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The Development of HGF Mimetics Using Human Antibodies with Agonist Effect Against c-Met

c-Met에 대하여 작용제 효과를 지닌 인간 항체를 이용한 간세포성장인자 모사체 개발

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이 지 혜

ABSTRACT

The Development of HGF Mimetics Using Human Antibodies with Agonist Effect Against c-Met

Ji Hye Lee

College of Pharmacy

Graduate School of Pharmacy

Seoul National University

Human Hepatocyte Growth Factor (HGF) is known to play important roles in various tissues such as the liver, lung, kidney, and nervous system. HGF binds to its receptor c-Met, and activates a signal cascade that leads to proliferation, differentiation, motility, and survival of the cell. As such, the HGF/c-Met signaling system plays crucial roles in organogenesis during embryonic development and tissue regeneration. It was also observed that administration of human recombinant HGF on animal models with degenerative diseases leads to better prognosis. HGF is in its natural form fairly

unstable, with serum levels dropping drastically several minutes after administration, making it difficult to use as a therapeutic molecule. To overcome these obstacles, we have identified and characterized antibodies A8 and A11 that bind to the HGF receptor, c-Met, with agonist properties that are able to induce the same signaling cascades. The identification of the antibody binding to c-Met was done by phage display screening and verified through ELISA and flow cytometry. The inducing of phosphorylation signals at catalytic and docking sites and the activation of Akt and Erk signals was seen when treated with A8 and A11 alone, indicating agonist properties of the antibodies. Proliferation was observed, proof that the induction of signals results in direct effect on the cells. We could also verify through adipose-derived mesenchymal stem cell culture that A8 and A11 could substitute the role of HGF in maintaining multipotency. We have thus verified c-Met agonist antibodies that hold the potential of being used as a substitute for HGF in mesenchymal stem cell culture for therapeutic purpose and possibly in treatments for chronic and degenerative diseases.

Key words: Hepatocyte growth factor (HGF), c-Met, agonist, phosphorylation, mesenchymal stem cell

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INTRODUCTION

HGF was first identified in 1984 as a putative hepatotrophic factor present in the serum of rats, which stimulated the proliferation of rat hepatocytes [1]. It is known to be the ligand of c-Met, a transmembrane receptor coded by the MET gene, which is a class IV receptor tyrosine kinase [2, 3]. This receptor not only plays important roles in the organogenesis and morphogenic events during embryonic development and adult life, but also in invasiveness during cancer development [4]. The receptor is a disulfide linked heterodimer formed by α/β subunits and is composed of an extracellular domain, intercellular domain, and the catalytic intracellular domain. The extracellular domain contains three subdomain types: the Sema domain, the PSI domain and the IPT domain [2-5]. The Sema domain and IPT domain were suggested to be important in the binding of HGF to the receptor, thereby inducing the conformational shift, resulting in the dimerization and activation of the c-Met signaling pathway [7]. The dimerization of c-Met induces phosphorylation of the catalytic site Tyr 1234 and the docking site Tyr 1349. Upon phosphorylation, molecules like Grb2 and Gab1 bind to the docking site of the receptor, and mediate mitogenic, angiogenic, morphogenic, motogenic, and anti-apoptotic

responses [1, 8]. Due to these vast array of responses, c-Met plays important roles in tumorigenesis and has been heavily studied as a potential target in anti-cancer therapies, especially in lung and gastric cancers [9-12]. Small molecules like inhibitors are commonly used as therapy [12], but also strategies using antagonist antibodies that target c-Met have been studied [13, 14]. Despite its important role in tumorigenesis, the HGF/c-Met signaling pathway has been found to play important roles in the proliferation and regeneration of non-cancerous cells and tissues [1]. Mice with HGF knock-out or c-Met receptor knock-out died in utero about 14 days after gestation, which was caused by the impairment of the placenta and organogesis [15]. Further, it was also observed in mice with *Met* gene knock-down in specific tissues, that the signaling pathway of HGF/c-Met play important roles in tissue-specific repairs, regeneration, protection, homeostasis of tissues, inhibition of chronic inflammation and fibrosis [15]. Further, it was observed that the role of this signaling pathway is important in a broad range of organs like the liver, lung, spinal cord, and even brain [17-21]. All these findings suggest a promising therapeutic potential for HGF as the ligand for c-Met activation. Phase I/II clinical trials for therapy using recombinant HGF are in progress [1]. But using HGF as a therapeutic molecule was reported to have many limitations. HGF undergoes a rather complicated process until maturation. The

growth factor is first secreted as an inactive single-chain precursor form that is proteolytically cleaved into the two-chain form comprising of the 4-kringle α-chain and the β-chain [22]. In addition, once it's produced, the molecule is very unstable and the yield is known to be very low, posing potential problems in the accessibility of HGF-based therapies even after being developed [23]. To overcome this problem, we have searched for an agonist with a more stable structure with effects against c-Met that mimic the role of HGF. We suggest that agonist antibodies against c-Met can induce the HGF mediated signaling pathways, with the advantages of higher stability and ease of production, opening a possibility to be efficiently used in therapy.

MATERIALS AND METHODS

Reagents

A549, MDA-MB231 cells were purchased from the American Type Culture Collection (ATCC, USA), and H596, SKBR-3 were purchased from the Korean Cell Line Bank (KCLB). The adipose derived mesenchymal cells were kindly provided by Xcell Therapeutics (Seoul, Korea). Chemically defined culture medium for mesenchymal stem cell culture was also provided by Xcell Therapeutics. The antigen used for screening was human c-Met recombinant protein from Sinobiological (China) that contained the aa 1-932 of the receptor, and the commercial anti-c-Met antibody used as control was from Abcam (USA).

Antibody screening

Phage display

Human recombinant c-Met protein (Sinobiological, China) was used as antigen for phage display screening. Human scFv library was used for screening hits binding to the c-Met extracellular region. Antigen was coated to immunotubes (Nunc, USA) at a concentration

10ug/ul and incubated O/N for binding. The immunotube and phages were blocked with blocking buffer (3% milk in PBST). The phages were put into the antigen-coated immunotubes for binding. After 1 hour, washing was done 4 times with PBST solution and 1 time with PBS. The phages were then eluted with 100mM TEA for 7~8 minutes and neutralized with Tris-HCl (pH 8) solution. The eluted phages were infected in E. coli and some grown on solid LA plates O/N to check the output titer. The rest of the phages were rescued using helper phage and the same cycle repeated 3 more times.

ELISA screening

After the 4th panning, single colonies resulted were each injected in 150ul SB (+ampicillin) in a 96-well plate. Cells were cultured at 37℃ in a shaking incubator until it turned blurry. After incubation, the culture solution was added to the master plate and incubated overnight at 30℃ after IPTG induction in a concentration of 1nM. c-Met recombinant protein was used as antigen (Sinobiological, China) and coated 1ug/ml in PBS, in an ELISA plate (Corning 3690) and incubated at 4℃ overnight. The next day, the plate with injected clones was centrifuged at 3000rpm for 15 min. The soup was disposed of, and the pellet was resuspended in 1X TES buffer at 37℃ for 5~7 minutes. The cells were lysed by osmotic shock by adding 0.2X TES and incubation at 4℃ for 30 minutes. Meanwhile, the antigen-coated plates were washed with 150ul of TBST for 3 times

and blocking proceeded using 3% skimmed milk. A control plate was also blocked using the same blocking buffer. The periplasmic extract was taken from the lysed cells and blocked in a new plate with 6% skimmed milk for 1 hour. Then the solution was added to the antigen-coated plate and incubated for 1 hour at RT. Washing was done 3 times with TBST. Anti-HA Hrp secondary antibody was added and incubated for 1 hour. After washing 3 times with TBST, 30ul TMB was used to start the reaction and then quenched using 1N H_2SO_4. Detection was done at 450nm.

Sequencing and IgG conversion

The hits selected from the first ELISA screening were sequenced (Cosmogenetech, Korea). The final hits selected after sequencing and ELISA screening were converted to human IgG. The scFv sequences were converted to human light and heavy chain sequences and integrated in pOptiVEC™-TOPO and pcDNA™3.3-TOPO (Thermofisher, USA) vectors by cloning. The plasmids were amplified by midi prep (Macherey Nagel, Germany).

Transient expression and antibody purification

The amplified plasmids were transiently expressed in Freestyle Expression System (Invitrogen, USA). Freestyle cells were thawed and cultured in Freestyle Expression Medium in erlenmeyer flasks (Corning, USA). Passaging was done every 2-3 days when cells reached a density of 3.0 x 10^6 cells/ml. After 4 rounds of passaging, cells were transfected using the heavy and light chain plasmids and FreeStyle™ MAX Transfection Reagent (Invitrogen, USA). After transfection, cells were incubated for 7 days on an orbital shaker in 37° C incubator with a humidified atmosphere of 8% CO_2. 7 days post transfection, cells were harvested, with only the supernatant taken and filtered. After filtration, the supernatant was applied to MabSelect SuRe protein A beads (GE healthcare. USA) in a chromatography column (Bio-rad, USA). The sizes were checked by SDS-PAGE and coomassie blue staining.

Flow Cytometry Analysis

A549, MDA-MB231, H596, and SKBR-3 cells were used for flow cytometric analyses. The cells were detached with cell dissociation buffer (Hyclone, USA), washed with PBS and put into separate tubes in 2.0 x 10^5 cells. Antibodies were diluted in DPBS (Wellgene, Korea) solution containing 2% FBS in a concentration of 1ug/tube, added to cells, and incubated for one hour. A commercial anti-c-

Met antibody was used as control. Cells were washed three times and incubated with FITC conjugated secondary antibodies for 40 minutes. After washing 3 times, cells were analyzed using FACS BD Calibur (BD, USA).

Octet analysis

Octet service was provided by PALL corp, Fortebio. His tagged human recombinant c-Met protein (Sinobiological, China) was used as antigen. The target was captured onto 12 Ni-NTA sensors at 20ug/ml on a bio-surface. PBS was used as ligand buffer and 1x Fortebio Kinetic buffer as analyte buffer. Once the ligand was fixed, control commercial c-Met antibody, A8, A11, and C8 were bound and the binding affinity assessed by analyzing K_{on} and K_{off} values. Total assay time was 60 minutes.

Cell culture and antibody treatment

H596 cells were thawed and passaged two times before use in assays. Cells were cultured in RPMI (Wellgene, Korea) supplemented with 10% FBS (Hyclone, USA), and 1% penicillin/streptomycin solution (Hyclone, USA). Cells were

passaged once every 3~4 days at a confluency of 70~80%.

To observe whether the antibodies could induce phosphorylation signals, cells were seeded in 6-well culture plates (SPL, Korea) in FBS supplied media. To avoid interference of signals due to FBS, cells were starved in only RPMI (Wellgene, Korea) O/N. The next day, media was replaced with solutions containing different dilutions of antibodies or HGF for 1 hour.

Western blot analysis

Cells were prepared as mentioned, and harvested with lysis buffer containing RIPA (Biosesang, Korea), protease inhibitor (Roche, USA), and phosphatase inhibitor (Roche, USA) and lysed using 1ml syringe. After lysis, cells were centrifuged at 14000rpm for 15 minutes. The supernatant was taken out and protein quantification done through BCA assay (Thermofisher, USA). The supernatant was mixed with 5x sample loading buffer and boiled for 10 minutes for complete denaturation of proteins. SDS-PAGE was done using acrylamide gels. Wet transfer was performed and the proteins transferred to activated polyvinylidene difluoride (PVDF) membranes (Bio-rad, USA). Membranes were blocked with 5% BSA, incubated with primary antibodies and then with Hrp-

conjugated secondary antibodies. Signals were detected using ECL (Amersham, USA) in a dark room.

Cell proliferation assay

To assess the effect of HGF and candidate anti-c-Met antibodies on proliferation, H596 cells were seeded in 96 well plates (SPL, Korea) at a confluency of 1.0 x 10^4 cells/well and incubated O/N. The next day, media was removed and replaced with solutions containing concentrations of HGF or anti-c-Met antibodies ranging from 39 picomolar to 10 nanomolar. Cells were incubated for 72 hours after treatments. After the respective times, media was replaced with WST solution (DoGen, Korea), incubated for 2-3 hours, and detected at 450nm using a multi-reader (Tecan, Switzerland).

Adipose-derived mesenchymal stem cell culture and tri-lineage differentiation

Cell culture

Cells were seeded in 6-well plates in chemically defined media supplied with growth factors. Separate media containing either HGF, no HGF, or different concentrations of A8 or A11 were used.

Subculture was done when cells reached 80-90% confluency until passage 10. Viability and population doubling time were assessed either by manual counting or using Cedex cell counter (Roche, USA) after trypan blue staining.

Adipogenic differentiation

After cells have been cultured until passage 10 and have reached about 90-100% confluency, culture media was removed and replaced with differentiation media. StemPro adipogenesis differentiation kit (Thermofisher, USA) was used as differentiation media. Medium was changed every 2~3 days for two weeks. After differentiation, cells were fixed with 10% formaldehyde solution and stained with Oil Red O to observe the presence of lipid droplets.

Chondrogenic differentiation

Cells were cultured until passage 10, and when they have reached 50% confluency, culture media was replaced with differentiation media. As differentiation media, the following was used: DMEM low glucose medium (Wellgene, Korea), supplied with FBS (Hyclone, USA), 1% ITS-X, 50ug/ml ascorbic acid, 100nM dexamethasone, and 10ng/ml TGF-β1. After three weeks, cells were fixed and stained with Alcian Blue solution.

Osteogenic differentiation

After cells were cultured until passage 10, culture media was replaced with differentiation media consisting of DMEM low glucose supplemented with FBS (Hyclone, USA), 100nM dexamethasone, 10nM glycerol-2-phosphate, 50ug/ml ascorbic acid, and 1% Glutamax. Differentiation was performed for three weeks, with media being replaced every 2~3 days. After differentiation, cells were fixed and stained with Alizarin Red solution.

RESULTS

Screening and identification of scFvs binding to c-Met

To identify antibodies that bind to c-Met, a screening of human scFv library was conducted using a human recombinant c-Met antibody containing only the extracellular domain (aa. 1-932) as antigen (Figure 1A). Screening was carried out by binding the antigen to an immunotube and repeated for four cycles. The output increased in the third and fourth panning cycles (Figure 1B and 1C) and samples from cycles 3 and 4 were tested for binding through ELISA (Figure 1D). By comparing to the control plate, hits that were observed to give signals were chosen and sequenced (Figure 2A). 31 hits with differing sequences were selected and binding verified through ELISA. Among these, 10 hits that showed the strongest binding signals were selected and converted to human IgG form (Figure 2B and 2C).

Verification of the human IgG form binding to native c-Met

After the hits with strongest binding were identified, they were

converted to human IgG form and transiently expressed in 293F cells (Figure 3A and 3B). They were purified by gravity-based protein chromatography using protein A beads and checked for size by SDS-PAGE. After confirmation of IgG conversion, cells positive for c-Met were selected based on the information from CCLE and flow cytometry analysis was performed. The binding pattern seemed to match the expression level of c-Met for each cell line, with highest shifts seen in A549 cells (Figure 4A). Based on data, the four antibodies that showed shifts were selected and tested on other cell lines. H596 cell line was replaced for the moderate expression cell line. SKBR-3 was selected as the negative cell line to test for specificity. Results were in correlation with the expression level of c-Met as seen in the previous experiment (Figure 4B). Binding shift was not seen for the c-Met negative cell line, suggesting specificity of the antibodies to the c-Met receptor.

Binding affinity analysis of the lead antibodies

After confirming binding to native c-Met, the leads that showed the highest shifts (A8, A11, and C8) were tested for binding affinity kinetics. The assay was performed in an Octet platform and the human c-Met recombinant protein was used as the antigen. The

antigen was fixed on a biosurface and the antibodies were left to flow and react with the antigen. The binding coefficient, K_{on}, and the dissociation coefficient, K_{off}, were used to calculate the K_D, a quantitative value for affinity. The higher the K_{on} the faster the antibody associates with the ligand, and conversely, the lower the K_{off} value, the slower the target will dissociate from the receptor. All the three leads showed higher values for affinity and lower values for dissociation compared to the commercial anti-c-Met antibody. K_D was calculated as the ratio between K_{off} and K_{on} (Figure 5). A11 showed the lowest K_D value of 0.0244 nM, indicating that it has the highest binding affinity for the ligand. Compared to the commercial antibody, the lead antibodies showed faster association and slower dissociation from the receptor.

The agonist effect of the c-Met antibodies mimicking HGF

As it was seen, the antibodies had high affinity for the c-Met receptor, so next we investigated their effect on the c-Met signaling pathway. To examine the effect on signal, we tested for cell lines that were positive for c-Met, but were dependent on HGF for signal activation. Several cell lines were tested for signal using western blot,

and H596 cell line was selected as no phosphorylation of c-Met catalytic and docking sides had occured in the absence of HGF (Figure 6A). Upon the addition of HGF, the catalytic and docking sites were phosphorylated (Figure 6B), showing a concentration dependent pattern, becoming saturated at about 500ng/ml. Having verified the HGF dependent c-Met receptor activation in H596 cell line, the same assay was done using anti-c-Met antibodies. H596 cells were seeded in 6-well plates, given starvation with only media (RPMI) O/N, and treated with nanomolar concentrations of HGF or lead antibodies (A8, A11, B10, C8) for one hour. After treatment, the cells were harvested, lysed, and assayed through western blot. HGF, A8, and A11 induced phosphorylation signals, including main downstream signals like phospho-Erk and phospho-Akt, while B10 and C8 did not (Figure 6C).

Based on these results, next we assessed whether the induction of signals resulted in direct effect on cells such as proliferation. Only A8 and A11 were selected for further testing, since B10 and C8 did not induce signal activation. H596 cells were seeded in 96-well plates, incubated O/N, and treated with different concentrations of HGF or antibodies ranging from 39 picomolar to 10 nanomolar. Cells were incubated for 72 hours. Proliferation was observed, though antibodies did not show the same proliferation rate as the original ligand HGF, the saturation point was higher (Figure 7).

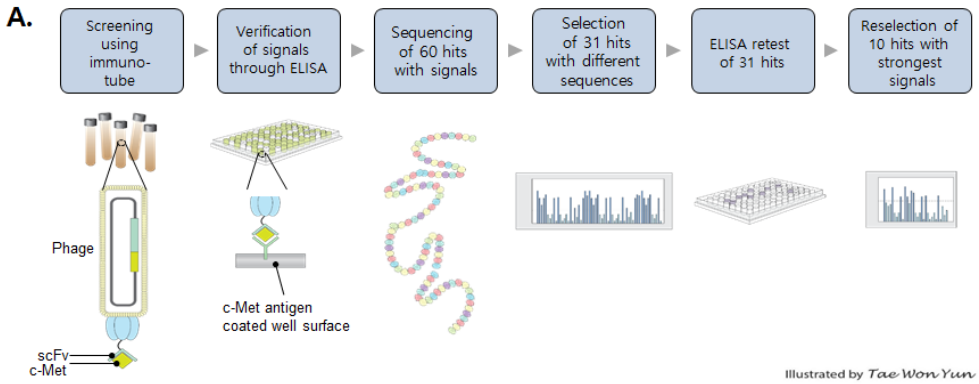
The effect of anti-c-Met antibodies on mesenchymal stem cells

To better assess the capacity of antibodies to mimic HGF, A8 and A11 were used as a replacement for HGF in mesenchymal stem cell culture. Adipose-derived- mesenchymal stem cells were cultured in chemically defined media without HGF, with HGF, A8 or A11. The antibodies were added in different concentrations to observe if there are any concentration dependent effects. Cells were cultured until passage 10 in the respective media, while assessing population doubling time, viability and cell morphology. Population doubling time and viability were assessed by counting and trypan blue staining. Morphology was observed by taking images on the light microscope. In population doubling time, the group with media that contained no HGF seemed to have a slightly longer population doubling time from passages 4 to 8 than other groups, but it was hard to see as significant. In terms of viability, the cells did not show any important difference in viability dependent on the condition of media, with the average viability not falling below 90% (Figure 8A and 8B). The anti-c-Met antibodies did not influence the culture morphology of the stem cells, suggesting no additional effect compared to HGF.

Since no difference was observed in routine cell culture, the ability to maintain multipotency for each media condition was assessed.

Cells were cultured in the various conditioned media as mentioned until passage 9. At passage 9, cells were seeded in 6-well plates and grown until full or about 50% confluency depending on the cell lineage to be differentiated. When the cells at passage 10 have reached the necessary confluency, culture media was replaced with differentiation media and maintained for 2 to 3 weeks, with media being renewed every 2 to 3 days. The cells were induced for adipogenic, chondrogenic, and osteogenic differentiation. After adipogenic differentiation, cells were stained with Oil Red O for fat vacuoles. Chondrogenic differentiation was verified through Alcian Blue which stains acidic polysaccharides in cartilages, and osteogenesis checked by Alizarin Red S staining. The stem cells had no difficulty in the differentiation to adipogenic lineage in all media conditions. In chondrogenic and osteogenic differentiation, the group that was cultured in media without HGF were unable to differentiate properly, suggesting an important role of HGF in maintaining stem cell multipotency. The groups that were cultured in media containing A8 or A11 were stained in similar density with the HGF control group, suggesting that the antibodies were able to mimic the role of HGF in mesenchymal stem cells.

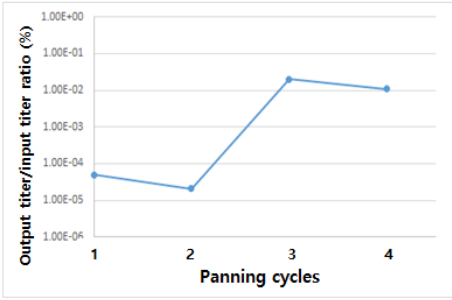
A.



B.

	1st	2nd	3rd	4th
Antigen	10ug	5ug	1ug	1ug
Wash	5 times	10 times	10 times	10 times
Input titter	$5.5x10^{12}$	$1.19x10^{12}$	$2.36x10^{11}$	$4.4x10^{12}$
Output titter	$2.7x10^{6}$	$2.5x10^{5}$	$4.76x10^{7}$	$4.81x10^{8}$

C.



D.

Control

	3rd						4th					
< >	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0810	0.0700	0.0710	0.0680	0.0950	0.0780	0.0840	0.0780	0.0870	0.3140	0.1010	0.1090
B	0.0760	0.0650	0.0660	0.0650	0.0790	0.5470	0.0830	0.0710	0.0870	0.1000	0.0970	0.1000
C	1.2170	0.0590	0.0600	0.0600	0.0730	0.0640	0.0700	0.0650	0.0780	0.0910	0.0910	0.0990
D	0.3710	0.0630	0.0680	0.0650	0.0840	0.0730	0.0800	0.0720	0.0880	0.0970	0.0900	0.0970
E	0.0700	2.0890	0.0620	0.0590	0.0780	0.0700	0.0790	0.0690	0.0830	0.0910	0.0860	0.0940
F	0.0790	0.0700	0.0730	0.1430	0.0920	0.0860	0.0930	0.0800	0.0950	0.1040	0.1010	0.1070
G	0.0820	0.0660	0.0700	0.0680	0.0900	0.0810	0.0880	0.0760	0.0920	0.1050	0.0940	0.1030
H	0.0790	0.3840	0.0690	0.0690	0.0850	0.0780	0.0850	0.0750	0.0900	0.1040	0.0920	0.0980

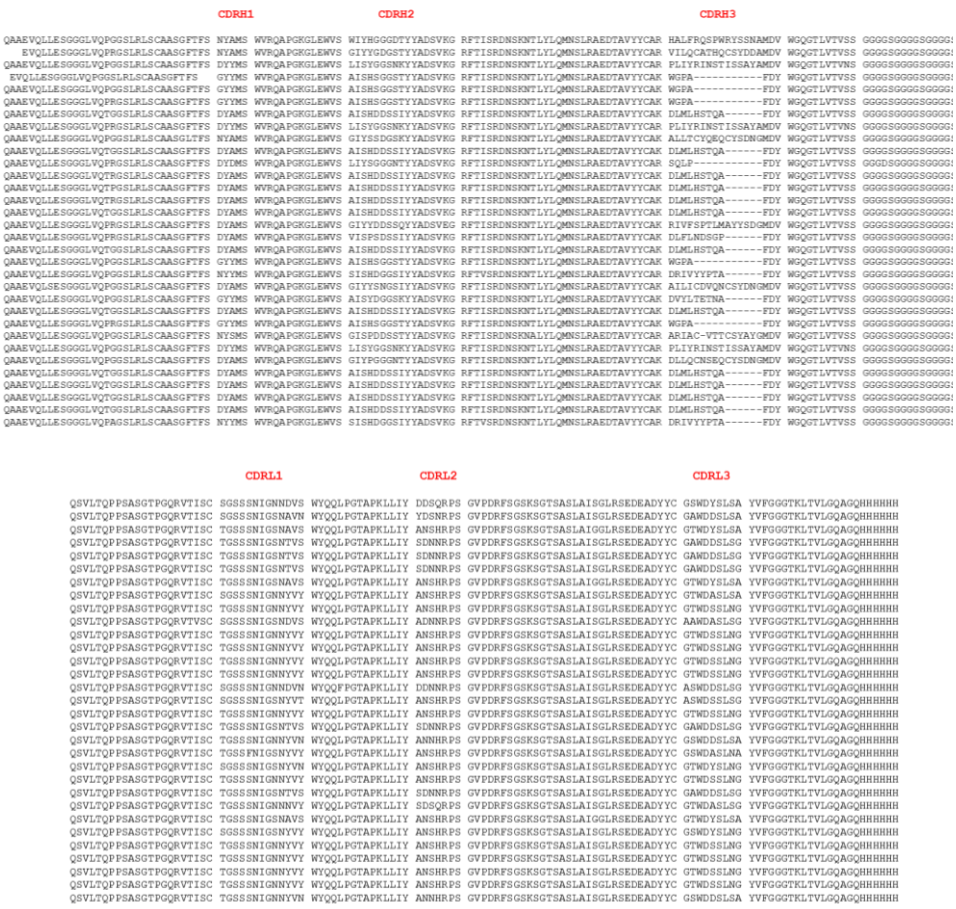
c-Met recombinant

< >	1	2	3	4	5	6	7	8	9	10	11	12
A	1.2520	1.4030	0.9280	1.1080	1.3100	1.2730	0.9760	0.2700	1.1030	1.3780	0.2960	0.2980
B	1.3120	1.2850	1.0420	0.9910	1.0830	1.4060	0.9510	0.8540	1.1790	0.9910	1.1070	1.2980
C	1.8630	1.0400	1.1560	0.8210	1.3490	1.0820	1.0600	0.6220	1.0540	0.8090	1.0350	0.2320
D	1.4240	0.9220	1.4780	1.2100	0.3290	1.0460	0.8690	1.2600	0.2790	0.7520	1.0360	1.0920
E	1.3200	2.3720	1.0530	1.7390	1.7170	1.7590	1.5010	1.4010	1.3940	0.9640	1.0890	0.8010
F	1.1260	1.3590	0.6080	1.4520	1.2930	1.0550	0.6250	0.2590	0.1830	1.1200	1.0350	0.4290
G	1.1110	1.2260	0.9390	1.0740	0.9590	0.9800	1.2140	0.8710	0.2640	0.6350	1.2070	1.8900
H	2.6790	1.6690	0.9230	1.2880	1.2450	0.0780	1.0730	0.9780	1.1200	1.0680	1.0130	0.0990

Figure 1. Screening by phage display and ELISA using human c-Met recombinant protein as antigen

Schematic representation of workflow (A). The c-Met recombinant protein comprising regions 1-932 of the extracellular domain was used as antigen. The concentration of antigen and wash methods were optimized for each panning cycle and the input and outputs were calculated (B). The ratio between output and input changed after each cycle (C). The binding of samples resulted from 3^{rd} and 4^{th} panning cycles were assayed through ELISA (D). Hits were selected by comparing results from the control plate and sequenced.

A.



B.

Selected hits

Controls

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.8930	2.6440	1.2660	1.1900	2.9510	2.9730	2.2170	2.3660	0.0810	0.0890	0.0900	0.0970
B	2.4350	2.6540	2.3590	2.4940	3.2030	2.3840	2.2050	2.9420	0.0790	0.0840	0.0830	0.0880
C	2.6890	0.5750	2.9400	2.7140	1.3170	2.8620	0.7430	1.8660	0.0750	0.0830	0.0770	0.0830
D	1.4230	2.2890	1.5880	1.9130	2.9050	2.2200	2.5530	0.7530	0.0800	0.0840	0.0850	0.0860
E	0.0680	0.0560	0.0670	0.0560	0.0750	0.0640	0.0730	0.0670	0.0770	0.0880	0.0820	0.0840
F	0.0780	0.0660	0.0680	0.0660	0.0840	0.0780	0.0810	0.0790	0.0860	0.0910	0.0890	0.0960
G	0.0770	0.0650	0.0650	0.0620	0.0830	0.0710	0.0790	0.0750	0.0820	0.0860	0.0890	0.0950
H	0.0870	0.0630	0.0710	0.0650	0.0810	0.0730	0.0800	0.0690	0.0860	0.0850	0.0840	0.0970

C.

Clones	CDRH1	CDRH2	CDRH3	CDRL1	CDRL2	CDRL3
A8	NYAMS	GIYYGDGSTYYADSVKG	VILQCATHQCSYDDAMDV	SGSSSNIGSNAVN	YDSNRPS	GAWDDSLSA
A9	DYYMS	LISYGGSNKYYADSVKG	PLIYRINSTISSAYAMDV	TGSSSNIGSNAVS	ANSHRPS	GTWDYSLSA
A11	GYYMS	AISHSGGSTYYADSVKG	WGPAFDY	TGSSSNIGSNTVS	SDNNRPS	GAWDDSLSG
B8	NYAMS	GIYSSDGSKYYADSVKG	ALLTCYQEQCYSDNGMDV	SGSSSNIGNNYVY	ANSHRPS	GTWDASLSA
B10	DYDMS	LIYSGGGNTYYADSVKG	SQLPFDY	SGSSSNIGSNDVS	ADNNRPS	AAWDASLSG
C8	DYAMS	GIYYDDSSQYYADSVEG	RIVFSPTLMAYYSDGMDV	SGSSSNIGNNDVN	DDNNRPS	ASWDDSLSG
C9	DYAMS	VISPSDSSIYYADSVKG	DLFLNDSGPFDY	SGSSSNIGSNYVT	ANSHRPS	ASWDSSLSG
D7	GYYMS	AISYDGGSKYYADSVKG	DVYLTETNAFDY	SGSSSNIGSNYVN	ANSHRPS	GTWDYSLNG
D12	DYAMS	GIYPGGGNTYYADSVKG	DLLQCNSEQCYSDNGMDV	SGSSSNIGSNYVY	ANSHRPS	GSWDYSLNG
E10	DYAMS	AISHDDSSIYYADSVKG	DLMLHSTQAFDY	TGSSSNIGNNYVY	ANSHRPS	GTWDSSLNG

Figure 2. Sequencing, selection of hits and IgG conversion

Based on the results from ELISA, hits were selected and sequenced (A) and 31 hits with matching sequences were identified. The identified hits were tested for binding through ELISA (B). Among the selected samples, 10 hits that showed the strongest binding signals were selected and converted to human IgG form (C).

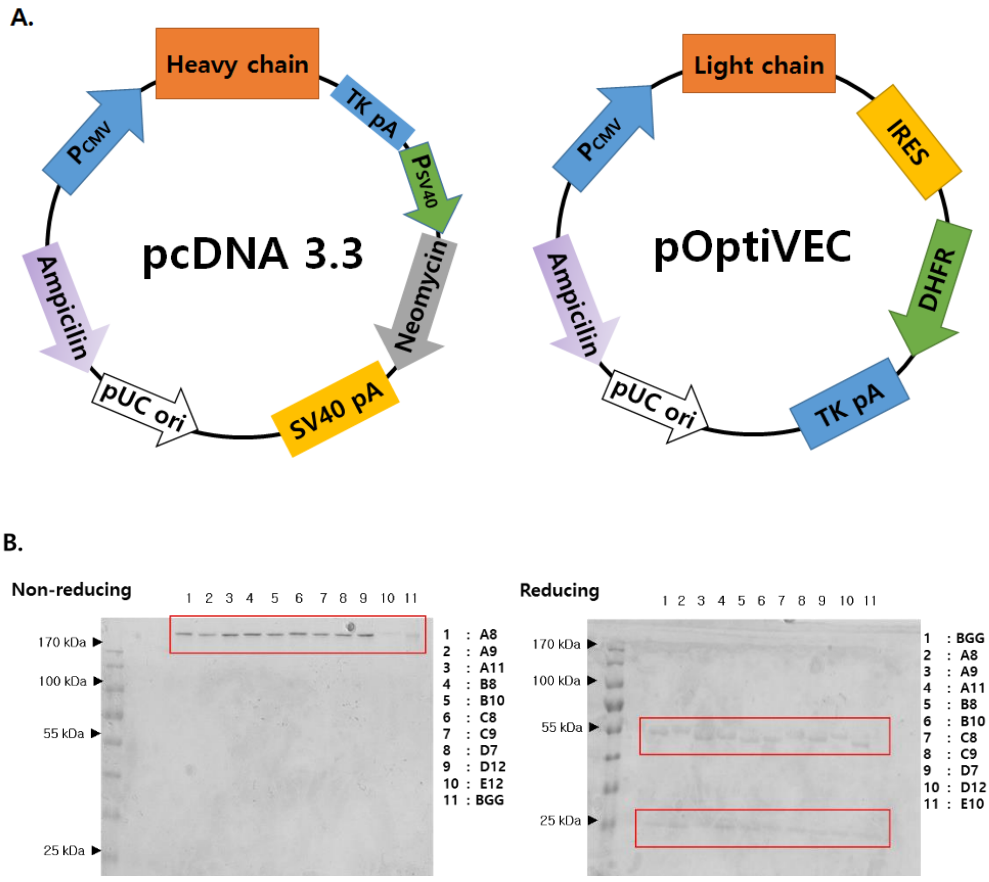


Figure 3. Antibody production and purification by gravity-based chromatography

Schematic representation of vectors used for expression of antibodies (A). The plasmids were transfected in 293F expression system, incubated for 7 days, harvested and the expressed antibodies were purified using protein A bead. The heavy chain and light chain sizes were checked through SDS-PAGE (B).

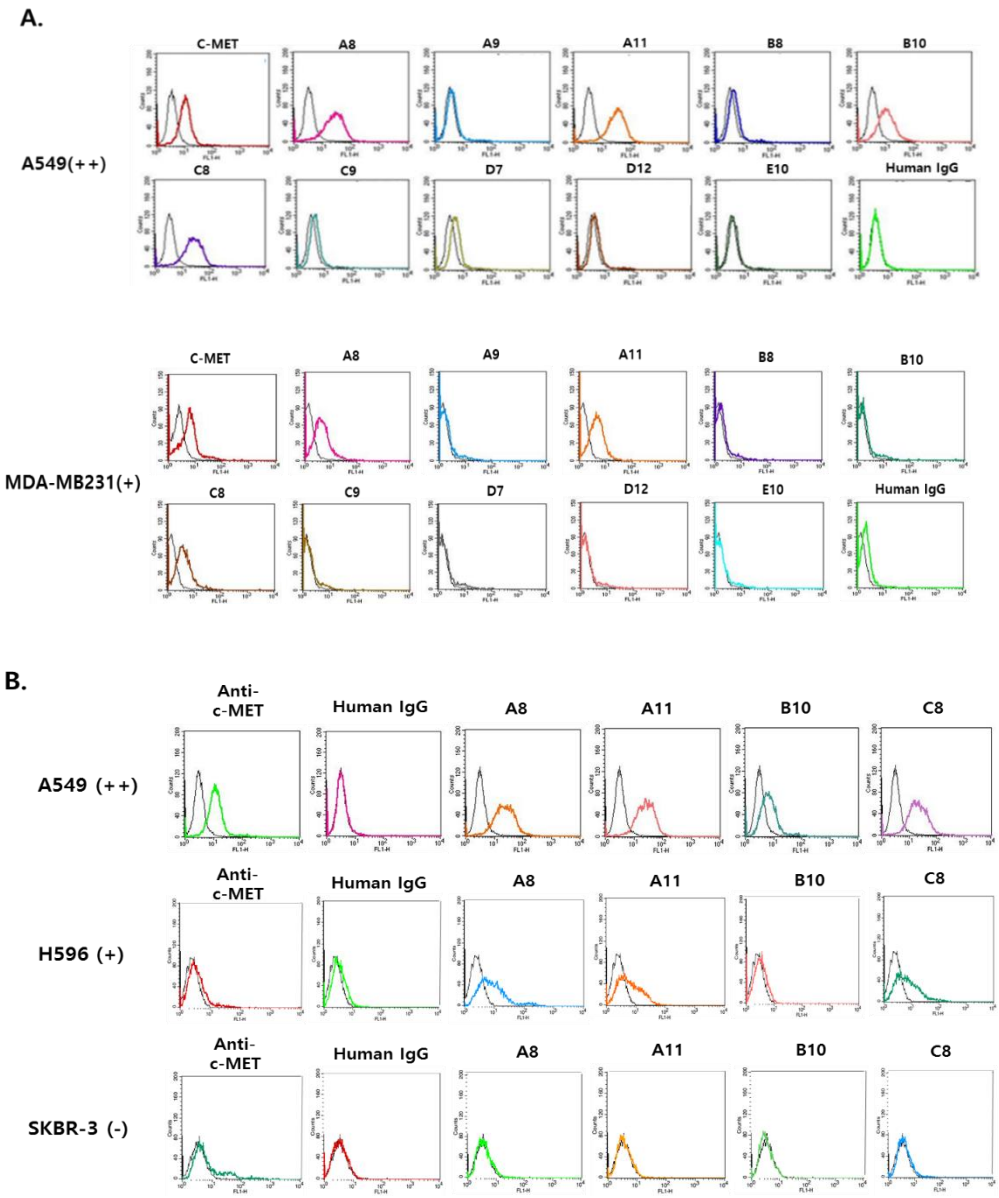
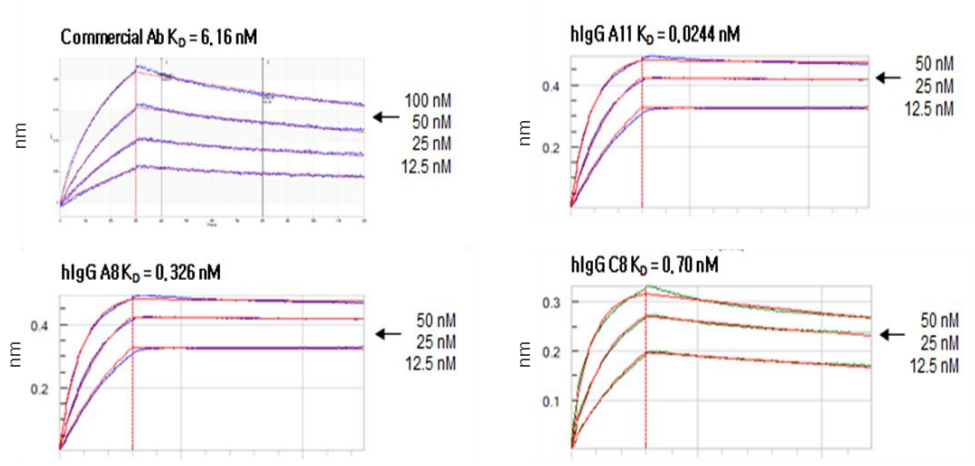


Figure 4. Verification of binding to native receptors by flow cytometry

Cells positive for c-Met (A549, MDA-MB231) were incubated with the IgG converted anti-c-Met antibodies at a concentration of

1ug/sample (A). The hits that showed binding shifts, A8, A11, B10, and C8, were selected as leads. The antibodies selected were tested again with different cell lines including a c-Met negative cell line SKBR-3 (B).



Sample	Kon(1/Ms)	Koff(1/s)	K_D(nM)
Anti-c-met	5.47E+04	3.37E-04	6.16
A8	9.53E+05	3.10E-04	0.326
A11	5.69E+05	0.139E-04	0.0244
C8	2.63E+05	1.85E-04	0.701

Figure 5. Assessment of binding affinity through Octet

The leads that showed the highest binding shifts were selected and tested for affinity through Octet system and compared to commercial anti-c-Met antibody. The human recombinant c-Met was used as antigen. The KD value was calculated from Kon and Koff values.

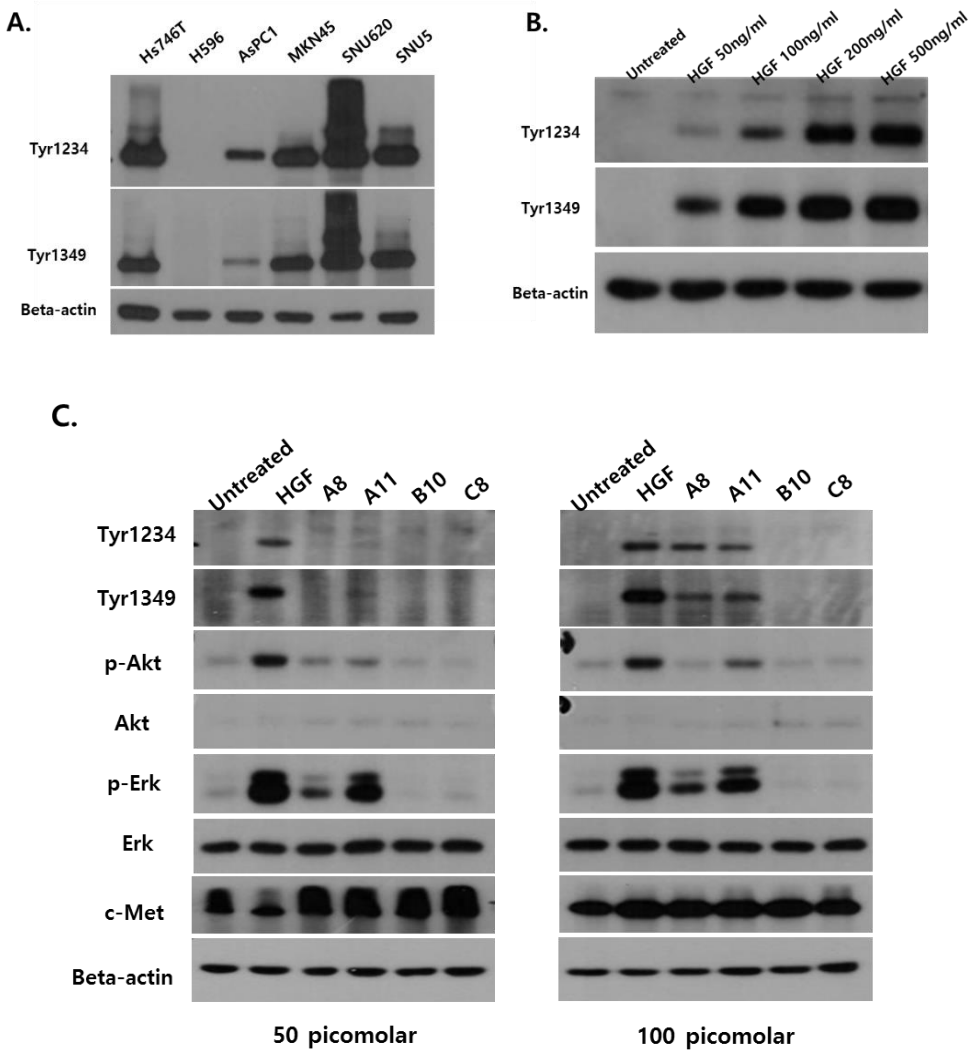


Figure 6. Analysis of c-Met signal patterns in c-Met positive cell lines through western blot

To verify the c-Met signal patterns, c-Met positive cell lines were selected and western blot was performed (A). H596 cell line did not show any phosphorylation when untreated. When H596 cells were treated with different concentration of HGF, activation of catalytic

and docking sites could be observed (B). Assessment of phosphorylation pattern and downstream signals upon HGF treatment, A8, A11, B10, and C8 (C). While A8 and A11 showed signal activation, B10 and C8 did not.

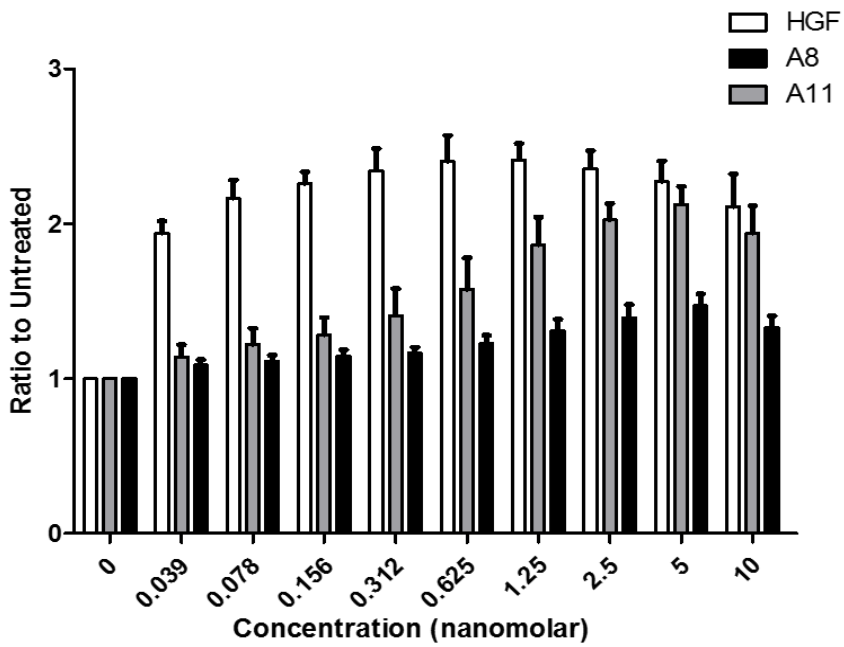


Figure 7. Proliferation assessment of anti-c-Met antibodies and HGF

H596 cells were seeded in 96 well plates and treated with different concentrations of anti-c-Met antibodies and HGF. The cells were incubated for 72 hours after treatment, and proliferation was assessed by WST.

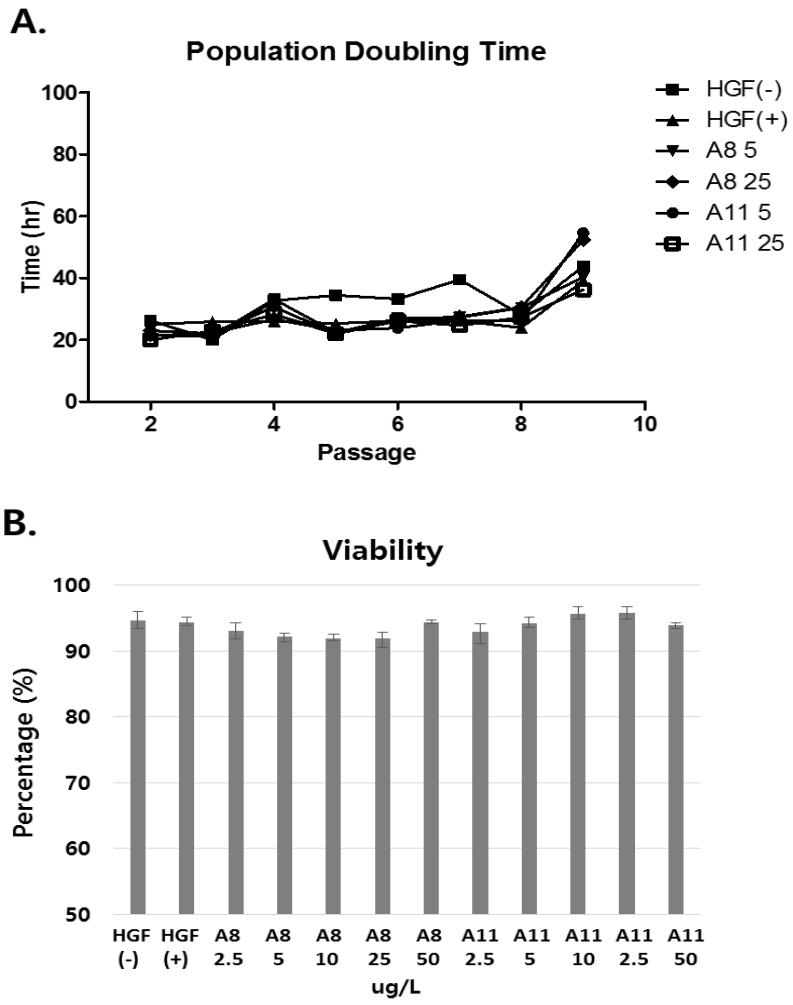


Figure 8. Mesenchymal stem cell culture in chemically defined media supplied with HGF or with selected anti-c-Met antibodies

Adipose-derived mesenchymal stem cells were cultured in chemically defined media without HGF, with HGF, or with different concentrations of anti-c-Met antibodies A8 and A11. Cells were cultured from passage 1 to 9 and tested for population doubling time (A) and viability (B).

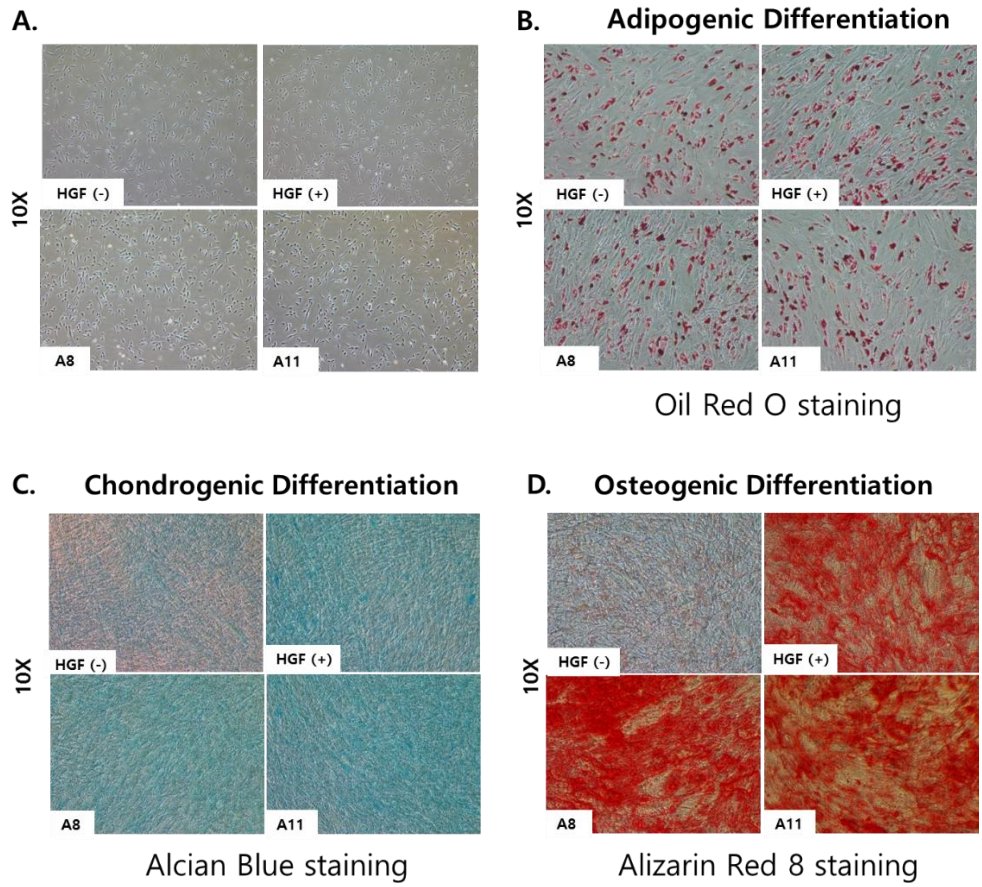


Figure 9. Comparison of culture morphology and trilineage differentiation ability in different media conditions

Morphology of cells during culture before differentiation (A). Cells were cultured until passage 10, then each media replaced with differentiation media. Adipogenic differentiation was done for two weeks then assessed using Oil Red O staining (B). Chondrogenic differentiation and osteogenic differentiation were also performed at passage 10, then each assessed with Alcian Blue and Alizarin Red

8 respectively (C and D).

DISCUSSION

There are currently many trials for applying the HGF/c-Met signaling pathway in the treatment of numerous diseases. Recombinant HGF is being tested clinically for spinal cord injury (Phase I/II trial), amyotrophic lateral sclerosis (Phase I trial), fulminant hepatitis (Phase I/II trial), and venous leg ulcers (Phase I/II trial) [24-27]. Although these trends may look promising for the treatment of these chronic diseases, difficulties related to the production of recombinant HGF, its quality control and poor stability still remain. In attempts to overcome these problems, several groups have taken a different approach by using gene therapy. The diseases to which this method was applied and are currently in clinical trial include peripheral arterial disease, critical limb ischemia, and amyotrophic lateral sclerosis [28-30]. Using DNA therapy may overcome to some degree the difficulty of HGF production, but still poses challenges in molecule stability.

Instead of using the growth factor, we tried to replace the cytokine with antibodies that provide a more stable structure with the ability to trigger the HGF/c-Met signaling pathway in a similar fashion as the original ligand. In the past, agonist antibodies were used to study the signaling threshold required for the invasiveness of cancer cells

[31]. There were several cases where the effectiveness of c-Met agonist antibodies were tested as substitutes of HGF in anti-apoptotic and tissue protective roles [32, 33]. Interestingly, attempts to further develop therapies using these agonist antibodies have not been seen. We screened the human scFv library using the extracellular region of the receptor, since it is the extracellular region that HGF binds to activate signals. As it was discovered that agonism directed against c-Met is dependent on mAb bivalence [6, 34], the selected scFvs were converted to human IgG form and tested for affinity. After affinity and specificity have been confirmed, we tested for the ability to induce signals. The H596 cell line, derived from NSCLC, was used as a model cell line for the signal transduction study. Unlike many other c-Met positive cell lines, H596 cell lines only contain the exon 14 deletion mutation that makes them cancerous, yet dependent on HGF for c-Met activation [35]. This characteristic made an easily reproducible model for our early study of c-Met signaling activation. When antibodies were tested for signaling activation, both of our candidates A8 and A11 were able to phosphorylate the catalytic and docking sites of c-Met. The phosphorylation resulted in the further activation of downstream signals like Erk/MAPK and Akt that play essential roles in proliferation and survival [36]. In line with these results, the antibodies induced proliferation of H596 cells and showed

concentration dependent patterns, though A11 was observed to induce higher rates of proliferation than A8. These results may be explained by looking at the difference in binding affinity of the two antibodies. Based on Octet results, A11 showed both faster binding and slower dissociation compared to A8. This kinetic pattern may influence the extent or intensity of the signal that the antibody induces. The effect of kinetics in the control of signal intensity and duration remain to be revealed.

After verifying the effects of antibodies in signal transduction and proliferation, we tried to see if the antibodies were applicable as substitutes of HGF in more sensitive conditions. Mesenchymal stem cells are known to be the main producers of HGF, but this cytokine also acts in an autocrine manner to induce signals essential for maintaining multipotency in stem cells. There have been previous studies which demonstrated that the differentiation of several cell lineages like osteogenesis require HGF [37, 38, 39]. Based on these studies, we have investigated the effect of the antibodies on mesenchymal stem cells compared to HGF. The media used for stem cell culture was chemically defined, containing no FBS and supplied with growth factors. To test the growth of mesenchymal stem cells in different conditions, 4 kinds of media were manufactured: no HGF, including HGF, A8, or A11. When we assessed population doubling time, viability, and morphology, the groups did not show significant

differences. On the other hand, when cells were induced to differentiation in three lineages (adipogenesis, chondrogenesis, and osteogenesis), a clear difference was seen in the group that was cultured without HGF. Because the mesenchymal stem cells were adipose-derived, there was no problem in the differentiation to adipocytes, the original lineage. As for chondrogenesis and osteogenesis, the cells that were cultured in media without HGF were not able to successfully differentiate. These results suggest a critical role for HGF/c-Met signaling in the multipotency of stem cells. A8 and A11 were also able to maintain stem cell multipotency, with no observable difference compared to the HGF group. Thus, antibodies A8 and A11 performed as successful substitutes for HGF in mesenchymal stem cell culture.

This study aimed to identify agonist antibodies binding to c-Met, with comparable effects on signaling and proliferation. We have showed that our agonist antibodies A8 and A11 are able to induce the activation of c-Met signaling pathway including main downstream signals, resulting in the proliferation of cells. When they were tested for equivalence with HGF in mesenchymal stem cell culture, the antibodies proved to have the same efficiency in maintaining multipotency and allowed differentiation to chondrogenic and osteogenic lineages. Antibodies also provide higher stability, ease of production and purification, overcoming the main difficulties related

to the recombinant HGF. Further studies are required to assess the effect of these antibodies in protection and restoration of damaged tissues in animal models in order to define their role as HGF substitutes in therapy.

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국문초록

Human Hepatocyte Growth Factor(HGF)는 간, 폐, 신장 및 심지어 신경을 포함한 다양한 조직에서 중요한 역할을 하는 것으로 알려져 있다. HGF는 리간드인 c-Met 수용체에 결합하여 Akt, Ras, MAPK와 같은 세포의 성장, 분화, 생존과 연관된 신호를 유도한다. 이러한 역할로 인해 HGF/c-Met 신호 전달 경로는 암의 원인이 될 수 있지만 정상적으로 작용할 때는 배아 발달 및 장기 회복에 매우 중요한 것으로 드러났다. HGF 유전자를 인위적으로 없앤 마우스 배아는 발달과정에서 사망하는 것이 보고되었다. 더 나아가 간, 심장 등의 만성 질환, 류머티즘 같은 퇴행성 질환에 재조합 HGF를 투여 시 개선효과가 있는 것이 발견되었다. 그러나 HGF는 혈중에서 불안정하여 투여 시 몇 분 안에 혈중농도가 급격히 떨어진다. 또한 합성이 매우 어렵고 치료용으로 쓰기 위한 QC의 확보가 큰 과제이다. 이 과제들을 해결하기 위해 HGF의 수용체인 c-Met에 결합을 하여 동일하게 신호 전달을 유발할 수 있는 agonist 항체의 기능을 연구하였다.

Phage display를 통해 c-Met의 extracellular domain에 결합하는 후보들을 1차적으로 선별하고 ELISA와 flow cytometry를 수행하여 결합력이 가장 높은 항체들을 2차적으로 선별하였다. 선별된 항체들이 c-Met에 결합을 하였을 때 신호전달을 유도할 수 있는지 확인을 하기 위해 H596 세포에 HGF와 항체들을 각각 다양한 농도에서 처리하였다. Western blot으로 신호를 확인한 결과 c-Met의 활성이

이루어졌고 Akt와 Erk같은 하위 신호도 함께 유도되는 것을 볼 수 있었다. 신호들이 유도됨에 따라, 이것이 실제로 세포 성장에 영향을 미치는지 WST로 세포의 성장속도를 확인하였고 HGF와 마찬가지로 항체만 단독으로 처리했을 때도 세포의 성장이 촉진되는 것을 관찰하였다.

또한 HGF는 mesenchymal stem cell 배양에 있어서 필수적인 성장인자로 알려져 있는데 같은 기능을 가진 항체가 HGF를 대체할 수 있는지 확인하기 위해 다양한 농도로 항체를 세포 배양액에 첨가하고 HGF를 배제하였다. 그 결과 항체만 첨가하였을 때도 본래의 조건에서 배양했을 때와 다르지 않은 morphology를 띄는 것을 확인할 수 있었다. 또한 줄기세포의 분화능을 유지하는데 있어 HGF와 동일한 효과를 보이는 것을 확인하였다.

본 연구는 치료제로 주목 받고 있는 HGF의 한계점을 극복하기 위해 이의 기능을 대체할 수 있는 항체를 확인하였고 치료용 mesenchymal stem cell 배양에 대체가 가능함과 더 나아가 만성 및 퇴행성 질환에 치료제로 연구될 가능성을 제시하였다.

주요어: Hepatocyte growth factor (HGF), c-Met, agonist, 인산화, 중간 엽 줄기세포,

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