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Inhibition of PDGFRα by MEDI-575 reduces tumor growth and stromal fibroblast content in a model of non-small cell lung cancer

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Running Title: Modulation of tumor stroma by MEDI-575

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Abbreviations: ELISA (enzyme-linked immunosorbent assay), SCID (severe combined immunodefient mice), huPDGFRα (human PDGFRα), huPDGFRα/SCID (SCID mice expressing human PDGFRα transgenically), sPDGFRα (soluble PDGFRα), mAb (monoclonal antibody), IHC (immunohistochemistry)

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Abstract

Platelet-derived growth factor receptor alpha (PDGFRα) is a receptor tyrosine kinase that promotes cell survival and is expressed in both the tumor and the stromal components of human cancers. We have developed a fully human monoclonal antibody, MEDI-575, that selectively binds to human PDGFRα with high affinity, with no observable affinity for murine PDGFRα. To more fully characterize the role of PDGFRα in the regulation of tumor stroma, we evaluated the *in vivo* antitumor effects of MEDI-575 in tumor-bearing SCID mice and in genetically altered SCID mice expressing human PDGFRα in place of murine PDGFRα. We utilized the Calu-6 non-small cell lung cancer model, because it lacks an *in vitro* proliferative response to PDGFRα activation. Antitumor activity was observed when the study was performed in mice expressing the human receptor, but no activity was observed in the mice expressing the murine receptor. Immunohistological analysis of tumors from mice expressing human PDGFRα showed a highly significant reduction in stromal fibroblast content and only minor changes in tumor proliferative index in tumors exposed to MEDI-575 compared to the results seen in vehicle-treated tumors or tumors from mice expressing murine PDGFRα. Additional *in vitro* studies indicated that exposure of primary cancer-associated fibroblasts to MEDI-575 can directly affect proliferation and key signaling pathways in these cells. These results highlight the potential for observing antitumor activity with MEDI-575 through modulation of the stromal component of tumors and confirm that the PDGFRα pathway can play a role in maintaining a tumor microenvironment conducive to tumor growth.

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Introduction

The platelet-derived growth factor (PDGF) receptors are Class III receptor tyrosine kinases that activate downstream signaling cascades that promote cellular survival and growth. Aberrant activation of platelet-derived growth factor receptor alpha (PDGFRα) has been observed in several human tumor types derived mainly from mesenchymal tissues, such as sarcomas, gastrointestinal stromal tumors, and glioblastomas. In these tumor types, increased signaling through tumor cell-expressed PDGFRα has been associated with overexpression, amplification, or mutation of the receptor (Clarke and Dirks, 2003; Joensuu et al., 2005; Lasota and Miettinen, 2006). In contrast, overexpression or mutation of PDGFRα in tumor tissues of epithelial origin is less common but has been observed (Matei et al., 2006; McDermott et al., 2009; Rikova et al., 2007).

Homodimeric ligands that specifically activate PDGFRα (i.e., PDGF-AA and PDGF-CC) are potent chemoattractants and mitogens for normal cells of mesenchymal origin. During development, paracrine signaling through PDGFRα is critical for development of the lung, skin, and gastrointestinal tract (Hoch and Soriano, 2003). In these tissues, PDGFRα-expressing mesenchymal cells migrate to sites of future epithelial folding and are required for such process. In the mouse, both PDGF-A and PDGFRα are required for postnatal alveolar septation in the lung and proper villus formation in the gastrointestinal tract, processes involving cross-talk between epithelial cells and PDGFRα-expressing mesenchymal cells (Hoch and Soriano, 2003; Klinghoffer et al., 2002). In addition to facilitating proper development of normal epithelial tissues, cross-talk between epithelial tumor cells and mesenchymal stromal cells can play a role in maintaining a tumor microenvironment that is conducive to increased tumor growth and angiogenesis. In this model, epithelial tumor cells

secrete multiple factors that promote the recruitment and activation of host mesenchymal stromal cells including fibroblasts (Bhowmick et al., 2004; De Wever et al., 2008). These stromal fibroblasts can in turn promote tumor growth and invasion by secreting multiple growth factors and chemokines that act in a paracrine or autocrine manner on multiple tumor components. The PDGF family of ligands, and PDGF-A and PDGF-C in particular, are key stromal support factors produced by some epithelial tumor cells. Forced overexpression of PDGF-C by xenografted melanoma cells was recently shown to increase tumor growth, which is associated with increased recruitment of PDGFRα-expressing fibroblasts into tumor stroma (Anderberg et al., 2009). Conversely, knockdown of tumor-cell-expressed PDGF-A, or neutralization of PDGF function, have been shown to reduce the growth of xenograft tumors that have little or no PDGF receptor expression on the tumor cells (Crawford et al., 2009; Reinmuth et al., 2009; Shikada et al., 2005; Tejada et al., 2006). Studies focused on the beta receptor of the PDGF family (PDGFRβ) have provided evidence supporting the role for PDGFRβ signaling in the function of pericytes and interstitial fluid pressure in tumors (Ostman, 2004; Shen et al., 2009; Furuhashi et al., 2009). These studies highlight the potential role of stromal PDGFR signaling in promoting the growth of epithelial tumors.

In order to directly probe the role of stromal PDGFRα function in supporting tumor growth, we generated a fully human antibody that neutralizes human PDGFRα, MEDI-575, and tested the antitumor effects of this antibody in the Calu-6 xenograft model of non-small cell lung cancer. This model was chosen because the tumor cells secrete PDGF-AA but do not exhibit a proliferative response to this ligand. In addition, Calu-6 cells elicit a stromal response when grown *in vivo*, resulting in tumor xenografts containing host stromal cells. In the current study, MEDI-575 was characterized as a selective inhibitor of human PDGFRα function with

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no detectable activity at the corresponding murine receptor or human platelet-derived growth receptor beta (PDGFRβ). The antitumor activity of MEDI-575 was evaluated in host mice expressing either murine or human PDGFRα to probe the specific role of host stromal PDGFRα in the growth of Calu-6 xenografts. Antitumor activity in this model was observed in the mice expressing human PDGFRα, but was not observed in the mice expressing murine PDGFRα, suggesting that modulation of the host PDGFRα was critical for effecting reduced tumor growth. We further characterized the effects of MEDI-575 exposure on cancer-associated fibroblasts (CAFs) *in vitro* and *in vivo*, and provide evidence to suggest that specific inhibition of PDGFRα function can reduce tumor growth through modulation of stromal fibroblasts in this model of lung cancer.

Materials and Methods

Generation of MEDI-575

Fully human monoclonal antibodies against PDGFRα were developed by immunizing mice (XenoMouse strain XM3C-1 [IgG4 kappa], Amgen, Inc.) with the extracellular domain of PDGFRα (sPDGFRα; R&D Systems) along with either Titermax Gold (Titermax) or aluminum phosphate gel adjuvant and ImmuneEasy Mouse Adjuvant (Qiagen) twice a week for 7 weeks. Lymph nodes were harvested and a B-cell suspension from these nodes was fused with myeloma cells (ATCC CRL-1580) using standard procedures. After clonal dilution, expansion the heavy and light chain variable domains of the lead IgG4 hybridoma line were sequenced, revealing three framework mutations (at amino acid positions 20, 49, and 107 using the Kabat numbering scheme) in the kappa light chain which utilized the VκO13 and Jκ5 human germline genes. Isotype switching was achieved by subcloning the variable domains into vectors with IgG2 isotype constant domains (Marks and Bradbury,

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2004). Germlining was performed by side-directed mutagenesis of the non-germline residues in the framework region of the light chain (Lo, 2004). MEDI-575 was produced using the 293F system (Invitrogen) and purified using protein A affinity chromatography.

Cell line authentication.

The authentication of all cell lines utilized in this study was performed using CellCheck services by RADIL (Missouri). For human cells, cell lines were verified using 9 different short terminal repeat loci, whereas the NIH-3T3 line was verified with a panel of murine-specific microsatellite markers and comparison to the original baseline profiles established for the cell line.

Statistical Analysis of Data

The graphs (Figures 1-6 and supplemental Figure 4) are derived from the average of multiple observations (cell data is in triplicate at a minimum; in vivo antitumor data used groups of 10-12 mice; image quantitation is described below) with error bars representing standard deviation from the mean. Student's t-test was used to evaluate the means from the two different groups, with a p value of ≤ 0.05 being significant.

PDGFRα phosphorylation assays.

Human osteosarcoma MG-63 cells were cultured in serum-free RPMI medium, and then MEDI-575 or buffer was then added to each well followed by PDGF-AA (R&D systems, 100 ng/mL).The primary CAFs were derived from a human male prostate cancer specimen and obtained from Asterand, Inc. CAFs were cultured in CAF medium (DMEM, 1 µg/mL hydrocortisone, 10 ng/mL epidermal growth factor, 5 µg/mL transferrin), followed by the addition of MEDI-575 or buffer, PDGF-AA (100 ng/mL). The level of human phosphorylated

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PDGFRα (pPDGFRα) in cell lysates was determined by ELISA utilizing immobilized goat anti-human PDGFRα antibody (R&D Systems), and antiphosphotyrosine-horseradish peroxidase-conjugated antibody (R&D Systems) for detection.

The murine PDGFR phosphorylation assay utilized NIH3T3 cells cultured in serum-free DMEM containing MEDI-575 or control IgG2 antibody, and then PDGF-BB was added (100 ng/mL). After a 10-minute incubation period, the medium was removed, and phosphorylated receptor was quantitated with elisa kits for the murine alpha and beta receptors (R&D Systems and Cell signaling, respectively).

Proliferation Assays

All tumor cell lines were obtained from the ATCC. Tumor cells were cultured in serum-free DMEM medium. For the primary human cancer-associated fibroblasts (CAF) proliferation assay, CAFs were plated in CAF medium without serum. For both CAFs and tumor cells, MEDI-575 or buffer was then added followed by PDGF-AA (100 ng/ml) or serum-free medium (control). After 72 hours incubation, relative proliferation rates were measured with the Alamar blue readout.

Western blotting of cell and tissue lysates.

Cell extracts were run on SDS-PAGE gels and the proteins were then transferred to a polyvinyl difluoride membrane according to standard procedures. Blots were probed with antibodies to human phosphorylated PLCγ1 (Tyr783), phosphorylated Akt (Ser473 in Akt1 and corresponding residues in Akt2 and Akt3) or phosphorylated MAPK (Thr202, Tyr204 in p44 and Thr185,Tyr187 in p42) (all from Cell Signaling), or a goat anti-human total

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PDGFRα antibody (R&D Systems) and then with peroxidase labeled secondary antibodies and developed. All blots were directly imaged using a Odyssey Imager (Licor).

Generation of homozygous huPDGFRα/SCID mice

For all studies with mice in this report, animals were treated in accordance with local and national animal welfare guidelines and the Institutional Animal Care and Use Committee guidelines. huPDGFRα expressing mice were generated and provided by the AstraZeneca Transgenics Centre, Discovery Sciences, Mölndal, Sweden. The linearized targeting vector was electroporated into R1 mouse embryonic stem (ES) cells (Andras Nagy, Toronto, Canada), and ES cell clones were selected for G418 resistance followed by PCR and extensive Southern blot analyses (data not shown). Correctly targeted ES cell clones were injected into C57BL/6-129 blastocysts (mice obtained from Harlan) to generate chimeras that were subsequently bred to C57BL/6-129 females. Germline transmission was confirmed by PCR genotyping using primers specific for the targeted or wild-type alleles (Supplemental Table 1). An F1 male heterozygous for the desired deletion and insertion was bred with multiple wild-type C57BL/6-129 females. Reverse transcription of liver-derived RNA was performed followed by PCR using primers specific for human (NM006206.4) or mouse (NM001083316.1) PDGFRα to confirm expression of the human PDGFRα transgene (Supplemental Table 1). Breeding of heterozygous mice and SCID mice (Charles River) was followed by intercross breeding to generate homozygous human PDGFRα transgenic mice in an immunodeficient background (huPDGFRα/SCID).

In vivo tumor growth studies and tissue harvesting

For all efficacy studies, animals were dosed with 10 mg/kg MEDI-575, control IgG2 or saline (vehicle), delivered intraperitoneally twice per week. For the U118 study, female CB17 SCID

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(Charles River) mice were implanted subcutaneously in the right flank with 13×10^6 U118 human glioblastoma cells combined with matrigel (1:1). For the Calu-6 xenograft studies, SCID mice (Charles River) were implanted subcutaneously in the right flank with 2×10^6 Calu-6 lung cancer cells. Transgenic mice (huPDGFRα/SCID) were implanted subcutaneously in the right flank with 2×10^6 Calu-6 lung cancer cells mixed with matrigel (1:1). Tumor volumes were calculated with caliper measurements using the formula :volume = (length × width^2)/2.When tumors reached an average of 300 mm^3 (U118) or 160 mm^3 (Calu-6) animals were randomized into groups of ten to twelve and dosing was initiated. Data points represent mean tumor volume +/- standard error (SEM).

Immunohistochemical analysis

Calu-6 tumor samples were harvested from mice, trimmed into 5 mm^3 pieces and immediately fixed in either zinc fixative or 10% neutral buffered formalin, transferred to 70% ETOH, embedded into paraffin blocks and sectioned (5 µm thickness). Slides were stained using optimized protocols on a Ventana system using various antibodies as follows: a rat anti-mouse CD31 antibody (BD Biosciences); a rabbit polyclonal smooth muscle actin (αSMA) antibody (Abcam); a rabbit polyclonal cleaved caspase-3 antibody (Cell Signaling); a rabbit polyclonal antibody to NG-2 chondroitin sulfate proteoglycan (Chemicon); a murine Ki67 mAb (Ventana). All slides were stained with diaminobenzidine (Ventana). Images were generated using the Aperio imaging system (ImageScope Version 8) and the signal density was analyzed using the Aperio color deconvolution imaging software (Version 8). The viable regions of the tumors were selected for Aperio analysis using the color deconvolution imaging software. The staining intensities from three entire sections representing different depths from each tumor

were quantitated. All images were postprocessed in Photoshop version 7 (Adobe) by employing a custom action to apply equal amounts of sharpening and curves to all images before conversion to JPEG format. The intensities of each stain in the vehicle and treated tumors were normalized such that 100% represented the average vehicle staining intensity.

Results

Generation and characterization of MEDI-575

Xenomouse mice (IgG4 strain) were immunized with sPDGFRα, and hybridomas were generated from animals that exhibited a positive titer for the presence of sPDGFRα-binding activity. A lead hybridoma line that exhibited potent binding to PDGFRα and did not bind to PDGFRβ was chosen for cloning and sequencing (data not shown). Human IgG4 antibodies have been shown to be susceptible to exchange of IgG half-molecules (one heavy and one light chain) under *in vivo* conditions resulting in bispecific antibodies with altered characteristics (Aalberse and Schuurman, 2002). MEDI-575 was generated by switching the isotype to IgG2, and then germlining to remove three framework mutations. The final antibody (MEDI-575) produced effective inhibition of human PDGFRα phosphorylation induced by PDGF-AA and PDGF-BB in a human osteosarcoma cell line (Figures 1a and 1b, respectively). In similar studies with NIH-3T3 cells, MEDI-575 did not inhibit ligand-induced phosphorylation of murine PDGFRa or PDGFRb (Figures 1c and 1d). The monovalent affinity of MEDI-575 for human sPDGFRα was determined using surface plasmon resonance (SPR) technology, with the results indicating that the equilibrium dissociation constant was 8.6 pM (supplemental Figure 1a). Since PDGFRα and PDGFRβ have distinct tissue expression profiles and different tissue-specific functions (Hoch and Soriano, 2003), it was important to understand the potential for MEDI-575 to bind to and modulate human PDGFRβ

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given that the original hybridoma generated antibody showed no interaction with human PDGFRb. SPR studies showed no detectable interaction of soluble human PDGFRβ with immobilized MEDI-575 (data not shown), likely due to the low homology (32% identity) between the extracellular domains of these two receptors. FACS titration analysis of cells overexpressing human PDGFRα was performed (Supplemental Figure 1b) which produced a K_d value of less than 1 nM.

PDGFRα signaling in cancer-associated fibroblasts

Fibroblasts are cells of mesenchymal origin that have been shown to express both PDGF receptors. Since stromal fibroblasts within human tumors can play a role in maintaining a tumor microenvironment that promotes tumor growth and angiogenesis, we evaluated the mitogenic response of cultured primary human cancer-associated fibroblasts (CAFs) to PDGF-AA exposure. The addition of this ligand to serum-starved CAFs produced nearly a two-fold increase in proliferation and over threefold increase in phosphorylated PDGFRαover untreated controls (data not shown), indicating that these cancer stromal cells expressed functional PDGFRα. MEDI-575 effectively reduced the mitogenic effect of PDGF-AA in these CAFs (Figure 1e). In addition, dose-dependent inhibition of ligand-induced PDGFRα phosphorylation in CAFs was observed (Figure 1e). It is known that signaling via PDGFRα can activate multiple downstream signaling pathways in cancer cells (Rikova et al., 2007); therefore, we investigated which of these pathways were activated in these CAFs after ligand-induced PDGFRα stimulation. Exposure of serum starved cells to PDGF-AA induced the phosphorylation of phospholipase Cγ, Akt, and mitogen-activated protein kinase (MAPK) (Figure 1f). In the presence of MEDI-575 there was a clear dose-dependent reduction in the ligand-induced phosphorylation of all of these signaling proteins, while expression of total

PDGFRα protein levels were not altered. These data indicate that activation of PDGFRα in CAFs can promote activation of multiple intracellular signaling cascades and proliferation, while exposure to MEDI-575 effectively abrogates these effects.

Taken together, these results identified MEDI-575 as a neutralizing mAb with a high affinity for human PDGFRα, with little or no detectable interaction with human PDGFRβ or murine PDGF receptors. This mAb was employed in selected *in vivo* studies as a specific antagonist of human PDGFRα function.

The expression and activation of PDGFRα in a tumor cell panel

In order to identify a tumor xenograft model that is apparently not dependent on autocrine PDGFRα signaling for tumor growth, we analyzed a panel of tumor cell lines for the expression of PDGFRα protein and proliferative response to PDGF-AA ligand (Figures 2a and 2b). The U118 glioma and MG-63 osteosarcoma lines expressed high levels of PDGFRα, consistent with the fact that they are derived from mesenchymal tissues that commonly express PDGFRα. High expression of PDGFRα was also detected in the NCI-H1703 lung cancer line, consistent with observations from a previous publication (Rikova et al., 2007). The level of PDGFRα expressed by the Calu-6 lung adenocarcinoma cell line was detectable but extremely low, whereas the other four cell lines expressed no detectable PDGFRα. Expression of PDGFRα tracked with the mitogenic response to PDGF-AA stimulation under serum-starved culture conditions (Figures 2a and 2b). Importantly, these studies identified the Calu-6 line as one that exhibited no detectable mitogenic response to PDGF-AA, confirming a previous report that these cells did not proliferate in response to PDGF-AA (Tejada et al., 2006). In the same study, it was shown that Calu-6 cells secret PDGF-AA, and that this ligand was the major fibroblast activation factor secreted by these cells when in culture. The Calu-6

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model was therefore selected for *in vivo* studies with MEDI-575, in order to probe the role of PDGFRα signaling in the stromal support of the growth of xenografted tumors from this cell line.

Since the U118 glioma cell line showed a proliferative response to PDGFRα activation, and previous studies reported that a selective antibody to human PDGFRα showed antitumor activity in xenograft studies with the U118 model (Loizos et al., 2005), we proceeded to test MEDI-575 for antitumor activity in this model. Treatment with MEDI-575 produced 101% inhibition of growth ($p < 0.0001$; Figure 2c), indicating that MEDI-575 can abrogate PDGFRα signaling *in vivo* in a model that is directly dependent on signaling through this receptor for tumor growth. Treatment with MEDI-575 did not induce changes in body weight compared to the mice treated with vehicle (supplemental Figure 4).

Characterization of huPDGFRα/SCID mice and Calu-6 tumors grown in either huPDGFRα/SCID or nude mice

Our studies identified a fully human mAb that antagonized human PDGFRα function and did not bind appreciably to, or modulate the activity of, murine PDGFRα. In order to evaluate the role of stromal PDGFRα in xenograft tumor growth in mice using MEDI-575, we generated genetically modified SCID mice expressing human PDGFRα in the place of murine PDGFRα (huPDGFRα/SCID) with expression of the human gene under the control of the promoter for murine PDGFRα (supplemental Figure 2). Germline transmission was confirmed by PCR genotyping of the F1 offspring (supplemental Figure 3) and RT-PCR analysis performed on the offspring from F1 intercrosses confirmed that the homozygous mice expressed only human PDGFRα (Figure 3a). These homozygous mice were both viable and did not exhibit most of the strong phenotypes associated with null mutations of PDGFRα in mice (Hoch and

Soriano, 2003; Klinghoffer et al., 2002), indicating that the human allele provided some compensation for the loss of the mouse gene.

Western analyses of selected tissues from the huPDGFRα/SCID mice and from nude mice were performed and the data confirmed that the human PDGFRα protein was expressed in the transgenic mice, while the corresponding murine receptor was expressed in nude mice (Figure 3b). Western analysis of Calu-6 xenograft tumors generated in nude mice and in the huPDGFRα/SCID mice was performed (Figure 3c). Calu-6 tumors expressed low levels of human PDGFRα when grown in nude mice, much lower than in a human U118 glioma xenograft, reflecting low tumor cell expression of human PDGFRα. Murine PDGFRα was detected in Calu-6 tumors from nude mice, indicating the presence of host PDGFRα-expressing cells within these tumors. These data provided evidence to suggest that Calu-6 tumors showed low tumor cell expression of human PDGFRα and contained PDGFRα-expressing stromal cells derived from the host, consistent with the observation that this model contained PDGFRα positive stromal cells (Tejada et al., 2006).

Antitumor activity in the Calu-6 model with MEDI-575 required host expression of human PDGFRα

In order to probe the role of PDGFRα in stromal support for tumor growth, we compared the effects of MEDI-575 on the growth of Calu-6 tumors hosted in 2 strains of mice expressing murine PDGFRα (Nude and SCID mice) to that observed in mice expressing human PDGFRα (huPDGFRα/SCID mice). Due to the observed specificity of MEDI-575 for human PDGFRα, modulation of PDGFRα function in the host tissues was expected to occur after treating the huPDGFRα/SCID mice, but not nude or SCID mice, with this antibody.

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Dosing with MEDI-575 resulted in reduced growth of Calu-6 tumors when hosted in the huPDGFRα/SCID mice (Figure 4a, 66% inhibition, p=0.0102) while no significant activity was observed when the same tumors were hosted in either SCID mice or nude mice (Figure 4b and 4c; p> 0.5 for both studies). Administration of MEDI-575 produced no noteworthy body weight loss in the mice expressing human PDGFRα for the duration of the studies (Supplemental Figure 4). The lack of activity in the SCID and nude mice, where MEDI-575 would be expected to modulate only tumor cell-derived PDGFRα, is consistent with the *in vitro* data (Figure 2b) indicating that Calu-6 cells do not exhibit a proliferative response to PDGFRα activation. The antitumor activity observed when the tumors were hosted in mice expressing human PDGFRα raised the possibility that the effects of MEDI-575 were mediated through modulation of the host-derived tumor stroma. We therefore investigated the histological changes associated with the reduced tumor growth after exposure to this antibody and compared the histology to the tumors from the SCID mice where antitumor efficacy was not observed.

Immunohistochemical analysis revealed modulation of stromal cells by MEDI-575

Calu-6 tumors from huPDGFRα/SCID mice were stained for αSMA to visualize the stromal myofibroblasts present in the tumors. Histological changes were observed in tumors harvested after 7 days of exposure to MEDI-575 or vehicle (Figure 5a). In the tumors from vehicle-treated mice, positive staining was noted throughout the body of the xenograft characterized by random foci of stellate to spindle shaped myofibroblast cells making close contacts with tumor cells and other myofibroblasts (Figure 5a, left panel). Tumors from mice treated with MEDI-575 showed an overall decrease in the density of αSMA staining (Figure 5a, top

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panels). Quantitative threshold image analysis of the αSMA staining indicated a significant reduction in total staining for αSMA in the treated tumors (Figure 5b).

The vascular content, proliferative index, and apoptosis levels in treated tumors were also determined via immunohistochemical analyses after 7 days of exposure to MEDI-575 or vehicle (Figure 5). A moderate but statistically significant reduction in vasculature content, as determined by CD31 staining density, was associated with MEDI-575 exposure. There were no apparent differences between the groups in their various proliferative indices as determined by Ki67 staining, supporting the notion that inhibition of PDGFRα function in the Calu-6 model does not directly affect tumor cell proliferation. However an increase (40%) in the staining for cleaved caspase-3 was observed after dosing with MEDI-575, suggesting that some cells within the tumor became apoptotic after exposure to this antibody. These results show that inhibition of PDGFRα with MEDI-575 produced a significant reduction in αSMA content and a smaller reduction (~30%) in vessel density in the tumors 7 days after the start of administration of MEDI-575. Importantly, these observed changes preceded major tumor growth delay, since there was little modulation in tumor size 7 days after the initiation of dosing (Figure 4a).

In order to determine if the stromal modulation that was observed in tumors after 7 days of exposure to MEDI-575 persisted during the 21 day time period associated with tumor growth delay, an IHC analysis was performed on tumors after 21 days of twice weekly dosing with this antibody. In this study, tumors were stained with two different markers that can stain both myofibroblasts and pericytes, NG-2 and αSMA. Tumors from the vehicle-treated group showed a similar pattern of stromal staining for these two markers, indicating the continued presence of stromal cells within vehicle-treated tumors during the study (Figure 6a, left

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panels). After 21 days of dosing tumor-bearing huPDGFRα/SCID mice with MEDI-575, the tumors showed a decrease in both NG-2 and αSMA staining characterized by a major reduction in the number and density of positively stained stromal cells (Figure 6a, right panels) and a statistically significant decrease in staining intensity for these two markers (Figure 6b). Consistent with the observations at Day 7, the staining for Ki67 was not altered in tumors from the huPDGFRα mice after 21 days of exposure to MEDI-575 (Figure 6b). Furthermore, analysis of Calu-6 tumors from standard SCID mice showed that treatment with MEDI-575 was not associated with significantly reduced αSMA staining after 17 days of exposure (Figure 6b, p=0.104), in contrast to the marked effects on SMA in tumors from the huPDGFRa SCID mice at both Day 7 and 21. Taken together, the studies performed on tumors after both 7 and 21 days of exposure to MEDI-575 indicated that tumor growth reduction was associated with reduced stromal myofibroblast content, while no observable changes to the total proliferative index of the tumor were observed.

In contrast to the moderate changes in vasculature content observed in tumors harvested 7 days after MEDI-575 dosing, quantitative analysis of CD31 staining showed no difference between vehicle and MEDI-575-treated tumors after 21 days of exposure to this mAb (data not shown). In addition, analysis of the intratumoral content of VEGF in these same tumors by an ELISA showed no significant difference between the two groups (data not shown). Therefore there was no sustained effect of MEDI-575 on the tumor vasculature density or VEGF levels in this study.

Discussion

Herein, we report the characterization of MEDI-575, a potent and selective antibody to human PDGFRα, and we provide evidence that targeting stromal fibroblasts with MEDI-575 can

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reduce tumor growth. Previous studies with another anti-human PDGFRα antibody, IMC-3G3, have indicated that targeting human PDGFRα can reduce the growth of human glioma and sarcoma xenografts (Loizos et al., 2005); both tumor types are of mesenchymal origin and likely derive a strong growth signal from autocrine PDGFRα signaling. We have confirmed these findings in the U118 Glioma models where robust growth inhibition with MEDI-575 was achieved indicating MEDI-575 might be a promising antibody for treating tumours driven by autocrine PDGFRa signaling.

Outside of the direct tumor growth drive provided by autocrine PDGFRα signaling, little is known about the additional mechanisms by which PDGFRα signaling may promote a microenvironment conducive to tumor growth. Multiple studies have highlighted the importance of various PDGF ligands in the recruitment of tumor stroma and subsequent effects on tumor growth, but the role of the specific PDGF receptors in these studies is unclear. In a xenograft model of breast carcinoma, it was shown that expression of dominant-negative PDGF abolishes desmoplasia, which is a host fibroblast-mediated response that can promote tumor growth (Shao et al., 2000). In a similar fashion, expression of dominant-negative PDGF reduced the *in vivo* growth of A549 lung xenograft tumors, which was associated with reduced content of stromal fibroblasts and pericyte coverage of the tumor vessels (Shikada et al., 2005). Since the dominant-negative PDGFs utilized in both of these studies reduced the function of both PDGF-A and PDGF-B, and because these ligands as homodimers or heterodimers can, in turn, activate PDGFRα and PDGFRβ, these studies did not directly address which PDGF receptor was critical for the effects observed.

The current studies examined the specific role of paracrine PDGFRα signaling in the stroma using the Calu-6 model of non-small cell lung cancer that is not responsive to or dependent on

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autocrine tumor cell PDGFRα activation for growth in culture. Decreased growth of Calu-6 xenograft tumors after intratumoral administration of adenoviral constructs encoding either soluble PDGFRα or PDGFRβ has been reported (Lo, 2004), suggesting some dependency on PDGF signaling for tumor growth in this model. The results reported here indicated that modulation of host PDGFRα by MEDI-575 is required for the antitumor effects observed, since reduced tumor growth was only observed when the host mouse expressed human PDGFRα in place of the murine receptor. Our results are in agreement with a report that treatment of Calu-6 xenograft bearing mice with an antagonistic antibody specific for murine PDGFRα results in reduced tumor growth, which suggested that host-specific PDGFRα modulation was the mechanism for antitumor activity (Gerber et al., 2012). In addition, we observed that the antitumor activity of MEDI-575 was associated with reduced tumor stromal fibroblast content, and there was evidence for altered morphology of the intratumoral fibroblasts that remained after exposure to this mAb. To our knowledge, this is the first direct preclinical evidence indicating that specific modulation of PDGFRα with an antibody can effect tumor growth reduction that is associated with significant modulation of stromal fibroblasts.

The data presented in the current study fits with the model that epithelial carcinoma cells can secrete soluble factors that attract and/or promote the survival and activation of fibroblasts within the tumor and that the fibroblasts in turn secrete multiple factors that induce epithelial tumor cell proliferation or promote angiogenesis and invasion (Bhowmick et al., 2004; De Wever et al., 2008; Anderberg et al., 2009; Crawford et al., 2009; Tejada et al., 2006). In the present study, MEDI-575 exposure reduced intratumoral stromal fibroblast content that was not associated with a sustained decrease in vessel content. These observations are in

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agreement with a study on another lung cancer xenograft model where neutralization of both PDGF-A and PDGF-B function reduced tumor growth but did not change the vessel count or VEGF content of the tumors (Reinmuth et al., 2009). Although MEDI-575 did not constitutively alter angiogenesis in the model used for the current study, these observations may be specific to the model used, since others have reported a link between PDGFRα signaling, VEGF levels, and angiogenesis in lung cancer models (Shikada et al., 2005).

In summary, the current study provides direct evidence indicating that targeting PDGFRα with MEDI-575 can reduce tumor growth by modulating stromal fibroblasts in a model of lung cancer where the tumor cells are not directly dependent on autocrine PDGFRα signaling for growth. These results underscore the potential importance of fibroblast-epithelial tumor cell crosstalk in promoting xenograft tumor growth and that disrupting this crosstalk can reduce tumor growth by targeting a mechanism that is distinct from existing cancer therapies such as chemotherapy and anti-angiogenic agents.

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Authorship Contributions

Participated in research design: Laing, McDermott, Hall, Reimer, Andersen, Cao, Blakey.

Conducted experiments: McDermott, Wen, Yang, Lawson, Collins, Hall, Andersén, Cao.

Contributed new reagents or analytic tools: Andersén, Snaith, Wang, Bedian, Cao.

Performed data analysis: Laing, McDermott, Wen, Yang, Collins, Reimer, Hall, Andersén, Wang, Cao.

Wrote or contributed to the writing of the manuscript: Laing, McDermott, Andersén, Snaith, Bedian, Blakey, Cao, Hall.

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Footnotes

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Figure Legends

Figure 1. Characterization of MEDI-575 in human and murine cells.

Medi-575 inhibits ligand-induced phosphorylation of human PDGFRα (h-PDGFRα) by PDGF-AA (**a**) and PDGF-BB (**b**) in human osteosarcoma (MG-63) cells. Murine PDGF receptor phosphorylation assays **(c and d)**. MEDI-575 did not significantly alter phosphorylation of murine PDGFRα (murine p-PDGFRα) (**c**), or murine PDGFRβ (murine p-PDGFRβ) (**d**) induced by PDGF-BB. Data in panels (a) to (e) is expressed relative to the signal for phosphorylation induced by ligand alone (100%) and cells not treated with ligand (0%). MEDI-575 modulates the response of CAFs to PDGF-AA exposure (**e and f**). Ligand-induced proliferation and phosphorylation of PDGFRα (p-PDGFRα) in human cancer-associated fibroblasts (CAFs) were inhibited by MEDI-575 in a dose-dependent manner**(e)**. Inhibition of PDGF-AA mediated phosphorylation of phospholipase Cγ (p-PLCγ), MAPK (p-MAPK), and Akt (p-Akt) in CAFs by western blot analysis (f), with the position of the closest molecular weight markers indicated (mwm). CAFs were exposed to the indicated concentrations of MEDI-575 prior to ligand addition. The levels of total PDGFRα were not reduced after exposure to MEDI-575.

Figure 2. PDGFRα expression and signaling in human CAFs and tumor cell lines and in vivo antitumor activity in the U118 model.

(a) Expression of PDGFRα (PDGFRa) protein in human tumor cell lines. The lines that were tested were Calu-6 (Calu); U118; MG-63 (MG63); NCI-H1703 (H17); IGROV-1 (IGR); Colo205 (Colo); MDA-MB231 (MB); and MCF-7 (MCF7). Western analysis for Hsp60 level was performed as a protein loading control. **(b)** The effects of PDGF-AA exposure on the

proliferation of serum-starved tumor cell lines. Data is reported as the fluorescent units from the proliferation readout for the serum starved cells with and without ligand addition`. **(c)** The effects of dosing tumor-bearing mice with MEDI-575 on the growth of U118 glioma xenografts. MEDI-575 or vehicle was administered starting on Day 21 for the duration of the study.

Figure 3. Analysis of PDGFRα expression in tissues from huPDGFRα/SCID and wild-type mice.(a) Gene expression (mRNA) analysis of various littermates during the generation of the huPDGFRα/SCID mice. The homozygous mice expressed human and not murine PDGFRa gene. The specificity of the primers utilized are noted (human or murine) and the exons targeted by the forward and reverse primers, respectively, are indicated within parentheses. The sizes of the observed band were consistent with the predicted sizes and are shown with each gel (Wt = wild type mouse , Ht = heterozygous , Hm = homozygous, -ve = no DNA). **(b)** Western analysis of multiple tissues from the huPDGFRα/SCID mice (H) and SCID mice (M). Positive controls included U118 xenograft lysate (U) for human PDGFRa, and soluble recombinant human PDGFRα (hRα) or murine PDGFRα (mRα). The lysates analyzed were from the following tissues: colon (Co), lung (Lu) skeletal muscle (Mu), skin (Sk) and spleen (Sp). **(c)** PDGFRα expression in Calu-6 tumors grown subcutaneously in nude mice (Calu-6 nude) compared to those grown in the huPDGFRα/SCID mice (Calu-6 KO/KI). Controls included U118 glioma xenograft (U118) for human PDGFRα and mRα for the murine receptor. For all tissue lysates except the soluble receptors, 20 µg protein was loaded into each well.

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Figure 4. Reduced growth of Calu-6 tumors with Medi-575 treatment of huPDGFRα/SCID mice with Medi-575. There was no significant antitumor activity observed after dosing standard SCID or nude mice bearing Calu-6 tumors with MEDI-575. Tumor growth vs time for Calu-6 xenograft tumors hosted in (**a**) huPDGFRα/SCID mice, (**b**) SCID mice and (c) nude mice. Twice weekly dosing of tumor-bearing mice with MEDI-575, control IgG (10 mg/kg) or vehicle was initiated on Day 7 (**a**) , Day 11 (b) or Day14 (**c**) and continued throughout the duration of the studies.

Figure 5. Evidence for tumor stromal modulation after 7 days exposure to MEDI-575. IHC staining of Calu-6 tumors harvested from the huPDGFRα/SCID mice after dosing mice with MEDI-575 or vehicle**.** Mice were dosed with vehicle or 10 mg/kg MEDI-575 on days 1 and 4, and the tumors were harvested on Day 7. **(a)** Representative images of sections stained with antibodies to detect αSMA (stromal myofibroblasts, pericytes); CD31 (vasculature), Ki67 (proliferation), and cleaved caspase-3 (cc-3; apoptosis) **(b)** Thresholded image quantitation of tumor staining are reported as a percentage of the vehicle control values. The p values generated from Student's t-test comparing the treated and control groups were αSMA (0.005), CD31 (0.020), Ki67 (0.65) and cleaved caspase-3 (0.005). The staining intensity for the vehicle group was set at 100% for quantitative comparison.

Figure 6. Stromal modulation after 21 days exposure to MEDI-575 in tumors from huPDGFRα mice. Mice were dosed twice weekly with vehicle or MEDI-575 and tumors were harvested for analysis after 21 (huPDGFRa/SCID mice) or 17 days (SCID mice). **(a)** Representative images of tumors from huPDGFRα/SCID mice, stained with antibodies to

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NG-2,αSMA and Ki67 are shown. Representative images of tumors from SCID mice stained with αSMA are shown in the lowest panels. **(b)** Threshold image quantitation of the stained tumor sections. The staining intensity for the vehicle groups were set at 100% for quantitative comparison. There was a significant difference between tumors from huPDGFRa/SCID mice treated with vehicle vs MEDI-575 for the NG-2 and αSMA staining, as determined by Student's t-test ($P = 0.0007$ and < 0.00001, respectively). In contrast, there was no significant change in αSMA staining of MEDI-575 treated compared to vehicle tumors from SCID mice ($P = 0.104$).

Fig. 1

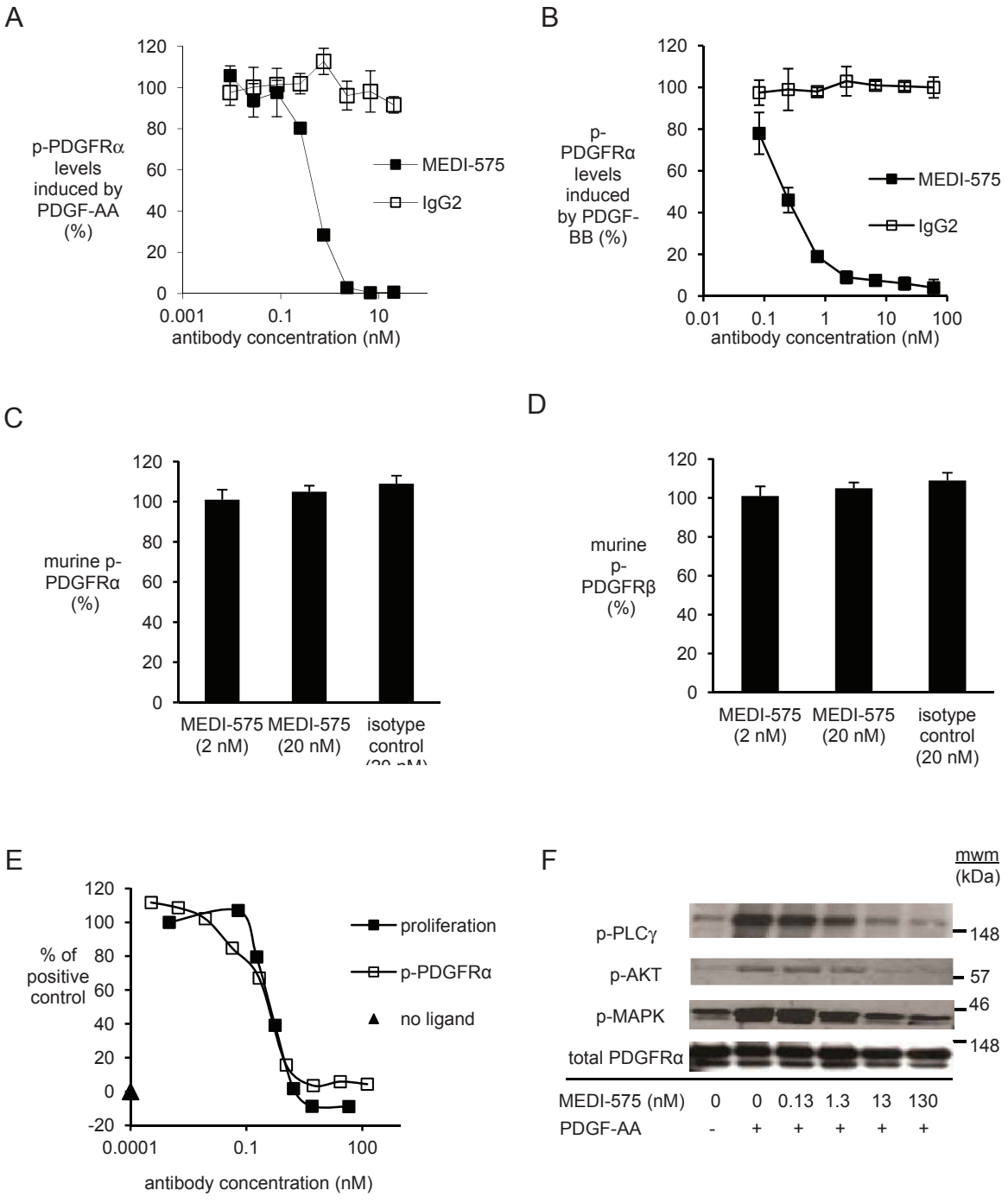

A
p-PDGFRα levels induced by PDGF-AA (%)
120
100
80
60
40
20
0
0.001
0.1
10
antibody concentration (nM)
MEDI-575
IgG2
B
p-PDGFRα levels induced by PDGF-BB (%)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
antibody concentration (nM)
MEDI-575
IgG2
C
murine p-PDGFRα (%)
120
100
80
60
40
20
0
MEDI-575 (2 nM)
MEDI-575 (20 nM)
isotype control
D
murine p-PDGFRβ (%)
120
100
80
60
40
20
0
MEDI-575 (2 nM)
MEDI-575 (20 nM)
isotype control (20 nM)
E
% of positive control
120
100
80
60
40
20
0
-20
0.0001
0.1
100
antibody concentration (nM)
proliferation
p-PDGFRα
no ligand
F
mwm (kDa)
p-PLCγ
p-AKT
p-MAPK
total PDGFRα
148
57
46
148
MEDI-575 (nM) 0 0 0.13 1.3 13 130
PDGF-AA - + + + + +

Fig. 2

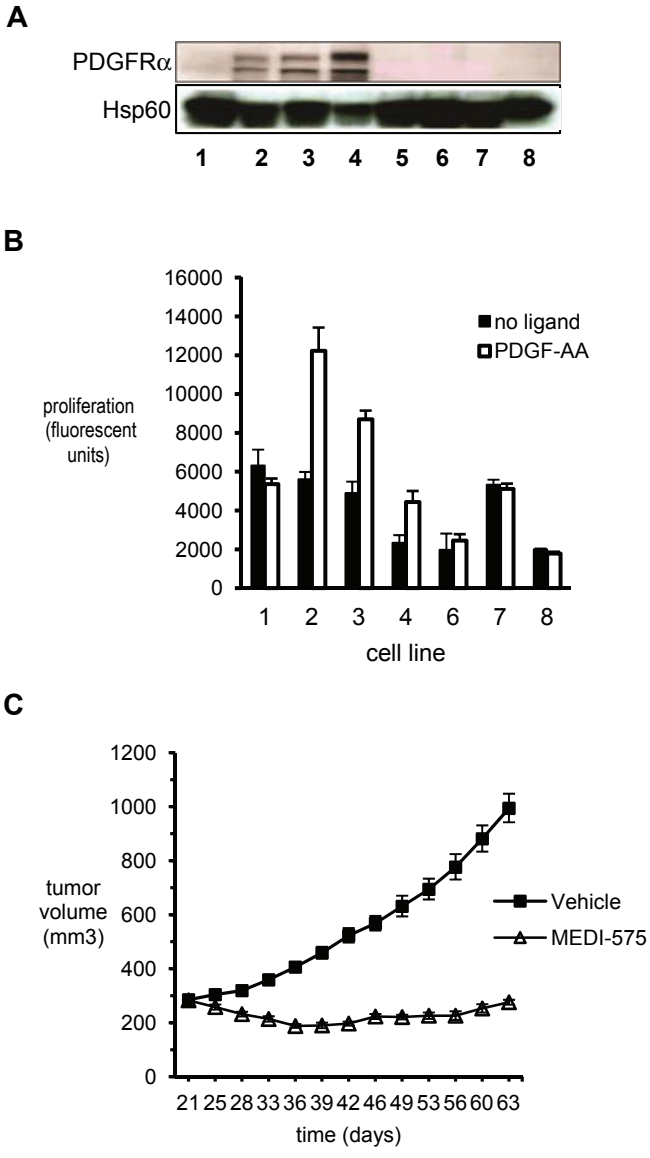

A
PDGFRα
Hsp60
1 2 3 4 5 6 7 8
B
16000
14000
12000
10000
8000
6000
4000
2000
0
proliferation (fluorescent units)
no ligand
PDGF-AA
1 2 3 4 6 7 8
cell line
C
1200
1000
800
600
400
200
0
tumor volume (mm3)
Vehicle
MEDI-575
21 25 28 33 36 39 42 46 49 53 56 60 63
time (days)

Fig. 3

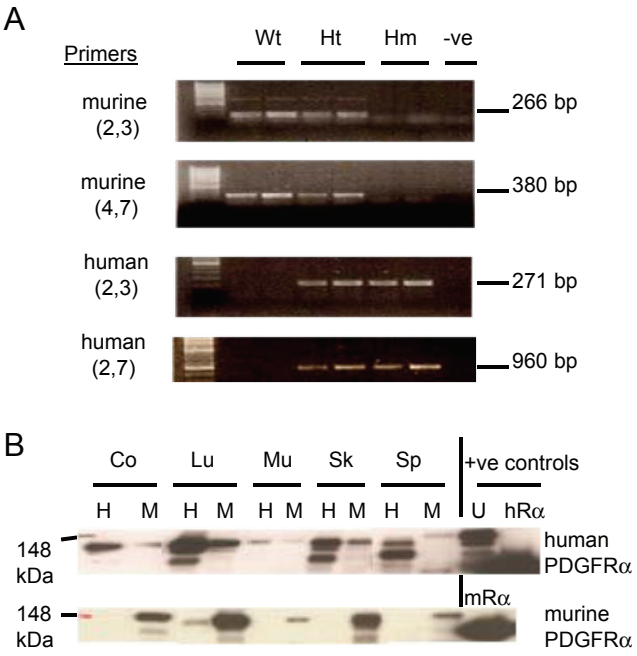

A
Primers
Wt
Ht
Hm
-ve
murine (2,3)
266 bp
murine (4,7)
380 bp
human (2,3)
271 bp
human (2,7)
960 bp
B
Co
Lu
Mu
Sk
Sp
+ve controls
H M H M H M H M H M U hRα
148 kDa
human PDGFRα
mRα
148 kDa
murine PDGFRα
C
Calu-6 nude
U118
Calu-6 KO/KI
U118
human PDGFRα
Calu-6 nude
mRα
Calu-6 KO/KI
mRα
murine PDGFRα

Fig. 4

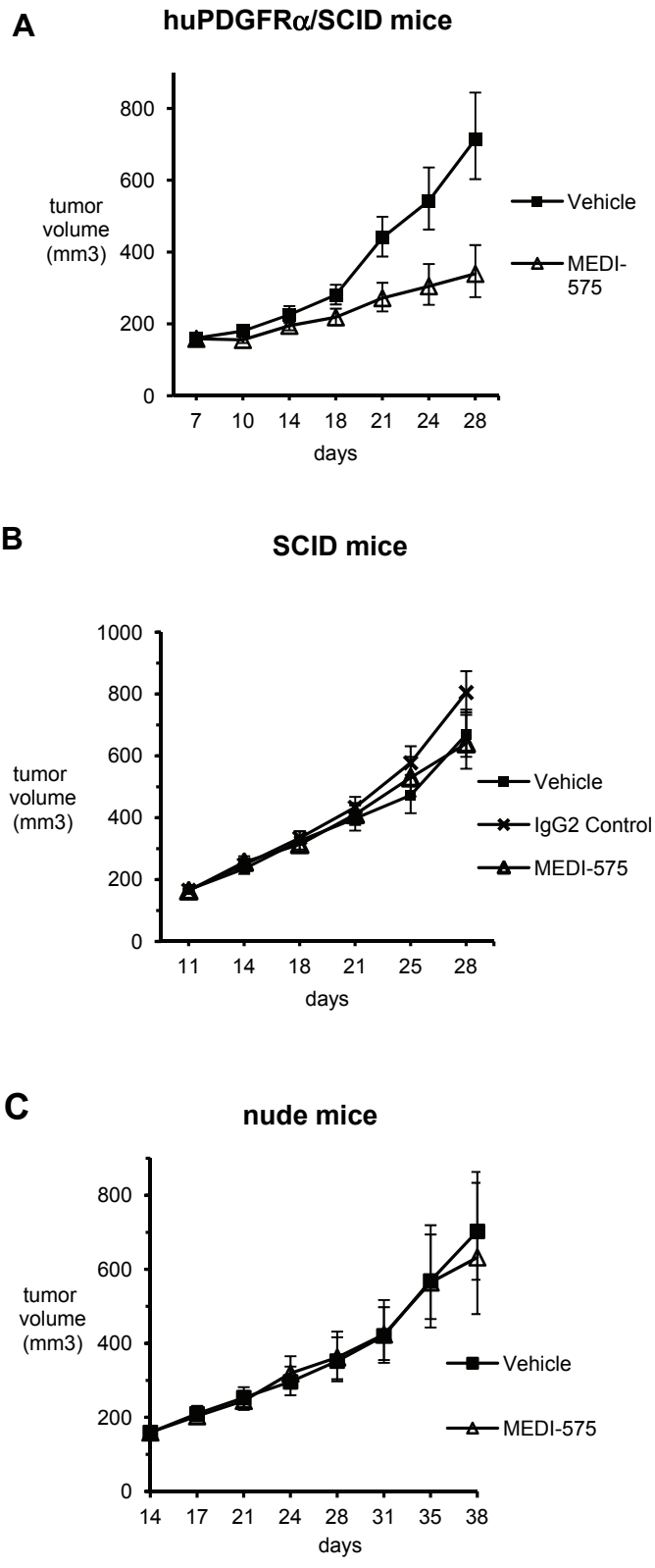
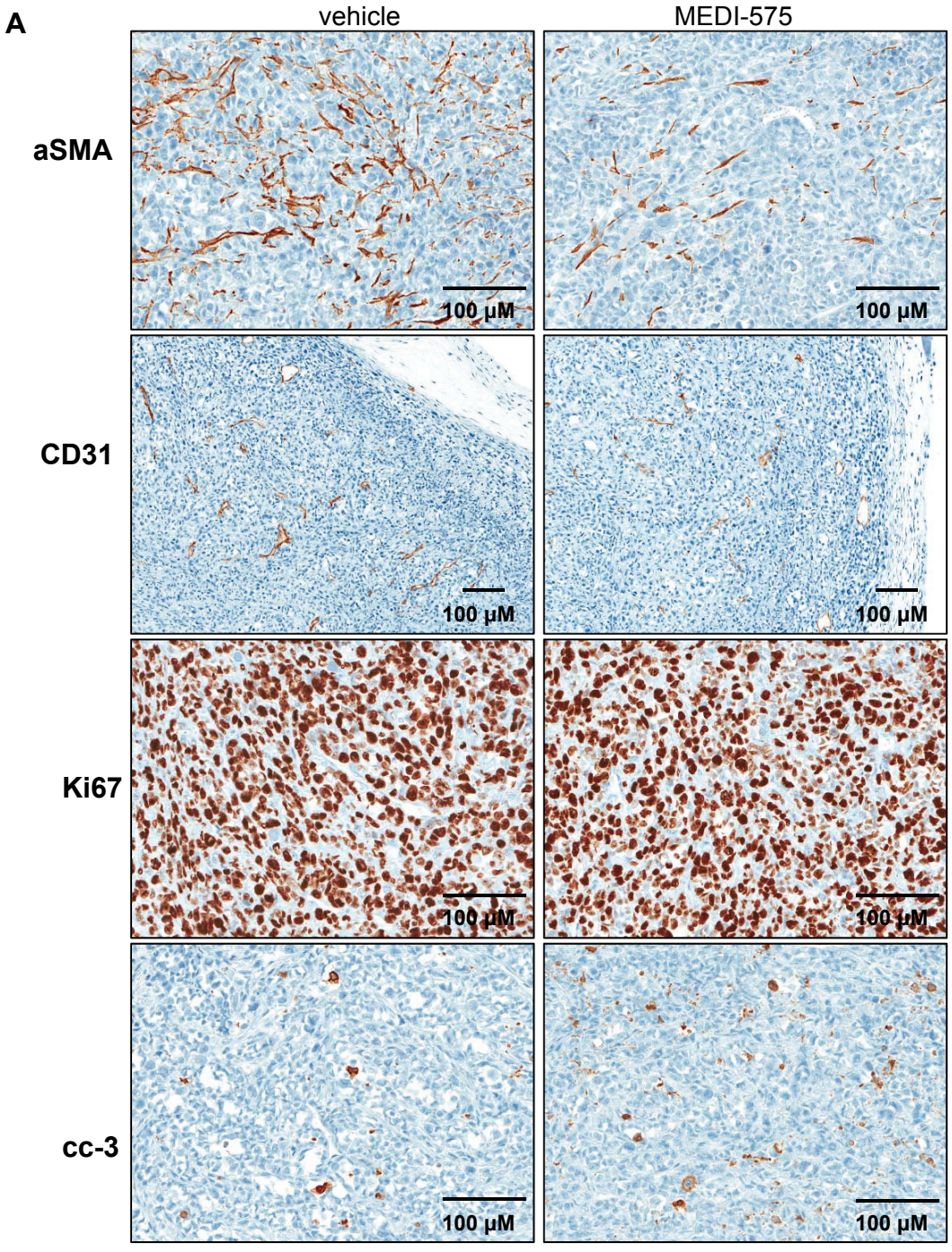

A
huPDGFRα/SCID mice
tumor volume (mm3)
800
600
400
200
0
7
10
14
18
21
24
28
days
Vehicle
MEDI-575
B
SCID mice
tumor volume (mm3)
1000
800
600
400
200
0
11
14
18
21
25
28
days
Vehicle
IgG2 Control
MEDI-575
C
nude mice
tumor volume (mm3)
800
600
400
200
0
14
17
21
24
28
31
35
38
days
Vehicle
MEDI-575

Fig. 5


A
vehicle
MEDI-575
aSMA
CD31
Ki67
cc-3
100 μM

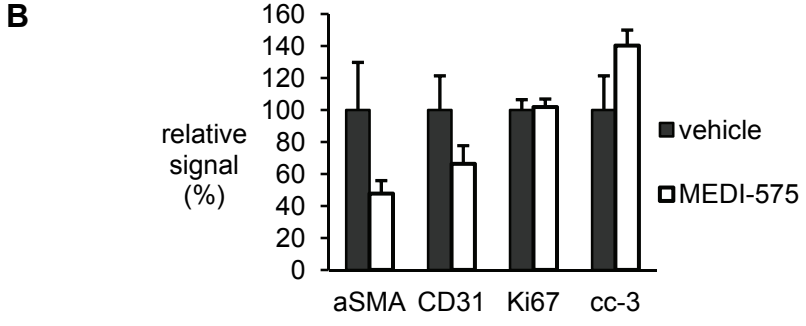
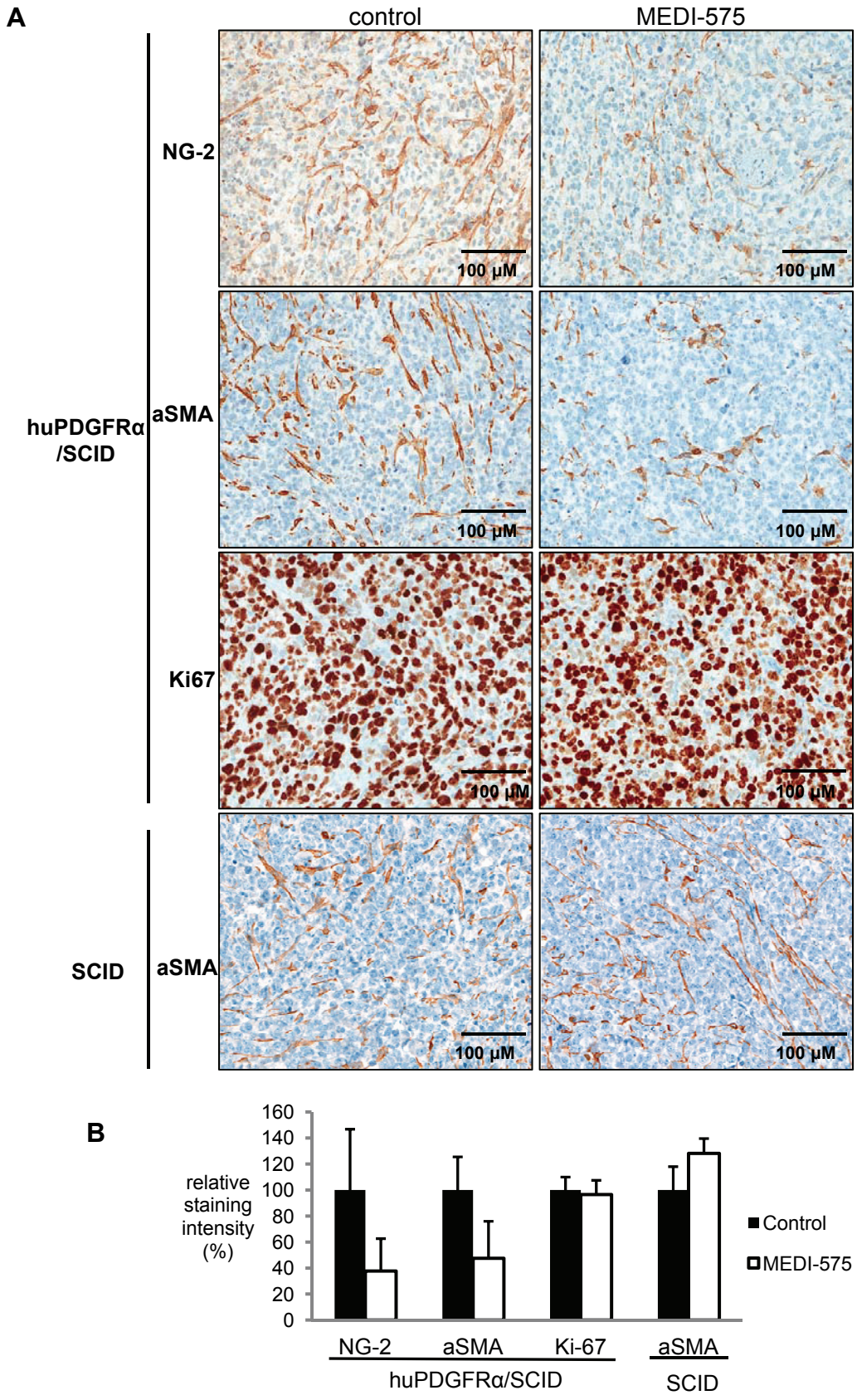

B
relative signal (%)
160
140
120
100
80
60
40
20
0
aSMA
CD31
Ki67
cc-3
vehicle
MEDI-575

Fig. 6


A
control
MEDI-575
NG-2
aSMA
Ki67
huPDGFRα /SCID
SCID
aSMA
100 μM
B
relative staining intensity (%)
160
140
120
100
80
60
40
20
0
NG-2
aSMA
Ki-67
aSMA
huPDGFRα/SCID
SCID
Control
MEDI-575