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Targeted therapy by gene transfer of a monovalent antibody fragment against the Met oncogenic receptor

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Abstract

Due to the key role played in critical sub-populations, Met is considered a relevant therapeutic target for glioblastoma multiforme and lung cancers. The anti-Met DN30 antibody, engineered to a monovalent Fab (Mv-DN30), proved to be a potent antagonist, inducing physical removal of Met receptor from the cell surface. In this study, we designed a gene therapy approach, challenging Mv-DN30 in preclinical models of Met-driven human glioblastoma and lung carcinoma. Mv-DN30 was delivered by a Tet-inducible-bidirectional lentiviral vector. Gene therapy solved the limitations dictated by the short half-life of the low molecular weight form of the antibody. In vitro, upon doxycycline induction, the transgene: (1) drove synthesis and secretion of the correctly assembled Mv-DN30; (2) triggered the displacement of Met receptor from the surface of target cancer cells; (3) suppressed the Met-mediated invasive growth phenotype. Induction of transgene expression in cancer cells—transplanted either subcutaneously or orthotopically in nude mice—resulted in inhibition of tumor growth. Direct Mv-DN30 gene transfer in nude mice, intra-tumor or systemic, was followed by a therapeutic response. These results provide proof of concept for a gene transfer immunotherapy strategy by a Fab fragment and encourage clinical studies targeting Met-driven cancers with Mv-DN30.

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Key message

- Gene transfer allows the continuous in vivo production of therapeutic Fab fragments.
- Mv-DN30 is an excellent tool for the treatment of Met-driven cancers.
- Mv-DN30 gene therapy represents an innovative route for Met targeting.

Keywords Cancer · Targeted therapy · Gene therapy · Met · Antibody · Lentiviral vector

Introduction

Targeted therapy, the new frontier of cancer treatment, employs pharmacological tools (drugs or antibodies) specifically blocking crucial gene products that sustain the transformed phenotype. For this therapeutic approach, one important point is the identification of responsive tumors based on their relevant genetic lesions [1].

Glioblastoma multiforme (GBM) is the most common and malignant glial tumor. GBM prognosis is very poor; after an initial response to conventional therapy, nearly all patients relapse and do not survive beyond one year. Given that GBM is essentially considered an incurable cancer; new therapies for its treatment are sought. It has been shown that the product of the *MET* oncogene, the hepatocyte growth factor receptor (HGFR/Met), actively supports the stem-like invasive phenotype of glioblastoma cells of the mesenchymal subtype [2].

Non-small cell lung cancer (NSCLC) is a complex and often fatal disease. As EGFR mutations are frequently responsible for the transformed phenotype, therapies targeting EGFR have

been effectively employed [3]. Unfortunately, a number of patients become refractory to therapy [4]. Studying the molecular mechanisms that underlie this resistance, it has been shown that a subset of refractory tumors takes advantage from the emergence of clones harboring *MET* gene amplification [5, 6].

Due to the clear role of the oncogene as a driver in these two genetically defined tumors, proof of evidence in favor of Met-targeted therapy is mandatory.

Until now, several molecules inhibiting Met signaling have been developed. These include competitive HGF inhibitors, small molecular weight kinase inhibitors, anti-HGF, and anti-Met antibodies. Currently, several Met kinase inhibitors are in the clinic [7]. Some drawbacks emerged, often related to off-target side effects. On the other hand, antibodies are highly specific and, being natural molecules, are generally well tolerated by the host. Unfortunately, due to their bivalent nature, anti-Met antibodies almost invariably behave as 'agonists' or 'partial agonists', mimicking HGF [8]. The mAb DN30 is directed against the extracellular moiety of the human Met receptor binding, with sub-nanomolar affinity, the immunoglobulin-like region [9]. Its bivalent form is a partial agonist promoting some, but not all, Met-mediated biological responses [8]. Nonetheless. it inhibits tumor growth and metastasis through a mechanism of receptor 'shedding' [10]. Receptor shedding is a physiological cellular mechanism of protein degradation. Antibody-induced Met shedding occurs in two steps: first, the metalloprotease ADAM-10 cleaves the Met extracellular domain, recognizing specific sequences upstream the trans-membrane region; then the residual trans-membrane fragment is cleaved by a second protease, γ-secretase, releasing the kinase domain from the membrane to the proteasome [11, 12]. DN30 'triggers' this mechanism, leading to a dramatic reduction in the number of Met receptors exposed at the cell surface. Concomitantly, DN30 releases a soluble 'decoy' ectodomain in the extracellular space. The latter inactivates the residual intact trans-membrane receptors, competing for ligand binding and generating inactive heterodimeric complexes [13]. This combined molecular mechanism results in strong inhibition of Met signaling and in prevention of downstream biological responses.

Recently, we demonstrated that the monovalent Fab fragment (Mv-DN30) is devoid of any agonistic activity while it maintains the ability to induce shedding, potentiating Met inhibition [14]. While Mv-DN30 could be very attractive for therapeutic applications, its clinical use is limited by short plasma half-life originated by high rate of renal clearance due to the small size of the molecule and to the lack of Fc domain-mediated recycling via the neonatal Fc Receptor salvage pathway. Systemic administration of antibody fragments has been addressed by different routes, with questionable results. Here, we propose an alternative to address the issue, describing a gene transfer delivery strategy and providing proof of principle for its therapeutic efficacy in two preclinical models of Met-driven cancers.

Materials and methods

Cell culture

EBC-1 human lung carcinoma cells were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). U87-MG human glioblastoma cells were obtained from F. Pentronzelli (Sigma-Tau, Pomezia, Italy). Cells were maintained as described [14]. When required, Dox (Sigma Life Science, St. Louis, Missouri) was added to the culture medium (200 ng/ml) and replaced every 3 days.

LV-rtTA and LV-Tet-Mv-DN30 generation

LV-rtTA: the synthetic gene for reverse Tet Transactivator2-S2 (rtTA2-S2, provided by H Bujard, ZMBH, Heidelberg, Germany) [15] was sub-cloned into the LV-TA1 vector [16] using the BamHI-SalI restriction sites, substituting the tTA2s c-DNA.

LV-Tet-Mv-DN30: the DNA fragment corresponding to the *tet* O7-mCMV operator was amplified using the primers: 5′-CGGCGATATCATCTGACGCGTCGTCGACC-3′ (forward) and 5′-ATCCCGGGAATTCCGCGGAGGCTGGATCG-3′ (reverse), on the template LV-R1 [16] and sub-cloned in the LV-MvDN30 [14] using the EcoRV-XmaI restriction sites, substituting the PGK promoter.

LV-mALB: the cDNA corresponding to mouse albumin was sub-cloned into the backbone pRRL.sin.PPT.CMV.eGFP.Wpre [17] using the BamHI-SalI sites, substituting the eGFP cDNA.

Lentiviral vectors

Vector stocks were produced by transient transfection and concentrated by ultracentrifugation as described [14]. p24 antigen concentration was determined by Alliance® HIV-1 p24 ELISA kit (Perkin Elmer Inc., Waltham, Massachusetts). Cells were transduced as described [17] with 100 ng/ml of p24 equivalent particles of LV-rtTA for 20 h, washed, and then transduced with 25 ng/ml p24 equivalent particles of LV-Tet-Mv-DN30 or LV-mALB for 24 h.

Mv-DN30 quantification in mice sera

Mv-DN30 serum concentrations were determined by ELISA as described [9]. Range of Mv-DN30 dilutions for the standard curve was 0–200 ng/ml. Colorimetric assay was revealed by the multilabel reader Victor™ X4 (Perkin Elmer Inc.).

Western blot analysis

Cells were lysed with Laemmli Buffer and protein separation was performed using standard SDS-PAGE electrophoresis method. Primary antibodies for Western blot detection were:

anti-FLAG clone M2 and anti-Vinculin clone hVIN-1 (mAbs, Sigma Life Sciences); anti-human Met mAb clone DL-21 [8]. Secondary anti-mouse IgG was from GE Healthcare (Freiburg, Germany). Western blot bands were quantified using ImageJ software.

Analysis of surface Met

Cells were detached with PBS-1 mM EDTA, collected, spun and resuspended in cold PBS-1%FBS plus anti-Met antibody (clone #95106) PE-conjugated (R&D System, Minneapolis, Minnesota) diluted 1:20. After incubation for 30 min on ice, cells were washed, stained with DAPI (Roche Applied Science, Indianapolis, Indiana) and analysed by flow cytometry using a $CyAN_{ADP}$ apparatus (Dako, Glostrup, Denmark). Gates were set using negative controls (unlabelled cells); DAPI positive cells were excluded from the analysis.

Biological assays

Anchorage-independent growth assays: transduced U87-MG cells were seeded in 24-well dishes (1,000 cells/well) in semisolid medium as described [14]. After 28 days of culture, colonies were stained by tetrazolium salts (Sigma Life Science) and scored by microscopy.

Anchorage-dependent growth assays: transduced EBC-1 cells were seeded in 96-well dishes as described [14]. After 16 h, medium was replaced with fresh one in presence or absence of Dox. Cell number was evaluated at the indicated time points using the CellTiter-Glo luminescent cell viability assay (Promega Corp., Madison, Wisconsin). Chemoluminescence was detected with Victor™ X4.

Animals experiments

All animal procedures were performed according to protocols approved by Ethical Committee for animal experimentation of the Fondazione Piemontese per la Ricerca sul Cancro and by Italian Ministry of Health. Mice (*nu/nu* females on Swiss CD-1 background) were purchased from Charles River Laboratories (Calco, Italy). When required Dox (1 mg/ml) plus 2.5 % sucrose were added to drinking water.

Tumorigenesis assays: EBC-1 or U87-MG cells, respectively 2×10^6 and 2.5×10^6 cells/mouse in 0.2 ml of Iscove Modified Dulbecco Medium (IMDM, Sigma Life Science), were injected subcutaneously into the right posterior flank of 7-week-old nude mice. Tumor size was evaluated periodically with a caliper. Tumor volume was calculated as described [14]. At the end of the experiments, mice were euthanized and tumors were extracted. Each tumor specimen was divided in two parts; one was frozen and the other was formalin fixed and paraffin embedded. For orthotopic tumorigenesis assay, U87-MG cells (3×10^5 cells in 5 μl of IMDM) were injected by a stereotactic apparatus into the forebrain (caudate/putamen) of 11-week-old athymic nude mice. When euthanized, mice were perfused with 4 % paraformaldehyde, brains were extracted, fixed, embedded in paraffin and processed for histology. Five-micrometre coronal sections were stained with Mayer's H&E (Bio Optica SpA, Milan Italy).

Intra-tumoral delivery of lentiviral vectors: subcutaneous palpable tumors, obtained as described above, were injected with 50 μl of lentiviral vector particles in PBS. In the experiments performed using LV-Mv-DN30 and LV-mALB, 1.7 μg of p24 equivalent particles/tumor were injected and the injection was repeated after 48 h. In the experiment performed using LV-Tet-Mv-DN30, 1 μg of p24 equivalents/tumor was delivered every day for five consecutive days.

Intra-tumoral delivery of Mv-DN30 as protein: subcutaneous palpable tumors, obtained as described above, were injected with 25 μg of pure Mv-DN30 in 50 μl of PBS three times per week for 2 weeks (days of treatment: 18, 20, 22, 25, 27, 29, 32). The same volume of vehicle was injected at the same times in the control animals.

Systemic delivery of lentiviral vectors: LV-Mv-DN30 particles (35 μg of p24 equivalents/mouse in 350 μl of PBS) were injected through tail vein in nude mice (4-week-old). Animals injected with the same volume of PBS were used as control. Blood was collected 4, 5, 6 weeks after LV delivery and Mv-DN30 serum concentrations were estimated by ELISA.

Quantification of vector DNA by real-time PCR

Integrated vector copies were quantified by real-time PCR in 100 ng of template genomic DNA extracted from the tumors with DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany). As primers were used: Forward: 5′-ACCATCATCACCATTG ACTCG-3′, annealing to the FLAG Tag located at the C-terminal of the Mv-DN30 heavy chain; Reverse: 5′-TCCACA TAGCGTAAAAGGAGC-3′, annealing to the Wpre region of the lentiviral vector backbone. Reactions were carried out according to manufacturer's instructions in the presence of the Power Syber® Green PCR master mix (Applied Biosystems, Carlsbad, California) and analysed by ABI Prism 7900HT sequence detection system (Applied Biosystems).

Statistical analysis

Averages, standard deviations, medians and *p* values obtained by Student's *t* test were calculated using Microsoft Office Excel 2003 software (Microsoft Corporation, Redmond, Washington). Data from ELISA assays were analysed and fit using GraphPad Prism software (GraphPad Software, San Diego, California).

Results

Mv-DN30 expression by gene transfer in vitro and in vivo

In the repertoire of human cancer cell lines, a few display constitutive activation of the Met receptor (i.e. tyrosine phosphorylation). This activation is the result of genetic lesions - mutations or amplifications - or of an autocrine loop somehow 'coherent' with a step of differentiation [2] (e.g. the neural stem cell). An example of the first instance is the NSCLC cell line EBC-1, in which the *MET* oncogene is amplified [18] and sustains the transformed phenotype in the absence of other relevant genetic alterations [19]. An example of the second instance is the anchorage independent growth of U87-MG glioblastoma, sustained by an autocrine loop of HGF/Met [20]. EBC-1 is a cell line that fulfills the paradigm of Met oncogenic addiction [18]. U87-MG cells are the golden standard used for measuring the responsiveness to specific Met/HGF inhibitors [21–23]. Both cell lines are tumorigenic when xenografted in nude mice. Due to the above listed features, EBC-1 and U87-MG cells were selected to test Mv-DN30 gene therapy.

Refining our previously developed [14] constitutive lentiviral vector (LV), we built a second-generation vector expressing Mv-DN30 under the control of a bidirectional tetracycline (Tet) -inducible promoter. A fine temporal control of transgene expression was achieved by the reverse Tet-dependent transcriptional activator (rtTA2-S2), provided *in trans* by a second LV (Tet-ON system: Fig. 1a). After gene transfer, U87-MG and EBC-1 efficiently synthesized, correctly assembled and secreted the genetically engineered recombinant Fab. In the absence of doxycycline (Dox), transgene transcription was barely detectable (Fig. 1b). Mv-DN30 produced by mean of gene transfer fully maintained Met-binding properties [14].

We performed direct in vivo gene transfer delivering to nude mice, by tail vein injection, LV carrying Mv-DN30 under the control of the bidirectional constitutive promoter [14]. LV systemic administration targets primarily liver and spleen [24]. These organs function as endocrine secretors of the exogenous therapeutic molecule. Four weeks after injection, all mice subjected to gene transfer showed measurable amounts of Mv-DN30 in the serum, ranging from 0.86 to 4.8 ng/ml. Mv-DN30 serum concentrations, were evaluated for further 2 weeks (Fig. 1c).

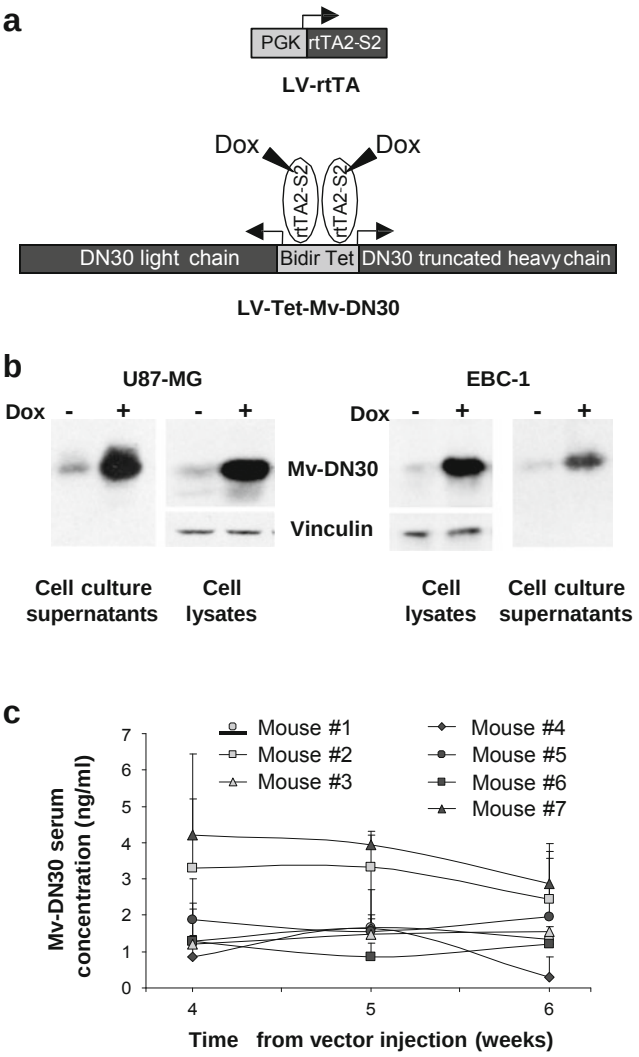


Fig. 1 Analysis of lentiviral vector-mediated Mv-DN30 expression. a Scheme of the expression cassettes inserted into the lentiviral vector (*LV*). *Upper* : expression cassette for reverse Tet transactivator (rtTA2-S2) driven by the human phosphoglycerate kinase promoter (PGK); *lower*: expression cassette for Mv-DN30. In the presence of Dox, rtTA2-S2 binds to the Tet-dependent bidirectional promoter (BidirTet) and activates transcription of two divergent RNAs, encoding for DN30 light chain and for DN30 heavy chain truncated at the C-terminal of the first constant region. b Mv-DN30 expression in U87-MG (*left*) and EBC-1 (*right*) cells transduced with LV-rtTA plus LV-Tet-Mv-DN30 and treated or not with Dox. Treatment was carried out for 6 days in U87-MG and 3 days in EBC-1. Synthesized (cell lysates) and secreted (cell culture supernatants) Mv-DN30 was revealed by western blot under non-reducing conditions, probing filters with anti-FLAG antibodies. As control, cell lysate filters were probed with anti-vinculin antibodies (*lower panels*). c Kinetic of Mv-DN30 expression in sera from nude mice tail vein injected with LV-Mv-DN30. Mice injected with PBS were completely negative (not shown). Data are representative of two experiments done

Mv-DN30 transgene expression down-regulates Met and inhibits Met-biological responses in vitro

U87-MG and EBC-1 cells were transduced first with LV-rtTA and then with LV-Tet-Mv-DN30 or LV carrying mouse albumin cDNA as control. The transduced cells expressing Mv-DN30 upon Dox treatment showed a significant Met receptor down-regulation as assessed by the analysis of total cell lysates (Fig. 2a). This was confirmed by cytofluorimetric analysis that showed, for both cell lines a 40 % reduction of Met receptor exposed at the cell membrane (Fig. 2b). Met down-regulation observed in the Dox-treated cells was comparable to what obtained by a 250 nM Mv-DN30 exogenous treatment and

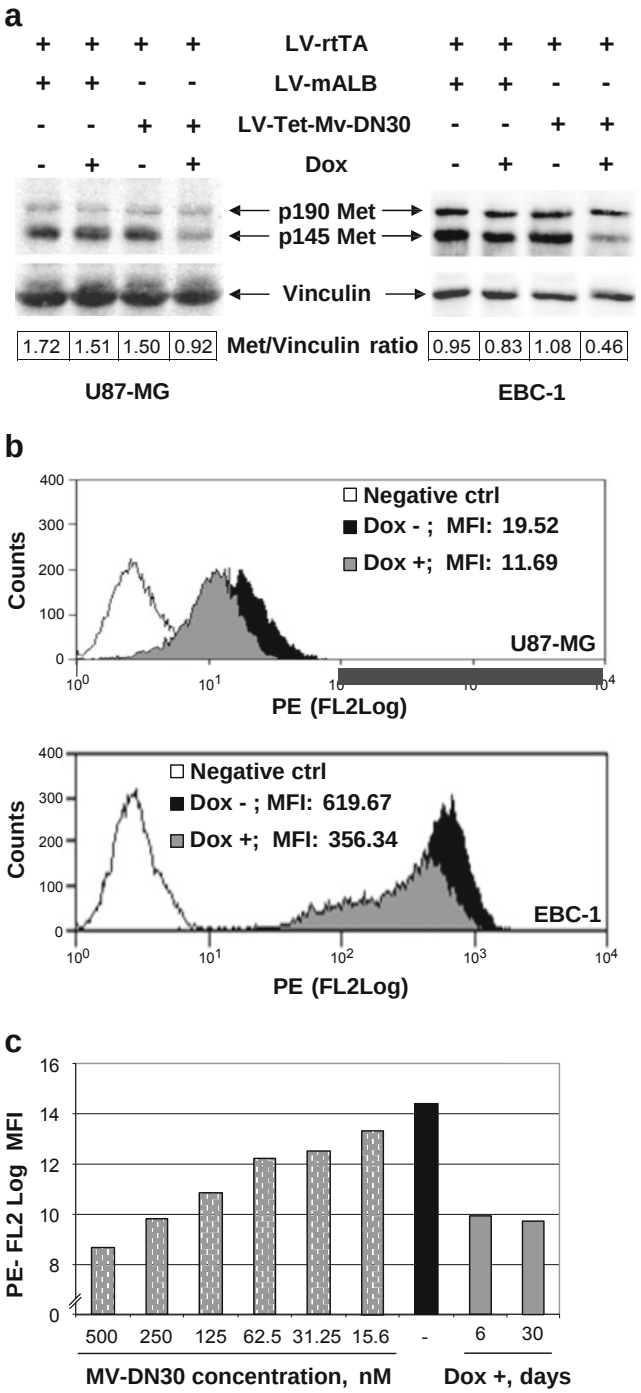


Fig. 2 Analysis of Met expression in cells producing Mv-DN30. Transgene expression was induced for 6 days in U87-MG and 3 days in EBC-1. a Total Met receptor in cell extracts from U87-MG (*right*) and EBC-1 (*left*) transduced with LV-rtTA plus LV-Tet-Mv-DN30 or LV-mALB, determined by western blot with anti-Met antibodies (*upper panels*). The two Met bands correspond to the unprocessed (p190 Met) and mature (p145 Met) form of the receptor. As control, the same filters were probed with anti-vinculin antibodies (*lower panels*). Bands were quantified; the reported values represent intensity of Met signal normalized against vinculin. b Surface Met receptor in transduced U87-MG (*top*) and EBC-1 (*bottom*) cells determined by cytofluorimeter, analysing intact cells labeled with anti-Met antibodies conjugated with phycoerythrin (*PE*). *Negative ctrl*: cells not treated with anti-Met; *MFI*: mean fluorescence intensity. c Surface Met receptor, determined by cytofluorimeter, in U87-MG wild-type cells treated with the indicated amounts of Mv-DN30 protein (*grey/white dashed bars*) for 3 days and in transduced U87-MG cells treated with Dox (*grey bar*) for the indicated period of time. *Black bar* : untreated cells; *MFI* : mean fluorescence intensity. Data reported in the figure are representative of at least two experiments done

was stable during time (Fig. 2c). Technical issue related to cross-competition between the antibodies used to induce Met down-regulation and Met staining was ruled out analysing by cytofluorimeter Met expression in cells pre-incubated for 30 min at 4 °C with increasing concentration of Mv-DN30 (data not shown).

As U87-MG glioblastoma is not a 'Met-addicted' cell line (see above), Mv-DN30 inhibitory activity on cell growth was scored in anchorage-independent condition. The transgene was turned on either at time 0 (immediately after seeding) or at day 14 (when the colonies were already formed). Mv-DN30 expression at time 0 resulted in a 43.3 % reduction on the number of colonies (p =0.03); expression from day 14 resulted in a reduction of 37.2 % (p =0.02; Fig. 3a). On the other hand, as EBC-1 have a 'Met-addicted' phenotype, transduced cells were tested for cell growth in adherent condition. It should be noted that these cells do not grow in anchorage-independent condition. As determined by the kinetic of growth reported in Fig. 3b, transgene expression slowed down cell growth after 3 days of Dox induction. After 7 days growth, inhibition was 65.3 % (p <0.001) compared to the same infected EBC-1 cells, not induced.

Mv-DN30 transgene expression inhibits GBM growth in vivo

U87-MG cells, transduced with LV-rtTA+LV-Tet-Mv-DN30, were transplanted subcutaneously in nude mice. One group (group A, n =10) was treated with Dox from the day of cell injection (*prevention trial* , Fig. 4a); control mice were left untreated (n =19). After 105 days, 85 % of controls developed the tumor, while the Dox-treated mice were 80 % tumor free (p <0.001). After 112 days, Dox was removed and tumor-free animals (n =8) were left untreated for further 100 days: seven out of eight (87.5 %) did not develop tumor. Only one mouse developed a slow-growing tumor very late (210 day). Thirty-nine days after cell injection, untreated mice that developed a tumor were divided in two groups: one was left untreated (group B, n =6) while the other (group C, n =5) was induced with Dox (*regression trial*). After 1 week of Dox treatment, mice were bled to evaluate Mv-DN30 transgenic expression in serum. Figure 4b shows the ratio between antibody concentration and tumor volume at the day of the bleeding. We scored a very low ratio, less than 0.08 ng/ml/mm^3 in all the untreated mice, consistent with the detectable grade of leakiness of the

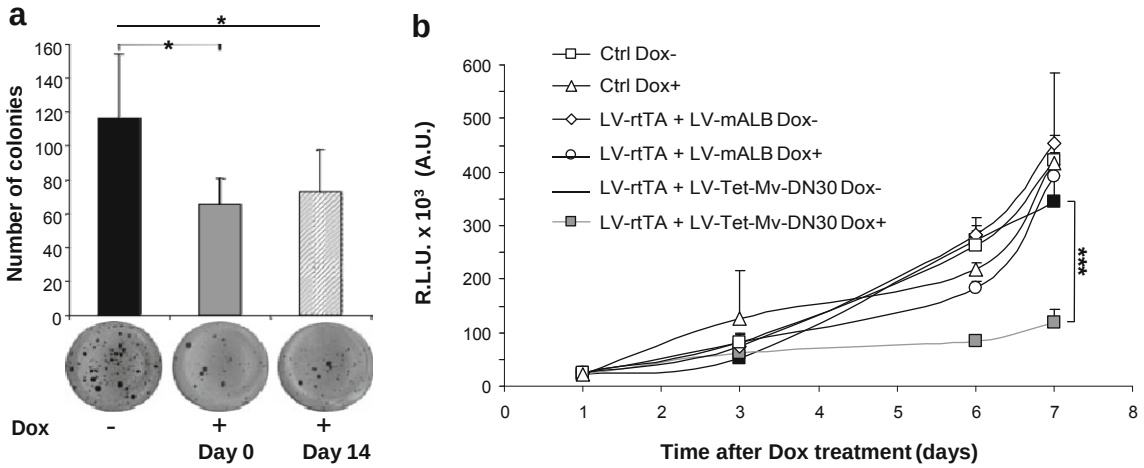


Fig. 3 Analysis of Met–mediated biological responses in cells expressing Mv-DN30. a Anchorage-independent growth of transduced U87-MG cells. *Graph* represents the number of colonies grown in semisolid medium. Samples are in triplicates, *bars* represent SD. Pictures are representative of samples from each group. * p <0.05, Student's t test. b Anchorage-dependent growth of transduced EBC-1. Kinetic of growth was determined using a luminescence-based ATP assay. Samples are in quadruplicates, *bars* represent SD. *RLU*: relative light unit; *AU*: arbitrary unit; ***p <0.001, Student's t test (LV-rtTA+LV-Tet-Mv-DN30 Dox+ cells versus LV-rtTA+LV-Tet-Mv-DN30 Dox- cells at day 7). Data reported in the figure are representative of at least two experiments done

regulated system observed in vitro (see Fig. 1b). Dox-treated mice showed a high Mv-DN30 concentration/tumor volume ratio (more than 0.199 ng/ml/mm^3), indicating that the inducible system was working correctly. The average tumor volume developed by the two groups is shown in Fig. 4c. Analysis of tumor growth in the single Dox-induced animals gave the following results (Fig. 4d): two mice displayed reduced tumor growth between day 10 and 30 of treatment; afterwards the tumors relapsed. Interestingly, these two mice showed the lowest Mv-DN30 concentration/tumor volume ratio scored among Dox-treated mice (Fig. 4b). In three mice, tumors regressed, never reappearing in two cases, and relapsing in one case, after 95 days. In the two mice showing complete remission, Dox was removed after 140 days; notably the tumor did not reappear until the end of experiment (269 days).

U87-MG cells carrying the LV-rtTA+LV-Tet-Mv-DN30 transgenes were also injected into the forebrain of nude mice using a stereotactic apparatus. Transplanted animals were randomized into two groups (n =8), one of which received Dox. Tumor growth into the brain was lethal thus mouse survival was measured. As shown in Fig. 5a, Mv-DN30 expression significantly increased mice survival: median survival of control group was 34 days while for the treated group was 70.5 days (p <0.001). Notably, 120 days after cells transplantation two mice in the Dox arm were still alive. Histological analysis of the brain showed that these mice were tumor-free (data not shown). The impairment of tumor onset was further confirmed in a second experiment conducted as above. In this experiment, the animals belonging to each group (n =4) were sacrificed 40 days from cells transplantation. Histological analysis of the brains showed that three out of four treated animals were tumor-free and one carried a small tumor, while all the untreated mice carried very large tumors (Fig. 5b).

Intra-tumor Mv-DN30 gene delivery inhibits GBM growth

To establish a gene therapy protocol, we attempted direct LV delivery. LV particles were injected into tumors developed in nude mice after subcutaneous transplantation of U87-MG cells. Group A (n =10) received LVs carrying mouse albumin cDNA (control); group B (n =10) received LV-Mv-DN30. In both cases, transgene expression was under the control of a constitutive promoter. Intra-tumor injection of LV-Mv-DN30 significantly inhibited tumor growth. At the end of the experiment, a 38.8 % reduction of the tumor masses was observed (average volume group A=1800.76±663.62 mm^3, average group B= 1102.32 ± 565.62 mm^3; p = 0.02). As shown in Fig. 6a, nine out of ten mice treated with gene therapy carried tumors smaller than the average of controls.

In a second experiment, LV-Tet-Mv-DN30 particles were directly injected into the tumor masses obtained after subcutaneous transplantation of U87-MG cells expressing rtTA2-S2. Mice were then randomized into two groups: one group was left untreated (group A, n =4) while the other was treated with Dox (group B, n =4). Dox induction of the transgene significantly slowed down tumor growth. At the end of the experiment (39 days after cell injection), a 56 % reduction in the average tumor mass was observed (group A=895.06± 234.16 mm^3, group B=388.97±272.11 mm^3; p =0.03) (Fig. 6b). Analysis of tumor growth in single Dox-induced animals gave the following results (Fig. 6c): in one mouse tumor growth was indistinguishable from controls; in two mice, growth was slowed down and in one mouse the tumor showed a clear regression. Interestingly, at the end of the experiment, the mouse that did not respond to therapy showed the lowest ratio between Mv-DN30 concentration and tumor volume (Fig. 6d). As expected the mouse with complete tumor

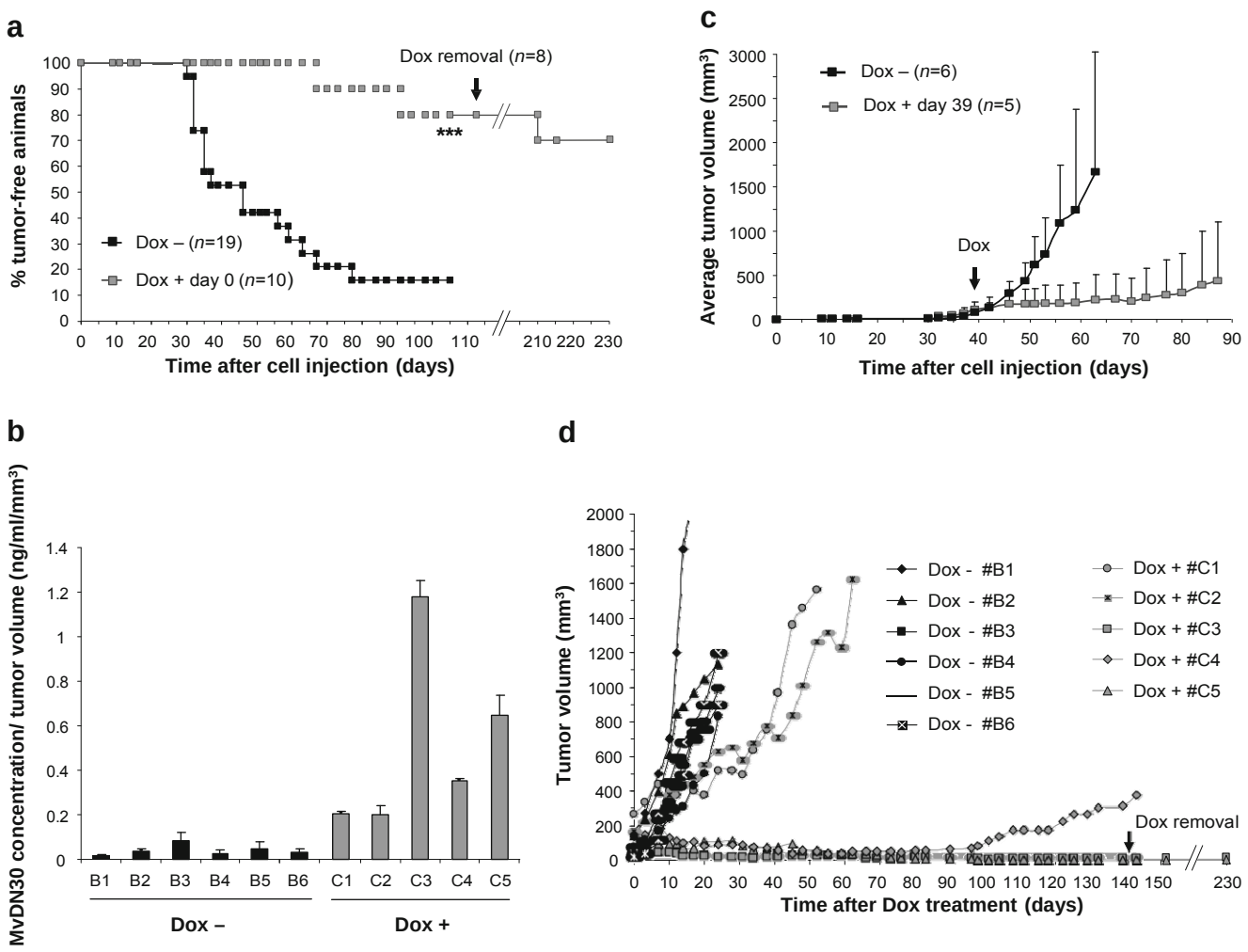


Fig. 4 Analysis of the in vivo tumorigenesis of Mv-DN30-expressing U87-MG cells: subcutaneous injection. U87-MG cells, transduced in vitro with LV-rtTA plus LV-Tet-Mv-DN30, were inoculated subcutaneously into the flank of nude mice. Data are representative of two experiments done. a *Prevention trial*: mice were treated with Dox from the day of cell injection. The graph reports percentage of tumor-free animals in function of the time. b, c, d *Regression trial*: mice were treated with Dox at the 39th day from cell injection. After 1 week (46th day), mice were bled. Graph in b reports the ratio between Mv-DN30 serum concentration and tumor volume measured at the day of bleeding. Each histogram *bar* represents the ratio in the indicated mouse. Group B (*black*): untreated mice; group C (*grey*): Dox-treated mice. c Graph reports tumor growth considering the average values of the groups. *Bars* represent SD. d Graph reports tumor growth of each mouse. Untreated mice, group B: *black*; Dox-treated mice, group C: *grey*

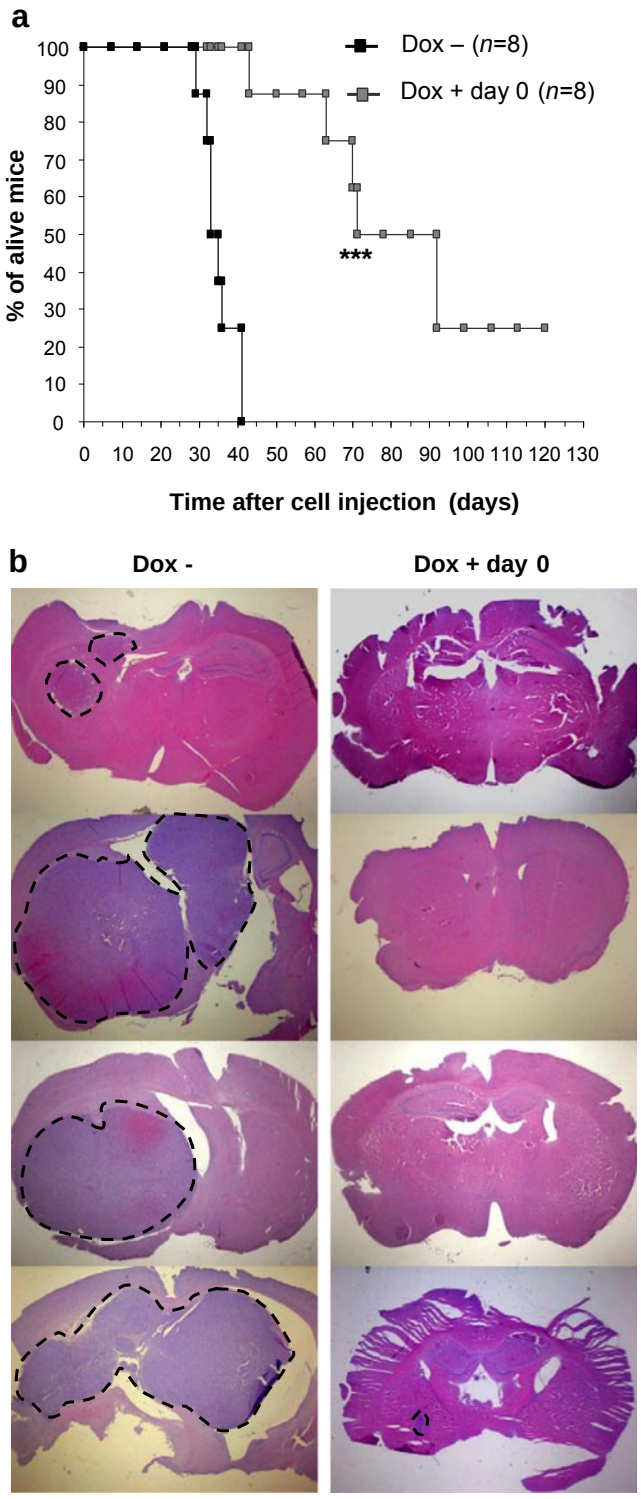
regression did not show detectable circulating Mv-DN30 since bleeding was possible only at the end of the experiment, when the Mv-DN30 transduced, tumor cells were not anymore present.

In a third experiment, the activity of Mv-DN30 administered intra-tumor by a conventional protein delivery was assessed. To this end, mice carrying U87-MG tumors were treated with repeated administrations of Mv-DN30 protein (group B, n =6) or with same volume of PBS (group A, n = 9). Fab fragment (25 μg/mouse) was delivered three times per week for 2 weeks. At the end of the experiment (33 days after cell injection), we observed a 27.8 % reduction in the tumor mass average of the treated animals (group A=1218.84± 402.27 mm^3, group B= 879.98 ± 579.26 mm^3) (Fig. 6e). However, although therapeutic efficacy was observed, the individual responses were heterogeneous (p=0.202).

Mv-DN30 transgene expression inhibits NSCLC growth in vivo

EBC-1 cells transduced with LV-rtTA+LV-Tet-Mv-DN30 were subcutaneously inoculated in nude mice. Immediately after transplantation, mice were randomized into three groups. Group A (n =12) was untreated (control). Group B (n =6) was treated with Dox from day 0 and group C (n =12) was left untreated until day 12 than treated with Dox until the end of the experiment.

Fig. 5 Analysis of the in vivo tumorigenesis of Mv-DN30-expressing U87-MG cells: orthotopic injection. U87-MG cells, transduced in vitro with LV-rtTA plus LV-Tet-Mv-DN30, were inoculated into the forebrain (caudate-putamen) of nude mice. Data are representative of two experiments done. Mice were treated with Dox from the day of cell injection (*prevention trial*). a Graph reports percentage of live animals in function of time. *Arrows* indicate the day of Dox treatment switch. ***p <0.001, Student's t test (median survival treated versus untreated animals). b Histological analysis of brains collected 40 days after cell transplantation. *Left panels* : brains from control mice not treated with Dox; *right panels*: brains from Dox-treated mice. *Black dots* : tumor border lines



When induced from day 0 (*prevention trail*), Mv-DN30 expression significantly delayed tumor onset (p <0.001): median of tumor appearance was 19 days for Dox-treated mice (group B) and 8 days for untreated mice (group A). At day 12, group B mice were all tumor-negative while group A mice were all tumor-positive (Fig. 7a). Moreover, Mv-DN30 expression slowed tumor growth: considering the average values, group A tumor volume increased 4.7-folds in 7 days (from day 12 to day 19) while the increment in 7 days (from day 19 to day 26) for the group B was only of 3.8-folds. Twenty-one days after tumor cell injection Dox-treated mice carried tumors 6.6-folds smaller than untreated mice (p <0.0001) (Fig. 7b).

After 12 days, all untreated mice were tumor positive: tumor volume average was 381 mm^3 for group A and 387 mm^3 for group C. Group C mice started Dox treatment (*regression trial*). After 1 week, tumor volumes were on average 1.8-folds smaller compared to their previous size, having, on average, a mass 8.3 times smaller than controls (p <0.0001). After this strong initial response, the Dox-treated tumors relapsed, proliferating with reduced growth rate (Fig. 7c). By real-time PCR, we measured Mv-DN30 copies integrated into the genomic DNA extracted from tumors after sacrifice. Both B and C groups, compared to group A, showed a decrease in average number of integrated transgene (Fig. 7d) (group B versus group A, p =0.02; group C versus group A, p =0.002), indicating that expression of Mv-DN30 was counter selected in the relapsing tumors.

Systemic Mv-DN30 gene delivery inhibits NSCLC tumor growth

As transduced EBC-1 cells showed a counter selection for Mv-DN30 transgenic expression, LV particles were delivered to nude mice systemically, by tail vein injection. One group was injected with PBS (n =9, control) and a second group (n = 7) was injected with LVs carrying Mv-DN30 under the control of the constitutive promoter. Six weeks after injection, the amount of transgenic antibody was measurable in the serum of six out of seven mice subjected to gene transfer, while in one was barely detectable (Fig. 8a). One week later, EBC-1 cells were transplanted and tumor growth was monitored. Two out of seven mice subjected to gene transfer did not develop tumor at all (Fig. 8b). Tumors grew slower in four animals (Fig. 8c) resulting, at the end of the experiment, in a 68.78 % inhibition of the average tumor volumes (average volume group A=1827.42±1040.39 mm^3, average four mice group B=570.56±415.02 mm^3; p =0.043). The tumor grown in the mouse with the lowest amount of circulating Mv-DN30 before

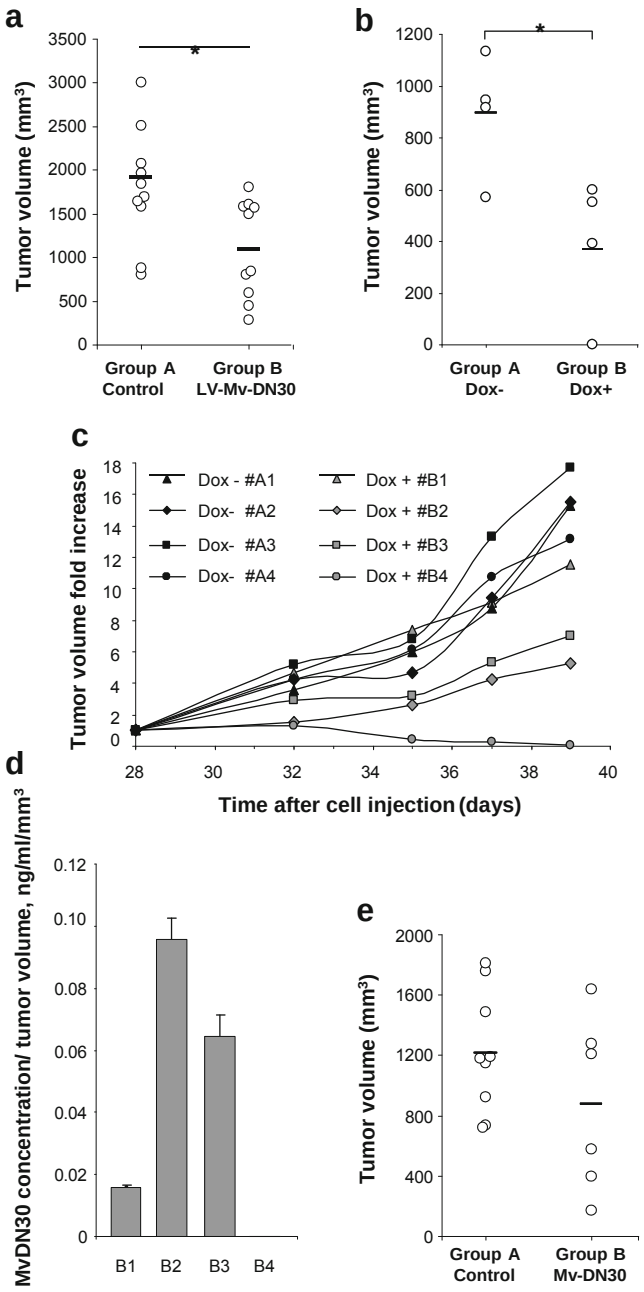


Fig. 6 Analysis of U87-MG tumor growth in mice subjected to Mv-DN30 gene transfer by intra-tumor lentiviral vector delivery. *Circles* in the graphs represent the volume of the tumor carried by each mouse at the end of the experiment, while the *black bars* represent the average volume of the group. *p <0.05, Student's t test. Data are representative of two experiments done. a Gene therapy with constitutive LV. Group A (control, n =10) received LV-mALB, group B (n =10) received LV-Mv-DN30. Lentiviral vector particles were inoculated 22 days after cell injection (average size of the tumors: group A, 36.29±21.41 mm^3; group B, 37.34±24.88 mm^3). Tumor volumes were monitored 41 days after cell injection. b, c, d Gene therapy with Tet-inducible LV. LV-Tet-Mv-DN30 particles were inoculated 28 days after cell injection, then mice were randomized into two groups (n =4; average size of the tumors: group A, untreated mice= 59.27± 20.48 mm^3; group B, Dox-treated mice=62.15±17.59 mm^3). Tumor volumes were monitored 39 days after cell injection. Graph in b reports tumor volumes at the end of the experiment. Graph in c reports tumor volume fold increase during time of each mouse. Untreated mice, group A: *black*; Dox-treated mice, group C: *grey*. Graph in d reports the ratio between Mv-DN30 serum concentration and tumor volume, both measured at the end of the experiment, for each Dox-treated mouse. e Protein therapy. Group A (control, n =9) received PBS, group B (n =6) received repeated intra-tumor administration of MvDN-30 (25 μg each injection) starting from day 18 (average size of the tumors: group A, 68.03±28.31 mm^3; group B, 70.47±46.36 mm^3). Tumor volumes were monitored 33 days after cell injection

tumor cell injection was as large as tumors developed by controls (Fig. 8c).

Discussion

Gene therapy landed in the field of cancer research after a period of lights and shades in a number of severe diseases. Far from being a standard option for cancer therapy, burgeoning proofs of concept highlight gene therapy as an attractive innovation to more toxic—or still absent—alternatives [25].

Strategies for high efficiency of gene transfer are crucial for the therapeutic response. So far, non-viral and viral vectors have been explored, yet the perfect system for any gene therapy application was not—and will not easily be—available [26, 27]. Tailoring the right tool to meet different specific needs is still a challenge.

LV has the peculiarity to integrate its gene cargo into the genome of non-proliferating cells [28]. Thus, to reach effective Met targeting, the choice of a lentiviral vector is rationale, as the therapeutic transgene should be integrated into cancer 'stem' cells that proliferate at an extremely low rate. In fact, in GBM, it has recently been shown that the Met oncogenic receptor is a functional marker of the cancer stem cell subpopulation sustaining the malignant growth [2]. In the case of lung cancer refractory to EGFR target therapy, it has been shown, as well, that resistance is likely due to the expansion of a pre-existing stem cell population driven by the amplified *MET* oncogene [6]. LVs integrate the transgene virtually without genotoxicity [29] and assure high, stable and long-term expression [28]. Concerns about LV biosafety have been overcome by a careful genetic engineering refinement of the third generation of these vectors [30] that met Food and Drug Administration approval for a number of clinical trials now ongoing (see www.clinicaltrial.gov).

A body of evidence from this laboratory has assessed, in preclinical setting, the therapeutic efficacy of the DN30 antibody, directed against the extracellular moiety of the human Met receptor [10]. In case of antibody directed against growth factor receptor—intrinsically activated by dimerization—construction of monovalent reagents is mandatory to obtain a full

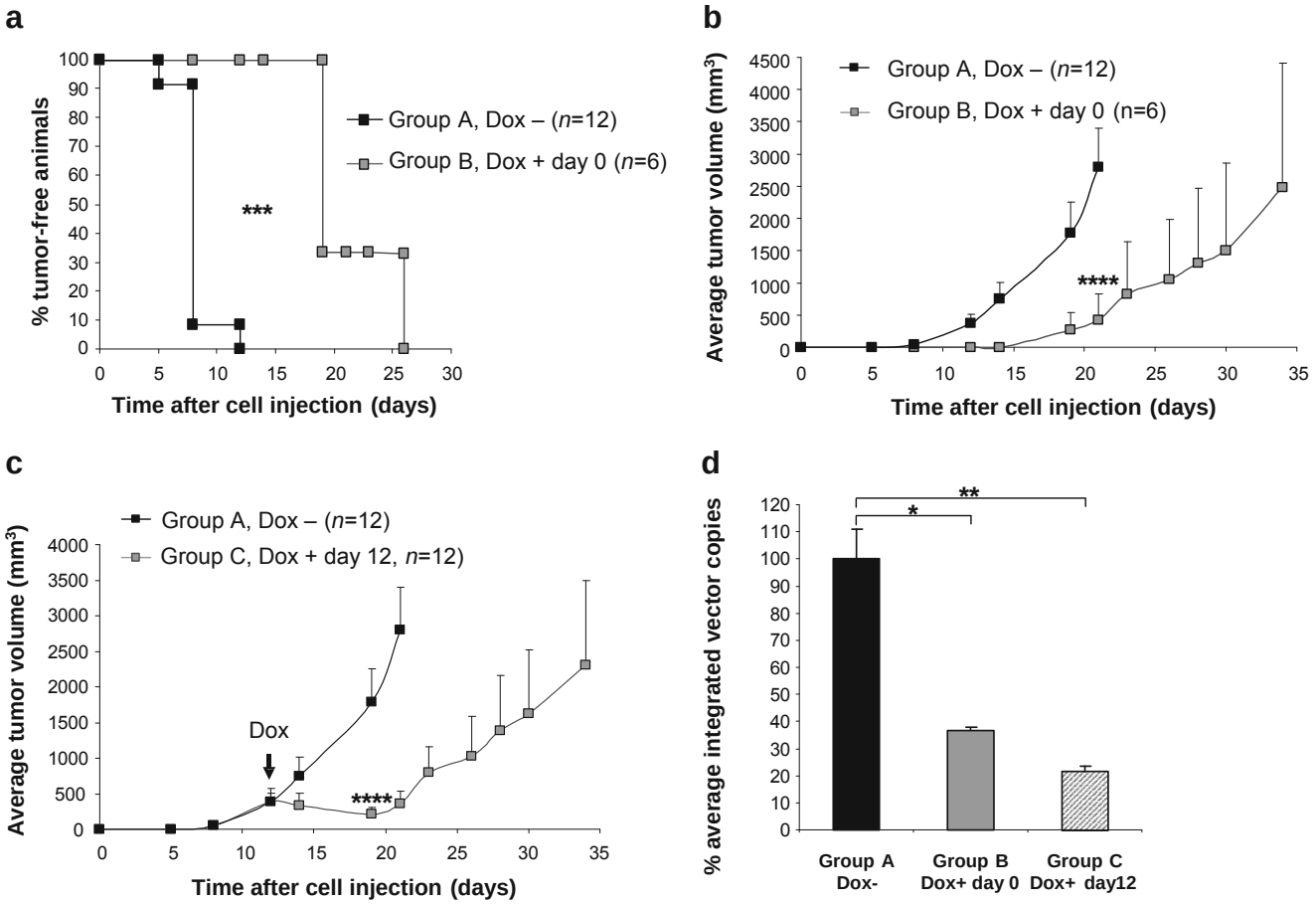


Fig. 7 Analysis of EBC-1 expressing Mv-DN30 tumorigenesis in vivo. EBC-1 cells, transduced in vitro with LV-rtTA plus LV-Tet-Mv-DN30, were inoculated subcutaneously into the flank of nude mice. Data are representative of two experiments done. a, b *Prevention trial*: mice were treated with Dox from the day of cell injection. In a tumor onset; in b tumor growth. *Bars* represent SD. c *Regression trial*: mice were treated with Dox at the 12th day from cell injection. Graph reports kinetic of tumor growth. *Bars* represent SD. ***p <0.001, Student's t test (median tumor free treated versus untreated animals); **** p <0.0001, Student's t test (treated versus untreated animals evaluated in b at day 21, in c at day 19). d Analysis of integrated LV-Mv-DN30 copies into the genomic DNA extracted from tumors of the regression trial

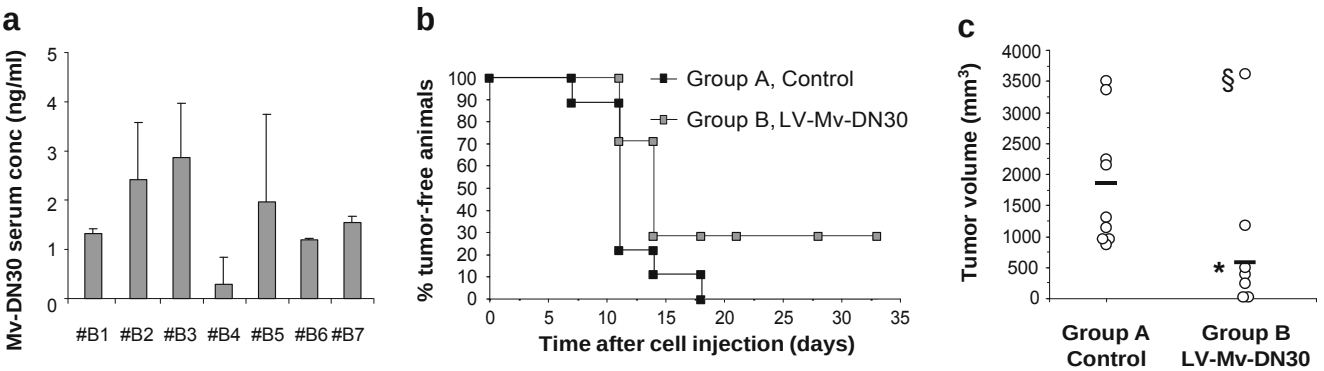


Fig. 8 Analysis of EBC-1 tumor growth in mice subjected to Mv-DN30 gene transfer by systemic lentiviral vector delivery. LV-Mv-DN30 particles were injected into the tail vein of nude mice. Data are representative of two experiments done. a Analysis of Mv-DN30 concentrations measured in the sera of each mouse subjected to gene transfer 3 weeks after vector injection. *Bars*: SD. b Kinetic of EBC-1 tumor onset. (Control group: n =9; LV-Mv-DN30 group: n =7). c Volume of the tumors at the end of the experiment (33 days after cell injection). Each circle represents the volume of the tumor carried by each mouse; *black bar* represents volume average (for the group B average was calculated on four mice, excluding tumor-negative mice and mouse #B4, see below). *p <0.05, Student's t test. *Section sign*, *circle* corresponding to the tumor volume carried by the mouse #B4 that secreted barely detectable amount of Mv-DN30 (seetext)

antagonist [14, 21]. The optimized bidirectional promoter used in this study generates stoichiometric amounts of heavy and light chains, giving rise to correctly assembled monovalent Fabs and sustained long-term secretion. The latter property, moreover, bypasses the intrinsic limitation due to the extremely short half-life of any antibody Fab fragment. In all experiments based on in vitro gene transfer, we used a lentiviral vector with Tet-dependent system of expression. The 'at will' regulation of transgene production allowed operations with a highly controlled system. Nevertheless, a vector with Tet-regulated expression can be transferred in vivo only in immune-compromised hosts because rtTA, a fusion protein derived from bacterial and viral sequences, elicits an immunological response. Moreover, the inducible system, based on the delivery of two separate vectors, cannot be easily handled for a direct in vivo gene transfer. Therefore, we performed the in vivo experiments with the constitutive lentiviral vector.

LVs are suitable both for local and systemic delivery. Local administration, such as intra-tumor infusion, has advantages, since toxicity due to 'off- target' effects is limited; the vector transduces cells surrounding the site of injection, avoiding spread into the organism. The same spatial limitation occurs also for the therapeutic protein, which accumulates and stays in the tumor. Although a relatively small number of cancer cells are infected, they secrete the transgenic Fab molecule, amplifying the response to the surrounding tissue ('bystander' effect). Moreover, in the case of GBM, delivery of the therapeutic agent in situ is not blood–brain barrier dependent, achieving higher concentrations at the tumor site. It is reasonable to hypothesize that Mv-DN30 protein administered locally accumulates at the tumor site, resulting in an efficacy comparable to that achieved by gene transfer. On the experimental ground—in our settings—comparison between protein and gene administration highlights the advantage of gene therapy.

Gene therapy by intra-tumor administration has drawbacks, as the therapeutic agent fosters a negative selection by destroying vector-infected cancer cells synthesizing the Fab. Negative selection was clearly seen in EBC-1 cells: in the early phase of therapy, all mice were highly responsive but, in a later phase, they eventually relapsed. Assessment of the integrated vector copy number indicated that a negative selection occurred, by self-elimination of cells producing the anti-Met Fab. This problem was circumvented by systemic administration of the vector as the therapeutic protein was produced far from its site of action, by cells shielded from suicide effects. This approach, far from being an 'out of the box' clinical protocol, gives a strong proof of concept that by systemic gene transfer, is possible to reach therapeutic efficacy with a low molecular weight protein, thanks to its continuous secretion in the circulation. In spite of the limited significance imposed by the small number of cases studied (due the technical struggle of vector particles production 'in house'), these results encourage exploring a number of alternative gene therapy approaches by transplantation of cells genetically modified ex vivo [31, 32]. It would be in any case hard to believe that gene therapy could be used to 'debulk' the tumor mass. Gene therapy would more rationally be used to prevent tumor relapse, a clinical condition mimicked by the prevention trials described in this paper.

Targeted therapy directed against the oncogenic Met receptor has now come of age and a rich armamentarium is available, including small molecule kinase inhibitors, antibodies and non-conventional reagents [7]. Combination of these reagents and radiotherapy proved to be very effective on a number of tumors including GBM [33, 34]. Gene therapy can represent an adjuvant to enforce effectiveness of any of them.

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Conflict of interest PMC and PM are consultants of Metheresis Translational Research SA. The other authors declare no potential conflict of interest.

References

1. Martini M, Secchione L, Siena S, Tejpar S, Bardelli A (2011) Targeted therapies: how personal should we go? Nat Rev Clin Oncol 9:87–97
2. De Bacco F, Casanova E, Medico E, Pellegatta S, Orzan F, Albano R, Luraghi P, Reato G, D'Ambrosio A, Porrati P et al (2012) The MET oncogene is a functional marker of a glioblastoma stem cell subtype. Cancer Res 72:4537–4550
3. Janku F, Stewart DJ, Kurzrock R (2010) Targeted therapy in non-small-cell lung cancer-is it becoming a reality? Nat Rev Clin Oncol 7: 401–414
4. Wheeler DL, Dunn EF, Harari PM (2010) Understanding resistance to EGFR inhibitors-impact on future treatment strategies. Nat Rev Clin Oncol 7:493–507
5. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, Gale CM, Zhao X, Christensen J et al (2007) MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 316:1039–1043
6. Bean J, Brennan C, Shih JY, Riely G, Viale A, Wang L, Chitale D, Motoi N, Szoke J, Broderick S et al (2007) MET amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib. Proc Natl Acad Sci U S A 104:20932–20937
7. Gherardi E, Birchmeier W, Birchmeier C, Vande Woude G (2012) Targeting MET in cancer: rationale and progress. Nat Rev Cancer 12: 89–103
8. Prat M, Crepaldi T, Pennacchietti S, Bussolino F, Comoglio PM (1998) Agonistic monoclonal antibodies against the Met receptor dissect the biological responses to HGF. J Cell Sci 111:237–247

9. Vigna E, Pacchiana G, Mazzone M, Chiriaco C, Fontani L, Basilico C, Pennacchietti S, Comoglio PM (2008) "Active" cancer immunotherapy by anti-Met antibody gene transfer. Cancer Res 68:9176–9183
10. Petrelli A, Circosta P, Granziero L, Mazzone M, Pisacane A, Fenoglio S, Comoglio PM, Giordano S (2006) Ab-induced ectodomain shedding mediates hepatocyte growth factor receptor down-regulation and hampers biological activity. Proc Natl Acad Sci U S A 103:5090–5095
11. Foveau B, Ancot F, Leroy C, Petrelli A, Reiss K, Vingtdeux V, Giordano S, Fafeur V, Tulasne D (2009) Down-regulation of the met receptor tyrosine kinase by presenilin-dependent regulated intramembrane proteolysis. Mol Biol Cell 20:2495–2507
12. Schelter F, Kobuch J, Moss ML, Becherer JD, Comoglio PM, Boccaccio C, Kruger A (2010) A disintegrin and metalloproteinase-10 (ADAM-10) mediates DN30 antibody-induced shedding of the met surface receptor. J Biol Chem 285:26335–26340
13. Michieli P, Mazzone M, Basilico C, Cavassa S, Sottile A, Naldini L, Comoglio PM (2004) Targeting the tumor and its microenvironment by a dual-function decoy Met receptor. Cancer Cell 6:61–73
14. Pacchiana G, Chiriaco C, Stella MC, Petronzelli F, De Santis R, Galluzzo M, Carminati P, Comoglio PM, Michieli P, Vigna E (2010) Monovalency unleashes the full therapeutic potential of the DN-30 anti-Met antibody. J Biol Chem 285:36149–36157
15. Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W (2000) Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. Proc Natl Acad Sci U S A 97:7963–7968
16. Vigna E, Cavalieri S, Ailles L, Geuna M, Loew R, Bujard H, Naldini L (2002) Robust and efficient regulation of transgene expression in vivo by improved tetracycline-dependent lentiviral vectors. Mol Ther 5:252–261
17. Follenzi A, Ailles LE, Bakovic S, Geuna M, Naldini L (2000) Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. Nat Genet 25:217–222
18. Lutterbach B, Zeng Q, Davis LJ, Hatch H, Hang G, Kohl NE, Gibbs JB, Pan BS (2007) Lung cancer cell lines harboring MET gene amplification are dependent on Met for growth and survival. Cancer Res 67:2081–2088
19. Bertotti A, Burbridge MF, Gastaldi S, Salimi F, Torti D, Medico E, Giordano S, Corso S, Rolland-Valognes G, Lockhart BP et al (2009) Only a subset of Met-activated pathways are required to sustain oncogene addiction. Sci Signal 2:er11
20. Chattopadhyay N, Butters RR, Brown EM (2001) Agonists of the retinoic acid- and retinoid X-receptors inhibit hepatocyte growth factor secretion and expression in U87 human astrocytoma cells. Brain Res Mol Brain Res 87:100–108
21. Martens T, Schmidt NO, Eckerich C, Fillbrandt R, Merchant M, Schwall R, Westphal M, Lamszus K (2006) A novel one-armed anti-c-Met antibody inhibits glioblastoma growth in vivo. Clin Cancer Res 12:6144–6152
22. Burgess T, Coxon A, Meyer S, Sun J, Rex K, Tsuruda T, Chen Q, Ho SY, Li L, Kaufman S et al (2006) Fully human monoclonal antibodies to hepatocyte growth factor with therapeutic potential against hepatocyte growth factor/c-Met-dependent human tumors. Cancer Res 66: 1721–1729
23. Zou HY, Li Q, Lee JH, Arango ME, McDonnell SR, Yamazaki S, Koudriakova TB, Alton G, Cui JJ, Kung PP et al (2007) An orally available small-molecule inhibitor of c-Met, PF-2341066, exhibits cytoreductive antitumor efficacy through antiproliferative and antiangiogenic mechanisms. Cancer Res 67:4408–4417
24. Follenzi A, Sabatino G, Lombardo A, Boccaccio C, Naldini L (2002) Efficient gene delivery and targeted expression to hepatocytes in vivo by improved lentiviral vectors. Hum Gene Ther 13:243–260
25. Gillet JP, Macadangdang B, Fathke RL, Gottesman MM, Kimchi-Sarfaty C (2009) The development of gene therapy: from monogenic recessive disorders to complex diseases such as cancer. Methods Mol Biol 542:5–54
26. Kaneda Y (2010) Update on non-viral delivery methods for cancer therapy: possibilities of a drug delivery system with anticancer activities beyond delivery as a new therapeutic tool. Expert Opin Drug Deliv 7:1079–1093
27. Warnock JN, Daigre C, Al-Rubeai M (2011) Introduction to viral vectors. Methods Mol Biol 737:1–25
28. Vigna E, Naldini L (2000) Lentiviral vectors: excellent tools for experimental gene transfer and promising candidates for gene therapy. J Gene Med 2:308–316
29. Montini E, Cesana D, Schmidt M, Sancito F, Ponzoni M, Bartholomae C, Sergi Sergi L, Benedicenti F, Ambrosi A, Di Serio C et al (2006) Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. Nat Biotechnol 24:687–696
30. Mátrai J, Chuah MK, VandenDriessche T (2010) Recent advances in lentiviral vector development and applications. Mol Ther 18:477–490
31. De Palma M, Mazzieri R, Politi LS, Pucci F, Zonari E, Sitia G, Mazzoleni S, Moi D, Venneri MA, Indraccolo S et al (2008) Tumor-targeted interferon-alpha delivery by Tie2-expressing monocytes inhibits tumor growth and metastasis. Cancer Cell 14:299–311
32. Sanz L, Compte M, Guijarro-Muñoz I, Álvarez-Vallina L (2012) Non-hematopoietic stem cells as factories for in vivo therapeutic protein production. Gene Ther 19:1–7
33. Lal B, Xia S, Abounader R, Laterra J (2005) Targeting the c-Met pathway potentiates glioblastoma responses to gamma-radiation. Clin Cancer Res 11:4479–4486
34. De Bacco F, Luraghi P, Medico E, Reato G, Girolami F, Perera T, Gabriele P, Comoglio PM, Boccaccio C (2011) Induction of MET by ionizing radiation and its role in radioresistance and invasive growth of cancer. J Natl Cancer Inst 103:645–661