Mediator *Med23* deficiency enhances neural differentiation of murine embryonic stem cells through modulating BMP signaling

Wanqu Zhu, Xiao Yao*, Yan Liang*, Dan Liang, Lu Song, Naihe Jing, Jinsong Li and Gang Wang‡

**ABSTRACT**

Unraveling the mechanisms underlying early neural differentiation of embryonic stem cells (ESCs) is crucial to developing cell-based therapies of neurodegenerative diseases. Neural fate acquisition is proposed to be controlled by a ‘default’ mechanism, for which the molecular regulation is not well understood. In this study, we investigated the functional roles of Mediator *Med23* in pluripotency and lineage commitment of murine ESCs. Unexpectedly, we found that, despite the largely unchanged pluripotency and self-renewal of ESCs, *Med23* depletion rendered the cells prone to neural differentiation in different differentiation assays. Knockdown of two other Mediator subunits, *Med1* and *Med15*, did not alter the neural differentiation of ESCs. *Med23* knockdown selectively inhibited endoderm differentiation, suggesting the specificity of cell fate control by distinctive Mediator subunits. Gene profiling revealed that *Med23* depletion attenuated BMP signaling in ESCs. Mechanically, *MED23* modulated *Bmp4* expression by controlling the activity of ETS1, which is involved in *Bmp4* promoter-enhancer communication. Interestingly, *med23* knockdown in zebrafish embryos also enhanced neural development at early embryogenesis, which could be reversed by co-injection of *bmp4* mRNA. Taken together, our study reveals an intrinsic, restrictive role of *MED23* in early neural development, thus providing new molecular insights for neural fate determination.

**KEY WORDS:***BMP signaling, ETS1, MED23, Mediator, Embryonic stem cells, Neural differentiation**

**INTRODUCTION**

Embryonic stem cells (ESCs) are derived from the inner cell mass of growing blastocysts and have been widely used in developmental biology, drug discovery and cell therapies (Crook and Kobayashi, 2008; Kirouc and Zandstra, 2008). Neural induction from ESCs is regulated by many transcription factors and signaling pathways. Two distinct molecular models have been proposed to describe this process. The default model states that neural induction is achieved in absence of inhibitory instruction from bone morphogenetic protein (BMP) signals (Muñoz-Sanjuán and Brivanlou, 2002). Recent studies suggest that fibroblast growth factor (FGF) signaling, collaborating with BMP inhibition, contributes to neural specification (Kunath et al., 2007; Stavridis et al., 2007). Despite multiple signals of neural induction having been identified, it remains unclear how they are internally controlled or interpreted by the cell. A detailed description of molecular and cellular events controlling the generation of neural cell types from ESCs is fundamental to our understanding of neural specification processes and for developing treatments for neurological diseases.

The Mediator complex is an evolutionarily conserved multi-protein complex, connecting transcriptional factors to the mRNA polymerase II apparatus, and contributes to regulating gene activities and biological processes (Kagey et al., 2010; Malik and Roeder, 2010). The Mediator subunit *med23* (*sur2*) was first identified as a genetic modulator for an abnormal viral phenotype in *Caenorhabditis elegans* that resulted from hyperactive Ras signaling (Singh and Han, 1995). Previously, we demonstrated that *MED23* physically interacts with the ternary complex factor ELK1 to transduce insulin-MAPK signaling (Wang et al., 2005, 2009). In the mesoderm development, *MED23* has been recently shown to function as a ‘two-way’ switch between Ras/Elk1 and RhoA/Mal signaling, which leads to cell fate preference between the SMC and adipocyte (Yin et al., 2012). Mice lacking *MED23* function showed embryonic lethality at around embryonic day (E) 10.5 and the homozygous mutant embryos were smaller than their wild-type littermates (Balamotis et al., 2009). Besides the apparent anomalies in blood vessel formation, E9.5 *Med23−/−* embryos exhibited severely disorganized nervous system and aberrant neural tube closure, indicating that *MED23* might play a crucial role in neural development. However, due to the complexity of the *in vivo* environment, the specific function and mechanism of *MED23* in early neural development remains to be elucidated.

In this study, we used murine *Med23−/−* (KO) ESCs (mESCs) as an *in vitro* model to investigate the molecular functions of *MED23* in pluripotency and cell lineage commitment. We found that *Med23* depletion did not change the pluripotency or self-renewal of ESCs, but significantly promoted neural lineage specification through attenuating BMP signaling. We further demonstrated that *MED23* functionally interacts with ETS1 to control BMP4 expression through both its promoter and enhancer functions. Interestingly, morpholino-mediated *med23* knockdown in zebrafish enhanced the neural development at a specific time window of the early development, which was reversible by co-injection of *bmp4* mRNA. These results suggest that *MED23* functions as a cell-intrinsic regulator of the early neural development through controlling the ETS1-driven *Bmp4* transcription. Our findings provide mechanistic insights into the transcriptional control of the most crucial factor of the neural ‘default’ model. This should help in understanding the intricate molecular regulation of neural fate and in developing cell-based therapies of neurodegenerative diseases.

**RESULTS**

*Med23* depletion does not change the stem cell pluripotency

The generation of *Med23* KO ESCs has been described in detail previously (Stevens et al., 2002), and is represented by a schematic diagram here (supplementary material Fig. S1A). To investigate the role of Mediator MED23 in early embryonic development, we...
first examined the self-renewal capability and pluripotency of Med23 KO ESCs in vitro. We initially observed that the KO ESCs formed colonies on MEF feeder cells, similar to wild-type ESCs, and all wild-type and KO ESC colonies were positive for alkaline phosphatase staining (Fig. 1A). Pluripotency marker gene expression, represented by Oct4 and Nanog, was comparable between wild-type and KO ESCs when analyzed by quantitative real-time PCR (qRT-PCR) (Fig. 1B). Global gene expression profiling showed that KO ESCs are similar to wild-type ESCs, with an \( r^2 \) value (square of linear correlation coefficient) between KO ESCs and wild-type ESCs of 0.99 (Fig. 1C). Embryoid body (EB) formation is a feature observed in ESCs. KO ESCs were able to grow into EBs similar to those of wild-type ESCs, suggesting that KO ESCs retain many features of ESCs (Fig. 1D). Accordingly, immunostaining revealed no obvious differences in the expression of the pluripotency marker proteins OCT4 and SSEA1 between wild-type and KO ESCs (Fig. 1E). Teratomas generated from either wild-type or KO ESCs implanted subcutaneously in nude mice also successfully differentiated into the three germ layers (Fig. 1F). Unbiased spontaneous differentiation of EBs derived from both wild-type and KO ESCs gave rise to the cell types of all three germ layers, as indicated by real-time PCR analysis of the marker gene expression (Fig. 2A). These results indicate that KO ESCs could differentiate into cells of the three primary germ layers, both in vivo and in vitro, similar to wild-type ESCs. Finally, considering that Med23 KO embryos were able to develop to E10.5 and form most tissues and organs (Balamotis et al., 2009), we conclude that the embryonic stem cell features of the Med23 KO ESCs are largely unchanged by loss of Med23.

**Med23 deficiency promotes neural lineage specification during spontaneous differentiation**

Despite the fact that Med23 deficiency does not significantly alter the self-renewal ability of ESCs, the full term development of Med23 KO mice was compromised, suggesting a possible defect in cellular differentiation. Indeed, neural cells appeared to be more abundant in Med23 KO teratomas than in wild type, and neural cells appeared to be more abundant in Med23 KO ESC-derived teratomas than in wild-type ESC-derived teratomas (data not shown). This observation prompted us to investigate the function of MED23 in ESC differentiation. Wild-type and KO ESCs were cultured under conditions that favored EB formation without leukemia inhibitory factor (LIF). We observed many rosette structures in KO cells at day 9, whereas rosettes rarely appeared in wild-type cells (supplementary material Fig. S1B,C). The expression of marker genes for all three germ layers was monitored over time by RT-PCR.
Compared with wild-type cells, the expression of the ectoderm-specific gene nestin (Nes) was more upregulated from day 6 to day 12, and β-tubulin III (Tubb3) was more upregulated at day 12 of spontaneous differentiation in KO cells. By contrast, there seemed to be no consistent, significant difference in the expression levels of the mesoderm-specific genes Gsc and Sm (Ighmbp2 – Mouse Genome Informatics) and of the endoderm marker genes Sox17 and Foxa2 between the differentiating KO and wild-type cells (Fig. 2A).

Similar results were obtained when immunostaining for the markers of neural progenitors (NES) and immature neurons (TUBB3), which both were significantly enhanced in KO cells at day 9 under differentiation condition (Fig. 2B). To quantitate the difference of wild-type and KO cell differentiation, immunostaining for NES combined with FACS analysis revealed that the percentage of NES+ cells was 13.16% in KO at day 12, compared with 5.27% in WT (Fig. 2C).

Wild-type and KO ESCs grown in N2B27 medium were harvested at days 1, 2, 3, 4 and 7. Consistent with our observations of the spontaneously differentiating cells, the expression levels of multiple neural lineage genes, such as Blbp (Fabp7 – Mouse Genome Informatics), Pou3f3, Cp, Fgfrs and Nes, were strikingly upregulated at days 4 and 7 in Med23 KO cells compared with wild-type cells (Fig. 3A). Moreover, immunofluorescence staining with antibodies against the neural markers NES, MAP2 (Fig. 3B) and BLBP (supplementary material Fig. S1D) revealed that the expression levels of these proteins were greatly enhanced in KO cells compared with wild-type cells.

To test whether the function of MED23 in neural differentiation is independent of the N2B27 medium and the monolayer culture, we performed an additional neural differentiation assay using the serum-free EB formation (SFEB) method (Watanabe et al., 2005). In the SFEB culture, both wild-type and KO ESCs were able to aggregate and form EBs. Gene expression levels in EBs were monitored using real-time PCR after 0, 2, 4, 6 and 8 days of SFEB culture. Consistent with monolayer differentiation, the expression levels of the ESC marker genes Oct4 and Nanog decreased more rapidly in Med23 KO EBs than in wild-type EBs. However, expression of neural cell markers, such as Sox1, Blbp, Pou3f3, Cp, Nes and Tubb3, increased significantly faster and to higher levels in Med23 KO EBs (supplementary material Fig. S2A), thus suggesting more efficient and rapid differentiation in Med23 KO EBs. Differentiating EBs
derived from wild-type and KO ESCs were harvested at day 6, sectioned and stained with antibodies specific against OCT4, SOX2, BLBP and NES. Co-immunostaining of OCT4 and SOX2 exhibited distinct expression patterns in the differentiating wild-type and KO EBs. OCT4+SOX2+ cells and OCT4−SOX2+ cells, indicating ESCs and neural precursor cells, respectively, were counted in multiple sections from multiple EBs. OCT4−SOX2+ cells represented 46.1% of the cells in KO EBs, compared with 7.3% of the cells in WT (supplementary material Fig. S2B,C), indicating a ∼6.5-fold increase in the number of neural precursor cells derived from the Med23-depleted cells. However, the numbers of OCT4+SOX2+ cells (ESCs) were less than onefold lower in KO EBs than in wild-type EBs. Moreover, co-immunofluorescent staining for BLBP and NES was enhanced by 35% and 48%, respectively, in KO EBs compared with wild-type EBs, as quantified by fluorescence intensity (data not shown). Thus, two independent methods of cell differentiation, using distinct culture conditions, demonstrate that depletion of Med23 promotes rapid and efficient neural differentiation of mouse ESCs.

To further verify the role of Med23 in neural differentiation, mouse Med23 was reintroduced into the KO cells using retroviral transduction. Exogenous expression of mouse MED23 in stable cell lines was examined by western blot (Fig. 4A). Only one line of cells showed the re-expression of MED23, and this line was used for further analysis. After cells were induced to differentiate, RT-PCR confirmed the greatly enhanced expression of neural markers in KO cells (Fig. 4B). Significantly, exogenous expression of mouse MED23 partially rescued the enhanced neural differentiation in KO cells, as assessed by the expression of Sox1, Nes, Blbp and Pou3f3 and by immunostaining for NES and BLBP (Fig. 4B,C). Collectively, these results, coupled with the experiments in both adherent monolayer and suspension cultures, demonstrate that Med23 is crucial for regulating neural differentiation of ESCs.

Two other Mediator components, MED1 and MED15, do not alter neural differentiation
Med23 depletion does not impair the integrity of the Mediator complex, as demonstrated in our earlier study (Wang et al., 2009). To ascertain the specificity of MED23 in neural differentiation among the Mediator subunits, we probed the functions of two other subunits, MED1 and MED15, during ESC differentiation.

A previous study showed that Mediator Med1 is required for PPARG2-mediated adipogenesis (Ge et al., 2002); however, whether it is required for neural specification is not yet known. We established stable cell lines by viral-mediated knockdown of Med1 and Med23 in R1 ESCs. Consistently, we observed that knocking down Med23 leads to enhanced neural differentiation. However, interference with the expression of another Mediator component, Med1, failed to change the speed and efficiency of neural differentiation (supplementary material Fig. S3).
A previous study found that MED15 contributes to mesendoderm specification in Xenopus (Kato et al., 2002), but it remains to be determined whether MED15 controls the early cell fates of ESCs. In this study, we observed that viral-mediated knockdown of Med15 did not affect the features of the ESCs (supplementary material Fig. S4A,B); however, consistent with the study in Xenopus, MED15 is specifically required for endoderm differentiation in mouse ESCs (supplementary material Fig. S4C). The specificity was shown by the fact that Med15 depletion resulted in reduction in endoderm markers, such as FOXA2, AFP and SOX17 (supplementary material Fig. S4C), but failed to affect neural differentiation (supplementary material Fig. S4D). These observations further emphasized the specific role of MED23 in neural lineage. Collectively, these results suggest that different Mediator subunits contribute to the distinct cell lineage regulation during early development.

**MED23 regulates neural specification by modulating BMP4 signaling**

Multiple signaling pathways have been shown to impact the neural induction from ESCs, such as BMP, FGF and WNT signaling at the gastrulation stage (Baker et al., 1999; Wilson et al., 2000; Stern, 2005). In this context, we examined whether MED23 affects these signal pathways. A transcriptome analysis was performed to compare the gene expression patterns between Med23 KO and wild-type ESCs under the standard ESC culture condition, which represents the starting point of ESC differentiation. Only very few genes from the WNT signaling pathway (the core β-catenin pathway and WNT/sFRP families) and the FGF/MAPK pathway [except Egr1, a prototype MED23-target gene (Wang et al., 2005)] that displayed strong defects as a result of the Med23 KO (Fig. 5A, columns 1, 2, 4 and 5). However, we observed the downregulation of multiple BMP/SMAD pathway member genes, such as Id1, Id2, Bmp4, Bmpr1a, Dusp9 and Coch (Fig. 5A, column 3) that have been described as BMP-target genes by previous studies (Li et al., 2012). In contrast to the significantly altered gene expression of the BMP4/SMAD pathway, the subunit genes of the TFIID complexes were only moderately changed by Med23 deletion (Fig. 5A, column 5), thereby suggesting that Med23 deficiency might specifically attenuate BMP4 signaling. As a genome-wide analysis also revealed that Bmp4 expression levels were largely reduced in KO ESCs, we examined the expression of BMP4-target gene Id1 and Id2 by qRT-PCR and found that expression of both genes was reduced (Fig. 5B). Importantly, qRT-PCR also verified that the expression of Bmp4 was greatly reduced in Med23 KO ESCs during the entire neural differentiation process in adherent monolayer culture (Fig. 5B). In addition, the level of phospho-Smad1/5/8, representing BMP signaling activity, was significantly reduced in both normal culture and serum starvation condition in KO ESCs compared with wild-type ESCs, suggesting that BMP signaling is reduced in KO ESCs (Fig. 5C). Therefore, Med23 deficiency impairs Bmp4 expression, which might be the crucial event that results in the downregulation of the entire pathway.

**Fig. 4. Enhanced neural differentiation is reversed by re-expression of Med23.** (A) Immunoblot of MED23 in wild-type, Med23 KO and Med23 KO+exogenous Med23 ESCs. (B) Expression of marker genes for ESC self-renewal and neural precursors were measured by qRT-PCR. Gapdh mRNA was used as internal control. (C) Immunostaining for NES and BLBP in differentiated cells derived from wild-type, Med23 KO and Med23 KO +exogenous Med23 ESCs, respectively. Scale bars: 50 µm.
BMP signaling has been previously shown to repress neural differentiation in mouse ESCs (Tropepe et al., 2001; Ying et al., 2003). Given that MED23 impacts neural differentiation through BMP4, addition of BMP4 should be able to repress the enhanced neural differentiation in the Med23 KO ESCs culture. Indeed, we observed that, whereas Med23 deficiency promoted ESC neural differentiation, addition of BMP4 significantly abrogated the enhanced neural differentiation (Fig. 5D). Consequently, neural differentiation became equivalent between wild-type and KO ESCs at day 6 of differentiation. To further define the time window during which BMP4 functions during neural differentiation, recombinant BMP4 was added to the N2B27 monolayer culture at different time points of the wild-type and KO ESC differentiation. We defined the day on which the ESCs were seeded as differentiation day 0 and examined the neural marker at day 6. We found that the most significant inhibition of neural differentiation was achieved when BMP4 was added during day 1-3, whereas the addition of BMP4 at other time points failed to change the course of differentiation (Fig. 5E). This suggests that ESCs are most sensitive to BMP4-mediated block of neural differentiation at the early phase of differentiation.

Fig. 5. Med23 deletion attenuates BMP4 signal.

(A) Difference in expression (log2 [KO/WT]) and statistical significance between Med23-depleted and wild-type ESCs. (B) qRT-PCR measured the expression of Id1 and Id2 in ESCs, and the expression of Bmp4 in ESCs and during differentiation in adherent monolayer culture. (C) Identification of phospho-Smad1/5/8 under ESC or serum starvation culture conditions. (D) Expression of pluripotency markers and neural markers was examined by qRT-PCR at day 6 of monolayer differentiation; the expression was normalized to Gapdh mRNA levels. (E) Cells cultured by N2B27, with recombinant hBMP4 added at different time points; pluripotency markers and neural markers were examined by qRT-PCR.

MED23 regulates Bmp4 expression through controlling the activity of the ETS family member ETS1

To understand the molecular mechanism by which Med23 deficiency inhibits Bmp4 expression, we first performed a promoter analysis for the Bmp4 gene. Sequence analysis of the Bmp4 promoter region revealed the presence of multiple putative binding sites for the transcription factor ETS1, an ETS family member. The putative ETS1-binding sites (EBS) could be separated as distal region 'A' and proximal region 'B', respectively (Fig. 6A). The transcription factor ETS family members are defined by a conserved DNA-binding domain and a common consensus target sequence, GGA(A/T) (Hollenhorst et al., 2011). Our previous study shows that the ETS family member ELK1 interacts with MED23 (Wang et al., 2009), we thus tested whether ETS1 could cooperate with MED23 to control the expression of Bmp4.
We first determined whether ETS1 regulates Bmp4 expression. The promoter region of murine Bmp4 was subcloned into a reporter vector with or without the deletions of the region ‘A’ and/or ‘B’. The following cloned luciferase constructs were used: the full-length (FL) promoter sequence from −1 to −1676 bp; FL with region ‘A’ deleted (FL-A), from −1 to −1311 bp; FL with region ‘B’ deleted, from −166 to −1676 bp; and FL with both region ‘A’ and ‘B’ deleted, from −166 to −1311 bp (Fig. 6C). The luciferase activity driven by FL, FL-A, FL-B and FL-AB was evaluated in co-transfection experiments in 293T cells. As control, pGL-Control was transfected with empty pGL3. The luciferase activity of the promoters FL-B and FL-AB was reduced by 80% compared with the FL promoter, whereas the reduction of luciferase activity of FL-A was only 21% (Fig. 6D), suggesting that region ‘B’ is more important than ‘A’ for the ETS1 activity. In addition, overexpression of ETS1 enhanced the luciferase activity of promoter FL and FL-A more than threefold, but the luciferase activity of FL-B and FL-AB could not be induced by ETS1 (Fig. 6D), thus again suggesting that the ETS1-binding site ‘B’ is crucial for ETS1 in activating Bmp4 promoter. Consistent with these results in 293T cells, we also observed that the luciferase activity of FL was lower in KO ESCs compared with WT, and that exogenous expression of ETS1 enhanced the luciferase activity of the promoter FL threefold in wild-type ESCs, but only onefold in KO ESCs (Fig. 6E). Furthermore, deletion of either ‘A’ or ‘B’ impaired...
promoter activity significantly (Fig. 6E), suggesting that the Bmp4 promoter regions ‘A’ and ‘B’ are both required for ETS1-driven Bmp4 promoter activity in ESCs.

We next examined whether both endogenous ETS1 and MED23 are required for Bmp4 transcription. Chromatin immunoprecipitation (ChIP) assays were performed to measure the binding of ETS1 and POL II (POLR2F - Mouse Genome Informatics) at the Bmp4 locus when cells were cultured under the serum-free condition that closely mimics the neural differentiation condition. We found that both ETS1 and Pol II constitutively bind to the Bmp4 promoter at both regions ‘A’ and ‘B’. However, ETS1 binding at both regions ‘A’ and ‘B’ of the Bmp4 promoter was greatly reduced in KO cells (Fig. 6F), despite the fact that the expression level of ETS1 protein seemed to be equal in both wild-type and KO ESCs (Fig. 6B).

We were intrigued by the observation that Pol II binding reduced significantly at distal promoter region ‘A’ (Fig. 6F), suggesting that ETS1 probably also regulates Bmp4 transcription through an enhancer. Previous studies indicated that the Mediator complex cooperates with the cohesin complex to contribute to chromatin architecture (Kagey et al., 2010), and collaborates with diverse transcription factors to establish a ‘super enhancer’ for cell fate determination (Whyte et al., 2013). This information prompted us to examine whether an enhancer might be involved in the Bmp4 gene regulation. Predicted by the ChIP-seq data of H3K27ac and P300 from ENCODE, a putative enhancer region −10 kb upstream of the Bmp4 transcription start site was identified (Fig. 6G). We then tested whether the Mediator subunit MED23 regulates this enhancer function of the Bmp4 gene. When comparing Pol II ChIP-seq signals on Bmp4 between wild-type and KO ESCs, we found that enrichment of Pol II was decreased in KO ESCS at both the promoter and the putative enhancer regions of Bmp4, suggesting that MED23 depletion might have disrupted the interplay between the promoter and enhancer regions of Bmp4 (Fig. 6G). To verify the effect of MED23 depletion on the enhancer activity, we performed ChIP assays to examine the possible changes of three typical enhancer markers: Pol II, P300 and H3K27ac at the Bmp4 putative enhancer region in the KO ESCs. The results showed that MED23 deficiency reduced their enrichments at the Bmp4 enhancer, suggesting that MED23 can alter promoter-enhancer loop confirmation on Bmp4 for its transcriptional control (Fig. 6H). By contrast, the enrichment of Pol II and H3K27ac at the Oct4 enhancer region was largely unchanged in either wild-type or KO cells (Fig. 6I), which is consistent with the fact that the Oct4 expression is independent of MED23 (Fig. 1B,C). Taken together, these data suggest that the presence of MED23 favors the ETS1 binding to facilitate promoter-enhancer communication on Bmp4, which might account for the higher Bmp4 expression in wild-type ESCs. We are currently unable to co-IP the endogenous ETS1 and MED23, probably due to the transient and dynamic nature of ETS1-MED23 interaction. However, based on the experimental evidence shown above, we propose that MED23 might functionally associate with ETS1, directly or indirectly, to mediate the enhancer-promoter interaction, thus controlling Bmp4 expression (Fig. 7F).

Knockdown of med23 in zebrafish results in enhanced neural development that is reversible with bmp4 mRNA co-injection

During the early development of zebrafish med23 is ubiquitously expressed (supplementary material Fig. S5A). Our recent study showed that med23 MO-injected embryos (morphants) showed abnormal phenotypes and precocious development of vascular smooth muscle (Yin et al., 2012). As Med23 KO mESCs are prone to neural differentiation, we repeated the med23 MO-injection experiment in zebrafish to explore the role of med23 in neural development. In this study, two antisense morpholino oligonucleotides (MO-1 and MO-2) were used to inhibit the expression of med23 in zebrafish as shown by western blot (Fig. 7D). We observed that med23 morphants generally had a survival time of 5-7 days post fertilization (supplementary material Fig. S5E) and an apophysis phenotype in the dorsal brain (supplementary material Fig. S5B). To determine whether Med23 regulates neural development in vivo, we monitored the early neural development in huC-gfp transgenic zebrafish, using the huC expression to indicate the pan-neural development. We found that
HuC-GFP signals can be detected as early as 12 h post fertilization (hpf) in med23 morphants, but not in the control MO embryos (12 hpf). Consistently, the total mRNA levels of endogenous huC and egr2b were also increased in both morphants compared with the control at 6 hpf and 9 hpf (supplementary material Fig. S5C), which was further verified by in situ hybridization for huC at 12 hpf (supplementary material Fig. S5D). Moreover, we observed that HuC-driven GFP expression was greatly enhanced in morphant brains at 18 hpf and 24 hpf compared with the control group (Fig. 7A). The enhanced GFP expression seems to localize mainly to a particular hindbrain region, as indicated by in situ hybridization for huC at 15 hpf (Fig. 7B) and for huC and egr2b at 24 hpf (Fig. 7E). Accordingly, the expression levels of endogenous anf (telencephalon), fli1 (diencephalon), egr2b and hoxb1a (hindbrain) were upregulated in morphants at 24 hpf compared with the control group, although some neural markers, such as cyp26b1 (hindbrain), six3a (forebrain) and otx2 (midbrain) were not changed after med23 knockdown (Fig. 7C). Notably, we also observed that the HuC-GFP signal became weaker in med23 morphants at 48 hpf compared with the control group, probably as result of precocious neuronal differentiation (Fig. 7A).

Previous studies have shown that BMP activity affects dorsoventral (DV) patterning at all anteroposterior (AP) levels of the neural plate and is required for patterning neurons at all DV levels of the central nervous system (CNS) (Barth et al., 1999), and Bmp signaling might regulate the acquisition of a DV and AP identity of a cell simultaneously (Tucker et al., 2008). In our zebrafish study, we observed that the DV axes of med23 morphants shortened and the AP axes elongated compared with the control group (Fig. 7E). Given that Med23 regulates BMP/SMAD signaling in mESCs, we are interested in whether the enhanced neural development in med23 morphants is also regulated by a Bmp4 signal. In situ hybridization revealed that the increased expression of huC in the brain and spinal cord of med23 morphants could be repressed after co-injection of low level bmp4 mRNA (3 pg/per embryo) to a level comparable to that of control MO zebrafish embryos (Fig. 7E). Interestingly, zebrafish embryos injected with a higher concentration of med23 MO presented a more severe phenotype; this phenotype was reversed (or rescued) when embryos were co-injected with bmp4 mRNA (Fig. 7E). This experiment suggests that Bmp4 is a crucial effector in Med23-modulated early neural development of zebrafish. Overall, the zebrafish data seem to be consistent with our observations on the role of MED23 in mESC differentiation, reinforcing the notion that MED23 impacts on early neural development via BMP4 signaling.

DISCUSSION

Previous studies demonstrated that all Mediator complex subunits are generally expressed at high levels in ESCs compared with differentiated fibroblasts, and Mediator contributes to the communication between the enhancer and proximal promoter regions of the pluripotency genes in ESCs (Kagey et al., 2010). This suggests that Mediator participates in the regulation of ESC function. Med23-depleted ESCs were shown to maintain the integrity of the Mediator complex, as evaluated through co-IP and gel filtration assays (Stevens et al., 2002; Wang et al., 2009). To understand the role of MED23 in regulating stem cell identity, we performed numerous assays, including gene profiling, pluripotency marker analysis, EB formation and teratoma formation, and we demonstrated that loss of Med23 did not alter the self-renewal and pluripotency of ESCs. As both wild-type and KO ESCs maintain the stem cell properties, we analyzed whether wild-type and KO ESCs might have different differentiation potentials, using an unbiased spontaneous EB differentiation assay. Interestingly, Med23 deficiency specifically promoted neural differentiation in an unbiased differentiation assay. Two distinct neural differentiation assays, N2B27 monolayer and KSR suspension culture, further verified this observation.

Neural specification from ESCs is regulated by multiple signals, such as WNT signaling, BMP/SMAD and FGF/MAPK pathway. By gene profiling, we detected that BMP/SMAD signaling was attenuated and Bmp4 expression was reduced in Med23 KO ESCs. An in-depth mechanistic study revealed that MED23 regulates ETS1-driven Bmp4 expression. Importantly, enhanced neural differentiation was attenuated when Bmp4 was added back to the KO ESC culture, or when the med23 MO was co-injected with bmp4 mRNA, suggesting that BMP4 is indeed an effector in MED23-regulated neural development. To further understand how exactly MED23 controls ETS1-driven Bmp4 expression, we employed ChIP and ChIP-seq assays. We showed that MED23 depletion might disrupt the connection between ETS1 and the transcription machinery, and compromise the promoter-enhancer interaction of Bmp4, thus resulting in the decreased Bmp4 expression and enhanced neural differentiation. Bmp4 is a well-known neural lineage blocker gene, although its own regulation during development is not well studied. Our work describes for the first time that how Bmp4 is regulated by MED23 during neural differentiation of ESCs. This expands our understanding of the function of the Mediator complex in long-distance gene regulation, and provides a novel mechanistic basis of how different Mediator subunits determine different cell lineages (Yin and Wang, 2014).

Besides the BMP pathway, multiple signaling pathways are involved in regulating neural differentiation from ESCs. Previous studies suggested that FGF/MAPK signaling is required for neural fate determination (Kunath et al., 2007; Stavridis et al., 2007; LaVaute et al., 2009) and that MED23 is a target of this pathway (Wang et al., 2005). We therefore also examined the activity of the MAPK pathway in Med23 KO ESCs during differentiation. Phospho-ERK1/2 levels, representing FGF/MAPK pathway activity, were analyzed by western blot and seemed to be similar in KO and wild-type ESCs. However, ERK1/2-phosphorylation showed a slight increase in KO cells at early differentiation (day 1), which is consistent with a previous study showing that FGF-induced MAPK/ERK activity is required for neural lineage commitment of ESCs (Kunath et al., 2007) (supplementary material Fig. S6A). In addition to FGF/MAPK, WNT signaling also extensively affects neuroectodermal differentiation from ESCs (Aubert et al., 2002; Sato et al., 2004; Lindsley et al., 2006). In our study, we observed that phospho-β-catenin was increased and total β-catenin was reduced in KO ESCs and in KO ESC-derived cells during differentiation (day 1-2) compared with WT, suggesting that the WNT signaling activity was probably attenuated (supplementary material Fig. S6A). Paradoxically, we found that the expression of the WNT target genes Axin2 and Sox3 were increased in KO cells compared with wild-type cells during differentiation (supplementary material Fig. S6B). Despite these observations, only a few genes from the WNT signaling pathway, as examined by gene profiling, displayed obvious defects (Fig. 5A, column 4), thus suggesting that WNT signaling activity was not changed as a result of Med23 depletion. Overall, our data showed that Med23 might regulate neural differentiation mainly through modulating BMP signaling.

Growing evidence supports the notion that the Mediator subunits control diverse development processes with subunit specificity (Kato et al., 2002; Wang et al., 2006; Yang et al., 2006; Lin et al., 2007; Chen
et al., 2010). Our previous studies demonstrated that Med23 deficiency reduced adipocyte differentiation and increased SMC differentiation (Wang et al., 2009; Yin et al., 2012), suggesting the important roles of Med23 in mesoderm development. In this study, however, in multiple ESC differentiation assays, we did not observe any dramatic and consistent changes for mesoderm markers, such as Smα and Flik1, which might be due to the ESC differentiation representing an early stage of development. In contrast to the mild changes of Flik1 and Smα, the expression of both Nes and Tubβ3 was greatly increased in KO ESCs in multiple differentiation assays. Therefore, we focused on the role of Med23 in neural differentiation using the ESC differentiation system. In this study, enhanced neural differentiation by Med23 depletion was observed in two independent ESC lines and under three distinct culture conditions, including spontaneous EB differentiation, N2B27 monolayer and KSR suspension culture. However, knockdown of two other Mediator components, Med1 or Med15, did not change the neural differentiation potentials (supplementary material Figs S3 and S4), and Med15 knockdown specifically blocked the endoderm differentiation (supplementary material Fig. S4). Using zebrafish to study the role of Med23 on neural development in vivo, we observed that med23 knockdown facilitated neural development ahead of schedule compared with control embryos, and led to precocious neuronal differentiation. Taken together, these data demonstrate that, despite the Mediator subunit MED23 having pleiotropic effects on multiple cell fate determination at different development stages, MED23 does play a specific role at an early stage of neural development.

Thus, targeting Mediator MED23 might provide a rapid and efficient method to generate a large amount of neural progenitor cells from ESCs or induced pluripotent stem cells (iPSCs), which might lead to new therapeutic strategies for neurodegenerative diseases.

**MATERIALS AND METHODS**

**Animals**

Wild-type zebrafish (*Danio rerio*) and HuC transgenic zebrafish were obtained and maintained in the National Zebrafish Resources of China (Shanghai, China). All zebrafish were maintained with an automatic fish-housing system (ESEN, China) at 28°C. Embryos and larvae were raised on a 14-10 h light-dark cycle in 10% Hank’s solution.

The nude mice were obtained from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. All animal handling procedures were approved by the Institutional Animal Care and Use Committee of Shanghai Institutes for Biological Sciences.

**ESC culture**

All ESCs were maintained in knockout Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 15% fetal bovine serum (Hyclone), 1× nonessential amino acids (Gibco), 1% L-glutamine (Gibco), 1% penicillin/streptomycin (Gibco), 1×10^5 units/ml LIF (ESGRO, Chemicon) and 0.1 mM β-mercaptoethanol (β-ME; Sigma). Cells were grown in a humidified incubator at 37°C and 5% CO2 for 10% media every other day. ESCs were fed daily. LW murine ESCs, 129-derived (a kind gift from Dr Hong Wu, UCLA, USA), were maintained on 0.2% gelatin-coated plates. R1 murine ESCs were maintained on a feeder cell layer of irradiated mouse embryonic fibroblasts.

**Spontaneous differentiation**

Undifferentiated ESCs were cultivated as EBs in hanging drops for 2 days, followed by culturing in suspension for an additional 3 days. The EBs were then plated onto gelatin-coated tissue culture plates. For immunostaining assays, cells were cultured in gelatin-coated 4-well chamber slides. Cells were cultured in ES medium lacking LIF, and the medium was changed every other day.

**Neural differentiation methods**

The method used for monoculture differentiation has been described previously (Pollard et al., 2006). Undifferentiated ESCs were dissociated and plated onto 0.2% gelatin-coated plates at a density of 0.8-1.2×10^5/cm^2 in N2B27 medium. After the first 2 days, medium was renewed daily. N2B27 is a 1:1 mixture of DMEM/F12 (Gibco) supplemented with modified N2 (25 μg/ml insulin, 100 μg/ml human transferrin, 6 ng/ml progesterone, 16 μg/ml putrescine, 30 nM sodium selenite and 50 μg/ml bovine serum albumin fraction V (Gibco)) and neurobasal medium (Gibco) supplemented with B27 (Gibco). For immunostaining, cells were transferred to 4-well chamber slides pre-treated with poly-L-ornithine (50 μg/ml) and laminin A (10 μg/ml). For SFEB culture, differentiation medium was prepared as follows: G-MEM supplemented with 5% knockout serum replacement (KSR) (Invitrogen), 2 mM glutamine, 1 mM pyruvate, 0.1 mM nonessential amino acids and 0.1 mM β-ME. Briefly, 5×10^4 cells per 1 ml differentiation medium were seeded into 6-cm bacterial dishes (5 ml). ESCs spontaneously aggregated to form EBs in serum-free suspension cultures within 1 day. Medium was changed every two days. RNA was extracted from differentiating EBs on days 2, 4, 6 and 8 for gene expression assays. EBs were fixed and sectioned for immunofluorescent staining on day 6. The day on which ESCs were seeded to differentiate was defined as day 0.

**Immunofluorescence**

Cells were washed with PBS twice, fixed in 4% paraformaldehyde and incubated for 1 h in blocking buffer (PBS, 2% BSA and 0.2% Triton X-100). Primary antibodies were diluted in blocking buffer and applied overnight at 4°C. After three washes in PBS, secondary antibodies diluted in blocking buffer were applied for 1 h at room temperature. After three washes by PBS, nuclei were stained with DAPI (1:1000) for 4 min. Antibodies used for immunostaining were as follows: anti-Nes (Santa Cruz, sc-21249; 1:50); anti-βB-LBP (Abcam, ab32423; 1:200); anti-Oct4 (Santa Cruz, sc-8628; 1:100); anti-SOX2 (Abcam, ab59776; 1:200); anti-MAP2 (Chemicon, MAB5622; 1:200) and anti-SESA1 (Santa Cruz, sc-21702; 1:100).

**Real-time PCR and western blotting**

Methods for real-time PCR and western blot were described previously (Wang et al., 2009). Total RNA was isolated from cells using TRIzol (Invitrogen). The first-strand cDNA was generated using MMLV transcriptase (Promega), and real-time PCR was performed in triplicate using a SYBR Green PCR master mix in an Eppendorf Mastercycler. All values were normalized to the level of EF2 or Gapdh mRNA, which is constitutively expressed and not changed during the time course of the experiments. Primer sequences used in the experiments are included in the supplementary material Table S1. The western blot was performed using an ECL kit (Pierce) based on the manufacturer’s recommendations. HRP-conjugated secondary antibodies were purchased from Jackson Laboratory.

**Teratoma formation**

After nude mice were anesthetized with diethyl ether, 1.5×10^6 ESCs were injected subcutaneously into the dorsal flank. Tumors were surgically dissected from the mice 26 days after injection. Samples were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin.

**Retroviral constructs and infection**

Establishing stable cell lines to knock down a gene of interest was based on the manufacturer’s recommendation (Clontech). For knockdown of Med23, Med15 and Med1 in ESCs, retrovirus-mediated siRNA expression was used. The targeted sequence was determined using the Whitehead Institute siRNA designing tool. As recommended by Clontech for the RNAi-Ready pSIREN-RetroQ system, two complementary oligos for each targeted sequence were designed, annealed and ligated into the BamHI/EcoRI-linearized pSIREN-RetroQ vector. The resulting construct, upon packaging into retroviruses, allows for the stable expression of the siRNA hairpin
specific for the target gene. The sequences of the oligonucleotides are provided in supplementary material Table S1. Generation of retroviruses has been described previously (Wang et al., 2009).

**Morpholino, mRNA synthesis and microinjection**

Morpholino antisense oligonucleotides (MO) were obtained from Gene Tools, and the sequence information of morpholinos is provided in supplementary material Table S1. To generate mRNAs, full-length bmp4 (zebrafish) genes were subcloned into a pCDNA3 vector (cloning primer sequences are listed in supplementary material Table S1) and synthesized using the mMessage mMACHINE kit (Ambion). For all microinjection experiments, a volume of 1 nl was injected into one- to two-cell stage embryos. Zebrafish maintenance and breeding were carried out as previously described (Westerfield, 1993). Embryos were raised at 28.5°C and staged as previously described (Kimmel et al., 1995).

**Whole-mount in situ hybridization**

RNA antisense probes were synthesized using a DIG RNA labeling kit (Roche, 11175029510). Zebrafish embryos of the appropriate stages were collected and dechorionated when needed. The embryos were fixed in PBS containing 4% PFA and dehydrated in methanol at –20°C. Whole-mount in situ hybridization (WISH) was performed as described previously (Yue et al., 2009) with some modifications. Hybridization signals were detected by the addition of an anti-DIG antibody conjugated with alkaline phosphatase (Roche, 11093274910) and the enzyme substrates BCIP/NBT (Vector Labs, SK5400).

**Microarray analysis**

Mouse genome-wide gene expression analysis was performed using Affymetrix Mouse Gene 430 2.0 array. Six arrays were probed with cDNA synthesized from total RNA isolated from wild-type and Med23 knockout ESCs. Data are deposited at GEO under accession number GSE64500.

**ChIP, ChIP-seq and data analysis**

ChIP assays were performed as described previously (Wang et al., 2005), and immunoprecipitated DNA was quantified by real-time PCR. The following antibodies were used: anti-ETSI (Santa Cruz, sc-350X; 1:100); anti-Pol II (Santa Cruz, sc-899; 1:100); anti-P300 (Santa Cruz, sc-585; 1:100); anti-H3K4me1 (Abcam, ab8895; 1:500) and anti-H3K27ac (Abcam, ab4729; 1:500). The primers for analyzing the ChIP DNA are provided in supplementary material Table S1. ChIPed DNA was prepared for ChIP-seq and data analysis as described previously (Huang et al., 2012).

**Acknowledgements**

We thank Dr Annie Berk (UCLA, Los Angeles, USA) for reagents and Dr Juilin Du (Institute of Neuroscience, Shanghai, China) for help with the zebrafish experiments.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

G.W. and W.Z. conceived and designed the experiments and wrote the manuscript. Data are deposited at GEO under accession number GSE64500.

**References**


mammalian neural stem cell stage acquired through a default mechanism. Neuron 30, 65-78.


