Targeting EphA3 Inhibits Cancer Growth by Disrupting the Tumor Stromal Microenvironment

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Abstract

Eph receptor tyrosine kinases are critical for cell–cell communication during normal and oncogenic tissue patterning and tumor growth. Somatic mutation profiles of several cancer genomes suggest EphA3 as a tumor suppressor, but its oncogenic expression pattern and role in tumorigenesis remain largely undefined. Here, we report unexpected EphA3 overexpression within the microenvironment of a range of human cancers and mouse tumor xenografts where its activation inhibits tumor growth. EphA3 is found on mouse bone marrow–derived cells with mesenchymal and myeloid phenotypes, and activation of EphA3+/CD90+/CD45−/CD11b−/CD11c−/CD117− mesenchymal/stromal cells with an EphA3 agonist leads to cell contraction, cell–cell segregation, and apoptosis. Treatment of mice with an agonistic α-EphA3 antibody inhibits tumor growth by severely disrupting the integrity and function of newly formed tumor stroma and microvasculature. Our data define EphA3 as a novel target for selective ablation of the tumor microenvironment and demonstrate the potential of EphA3 agonists for anticancer therapy. Cancer Res; 74(16); 4470–81. ©2014 AACR.

Introduction

The intimate communication between cancer cells and host-derived stromal and myeloid cells (1), which are recruited from the bone marrow (2) and constitute the vascularized tumor microenvironment (TME), is critical for primary tumor growth, invasion, and metastasis (3). Among the proteins considered instrumental in establishing the TME (1), Ephs and their cell-associated ephrin ligands are implicated in neoangiogenesis and invasive tumor growth, and are increasingly being recognized as therapeutic targets entering clinical trials (4).

Ephs RTKs comprise A- and B-type receptors that interact preferentially with GPI-linked type-A ephrins, and transmembrane type-B ephrins, respectively. Their activation triggers context-dependent signaling pathways that control cell spreading, adhesion, cell migration, and (stem) cell proliferation. Ephs function also in the absence of kinase activity, usually with the opposite outcome, promoting cell-spreading, cell–cell adhesion, and supporting stem cell proliferation (5, 6).

Ephs and ephrins are commonly overexpressed in a broad range of cancers, where their oncogenic roles often reflect their dichotomous developmental activities. Thus, depending on tumor type and disease stage, overexpressed Ephs can promote or inhibit tumor progression (7–9). Importantly, oncogenic Eph expression often coincides with low or absent ephrin expression and kinase-independent functions (7, 10), whereas Eph activation by exogenous agonists typically inhibits proliferation, survival, and tumor growth (4, 5).

EphA3 is found in mesenchymal tissues of developing axial muscles, lung, kidney, and heart (11), is implicated in mesoderm (12), neural patterning (13), and is essential for endothelial-to-mesenchymal transition (EndMT) during heart development (14). There is very little evidence for physiologic adult expression or function, but EphA3 is overexpressed in solid and hematopoietic tumor cells (9, 15–17), and implicated in maintaining tumor-initiating cells in glioblastoma (9) and leukemia (18). Frequent somatic mutations of EphA3 in various metastatic cancers are thought to indicate a tumor suppressor role of the kinase-active receptor (19–22).

We now demonstrate, for the first time, conspicuous EphA3 expression predominantly in the stromal TME in a broad range of human solid tumors and mouse tumor xenografts. The
EphA3–activating mAb IIIA4 (23) targets bone marrow–derived mesenchymal/stromal and myeloid EphA3+ cells in the tumor microenvironment, and EphA3+/CD29+/CD90+/Sca1low mesenchymal–stromal/stem cells (MSC) respond to IIIA4 with EphA3 phosphorylation, rapid cell contraction, and apoptosis. In xenograft models, IIIA4 treatment significantly inhibits tumor growth by disrupting the overall stromal and vascular tissue architecture and function.

The prominent expression and function of EphA3 in the TME but not in normal tissues, together with favorable clinical opportunities for solid tumor treatment.

In xenograft models, IIIA4 treatment significantly inhibits tumor growth by disrupting the overall stromal and vascular tissue architecture and function.

Materials and Methods

Antibodies and reagents

A list of antibodies and reagents is provided in the Supplementary Data.

Cell lines and culture

Human DU145 (ATCC HTB-81), 22RV1 (ATCC CRL-2505), and LnCaP (ATCC CRL-1740), and mouse LLC (ATCC CRL-1642) are authenticated, karyotyped cell lines from ATCC. EphA3/293 cells were derived (23) from HEK293T cells (ATCC CRL-3216) and maintained in DMEM 10% FCS/G418/zeocin, 5% CO2, LIM2550 cells (25), generated at the Ludwig Institute (Melbourne, Australia), and all ATCC cell lines were maintained in RPMI/10% FCS/10% CO2. Cell lines were kept in continuous culture for <10 passages and tested (PCR, FACS) before experimental use for phenotype and expression of relevant proteins.

Mouse xenografts, antibody treatment, and GFP bone marrow reconstitution

Animal procedures were according to Monash University and Austin Hospital Animal Ethics Committee guidelines. BALB/cnu mice (4–6 weeks, ARC), subcutaneously injected with 1 × 107 DU145, 22Rv1, or LIM2550 cells, were treated by intraperitoneal injection (2/week) of antibodies or PBS when tumors reached approximately 100 mm3. For bone marrow transplantation, BALB/cnu mice (4 weeks old), within 48 hours of irradiation (2 × 5.25 Gy), were intravenously injected with 1 × 107 freshly isolated bone marrow cells from BALB/cnu or GFP transgenic mice [Tg(CAG-EGFP)1Osb/J, Jackson Laboratory], 4 to 6 weeks before tumor xenografting. Bone marrow cells were depleted by MACS (Miltenyi Biotec) or FACS from EphA3+ cells using Alexa647-chIII4; control bone marrow was prepared by applying unlabeled cells to the MACS.

Hyposxia measurement

Mice were injected, 30 minutes before analysis, with 60 μg/g pimidazole (Hypoxyprobe HPI, Inc.), optimum cutting temperature (OCT)-frozen tumor sections analyzed with [FITC]-α-pimidazole/HRP/α-FITC antibodies. Olympus CellSens software was used for quantitation (n ≥ 3 mice/group, 10 fields of view).

In vivo multiphoton and live cell microscopy

For intravital multiphoton imaging (Leica SP5, 20× Plan Apo-1.0-NA water lens, 4 external detectors), subcutaneous tumors were exposed by generating skin flaps through incisions along the ventral midline. Qdor647-chIII4A (0.025 mg) was tail vein injected 48 hours before imaging, and 0.1 mL Ricinus Communis Agglutinin (RCA)-lectin[FTTC]; 5 mg/mL was added immediately before imaging to label blood vessels (26). Intrinsic Second Harmonic Generation (SHG) signals were used to reveal collagen fibers (27). To monitor vasculature integrity, mice were injected 30 minutes before imaging with 0.1 mL [FITC]lectin and 15 μL Qtracker 655.

Leica SP5 inverted confocal microscopes (63× glycerol objective, NA 1.3) were used for imaging live cells or 4% PFA-fixed cells.

3D image analysis

Imaris 7.3.1 Software (Bitplane) was used for three-dimensional (3D) reconstruction and image analysis of 2-photon microscope Z-stacks (1–5 μm sections). Total fluorescent isosurfaces and ratios between fluorescent channels were determined by thresholding of individual channels, volume rendering of Z-stacks, and applying isosurfaces to thresholded regions (constant threshold values for all samples in one experiment). A minimum of 3 to 10 regions, 3 mice per experiment were analyzed.

Tissue immunohistochemistry and immunofluorescence microscopy

OCT-embedded fresh-frozen human tissue samples from surgical biopsies between 1995 and 2007 (Austin Health Tissue Bank), or from resected tumor xenografts, sectioned (6 μm) and fixed (10 minutes, acetone) were stained (Vector Labs ABC Kit), Hematoxylin counterstained and imaged (Olympus Dot- Slide). GFP+ tumor tissues were fixed in 4% PFA overnight at 4°C and transferred to 30% sucrose overnight before freezing in OCT and sectioning. For immunofluorescence microscopy (IFM), directly conjugated primary or secondary antibodies were used, nuclei counterstained with Hoechst, and imaged on Leica AF6000 or SP5 microscopes.

2D image analysis

Microscopic images (Leica AF6000, 40× objective) of tumor sections (≥10 fields of view/tumor, ≥3 mice/group) and fluorescent live cell images were quantitated (ImageJ) using thresholded fluorescent channels. ImageJ macros were adopted into a Kepler workflow software (28) for unbiased quantification to threshold and calculate total fluorescent area per field (for tenascin and nuclei) or binarized for particle detection to count individual vessels per field.

Tumor MSC isolation

Single-cell suspensions, filtered (40- and 20-μm sieves) from Collagenase Type 3/Deoxyribonuclease 1 (Worthington)- digested tumors and treated with Red Blood Cell Lysis Buffer (Sigma-Aldrich), were FACsSed (Influx, BD Biosciences) for EphA3/CD90/Sca-1 expression and sorted cells maintained (10,000 cells/well, 6-well plate) at 2% O2/5% CO2/37°C in
DMEM/10% FBS/penicillin/streptomycin, leukemia inhibitory factor (1,000 U/mL: Millipore), EGF (10 ng/mL: Invitrogen), PDGF (10 ng/mL: Invitrogen). Osteogenic, adipogenic, and chondrogenic MSC differentiation media were from Invitrogen.

Viability assay

Tumor MSCs were treated as indicated in figures and viability assessed by propidium Iodide exclusion. Apoptosis of adherent cells was measured using a TUNEL kit (In Situ Cell Death Detection Kit, Roche). MSCs on fibronectin-coated polystyrene slides were treated as described in the figure. Fractions of apoptotic nuclei were determined from “thresholded”, binarized fluorescence channels using particle counting (ImageJ, version 1.46r).

Immunoprecipitation and Western blotting

III4 α-EphA3 mAb (23) immunoprecipitates from (0.15 mg total protein) tumor cell lysates or frozen tumor homogenates were Western blotted with indicated antibodies, and actin blots of total protein lysate (10 μg) were used as a loading control. Blots were visualized using ECL (Supersignal, Thermo Fisher Scientific).

Flow cytometry

ALSRII flow cytometer (Becton Dickinson) was used for flow cytometry, dead cells detected with propidium iodide, and FLOWJO software (TreeStar) used for raw data analysis and multivariate compensation.

Statistical analysis

GraphPad and Microsoft Excel (Microsoft) were used to estimate mean ± SE, unpaired two-tailed t tests (two variables) and two-way ANOVA (multiple parameters). For tumor treatments, one-way ANOVA with a Bonferroni t test as pairwise post hoc analysis was used.

Results

Preferential expression of EphA3 in tumor stroma and vasculature

Compared with other Ephs, the expression profile of EphA3 in tumors has remained poorly characterized. Using the III4 mAb, previously used for the isolation and functional characterization of EphA3 (29–31), our immunohistochemical expression analysis revealed EphA3-specific staining in 95% (154 of 162) of human tumor tissues from a broad range of cancer types (Supplementary Table S1). α-EphA3 and α-CD31 immunohistochemistry (IHC) of successive tumor tissue sections, and IFM with combinations of III4 and α-CD31 or α-Vimentin antibodies (Fig. 1A and Supplementary Fig. S1A), showed that in most cases EphA3 is present in the vascularized (89%) or stromal (71%) TME (Supplementary Table S1), but less frequent in tumor cells. Organ-matched nontumor samples from all patients showed little detectable III4A staining (Fig. 1A, insets; Supplementary Fig. S1A). Indeed, immunoprecipitation (IP) analysis of human tumor cell lines indicated the lack of EphA3 in all tested colon and lung carcinoma cells, while in agreement with previous studies (16), several primary melanoma cell lines were EphA3+ (Supplementary Fig. S1D). Also, analysis of experimental tumors from EphA3+ mouse lung carcinoma (LLC) and human prostate (DU145) and colon carcinoma cells (LIM2250) indicated EphA3 in tumor lysates, but not in the corresponding cell lines (Fig. 1B and C). IHC confirmed α-EphA3 immunoreactivity in areas of the tumor xenografts that stained with antibodies against stromal and vascular markers, particularly within the rim of EphA3+ (DU145, LIM2550) and EphA3+ 22RV1 tumor xenografts (Fig. 1D). In contrast, nonstromal DU145 tumor tissue, all of the tested normal adult mouse tissues, and established mouse endothelial cell lines (not shown) lacked EphA3 expression (Supplementary Figs. S1C and S2A–S2C).

The chIII4A α-EphA3 mAb selectively targets tumor stroma and vasculature

We studied therapeutic targeting of the EphA3+ tumor stroma by comparing EphA3+ 22RV1 and EphA3– DU145 prostate carcinoma xenografts, the latter displaying EphA3 stromal and vascular staining reminiscent of that observed in human prostate carcinomas (Fig. 1A and D and Supplementary Fig. S1C). For ease of EphA3 detection in these and other experiments, we used a recombinant chimeric (ch) version of the III4A mAb (23), where the mouse III4 variable IgG region is fused to human IgG1 and IgG constant regions, retaining the affinity (Supplementary Table S2), specificity, and tumor-targeting capacity (Supplementary Fig. S3A–S3C) of mouse III4. IFM of tumors from chIII4A-injected mice confirmed its dose-dependent accumulation, significantly stronger in EphA3+ 22RV1 xenografts than in EphA3– DU145 xenografts (Supplementary Fig. S4A–S4C).

We assessed chIII4A tumor targeting in vivo, by imaging subcutaneous Du145 xenografts in mice injected with 655Qdot-chIII4A, and with FITC-tagged RCA lectin (FITC) lectin marking blood-perfused vessels (5, 32). Intravital Multiphoton microscopy revealed 655Qdot-chIII4A within stromal tissue and around blood vessels within the tumor (Fig. 2A, tumor), but not within the adjacent normal mouse skin (Fig. 2A, skin). Confocal microscopy confirmed binding of the injected chIII4A (Fig. 2B), but not of an isotype-matched control mAb (Fig. 2C), to some CD31+ tumor vessels, whereas flow cytometry of cell suspensions revealed that 13.6% of the EphA3+ cells in these tumors (14.2% of total) were also [FITC]lectin+ (Fig. 2D). Furthermore, confocal microscopy indicated that also in 22RV1 xenografts, Tenascin-C+ stromal cells and some [FITC]lectin-labeled tumor vessels are targeted by chIII4A (Fig. 2E).

EphA3 is expressed on bone marrow–derived MSCs that promote tumor growth

We next studied the phenotype and tissue origin of EphA3+ stromal cells. FACS analysis of single-cell suspensions from day 21 (~0.2 cm3) DU145 or LIM2550 (not shown) tumors yielded a population of EphA3+ cells expressing myeloid, MSC, and endothelial surface markers (Supplementary Fig. S3A). To isolate tumor stroma–derived cells capable of ex vivo expansion, sorting for EphA3+/CD90+/Sca-1+ cells consistently yielded a small population of adherent cells (Supplementary Fig. S3B and C), the only population that allowed propagation...
at clonal seeding density (10 cells/well, 18%–30% of wells). The expanded adherent cells expressed typical MSC cell surface markers (33, 34), including Sca-1, CD29, CD90, CD105, CD106, CD146, and CD44low, but lacked typical myeloid and endothelial markers (Supplementary Fig. S5D). Importantly, the cell surface marker expression profile of these tumor-derived MSCs was almost identical to that of bone marrow–derived MSCs (33) isolated from crushed femurs of the same DU145 tumor-bearing mice (Fig. 3A), and both MSC populations differentiated in corresponding culture media into osteogenic,
chondrogenic, or adipogenic lineages (Fig. 3B), confirming
their multilineage differentiation potential. Supporting our
conclusion from these experiments that EphA3+ tumor MSCs
are of bone marrow origin, we found elevated levels of EphA3+
cells in bone marrow and in peripheral blood of tumor-bearing
mice (Supplementary Fig. S5E).

To further assess the bone marrow origin of the EphA3+
tumor stromal cells, we analyzed tumors from mice that had
received bone marrow transplants from transgenic donor mice
with ubiquitous GFP expression. Flow cytometry of cell sus-
spensions from early (~0.2 cm3) tumors revealed a large pro-
portion of EphA3+ cells as part of a tumor-infiltrating GFP+,
cell population (Fig. 3C), coexpressing previously assigned cell
surface markers, including CD90, CD105, CD106, CD146, CD34,
and Sca-1 (Supplementary Fig. S6A). Notably, MACS depletion
of EphA3+ cells from the donor bone marrow used for adoptive
transfer slowed tumor growth significantly (Fig. 3D). To allow
detection of the bone marrow–derived cells in tumor
xenografts, we generated chimeric GFP+/EphA3+ bone mar-
row for adoptive transfer, by replacing the EphA3+ cells in the
donor bone marrow with FACS-isolated transgenic GFP+/EphA3+
cells (Supplementary Fig. S6B and S6C). The tumor
growth rate in mice reconstituted with EphA3-depleted bone
marrow is reduced compared with mice receiving chimeric
donor bone marrow complemented with GFP-EphA3+ cells
(Supplementary Fig. S6D). Analysis of tumors from these mice
revealed matching immunohistochemical profiles of GFP+ and
EphA3+ cells, particularly in the tumor rim (Fig. 3E). Fur-
thermore, confocal microscopy of tumor tissue sections revealed
GFP- and EphA3-expressing cells in stromal and perivascular
regions of the tumor, which were absent in tumor sections
from mice transplanted with non-chimeric bone marrow (Fig.
3F). Together, these findings indicate that EphA3+ bone mar-
row–derived cells are recruited into the TME and promote
tumor growth.

**Tumor MSCs respond to EphA3+ activation by cell
contraction and apoptosis**

Because oncogenic Eph expression frequently coincides
with low or absent ephrin expression, while Eph activation is
typically tumor suppressive (7, 10, 35), we explored the
response of tumor–resident MSCs to EphA3 activation. Anal-
ysis of DU145 tumors with EphA3-Fc, a fusion protein that
binds EphA3 ligands (36, 37). confirmed lack of EphA3-inter-
acting ephrins in the areas of EphA3 expression and other parts
of the tumor (Supplementary Fig. S7A–S7C), suggesting that
EphA3 is not ligated in tumor–resident stromal cells.

*In vitro* activation of Ephs is achieved with preclustered
ephrin-Fc fusion proteins (5, 6), but we also demonstrated
previously that clustered IIIA4 or synergistic binding of IIIA4
and ephrin-A5 Fc triggers EphA3 activation and cellular
responses (23). Thus, while binding of unclustered Alexap+III-
A4 to cultured EphA3+ MSCs was barely visible, its
clustering with α-huFc antibodies significantly increased the
cell-bound fluorescence and caused pronounced cell con-
traction (Fig. 4A–C and Supplementary Fig. S7D). Confocal
and Western blot analysis confirmed that IIIA4 clustering, or its
combination with ephrin-A5-Fc, elicits robust EphA3

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**Figure 2.** Targeting profile of chIIIA4 antibody in prostate tumor
grafts. A, intravital multiphoton 3D Z-stacks of the rim of DU145
grafts and adjacent skin, injected 48 hours before imaging with
Quantum dot-labeled chIIIA4 antibody (red) and with [FITC]lectin (green),
labeling blood vessels. Collagen fibers, detected by second harmonic
generation (SHG) signals, are shown in blue. Bottom, adjacent normal
mouse from the same mouse; scale bars, 50 μm. B and C, DU145
graft tissue sections from mice treated with chIIIA4 (B) or a
nonrelevant colon–specific humanized mAb (C) were analyzed with
Alexa594-α-human antibodies and FITC-α-CD31 antibodies to assess
vascular targeting; scale bars, 25 μm. Bottom, specific binding of the
control antibody, but not of IIIA4, to colonic epithelium is illustrated.
D, flow cytometry of cell suspensions from DU145 tumor–bearing mice,
intravenously injected with [FITC]lectin to label blood-perfused vessels:
EphA3+ and [FITC]lectin+ cells as fraction of the total population, lectin+ cells as fraction of the EphA3+ cell population were quantitated; mean
and SEM are shown (n ≤ 3 mice/group). E, 22RV1 xenograft tissue
sections from mice treated with chIIIA4 (2 × 100 μg) were costained
with Alexa594-α-human antibodies, rat α-TNC/Alexa647-α-rat antibodies,
and RCA-lectin[FITC]; scale bars, 50 μm.
Figure 3. Bone marrow–derived EphA3⁺ MSCs contribute to the TME. A, comparison by flow cytometry of tumor-derived EphA3⁺/CD90⁺/Sca1 cells, isolated from DU145 tumors (see Supplementary Fig. S5A–S5D), and of EphA3⁺ BM-MSCs from corresponding tumor-bearing mice. EphA3 coexpression with indicated cell surface markers: chIII4-labeled cells (blue) are compared with unstained cells (red). Histograms are representative of three independent experiments. B, multidifferentiation potential of tumor- or bone marrow–derived EphA3⁺ MSCs in control maintenance medium (ctr) or in osteogenic, adipogenic, or chondrogenic differentiation (diff) culture media; staining for osteocyte (alizarin red), adipocyte (oil red O), or chondrocyte (alcian blue) products, respectively. Scale bars, 200 μm. C, single-cell suspensions from DU145 tumors grown in mice after adoptive transfer with EGFP-transgenic bone marrow were assessed for GFP⁺/EphA3⁺ cells by flow cytometry; panels illustrate samples unlabeled (left) and Alexa647-chIII4-labeled cells (right). D, sublethally irradiated mice were reconstituted with bone marrow depleted of EphA3⁺ cells using Alexa647-chIII4 as capture mAb. Control bone marrow was applied to the MACS system without capture mAb. Tumor growth curves (blue, EphA3-depleted bone marrow; red, control bone marrow) and individual tumor volumes 27 days after grafting are shown; data are representative of three independent experiments. E, tissue sections from Du145 tumor xenografts in mice, transplanted with wild-type (W/t) bone marrow containing FACS-isolated EphA3⁺ bone marrow cells from GFP⁺ transgenic mice (see Supplementary Fig. S6B) or W/t bone marrow passed through the FAC sorter without selection. IHC with indicated antibodies (scale bar, 500 μm); insets, boxed areas at 10× resolution (scale bars, 50 μm). F, confocal images of tumor sections from the same group of mice injected with [Rhodamine]lectin to reveal blood-perfused vessels (white pseudo color); sections stained with Alexa647-sheep-α-EphA3 (red pseudo color), counterstained with Hoechst (blue, nuclei). GFP⁺ cells are green (scale bar, 40 μm). Top right, boxed area at increased magnification (scale bar, 20 μm); bottom left, tumor section from W/t nonchimeric bone marrow transplanted mouse; bottom right, control section stained with secondary antibodies (Alexa647-sheep-α-EphA3) only.
phosphorylation, cytoskeletal contraction, and cell rounding (Fig. 4D and E). Furthermore, in cocultures of MSCs and DU145 tumor cells, EphA3 activation resulted in a marked dispersion of MSC cell strands (Supplementary Fig. S7E).

We next assessed whether the treatment of EphA3⁺ tumor MSCs with chIIIA4 would affect their viability. Continuous exposure (48 hours) to preclustered chIIIA4 resulted in a dose-dependent decrease in EphA3⁺ MSC viability (Fig. 4F). Importantly, this effect was not observed with nonclustered chIIIA4 or a clustered control mAb, where TUNEL staining of chIIIA4-treated MSCs confirmed apoptosis as the underlying mechanism (Fig. 4G and Supplementary Fig. S7F).

ChIIIA4 treatment inhibits tumor growth and disrupts the tumor stromal architecture

To assess the effect of EphA3 targeting in vivo, we compared the therapeutic effect of chIIIA4 treatment on established xenografts from EphA3-negative (DU145) or EphA3-positive (22RV1) carcinoma cell lines. Considering that the agonistic activity of chIIIA4 relies on its preclustering with α-Fc antibodies (23), we initially assessed that clustering of cell-bound chIIIA4 by Fc-γ receptor-mediated binding of mouse effector cells (monocytes/macrophages, neutrophils) would cause EphA3 activation. Indeed, chIIIA4 bound to peripheral blood monocytes isolated from BALB/c mice in an FcγII/III-
specific manner (Supplementary Fig. S8A), whereas combined treatment of EphA3+ MSCs with chIII4 and effector cells caused pronounced EphA3 activation and cell contraction (Supplementary Fig. S8B).

Importantly, chIII4 treatment of the mice caused a dose-dependent inhibition of both, 22RV1 and DU145 xenograft growth (Fig. 5A and B). IHC and IFM of treated tumors indicated a pronounced disruption of the stromal tumor architecture, where loss of defined stromal strands resulted in dispersed tenascin staining (Fig. 5C). Not surprisingly, chIII4 treatment had a dramatic effect on cell viability, and comparative analysis revealed a significant increase in apoptotic cells, particularly in the margin of chIII4-treated tumors (Fig. 5D).

In view of its preferential targeting of the vascularized TME, we assessed whether the antitumor effect of IIIA4 could be enhanced by combination treatment with bevacizumab, an approved humanized VEGF-neutralizing and antiangiogenic antibody (38). Indeed, at bevacizumab concentrations, only partially inhibiting DU145 tumor growth, addition of hIII4, the humanized version of chIII4 (24), enhanced the antitumor activity and almost completely inhibited DU145 xenograft growth at a ratio of 1:10 mg/kg bevacizumab/hIII4 (Fig. 5E).

**ChIII4 disrupts the architecture and function of tumor microvessels**

We next analyzed the effect of chIII4 on the tumor microvasculature. IHC and IFM of tumor sections revealed a significantly reduced microvascular density and frequently collapsed vessels (Fig. 6A and Supplementary Fig. S8B and S8D). We therefore used multiphoton microscopy to monitor treatment effects on stromal and microvascular integrity in vivo. Imaging of PBS-treated tumors confirmed prominent binding of Qdot655-chIII4 to [FITC]lectin-labeled tumor microvessels and to collagen fibers. In contrast, the tumor
capsule of chIIIA4-treated tumors was “collapsed,” had barely discernible collagen fibers, and severely disrupted tumor microvessels (Fig. 6B), whereas minimal Qdot655 chIIIA4 staining suggested that therapeutic chIIIA4 was occupying most of the EphA3-binding sites (Supplementary Fig. S4C).

We examined the vascular disruption suggested from these experiments, by using nonconjugated Qdots655 (Qtracker; Invitrogen) to track the blood perfusion in lectin-labeled tumor microvessels after a two-week chIIIA4 treatment. Compared with PBS-treated control mice with a typical contorted tumor microvasculature, the tumor microvessels of treated mice were collapsed, unevenly stained with [FITC]lectin and only partially perfused (Fig. 6C). An approximate doubling in the ratio of [FITC]lectin/Qtracker-fluorescence in treated tumors indicated significantly reduced perfusion of tumor microvessels in chIIIA4-treated mice. In agreement with the extensive damage to the microvessels in the tumor rim suggested from these experiments, we observed a significant increase in hypoxic tumor tissue particularly in the tumor margin, potentially reflecting the reduced oxygenation in regions with compromised tumor vasculature (Fig. 6D).

**ChIIIA4 treatment causes a significant reduction in tumor MSCs**

We next characterized the in vivo response to chIIIA4 in the TME at a cell/biochemical level. In agreement with the observation that clustered chIIIA4 activates EphA3+ tumor MSCs in vitro, IP/Western blot analysis of tumor lysates revealed that chIIIA4 treatment leads to transient EphA3 phosphorylation.
within tumors 2 hours after injection (Fig. 7A). Interestingly, after 1 week of chIIIA4 treatment, the overall EphA3 protein levels in DU145 tumors were notably reduced and did not reveal detectable EphA3 phosphorylation (Fig. 7B and Supplementary Fig. S8E). We therefore assessed the cell type within the TME that was mainly targeted by chIIIA4. After 2-week treatment, we noted a significant reduction in the fraction of EphA3+ Sca-1+ CD90+ CD45−/C3/C3 cells (Fig. 7C), consistent with IIIA4 affecting the viability of EphA3+ MSCs in vitro (Fig. 4F and G). Overall, this resulted in a slight decrease in EphA3+ cells. In agreement with the effect of chIIIA4 on EphA3+ MSC viability in vitro and in vivo, and on the overall integrity of the tumor stroma, IHC of xenograft sections revealed notably disrupted patterns of stromal (CD90), perivascular (α-smooth muscle actin, α-NG2), and myeloid (α-F4/80) markers; insets show overviews and the positions of magnified areas are boxed (scale bars, 1 mm, 50 μm).

Discussion

Eph receptors are implicated in growth and progression of a large range of cancers (4, 5). We now demonstrate for the first time the prominent expression and function of EphA3 in the TME of tumor xenografts and human sections, but virtually undetectable expression in normal adult tissues and organs. We show that bone marrow–derived EphA3+ stromal cells are recruited into the TME where they contribute to nascent vascular and stromal tissues. Treatment with chIIIA4, a highly specific mAb and selective EphA3 agonist (29–31), elicits EphA3 kinase activation, cell contraction, and apoptosis of tumor-resident MSCs, and inhibits tumor growth by disruption of the architecture and function of the vascularized TME.

EphA3 activation inhibits tumor growth

Our demonstration that activation of EphA3 with chIIIA4 effectively inhibits tumor growth seems at odds with a range of reports where its overexpression in various cancers has been correlated with disease progression and poor prognosis (9, 15–17). However, Ephs are known for their context-dependent, dichotomous functions (4, 5): typically, kinase-dormant Ephs promote cell–cell adhesion, invasion, and tumor (stem cell) maintenance and are regarded oncogenic (4–6, 39), whereas Eph kinase activation is tumor suppressive by causing cell–cell segregation and reduced viability (7, 32, 40). Thus, overexpression of kinase-dormant EphA3 in glioblastoma acts to maintain tumor cells in a dedifferentiated, tumorigenic state, whereas activation of its kinase inhibits glioma cell proliferation (9). In agreement, our studies suggest that EphA3, overexpressed in tumor MSCs and other stromal cells with a dormant kinase, functions in the development of the TME and that its pharmacologic activation results in TME disruption and suppressed tumor growth. Necessarily, this kinase-dormant EphA3 function implies the absence of interacting ephrins, which is in agreement with previous reports,
suggesting exclusive oncogenic overexpression of either Ephs or ephrins (7, 10). We found no evidence for EphA3-binding ephrins in the analyzed tumors.

The IIIA4 mAb targets tumor-derived MSCs and disrupts tumor stromal and vascular integrity

Considering the reported expression of EphA3 in a range of tumor cells (9, 15–17, 41), the most prominent tumor stromal EphA3 expression described here is noteworthy. Thus, while our analysis of human tumor tissues confirmed the previously reported tumor cell expression in glioblastoma and colon cancer (9, 41), our survey revealed significantly more pronounced and frequent EphA3 expression in the tumor stroma of all analyzed cancers, a pattern that is consistent with its involvement in the formation of the TME. Consistent with this previously unrecognized role, an interim report from ongoing clinical studies of human engineered IIIA4 (KB004) in leukemias shows responses in one of the patients that also suggest targeting of the stromal/fibrotic TME (24).

Our experiments demonstrate EphA3 expression on bone marrow–derived MSCs and myeloid cell types. Considering the multilineage potential and phenotypic plasticity of MSCs, allowing their differentiation into mesenchymal and vascular lineages, and their direct contribution to adult neovascularization (42, 43), it is tempting to speculate that the EphA3+ MSCs in our studies may be progenitors for several or all of the tumor-infiltrating stromal cell types. EphA3 was previously identified among the hypoxia-regulated genes of human bone marrow–derived MSCs (44), and the notion of EphA3+ mesenchymal progenitor cells, differentiating within the TME into different EphA3+ stromal cell populations, concurs with its embryonic expression on mesodermal (12), vascular, and mesenchymal tissues (11, 45) and its essential role in EndMT during heart valve development (14). In support of this premise, we found that bone marrow–derived and tumor-derived EphA3+ MSCs from tumor-bearing mice have the same phenotypic and pluripotent functional characteristics, and confirmed by adoptive bone marrow transfer the bone marrow origin of tumor-resident EphA3+ MSCs.

Together with the notable lack of EphA3 expression in non-tumor adult tissues, our data suggest EphA3 as a novel marker, both of MSCs and of tumor stromal tissue. Considering its developmental role in EndMT (14) and the recently reported involvement of EphB2 signaling in MSC adhesion, migration, and differentiation (46), it will be of interest to understand the potential involvement of EphA3 in MSC mobilization, recruitment into tumors, and multilineage differentiation capacity.

In conclusion, the notable stromal expression of EphA3 in the vast majority of analyzed human tumor samples and lack of expression in normal adult tissues, together with the targeting specificity and antitumor properties of the chIIIA4 mAb, define EphA3 as an attractive target for antibody-based anticancer therapies. The promising clinical responses of the human engineered therapeutic IIIA4 antibody, KB004, in ongoing phase I/II trials (NCT01211691; ref. 24) therefore suggest immediate therapeutic opportunities for the treatment of solid tumors.

References

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