







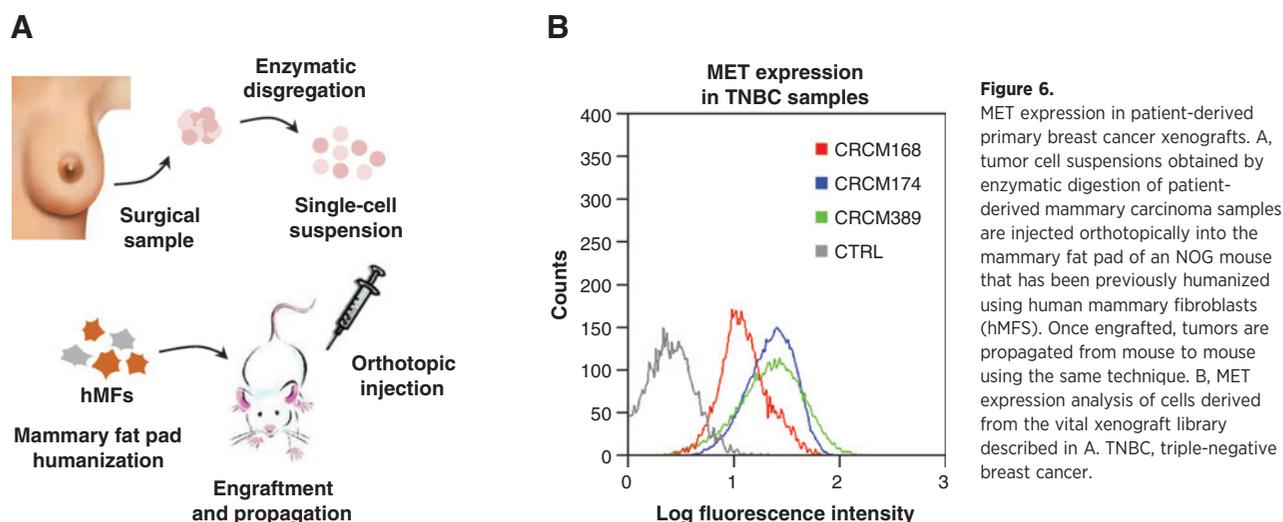








Hultberg et al.



**Figure 6.** MET expression in patient-derived primary breast cancer xenografts. A, tumor cell suspensions obtained by enzymatic digestion of patient-derived mammary carcinoma samples are injected orthotopically into the mammary fat pad of an NOG mouse that has been previously humanized using human mammary fibroblasts (hMFs). Once engrafted, tumors are propagated from mouse to mouse using the same technique. B, MET expression analysis of cells derived from the vital xenograft library described in A. TNBC, triple-negative breast cancer.

JNJ-38877605 used in these experiments achieved complete MET kinase blockade in all cells (Supplementary Fig S6C).

#### ARGX-111 kills MET-expressing cancer cells in the presence of NK cells

We investigated whether ARGX-111 could kill cancer cells by ADCC using a representative panel of human tumor cell lines expressing different MET levels. Cells were incubated with increasing concentrations of ARGX-111 (0–1,000 nmol/L) in the presence of NK cells, and tumor cell lysis was determined by a standard  $^{51}\text{Cr}$ -release assay. An irrelevant IgG1 and the fucosylated G52 antibody were used as controls. Each assay was repeated using effector cells derived from three different healthy donors (Supplementary Table S6). This analysis revealed that ARGX-111 induces dose-dependent lysis of all tumor cell lines tested with an efficacy directly proportional to MET protein expression levels. The fucosylated G52 antibody displayed only a modest ADCC activity on MKN-45 cells, which express very high MET levels, but did not have any effect on the other cell lines tested (Supplementary Fig. S7A). We also analyzed ARGX-111-induced antibody-dependent cellular phagocytosis (ADCP) using MKN-45 cells or 786-O human renal cell carcinoma cells and human monocyte-derived macrophages. Consistent with our finding that afucosylation does not significantly change antibody affinity for FcγRI (Supplementary Table S4), ARGX-111 did not display increased ADCP compared with G52 in any condition tested (Supplementary Fig. S7B).

#### MET-targeted ADCC kills mammary carcinoma stem cells

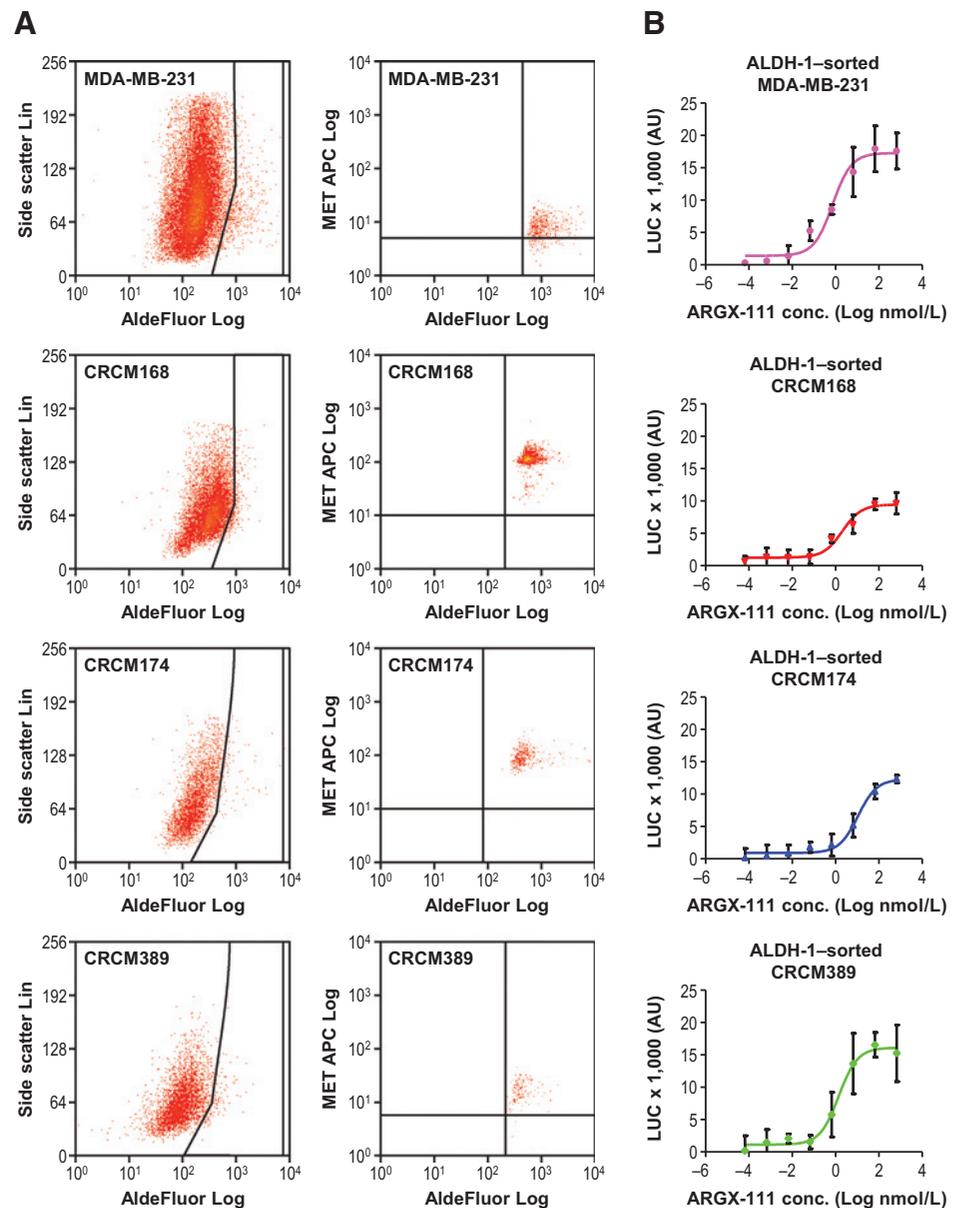
The results obtained in the orthotopic mammary carcinoma models indicate that MET-targeted ADCC impairs the ability of CTCs to colonize distant organs and to give rise to metastases. Because only tumor cells with stem-like characteristics are competent to originate secondary colonies, we hypothesized that the mammary carcinoma stem cell population may express MET. To test this hypothesis, we analyzed coexpression of MET with established breast cancer stem cell markers (33) in MDA-MB-231 tumors as well as in a vital library of patient-derived breast xenografts (CRCM tumors; ref. 25) generated by implantation of primary mammary carcinomas into immunodeficient mice (Fig. 6A). Interestingly, all triple-negative tumors from this library

expressed MET (Fig. 6B). Flow-cytometry analysis of CD24, CD44 and MET unveiled that the majority of cells in the CD44<sup>+</sup>/CD24<sup>lo</sup> population express MET in both the MDA-MB-231 cell line (99%) and the primary breast carcinoma samples (78%–98%; Supplementary Table S7). Similarly, dual staining with AldeFluor reaction and anti-MET antibodies revealed that ALDH-1-positive tumor cells express MET in all samples analyzed (Fig. 7A). Prompted by these data, we determined whether MET-targeted ADCC could kill mammary carcinoma stem cells *in vitro*. To this end, cells derived from MDA-MB-231, CRCM168, CRCM174, and CRCM389 tumors were stained with AldeFluor and then sorted by FACS. Sorted cells were incubated with increasing ARGX-111 concentrations (0–667 nmol/L) in the presence of bioluminescent ADCC reporter cells (34), and ADCC was determined by analyzing luciferase activity. Remarkably, ARGX-111 promoted dose-dependent ADCC of all stem cell populations analyzed (Fig. 7B). We conclude that MET-targeted ADCC may be an effective method to eliminate breast cancer stem cells.

## Discussion

HGF and its receptor MET have been attracting increasing interest in the last two decades as appealing targets for cancer therapy. This interest has led to the development of numerous targeted drugs, some of which have reached the clinic. The initial enthusiasm has been partially mitigated, however, by the realization that anti-HGF/MET drugs display cytostatic rather than cytotoxic activity in most systems, including cell-based assays, mouse models of cancer, and oncologic patients. In this study, we report the engineering of an antagonistic anti-MET antibody that, in addition to inhibiting HGF/MET signaling, kills MET-expressing tumor cells by enhanced ADCC. The data presented here suggest that depleting MET-positive cancer cells is therapeutically more effective than simply inhibiting HGF/MET signaling.

These results must be interpreted at the light of the role played by MET in cancer biology. It has been shown that tumors take advantage of HGF/MET signaling in two different situations, determined by the number of *c-MET* gene copies contained in their genome (22). Tumors bearing normal *c-MET* gene copy-number exploit paracrine HGF stimulation to proliferate in adverse

**Figure 7.**

MET-targeted ADCC kills mammary carcinoma stem cells. A, flow cytometry analysis of MET expression in ALDH-1<sup>+</sup> mammary carcinoma cells. B, ADCC assay using ALDH-1-sorted cells derived from enzymatic digestion of the tumors described in A. ADCC was determined using bioluminescent ADCC reporter cells. LUC, luciferase; AU, arbitrary units.

environmental conditions and to invade adjacent tissues, but do not strictly depend on HGF/MET signaling for their own survival. In this setting, inhibition of HGF/MET signaling attenuates proliferation and invasion, but does not eliminate MET-expressing cancer cells per se. On the other hand, tumors displaying high-grade focal *c-MET* amplification totally rely on constitutive HGF-independent MET signaling, resulting from MET protein overexpression, to sustain their proliferation and EMT phenotype (35, 36). In these tumors, MET inhibition leads to G<sub>1</sub> arrest and to transient reversion of EMT; however, both proliferation and EMT can be resumed upon removal of MET blockade (20). Therefore, whether we are hindering HGF-mediated oncogenic "expedience" in tumors bearing normal *c-MET* gene copy-number or MET-dependent oncogenic "addiction" in *c-MET*-amplified lesions, we are temporarily preventing tumor cells to carry out their malignant program, but we are not eradicating them.

This notion acquires high therapeutic relevance in light of the emerging evidence that MET is expressed and functionally required in the stem cell compartment of several tumor types, including head and neck carcinoma (37), colorectal carcinoma (38), prostate cancer (39), breast cancer (40), and glioblastoma multiforme (41). In analogy with normal tissue stem cells, cancer stem cells represent a multipotent tumor cell subpopulation that possesses the ability to replicate indefinitely and to give rise to secondary tumor colonies with the full differentiation spectrum of the primary lesion. Circulating cancer cells with stem-like characteristics are the only cell type that can give rise to metastases and are often drug resistant (42). Following therapy interruption, residual cancer stem cells that survived treatment may resume their proliferative cycle, reconquer space in the host organism, and give rise to malignant lesions, resulting in new metastases or tumor recurrence (43). From a therapeutic viewpoint, killing

rather than simply inhibiting the proliferation of cancer stem cells may be associated with a greater patient benefit.

The possibility to intercept MET-positive CTCs with stem-like properties generates a new paradigm for the treatment of MET-positive tumors. So far, MET-targeted therapy has been envisioned either for causing the regression of established HGF/MET-dependent lesions or for overcoming HGF/MET-driven resistance to a second anticancer drug (44). The data presented here suggest that these strategies may not exploit the full therapeutic potential of an ADCC-enhanced anti-MET antibody, and point at the neoadjuvant and adjuvant settings as valid alternative approaches. Indeed, CTCs are ultimately responsible for tumor recurrence or metastasis onset following surgery, independently of how well the original lesion has responded to a previous therapy. On the basis of the results obtained in our neoadjuvant and adjuvant orthotopic breast cancer models, it would be reasonable to expect that eradication of CTCs by MET-targeted ADCC is associated with prolonged disease-free survival. Importantly, this opportunity is unique to an ADCC-enhanced anti-MET antibody. In fact, inhibition of HGF/MET signaling using a conventional HGF/MET-targeted drug, whether an antibody or a small molecule, would probably be able to prevent the escape of cancer cells from a primary lesion, but would have no effect on the survival of metastasis-initiating cells once they are in circulation, especially if these cells have become resistant to anoikis during their journey from the home epithelium to the breakout vessel.

Although MET plays its most relevant physiologic role during embryo development and remains mostly silent during the adult stage, it is widely expressed in epithelial and endothelial tissues of virtually all organs. This broad expression pattern could raise the concern that MET-targeted ADCC may cause damage to normal tissues, resulting in systemic toxicity. However, toxicologic studies conducted in cynomolgus monkeys (*Macaca fascicularis*) indicate that this is not the case. In fact, although ARGX-111 does not cross-react with mouse MET, it binds with high affinity to simian MET and to simian FcγRIIIa (Supplementary Table S4). In a large study involving 40 adult animals, the no-observed adverse event level of ARGX-111 corresponded to a dose of 30 mg/kg administered weekly (data on file). These results suggest that MET-targeted ADCC may be well tolerated by normal tissues expressing MET and may, therefore, not interfere with housekeeping physiologic functions at doses that are active against MET-dependent tumors. More definitive data on ARGX-111 safety

will emerge from the phase I study that has started in January 2014 (NCT02055066).

### Disclosure of Potential Conflicts of Interest

A. Hultberg is a senior scientist and has ownership interest (including patents) in arGEN-X. C. Blanchetot is an employee in arGEN-X. M. Saunders is a senior director in arGEN-X. A. Thibault has ownership interest (including patents) in arGEN-X. H. De Haard is a CSO in arGEN-X BVBA. P. Michieli is a consultant/advisory board member in arGEN-X BVBA. No potential conflicts of interest were disclosed by the other authors.

### Authors' Contributions

**Conception and design:** A. Hultberg, V. Morello, C. Blanchetot, F. Maina, P. Brouckaert, M. Saunders, A. Thibault, T. Dreier, H. de Haard, P. Michieli

**Development of methodology:** A. Hultberg, V. Morello, L. Huyghe, N. De Jonge, C. Blanchetot, F. Lamballe, F. Maina, P. Brouckaert, H. de Haard, P. Michieli

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** A. Hultberg, V. Morello, L. Huyghe, V. Hanssens, K. Silence, R. Heukers, B. Roux, F. Lamballe, C. Ginestier, E. Charafe-Jauffret, P. Brouckaert, H. de Haard, P. Michieli

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** A. Hultberg, V. Morello, L. Huyghe, G. De Boeck, K. Silence, E. Festjens, F. Lamballe, F. Maina, P. Brouckaert, H. de Haard, P. Michieli

**Writing, review, and/or revision of the manuscript:** A. Hultberg, V. Morello, N. De Jonge, V. Hanssens, K. Silence, R. Heukers, F. Maina, P. Brouckaert, M. Saunders, A. Thibault, H. de Haard, P. Michieli

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** V. Hanssens, E. Festjens, H. de Haard

**Study supervision:** A. Hultberg, F. Maina, H. de Haard, P. Michieli

### Acknowledgments

The authors thank Brigitte Bonnin and John Wijdenes for the ADCP and ADCC assays, Sylvie Richelme for HGF-dependent assays, Sjudry-Ilona Osepa for llama-human chimera construction, and U-protein Express for production and purification of antibodies.

### Grant Support

This work was supported by IWT (Agentschap voor Innovatie door Wetenschap en Technologie) grants # IWT090297 and IWT100440, by Italian Association for Cancer Research (AIRC 2010 Special Program in Molecular Clinical Oncology 5% Project n. 9970 and AIRC 2012 IG grant n. 12798), and the University of Torino/Compagnia di San Paolo (Progetti di Ricerca di Ateneo 2012).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 9, 2015; revised May 11, 2015; accepted May 29, 2015; published OnlineFirst July 3, 2015.

### References

- Trusolino L, Bertotti A, Comoglio PM. MET signalling: principles and functions in development, organ regeneration and cancer. *Nat Rev Mol Cell Biol* 2010;11:834–48.
- Nakamura T, Mizuno S. The discovery of hepatocyte growth factor (HGF) and its significance for cell biology, life sciences and clinical medicine. *Proc Jpn Acad Ser B Phys Biol Sci* 2010;86:588–610.
- Birchmeier C, Birchmeier W, Gherardi E, Vande Woude CF. Met, metastasis, motility, and more. *Nat Rev Mol Cell Biol* 2003;4:915–25.
- Comoglio PM, Trusolino L. Invasive growth: from development to metastasis. *J Clin Invest* 2002;109:857–62.
- Nakamura T, Mizuno S. The discovery of hepatocyte growth factor (HGF) and its significance for cell biology, life sciences and clinical medicine. *Proc Jpn Acad Ser B Phys Biol Sci* 2010;86:588–610.
- Trusolino L, Comoglio PM. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. *Nat Rev Cancer* 2002;2:289–300.
- To CT, Tsao MS. The roles of hepatocyte growth factor/scatter factor and met receptor in human cancers. *Oncol Rep* 1998;5:1013–24.
- You WK, McDonald DM. The hepatocyte growth factor/c-Met signaling pathway as a therapeutic target to inhibit angiogenesis. *BMB Rep* 2008;41:833–9.
- Gherardi E, Birchmeier W, Birchmeier C, Vande Woude G. Targeting MET in cancer: rationale and progress. *Nat Rev Cancer* 2012;12:89–103.
- Cappuzzo F, Marchetti A, Skokan M, Rossi E, Gajapathy S, Felicioni L, et al. Increased MET gene copy number negatively affects survival of surgically resected non-small cell lung cancer patients. *J Clin Oncol* 2009;27:1667–74.

11. De Oliveira AT, Matos D, Logullo AF, DA Silva SR, Neto RA, Filho AL, et al. MET Is highly expressed in advanced stages of colorectal cancer and indicates worse prognosis and mortality. *Anticancer Res* 2009;29:4807–11.
12. Zagouri F, Bago-Horvath Z, Rössler F, Brandstetter A, Bartsch R, Papadimitriou CA, et al. High MET expression is an adverse prognostic factor in patients with triple-negative breast cancer. *Br J Cancer* 2013;108:1100–5.
13. Tuynman JB, Lagarde SM, Ten Kate FJ, Richel DJ, van Lanschot JJ. Met expression is an independent prognostic risk factor in patients with oesophageal adenocarcinoma. *Br J Cancer* 2008;98:1102–8.
14. Graziano F, Galluccio N, Lorenzini P, Ruzzo A, Canestrari E, D'Emidio S, et al. Genetic activation of the MET pathway and prognosis of patients with high-risk, radically resected gastric cancer. *J Clin Oncol* 2011;29:4789–95.
15. Rocci A, Gambella M, Aschero S, Baldi I, Trusolino L, Cavallo F, et al. MET dysregulation is a hallmark of aggressive disease in multiple myeloma patients. *Br J Haematol* 2014;164:841–50.
16. Olmez OF, Cubukcu E, Evrensel T, Kurt M, Avcı N, Tolunay S, et al. The immunohistochemical expression of c-Met is an independent predictor of survival in patients with glioblastoma multiforme. *Clin Transl Oncol* 2014;16:173–7.
17. Underiner TL, Herberich T, Miknyoczki SJ. Discovery of small molecule c-Met inhibitors: evolution and profiles of clinical candidates. *Anticancer Agents Med Chem* 2010;10:7–27.
18. Cecchi F, Rabe DC, Bottaro DP. Targeting the HGF/Met signaling pathway in cancer therapy. *Expert Opin Ther Targets* 2012;16:553–572.
19. Smyth EC, Sclafani F, Cunningham D. Emerging molecular targets in oncology: clinical potential of MET/hepatocyte growth-factor inhibitors. *Onco Targets Ther* 2014;7:1001–1014.
20. Bertotti A, Burbridge MF, Gastaldi S, Galimi F, Torti D, Medico E, et al. Only a subset of Met-activated pathways are required to sustain oncogene addiction. *Sci Signal* 2009;2:ra80.
21. Lai AZ, Cory S, Zhao H, Gigoux M, Monast A, Guiot MC, et al. Dynamic reprogramming of signaling upon met inhibition reveals a mechanism of drug resistance in gastric cancer. *Sci Signal* 2014;7:ra38.
22. Comoglio PM, Giordano S, Trusolino L. Drug development of MET inhibitors: targeting oncogene addiction and expedience. *Nat Rev Drug Discov* 2008;7:504–516.
23. Michieli P, Cavassa S, Basilico C, De Luca A, Mazzone M, Asti C, et al. An HGF-MSP chimera disassociates the trophic properties of scatter factors from their pro-invasive activity. *Nat Biotechnol* 2002;20:488–495.
24. Basilico C, Hultberg A, Blanchetot C, de Jonge N, Festjens E, Hanssens V, et al. Four individually druggable MET hotspots mediate HGF-driven tumor progression. *J Clin Invest* 2014;124:3172–3186.
25. Charafe-Jauffret E, Ginestier C, Bertucci F, Cabaud O, Wicinski J, Finetti P, et al. ALDH1-positive cancer stem cells predict engraftment of primary breast tumors and are governed by a common stem cell program. *Cancer Res* 2013;73:7290–7300.
26. Armour KL, Clark MR, Hadley AG, Williamson LM. Recombinant human IgG molecules lacking Fcγ receptor I binding and monocyte triggering activities. *Eur J Immunol* 1999;29:2613–2624.
27. Lazar GA, Dang W, Karki S, Vafa O, Peng JS, Hyun L, et al. Engineered antibody Fc variants with enhanced effector function. *Proc Natl Acad Sci U S A* 2006;103:4005–4010.
28. Martens T, Schmidt NO, Eckerich C, Fillbrandt R, Merchant M, Schwall R, et al. A novel one-armed anti-c-Met antibody inhibits glioblastoma growth in vivo. *Clin Cancer Res* 2006;12:6144–6152.
29. Pennacchietti S, Cazzanti M, Bertotti A, Rideout WM III, Han M, Gyuris J, et al. Microenvironment-derived HGF overcomes genetically determined sensitivity to anti-MET drugs. *Cancer Res* 2014;74:6598–6609.
30. Vaccaro C, Bawdon R, Wanjie S, Ober RJ, Ward ES. Divergent activities of an engineered antibody in murine and human systems have implications for therapeutic antibodies. *Proc Natl Acad Sci U S A* 2006;103:18709–18714.
31. Masuda K, Kubota T, Kaneko E, Iida S, Wakitani M, Kobayashi-Natsume Y, et al. Enhanced binding affinity for FcγRIIIb of fucose-negative antibody is sufficient to induce maximal antibody-dependent cellular cytotoxicity. *Mol Immunol* 2007;44:3122–3131.
32. Basilico C, Pennacchietti S, Vigna E, Chiriaco C, Arena S, Bardelli A, et al. Tivantinib (ARQ197) displays cytotoxic activity that is independent of its ability to bind MET. *Clin Cancer Res* 2013;19:2381–2392.
33. Iqbal J, Chong PY, Tan PH. Breast cancer stem cells: an update. *J Clin Pathol* 2013;66:485–490.
34. Parekh BS, Berger E, Sibley S, Cahya S, Xiao L, LaCerte MA, et al. Development and validation of an antibody-dependent cell-mediated cytotoxicity-reporter gene assay. *MAbs* 2012;4:310–318.
35. Smolen GA, Sordella R, Muir B, Mohapatra G, Barmettler A, Archibald H, et al. Amplification of MET may identify a subset of cancers with extreme sensitivity to the selective tyrosine kinase inhibitor PHA-665752. *Proc Natl Acad Sci U S A* 2006;103:2316–2321.
36. McDermott U, Sharma SV, Dowell L, Greninger P, Montagut C, Lamb J, et al. Identification of genotype-correlated sensitivity to selective kinase inhibitors by using high-throughput tumor cell line profiling. *Proc Natl Acad Sci U S A* 2007;104:19936–19941.
37. Lim YC, Kang HJ, Moon JH. C-Met pathway promotes self-renewal and tumorigenicity of head and neck squamous cell carcinoma stem-like cell. *Oral Oncol* 2014;50:633–639.
38. Luraghi P, Reato G, Cipriano E, Sassi F, Orzan F, Bigatto V, et al. MET signaling in colon cancer stem-like cells blunts the therapeutic response to EGFR inhibitors. *Cancer Res* 2014;74:1857–1869.
39. Nishida S, Hirohashi Y, Torigoe T, Inoue R, Kitamura H, Tanaka T, et al. Prostate cancer stem-like cells/cancer-initiating cells have an autocrine system of hepatocyte growth factor. *Cancer Sci* 2013;104:431–436.
40. Baccelli I, Schneeweiss A, Riethdorf S, Stenzinger A, Schillert A, Vogel V, et al. Identification of a population of blood circulating tumor cells from breast cancer patients that initiates metastasis in a xenograft assay. *Nat Biotechnol* 2013;31:539–544.
41. De Bacco F, Casanova E, Medico E, Pellegatta S, Orzan F, Albano R, et al. The MET oncogene is a functional marker of a glioblastoma stem cell subtype. *Cancer Res* 2012;72:4537–4550.
42. Meacham CE, Morrison SJ. Tumour heterogeneity and cancer cell plasticity. *Nature* 2013;501:328–337.
43. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. *Nat Rev Cancer* 2013;13:714–726.
44. Parikh RA, Wang P, Beumer JH, Chu E, Appleman LJ. The potential roles of hepatocyte growth factor (HGF)-MET pathway inhibitors in cancer treatment. *OncoTargets Ther* 2014;7:969–983.