Dynamic and Nuclear Expression of PDGFRα and IGF-1R in Alveolar Rhabdomyosarcoma

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Abstract

Since the advent of tyrosine kinase inhibitors as targeted therapies in cancer, several receptor tyrosine kinases (RTK) have been identified as operationally important for disease progression. Rhabdomyosarcoma (RMS) is a malignancy in need of new treatment options; therefore, better understanding of the heterogeneity of RTKs would advance this goal. Here, alveolar RMS (aRMS) tumor cells derived from a transgenic mouse model expressing two such RTKs, platelet-derived growth factor (PDGFR)α and insulin-like growth factor (IGF)-1R, were investigated by fluorescence-activated cell sorting (FACS). Sorted subpopulations that were positive or negative for PDGFRα and IGF-1R dynamically altered their cell surface RTK expression profiles as early as the first cell division. Interestingly, a difference in total PDGFRα expression and nuclear IGF-1R expression was conserved in populations. Nuclear IGF-1R expression was greater than cytoplasmic IGF-1R in cells with initially high cell surface IGF-1R, and cells with high nuclear IGF-1R established tumors more efficiently in vivo. RNA interference–mediated silencing of IGF-1R in the subpopulation of cells initially harboring higher cell surface and total IGF-1R resulted in significantly reduced anchorage-independent colony formation as compared with cells with initially lower cell surface and total IGF-1R expression. Finally, in accordance with the findings observed in murine aRMS, human aRMS also had robust expression of nuclear IGF-1R.

Implications: RTK expression status and subcellular localization dynamics are important considerations for personalized medicine. Mol Cancer Res; 11(11); 1303–13. ©2013 AACR.

Introduction

Rhabdomyosarcoma is the most frequent soft tissue sarcoma of childhood. Of the common subtypes for this aggressive muscle cancer, patients with alveolar rhabdomyosarcoma have the most dismal outcome despite maximal therapeutic intervention (1–3). We have developed and validated a genetically engineered, conditional mouse model of alveolar rhabdomyosarcoma (4, 5) and therein identified the receptor tyrosine kinase (RTK) targets platelet-derived growth factor receptor-α (PDGFRα) and insulin-like growth factor-1 receptor (IGF-1R) as potential therapeutic targets (6, 7). In agreement with our mouse model studies, careful work by other groups has ascribed biologic and clinical significance to the expression of these and other RTKs for alveolar rhabdomyosarcoma (8, 9).

In the current era of biomarker-stratified and personalized cancer therapy clinical trials, great importance is being ascribed to biomarker expression from the solitary biopsy sample taken from each patient at a single time point (10). However, intra and intertumor heterogeneity for individual patients is increasingly recognized (11). Several models developed to interpret the heterogeneity of individual cells that compose a tumor have been proposed, with strong evidence that solid tumors consist of subsets of cells distinct at the genetic and phenotypic level (11–15). However, the concept of isolating unique populations of cells by expression of surface membrane proteins lends itself toward controversy because cells reverting to a state reflecting the heterogeneity of the original tumor have been described as well (16). Thus, the ability to profile cell populations using cell surface markers,
particularly RTKs expressed by only a subpopulation of tumor cells, might be informative if RTK expression is stable. Otherwise, understanding mechanisms that dynamically modulate subcellular localization of RTKs is necessary (17–23). In this regard, the mechanism of intracellular (nuclear) trafficking of RTKs has been described with most details for the EGF receptor (EGFR; refs. 18, 20, 21). Other RTKs, such as FGFR, IGF-1R, and ErbB4 have also been shown to localize to the cell nucleus in cancer (17, 19, 22).

Because PDGFRα and IGF-1R are reported to act in concert for resistance to IGF-1R small-molecule inhibition in rhabdomyosarcoma (24), we sought to understand whether tumor cell subpopulations can be subdivided into distinct RTK profiles and whether isolated cells behave as phenotypically static or dynamic populations. Furthermore, we aimed to determine whether differential subcellular localization of these RTKs had a biologically significant role in tumor establishment.

Materials and Methods

Cell culture
Mouse tumor cell lines were established and cultured previously described (7). Cells were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS and penicillin (100 U/mL)/streptomycin (100 μg/mL; Invitrogen) in 5% CO2 in air at 37°C.

Flow cytometry
Murine alveolar rhabdomyosarcoma cells were grown to approximately 50% confluency then trypsinized, washed with PBS, and stained with primary APC-conjugated antibody to PDGFRα (1:40, eBiosciences) in fluorescence-activated cell sorting (FACS) buffer (2% FBS in PBS) on ice for 30 minutes, washed in FACS buffer, then stained with IGF-1R (1:100, ab32823, Abcam) primary antibody for 30 minutes on ice. Cells were again washed in FACS buffer, then stained with anti-chicken secondary antibody conjugated to Cy3.5 (1:80, ab97146, Abcam) for 30 minutes on ice. Cells were again washed in FACS buffer, and resuspended in buffer and analyzed by a BD FACSaria III Cell Sorter (BD Biosciences). As a control, cells were stained with isotype antibody conjugated to APC at the same concentration as PDGFRα antibody (#17-1401-81, eBioscences) and subsequently with secondary antibody conjugated to Cy3.5 only, at the aforementioned concentration. Stained cells were gated according to control and sorted by quadrant for PDGFRα and IGF-1R expression. Once cells were sorted, reanalysis by FACS was done to assess purity of sorted populations before cells were recounted and allowed to recover in culture before further experiments were carried out. Analysis of .fcs files was done with FlowJo (Tree Star, Inc., PC version 7.6).

Immunoblotting and immunoprecipitation
Cell lysates were prepared using Cell Lysis Buffer (#9803, Cell Signaling Technology) supplemented with the appropriate protease and phosphatase inhibitors (Sigma-Aldrich), and standard immunoblotting procedures were subsequently conducted and proteins visualized with chemiluminescent signal using Super Signal Western Pico or Dura Substrate (Pierce Biotechnology). Mouse tumors were washed in PBS, and then lysed by radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors. Lysates were homogenized and centrifuged at maximum speed for 10 minutes at 4°C and supernatant used for Western blotting. Antibodies used for immunoblotting were PDGFRα (1:1000, #3164, Cell Signaling Technologies), IGF-1Rβ (1:1000, sc-713, Santa Cruz Biotechnology), p-IGF-1Rβ recognizing phospho-Tyr 1161 (1:1000, sc-101703, Santa Cruz Biotechnology), phospho and total p42/44 MAP K from Cell Signaling Technology (used at 1:1000), β-actin (1:10,000, Sigma), α-tubulin (1:10,000, Sigma), and Sp1 (1:1000, Millipore) and pan-Tyr antibody (1:10,000, 4G-10, Upstate Biotechnology). Immunoprecipitation was conducted by standard protocol using Protein A beads for rabbit immunoglobulin G (IgG; Invitrogen).

Cytosolic and nuclear fractionation
The nuclear fraction of cells was isolated by the following protocol: cells were lifted from a 100-mm culture dish and washed with PBS once. Cell pellet was resuspended in 600 μL buffer 1 (25 mmol/L HEPES, pH 7.9, 5 mmol/L KCl, 0.5 mmol/L MgCl2, 1 mmol/L dithiothreitol, 100 mmol/L phenylmethylsulfonylfluoride in H2O), then 600 μL of buffer 2 was added (buffer 1 + 1% Nonidet-P40), and then centrifuged at 2,600 rpm for 5 minutes. The supernatant (cytoplasmic fraction) was transferred to a new tube. Hundred microliter μL of buffer 3 (1:1 mix of buffer 1 and 2) was added to the cell pellet and gently mixed, centrifuged at 2,600 rpm, and supernatant added to cytoplasmic fraction. Cell pellet was then washed two to three times with buffer 1 and supernatant discarded for purity of subsequent soluble nuclear protein extraction. Five hundred microliter of buffer 4 (25 mmol/L HEPES, pH 7.9, 360 mmol/L NaCl, 10% dextrose, 0.05% Nonidet-P40) was added to cell pellet and tube rotated for 1 hour at 4°C. Tube was centrifuged at 13,200 rpm for 10 minutes and supernatant (soluble nuclear fraction) was transferred to a new tube. At this point, both the cytoplasmic and nuclear fractions were clarified again by centrifuged at 13,200 rpm and transferring supernatant to a new tube. Protein was quantified using BCA Protein Assay Reagent (Pierce) and immunoblotting was conducted as mentioned above.

RNA interference mediated silencing of IGF-1R and anchorage-independent colony formation assay
For RNA interference (RNAi) studies, siRNA targeted to IGF-1R (17-10074017) and nonspecific siRNA (#1027280) were purchased from Qiagen. Experiments with siRNA were carried out at 100 nmol/L concentration out using Lipofectamine 2000 in Opti-MEM Reduced Serum Media (Invitrogen). Cells were transfected with nonspecific siRNA or siRNA-targeting IGF-1R and on day 2, trypsinized and in each well of a 6-well plate, 6,000 cells were suspended in 1.5 mL of 0.7% agarose (at 57°C) in DMEM with 10% fetal calf serum. The cells in agarose were plated atop a 1.4% agarose...
layer and were allowed to grow for 2 weeks before visualizing the colonies by light microscopy.

**Immunohistochemistry**

Human alveolar rhabdomyosarcoma tissue microarrays were obtained from the COG Biorepository (TMA #3000-30-P6897). Histology was conducted using standard protocol. Briefly, formalin-fixed paraffin-embedded sections (FFPE) were dewaxed and dehydrated in xylene and graded alcohol concentrations. Heat-induced epitope retrieval was used with sodium citrate buffer (pH 6.0) for 20 minutes, slides blocked with 2% normal goat serum (NGS) and for endogenous biotin using an avidin/biotin blocking kit (Vector Labs). Primary antibody for IGF-1R (Santa Cruz) was used at 1:50 overnight in 2% NGS. VECTASTAIN Elite ABC kit (Rabbit IgG) and ImmPACT DAB Peroxidase Substrate kit was used according to manufacturer’s protocol to visualize staining (Vector Lab). Slides were counterstained with hematoxylin for 5 minutes, rinsed, dehydrated, and mounted with xylene-based mounting medium.

**Immunocytochemistry**

Confocal microscopy was conducted as follows: cells were allowed to recover postsort in culture for 2 to 3 days then replated in 8 chamber slides (BD Biosciences) at low confluence and allowed to incubate for 2 to 3 days. Cells were then fixed in 4% paraformaldehyde in PBS at room temperature for 20 minutes. Slides were carefully washed with PBS three times and incubated at room temperature in 5% NGS (Invitrogen) and 0.01% Triton-X in PBS for 1 hour to inhibit nonspecific binding of antibodies. Cells were washed again, and primary antibody diluted in 5% NGS in PBS was added overnight at 4°C. Alexafluor 488-conjugated or Alexafluor 594-conjugated anti-rabbit IgG antibody (Invitrogen) at 1:200 was added, and cells were incubated for 1 hour at room temperature. Slides were mounted using the VECTASHIELD Mounting Medium with 4',6-diamidino-2-phenylindole (Vector Laboratories) and visualized with a Zeiss LSM 700 confocal microscope. PDGFRα antibody was used at 1:100 (#3164, Cell Signaling Technologies) and IGF-1Rβ at 1:50 (sc-713, Santa Cruz Biotechnology). Images were taken at ×40 and ×63 oil immersion for further magnification.

**In vivo studies**

All animals were treated humanely and the experiments were carried out in accordance with the Institutional Animal Care and Use Committee approved protocols. SCID/hairless/ outbred (Crl:SHO-Prkdc<sup>+/−</sup>Hph<sup>+</sup>) mice purchased from Charles River at 8 weeks of age were injected with 1 × 10<sup>5</sup> U23674 cells into the gastrocnemius muscle that had been preinjured 24 hours prior with 0.85 µg/mouse cardiotoksin (from *Naja mossambica*, Sigma). Tumor volumes (cm<sup>3</sup>) were calculated from formula (π/6) × length × width × height, assuming tumors to be spheroid. Once tumor volume reached 2 cm<sup>3</sup>, mice were euthanized and the tumor was harvested at necropsy.

**Results**

Murine alveolar rhabdomyosarcomas express PDGFRα and IGF-1R by FACS and can be isolated by cell sorting

The murine alveolar rhabdomyosarcoma primary cell culture, U23674, was derived from a left upper extremity primary tumor that developed in a 46-day-old mouse from our genetically engineered, conditional mouse model of alveolar rhabdomyosarcoma harboring the *Pax3:Fkhr oncoprotein* and homozygous p53 deletion (4, 5). This lesion was histologically diagnosed as an alveolar rhabdomyosarcoma tumor and used for these studies at low passage number (P < 10). Initially, U23674 cells were stained for PDGFRα (APC) and IGF-1R (Cy3.5) and analyzed by FACS, indicating that approximately 35% of cells express both PDGFRα and IGF-1R, but approximately 15% of cells express either receptor exclusively (Fig. 1A). However, tumor cells displayed a continuum of expression of PDGFRα and IGF-1R rather than neatly distinct populations expressing either RTK individually or together. Similar results were seen in two other murine alveolar rhabdomyosarcoma cell lines analyzed by flow cytometry for cell surface expression of PDGFRα and IGF-1R (Supplementary Fig. S1).

Next, after U23674 cells were stained for PDGFRα and IGF-1R, the cells were sorted by expression of each receptor into four populations. Gates were determined using control cells stained with isotype control antibody in addition to a Cy3.5 conjugated secondary antibody as a control. Cells positive or negative for PDGFRα or IGF-1R by the assigned gates were designated as PDGFRα<sup>hi</sup>/IGF-1R<sup>hi</sup>, PDGFRα<sup>lo</sup>/IGF-1R<sup>hi</sup>, PDGFRα<sup>lo</sup>/IGF-1R<sup>lo</sup>, or PDGFRα<sup>hi</sup>/IGF-1R<sup>lo</sup>, respectively (Fig. 1B). For instance, cells sorted for high IGF-1R expression, but negative for PDGFRα were denoted as PDGFRα<sup>−</sup>/IGF-1R<sup>hi</sup>. Immediately following sorting, cells were reanalyzed by flow cytometry to assess purity of the sort (Fig. 1B). Figure 1C shows distinct morphology of cells grown in culture for 7 days postsort. IGF-1R<sup>hi</sup> expression, but not PDGFRα<sup>hi</sup> expression, was associated with spindle morphology.

**PDGFRα and IGF-1R cell surface expression is dynamic**

Once U23674 cells were sorted by PDGFRα and IGF-1R expression, cells were allowed to recover postsort in culture and reanalyzed by flow cytometry for surface expression of both RTKs at serial time points. FACS analysis revealed that cell surface expression of both PDGFRα and IGF-1R varied dramatically as cells were grown in culture postsort. Two such time points are shown in Fig. 2 at 2 days and 14 days postsort, compared with day 0 (day 7 was similar to day 14; Supplementary Fig. S2). These results illustrated an initial dramatic change in expression of PDGFRα and IGF-1R postsort within a 48-hour time period, tending toward a presort profile. With the observation that cell surface expression profile shifted considerably within a short period of time, we sought to understand whether altered receptor expression was a function of many or few rounds of replication. Thus, after a purity check was conducted for sorted populations postsort, cells were recounted and plated at a known cell number. Exactly 48 hours after the initial
sort, cells were recounted and FACS analysis for PDGFRα and IGF-1R expression was done. The number of cells comprising each of the four populations was calculated from the FACS profile of the initial sort and 48-hour postsort (Fig. 2). Doubling times were on the order of 30 to 42 hours (Supplementary Table S1); therefore, dynamic alterations of PDGFRα and/or IGF-1R cell surface expression seem to start after 0 to 1 cell divisions.

Figure 1. Murine alveolar rhabdomyosarcoma isolated by PDGFRα and IGF-1R expression. A, U23674 (murine alveolar rhabdomyosarcoma) cell expression profile by FACS of PDGFRα (APC) and IGF-1R (Cy3.5) gated with respect to isotype and secondary only control. B, purity of isolated cells by FACS shown. Isolated populations according to cells positive and negative for each receptor designated as hi and lo, respectively. C, phase contrast microscopy of sorted populations showing differential morphology of cells grown in culture 7 days postsort. Scale bar, 200 μm.

Total expression of PDGFRα and IGF-1R by immunoblot analysis in sorted cells differs from cell surface expression profile by FACS

To determine whether total expression of either PDGFRα or IGF-1R in the four sorted cell populations reflected the surface expression profile of each RTK by FACS, we conducted immunoblot analysis on cells grown in culture for 7 days postsort. Surprisingly, total cellular PDGFRα expression
seemed to vary dramatically among the sorted populations (Fig. 3A). Higher PDGFRα expression at day 7 correlated with the populations, which were sorted for high PDGFRα expression by FACS initially. Conversely, total IGF-1R expression by immunoblot analysis did not show a dramatic difference in expression among sorted populations (Fig. 3A). In terms of activation, phosphorylation of PDGFRα was elevated in the PDGFRα^hi^IGF-1R^lo^ and PDGFRα^hi^IGF-1R^hi^ populations (Fig. 3A). Despite lack of differential expression for total IGF-1R, IGF-1R phosphorylation was increased in the PDGFRα^lo^IGF-1R^lo^ populations, as well as (modestly) in the PDGFRα^lo^IGF-1R^hi^ population. Because we observed that activated receptor levels varied across sorted populations, we looked at activation of common cell survival and proliferation pathways downstream of both PDGFRα and IGF-1R such as Akt and extracellular signal-regulated kinase (Erk)-1/2, respectively. Although we did not see a difference in Akt phosphorylation levels between the sorted cells (data not shown), Erk1/2 phosphorylation levels were remarkably elevated in the PDGFRα^hi^IGF-1R^hi^ population and conversely decreased in the PDGFRα^lo^IGF-1R^lo^ population; p-Erk 1/2 levels seemed to correlate with the levels of phosphorylated PDGFRα and IGF-1R.

Thus far, our results indicated that PDGFRα and IGF-1R in tumor cells are expressed in a dynamic manner with respect to surface expression profile by FACS, as well as total protein expression by immunoblot analysis. However, the dramatic difference in total PDGFRα expression (Fig. 3A), for example, did not reflect the percentage of cells positive for PDGFRα by FACS analysis at the same time point (day

Figure 2. Murine alveolar rhabdomyosarcoma show dynamic expression of PDGFRα and IGF-1R by FACS in a temporal manner. A, FACS profile of PDGFRα and IGF-1R expression in U23674 cells at day of sort (Day 0), and 2 and 14 days postsort. Day 7 was similar to Day 14.
7; Fig. 2). Therefore, to determine whether the amount of PDGFRα or IGF-1R expressed on the surface membrane of the sorted cells was reflective of total expression of each receptor, we looked at mean fluorescence intensity (MFI) of cells stained for PDGFRα and IGF-1R at day 7. Figure 3B shows MFI of both RTKs in each of the four sorted populations. The MFI values indicated that cells sorted initially for PDGFRα or IGF-1R displayed a higher cell surface expression of each receptor 1 week postsort. Taken together, these results showed that surface expression did not completely correlate with total expression level of either receptor, indicating, among other possibilities, differential subcellular localization of these RTKs.

Total PDGFRα and IGF-1R expression in vivo reflects in vitro behavior of tumor cells and IGF-1Rhi-lo-sorted cells establish tumors in vivo earlier than IGF-1Rlo-hi cells

To determine whether tumor cells retained an expression profile of PDGFRα and IGF-1R in vivo reflective of in vitro behavior (Fig. 3A), we used an orthotopic allograft in vivo model of alveolar rhabdomyosarcoma and implanted cells immediately postsort into the right gastrocnemius muscle of immunocompromised mice. Immunoblot analysis of tumors formed in mice showed that PDGFRα and IGF-1R expression of subpopulations (Fig. 4A) mirrored the expression of both receptors in vitro (Fig. 3A). Leg measurements taken daily to monitor tumor growth revealed that the PDGFRαhI-IGF-1Rlo and PDGFRαlo-IGF-1Rhi-sorted cells more quickly established tumors in mice compared with IGF-1Rlo-lo and IGF-1Rhi-hi cells (Fig. 4B and C). The control group of mice implanted with unsorted U23674 murine alveolar rhabdomyosarcoma cells established tumors with the same latency as PDGFRα-lo-IGF-1R-lo-sorted cells (Fig. 4D).

To confirm the results that IGF-1Rlo-hi-sorted cells establish tumors in vivo earlier than IGF-1Rlo-hi cells, we sorted the independent murine alveolar rhabdomyosarcoma primary cell culture U21459 into IGF-1Rlo-lo and IGF-1Rhi-hi cells and implanted the cells immediately postsort in recipient nonobese diabetic/severe combined immunodeficient (NOD/SCID) host mice. Compared with IGF-1Rhi-lo cells, IGF-1Rlo-hi cells sorted from U21459 engrafted with decreased latency and led to shorter survival (Supplementary Fig. S3A–S3C).

PDGFRα and IGF-1R show nuclear localization in PDGFRα-lo and IGF-1R-hi-sorted cells and nuclear IGF-1R is associated with increased anchorage-independent colony formation ability

On the basis of the observation that cells sorted for either PDGFRα or IGF-1R exhibited an apparent discrepancy between cell surface and total expression of each receptor, we hypothesized that differential cellular localization of PDGFRα and IGF-1R was the etiology for these disparate results. We first conducted confocal microscopy on sorted cells looking at PDGFRα and IGF-1R localization. Interestingly, we observed that in PDGFRα-lo-IGF-1Rlo and PDGFRαhi-IGF-1Rhi cells, PDGFRα was predominantly localized to the nucleus, while chiefly cytosolic or membranous in PDGFRα-lo-IGF-1Rhi and PDGFRα-lo-IGF-1Rhi cells (Fig. 5 and Supplementary Fig. S3). Similarly, in PDGFRα-hi-IGF-1Rhi and PDGFRα-lo-IGF-1Rhi cells, IGF-1R seemed to be principally localized to the nucleus, whereas more abundant in the cytosolic compartment/cell membrane of PDGFRα-lo-IGF-1Rlo and PDGFRα-lo-IGF-1Rhi.
cells (Fig. 5 and Supplementary Fig. S4). In the biologically independent U21459 cell culture, immunocytochemistry also revealed that IGF-1R was commonly localized to the nucleus in IGF-1R hi cells and IGF-1R lo cells, respectively (Supplementary Fig. S3D). These results subsequently led us to question whether the ability of IGF-1R hi cells to establish tumors earlier in vivo compared with IGF-1R lo cells was due to higher nuclear expression of IGF-1R in these cells. Focusing our efforts on IGF-1R because of the orthotopic allograft results, we next confirmed whether IGF-1R was indeed expressed at higher levels in the nucleus in IGF-1R hi cells, compared with IGF-1R lo cells, by separating cytosolic and nuclear proteins of all four sorted populations 7 days following isolation of cells by FACS. We subsequently immunoblotted for IGF-1R, using Sp1 and α-tubulin as confirmation of enrichment of nuclear and cytosolic cell fractions, respectively (Fig. 6A). These results confirmed a higher nuclear expression of IGF-1R in IGF-1R hi cells compared with IGF-1R lo cells.

To confirm our hypothesis that nuclear expression of IGF-1R contributed to the ability of tumor cells to establish more aggressive tumors in vivo, we used RNAi to silence IGF-1R in PDGFRα+/IGF-1R hi and PDGFRα−/IGF-1R lo cells. We then assessed the effect of IGF-1R knockdown on these cells’ ability to form colonies in an anchorage-independent colony formation assay (Fig. 6B). Knockdown efficiencies of IGF-1R in both sorted cells are shown (Fig. 6B). Colony formation ability of PDGFRα+/IGF-1R hi was decreased approximately 45% compared with control (nonspecific siRNA) when IGF-1R was silenced by RNAi, whereas PDGFRα+IGF-1R lo cells show only a approximately 27% reduction in colony formation ability with IGF-1R knockdown. Similarly, studies with the nuclear IGF-1R-expressing murine alveolar rhabdomyosarcoma primary cell culture U21459 showed that three independent siRNA for IGF-1R suppressed colony formation in soft agar (Supplementary Fig. S3E). Taken together, these results indicate that nuclear expression of IGF-1R confers an increased tumorigenic phenotype to murine alveolar rhabdomyosarcoma cells.

Normal human tissue expresses cytosolic IGF-1R, whereas human alveolar rhabdomyosarcoma has high frequency of nuclear IGF-1R by immunohistochemistry

To determine whether nuclear IGF-1R occurs in human alveolar rhabdomyosarcoma, we conducted immunohistochemistry for IGF-1R on a human alveolar...
rhabdomyosarcoma tumor microarray with 31 sections of 19 unique cases. We observed that 16 out of 19 (~84%) specimens had nuclear expression of IGF-1R (>10% nuclei of section positive). Tumor sections showed either cytosolic (Fig. 7A), or predominantly nuclear staining of IGF-1R (Fig. 7B). Normal human tissue (pancreatic and skeletal muscle) was used as positive controls for IGF-1R, and showed only cytosolic staining of IGF-1R; no nuclear IGF-1R was seen in these sections (data not shown). Details of sample scores are given in Supplementary Table S2.

Discussion

In this report, we describe that the RTK PDGFRα and IGF-1R exhibit a dynamic cell surface expression profile in murine alveolar rhabdomyosarcoma cells. Although we were able to isolate relatively pure populations of cells expressing high or low PDGFRα and/or IGF-1R, all sorted populations rapidly and dynamically alter their cell surface expression profile before the second cell division. Kept isolated in culture, sorted populations eventually equilibrated their cell surface expression profile toward a presort profile. In the context of previous studies illustrating the phenotypically dynamic state of cancer cells, including stem and non-stem cancer cells (16, 25), our study is a key next step into understanding the complexity and potential pitfalls of assigning therapy based on the surface expression profiles or other static biomarkers. Previously, several studies have used cell surface markers (e.g., CD133, CD44, CD24, etc.) as stable markers for a specific cell population (12, 15, 26). RTKs, which are known to undergo ligand-dependent subcellular trafficking and recycling (18, 27, 28), may however, be particularly undesirable as cell surface biomarkers. Nevertheless, our results then pointed us to differences in PDGFRα and IGF-1R subcellular localization that paralleled membrane expression. IGF-1R nuclear localization, in particular, exhibited an important functional effect in contributing toward a more aggressive tumor phenotype in vivo (summarized in Supplementary Fig. S5).

Nuclear localization of RTKs or their substrates is an interesting phenomenon, which has been described in select early reports (19–22, 29). IGF-1R had previously been shown to be a poor prognostic indicator in clear cell renal cell carcinoma (30), and a new study was able to further show that among renal tumors expressing IGF-1R, intense or widespread nuclear expression by immunohistochemistry portends a decrease in survival probability (17). Interestingly, nuclear localization of the IGF-1R holoreceptor was dependent upon tyrosine phosphorylation of putative

Figure 5. Nuclear localization of PDGFRα and IGF-1R correlates with cells sorted by FACS for surface expression of the respective receptor. Representative confocal images of U23674 sorted populations showing localization of PDGFRα and IGF-1R (>40) with inset box enlarging image of a single cell to appreciate localization. Scale bar, 100 μm. Larger magnifications of insets are given in Supplementary Fig. S3.
residues on the IGF-1Rβ subunit of the receptor (31). In our sorted populations, the IGF-1R hi cells, which displayed a higher nuclear expression of IGF-1R, also had increased phosphor-IGF-1Rβ. A typical biologic response when an RTK is stimulated by its respective ligand is receptor internalization and subsequent degradation (31). However, phosphorylation of IGF-1Rβ is one of the two necessary posttranslation modifications of the receptor required for
nuclear trafficking. SUMOylation of K1025, K1100, and K1120 residues in the kinase domain of IGF-1Rβ is also critical to nuclear import of the holoreceptor (32). Further investigation has shown that nuclear IGF-1R originates from surface IGF-1R only (33). Of note, we used an antibody to sort cells based on surface IGF-1R expression (the α subunit of IGF-1R) and a different antibody to detect IGF-1Rβ at the nuclear fraction by immunoblot analysis, but given the covalent assembly of α and β subunits in the mature receptor, we do not expect this to have altered our results. Another intriguing aspect of our studies was that nuclear PDGFRα did not seem to have an adverse functional role with respect to tumor establishment in vivo.

Although we were able to identify a difference in receptor localization among the sorted populations of murine alveolar rhabdomyosarcoma, we did not observe a differential sensitivity of these cells to small-molecule inhibitors which target PDGFRα or IGF-1R (data not shown). An interesting question these results raise is whether small-molecule inhibitors designed to inhibit kinase activity of RTKs are able to inhibit the function of these RTKs when localized to the nucleus. Understanding whether kinase activity contributes to the role these receptors play in the nucleus would be one step toward answering this question. Presumably, small-molecule inhibitors would have an advantage over neutralizing antibodies in this respect. Prior studies have already shown that certain RTKs, such as EGFR, interact with DNA-binding proteins to directly regulate the transcription of putative gene targets (34). The exact role of IGF-1R in the nucleus of alveolar rhabdomyosarcoma will be an active area of further investigation, particularly given the potential translation from mouse to human and the expression of nuclear IGF-1R in clinical FFPE samples. The mechanisms by which nuclear IGF-1R contributes to the establishment of new (metastatic) tumors will be a key subject of further study and perhaps an operative step toward a temporal biomarker-driven and personalized medicine approach in cancer therapy with regards to IGF-1R inhibition.

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

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Grant Support
This work was supported by SR01CA133229-05 and -06 to C. Keller. The studies were also supported, in part, from a gift from the Erhan Jostad Foundation for Childhood Cancer. Pilot studies leading to this work were supported by an innovation award from Alex’s Lemonade Stand. J.W. Tyner is supported by grants from the William Lawrence and Blanche Hughes Foundation, Leukemia & Lymphoma Society, and the National Cancer Institute. M.I. Aslam is a Howard Hughes Medical Institute Medical Research Fellow. B.J. Draker is an investigator of the Howard Hughes Medical Institute.

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Received October 17, 2012; revised June 25, 2013; accepted July 15, 2013; published OnlineFirst August 8, 2013.

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Mol Cancer Res; 11(11) November 2013 Molecular Cancer Research
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