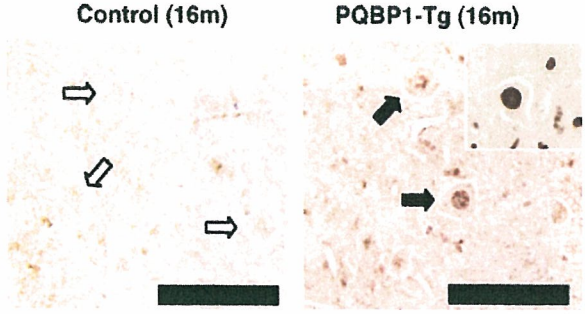

Fig. 6 p53 is increased in spinal motor neurons of PQBP-1 Tg mice. Expression of p53 in the nuclei of spinal motor neurons of PQBP-1 Tg mice at 16 months. Paraffin sections of the spinal cord were stained with p53 antibody. Motor neurons (largest neurons in the anterior horn) with nuclear staining were clearly increased in PQBP-1 Tg mice (right panel). The inlay shows immunohistochemistry with anti-phospho-p53 (Ser389) antibody, indicating that the nuclear staining corresponded to an increase in p53 phosphorylated at Ser389. Unstained neurons in control mice and stained neurons in PQBP-1 Tg mice are indicated with white and black arrows respectively. Scale bars 10 μm.

and calculated the survival ratio, i.e. the total number of neurons on the injured side as a percentage of that on the uninjured side in the same animal. By using a nucleolus with a diameter smaller than 36 μm as a hallmark of a neuron, we excluded overlapped counting of neurons in serial sections. A remarkable reduction in neurons was found throughout the facial nucleus on the lesioned side, supporting the accuracy of the nerve section model. The number of residual neurons was decreased to about 9% of that on the control side in mock-treated animals. The loss of facial motor neurons was clearly repressed by HDGF (Fig. 5). Although residual neurons became small and pyknotic, the survival rate was more than 20% with HDGF and BDNF treatments (Fig. 5). These results support the trophic effect of HDGF on motor neurons *in vivo*.

p53 is increased in spinal motor neurons under degeneration

We next investigated the molecular mechanisms underlying the up-regulation of HDGF in spinal motor neurons. Because several studies have reported an increase in p53 immunoreactivity in spinal motor neurons in patients with ALS (see review by Sathasivam and Shaw 2005) and because we previously found mitochondrial dysfunction and delayed activation of caspase 3 in PQBP1 Tg mice (Marubuchi *et al.* 2005), we focused on this molecule and examined whether p53 is increased in presymptomatic PQBP-1 Tg mice at 12 months by using p53 antibody. Spinal motor neurons of Tg mice showed an increase in p53 from 12 to 16 months. At 16 months, we found that p53 strongly stained the nuclei of motor neurons of PQBP-1 Tg mice (Fig. 6).

Furthermore, we examined spinal cord tissues with antibodies against phosphorylated p53: anti-phospho-Ser15, -Ser20 and -Ser 389 antibodies. Anti-phospho-Ser389 antibody clearly stained the nuclei of spinal motor neurons in the Tg mice at 16 months (Fig. 6, inlay). Because phosphorylation at Ser389 is known to regulate transcriptional activity of p53 (Bruins *et al.* 2004), these findings suggest the involvement of p53 in the transcriptional regulation of HDGF in spinal motor neurons.

p53 up-regulates *HDGF* gene expression

We searched the upstream region of the *HDGF* gene for consensus binding sequences of transcription factors, and found five sequences that partially match the p53-binding consensus (e1–e5; Fig. S2a). We synthesized double-strand oligonucleotides (Fig. S2b) and tested binding of purified recombinant p53 to these sequences in gel mobility shift assays. p53 bound to the e2 sequence with a similar affinity to the p53 consensus sequence in the upstream of the *p21/waf1* gene (Figs 7a and b). The e1 sequence including the e2 sequence showed a weak interaction in which the shift of the e1 probe was similar to that of the e2 probe (Figs 7a and b), indicating that the binding core motif in the e1 probe is the e2 sequence.

We then constructed a CAT reporter plasmid containing the e1/2 sequence in the upstream of the *IFN* promoter and *CAT* gene (Fig. S2a). CAT assay with the reporter plasmid clearly showed that p53 up-regulated transcription of the *HDGF* gene through this *cis* element both in P19 and HEK293 cells (Figs 7c and d). These data suggest that p53 is a factor regulating *HDGF* gene expression and that the increase in p53 in spinal motor neurons of PQBP-1 Tg mice up-regulates transcription of the *HDGF* gene.

HDGF is increased in CSF

Finally, we investigated the connection between the findings that HDGF is a trophic factor for motor neurons (Figs 3–5) and that HDGF is increased in spinal motor neurons of PQBP-1 Tg mice (Figs 1 and 2). HDGF was originally purified from conditioned medium of the human hepatoma-derived cell line, HuH-7 (Nakamura *et al.* 1994), indicating that HDGF is released from cells although HDGF does not possess any signal sequence for secretion. In addition, Zhou *et al.* showed that HDGF is released from primary hippocampal neurons into the medium (Zhou *et al.* 2004). Therefore, we examined at 12 months whether the up-regulation of HDGF leads to an increase in CSF HDGF levels. Western blot analysis of age-matched littermates and Tg mice clearly showed an increase in HDGF in CSF (Fig. S3), suggesting that the increase in HDGF in spinal cord tissue leads to an increase in the CSF. The increase in HDGF in the CSF might directly lead to an increase in HDGF in the extracellular fluid of the CNS inside the blood–brain barrier, and it may support spinal motor neurons. We performed a similar experiment with PQBP-1 Tg mice at 2 months, but HDGF was not yet increased at this age

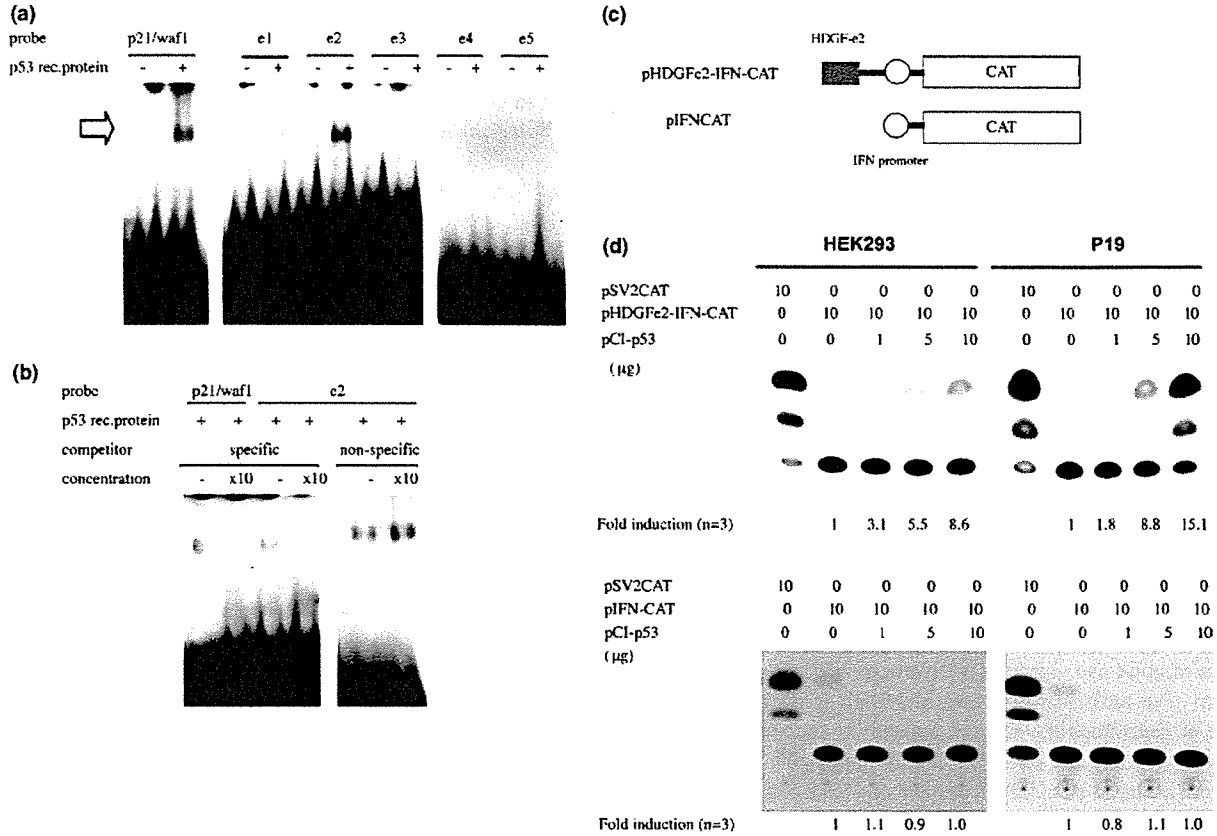

Fig. 7 p53 enhances gene expression via a binding site in the upstream region of the *HDGF* gene. (a) Gel mobility shift assays show definite binding of p53 to e2 and weak binding to e1. Human recombinant p53 protein (5 ng) was incubated with 10 000 cpm of each probe. The p53 consensus sequence in the upstream of *p21/waf1* gene was used as a positive control. (b) Competition by cold probe (10 times the concentration of hot probe) showed that the affinity of p53 for e2 was similar to that for the consensus from the *p21/waf1* gene. Non-specific cold probe did not impair the binding of p53 to e2. (c) Structures of pHDGFe2-IFN-CAT and pIFN-CAT. (d) CAT assays with P19 and HEK293 cells revealed that p53 transactivates gene expression from pHDGFe2-IFN-CAT (upper panels) but not from pIFN-CAT (lower panels). Together with gel mobility shift assay data, the CAT assay showed that p53 transactivated the *HDGF* gene through the e2 element. Total amounts of plasmids used for transfection were adjusted to 21 μg by addition of pBluescriptKS+. Three independent transfections were performed and the fold increase in p53 expression is shown beneath each panel.

(Fig. S3). This means that PQBP-1 expression at 2 months does not promote spinal neurons to up-regulate HDGF. We also tried to obtain CSF from PQBP-1 Tg mice at 20 months, but this was technically impossible because they were unable to tolerate the surgical procedure.

Discussion

We have shown in this study that HDGF is a novel trophic factor for motor neurons *in vitro* and *in vivo*. The effects of HDGF on survival and neurite extension of motor neurons (Figs 3 and 4) are equivalent to those of well known neurotrophic factors including CNTF and BDNF (Arakawa *et al.* 1990; Sendtner *et al.* 1990, 1992; Oppenheim *et al.* 1991, 1992; Yan *et al.* 1992; Henderson *et al.* 1993; Koliatsos *et al.* 1993; Mitsumoto *et al.* 1994). In addition, HDGF clearly supports survival of facial motor neurons in newborn rats after facial nerve section (Fig. 5). As far as we know, this is the first report that has proved HDGF to be a trophic factor for motor neurons.

HDGF is homologous to HMGB1 protein that is released from necrotic cells (Scaffidi *et al.* 2002). Zhou *et al.* recently reported that HDGF is released from primary hippocampal neurons under physiological conditions and that the release is accelerated in necrosis (Zhou *et al.* 2004). Like other HMG family proteins (Scaffidi *et al.* 2002), HMGB1 is not released in apoptosis (Zhou *et al.* 2004), suggesting that HMGB proteins have an extracellular function in a limited type of cell death. HDGF might also be released from neurons in a specific type of cell death distinct from apoptosis.

Regarding the pathological relevance of HGDF to motor neuron degeneration in PQBP-1 Tg mice, we have shown

that (i) HDGF is increased in the cytoplasm of spinal motor neurons of presymptomatic PQBP-1 Tg mice, (ii) HDGF protects spinal motor neurons *in vitro* and *in vivo*, and (iii) the release of HDGF into CSF is increased in PQBP-1 Tg mice. Collectively these data suggest that HDGF seems to play a protective role against motor neuron degeneration in presymptomatic PQBP-1 Tg mice.

We used mice of several ages of (2, 7, 12, 16 and 20 months) and obtained the following data. At 2 months, microarray analysis did not show an increase in HDGF (Marubuchi *et al.* 2005). The increase in HDGF was detected by microarray at 12 months (Fig. 1) and supported by immunohistochemistry at 12 and 16 months (Fig. 2). After 20 months, the increase in HDGF was not confirmed because of the neuronal loss (data not shown). Therefore, HDGF seems to increase transiently during the presymptomatic period and the transient increase might support survival of motor neurons. We also found expression of HDGF in motor neurons of mutant SOD1 Tg mice. However, general pathological roles of HDGF in other motor neuron neurodegenerations remain unknown, and we need further analyses to answer these questions.

The receptor and signaling pathways of HDGF are not yet known. The molecular structure of HDGF is distinct from those of neurotrophins, and the receptor is supposed to be completely different from trk family membrane tyrosine kinases (Barbacid *et al.* 1991; Meakin and Schooter 1992; Ip and Yancopoulos 1994; Chao 2003; Reichardt and Mobley 2004). Meanwhile, RAGE, the receptor for advanced glycation end-products, is known to be the receptor of HMGB proteins that are partially homologous to HDGF (Hori *et al.* 1995). RAGE activates intracellular signaling molecules such as extracellular signal-regulated kinases, p38 mitogen-activated protein kinase, c-Jun N-terminal kinase (JNK) and nuclear factor-κ (Simm *et al.* 2004). The binding of HMGB proteins to the RAGE induces the release of various cytokines including IL-6, tumor necrosis factor-α and transforming growth factor-β from macrophage/monocytes, fibroblasts and vascular smooth muscle cells (Rauvala *et al.* 1988), leading to transcriptional up-regulation of genes involved in apoptosis. Interestingly, however, RAGE-mediated signals also support neurite extension (Rauvala *et al.* 1988; Parkkinen and Rauvala 1991). However, it is still possible that HDGF, which lacks the HMG box conserved among HMG family proteins, interacts with completely different receptors and mediates totally distinct cell signals. HDGF does not possess any typical signal peptide; thus, although the detail is not known, secretion of HDGF from cells might be analogous to that of CNTF without signal peptide.

It is also possible that HDGF is taken up by some membrane transporter(s) and further transferred to the nucleus. We tested this hypothesis by immunostaining motor and cortical neurons cultured with recombinant HDGF or GST-HDGF fusion protein. However, no definite increase in nuclear staining was observed by immunocytochemistry with anti-HDGF or anti-GST antibody (data not shown), refuting this possibility. However, further analyses are required to enable us to understand the molecular mechanisms underlying the biological functions of HDGF.

p53 is implicated in various neurodegenerative disorders including ALS, a typical motor neuron degeneration (de la Monte *et al.* 1998; Martin 2000; Sathasivam and Shaw 2005). It is of interest that spinal motor neurons of presymptomatic PQBP-1 Tg mice showed a similar increase in p53 proteins in this study (Fig. 6) to those of patients with ALS (de la Monte *et al.* 1998; Martin 2000). The mechanisms underlying the increase in p53 protein in ALS are not yet known. The increase in p53 in PQBP-1 Tg mice was not due to transcriptional up-regulation because the level of p53 mRNA was not changed more than 1.5 fold at any time point (data not shown). Therefore, post-transcriptional regulation including translation and/or protein degradation might lead to the increase in p53 in PQBP-1 Tg mice. At 16 months, total p53 and Ser389-phosphorylated p53 (equivalent to phophorylation of human p53 at Ser392) were increased in the nuclei of spinal motor neurons of PQBP-1 Tg mice (Fig. 6). Phospho-p53 was weakly stained at 12 months (data not shown). Collectively, these results suggest post-transcriptional modification and/or degradation of p53 to increase expression of HDGF.

The role of p53 in motor neuron degeneration is still under debate (see review by Sathasivam and Shaw 2005). Increased p53 might trigger apoptotic cell death signaling, whereas cross-breeding of mutant SOD1 Tg mice with p53 knockout mice did not show any protective effect on neurodegeneration (Kuntz *et al.* 2000; Prudlo *et al.* 2000). On the other hand, cell-protective roles of p53 in various types of DNA repair have been recognized recently (see review by Sengupta and Harris 2005). Our finding that increased p53 in motor neurons up-regulates a trophic factor may shed light on the role of p53 in neurodegeneration and may promote understanding of the controversial functions of p53 in neurodegenerative disorders.

In summary, starting with microarray analysis of a mouse model of motor neuron disease, we found a novel trophic factor for motor neurons, HDGF, that is endogenously up-regulated in the affected neurons. The change in spinal motor neurons and CSF of presymptomatic Tg mice suggest that HDGF plays a role in the motor neuron degeneration in this mouse model. In addition, some of our results suggest a relevance of HDGF to ALS. Our data suggest that HDGF and its derivatives may be developed as candidate drugs to inhibit progression of motor neuron degeneration.

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Supplementary material

The following material is available for this paper online.

Fig. S1 (a) Preparation of recombinant mouse HDGF. Samples at various steps of production and purification were stained with CBB. G, GST protein expressed in E. coli; M, molecular weight marker; F, GST-HDGF fusion protein. GST-HDGF fusion protein was digested by Factor X from 3 to 16 h and recovered by heparin column. FT, flow through; W, wash; E, elute; PE, postelution wash. Arrow indicates recombinant HDGF. (b) Four antibodies against HDGF were characterized by western blotting using extracts of either fusion protein-expressing or non-expressing bacteria (upper panel). Arrow indicates the specific band of HDGF. The lower panel is a Coomassie-stained gel showing the amounts of sample blotted. C, non-transformed E. coli cells; H, recombinant HDGF-expressing E. coli cells.

Fig. S2 (a) Schematic structure of the upstream region of the mouse HDGF gene. Five possible binding sites for p53 (e1–e5) are indicated by white boxes. Exon 1 is indicated as a black box. (b) The sequences of e1–e5 used for the gel mobility shift assay are shown. The p53 consensus sequence in the upstream of the p21/waf1 gene was used as positive control.

Fig. S3 Western blot analysis of CSF protein with anti-HDGF antibody showed that HDGF is increased in the CSF of PQBP-1 Tg mice. CSF was centrifuged to exclude cells and then separated by SDS–polyacrylamide gel electrophoresis. We performed western blot with three Tg and littermate (C) mice. Hela cells expressing HDGF were used as a positive control (PC). (b) Band intensities were quantified by image analyzer and corrected with respect to those for β-actin. A representative case is shown in the left panel. The fold increase of HDGF in Tg mice ($n = 3$, 12 months) was calculated by defining the mean intensity of HDGF in the control group as 1.0. Right panel shows the mean ± SEM fold increase in HDGF in CSF of Tg mice.

This material is available as part of the online article from http://www.blackwell-synergy.com

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