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Paracrine Placental Growth Factor Signaling in Response to Ionizing Radiation Is p53-Dependent and Contributes to Radioresistance

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Placental growth factor regulation in response to ionizing radiation is p53-dependent

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Running title: Placental growth factor and ionizing radiation

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Abstract

Placental growth factor (PlGF) is a pro-angiogenic, N-glycosylated growth factor, which is secreted under pathological situations. Here, we investigated the regulation of PlGF in response to ionizing radiation (IR) and its role for tumor angiogenesis and radiosensitivity. Secretion and expression of PlGF was induced in multiple tumor cell lines (medulloblastoma, colon and lung adenocarcinoma) in response to irradiation in a dose- and time-dependent manner. Early upregulation of PlGF expression and secretion in response to irradiation was primarily observed in p53 wildtype tumor cells, whereas tumor cells with mutated p53 only showed a minimal or delayed response. Mechanistic investigations with genetic and pharmacological targeting of p53 corroborated regulation of PlGF by the tumor suppressor p53 in response to irradiation under normoxic and hypoxic conditions, but with so far unresolved mechanisms relevant for its minimal and delayed expression in tumor cells with a p53-mutated genetic background. Probing a paracrine role of IR-induced PlGF secretion in vitro, migration of endothelial cells was specifically increased towards irradiated PlGF wildtype but not towards irradiated PlGF knockout medulloblastoma cells. Tumors derived from these PlGF-ko cells displayed a reduced growth rate, but similar tumor vasculature formation as in their wildtype counterparts. Interestingly though, high-dose irradiation strongly reduced microvessel density with a concomitant high rate of complete tumor regression only in the PlGF-ko tumors.

Implications: Our study shows a strong paracrine vasculature-protective role of PlGF as part of a p53-regulated IR-induced resistance mechanism and suggest PlGF as a promising target for a combined treatment modality with RT.

Introduction

Radiotherapy (RT) is widely used alone or as part of a combined treatment modality with surgery and chemo-/immunotherapy for the management of solid tumors (1-5). In addition to DNA damage and genome instability, ionizing radiation (IR) also leads to stress responses in the irradiated tumor cells by activating signal transduction pathways and inducing secretion of several auto- and paracrine factors (6, 7). We recently performed an exhaustive semi quantitative dot blot secretome analysis from irradiated tumor cells and identified among others that placental growth factor (PlGF) is secreted from tumor cells in response to irradiation. A secondary analysis revealed that multiple of these factors, but not PlGF, are substrates of the matrix metalloproteinase ADAM17 and that irradiation enhances its sheddase activity in a dose-dependent way (8).

PlGF is an N-glycosylated, homodimeric protein and belongs to the vascular endothelial growth factor (VEGF)-family, with 53% similarity to the platelet-derived growth factor (PDGF)-like region of VEGF (9). The PlGF gene contains seven exons and due to alternative splicing exists in four isoforms consisting of 131, 152, 203 and 224 amino acids (10, 11). Under normal conditions, PlGF expression is low in organs such as heart, skeletal muscle and lungs (12-14). Binding of PlGF to its receptor vascular endothelial growth factor receptor 1 (VEGFR1) leads to phosphorylation of the receptor, activation of downstream pro-angiogenic signaling pathways and a crosstalk between VEGFR-1 and 2 (15-18). In comparison to VEGF-A, PlGF is not extensively investigated and its regulation and role in response to irradiation is poorly understood. PlGF is highly expressed in the placenta, however, its deletion does not affect embryonic development in mice (16). Interestingly, while PlGF-deficient mice develop normally, lack of PlGF results in reduced tumor vascularization and tumor growth (16, 17). Several studies demonstrate the contribution of PlGF to tumor angiogenesis under pathological conditions. Tumor stage, metastasis and poor overall survival correlate to increased PlGF levels

in different tumors entities (19-24). Of note inhibition of VEGF and its receptors leads to increased PlGF levels, probably contributing to escape and resistance against these treatment modalities (24-27). On the preclinical level as investigated in several mouse models, blocking of PlGF by RNA interference, neutralizing antibodies or gene silencing resulted in decreased angiogenesis, reduced tumor growth and dissemination (16, 24, 28-32). Interestingly, while blockage of PlGF normalized tumor vessels in some tumor models (28, 33), tumor vessel normalization was also observed in PlGF overexpressing tumors (34).

Expression of PlGF is regulated by several transcription factors, however contradictory results exist on the regulation of PlGF by the tumor suppressor p53 and the hypoxia-inducible factor HIF-1 α (9, 16, 18, 29, 35-37). Furthermore, almost no data exist on the regulation of PlGF in response to irradiation (38). Here we investigate expression and secretion of PlGF across multiple tumor cell lines in response to irradiation and its role for tumor angiogenesis in response to radiotherapy.

Materials and methods

Cell cultures and treatments

Human lung adenocarcinoma cells (A549, H460, H358, H292, H125, HCT116) (ATCC, Manassas, VA, USA) were cultured in RPMI1640 media (Gibco, ref. no. 22409-015), medulloblastoma cells (D341, D425, DAOY, UW228) (kindly provided by Martin Baumgartner, Children's Hospital Zurich) in improved MEM media (Richter's modification) (Gibco,) and head and neck squamous cell carcinoma cells (FaDu) (ATCC, Manassas, VA, USA) in DMEM media (Gibco,). All media were supplemented with 10% (v/v) fetal calf serum (Gibco, ref. no. 10270-106), 1% (v/v) penicillin-streptomycin (Gibco) and 1% (v/v) Glutamax (Gibco) and cells were kept at 37 °C in 5% CO₂. HUVECs, Endothelial Cell Growth Medium (ECGM) and supplementary growth factors were purchased from Promocell (Heidelberg,

Germany). Human primary lung fibroblasts, fibroblast basal medium and fibroblast growth kit were purchased from ATCC. All cells were regularly tested for mycoplasma infection (MycoAlert Mycoplasma Detection Kit #LT07-118, Lonza). Cells were pretreated with Pifithrin- α (S2929), MI-773 (S7649), BAY87-2243 (S7309) (all purchased from Selleckchem, Houston, TX, USA) in given concentrations 1-2 hours prior to irradiation with an Xstrahl 200kV X-ray unit at 1Gy/min or an RS-2000 225kV irradiator at 4.2Gy/min (Rad Source, Buford, GA, USA). Hypoxia experiments were performed with cells irradiated and/or treated with pharmacological agents followed by incubation at 1% O₂.

Bio-Plex assay

Bioplex Biomarker Cancer Panel assay (32 biomarkers) was performed with undiluted conditioned medium samples according to the manufacturer protocol (Bio-Rad, Hercules, CA, USA). Briefly, beads covalently coupled to a capture antibody directed against specific biomarker were incubated with standards or samples (supernatant derived from non-irradiated and irradiated A549 cells). After a washing step, beads are incubated with biotinylated detection antibodies. The unbound biotinylated antibodies were washed away and the beads were incubated with a fluorescence reporter streptavidin-phycoerythrin conjugate (SA-PE), forming the detection complex. The fluorescence of each bead with bound SA-PE was measured when the beads were passed through the two laser Bio-Plex reader. The data are presented as median fluorescence intensity (MFI) and concentration (pg/ml) using a Bio-Plex ManagerTM software, where concentration is proportional to MFI of the reporter.

ELISA

Secreted PIGF concentration was detected in filtered conditioned media at the indicated time points after irradiation using a PIGF DuoSet ELISA kit according to manufacturer's guidelines

(R&D Systems, Minneapolis, MN, USA). The absorbance was determined with the plate reader EL808 Ultra Microplate Reader (Bio–Tek Instruments, Inc., Winooski, VT, USA) at 450nm excitation and 570nm emission, and normalized to live cell count using EVE Automatic cell counter (NanoEnTek, Seoul, South Korea).

qRT-PCR

Sample mRNA expression was determined at 4 hours and 24 hours after irradiation. Cell lysate collection and RNA isolation were performed using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's guidelines. RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Bioscience, Waltham, MA, USA) and cDNA was amplified using SYBR Green Master Mix (Roche, Basel, Switzerland) with the following primers: (5'-3'): GAPDH forward: AACGGATTTGGTCGTATTGGGC; GAPDH reverse: TTGATTTTGGAGGGATCTCG; PIGF forward: TGTCACCATGCAGCTCCTAA; PIGF reverse: AGCATCGCCGCACCTTTC; p53 forward: CCCTTCCCAGAAAACCTA; p53 reverse: CTCCGTCATGTCCTGTGA. qRT-PCR was performed on LightCycler 480 (Roche, Basel, Switzerland).

siRNA transfection

Transfection was performed by using reverse transfection with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) in antibiotic-free medium. Cells were re-seeded into 6-well plates with fresh medium supplemented with antibiotics 24h thereafter. siLuc-RNA was synthesized by Microsynth (Balgach, Switzerland) with the following sequence: (5'-3'): CGUACGCGGAAUACUUCGATT. sip53-RNA was purchased as a pool of four siRNAs (Dharmacon, Lafayette, CO, USA). Irradiation was performed 48 hours after siRNA transfection.

Western Blotting

Whole cell extracts were collected using Laemmli buffer and samples were boiled for 5 minutes at 95 °C. 40-50µg of protein were separated by SDS-PAGE and transferred to PVDF membranes. The membrane was then blocked in 5% non-fat milk and incubated with primary antibodies (rabbit polyclonal anti-p53 (GTX102965, GeneTex, Irvine, CA, USA), rabbit monoclonal anti-p21 (2947S, Cell Signaling, Danvers, MA, USA), rabbit monoclonal anti-HIF-1 α (D2U3T, Cell Signaling, Danvers, MA, USA), mouse monoclonal anti- β -actin, (A1978, Sigma, St. Louis, MO, USA)) at 4°C overnight. After three washings, HRP conjugated secondary antibodies (mouse anti-rabbit (SC-2357, Santa Cruz, Dallas, TX, USA) or sheep anti-mouse (NA931V, GE Healthcare, Chicago, IL, USA)) were added for one hour at room temperature followed by additional washing steps. The membrane was developed with the ECL system (Amersham Bioscience, Amersham, England) according to manufacturer's protocol in Fusion FX (Vilber, Collegien, France).

Genomic deletion of *PlGF* and *p53* via CRISPR/Cas9 in D341 cells

Two separate sgRNAs targeting different regions of the third exon of the *PGF* gene (target sequence 1: GAATCTGCACTGTGTGCCGG; target sequence 2: CGTGTCCGAGTACCCCAGCG) were selected a using freely available online tool (CRISPOR). Likewise two separate sgRNAs targeting different regions of the third exon of the *TP53* gene (target sequence 1: GAATCTGCACTGTGTGCCGG; target sequence 2: CGTGTCCGAGTACCCCAGCG) were selected. SgRNA oligos were synthesized by Microsynth (Balgach, Switzerland). The single-stranded oligomers were annealed and ligated into GFP and Cas9 expressing pLentiCRISPR-EGFP plasmid (#75159, Addgene, Cambridge, MA, USA) using the Golden Gate assembly cloning strategy. The ligation mix was transformed

into competent bacteria by heat shock. Clones were identified by colony-PCR using an U6 promoter specific forward primer (5' –GACTATCATATGCTTACCGT- 3') and the reverse sgRNA oligo as reverse primer. Positive colonies were inoculated for mini-prep (Sigma, St. Louis, MO, USA) culture. HEK293T (ATCC, Manassas, VA, USA) cells were used for production of lentiviral constructs according to guidelines from Collecta (Mountain View, CA, USA). Filtered lentiviral supernatant was used to transfect the target cells (D341). To prepare cells for flow cytometry, live cells were harvested, re-suspended in PBS with 2% FBS and filtered using a 40µm cell strainer (BD Falcon, Franklin lakes, NJ, USA). Cell sorting was performed with a FACS Aria™ III Cytometry System (BD Biosciences, Franklin lakes, NJ, USA). Sorted cells were further subcloned through limited dilution and sequenced before further experiments. Each subclone was named after their respective well number, i.e. control cell lines C4, C6, and knock out cell lines B5, B7, G7 and F8.

Migration Assay

For the transwell migration assay, 24-well transwell units (6.5mm) with 1µm pore size PET membranes (Greiner Bio-One, 662610, Kremsmuenster, Austria) were used according to the manufacturer's instructions. Briefly, 3×10^5 attracting cells (D341 PIGF-wt or PIGF-ko) were plated into the lower chamber of the transwell containing 1000µl ECGM supplemented with 1% FBS and without growth factors and allowed to attach for a minimum of 6h. The plate was sham irradiated or irradiated with 5Gy. Next, 3×10^4 endothelial cells in 200µl of the same ECGM medium as above were seeded into the upper chamber of the transwell inserts and allowed to attach for a minimum of 6h. Thereafter, the inserts were irradiated with 5Gy and immediately placed on the wells harboring the attracting cells. The co-culture was maintained at 37°C in 5% CO₂ for 48 hours. For quantification, cells from the upper side of the insert were scraped away with a cotton swap and inserts were then fixed in Methanol/Acetic Acid

(75%/25%, v/v), dried and stained with DAPI (1:25000) in 99% MetOH. Fluorescent microscopy pictures were taken (Leica 7000DT, Wetzlar, Germany) and the migrated cells were counted manually in at least 3 images at 20x magnification per insert.

In vivo experiments

D341 PlGF control and PlGF knockout subclones (4×10^6 cells in 150 μ l PBS) were injected subcutaneously in the back of 8-week old, female athymic CD1 nude mice (Charles River, Wilmington, MA, USA). Tumor volumes were determined with caliper according to the formula $(L \times l^2)/2$. Treatment started when tumors reached a volume of $200\text{mm}^3 \pm 20\%$. Tumors were sham-irradiated or irradiated with a single dose of 5 or 10Gy using an image-guided small animal radiotherapy platform (Precision X-Ray, X-Rad SmART, North Brandford, CT, USA) 225kV unit with a dose rate of 3Gy/min, equipped with a cone beam CT (CBCT) scanner. Precise irradiation plans were designed with the corresponding SmARTPlan software. Radiation therapy was applied with two opposing fields. Animals were kept under 3% isoflurane anesthesia for imaging and treatment. 8 days after treatment, tumors were harvested and immediately fixed in formalin. All in vivo experiments were performed according to guidelines for the welfare and use of animals of the Veterinäramt Kanton Zurich, Switzerland.

Immunohistochemistry

Immunohistological endpoints were analyzed on formalin-fixed paraffin-embedded (FFPE) 4 μ m tissue sections derived from PlGF control or PlGF ko-derived tumor xenografts for hematoxyline and eosine (H&E), smooth muscle actin (SMA) (1:50, M0851, Dako, Glostrup, Denmark) and CD31 (1:10, M0823, Dako, Glostrup, Denmark). Images were taken on a wide-field Nikon Eclipse TI microscope (Minato, Tokyo, Japan). Amount of vessels were counted in at least 10 different fields in each xenograft.

Statistical analysis

Cellular experiments were performed at least three times and statistical analysis was performed using GraphPad Prism v.8. Data was tested for normality using the Shapiro-Wilk test. Tumor volumes over time were evaluated by using linear regression and the slopes of the best linear fit line were statistically analysed. In all cases, two groups are compared by a two-side t-test (or Mann-Whitney test for data not following the normal distribution) and multiple groups are compared by one-way ANOVA (or Kruskal-Wallis test for data not following the normal distribution), followed by Fisher's LSD test in the case of planned comparisons and the Holm-Sidak correction otherwise. For all experiments data are represented as mean $\pm$ SD, * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$. P-values are indicated for $P < 0.05$.

Results

Ionizing radiation increases PlGF expression and secretion across multiple cancer cell lines

Secretome analysis by semiquantitative antibody array screening previously indicated that PlGF is secreted in response to irradiation [8]. Additional bioplex-based quantification of 32 major cancer biomarkers in the supernatant from irradiated A549 lung adenocarcinoma cells now revealed that PlGF is most strongly increased among these cancer biomarkers in response to irradiation (**Fig. 1A**). In order to analyze irradiation-induced PlGF-expression in more detail and across multiple tumor entities (medulloblastoma, lung adenocarcinoma, colon carcinoma; D341, DAOY, H125, A549, HCT116), tumor cells were irradiated, supernatants collected at multiple time points after irradiation and analyzed for PlGF secretion by ELISA. IR-enhanced PlGF-secretion was identified to different extents in the conditioned media (CM) from these cell lines in a time- and IR-dose-dependent manner (**Fig. 1B, C** for the medulloblastoma cell lines D341 and DAOY, and Supplementary Fig. S1B-D for the cell lines derived from other

tumor entities). Similar to the original bioplex analysis, PlGF secretion was also most strongly enhanced in the D341 medulloblastoma cell line relative to other cancer biomarkers determined (Supplementary Fig. S1A)

Enhanced secretion in response to irradiation might be related to IR-induced *PGF*-transcription. Upregulation of PlGF mRNA was observed in several cancer cell lines as early as 4 hours after irradiation (D341, A549), while only minimally or only observed in a delayed way in other cell lines 24 hours after irradiation (DAOY) (**Fig. 2 A-F**) (see Supplementary Fig. S2 for other cell lines, H292, H460, H358, FaDu, UW228, H125, HCT116, HUVEC, HPLFB). Based on a literature search on the mutational status of Kras and p53 in these cell lines (Supplementary Table S1), this differential IR-induced expression pattern of PlGF could be correlated with the mutational status of the tumor suppressor and transcription factor p53.

Interestingly, cells expressing wildtype p53 demonstrated IR-induced PlGF transcription as early as 4 hours after irradiation, which correlated with early PlGF secretion. In contrast cells expressing mutant p53 did not show any changes in the PlGF mRNA level at this early time point after irradiation, suggesting that p53 regulates immediate IR-induced PlGF expression. Of note, delayed expression of PlGF in the p53 mutated cells in response to irradiation correlated with delayed PlGF secretion into the CM of the respective cells (48 hour time point) (see above Fig. 1). Due to their high PlGF expression and secretion levels, and that treatment of medulloblastoma patients includes radiotherapy (39), further experiments were primarily performed with p53 wildtype D341 cells.

p53 is the main regulator of PlGF in p53 wildtype cancer cells

To determine regulation of PlGF-expression and secretion by p53, D341 and DAOY medulloblastoma cells were treated with increasing concentrations of the MDM2-inhibitor MI-773. MDM2 downregulates the transcriptional activity of p53 by direct binding to the tumor suppressor p53, its ubiquitination and subsequently inducing its proteasomal degradation (40).

Thereby, pharmacological disturbance of the MDM2-p53 interaction might lead to p53 stabilization and increased transcriptional activity independent of irradiation. Cellular treatment with MI-773 rapidly induced PlGF-expression in the D341 but not in the DAOY cells and resulted in enhanced PlGF levels in the CM of D341 cells as determined 24 and 48 hours after treatment start (**Fig. 3A-D**). MI-773-induced PlGF-expression and secretion were confirmed with p53-wt HCT116 colon carcinoma cells (Supplementary Fig S3A, B).

Putative regulation of PlGF by p53 was more specifically investigated using p53-directed siRNA. p53 mRNA and protein levels were downregulated on siRNA-treatment for up to 96 hours. Likewise, expression of the well-known p53 downstream target p21 was also downregulated (Supplementary Fig. S3C, D). Tumor cells were irradiated 48 hours after siRNA transfection, and PlGF secretion was determined in the CM 48 hours after irradiation. PlGF levels were strongly enhanced in response to irradiation in siLuc-pretreated cells, but basal and irradiation-induced PlGF-levels were significantly decreased in CM of sip53-pretreated cells (**Fig. 3E**). Eventually, D341 p53 knockout cells were generated. IR-induced PlGF expression and secretion was completely abrogated in p53-ko D341 tumor cells (**Fig 3F, G**).

MDM2 has additional binding targets besides p53 and thereby MI-773 might reactivate also other transcriptional activities besides p53 leading to enhanced PlGF-expression. Therefore, PlGF-expression and secretion was also analyzed in siRNA-p53-downregulated and D341 p53-ko cells on treatment with MI-773 for 4 and 24 hours. Neither expression nor secretion of PlGF were enhanced by MI-773 in these cells (**Fig. 4A-C**), (see also Supplementary Figure S3D-H for colon carcinoma cells).

To analyze dependence on p53 transcriptional activity, additional experiments were performed with the p53-inhibitor Pifithrin (PFT), which prevents nuclear translocation of p53 and transcriptional activation of downstream genes (41, 42). Prior to irradiation, cells were preincubated with Pifithrin for 2 hours and the PlGF level determined 24 hours thereafter.

Similar to the experiments performed with the MDM2-inhibitor and p53-directed siRNA, decreased PlGF levels in Pifithrin-pretreated cells indicate p53-mediated PlGF regulation in response to irradiation (**Fig. 4D, E**) (see also Supplementary Figure S3I for colon carcinoma cells). Overall, these complementary experiments strongly demonstrate that p53 is a major transcriptional regulator for PlGF expression.

To determine whether p53 truly binds to the promoter region of PlGF, we performed additional ChIP-qPCR assay. Unfortunately, we could not identify a p53 binding site on the *PGF* promoter using p53-antibodies and PlGF site-specific primers mentioned by Rashi-Elkeles et al. (38).

Irradiation-induced PlGF secretion under hypoxia

Both p53 and the hypoxia inducible transcription factor-1 (HIF-1) regulate cellular transcriptional responses to hypoxia and to ionizing radiation. At the same time, an intriguing interplay exists between p53 and HIF-1 including transregulation and competition for limiting co-factors (43). Of note, contradictory results exist on the expression of PlGF under hypoxic stress conditions, which might be cell type and genetic background dependent (35-37). To investigate p53-dependent and IR-induced PlGF-secretion under hypoxic conditions, D341 medulloblastoma cells were irradiated followed by incubation under normoxic and hypoxic (1% O₂) conditions. In addition, cells were pre-incubated with increasing concentrations of BAY 87-2243 (0nM, 10nM, 50nM) one hour prior to IR. BAY87-2243 is a potent inhibitor for hypoxia-induced, HIF-1 α and HIF-2 α -mediated gene activation (44). Hypoxia minimally enhanced basal and IR-induced PlGF secretion, and cellular pretreatment with BAY 87-2243 did not interfere with PlGF secretion neither under normoxic nor hypoxic conditions (**Fig. 5A**). PlGF-secretion was also determined in CM derived from D431 cells transfected with p53-oriented siRNA and incubated under hypoxic conditions (1% O₂). Similar to the experiments performed under normoxic condition (see above), downregulation of p53 by p53-directed

siRNA strongly decreased irradiation-induced PlGF secretion also under hypoxic condition (**Fig. 5B**). Basal PlGF-secretion was only minimally increased under hypoxic conditions in comparison to normoxic conditions in both D341-p53-wt and D341-p53-ko cells. Irradiation increased PlGF-secretion only from D341 p53-wt cells under hypoxic conditions, which was not affected by cellular pretreatment with the HIF-1 inhibitor BAY 87-2243 (**Fig 5C**). Detection of HIF-1 α in non-irradiated and irradiated cells by western blotting demonstrated hypoxia-upregulated HIF-1 α under hypoxic conditions in both D341-p53-wt and D341-p53-ko cells, which was absent in cells pretreated with BAY 87-2243 (**Fig. 5D**). These results strongly corroborate that regulation of PlGF-secretion under normoxic and hypoxic conditions is primarily p53- and not HIF-signaling dependent.

Angiogenesis is inhibited in PlGF-ko cells

To further investigate auto- and paracrine effects of IR-induced secretion of PlGF, D341 PlGF knockout cells were generated using two different exon 3-targeting single guide RNAs of the *PGF* gene and cloned into a GFP and Cas9 expressing pLentiCRISPR plasmid. Sequencing of the targeting region of exon 3 in our cell clones confirmed successful *PGF*-gene targeting (supplementary fig. S4A). IR-induced PlGF secretion was determined in the cell supernatants derived from a control cell clone and a selected knock out cell clone 24 hours and 48 hours after irradiation. PlGF was undetectable in the supernatants of PlGF knockout cell lines and IR-enhanced PlGF-levels were only determined in the supernatants of the control cell line (PlGFwt) (Supplementary Fig. S4B). In order to investigate a paracrine effect of PlGF, we performed a Boyden chamber migration assay with irradiated human umbilical vein endothelial cells (HUVECs) seeded in the upper chamber migrating towards non-irradiated and irradiated D341 PlGF-wt and D341 PlGF-ko cells, respectively. No quantitative difference in HUVEC migration towards unirradiated PlGF-wt versus unirradiated PlGF-ko cells could be

determined. However, significantly increased migration of HUVECs towards irradiated PlGF-wt cells versus irradiated PlGF-ko cell could be observed (**Fig. 6A**).

Prior to *in vivo* experiments, proliferative activity and clonogenicity of PlGF-wt and PlGF-ko cell clones were determined and revealed only minor and PlGF-independent differential readouts (Supplementary Fig. S4 C-D).

To determine a role of IR-regulated PlGF-expression on the endothelial compartment *in vivo*, medulloblastoma xenografts were developed from subcutaneously-injected PlGFwt and PlGF-ko tumor cells. Overall, 83-87% of mice injected with PlGF-wt cells and 52-58% of mice injected with PlGF-ko cells developed tumors. Days to reach the starting volume ranged from 31-37 days for PlGF-wt and 36-50 days for PlGF-ko cells respectively. Tumors were irradiated with increasing doses of IR (0Gy, 5Gy, 10Gy) and mice were sacrificed eight days following irradiation. Tumor sections were stained for H&E, CD31 and SMA and the amount of vessels were counted in at least ten fields for each tumor. Microvessel densities (MVD) (CD31-staining) in PlGF-wt and PlGF-ko medulloblastoma xenografts were comparable, and MVD only minimally changed in PlGF-wt tumor xenografts in response to irradiation. Interestingly though, a decrease in MVD was observed in tumor xenografts derived from PlGF-ko-medulloblastoma cells irradiated with 5Gy and MVD was strongly reduced on irradiation with 10Gy (**Fig. 6B**). A quantitative similar outcome was determined on smooth muscle actin (SMA) staining as an indicator of pericyte coverage and vessel functionality (**Fig. 6C**). Representative staining of tumor sections are shown in **Fig. 6D**.

PlGF-wt and PlGF-ko cells displayed comparable proliferative activity and radiosensitivity *in vitro* (see above). Interestingly, the determination of a short-time efficacy-oriented endpoint *in vivo* revealed statistically significantly enhanced tumor shrinkage of PlGF-ko-tumors in comparison to PlGF-wt tumors in response to the same dose of irradiation (**Fig. 7**). Furthermore 3 out of 5 PlGF-ko tumors (60%) in each treatment group (5 and 10 Gy) showed

complete tumor regression in comparison to none of PlGF-wt tumors in the group of mice treated with 5Gy and only 1 PlGF-wt tumor in the group of mice treated with 10 Gy of IR. These results demonstrate enhanced radiosensitivity of PlGF-ko tumors in comparison to PlGF-wt tumors and suggest that the vasculature protective effect of tumor-derived PlGF co-determines tumor radiosensitivity *in vivo*.

Overall, these results suggest that tumor-derived PlGF has a tumor vasculature protective effect and thereby co-determines tumor radiosensitivity *in vivo*.

Discussion

Anti-angiogenic agents are mainly directed against the VEGF/VEGFR signaling pathway. However, targeting the VEGF/VEGFR pathway eventually leads to resistance and to an angiogenic switch to other growth factors, such as PlGF (29). The expression level of placental growth factor is increased in several cancer types and correlates with poor survival, cancer progression and resistance to therapy (19-22). Patients with metastatic colorectal cancer have shown increased PlGF levels after combined treatment with bevacizumab, chemotherapy and radiotherapy (45, 46). Furthermore, increased PlGF expression has been linked to increased vessel number, size and permeability (24) and targeting of PlGF demonstrated reduced tumor vascularization in VEGFR-inhibitor-resistant tumor models (29). However, clinical studies with PlGF neutralizing antibodies did not show improved survival in combination with bevacizumab in previously treated glioblastoma patients and therefore the strategy to target PlGF was discontinued, despite minimal normal tissue toxicities (47-49).

Here, we investigated PlGF expression and secretion in multiple cancer cell lines with differential genetic backgrounds in response to increasing doses of IR. Early, treatment-induced PlGF expression could be directly linked to the p53-status of the irradiated cells. Other

transcription factors, could play a role in PlGF regulation in cells with mutated p53 and could be relevant for delayed expression in response to irradiation.

The PlGF promoter contains binding sites for multiple transcription factors including p53. It has four NF- κ B, five metal transcription factor 1 (MTF1), three Sp1, 1 BF2 binding sites and a predicted hypoxia responsive element (HRE) in its promoter. However, all the binding sites were identified in different cell types (HEK, immortalized mouse embryonic fibroblasts, HeLa cells) and under different conditions (37, 50, 51). First indications that p53 might regulate PlGF-expression derive from a wide-scale transcriptome study in normal human B-lymphoblastoid cells (TK6 cells) investigating the transcriptional activity of p53 in response to ionizing radiation. Binding of p53 to the PlGF-promoter was also confirmed by ChIP assay but has not been further investigated beyond these transcriptional studies (38). Despite several attempts we could not perform successful ChIP assays demonstrating direct binding of p53 to the promoter site of PlGF in the medulloblastoma. This might be due to technical limitations or the intrinsic challenge to detect direct promoter binding of p53 due to the multiple layers of different transcriptional regulation of p53 downstream targets in response to stress (52). Nevertheless, our expression and secretion studies using different p53-interfering agents and performed in multiple cell lines clearly demonstrate that p53 plays a major role in PlGF-regulation in response to ionizing radiation.

Of note, cellular treatment with the MDM2 inhibitor MI-773 stabilized p53 and subsequently resulted in increased PlGF expression and secretion with similar kinetics to IR-induced PlGF-expression and secretion in these p53-wt tumor cells. The *PGF* promoter has additional binding sites for other transcription factors than p53, that could also be affected by MDM2-inhibitor. Control experiments with the MDM2-inhibitor with concomitant downregulation of p53 and in p53 knockout cells indicated, that p53 is the major transcription factor relevant for IR-mediated regulation of *PGF* in p53-wildtype tumor cells. IR-induced and p53-mediated

secretion of PlGF corresponds to the categorization of PlGF into the class of SASP (senescence-associated secretory phenotype)-related cytokines (53). However, SASP was not further investigated in this report as several of the investigated cell lines do not undergo senescence in response to irradiation.

Due to the interplay between p53 and the hypoxia inducible factor-1, we hypothesized that other transcription factors, mainly HIFs, could be additional regulators for PlGF-expression in irradiated tumor cells lacking p53, in particular under hypoxic conditions. Therefore PlGF secretion was determined in non-irradiated and irradiated p53-wildtype, p53-downregulated and p53-knockout tumor cells, respectively, both under normoxic and hypoxic conditions. Basal and IR-induced PlGF-secretion was comparable under normoxic and hypoxic conditions, and IR-enhanced PlGF secretion under hypoxic conditions was also strongly p53-dependent. HIF-1-oriented inhibitory studies with BAY87-2243 confirmed that the transcription factor p53 and not HIFs is most relevant for IR-induced PlGF-regulation. Our results relate to investigations performed by others, showing though increased PlGF expression under hypoxic conditions, but in a HIF-1-independent way (50, 51, 54). In-depth mechanistic investigations are now required to identify additional, but so far unresolved mechanisms relevant for its minimal and delayed expression in tumor cells with a p53-mutated genetic background.

Tumor growth rate in the PlGF-knockout medulloblastoma tumor xenografts was only slightly delayed, corresponding to previous reports on the influence of PlGF on basal tumor growth (16). However, PlGF-knockout tumor xenografts were more radiosensitive with an increased rate of total remission in response to single high doses of IR. Reduced tumor growth and enhanced radiosensitivity, though, was not due to a differential proliferative activity and radiation response on the tumor cell level but might be linked to an enhanced secondary effect in response to irradiation on the tumor-microenvironment (55) due to the lack of tumor cell-derived PlGF. Such PlGF-dependent, paracrine-mediated effects in response to irradiation

could also be observed *in vitro* determining the migratory capacity of endothelial cells. Furthermore the formation of the tumor vasculature was comparable in the growing PlGF-knockout and -wildtype tumor cell-derived xenografts. However, the integrity of the vasculature was strongly reduced in the PlGF-knockout tumor xenografts on irradiation and correlated with enhanced radiosensitivity of the PlGF-knockout tumors in comparison to their wildtype counterparts.

Overall, these results indicate that PlGF plays a minor role for the formation of an intact vasculature during tumor growth, which might explain the lack of a therapeutic effect by PlGF-oriented inhibitory antibodies (47-49). On the other hand, PlGF has a strong tumor vasculature protective role in response to irradiation, which might be mediated by IR-induced secretion of PlGF from the irradiated tumor cells. Lack of this protective effect contributes to a microenvironment-mediated increase of tumor radiosensitivity. Thus, targeting of PlGF still represents an interesting rationale but might be more effective not as part of a single but as part of a combined treatment modality with radiotherapy, thereby overcoming a radiotherapy-induced treatment resistance mechanism, namely radiotherapy-induced PlGF-secretion.

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Figure Legends

Figure 1. PlGF secretion is increased in multiple cell lines after irradiation. **A**, Bioplex-based quantification of 32 cancer biomarkers in the supernatant from irradiated A549 lung adenocarcinoma cells with strongly increased PlGF levels determined 24 hours after irradiation. **B – C**, Irradiation-induced PlGF-secretion was investigated in the medulloblastoma cell lines D341 and DAOY. Supernatants were collected and analyzed for PlGF secretion at the indicated time points after irradiation. IR-enhanced PlGF-secretion was identified in the conditioned media (CM) by ELISA in a time- and IR-dose-dependent way. Level of PlGF secretion differed between the different cell lines and therefore graphs show adjusted y-axis for each cell line. Bar graphs represent concentration of PlGF normalized to viable cell count $\pm$ SEM. Students t-test was used for statistical analysis.

Figure 2. PlGF mRNA expression is increased in response to IR. qRT-PCR-based relative PlGF mRNA expression determined in lung adenocarcinoma (A549) cells (**A-B**) and medulloblastoma cells (D341 and DAOY) (**C-F**) 4 hours and 24 hours after irradiation with increasing doses of ionizing radiation. Bar graphs show PlGF mRNA expression relative to GAPDH $\pm$ SEM. Students t-test was used for statistical analysis.

Figure 3. p53 is a major regulator of PlGF-expression in response to IR. Treatment of D341 (**A – B**) and DAOY cells (**C – D**) with increasing doses of the MDM2-inhibitor MI-773. MI-773 induced PlGF-expression at 4 hours and resulted in enhanced PlGF levels in the supernatant 24 hours after treatment start of D341 but not of DAOY cells. **E**, Downregulation of p53 with p53-directed siRNA abolished IR-induced PlGF-secretion. PlGF secretion was determined in the CM 48 hours after irradiation. siLuc treated cells demonstrated a strong increase in PlGF secretion upon irradiation. **F, G** Basal and IR-induced PlGF mRNA secretion in D341-p53-wt and D341-p53-ko cells. Bar graphs show PlGF mRNA expression relative to GAPDH and PlGF secretion $\pm$ SEM. Students t-test was used for statistical analysis.

Figure 4. The MDM2-p53 axis specifically regulates PlGF expression. PlGF expression and secretion in p53-downregulated D341 (A – B) and D341-p53-ko cells (C) on treatment with increasing doses of MI-773 for 4 and 24 hours. D-E, PlGF expression and secretion of Pifithrin (PFT)-pretreated cells 24 hours after irradiation. PFT was added to cells 2 hours prior to irradiation. Bar graphs show PlGF mRNA expression relative to GAPDH and PlGF secretion $\pm$ SEM. Students t-test was used for statistical analysis.

Figure 5. PlGF secretion is HIF-independent. A, p53 wildtype D341 medulloblastoma cells were pretreated with increasing doses of BAY 87-2243 one hour prior to IR. Following irradiation, cells were incubated under normoxic and hypoxic (1% O₂) conditions for 48 hours and PlGF was determined in the CM by ELISA. B, PlGF-secretion in sip53-pretreated D341 cells under hypoxia. C, PlGF-secretion of D341-p53-wt and D341-p53-ko cells pretreated with BAY 87-2243 under hypoxia. D, HIF-1 α protein level in BAY 87-2243 pretreated, non-irradiated and irradiated D341-p53-wt and D341-p53-ko cells under normoxic and hypoxic conditions and determined in cellular lysates by western blotting. Graphs show secreted PlGF levels $\pm$ SEM. Students t-test was used for statistical analysis.

Figure 6. PlGF protects the tumor vasculature in response to irradiation. A, The paracrine effect of PlGF on HUVECs was investigated by a Boyden chamber migration assay. HUVECs were seeded in the upper chamber and irradiated, and the amount of migrated HUVECs towards non-irradiated and irradiated D341 PlGF-wt (C4,C6) and PlGF-ko (B5,B7,G7,F8) cells, respectively, was determined by DAPI staining. B – C, Microvessel densities were determined in tissue sections from tumor xenografts derived from PlGF-wt and PlGF-ko D341 medulloblastoma cells. Tumors were harvested 8 days following irradiation and tissue sections were stained for CD31- and SMA. The amount of vessels was counted in at least ten fields for each tumor. Each dot shows amount of vessels in one field. D, Representative tissue sections for each treatment group. Scale bar shows 50 μ m. One-way ANOVA and Students t-test were used for statistical analysis.

Figure 7. PlGF determines medulloblastoma radiosensitivity. **A,** Tumor volumes over time were evaluated by using linear regression and the slopes of the best linear fit line were statistically analysed. Slopes of tumor growth over time (PlGF-wt versus PlGF-ko) were compared between different treatment groups (0, 5 and 10Gy). **B)** Tumor xenografts derived from PlGF-wt and PlGF-ko D341 medulloblastoma cells were sham-irradiated or irradiated with 5 and 10 Gy when tumors reached a volume between 150mm³ and 200mm³. The responses of the individual tumors are depicted. In response to 5 and 10Gy, 3 out of 5 PlGF-ko tumors of each irradiated group (60%) showed complete tumor regression in comparison to only 1 PlGF-wt tumor in the group of mice treated with 10 Gy of IR.

Figure 1

Figure 2

Figure 3

A)

B)

C)

D)

E)

F)

G)

Figure 4

Figure 5

A)

B)

C)

D)

Figure 6

Figure 7

Supplementary Figure S1

A)

B)

C)

D)

Supplementary Figure S1. PIGF secretion is increased in multiple cell lines after irradiation. A, IR-induced secretion of different cytokines from D341 medulloblastoma cells 24 hours after irradiation (ND: not detectable) B–D, Irradiation-induced PIGF-secretion was investigated in tumor cell lines derived from lung adenocarcinoma (A549 and H125) and colon carcinoma (HCT116). IR-enhanced PIGF-secretion was identified in the conditioned media (CM) by ELISA in a time- and IR-dose-dependent manner. Bar graphs represent PIGF concentrations normalized to viable cell count $\pm$ SEM. Students t-test was used for statistical analysis.

Supplementary Figure S2

K)

L)

M)

N)

O)

P)

Q)

R)

Supplementary Figure S2. PIGF mRNA expression is increased in response to IR. A – F, qRT PCR-based relative PIGF mRNA expression was determined in lung adenocarcinoma (H292 and H460) and colon carcinoma (HCT116) cells 4 hours and 24 hours after irradiation with increasing doses of ionizing radiation. G – N, Minimal or no change in relative PIGF mRNA expression in head and neck squamous

carcinoma (FaDu), medulloblastoma (UW228) and lung adenocarcinoma (H125 and H358) cells after irradiation. O-R, Minimal or no change in relative PlGF mRNA expression in human umbilical vein endothelial cells (HUVECs) and increase in relative PlGF mRNA expression with increasing doses of ionizing radiation in human pulmonary lung fibroblasts (HPLFBs). Bar graphs show PlGF mRNA expression relative to GAPDH $\pm$ SEM. Students t-test was used for statistical analysis.

Supplementary Figure S3

Supplementary Figure S3. P53 is a major regulator of PlGF in response to IR. **A-B**, MI-773-induced PlGF-expression and secretion were confirmed with p53-wt HCT116 colon carcinoma cells. **C**, Relative p53 mRNA expression was determined 48-96 hours after p53 downregulation in D341 cells. **D**, p53 protein level determined in cell lysates of D341 cells 48-96 hours after sip53 treatment start. As a control and downstream target of p53, p21 levels are represented following sip53 treatment. **E-H**, Relative p53 mRNA expression was determined 48-96 hours after p53 downregulation in HCT116 colon carcinoma cells and corresponding PlGF secretion. Neither expression nor secretion were enhanced by MI-773 in the sip53 pretreated HCT116 cells. **I**, Decrease in PlGF secretion in Pifithrin-pretreated HCT116 cells. Graphs show p53 mRNA expression relative to GAPDH $\pm$ SEM.

A)

wt	200	GTGGTACCCCTTCCAGGAAGTGTGGGGCCGCAGCTACTGCCGGGCGCTGGAGAGGCTGGTG	259
Ct	1	GTGGTACCCCTTCCAGGAAGTGTGGGGCCGCAGCTACTGCCGGGCGCTGGAGAGGCTGGTG	60
	260	GACGTCGTGTCCGAGTACCCAGCGAGGTGGAGCACATGTTTCAGCCCATCCTGTGTCTCC	319
	61	GACGTCGTGTCCGAGTACCCAGCGAGGTGGAGCACATGTTTCAGCCCATCCTGTGTCTCC	120
	320	CTGCTGCGCTGCACCGGCTGCTGCGGCGATGAGAATCTGCACTGTGTGCCGGTGGAGACG	379
	121	CTGCTGCGCTGCACCGGCTGCTGCGGCGATGAGAATCTGCACTGTGTGCCGGTGGAGACG	180
	380	GCCAAATGTCACCATGCAGGTAGGTCCATAACCCTGCCCAGGGGGCCACCGAAATCTGCCAC	439
	181	GCCAAATGTCACCATGCAGGTAGGTCCATAACCCTGCCCAGGGGGCCACCGAAATCTGCCAC	240
	440	TAGAAGGTACCTGGAGAGGGAAGAAGATAGGCCAGGT	477
	241	TAGAAGGTACCTGGAGAGGGAAGAAGATAGGCCAGGT	278
wt	214	GGAAGTGTGGGGCCGCAGCTACTGCCGGGCGCTGGAGAGGCTGGTGGACGTCGTGTCCGA	273
KO	1	GGAAGTNTGGGGCCGCAGCTACTGCCNGGCGCTGGAGAGGCTGGTGGACGTCGTGTCCGA	60
	274	GTACCCC-AGCGAGGTGGAGCACATGTTTCAGCCCATCCTGTGTCTCCCTGCTGCGCTGCA	332
	61	GTACCCCAAGCAGGTTGGAGCACATGTTTCAGCCCATCCTGTGTCTCCCTGCTGCGCTGCA	120
	333	CCGGCTGCTGCGGCGATGAGAATCTGCACTGTGTGCCGGTGGAGACGGCCAAATGTCACCA	392
	121	CCGGCTGCTGCGGCGATGAGAATCTGCACTGTGTGCCGGTGGAGACGGCCAAATGTCACCA	180
	393	TGCAGGTAGGTCCATAACCCTGCCCAGGGGGCCACCGAAATCTGCCCACTAGAAGGTACCTG	452
	181	TGCAGGTAGGTCCATAACCCTGCCCAGGGGGCCACCGAAATCTGCCCACTAGAAGGTACCTG	240
	453	GAGAGGGAAGAAGATAGGCCAGGT	477
	241	GAGAGGGAAGAAGATAGGCCAGGT	265

B)

C)

D)

Supplementary Figure S4. Generation of PIGF knockout cell lines using CRISPR/Cas9 system. A, A non-targeting (Ct) and an exon 3-targeting single guide RNA of the PIGF gene were separately cloned into the Cas9 and EGFP expressing pLentiCRISPR plasmids. Sequencing of the targeted region of exon 3 revealed successful PIGF-gene targeting. Aligned sequences of exon 3 of wildtype (upper sequence) and

transfected cell lines (lower sequence) was compared. Successful knockout is shown by an insertion of a nucleotide in the knockout cell line (red square). **B**, IR-induced PlGF secretion was determined in the supernatants of the cell clones 24 hours and 48 hours after irradiation. **C**, Clonogenicity and **D**, proliferative activity of PlGF-wt and PlGF-ko cell clones in response to irradiation.

Supplementary Table S1. Correlation of genetic background to tumor cell lines to early PLGF expression in response to IR.

CELL LINES	SOURCE/DISEASE	KRAS STATUS	P53 STATUS	PLGF EXPRESSION AT 4H	REFERENCES
H460	Lung /carcinoma	Q61H	Wild-type	Increased	den Dunnen JT 2000, Leroy B 2017
H292	Lung/carcinoma	Wild-type	Wild-type	Increased	Lu W 2017, Acunzo M 2017
H358	Lung/carcinoma	G12C	Null	Unchanged	den Dunnen JT 2000, Tung MC 2015
FADU	Head and neck/ squamous cell carcinoma	Wild-type	R248L	Unchanged	Somers KD 1992, Hoa M 2002
DAOY	Cerebellum/Medulloblastoma	Wild-type	C242F	Unchanged	Saylors RL 1991
D341	Cerebellum/Medulloblastoma	Wild-type	Wild-type	Increased	Saylors RL 1991
A549	Lung/carcinoma	G12S	Wild-type	Increased	den Dunnen JT 2000, Leroy B 2017
H125	Lung/carcinoma	Wild-type	Frameshift	Unchanged	Phelps RM 1996, Padanad MS 2016
UW228	Cerebellum/Medulloblastoma	Mutated	T155N	Unchanged	Lacroix 2014
HCT116	Colon carcinoma	G13D	Wild-type	Increased	Liu Y 2005, Alves S 2015