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Generation and characterization of multi-specific antibodies for therapeutic applications in oncology

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To all the people who supported me in this project

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1. Introduction

1.1 The pathogenesis of cancer

Cancer is a complex disease which develops in multiple steps. Its occurrence and progression are typically characterized by aberrations of several molecular and subsequent phenotypic cellular mechanisms. These 'hallmarks', as defined by Hanahan and Weinberg {Hanahan et al., 2011; Hanahan et al., 2000; see Figure 1}, describe the complexity of tumor pathogenesis in the context of both cancer cells and their surrounding microenvironment.

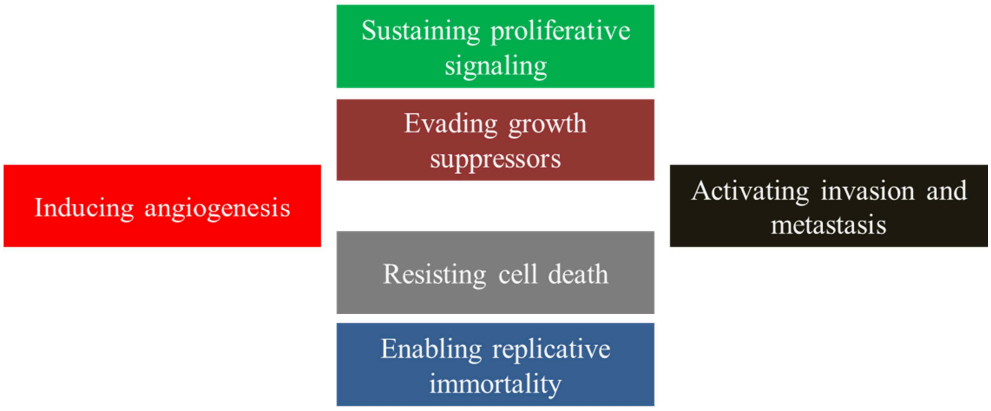

Figure 1. The six hallmarks of cancer as proposed by Hanahan and Weinberg {modified from Hanahan et al., 2011}.

In the context of this work two major characteristics of tumors will be discussed in more detail: the inherent ability of transformed cells to sustain proliferation and become motile under certain circumstances as well as the impact of the tumor microenvironment on the properties of tumor cells.

Tumor cells can maintain proliferative signaling through stimulation of ligand secretion by the surrounding stromal cells, i.e. paracrine activation, as well as through autocrine

production of growth factors {Lemmon et al., 2010; Witsch et al., 2010; Hynes et al., 2009; Perona, 2006; Cheng et al., 2008; Bhowmick et al., 2004}. An additional mechanism which maintains continuous tumor cell proliferation is the over-expression or gene amplification of oncogenic receptors which are responsible for the cellular response to external stimuli (i.e. pro-survival and anti-apoptotic signaling). These receptors are often mutated in cancer cells and can, under certain conditions, become capable of ligand independent signaling, transducing the signal to the downstream effector molecules without prior ligand binding. Cytoplasmic proteins implicated in cellular signaling and growth may also bear activating mutations, thus ensuring a sustained proliferative signaling independently from receptor activation. Prominent examples include the catalytic subunit of the phosphoinositide 3-kinase (PI3K) {Jiang et al., 2009; Yuan et al., 2008} or the signaling molecule B-Raf, which is mutated in up to 70% of human melanomas at position Val600 {Davies et al., 2010}. Cellular proliferative signaling is also affected by negative feedback mechanisms which are essential for the maintenance of homeostasis. These negative feedback loops are often compromised in tumor signaling. Loss of phosphatase and tensin homolog (PTEN) function, for example, causes continuous activation of the PI3K–AKT molecular pathway and contributes to the deregulation of normal cellular growth and tumor formation {Jiang et al., 2009; Yuan et al., 2008}. Cancer cells may additionally sustain their proliferative phenotype by evading cellular checkpoint proteins involved in cell cycle progression and regulation as well as by counteracting the apoptotic machinery. These mechanisms would otherwise protect the body from highly mutated and malignant cells {Burkhart et al., 2008; Deshpande et al., 2005; Sherr et al., 2002}.

Carcinoma cells can acquire an invasive phenotype which is defined by the ability of the tumor cell to overcome contact inhibition. A usually required step is the loss of expression of

the adhesion molecule E-cadherin {Berx et al., 2009; Cavallaro et al., 2004}. The underlying regulatory program is called epithelial to mesenchymal transition (EMT) {Klymkowsky et al., 2009; Polyak et al., 2009; Thiery et al., 2009; Yilmaz ez al., 2009; Barrallo-Gimeno et al., 2005}. This process is triggered by different transcription factors which are involved, among others, in the expression of matrix-degrading enzymes and enhanced motility {Micalizzi et al., 2010; Taube et al., 2010; Yang et al., 2008}. The tumor microenvironment also plays an active role during metastasis induction, for instance by stimulating the tumor cells to express such transcription regulators {Karnoub et al., 2006-2007; Brabletz et al., 2001} and by supplying soluble factors under the direct stimulation of cancer cells {Qian et al., 2010; Karnoub et al., 2007; Wyckoff et al., 2007}.

Other tumor growth promoting mechanisms include the reactivation of quiescent vasculature which is essential for provision of nutrients and oxygen to the growing neoplastic tissue {Hanahan et al., 1996}. This can be driven by oncogenes responsible for the up-regulation of angiogenic factors as well as by the tumor microenvironment. The latter plays a pivotal role in tumor progression by supporting tumor growth and invasion, protecting the tumor from host immunity, fostering therapeutic resistance, and providing niches for dormant metastases to thrive {Swartz et al., 2012}.

Overall, this plethora of mechanisms involved in cancer formation and progression has made therapeutic interventions against tumors a highly challenging task, which has so far not lead to a cure for cancer.

1.2 Cancer drug treatments: molecular targeted therapies

Therapeutic intervention in cancer therapy includes surgery, chemotherapy and radio-therapy. However, thanks to the improvement in the knowledge of cancer biology, molecular targeted therapies have rapidly emerged in the last decades and are now complementing the classical pillars of tumor treatments {Cepero et al., 2010; Imai et al., 2006}. These are based on drugs which specifically target molecules responsible for tumor formation or progression. Such a therapeutic strategy is applicable when a molecular target is known to be expressed and altered in a specific tumor type, or when it has been demonstrated that its inhibition can efficiently reduce cancer growth, without affecting the physiology of normal tissues {Cepero et al., 2010}. Mutations, amplification, or over-expression of the target molecule are usually the parameters used to select the tumor indication and the patient population which may respond to the targeted treatment.

The concept and clinical adoption of targeted therapies is complicated by the notion that tumors are highly heterogeneous. In fact, many different factors are involved in cancer progression, from the alteration of normal cell growth and survival to the interaction between tumor cells and the surrounding environment. Due to this complexity, during the early days of the discovery and application of targeted therapies, oncogenes regulating cellular proliferation and anti-apoptotic pathways have been the most addressed targets. The majority of them include membrane-embedded receptor tyrosine kinase proteins (RTKs) {Carlomagno et al., 2005; Zwick et al., 2002; Robinson et al., 2000}. This class of protein receptors can be amplified, over-expressed or mutated, becoming capable of triggering proliferative signals inside the cells in a ligand dependent or independent fashion.

Drugs already approved or in development for the blockade of RTKs are either monoclonal antibodies (mAbs) or small molecules kinase inhibitors. Both have distinct mechanisms of action: mAbs can bind and block the activity of surface receptors or secreted molecules; small molecules, in contrast, can easily permeate the cell membrane and inhibit the activity of the intracellular protein kinases involved in cellular growth and proliferation {Zhang et al., 2009; see Figure 2}. The latter are generally ATP competing enzyme blockers. They act by binding to the ATP binding site of the target kinase molecule's catalytic domain, thus inhibiting its enzymatic activity. Since the catalytic domains of protein kinases are highly conserved, these drugs are often multi-specific and able to block multiple targets. Allosteric protein kinase inhibitors, instead, bind to other regions of the target molecules and are specific for unique targets {Imai et al., 2006}.

To cite some examples, the antibody cetuximab (Erbitux) binds and blocks the activity of the epidermal growth factor receptor (EGFR) on the surface of cancer cells, by competitively inhibiting binding to the endogenous ligands epidermal growth factor (EGF) and transforming growth factor-alpha (TGFα). This results in inhibition of cell proliferation, enhanced apoptosis, and reduced angiogenesis, invasiveness and metastasis. Additionally, binding of cetuximab to EGFR induces internalization of the antibody-receptor complex, contributing to the down-regulation of EGFR expression {Harding et al., 2005}. Cetuximab is approved for the treatment of patients with metastatic colorectal cancer and head and neck squamous-cell carcinoma in combination with conventional therapy. Another example is the monovalent (one-armed) monoclonal antibody Onartuzumab (MetMAb) which inhibits the hepatocyte growth factor (HGF) receptor Met. MetMab binds to the Sema domain (e.g. structural domain of semaphorins) of Met, which is located in the extracellular portion of the receptor and is responsible for the interaction with its ligand {Kong-Beltran et al., 2004}. By

blocking the ligand-induced Met-dimerization and activation of the intracellular kinase domain, this therapeutic antibody prevents Met-driven tumor cell growth and metastasis {Martens et al., 2006}. Among the small molecules, erlotinib (Tarceva) is an EGFR specific protein kinase inhibitor approved for the treatment of patients affected by non-small cell lung cancer (NSCLC) and pancreatic cancer {Shepherd et al., 2005}. Erlotinib binds in a reversible fashion to the ATP binding site of EGFR thus inhibiting trans-phosphorylation of the receptor homodimers which is required for signal transmission to the downstream pathway.

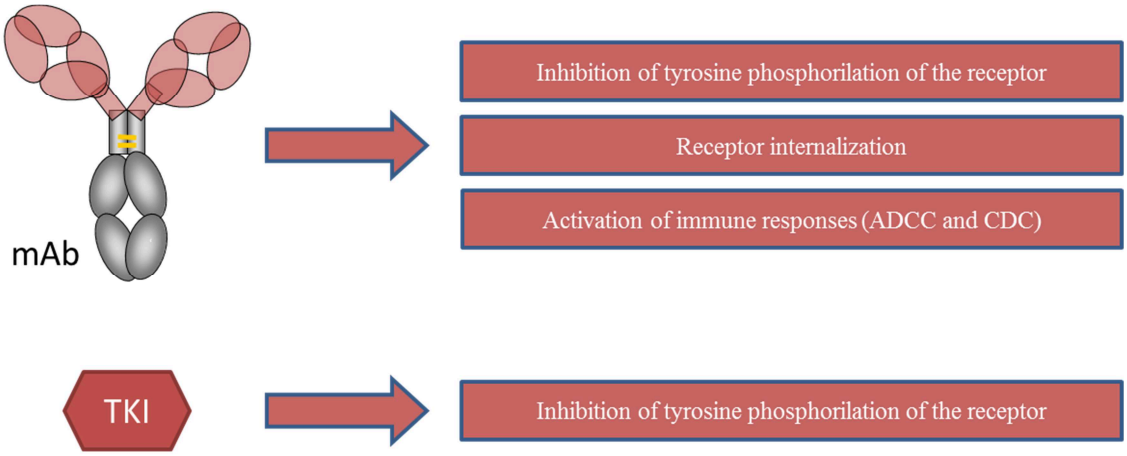

Figure 2. Schematic representation of distinct mechanisms of a therapeutic antibody and a small-molecule inhibitor for targeting a receptor tyrosine kinase protein - such as EGFR - in cancer cells {the scheme is modified from the publication by Imai et al., 2006}. TKI: small-molecule tyrosine kinase inhibitor (example: erlotinib); mAb: therapeutic monoclonal antibody (example: cetuximab).

These examples illustrate the potential of targeted therapies but also underline their limitations, since a pre-requisite for the success of such therapies is an in-depth knowledge of the tumor type and its underlying molecular mechanisms. However, both solid and hematological tumors rely on more than one oncogene during the course of their development and thus a single, targeted treatment may prove insufficient. 'Inherent' resistance is often due to the constitutive activation of downstream pathways, which circumvent the upstream

receptor blockade by the therapeutic inhibitor. In colorectal cancer, for example, mutations in K-Ras (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), which are present in about 40% of colon tumors, play a profound role in the intrinsic resistance to cetuximab {Weickhardt et al., 2010}. In addition, tumors frequently develop 'acquired' resistance mechanisms, which allow them to escape from targeted treatments. Possible acquired resistance mechanisms include up-regulation and signaling via other RTKs, or additional aberrations of the target, as the secondary mutation T790M in EGFR, which has been correlated to the clinical resistance to erlotinib in NSCLC patients {Engelman et al., 2008}. Nevertheless, the basis of tumor resistance is still poorly understood {Scott et al., 2012; Pillay et al., 2009}.

1.3 The biology of the receptor tyrosine kinases EGFR and Met: two molecular targets for anti-cancer drugs

Among the different known RTKs, EGFR and Met receptors are relevant proteins playing a role in epithelial tumor formation. For this reason, they are both well described targets for targeted therapeutics.

1.3.1 The epidermal growth factor receptor (EGFR/HER1) a member of the HER family of receptor tyrosine kinases

EGFR is the first discovered member of the HER family of receptor tyrosine kinases, which additionally includes HER2 (ErbB2), HER3 (ErbB3) and HER4 {Yarden, 2001; Hynes et al., 2009}. It is a single-pass, type I trans-membrane protein, composed by an N-terminal extracellular ligand binding domain (ectodomain), a hydrophobic region which resides in the

plasma membrane and a C-terminal cytoplasmic domain responsible for the catalytic activity of the receptor (Figure 3).

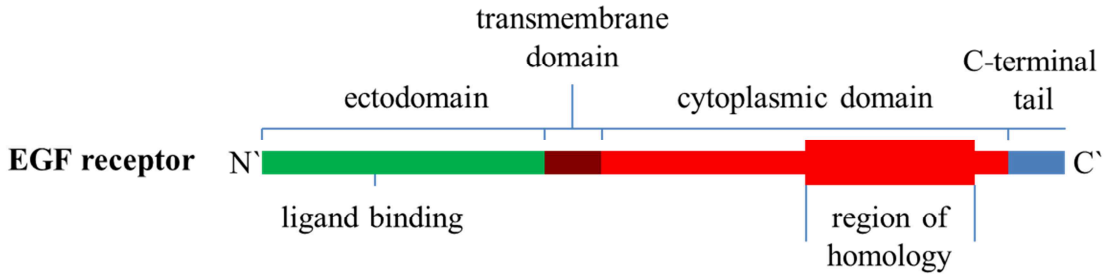

Figure 3. Schematic representation of the EGF receptor structure (modified from The Biology of Cancer; RA Weinberg; Chapter 5; 2007).

Several ligands have been described which may lead to homo- or hetero-dimerization of the HER receptors. With respect to EGFR, following EGF binding to the ectodomain, the monomeric receptor homo-dimerizes with another receptor partner. The two molecules phosphorylate each other in the intracellular portion called activation loop which normally prevents the activity of the catalytic domain. This 'trans-phosphorylation' induces a conformational change in the homo-dimers which allows the kinase domain to become active and phosphorylate its intracellular substrate molecules. Tyrosine residues in the C-terminal tail of the receptor are thereby phosphorylated and serve as docking site for adapter proteins which stimulate downstream signal transduction cascades. Downstream targets include the mitogen-activated protein kinase (MAPK) pathway or the PI3K pathway, which induce expression of genes ultimately responsible for cell proliferation, survival and migration. Internalization via endocytosis of the receptor-ligand complex is the physiologic mechanism of EGFR signal inactivation {Ullrich et al., 1990; Wells, 1999}.

Constitutive EGFR signaling plays a role in tumor biology by promoting survival and proliferation of tumor cells. This is achieved by specific genetic changes which have been reported in lung, breast, colon, head and neck cancer, as well as in glioblastoma multiforme {Holbro et al., 2004; Pines et al., 2010}. These genetic alterations include activating mutations in the kinase domain (i.e. exon 18-21), gene amplification leading to protein over-expression and deletions in the extracellular domain {Shigematsu et al., 2006; Sharma et al., 2009; Jorissen et al., 2003}. Approved EGFR-inhibitors for cancer treatment include two monoclonal antibodies (cetuximab and panitumumab), and the two small molecular weight compounds gefitinib and erlotinib. Gefitinib and erlotinib have been found to be particularly efficacious in lung cancer patients bearing tumors characterized by mutated forms of EGFR {Nedergaard et al., 2012; Johnson et al., 2005}.

HER receptors have been targets for pharmacological intervention for many years in cancer research. Their role in tumor progression has been thoroughly dissected together with some of the underlying hypotheses of molecular resistance and tumor escape occurring when HER receptors are inhibited. As mentioned before, escape mechanisms of cancer cells developing in response to inhibition of a specific signaling node are often the cause of the limited efficacy of targeted single agent therapies {Alexander et al., 2012}. Understanding both acquired as well as inherent resistance mechanisms in tumor biology is crucial for future rational combination therapies.

1.3.2 The HGF receptor Met and its role in tumor resistance to EGFR inhibition

The receptor Met belongs to the HGF family of receptor tyrosine kinases together with Ron (recepteur d'origine nantais) and Sea. Although Met and Ron are activated by different

ligands, they share a common structure and are both involved in processes such as cell dissociation and motility {Comoglio et al., 1996}. Met and its ligand HGF are important mediators of tumor growth, survival, and metastasis {Trusolino et al., 2010; Gherardi et al., 2012; Birchmeier et al., 2003}. Stimulated by HGF, Met triggers activation of several downstream signaling molecules responsible for proliferation, cell cycle progression, migration and invasion.

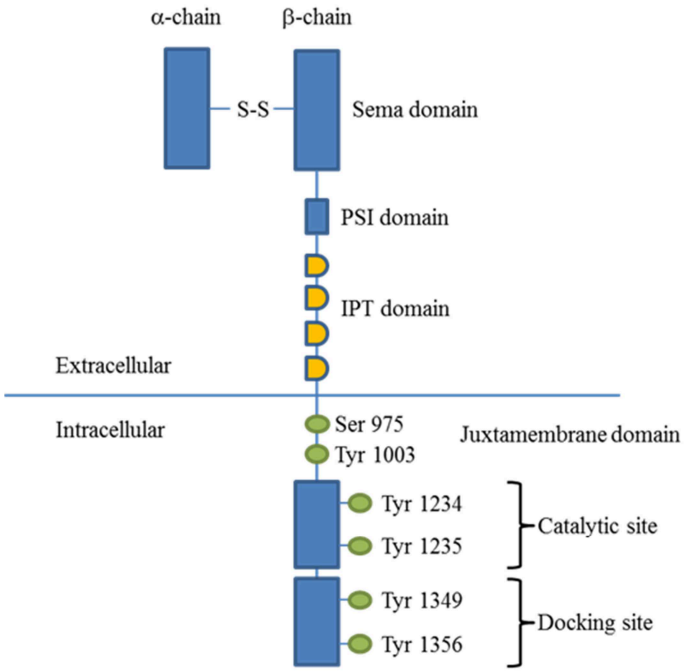

Figure 4. Schematic representation of the HGF receptor Met {modified from Jung et al., 2012}.

The mature Met receptor is a single-pass, type I disulphide-linked heterodimer trans-membrane protein consisting of a 45 kDa extracellular α-chain and the membrane spanning 140 kDa β-chain. The β-subunit, which is also required for HGF binding, additionally comprises a trans-membrane region and the cytoplasmic domain where the catalytic ATP binding site and a C-terminal tail are located (Figure 4). The phospho-tyrosine residues of the C-terminal docking site interact with signaling adapter proteins such as the growth factor

receptor bound protein 2 (Grb2) and Grb2 associated binding protein 1 (Gab1) {Trusolino et al., 2010; Ponzetto et al., 1994; Weidner et al., 1996}. Phosphorylation of Gab1 induces among others the recruitment of the PI3K, which can also bind the receptor directly via its regulatory subunit p85. A negative regulator domain of Met, which includes a specific phospho-tyrosine residue in the juxtamembrane domain, is responsible for Met degradation and signal inactivation through the binding to the E3 Ubiquitin ligase Casitas B-lineage lymphoma (Cbl) {Jeffers et al., 1997}.

Epithelial cells respond to Met activation by scattering, increased motility and by undergoing EMT {Weidner et al., 1990}. Both the MAPK and the PI3K pathways have been found to be involved in the motility phenotype driven by Met {Potempa et al., 1998} while survival is mainly dictated by the PI3K pathway via AKT {Xiao et al., 2001}. The expression of Met in healthy adult tissues is very low under physiological conditions. Its activation in cancer is often times HGF-dependent but it may also occur in a ligand-independent way, if the receptor is over-expressed on the surface of tumor cells. Known genetic changes which involve Met in cancer progression include duplication of a mutant Met allele {Zhuang et al., 1998}, gene amplification, structural rearrangement and somatic mutations which have been found in different human cancers {Ma et al., 2008}. As for EGFR, a multitude of inhibitors, both low molecular weight compounds and monoclonal antibodies directed against Met, e.g. tivantinib (ARQ-197) or onartuzumab (MetMAb), are currently tested in clinical trials {Yap et al., 2011; Jung et al., 2012}.

Met interacts with several other membrane proteins, such as EGFR {Guo et al., 2008; Hammond et al., 2010}. A cross-talk between EGFR and Met in transformed cells has been reported {Jo et al., 2000}. Furthermore, Met gene amplification has been demonstrated to

contribute to the EGFR inhibitor-mediated acquired resistance observed in approximately 10% of NSCLC patients, presumably achieved through the PI3K pathway {Engelman et al., 2007; Bean et al., 2007; Yamada et al., 2010; Engelman et al., 2008}. Recently, other studies have demonstrated that HGF-dependent activation of Met can induce EGFR tyrosine kinase inhibitors (TKI) resistance by restoring downstream MAPK and PI3K/AKT signaling {Okamoto et al., 2010; Donev et al., 2011}. In this context, transient inhibition of PI3K/AKT pathway by the PI3K inhibitor PI-103 and gefitinib has been shown to overcome this phenomenon by inducing apoptosis in EGFR mutant lung cancer cells {Donev et al., 2011}. The combination of Met and EGFR inhibitors is currently under pre-clinical and clinical evaluation. Co-treatment of erlotinib and SGX523 (a small molecule inhibitor of Met) has been tested in transgenic mice expressing human HGF and has been found to be more efficacious than the administration of either single agent {Zhang et al., 2010}. In a recent study, the simultaneous administration of WZ4002, a mutant EGFR-TKI and E7050, a mutant selective dual inhibitor of Met and vascular endothelial growth factor receptor -2 (VEGFR-2) was shown to inhibit tumor growth in erlotinib resistant NSCLC cells *in vitro* and *in vivo*. This combination resulted in the successful inhibition of EGFR, Met, and the downstream PI3K-AKT pathway {Nakagawa et al., 2012}. In light of these preclinical data, early stage clinical trials combining EGFR and Met inhibitors are underway.

The biology and cross-talk of HER family members with other RTKs (which can mediate clinically-relevant resistance) constitute a fundamental field of research aimed at generating novel anti-cancer compounds with increased clinical efficacy. EGFR and Met have been further characterized in this thesis for their pivotal role in cancer biology and resistance to targeted treatments. We have exploited the possibility to modulate these targets by means of antibody engineering technology.

1.4 Antibodies as drugs

Antibodies are long established in clinical practice with more than 25 monoclonal antibodies currently approved by the FDA {An, 2010; Trikha et al., 2002; Adams et al., 2005}, half of which have been developed for the treatment of cancer {Nieri et al., 2009, Carter, 2001}. A therapeutic mAb is an IgG molecule, which in contrast to the native antibody repertoire, recognizes a self-antigen. The majority of approved mAbs belong to the IgG_1 subclass characterized by a long half-life and potent molecule-associated effector functions (i.e. antibody-dependent cellular cytotoxicity, ADCC). A regular IgG contains two light chains (composed of one variable and one constant region) and two heavy chains (each composed of one variable and three constant regions). Complementarity-determining regions (CDRs) are responsible for antigen recognition and reside in the variable fragment (Fv) portion of the antigen-binding fragment (Fab). A therapeutic monoclonal antibody can contain CDRs which bind with high affinity to a tumor–associated target or molecule involved in tumor formation and progression {Beck et al., 2010}.

Over the last years, antibody engineers have become more and more proficient in generating and improving the features of such molecules. The field has grown from the use of conventional hybridoma technology to produce murine monoclonal antibodies to the application of genetic engineering techniques to generate chimeric antibodies (containing mouse antigen-binding variable regions joined to human constant domains). A further improvement in minimizing immunogenicity to the antibody consisted in producing humanized antibodies in which the non-human CDRs constitute the only rodent sequences and are ‘grafted’ into a human IgG {Nieri et al., 2009; Carter 2001}. Generation of fully human antibodies is now possible by phage display (to screen libraries for human antibody

binding domains with high affinity to specific antigens) {Hoogenboom et al., 1998} as well as by transgenic technology {Brueggemann et al., 1997}.

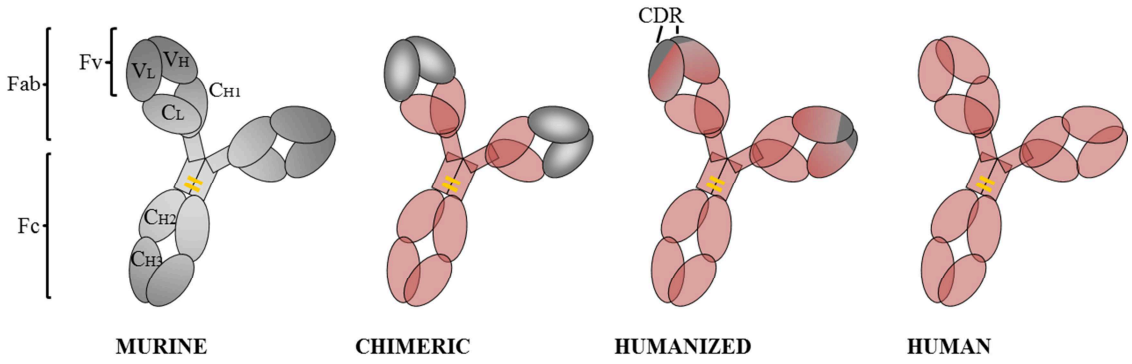

Figure 5. Schematic representation of murine, chimeric, humanized and human IgG {modified from Imai et al., 2006}.

The modes of action of a monoclonal antibody generated as cancer therapeutic include inhibition of target RTKs, {Izumi et al., 2002; Harding et al., 2005}, apoptosis induction, pay-load delivery {Hudson et al., 2003}, or immune effector cell activation (i.e. ADCC). Nevertheless, as discussed above, inhibition of an oncogenic driver protein by a mono-specific therapeutic antibody often results in rapid emergence of resistance, rendering the treatment ineffective {Pillay et al., 2009}. Therefore, it is not surprising that combinations of more than one antibody {Scheuer et al., 2009} or generation of alternative antibody scaffolds designed at targeting simultaneously more than one antigen are under evaluation.

1.4.1 Bi- and multi–specific antibodies

A native IgG is bivalent and monospecific because it contains two identical Fabs, both recognizing the same antigen. Antibodies in bi- or multi-specific formats can be generated to bind two or more different epitopes (usually on distinct antigens) within a single molecule. Depending on their design, multi-specific antibodies can be monovalent or bivalent for each

of the targets, if they are engineered to recognize their specific antigens with one or with two separate binding arms.

Bi-specificity can be simply achieved by engineering molecules whereby the two binding arms can simultaneously recognize different antigens, with a structure and molecular weight resembling the ones of a regular IgG {Bostrom et al., 2009}. However, with respect to the structural properties and possible formats, a variety of bispecific constructs have been described in the past (Kontermann, 2010), produced by different technologies. The strategy used for the generation of bi- and multi-specific antibodies in this thesis is based on the "knobs into holes" approach {Ridgway et al., 1996; Carter, 2001; Merchant et al., 1998}, combined with single chain Fabs (ScFabs) and single chain Fvs (ScFvs) fusions at the N- and C- terminal of the IgG molecule. While developing a bispecific antibody in an IgG format constituted by two different heavy chains, it is essential to avoid generation of chain mispairing contaminants. A good approach consists in engineering the C_{H3} domain (the main part of the Fc domain responsible for the heavy chains interaction) in order to force hetero-dimerization and reduce the formation of homo-dimers. The knobs into holes technique consists in replacing a small amino acid with a larger one in one of the C_{H3} domains ('knob') and simultaneously introducing smaller amino acids ('hole') into the C_{H3} domain of the second heavy-chain. By doing this, the residues of the C_{H3} of one chain can easily accommodate into the other one. The resulting hetero-dimeric Fc part can be further stabilized by artificially introduced disulfide bridges. Antibodies designed with this approach retain their effector functions {Carter, 2001} and a potentially extended half-life. In addition, since these mutations are hidden in the antibody structure, they are not expected to induce immunogenicity once such molecules are administered to patients. To increase the number of specificities, entities specific for additional targets can be fused at the N- or C-termini of the

antibody (single-chain disulfide stabilized Fvs or Fabs) {Metz et al., 2011; Schanzer et al., 2011; Croasdale et al., 2012}. The use of ScFabs and ScFvs fusions additionally solves the problem of light chain mispairing. Exemplary schematic representations of the knobs into holes mutations, an N-terminal ScFab and a C-terminal ScFv fusion introduced to avoid light chain mispairing and increase specificities are shown are Figure 6.

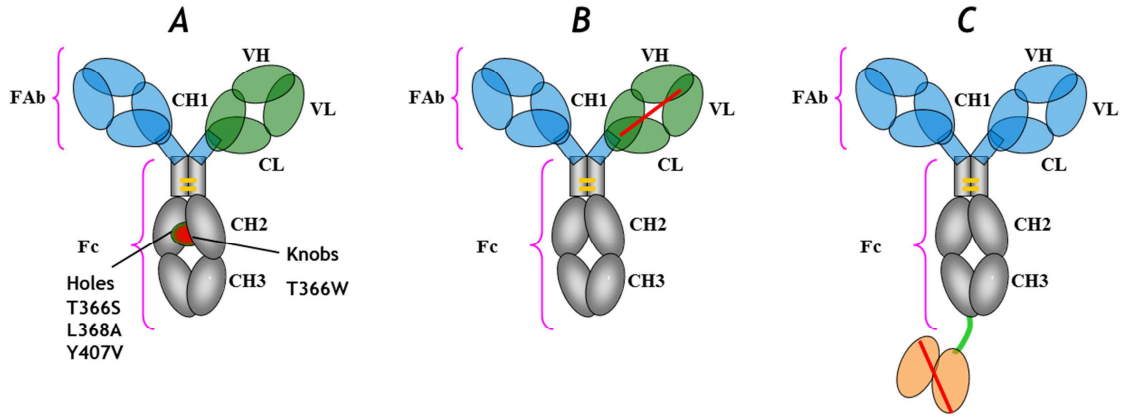

Figure 6. A): Knobs into holes mutations. B): N-terminal ScFab (VL-CL-Linker -VH-CH1). C): C-terminal ScFv fusion (VH-Linker-VL).

Binding of bi- or multi-specific antibodies to their antigens depends on biochemical properties (i.e. affinity and avidity) as well as on the relative expression of the targets on the tumor cells. By blocking two or more pathways at the same time, multi-specific antibodies may provide potential synergistic effects compared to the combinations of single agents. The simultaneous targeting of at least two molecules may in fact improve their binding characteristics. Besides the relative affinity to the single targets which plays an essential role, once the first arm is bound to the respective antigen, the other arm/s is/are brought in close proximity to the plasma membrane. This condition favors a much faster interaction to the second/additional antigens. This phenomenon is called avidity {Dower et al., 1981 1 and 2} and represents a clear advantage of a multi-specific antibody versus the combination of single

agents. It can occur when one of the antigens, which is expressed at higher level, recruits the antibody to the cell surface, resulting in increased potency of the antibody itself towards the less expressed targets {Fitzgerald et al., 2011}. Since most of the targets are not 'tumor-specific' but rather 'tumor associated', monovalent binding of certain multi-specific antibodies would additionally preserve the healthy cells which may express the targets at lower density from potential side effects of the therapeutic antibody targeting {Marvin et al., 2006}. Avidity can also be influenced by the number of valencies of the antibody for the respective antigen (i.e. an antibody binding an antigen with two arms will show a higher potency compared to monovalent binding). At the same time, by bringing different receptor tyrosine kinases in close proximity on the surface of a tumor cell, a multi-specific antibody may show unwanted agonistic activity. For therapeutic applications, the selection of an appropriate targeting moiety is usually directed by the underlying biology, i.e. the expression of the targets of interest on the tumor cells. In the case of a multi-specific molecule, this should be combined with a deep biochemical characterization of the lead compound {Filpula, 2007; Mansi et al., 2010} with a precise analysis of the antigen binding properties since these may strongly influence clinical efficacy.

Only antibodies in bispecific formats are now emerging as effective therapeutics {Chames et al., 2009; Thakur et al., 2010}: some of these, (such as MM-111, targeting HER2/ErbB3 heterodimers, and MEHD7945A, targeting EGFR/ErbB3 heterodimers) are in development for the treatment of diseases where HER-receptors dimerization is proposed to play a central role {Schaefer et al., 2011; Nielsen et al., 2008}. There is currently only one approved bispecific antibody: the EpCAMxCD3 mouse-rat chimeric catumaxomab, for the treatment of malignant ascites {Linke et al., 2010; Seimetz et al., 2010; Ströhlein et al., 2010}. Until recently, only bispecific effector cell recruiters entered clinical investigations. However,

compounds targeting angiogenic factors (VEGF and Angiopoietin 2) or RTKs, as the ones mentioned above, are currently under investigation {McDonagh et al., 2012; Doppalapudi et al., 2010}.

Based on the currently known resistance mechanisms in HER signaling, namely activation of other RTKs such as Met and IGF1R (insulin-like growth factor 1 receptor) {Hynes et al., 2005}, this thesis has been dedicated to the characterization of novel bi- and multi-specific antibodies which are either mono-, or bivalent for some of these targets. Simultaneous binding to all antigens, avidity properties, ability to inhibit targets and downstream molecules phosphorylation, as well as invasion and tumor cell growth both *in vitro* and *in vivo* were investigated to evaluate the feasibility of generating such molecules for future applications as anti-cancer agents in various therapeutic areas.

1.5 Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
ATP	Adenosine-5'-triphosphate
Cbl	Casitas B-lineage lymphoma
CD3	Cluster of differentiation 3
CDC	Complement-dependent cytotoxicity
CDRs	Complementarity-determining regions
C_H	Constant heavy
DAF	Dual action Fab
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
Fab	Fragment, antigen binding
Fc region	Fragment crystallizable region
FDA	US Food and drug administration
Fv	Fragment, variable
Gab1	Grb2 associated binding protein 1
Grb2	Growth factor receptor bound protein 2
HER	Human epidermal growth factor receptor
HGF	Hepatocyte growth factor
IGF1R	Insulin-like growth factor 1 receptor
IgG	Immunoglobulin G
kDa	Kilo-Dalton
K-Ras	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
NSCLC	Non-small cell lung cancer
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homolog
RB	Retinoblastoma protein
Ron	Recepteur d'origine nantais

RTK	Receptor tyrosine kinase protein
ScFab	Single chain Fab fragment
ScFv	Single chain variable fragment
TGF-α	Transforming growth factor-alpha
TKI	Tyrosine kinase inhibitor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

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2. Summary of the presented publications

Cancer is a complex and heterogeneous disease characterized by specific tumor and individual genotypic and phenotypic alterations which accumulate during its development, treatment and progression. The importance of linking an associated tumor antigen with a targeted molecular treatment is becoming more and more evident and necessary from a clinical perspective. However, tumors are frequently hyper-mutated, driven by multiple molecular aberrations and adopt differentially defined escape mechanisms during the course of treatment. Such mechanisms are often based on modulation of one or more alternative molecular targets or pathways, alone or simultaneously.

In the first paper, we studied and dissected in detail a mechanism whereby epithelial tumors escape targeted therapies: the cross-talk of receptor tyrosine kinases on the membrane surface of tumor cells, in this particular case the hepatocyte growth factor (HGF) receptor - Met - and the epidermal growth factor (EGF) receptor EGFR. We observed that treatment with EGFR inhibitors of various tumor cells stimulated with HGF and EGF, results in transient up-regulation of phosphorylated AKT accompanied by a pro-invasive phenotype. Additionally, co-treatment with an AKT inhibitor strongly reverts the invasive phenotype, suggesting a connection between signaling and functional data. These findings imply that during treatment of tumors a balanced ratio of EGFR and Met inhibition is required to counteract both pathways. Hence, we employed antibody engineering technology to address the observed resistance phenomenon by designing a therapeutic compound targeting EGFR and Met (MetHer1). The bispecific antibody MetHer1 proved to inhibit tumor cell proliferation and displayed high potency in a migration assay. We showed that the bispecific construct suppresses invasive growth when both Met and EGFR pathways are active, in contrast to

treatment with the EGFR inhibitors alone. In an *in vivo* experiment MetHer1 bispecific antibody potently inhibits tumor growth in a non-small cell lung cancer xenograft model. These findings support a combination treatment with EGFR and Met inhibitors and further evaluation of clinically–relevant resistance mechanisms to EGFR inhibition in the context of an active ligand-dependent Met signaling.

To go beyond bi-specificity, given that tumors may escape therapies by simultaneous up-regulation of multiple targets, in the second paper we demonstrated the feasibility to generate multi-specific (i.e. tri-specific) antibodies for cancer therapy. By surface plasmon resonance techniques, we showed antigen-binding kinetic profiles comparable to the parental antibodies and proved that simultaneous binding to three large extracellular domains is feasible. By performing cellular and chip surface competition experiments we proved that the molecules display avidity and retain their effector cell recruitment potential. Agonistic activity might be expected from bringing different receptor tyrosine kinases in close proximity on the surface of a tumor cell. Therefore, we verified that the newly generated multi-specific molecules are devoid of this effect by testing their inhibitory profile both on the targets as well as on tumor cell proliferation. We hypothesize that administration of such molecules to patients – whose tumors would first need to be molecularly classified according to the expression of the respective targets - may result in effective therapy.

This thesis demonstrates that generation and application of multi-target specific therapeutic antibodies – combined with in-depth biological understanding of the molecular features that grant tumor resistance to current targeted therapeutics - represent a powerful venue towards the discovery and development of novel anti-cancer drugs. Such biological drugs would

potently inhibit tumors and prevent resistance by addressing disease-associated *de novo* and acquired escape mechanisms.

3. Zusammenfassung der präsentierten Arbeiten

Krebs ist eine komplexe und heterogene Erkrankung, die durch tumorspezifische aber auch individuell vererbte genotypische Veränderungen charakterisiert wird. Aus klinischer Sicht ist eine Behandlung, welche nur den Tumor trifft, wünschenswert, um den therapeutischen Effekt zu maximieren. Dies wird über assoziierte Tumorantigene als Zielstrukturen für eine gerichtete molekulare Behandlung erreicht. Allerdings sind Tumore, bedingt durch die zahlreichen molekularen Aberrationen, vergleichsweise leicht in der Lage, einer gerichteten Behandlung, durch Nutzung alternativer Signaltransduktionswege, auszuweichen (*escape*-Mechanismus).

In der ersten Publikation haben wir einen solchen *escape*-Mechanismus am Beispiel der Therapie von epithelialen Tumorzellen detailliert untersucht: den sogenannten *cross-talk* von Rezeptor-Tyrosin-Kinasen an der Membranoberfläche von Tumorzellen. Konkret wurde dies für den Hepatozyten-Wachstumsfaktor (HGF)-Rezeptor Met und den epidermalen Wachstumsfaktor (EGF)-Rezeptor EGFR betrachtet. Eine Behandlung von unterschiedlichen, mit HGF und EGF stimulierten, Tumorzellen mit EGFR-Inhibitoren induziert eine transiente Erhöhung von phosphoryliertem AKT über das Maß hinaus, welches mit den Wachstumsfaktoren allein beobachtet wird. Dies geht einher mit einem pro-invasiven Phänotyp. Die gleichzeitige Behandlung mit einem AKT-Hemmer kehrt den invasiven Phänotyp um, was auf eine Verbindung zwischen Signal- und funktionellen Eigenschaften hinweist. Diese Ergebnisse deuten darauf hin, dass bei einer Tumortherapie ein ausgewogenes Verhältnis von EGFR und Met Hemmung erforderlich ist, um beide Signalwege zu inhibieren. Ob jedoch Gabe von EGFR-Inhibitoren in der Gegenwart von aktivem Met Signalweg auch klinisch nachteilig ist, kann mit den erhobenen Daten nicht

geklärt werden. Um eine simultane Inhibition zu erreichen, wurde ein bispezifischer Antikörper, welcher EGFR und Met gleichzeitig binden kann (MetHer1), hergestellt. Dieser bi-spezifische Antikörper MetHer1 war in der Lage, die Proliferation der Tumorzellen zu hemmen und zeigte eine hohe Wirksamkeit in einem *in vitro* Migrations-Assay. Wir konnten ebenfalls zeigen, dass der bi-spezifische Antikörper invasives Wachstum unterdrückt, wenn sowohl der Met als auch der EGFR Signalweg aktiv sind, wohingegen EGFR-Inhibitoren allein dazu nicht in der Lage sind. In einem nicht-kleinzelligen Lungenkarzinom-Xenotransplantat-Modell zeigte der bi-spezifische MetHer1 Antikörper eine starke Inhibition des Tumorwachstums. Diese Ergebnisse unterstützen die Kombination von EGFR- und Met-Hemmer sowie weitere Untersuchungen zu klinisch relevanten Resistenzmechanismen gegen EGFR-Hemmung im Kontext eines aktiven Met-abhängigen Signalweges.

Da Krebszellen einer gerichteten Therapie durch gleichzeitige Hochregulation mehrerer Signalwege entkommen können, haben wir die Generierung und den Einsatz multi-spezifischer (z.B. tri-spezifischer) Antikörper für die Krebstherapie in der zweiten Publikation evaluiert. Durch Plasmonresonanz (SPR)-Spektroskopie konnten wir zeigen, dass die Bindungs-Kinetiken der multispezifischen Antikörper mit denen der monospezifischen Ursprungs-Antikörper vergleichbar sind. Außerdem wiesen wir nach, dass die gleichzeitige Bindung von drei großen extrazellulären Protein-Domänen möglich ist. Durch zelluläre und Chip-Oberflächen Kompetitions-Experimente konnten wir zeigen, dass die hergestellten Moleküle eine entsprechende Avidität zeigen, und ihre Fähigkeit Immunzellen zu rekrutieren, beibehalten haben. Durch die induzierte räumliche Nähe unterschiedlicher Rezeptor-Tyrosin-Kinasen auf der Oberfläche einer Tumorzelle wäre eine agonistische Aktivität zu erwarten gewesen. Daher wurde in Proliferations-Assays ausgeschlossen, dass die hergestellten multi-spezifischen Moleküle einen agonistischen Effekte besitzen .Wir glauben daher, dass die

klinische Verabreichung von tri-spezifischen Antikörpern eine wirksame Therapie besonders bei jenen Patienten darstellen kann, deren Tumoren eines oder mehrere der Antigene exprimieren.

Diese Arbeit belegt, dass die Herstellung und *in vitro* Charakterisierung von multi-spezifischen therapeutischen Antikörpern, kombiniert mit dem fundierten biologischen Verständnis der molekularen *escape*-Mechanismen, eine potente Plattform für die Entwicklung und Evaluation neuartiger Krebsmedikamente darstellt. Multi-spezifische Proteinbasierte Arzneimittel haben das Potenzial, das Tumorwachstum stark zu hemmen und die Entstehung von therapieresistenten Erkrankungen zu verhindern.

4. Results (publications)

4.1 Publication: Castoldi R et al. Oncogene. 2013

A novel bispecific EGFR/Met antibody blocks tumor-promoting phenotypic effects induced by resistance to EGFR inhibition and has potent antitumor activity

Castoldi R, Ecker V, Wiehle L, Majety M, Busl-Schuller R, Asmussen M, Nopora A, Jucknischke U, Osl F, Kobold S, Scheuer W, Venturi M, Klein C, Niederfellner G, Sustmann C.

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ORIGINAL ARTICLE

A novel bispecific EGFR/Met antibody blocks tumor-promoting phenotypic effects induced by resistance to EGFR inhibition and has potent antitumor activity

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Simultaneous targeting of epidermal growth factor receptor (EGFR) and Met in cancer therapy is under pre-clinical and clinical evaluation. Here, we report the finding that treatment with EGFR inhibitors of various tumor cells, when stimulated with hepatocyte growth factor (HGF) and EGF, results in transient upregulation of phosphorylated AKT. Furthermore, EGFR inhibition in this setting stimulates a pro-invasive phenotype as assessed in Matrigel-based assays. Simultaneous treatment with AKT and EGFR inhibitors abrogates this invasive growth, hence functionally linking signaling and phenotype. This observation implies that during treatment of tumors a balanced ratio of EGFR and Met inhibition is required. To address this, we designed a bispecific antibody targeting EGFR and Met, which has the advantage of a fixed 2:1 stoichiometry. This bispecific antibody inhibits proliferation in tumor cell cultures and co-cultures with fibroblasts in an additive manner compared with treatment with both single agents. In addition, cell migration assays reveal a higher potency of the bispecific antibody in comparison with the antibodies' combination at low doses. We demonstrate that the bispecific antibody inhibits invasive growth, which is specifically observed with cetuximab. Finally, the bispecific antibody potently inhibits tumor growth in a non-small cell lung cancer xenograft model bearing a strong autocrine HGF-loop. Together, our findings strongly support a combination treatment of EGFR and Met inhibitors and further evaluation of resistance mechanisms to EGFR inhibition in the context of active Met signaling.

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Keywords: EGFR; Met; HGF; EGF; bispecific antibody

INTRODUCTION

Escape mechanisms occurring in cancer cells and which develop in response to inhibition of a specific signaling pathway often limit efficacy of targeted single-agent therapies.[1] Understanding the biology of such acquired but also intrinsic resistance mechanisms in tumors is pivotal for devising future rational combination therapies. The inhibition of a single receptor tyrosine kinase signaling presents a good example of molecular networks, which mediate tumor escape.[2] A cross-talk of epidermal growth factor receptor (EGFR) and Met in transformed cells was already described in 2000 by Strom *et al.*[3] EGFR is a member of the ErbB family of receptor tyrosine kinases consisting of EGFR (ErbB1), HER2/neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4).[4] Constitutive EGFR signaling has a role in tumor biology by promoting survival and proliferation of cancer cells. Several EGFR-specific small molecular weight inhibitors (for example, gefitinib or erlotinib) as well as antibody modulators (cetuximab or panitumumab) have been developed and are approved for clinical use.[5] Met and its ligand hepatocyte growth factor (HGF) are important mediators of tumor growth, survival and metastasis.[6,7] Similarly as for EGFR, a multitude of inhibitors, small molecules or monoclonal antibodies directed against Met (for example, tivantinib or onartuzumab (MetMAb)), are currently tested in clinical trials.[8] Increased HGF/Met signaling can limit the effect of EGFR pathway inhibition and has been linked with acquired resistance to EGFR-targeted drugs in EGFR-mutant lung tumors.[9,10] Although the incidence of acquired resistance, as observed in non-small cell lung cancer, is only about 10%, Met is considered to be a major escape route for EGFR-targeted therapies.[11,12] Not surprisingly, ErbB family members may also confer resistance to Met tyrosine kinase inhibition.[13,14] Perturbation of both receptors' activity suggests that EGFR and Met signaling nodes are highly and dynamically interconnected.[15,16] These findings are further substantiated in various cellular models and as such, may reflect a general phenomenon.[17–21] As murine HGF is only weakly cross-reactive to human Met, a combination of erlotinib and SGX523, a small molecule inhibitor of Met, was assessed in transgenic mice expressing human HGF and found to be superior to both single-agent treatment.[22] In addition, results of a combination study of erlotinib and onartuzumab strengthen the co-targeting rationale.[23] In this study, we demonstrate for the first time that, under conditions of active EGFR and Met signaling, treatment with specific EGFR inhibitors induces an increase in phosphorylated AKT and most importantly enhances the invasive properties of tumor cells. To test the hypothesis that combined inhibition of both receptor activities is required to suppress invasiveness, we generated a bispecific antibody based on the anti-EGFR antibody cetuximab and the Met-specific 5D5 antibody. The selected format

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for the bispecific antibody was that of a 2 + 1 molecule, which then allows fixed stoichiometry and consequent balanced inhibition of both receptors.

RESULTS

EGFR inhibition triggers p-AKT and induces invasion in HGF-stimulated tumor cell lines

H596 cells, on stimulation with EGF and HGF and treatment with cetuximab, displayed an increase in phospho-AKT compared with untreated stimulated cells. This effect was also observed with the two alternative EGFR inhibitors panitumumab and erlotinib (Figures 1a and b). This observation, made consistently in the background of potent stimulation by HGF and EGF, was consistent and reproducible over a large set of experiments with a mean increase of 1.62 (Figure 1c). The EGFR inhibitors did not affect phosphorylation by themselves in the absence of EGF and HGF stimulation (Supplementary Figures S1A and B). Treatment with HGF and cetuximab led to a very modest increase of phospho-AKT in comparison with HGF-treatment only (Supplementary Figure S1C). Furthermore, spatially restricted increase of AKT phosphorylation was clearly observed in the membrane proximal region of A549-stimulated cells as described for H596 cells and in the context of EGFR inhibition (Figure 1d), which might be indicative for a potential role in migration and invasion events. In order to explore possible functional consequences, we tested the effect of EGFR inhibitors in an invasion assay using HGF and EGF-stimulated cells. Experiments were performed with A431 cells, as this cell line is a good model to study motility in Matrigel chambers, it responds to cetuximab treatment with an increase in phospho-AKT when stimulated with HGF and also displays increased invasion on treatment with HGF and/or EGF (data not shown). Cetuximab treatment after stimulation with EGF and HGF increased the invasive phenotype of A431 cells in a statistically significant manner ($P<0.001$) and this effect was dose-dependently reverted by co-treatment with an AKT inhibitor (AKTi-1/2 VIII; Figures 2a and c). A similar—albeit smaller—increase in invasion was induced by panitumumab and erlotinib treatments, and it was similarly impaired by the addition of an AKT inhibitor (Figures 2b and c). The AKT inhibitor was used at 1 μM: at this concentration it abrogated Ser473 phosphorylation, which is an activation marker, and was not cytotoxic in the assay (Supplementary Figures S2A and B).

MetHer1 impairs proliferation in selected cell lines

To test the hypothesis whether the increase in phospho-AKT and the accompanying invasive phenotype, potentially mediating resistance to EGFR inhibition in the presence of HGF, could be reverted by the simultaneous inhibition of the HGF receptor Met, we generated a bispecific antibody construct capable of blocking EGFR and Met (MetHer1) (Supplementary Figures S3A–C). This was achieved by cloning the variable regions of cetuximab into an immunoglobulin G1 (IgG1) antibody backbone with a monovalent anti-Met single chain Fab similar to the one-armed 5D5 (onartuzumab) fused at the C-terminus of one of the heavy chains. Correct heavy chain hetero-dimerization was enforced using the knobs-into-holes technology.[24] The final product had a purity >98% and was able to simultaneously bind to both antigens (Supplementary Figures S3D and E), displaying binding kinetics for each antigen in the nM range, comparable to those of the parental monospecific antibodies (Supplementary Figures S4A and B). As a side-product resulting in a bispecific antibody with two single chain Fab fusions would be agonistic, the activating marker phospho-Met was monitored in the presence of MetHer1

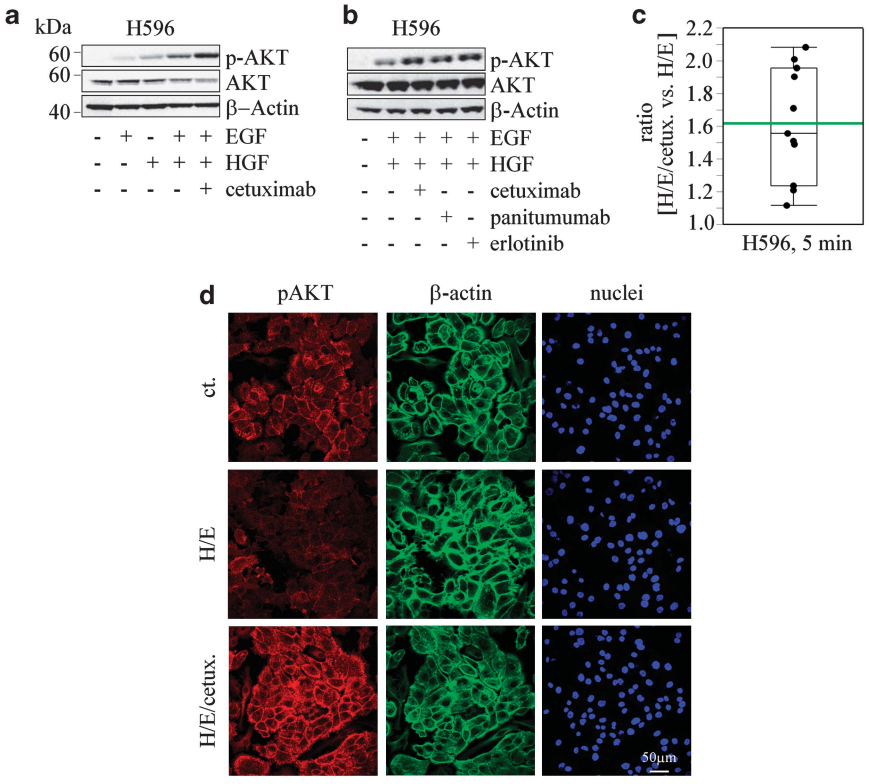

Figure 1. EGFR inhibition under EGF and HGF-stimulated conditions induces an increase in AKT phosphorylation. (**a**) AKT status in stimulated H596, in the presence or absence of cetuximab. (**b**) AKT status after treatment with cetuximab, panitumumab or erlotinib. (**c**) Box plot presentation of cetuximab-dependent pAKT stimulation. Analysis of the ratio of HGF/EGF (H/E) treatment versus H/E treatment in the presence of cetuximab (n 11 biological replicates). The box indicates 25th, 50th (median) and 75th percentiles, as well as mean (green bar). (**d**) Confocal microscopy at × 63 magnification of phospho-AKT and β-actin-stained A549 cells.

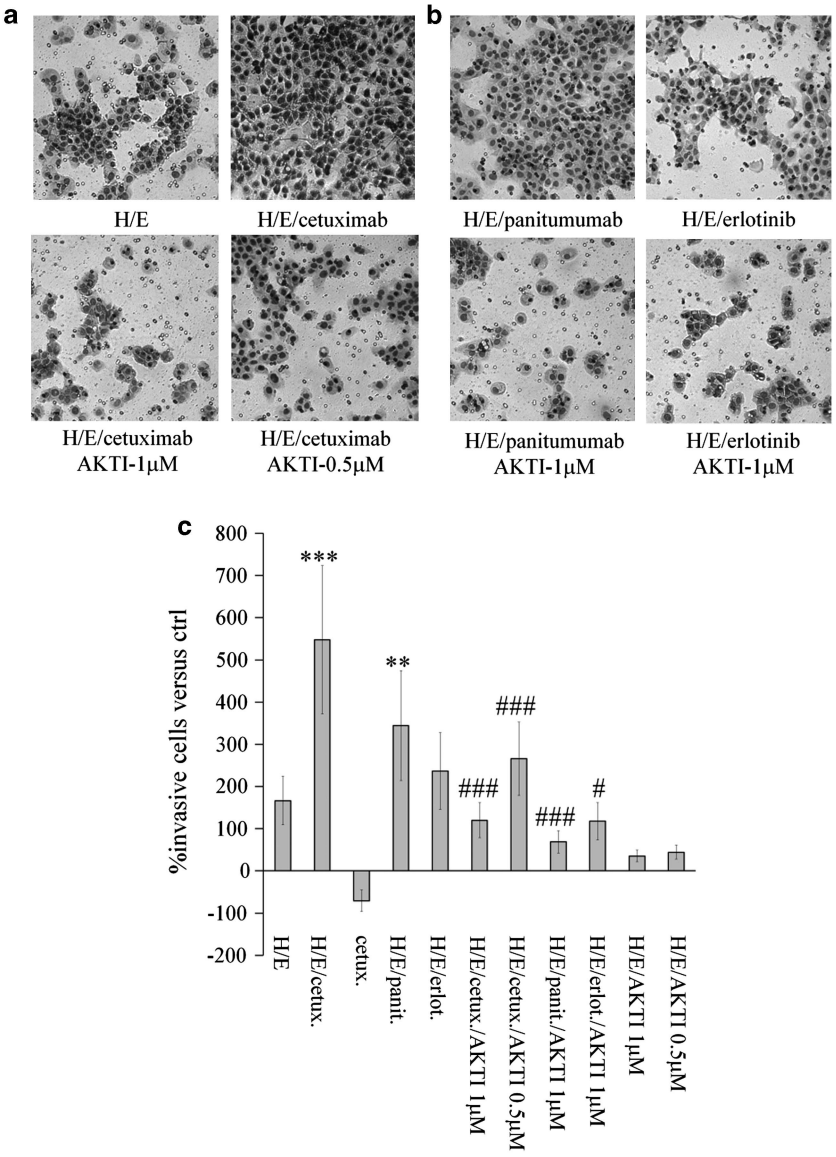

Figure 2. EGFR inhibition in the presence of HGF and EGF induces an invasive phenotype. (**a**, **b**) Invasive phenotype of HGF/EGF-stimulated A431 after treatment with cetuximab, panitumumab and erlotinib ± AKTi -1/2VIII. (**c**) Quantitation of percentage invasive cells compared with untreated cells (statistics: treated versus untreated (*) or treated versus treated plus AKTi-1/2VIII ($^{\#}$) ($^{x}P<0.05$; $^{xx}P<0.01$; $^{xxx}P<0.001$, where x, xx, xxx are either * or #)).

and in comparison with the bivalent and agonistic Met antibody. No agonism could be seen for MetHer1 (Supplementary Figures S3F).

MetHer1 was further characterized *in vitro* for its effect on viability in basal conditions in A431, H596 and H322M cell lines and efficacy was compared with the two parental antibodies given as monotherapy or in combination (Figure 3a). Cells were cultivated in medium supplemented with 10% fetal calf serum (FCS) and HGF was added for comparison as it is essential for the functionality of the ligand-dependent 5D5 component of MetHer1. Treatment only with cetuximab was already efficacious in A431 cells, which are known to be EGFR addicted, but efficacy was completely lost on addition of HGF. In this setting, 5D5 antibody alone had no effect as well, whereas only MetHer1 or the combination of both parental antibodies induced a clear and significant reduction in cell viability (approximately 40%). This suggests that only inhibiting both receptors simultaneously may have therapeutic potential in tumor cells where both pathways are active. A very similar result was obtained with H322M, with MetHer1 showing a 60% growth inhibition. In this cell line as well, addition of HGF *per se* did not enhance proliferation, which 5D5 alone could also not block. However, addition of HGF impaired the anti-proliferative effect of cetuximab and only treatment with the combination of cetuximab and 5D5 or with MetHer1 restored growth inhibition. mRNA profiling data suggest a very low expression of Met in this particular cell line, compared with the other two (data not shown) and our results imply that the growth inhibition induced by MetHer1 occurred mainly via the EGFR-specific arm. Nevertheless, a comparable effect was not observed, when HGF-stimulated cells were treated with cetuximab alone.

In H596 cells stimulated with HGF, MetHer1 mediated 60% growth inhibition, which was significantly greater than that induced by 5D5 alone ($P<0.001$). Co-culture of H596 with normal and tumor lung fibroblasts resulted in a higher proliferation rate after 5 days, which was significantly reduced by treatment with

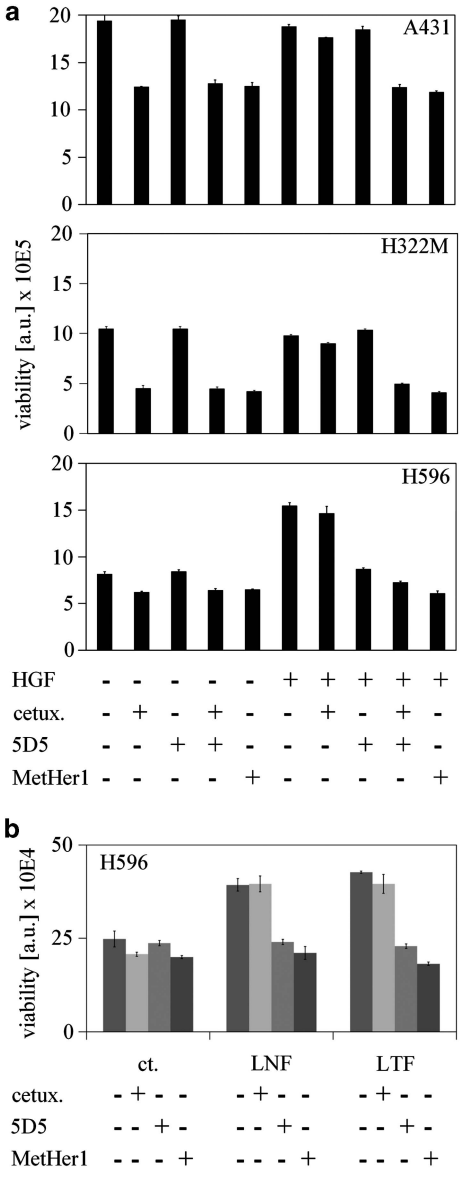

Figure 3. MetHer1 efficacy *in vitro*: effect on tumor cell proliferation. (**a**) Viability of indicated cell lines on antibody treatment. (**b**) Viability of H596 cultivated alone (ct.), or in the presence of normal (LNF) and tumor (LTF) lung fibroblasts. Cells were treated with MetHer1 and parental antibodies for comparison.

5D5 and MetHer1, but not by cetuximab (Figure 3b). The effect was probably dependent on fibroblasts producing HGF (Supplementary Figure S5A).

The anti-proliferative effect of MetHer1 was also evaluated in combination with a sub-optimal dose of the chemotherapeutic agent cisplatin in H596 and BxPC3. BxPC3 represents a pancreatic model in which the bispecific showed only a weak effect on viability (Supplementary Figure S5B). Nevertheless, combined treatment was superior to the effect of cisplatin alone ($P<0.001$) with an overall percentage growth inhibition of $>60\%$. A combination of MetHer1 and cisplatin in H596, which already responded well to MetHer1 mono-treatment, had no additional effect. This supports the rationale that a combination of bispecific antibody with reduced and thus better tolerated doses of a chemotherapeutic can improve efficacy and safety, particularly in tumor models, which are less dependent on signaling (that is, BxPC3).

MetHer1 prevents HGF-induced scattering

HGF is also a known motility factor, which induces scattering and invasion of epithelial cells. This is phenotypically characterized by a change in cell shape and the effect can be macroscopically observed in Figure 4a showing DU145 after 24 h of treatment with HGF. Cellular migration can be semi-quantitatively evaluated with a real-time cell analyzer (RTCA system), which measures impedance changes as surrogate parameter of cell adhesion. As reported in Figure 4a, HGF-induced cell motility and dissemination of DU145 cells, thus reducing the measured impedance, when compared with control. Scattering was quantified in a graph where a normalized cell index (compound addition) was plotted against time. DU145 were treated with cetuximab and 5D5, the combination of both and MetHer1 (at 200 and 10 nM) and stimulated with EGF and HGF. At high dose, MetHer1 could completely revert the HGF-induced scattering and to a smaller extent also at the low dose. In the latter case, no efficacy was seen instead for the combination of the monospecific antibodies. Efficacy of 5D5 alone was reduced by the influence of EGF treatment, which *per se* also showed an effect on cell adhesion (Figure 4b). Viability analysis displayed no differences between treatments, excluding any influence of cell viability or proliferation on the interpretation of the results (data not shown). A human IgG control antibody did not influence cellular scattering (Supplementary Figures S6C and D), suggesting specificity of the reported data. The potential superiority of MetHer1 at low doses was further evaluated in a dose-response scatter experiment. The percentage scatter inhibition for MetHer1 or the combination (Combo) was calculated and the ratio of both determined. MetHer1 displayed superior inhibitory activity over three logs of antibody concentration with a sevenfold higher potency at doses as low as 1 nM (Figure 4c).

To better assess the superiority of MetHer1 versus the combination in preventing growth factor-induced cell dissociation at a low dose, the kinetics of internalization of the two single agents in comparison with MetHer1 was evaluated in a fluorescence-activated cell sorting assay. Presence of the receptors on the cell surface was measured after binding with the respective antibodies for 2 h, versus t0 (Supplementary Figure S6A). The amount of antigen–antibody complex on the cell surface was unchanged within this time. Intracellular staining was only visible as speckle-like structures after 4 h of incubation with fluorescently labeled antibodies by confocal microscopy (Figure 4e, Supplementary Figure S6B). Cetuximab binding appeared to be stronger compared with 5D5, which may be a consequence of differential antigen expression (Figure 4d). There was no difference in the kinetics of internalization between the molecules. Therefore, superiority of MetHer1 in the scatter assay could not be explained by differential internalization.

MetHer1 inhibits EGFR and Met-related pathways

MetHer1 efficacy in proliferation experiments was accompanied by a strong decrease of target phosphorylation in A431 and H596 (Figure 5a), as well as in other *in vitro* models (Supplementary Figure S7A). In A431, phospho-ERK1/2 was blocked by MetHer1 but not or only minimally by treating with the single parental antibodies. The level of phospho-AKT, which was found to be increased in HGF/EGF-stimulated cells after treatment with cetuximab alone reverted back to basal untreated values in the presence of MetHer1 in five cancer cell lines of different tissue origins (Figures 5a and b). In BxPC3, we observed phosphorylation of Met after stimulation of cells with EGF, which might be due to a cross-talk between EGFR and Met. MetHer1 also reduced invasion induced by HGF and EGF and significantly counteracted the effect induced by cetuximab parental antibody in equal settings (Figures 5c and d). The effect of simultaneous treatment with cetuximab and 5D5 is additionally shown for comparison.

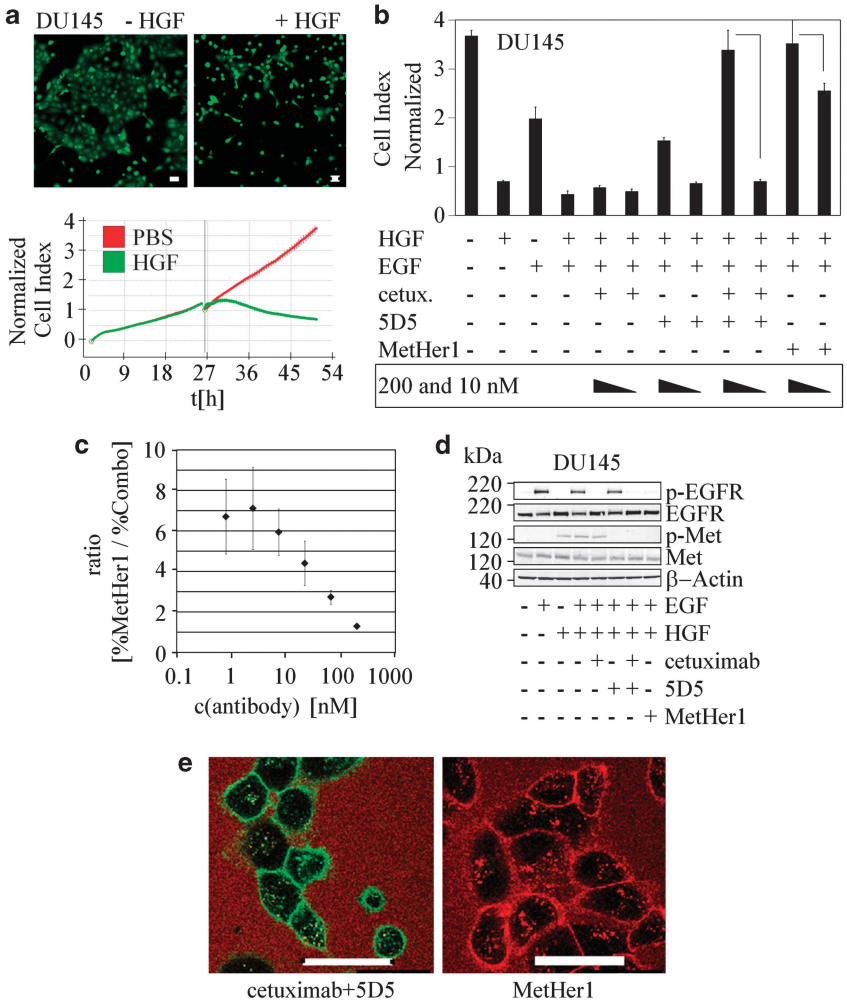

Figure 4. MetHer1 effect on HGF-induced motility. (**a**) DU145 after 24-h treatment with 30 ng/ml HGF. Confocal microscopy analysis of calcein-stained cells and effect on impedance measured by RTCA (white bar x, y: 50 μm). (**b**) Quantitation of MetHer1 effect on HGF-induced DU145 scattering. (**c**) Dose-response curve analysis of scatter assay in DU145. The efficacy of bispecific antibody and cetuximab + 5D5-mediated inhibition of cell dissemination was determined after 24 h and the ratio of both calculated. (**d**) Basal and on-treatment receptor status of EGFR and Met. (**e**) Internalization of fluorescently labeled antibodies evaluated in DU145 cells after 4 h of incubation (white bar x, y: 50 μm).

MetHer1 has a potent antitumor effect *in vivo*

To test the efficacy of MetHer1 in a mouse model, an A549 tumor cell line overexpressing HGF was generated by viral transduction with a vector-encoding human HGF to overcome the issue of non-cross-reactivity of murine HGF to human Met and ensure an efficacy contribution by the 5D5 component. Several clones were generated and their ability to produce HGF in the presence and absence of selection pressure was evaluated by enzyme-linked immunosorbent assay over a period of 29 days to ascertain stable expression (Supplementary Figure S8A). Clone20 was selected because of high secretion levels of HGF and constitutive Met phosphorylation (Supplementary Figures S8A and C). The RTK signaling network in this clone was compared with parental A549 by using a phospho-RTK array and affymetrix profiling. Overall, A549 clone20 was comparable in its mRNA expression profile but displayed a slightly different activation pattern of receptor tyrosine kinases (Supplementary Figure S8B and data not shown). HGF-producing A549 clone20 was characterized by cell surface binding of fluorescently labeled MetHer1, 5D5 and cetuximab. Although cetuximab and MetHer1 displayed a strong binding capacity, 5D5 binding was found to be reduced in the HGF-overexpressing clone compared with un-transduced cells (Supplementary Figure S8E). This might be a consequence of competition with ligand and/or lower steady-state Met cell surface expression levels because of constitutive internalization induced by the ligand HGF (Supplementary Figure S8C). MetHer1 inhibited *in vitro* phosphorylation of both EGFR and Met to the same extent as the parental antibodies. When subcutaneously implanted into mice, tumors produced HGF (7.4 ± 2.71 ng/ml: average of 10 animals), which was further confirmed *ex vivo*, in tumor lysates (Supplementary Figure S8D). MetHer1 efficacy was tested *in vivo* in the subcutaneous setting and compared with the parental antibodies, which were administered in an equimolar ratio as monotherapy or in combination. Tumor growth inhibition at the end of study was with 75% higher for MetHer1 but not statistically significantly different from the combination (55%) after three weekly cycles of treatment (tumor growth inhibition for cetuximab and 5D5: 11% and 51%). Data are presented as tumor growth inhibition and nonparametric treatment-to-control-ratio graph (Figures 6a and b). Near infrared fluorescence analysis with fluorescently labeled antibodies confirmed *in vivo* binding, as shown with two representative animals per group (Figure 6c). Human HGF measured in the tumors was strongly reduced in the MetHer1 treatment group compared with the vehicle group,

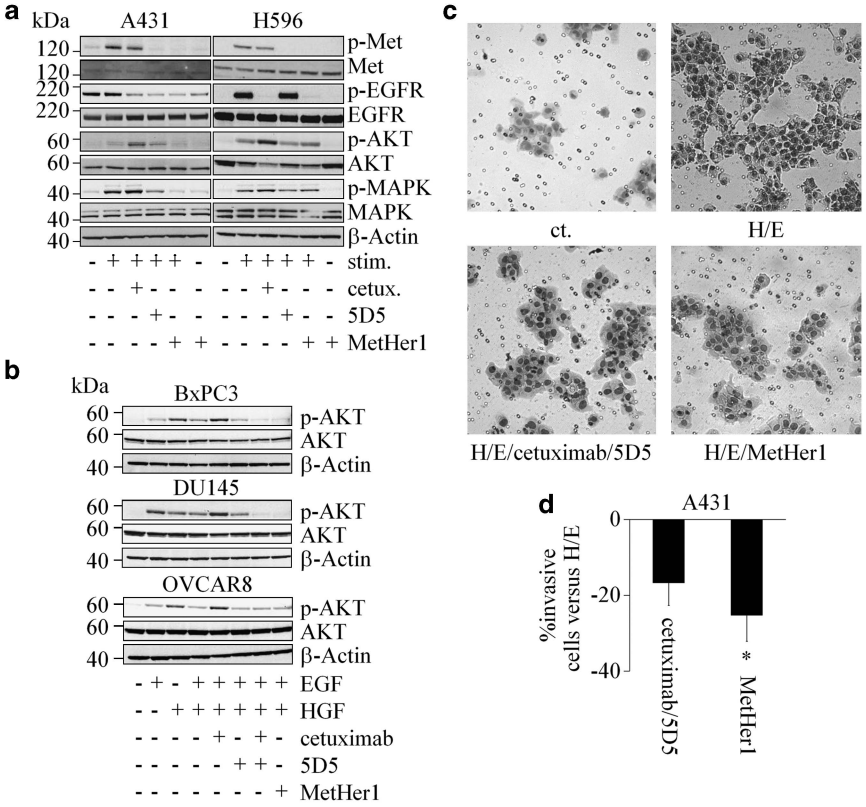

Figure 5. MetHer1 inhibits downstream signaling and invasion. (**a**) Expression and phosphorylation status of indicated proteins in A431 and H596 on treatment. A431 were stimulated with HGF, H596 with HGF and EGF. (**b**) Phosphorylation status of AKT in indicated cell lines after antibody treatment. (**c**) Invasive A431 cells after treatment with MetHer1 (H/E HGF and EGF). (**d**) Percentage of invasive A431 after stimulation with HGF/EGF and treatment with indicated antibodies. *P*-values were calculated versus stimulated cells; $*P < 0.05$.

probably as a consequence of smaller tumor sizes (Figure 6d). The low efficacy observed after treatment with cetuximab was expected because of mutant KRAS status. To predict the effect of a putative combination of MetHer1 with a MEK inhibitor, which would block the pathway downstream of KRAS, the effect of MetHer1 and the MEK inhibitor UO126 on proliferation was tested *in vitro* in A549 clone20 cells. Figure 6e shows the results obtained when UO126 was administered at the sub-optimal dose of 5 μM alone or in combination with MetHer1 (UO126 IC50 for this cell line: 12.7 μM; data not shown). In combination with the MEK inhibitor, a fourfold increase in the percentage inhibition was observed, supporting that the KRAS mutation strongly influences treatment efficacy.

DISCUSSION

In this study, we investigated in detail the counterbalancing mechanisms mediated by Met that confer resistance to targeted inhibition of EGFR. We confirmed in tumor cell lines from different origins that treatment with EGFR inhibitors results in a transient upregulation of phospho-AKT under conditions of co-activation of the EGFR and Met pathways. In the presence of active Met signaling, EGFR inhibition also enhanced invasiveness (Figure 2a). Invasive growth of tumor cells on stimulation with EGF or HGF is well known.[25] Although a variety of studies on the cross-talk of the two receptors and their inhibition have been published,[19,21,26] it has not been previously described that addition of EGFR inhibitors to HGF-stimulated cells can increase invasiveness in comparison with growth factor treatment only. Bonine-Summers *et al.*[27] previously published that the EGFR inhibitor gefitinib also inhibits Met signaling, which is in contrast to our findings. It has been shown that gefitinib very potently targets cyclin-G-associated kinase also.[28] Meanwhile, it is known that cyclin-G-associated kinase regulates PP2A and clathrin-mediated endocytosis, both also important for Met regulation, which might explain the authors' findings.[29,30] A very comprehensive study by Gusenbauer *et al.*[31] demonstrates the intricate cell surface network for EGFR and Met but also for a variety of membrane proteins, which are involved in this signaling node. Interference by our EGFR inhibitors, especially antibodies binding EGFR, might shift the balance between these signaling nodes and thus produce the observed effects.

Maseki *et al.*[32] reported that gefitinib-resistant head and neck squamous cell carcinoma can acquire an epithelial to mesenchymal transition phenotype, which is accompanied by an increase of phospho-AKT. A similar epithelial to mesenchymal transition process might occur in our experimental setting, accompanied by Twist and Snail-mediated repression of E-cadherin.[33] Alternatively, phosphatidylinositol 3 kinase/AKT signaling could directly act on focal adhesion kinase.[34] Focal adhesion kinase and Src are known to modulate E-cadherin and thereby promote cancer cell invasion.[35] Further addition of an AKT inhibitor reversed the invasive phenotype similarly to the combined inhibition of EGFR and Met (Figures 2a, 5c and d). This implies, but does not unambiguously prove, that the transient increase of phospho-AKT is causally linked to the increase in invasiveness. In this context, it is an intriguing recent experimental finding that an artificial increase of phospho-AKT results in loss of cetuximab sensitivity in various lung cancer cell lines.[36]

Our findings could be clinically relevant in the setting of an adjuvant anti-EGFR therapy given that, independently from the

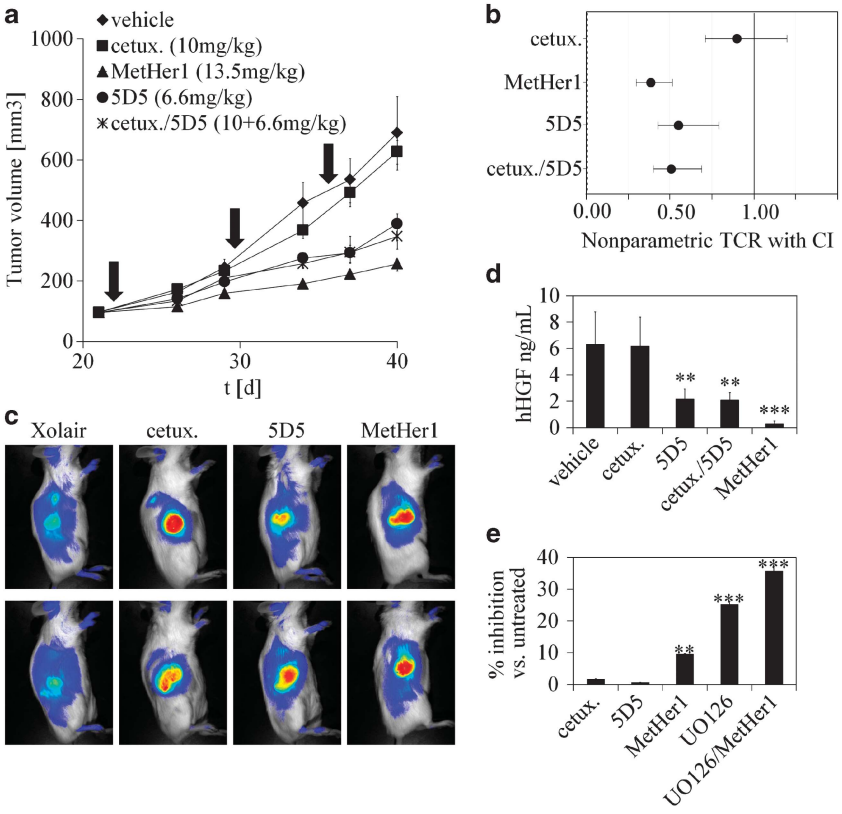

Figure 6. MetHer1 is efficacious *in vivo* in a HGF-overexpressing A549 human lung adenocarcinoma xenograft model. (**a**) Mean tumor volume (arrows treatment). The anti-IgE antibody Xolair was used as control antibody. (**b**) Nonparametric treatment-to-control-ratio (TCR) of tumor growth inhibition at the end of study. (**c**) *In vivo* imaging in two representative animals per group. (**d**) Quantitation of human HGF from serum samples at the end of study. (**e**) Growth inhibition of A549 cl.20 *in vitro*, with a sub-optimal dose of the MEK inhibitor UO126 and indicated antibodies (**$P<0.01$, ***$P<0.001$).

well-known autocrine or paracrine HGF supply by tumor cells and/or tumor associated fibroblasts, it has been shown that HGF serum levels are elevated after surgery as part of the wound-healing process.[37–39] However, the duration of this process in patients is unclear. Targeted EGFR inhibition in lung cancer, in an adjuvant setting, has already been studied.[40] In 2002, the JBR.19 trial investigated gefitinib as maintenance treatment in resected non-small lung cancer. However, this trial was prematurely stopped because of negative results of the ISEL and SWOG 0023 trials with gefitinib. The ongoing RADIANT trial with erlotinib is primed to demonstrate whether EGFR inhibition in the adjuvant setting is beneficial.

Clinical trials with combinations of EGFR and Met inhibitors are ongoing. In this co-targeting setting, our data suggest that an imbalance of EGFR and Met-targeting activities in tumor samples may pose the risk of increased tumor spread. This could be of special concern if low molecular weight and antibody inhibitors with different pharmacodynamics are co-administered, thus making a stronger case for the development of the bispecific antibody we described. We have generated a bispecific antibody consisting of cetuximab and 5D5 in a 2+1 format under the assumption that a fixed stoichiometry of both targeting compounds should ensure simultaneous inhibition of both targets even in poorly accessible solid tumors. Mechanistically, such a bispecific antibody might for instance display differential avidity, clustering and internalization or antibody-dependent cell-mediated cytotoxicity properties in comparison with the combination of two antibodies. To our knowledge, MetHer1 presents the first bispecific IgG-like antibody targeting Met and EGFR. The antibody is non-agonistic and proves the concept of targeting both receptors simultaneously with a bispecific antibody. Previously, bispecific antibodies targeting EGFR and IGF-1R have been described with a similar co-targeting approach[41–44] as well as EGFR was used as targeting moiety for effector cell recruitment or payload delivery.[45,46] MetHer1 displays no agonistic activity in cellular assays and the overall activity was mostly similar to the combination of the parental antibodies cetuximab and 5D5. We observed differences in cell dissemination in the presence of low inhibitor concentrations. This could possibly be explained by an avidity effect, which raises the local Met inhibitor concentration and thereby enhances efficacy. We propose that in the presence of EGFR binding, the Met component of MetHer1 is enriched on the cell membrane and can better inhibit Met activity. A close proximity of both receptors has been previously shown by co-immunoprecipitation.[3]

In a ligand-dependent animal model, the overall activity of MetHer1 was superior but not significantly better than the combination of the parental antibodies cetuximab and 5D5 (Figure 6a). Efficacy of cetuximab is greatly impaired by the KRAS mutation found in A549. Although the mAb does not confer much antitumor activity, in the MetHer1construct, cetuximab could function as targeting moiety leading to more efficient 5D5 recruitment. This could explain the modest superiority of MetHer1 over the combination of parental antibodies but needs further investigation. The hypothesis is supported by our *in vitro* cell dissemination experiments whereby at lower doses MetHer1 was also more efficacious than the parental antibody combination (Figures 4b and c).

In contrast to onartuzumab, MetHer1 is a fully glycosylated human IgG1 antibody. Thus, MetHer1 retains effector function

abilities and these are not affected by the C-terminal fusion of the 5D5 single chain Fab (data not shown). Cetuximab and 5D5, as used herein, also have a glycosylated human IgG1 Fc-part. In the A549 clone20 *in vivo* model, immune effector functions, for example, by residual macrophages, may have a role. However, theoretically, these effects should be stronger in the combination group, as the total Fc load per tumor cell is presumably higher than for the MetHer1 group.

Although inhibition of tumor growth is a primary parameter, it remains to be shown if the number of metastases is affected in models, which display stronger tumor spread, especially after excision of the primary tumor. A major hurdle is the availability of human HGF to activate Met in such a model. Transgenic mice producing human HGF have been described in the past and might help to address this problem.

In summary, the findings reported here highlight the complexity of perturbing regulatory networks by the use of targeted therapies, especially if multiple activating signals are present, which is the case in the majority of solid tumors, either *de novo* or as consequence of acquired resistance. Bispecific antibodies—as exemplified by MetHer1—facilitate targeting of two pathways without the risk of under-dosing one compound, efficiently counteract resistance mechanisms at the molecular level and yet retain the ability to effectively mediate antibody effector functions. Potential liabilities of such bispecific antibodies, for instance, cumulative toxicities or unanticipated modes of action, would need to be carefully evaluated during the development process.

MATERIALS AND METHODS

Cell culture

A431, A549 and BxPC3 were obtained from ATCC (Manassas, VA, USA); DU145, OVCAR8 and H322M from the NCI (Bethesda, MD, USA); H596 from Chugai Pharmaceuticals Co., Ltd. (Tokyo, Japan) and lung normal and tumor fibroblasts from Asterand plc (Royston, Herts, UK). Except H596, all cells were maintained in RPMI 1640 medium, supplemented with 10% FCS, non essential amino acids, sodium pyruvate and L glutamine (Gibco, Darmstadt, Germany). H596 were maintained in RPMI high glucose, supplemented with L glutamine, 1 mM sodium pyruvate, 10 mM HEPES (PAN Biotech, Aidenbach, Germany) and 10% FCS. Cells were propagated according to standard cell culture protocols.

Proteins and inhibitors

The variable heavy and light chain domain sequences of cetuximab and 5D5.v2, herein referred as 5D5, were cloned based on published sequences via gene synthesis in mammalian expression vectors. For cetuximab, a human IgG1 framework and kappa light chain backbone was used. For 5D5, two heavy chain 5D5 plasmids were used which carried the knobs into hole mutations[47] and in which one was missing the VH CH1 domain. MetHer1 was constructed from cetuximab with a human IgG1 backbone with knobs into hole and a single chain Fab fusion of Met at the knob heavy chain. Light and heavy chains were co transfected in HEK 293F (Invitrogen/Life Technologies GmbH, Darmstadt, Germany) resulting in full glycosylation of all antibodies, then purified as previously described.[45] Purity was analyzed using an Agilent HPLC 1100 (Agilent Technologies, Oberhaching, Germany) with a TSK GEL G3000SW column (Tosoh Corp., Tokyo, Japan). Identity was confirmed by mass spectrometry and binding properties characterized by surface plasmon resonance (SPR). Cetuximab parental antibody was purchased from Merck Serono (Darmstadt, Germany), panitumumab from Amgen Inc. (Thousand Oaks, CA, USA). Met and EGFR ectodomains were transiently expressed and purified from HEK 293F supernatants. Recombinant huHGF and huEGF were obtained from R&D Systems (Minneapolis, MN, USA) and Gibco. AKTi 1/2 VIII and UO126 were bought from Calbiochem/Merck KgaA (Darmstadt, Germany). Other antibodies: pEGFR, pAKT1 (Epitomics, Burlingame, CA, USA), EGFR (Millipore/Merck KgaA, Darmstadt, Germany), pMet, Met, pMAPK, MAPK, AKT (Cell Signaling Technology Inc., Danvers, MA, USA) and β actin (Abcam, Cambridge, UK).

Immunoblot

Cells (5 8×10^5 per well) were seeded in a six well plate in medium with 0.5% FCS and treated the following day with 0.07 μM of cetuximab, panitumumab, 5D5 and MetHer1 and 5 μM erlotinib for 30 min (1 h for erlotinib) prior stimulation (HGF 30 ng/ml and EGF 50 ng/ml). After 5 or 15 min of incubation at 37 °C, cells were washed with phosphate buffered saline, lysed and subjected to immunoblot analysis. For statistical analysis, a box plot analysis was applied.

Invasion assay

A431 (50 000 cells per well) were pre incubated for 15 min at 37 °C with 0.2 μM antibodies, 5 μM erlotinib or AKT inhibitor in medium with 0.5% FCS and seeded in Matrigel chambers (BD Biocoat Matrigel Invasion Chambers, BD Biosciences, Heidelberg, Germany), which were beforehand rehydrated and immersed in 24 well companion plates in medium with 10% FCS and/or growth factors plus treatment. HGF and EGF were added in the chambers before incubation for 43 h at 37 °C. Non invading cells were removed from the upper surface of the membrane by scrubbing and cells were fixed and stained (Diff Quick stain). Pictures were taken at a magnification of × 100 and invasive cells counted in four different fields each of quadruplicate membranes of two independent experiments. Standard deviation was calculated as average of all values. In parallel, 100 μl of the medium were used for a cytotoxicity assay (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Proliferation assays

Cells (A431, H322M: 2500 cells per well; H596: 5000 cells per well; A549 clone20: 1000 cells per well) were seeded in medium with 10% FCS and treated the following day with 0.2 μM of the antibodies for 15 min before stimulation with HGF 30 ng/ml. Viability was measured via Cell Titer Glo (Promega) at 5 days (A431, H322M and H596) and 4 days after treatment for A549 clone20. UO126 was added at 5 μM 24 h before measuring.

Migration assay

Changes in cell morphology were monitored using xCelligence (Roche Applied Science, Mannheim, Germany). DU145 (3000 cells per well) were seeded in a 96 well E plate in medium supplemented with 0.5% serum and treated the following day with antibodies (200 and 10 nM) for 15 min before HGF and EGF stimulation (30 and 50 ng/ml).

Xenograft study

To generate primary tumors, 1×10^7 tumor cells in a volume of 100 μl phosphate buffered saline were injected subcutaneously into the right flank of the mice. Animals were controlled 5 × per week for their health status. Tumor dimensions were measured by caliper on the staging day, and twice weekly for the treatment period. Animals were treated on study day 21, 28 and 35. All experiments were approved by the local regulatory agency. Nonparametric treatment to control ratios based on end point analysis and the two sided nonparametric confidence intervals compared with vehicle group were calculated to assess statistical significance.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)

Castoldi et al.: Supplemental Data for "A novel bispecific EGFR/Met antibody blocks tumor promoting phenotypic effects induced by resistance to EGFR inhibition and has potent anti-tumor activity"

Supplemental Figure Legends

Supplemental Figure S1

(**A, B**) Analysis of AKT phosphorylation upon treatment of H596 and A431 cells with the indicated antibodies. HGF and EGF stimulation was used as positive control. (**C**) Analysis of cetuximab treatment in H596 in the presence of one or both growth factors. Numbers below the immunoblot indicate relative quantitation of signals with HGF treatment only set to 1.0.

Supplemental Figure S2

(**A**) Cytotoxicity is presented versus untreated A431 after 43h of stimulation with HGF and EGF together with the indicated treatments. Cytotoxicity was directly determined in medium obtained from matrigel chambers before cell fixation. 3 μM Staurosporine was used as positive control for the assay. (**B**) Immunoblot of phosphorylated and total AKT in A431 cells stimulated for 10 min with HGF and EGF after 100 min treatment with two concentrations of AKT inhibitor (0.5 and 1μM).

Supplemental Figure S3

Generation of MetHer1. (**A**) Schematic presentation of MetHer1. KiH and disulfide bridges are indicated. scFab: V_L-C_L-$(G_4S)_6$GG-V_H-$C_{H1,}$ fusion via a $(G_4S)_3$ connector. (**B**) SDS-PAGE

analysis of purified MetHer1 under non-reducing (NR) and reducing (R) conditions. (**C**) Size exclusion purification of MetHer1. (**D**) Analytical HPLC of MetHer1. (**E**) Simultaneous binding to recombinant Met and EGFR (Biacore). EGFR was amine-coupled to the chip surface. (**F**) Immunoblot analysis of Met phosphorylation by indicated antibodies in H596 after 30 min of incubation.

Supplemental Figure S4

SPR analysis of binding kinetics. (**A**) Concentration series of soluble receptor binding to the respective antibodies (cetuximab, 5D5, MetHer1). Sensorgrams were fitted to a Langmuir 1:1 model, RI=0 (black lines). (**B**) Kinetic constants for soluble receptor binding to cetuximab, 5D5 and MetHer1.

Supplemental Figure S5

(**A**) HGF concentrations in medium upon cultivation of H596 with or without lung normal fibroblasts (LNF) or lung tumor fibroblasts (LTF) for two (D2) and six (D6) days. All cells were maintained in the same volume of medium. (**B**) Percentage growth inhibition versus control of BxPC3 and H596 cell lines after treatment with a sub-optimal dose of cisplatin alone or in combination with MetHer1.

Supplemental Figure S6

(**A**) *Left:* Internalization of indicated antibodies measured by FACS as DU145 cell surface binding after two hours of incubation at 37°C. *Right:* Mean fluorescence intensity (MFI) values of the same experiment including also the time points 30 and 60 min. (**B**) Internalization of fluorescently-labeled antibodies evaluated in DU145 cells with confocal microscopy after 4h of

incubation (white bar x,y: 50µm). (**C**) RTCA-based analysis of DU145 scattering in presence and absence of HGF. Addition of human IgG control in the absence of HGF was compared to PBS. (**D**) Effect of human IgG control antibody (0.2µM) on scattering induced by HGF and HGF+EGF in DU145 cells measured by RTCA.

Supplemental Figure S7

(**A**) Expression and phosphorylation of EGFR, HER2, HER3, Met, and the downstream signaling proteins MAPK and AKT in BxPC3, DU145, OVCAR8 cells after treatment with MetHer1, and parental antibodies cetuximab and 5D5 in the presence of HGF and EGF stimulation. This figure contains data already presented in Figure 4D and 5B.

Supplemental Figure S8

(**A**) Relative HGF amounts in the culture medium of A549 clones (numbers x-axis) stably transfected with human HGF after 29 days of *in vitro* culture. Cells maintained HGF expression in presence (black) and absence (grey) of neomycin. (**B**) Phospho-RTK array of HGF-transfected A549 clone20, versus A549 wild type (wt) cells. Receptors with visible differences in phosphorylation are shown in the squares. (**C**) Expression and phosphorylation of Met and EGFR in HGF-transfected A549 clone20, with respect to wild type A549 after treatment with MetHer1, and parental antibodies cetuximab and 5D5. (**D**) Human HGF levels in 10 untreated animals from the HGF-overexpressing subcutaneous A549 human lung adenocarcinoma xenograft model. *Ex vivo* immunoblot analysis of tumor lysates confirmed presence of HGF in A549 clone20 but not in wild type (wt) cells. (**E**) *In vitro* binding of Cy5-labeled MetHer1, cetuximab and 5D5 to A549 clone20 versus A549 wild type cells.

Supplemental Data: Materials and Methods

Co-cultures

H596 were seeded as mono-culture (5000 cells/well) or in co-culture with lung normal and tumor (adenocarcinoma) primary fibroblasts - 2000 cells/well with 3000 cells/well fibroblasts - in 5% PANEXIN NTA RPMI medium in 96 well-polyhema-coated plates, where cells could form spheroids and grow in suspension. Cells were treated with 30µg/mL antibodies and viability was measured after 5d.

Confocal microscopy

DU145 ($1x10^4$/well) were seeded in ibidi 8-well µ slides and treated the following day either for 24h with HGF (30ng/mL) or for 4h with 10µg/mL cetuximab-Alexa488, 5D5-Cy5, MetHer1-Cy5. Images were acquired by confocal laser scanning microscope (Nikon Eclipse TE-2000-E, Nikon D-Eclipse C1, 4 Laser System). Calcein (3µg/mL) was added 30min before analysis at T=37°C.

Surface Plasmon Resonance

Experiments were performed with a Biacore T200 instrument via standard amine-coupling to EDC/NHS activated chip surfaces. PBS 0.05% (v/v) Tween20 was used as running buffer and dilution buffer (with further addition of 1mg/mL BSA). For kinetic characterization of single antigen binding to MetHer1, the bispecific and parental antibodies were captured with an amine-coupled goat anti-human IgG (CM5 chip). Dilution series (c ~ 4-1200nM Met, EGFR) were analyzed in duplicates with an association phase of 180s and a dissociation phase of 1200s, at T=37°C and with a flow rate of 50µL/min. Signals were double referenced against a flow cell containing only dilution buffer. Kinetic constants were calculated from fitting to Langmuir 1:1

model (RI=0). For simultaneous binding evaluation, MetHer1 antibody (c=15nM) was captured by amine-coupled EGFR. Met (c=200/400nM) was subsequently injected at 25°C to minimize dissociation.

Relative quantitation of HGF

Relative quantitation of HGF levels out of serum from mice or cell culture medium was performed with a HGF ELISA (R&D Systems). Experiments were carried out as recommended by the manufacturer. Different dilutions were measured against an internal calibrator standard provided by the kit.

Proliferation Assay (MetHer1 + Cisplatin)

BxPC3, 2500 cells/well, and H596, 5000 cells/well, were seeded in medium with 10% FCS and treated the following day with 0.2µM MetHer1 for 15 min before stimulation with 30ng/mL HGF. Cisplatin (7µM and 14µM for BxPC3 and H596 respectively) was added 48h prior to measuring. Viability was evaluated via Cell Titer Glo, according to the manufacturer's specifications (Promega) 5 days after treatment.

FACS internalization assay

DU145 cells ($5x10^5$) were diluted in 50µL of the different antibody solutions (10µg/mL) and incubated at 37°C for 30, 60 or 120 min. Cells were kept on ice and stained with 5µg/mL anti-human IgG AlexaFluor-488 (Invitrogen). Samples were fixed and measured (BD, FACS Canto).

Generation of A549 cell line stably expressing human HGF

The coding sequence of human hepatocyte growth factor was cloned in the retroviral pLXSN vector (Clontech). The pLXSN vector contains a neomycin cassette which allows selection of

infected cells. Virions were generated by transient transfection of this plasmid with Fugene HD (Roche) in the PA317 (ATCC, CRL-9078) cell line. Cell supernatants were harvested after 3d and sterile filtered through 0.45µm cellulose acetate filters (Nalgene). A549 cells were seeded at $1x10^6$ cells per 10 cm cell culture plate and allowed to attach overnight. The following day, medium was replaced by 4 mL of a logarithmic dilution series of the cell supernatant containing virions in medium containing 8µg/mL polybrene (Millipore). After 24h of infection, cells were washed and fresh complete medium without virions or polybrene was added. After additional 48h, selection medium containing 0.5 mg/mL G418 (Life Technologies) was added. Stable clones were picked using cloning rings and maintained under G418 selection. Absence of virion particles was confirmed by PCR on reverse transcribed mRNA of isolated clones.

Phospho-RTK array with stably expressing HGF A549 clones

A549 clones were maintained in medium containing 0.5 mg/mL G418. 80-90% confluent clones were harvested by detachment of cells with Accutase (Invitrogen). Cells were lysed in buffer containing 1% NP-40, 20mM Tris-HCl (pH 8.0), 137mM NaCl, 10% glycerol, 2mM EDTA, 1mM sodium orthovanadate and protease inhibitors. 100µg of cell lysate, as quantified by the BCA method, were incubated overnight with a phospho RTK membrane (R&D Systems). Detection and image acquisition was performed as recommended by the manufacturer.

Labeling of antibodies

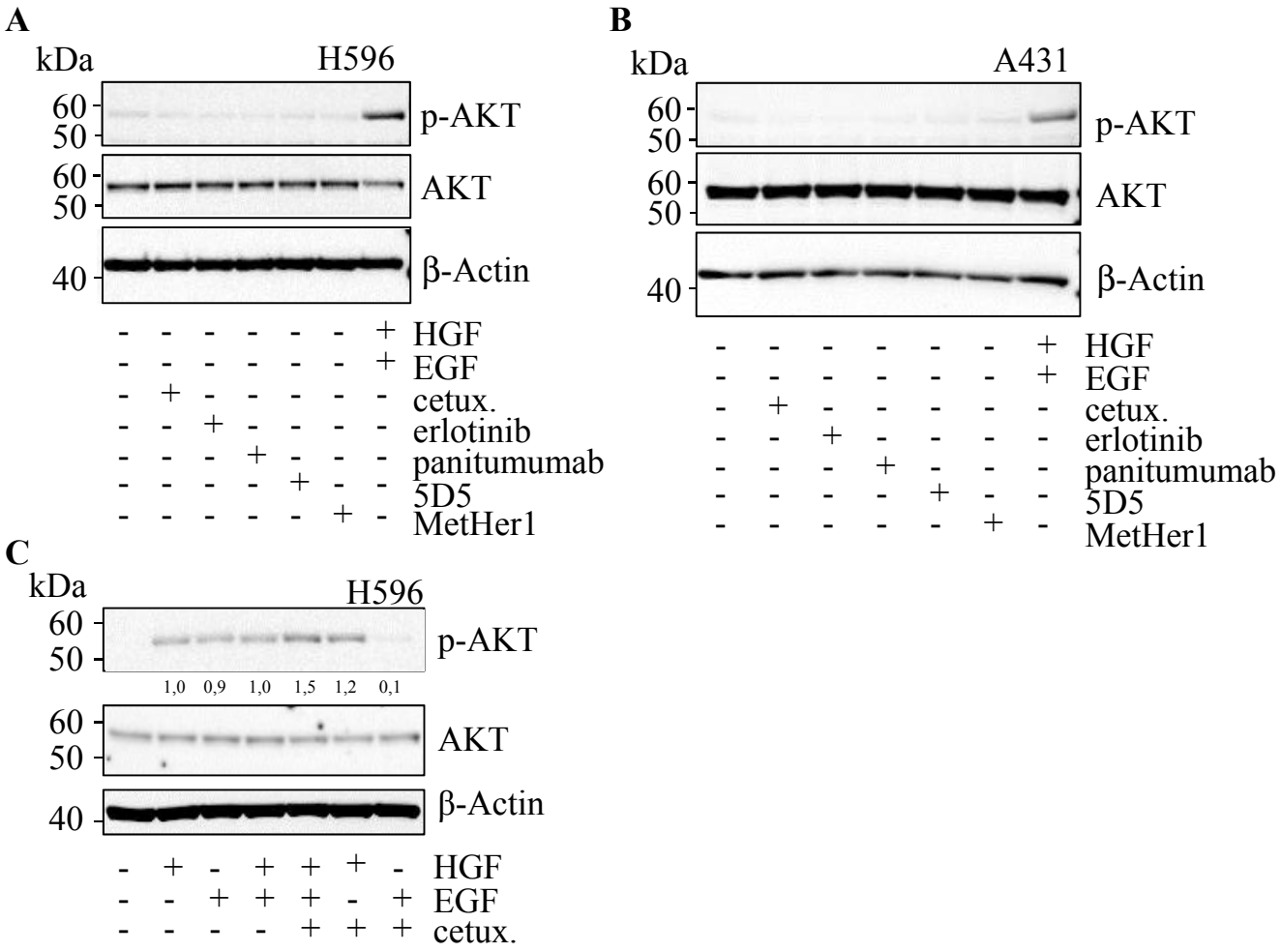
Fluorescently labeled antibodies were obtained by lysine linker chemistry. The antibody to fluorophore ratio was about 1:3 for all antibodies. Before use, fluorescently labeled antibodies were evaluated in Biacore to confirm that binding properties were unaltered.

Imaging

Tumor bearing SCID beige mice were injected i.v. with 50µg of Cy5-labeled Xolair and cetuximab, 33.3µg of 5D5 and 66.7µg of MetHer1 to assure equal molarity. NIRF signal was measured 48h after i.v. injection with the Maestro System (CRI) at optimal acquisition times. Images were processed and normalized to obtain optimal comparability.

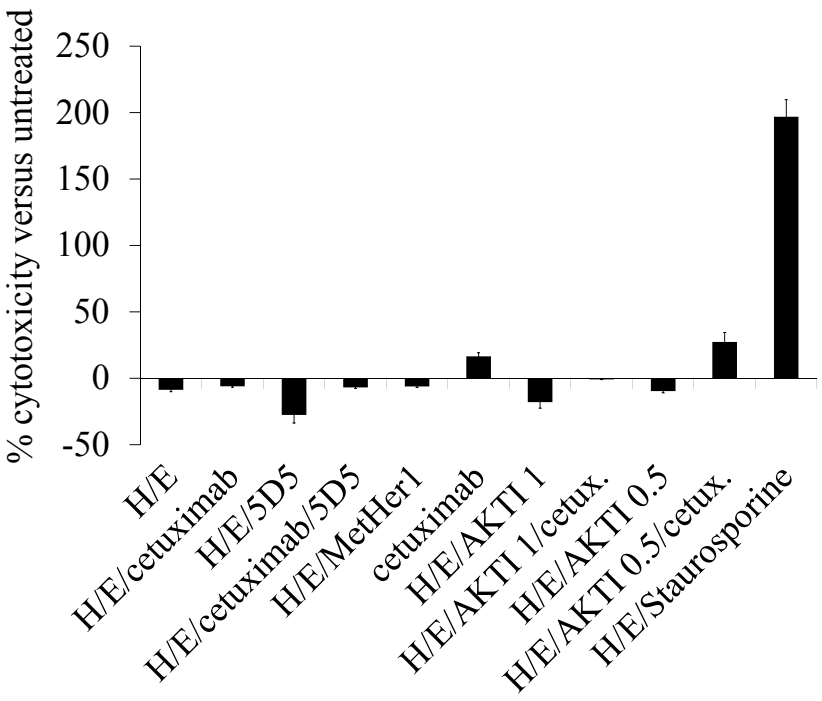
HGF-expressing A549 clone20 and A549 wild-type in vitro staining

Wild type and HGF-expressing A549 clone20 cells were seeded at a concentration of $2x10^6$ cells/mL into µ-slides VI (ibidi). After 24h, cells were washed with PBS and incubated for 30 min with 50 µl of 2.5 mg/mL Cy5-labeled antibodies, as reported; nuclei were subsequently stained with 50 µl of a HOECHST33342 solution (10µg/ml) for 15 min. Slides were imaged multi-spectrally with the Nuance-System (CRi) and analyzed. The displayed pictures were normalized for optimal comparability.



Castoldi et al. , Supplemental Figure 1

A



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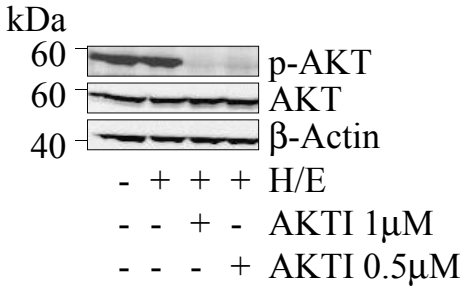

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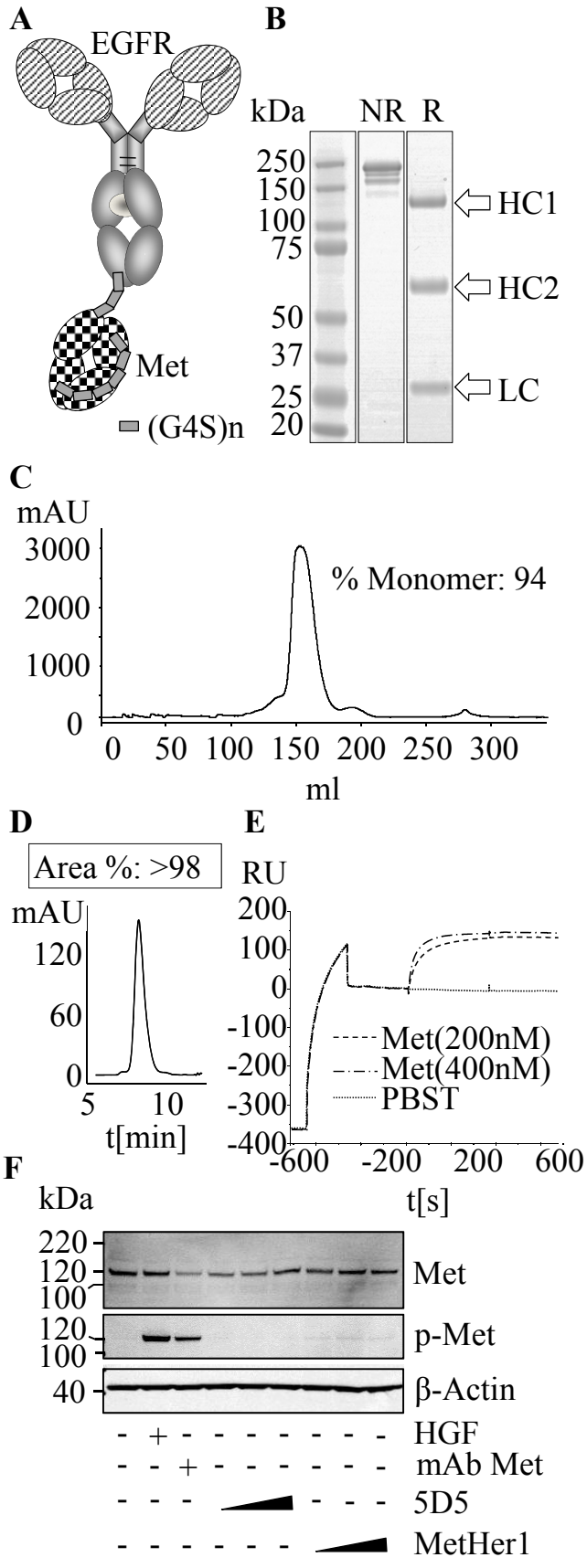

Castoldi et al. , Supplemental Figure 3

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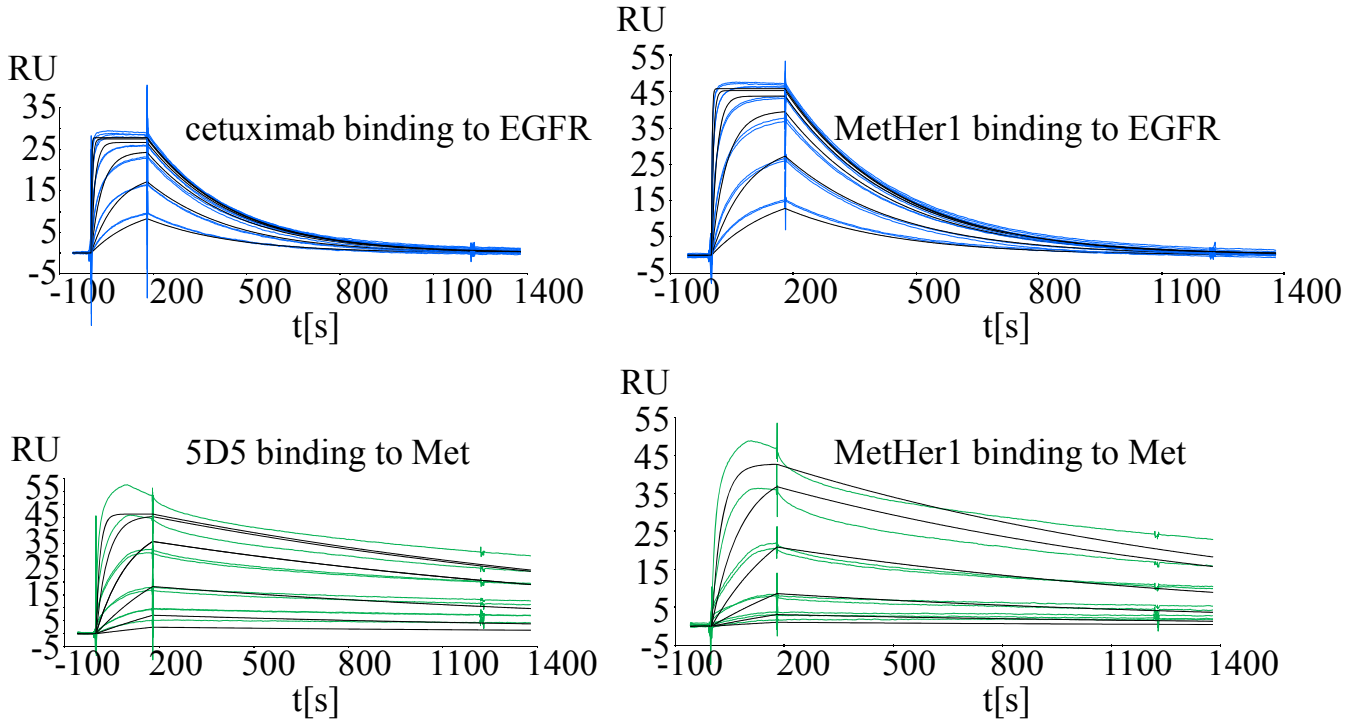
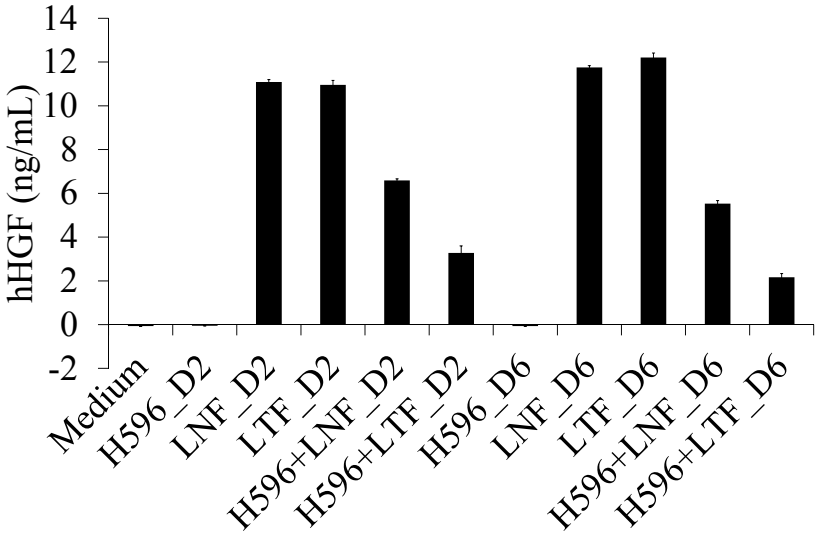


B

Antibody	Analyte	ka [$M^{-1}s^{-1}$]	kd [s^{-1}]	t (1/2) [min]	K_D [M]
MetHer1	EGFR	6.3E+05	3.7E-03	3.1	5.8E-09
cetuximab	EGFR	6.7E+05	3.6E-03	3.2	5.4E-09
MetHer1	Met	2.9E+04	7.1E-04	16.3	2.4E-08
5D5	Met	6.6E+04	5.3E-04	16.8	8.0E-09

Castoldi et al. , Supplemental Figure 4

A



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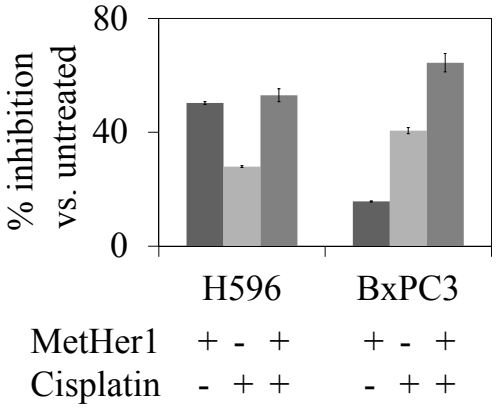

Castoldi et al. , Supplemental Figure 5

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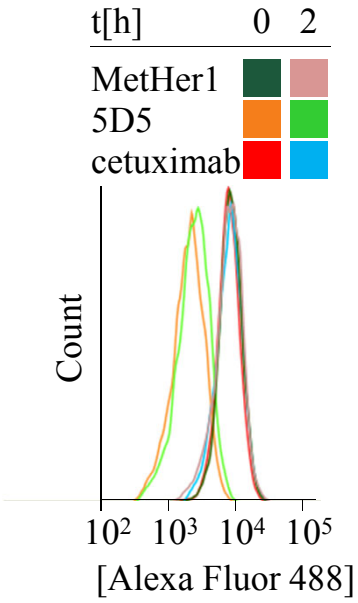
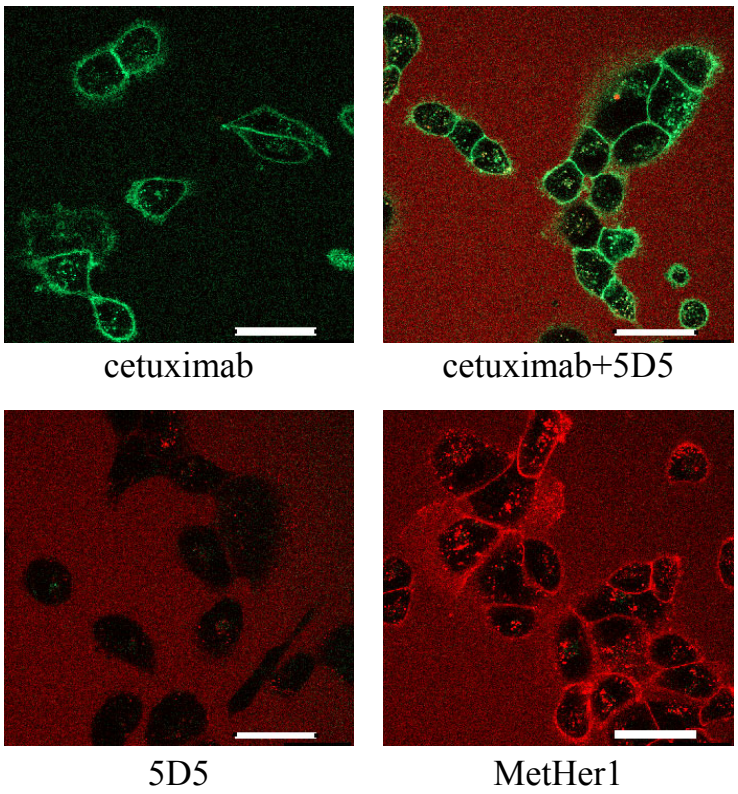


	Mean Fluorescence Intensity (MFI) Alexa-488			
	0 min	30 min	60 min	120 min
cetuximab	8603	10100	12600	13000
5D5	2338	2499	2874	3352
cetuximab+5D5	10400	11400	14000	15200
MetHer1	9351	11000	13000	13200
ctrl	143	171	198	205

B



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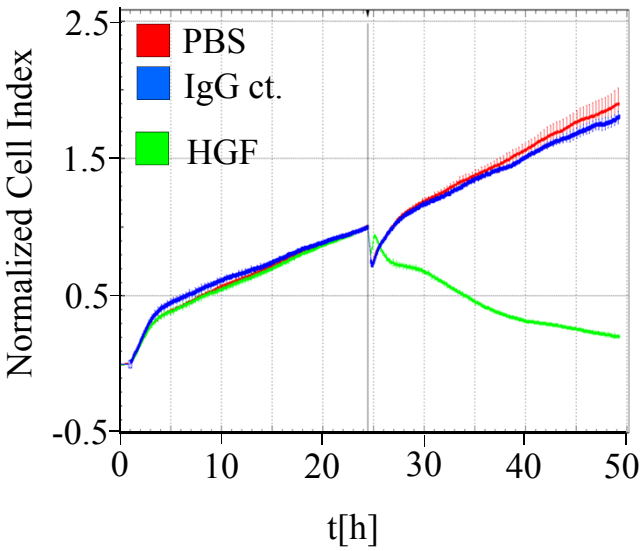

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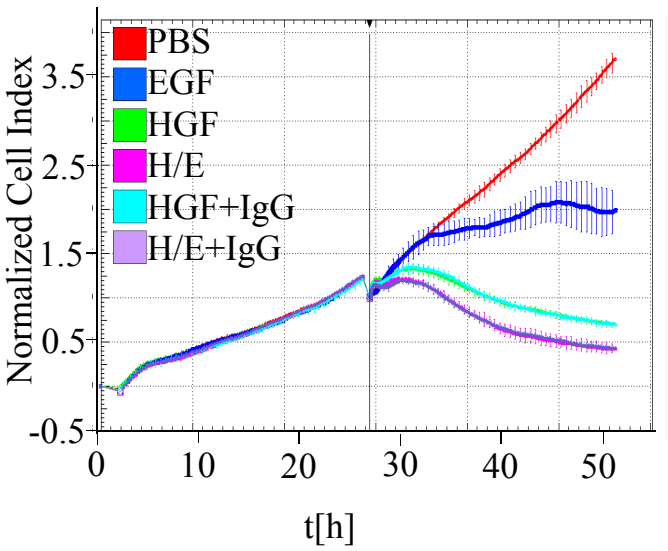

Castoldi et al. , Supplemental Figure 6

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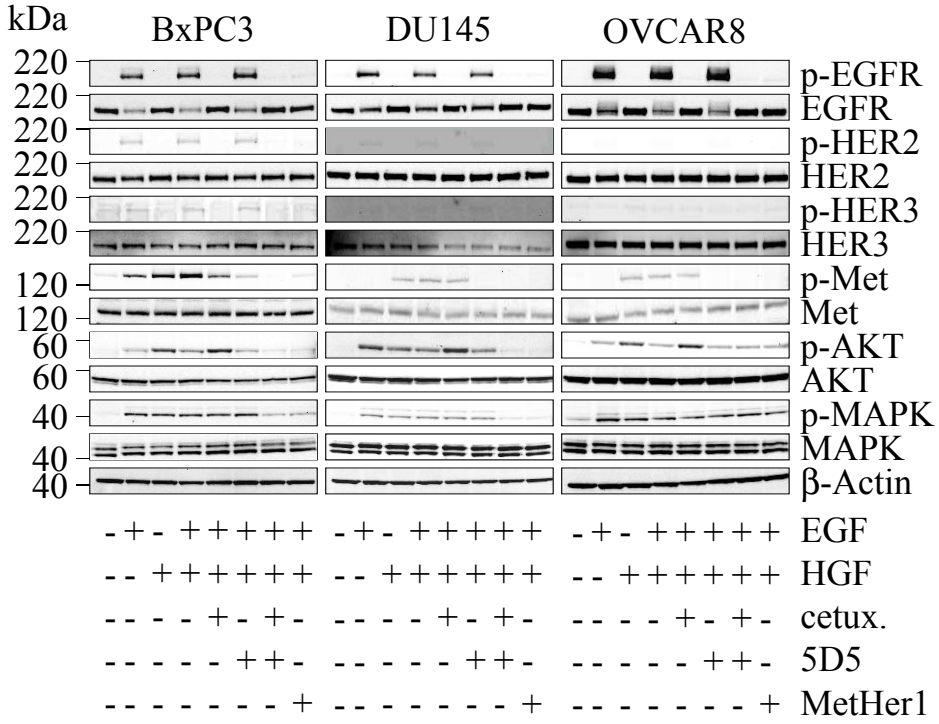
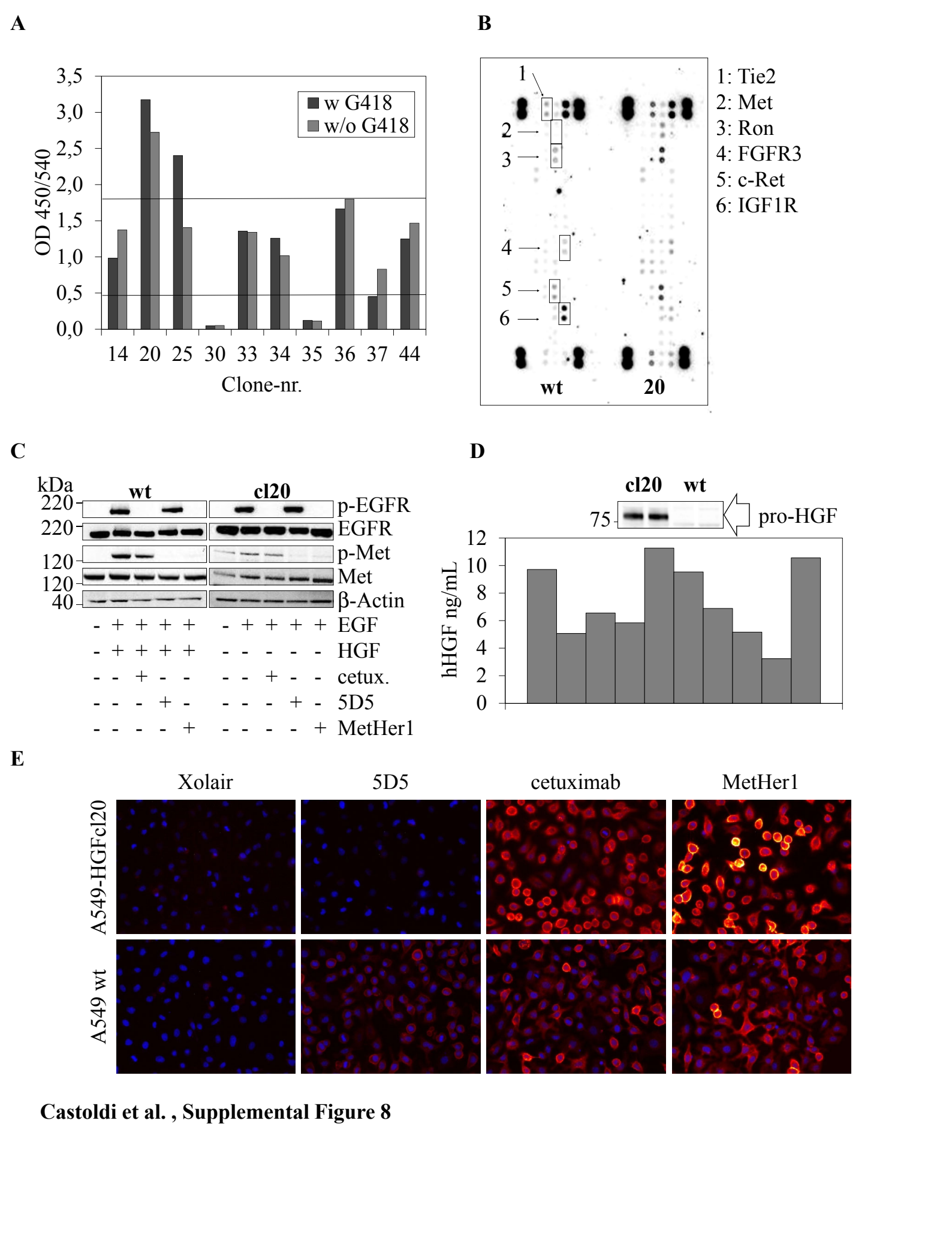


Castoldi et al. , Supplemental Figure 7



Castoldi et al. , Supplemental Figure 8

4.2 Publication: Castoldi R, Jucknischke U et al. Protein Eng Des Sel. 2012

Molecular characterization of novel trispecific ErbB-cMet-IGFIR antibodies and their antigen-binding properties

Castoldi R, Jucknischke U, Pradel LP, Arnold E, Klein C, Scheiblich S, Niederfellner G, Sustmann C.

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Molecular characterization of novel trispecific ErbB-cMet-IGF1R antibodies and their antigen-binding properties

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Therapeutic antibodies are well established drugs in diverse medical indications. Their success invigorates research on multi-specific antibodies in order to enhance drug efficacy by co-targeting of receptors and addressing key questions of emerging resistance mechanisms. Despite challenges in production, multi-specific antibodies are potentially more potent biologics for cancer therapy. However, so far only bispecific antibody formats have entered clinical phase testing. For future design of antibodies allowing even more targeting specificities, an understanding of the antigen-binding properties of such molecules is crucial. To this end, we have generated different IgG-like TriMAbs (trispecific, trivalent and tetravalent antibodies) directed against prominent cell surface antigens often deregulated in tumor biology. A combination of surface plasmon resonance and isothermal titration calorimetry techniques enables quantitative assessment of the antigen-binding properties of TriMAbs. We demonstrate that the kinetic profiles for the individual antigens are similar to the parental antibodies and all antigens can be bound simultaneously even in the presence of FcγRIIIa. Furthermore, cooperative binding of TriMAbs to their antigens was demonstrated. All antibodies are fully functional and inhibit receptor phosphorylation and cellular growth. TriMAbs are therefore ideal candidates for future applications in various therapeutic areas.

Keywords: ITC/receptor tyrosine kinase/SPR/therapeutic antibodies/trispecific antibodies

Introduction

Monoclonal antibodies (MAbs) are well established in clinical practice and more than 25 MAbs are currently approved by the Food and Drug Administration (An, 2010). Of these, about half are in use for treatment of cancer (Nieri *et al.*, 2009). Despite these clinical successes, inhibition of an oncogenic driver protein with a therapeutic antibody often results in rapid emergence of resistance, rendering treatment ineffective (Pillay *et al.*, 2009). A paradigm illustrating this concept is the ErbB receptor family, consisting of EGFR, Her2/ErbB2, Her3/ErbB3 and ErbB4, which propagate pro-survival signals by forming homo- or hetero-dimeric complexes on the cell surface. Inhibition of one of these receptors is often compensated by other human epidermal growth factor receptor family members or activation of other receptor tyrosine kinases (Yarden and Sliwkowski, 2001; Hynes and Lane, 2005; Hynes and MacDonald, 2009). To counter such tumor escape from single agent therapy, combinations of targeted therapies, as well as multi-specific low molecular weight inhibitors are being developed and have already entered clinical trials (Pivot *et al.*, 2011).

Bispecific antibodies (BiAbs) provide another option to combine two tumor treatment approaches in a single therapeutic molecule. Using multi-specific antibodies rather than exploiting the polypharmacology of certain small molecule kinase inhibitors has the clear advantage that the target combination can be freely chosen and is clearly defined, whereas the combination of kinases that are hit by the same ATP-competitive small molecule inhibitor is dictated by similarities in sequence and structure of the ATP-binding site (Vieth *et al.*, 2005). While there is already clinical proof of concept for BiAbs recruiting immune effector cells, like bispecific T-cell engaging antibodies, BiAbs aimed at inhibiting signaling of two different tumor cell surface targets are just emerging in clinical trials (Chames and Baty, 2009a,b; Thakur and Lum, 2010). This delay is due to our still incomplete understanding of the complex biology of signaling networks that allows tumors to escape from targeted therapy by using certain alternative signaling routes. For treatment of diseases where ErbB receptor signaling is supposed to play a role, MM-111, targeting Her2/ErbB3 heterodimers (Nielsen *et al.*, 2008), and MEHD7945A, targeting EGFR/ErbB3 heterodimers (Schaefer *et al.*, 2011), are considered promising combinations and both molecules have entered clinical trials (cf. clinicaltrials.gov).

Regarding the structural properties and possible formats of such molecules, a variety of bispecific constructs have been described in the past (Nieri *et al.*, 2009; Kontermann, 2010; Thakur and Lum, 2010). It has also been demonstrated that BiAbs can bind to both antigens as well as FcγR family members simultaneously and therefore retain effector functions (Seimetz *et al.*, 2010). For therapeutic applications, the selection of an appropriate format is directed by the biology of the targets (e.g. inhibitory, agonistic or downregulating

[†]Both authors contributed equally to this work.

antibody), as well as technical developability (Filpula, 2007; Mansi *et al.*, 2010). As a consequence of this, the complexity of analyzing the binding properties of bi- or multi-specific antibodies increases with each additional specificity. Yet a thorough understanding of the binding properties is important since they affect efficacy.

In this work, we examined currently known resistance mechanisms in ErbB signaling, namely activation of the receptor tyrosine kinases cMet and IGF1R (Hynes and Lane, 2005), and evaluated the feasibility of generating novel trispecific antibodies which are either mono- or bivalent for some of these targets. For inhibition of ErbB signaling, inhibitory antibodies against EGFR and Her3 were selected (Yarden and Sliwkowski, 2001). They were combined with an antagonistic IGF1R antibody, since IGF1R can compensate for inhibition of EGFR (Hendrickson and Haluska, 2009; van der Veeken *et al.*, 2009). We also combined them with a c-Met targeting antibody, since pre-clinical and clinical findings underscore the importance of cMet activation in ErbB signaling compromised tumor cells (Karamouzis *et al.*, 2009; Bonanno *et al.*, 2011). To fully exploit all antibody properties, Fc-containing scaffolds were chosen as these retain all possible effector functions and maintain the regular long serum half-life of an IgG antibody (Roopenian and Akilesh, 2007; Nimmerjahn and Ravetch, 2008).

By means of comprehensively analyzing their molecular features, we demonstrate the feasibility of generating trispecific antibody molecules, and investigate their simultaneous binding to all antigens, as well as provide evidence that these antibodies can bind at least with two specificities simultaneously on cells. Finally, the trispecific antibodies maintain all features of their parental antibodies and inhibited receptor activation equivalent to the parental antibodies, which makes them ideal candidates for future applications as anti-cancer agents.

Materials and methods

Cell culture

BxPc3 were obtained from ATCC. Cells were maintained in RPMI1640 medium supplemented with 10% fetal calf serum (FCS), non-essential amino acids and 2 mM L-glutamine (Gibco). Propagation of cells followed standard cell culture protocols.

Antibodies and reagents

For immunoblot analysis p-EGFR (Epitomics), EGFR (Millipore), Her3, IGF1R (Santa Cruz), p-Her3, p-cMet, cMet, p-IGF1R (CST) and β-actin (Abcam) were purchased. For fluorescence-activated cell sorting (FACS) analysis human IgG_1 Mab versions of the TriMAbs were used for the determination of cell surface receptor expression. An α-human Alexa488 antibody (Invitrogen) was used as secondary antibody. Ectodomain Fc-chimera of EGFR, Her3, cMet with C-terminal His tag and FcγRIIIa were purified from cell culture supernatants of transiently transfected eukaryotic cells. Recombinant IGF1R was purchased (R&D). Human growth factor (HGF), heregulin, epidermal growth factor (EGF) and insulin-like growth factor (IGF) were purchased. Antibody sequences were derived from available patents (Kuenkele *et al.*, 2005; Dennis *et al.*, 2007; Bossenmaier *et al.*, 2011; Umana and Mossner, 2011).

Design, cloning and production of TriMAbs

Sequences containing variable regions were ordered as gene synthesis with flanking restriction sites (GeneArt). Sequences were cloned in mammalian expression vectors with a cDNA organization of the antibody backbone. Antibody chains were transiently co-transfected in HEK-293F cells (Invitrogen) and purified as described (Metz *et al.*, 2011). Antibody homogeneity was analyzed using an Agilent HPLC 1100 (Agilent Technologies) with a TSK-GEL G3000SW column (TosoHaas Corp.). Individual specificities of the MAbs are indicated by MAb <specificity>.

Dynamic light scattering analysis of TriMAbs

Molecule stability was determined by dynamic light scattering (DLS) using a DynaPro Plate Reader. Samples were filtered through a 0.45 μm 384-well filter plate into a 384-well clear bottom plate and covered with 15 μl of paraffin oil followed by a centrifugation step (1 min/1000 × *g*). Five acquisitions with 10 s acquisition time and five acquisitions with 20 s acquisition time were performed for temperature ramping and temperature stability experiments, respectively.

FACS competition experiments

For competition experiments, a 3-fold dilution series of either unlabeled Fabs or TriMAbs ranging from 100 to 0.002 μg/ml was prepared which also contained 1 μg/ml of AlexaFluor647 (Invitrogen) labeled MAbs. This mixture was added to a suspension of 2×10^5 BxPc3 cells. After 45 min of incubation cells were washed twice and subjected to flow cytometry (BD, FACS Canto).

Immunoblot

A total of 7×10^5 BxPc3 cells were seeded the day prior the experiment in starvation medium containing 0.5% FCS. The following day, cells were pre-incubated 30 min with 0.07 μM of the indicated antibodies upon which stimulation for 10 min with growth factors EGF (50 ng/ml), HGF (30 ng/ml), IGF (50 ng/ml) and Heregulin (500 ng/ml) followed. Upon cell lysis protein lysates were subjected to immunoblot analysis.

Proliferation assay

A total of 2500 BxPc3 cells per well were seeded the day prior to the experiment in 96-well plates in medium with 10% FCS. The following day, TriMAbs were added in the indicated concentrations and cells were maintained for a total of 144 h after antibody addition at 37°C/5% CO_2. Proliferation was assessed by cell titer glow assay (Promega) in an Infinite M200 reader (Tecan).

Surface plasmon resonance

All experiments were performed on Biacore B3000, T100 and T200 instruments in running buffer phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween20. Dilution buffer consisted of running buffer supplemented with 1 mg/ml bovine serum albumin. Standard amine coupling to 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride/N-hydroxysulfosuccinimide activated chip surfaces were performed as recommended by the provider GE Healthcare.

Kinetic characterization of single antigen binding to TriMAbs

Signals were double referenced against blank buffer and a flow cell containing no ligand. Kinetic constants were calculated from fitting to a 1 : 1 Langmuir-binding model (RI 0). TriMAbs or MAbs were captured via α-human kappa light chain (Dako), human Fab binder (GE Healthcare) or α-human Fc (JIR). Series with increasing antigen concentrations were analyzed with an association phase of 180 s and dissociation phase of 800 1800 s depending on the k_d-rate. Capture antibodies were regenerated with 10 mM glycine, pH 1.5 (25°C) or 1.75 (37°C), or for human Fab binder as recommended by the vendor. Monomeric cMet, Her3 and EGFR were analyzed in concentrations from 4.94 to 1200 nM in triplicates on a CM5 sensor chip at 37°C. For the dimeric antigen IGF1R a sensor chip C1 was used at 25°C, with concentrations of 2.7 400 nM, one of these as duplicate.

Simultaneous in-solution binding of all antigens to TriMAbs

TriMAbs were captured via α-human Fc on a C1-Chip. Four antigens were injected consecutively using two dual injects with a contact time of 180 s each. The antigen concentration was chosen for each antigen close to saturation (~90%) as observed in the kinetics experiment. As control a second inject of the identical antigen did not raise response level, demonstrating equilibrium was reached (cMet: 1200 nM, EGFR: 1000 nM, Her3: 1000 nM and IGF1R: 400 nM). A temperature of 25°C was chosen to minimize dissociation.

Binding of FcγRIIIa to TriMAbs in presence of all antigens

EGFR was amine coupled on a C1 sensor chip. TriMAb/MAbs-binding EGFR were injected, followed by a dual inject of the remaining antigens (first inject: Mix Her3/cMet, second inject: IGF1R). The binding of FcγRIIIa was measured by a subsequent inject with 180 s association and 600 s dissociation phase at 25°C. Regeneration was performed with 15 mM NaOH.

Cooperative binding of TriMAbs to mixture of antigens on the chip surface

PentaHis antibody (Qiagen) was immobilized on a CM5 sensor chip with high ligand density (15 000 RU). His-tagged IGF1R and His-tagged Fc chimera of cMet, EGFR and Her3 were captured either as single antigens or a 1 : 1 : 1 : 1 mixture by volume. Single antigen concentrations were adjusted by a 1 : 3 dilution with buffer. MAbs and TriMAbs were injected as analytes (c 30 nM) with an association phase of 180 s and a dissociation phase of 1800 s. To obtain faster dissociation and clear avidity effects the experiment was performed at 37°C. Regeneration: 10 mM glycine pH 2.0

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) experiments were carried out using an iTC200 from MicoCal Inc. (Northampton, MA, USA) at 25°C. To avoid buffer artifacts all protein samples were dialyzed against PBS at 4°C. For further reference purposes the calorimetric dilution effect of dialyzed buffer as well as every other particular titrant was evaluated in advance. Eighteen automatically defined injections of 2 μl over 5 s and a syringe stirring of 600 rpm were used as overall settings. While highest possible concentrations (15 38 μM) were used for the soluble receptor titrants in the syringe, 1.5 1.8 μM of the particular MAb in the mess cell were applied. Data analysis was performed with 'Origin' (supplied by Microcal Inc.). Data points were fitted to a theoretical titration curve, resulting in ΔH (binding enthalpy in kcal mol ^{1}), K_A (association constant) and n (number of binding sites per monomer). In consecutive injects of several titrants alterations in mess cell concentrations were corrected (for any further titrant) by defining end

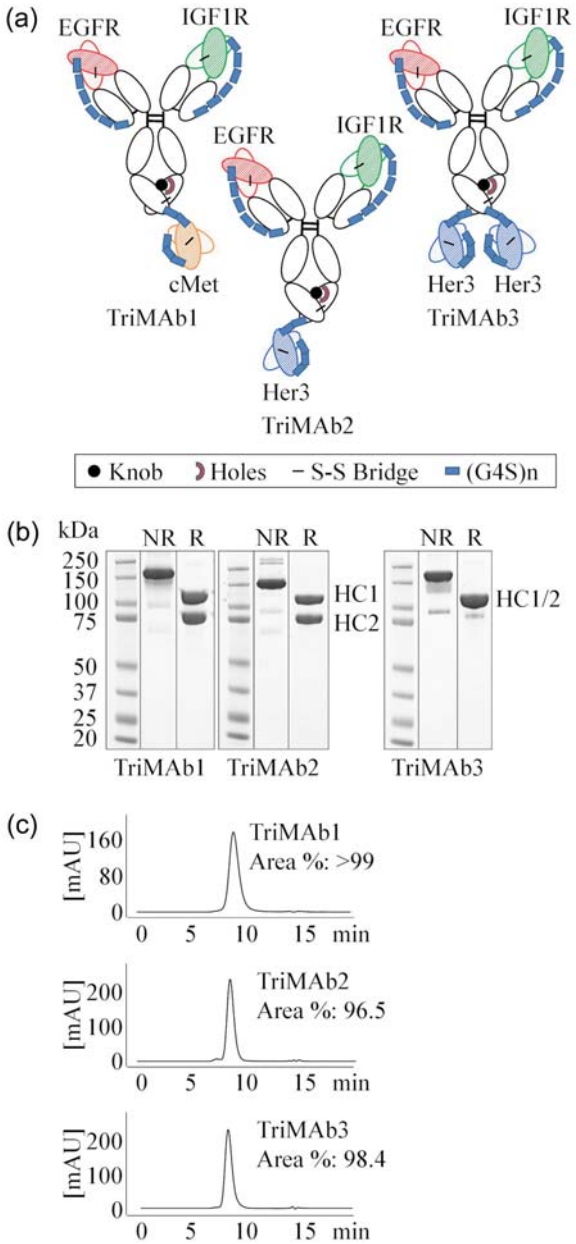

Fig. 1. Production of trispecific antibodies based on scFab and scFv. (**a**) Schematic presentation of trispecific antibodies. HCs are distinguished by knobs into hole technology. scFabs were constructed by V_L C_L $(G_4S)_6$ V_H C_{H1} fusion to the constant regions of human IgG_1. scFv were fused with a $(G_4S)_2$ connector to the C terminus of the HC in the order of V_H $(G_4S)_3$ V_L. (**b**) Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of protein A and size exclusion purified TriMAbs under non reducing (NR) and reducing (R) conditions. (**c**) Analytical HPLC of TriMAbs. A colour version of this figure is available as supplementary data at *PEDS* online.

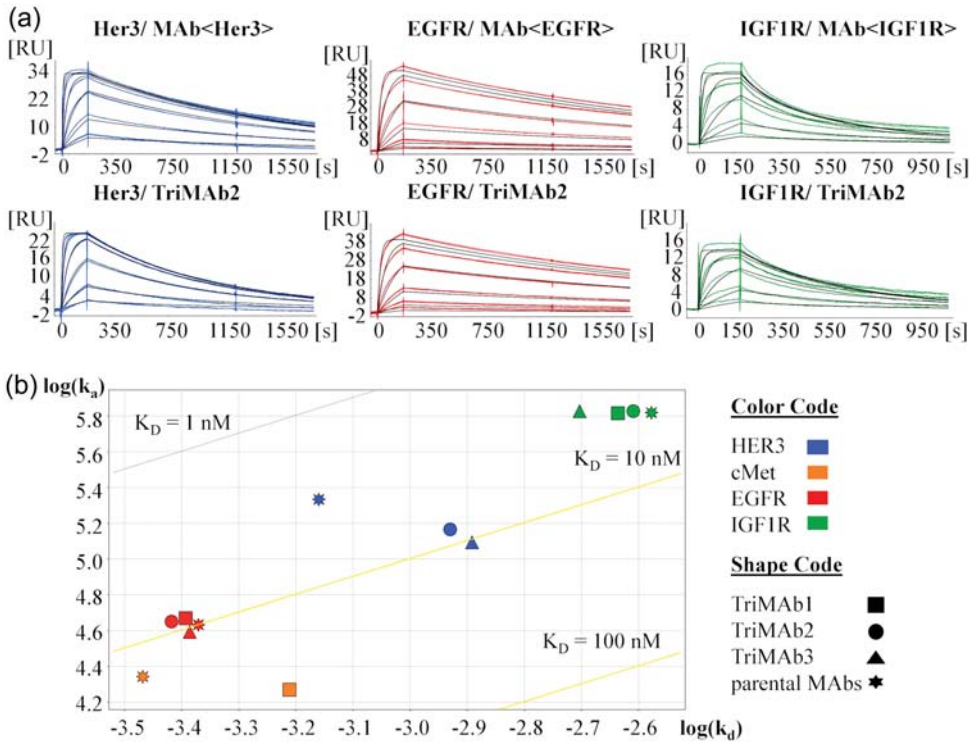

Fig. 2. (**a**) SPR sensorgrams (concentration series) of soluble receptor binding to parental MAb<Her3, EGFR, IGF1R> and TriMAb2. Sensorgrams were fitted to a Langmuir 1 : 1 model, RI = 0 (black lines). (**b**) Plot of the kinetic constants of TriMAb1, 2, 3 and their corresponding parental MAbs for binding soluble receptors Her3, cMet, EGFR and IGF1R, as measured by SPR. Diagonals depict iso affinity lines. A colour version of this figure is available as supplementary data at *PEDS* online.

Table I. Kinetic constants for binding of soluble receptors to parental MAb<Her3, EGFR, IGF1R> and TriMAbs

Ligand	Analyte	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	t(1/2) (min)	KD (M)	% SE (k_a) (%)	% SE (k_d) (%)	T (°C)
MAb<Her3>	Her3	2.1E + 05	6.9E 04	16.7	3.2E 09	0.2	0.1	37
TriMAb2	Her3	1.5E + 05	1.2E 03	9.8	8.0E 09	0.2	0.1	37
TriMAb3	Her3	1.2E + 05	1.3E 03	9.0	1.0E 08	0.2	0.1	37
Mab<cMet>	cMet	2.2E + 04	3.4E 04	33.9	1.6E 08	0.1	0.2	37
TriMAb1	cMet	1.9E + 04	6.1E 04	18.8	3.3E 08	0.2	0.2	37
Mab<EGFR>	EGFR	4.3E + 04	4.3E 04	27.1	1.0E 08	0.2	0.3	37
TriMAb1	EGFR	4.7E + 04	4.0E 04	28.6	8.7E 09	0.2	0.2	37
TriMAb2	EGFR	4.5E + 04	3.8E 04	30.2	8.5E 09	0.2	0.3	37
TriMAb3	EGFR	3.9E + 04	4.1E 04	28.1	1.1E 08	0.2	0.3	37
Mab<IGF1R>	IGF1R	6.6E + 05	2.6E 03	4.4	4.0E 09	2.3	1.9	25
TriMAb1	IGF1R	6.6E + 05	2.3E 03	5.0	3.5E 09	1.8	1.5	25
TriMAb2	IGF1R	6.7E + 05	2.5E 03	4.7	3.7E 09	2.1	1.8	25
TriMAb3	IGF1R	6.7E + 05	2.0E 03	5.8	3.0E 09	1.3	1.1	25

point concentrations of one titration as starting concentrations for the next titration.

Results

Generation of trispecific antibodies

We selected one TriMAb format which enabled monovalent binding to each antigen and one which was bivalent for Her3 (Fig. 1a). To this end, the knobs-into-holes technology was used to differentiate the IgG_1 heavy chains (HCs) (Ridgway *et al.*, 1996; Atwell *et al.*, 1997). Light chain mispairing was prevented by employing the single chain Fab (scFab) and single chain Fv (scFv) technology (Fig. 1a). scFab and scFv formats have been described in the past (Kontermann, 2010). Antibodies were transiently expressed in HEK-293F and purified by standard Protein A and size-exclusion chromatography. Gel electrophoresis, analytical high-performance liquid chromatography and mass spectroscopy (Fig. 1b, c and data not shown) confirmed homogeneity greater than 95%.

Kinetic characterization of single antigen binding to TriMAbs

For each of the four different antigens cMet, Her3, IGF1R and EGFR recognition by the TriMAbs1, 2 and 3 was compared with the corresponding parental antibodies using surface plasmon resonance (SPR). TriMAbs were captured on a sensor chip and the binding kinetics of the soluble receptors was measured using a concentration series for each antigen in separate runs. To verify that antigen binding was not impaired by the capture method, three different setups were examined. To this end, human specific antibodies

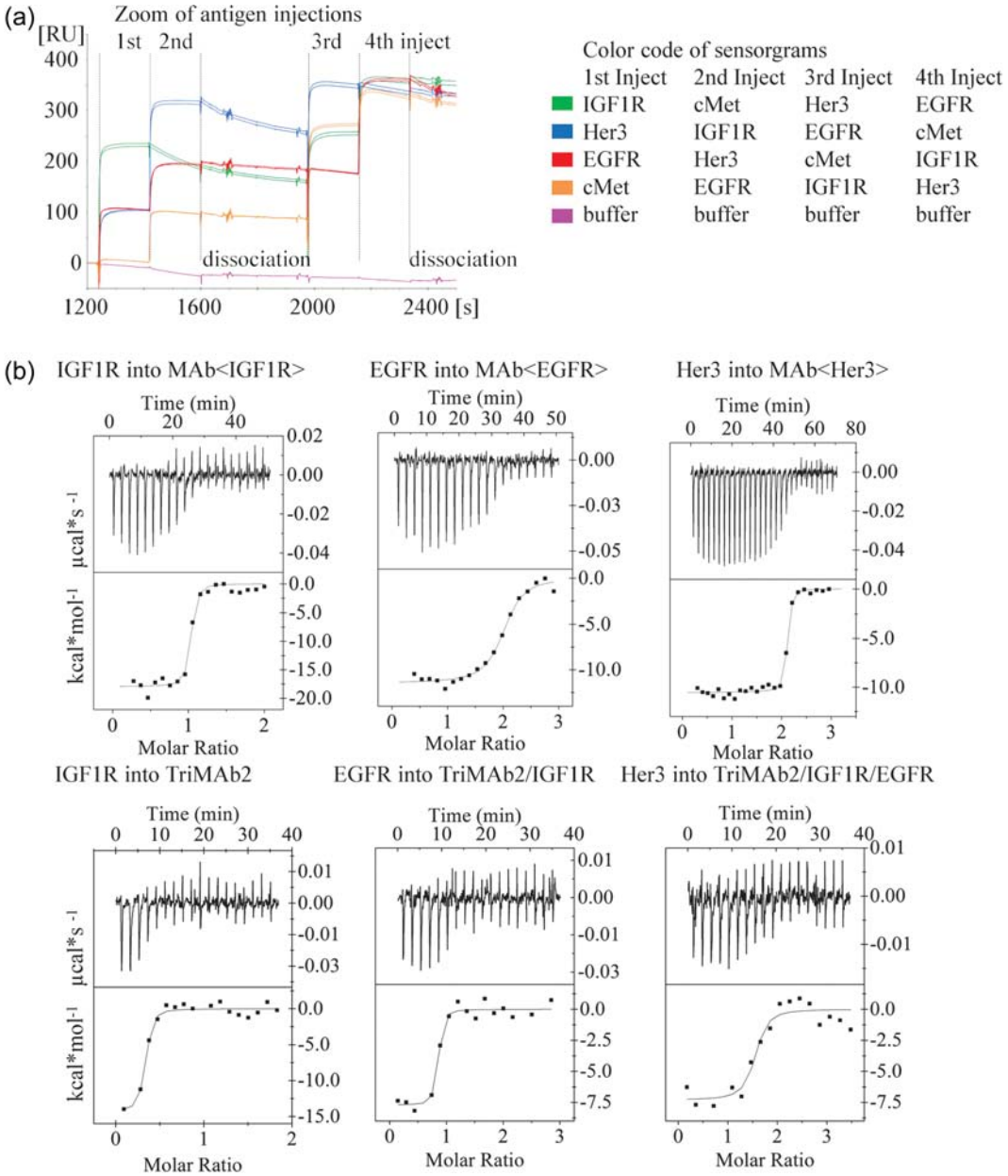

Fig. 3. (**a**) Overlay of SPR sensorgrams showing simultaneous binding of EGFR, IGF1R and Her3 (plus cMet as negative control) to TriMAb2. TriMAb2 was captured onto the sensor chip and binding of antigens studied by four consecutive injects (with a technical lack phase after second inject) of soluble receptors, permutating the order in different runs. Each run is highlighted by a different color. (**b**) Heat of receptor binding to MAbs measured by ITC and fitted to a 1 : 1 binding event curve. Top panel: soluble receptors titrated into a solution of their corresponding parental MAb in three independent experiments. Bottom panel: the three receptors titrated one after the other into the same solution of TriMab2. The consecutive titrations are evaluated and depicted separately. A colour version of this figure is available as supplementary data at *PEDS* online.

against kappa light chain, the Fab moiety or the constant Fc were used for antibody capturing. Exemplary binding of one of the antigens (EGFR) showed only minimal changes in the kinetic constants (data not shown). For IGF1R the assay setup was modified to account for its homo-dimeric structure. To obtain monovalent binding, a C1 chip with very low ligand density and thus capture level of the antibodies was chosen (~8 RU). In a control experiment it was demonstrated with the Fab fragment of the parental IgG_1 antibody that the k_d-rates of both are comparable under the selected conditions. Similar results were obtained in a reversed assay format with amine-coupled receptor and Fab fragment (data not shown). For quantitation of IGF1R binding kinetics the temperature was reduced to 25°C to obtain k_a-rates within the instrument limitations, since the parental MAb<IGF1R> has a very high k_a. Upon capturing of TriMAbs by Fc-specific antibodies, it was found that all TriMAbs were functional in binding each of the single antigens and moreover retained kinetic profiles comparable to that of their parental MAbs as exemplarily shown for TriMAb2 (Fig. 2a). To better visualize this and allow relative comparison of all three TriMAbs we chose to deconvolute kinetics in a log(k_a) log(k_d) plot (Fig. 2b). Whereas the scFab moieties bound EGFR and IGF1R with virtually the same affinity as the parental MAbs, we found that the affinity of the scFv moieties for Her3 or cMet was slightly reduced

Table II. Quantification of receptor molecules simultaneously bound by TriMAbs

TriMAb	First antigen bound (%)	Second antigen bound (%)	Third antigen bound (%)	Fourth antigen bound (%)
	IGF1R	cMet	Her3	EGFR
TriMAb1	73	73	0	90
TriMAb2	72	0	89	108
TriMAb3	69	0	88	82
	Her3	IGF1R	EGFR	cMet
TriMAb1	0	71	89	89
TriMAb2	99	69	85	0
TriMAb3	95	64	84	0
	EGFR	Her3	cMet	IGF1R
TriMAb1	97	0	97	61
TriMAb2	97	90	0	71
TriMAb3	97	88	0	49
	cMet	EGFR	IGF1R	Her3
TriMAb1	103	87	60.5	0
TriMAb2	0	93	63	63
TriMAb3	0	92	61	59

Hundred percent theoretical maximum is deducted from the known capture level of TriMAbs in this experiment, where response units are directly proportional to molecular weight. A second inject of the same receptor did not increase binding (not shown).

by a factor of 2 3 (Table I). Slight deviation from Langmuir 1 : 1 fits (RI 0) or exceeding of the theoretical R_{max} observed in some cases was most likely due to small amounts of aggregates in the antigen batches used, but no difference between the parental and the TriMAbs was observed. Deviations from Langmuir 1 : 1 binding were most apparent for the IGF1R specificity, but are likely intrinsic to the antibody clone as a different control antibody binding the same epitope region did not show this phenomenon (data not shown). Thus, we could demonstrate that all TriMAbs have similar monospecific-binding properties like the corresponding MAbs.

Simultaneous in-solution binding of all antigens to TriMAbs

Having shown that all antigen-binding moieties of the TriMAbs were *per se* functional, we next addressed the question whether several of the antigens could be bound simultaneously or whether steric hindrance between the large receptor molecules would impede this. Antibodies were captured via their Fc part and exposed to soluble receptor injected as analyte. Analyte concentrations were set to achieve near saturation (>90% of theoretical R_{max}) of all MAb-binding sites during the ~180 s association phase. Immediately following the association of the first receptor, the second receptor was injected in 'dual injection mode' leading to a ternary complex with the MAb. Finally, the third receptor was injected leading to a stepwise rise in the SPR signal (Fig. 3a). In several runs, the sequence of antigen injections was permutated as exemplary shown for TriMAb2 (Fig. 3a). At 25°C the concurrent dissociation of the first antigens during the course of these experiments was generally low and therefore a qualitative interpretation of the events was possible. TriMAbs 1, 2 and 3 showed subsequent binding of all three antigens (Table II). SPR signals were in all cases close to the theoretical R_{max}, which indicated that binding of the second and third antigen was not significantly hindered by already bound antigen. This was also valid for TriMAb3 which can in theory bind a total of four receptor molecules (IGF1R, EGFR, 2× Her3). Only with IGF1R, which is a naturally cysteine-bridged homo-dimer, a slight effect on subsequent cMet binding was observed for TriMAb1. The findings have been summarized in a quantitative manner for all TriMAbs in Table II. It is of note that for homo-dimeric IGF1R the theoretical R_{max} is between 50 and 100% since a significant portion of this antigen is bound bivalently by two neighboring TriMAb molecules at the chosen ligand density. In summary, we demonstrate that all TriMAbs can simultaneously bind to all antigens.

Antigen binding to TriMAbs in solution via ITC

SPR-binding experiments were complemented by ITC which yields a more direct measurement of the stoichiometry. TriMAb2 in solution was titrated consecutively with all three antigens, and compared with corresponding titrations of the parental MAbs. Fitting of the observed heat effect to 1 : 1 binding events confirmed the simultaneous binding of all three receptors to TriMAb2 (Fig. 3b). Binding enthalpies were similar to those of the parental MAbs and in the same range for all antigens. The dimeric IGF1R showed a molar ΔH which was approximately twice that of the other monomeric receptors.

Cooperative binding of TriMAbs to a mixture of antigens

The aforementioned experiments confirmed that TriMAbs are able to bind all their antigens simultaneously. On cells the conformational freedom is much more restricted and antibody antigen interactions are limited to certain geometries. To better approximate the steric situation on a cell surface, we looked at cooperative binding of soluble MAbs to different receptor molecules fixed on the sensor chip surface. Cooperative binding should be detectable as much lower dissociation rate of the MAb due to an avidity effect, compared with monovalent binding of only a single antigen. A roughly equimolar mixture of all receptor ectodomains (IGF1R, EGFR, Her3 and cMet) or binary mixtures (IGF1R and Her3, IGF1R and cMet) were captured onto the chip via their His tag by a PentaHis-antibody. As control, single antigens were captured on other flow cells. To demonstrate that the chosen antigen density was high enough to allow avid binding, each of the parental antibodies was analyzed as positive control. The parental IgG antibodies indeed bound bivalently to both their single antigen and the mixture of all antigens, as judged by the observed low k_d-rates compared with previous experiments (Table I). TriMAbs 1 and 2 on the contrary are only able to bind monovalently to each antigen and showed marked dissociation (Fig. 4a) from single antigen surfaces. In contrast, when a mixture of the antigens was presented, the TriMAbs showed the expected avidity effect and a significantly decreased dissociation rate constant k_d. These results imply cooperative binding of at least two antigens. We sought to confirm these findings on cells. A cell line expressing all four receptors, preferably with one of the receptors, which can mediate the avidity effect, in excess, was selected. BxPc3 cells were selected by their mRNA profile and receptor expression confirmed by flow cytometric analysis (Fig. 4b). To a suspension of these cells, a dilution series containing a constant concentration of labeled bivalent MAb<IGF1R> and increasing amounts of

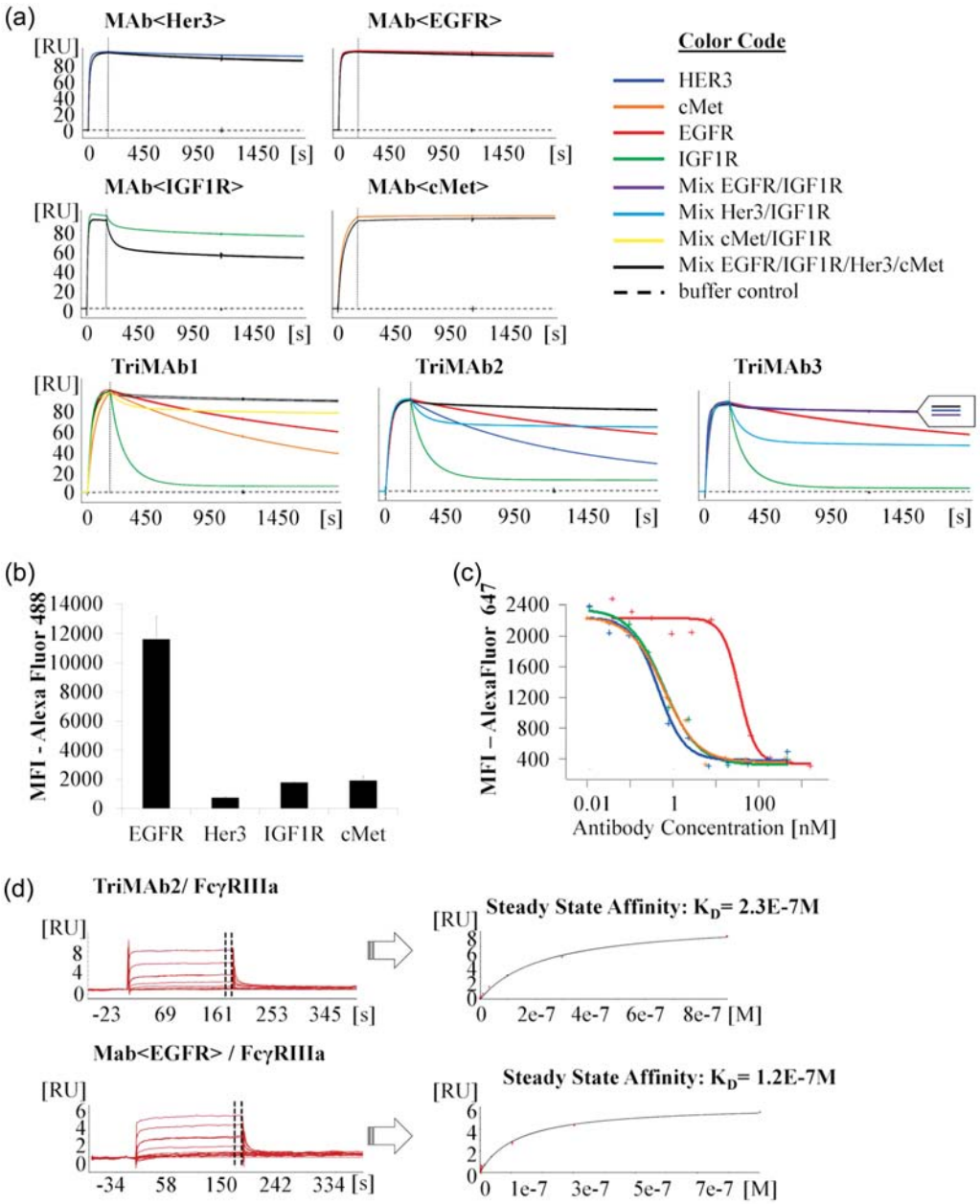

Fig. 4. (**a**) SPR sensorgrams showing association and dissociation of parental MAbs and TriMAbs to chip surfaces coated with single antigens or mixtures of antigens in high density, as indicated by color code. Bivalent MAbs bind with avidity effect and dissociate slowly from either surface. TriMAbs dissociate slowly only from surfaces with mixtures of antigens, indicating cooperative binding to different antigens. (**b**) FACS based analysis of cell surface receptor expression in BxPc 3 cells (mfi = mean fluorescence intensity). (**c**) FACS based avidity assay in BxPc 3 cells (red = Fab<IGF1R>; green = TriMAb1; blue = TriMAb2 and orange = TriMAb3). (**d**) SPR sensorgrams of FcγRIIIa binding to TriMAb2 and parental MAb<EGFR> in the presence of antigens. FcγRIIIa association and dissociation was detected in a rising concentration series. Because of very fast k_a and k_d rates, K_D was calculated from steady state. Averaged equilibrium response R(eq) at the indicated time point of the association phase were plotted against concentration of FcγRIIIa (fitted with steady state model). A colour version of this figure is available as supplementary data at *PEDS* online.

unlabeled Fab or TriMAb molecules was added. The assumption was that TriMAbs will much more efficiently compete for IGF1R binding than the Fab<IGF1R> due to additional avidity mediated by the EGFR, Her3 or cMet specificity. As expected, a 56-fold reduction in the EC50 for the TriMAbs was found which implies avid binding on the cell surface (Fig. 4c). These data were in accordance with the findings on the sensor chip in which a strong avidity effect was observed for the EGFR/IGF1R mixture in contrast to IGF1R only. Finally, we obtained similar findings on cells, if Her3 or cMet were targeted instead of the IGF1R (Supplementary Fig. S1A and B). Thus, the artificial setup on a sensor chip can mimic effects found on living cells.

Simultaneous in-solution binding of antigens and FcγRIIIa to TriMAbs

To examine whether simultaneous complexation of several antigens would impair binding of TriMAbs to FcγRIIIa, a soluble construct of the FcγRIIIa ectodomain was injected as the last analyte, subsequently to saturating the TriMAbs with all other antigens. For this, the first antigen, EGFR, was immobilized on the sensor chip and used to capture the

TriMAbs, since capturing via anti-human Fc antibodies partially blocked the FcγRIIIa-binding site on the Fc part of the IgG MAbs. After complexation of all antigens, the TriMAbs still displayed high nanomolar affinity for FcγRIIIa (TriMAb1/2/3 K_D: 222, 232, 254 nM) which is in the range of standard IgG_1 antibodies (MAb<EGFR> K_D: 120 nM) (Fig. 4d). Thus, according to the nomenclature of Triomabs (Trion) our TriMAbs could be called tetraspecific (Seimetz *et al.*, 2010).

Inhibition of receptor signaling and cellular growth by TriMAbs

Cell surface expression and activation status of all receptors was confirmed in BxPc3 in the presence or absence of supplemented growth factors (Fig. 5a). Addition of TriMAbs inhibited ligand-dependent receptor phosphorylation. To further address the functional activity of TriMAbs a proliferation assay was performed and activity of individual antibodies or combinations was compared with TriMAb activity (Fig. 5b). We observed significant growth inhibitory effects for combined targeting of EGFR, IGF1R and Her3 but not for TriMAb 1 containing a cMet specificity. Neither of the single parental antibodies had significant effects on proliferation in BxPc3 (data not shown). In conclusion, TriMAbs were as efficacious as the combination of all three parental antibodies (Fig. 5b).

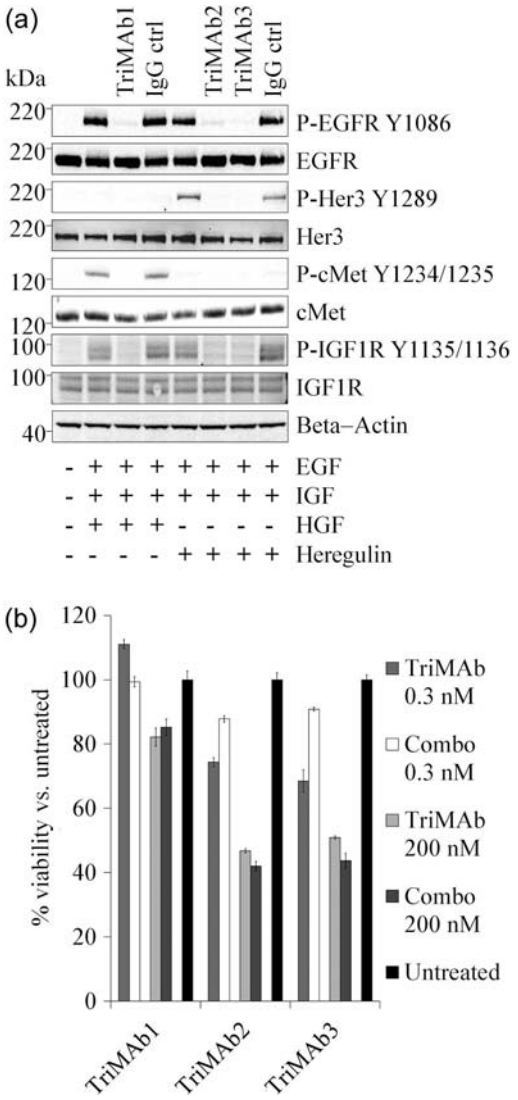

Fig. 5. Functional analysis of TriMAbs in BxPc3. (**a**) Immunoblot analysis of receptor expression and phosphorylation in BxPc3. (**b**) Proliferation assay with TriMAbs in comparison to the relative combinations of the three parental antibodies. Percentage viability was evaluated versus controls (set to 100%). Presented is the mean of two independent experiments.

Discussion

We present here the generation of trispecific antibodies for cancer therapy. The chosen antibody scaffold admittedly poses some challenges with regard to production and characterization. Which titers and purity can be obtained in stable chinese hamster ovary production cell lines remains to be seen as this cannot be predicted from our results with transient expression in HEK-293F. Stability analysis of the generated TriMAbs revealed that TriMAb1 had a melting curve well above 60°C and displayed long-term stability at elevated temperatures (Supplementary Fig. S2A and B). The other two TriMAbs were less stable with partial unfolding already at 45°C. Since stable and less stable TriMAbs only differed in the Her3 scFv, clone specific variable region differences seem to have affected the stability of our TriMAbs. Such clonal variation is also observed for regular MAbs and does therefore not pose a special threat for further development of this antibody format.

The characterization of TriMAbs with regard to their binding and functional properties presents additional challenges in comparison to BiAbs. First and foremost, the analysis of antigen binding is more complex and the important question, whether such molecules indeed have the capacity to simultaneously bind to different tumor antigens has to be addressed for each combination individually. Our findings that simultaneous binding to three large extracellular domains of receptor tyrosine kinases is in principle feasible implies that there is a surprisingly high flexibility in the binding of multiple antigens. Nevertheless, simultaneous binding of three soluble target proteins certainly poses less steric constraints than simultaneous binding to three membrane anchored antigens on a living cell.

On the cell surface, lateral diffusion, steric hindrance by other proteins or variable antigen availability due to endocytosis or receptor shedding might impair accessibility. In order to more closely mimic cell membrane conditions, we developed an experimental approach in which different antigens are simultaneously bound to a chip surface. We challenged this artificial setup by comparison with a cell line expressing all receptors. In this cellular competition experiment we found a good correlation with the data obtained by SPR and could additionally demonstrate that our TriMAbs display avidity due to at least bispecific binding. This suggests that a mixture of antigens bound to the chip surface can serve as a surrogate setup for direct cell surface analysis.

Furthermore, we confirmed binding of FcγRIIIa ectodomain to the Fc part of TriMAbs. It is particularly interesting that the FcγRIIIa Fc interaction was not only unimpaired by scFv fusions at the C-terminus of the HCs, but also tolerated the concomitant presence of all three antigens. Hence, it can be expected that TriMAbs retain their effector cell recruitment potential also in cellular assays.

Based on these findings, we propose a novel technical approach whereby a combination of SPR, ITC and cellular

avidity assays quickly and accurately sheds light onto the binding properties of a chosen TriMAb combination; such data are of a quantitative nature when the first two methods are applied and of a semi-quantitative nature for the cellular assay setup. This novel approach can for instance support format optimization by permutation of the order of antigen-specificities on the Fab arms or on the HC fusion sides.

Interestingly, the presented TriMAbs did not exhibit agonistic activity as might have been expected from bringing different receptor tyrosine kinases in close proximity. This suggests that receptor cross-activation either requires a very specific spatial orientation of the interacting partners or that the receptors have to adopt an active conformation not compatible with TriMAb binding. From the perspective of therapeutic benefit and health care costs TriMAbs appear attractive, since we obtained similar functional activity with them as a single therapeutic agent as with a combination of three MAbs. However, other potential challenges that are outside the scope of this study, like their technical developability, potential immunogenicity and adverse effects, need to still be addressed before tri- or tetraspecific antibody formats can enter into clinical trials. In conclusion, a combined analysis of our data strongly supports the notion that TriMAbs present a powerful avenue to follow on the way to drugs which potently inhibit tumor and associated *de novo* escape mechanisms.

Supplementary data

Supplementary data are available at *PEDS* online.

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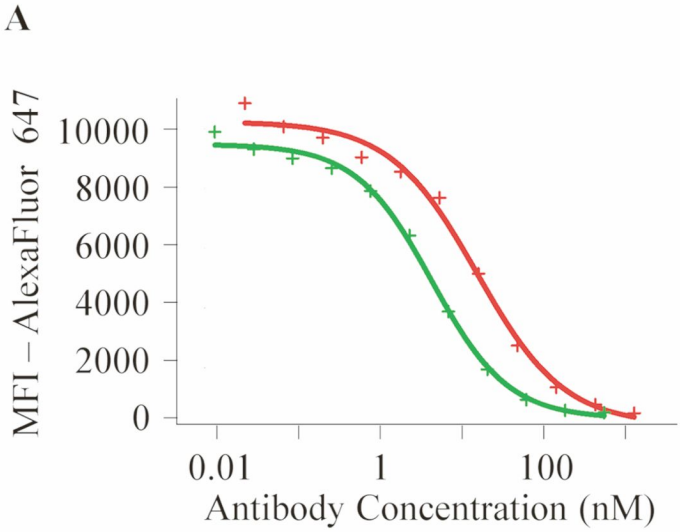
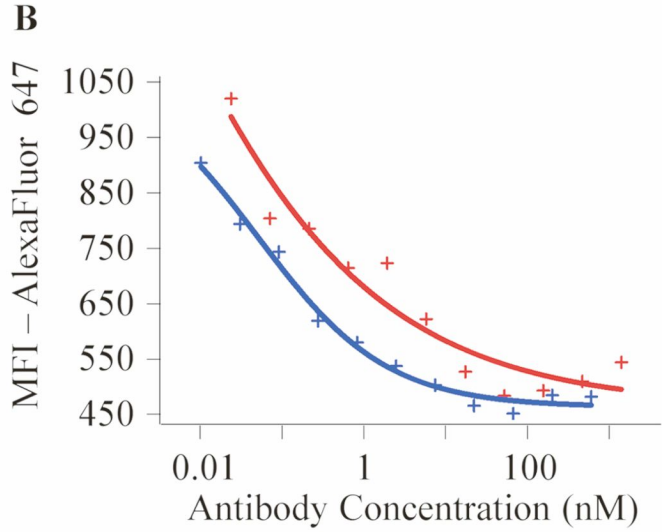
Supplemental Figure 1

FACS-based avidity assay in BxPc-3. (**A**) Competition of a dilution series of Fab<cMet> (red) against a constant concentration of bivalent MAb<cMet>. The competition curve for TriMAb1 (green) against a constant concentration of bivalent MAb<cMet> is shown. (**B**) Competition of a dilution series of Fab<Her3> (red) against a constant concentration of bivalent MAb<Her3>. The competition curve for TriMAb2 against a constant concentration of bivalent MAb<Her3> (blue) is shown.

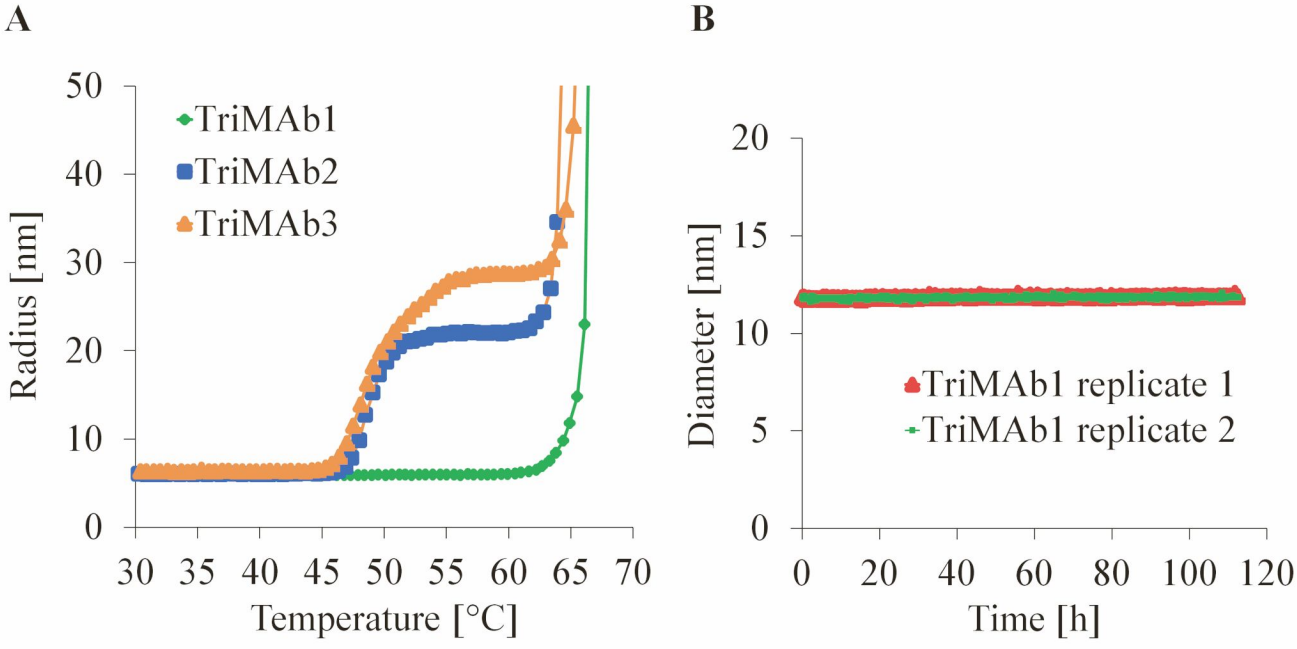
Supplemental Figure 2

Stability of TriMAbs measured by dynamic light scattering. (**A**) Plot of the change of the hydrodynamic radius of TriMAb1, TriMAb2 and TriMAb3 upon incremental (0.05°C/min) increase of temperature. (**B**) Temperature stability of TriMAb1 was measured over a period of 112 h at 50°C.





Castoldi et al., Supplemental Figure 1



Castoldi et al., Supplemental Figure 2

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6. Publications

6.1 Papers

1. **Castoldi R**, Ecker V, Wiehle L, Majety M, Busl-Schuller R, Asmussen M, Nopora A, Jucknischke U, Osl F, Kobold S, Scheuer W, Venturi M, Klein C, Niederfellner G, Sustmann C. A novel bispecific EGFR/Met antibody blocks tumor-promoting phenotypic effects induced by resistance to EGFR inhibition and has potent antitumor activity.
   Oncogene. 2013 Dec 12;32(50):5593-601. Epub 2013 Jul 1.

2. **Castoldi R**, Jucknischke U, Pradel LP, Arnold E, Klein C, Scheiblich S, Niederfellner G, Sustmann C. Molecular characterization of novel trispecific ErbB-cMet-IGFIR antibodies and their antigen-binding properties.
   Protein Eng Des Sel. 2012 Oct;25(10):551-9. Epub 2012 Aug 29.

3. Kobold S, Steffen J, Grassmann S, Henkel J, **Castoldi R**, Zeng Y, Schmollinger J, Schnurr M, Rothenfußer S, Sustmann C, Niederfellner G, Klein C, Bourquin C, Endres S. A new anti-EGFR anti-EpCAM bispecific antibody enhances the efficacy of adoptive T-cell therapy in a syngeneic gastric cancer-derived tumor model.
   2013, submitted to J Natl Cancer Inst; under revision.

6.2 Posters and presentations at conferences

1. **Castoldi R**, Weininger D, Gassner C, Klein C, Niederfellner G, Sustmann C. Generation and *in vitro* characterization of bispecific c-Met – HER1/HER2 antibodies for the treatment of cancer.
   PEGS Europe. October 2010 - Hannover (Germany) - Poster selected for oral presentation.

2. Kobold S, Steffen J, Grassmann S, Henkel J, **Castoldi R**, Schmollinger JC, Sustmann C, Niederfellner G, Klein C, Bourquin C, Endres S. A new EGFR x EpCAM bispecific antibody enhances the efficacy of adoptive T-cell therapy in a murine gastric tumor model.
   Cancer Immunology and Immunotherapy - Keystone Symposia. January 2013 – Vancouver (Canada).

6.3 Patent applications

1. **Castoldi R**, Haas A, Klein C, Schaefer W, Sustmann C. Multispecific antibodies. WO2013174873 (A1) – 2013-11-28.

2. Bourquin C, **Castoldi R**, Endres S, Klein C, Kobold S, Niederfellner G, Sustmann C. Bispecific antibody molecules with antigen-transfected T-cells and their use in medicine. WO2013113615 (A1) – 2013-08-08.

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Castoldi Raffaella

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