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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 June 2014 (19.06.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/090948 A1

(51) International Patent Classification:
A61K 38/55 (2006.01) *A61P 35/04* (2006.01)

(21) International Application Number:
PCT/EP2013/076389

(22) International Filing Date:
12 December 2013 (12.12.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
12306580.7 13 December 2012 (13.12.2012) EP

(71) Applicant: **INSERM (INSTITUT NATIONAL DE LA SANTÉ ET DE LA RECHERCHE MÉDICALE)** [FR/FR]; 101, rue de Tolbiac, F-75013 Paris (FR).

(72) Inventor: **KHATIB, Abdel-Majid**; Inserm U1029, Université des Sciences et Techniques, Avenue des Facultés, Bâtiment B2, F-33405 Talence (FR).

(74) Agent: **HIRSCH, Denise**; Inserm Transfert, 7 rue Watt, F-75013 Paris (FR).

(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, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, 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, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, 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, 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))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2014/090948 A1

(54) Title: SERPIN SPN4A AND BIOLOGICALLY ACTIVE DERIVATIVES THEREOF FOR USE IN THE TREATMENT OF CANCER

(57) Abstract: The invention relates to an isolated Serpin Spn4A polypeptide or a biologically active derivative thereof for use in the prevention or treatment of cancer and related metastases. The invention also relates to a pharmaceutical composition for use in the prevention or treatment of cancer and related metastases comprising at least one polypeptide according to the invention, or at least one acid nucleic according to the invention, or at least one expression vector according to the invention, or at least one host cell according to the invention and a pharmaceutically acceptable carrier.

SERPIN SPN4A AND BIOLOGICALLY ACTIVE DERIVATIVES THEREOF FOR USE IN THE TREATMENT OF CANCER

FIELD OF THE INVENTION:

The invention relates to the field of oncology. More particularly, the invention relates to an isolated Serpin Spn4A polypeptide or a biologically active derivative thereof for use in the prevention or treatment of cancer and related metastases.

BACKGROUND OF THE INVENTION:

Proprotein convertases (PCs) located along the constitutive secretory pathway (Furin, PACE4, PC5 and PC7) are involved in the proteolytic cleavage and/or expression of various neoplasia-related mediators, making them promising targets in cancer therapy.

Indeed, by inducing cleavage and/or expression of various protein precursors, PCs play a key role in cancer progression and metastasis (1-8). To date, a wide range of these substrates and/or downstream effectors comprise growth factors and receptors, adhesion receptors and proteases. For example, proteolytic processing by PCs of VEGF-C, -D (3, 4), PDGF A, -B (5, 6), IGF-1 receptor (7) and MT1-MMP (8) was found to be required for their ability to mediate tumor cell proliferation and/or invasion. Similarly, hepatic cytokine and E-selectin induction by metastatic colon cancer cells during liver colonization was reported to require functional PCs in invading tumor cells (9). The PCs activate their substrates by cleavage at the consensus sequence (K/R)-(X)n-(K/R)↓, where n = 0, 2, 4, or 6 and X is any amino acid except Cys (1, 2). This conversion is mediated by one or more of the PC family members. These include PC1 and PC2 found within dense core secretory granules that process proteins secreted by the regulated secretory pathway, and Furin, PC4, PC5, PACE4 and PC7 that are involved in protein precursors processing secreted via the constitutive secretory pathway (1-9). Previously, altered levels of PCs were reported to be associated with enhanced invasion and proliferation of colon cancer cells (10, 11). Conversely, inhibition of PCs by the bioengineered inhibitor α1-PDX in colon carcinoma cells resulted in PC substrates processing blockade (7, 9, 11).

Maturation of protein precursors by PCs was described in various organisms and species including plants and eukaryotes, ranging from Hydra to mammals (12). However, although the distribution and conservation of PCs within the secretory pathway predicts the existence of endogenous inhibitors and/or mechanisms able to regulate their activity (1, 2), to date only three naturally occurring inhibitors have been identified; including 7B2 (13),

proSAAS (14), and CRES (15). All of these inhibitors are directed against regulated secretory pathway convertases PC2 and PC1/3, but they do not exhibit a direct effect on secretory pathway PCs (13-15).

Unfortunately until now, no naturally occurring inhibitor is known that efficiently inhibits all secretory pathway PCs, because such inhibitor would be of great interest for preventing or treating cancer, tumoregenesis and related metastasis (e.g. by inhibiting proliferation, migration and invasion of cancer cells).

Serpins belong to a superfamily of protease inhibitors that play a regulatory role in blood coagulation, and inflammation. Mutations in these proteins were found to cause diseases including blood coagulation disorders, cirrhosis, emphysema, and dementia (16-18). To date, more than 800 serpins have been identified in plants, animals, viruses and prokaryotes (16-18). In *Drosophila melanogaster*, 29 serpin genes are known, a high number as compared to human. They are involved in maintaining enzymatic homeostasis of various proteases (18-21).

SUMMARY OF THE INVENTION:

In a first aspect, the present invention relates to an isolated Serpin Spn4A polypeptide or a biologically active derivative thereof for use in the prevention or treatment of cancer and related metastases.

In a second aspect, the present invention also relates to a nucleic acid encoding a polypeptide of the invention, or an expression vector comprising a nucleic acid of the invention, or a host cell comprising an expression vector for use in the prevention or treatment of cancer and related metastases.In a third aspect, the present invention further relates to a pharmaceutical composition comprising an isolated Serpin Spn4A polypeptide or a biologically active derivative thereof, or a nucleic acid encoding thereof, or an expression vector comprising thereof, or a host cell comprising thereof and a pharmaceutically acceptable carrier.

In another aspect, the present invention relates to a kit-of-part composition comprising an isolated Serpin Spn4A polypeptide or a biologically active derivative thereof, or a nucleic acid encoding thereof, or an expression vector comprising thereof, or a host cell comprising thereof and an additional therapeutic agent.

In still another aspect, the present invention relates to a pharmaceutical composition or a kit-of-part composition for use in the prevention or treatment of cancer and related metastases.

DETAILED DESCRIPTION OF THE INVENTION:

The invention is based on the discovery that *Drosophila melanogaster* serpin Spn4A, which is not conserved in human genome, inhibits the entire constitutive secretory pathway PCs (Furin, PC4, PC5, PACE4 and PC7), the proliferation as well as the metastatic potential of cancer cells (e.g. colon cancer cells). As disclosed herein, the serpin Spn4A is able, when expressed in colon carcinoma cells, to inhibit processing of PC substrates and reduced anchorage-independent growth, invasiveness and enhanced chemosensitivity. The latter is associated with reduced expression of Bcl-2 and increased Caspase-3 activity. As also shown herein, Spn4A repressed *in vivo* tumor development and formation of liver metastases in response to intrasplenic/portal inoculation of colon cancer cells. Furthermore, the tumor-suppressor function of Spn4A was linked to increased expression of molecules with anti-metastatic functions and inhibited expression of pro-tumorigenic molecules.

Thus, blocking tumor growth, tumorigenesis and increasing chemosensitivity by using Spn4A can be effective for prevention or treatment of cancer and tumor metastasis.

Therapeutic methods and uses:

The invention provides methods and compositions (such as pharmaceutical compositions) for preventing or treating cancer and related metastasis. The invention also provides methods and compositions for inhibiting or preventing proliferation, migration and invasion of cancer cells. The invention further provides methods and compositions for increasing chemosensitivity to chemotherapeutic compound such as tyrosine kinase inhibitors.

A first aspect of the invention relates to an isolated Serpin Spn4A polypeptide or a biologically active derivative thereof for use in the prevention or treatment of cancer and related metastases.

As used herein, the term "Serpin Spn4A" (Spn4a) refers to a Serine Proteinase Inhibitor of *Drosophila melanogaster*. Serpin Spn4A refers to any native or variant (whether native or synthetic) polypeptide of 392 amino acids provided in the GenBank database under accession number NM_165496.2 and is shown as follows (SEQ ID NO: 1):

MADAAHQEFARRLALFSINVYGKLSGQKPGENIVFSPFSIQTCAAMARLGAEN
ETATQLDQGLGLASSDPEQIAHSFHQVLAAYQDSQILRIANKIFVMDGYQLRQEFDQL
LSKQFLSAAQSVDFSKNVQAAATINNWVEQRTNHLIKDLVPADVLNSESRLVLVNAI
HFKGTWQHQFAKHLTRPDTFHLDGERTVQVPMMSLKERFRYADLPALDAMALELP
YKDSDLSMLIVLPNTKTGLPALEEKLRLTTLSQITQSLYETKVALKLPRFKAEFQVELS
EVFQKLGMSKMFSDQAEFGKMLQSPEPLKVSAFIHKAFIEVNEEGTEAAAATGMAV
RRKRAIMSPEEPIEFFADHPFTYVLVHQKDLPLFWGSVVRLEENTFASSEHDEL

As used herein, the term "polypeptide" refers to a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically, having no specific length. Thus, peptides, oligopeptides and proteins are included in the definition of "polypeptide" and these terms are used interchangeably throughout the specification, as well as in the claims. The term "polypeptide" does not exclude post-translational modifications that include but are not limited to phosphorylation, acetylation, glycosylation and the like. The term also applies to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

By an "isolated" polypeptide, it is intended that the polypeptide is not present within a living organism, e.g. within human body. However, the isolated polypeptide may be part of a composition or a kit. The isolated polypeptide is preferably purified. Such polypeptide is essentially free from contaminating cellular components, such as carbohydrate, lipid, or other proteinaceous impurities associated with the polypeptide in nature. Typically, a preparation of isolated polypeptide contains the polypeptide in a highly purified form, i.e., at least about 80% pure, at least about 90% pure, at least about 95% pure, greater than 95% pure, such as 96%, 97%, or 98% or more pure, or greater than 99% pure. One way to show that a particular protein preparation contains an isolated polypeptide is by the appearance of a single band following SDS-polyacrylamide gel electrophoresis of the protein preparation and Coomassie Brilliant Blue staining of the gel.

A "native sequence" polypeptide refers to a polypeptide having the same amino acid sequence as a polypeptide derived from nature. Thus, a native sequence polypeptide can have the amino acid sequence of naturally-occurring polypeptide from *Drosophila melanogaster*. Such native sequence polypeptide can be isolated from nature or can be produced by

recombinant or synthetic means. The term "native sequence" polypeptide specifically encompasses naturally-occurring forms of the polypeptide (e. g., a proprotein), naturally-occurring variant forms (e. g., alternatively spliced forms) and naturally-occurring allelic variants of the polypeptide. Serpin Spn4A variants disclosed herein include, but are not limited to, those described in Brüning et al., 2007 (27).

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a query amino acid sequence of the present invention, it is intended that the amino acid sequence of the subject polypeptide is identical to the query sequence except that the subject polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the query amino acid sequence. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a query amino acid sequence, up to 5% (5 of 100) of the amino acid residues in the subject sequence may be inserted, deleted, or substituted with another amino acid.

In the frame of the present application, the percentage of identity is calculated using a global alignment (i.e., the two sequences are compared over their entire length). Methods for comparing the identity and homology of two or more sequences are well known in the art. The "needle" program, which uses the Needleman-Wunsch global alignment algorithm (Needleman and Wunsch, 1970 J. Mol. Biol. 48:443-453) to find the optimum alignment (including gaps) of two sequences when considering their entire length, may for example be used. The needle program is for example available on the ebi.ac.uk world wide web site. The percentage of identity in accordance with the invention is preferably calculated using the EMBOSS::needle (global) program with a "Gap Open" parameter equal to 10.0, a “Gap Extend” parameter equal to 0.5, and a Blosum62 matrix.

Polypeptides consisting of an amino acid sequence "at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical" to a reference sequence may comprise mutations such as deletions, insertions and/or substitutions compared to the reference sequence. The polypeptide consisting of an amino acid sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to a reference sequence may correspond to an allelic variant of the reference sequence. It may for example only comprise substitutions compared to the reference sequence. The substitutions preferably correspond to conservative substitutions as indicated in the table below.

Conservative substitutions	Type of Amino Acid
Ala, Val, Leu, lle, Met, Pro, Phe, Trp	Amino acids with aliphatic hydrophobic side chains
Ser, Tyr, Asn, Gln, Cys	Amino acids with uncharged but polar side chains
Asp, Glu	Amino acids with acidic side chains
Lys, Arg, His	Amino acids with basic side chains
Gly	Neutral side chain

As used herein, the term "biologically active derivatives of the Serpin Spn4A polypeptide" includes the variants and the fragments of the polypeptide to which it refers (i.e. the Serpin Spn4A polypeptide) and that retain the biological activity and the specificity of the parent polypeptide. Therefore, the "derivatives of the Serpin Spn4A polypeptide" include variants and fragments of the polypeptide represented by SEQ ID NO: 1.

A polypeptide "fragment", as used herein, refers to a biologically active polypeptide that is shorter than a reference polypeptide (e.g. a fragment of the Serpin Spn4A polypeptide). Thus, the polypeptide according to the invention encompasses polypeptides comprising or consisting of fragments of Spn4A, provided the fragments are biologically active. In the frame of the invention, the biologically active fragment may for example comprise at least 15, 25, 50, 75, 100, 150 200, 250, 300 or 350 consecutive amino acids of the Spn4A polypeptide.

A "biologically active derivative polypeptide" of the Serpin Spn4A polypeptide refers to a polypeptide exhibiting at least one, preferably all, of the biological activities of the reference polypeptide, provided the biologically active derivative retains the capacity of reducing malignant phenotype of cancer cells and/or reducing the tumor growth and/or increasing the chemosensitivity. The biologically active derivative may for example be characterized in that it is capable of inhibiting the proteolytic activity of PCs such as Furin, PACE4, PC5A, PC5B and/or PC7 (see Example "Measurement of proprotein convertases activity"). Such proteolytic activity may for instance be assessed *in vitro* by conventional techniques such as activity assays using fluorogenic substrates (e.g. the fluorogeneic peptide pERTKR-MCA) as previously described in Lalou et al. 2010). Preferably, said inhibition of proteolytic activity has to be of at least 50%, preferably at least 70%, more preferably at least

80%, and even more preferably at least 90% of the proteolytic activity of the parent polypeptide.

The skilled in the art can easily determine whether a derivative of the Serpin Spn4A polypeptide is biologically active. To check whether the newly generated polypeptides reduce the tumorigenicity of cancer cells and/or increase the chemosensitivity in the same way than the initially characterized polypeptide Spn4A, a proteolytic activity assay (see in Example), a cell proliferation assay (see in Example), a cell migration assay (see in Example), a cell invasion assay (see in Example), an apoptosis assay (see in Example) may be performed with each polypeptide. Additionally, a time-course and a dose-response performed *in vitro* or *in vivo* (e.g. by using a liver metastasis assay as described in the Examples section) will determine the optimal conditions for each polypeptide.

In one embodiment, the polypeptides of the invention may comprise a tag. A tag is an epitope-containing sequence which can be useful for the purification of the polypeptides. It is attached to by a variety of techniques such as affinity chromatography, for the localization of said polypeptide within a cell or a tissue sample using immunolabeling techniques, the detection of said polypeptide by immunoblotting etc. Examples of tags commonly employed in the art are the GST (glutathion-S-transferase)-tag, the FLAG™-tag, the Strep-tag™, V5 tag, myc tag, His tag (which typically consists of six histidine residues), etc.

In another embodiment, the polypeptides of the invention may comprise chemical modifications improving their stability and/or their biodisponibility. Such chemical modifications aim at obtaining polypeptides with increased protection of the polypeptides against enzymatic degradation *in vivo*, and/or increased capacity to cross membrane barriers, thus increasing its half-life and maintaining or improving its biological activity. Any chemical modification known in the art can be employed according to the present invention. Such chemical modifications include but are not limited to:

- replacement(s) of an amino acid with a modified and/or unusual amino acid, e.g. a replacement of an amino acid with an unusual amino acid like Nle, Nva or Orn; and/or
- modifications to the N-terminal and/or C-terminal ends of the peptides such as e.g. N-terminal acylation (preferably acetylation) or desamination, or modification of the C-terminal carboxyl group into an amide or an alcohol group;
- modifications at the amide bond between two amino acids: acylation (preferably

acetylation) or alkylation (preferably methylation) at the nitrogen atom or the alpha carbon of the amide bond linking two amino acids;

- modifications at the alpha carbon of the amide bond linking two amino acids such as e.g. acylation (preferably acetylation) or alkylation (preferably methylation) at the alpha carbon of the amide bond linking two amino acids.
- chirality changes such as e.g. replacement of one or more naturally occurring amino acids (L enantiomer) with the corresponding D-enantiomers;
- retro-inversions in which one or more naturally-occurring amino acids (L-enantiomer) are replaced with the corresponding D-enantiomers, together with an inversion of the amino acid chain (from the C-terminal end to the N-terminal end);
- azapeptides, in which one or more alpha carbons are replaced with nitrogen atoms; and/or
- betapeptides, in which the amino group of one or more amino acid is bonded to the β carbon rather than the α carbon.

In another embodiment, adding dipeptides can improve the penetration of a circulating agent in the eye through the blood retinal barrier by using endogenous transporters.

Another strategy for improving drug viability is the utilization of water-soluble polymers. Various water-soluble polymers have been shown to modify biodistribution, improve the mode of cellular uptake, change the permeability through physiological barriers; and modify the rate of clearance from the body. To achieve either a targeting or sustained-release effect, water-soluble polymers have been synthesized that contain drug moieties as terminal groups, as part of the backbone, or as pendent groups on the polymer chain.

Polyethylene glycol (PEG) has been widely used as a drug carrier, given its high degree of biocompatibility and ease of modification. Attachment to various drugs, proteins, and liposomes has been shown to improve residence time and decrease toxicity. PEG can be coupled to active agents through the hydroxyl groups at the ends of the chain and via other chemical methods; however, PEG itself is limited to at most two active agents per molecule. In a different approach, copolymers of PEG and amino acids were explored as novel biomaterials which would retain the biocompatibility properties of PEG, but which would have the added advantage of numerous attachment points per molecule (providing greater drug loading), and which could be synthetically designed to suit a variety of applications.

Those of skill in the art are aware of PEGylation techniques for the effective modification of drugs. For example, drug delivery polymers that consist of alternating polymers of PEG and tri-functional monomers such as lysine have been used by VectraMed (Plainsboro, N.J.). The PEG chains (typically 2000 daltons or less) are linked to the a- and e-amino groups of lysine through stable urethane linkages. Such copolymers retain the desirable properties of PEG, while providing reactive pendent groups (the carboxylic acid groups of lysine) at strictly controlled and predetermined intervals along the polymer chain. The reactive pendent groups can be used for derivatization, cross-linking, or conjugation with other molecules. These polymers are useful in producing stable, long-circulating pro-drugs by varying the molecular weight of the polymer, the molecular weight of the PEG segments, and the cleavable linkage between the drug and the polymer. The molecular weight of the PEG segments affects the spacing of the drug/linking group complex and the amount of drug per molecular weight of conjugate (smaller PEG segments provides greater drug loading). In general, increasing the overall molecular weight of the block co-polymer conjugate will increase the circulatory half-life of the conjugate. Nevertheless, the conjugate must either be readily degradable or have a molecular weight below the threshold-limiting glomular filtration (e.g., less than 60 kDa).

In addition, to the polymer backbone being important in maintaining circulatory half-life, and biodistribution, linkers may be used to maintain the therapeutic agent in a pro-drug form until released from the backbone polymer by a specific trigger, typically enzyme activity in the targeted tissue. For example, this type of tissue activated drug delivery is particularly useful where delivery to a specific site of biodistribution is required and the therapeutic agent is released at or near the site of pathology. Linking group libraries for use in activated drug delivery are known to those of skill in the art and may be based on enzyme kinetics, prevalence of active enzyme, and cleavage specificity of the selected disease-specific enzymes. Such linkers may be used in modifying the polypeptides described herein for therapeutic delivery.

In still another embodiment, the polypeptides of the invention may be fused to a heterologous polypeptide (i.e. polypeptide derived from an unrelated protein, for example, from an immunoglobulin protein).

As used herein, the terms "fused" and "fusion" are used interchangeably. These terms refer to the joining together of two more elements or components, by whatever means

including chemical conjugation or recombinant means. An "in-frame fusion" refers to the joining of two or more polynucleotide open reading frames (ORFs) to form a continuous longer ORF, in a manner that maintains the correct translational reading frame of the original ORFs. Thus, a recombinant fusion protein is a single protein containing two or more segments that correspond to polypeptides encoded by the original ORFs (which segments are not normally so joined in nature. Although the reading frame is thus made continuous throughout the fused segments, the segments may be physically or spatially separated by, for example, in-frame linker sequence.

As used herein, the term "fusion protein" means a protein comprising a first polypeptide linearly connected, via peptide bonds, to a second, polypeptide.

As used herein, the term "Spn4A fusion protein" refers to a polypeptide comprising the Spn4A polypeptide or a derivative thereof fused to heterologous polypeptide. The Spn4A fusion protein will generally share at least one biological property in common with the Spn4A polypeptide (as described above).

An example of a Spn4A fusion protein is a Spn4A immunoadhesin.

As used herein, the term "immunoadhesin" designates antibody-like molecules which combine the binding specificity of a heterologous protein (an "adhesin") with the effector functions of immunoglobulin constant domains. Structurally, the immunoadhesins comprise a fusion of an amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site of an antibody (i.e., is "heterologous"), and an immunoglobulin constant domain sequence. The adhesin part of an immunoadhesin molecule typically is a contiguous amino acid sequence comprising at least the binding site of a receptor or a ligand. The immunoglobulin constant domain sequence in the immunoadhesin may be obtained from any immunoglobulin, such as IgG-1, IgG-2, IgG-3, or IgG-4 subtypes, IgA (including IgA-1 and IgA-2), IgE, IgD or IgM.

The immunoglobulin sequence preferably, but not necessarily, is an immunoglobulin constant domain (Fc region). Immunoadhesins can possess many of the valuable chemical and biological properties of human antibodies. Since immunoadhesins can be constructed from a human protein sequence with a desired specificity linked to an appropriate human immunoglobulin hinge and constant domain (Fc) sequence, the binding specificity of interest can be achieved using entirely human components. Such

immunoadhesins are minimally immunogenic to the patient, and are safe for chronic or repeated use. In one embodiment, the Fc region is a native sequence Fc region. In another embodiment, the Fc region is a variant Fc region. In still another embodiment, the Fc region is a functional Fc region. The Spn4A portion and the immunoglobulin sequence portion of the Spn4A immunoadhesin may be linked by a minimal linker. The immunoglobulin sequence preferably, but not necessarily, is an immunoglobulin constant domain. The immunoglobulin moiety in the chimeras of the present invention may be obtained from IgG1, IgG2, IgG3 or IgG4 subtypes, IgA, IgE, IgD or IgM, but preferably IgG1 or IgG3.

As used herein, the term "Fc region" is used to define a C-terminal region of an immunoglobulin heavy chain, including native sequence Fc regions and variant Fc regions. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof.

A "functional Fc region" possesses an "effector function" of a native sequence Fc region. Exemplary "effector functions" include Clq binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc.

A "native sequence Fc region" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. Native sequence human Fc regions include a native sequence human IgGi Fc region (non-A and A allotypes); native sequence human IgG2 Fc region; native sequence human IgG3 Fc region; and native sequence human IgG4 Fc region as well as naturally occurring variants thereof.

A "variant Fc region" comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one amino acid modification, preferably one or more amino acid substitution(s). Preferably, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or to the Fc region of a parent polypeptide, e.g. from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide. The variant Fc region herein will preferably possess at least about 80% homology with a native sequence Fc region and/or with an Fc region of a parent polypeptide, and most preferably at least about 90% homology therewith, more preferably at least about 95% homology therewith.

Another example of a Spn4A fusion protein is a fusion of the Spn4A polypeptide with human serum albumin -binding domain antibodies (AlbudAbs) according to the AlbudAb™ Technology Platform as described in Konterman et al. 2012 AlbudAb™ Technology Platform–Versatile Albumin Binding Domains for the Development of Therapeutics with Tunable Half-Lives

The polypeptides of the invention may be produced by any suitable means, as will be apparent to those of skill in the art. In order to produce sufficient amounts of a Spn4a or functional equivalents thereof, or a Spn4A fusion protein such as a Spn4A immunoadhesin for use in accordance with the invention, expression may conveniently be achieved by culturing under appropriate conditions recombinant host cells containing the polypeptide of the invention. Preferably, the polypeptide is produced by recombinant means, by expression from an encoding nucleic acid molecule. Systems for cloning and expression of a polypeptide in a variety of different host cells are well known.

When expressed in recombinant form, the polypeptide is preferably generated by expression from an encoding nucleic acid in a host cell. Any host cell may be used, depending upon the individual requirements of a particular system. Suitable host cells include bacteria mammalian cells, plant cells, yeast and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells. HeLa cells, baby hamster kidney cells and many others. Bacteria are also preferred hosts for the production of recombinant protein, due to the ease with which bacteria may be manipulated and grown. A common, preferred bacterial host is *E coli.*

Moreover, it should be noted that the majority of protein-based biopharmaceuticals bare some form of post-translational modification which can profoundly affect protein properties relevant to their therapeutic application. Protein glycosylation represents the most common modification (about 50% of human proteins are glycosylated). Glycosylation can introduce considerable heterogeneity into a protein composition through the generation of different glycan structures on the proteins within the composition. Such glycan structures are made by the action of diverse enzymes of the glycosylation machinery as the glycoprotein transits the Endoplasmatic Reticulum (ER) and the Golgi-Complex (glycosylation cascade). The nature of the glycan structure(s) of a protein has impact on the protein's folding, stability,

life time, trafficking, pharmaco-dynamics, pharmacokinetics and immunogenicity. The glycan structure has great impact on the protein's primary functional activity. Glycosylation can affect local protein structure and may help to direct the folding of the polypeptide chain. One important kind of glycan structures are the so called N-glycans. They are generated by covalent linkage of an oligosaccharide to the amino (N)-group of asparagin residues in the consensus sequence NXS/T of the nascent polypeptide chain. N-glycans may further participate in the sorting or directing of a protein to its final target: the N-glycan of an antibody, for example, may interact with complement components. N-glycans also serve to stabilize a glycoprotein, for example, by enhancing its solubility, shielding hydrophobic patches on its surface, protecting from proteolysis, and directing intra-chain stabilizing interactions. Glycosylation may regulate protein half-life, for example, in humans the presence of terminal sialic acids in N-glycans may increase the half-life of proteins, circulating in the blood stream.

As used herein, the term "glycoprotein" refers to any protein having one or more N-glycans attached thereto. Thus, the term refers both to proteins that are generally recognized in the art as a glycoprotein and to proteins which have been genetically engineered to contain one or more N-linked glycosylation sites. As used herein, the terms "N-glycan" and "glycoform" are used interchangeably and refer to an N-linked oligosaccharide, for example, one that is attached by an asparagine-N- acetylglucosamine linkage to an asparagine residue of a polypeptide. N-linked glycoproteins contain an N-acetylglucosamine residue linked to the amide nitrogen of an asparagine residue in the protein. The predominant sugars found on glycoproteins are glucose, galactose, mannose, fucose, N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc) and sialic acid (e.g., N- acetyl-neuraminic acid (NANA)). The processing of the sugar groups occurs co-translationally in the lumen of the ER and continues post-translationally in the Golgi apparatus for N~linked glycoproteins.

A number of yeasts, for example, Pichia pastoris, Yarrowia lipolytica and Saccharomyces cerevisiae are recently under development to use the advantages of such systems but to eliminate the disadvantages in respect to glycosylation. Several strains are under genetical development to produce defined, human-like glycan structures on a protein. Methods for genetically engineering yeast to produce human-like N- glycans are described in U.S. Patent Nos. 7,029,872 and 7,449,308 along with methods described in U.S. Published Application Nos. 20040230042, 20050208617, 20040171826, 20050208617, and

20060286637. These methods have been used to construct recombinant yeast that can produce therapeutic glycoproteins that have predominantly human-like complex or hybrid N- glycans thereon instead of yeast type N-glycans. As previously described, human-like glycosylation is primarily characterized by "complex" N-glycan structures containing N-acetylglusosamine, galactose, fucose and/or N-acetylneuraminic acid. Thus, several strains of yeasts have been genetically engineered to produce glycoproteins comprising one or more human-like complex or human-like hybrid N-glycans such as GlcNAcMan3GlcNAc2.

Alternatively, a nucleic acid encoding a polypeptide of the invention (such as the Serpin Spn4A polypeptide as shown in SEQ ID NO: 1) or a vector comprising such nucleic acid or a host cell comprising such expression vector may be used in the prevention or treatment of cancer and related metastases.

Accordingly, another aspect of the invention relates to a nucleic acid encoding an amino acids sequence comprising SEQ ID NO: 1 as described here above for use in the prevention or treatment of cancer and related metastases.

In one embodiment, said nucleic acid encoding an amino acid sequence consisting on SEQ ID NO: 1.

Nucleic acids of the invention may be produced by any technique known per se in the art, such as, without limitation, any chemical, biological, genetic or enzymatic technique, either alone or in combination(s).

Another aspect of the invention is an expression vector comprising a nucleic acid sequence encoding an amino sequence comprising SEQ ID NO: 1 as described here above for use in the prevention or treatment of cancer and related metastases.

In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of a nucleic acid to the cells and preferably to cancerous cells. Preferably, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the nucleic acid sequences of interest. Viral vectors are a preferred type of vector and include, but are not

limited to nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rouse sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses (e.g., lentivirus), the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes *in vivo*. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in KRIEGLER (A Laboratory Manual," W.H. Freeman C.O., New York, 1990) and in MURRY ("Methods in Molecular Biology," vol.7, Humana Press, Inc., Cliffton, N.J., 1991).

Preferred viruses for certain applications are the adeno-viruses and adeno-associated viruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy. The adeno-associated virus can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that

the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well known to those of skill in the art. See e.g., SANBROOK et al., "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells *in vivo*. They are particularly advantageous for this because they do not have the same safety concerns as with many of the viral vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUCl9, pRC/CMV, SV40, and pBlueScript. Other plasmids are well known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, intradermal, subcutaneous, or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold particles or in association with another DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

Another aspect of the invention is a host cell comprising an expression vector as described here above for use in the prevention or treatment of cancer and related metastases.

According to the invention, examples of host cells that may be used are human dendritic cells or monocytes (particularly those obtained from the subject to be treated).

The means by which the vector carrying the gene may be introduced into the cells include, but are not limited to, microinjection, electroporation, transduction, or transfection using DEAE-dextran, lipofection, calcium phosphate or other procedures known to one skilled in the art.

In a particular embodiment, polypeptides, nucleic acids, expression vector or host cells of the invention are used advantageously for the prevention or treatment of cancers and related metastases including, but not limited to, carcinoma, including that of the bladder, breast, colon, kidney, liver, lung, ovary, prostate, pancreas, stomach, cervix, thyroid and skin, including squamous cell carcinoma; hematopoietic tumors of lymphoid lineage, including leukemia, acute lymphocytic leukemia, acute lymphoblastic leukemia, B-cell lymphoma, T-cell lymphoma, Hodgkins lymphoma, non-Hodgkins lymphoma, hairy cell lymphoma and Burketts lymphoma; hematopoietic tumors of myeloid lineage, including acute and chronic myelogenous leukemias and promyelocytic leukemia; tumors of mesenchymal origin, including fibrosarcoma and rhabdomyoscarcoma; other tumors, including neuroblastoma and glioma; tumors of the central and peripheral nervous system, including astrocytoma, neuroblastoma, glioma, and schwannomas; tumors of mesenchymal origin, including fibrosarcoma, rhabdomyoscaroma, and osteosarcoma; and other tumors, including melanoma, xeroderma pigmentosum, keratoacanthoma, seminoma, thyroid follicular cancer and teratocarcinoma.

By "metastasis" or "tumor metastasis" is meant the spread of cancer from its primary site to other places in the body. Cancer cells can break away from a primary tumor, penetrate into lymphatic and blood vessels, circulate through the bloodstream, and grow in a distant focus (metastasize) in normal tissues elsewhere in the body. Metastasis can be local or distant. Metastasis is a sequential process, contingent on tumor cells breaking off from the primary tumor, traveling through the bloodstream or lymphatics, and stopping at a distant site. At the new site, the cells establish a blood supply and can grow to form a life-threatening mass. In certain embodiments, the term metastatic tumor refers to a tumor that is capable of metastasizing, but has not yet metastasized to tissues or organs elsewhere in the body. In certain embodiments, the term metastatic tumor refers to a tumor that has metastasized to tissues or organs elsewhere in the body.

By "primary tumor" or "primary cancer" is meant the original cancer and not a metastatic lesion located in another tissue, organ, or location in the subject's body.

In one particular embodiment, the cancer is a colorectal cancer. Accordingly, in one preferred embodiment, the related metastases are liver colorectal metastases.

In another particular embodiment, the cancer is a skin cancer (e.g. melanoma). Accordingly, in one preferred embodiment, the related metastases are pulmonary melanoma metastases. In still another particular embodiment, the cancer is a breast cancer. Accordingly, in one preferred embodiment, the related metastases are pulmonary breast metastases.

Another aspect of the invention relates to a method for preventing or treating cancer and related metastases comprising administering to a subject in need thereof a therapeutically effective amount of a polypeptide or a derivative thereof as described above, or a nucleic acid of the invention, or an expression vector of the invention or a host cell of the invention.

In one embodiment, the invention relates to a method for preventing or treating cancer and related metastases comprising administering to a subject in need thereof a therapeutically effective amount of a polypeptide of SEQ ID NO: 1 as above described.

In one embodiment, the cancer is a colorectal cancer. Accordingly, in one particular embodiment, the related metastases are liver colorectal metastases.

As used herein, the term "therapeutically effective amount" is intended for a minimal amount of active agent, which is necessary to impart therapeutic benefit to a subject. For example, a "therapeutically effective amount of the active agent" to a subject is an amount of the active agent that induces, ameliorates or causes an improvement in the pathological symptoms, disease progression, or physical conditions associated with the disease affecting the subject.

As used herein, the term "treating" a disorder or a condition refers to reversing, alleviating or inhibiting the process of one or more symptoms of such disorder or condition.

As used herein, the term "preventing" a disorder or a condition refers to keeping from occurring, or to hinder, defend from, or protect from the occurrence of a disorder or a condition or phenotype, including a symptom.

As used herein, the term "subject" denotes a mammal, such as a rodent, a feline, a canine, and a primate. Preferably, a subject according to the invention is a human.

In one embodiment, the subject in need thereof may have developed or be at risk for developing metastasis (e.g. affected with a cancer such as a colorectal cancer).

Pharmaceutical compositions:

Another aspect of the invention is a pharmaceutical composition comprising:

a) an isolated Serpin Spn4A polypeptide or a biologically active derivative thereof according to the invention; or
b) an acid nucleic according to the invention; or
c) an expression vector according to the invention; or
d) an host cell according to the invention;
e) and a pharmaceutically acceptable carrier.

In one embodiment, said pharmaceutical composition comprises an isolated Serpin Spn4A polypeptide having the sequence SEQ ID NO: 1.

Any therapeutic agent of the invention as above described may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

"Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

The form of the pharmaceutical compositions, the route of administration, the dosage and the regimen naturally depend upon the condition to be treated, the severity of the illness, the age, weight, and sex of the patient, etc.

The pharmaceutical compositions of the invention can be formulated for a topical, oral, intranasal, intraocular, intravenous, intramuscular or subcutaneous administration and the like.

Preferably, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry,

especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

The doses used for the administration can be adapted as a function of various parameters, and in particular as a function of the mode of administration used, of the relevant pathology, or alternatively of the desired duration of treatment. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Preferably, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the symptomatic adjustment of the dosage to the subject to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, preferably from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

To prepare pharmaceutical compositions, an effective amount of a polypeptide or a nucleic acid according to the invention may be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The polypeptides thereof or the nucleic acid according to the invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or

such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetables oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The preparation of more, or highly concentrated solutions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small tumor area.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution may be suitably buffered and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous,

intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

In addition to the compounds formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; time release capsules; and any other form currently used.

Pharmaceutical compositions of the invention may comprise an additional therapeutic agent.

In one embodiment, said additional therapeutic active agent is a chemotherapeutic agent.

Non-limiting examples of chemotherapeutic compounds which can be used in combination treatments of the present invention include, for example, conventional chemotherapeutic, radiotherapeutic and anti-angiogenic agents.

In one particular embodiment, said chemotherapeutic agent is a tyrosine kinase inhibitor (TKI).

A number of other TKIs are in late and early stage development for treatment of various types of cancer. Examplary TKIs include, but are not limited to: BAY 43-9006 (sorafenib, Nexavar®) and SU11248 (sunitinib, Sutent®), Imatinib mesylate (Gleevec®, Novartis); Gefitinib (Iressa®, AstraZeneca); Erlotinib hydrochloride (Tarceva®, Genentech); Vandetanib (Zactima®, AstraZeneca), Tipifarnib (Zarnestra®, Janssen-Cilag); Dasatinib (Sprycel®, Bristol Myers Squibb); Lonafarnib (Sarasar®, Schering Plough); Vatalanib succinate (Novartis, Schering AG); Lapatinib (Tykerb®, GlaxoSmithKline); Nilotinib (Novartis); Lestaurtinib (Cephalon); Pazopanib hydrochloride (GlaxoSmithKline); Axitinib (Pfizer); Canertinib dihydrochloride (Pfizer); Pelitinib (National Cancer Institute, Wyeth); Tandutinib (Millennium); Bosutinib (Wyeth); Semaxanib (Sugen, Taiho); AZD-2171

(AstraZeneca); VX-680 (Merck, Vertex); EXEL-0999 (Exelixis); ARRY-142886 (Array BioPharma, AstraZeneca); PD-0325901 (Pfizer); AMG-706 (Amgen); BIBF-1120 (Boehringer Ingelheim); SU-6668 (Taiho); CP-547632 (OSI); (AEE-788 (Novartis); BMS-582664 (Bristol-Myers Squibb); JNK-401 (Celgene); R-788 (Rigel); AZD-1152 HQPA (AstraZeneca); NM-3 (Genzyme Oncology); CP-868596 (Pfizer); BMS-599626 (Bristol-Myers Squibb); PTC-299 (PTC Therapeutics); ABT-869 (Abbott); EXEL-2880 (Exelixis); AG-024322 (Pfizer); XL-820 (Exelixis); OSI-930 (OSI); XL-184 (Exelixis); KRN-951 (Kirin Brewery); CP-724714 (OSI); E-7080 (Eisai); HKI-272 (Wyeth); CHIR-258 (Chiron); ZK-304709 (Schering AG); EXEL-7647 (Exelixis); BAY-57-9352 (Bayer); BIBW-2992 (Boehringer Ingelheim); AV-412 (AVEO); YN-968D1 (Advenchen Laboratories); Staurosporin, Midostaurin (PKC412, Novartis); Perifosine (AEterna Zentaris, Keryx, National Cancer Institute); AG-024322 (Pfizer); AZD-1152 (AstraZeneca); ON-01910Na (Onconova); and AZD-0530 (AstraZeneca).

Chemotherapeutic agents have different modes of actions, for example, by influencing either DNA or RNA and interfering with cell cycle replication. Examples of chemotherapeutic agents that act at the DNA level or on the RNA level are anti-metabolites (such as Azathioprine, Cytarabine, Fludarabine phosphate, Fludarabine, Gemcitabine, cytarabine, Cladribine, capecitabine 6-mercaptopurine, 6-thioguanine, methotrexate, 5-fluoroouracil and hyroxyurea; alkylating agents (such as Melphalan, Busulfan, Cis-platin, Carboplatin, Cyclophosphamide, Ifosphamide, Dacarabazine, Procarbazine, Chlorambucil, Thiotepa, Lomustine, Temozolamide); anti-mitotic agents (such as Vinorelbine, Vincristine, Vinblastine, Docetaxel, Paclitaxel); topoisomerase inhibitors (such as Doxorubincin, Amsacrine, Irinotecan, Daunorubicin, Epirubicin, Mitomycin, Mitoxantrone, Idarubicin, Teniposide, Etoposide, Topotecan); antibiotics (such as actinomycin and bleomycin); asparaginase; anthracyclines or taxanes.

Additional chemotherapeutic agent may be selected from, but are not limited to, cytokines, chemokines, growth factors, growth inhibitory factors, hormones, soluble receptors, decoy receptors, monoclonal or polyclonal antibodies, mono-specific, bi-specific or multi-specific antibodies, monobodies, polybodies.

Exemplary biologics drugs include, but are not limited to: anti-angiogenic agents such as Bevacuzimab (mAb, inhibiting VEGF-A, Genentech); IMC-1121B (mAb, inhibiting

VEGFR-2, ImClone Systems); CDP-791 (Pegylated DiFab,VEGFR-2, Celltech); 2C3 (mAb, VEGF-A, Peregrine Pharmaceuticals); VEGF-trap (soluble hybrid receptor VEGF-A, PlGF (placenta growth factor) Aventis/Regeneron).

In another aspect, the invention also relates to a kit-of-part composition comprising a polypeptide or a derivative thereof, or a nucleic acid, or a vector, or a host cell according to the invention and an additional therapeutic active agent.

In one embodiment, said additional therapeutic active agent is a chemotherapeutic agent as described above (e.g. a tyrosine kinase inhibitor).

Another aspect of the invention is a pharmaceutical composition for use in the prevention or treatment of cancer and related metastases comprising an isolated Serpin Spn4A polypeptide or a biologically active derivative thereof according to the invention; or an acid nucleic according to the invention; or an expression vector according to the invention; or an host cell according to the invention and a pharmaceutically acceptable carrier.

In another aspect, the invention also relates to a kit-of-part composition comprising an isolated Serpin Spn4A polypeptide or a derivative thereof, or a nucleic acid, or a vector, or a host cell according to the invention and a further therapeutic active agent for simultaneous, separate or sequential use in the prevention or treatment of cancer and related metastases.

The terms "kit", "product" or "combined preparation", as used herein, define especially a "kit of parts" in the sense that the combination partners as defined above can be dosed independently or by use of different fixed combinations with distinguished amounts of the combination partners, i.e. simultaneously or at different time points. The parts of the kit of parts can then, e.g., be administered simultaneously or chronologically staggered, that is at different time points and with equal or different time intervals for any part of the kit of parts. The ratio of the total amounts of the combination partners to be administered in the combined preparation can be varied. The combination partners can be administered by the same route or by different routes. When the administration is sequential, the first partner may be for instance administered 1, 2, 3, 4, 5, 6, 12, 18 or 24 h before the second partner.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1: Inhibition by Spn4A of cellular Furin, PACE4, PC5A, PC5B and PC7 activity. (**A**), Effect of PCs expression on PDGF-A precursor (ProPDGF-A) processing in PC-deficient CHOFD11 cells. ProPDGF-A processing was analyzed by Western blotting of conditioned media obtained from CHOFD11 transfected with either empty vectors (lane 1), empty pIRES2-EGFP vector and pIRES2-EGFP vector containing proPDGF-A (lane 2), or with pIRES2-EGFP vector containing proPDGF-A and pIRES2-EGFP vector that expresses full-length PCs (Lanes 3-7). An anti-V5 antibody was used for detection. Note that all indicated PCs are able to rescue proPDGF-A processing in these PC-deficient cells. (**B**) Effect of Spn4A on PC-mediated proPDGF-A processing. CHOFD11 cells stably transfected with each of the indicated PCs were transiently transfected with either the empty vectors (lane 1), empty pIRES2-EGFP vector and pIRES2-EGFP vector containing proPDGF-A (lanes 2, 4, 6, 8, 10) or with the pIRES2-EGFP vector containing proPDGF-A and pIRES2-EGFP-V5 vector that expresses full-length Spn4A (Lanes 3, 5, 7, 9, 11). Note that Spn4A expression inhibited proPDGF-A processing mediated by PCs. Bars denote the corresponding percentages of pro-PDGF-A processing. Values are mean ± SD (n = 3 per group). (**C**). Effect of Spn4A on human recombinant Furin was assessed by evaluating Furin (0.2x10-4 U) ability to digest the fluorogenic peptide pERTKR-MCA at the indicated time points. (**D**) Results shown in the bar graph represent Furin activity after 20 min of incubation. Results are representative of three experiments and data are mean ± S.D performed in triplicate. **$P < 0.001$. *** $P < 0.0001$.

Figure 2: Inhibition of endogenous PCs activity in colon cancer cells by Spn4A. (**A-B**), Using specific primers, the expression of Furin, PACE4, PC5A, PC5B and PC7, was assessed by Real time-PCR analysis on RNA extracted from the colon cancer cell line HT-29 (**A**) and CT-26 (**B**). All these PCs were present in HT-29 cells, whereas CT-26 cells lacked the expression of PC5 and PACE4. (**C–F**), Endogenous PCs activity was assessed by ability of indicated cells to process the PC substrates proPDGF-A and proIGF-1 receptor. proPDGF-A and proIGF-1R processing was analyzed by Western blotting using an anti-V5 (**C and D**) and anti–IGF-1R antibody (**E and F**), respectively. Note that in HT-29 and CT-26 cells expressing Spn4A, the processing of these PC substrates is inhibited. Bars denote the

corresponding percentages of pro-PDGF-A and pro-IGF-1R processing. Values are mean ± SD (n = 3 per group). *P < 0.05; **P < 0.001. *** P < 0.0001.

Figure 3: Inhibition of anchorage-independent growth and proliferation of colon cancer cells by Spn4A. (**A-B**), Control (CTL) and tumor cells stably expressing Spn4A (Spn4A) were seeded in triplicate in six-well plates in soft agar as described under "Experimental Procedures". After 2 weeks, colonies ≥100 μm in diameter were counted. Results are shown as means ± S.D. of three experiments performed in triplicate. (**C-D**), Starved control or Spn4A-expressing cells were cultured for 6 days under standard conditions and cell proliferation was assessed using Cell Titer96 non-radioactive cell proliferation assay. Each value results are shown as means ± S.D. of three experiments performed in triplicate. *P < 0.05; **P < 0.001. *** P < 0.0001.

Figure 4: Inhibition of PCs by Spn4A alters tumor cells survival and chemosensitivity. (**A**) Percentages of apoptotic cells, under these conditions are indicated. (**B**), tumor cells were incubated for 6 h with H_2O_2 (5 mM) or staurosporin (1 μM) and caspase-3 activity was evaluated using the Caspase-3 Fluorescence assay Kit. Note that H_2O_2 and staurosporin caused an increased in the percentage of apoptotic cells that are associated with increased caspase-3 activity. This effect was exacerbated in cells-expressing Spn4A. Data shown represents the mean±SD from at least three independent experiments. (**C-D**), Data derived from analysis of total RNA analyzed by RT^2 Profiler PCR array PAHS-502C for oncogenes and tumor suppressor genes that contains probes for *BCL2* and *TNF* genes. For each well, the results are expressed relative to control cells transfected with empty vector which was assigned a value of 1. Values are shown as means ± S.D. *P < 0.05; **P < 0.001. *** P < 0.0001.

Figure 5: Inhibition of tumor growth by Spn4A is associated with reduced PCs activity and induced apoptosis in developed tumors. (**A-B**), Control (CTL) and colon tumor cells stably expressing Spn4A (Spn4A) (1×10^6) were injected subcutaneously into 4-week-old nude mice. The animals were monitored for tumor formation every 2-3 days. Note the smaller size of tumors induced by tumor cells expressing Spn4A. Results are representative of three experiments. Values are mean ± SD (n = 6 per group). *P < 0.05; **P < 0.001. (**C-D**), Subcutaneously developed tumors were removed and lysed in lysis buffer. Protein extracts were incubated with the fluorogenic peptide PC substrate pERTKR-MCA.

Substrate cleavage was evaluated as raw fluorescence intensity (RFI) at indicated time periods. Results shown in the bar graph represent PCs activity after 2 hours of incubation. (**E-F**), Developed tumors derived from control and Spn4A-expressing tumor cells were analyzed for apoptosis using TumorTACS™ in situ apoptosis detection Kit (Trivigen). The number of intra-tumoral apoptotic cells was counted in ten different fields for each tumor. Percentages of apoptotic populations are shown. Data are presented as mean ± SD. ***P < 0.0001. Original magnification × 360.

Figure 6: Effect of Spn4A on tumor cell migration and invasion. (**A-B**), Control tumor cells and cells expressing Spn4A were incubated for 24 hours in a microchemotaxis chamber alone (**A**) or pre-coated with collagen IV (**B**), for cell migration and invasion, respectively. Three independents experiments were done (n = 3) and the results are represented as the percentage of migrating and invading cells. (**C**), Serum-free media derived from the indicated cells were collected and analyzed for gelatinase enzymatic activity. The corresponding percentage of MMP-2 cleavage calculated from the ratio of band intensities of MMP-2/(proMMP-2+MMP-2) is indicated. Note that Spn4A expression inhibited the activity of MMP-2 and reduced processing of pro-MMP-2. Values are mean ± SD (n = 3 per group). **P < 0.001. *** P < 0.0001.

EXAMPLE:

Material & Methods

Cell culture and transfection: The human HT-29 colon adenocarcinoma cell line, murine CT-26 colon adenocarcinoma cells and PC-deficient CHOFD11 cells were maintained in DMEM, RPMI and DMEM/F12 media, respectively, supplemented with 10% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Prior cell transfection, Spn4A cDNA was cloned into SacII/EcoRI-digested pIRES2-EGFP-V5 (6, 7). Cells were stably transfected with empty pIRES2-EGFP-V5 vector (HT-29/CTL, CT-26/CTL and CHOFD11/CTL) or with pIRES2-EGFP-V5 vector containing full-length Spn4A cDNA (HT-29/Spn4A, CT-26/Spn4A and CHOFD11/Spn4A). To generate single-mixture cells expressing Spn4A, HT-29/Spn4A and CT-26/Spn4A cells were cultured in presence of *Pseudomonas* exotoxin A. This toxin induces cells death only after its cleavage by PCs (26). The expression of Spn4A-V5 was assessed by immunoblotting and cells were grown in their adequate media supplemented with 200 µg/ml G418. To evaluate the effect of Spn4A on PC substrates, processing by each of individual PC found in the secretary pathway, CHOFD11

cells were stably transfected with Furin, PACE4, PC5A, PC5B or PC7. In other experiments, CHOFD11, HT-29, CT-26, HT-29/Spn4A and CT-26/Spn4A cells were transiently transfected with empty pIRES2-EGFP-V5 vector or with the same vector containing full-length PDGF-A cDNA to study proPDGF-A processing. All transfections were carried out using Lipofectamine reagent (Invitrogen), as recommended by the manufacturer.

Real-time PCR: Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. One µg of total RNA was subjected to cDNA synthesis using the high capacity cDNA reverse transcription kit (Applied Biosystems, Courtaboeuf, France). The relative quantification of specific mRNAs was performed by real-time PCR using the StepOnePlus™ Real-Time PCR System and PCR Master Mix (Applied Biosystems, Courtaboeuf, France) according to the manufacturer's instructions. Briefly, the reaction mixture (20 µl) contained 2 µl of cDNA resulting of 5-fold dilution of RT mixture product, 2 x TaqMan Universal PCR Master Mix, 0.3 µM of the probe and 0.9 µM of the forward and reverse primers. PCR reactions were performed at 94°C for 15 s and at 60°C for 1 min during 40 cycles. hsB2m and mHPTRT1 transcriptions levels evaluated in each sample, were used as endogenous controls for human and mouse cells, respectively.

RT^{2}Profiler PCR Array: The RT^2 Profiler PCR Array was used as a method of combining real-time PCR performance with a simultaneous analysis of a panel of genes related to human oncogenes and tumor suppressor genes (Array PAHS-502C) and to human extracellular matrix and adhesion molecules (array PAHS-013C). Preparation and analysis of samples were carried out in accordance with the manufacturer's recommendations (SABiosciences).

Immunoblotting: Cells were lysed in Triton buffer (50 mmol/l Tris–HCl (pH=7.4), 250 mmol/l NaCl, 1 mmol/l EDTA (pH=8), 0.1% Triton) containing protease inhibitors (Roche). Media or lysates were subjected to SDS-polyacrylamide gel electrophoresis and proteins were blotted onto nitrocellulose membranes. The primary antibodies used were anti-IGF-I receptor (Santa Cruz Biotechnology) and anti-V5, for detection of PDGF-A-V5 and Spn4A-V5 (Invitrogen), respectively. Primary antibodies were revealed by horseradish peroxidase-conjugated secondary antibodies (Amersham, Pharmacia Biotech) and Enhanced Chemiluminescence (ECL+Plus, Amersham Pharmacia Biotech) according to the manufacturers' instructions.

Measurement of proprotein convertases activity: The effect of Spn4A on PCs activity in cells and tissues was assessed by the evaluation of the enzymes ability to digest the universal PC substrate, the fluorogenic peptide pERTKR-MCA, as previously described (24). In brief, cancer cells, and tumor tissues extracts were incubated with pERTKR-MCA (100 μM) during the indicated time periods in the presence of 25 mM Tris (pH 7.4), 25 mM methyl-ethane-sulfonic acid, and 2.5 mM CaCl2, at 37°C, and the fluorometric measurements were performed using a spectrofluorometer (Tecan Infinite® F200 PRO, Tecan Group Ltd. France).

Proliferation assay: Tumor cells were plated in triplicate on 96 wells plate ($2x10^3$/well) under serum free conditions for 24 h. The starved cells were then cultured during the indicated periods. Proliferation levels of cells was evaluated every two days using the Cell Titer96 non-radioactive cell proliferation assay kit (Promega) according to the manufacture's protocol.

Soft agar assay: To assay anchorage-independent colony formation, HT-29/CTL, CT-26/CTL, HT-29/Spn4A or CT-26/Spn4A cells (4 x 10^3 cells/well) were suspended in complete medium containing 0.8% agar and seeded in triplicate in six-well plates onto a basal layer of complete medium containing 3% agar. Complete medium was added every three days. After two weeks of cell growth, colonies >100 μm in diameter were counted using inverted microscopy and the results were represented as number of colonies formed, as previously described (7).

Apoptosis assays and caspase-3 activity measurement: For apoptosis assays, tumor cells were grown to 70% confluency and cells were washed repeatedly to remove serum and then incubated for 6 h in media containing or not 5 mM H_2O_2 or 1 μM staurosporin. Cells were washed and stained with Phycoerythrin (PE)-labeled annexin V (AN) and 7-amino-actinomycin D (7AAD) using the PE Annexin V Apoptosis Detection Kit I (BD Pharmingen™) as instructed by the manufacturer. Cells were analyzed by flow cytometry (FACS CamptoII). The populations AN-/7AAD-, AN+/7AAD-, AN-/7AAD+, and AN+/7AAD+ that correspond to live cells, early apoptotic cells, necrotic cells and late apoptotic cells, respectively, were enumerated. Caspase-3 activity was determined in control and Spn4A-expressing cells following incubation for 6 h in media containing or not 5 mM

H_2O_2 or 1 μM staurosporin, using the Caspase-3 Fluorescence assay Kit, as described by the manufacture (Cayman Chemical Company).

Cell migration and invasion assays: Cell migration and invasion were determined using 24-well microchemotaxis chambers alone or precoated with 7.5 μg collagen type IV (Becton Dickinson Labware), respectively (7, 25). Control tumor cells or cells expressing Spn4A cDNA were resuspended in serum-free media and loaded into upper chamber of each well. Cells were incubated at 37°C for 24 h, after which, the filters were fixed and stained with Diff-Quik (Medion Diagnostic). Cell migration and invasion were quantified by determination of the number of cells that migrated directly through the membrane toward the medium containing 10% serum that was used as a chemoattractant. Cells detected in each well were counted and the results were represented as (number of migrated cells/number of total cells) x 100%.

Gelatin zymography: Zymography assay was done as previously described (25), on serum-free conditioned media derived from control cells or cells stably transfected with Spn4A cDNA. SDS-PAGE gels were copolymerized with gelatin and samples were loaded onto gels without boiling. The gels were washed at room temperature in renaturing solution and in 10 mM of Tris-HCl (pH 8). For enzymatic reaction to take place, gels were incubated at 37°C in a solution of 50 mM of Tris-HCl (pH 8) containing 10 mM of CaCl2. The gels were then stained in Coomassie blue R250 solution and regions without staining were indicative of gelatin lysis.

Tumorigenicity assay: Ethical approval for all animal studies was obtained from the Institutional Animal Care and Use Committee of the INSERM Institute in accordance with the National Advisory Committee for Laboratory Animal Research Guidelines licensed by the French Authority. Female 4- to 6-week-old nu/nu mice from Janvier Laboratories, housed in a pathogen-free facility, were used for all of the experiments. To assess the effect of PCs inhibition by Spn4A on tumor growth, $1x10^6$ HT-29, CT-26, HT-29/Spn4A, or CT-26/ Spn4A cells were injected subcutaneously into nude mice, tumor formation was monitored every 2-3 days, and mice were sacrificed in the end of the experiments. Tumor volume was calculated as previously described (7, 9).

Liver metastasis assay: Experimental liver metastases were generated by intrasplenic/portal injection of HT-29/CTL, HT-29/ Spn4A, CT-26/CTL or CT-26/Spn4A cells, as previously described (9). Because of high aggressiveness of CT-26 colon cancer cells, CT-26/CTL and CT-26/Spn4A cells-injected nude mice were sacrificed two weeks after the injection, whereas HT-29- and HT-29/Spn4A cells-injected mice were sacrificed four weeks later. Livers were removed and metastases were enumerated, without prior fixation.

Immunohistochemical analyses: Developed tumors derived from injection of HT-29/CTL, HT-29/Spn4A, CT-26/CTL or CT-26/Spn4A cells, were analyzed for in vivo apoptosis using TumorTACS™ In Situ Apoptosis Detection Kit (Trivigen), as instructed by the manufacturer. The assay allows detection of chromosomal DNA fragments in the presence of deoxynucleotidyl transferase (TdT) that incorporated biotinylated nucleotides. The latter are visualised as dark brown precipitate through their biding to streptavidin-horseradish peroxydase and interaction with diaminobenzidine (DAB).

Statistics: Unless otherwise indicated, Student's t test was used to determine the statistical significance of differences between the means of several experiments. A probability value less than 0.05 was considered to be statistically significant.

Results

Inactivation of cellular Furin, PACE4, PC5A, PC5B and PC7 by Spn4A: In contrast to the regulated secretory pathway enzymes PC1 and PC2, to date, there is no naturally occurring inhibitor known that regulates the activity of PCs found in the constitutive secretory pathway (Furin, PACE4, PC5 and PC7). Interestingly, in *Drosophila melanogaster* that encodes two Furin homologs (12, 21), and one PC2-like enzyme (17), the use of *in vitro* enzymatic digestion assays revealed that recombinant *Drosophila melanogaster* Spn4A was able to efficiently inactivate Furin *in vitro* (19, 20). To evaluate the effect of Spn4A on enzymatic activity of all constitutive secretory pathway PCs *in vivo*, the inventors first assessed in cells stably expressing Spn4A, the ability of Furin, PACE4, PC5A, PC5B and PC7 to convert human PDGF-A (tagged with V5 epitope) into the mature form (6). Previously, proPDGF-A was reported to be processed by these PCs (6). Thereby, inhibition of proPDGF-A processing is a good indicator of lack of PCs activity. Using an anti-V5 antibody, immunoblotting analysis revealed that media derived from PC-deficient CHOFD11 cells (23) cotransfected with a vector encoding proPDGF-A and control vector, showed only one band

(~24 kDa) corresponding to the intact proPDGF-A precursor (Figure 1A, Lane 2). Transfection of CHOFD11 cells that stably express Furin, PC5A, PC5B, PC7 or PACE4 with vector encoding proPDGF-A demonstrated reduction of the precursor protein, and the appearance of a ~16 kDa product, corresponding to mature form of PDGF-A (Figure 1A, Lanes 3-7). In contrast, when Spn4 was cotransfected together with pro-PDGF-A processing of pro-PDGF-A was inhibited (Figure 1B, lanes 3, 5, 7, 9, 11). These data revealed that Spn4A is able to repress all PCs found in the constitutive secretory pathway when expressed in cells. Using an in vitro enzymatic digestion assay with the fluorogenic peptide pERTKR-MCA as substrate (24), they confirmed inhibition of PCs by Spn4A. The enzymatic activity of recombinant human Furin (0.2x10-4 U) (Figures 1C, 1D) and of media derived from PC deficient CHOFD11 cells, stably transfected with a vector expressing Furin (FD11/Furin), PACE4 (FD11/PACE4), PC5A (FD11/PC5A), PC5B (FD11/PC5B) or PC7 (FD11/PC7) was inhibited by recombinant Spn4A.

Inhibition of endogenous PCs activity in colon cancer cells by Spn4A: To assess the possibility that the endogenous proteolytic activity of PCs is inhibited by Spn4A, they analysed in colon carcinoma cells HT-29 and CT-26 stably transfected with Spn4A ability to mediate maturation of PC substrates. HT29 cells express all convertases while CT-26 lacks PACE4 and PC5 (Figures 2A, 2B). They next analysed the processing of two PC substrates, PDGF-A (Figures 2C, 2D) and IGF-1R (Figures 2E, 2F). Both cell lines express IGF-1R while lacking PDGF-A expression. Immunoblotting of media derived from cells transfected with proPDGF-A cDNA revealed that proPDGF-A was significantly processed (Figures 2C lane 3, 2D lane 3). In contrast, when Spn4A is expressed, in addition, in these cells, the maturation of proPDGF-A was inhibited, as demonstrated by the accumulation of its unprocessed form (~24 kDa) and a reduction of its mature form (~16 kDa) (Figures 2C lane 4, 2D lanes 4). Similarly, using a specific IGF-1R antibody, they found that the processing of the endogenous proIGF-1R β-subunit was also blocked in HT-29 and CT26 cells expressing Spn4A (Figure 2E-F), as evidenced by the accumulation of the precursor form (~250 kDa) and the reduction of the mature form (~105 kDa) of IGF-1R.

Inhibition of anchorage-independent growth and proliferation of colon cancer cells by Spn4A: They first investigated whether anchorage-independent growth of colon cancer cells was inhibited by Spn4A. Tumor cells expressing Spn4A exhibited more than 80% reduction in their anchorage-independent growth in comparison to control cells (Figures 3A,

3B). Furthermore, control cells but not Spn4 transfected cells grew well in presence of serum. This indicates that cell proliferation is inhibited by Spn4 (Figures. 3C, 3D).

Inhibition of PCs activity by Spn4A alters survival of colon cancer cells and chemosensitivity: The inventors then investigated the effect of Spn4 on apoptosis by flow cytometry in colon cancer cells HT-29 under normal conditions, and after treatment with H_2O_2 (5 mM) or staurosporin (1 μM). Using annexin V and 7AAD as markers, flow cytometric analysis, identified four cell populations: viable (negative for both dyes), early apoptotic (Annexin+/7AAD–), necrotic (Annexin-/7AAD+), and late apoptotic cells (Annexin+/7AAD+). The percentages of these populations under normal condition or after treatment with H_2O_2 or staurosporin, are shown in Figure 4A. As illustrated, incubation of control cells with H_2O_2 or staurosporin for 6h caused an increase in apoptotic cells which was associated with increased Caspase-3 activity (Figure 4B). This effect was exacerbated in tumor cells-expressing Spn4A, indicating a higher chemosensitivity. Similar results were obtained with the colon carcinoma cells CT-26. Real-time PCR array revealed a downregulated expression of the anti-apoptotic gene *BCL2* in Spn4A-expressing tumor cells and an increased expression of the pro-apoptotic gene *TNF* (Figure 4D, Table 2).

Tumorigenicity of Spn4A-expressing colon cancer cells: To assess the effect of PCs inhibition by Spn4A on tumorigenicity of colon cancer cells, four groups of nude mice (n=6) were subcutaneously inoculated with HT-29/CTL, CT-26/CTL, HT-29/Spn4A or CT-26/Spn4A cells. Tumors size was measured at various intervals, and data are summarized in Figures 5A, 5B. Animals injected with Spn4A-expressing tumor cells exhibited a delay in tumor appearance (day 7 for HT-29/Spn4A as compared with day 2 for HT-29/CTL and day 12 for CT-26/Spn4A, as compared with day 5 for CT-26/CTL). Tumor volume, measured at the end of the experiment, was significantly smaller for HT-29/Spn4A and CT-26/Spn4A-derived tumors than for control tumors. In agreement with these finding, the expression of several genes associated with tumor initiation and/or progression including FOXD3, MEN1, and S100a4 was upregulated in Spn4A-expressing cells. However, the expression of some other genes involved in tumorigenesis such as c-REL and JUNB was down regulated in these cells (Table 2).

Reduced PCs activity in colon cancer cells expressing Spn4A-derived tumors: To evaluate the effect of Spn4A on PCs activity in tumors derived from colon cancer cells,

control and Spn4A-expressing colon cancer cells were subcutaneously injected in mice. Tumors were removed 2-4 weeks later. PCs activity was analyzed by assessing the ability of tumor-derived protein extracts to cleave the PCs substrate pERTKR-MCA (24). The results in Figures 5C and 5D revealed that the extent of substrate cleavage by protein extracts of HT-29/CTL- or CT-26/CTL-derived tumors was higher than that of HT-29/Spn4A- or CT-26/Spn4A-derived tumors. A significant reduction of up to 50% of total PCs activity in tumor cells expressing Spn4A-derived tumors was observed.

Immunohistochemical analysis of tumor apoptosis: Apoptotic cells in control and Spn4A-expressing cells-derived tumors were analyzed using TumorTACS™ *in situ* apoptosis detection assay. A marked increase (>5-fold) in apoptotic cells in HT-29/Spn4A- and CT-26/Spn4A cell-derived tumors was observed, as revealed by the increased dark brown precipitate within the cells (Figures 5E, 5F).

Effect of PCs inhibition by Spn4A on migration and invasion of colon cancer cells: To evaluate whether inhibition of PCs in tumor cells can affect cell migration and invasion, cells were incubated for 24 hours in a microchemotaxis chamber alone (Figures 6A), or in a chamber pre-coated with collagen IV (Figure 6B). Expression of Spn4A in these cells clearly decreased their ability to migrate and to invade. Furthermore, gelatinase activity in 48h-conditionned serum free media revealed that media derived from control tumor cells presented high MMP-2 and MMP-9 activity. Expression of Spn4A in these cells resulted in decreased MMP-2 and MMP-9 activity. In Spn4A-expressing cells, processing of MMP-2 was also reduced, as revealed by the accumulation of pro-MMP-2 (Figure 6C). The inventors then analyzed mRNA expression of these MMPs and of their naturally occurring inhibitors TIMP-1 and TIMP-2, using real-time PCR. They found that while expression of MMP-2 and MMP-9 was reduced in Spn4A-expressing tumor cells, mRNA expression levels of TIMP-1 and TIMP-2 was significantly increased. Evaluation of MMPs/TIMPs ratios indicated that compared to control cells, these ratios were significantly decreased in Spn4A-expressing tumor cells. Similarly, since the uPA/uPAR/PAI-1 system is also involved in cell invasion, they analyzed the effect of PCs inhibition by Spn4A on expression levels of these molecules by real-time PCR. In Spn4A-expressing cells, the level of uPA was reduced significantly; PAI-1 in contrast was increased, whereas the level of uPAR mRNA remained unchanged. Evaluation of uPA/PAI-1 ratios indicated that, compared to control cells, these ratios were significantly decreased in Spn4A-expressing tumor cells. Similarly, further analysis revealed

that the expression of other genes involved in extracellular matrix (ECM) degradation including MMP11, MMP12, MMP13, MMP15 and ADAMTS1 was reduced. MMP-8 expression in contrast was increased (Table 2), suggesting the contribution of this global effect of PCs inhibition by Spn4A on cell migration and invasion.

PCs blockade by Spn4A prevents experimental colorectal liver metastasis: To evaluate the effect of PCs inhibition by Spn4A on the ability of colon cancer cells to colonize the liver, HT-29/CTL, CT-26/CTL, HT-29/Spn4A, and CT-26/Spn4A cells, were injected in mice through the intrasplenic/portal route. At 2 weeks after injection of CT-26/CTL and CT-26/Spn4A cells, and 4 weeks after injection of HT-29/CTL and HT-29/Spn4A cells, livers were removed and the number of metastases was determined. In mice inoculated with tumor cells, the number of hepatic metastases was reduced by up to 75% ($P < 0.001$) in HT-29/Spn4A- and CT-26/Spn4A-injected mice relative to control animals (Mann-Whitney test, Table 1). Similarly, PCR Array analysis of ECM proteins and adhesion molecules, known to be involved in invasion and metastasis revealed their reduced expression in Spn4A-expressing cells. These include COL6A1, CTNND2, TNC, HAS1, ITGA4, ICAM1, NCAM1, VCAM1, CLEC3B and LAMA3 (Table 2).

Table 1: Metastasis incidence and prevalence and liver weight

Cell line	Metastasis incidence	No metastases per liver (range)	liver weight (g)
HT-29/CTL	100%	36-185	2.5 1
HT-29/Spn4A	25%	2-3	1.6 0.3
CT-26/CTL	100%	54-535	3.7 1.2
CT-26/Spn4A	25%	7-20	1.9 0.6

PC activity in colon cancer cells-derived hepatic metastases: To evaluate the effect of PCs activity in metastatic livers, tumor cells were injected in mice through the intrasplenic/portal route. After 2 or 4 weeks, the livers were removed and lysed. PCs activity was analyzed by assessing their ability to digest pERTKR-MCA (24). It was shown that PCs activity in HT-29/Spn4A- and CT-26/Spn4A-derived metastases were reduced as compared to HT-29/CTL- and CT-26/CTL-derived metastatic livers.

Table 2: List of genes regulated by Spn4A in the colon carcinoma cells.

Genes listed are those differentially regulated after expression of Spn4A in colon cancer cells HT-29 as compared to control HT-29 cells. The cut-off limit in the analysis was set to two-fold for both induced (+) and repressed (-) genes. The Gene Bank accession numbers are indicated.

Gene Symbol	Gene banque reference	Name	Fonction	Fold regulation	Relevance in cancer
ADAMTS1	NM_006988	ADAM metallopeptidase with thrombospondin type 1 motif, 1	Extramolecular Matrix Proteins:ECM Protease	-7.5	Involved in cell invasion and tumor progression
COL11A1	NM_080629	Collagen, type XI, alpha 1	Extramolecular Matrix Proteins: Collagens and ECM structural constituants; protumoral properties	+2.7	Involved in cell invasion
COL12A1	NM_004370	Collagen, type XII, alpha 1	Extramolecular Matrix Proteins: Collagens and ECM structural constituants; protumoral properties	+3.8	Marker of tumor invasion fronts
COL16A1	NM_001856	Collagen, type XVI, alpha 1	Extramolecular Matrix Proteins: Collagens and ECM	-1.8	Involved in cell invasiveness

			structural constituants; protumoral properties		
CTNND2	NM_001332	Catenin (cadherin-associated protein),delta 2 (neural plakophilin-related arm-repeat protein)	Extramolecular Matrix Proteins: Collagens and ECM structural constituants; protumoral properties	-54.5	Involved in malignant progression and promotes cell invasion
ECM1	NM_004425	Extracellular matrix protein 1	Extramolecular Matrix Proteins ; protumoral and prometastatic properties	-4.4	Implicated in cell proliferation, angiogenesis and differentiation
HAS1	NM_001523	Hyaluronan synthase 1	Extramolecular Matrix Proteins ; protumoral and prometastatic propertises	-13	Facilitate tumor progression by enhancing invasion, growth, angiogenesis and metastasis
ICAM1	NM_000201	Intercellular adhesion molecule 1	Cell adhesion molecules: protumoral propertises	-4.8	Prometastatic effect
ITGA1	NM_181501	Integrin, alpha 1	Cell-Matrix adhesion molecules: protumoral properties	+3.6	Implicated in cell adhesion. Have a high metastatic abilities
ITGA4	NM_000885	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4	Cell-Matrix adhesion molecules: protumoral properties	-9.4	Involved in cell spreading and migration

		receptor)			
LAMA3	NM_000227	Laminin, alpha 3	Extramolecular Matrix Proteins: basement membrane constituent protumoral properties	-13.5	Play a role in tumor progression and metastasis
MMP11	NM_005940	Matrix metallopeptidase 11 (stromelysin 3)	Extramolecular Matrix Proteins: ECM Protease	-5	Associated with tumor progression and poor prognosis
MMP12	NM_002426	Matrix metallopeptidase 12 (macrophage elastase)	Extramolecular Matrix Proteins: ECM Protease	no expression in Spn4A expressing cells	Overexpression correlate with presence of venous infiltration, high serum AFP level, early tumor recurrence and poor overall survival
MMP13	NM_002427	Matrix metallopeptidase 13 (collagenase 3)	Extramolecular Matrix Proteins: ECM Protease	-9.2	Frequently present and active in colorectal cancer. Its activity is associated with poorer survival
MMP15	NM_002428	Matrix metallopeptidase 15 (membrane-inserted)	Extramolecular Matrix Proteins: ECM Protease	-3.4	Seems to be up-regulated during tumorogenesis

MMP8	NM_002424	Matrix metallopeptidase 8 (neutrophil collagenase)	Extramolecular Matrix Proteins: ECM Protease	+11.1	Loss-of-function enhances the progression of melanoma. Expression in breast cancer associated with diminished risk of lymph node metastasis
NCAM1	NM_000615	Neural cell adhesion molecule 1	Cell adhesion molecules: protumoral properties	-10.1	Has anti apoptotic properties, mediate cell-cell cohesion, enabling the formation of cell aggregates
SPARC	NM_003118	Secreted protein, acidic, cysteine-rich (osteonectin)	Cell-Matrix adhesion molecules: anti-tumoral properties	-2.1	Inhibits cell proliferation, spreading and migration
TIMP3	NM_000362	TIMP metallopeptidase inhibitor 3	Extramolecular Matrix Proteins: ECM Protease inhibitor	-12.8	Inhibits tumor growth and cell invasion
CLEC3B	NM_003278	C-type lectin domain family 3, member B	Extramolecular Matrix Proteins: protumoral properties	-117.3	Promote invasion, have a potential role in cell survival
TNC	NM_002160	Tenascin C	Cell adhesion molecules: protumoral properties	-5.8	Promote cancer cell growth, migration, adhesion, invasion and metastasis
VCAM1	NM_001078	Vascular cell adhesion molecule 1	Cell adhesion molecules: protumoral properties	-20.2	Implicated in cell adhesion and metastasis

BCL2	NM_000633	B-cell CLL/lymphoma 2	Tumor suppressor, anti apoptotic properties	-3.9	Anti-apoptotic early expressed in colon carcinoma
ESR1	NM_000125	Estrogen receptor 1	Both oncogene and tumor suppressor properties	+2.3	Hypermethylation in colorectal cancer associated with risk of recurrence
FOXD3	NM_012183	Forkhead box D3	Tumor suppressor	+6.5	Down regule migration and invasion; Required for cell differentiation; Negative cell cycle regulator
JUNB	NM_002229	Jun B proto-oncogene	Oncogene	-2.3	Increase cell proliferation; Induce morphological and anchorage-independant cell growth consistent with a transformation like phenotype
MEN1	NM_000244	Multiple endocrine neoplasia I	Tumor suppressor	+2.4	Tumor suppressor. Mutation leads to cell proliferation
MYB	NM_005375	V-myb myeloblastosis viral oncogene homolog (avian)	Noth oncogene and tumor suppressor propertises	+8.1	Blok differentiation. Regulator of stem and progenitor cells in born marrow
REL	NM_002908	V-rel reticuloendotheliosis viral oncogene homolog	Oncogene	-4	Overexpression contribute to inhibition of growth arrest and

		(avian)			apoptosis of tumor cells
S100A4	NM_002961	S100 calcium binding protein A4	Tumor suppressor	+3.99	Interact with p53 tumor suppressor protein and induce apoptosis
TNF	NM_000594	Tumor necrosis factor	Both oncogene and tumor suppressor properties, anti apoptotic properties	+9.88	Expression is down regulated in high stage colorectal tumors. Stimulate cell death/apoptosis

REFERENCES:

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

1. Thomas G. Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nat Rev Mol Cell Biol* 2002; 3:753-766.
2. Couture F, D'Anjou F, Day R. On the cutting edge of proprotein convertase pharmacology: from molecular concepts to clinical applications. *Biomol Concepts* 2011; 2:421-438.
3. Siegfried G, Basak A, Cromlish JA, Benjannet S, Marcinkiewicz J, Chretien M, Seidah NG, et al. The secretory proprotein convertases furin, PC5, and PC7 activate VEGF-C to induce tumorigenesis. *J Clin Invest* 2003;111:1723-1732.
4. Harris NC, Paavonen K, Davydova N, Roufail S, Sato T, Zhang YF, Karnezis T, et al. Proteolytic processing of vascular endothelial growth factor-D is essential for its capacity to promote the growth and spread of cancer. *FASEB J* 2011; 25:2615-2625.
5. Siegfried G, Basak A, Prichett-Pejic W, Scamuffa N, Ma L, Benjannet S, Veinot JP, et al. Regulation of the stepwise proteolytic cleavage and secretion of PDGF-B by the proprotein convertases. *Oncogene* 2005; 24:6925-6935.
6. Siegfried G, Khatib AM, Benjannet S, Chretien M, Seidah NG.The proteolytic processing of pro-platelet-derived growth factor-A at RRKR(86) by members of the proprotein convertase family is functionally correlated to platelet-derived growth factor-A-induced functions and tumorigenicity. *Cancer Res* 2003; 63:1458-1463.
7. Khatib AM, Siegfried G, Prat A, Luis J, Chretien M, Metrakos P, Seidah NG. Inhibition of proprotein convertases is associated with loss of growth and tumorigenicity of HT-29 human colon carcinoma cells: importance of insulin-like growth factor-1 (IGF-1) receptor processing in IGF-1-mediated functions. *J Biol Chem* 2001; 276:30686-30693.
8. Golubkov VS, Chernov AV, Strongin AY. Intradomain cleavage of inhibitory prodomain is essential to protumorigenic function of membrane type-1 matrix metalloproteinase (MT1-MMP) in vivo. *J Biol Chem* 2011; 286:34215-34223.

9. Scamuffa N, Siegfried G, Bontemps Y, Ma L, Basak A, Cherel G, Calvo F, et al. Selective inhibition of proprotein convertases represses the metastatic potential of human colorectal tumor cells. *J Clin Invest* 2008; 118:352-363.

10. Tzimas GN, Chevet E, Jenna S, Nguyen DT, Khatib AM, Marcus V, Zhang Y, et al. Abnormal expression and processing of the proprotein convertases PC1 and PC2 in human colorectal liver metastases. *BMC Cancer* 2005; 5:149.

11. Khatib AM, Siegfried G, Chretien M, Metrakos P, Seidah N.G. Proprotein convertases in tumor progression and malignancy: novel targets in cancer therapy. *Am J Pathol* 2002; 160:1921-1935.

12. Rockwell, N.C., Krysan, D.J., Komiyama, T., and Fuller, R.S. 2002. Precursor processing by kex2/furin proteases. *Chem Rev* 102:4525-4548.

13. Mbikay M, Seidah N.G, Chretien M. Neuroendocrine secretory protein 7B2: structure, expression and functions. *Biochem J* 2001; 357:329-342.

14. Fortenberry Y, Hwang JR, Apletalina EV, Lindberg I. Functional characterization of ProSAAS: similarities and differences with 7B2. *J Biol Chem* 2002; 277:5175-5186.

15. Cornwall GA, Cameron A, Lindberg I, Hardy DM, Cormier N, Hsia N. The cystatin-related epididymal spermatogenic protein inhibits the serine protease prohormone convertase 2. *Endocrinology* 2003; 144:901-908.

16. Gettins PG. Serpin structure, mechanism, and function. *Chem Rev* 2002; 102:4751-4804.

17. van Gent D, Sharp P, Morgan K, Kalsheker N.. Serpins: structure, function and molecular evolution. *Int J Biochem Cell Biol* 2003; 35:1536-1547.

18. Kruger O, Ladewig J, Koster K, Ragg H. Widespread occurrence of serpin genes with multiple reactive centre-containing exon cassettes in insects and nematodes. *Gene* 2002; 293:97-105.

19. Oley M, Letzel MC, Ragg H. Inhibition of furin by serpin Spn4A from Drosophila melanogaster. *FEBS Lett* 2004; 577:165-169.

20. Richer MJ, Keays CA, Waterhouse J, Minhas J, Hashimoto C, Jean F. The Spn4 gene of Drosophila encodes a potent furin-directed secretory pathway serpin. *Proc Natl Acad Sci U S A* 2004; 101:10560-10565.

21. De Bie I, Savaria D, Roebroek AJ, Day R, Lazure C, Van de Ven WJ, Seidah N.G. Processing specificity and biosynthesis of the Drosophila melanogaster convertases dfurin1, dfurin1-CRR, dfurin1-X, and dfurin2. *J Biol Chem* 1995; 270:1020-1028.

22. Siekhaus DE, Fuller RS. A role for amontillado, the Drosophila homolog of the neuropeptide precursor processing protease PC2, in triggering hatching behavior. *J Neurosci* 1999; 19:6942-6954.

23. Gordon VM, Rehemtulla A, Leppla SH. A role for PACE4 in the proteolytic activation of anthrax toxin protective antigen. *Infect Immun* 1997; 65:3370-3375.

24. Lalou C, Scamuffa N, Mourah S, Plassa F, Podgorniak MP, Soufir N, Dumaz N, et al. Inhibition of the proprotein convertases represses the invasiveness of human primary melanoma cells with altered p53, CDKN2A and N-Ras genes. *PLoS One* 2010; 5:e9992.

25. Lapierre M, Siegfried G, Scamuffa N, Bontemps Y, Calvo F, Seidah NG, Khatib AM. Opposing function of the proprotein convertases furin and PACE4 on breast cancer cells' malignant phenotypes: role of tissue inhibitors of metalloproteinase-1. Cancer Res 2007; 67:9030-9034.

26. Gu M, Gordon VM, Fitzgerald DJ, Leppla SH.. Furin regulates both the activation of Pseudomonas exotoxin A and the Quantity of the toxin receptor expressed on target cells. Infect Immun 1996; 64:524-527.

27. Brüning M, Lummer M, Bentele C, Smolenaars MM, Rodenburg KW, Ragg H; The Spn4 gene from Drosophila melanogaster is a multipurpose defence tool directed against proteases from three different peptidase families; Biochem J. 2007 Jan 1;401(1):325-31

CLAIMS:

1. An isolated Serpin Spn4A polypeptide or a biologically active derivative thereof for use in the prevention or treatment of cancer and related metastases.

2. A nucleic acid encoding a polypeptide according to claim 1 for use in the prevention or treatment of cancer and related metastases.

3. An expression vector comprising a nucleic acid according to claim 2 for use in the prevention or treatment of cancer and related metastases.

4. A host cell comprising an expression vector according to claim 3 for use in the prevention or treatment of cancer and associated metastases.

5. The polypeptide or the derivative thereof, the nucleic acid, the expression vector or the host cell for use according to any one claims 1 to 4, wherein the cancer is a colorectal cancer.

6. The polypeptide or the derivative thereof, the nucleic acid, the expression vector or the host cell for use according to claim 5, wherein the related metastases are liver colorectal metastases.

7. A pharmaceutical composition comprising an isolated Serpin Spn4A polypeptide or a biologically active derivative thereof, or a nucleic acid encoding thereof, or an expression vector comprising thereof, or a host cell comprising thereof according to any one claims 1 to 6; and a pharmaceutically acceptable carrier.

8. The pharmaceutical composition according to claim 7, further comprising an additional chemotherapeutic agent.

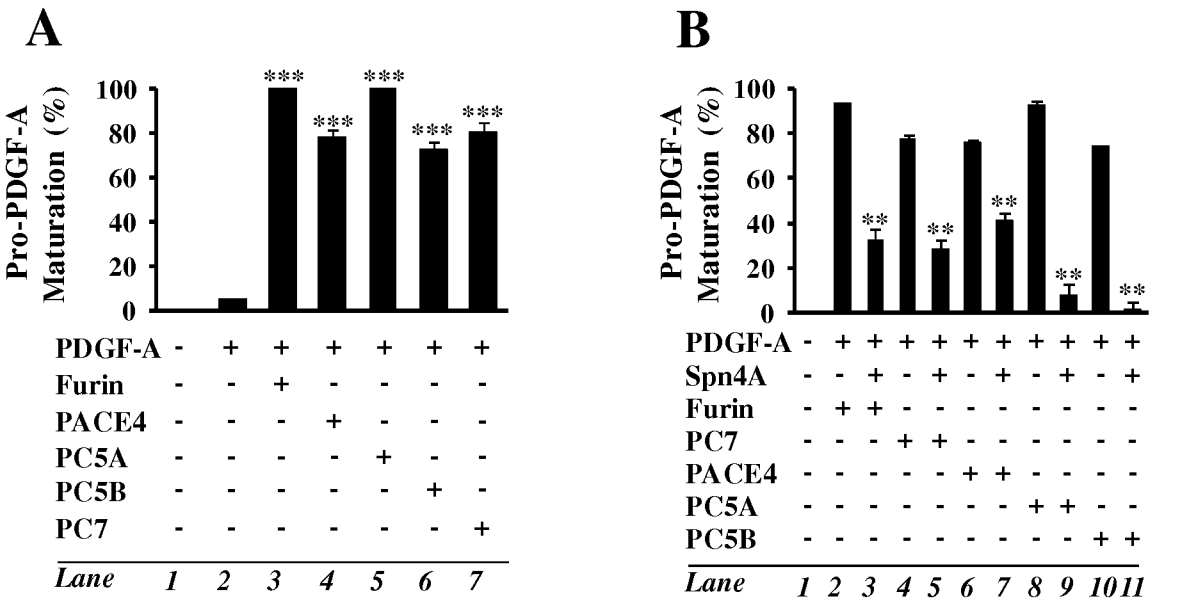
9. A kit-of-part composition comprising an isolated Serpin Spn4A polypeptide or a biologically active derivative thereof, or a nucleic acid encoding thereof, or an expression vector comprising thereof, or a host cell comprising thereof according to any one claims 1 to 6; and an additional therapeutic agent.

10. The pharmaceutical composition according to claim 8 or the kit-of-part composition according to claim 9, wherein said additional therapeutic agent a chemotherapeutic compound.

11. The pharmaceutical composition or the kit-of-part composition according to claim 10, wherein said additional chemotherapeutic compound is a tyrosine kinase inhibitor.

12. The pharmaceutical composition according to any one claims 7 to 11 for use in the prevention or treatment of cancer and related metastases.

13. The kit-of-part composition according to any one claims 7 to 11 for simultaneous, separate or sequential use in the prevention or treatment of cancer and metastases.


A
Pro-PDGF-A Maturation (%)
100
80
60
40
20
0
***
PDGF-A - + + + + + +
Furin - - + - - - -
PACE4 - - - + - - -
PC5A - - - - + - -
PC5B - - - - - + -
PC7 - - - - - - +
Lane 1 2 3 4 5 6 7
B
Pro-PDGF-A Maturation (%)
100
80
60
40
20
0
**
PDGF-A - + + + + + + + + + +
Spn4A - - + - + - + - + - +
Furin - + + - - - - - - - -
PC7 - - - + + - - - - - -
PACE4 - - - - - + + - - - -
PC5A - - - - - - - + + - -
PC5B - - - - - - - - - + +
Lane 1 2 3 4 5 6 7 8 9 10 11

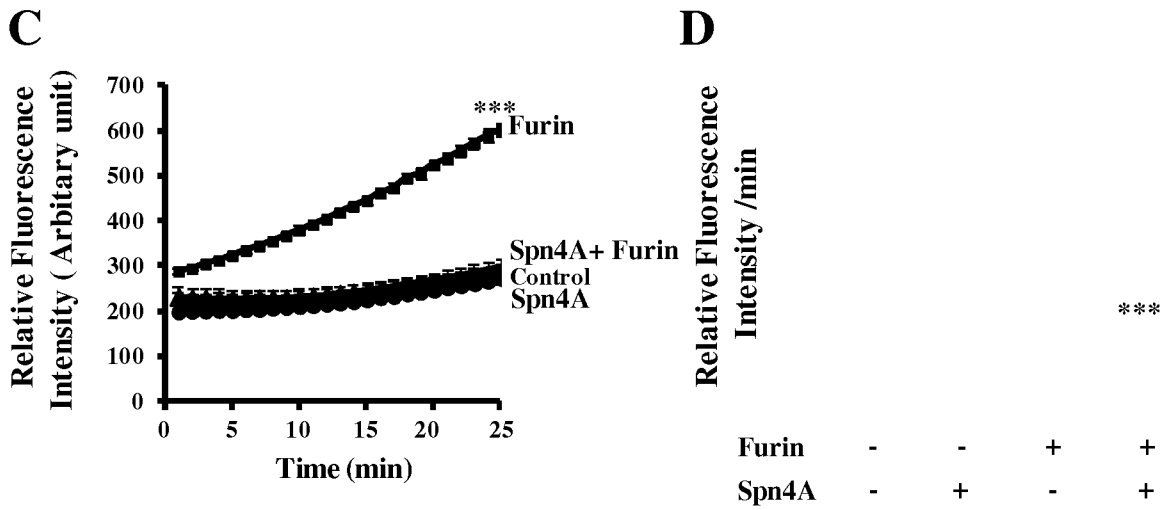

C
Relative Fluorescence Intensity (Arbitary unit)
700
600
500
400
300
200
100
0
0 5 10 15 20 25
Time (min)
***
Furin
Spn4A+ Furin
Control
Spn4A
D
Relative Fluorescence Intensity /min
***
Furin - - + +
Spn4A - + - +

Figure 1

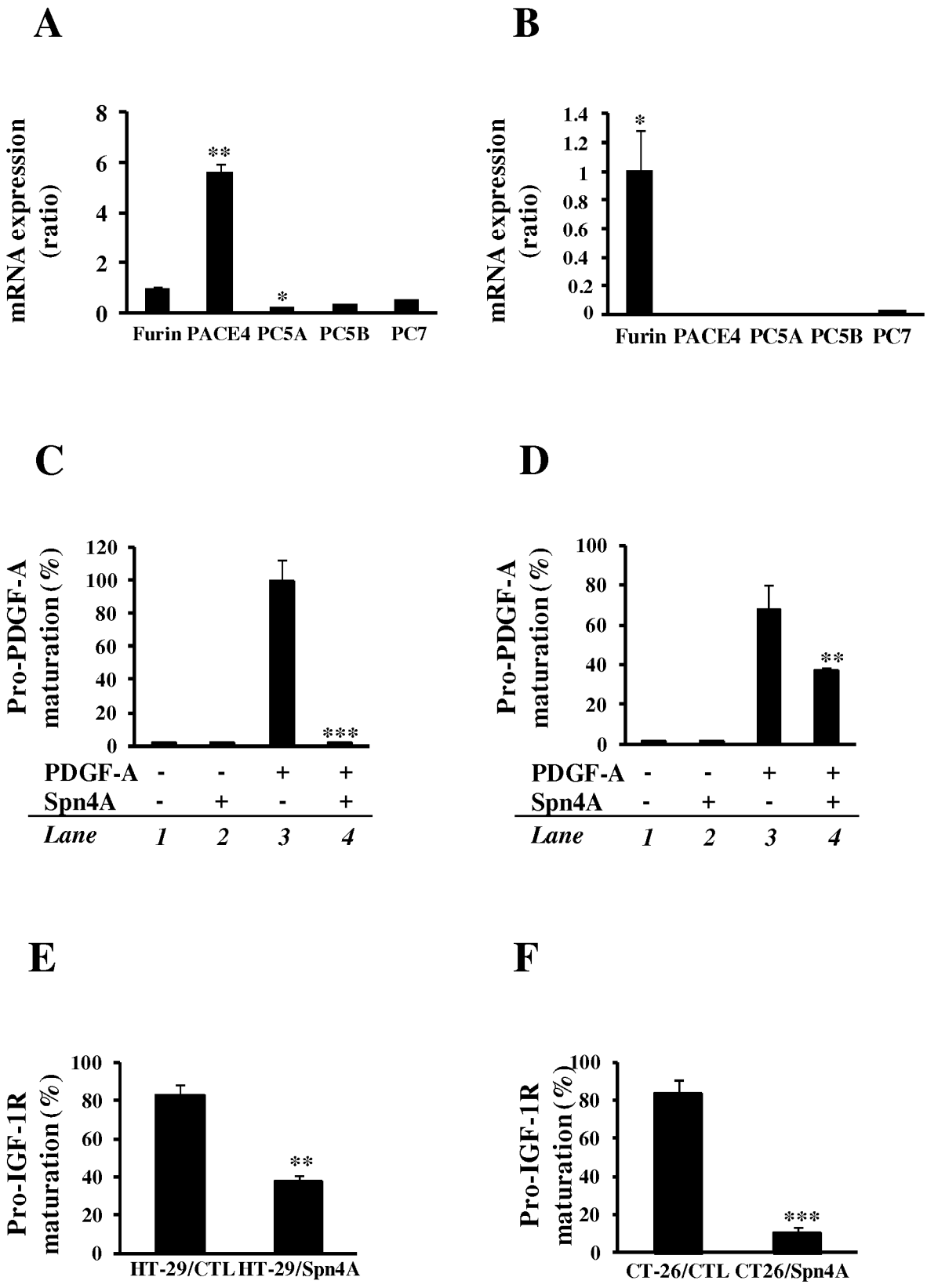

A
mRNA expression (ratio)
8
6
4
2
0
**
*
Furin PACE4 PC5A PC5B PC7
B
mRNA expression (ratio)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Furin PACE4 PC5A PC5B PC7
C
Pro-PDGF-A maturation (%)
120
100
80
60
40
20
0
***
PDGF-A - - + +
Spn4A - + - +
Lane 1 2 3 4
D
Pro-PDGF-A maturation (%)
100
80
60
40
20
0
**
PDGF-A - - + +
Spn4A - + - +
Lane 1 2 3 4
E
Pro-IGF-1R maturation (%)
100
80
60
40
20
0
**
HT-29/CTL HT-29/Spn4A
F
Pro-IGF-1R maturation (%)
100
80
60
40
20
0
***
CT-26/CTL CT26/Spn4A

Figure 2

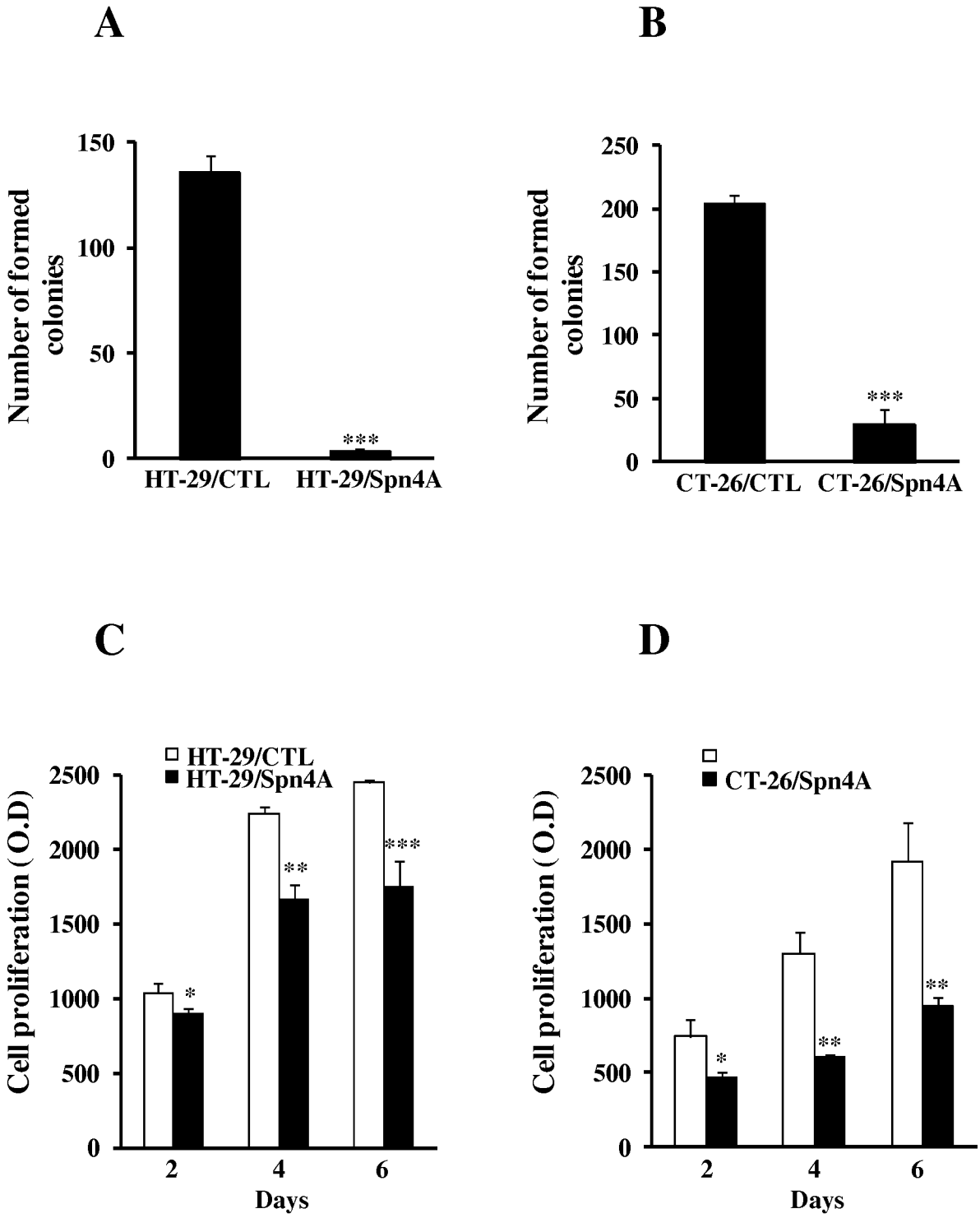

A
Number of formed colonies
150
100
50
0
***
HT-29/CTL
HT-29/Spn4A
B
Number of formed colonies
250
200
150
100
50
0
***
CT-26/CTL
CT-26/Spn4A
C
HT-29/CTL
HT-29/Spn4A
Cell proliferation (O.D)
2500
2000
1500
1000
500
0
*
**
***
2
4
6
Days
D
CT-26/Spn4A
Cell proliferation (O.D)
2500
2000
1500
1000
500
0
*
**
**
2
4
6
Days

Figure 3

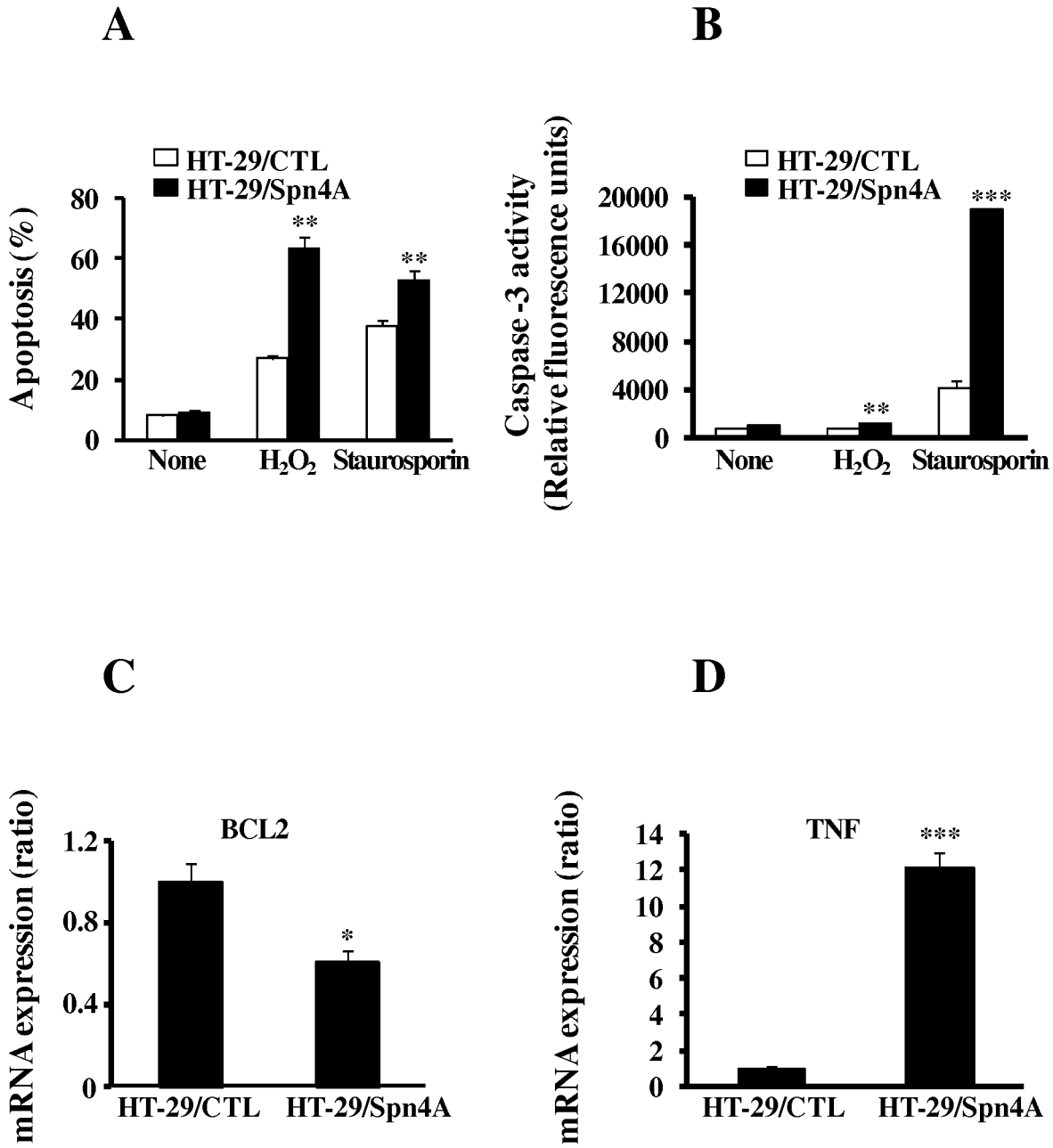

A
HT-29/CTL
HT-29/Spn4A
Apoptosis (%)
80
60
40
20
0
**
**
None
H2O2
Staurosporin
B
HT-29/CTL
HT-29/Spn4A
Caspase -3 activity
(Relative fluorescence units)
20000
16000
12000
8000
4000
0
**
***
None
H2O2
Staurosporin
C
BCL2
mRNA expression (ratio)
1.2
0.8
0.4
0
*
HT-29/CTL
HT-29/Spn4A
D
TNF
mRNA expression (ratio)
14
12
10
8
6
4
2
0
***
HT-29/CTL
HT-29/Spn4A

Figure 4

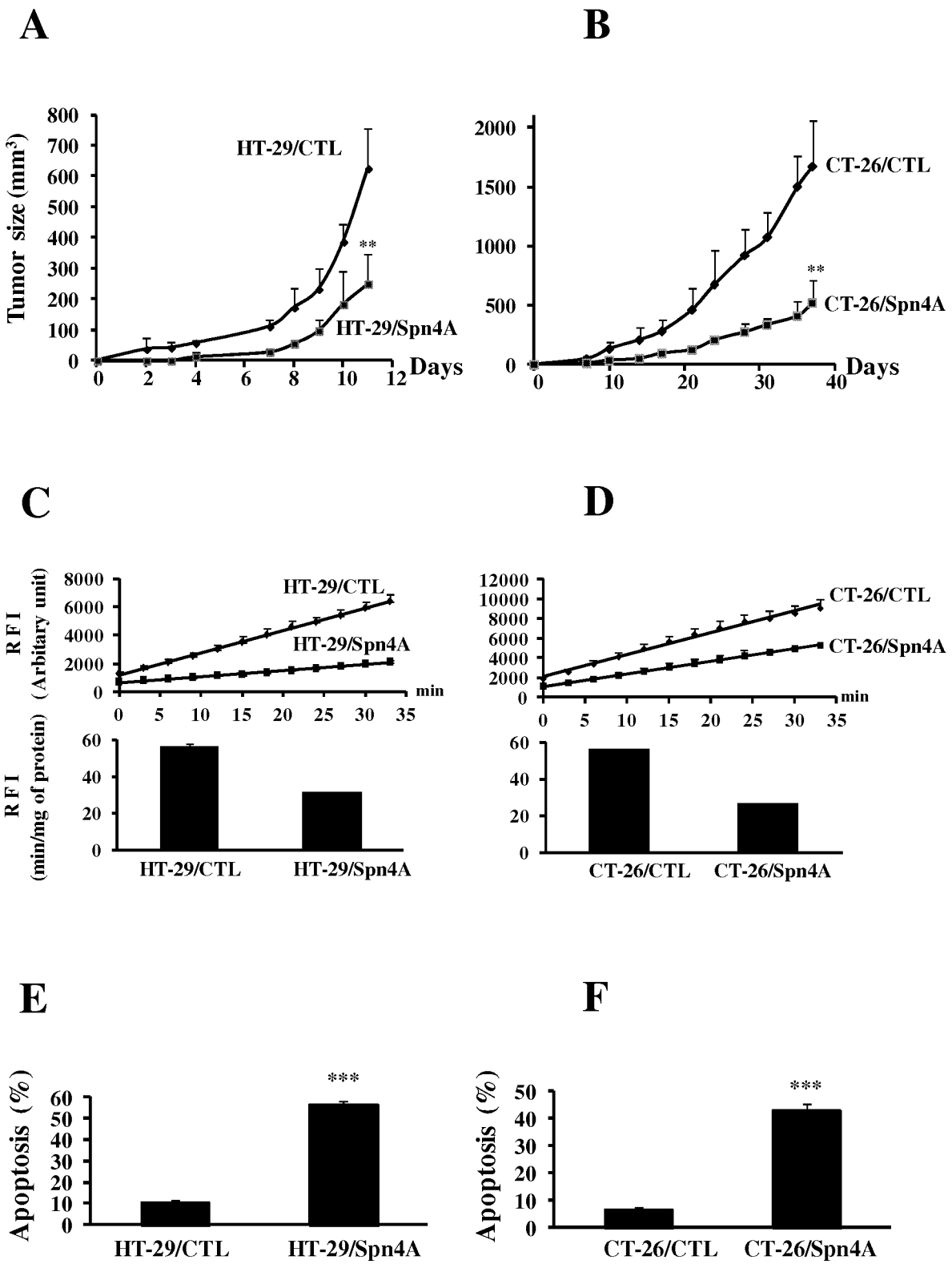

A
B
Tumor size (mm3)
HT-29/CTL
HT-29/Spn4A
CT-26/CTL
CT-26/Spn4A
**
Days
C
D
RFI (Arbitary unit)
RFI (min/mg of protein)
min
HT-29/CTL
HT-29/Spn4A
CT-26/CTL
CT-26/Spn4A
E
F
Apoptosis (%)
***

Figure 5

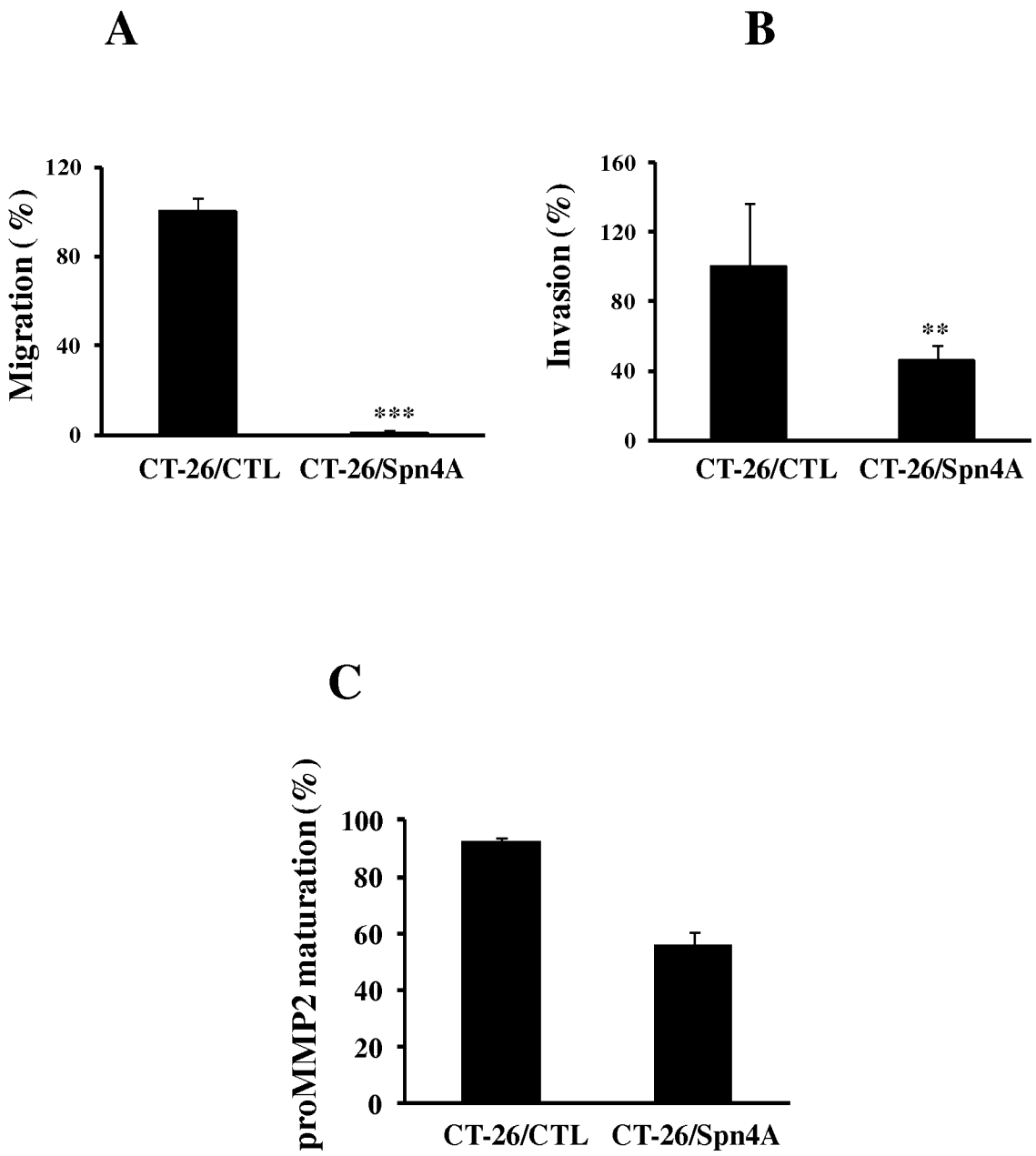

A
Migration (%)
120
80
40
0
***
CT-26/CTL
CT-26/Spn4A
B
Invasion (%)
160
120
80
40
0
**
CT-26/CTL
CT-26/Spn4A
C
proMMP2 maturation (%)
100
80
60
40
20
0
CT-26/CTL
CT-26/Spn4A

Figure 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/076389

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/55 A61P35/04
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)
A61K

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, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 01/18057 A2 (UNIV CAMBRIDGE TECH [GB]; GUBB DAVID CHRISTOPHER [GB]; REICHHART JEAN) 15 March 2001 (2001-03-15) *cf. page 1, lines 5-20, page 4, lines 19-28, page 11, lines 4-24, page 26, lines 23-28, claims 37-41*	1-13
Y	JEAN FRANCOIS ET AL: "Drosophila Spn4A, the first natural furin-directed secretory pathway serine protease inhibitor (SERPIN)", FASEB JOURNAL, FED. OF AMERICAN SOC. FOR EXPERIMENTAL BIOLOGY, US, vol. 18, no. 8, Suppl. S, 1 May 2004 (2004-05-01), page C179, XP009169374, ISSN: 0892-6638 *cf. page C179, no. 76.7*	1-13

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 February 2014	06/03/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Stoltner, Anton

1

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/076389

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RICHER MARTIN J ET AL: "The Spn4 gene of Drosophila encodes a potent furin-directed secretory pathway serpin", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, NATIONAL ACADEMY OF SCIENCES, US, vol. 101, no. 29, 20 July 2004 (2004-07-20), pages 10560-10565, XP009169373, ISSN: 0027-8424, DOI: 10.1073/PNAS.0401406101 [retrieved on 2004-07-09] *cf. summary part*	1-13
A	OLEY M ET AL: "Inhibition of furin by serpin Spn4A from Drosophila melanogaster", FEBS LETTERS, ELSEVIER, AMSTERDAM, NL, vol. 577, no. 1-2, 5 November 2004 (2004-11-05), pages 165-169, XP004622746, ISSN: 0014-5793, DOI: 10.1016/J.FEBSLET.2004.10.003 *cf. abstract, introduction at page 165*	1-13

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2013/076389

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0118057 A2	15-03-2001	AU 7024900 A	10-04-2001
		WO 0118057 A2	15-03-2001