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(54) Titre : COMBINAISON D'ANTICORPS ANTI-HGFR ET HEGFR POUR LE TRAITEMENT D'UNE TUMEUR ET/OU D'UNE METASTASE

(54) Title: COMBINATION OF ANTI-HGFR ANTIBODY AND HEGFR FOR THE TREATMENT OF A TUMOR AND/OR METASTASIS

(57) Abrégé/Abstract:
An anti-HGFR antibody fragment in combination with an extracellular portion of human HGFR for use in the treatment of a patient suffering from a tumor and/or metastasis, wherein: (i) the anti-HGFR antibody fragment has only one paratope able to bind to an epitope of the extracellular portion of human HGFR and has antagonist activity towards HGFR, (ii) the extracellular portion of human HGFR is capable of binding to HGF in a stable manner and contains at least one amino acid mutation at the epitope recognized by the anti-HGFR antibody fragment to prevent binding of the anti-HGFR antibody fragment thereto, and (iii) the anti-HGFR antibody fragment and the extracellular portion of human HGFR are suitable for administration to the patient are suitable for administration to the patient (a) in a protein form or (b) in a nucleic acid form.

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(54) Title: COMBINATION OF ANTI-HGFR ANTIBODY AND HEGFR FOR THE TREATMENT OF A TUMOR AND/OR METASTASIS

(57) Abstract: An anti-HGFR antibody fragment in combination with an extracellular portion of human HGFR for use in the treatment of a patient suffering from a tumor and/or metastasis, wherein: (i) the anti-HGFR antibody fragment has only one paratope able to bind to an epitope of the extracellular portion of human HGFR and has antagonist activity towards HGFR, (ii) the extracellular portion of human HGFR is capable of binding to HGF in a stable manner and contains at least one amino acid mutation at the epitope recognized by the anti-HGFR antibody fragment to prevent binding of the anti-HGFR antibody fragment thereto, and (iii) the anti-HGFR antibody fragment and the extracellular portion of human HGFR are suitable for administration to the patient are suitable for administration to the patient (a) in a protein form or (b) in a nucleic acid form.

COMBINATION OF ANTI-HGFR ANTIBODY AND HEGFR FOR THE TREATMENT OF A TUMOR AND/OR METASTASIS

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Field of the invention

The disclosure concerns a novel combination of therapeutic agents for the treatment of a tumor and/or metastasis, preferably metastasis.

Background of the invention

Metastatic spreading is based on the ability of cancer cells to disrupt cell-to-cell interactions, migrate through the extracellular matrix, survive and proliferate in tissues other than their site of origin. The physiological counterpart of this complex program – known as 'invasive growth' – is at the basis of embryogenesis and accounts for wound healing and organ regeneration during adult life. Invasive growth is tightly regulated by specific extracellular signals, one of which is Hepatocyte Growth Factor (HGF), the ligand for the receptor encoded by *MET* oncogene. In conditions of aberrant activation, HGF/MET signaling drives tumor onset, progression and metastasis in a broad spectrum of human malignancies. In a minority of events, *MET* behaves as a 'driver' oncogene and tumor cells are dependent on constitutive MET signaling for growth and survival ('*MET addiction*'). This condition relies on the presence of genetic lesions, mostly increased gene copy number or -less frequently- mutations[2] that result in constitutive ligand-independent receptor activation. In this context, treatment with MET inhibitors is highly effective, inducing block of cell proliferation and cell cycle arrest *in vitro* and inhibition of tumor growth *in vivo*. The co-expression of ligand and receptor within the

same cell is another strategy exploited by cancer to achieve continuous MET activation, and has been described mainly in non-epithelial human cancers, such as osteosarcomas, glioblastomas and multiple myelomas. In most cases, however, aberrant MET activation in tumors originates from receptor over-expression, due to transcriptional upregulation of the wild-type gene, triggering cancer cell sensitization to ligand stimulation[3]. In the latter case, MET signaling –which results in pro-invasive and anti-apoptotic responses– is exploited by cancer cells as a strategy to bypass stress conditions and boosts the malignant phenotype (‘*MET expedience*’[6]). In the absence of specific genetic lesions, *MET* is not strictly necessary for tumor growth, but the presence of the ligand sustains receptor activation, enhancing the malignant phenotype. Finally, MET behaves as a functional marker of cancer ‘stem-progenitor’ cells in glioblastomas[8], and supports the ‘stem’ phenotype in colorectal and breast cancers[12,15]. Moreover, it has been shown that stromal-derived HGF sustains the WNT self-renewal pathway of colorectal cancer stem cells and promotes proliferation of colon cancer initiating cells, triggering resistance to anti-EGFR therapy[27].

A number of strategies targeting MET or HGF – either small molecule inhibitors, antibodies or recombinant proteins- have been designed and are currently under investigation. Among them, the MvDN30 antibody is a monovalent chimeric Fab fragment that binds to the extracellular domain of MET, inducing proteolytic cleavage (‘*shedding*’) of the receptor from the cell surface[21, 28]. DecoyMET is a recombinant soluble receptor encompassing the whole extracellular region of MET; it binds HGF with high affinity and inhibits ligand-driven biological activities *in vitro* and *in*

vivo when expressed by lentiviral vector technology[17] or as Fc-fusion protein[7,26]. As in the majority of biological systems, hitting a single element of a signal transduction chain unlikely results in complete shut-off of the response. Thus, in the case of MET, every molecule will never achieve 100% inhibition, leaving residual activity sensitive to HGF stimulation.

Summary of the invention

The object of this disclosure is to provide novel combination of anti-tumor agents useful in the treatment of oncologic patients.

According to the invention, the above object is achieved thanks to the subject matter recalled specifically in the ensuing claims, which are understood as forming an integral part of this disclosure.

The present invention provides an anti-Hepatocyte Growth Factor Receptor (HGFR) antibody fragment in combination with an extracellular portion of human HGFR for use in the treatment of a patient suffering from a tumor and/or metastasis, preferably metastasis, wherein:

(i) the anti-HGFR antibody fragment has only one paratope able to bind to an epitope of the extracellular portion of human HGFR and has antagonist activity towards HGFR,

(ii) the extracellular portion of human HGFR is capable of binding to Hepatocyte Growth Factor (HGF) in a stable manner and contains at least one amino acid mutation within the epitope recognized by the anti-HGFR antibody fragment to prevent binding of the anti-HGFR antibody fragment thereto, and

(iii) the anti-HGFR antibody fragment and the extracellular portion of human HGFR are suitable for

administration to the patient (a) in a protein form or (b) in a nucleic acid form.

Brief description of the drawings

The invention will now be described in detail, purely by way of illustrative and non-limiting example, with reference to the attached figures, wherein:

- Figure 1. Generation of a mutated decoyMET receptor that does not bind the DN30 antibody.

(A) Comparison of the aminoacid sequences of the third and fourth IPT domains of human, mouse, rat and dog MET. Residues that are changed exclusively in the mouse sequence are highlighted in in bold, white on black background, aminoacids that are changed also (or only) in the rat or dog sequences are highlighted in bold. Only the IPT-3/IPT-4 boundaries are shown. **(B)** DecoyMET receptors carrying single aminoacid substitutions were incubated with the DN30 antibody. The complexes were immunoprecipitated with protein A -that binds to the antibody- and revealed with HRP-conjugated streptactin -that binds to the strep-tag in the decoy (left panel). 30 µl of normalized supernatants used for the immunoprecipitation were run on SDS PAGE to verify decoyMET receptors loading (right panel). **(C)** ELISA binding analysis. DN30 mAb was in liquid phase, wild-type decoyMET or decoyMET^{K842E} in solid phase. Antibody binding was detected using an HRP-conjugated anti-mouse antibody. OD, optical density at 450 nm. Each point is the mean of values in triplicate ± SD.

- Figure 2. DecoyMET^{K842E} binds HGF at high affinity and inhibits HGF-induced MET phosphorylation.

(A) ELISA binding analysis. Wild-type decoyMET or decoyMET^{K842E} were in solid phase; increasing concentrations of HGF were added in liquid phase. HGF

binding was detected using a biotinylated anti-HGF antibody. Each point is the mean of values in triplicate ± SD. **(B)** HGF-induced MET phosphorylation. A549 cells were incubated with wild-type decoyMET or decoyMET^{K842E} (2 μM) and stimulated with HGF (50 ng/ml). Total cell lysates were immunoblotted with anti-phosphoMET (upper panel), anti-MET antibodies (middle panel) or with anti-vinculin antibodies (lower panel). p145, mature form of MET; p190, single-chain precursor of MET; p117, vinculin.

- Figure 3. MvDN30 and DecoyMET^{K842E} cooperate in reducing HGF-induced MET phosphorylation and MET-driven biological activities.

(A) HGF-induced MET phosphorylation. A549 human lung adenocarcinoma cells were incubated with 2 μM decoyMET^{K842E} , 125 nM MvDN30 or the combination of the two, and stimulated or not with 50 ng/ml HGF. Total cell lysates were immunoblotted with anti-phosphoMET (upper panel), anti-MET antibodies (middle panel) or anti-vinculin (lower panel). p145, mature form of MET; p190, single-chain precursor of MET; p117, vinculin. **(B)** HGF-induced ERK and AKT phosphorylation. A549 human lung adenocarcinoma cells were incubated with 500 nM MvDN30, 2 μM decoyMET^{K842E} or the combination of the two, and stimulated or not with 100 ng/ml HGF. Total cell lysates were immunoblotted with anti-phosphoERK, anti-ERK, anti-phosphoAKT, anti-AKT, anti-GAPDH. p42/44, ERK; p60, AKT; p36, GAPDH. **(C)** Anchorage-independent growth sustained by autocrine HGF stimulation. U87-MG human glioblastoma cells, expressing both HGF and MET proteins, were treated with 0.5 μM MvDN30 or 1 μM decoyMET^{K842E}, alone or in combination. Graph represents percentage of average colony growth for each treatment compared to the untreated control. **(D)** Anchorage-independent growth

sustained by paracrine HGF stimulation. A549 cells were stimulated with 30 ng/ml HGF and treated with 1µM MvDN30 or 1 µM decoyMET^{K842E}, alone or in combination. Graph represents percentage of average colony growth for each treatment compared to the HGF-stimulated control. **(E)** Transwell invasion assay. HPAF-II human pancreatic adenocarcinoma cells were stimulated with 12.5 ng/ml HGF and treated with 0.5 µM MvDN30 or 1 µM decoyMET^{K842E}, alone or in combination. Graph represents the percentage of invasion in comparison to the HGF-stimulated control. Right panel, one representative image/group of the cells migrated through the matrigel layer. n.t., not treated cells. Magnification, 200x. Each point is the mean of values in triplicate ± SD. ***= $P \leq 0.001$; **= $P \leq 0.01$; * = $P \leq 0.05$.

- **Figure 4. MvDN30 and decoyMET^{K842E} in combination inhibit HGF-dependent MET phosphorylation.**

U87MG **(A)**, A549 **(B)** and HPAF-II **(C)** cells were treated with MvDN30, decoyMET^{K842E} or the combination of the two (COMBO). HPAF-II and A549 cells were stimulated or not with HGF; n.t., not treated cells. Left panels: representative confocal sections showing anti-phosphoMET (top rows) and phalloidin (bottom rows). Graphs on the right report the Mean Fluorescence Intensity (MFI) of phosphoMET, background subtracted and normalized on phalloidin. Each point is the mean of 5 values ± SEM. Bar is 50 µm. ***= $P \leq 0.001$; **= $P \leq 0.01$; * = $P \leq 0.05$.

- **Figure 5. MvDN30 and DecoyMET^{K842E} synergize to inhibit HGF-dependent cell scattering.**

(A) Analysis of cell motility. HPAF-II cells were pre-incubated with different concentrations (0.06, 0.25, 1 or 4 µM) of MvDN30 or decoyMET^{K842E}, alone or in 1:1 combination, and then stimulated with 6.25 ng/ml HGF. Cell scattering was monitored in real time using

an X-CELLigence RTCA device and is expressed as Normalized Cell Index. Each graph refers to one treatment concentration. **(B)** Representative images of HPAF-II cells pre-incubated with 1 µM MvDN30 or 1 µM decoyMET^{K842E}, alone or in combination, and then stimulated with 6.25 ng/ml HGF. n.t., not treated cells. **(C)** Cell motility curve. Effect = Cell index values measured at the end of the experiment for each dose of treatment, normalized on the values obtained with HGF alone and expressed as [1-x]. **(D)** Drug combination analysis. Values from the cell motility curve were elaborated with the Calcusyn software to calculate the Combination Index (CI) for each concentration of MvDN30 and decoyMET^{K842E}. CI = 1, cooperation; CI <1, synergism; CI>1, antagonism.

- Figure 6. MvDN30 and decoyMET^{K842E} in combination reduce the invasive phenotype of subcutaneous U87-MG tumors.

U87-MG cells were injected subcutaneously in NOD-SCID mice. When the tumors reached a volume of 80-100 mm^3, mice were stratified in four homogeneous groups: VEHICLE (*n*=10); MvDN30 (*n*=9); K842E (*n*=9); the combination of the two (*n*=10). **(A)** Tumor volume at sacrifice, expressed as fold increase. Each bar is the mean of the group ± SEM. **(B)** Histochemical analysis of tumor burden. Representative images of hematoxylin-eosin stained tumor sections. Arrows point to the boundary between the tumor and the surrounding tissue. Magnification 100x.

- Figure 7. Effect of MvDN30 or decoyMET^{K842E}, alone and in combination, on the proliferation and apoptosis of pancreatic cancer cells injected orthotopically in hHGF-Ki mice.

Luciferase-expressing HPAF-II cells were injected in the pancreas of hHGF-Ki mice and stratified into

four homogeneous groups: VEHICLE (*n*=10), MvDN30 (*n*=6), decoyMET^{K842E} (*n*=6), the combination of the two (*n*=6). **(A)** Tumor bioluminescence detected by IVIS Spectrum. Numbers indicate the average values of total flux of bioluminescence (photons/second x 10^8) of each experimental group ± SEM. **(B)** Analysis of tumor cell proliferation measured by Ki67 immunohistochemistry. Left panel, representative images of each experimental group. Magnification, 200x. Graph on the right reports average values obtained by the analysis of five images per tumor ± SEM. Proliferation Index is calculated as Ki67 positive cells/total number of cells. **(C)** Analysis of tumor cell apoptosis measured by cleaved Caspase-3 immunofluorescence. Left panel, representative images of each experimental group. Bar is 50 µm. Graph on the right reports average values obtained by the analysis of 8 images per tumor ± SEM. Apoptotic index is calculated as cleaved Caspase-3 positive cells/total number of cells. Bar is 50 µm. *** = *P* value < 0.001; **= *P* value < 0.01; *= *P* value < 0.05.

- Figure 8. MvDN30 and DecoyMET^{K842E} reduce MET phosphorylation and metastatic dissemination of pancreatic cancer cells in hHGF-Ki mice.

Luciferase-expressing HPAF-II cells were injected in the pancreas of hHGF-Ki mice and stratified into four homogeneous groups: VEHICLE (*n*=10), MvDN30 (*n*=6), decoyMET^{K842E} (*n*=6), the combination of the two (*n*=6). **(A)** PhosphoMET status within tumors measured by immunofluorescence. Left panel, representative confocal sections of each experimental group showing anti-phosphoMET. Graph on the right reports the Mean Fluorescence Intensity (MFI) of phosphoMET, background subtracted and normalized on DAPI. Each point is the mean of 12 values ± SEM. Bar is 50 µm. **(B)** Evaluation of the EMT phenotype of HPAF-II tumors by

immunofluorescence analysis of E-cadherin and vimentin expression. Left panel, representative images of each experimental group. Anti-E-cadherin (top row), anti-vimentin (lower row). Bar is 50 μm. Graph on the right reports average values obtained by the analysis of 6 images per each tumor ± SEM. EMT phenotype is expressed as Vimentin/E-cadherin ratio. **(C)** Metastatic nodules in the lungs evaluated by histochemical HE staining. Graph on the left: number of metastatic lesions; each point represents the number of lesions scored for each mouse. Graph on the right: area of metastatic lesions; each point represents the average area of metastases measured for each mouse. Ten slides/mouse were analyzed; metastatic lesions were scored and their area quantified with ImageJ. ***= $P \leq 0.001$; **= $P \leq 0.01$; * = $P \leq 0.05$.

- **Figure 9. Schematic representation of one embodiment of the fusion proteins containing an anti-HGFR antibody fragment and an extracellular portion of human HGFR.**

(A) Draw of a molecule generated by the in frame fusion of decoyMET^{K842E} at the N-terminal and scMvDN30 at the C-terminal. **(B)** Draw of a molecule generated by the in frame fusion of scMvDN30 at the N-terminal and decoyMET^{K842E} at the C-terminal. Between the two moiety a linker has been inserted. The sequence of the linker can be selected from the ones listed in **(C)**. SEMA, PSI, IPT 1-4 are the regions of decoyMET. The star in the IPT 4 represents the mutation K→E at position 842. The anti-HGFR antibody fragment is constituted by two chains jointed by a second linker. VL, variable light region; CL, constant light region; VH, variable heavy region; CH, constant heavy region. The black square represents the Strept-His TAGs included in the recombinant protein for purification/detection purpose.

- Figure 10. Binding analysis of the fusion proteins containing an anti-HGFR antibody fragment and an extracellular portion of human HGFR to MET or to HGF.

(A) Binding analysis to HGF by ELISA. Left panel, binding of the fusion proteins containing decoyMET^{K842E} at the N-terminal and scMvDN30 at the C-terminal. Right panel, binding of the fusion proteins containing scMvDN30 at the N-terminal and decoyMET^{K842E} at the C-terminal. The fusion proteins or decoyMET^{K842E} (positive control) were in solid phase. Increasing concentrations of HGF were added in liquid phase. HGF binding was detected using a biotinylated anti-HGF antibody. Each point is the mean of values in triplicate ± SD. **(B)** Binding analysis to MET by ELISA. Left panel, binding of the fusion proteins containing decoyMET^{K842E} at the N-terminal and scMvDN30 at the C-terminal. Right panel, binding of the fusion proteins containing scMvDN30 at the N-terminal and decoyMET^{K842E} at the C-terminal. Wild-type decoyMET was in solid phase and increasing concentrations of the different fusion proteins were in liquid phase; as positive control scMvDN30 was included in the assay. Antibody binding was detected using an HRP-conjugated anti-human k chain antibody. OD, optical density at 450 nm. Each point is the mean of values in triplicate ± SD.

- Figure 11. Inhibition of tumor cell growth upon treatment with the fusion proteins containing an anti-HGFR antibody fragment and an extracellular portion of human HGFR.

(A) Growth of EBC-1 lung carcinoma cells treated with increasing concentrations of the fusion proteins containing decoyMET^{K842E} at the N-terminal and scMvDN30 at the C-terminal. **(B)** Growth of EBC-1 lung carcinoma cells treated with increasing concentrations of the

fusion proteins containing scMvDN30 at the N-terminal and decoyMET^{K842E} at the C-terminal. As positive control equimolar combinations of scMvDN30 and decoyMET^{K842E} (Combo) were included in the assay. Cells were analyzed after 3 days of treatment. Samples are in triplicates, bars represent SD. **(C)** Table showing IC_{50} values for each fusion protein calculated as the average value from at least three independent experiments, in comparison to the inhibitory activity exerted by MvDN30/decoyK842E in combination.

- Figure 12. Inhibition of tumor cell motility upon treatment with the fusion proteins containing an anti-HGFR antibody fragment and an extracellular portion of human HGFR.

HPAF-II cells were pre-incubated with the different fusion proteins (3 µM) and then were stimulated with 6.25 ng/ml HGF. Cell scattering was monitored in real time using an X-CELLigence RTCA device and is expressed as Normalized Cell Index. Samples were in triplicates. **(A)** Cells treated with the fusion proteins containing decoyMET^{K842E} at the N-terminal and scMvDN30 at the C-terminal. **(B)** Cells treated with the fusion proteins containing scMvDN30 at the N-terminal and decoyMET^{K842E} at the C-terminal. As positive control equimolar combinations of scMvDN30 and decoyMET^{K842E} (COMBO) were included in the assay. **(C)** Table showing the percentage of cell motility calculated as the average value from two independent experiments, versus the cells treated with HGF.

- Figure 13. Fusion proteins containing an anti-HGFR antibody fragment and an extracellular portion of human HGFR reduce metastatic dissemination of pancreatic cancer cells in hHGF-Ki mice.

Luciferase-expressing Capan-1 cells were injected in the pancreas of hHGF-Ki mice and stratified into

seven homogeneous groups: VEHICLE (*n*=14), decoyMET^{K842E} and scMvDN30 in 1:1 combination (two groups), and four groups to test different fusion proteins, containing either decoyMET^{K842E} at the N-terminal and scMvDN30 at the C-terminal or containing scMvDN30 at the N-terminal and decoyMET^{K842E} at the C-terminal. Two different linkers (L60 and L134) were analyzed. COMBO 1 (*n*=4): MvDN30 delivered 7x week and decoyMET^{K842E} delivered 2x week; COMBO 2 (*n*=6): MvDN30 delivered 4x week and decoyMET^{K842E} delivered 2x week; K842E_scMvDN30_L60 (*n*=4); scMvDN30_K842E_L60 (*n*=5); K842E_scMvDN30_L134 (*n*=5); K842E_scMvDN30_L134 (*n*=6). All the fusions were delivered 2x week. **(A)** Table showing the number of mice carrying liver metastasis versus total mice analyzed. Mice were considered positive when bioluminescence detected in the liver by IVIS Spectrum was higher than 10^5 photons/second. **(B)** Graph showing the percentage of bioluminescence detected by IVIS Spectrum in the liver of each experimental group versus vehicle treated group.

Detailed description of the invention

In the following description, numerous specific details are given to provide a thorough understanding of embodiments. The embodiments can be practiced without one or more of the specific details, or with other methods, components, materials, etc. In other instances, well-known structures, materials, or operations are not shown or described in detail to avoid obscuring aspects of the embodiments.

Reference throughout this specification to "one embodiment" or "an embodiment" means that a particular feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment. Thus, the appearances of the phrases

"in one embodiment" or "in an embodiment" in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

The headings provided herein are for convenience only and do not interpret the scope or meaning of the embodiments.

The instant disclosure concerns a novel combination of therapeutic agents for the treatment of a tumor and/or metastasis.

The present invention relies on the idea that a higher therapeutic robustness in the treatment of tumors and/or metastasis, preferably metastasis, can be achieved by targeting both HGF and HGFR. The experimental data witnessed herein actually show that vertical inhibition of the MET/HGF axis effectively hinders tumor cell growth, motility and invasion *in vitro* and significantly reduces metastatic spreading *in vivo*, providing a combined targeted therapy in a broad spectrum of cancers expressing wild-type MET.

In an embodiment, the present invention concerns an anti-Hepatocyte Growth Factor Receptor (HGFR) antibody fragment in combination with an extracellular portion of human HGFR for use in the treatment of a patient suffering from a tumor and/or metastasis, preferably metastasis, wherein:

(i) the anti-HGFR antibody fragment has only one paratope able to bind to an epitope of the extracellular portion of human HGFR and has antagonist activity towards HGFR,

(ii) the extracellular portion of human HGFR is capable of binding to Hepatocyte Growth Factor (HGF) in a stable manner and contains at least one amino acid

mutation within the epitope recognized by the anti-HGFR antibody fragment to prevent binding of the anti-HGFR antibody fragment thereto, and

(iii) the anti-HGFR antibody fragment and the extracellular portion of human HGFR are suitable for administration to the patient (a) in a protein form or (b) in a nucleic acid form.

In a preferred embodiment, the patient subject to the therapeutic treatment with the combination therapy herein disclosed carries a wild-type *MET* oncogene (i.e. he/she does not carry genetic alterations in the *MET* oncogene).

In one or more embodiments, the anti-HGFR antibody fragment contains one light chain variable domain (VL) and one heavy chain variable domain (VH), wherein the light chain and heavy chain variable domains are non-human or humanized, and wherein the light chain variable domain (VL) contains Complementary Determining Regions (CDRs) having the amino acid sequences set forth in SEQ ID No.: 1 to 3, and the heavy chain variable domain (VH) contains CDRs having the amino acid sequences set forth in SEQ ID No.: 4 to 6.

In a further embodiment, the anti-HGFR antibody fragment further contains one human light chain constant domain (CL) and one human heavy chain CH1 constant domain (CH1), the light chain variable domain (VL) being fused to the human light chain constant domain (CL) in the N- to C-terminal direction (thus generating a VL-CL light chain), the heavy chain variable domain (VH) being fused to the human heavy chain CH1 constant domain in the N- to C-terminal direction (thus generating a VH-CH1 heavy chain). In a preferred embodiment, the VL-CL light chain contains, preferably consists of, an amino acid sequence as set forth in SEQ ID No.: 7, and the VH-CH1 heavy chain

contains, preferably consists of, an amino acid sequence as set forth in SEQ ID No.: 8.

In different embodiments encompassed by the present invention, the anti-HGFR antibody fragment is in a nucleic acid form. In such a case, the anti-HGFR antibody fragment is encoded by a first and a second nucleic acid molecule, wherein:

(i) the first nucleic acid molecule encodes one light chain variable domain (VL) containing CDRs having the nucleic acid sequences set forth in SEQ ID No.: 9, 10 and 11, and wherein the light chain is non-human or humanized; and

(ii) the second nucleic acid molecule encodes one heavy chain variable domain (VH) containing CDRs having the nucleic acid sequences set forth in SEQ ID No.: 12, 13 and 14, and wherein the heavy chain is non-human or humanized.

In a further preferred embodiment, the anti-HGFR antibody fragment is encoded by a first and a second nucleic acid molecule, wherein:

(i) the first nucleic acid molecule encodes (a) one light chain variable domain (VL) containing CDRs having the nucleic acid sequences set forth in SEQ ID No.: 9, 10 and 11, wherein the light chain is non-human or humanized, and (b) one human light chain constant domain (CL) in the 5′- to 3′-terminal direction (i.e. a VL-CL light chain); and

(ii) the second nucleic acid molecule encodes (a) one heavy chain variable domain (VH) containing CDRs having the nucleic acid sequences set forth in SEQ ID No.: 12, 13 and 14, wherein the heavy chain is non-human or humanized, and (b) one human heavy chain CH1 constant domain (CH1) in the 5′- to 3′-terminal direction (i.e. a VH-CH1 heavy chain).

In a preferred embodiment, the first nucleic acid molecule (encoding the VL-CL light chain) contains, preferably consists of, a nucleic acid sequence as set forth in SEQ ID No.: 15, and the second nucleic acid molecule (encoding the VH-CH1 heavy chain) contains, preferably consists of, a nucleic acid sequence as set forth in SEQ ID No.: 16.

In a further embodiment encompassed by the present invention, the anti-HGFR antibody fragment is an anti-HGFR single-chain Fab fragment. The anti-HGFR single-Fab fragment contains, preferably consists of, an amino acid sequence as set forth in SEQ ID No.:_17 or the anti-HGFR single-Fab fragment is encoded by a nucleic acid molecule containing, preferably consisting of, a nucleic acid sequence as set forth in SEQ ID No.: 18.

In one or more embodiments, the present invention provides for the extracellular portion of human HGFR containing the SEMA, PSI, IPT-1, IPT-2, IPT-3 and IPT-4 domains.

In a preferred embodiment, the extracellular portion of human HGFR contains, preferably consists of, an amino acid sequence as set forth in SEQ ID No.: 19, wherein one or more of the amino acids between position 797 and position 875 of SEQ ID No.: 19 are mutated. According to the present disclosure, the extracellular portion of human HGFR has a sequence mutated according to the knowledge concerning the anti-HGFR antibody and HGF binding sites on HGFR, in a way that the antibody do not interact with the HGFR, while HGF retains its binding ability to the extracellular portion of human HGFR. In a further preferred embodiment, the extracellular portion of human HGFR has the sequence as set in SEQ ID No.: 20.

In a further embodiment encompassed by the present invention, the extracellular portion of human HGFR is

in form of a nucleic acid molecule, wherein the nucleic acid molecule contains, preferably consists of, the nuclei acid sequence as set forth in SEQ ID No.:_21 wherein one or more of the nucleic acids between position 2391 and position _2625 of SEQ ID No.: 21 are mutated. According to the present disclosure, the nucleic acid molecule encodes an extracellular portion of human HGFR having a sequence mutated according to the knowledge concerning the anti-HGFR antibody and HGF binding sites on HGFR, in a way that the antibody do not interact with the HGFR, while HGF retains its binding ability to the extracellular portion of human HGFR. In a further preferred embodiment, the nucleic acid molecule encoding the mutated extracellular portion of human HGFR has the sequence as set in SEQ ID No.: 22.

In different embodiments encompassed by the present invention, the anti-HGFR antibody fragment is conjugated by means of a linker to the extracellular portion of human HGFR in the N- to C-terminal direction, thus generating an anti-HGFR antibody fragment-linker-extracellular portion of human HGFR fusion protein. Alternatively, the extracellular portion of human HGFR is conjugated by means of a linker to the anti-HGFR antibody fragment in the N- to C-terminal direction thus generating an extracellular portion of human HGFR-linker-anti-HGFR antibody fragment fusion protein.

In different embodiments encompassed by the present invention, the nucleic acid molecule encoding the anti-HGFR antibody fragment is linked by means of a nucleic acid molecule encoding a linker to the nucleic acid molecule encoding the extracellular portion of human HGFR in the 5′- to 3′-terminal direction, thus encoding an anti-HGFR antibody fragment-linker-

extracellular portion of human HGFR fusion protein. Alternatively, the nucleic acid molecule encoding the extracellular portion of human HGFR is linked by means of a nucleic acid molecule encoding a linker to the nucleic acid molecule encoding the anti-HGFR antibody fragment in the 5'- to 3'-terminal direction, thus encoding an extracellular portion of human HGFR-linker-anti-HGFR antibody fragment fusion protein.

The instant invention therefore provides for a fusion protein comprising in the N- to C-terminal direction:

(i) an anti-HGFR antibody fragment, a linker, and an extracellular portion of human HGFR; or

(ii) an extracellular portion of human HGFR, a linker, and an anti-HGFR antibody fragment.

In a preferred embodiment, the fusion protein as defined above contains, preferably consists of, an amino acid sequence selected from any one of SEQ ID No.: 23 to 28.

The instant invention therefore encompasses nucleic acid molecules encoding a fusion protein comprising in the N- to C- terminal direction:

(i) an anti-HGFR antibody fragment, a linker, and an extracellular portion of human HGFR; or

(ii) an extracellular portion of human HGFR, a linker, and an anti-HGFR antibody fragment.

In a preferred embodiment, the nucleic acid molecule encoding the fusion protein as defined above contains, preferably consists of, a nucleic acid sequence selected from any one of SEQ ID No.: 29 to 34.

In a further embodiment, the present disclosure concerns a pharmaceutical product comprising, in a single bottle or in two bottles, (a) an anti-Hepatocyte Growth Factor Receptor (HGFR) antibody fragment and a pharmaceutically acceptable vehicle, and (b) an

extracellular portion of human HGFR and a pharmaceutically acceptable vehicle, wherein:

(i) the anti-HGFR antibody fragment has only one paratope able to bind to an epitope of the extracellular portion of human HGFR and has antagonist activity towards HGFR,

(ii) the extracellular portion of human HGFR is capable of binding to Hepatocyte Growth Factor (HGF) in a stable manner and contains at least one amino acid mutation within the epitope recognized by the anti-HGFR antibody fragment to prevent binding of the anti-HGFR antibody fragment thereto,

(iii) the anti-HGFR antibody fragment and the extracellular portion of human HGFR are either in a protein form or in a nucleic acid form.

The pharmaceutical product therefore comprises the anti-HGFR antibody fragment and the extracellular portion of human HGFR having the features, alone or in any respective combination, disclosed above.

In one aspect, the invention provides a method of treating a tumor and/or metastasis in a subject, said method comprising administering to the subject an effective amount of an anti-HGFR antibody fragment in combination with an extracellular portion of human HGFR, whereby said condition is treated.

In one aspect, the invention provides a method of inhibiting the growth of a cell that expresses HGFR, said method comprising contacting said cell with an anti-HGFR antibody fragment in combination with an extracellular portion of human HGFR of the invention thereby causing an inhibition of growth of said cell.

In one aspect, the invention provides a method of therapeutically treating a mammal having a cancerous tumor and/or metastasis, said method comprising administering to said mammal an effective amount of an

anti-HGFR antibody fragment in combination with an extracellular portion of human HGFR, thereby effectively treating said mammal.

In one aspect, the invention provides a method for treating a cell proliferative disorder, said method comprising administering to a subject in need of such treatment an effective amount of an anti-HGFR antibody fragment in combination with an extracellular portion of human HGFR, thereby effectively treating or preventing said cell proliferative disorder.

In one aspect, the invention provides a method of therapeutically treating a tumor and/or metastasis in a mammal, wherein the growth of said tumor is at least in part dependent upon a growth potentiating effect of the system HGFR/HGF, as the consequence of either an increase in cell proliferation, a protection from apoptosis, or both. Said method comprises contacting a tumor cell with an effective amount of an anti-HGFR antibody fragment in combination with an effective amount of an extracellular portion of human HGFR of the invention, thereby effectively treating said tumor and/or metastasis.

The tumor, that can be effectively treated with the combination therapy of the present invention, are selected from breast, colorectal, lung, colon, pancreatic, prostate, ovarian, cervical, central nervous system, renal, hepatocellular, bladder, gastric, head and neck tumor cell, papillary carcinoma (e.g. the thyroid gland), melanoma, lymphoma, myeloma, glioma/glioblastoma (e.g. anaplastic astrocytoma, multiforme glioblastoma, anaplastic oligodendroglioma, anaplastic oligodendroastrocytoma), leukemia cell, sarcoma, rhabdomyosarcoma, or a tumor from a cancer of Unknown Primary origin (CUP).

In one embodiment, a cell that is therapeutically targeted in a method of the invention is a hyperproliferative and/or hyperplastic cell. In one embodiment, a cell that is targeted in a method of the invention is a dysplastic cell. In yet another embodiment, a cell that is targeted in a method of the invention is a metastatic cell. In a further embodiment, a cell that is targeted in a method of the invention is a HGFR expressing cell belonging to the microenvironment sustaining the tumor and/or the metastasis.

The therapeutic methods of the invention can further comprise additional treatment steps. For example, in one embodiment, the therapeutic method further comprises a step wherein a targeted tumor cell and/or tissue is exposed to a radiation treatment or a chemotherapeutic treatment. In a further embodiment, a targeted tumor cell and/or tissue is treated, in addition to an anti-HGFR antibody fragment in combination with an extracellular portion of human HGFR of the invention, with molecules specifically hitting other targets relevant in the maintenance of the transformed phenotype (i.e. anti-EGFR molecules).

The expression "antagonist activity" of the anti-HGFR antibody fragment as used herein refers to the antibody that is able to quench the intracellular signaling elicited in a cell upon HGFR activation. The antagonistic activity of the said antibody can be measured by the evaluation of the HGFR level of expression and/or phosphorylation by conventional techniques such as western blot, immunofluorescence, immunohistochemistry, ELISA, cytofluorimeter analysis or any other method that includes the use of an antibody that recognize specifically HGFR, or the HGFR residues $Tyr^{1234-1235}$ if phosphorylated (ie the major

phosphorylation site of MET[9], or the HGFR residues Tyr [1349/1356] if phosphorylated (ie the docking site of MET[22].

As used herein, the expression "the extracellular portion of human HGFR is capable of binding to human HGF in a stable manner" means that the extracellular portion of human HGFR binds to HGF with a calculated Kd not higher than 100 nM.

The expression "the extracellular portion of human HGFR contains at least one amino acid mutation at the epitope recognized by the anti-HGFR antibody fragment", as used herein, means the presence of one or more mutations (i.e. aminoacid substitutions and/or deletions and/or insertions) within the extra-cellular portion of HGFR, able to induce a modification within the extra-cellular portion of HGFR that prevents the engagement of the above region by the antibody variable domains. The skilled man in view of his common general knowledge (represented i.a. by the possibility to generate a cDNA including a single nucleotide change in a given DNA sequence using specific primers during DNA duplication see Maniatis T. Molecular cloning: A laboratory manual Cold Spring Harbor Laboratory (1982)) does not need further details about the realization of a mutated form of the extracellular portion of human HGFR retaining the ability to bind to human HGF but not to the anti-HGFR antibody fragment.

The present invention must not therefore be interpreted as encompassing only the mutated extra-cellular portion of human HGFR as disclosed herein (i.e. SEQ ID No.: 8), since the skilled man in view of the common general knowledge can produce further mutated versions of the extra-cellular portion of human HGFR having SEQ ID No.: 19 that prevent the bind of the anti-HGFR antibody thereto.

As used herein, the term "single-chain Fab fragment" refers to a single polypeptide encoding for VL, CL, VH, CH1 domains of an antibody, wherein VL and CL can be positioned at the N-terminus of the said polypeptide and joined to the VH and CH1 domains by a flexible linker or VH and CH1 domains can be positioned at the N-terminus of the said polypeptide and linked to VL and CL domains by a flexible linker.

The terms "SEMA", "PSI", "IPT-1", "IPT-2", "IPT-3" and "IPT-4" refer to the HGFR domains constituting the extracellular region of HGFR. Such domains names belong to the common general knowledge of a skilled man as represented i.a. by [4, 13]. SEMA domain is a protein interacting module in common to semaphorins and plexins encompassing the region comprised between aminoacids 25-516 of MET (SEQ ID No.: 19); PSI is a domain in common with plexins, semaphorins, integrins encompassing the region comprised between aminoacids 519-561 of MET (SEQ ID No.: 19); the IPT domain - repeated four times - is a region Immunoglobulin Like in common with plexins and transcription factors, encompassing the region comprised between amino acids 563-934 of MET (SEQ ID No.: 19. In detail IPT repeat 1 covers the amino acid positions 563-656 of SEQ ID No.: 19, IPT repeat 2 covers the amino acid positions 657-740 of SEQ ID No.: 19, IPT repeat 3 covers the amino acid positions 741-837 of SEQ ID No.: 19, IPT repeat 4 covers the amino acid positions 838-934 of SEQ ID No.: 19.

The fusion proteins disclosed herein can be easily manufactured either in the form of proteins or in the form of nucleic acid molecules encoding the fusion proteins by a skilled man in view of the common general knowledge of the field related to the recombinant DNA technology, as represented i.a. by Maniatis T.

Molecular cloning: A laboratory manual Cold Spring Harbor Laboratory (1982). As example the following standard procedure can be followed: (i) synthesis of the corresponding cDNA sequence, (ii) insertion of the cDNA into a plasmid suitable for expression in mammalian by conventional recombinant DNA methods, (iii) transient or stable transfection with the above mentioned plasmid of a mammalian cell line, (iv) collection of the culture supernatant, (v) purification by affinity chromatography of the fusion protein. The linkers used to conjugate the two protein sequences, i.e. the anti-HGFR antibody fragment and the extracellular portion of human HGFR, can have different length and/or aminoacid composition, being flexible, rigid or a combination of flexible and rigid regions. The linkers employed in the production of the fusion proteins can be selected from, but not limited to, any one of the amino acid sequences as set forth in SEQ ID No.: 35, 36 and 37. The linkers employed in the production of the nucleic acid molecules encoding the fusion proteins can be selected from, but not limited to, any one of the nucleic acid sequences as set forth in SEQ ID No.: 38, 39 and 40.

In the following the instant invention will be exemplified by referring to a combination of the anti-HGFR antibody fragment containing the CDRs of SEQ ID No.: 1 to 6 belonging to the DN30 monoclonal antibody disclosed in WO 2007/090807 and the extracellular portion of HGFR containing the SEMA, PSI, IPT-1 to IPT-4 domains, briefly named in the following DecoyMET.

The present inventors unexpectedly discovered that monovalent DN30 antibody fragment (MvDN30) and DecoyMET, used in combination, allow dual targeting of ligand and receptor, acting simultaneously on MET-expressing cancer cells and on HGF-secreting tumor

stroma and exerting a synergistic effect in the treatment of tumors and/or metastasis.

Pharmacological inhibition of the MET tyrosine kinase receptor in oncogene 'addicted' cancer cells extinguishes cell proliferation and invasion. Accordingly, patients with *MET* amplified advanced NSCLC, metastatic gastric or esophageal cancer respond to anti-MET therapy[5, 14, 19]. On the other hand, cancer cells without *MET* genetic alterations exploit the 'physiological' program triggered by the oncogene as an 'expedient' to boost the malignant phenotype[6]. '*Expedience*' requires stimulation of wild-type MET by its ligand HGF. In this respect, the contribution of tumor microenvironment to cancer progression and metastasis is becoming increasingly relevant, as experimental evidences suggest that the malignant phenotype does not develop in a strictly cell-autonomous way, but in a rather complex interplay between cancer cells and host stroma. The tumor microenvironment is a significant source of HGF, secreted by stromal cells of mesenchymal origin as an inactive precursor (proHGF). The latter is stored in the extracellular matrix, thanks to its avidity for heparansulfates, and is activated by specific proteases produced either by tumor or stromal cells. Therefore, an excess of biologically active ligand is readily available for binding the MET receptor and triggers the invasive growth signaling cascade in 'non-addicted' cells. The data provided herein show that in conditions of MET '*expedience*', a concomitant intervention hitting both sides of the MET/HGF axis results in improved inhibitory activity. Simultaneous targeting was achieved combining a monovalent MET antibody, MvDN30, with a recombinant soluble receptor, decoyMET^{K842E}. The data provided herein indicate that there is no

redundancy in targeting the same pathway with complementary tools. The two inhibitors were selected on the basis of their mechanisms of action: the antibody induces the physical removal of MET from the cell surface by 'shedding' of the ectodomain. The latter is released in the extracellular environment and acts as 'decoy' for HGF. Exogenous supply of recombinant decoyMET reinforces the HGF-sequestering activity of the endogenous decoyMET generated by MvDN30. To enable the concomitant use of MvDN30 and decoyMET, a modified soluble receptor was generated (decoyMET^{K842E}), deficient in MvDN30 interaction but endowed with high affinity binding properties to HGF. The two agents in combination cooperate in a variety of cancer cells, reducing the effective therapeutic dose. Moreover this 'dual strategy' displays a strong synergistic effect, potentially exerting a superior anti-tumor efficacy. MET expression in a sub-population of stem/progenitor cancer cells has been defined as MET '*inherence*', *i.e.* the physiologic (inherent) HGF-induced intracellular response activated in cancer stem cells - in the absence of genetic lesions- responsible for resistance to targeted therapies, such as Epidermal Growth Factor Receptor (EGFR) inhibitors in colorectal cancer. The notion linking cancer stem cells and resistance to conventional therapies is largely accepted, and the role of microenvironmental HGF in maintaining the stem phenotype of MET-expressing progenitor cells is becoming more and more established. An effective anti-MET treatment, as the combination of MvDN30 and decoyMET^{K842E}, represents a therapeutic support to blunt cancer stem cells and to oppose the onset of resistance to targeted therapies.

The role of host microenvironment is difficult to investigate in mouse xenografts due to the limited

cross-reactivity between murine stromal-derived factors and specific targets on human cancer cells. This is particularly significant in the case of the HGF/MET system, because murine HGF does not activate human MET. The development of genetically modified mouse strains expressing the Knocked-in human HGF gene (hHGF-Ki mice) circumvented this problem. In this transgenic model, it is shown that concomitant targeting of environmental HGF and its receptor on cancer cells may be an effective therapeutic strategy to hinder malignant progression and metastasis.

Xenografts of pancreatic adenocarcinoma are characterized by precocious metastatic dissemination, occurring very early during tumor development, and are sustained by an abundant stromal compartment. Recently, HGF secreted by pancreatic stellate cells was identified as a factor playing a relevant function in tumor-stroma interaction in this type of malignancy. In an orthotopic mouse model of human pancreatic adenocarcinoma grafted in hHGF-Ki mice, MvDN30 and decoyMET^{K842E} in combination slightly delayed tumor growth, as expected in a model of '*expedience*' where MET is not the driver oncogene. On the other hand, the combo treatment proved to be very effective in reducing the metastatic spread, suggesting a possible therapeutic application in non-addicted cancer cells featuring wild-type MET. Epidemiological data show that only 2-3% of epithelial cancers rely on *MET* oncogenic addiction, either because of gene amplification, rearrangement or mutation[29]. For this reason, a number of clinical trials -addressing unselected populations of cancer patients- failed. On the other hand, the vast majority of carcinomas exploit ligand-dependent wild-type MET activation to unleash the invasive metastatic phenotype in response to hypoxia, ionizing radiation or

chemotherapy. Thus these findings suggest that a large cohort of patients -currently unfit to MET targeted therapy due to the absence of a specific genetic lesion- should benefit from treatments encompassing a dual antibody-decoy strategy, that allows optimal blockade of the HGF-driven MET signaling.

Therapeutic compositions comprising the active ingredients of the instant invention, i.e. an anti-HGFR antibody fragment and an extracellular portion of human HGFR, can be prepared either as a single preparation containing the two active ingredients mixed together or as separate preparations, one containing an anti-HGFR antibody fragment and the other an extracellular portion of human HGFR. The active ingredients are prepared for storage by mixing the active ingredient(s) having the desired degree of purity with physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of aqueous solutions, lyophilized or other dried formulations. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers; antioxidants; preservatives; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids; monosaccharides, disaccharides, and other carbohydrates; chelating agents; sugars; salt-forming counter-ions; metal complexes and/or non-ionic surfactants. The formulations disclosed herein may also contain other active compound(s) as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect the therapeutic activity of the anti-HGFR

antibody fragment and of the extracellular portion of human HGFR. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredient(s) may also be entrapped in microcapsules prepared by means of techniques disclosed i.a. in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

The formulations to be used for *in vivo* administration must be sterile.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the active ingredients of the invention, which matrices are in the form of shaped articles, e.g. films, or microcapsule.

The active ingredients of the invention can be used either alone or in combination with another antibody, chemotherapeutic agent(s) (including cocktails of chemotherapeutic agents), other cytotoxic agent(s), anti-angiogenic agent(s), cytokines, and/or growth inhibitory agent(s). Such combined therapies noted above include combined administration (where the two or more agents are included in the same or separate formulations), and separate administration, in which case, administration of the antibody of the invention can occur prior to, and/or following, administration of the adjunct therapy or therapies.

The active ingredients of the present invention (and adjunct therapeutic agent(s)) are administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. The active ingredients of the instant invention can be suitably administered by pulse

infusion, particularly with declining doses of the active ingredients. Dosing can be by any suitable route, e.g. by injections, such as intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic. The active ingredients of the invention can be also delivered by gene transfer by mean of viral vectors (i.e. lentiviral vectors), administered locally or systemically or by mean of cell therapy, *i.e.* delivery by intravenous of local injection of human cells genetically modified by viral vectors transduction (i.e. lentiviral vectors) to express an anti-HGFR antibody fragment and an extracellular portion of human HGFR.

The active ingredients of the invention will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The active ingredients of the invention need not be, but may optionally be formulated with one or more agents currently used to prevent or treat the disorder in question. The effective amount of such other agents depends on the amount of active ingredients of the invention present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as used hereinbefore or about from 1 to 99% of the heretofore employed dosages.

For the treatment of disease, the appropriate dosage of the active ingredients of the invention (when

used alone or in combination with other agent(s) such as chemotherapeutic agent(s)) will depend on the type of disease to be treated, the severity and course of the disease, whether the active ingredients are administered for preventive or therapeutic purposes, the patient's clinical history and response to the active ingredients of the invention are duly taken into consideration, and at the discretion of the attending physician.

The antibody fragment is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 mg/kg to 15 mg/kg of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. One typical daily dosage might range from about 1 mg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. One exemplary dosage of the antibody fragment would be in the range from about 0.05 mg/kg to about 20 mg/kg. Thus, one or more doses of about 0.5 mg/kg, 2.0 mg/kg, 4.0 mg/kg or 10 mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, e. g. every week or every three weeks (e. g. such that the patient receives from about two to about twenty, e. g. about six doses of the antibody). An initial higher loading dose, followed by one or more lower doses may be administered. An exemplary dosing regimen comprises administering an initial loading dose of about 4 mg/kg, followed by a weekly maintenance dose of about 2 mg/kg of the

antibody. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays. The extracellular portion of human HGFR must be delivered at the same time of the antibody fragment and must be dosed in proportion to the antibody fragment, preferentially but not limited to, the molar ratio 1:1 antibody fragment:decoyMET, depending on the type and severity of the disease.

RESULTS

Site-directed mutagenesis of the DN30-binding epitope in decoyMET

To exploit the activity of MvDN30 antibody and decoyMET in combination, it is mandatory to prevent interaction between the two molecules that would result in mutual neutralization. It has been shown previously that MvDN30 recognizes an epitope within the IPT-4 domain of MET extracellular region at the boundary with the IPT-3 domain[28]. Former studies showed that the parental DN30 antibody, that binds the human receptor with picomolar affinity, also interacts with dog and rat MET[11,23], while it does not cross-react with mouse[21]. Upon alignment of the IPT-3 and IPT-4 aminoacid sequences of the above-mentioned mammalian species, a number of residues were identified that are selectively changed in the mouse (Fig. 1A). To test if human-to-mouse swapping of single aminoacid residues could impair antibody binding, soluble receptors carrying point mutations in the IPT 3-4 domain were generated and tested against the DN30 antibody. Substitution of lysine 842 with glutamic acid generated decoyMET^{K842E}, a modified soluble receptor not recognized anymore by the antibody, while all other mutations did not affect the interaction (Fig. 1B). The inability of the antibody to

interact with decoyMET^{K842E} was confirmed in ELISA assays, performed with affinity-purified decoys in solid phase and DN-30 antibody in liquid phase (Fig. 1C). Hence, substitution of a basic amino acid at position 842 with an acidic residue resulted in critical perturbation of the binding site, hampering decoyMET^{K842E}-antibody interaction.

DecoyMET^{K842E} binds HGF with high affinity

The present inventors then investigated if the K842E amino acid substitution interferes with binding of HGF. In ELISA binding assays, the affinity constant of decoyMET^{K842E} for HGF (K_d = 1.04 ± 0.05 nM) was superimposible with the K_d of 1.44 ± 0.07 nM measured for the wild-type decoyMET (Fig. 2A). Finally, the inhibitory activity of decoyMET^{K842E} was tested in an HGF-induced MET phosphorylation assay. As shown in Fig. 2B, wild-type decoyMET and decoyMET^{K842E} inhibited HGF-dependent MET phosphorylation in A549 lung cancer cells with comparable potency. Thus, the K842E substitution does not interfere with the formation of a stable complex with HGF, and leaves the inhibitory activity of the decoy unaffected.

MvDN30 and decoyMET^{K842E} cooperate in inhibition of MET phosphorylation and downstream biological responses

To assess the inhibitory activity elicited by concomitant targeting of the ligand and the receptor on MET signal transduction, MET phosphorylation was tested in the presence of MvDN30 and decoyMET^{K842E}, either alone or in combination. To this end, A549 cells, expressing a wild-type MET receptor, were stimulated with nanomolar concentrations of HGF and MET activation was measured by phosphoMET antibodies. Both molecules displayed inhibitory activity, and the combination of

the two was more efficient (Fig. 3A). Analysis of downstream signaling transducers confirmed that the combination achieved the most effective inhibition of ERK and AKT activation (Fig. 3B).

On the biological ground, MvDN30 and decoyMET^{K842E} in combination strongly inhibited anchorage-independent colony growth, in cellular models of both autocrine or paracrine HGF stimulation. In the former, U87-MG glioblastoma cells -displaying very efficient colony growth in soft agar due to a MET/HGF autocrine loop- were inhibited by 75% when MvDN30 and decoyMET^{K842E} were used in combination, while colony growth inhibition never exceeded 40% when the two molecules were used as single agents (Fig. 3B). Likewise, the combo treatment completely blocked the formation of A549 soft agar colonies induced by nanomolar concentrations of exogenously-administered HGF, while MvDN30 and decoyMET^{K842E} alone achieved a partial though significant inhibition of colony growth (65% and 74%, respectively, Fig. 3C). Similar results were obtained in invasion assays performed in Matrigel-coated chambers: MvDN30 and decoyMET^{K842E} in combination reduced HGF-driven invasion of HPAF-II human pancreatic adenocarcinoma cells by 85%, while as single agents achieved only 59% and 52% inhibition, respectively (Fig. 3D). In all these biological systems, MvDN30 and decoyMET^{K842E} combination impaired MET phosphorylation more efficaciously than the single treatments (Fig. 4).

To assess if the effect of the two inhibitors is additive or synergistic, a quantitative motility assay was performed. HPAF-II human pancreatic adenocarcinoma cells, expressing wild-type MET, were induced to scatter by HGF. Cell scattering was quantified by measuring the variations of electrical impedance of cell-covered electrodes (X-CELLigence Real Time Cell

Analyzer). The two molecules in combination reduced HGF-dependent cell scattering in dose-dependent fashion, starting inhibition at 250 nM and achieving complete blockage in the micromolar range (Fig. 5 A and B). The Cell Index values measured at the end of the experiment (time = 48h) were normalized on HGF (Fig. 5C) and elaborated with the CalcuSYN software to assess synergism (Fig. 5D): for all concentrations examined, the calculated Combination Index (CI) was well below 0.5 (CI = 0.1, 0.09, 0.17 and 0.38 for 0.06, 0.25, 1 and 4 μM, respectively), indicating that MvDN30 and decoyMET^{K842E} display a synergistic behavior[10].

MvDN30 and decoyMET^{K842E} attenuate the invasive phenotype and reduce metastatic spread

The inhibitory activity of MvDN30 and decoyMET^{K842E} in combination was assessed *in vivo* in mouse models of ligand-driven MET stimulation.

The U87-MG glioblastoma xenograft tumor model of autocrine HGF stimulation was investigated. Cells were injected subcutaneously in NOD-SCID mice; when tumors reached a volume of 80-100 mm^3, mice were stratified in homogeneous groups and randomly assigned to 4 treatment arms: Vehicle, MvDN30, decoyMET^{K842E} or the combination of the two. After 22 days of treatment, mice were sacrificed and primary tumors excised for histological examination. While the combo treatment induced only a marginal inhibition of growth (Fig. 6A), reduction of phenotypic hallmarks of tumor invasion was observed (Fig. 6B).

The combo treatment was also challenged in a paracrine model of HGF stimulation. As previously reported, mouse HGF does not activate the human MET receptor[24, 30]. Hence, to test the inhibitory activity of decoyMET^{K842E} in xenografts of human tumors, we

exploited a transgenic SCID mouse where the mouse HGF gene was replaced by the human gene (hHGF-Ki)[20]. Human pancreatic adenocarcinoma cells (HPAF-II) were labeled by transduction with the luciferase gene and injected orthotopically in the pancreas of hHGF-Ki mice. Engraftment was checked by analysis of total body luminescence; mice were stratified into homogeneous groups on the basis of bioluminescence values, and randomly assigned to 4 treatment groups: VEHICLE, MvDN30, decoyMET^{K842E} or the combination of the two. Tumor growth was monitored by total body luminescence (Fig. 7A). At sacrifice, 5 weeks after cell injection, tumors were excised and analyzed for MET phosphorylation, proliferation, apoptosis and vimentin/E-cadherin expression (markers of epithelial-mesenchimal transition). Concurrently, lungs were collected for histochemical evaluation of metastatic nodules. The phosphorylated status of MET at Tyrosines 1234-1235 was inhibited by either agents, and the combo treatment elicited a dramatic effect (Fig. 8A). As expected in this model of *MET* oncogene '*expedience*' (*i.e.* expression of wild-type MET), proliferation as well as apoptosis were modestly affected, and only by the combo treatment (Fig. 7B and C). Analysis of the ratio between vimentin and E-cadherin showed that the combination treatment pushed cancer cells towards a more epithelial phenotype (Fig. 8B). Accordingly, concomitant administration of MvDN30 and decoyMET^{K842E} significantly inhibited MET-driven metastatic dissemination to the lung (Fig. 8C).

Generation of a panel of fusion proteins containing an anti-HGFR antibody fragment and an extracellular portion of human HGFR

To generate a single cDNA encoding the fusion

proteins containing an anti-HGFR antibody fragment and an extracellular portion of human HGFR the present inventors first designed a single chain MvDN30 (ScMvDN30), introducing a linker between the light chain (VL-CL) and the heavy chain (VH-CH1). The linker was flexible, rich in glycine/serine residues and had a length that allowed preferentially the generation of monomeric molecules, constituted by the association of antibody light and heavy chains belonging to the same polypeptide and not from separated polypepdites thus generating dimers and/or multimers. The preferred amino acid sequence of the linker is reported in SEQ ID No.: 36; the corresponding nucleotide sequence is reported in SEQ ID No.: 39. Then ScMvDN30 cDNA was fused in frame with Decoy MET^{K842E}. Two groups of fusion proteins has been generated: i) $decoyMET^{K842E}$ was located at the N-terminal and ScMvDN30 at the C-terminal, and ii) scMvDN30 was located at the N-terminal and $decoyMet^{K842E}$ at the C-terminal. To guarantee a high grade of freedom to the entire structure the present inventors introduced a second linker between the two moieties. Three different linkers, modified in amino acid composition and/or length, has been employed: i) L45: a rigid linker of 45 amino acids constituted of two repeats of a sequence rich in alanine and charged residues, SEQ ID No.: 35, the corresponding nucleotide sequence is reported in SEQ ID No.: 38; ii) L60, a flexible linker rich in glycine/serine residues of 60 amino acids SEQ ID No.: 36, the corresponding nucleotide sequence is reported in SEQ ID No.: 39; iii) L134, a combination of flexible and rigid regions of 134 amino acids SEQ ID No.:37, the corresponding nucleotide sequence is reported in SEQ ID No.: 40. A schematic representation of the fusion proteins is reported in Fig. 9.

Fusion proteins containing scMvDN30 at the N-terminal and decoyMET^{K842E} at the C-terminal bind with high affinity both MET and HGF.

The present inventors investigated by ELISA assays if the new generated fusion proteins maintained the ability of binding both HGF and MET. All the fusion proteins interacted with HGF with an affinity superimposable with the one of decoyMET^{K842E} (Fig. 10A), while the fusion proteins containing scMvDN30 at the C-terminal recognized MET with a lower affinity with respect to the fusions with the antibody located at the N-terminal. The Kd values measured for this latter group of molecules were comparable to the one measured for scMvDN30 alone (Fig. 10B).

The fusion proteins efficiently inhibited tumor cell growth.

The present inventors assessed the inhibitory properties of the fusion proteins measuring cell growth impairment after three days of treatment in comparison with scMvDN30 combined with decoyMET^{K842E} at equimolar ratio. All the fusion proteins displayed inhibitory properties in a dose-dependent manner. In the reported experiment the fusions with L60 linker were less potent than the other molecules, while L45 and L134 proteins displayed an activity similar to scMvDN30-decoyMET^{K842E} in combination (Fig. 11).

The fusion proteins efficiently inhibited HGF-induced cell motility.

To assess the effect of the fusion proteins in inhibiting HGF-driven cell motility the present inventors performed the quantitative motility assay with the X-CELLigence Real Time Cell Analyzer using as

model the HPAF-II human pancreatic adenocarcinoma cells treated with HGF. All the fusions were able to reduce HGF-dependent cell scattering (Fig. 12).

The fusion proteins reduced metastatic spread more potently than MvDN30 and decoyMET^{K842E} in combination

The inhibitory activity the fusion proteins was assessed *in vivo* in the hHGF-Ki SCID mouse model. Human pancreatic adenocarcinoma cells (Capan-I) were labeled by transduction with the luciferase gene and injected orthotopically in the pancreas of the mice. Engraftment was checked by analysis of total body luminescence; mice were stratified into homogeneous groups on the basis of bioluminescence values, and treated with the fusion proteins including the linkers L60 or L134. As references MvDN30 and decoyMET^{K842E} in 1:1 combination and vehicle were included in the experimental groups. At sacrifice, 5 weeks after cell injection, livers were excised and analyzed by IVIS Spectrum to score metastasis. Concomitant administration of MvDN30 and decoyMET^{K842E} significantly inhibited MET-driven metastatic dissemination to the liver; a high efficacy was scored if MvDN30 administration was done daily (COMBO 1 group), while the therapeutic response was significantly reduced if less frequent administrations were applied (COMBO 2 group). This limitation was not scored in the mice treated with the fusion proteins. In fact all of them reduced metastatic dissemination to the liver with high efficacy, even if the delivery was done twice a week (Fig.13).

MATERIAL AND METHODS

Cell culture

A549 human lung adenocarcinoma cells, HPAF-II and Capan-1 human pancreatic adenocarcinoma cells, and U87-

MG human glioblastoma cells were obtained from ATCC/LGC Standards S.r.l. (Sesto San Giovanni, Italy); EBC-1 human lung carcinoma cells were from the Japanese Collection of Research Bioresources. All the cells were cultured as suggested by the supplier. All cell cultures were tested for mycoplasma contamination.

Generation, expression and purification of the anti-HGFR antibody fragment

The cDNAs encoding the light chain (VL-CL) and the heavy chain (VH-CH1) of the anti-HGFR antibody fragment able to the recognize an epitope of the extracellular portion of HGFR were generated by gene synthesis performed in outsourcing by Invitrogen GeneArt Gene Synthesis (ThermoFisher) according to the sequences reported in SEQ ID No.: 7 and SEQ ID No.: 8. Antibody fragments were produced by transient transfection of HEK-293T cells with pcDNA3.1 plasmids (cat.# V79020 Invitrogen Corporation, Camarillo, CA) expressing cDNAs encoding for the light chain (VL-CL) and the heavy chain (VH-CH1). Transfected cells were starved for three days and cell culture supernatants containing the soluble receptors were collected. Purification of the recombinant proteins was done by affinity chromatography using HisTrap HP columns (cat.# 17524701 GE Healthcare, Freiburg, Germany) according to manifacturer's instructions. Large-scale protein production and purification were performed by U-Protein Express BV (Utrecht, The Netherlands)._

Generation, expression and purification of mutated MET ectodomains

cDNA sequences of human MET ectodomains (decoyMET) carrying single aminoacid substitutions were generated using the QuickChange II Site-Directed Mutagenesis Kit

(cat.# 200524 Agilent Technologies, Santa Clara, CA), following the instruction of the manufacturer. The procedure requires the design of sense and antisense oligonucleotides, that include the desired point mutation. The following oligos has been employed:

- mutation K842E:

sn. 5′-gtacataatcctgtgtttgagcctttgaaaagccagtg-3′ (SEQ ID No.: 41)

as. 5′-cactggcttttcaaaaggctcaaacacaggattatgtac-3′ (SEQ ID No.: 42)

- mutation Q807K:

sn. 5′-ccactccttccctgaaacagctgaatctgcaactcc-3′ (SEQ ID No.: 43)

as. 5′-ggagttgcagattcagctgtttcagggaaggagtgg-3′ (SEQ ID No.: 44)

- mutation D864N:

sn. 5′-ctggaaattaagggaaataatattgaccctgaagcagttaaagg-3′ (SEQ ID No.: 45)

as. 5′-cctttaactgcttcagggtcaatattatttcccttaatttccag-3′ (SEQ ID No.: 46).

Engineered soluble receptors were produced by transient transfection of HEK-293T cells with pcDNA3.1 plasmids (cat.# V79020 Invitrogen Corporation, Camarillo, CA) expressing cDNAs encoding for wild-type decoyMET or decoyMET mutants. Transfected cells were starved for three days and cell culture supernatants containing the soluble receptors were collected. Purification of the recombinant proteins was done by affinity chromatography using HisTrap HP columns (cat.# 17524701 GE Healthcare, Freiburg, Germany) according to manifacturer's instructions. Large-scale protein production and purification were performed by U-Protein Express BV (Utrecht, The Netherlands).

MET phosphorylation assays

Serum-starved A549 were incubated for 24 h with 125 nM MvDN30 or 2 µM decoyMET^{K842E}, alone or in combination, and then stimulated with 50 ng/ml HGF (cat.# 294-HG-025 R&D Systems) for 2 h at 4°C. Total cellular lysates were analyzed by Western blot using the following primary antibodies: anti-MET phospho-Tyr$^{1234/1235}$ (D26, cat.# 3077 Cell Signaling Technology, Beverly, MA); anti-MET (3D4, cat.# 08-1366 Invitrogen Corporation); anti-vinculin (clone hVIN-1, cat.# V9131 Sigma Life Science, Saint Louis, MO). Anti-mouse IgG1 (cat.# JI115035003) and anti-rabbit IgG (cat.# JI111035003) HRP-conjugated secondary antibodies were from Jackson ImmunoResearch. (West Grove, PA).

ELISA Binding Assays

For analysis of the interaction between decoyMET and the DN30 mAb, affinity-purified soluble receptors (wild-type decoyMET or decoyMET^{K842E}, 100 ng/well) were immobilized on ELISA plates and increasing concentrations of the antibody (0 – 100 nM) were added in liquid phase. Binding was revealed using HRP-conjugated anti-mouse antibodies (cat.# NA931 GE Healthcare). For analysis of the interaction between and the fusion proteins, affinity-purified decoyMET wild type (100 ng/well) was immobilized on ELISA plates and increasing concentrations of the fusion proteins or scMvDN30 (0 – 1000 nM) were added in liquid phase. Binding was revealed using HRP-conjugated anti-human k chain antibody (cat.# A7164 Sigma-Aldrich). For analysis of decoyMET or fusion proteins binding to HGF, soluble receptors (100 ng/well) were immobilized on ELISA plates and incubated with increasing concentrations of HGF (0-11 nM) in solution. Binding was detected using the anti-HGF biotinylated antibody (cat.# BAF294 R&D Systems) and revealed with HRP-

conjugated streptavidin (cat.# RPN 1231 GE Healthcare).

Colorimetric assay was quantified by the multi-label plate reader VICTOR-X4 (Perkin Elmer Instruments INC., Whaltman, MA). Data were analyzed and fit using Prism software (GraphPad).

***In vitro* biological assays**

For anchorage-independent growth assays, cells were suspended in the appropriate culture medium supplemented with 2% FBS and 0.5% Seaplaque agarose (cat.# 50100 BMA, Rockland, ME), and seeded in 48-well plates (500 cells/well) on top of 1% agarose. Fresh medium containing the treatments was supplied twice weekly. A549 cells were treated with 1 µM MvDN30 or 1 µM decoyMET^{K842E}, alone or in combination, in the presence of 30 ng/ml HGF. U87-MG cells were treated with 0.5 µM MvDN30 or 1 µM decoyMET^{K842E}, alone or in combination. Colonies were stained with tetrazolium salts (cat.# T01380 Sigma-Aldrich) after 12 days of culture. Colony growth was determined using Metamorph software (Molecular Devices, Sunnyvale, CA). For cell invasion assays, HPAF-II cells (1.5 X 10^5/well) were suspended in serum-free culture medium in the presence of 0.5 µM MvDN30 or 1 µM decoyMET^{K842E}, alone or in combination, and seeded on the upper compartment of transwell chambers pre-coated with 30 µg/well of Matrigel Matrix (cat.# 354234 Corning Incorporated, NY). Culture medium supplemented with 2% FBS and 12.5 ng/ml HGF was added to the lower compartment of the chamber. After 24 h, cells on the upper side of the transwell filters were mechanically removed, while cells migrated through the membrane were fixed with 11% glutaraldehyde (cat.# 340855 Sigma-Aldrich) and stained with 0.1% Crystal Violet (cat. # C3886 Sigma-Aldrich). Cell invasion was quantified with Image-J software. For

cell scattering assays, HPAF-II cells (8000/well) were seeded in 96-well plates in complete culture medium. After 6 h, increasing concentrations (0 - 4 μM) of MvDN30 or decoyMET^{K842E}, alone or in 1:1 combination, were added. After additional 24 h, cells were stimulated with 6.25 ng/ml HGF for 20 h. Cells were fixed with 11% glutaraldehyde and stained with 0.1% Crystal Violet). For real-time cell motility assay, HPAF-II cells were seeded in E-plates (8000/well; Roche Diagnostics, Mannheim, Germany) and treated as above; when applied, the fusion proteins were tested at a concentration of 3μM. Electrical impedance was monitored continuously for 48 h, with data recording every ten minutes, using a X-Celligence RTCA device (Roche Diagnostic). Values are expressed as cell index normalized at the instant of HGF addition.

For anchorage-dependent cell growth, EBC-1 cells were seeded 2000 cells/well in a 96 well plate in 5% FBS culture medium. After 24 hrs, medium was replaced with a fresh one with 5% FBS plus the molecules to be tested (increasing concentrations - from 0,001 to 3 μM). Cell viability was evaluated after 72 hrs using the CellTiter-Glo (cat.# G7573 Promega Corp., Madison, WI), according to the manufacturer's instructions. Chemoluminescence was detected with VICTOR X4.

Immunofluorescence

Immunofluorescence analysis on tumor cells and tissues was performed as described[16,25]. Staining was done with an anti-MET phospho-Tyr$^{1234/1235}$ primary antibody (D26) and revealed by Alexa Fluor 555-conjugated secondary antibody (cat.# A31570 Thermo Fisher Scientific). Cells were counterstained with 488-conjugated phalloidin (cat.# A12379 Thermo Fisher Scientific). All images were captured with a Leica TCS

SP5 AOBS confocal laser-scanning microscope (Leica Microsystems). Immunofluorescence acquisition settings were kept constant within each cell line or tumor tissue. Mean Fluorescence Intensity (MFI) was evaluated with ImageJ software, measuring the mean pixel intensity in each channel, background subtracted. For cell lines, phosphoMET MFI was normalized on phalloidin, while for tumors all the signals were normalized on DAPI.

***In vivo* experiments**

All animal procedures were performed according to protocols approved by Ethical Committee for animal experimentation of the Fondazione Piemontese per la Ricerca sul Cancro and by Italian Ministry of Health. NOD-SCID mice were purchased from Charles River (Calco, Italy); hHGF-Ki SCID mice) were obtained from AVEO Pharmaceuticals, Cambridge, MA.

U87-MG cells were injected subcutaneously ($2x10^6$/mouse) in the right flank of female NOD-SCID mice. Tumor growth was monitored by caliper measurement twice weekly. Tumor volume was calculated using the formula: $V= 4/3\ \pi\ (x/2)(y/2)(z/2)$, where x, y and z are height, width and depth of the tumor mass. When the tumors reached a volume of 80-100 mm^3 (day 0), mice were stratified in four homogeneous groups and treated twice weekly by intratumor injection with: vehicle (n=10); MvDN30, 12.5 µg (n=9); K842E, 125 µg (n=9); the combination of the two (n=10). After 22 days of treatment, mice were sacrificed and tumors were excised and embedded in paraffin for histological analysis. Tumor volume fold increase was calculated as the ratio between the value at day 22 and the value at day 0.

HPAF-II or Capan-1 cells were transduced with 100 ng/ml p24 of lentiviral vectors carrying the luciferase

gene under the control of the CMV promoter as described[1]. Luciferase-expressing HPAF-II cells (10^4/mouse) were injected in the pancreas of 4- to 6-week-old female hHGF-KI SCID mice. After two days, mice were injected intraperitoneally with XenoLight D-Luciferin (150 mg/kg; cat.# 122799 Perkin Elmer), stratified into homogeneous groups on the basis of the bioluminescence signal using an IVIS Spectrum CT apparatus (Perkin Elmer), and randomly assigned to 4 treatment arms: vehicle (*n*=10); MvDN30 (10 mg/kg, *n*=6); decoyMET^{K842E} (10 mg/kg, *n*=6); MvDN30 + decoyMET^{K842E} (10 + 10 mg/kg , *n*=6). Treatments were administered daily (MvDN30) or every two days (decoyMET^{K842E}) by intraperitoneal injection. At sacrifice, after five weeks of treatment, tumors and lungs were explanted. Tumors were embedded in paraffin or OCT and processed for immunohistochemical or immunofluorescence analysis, respectively. Proliferation of tumor cells was determined using a monoclonal anti-Ki67 antibody (MIB-1, cat.# M724001-2 Agilent Technologies) as previously described[18]. Lungs were processed for histochemical analysis and micrometastases were evaluated by light microscopy on paraffin-embedded, HE-stained non sequential sections. For each mouse, ten slides were analyzed; metastatic lesions were scored and their area quantified with ImageJ software.

Luciferase-expressing Capan-1 (10^6/mouse) were injected in the pancreas of 4- to 6-week-old female hHGF-Ki SCID mice. Mice stratification was performed as above. Treatment arms: Vehicle (*n*=14), MvDN30 and decoyMET^{K842E} in combination (10 + 10 mg/kg; group 1, *n*=4; group 2 *n*=6), scMvDN30_K842E_L60 (10 mg/kg; *n*=5), K842E scMvDN30_L60 (10 mg/kg; n=4), scMvDN30_K842E_L134 (10 mg/kg; *n*=6), K842E_scMvDN30_L134 (10 mg/kg; *n*=5). Treatments were

administered daily (MvDN30) or every two days (decoyMET^{K842E} and recombinant fusion proteins) by intraperitoneal injection. After five weeks of treatment, animals were sacrificed and livers were collected to measure bioluminescence signals by IVIS spectrum. Mice were considered metastasis positive when bioluminescence detected in the liver by IVIS Spectrum was higher than 10^5 photons/second.

Statistical analysis

Average, standard deviation (SD) and standard error of the mean (SEM) were calculated using Microsoft Office Excel 2010 software (Microsoft Corporation, Redmond, Washington). To calculate K_d values, data from ELISA assays were analyzed and fitted according to nonlinear regression, one site binding hyperbola curve, using GraphPad Prism software (GraphPad Software, San Diego, California). To calculate IC_{50} values, data from proliferation assays were analyzed and fitted according to nonlinear regression, sigmoidal dose-response curve, using GraphPad Prism software. Statistical significance was determined using a two-tailed Student's *t* test. All experiments were repeated at least three times (biological replicates). The *in vivo* experiments were repeated two times. Figures show one representative experiment.

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Claims

1. An anti-Hepatocyte Growth Factor Receptor (HGFR) antibody fragment in combination with an extracellular portion of human HGFR for use in the treatment of a patient suffering from a tumor and/or metastasis, preferably metastasis, wherein:

(i) the anti-HGFR antibody fragment has only one paratope able to bind to an epitope of the extracellular portion of human HGFR and has antagonist activity towards HGFR,

(ii) the extracellular portion of human HGFR is capable of binding to Hepatocyte Growth Factor (HGF) in a stable manner and contains at least one amino acid mutation within the epitope recognized by the anti-HGFR antibody fragment to prevent binding of the anti-HGFR antibody fragment thereto, and

(iii) the anti-HGFR antibody fragment and the extracellular portion of human HGFR are suitable for administration to the patient (a) in a protein form or (b) in a nucleic acid form.

2. The anti-HGFR antibody fragment in combination with the extracellular portion of human HGFR for use according to claim 1, wherein the anti-HGFR antibody fragment contains one light chain variable domain (VL) and one heavy chain variable domain (VH), wherein the light chain and heavy chain variable domains are non-human, or humanized, and wherein the light chain variable domain contains Complementarity Determining Regions (CDRs) having the amino acid sequences set forth in SEQ ID No.: 1 to 3, and the heavy chain variable domain contains CDRs having the amino acid sequences set forth in SEQ ID No.: 4 to 6.

3. The anti-HGFR antibody fragment in combination with the extracellular portion of human HGFR for use according to claim 1 or claim 2, wherein the anti-HGFR antibody fragment further contains one human light chain constant domain and one human heavy chain CH1 constant domain, the light chain variable domain being fused to the human light chain constant domain in the N- to C-terminal direction, and the heavy chain variable domain being fused to the human heavy chain CH1 constant domain in the N- to C-terminal direction.

4. The anti-HGFR antibody fragment in combination with the extracellular portion of human HGFR for use according to claim 3, wherein the anti-HGFR antibody fragment is a single-chain Fab fragment.

5. The anti-HGFR antibody fragment in combination with the extracellular portion of human HGFR for use according to any one of the preceding claims, wherein the extracellular portion of human HGFR contains SEMA, PSI, IPT-1, IPT-2, IPT-3 and IPT-4 domains.

6. The anti-HGFR antibody fragment in combination with the extracellular portion of human HGFR for use according to any one of the claims 1 to 5, wherein the extracellular portion of human HGFR contains a sequence as set forth in SEQ ID No.: 19, wherein at least one of the amino acids between position 797 and position 875 of SEQ ID No.: 19 are mutated in order to prevent binding of the anti-HGFR antibody fragment thereto.

7. The anti-HGFR antibody fragment in combination with the extracellular portion of human HGFR for use according to any one of the preceding claims, wherein

the extracellular portion of human HGFR has the sequence set forth in SEQ ID No.: 20.

8. The anti-HGFR antibody fragment in combination with the extracellular portion of human HGFR for use according to any one of the preceding claims, wherein:

(i) the anti-HGFR antibody fragment is conjugated by means of a linker to the extracellular portion of human HGFR in the N- to C-terminal direction, thus generating an anti-HGFR antibody fragment-linker-extracellular portion of human HGFR fusion protein; or

(ii) the extracellular portion of human HGFR is conjugated by means of a linker to the anti-HGFR antibody fragment in the N- to C-terminal direction, thus generating an extracellular portion of human HGFR-linker-anti-HGFR antibody fragment fusion protein.

9. The anti-HGFR antibody fragment in combination with the extracellular portion of human HGFR for use according to any one of the preceding claims, wherein:

(i) the nucleic acid molecule encoding the anti-HGFR antibody fragment is linked by means of a nucleic acid molecule encoding a linker to the nucleic acid molecule encoding the extracellular portion of human HGFR in the 5′- to 3′-terminal direction, thus encoding an anti-HGFR antibody fragment-linker-extracellular portion of human HGFR fusion protein; or

(ii) the nucleic acid molecule encoding the extracellular portion of human HGFR is linked by means of a nucleic acid molecule encoding a linker to the nucleic acid molecule encoding the anti-HGFR antibody fragment in the 5′- to 3′-terminal direction, thus encoding an extracellular portion of human HGFR-linker-anti-HGFR antibody fragment fusion protein.

10. The anti-HGFR antibody fragment in combination with the extracellular portion of human HGFR for use according to any one of the claims 8 or 9, wherein:

(i) the anti-HGFR antibody fragment-linker-extracellular portion of human HGFR fusion protein contains an amino acid sequence selected from SEQ ID No.: 26, 27 and 28; or

(ii) the extracellular portion of human HGFR-linker-anti-HGFR antibody fragment fusion protein contains an amino acid sequence selected from SEQ ID No.: 23, 24 and 25.

11. The anti-HGFR antibody fragment in combination with the extracellular portion of human HGFR for use according to any one of the preceding claims, wherein the patient carries a wild-type *MET* oncogene.

12. A pharmaceutical composition containing an anti-Hepatocyte Growth Factor Receptor (HGFR) antibody fragment and an extracellular portion of human HGFR according to any one of the preceding claims and a pharmaceutically acceptable vehicle.

13. A fusion protein comprising in the N- to C-terminal direction:

(i) an anti-HGFR antibody fragment, a linker, and an extracellular portion of human HGFR; or

(ii) an extracellular portion of human HGFR, a linker, and an anti-HGFR antibody fragment,

wherein the anti-HGFR antibody fragment has only one paratope able to bind to an epitope of the extracellular portion of human HGFR and has antagonist activity towards HGFR, and

wherein the extracellular portion of human HGFR contains at least one amino acid mutation within the

epitope recognized by the anti-HGFR antibody fragment to prevent binding of the anti-HGFR antibody fragment thereto.

14. The fusion protein according to claim 13, wherein the fusion protein contains an amino acid sequence selected from any one of SEQ ID No.: 23 to 28.

15. A nucleic acid molecule encoding the fusion protein according to claim 13 or claim 14.

16. A pharmaceutical composition containing a fusion protein according to claim 13 or 14 or a nucleic acid molecule according to 15 and a pharmaceutically acceptable vehicle.

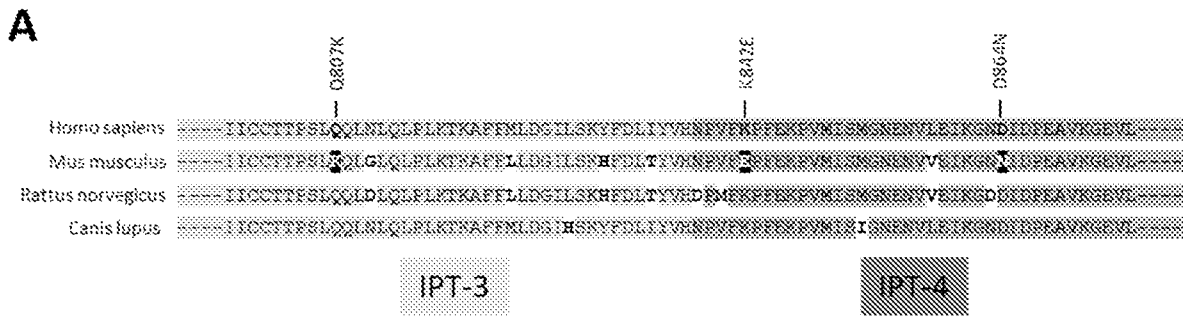
17. A pharmaceutical product comprising, in a single bottle or in two bottles, (a) an anti-Hepatocyte Growth Factor Receptor (HGFR) antibody fragment and a pharmaceutically acceptable vehicle, and (b) an extracellular portion of human HGFR and a pharmaceutically acceptable vehicle, wherein:

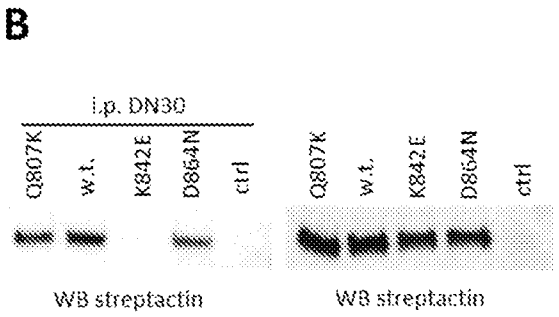
(i) the anti-HGFR antibody fragment has only one paratope able to bind to an epitope of the extracellular portion of human HGFR and has antagonist activity towards HGFR,

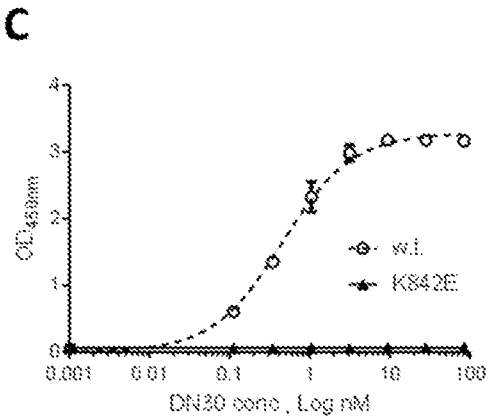
(ii) the extracellular portion of human HGFR is capable of binding to Hepatocyte Growth Factor (HGF) in a stable manner and contains at least one amino acid mutation within the epitope recognized by the anti-HGFR antibody fragment to prevent binding of the anti-HGFR antibody fragment thereto,

(iii) the anti-HGFR antibody fragment and the extracellular portion of human HGFR are either in a protein form or in a nucleic acid form.

Figure 1

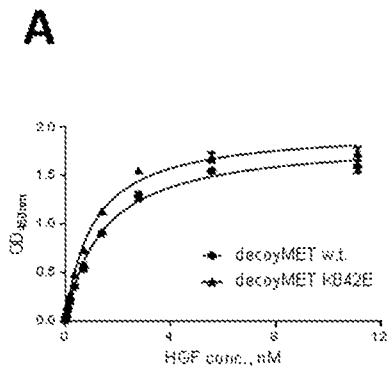






	w.t.	K842E
K_D nM	0.446 ± 0.027	n.a.

Figure 2



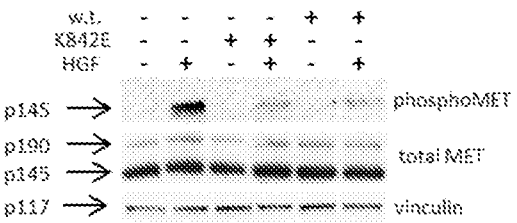


Figure 3

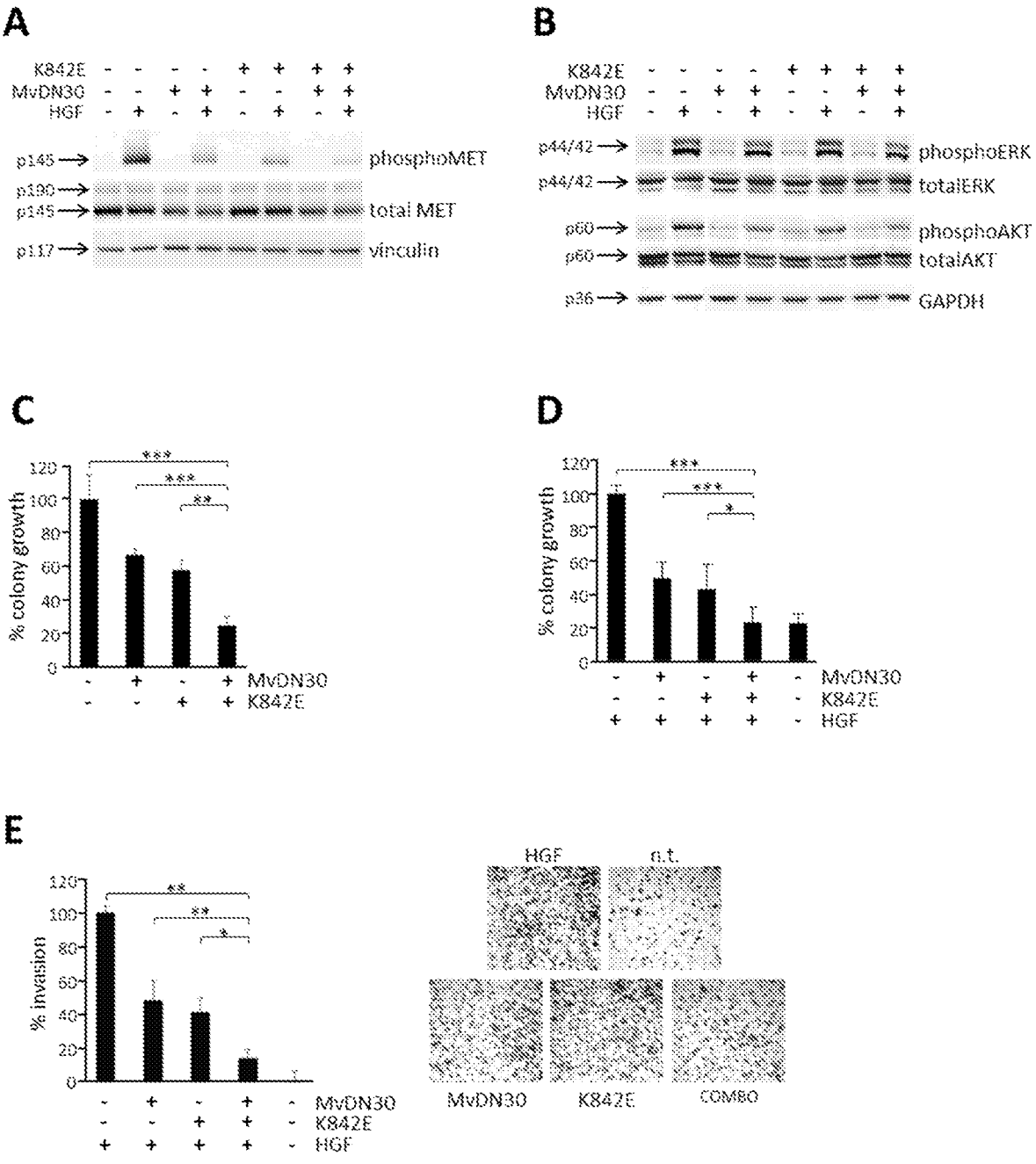

A
K842E
MvDN30
HGF
p145
phosphoMET
p190
p145
total MET
p117
vinculin
B
K842E
MvDN30
HGF
p44/42
phosphoERK
p44/42
totalERK
p60
phosphoAKT
p60
totalAKT
p36
GAPDH
C
% colony growth
MvDN30
K842E
D
% colony growth
MvDN30
K842E
HGF
E
% invasion
MvDN30
K842E
HGF
HGF
n.t.
MvDN30
K842E
COMBO

Figure 4

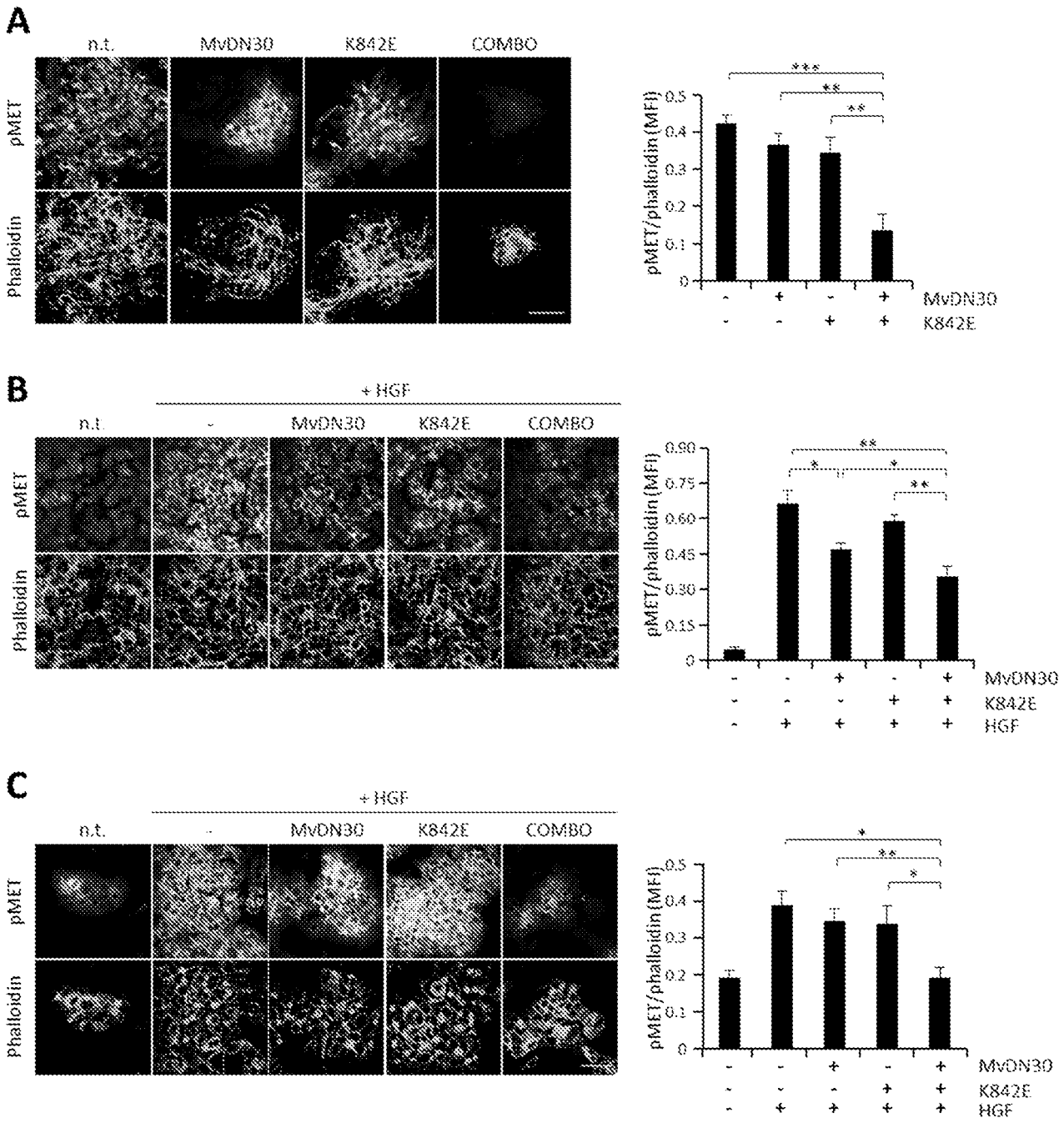

A
n.t.
MvDN30
K842E
COMBO
pMET
Phalloidin
pMET/phalloidin (MFI)
MvDN30
K842E
B
+ HGF
n.t.
-
MvDN30
K842E
COMBO
pMET
Phalloidin
pMET/phalloidin (MFI)
MvDN30
K842E
HGF
C
+ HGF
n.t.
-
MvDN30
K842E
COMBO
pMET
Phalloidin
pMET/phalloidin (MFI)
MvDN30
K842E
HGF

Figure 5

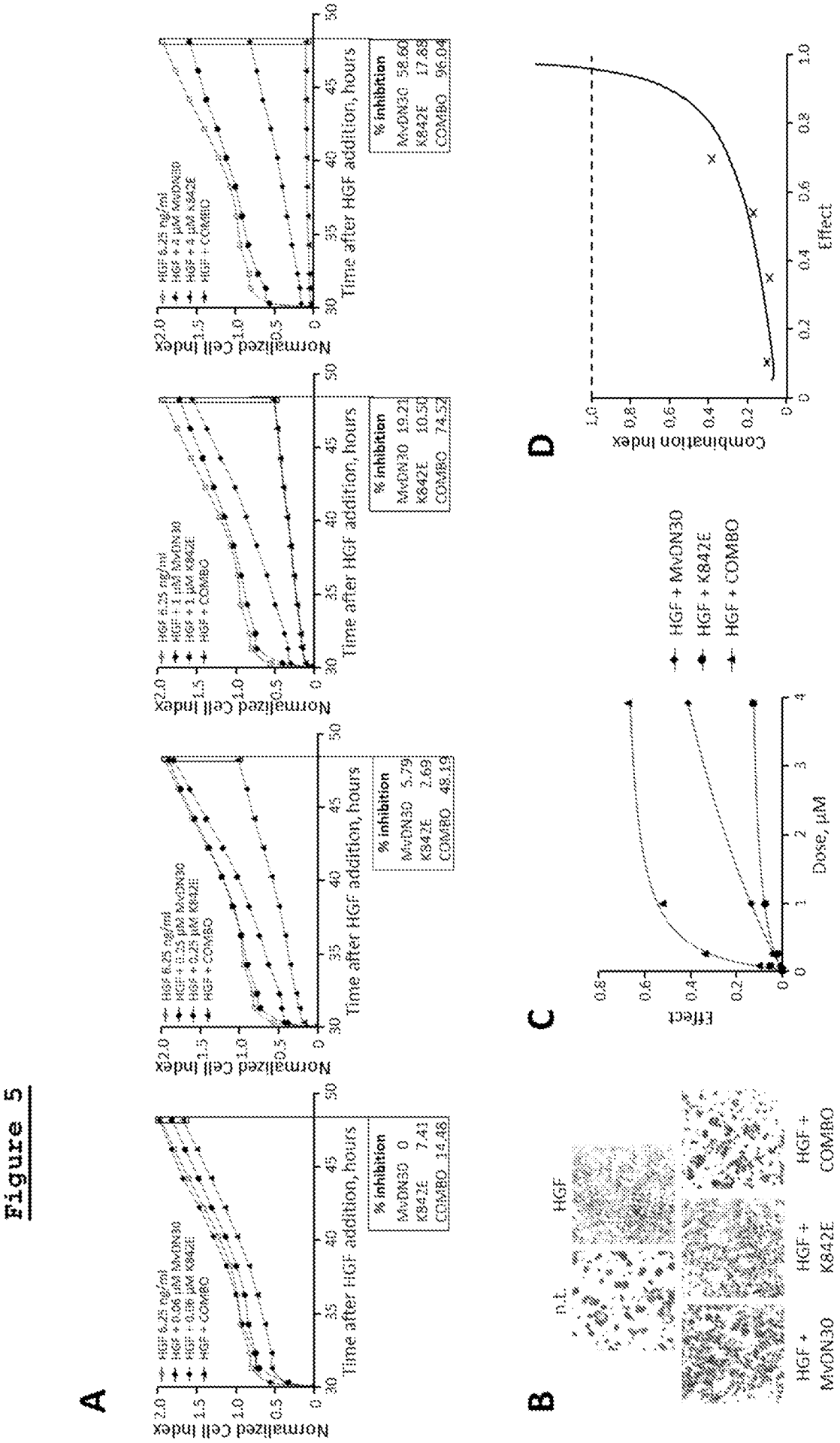
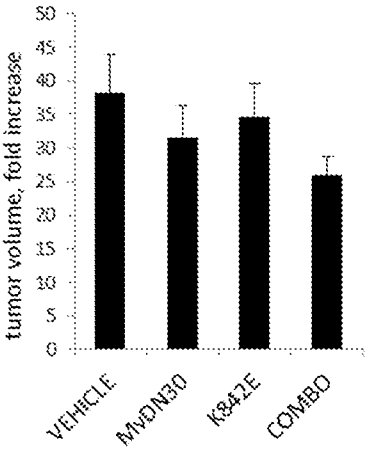

A
B
C
D
Normalized cell index
Time after HGF addition, hours
HGF 6.25 ng/ml
HGF + 0.06 µM MvDN30
HGF + 0.16 µM K842E
HGF + COMBO
% inhibition
MvDN30 0
K842E 7.41
COMBO 14.46
HGF + 0.25 µM MvDN30
HGF + 0.25 µM K842E
% inhibition
MvDN30 5.79
K842E 2.69
COMBO 48.19
HGF + 1 µM MvDN30
HGF + 1 µM K842E
% inhibition
MvDN30 19.23
K842E 10.50
COMBO 74.52
HGF + 2 µM MvDN30
HGF + 4 µM K842E
% inhibition
MvDN30 58.60
K842E 17.68
COMBO 96.04
n.t.
HGF
HGF + MvDN30
HGF + K842E
HGF + COMBO
Effect
Dose, µM
Combination index
Effect

Figure 6

A


tumor volume, fold increase
50
45
40
35
30
25
20
15
10
5
0
VEHICLE
MvON30
K842E
COMBO

B

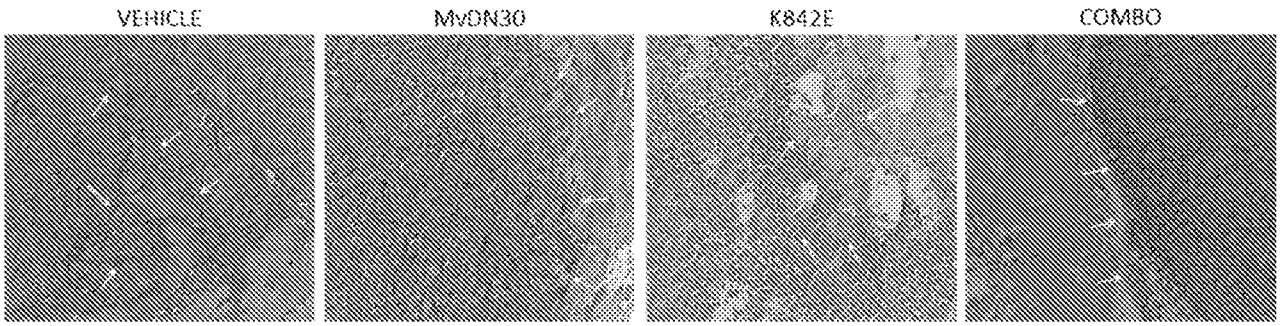
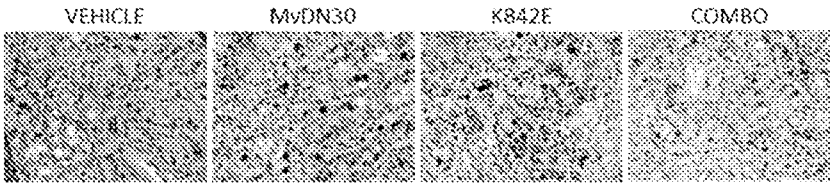

VEHICLE
MvON30
K842E
COMBO

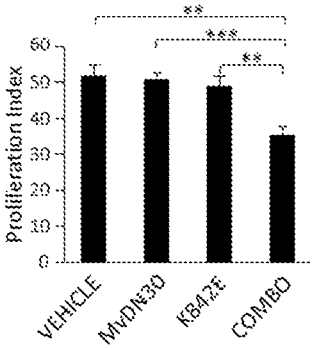
Figure 7

A

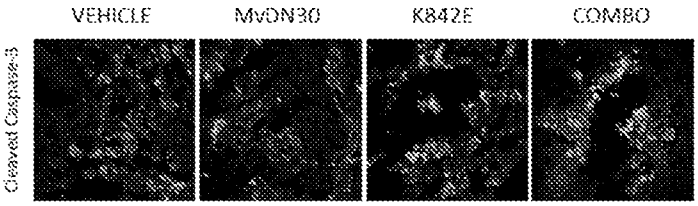
	VEHICLE	MvDN30	K842E	COMBO
Tot. Flux (P/s x 10^6)	4.17 ± 0.78	4.06 ± 1.44	3.95 ± 1.18	2.70 ± 0.68

B





C



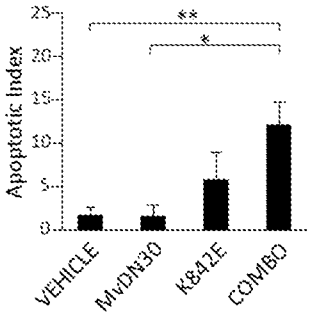


Figure 8

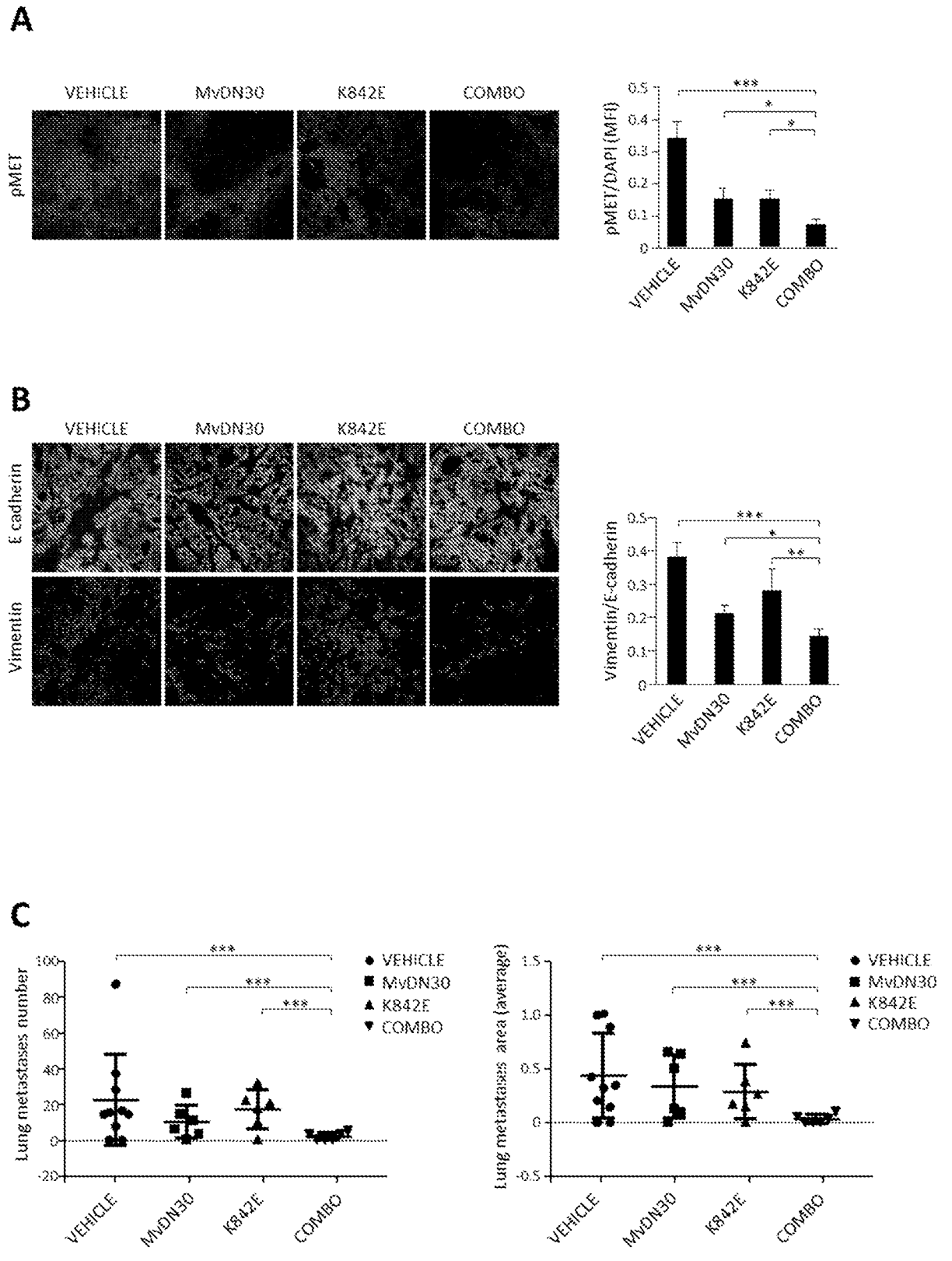
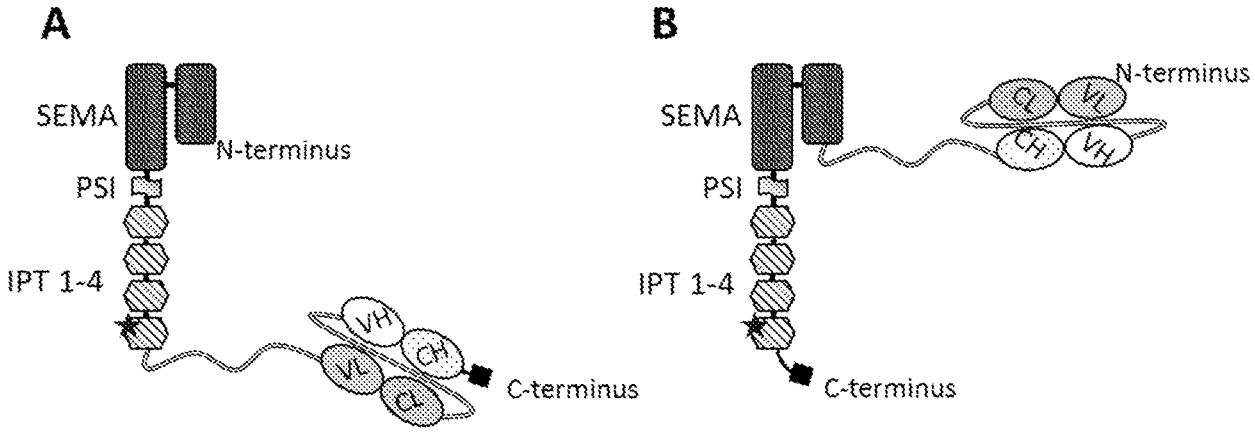

A
VEHICLE
MvDN30
K842E
COMBO
pMET
pMET/DAPI (MFI)
B
E cadherin
Vimentin
Vimentin/E-cadherin
C
Lung metastases number
Lung metastases area (average)
***
*
**

Figure 9



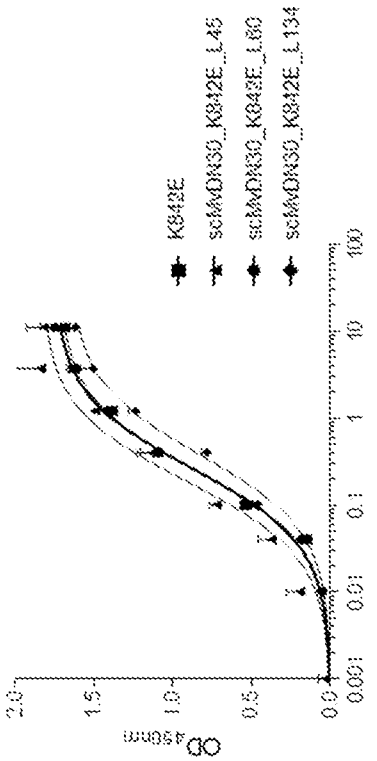
C

Linkers for fusion proteins

name	#aa	aa sequence	repeats	features
L45	45	AEAAAKEAAAKEAAAKEAAAKAAAEAAAKEAAAKEAAAKEAAAKA	A(EAAAK)$_3$-AAA-(EAAAK)$_3$A	rigid (α-helix)
L60	60	GGSSGSESGSTGTSSSGTGTSAGTTGTSASTSESGSGGSGGSGGGSAGSTA TAGASSGS	N/A	flexible
L134	134	GGGGSGGGGSGGGGSAEAWYNLGNAYYKQGDYQKAIEYYQKALELDPNNA EAWYNLGNAYYKQGDYQKAIEYYQKALELDPNNAEAWYNLGNAYYKQGDY QKAIEDYQKALELDPNNGGGGSGGGGSGGGGSGG	(G$_4$S)$_3$-TPR3-(G$_4$S)$_3$GG	mixed (flexible + α-helix)

Figure 10

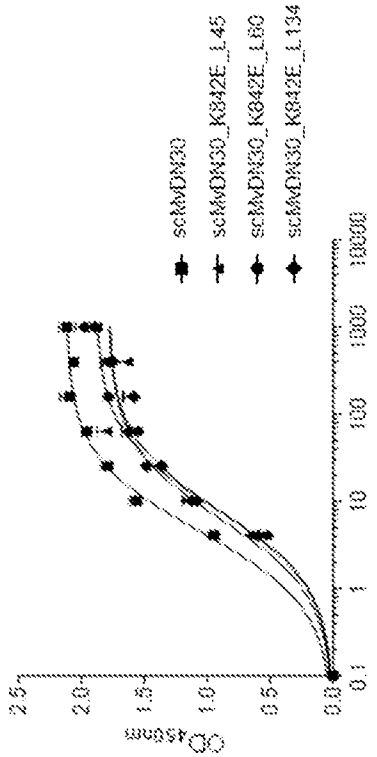
A



	K842E_scMvDN30_L45	K842E_scMvDN30_L60	K842E_scMvDN30_L134
K_d	0.148	0.174	0.125

	scMvDN30_K842E L45	scMvDN30_K842E L60	scMvDN30_K842E L134
K_d	0.187	0.262	0.374

B

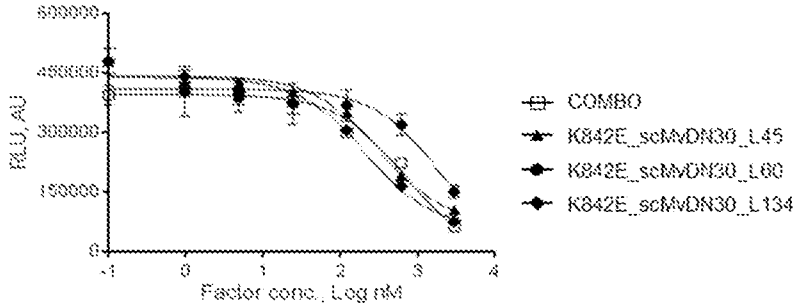


	K842E_scMvDN30_L45	K842E_scMvDN30_L60	K842E_scMvDN30_L134
K_d	29.39	34.33	39.61

	scMvDN30_K842E L45	scMvDN30_K842E L60	scMvDN30_K842E L134
K_d	6.214	8.442	7.679

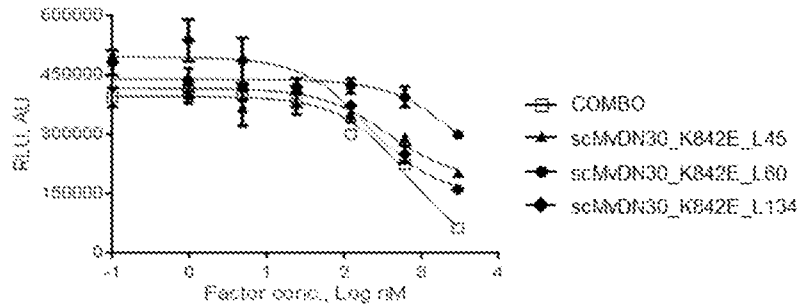
Figure 11

A



	COMBO	K842E_scMvDN30 L45	K842E_scMvDN30 L60	K842E_scMvDN30 L134
IC50	632	309	1905	205

B

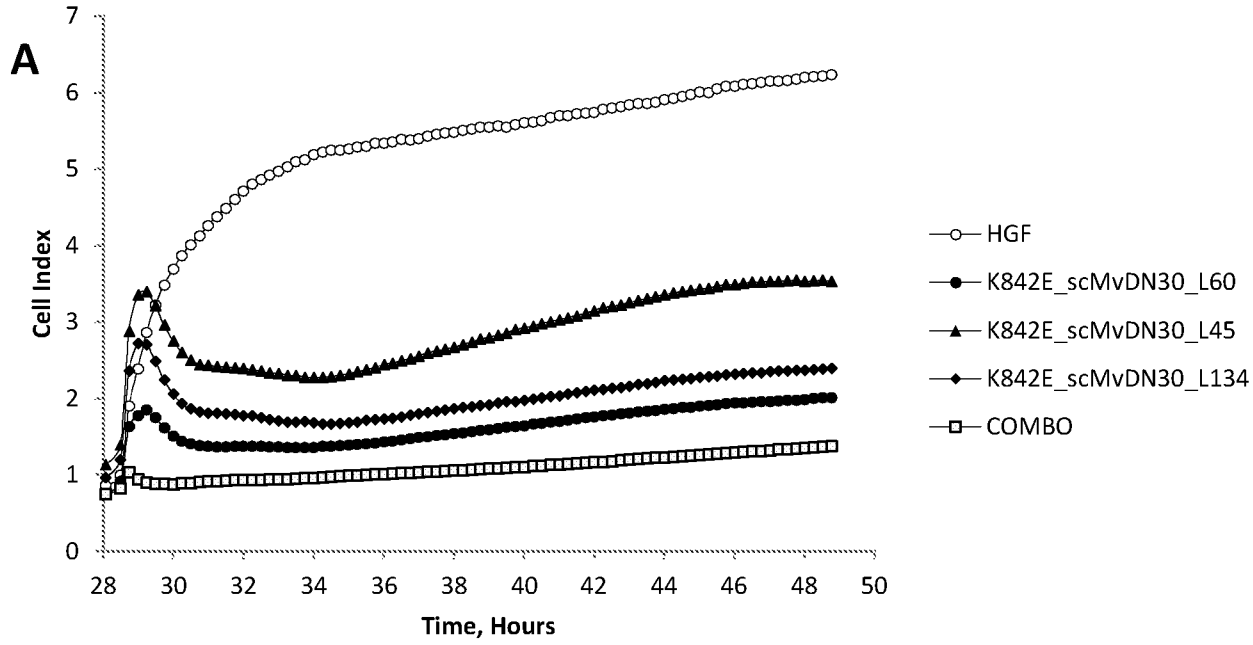


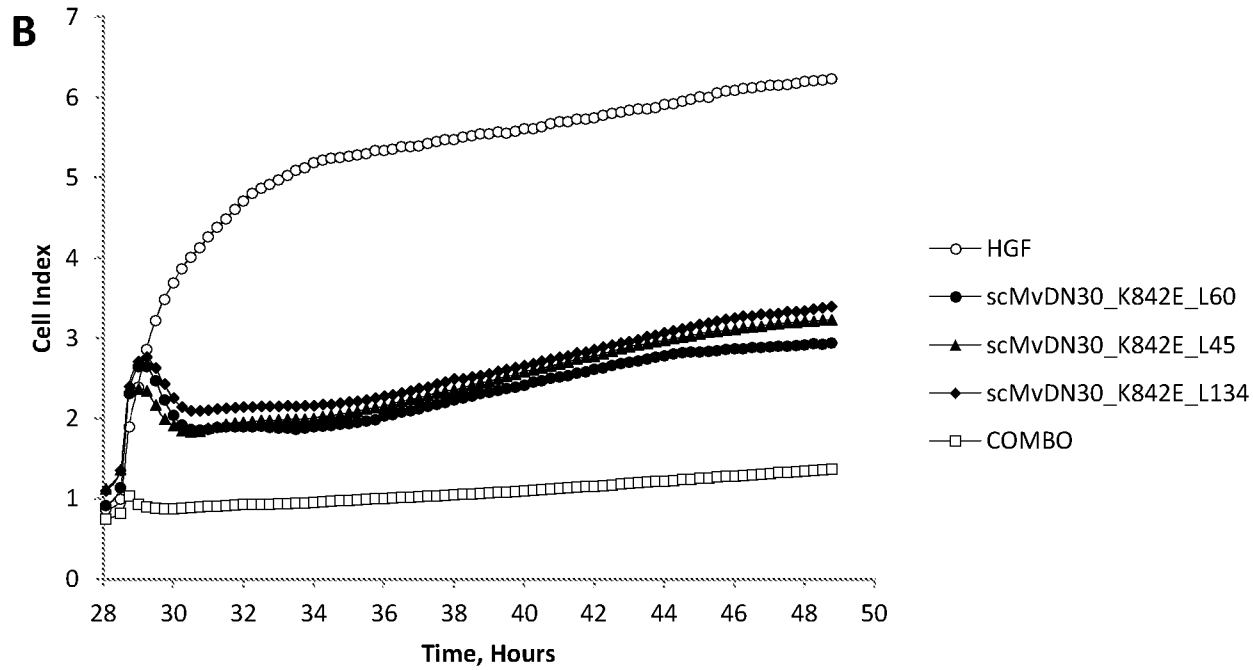
	COMBO	scMvDN30_K842E L45	scMvDN30_K842E L60	scMvDN30_K842E L134
IC50	632	385	2726	204

C

	K842E_scMvDN30			scMvDN30_K842E			Combo
	L60	L45	L134	L60	L45	L134	
IC50 (nM)	4330,67	6535,68	4969,85	8919,07	4990,27	3530,98	662,20

Figure 12





C

	HGF							
		K842E_scMvDN30			scMvDN30_K842E			Combo
		L60	L45	L134	L60	L45	L134	
% motility versus HGF	100	32,73	46,19	56,80	49,23	33,06	52,90	6,17

Figure 13

A

	N
VEHICLE	8/14
COMBO 1	0/4
COMBO 2	4/6
K842E_scMvDN30_L60	0/4
scMvDN30_K842E_L60	0/5
K842E_scMvDN30_L134	0/5
scMvDN30_K842E_L134	0/6

B