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LRH1 as a driving factor in pancreatic cancer growth

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Abstract

Liver receptor homolog 1 (LRH1), directs the development and differentiation of embryonic pancreas, and is overexpressed in pancreatic cancer (PC). We hypothesized that LRH1 promotes PC growth. Cell proliferation and tumorigenicity in nude mice were compared between empty vector-transfected (control) and stable LRH1-overexpressed PC cell lines. The subsequent tumor burden, vasculature development, and histologic features were evaluated. LRH1 overexpression enhanced the expression of downstream target genes (cyclin D1/E1) and stimulated cell proliferation in PC cell lines. LRH1 upregulated cyclin E1 truncated T1/T2 isoforms expression which may occur through ERα—calpain1 signaling. Compared with the control, LRH1 overexpressing stable cells generated tumors with increased weight, proliferation index and enhanced angiogenesis. Cyclin D1/E1 and calpain1 were overexpressed in human PC tumors compared to adjacent normal pancreas. These observations demonstrate that LRH1 promotes PC growth and angiogenesis, suggesting that LRH1 is a driving factor in tumorigenesis and may serve as a potential therapeutic target.

Keywords

liver receptor homolog 1; cyclin D1; cyclin E1; pancreatic cancer

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CONFLICTS OF INTEREST
We have no conflict of interest.

1. INTRODUCTION

Pancreatic cancer (PC) is the fourth-leading cause of cancer mortality in the United States, with an estimated 43,920 new cases and 37,390 deaths in 2012.[12] PC has the lowest 5-year survival rate of all malignancies due to aggressive progression and multiple-drug resistance.[12] Previous studies have revealed that signaling pathways involved in the development of the embryonic pancreas also play major roles in the malignant transformation of the adult pancreas.[9, 13] We hypothesize that liver receptor homolog 1 (LRH1) (also called Nuclear Receptor Subfamily 5 Group A Member 2 [NR5A2]) is a driving factor that is shared between pancreatic embryogenesis and oncogenesis.[7] LRH1 is a promoter binding factor and an alpha-fetoprotein transcriptional factor. LRH1 is a key downstream target of the pancreatic and duodenal homeobox 1 regulatory cascade that regulates pancreatic development, differentiation, and function.[1]

Importantly, accumulating evidence indicates participation of LRH1 in the pathogenesis of multiple tumors, such as gastric, [15] breast, [14] colon, [11] and PC.[4, 10] For example, LRH1 affects tumor response to anti-estrogen drugs by regulating the expression of estrogen receptor in breast cancer cells.[14] In the intestine, LRH1 and β-catenin/Tcf4 signaling induces G1/S-specific cyclin D1/E1-mediated cell proliferation and self-renewal.[5] LRH1 contributes to intestinal tumor formation by affecting both cell cycle control and the inflammatory process.[11] Notably, a recent genome-wide association study identified single nucleotide polymorphisms of the *LRH1* gene associated with PC risk.[10] LRH1 mRNA level was higher in PC cell lines compared to a normal pancreatic ductal epithelium derived cell line, and is associated with increased protein levels in pancreatic tumors.[4] LRH1 expression upregulated mRNA levels of cyclin D1/E1 in PC cell lines.[4] Accordingly, selective blocking of LRH1 by siRNA decreased mRNA levels of cyclin D1/E1.[4] However, the mechanism by which LRH1 promotes PC development remains unclear. To explore the oncogenic property of LRH1, we investigated how LRH1 contributes to PC growth using cell-based assays, xenografts in nude mice, and tissue specimens of PC.

2. MATIERIALS AND METHODS

2.1 Cell culture

The human PC cell lines AsPC-1, BxPC-3, Capan-1, HPAF-II, Hs 766T, MIA PaCa-2, PL45 and Panc-1, liver hepatocellular carcinoma cell line HepG2 (ATCC), and non-neoplastic human pancreatic duct epithelial cell line HPDE (Applied Biological Materials Inc.) were employed. All the cell lines have been authenticated by short tandem repeat (STR) profiling to reduce the frequency of cell misidentification.[8] Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in corresponding medium supplemented with 10% FBS and antibiotics (penicillin and streptomycin). Cells were passaged when they reached 80% confluence.

2.2 Lentiviral infected stable PC cell lines with overexpression of LRH1

We established two stable Capan-1 and AsPC-1 cell lines with constitutive expression of LRH1 using the lentiviral expression system (GeneCopoeia, #EX-Z2607-Lv105). After 48 h of transfection with packaging plasmids and pLentiviral plasmids of target genes in 293T cells, the supernatants containing lentiviral particles were filtered by 0.45 μm nitrocellulose membrane and mixed with polybrene at 8 μg/ml. Twenty-four hours after lentiviral infection, cells were incubated with the corresponding media for 24 h, then selected with 8 μg/mL (for Capan-1) or 3 μg/mL (for AsPC-1) puromycin overtime to eliminate un-infected cells. The LRH1 protein level in stable PC cell lines was confirmed by Western blot. The

selected cell lines were routinely cultured in corresponding puromycin media until two days before the experiments, the cells were cultured in media without puromycin.

2.3 Western Blot Analysis

Cell lysates were treated with ConA-sepharose beads overnight followed by centrifugation to remove cadherin-bound β-catenin. Total Cell lysates and non-membrane bound cell lysates were separated by SDS PAGE and transferred to nitrocellulose membranes. Western blot analysis was performed using primary antibodies against LRH1 (Abcam, ab18293), cyclin D1 (Santa Cruz, sc-8396), cyclin E1 (Santa Cruz, sc-247), calpain1 (Santa Cruz, sc-7531), β-catenin (Cell signaling Technology, #9562), estrogen receptor alpha (ERα) (Santa Cruz, sc-7207). Protein bands were visualized by IRDye® 680RD Infrared Dye and IRDye® 800CW Infrared Dye and exposed on Odyssey image system (LI-COR).

2.4 MTT Assay

PC cells (vectors vs. LRH1 overexpressing Capan-1 and AsPC-1) (1.7×10^4 cells per well) were seeded in 24 well plates and cultured for 4 days. Cells were incubated with MTT solution (Sigma-Aldrich) in medium (10% v/v) at 37 °C for 3 h. Then 0.1 N HCl isopropanol was added. Plates were analyzed daily using a plate reader at a wavelength of 570 and 690 nm, respectively. The background absorbance of multiwell plates measured at 690 nm was subtracted from the measurement at 570 nm.

2.5 Subcutaneous tumor model[17]

Athymic BALB/c female nude mice (Charles River laboratory) were housed in laminar flow cabinets under specific pathogen-free conditions and used at 6–8 weeks of age. All animal protocols were approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital. Vectors and LRH1 stable Capan-1 cells in exponential growth phase were harvested, washed, and resuspended in Ca^{2+}/Mg^{2+}-free HBSS to the desired cell concentration. Cell viability was determined by trypan blue exclusion and single-cell suspensions of >90% viability was used. In the subcutaneous (s.c.) model, tumor cells (5×10^6 in 0.2 mL of HBSS) were injected subcutaneously into the left (vector) and right (LRH1) shoulder of the nude mice (n = 7), respectively. The mice were sacrificed after 2 weeks. The tumors were harvested, weighed and fixed with 10% neutral buffered formalin. To detect nuclear pleomorphism, the section was stained with H&E. IHC for cell proliferation marker Ki67 and vascular proliferation marker CD34 (Cell Signaling Technology, #9449 and #3569, respectively) was performed on 4-μm sections of s.c. tumors. The Ki67 Index (percentage of Ki67-positive pancreatic cancer cell nuclei) was determined by counting positive and negative staining nuclei.

2.6 Immunohistochemistry (IHC)

Tissue specimens from 18 patients with stage I/II pancreatic ductal adenocarcinoma were studied. The study was approved by the Institutional Review Board of Lifespan Rhode Island and The Miriam Hospital. Immunohistochemical staining was conducted on 4-μm formalin-fixed paraffin-embedded (FFPE) unstained sections using antibodies to LRH1 (Abcam, ab18293), cyclin D1 (Cell Signaling Technologym #2978), cyclin E1 (Abcam, ab135380), and calpain1 (Abcam, ab39171). The sections were deparaffinized in xylene and rehydrated in a descending ethanol gradient. Antigen retrieval was performed using Citric Acid Based Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA) in a microwave pressure cooker (Nordic Ware, Minneapolis, MN) for 6 minutes at full power, followed by a 30 minutes cool down process. Endogenous peroxidase activity was quenched by a 30-min treatment with 3% hydrogen peroxide in methanol. The remaining steps of the staining procedure, including blocking, secondary antibody incubation, and ABC reagent

incubation, were performed using the VECTASTAIN Elite ABC Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. Primary antibodies were diluted in TBS/0.1% Tween-20 with 5% normal goat serum and were incubated at 4°C overnight. Color development was done using DAB Tablet (Wako Chemicals, Richmond, VA) as a substrate per manufacturer's instruction. Finally, the sections were counterstained by hematoxylin, dehydrated in an ascending ethanol gradient, and mounted with VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA).

2.7 Statistical analysis

All obtained data were calculated and expressed as mean ± SD. Statistical analyses were performed with the two-tailed Student's or paired t-test using SPSS software. A $p<0.05$ was considered statistically significant.

3. RESULTS

3.1 Expression of LRH1 in PC cells cultured *in vitro*

Western blot detected a band of approximately 57 kDa (predicted molecular weight of LRH1 is 61 kDa). LRH1 was highly expressed in MIA PaCa-2 and to a lesser degree in Hs766T PC cells, compared with HPDE cells (negative control), as well as HepG2 and HEK-293 cells transfected with pcDNA3-LRH1 (positive controls) (Fig. 1A). LRH1 protein was undetectable/very weak in AsPC-1, BxPC-3, Capan-1, HPAF-II, PL45 and Panc-1 cells. Because of the apparent absence of the LRH1 receptor in these cells, we used AsPC-1 and Capan-1 to establish stable cell lines for further experiments.

3.2 LRH1 upregulates downstream target genes cyclin D1/E1, ERα and Calpain1

We examined the potential mechanisms of LRH1 involvement in PC by measuring its downstream target genes. Western blot analysis revealed that cyclin D1 and cyclin E1 (full-length [FL] and truncated T1/T2 isoforms) were upregulated by LRH1 in stable PC cells (Fig. 1A). Cyclin E is converted to T1/T2 by calpain1 through post-translational processing. LRH1-mediated cyclin E T1/T2 overexpression was attributed to upregulation of ERα—Calpain1 signaling (Fig. 1B).

3.3 LRH1 promotes PC cell proliferation

LRH1 overexpression resulted in a significant increase in PC cell proliferation compared with the control (Fig. 2). Both AspC-1 and Capan-1 cells expressing LRH1 obtained a selective growth advantage and presented a higher proliferation rate than the control.

3.4 Tumorigenicity of LRH1 *in vivo*

In vitro observations suggest that LRH1 expression is important for PC cell proliferation. Therefore, we explored its oncogenic role using an *in vivo* model (Fig. 3). We measured the resulting tumor growth in the immune deficient mice after injection of parental and LRH1-transfected PC cells. Following introduction and expression of LRH1, Capan-1 cells generated significantly enhanced s.c. tumor growth characterized by a higher tumor weight compared to vectors (895 ± 135 mg vs. 514 ± 151 mg, $p<0.01$) (Fig. 3A). Histologically, in the tissue of s.c. tumor derived from LRH1 expressing cells was characterized by hyperchromatic, pleomorphic and irregular nuclei with prominent nucleoli and abundance of large cells with clear cytoplasm reminiscent of "signet ring" cells (Fig. 3B). The tumors derived from LRH1 expressing cells displayed a higher Ki67 Index (27.9% vs. 18.9%, $p<0.0001$) (Fig. 3C) and formed highly vascularized tissue compared with tumor from control cells as shown by enhanced CD34 staining of endothelial cells depicted by a representative example in Fig. 3D (n=7).

3.5 Expression of LRH1, cyclin D1, cyclin E1, and calpain1 in patients with PC

We hypothesize that downstream target genes are activated and upregulated corresponding to LRH1 overexpression in PC patients. The expression profiling of LRH1-related genes in human pathologic specimens was examined. LRH1 was overexpressed in 15/18 (83.3%) of tumors in PC patients (Fig. 4). Consistent with the *in vitro* findings of LRH1 overexpression in PC cell lines, cyclin D1, cyclin E1, and calpain1 were also overexpressed in tumorous tissues as well compared with adjacent normal pancreas derived from PC patients (Fig. 4).

4. DISCUSSION

We have found that LRH1 overexpression enhanced upregulation of downstream targets and contributed to cell proliferation, as well as tumor formation and angiogenesis in nude mice. Based on these observations, LRH1 is proposed to act as a driving factor for PC growth as detailed by the diagram presented in Fig. 5.

LRH1 promotes PC cell proliferation by inducing G1 cyclin D1/E1 and thus cell cycle transition from G1 to S phase.[4] Cyclins promote DNA synthesis by initiating cyclin-dependent kinase activation, retinoblastoma phosphorylation and E2F transcriptional factors activation. LRH1 upregulates the expression of downstream targets (cyclin D1/E1) in PC cells, which is also consistent with the results of previous studies.[4, 5, 11] The cell cycle regulators cyclin D1/E1 are frequently overexpressed in gastrointestinal tumors [11] and contribute to oncogenesis in animal models.[16]

LRH1 upregulates the expression of cyclin E truncated T1/T2 isoforms in PC cells. Cyclin E is up-regulated in a variety of malignancies such as breast cancer [6] and melanoma.[2] In tumors, cyclin E deregulation produces five low molecular weight (LMW) isoforms via post-translational processing by calpain1/2. LMW cyclin E (LMW-E), which is not found in normal tissue, caused genomic instability, induced resistance to p21/p27-mediated apoptosis and anti-estrogen therapy, and correlated with decreased survival of patients with breast cancer.[6] Both primary tumor formation and metastasis were increased in LMW-E transgenic mice compared to the full-length cyclin E (EL). Moreover, LMW-E selects for spontaneous inactivation of the ARF-p53 pathway by loss of heterozygosity, blocking its protective checkpoint function and accelerating progression to malignancy. LMW-E is also overexpressed in metastatic melanoma and primary invasive melanoma, but not in benign nevi. Overexpression of cyclin E T1/T2 generated more angiogenic melanoma with prominent perineural invasion and displayed as a large increase in lung metastases compared with EL.[2] Using frozen tissue samples from PC patients, we detected overexpression of LRH1 and cyclin E T2 in tumors compared to the normal pancreas. The results support the critical involvement of upregulated cyclin E T2 signaling in contributing to tumor growth and angiogenesis of PC.

Structure-based discovery of LRH1 antagonists has identified ligands that inhibit LRH1 transcriptional activity and diminish expression of the receptor's target genes.[3] In this regard, a number of potential LRH1 antagonists are under investigation for analyses of the receptor's biological mechanisms.

In summary, our study revealed that overexpressed LRH1 promotes cell proliferation, tumor formation and angiogenesis. Our observations demonstrate that the LRH1 receptor may play a role in driving tumor growth of PC (Fig. 5).

Acknowledgments

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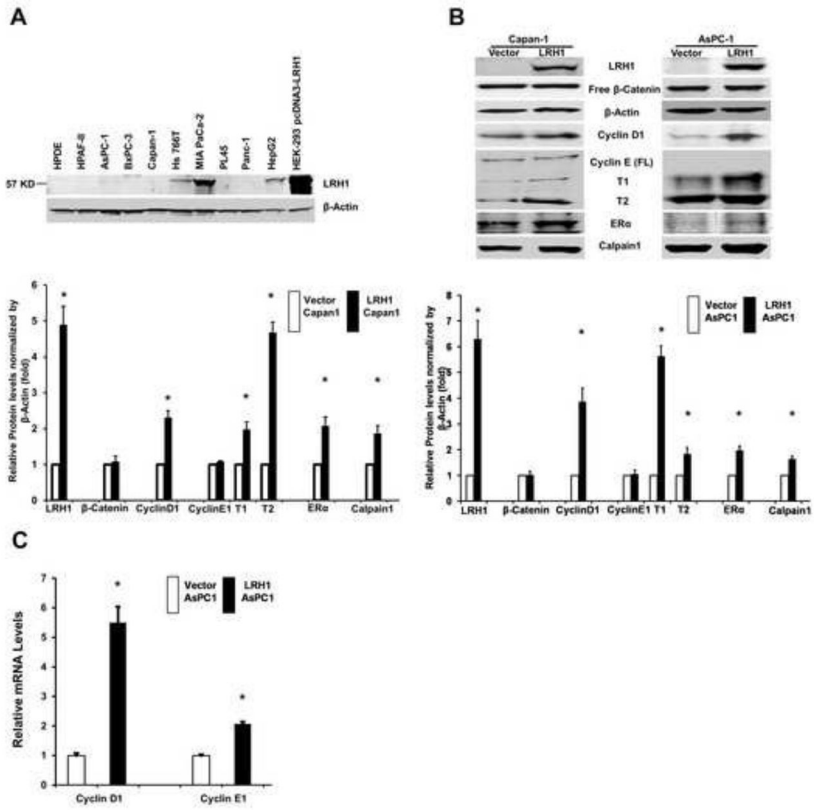

Fig. 1.

LRH1 upregulated downstream target genes in PC. (**A**) Endogenous expression of LRH1 in PC cell lines. LRH1 was highly expressed in MIA PaCa-2 and Hs766T, compared with HPDE (negative control), as well as HepG2 and HEK-293 cells transfected with pcDNA3-LRH1 (positive controls). (**B**) Expression levels of cyclin D1 and cyclin E1 truncated T1/T2 isoforms were significantly upregulated in LRH1 overexpressing stable Capan-1 and AsPC-1 cells than in controls. Cyclin E1 (FL), full length cyclin E1. (**C**) The mRNA levels of cyclin D1/E1 were upregulated in PC cell line.

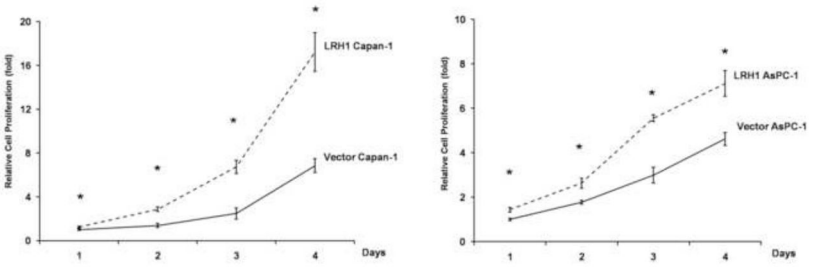

Fig. 2.

Growth curves of stable PC cells with LRH1 overexpression. Accelerated cell growth was observed in LRH1 stable transfected Capan-1 and AsPC-1 cells than in controls (Student t test, 2-tailed, *p<0.01).

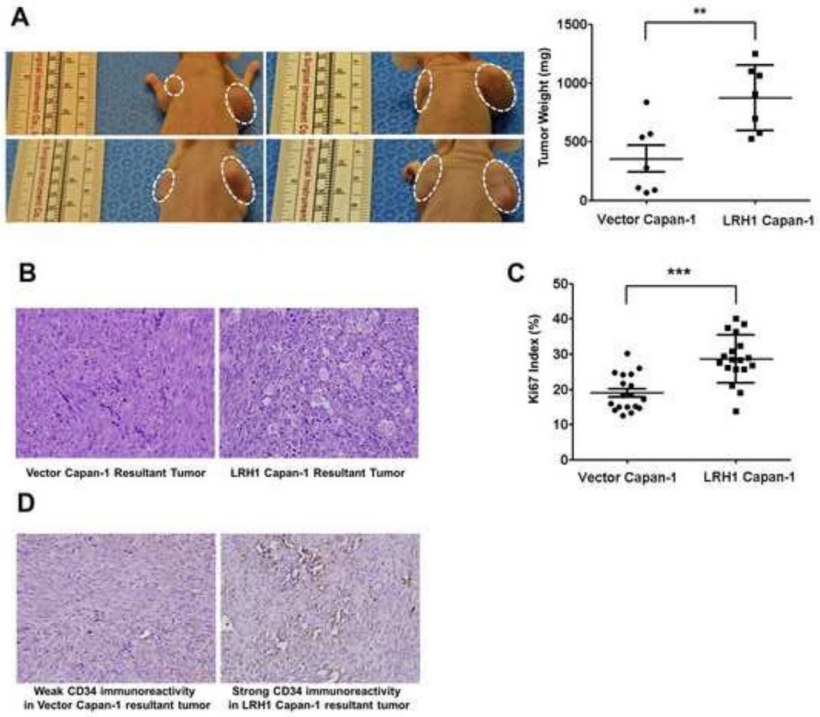

Fig. 3.

LRH1 promoted PC tumor growth in nude mice. (**A**) Representative established subcutaneous (s.c.) tumors by inoculation of 1×10^7 Capan-1 cells transfected with LRH1 or vector control. Right shoulder tumors were derived from LRH1-transfected cells, left shoulder tumors were derived from vector control cells. Tumor weights were analyzed within 3 weeks after inoculation of Capan-1 cells stably transfected with LRH1 or vectors (n=7). Compared to vectors, LRH1 expressing stable Capan-1 cells produced much larger tumors (paired t test, 2-tailed, $^{**}p<0.01$). Vertical bars, standard error. (**B**) H&E staining of two subcutaneous tumors. (**C**) Proliferation Index as measured by positive Ki67 staining of tumor nuclei (Student t test, 2-tailed, $^{***}p<0.0001$). (**D**) Examples of enhanced CD34 expression in endothelial indicating increased vascularity in two LRH1 induced tumors.

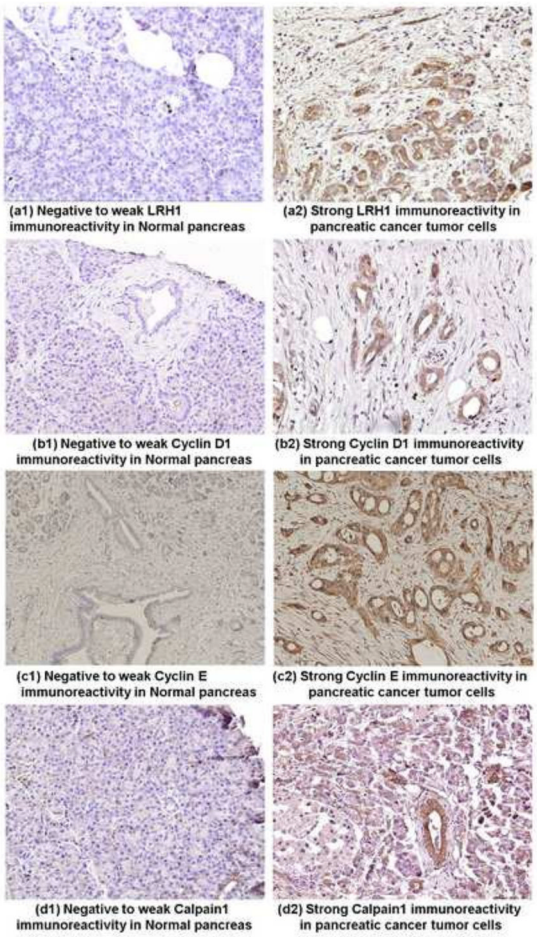

Fig. 4.

Representative examples of LRH1 related genes expression in PC. Weak-negative staining for LRH1 (a1), cyclin D1 (b1), cyclin E1 (c1), and calpain1 (d1) was observed for all components of normal exocrine pancreas. In human PC tumor cells, elevated levels of LRH1 (a2), cyclin D1 (b2), cyclin E1 (c2), and calpain1 (d2) were detected. The original magnification (×40) is specified.

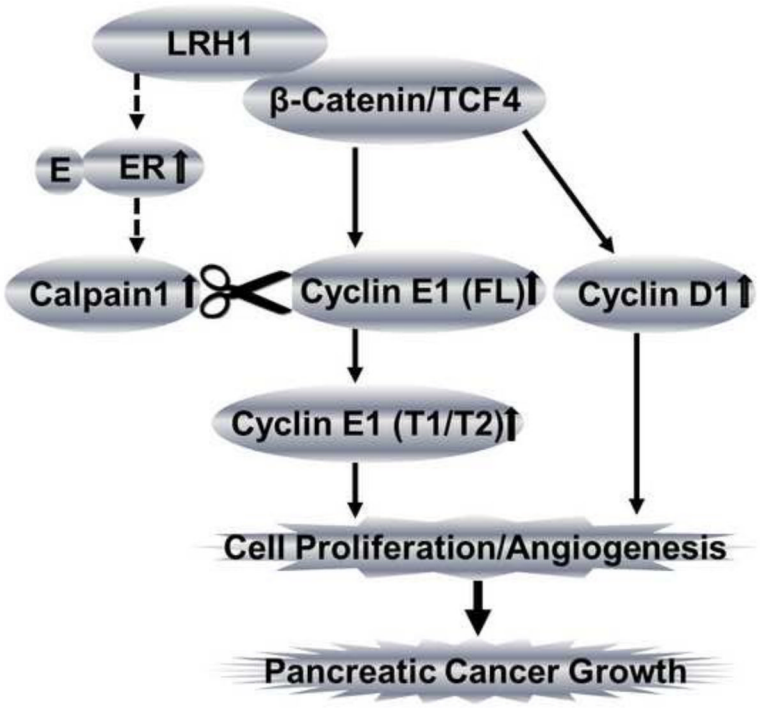

Fig. 5.

Hypothesized oncogenic role of LRH1 in PC. We suggest that LRH1 promotes PC tumor growth and angiogenesis by upregulating cyclin D1/E1. In combination with estrogen (E) and estrogen receptor (ER), LRH1 acts to upregulate caplain1. Calapin1 is known to convert full-length cyclin E1 into truncated T1/T2 isoforms through post-translational processing [2, 6]. These isoforms may exhibit enhanced oncogenic and angiogenic properties [2, 6].