Transcription regulation of human phospholipid scramblase 1 (hPLSCR1) by a Sry related high mobility group transcription factor, S-SOX5

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Research Article

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Abstract

Objectives Human phospholipid scramblase 1 (hPLSCR1) is a multifunctional protein so it is vital to understand the regulation of its expression and transcription factors play critical role in regulating any gene.

Results The transcription factor (TF) prediction tool ConSite identified one S-SOX5 binding site on the promoter region of hPLSCR1. Luciferase assays determined the increase in hPLSCR1 promoter activity with the increase in S-SOX5 concentration in a dose dependent manner. The deletion and the site-directed mutagenesis constructs exhibited a decrease in the hPLSCR1 promoter activity as compared to control. Also, transcriptional regulation of hPLSCR1 by S-SOX5 was checked by western blotting using S-SOX5 and hPLSCR1 specific antibodies. Further, Chromatin Immunoprecipitation (ChIP) assay confirmed the in-vivo interaction of S-SOX5 with hPLSCR1 promoter.

Conclusions The expression of hPLSCR1 in HEK293T cells was shown to be upregulated by one S-SOX5 binding site present on the hPLSCR1 promoter suggesting that S-SOX5 as the potent regulator of hPLSCR1 expression.

Introduction

The human phospholipid scramblases (hPLSCRs) are a group of homologs that are ATP independent and Ca^{2+} dependent lipid transporters mediating the bi-directional transport of PLs across the cell membrane (Sahu et al. 2007). hPLSCRs constitute a family of five homologous proteins named hPLSCR1–hPLSCR5. Among all the hPLSCRs, hPLSCR1 is the most extensively studied scramblase and exhibited 59, 47, and 46% sequence homology to hPLSCR2, hPLSCR3, and hPLSCR4 respectively (Wiedmer et al. 2000). hPLSCR1 is expressed in all tissues except the brain and is localized to PM when palmitoylated and nucleus when un-palmitoylated (Wiedmer et al. 2003). hPLSCR1 is a 37 kDa multifunctional protein known to regulate many vital functions in various cellular processes such as autoimmune diseases (Suzuki et al. 2010), cell proliferation and differentiation (Zhou et al. 2002), cell signaling (Nanjundan et al. 2003; Amir-Moazami et al. 2008), anti-viral defense (Dong et al. 2004) and apoptosis (Frasch et al. 2000; Yu et al. 2003; Bailey et al. 2005). It also plays an active role in cancer pathways like colorectal cancer, leukemia, hepato-pancreatic cancer, breast cancer, and ovarian cancer (Silverman et al. 2002; Kuo et al. 2011; Behuria et al. 2022). hPLSCR1 acted as TF and up-regulated the expression of the IP3R1 gene by directly binding to its 5'-promoter region (Zhou et al. 2005). The expression of hPLSCR1 was found to be mediated by interferon-α at the transcriptional level. (Zhou et al. 2000). Interferon inducible hPLSCR1 plays an important role in anti-viral defence against encephelomyocarditis virus, vesicular stomatitis virus (Dong et al. 2004) and HTLV1 virus (Kusano et al. 2012).

Overexpression of hPLSCR1 regulates apoptosis in Chinese hamster ovary cells (Yu et al. 2003) and suppresses tumorigenesis in HEYIB cell lines (Silverman et al. 2002). PKC-δ enhanced the hPLSCR1

scramblase activity and surface PS exposure during cell stimulation and apoptosis (Frasch et al. 2000). However, the reason for overexpression of hPLSCR1 is not yet elucidated. hPLSCR1 is involved in several cellular and cancer pathways, so the expression of such a multifunctional protein should be under stringent regulation. TF plays a vital role in the transcriptional regulation of the expression of any gene. Transcriptional regulation helps in a better understanding of the function and expression of a protein. Our two reports suggested transcriptional regulation of hPLSCR1, where the expression of hPLSCR1 was down-regulated by Snail (Francis et al. 2014) and up-regulated by c-Myc (Vinnakota et al. 2016). In addition to c-Myc and Snail, there could be several other TFs that might regulate the expression of hPLSCR1. SOX5 plays an essential role in gliogenesis, nervous system development, neurogenesis, organogenesis, and chondrogenesis (Kwan et al. 2008; Liu et al. 2015; Lee et al. 2017; Stevanovic et al. 2021). SOX5 transcriptionally regulates the expression of SPAG6 and SPAG16 genes, which are involved in the formation of motile cilia/flagella (Kiselak et al. 2010; Zhang et al. 2017). Sox5 controls the expression of the catsper1 gene in association with SOX9; catsper1 plays a central role in male fertility (Mata-Rocha et al. 2014). Contrary to this, SOX5 expression levels contribute to various cancers like lung adenocarcinoma, prostate cancer, nasopharyngeal carcinoma (Huang et al. 2008; Chen et al. 2018; Hu et al. 2018; Grimm et al. 2020). The SOX5 haploinsufficiency causes Lamb-Shaffer syndrome (LAMSHF), a neurodevelopmental disorder characterized by intellectual disability, language and motor impairment, and distinct facial features (Zawerton et al. 2020). Apart from this, SOX5 is involved in many diseases like type 2 diabetes, back pain, and rheumatoid arthritis (RA) (Feng et al. 2016; Axelsson et al. 2017; Suri et al. 2018; Shi et al. 2018).

TF prediction tool ConSite (Sandelin et al. 2004) proposed one S-SOX5 binding site (SBS) in the hPLSCR1 promoter at a 100% cutoff. According to the literature and results from prediction tools, we assumed that S-SOX5 might transcriptionally regulate the expression of hPLSCR1. A few experiments like transcription reporter assays, deletion and mutation studies of S-SOX5, and ChIP assay were conducted to prove our assumption. The results indicated that S-SOX5 TF transcriptionally regulates the expression of hPLCR1. This study about the transcriptional regulation of hPLSCR1 will provide more information about the role of SOX5 in hPLSCR1 expression and function.

Materials And Methods

Cell culture

HEK293T cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) high glucose media (Thermo Scientific USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA) and 1% Antibiotic-Antimycotic (Thermo Fisher Scientific, USA). The cells were grown at 37°C and 5% CO_2 in an incubator.

Bioinformatic analysis

The promoter of hPLSCR1 (~1.52 kb) was screened at a 100% cutoff for the presence of putative transcription factor binding sites using a TF prediction tool called ConSite (http://consite.genereg.net/). Only one SBS was predicted on the promoter region of hPLSCR1. The results were then checked with other TF prediction tools like Tfsitescan (http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl) and PROMO (http://alggen.lsi.upc.es/cgibin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3).

Preparation of hPLSCR1 promoter reporter and S-SOX5 expression constructs

The promoter of hPLSCR1 (1.525 kb) was PCR amplified from human genomic DNA using Phusion high fidelity polymerase (New England Biolabs, USA). hPLSCR1 specific primers were designed containing sites of *Kpn*I and *Xho*I restriction enzymes (Supplementary Table 1). The amplified fragments were resolved on a 1% agarose gel and visualized under a UV transilluminator. The PCR product was then digested with *Kpn*I and *Xho*I enzymes and cloned in pGL3 basic vector. The clone was further confirmed by DNA sequencing. To amplify S-SOX5 cDNA, SOX5-specific primers were designed containing sites for *Bam*HI and *Eco*RI restriction enzymes (Supplementary Table 1). The PCR amplified fragments were run on a 1% agarose gel and visualized under UV transilluminator. The amplified fragment was digested with restriction enzymes and was specifically cloned between *Bam*HI and *Eco*RI restriction sites of pcDNA3.1 expression vector. Finally, the clone was further confirmed by DNA sequencing.

Preparation of deletion and site-directed mutagenesis constructs

One deletion construct lacking the SBS region was prepared by employing hPLSCR1 F.P. and Deletion R.P. primers (Supplementary Table 1). The amplified DNA fragment was cloned between the *Kpn*I and *Xho*I sites of pGL3 basic vector. Point mutations were induced in SBS on hPLSCR1 promoter by site-directed mutagenesis. The SDMSC1 F.P. and SDMSC1 R.P. primers (Supplementary Table 1) carrying mutations in SBS were designed. The PCR product was then checked for the presence of desired size band (6.343 kb) on 1% agarose gel and digested with *Dpn*I restriction enzyme (New England Biolabs, USA) overnight. The resulting digested product was transformed into DH5α cells and screened for the positive clones. The clones were then subjected to DNA sequencing for mutation confirmation.

Transient transfections and promoter reporter assays

HEK293T cells (60–70% confluent) were transfected with hPLSCR1 promoter reporter construct, S-SOX5 expression plasmid, and β-galactosidase plasmid using the calcium phosphate method (Kingston et al. 2003) in 24 well plates. After 24h of transfection, cells were lysed in cell lysis buffer [100 mM phosphate

buffer (pH 7.8), 1 mM DTT, 0.1% (w/v) Triton-×100] and luciferase activity was assayed in assay buffer [100 mM Tris-acetate (pH 7.8), 10 mM magnesium acetate, 1 mM EDTA, 2 mM ATP (Bio Basic, Canada) and 1 mM D-luciferin (Abcam, UK). The relative luminescence readings were measured in a luminometer (Berthold Detection Systems, Germany). Cells were simultaneously assayed for β-galactosidase activity by measuring the absorbance at 420 nm after incubating with the substrate ONPG (Sigma Aldrich, USA) at 37 °C for 10–15 min.

Western blot analysis

HEK293T cells were transfected with S-SOX5 expression plasmid and pcDNA3.1 as a control plasmid. 24 h post-transfection, cells were lysed in RIPA buffer [150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl (pH 7.4), 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM PMSF and protease inhibitor cocktail (Sigma-Aldrich, USA)] and the total protein was estimated using BCA kit (Sigma-Aldrich, USA). 30–50 µg of total cell lysate was loaded on 12% SDS-PAGE and then transferred onto a nitrocellulose membrane (Pall life sciences, USA). The nitrocellulose membrane was then blocked for 2 h at 25 °C in blocking buffer [10 mM Tris-HCl (pH 7.6), 150 mM NaCl and 0.2% (v/v) Tween-20 with 3% (w/v) BSA]. The blots were incubated using primary goat anti-hPLSCR1 antibody (Santa Cruz, USA) and primary rabbit anti-SOX5 antibody (Santa Cruz, USA) at 4°C overnight. The resulting blot was washed thrice with TBST and probed with mouse anti-goat secondary antibody (Santacruz, USA) and mouse anti-rabbit secondary antibodies (Santacruz, USA), respectively. The blots were simultaneously probed for β-Actin using a mouse anti-Actin primary antibody. The immunoblots were developed using a chemiluminescence kit (Bio-Rad, USA).

Chromatin immunoprecipitation (ChIP) assays

ChIP assay was performed as per the method described earlier (Das et al. 2004). HEK293T cells were fixed with 1% (v/v) formaldehyde solution. The fixed cells were lysed and sheared to small fragments (~500 to 800 bp) by sonication (Branson 450 sonifier, USA). Anti-SOX5 antibody (5 µg) was used to pull down protein DNA complexes. The pull-down was carried out overnight at 4°C. An anti-IgG antibody (Sigma-Aldrich) was used as a negative control. The DNA, before pulled down was considered as positive control called Input. The region corresponding to SBS was amplified using CHIP Sox5 F.P. and CHIP Sox5 R.P. primers (Supplementary Table 1). PCR fragments were visualized on 1% agarose gel stained with ethidium bromide under a U.V. transilluminator.

Statistical analysis

All the experiments were performed at least three independent times, and an unpaired two-tailed t-test (Graph pad prism 3.0) was used to determine the statistical significance. P< 0.05 was considered to be statistically significant.

Results

Prediction of SOX5 TF and endogenous expression of hPLSCR1 and S-SOX5

TF prediction tool ConSite predicted binding sites for various TFs with varied functions, including HFH-3, C-FOS, HNF 3β, AML-1, Snail, SOX5, AML-1, and Spz-1 at 100 % cutoff on hPLSCR1 promoter (Fig. 1a). Based on this, we hypothesized that the hPLSCR1 promoter harbored only one SBS (Fig. 1a). The result of the western blot clearly showed the endogenous expression of S-SOX5 and hPLSCR1 in HEK293T cells (Fig. 1b). Anti-SOX5 and anti-hPLSCR1 antibodies were used to probe S-SOX5 and hPLSCR1, respectively whereas an anti-actin antibody was used to probe actin as an internal control (Fig. 1b).

S-SOX5 up-regulates the expression of hPLSCR1

ConSite predicted one SBS with a recognition sequence of GAACAAT (-340 to -347) on the promoter region of hPLSCR1 (Fig. 2a). Luciferase assays were carried out to determine the effect of the concentration of S-SOX5 on the expression of hPLSCR1 at the transcriptional level. For this, co-transfection of HEK cells was done with 0.10 ug hPLSCR1 promoter-reporter construct (pGL3-hPLSCR1) with increasing doses of S-SOX5 expression plasmid (pcDNA3-S-SOX5) ranging from 0.01 ug to 0.10 ug. The RLU values obtained from the luciferase assays indicated that the promoter activity of hPLSCR1 (pGL3-hPLSCR1) proportionately increased with an increase in the amount of pcDNA3-S-SOX5 plasmid (Fig.2b). The RLU values were normalized with β-galactosidase, which was used as transfection control. Further, the increase in the endogenous expression of hPLSCR1 with the increase in S-SOX5 expression was confirmed by western blot (Fig. 2c). These results indicated that S-SOX5 mediated the up-regulation of hPLSCR1 at the transcriptional level.

Analysis of S-SOX5 binding region by deletion construct

To determine the actual SBS in the promoter region of hPLSCR1, deletion construct (pGL3-DEL) lacking SBS was generated (Fig. 3a). The luciferase assays showed that the promoter activity of deletion construct (pGL3-DEL) was reduced by 44% when compared with the full-length promoter reporter construct (pGL3-hPLSCR1) when transfected with the expression construct (pcDNA3-S-SOX5) (Fig. 3b). The promoter activity of the deletion construct was almost equal to the full-length construct without transfection with pcDNA3-S-SOX5 construct (Fig. 3b). Further, the western blot analysis showed a

decrease in hPLSCR1 protein expression levels when cells were transfected with deletion construct as compared to control (Fig. 3c) and thus confirming S-SOX5 mediated up-regulation of hPLSCR1. Actin protein expression was used as an internal control in western blot assay for which the expression is same in all the samples (Fig. 3c). These results clearly showed that SBS is crucial for up-regulating the hPLSCR1 expression.

Confirmation of S-SOX5 binding site by Site directed mutagenesis

The actual SBS on the promoter of hPLSCR1 was confirmed by introducing point mutations in SBS where -GAAC**AA**T- was mutated to -GAAC**GG**T- (pGL3-SDM) (Fig. 3d). Luciferase assays revealed that mutated SBS (-347 GAACGGT -340) (pGL3-SDM) displayed a 56 % decrease in the promoter activity when compared to the wild type (pGL3- hPLSCR1) (Fig. 3e). This confirms that the predicted SBS on the promoter of hPLSCR1 is indeed the actual binding site for S-SOX5. Further, the expression of protein by western blot validated that the expression of endogenous hPLSCR1 was reduced when cells were transfected with pGL3-SDM construct in comparison to the wild type (Fig. 3f) where actin was represented as an internal control. Based on this, we hypothesized that SBS might help in regulating the expression of hPLSCR1.

In-vivo interaction of S-SOX5 with hPLSCR1 promoter

ChIP assay was performed to study the endogenous interaction of S-SOX5 with hPLSCR1 promoter at chromatin level. Formaldehyde cross-linked chromatin fragments of HEK cells were immunoprecipitated by SOX5 antibody and control IgG antibody. The DNA purified from the immunoprecipitated fragments was amplified by PCR using CHIP primers. An expected amplicon of 222 bp was obtained with DNA pulled down by SOX5 antibody (Fig. 4). Anti-IgG antibody was used as a negative control, and so no amplicon was detected with DNA which was pulled down by IgG antibody (Fig. 4). The total chromatin prior to immunoprecipitation called input was used as a positive control and amplified a band of 222 bp (Fig. 4). These results indicate the interaction of S-SOX5 with hPLSCR1 promoter at *in-vivo* level and showed that SBS is highly specific in nature.

Discussion

The SOX (SRY box-containing) group of TFs, share a high-mobility group (HMG) DNA-binding domain with mammalian Y-linked testis-determining gene (Sry) (wunderle et al. 1996; Ikeda et al. 2002). Sox proteins has been characterized into six subfamilies (wunderle et al. 1996). SOX5 belongs to the group D subfamily of Sox proteins having a consensus recognition sequence of 5’-AACAAT-3’ (Ikeda et al. 2002). The group D proteins were characterized by a highly conserved HMG box located at the C-terminal, and the other domains located at the N-terminal called lucine-zipper, coiled coil and Q box (Ikeda et al. 2002).

Human SOX5 was cloned from the human testis and is highly conserved with mouse Sox5 (Wunderle et al. 1996) which suggests that their functions may also be similar. SOX5 has two forms: short (S-SOX5) (48 kDa) and long (L-SOX5) (84 kDa), which further has many isoforms and variants (Ikeda et al. 2002). Preliminary bioinformatics analysis of hPLSCR1 promoter revealed the presence of many putative TF binding sites like Snail, Spz-1, HNF 3β, and AML-1 at 100% cutoff. However, only one SBS with a recognition sequence of -GAACAAT- was found at a 100% cutoff (Fig. 3a). Other TF prediction tools such as Tfsitescan and PROMO also proposed identical SBS on hPLSCR1 promoter. Based on this, we tried to discover the regulation of hPLSCR1 by S-SOX5. Our results undoubtedly indicate that S-SOX5 up-regulated the expression of hPLSCR1. The results supporting up-regulation of hPLSCR1 expression include (i) dose dependent studies exhibited an up-regulation of hPLSCR1 at transcriptional and translational levels (Fig. 2), (ii) deletion and mutation studies showed the importance of SBS (-347 GAACAAT -341) in transcriptionally regulating the expression of hPLSCR1 (Fig. 3) and (iii) ChIP assay confirmed the *in-vivo* interaction of S-SOX5 with hPLSCR1 promoter (Fig. 4).

hPLSCR1 was reported to play crucial role in apoptosis (Sivagnanam et al. 2017). The PM surface PS exposure is the hallmark of apoptosis (Sivagnanam et al. 2017). hPLSCR1 mediates apoptosis by interacting with proteins like PKC- δ and thus enhancing PS exposure (Frasch et al. 2000; Sivagnanam et al. 2017). The increased expression level of hPLSCR1 in CHO-K1 cell lines elevated the UV-induced apoptosis (Yu et al. 2003; Bailey et al. 2005). The UV-induced apoptosis increases primarily by augmentation of the intrinsic apoptotic pathway and not through the phosphorylation of hPLSCR1 by PKC-δ (Bailey et al. 2005). Overexpression of hPLSCR1 in CHO-K1 cells activated caspase-3 and stimulated the PS biosynthesis (Yu et al. 2003). On the other hand, the overexpression of hPLSCR1 showed an important role in various cancer types (Silverman et al. 2002; Kuo et al. 2011; Behuria et al. 2022). hPLSCR1 helps in blood cell differentiation. The Overexpression of PLSCR1 induces apoptosis and inhibits protective autophagy of NB4 leukemic cells and thus acts as a analytical marker for acute myelogenous leukemia (AML) (Behuria et al. 2022). hPLSCR1 is a regulatory protein in Colorectal Carcinoma, Hepato-Pancreatic Carcinoma, Ovarian Cancer, and breast cancer (Silverman et al. 2002; Kuo et al. 2011; Behuria et al. 2022). The overexpressed hPLSCR1 suppresses the tumorigenesis in HEY1B cell lines. Also, the tumor morphology was changed along with increased infiltration of macrophages and neutrophils (Silverman et al. 2002). SOX5 regulates the cell proliferation, migration and apoptosis in KSHV-infected cells (Yuan et al. 2021). Apart from apoptosis, SOX5 plays a vital role in solid tumors and metastasis (Huang et al. 2008; Chen et al. 2018; Hu et al. 2018; Grimm et al. 2020). SOX5 acts as inducer of angiogenesis in lung adenocarcinoma by inducing the expression of VEGF through STAT3 signalling (Chen et al. 2018). The increased SOX5 expression activated the Twist1 gene, which initiated TGF-β-induced EMT. Thus, targeting SOX5 could inhibit prostate cancer progression (Hu et al. 2018). SOX5 enhances the progression of nasopharyngeal carcinoma by down-regulating the expression of the SPARC gene (Huang et al. 2008). Transcriptional regulation is the one of the several possible mechanisms for the overexpression of hPLSCR1 during apoptosis and various cancer types.

Based on our results and literature reports, we hypothesized that regulation of hPLSCR1 gene by S-SOX5 might be one of the reasons for the overexpression of hPLSCR1 during the process of apoptosis and

various cancers. However, there may be several other TFs regulating the expression of hPLSCR1 like Snail and c-Myc (Francis et al. 2014; Vinnakota et al. 2016). In the present study, our focus was to decipher the role played by Sox5 in regulating hPLSCR1 expression. The details of Sox5 regulating the expression of hPLSCR1 during apoptosis, cancer, and cell differentiation are yet to be ascertained. Nevertheless, this is the first evidence to show transcriptional up-regulation of hPLSCR1 by S-SOX5.

Declarations

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Declaration of competing interest

The authors declare no competing interests.

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References

Amir-Moazami O, Alexia C, Charles N, Launay P, Monteiro RC, Benhamou M (2008) Phospholipid scramblase 1 modulates a selected set of IgE receptor-mediated mast cell responses through LAT-dependent pathway. J Biol Chem 283(37):25514-25523. https://doi.org/10.1074/jbc.M705320200

Axelsson AS, Mahdi T, Nenonen HA, Singh T, Hänzelmann S, Wendt A, Bagge A, Reinbothe TM, Millstein J, Yang X, Zhang B (2017) Sox5 regulates beta-cell phenotype and is reduced in type 2 diabetes. Nature communications 8(1):1-16. https://doi.org/10.1038/ncomms15652

Bailey K, Cook HW, McMaster CR (2005) The phospholipid scramblase PLSCR1 increases UV induced apoptosis primarily through the augmentation of the intrinsic apoptotic pathway and independent of direct phosphorylation by protein kinase C δ. Biochim Biophys Acta (BBA)-Molecular and Cell Biology of Lipids 1733(2-3):199-209. https://doi.org/10.1016/j.bbalip.2004.12.013

Behuria HG, Dash S, Sahu SK (2022) Phospholipid Scramblases: Role in Cancer Progression and Anticancer Therapeutics. Frontiers in Genetics 13. https://doi.org/10.3389/fgene.2022.875894

Chen X, Zheng Q, Li W, Lu Y, Ni Y, Ma L, Fu Y (2018) SOX5 induces lung adenocarcinoma angiogenesis by inducing the expression of VEGF through STAT3 signaling. OncoTargets and therapy 11:5733. https://doi.org/10.2147/OTT.S176533

Das PM, Ramachandran K, VanWert J, Singal R (2004) Chromatin immunoprecipitation assay. Biotechniques 37(6):961-969. https://doi.org/10.2144/04376RV01

Dong B, Zhou Q, Zhao J, Zhou A, Harty RN, Bose S, Banerjee A, Slee R, Guenther J, Williams BR, Wiedmer T (2004) Phospholipid scramblase 1 potentiates the antiviral activity of interferon. J Virol 78(17):8983-8993. https://doi.org/10.1128/JVI.78.17.8983-8993.2004

Feng X, Shi Y, Xu L, Peng Q, Wang F, Wang X, Sun W, Lu Y, Tsao BP, Zhang M, Tan W (2016) Modulation of IL-6 induced RANKL expression in arthritic synovium by a transcription factor SOX5. Scientific reports 6(1):1-10. https://doi.org/10.1038/srep32001

Francis VG, Padmanabhan P, Gummadi SN (2014) Snail interacts with hPLSCR1 promoter and down regulates its expression in IMR-32. Biochem Biophys Res Commun 450(1):172-177. https://doi.org/10.1016/j.bbrc.2014.05.094

Frasch SC, Henson PM, Kailey JM, Richter DA, Janes MS, Fadok VA, Bratton DL (2000) Regulation of phospholipid scramblase activity during apoptosis and cell activation by protein kinase Cδ. J Biol Chem 275(30):23065-23073. https://doi.org/10.1074/jbc.M003116200

Grimm D, Bauer J, Wise P, Krüger M, Simonsen U, Wehland M, Infanger M, Corydon TJ (2020) The role of SOX family members in solid tumours and metastasis. In Seminars in cancer biology 67:122-153. Academic Press. https://doi.org/10.1016/j.semcancer.2019.03.004

Hu J, Tian J, Zhu S, Sun L, Yu J, Tian H, Dong Q, Luo Q, Jiang N, Niu Y, Shang Z (2018) Sox5 contributes to prostate cancer metastasis and is a master regulator of TGF-β-induced epithelial mesenchymal transition through controlling Twist1 expression. British journal of cancer 118(1):88-97. https://doi.org/10.1038/bjc.2017.372

Huang DY, Lin YT, Jan PS, Hwang YC, Liang ST, Peng Y, Huang CY, Wu HC, Lin CT (2008) Transcription factor SOX-5 enhances nasopharyngeal carcinoma progression by down-regulating SPARC gene expression. The Journal of pathology 214(4):445-455. https://doi.org/10.1002/path.2299

Ikeda T, Zhang J, Chano T, Mabuchi A, Fukuda A, Kawaguchi H, Nakamura K, Ikegawa S (2002) Identification and characterization of the human long form of Sox5 (L-SOX5) gene. Gene 298(1):59-68. https://doi.org/10.1016/S0378-1119(02)00927-7

Kingston RE, Chen CA, Rose JK, Calcium phosphate transfection, in: F.M. Ausubel, et al. (Eds.), Current protocols in molecular biology, J. Wiley & Sons, New York, 2003, 9.1.4-9.1.11.

Kiselak EA, Shen X, Song J, Gude DR, Wang J, Brody SL, Strauss JF, Zhang Z (2010) Transcriptional regulation of an axonemal central apparatus gene, sperm-associated antigen 6, by a SRY-related high mobility group transcription factor, S-SOX5. J Biol Chem 285(40):30496-30505. https://doi.org/10.1074/jbc.M110.121590

Kuo YB, Chan CC, Chang CA, Fan CW, Hung RP, Hung YS, Chen KT, Yu JS, Chang YS, Chan EC (2011) Identification of phospholipid scramblase 1 as a biomarker and determination of its prognostic value for colorectal cancer. Mol Med 17(1):41-47. https://doi.org/10.2119/molmed.2010.00115

Kusano S, Eizuru Y (2012) Human phospholipid scramblase 1 interacts with and regulates transactivation of HTLV-1 Tax. Virology 432(2):343-352. https://doi.org/10.1016/j.virol.2012.06.019

Kwan KY, Lam MM, Krsnik Ž, Kawasawa YI, Lefebvre V, Šestan N (2008) SOX5 postmitotically regulates migration, postmigratory differentiation, and projections of subplate and deep-layer neocortical neurons. Proc Natl Acad Sci USA 105(41):16021-16026. https://doi.org/10.1073/pnas.0806791105

Lee WJ, Chatterjee S, Yap SP, Lim SL, Xing X, Kraus P, Sun W, Hu X, Sivakamasundari V, Chan HY, Kolatkar PR (2017) An integrative developmental genomics and systems biology approach to identify an in vivo sox trio-mediated gene regulatory network in murine embryos. BioMed research international 2017. https://doi.org/10.1155/2017/8932583

Liu CF, Lefebvre V (2015) The transcription factors SOX9 and SOX5/SOX6 cooperate genome-wide through super-enhancers to drive chondrogenesis. Nucleic acids research 43(17):8183-8203. https://doi.org/10.1093/nar/gkv688

Mata-Rocha M, Hernández-Sánchez J, Guarneros G, de la Chesnaye E, Sánchez-Tusié AA, Treviño CL, Felix R, Oviedo N (2014) The transcription factors Sox5 and Sox9 regulate Catsper1 gene expression. FEBS letters 588(18):3352-3360. https://doi.org/10.1016/j.febslet.2014.07.024

Nanjundan M, Sun J, Zhao J, Zhou Q, Sims PJ, Wiedmer T (2003) Plasma membrane phospholipid scramblase 1 promotes EGF-dependent activation of c-Src through the epidermal growth factor receptor. J Biol Chem 278(39):37413-37418. https://doi.org/10.1074/jbc.M306182200

Sahu SK, Gummadi SN, Manoj N, Aradhyam GK (2007) Phospholipid scramblases: an overview. Arch Biochem Biophys 462(1):103-114. https://doi.org/10.1016/j.abb.2007.04.002

Sandelin A, Wasserman WW, Lenhard B (2004) ConSite: web-based prediction of regulatory elements using cross-species comparison. Nucleic Acids Res 2004 32(2):W249-252. https://doi.org/10.1093/nar/gkh372

Shi Y, Wu Q, Xuan W, Feng X, Wang F, Tsao BP, Zhang M, Tan W (2018) Transcription factor SOX5 promotes the migration and invasion of fibroblast-like synoviocytes in part by regulating MMP-9 expression in collagen-induced arthritis. Frontiers in immunology 9:749. https://doi.org/10.3389/fimmu.2018.00749

Silverman RH, Halloum A, Zhou A, Dong B, Al-Zoghaibi F, Kushner D, Zhou Q, Zhao J, Wiedmer T, Sims PJ (2002) Suppression of Ovarian Carcinoma Cell Growth in Vivo by the Interferon-inducible Plasma Membrane Protein, Phospholipid Scramblase. Cancer Res 62(2):397-402.

Sivagnanam U, Palanirajan SK, Gummadi SN (2017) The role of human phospholipid scramblases in apoptosis: An overview. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1864(12):2261-2271. https://doi.org/10.1016/j.bbamcr.2017.08.008

Stevanovic M, Drakulic D, Lazic A, Ninkovic DS, Schwirtlich M, Mojsin M (2021) SOX transcription factors as important regulators of neuronal and glial differentiation during nervous system development and adult neurogenesis. Frontiers in molecular neuroscience 14:654031. https://doi.org/10.3389/fnmol.2021.654031

Suri P, Palmer MR, Tsepilov YA, Freidin MB, Boer CG, Yau MS, Evans DS, Gelemanovic A, Bartz TM, Nethander M, Arbeeva L (2018) Genome-wide meta-analysis of 158,000 individuals of European ancestry identifies three loci associated with chronic back pain. PLoS genetics 14(9):1007601. https://doi.org/10.1371/journal.pgen.1007601

Suzuki E, Amengual O, Atsumi T, Oku K, Hashimoto T, Kataoka H, Horita T, Yasuda S, Ieko M, Fukushima K, Koike T (2010) Increased expression of phospholipid scramblase 1 in monocytes from patients with systemic lupus erythematosus. J Rheumatol 37(8):1639-1645. https://doi.org/10.3899/jrheum.091420

Vinnakota JM, Gummadi SN (2016) Two c-Myc binding sites are crucial in upregulating the expression of human phospholipid scramblase 1 gene. Biochem Biophys Res Commun 469(3):412-417. https://doi.org/10.1016/j.bbrc.2015.11.131

Wiedmer T, Zhao J, Nanjundan M, Sims PJ (2003) Palmitoylation of phospholipid scramblase 1 controls its distribution between nucleus and plasma membrane. Biochemistry 42(5):1227-1233. https://doi.org/10.1021/bi026679w

Wiedmer T, Zhou Q, Kwoh DY, Sims PJ (2000) Identification of three new members of the phospholipid scramblase gene family. Biochim Biophys Acta (BBA)-Biomembranes. 1467(1):244-253. http://doi:10.1016/s0005-2736(00)00236-4

Wunderle VM, Critcher R, Ashworth A, Goodfellow PN (1996) Cloning and Characterization of SOX5, a New Member of the Human SOX Gene Family. Genomics 36(2):354-

358.https://doi.org/10.1006/geno.1996.0474

Yu A, McMaster CR, Byers DM, Ridgway ND, Cook HW (2003) Stimulation of phosphatidylserine biosynthesis and facilitation of UV-induced apoptosis in Chinese hamster ovary cells overexpressing phospholipid scramblase 1. J Biol Chem 278(11):9706-9714. https://doi.org/10.1074/jbc.M204614200

Yuan WM, Fan YG, Cui M, Luo T, Wang YE, Shu ZJ, Zhao J, Zheng J, Zeng Y (2021) SOX5 Regulates Cell Proliferation, Apoptosis, Migration and Invasion in KSHV-Infected Cells. Virologica Sinica 36(3):449-457. https://doi.org/10.1007/s12250-020-00313-3

Zawerton A, Mignot C, Sigafoos A, Blackburn PR, Haseeb A, McWalter K, Ichikawa S, Nava C, Keren B, Charles P, Marey I (2020) Widening of the genetic and clinical spectrum of Lamb–Shaffer syndrome, a neurodevelopmental disorder due to SOX5 haploinsufficiency. Genetics in Medicine 22(3):524-537. https://doi.org/10.1038/s41436-019-0657-0

Zhang L, Liu Y, Li W, Zhang Q, Li Y, Liu J, Min J, Shuang C, Song S, Zhang Z (2017) Transcriptional regulation of human sperm-associated antigen 16 gene by S-SOX5. BMC molecular biology 18(1):1-9. https://doi.org/10.1186/s12867-017-0082-3

Zhou Q, Ben-Efraim I, Bigcas JL, Junqueira D, Wiedmer T, Sims PJ (2005) Phospholipid scramblase 1 binds to the promoter region of the inositol 1, 4, 5-triphosphate receptor type 1 gene to enhance its expression. J Biol Chem 280(41):35062-35068. https://doi.org/10.1074/jbc.M504821200

Zhou Q, Zhao J, Al-Zoghaibi F, Zhou A, Wiedmer T, Silverman RH, Sims PJ (2000) Transcriptional control of the human plasma membrane phospholipid scramblase 1 gene is mediated by interferon-α. Blood, The Journal of the American Society of Hematology 95(8):2593-2599. https://doi.org/10.1182/blood.V95.8.2593

Zhou Q, Zhao J, Wiedmer T, Sims PJ (2002) Normal hemostasis but defective hematopoietic response to growth factors in mice deficient in phospholipid scramblase 1. Blood, The Journal of the American Society of Hematology 99(11):4030-4038. https://doi.org/10.1182/blood-2001-12-0271

Figures

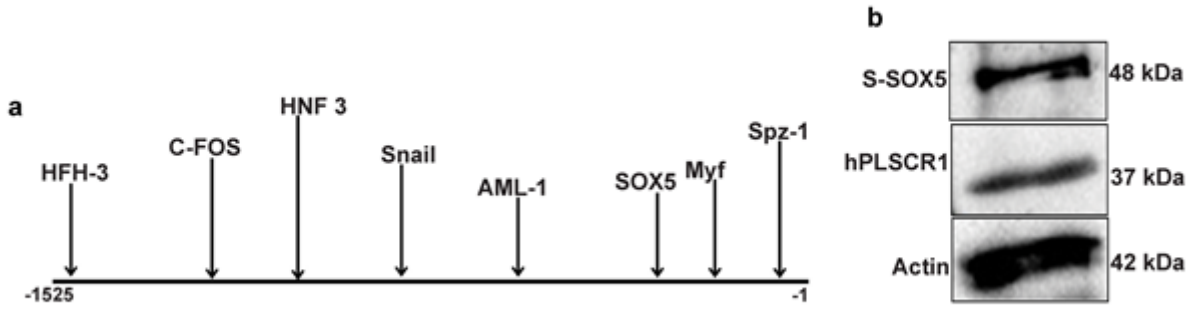

Figure 1

Prediction of putative TF binding sites on hPLSCR1 promoter. **a** TF prediction tool ConSite predicted the presence of many putative TFs on the promoter of hPLSCR1 at 100% cut-off. **b** Western blot showed endogenous expression of S-SOX5 and hPLSCR1 in HEK293T cells when probed with anti-Sox5 and anti-hPLSCR1 antibodies. Actin was considered as internal control and probed with anti-actin antibody.

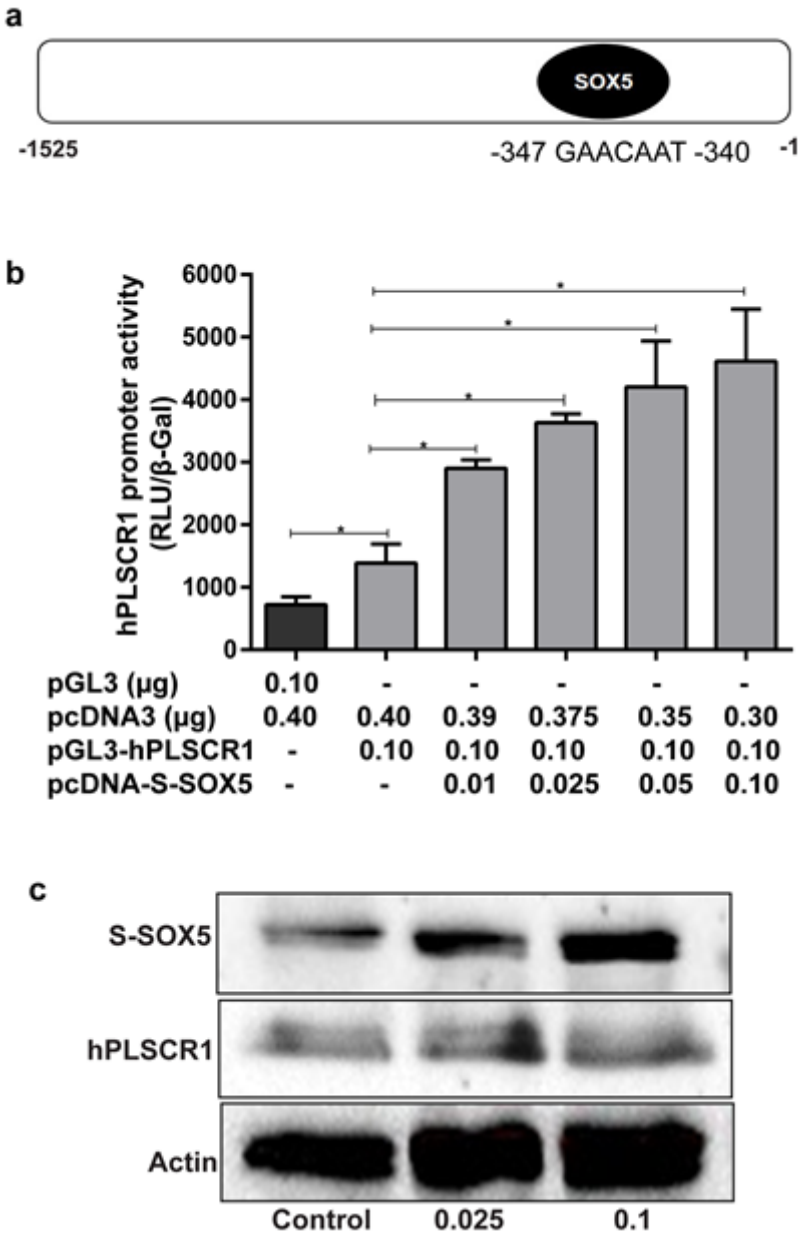

Figure 2

Effect of over expression of S-SOX5 TF on hPLSCR1 expression. **a** Schematic representation of position of one SBS (–GAACAAT-) on the promoter of hPLSCR1 at 100% cut-off. **b** Luciferase assay depicting an increase in hPLSCR1 promoter activity with an increase in S-SOX5 expression plasmid concentration. β-galactosidase was used as a transfection control for normalization. Data presented are indicative of three independent experiments and error bars represent standard error mean. **c** Western blot representing an increase in the endogenous hPLSCR1 expression with increasing doses of S-SOX5 plasmid when probed with anti-Sox5 and anti-hPLSCR1 antibodies. Actin used as control.

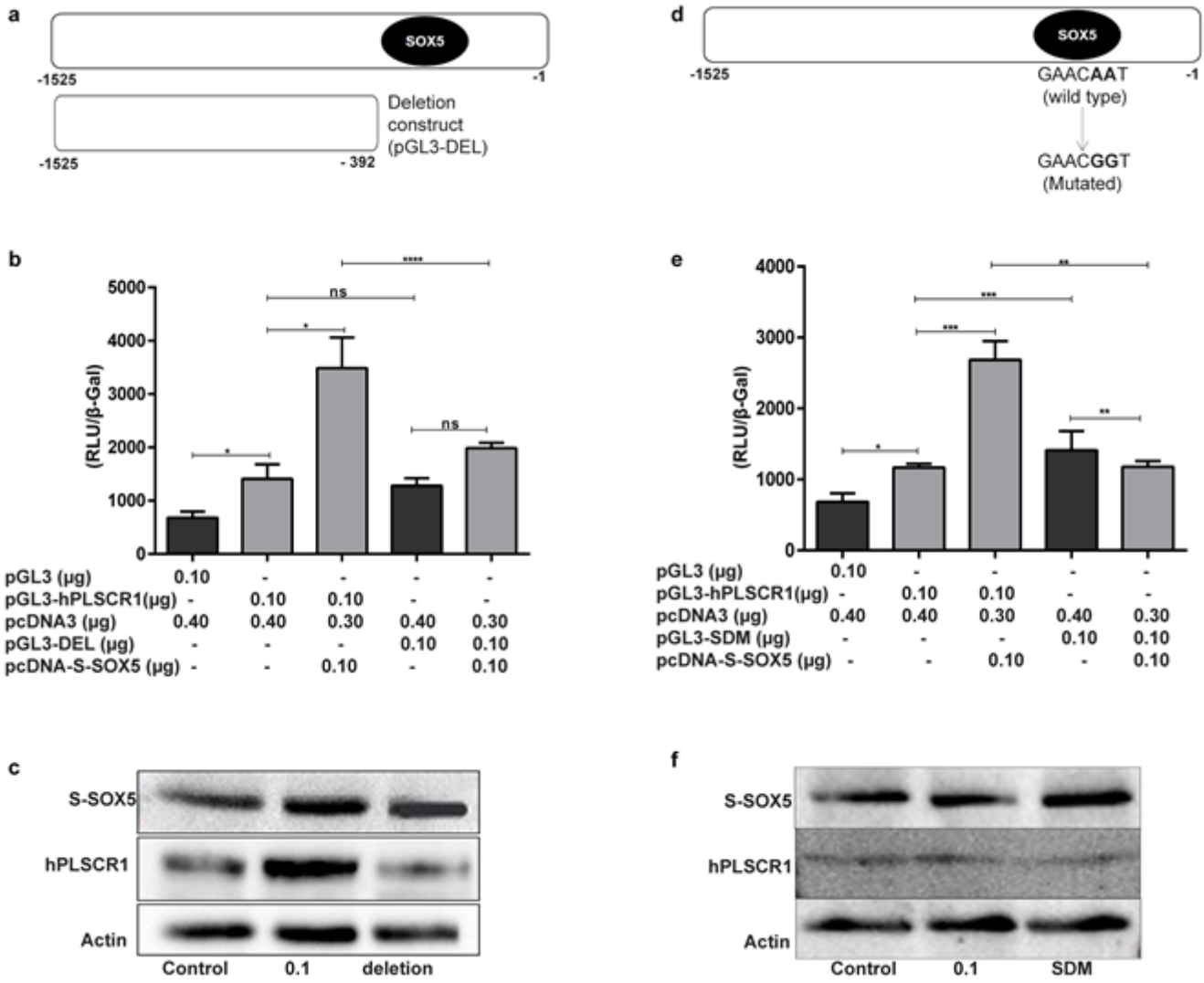

Figure 3

Deletion and mutation studies of S-SOX5 binding site. **a** Schematic depiction of deletion construct (pGL3-DEL) lacking S-SOX5 binding site. **b** Deletion construct was co-transfected with S-SOX5 expression plasmid or control plasmid. Luciferase assay depicting a decrease in the promoter activity of deletion construct in comparison with full length hPLSCR1 construct when co-transfected with S-SOX5 expression plasmid. The bars represent the statistically non-significant (ns) promoter activities of hPLSCR1 and deletion construct transfected with control plasmid (pcDNA3). **c** Western blot displaying a decrease in the endogenous hPLSCR1 expression as compared to control. Actin used as an internal control. **d** Schematic representation of point mutations induced in S-SOX5 binding site of hPLSCR1 promoter. **e** Mutated hPLSCR1 construct and wild type hPLSCR1 construct were co-transfected with S-SOX5 expression plasmid and control plasmid. Luciferase assay representing a decrease in hPLSCR1 mutant promoter activity upon comparison with the wild type construct. **f** Western blot showing a decrease in the endogenous hPLSCR1 expression as compared to control. Actin considered as internal control. Data

presented as mean of three independent experiments and error bars represent standard error mean in both the graphs (**b** and **e**).

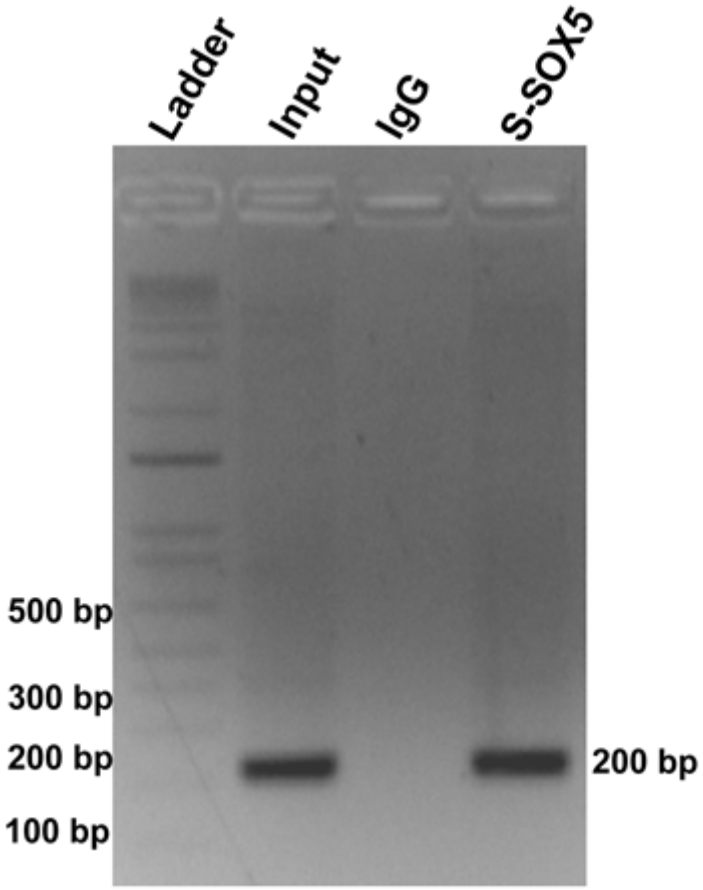

Figure 4

In-vivo interaction of S-SOX5 and hPLSCR1 promoter. The DNA-protein complexes from HEK cells were specifically pulled down by Sox5 antibody. Anti-IgG antibody was used a negative control. DNA prior to pull down (Input) was used as positive control. DNA stretch including S-SOX5 binding site was amplified in Input and S-SOX5 lanes. A 222 bp amplicon was amplified from immunoprecipitated complexes of HEK cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.docx