

Retinoic acid treatment and cell aggregation independently regulate alternative splicing in P19 cells during neural differentiation

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Abstract

To induce neural differentiation of P19 cells, two different treatments, RA (retinoic acid) and cell aggregation, are required. However, there has been no report that RA treatment alone or cell aggregation alone could control alternative splicing regulation in P19 cells. Therefore, we focused on alternative splicing effects by neural induction (RA treatment and/or cell aggregation) in P19 cells. We analysed the splicing patterns of several genes, including *5-HT3R-A* (5-hydroxytryptamine receptor), *Actn1* (actinin alpha1), *CUGBP2* (CUG-binding protein) and *PTB* (polypyrimidine track-binding protein), which showed different responses during the early neural induction of P19 cells. We show here that RA treatment alone changes the alternative splice mechanism of *5-HT3R-A*. Cell aggregation alone controls alternative splicing regulation of *Actn1*. Both treatments (RA and cell aggregation) compensate and regulate the alternative splicing mechanism of *CUGBP2*. However, *PTB* is independent of RA and cell aggregation. Taken together, our results suggest that RA treatment and cell aggregation independently regulate the alternative splicing mechanism in the early stage of P19 cells during neural differentiation.

Keywords: P19 embryonic carcinoma cell; neural differentiation; retinoic acid; cell aggregation; alternative splicing

1. Introduction

The differentiation of mammalian neurons during development is a highly complex process, related to regulation and coordination of gene expression. The study of neural differentiation of stem cells has generated considerable interest in the last few years. These studies facilitated a better understanding of the fundamental aspects of neurogenesis. Since the first report on derivation of human ES (embryonic stem) cells, a number of studies have explored the possibility of directing the differentiation of ES cells towards neural development (Suter and Krause, 2008). When cultured in a non-adhering dish, pluripotent stem cells, such as ES cells and EC (embryonic carcinoma) cells, can spontaneously form multicellular aggregates that are similar to post-implantation embryonic tissues *in vivo*. Mouse EC P19 cells were isolated from a teratocarcinoma in C3H/He mice (McBurney and Rogers, 1982). P19 cells are pluripotent and can be induced to differentiate by chemical inducers and by culture conditions (McBurney and Rogers, 1982; Jones-Villeneuve et al., 1982; McBurney et al., 1982; McBurney, 1993; van der Heyden and Defize, 2003). Aggregates of P19 cells exposed to DMSO differentiate into endodermal and mesodermal derivatives, including cardiac and skeletal muscle. RA (retinoic acid) treatment and cell aggregation can induce P19 cells to differentiate into neurons and glial cells (McBurney et al., 1982; Jones-Villeneuve et al., 1982, 1983); therefore, P19 cells have been used as a model to study neural differentiation. Neural differentiation depends not only on intrinsic

factors but also on extrinsic signals. Signals come from molecules, such as FGFs (fibroblast growth factors), BMPs (bone morphogenic proteins), Wnt proteins, RA and cell aggregation, and these molecules regulate the differentiation of the different neural progenitor and precursor cells (Sasai and De Robertis, 1997; Frade and Rodriguez, 2000). Various factors, including transcriptional factors such as Mash-1, Ngn-1 and Sox6, are induced by the RA signalling pathway during neural differentiation of P19 cells (Itoh et al., 1997; Nakakura et al., 2001; Hamada-Kanazawa et al., 2004).

Although both treatments, RA and cell aggregation, are needed for complete neural differentiation of P19 cells, Teramoto et al. (2005) monitored gene expression in P19 cells, and their results show that RA treatment alone or cell aggregation alone, or a combination of both, control gene expression to a certain extent. RA treatment alone up-regulates the expression of *NeuroD* and *GDFNRβ* (glial cell line-derived neurotrophic factor receptor) genes, and also activates JNK and PBX proteins, which are necessary for neural differentiation of P19 cells (Wang et al., 2001; Qin et al., 2004; Teramoto et al., 2005). Cell aggregation alone up-regulates the expression of *Ngn1*, *Wnt-1*, *Fgf8* and *ADAM23* genes, whereas it down-regulates the expression of the *Id1* gene in P19 cells during neural differentiation (Kim et al., 2004; Teramoto et al., 2005; Wang et al., 2006; Sun et al., 2007). Both treatments up-regulate the expression of *AChE* (acetylcholinesterase), *int-1* proto oncogene and *ArpNα* (actin-related protein) gene, whereas both treatments down-regulate the

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Abbreviations: *5-HT3R-A*, 5-hydroxytryptamine receptor; *Actn1*, actinin alpha1; EC, embryonic carcinoma; ES, embryonic stem; EtBr, ethidium bromide; FGFs, fibroblast growth factors; FR- α , folate receptor- α ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NCBI, National Center for Biotechnology Information; NM, non-muscle; RA, retinoic acid; RARs, retinoic acid receptors; RT, room temperature; RT-PCR, reverse transcription-PCR; SM, smooth muscle; SR, serine/arginine-rich proteins; ss, splice site.

expression of *Oct-3* gene in P19 cells during neural differentiation (Schuurin et al., 1989; Coleman and Taylor, 1996; Teramoto et al., 2005). Recently, Boutz et al. (2007) reported that both treatments change the expression of alternatively spliced isoforms of several genes in neural differentiation of P19 cells. For instance, both treatments up-regulate alternative splicing of *Src N1* (a member of the Src family of protein tyrosine kinase), *Smad1* (stromal membrane-associated protein 1), *CICB EN*, *Kif1b* (kinesin intermediate filament) and *Dst3* (dystonin) genes. In contrast, both treatments down-regulate alternative splicing of *Mtap2* (microtubule-associated protein 2) and alternative splicing of the *Dst2* gene in P19 cells during neural differentiation. However, the independent effects of RA treatment and cell aggregation on alternatively spliced isoforms of the genes are not well understood. Although there has been no report that RA treatment alone or cell aggregation alone controls alternative splicing regulations in P19 cells, a little is known about the regulation of alternative splicing by RA treatment in cells and tissues. For instance, RA treatment up-regulates the RAR (RA receptor) α and γ isoforms in embryo and adult rats, and it also up-regulates the isoforms of FR- α (folate receptor- α) in ES cells (Takeyama et al., 1996; Bolton et al., 1999). In contrast, RA treatment down-regulates the expression of alternatively spliced isoforms of hTERT in HEN-16-2 and HEN-16-2/CDDP cells (Ding et al., 2002), and it also down-regulates the alternatively spliced isoforms, CREM τ (cAMP-responsive element modulator) in rat testes (Matsuda et al., 2005). Therefore, clarification of the regulation mechanism of alternative splicing of the genes during neural differentiation of P19 cells by RA treatment alone or cell aggregation alone would be important. To clarify the mechanism of alternative splicing, first, we randomly checked alternative splicing events using UCSC blat search and found 55 alternative splicings among 36 genes. We examined all these splicings in neural differentiation of P19 cells. Among the genes examined, three genes (three alternative splicings) such as *5-HT3R-A* (5-hydroxytryptamine receptor), *Actn1* (actinin alpha1) and *CUGBP2* (CUG-binding protein) were clearly found to be changed within day 1 (24 h), after RA induction. Moreover, the alternative splicing of *PTB* (polypyrimidine track-binding protein) changed slightly within 24 h. Therefore, we decided to perform a detailed investigation of these four genes (including *PTB*) in early neural induction (by RA treatment and/or cell aggregation) of P19 cells.

From previous reports, and the database from NCBI (National Center for Biotechnology Information) (Altschul et al., 1997), it is obvious that *5-HT3R-A*, *Actn1*, *CUGBP2* and *PTB* genes are alternatively spliced. *5-HT3R-A* gene generates two isoforms, 5-HT3R-A long and short (5-HT3R-AL and 5-HT3R-AS) (Hope et al., 1993; Werner et al., 1994), by alternative use of 3' splice site selection of exon 9 (Figures 1A and 1B). *Actn1* gene has a pair of exons, NM (non-muscle) and SM (smooth muscle) exons, which are joined to the upstream EF1a exon and downstream EF2 exon (Figure 1C) (Southby et al., 1999), and this generates two isoforms, NM type and SM type, in which the upstream NM exon and the downstream SM exon are used in a mutually exclusive manner (Figure 1D) (Waites et al., 1992; Southby et al., 1999; Kremerskothen et al., 2002). The *CUGBP2* gene has 5' and 3' splice site selections of exon 11 and exon 12 (Figure 1E). Alternative usages of 5' and 3' splice site selections produce four isoforms of different

lengths, the longest isoform (α), the second longest isoform (β), the second shortest isoform (γ) and the shortest isoform (δ) (Figure 1F). *PTB* gene has a skipping exon (exon 8) (Figure 1G), and it generates two isoforms, (PTB4 and PTB1) using either inclusion or exclusion of exon 8 (Figure 1H).

By analysis of the splicing patterns of various genes during induced differentiation of P19 cells, we found that four different genes, *5-HT3R-A*, *Actn1*, *CUGBP2* and *PTB* were regulated independently. In this study, we show that RA treatment alone enhances the expression of the alternatively spliced 5-HT3R-AL isoform of *5-HT3R-A*; on the other hand, RA treatment alone decreases the 5-HT3R-AS isoform of *5-HT3R-A*. Cell aggregation alone down-regulates significantly the NM type of *Actn1*, whereas it up-regulates significantly the SM type of *Actn1*. Both treatments up-regulate significantly the γ isoform of *CUGBP2* and, in contrast, down-regulate significantly the δ isoform of *CUGBP2*. Taken together, our results demonstrate that cell aggregation and RA treatment independently change the mechanism of alternative splicing in early stage P19 cells during neural differentiation.

2. Materials and methods

2.1. Database analysis for *5-HT3R-A*, *Actn1*, *CUGBP2* and *PTB* genes

The information regarding mouse *5-HT3R-A* (NM_013561), *Actn1* (NM_134156), *CUGBP2* (NM_010160) and *PTB* (NM_008956 and NM_001077363) genes from the NCBI was used for nucleotide sequence analysis. The RefSeq (reference sequences) of *5-HT3R-A*, *Actn1*, *CUGBP2* and *PTB* genes were used in a mouse blat search (<http://www.ncma.org/glocal/cgi-bin/hgBlat?hgside=4>), and different mRNAs were found. From this database, we found an alternative 3' splice site selection in *5-HT3R-A*, mutually exclusive exons in *Actn1*, alternative 3' and 5' splice site selections in *CUGBP2*, and a skipping exon in *PTB*. We prepared the forward and reverse primers using the primer3 (<http://fokker.wi.mit.edu/primer3/input.htm>) program.

2.2. P19 cell culture and cell differentiation

Pluripotent mouse EC P19 cells were cultured essentially as described elsewhere (Runnicki and McBurny, 1987; Komatsu et al., 1999; Alam et al., 2009). To induce neural differentiation, P19 cells were allowed to aggregate in bacteriological-grade Petri dishes (Falcon) at a seeding density of 1×10^5 cells/ml in the presence of 1 μ M all-*trans*-retinoic acid (RA, Sigma) in α -MEM (minimum essential medium, Wako) supplemented with 10% FBS (fetal bovine serum, Sigma). After 4 days of aggregation, cells were dissociated into single cells by 0.25% EDTA (Sigma) solution and were replated in a tissue culture dish at a density of $3\text{--}6 \times 10^5$ cells/ml. The cells were then allowed to adhere and cultured in the absence of RA for 10 days. All media were replaced every 48 h.

To examine the effects of the different treatments, P19 cells were cultured in four different conditions, such as standard culture (no treatment), RA treatment and cell aggregation, cell aggregation alone and RA treatment alone. In the case of RA treatment

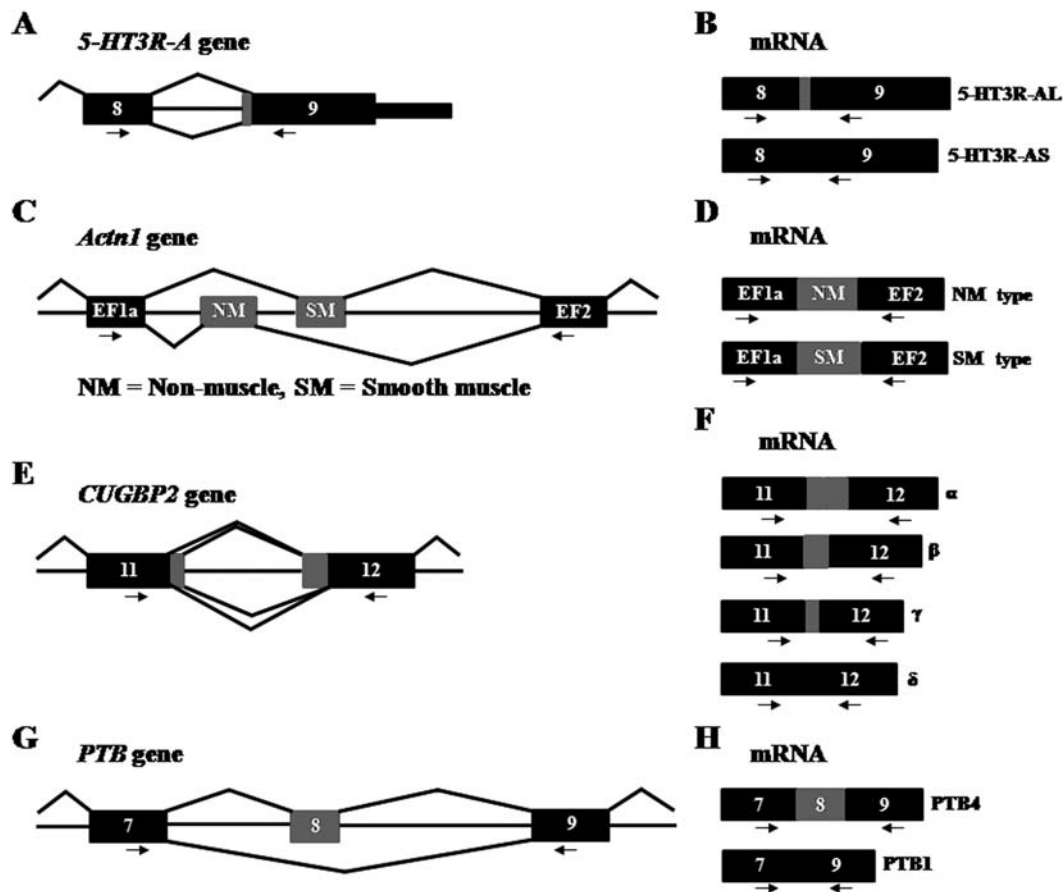


Figure 1 Alternative splicings of *5-HT3R-A*, *Actn1*, *CUGBP2* and *PTB* genes

Exons are indicated as black boxes with alternative splicing events depicted as grey boxes. Introns are shown as narrow central lines. The arrows indicate the primer annealing sites. (A) Schematic representation of alternative exons along with 3' ss selection of exon 9 in *5-HT3R-A*. (B) The 5-HT3R-AS isoform consists of the distal 3' ss selection of exon 9. The 5-HT3R-AL isoform differs from 5-HT3R-AS in that 5-HT3R-AL also includes the proximal 3' ss selection of exon 9. (C) Schematic representation of alternative exons along with mutually exclusive NM and SM exons in *Actn1*. (D) The NM and SM isoforms consist of the upstream EF1a exon and downstream EF2a exon using either the NM exon or SM exon, respectively. (E) Schematic representation of alternative exons along with 5' and 3' ss selections of exons 11 and 12 in *CUGBP2*. (F) The δ isoform consists of the distal 5' and 3' ss selections of exons 11 and 12. The α , β and γ differ from the δ isoform in that they also include proximal 5' and 3' ss selections of exons 11 and 12, proximal 3' ss selection of exon 12 and proximal 5' ss selection of exon 11. (G) Schematic representation of alternative exons along with skipping exon 8 in *PTB*. (H) PTB4 and PTB1 isoforms consist of either inclusion or exclusion of exon 8.

alone, P19 cells were cultured as a monolayer in tissue culture dishes, and cell samples were collected at 12 and 24 h. During cell aggregation alone, P19 cells were cultured in suspension in bacteriological-grade Petri dishes, and cell samples were collected at 12 and 24 h. For both treatments (RA and cell aggregation), P19 cells were cultured in suspension in bacteriological-grade Petri dishes, and cell samples were collected at five separate intervals (3, 6, 12, 18 and 24 h).

2.3. RNA isolation and first-strand cDNA synthesis

Total RNAs were isolated from P19 cells by TRIzol reagent (Invitrogen) after the cells were washed three times in ice-cold PBS. Total RNAs (2 μ g) were digested for 15 min at RT (room temperature) with 1 μ l (1 unit/ μ l) of DNase I (Roche) and 1 \times DNase I reaction buffer in 10 μ l reaction volume. After digestion, the activity of DNase I was stopped by the addition of 2.5 mM EDTA (pH 8.0) solution to the reaction mixture and by heating at 65°C for

15 min. To purify and concentrate the total RNAs, phenol-chloroform extraction followed by ethanol precipitation were performed. For cDNA synthesis, 1 μ g of purified total RNAs was used in a total volume of 20 μ l in the presence of 0.5 μ g of oligo (dT)₁₅ primer (Promega), 0.5 mM dNTPs, 1 \times first-strand buffer, 5 mM DTT (dithiothreitol), 2 units of RNaseOUT (40 unit/ μ l) and 10 units of SuperScript III reverse transcriptase (200 unit/ μ l) (Invitrogen). The reaction was incubated for 1 h at 50°C, and the reaction was stopped by heating at 70°C for 15 min.

2.4. PCR

The first-strand cDNA was amplified by PCR with specific primers for sequences of the genes. The primer names and sequences were as follows: exon 8 forward 5'-GGCACCTGGTCCTAGACAGA-3' and exon 9 reverse 5'-GTGGTGAAGAGGGCTACCT-3' for *5-HT3R-A*, exon EF1a forward 5'-CGCCTCTTCAACCAC-TTTG-3' and exon EF2 reverse 5'-TCATGATTCGGGCAAACCTCT-

3' for *Actn1*, exon 11 forward 5'-GAGCCACTGTCCGATTGAATA-3' and exon 12 reverse 5'-GCGCCAAGTCCTCCATTC-3' for *CUGBP2*, and exon 7 forward 5'-GCATCGACTTCTCCAAGCTC-3' and exon 9 reverse 5'-GGACAGAATCCAGCACC-3' for *PTB*. The PCR mixture contained 1 μ l of template cDNA, 0.25 units of Go Taq Flexi DNA polymerase (Promega), 1 \times Go Taq Flexi Buffer, 2.5 mM MgCl₂, 0.2 mM dNTPs and 2 pmol of each primer in 10 μ l of reaction mixture. The PCR conditions were initially denaturation at 95°C for 3 min, followed by a cycle of denaturing at 95°C for 30 s, annealing at 58–60°C for 30 s and extension at 72°C for 30 s. From 27 to 32 cycles of amplification were performed, depending on the target genes. The PCR reactions were completed by a final 7-min extension at 72°C. β -Actin was used as an internal control and was detected by PCR. The primer sequences for β -actin were forward 5'-CAACGAGCGGTTCCGAGT-3' and reverse 5'-GCCA-CAGGATTCCATACCCA-3'. The same thermal profile was used for β -actin, except the annealing temperature was 56°C for only 20 s, and the PCR reaction was repeated for 28 cycles. The PCR products of these genes and β -actin were electrophoresed in 6% polyacrylamide gel. The gels were stained with EtBr (ethidium bromide), and the images were visualized in UV-trans illuminator (Vilber Lourmat). Relative mRNA transcription levels were determined by densitometric analysis of EtBr-stained bands of PCR products in acrylamide gel using Multi Gauge ver3.0 software (Fujifilm). β -Actin transcript was used to normalize the expression levels.

2.5. Cloning and sequencing

The PCR products were purified using the LaboPass Gel extraction kit (Cosmo), followed by phenol–chloroform extraction and ethanol precipitation. The purified PCR products were inserted into pGEM-T Easy vector (Promega). The plasmid DNA containing the insert was purified using the QIAprep Spin Miniprep Kit (Promega). The plasmid DNA was sequenced in the presence of M13 forward and reverse primers, using the Big Dye Terminator v3.1 DNA Sequencing Kit (Applied Biosystems). Sequences were determined on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Confirmation of sequence identities was obtained using BLAST software (<http://blast.ncbi.nlm.nih.gov/bl2seq/wblast2.cgi>) and EMBOSS pairwise alignment algorithm (<http://www.ebi.ac.uk/Tools/emboss/align/index.html>) tools.

2.6. Minigene construction

Three minigenes, *5-HT3R-A*, *Actn1* and *CUGBP2*, were constructed for *in vivo* analysis (Figures 5A, 5C and 5E). Minigene *5-HT3R-A* (Figure 5A) was created from mouse genomic DNA, amplified as one 1.6-kb fragment. To obtain this fragment, we prepared forward primer (5'-CTTCTCCGGCTTAGGTG-3') from downstream intron 8, and introduced TGTACTIONGAG nucleotides into the forward primer as an Xho1 cut site, and prepared reverse primer (5'-TGAATACAAATGGTCACAGACAGA-3') from upstream intron 10 and introduced CCGTTCTAGA nucleotides into the reverse primer as an Xba1 cut site. This amplified DNA was subcloned into pCS2+vector, which was linearized with Xho1 and Xba1. For *Actn1* minigene (Figure 5C), the genomic fragment

between the upstream exon of NM exon and the downstream exon of SM exon was amplified using forward primer (5'-ATATCCGTGTGGGCTGG-3') along with TGCTGGATCC nucleotides introduced into the forward primer as a BamH1 cut site, and reverse primer (5'-CTTATCTCCAGCCAGGATCTTG-3') along with CACTACTCGAG nucleotides introduced into the reverse primer as an Xho1 cut site. The amplified fragment was inserted into pCS2+vector, which was linearized with BamH1 and Xho1. Minigene *CUGBP2* (Figure 5E) was created from mouse genomic DNA, amplified in two fragments as 0.5 and 1.4 kb. The first fragment includes downstream intron 11 to upstream intron 12 using forward primer (5'-TTCAGTGGCTGCTTCAACC-3') and reverse primer (5'-CCTCCTGAGTGTGGGATTA-3'), and the second fragment includes downstream intron 12 to upstream intron 13 using forward primer (5'-TTCCTTACCCAGCCTCTAG-AA-3') and reverse primer (5'-AGGCCCTGGGTACAGATAG-3'). Both the fragments were blunted with mung bean nuclease and subcloned into pCS2+vector.

2.7. Transfection

Three minigenes were transiently transfected into P19 cells using Lipofectamine 2000 (Invitrogen), based on the manufacturer's instructions. Twenty hours post-transfection, the cells were treated with four different treatments, such as no treatment, RA treatment alone, cell aggregation alone and both RA and cell aggregation for 24 h. RNA was extracted with Trizole reagent (Invitrogen). To analyse splicing products, cDNA was prepared using T7 primer specific to pCS2+vector, and RT-PCR (reverse transcription-PCR) was performed using 5-HT3R-A, *CUGBP2* and *Actn1* exon forward and reverse primers. The number of PCR cycles was 30 for *5-HT3R-A*, 25 for *CUGBP2* and 27 for *Actn1*. The PCR products were electrophoresed in 6% polyacrylamide gels, and stained with EtBr. The images were visualized using UV transilluminator. Densitometry was performed using Multi Gauge ver3.0 software (Fuji Film). Each transfection experiment was performed more than three times, and similar results were obtained.

2.8. Western blotting

The P19 cells were treated with four different treatments, such as no treatment, RA treatment alone, cell aggregation alone and both RA and cell aggregation for 24 h. Cells were washed with ice-cold PBS and collected in 1 \times buffer D. The protein lysates were sonicated for 20 s on ice four times. Two micrograms of protein lysates were analysed in SDS/PAGE to detect GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Western blotting was performed as described before (Fushimi et al., 2005). In brief, 25 μ g of protein lysates was analysed in 8% SDS/polyacrylamide gels, and proteins were transferred to nitrocellulose by semidry blotting. For phospho-Smad1/5, phospho-Smad2, Smad5 and Smad2 detection, membranes were blocked in TBS-T [0.1 M Tris/HCl (pH 8.0), 1.5 M NaCl, 0.5% (v/v) Tween 20] containing 5% (w/v) non-fat dry (skim) milk for 1 h at RT. Membranes were briefly rinsed with TBS-T and incubated with primary antibody against phospho-Smad1/5 (1:1000, Cell Signaling), phospho-Smad2 (1:1000, Cell Signaling), Smad5 (1:1000, Cell Signaling) and

Smad2 (1:1000, Cell Signaling) in TBS-T/5% skim milk for 1 h at RT. Blots were washed with TBS-T and incubated with anti-rabbit IgG-HRP (horseradish peroxidase; 1:2000, Cell Signaling) in TBS-T/5% skim milk for 1 h at RT. For GAPDH detection, blot was blocked in TBS-T/5% skim milk and incubated with goat anti-GAPDH antibody (1:1000) in TBS-T/5% skim milk for 1 h at RT. The blot was washed with TBS-T, and incubated with swine anti-goat Ig's HRP conjugated (1:3000, Invitrogen). The membranes were treated with ECL kit (GE), and the images were analysed in LAS-3000 (Fuji Film). Densitometry was performed using Multi Gauge ver3.0 software (Fuji Film). Each Western blot experiment was performed more than three times, and similar results were obtained.

2.9. Statistics

Each experiment was repeated at least three times, and similar results were obtained. The data are expressed as means \pm S.E.M. Significance was determined by Student's *t* test ($*P < 0.05$).

3. Results

3.1. Expression of alternatively spliced isoforms in P19 cells during neural differentiation

To clarify changes of splicing machinery during neural differentiation, first, we studied splicing patterns of various genes during the differentiation of P19 cells. Among several genes examined, semiquantitative RT-PCR results of four genes are shown in Figure 2(A). The expression of alternatively spliced isoforms of *5-HT3R-A*, *Actn1* and *CUGBP2* was found to be changed within 1 day after the induction of P19 cells. In the case of *5-HT3R-A*, the 5-HT3R-AL isoform is not detected in undifferentiated P19 cells, but it is detected on day 1 in induced P19 cells, whereas the 5-HT3R-AS isoform is detected in both undifferentiated and induced P19 cells. The 5-HT3R-AL and 5-HT3R-AS isoforms of *5-HT3R-A* are detected on day 7 (neural stage), but a lower level of 5-HT3R-AS isoform of *5-HT3R-A* was detected on day 7 after the start of RA treatment (4 days RA treatment+3 days more culture in the absence of RA) than on day 1 (induction stage) (Figure 2A).

The SM type of *Actn1* is not detected in undifferentiated P19 cells, but it can be seen on day 1 in induced P19 cells. On the other hand, the NM type of *Actn1* is detected in both undifferentiated and induced P19 cells. On day 7, both the NM and SM types of *Actn1* were detected, and a higher level of SM type of *Actn1* was detected on day 7 than on day 1 (Figure 2A). Our results suggest that the 5-HT3R-AL isoform of *5-HT3R-A* and the SM type of *Actn1* are alternatively spliced in a differentiation-dependent manner and might involve neural differentiation of P19 cells.

In *CUGBP2*, the α , β and δ isoforms are down-regulated, whereas the γ isoform is up-regulated on day 1, after the induction of P19 cells, which suggests that alternatively spliced isoforms of *CUGBP2* are regulated in the early stage of P19 cells during neural differentiation. All the isoforms, α , β , γ and δ of *CUGBP2* are

expressed on day 7 (Figure 2A). In *PTB*, the PTB4 and PTB1 isoforms were up-regulated within 1 day; in contrast, they were down-regulated on day 7 compared to day 1 (Figure 2A).

Densitometric analysis reveals that the relative amount of 5-HT3R-AS mRNA of *5-HT3R-A* is higher than that of 5-HT3R-AL mRNA of *5-HT3R-A*, that the relative amount of NM mRNA of *Actn1* is higher than that of SM mRNA of *Actn1*, and that the relative amount of γ mRNA of *CUGBP2* is higher than that of δ mRNA of *CUGBP2* during neural differentiation of P19 cells (Figure 2B). Neither isoform of *5-HT3R-A* is detected on day 4, after RA induction, but both are detected on day 10 (glial differentiation). SM type is not detected, and NM type has been down-regulated on day 4 when compared with undifferentiated P19 cells. In the glial stage, both NM and SM types are detected. All the isoforms, α , β , γ and δ of *CUGBP2* are up-regulated on days 4 and 10. Both PTB1 and PTB4 are down-regulated on days 4 and 10 (Figures 2A and 2B).

3.2. Expression of alternatively spliced isoforms in early stage of P19 cells

Expression of alternatively spliced isoforms of *5-HT3R-A*, *Actn1* and *CUGBP2* was changed within 1 day after the induction of P19 cells. Therefore, we examined the expression of alternatively spliced isoforms of these genes in RA treatment and aggregation of P19 cells at various times within day 1 by semiquantitative RT-PCR. β -Actin transcript was used to normalize the expression levels. Total RNAs were extracted from undifferentiated P19 cells (0 h) as control, and aggregated P19 cells treated with RA at 3, 6, 12, 18 and 24 h. In the case of *5-HT3R-A*, the 5-HT3R-AL isoform is up-regulated from 3 to 12 h, followed by down-regulation from 12 to 24 h, whereas the 5-HT3R-AS isoform is up-regulated from 3 to 6 h, followed by down-regulation from 6 to 12 h and again up-regulated from 12 to 24 h (Figure 3A). No change of the relative amount of 5-HT3R-AL mRNA of *5-HT3R-A* is detected in undifferentiated P19 cells, but a change is detected on day 1, after the induction of P19 cells, and the amount reaches 16% of total *5-HT3R-A* (L and S) (Figure 3B; 5-HT3R-AL 3.67, 12 h/0 h) when compared with undifferentiated P19 cells.

In *Actn1*, the NM type was down-regulated from 3 to 12 h, followed by up-regulation from 12 to 24 h; in contrast, the SM type was up-regulated from 3 to 12 h, followed by down regulation from 12 to 24 h (Figure 3A). No change of relative amount of SM mRNA of *Actn1* is detected in undifferentiated P19 cells, but is detected on day 1 in induced P19 cells and reaches 19% of total *Actn1* (NM and SM) (Figure 3B; SM type 4.17, 12 h/0 h) when compared with undifferentiated P19 cells.

In *CUGBP2*, the γ isoform was up-regulated from 3 to 12 h, followed by down-regulation from 12 to 24 h. On the other hand, the α , β and δ isoforms of *CUGBP2* were down-regulated from 3 to 12 h, followed by up-regulation from 12 to 24 h (Figure 3A). The changes of amount of γ and δ mRNAs of *CUGBP2* reached 14% of total *CUGBP2* (α , β , γ and δ), respectively (Figure 3B; *CUGBP2* 1.47, 12 h/0 h), when compared with undifferentiated P19 cells. No significant change was observed in the isoforms of the *PTB* gene (Figure 3A). Our results suggest that the relative amounts of the NM type of *Actn1*, and α , β and δ isoforms of *CUGBP2* were

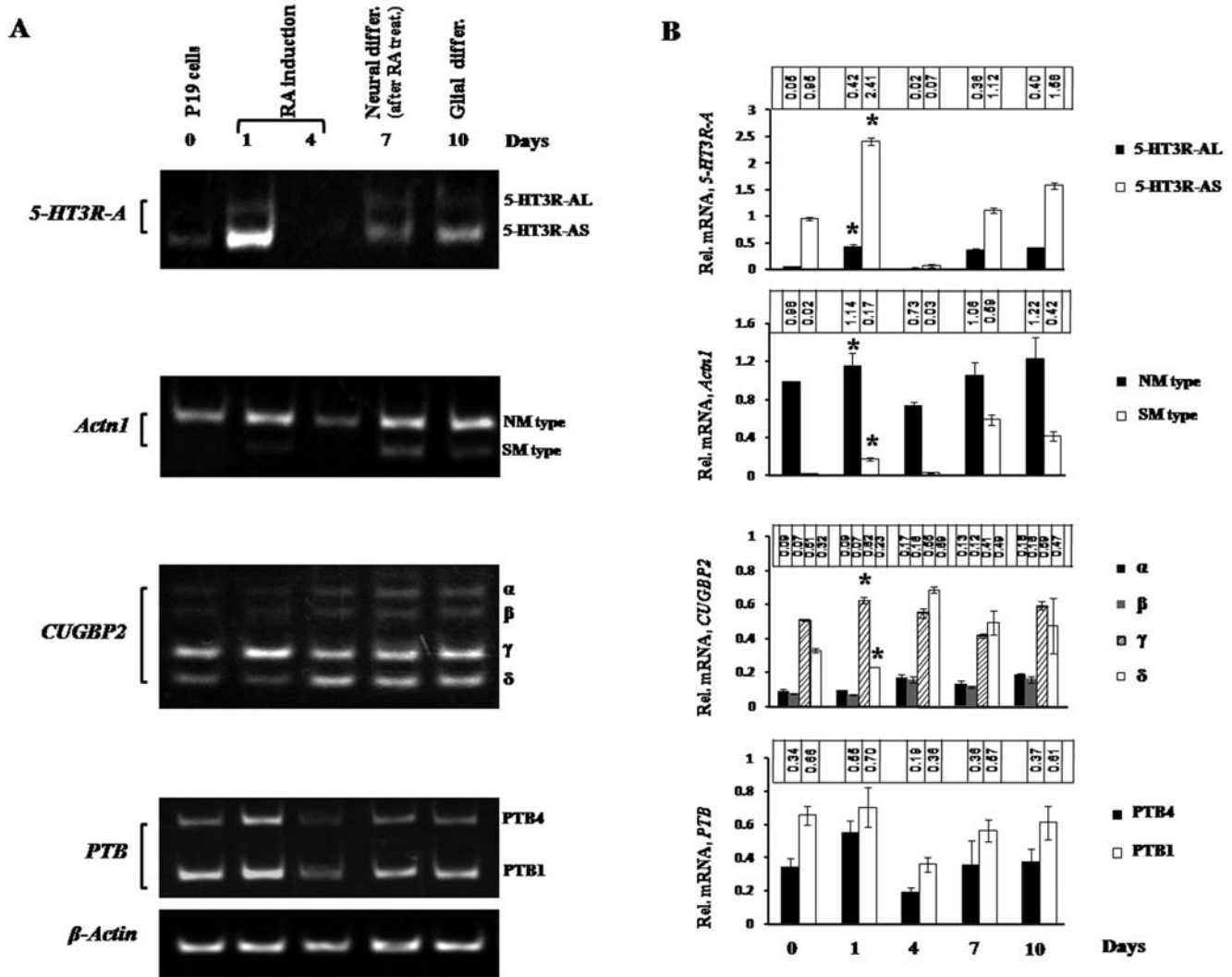


Figure 2 Expression of *5-HT3R-A*, *Actn1*, *CUGBP2* and *PTB* genes in P19 cells during neural differentiation (A) Expression of these genes was analysed by semiquantitative RT-PCR in undifferentiated, RA-induced differentiated, neural stage (after RA treatment) and glial stage of P19 cells. The positions of the genes are indicated on the left, and the isoforms of the respective genes are indicated on the right. The bottom panel shows PCR products of β -actin as a control. β -Actin transcript was used to normalize the expression levels. All experiments were repeated more than three times, and similar results were obtained. (B) Relative expression of *5-HT3R-A*, *Actn1*, *CUGBP2* and *PTB* isoforms was determined by a densitometric method. Error bars indicate the S.D. from three different experiments. *P*-values were determined by Student's *t* testing (**P*<0.05). The asterisk values were determined compared with day 0. The upper bold boxes indicate values of relative ratios of the isoforms.

down-regulated at 12 h and were up-regulated at 24 h. In contrast, the relative amounts of the SM type of *Actn1* and γ isoform of *CUGBP2* were up-regulated at 12 h and were down-regulated at 24 h. All together, our results indicate that the alternatively spliced isoforms of three genes are regulated in a time-dependent manner and might play important roles in the early stages of neural differentiation of P19 cells.

3.3. Independent effects of RA treatment and cell aggregation on alternative splicing in P19 cells

To know whether the changes in expression of alternatively spliced isoforms of *5-HT3R-A*, *Actn1*, *CUGBP2* and *PTB* were induced by RA treatment alone, by cell aggregation alone or by a

combination of both treatments, we analysed the independent effects of RA treatment and cell aggregation on alternatively spliced isoforms of these genes in P19 cells. The expression patterns of the isoforms of their respective genes were different at 12 and 24 h, after the induction of P19 cells (Figure 3A). The cells were cultured under the following four conditions: standard culture (no treatment) as control, RA treatment combined with cell aggregation, cell aggregation alone and RA treatment alone. Total RNAs were collected at 0, 12 and 24 h for each treatment condition, after which total RNAs were subjected to semiquantitative analysis. β -Actin transcript was used to normalize the expression levels. 5-HT3R-AL of *5-HT3R-A* was not detected, and 5-HT3R-AS of *5-HT3R-A* was detected in undifferentiated (no treatment) P19 cells. Both treatments up-regulate the transcripts

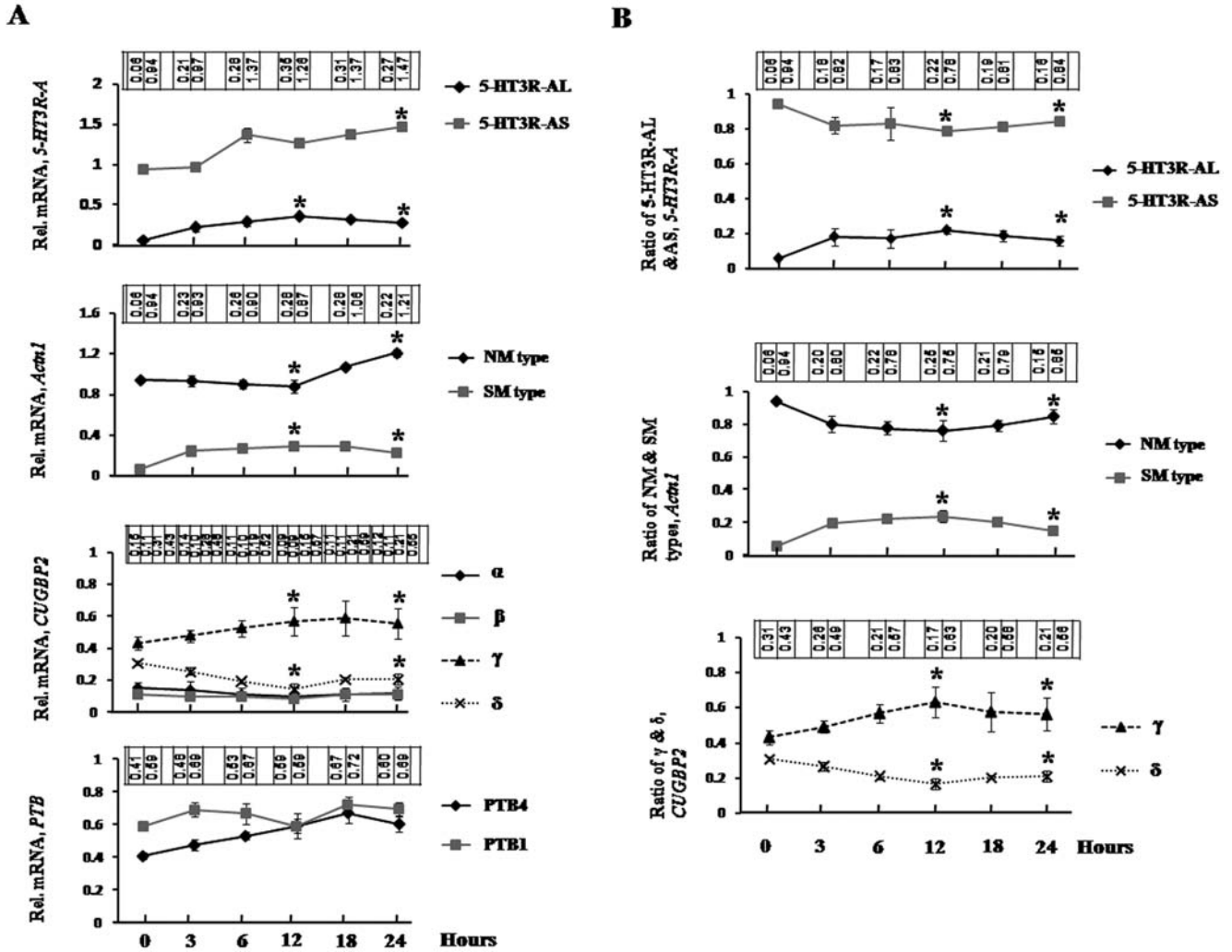


Figure 3 Expression of isoforms in early stage of P19 cells during neural differentiation (A) The relative amounts of the isoforms of *5-HT3R-A*, *Actn1*, *CUGBP2* and *PTB* were determined in undifferentiated cells (0 h) as control, and at 3, 6, 12, 18 and 24 h in induced differentiated P19 cells. (B) The ratios of isoforms of *5-HT3R-A*, *Actn1* and *CUGBP2* are shown. Error bars indicate the S.D. from three different experiments. *P*-values were determined by Student's *t* testing (**P* < 0.05). The asterisk values were determined compared with 0 h. The upper bold boxes indicate values of relative ratios of the isoforms.

5-HT3R-AL and 5-HT3R-AS of *5-HT3R-A* at 12 h (Figure 4A) when compared with no treatment. In contrast, cell aggregation alone down-regulates the alternatively spliced 5-HT3R-AS isoform of *5-HT3R-A*, and 5-HT3R-AL of *5-HT3R-A* is not detected in cell aggregation. RA treatment alone enhances significantly the alternatively spliced 5-HT3R-AL isoform of *5-HT3R-A* (Figure 4A) and acts as a compensatory action, which suggests that RA treatment alone changes alternative splicing regulation of *5-HT3R-A* in P19 cells. Moreover, 5-HT3R-AL of *5-HT3R-A* shows differential alternative splicing with and without RA treatment. The amount of 5-HT3R-AL mRNA increases 14% with RA treatment alone, and increases 11% with both treatments when compared with no treatment at 12 h (Figure 4B; 5-HT3R-AL 3.33, RA/day 0). We found that the effect of RA on the expression patterns of *5-HT3R-A* was the same at 24 h (data not shown).

In the case of *CUGBP2*, both treatments up-regulate significantly the γ isoform of *CUGBP2* and significantly down-regulate

the δ isoform of *CUGBP2* at 12 and 24 h (Figures 4A and 4C) when compared with no treatment. RA treatment alone or cell aggregation alone also up-regulates significantly the γ isoform of *CUGBP2* and down-regulates significantly the δ isoform of *CUGBP2* at 12 and 24 h (Figures 4A and 4C). Therefore, each treatment compensates and controls alternatively spliced isoforms, γ and δ , of *CUGBP2*. The relative changes of γ and δ mRNAs are up-regulated and down-regulated almost 25% with both treatments, with RA treatment alone, and with cell aggregation alone when compared with no treatment. (Figures 4B and 4D; *CUGBP2* 2.0, RA and/or cell aggregation/day 0).

In *Actn1*, RA treatment alone, and also both treatments, up-regulate the NM and SM transcripts of *Actn1* at 24 h (Figure 4C) when compared with no treatment. Interestingly, cell aggregation alone down-regulates significantly the NM type of *Actn1* and significantly up-regulates the alternatively spliced SM type of *Actn1* (Figure 4C) when compared with no treatment, which

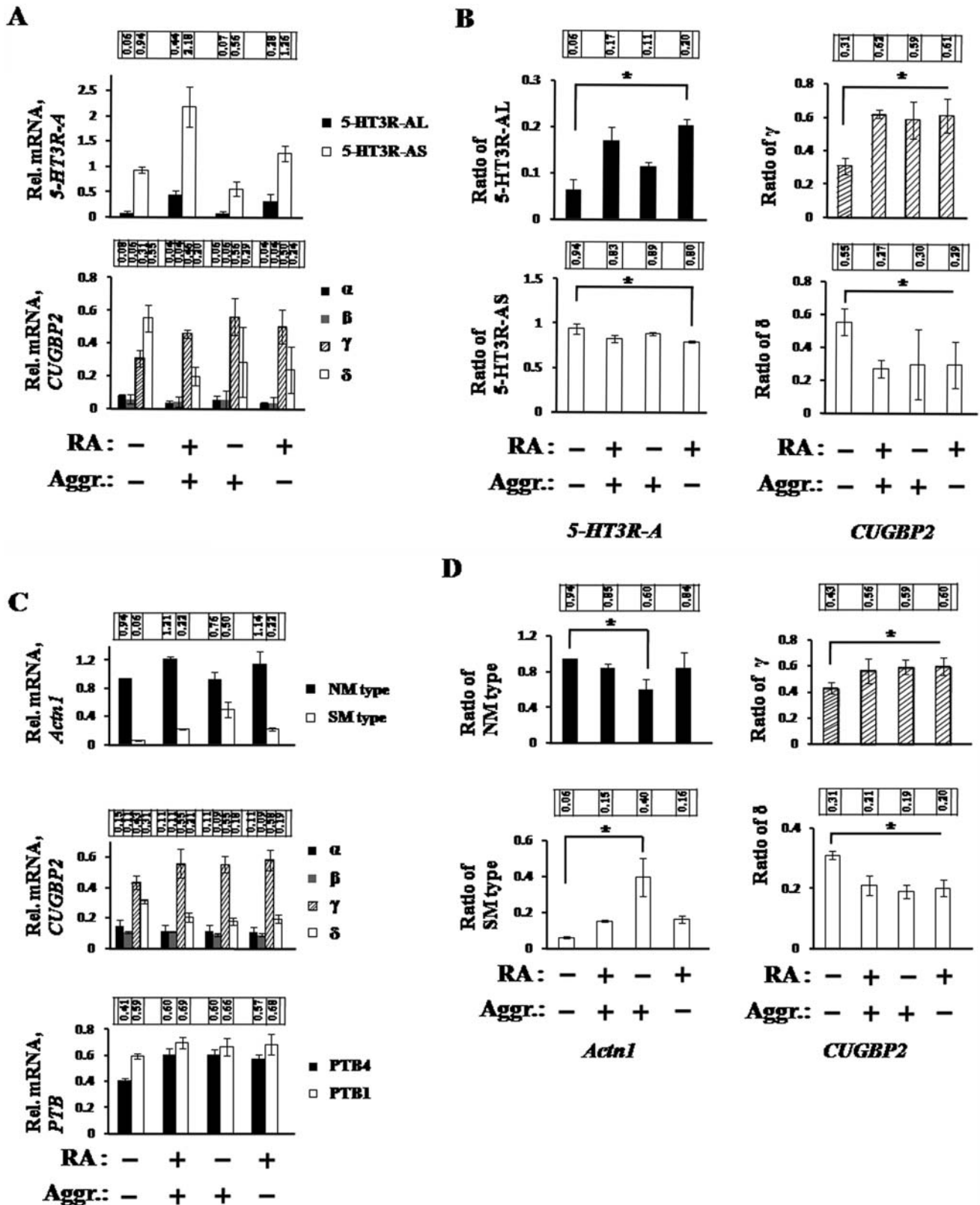


Figure 4 Effects of RA treatment and cell aggregation on alternatively spliced isoforms of *5-HT3R-A*, *Actn1*, *CUGBP2* and *PTB* in P19 cells
P19 cells were cultured in four different conditions, such as no treatment [(-) RA (-) aggregation] as control, RA treatment combined with cell aggregation [(+) RA (+) aggregation], cell aggregation alone [(-) RA (+) aggregation], and RA treatment alone [(+) RA (-) aggregation]. (A) Total RNAs were extracted from the cells at 0 and 12 h for each treatment condition mentioned above. The relative amounts of the isoforms of *5-HT3R-A* and *CUGBP2*

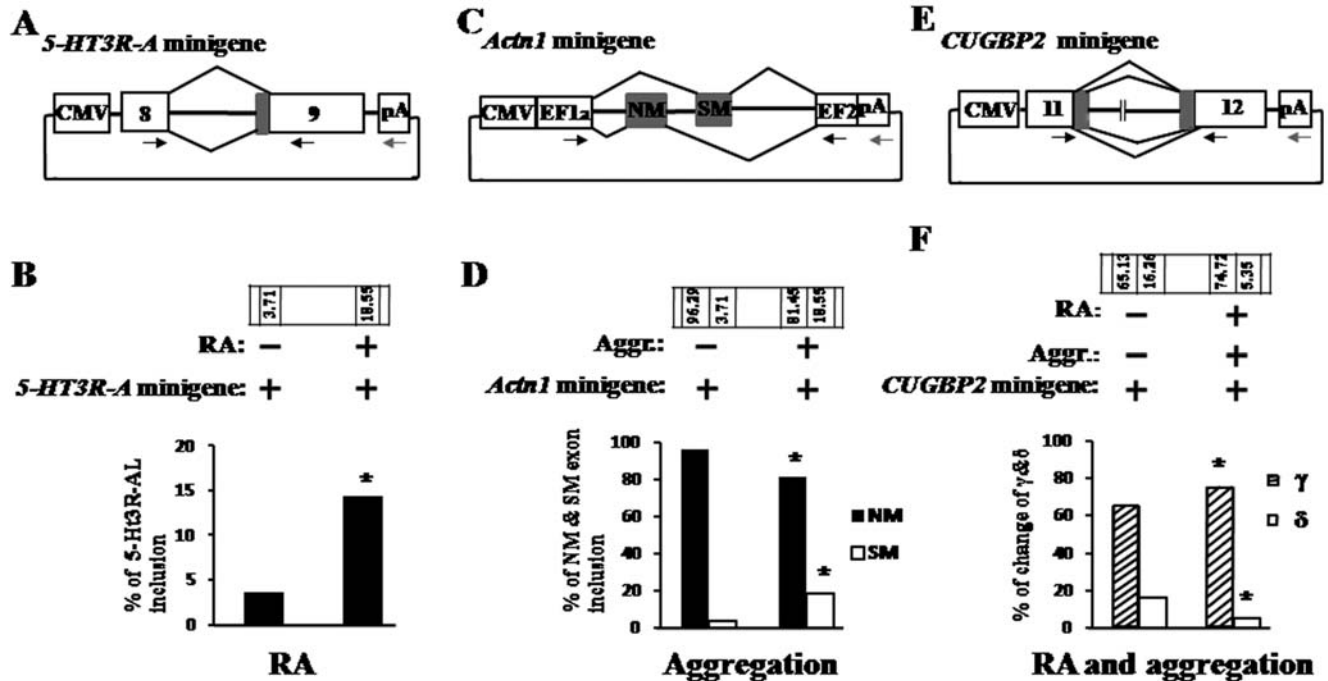


Figure 5 Alternative splicing analysis using minigenes by transfection experiment in P19 cells (A, C, E) Schematic representation of *5-HT3R-A*, *Actn1* and *CUGBP2* minigenes. The mouse genomic fragments for minigenes were inserted between CMV promoter and SV40 poly(A) site in a mammalian expression vector. The black arrows indicate the positions of primers used for amplification of alternatively spliced products, and the grey arrow indicates the position of T7 primer for cDNA synthesis. Five micrograms of each minigene containing expression vector were transiently transfected in P19 cells and were cultured in different treatment conditions for 24 h. (B) The transfected cells were cultured in the absence or presence of RA treatment. The percentage of inclusion of 5-HT3R-AL was determined by densitometry. (D) In the case of *Actn1*, the cells were cultured in the absence or presence of aggregation. The NM and SM exon inclusions were determined by densitometry. (F) For *CUGBP2*, the cells were treated in the absence or presence of RA treatment, and the absence or presence of cell aggregation. By using densitometry, the percentage change of γ and δ isoforms was determined. In each case, the percentage of each splice product was calculated over the total of spliced products. All the experiments were repeated more than three times, and similar results were obtained. *P*-values were determined by Student's *t* testing ($*P < 0.05$). The asterisk values were determined compared with no treatment. The upper bold boxes indicate values of relative ratios of the isoforms.

suggests that cell aggregation alone controls the expression of alternatively spliced NM and SM type of *Actn1* in P19 cells. Moreover, NM and SM type of *Actn1* show differential alternative splicing with and without RA treatment. NM mRNA is down-regulated 34%, and SM mRNA is up-regulated 34% with cell aggregation alone when compared with no treatment at 24 h (Figure 4D; SM type 6.67, cell aggregation/day 0). We found that the effect of cell aggregation on the expression patterns of *Actn1* was similar at 12 h (data not shown). The transcripts, PTB1 and PTB4 of *PTB* were up-regulated when treated with either RA treatment alone or cell aggregation alone, or with both treatments (Figure 4D) compared with no treatment, which suggests that these transcripts display independent effects. Taken together, our results suggest that RA treatment alone or cell aggregation alone individually, changes alternatively spliced isoforms, and these isoforms may be involved in the early stage of P19 cell neural differentiation.

3.4. RA treatment and cell aggregation independently change alternative splicing *in vivo*

To investigate the effects of RA treatment alone and cell aggregation alone on alternative splicing *in vivo*, we performed transient transfection experiments with different treatment conditions (e.g. no treatment as control, RA treatment alone specific for *5-HT3R-A*, cell aggregation alone specific for *Actn1* and both treatments specific for *CUGBP2*) in P19 cells. In the *5-HT3R-A* minigene, we observed that RA treatment alone increases 5-HT3R-AL, on the other hand, it decreases 5-HT3R-AS when compared with the absence of RA treatment (Figure 5B; 5-HT3R-AL 5.0, RA/no treatment). In the case of *Actn1*, cell aggregation alone increases SM type; in contrast, it decreases NM type of *Actn1* when compared with the absence of cell aggregation (Figure 5D; SM type 5.0, cell aggregation/no treatment). We also

were determined by a densitometric method. (B) Ratios of isoforms of *5-HT3R-A* and *CUGBP2* are shown. (C) Total RNAs were extracted from the cells at 0 and 24 h for each treatment condition mentioned above. The relative amounts of the isoforms of *Actn1*, *CUGBP2* and *PTB* were determined by a densitometric method. (D) Ratios of isoforms of *Actn1* and *CUGBP2* are shown. Error bars indicate the S.D. from three different experiments. *P*-values were determined by Student's *t* testing ($*P < 0.05$). The asterisk values were determined compared with day 0. The upper bold boxes indicate values of relative ratios of the isoforms.

performed a transfection experiment using *CUGBP2* minigene with both treatments. We found that both treatments increase the γ isoform, whereas they decrease δ isoform when compared with no treatment (Figure 5F; *CUGBP2* 1.15, both treatments/no treatment).

3.5. RA and cell aggregation independently induce early stage neural differentiation via SMADs phosphorylation

To elucidate the signalling pathways including splicing regulation, *in silico* analysis using KeyMolnet software (IMMD) was performed (Sato et al., 2005). Because FGF8-mediated SMADs signalling was found in the analysis by Sato et al. (2005), and it was known that induced expression of FGF8 (Wang et al., 2006) was caused by cell aggregation in P19 cells, we analysed RA and cell aggregation signalling through SMADs by Western blotting in P19 cells treated under four conditions (Figure 6A, and see the Materials and methods). The phosphorylation of Smad1/5 level was increased by a combination of both treatments compared with no treatment (Figures 6A and 6B; p-Smad1/5 15.68, both treatments/no treatment). When cells were treated with RA and cell aggregation separately, RA treatment alone increased phosphorylation of Smad1/5 level; in contrast, phosphorylation of Smad1/5 level did not change due to cell aggregation alone compared with no treatment (Figures 6A and 6B; p-Smad1/5 14.79, RA/no treatment). Non-phosphorylated Smad5 level did not change in different treatment conditions compared with no treatment (data not shown). In the case of phospho-Smad2, equal increased levels of phosphorylation of Smad2 were observed due to a combination of both treatments, cell aggregation alone and RA treatment alone, when compared with no treatment (Figures 6A and 6B; p-Smad2 6.19, cell aggregation/no treatment). We could not see any change of non-phosphorylated

Smad2 level under different treatment conditions compared with no treatment (Figures 6A and 6B). We also examined GAPDH levels under in these four conditions as positive control and found that the GAPDH level was equal under all the conditions. Analysis of three more separate experiments using band densitometry confirmed these findings (Figure 6B).

4. Discussion

Transcriptional factors such as Mash-1, Ngn-1 and Sox6, are induced by the RA signalling pathway during neural differentiation of P19 cells (Itoh et al., 1997; Nakakura et al., 2001; Hamada-Kanazawa et al., 2004). RA treatment alone affected not only transcriptional levels of *5-HT3R-A* and *Actn1*, but also affected some alternative splicings in our results. For instance, with cell aggregation, the expression of 5-HT3R-AL of *5-HT3R-A* is not detected, but 5-HT3R-AS of *5-HT3R-A* is detected. However, with RA treatment, the alternatively spliced isoform, 5-HT3R-AL of *5-HT3R-A* is detected (Figures 4A and 7). Moreover, RA treatment alone affects the γ and δ isoforms of *CUGBP2* (Figures 4A, 4C and 7). It has already been reported that RA treatment alone controls the alternatively spliced isoforms of RAR, FR- α , hTERT and CREM in different cells and tissues (Takeyama et al., 1996; Bolton et al., 1999; Ding et al., 2002; Matsuda et al., 2005). Now we have found that RA treatment alone changes alternatively spliced isoforms of *5-HT3R-A* and *CUGBP2* in P19 cells. Cell aggregation alone decreases significantly the NM type of *Actn1* and significantly increases the alternatively spliced SM type of *Actn1* (Figures 4C and 7), even though RA treatment up-regulates the transcriptional levels of *Actn1*. Since there was no report of the effects of cell aggregation on alternative splicing, we checked this treatment separately and found cell aggregation alone changes the alternatively spliced isoforms, NM and SM type of *Actn1*. Interestingly,

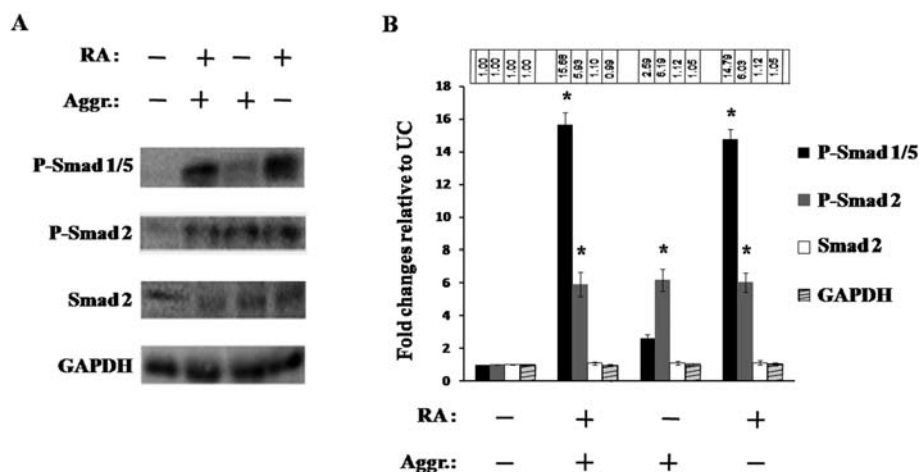


Figure 6 RA and cell aggregation signalling via phosphorylated SMADs (A) Western blot experiments were performed as detailed in the Material and methods section. Cells were treated under four different treatment conditions for 24 h. Twenty-five micrograms of lysates were applied in each lane to detect phosphorylated Smad1/5, phosphorylated Smad2 and non-phosphorylated Smad2. Two micrograms protein lysates were applied in each lane for GAPDH. (B) Fold changes relative to untreated control (UC) [(−RA) (−) aggregation] were determined by a densitometric method. All the experiments were repeated more than three times, and similar results were obtained. The asterisk values were determined compared with no treatment. The upper bold boxes indicate values of relative ratios of the phosphosmads.

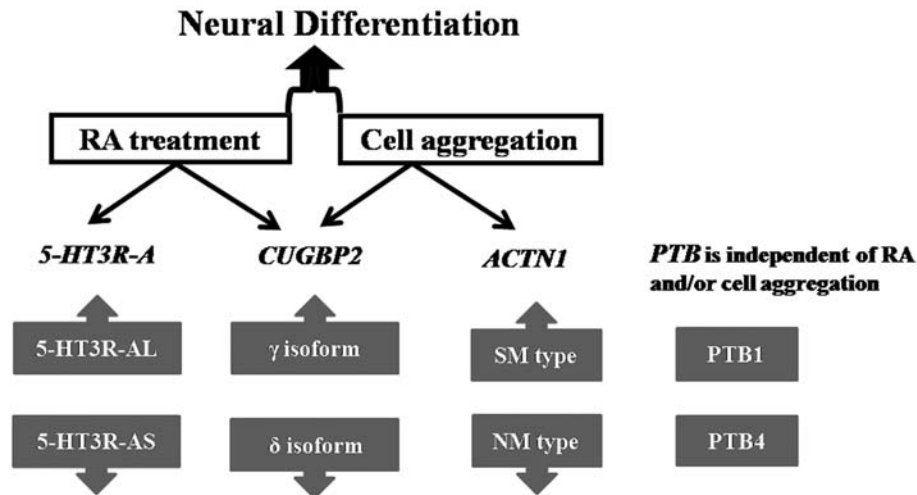


Figure 7 Schematic representation of our study
Even though RA treatment and cell aggregation are both needed for complete neural differentiation of P19 cells, RA treatment alone and cell aggregation alone individually regulate alternative splicing mechanism in P19 cells.

both RA treatment and cell aggregation individually affect the alternative splicing of *CUGBP2* in a similar manner (Figures 4A and 4C). This result suggests that either RA treatment or cell aggregation regulates alternative splicing in *CUGBP2*.

By doing *in vivo* analysis, we show that RA treatment regulates alternative splicing of *5-HT3R-A*. Cell aggregation controls splicing mechanism of *Actn1*. A combination of both treatments regulates alternative splicing of *CUGBP2* (Figure 5). All of these *in vivo* results are consistent with our *in vitro* results, which suggest that RA treatment and cell aggregation independently regulate alternative splicing in neural differentiation of P19 cells. Sapkota et al. (2007) reported that phosphorylation of Smad1/5 and phosphorylated Smad2, whereas cell aggregation alone only promotes phosphorylated Smad2 (Figure 6). These phosphorylated Smads might be involved in early neural differentiation of P19 cells, but the molecular mechanism is still unknown.

The effects of RA are mediated by two classes of ligand-dependent transcription factors, the nuclear RARs and the RXRs (retinoid X receptors) (Kastner et al., 1995). Taneja et al. (1997) reported that phosphorylation is important for proper functioning of RAR α , and this phosphorylation is required for the RA-mediated differentiation of F9 cells into parietal endoderm. RA reduces phosphorylation of RAR α by inhibiting cyclin H and cdk7 expression (Crowe and Kim, 2002).

The regulation of alternative splicing requires *cis*-acting and *trans*-acting elements. *Cis*-acting elements interact directly or indirectly with *trans*-acting elements (activator or repressor of splicing) which regulate alternative splicing (Garcia-Blanco et al., 2004). There are mainly two types of *trans*-acting factors which are involved in alternative splicing regulation, such as general splicing factors [such as SR (serine/arginine-rich) proteins and snRNP (small nuclear ribonucleoproteins)] and tissue-specific splicing factors (e.g. PTB, Fox-1 and Nova). It has been reported

that several alternatively spliced genes are down-regulated by PTB and up-regulated by nPTB in neural differentiation, suggesting that PTB and nPTB switch post-transcriptional mechanism for alternative splicing in later stage of neural differentiation (Boutz et al., 2007; Grabowski, 2007; Coutinho-Mansfield et al., 2007). Since our results show changes in the early stage of neural differentiation (within day 1), we speculate that alternative splicing mechanism might not be affected by PTB. Nova proteins regulate neural-specific alternative splicing, whereas Fox-1 regulates both neural and muscular type alternative splicing through specific binding sequences (Jin et al., 2003; Ule et al., 2006).

SR proteins (RNA-binding domain and RS domain) are required for pre-mRNA splicing. SR proteins regulate constitutive splicing and are also involved in splicing site selection (Krainer et al., 1990). Generally, hnRNP (hetero nuclear ribonucleoprotein) favours distal 5' splice site, whereas SF2 (splicing factor 2), a prototype member of the SR protein family, results in utilization of proximal 5' splice site (Mayeda and Krainer, 1992). SR proteins are highly phosphorylated and are necessary for initiation of spliceosome assembly, and SR protein dephosphorylation appears to be needed for splicing catalysis (Cao et al., 1997). The levels of SR protein phosphorylation are critical, since both hyperphosphorylation and hypophosphorylation inhibit splicing activity (Kanopka et al., 1998; Prasad et al., 1999) in both constitutive and activated alternative splicing.

In our study, we showed that the alternatively spliced isoforms of *Actn1*, *5-HT3R-A* and *CUGBP2* were regulated differentially and in a time-dependent manner (Figures 2A and 3A), and changed their expression patterns very quickly. The expression of the alternatively spliced isoform, 5-HT3R-AL of *5-HT3R-A*, was changed within 12 h; both the NM and SM types of *Actn1* were changed within 24 h. The expression of alternatively spliced isoforms, α , β , γ and δ of *CUGBP2* was changed at 12 and 24 h. Since the changes in our results are quick, SR protein phosphorylation may be involved in alternative splicing regulation. Even

though we cannot deny accumulation of cell-specific factors, it is easy to consider the possibility of alternative splicing regulation by the phosphorylation state of SR proteins. Therefore, further study is necessary to find out which SR proteins are involved in this process. Previously, we identified an SR-related protein, NSSR (neural-salient serine/arginine-rich protein), also known as SRp38 and TASR (Komatsu et al., 1999; Yang et al., 2000; Shin and Manley, 2002). Like other SR proteins, NSSR also acts as a splicing regulator (Shin and Manley, 2002; Fushimi et al., 2005). So, the phosphorylation state of NSSR or other splicing regulators might be involved in regulation of alternative splicing mechanism in early stage P19 cell neural differentiation.

Past studies have stated that both treatments, RA and cell aggregation, are required to induce neural differentiation of P19 cells. However, in this paper, we have shown that RA treatment and cell aggregation independently induce neural differentiation of P19 cells and regulate alternative splicing in the early stage of P19 cells during neural differentiation, but the molecular mechanism is unclear. Further study is necessary to confirm the alternative splicing regulation mechanism by RA treatment alone or cell aggregation alone at the molecular level in early neural differentiation of P19 cells.

Author contribution

AHM Khurshid Alam performed all the experiments and prepared all of the Figures as well as the manuscript. Toshifumi Tsukahara was the team leader. He organized and supervised the study. The other co-author, Hitoshi Suzuki also supervised this study.

Acknowledgements

We thank Mary Ann Mooradian, Lecturer, JAIST Global Communication Center, for grammatical corrections.

Funding

This work was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Received 23 October 2009/5 March 2010; accepted 16 March 2010

Published as Immediate Publication 16 March 2010, doi 10.1042/CBI20090332