PRL-3 Initiates Tumor Angiogenesis by Recruiting Endothelial Cells In vitro and In vivo

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Abstract

We show here that PRL-3 protein is expressed in fetal heart, developing blood vessels, and pre-erythrocytes but not in their mature counterparts. These observations imply that PRL-3 may be involved in the early development of the circulatory system. Because PRL-3 mRNA had been reported to be consistently elevated in metastatic samples derived from colorectal cancers, we attempted to investigate if PRL-3 might be involved in tumor angiogenesis and if PRL-3-expressing cells could cross-talk to human umbilical vascular endothelial cells (HUVEC) by using an in vitro coculture system. HUVECs were grown with fibroblasts, which were later overlaid with PRL-3-expressing cells. We observed that both PRL-3-expressing Chinese hamster ovary (CHO) cells and PRL-3-expressing DLD-1 human colon cancer cells could redirect the migration of HUVECs toward them; in addition, PRL-3-expressing DLD-1 cells could enhance HUVEC vascular formation. In vivo injection of PRL-3-expressing CHO cells into nude mice to form local tumors resulted in the recruitment of host endothelial cells into the tumors and initiation of angiogenesis. We further showed that PRL-3-expressing cells reduced interleukin-4 (IL-4) expression levels and thus attenuated IL-4 inhibitory effects on the HUVEC vasculature. Our findings provide direct evidence that PRL-3 may be involved in triggering angiogenesis and establishing microvasculature and it may serve as an attractive therapeutic target with respect to both angiogenesis and cancer metastasis. (Cancer Res 2006; 66(19): 9625-35)

Introduction

Cancer metastasis is the leading cause of cancer-related deaths worldwide. Endothelial cell recruitment and new blood vessel formation play central roles in tumor progression and cancer metastasis. Key events in metastasis include the ability of tumor cells to survive in the circulatory system and then extravagate into a new tissue, to begin and maintain growth and then form preangiogenic micrometastases in this tissue, and finally to develop a blood supply that enables the formation of macroscopic tumors. The final step is the most difficult and inefficient but important step in the whole event of metastatic process (1–5). However, how tumors build up their blood supply circuits is largely unclear. Inhibiting tumor angiogenesis will be crucial in thwarting cancer development and metastasis, but the ability to do this will depend on our understanding of how angiogenesis is initiated.

PRL-1 (6), a phosphatase induced in regenerating liver, was originally identified as an immediate early gene whose transcript is induced after partial hepatectomy. Searching expressed sequence tagged databases with the mouse PRL-1 sequence led us to identify two homologues: PRL-2 and PRL-3 (7). The three PRL protein tyrosine phosphatase members (PRL-1, PRL-2, and PRL-3) comprise a distinct subclass of protein tyrosine phosphatases with a COOH-terminal prenylation motif. Abnormal regulation of tyrosine phosphorylation or dephosphorylation can result in human diseases, such as cancer (8, 9). PRL-1 is expressed at high levels in proliferating cells and in several human tumor cell lines, including HeLa cells (6, 10). PRL-2 is associated with prostate tumor progression (11). Overexpression of PRL-3 in human embryonic kidney fibroblast HEK293 enhances its growth rate (12). PRL-3 is first linked to metastasis from genome-wide transcriptional analysis of colorectal cancer samples (13). PRL-3 mRNA expression is consistently elevated in most metastatic lesions derived from colorectal cancer regardless of the targeted organs (14). PRL-3 is rapidly gaining interest as a putative prognostic factor and as a therapeutic target for metastatic tumors (15). We used a PRL-3-specific monoclonal antibody to assess >280 human colorectal cancer tissues and showed that 11% of them express PRL-3, indicating that PRL-3 may prime the metastatic process (16). PRL-3 mRNA is highly expressed in metastatic melanoma B16-BL6 cells but not in its less metastatic parental cell line (17). PRL-3 expression might also play a significant role in invasion and metastasis of gastric carcinoma (18). PRL-3 phosphatase is implicated in ovarian cancer growth (19). PRL-3 is a potential marker for liver metastasis of colorectal cancer cells, and its level correlates negatively with the prognosis of colorectal cancer patients (20). Taken together, these data suggest that the PRLs may be major players involved in oncogenic and metastatic processes, but how these phosphatases spur cancer metastasis still remains a complex and largely unresolved matter (21).

We had previously shown that overexpression of PRL-1 and PRL-3 in Chinese hamster ovary (CHO) cells promoted cell migration, invasion, and metastasis (22). Our observations that some PRL-3 metastatic tumors sprout and grow inside blood vessels reveal a potential role for PRL-3 in tumor-related angiogenesis (23). PRL-3 is expressed predominantly in the vasculature of invasive breast cancers and is able to enhance the migration of endothelial cells in vitro (24). Tumor and normal endothelia are distinct at the molecular level (25), and PRL-3 is detected in a subset of endothelial cells in some human colon samples (14). There is evidence that PRL-3 is functional in both endothelial and malignant cells (26). Together, these observations suggest that PRL-3 may be associated with tumor angiogenesis.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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In the present study, we elucidate an important role that PRL-3 might play during the early development of the cardiovascular system. Genes that are important in embryonic development are frequently found to be culprits in cancer (27). Our findings that cross-talk between PRL-3-expressing cancer cells and endothelial cells results in the reorganization and migration of the latter toward tumor cells provide compelling evidence to suggest that PRL-3 may be involved in angiogenesis during tumor development and imply the importance of PRL-3 in initiating tumor angiogenesis. PRL-3 might be suitable as a novel anticancer target for anti-invasive cancer therapy.

Materials and Methods

Human multiple tissue arrays. To explore PRL-3 RNA tissue-specific expression patterns, we used human multiple tissue arrays that were obtained from BD Biosciences (San Jose, CA). Each array contains 73 mRNAs derived from 65 different human tissues and 8 human cell lines (Supplementary Fig. S1A). The dot blots were probed with human PRL-1, PRL-2, or PRL-3 cDNAs that were radiolabeled with [32P]dCTP according to the manufacturer’s instructions (Roche, Mannheim, Germany). PRL mRNA expression patterns are shown in Supplementary Fig. S1B.

Immunohistochemical analysis of PRL-3 protein levels in rat and human tissues. We examined PRL-1, PRL-2, and PRL-3 protein expressions in rat fetal and adult hearts, in human adult heart, in blood vessels using normal and colorectal cancer arrays TS235790D (BioChain Institute, Inc., Hayward, CA) and TS-12405-05 and TS124050903 (BioGenex, San Ramon, CA), and in normal human bone marrow tissues TS-4007-05 (BioGenex). Using DAKO EnVision Systems K 1395 (DAKO, Carpinteria, CA), immunohistochemistry was done on the above samples. Formalin-fixed, paraffin-embedded slides were dewaxed in fresh xylene for 5 minutes, and the step was repeated. The slides were subjected to rehydration with sequential

100%, 95%, 80%, and 75% ethanol and then PBS (2 minutes for each change) followed by antigen retrieval with 200 μl (2.5 mg/ml) pepsin (EK0000-10K, BioGenex) on each slide in a 37°C water bath for 5 minutes. The detailed steps of immunohistochemistry and generations of PRL-1-specific (clone 269) and PRL-3-specific (clone 223) monoclonal antibodies were as described previously (16). We preincubated rabbit anti-PRL antibodies with 2 μg each of recombinant PRL-1 and PRL-3 antigens for 1 hour so that the resulting antibodies will be more selective for PRL-2.

To further show that PRL-3 is expressed in developing blood cells, we fractionated each hematopoietic lineage from mouse bone marrow using FACS Vantage (Becton Dickinson, San Jose, CA). Ter119 and CD61 (29G2) antibodies were purchased from PharMingen (San Diego, CA). Total RNAs were extracted by Trizol (Life Technologies, Gaithersburg, MD), and cDNAs were synthesized with an oligo(dT)12-18 primer and SuperScript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). To amplify the mPRL-3 cDNA fragment (457 bp), a forward primer (5’-CACATGCCGGCTTCCTCATCA-3’) and a reverse primer (5’-TCTTGTGCT- CTGGTGAGGCTTTT-3’) were used for the PCR (95°C, 53°C, and 72°C, 32 cycles). To amplify the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) fragment (450 bp), a forward primer (5’-ACCAGGTCCATGC-CATCAC-3’) and a reverse primer (5’-TCCACACCCCTGCTGATGA-3’) were used as controls for the PCR (95°C, 55°C, 72°C, 32 cycles).

Generation of CHO or DLD-1 cell pools stably expressing EGFP-PRL-3 or EGFP-PRL-3 (C104S). The EGFP-PRL-3 or EGFP-PRL-3 (C104S) expression constructs were made and confirmed by DNA sequencing and then transfected into CHO-K1 cells (ATCC CCL-61; American Type Culture Collection, Manassas, VA) using LipofectAMINE 2000 (Invitrogen Life Technologies). CHO cell pools stably expressing EGFP-PRL-3 or EGFP-PRL-3 (C104S) were as described in a previous study (22). The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and selected in 1 mg/mL G418 for 20 to 30 days to establish stable cell pools. The two stable pools (10⁶ cells/mL) were then subjected to enhanced green fluorescent protein (EGFP) sorting by FACS Vantage SE

Figure 1. PRL-3 protein is detected in fetal heart and developing blood vessels. Positive staining for PRL-3 (red, with Fast Red chromogen, brown, with 3,3-diaminobenzidine chromogen). A, PRL-3 protein was detected in rat fetal heart (a, E16 and b, E18 (×200 vertical view) but neither in rat adult heart (d, ×400 vertical view) nor in human adult heart (c, ×400 longitudinal view). B, in normal colorectal sample (a, ×200), PRL-3 protein was expressed in developing blood vessel (arrow) but not in the mature large blood vessels (BV). In human colorectal cancer samples (b, ×200; c and d, ×400), PRL-3 protein was also expressed in developing blood vessels (arrows) but not in the large blood vessels (b, BV), C, a and c and b and d, adjacent sections. CD31 was used as a human endothelial marker to stain blood vessels in colorectal cancer samples. Black arrows, PRL-3+ and CD31+ blood vessels; red arrows, PRL-3- but CD31+ blood vessels. Magnification, ×200. D, percentages of PRL-3+/CD31+ blood vessels in 40 normal and 50 cancer colorectal samples were studied. In normal colon samples, 23 (PRL-3+ blood vessels) of 403 (CD31+ blood vessels) expressed PRL-3 protein in endothelial cells (5.7%), whereas in human colorectal cancer samples, 43 (PRL-3+ blood vessels) of 379 (CD31+ blood vessels) expressed PRL-3 protein in endothelial cells (11.3%).
mode. A similar approach was done to generate DLD-1 cell (ATCC CCL-221; American Type Culture Collection) pools stably expressing EGFP-PRL-3 or EGFP-PRL-3 (C104S).

Generation of AT-2 and AT-3 tumor cell lines stably expressing EGFP-PRL-3. To obtain EGFP-PRL-3 tumors, 8-week-old female nude mice (The Jackson Laboratory, Bar Harbor, ME) were each injected via the tail vein with EGFP-PRL-3-expressing cells (5 × 10^5). Mice were sacrificed at 3 weeks after the tail vein injection. Lungs carrying EGFP-PRL-3 tumors were removed. EGFP-PRL-3 tumors were dissected out individually under the fluorescent microscope (M2 Bio Quad, Zeiss, Jena, Germany). To generate cell lines derived from these tumors, each EGFP-PRL-3 tumor was washed twice in PBS in a tissue culture dish under sterile conditions. The tumor was cut into tiny pieces and cultured at 37°C with 5% CO₂ with RPMI 1640, 10% FBS, and 1% antibiotics (Sigma-Aldrich, St. Louis, MO). The cell lines derived from tumor 2 or 3 were named AT-2 or AT-3. The cells were trypsinized and split at 1:3 ratio into new dishes. The cell lines are now homogeneously expressing EGFP-PRL-3 as confirmed under fluorescent microscope.

In vitro angiogenesis assay to study the influence of PRL-3 expressing cells on human umbilical vascular endothelial cells. The human angiogenesis model kit ZHA-1000 was purchased from TCS CellWorks (Buckingham, United Kingdom). The assay was supplied as a growing human umbilical vascular endothelial cell (HUVEC) coculture at the earliest stages of tubule formation in a 24-well plate format. The resulting effects on HUVEC tubule formation can then be monitored. An angiogenesis control reagent kit (ZHA-1300) containing positive compound vascular endothelial growth factor (VEGF; 2 µg/mL, ×200) and negative compound suramin (2 mmol/L, ×20) was purchased from the same company. Mouse interleukin (IL)-12 was purchased from PeproTech (London, United Kingdom), whereas mouse and human IL-4 were purchased from R&D Systems (Minneapolis, MN). The angiogenesis kit (AngioKit) is designed so that test compounds, conditioned medium, or tissue explants can be added to individual wells to do the angiogenesis assay. In each well, HUVECs were grown on a matrix that is laid down in situ by fibroblasts. To assess the relationship between PRL-3-expressing cells and HUVECs, we carefully overlaid, respectively, 5 × 10^4 EGFP-PRL-3 (C104S), EGFP-PRL-3, or AT-3 cells onto HUVECs in each AngioKit well. The PRL-3-expressing cells-HUVECs were further cocultured with optimized medium mixed 1:1 with RPMI 1640 and FBS-free medium and always maintained in an incubator at 37°C with 5% CO₂ in a humidified atmosphere. Culture media were changed every 2 to 3 days. On day 11, cell sheets in each well were carefully fixed and stained for visualization. EGFP-PRL-3 cell patches and AT-3 bindles were shown in green. The HUVEC threadlike structures were revealed in red by doing indirect immunofluorescence with mouse antibody against CD31 (PECAM-1) followed by anti-mouse IgG-conjugated Texas Red (Sigma).

Confocal microscopy and analysis of in vitro angiogenesis. On day 11, cells cocultured in the AngioKit plate were washed once with PBS.
containing 1 mmol/L MgCl₂ and 1 mmol/L CaCl₂ (PBSCM). PBS was carefully aspirated from each well to avoid damaging the cell sheet, which was then fixed in 2.7% paraformaldehyde for 20 minutes at room temperature. After two more washes with PBSCM, the cells were permeabilized for 15 minutes with 0.12% saponin in PBSCM and incubated with mouse anti-CD31 (ZHA-1200, TCS CellWorks) for 1 hour at room temperature and then overnight at 4°C. The cells were washed gently thrice with PBSCM and incubated with anti-mouse IgG conjugated with Texas Red for 4 hours at room temperature. The 24-well plate was washed three more times. EGFP-PRL-3-expressing cells are green, whereas HUVECs are red. Confocal imaging was done (Zeiss LSM 510 image browser).

Using serial sections to reveal three-dimensional structures formed by AT-3 cells and HUVECs. It seems that cell lines (AT-2 and AT-3) derived from tumors might carry three-dimensional information of the parental tumor. They automatically form bundles and stack up to 350 μm in thickness and —1,500 μm in length in AngioKit wells. Serial imaging was done to reveal a detailed relationship between these EGFP-PRL-3 and HUVEC three-dimensional structures. Consecutive serial images were taken.

Figure 3. Indirect immunofluorescence reveals that EGFP-PRL-3-expressing CHO and DLD-1 cells recruit HUVECs; in addition, EGFP-PRL-3-expressing DLD-1 cells enhance HUVEC vascular formation in vitro. A, a simplified scheme shows steps of coculture in vitro system. B, note that EGFP-PRL-3-expressing CHO cells (green) did not grow evenly but instead formed patches in the matrix-coated dishes (a, d, and g). The vast majority of HUVECs (red) were also rearranged as patches (b, e, and h) apparently under the influence of each other. Image c was merged with a and b. Image f was merged with d and e. Image i was merged with g and h. c, f, and i, the merged images show that HUVEC clusters were well coordinated or matched within PRL-3-expressing cells. Bar, 200 μm (a). C, in contrast, when HUVECs were overlaid with EGFP-PRL-3(C104S) CHO cells (5 × 10⁵), the phenomenon of coordinated arrangement in (B) was not observed. The mutant PRL-3-expressing cells (a and d) and HUVECs (b and e) migrated independently as shown in the merged image (c and f).
of PRL-3 expression on tumor growth and tumor angiogenesis in vivo
we mixed 0.1 mL Matrigel (BD Biosciences) with 0.1 mL PBS, respectively,
forming tumor masses and vascular densities. To investigate the effect
of PRL-3 expression on tumor growth and tumor angiogenesis in vivo
(28), we mixed 0.1 mL Matrigel (BD Biosciences) with 0.1 mL PBS, respectively,
containing 1 × 10^6 of EGFP-PRL-3 (C104S), EGFP-PRL-3, or AT-3 tumor
cells. We then injected the mixes into the hips of nude mice to assess their
effects on tumor growth and angiogenesis. On day 10, the mice were
sacrificed and the sizes of the tumors were carefully measured. Each tumor
mass was sectioned at 10 μm in thickness. Indirect immunofluorescence
was done on the cryosections to study the intensities of blood vessels
within the tumors. The cryosections were fixed with 2.7% paraformalde-
hyde for 20 minutes at room temperature. After three more washes with
PBS CM, the sections were permeabilized for 20 minutes with 0.1% saponin
and 0.1% Triton X-100 in PBS CM. The slides were washed thrice with
PBS CM followed by rabbit anti–von Willebrand factor (vWF) and then with
anti-rabbit IgG conjugated with Texas Red. The EGFP tumor areas were
visualized in green. The slides were then washed four times with PBS CM
and mounted onto a glass slide with one drop of antifade reagent in PBS
glycerol (Biomeda Corp., Foster City, CA). Confocal imaging was done using
a Zeiss Axiophot fluorescence microscope with laser scanning (Zeiss LSM
510 image browser).

Transignal human cytokine antibody array to analyze culture
medium collected from PRL-3-expressing cells. The human cytokine
antibody array (Supplementary Fig. S2A) was purchased from Panomics,
Inc. (Redwood City, CA). Thirty-three of 36 human cytokine antibodies
cross-react with rodent cytokines (Supplementary Fig. S2, 1). Equal cell
numbers (1 × 10^6) respectively from the four cell types [type 1, CHO; type 2,
CHO expressing PRL-3 (C104S); type 3, CHO expressing PRL-3; and type 4,
CHO AT-3 tumor cell] were cultured in 4.5 mL serum-free RPMI 1640 for 6
hours. The culture media collected from the four above-mentioned cell
types were then used to bind to the antibodies immobilized on the array;
the secondary biotin-labeled detection antibodies were added to the arrays.
With this sandwich ELISA method, we can rapidly and accurately profile
the expression of multiple cytokines at the protein level in pg/mL to
determine any changes among the 33 cytokines examined in the medium.
The manufacturer’s instructions were carefully followed. By comparing
signal intensities, the relative expression levels of cytokines can be
determined. The dots were quantified with a GS-800 calibrated densitom-
eter (Bio-Rad, Hercules, CA) as follows: (a) subtract background on the
whole image; (b) mark the dots individually with circles of identical size;
and (c) quantify and calculate the density of dots. The data were displayed
with Microsoft Excel.

Results

PRL-3 protein is detected in fetal heart, developing blood
vessels, and developing RBCs but not in their mature
counterparts. Using the PRL-3-specific monoclonal antibody
cloned to 223 (16) to assess its protein expression in paraffin-
embedded tissue sections of rat and human hearts, we detected
PRL-3 protein only in rat fetal heart (E16, Fig. 1A, a; E18,
Fig. 1A, b) but neither in rat (Fig. 1A, d) nor in human adult heart
(Fig. 1A, c). To confirm this result, we used human multiple tissue
mRNA dot blots containing 73 mRNAs derived from 65 different
human tissues and 8 human cell lines to study PRL mRNA
expression patterns (Supplementary Fig. S1B). Again, only PRL-3
mRNA (Supplementary Fig. S1B, red arrow) but not PRL-1 or
PRL-2 mRNA (Supplementary Fig. S1B, black arrows) was
detected in fetal heart (Supplementary Fig. S1B, dot 11B).

We next carefully studied PRL-3 protein expression in blood
vessels. PRL-3 protein was detected in endothelia of some chaotic
architecture of tumor blood vessels or premature small blood
vessels (Fig. 1B, b-d, arrows). PRL-3 was not expressed in mature
blood vessels (BV and BV; Fig. 1B, a and b) in colorectal samples.
PRL-3 was not expressed in mature RBCs (within BV) as
illustrated in Fig. 1B, a. Using CD31 as an endothelial marker
(Fig. 1C, a and b), we used human colorectal tissue arrays
T8235790D and TS4205903. In 40 normal colon samples, 23 of
403 (CD31+) examined blood vessels are PRL-3+ (5.7%), whereas in
50 human colorectal cancer samples, 43 of 379 (CD31+) examined
blood vessels are PRL-3+ (11.3%). The increased ratios of PRL-3+/CD31+
blood vessels from 5.7% in normal to 11.3% in cancer
samples represent an active angiogenic phenotype in cancer
samples (Fig. 1D). PRL-3 might be involved in establishing new
blood vessel formation during cancer development. These results

![Figure 3](image-url) Continued. D, again, DLD-1 cells
expressing EGFP-PRL-3 (d and d) showed that HUVEC clusters were well coordinated
or matched within PRL-3-expressing cells. a and c, DLD-1 cells expressing EGFP-PRL-3
could enhance HUVEC vascular formation. Neither DLD-1 wild-type cells (note that DLD-1
control cells are invisible as they do not
express EGFP; e and f) nor DLD-1 cells
expressing EGFP-PRL-3 (C104S) have the
same properties. Bar, 200 μm.
are consistent with a previous report that PRL-3 mRNA is present in a subset of endothelial cells (15, 26).

As normal hematopoiesis occurs largely in the bone marrow, it was perhaps not surprising that we detected PRL-3 protein expression in clusters of pre-erythrocytes (Ter119-positive cells; Fig. 2A, black arrows) but was undetectable in megakaryocytes (CD61-positive cells; Fig. 2A, red arrows). Although these two different cell types are both derived from the same progenitors, megakaryocyte/erythrocyte progenitors (29), Ter119-positive cells will later develop and produce mature erythrocytes, whereas CD61-positive cells will later produce platelets (30). We further validated our results by PCR using cDNAs respectively from total bone marrow, cells, Ter119 cells, and CD61 cells. Our PCR data clearly show that PRL-3 is expressed in both total bone marrow and Ter119-positive cell fractions but not in CD61-positive cell fraction (note stronger signals in Ter119 + compared with total bone marrow; Fig. 2B). The specific PRL-3 mRNA expression in Ter119 cells further suggested that PRL-3 expression is specifically associated with pre-erythrocytes. In addition, we showed that Ter119 and PRL-2, but not PRL-1, were expressed in these pre-erythrocytes shown in Fig. 2C.

EGFP-PRL-3-expressing CHO and DLD-1 cells recruit HUVECs; in addition, EGFP-PRL-3-expressing DLD-1 cells enhance HUVEC vascular formation in vitro. Using an in vitro AngioKit, HUVECs initially formed small islands under the bone marrow; Fig. 2A, red arrows). Although these two types of cell migrate independently. The above phenomenon of coordinated movement was not seen (Fig. 3C); HUVECs and mutant PRL-3-expressing cells migrated independently. These intriguing observations suggest that the EGFP-PRL-3-expressing cells can attract and recruit HUVECs, and these properties are highly dependent on the phosphatase activity of PRL-3. To provide semiquantitative information for these phenomena, we randomly scored 50 microscopic fields for each coculture. Positives were scored if the majority (≥90%) of HUVECs were located and distributed within PRL-3 cell patches (Fig. 3B). Otherwise, they were scored negative (Fig. 3C). Based on this criterion, a plot was made by using Microsoft Excel. The abilities of recruiting HUVECs by AT-3, EGFP-PRL-3, and EGFP-PRL-3 (C104S) cells were assayed for their bindle formation in vitro. They showed 30% of the tumor cells expressing PRL-3 and the other showed 5% PRL-3-expressing levels, modified AngioKit wells were specially designed. Three types of coculture wells were made: type 1 wells contain HUVECs and fibroblasts, type 2 wells contain HUVECs only, and type 3 wells contain fibroblasts only. We overlaid AT-3 cells respectively on top of these three types of well. We found that AT-3 bindles were only formed in type 1 wells but neither in type 2 nor in type 3 wells, suggesting that AT-3 bindle formation has to depend on both HUVECs and fibroblasts (Supplementary Fig. 5A). We then asked if these bindle formations depend on PRL-3 expression levels. Using similar approach of AT-3 cell line generation, we generated two more tumor cell lines. One showed 30% of the tumor cells expressing PRL-3 and the other showed 5% PRL-3-positive cells by doing indirect immunofluorescence. They were assayed for their bindle formation in vitro angiogenesis system. We found that both low PRL-3-expressing tumor cell lines could not form good bindles (Supplementary Fig. 5B), indicating that the expression of PRL-3 is also important in AT-3 bindle formation. The capability of AT-3 tumor cells to form three-dimensional bindles in vitro is critically important, as the formation of these bindles in vitro might reveal and mimic the phenomena of microtumors forming macrotumors in vivo.

AT-3 tumor-derived cell line synergistically recruits endothelial cells and forms three-dimensional bindles that are inhibited by suramin in vitro. Cell lines derived from PRL-3-expressing metastatic tumors were expected to be more homogeneous in the property of regulating HUVECs. Using mice as a "cell sorter" to select for cells with high metastatic ability (31), a cell line (AT-3) was generated from a lung metastatic tumor of a nude mouse that was injected with EGFP-PRL-3-expressing cells via its tail vein. Similar coculture experiments were done with AT-3 cells. Figure 4A is a simplified scheme to show these coculture steps. HUVECs were grown 2 days in advance with fibroblasts that form a matrix in situ. On day 3, they were overlaid with 5 × 10⁴ AT-3 tumor cells. Surprisingly, on day 11, the tumor-derived cell lines AT-3 or AT-2 (data not shown) automatically piled up `tiers' stacking into multiple bindles of ~300 to 350 μm in thickness and ~1,500 μm in length as solid cords in these AngioKit wells (Fig. 4B; cells in green). HUVECs also form threadlike networks of 150 to 200 μm in thickness and ~1,400 μm in length, which are inserted into the center area of AT-3 bindles (Fig. 4B, d and h). We further analyzed these AT-3 cord-tubules by serial sectioning (Supplementary Serial Sections 1-3) and found that the endothelial threadlike vascular networks (in red) were well coordinated with AT-3 cells (in green), located at the center area of the AT-3 bindles. To examine the function of HUVECs on the formation of AT-3 bindles, on the other hand, we added 100 μmol/L/mL suramin (an angiogenesis inhibitor) in culture medium (from days 3 to 11). We found that the growth of HUVECs was significantly inhibited by suramin and AT-3 tumor cells were spread into monolayer cell sheets on AngioKit wells (Fig. 4C), suggesting that the formation of AT-3 bindles might depend on the support of HUVECs in this system.

AT-3 bindle formation is dependent on HUVECs, fibroblasts, and PRL-3 expression levels in vitro. To further investigate if AT-3 bindle formation is dependent on HUVECs, fibroblasts, or PRL-3-expressing levels, modified AngioKit wells were specially designed. Three types of coculture wells were made: type 1 wells contain HUVECs and fibroblasts, type 2 wells contain HUVECs only, and type 3 wells contain fibroblasts only. We overlaid AT-3 cells respectively on top of these three types of well. We found that AT-3 bindles were only formed in type 1 wells but neither in type 2 nor in type 3 wells, suggesting that AT-3 bindle formation has to depend on both HUVECs and fibroblasts (Supplementary Fig. 5A). We then asked if these bindle formations depend on PRL-3 expression levels. Using similar approach of AT-3 cell line generation, we generated two more tumor cell lines. One showed 30% of the tumor cells expressing PRL-3 and the other showed 5% PRL-3-positive cells by doing indirect immunofluorescence. They were assayed for their bindle formation in vitro angiogenesis system. We found that both low PRL-3-expressing tumor cell lines could not form good bindles (Supplementary Fig. 5B), indicating that the expression of PRL-3 is also important in AT-3 bindle formation. The capability of AT-3 tumor cells to form three-dimensional bindles in vitro is critically important, as the formation of these bindles in vitro might reveal and mimic the phenomena of microtumors forming macrotumors in vivo.
PRL-3-expressing tumor cells promote tumor growth and enhance angiogenesis in vivo. To investigate the effect of PRL-3 expression on tumor growth and angiogenesis in vivo, equal cell numbers (1 × 10^6/0.1 mL PBS) of EGFP-PRL-3 (C104S), EGFP-PRL-3, and AT-3 were each premixed with 0.1 mL Matrigel. The 0.2 mL of cell-Matrigel mixes was then each introduced into the hips of nude mice via s.c. injection. After 10 days, the animals were sacrificed for the examination of tumor sizes and blood vessel densities in fresh-frozen xenograft tumor sections. The average sizes (n = 5; 0.5, 0.9, and 1.5 cm in length) of EGFP-PRL-3 (C104S), EGFP-PRL-3, and AT-3 tumors are shown respectively in Fig. 5, a, e, and i. The blood vessel densities were examined using serial sections from these tumors by indirect immunofluorescence via staining with an antibody against vWF, a common marker for endothelial cells. We detected marked reduction of vWF-positive cells (Fig. 5, c) in poor tumors (Fig. 5, a) formed by EGFP-PRL-3 (C104S) cells, which showed weak EGFP signals (Fig. 5, b). This might indicate that EGFP-PRL-3 (C104S) cells could not cope with new environment, resulting in poor growth and forming small as well as less tumors. In contrast, the AT-3 tumor displayed robust angiogenesis and exhibited the highest degree of vascularization based on numbers of vWF + cells in tumor sections (Fig. 5, k). A statistical comparison of degrees of tumor angiogenesis was made. We cut sections from the three tumor types and did immunofluorescence. Total mouse endothelial cells (vWF-positive cells) were counted from 10 random microscopic fields (×200) and averaged. For each tumor type, three independent tumors were used for the quantification. The results are shown in Fig. 5B. The degrees of vascularization in EGFP-PRL-3 (C104S), EGFP-PRL-3, and AT-3 tumor sections were as follows: EGFP-PRL-3 (C104S) < EGFP-PRL-3 < AT-3. These results indicate that PRL-3 initiated tumor angiogenesis by recruiting host endothelial cells to the tumor sites and facilitated tumor growth, whereas phosphatase-dead PRL-3-expressing tumors displayed weak or no ability to recruit host endothelial cells.

Figure 4. AT-3 tumor cells recruit HUVECs and depend on HUVECs to form three-dimensional bindles in AngioKit dishes. A, a simplified scheme to show steps in coculture of AT-3 cells with HUVECs and fibroblasts in vitro. B, three top views of the respective crossing lines drawn in e, f, and g are shown in a, b, and c to reveal solid structures on these cord-tubules. d, HUVEC solid structure only. Serial sections were taken with a confocal Zeiss LSM 510. Each slice was cut at thicknesses of 2.7 μm with a total of 103 slices. Slices 30 (e), 50 (f), and 70 (g). Serial sections were photographed on other bindles. Each slice was 3.9 μm in thickness, and a total of 45 slices was obtained for each tubule. Slices 5 (i), 15 (j), and 25 (k). The serial images were converted to a QuickTime movie to show that the HUVECs (red) were recruited into AT-3 bindles (green; Supplementary Serial Sections 1-3). C, a and d, formation of bindles is inhibited by 100 μmol/L/mL suramin. HUVECs are much reduced by suramin. The inhibition of endothelial cell proliferation prevented AT-3 cells from forming bindles. Instead, they formed two-dimensional monolayers on these coculture dishes (b and e). Image c was merged with a and b, whereas image f was merged with d and e. Bar, 200 μm.
endothelial cells for vascularization. Therefore, PRL-3 plays a crucial role in initiating tumor angiogenesis both in vitro and in vivo.

Specific down-regulation of IL-4 as a result of PRL-3 expression in vitro. To uncover the possible mechanism of action of PRL-3 on HUVECs, we searched for connections between PRL-3-expressing cells and HUVECs by examining secreted factors in the medium collected from the following four cell types: type 1, CHO cells as control; type 2, CHO expressing EGFP-PRL-3 (C104S); type 3, CHO expressing EGFP-PRL-3; and type 4, the AT-3 tumor cell line. We speculate that PRL-3-expressing cells might secrete angiogenic factors that penetrate into their microenvironment to recruit HUVECs and initiate tumor angiogenesis. Alternatively, PRL-3-expressing cells might reduce secretion of angiogenesis inhibitors and therefore facilitate the angiogenesis process. We collected culture medium from the above four cell types and screened changes in expression levels of 36 cytokines, which are known to play important roles in angiogenesis. The reduction in IL-4 levels was observed in culture medium from types 3 and 4 cells (Fig. 6A). IL-4 was the only cytokine whose level was changed as a result of PRL-3 expression. Because IL-12 levels were not changed yet IL-12 is located nearby IL-4 on the grid, we used IL-12 levels to normalize IL-4/IL-12 ratios. We detected decreasing IL-4/IL-12 ratios (i.e., 0.82, 0.54, 0.35, and 0.19) among the type 1, 2, 3, and 4 cells, respectively (Fig. 6B).

To further examine the functions of these two cytokines in the angiogenesis bioassay. By adding IL-4 or IL-12 (at 0.04, 1.0, and 25.0 ng/mL) respectively into the AngioKit culture medium, we found that IL-4 indeed reduced the formation of HUVEC vascular networks and reduced AT-3 bindle

Figure 5. PRL-3-expressing cells promote tumor growth by recruiting host endothelial cells into tumor sites. A, EGFP-PRL-3 (C104S), EGFP-PRL-3, or AT-3 cells were premixed with Matrigel and injected into the hips of nude mice to assess the effects on tumor growth and angiogenesis in vivo. a, e, and i, 10 days after injection, tumor masses were dissected and displayed. b, f, and j, green, each EGFP tumor mass was then cut at thicknesses of 10 μm. The same cryosections were visualized by immunofluorescence with rabbit anti-vWF and then anti-rabbit IgG conjugated with Texas Red to display endothelial cells (red, vWF-positive cells), showing densities of the newly forming blood vessels in the same EGFP areas of the respective sections. c, g, and k, grades of vascularization in EGFP-PRL-3 (C104S), EGFP-PRL-3, and AT-3 tumor sections. k, AT-3 tumor cells displayed robust angiogenesis and showed the highest degree of vasculisation. Bars, 5 mm (a) and 125 μm (b–i). B, a statistical comparison of grades of tumor angiogenesis was made. Total mouse endothelial cells (vWF-positive cells) were counted from 10 random microscopic fields (×200) and averaged. For each tumor type, three independent tumors were used for the quantification analysis. The total vWF cells in 10 random microscopic fields for EGFP-PRL-3 (C104S), EGFP-PRL-3, and AT-3 tumor sections were 44, 202, and 423, respectively. The degrees of vascularization were as follows: EGFP-PRL-3 (C104S) < EGFP-PRL-3 < AT-3.
formation, whereas IL-12 did not show similar effects (Fig. 6C) in the same system. The effects of IL-4 in reducing AT-3 bindle formation are shown under low magnification in Supplementary Fig. S4C.

Discussion

The growth of solid tumors is dependent on angiogenesis, as a tumor requires an ample blood supply for expansive growth (32). Tumors have to be located within 150 μm of blood vessels to obtain adequate oxygen and nutrients (33). Without blood vessels, they cannot grow beyond a critical size or metastasize to another organ. They must establish new blood vessels by vasculogenesis and angiogenesis (34). The exact molecular events driving tumor angiogenesis still remain to be elucidated (35). Understanding the mechanism of angiogenesis should provide new approaches to the treatment of a wide range of pathologies. Targeting tumor angiogenesis will effectively combat tumor growth and cancer metastasis.

We investigated the functions of PRL-3 in physiologic angiogenesis during development (Figs. 1 and 2). Although PRL-3 protein is expressed in fetal heart, developing blood vessels, and immature erythrocytes, it is fortunate that PRL-3 protein is not expressed in mature heart, blood vessels, and RBCs. These differences in PRL-3 protein expression suggest an essential role in early angiogenic events. An inhibitor of PRL-3 should selectively act on newly active angiogenesis.

Tumor angiogenesis largely reflects normal physiologic neovascularization (36). We further investigated the functions of PRL-3 in tumor angiogenesis by using an in vitro angiogenesis assay. We carefully overlaid PRL-3-expressing cells onto HUVECs, which had grown on a matrix laid down in situ by human fibroblasts. After 8 days of coculturing, the cocultured cells were fixed and stained for CD31 to reveal HUVECs. The animated images were

![Figure 6](image_url)
taken to show the cross-talk between PRL-3-expressing cells and HUVECs. We show for the first time that PRL-3-expressing CHO or DLD-1 cells can recruit and remodel the migration of HUVECs (direct contact communication); in addition, PRL-3-expressing DLD-1 cells might also be able to secrete angiogenic factors to distant HUVECs and enhance vascular network formation (remote communication). We suggest that the in vitro angiogenesis bioassay might be useful for the screening and discovery of PRL-3 inhibitors. The compounds that can break down the linkage between PRL-3-expressing cells and HUVECs in culture might be crucial candidates as new pharmacologic agents. As previously, the metastasis-promoting property is also dependent on the phosphatase activity of PRL-3 (23). The phosphatase activity of PRL-3 might be an ideal target for drug development in inhibiting angiogenesis because EGFP-PRL-3 (C104S) lost its ability in directing and recruiting endothelial cells (Fig. 3C). Here, we show that the role of PRL-3 in angiogenesis is mainly dependent on its catalytic domain. We suggest that inhibition of this catalytic domain represents a relevant therapeutic target for antiangiogenic therapy. Importantly, the formation of AT-3 bindles might depend on angiogenesis and can be blocked by 100 μM/L/mL of an angiogenesis inhibitor, suramin (Fig. 4C). The formation of AT-3 tumor cells as three-dimensional bindles in vitro might mimic the development of microtumors to macrotumors in vivo.

In our in vivo angiogenesis assay, PRL-3-expressing cells exert a unique ability in communicating with host endothelial cells, recruiting them into PRL-3 tumor areas to deliver nutrition and facilitate tumor growth. The results may explain how PRL-3 can evoke cancer metastasis. It still remains an open question whether HUVECs in vitro and endothelial cells in vivo respond to a PRL-3-specific cell surface protein or to factors secreted by PRL-3 cells. We favor the latter hypothesis and believe that EGFP-PRL-3-expressing cells might trigger angiogenesis by releasing diffusible angiogenic factors or reducing angiogenesis inhibitors into their surrounding microenvironment to co-opt their normal neighbors. IL-4 had been reported to play a role in normal physiology and contribute significantly to antitumor activity by inhibiting the migration of endothelial cells and angiogenesis (37, 38). PRL-3-reduced IL-4 secretion could therefore be a prerequisite for the migration of HUVECs to form a vascular network. Adding IL-4 into this in vitro system indeed caused marked inhibition on vascularization (Fig. 6C, e and g), whereas IL-12 did not have the effect (Fig. 6C, h). How PRL-3 reduces IL-4 secretion and how IL-4 inhibits the vascular network of HUVECs are critical questions to be addressed in the future.

Four major conclusions from this study may be summarized. First, PRL-3 protein is expressed in the developing cardiovascular system, including pre-erythrocytes, immature blood vessels, and fetal hearts but not in their mature counterparts. Second, when PRL-3-expressing cells are cocultured with HUVECs and fibroblasts, the PRL-3-expressing cells play a vigorous role in governing and redirecting the migration of HUVECs in vitro; in return, HUVECs provide strong support for PRL-3 tumor cells to grow rapidly. Third, using an in vivo (Matrigel) tumor angiogenesis assay, tumors formed by PRL-3-expressing cells showed increased blood vessel densities and tumor masses. It seems likely that PRL-3 expression has the property of attracting and remodeling/assembling host endothelial cells into its tumor area, and as a consequence, new vasculature may be initiated to support the development of a macroscopic tumor. PRL-3 mutant-expressing cells exhibited dramatically attenuated phenotypes both in vivo and in vitro. Fourth, PRL-3 expression somehow reduces the secretion of IL-4, which acts as a negative regulator of HUVEC vasculogenesis by preventing endothelial assembly. Overall, our studies provide unique insights into angiogenesis initiation driven by PRL-3 at the cellular and molecular levels. PRL-3 is undoubtedly a novel target for abrogating tumor angiogenesis and thwarting cancer spread in clinical trials.

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# PRL-3 Initiates Tumor Angiogenesis by Recruiting Endothelial Cells \textit{In vitro} and \textit{In vivo}

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