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The oncoprotein HBXIP upregulates PDGFB *via* activating transcription factor Sp1 to promote the proliferation of breast cancer cells

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ABSTRACT

We have reported that the oncoprotein hepatitis B virus X-interacting protein (HBXIP) acts as a novel transcriptional coactivator to promote proliferation and migration of breast cancer cells. Previously, we showed that HBXIP was able to activate nuclear factor-κB (NF-κB) in breast cancer cells. As an oncogene, the platelet-derived growth factor beta polypeptide (PDGFB) plays crucial roles in carcinogenesis. In the present study, we found that both HBXIP and PDGFB were highly expressed in breast cancer cell lines. Interestingly, HBXIP was able to increase transcriptional activity of NF-κB through PDGFB, suggesting that HBXIP is associated with PDGFB in the cells. Moreover, HBXIP was able to upregulate PDGFB at the levels of mRNA, protein and promoter in the cells. Then, we identified that HBXIP stimulated the promoter of PDGFB through activating transcription factor Sp1. In function, HBXIP enhanced the proliferation of breast cancer cells through PDGFB *in vitro*. Thus, we conclude that HBXIP upregulates PDGFB *via* activating transcription factor Sp1 to promote proliferation of breast cancer cells.

1. Introduction

Hepatitis B X-interacting protein (HBXIP) is a cellular 18 KD protein among mammalian species. It was originally identified by its interaction with the C-terminus of the hepatitis B virus X protein (HBx) [1]. Accumulating evidence shows that HBXIP has aboard roles in cancers. HBXIP can regulate centrosome duplication, causing excessive centrosome production and multipolar mitotic spindles in HeLa cells [2,3] and suppress apoptosis in hepatoma cells [4]. It has been demonstrated that HBXIP is a component of Ragulator, which is a guanine nucleotide exchange factor for the Rag GTPases that signal amino acid levels to mTORC1 [5]. Our previous studies reported that HBXIP was over-expressed in clinical breast cancer tissues and could promote proliferation and migration through inducing NF-κB, MAPK/ERK or PI3K/AKT signaling pathway in breast cancer cells [6–8]. Recently, we have discovered that HBXIP functions as a novel oncogenic coactivator of some transcription factors, such as STAT4, Sp1 and E2F1, to transactivate S100A4, LMO4 and Skp2 in promotion of cell proliferation and migration of breast cancer cells [8–10]. Therefore, HBXIP should transactivate more important genes to promote proliferation of cancer cells as a transcriptional coactivator.

Growth factors have important roles in the regulation of many biological processes including embryonic development, angiogenesis, cell proliferation and differentiation, and contribute to the pathophysiology of some diseases, including the cancer [11]. Platelet-derived growth factor (PDGF) is a ubiquitous, potent mitogen and chemotactic factor for many connective tissue cells [12] and stimulates tumor growth and progression by affecting tumor and stromal cells [13]. Although there are several PDGF family members (A, B, C, and D), PDGFB which is also called c-sis proto-oncogene, is a prime candidate to study, because a functional, contributory role for PDGFB in the development and maintenance of cancer cells is supported by several observations [14]. PDGF family members could form homo or hetero dimers such as PDGF-AA, PDGF-BB or PDGF-AB [15]. Among them, PDGF-BB exhibits the strongest activity [15]. High levels of PDGF-BB expression have been found in breast cancer tissues [16] and the PDGF-BB molecule has been described to activate the NF-κB pathway, which is well recognized for its role in regulating cytokine production [17]. PDGF-BB can promote fibroblast proliferation and enhance extracellular matrix [18], synthesis regulates pericyte and fibroblast functions in the supporting matrix of tumors [19]. The fact that high level expression of the cellular form of the PDGFB oncogene is sufficient for oncogenesis, implying that loss of its transcriptional regulation provides a contributory step in neoplastic

Abbreviations: HBXIP, hepatitis B X-interacting protein; PDGFB, platelet-derived growth factor beta polypeptide; NF-κB, nuclear factor-κB; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EdU, 5-ethynyl-2′-deoxyuridine.

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transformation [20]. Therefore, accumulated evidence suggests that PDGFB displays a proto-oncogenic role in cancer.

In the present study, we try to gain insight into the effect of HBXIP on the regulation of PDGFB in promotion of proliferation of breast cancer cells. Interestingly, our data support the notion that HBXIP is able to upregulate oncoprotein PDGFB through serving as a coactivator of Sp1 in the cells. Our finding provides new insights into the mechanism by which HBXIP promotes the proliferation of breast cancer cells.

2. Materials and methods

2.1. Cell lines

The breast cancer cell lines, such as MDA-MB-231, MCF-7, SK-BR3 and LM-MCF-7 (a metastatic subclone of MCF-7 breast cancer cell line) were maintained in RPMI 1640 (Gibco, CA, USA) medium containing 10% fetal bovine serum (FBS) (Gibco) [8].

2.2. Reagents and siRNA

Human PDGF-BB was purchased from sigma (USA). The cells were treated with 10 nM PDGFB for 8–72 h. The siRNAs directed against HBXIP, PDGFB, Sp1 and control siRNAs were purchased from Riobio Company (Guangzhou, China). The cells were transfected with HBXIP siRNA or PDGFB siRNAs in different doses (0.04–0.08 nmol).

2.3. Total RNA isolation, reverse-transcription PCR (RT-PCR)

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, USA) according to the instructions. First-strand cDNA was synthesized with the PrimeScript reverse transcriptase Kit (TaKaRa Bio, China). To examine the mRNA level, the PDGFB primers used were as follows: forward, 5′-GGCCTTCTTAAAGATTGGCTTCT-3′; and reverse, 5′-GCCTCATAGACCGCACCAAC-3′. The primers for HBXIP and GAPDH were described previously [7]. PCR conditions were 35 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min.

2.4. Transfection and luciferase reporter gene assay

The promoter region (from −250 to +80 nt) of PDGFB gene was amplified by PCR from genomic DNA of MCF-7 cells and was inserted into the KpnI/XhoI site in the pGL3-basic vector [21]. For luciferase reporter gene assays, MCF-7 or SK-BR3 cells (2×10^4 cells per well in 24-well plates) were transfected with NF-κB or PDGFB luciferase reporter plasmids (0.3 μg) and the pRL-TK normalization construct (0.1 μg) by using Lipofectamine 2000 (Invitrogen, USA). In addition, the plasmid pCMV-HBXIP [6] (100–300 ng) was co-transfected with reporter plasmids. Otherwise, siRNA targeting HBXIP mRNA or Sp1 mRNA (0.04–0.08 nmol) was co-transfected with reporter plasmids. Luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega, USA) 24 h after transfection. Data were presented as firefly luciferase activity normalized to Renilla luciferase activity for each set of triplicate samples. All experiments were performed at least three times.

2.5. Chromatin immunoprecipitation (ChIP)

The ChIP assay was performed according to the manufacturer's instructions from Epigentek Company (USA) [8]. The DNA that was pulled down by antibodies was amplified by PCR. The primers for the PDGFB promoters were forward 5′-CGGTGGGTCACCCC-TAGTTC-3′, reverse 5′-TGAAAAATGGGCGCTGGCGG-3′ and primers for the negative controls were described previously [8].

2.6. Western blot analysis

The protocol was described previously [6]. Primary antibodies used were rabbit anti-HBXIP (Santa Cruz, USA), rabbit anti-PDGFB (Santa Cruz, USA), rabbit anti-Sp1 (Epitomics, USA) and mouse anti-β-actin antibodies (Sigma, USA).

2.7. The MTT assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as previously described [8]. In brief, cells were harvested from exponential-phase cultures, counted, and plated in 96-well plates at 3×10^3 cells per well. 24 h later, the cells were incubated with the MTT substrate (20 mg/ml) for 4 h. After incubation, the culture medium was removed, and DMSO was added. Optical density was measured at 490 nm. Cell proliferation was measured by MTT assays each day for 3 days.

2.8. EdU assay

Five-ethynyl-2′-deoxyuridine (EdU) can be used to label cells undergoing DNA replication [22]. The EdU assay was performed

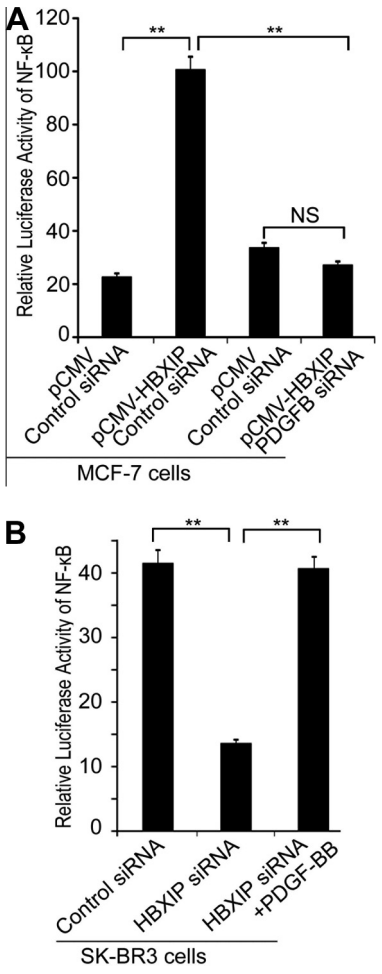

Fig. 1. HBXIP is associated with PDGFB in breast cancer cells. (A) The transcriptional activity of NF-κB was examined by luciferase reporter gene assays in MCF-7 cells transfected with pCMV-HBXIP plasmids. Luciferase activities were measured 24 h after transfection. Bars: means ± SEM ($n = 3$). Student's t test, $^{**}p < 0.01$. (B) The transcriptional activity of NF-κB was examined by luciferase reporter gene assays in SK-BR3 cells when the cells transfected with HBXIP siRNAs. Luciferase activities were measured 24 h after transfection. The cells were treated with 10 nM PDGF-BB for 8 h. Bars: means ± SEM ($n = 3$). Student's t test, $^{**}p < 0.01$.

using the Cell-Light TM EdU imaging detection kit (RiboBio, China) according to the manufacturer's instructions.

2.9. Statistical analysis

The data were analyzed by the Student's *t* test. Statistically significant *p* values were indicated in figures as follows: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ using the SPSS software program (SPSS, Chicago, USA). Each experiment was repeated at least three times.

3. Results

3.1. HBXIP is associated with PDGFB in breast cancer cells

Our previous data showed that the oncoprotein HBXIP was highly expressed in breast cancer tissues and promoted the proliferation of breast cancer cells [7–10]. It has been reported that PDGFB is highly expressed in breast cancer tissues and contributes to the proliferation of breast cancer cells [16]. Accordingly, we examined the relationships between HBXIP and PDGFB in breast cancer cell lines including MDA-MB-231, LM-MCF-7, SK-BR3 and MCF-7 by RT-PCR and Western blot assay, respectively (Fig. S1). Our data revealed that the expression of PDGFB was relevant to that of HBXIP in these cell lines. It has been reported that NF-κB is activated by PDGFB [17]. Thereby, we hypothesized that HBXIP was able to activate NF-κB through PDGFB in breast cancer cells. Interestingly, we found that the knockdown of PDGFB was able to block the HBXIP-increased transcriptional activities of NF-κB in MCF-7 cells by luciferase reporter gene assays (Fig. 1A). Moreover, the treatment of PDGF-BB was able to rescue the HBXIP siRNA-decreased transcriptional activities of NF-κB in SK-BR3 cells by luciferase reporter gene assays (Fig. 1B), implying that PDGFB is involved in the transcriptional activities of NF-κB mediated by HBXIP. Therefore, our data suggest that HBXIP is associated with PDGFB in breast cancer cells.

3.2. HBXIP is able to upregulate the expression of PDGFB in breast cancer cells

Accordingly, we examined the effect of HBXIP on PDGFB in breast cancer cells. Our data showed that the over-expression of HBXIP was able to upregulate the expression of PDGFB at the

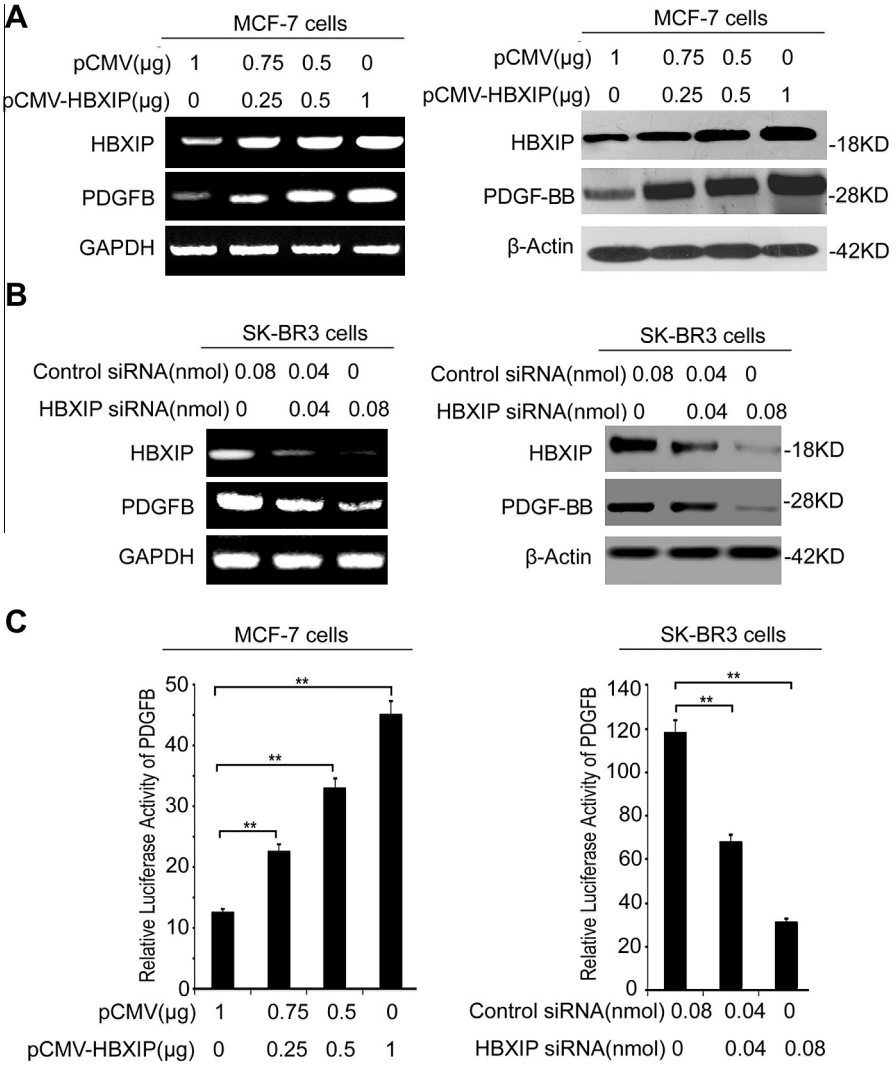

Fig. 2. HBXIP is able to upregulate the expression of PDGFB in breast cancer cells. (A) MCF-7 cells were transfected with pCMV-HBXIP plasmids. The expression of HBXIP and PDGFB at the levels of mRNA and protein was detected by RT-PCR and Western blot analysis, respectively. (B) SK-BR3 cells were transfected with HBXIP siRNAs. The expression of HBXIP and PDGFB at the levels of mRNA and protein was detected by RT-PCR and Western blot analysis, respectively. (C) The promoter activity of PDGFB was examined by luciferase reporter gene assays in MCF-7 cells transfected with pCMV-HBXIP plasmids. SK-BR3 cells were transfected with HBXIP siRNAs. Luciferase activities were measured 24 h after transfection. Bars: means ± SEM ($n = 3$). Student's *t* test, $^{**}p < 0.01$.

mRNA and protein levels in MCF-7 cells in a dose-dependent manner (Fig. 2A). While, the knockdown of HBXIP showed the opposite results in SK-BR3 cells (Fig. 2B). To assess the effect of HBXIP on PDGFB promoter activity, we cloned the promoter region of PDGFB (from −250 to +80 nt) into the pGL3-basic plasmid according to the report [21]. Luciferase reporter gene assays revealed that the over-expression of HBXIP was able to increase the promoter activity of PDGFB in MCF-7 cells in a dose-dependent manner. While, the knockdown of HBXIP showed the opposite results in SK-BR3 cells (Fig. 2C). Overall, we conclude that HBXIP is able to upregulate the expression of PDGFB in breast cancer cells.

3.3. HBXIP activates the PDGFB promoter via transcription factor Sp1

Next, we try to unravel the mechanism by which HBXIP upregulates PDGFB. It has been reported that there is a transcription factor Sp1 binding site, named the SIS proximal element (SPE), at the promoter of PDGFB [21] (Fig. 3A). Our recent report posits that HBXIP acts as a novel coactivator to transactivate LMO4 through interacting with transcription factor Sp1 in promotion of cell proliferation of breast cancer cells [9]. Thus, we speculated that HBXIP might stimulate the promoter of PDGFB through Sp1 in the same way. Chromatin-immunoprecipitation (ChIP) showed that HBXIP was able to bind to the promoter of PDGFB in MCF-7 cells (Fig. 3B). Moreover, the knockdown of Sp1 by siRNA abolished the interaction of HBXIP with the promoter of PDGFB (Fig. 3C). Our previous report revealed that HBXIP could bind to Sp1 in the cells [9]. It suggests that HBXIP may form a complex with Sp1 to bind to the PDGFB promoter. Interestingly, luciferase reporter gene assays showed that HBXIP failed to increase the activity of PDGFB promoter when Sp1 was knockdown by siRNA in MCF-7 cells (Fig. 3D). Meanwhile, we validated that HBXIP failed to upregulate the expression of PDGFB at the levels of mRNA and protein when Sp1 was knockdown by siRNA in the cells (Fig. S2A and S2B). Thus,

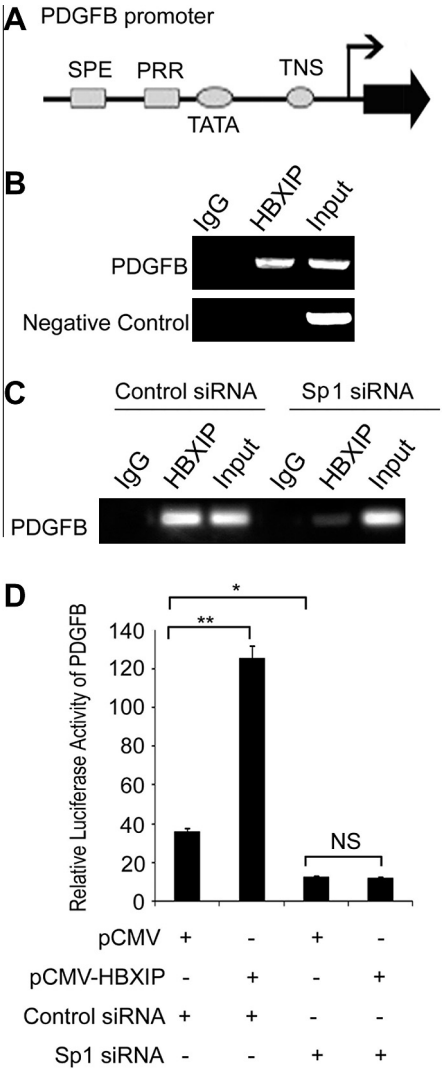

Fig. 3. HBXIP activates the PDGFB promoter *via* transcription factor Sp1. (A) A model of PDGFB promoter. The black line represents the PDGFB promoter. The gray boxes represent the binding sites of transcription factors. The gray circle means TATA boxes. (B) The 5 μg HBXIP antibodies were used for each ChIP samples. The 0.5% cells nuclear lysis was as input. The ChIP assay was performed in MCF-7 cells. (C) MCF-7 cells were transfected with Sp1 siRNA. ChIP assays were measured 24 h after transfection. (D) MCF-7 cells were transfected with Sp1 siRNA. Luciferase reporter gene assays were measured 24 h after transfection. Bars: means ± SEM ($n = 3$). Student's t test, $^{*}p < 0.05$, $^{**}p < 0.01$.

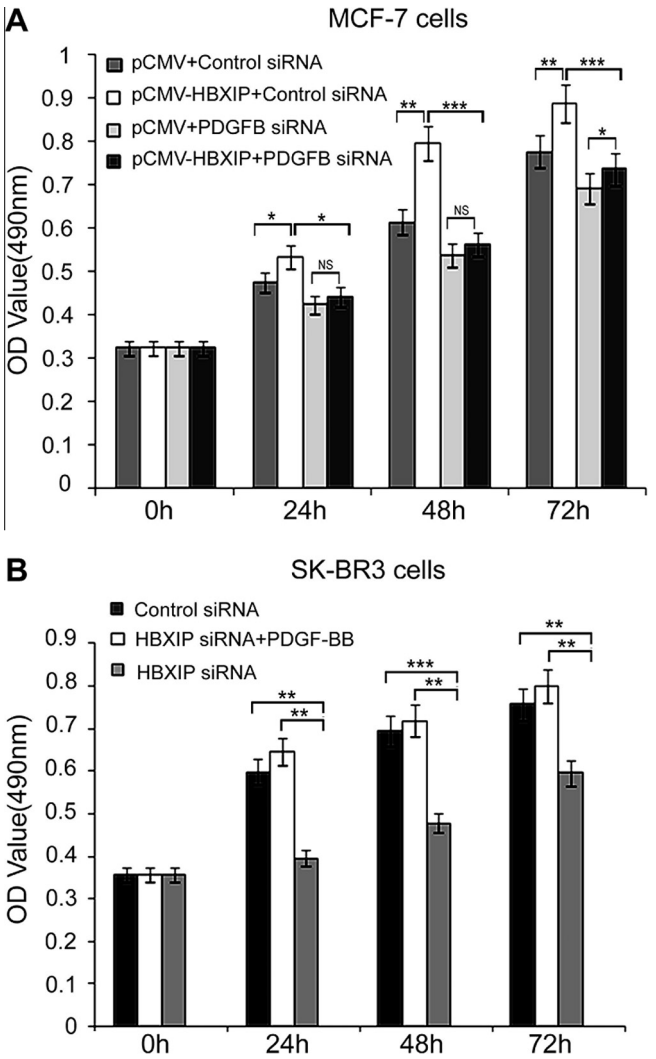

Fig. 4. HBXIP promotes the proliferation of breast cancer cells *via* upregulating PDGFB. (A) MCF-7 cells were transfected with pCMV-HBXIP plasmids or PDGFB siRNAs. The effect of HBXIP and PDGFB on the proliferation of MCF-7 cells was measured by MTT assays. Bars: means ± SEM ($n = 3$). Student's t test, $^{*}p < 0.05$,$^{**}p < 0.01$, $^{***}p < 0.001$. (B) SK-BR3 cells were transfected with HBXIP siRNAs or treated with 10 nM PDGF-BB. The effect of HBXIP and PDGFB on proliferation of SK-BR3 cells was measured by MTT assays. Bars: means ± SEM ($n = 3$). Student's t test, $^{**}p < 0.01$, $^{***}p < 0.001$.

we conclude that HBXIP is able to stimulate the promoter of PDGFB through activating transcription factor Sp1.

3.4. HBXIP promotes the proliferation of breast cancer cells via upregulating PDGFB

Our recent studies have showed that HBXIP is able to enhance the proliferation of breast cancer cells [7–10]. Notably, PDGFB functions in promotion of proliferation of breast cancer cells [15]. Thus, we are interested in whether HBXIP promotes the proliferation of breast cancer cells *via* upregulating PDGFB. Interestingly, MTT assay showed that PDGFB siRNA was able to block the HBXIP-enhanced proliferation of MCF-7 in a time-course manner (Fig. 4A). While, the treatment of PDGF-BB was able to rescue the HBXIP siRNA-decreased proliferation of SK-BR3 cells (Fig. 4B). EdU assay could repeat the data in the same cell lines (Fig. S3A and S3B). Overall, our data suggest that HBXIP promotes the proliferation of breast cancer cells *via* upregulating PDGFB *in vitro*.

4. Discussion

Our recent reports posit that HBXIP is an important oncoprotein and dramatically enhances the proliferation and migration of breast cancer cells [7–10]. However, the mechanisms by which HBXIP contributes to the proliferation of breast cancer cells have not been fully elucidated. PDGFB has been implicated in the pathogenesis of breast cancer by regulating the cellular proliferation and invasion [20,23]. Accordingly, we are certainly concern whether PDGFB is involved in the aggressive accumulation of breast cancer cells mediated by HBXIP.

Clearly, growth factors not only play crucial roles in the regulation of many biological processes including embryonic development, angiogenesis, cell proliferation and differentiation, but also contribute to the pathophysiology of some diseases, including the cancer [11]. PDGF is a potent mitogen and chemoattractant that functions as an important mediator in the pathogenesis of vascular disease [24]. It has been reported that the oncoprotein PDGFB is required for the proliferation and migration of cancer [19]. In this study, we are interested in the role of PDGFB in promotion of proliferation of breast cancer cells mediated by HBXIP. Strikingly, we discovered that HBXIP was associated with PDGFB in breast cancer cells. Then, we found that HBXIP was able to dramatically upregulate the expression of PDGFB at the levels of mRNA, protein and promoter in the cells. Next, we try to identify the mechanism by which HBXIP upregulates PDGFB. We have reported that HBXIP acts as a transcriptional coactivator [8–10]. Thereby, we speculated that HBXIP served as a coactivator to transactivate PDGFB through interacting with transcription factors. It has been reported that there is a transcription factor Sp1 binding site, named the SIS proximal element (SPE), at the position −58 to −39 relative to the PDGF-B mRNA initiation site that is essential for the TPA-induced activation [25]. Sp1 is a ubiquitous transcription factor that activates a broad and diverse spectrum of mammalian genes. Recently, we have reported that HBXIP activates transcriptional coregulatory protein LMO4 *via* Sp1 to promote proliferation of breast cancer cells [9]. Moreover, Sp1 plays a critical role in the growth and metastasis of many tumor types including breast cancer by regulating several genes associated with cell growth. Interestingly, in this study we found that the transcription factor Sp1 was required for the binding of HBXIP to BPGFB promoter, resulting in the transcriptional activation of PDGFB gene. This finding provides more evidence that HBXIP serves as a coactivator of transcription factors. In function, we demonstrated that HBXIP promoted the proliferation of breast cancer cells through upregulating PDGFB in breast cancer cells. Therefore, our observation suggests that the abnormality of HBXIP expression is oncogenic and may facilitate carcinogenesis and tumor progression for abnormally activating growth factors in breast cancer.

Collectively, our data presented here point towards a novel mechanism by which the oncoprotein HBXIP promotes the breast tumor progression through upregulating PDGFB involving transcription factor Sp1. This finding provides new insights into the mechanism of HBXIP in promotion of proliferation of breast cancer cells. Therapeutically, HBXIP may serve as a new target of breast cancer.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.123.

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