2015년 2월 석사학위 논문 HSF2 regulates its own expression via a transcriptional auto-regulatory mechanism 박 선 미

2015년 2월

석사학위 논문

HSF2 regulates its own expression via a transcriptional auto-regulatory mechanism

조 선 대 학 교 대 학 원

치의생명공학과

박 선 미

HSF2 regulates its own expression via a transcriptional auto-regulatory mechanism

HSF2의 전사적 자동 조절 기전 규명

2015년 2월 25일

조선대학교 대학원

치의생명공학과

박 선 미

HSF2 regulates its own expression via a transcriptional auto-regulatory mechanism

지도교수 안 상 건

이 논문을 이학 석사학위신청 논문으로 제출함

2014년 10월

조선대학교 대학원

치의생명공학과

박 선 미

박선미의 석사학위 논문을 인준함

위원장 조선대학교 교 수 국 중 기 (인)

위 원 조선대학교 교 수 김 도 경 (인)

위 원 조선대학교 교 수 안 상 건 (인)

2014년 11월

조 선 대 학 교 대 학 원

CONTENTS

LIST OF FIGURES

ABSTRACT

HSF2의 전사적 자동 조절 기전 규명

박 선 미

지도교수 : 안 상 건

조선대학교 대학원 치의생명공학과

Heat shock transcription factors (HSFs)는 다양한 세포내외부의 병리 생리학적 스트레스에 대해 세포를 보호하기 위한 생체방어기전을 조절한다. 그중 HSF2는 발생과 분화 및 유비퀴틴-프로테아좀 과정에서 중요한 조절기전이 있음이 알려지고 있다. 이러한 중요성에도 불구하고, HSF2의 발현에 대한 조절기전은 아직 명확하지 않다. 따라서, 본 연구에서는 HSF2의 전사적 조절 기전에 대해 규명하고자 한다. 본 연구 결과, 사람 적백혈병 세포인 K562 세포에서 과발현 시킨 HSF2에 의해 endogenous HSF2의 mRNA 발현이 RT-PCR과 real time RT-PCR을 통해 감소된 결과를 보였고, HSF2의 전사적 활성을 확인하기 위해 HSF2의 promoter (-2.86 kb)를 이용한 luciferase reporter assay 한 결과 HSF2의 과발현이 농도 의존적으로 HSF2의 promoter 활성을 억제시킴을 확인하였다. 이러한 결과는 chromatin immunoprecipitation assay (ChIP)를 통해 과발현 시킨 HSF2의 단백질이 HSF2 promoter (-1.5kb)내 heat shock binding element (HSE)에 결합하여 HSF2의 발현을 조절함을 확인할 수 있었다. 또한, 세포내 HSF2의 전사조절이 HSF1 단백질에 의해서도 조절됨을 규명하였고 이는 HSFs들 간에 높은 유사성을 가지고 상호 조절기전을 가지고 있음을 보여주고 있다.

본 연구는 HSF2의 새로운 발현 조절 기전을 규명하였고, 이는 앞으로 퇴행성 뇌질환, 암 및 노화 연구를 좀 더 이해하는데 기여할 수 있을 것으로 사료된다.

Ⅰ. Introduction

In response to various stimuli under the physiological or stress conditions such as elevated temperatures, oxidants, heavy metals and baterial and viral infection, the heat shock transcription factors (HSFs) regulate the dynamic expression of different heat shock proteins (HSPs) which are responsible for the subsequent downstream effects including stress-related cytoprotective functions, folding and assembling of nascent polypeptides and intracellular transport of proteins (1-4).

Heat shock factor 2(HSF2), belonging to the family of HSFs, had been proved to play a key role in regulating the ubiquitin proteasome, development and differentiation (1,2,5). HSF2 is expressed abundantly and is activated in stem cells and embryonic carcinoma cells and during embryogenesis and spermatogenesis (1,6,7). The transcription of HSF2 is complicated by the existence of two isoforms generated by alternative splicing events, HSF2-α and HSF2-β. The ratio of HSF2-α and HSF2-β isoforms varies significantly between different adult tissues, such as brain, heart, and testis, suggesting that these two proteins are functionally distinct (1,4). Recently, like HSF1, HSF2 is activated during heat shock and induced upon exposure to proteasome inhibitors, and its deficiency increased the sensitivity of vertebrate cells to heat shock (8-11). Although HSF2 generally functions as a transcription factor, it also induces gene bookmarking such as hsp70i gene, as demonstrated in mitotic cells (12). Additionally, HSF2 has modulated expression of the heat-shock genes by interacting directly with HSF1 or HSF4 (13-16). Hsf2-/- mice display male hypofertile phenotype that was characterized by reduced testis size and brain abnormalities characterized by enlarged ventricles (6,17). However, little is known about the exact transcriptional regulation of HSF2 during cellular processes.

These results present the first direct evidence that HSF2 transcription is inhibited and regulated by an auto-regulatory mechanism through its own promoter regions, thus providing a new mechanism for the regulation of HSF2 from various cellular signals.

Ⅱ. Materials and Methods

1) Cell culture and reagents

Human K562 erythroleukemia cells were cultured in 5% CO_2 atmosphere at 37℃ in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). HEK 293 embryonic kidney cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) fetal bovine serum(FBS) as the growth medium. Hemin was purchased from Sigma (St Louis, MO). HSF1-/- and HSF2−/− MEF cells were maintained in DMEM with 10% FBS. HSF2−/− MEF cells were kindly provided by Dr. Valérie LALLEMAND-MEZGER(University Paris Diderot).

2) Plasmid constructs

Human HSF1 and HSF2 were generated by PCR amplification and subcloned into pcDNA3.1 plasmid. The deletion fragments of HSF2 promoter were constructed using PCR with human genomic DNA as the template. The deleted HSF2 promoters were PCR-amplified using the primers listed below. Forward primers: pGL3-HSF2-P1, 5′- CTAGCTAGCGCCAGTAGCATCTGCGTCATCT-3′; pGL3-HSF2-P2, 5′- GGAAAGGGCACATACTTTTGAGCTC-3′; pGL3-HSF2-P3, 5′- CTAGCTAGCACTCTCCCATTTACTTGCTGTGACTG-3′; pGL3-HSF2-P4, 5′- CTAGCTAGCCTAGTTCATTGGGTTGTTGTGAGGATTC-3′. Reverse primer 5′-AGCTCATTAGCCAAATGCATGAGCCTC-3′. The pGL3-HSF2-P5 was created using *HindIII* digestion as a template with pGL3-HSF2-P1.

3) Promoter-luciferase reporter assay

The pGL3-HSF2 (-2.68kb) promoter construct served as the template to generate a deletion construct in which the putative HSF-binding element (HSE) was deleted. Luciferase reporter assays were performed as described previously (15). The cells were grown in 12-well plates and cotransfected with HSF2 promoter-luciferase plasmid DNA and either pcDNA3.1-HSF1 or -HSF2. After 48 h transfection, cell lysates were analyzed for luciferase activities. The luciferase reporter assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) following the manufacturer's instructions. Each experiment was performed in triplicate and repeated twice.

4) RT-PCR and quantitative real-time RT-PCR

Total RNA was prepared using the TRIzol Reagent (Invitrogen, CA, USA). Total RNA (1 μg) was treated with DNase (Promega, Madison, WI, USA) and reverse transcribed using Maxime RT PreMix (iNtRON, Korea). Following were primers used for RT-PCR analysis: hHSF2-ORF- forward, 5'-TAGAGAACCCACTGCTTACTGG-3'; hHSF2-ORF-reverse 5'-GTTGCTCATCCAAGACCAGAA-3'; hHSF2-endo-forward, 5'-CCCCAGGAAGTGGACTTTACATGTA-3'; hHSF2-endo-reverse, 5'-TATGGAGCTGGAACCCTATCAGACA-3'. The following primers were used for quantitative real-time RT-PCR: hHSF2-ORF-forward, 5'-ATTCAGAGTGGAGAGCAGAATG-3'; hHSF2-ORF-reverse 5'-CTGGACAGCACTAGACATGAGA-3'; hHSF2-endo-forward, 5'-CCGCGTTAACAATGAAGCAG-3'; hHSF2-endo-reverse, 5'-CATTCTGGCTCCAGGTGATG-3'. After the reaction mixture was loaded into a glass capillary, the following cycling conditions were used initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing

at 55°C for 30 s and extension at 72°C for 10 s. In the final cycle, the melting curve was obtained by initially heating to 95°C and subsequently cooling to 40°C for 30 s. Our method was optimized for the relative quantification module of the LightCycler Software 4.0.

5) Immunoblotting

Cells were treated with hemin for indicated time or transfected transiently with pcDNA3 plasmids containing HSF1 or HSF2. Cells were then washed with PBS and harvested in lysis buffer. Samples containing equal amounts of protein were loaded into each lane of an SDS-polyacrylamide gel for electrophoresis and subsequently transferred onto a polyvinylidene difluoride membrane. The membranes were blocked and then incubated with antibodies. Antibodies against HSF1 and HSF2 antibodies were from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

6) Chromatin immunoprecipitation assay

The K562 cells were grown to nearly 80% confluence and cross-linked with formaldehyde (Sigma, St Louis, MO, USA) at room temperature for 10 min. The cross-linked chromatin was prepared with a commercial ChIP assay kit (EZ-Magna ChIP; Millipore, Billerica, MA, USA) and immunoprecipitated using 4 μg of normal rabbit anti-IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 4 μg of anti-HSF2 antibody (Santa Cruz Biotechnology). The HSF2-binding site was PCR-amplified using the input DNA or DNA isolated from the precipitated chromatin as the template in combination with primers flanking the putative HSF-binding sites in the HSF2 promoter. The forward primer sequence was 5′-CTCTCCCATTTACTTGCTGTGACTGAAG-3′, and the reverse primer sequence was 5′- GAGCCCTTATATATGCCAAGGGCTTTAC-3′.

7) Purification of TAT-HSF2 fusion proteins

TAT-HSF2 protein was expressed in E. coli BL21(DE3) pLysS cells (Invitrogen, CA, USA) and was purified using the urea-denaturing protein purification method [19]. The cells were lysed via sonication in lysis buffer (1 mM imidazole, 100 mM NaCl, 20 mM HEPES, pH 8.0) containing 8M urea. The cell lysates were centrifuged at 12000 g for 30 min at 4℃ and 1 ml of Ni^{2+}-NTA agarose was added to the cleared supernatant. After 2h of gentle mixing at 4℃, the resins were transferred into a column and subsequently washed three times with 10 ml of washing buffer (20 mM imidazole, 300 mM NaCl, 50 mM phosphate buffer, pH 8.0). The proteins were eluted four times with 1 ml of elution buffer (500 mM imidazole, 300 mM NaCl, 50 mM phosphate buffer, pH 8.0). The urea denaturant was removed with a Mono-Q ionic exchange column and desalinated with a PD10 Sephadex size exclusion column. The protein concentration was quantified via the Bradford assay and confirmed by SDS - polyacrylamide gel electrophoresis.

8) Statistical analysis

All experiments were performed at least three times. All statistical analyses were carried out using Excel software. Mean values for experiments are expressed as mean ± SEM. A p-value <0.05, 0.01, 0.001 was regarded as statistically significant.

Ⅲ. Results

1) HSF2 transcription is negatively down-regulated by overexpression of HSF2

To examine whether HSF2 could directly regulate its own expression, K562 cells were transiently transfected with HSF2 expression plasmid and the expression levels of endogenous HSF2 mRNA were measured by RT-PCR and/or real time quantitative RT-PCR. The primer set used for endogenous HSF2 mRNA was designed to detect the 5'-UTR region of HSF2 mRNA. As shown in Fig. 1A, overexpression of HSF2 importantly inhibited the endogenous HSF2 mRNA. Hemin is one of the well-established inducers for HSF2 in K562 cells. Consistent with previous study, hemin treatment remarkably induced the expression of endogenous HSF2 mRNA. Additionally, K562 cells with HSF2 overexpression under hemin treatment displayed inhibited levels of hemin-induced endogenous HSF2 mRNA. However, the levels of the open reading frame (ORF) HSF2 presented similar expression levels compared with HSF2 overexpressed or hemin treated cells (Fig. 1A).

To confirm this observation, we performed quantitative real-time RT-PCR with prepared total RNA. There was no significant change in the expression levels of the ORF HSF2 mRNA in HSF2 overexpressed and/or hemin treated cells (Fig. 1B). Whereas increased endogenous HSF2 levels by hemin were significantly decreased in HSF2 transfected cell (Fig. 1C). These data revealed that HSF2 has an ability to regulate its own transcriptional activity.

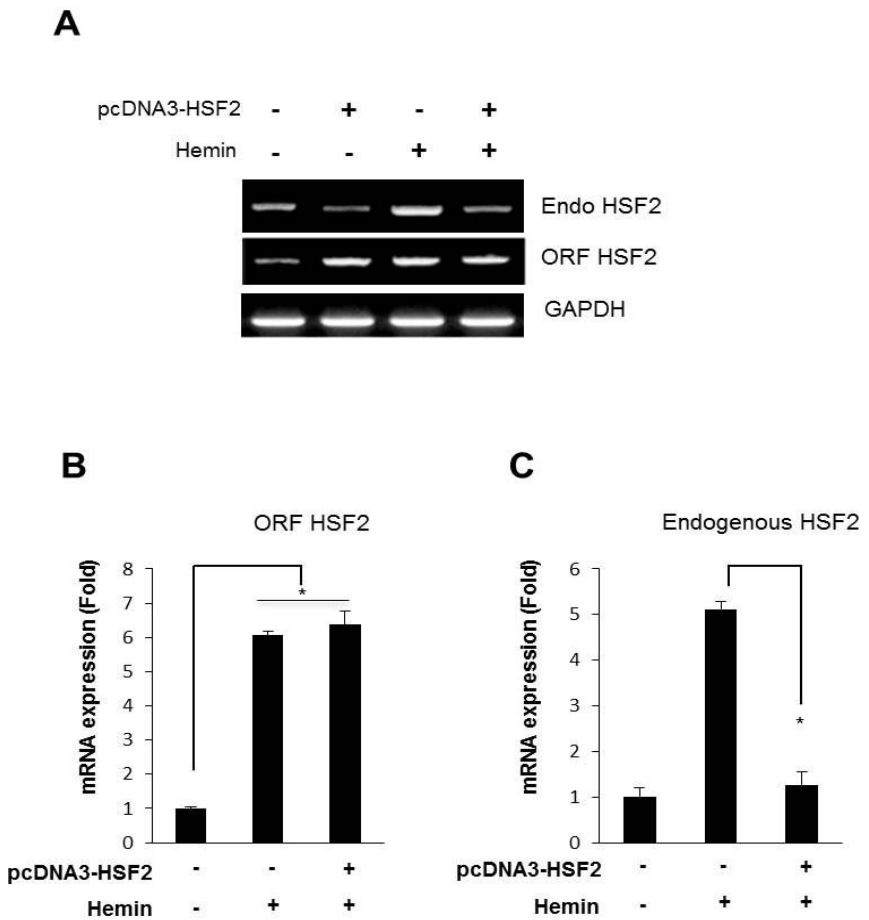

Figure 1. Enforced expression of HSF2 inhibits the endogenous HSF2 mRNA. (A) Expression levels of the endogenous HSF2 mRNA. K562 erythromyeloblastoid leukemia cells were transiently transfected with pcDNA3-HSF2 or/and treated with hemin. Forty-eight hours after transfection, total RNA were prepared and subjected to RT-PCR. For RT-PCR, GADPH was used as an internal control. (B, C) Quantitative real-time RT-PCR. Total RNA were prepared as in (A) and subjected to quantitative real-time RT-PCR to examine the expression levels of the exo- and endogenous HSF2 mRNA. The data are expressed as the mean ±SEM of the results from three separate experiments($* P<0.05$).

2) HSF2 regulates its own promoter

To investigate the effect of HSF2 on its own promoter, a plasmid expressing HSF2 was co-transfected with the human HSF2 promoter-luciferase construct into K562 cells. As shown in Fig. 2A, HSF2 protein levels increased in HSF2 transfected cells compared to control.

Overexpression of HSF2 strongly reduced the hemin-induced HSF2 promoter activity, indicating its own promoter-specific repression by HSF2 (Fig. 2B).

To further analyze concentration dependent activation of the HSF2 promoter by HSF2 overexpression, K562 cells were co-transfected with pcDNA-HSF2 and pGL3-HSF2 (2.68kb/+19). Co-transfection with a fixed amount of pGL3-HSF2 and increasing amounts of the expression plasmid pcDNA-HSF2 resulted in inhibited luciferase activity in a concentration-dependent manner. Similarly, cells transiently increasing amounts of the expression plasmid pcDNA3-HSF2 exhibited a marked reduction of the activity of HSF2 promoter compared with the cells treated hemin (Fig. 2C). These results strongly suggest that the promoter of HSF2 (at position 2.68kb/+19) contains a HSF2-responsive region, promoter activity is regulated by HSF2 in concentration-dependent manner.

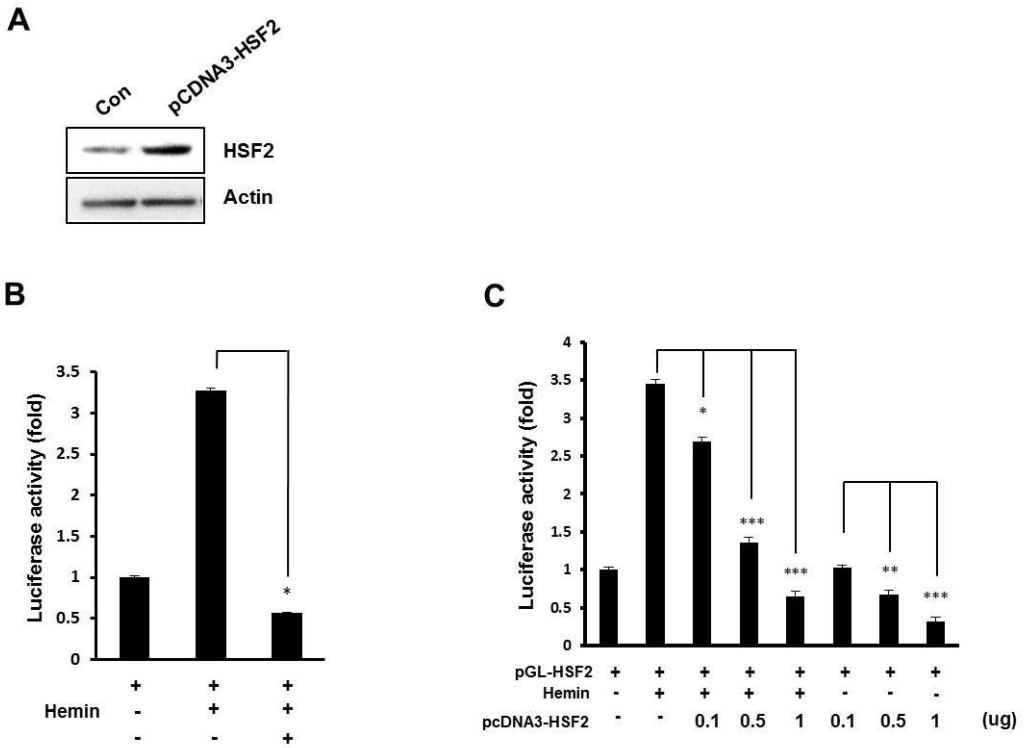

Figure 2. Endogenous HSF2 transcription is repressed by ectopic expression of HSF2 in K562 cells. (A) Expression levels of the pcDNA3-HSF2. K562 cells were transiently transfected with pcDNA3-HSF2 plasmid. Forty-eight hours after transfection, whole cell lysates were prepared and subjected to immunoblotting. Actin was used as a loading control. (B, C) Luciferase reporter assay. K562 cells were transfected with the pGL3-HSF2 (-2.68kb/+19) and Renilla luciferase reporter plasmid (pRL-TK) together with control vector or pcDNA3-HSF2 expression vector as indicated. Forty-eight hours after transfection, cells were lysed and their luciferase activities were measured. Firefly luminescence signal was standardized by the Renilla luminescence signal. Results are shown as fold induction of the firefly luciferase activity compared with control cells transfected with the empty plasmid. The data are expressed as the mean ±SEM of the results from three separate experiments(* P<0.05, * * P<0.01, * * * P<0.001).

3) HSF2 binds to the HSE sites of its Own Promoter

Since HSF2 is known as a transcriptional factor with a DNA-binding activity (18), It was then examined whether the HSF2 promoter contains typical HSF binding site. Sequence analysis of the HSF2 promoter has revealed one potential HSE sites at -1500/-1484 of the promoter region. It was then tested whether this HSE site play a role in negative regulation of HSF2 promoter. The HSF2 promoter reporter plasmid containing truncated HSE sites was constructed (Fig. 3A). The wild-type and mutated-type reporters were transfected into K562 cells for the promoter activity analysis. Interestingly, deletion of HSE site reduced the promoter activity to 60% relative to the wild-type promoter, indicating that this HSE sites act as critical region (Fig. 3B). Overexpression of HSF2 significantly decreased the luciferase activity expressed from the wild-type promoter, but not the HSE truncated- promoter (pGL3-HSF2-P4 and pGL3-HSF2-P5), indicating that HSE sites still contribute to the responsiveness to HSF2-mediated repression.

To further confirm these results, we performed a chromatin immunoprecipitation (ChIP) assay. The cross-linked genomic DNA prepared from HSF2 transfected K562 cells was subjected to ChIP assay. As clearly shown in Fig. 3C, PCR product containing the putative HSE region was specifically amplified, indicating that the exogenous HSF2 directly binds to the HSE site. IgG was employed as a positive control for this experiment. Additionally, the HSF2 promoter reporter (pGL3-HSF2-P1) plasmid containing a mutation of the HSE site was constructed (Fig. 3D). The mutated reporters were transfected into K562 cells to analyze promoter activity. Interestingly, the pGL3-HSF2 HSE mutant was not significantly reduced promoter activity by HSF2 overexpression (Fig. 3E). Accordingly, It was speculated that a binding to this HSE sites may mediate repression effect of HSF2 on its own promoter.

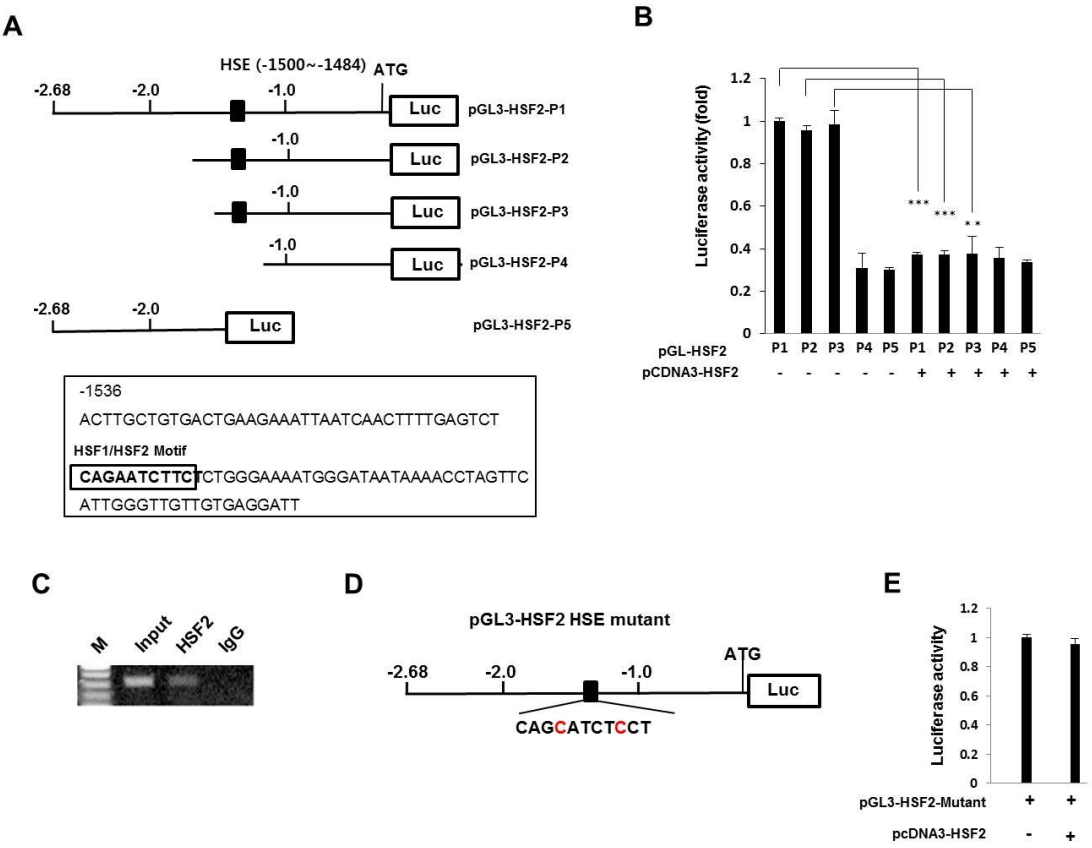

Figure 3. Transcriptional activity of HSF2 promoter deletion mutants. (A) Schematic representation of luciferase constructs containing various lengths of the HSF2 promoter region is shown on the left. Putative HSF transcription factor binding sites (HSE) are shown on the diagram. (B) Luciferase activity was measured using the Luc ELISA kit, normalized with luciferase activity derived from pCMV. The data are expressed as the mean ±SEM of the results from three separate experiments($*P<0.05$, $**P<0.01$, $***P<0.001$). (C) Chromatin immunoprecipitation-PCR analysis. Chromatin was prepared and immunoprecipitated with specific antibodies against HSF2 or IgG. The input DNA and DNA isolated from the precipitated chromatin were amplified by PCR and separated on a 1.5% agarose gel. Lanes: M, marker; 1, input; 2, HSF2 antibody; 3, IgG (negative control). (D, E) pGL3-HSF2 promoter assay with HSE motif point mutation constructs in K562 cells. Cells were transfected with wild-type pGL3-HSF2 promoter or point mutated pGL3-HSF2 (as indicated). pRL (Renilla luciferase) plasmid was co-transfected as an internal control. The cells were harvested 48 h after transfection. The promoter activity of each preparation was normalized to the Renilla value. The relative promoter activity is averaged from at least three independent experiments.

4) HSF1 is partially involved in the regulation of HSF2 promoter activity

HSF1 is a transcription factor that contains a DNA binding domain and has DNA-binding activity at the same DNA sequences (HSE) as HSF2 (18). To further analyze transcriptional regulation of the HSF2 promoter by HSF1, cells were co-transfected with pcDNA-HSF1 or -HSF2 and pGL3-HSF2-P1. First, K562 cells were transfected by HSF1 expression plasmid and subjected to RT-PCR and western blot analyses. The levels of HSF1 protein increased in HSF1 transfected cells compared to vector transfected cells (Fig. 4A). As shown in Fig. 4B, similar to HSF2 overexpression, HSF1 also inhibited the levels of endogenous HSF2 mRNA. In addition, HSF1 overexpressed cells with hemin treatment inhibited the levels of hemin-induced endogenous HSF2 mRNA. In accordance with the results from RT-PCR experiment, cells expressing HSF1 exhibited a marked reduction of the promoter activity of HSF2 (Fig. 4C). Overexpression of HSF2 resulted in a stronger its own promoter inhibition compared to HSF1. These results show that HSF2 is a more potent transcriptional inhibitor of HSF2 than the HSF1. In addition, like HSF2, increasing amounts of the expression plasmid pcDNA-HSF1 resulted in inhibited luciferase activity in a concentration-dependent manner (Fig. 4D).

To examine whether the sequence containing HSE site of HSF2 promoter can be recognized by HSF1, It was performed the ChIP assays. The characterized anti-HSF1 antibody were used to immunoprecipitate chromatin from HSF1 transfected cells, and the associated DNA fragments were amplified using primers flanking the HSE sites contained in the HSF2 promoter. An expected band was observed, thus suggesting that HSF1 is also associated with its putative HSE site (Fig. 4E).

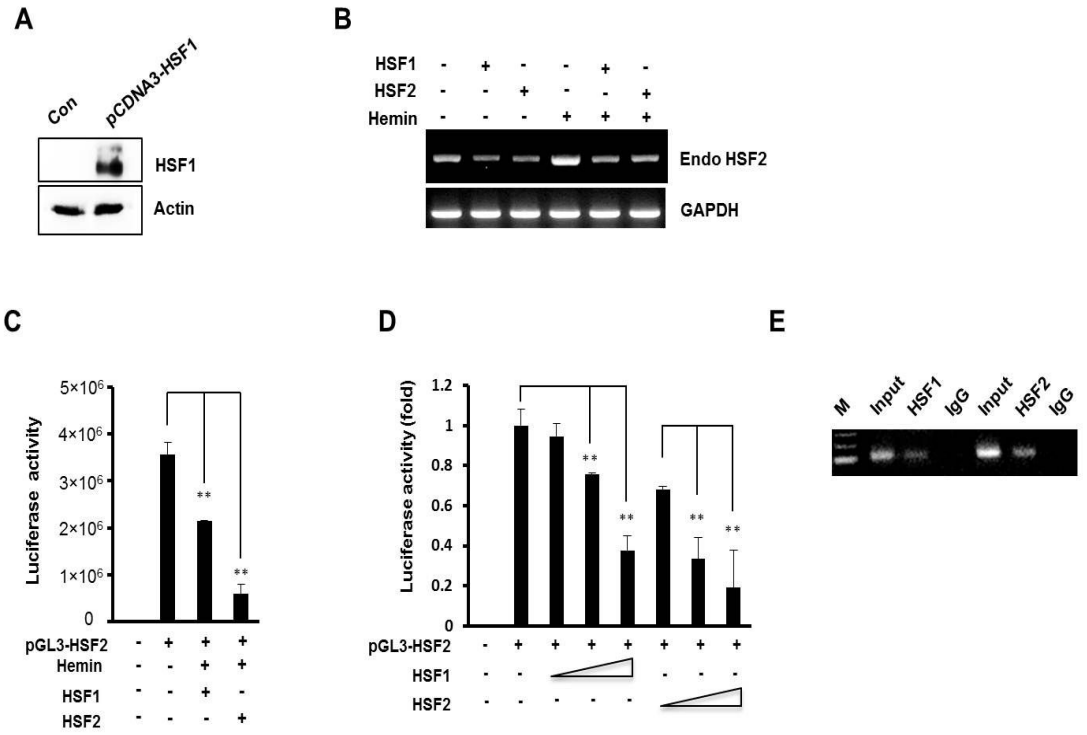

Figure 4. Endogenous HSF2 is also inhibited by overexpression of HSF1. (A) Expression levels of the pcDNA3-HSF1. K562 cells were transiently transfected with pcDNA3-HSF1 plasmid. Total lysates as described above were subjected to Western blot analysis using antibodies against HSF1. (B) Expression effects of endogenous HSF2 by HSF1. Total RNA were prepared as in Fig.1 and subjected to RT-PCR to examine the expression levels of the endogenous HSF2 mRNA. (C, D) Effects of HSF1 and HSF2 on HSF2 promoter assay. K562 cells were transfected with the pGL3-HSF2 (-2.68kb/+19) and pCMV-β-Gal together with control vector or pcDNA3-FLAG-HSF1 or HSF2 expression vector as indicated. After 48 h transfection, the luciferase activity was normalized by Renilla luminescence activity in each sample. The data are expressed as the mean ±SEM of the results from three separate experiments(* * $P<0.01$). (E) ChIP assays to detect in vivo binding of HSF1 to HSF2 promoter. Chromatin was prepared and immunoprecipitated with specific antibodies against HSF1 or IgG. The input DNA and DNA isolated from the precipitated chromatin were amplified by PCR and separated on a 1.5% agarose gel. HSF2 used as a positive control).

5) Both HSF1 and HSF2 are recruited to the HSF2 promoter and regulate its promoter activity

In previous study, it was shown that HSF1 and HSF2 can form heterotrimers and bind to DNA under a proteasome inhibitor treatment (13,14). It was raised the question whether HSF2 transcriptional activity is synergistically inhibited by complexes of HSF1 and HSF2 protein. The expression plasmids, pcDNA-HSF1 and/or pcDNA-HSF2, were co-transfected with HSF2 promoter genes in HSF1(-/-) or HSF2(-/-) MEFs. As expect results, in MEF wild type cells, HSF2 transcriptional activity was significantly inhibited approximately 40 % and 80 % in overexpression of HSF1 or HSF2, respectively. However, it was decreased more than HSF2 transfection alone after co-transfection with HSF1 and HSF2 (Fig. 5A). Similar results were also seen in the MEF HSF1(-/-) cells that were transfected with HSF2 promoter luciferase construct (Fig. 5B). Likewise, HSF2 transcriptional activity was strongly inhibited when transfected with HSF2 in MEF HSF2 (-/-) cells (Fig. 5C). Especially, it was significantly decreased the HSF2 transcriptional activity after co-transfection with HSF1 with HSF2 in all MEF cells. Clearly, these results suggested that HSF2 negatively auto-regulates its own promoter through interplaying with or without HSF1.

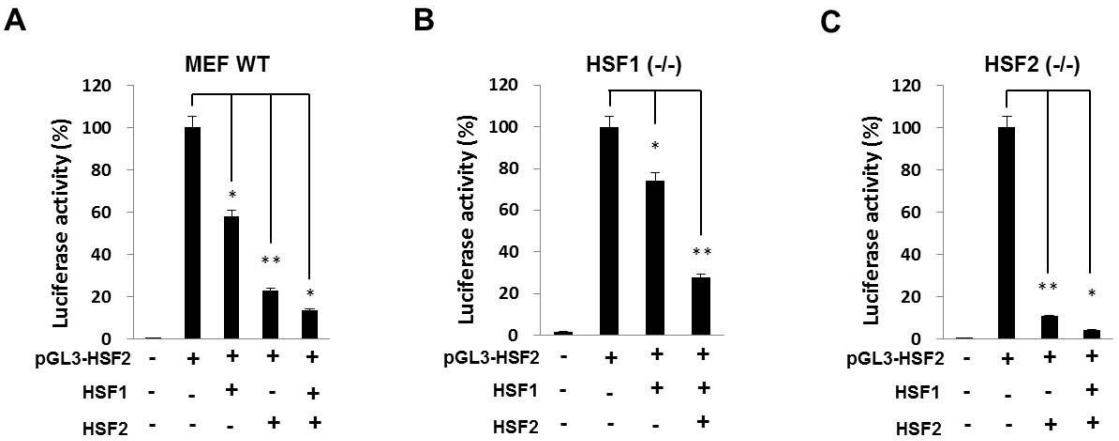

Figure 5. Relative luciferase activity of HSF2 promoter with or without HSF1 or HSF2 in MEF cells. (A) MEF wild type, (B) MEF HSF1(-/-), and (C) MEF HSF2(-/-) cells were transfected with the pGL3-HSF2 (-2.68kb/+19) together with control vector or pcDNA3-HSF1 and HSF2 expression vector as indicated. Forty-eight hours after transfection, cells were lysed and their luciferase activities were measured. The data are expressed as the mean ±SEM of the results from three separate experiments(* P<0.05, * * P<0.01).

6) Transduction of Tat-HSF2 fusion protein functionally induces suppression of HSF2 transcriptional activity

To determine whether the HSF2 transcriptional activity is functionally inhibited by HSF2 protein, it was assessed the effects of TAT-HSF2 protein in MEF cells. The purified TAT-HSF2 fusion protein was added to the MEF cells for 24 h and then the level of transduced HSF2 was determined by western blot analysis. As shown in Fig. 6A, TAT-HSF2 was delivered successfully into the MEF cells in a dose-dependent manner. Consistent with Fig. 5, hemin alone exerted the induction of HSF2 transcriptional activity. However, the TAT-HSF2 transduced cells inhibited their transcriptional activity by hemin treatment (Fig. 6B).

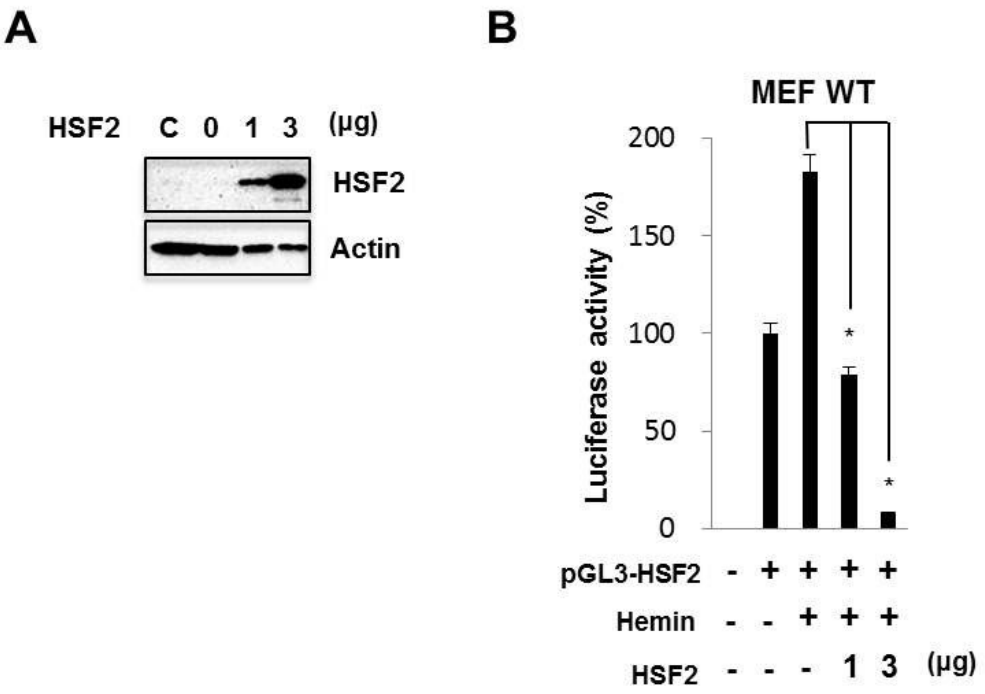

Figure 6. Transduction of purified TAT-HSF2 protein inhibits HSF2 promoter activity in MEF cells. (A) MEF wild type cells were transduced with 1 or 3 μg of TAT-HSF2 protein for 24 h. Transduced TAT-HSF2 was detected by immunoblotting. (B) Cells were pre-treated with TAT-HSF2 for 6 h and exposed to hemin for 24 h. Luciferase activity of HSF2 promoter was measured. The data are expressed as the mean ±SEM of the results from three separate experiments(* P<0.05).

Ⅳ. Discussion

HSF2 is a transcription factor that displays a tightly regulated gene expression. Its expression can be stimulated by physiological signals triggered by differentiation or development of the tissues (1, 19, 20), but also by environmental stress conditions, such as heat shock or proteasome inhibition (10, 11). The HSF2 gene promoter contains many putative responsive elements (21). Nevertheless, the precise transcription factors involved in the regulation of the HSF2 transcription during various stimuli remained unknown.

In this study, it was showed that HSF2 transcription is auto-regulated by its overexpression in a negative manner. However, unlike other transcription factors, here it was shown that the expression of HSF2 regulates its own promoter through its interaction with HSE site. These results also suggest that HSF2 negatively modulates HSE-mediated transcription through HSF1-HSF2 protein interaction or HSF2 alone with direct binding to its recognition sequence. On the other hand, HSF2 transcription is partially repressed by HSF1. Chromatin immunoprecipitation assays showed that HSF2 can bind to its own promoter, thus providing evidence for the hypothesis of an auto-regulatory mechanism at the transcriptional level, and promoter reporter analysis confirmed that the binding of HSF2 to promoter was mediated by one the putative HSE sites.

The previous study has shown that HSF4a was able to inhibit hemin-induced HSF2 mRNA and protein expression (15). Based on the results of previous study, it was suggested that HSF2 expression could be regulated by HSFs family and regulated by transcriptional and/or functional relationship between the HSFs. It is also possible that overexpressed HSFs may regulate HSF2 expression by preventing HSF2 inducer or by the expression of other factors controlled by HSF4a-mediated signaling. An alternative explanation could denote the presence of post-translational modifications, such as phosphorylation and/or sumolyation that may stabilize the binding of HSF2 to promoter (22).

It was reported that HSF1-HSF2 heterocomplexes provided a switch that incorporates transcriptional activation in response to stress and developmental stimuli (13). Previous studies showed that HSF2 is associated with HSF1 to activate the HSP70 promoter *in vitro* and *in vivo* (14,16). It has been shown that, under various stimuli, HSF1 and HSF2 may interact and form heterocomplexes that could be recruited to specific promoters (15). For example, in endothelial cells, AIRAP transcriptional level is regulated by HSF1-HSF2 heterotrimeric complexes after anticancer drug bortezomib treatment, proposing a close functional relationship for these two factors. In addition, it was also suggested that HSF2 alone can regulate bortezomib induced AIRAP expression negatively (23). Proteasome inhibitor MG132 or the amino acid analogue AZC (L-azetidine-2-carboxylic acid) treatments induced the formation of a HSF1/HSF2 heterocomplex that bound to clusterin element, and increased both clusterin protein and mRNA levels in human glial cell line U-251 MG (24).

In this study, it is also showed that, under overexpression condition, both HSF1 and HSF2 are recruited to the HSF2 promoter. HSF2 promoter activity is more decreased by both HSF1 and HSF2 overexpression than each HSF1 or HSF2. It is suggest that HSF1 and HSF2 can interplay directly and form heterocomplexes to bind HSF2 promoter. In addition, HSF1 or HSF2 have already been shown to be capable of interacting together, and the trimerzation domains of HSF1 and HSF2 were found necessary for this physical interaction, suggesting that HSF1-HSF1 or HSF2-HSF2 complexes can be homotrimers (18,25).

The fact that HSF1 is able to inhibit HSF2 promoter activity in MEF HSF2 (-/-) cells indicates that HSF1 has an important role in HSF2 transcription that cannot be compensated by HSF2. Although the potential impact of HSF1 on stress-regulated HSF2 transcriptional expression is not yet well defined, at the transcriptional level, HSFs could positively and/or negatively modulate the expression of specific target genes as well as its own gene.

In conclusion, it has provided molecular evidence of an auto-regulatory mechanism that allows HSF2 to control its own expression. These findings may provide new sight to understanding of transcriptional adaptation of HSF2 involved

in neurodegenerative diseases associated with protein misfolding diseases such as huntington's disease and in ulcerative colitis.

Ⅴ. References

1. Akerfelt M, Trouillet D, Mezger V, Sistonen L. Heat shock factors at a crossroad between stress and development. Ann N Y Acad Sci. 1113:15-27, 2007.
2. Fujimoto M, Nakai A. The heat shock factor family and adaptation to proteotoxic stress. FEBS J. 277:4112-25, 2010.
3. Akerfelt M, Morimoto RI, Sistonen L. Heat shock factors: integrators of cell stress, development and lifespan. Nat Rev Mol Cell Biol. 11:545-55, 2010.
4. Pirkkala L, Nykänen P, Sistonen L. Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. FASEB J. 15:1118-31, 2001.
5. Pirkkala L, Alastalo TP, Zuo X, Benjamin IJ, Sistonen L. Disruption of heat shock factor 1 reveals an essential role in the ubiquitin proteolytic pathway. Mol Cell Biol. 20:2670-5, 2000.
6. Wang G, Zhang J, Moskophidis D, Mivechi NF. Targeted disruption of the heat shock transcription factor (hsf)-2 gene results in increased embryonic lethality, neuronal defects, and reduced spermatogenesis. Genesis 36:48 - 61, 2003.
7. Choi MR, Jung KH, Park JH, Das ND, Chung MK, Choi IG, Lee BC, Park KS, Chai YG. Ethanol-induced small heat shock protein genes in the differentiation of mouse embryonic neural stem cells. Arch Toxicol. 85:293-304, 2011.
8. Shinkawa T, Tan K, Fujimoto M, Hayashida N, Yamamoto K, Takaki E, Takii R, Prakasam R, Inouye S, Mezger V, Nakai A. Heat shock factor 2 is required for maintaining proteostasis against febrile-range thermal stress and polyglutamine aggregation. Mol Biol Cell. 22:3571-83, 2011.
9. Trinklein ND, Chen WC, Kingston RE, Myers RM. Transcriptional regulation and binding of heat shock factor 1 and heat shock factor 2 to 32 human heat shock genes during thermal stress and differentiation. Cell Stress Chaperones. 9:21-8, 2004.
10. Mathew A, Mathur SK, Morimoto RI. Heat shock response and protein

degradation: regulation of HSF2 by the ubiquitin-proteasome pathway. Mol Cell Biol. 18:5091-8, 1998.

11. Sistonen L, Sarge KD, Phillips B, Abravaya K, Morimoto RI. Activation of heat shock factor 2 during hemin-induced differentiation of human erythroleukemia cells. Mol Cell Biol. 12:4104-11, 1992.
12. Xing H, Wilkerson DC, Mayhew CN, Lubert EJ, Skaggs HS, Goodson ML, Hong Y, Park-Sarge OK, Sarge KD. Mechanism of hsp70i gene bookmarking. Science. 307:421-3, 2005.
13. Sandqvist A, Björk JK, Akerfelt M, Chitikova Z, Grichine A, Vourc'h C, Jolly C, Salminen TA, Nymalm Y, Sistonen L. Heterotrimerization of heat-shock factors 1 and 2 provides a transcriptional switch in response to distinct stimuli. Mol Biol Cell. 20:1340-7, 2009.
14. Ostling P, Björk JK, Roos-Mattjus P, Mezger V, Sistonen L. Heat shock factor 2 (HSF2) contributes to inducible expression of hsp genes through interplay with HSF1. J Biol Chem. 282:7077-86, 2007.
15. Kim SA, Yoon JH, Ahn SG. Heat shock factor 4a (HSF4a) represses HSF2 expression and HSF2-mediated transcriptional activity. J Cell Physiol. 227:1-6, 2012.
16. Haiying He, Fabrice Soncin, Nicholas G., Youlin Li, Aliki Siganou, Jianlin Gong, Steven A. Brown, Robert E. Kingston, Stuart K. Calderwood. Elevated Expression of Heat Shock Factor(HSF) 2A Stimulates HSF1-induced Transcription during Stress. J. Biol Chem. 278:35465-35475, 2003.
17. Kallio M., Chang Y., Manuel M., Alastalo T.-P., Rallu M., Gitton Y., Pirkkala L., Loones M.-T., Paslaru L., Larney S., et al. Brain abnormalities, defective meiotic chromosome synapsis and female subfertility in HSF2 null mice. EMBO J. 21:2591 - 2601, 2002.
18. Ahn SG, Liu PC, Klyachko K, Morimoto RI, Thiele DJ.The loop domain of heat shock transcription factor 1 dictates DNA-binding specificity and responses to heat stress. Genes Dev. 15:2134-45, 2001.
19. Morange M. HSFs in development. Handb Exp Pharmacol. 172:153-69, 2006.
20. Goodson ML, Park-Sarge OK, Sarge KD. Tissue-dependent expression of heat

shock factor 2 isoforms with distinct transcriptional activities. Mol Cell Biol. 15:5288-93, 1995.

21. Nykänen P[1], AlastaloTP, AhlskogJ, Horelli-KuitunenN, PirkkalaL, SistonenL. Genomic organization and promoter analysis of the human heat shock factor 2 gene. Cell Stress Chaperones. 6:377-85, 2001.
22. Yan-Ming Xu, Dong-Yang Huang, Jen-Fu Chiu, Andy T. Y. Lau. Post-translational modification of human heat shock factors and their functions: a recent update by proteomic approach. J. Proteome Res. 11:2625-2634, 2012.
23. Antonio Rossi, Anna Riccio, Marta Coccia, Edoardo Trotta, Simone La Frazia, M. Gabriella Santoro. The proteasome inhibitor bortezomib is a potent inducer of zinc-finger AN1-type domain 2a gene expression: role of HSF1/HSF2 heterocomplexes. J. Biol. Chem. 289:12705-15, 2014.
24. Loison F, Debure L, Nizard P, le Goff P, Michel D, le Dréan Y.Up-regulation of the clusterin gene after proteotoxic stress: implication of HSF1-HSF2 heterocomplexes. Biochem J. 395:223-31, 2006.
25. Ahn SG, Liu PC, Klyachko K, Morimoto RI, Thiele DJ.The loop domain of heat shock transcription factor 1 dictates DNA-binding specificity and responses to heat stress.Genes Dev. 15:2134-45, 2001.