(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 December 2020 (30.12.2020)

(10) International Publication Number
WO 2020/257942 A1

(51) International Patent Classification:
*C12N 15/67* (2006.01) *C12N 5/10* (2006.01)
*C07K 14/51* (2006.01) *C12N 9/64* (2006.01)
*C12N 15/12* (2006.01) *C12P 21/02* (2006.01)
*C12N 15/57* (2006.01) *C12Q 1/68* (2018.01)
*C12N 15/85* (2006.01)

(21) International Application Number: PCT/CA2020/050893

(22) International Filing Date: 26 June 2020 (26.06.2020)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/868,602 28 June 2019 (28.06.2019) US

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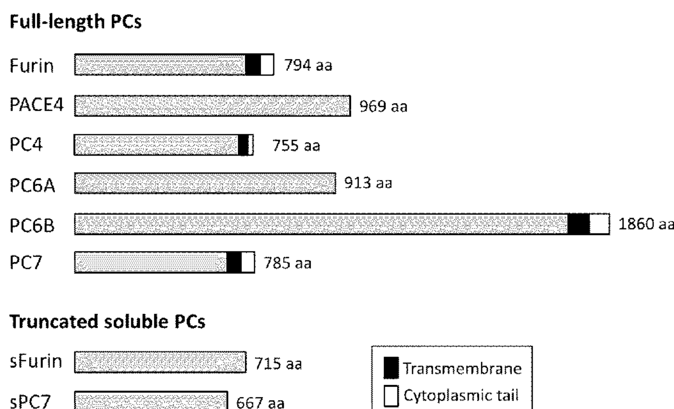
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

**Published:**
— *with international search report (Art. 21(3))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

(54) **Title:** A METHOD FOR INCREASING YIELDS AND THE SPECIFIC ACTIVITY OF CERTAIN RECOMBINANT PROTEINS IN MAMMALIAN CELLS BY CO-EXPRESSING FULL-LENGTH FURIN

**Figure 1**




(57) **Abstract:** The present invention teaches a method for increasing the yeild of a recombinant protein of interest comprising, transfecting a mammalian cell with both the protein of interest and with full length furin.

WO 2020/257942 A1

# A METHOD FOR INCREASING YIELDS AND THE SPECIFIC ACTIVITY OF CERTAIN RECOMBINANT PROTEINS IN MAMMALIAN CELLS BY CO-EXPRESSING FULL-LENGTH FURIN

## FIELD OF THE INVENTION

**[0001]** The present invention relates to a system and method for increasing yields and the specific activity of certain recombinant proteins in mammalian cells by co-expressing full-length Furin in those cells.

## BACKGROUND OF THE INVENTION

**[0002]** With the advent of recombinant DNA technology, more than 200 recombinant proteins for therapeutic use, including hBMP-2 and hBMP-7, have been successfully produced recombinantly (Demain and Vaishnav 2009; Leader et al. 2008). Some well recognized therapeutic recombinant proteins include insulin (for diabetes), Factors VII, VIII, and IX (for blood clotting disorders), luteinizing hormone (for infertility), erythropoietin (for anemia and renal disease), lysozyme (for metabolic disorder), platelet derived growth factor (for diabetic ulcers), and a large number of monoclonal antibodies (to treat a variety of different conditions including arthritis, osteoporosis, and cancer).

**[0003]** An array of expression systems has been developed to produce recombinant proteins. The systems include single cell expression systems which use bacteria, yeasts, baculovirus infected insect cells, mammalian cells, and multi-cellular expression systems that use transgenic plants or animals to produce transgenic proteins. There are many important factors one needs to consider when selecting an appropriate expression system to produce a biologically active recombinant protein for therapeutic use, they mainly include: (1) protein quality, functionality, and quantity, (2) the cost of manufacturing and purification, and (3) the time required from gene to purified protein (Brondyk 2009).

**[0004]** Mammalian cell expression has become the dominant recombinant protein production system to produce recombinant proteins for clinical applications. More than half of therapeutic proteins approved and on the market are produced using mammalian cells (Zhu 2012). A major advantage of mammalian cells is that they are able to synthesize proteins which are very similar to native human proteins in terms of physical, biochemical and physiological properties, due to the proper protein folding, assembly, processing, and posttranslational modifications. As the result, the quality and efficacy of the proteins produced from mammalian systems are typically superior to that of the same proteins expressed in bacteria, yeasts or insects. In addition, when a well-characterized mammalian cell line is used, the scale-up is moderately straightforward because the cell line has well known growth characteristics and nutritional requirements (Wurm 2004).

1 **[0005]** The most common choice of mammalian host cells for therapeutic recombinant
2 protein production is the Chinese hamster ovarian (CHO) cell line (Jostock and Knopf 2012).
3 CHO cell lines are well characterized, allowing for efficient transfection, gene amplification,
4 and selection of highly productive clones (Jostock and Knopf 2012). Other popular
5 mammalian cell lines for recombinant protein production include human embryonic kidney
6 (HEK-293), mouse myeloma (NS0), and baby hamster kidney (BHK) (Jostock and Knopf
7 2012; Zhu 2012) and more recently the PER.C6 cell line which was derived from human
8 embryonic retinal cells.
9 **[0006]** Mammalian expression systems are relatively costly to maintain compared to other
10 expression systems and typically have lower recombinant protein yields. Further, while
11 typical yields of recombinant monocolonal antibodies in mammalian cells are in the g/L
12 range with specific productivities of 90 pg/cell/day (Wurm 2004), for other proteins reported
13 yeilds are lower. For example it has been reported that yields for rhBMP-2 and rhBMP-7
14 remain low (Roe et al. 2004; Swencki-Underwood et al. 2008) and it is believed that the
15 commercial manufacturing proceses used to produce rhBMP-2 and rhBMP-7 are in the 10
16 to 30 mg/L range with an estimated specific productivity between 2 to 4 pg/cell/day, (Peel,
17 S., personal communication).
18
19 **Strategies to increase yeilds of difficult to express recombinant proteins**
20 **[0007]** Strong promoters, such as the CMV promoter, are routinely integrated in the
21 expression vectors to drive high recombinant protein gene expression. Site-specific
22 transgene integration strategy using the CHO-FlpIn system (Invitrogen) has also been used
23 to incorporate BMP gene into a highly transcribed site, and hence increase the rate of
24 transgene expression (Zhou et al. 2009).
25 **[0008]** Further, multiple rounds of gene amplification using methotrexate (MTX) on DHFR-
26 deficient CHO cells to maximize the copy numbers of the recombinant protein gene
27 incorporated into the cells is routinely used to increase expression.
28 **[0009]** In studies using a strong CMV promoter and five rounds of MTX amplification, it was
29 estimated that the number copy of BMP increased at least 70-fold (from 1 copy to 70+
30 copies per cell) and the yield of BMP-2 increased from µg/mL to mg/mL (Peel et al,
31 unpublished data). However, additional rounds of MTX amplification (past round 5) did not
32 further increase rhBMP-2 yield (Peel et al, unpublished). Despite a significant increase
33 compared to unamplified cells, the rhBMP-2 yield is still far below the g/L level achieved for
34 recombinant monoclonal antibodies. It would appear that increasing gene expression or

mRNA levels alone are not sufficient to increase the overall protein yield to the g/L range for some proteins including rhBMPs.

**[0010]** Accordingly, it is desired to have methods other than increasing gene and mRNA expression that will increase the yields of certain recombinant proteins in mammalian cells.

**[0011]** In recent years, pro-forms of proteins have received intensive scientific attention because of the roles pro-domains have in the maturation and processing of the precursor proteins (Harrison et al. 2011). The pro-domain of a protein is well known to confer latency to the mature protein.

**[0012]** They can also affect the secretion of recombinant pro-proteins. In-frame deletion of the pro-peptide of BMP-2 yielded a polypeptide that was not secreted from the cell (Israel et al. 1992). Dick et al. constructed a mutated BMP-7 in which the amino acid residues Val and Gly were exchanged (Val to Gly) in the pro-domain; it was found that this dramatically reduced the amount of pro-domain and the mature protein secreted into the conditioned medium (Dick et al. 2000). ProBMP-2 contains two proprotein convertase (PC) recognition sites (S1 and S2). We have demonstrated that cell lines expressing proBMP-2, with a silenced S1 site (mS1) that inhibited PC cleavage, secreted the 20-kDa form BMP-2, while cells expressing wild type (wt) BMP-2 secreted 18- and 20-kDa mature BMP-2 N-terminal isoforms. The mS1 cells secreted 15-fold more mature BMP-2 than the wild type, despite their similar mRNA levels (Zhou et al. 2012).

**[0013]** Some studies have demonstrated that expressing a full-length furin in several mammalian cell lines improved the processing of von Willebrand factor (vWF) and transforming growth factor-ß1 (TGF-ß1) (Ayoubi et al. 1996). It was also shown that overexpression of a soluble paired basic amino acid cleaving enzyme (PACE or furin) improved rhBMP-2 processing and yield of rhBMP-2 (Roe et al. 2004). However, another study with BMP-7 suggested that co-expression of full-length furin with BMP-7 did not have a significant impact on processing success (Swencki-Underwood et al. 2008). Therefore it remains unclear whether the overexpression of full-length and/or soluble furin or other similar pro-protein convertases would help improve the yield of recombinant BMPs and related proteins.

**SUMMARY OF THE INVENTION**

**[0014]** According to one aspect of the invention a mammalian cell is transfected with both the protein of interest and with full-length furin to increase yeilds of the recombinant protein of interest.

**[0015]** According to another aspect of the invention selected clones of mammalian cells that have been transfected with the protein of interest and undergone mutliple rounds of gene amplification, then undergo a second round of transfection with full-length furin to further increase protein yeilds.

**[0016]** According to another aspect of the invention mammalian cells are first transfected with full-length furin and they are screened to identify clones with the appropriate level of furin activity to generate a parent cell-line that can be used to transfect with the recombinant protein of interest where increased furin activity would be beneficial.

**[0017]** According to another aspect of the invention selected clones of mammalian cells that have been transfected with the protein of interest and undergone mutliple rounds of gene amplification, then undergo a second round of transfection with full-length furin to further increase protein processing to reduce protein N-terminal heterogeneity.

**[0018]** According to another aspect of the invention selected clones of mammalian cells that have been transfected with the protein of interest and undergone mutliple rounds of gene amplification, then undergo a second round of transfection with full-length furin to further increase protein processing to increase the specific activity of the recombinant protein.

**[0019]** According to another aspect of the invention clones are selected for use following transfection with full length furin based on the level of furin expressed.

**[0020]** Other features and advantages of the present invention are described more fully below.

## Brief Description of the Drawings

**[0021]** Preferred embodiments of the present invention will now be described, by way of example only, with reference to the attached Figures, wherein:

Figure 1: Schematic diagram of full-length and truncated PCs.
The primary structures of six full-length human proprotein convertases (PCs) and two truncated soluble PCs: soluble Furin (sFurin) and soluble PC7 (sPC7), both generated by deleting their transmembrane domains and cytoplasmic tails.

Figure 2: FACS of CHO cells post PC transfection.
(A) CHO cells transfected with tGFP tagged PCs were gated on size and granularity. Doublets were discriminated using forward scatter and size scatter height and width parameters (FSC-H versus FSC-W). The efficiency of cells transfected with (B) Furin-tGFP, (C) sFurin-tGFP, and (D) no DNA plasmids (as control) were sorted on the basis of their

tGFP expressions. Cells were gated into tGFP negative (Neg), low positive (Low), mid positive (Mid), and high positive (High) groups. Cells from each group were sorted into a 96-well plate at 2000 cells per well (n = 2 to 4). The experiment was repeated at least three times for each PC (N ≥3).

Figure 3: Transient overexpression of PC-tGFP fusion protein on rhBMP-2 yield.
(A) Transfection efficiency of PCs determined by their tGFP expression by FACS analysis.
(B) BMP-2 secreted by CHO cells expressing membrane-bound PCs: Furin, PC4, PC6B, and soluble PCs: sFurin, sPC7, PC6A, and PACE4 sorted into tGFP negative (Neg), low- (Low), mid- (Mid), and high-positive (High) groups. If the transfection efficiency of a PC was low, the cells were sorted into Neg, Low, and High positive groups (i.e. PC6A) or simply Neg and Pos groups (i.e. PC4 and PACE4). All data are shown as mean ± SE. At least three sets of independent experiments were carried out for each of the PCs (N = 3). Results also showed that cells over expressing lower levels of Furin produced more rhBMP-2 than cells overexpressing high levels of Furin

Figure 4: Overexpression of PCs with or without tGFP tag on mature rhBMP-2 yield.
(A) Transfection efficiency of PCs determined by their tGFP expression by FACS analysis.
(B) BMP-2 secreted by CHO cells expressing various levels of the PCs. Furin, sFurin, and PC7 were tagged with (B, top row) or without (B, bottom row) tGFP. All data are shown as mean ± SE (*P<0.05, **P <0.01, ***P<0.001). Three sets of independent experiments were carried out for each of the PCs (N = 3). Results showed that only the cells overexpressing full length furin resulted in increased yeilds. Results also showed that cells over expressing lower levels of Furin produced more rhBMP-2 than cells overexpressing high levels of Furin

Figure 5 Bioactivity of rhBMP-2 produced by Furin transfected cells.
Biological activity of equal amounts of rhBMP-2 secreted by cells overexpressing Furin (BMP-2(Furin), open squares) or negatively transfected cells (BMP-2(NC), filled circles), tested by induction of ALP activity in myogenic C2C12 cells. Data are shown as mean ± SE. Two independent experiments were carried out. Resuls showed that the more fully processed rhBMP-2 prodcued by the cells overexpressing Furin had a higher specific activity.

1 Figure 6 Furin and sFurin expression in transfected cells.
2 Furin overexpressed in (A) conditioned media and in (B) cell lysates was detected by
3 Western blot analysis probed with anti-furin antibody and quantified by furin ELISA. The
4 amount of Furin and sFurin was normalized to the protein content of the cells. NC (negative
5 control): cells not overexpressing Furin or sFurin; Furin: full-length furin (794 aa); sFurin:
6 soluble Furin lacking the transmembrane and cytoplasmic domains (715 aa). Data are
7 shown as mean ± standard deviation (SD) (* $P<0.05$, ** $P<0.01$).
8
9 Figure 7 BMP processing in untransfected and Furin and sFurin overexpressing cells
10 BMP-2 proteins secreted in (A) conditioned media and in (B) cell lysates were characterized
11 by Western blot analysis and quantified by ELISA. BMP-2 concentration was normalized to
12 the protein content of the cells. NC (negative control): cells not overexpressing Furin or
13 sFurin; Furin: full-length furin (794 aa); sFurin: soluble Furin lacking the transmembrane and
14 cytoplasmic domains (715 aa). Data are shown as mean ± SD (* $P<0.05$).
15
16 Figure 8 The effect of extracellular furin treatment on BMP-2 yield.
17 (A) CHO cells were cultured with cell culture media with or without added furin for 48 h, after
18 which the BMP-2 in the conditioned media and the cell lysates were measured by BMP-2
19 ELISA. (B) Various concentrations of extracellular furin were added directly to cell-free,
20 BMP-2 containing conditioned media for 24 h. BMP-2 concentration was by BMP-2 ELISA.
21 Data are shown as mean ± SE (N = 2).
22
23 Figure 9 differences in Furin protein levels in different cell types
24 Furin concentration in cell lysates was measured by ELISA method and normalized to
25 protein concentration (ng Furin/μg protein). Bars represent the mean ± SEM (n=3).
26
27 Figure 10 differences in proprotein convertase activity in different cell types
28 PC activity assay results CHO and HEK cells. Activity is measured by fluorescence
29 proportional to the concentration of released AMC from substrate. AMC concentration was
30 normalized by total cellular protein as determined by Bradford Assay, and further normalized
31 by time. Thus activity is defined as [AMC] (uM)/ [protein] (ug/ml) /time (minutes). Bars
32 represent the mean ± SEM (n=3) (*** $P < 0.001$).
33
34
35

## DETAILED DESCRIPTION OF THE INVENTION

**[0022]** The system and method of the invention can be used for increasing yeilds of difficult to express recombinant proteins that undergo processing by pro-protein convertases

**[0023]** The system and method of the invention can be used for increasing the specific activity of recombinant proteins that undergo incomplete processing by proprotein convertases in mammalian expression systems with insufficient proprotein convertase activtiy.

**[0024]** The system and method of the invention can be used to generate a parent cell line that can be used for production of recombinant proteins that undergo processing by proprotein convertases.

## Definitions

**[0025]** Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

**[0026]** As used herein the term “recombinant” means a protein produced by a transiently transfected, stably transfected, or transgenic host cell or animal as directed by an expression construct containing the cDNA for that protein. The term “recombinant” also encompasses pharmaceutically acceptable salts of such a polypeptide

**[0027]** As used herein, the term “polypeptide” or “protein” refers to a polymer of amino acid monomers that are alpha amino acids joined together through amide bonds. Polypeptides are therefore at least two amino acid residues in length, and are usually longer. Generally, the term “peptide” refers to a polypeptide that is only a few amino acid residues in length. A polypeptide, in contrast with a peptide, may comprise any number of amino acid residues. Hence, the term polypeptide included peptides as well as longer sequences of amino acids.

**[0028]** As used herein, the terms “bone morphogenetic protein” or “bone morphogenic protein” or “BMP” are used interchangeably and refer to any member of the bone morphogenetic protein (BMP) subfamily of the transforming growth factor beta (TGFβ) superfamily of growth and differentiation factors, including BMP-2, BMP-3 (also known as osteogenin), BMP-3b (also known as growth and differentiation factor 10, GDF-10), BMP-4, BMP-5, BMP-6, BMP-7 (also known as osteogenic protein-1, OP-1), BMP-8 (also known as osteogenic protein-2, OP-2), BMP-9, BMP-10, BMP-11 (also known as growth and differentiation factor 8, GDF-8, or myostatin), BMP-12 (also known as growth and differentiation factor 7, GDF-7), BMP-13 (also known as growth and differentiation factor 6, GDF-6), BMP-14 (also known as growth and differentiation factor 5, GDF-5), and BMP-15.

[0029] The terms “bone morphogenetic protein” and “BMP” also encompass allelic variants of BMPs, function conservative variants of BMPs, and mutant BMPs that retain BMP activity. The BMP activity of such variants and mutants may be confirmed by any of the methods well known in the art (see the section Assays to measure BMP activity, below).

[0030] In preferred embodiments, the BMP is BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8 or BMP-9. In particularly preferred embodiments the BMP is BMP-2, BMP-4 or BMP-7.

[0031] In preferred embodiments the BMP is a mammalian BMP (e.g., mammalian BMP-2 or mammalian BMP-7). In particularly preferred embodiments, the BMP is a human BMP (hBMP) (e.g. hBMP-2 or hBMP-7).

[0032] As used herein, the term full-length Furin (also called Furin or PACE) refers to the product of the "FURIN” gene (NM_002569) a 794 amino acid membrane associated proprotein convertase subtilisin/kexin type 3; dibasic processing enzyme (EC_number="3.4.21.75") and varients thereof that are membrane associated, in contrast to varients that are missing part or all of the C-terminal domain (amino acid residues 683 to 794) and are therefore soluble or shed rather than membrane associated, which are termed sFurin (also called sPACE).

## EXAMPLES

[0033] The invention is next described by means of the following examples. However, the use of these and other examples anywhere in the specification is illustrative only, and in no way limits the scope and meaning of the invention or of any exemplified form. Likewise, the invention is not limited to any particular preferred embodiments described herein. Indeed, many modifications and variations of the invention may be apparent to those skilled in the art upon reading this specification, and can be made without departing from its spirit and scope. The invention is therefore to be limited only by the terms of the appended claims, along with the full scope of equivalents to which the claims are entitled.

## EXAMPLE 1

[0034] PC cDNA plasmids

[0035] The cDNA plasmids expressing full-length human proprotein convertases (PC) including Furin (NM_002569), PACE4 (NM_138322), PC4 (NM_017573), PC6A (NM_006200), PC6B (NM_001190482), and PC7 (NM_004716) tagged with C-terminal turbo green fluorescent protein (tGFP) tag were obtained (Origene, Rockville, MD, USA). Soluble forms of Furin (sFurin) and PC7 (sPC7) were generated by deleting the cDNA encoding the predicted transmembrane domain and cytoplasmic tail from the full-length proteins using the

1 primers listed in Table 1 (Origene). Seventy-eight amino acid (aa) residues (717 to 794) and
2 117 aa residues (669 to 785) were removed from the C-termini of the full-length Furin and
3 PC7 to construct the soluble furin (sFurin) and PC7 (sPC7), respectively (Figure 4-1). The
4 soluble PC plasmids were sequence-verified. Non tGFP-tagged Furin, sFurin, and PC7
5 plasmids containing an internal ribosome entry site (IRES) as part of the bicistronic transcript
6 were also obtained from Origene. All plasmids contain a CMV promoter and a bacterial
7 ampicillin resistance gene.
8
9 TABLE 1
10 **[0036]** Mutant Primer sequence: Sense/antisense
11 **[0037]** sFurin 5'-GACATCCTCACCGAGCCCAAAGACA-3'
12 **[0038]** 5'-TTAAGGGGGCAGAGGCTGAGGTGC-3'
13 **[0039]** sPC7 5'-GCGATCGCCATGCCGAAGGGGAGGCAGAAAGTG-3'
14 **[0040]** 5'-ACGCGTGGTCTTGAGGGTGTTGGGGGTGAT-3'
15 **[0041]** Table 1 Primers used to generate soluble furin (sFurin) and PC7 (sPC7).
16 **[0042]** The transmembrane domain and cytoplasmic tail of the full-length furin and PC7
17 were removed to generate soluble furin (sFurin) and PC7 (sPC7).
18
19 Cell culture
20 **[0043]** Chinese hamster ovarian (CHO-DG44) cells stably expressing hBMP2 gene were
21 cultured with chemically defined medium CD OptiCHO™ (Invitrogen, Burlington, ON,
22 Canada) plus 2 mM GlutMAX™ I (Invitrogen) and methotrexate (MTX), in 50 ml TubeSpin
23 bioreactors (TPP, Trasdingen, Switzerland) on an orbital shaker rotating at 250 rpm. The
24 cells were incubated at 37°C with 5% CO2. CHO cells were transfected and then sorted, as
25 described below. Post cell sorting, the transfected cells were cultured for 48 h and the
26 conditioned media was harvested and centrifuged (500g for 5 min) to remove any debris.
27 The cells were lysed (CelLytic M, Sigma-Aldrich, St. Louis, MO), homogenized, and the cell
28 lysates were collected.
29
30 Cell transfection
31 **[0044]** CHO cells were cultured in CD OptiCHO medium without MTX. The cDNA plasmids
32 was transfected into the cells via electroporation using Amaxa cell line nucleofector kit V
33 (Lonza, Basel, Switzerland), following manufacturer's instructions. Briefly, every $1x10^6$ CHO
34 cells were transfected with 2.5 µg of the plasmid and cultured in a 24-well plate (at $1x10^6$
35 cells per well) for 24 hr before cell sorting.

Fluorescence-activated cell sorting (FACS)

**[0045]** Cellular expression of proprotein convertase (PC) was determined by its tGFP expression. Twenty-four hour post-electroporation, transfection efficiency was determined and the cells were sorted by FACS using BD FACSAria II or III (Becton Dickinson, Mississauga, ON, Canada) equipped with 488-nm blue laser for detecting GFP. Cells were gated on size and granularity. Doublets were discriminated using forward scatter and size scatter height and width parameters (FSC-H versus FSC-W). Cells were gated into tGFP negative, low, mid, and high positive groups based on their tGFP expression level. Subsequently, the cells were sorted into a 96-well plate at 2000 cells per well (n = 2 to 4 depending on the transfection efficiency, 200 µl of fresh medium per well) or into 5 ml collection tubes and then plated into a 24-well plate at $5\times10^5$ cells per well (n = 2).

Enzyme-linked immunosorbent assay (ELISA)

**[0046]** The concentration of mature rhBMP-2 secreted in the conditioned media or in the cell lysates of the PC transfected cells was quantified by BMP-2 immunoassay (R&D Systems, Mineapolis, MN) as described previously (Zhou et al. 2012a). Briefly, the conditioned media samples were loaded into a 96-well microplate pre-coated with monocloncal anti-BMP2 antibodies. After 2hr incubation, the captured rhBMP-2 proteins were detected using polyclonocal anti-BMP2 antibodies. Recombinant human BMP-2 was used to generate the standard curve. Similarly, the amount of furin in the conditioned media and the cell lysates were determined using a furin DuoSet ELISA Development System (R&D Systems), following manufacturer’s instructions.

**[0047]**

Western blot analysis

**[0048]** The conditioned media and the cell lysate samples were resolved by 4-12% LDS-PAGE (Invitrogen) and blotted to a nitrocellulose membrane (Invitrogen). The membrane was blocked in 5% fat-free milk and probed with anti-BMP-2 (Abcam, Cambridge, MA; 2 µg/ml), anti-Furin (R&D Systems; 2 µg/ml) or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam; 1 µg/ml) antibodies, followed by horseradish peroxidase-linked secondary antibodies. Signal was visualized by incubating with SuperSignal chemiluminescent substrate (Pierce, Thermo Fisher Scientific, Rockford, IL) according to the manufacturer’s instructions. Image detection was performed with ChemiDox XRS and

Quantity One software (Bio-Rad, Hercules, CA). The molecular weight of the immuno-reactive bands was determined from the log plot of the relative migration of the protein standards.

BMP-2 bioactivity assay

**[0049]** A murine myogenic cell line C2C12 (American Type Culture Collection, ATCC, Manassas, VA) was seeded at $2.5 \times 10^4$ cells per well in 500 µl α-minimum essential medium (α-MEM, Invitrogen) plus 10% (v/v) fetal bovine serum (FBS, Invitrogen) in 48-well plates. After 24 h culture, the media was replaced with 500 µl of test samples per well. After 48 h incubation, the cells were lysed (CelLytic M, 200 µl/well; Sigma-Aldrich, St. Louis, MO), homogenized, and the alkaline phosphatase (ALP) enzymatic activity was determined using paranitrophenyl phosphate (pNPP) (Sigma-Aldrich) as previously described (Zhou et al. 2012b). The ALP level was normalized to the amount of cellular protein, measured by Bradford assay.

Extracellular furin cleavage

**[0050]** BMP-2 expressing CHO cells were seeded in a 24-well plate at $5 \times 10^4$ cells per well and cultured with 1 ml of fresh medium spiked with 0, 4, 40, or 400 ng/ml of recombinant human furin (R&D Systems) under standard cell culture conditions. After 48 h incubation, the conditioned media were collected and cell lysates were prepared (as described in the previous section BMP-2 bioactivity assay), and the amount of BMP-2 in the samples was measured by BMP-2 ELISA. In addition, some conditioned media from CHO cell culture were pipetted into a 96-well plate at 200 µl per well. Recombinant human furin (R&D Systems) was added to the conditioned media at 0, 16, 80, 400, or 2000 ng/ml and incubated at 37°C for 24 h. Following the incubation, the conditioned media were collected and the amount of BMP-2 was measured by BMP-2 ELISA.

ProBMP-2 ELISA Protocol

Materials

**[0051]** DuoSet ® rhBMP-2 ELISA kit (R&D Systems, cat #DY335)

**[0052]** Anti-ProBMP-2 antibody (R&D Systems, MAB2260 or Abcam, ab 14933)

Plate Preparation

[0053] Transfer 100 μL/well of the anti-proBMP-2 antibody (5 μg/ml) as the capture antibody (diluted to the appropriate concentration in PBS, use immediately) to an ELISA plate. Seal plate and incubate overnight at room temperature.

[0054] Aspirate each well and wash with Wash Buffer, repeating the process for a minimum of 3 washes. Wash by forcefully filling each well with Wash Buffer (400 μL) using an automatic washer. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating and by inverting the plate and blotting it against clean paper toweling.

[0055] Block plates by adding 300 μL of recommended Blocking Buffer to each well. Incubate at room temperature for a minimum of 1 hour.

[0056] Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition. Alternatively, the blocking buffer can be aspirated after step 3 and the plates can be dried under vacuum. When sealed with desiccant, the plates can be stored at 4 to 8° C for at least 2 months.

[0057] Assay Procedure

[0058] Add100 μL of sample or standards in an appropriate diluent, per well. Mix by gently tapping the plate frame for 1 minute. Cover with an adhesive strip and incubate 2 hours at room temperature.

[0059] Repeat the aspiration/wash as in step 2 of Plate Preparation.

[0060] Add 100 μL of the biotinylated detection antibody, diluted in appropriate diluent, to each well. Cover with a new adhesive strip and incubate 2 hours at room temperature.

[0061] Repeat the aspiration/wash as in step 2 of Plate Preparation.

[0062] Add 100 μL Streptavidin-HRP (R&D Systems, Catalog # DY998, dilute according to the directions) to each well. Cover the plate and incubate for 20 minutes at room temperature. An alternate detection system may be used. Avoid placing the plate in direct light.

[0063] Repeat the aspiration/wash as in step 2 of Plate Preparation.

[0064] Add 100 μL Substrate Solution (R&D Systems, Catalog # DY999) to each well. Incubate for20 - 30 minutes at room temperature. Avoid placing the plate in direct light.

[0065] Add 50 μL Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

[0066] Determine the optical density (O.D.) of each well within 30 minutes. Set the micro-titer plate reader to 450 nm. If the wavelength correction is available, set it to 540 nm.

1 RESULTS

2 **[0067]** To investigate the effect of PC overexpression on BMP-2 production and processing
3 in the CHO expression system, cells were transfected with one of the seven tGFP-tagged
4 full-length proprotein convertases (PCs: Furin, PACE4, PC6A, PC6B, and PC7) or their
5 soluble forms (sFurin, soluble furin; sPC7: soluble PC7) (Figure 1). Several attempts were
6 made to transfect these suspension CHO cells by Lipofectamin (Invitrogen), but the
7 transfection efficiency was very low (only 5-10%, data not shown). When electroporation was
8 used, the transfection efficiency improved greatly with majority of the PCs transfected at 60
9 to 80%, except for PACE4 (23%) and PC6A (32%) (Figure 3A). For PCs with high
10 transfection efficiency, the transfected cells (or positive cells) were further sorted into low,
11 mid, and high positive groups (Figure 2). For PCs with low transfection efficiency, cells were
12 sorted into negative and positive transfected groups.

13 **[0068]** The conditioned media of the transfected cells was collected 24 h post sorting and
14 the amount of BMP-2 secreted was measured by ELISA.

15 **[0069]** Among all the PCs tested, only the cells overexpressing full-length Furin
16 demonstrated a significant increase in their secreted mature BMP-2 ($P$ <0.0001) (Figure 3B).
17 With the low and mid level Furin expressions, the cells secreted up to 8-fold ($P$ <0.001) and
18 5-fold ($P$ <0.01) more BMP-2 respectively than cells not overexpressing furin (negative
19 control). Surprisingly, cells expressing the highest level of Furin showed lower rhBMP-2 yield
20 than cells expressing low- and mid-level of Furin.

21 **[0070]** PCs can be membrane-bound or soluble depending on whether they have a
22 transmembrane domain in their sequences (Figure 1). PC6 has two naturally occurring
23 variants: soluble PC6A and membrane-bound PC6B. PACE4 does not contain a
24 transmembrane domain and hence is naturally soluble. The soluble furin (sFurin) and PC7
25 (sPC7) were constructed by deleting their transmembrane domains and the cytoplasmic tails
26 from the full-length PCs. Cells overexpressing any one of the soluble PCs did not show any
27 significant impact on the amount of mature BMP-2 secreted (Figure 3B).

28

29 **[0071]** To ensure the tGFP tag does not interfere with the PC activity, plasmids expressing
30 PCs and tGFP individually as bicistronic transcripts via an internal ribosome entry site
31 (IRES) located between the two proteins were used. The transfection efficiencies for the
32 plasmids expressing PCs tagged with or without tGFP differ significantly for sFurin ($P$
33 =0.0317) and PC7 ($P$ =0.0170) (Figure 4A). However, despite the difference in their
34 transfection efficiencies, overexpression of Furin, sFurin, or PC7 showed little difference on
35 the amount BMP-2 secreted in their matching partners (with vs. without tGFP tag) (Figure

1 4B). Regardless of the tGFP tag, the overexpression of Furin significantly increased BMP-2
2 yields by approximately 5-6 fold
3
4 **[0072]** The alkaline phosphatase (ALP) level, a known marker of osteoblast differentiation,
5 was measured in the C2C12 cell line to assess the biological activity of mature BMP-2
6 expressed by cells overexpressing Furin *in vitro*. In the absence of BMP, myogenic C2C12
7 cells express very low levels of ALP. In the presence of BMP, ALP activity of the C2C12
8 cells increases in a dose-dependent manner (Katagiri et al. 1994; Zhou et al. 2012b). This
9 was observed when the mature BMP-2 secreted by cells overexpressing Furin were tested,
10 indicating that the secreted BMP-2 is biologically active *in vitro* (Figure 5). The rhBMP-2
11 secreted by cells overexpressing Furin also induced a significantly greater amount of ALP
12 activity compared with the untransfected cells (negative control, NC) at concentrations up to
13 8 nM BMP-2 (paired t-test, $P$ =0.0319)(Figure 5).
14
15 **[0073]** To investigate the effect of Furin overexpression on BMP-2 post-translational
16 processing, CHO cells were transfected with Furin or sFurin tagged with tGFP. Twenty-four
17 hours post transfection, positively transfected and negative cells (cells that did not take up
18 PC plasmid and hence expressed little or no GFP) were sorted and cultured for 48 h. After
19 incubation, the conditioned media and cell lysates were collected and analyzed by Western
20 blot. Probing with an anti-Furin antibody, a strong immunoreactive band at 80 kDa was
21 observed in the conditioned media of cells overexpressing Furin, presumably representing
22 furin shed from the plasma membrane (called shed furin), but no band in the lanes of the
23 negative control (NC) or cells overexpressing sFurin (Figure 6A). In the cell lysates, three
24 furin immunoreactive bands were observed at 80, 90 and 116 kDa in cells overexpressing
25 Furin and sFurin, but not the in negatively transfected cells. The amount of Furin and sFurin
26 overexpressed was quantified by furin ELISA, which showed significant increases in their
27 levels in both the conditioned media and cell lysates compared to the negatively transfected
28 cells with undetectably low amount of furin (Figure 6B).
29 **[0074]** The secreted and intracellular BMP-2 from cells transfected with Furin or sFurin were
30 characterized by Western blot and quantified by BMP-2 ELISA. Using an anti-BMP-2
31 antibody, cells overexpressing Furin showed a single immunoreactive band at 18 kDa in the
32 conditioned media, whereas the control and cells overexpressing sFurin both expressed two
33 BMP-2 immunoreactive bands at 18 and 20 kDa (Figure 7A). Cells overexpressing sFurin
34 also secreted a higher molecular mass BMP-2 immunoreactive band at 55 kDa, believed to
35 be the incompletely processed proBMP-2, which was not present in the conditioned media

from cells overexpressing Furin. In the cell lysates, the 18 and 55 kDa bands were present in all three samples. In addition, a faint 20 kDa band was observed in the untransfected cells. BMP-2 ELISA showed significantly increased amount of extracellular ($P < 0.05$, ANOVA) and intracellular BMP-2 ($P < 0.05$) in cells overexpressing Furin than those overexpressing sFurin or the untransfected cells (Figure 7B). Cells overexpressing sFurin expressed similar amount of BMP-2 to the untransfected cells extracellularly and intracellularly. These data demonstrates that the overexpression of full-length membrane bound Furin, but not the soluble sFurin, reduced the level of partially processed or unprocessed proBMP-2 and the N-terminal heterogeneity of the mature BMP-2.

**[0075]** To further investigate whether the Furin in the conditioned media alters the BMP-2 yield, CHO cells were cultured in media containing 0, 4, 40, and 400 ng/ml of recombinant human furin for 48 h. The data showed that the amount of BMP-2 secreted in the conditioned media or expressed in the cell lysates showed an increasing trend with the increasing the concentration of extracellular furin in the cell culture media (Figure 8A). With increased furin concentration up to 400 ng/ml (which was 100 times higher than required to affect the BMP-2 yields), the amount of BMP-2 in the conditioned media and the cell lysates only showed a slight, but not statistically significant increase. In addition, instead of culturing CHO cells in media with furin, up to 2000 ng/ml of furin were added to the BMP-containing conditioned media and incubated for 24 h. All furin treated groups, even at 2000 ng/ml, which is 500 times higher than required to affect the BMP-2 yield, did not show any significant change in BMP-2 yield compared to the untreated group (Figure 8B).

Discussion

**[0076]** The transmembrane and the cytoplasmic domains of furin have been found to be dispensable for the functional activity of furin. High levels of expression of full-length recombinant furin have resulted in the natural secretion of a truncated furin, called “shed” furin, lacking those C-terminal domain (aa 683 to 794). Mutant form of soluble furin constructed by deleting the transmembrane domain and cytoplasmic tail (aa 716-794) has been shown to still exhibit activity. When we incubated the purified proBMP-2 secreted by CHO cells with a commercially available recombinant soluble human furin (purchased from R&D Systems), proBMP-2 was cleavage into mature BMP-2 over 24 h in vitro (data not shown). Hence, in theory the soluble sFurin should be able to process proBMP-2. We also attempted measuring the activity of the soluble sFurin in the cell lysate by incubating it with a fluorogenic substrate BOC-RVRR-AMC containing the predicted PC clevage site RVRR in

vitro. Upon cleavage, the fluorescent AMC is liberated and the fluorescence can be detected. When this fluorometric activity assay was performed on sFurin in the cell lysate, we were unable to detect a fluorescent signal, most likely due to the limited sensitivity of the assay. Although we were unable to demonstrate the activity of sFurin expressed by CHO cells, we suspect that the inactivity of sFurin is unlikely the reason that its overexpression did not result in increased rhBMP-2 yield.

**[0077]** Although the cells transfected with Furin and sFurin had comparable transfection efficiency (60-70%, Figure 3A) and expressed similar amounts of intracellular furin (Figure 6B), only the full-length Furin was able to significantly improve rhBMP-2 yield

**EXAMPLE 2 Measurement of Furin content and Proprotein Convertase activity in various cell lines**

**[0078]** In this study, the levels of Furin expression and proprotein convertase (PC) activity in CHO and HEK lines were investigated.

MATERIALS AND METHODS

Cell Culture

**[0079]** HEK 293 Flp-In cell lines and CHO DG44 cells expressing rhBMP-2 or varients were created. HEK cells were stratified into 3 groups: FRT parent cell line; wild type BMP (WT); or mutant BMP (ms1).

**[0080]** CHO m713 cells express the equivalent of the BMP WT preform – they are a 7X amplified wild type gene. ‘CHO m501 is similar, but the gene is only 5X amplified. CHO EGFP expresses green fluorescent protein and acts as a control since it is not overexpressing BMP2. CHO cells were grown with CD OptiCHO medium (Invitrogen) in T75 flasks at 37°C.

Lysis and Storage

**[0081]** All cell lines were cultured in T-75 flasks under the same conditions. At confluence, cultures were terminated and harvested. The medium was removed, 10 ml of trypsin (Invitrogen) was added, and incubated at 37°C for 5 min. Cells were checked under a microscope to confirm separation from flask surface. 10 ml of fresh medium was added to stop trypsin digestion. Cell counts were performed on a sample of medium using a ViCell Cell Viability Analyzer (Beckman). The remaining medium was made into 20 x $10^6$ cell aliquots. Each aliquot was centrifuged at 10,000 x g for 10 min to pellet the cells. The cell pellet was washed with PBS. 1 ml of CelLytic M Cell Lysis Reagent (Sigma-Aldritch) was added to the washed pellet and mixed to re-suspend the cells. The mixture was

1 homogenized using PowerGen 125 (Fischer-Scientific) and then centrifuged at 16,000 x g
2 for 10 min. The supernatant was collected and stored at -80°C.
3
4 Proprotein Convertase (PC) Activity Assay
5 **[0082]** PC activity was determined by measuring the concentration of a fluorescent, cleaved
6 product produced after incubation of a flourogenic substrate in the presence of Furin. Cell
7 lysates were diluted in 1 mM CaCl2, 0.5%(w/v) Brij-35 in 25mM Tris. 50uL of fluorogenic
8 BOC-RVRR-AMC substrate (Bachem) was added to 50uL of each cell lysate dilution, in
9 duplicate and incubated at 37°C. We prepared undiluted samples as well as 10-fold
10 dilutions. Stock rhFurin (R&D Systems) was treated identically as a positive control. 7-
11 amino-4-methyl-coumarin (AMC) (Aldrich Chem Co) was prepared as a serial dilution from
12 128uM to 0.25uM but was not treated with substrate. Fluorescence was measured at
13 wavelengths 360nm for excitation and 460nm for emission, using black 96 well plates with a
14 clear bottom on an FL600 Fluorescence Plate Reader (BioTek Instruments). Readings were
15 taken every 30 minutes. Activity was defined as released AMC concentration per minute,
16 and released AMC concentration was derived by back calculation from the AMC standard
17 curve. Activity was normalized for variations in furin amount by dividing the result by the total
18 cellular protein concentration, which was determined by a Bradford assay.
19
20 Bradford Assay
21 **[0083]** To determine total cellular protein amount, cell lysates were thawed at room
22 temperature and diluted in CelLytic M Cell Lysis Reagent (Sigma). We prepared undiluted
23 samples as well as 10, 100, and 500 fold dilutions for each HEK and CHO cell line. 20uL of
24 lysate was treated with 200uL of Coomassie Plus Protein Assay Reagent G250 (Pierce), in
25 quadruplicate. Bovine Serum Albumin (BSA) (Sigma) was treated similarly, but serially
26 diluted from 400ug/mL to 1.625ug/mL to produce a standard curve, as BSA was the positive
27 control. BSA standards were run in duplicate rather than quadruplicate. Samples were
28 incubated for 10 minutes then measured in a spectrophotometer at wavelength 595nm.
29 Protein concentration of each unknown lysate was determined by back calculation via
30 regression analysis from the BSA standard curve.
31
32 Furin ELISA
33 **[0084]** The purpose of this experiment is to determine the specific amount of Furin in HEK
34 and CHO cell lysates. We used the R&D Systems Furin ELISA Duoset Kit. 96 well plates
35 were coated overnight with 100µL per well of 2µg/mL mouse anti-human Furin antibody

(capture antibody) in PBS. The plates were washed 3 times with 400uL wash buffer (0.05% Tween 20 in 1XPBS) then blocked for 1 hour with 300µL of reagent diluent (1% fraction V BSA in 1XPBS). Plates were washed again, then incubated for 2 hours with 100µL of diluted sample or rhFurin standard. Samples were undiluted or 3 fold dilutions of HEK and CHO cell lysate in reagent diluent. The plates were washed after incubation and coated with 100µL of 100ng/mL biotinylated goat anti-human Furin antibody (detection antibody). A 2 hour incubation period passed before another wash step, and successive addition of 100µL of streptavidin-HRP. After 20 minutes and a final wash step, 100µL of substrate (50% tetramethylbenzidine, 50% hydrogen peroxide) was added and left to incubate for 20 minutes on a plate shaker. 50µL of 2N $H_2SO_4$ was added to stop the reaction. Readings were taken using a spectrophotometer at wavelengths 450 and 540. Furin levels were normalized by total protein amount for analysis.

**[0085]** Results are presented as the mean ± standard error of the mean (SEM); n=3. Statistical analysis was performed using one-way analysis of variance (ANOVA) or a paired t test. Post hoc analyses used Dunnett's and Tukey's multiple comparison tests. Microsoft Excel 2010 and IBM PASW 18 softwares were used for graphical and statistical representation. Critical P is as denoted, but typically $P < 0.05$ was considered significant.

RESULTS

Furin concentrations in HEK and CHO cells

**[0086]** Furin ELISA results for each cell line variant demonstrated no significant difference between cell lines from the same parent (HEK or CHO) (Figure 9). However, there is a significant difference HEK to CHO cell lines when combining results from all CHO vs all HEK cell lines (ANOVA, $P < 0.05$).

PC activity in HEK cells and CHO cells

**[0087]** PC activity assay data revealed a significant difference between HEK and CHO cell lysates (Figure 10). PC activity was similar, with no significant difference between variants of the same cell line ($P<0.05$). Notably, there is no difference in PC activity between cell lines containing a control vector, versus those that contain a BMP vector. For this reason, all HEK cell line variants may be considered one group, and likewise all CHO cell line variants may be considered a separate group. Rank ordered ANOVA demonstrated that a significant difference does exist between these two cell lines ($P<0.001$).

**[0088]** These results demonstrate methods by which is it possible to identify cell lines with varying expression levels of furin and PC activity.

## CLAIMS

We claim:

1. A method for increasing the yeild of a recombinant protein of interest comprising, transfecting a mammalian cell with both the protein of interest and with full length furin.

2. The method of claim 1 wherein the transfection with the protein of interest is carried out before the transfection with full length furin.

3. The method of claim 2 wherein the mammalian cells further undergo one or more rounds of gene amplification between the transfection with the protein of interest and the transfection with full length furin.

4. A method for increasing the yield of a recombinant protein of interest comprising: transfecting mammalian cells with full length furin,
screening the transfected cells and selecting the cells expressing a suitable level of furin to generate a parent cell line,
transfecting cells of the parent cell line with a protein of interest.

5. The method of claim 4 wherein a suitable level of furin expression is a low level or mid- level of expression.

6. The method of any one of claims 1-3 wherein after transfection with full length furin the furin expression is a low to mid-level of expression.

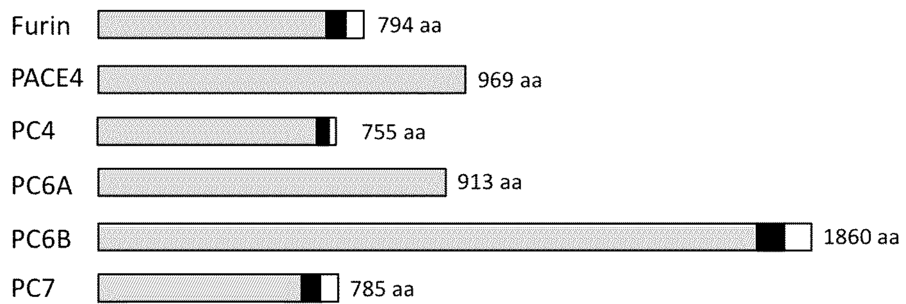
7. The method of any one of claims 1-6 wherein the protein of interest is BMP-2 or BMP-7.

8. The method of any one of claims 1-7 wherein the mammalian cell is HEK or CHO.

9. A method of preparing a parent cell line for improving the yield of a recombinant protein of interest when the cell line is transfected with the protein of interest, comprising, transfecting mammalian cells with full length furin, screening transfected cells and selecting the cells expressing a suitable level of furin to generate a parent cell line.

10. A parent cell line having a suitable level of furin expression to increase the yield of a recombinant protein of interest when the cell line is transfected with the protein of interest prepared by the method of claim 9.

**Figure 1**



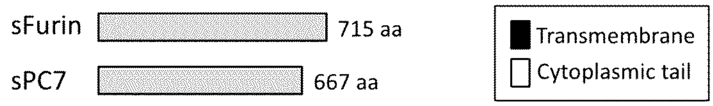



**Figure 2**

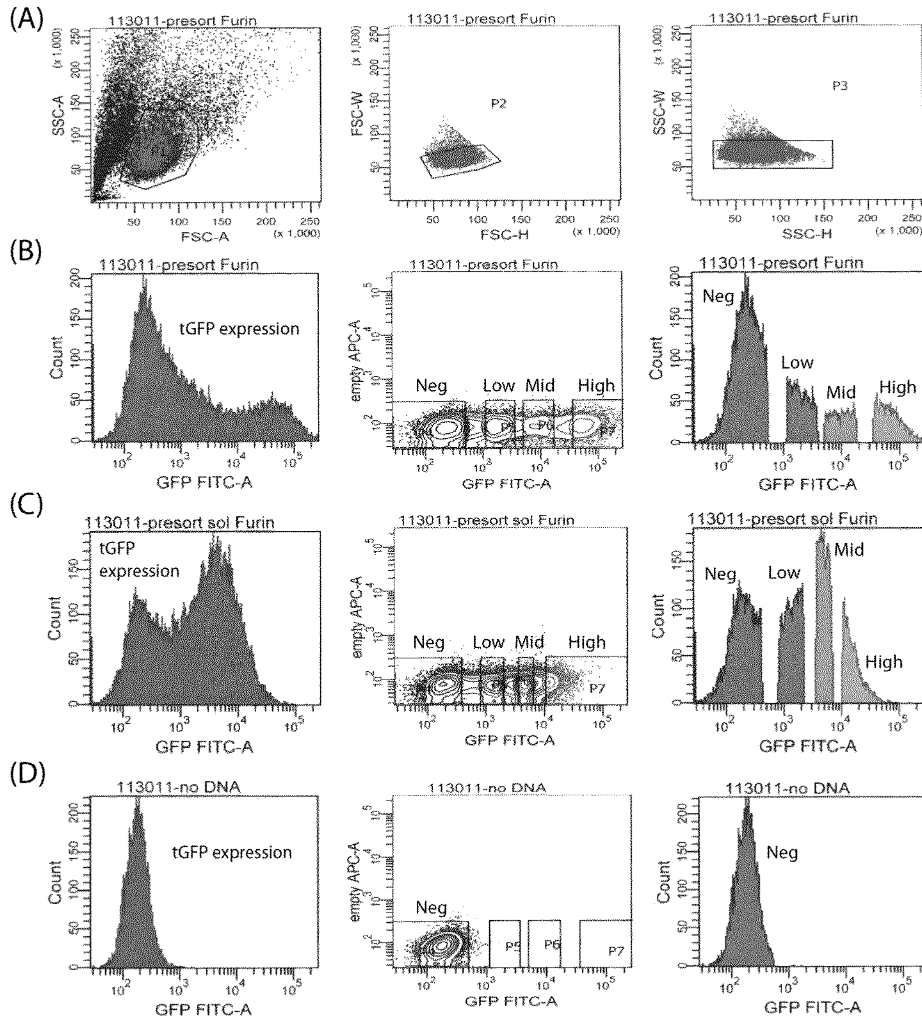


**Figure 3**

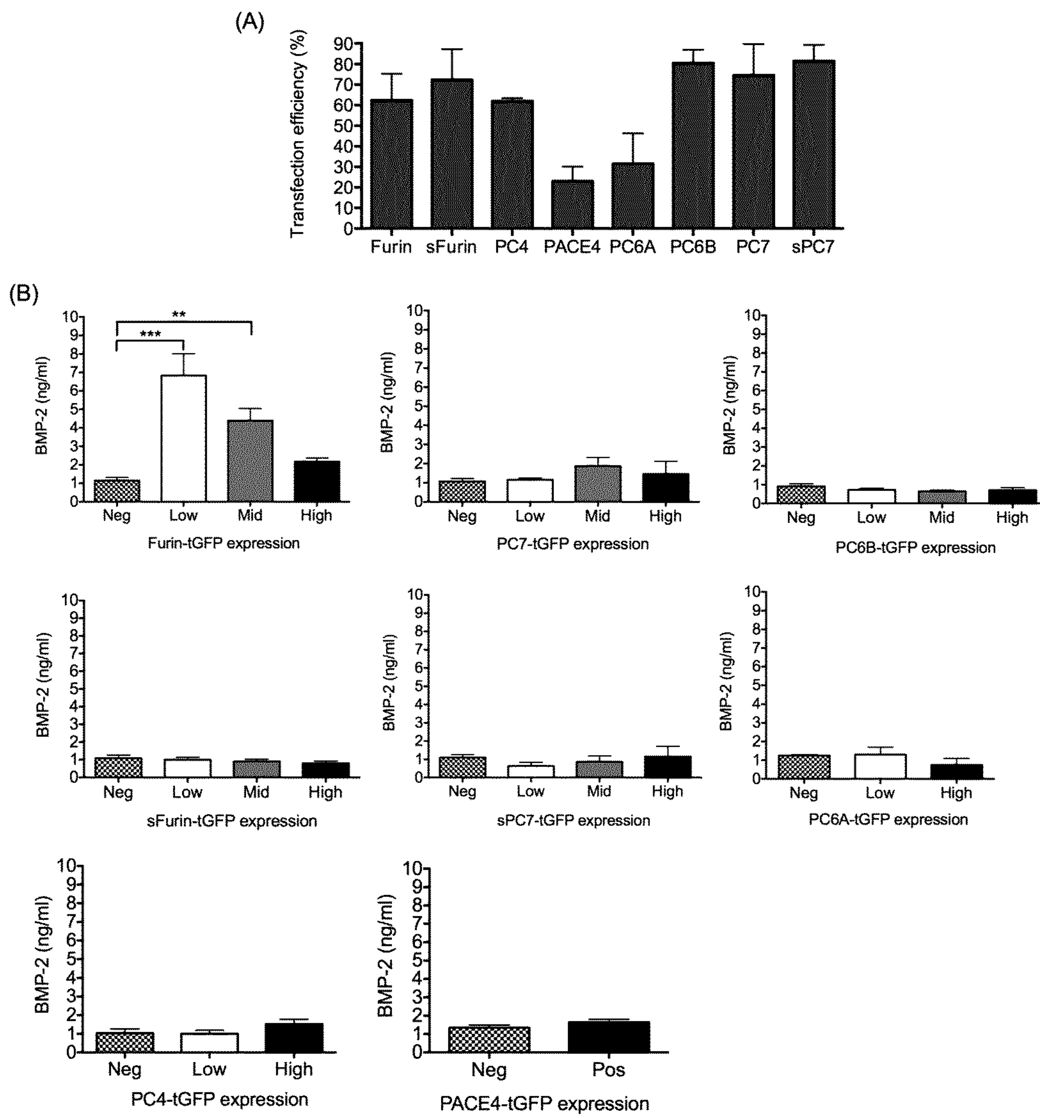


**Figure 4**

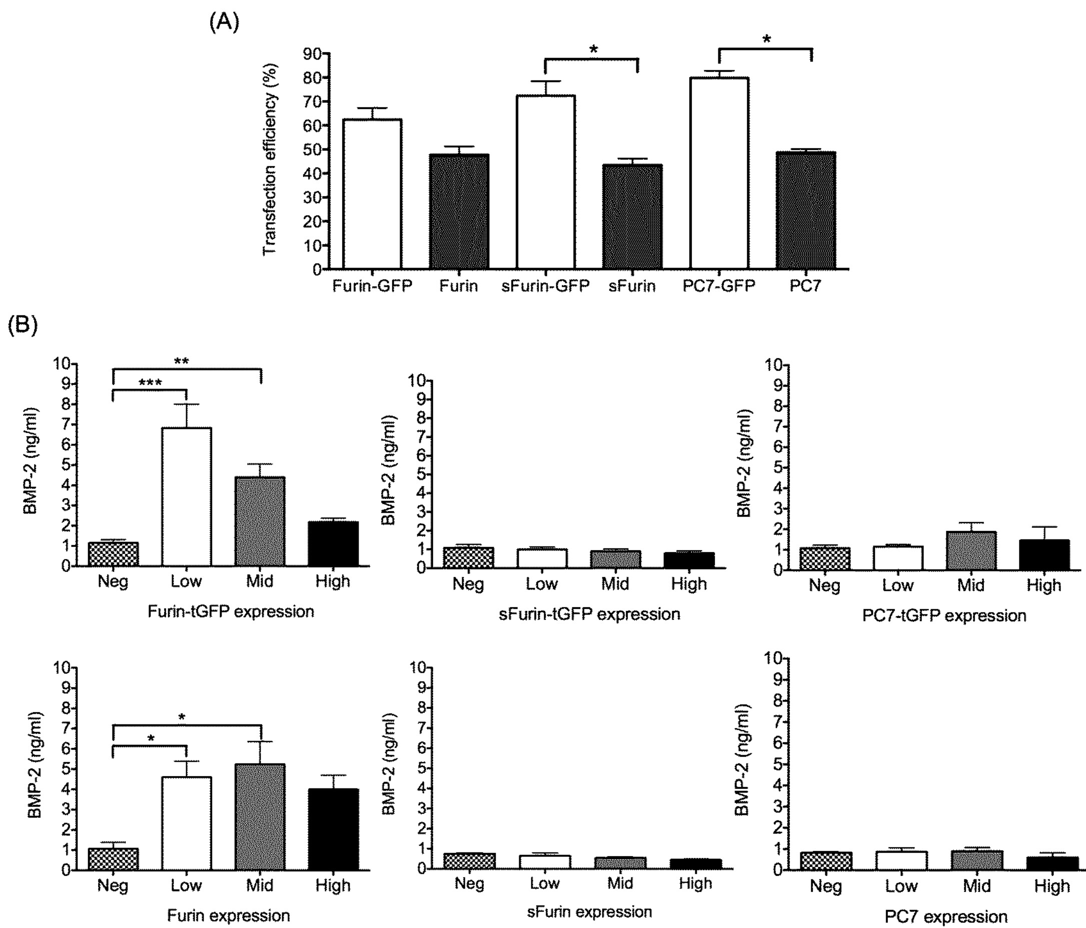


**Figure 5**

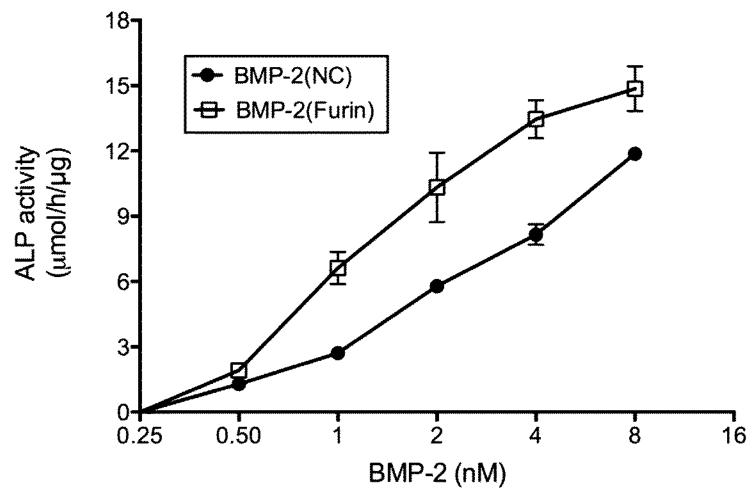


Figure 6

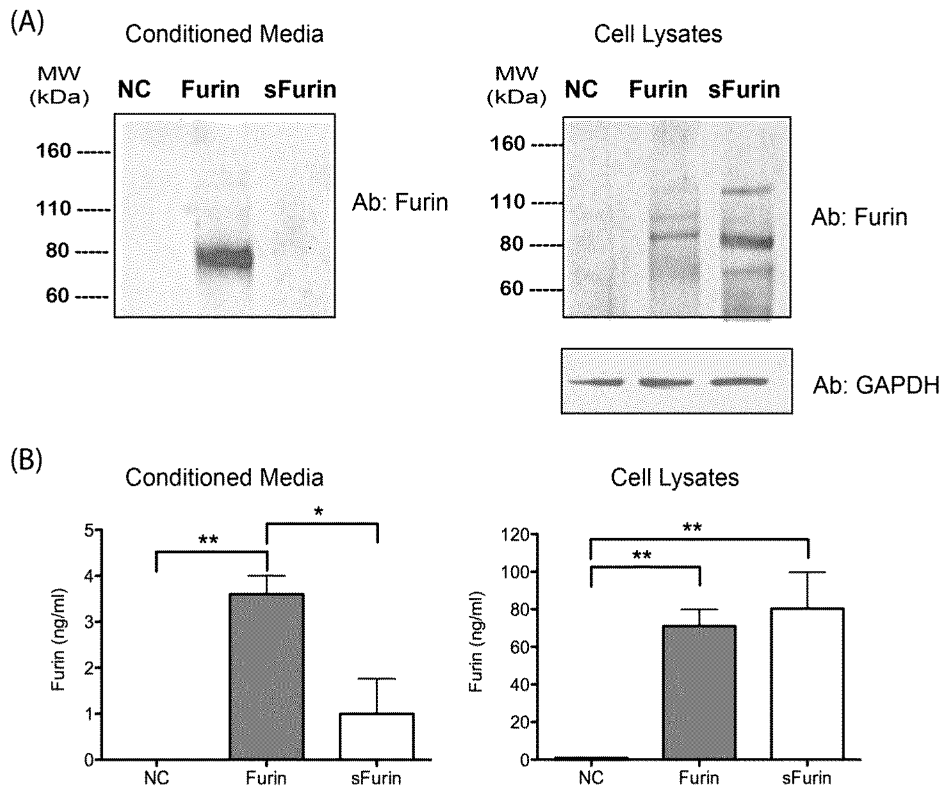


**Figure 7**

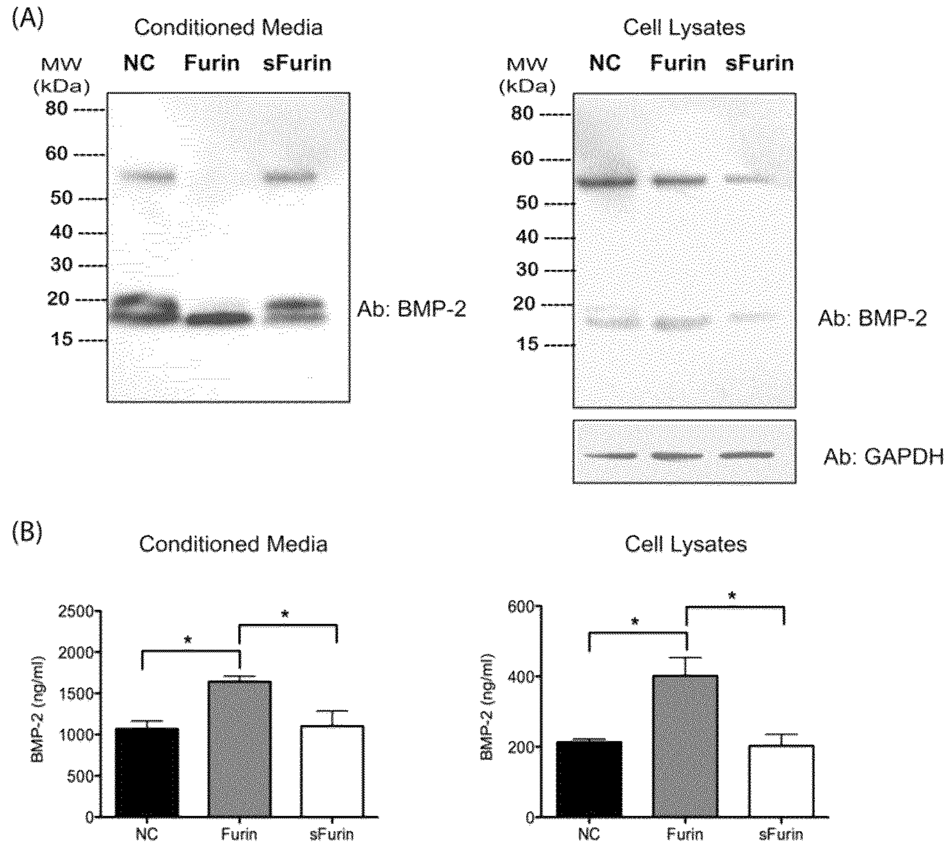


**Figure 8**

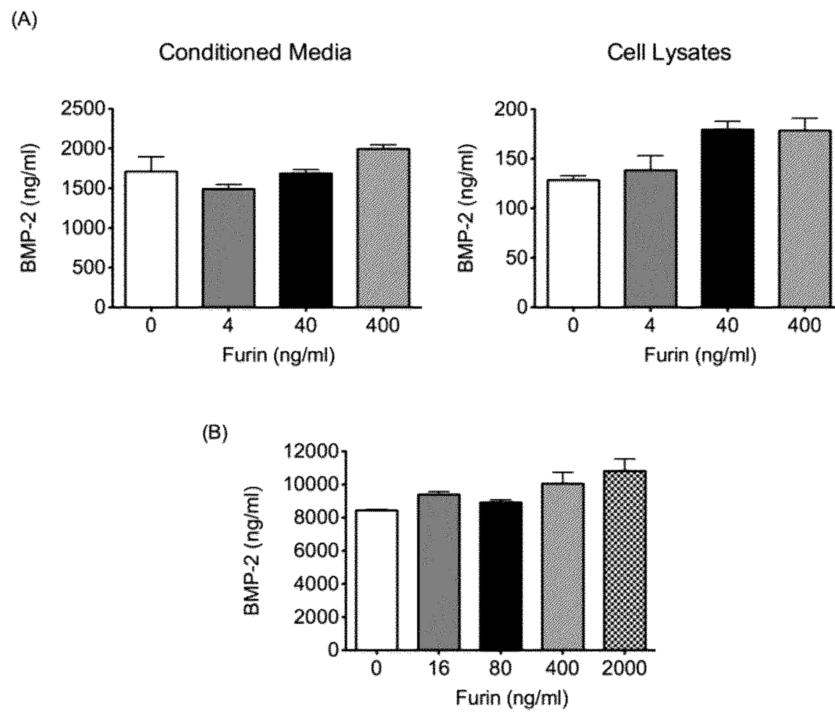


**Figure 9**

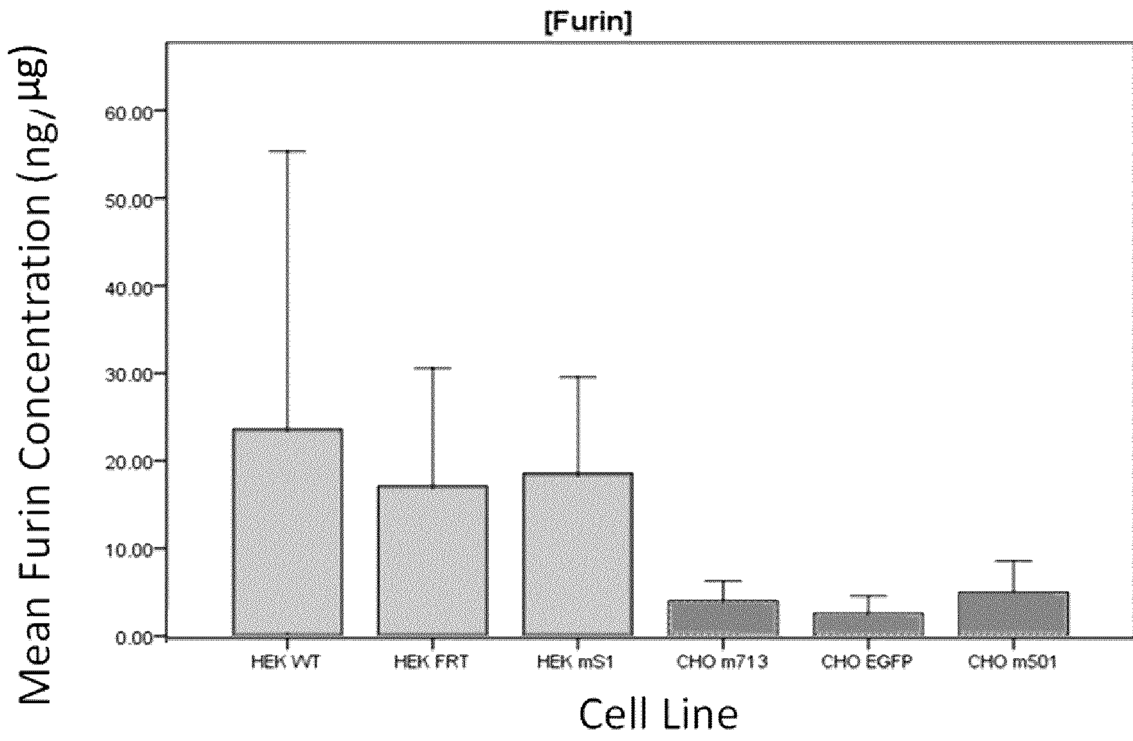
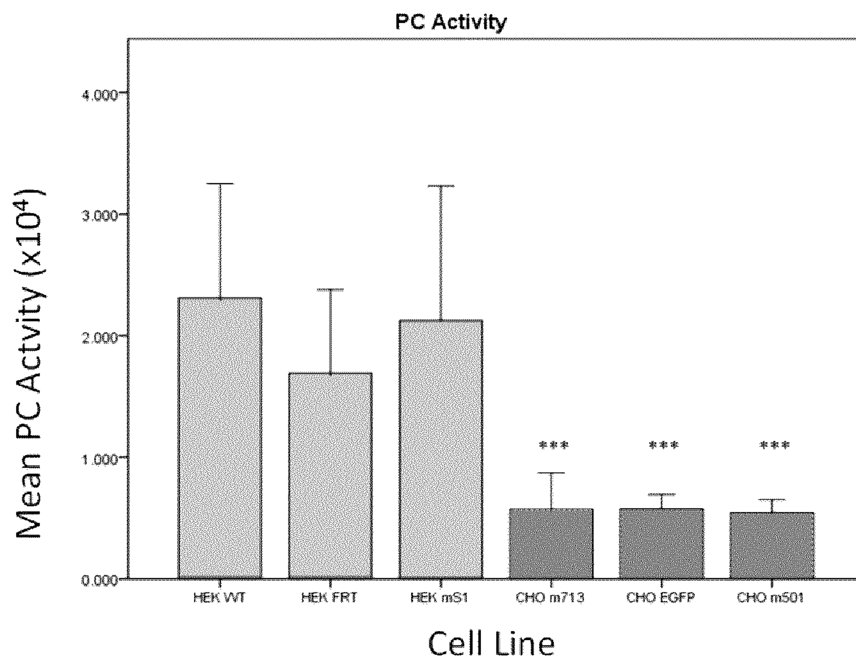


**Figure 10**



A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C12N 15/67*** (2006.01) , ***C07K 14/51*** (2006.01) , ***C12N 15/12*** (2006.01) , ***C12N 15/57*** (2006.01) , ***C12N 15/85*** (2006.01) , ***C12N 5/10*** (2006.01) (more IPCs on the last page)

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)

"Keywords used across the whole IPC"

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

none

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

CIPO Library Discovery Tool, Scopus, Questel
Keywords: Furin and recombinant protein yield, furin-co-expression, bone morphogenetic protein expression, BMP-2, BMP-7

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| X | Bingo M et al., Recombinant Factor X using a chimeric construct containing the prothrombin propeptide under co-expression of furin leads to high yield and matured protein expressions in 293T Cells. 2019, Rinsho Byori Vol. 67, pages 24-31, ISSN: 0047-1860. | 1, 8 |
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☑ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "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
08 September 2020

Date of mailing of the international search report
25 September 2020 (25-09-2020)

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| | | TWI666319B | 21 July 2019 (21-07-2019) |
| | | WO2016025615A1 | 18 February 2016 (18-02-2016) |

***C12N 9/64*** (2006.01) , ***C12P 21/02*** (2006.01) , ***C12Q 1/68*** (2018.01)