Basic Fibroblast Growth Factor is Involved in Bisphenol S Induced Proliferation of Hemangioma Cells

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Abstract

Background

The hyperproliferation of mesoblastic vascular tissues can lead to the incidence of hemangioma (IHA), which is the most common benign tumor in infants. Estrogenic signals can trigger the progression of HA via activation of gene transcription.

Results

We found that bisphenol S (BPS), one widely spread endocrine disrupting compound (EDC), can induce the proliferation of HA cells and trigger the G1 to S transition of cell cycle. Among the tested cytokines, BPS can up regulate of basic fibroblast growth factor (bFGF). Targeted inhibition of bFGF via its neutralization antibody can reverse BPS induced cell proliferation. Mechanistically, BPS can increase the mRNA expression of bFGF via increasing the transcription and mRNA stability. The activation of p65 and down regulation of miR-155-5p were responsible for BPS induced transcription and mRNA stability of bFGF, respectively.

Conclusions: BPS can increase the expression of bFGF via activation of p65 and down regulation of miR-155-5p, which resulted in the proliferation of HA cells.

Background

Infantile hemangioma (IHA), caused by hyperproliferation of mesoblastic vascular tissues, is the most common benign tumor in infants at present (Mabeta and Pepper, 2011). HA can cause a heavy physical and mental burden to patients, particularly for patients with disfigured skin lesions (Chibbaro et al., 2018). Pharmacotherapy and surgery are major approaches for HA therapy (Chen et al., 2018). However, there are still 25% HA patients undergoing resection had persistence of symptoms (Haggstrom et al., 2007). The understanding about initiation and risk factors for HA is warranted for discovery of novel treatment approaches and prevent of HA.

It has been reported that about 75–80% of HA patients are females (Chibbaro et al., 2018). The detailed cause of the female preponderance is not yet understood. It has been reported that level of estradiol (E2) in healthy children was significantly lower than that in HA patients (Sasaki et al., 1984). The serum levels of E2 in proliferating HA tissues are greater than that in involuting phase (Yu et al., 2006). Laboratory studies indicated that estrogen can promote the *in vitro* proliferation of HA cells, which may depend on certain growth factors (GFs) and be inhibited by tamoxifen (Xiao et al., 1999). Further, estrogen and VEGF had synergistic effects on proliferation of HA cells (Xiao et al., 2004). All these data suggested the positive roles of estrogenic signals on HA progression.

Endocrine disrupting compounds (EDCs) are environmental compounds which have similar characteristics with E2 (Rubin, 2011). They can influence multiple endocrine related pathways and then

disrupt hormone functions (Henley and Korach, 2006). Bisphenol A (BPA) is a typical EDC banned from many human consumer products due to the negative effects on human health (Lu et al., 2013). Its analog bisphenol S (BPS) are widely used as substitutes for industrial application particularly in many commercial products labeled “BPA-free” (Rochester and Bolden, 2015). Recently, numerous studies indicated that BPS can also accumulate in human body and is an urgent issue for public health (Rochester and Bolden, 2015). For example, BPS can reduce the steroid hormone synthesis and down regulate steroidogenic gene transcripts (Feng et al., 2016). BPS also has a comparable estrogenic activity as BPA (Kuruto-Niwa et al., 2005). It can induce epithelial-mesenchymal transition (EMT) in HA cells via induction of Snail (Zhai et al., 2016). While the potential effects of BPS on the progression of HA are not investigated.

The cytokines such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are critical for HA progression (Fu et al., 2017; Przewratil et al., 2010). For example, VEGFA can facilitate the growth of HA-derived endothelial cells (HemECs) (Jinnin et al., 2008). While targeted inhibition of VEGF can inhibit the proliferation of HA cells (Pan et al., 2015). As to bFGF, its expression was associated with incidence of HA (Bielenberg et al., 1999) and proliferation of HA cells (Przewratil et al., 2010). However, the regulators about bFGF and other cytokines in HA were not well illustrated.

Our present study found that nanomolar BPS can trigger the proliferation of HA cells via increasing the expression of bFGF. While the neutralization antibody of bFGF can abolish BPS-induced proliferation of HA cells. Mechanistically, BPS increased the transcription of bFGF via activation of p65 and stabilized the mRNA of bFGF via decreasing miR-155-5p.

Results

1 BPS triggers the proliferation and cell cycle transition of HA cells

To evaluate the potential functions of BPS on progression of HA, we treated cells with increasing concentration of BPS for 48 h. CCK-8 analysis showed that 10 nM BPS can significantly trigger the proliferation of HDEC cells (Fig. 1A), however, BPS with the concentrations greater than 1 mM can suppress proliferation of HDEC cells. Similarly, nanomolar concentrations of BPS can also trigger the proliferation of CRL-2586 cells (Fig. 1B). Considering that nanomolar was more closely to concentration of BPS observed in human body, we only focused on the potential roles of nanomolar BPS in next studies. Colony formation analysis showed that 10 nM BPS can also significantly trigger the colonization of both HDEC and CRL-2586 cells (Fig. 1C). Cell cycle analysis showed that population of the cells in G0/G1 was decreased in the groups treated with BPS. The distribution of S phase was increased by treatment with BPS (Sun et al., 2018). It indicated that BPS can trigger the proliferation of HA cells via inducing cell cycle transition.

2 BPS increases the expression of bFGF in HA cells

Growth factors (GFs) such as EGF and hepatocyte growth factor (HGF) are suggested to play important roles in epithelial cell proliferation (Han et al., 2011; Zelenka and Arpitha, 2008). We then tested the potential effects of BPS on the expression of various GFs including acidic fibroblast growth factor (aFGF), bFGF, FGF3, Insulin-like growth factor-1 (IGF-1), HGF, VEGF, and transforming growth factor beta (TGF-β). Our data showed that BPS can increase the expression of bFGF and VEGF in HDEC cells (Fig. 2A). However, BPS can only increase the expression of bFGF in CRL-2586 cells (Fig. 2B). Consistently, BPS can increase the expression of bFGF in HDEC cells via both time (Fig. 2C) and concentration (Fig. 2D) manners. ELISA confirmed that BPS increased expression of bFGF in both HDEC and CRL-2586 cells (Fig. 2E). All these results suggested that BPS increased the expression of bFGF in HA cells.

3 bFGF is involved in BPS induced proliferation of HA cells

The potential roles of bFGF were measured in BPS induced proliferation of HA cells. We found that neutralization antibody of bFGF can attenuate BPS induced proliferation of HDEC cells (Fig. 3A). However, the neutralization antibody for VEGF, which was also upregulated by BPS, had no significant effect on BPS induced proliferation of HDEC cells (Fig. 3B). Consistently, anti-bFGF also reversed BPS induced proliferation of CRL-2586 cells (Fig. 3C). In addition, anti-bFGF also partially attenuated BPS decreased proportions of G0/G1 phase (Fig. 3D) while increased proportions of S phase (Fig. 3E) in HDEC cells. All these data suggested that bFGF was involved in BPS induced proliferation of HA cells.

4 BPS can increase the transcription and mRNA stability of bFGF

We then further investigated the mechanisms responsible for upregulation of bFGF. Our data showed that BPS treatment can increase the mRNA of bFGF since treatment for 4 h. We then analyzed the promoter activity of bFGF in BPS treated HA cells by use of promoter luciferase assay. Our data showed that BPS can increase the promoter activity of bFGF in both HDEC and CRL-2586 cells (Fig. 4A). Further, BPS can increase the half-life of bFGF mRNA in HDEC cells (Fig. 4B). However, BPS had no effect on mRNA distribution in cellular fractions such as cytoplasm and nucleus in HDEC cells (Fig. 4C). Further, BPS had no effect on protein stability of bFGF in HDEC cells (Fig. 4D). These data suggested that bFGF can increase the transcription and mRNA stability of bFGF.

5 NF-κB is involved in BPS induced expression of bFGF in HA cells

Previous studies indicated that AP-1 and NF-κB can regulate the transcription of bFGF (Wu et al., 2016). We therefore investigated whether AP-1 or NF-κB was involved in BPS induced transcription of bFGF. We found that BPS can increase the phosphorylation of p65, while not c-Fos or c-Jun, in HDEC cells (Fig. 5A). Consistently, BPS also increased the phosphorylation of p65 in CRL-2586 cells (Fig. 5B). Further, BAY, the

inhibitor of NF-κB, can reverse BPS induced upregulation of *bFGF* in HDEC cells (Fig. 5C). Consistently, BAY also partially attenuated BPS induced proliferation of HDEC cells (Fig. 5D). These data suggested that NF-κB was involved in BPS induced expression of bFGF in HA cells.

6 miR-155-5p is involved in BPS increased mRNA stability of bFGF

miRNAs can target the 3'UTR of mRNA and then lead to degradation of its target (Mohajeri et al., 2018). Based on the prediction results of miRTarBase (Chou et al., 2016), miRecords (Xiao et al., 2009) and starBase version 2.0 (Li et al., 2014), miR-16-5p, miR-140-5p, miR-503-5p, and miR-155-5p can directly target the 3'UTR of bFGF to regulate its expression. Our data showed that BPS can significantly inhibit the expression of miR-155-5p, while not others, in HDEC cells (Fig. 6A). Consistently, our data showed that BPS can also decrease the expression of miR-155-5p in CRL-2586 cells (Fig. 6B). The mimic of miR-155-5p can attenuate BPS induced upregulation of bFGF in HDEC cells (Fig. 6C). Consistently, mimic of miR-155-5p can also partially attenuate BPS induced proliferation of HDEC cells (Fig. 6D). All these data indicated that miR-155-5p is involved in BPS increased mRNA stability of bFGF.

Discussion

Recent studies indicated that BPA can increase the progression of HA via induction of EMT and upregulation of Snail (Zhai et al., 2016). Our present study found that BPS, the "safety" analog of BPA, can trigger the proliferation of HA cells and its cell cycle transition via upregulation of bFGF. Further, BPS can increase the mRNA stability of bFGF via decreasing the expression of miR-155-5p. As well, BPS can increase the transcription of bFGF via activation of NF-κB.

BPS have existed estrogenic and cellular functions in many recent studies (Chin et al., 2018; Kinch et al., 2015; LaPlante et al., 2017). As to cell proliferation, BPS significantly promoted the proliferation of ERα positive MCF-7 cells, but failed to promote the proliferation of ERα negative MDA-MB-231 and SK-BR-3 cells (Lin et al., 2019), which was consistent with our present study that BPS at nanomolar can significantly promote proliferation of HA cells. Our results also observed that BPA can increase S phase and decrease G0/G1 phase of HA cell, which was also consistent with previous data that EDCs such as BPA(Wu et al., 2012), Perfluorooctanoic acid (PFOA) (Pierozan et al., 2018), and polybrominated diphenyl ethers (Li et al., 2012) can reduce the percentage of cells at G0/G1 phase and increase percentage of cells at S phase to trigger the cell cycle transition and cell proliferation. In MCF-7 cells, BPS treatment also resulted in an acceleration of G1-S phase transition (Lin et al., 2019). Since BPS existed comparable estrogenic potency to E_2 (Vinas and Watson, 2013) and HA cells can be exposed to BPS via blood circulation, the potential effects of BPS on HA progression need further study.

We found the upregulation of bFGF was involved in BPS induced proliferation of HA cells. The expression of bFGF and its receptor are closely associated with proliferation of infantile cutaneous hemangioma (Przewratil et al., 2010). *In situ* hybridization and immunohistochemical analysis confirmed that the

expression of bFGF is closely correlated with incidence of hemangioma (Bielenberg et al., 1999). The bFGF is an effective stimulator of breast epithelial cells proliferation and differentiation (Korah et al., 2000). Our present study found that the neutralization antibody against bFGF can suppress the proliferation of HA cells and block promotion effect of BPS on cells proliferation, which further confirmed the essential roles of bFGF in HA progression.

We found that activation of p65 and down regulation of miR-155-5p were responsible for BPS induced transcription and upregulation mRNA stability of bFGF, respectively, in HA cells. In bovine mammary epithelial cells (BMEC), the expression of bFGF is dependent on the NF-κB and AP-1 signaling pathways (Wu et al., 2018). While BPS had no significant effect on the phosphorylation of AP1 in HA cells. Further, activation of p65 was also involved in BPA induced migration of cervical cancer cells (Ma et al., 2015). In male zebrafish, BPS exposure can change the expression of 14 miRNAs involved in hematopoiesis, lymphoid organ development, and immune system development (Lee et al., 2018). In pheochromocytoma PC12 cells, BPS can regulate the expression of miR-10b to inhibit the expression of KLF4 and induce cell migration (Jia et al., 2018). It has been reported that miR-15a (Zhu et al., 2017), miR-146a (Liu et al., 2016), and miR-195 (Wang et al., 2017) can also regulate the mRNA stability and expression of bFGF. Whether these miRNAs are involved in BPS regulated expression of bFGF needs further study.

Conclusion

Nanomolar BPS can induce the proliferation of HA cells via induction of bFGF through p65 induced transcription and miR-155 regulated mRNA stabilization. It indicated the potential risks about BPS on HA progression and hormone related diseases need more attention.

Materials And Methods

1 Cell culture and treatment

The primary HA-derived endothelial cell (HDEC) and CRL-2586 cells were maintained in our laboratory and cultured in DMEM supplied with FBS to a final concentration of 10% and streptomycin sulfate and penicillin (Life Technologies, Inc, Gaithersburg, Maryland) at 37 °C under 5% CO_2. Bisphenol S (99%, 4,4'-sulfonyldiphenol) was bought from Molecular Probes (USA) and dissolved in DMSO to get a stock solution of 100 mM. Medium contains the same amount (less than 0.5%) of DMSO, which had no toxic effect on cells, was used as control.

2 Cell proliferation assay

The effects of BPS on proliferation of HA cells were tested by use of CCK-8 kit according to the previous study (Pang et al., 2019). Briefly, cells (2×10^3 cells/well) seeded in the 96-well plates were treated with different concentrations of BPS for the indicated time periods. At the end of experiments, 10 μL of CCK-8 solution was added to each well and incubated with 2 h at 37 °C. The absorbance at 450 nm was measured using a microplate reader (PerkinElmer, USA).

3 Colony formation assay

Cells (2×10^2 cells/well) seeded in six-well plates were treated with or without BPS as the indicated conditions. Then cells were allowed to grow for 5–14 days with media changed every 5 days. The formation of colony was stained and counted according to the previous study (Kalailingam et al., 2019).

4 Cell cycle analysis

Cells were treated with or without BPS before cell cycle analysis. After treatment, cells were fixed with cold 70% ethanol for 4 h, stained with propidium iodide (PI), and analyzed by flow cytometry using a FACSort Flow Cytometer (San Jose, CA) equipped with CellQuest Software according to the previous study (Yu et al., 2019). For each assay, 10,000 single cells were collected for analysis by use of ModFit software to determine each phase of cell cycle and are reported as percentage of G0/G1, S, and G2/M for each sample.

5 Real-time RT-PCR analysis

After treatment, cells were harvested with TransZolUp (TransGen Biotech, Beijing, China) and extracted with an RNA Purification Kit (Qiagen, Valencia, CA). Then, 1000 ng of total RNA was used to generate cDNA by use of reverse transcription reagent kit (Takara Biotechnology, Kusatsu, Shiga, Japan) for mRNA and miRNA reverse transcription kit (Promega Corporation, Madison, WI, USA) for miRNAs, respectively. The qRT-PCR was conducted by use of an iCycler (Bio-rad, Hercules, USA) with the primers as follow: VEGFA, 5'- AGGGCAGAATCATCACGAAGT – 3' and 5'- AGGGTCTCGATTGGATGGCA – 3'; aFGF, 5'-CTCCCGAAGGATTAAACGACG – 3' and 5'- GTCAGTGCTGCCTGAATGCT – 3'; bFGF, 5'-AGAAGAGCGACCCTCACATCA-3' and 5'- CGGTTAGCACACACTCCTTTG – 3'; FGF3, 5'-GGCGTCTACGAGCACCTTG – 3' and 5'- CCACTGCCGTTATCTCCAAAA-3'; IGF1, 5'-GCTCTTCAGTTCGTGTGTGGA – 3' and 5'- GCCTCCTTAGATCACAGCTCC-3'; HGF, 5'-GCTATCGGGGTAAAGACCTACA – 3' and 5'- CGTAGCGTACCTCTGGATTGC – 3'; TGFB1, 5'-GGCCAGATCCTGTCCAAGC – 3' and 5'- GTGGGTTTCCACCATTAGCAC – 3'; GAPDH, forward 5'-GCA CCG TCA AGG CTG AGA AC-3' and reverse 5'-TGG TGA AGA CGC CAG TGG A-3'. GAPDH and U6 were used to normalize the level of mRNAs and miRNAs, respectively. The results were based on the ΔΔCt method and expressed as relative changes as compared to untreated control groups.

6 Western blot analysis

The protein expression was checked by western blot analysis according to the previous study (Song et al., 2019). The primary antibodies were listed as follows: bFGF (ab126861, Abcam, 1:500); GAPDH (ab181603, Abcam, 1:500); p-p65 (ab76302, Abcam, 1:500); p65 (ab16502, Abcam, 1:500); p-c-fos (ab27793, Abcam, 1:500); c-fos (ab208942, Abcam, 1:500); p-c-Jun (ab32385, Abcam, 1:500); c-Jun (ab32137, Abcam, 1:500). GAPDH was used as an internal reference.

7 Enzyme-linked immunosorbent assay (ELISA)

The levels of bFGF in medium of cells treated with or without BPS were measured by use of ELISA kit according to the manufacturer's protocol (USCN Business Co. Ltd., Wuhan, China).

8 **Promoter activity assay**

The promoter (1 kb upstream of TSS) of bFGF was cloned into the pGL-Basic plasmid to generate pGL-bFGF-Basic plasmid. Effects of BPS on the promoter activity of bFGF were tested by luciferase assay according to previously described protocol (Jiang et al., 2013). Briefly, cells were transfected with pGL-bFGF-basic and pRL-TK for 12 h and then further treated with or without BPS for the indicated time periods. The luciferase was measured by Dual-Glo Luciferase Assay system (Promega). Renilla Luciferase (R-luc) was used to normalize firefly luciferase (F-luc) activity to evaluate reporter translation efficiency.

9 **Statistical analysis**

All values are expressed as the mean ± standard deviation (SD). Data were analyzed by use of SPSS 14.0 software (SPSS, Inc., Chicago, IL, USA). The student *t* test was used to assess the difference between two groups. $P \leq 0.05$ was considered as statistically significant.

List Of Abbreviations

bFGF, basic fibroblast growth factor; BMEC, bovine mammary epithelial cells; BPA, Bisphenol A; BPS, bisphenol S; E2, estradiol ; EDC, endocrine disrupting compound; ELISA, Enzyme-linked immunosorbent assay; EMT, epithelial-mesenchymal transition; F-luc, firefly luciferase; GFs, growth factors; HDEC, HA-derived endothelial cell; HemECs, HA-derived endothelial cells; IGF-1, Insulin-like growth factor-1; IHA, hemangioma; PFOA, Perfluorooctanoic acid; PI, propidium iodide; SD, standard deviation; TGF-β, transforming growth factor beta; VEGF, vascular endothelial growth factor

Declarations

Ethics approval and consent to participate

Nohuman/animal study was included in the present study.

Consent for publication

All authors give the consent for the publish of this study.

Availability of data and material

All data and material are available.

Disclosure of potential conflicts of interest

The authors declare no conflict of interest.

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Authors' contributions

Data collecting: Dahai Liu, Yubo Hui, Junrong Wang

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Figures

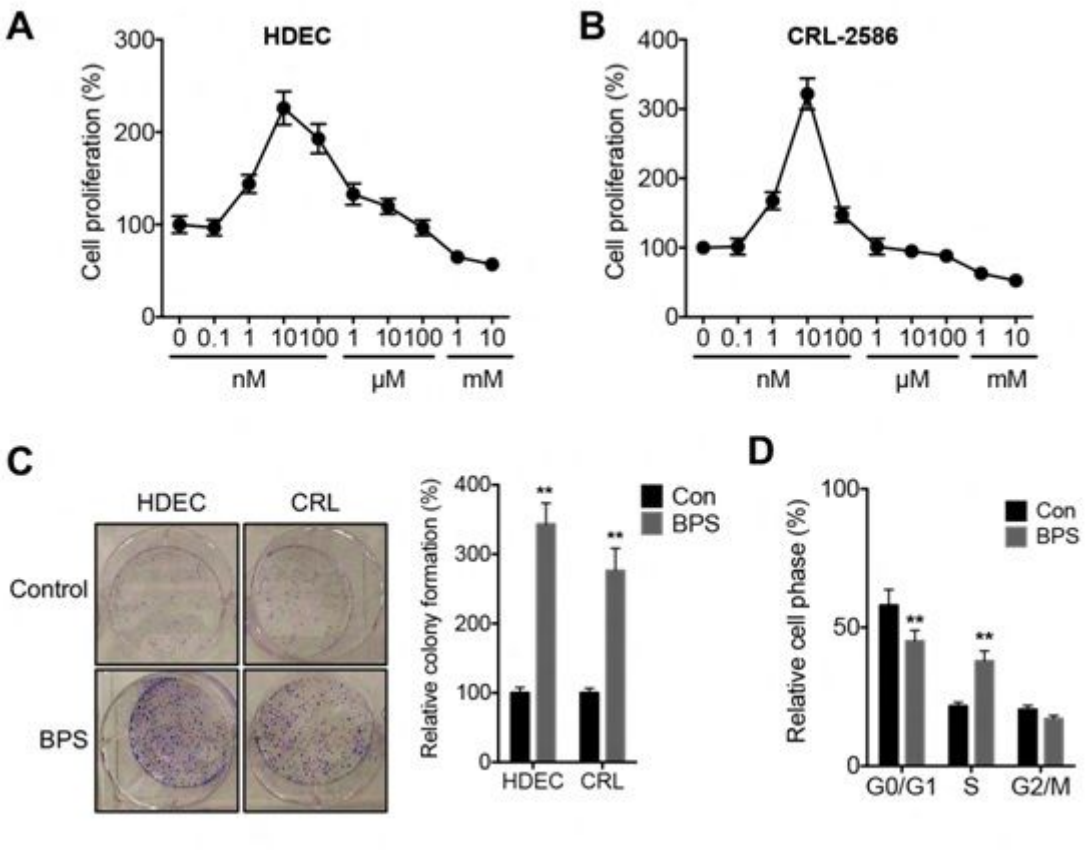

Figure 1

BPS triggers the proliferation and cell cycle transitionof HA cells. HDEC (A) or CRL-2586 (B) cells were treated with increasing concentrations of BPS for 48 h,cell proliferation was tested by CCK-8 kit; (C) Cells (2 × l05) treated with or without 100 nM BPS were cultured in 6-well plates for two weeks before colonies were counted; (D) HDEC cells were treated with or without 100 nM BPS for 24 h, the cell cycle was detected using flow cytometry. Data are shown as means ± SD. **$p < 0.01$ compared to control.

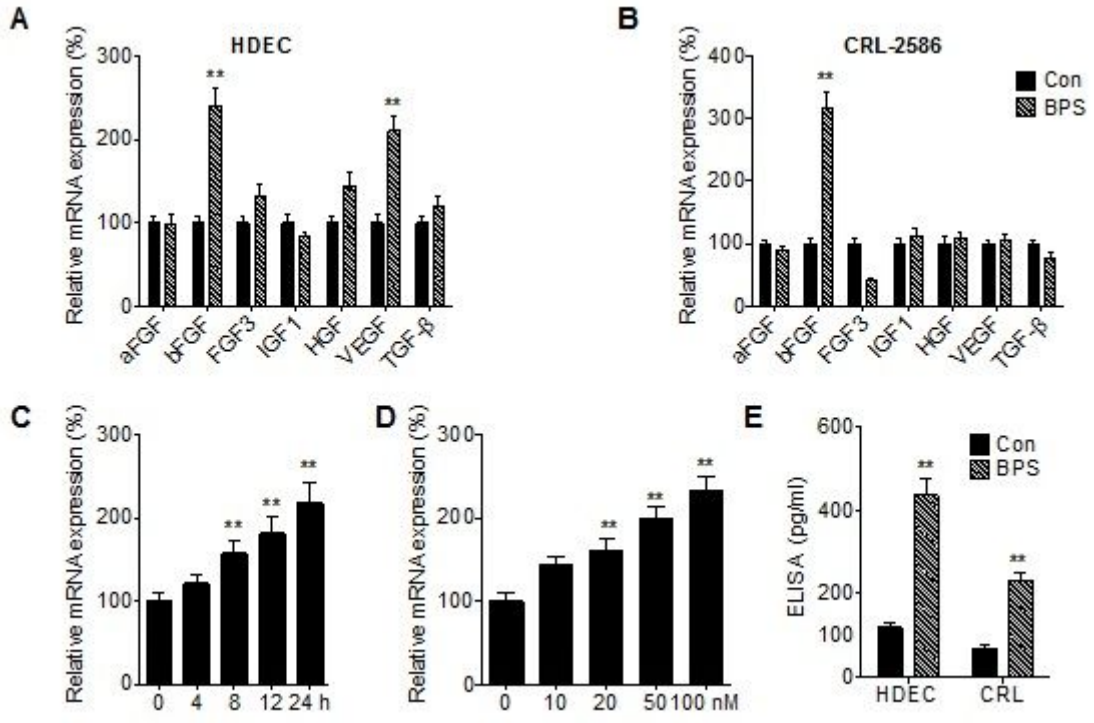

Figure 2

BPS increases the expression of bFGF in HA cells. HDEC (A) or CRL-2586 (B) cells were treated with or without 100 nM BPS for 24 h, the expression of GFs was measured by qRT-PCR; HDEC cells were treated with 100 nM BPS for the increased time periods (C) or increasing concentrations of BPS for 24 h (D), the expression of bFGF was checked by qRT-PCR; (E) Cells were treated with or without 100 nM BPS for 24 h, the expression of bFGF was checked by ELISA. Data are shown as means ± SD. **$p < 0.01$ compared to control.

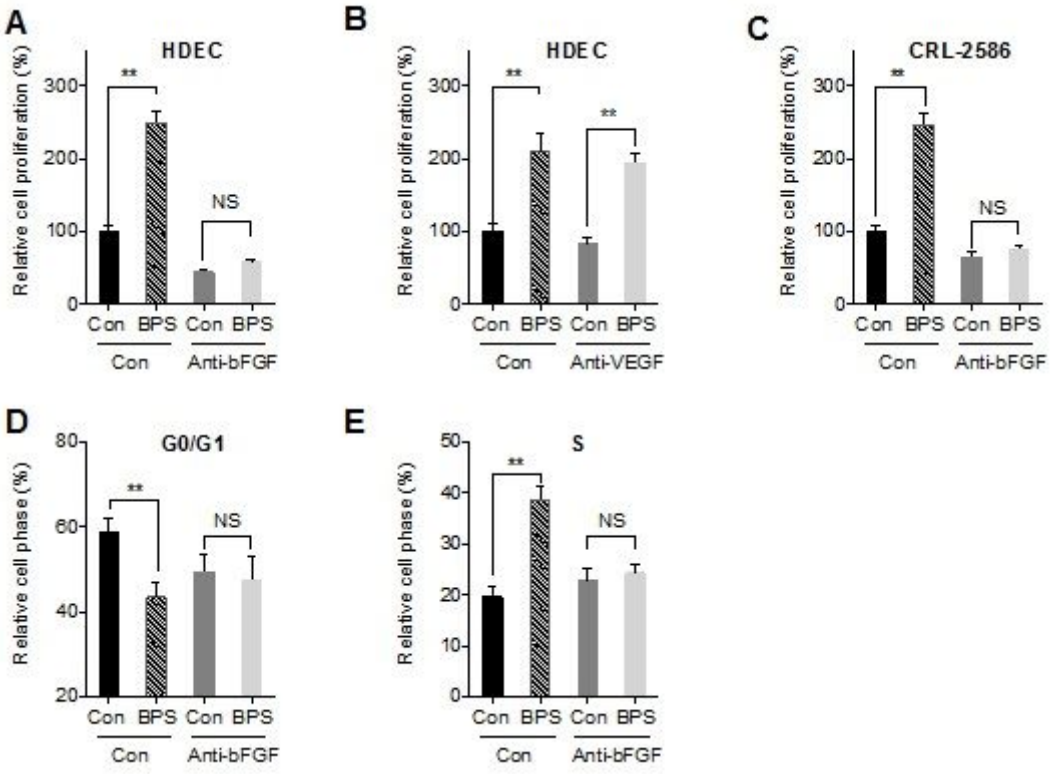

Figure 3

bFGF is involved in BPS induced proliferation of HA cells. HDEC cells were pre-treated with anti-bFGF (A) or anti-VEGF (B) (100 ng/ml) for 2 h and then further treated with or without 100 nM BPS for 24 h, the cell proliferation was tested by CCK-8 kit; (C) CRL-2586 cells were pre-treated with or without anti-bFGF for 2 h and then further treated with or without 100 nM BPS for 24 h, the cell proliferation was tested by CCK-8 kit; HDEC cells were pre-treated with or without anti-bFGF and then further treated with or without 100 nM BPS for 24 h, the cell cycle of G0/G1 (D) or S (E) phase of cells were detected using flow cytometry. Data are shown as means ± SD. **$p < 0.01$ compared to control.

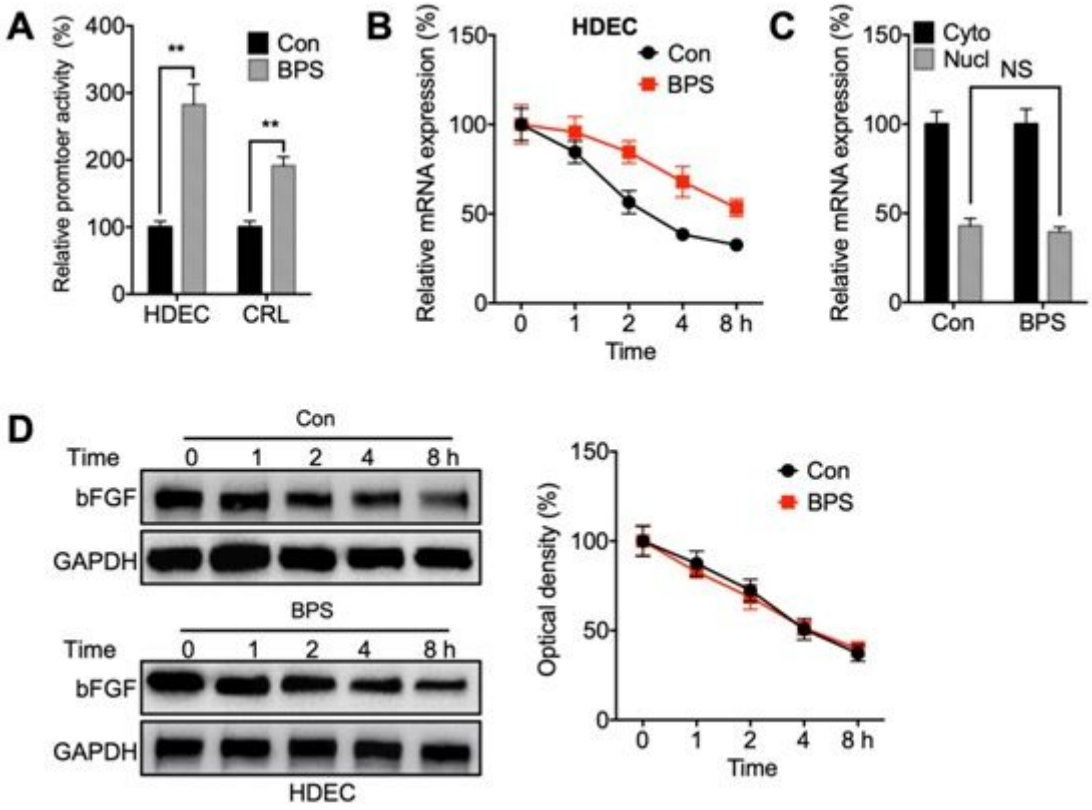

Figure 4

bFGF can increase the transcription and mRNA stability of bFGF. (A) Cells were treated with or without 100 nM BPS for 24 h, the luciferase activities of bFGF promoter were measured by dual-luciferase assay; (B) HDEC cells pretreated with or without 100 nM BPS for 24 h were further treated with or without transcriptional inhibitor Act-D for the indicated times, the mRNA of bFGF was checked by qRT-PCR; (C) After treated with or without 100 nM BPS for 24 h, the mRNA of bFGF in cytoplasm and nucleus of HDEC cells was checked by qRT-PCR; (D) HDEC cells were pre-treated with or without 100 nM BPS for 24 h and further treated with CHX (100 μg/ml) for increasing time periods, the expression of bFGF was recorded (left) and quantitatively analyzed (right). Data are shown as means ± SD. **$p < 0.01$ compared to control.

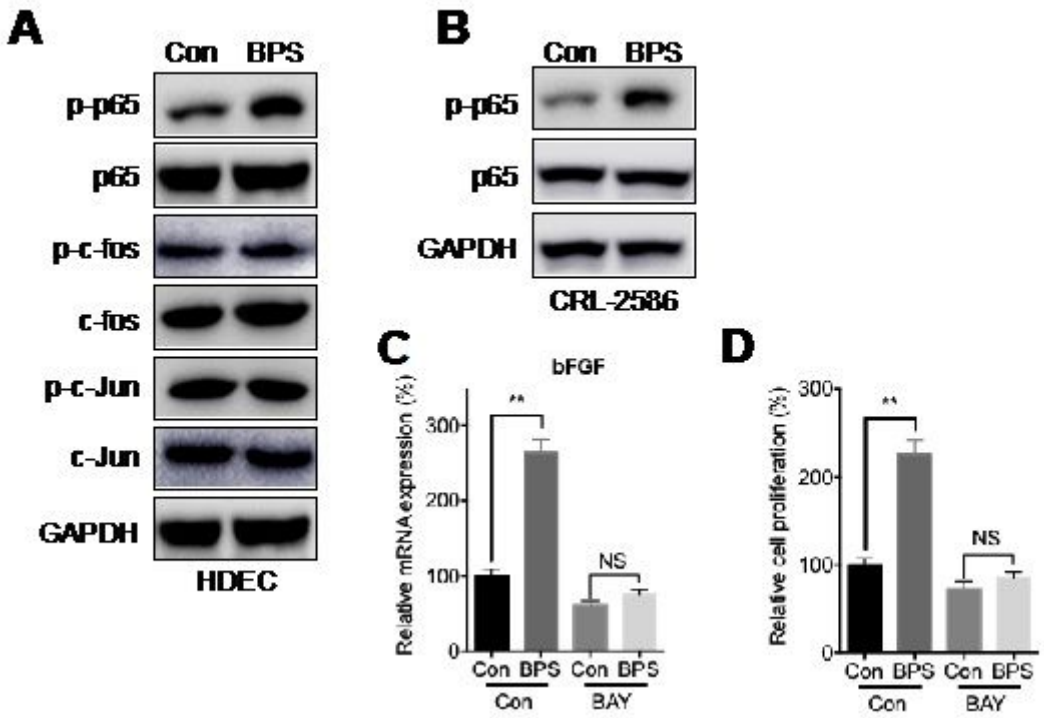

Figure 5

NF-κBis involved in BPS induced expression of bFGF in HA cells.HDEC (A) or CRL-2586 (B) cells were treated with or without 100 nM BPS for 15 min, the phosphorylation and total expression of p65, c-fos and c-Jun was measured by western blot analysis; HDEC cells were pretreated with or without BAY, the inhibitor of NF-κB, for 30 min, and then further treated with or without 100 nM BPS for 24 h, the mRNA expression of bFGF was tested by qRT-PCR (C), and cell proliferation was tested by CCK-8 kit (D). Data are shown as means ± SD. **$p < 0.01$ compared to control.

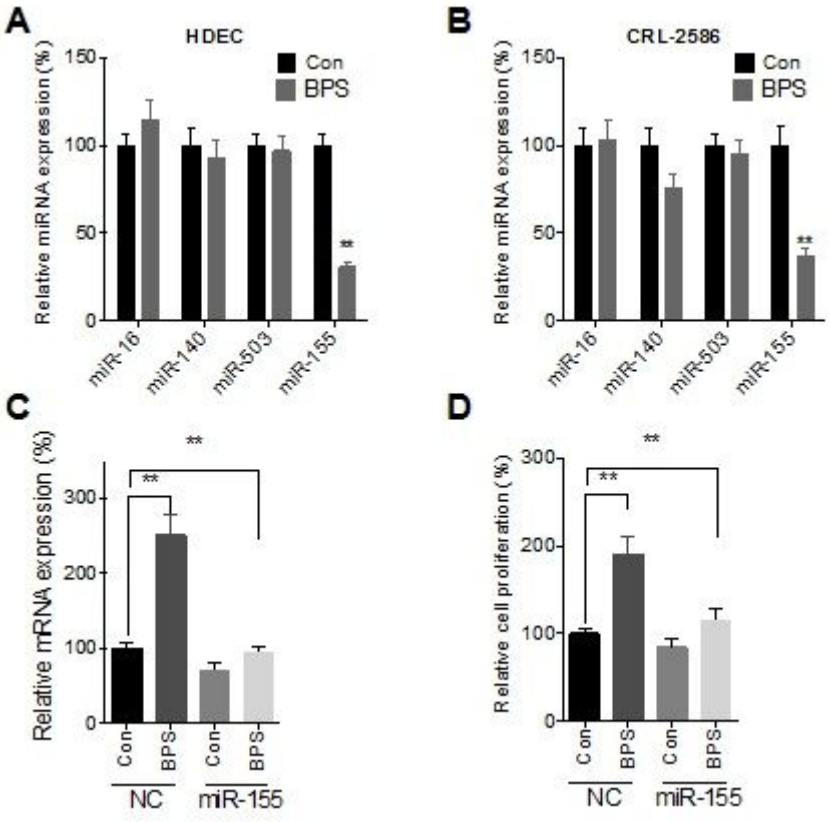

Figure 6

miR-155-5p is involved in BPS increased mRNA stability of bFGF.HDEC (A) or CRL-2586 (B) cells were treated with or without 100 nM BPS for 15 min, the expression of miRNAs was checked by qRT-PCR; HDEC cells pretreated with scramble control or miR-155 mimic and then further treated with or without 100 nM BPS for 24 h, the mRNA expression of bFGF was checked by qRT-PCR (C), the cell proliferation was detected by CCK-8 kit (D). Data are shown as means ± SD. **$p < 0.01$ compared to control.