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Grp78 is a Novel Downstream Target Gene of Hoxc8 Homeoprotein

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Previously, we have identified 14 putative downstream target genes of Hoxc8 homeoprotein in F9 murine embryonic teratocarcinoma cells through proteomics analysis. Among those, we tested a possibility of a DNA-k type molecular chaperone, *Grp78*, as a direct downstream target of Hoxc8, by cloning a 2.4 kb upstream region of murine *Grp78* into a reporter plasmid and by testing if Hoxc8 can regulate its expression. We observed that Hoxc8 proteins could transactivate the reporter gene, which was affected by small interference RNAs (siRNAs) against to *Hoxc8*, suggesting that *Grp78* is a novel downstream target of Hoxc8 *in vivo*.

Key Words: Hoxc8, Homeoprotein, Downstream target gene, Grp78, Luciferase reporter, siRNA

INTRODUCTION

Hox proteins are a subgroup of transcription factors carrying the DNA-binding homeodomain and play important roles in a variety of developmental processes during embryogenesis (Gehring and Hiromi, 1986; Hombria and Lovegrove, 2003). Nevertheless, only a few downstream targets that are directly regulated by Hox proteins have been identified, which include an upregulation of the progesterone receptor gene by Hoxa5, and the hair keratin by Hoxc13 during early trichocyte differentiation (Jave-Suarez et al., 2002), and an attenuation of *transglutaminase I* by Hoxa7 during keratinocyte differentiation (La Celle and Polakowska, 2001), and proliferating cell nuclear antigen (Pcna) and mouse homologue of the Schizophrenia susceptibility gene ZNF804A and Smad6 by Hoxc8 (Min et al., 2010; Chung et al., 2010; Kang et al., 2010).

As an effort to identify downstream targets of Hox proteins, we have conducted the proteomic analysis, focusing on Hoxc8, one of three members of the paralogous group VIII of *Hox* genes (Kwon et al., 2003). Similar to other Hox proteins, Hoxc8 has been shown to play a critical role in providing axial identities to the embryonic tissues that normally express *Hoxc8* (Le Mouellic et al., 1992; Tiret et al., 1998). In addition, Hoxc8 has also been shown to be involved in cartilage differentiation (Yueh et al., 1998), hematopoiesis (Shimamoto et al., 1999), and osteoblast differentiation (Yang et al., 2000). Through the proteomic analysis, we identified 14 putative downstream target genes that might be regulated by Hoxc8 (Kwon et al., 2003). Among those, we noticed that the expression patterns of *Glucose-regulated protein 78* (*Grp78*) are similar to those of *Hoxc8* during mouse embryogenesis (Kang et al., 2005), suggesting that *Grp78* may be a direct downstream target of Hoxc8 *in vivo*.

In this study, we tested whether Hoxc8 could directly regulate the expression of *Grp78*. We observed that *Grp78* mRNA expression was increased by overexpression of Hoxc8 in F9 murine embryonic teratocarcinoma cells. Thus, we further examined if Hoxc8 could regulate the expression of a reporter whose transcription is under the control of the 2.4 kb regulatory sequence of murine *Grp78*. Our results showed that the reporter expression was up-regulated by Hoxc8 in F9 cells, and the upregulation was abolished when the upstream sequence of *Grp78* was inserted in reverse

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orientation. In addition, the transcriptional activity of Hoxc8 on the *Grp78* reporter construct was partially attenuated by applying the siRNA against to *Hoxc8*. These results strongly suggest that Hoxc8 directly regulates transcription of *Grp78* by acting on the 5' upstream regulatory domain of *Grp78 in vivo*.

MATERIALS AND METHODS

Expression plasmids and siRNAs

The plasmid expressing murine Hoxc8 has been described previously (Kwon et al., 2003). To construct Grp78-Luc reporter plasmids, the 2.4 kb DNA fragment containing 2.2 kb 5' upstream sequence and 0.2 kb coding sequence of murine *Grp78* was amplified by PCR from mouse genomic DNA and cloned into the pGL2 promoter vector (Promega) either in the correct or reversed orientation (Fig. 3). *Hoxc8* siRNAs were designed and purchased from Samchully Pharm. co., LTD. (Seoul, Korea). Sequences of the siRNAs are as follows: siHoxc8-1 sense 5' - AGA CGC CUC CA AUU CUA U - 3', antisense 5' - AUA GAA UUU GG GGC GUC U - 3', siHoxc8-2 sense 5' - GUA UCA GAC CUU GGA ACU A - 3', antisense 5' - UAG UUC CCA GGU CUG AUA C - 3'.

Cell culture and transfection

Murine embryonic teratocarcinoma F9 cells were maintained in Dulbecco's modified Eagle's medium (DMEM: Gibco BRL, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL) at 37℃ in a 5% CO_2 incubator. Transfection was performed using a Lipofectamine Plus™ Agent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

Luciferase reporter assay

F9 cells (1×10^5 cells per well of a 12 well plate) were transiently transfected with 10 ng renilla luciferase control vector (for adjusting transfection efficiency), 0.5 μg Hoxc8 expression plasmid, and 0.5 μg luciferase reporter plasmid with or without 10 nM siRNAs, as indicated. Luciferase activities were assayed 24 h after transfection by using the luciferase assay kit (Promega) according to the manufacturer's instructions. The results were obtained from three independent experiments, each with duplicates.

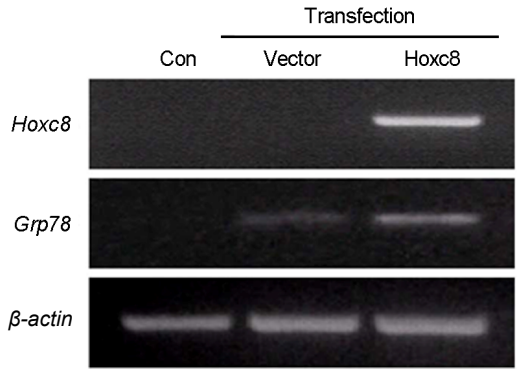


Fig. 1. Upregulation of *Grp78* by overexpression of *Hoxc8* in F9 cells. Murine embryonic teratocarcinoma F9 cells were transfected with either an empty vector (pcDNA3) or with the vector encoding murine *Hoxc8*. Expression levels of *Hoxc8* or *Grp78* were analyzed by semi-quantitative RT-PCR. Increased expression of *Hoxc8* was obvious in the cells transfected with Hoxc8 plasmids. *Grp78* expression was upregulated in the cells transfected with Hoxc8 plasmids, compared to the cells transfected with the empty vector or untransfected. There was no change in expression levels of *β-actin* in all conditions.

RESULTS AND DISCUSSION

In order to test whether *Grp78* is a downstream target of Hoxc8, we first observed if *Grp78* expression could be enhanced by overexpression of Hoxc8 proteins. F9 cells were transfected with the plasmid expressing Hoxc8, and expression levels of *Grp78* were accessed by semi-quantitative reverse transcriptase RCR (RT-PCR). We observed that *Grp78* expression was increased in the cells that were transfected with the Hoxc8 expression vector compared to those either transfected with an empty vector or untransfected (Fig. 1), indicating that overexpression of Hoxc8 proteins can activate the transcription of *Grp78* in F9 cells.

This observation prompted us to analyze the upstream regulatory sequence of *Grp78* and examine if the consensus Hox binding motifs are present in that region. There are several putative Hox binding motifs (TAAT, ATTA) within the 2.2 kb 5' upstream regulatory domain of murine *Grp78* (Fig. 2). We also found a Hox/PBC consensus binding sequence TGATNNA[g/t][g/a], to which Hox homeoproteins

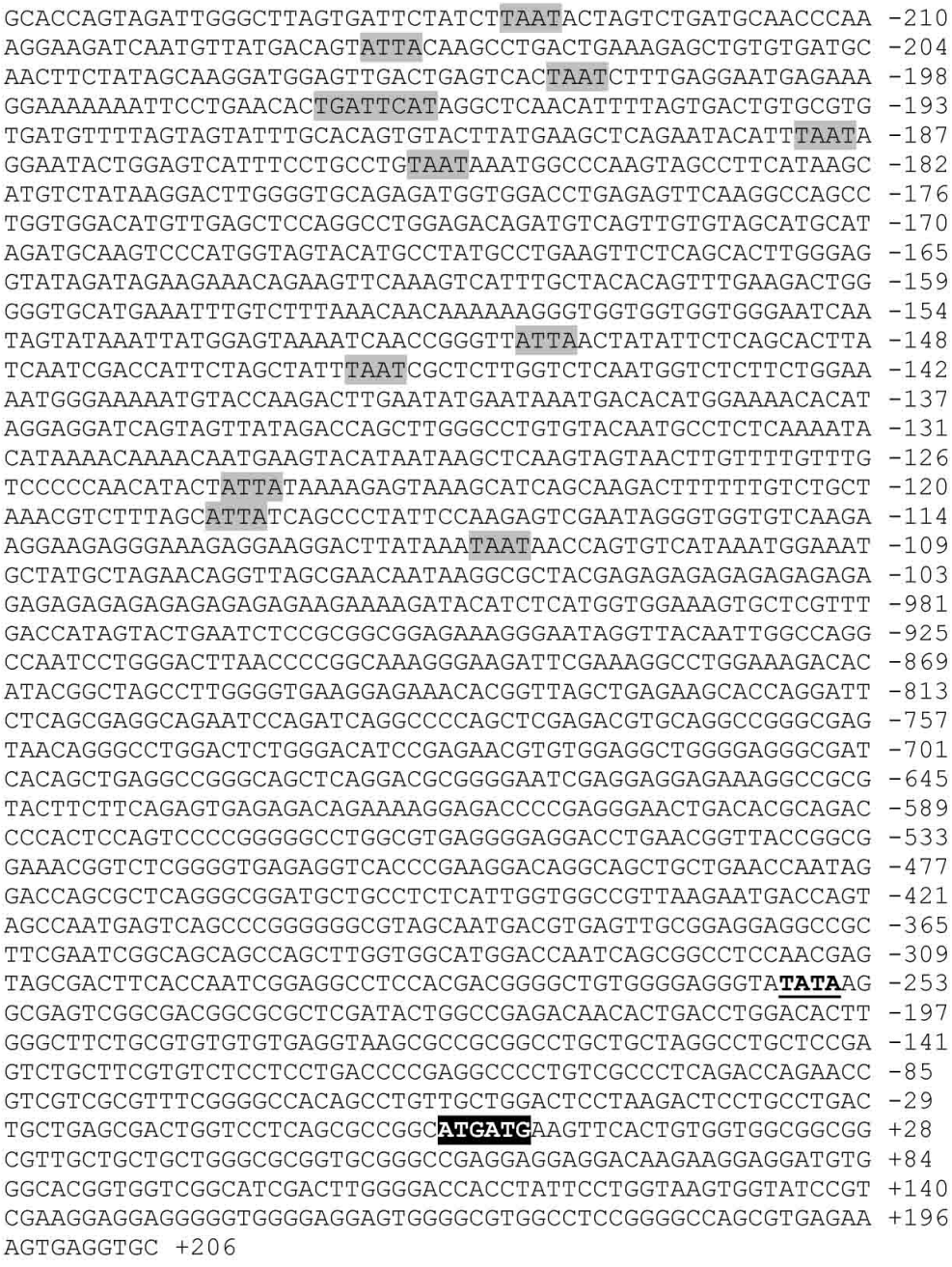


Fig. 2. Sequence analysis of the upstream regulatory region of murine *Grp78* gene. Putative Hox binding core motifs and the start codon are designated either by gray or black boxes. The TATA box is underlined.

and their cofactors such as PBC family proteins cooperatively bind (Mann and Affolter, 1998). In order to directly test if Hoxc8 can regulate gene expression controlled by the upstream regulatory sequence of *Grp78*, we amplified a 2.4 kb DNA fragment encompassing 2.2 kb *Grp78* upstream sequence as well as 0.2 kb *Grp78* coding sequence (Fig. 3). The 2.4 kb regulatory sequence was inserted in the pGL plasmid (Grp78-Luc), in which the expression of fruitfly *luciferase* gene is under the control of the inserted promoter sequence (Fig. 3B). The reporter plasmid carrying the *Grp78* upstream sequence in a reverse orientation (revGrp78-Luc) was also constructed (Fig. 3B). We observed that the luciferase activity was increased by Hoxc8 in the cells transfected with the Grp78-Luc reporter plasmid, whereas

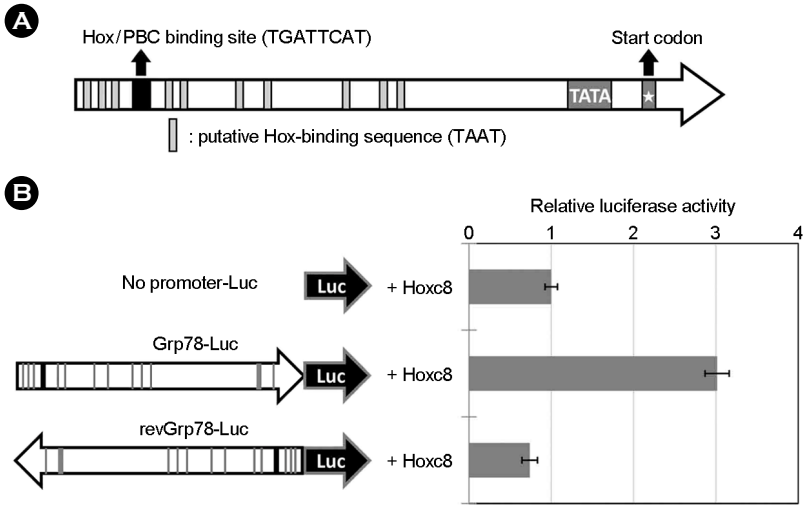
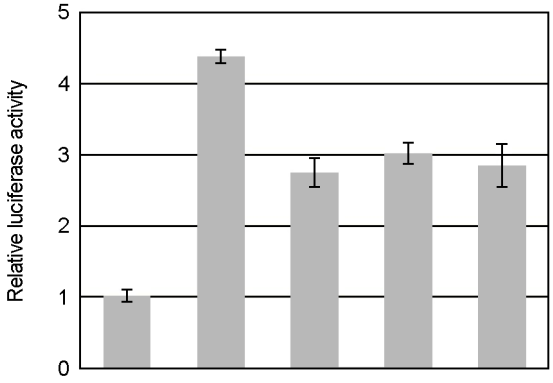


Fig. 3. Transcriptional activity of the *Grp78* upstream region by Hoxc8 in F9 cells. (A) Schematic diagrams of the 2.4 kb *Grp78* upstream sequence, showing the location of putative Hox binding sites, TATA box, and the start codon of *Grp78*. (B) The *Grp78* upstream sequence was inserted in pGL plasmid (Grp78-Luc), in which the expression of the reporter, *luciferase*, is subject to the control of the inserted promoter sequence.

there was no increase in the cells transfected with the reporter carrying the *Grp78* upstream sequence in a reversed orientation (Fig. 3B). These results indicate that Hoxc8 can directly regulate the downstream genes under the control of *Grp78* upstream promoter region.

To test whether the activation of the Grp78-Luc reporter by Hoxc8 is mediated through the specific transcriptional activation by Hoxc8, we knocked down the expression level of *Hoxc8* using the small interference RNA (siRNA). We designed two different siRNA molecules against two regions within the homeodomain of Hoxc8. Treating with either of the siRNAs reduced the increased luciferase expression by Hoxc8, suggesting that the increased expression of the Grp78-Luc reporter was mediated by the transcriptional activity of Hoxc8. There was no additive effect of treating both of the siRNAs, suggesting that either one of the siRNAs is sufficient to exert its maximum inhibitory effect on *Hoxc8* expression.

Previously, we have identified 14 proteins that are either up- or down-regulated by Hoxc8 overexpression in F9 cells through the proteome analysis, Grp78 protein being one of them (Kwon et al., 2003; Min et al., 2010). Nevertheless, mRNA expressions of most of the identified proteins were not regulated by overexpression of Hoxc8 in F9 cells (data not shown), suggesting that Hoxc8 may not regulate their mRNA expressions directly, but rather indirectly via regulating other transcription factors. Among the proteins identified, *Grp78* appeared to be regulated by Hoxc8 (Fig. 1).



	Luc	Grp78-Luc			
Hoxc8	+	+	+	+	+
siHoxc8-1	-	-	+	-	+
siHoxc8-2	-	-	-	+	+

Fig. 4. Effects of *Hoxc8* siRNAs on the *Grp78* promoter expression in F9 cells. F9 cells were transfected with the Grp78-Luc reporter, Hoxc8 expression vector, together with or without *Hoxc8* specific siRNAs. Two different siRNAs were designed to hybridize to the homeoxox sequence of *Hoxc8*.

In addition, *Grp78* mRNA expression patterns during mouse embryogenesis are similar to those of *Hoxc8* temporally and spatially (Kang et al., 2005). Consistently, we observed several putative Hox-binding motifs within the *Grp78* upstream regulatory domain (Fig. 2). We also observed that Hoxc8 was able to upregulate the reporter gene whose expression is under the control of *Grp78* upstream regulatory sequence, and this regulation was dependent on the

correct orientation of the regulatory sequence (Fig. 3). Based on our observations, we propose that the upregulation of Grp78 proteins by Hoxc8 overexpression in F9 cells and the similar expression patterns between *Grp78* and *Hoxc8* during mouse embryogenesis are mediated by a direct transcriptional regulation of *Grp78* gene by Hoxc8 homeoprotein.

Grp78 is a major endoplasmic reticulum (ER) chaperone, which transiently and non-covalently binds to nascent polypeptides to facilitate proper folding and assembly, and also to prevent deleterious aggregations (Ellis and van der Vies, 1991). In addition, Grp78 plays a role in the cellular response to stress such as glucose deprivation or depletion of ER Ca^{2+} (Hightower, 1991). Recently, Grp78 has also been shown to be related to cancer and suggested as a therapeutic target of human cancers (Lee, 2007). Thus far, it is not clear how the regulation of *Grp78* expression by Hoxc8 homeoprotein contributes to such functions in the body. Detailed analyses of expression patterns of *Hoxc8* and *Grp78* during embryonic stages will provide insights into possible functional relationships between Hoxc8 homeoprotein and its putative downstream target Grp78 during embryogenesis.

Acknowledgements

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