Cell Aggregation-induced FGF8 Elevation Is Essential for P19 Cell Neural Differentiation[□]

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FGF8, a member of the fibroblast growth factor (FGF) family, has been shown to play important roles in different developing systems. Mouse embryonic carcinoma P19 cells could be induced by retinoic acid (RA) to differentiate into neuroectodermal cell lineages, and this process is cell aggregation dependent. In this report, we show that FGF8 expression is transiently up-regulated upon P19 cell aggregation, and the aggregation-dependent FGF8 elevation is pluripotent stem cell related. Overexpressing FGF8 promotes RA-induced monolayer P19 cell neural differentiation. Inhibition of FGF8 expression by RNA interference or blocking FGF signaling by the FGF receptor inhibitor, SU5402, attenuates neural differentiation of the P19 cell. Blocking the bone morphogenetic protein (BMP) pathway by overexpressing Smad6 in P19 cells, we also show that FGF signaling plays a BMP inhibition-independent role in P19 cell neural differentiation.

INTRODUCTION

Neural induction is the process by which part of the ectoderm is specified and set aside to become the embryonic neural plate (Waddington and Schmidt, 1933). For the mechanism of neural induction, Spemann and Mangold (1924) proposed that the organizer, the dorsal lip of blastopore, instructs neighboring nascent embryonic ectoderm cells to adopt neural fates. Since the last decade, the default model proposes that ectodermal cells acquire their neural identity autonomously in the absence of inhibitory bone morphogenetic protein (BMP) signals. The organizer secretes BMP antagonists to block BMP signaling, which allows the ectoderm to differentiate into neural tissue in a default way (Hemmati-Brivanlou and Melton, 1997a, 1997b). Recently, however, studies in chicks show that fibroblast growth factor (FGF) signaling is essential for neural induction by repressing BMP mRNA expression and also by a BMP repression independent pathway with an unknown mechanism. Wnt signaling is also involved in this process, suggesting a more complicated mechanism (Wilson *et al.*, 2000, 2001; Wilson and Edlund, 2001). In *Xenopus*, FGF and an insulin-like growth factor (IGF) play positive roles in neural induction through receptor tyrosine kinases, and FGF promotes neural induction through phosphorylating the linker region of Smad1 to inhibit BMP signaling (Streit *et al.*, 2000; Pera *et al.*,

2003; Delaune *et al.*, 2005; Kuroda *et al.*, 2005). However, several questions, like whether BMP inhibition is really sufficient to specify neural fate and whether FGF signaling plays any direct and BMP independent role(s) in neural induction, remain obscure (Stern, 2005).

Mouse embryonic carcinoma (EC) P19 cells can be induced to differentiate into neurons and glial cells when aggregated in the presence of retinoic acid (RA; Jones-Villeneuve *et al.*, 1982, 1983; Mcburney *et al.*, 1982). Exposure of monolayer cultured P19 cells to RA leads to formation of endoderm- and mesoderm-like cells (Jones-Villeneuve *et al.*, 1982, 1983; den Hertog *et al.*, 1993), whereas aggregation of P19 cells without chemical inducers results in the differentiation of the extraembryonic endoderm (Smith *et al.*, 1987; Mummery *et al.*, 1991), implying that both RA (diffusible signals) and cell aggregation (cell–cell contact) are essential for P19 cell neural differentiation. Moreover, overexpression of RA induced genes could trigger P19 cell neural differentiation without RA treatment, but the cell aggregation is still needed (Gao *et al.*, 2001; Tang *et al.*, 2002). These observations suggest that cell aggregation might mediate an independent pathway, which is parallel to RA signaling during P19 cell neural differentiation. To identify the molecule(s) involved in P19 cell aggregation and to uncover its relationship with neural fate determination would be interesting.

FGF8, a member of the FGF family, has been shown to be essential for multiple developmental processes. It is expressed in several regions of mouse embryo: i.e., the epiblast, primitive streak, surface ectoderm of branchial arches, apical ectodermal ridge of the limb bud, isthmus of midbrain-hindbrain junction, and forebrain (Crossley and Martin, 1995). Consistent with its expression pattern, FGF8 is important for induction and patterning of the embryo during gastrulation, limb development, and midbrain-hindbrain formation.

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Table 1. PCR primers and reaction parameters

	5' primer	3' primer	T _m (°C)	Cycles (bp)	Size
FGF8	caggtgaggagcagagcct	agttgttctccagcacgac	61	31	310
FGF1	tatcacgtcactgtgtgcttgg	ttcatttgaacagcattctctgg	52	40	244
FGF2	caagcggctctactgcaaga	cagctcttagcagacattgg	58	35	381
FGF4	cgaccacaggggacgctgctg	actccgaagatgctcaccacg	58	38	364
FGF5	aaagtcaatggctcccacgaa	cttcagctgtgacttactg	53	35	464
FGF10	cattgtgcctcagccttcc	tcattttctctatctctc	52	38	461
IGF	tactcaacaagcccacagg	tccttctccttgcagcttc	55	35	239
EGF	tctggatggctccaacgcc	ccatgatttcagccactag	56	40	479
FGFR1	atgtggggctggaagtgcctct	gtacgggtgctctcaccagctg	61	27	235
FGFR2	gcgcttcac tgcctggct	agtctctagggtggtggcact	56	27	289
Brachyury	aagaacgaccacaagatg	tggtaccattgctcacagac	60	33	410
Goosecoid	agcatgttcagcatcgacaa	cgtagaagtagctgtttag	54	33	247
HNF3β	actggagcagctactacg	cccacataggatgacatg	60	33	160
GATA6	agacataaacattctcgatgcg	ttccaagtgcacctcagacg	56	33	504
Sox1	tgaggaggcacagctggcctac	tgccgccaccgccgagttctgg	60	30	281
Sox2	acctacagcatgtctactcg	gggcagtggtccgttaattgg	54	30	276
Smad6	cgactttggcgaagctgtgt	tacgtgaccgtcttgagctc	52	40	550
β-actin	tcgtcgacaacggctccgcatgt	ccagccagggtccagacgaggat	56	23	520
GAPDH	gccatcaacgaccttcac	gctgtcttcaccaccttc	56	23	701

FGF8 knockout mice could not survive beyond embryonic day 9.5 (E9.5), because of defects in epithelial-mesenchymal transition (Sun *et al.*, 1999). FGF8 also induces, initiates, and sustains the formation of limb buds by promoting sonic hedgehog expression (Crossley *et al.*, 1996a). Implantation of FGF8 beads into the caudal diencephalons could mimic the isthmus organizer to induce a supernumerary midbrain and isthmus tissue (Crossley *et al.*, 1996b). However, little is known if FGF8 plays any role in the neural induction of early mouse embryos. In this study, we identified FGF8 as an early responsive molecule to the aggregation of P19 cells, and showed that FGF signaling is essential for P19 cell neural differentiation.

MATERIALS AND METHODS

Plasmids Construction

The coding region of FGF8 cDNA (a generous gift from Dr. Martin) was inserted into Myc-pcDNA3 vector to construct the Myc-FGF8 fusion protein expression vector, Myc-FGF8-pcDNA3, as described (Tang *et al.*, 2002). Sequence corresponding to FGF8 mRNA 733–751 base pairs was chosen as the RNA interference (RNAi) target and inserted into the BglII/HindIII sites of PPE, a modification of pSuper (Zhang *et al.*, 2004), to generate the FGF8 RNAi expression vector.

P19 Cell Neural Differentiation

P19C6, a subclone of the mouse embryonic carcinoma P19 cell line, was used in this study, and the RA-induced P19 cell neural differentiation was performed as previously described (Gao *et al.*, 2001; Tang *et al.*, 2002).

The monolayer P19 cells (30–40% confluent) were transiently transfected with Myc-FGF8-pcDNA3 with calcium phosphate (Sambrook and Russell, 2001) and were cultured in 10% fetal bovine serum (FBS)/DMEM/F12 in the presence of 10^{-6} M RA for 2 d, and then the medium was changed to N2 medium for 5 d before immunostaining analysis. For neural induction with growth factors, the monolayer P19 cells were seeded at a density of 1×10^5 cells/well in six-well plates containing 10% FBS/DMEM/F12. The next day, the medium was changed to 10% FBS/DMEM/F12 supplemented with growth factors (10 ng/ml, PeproTech, London, United Kingdom), RA (10^{-6} M), and heparin (2 μ g/ml). The cells were cultured for 2 d and then trypsinized and fixed for immunostaining analysis.

The P19 cell lines stably transfected with Myc-FGF8-pcDNA3 expression vector (FGF8/P19) were established as previously described (Gao *et al.*, 2001; Tang *et al.*, 2002). Monolayer FGF8/P19 cells (60–70% confluent) were cultured in 10% FBS/DMEM/F12 with 10^{-6} M RA for 2 d, and medium was changed into N2 medium for up to 2 wk. Every other day, cells were trypsinized and fixed for immunostaining analysis.

The P19 cell lines stably transfected with Flag-Smad6 expression vector (Smad6/P19) were established as above. Smad6/P19 cells were maintained in

10% FBS/DMEM/F12. For neural differentiation, cells were switched into serum-free DMEM/F12 medium and cultured for 3 d with the medium changed daily. BMP2 (10 ng/ml, PeproTech), SU5402 (5 μ M, Calbiochem, La Jolla, CA; Mueller *et al.*, 2002; Gunhaga *et al.*, 2003) or U0126 (5 μ M, Calbiochem; Kretschmar *et al.*, 1997) were added at the beginning of serum starvation and maintained for the first 2 d.

RT-PCR

Total RNAs were extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed with 5 μ g of total RNA using SuperScript II reverse transcriptase (Invitrogen). The PCR reaction consisted of denaturation at 94°C for 45 s, annealing for 45 s, and extension at 72°C for 45 s. PCR primers and reaction parameters for each gene are shown in Table 1.

Northern Blot

Northern blot analysis was performed as described previously (Hou *et al.*, 2004).

Immunostaining

Immunocytochemistry was performed as described (Gao *et al.*, 2001). The following antibodies were used: monoclonal: FGF8 (R&D Systems, Minneapolis, MN), Smad1 (Santa Cruz Biotechnology, Santa Cruz, CA), MAP2 (Sigma, St. Louis, MO), Tuj1 (Sigma), GFAP (Sigma), Oct4 (Santa Cruz); and polyclonal: c-terminal phosphorylated Smad1 (Cell Signaling Technology, Beverly, MA), group B1 Sox proteins (Sox1/(2)/3; Okada *et al.*, 2004; Tanaka *et al.*, 2004). Sox1/(2)/3 antibody has a preference for Sox1 and Sox3 over Sox2. Goat anti-GATA4 or AFP antibodies were from Santa Cruz. FITC- and Cy3-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Normal mouse and rabbit IgG (Zymed, South San Francisco, CA) were used as the negative control. The Smad1 subcellular localization images were captured with a confocal microscopy (TCS SP2, Leica, Heidelberg, Germany) with a 100 $\times$ objective lens. Other images were taken with Olympus BX50 fluorescence microscopy (Tokyo, Japan).

FGF8 Protein Quantification

The monolayer P19 cells and P19 cell aggregates were washed twice with phosphate-buffered saline (PBS) and fixed with 4% PFA. Monolayer cells were permeated and blocked with 0.1% Triton X-100, 0.5% normal goat serum, and 1% BSA in PBS at RT for 1 h and immunostained with anti-FGF8 antibody. Cell aggregates were frozen sectioned as 10- μ m sections and immunostained as above. The immunofluorescence intensity was taken with Leica confocal microscopy (TCS SP2) as described (Thompson *et al.*, 1998; Fernandez *et al.*, 2000). In each experiment, at least 20 cells or cell aggregate sections were quantified and the fluorescence intensity data were used for statistical analysis. For data comparison, all parameters for each image taken were tightly fixed.

Luciferase Assay

Luciferase assays were performed as described previously (Cheng *et al.*, 2004). For analyzing the inhibitory effect of Smad6, Vector/P19 cells and Smad6/P19 cells were transiently cotransfected with Vent2-Lux with or without constitutively active BMP receptor 1B (ca-BMPR-1B; Beall and Pearce, 2001; Ulloa and Tabibzadeh, 2001). Eight hours after transfection, medium was changed to DMEM/F12. Data were obtained 24 h after serum starvation.

Statistics

Each experiment was repeated at least three times. Data shown were expressed as mean $\pm$ SEM. Student's *t* tests were used to compare the effects of all treatments. Differences were considered statistically significant as follows: **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (see Figures 1–6).

RESULTS

FGF8 Expression Is Up-regulated during P19 Cell Aggregation

During RA-induced P19 cell neural differentiation, Northern blot analysis showed that FGF8 mRNA had a basal expression in the noninduced P19 cells and was increased significantly in the first day of RA induction and aggregation (Figure 1A). To distinguish whether FGF8 expression was induced by RA treatment or by cell aggregation, FGF8 expression was further analyzed in RA-treated monolayer P19 cells and in non-RA-treated P19 cell aggregates. FGF8 mRNA was up-regulated in the cell aggregates in the absence of RA and remained unchanged in the monolayer P19 cells with RA treatment (Figure 1B). Immunostaining showed that FGF8 protein was evenly distributed in the cytoplasm of all cells within the aggregate sections, and the fluorescence intensities of cell aggregate sections were higher than that of monolayer P19 cells (Figure 1C). Quantitative analysis of fluorescence intensity showed that the FGF8 protein increased significantly in the first 2 d during P19 cell aggregation in the presence or absence of RA, compared with control monolayer P19 cells (Figure 1D). Western blots were used to detect FGF8 protein expression, and the result was inconsistent probably because of the diffusible nature of the FGF8 protein, the interference from the serum, or instability of the FGF8 antibody (unpublished data).

The expression of other FGF family members, IGF-1, EGF, and FGF receptors (FGFRs), were also examined during P19 cell neural differentiation. FGF8 was the only factor whose expression was induced by P19 cell aggregation. Other factors were mainly expressed in noninduced P19 EC cells (FGF5) or in later stages of P19 cell neural differentiation (FGF4, FGF2, IGF-1; Supplementary Figure S1). FGFRs, FGFR1 and FGFR2, were expressed at high levels in noninduced P19 cells and the early stages of P19 cell neural differentiation, whereas FGFR3 and FGFR4 were barely detectable (unpublished data), which was consistent with ES cell differentiation (Esner *et al.*, 2002). Taken together, our results show that the aggregation of P19 cells could rapidly increase FGF8 mRNA and protein expression.

Aggregation-dependent FGF8 Elevation Is Pluripotent Stem Cell Related

To determine whether the cell aggregation-induced FGF8 up-regulation is an universal phenomenon or a cell type-specific event, we analyzed pluripotent stem cells such as, Wnt-1 overexpressing P19 (Wnt-1/P19) cells, the aggregation of which could lead to neural differentiation without RA treatment (Tang *et al.*, 2002), the mouse embryonic stem (ES) cell line, D3, and other non-ES/EC cell-related cells. As shown in Figure 2, aggregation of Wnt-1/P19 cells could increase FGF8 mRNA expression (Figure 2A), and this was also true for D3 ES cells, in which FGF8 mRNA was up-

Figure 1. FGF8 expression was induced by P19 cell aggregation. (A) Northern blot of total RNA (30 μ g/lane) from different days of RA-induced P19 cell neural differentiation showed that FGF8 mRNA was transiently elevated in the first day of P19 cell aggregation and RA induction. (B) Northern blot analysis showed FGF8 expression was up-regulated by P19 cell aggregation, whereas it remained unchanged in monolayer P19 cells with RA treatment. (C) Immunostaining showed the FGF8 protein was distributed evenly in cells within the section of aggregates, and the fluorescence intensities of cell aggregate sections were higher than that of monolayer P19 cells. (D) Quantification of the FGF8 protein in different days by measurement of FGF8 fluorescence intensity with confocal microscopy. FGF8 protein expressions in the first 2 d of P19 cell aggregation with or without RA treatment were higher than that of monolayer P19 cells. Agg, aggregation; A1, aggregation day 1. Scale bar, 10 μ m.

regulated in the first day of cell aggregation and started to down-regulate in the third day (Figure 2B). There was no FGF8 expression change in the aggregation of other non-ES/EC cells such as human neuroblastoma SH-SY5Y cells, rat glioma C6 cells, and mouse mammary tumor GR2H6 cells (Figure 2, C–E). These data suggest that the aggregation-induced FGF8 up-regulation is a pluripotent stem cell-related event and might be involved in the differentiation of these cells.

Overexpression of FGF8 Could Promote RA-induced Monolayer P19 Cell Neural Differentiation

To test whether overexpression of FGF8 could bypass cell aggregation and promote neural differentiation of mono-

Figure 2. Aggregation-dependent FGF8 elevation was pluripotent stem cell related. Pluripotent stem cells, Wnt-1/P19, D3 ES cells, and other non-ES/EC cells were aggregated in the absence of RA for 2–7 d, and total RNAs were collected for Northern blot and RT-PCR analysis. FGF8 mRNA was up-regulated during aggregation of Wnt-1/P19 cells (A) and D3 ES cells (B). However, no FGF8 elevation was observed in the aggregation of rat glioma C6 cells (C), human neuroblastoma SH-SY5Y cells (D), and mouse mammary tumor GR2H6 cells (E).

layer P19 cells, Myc-FGF8-pcDNA3, or control plasmid was transiently transfected into monolayer P19 cells with or without RA treatment (Figure 3). MAP2 was used as a neuronal marker, and RA-induced P19 cell aggregates served as the positive control (Figure 3E). FGF8-transfected monolayer P19 cells in the presence of RA could differentiate into MAP2-positive neuron-like cells (Figure 3D), whereas the monolayer P19 cells transfected with pcDNA3 in the absence (Figure 3A) or in the presence of RA (Figure 3B) and FGF8-transfected monolayer P19 cells without RA treatment (Figure 3C) could not differentiate into neurons. The percentages of MAP2-positive cells were similar in aggregated and FGF8-transfected monolayer P19 cells treated with RA (Figure 3F). RA-induced monolayer P19 cells transfected with FGF8 also expressed other neuronal markers such as type III β -tubulin (TuJ1), neurofilament, and GAP43 (unpublished data).

FGF8 stably transfected P19 cell lines (FGF8/P19) were established, and monolayer FGF8/P19 cells could differentiate into neural cells upon RA induction. The MAP2-positive cells appeared at the first day of differentiation, and ~45% of cells became MAP2-positive by day 9. GFAP-positive cells were detected at day 7 of differentiation and maintained at a constant level (10%) during the next 5 d. In contrast, control monolayer pcDNA3/P19 cells could differentiate into neither neurons nor astrocytes (Figure 3G). Because FGF8 is a secreted protein, it can be added with RA into the medium of monolayer P19 cells. As shown in Figure 3H, FGF8 and RA together could promote the differentiation of pluripotent P19 EC cells ($\text{Oct4}^+\text{Sox}^+$) into neural stem cells ($\text{Oct4}^-\text{Sox}^+$; Wood and Episkopou, 1999). Other FGF family members as well as IGF-1 had similar effects, probably due to activation of the same FGF signaling pathway (Pera *et al.*, 2003).

The monolayer P19 cells induced by RA differentiated into the endoderm and mesoderm-like cells (Jones-Villeneuve *et al.*, 1982, 1983; den Hertog *et al.*, 1993). We would like to know whether FGF8- and RA-induced monolayer P19 cells could differentiate into the endoderm and mesoderm cell lineages. As shown in Figure 3I, RA-induced monolayer P19 cells (lane 3) expressed endoderm marker HNF3 β and GATA6 (Levinson-Dushnik and Benvenisty, 1997; Li *et al.*, 2004) and the mesoderm marker Brachyury (Bra; Vidricaire *et al.*, 1994; Leahy *et al.*, 1999), whereas FGF8-induced monolayer P19 cells (lane 4) expressed the mesoderm marker Bra and Goosecoid (Gsc; Artinger *et al.*, 1997; Yasunaga *et al.*, 2005). Besides the endoderm and mesoderm markers, the neural stem cell marker, Sox1, only expressed in FGF8 plus RA-induced monolayer P19 cells (lane 5) and in RA-induced P19 cell aggregates (lane 6). Proteins of two other endoderm markers, AFP and GATA4, could also be detected in RA- and FGF8-induced monolayer P19 cells (Supplementary Figure S2).

Together, these results suggest that the cell aggregation-induced FGF8 elevation is sufficient for RA-induced neural differentiation of P19 cells, and this neural differentiation is accompanied by mesoderm and endoderm cell lineages.

Inhibition of FGF8 Expression by RNAi and Blocking FGF Signaling by SU5402 Lead to Reduced P19 Cell Neural Differentiation

Given that FGF8 overexpression could promote monolayer P19 cell neural differentiation, we wonder whether inhibition of FGF8 elevation could impair the RA-induced P19 cell neural differentiation. Twenty P19 cell clones with stable transfection of the FGF8 RNAi plasmid (FGF8 RNAi/P19) were generated, and five of them showed a significant reduction of FGF8 mRNA in monolayer state by RT-PCR; clones 8 (FGF8 RNAi-8/P19) and 18 (FGF8 RNAi-18/P19) were chosen for further study (Figure 4A). The empty RNAi vector-transfected P19 cell line (PPE/P19) served as the negative control. The inhibition of the aggregation-induced FGF8 up-regulation was evident at both FGF8 mRNA (Figure 4B) and protein (Figure 4C) levels in FGF8 RNAi/P19 cells, compared with the control PPE/P19 cells.

Inhibition of FGF8 expression by RNAi could significantly reduce the number of MAP2-positive cells in RA-induced FGF8-RNAi/P19 cells (Figure 4E, a and b, and F). Similarly, blocking FGF signaling by the FGFR inhibitor, SU5402 (5–10 μM), could also reduce the MAP2-positive cells differentiated from RA-induced monolayer FGF8/P19 cells (Figure 4, E, c and d, and G). To block the activities of FGF factors, we also added soluble FGFR-Fc chimeric molecules into the cell culture medium in the presence of RA for 4 d. P19 cell aggregates were sectioned and $\text{Oct4}^-\text{Sox}^+$ cells were determined. FGFR3-Fc could significantly reduce the $\text{Oct4}^-\text{Sox}^+$ cells, whereas other FGFR-Fc molecules had no effect (Supplementary Figure S3; Ye *et al.*, 1998; Fukuchi and Grove, 2003). These results show that inhibition of FGF signaling by repressing FGF8 expression, blocking FGFR signaling, or neutralizing FGF8 protein activity could attenuate the RA-induced P19 cell neural differentiation, supporting the view that both cell aggregation-induced FGF8 elevation and intact FGF signaling are necessary for RA-induced P19 cell neural differentiation.

Because RA and aggregation could induce a transient FGF8 up-regulation followed by a sustained expression of FGF4 during P19 cell neural differentiation (top panel of Figure 4D and Supplementary Figure S1), we would like to distinguish the different roles between FGF8 and FGF4 in

Figure 3. FGF8 overexpression promoted RA-induced monolayer P19 cell neural differentiation. MAP2 immunostaining showed that monolayer P19 cells could not differentiate into MAP2-positive neuronal cells by transfection pcDNA3 vector (A), or pcDNA3 transfection with 10^{-6} M RA treatment (B), or FGF8 transfection alone (C). However, FGF8 transfection together with RA could induce P19 cell neural differentiation (D). MAP2 staining of RA-induced P19 cell aggregation was used as the positive control (E). The MAP2-positive cells were determined in these conditions (F). (G) Time course of MAP2-positive and GFAP-positive cells differentiated from FGF8 or pcDNA3 stably transfected P19 cell lines. —◆—, MAP2⁺ cells in FGF8/P19 cells; —◆—, MAP2⁺ cells in pcDNA3/P19 cells; —▲—, GFAP⁺ cells in FGF8/P19 cells; —▲—, GFAP⁺ cells in pcDNA3/P19 cells. (H) Different FGF factors and EGF and IGF1 (10 ng/ml) were added into RA-induced monolayer P19 cells, and double staining of Oct4 and Sox showed that FGF2, FGF4, FGF10, and IGF1 could promote P19 cell neural differentiation. The percentage of Oct4⁺Sox⁺ (□) and Oct4⁺Sox⁺ (■) cells were determined. (I) RT-PCR analysis of expression of endoderm, mesoderm and ectoderm markers from noninduced P19 cells (lane 1), monolayer P19 cells without induction (lane 2), or induced by FGF8 (lane 3), RA (lane 4), FGF8 plus RA (lane 5), and RA-induced P19 cell aggregation (lane 6). Scale bar, 100 μ m.

this process. In the FGF8-RNAi/P19 cells aggregation stage, the weak FGF4 expression could still be detected, implying the specificity of the FGF8 RNAi construct, but its expression was ceased in the differentiation stage (middle panel of Figure 4D). Interestingly, if FGF signaling was inhibited transiently by adding SU5402 only in the first day of P19 cell aggregation, it inhibited both FGF8 and FGF4 expression and also impaired neural differentiation (bottom panel of

Figure 4D and Figure 4E, e and f, and F). These results suggest that there are two waves of FGF expression during RA-induced P19 cell neural differentiation, a transient FGF8 elevation and a sustained FGF4 up-regulation. The FGF8 expression depends on the intact FGF signaling, and the sustained FGF4 expression depends on FGF8 elevation. Both FGF8 and FGF4 inhibition are responsible for reduced P19 cell neural differentiation.

Figure 4. Inhibition of FGF8 expression by RNAi and blocking of FGFR signaling by SU5402 impaired P19 cell neural differentiation. RT-PCR analysis showed that FGF8 mRNA was significantly down-regulated in the monolayer (A) and aggregated (B) FGF8-RNAi/P19 cell lines. (C) Quantification of FGF8 protein with fluorescence intensity showed that FGF8 protein levels in the monolayer and aggregated FGF8 RNAi/P19 cells were down-regulated. FGF8-RNAi/P19; control PPE/P19 cells were aggregated in the presence of RA for 4 d, differentiated in N2 serum-free medium for 5 d, and then immunostained with MAP2 antibody (E, a and b, and F). Monolayer FGF8/P19 and control pcDNA3/P19 cells were induced by RA for 2 d with SU5402 or dimethyl sulfoxide (DMSO) differentiated in N2 medium for 5 d, and then immunostained with MAP2 antibody (E, c and d, and G). To inhibit the transient FGF8 elevation in the first day of P19 cell aggregation, DMSO or SU5402 was added in the first day of normal P19 cell aggregation and induction and then washed away (DMSO 1 d and SU5402 1 d). Treated cell aggregates were cultured in suspension for another 3 d, differentiated in N2 medium for 5 d, and immunostained as above (E, e and f, and F). (D) Expression of FGF8 and FGF4 during the RA-induced neural differentiation of normal P19 cells (top panel), FGF8-RNAi/P19 cells (middle panel), and SU5402 1d/P19 cells (bottom panel). Note that inhibition of FGF8 expression or FGF signaling in the first day of P19 cell aggregation resulted in the reduced FGF4 expression. Scale bar, 100 μ m.

Figure 5. FGF signaling was directly involved in neural differentiation of Smad6/P19 cells. (A) RT-PCR analysis of overexpressed Smad6 in Smad6/P19 cells. (B) Down-regulation of Vent2-luciferase reporter activity in Smad6/P19 cells. (C) Distribution of endogenous Smad1 (a–d) and c-terminal phosphorylated Smad1 (e–h) in serum containing (a, c, e, and g) and serum-free medium (b, d, f, and h) of Vector/P19 (a–b and e–f), and Smad6/P19 (c–d and g–h) cells. (D) Vector/P19 cells cultured in serum-containing (a) and serum-free medium (c) as well as Smad6/P19 cells cultured in serum-containing medium (b) could not differentiate into TuJ1-positive neuronal cells. However, Smad6/P19 cells in serum-free medium could spontaneously differentiate into TuJ1-positive neurons (d). This neural differentiation could be inhibited by BMP2 (10 ng/ml, e), SU5402 (5 μ M, f), or U0126 (5 μ M, g). TuJ1 was used as a neuronal marker (c'–g'), and the TuJ1-positive cells were determined (h). Scale bar for (C), 5 μ m, and (D), 50 μ m.

FGF Signaling Is Directly Involved in the Neural Differentiation of P19 Cells

Having established a crucial role for the cell aggregation-induced FGF8 elevation in P19 cell neural differentiation, we wanted to learn the molecular mechanism(s) involved in this process. It has been shown that FGF signaling can block BMP pathway by phosphorylating the linker region of Smad1 and inhibiting its nuclear translocation (Kretzschmar

et al., 1997; Pera *et al.*, 2003; Kuroda *et al.*, 2005). Consistent with these observations, we also found that FGF8 inhibited BMP signaling through phosphorylating the linker region of Smad1 and blocking its nuclear translocation in P19 cells, and Erk1/2 activation was involved in this process (Supplementary Figure S4).

To further investigate whether FGF signaling plays any BMP-inhibition-independent role in P19 cell neural differ-

entiation, we overexpressed an inhibitory Smad, Smad6, to block the BMP pathway in P19 cells and examined its neural differentiation. Smad6 was up-regulated in an Smad6 stably transfected P19 cell line (Smad6/P19; Figure 5A), and BMP signaling was inhibited in the Smad6-overexpressing cells (Figure 5B). Immunostaining showed that less endogenous Smad1 localized in the nucleus of Smad6/P19 cells, compared with the control Vector/P19 cells, in both serum-containing and serum-free medium (Figure 5C, a–d). Immunostaining of c-terminal phosphorylated Smad1 showed that the activated Smad1 could be found in Vector/P19 cells and Smad6/P19 cells with serum containing medium, but could hardly be detected in Smad6/P19 cells in serum-free medium (Figure 5C, e–h). Together, these results suggest that there are BMP activities in Vector/P19 cells and Smad6/P19 cells with serum-containing medium, but not in Smad6/P19 cells with serum-free medium.

To examine the neural differentiation of Smad6/P19 cells, both cell lines were cultured in serum containing and serum-free medium, and TuJ1 was used as the neuronal marker (Figure 5D). We could not detect TuJ1-positive cells in Vector/P19 and Smad6/P19 cells in serum-containing medium as well as control Vector/P19 cells in serum-free medium (Figure 5D, a–c). Smad6/P19 cells, however, could spontaneously differentiate into TuJ1-positive neurons after being cultured in serum-free medium for 3 d (Figure 5D, d and h). Because the trace amounts of BMP in serum could block ES cell neural differentiation (Ying *et al.*, 2003a, 2003b), we speculated that this was the reason the Smad6/P19 cells could not differentiate into neural cells in serum-containing medium. To confirm this, we added BMP2 (5–10 ng/ml) into the serum-free medium and found that it severely impaired Smad6/P19 cell neural differentiation (Figure 5D, e and h). To prove that endogenous FGF signaling is directly involved in P19 cell neural differentiation, we treated Smad6/P19 cells with SU5402 (5 μ M) or U0126 (5 μ M) and found that these cells could hardly differentiate into TuJ1-positive cells in serum-free medium (Figure 5D, f–h). These results suggest that besides inhibiting BMP signaling, FGF signaling coordinates other pathways via the MEK-ERK pathway to induce P19 cell neural differentiation.

DISCUSSION

Compared with monolayer cultured cells, cell-cell/cell-ECM interaction is enhanced in cell aggregates, which is vital for efficient differentiation of embryonic stem cells (Campione-Piccardo *et al.*, 1985; Smith *et al.*, 1987; Mummery *et al.*, 1991; Armour *et al.*, 1999). Understanding the mechanisms underlying aggregation would be helpful to dissect the relationship between cell-cell/cell-ECM interaction and cell fate determination. FGF signaling is involved in cell contact-associated events. The neural cell adhesion mediated by NCAM leads to activation of FGF signaling. Activated FGFR-Ras-MAPK and FGFR-PI3k-Akt pathways promote neurite outgrowth and neuronal survival (Kolkova *et al.*, 2000). ES cells overexpressing dominant negative FGFR2 show impaired epithelial differentiation with synthesis defects of laminin and type IV collagen, because regulation of embryoid body (EB) basement membrane formation depends on FGF-Akt/PKB (Li *et al.*, 2001). Consistent with these observations, we found that FGF8 expression was elevated in the first day of P19 cell aggregation, and FGF8 was the only factor within FGF family responding to P19 cell aggregation (Figure 1 and Supplementary Figure S1). Importantly, the aggregation-induced FGF8 elevation is pluripotent stem cell related, suggesting its potential function in

Figure 6. A model of cell aggregation induced FGF8 elevation and the distinct mechanisms of FGF signaling in neural induction of P19 cells.

stem cell fate determination (Figure 2). Through gain and loss of function assays, we showed that FGF8 and FGF signaling were both sufficient and necessary for the cell aggregation related neural fate determination of P19 cells (Figures 3 and 4).

FGF8 mRNA is expressed in the proximal-posterior epiblast of E6.0–E6.5 mouse embryos, and in the primitive streak of E7.5 embryos (Crossley and Martin, 1995). FGF4 transcript is first detected in the inner cell mass of E4.5 embryos, and its expression overlaps with FGF8 in the epiblast of E6.5 and the primitive streak of E7.5 embryos (Niswander and Martin, 1992). FGF4 expression in the primitive streak is dependent on FGF8, because in E7.5 FGF8 mutant embryo the FGF4 expression is lost in this region, which causes the failure of cell migration and a defect of posterior neural induction (Sun *et al.*, 1999). Consistent with these observations, we found two waves of FGF expression during RA-induced P19 cell neural differentiation: a transient FGF8 elevation and a sustained FGF4 expression. FGF4 expression depended on the FGF8 elevation and intact FGF signaling. Both FGF8 expression inhibition and FGF signaling interruption were responsible for the reduced P19 cell neural differentiation (Figure 4). FGF signaling has been shown to be involved in the vertebrate neural induction, but the exact FGF factor(s) engaged in this process remains unclear. The similarities of FGF8 and FGF4 expression pattern between E7.5 mouse embryo and P19 cell neural differentiation promote us to speculate that FGF8 and FGF4 are the possible candidates involved in neural induction in vivo.

Inhibition of BMP signaling is very important for neural induction (Hemmati-Brivanlou and Melton, 1997a, 1997b), and BMP levels must be tightly controlled for ES cell neural induction (Tropepe *et al.*, 2001; Ying *et al.*, 2003a). In our study, we induced P19 cell neural differentiation in serum-containing medium. Because serum contains trace amounts of BMPs to maintain stem cell pluripotency and restrict differentiation, BMP signals have to be reduced for stem cell neural differentiation (Ying *et al.*, 2003a, 2003b). We showed that treatment of monolayer P19 cells with FGF8 could effectively inhibit BMP pathway (Supplementary Figure S4; Kretzschmar *et al.*, 1997, 1999; Pera *et al.*, 2003; Kuroda *et al.*, 2005). In addition to BMP inhibition, FGF signaling has also been suggested to be responsible for generating the nervous system (Linker and Stern, 2004; Delaune *et al.*, 2005). In

chick, FGF is involved in both BMP inhibition-dependent and -independent functions in neural induction (Wilson *et al.*, 2000). However, these assays are conducted by tissue explant or injecting plasmids at very early stages of development, and it cannot exclude altering normal cell movement patterns or initiating multiple cascades of other signaling events (Stern, 2005). It is also unknown whether FGF is involved in a BMP inhibition-independent pathway of neural induction in the mouse embryo. To tackle this question, we inhibited endogenous BMP signaling by overexpressing Smad6, a universal inhibitor of BMP signaling (Linker and Stern, 2004) in P19 cells and found that these cells could spontaneously differentiate into neurons in serum-free medium (Figure 5). In this BMP negative circumstance, Smad6/P19 cell neural differentiation was impaired by blocking the FGF pathway with SU5402 or U0126, which suggested an obligate role for FGF signaling in P19 cell neural differentiation.

On the basis of these results, we proposed that as in *Xenopus* and chick, BMP inhibition remains central in P19 cell neural fate determination (Figure 6). FGF8 expression, elevated during P19 cell aggregation, results in intracellular Erk1/2 activation and inhibition of Smad1 nuclear translocation. In addition, FGFs exerts direct effects on P19 cell neural differentiation through Erk1/2 phosphorylation. In BMP negative condition, endogenous FGF signaling is sufficient to promote P19 cell neural induction; although in BMP positive conditions, endogenous FGFs are not sufficient and additional FGFs are required to inhibit the BMP pathway. Moreover, much still remains unknown on the direct target of BMP-independent action of FGF activity, which was only addressed in ascidian (Bertrand *et al.*, 2003). Understanding how aggregation induces FGF8 expression will provide insights into the relationship between cell-cell interaction and growth factor secretion, which are both essential for embryonic development.

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