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XPLN is modulated by HDAC inhibitors and negatively regulates SPARC expression by targeting mTORC2 in human lung fibroblasts

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ABSTRACT

Pathogenesis of idiopathic pulmonary fibrosis (IPF) remains unclear. Secreted protein acidic and rich in cysteine (SPARC) is a matricellular protein that participates in the assembly and turnover of the extracellular matrix, whose expression is regulated by transforming growth factor (TGF)-β1 through activation of mammalian target of rapamycin complex 2 (mTORC2). Exchange factor found in platelets, leukemic, and neuronal tissues (XPLN) is an endogenous inhibitor of mTORC2. However, whether XPLN modulates SPARC expression remains unknown. Herein, we investigated the regulatory mechanisms of XPLN in human lung fibroblasts. Effect of XPLN on mTORC2 activity was evaluated by silencing XPLN in human foetal lung fibroblasts (HFL-1 cells), using small interfering RNA. SPARC expression was quantified by quantitative real-time RT-PCR and western blotting. Fibroblasts were treated with TGF-β1, histone deacetylase (HDAC) inhibitors, entinostat, or vorinostat, to assess their effects on XPLN expression. Moreover, the effect of mTORC1 inhibition on SPARC and XPLN was examined. XPLN depletion stimulated SPARC expression and Akt phosphorylation on Ser473. TGF-β1 treatment down-regulated XPLN via Smad 2/3. *XPLN* mRNA expression was up-regulated upon treatment with HDAC inhibitors in a concentration-dependent manner, and TGF-β1-induced SPARC expression was reversed by entinostat treatment. mTORC1 inhibition by rapamycin and Raptor depletion stimulated SPARC expression. In conclusion, this is the first study describing the involvement of XPLN in the regulation of SPARC. These findings may help uncover the regulatory mechanisms of the mTORC2-SPARC axis. The up-regulation of XPLN by HDAC inhibitors may be a novel therapeutic approach in patients with IPF.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic disease characterised by progressive scarring of the lung parenchyma and the histopathological pattern of usual interstitial pneumonia [1]. The course of the disease progression is heterogeneous and difficult to predict. However, it generally involves progressive and inexorable lung deterioration with a median survival time of 2.5–3.5 years following diagnosis, and curative therapy to quell ongoing fibrosis is currently unavailable [2]. Although the pathological processes underlying disease progression are not fully understood, recent evidence suggests a degenerative process of alveolar epithelial cell injury and deregulated repair, leading to aberrant expansion and persistent proliferation of myofibroblasts. The myofibroblasts secrete extracellular matrix (ECM) proteins, which are linked to the

Abbreviations: ADHP, 10-Acetyl-3,7-dihydroxyphenoxazine; DAPI, 4′,6-diamidino-2-phenylindole; ECM, extracellular matrix; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GEF, guanine nucleotide exchange factor; H_2O_2, hydrogen peroxide; HDAC, histone deacetylase; HFL-1, human foetal lung fibroblast; HRP, horseradish peroxidase; IgG, immunoglobulin G; IPF, idiopathic pulmonary fibrosis; MAPK, mitogen-activated protein kinase; mTORC, mammalian target of rapamycin complex; PBS, phosphate-buffered saline; PI3K, phosphoinositide 3-kinase; qRT-PCR, quantitative real-time reverse transcription PCR; RIPA buffer, radioimmunoprecipitation assay buffer; SAHA, suberoylanilide hydroxamic acid; siRNA, small interfering RNA; SPARC, secreted protein acidic and rich in cysteine; TGF-β1, transforming growth factor-β1; XPLN, exchange factor found in platelets, leukemic, and neuronal tissues.

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thickening of the alveolar walls, remodelling of the lung architecture, and impairment of lung function.

Mammalian target of rapamycin (mTOR) complexes are serine/threonine kinases that control cellular survival, growth, and metabolism [3,4]. It is increasingly apparent that deregulation of the mTOR pathway occurs in common diseases, including cancer and diabetes, emphasizing the importance of identifying and understanding the function of components of the mTOR signalling network [5]. In mammalian cells, mTOR resides in two physically and functionally distinct multi-protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Rapamycin and related rapalogs are known inhibitors of mTORC1, but do not generally directly inhibit mTORC2 [6]. The mTORC2 complex mediates the phosphorylation of Akt on Ser473 and, thereby, activates the downstream Akt pathway, which regulates multiple cellular responses [7]. Recently, dual mTOR inhibitors such as MLN0128 or pp242, which simultaneously inhibit both mTORC1 and mTORC2, have been developed [8]. Chang and colleagues demonstrated that they inhibit pro-fibrotic components, which are downstream of mTORC2, exhibiting anti-fibrotic properties [6].

Secreted protein acidic and rich in cysteine (SPARC) is one of the molecules related to fibrosis formation, whose expression is controlled by transforming growth factor (TGF)-β-mediated activation of mTORC2 signal transduction [6]. It is a 43-kDa matricellular protein secreted into the extracellular space, where it binds directly to ECM proteins such as collagen, participating in ECM assembly and turnover, but does not have a structural function in the ECM [9,10]. Several studies showed that inhibition of SPARC expression decreases fibrosis [11]. Although SPARC expression is undetectable in adult human lungs, it increases in patients with pulmonary fibrosis [12]. Chang and colleagues demonstrated that SPARC is overexpressed in IPF fibroblasts [13]. However, to investigate the direct effect of mTORC2 on SPARC expression, a selective inhibitor targeting mTORC2 is needed, although it has yet to be developed.

*E*xchange factor found in *p*latelets, *l*eukemic, and *n*euronal tissues (XPLN), which is a guanine nucleotide exchange factor (GEF) for Rho GTPases, has recently been identified as an interacting partner of mTOR. XPLN reportedly interacts with mTORC2, but not with mTORC1, and this interaction is dependent on Rictor [14]. While an mTORC2 inhibitor is currently unavailable, determining the effect of an endogenous mTORC2 inhibitor on the downstream signalling appears to be a favourable surrogate strategy.

Here, we hypothesised that modulation of XPLN could alter TGF-β1-induced SPARC expression via the down-regulation of mTORC2 activity and affect the architectural changes in fibrosis. To test this hypothesis, we silenced XPLN, using small interfering RNA (siRNA), and evaluated the effect on SPARC expression in human lung fibroblasts. We also investigated the modulation of XPLN expression by TGF-β1. Furthermore, we also tested whether histone deacetylase (HDAC) inhibitors affect TGF-β1-induced SPARC expression through modulation of XPLN, and investigated the effect of mTORC1 inhibition on SPARC and XPLN expression. Findings from our preliminary studies were reported in an abstract form at a meeting of the American Thoracic Society [15].

2. Materials and methods

2.1. Chemicals and reagents

TGF-β1 was purchased from R&D Systems Inc. (Minneapolis, MN, USA). The antibody against XPLN was purchased from Abnova (Taipei, Taiwan). Antibodies against SPARC, Akt, and phosphorylated-Akt were purchased from Cell Signalling Technology, Inc. (Danvers, MA, USA). The selective Akt inhibitor (MK-2206 2HCl), p38 mitogen-activated protein kinase (MAPK) inhibitor (SB202190), and phosphoinositide 3-kinase (PI3K) inhibitor (LY294002) were purchased from Selleck Chemicals (Houston, TX, USA). A mouse monoclonal anti-β-actin antibody (clone AC-74), rapamycin, and pp242 were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA).

Biotin-conjugated anti-rabbit immunoglobulin G (IgG) was purchased from Dako (Glostrup, Denmark). Alexa Fluor 488-conjugated streptavidin was purchased from Life Technologies (Carlsbad, CA, USA). VECTASHIELD Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Vector Laboratories, Inc. (Burlingame, CA, USA). Radio-Immunoprecipitation Assay (RIPA) Buffer and horseradish peroxidase (HRP) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Entinostat (MS-275), vorinostat (SAHA; suberoylanilide hydroxamic acid) and 10-Acetyl-3,7-dihydroxyphenoxazine (ADHP) were purchased from Cayman Chemical (Ann Arbor, MI, USA).

2.2. Cell culture

The widely used human foetal lung fibroblast strain (HFL-1) was obtained from the RIKEN BRC CELL BANK (RCB0521; Ibaraki, Japan). HFL-1 cells were cultured in 75-cm^2 Nunc EasyFlask (Thermo Fisher Scientific Inc., Waltham, MA, USA) with Roswell Park Memorial Institute (RPMI)-1640 medium, supplemented with 10% foetal calf serum, 100 μg/mL penicillin, and 250 μg/mL streptomycin sulphate (Wako Pure Chemical Industries, Ltd.). Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Sub-confluent cells were serum-starved for 24 h and then, used for the subsequent experiments. HFL-1 cells between the 14th and 18th passage were used.

2.3. RNA extraction and quantitative real-time reverse transcription PCR

Total RNA was extracted from cultured HFL-1 cells, using ISOGEN reagents with spin columns (Nippon Gene, Tokyo, Japan), and converted to complementary DNA, as described elsewhere [16]. *SPARC*, *XPLN*, and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA expression was measured by quantitative real-time reverse transcription PCR (qRT-PCR) using TaqMan Gene Expression Assay (Applied Biosystems Japan, Ltd., Tokyo, Japan). THUNDERBIRD Probe qPCR Mix was purchased from Toyobo (Osaka, Japan). Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems) was used. The relative amounts of *SPARC* and *XPLN* mRNA in the original samples were normalised against the housekeeping gene, *GAPDH,* mRNA expression levels.

2.4. Western blot analysis

Cells from sub-confluent cultures were washed twice with phosphate-buffered saline (PBS), scraped into PBS, and pelleted by centrifugation. Whole-cell lysates were prepared in RIPA buffer for immunoblotting experiments. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol and by using bovine serum albumin as a standard. Samples were resolved by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in Tris-buffered saline (0.15 M NaCl, 0.05 M Tris-HCl [pH 8.0], and 0.05% [vol/vol] Tween 20) containing 5% skim milk and incubated with the indicated antibodies at the manufacturer's recommended dilutions. An anti-β-actin antibody was used to confirm equal protein loading and