Distinct Effects of Ligand-Induced PDGFRα and PDGFRβ Signaling in the Human Rhabdomyosarcoma Tumor Cell and Stroma Cell Compartments

Monika Ehnman1,2, Edoardo Missiaglia3,4, Erika Folestad2, Joanna Selfe3, Carina Strell1, Khin Thway3, Bertha Brodin1, Kristian Pietras5, Janet Shipley3, Arne Östman1, and Ulf Eriksson7

Abstract

Platelet-derived growth factor receptors (PDGFR) α and β have been suggested as potential targets for treatment of rhabdomyosarcoma, the most common soft tissue sarcoma in children. This study identifies biologic activities linked to PDGFR signaling in rhabdomyosarcoma models and human sample collections. Analysis of gene expression profiles of 101 primary human rhabdomyosarcomas revealed elevated PDGF-C and -D expression in all subtypes, with PDGF-D as the solely overexpressed PDGFRβ ligand. By immunohistochemistry, PDGF-CC, PDGF-DD, and PDGFαβ were found in tumor cells, whereas PDGFRβ was primarily detected in vascular stroma. These results are concordant with the biologic processes and pathways identified by data mining. While PDGF-CC/PDGFRα signaling associated with genes involved in the reactivation of developmental programs, PDGF-DD/PDGFRβ signaling related to wound healing and leukocyte differentiation. Clinicopathologic correlations further identified associations between PDGFRβ in vascular stroma and the alveolar subtype and with presence of metastases. Functional validation of our findings was carried out in molecularly distinct model systems, where therapeutic targeting reduced tumor burden in a PDGFR-dependent manner with effects on cell proliferation, vessel density, and macrophage infiltration. The PDGFR-selective inhibitor CP-673,451 regulated cell proliferation through mechanisms involving reduced phosphorylation of GSK-3α and GSK-3β. Additional tissue culture studies showed a PDGFR-dependent regulation of rhabdosphere formation/cancer cell stemness, differentiation, senescence, and apoptosis. In summary, the study shows a clinically relevant distinction in PDGFR signaling in human rhabdomyosarcoma and also suggests continued exploration of the influence of stromal PDGFRs on sarcoma progression. Cancer Res; 73(7); 2139–49. © 2013 AACR.

Introduction

Rhabdomyosarcomas are the most common pediatric soft tissue sarcoma, with 2 main histologic subtypes, embryonal (ERMS) and alveolar (ARMS; ref. 1). ERMS has the most favorable prognosis, but the estimated 3-year overall survival rate is still only 47% in children and adolescents with metastatic disease (2). The ARMS is typically associated with frequent expression of oncogenic PAX3-FOXO1 or PAX7-FOXO1 gene fusion products and a high propensity to metastasize (2). Both subtypes display features of developing skeletal muscle (3) and their genetic signatures and presence of PAX3-FOXO1 have been proposed to be useful for patient stratification into low- and high-risk groups (4–7). However, the oncogenic heterogeneity of rhabdomyosarcomas makes the identification of suitable molecular targets for directed therapies challenging.

A potential therapeutic candidate for rhabdomyosarcoma is the tyrosine kinase platelet-derived growth factor receptor (PDGFR)α. PDGFRα is a direct target of the PAX3-FOXO1 fusion protein in p53-deficient cells (8, 9), and its expression has been reported to correlate with decreased failure-free survival in patients and increased tumorigenicity in mice (9–11). So far, there has been little evidence for other PDGFR family members contributing to the biology of rhabdomyosarcoma. Classically, tumorigenic PDGFR signaling follows as a consequence of activating point mutations, amplifications, or translocations, often resulting in autocrine stimulatory loops (12, 13). PDGFR ligand production is another mechanism of action to promote both autocrine signaling and paracrine crosstalk with infiltrating stromal cells in various tumors (14).

www.aacrjournals.org
It has been shown that the 5 homo- or heterodimeric ligands (PDGF-AA, -BB, -AB, -CC, and -DD) have different receptor affinity in vivo; both PDGF-AA and PDGF-CC signal via PDGFRα, whereas PDGF-BB and PDGF-DD act preferentially via PDGFRβ (12). Moreover, PDGF-CC and PDGF-DD are secreted as latent growth factors, subsequently proteolytically activated by plasminogen activators before PDGFR binding (15–18). Accordingly, it is becoming increasingly evident that PDGF signaling is regulated at multiple levels in a context-dependent manner.

In this study, autocrine and paracrine PDGF signaling events are systematically analyzed with particular attention to the heterogeneity among rhabdomyosarcomas. The data illustrates that PDGF activity is linked to microenvironmental changes with distinct cellular responses observed not always predictable from PDGFR expression levels.

Materials and Methods

Gene expression profiles of rhabdomyosarcoma patients

The ITCC/CIT (Innovative Therapies for Children with Cancer/Carte d’Identité des Tumeurs) gene expression profile dataset has been previously described (6, 7). Additional information about the gene expression profiling and data handling can be found in Supplementary Materials and Methods.

Tissue microarrays and clinicopathological correlations

Three independent tissue microarrays were used for analysis of PDGF ligand and receptor expression in human rhabdomyosarcoma specimens. First, a commercially available array (Tissue Array Networks), representing rhabdomyosarcoma and leiomyosarcoma samples from 36 patients of various ages, was used to assess tumor compartment–specific protein localization. For clinicopathologic correlations, only pediatric material was used. The second array has previously described and contained material from 60 alveolar and 171 embryonal cases with a mean age of 6.3 years (19). The third array, also previously described, consisted of 25 alveolar and 54 embryonal cases with a mean age of 3.8 years (20). Immunostaining for PDGFRα and β was scored by a consultant pathologist (K. Thway). Each tissue core was assigned a staining intensity score, where 0, 1, 2, and 3 indicated negative, weak, moderate, and strong staining, respectively. The score for each patient was derived from the maximal score in all available cores for a patient where the percentage of cells stained was above 10%. Kaplan–Meier survival analysis of PDGFR expression was conducted separately for tumor cells and tumor stroma, using 1% positively stained cells as cutoff for the latter. Survival characteristics were analyzed on the basis of overall survival as clinical outcome using date of diagnosis as time zero.

Cell culture

Cells were kept at 37°C in a humidified 5% CO2 atmosphere. Dulbecco’s Modified Eagle Medium (DMEM) was used for all cell lines with the exception of RMS-YM, which was maintained in Roswell Park Memorial Institute (RPMI)-1640 medium. Supplements included 10% FBS, 2 mmol/L glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin unless otherwise stated.

Characterization of cell lines

Gene expression profiles for cell lines were available (21) and analyzed to evaluate PDGF-C and -D expression levels. PDGFRα expression was analyzed by immunoblotting of cell lysates using a rabbit antibody (Cell Signaling). Equal sample loading was confirmed with a calnexin-targeting goat antibody (Santa Cruz Biotechnology).

Modulation of PDGF activity in vitro

Tyrosine phosphorylation of PDGFRα was investigated as previously described (16). Following PDGF treatment for 7 minutes at 37°C, pAkt(Thr308) was detected with a rabbit antibody (Cell Signaling). Phosphorylated GSK-3α/β(Ser9) and pAkt(Thr308) were likewise detected with a rabbit antibody (Cell Signaling) after a 1 hour pretreatment at 37°C with 0.5 µmol/L CP-673,451 or vehicle. CP-673,451 was chosen for its reported ability to inhibit PDGFR phosphorylation with an otherwise limited substrate crossreactivity (22, 23).

Cell proliferation/viability was analyzed using the CyQuant cell proliferation assay (Life Technologies). Prestarved cells were treated every 24 hours with vehicle (dimethyl sulfoxide) or 0.5 µmol/L CP-673,451 diluted in serum-reduced medium (1.5% FBS) for 96 hours. The amount of nucleic acid present in lysed cells was normalized to the amount when treatment was initiated. Cell proliferation/viability in response to 300 ng/ml PDGF-CC (16) was likewise analyzed, but cells were then kept in serum-free medium and treated twice during a 48-hour period.

Apoptosis was studied after 96-hour treatment as above. Cells were then enzymatically detached and labeled using the Annexin-V-FLUOS Staining Kit (Roche) according to the manufacturer’s instructions. Samples were analyzed with the BD FACScalibur flow cytometer and the CellQuest software (BD Biosciences).

A cell-cycle analysis was likewise conducted after 96-hour treatment. Cell membranes were lysed with a hypotonic buffer (4 mmol/L sodium citrate, 0.1% Triton X-100) containing 0.1% RNaseA (Life Technologies) for 20 minutes at 4°C. Aggregates were removed by filtration through a 40-µm cell strainer. The total nuclei fluorescence, FL2-A, was measured under exclusion of debris/aggregates via the FL2-W versus FL2-A plot. Data were analyzed with the ModFIT LT Cell Cycle analysis software (Verity Software House).

Rhabdosphere-forming capacity was analyzed in a limited dilution assay with or without pretreatment with 0.5 µmol/L CP-673,451 or vehicle for 72 hours. The cells were filtered through a 40-µm cell strainer to generate a single-cell suspension and then seeded in a descending cell number/well (16, 8, 4, 2, 1) in ultra-low attachment 96-well plates (Coming) in 75 µL complete stem cell medium (Neurocult, LifeTechnologies) supplemented with 2 µg/ml heparin (STEMCELL Technologies), 2 × 10−7 M sodium butyrate without vitamin A (Life Technologies), 10 ng/ml EGF, and 10 ng/ml basic fibroblast growth factor (bFGF; both R&D Systems) containing either 0.5 µmol/L CP-673,451 or

Ehnman et al.
vehicle. Fresh medium, 20 μL/well of 0.5 μmol/L CP-673,451 or vehicle, was added every day. RD rhabdospheres larger than 100 μm were counted after 10 days. RUCH2 rhabdospheres larger than 200 μm were counted after 14 days. Results were analyzed using the online Extrem Limited Dilution Analysis software (24).

Xenograft studies
Experimental procedures were approved by the local committee for animal experiments. Two million RUCH2 cells in PBS were implanted subcutaneously into the flank of 8-week-old females and those with palpable tumors were then stratified into 2 groups based on tumor size (width2 × length × 0.52). The first cohort of severe combined immunodeficient (SCID) mice included 13 tumor-bearing animals and the second, 6 animals. Freshly prepared CP-673,451, or PEG-400 as vehicle, was given once daily by oral gavage for 18 days, and the animals were sacrificed 4 hours after the last treatment. After perfusion with PBS followed by 2% paraformaldehyde (PFA), tumors were kept in 30% sucrose at 4°C overnight and then embedded for cryopreservation, or alternatively, postfixed in 2% PFA at 4°C overnight, dehydrated, and paraffin-embedded. For xenografts derived from the RMS cell line, 5 million cells in PBS/Matrigel (BD Biosciences) were implanted into 20 SCID mice and CP-673,451 or vehicle was given for 9 days. The sorafenib treatment has been described elsewhere (25).

Immunostaining and immunoquantifications
Paraffin-embedded sections were deparaffinized, and endogenous peroxidase activity was quenched by 3% H2O2 for 10 minutes at room temperature. Immunostaining was then conducted as previously described for PDGFRα and β (26) as well as PDGF-DD (18). For detection of PDGF-CC, the latter staining protocol was used with the exception of including an affinity-purified polyclonal rabbit anti-human PDGF-CC antibody. Staining specificities were confirmed in pelleted and paraffin-embedded COS-1 cells transiently transfected with human full-length PDGF-C or -D as previously described (refs. 16, 18; Supplementary Fig. S1). The PDGF antibodies were chosen based on the results from a recent antibody screen, where PDGFR isomorph-specific reactivities were investigated (27). Both unphosphorylated and phosphorylated receptors were recognized.

For immunofluorescence-based detection, cryosections were fixed in 4% PFA (Ki67) or ice-cold acetone (F4/80, CD31/PECAM, pPDGFRs). Paraffin-embedded sections were deparaffinized and heat-induced antigen retrieval conducted in citrate (Vector Laboratories) or DAKO target retrieval solution, high pH buffer (Dako) prior staining (podocalyxin and PDGFRβ, respectively). Slides were incubated with blocking solution (Dako) and primary antibodies in TNB buffer (PerkinElmer Life Sciences) overnight. Ki67 was detected with a rabbit antibody according to the manufacturer’s instructions (Novocastra Laboratories Ltd.). For recognition of macrophages, a rat F4/80 antibody (Serotec) was used. CD31/PECAM was detected with a rat antibody (BD Pharmingen) and pPDGFRs with a cross-reacting mouse antibody against PDGFRβ (PY7/51) (Cell Signaling). Podocalyxin was detected with a goat antibody (R&D Systems) and PDGFRβ with the same antibody as above. Appropriate secondary antibodies (Life Technologies) were applied before mounting with Vectorshield mounting medium with DAPI (Vector Laboratories). The AxioVision Rel. 4.6 Software (Carl Zeiss) was used for automated quantifications of immunostaining from typically 6 RUCH2 tumors per group. Vessel density in RMS tumors was analyzed in 3 tumors/group.

Results

PDGF ligands and receptors are expressed in human rhabdomyosarcoma
PDGF ligand and receptor expression was systematically assessed by microarray analysis of patient material from 101 rhabdomyosarcomas and 36 skeletal muscle samples. For comparison, other small round blue cell tumors (SRBCT) and mesenchymal stem cells were also included. PDGF-C and -D were the only PDGF ligands consistently overexpressed compared with skeletal muscle, whereas PDGF-B was consistently underexpressed (Fig. 1A). Only PDGFRβ, among the receptors, showed significant overexpression in both ERMS and ARMS P3F-positive samples (Fig. 1B). Altogether, these data are indicative of a ligand-mediated PDGFR activation in rhabdomyosarcoma, which is also supported by the lack of identified PDGFR-activating mutations reported so far (28, 29).

PDGF ligands are confined to the rhabdomyosarcoma tumor cell compartment, while PDGFRs are expressed by both tumor cells and infiltrating stromal cells
Tumor compartment–specific expression of PDGF family members was analyzed by immunostaining of 3 different tissue microarrays. On the basis of the results from the RNA expression profiling data, PDGF-CC was selected as the only consistently overexpressed PDGFRα ligand and PDGF-DD as the only overexpressed PDGFRβ ligand. Production of PDGF-CC and PDGF-DD was shown to be almost exclusively located to tumor cells, whereas PDGFRα was frequently expressed in both the tumor and stromal cell compartment (Fig. 2A and C). PDGFRβ was occasionally detected in tumor cells but mainly expressed in vascular stroma (Fig. 2A–C).

Stromal PDGFRβ expression associates with the alveolar subtype
Protein levels of PDGFRs were assessed in 2 different tissue microarrays for clinicopathologic correlations. PDGFRβ was again rarely detected in tumor cells, whereas its stromal staining was associated with the alveolar subtype (Table 1). PDGFRα expression was observed in tumor cells and stroma and was in both cases associated with the embryonal subtype ($P < 0.0033$ and $P < 0.0329$).

The material in the third and largest array (19) was separately analyzed for associations with proliferation and metastatic status. No significant association was seen between either PDGFR staining and the proliferation marker Ki67 (evaluated by the reference monoclonal antibody MIB-1, data not shown). Stromal expression of PDGFRα and PDGFRβ was, respectively, negatively and positively associated with the presence of metastases at diagnosis (Table 1).
**PDGF-C expression is associated with genes involved in developmental programs, while PDGF-D is associated with those involved in wounding and immune system processes**

To further investigate ligand-dependent PDGFR activation, the gene expression profile of the primary tumors was analyzed to identify sets of genes, which correlated to either PDGF-C or PDGF-D. On the basis of Gene Ontology (GO) enrichment analysis, several genes positively correlating with PDGF-C expression were associated with various developmental processes (Table 2). This suggests that PDGF-C expression is linked with the reactivation of embryonic signaling pathways, which could facilitate tumor progression. PDGF-D expression correlated with genes associated with wounding, leukocyte differentiation, and cell adhesion. When individual genes were investigated, PDGF-C was found to correlate with, for example, HOX genes, GLI3 and FZD7 (Supplementary Table S1). PDGF-D, on the other hand, correlated with genes such as CXCL12, VWF, and CD302. Our findings are consistent with some of the previously reported activities mediated by PDGFRα and PDGFRβ signaling (30).

---

**Figure 1.** PDGF ligands and receptors are expressed in human rhabdomyosarcomas. Box and whisker plots representing the expression of PDGF-A, PDGF-B, PDGF-C, PDGF-D (A) and their cognate receptors, PDGFRα and PDGFRβ (B), in 36 skeletal muscle control samples (Sk. muscle), 50 neuroblastomas (NB), 18 Ewing sarcomas (ES), 20 Wilms tumors (WT), 39 medulloblastomas (MBL), 36 ERMS (ERMS), 34 ARMS fusion-negative (ARMS_NEG), 10 ARMS fusion-positive (ARMS_P3F), and 20 ARMS fusion-negative (ARMS_P7F), and 20 ARMS fusion-negative (ARMS_P7F) samples based on gene expression profiling using the Affymetrix chip HG-133plus2 (*, P < 0.05; **, P < 0.01; ****, P < 0.001; Wilcoxon rank-sum test between Sk. muscle and each rhabdomyosarcoma subtype).
To further analyze PDGF-DD/PDGFRβ signaling, patients with tumors with high (top quartile) and low (bottom quartile) PDGF-D expression were compared using predefined gene sets (31). High expression of PDGF-D was associated with expression of genes involved in cell migration (Supplementary Fig. S2A), leukocyte transendothelial migration, and vascular smooth muscle contraction (Supplementary Fig. S2B). These associations were not seen in the corresponding PDGF-C expression analysis (data not shown).

Therapeutic targeting of PDGFR signaling results in decreased cell proliferation, cell-cycle arrest, and apoptosis in a cell-type–specific manner

To explore ligand-dependent PDGFR activity, cell lines were screened for PDGF-C and -D expression (Supplementary Table S2), and a subset (Supplementary Table S3) of high- and low-expressing cell lines was analyzed for PDGFRα protein expression. RD and RUCH2 cells displayed high PDGFRα expression and were responsive to PDGF stimuli (Fig. 3A and B, Supplementary Fig. S3A and S3B). By qRT-PCR analysis (Supplementary Table S4), these 2 cell lines displayed the highest expression levels of PDGF-C and -D compared with PDGF-A and -B (Supplementary Fig. S3C). They also decreased their proliferation rate in response to the PDGFR inhibitor CP-673,451, whereas the PDGFR-negative cell line RMS did not (Fig. 3C). Furthermore, Akt and the downstream key regulators GSK-3α and GSK-3β were identified as targets after ligand-induced PDGFRα stimulation (Fig. 3D and E). This phosphorylation response was completely abolished when cells were pretreated with CP-673,451.

![Figure 2](image_url)

**Figure 2.** PDGF ligands are confined to the rhabdomyosarcoma tumor cell compartment, whereas PDGFRs are expressed by both tumor cells and infiltrating stromal cells. A, immunostaining of PDGF-CC, PDGF-DD, PDGFRα, and PDGFRβ in consecutive sections of human ERMS tissue cores. B, PDGFRβ expression in vascular stroma (top) and in tumor cells (bottom). C, stromal expression of PDGFRα and PDGFRβ in ERMS and ARMS, respectively, used for scoring and clinicopathologic correlations. PDGFRα was notably also found in tumor cells.

### Table 1. Associations of PDGFR expression in tumor stroma with metastatic status and subtype using a χ² test for trend

<table>
<thead>
<tr>
<th>PDGFRα in tumor stroma</th>
<th>Staining intensity score</th>
<th>Metastasis</th>
<th>Subtype distribution</th>
<th>Subtype distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>Yes</td>
<td>ERMS</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>88</td>
<td>30</td>
<td>125</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>14</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>16</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>4</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

PDGFRα stromal staining is negatively associated with metastasis.

<table>
<thead>
<tr>
<th>PDGFRβ in tumor stroma</th>
<th>Staining intensity score</th>
<th>Metastasis</th>
<th>Subtype distribution</th>
<th>Subtype distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>Yes</td>
<td>ERMS</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>76</td>
<td>9</td>
<td>121</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>23</td>
<td>8</td>
<td>36</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>29</td>
<td>16</td>
<td>44</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>12</td>
<td>4</td>
<td>12</td>
</tr>
</tbody>
</table>

PDGFRβ stromal staining is positively associated with metastasis.

χ² test for trend statistic

6.4005; P = 0.0114

NOTE: Metastatic status implies that the patient presented at stage IV with metastasis.
673,451. A striking heterogeneity was observed between the 2 PDGFR-positive cell lines: the RUCH2 cell line displayed an apoptotic response accompanied by a G2–M cell cycle arrest, whereas RD cells only responded with a modest S-phase arrest (Fig. 3F and G).

**Impaired rhabdosphere-forming capacity and differentiation in cells devoid of PDGF activity**

So far, the data indicated that PDGF-CC/PDGFRα signaling is involved in the reactivation of developmental pathways. Stemness potential in the presence of CP-673,451 or vehicle was therefore analyzed in an anchorage-independent growth assay. CP-673,451 impaired rhabdsosphere-forming capacity in both RD and RUCH2 cultures (Fig. 4A) and this was likewise observed in a rhabdsosphere-forming assay where adherent RUCH2 cells were pretreated for 72 hours with CP-673,451 before seeding without treatments (Fig. 4B). In light of previous studies, RD cells were also used as a model system for rhabdomyosarcoma differentiation (32). In the presence of CP-673,451 under conditions facilitating cellular differentiation, hallmarks of senescence, that is, enlarged cell size and presence of perilnuclear senescence-associated βgalactosidase (SA-βgal) activity, were observed (Supplementary Fig. S4A). The absence of elongating cells initiating myogenesis suggested dysfunctional differentiation (Supplementary Fig. S4B) further supported by an expression analysis of the myogenic markers MYL1 and MYOGENIN and the myogenic repressor HEY-1 (ref. 33; Supplementary Fig. S4C).

**PDGFR inhibition reduces tumor growth and stromal cell infiltration**

On the basis of the in vitro results, RUCH2 cells expressed PDGF-C and -D and showed PDGFR dependency. Xenograft-bearing mice were treated with CP-673,451 or vehicle and growth inhibition due to the therapeutic regimen was detected after 10 days of treatment (Fig. 5A). Tumor volume after dissection was analyzed in both cohorts as a separate endpoint. Control tumors had then reached an average size of 66 mm³ and tumors from mice treated with the active compound had reached a size of 32 mm³ (Student t test, \( P = 0.017, n \geq 9 \) mice/group). No metastases were detected.

Immunostaining revealed a decreased number of cells in the proliferative phase in CP-673,451-treated animals (Fig. 5B). The effects on PDGFR phosphorylation were specifically visualized by immunostaining for phosphorylated PDGFRα. A reduced staining intensity was observed in sections from animals treated with the active compound (Fig. 5C).

Before evaluating the therapeutic effect on host-derived stroma, it was confirmed that human PDGF-DD (34) could activate mouse PDGFRβ (Supplementary Fig. S5). Thereafter, immunostaining of RUCH2 xenografts revealed that CP-673,451 reduced the number of F4/80-positive macrophages (Fig. 5D) and CD31-positive vessels (Fig. 5E). Fibrotic streaks composed of collectively migrating fibroblasts were not found and consequently not quantified (data not shown).

**Vessel density in tumors derived from the RMS cell line is reduced by the multtargeting tyrosine kinase inhibitor sorafenib but not by CP-673,451**

In primary tumors, PDGF-D expression correlated to sets of genes involved in blood vessel function. Vessel density was therefore separately analyzed in tumors derived from the RMS cell line after treatment with CP-673,451 or sorafenib. PDGFRs were detected in infiltrating stroma

<table>
<thead>
<tr>
<th>GOBPID</th>
<th>P</th>
<th>OR</th>
<th>Exp count</th>
<th>Count</th>
<th>Size</th>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0009954</td>
<td>0.000</td>
<td>32.961</td>
<td>0</td>
<td>6</td>
<td>18</td>
<td>Proximal/distal pattern formation</td>
</tr>
<tr>
<td>GO:0009953</td>
<td>0.000</td>
<td>7.919</td>
<td>1</td>
<td>10</td>
<td>95</td>
<td>Anterior/posterior pattern formation</td>
</tr>
<tr>
<td>GO:0045110</td>
<td>0.000</td>
<td>193.875</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>Intermediate filament bundle assembly</td>
</tr>
<tr>
<td>GO:0048856</td>
<td>0.000</td>
<td>2.228</td>
<td>25</td>
<td>46</td>
<td>1695</td>
<td>Anatomical structure development</td>
</tr>
<tr>
<td>GO:0007275</td>
<td>0.000</td>
<td>2.145</td>
<td>25</td>
<td>44</td>
<td>1705</td>
<td>Multicellular organismal development</td>
</tr>
<tr>
<td>GO:0007389</td>
<td>0.000</td>
<td>4.427</td>
<td>3</td>
<td>11</td>
<td>178</td>
<td>Pattern specification process</td>
</tr>
<tr>
<td>GO:0007155</td>
<td>0.000</td>
<td>6.203</td>
<td>3</td>
<td>12</td>
<td>477</td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>GO:0030318</td>
<td>0.000</td>
<td>75.597</td>
<td>0</td>
<td>3</td>
<td>11</td>
<td>Melanocyte differentiation</td>
</tr>
<tr>
<td>GO:0009611</td>
<td>0.000</td>
<td>6.940</td>
<td>2</td>
<td>9</td>
<td>302</td>
<td>Response to wounding</td>
</tr>
<tr>
<td>GO:0050878</td>
<td>0.000</td>
<td>15.424</td>
<td>0</td>
<td>5</td>
<td>73</td>
<td>Regulation of body fluid levels</td>
</tr>
<tr>
<td>GO:0042246</td>
<td>0.000</td>
<td>60.464</td>
<td>0</td>
<td>3</td>
<td>13</td>
<td>Tissue regeneration</td>
</tr>
<tr>
<td>GO:0002763</td>
<td>0.000</td>
<td>54.961</td>
<td>0</td>
<td>3</td>
<td>14</td>
<td>Positive regulation of myeloid leukocyte differentiation</td>
</tr>
</tbody>
</table>

**NOTE:** A gene to GO BP conditional test for overrepresentation, where Exp Count is the expected number of genes to be found associated with each specific process (term). This is obtained considering the number of genes (size) associated with that process and how many of those being selected by the analysis (count). The first 6 categories associated with each gene are shown.
**Discussion**

Therapeutic inhibition of PDGF activity has proven beneficial in several types of sarcomas (35–37). PDGFRα was also recently associated with acquired resistance to IGF-1R antibody therapy in rhabdomyosarcoma (38). However, little is...
known about the pathobiology associated with PDGF signaling in rhabdomyosarcoma. We have therefore systematically analyzed PDGF ligand and receptor expression in human rhabdomyosarcoma samples and used animal and cell culture models for functional studies. The analyses suggest the existence of tumor compartment–specific effects of ligand-dependent PDGFRα and PDGFRβ signaling. An important aspect of the study is the indication of clinically relevant variations in the stromal compartment of rhabdomyosarcoma. Stromal PDGFR signaling has previously been extensively described in tumors of epithelial origin (27, 39, 40).

Supported by an analysis of the gene expression profile of 101 pediatric primary tumors and 36 normal skeletal muscle samples, PDGF-C and PDGF-D were identified as the only PDGF ligands with consistently elevated expression relative to skeletal muscle. Accordingly, we confirmed their presence in the hyperchromatic tumor cell compartment. PDGFRα seemed to be the predominating receptor isoform expressed by tumor cells, whereas PDGFRβ was mainly found in vascular stroma. Consistent with our findings, autocrine PDGFRα signaling has recently been associated with both rhabdomyosarcoma tumor cell survival and regulation of differentiation in pediatric malignancies (9, 41), whereas PDGFRβ is classically associated with blood vessel morphogenesis (42–44). Accumulating data hereby suggest that PDGF signaling in rhabdomyosarcoma includes both autocrine and paracrine signaling in different cell types.

The observed compartmentalization of PDGFRα and PDGFRβ expression urged us to elucidate clinical parameters associated with their expression in tumor stroma. PDGFRα was then found to associate with the embryonal subtype, whereas PDGFRβ associated with the alveolar subtype. This difference was not detected in our gene expression analysis of tumor lysates but is likely explained by the relatively small proportion of infiltrating cells compared with the tumor cell mass. In light of previous findings about PDGF signaling in blood vessel morphogenesis (43) and metastatic spread of rhabdomyosarcoma cells (9, 10), associations with the presence of metastases were analyzed in a similar way. Stromal PDGFRα was then found to negatively associate with metastasis, whereas PDGFRβ positively associated with metastasis. These findings could reflect the difference in PDGF expression observed between ERMS and ARMS and the higher propensity of the latter to metastasize. Accordingly, multivariate analyses of data from even larger cohorts are needed to clarify whether the investigated correlations are independent of histology.

By analyzing gene expression profiling of primary tumor samples, tumorigenic PDGF activities were characterized; GO enrichments for biologic processes suggested that PDGF-C may be involved in developmental processes, whereas PDGF-D associated with genes active in cell adhesion, wounding, and immune system processes. These findings support a role of PDGF-DD/PDGFRβ signaling in stromal cell recruitment and are in line with previous studies on PDGFRβ as a regulator of interstitial fluid pressure, a well-established mechanism involving stromal cells and extracellular matrix constituents with direct implications for the delivery of therapeutic agents to tumor cells (45–47). To explore this further, we compared high PDGF-D–expressing tumors with low PDGF-D–expressing tumors on the basis of metagenes generated using GO and KEGG annotations. In this analysis, we observed enrichment for terms such as cell adhesion molecules, leukocyte transendothelial migration, and vascular smooth muscle contraction. This was detected for PDGF-D, and not PDGF-C, once again highlighting in vivo differences between PDGFRα and PDGFRβ signaling.

To mechanistically explore autocrine PDGFRα signaling, cell lines were screened for PDGF-C and -D expression. High PDGFRα protein expression was confirmed in RD and RUCH2 cells. These 2 cell lines were responsive to PDGF-CC stimulation and they displayed decreased cell proliferation/survival after PDGFR targeting by the PDGFR tyrosine kinase inhibitor CP-673,451. However, in line with our gene set enrichments based on human material, our in vitro data revealed a heterogeneity associated with PDGF signaling in rhabdomyosarcoma. Following PDGFR inhibition, the RUCH2 cell line morphologically displayed clear signs of cell death, mechanistically identified as an increase in apoptosis and a G1–M cell-cycle arrest. RD cells, on the other hand, were largely unaffected under proliferative conditions. Under conditions facilitating myogenic differentiation; however, morphologic changes, including signs of senescence, were observed in the presence of CP-673,451. An impaired differentiation capacity was also
evident, which is in line with a recent study describing the need for active PDGFRα signaling in neuroblastoma differentiation (41). This is supportive of PDGF-CC/PDGFRα being involved in the intricate interplay between proliferation and differentiation as our findings from the data mining suggested. At the same time, in an anchorage-independent stem cell assay, both RD and RUCH2 cells displayed impaired ability to form rhabdospheres in the presence of CP-673,451, indicative of PDGFRα signaling being required for the maintenance of stemness characteristics of embryonal rhabdomyosarcoma cells. Together these results suggest that PDGFRα signaling regulates both cancer cell stemness and myogenic differentiation.

For further validation of the identified correlations in the rhabdomyosarcoma patient dataset, the RUCH2 cell line, with comparably high PDGF-D expression, was selected for development of a mouse xenograft model. These cells displayed the highest response to PDGF-CC stimulation in vitro and in the corresponding in vivo model, therapeutic targeting of PDGFRs decreased tumor burden. Additional effects were noted on vessel density and the number of infiltrating macrophages. Whether our therapeutic regimen mechanistically targeted PDGFRβ-positive pericytes involved in the angiogenic process (22, 48), or alternatively, downregulated VEGF expression in tumorigenic cells (49) is not known. It is however likely that both these previously characterized PDGF/PDGFR-driven processes would explain how PDGFR signaling contributes to angiogenesis in RUCH2 xenografts.

Figure 5. PDGFR inhibition reduces tumor growth and stromal cell infiltration. A, growth of RUCH2 xenografts in response to CP-673,451 or vehicle (n ≥ 6 mice/group). B, quantification of Ki67 immunopositive cells in RUCH2 tumor sections. C, quantification of pPDGFR immunopositive cells in RUCH2 tumor sections. D, quantification of F4/80 immunopositive macrophages in RUCH2 tumor sections. E, quantification of vessel density in RUCH2 tumor sections immunostained for CD31/PECAM. F and G, quantification of vessel density in tumors derived from the RMS cell line and treated with CP-673,451, sorafenib, or vehicle. Sections were immunostained for the vessel marker podocalyxin. A compensatory threshold was set to disregard the autofluorescence generated from massive cell death in tumors treated with sorafenib. Data are presented as SD (*, P < 0.05; **, P < 0.01; ***, P < 0.001, Student t test).
Angiogenesis was also explored in xenografts derived from the RMS cell line. These cells were not responsive to PDGFR inhibition in vitro or in vivo. Blood vessel density was also not altered in these tumors following treatment with CP-673,451. PDGFRβ expression was only detected in stroma, and consequently, these results indicate that targeting of PDGFR signaling in stroma is insufficient to affect rhabdomyosarcoma tumor growth. They further support that if PDGFR signaling regulates blood vessel characteristics, it is most likely via an intimate crosstalk between PDGFR-positive mural cells and endothelial cells, mechanistically distinct from VEGF-driven angiogenesis acting directly on the vascular endothelial cells. In a comparative analysis, tumors derived from the RMS cell line were in contrast highly responsive to sorafenib, known for its ability to target VEGFRs. 

Whether or not vascular PDGFRs are transiently expressed during rhabdomyosarcoma tumor formation and progression is still unclear, but this was at least not evident from a therapeutic perspective in our study. Overall, very little is known about stromagenesis, including blood vessel morphogenesis and immunoregulatory processes, in rhabdomyosarcoma biology. Macrophage infiltration has been linked to PDGF-D expression in skeletal muscle (50), and our findings indicate that PDGF-D has similar immunoregulatory functions in the corresponding tumor tissue. Immunomodulation was also recently investigated in a conditional mouse model of rhabdomyosarcoma (9). PDGFRα expression was in these mice linked to disease progression but did not regulate immune responses. This is in line with our results that rather suggest PDGFRβ to be involved in this regulation not PDGFRα.

Taken together, through analysis of gene expression profiling of 101 rhabdomyosarcoma patient samples and subsequent in vitro and in vivo validation, we found that PDGF activity can support rhabdomyosarcoma growth and regulate fundamental cellular behavior. This includes tumor cell proliferation/survival, apoptosis/senescence, cancer cell stemness/differentiation, immune system processes and blood vessel characteristics—typical processes known to require extracellular matrix remodeling. We could also link these activities to PDGF-CC/PDGFRα signaling and PDGF-DD/PDGFRβ signaling in a tumor compartment-specific manner. Our findings suggest that stromal PDGFR signaling should be further studied in rhabdomyosarcoma subtypes and other sarcomas.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M. Ehnman, K. Pietras, A. Östman, U. Eriksson
Development of methodology: (A. Östman, K. Pietras, M. Ehnman)
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.) B. Brodin
Writing, review, and/or revision of the manuscript: M. Ehnman, E. Missiaglia, C. Strell, K. Pietras, J. Shipley, A. Östman, U. Eriksson
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Ehnman, E. Folestad, J. Selfe, J. Shipley
Study supervision: M. Ehnman, J. Shipley, A. Östman, U. Eriksson

Acknowledgments
The authors thank Inger Bodin for technical assistance and, Carina Hellberg and Carl-Henrik Hedén (Ludwig Institute for Cancer Research Ltd.) who kindly provided PDGF-βB and antisense against human PDGFRs. CP-673,451, owned by Arog Pharmaceuticals LLC, was a valuable gift from Pfizer. The Children's Cancer and Leukaemia Group assisted with tumor collection.

Grant Support
This study was supported by the Ludwig Institute for Cancer Research Ltd. (M. Ehnman, E. Folestad, U. Eriksson), The Swedish Cancer Society (U. Eriksson, A. Östman, K. Pietras), The Childhood Cancer Foundation (M. Ehnman, K. Pietras), The Swedish Research Council Linnaeus grant to the STARGET consortium (U. Eriksson, A. Östman, K. Pietras), Karolinska Institutet (U. Eriksson), The Chris Lucas Trust (J. Selfe, E. Missiaglia), The Institute of Cancer Research (J. Shipley), The Royal Marsden Hospital Charity Panel (K. Thway), and Anders Otto Swards stiftelse (E. Folestad). Cancer Research UK (J. Shipley C3866/A10399) funded tumor collection and the Carte d’Identite Program of the Ligue Nationale Contre le Cancer supported the expression profiling. The authors thank NHS funding to the NIHR Biomedical Research Centre. The costs of publication of this article were partially paid by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 2, 2012; revised November 6, 2012; accepted November 26, 2012; published OnlineFirst January 21, 2013.

References

2148 Cancer Res; 73(7) April 1, 2013 Cancer Research

Downloaded from cancerres.aacrjournals.org on April 13, 2017. © 2013 American Association for Cancer Research.
Distinct Effects of Ligand-Induced PDGFR\(\alpha\) and PDGFR\(\beta\) Signaling in the Human Rhabdomyosarcoma Tumor Cell and Stroma Cell Compartments

Monika Ehnman, Edoardo Missiaglia, Erika Folestad, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-12-1646

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2013/01/21/0008-5472.CAN-12-1646.DC1

Cited articles
This article cites 50 articles, 26 of which you can access for free at:
http://cancerres.aacrjournals.org/content/73/7/2139.full.html#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
/content/73/7/2139.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.