Grp78 is a Novel Downstream Target Gene of Hoxc8 Homeoprotein

Jin Joo Kang¹, Jinwoong Bok¹ and Myoung Hee Kim†

Department of Anatomy, Embryology Lab., Brain Korea 21 Project for Medical Science
Yonsei University College of Medicine, Seoul 120-752, Korea

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
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 5' upstream 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°C in a 5% CO2 incubator. Transfection was performed using a Lipofectamine Plus™ Agent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

Luciferase reporter assay

F9 cells (1 × 10³ 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.

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 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.

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.

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 TGATNNAg(T|G)a, to which Hox homeoproteins
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 of Grp78 upstream sequence as well as 0.2 kb of Grp78 coding sequence (Fig. 3).

The 2.4 kb regulatory sequence was inserted in the pGL3 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. 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.](image)
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).

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

This work was supported by grants from 2008-0058561 (2010-0000155) and 2010-0026759 from National Research Foundation (NRF), and partly by 20070401-034-030 from the BioGreen21 Program, RDA, Korea.

REFERENCES