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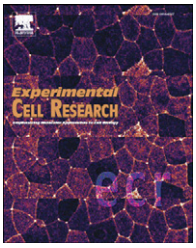

Research Article

Stanniocalcin-2 is a HIF-1 target gene that promotes cell proliferation in hypoxia

*Alice Y.S. Law, Chris K.C. Wong**

Department of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong

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ABSTRACT

Stanniocalcin-2 (STC2), the paralog of STC1, has been suggested as a novel target of oxidative stress response to protect cells from apoptosis. The expression of STC2 has been reported to be highly correlated with human cancer development. In this study, we reported that STC2 is a HIF-1 target gene and is involved in the regulation of cell proliferation. STC2 was shown to be up-regulated in different breast and ovarian cancer cells, following exposure to hypoxia. Using ovarian cancer cells (SKOV3), the underlying mechanism of HIF-1 mediated STC2 gene transactivation was characterized. Hypoxia-induced STC2 expression was found to be HIF-1α dependent and required the recruitment of p300 and HDAC7. Using STC2 promoter deletion constructs and site-directed mutagenesis, two authentic consensus HIF-1 binding sites were identified. Under hypoxic condition, the silencing of STC2 reduced while the overexpression of STC2 increased the levels of phosphorylated retinoblastoma and cyclin D in both SKOV3 and MCF7 cells. The change in cell cycle proteins correlated with the data of the serial cell counts. The results indicated that cell proliferation was reduced in STC2-silenced cells but was increased in STC2-overexpressing hypoxic cells. Solid tumor progression is usually associated with hypoxia. The identification and functional analysis of STC2 up-regulation by hypoxia, a feature of the tumor microenvironment, sheds light on a possible role for STC2 in tumors.

Introduction

Human stanniocalcin-2 (STC2) is a glycoprotein hormone with limited sequence homology to its paralog STC1 [1-4]. It has recently been suggested that the hormone may play a role in human carcinogenesis, as altered expression profiles in cancerous tissues were detected [5]. Intriguingly STC2 was identified as an estrogen responsive gene and was found to co-express with estrogen receptor in human breast cancers or breast cancer cell lines [6,7]. As a result of all these findings, the use of STC2 as a prognostic marker to renal, ovarian and breast cancers has been suggested [8-13].

Hypoxia is a common feature of most solid tumors. Solid tumor progression is usually associated with hypoxia and endoplasmic reticulum (ER) stress. This microenvironmental factor has profound consequence to tumor growth characteristics and to the responses in cancer therapy. The general hypoxia-driven responses in tumor cells include angiogenesis, anaerobic glycolysis and the reduction of macromolecule synthesis [14,15]. This adaptative response is basically mediated by the activation of HIF-1 responsive genes and the phosphorylation of the translation initiation factors [14,16], resulting in the development of resistance to apoptosis and an increased risk of metastasis. Therefore the identification of genes associated in hypoxic tolerance may be useful in diagnostic prediction and therapeutic treatment. In deciphering the regulation and function of STC2, it was reported that the gene was epigenetically regulated in cancer cells [17]. The stimulatory effects of hypoxia [17,18] and ER stress [19] to STC2

* *Corresponding author.*
E-mail address: ckcwong@hkbu.edu.hk (C.K.C. Wong).

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expression were demonstrated, and functionally, STC2 was related to the unfold protein response (UPR).

Clinically, STC2 has been proposed to be a biomarker for ovarian cancers, in association with the formation of tumor neovessels [8]. The growth and the spread of the ovarian cancer cells throughout peritoneal cavity depend on the outcomes of hypoxic adaptation (i.e., angiogenesis and proliferative/invasive capability). Since hypoxic cells often demonstrate resistance to therapeutic treatment, peritoneal invasion of ovarian cancer cells generally leads to poor patient outcome. Hypoxia regulated STC2 expression was demonstrated and was found to be HIF-1 dependent [17,18,20]. However the direct transactivation role of HIF-1α and the location of the functional hypoxia responsive element (HRE) on STC2 gene promoter had not been characterized. Additionally, the clinical relevance of STC2 is not clear. In the present study, we demonstrated the direct transactivation role of HIF-1α and identified the functional HRE on human STC2 gene promoter. The HIF-1 transactivation of STC2 promoter was found to require two consecutive HRE binding sites and the recruitment of the coactivators p300 and HDAC7. We also identified that STC2 stimulated cell proliferation under hypoxia. The stimulation was correlated to the increasing cellular levels of phosphorylated form of retinoblastoma (Rb) and cyclin D.

Materials and methods

Effects of hypoxia and drug treatments to the expression of STC2, and the binding of HIF-1α to STC2 promoter

The human breast cancer cells (i.e., MCF7, T47D and MDA-231) and the human ovarian cancer cell lines (SKOV3, OVCAR3 and CaOV3) were maintained in their respective media and exposed to hypoxic condition for 24 h. Total RNA was extracted for the determination of STC2 mRNA by real-time PCR. Significant inductions of STC2 were observed in all the tested cell lines. Among those SKOV3 produced the most significant response in STC2 expression at hypoxia. Therefore SKOV3 cells were used in the subsequent experiments for the characterization of HIF-1 transactivation of STC2 promoter.

The human ovarian cancer cell line SKOV3 was grown in McCoy's 5A (Sigma) supplemented with 10% FBS and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) (Invitrogen) at a density of 4×10^4 or 8×10^4 per well in 12-well or 6-well plates (Falcon). The cells were incubated in 5% CO_2/95% air at 37 °C. After overnight incubation, the cells were exposed to either normoxic or hypoxic condition for 24 h as described previously [21]. To achieve condition of O_2 depletion, the cultures were maintained in an air-tight modular incubation chamber (Billups-Rothenberg, Inc., Del Mar, CA) infused with a preanalyzed gas mixture (5% CO_2/95% N_2). The O_2 content in the incubation chamber was maintained at about 1%. The O_2 content was measured by a gas analyzer mounted with an O_2 sensor (Quest Technologies, Oconomowoc, WI). In some experiments, the cells incubated in the hypoxic chamber were cotreated with the p300 inhibitor chetomin (100 nM, CalBiochem) or the inhibitor of histone deacetylase (HDAC) trichostatin A (500 nM, CalBiochem) for 24 h. Total RNA and cell lysates were collected for real-time PCR and Western blotting. For re-ChIP assay, the cells were incubated in 2 mM of disuccinimidyl glutarate (Sigma) for 45 min before a post-fixation with 1% formaldehyde (Sigma).

Effects of HIF-1α overexpression to STC2 promoter-driven luciferase activity in SKOV3

The day before transfection, the cells were plated into 6-well tissue culture dishes at a density reaching 70–80% confluence by the time of transfection. Transfection was performed using LipofectAMINE™ 2000 reagent (Invitrogen). For promoter studies, the pGL3-basic vector (Promega) or the vector containing 0.75 kb (−689/+59) or 2 kb (−1901/+59) of human STC2 promoter was cotransfected with an expression vector encoding the wild-type form of HIF-1α (pCMV-HIF-1α, OriGene) or pCMV-empty vector and an internal control, pRL-SV40 plasmid (Promega). In the subsequent experiments, SKOV3 cells were cotransfected with pCMV-HIF-1α and pGL3-SV40-empty vector or pGL3-SV40 vector containing different STC2 promoter deletion constructs, with putative HRE sites located at −1901/−1309, −1901/−1702, −1519/−1309, −1519/−1397 and −1397/−1309. Six hours after transfection, the transfection medium was replaced by a complete medium and the cells were then incubated overnight in 5% CO_2/95% air at 37 °C. The cells were then lysed in a passive lysis buffer (Promega) for the dual-luciferase assay. Twenty microliters of the supernatant was used to assay the luciferase activities. Firefly and renilla luciferase activities were sequentially measured in the same sample using the Dual-Luciferase reporter assay system (Promega) and an Infinite™ F200 luminometer (TECAN).

Site directed mutagenesis

Mutations in the STC2 promoter plasmid constructs were introduced using the QuickChange® II Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The two putative HRE sequences at −1369 to −1366 and −1363 to −1360 of the STC2 promoter were mutated by replacing the CG in the 5′-CGTG-3′ motifs by AA. The sequence of the mutagenic primers were CCCGGCGCGGGGGAGAAATGAGCGTGCA-forward and TGCACGCT-CATTTCTCCCCCGCGCCGGG-reverse for the mutation of the first putative HRE, CGGGGGAGACGTGAGAATGCACACGTACACAC-forward and GTGTGTACGTGTGCATTCTCACGTCTCCCCCG-reverse for the mutation of the second putative HRE and GCCCCGGCGCGGGG-GAGAAATGAGAATGCACACGTACAC-forward and GTGTACGTGTG-CATTCTCATTTCTCCCCCGCGCCGGGGC-reverse for the mutations of both putative HREs. The mutation was confirmed by sequencing.

Chromatin immunoprecipitation (ChIP) and re-ChIP assays

ChIP assay was conducted using the ChIP assay kit according to the manufacturer's instruction (Upstate) and rabbit antibody against human HIF-1α protein (Abcam®) as described in our previous studies [17,22]. Re-ChIP assays were performed with modifications of the procedure described by Metivier et al. [23]. Briefly HIF-1α ChIP complexes were eluted by incubation for 30 min at 37 °C in 25 μl of 10 mM dithiothreitol (CalBiochem). After centrifugation, the supernatant was diluted with a re-ChIP buffer (20 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA and 1% Triton X-100, pH 8.0). The diluted complexes were then subjected to immunoprecipitation by mouse against human p300 antibody (BD Pharmingen) or rabbit against human HDAC7 antibody (Cell Signaling Technology®). The immunoprecipitated chromatin was analyzed in triplicate by PCR using the primers (AGTGCGCGCCAACGCCG-forward and AAGTTGGGCGCCGCTTGGA-reverse) for human STC2 promoter.