

UNIVERSITA' DEGLI STUDI DI PERUGIA
DOTTORATO DI RICERCA IN “*BIOLOGIA E
BIOTECNOLOGIA MOLECOLARE*”
“XXII” CICLO
settore scientifico disciplinare “*BIO/10*”

Generation of Antagonistic Monoclonal Antibodies
Specific for the Human Receptor Tyrosine Kinase RON as
Tools to Dissect the Ligand-Dependent Role of the
Receptor in Tumor Cell Migration and Tumor Growth

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A. A. 2009/10

ACKNOWLEDGEMENTS

For me it is an honour apart from being a moral debt thanking to Prof. Riccardo Cortese, who opened the way to my studies and adventure in Italy and has always been a perfect example as a scientist but more importantly as a example-to-take person with all his personal aspects and his standing in life.

I am deeply grateful to Alessandra Vitelli, who has supervised my work at IRBM and has been a perfect model to follow as a complete woman in science, as a supervisor and as a friend.

Special thanks to Prof. Gennaro Ciliberto, who has followed and supervised this work and gave me the chance to complete it.

I would like to thank to Silvia Benvenuti from IRCC, who has shared kindly her cell lines with me and was a great collaborator to discuss science.

Helenia Ansuini , I have learned a lot from you; thank you for the elegance and ease of doing things even when you were handling very complicated issues. Your help made my way less difficult than it would be.

Valentina Viti, you have been a great lab-mate, colleague, friend, sister. These four yours would not be as they are without you neither in lab nor in personal life.

It will be impossible not to mention Nunzia Langella, Alessia Noto, Lionello Ruggeri, Stefano Acali, and Monica Pezzanera for their contribution as colleagues who had been more than colleagues but real friends ready to help in any occasion.

My mother Suheyra Dogan and my father Mutlu Gunes, you have been great parents who supported me in every decision of mine, you are a real treasure one can have and I am the most lucky daughter on this earth for having you both.

At last but not at least, thanks to Luca Di Marcoberardino for giving me strength to dream and being on my side while those dreams become reality.

Zeynep Gunes

I. INTRODUCTION	4
A) CANCER AS A COMPLEX GENETIC DISEASE	5

B)METASTASIS	6
<i>B.1) Molecular Mechanism of Cell Migration.....</i>	<i>9</i>
<i>B.2)Cancer Cell Migration.....</i>	<i>13</i>
C)RON RTK	15
<i>C.1) Chromosomal Location.....</i>	<i>15</i>
<i>C.2) Structure and expression pattern.....</i>	<i>16</i>
<i>C.3) Ligand of RON, MSP</i>	<i>18</i>
<i>C.4) Biological Functions of RON</i>	<i>20</i>
<i>C.5) Oncogenic Signalling Pathways activated through ligand dependent activation of RON</i>	<i>21</i>
<i>C.6) Crosstalk of RON with EGFR</i>	<i>22</i>
II) MATERIALS AND METHODS.....	25
A)CELL CULTURE	25
C)ScFv PHAGE SELECTIONS	25
<i>C.1)Solid Phase Selection (Panning)</i>	<i>27</i>
<i>C.2)Phage Rescue.....</i>	<i>27</i>
<i>C.3)Liquid Phase Selections</i>	<i>28</i>
C.3.a)SW480 Cells.....	28
C.3.b)RON ECD-Fc	29
D)IDENTIFICATION OF POSITIVE CLONES THROUGH PHAGE ELISA	30
E)SEQUENCING PHAGE CLONES	30
F)ScFv TO IgG1 CONVERSION	31
G)FLOW CYTOMETRY	36
<i>G.1)FACS analysis of Phage ScFvs</i>	<i>36</i>
<i>G.2)FACS analysis of IgG.....</i>	<i>37</i>
H) IP AND MALDI-TOF-MS	37
<i>H.1)Labelling and preparation of native membrane proteins (NMPs) </i>	<i>37</i>
<i>H.2) Immunoprecipitation of the target antigen.....</i>	<i>38</i>
<i>H.3) Mass spectrometry and database search</i>	<i>39</i>
I) WESTERN BLOT ANALYSES TO STUDY RON DOWNSTREAM SIGNALING. 39	
<i>I.1)Induction with MSP</i>	<i>39</i>
<i>I.2) Testing RON IgGs' ability to block MSP-driven induction.....</i>	<i>40</i>
<i>I.3)Time course experiment with RON mAbs</i>	<i>40</i>
<i>I.4) Tumor lysate preparation</i>	<i>40</i>
<i>I.5) Cell lysate preparation and Western blot analysis</i>	<i>41</i>
J) Kd DETERMINATION VIA FACS ANALYSIS.....	42
K)CELL MIGRATION ASSAY	43

K.1) Migration in 2% FBS Conditioned Medium	43
K.2) Migration in 0%FBS on Collagen Coated Chambers	43
K.3) Testing RON mAbs	44
K.4) Quantification	44
L) CROSS COMPETITION / EPITOPE OCCUPANCY ASSAY	44
M) CELL CYTOTOXICITY ASSAY	45
N) IN VIVO MOUSE MODELS	45
N.1) T47D orthotopic model	45
N.2) BxPC3 and HT29 xenograft models	46
N.4) T47DtetRron orthotopic model	47
O) RTK PROTEOME PROFILER ASSAY	47
A) SELECTION OF RON-BINDING PHAGE-SCFVS FROM PHAGE LIBRARIES	49
B) IDENTIFYING RON MABS THAT SELECTIVELY BIND TO RON	49
C) MABS 4, 7 AND 10 ARE ABLE TO IMMUNOPRECIPITATE RON	53
D) MSP STIMULATION ACTIVATES DOWNSTREAM SIGNALLING PATHWAYS AND CAUSES AKT AND ERK PHOSPHORYLATION	54
E) RON MABS SHOW ANTAGONISTIC PROPERTIES	55
F) RON IGGs HAVE SUB-NANOMOLAR AFFINITIES	57
G) ANTAGONISTIC RON MABS DO NOT CAUSE RECEPTOR DOWN-REGULATION	59
H) HIGH SERUM CONCENTRATIONS HAS A NEGATIVE EFFECT ON MSP-DRIVEN MIGRATION	59
I) MSP INCREASES T47D CELL MIGRATION	61
J) RON SILENCING ABROGATES MSP DRIVEN MIGRATION	62
K) RON MABS ARE ABLE TO BLOCK MSP DRIVEN CELL MIGRATION	63
L) RON MABS BIND TO DIFFERENT REGIONS ON RON	64
M) RON MABS SHOW INCREASED ACTIVITY WHEN USED IN COMBINATION	65
N) IGG4, IGG7, AND IGG10 DO NOT DISPLAY ANTITUMOR ACTIVITY IN VIVO	67
O) RON SILENCING CAUSES DECREASED TUMOR GROWTH IN VIVO	69
O.1) HCT116tetRRON model	70
O.2) T47DtetRron orthotopic in vivo model	73
P) MSP CAUSES EGFR PHOSPHORYLATION	74
V) DISCUSSION	76
VI) REFERENCES	90

VII)CV

VIII) Published Article: Gunes *et.al.*(2011) Isolation of Fully Human Antagonistic RON Antibodies Showing Efficient Block of Downstream Signaling and Cell Migration. Translational Oncology **4**(1),38-46

I. INTRODUCTION

A) Cancer as a complex genetic disease

Cancer is a generic term for a large group of diseases that can affect any part of the body. One defining feature of cancer is the rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs.

Cancer arises from one single cell; and as other complex diseases, it comes up from combinations of changes that accumulate in the same cell over a period of time. The transformation from a normal cell into a tumor cell is a multistage process, typically a progression from a pre-cancerous lesion to malignant tumor. These changes are the result of the interaction between a person's genetic factors and external agents.

Being a leading cause of death worldwide, cancer accounted for 7.4 million deaths (around 13% of all deaths) in 2004.¹ Lung, stomach, colorectal, liver, and breast cancers cause the most of the cancer deaths each year .(Table 1)

Although currently cancer is the second most common cause of death in the United States, it is likely to become the most common cause of death in the near future. In contrast with the large investments in cancer research by public and private institutes and pharmaceutical industry, cancer remains to be one of the major diseases which is minimally controlled by modern medicine, especially when compared with other major diseases.² Indeed, the age-adjusted mortality rate for cancer is about the same in the 21st century as it was 50 years ago, whereas the death rates for cardiac, cerebrovascular, and infectious diseases have declined by about two-thirds.³

Cancer type	Deaths/year
Lung	1.3 million
Stomach	803 000
Colorectal	639 000
Liver	610 000
Breast	519 000

Table-1 Annual overall cancer mortality due to main types of cancer. ¹

B)Metastasis

The dissemination of cancer cells from the primary tumor to a distant organ is referred to as metastasis. Metastases are the major cause of death from cancer, being responsible of 90% of deaths from solid tumors.⁴ Thus it is very important to study and understand the metastasis mechanisms in order to combat cancer lethality.

Although molecular mechanisms are highly complex and variable, several discrete steps are discernable in the biological cascade of metastasis: loss of cellular adhesion, increased motility and invasiveness, entry and survival in the circulation, exit into new tissue, and eventual colonization of a distant site.^{5,6} During these steps, metastasis proceeds through the progressive acquisition of traits which allow malignant cells originating in one organ to disseminate and colonize a secondary site.⁷ (Figure 1)

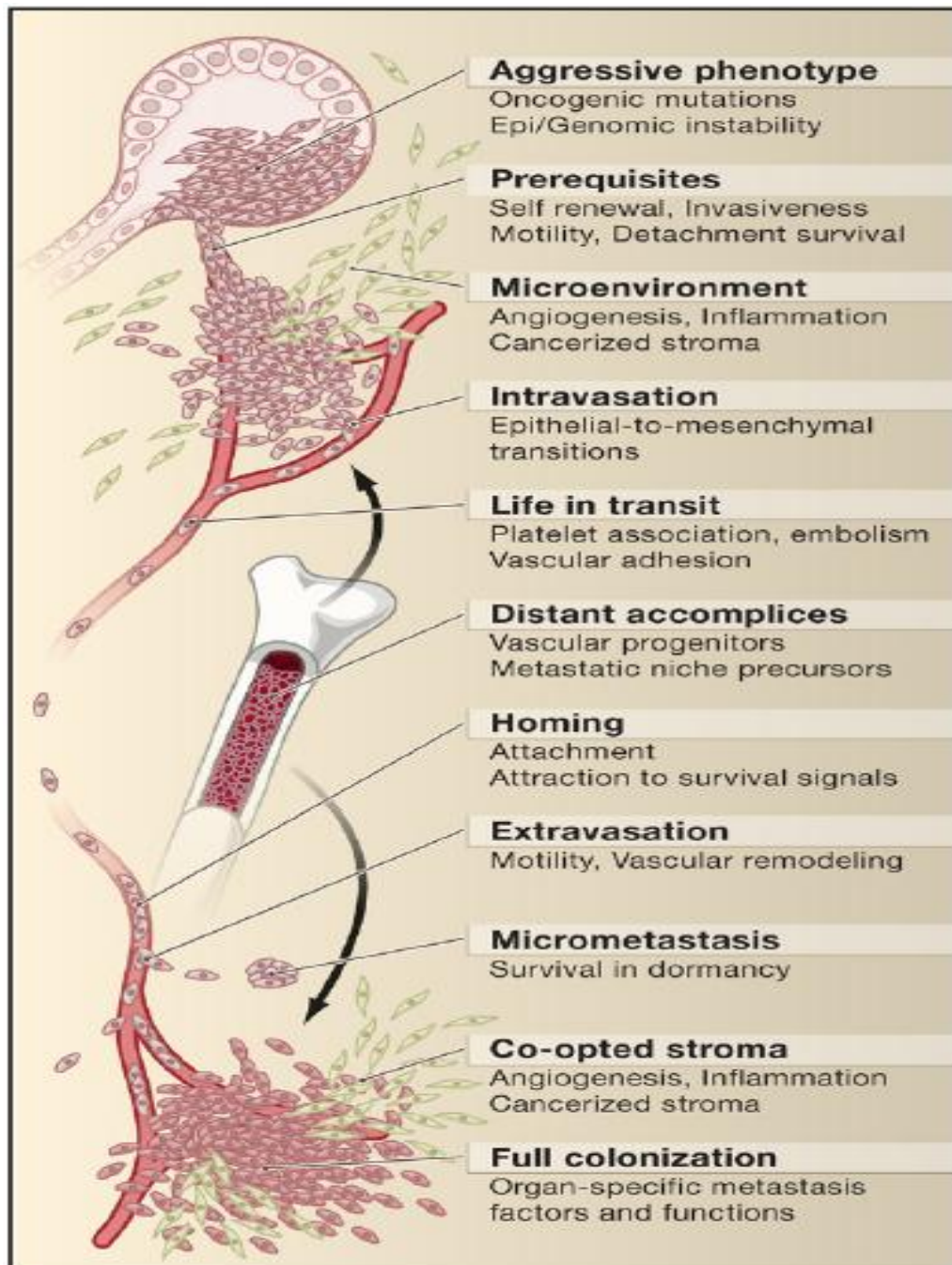


Figure 1. Stages of Metastatic Progression .Metastasis proceeds through the progressive acquisition of traits that allow malignant cells originating in one organ to disseminate and colonize a secondary site. Although these traits are depicted as part of a contiguous biological sequence, their acquisition during metastatic progression need not follow this particular order. Although in some cases several factors may be necessary to implement a single step in this cascade, other mediators of metastasis may facilitate execution of multiple stages simultaneously. Similarly, the specific steps of this sequence that are rate limiting for metastatic progression may also vary from one tumor to the next.⁸

One of the early changes among these is altered cellular adhesion. Compared to normal epithelia, carcinoma cells almost invariably show diminished intercellular adhesiveness.⁹ In many instances, epithelial tumors lose E-cadherin mediated adhesions as they progress towards malignancy.⁹ Additionally, E-cadherin expression can be repressed as part of a broader program resembling an epithelial-to-mesenchymal transition.

Another important mediator of cellular adhesion and malignant phenotype are Integrins. As we will see in the following part, apart from mediating adhesion at stationary phase, cell motility is achieved by active participation and cycling of Integrins. Particularly $\alpha_6\beta_4$ integrin, which binds to ECM protein laminin, forms signaling complexes with oncogenic receptor tyrosine kinases including Met (which is closely related to RON), EGFR and Her2.¹⁰ $\alpha_6\beta_4$ integrin is upregulated in carcinomas such as papillomas¹¹, and is mobilized from hemidesmosomes to migratory regions of the cell in others.¹² $\alpha_3\beta_1$ and $\alpha_v\beta_3$ integrins have been implicated in the later stages of metastasis, especially during adhesion of circulating tumor cells to the vasculature.¹³ (fig 1, extravasation) However, while the expression of some Integrins such as $\alpha_6\beta_4$ and $\alpha_v\beta_3$ increases, expression of other integrins decreases. One example is $\alpha_5\beta_1$ which disappears from the surface of cell lines that are transformed with Rous sarcoma virus.¹⁴ Apart from changes in expression levels, increasing the affinity of Integrins for their ligand is another mechanism by which cells can alter their adhesive profile to assume a more migratory phenotype. As instance, $\alpha_v\beta_3$ affinity has been reported to be modulated in a number of cells¹⁵ and selectively blocking high-affinity $\alpha_v\beta_3$ has been reported to impair the directed migration of endothelial cells.¹⁶

Another study showed that inhibition of cellular adhesion to the matrix by integrin antibodies reduced the invasive potential of breast cancer cell lines.¹⁷ So cells that assume a more invasive and migratory phenotype might modulate their adhesive capacity and intracellular signaling activities by altering their integrin expression and affinity profiles. Also changes in the ECM components that integrins bind might as well contribute to regulation of metastatic potential always by modulating adhesive capacity of a cell.

B.1) Molecular Mechanism of Cell Migration

Cell migration is a highly incorporated multistep process that coordinates embryonic morphogenesis; contributes to tissue repair and regeneration; and drives disease progression in mental retardation, atherosclerosis, arthritis and most importantly in cancer.

Metastasis fundamentally involves the movement of cells from one site to another. Thus, cell motility is a very important step of metastasis being a prerequisite during detachment of cells from the primary tumor site and also during extravasation.

Complex regulatory pathways contribute to formation of the migration process. On the whole, cell migration can be conveniently conceptualized as a cyclic process.¹⁸

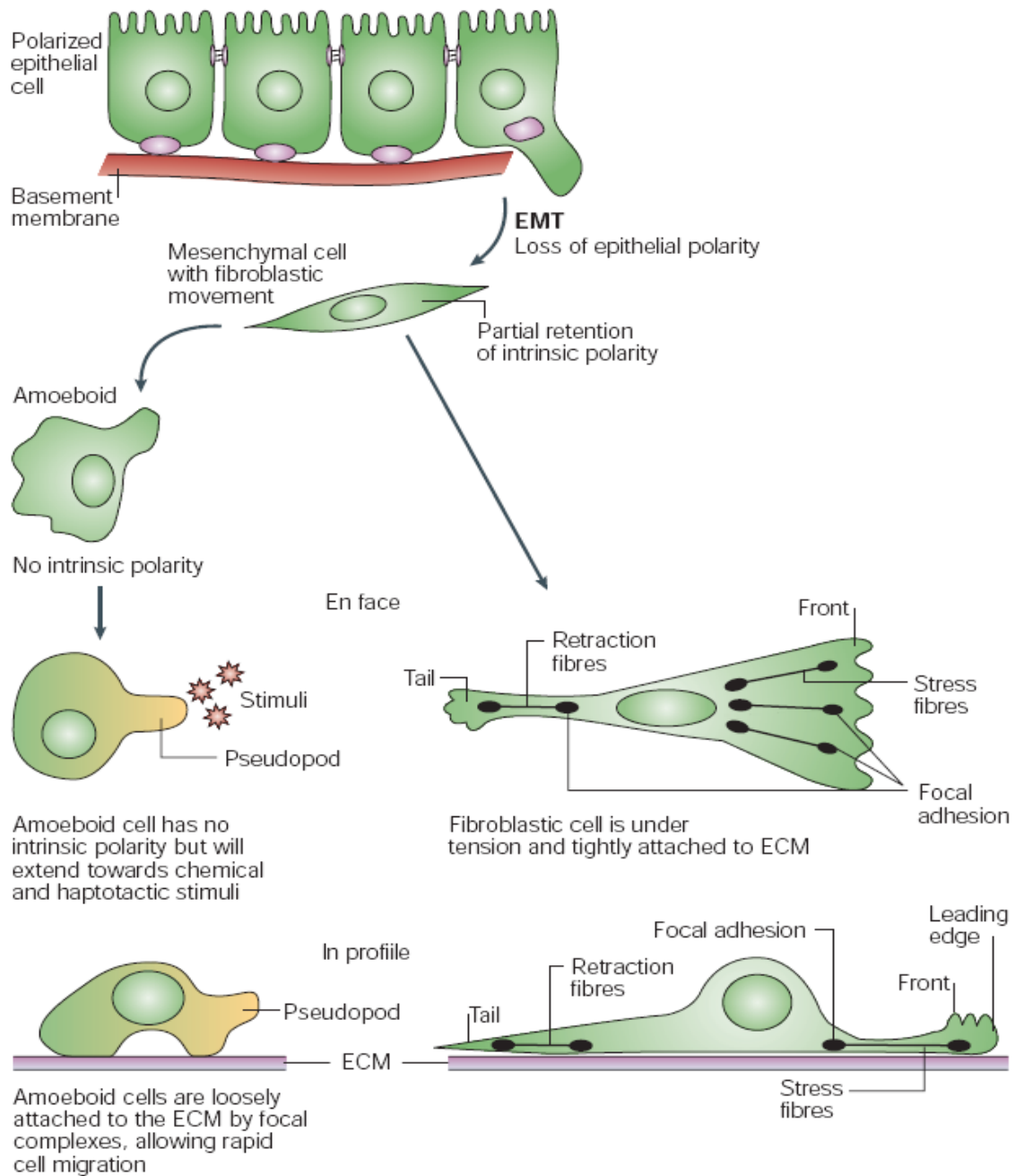


Figure 2. **Definitions of types of cell movement.** During EMT, tumor cells undergo a loss of intrinsic polarity and lose cell-cell junctions that are required for the integrity of the epithelium. Cells that partially retain their polarity still have a tail that contains retraction fibres and a front that contains stress fibres and can form focal adhesions. They can still attach to the extracellular matrix (ECM) and carry out fibroblastic movement. Conversely, cells that lose all polarity are characterized as 'amoeboid'. These cells can chemotax towards stimuli and are only loosely attached to the ECM, allowing for rapid migration.¹⁹

However depending on the cell type and extracellular conditions different types of cell movement and morphology are adopted by the cells. In addition to fibroblast-like cell motility which forms the base for current models of cell migration, there are other forms of cell motility such as amoeba-like movement that is seen in leukocytes and tumor cells that has undergone epithelial to mesenchymal transition, or gliding motion which is displayed by keratocytes.²⁰

Figure 2 exemplifies two most common types of motility observed in human cells: fibroblast-like motility and amoeboid motility.

The initial reaction of a cell to a migration-promoting agent is to polarize and extend protrusions in the direction of migration. These protrusions extended by the cell vary in size and shape. They can be large, broad lamellipodia(on 2-D surfaces), finger like pseudopodia or spike-like filopodia; and are driven by actin polymerization. Growing actin filaments connect to adaptor proteins and push the cell membrane in an outward direction. As a result of this polarization a leading edge and a trailing edge emerges. (fig x) This first step is common to both fibroblastic and amoeboid motility.²¹

In the next step, the formation of adhesions stabilizes and attaches the protrusions to the substratum on which the cell is migrating (ECM or adjacent cells) via transmembrane receptors linked to the actin cytoskeleton.^{21,22} These adhesions serve, in part, as traction points for migration, and they also initiate signals that regulate adhesion dynamics and protrusive activity. As the cell moves forward over them, adhesions assemble at the leading edge and disassemble at the trailing edge(rear), allowing the cell to detach.

However , adhesions also disassemble at the front during protrusion and feed components to nascent adhesions at the leading edge in a process called adhesion turnover.²³ The description of the cell migration above is based on a classical model of cell migration, an individual fibroblast-like cell migrating on a 2-D environment. Even though many aspects of this picture are shared among different cell types, the details can differ greatly.

For example, these steps are observed most distinctly in slow-moving cells such as fibroblasts, but are not as obvious in fast-moving cells such as neutrophils, which seem to glide over the substratum, protruding and retracting smoothly without forming noticeable attachments. Furthermore when cells move in 3-D such as in tissues, there is an additional step in migration process which is the proteolytic remodeling of the ECM. Figure 3 describes a detailed model for 3-D migration which shares the molecular mechanisms with 2-D migration with an exception of step3 .

Cancer cells can modify their morphology and nature of migration in response to environmental changes. *In vitro* and *in vivo* observations showed that tumor cells infiltrate neighbouring tissue matrices in diverse patterns.¹⁹ They can disseminate as individual cells referred to as ‘individual cell migration’ or expand in solid cell strands, sheets, files or clusters called collective migration. Furthermore in response to the changes in their molecular migration program they can switch between these different migration patterns.

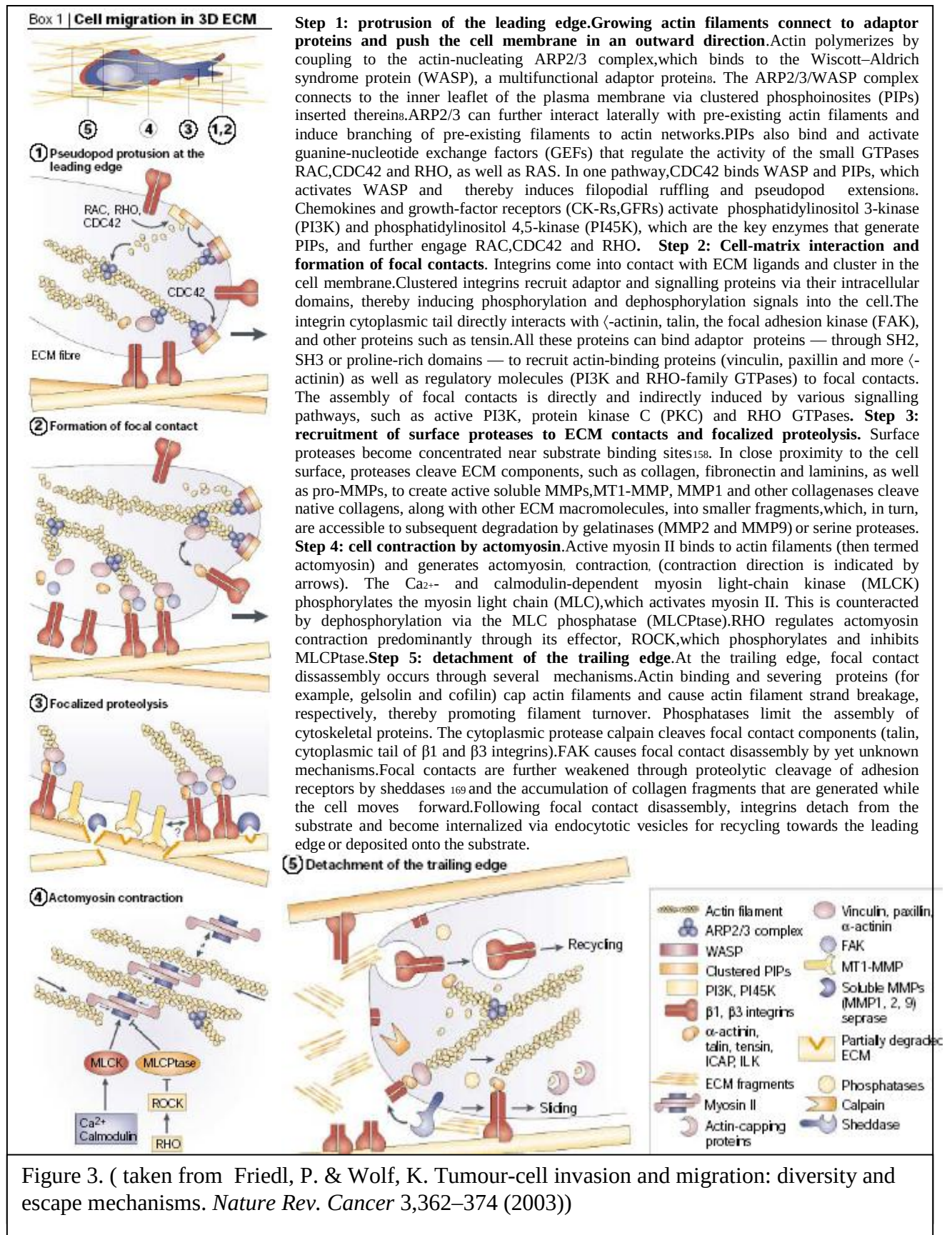


Figure 3. (taken from Friedl, P. & Wolf, K. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nature Rev. Cancer* 3,362–374 (2003))

Intravital imaging of cell movement in tumors revealed many differences among *in vivo* and *in vitro* migration. For example, in mammary tumors, carcinoma cells migrate as solitary amoeboid cells on collagen containing fibers. (Figure 4) They have not been observed to migrate as collections of attached cells as observed in several *in vitro* models of invasion.⁷ Another striking difference between migration of adenocarcinoma cells *in vivo* and *in vitro* is the difference in velocity. *In vivo*, adenocarcinoma cells migrate ten fold faster than cells moving in 2-D culture²⁴ and as much as 30 fold faster than cells moving in 3-D networks of ECM *in vitro*²⁵

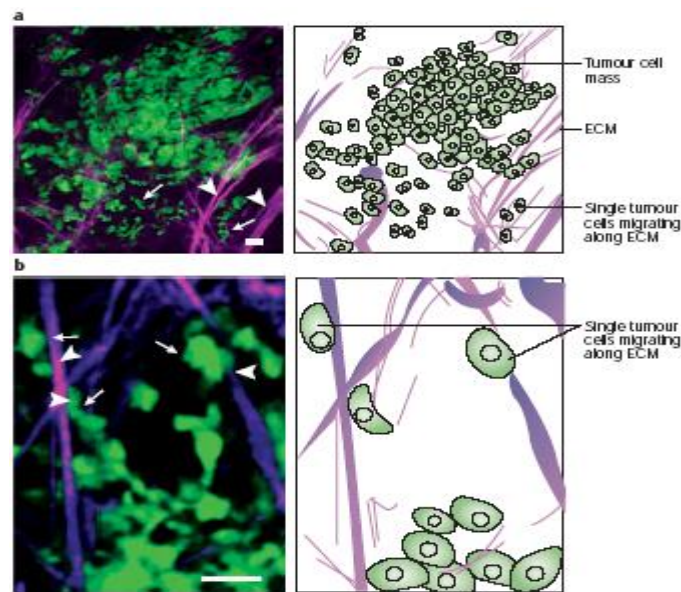


Figure 4 Carcinoma cells in primary mammary tumours move along collagen containig ECM fibres. Multiphoton microscopy shows that carcinoma cells (green) move on extracellular-matrix (ECM) fibres (purple) and do not seem to be constrained by the ECM networks. a) A low-magnification image that shows, in the lower third of the image, single carcinoma cells (arrows) invading away from the central tumour mass and accompanying ECM fibres (arrowheads). b) A highermagnification image — visualized by second-harmonic generation — that shows carcinoma cells (arrows) in a tumour contacting collagen-containing fibres. Arrowheads point to cell–matrix interactions. Scale bar = 25 μm . (From, Wang, W. *et al.* Single cell behavior in metastatic primary mammary tumors correlated with gene expression patterns revealed by molecular profiling. *Cancer Res.* **62**, 6278–6288 (2002))

C)RON RTK

Also being called as MST1R or HGFLR the **R**ecepteur d'**O**rigine Nantais (RON) is a member of the MET protooncogene family of RTK's.²⁶ Human RON cDNA was initially identified and isolated through the screening of cDNA libraries prepared from human foreskin keratinocytes.²⁷ The mouse RON cDNA was cloned from bone marrow cells and named as stem-cell derived tyrosine kinase (STK).²⁸ RON was classified based upon its homology to Met and also by its homology to the Sea receptor that is found in chicken. The c-Sea receptor is the cellular homolog of the avian v-sea oncoprotein. Moreover it is structurally similar to RON and Met.^{29,30}

Up to now, homologs of RON and its ligand have been identified in many mammalian species such as common chimpanzee(*Pan troglodytes*), cow (*Bos Taurus*), rat (*Rattus norvegicus*), dog (*Canis lupus familiaris*), horse (*Equus caballus*) and rhesus monkey (*Macaca mulatta*) as well as non-mammalian species such as chicken (*gallus gallus*), African clawed frog (*Xnopus laevis*).³¹ RON is evolutionarily conserved in multiple species, which suggests a vital function for this receptor.

C.1) Chromosomal Location

The human RON gene maps to 3p21.3 region.³² The human chromosome 3p21 region has been frequently observed to undergo loss of heterozygosity in cancer specimens and cell lines, suggesting that this region may harbor tumor suppressor genes. 3p21 region is also known to be altered in lung cancer .^{32, 33} Amplification of 3p is a common event in lung cancer cell lines, renal and breast carcinoma biopsy material, occurring in 15-42.5% of

the samples examined³⁴ This amplification of the chromosome region containing RON is consistent with the overexpression of RON seen in many human tumor types.

C.2) Structure and expression pattern

The human RON transcript consists of 20 exons and codes for 1400 amino acids.²⁷ RON is synthesized as a single chain glycosylated precursor (pro-RON) which is further processed by furin-like proteases before being delivered as a mature receptor to the cell surface. Mature RON is a 180 kDa disulfide linked heterodimeric protein composed of a 35 kDa extracellular α -chain and a 150 kDa transmembrane β -chain with intrinsic kinase activity and regulatory elements.^{26, 35,36}

Structural domains of RON include the SEMA domain which is unique to this receptor family, the plexin-semaphorin-integrin (PSI) domain and four immunoglobulin-like IPT domains in the extracellular portion followed by a single pass transmembrane domain and an intracellular tyrosine kinase domain.^{35, 37}(Figure 5)

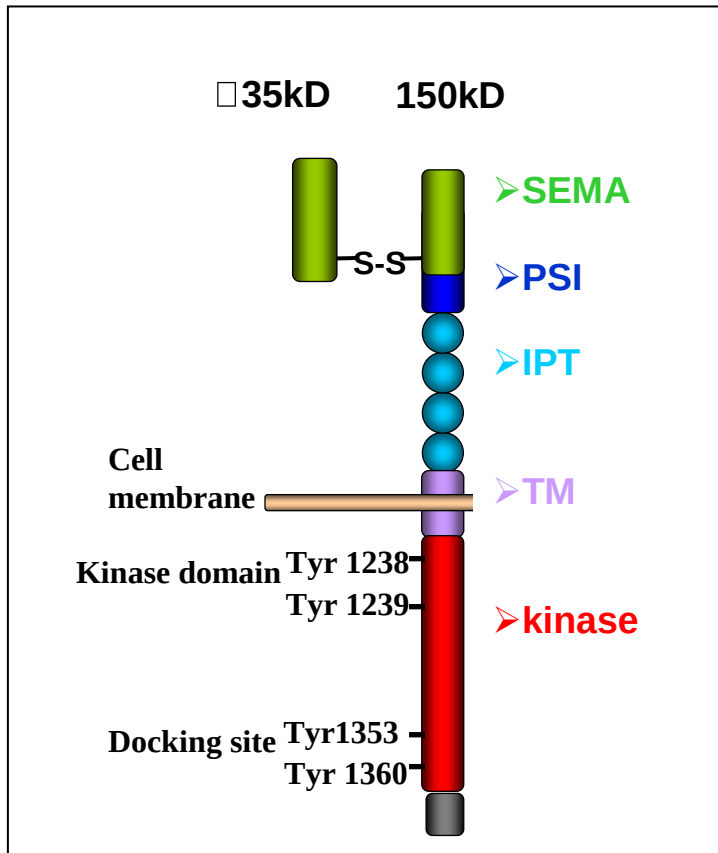


Figure 5. Structural domains of the mature full-length RON receptor. RON is a transmembrane receptor consisting of a 150 kDa β chain and 35kDa α -chain linked by a disulfide bridge. Structural domains include SEMA, PSI (plexin-semaphorin-Integrin), four IPT(1-4, Immunoglobulin-like fold shared by Plexins and Transcriptional factors), transmembrane (TM) and kinase domains.

RON is expressed on many different cell types such as tissue macrophages, osteoclasts, neurons, epithelial, endothelial, mesangial and hematopoietic cells and bone marrow megakaryocytes.³⁷ High expression of RON is seen in epithelial tumors and tumor cell lines of breast, colon, ovary, bladder, pancreas, lung, liver and kidney.^{35,38,39,40} (Figure 6)

Tumor type	Positive cell lines	Negative cell lines
Colon	DiFi, HCT-8, HCT-15, HCT-116 HCC-2998, Colo205, Colo-201 DLD-1, GEO, T84, HT-29, KM12 Sw480, Sw620	SNU-C1, LoVo, CaCo-2
Lung	NCI-H1650, NCI-H292, NCI-H1975, NCI-H358, NCI-H1666, NCI-H226, NCI-H441, NCI-H1650, NCI-H727, NCI-H596, NCI-H322M, EKVX, A549	NCI-H460, NCI-H23, NCI-H522 Hop-62, Hop-92
Pancreas	BXPC-3, Capan-2, HPAC, HPAFII L3#7.p1, Hs766T, ASPC-1, CFPAC-1	MIA-PACA-2
Breast	T47D, HCC-1937, MDA-MB-468, BT20, DU4475	MDA-MB-231, MDA-MB-435, MCF-7, BT-474, SKBR3, Hs578T
Ovary	SKOV3, OVCAR3, OVCAR5, MDAH-2774, IGOV-1, OV90	OVCAR8, CaCOV3, CaCOV4
Prostate	PC-3, DU145, LnCAP	22RV.1
Stomach	NCI-N87, AGS	SNU-1, SNU-16, KKVR
Liver	HepG2	SNU-182, SNU-449, SNU-475 SNU-398, Hep3B, PLC/PRF/5
Leukemia		EOL, JM-1, HEL, Jurkat, TF-1 U937, HL-60
Kidney		A498, Caki-1, SKRC29, 786-0, ACHN, RXF-393, SN12C, TK-10, UO-31

Figure 6. Flow cytometric, Western blot and Gene Logic analyses of RON expression in cancer cell lines. (From reference 40)

C.3) Ligand of RON, MSP

Macrophage Stimulating Protein (MSP), the ligand of RON, is a growth and motility factor also named as Hepatocyte Growth Factor Like (HGFL) protein or Scatter Factor 2 (SF-2) that belongs to plasminogen-related growth factor family.^{35, 41}

MSP was originally identified as a serum protein that stimulated murine resident peritoneal macrophage chemotaxis in response to complement factor C5a.³⁶ By screening for the characteristic kringle domains present in prothrombin and several other proteins in the blood coagulation system, HGFL protein was cloned and named after HGF due to the structural similarity between these two proteins. Later on, MSP and HGFL were determined to be identical.⁴² Following this, two independent groups determined MSP to be the ligand for RON receptor.^{26,43}

MSP maps to human chromosome 3p21, like RON, which is a region that often displays loss of heterozygosity in cancerous tissue. It is synthesized mainly by hepatocytes as a biologically inactive single chain precursor (pro-MSP) with a putative signal peptide followed by a pre-activation segment, four kringle domains and a serine-protease-like domain with the substitution of three amino acids in the active site.³⁵ The amount of pro-MSP in serum is approximately 2 to 5 nmol/L.³⁵ Pro-MSP is converted to its biologically active form in extravascular sites through cleavage at Arg483/ Val484 to yield a disulfide linked heterodimer of a 50 kDa α -chain and a 35 kDa β chain. Proteases of the coagulation cascade such as factor XIa, factor XIIa and kallikrein are capable of cleaving pro-MSP into MSP.³⁶ The specific membrane-bound protease that is responsible for the activation of pro-MSP at the cell surface has been identified through the transcriptional profiling of cancer cell lines, multiple types of cancer tissues and normal tissues; and validated by biochemical and functional testing. This enzyme is known as Matriptase or membrane type serine protease 1 (MT-SP1).⁴⁴

MSP has two independent RON-binding sites. The high affinity binding site resides on β -chain and the low-affinity binding site is on the α -chain with EC50 values of 0.25 and 16.9 nM respectively.⁴¹

Mature MSP acts on several cell types including epithelia, hematopoietic cells and macrophages.^{36,41} It induces motility, adhesion, proliferation and survival of epithelial cells; whereas in macrophages induces motility and inhibition of iNOS upregulation by inflammatory stimuli and regulates cytokine production.^{41,45}

C.4) Biological Functions of RON

RON has been shown to be important for embryological development of epithelial, bone and neuron-endocrine tissues.^{36,46,47} Its expression in embryonic tissue and RON knockout lethality of mice supports RON's role in embryogenesis.³⁷ Moreover, RON is involved in regulation of IFN- γ production and responses in innate immunity.⁴⁸

Another prominent function of RON is its involvement in cellular adhesion. MSP increased cell adhesion to human fibronectin only in cells expressing RON. And this increased cell adhesion requires integrin function.⁴⁹

Studies have been demonstrated that RON is involved in cell motility and invasion. Once transfected with RON cDNA, normal colonic epithelial cells showed increased migration and invasion after MSP treatment in a dose-dependent way.⁵⁰

Increased cell migration and invasion have also been demonstrated in human cancer cells that endogenously express RON.^{50,51}

Furthermore, RON activation is found to cause changes associated with epithelial to mesenchymal transition (EMT)⁵², which is a unique process observed in embryonic development and tumorigenesis in which cells lose epithelial characteristics and gain mesenchymal properties.⁵³ EMT due to MSP that is mediated by MEK MAPK was observed as a 50% reduction in E-cadherin levels upon 48h of MSP exposure in MDCK cells transfected with RON cDNA; redistribution of E-cadherins, B-catenin and actin.⁵² Changes

associated with EMT due to RON activation further support the ability of RON to increase cellular migration and invasion, as well as implicating RON in tumor progression and metastasis.

RON expression and activation by MSP can enhance the ability of cells to avoid apoptosis.³⁶ It was observed that MSP treatment prevented apoptosis caused by serum starvation or anoikis (cell death caused upon detachment of cells).^{52,54}

Cell transformation as a result of RON overexpression has been elegantly demonstrated in transgenic mouse models. Tissue specific over-expression of RON resulted in aggressive tumor formation in transgenic mouse models of lung and breast cancer.^{55, 56}

C.5) Oncogenic Signalling Pathways activated through ligand dependent activation of RON

Under physiological conditions MSP binding to RON gives rise to receptor autophosphorylation and up regulation of RON kinase activity, which in turn stimulates a number of intracellular pathways mediating MSP effects. In normal cells MSP-induced activation is a transient event whereas in tumor cells RON activity is often up regulated.

As with other RTKs, activation of the kinase domain of RON is thought to depend on receptor dimerization through ligand binding and trans-autophosphorylation of two tyrosine residues in the docking site. (Figure 5)

RON is a strong inducer of both PI3-K and MAPK signaling pathways. Stimulation of RON by MSP causes phosphorylation of both RON and PI3-K and promotes association between these two molecules.³⁷ PI3-K directly interacts with C-terminal tyrosines (Y1353/Y1360 in human) when they are in phosphorylated form. Small GTP-binding proteins are down-stream components of the PI3-K pathway that mediate cell motility, whereas AKT which is as well activated through PI3-K, is essential for protection from apoptosis. Indeed, it has been shown that PI3-K mediates MSP induced cell survival and a PI3-K inhibitor wortmannin blocks MSP-induced motility effects such as adhesion, shape changes and migration.³⁷

Another oncogenic pathway activated by RON is the MAPK pathway. MAPK is activated in cells treated with MSP and regulates proliferative and anti-apoptotic MSP/RON induced signals. Ras and c-Src may represent either one or two independent pathways leading to MAPK activation.³⁷ It has been also shown that MSP activates JNK and by increasing IL-6 secretion leads activation of Jak/Stat oncogenic pathway.³⁷

Constitutive activation of intracellular signaling pathways above may underlie the mechanism of RON-mediated oncogenic cell transformation.

C.6) Crosstalk of RON with EGFR

Both direct and indirect evidence suggests that RON is spatially and functionally associated with other transmembrane molecules such as adhesion receptors and growth factor receptors. This interaction between

receptors of different types has been termed as receptor cross-talk. Interaction between dissimilar receptors might result in ligand-independent activation of one or both receptors as well as causing modifications of responses to ligand-dependent activation. In case of tumor progression, receptor cross-talk becomes highly important as many different receptors might be upregulated in tumors.

EGFR is one of the growth factor receptors which are frequently overexpressed in tumors. It has been shown that RON and EGFR co-immunoprecipitate both in the presence and absence of either MSP or EGF(ligand of EGFR) and the interaction between two receptors showed to be ligand-independent.^{57,58} Transphosphorylation of both receptors were induced upon stimulation with either EGF or MSP.⁵⁷

By expressing a dominant-negative form of EGFR in cells stably expressing mouse Ron, scattering response to MSP was significantly reduced indicating a biological function for the interaction of two receptors.⁵⁷

D) CaT scFv Phage Display Technology

scFvs are engineered antibody parts that are formed of variable regions of the heavy and light chain of immunoglobulins connected to each other by a glycine linker. VL and VH sequences are obtained by reverse transcription from B cells of healthy donors.⁵⁹ After fragments for scFv are assembled, they are introduced into a phagemid vector in frame with gene III of the filamentous phage, which codes for surface protein pIII. By this way scFvs are expressed on the surface of the phage, thanks to their fusion to pIII. By

this way a collection of scFv fragments bound to phages, named a phage display library is formed.(Figure 7)

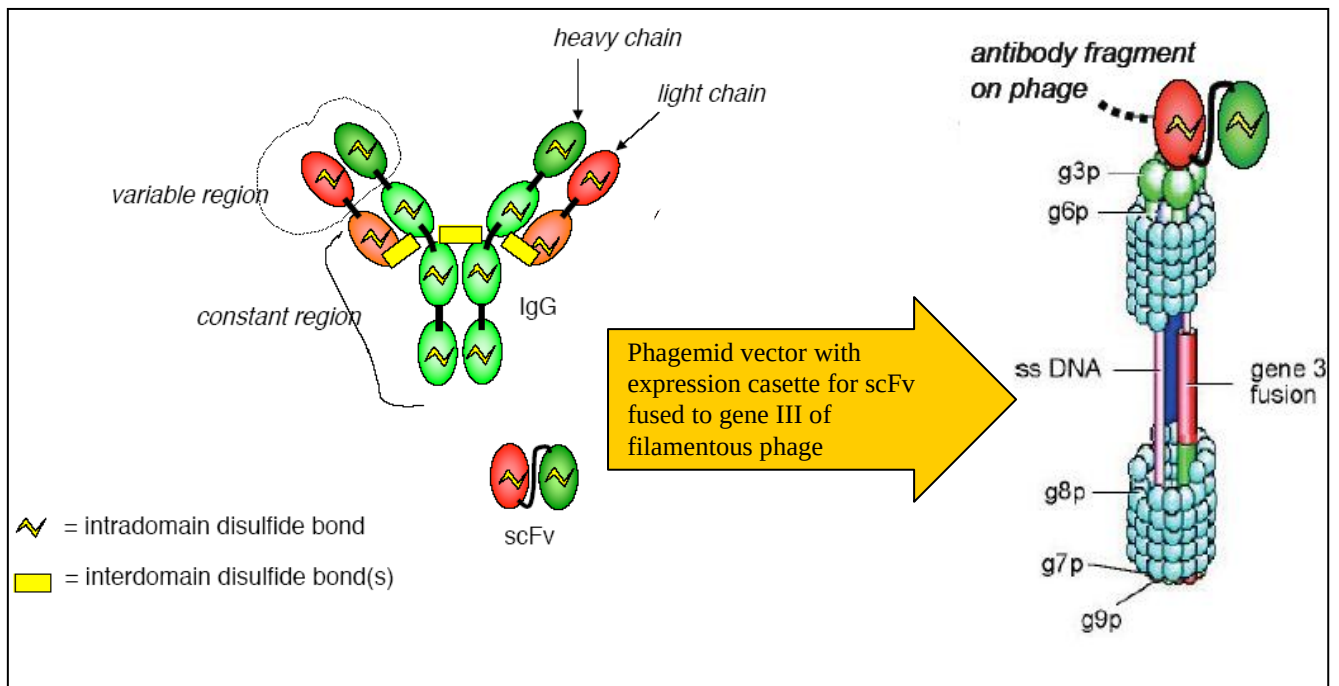


Figure7. Schematic illustration of scFv phage display library.

II) MATERIALS AND METHODS

A)Cell Culture

T47D, BxPC3, ZR-75, HT29, MDA-MB-231, DU145, SW480, HPAC, HEK293, and 293-EBNA cells were purchased from ATCC (Milan, Italy) and grown as recommended by the manufacturer except T47D cells, which were grown in Dulbecco modified Eagle medium F-12 Glutamax medium (GIBCO-Invitrogen, Carlsbad, CA), enriched with 10% FBS at 37°C and 5% CO₂. To generate cell lines that overexpress Ron receptor, complementary DNA (cDNA) encoding the full-length RON protein was cloned into the Gateway-pcDNA-Dest40 expression vector (Invitrogen, Carlsbad, CA) and into the doxycycline-inducible expression vector pCEP/TetO-MCS. CHO cells were transfected with Gateway-pcDNADest40-RON construct, and HEK 293 cells were transfected with pCEP/TetO-MCS-RON construct using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Cells expressing high RON levels were FACS-sorted and grown under appropriate antibiotic selection to obtain stable clones (Geneticin for pDest 40 -RON, Hygromycin for pCEP/TetO-MCS-RON). T47DtetRronWT, HCTtetRton WT and HCTtetRronMUT cell lines which bear a doxycycline inducible shRNA sequence against RON are kind gift of Silvia Benvenuti.(IRCC)

C)ScFv Phage Selections

DP47, CS and BMV ScFv are non-immunized, human ScFv phage-display libraries with high complexity (over 10¹¹ different clones), derived from B cells of healthy donors are obtained from Cambridge Antibody Technology

Group plc (CaT) ,(Melbourn, Cambridgeshire, SG8 6JJ. England) Each CAT phage scFv library has undergone three rounds of selection to enrich the clones that bind antigen.(Figure 8) First round of selection was carried out by panning (solid phase selection) the phage library on recombinant RON SEMA-PSI domain. Liquid phase selections with SW480 cells naturally expressing RON and RON ECD-Fc fusion protein was carried out as second and third rounds of selection. Phage rescue was carried out between subsequent selections.

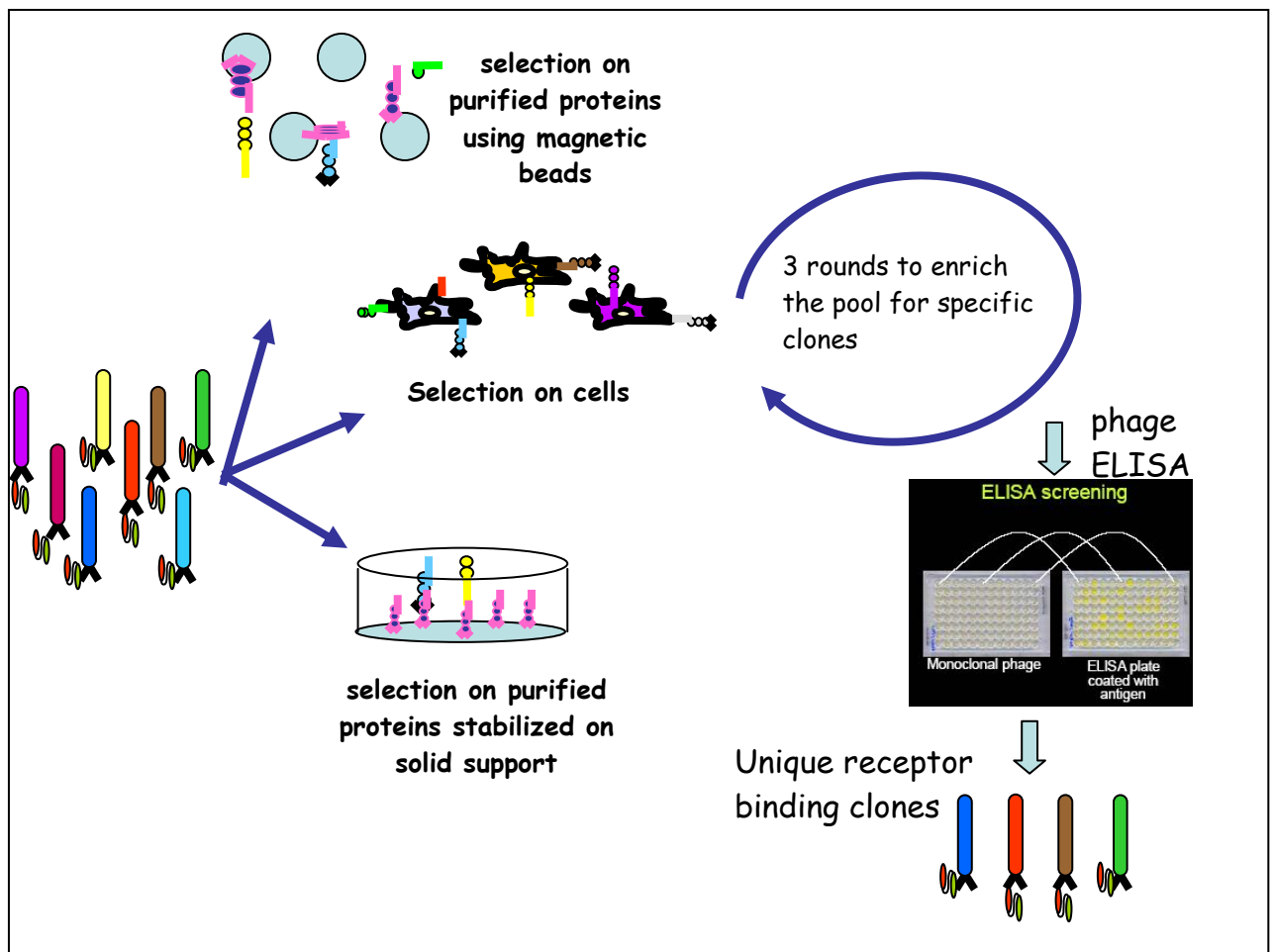


Figure 8. Selection process for scFv phages that bind to RON RTK.

C.1)Solid Phase Selection (Panning)

50ml of 2xTY was inoculated in a 250 ml flask with a single colony of *E.coli* TG1 cells taken from a freshly grown minimal media plate. 100µl of 10µg/ml RON SEMA-PSI domain (R&D, cat.no 1947-MS) in PBS was distributed to each well of NUNC Maxisorp ELISA plate(cat.no 442404). Binding is allowed by overnight incubation without shaking. Plates were washed 3 times with PBS to remove excess antigen. Surface of the wells were blocked by 3% (w/v) skimmed milk powder in PBS. Plates were incubated for 1h at 37°C. 50µl phage aliquot was added to 50µl of 6% (w/v) skimmed milk powder in 2xPBS (this gives 10¹² phage in 100µl) and was incubated at RT for 30 minutes. Maxisorp plate is washed 3 times with PBS. 1µl pre-blocked phage was added to the coated microtiter plate well. Plate was incubated for 1 hour at RT(stationary). Plate was washed 5 times with PBS/0.1% Tween and 5 times with PBS. Bound phage was eluted using Trypsin. To 10 ml of TG1 cells that reached mid-logarithmic growth phase (O.D.₆₀₀: 0.5), add eluted phage. Culture was shaken at 150 rpm, 37°C for 1 hour. Cells are centrifuged at 2000 rpm for 5 minutes. Supernatant was discarded and cells were resuspended in 500 µl of 2xTYAG media and spreaded onto a 2xTYAG agar bioassay plate and grow overnight at 30°C.

C.2)Phage Rescue

10ml 2xTYAG was added per bioassay plate. Cells were resuspended with a spreader, mixed and transferred to a 50 ml falcon tube. (To preserve these cells as long term glycerol stocks add half volume of 50% glycerol and

freeze them at -70°C as 1 ml aliquots). 50 ml of 2xTYAG was inoculated with 50 μl of the cells and grown at 37°C to O.D.₆₀₀: 0.5 at 280 rpm.(approximately 10^8 cells/ml).

MK13K07 helper phage was added to a multiplicity of infection (m.o.i) of 10.(10 phage per cell). Infected culture was incubated at 37°C 30 minutes stationary and then at 37°C for 30 minutes shaking at 150 rpm. Cells are transferred to a 50ml polypropylene centrifuge tube and spun at 3200 rpm for 10 minutes. Supernatant was removed and cells were resuspended in 50 ml 2xTYAK. Cells were grown overnight at 25°C at 280 rpm. Cultures were centrifuged at 4700 rpm for 45 minutes at 4°C . Supernatant was collected and 15ml of cold 20% (w/v) PEG 8000/2.5M NaCl was added to each sample. Tubes are incubated on ice at least for 1 hour ,after which precipitated phage antibodies were centrifugated at 4700 rpm for 45 minutes at 4°C . Pellet was resuspended in 8 ml of PBS. After adding 2 ml of cold 20% PEG/NaCl, incubation was continued for an additional hour on ice. After centrifugation at 4700 rpm for 45 minutes at 4°C , the pellet was resuspended in 850 μl of RPMI containing 2% BSA. The suspension was centrifuged at 13,000rpm for 5 min to pellet debris, and the supernatant was added to a tube containing 150 ml glycerol. Phage-ScFvs were stored at -20°C until further use in selection rounds .

C.3)Liquid Phase Selections

C.3.a)SW480 Cells

50ml of 2xTY was inoculated in a 250 ml flask with a single colony of E.coli TG1 cells taken from a freshly grown minimal media plate. Phage

rescued from previous round of selection was blocked by 30 minutes RT incubation with 3% (w/v) skimmed milk powder in PBS and de-selected on 10^7 CHO cells that don't express RON RTK by 1 hour of incubation, shaking. Blocked and de-selected phage was incubated with 10^7 SW480 cells naturally expressing RON RTK for 30 min at room temperature (RT) with shaking. After extensive washing with PBS, cell-bound phage were centrifuged 5 min at 2 k rpm and re-suspended in 800 mL of trypsin solution (1 mg/ml in sodium phosphate). *E. Coli* TG1 cells were infected with eluted phage and plated on 2XTYAG agar. Phage rescue and amplification was carried out as described before.

C.3.b)RON ECD-Fc

50ml of 2xTY was inoculated in a 250 ml flask with a single colony of *E.coli* TG1 cells taken from a freshly grown minimal media plate. Phage rescued from previous round of selection was blocked by 30 minutes RT incubation with 3% (w/v) skimmed milk powder in PBS. RON ECD-Fc fusion protein was incubated 1h at RT with pre-blocked phage. Biomag ProteinA magnetic beads (QIAGEN, cat. no: 311802) were added to the phage/RON ECD-Fc mixture and incubated at RT for 10 minutes. With the help of a magnetic separator phage bound to RON ECD-Fc, which was in turn bound to the beads were separated from the solution. After extensive washes with PBS, phage were eluted by 800 μ l trypsin. *E. Coli* TG1 cells at OD₆₀₀: 0.5. were infected with eluted phage and plated on 2XTYAG agar bioassay plates. Phage rescue and amplification was carried out as described before.

D)Identification of positive clones through Phage Elisa

Individual phage clones were randomly chosen from the pool of selected phage and tested by phage ELISA for their binding capability to immobilized RON-ECD-Fc through the use of an anti-Fc Ab (immunoPure Goat anti hIgG (Fc) Pierce) directly bound to the plate. Phage supernatants, after blocking with 3% (w/v) skimmed milk powder in PBS, were added to each well and incubated for 1 hour at RT. After PBS washing, binding of phage antibodies was revealed with HRP-conjugated anti M13 monoclonal antibody (Amersham Biosciences cat.no 27-9421-01) in 3% (w/v) skimmed milk powder in PBS using tetramethylbenzidine (Sigma Aldrich cat.no T8665) as substrate. Phage- Abs whose binding to the target measured by $A_{450}-A_{620}$ was at least three fold superior to that observed with an unrelated phage and higher than 0.5 units were defined as positive. These phage-Abs has been completely sequenced and converted into IgG1 format.

E)Sequencing Phage Clones

Phagemid vector that was used in the construction of the CaT scFv library, pCANTAB6 is a 4544bp phagemid vector (approximately 5.3 kb with a scFv inserted) containing an expression cassette for scFv fused to gene III of the filamentous phage fd. Sequencing templates are prepared by a primary PCR reaction of an aliquot of cells grown overnight. Primers puc reverse: 5'AGCGGATAACAATTCACACAGG and fdtetseq24: 5'TTTGTCGTCTTTC CAGACGTTAGT were used for this primary PCR

reaction. For the sequencing reaction, the primers gene3leader : 5'TTATTATTCGCAATTCCTTTAGTTGT TCCT (sequencing from the synthetic geneIII leader sequence through the heavy chain sequence) and mycseq :5' CTCTTCTGAGATGAGTTTTTG (from the myctag through the light chain sequence) were used. An annealing temperature of 55⁰C and 30 cycles were used.

F)ScFv to IgG1 conversion

Individual phage clones positive to all criteria have been converted into IgG1 format, produced and purified as follows.

PCR amplification of Variable Regions

VH and VL sequences were PCR amplified from pCANTAB6 phagemid containing scFv sequences. Primers used during amplifications are designed to add attB recombination sites to the PCR products at both ends in order to be able to insert these PCR products into mammalian expression plasmids with attP sites using BP clonase reaction.

The PCR for light and heavy chain amplification was carried out using: 20-30 ng DNA template; 0.16 µM each Forward and Reverse primers; 0.4 mM dNTPs (Bioline); 1U Taq and Buffer 10X of Advantage cDNA PCR Kit (Clontech).

The programs for the light and heavy chain amplification, optimized for each primer couple, are the following:

Light Chain

- 95°C for 2min;
- 10 cycles: 95°C for 30''
53°C for 30''
68°C for 30''
- 10 cycles: 95°C for 30''
60°C for 30''
68°C for 30''
- 10 cycles: 95°C for 30''
68°C for 30''
68°C for 30''
- 68°C for 7 min.

Heavy Chain

- 95°C for 5 min;
- 30 cycles: 95°C for 30''
68°C for 30''
- at 68°C for 7 min.

All final PCR products were checked on 2% agarose gels and both inserts were purified using QIAquick PCR purification KIT (Qiagen)

Expression Vectors

The vectors used in this work are the result of a combination carried out between the GATEWAY technology (Invitrogen, Carlsbad, CA) and CAT methodologies. The vectors pEU1.2, pEU3.2 and pEU4.2 were provided by CAT, and represent an integrated vector expression system for the eukaryotic expression of antibodies or their fragments after selection from phage display libraries.⁶⁰

pEU1.2 bears an expression cassette for constant region for human IgG heavy chain (γ_1 isotype). pEU3.2 contains a cassette for expression of

constant region for human IgG κ light chain(km³ allotype) and pEU4.2 has an expression cassette for constant region of human λ isotype light chain.

The ccdB suicide gene flanked by two attP sites were introduced into above vectors using pDONRP2R-P3(Invitrogen) for pEU1.2 and pDONR221(Invitrogen) for pEU3.2 and pEU4.2 by homologous recombination. By this way gateway technology was transferred to the pEU expression vectors.

Final versions of vectors, which are used as donor vectors, are named as 1.2gateway, 3.2gateway and 4.2gateway. They contain expression cassettes for the constant region of corresponding heavy or light chain, the suicide gene ccdB flanked by two attP recombination sites in order to be used as insertion site for VH or VL fragments flanked by attB sites, ampicillin resistance gene and the origin of replication for amplification in bacteria.

PCR amplified VH regions were cloned into 1.2gateway, whereas depending on the isotype VL regions are cloned into 3.2gateway or 4.2gateway using BP clonase(Invitrogen) according to manufacturers protocol. (Figure 9)

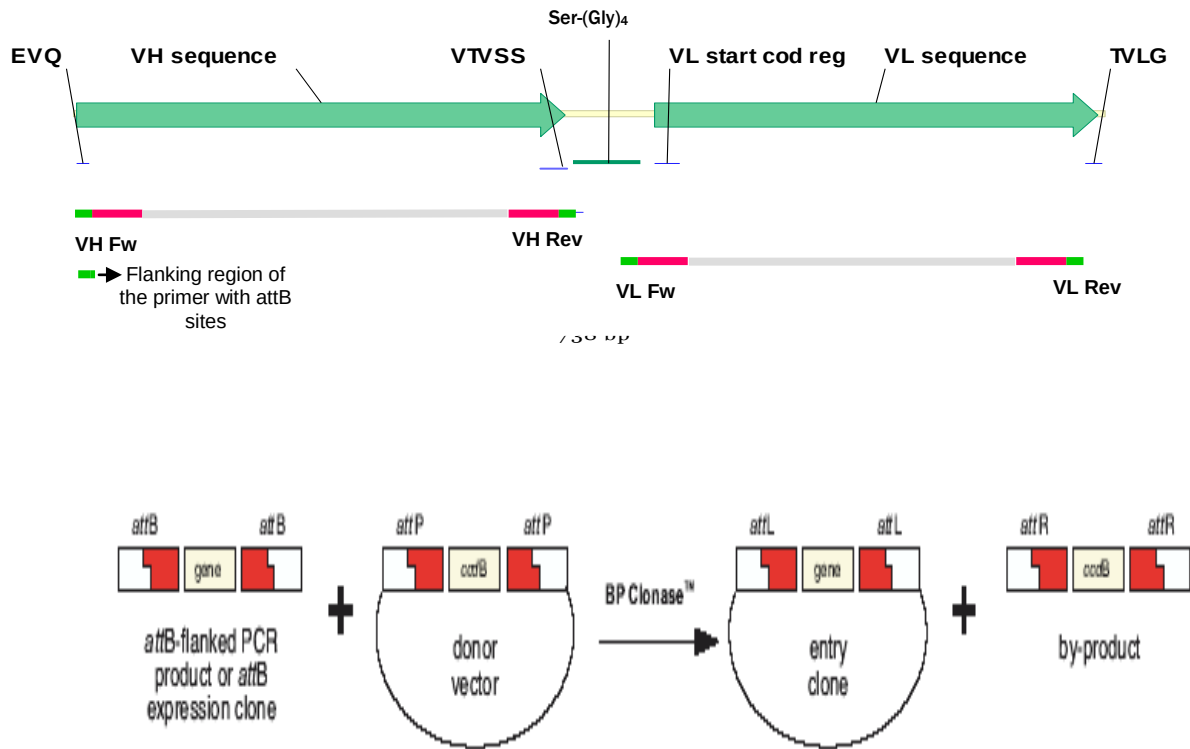


Figure 9. (up) VH and VL from phagemids were amplified separately, using primers in part complementary to VH and VL sequences (pink regions) with flanking attB sites (green regions). (down) BP clonase reaction permits recombination between the VH or VL sequences of interest flanked by attB sites and ccdB gene in the donor vector flanked by attP sites, resulting in expression vector with VH or VL sequences and a by-product of ccdB gene fragment.

Aliquots of the BP reaction were used to transform *ccdB*-sensitive DH5 α competent cells (Invitrogen) and, *ccdB*-negative bacteria were directly grown, shaking over night at 37°C, in 5ml liquid medium, supplemented with 100 μ g/ml ampicillin. Those bacteria that receive the plasmids in which recombination wasn't successful are eliminated due to presence of the suicide gene, permitting the selection of vectors with VH/VL insert. Subsequently, plasmidic DNA was purified from each culture using the minipreps Ultraplasmid kit (Qiagen) and, checked by

restriction analyses. Final digested products were checked on 2% agarose gels.

IgGs expression and purification

The 293EBNA cells were maintained in Dulbecco's Modified Eagle Medium (DMEM;Gibco), containing 10% foetal bovine serum (Perbio); penicillin (100U/ml), streptomycin (100 µg/ml), 250 µg/ml geneticin (Invitrogen), 2mM glutamine and 1% non-essential amino-acids (Invitrogen). The two plasmids were co-transfected into 293-EBNA cells using lipofectamin 2000 (Invitrogen) as described by manufacturer. 6-8 hours after of transfection, the old medium was replaced with 10 ml of fresh CD-CHO medium(Gibco), containing 8mM glutamine, 1X penicillin/streptomycin supplement, 1x hypoxantine/ thymidine supplement (Invitrogen). The cells were incubated in the above medium at 37°C, after 7 days the supernatant was collected.

The expression of soluble IgGs was checked by SDS PAGE of the growth medium. After a brief centrifugation to spin down any debris, 20 µl of the growth medium from each transfection, denatured and reduced in loading buffer, were loaded on a denaturing minigel with a 4-12% acrylamide linear gradient (NuPAGE 4-12% from Invitrogen) and subjected to electrophoresis for one hour at 180 V. The presence of IgGs was detected staining the gel with Coomassie blue as two major bands of 50 kDa (heavy chain) and 25 kDa (light chain) respectively.

For purification, the supernatants subjected to centrifugation and filtration on 0.2 μm filters and then loaded on a Protein A Sepharose High Performance prepacked column (HiTrap Protein A HP 1ml, GE Healthcare) for affinity chromatography on an AKTApurifier apparatus (GE Healthcare). Typically, about 40 ml of supernatant were loaded on a 1 ml column equilibrated in buffer A (50 mM TrisHCl pH 8.0, 250 mM NaCl), and after a wash with 10 column volumes of buffer A the IgGs were eluted with buffer B (100 mM Sodium Citrate pH 3.0) and collected in fractions neutralized by adding 1M Tris, pH 9.0. The eluted fractions containing, from inspection of the chromatogram, the IgGs were pooled and concentrated by ultrafiltration in spin column concentrators (Vivaspin 4 ml, VivaScience). The same devices were used for desalting/buffer exchange, and the purified IgGs, in final PBS buffer, were quantified spectrophotometrically using a calculated extinction coefficient at 280 nm of $1.49 \text{ cm}^{-1} \times \text{mg}^{-1}/\text{ml}^{-1}$. IgGs prepared as described can be frozen and stored at -80°C for several months.

G)Flow cytometry

G.1)FACS analysis of Phage ScFvs

PEG-precipitated phage-Abs were re-suspended in 2%BSA in PBS and incubated for 30 min at RT. SW480cells were incubated with phage-Ab solution 60min at 4°C (2×10^5 cells with pre-blocked phage in 100 mL). After washing with 2%BSA in PBS, cells were incubated with biotin-conjugated anti-M13 phage monoclonal antibody (Progen Biotechnik, Heidelberg, Germany) for 30 min at 4°C . then washed and incubated with

streptavidin-APC (Molecular Probes/Invitrogen, Carlsbad, CA) 30 min at 4°C. Finally, cells were washed and fixed with 1% PAF in PBS and analysed using BD FACS Canto II cytometer and BD Diva analysis software.

G.2)FACS analysis of IgG

MAbs were labeled by incubation with Zenon Human IgG Alexa Fluor 488 labeling kit (Molecular Probes/Invitrogen, Carlsbad, CA) according to manufacturer's guidelines. Cells were then incubated with 50 ug/ml pre-labeled mAb solution for 30 min at RT (2×10^5 cells in 100 mL of PBS, 2%BSA for 30 min). Cells were then washed and fixed with 1% paraformaldehyde in PBS. Analysis was done using BD FACS Canto II cytometer and BD Diva analysis software.

H) IP and MALDI-TOF-MS

H.1)Labelling and preparation of native membrane proteins (NMPs)

T47D cells were grown in at 70-80% confluency. The labeling with Cy5 dye (Amersham, cat. no. PA25001) of living cells were performed only on 20 % of total dishes/flasks (i.e. 2 out of 10 flasks) while for the rest of the dishes/flasks the NMPs were extracted as unlabelled. Cells were washed with PBS and then incubated for 30 minutes at 4°C in the presence of Cy5 resuspended in PBS. Normally one vial of Cy5 mono-reactive dye was resuspended in 24mL of PBS and 6mL of this solution was added to each 150mm Petri dish. The Cy5 solution was then removed and the cells were washed again with PBS. The NMPs preparation was then performed either

on labeled and unlabelled cells by adding 4.5mL/plate of lysis buffer Tris-HCl 50mM, pH=7.5, NaCl 150mM, Triton-X100 1%, Protease Inhibitors (Amersham, cat. n° 80-6501-23) according to manufacturer dilution. After 30 minutes at +4°C the supernatant was recovered and cleared by centrifuging 15' at 13.000 RPM, +4°C.

H.2) Immunoprecipitation of the target antigen

Antigens recognized by monoclonal antibodies were identified by immunoprecipitation experiments. After cell surface labeling by Cy5 fluorescent dye and solubilization in non-denaturing buffer, membrane-bound antigens were immunoprecipitated with the selected antibodies covalently bound to sepharose beads and analyzed by 1D-PAGE. Dual-wavelength scan against Cy5 and Sypro Ruby identified membrane proteins as distinct from non-specific binders. The immunoprecipitated bands were excised from the gel and subjected to *in-gel* digestion. The antibodies were immobilized onto CNBr-activated Sepharose Beads (Amersham, cat. n° 17 0430 01) accordingly to manufacturer instructions to have mAb and beads 1:100 (w/w). Briefly the beads were rehydrated and washed in HCl 1 mM at a concentration of 100mg/ml and equilibrated in the coupling buffer (NaHCO₃ 100 mM, NaCl 500mM pH=8.3). The mAb was added to the proper final volume and incubated on the beads 2 hours at +37°C with shaking. The beads were then washed with Tris-HCl 100mM, NaCl 500mM pH=8.0 and incubated in the same buffer 2 hours at +37°C with shaking to end-cap reactive sites on their surface. The mAb-coated beads were finally washed with PBS and stored at +4°C until use. When needed the mAb-coated beads were stored in the presence of sodium azide or in 20% Ethanol

according to the manufacturer. The immunoprecipitation was carried out by incubating the mAb-coated beads in the presence of the NMPs at +4°C for 2h in a tube rotator. The beads were then washed with lysis buffer. IP proteins were eluted directly in reducing loading buffer and analyze them by 1D-PAGE.

H.3) Mass spectrometry and database search

After overnight digestion the supernatant was recovered and the remaining tryptic peptides were further extracted from the gel pieces by incubation in 50µl of 5% Formic Acid in water (15min). The supernatants were pooled together and dried down in a SpeedVac concentrator. Peptides were finally resuspended in 0.1% Trifluoroacetic Acid and subjected to µC₁₈ ZipTip (Millipore) desalting prior to Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) for protein identification. Peptide mass fingerprint experiments (MALDI-TOF-MS) were performed using a Voyager sSTR mass spectrometer (Applera, USA) and the MASCOT software package was used to identify proteins from independent non-redundant sequence databases. Data were elaborated using the TurboSequest software provided by the manufacturer. Candidates with Xcorr values >2.5 were considered identified with high confidence.

I) Western Blot Analyses to study RON downstream signaling

I.1)Induction with MSP

Subconfluent RON-expressing cell lines (HT29, MDA-MB-231, Bx-Pc3, ZR-75, T47D, DU145, SW480, and HPAC) were serum-starved for 18

hours. Wild-type MSP (10 nM; R&D) was used to induce cells for 30 minutes at 37°C.

I.2) Testing RON IgGs' ability to block MSP-driven induction

After 18 hours of serum starvation, 100 µg/ml of RON IgGs was incubated with subconfluent BxPC3, T47D, and ZR-75 cell lines for 1 hour at 37°C before 30 minutes of induction with MSP. ID1 commercial blocking antibody CD136 (RON; Beckman Coulter, Milan, Italy) against RON was used as a positive control, and an unrelated antibody (IgG1, Lambda, from human myeloma plasma; Sigma) was used as a negative control. RON IgGs dose-response experiment. Subconfluent T47D cells that were serum-starved for 18 hours were incubated with increasing concentrations of RON mAbs (2.4, 7.3, 22, and 66 nM) for 1 hour. MSP, 10 nM, was used to induce T47D cells for 30 minutes at 37°C.

I.3) Time course experiment with RON mAbs

A total of 4.5×10^5 T47D cells per well in a six-well plate for each point were used. The day after plating, cells were treated with 20 µg/ml of RON mAbs 4, 7, and 10 for 30 minutes, 1 hour, 4 hours, or 24 hours.

I.4) Tumor lysate preparation

Frozen tumors were cut into small pieces and transferred into TPER bugger (Pierce, cat.no 78510) supplemented by 0.5 uM okadaic acid (calbiochem cat. No 495604), complete protease inhibitor cocktail (Roche, Milan, Italy), halt phosphatase , inhibitor cocktail [Thermo Scientific, Milan, Italy]. Approximately, 50-100 mgs of tumor were lysed in 600 ul of buffer by adding 5mm stainless steal bead and shaking the vials twice in Mixer Mill

MM300 homogenizer(QIAGEN) for 2 min. at 30HZ. Samples were centrifuged for 10 minutes at 14000 rpm at 4⁰C. The supernatant was recovered and kept in aliquots at -80⁰C.

I.5) Cell lysate preparation and Western blot analysis

Cells were lysed using lysis buffer (50 mM Tris, 1% Triton, 150 mM NaCl, complete protease inhibitor cocktail (Roche, Milan, Italy), halt phosphatase inhibitor cocktail [Thermo Scientific, Milan, Italy]). Total protein concentration was determined by Bradford method, 20 µg of total protein per sample was loaded on 4% to 12% NuPage BisTris Gels, and SDS-PAGE was performed. Proteins were transferred on to a 22-µm nitrocellulose membrane that was then blocked with 5% nonfat dry milk in 0.05% Tween in TBS. For determination of extracellular signal-regulated kinase (ERK) (mitogen-activated protein kinase, MAPK) and AKT phosphorylation, p44/42 MAPK Thr202/Tyr204 rabbit mAb (1:1000; Cell Signaling, Milan, Italy) and phospho-Akt/ Ser473 (1:500; Cell Signaling) were used. Protein-blotted membranes were incubated overnight at 4°C with primary antibodies in 5% BSA in 0.05% Tween in TBS. HRP-conjugated rabbit (Abcam, Cambridge, UK) and mouse (Promega, Madison, WI) secondary antibodies were used as 1:1500 and 1:3000, respectively, carrying out 1 hour of incubation at RT. To verify equivalent sample loading, blots were probed for β-actin (NeoMarkers, Fremont, CA). Blots were developed using ECL reagent (GE Healthcare, Milan, Italy). Images were acquired by luminescent image analyzer LAS3000 and then quantified by MultiGauge version 2.2 software (FUJIFilm, Düsseldorf, Germany). To

determine expression levels of total ERK and total AKT, these membranes were stripped with Restore Western Blot Stripping Buffer for 15 minutes at room temperature (Pierce) and reprobed with p44/42 MAPK (1:1000; Cell Signaling) or anti-AKT antibody (1:2000; Cell Signaling). After washing and incubating with the secondary antibody, development and image analysis were carried out as before. For determination of RON protein levels, Ron (C-20) sc-322 rabbit polyclonal IgG and HRP-conjugated rabbit (Abcam) were used.

J) Kd Determination via FACS Analysis

4×10^5 T47D cells per sample were incubated with related RON IgGs with increasing concentrations from 37pmol/ml to 9 μ g/ml in FACS buffer(0.2%BSA, 10mM HEPES) for 1 hour at RT. Cells were washed once with 4 ml FACS buffer, and were centrifuged at 1200 rpm 6 minutes at 4°C. Cells were incubated for 40 minutes at RT with APC/F(ab')₂ Fragment Goat Anti-Human IgG Fc γ fragment specific ,with 1:500 dilution(Jackson ImmunoResearch , cat.no:109-136-170). Cells were washed once with 4 ml FACS buffer, and were centrifuged at 1200 rpm 6 minutes at 4°C. Cells were resuspended in 400 μ l FACS buffer and analysed using BD FACS Canto II cytometer and BD Diva analysis software. Kd values were determined using SigmaPlot 10 software.

K)Cell Migration Assay

24 well HCT Fluoroblok Insert Systems with fluorescence blocking PET membranes were used as migration chambers. (BD Biosciences)

K.1)Migration in 2% FBS Conditioned Medium

T47D cells were plated in 15cm culture plates. When they reached 70% confluency conditioned medium over the cells diluted into 2% FBS with serum-free medium.

24 hours later the conditioned medium(CM) over the cells were collected. Cells are harvested using Accutase(PAA) and filtered through 40 μm filter. 5×10^4 cells were placed in each upper chamber in 300 μl of CM. 1ml CM placed in each bottom chamber. Recombinant hMSP C672A(R&D Systems) was added at a final concentration of 2nM to the bottom chambers. Cells were allowed to migrate for 24 hours.

K.2) Migration in 0%FBS on Collagen Coated Chambers

T47D cells were serum starved overnight. The fluoroblok migration chambers were coated with collagen type 1 (Rat tail, BD biosciences). 500 μl of 1 $\mu\text{g/ml}$ collagen in 3 % acetic acid was added to both upper and lower chambers.

Migration chambers were incubated at 37⁰C, 5%CO₂ for 1h. Collagen solution was aspirated, chambers were washed three times with PBS and used immediately. 5×10^4 cells were placed in each upper well in 300 μl of 0% FBS starving medium. 1ml of starving medium was placed in the corresponding bottom chambers.

K.3) Testing RON mAbs

RON mAbs, unrelated human IgG(IgG1,Lambda,from human myeloma plasma, Sigma) and ID1(as a positive control) were added in both upper and lower chambers at a final concentration of 20 ug/ml. After 45 minutes of incubation at 37⁰C, 5%CO₂, MSP at a final concentration of 2nM was added to the bottom chambers. Cells were allowed to migrate for 24 h.

K.4) Quantification

Medium in both chambers were aspirated and 500 ul of 4μg/ml Calcein-AM (Fluka) in HBSS was added to each bottom chamber. After 30 minutes of incubation at 37⁰C, 5%CO₂, cells that migrated to the bottom side of the membrane was quantified using the fluorescence plate reader(VICTOR²). After background subtraction results were elaborated as the fold fluorescence units of basal condition.

L)Cross Competition / Epitope Occupancy Assay

5x10⁵ cells per sample were washed once with PBS 1%BSA and incubated with 100 ug/ml of saturating mAb or unrelated human IgG(IgG1,Lambda,from human myeloma plasma, Sigma) for 40 minutes at room temperature on orbital shaker. Second antibody to be tested was labeled with AlexaFluor 647 using Zenon AlexaFluor 647 for human IgG (Molecular Probes cat. n.Z 25408) . After a wash with PBS 1%BSA, cells saturated for the first antibody were incubated with different concentrations of the labeled antibody for 40 min. at room temperature on orbital shaker.

(50 , 10 , 2 , 0.4 and 0.08 ug/ml). After a wash with PBS 1%BSA cells were fixed in 1%formaldehyde in PBS and read at FACS.

M) Cell Cytotoxicity Assay

T47D cells were serum-starved for 18 hours and were plated in 96-well plates that had been previously coated with collagen (rat tail collagen type I; BD Biosciences) Ron mAbs or an unrelated human IgG in combination with MSP (2-nM recombinant hMSP C672A; R&D Systems) were added to the cells in serum-free medium. After 24 hours of incubation at 37°C and 5% CO₂, ATP levels of cells 40 Novel RON Antagonist Monoclonal Antibodies Gunes et al. Translational Oncology Vol. 4, No. 1, 2011 were measured using ATPlite 1 step reagent (Perkin Elmer) using Perkin Elmer TopCount microplate luminescence counter.

N) In Vivo Mouse Models

N.1) T47D orthotopic model

In the T47D model, 17 β -estradiol pellets (0.36 mg per pellet, 60-day release) were implanted in the dorsal midline of 5-week-old female NOD/SCID mice. After 1 week, 2×10^6 T47D cells in Matrigel were injected in the fourth mammary fat pad. Nineteen days after injection, mice with tumors greater than 30 mm³ were allocated randomly in five groups. Mice were treated weekly with 25 mg/kg intraperitoneally with RON mAbs

and isotypic control human IgG1. On day 29 after allocation, mice were killed, and tumors were excised, weighed, and frozen in liquid nitrogen.

N.2) BxPC3 and HT29 xenograft models

5-week-old female athymic nude mice (Charles River, Calco, Como, Italy) were injected subcutaneously with 5×10^6 BxPC3 cells in Matrigel or intraperitoneally with HT29 cells in Matrigel. After tumors reached an average size of 150 to 200 mm³, 25-mg/kg intraperitoneal treatment with RON mAbs or histidine buffer was carried out biweekly. At day 26 after treatment, mice were killed, and tumors were excised, weighed, and frozen in liquid nitrogen.

N.3) HCT116tetRron xenograft model (dox induced RON silencing through shRNA)

HCT116tetRron WT cell line which bear a doxyxiline inducible shRNA and HCT116tetRron mut with a mutated shRNA for RON under control of tetO cassette which do not cause silencing but used as a control are a kind gift from Silvia Benvenuti. (IRCC, Turin). Doxycycline was administered in drinking water of the tested groups throughout the study starting from 1 week before implantation. Cells to be implanted in mice were treated with 100ng/ml doxycycline starting from 72 hours prior to implantation. 5-week-old female athymic nude mice (Charles River, Calco, Como, Italy) were injected subcutaneously with 4×10^6 cells in Matrigel. (HCT116tetRron WT, HCT116tetRron mut, HCT116 WT). Tumor growth was monitored.

N.4) T47DtetRron orthotopic model

T47D teRronWT cell line which bear a shRNA preceded by TetO cassette is a kind gift from Silvia Benvenuti. (IRCC, Turin). Doxycycline was administered in drinking water of the tested groups throughout the study starting from 1 week before implantation. Cells to be implanted in mice were treated with 100ng/ml doxycycline starting from 72 hours prior to implantation. 17 β -estradiol pellets (0.36 mg per pellet, 60-day release) were implanted in the dorsal midline of 5-week-old female NOD/SCID mice. After 1 week, 2×10^6 T47DtetRron cells in Matrigel were injected in the fourth mammary fat pad. Tumor growth was monitored.

O) RTK Proteome Profiler Assay

Proteome Profiler Assay was purchased from R&D Systems (Cat.No ARY001). It is a screen which allows the determination of the relative level of tyrosine phosphorylation of 42 different human RTK's. Assay was carried out according to manufacturer's protocol.

Briefly principle of the assay is as follows. Capture and control antibodies have been spotted in duplicate on nitrocellulose membranes. Cell lysates are diluted and incubated with the human Phospho-RTK Array. After binding the extracellular domain of both phosphorylated and unphosphorylated RTK's unbound material is washed away. A pan-antiphosphotyrosine antibody conjugated to horse reddish peroxidase (HRP) is then used to detect phosphorylated tyrosines on activated receptors by chemiluminescence.

II) RESULTS

In order to select RON mAbs with desired properties following steps listed in the screening funnel depicted in Figure 10 were followed.

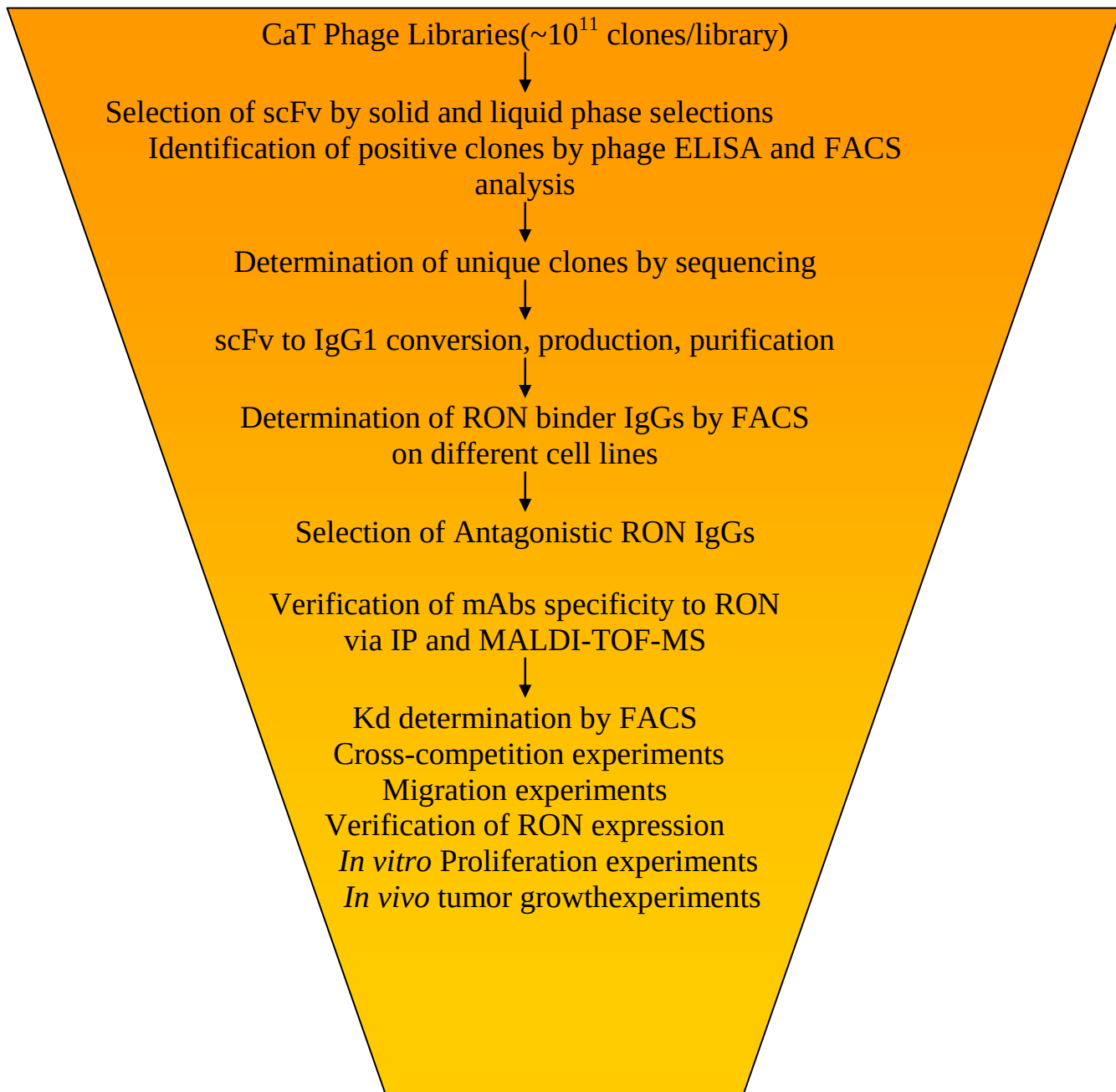


Figure 10. Screening Funnel for antagonistic RON IgGs.

A) Selection of RON-binding phage-ScFvs from phage libraries

In order to obtain phage-Abs that specifically bind to RON extracellular domain we used ScFv CAT (Cambridge Antibodies Technology) libraries. Three rounds of selections have been carried out using as selectors SW480 cells endogenously expressing RON, the RON-ECD-Fc protein as well as recombinant human RON SEMA-PSI domain. In case of RON SEMA-PSI domain solid phase selection was carried out, whereas with SW480 cells or RON-ECD-Fc proteins, liquid phase selections were done. Using different methods of selection at each round, especially two different phase systems, it is aimed to decrease unspecific binders. Background due to unspecific binding to beads or plate despite of blocking, thus might have been eliminated further due to alternation of the selection method.

After enriching the phage population for the percentage of sufficient RON binders through 3 rounds of selection, individual phage clones were recovered. Among them randomly selected clones were screened by phage-ELISA and FACS analysis. Positive clones, which are capable of binding to RON-ECD-Fc and SW480 cells were selected. Sequencing of these positive clones revealed 102 individual clones. 75 of these clones have been successfully converted into IgG1 format, produced and purified.

B) Identifying RON mAbs that selectively bind to RON

Following conversion to IgG1 format, RON IgGs were challenged in a FACS assay to eliminate those that lost their binding capability during

conversion, production and purification steps and to select those that selectively bind to RON. For this assay tumor cell lines endogenously expressing RON (SW480, T47D, HCT116) as well as 293(HEK293) cell lines that express RON upon doxycycline induction (in the presence or absence of induction), CHOwt and CHO RON cells were used.

RON IgGs were selected as RON binders, when mean fluorescence values of the P1 population shows a clear shifting to higher values in case of doxycycline induced 293 cells and CHO RON cells when compared with 293 cells not induced with doxycycline or CHO wt cells.

As can be seen from the representative FACS results in Figure 11, RON IgG54 binds selectively to 293 cells that are doxycycline induced, thus expressing RON. Figure 12 summarizes values obtained for the three RON antibodies that were selected at the end of this study to study MSP dependent migration as an example for the IgGs resulted positive for selection at this step.

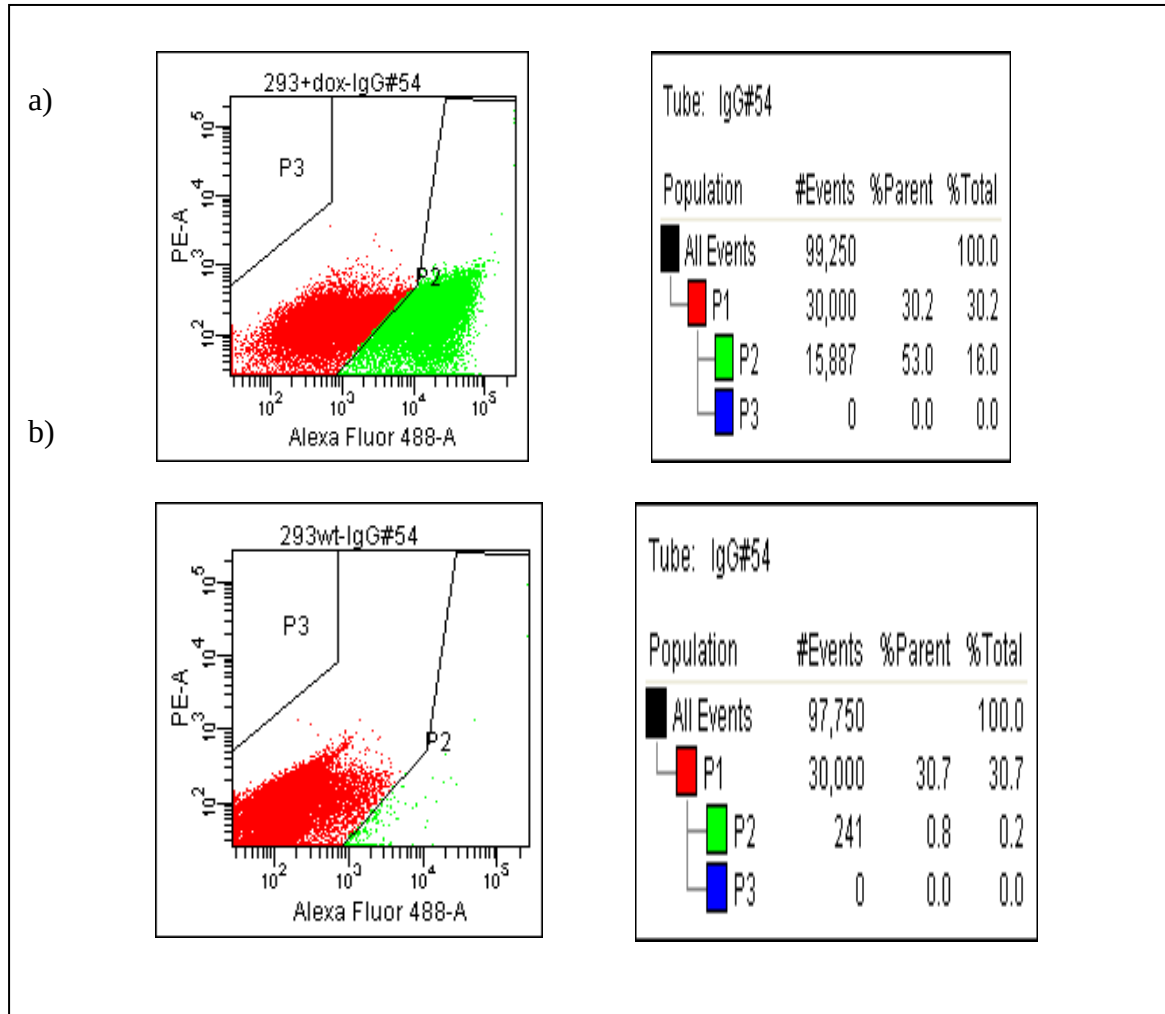


Figure 10 .An example of differential binding of anti RON Ig-G 54 on 293 cells transfected with the Doxycycline inducible RON ECD-TM expression vector, either in presence (a) or absence (b) of the inducer.

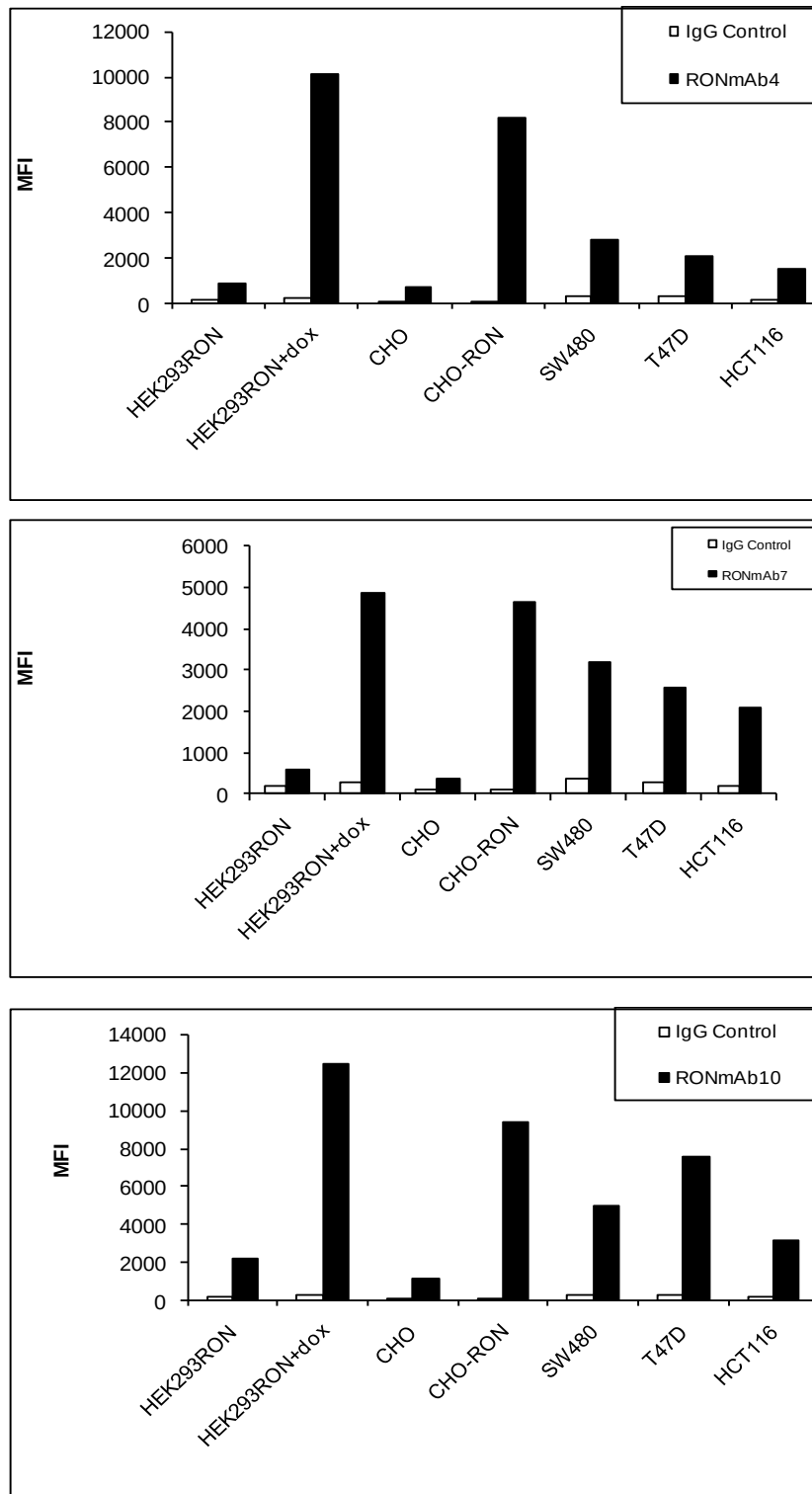


Figure 11. RON mAbs selectively bind to RON positive cell lines. 50 ug/ml of RON mAbs were incubated with RON negative (HEK293RON not induced, CHO) or RON positive (HEK293RONdoxycycline induced, CHO-RON, SW480, T47D, HCT116) cell lines. Binding of RONmAbs to cells were quantified by FACS analysis. Unrelated human IgG1 was used as a negative control. Mean fluorescence Index of P1 population was plotted in Y axis.

C) mAbs 4, 7 and 10 are able to immunoprecipitate RON

Further confirmation for the specificity of RON mAbs was obtained by IP of fluorescently labeled membrane proteins with RON mAbs followed by in-gel digestion of protein bands and peptide identification by matrix-assisted laser desorption ionization - time of flight - mass spectrometer (MALDI-TOF-MS). All RON mAbs specifically immunoprecipitated two major bands in polyacrylamide gel, which were identified as RON α and β chains (Figure 12).

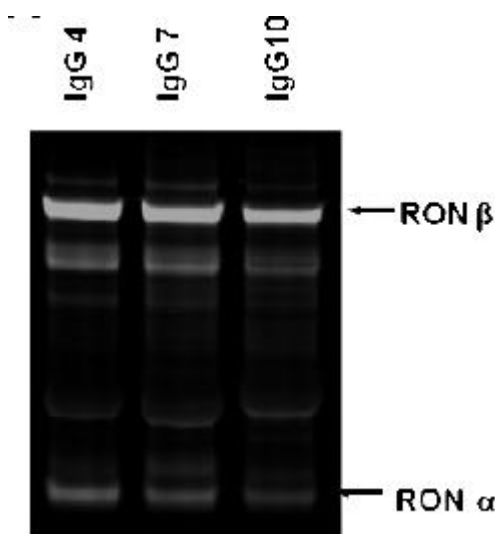


Figure 12. RON IgGs are able to immunoprecipitate RON. Immunoprecipitation with RON IgG4, IgG7, and IgG10 was carried out on fluorescently labeled membrane proteins. One-dimensional PAGE was followed by in-gel digestion of immunoprecipitated protein bands and identification of the protein bands using MALDI-TOF-MS. Data were elaborated using the TurboSequest software. Candidates with Xcorr values greater than 2.5 were considered identified with high confidence.

D) MSP stimulation activates downstream Signalling Pathways and causes AKT and ERK phosphorylation

A panel of human cancer cell lines (HT29, MDA-MB-231, Bx-Pc3, ZR-75, T47D, DU145, SW480, HPAC) were compared by Western Blot analysis for RON downstream signaling. Breast carcinoma cell lines T47D and ZR-75, and pancreas carcinoma cell line BxPC3 showed a clear MSP-dependent phosphorylation of both AKT and ERK.(Figure 14) Kinetics of the treatment of RON-expressing cells with the ligand MSP showed that the maximum phosphorylation of ERK and AKT was reached after 30 minutes of incubation.(Figure 13) Among these three cell lines T47D breast carcinoma cell line gave the clearest MSP dependent phosphorylation of AKT and ERK , by having least phosphorylation of these proteins in unstimulated state and giving a strong phosphorylation upon MSP stimulation.

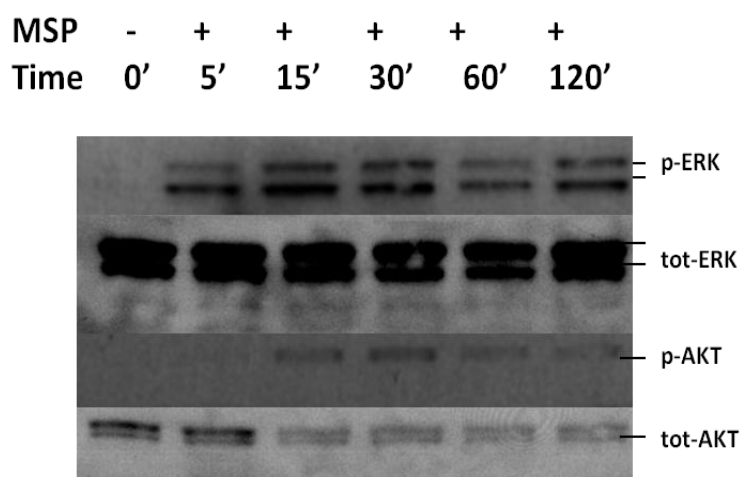


Figure 13. ZR-75 cells time course experiment for ERK and AKT phosphorylation in response to MSP stimulation. The highest phosphorylation levels were obtained at 30 minutes.

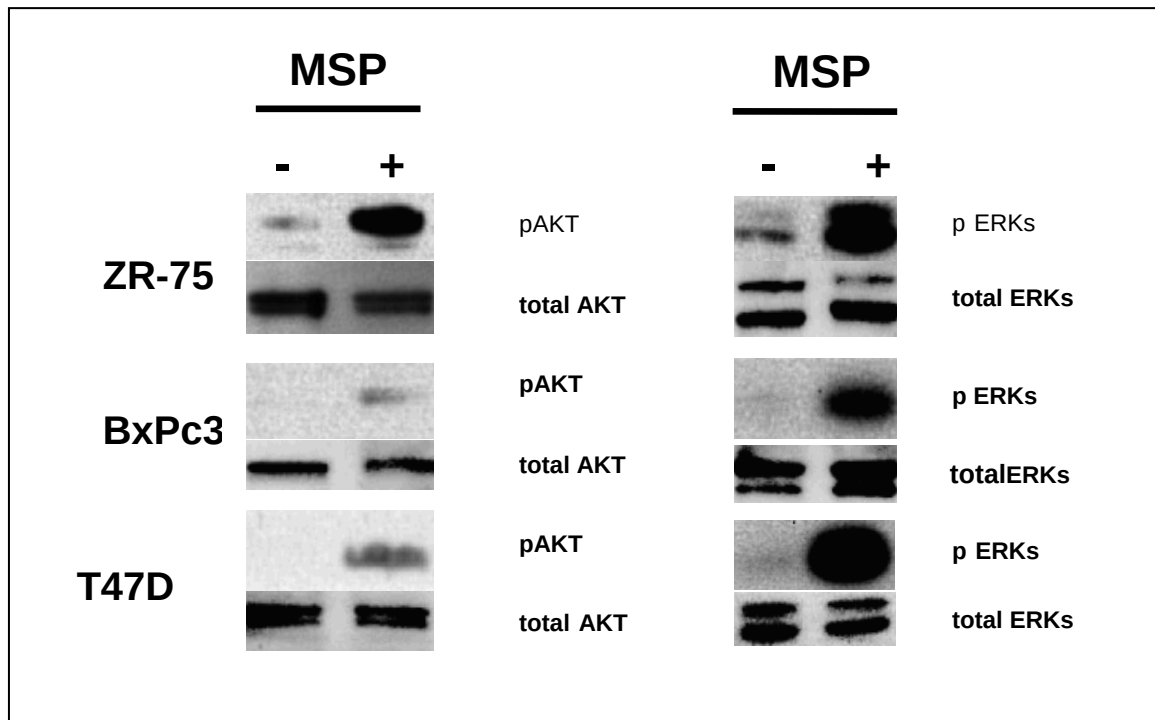


Figure 14. Activation of downstream signaling pathways in ZR75, BxPC3 and T47D cell lines upon MSP stimulation. MSP stimulation causes ERK and AKT phosphorylation in these cell lines

E) RON mAbs show antagonistic properties

The antagonistic activity of the RON mAbs were evaluated on the basis of their ability to reduce MSP induced AKT and ERK phosphorylation in breast cancer cell line T47D.

Among 36 RON mAbs tested, 6 showed a decrease in ERK and AKT phosphorylation. RON IgGs # 1, 3, 4, 7, 10, 32 were shown to have antagonistic activity and were chosen to be tested in the subsequent experiments.(Figure 15)

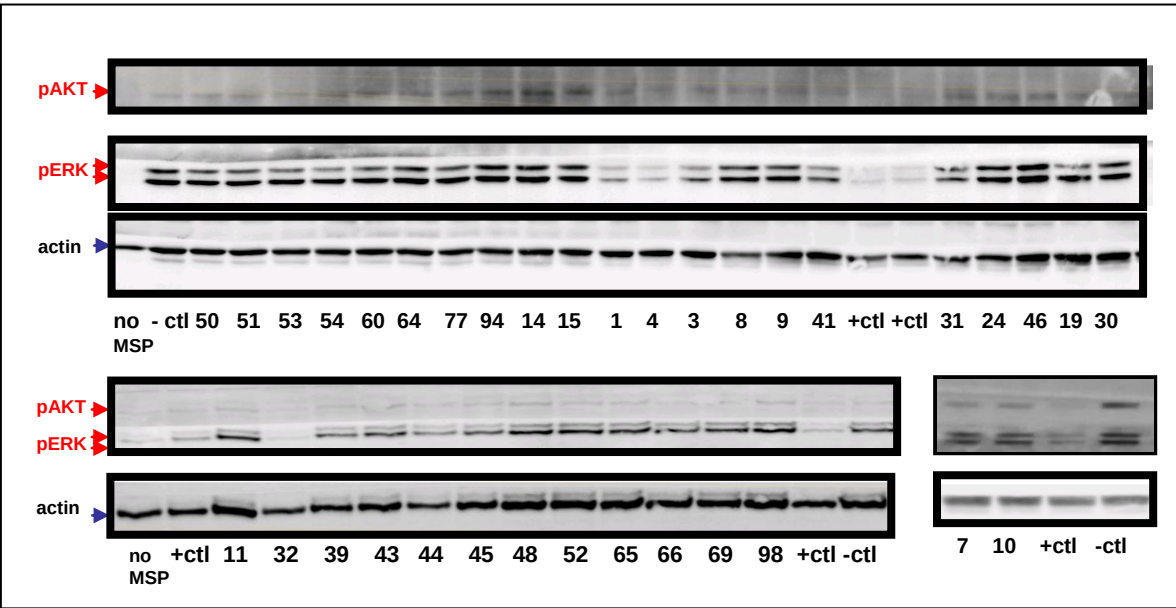


Figure 15. RON IgGs 1,3,4,7,10 and 32 show antagonistic activity by means of decrease in MSP driven ERK phosphorylation.

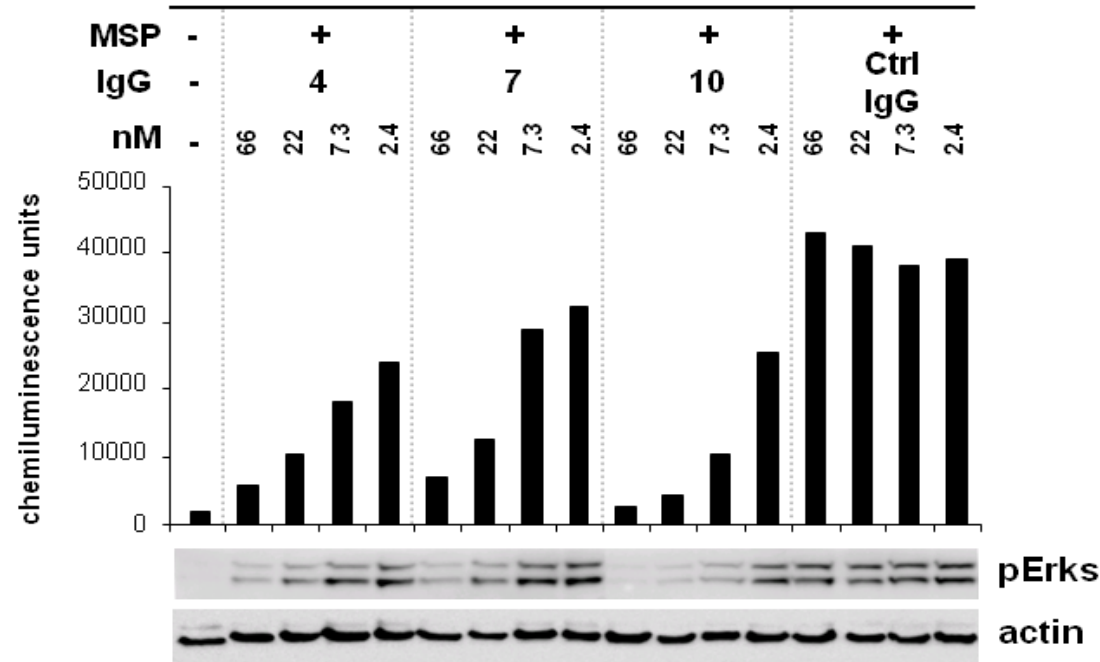


Figure 16. Western blot analysis of cell lysates of T47D cells treated with increasing concentration of IgG4, IgG7, and IgG10 before MSP stimulation. Signal intensities were quantified by densitometric scanning and expressed as chemiluminescence units. An antibody directed to actin was used as a loading control.

Antagonistic RON mAbs were tested in a dose response experiment where they were used in four different concentrations (2,4 nM, 7,3 nM, 22nM, 66nM). In general, with increasing concentrations of RON mAbs a stronger reduction in ERK phosphorylation was observed. Figure 16 shows the dose-response experiment done with 3 RON IgGs that show decrease in ERK phosphorylation with increasing antibody concentration.

F)RON IgGs have sub-nanomolar affinities

In the next step we have determined affinity of IgGs for RON via FACS analysis. Figure 17 shows sigma-plot graphs of the three selected RON IgGs that came out from the screening funnel and tested in migration assays. RON mAbs 4,7 and 10 have sub-nanomolar K_d's indicating that they are high affinity RON binders. Average K_d values were listed for mAbs 4, 7 and 10 in Table 2.

RON IgG	EXP #1	EXP #2	AVERAGE
4	0.23nM	0.1053nM	0.168 nM
7	0.31nM	0.544nM	0.427 nM
10	1.07nM	0.202nM	0.636 nM

Table 2. K_d values obtained for antibody 4,7 and 10. Two independent experiments were conducted and average of two values were accepted as K_d.

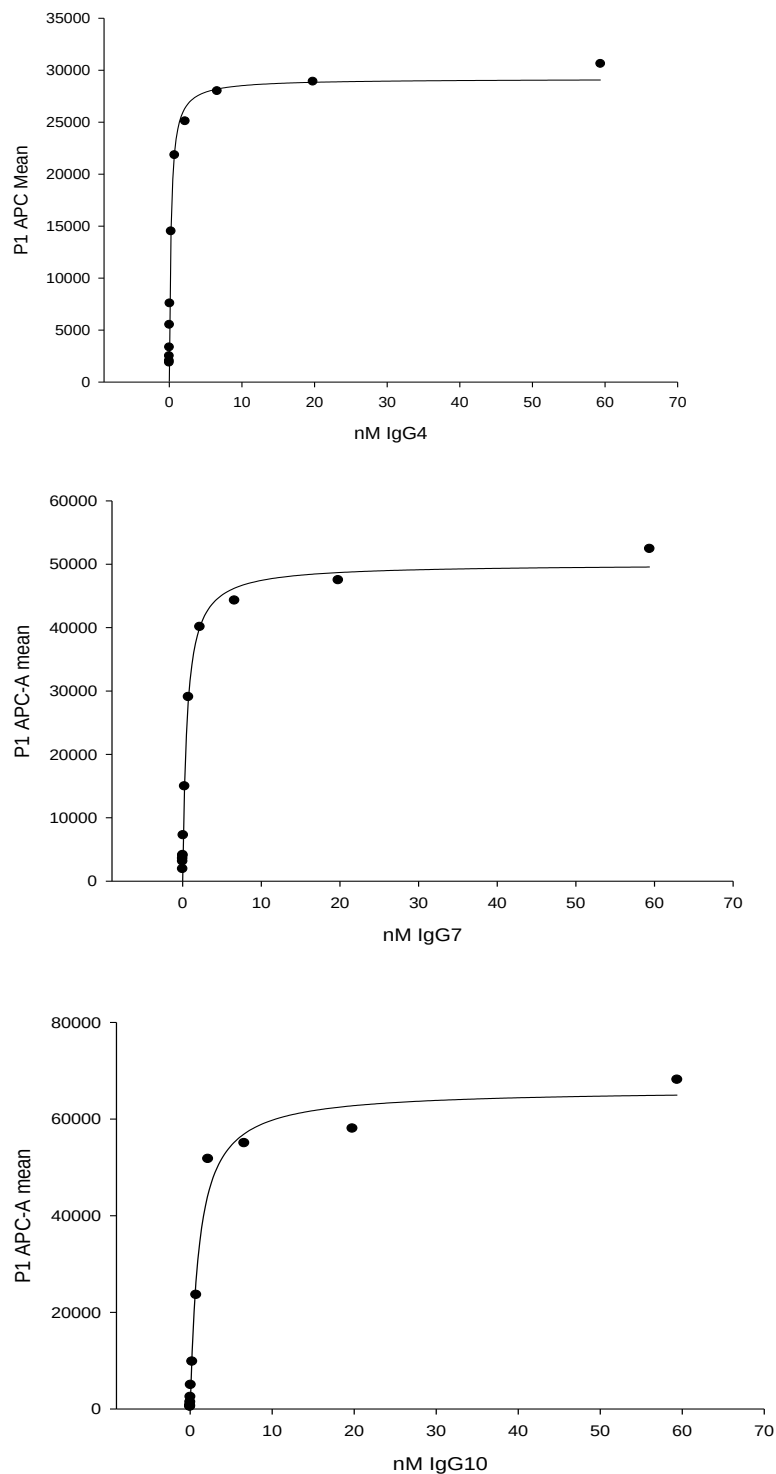


Figure 17. RON IgGs have sub-nanomolar affinity.

From top to bottom , sigma-plot graphs of RON IgGs 4, 7 and 10. P1 APC mean-fluorescence in the y axis versus nM RON IgG in the x axis was plotted. Sigma-Plot software used for graphs and Kd calculation.

G)Antagonistic RON mAbs do not cause receptor down-regulation

In a time-course experiment, effect of RON mAbs on RON protein levels were assessed using a western blot analyses. T47D cells were incubated with RON IgGs 4, 7 or 10 for 30min, 1h,6h or 24 h. It can be seen in Figure 18 that RON protein levels do not change with any of the RON IgGs at any time point. This suggests that RON mAbs do not cause receptor down-regulation.

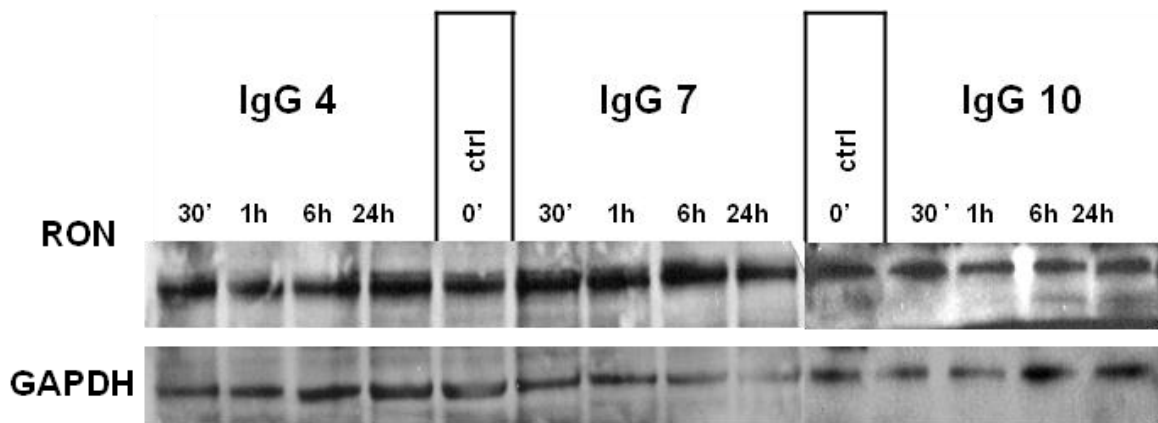


Figure 18. RON IgG's do not trigger RON downregulation.

H)High Serum Concentrations has a negative effect on MSP-driven Migration

After screening 5 tumor cell lines (A431, BxPC3, HCT116, HEK293, T47D) T47D breast cancer cell line was chosen as the model system for MSP driven migration. During screening and setting up it is observed that it was hard to observe the effect of MSP with highly motile cell lines such as

BxPC3, where effect of MSP was probably overcome by basal motility of cells. T47D cell line having low motility allowed us to observe MSPs effect clearly.

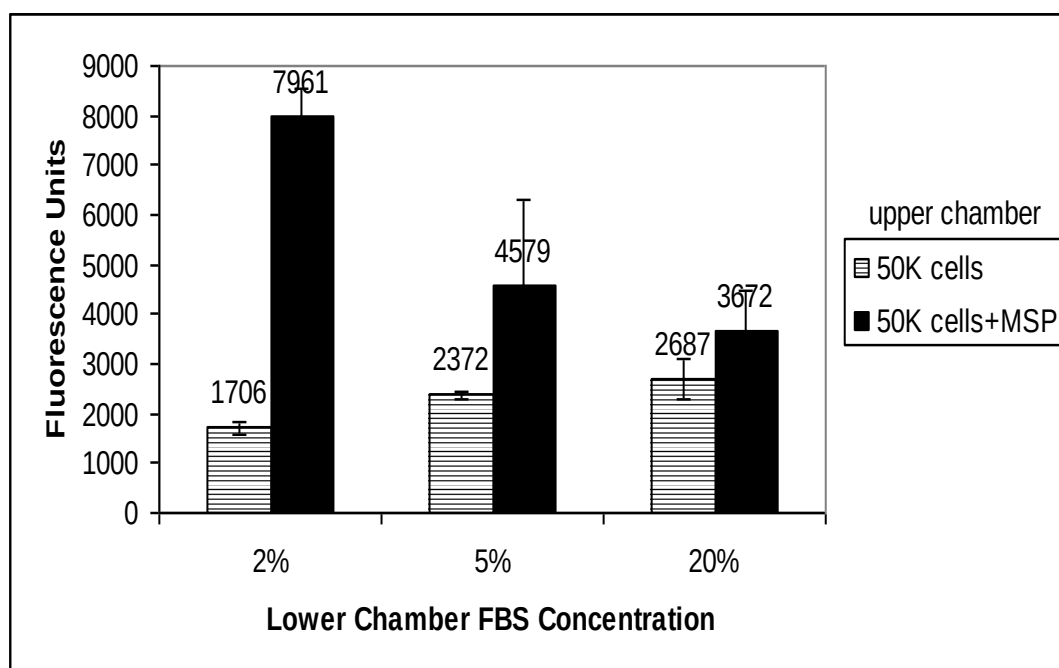


Figure 19. MSP effect is observed best at low serum concentrations.

Different serum concentrations were tried and it is observed that at lower serum concentrations such as 2% or 5% FBS, MSP stimulation increases T47D migration. Whereas at 20%FBS, MSP driven T47D migration diminishes.(Figure 19) To decrease the negative effect of serum, we have tried 0%FBS and 2%FBS conditioned medium (CM) in the lower chamber. MSP was added either to lower or to the upper well. 2 %FBS CM was the most suitable condition for MSP driven migration. (Figure 20)

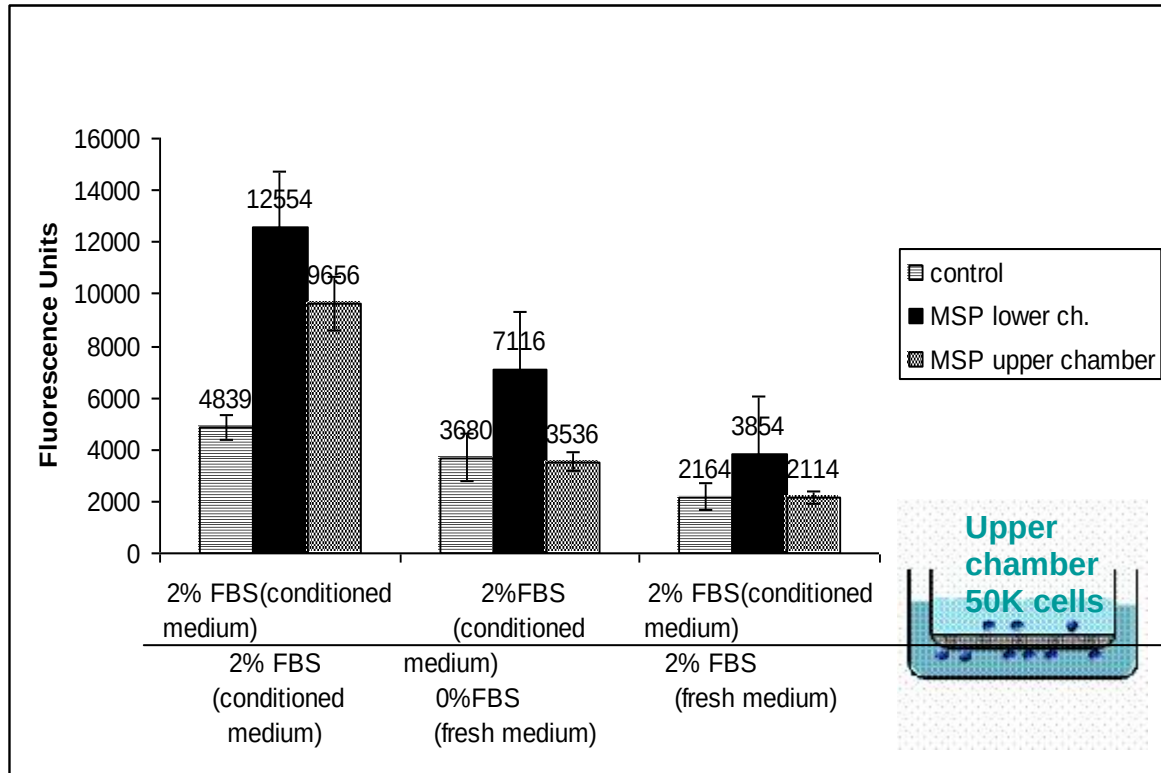


Figure 20. Higher MSP driven migration was observed with MSP in the lower chamber with 2% FBS(conditioned medium) in both chambers.

MSP induction was more effective on migration when it is placed in the lower chamber than the upper chamber. When serum free medium was used in both chambers no migration was seen. (data not shown). In order to eliminate serum totally, collagen coating was tried to increase T47D motility.

I)MSP Increases T47D cell Migration

MSP stimulation caused increased cell migration under two different conditions. (Figure 21) In serum free medium, MSP increased migration of T47D cells once the chambers were coated with Collagen I. MSP presence

in the lower well caused a cell migration almost five fold of basal condition. In 2%FBS conditioned medium, MSP caused a cell migration which is around 2.5 fold of cell migration in the basal condition.

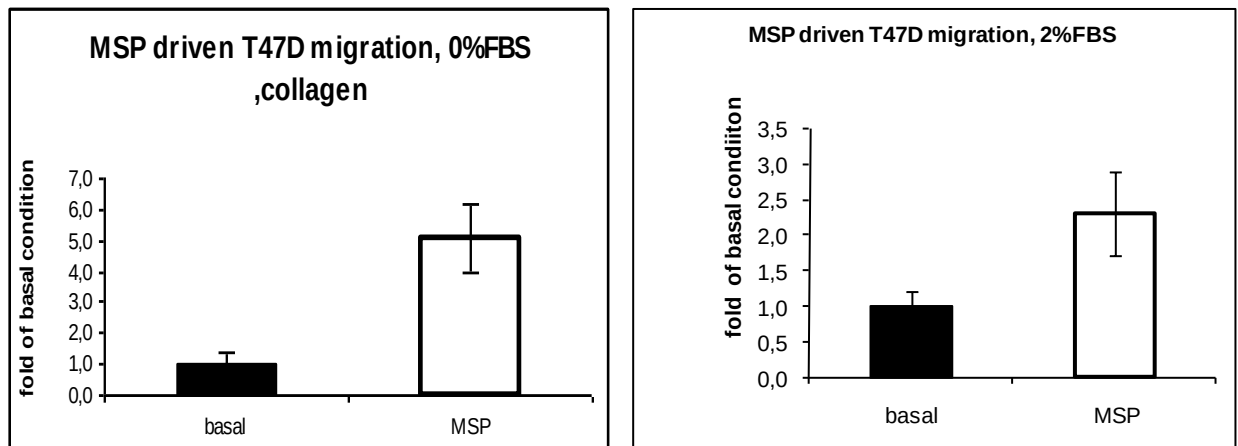


Figure 21. MSP induction increases T47D cell migration . (A) T47D cells were induced with 2nM MSP in the migration chambers and allowed to migrate for 24 hours in 2%FBS conditioned medium. (B) Migration chambers were coated with Collagen I and T47D cells were allowed to migrate for 24 hours with or without induction with 2nM MSP. [Black: basal condition, white: MSP in the lower well]

J) RON Silencing abrogates MSP driven migration

To study the role of RON in MSP driven migration better, we used silencing of the receptor in a cell line inducible with doxycycline. T47DtetRonWT is a kind gift of Silvia Benvenuti (IRCC). This cell line has a doxycycline inducible shRNA expression cassette. Thus upon doxycycline treatment shRNA mediated RON silencing takes place.

As seen in Figure 22 in cell line not treated with doxycycline where RON is present, MSP increases T47D cell migration. In the doxycycline treated cells however MSP treatment do not cause any increase in migration, showing the specificity of MSP action on RON RTK for migration increasing action.

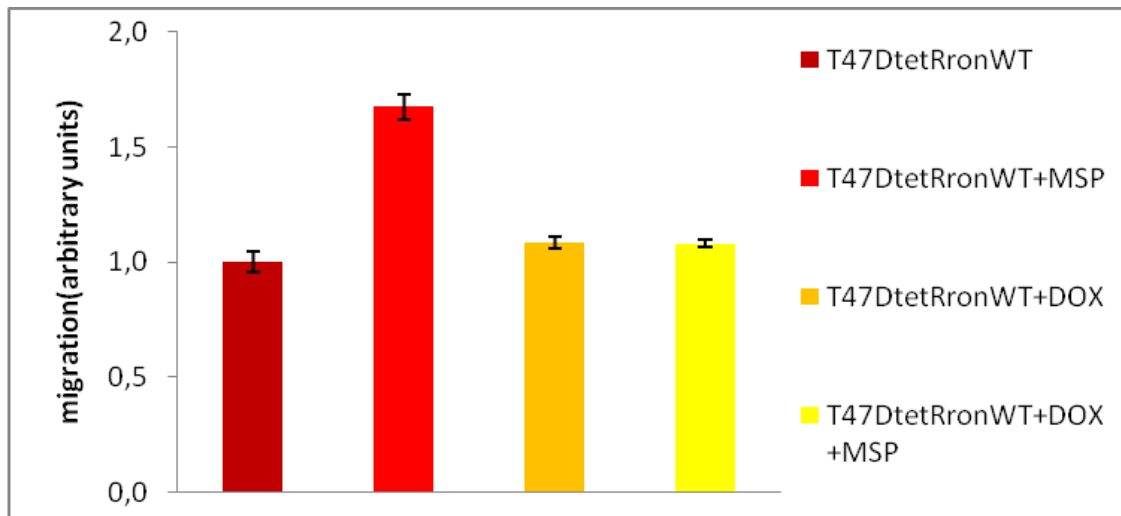


Figure 22. RON silencing abrogates MSP driven T47D migration.

K)RON mAbs are able to block MSP driven cell migration

After verifying that MSP increases migration, have challenged our mAbs in the migration assay for two reasons: Firstly, to see if migration increase caused by MSP is due to MSP-RON interaction. Secondly if this increase can be blocked or reverted by RON mAbs, thus verifying their capability to block migration caused by MSP. We have tested our antibodies in both conditions and showed that they all decrease MSP driven migration.(Figure 23)

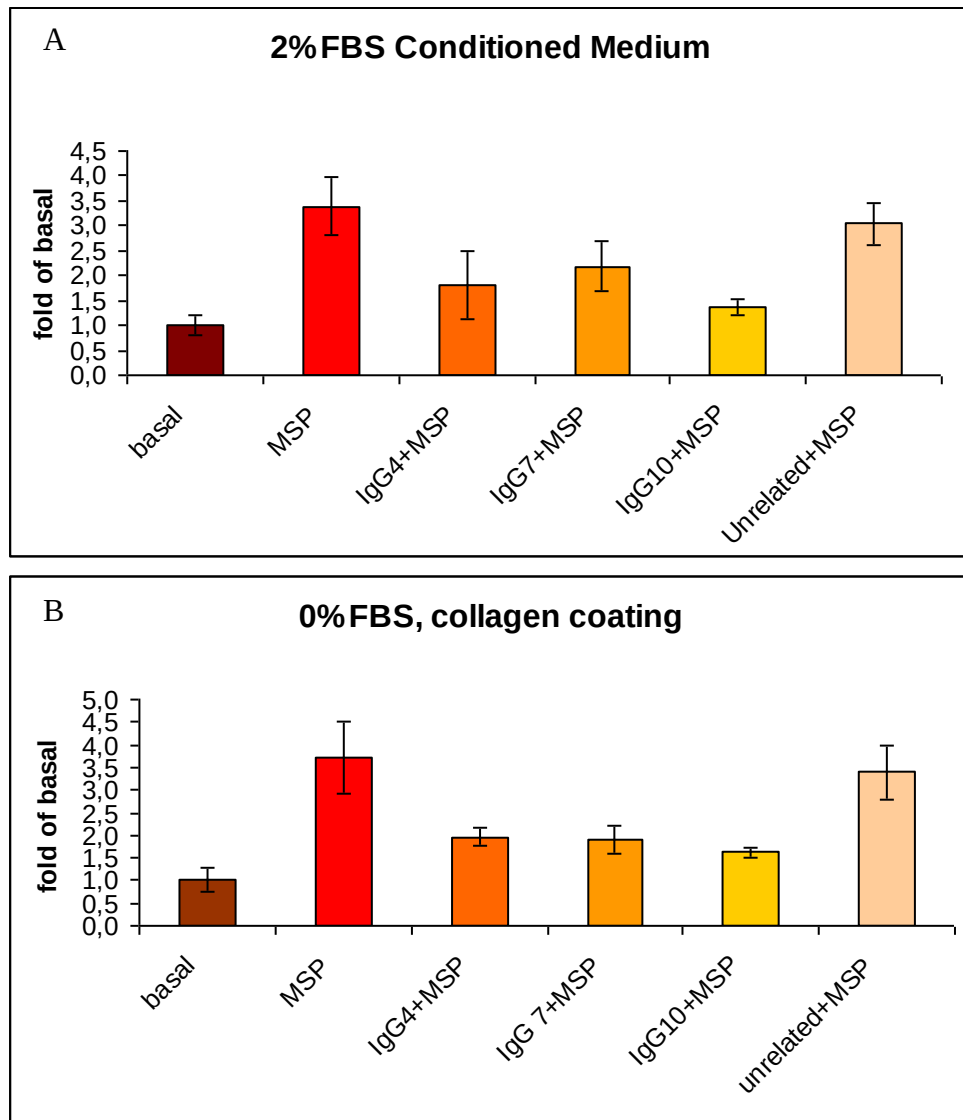


Figure 23. RON IgGs are able to block MSP driven migration. T47D cells treated with RON mAbs before 2nM MSP stimulation and allowed to migrate for 24 hours. (A) In 2%FBS conditioned medium (B) In 0%FBS medium using collagen I coated migration chambers. An unrelated antibody used as a negative control.

L)RON mAbs bind to different regions on RON

According to cross-competition results RON IgGs 4, 7 and 10 do not interfere with one another by means of binding to RON. This is revealed by incubating T47D cells with saturating concentrations of one antibody and

following the binding of the other antibody which is fluorescently tagged. An unrelated antibody was used as a negative control. It is clearly seen that neither of the antibodies interfere with the binding of the other to RON on T47D cells. (Figure 24)

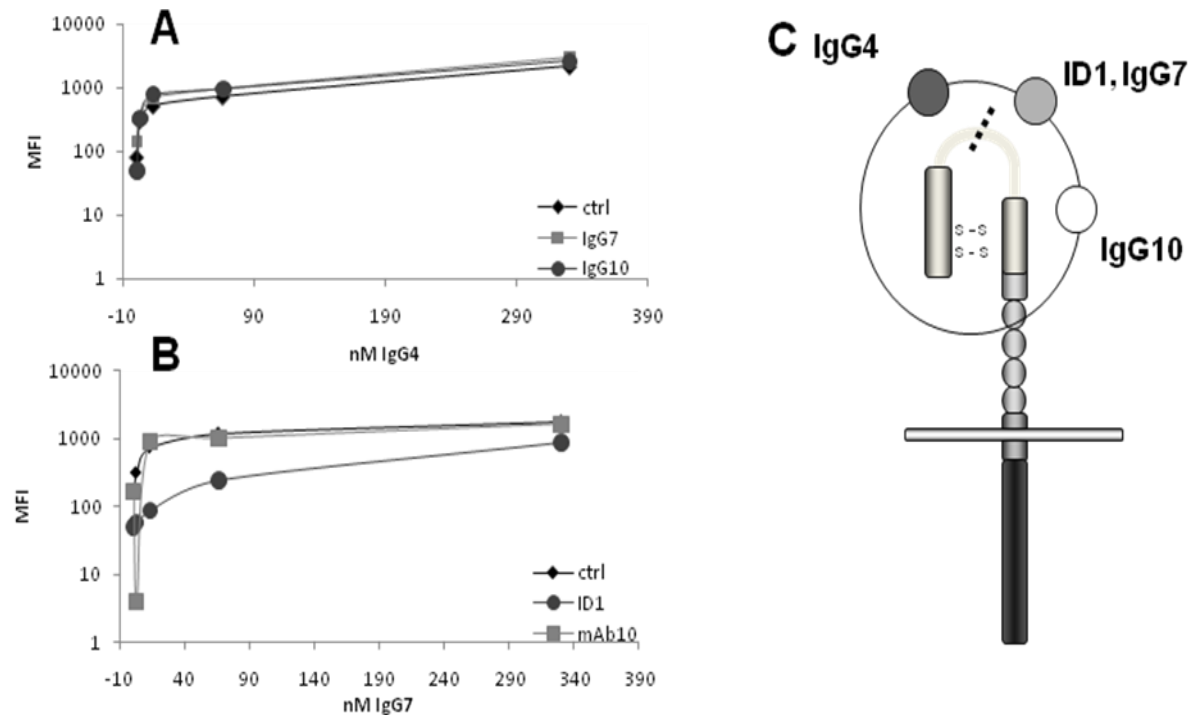


Figure 24. RON IgGs bind to different regions of the Sema domain. (A) T47D cells were incubated with increasing concentrations of labeled mAb4 in the presence of saturating amounts of mAb7, mAb10, or control isotypic IgG1 antibody (Ctrl IgG) as negative control. (B) T47D cells were incubated with increasing concentrations of labeled mAb7 in the presence of saturating amounts of mAb10, ID1 and control IgG. IgG4, IgG7, or IgG10 did not interfere with any other's binding, indicating that they recognize different regions, whereas ID1 blocks mAb7 binding, indicating that they recognize the same region. (C) Schematic cartoon summarizing the results of crosscompetition experiments.

M) RON mAbs show increased activity when used in combination

Due to the fact that our RON mAbs were recognizing different regions, we have tested them in combinations to observe a possible increase in the

capability to block MSP driven migration. When used together RON mAbs show an increased potency to block MSP effect. (Figure 25) Combinatorial effect of RON mAbs are more evident in the migration conditions where collagen coating and medium without serum was used. In this condition spontaneous motility of T47D cells are inhibited.

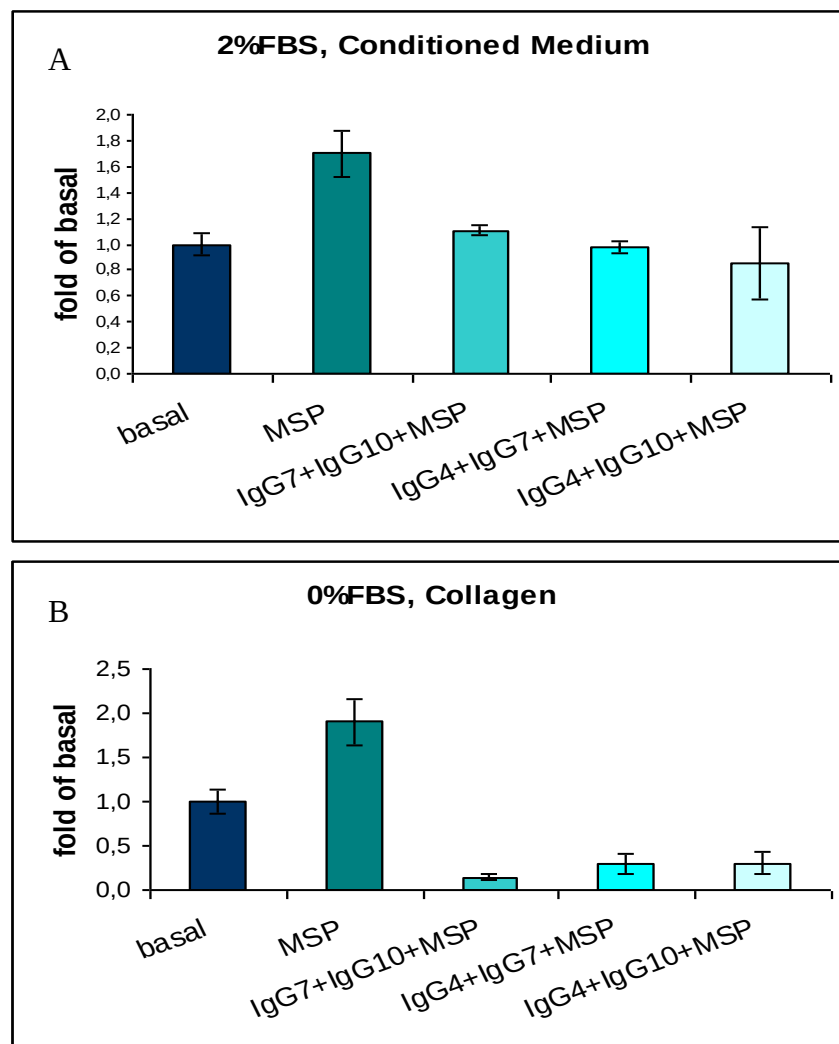


Figure 25. RON mAbs used in combinations to treat T47D cells before 2nM MSP stimulation. Migrant cells are quantified after 24 hours of migration. (A) In 2%FBS conditioned medium (B) In 0%FBS medium using collagen I coated migration chambers.

N) IgG4, IgG7, and IgG10 Do Not Display Antitumor Activity *In Vivo*

We have generated an orthotopic mammary tumor xenograft model with implanted T47D cells to study the efficacy of IgG4, IgG7, and IgG10 *in vivo*. Groups of seven mice were randomly allocated at day 19 after inoculation based on mean tumor sizes of 150 to 200 mm³. Mice were treated weekly with 25 mg/kg of RON mAbs or an isotypic control IgG. On day 29 after treatment, the experiment was terminated. The minor reduction observed was not statistically significant with P values of .17 for IgG4, .23 for IgG7, and .23 for IgG10, with respect to control IgG on day 29 (Figure 26).

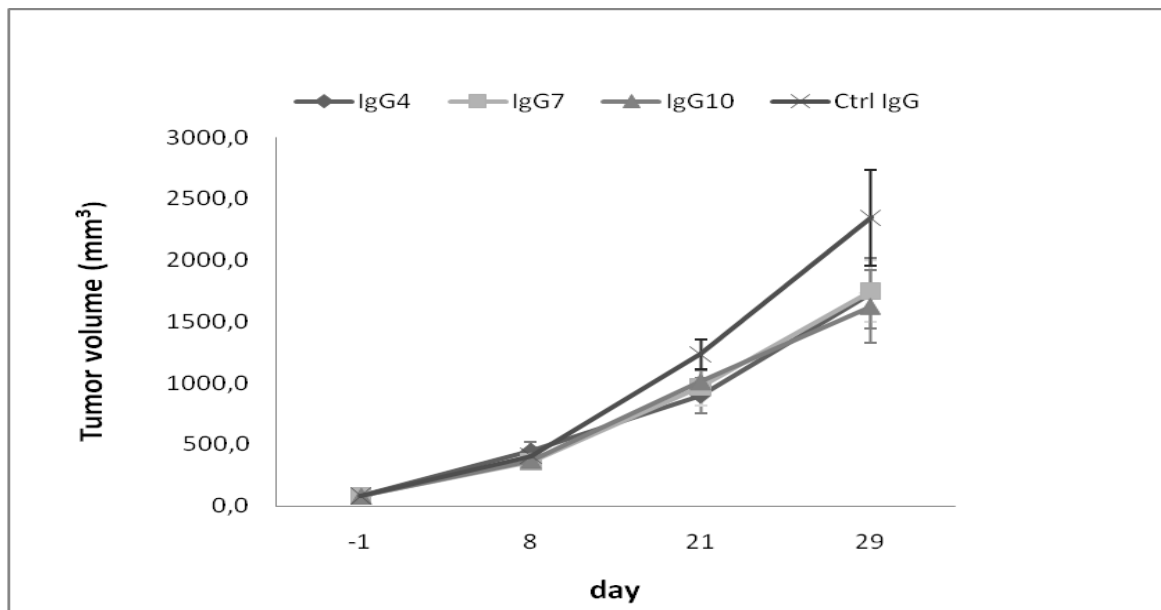


Figure 26. IgG4, IgG7, and IgG10 do not display antitumor efficacy in orthotopic T47D xenograft. Groups of 10 mice were treated weekly with 25 mg/kg of IgG starting when the mean tumor size was between 150 and 200 mm³. The minor reduction observed was not statistically significant with P values of .17 for IgG4, .23 for IgG7, and .23 for IgG10 with respect to control IgG on day 29.

To verify whether the lack of antitumor effect was not limited to a single xenograft model, the selected IgGs were tested in mice injected with BxPC3 or HT29 cell lines. Recent studies have shown that mAb IMC-41A10 that blocks RON-mediated signaling also inhibits growth of these tumor cell lines *in vivo*.⁴⁰ Groups of 10 mice were treated with 25 mg/kg of the antibody. A control group was treated with histidine buffer. Antibody treatment was initiated when the tumors were 85 to 130 mm³, and intraperitoneal injections were repeated twice weekly during the study.

Dosing schedule was established based on preliminary mAb pharmacokinetic analyses in mice where the trough level of the antibodies remained above the concentration needed for receptor saturation in serum withdrawn 72 hours after mAb administration (data not shown).

In the BxPC3 xenograft, we could use an antihuman IGF1R antibody as an internal positive control, which has antitumor activity in this xenograft model.

Unlike the reported antitumor effects observed with mAb IMC-41A10, none of the antagonistic RON mAbs displayed an antitumor effect in either BxPC3 xenograft(Figure 27) or HT29(Figure 28), whereas the positive control antihuman IGF1R successfully inhibited tumor growth in BxPC3 model . The histopathology and the weight of the tumors from treated and untreated mice were also not different.

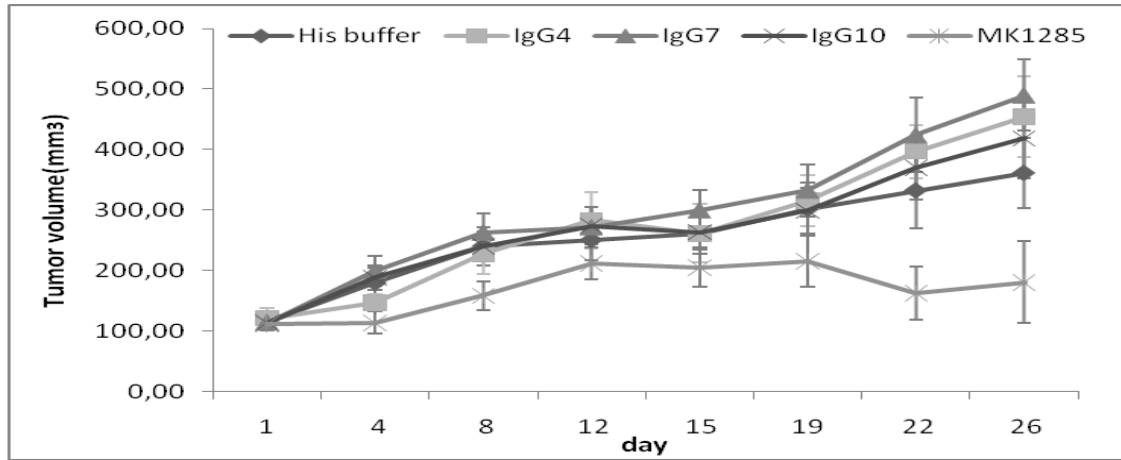


Figure 27. RON IgGs do not have antitumor activity in BxPC3 xenograft. Ten athymic nude mice per group were treated biweekly with 25 mg/kg of IgG starting when the mean tumor size was between 150 and 200 mm³. An internal positive control MK1285, anti-IGF-1R antibody, has antitumor activity in this model.

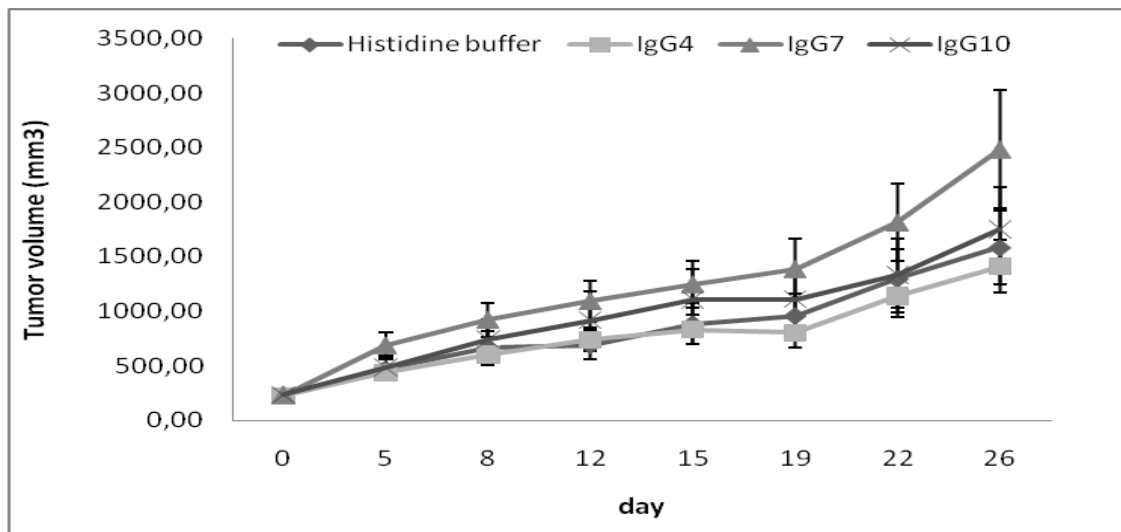


Figure 28. RON IgGs do not have antitumor activity in HT29 xenograft. Ten athymic nude mice per group were treated biweekly with 25 mg/kg of IgG starting when the mean tumor size was between 150 and 200 mm³.

O) RON Silencing causes decreased tumor growth *in vivo*

Since no antitumor effect was obtained with any of the RON IgGs, a better understanding of the role of RON RTK needed by silencing RON and studying the effect of this phonemanon on tumor growth.

O.1) HCT116tetRRON model

We tested the effect of RON silencing in murine xenograft models of HCT116 and T47D. In HCT116 model the doxycycline inducible cell line where Doxycycline treatment results in RON silencing was used. In addition we have used a control cell line with a mutated RON shRNA that do not result in RON silencing to be sure the effect observed is not due to shRNA related toxicity. As can be seen in Figure 29, RON silencing significantly decreases tumor growth.

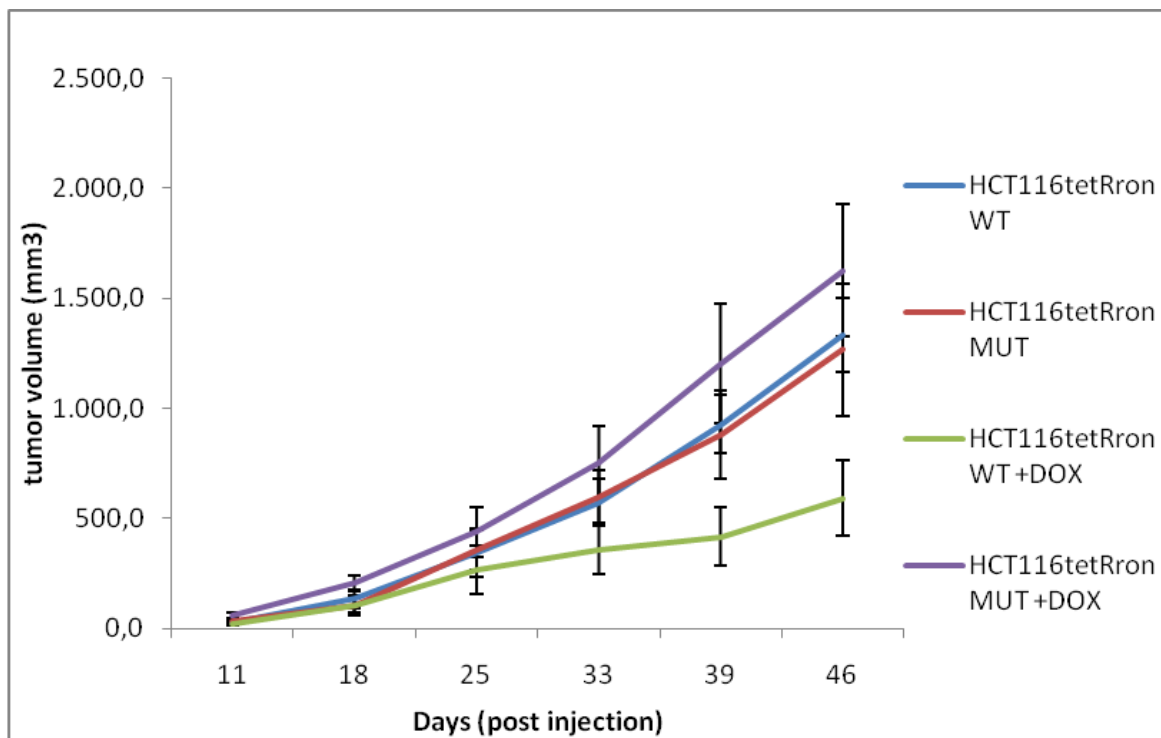


Figure 29. RON silencing upon doxycycline treatment decreases tumor growth in HCT116tetRronWT cells. No decrease was observed upon doxycycline treatment in HCT116 cells with the mutant shRNA construct. (HCT116tetRronMUT)

During the study body weight of the mice among experimental groups did not show significant differences(Figure 30), thus the possible influence of body-weight fluctuations on tumor size can be ruled out.

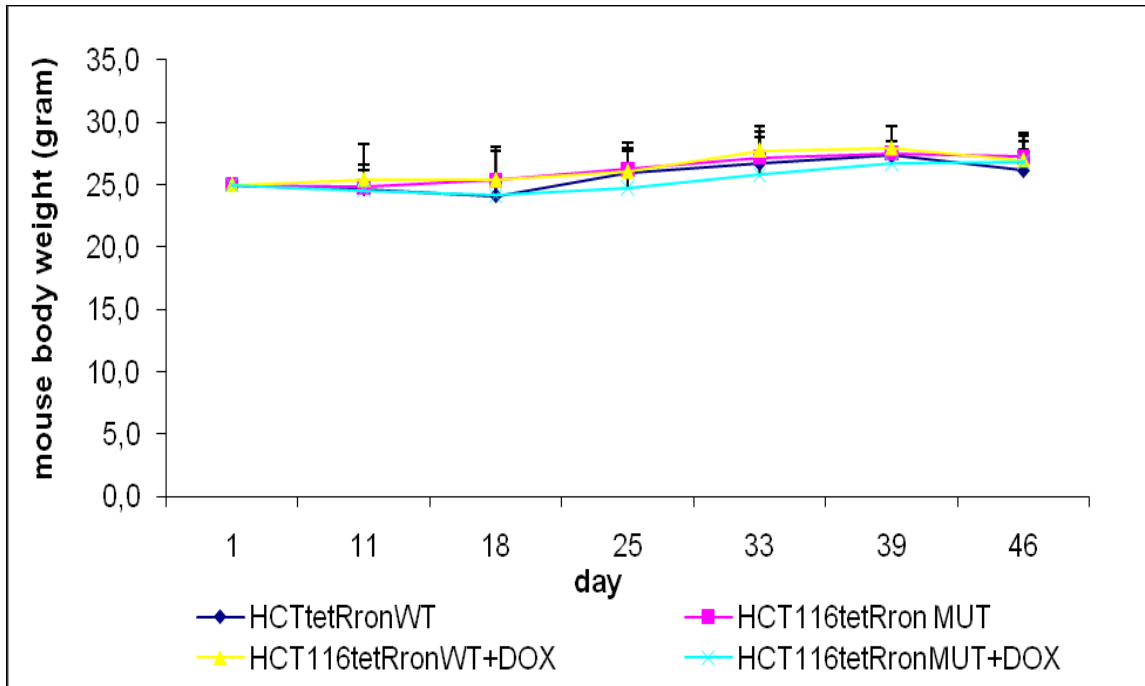


Figure 30. HCT116tetRron xenograft. Average body weight of the mice in experimental groups do not differ from group to group.

To ensure that the Doxycycline treatment resulted in RON silencing, at day 39 and day 46, randomly chosen mice from each group were sacrificed and tumors were excised. A western blot analysis was carried out from the tumor lysates. RON protein expression levels were deduced from the blots, where chemiluminescence of the bands corresponding to RON was quantified. Figure 31 shows that on day 39 and 46, RON protein is clearly downregulated in tumor coming from HCT116tetRronWT+DOX mice when compared to other three experimental groups.

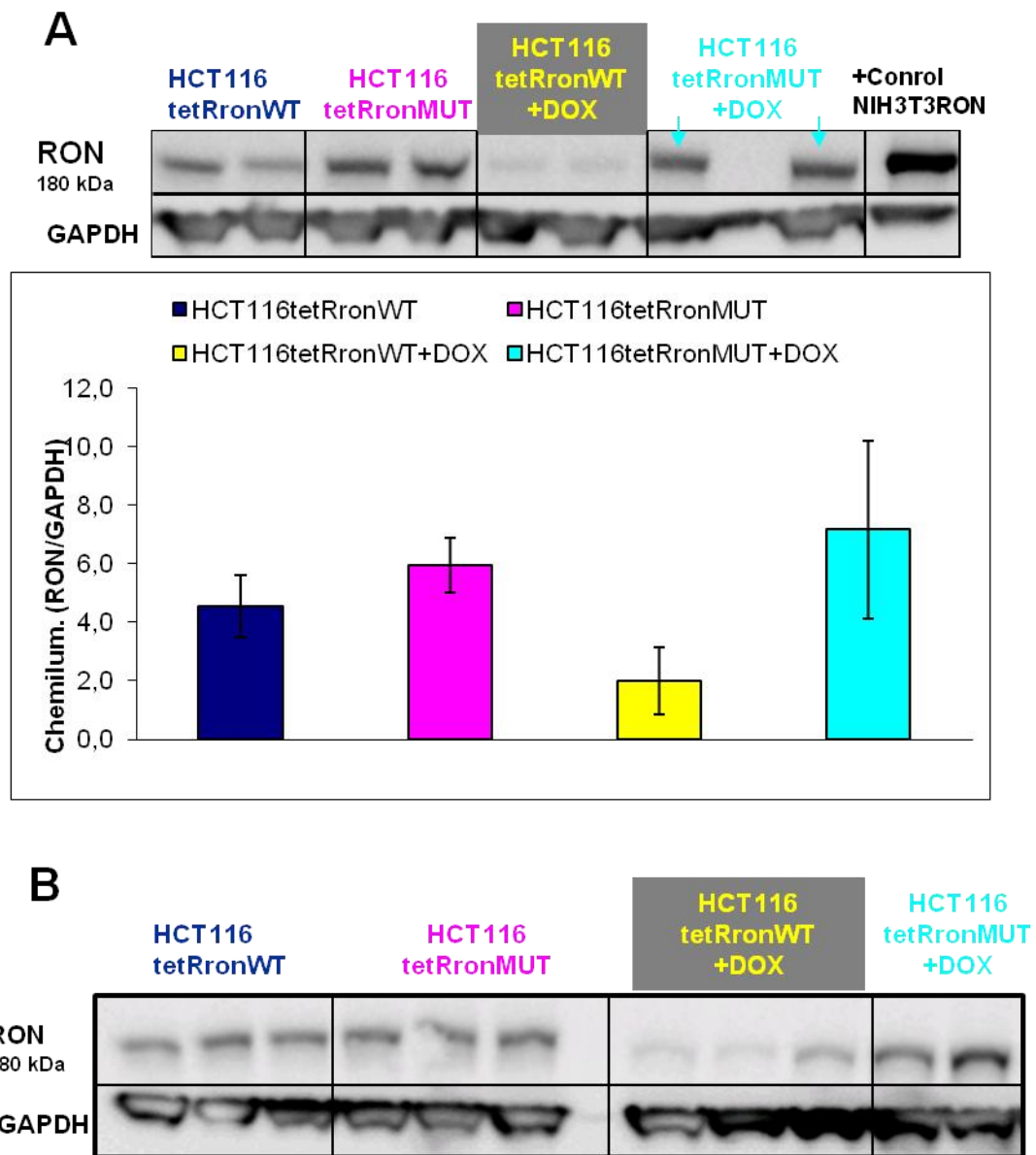


Figure 31. Doxycycline treatment successfully induces RON silencing, as a consequence RON protein levels in HCT116tetRronWT+DOX tumors are nearly absent and much lower than other tumor groups. A) RON protein expression on day 39 shown by WB (on top), and quantitative measurement of chemiluminescence (below). B) RON protein expression on day 46 shown by WB.

O.2) T47DtetRron orthotopic *in vivo* model

After HCT116tetRron model, T47DtetRron model was studied to ulteriorly verify the effect of RON silencing on tumor growth. The difference of this model being orthotopic, the cells were implanted into the 4th mammary fat pad of female mice which were previously implanted by estradiol pellets. Again in this model RON silencing significantly reduces tumor growth.(Figure 32)

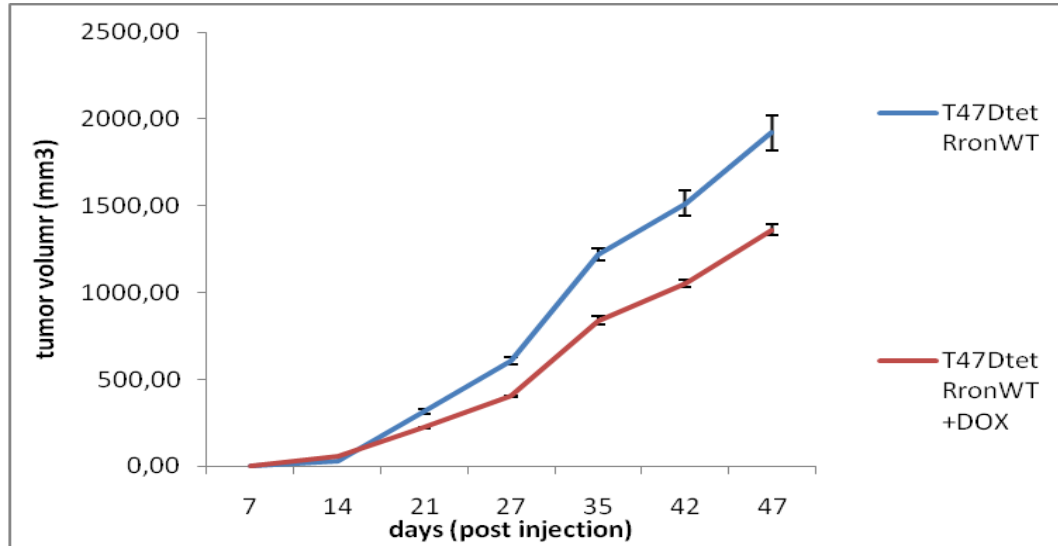


Figure 32. RON silencing via shRNA induced by doxycycline significantly reduces tumor growth in T47D cells.

As control, bodyweight of the mice were monitored to eliminate the possibility of interference of the body weight fluctuations among groups. As can be seen in Figure 33 there is not a significant different between average body weight of mice in two experimental groups.

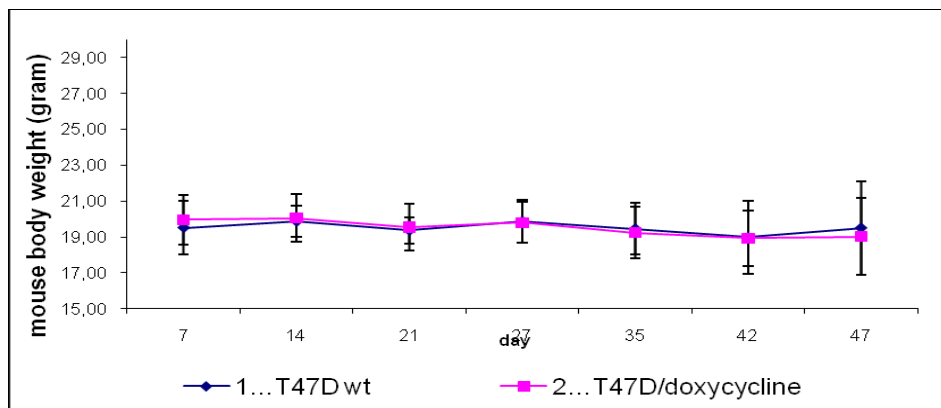


Figure 33. Average mouse body weight in T47DtetRronWT model do not differ among experimental groups.

As a further proof of the reducing effect of ron silencing on tumor growth, mice were sacrificed and tumors were weighed at the end of the in vivo study in T47DtetRronWT model. Figure 34 shows that the reduction in tumor volume is well reflected to tumor weight. Clearly RON silencing caused a reduction in tumor weight as well as tumor volume.

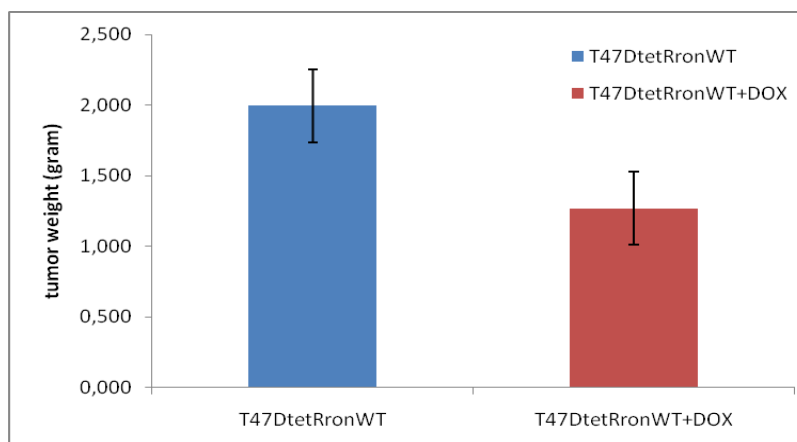


Figure 34. RON silencing causes decrease in tumor weight in T47DtetRronWT model.

P) MSP causes EGFR phosphorylation

In light of in vivo studies we suspected that RON RTK is important in tumor growth through receptor cross-talk events. Thus, we decided to study

the other RTK's that respond to RON activation. For this purpose we used p-RTK Proteome profiler array. This array bears 42 different human RTKs. T47D cells that were treated with MSP and in later experiments with EGFR were subject to analysis in this array to fish-out the RTK's that are activated thanks to RON RTK. MSP stimulation principally resulted in phosphorylation of Epidermal Growth Factor Receptor (EGFR). MSP stimulation had an additive effect on EGFR phosphorylation as EGF alone caused less phosphor-EGFR than stimulation with EGF and MSP together. Surprisingly in this cell line MSP caused more p-EGFR than EGF did. (Figure 35)

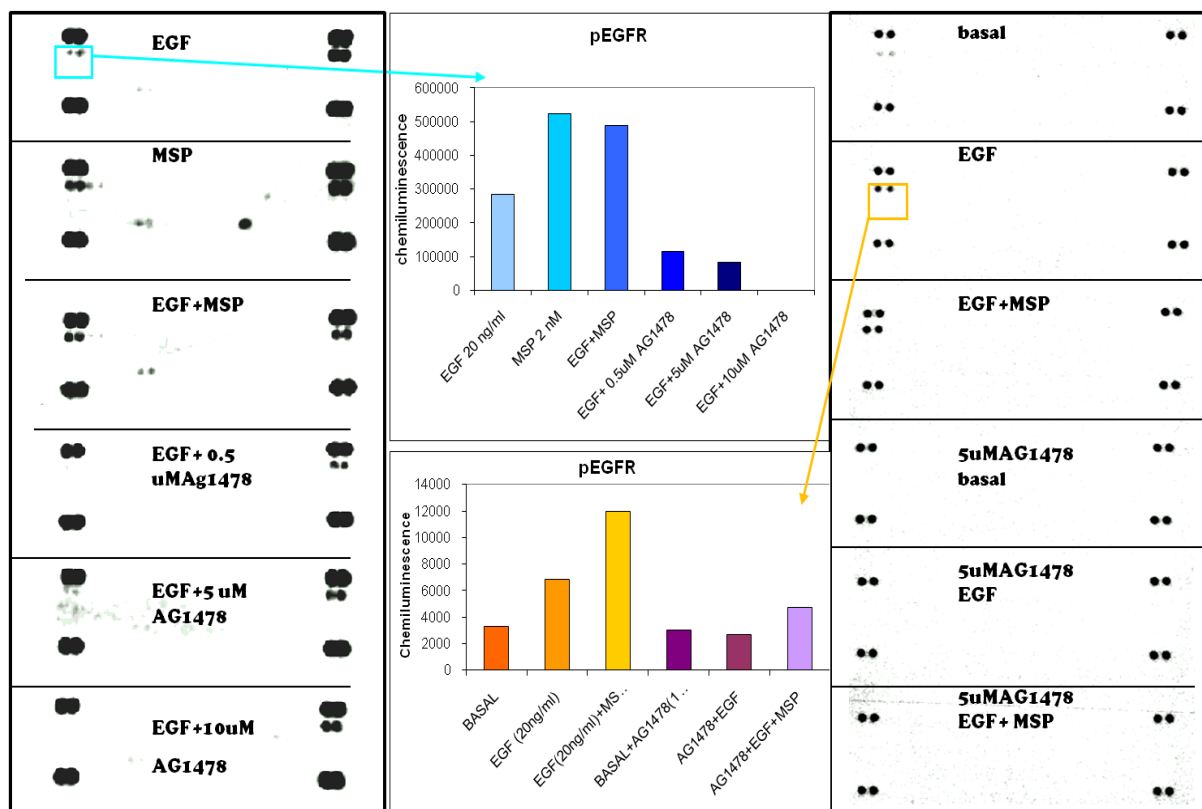


Figure 35. MSP causes EGFR phosphorylation. T47D cell lysates after EGF, MSP and AG1478 treatment were subject to pRTK proteome profiler array.

V)DISCUSSION

Although less extensively studied than the family member MET, accumulating evidence suggest that RON RTK is important for cancer progression.^{36,36,39,40,61} The pathways by which RON conveys signals to the intracellular environment have been studied; however, the relationships of these different pathways to the specific biologic responses that are relevant to tumor formation are still poorly defined.⁶² RON and MET tyrosine kinase domains exhibit 63% sequence identity.⁶³ Because of the high similarity with MET tyrosine kinase domain, the development of potent and selective inhibitor drugs targeting RON has so far been very challenging.^{36,64,65} Biologic drugs such as monoclonal antibodies or small interfering RNA (siRNA) are therefore highly desirable for the high specificity for the target. Biodrugs that target the RON receptor are in the early stage of development. The extracellular region of the RON receptor containing the Sema domain was able to block the binding of MSP to RON and to inhibit the growth of HCT116 colon cancer cells.⁶⁶ Silencing using siRNA against RON reduced cancer cell proliferation and motility, increased apoptotic susceptibility of the cells *in vitro*, and significantly reduced lung metastasis *in vivo*.⁶⁷ Neutralizing monoclonal antibodies that block the interaction of RON with MSP and diminish RON phosphorylation and downstream signaling have been developed.^{40,64,68} Nevertheless, so far, only one humanized antibody against RON (IMC-41A10) has been found to significantly decrease tumor growth of murine xenografts from subcutaneously injected lung, colon, and pancreatic cancer cell lines in nude mice.^{40,69}

In this study, we describe the selection and characterization of three fully human mAbs that are potent antagonists of the human RON RTK. All three mAbs are highly specific for the human RON receptor as shown by IP followed by mass spectrometry (IP-MS) and highly selective; in fact, they do not cross react with mouse RON nor recognize human MET. Moreover, they all show high affinity binding to RON with measured K_d in the low-nanomolar range (mAb4 = 0.17 nM, mAb7 = 0.43 nM, and mAb10 = 0.64 nM; Table 2, Figure 17) and do not compete with each other for receptor binding, indicating that they recognize different regions of the RON extracellular domain (Figure 24). AKT and ERK are key downstream effector molecules very important in cancer progression and are also known to be modulated through RON receptor signaling^{36,40}. For this reason, we were keen to evaluate whether the novel RON mAbs could inhibit AKT and ERK phosphorylation and subsequent activation. Consistent with their high affinity to RON, we found that IgG4, IgG7, and IgG10 potently inhibited MSP-induced ERK phosphorylation in the human mammary tumor cell line T47D in a dose-dependent manner (Figure 16).

The effect of the selected IgGs on RON-mediated signaling was assessed in transwell migration assays. All the three RON mAbs strongly inhibited MSP-dependent migration of T47D cells, giving a functional confirmation of their antagonistic activity and the role of RON RTK on cellular migration (Figure 23). We have further showed that sh-RNA mediated silencing of RON abrogates the MSP driven migration of T47D cells. Thus MSP's migratory action is showed to be through RON RTK. (Figure 22)

Blocking different epitopes in a recognition site can modulate the inhibitory effect on receptor-mediated signaling. Because our mAbs bind to different

epitopes, we hypothesized that, by occupying different sites present on the receptor, we might interfere with the interaction of RON with other cell membrane elements and obtain a greater antimigratory effect. Indeed, when cells were treated with a combination of two mAbs, a significant reduction of T47D spontaneous motility was observed (Figure 25). Importantly, no effect on cell viability was observed on treatment of the cells with the antibodies alone or in combination. A recent work supporting the role of RON in spontaneous cell motility showed that siRNA-mediated knockdown of RON resulted in inhibition of ligand-independent motility of human colon cancer cells.⁶⁷ Inhibition of the spontaneous motility by combinatorial treatment with Sema-binding mAbs might be due to a more efficient blocking of the Sema domain leading to an impairment of RON RTK interaction with different surface proteins or receptors leading to reduction of spontaneous migration as well as MSP-driven migration. This conclusion is supported by the observation that, in addition to its ligand, RON may be activated through cross talk with other surface proteins such as MET, integrins, type B plexins, and EGFR.^{57,58,70,71}

For further understanding of RON pathway and its role in tumor progression we carried out *in vivo* studies. Because RON IgG4, IgG7, and IgG10 shared similar characteristics but bound to distinct regions of the receptor, they all were tested *in vivo* to assess their antitumor potency. To this purpose, we established different xenograft models used based on cell lines derived from breast (T47D), colon (HT29), or pancreas (BxPC3) tumors. Despite the potent inhibitory effect on RON signaling, none of the mAbs had therapeutic efficacy in any of the three xenograft models.(Figure 26, Figure

27, Figure 28) This observation leads us to conclude that blocking MSP-dependent signal transduction is not sufficient to inhibit tumor growth.

After these results, we decided to question the role of RON RTK in a more direct manner. For this purpose we used shRNA mediated silencing of Ron RTK in two *in vivo* xenograft models of breast (T47DtetRron) and colon(HCT116tetRron). In these models, silencing of RON RTK caused a significant decrease in tumor growth.

There are several possible explanations for our *in vivo* findings. In the first instance, it is entirely possible that RON signaling *in vivo* is activated by the interaction with other membrane receptors. Previous studies had shown that the physical interaction between RON and EGFR is ligand independent and that transphosphorylation of both receptors can be induced on stimulation with either EGF or MSP.^{57,58} We have also nicely demonstrated that MSP stimulation causes a very strong EGFR phosphorylation.(Figure 35). Apart from slight Ephrin RTK phosphorylation EGFR was the only RTK which was phosphorylated upon MSP stimulation in our experiment. This result taken together the well-established role of EGFR in tumor growth makes us think EGFR as a possible collaborator of RON RTK for tumor growth.

Thus, for an antitumor activity, not only antagonistic ability but also interfering RON's interaction with other surface proteins such as EGFR might be needed. It is possible that RON RTK play a role in tumor growth not through its signaling pathway but through phosphorylation of other RTKs such as EGFR or being a docking protein for other RTKs. A good example for this kind of relation among RTKs come from the ErbB family

members. ErbB2, which do not have known ligand, apart from forming homodimers forms heterodimers with ErbB3. This heterodimer apart from exerting a potent signaling through ErbB3, is thought to be responsible of the ErbB tyrosine kinase inhibitors' failure to induce sustained inhibition of HER3 signalling in HER2-driven breast cancer cells.⁷² If we are to look at the two recent antibodies against ErbB2, namely Trastuzumab (Herceptin) and Pertuzumab, we can see that they vary greatly in their mode of action and tumor growth inhibition once studied in detail. While Trastuzumab is not able to block ligand induced heterodimers and result in low ability to block tumor growth through ErbB2-ErbB3 driven signaling, Pertuzumab sterically blocks HER-2 dimerization with other HER receptors and blocks ligand-activated signaling from HER-2/EGFR and HER-2/HER-3 heterodimers.^{73, 74}

A second possibility is that the primary role of the RON receptor in tumors might not be the activation of antiapoptotic pathways but rather the control of motility and migration. This hypothesis cannot be easily ruled out in the xenograft models used in this study where tumor growth at the primary site of inoculation is the main parameter followed, but it could be better studied in mouse models of metastasis formation. Indeed, we plan to test the efficacy of the combinatorial administration of RON mAbs in metastatic models. Finally, it should be kept in mind that, for in vivo activity of therapeutic mAbs, Fc receptor-dependent mechanisms such as antibody-dependent cell-mediated cytotoxicity are important contributors.⁷⁵ We cannot exclude that RON IgG4, IgG7, and IgG10 are not efficient in activating these mechanisms, which might render them less effective in vivo.

In conclusion, RON IgG4, IgG7, and IgG10, although being potent antagonists of RON, do not seem to inhibit receptor-specific signaling that may be relevant for tumor progression. These results have important implications for future selection strategies of therapeutic mAbs for RON and, more in general, for RTK involved in tumor development and progression, in that properties other than blocking receptor signaling and inhibiting in vitro functions of RON are needed to generate IgGs with in vivo antitumor activity. Furthermore, RON is important in cell migration and tumor growth. However, although cell migration seems to be directly related with RON downstream signaling; in vivo tumor growth seems not to be directly controlled by RON downstream signaling but through receptor cross-talk events such as RON-EGFR interaction.

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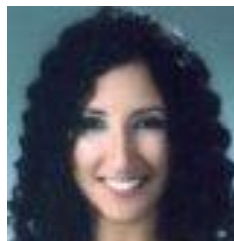
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VI) REFERENCES

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Strong oral and written communication skills – Strong interpersonal skills-Innovative - Challenging - Fast learning

Research Experience

- 2010/current** Visiting Scientist at **La Sapienza University**, Rome (Italy) Department of clinical and molecular medicine, working on development of antagonistic mAbs against ErbB3 receptor under supervision of Prof. Gennaro Ciliberto and Prof. Maurizio Alimandi.
- 2006/2010** **PhD Fellow at Istituto di Ricerche di Biologia Molecolare (IRBM) "P.Angeletti" (Merck Research Laboratories (MRL))**, in Rome (Italy) under the supervision of Dr. Alessandra Vitelli (Oncology department) working on the development of monoclonal antibodies against new targets in cancer main focus being RON receptor tyrosine kinase and Ephrine receptor.
- Fall 2005** Undergraduate research assistant at **Middle East Technical University, department of biological sciences** (Ankara, Turkey) under the supervision of Prof. Emel Arinc studied TPMT*3C Polymorphism in Turkish population and its clinical importance in Acute Lymphoblastic Leukemia
- Summer 2005** Summer student at **Istituto di Ricerche di Biologia Molecolare (IRBM) "P.Angeletti" (Merck Research Laboratories (MRL))**, in Rome (Italy) under the supervision of Dr Stefano Colloca (biotechnology department) worked on design of a antibody-protamine fusion protein for antibody mediated delivery of

siRNAs.

Fall 2004 **Undergraduate Research assistant at Middle East Technical University**
,department of biological sciences (Ankara, Turkey), under the supervision of
Prof. Feride Severcan participated in the research project “An FTIR Approach to
Investigate Molecular Changes Induced by Epilepsy”

Technical skills

Cellular Biology

Monolayer and suspension culture, transient and stable transfection techniques, virus preparation (HIV/SIV, HSV-2, lentivectors) and concentration, infection of monolayer and suspension culture of cell lines and primary ,detection of cytokines and chemokines (ELISA), proliferation and clonogenic assays, migration assays GUAVA, FACS, InCytosol immunofluorescence, apoptosis assays. Protein lysate preparation from tumor tissues. Cancer stem cells isolation and culturing. Mamosphere and neurosphere cultures.

Molecular Biology

Screening of phage libraries, phage recovery, liquid and solid phase panning. ELISA. Humanization of mAbs of mouse origin. Antibody labelling. DNA and RNA extraction. real-time quantitative PCR, cloning and subcloning techniques, amplification and purification of plasmid DNA, PCR, homologous recombination, electrophoresis, analysis of cellular proteins (whole and fraction cell extracts), western blot, immunofluorescence. constitutive or inducible gene silencing by lentiviral vectors coding shRNA. Protein quantification(BCA/Bradford). Quantitative Image analysis (LAS3000). Immunoprecipitation. In Vivo xenograft mouse models study design and follow-up. Genetically engineered mouse models design, application and follow-up.

Academic Qualifications

2006/2010 **PhD in Molecular Biology and Biotechnology** at University of Perugia
Department of Experimental Medicine and Biochemical Sciences , Prof
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2002/2006 **B.Sc. in Molecular Biology and Genetics** at Middle East Technical

University, Faculty of Arts and Sciences, Ankara, Turkey. Honour student CGPA: 3.31/4

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Skills acquired: Time management, organization, respecting tight deadlines, working under pressure.

Giving and attending seminars and conferences. Reviewing the literature and creating bibliographic libraries.

Original analytical thinking, perspicacity, multi-task and multidisciplinary abilities.

Innovative at problem solving and conceptualization

Autonomy, working independently and as part of a team, teaching bench work.

Languages and other Skills

Languages	English: Advanced(TOEFL Computer based grade 287/300)
	Italian: Advanced in reading and speaking, upper intermediate in writing.
	German: Beginner
	Turkish: Native
IT	Proficient in the use of the Internet, PCs and MACs computer systems and programs. Microsoft Office, SigmaPlot, Vector NTI.

Publications

1. **Gunes Z**, Zucconi A, Cioce M, Meola A, Pezzanera M, Acali S, Zampaglione I, De Pratti V, Bova L, Talamo F, Demartis A, Monaci P, La Monica N, Ciliberto G, Vitelli A. (2011) Isolation of fully human antagonistic RON antibodies showing efficient block of downstream signaling and cell migration. Translational Oncology 4(1),38-46
2. Ansuini H, Meola A, **Gunes Z**, Paradisi V, Pezzanera M, Acali S, Santini C, Luzzago A, Mori F, Lazzaro D, Ciliberto G, Nicosia A, La Monica N and Vitelli A. Anti-EphA2 antibodies with distinct in vitro properties have equal in vivo efficacy in pancreatic cancer. *J.Oncol.* 2009;2009:951917. Epub 2010 Jan 14.
3. Turker Görgülü S, Ateş N, İlbağ G, **Güneş Z**, Kara N, Severcan F. An FTIR Approach to investigate molecular changes induced by epilepsy. WSAS

Transactions on Biology and Biomedicine Issue 4, Vol. 2, October 2005
(<http://www.worldses.org/journals/biology/biology-october2005.doc>)

Meetings and courses participated

- September 2009** Horizons in Molecular Biology- 6th International PhD Student Symposium
Max Planck Research School, Göttingen(9-12 September)
- June 2008** FEBS Advanced practical course: QCM, SPR/Ellipsometer & AFM as
novel Biosensors &
Imaging Systems. Ankara/Turkey
- May 2007** GRC Symposium: Cell Contact & Adhesion, Lucca/Italy (May27-June1)
- September 2004** BIOMED-2004 Symposium, Hacettepe University, Ankara/Turkey

Scholarships and Awards

- 2006-2010** PhD Fellowship from IRBM/ Merck Research Laboratories
- 2002-2006** ODTÜ Temel Bilim Bursu (METU Basic Sciences Scholarship)
Given upon being among top 500 students(top 0.03%) in University
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- 1999-2002** Antalya College Scholarship (based on merit)

Examinations Passed

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References

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Privacy

Concerning the Italian privacy policy (**Legge 675, 31-12-1996 and D.lgs. 196/2003**), I hereby approve the use of the privacy protected content in this CV.

Isolation of Fully Human Antagonistic RON Antibodies Showing Efficient Block of Downstream Signaling and Cell Migration¹

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Abstract

RON belongs to the c-MET family of receptor tyrosine kinases. As its well-known family member MET, RON and its ligand macrophage-stimulating protein have been implicated in the progression and metastasis of tumors and have been shown to be overexpressed in cancer. We generated and tested a large number of human monoclonal antibodies (mAbs) against human RON. Our screening yielded three high-affinity antibodies that efficiently block ligand-dependent intracellular AKT and MAPK signaling. This effect correlates with the strong reduction of ligand-activated migration of T47D breast cancer cell line. By cross-competition experiments, we showed that the antagonistic antibodies fall into three distinct epitope regions of the RON extracellular Sema domain. Notably, no inhibition of tumor growth was observed in different epithelial tumor xenografts in nude mice with any of the antibodies. These results suggest that distinct properties beside ligand antagonism are required for anti-RON mAbs to exert antitumor effects *in vivo*.

Translational Oncology (2011) 4, 38–46

Introduction

Monoclonal antibodies (mAbs) are attractive therapeutic drug candidates in cancer therapy thanks to their potential for disease-specific targeting and low-toxicity profiles. Significant progress has been made in recent years in this field. However, there is substantial difficulty in predicting the efficacy of mAbs because the molecular pathways leading to their therapeutic effect are not clearly understood. Although many mechanisms have been proposed to account for the antitumor activities of therapeutic antibodies as a consequence of the interference with signaling pathways, antibody-dependent cell cytotoxicity stands also as a crucial component of the *in vivo* antitumor effect exerted by antibodies targeting tumor antigens [1].

Receptor tyrosine kinases (RTKs) are preferred target candidates for new mAbs because they fine-tune cellular pathways that control events involved in cellular growth and migration that are important in cancer development and progression.

The recepteur d'origine nantaïs (RON), also known as macrophage stimulating 1 receptor, is a member of the MET proto-oncogene family of RTKs and has been shown to be important in cancer [2–6]. It is a 180-kDa disulfide-linked heterodimeric protein composed of a 35-kDa extracellular α -chain and a 150-kDa transmembrane β -chain with intrinsic kinase activity and regulatory elements [2,7]. The extra-

cellular segment contains a Sema domain that constitutes the distinctive structural and functional elements of semaphorins, their plexin receptors, and the RTK MET [8–10]. This domain seems to provide a common structural scaffold that can be adopted to mediate a diverse range of protein-protein interactions such as dimerization,

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¹This article refers to supplementary materials, which is designated by Figures W1 to W6 and are available online at www.transonc.com.

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Received 5 August 2010; Revised 22 October 2010; Accepted 1 November 2010

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semaphorin-plexin binding, and receptor-ligand binding. RON's Sema domain is considered to be the ligand-binding region [2,9,10].

RON is mainly expressed in epithelial cells and is highly expressed and activated in most primary breast, colon, and pancreatic samples and tumor cell lines [2,4,11–15]. Ligand of RON is the macrophage-stimulating protein (MSP), which is a 78-kDa growth and motility factor [12,16,17]. RON and its ligand MSP have been implicated in the progression and metastasis of tumors. On binding to its ligand MSP, RON becomes phosphorylated at key intracellular tyrosine residues that provide docking sites for downstream signaling adapter molecules and triggers activation of several signaling pathways that include the Ras/MAPK, PI3K/Akt, and FAK pathways [18,19]. On stimulation with MSP, RON enhances invasion and cell motility in various epithelial cell lines [13,20]. A recent study demonstrated RON as a novel molecular target of hypoxia-inducible factor 1 and suggested a potential therapeutic role for RON TK inhibitors in the blockade of RON tyrosine kinase-mediated invasion of carcinoma cells [21].

RON is in cross talk with other RTKs, cell surface proteins and growth factors such as epidermal growth factor receptor (EGFR), MET, integrins, and transforming growth factor β [22–26]. RON activation can enhance signaling through c-MET and EGFR, both associated with tumorigenesis [25,27]. The changes associated with the epithelial-to-mesenchymal transition due to RON activation together with the ability of MSP-activated RON to increase cellular migration further support a role for RON in metastasis [23]. Tissue-specific overexpression of RON resulted in aggressive tumor formation in transgenic mouse models of lung and breast cancer [28,29].

There is a body of evidence that suggest that RON should be considered as target for cancer therapy [30]. Efforts to develop a cancer therapeutic specific for this RTK have generated a variety of agents that block RON signaling and alter oncogenic phenotypes [3,6,9,31]. Recently, a mAb, IMC-41A10, was reported to bind human RON with high affinity and to inhibit MSP binding as well as MSP-mediated RON, MAPK, and AKT phosphorylation and MSP-dependent *in vitro* cell migration. IMC-41A10 inhibited growth of tumor xenografts in nude mice, and its antitumor efficacy was enhanced when administered in combination with Erbitux [12].

In this study, we have extensively characterized three mAbs against RON, which were identified through the screening of a human scFv phage displayed library. These novel mAbs bind with high affinity to human RON, recognize distinct sites on the Sema domain, block downstream signaling mediated by MSP binding, and inhibit MSP-driven migration of T47D cells. Despite these characteristics being similar to those of IMC-41A10, no *in vivo* efficacy was detected with any of our antagonistic RON mAbs. Our results indicate that different mechanism(s) other than ligand antagonism and inhibition of downstream signaling are needed for a RON mAb to have a therapeutic potential in cancer.

Materials and Methods

Cell Lines

T47D, BxPC3, ZR-75, HT29, MDA-MB-231, DU145, SW480, HPAC, HEK293, and 293-EBNA cells were purchased from ATCC (Milan, Italy) and grown as recommended by the manufacturer except T47D cells, which were grown in Dulbecco modified Eagle medium F-12 Glutamax medium (GIBCO-Invitrogen, Carlsbad, CA), enriched with 10% FBS at 37°C and 5% CO₂.

To generate cell lines that overexpress Ron receptor, complementary DNA (cDNA) encoding the full-length RON protein was cloned into the Gateway-pcDNA-Dest40 expression vector (Invitrogen, Carlsbad, CA) and into the doxycycline-inducible expression vector pCEP/TetO-MCS. CHO cells were transfected with Gateway-pcDNA-Dest40-RON construct, and HEK 293 cells were transfected with pCEP/TetO-MCS-RON construct using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Cells expressing high RON levels were FACS-sorted and grown under appropriate antibiotic selection to obtain stable clones (Geneticin for pDest 40 -RON, Hygromycin for pCEP/TetO-MCS-RON).

Identification of Human Anti-RON ScFv Antibodies from Phage Display Libraries

Human ScFv phage-display libraries with high complexity ($>10^{11}$ different clones) were obtained from Cambridge Antibody Technology Group plc (CaT). Each CAT library has undergone two rounds of selection. The first round was carried out by panning the phage library on recombinant RON SEMA-PSI domain (R&D Systems, Minneapolis, MN) followed by a second-round selection with SW480 cells naturally expressing RON. Phage rescue and amplification were carried out between subsequent selections as described [32]. After two rounds of selection, individual phage clones were examined by phage ELISA for their binding capability to immobilized RON-Fc (Sigma-Aldrich, St Louis, MO) through the use of an anti-Fc Ab (immunoPure Goat anti hIgG [Fc]; Pierce, Milan, Italy). Bound phage was revealed with HRP-conjugated anti M1 monoclonal antibody (Amersham Biosciences, Milan, Italy) using tetramethylbenzidine (Sigma-Aldrich) as substrate. Positive clones were converted from an ScFv to an IgG1 format as previously described [33]. Positive clones were also tested by phage ELISA for their binding to human recombinant MET-Fc (R&D Systems) and to the recombinant mouse RON (R&D). The scFv VH and VL sequences were introduced into mammalian expression plasmids to produce IgG1 heavy or light chain using the Gateway technology (Invitrogen, Carlsbad, CA). The two plasmids were cotransfected into 293-EBNA cells using Lipofectamine (Invitrogen). Secreted IgG was purified from the medium by affinity chromatography on a Protein A Sepharose High Performance prepacked column (1 ml of HiTrap Protein A HP; GE Healthcare, Milan, Italy).

Identification of mAbs That Bind Selectively to RON Using FACS Analysis

Each purified RON mAb was tested for its binding to RON-positive cells (CHO/RON, HEK293/RON + doxycycline, SW480, T47D, HCT116) or RON-negative cells (CHO wt, HEK293/RON-doxycycline) using FACS analysis. MAbs were labeled by incubation with Zenon Human IgG Alexa Fluor 488 Labeling Kit (Molecular Probes/Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines. Cells were then incubated with 50 μ g/ml pre-labeled mAb solution for 30 minutes at room temperature (RT; 2×10^5 cells in 100 ml of phosphate-buffered saline (PBS), 2% BSA for 30 minutes), washed, and fixed with 1% paraformaldehyde in PBS. Analysis was done using BD FACS Canto II cytometer and BD Diva analysis software (BD Biosciences, Milan, Italy).

Immunoprecipitation/Mass Spectrometry Experiment—Identification of Target Receptors

Experiments was carried out as before [34]. Shortly, immunoprecipitation (IP) with RON IgGs 4, 7, and 10 was carried out on fluorescently

labeled (Cy5) membrane proteins. One-dimensional PAGE was followed by in-gel digestion of immunoprecipitated protein bands and identification of the protein bands using matrix-assisted laser desorption ionization–time of flight–mass spectrometer (MALDI-TOF-MS). Data were elaborated using the TurboSequest software (Thermo Electron Rodano, Milan, Italy). Candidates with Xcorr values greater than 2.5 were considered identified with high confidence.

K_d Determination Through FACS Analysis

A total of 4×10^5 T47D cells were incubated with related RON IgGs with increasing concentrations from 3.4×10^{-4} to 60 nM in FACS buffer (0.2% BSA, 10 mM HEPES) for 1 hour at RT followed by 40 minutes of incubation with APC/F(ab')₂ fragment goat anti-human IgG Fcγ fragment specific (Jackson ImmunoResearch, Milan, Italy) at RT. Analysis was carried out using BD FACS Canto II cytometer and BD Diva analysis software. *K_d* values were determined using Sigma Plot 10 software (Systat Software, San Jose, CA).

Cross-competition Assay

A total of 5×10^5 T47D cells per sample were washed once with PBS 1% BSA and incubated with 100 µg/ml of competitor IgG or unrelated human IgG (IgG1, Lambda, from human myeloma plasma; Sigma) for 40 minutes at room temperature on an orbital shaker. The IgG to be tested was labeled with AlexaFluor 647 using Zenon AlexaFluor 647 for human IgG (cat. no. Z25408; Molecular Probes). After a wash with PBS 1% BSA, cells saturated for the first IgG were incubated with different concentrations of the labeled antibody (330, 66, 13, 2.6, 0.5, and 0 nM) for 40 minutes at RT on an orbital shaker. After a wash with PBS 1% BSA, cells were fixed in 1% formaldehyde in PBS. Analysis was done using BD FACS Canto II cytometer and BD Diva analysis software. Binding curves were prepared using Sigma Plot 10 software.

Western Blot Experiments

Induction with MSP. Subconfluent RON-expressing cell lines (HT29, MDA-MB-231, Bx-Pc3, ZR-75, T47D, DU145, SW480, and HPAC) were serum-starved for 18 hours. Wild-type MSP (10 nM; R&D) was used to induce cells for 30 minutes at 37°C.

Testing RON IgGs' ability to block MSP-driven induction. After 18 hours of serum starvation, 100 µg/ml of RON IgGs was incubated with subconfluent BxPC3, T47D, and ZR-75 cell lines for 1 hour at 37°C before 30 minutes of induction with MSP. ID1 commercial blocking antibody CD136 (RON; Beckman Coulter, Milan, Italy) against RON was used as a positive control, and an unrelated antibody (IgG1, Lambda, from human myeloma plasma; Sigma) was used as a negative control.

RON IgGs dose-response experiment. Subconfluent T47D cells that were serum-starved for 18 hours were incubated with increasing concentrations of RON mAbs (2.4, 7.3, 22, and 66 nM) for 1 hour. MSP, 10 nM, was used to induce T47D cells for 30 minutes at 37°C.

Time course experiment with RON mAbs. A total of 4.5×10^5 T47D cells per well in a six-well plate for each point were used. The day after plating, cells were treated with 20 µg/ml of RON mAbs 4, 7, and 10 for 30 minutes, 1 hour, 4 hours, or 24 hours.

Cell lysate preparation and Western blot analysis. Cells were lysed using lysis buffer (50 mM Tris, 1% Triton, 150 mM NaCl, complete protease inhibitor cocktail (Roche, Milan, Italy), halt phosphatase inhibitor cocktail [Thermo Scientific, Milan, Italy]). Total protein concentration was determined by Bradford method, 20 µg of total protein per sample was loaded on 4% to 12% NuPage BisTris Gels, and SDS-PAGE was performed. Proteins were transferred on to a 22-µm nitrocellulose membrane that was then blocked with 5% nonfat dry milk in 0.05% Tween in TBS. For determination of extracellular signal-regulated kinase (ERK) (mitogen-activated protein kinase, MAPK) and AKT phosphorylation, p44/42 MAPK Thr202/Tyr204 rabbit mAb (1:1000; Cell Signaling, Milan, Italy) and phospho-Akt/Ser473 (1:500; Cell Signaling) were used. Protein-blotted membranes were incubated overnight at 4°C with primary antibodies in 5% BSA in 0.05% Tween in TBS. HRP-conjugated rabbit (Abcam, Cambridge, UK) and mouse (Promega, Madison, WI) secondary antibodies were used as 1:1500 and 1:3000, respectively, carrying out 1 hour of incubation at RT. To verify equivalent sample loading, blots were probed for β-actin (NeoMarkers, Fremont, CA). Blots were developed using ECL reagent (GE Healthcare, Milan, Italy). Images were acquired by luminescent image analyzer LAS3000 and then quantified by MultiGauge version 2.2 software (FUJIFILM, Düsseldorf, Germany). To determine expression levels of total ERK and total AKT, these membranes were stripped with Restore Western Blot Stripping Buffer for 15 minutes at room temperature (Pierce) and reprobed with p44/42 MAPK (1:1000; Cell Signaling) or anti-AKT antibody (1:2000; Cell Signaling). After washing and incubating with the secondary antibody, development and image analysis were carried out as before. For determination of RON protein levels, Ron (C-20) sc-322 rabbit polyclonal IgG and HRP-conjugated rabbit (Abcam) were used.

Cell Migration Assay

Twenty-four-well HCT FluoroBlok Insert Systems with fluorescence blocking PET membranes were used as migration chambers (BD Biosciences). T47D cells were serum starved for 18 hours and then plated as 5×10^4 cells per well in serum-free medium on previously collagen coated Fluoroblok chambers (rat tail collagen type I; BD Biosciences). Recombinant hMSP C672A (R&D Systems) was added at a final concentration of 2 nM to the bottom chambers. (Recombinant MSP C672A has increased bioactivity than recombinant MSP [35] because of the limited incorrect disulfide bond formation, so the concentration used in comparison to the previous experiments is lower than previously made signaling experiments as we were furnished with MSP C672A by R&D only during half of this study.) RON mAbs or an unrelated human IgG (IgG1, Lambda, from human myeloma plasma; Sigma) were added in both upper and lower chambers at a final concentration of 20 µg/ml before stimulation with MSP. After 2-nM MSP stimulation, cells are allowed to migrate for 24 hours at 37°C and 5% CO₂. Migrated cells were quantified after fluorescent labeling with 4 µg/ml Calcein-AM (Fluka, Milan, Italy) and by using fluorescence plate reader VICTOR² (Perkin Elmer, Waltham, MA).

Cell Cytotoxicity Assay

T47D cells were serum-starved for 18 hours and were plated in 96-well plates that had been previously coated with collagen (rat tail collagen type I; BD Biosciences) Ron mAbs or an unrelated human IgG in combination with MSP (2-nM recombinant hMSP C672A; R&D Systems) were added to the cells in serum-free medium. After 24 hours of incubation at 37°C and 5% CO₂, ATP levels of cells

were measured using ATPlite 1 step reagent (Perkin Elmer) using Perkin Elmer TopCount microplate luminescence counter.

In Vivo Mouse Models

In the T47D model, 17 β -estradiol pellets (0.36 mg per pellet, 60-day release) were implanted in the dorsal midline of 5-week-old female NOD/SCID mice. After 1 week, 2×10^6 T47D cells in Matrigel were injected in the fourth mammary fat pad. Nineteen days after injection, mice with tumors greater than 30 mm³ were allocated randomly in five groups. Mice were treated weekly with 25 mg/kg intraperitoneally with RON mAbs and isotypic control human IgG1. On day 29 after allocation, mice were killed, and tumors were excised, weighed, and frozen in liquid nitrogen.

In the following two *in vivo* models, 5-week-old female athymic nude mice (Charles River, Calco, Como, Italy) were injected subcutaneously with 5×10^6 BxPC3 cells in Matrigel or intraperitoneally with HT29 cells in Matrigel. After tumors reached an average size of 150 to 200 mm³, 25-mg/kg intraperitoneal treatment with RON mAbs or histidine buffer was carried out biweekly. At day 26 after treatment, mice were killed, and tumors were excised, weighed, and frozen in liquid nitrogen.

Results

Identification and Characterization of RON Specific mAbs

To identify RON-specific mAbs, we screened a human scFv phage-displayed library obtained from CaT with the following selection strategy: a first round of selection was carried out on the recombinant RON Sema-PSI domain, and the recovered phages were screened by a second round of selection on SW480 cells that express high levels of endogenous RON on the cell surface. Finally, individual scFv phages specific for RON were screened for binding to the closely related family member human recombinant MET-Fc and to the recombinant mouse RON. The restricted pool of RON-specific phages was found to bind selectively to human RON but not to mouse RON or human MET (data not shown). Individual phage clones were converted into a human type 1 IgGs and subjected to a further round of screening in a whole-cell binding FACS assay using RON-positive and -negative cell lines. IgG4, IgG7, and IgG10 were found to bind specifically to CHO/RON when compared with CHO wt (Figure W1B). We further confirmed the specificity of RON mAbs by IP of fluorescently labeled membrane proteins with RON mAbs followed by in-gel digestion of protein bands and peptide identification by MALDI-TOF-MS mass spectrometry (IP-MS). All RON mAbs specifically immunoprecipitated two major bands in polyacrylamide gel, which were identified as RON α and β chains (Figure W1A).

IgG4, IgG7, and IgG10 Bind RON with High Affinity

To determine the mAbs' affinity toward RON receptor, we performed whole-cell FACS binding assays on T47D cells endogenously expressing RON. IgG4, IgG7, and IgG10 all displayed high affinity toward RON (Figure 1) with subnanomolar K_d values of 0.17, 0.43, and 0.64 nM, respectively.

Antagonistic RON mAbs Bind to Different Epitopes on Sema Domain

Because of the strategy used to select RON-specific antibodies, the novel RON mAbs IgG4, IgG7, and IgG10 bound to the Sema domain of RON. To ensure that these mAbs recognized different regions

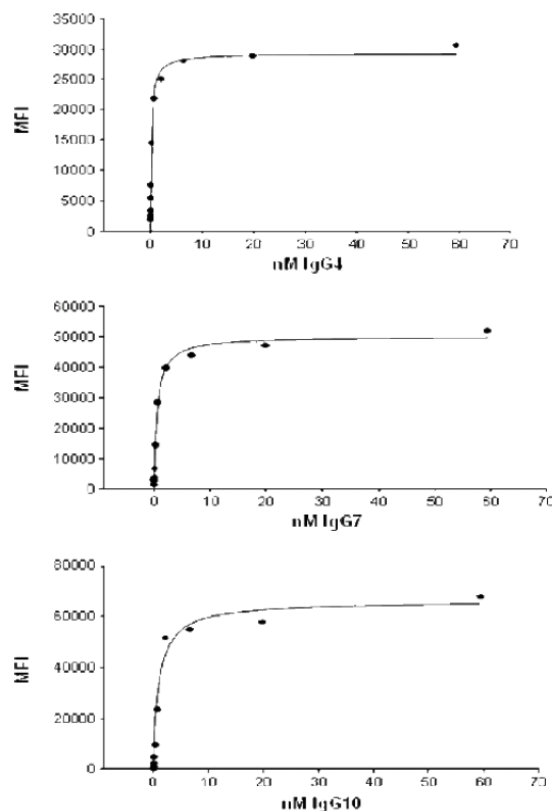


Figure 1. IgG4, IgG7, and IgG10 bind RON with high affinity. Binding saturation curves determined by whole-cell FACS binding assay are represented as mean fluorescence intensities (MFIs) over antibody concentration (nM). Calculated K_d values for IgG4, IgG7, and IgG10 were 0.17, 0.43, and 0.64 nM, respectively.

of the Sema domain, the epitope binding specificity was evaluated. A FACS-based cross-competition experiment was performed in which the binding of one tagged antibody to RON-displaying cells was measured in the presence of a potential competitor antibody or an unrelated IgG1 isotypic control at saturating concentration. As a reference, the commercially available blocking mAb ID1 was also included. This antibody is known to bind to the RON extracellular domain [36].

Notably, IgG4, IgG7, and IgG10 did not cross compete with each other, as the extent of binding was not altered by the presence of another mAb (Figure 2, A and B). Only mAb ID1 and IgG7 cross-competed with each other, as IgG7 binding to RON was strongly inhibited in the presence of ID1 (Figure 2B). As shown by the simplified cartoon in Figure 2C, these results indicate that IgG4, IgG7, and IgG10 bind to different regions of the RON Sema domain, whereas ID1 and 7 bind to the same or to closely positioned regions.

RON mAbs Block Downstream Signaling

Overexpression of the receptor as a consequence of transfection of the RON cDNA often results in autophosphorylation of this RTK regardless of MSP stimulation [37]. Therefore, to study the effect of RON mAbs on ligand-dependent receptor signaling, different cell

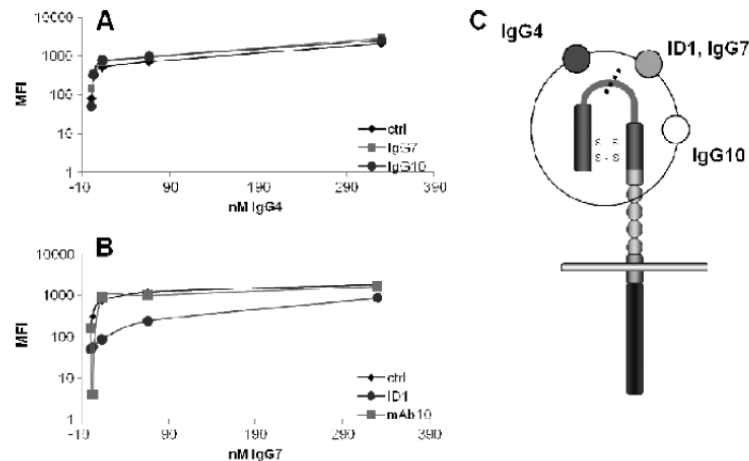


Figure 2. RON IgGs bind to different regions of the Sema domain. (A) T47D cells were incubated with increasing concentrations of labeled mAb4 in the presence of saturating amounts of mAb7, mAb10, or control isotypic IgG1 antibody (Ctrl IgG) as negative control. (B) T47D cells were incubated with increasing concentrations of labeled mAb7 in the presence of saturating amounts of mAb10, ID1, and control IgG. IgG4, IgG7, or IgG10 did not interfere with any other's binding, indicating that they recognize different regions, whereas ID1 blocks mAb7 binding, indicating that they recognize the same region. (C) Schematic cartoon summarizing the results of cross-competition experiments.

lines were used that endogenously express RON. In addition, to evaluate the ability of the RON mAbs to inhibit phosphorylation of ERK and AKT, an extensive screening of tumor cell lines was carried out to identify cells that expressed RON and at the same time showed ERK and AKT phosphorylation on MSP binding. As shown in Figure 3A, the pancreatic cancer cell line BxPC3 and the breast cancer cell lines ZR-75 and T47D exhibited a strong MSP-dependent phosphorylation of AKT and ERK. Kinetics of the treatment of RON-expressing cells with the ligand MSP showed that the maximum phosphorylation of ERK and AKT was reached after 30 minutes of incubation (Figure W2). To measure the inhibitory potency of the different antibodies, dose-response experiments were carried out in the T47D cells. Increasing concentrations of IgG4, IgG7, and IgG10 or unrelated

IgG1 isotypic control were incubated with T47D cells before MSP stimulation. All mAbs inhibited both ERK (Figure 2B) and AKT (data not shown) phosphorylation in a dose-dependent manner. Because the intensity of the pERK signal was stronger than pAKT, the half-maximal inhibitory concentration (IC_{50}) for each IgG was calculated on the basis of the extent of pERK inhibition. The measured IC_{50} was 3 nM for IgG4 and IgG10 and 50 nM for IgG7. Thus, these data demonstrate that IgG4, IgG7, and IgG10 are high-affinity antibodies, specific for human RON, which have ligand antagonistic activity. Finally, we investigated the cytotoxic potential of the antagonist RON mAbs 4, 7, and 10 in *in vitro* proliferation assays on T47D cells. Treatment with the antibodies did not induce an antiproliferative effect, whereas a known cytotoxic drug, cisplatin, clearly reduces proliferation (Figure W6).

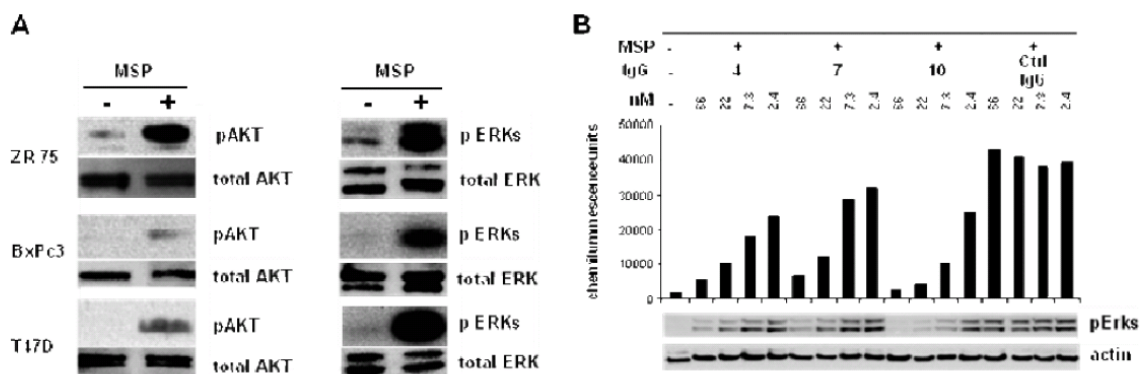


Figure 3. RON IgGs block MSP-activated signaling. (A) Cell lysates of ZR75, T47D, and BxPC3 were assayed by Western blot analysis with anti-phospho-Akt and anti-phospho-ERK antibodies. Antibodies directed to total Akt and total ERK were used as loading controls. (B) Western blot analysis of cell lysates of T47D cells treated with increasing concentration of IgG4, IgG7, and IgG10 before MSP stimulation. Signal intensities were quantified by densitometric scanning and expressed as chemiluminescence units. An antibody directed to actin was used as a loading control.

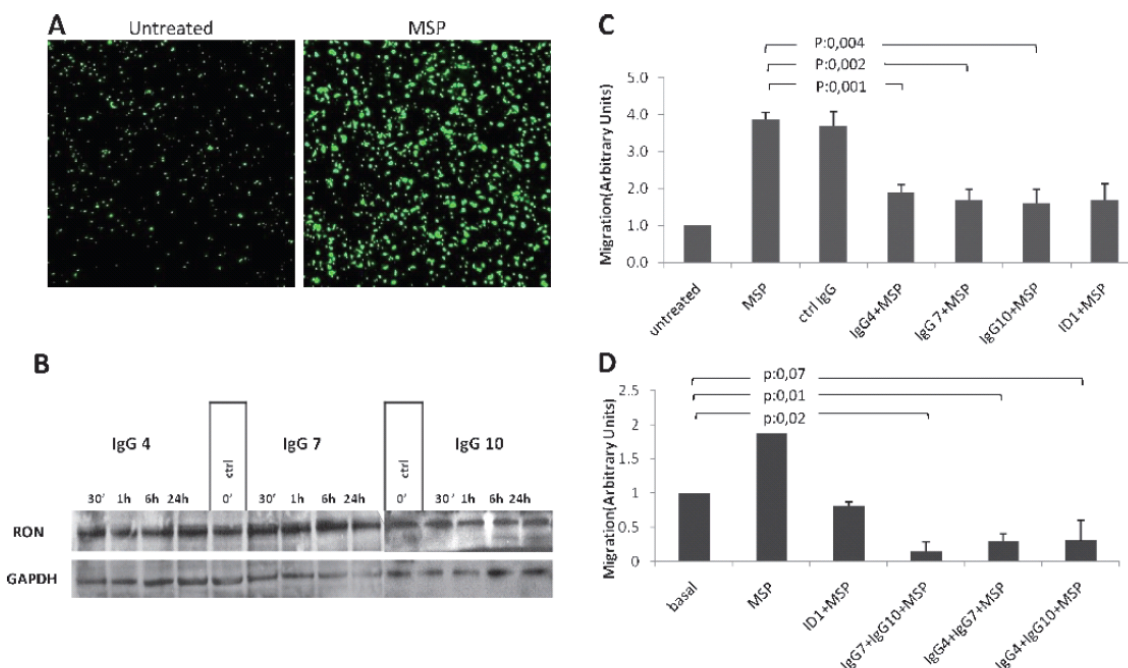


Figure 4. RON IgGs significantly block MSP-driven migration. (A) Representative images of fluorescently labeled T47D migrated to the lower side of the PET membrane in a Boyden chamber after 24 hours with or without MSP stimulation. (B) RON IgG4, IgG7, and IgG10 do not downregulate the RON receptor. T47D cells were treated with RON IgGs for 30 minutes, 1 hour, 4 hours, and 24 hours. (C) Migration activity of T47D cells was measured by a 24-well HCT FluoroBlok assay and expressed as arbitrary units. MSP stimulation (2 nM) caused up to a four-fold increase in migration with respect to untreated control. IgG4, IgG7, and IgG10 significantly blocked MSP-driven migration ($P = .001$, $P = .002$, and $P = .004$, respectively) comparable to the commercially available mAb ID1. (D) Combination of RON IgGs leads to an impairment of T47D spontaneous motility. Combination of IgG7-IgG10 and IgG4-IgG7 MSP inhibited spontaneous motility of T47D cells by 75% to 80% ($P = .02$ and $P = .01$, respectively). Combination of IgG4-IgG10 also reduces the motility of T47D although not significantly ($P = .07$). The experiments shown in the figures are representative of at least three different experiments.

RON IgG4, IgG7, and IgG10 Inhibit MSP-Driven Migration

It has previously been shown that on stimulation with MSP, RON enhances invasion and cell motility in various epithelial cell lines [13,20,27]. To assess the effect of the selected mAbs on cell motility, a fluorimetric transwell migration assay was set up with T47D cells where cells were allowed to migrate to the bottom chamber for 24 hours through collagen-coated membranes in the presence or absence of MSP. When the ligand was absent, a limited number of cells spontaneously migrated to the bottom chamber after 24 hours because of intrinsic cell motility. MSP stimulation caused up to a four-fold increase in the extent of migration with respect to the unstimulated condition. Migration of the cells to the bottom chamber was equally inhibited by IgG4, IgG7, and IgG10 when the mAbs were incubated with cells before MSP stimulation (Figure 4, A and C).

Because the three mAbs bind to the Sema domain, which is involved in RON, cross talk with other membrane proteins and because they do not interfere with each other's binding, we tested the effect of antibodies when used in combination. Notably, when IgG4, IgG7, and IgG10 were used in combination, they showed a stronger inhibiting effect on T47D migration, which not only was limited to inhibition of MSP-driven migration but also blocked the spontaneous motility of T47D cells. A significant effect was observed with IgG7-IgG10 or IgG4-IgG7 combinations, whereas IgG4-IgG10

exerted a lesser inhibitory effect. Nonetheless, all three combinations were more efficient than ID1 alone (Figure 4C). These results suggest that RON has a role in migratory machinery of T47D cells also independently from MSP stimulation. To exclude the possibility that the RON mAbs inhibitory effect on migration was due to receptor internalization, we carried out a time course experiment. T47D cells were treated for 30 minutes, 1 hour, 4 hours, or 24 hours with RON mAbs, and whole-cell lysates were evaluated for RON protein amount with Western Blot. As clearly shown in Figure 4B, there was no reduction in RON protein amounts during treatment with any of the RON mAbs. To control that the strong inhibitory effect was not due to mAb's induced cytotoxicity, cell viability in the migration assay conditions was measured in the presence of the mAbs either as single agents or in combination. No effect on cell viability due to the presence of the antibodies was observed (Figure W5). Thus, we concluded that the combinatorial effect of the RON mAbs was due to an enhanced block in receptor signaling.

IgG4, IgG7, and IgG10 Do Not Display Antitumor Activity In Vivo

We have generated an orthotopic mammary tumor xenograft model with implanted T47D cells to study the efficacy of IgG4, IgG7, and IgG10 *in vivo*. Groups of seven mice were randomly allocated at

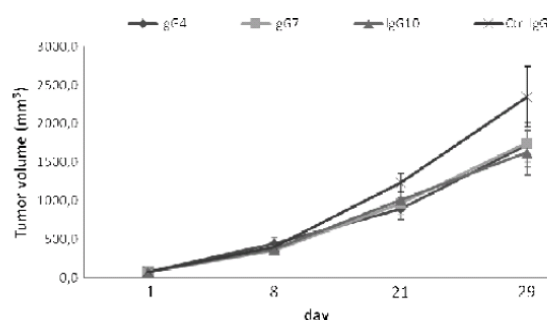


Figure 5. IgG4, IgG7, and IgG10 do not display antitumor efficacy in orthotopic T47D xenograft. Groups of 10 mice were treated weekly with 25 mg/kg of IgG starting when the mean tumor size was between 150 and 200 mm³. The minor reduction observed was not statistically significant with *P* values of .17 for IgG4, .23 for IgG7, and .23 for IgG10 with respect to control IgG on day 29.

day 19 after inoculation based on mean tumor sizes of 150 to 200 mm³. Mice were treated weekly with 25 mg/kg of RON mAbs or an isotypic control IgG. On day 29 after treatment, the experiment was terminated. The minor reduction observed was not statistically significant with *P* values of .17 for IgG4, .23 for IgG7, and .23 for IgG10, with respect to control IgG on day 29 (Figure 5).

To verify whether the lack of antitumor effect was not limited to a single xenograft model, the selected IgGs were tested in mice injected with BxPC3 or HT29 cell lines. Recent studies have shown that mAb IMC-41A10 that blocks RON-mediated signaling also inhibits growth of these tumor cell lines *in vivo* [12]. Groups of 10 mice were treated with 25 mg/kg of the antibody. A control group was treated with histidine buffer. Antibody treatment was initiated when the tumors were 85 to 130 mm³, and intraperitoneal injections were repeated twice weekly during the study. Dosing schedule was established based on preliminary mAb pharmacokinetic analyses in mice where the trough level of the antibodies remained above the concentration needed for receptor saturation in serum withdrawn 72 hours after mAb administration (data not shown). In the BxPC3 xenograft, we could use an antihuman IGF1R antibody as an internal positive control, which has antitumor activity in this xenograft model. Unlike the reported antitumor effects observed with mAb IMC-41A10, none of the antagonistic RON mAbs displayed an antitumor effect in either HT29 or BxPC3 xenograft, whereas the positive control antihuman IGF1R successfully inhibited tumor growth (Figures W3 and W4). The histopathology and the weight of the tumors from treated and untreated mice were also not different.

Discussion

Although less extensively studied than the family member MET, accumulating evidence suggest that RON RTK is important for cancer progression [2,3,12,14,38]. The pathways by which RON conveys signals to the intracellular environment have been studied; however, the relationships of these different pathways to the specific biologic responses that are relevant to tumor formation are still poorly defined [11].

RON and MET tyrosine kinase domains exhibit 63% sequence identity [39]. Because of the high similarity with MET tyrosine kinase domain, the development of potent and selective inhibitor drugs

targeting RON has so far been very challenging [3,40,41]. Biologic drugs such as monoclonal antibodies or small interfering RNA (siRNA) are therefore highly desirable for the high specificity for the target. Biologics that target the RON receptor are in the early stage of development. The extracellular region of the RON receptor containing the Sema domain was able to block the binding of MSP to RON and to inhibit the growth of HCT116 colon cancer cells [9]. Silencing using siRNA against RON reduced cancer cell proliferation and motility, increased apoptotic susceptibility of the cells *in vitro*, and significantly reduced lung metastasis *in vivo* [31]. Neutralizing monoclonal antibodies that block the interaction of RON with MSP and diminish RON phosphorylation and downstream signaling have been developed [12,36,40]. Nevertheless, so far, only one humanized antibody against RON (IMC-41A10) has been found to significantly decrease tumor growth of murine xenografts from subcutaneously injected lung, colon, and pancreatic cancer cell lines in nude mice [12,42].

In this study, we describe the selection and characterization of three fully human mAbs that are potent antagonists of the human RON RTK. All three mAbs are highly specific for the human RON receptor as shown by IP followed by mass spectrometry (IP-MS) and highly selective; in fact, they do not cross react with mouse RON nor recognize human MET. Moreover, they all show high affinity binding to RON with measured *K_d* in the low-nanomolar range (mAb4 = 0.17 nM, mAb7 = 0.43 nM, and mAb10 = 0.64 nM; Figure 1) and do not compete with each other for receptor binding, indicating that they recognize different regions of the RON extracellular domain (Figure 3).

AKT and ERK are key downstream effector molecules very important in cancer progression and are also known to be modulated through RON receptor signaling [3,12]. For this reason, we were keen to evaluate whether the novel RON mAbs could inhibit AKT and ERK phosphorylation and subsequent activation. Consistent with their high affinity to RON, we found that IgG4, IgG7, and IgG10 potently inhibited MSP-induced ERK phosphorylation in the human mammary tumor cell line T47D in a dose-dependent manner (Figure 2).

The effect of the selected IgGs on RON-mediated signaling was assessed in transwell migration assays. All the three RON mAbs strongly inhibited MSP-dependent migration of T47D cells, giving a functional confirmation of their antagonistic activity (Figure 4). Blocking different epitopes in a recognition site can modulate the inhibitory effect on receptor-mediated signaling. Because our mAbs bind to different epitopes, we hypothesized that, by occupying different sites present on the receptor, we might interfere with the interaction of RON with other cell membrane elements and obtain a greater antimigratory effect. Indeed, when cells were treated with a combination of two mAbs, a significant reduction of T47D spontaneous motility was observed (Figure 5). Importantly, no effect on cell viability was observed on treatment of the cells with the antibodies alone or in combination. A recent work supporting the role of RON in spontaneous cell motility showed that siRNA-mediated knockdown of RON resulted in inhibition of ligand-independent motility of human colon cancer cells [31]. Inhibition of the spontaneous motility by combinatorial treatment with Sema-binding mAbs might be due to a more efficient blocking of the Sema domain leading to an impairment of RON RTK interaction with different surface proteins or receptors leading to reduction of spontaneous migration as well as MSP-driven migration. This conclusion is supported by the observation that, in addition to its ligand, RON may be activated through

cross talk with other surface proteins such as MET, integrins, type B plexins, and EGFR [22,25,26,43].

Because RON IgG4, IgG7, and IgG10 shared similar characteristics but bound to distinct regions of the receptor, they were tested *in vivo* to assess their antitumor potency. To this purpose, we established different xenograft models used based on cell lines derived from breast (T47D), colon (HT29), or pancreas (BxPC3) tumors. Despite the potent inhibitory effect on RON signaling, none of the mAbs had therapeutic efficacy in any of the three xenograft models. This observation leads us to conclude that blocking MSP-dependent signal transduction is not sufficient to inhibit tumor growth. There are several possible explanations for this finding. In the first instance, it is entirely possible that RON signaling *in vivo* is activated by the interaction with other membrane receptors. Previous studies had shown that the physical interaction between RON and EGFR is ligand independent and that transphosphorylation of both receptors can be induced on stimulation with either EGF or MSP [25,26]. Thus, for an antitumor activity, not only antagonistic ability but also interfering RON's interaction with other surface proteins such as EGFR might be needed. A second possibility is that the primary role of the RON receptor in tumors might not be the activation of antiapoptotic pathways but rather the control of motility and migration. This hypothesis cannot be easily ruled out in the xenograft models used in this study where tumor growth at the primary site of inoculation is the main parameter followed, but it could be better studied in mouse models of metastasis formation. Indeed, we plan to test the efficacy of the combinatorial administration of RON mAbs in metastatic models. Finally, it should be kept in mind that, for *in vivo* activity of therapeutic mAbs, Fc receptor-dependent mechanisms such as antibody-dependent cell-mediated cytotoxicity are important contributors [1]. We cannot exclude that RON IgG4, IgG7, and IgG10 are not efficient in activating these mechanisms, which might render them less effective *in vivo*.

In conclusion, RON IgG4, IgG7, and IgG10, although being potent antagonists of RON, do not seem to inhibit receptor-specific signaling that may be relevant for tumor progression. These results have important implications for future selection strategies of therapeutic mAbs for RON and, more in general, for RTK involved in tumor development and progression, in that properties other than blocking receptor signaling and inhibiting *in vitro* functions of RON are needed to generate IgGs with *in vivo* antitumor activity.

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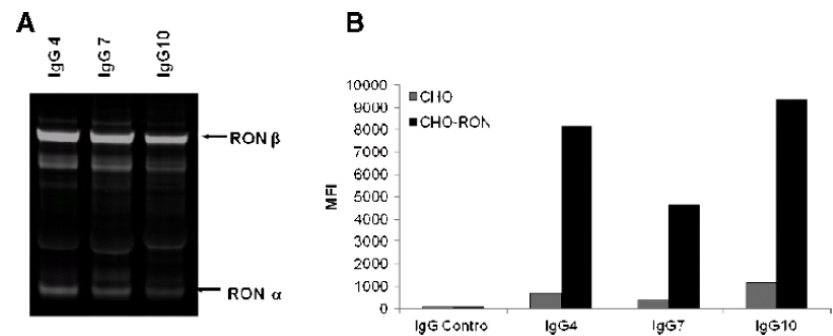


Figure W1. (A) RON IgGs are able to immunoprecipitate RON. Immunoprecipitation with RON IgG4, IgG7, and IgG10 was carried out on fluorescently labeled membrane proteins. One-dimensional PAGE was followed by in-gel digestion of immunoprecipitated protein bands and identification of the protein bands using MALDI-TOF-MS. Data were elaborated using the TurboSequest software. Candidates with Xcorr values greater than 2.5 were considered identified with high confidence. (B) Whole-cell binding FACS analysis was carried out for CHO wt or CHO-RON cells that are stably expressing RON full-length construct. IgG4, IgG7, and IgG10 specifically binds to CHO-RON cells, whereas unrelated IgG used as control does not bind any of the cells as expected.

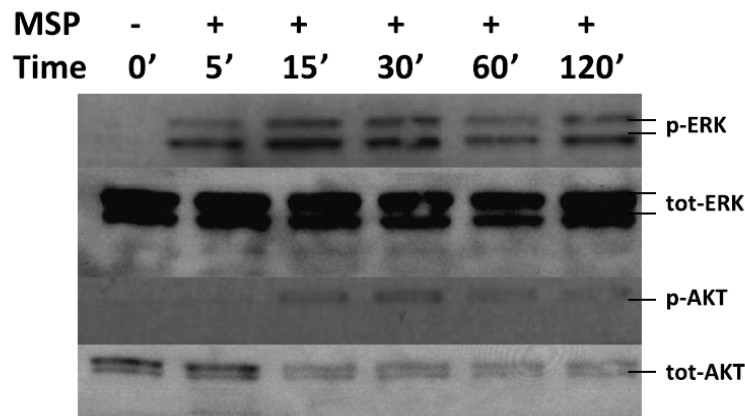


Figure W2. ZR-75 cells time course experiment for ERK and AKT phosphorylation in response to MSP stimulation. The highest phosphorylation levels were obtained at 30 minutes.

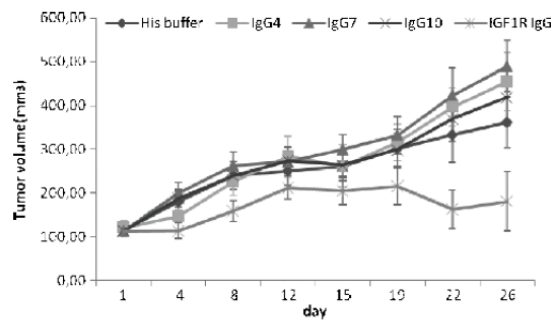


Figure W3. RON IgGs do not have antitumor activity in BxPC3 xenograft. Ten athymic nude mice per group were treated biweekly with 25 mg/kg of IgG starting when the mean tumor size was between 150 and 200 mm³. An internal positive control MK1285, anti-IGF-1R antibody, has antitumor activity in this model.

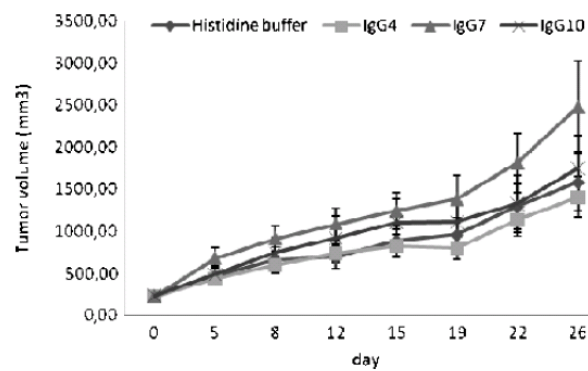


Figure W4. RON IgGs do not have antitumor activity in HT29 xenograft. Ten athymic nude mice per group were treated biweekly with 25 mg/kg of IgG starting when the mean tumor size was between 150 and 200 mm³.

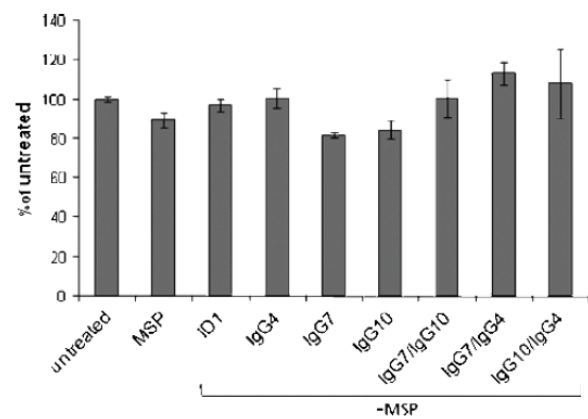


Figure W5. RON IgGs do not interfere with cell viability when used alone or in combination. T47D cell viability was determined using ViaLight Assay (Lonza, Milan, Italy), and values were expressed as percentages relative to untreated control.

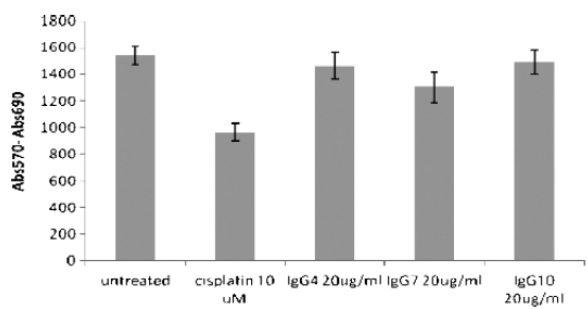


Figure W6. RON IgGs do not block T47D cell proliferation *in vitro*.

