO-derived cell lines (Lec cells), HeLa, COS, HEK293 and HEK-EBNA cells, amphibian cells such as *Xenopus* oocytes, insect cells such as *Trichoplusia ni*, Sf9 and Sf21
- 25 and yeast cells, such as *Pichia pastoris*.

- Techniques for the introduction of nucleic acid into cells are well-established in the art and any suitable technique may be employed, in accordance with the particular circumstances. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-
- 30 Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. adenovirus, AAV, lentivirus or vaccinia. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage.

- 35 Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing nucleic acid of interest, as is well-known in the art.

The introduced nucleic acid may be on an extra-chromosomal vector within the cell or the nucleic acid may be integrated into the genome of the host cell. Integration may be promoted by inclusion of sequences within the nucleic acid or vector which promote recombination with the genome, in accordance with standard techniques.

5

The introduction may be followed by expression of the nucleic acid to produce the encoded modified activin. In some embodiments, host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) may be cultured *in vitro* under conditions for expression of the nucleic acid, so that the encoded
10 activin polypeptide is produced. When an inducible promoter is used, expression may require the activation of the inducible promoter.

In some preferred embodiments, the modified activin is expressed in a prokaryotic system, such as *E. coli*, in the form of insoluble inclusion bodies. Following expression, the inclusion
15 bodies may be extracted from the prokaryotic cells and the expressed polypeptide solubilised from the purified inclusion bodies and refolded using a denaturant, such as guanidine hydrochloride. Suitable techniques for solubilising and refolding expressed polypeptides are well-known in the art.

20 The expressed polypeptide comprising or consisting of the modified activin may be isolated and/or purified, after production. This may be achieved using any convenient method known in the art. Techniques for the purification of recombinant polypeptides are well known in the art and include, for example ion exchange chromatography, reverse phase chromatography (RPC), HPLC, FPLC or affinity chromatography. In some embodiments, purification may be
25 performed using an affinity tag on the polypeptide as described above.

In some preferred embodiments, the refolded protein may be purified by reverse phase chromatography, followed by ion exchange chromatography, and finished with a high-resolution reverse phase chromatography. Suitable methods are described in detail below.

30

Another aspect of the invention provides a method of producing a modified activin comprising expressing a nucleic acid encoding a modified activin as described above in a host cell and optionally isolating and/or purifying the modified activin thus produced.

35 Polypeptides comprising or consisting of a modified activin produced as described may be investigated further, for example the pharmacological properties and/or activity may be determined. Methods and means of protein analysis are well-known in the art.

Another aspect of the invention provides a method of producing a modified activin comprising

- expressing a nucleic acid as described above and;
- 5 and isolating the modified activin expressed by the nucleic acid.

Another aspect of the invention provides a cell culture medium or a culture medium supplement comprising a modified activin as described above.

- 10 The cell culture medium may be suitable for the culture, expansion, differentiation or forward programming of stem cells, including embryonic stem cells and iPSCs.

The cell culture medium may comprise a basal medium, such as RPMI-1640 supplemented with additional factors, such as glucose, amino acids such as glutamine, HEPES, pH 7.2, antibiotics, such as penicillin and streptomycin, and/or β -mercaptoethanol.

In some embodiments, the cell culture medium may be a chemically defined medium. A CDM is a nutritive solution for culturing cells which contains only specified components, preferably components of known chemical structure. A CDM is devoid of components which are not fully defined, for example serum or proteins isolated therefrom, such as Foetal Bovine Serum (FBS), Bovine Serum Albumin (BSA), and feeder or other cells. In some embodiments, a CDM may be humanised and may be devoid of components from non-human animals. Proteins in the CDM may be recombinant human proteins. Suitable CDMs are well known in the art and described in more detail below.

25 The cell culture medium may comprise a chemically defined basal media, such as Johansson and Wiles CDM (Johansson and Wiles (1995) Mol Cell Biol 15, 141-151) which is supplemented with polyvinyl alcohol, insulin, transferrin and defined lipids. Johansson and Wiles CDM consists of: 50% IMDM (Gibco) plus 50% F12 NUT-MIX (Gibco); 7 μ g/ml insulin; 30 15 μ g/ml transferrin; 1 mg/ml polyvinyl alcohol (PVA); 1% chemically defined lipid concentrate (Invitrogen); and 450 μ M 1-thioglycerol.

The medium may be supplemented with serum or a serum substitute.

- 35 Optionally, the medium may be supplemented with recombinant FGF2 (also called bFGF or basic FGF), BMP-2, BMP-4, IL-2 or other growth factors and cytokines.

Basal media and media components may be obtained from commercial sources (e.g. Life Technologies, Roche, Sigma, Europabioproducts, Cellgenix, Life Sciences).

5 The culture medium may be formulated in deionized, distilled water. The culture medium will typically be sterilized prior to use to prevent contamination, e.g. by ultraviolet light, heating, irradiation or filtration. The culture medium may be frozen (e.g. at -20°C or -80°C) for storage or transport. The culture medium may contain one or more antibiotics to prevent contamination.

10 The culture medium may be a 1x formulation or a more concentrated formulation, e.g. a 2x to 250x concentrated medium formulation. In a 1x formulation each ingredient in the medium is at the concentration intended for cell culture, for example a concentration set out above. In a concentrated formulation one or more of the ingredients is present at a higher
15 concentration than intended for cell culture. Concentrated culture media are well known in the art. Culture media can be concentrated using known methods e.g. salt precipitation or selective filtration. A concentrated medium may be diluted for use with water (preferably deionized and distilled) or any appropriate solution, e.g. an aqueous saline solution, an aqueous buffer or a culture medium.

20 The culture medium may be contained in hermetically-sealed vessels. Hermetically-sealed vessels may be preferred for transport or storage of the culture medium, to prevent contamination. The vessel may be any suitable vessel, such as a flask, a plate, a bottle, a jar, a vial or a bag.

25 Another aspect of the invention provides the use of a culture medium comprising a modified activin as described above for the culture of mammalian cells.

30 Another aspect of the invention provides a method of culturing mammalian cells comprising exposing said cells to a culture medium comprising modified activin as described above.

The activity of the modified activin may be maintained in the culture medium for at least 1 day, at least 2 days, at least 3 days, at least 4 days, at least 5 days, at least 6 days, at least 7 days following exposure to mammalian cells.

35 The mammalian cells may be stem cells, including hESCs and iPSCs, and progenitor and precursor cells that are not fully differentiated.

In some embodiments, a culture medium comprising a modified activin may be used for the differentiation of mammalian cells. Suitable mammalian cells may include differentiated or differentiating mammalian cells.

5

Another aspect of the invention provides a kit for the culture of mammalian cells comprising a modified activin as described herein.

Modified activin is described above.

10

The kit may further comprise a chemically defined basal medium, a supplement as described above and/or one or more other cell culture ingredients selected from the group consisting of amino acids, vitamins, inorganic salts, carbon energy sources, buffers, FGF, transferrin, 1-thioglycerol, defined lipids, polyvinyl alcohol and optionally insulin.

15

The components of the kit may be contained in separate hermetically-sealed vessels.

A kit may further comprise a cell culture vessel. Suitable cell culture vessels, such as flasks, single or multiwell plates, single or multiwell dishes, bottles, jars, vials, bags and bioreactors, are well-known in the art.

20

Modified activin as described above may also be useful in therapeutic applications, such as wound healing and hair growth.

25

An aspect of the invention provides a modified activin for use in therapy.

Other aspects of the invention provide a method of wound healing and/or hair growth comprising administering a modified activin as described above to an individual in need thereof; a modified activin as described above for use in a method of wound healing and/or hair growth, and; the use of a modified activin as described above in the manufacture of a medicament for use in a method of wound healing and/or hair growth.

30

Other aspects and embodiments of the invention provide the aspects and embodiments described above with the term "comprising" replaced by the term "consisting of" and the aspects and embodiments described above with the term "comprising" replaced by the term "consisting essentially of".

35

It is to be understood that the application discloses all combinations of any of the above aspects and embodiments described above with each other, unless the context demands otherwise. Similarly, the application discloses all combinations of the preferred and/or optional features either singly or together with any of the other aspects, unless the context demands otherwise.

Modifications of the above embodiments, further embodiments and modifications thereof will be apparent to the skilled person on reading this disclosure, and as such, these are within the scope of the present invention.

All documents and sequence database entries mentioned in this specification are incorporated herein by reference in their entirety for all purposes.

“and/or” where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. For example “A and/or B” is to be taken as specific disclosure of each of (i) A, (ii) B and (iii) A and B, just as if each is set out individually herein.

Certain aspects and embodiments of the invention will now be illustrated by way of example and with reference to the figures described above.

Experiments

1. Materials and Methods

1.1 Cloning, expression and protein refolding

All expression constructs, encoding for wild type or mutated mature domain of human inhibin β B chain, were cloned into the pBAT4 expression vector. All mutagenesis was done using overlapping extension mutagenesis and sequences verified by dideoxy sequencing. Proteins were produced in BL21(DE3) strain of *E. coli* by growing the bacteria carrying the appropriate expression plasmid in 2xYT media with 100 mg/l of ampicillin until OD₆₀₀ reached 0.8-1.0 and protein expression was induced by addition of 400 μ M isopropyl- β D-galactopyranoside to media. The proteins were expressed for 3 h at 37°C after which the cells were collected by centrifugation. Cell pellet was resuspended in MilliQ water and stored at -20°C. Cells were lysed in 50 mM Tris-HCl pH 8.0 and insoluble inclusion bodies containing activin A were isolated by 20 min centrifugation at 15,000 $\times g$. The inclusion bodies were washed with 50 mM Tris HCl, 1 mM EDTA, 10 mM dithiothreitol. 1% Triton-X-100 once, and inclusion bodies were isolated by centrifugation. Second wash was with 50 mM Tris HCl, 1 M NaCl, 1 mM EDTA, 10 mM dithiothreitol and final wash was with 50 mM

Tris HCl 1 mM EDTA, 10 mM dithiothreitol. Washed inclusion bodies were solubilised in 6 M guanidine hydrochloride, 50 mM Tris, 5 mM tricarboxyethyl phosphine (TCEP) and insoluble material removed by 20 min centrifugation at 15,000 xg. Solubilised activin A was buffer exchange using Sephadex G25 column to 10 mM HCl, 6 M Urea and diluted to 100 ml of this solution per liter of *E. coli* culture that was used for expression of the protein. Denatured
5 activin A in urea was diluted 10-fold by rapid dilution into cold, degassed 1 M pyridinyl propyl sulfonate, 50 mM Tris, 50 mM ethanolamine, 2 mM cysteine, 0.2 mM cystine, 1 mM EDTA. Activin A refolding was let to proceed for up to 2 weeks in cold.

10 1.2 Protein purification

Refolded activin A solution was filtered to remove insoluble material and loaded directly onto 10 ml Source RPC column, one liter at a time. After binding, column was washed with 10 % acetonitrile, 0.1% trifluoroacetic acid and bound proteins eluted with linear gradient to 50% acetonitrile, 0.1% trifluoroacetic acid. Fractions containing dimeric activin A were pooled and
15 loaded into 6 ml ResourceS column, equilibrated with 6 M urea, 10 mM HCl. Bound proteins were eluted with a linear gradient to 6 M urea, 10 mM HCL, 1 M NaCl. Fractions with dimeric activin A were pooled and loaded into 10x250 mm ACE C4 10 um 300A reverse phase chromatography column, equilibrated with 10 % acetonitrile, 0.1% trifluoroacetic acid. Bound proteins were eluted with a gradient to 50% acetonitrile, 0.1% trifluoroacetic acid and pure
20 activin A dimer was dried under vacuum and stored at -80°C.

1.3 Luciferase assay

To analyse the signalling activity of activin A variants, a cell-based luciferase assay was established. HEK293T cells (ATCC, cat. CRL-3216) were cultured in 96-well flat-bottom cell
25 culture plates using Dulbecco's Modified Eagle Medium (DMEM) (Life technologies) with 10% (v/v) fetal bovine serum (FBS) (Life technologies) at 37°C in a humidified incubator with 5% CO₂. When the confluence of cells reached 80%, 33 ng of pGL3-CAGA (carrying activin A responsive firefly luciferase gene) and 17 ng of pRL-SV40 (Promega, with constitutively expressed *Renilla* luciferase) plasmids were transfected into the cells in each well using 0.2
30 µl of FuGENE HD transfection reagent (Promega). After overnight incubation, the cells were washed with sterile PBS and cultured in DMEM with 0.5% FBS. Serial dilutions of activin A and its mutants in DMEM with 0.5% FBS were added into the cell culture and the experiments were performed in triplicate. In the follistatin inhibition assay, follistatin 288 was diluted in DMEM containing 0.5% FBS and 100 pM activin A or its mutants. The mixture
35 samples were then added into the cell culture in triplicate. In the analysis of the residual activin A signaling activity of the iPSC cell culture media, the medium samples collected each day were diluted four times in DMEM with 0.5% FBS and then added into the HEK293T

cell culture in duplicate. After overnight incubation, cells were washed with PBS and lysed using 20 µl of passive lysis buffer (Promega). 5 µl of cell lysate in each well was transformed into Corning 96-well flat-bottom white plate (Sigma) and was mixed with 25 µl of Dual-Glo® Luciferase Reagent (Promega). After incubation with shaking for 30 minutes, the firefly luciferase was measured using PHERstar microplate reader (BMG LABTECH). 25 µl of Dual-Glo® Stop & Glo® Reagent (Promega) was then added into each well to quench the signal from firefly luciferase and to provide substrate for the *Renilla* luciferase. The luminescence was measured using BMG PHERAstar microplate reader. The signaling response was normalised using the firefly luminescent signal divided by the *Renilla* luminescent signal. The normalized signal ratio was then converted to the range of 0-100%, where 0% is defined by the signal ratio of untreated cells and 100% is defined by the maximum signal ratio for each data set.

1.4 iPSC culture

The induced pluripotent stem cell (FSPS 13B), was cultured in 6-well plate using DMEM-F12 medium with 0.22 mM L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate, 2x Insulin-Transferrin-Selenium, 0.054% Sodium Bicarbonate solution, 100 U/ml penicillin/streptomycin, 25 ng/ml bFGF, and 10 ng/ml activin A or engineered activin A (Asp27Asn Gln98Arg) (frActA001) (also termed “E8 medium”). The medium of the cell culture was not changed since day 0 and the medium samples were taken from the culture every day for seven days. The supernatant of the samples was collected after centrifugation and frozen at -20 °C until further analysis by ELISA and cell-based luciferase assay.

1.5 ELISA analysis

The concentrations of total activin and total follistatin in the iPSC culture media were measured using Activin A ELISA (Ansh Labs, AL-110) and Follistatin ELISA (Ansh Labs, AL-117), respectively.

1.6 Biolayer interferometry

To analyse interactions between follistatin (FST) and activin A variants, biolayer interferometry (BLI) experiments were performed using Octet RED96 (ForteBio) instrument at 30 °C. Anti-Mouse IgG Fc Capture (AMC) biosensors were pre-treated with 2.5 µg/ml anti-FST antibodies (Ansh Labs, 4/2D) for 120 seconds and then immobilised with 25 nM FST-288 for 80 seconds. After a baseline measurement of 60 seconds, the association phase of binding was performed by immersing the FST-288 immobilised biosensors into the solutions containing activin A or its mutants at different concentrations for 300 seconds, followed by the dissociation phase for 300 seconds. The biosensors were regenerated 5 seconds in 10

mM glycine pH 1.7 for three times with neutralisation in the kinetic buffer in between.

The data was analysed and fitted by Prism 6 software (GraphPad) using kinetic model as follows;

5 Dissociation model:
$$R = R_0 e^{-k_{off}(t-t_0)},$$

where R is BLI response, t is time, R₀ and t₀ are the starting response and time of dissociation phase.

Association model:
$$R = \frac{k_{on} C R_{max}}{k_{on} C + k_{off}} (1 - e^{-(k_{on} C + k_{off})t}),$$

10 where C is the concentration of analyte, R_{max} is the maximum response at the equilibrium with the maximum concentration of analyte.

2. Results

2.1 Generation of Mutant Activins

15 In order to reduce the affinity of activin A to FST-288 while remaining its signaling activity, the receptor binding sites, both the convex and concave surfaces of activin A were left untouched. Residues Asp27 and Gln98 were mutated both individually and as a pair to reduce their interaction with FST-288. Mutation of Asp27 to alanine or asparagine breaks the charge-charge interaction with the side chain of Arg192. Similarly, mutation of Gln98 to alanine abolishes hydrogen bonding with Arg192 and mutation of Gln98 to arginine provides
20 an additional positive charge that repulses Arg192. However, mutations of either Asp27 or Gln98 individually were found to result in little or no measurable difference in binding to follistatin. Surprisingly however, simultaneous mutation of both residues was found to be highly effective in reducing binding to follistatin and the double mutant Asp27Asn/Gln98Arg (SEQ ID NO: 11) was chosen for more detailed analysis.

25 2.2 Crystal structure and signaling activity of frActA001

FrActA001 (SEQ ID NO: 11) was successfully produced by refolding of over-expressed inclusion bodies in *E. coli* and purified to homogeneity. Rod-shaped crystals were obtained by crystallizing this protein in the similar condition as for the wild-type activin A (100 mM
30 HEPES pH 7.8, 3% v/v PEG 300, 1.44 M ammonium sulphate)². These crystals were collected and cryo-protected by 3.0 M ammonium sulphate, diffracting up to 2.6 Å. Datasets were collected at the ESRF synchrotron ID23. The final structure of frActA001 at the resolution of 2.6 Å was solved by molecular replacement using the wild-type activin A as the starting model.

The crystal structure of frActA001 shows a similar conformation as in that of wild-type activin A (Fig. 1a), suggesting that the mutations of Asp27 and Glu98 do not affect the overall structure of the protein.

- 5 Further activity analysis by cell-based luciferase assay shows that the frActA001 has marginally higher signaling activity as the wild-type one with the EC₅₀ values of 16 ± 3 pM and 25 ± 4 pM, for mutant and wild-type proteins, respectively (Fig. 1b, Table 1).

2.3 Follistatin inhibition and its interaction with frActA001

- 10 FST-288 inhibition of frActA001 in cell culture condition was analyzed using activin-responsive luciferase assay. HEK293T cells were treated with a mixture of 100 pM activin A (wild-type or mutant) and different concentrations of FST-288 and activin-induced luciferase activity was measured. The inhibition curves for wild-type and the mutant activin A revealed IC₅₀ values of 252 ± 8 pM and 643 ± 40 pM, respectively (Fig. 2a, Table 1). frActA001 shows
15 2.5x reduced inhibition by FST-288.

- To further characterize the interaction between frActA001 and FST-288, the kinetic analysis was performed by biolayer interferometry (BLI) experiment³. FST-288 was immobilized on the AMC biosensor tips through anti-FST antibodies and activin A or its mutant was diluted
20 in the solution at different concentrations. By analysing the association and dissociation phases of these two components, the affinity of FST-288 binding to activin A was determined with a K_D value of 146 ± 2 pM for the wild-type activin A and 454 ± 4 pM for frActA001 (Fig. 2b and 2c, Table 1). FST-288 shows a three-fold weaker affinity to the engineered activin A compared to the wild-type one, which is in agreement with the result of cell-based inhibition
25 assay.

2.4 Further mutagenesis to improve the follistatin-resistance of activin A

- Further mutagenesis was found to improve the follistatin-resistance of the engineered activin A. Additional mutations include truncation of N-terminus of activin A, and mutating residues
30 Arg87, Lys85, Asp95 and Lys103.

- The truncation of activin A N-terminus was designed to improve the yield and the stability of the protein. Activin A has a pair of cysteine residues that form a disulphide-linked loop at the N-terminus of each subunit. However, this N-terminal loop is not presented in the majority
35 members of TGF- β superfamily and is not located in the receptor-binding sites. Truncation of the N-terminus of activin A should not interfere with the receptor interaction, but help trim the protein to have a more compact structure. The removal of the N-terminal disulphide bond

that does not contribute to the core protein construction also helps improve the refolding efficiency.

The other additional mutation sites are involved in the polar interactions with follistatin, but not in the receptor binding sites. Therefore, these additional mutations should improve the follistatin-resistance, but not interfere the signaling activity of activin A.

All of these engineered activin A were successfully refolded and purified. The signaling activities of these activin A variants were analyzed using the cell-based luciferase assay.

Their EC_{50} values have less than 50% variations from that of the wild-type activin A (25 pM), confirming that these activin A variants have similar signaling activities as the wild-type activin A (Fig 3a and 3b, Table 1).

The ability of FST-288 inhibiting the signalling of these variants was also analysed using the cell-based luciferase assay. The result shows that the further mutagenesis leads to different level of increase in IC_{50} values of FST-288 inhibition with the highest IC_{50} value of 53.2 nM for frActA004 (Fig 3c and 3d, Table 1), confirming that these additional mutations improve the follistatin-resistance of engineered activin A.

The interactions between the engineered activin A and FST-288 were further analysed by BLI experiments (Fig 3e and 3f, Table 1). All of the engineered activin As with additional mutations show improvement in resisting FST-288 binding compared to the frActA001.

2.5 Effect of mutations on activin A bioactivity in prolonged stem cell cultures

iPSCs were grown in standard conditions for pluripotency maintenance with 10ng/ml of activin A or frActA001 without changing medium for up to seven days. Samples were taken each day for analysis of follistatin and activin A concentrations in the medium by ELISA and for measurements of activin A bioactivity by luciferase assay. The concentration of activin A was constant, while the follistatin concentration was found to increase continuously over the experiment (Fig 4). At day 1, the molar ratio of follistatin to activin A almost reached 2:1, which equals the association stoichiometry between follistatin and activin A. The signaling activity of the activin A-containing iPSC culture medium was also found to decrease to 60% at day 1 and almost reach 0% from day 2 (Fig 5). These results show clearly that the activin A signaling is negatively regulated by iPSC through the expression and secretion of endogenous follistatin.

The analysis of the medium of iPSC culture grown with frActA001 also shows an increasing

concentration of follistatin, which is even higher than the follistatin concentration in the activin A-containing culture medium (Fig 4). The signaling activity of this frActA001-containing iPSC culture medium, however, maintains at 92% at day 1 and remains above 60% until day 5 (Fig 5). FrActA001 would therefore provide a significantly more consistent

5 signaling activity over the course of a 7 day IPSC culture than wild type activin A when the medium is changed every day (Fig 6) or every other day (Fig 7). These results provide

indication that the frActA001, benefiting from its resistance to follistatin, has a prolonged signaling activity in iPSC culture. Moreover, the marginal decrease of signalling activity of frActA001 medium compared to that of wild type activin A after one-day culturing indicates

10 this follistatin-resistant activin A may have more consistent effects on stem cell culture even with daily medium changes.

Variant	Mutation sites	EC ₅₀ (pM)	IC ₅₀ (pM)	K _D (pM)
ActA	Wild type (SEQ ID NO: 6)	25 ± 4	252 ± 7	146 ± 2
frActA001	Asp27Asn/Gln98Arg (SEQ ID NO: 11)	16 ± 3	643 ± 39	454 ± 4
frActA003	Asp27Asn/Arg87Asn/Gln98Arg (SEQ ID NO: 10)	24 ± 4	1644 ± 142	628 ± 5
frActA004	Asp27Asn/Asp95Ala/Gln98Arg (SEQ ID NO: 9)	31 ± 4	53211 ± 5609	1244 ± 9
frActA005	Asp27Asn/Lys85Ser/Gln98Arg (SEQ ID NO: 14)	27 ± 3	1782 ± 180	567 ± 5
frActA007	Asp27Asn/Gln98Arg/Lys103Asn (SEQ ID NO: 15)	18 ± 3	1656 ± 231	752 ± 6
frActA008	Asp27Asn/Lys85Ser/Arg87Asn/Asp95Ala/Gln98Arg (SEQ ID NO: 16)	36 ± 5	664 ± 33	2015 ± 15

Table 1

			85 87 95 98
	SEQ ID NO: 4 ActA_D27A	TSGSSLSFHSTVINHYMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNI	
	SEQ ID NO: 5 ActA_D27N	TSGSSLSFHSTVINHYMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNI	
5	SEQ ID NO: 6 ActA	TSGSSLSFHSTVINHYMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNI	
	SEQ ID NO: 7 ActA_Q98A	TSGSSLSFHSTVINHYMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNI	
	SEQ ID NO: 8 ActA_Q98R	TSGSSLSFHSTVINHYMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNI	
	SEQ ID NO: 9 ActA_D27N_D95A_Q98R	TSGSSLSFHSTVINHYMRGHSPFANLKSCCVPTKLRPMSMLYYADGRNI	
10	SEQ ID NO:10 ActA_D27N_R87N_Q98R	TSGSSLSFHSTVINHYMRGHSPFANLKSCCVPTKLRPMSMLYYDDGRNI	
	SEQ ID NO:11 ActA_D27N_Q98R	TSGSSLSFHSTVINHYMRGHSPFANLKSCCVPTKLRPMSMLYYDDGRNI	
	SEQ ID NO:12 Nd-ActA	TSGSSLSFHSTVINHYMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNI	
	SEQ ID NO:13 Nd-ActA_D27N_Q98R	TSGSSLSFHSTVINHYMRGHSPFANLKSCCVPTKLRPMSMLYYDDGRNI	
	SEQ ID NO:14 ActA_D27N_K85S_Q98R	TSGSSLSFHSTVINHYMRGHSPFANLKSCCVPTSLRPMSMLYYDDGRNI	
	SEQ ID NO:15 ActA_D27N_Q98R_K103N	TSGSSLSFHSTVINHYMRGHSPFANLKSCCVPTKLRPMSMLYYDDGRNI	
15	SEQ ID NO:16 ActA_D27N_K85S_R87N_D95A_Q98R	TSGSSLSFHSTVINHYMRGHSPFANLKSCCVPTSLNPMSMLYYADGRNI	
	SEQ ID NO: 3 ActivinB	VPGSASSFHTAVVNQYRMRGLNP-GTVNSCCIPTKLSTMSMLYFDDEYNI	
		103	
	SEQ ID NO: 4 ActA_D27A	IKKDIQNMIVEECGCS	
20	SEQ ID NO: 5 ActA_D27N	IKKDIQNMIVEECGCS	
	SEQ ID NO: 6 ActA	IKKDIQNMIVEECGCS	
	SEQ ID NO: 7 ActA_Q98A	IKKDIQNMIVEECGCS	
	SEQ ID NO: 8 ActA_Q98R	IKKDIQNMIVEECGCS	
	SEQ ID NO: 9 ActA_D27N_D95A_Q98R	IKKDIQNMIVEECGCS (frActA004)	
25	SEQ ID NO:10 ActA_D27N_R87N_Q98R	IKKDIQNMIVEECGCS (frActA003)	
	SEQ ID NO:11 ActA_D27N_Q98R	IKKDIQNMIVEECGCS (frActA001)	
	SEQ ID NO:12 Nd-ActA	IKKDIQNMIVEECGCS	
	SEQ ID NO:13 Nd-ActA_D27N_Q98R	IKKDIQNMIVEECGCS	
	SEQ ID NO:14 ActA_D27N_K85S_Q98R	IKKDIQNMIVEECGCS (frActA005)	
30	SEQ ID NO:15 ActA_D27N_Q98R_K103N	IKKDIQNMIVEECGCS (frActA007)	
	SEQ ID NO:16 ActA_D27N_K85S_R87N_D95A_Q98R	IKKDIQNMIVEECGCS (frActA008)	
	SEQ ID NO: 3 ActivinB	VKRDPVPMNIVEECGCA	
35			

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- 5 3. Concepcion, J. et al. *Comb. Chem. High Throughput Screen.* **12**, 791–800 (2009).
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Claims

1. A modified activin comprising mutations at Asp27 and Gln98.
5
2. A modified activin according to claim 1 wherein said mutations reduce the binding affinity of the modified activin for follistatin.
3. A modified activin according to any one of the preceding claims wherein said
10 mutations reduce the inhibition of modified activin by follistatin.
4. A modified activin according to any one of the preceding claims wherein the Asp27 is substituted for a different amino acid residue.
- 15 5. A modified activin according to claim 4 wherein the Asp27 is substituted for a non-charged amino acid residue
6. A modified activin according to claim 5 wherein the Asp27 is substituted for Asn.
- 20 7. A modified activin according to any one of the preceding claims wherein Gln98 is substituted for a different amino acid residue.
8. A modified activin according to claim 7 wherein the Gln98 is substituted for a charged amino acid residue
25
9. A modified activin according to claim 8 wherein the Gln98 is substituted for Arg.
10. A modified activin according to any one of the preceding claims wherein the modified activin further comprises a mutation at Asp95.
30
11. A modified activin according to claim 10 wherein Asp95 is substituted for a different amino acid residue.
12. A modified activin according to claim 11 wherein the Asp95 is substituted for a
35 hydrophobic amino acid residue
13. A modified activin according to claim 8 wherein the Asp95 is substituted for Ala.

14. A modified activin according to any one of the preceding claims wherein the modified activin further comprises a mutation at Arg87.

5 15. A modified activin according to claim 14 wherein Arg87 is substituted for a different amino acid residue.

16. A modified activin according to claim 15 wherein the Arg87 is substituted for a hydrophobic amino acid residue

10

17. A modified activin according to claim 16 wherein the Asp95 is substituted for Ala.

18. A modified activin according to any one of the preceding claims wherein the modified activin further comprises a mutation at Lys85.

15

19. A modified activin according to claim 18 wherein Lys85 is substituted for a different amino acid residue.

20. A modified activin according to claim 19 wherein the Lys85 is substituted for a non-positively charged amino acid residue.

20

21. A modified activin according to claim 20 wherein the Lys85 is substituted for Ser.

22. A modified activin according to any one of the preceding claims wherein the modified activin further comprises a mutation at Lys103.

25

23. A modified activin according to claim 22 wherein Lys103 is substituted for a different amino acid residue.

24. A modified activin according to claim 23 wherein the Lys103 is substituted for a non-positively charged amino acid residue

30

25. A modified activin according to claim 24 wherein the Lys103 is substituted for Asn.

26. A modified activin according to any one of the preceding claims wherein the modified activin comprises the amino acid sequence of human activin with 30 or fewer of said residues mutated.

35

27. A modified activin according to any one of the preceding claims wherein the modified activin comprises an amino acid sequence which has at least 80% sequence identity with the amino acid sequence of human activin.

5

28. A modified activin according to any one of the preceding claims wherein the amino acid sequence of human activin is SEQ ID NO: 6.

10

29. A modified activin according to any one of claims 1 to 27 wherein the amino acid sequence of human activin is SEQ ID NO: 3.

15

30. A modified activin according to any one of the preceding claims wherein the modified activin comprises the amino acid sequence of human activin with residues Asp27 and Gln98 substituted for different residues

31. A modified activin according to claim 30 comprising the amino acid sequence of SEQ ID NO: 11 or SEQ ID NO: 13.

20

32. A modified activin according to any one of claims 1 to 29 wherein the modified activin comprises the amino acid sequence of human activin with residues Asp27, Gln98 and Asp95 substituted for different residues.

25

33. A modified activin according to claim 32 comprising the amino acid sequence of SEQ ID NO: 9.

34. A modified activin according to any one of claims 1 to 29 wherein the modified activin comprises the amino acid sequence of human activin with residues Asp27, Gln98 and Arg87 substituted for different residues.

30

35. A modified activin according to claim 34 comprising the amino acid sequence of SEQ ID NO: 10.

35

36. A modified activin according to any one of claims 1 to 29 wherein the modified activin comprises the amino acid sequence of human activin with residues Asp27, Gln98 and Lys85 substituted for different residues.

37. A modified activin according to claim 36 comprising the amino acid sequence of SEQ ID NO: 14.

38. A modified activin according to any one of claims 1 to 29 wherein the modified activin comprises the amino acid sequence of human activin with residues Asp27, Gln98 and Lys103 substituted for different residues.

39. A modified activin according to claim 38 comprising the amino acid sequence of SEQ ID NO: 15.

40. A modified activin according to any one of claims 1 to 29 wherein the modified activin comprises the amino acid sequence of human activin with residues Asp27, Gln98, Lys85, Arg87 and Asp95 are substituted for different residues.

41. A modified activin according to claim 40 comprising the amino acid sequence of SEQ ID NO: 16.

42. An isolated nucleic acid encoding a modified activin according to any one of claims 1 to 41.

43. A vector comprising a nucleic acid according to claim 42.

44. A recombinant cell comprising a vector according to claim 43.

45. A method of producing a modified activin comprising expressing a nucleic acid according to claim 42 in a host cell, and isolating the modified activin expressed by the nucleic acid.

46. A cell culture medium comprising a modified activin according to any one of claims 1 to 41.

47. Use of a medium according to claim 46 for the culture of mammalian cells.

48. A method of culturing mammalian cells comprising; culturing said mammalian cells in a medium according to claim 46.

a



b

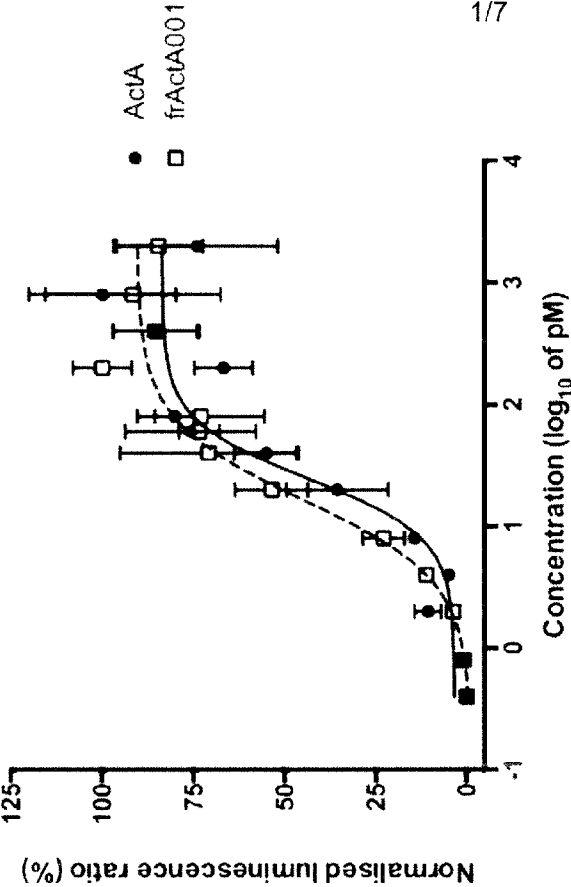


Figure 1

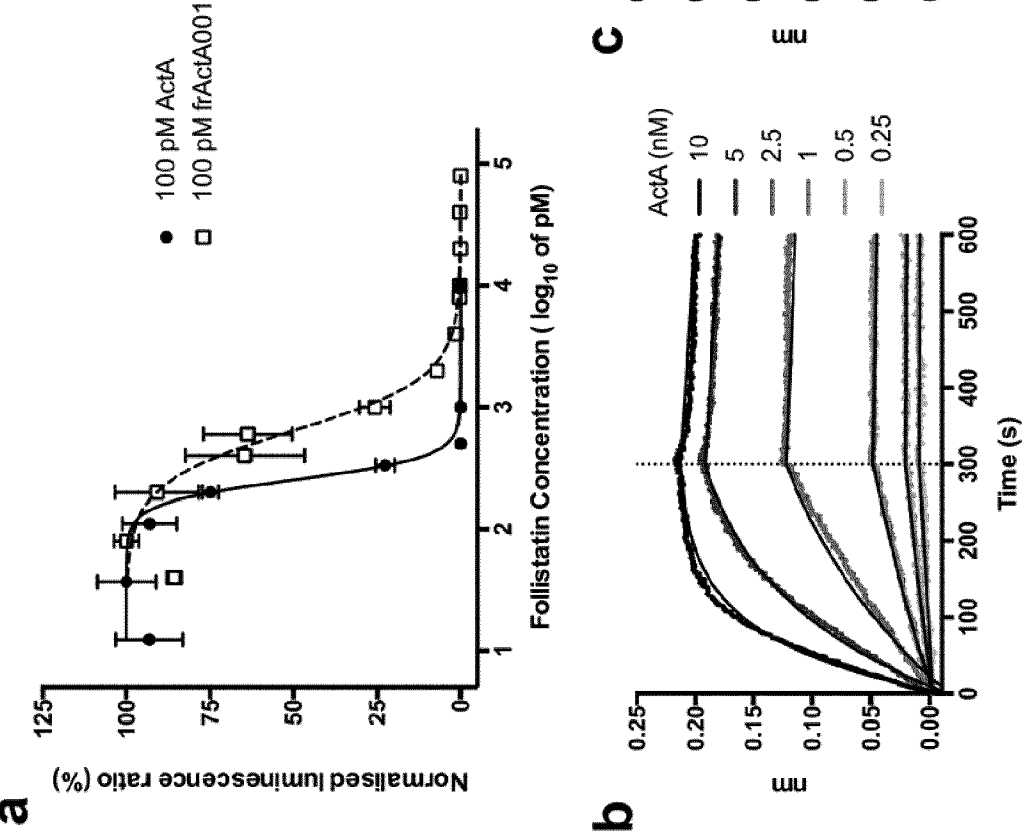


Figure 2

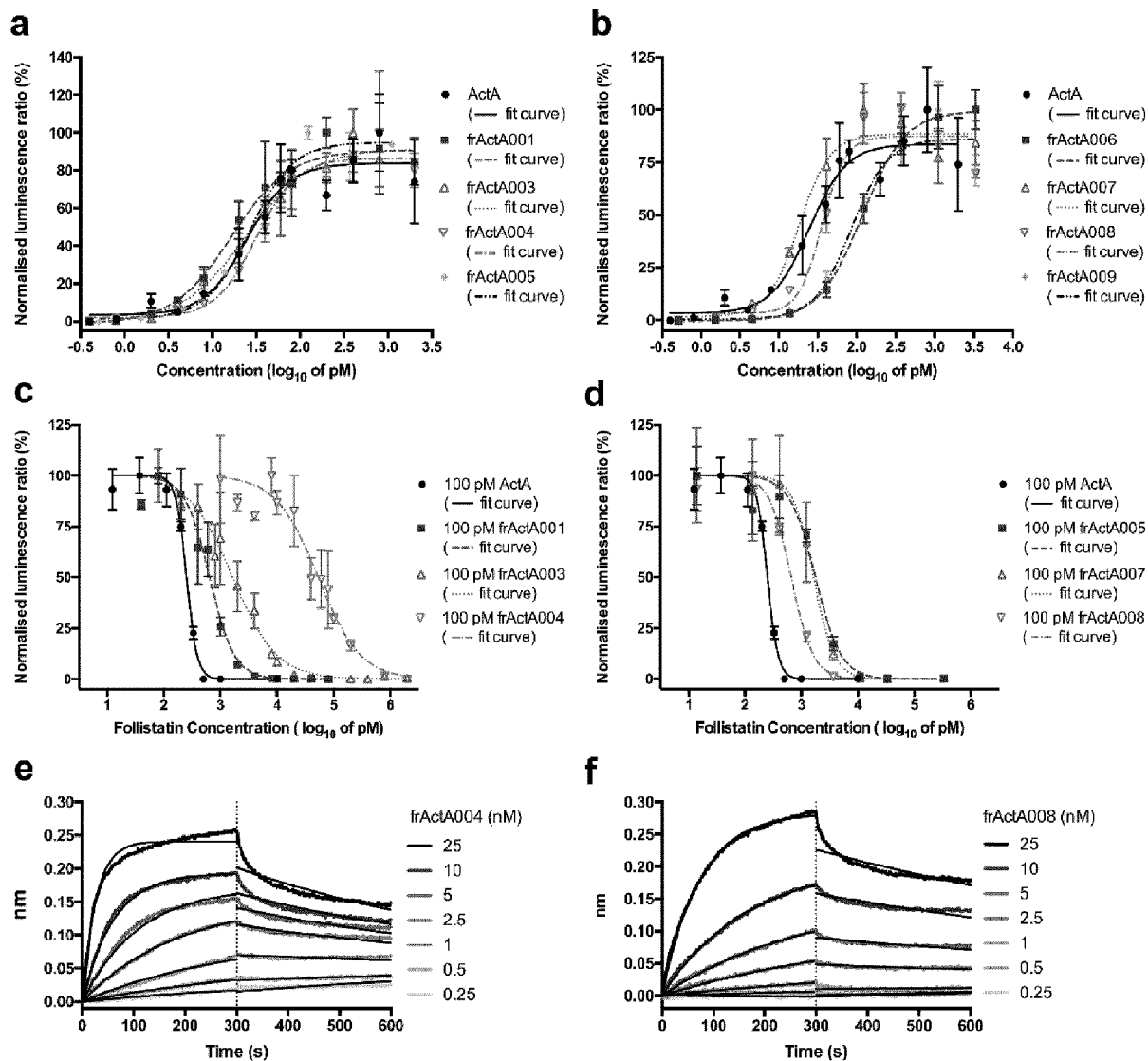


Figure 3

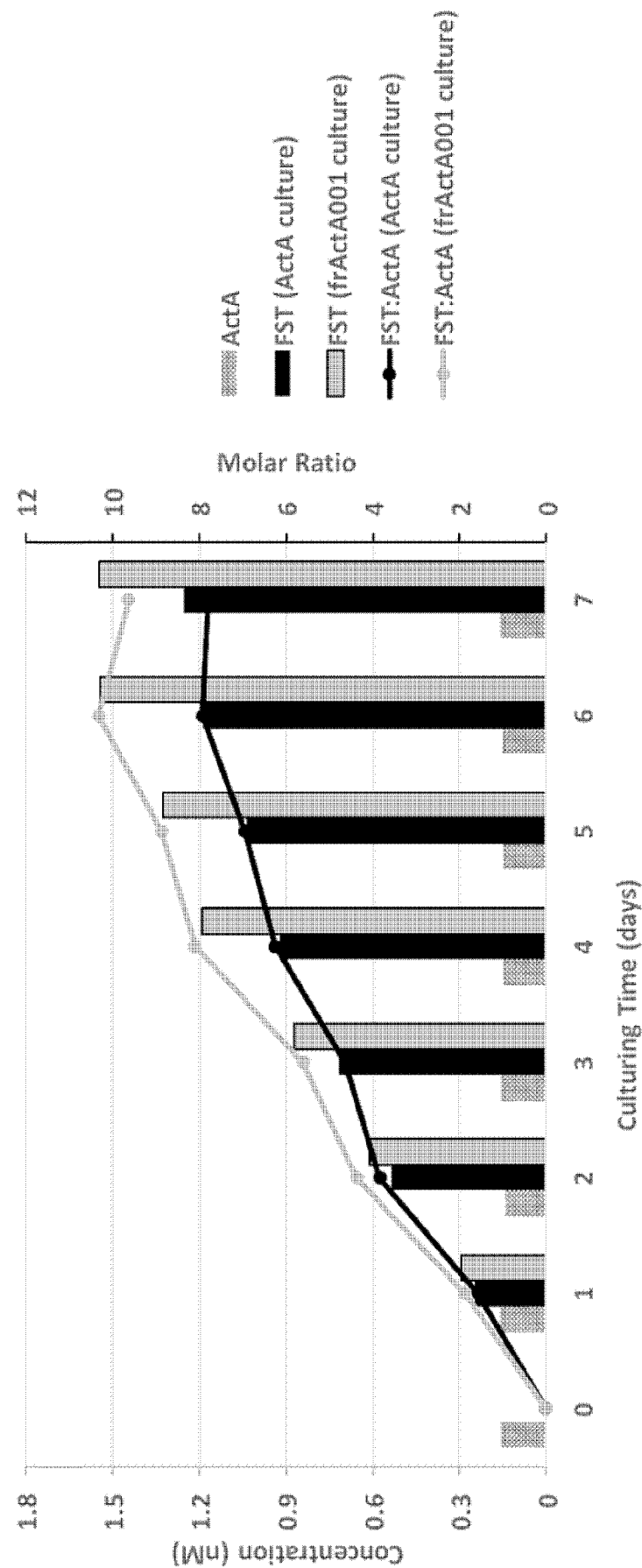


Figure 4

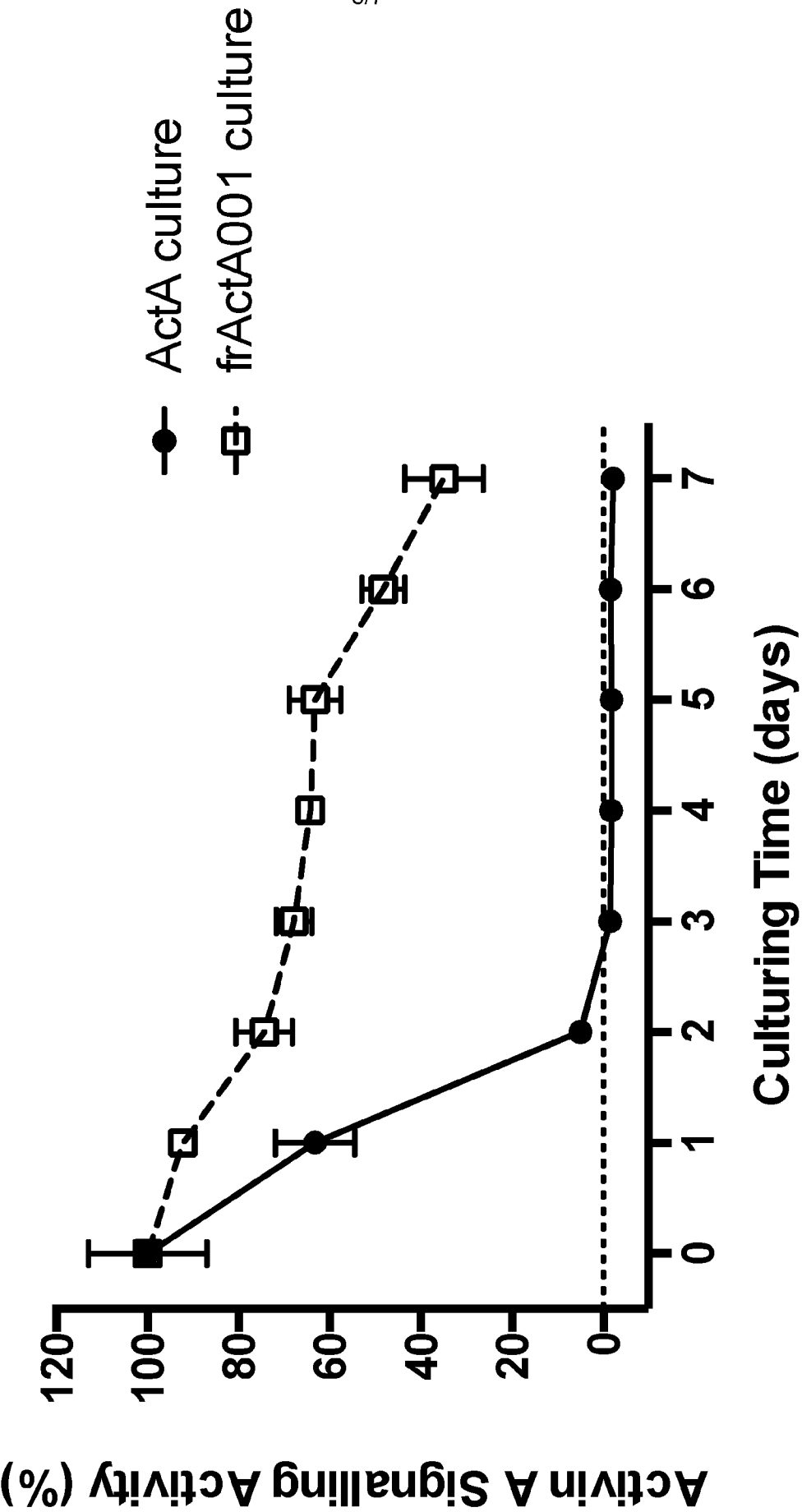


Figure 5

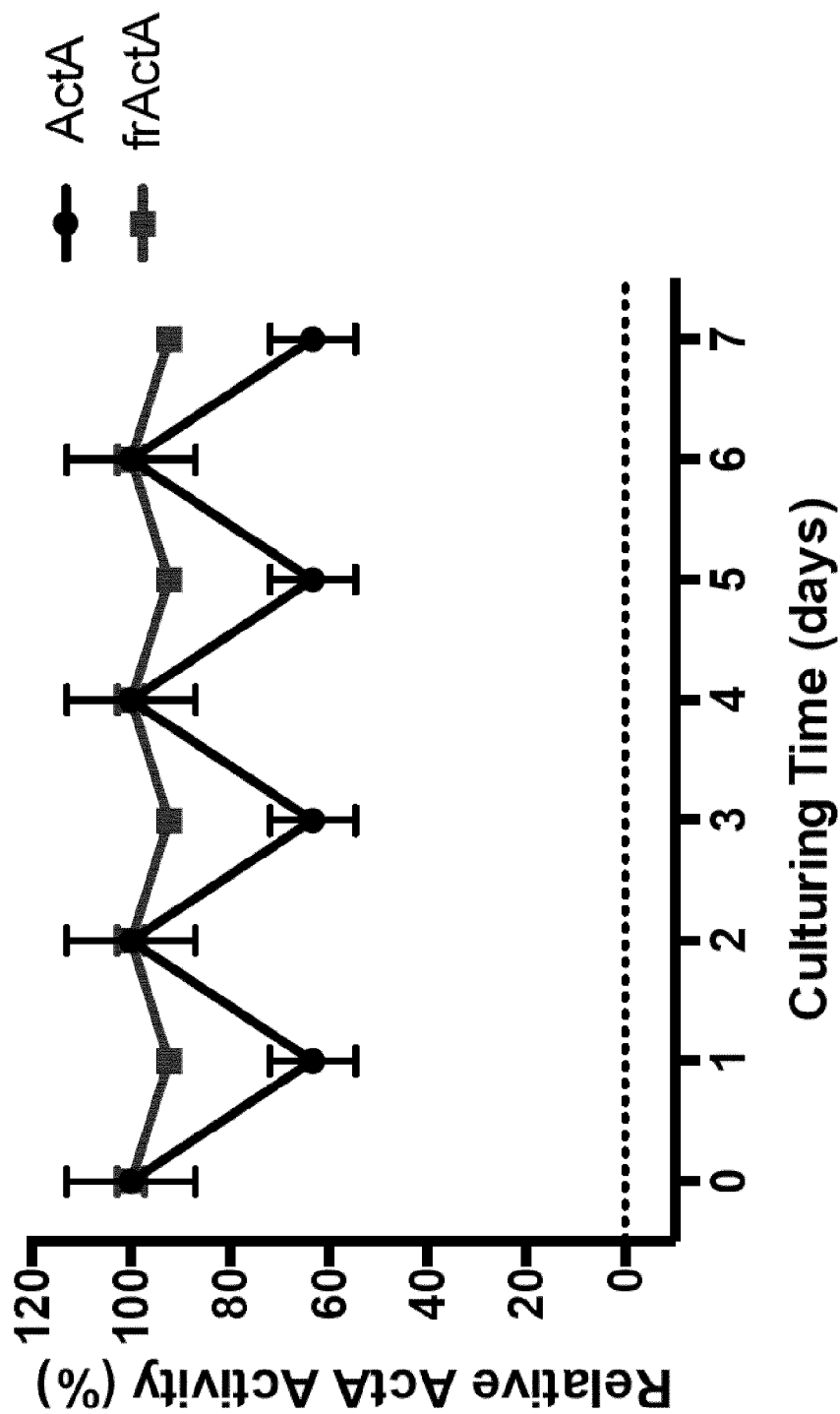


Figure 6

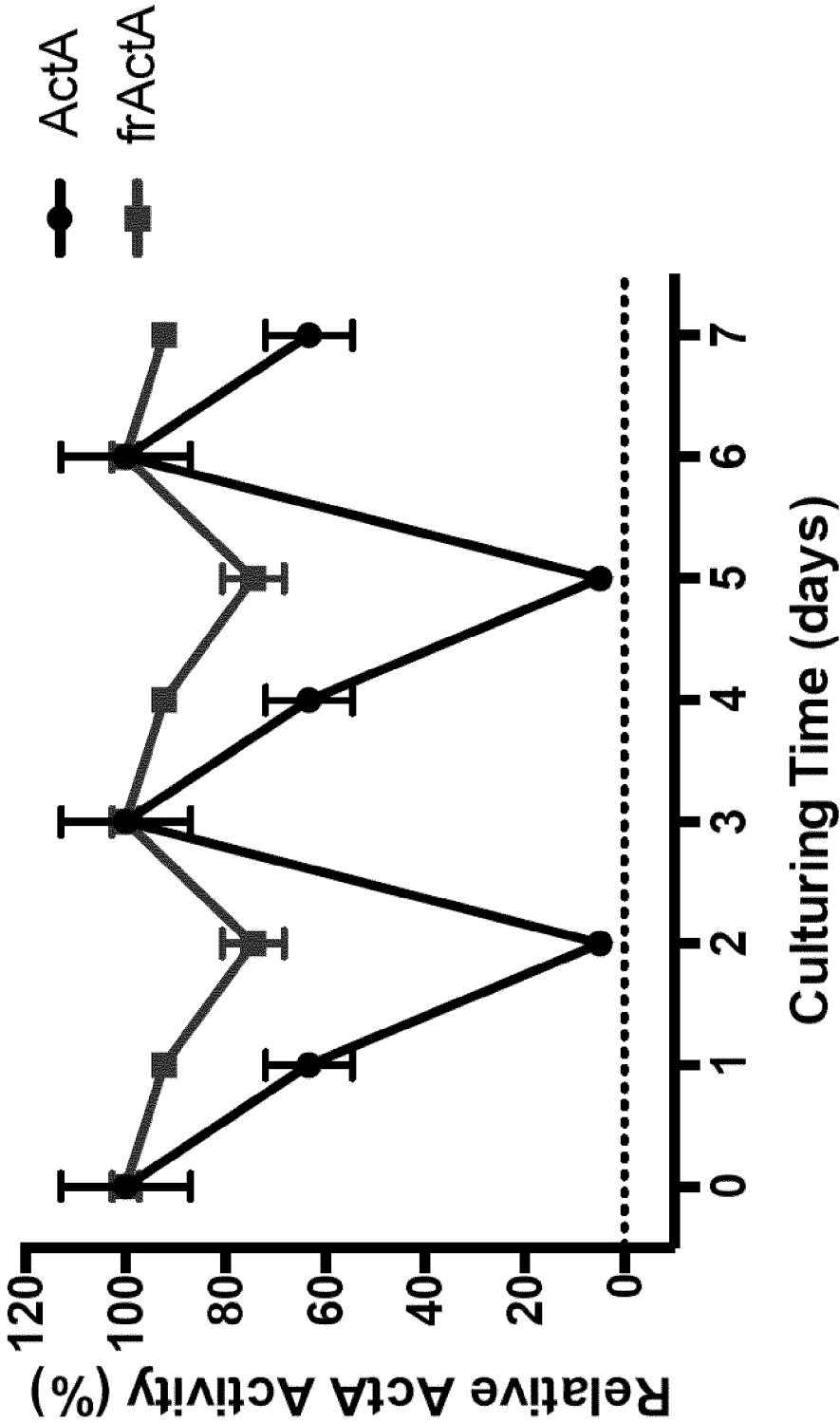


Figure 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/065857

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/71
ADD.

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)
C07K

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)

EPO-Internal, BIOSIS, Sequence Search, EMBASE, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE Protein [Online] 29 November 2016 (2016-11-29), "PREDICTED: inhibin beta B chain [Larimichthys crocea].", XP002783211, retrieved from NCBI accession no. Reference Sequence: XP_010755273.1 Database accession no. XP_010755273 the whole document ----- -/--	1-7,14, 15,22,23 8-13, 16-21, 24-48
A		



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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"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

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Date of the actual completion of the international search

20 July 2018

Date of mailing of the international search report

13/08/2018

Name and mailing address of the ISA/

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Authorized officer

Ury, Alain

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/065857

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HARRISON CRAIG A ET AL: "Activin-A binds follistatin and type II receptors through overlapping binding sites: Generation of mutants with isolated binding activities", ENDOCRINOLOGY, vol. 147, no. 6, Suppl. S, 9 March 2006 (2006-03-09), pages 2744-2753, XP002783212, ISSN: 0013-7227 the whole document	1-48
A	----- WALTON KELLY L ET AL: "New insights into the mechanisms of activin action and inhibition", MOLECULAR AND CELLULAR ENDOCRINOLOGY, vol. 359, no. 1, 8 July 2011 (2011-07-08), pages 2-12, XP028926475, ISSN: 0303-7207, DOI: 10.1016/J.MCE.2011.06.030 the whole document	1-48
A	----- WO 2005/116052 A2 (RES DEV FOUNDATION [US]; HARRISON CRAIG [AU]; GRAY PETER [US]; FISCHER) 8 December 2005 (2005-12-08) the whole document -----	1-48

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2018/065857

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2005116052 A2	08-12-2005	AU 2005247871 A1	08-12-2005
		CA 2563374 A1	08-12-2005
		EP 1740609 A2	10-01-2007
		US 2006008846 A1	12-01-2006
		WO 2005116052 A2	08-12-2005
