The Low-Density Lipoprotein Receptor–Related Protein Regulates Cancer Cell Survival and Metastasis Development

Valérie Montel, Alban Gaultier, Robin D. Lester, W. Marie Campana, and Steven L. Gonias

Abstract

Low-density lipoprotein receptor–related protein-1 (LRP-1) is a multifunctional receptor involved in receptor-mediated endocytosis and cell signaling. In this study, we show that LRP-1 is abundantly expressed in severe combined immunodeficient (SCID) mouse xenografts by various human cancer cell lines that express very low or undetectable levels of LRP-1 when cultured in 21% O₂ in vitro (standard cell culture conditions). To test whether LRP-1 expression in vivo may be explained by hypoxia in the xenografts, CL16 cells, which are derived from the MDA-MB-435 cell line, were cultured in 1.0% O₂. A substantial increase in LRP-1 expression was observed. To test the activity of LRP-1 in cancer progression in vivo, LRP-1 expression was silenced in CL16 cells with short hairpin RNA. These cells formed tumors in SCID mice, in which LRP-1 expression remained silenced. Although LRP-1 gene silencing did not inhibit CL16 cell dissemination from the primary tumors to the lungs, the pulmonary metastases failed to enlarge, suggesting compromised survival or growth at the implantation site. In cell culture experiments, significantly increased cell death was observed when LRP-1–silenced CL16 cells were exposed to CoCl₂, which models changes that occur in hypoxia. Furthermore, LRP-1–silenced cells expressed decreased levels of vascular endothelial growth factor in response to 1.0% O₂. These results suggest mechanisms by which LRP-1 may facilitate the development and growth of cancer metastases in vivo.

Introduction

The low-density lipoprotein (LDL) receptor-related protein-1 (LRP-1) is a member of the LDL receptor gene family, expressed by diverse cell types, including fibroblasts, hepatocytes, macrophages, and neurons (1). LRP-1 is synthesized as a large transmembrane protein (500 kDa) and processed in the trans-Golgi by a furin-like protease into the mature two-chain structure, which includes the 515-kDa α-chain, which is entirely extracellular, and the 85-kDa β-chain, which has the transmembrane domain (2). LRP-1 is first characterized as an endocytic receptor for apolipoprotein E–containing lipoprotein particles (3) and for the protease inhibitor, α2-macroglobulin (4). Since then, >40 ligands have been identified, including proteases, protease inhibitors, growth factors, extracellular matrix proteins, and foreign toxins (5). By binding bifunctional extracellular ligands and intracellular signaling–adaptor proteins, LRP-1 may promote the internalization and catabolism of other receptors with cell signaling activity (6). By binding adaptor proteins, LRP-1 also directly regulates the activity of various cell signaling enzymes, including extracellular signal–regulated kinase (ERK)/mitogen-activated protein (MAP) kinase, phosphatidylinositol 3-kinase (PI3K), and c-Jun NH₂-terminal protein kinase (JNK; ref. 7). By this mechanism, LRP-1 may promote cell survival (8, 9).

The diverse activities of LRP-1 suggest a model in which this receptor functions as a “sensor” of the cellular microenvironment. Such activity should be highly relevant to cancer because it is now widely accepted that a tumor and its microenvironment actively and reciprocally interact at all stages of cancer progression (10). LRP-1 is expressed by human glial cell tumors (11), by low-grade melanocytic tumors (12), and by fibrosarcoma cell lines (13). However, in epithelial cell malignancies, LRP-1 expression is less certain. Instead, these cells may express the VLDL receptor, which has functional properties that are partially overlapping with LRP-1 (14, 15). Differences in reported levels of LRP-1 in cancer cells in vitro may reflect the effects of culture confluency (16) and/or LRP-1 degradation by membrane-anchored metalloproteases (17). Kancha et al. (18) reported an inverse relationship between LRP-1 and cancer cell invasion in vitro in prostate and breast cancer cells. These results are consistent with our previous observation that LRP-1 inhibits HT 1080 cell migration and invasion in vitro by suppressing cell signaling downstream of the urokinase receptor (uPAR; ref. 13). However, the activity of LRP-1 as a regulator of cancer progression in vivo remains incompletely understood.

Because cancers typically outgrow their blood supply, cancer angiogenesis is considered essential for cancer progression. Development of novel agents that counteract angiogenesis has become a major focus for cancer therapeutics design (19, 20). Angiogenesis is stimulated by hypoxia, which is a hallmark of diverse neoplasms (21). Hypoxia activates complex genetic expression programs downstream of transcription factors, such as hypoxia-inducible factor-1α (HIF-1α; refs. 22, 23) and cyclic AMP–responsive element binding protein (24). Unfortunately, these programs may select cancer cells with aggressive phenotypes (25, 26). When HIF-1α is conditionally deleted in the mammary epithelium of transgenic mice that are engineered to develop breast cancer, onset and growth of breast cancer are delayed and metastasis is decreased (27). Thus, understanding the molecular changes that occur in hypoxic cancer cells is an important goal.

LRP-1 mRNA expression is increased by hypoxia in a number of cancer cell lines (28–30). In this study, we report that cancer cells, which express little or no LRP-1 protein in vitro, show greatly increased levels of LRP-1 in xenografts in severe combined immunodeficient (SCID) mice. To probe the responsible mechanism, we cultured CL16 cells, which are derived from the MDA-MB-435 cell line (31), in 1.0% O₂, as opposed 21% O₂ (standard cell culture conditions). We also treated these cells with CoCl₂, which stabilizes HIF-1α (32). Both treatments substantially increased LRP-1 protein levels. To study the role of LRP-1 in cancer
progression, LRP-1 expression was silenced in CL16 cells with short hairpin RNA (shRNA). The resulting cells showed increased cell death and decreased expression of vascular endothelial cell growth factor (VEGF) in response to hypoxia in vitro. Although LRP-1–silenced cells formed tumors in SCID mice and metastasized to the lungs, the metastases failed to enlarge, suggesting a deficiency in cell growth or survival. These results support a model in which LRP-1 may play a critical role in the development of cancer metastases.

Materials and Methods

Antibodies and reagents. HIF-1α–specific monoclonal antibody was purchased from BD Transduction Laboratories. In control experiments, we showed that this antibody recognizes HIF-1α in human cell lines exposed to 1.0% O2 but not in murine cells (RAW 264.7), treated equivalently. Monoclonal antibody 8G1, which recognizes the α-chain of human LRP-1 but does not cross-react with murine LRP-1 (33), was purified from hybridoma-conditioned medium. Monoclonal antibody directed against lamin and polyclonal antibody that recognizes total ERK/MAP kinase were from Upstate. Matrigel was obtained from Sigma. Quantitative PCR (qPCR) reagents, including primers and probes for human LRP-1, VEGF, and hypoxanthine phosphoribosyltransferase (HPRT-1), were from Applied Biosystems.

Cell culture. 2C5 cells and CL16 cells, which are subclones of MDA-MB-435 cells (31), were generously provided by Dr. David Tarin (University of California, San Diego, CA). HEK 293 cells were obtained from the American Type Culture Collection (ATCC). These cells were cultured in RPMI 1640 (HyClone) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. MDA-MB-468 were obtained from the ATCC and cultured in DMEM (HyClone) supplemented with 10% FBS, penicillin, and streptomycin. All cell cultures were passaged at subconfluence and maintained in 21% O2 and 5% CO2 for the indicated times before performing experiments. These standard conditions are referred to as normoxic cell culture. In hypoxic cell culture conditions, the O2 level was adjusted to 1.0%. CoCl2 (200 μM/l) was added to some cultures under normoxic conditions. CoCl2 stabilizes HIF-1α and thus induces many of the changes observed in hypoxia (32).

LRP-1 gene silencing. Silencing of LRP-1 gene expression in CL16 cancer cells was accomplished using the pSUPER vector system, which expresses shRNA (Oligoengine). The human LRP-1–derived sequence, GACCTGAGCCCCAAGCAGTGAAGACTGCTTGGGGCTGCAAGTC, which includes a central loop (underlined) was cloned into pSUPER-puro. CL16 cells were transfected with this construct or with the empty vector using the Nucleofector system from Amaxa. Transfected cells were selected with puromycin (1 μg/mL) and cloned by serial dilution. Clones were screened for LRP-1 expression by qPCR under normoxic and hypoxic conditions. pSUPER-2 cells were prepared as a control by transfecting CL16 cells with empty vector. The cells were then selected and cloned by serial dilution.

qPCR. Cells in 60-mm dishes were cultured in complete medium for 24 h, in 1.0% or 21% O2. Total RNA was extracted using TRIzol (Invitrogen), as directed by the manufacturer. cDNA was synthesized from 2 μg of total RNA using the iScript cDNA synthesis kit (Bio-Rad), qPCR was done using a System 7300 instrument (Applied Biosystems) and a one-step program: 95°C, 10 min; 95°C, 30 s, 60°C, 1 min for 40 cycles. LRP-1 and VEGF mRNA levels were measured in triplicate and normalized against human HPRT-1 mRNA. LRP-1 and VEGF mRNA expression was determined by the relative quantity method.

Immunoblot analysis. To prepare total cell extracts, cells in culture were washed once with ice-cold 20 mM/L sodium phosphate, 150 mM/L NaCl (pH 7.4; PBS), and then recovered by scraping into radioimmunoprecipitation assay (RIPA) buffer [20 mM/L sodium phosphate, 150 mM/L NaCl (pH 7.4), 1% NP40, 0.1% SDS, 0.5% deoxycholic acid] containing complete protease inhibitor cocktail (Roche). To analyze proteins released into the medium, cells were washed thrice with PBS and then cultured for 16 h in serum-free medium. The cell culture supernatant was cleared by centrifugation (5,000 × g at 4°C for 5 min) and concentrated ~200-fold using Centricon devices (Millipore). Tissue samples recovered from xenografts were minced with a razor blade, suspended in RIPA buffer, and then homogenized by six strokes in a Dounce homogenizer. Incubations in RIPA buffer were allowed to proceed for 30 min on ice. Samples were then subjected to centrifugation at 15,000 × g for 20 min at 4°C and the supernatants were collected for analysis. To prepare nuclear extracts, cultured cells or tissues were processed using the Nuclear Extraction Kit from Panomics. Protein concentration was determined by bicinchoninic acid assay (Sigma-Aldrich). Equal amounts of cellular protein were loaded on 4% to 12% polyacrylamide gels (Invitrogen) for SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes and probed with antibodies that detect human HIF-1α, human LRP-1, total ERK/MAP kinase, and lamin (the latter two are loading controls).

Xenografts. All animal experimentation was done in accordance with protocols approved by the University of California San Diego Animal Care Program. Eight-week-old C.B.17/IcrCr-stdcR mice (Charles River Laboratories) were used. To generate xenografts, 2 × 106 cells suspended in 50 μL of Matrigel (Sigma) were inoculated into the fourth right mammary fat pad of anesthetized mice (pSUPER-2, n = 11; shLRP1-1, n = 10; shLRP1-2, n = 11, two separate experiments). Primary tumor growth was monitored every 2 to 3 days, until the tumors were ~1 to 1.2 cm in maximum diameter, as estimated using calipers from above the surface of the skin. The mice were then euthanized and the xenografts recovered for analysis. Estimated tumor masses were determined by fluorescent stereomicroscopy (Wetzlar), first in the whole animal at necropsy and then, in each lobe of the lungs. Pulmonary metastatic load was determined by counting the number of fluorescent tumor foci visible on the ventral surface of each lobe at ×8 magnification. We arbitrarily defined three size categories of fluorescent masses: masses ranging between 100 and 500 μm2 were counted as “metastasis.” Masses larger than 0.5 mm in diameter were considered “macrometastases.” Fluorescent masses smaller than 100 μm were considered “micrometastases.” Green-fluorescing metastases were confirmed by histology.

In a separate set of experiments, xenografts were established with the same GFP-expressing cell lines; however, the tumor-bearing animals were euthanized earlier (18–25 days), when the tumors had grown to ~150 mm3 in volume (pSUPER, n = 4; shLRP1-1, n = 4; shLRP1-2, n = 5). After euthanasia, necropsies were done and the lungs were recovered. The total number of superficial green-fluorescing cells/cell clusters visible on the ventral surfaces of each pulmonary lobe (five lobes per animal) was determined by fluorescent stereomicroscopy at ×63 magnification.

Histology. Formalin-fixed tissues specimens were collected during necropsies and paraffin embedded. Serial 20-μm sections were obtained and stained with H&E.

Cell death ELISA. pSUPER-2 cells, shLRP1-1 cells, and shLRP1-2 cells were plated at 10,000 per well in 96-well plates in 10% FBS-containing medium and cultured overnight. The cells were then treated with 200 μM/L CoCl2 or with vehicle for 24 h. Cell death was determined by the Cell Death ELISA (Roche), as previously described (34). The assay detects intracytoplasmic oligonucleosomes.

Results

LRP-1 protein expression is greatly increased in cancer cells in vivo. To study LRP-1 expression in vivo, we established xenografts in SCID mice using a weakly metastatic cell line (2C5) and its highly metastatic counterpart (CL16), which are derived from the MDA-MB-435 cancer cell line (31). We also established xenografts with MDA-MB-468 breast cancer cells and with HEK 293
LRP-1 conditions, may indeed express significantly increased levels of LRP-1 in vivo under normoxic conditions (21% O2), LRP-1 was detected in two of three MDA-MB-468 cell xenografts, and in all three CL16 cell xenografts. Each number (#1–#3) refers to a primary tumor resected from a separate mouse. B, extracts were prepared from nonmetastatic 2C5 cells and metastatic CL16 cancer cells. Each number (#1–#3) refers to a primary tumor resected from a separate mouse. C, extracts were prepared from MDA-MB-468 breast cancer cells in normoxic cell culture and from three separate xenografts. C, extracts are shown for HEK 293 cells that were maintained in culture or grown in five separate xenografts in different mice. In all cases, 100 μg of protein was subjected to SDS-PAGE before immunoblotting. Membranes were reprobed for total ERK/MAP kinase as a control for loading. D, H&E-stained paraffin sections of xenografts established with MDA-MB-468 cells (left) and HEK 293 cells (right) show boundaries between viable tumor and areas of necrosis (N). Large areas of necrosis were present in the xenografts, as anticipated. Magnification, ×400.

Figure 1A shows that human LRP-1 was readily detected in two of three 2C5 cell xenografts and in all three CL16 cell xenografts. LRP-1 was detected in two of three MDA-MB-468 cell xenografts (Fig. 1B) and in all five HEK 293 cell xenografts (Fig. 1C). By contrast, in cell culture under normoxic conditions (21% O2), LRP-1 was not detectable in any of the cell lines. We conclude that cell lines, which express little or no LRP-1 under standard cell culture conditions, may indeed express significantly increased levels of LRP-1 in vivo. Because our results showed that cancer cells express LRP-1 in vivo, we hypothesized that LRP-1 may function as a regulator of cancer progression.

Substantial variability was noted in the level of LRP-1 detected in extracts of xenografts, derived from the same cell line, in different mice. We also noted differences in our loading control (ERK/MAP kinase), although equal amounts of xenograft protein were subjected to SDS-PAGE in every lane. We hypothesized that the variability resulted at least in part from the fraction of viable tumor versus inflammatory infiltrate and necrotic tissue in the samples that were subjected to SDS-PAGE. To test this hypothesis, we analyzed the xenografts by H&E histology. All of the xenografts included regions of viable tumor that were intermixed with large areas of inflammation and necrosis. Figure 1D shows boundary areas in xenografts formed with MDA-MB-468 and HEK 293 cells. The necrosis probably altered the fraction of xenograft protein that was cellular protein. The inflammation altered the fraction of human tumor cells that express LRP-1, which can be detected with antibody 8G1.

LRP-1 expression is increased by hypoxia in vitro. We hypothesized that hypoxia within the xenograft microenvironment may contribute to the increase in LRP-1 expression observed in the human cancer cells. To test whether hypoxic conditions exist in the xenografts, samples of three tumors, formed with CL16 cells, were extracted using a method that selectively recovers nuclear protein. The same method was applied to CL16 cells that were maintained in normoxic cell culture. As shown in Fig. 2A, HIF-1α was significantly increased in the xenograft extracts. By contrast, HIF-1α was not detectable in CL16 cultures maintained in 21% O2 in vitro.

The concentration of oxygen in conventional cell culture corresponds to a P02 of 150 mm Hg, well above the O2 level in even well-perfused tissues in vivo (40 mm Hg; ref. 36). To test
whether oxygen concentration regulates LRP-1 expression in CL16 cancer cells, we cultured the cells in 1.0% or 21% O_2 for 24 h. As a second test, we exposed CL16 cells to CoCl_2 (200 μmol/L), which stabilizes HIF-1α (32) and, thus, replicates many of the changes observed in hypoxia. As shown in Fig. 2B, LRP-1 protein expression was substantially increased by hypoxia and, to a somewhat lesser extent, by CoCl_2. HIF-1α protein expression was also increased by these treatments, as anticipated.

Because cancer cells may enter the bloodstream and implant in the lungs where the O_2 concentration is higher, we cultured CL16 cells in 1.0% O_2 for 24 h and then transferred the cultures to 21% O_2. LRP-1 protein expression was compared with that observed in cultures that were maintained in 21% O_2 throughout. As shown in Fig. 2C, the effects of hypoxia on LRP-1 expression remained for at least 8 h after transfer to 21% O_2. By 24 h, LRP-1 expression equalized. A slight increase in CL16 cell LRP-1 expression was observed under normoxic conditions after 48 h in culture in multiple experiments and seemed to be related to prolonged maintenance at high culture confluency (16).

**LRP-1 gene silencing is sustained in vitro and in vivo.** CL16 cells are known to metastasize from orthotopic xenografts in SCID mice (31). To assess the role of LRP-1 in CL16 cell metastasis, GFP-expressing cells were transfected to express LRP-1–specific shRNA and subjected to single-cell cloning. Multiple subclones were identified in which LRP-1 expression was silenced, compared with cells that were transfected with empty vector (pSUPER-2 cells). Figure 3A shows qPCR analysis of the cell lines shLRP1-1 and shLRP1-2, in which LRP-1 mRNA was decreased under normoxic cell culture conditions by 76 ± 3% and 69 ± 7%, respectively, compared with that present in pSUPER-2 cells. In 1.0% O_2, the decrease in LRP-1 mRNA was 87 ± 5% and 90 ± 3%, respectively.

Immunoblot analysis of pSUPER-2 cells, which were maintained in normoxic cell culture, showed only low levels of LRP-1 protein; however, in 1.0% O_2, the level of LRP-1 protein increased substantially (Fig. 3B). LRP-1 was also recovered in conditioned serum-free medium from pSUPER-2 cells that were cultured in 1.0% O_2. Shed LRP-1 α-chain has been previously detected in plasma and conditioned medium from astrocytic cells (37, 38). By contrast, cell extracts and conditioned medium from shLRP1-1 and shLRP1-2 cells showed very low or undetectable levels of LRP-1, under normoxic and hypoxic conditions.

Xenografts were established in SCID mice by injecting of 2 × 10^6 cells suspended in Matrigel into mammary fat pads (n = 10–11/cell line). The two cell lines in which LRP-1 was silenced (shLRP1-1 and shLRP1-2) and the control cells (pSUPER-2) readily generated tumors, which were allowed to grow until the maximum diameter was 1.0 to 1.2 cm, as estimated in the living mice with calipers. As shown in Fig. 3C, on average, tumors generated with shLRP1-1 cells grew slightly faster than the control tumors, whereas tumors generated with shLRP1-2 cells grew slightly slower; however, the differences in growth rates were not statistically significant, as determined by repeated measures ANOVA. Because we sacrificed the mice based on tumor size, we assessed each individual group to determine whether the number of metastases (discussed below) correlated with the age of the tumor at euthanasia; no correlation was observed (correlation coefficients < 0.4).

To determine whether LRP-1 gene silencing remained effective in vivo, extracts of primary tumors, recovered at necropsy, were subjected to immunoblot analysis with antibody 8G1. LRP-1 was readily detected in extracts of three separate tumors formed with pSUPER-2 cells (Fig. 3D). By contrast, LRP-1 was absent in extracts from three tumors formed with shLRP1-1 cells and from three tumors formed with shLRP1-2 cells. Thus, LRP-1 was expressed selectively by the pSUPER-2 cells in vivo.

**Metastasis development is inhibited when LRP-1 is deficient.** At necropsy, fluorescent imaging of intact animals suggested that...
metastases were restricted primarily to the lungs. To determine whether LRP-1 regulates metastasis, all five lobes from the lungs of each experimental animal \( (n = 10–11/\text{cohort}) \) were examined at \( \times 8 \) magnification for fluorescent tumors. Numerous green-fluorescing tumors were found in the lungs of all of the animals; however, the total number of metastases >500 µm in size and in the range from 100 to 500 µm was significantly decreased \( (P < 0.05) \) in mice that were inoculated with shLRP1-1 cells or shLRP1-2 cells, compared with mice that were inoculated with pSUPER-2 cells (Fig. 4A and B).

Our GFP fluorescence imaging studies were confirmed by analyzing H&E-stained sections of the lungs from the mice. Figure 4C shows representative images of metastases formed with pSUPER-2 cells. In some cases, the tumor cells nestled clearly within the lung parenchyma (e.g., ii). In other cases, clusters of tumor cells are apparent within lymphatics or blood vessels (e.g., iii).

To test why LRP-1 gene silencing inhibits growth and development of CL16 metastases in the lungs, we did an additional series of experiments in which SCID mice, which were inoculated with the three cell lines, were euthanized at an earlier time (when the tumors were \( \sim 150 \text{ mm}^3 \) in volume). At necropsy, the lung surfaces were scanned by fluorescent stereomicroscopy at \( \times 63 \) and the total number of fluorescent foci, almost all of which appeared to be composed of individual cells or small cell clusters, were determined. As shown in Table 1, LRP-1 gene silencing had no effect on the number of metastatic foci identified in the lungs at this stage.

**LRP-1 promotes cancer cell survival and VEGF expression.** Next, we did *in vitro* experiments to identify LRP-1 activities that may explain why LRP-1 deficiency inhibits the development of CL16 cell pulmonary metastases *in vivo*. LRP-1 supports the survival of nonneoplastic cells, including Jurkat cells, cerebellar

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**Figure 4.** The role of LRP-1 in cancer metastasis from xenografts in mice. A, xenografts were established in mice, using the two cell lines in which LRP-1 was silenced and control pSUPER-2 cells. All three cell lines expressed GFP. At necropsy, the lungs were removed and each lobe was assessed by fluorescence stereomicroscopy for surface metastases. Representative lobes are shown. Dashed line, the outline of the lobe. Arrows, metastases that are >500 µm in maximum diameter. B, lung metastases (mets) on the surfaces of each lobe were counted. The number of macrometastases (macro-mets; >500 µm in maximum diameter) and metastases (100–500 µm in diameter) were determined. Columns, mean; bars, SE (*, \( P < 0.05 \), compared with the matched control with pSUPER-2 cells; \( n = 10–11 \)). C, H&E-stained sections of lungs showing representative metastases formed by pSUPER-2 cells. In (iii), tumor may be filling a blood vessel or lymphatic vessel with clear extravascular invasion.
neurons, and Schwann cells (8, 9). To determine whether LRP-1 regulates CL16 cancer cell survival, pSUPER-2 cells, shLRP1-1 cells, and shLRP1-2 cells were cultured for 24 h in 10% FBS-supplemented medium, with or without 200 μmol/L CoCl2. Cell death was determined using the Cell Death ELISA kit. As shown in Fig. 5A, pSUPER-2 cells showed minimum cell death when treated with CoCl2. By contrast, the shLRP1-1 and shLRP1-2 cells showed significantly increased cell death (P < 0.05). Thus, LRP-1 may function as a cell survival receptor in cancer, especially under hypoxic conditions.

Next, we examined VEGF mRNA expression in response to hypoxia. We hypothesized that decreased hypoxia-induced VEGF expression, in LRP-1–silenced cells, may limit metastasis growth in vivo. As shown in Fig. 5B, pSUPER-2 cells expressed increased VEGF mRNA when cultured for 24 h in 1.0% O2, as anticipated (23). Hypoxia-induced VEGF mRNA expression was significantly decreased in the shLRP1-2 cells (P < 0.05).

Discussion

In this study, we show that 2C5, CL16, MDA-MB-468, and HEK 293 cells express abundant LRP-1 in xenografts. Because the same cells express very low or undetectable levels of LRP-1 in cell culture, conditions present in the xenograft must favor expression. Previous studies have identified LRP-1 as a hypoxia-regulated gene, based on analysis of LRP-1 mRNA (28–30). Our results confirm and extend these previous studies by showing that LRP-1 protein expression is increased in CL16 cells by hypoxia or CoCl2. We further show that significant hypoxia exists in CL16 cell xenografts by demonstrating increased levels of human HIF-1α protein. Interestingly, the increase in LRP-1 antigen, observed in hypoxic cell culture, was substantial, whereas the increase in LRP-1 mRNA was more modest. This result may suggest that posttranslational mechanisms contribute to the increase in LRP-1 protein observed in hypoxia. Although multiple mechanisms may be responsible for the selective expression of LRP-1 by cells in xenografts and not in cell culture, our data suggest a role for hypoxia.

Given our results demonstrating that LRP-1 is expressed by cancer cells in xenografts, we undertook studies to determine whether LRP-1 regulates cancer progression. Possible mechanisms by which LRP-1 may regulate cancer invasion and metastasis are abundant, including its function as a endocytic receptor for proteases such as uPA, matrix metalloproteinase (MMP)-2, and MMP-9; its ability to regulate cellular expression and catabolism of diverse extracellular matrix proteins; and its ability to regulate cell signaling either directly or indirectly, by regulating the activity of other receptors such as uPAR and the platelet-derived growth factor (PDGF) β-receptor (5, 7). Some LRP-1 activities relevant to cancer metastasis seem to be context dependent. For example, cell migration may be promoted or inhibited by LRP-1. In murine embryonic fibroblasts and in certain cancer cells, LRP-1 inhibits cell migration and Matrigel invasion in vitro by suppressing uPAR-dependent cell signaling to ERK/MAP kinase and Rac1 (39). LRP-1 also inhibits vascular smooth muscle cell migration in vivo, in this case by controlling cell signaling downstream of the PDGF β-receptor (40, 41). By contrast, LRP-1 promotes macrophage migration by regulating the interaction of Mac-1 with immobilized fibrinogen (42) and endothelial cell migration, by a pathway that involves thrombospondin (43). LRP-1 has been reported to support survival of nonneoplastic cells based on its ability to regulate cell signaling to PI3K and JNK (8, 9). LRP-1 may support cell proliferation by sustaining activation of ERK/MAP kinase in response to uPAR ligation (44) or inhibit growth by suppressing PDGF β-receptor–initiated cell signaling (40, 41). Thus, the function of LRP-1 in cancer is most likely determined by other receptors expressed by the cancer cell, which may function in common systems with LRP-1, and by the cancer cell microenvironment.

To study LRP-1, we silenced LRP-1 expression in CL16 cells and subcloned new lines from single cells. CL16 cells are derived from MDA-MB-435 cells (31), which for many years were considered a model of breast cancer. However, recent evidence suggests that

| Table 1. Number of fluorescent foci on the ventral surfaces of the lungs in mice bearing tumors formed with pSUPER-2, shLRP1-1, or shLRP1-2 cells, which were allowed to grow to a volume of 150 mm3 |
|-----------------|-----------------|-----------------|-----------------|
| **Xenograft**   | **Animal number/cohort** | **Mean tumor volume (mm3)** | **Pulmonary tumor foci** |
| pSUPER-2       | 4               | 207 ± 20        | 77 ± 7          |
| shLRP1-1       | 4               | 197 ± 19        | 83 ± 11         |
| shLRP1-2       | 5               | 217 ± 11        | 70 ± 15         |

Figure 5. Effects of LRP-1 gene silencing on CL16 cell viability and VEGF mRNA expression. A, shLRP1-1 cells, shLRP1-2 cells, and pSUPER-2 cells were cultured in serum-containing medium, in the presence of 200 μmol/L CoCl2 or in the absence of CoCl2 (Control) for 24 h. Cell death was determined by ELISA for intracytoplasmic oligonucleosomes. Columns, mean; bars, SE (*, P < 0.05 as determined by t test; n = 3 separate experiments). B, shLRP1-2 cells and control pSUPER-2 cells were cultured in 1.0% or 21% O2 for 24 h. VEGF mRNA expression was determined by qPCR and normalized against HPRT1 mRNA levels. The mRNA levels are expressed relative to that present in pSUPER-2 cells, under normoxic culture conditions. Columns, mean; bars, SE (*, P < 0.05, n = 4).

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MDA-MB-435 cells may represent melanoma (45). Thus, the exact nature of the CL16 cancer cells awaits resolution of this discrepancy regarding the MDA-MB-435 cells. LRP-1 gene silencing did not significantly alter the growth of primary tumors formed with CL16 cells. Furthermore, when the lungs were analyzed at an early time point, LRP-1 silencing did not inhibit the ability of the tumor cells to relocalize from the primary tumor to implantation sites in the lungs. However, both cell lines in which LRP-1 was silenced showed a decreased capacity to sustain and grow metastases. As a result, the number of larger metastases (>500 μm and 100–500 μm) was significantly decreased.

To determine mechanisms that may be responsible for the activity of LRP-1 in CL16 cancer cell metastasis, we conducted experiments using in vitro cell culture systems. We showed for the first time that LRP-1 may promote cancer cell survival, building on earlier studies suggesting prosurvival activity for LRP-1 in non-neoplastic cells (8, 9). Although cancer cells frequently appear in the bloodstream relatively soon after primary tumors are established, survival of cancer cells in the bloodstream and at secondary growth sites represents a major challenge (46). We hypothesize that survival of the CL16 cell–derived subclones in lung parenchyma may depend, in part, on whether LRP-1 is expressed and that this LRP-1 activity may contribute to the ability of LRP-1–expressing cells to form large metastases.

We also showed that the increase in VEGF mRNA expression in response to hypoxia is inhibited when LRP-1 is silenced. VEGF expression is increased in response to HIF-1 (23). Although increased VEGF expression may explain the ability of LRP-1–expressing cells to develop larger metastases in vivo, it is important to note that this property of LRP-1–expressing cells was not associated with an increase in the growth rate of the primary xenografts or with an apparent decrease in the extent of xenograft necrosis. Regulation of gene expression by LRP-1 has been shown previously (47). Among the gene products regulated in fibroblasts is the antiangiogenic factor, pigment epithelium-derived factor, which is suppressed by LRP-1. LRP-1 also has been implicated in the regulation of expression of type III collagen, which contributes to the structural integrity of blood vessels (47).

One question that emerges involves the relationship of LRP-1 expression in the hypoxic CL16 cell xenografts to the behavior of the same tumor cells in the lungs. While traversing the bloodstream and within the lungs, the oxygen concentration should be higher and, thus, possibly suppress LRP-1 expression. With this in mind, we conducted in vitro studies and showed that the increase in LRP-1 induced by hypoxia is retained for at least 8 h after the cells are returned to 21% O2. Whether the elevated level of LRP-1 in pSUPER-2 cells, resulting from exposure to hypoxia in the xenograft, contributed to metastasis development after implantation in the lungs is a topic for further study.

The spectrum of receptors that may be expressed at increased levels and/or activated in hypoxic tumors continues to grow. In many tumor cells, the proto-oncogene met is activated and is responsible for increased cancer invasion (48). In MCF-7 breast cancer cells, increased erythropoietin expression leads to increased ERK/MAP kinase activation and cell migration (49). Furthermore, in other cancer cells, uPAR expression is increased (50); hypoxia-induced uPAR expression in breast cancer cells induces epithelial mesenchymal transition (50). How the activities of these various receptors and LRP-1 are integrated in the cancer cell is an emerging question. Given the ability of LRP-1 to promote uPAR internalization and thereby decrease cell surface uPAR (13, 15), the relationship between these two receptors may be of particular interest. In MDA-MB-435 cells, uPAR expression is very low and in response to hypoxia, uPAR expression is not increased (50). Nevertheless, because uPAR functions as a prosurvival receptor, we cannot, at this point, rule out the possibility that increased cell surface uPAR expression, resulting from LRP-1 gene silencing, partially offsets or compensates for the otherwise more severe effects of LRP-1 deficiency on cancer cell survival.

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