Meteorin promotes the formation of GFAP-positive glia via activation of the Jak-STAT3 pathway

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Summary

Meteorin is an orphan ligand which has been previously reported to control neuritogenesis and angiogenesis, as well as gliogenesis. However, the precise function of this factor in CNS development and the underlying molecular mechanisms are poorly understood. Here, we demonstrate that meteorin is involved in GFAP-positive glial differentiation through activation of the Jak-STAT3 pathway, by using neurosphere and retinal explant culture systems. During embryonic brain development, meteorin is highly expressed in neural stem and radial glia cells of the ventricular zone and immature neurons outside the ventricular zone but its expression disappears spontaneously as development proceeds except in GFAP-positive astrocytes. In cultured neurospheres, meteorin activates STAT3, and in turn increases the transcriptional activity of GFAP by enhancing the binding of STAT3 to the promoter. By meteorin stimulation, differentiating neurospheres show increased numbers of GFAP-positive cells, but the effect is abrogated by a blockade of the Jak-STAT3 pathway using either a Jak inhibitor or STAT3 siRNA. Furthermore, we expand our findings to the retina, and show that meteorin increases GFAP expression in Müller glia. Together, our results suggest that meteorin promotes GFAP-positive glia formation by mediating the Jak-STAT3 signaling pathway during both cortical stem cell differentiation and retinal glia development.

Key words: Neural stem cell, GFAP, Astrocyte, Gliogenesis, Müller glia, Retina

Introduction

During the development of mammalian cerebral cortex, three major cell types, neurons, astrocytes and oligodendrocytes, are sequentially generated from the same progenitor cells of the neuroepithelium (Temple, 2001). The cell fate decision of a neural stem cell is controlled by the combination of intrinsic regulatory mechanisms and extrinsic signaling cues. In the early stage of cortical development, at around E14 in rats, basic helix-loop-helix (bHLH) transcription factors such as neurogenins play major roles (Sun et al., 2001), and extracellular cues such as nerve growth factor and bone morphogenetic protein (BMP) signal to the neural stem cells (NSCs) to turn on neurogenesis (Hall and Miller, 2004; Sauvageot and Stiles, 2002). However, at a later stage of development, around E17 to the perinatal stage, neurogenic programs are reduced; instead, astrogliogenic signals are turned on. In this transition, the levels of bHLH transcription factors are reduced, and the epigenetic inhibitions of gliogenesis disappear, which then allows the uptake of astrogliogenic signals from extracellular trophic factors (Nieto et al., 2001; Takizawa et al., 2001).

The Janus kinase (Jak)-signal transducer and activator of transcription (STAT) signaling pathway is a key mediator of astrocyte differentiation in NSCs. Interleukin-6 (IL-6) family cytokines, including leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1) and neuropoietin-1 (NP-1), are widely known to activate this pathway. They activate STAT3 through their co-receptor gp130, and activated STAT3 translocates to the nucleus, binds to the GFAP promoter, and finally promotes the transcription of GFAP (Nakashima and Taga, 2002; Sauvageot and Stiles, 2002; Taga and Fukuda, 2005). Besides IL-6 family cytokines, BMPs, Notch and basic fibroblast growth factor (bFGF) signaling pathways are also involved in astrogliogenesis (Ge et al., 2002; Song and Ghosh, 2004). In addition, crosstalk between those pathways and the Jak-STAT pathway is another crucial mechanism that synergizes the onset of gliogenesis. For example, SMAD protein, an effector molecule of BMP signaling forms a transcriptional complex with STAT3 via the p300 cofactor bridge (Nakashima et al., 1999b).

As a part of central nervous system (CNS), the retina is a useful model for studies of CNS development, blood-neural barrier and related disorders because it has advantages such as accessibility and structural simplicity. Moreover, the retina shares many common features with the brain; in particular, neuro-glial cell fate specification occurs sequentially via intrinsic and extrinsic regulations in the retinal progenitor pool as it does in the brain (Turner and Cepko, 1987). Most mammalian retinas have two types of macroglia: astrocytes and Müller glia cells. Retinal astrocytes are generated from brain-derived astrocytes that migrate via optic nerve, whereas Müller glia originate from retinal progenitor cells. Moreover, cytokine pathways that participate in brain astrogliogenesis function similarly in retinal glia development. For example, CNTF has been reported to promote Müller glia differentiation of retinal precursor cells with an increase in GFAP expression (Bhattacharya et al., 2008; Goureau et al., 2004).

Meteorin was first identified in the pluripotent P19 embryonal carcinoma cells through its all-*trans*-retinoic acid (RA)-inducible gene, and was suggested to be involved in glial differentiation and axonal extension (Nishino et al., 2004). In addition, astrocyte-

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derived meteorin inhibits angiogenesis via positive regulation of the anti-angiogenic factor TSP-1/2 (Park et al., 2008). However, very few papers about meteorin have been published so far, and many of its roles and underlying mechanisms are still unclear. In particular, because this factor does not contain any motif homologous to other known factors, and its receptor has not been identified yet, none of the previous reports has revealed downstream pathways that mediate its function. Hence, the present study investigates the role of meteorin during embryonic cortical development and retinal glia differentiation and explores the molecular mechanism directing its function. We found that meteorin increases GFAP-positive glial populations in all systems used in this study and the Jak-STAT3 pathway mediates the gliogenic activity of meteorin.

Results

Meteorin is highly expressed in the neural-stem–astrocyte-lineage cells and immature neurons of the embryonic mouse brain

To investigate the function of meteorin during cortical development, we first explored its expression pattern using cortical sections of the developing mouse brain. In-situ hybridization performed with an antisense meteorin riboprobe showed that meteorin transcripts were enriched in the ventricular zone (VZ) of the lateral ventricles of the cerebrum isolated from E18 (Fig. 1A,B) and early postnatal brains of mice (data not shown). These results agree with previous findings in developing mouse retina, spinal cord, and brain, which revealed meteorin expression in the neural progenitor cells of most neural tissues (Nishino et al., 2004). We additionally found that meteorin transcripts were present in the intermediate zone (Fig. 1B) and cortex (Fig. 1C). To verify the identity of the meteorin-positive cells, we next performed double immunofluorescence staining of the α-meteorin antibody with markers for NSCs (nestin), radial glia (GLAST) or immature neurons (tubulinβIII; also known as tubulin β3). The α-meteorin antibody specificity was confirmed by western blotting as well as immunocytochemistry against transfected recombinant meteorin (supplementary material Fig. S1). At E18, we detected dense immunoreactivity for meteorin in the VZ where the cell bodies of nestin-positive NSCs are located (Fig. 1E). The meteorin-expressing cells of the VZ were found to be positive also for GLAST indicating that these cells have radial glia identity as well (Fig. 1F) (Shibata et al., 1997). In addition to

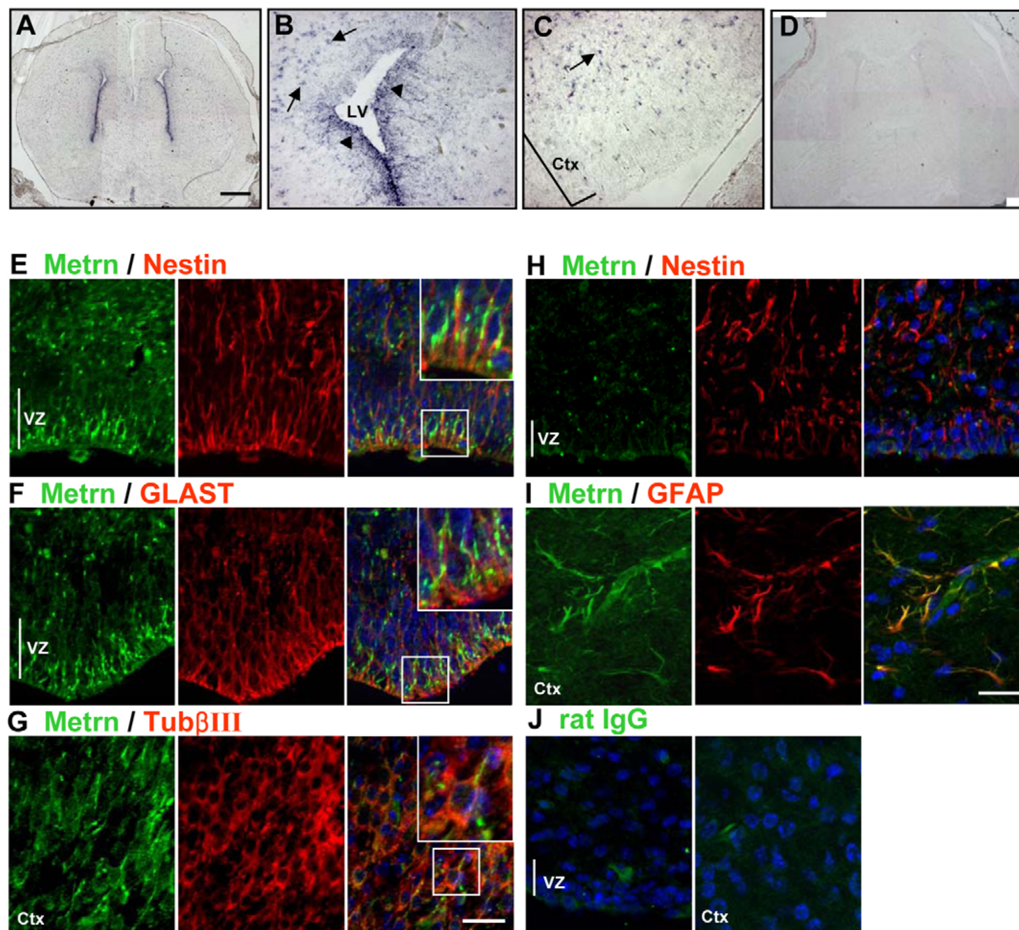

Fig. 1. Expression profiles of meteorin transcripts and protein in the developing mouse cerebral cortex. (**A-C**) In-situ hybridization of meteorin mRNA in transverse sections of E18 mouse cerebrum. The meteorin transcripts were enriched in the ventricular zone (VZ) of the lateral ventricle (LV) (arrowhead) and some cells outside the VZ (arrows). A higher magnification of the VZ is shown in B and of the cortex (Ctx) in C. (**D**) A sense probe revealed no signal. Scale bar: 500 μm. (**E-G**) E18 mouse brain sections showing double immunofluorescence staining of meteorin (Metrn) with antibodies against nestin (E), GLAST (F) and tubulinβIII (TubβIII; G). (**H**,**I**) P7 mouse brain sections showing double immunofluorescence staining of meteorin with antibodies for nestin (H) and GFAP (I). (**J**) Control staining for rat IgG shows no detectable fluorescence in either the VZ or cortex. The panel on the right of each set shows the merged images including DAPI staining. Higher magnifications of the boxed area are shown in the upper right corner of E-G. Scale bars: 25 μm.

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the signals in the VZ, we found dispersed meteorin signals outside the VZ, detected by in-situ hybridization. These cells were likely to be immature neurons positive for tubulinβIII (Fig. 1G). The meteorin expression in the VZ weakens progressively as development proceeds so that in P7 brains we could detect very faint signals (Fig. 1H). However, its expression persisted in astrocytes at P7 (Fig. 1I) and adult stage (data not shown) similarly to the previous reports (Jorgensen et al., 2009; Park et al., 2008).

Meteorin induces GFAP-positive glial differentiation of P19 cells and neurospheres

Meteorin was previously reported to induce glial differentiation (Nishino et al., 2004), and our immunofluorescence staining as well as in-situ hybridization results also suggest a possible role in neural stem cell differentiation. To investigate its role in differentiation, we used P19 embryonal carcinoma cells, which are capable of inducing neural lineage differentiation by treatment with RA (Jones-Villeneuve et al., 1982; McBurney, 1993). We first induced neuro-glial differentiation of the P19 cells and checked for the meteorin expression by semi-quantitative RT-PCR analysis. As shown in Fig. 2A, meteorin was upregulated during RA-induced aggregation formation but was reduced after replating when glial differentiation occurred. We generated a P19 cell line that stably expressesed meteorin (P19-Metrn) and examined the differences from a mock-transfected P19 cell line (P19-Mock). After the induction of neuro-glial differentiation the P19-Metrn population had 13% GFAP-positive cells and 4% MAP2-positive neurons, whereas in the P19-Mock population about 4% of cells showed GFAP-positive glia differentiation and 11% neuronal differentiation (Fig. 2B,C). The GFAP protein level was also increased in the P19-Metrn cells (Fig. 2D).

To further confirm the phenomenon caused by meteorin in the P19 cells, we used neuropheres formed from the E13-14 mouse cerebral cortex. For in vitro differentiation, neurospheres were dissociated as single cells and plated onto poly-L-ornithine-coated dishes and recombinant meteorin was added after growth factor withdrawal as described in Materials and Methods. The concentration of recombinant meteorin protein used was 200 ng/ml based on a previous report in which meteorin showed maximal activity in neurite outgrowth at around 100 to 300 ng/ml concentration (Nishino et al., 2004). To rule out the possibility of the effect being caused by co-purified contaminant protein, we performed parallel purification with CHO cells bearing empty plasmid, and treated the same volume of resultant product (rHis-empty), but there was no significant effect of any of the markers used in this experiment (Fig. 3B). By contrast, meteorin treatment promoted GFAP-positive glial differentiation of the neurospheres (Fig. 3A,B) similar to the result from the P19 cells. The GFAP protein level was also increased by meteorin (Fig. 3C). Another astrocyte marker, S100β, was also increased with GFAP (Fig. 3A,B), indicating that meteorin indeed promoted astrocyte lineage commitment of the NSCs. However, neuronal differentiation was not altered in neurospheres exposed to meteorin (Fig. 3A-C), which is different from P19 cells. These differences can be explained by the different differentiation levels. Our P19 stable cells expressed meteorin from the undifferentiated state even before neurogenesis was induced. By contrast, the neurospheres were derived from the E14 mouse brain and much of the neuronal fate is already determined at this stage. Therefore, meteorin was probably capable of reducing neurogenesis of the P19 cells while it had no effect on the neuronal differentiation of the neurospheres. Interestingly, the nestin-positive population was unchanged by meteorin treatment (Fig. 3A,B) even though ~50% of the cells have been transformed to GFAP-expressing cells. This result suggests that the GFAP-positive cells induced by meteorin still express nestin. To clarify the identity of the GFAP-positive cells in meteorin-treated neurospheres, we double-stained these cells with the neural stem cell markers, Sox2 and nestin. As shown in Fig. 3D, most of the GFAP-positive cells were positive for either nestin (Fig. 3D, upper) or Sox2 (Fig. 3D, middle) at 3 days of meteorin treatment. Surmising from the result that these cells are positive for S100β as well (Fig. 3D, lower), meteorin probably promotes glial

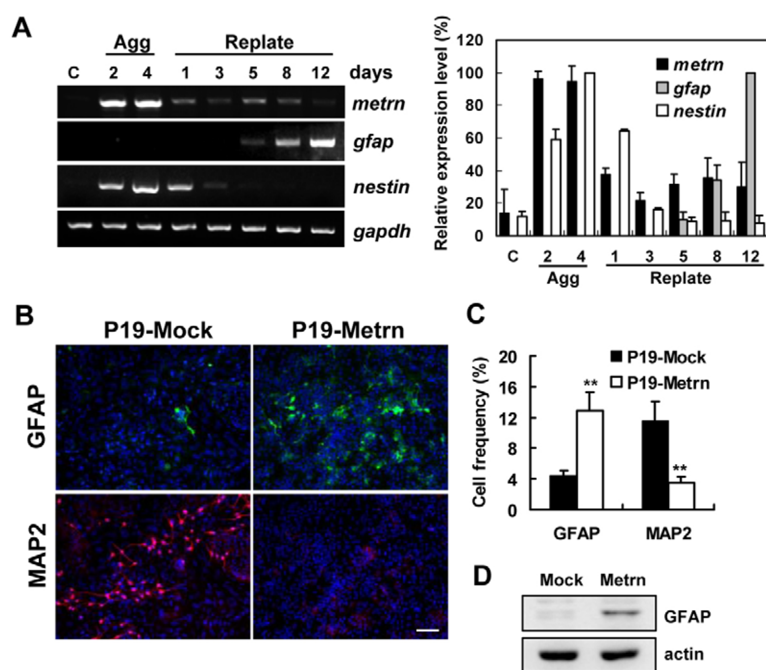

Fig. 2. **Meteorin promotes glial differentiation of P19 cells.** (**A**) Neuro-glial differentiation of P19 cells was induced, as described in the Materials and Methods. The levels of meteorin (*metrn*), *Gfap* (*gfap*), nestin (*nestin*) and *Gapdh* (*gapdh*) transcripts were measured by semi-quantitative RT-PCR during RA-stimulated aggregation formation (Agg) and after the replating of aggregates (Replate). Graph shows the relative expression levels of meteorin, *Gfap* and nestin mRNA analyzed by densitometry. Three independent experiments were used for statistical analyses. Error bars indicate the s.e.m. (**B**-**D**) Astrocyte and neuron cell populations were analyzed in mock- (P19-Mock) and meteorin-expressing P19 stable cell line (P19-Metrn) after 12 days of differentiation. Immunostaining for GFAP (green) and MAP2 (red) in P19-Mock and P19-Metrn cells is shown in B, and their frequencies are given in C (n=5, error bars indicate the s.e.m., **P<0.01). (D) The protein levels of GFAP in the P19-Mock and P19-Metrn cells were detected by western blotting.

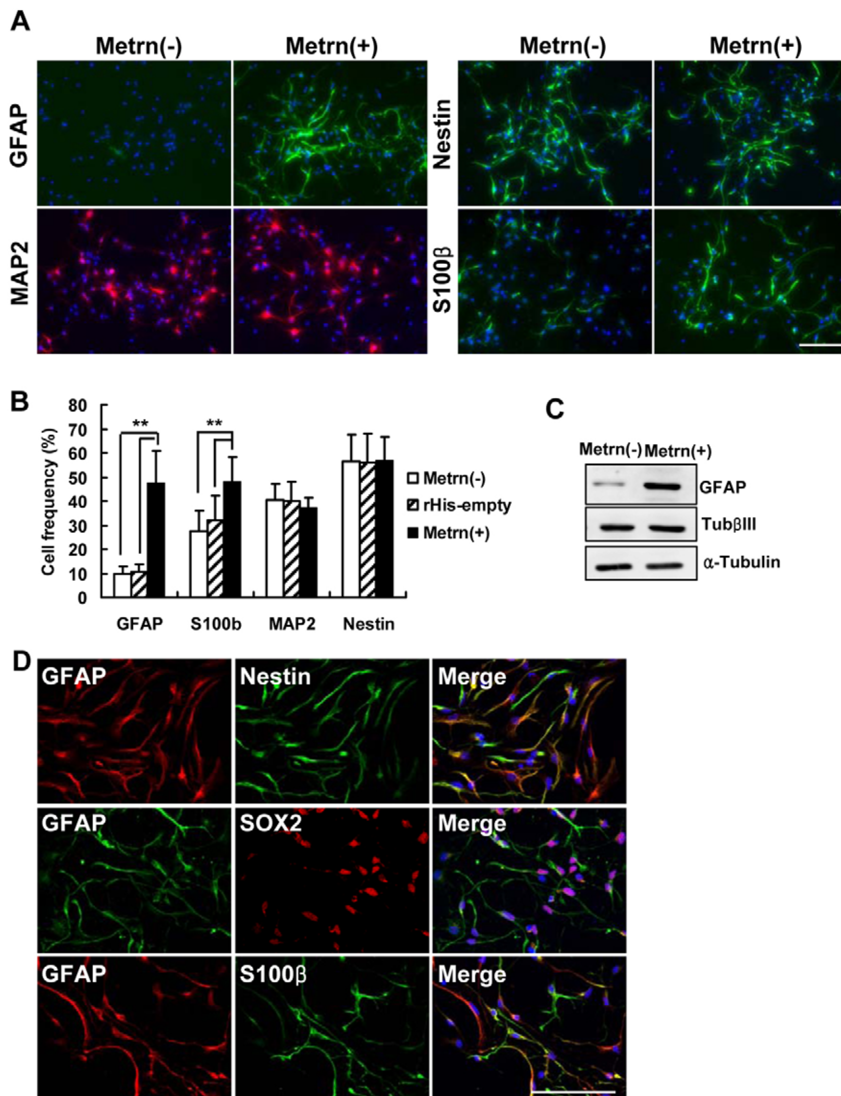

Fig. 3. Meteorin promotes GFAP-positive glia formation of neurospheres which are characterized as glial progenitor/radial glial cells. (**A-D**) The neurospheres were left untreated or treated with purified recombinant meteorin (200 ng/ml; Metrn) for 3 days. (A) Differentiated cells were detected by immunostaining for GFAP, nestin, S100β (green) and MAP2 (red). (B) The frequencies of each marker-positive cells were counted in untreated cells [Metrn(–)] and in cells treated with purification product from empty plasmid-transfected cells (rHis-empty), or recombinant meteorin [Metrn(+)] ($n \geq 7$, error bars indicate the s.e.m., $**P<0.01$). (C) Protein levels of GFAP and tubulinβIII (tubβIII) were detected by western blotting. (D) Meteorin-treated neurospheres were double stained for nestin (upper), Sox2 (middle) and S100β (lower). The right-hand panels show merged images including DAPI staining. Scale bars: 100 μm.

commitment of the NSCs, but it seems insufficient by itself to induce maturation to astrocytes. Overall, the cells can be characterized as glial progenitor or radial glia rather than mature astrocytes.

Meteorin activates tyrosine phosphorylation of STAT3 as well as gp130 co-receptor, which in turn promotes GFAP transcription

The IL-6 family of cytokines is the major factor that induces gliogenesis of NSCs. These cytokines activate the common downstream signaling machinery, the Jak-STAT pathway. Although meteorin does not have any homologous motif to IL-6 family cytokines, its gliogenic activity seems quite similar to those cytokines, such as CNTF and NP-1. Based on this similarity, we investigated whether meteorin activates the Jak-STAT pathway as part of its molecular mechanism. As shown in Fig. 4, meteorin treatment induced tyrosine phosphorylation of STAT3 in both P19 cells (Fig. 4A) and neurospheres (Fig. 4B) but not serine phosphorylation. The tyrosine residue of STAT3 (Tyr705) is mainly phosphorylated by activated Jaks whereas phosphorylation of the serine (Ser727) occurs by other signaling pathways such as MAPK, mTOR and PI3K-AKT pathways (Decker and Kovarik, 2000). Therefore, our results suggest that meteorin activates STAT3 via Jak activation and other pathways are unlikely to be involved in its function. Like other cytokines that activate STAT3 (Barnabe-Heider et al., 2005), meteorin also induced phosphorylation of Erk1/2 in neurospheres (Fig. 4B), although we could not detect phosphorylated Erk1/2 in P19 cells (Fig. 4A). In the Jak-STAT3 pathway, the activated STAT3 forms a homodimer and translocates to the nucleus to function as a transcription factor. We also detected a nuclear concentration of STAT3 after meteorin stimulation in neurospheres (Fig. 4C). This pathway promotes astrocyte generation because the GFAP promoter contains a STAT3 binding element. Thus, we examined whether the activation of STAT3 by meteorin results in GFAP transcription. We checked the binding of STAT3 to the GFAP promoter region by chromatin immunoprecipitation assay and found that only in the meteorin-treated neurospheres, was the GFAP promoter immunoprecipitated with α-STAT3 antibody (Fig. 4D). To further confirm the transcriptional activation of GFAP by meteorin, a luciferase assay was performed with a

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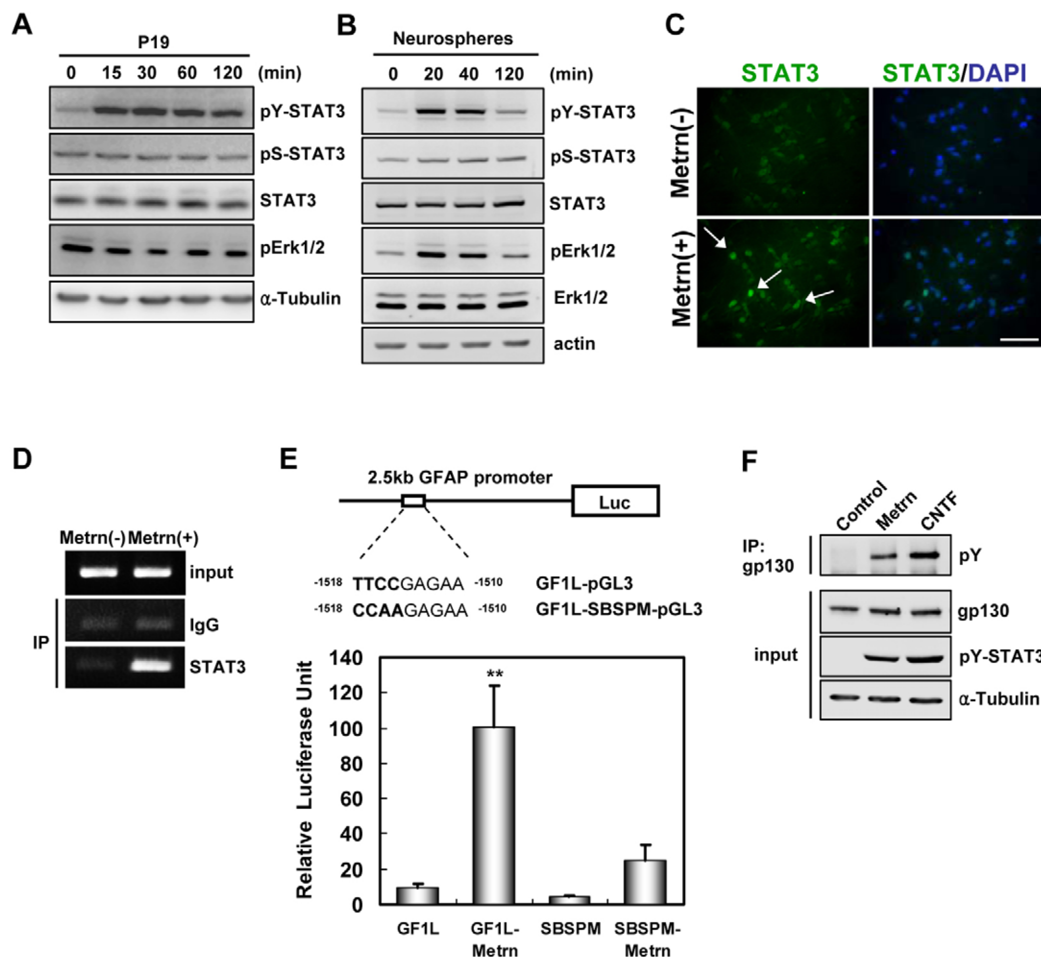

Fig. 4. Meteorin induces the activation of STAT3 by recruiting gp130 co-receptor, and improves GFAP transcriptional activity by enhancing the binding of STAT3 to its cognate sequence within the GFAP promoter. (**A**,**B**) Western blots showing the phosphorylation of STAT3 (pY-STAT3, pS-STAT3) and Erk1/2 (pErk1/2) after treatment with recombinant meteorin (200 ng/ml) in P19 cells (A) and neurospheres (B) for the indicated time periods. STAT3 and Erk1/2 total proteins were also blotted to show that the total amount was unchanged. (**C**) Immunocytochemistry of neurospheres was performed with an anti-STAT3 antibody after treatment with meteorin (200 ng/ml) for 40 min. Arrows indicate a nuclear localization of STAT3 after meteorin treatment. Scale bar: 50 μm. (**D**) Chromatin immunoprecipitation analysis showing the binding of STAT3 to the GFAP promoter in the neurospheres after stimulation with meteorin for 40 minutes .
(**E**) Schematic representation of a reporter plasmid containing a wild-type STAT3 binding sequence (GF1L-pGL3) or a mutant sequence (GF1L-SBSPM-pGL3; upper part) and a graph showing a luciferase assay performed in neurospheres with a wild type (GF1L) or mutant (SBSPM) GFAP promoter, in the presence or absence of meteorin (n=5, error bars indicate the s.e.m., **P<0.01). (**F**) Phosphorylation status of gp130 co-receptor was documented by immunoprecipitation followed by western blotting for pan phosphotyrosine antibody (pY). P19 cells were treated with either meteorin (200 ng/ml) or CNTF (100 ng/ml) for 10 minutes. Total levels of gp130, pY-STAT3 and α-tubulin were determined by western blot (input).

reporter plasmid containing a wild-type 2.5-kb GFAP promoter (GF1L-pGL3), or a mutant promoter containing substituted nucleotides, ~1.5 kb upstream of transcription start site, which is a previously reported STAT3 binding site (GF1L-SBSPM-pGL3; Fig. 4E, upper part) (Miura et al., 1990; Nakashima et al., 1999b). As shown in Fig. 4E, the wild-type promoter was transcriptionally activated in response to meteorin stimulation. By contrast, the mutant construct showed only slight induction upon meteorin stimulation. These data indicate that activation of the Jak-STAT3 pathway by meteorin, in turn, increases the transcriptional activity of the GFAP promoter, which is a crucial event for glial differentiation.

These results led us to consider whether meteorin utilizes the gp130 co-receptor of IL-6 family cytokines, as an upstream transducer of Jak-STAT3 signaling. It is well known that the IL-6 family cytokines recruit gp130 to deliver their signal into the cells even though most of them have their own recognizing receptor (Heinrich et al., 1998). Since the gp130 receptor is phosphorylated by Jak when it participates in signaling, we checked its phosphorylation status after meteorin treatment. As shown in Fig. 4F, immunoprecipitated gp130 was detected by phosphotyrosine antibody (pY) in response to meteorin as well as CNTF in P19 cells (Fig. 4F) and neurospheres (data not shown). This result suggests that meteorin recruits gp130 when it activates the Jak-STAT3 pathway. Additionally, these data also indicate that meteorin shares intracellular signaling machinery with IL-6 family cytokines. Therefore, if this is true, meteorin would hardly show a synergistic effect with IL-6 family cytokines such as LIF or CNTF. To confirm this hypothesis, we first determined the concentration of meteorin that would produce maximal activity by using luciferase assay

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because it might fully activate down stream pathway at this concentration. There was significant induction of the GFAP promoter at 100 ng/ml but its activity was spontaneously increased as concentration increased, reaching a maximum at ~5 μg/ml concentration (supplementary material Fig. S2A). Maximal activity of CNTF occurred at 200 ng/ml concentration (data not shown). Thus we treated 5 μg/ml meteorin and 200 ng/ml CNTF simultaneously but the result was similar to the single treatment of meteorin (supplementary material Fig. S2B). Collectively, these results indicate that meteorin does not have synergistic function with IL-6 family cytokines and suggest that meteorin might have a complementary function to those cytokines by sharing downstream signaling machinery.

Activation of the Jak-STAT3 pathway is essential for meteorin-induced gliogenesis

Our results obtained from the neurospheres and P19 cells reveal that meteorin activates the Jak-STAT3 pathway, and, as a result, promotes GFAP-positive glia formation. Thus, we next verified whether the Jak-STAT pathway is truly essential for meteorin-induced gliogenesis by blockade of this pathway. We first checked the effect of inhibitors specific for the Jak-STAT pathway and the MEK-Erk pathway. Neurospheres were pre-treated with Jak inhibitor I (JAKiI) or MEK inhibitor U0126 for 2 hours, and then meteorin was added to the culture medium. After 3 days in culture, the glial differentiation induced by meteorin was obviously impaired in the Jak-inhibitor-treated group, whereas the MEK inhibitor U0126 did not affect meteorin-induced gliogenesis (Fig. 5A,B). This result was in agreement with the protein levels of GFAP detected by western blot (Fig. 5C). Neurogenesis was not altered by the combined treatment of meteorin with JAKiI or by treatment with meteorin alone, but U0126 slightly reduced the portion of neurons (Fig. 5A,B). The specificity of those inhibitors was confirmed by determining phosphorylation levels of STAT3 and Erk1/2 (Fig. 5D). U0126 inhibited Erk1/2 phosphorylation but had no effect on the phosphorylation of STAT3. By contrast, JAKiI affected phosphorylation of both the STAT3 and Erk1/2, which means that the MEK-Erk pathway is located downstream of the

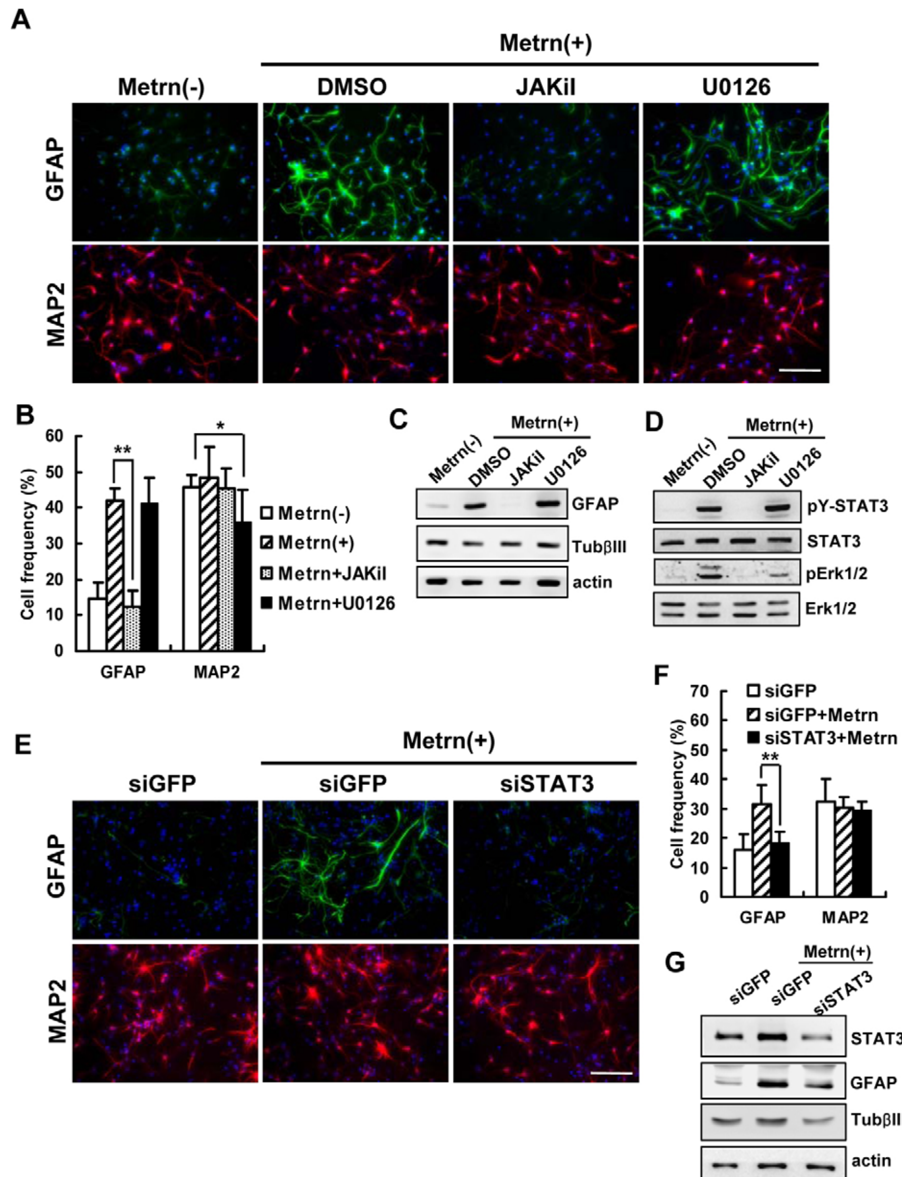

Fig. 5. Inhibition of the Jak-STAT pathway, but not Erk1/2, impairs meteorin-induced glial differentiation. (**A**-**D**) The neurospheres were treated with recombinant meteorin (200 ng/ml) in the presence or absence of Jak inhibitor I (JAKiI; 1 μM) and Erk inhibitor (U0126; 1 μM) for either 3 days (A-C) or 30 minutes (D). (A) Immunocytochemistry for GFAP (green) and MAP2 (red) was performed to identify the differentiated cells. (B) The cell frequencies of cells positive for GFAP and MAP2 in each group ($n\geq 8$, error bars indicate the s.e.m., $^{**}P<0.01$, $^{*}P<0.05$). Cell lysates were western blotted for GFAP, tubulinβIII (tubβIII), and actin (C) and for phosphorylated STAT3, Erk1/2, and total STAT3 and Erk1/2 (D). (**E**-**G**) siRNA against non-targeting GFP (siGFP) or STAT3 (siSTAT3) was transfected into neurospheres as described in the Materials and Methods, and differentiation was induced in the absence or presence of recombinant meteorin (200 ng/ml). (E) Immunocytochemistry for GFAP (green) and MAP2 (red) shows the differentiated glia and neurons, respectively. The cell frequencies are shown in F ($n\geq 8$,; error bars indicate the s.e.m., $^{**}P<0.01$). (G) Western blots showing the protein levels of STAT3, GFAP, tubβIII and actin. Scale bars: 100 μm.

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Jak-STAT pathway in this context. However, the phenomenon that Jak inhibitor does not affect neurogenesis even if it were to block Erk1/2 phosphorylation does not matched the data in which U0126 slightly reduced neurogenesis (Fig. 5B). The MEK-Erk pathway was previously reported to regulate neuronal differentiation of NSCs (Menard et al., 2002), therefore it seems reasonable that blockade of this pathway impairs neurogenesis. By contrast, meteorin did not affect neurogenesis despite it increased Erk phosphorylation. Hence, the possible explanation is that Erk inhibition by upstream STAT3 inhibitor had no effect on neurogenesis because it blocks Erk phosphorylation only in STAT3-activating cells, which might be programmed to differentiate to glia preferentially. By contrast, U0126 might broadly inhibit the MEK-Erk pathway in all NSCs, such that some of them were affected regarding their neural fate.

To further confirm the effect of the blockade of the Jak-STAT3 pathway, we next addressed the effect of genetic ablation of STAT3 by using siRNA. siRNAs against STAT3, or GFP as a non-targeting control, were transfected into the cells just before replating the neurospheres, and differentiation was induced for 3 days. As predicted, silencing STAT3 by siRNA weakened the effect of meteorin on gliogenesis, as confirmed by the immunostaining for GFAP and MAP2 (Fig. 5E), their quantification (Fig. 5F), and western blots for GFAP and tubulinβIII (Fig. 5G).

Meteorin increases the expression of GFAP in Müller glia in the retinal explant culture via the Jak-STAT3 pathway

To expand our findings about meteorin function to another part of the CNS, we chose the retina. Retinal neurons are generated from retinal precursor cells, from which Müller glia are also generated. As several previous reports have demonstrated, the mechanisms neuro- and gliogenesis in the retina and the brain are similar; for example, CNTF influences either retinal astrocyte differentiation or glial fate determination of retinal precursor cells (Goureau et al., 2004). These facts led us to hypothesize that meteorin has additional functions in retinal glia development. Therefore, we first examined meteorin expression in the retinas of P7 mice and detected positive signals in Müller glia (Fig. 6A). Moreover, we observed an increase in meteorin expression in CNTF-treated retinal explants, which induced Müller glia activation (supplementary material Fig. S3), confirming previous findings (Goureau et al., 2004). This result suggests that meteorin is upregulated in activated Müller cells. To understand its role in retinal glia development, we next performed retinal tissue culture with P0 mice eyes, in which retinal lamination and precursor differentiation is not yet complete. We detected almost normal lamination in the retinal explants cultured for 9 days in vitro, similar to that seen in the P9 mice eyes (data not shown), which means that our tissue culture system mimics in vivo development. We therefore added meteorin to the medium of explant cultures for 9 days and examined the alterations in retinal glia by immunostaining for GFAP, and the cellular retinaldehyde-binding protein (CRALBP; also known as RLBP1) as a marker of Müller glia. Hypothesizing that meteorin may play a role in retina in a similar manner to brain, we used the same concentration of the recombinant protein as used in the neurosphere experiment. We detected stronger GFAP signals in CRALBP-positive Müller glia of the meteorin-treated explants (Fig. 6B, middle) compared with the control cultures (Fig. 6B, upper part). As a positive control, we treated cultures with CNTF and observed increased GFAP signals in Müller glia as well (Fig. 6B, lower). These results mean that the upregulation of GFAP by meteorin can be recapitulated in the

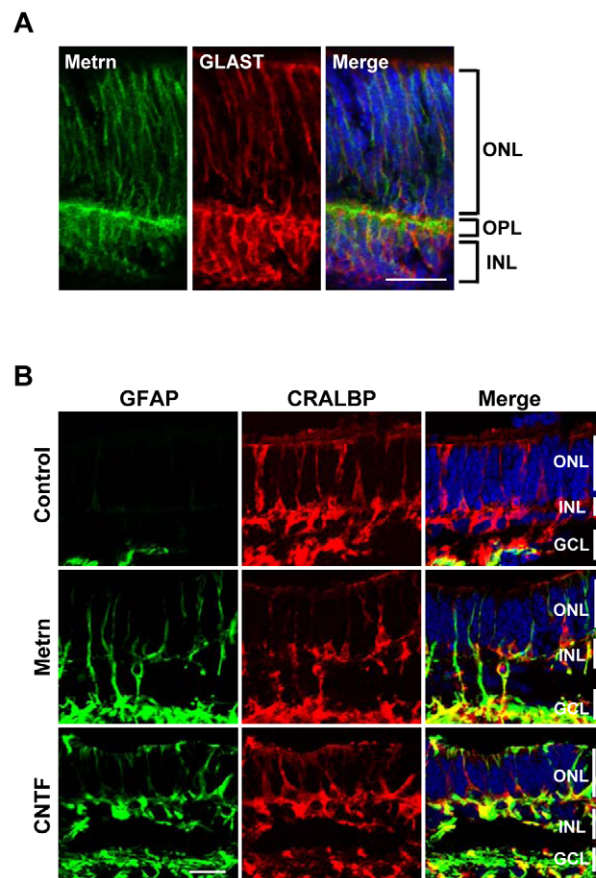

Fig. 6. Meteorin is expressed in Müller glia of the mouse retina, and its exogenous application increases GFAP expression of Müller cells in retinal explant culture. (**A**) Double immunofluorescence staining of P7 mouse retina with antibodies against meteorin (green) and GLAST (red). The panel on the right is a merged image, including DAPI staining, and the retinal layers are indicated. (**B**) P0 mice retinas were cultured as explants for 9 days with or without meteorin (200 ng/ml) and CNTF (100 ng/ml). GFAP staining shows activated Müller glia (green, left) and CRALBP shows Müller glia (red, middle). Merged images (right) indicate that GFAP-expressing cells are CRALBP-positive Müller glia (right). Scale bars: 25 μm (GCL, ganglion cell layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer).

retinal Müller glia as in cortical NSCs. However, there was no alteration in the size of the CRALBP-positive population with either meteorin or CNTF treatment, indicating that neither factor affects Müller gliogenesis of retinal precursor cells, but they do activate Müller glia to express GFAP.

On the basis of functional similarity of meteorin in cortical NSCs and retinal Müller cells, we next addressed the involvement of the Jak-STAT3 pathway in Müller glia activation. As shown in Fig. 7A, exogenous treatment of P0 retinal explants with meteorin protein promoted immediate STAT3 phosphorylation of the cells in outer neuroblast layer (NBL; Fig. 7A, middle). We also detected phosphorylated STAT3 in CNTF-treated explants in the same region as in the meteorin-treated retina (Fig. 7A, right). Furthermore, co-treatment with the chemical inhibitor of Jak (JAKiI) and either meteorin or CNTF failed to induce GFAP expression in the retinal Müller glia (Fig. 7B). These results suggest

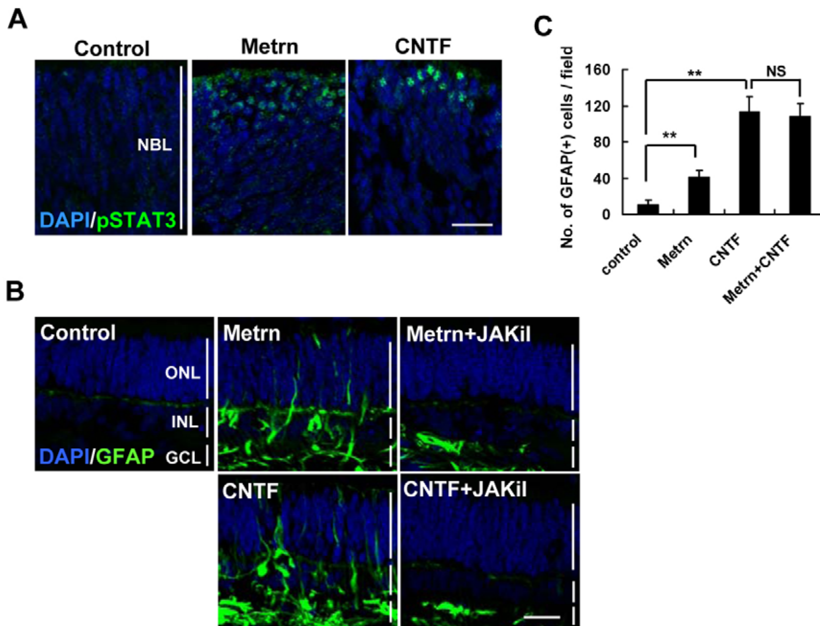

Fig. 7. The Jak-STAT3 pathway mediates meteorin-induced GFAP expression in Müller glia. (**A**) Tyrosine phosphorylation of STAT3 (pSTAT3, green) was induced by treatment with meteorin (200 ng/ml) or CNTF (100 ng/ml) for 30 minutes in explants of the P0 mice retinas. (**B**) P0 retinal explants were cultured for 8 days and treated with meteorin (200 ng/ml) or CNTF (100 ng/ml) and with or without Jak inhibitor I (JAKiI, 1 μM) as described in Materials and Methods. Each retina was then sectioned and stained with GFAP (green) and DAPI (blue) to show retinal layers. Scale bars: 25 μm (NBL, neuroblast layer; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer). (**C**) Retinal explants were cultured for 8 days with meteorin (200 ng/ml) or CNTF (200 ng/ml) or both. Explants were then dissociated into single cells and subjected to immunocytochemistry for GFAP and numbers of GFAP-positive cells were counted in the same area ($n \geq 8$, error bars represent the s.e.m., $^{**}P<0.01$; NS, not significant).

that meteorin has the same effect in Müller glia as in cerebral NSCs and its roles are similar to those of IL-6-related cytokines, not only for cerebral glia differentiation but also for Müller glia activation. Probably because of downstream sharing of signaling, no synergism was observed in co-treatment of CNTF and meteorin in retinal explants (Fig. 7C), consistent with the result from the reporter assay performed with neurospheres (supplementary material Fig. S3B).

Discussion

Cell fate determination is a complex event controlled spatiotemporally by fine orchestration of intrinsic and extrinsic factors. A massive amount of work has been done previously, and, as a result, several factors and pathways that modulate the fate of NSCs have been identified. Such factors include BMPs, FGF, Notch and IL-6 family members, and each of them activates different signaling pathways via the binding to its cognate receptors. The present study introduces meteorin as an important factor determining the fate of neural progenitor cells. We found that meteorin promotes formation of GFAP-positive P19 cells and neurospheres in the in vitro differentiation process (Figs 2 and 3). However, these cells were not mature astrocytes but glial progenitor or radial glial cells still bearing stem cell properties. This result coincides with the previous data in which meteorin transformed cerebellar astrocytes to radial glia (Nishino et al., 2004). Similarly, meteorin might possibly be capable of transforming cortical astrocytes into reactive glia because reactive astrocytes share molecular repertoires with radial glia (Gotz and Barde, 2005) and meteorin increased the population expressing those molecules (e.g. S100β, nestin, Sox2 and GFAP).

Sequence-based analysis of meteorin showed no homologous motif with other cytokines or growth factors. Nonetheless, our results demonstrate that this factor shares the downstream pathway with IL-6 family cytokines. Meteorin promoted STAT3 phosphorylation, nuclear translocation and transcriptional ability of the GFAP promoter. Moreover, we observed functional similarities between meteorin and those cytokines, including the promotion of gliogenic potential in neurospheres and GFAP upregulation in Müller glia. Diverse extracellular cues are involved in the downstream transduction pathway, for instance, there have been reported at least seven IL-6 family cytokines that promote astroglial differentiation of the cortical precursor cells (Derouet et al., 2004; Taga and Fukuda, 2005). In various combinations these related receptors all activate the Jak-STAT signaling pathway, which in turn increase GFAP transcription. They complement each other, as verified by knock-out studies in which knock-out of any one of those cytokines caused only mild effects, whereas deficiency of a co-receptor of the IL-6 family cytokines, gp130, resulted in more serious defects in astrocyte differentiation (Nakashima et al., 1999a). Therefore, it seems likely that meteorin has compensatory functions to other astrogliogenic factors, but identification of its receptor is required to verify this. Related to this issue, it is interesting that meteorin recruits gp130 co-receptor to activate the Jak-STAT3 pathway (Fig. 4F). Meteorin has been characterized as a novel family of neurotrophic factors based on a sequence-based homology search. Therefore, it is supposed that meteorin has a unique receptor and through this, recruits gp130.

Functions of meteorin that have been revealed so far are as follows. First, meteorin promotes glial differentiation as observed here and previously (Nishino et al., 2004). Second, it induces neurite outgrowth, and third, it inhibits angiogenesis via upregulation of TSP-1 (Nishino et al., 2004; Park et al., 2008). The latter two were revealed as autocrine effects in which meteorin stimulates the astrocyte itself to modulate other factor(s) of the astrocyte, which in turn signals neurons to induce neurite outgrowth and/or stimulates blood vessels to cease angiogenesis. Nevertheless, it is still unclear whether the gliogenic role of meteorin is regulated in the same way. To clarify the cellular identity of meteorin-expressing cells, we performed immunofluorescence staining with α-meteorin antibody and observed positive signals not only in the neural stem cell-astrocyte lineage but also in early neurons. Thus, we assume that meteorin has both an autocrine and paracrine signaling function during the cell fate determining period, in which NSCs, radial glia and neurons express meteorin, thereby stimulating

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precursor cells to express GFAP. However, most of them lose meteorin expression after completion of glial differentiation, with the exception of mature astrocytes. Astrocyte-derived meteorin then might function mainly in an autocrine manner.

Based on the results obtained from P19 and neurosphere culture, we then examined meteorin function in the retina. Its expression pattern in Müller glia led us to hypothesize that it may have a role in Müller glia differentiation. We revealed an enhanced Müller glia activation using explant culture of P0 mouse retinas, in which GFAP expression in Müller glia was increased by exogenous meteorin (Fig. 6). These results also seem similar to the effect of CNTF on Müller glia (Goureau et al., 2004), which demonstrated that CNTF increases GFAP expression of Müller glia in P0 mouse retinal explant cultures, and at the same time, it increases the CRALBP-positive Müller glia cell population itself, indicating that CNTF is involved in differentiation of retinal precursor cells into Müller glia. However, we could not detect any change in Müller glial cell number in cultured retina either by meteorin or CNTF treatment. Thus it seems likely that meteorin activates Müller glia rather than directs their differentiation, although we still cannot rule out the possibility of differences among experimental conditions because we could not observe an increased Müller glia population even in the CNTF-treated group. Muller glia do not express GFAP under normal conditions but begin to do so when they are activated by certain stimuli. GFAP expression is a hallmark of reactive gliosis not only in Müller glia of the retina but also in CNS astrocytes. The activated glia, then play either protective or detrimental roles in the injured tissues. In the former circumstances, they breakdown neurotoxic metabolites and release neuroprotective factors which in turn help neurons to survive. In the latter case, they lose some of their homeostatic functions or produce pro-inflammatory cytokines and as a result, are detrimental in overcoming injury (Bringmann et al., 2006). Interestingly, cytokines such as LIF and CNTF are known to have neuroprotective roles in the diseased brain and retina (Burgi et al., 2009; Tao et al., 2002). In this context, our results suggest that meteorin probably has specialized roles in a variety of CNS disorders accompanying reactive gliosis.

Materials and Methods

Cell culture

Neurospheres were prepared from E13-14 ICR mice forebrain purchased from a local supplier (Samtako, Daejeon, Korea). In brief, the neuroepithelium was isolated from brains of embryos and transferred to Hank's buffered salt solution (HBSS) and dissociated with a glass pipette. The neurospheres were formed in complete neurobasal medium (Gibco, Carlsbad, CA) containing B27 (Gibco), human EGF (20 ng/ml, R&D Systems, Minneapolis, MN), and bFGF (10 ng/ml, Invitrogen) for 4 days by plating neuroepithelial cells on non-coated culture dishes. For in vitro differentiation, neurospheres were dissociated with trypsin-EDTA (Cellgro, Herndon, VA) and plated onto poly-L-ornithine (Sigma, St Louis, MO)-coated dishes in complete medium. 24 hrs later, the medium was changed to neurobasal medium containing B27 without growth factors. Recombinant meteorin was then treated after overnight deprivation of growth factors for the indicated time periods.

P19 cells were maintained in alpha-MEM (minimal essential medium-α; Gibco) containing 1% antibiotic-antimicotic (Cellgro) and 10% FBS (Gibco). Embryonic-body-like aggregation and neuroglial differentiation were induced as described previously (Santiago et al., 2005). To generate a meteorin-expressing stable cell line, the cells were transfected with either an empty pcDNA3.1 plasmid (mock) or a pcDNA3.1-mmeteorin-myc plasmid (Metrn), and the transfected colonies were selected by geneticin (800 μg/ml). After colony formation, the expression levels of meteorin in each colony was analyzed by western blot, and the highest expressing line was used in the experiment.

Purification of recombinant meteorin

His_6-tagged recombinant mouse meteorin was purified from conditioned medium of CHO-K1 cells stably expressing meteorin, as described previously (Park et al., 2008). For a convincing negative control, parallel purification was performed with CHO-K1 cells harboring empty plasmid (pcDNA3.1-mychis) and the same volume of the product (rHis-empty) was treated as recombinant meteorin protein.

Inhibitor treatment and transfection of neurospheres

Neurospheres plated on poly-L-ornithine-coated dishes were treated with Jak inhibitor I (1 μM; Calbiochem, La Jolla, CA) and U0126 (1 μM, Promega, Madison, WI) 2 hours prior to meteorin treatment. To detect the efficacy of the inhibitors, the cells were subjected to western blot after a 30 minutes of meteorin treatment. To check the effect on differentiation, cells were treated with inhibitors for 3 days in the presence or absence of meteorin. siRNA against mouse STAT3 (target sequence 5′-GAGTCACATGCCACGTTGG-3′; Dharmacon, Lafayette, CO) or non-targeting GFP siRNA (Dharmacon) were transfected into neurospheres just before replating them using a mouse NSC nucleofector kit (Amaxa, Cologne, Germany) according to the manufacturer's protocol.

In-situ hybridization and immunofluorescence staining

In-situ hybridization was performed with probes specific for mouse meteorin mRNA using a modified protocol supplied from Roche Applied Science. A digoxigenin-labeled riboprobe was synthesized using a DIG RNA labeling kit (Roche, Indianapolis, IN). To prepare the tissue sections, embryonic day (E) 14, E18 and postnatal day (P) 0 ICR mice were fixed by immersion in 4% paraformaldehyde (PFA), dehydrated with serial grades of sucrose-PBS solutions, and embedded in OCT compound. Frozen samples were sectioned (10 μm) and placed on silane-coated slides (Muto-Glass, 5116-20F, Tokyo, Japan). After a wash with PBS plus 0.1% Tween 20, the tissues were treated with RNase-free proteinase K (1 μg/ml) and post-fixed with 4% PFA. The sections were then prehybridized at 58°C for 1 hour and hybridized with a probe at 60°C overnight in a humid chamber. The extra probe was removed through a series of SSC and PBS-Tween 20 washes, and the hybridized probe was detected with an anti-DIG-AP antibody (Roche) and NBT-BCIP stock solution (Roche). Immunofluorescence staining was performed using the previously reported antigen retrieval method. In brief, tissue sections were boiled in the 0.8 M urea solution for 5 minutes twice, using a microwave oven. After five washes with PBS, sections were permeabilized with PBS-0.1% Triton X-100, blocked with 2% blocking reagent (Roche) and incubated overnight at 4°C with following antibodies to: nestin (1:200, mouse; BD Bioscience, Palo Alto, CA), GLAST (1:200, rabbit; Abcam, Cambridge, UK), tubulinβIII (1:2000, mouse; Covance, Princetone, NJ), GFAP (1:1000, rabbit; Dako, Carpinteria, CA) and meteorin (1:100, rat; R&D).

Immunocytochemistry

1×10^5 cells were plated onto 12-mm round coverslips in a 24-well culture plate and fixed with 4% PFA, permeabilized with PBS-0.1% Triton X-100, and blocked with 1% blocking reagent (Roche). Primary antibodies for GFAP (1:500, rabbit, Dako), MAP2 (1:200, mouse; Santa Cruz, CA), nestin (1:200, mouse; BD Bioscience), S100β (1:100, mouse; Sigma-Aldrich, St Louis, MO), Sox2 (1:500, rabbit; Millipore, Billerica, MA) were treated in blocking solution at 4°C overnight. The cells were then incubated with secondary antibodies (1:1000, goat, Alexa Fluor; Invitrogen) for 1 hour at room temperature, counterstained with DAPI, and mounted with FluorSave Reagent (Calbiochem). For quantification, at least five random fields were captured, and the labeled cells were counted. The frequencies were calculated as the ratio of positive cells to the total number of DAPI-positive cells.

Semi-quantitative RT-PCR and western blot analysis

Total RNA was extracted using TRIzol reagent (Invitrogen). For cDNA synthesis, 2 μg of RNA was reverse transcribed with M-MLV reverse transcriptase (Promega), and each cDNA was used as a template for PCR with primers for meteorin (forward: 5′-atgctggtagccacgcttcttt-3′; reverse: 5′-gtccagtgccatctcacatggg-3′), *Gfap* (forward: 5′-ggccggggcgctcaa-3′; reverse: 5′-gccgactcccgcgcat-3′), nestin (forward: 5′-ggagagtcgcttagaggtgc-3′; reverse: 5′-tcaggaaagccaagagaagc-3′), and *Gapdh* (forward: 5′-accacagtccatgccatcac-3′; reverse: 5′-tccaccaccctgttgctgta-3′).

For western blot analysis, the cells were lysed with 1× cell lysis buffer (Cell Signaling, Beverly, MA), and 40 μg of the lysates was used in the western blot analysis with antibodies against phospho-Stat3 (Tyr705) (1:1000, rabbit; Cell Signaling), phospho-Stat3 (Ser727) (rabbit, 1:1000; Cell Signaling), Stat3 (1:5000, rabbit; Santa Cruz), phospho-p42/44 MAPK (1:1000, rabbit; Cell Signaling), p42/44 MAPK (1:5000, rabbit; Cell Signaling), GFAP (1:3000, rabbit; Dako), tubulinβIII (1:10,000, mouse; Covance) and actin (1:10,000, rabbit; Sigma). To detect phosphorylated gp130, immunoprecipitation was performed with anti-gp130 antibody (rabbit, Santa Cruz) in 1× cell lysis buffer and followed by western blotting with anti-phosphotyrosine antibody (1:1000, mouse; Cell Signaling).

Luciferase assay and chromatin immunoprecipitation assay

The reporter plasmids containing the wild-type (GF1L-pGL3) and mutant stat3 binding elements (GFlL-SBSPM-pGL3) of the GFAP promoter upstream of the luciferase gene were kindly provided by Dr Kinich Nakashima of Nara Institute of Science and Technology (Miura et al., 1990; Nakashima et al., 1999b). The luciferase plasmids and β-galactosidase were co-transfected into the neurospheres using FuGENE HD transfection reagent (Roche). The luciferase activity was measured using a luciferase assay kit (Promega) and normalized to β-galactosidase activity, as measured with *O*-nitrophenyl β-D-galactopyranoside.

The Chromatin immunoprecipitation assay was performed according to the protocol supplied by Upstate Biotechnology, with some modifications (Miao and Natarajan, 2005). Briefly, the neurospheres were incubated with recombinant meteorin (200 ng/ml) for 40 minutes and cross-linked with 1% formaldehyde for 10 minutes at room temperature. An antibody against STAT3 (rabbit, C-20; Santa Cruz) was used in the immunoprecipitation, and PCR was done with primers for the GFAP promoter region (forward primer: 5′-gcctgcttcccgctgtgctc-3′; reverse primer: 5′-ccccca-acccctgccttc-3′).

Mouse retinal explant culture

Mouse retinal explant culture was performed with a modified version of a previous method (Kretz et al., 2007). Eyes of P0 embryos were rapidly isolated and placed in prewarmed DMEM-F12 containing 1% FBS and N2 supplement. An opening was carefully made at the center of the cornea by mechanical force with forceps that let the lens and iris pop up through the hole of the cornea. The sclera and remaining debris were removed with forceps and the intact dissected retina was transferred to a membrane disc (Whatman, Maidstone, UK) floating in the medium, using a glass pipette. During 9 days culture, retinal explants were covered daily with 20 μl of fresh medium and treated with ciliary neurotrophic factor (CNTF; 100 ng/ml; Peprotech, Rocky Hill, NJ) every 3 days or meteorin (200 ng/ml) every day. Explanted retinal tissues were then fixed in 4% paraformaldehyde solution, cryopreserved in OCT compound and subjected to immunostaining after sectioning at 10 μm. To detect STAT3 phosphorylation, P0 retinas were explanted and treated for 30 minutes with meteorin or CNTF 1 day after culture. Explants were then immunostained with antibody against phospho-Stat3 (Tyr705, 1:100, rabbit; Cell Signaling). For inhibitor experiments, P0 retinal explants were left untreated or treated with Jak inhibitor I (1 μM; Calbiochem) 2 hours prior to meteorin or CNTF treatment. Cultures were maintained for 8 days and treated every day with meteorin (200 ng/ml) and every 3 days with CNTF (100 ng/ml) while inhibitor was treated once.

To quantify the number of marker-expressing cells, 8-day-cultured explants were dissociated as single cells by trypsine-EDTA treatment and plated onto poly-D-lysine-coated coverslip, one explant was seeded onto two coverslips. Immunocytochemical staining was then performed 3 hours after plating as described above. At least eight random fields were captured where the cells have similar distribution, and the numbers of marker-positive-cells were counted in each field.

Statistics

Error bars indicate standard error of the mean (s.e.m.) and statistics were performed using the unpaired Student's *t*-test.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/11/1959/DC1

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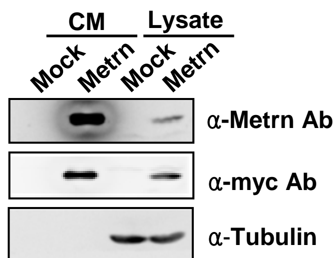

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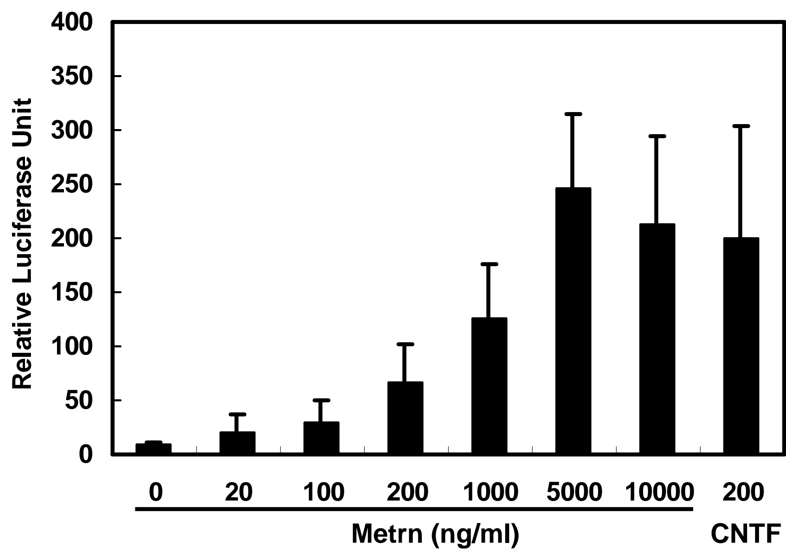

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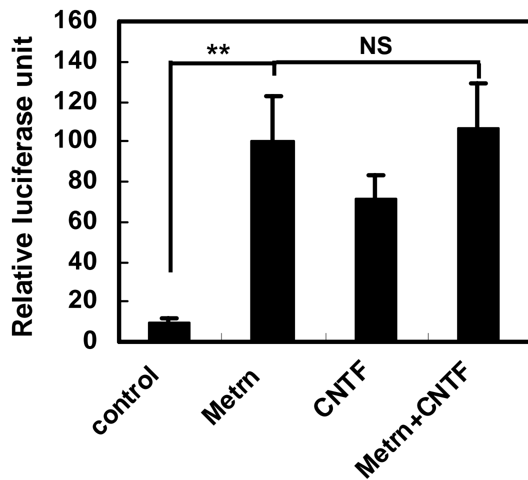

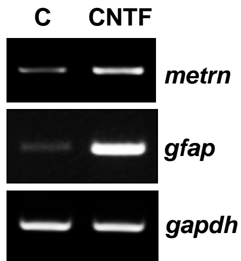
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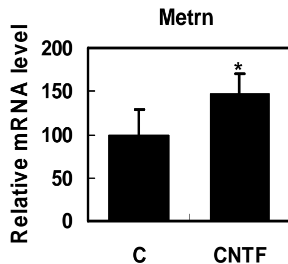
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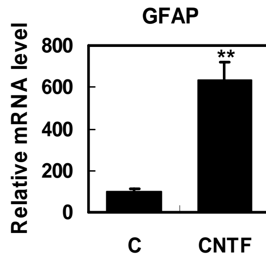
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