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Development of effective isolation method of ES cells for analysis of differentiation

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ABSTRACT

Neuroectoderm development is a milestone of vertebrate neurogenesis. However, the molecular mechanism underlying the differentiation of neuroectoderm is still unclear, especially in mammals. ES cells co-cultured with PA6 cells can differentiate to neuroectoderm by the stromal cell-derived inducing activity method (SDIA method), but contamination of PA6 cells is an obstacle to the analysis of molecular mechanisms of differentiation. Here we describe a novel method by which differentiated ES cells are easily isolated from PA6 cells. We attempted to induce the differentiation of ES cells using paraformaldehyde-fixed PA6 cells. RT-PCR and DNA microarray analysis revealed that the background noise derived from contaminated PA6 cells disappeared when fixed PA6 cells were used. Furthermore, genes up-regulated during the differentiation of ES cells were expressed in a developing mouse embryo. Thus, our newly developed method will be very useful for identifying novel genes associated with mouse neuroectoderm development *in vitro* and *in vivo*.

Introduction

Mouse neurogenesis begins with the induction of anterior neuroectoderm (ANE) on embryonic day 7.5 (E7.5) [1]. It is known that in the induction of ANE two groups of factors, (i) Nodal antagonists and (ii) Wnt antagonists, have to be secreted from the anterior visceral endoderm (AVE) [1]. In addition to these antagonists, several transcription factors, such as Otx2 and Zic family proteins, are expressed in the developing neuroectoderm [1,2]. However, the functions of these factors in neuroectoderm differentiation are still unclear, due to the lack of suitable model systems which well mimic the developing mouse embryo.

Embryonic stem (ES) cells are pluripotent stem cells derived from the inner cell mass (ICM). In the last decades, numerous researchers have developed a method of induction of various functionally differentiated cells from ES cells. These studies demonstrated that ES cells could differentiate to various cells in an *in vitro* culture system, and that the differentiation of ES cells partially mimics embryogenesis [3], indicating the usefulness of ES cells as a model of the developing mouse embryo. In particular, many efforts to induce neurons from ES cells have been made by various methods. Embryoid bodies (EBs) are formed in a floating culture of ES cells without LIF, and retinoic acid efficiently promotes neural differentiation of EBs [4]. Lee et al. have improved this method to induce dopaminergic and serotonergic neurons by the selection and expansion of neural precursors in EBs [5]. Watanabe et al. have developed an optimized serum-free suspension culture of ES cells to induce telencephalic precursors [6]. However, only neurons with limited characteristics were induced by these methods. Thus, it is not clear whether the neuroectoderm, which has very similar characteristics to the neuroectoderm in the developing mouse embryo, has been induced by these methods. To study the molecular events occurring in mammalian neuroectoderm development, a proper induction method is required.

By using the stromal cell-derived inducing activity (SDIA) method, which was first reported by Kawasaki et al. [7], mouse ES cells are induced to differentiate to most ectodermal lineage cells. In addition, various neurons through the rostral–caudal and dorsal–ventral axes could be induced in response to various signaling molecules such as BMP4, sonic hedgehog, and retinoic acid in association with the generation of neuronal identity *in vivo* [8]. Considering these findings, it has been postulated that the SDIA method is a good model system that mimics neural development *in vivo* embryogenesis. However, one difficulty has arisen in obtaining the accurate gene expression profiling of ES cells under the differentiation process, because ES cells are co-cultured with PA6 cells in this method, and the isolation of ES cells which are free from contamination with PA6 cells is practically impossible. In particular, contamination with PA6 cells has become a crucial problem in identifying unknown genes during the differentiation of ES cells.

To overcome this problem, we explored a method of isolating ES cells without contamination with PA6 cells. It was shown to be important that ES cells directly contact the PA6 feeder layer for

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efficient differentiation. Furthermore, ES cells equally differentiated to Tuj1-positive neurons, even on 4% paraformaldehyde (PFA)-fixed PA6 cells [7]. Thus, we speculated that fixed PA6 cells are fully resistant to the trypsin treatment, and we should have a good chance to obtain a pure population of differentiated ES cells by the use of a PFA-fixed feeder layer. By adopting this idea, we successfully developed an efficient method to detach ES cells from PFA-fixed PA6 cells, and this method enabled us to accurately analyze gene expression during the differentiation of ES cells without the background derived from the contamination of PA6 cells. Furthermore, we tried to identify the genes associated with mouse neuroectoderm development. Interestingly, the expression of genes which were up-regulated during the differentiation of ES cells was similar to that during mouse embryogenesis. These results indicate that the differentiation of ES cells on fixed PA6 cells mimics neuroectoderm development *in vivo*, and so the method we developed can be a powerful tool to elucidate the molecular mechanism underlying neuroectoderm differentiation *in vitro* and *in vivo*.

Materials and methods

Mouse ES cell culture and differentiation induction by the SDIA method. D3 ES cells were cultured as previously described [9]. Differentiation induction of ES cells, isolation of differentiation colonies from normal PA6 cells, and BMP4 treatment were performed as previously described [7]. Composition of medium was changed: the concentration of L-glutamine was changed to 4 mM.

Preparation of PFA-fixed PA6 cells. PA6 cells were cultured on a gelatin-coated dish until a confluent monolayer was formed. The PA6 monolayer was then rinsed with PBS (−) and incubated in 4% PFA for 15 min at room temperature. After incubation, the dish was washed three times with PBS (−) and medium for differentiation was added.

Isolation of differentiated ES cells from PFA-fixed PA6 cells and flow cytometry analysis. ES cells were cultured on 4% PFA-fixed PA6 cells at 37 °C for 4 days. After washing with PBS (−), ES cells were incubated in 0.05% Trypsin–EDTA (Invitrogen, Carlsbad, CA) for 3 min at 37 °C. After incubation, the dish was rocked gently, the supernatant was collected, and cells were collected by centrifugation. The cells were then washed with PBS (−) and the fluorescent intensity was analyzed by FACS Calibur (BD biosciences Mountain View, CA).

RT-PCR and DNA microarray analysis. RNA isolation and cDNA synthesis were performed as previously described [9]. Primers for RT-PCR analysis are shown in Supplementary Table 1 and were previously described [8]. TF oligo DNA microarray and 32k oligo DNA microarray analysis and qRT-PCR analysis were performed as previously described [9,10].

Antibodies. Mouse anti-Tuj1 (Covance, Princeton, NJ) was used, and Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) was used as a secondary antibody. Cells were counterstained with DAPI (Wako, Osaka, Japan).

Results and discussion

Development of the method for isolation of differentiated ES cells from PA6 cells

In order to develop a method to isolate ES cells from PA6 cells, we first prepared ES cells which stably express EGFP (EGFP-ES cells) by introducing the construct containing the EGFP expression unit driven by the CAG promoter. EGFP-ES cells express EGFP through differentiation, and the intensity of fluorescence was mostly uniform (data not shown). Next, we seeded EGFP-ES cells on PA6 cells and incubated them for 48 h. After incubation, we isolated EGFP-ES cells by various methods and analyzed fluorescence intensity with flow cytometry in order to determine the extent of contamination of PA6 cells (Fig. 1A and B). Although isolation of ES cells from the feeder layer with Papain was previously reported [7], contamination with a high percentage of PA6 cells was detected under this condition (Fig. 1E). It was reported that ES cells equally differentiated to Tuj1-positive neurons even on 4% PFA-fixed PA6 cells [7]. We tried to isolate EGFP-ES cells on 4% PFA-fixed PA6 cells using trypsin. As a result, EGFP-ES cells were isolated with very little PA6-cell contamination (Fig. 1F). In addition, EGFP-ES cells were similarly isolated through 4 days of culture (Fig. 1G). These results suggest that the normal ES cells can be isolated with almost no PA6-cell contamination by using PFA-fixed PA6 cells. Furthermore, we investigated whether the expression of *Inhβa* and *Bmp4*, which are expressed in PA6 cells [11], were detected in ES cells differentiated on normal PA6 cells or fixed PA6 cells after 4 days of differentiation induction. As a result, *Inhβa* was detected in ES cells differentiated on normal PA6 cells but not in ES cells differentiated on PFA-fixed PA6 cells (Fig. 1H). Although *Bmp4* was slightly detected in ES cells differentiated on fixed PA6 cells, the expression was decreased compared with that in ES cells differentiated on normal PA6 cells. These data suggest that the contamination of PA6 cells in the isolated ES cells prevents accurate gene expression analysis by RT-PCR during the differentiation of ES cells.

It was previously reported that the efficiency with which PFA-fixed PA6 cells induced Tuj1-positive cells was the same as in the case of normal PA6 cells [7]. If the differentiation process observed with PFA-fixed PA6 cells is identical to that observed with non-fixed PA6 cells, we will be able to easily obtain differentiating ES cells, which may lead to the discovery of numerous key factors involved in the differentiation mechanism of ES cells. In the previous report, neither the expression profiles of differentiation marker genes other than Tuj1 nor the differentiation timing were analyzed [7]. In the present study, we first examined the expression of differentiation marker genes in differentiated ES cells on PFA-fixed PA6 cells by semi-quantitative RT-PCR (Supplementary Fig. 1A: Fig. S1A). ES cells which differentiated on PFA-fixed PA6 cells expressed several developmental markers as ES cells differentiated on normal PA6 cells. In addition, it was reported that the identity of the differentiated ES cells turned to a caudal state with the addition of retinoic acid to the differentiation medium [8]. ES cells differentiated with 0.2 μM retinoic acid expressed spinal cord marker *Hoxb4* when both normal and PFA-fixed PA6 cells were used (Fig. S1A). On the other hand, *Otx2* and *Rx* expression were suppressed with the addition of retinoic acid. These results indicate that the expression profiles of the differentiation marker genes in differentiated ES cells obtained from PFA-fixed PA6 cells are almost identical to those in ES cells which were differentiated with non-fixed PA6 cells.

We then analyzed the effect of the addition of BMP4 to the differentiation process of ES cells. ES cells are known to differentiate to epidermal lineage by the SDIA method in the presence of 0.5 nM BMP4 until 3–4 days of differentiation [7]. The anti-neutralizing activity of BMP4 in the SDIA method clearly continued until 3–4 days of culture, and this result was very similar to the result obtained using normal PA6 cells (Fig. S1C). This result indicates that PFA fixation of the feeder layer has almost no effect on the differentiation timing of ES cells.

Next, in order to assess the influence of the contamination of PA6 cells on gene expression analysis, we analyzed the gene expression changes of ES cells differentiated on normal or fixed PA6 cells with DNA microarray. We compared the gene expression profiles of PA6 cells, ES cells differentiated on normal PA6 cells, and ES cells differentiated on fixed PA6 cells with undifferentiated ES cells using TF oligo DNA microarray, which contains the probes of 1562 transcriptional regulatory factors of mice [9]. The Venn

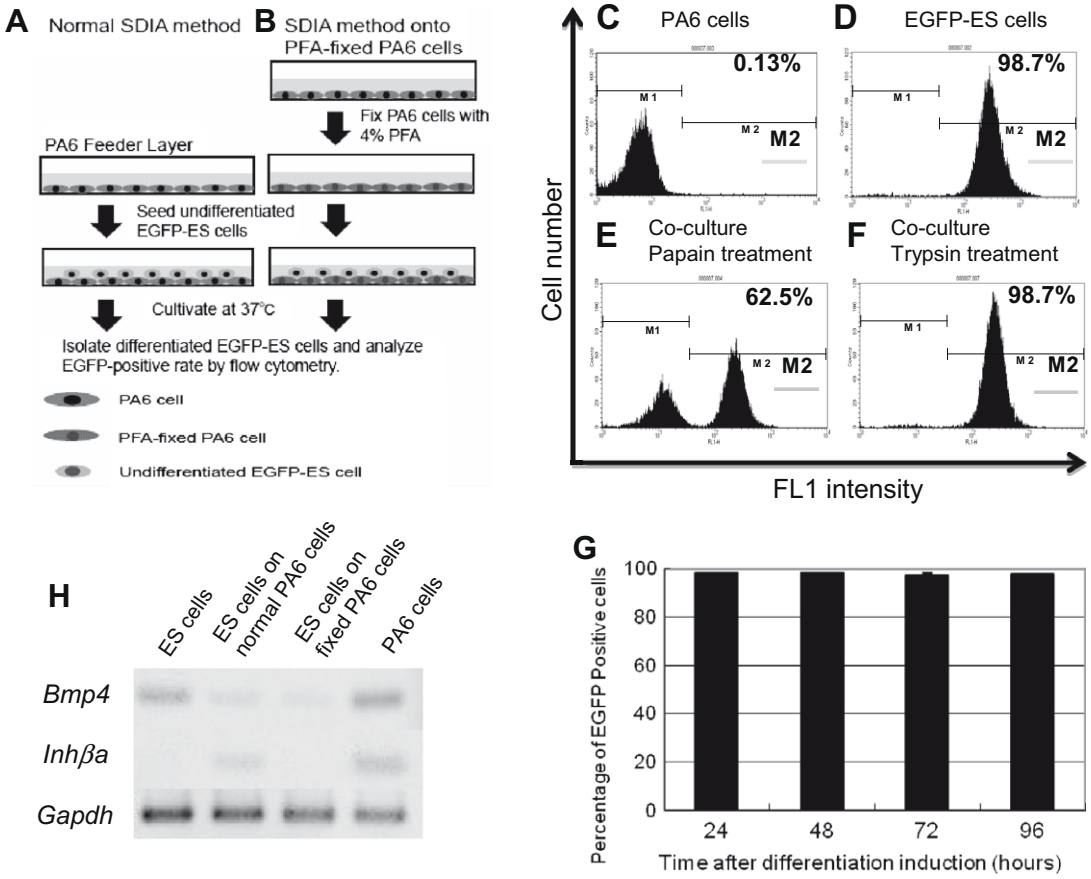

Fig. 1. Validation of isolation method of ES cells. (A,B) The validation scheme of the isolation method. (C–F) Fluorescence intensity of isolated cells. The histogram in the panel demonstrates the EGFP fluorescence intensity of PA6 cells (C), EGFP-ES cells (ES cells that expressed EGFP stably) (D), EGFP-ES cells on PA6 cells (E) and EGFP-ES cells on 4% PFA-fixed PA6 cells (F). After 48 h from differentiation induction, EGFP-ES cells were isolated by Papain (E) or Trypsin (F) and the fluorescence intensity was analyzed by flow cytometry. (G) The EGFP-positive rate of isolated cells on fixed PA6 cells after 24–96 h from differentiation induction. Vertical bars indicate the standard deviation. (H) RT-PCR analysis of *Bmp4* and *Inhβa* expression in undifferentiated ES cells, isolated ES cells on normal PA6 cells, ES cells on fixed PA6 cells, and PA6 cells.

diagram shown in Fig. 2A represents the number of genes whose expression levels changed more than 1.5-fold in PA6 cells, ES cells differentiated on normal PA6 cells, and ES cells differentiated on fixed PA6 cells, while the Venn diagram shown in Fig. 2C represents the number of genes which were expressed lower than 0.67-fold in PA6 cells, ES cells differentiated on normal PA6 cells, and ES cells differentiated on fixed PA6 cells. The numbers of genes which were highly expressed in PA6 cells and ES cells differentiated on normal PA6 cells were six times the numbers of genes which were highly expressed in PA6 cells and ES cells differentiated on fixed PA6 cells (Fig. 2A). Additionally, the numbers of genes with low expression in PA6 cells and ES cells differentiated on normal PA6 cells were approximately four times the numbers of genes with low expression in PA6 cells and ES cells differentiated on fixed PA6 cells (Fig. 2C). These results indicate that the expression of mRNA derived from contaminated PA6 cells is detected as a false positive or false negative. Additionally, the genes which were up-regulated only in ES cells differentiated on fixed PA6 cells were less expressed in PA6 cells than in undifferentiated ES cells (Fig. 2B). In addition, the genes which were down-regulated only in ES cells differentiated on fixed PA6 cells were expressed more highly in PA6 cells than in undifferentiated ES cells (Fig. 2D). Thus, the expression of these genes was not detected in ES cells differentiated on normal PA6 cells because the expression of these genes was masked by the opposite expression of mRNA derived from contaminated PA6 cells. *Rest* [12] and *Bmp4* [13], which were known to be down-regulated during the differentiation of ES cells, were detected only in ES cells differentiated on fixed PA6 cells. These data indicate that the contamination of PA6 cells can lead to inaccurate results in DNA microarray analysis. Thus, our developed method should be very effective for a sensitive analysis such as DNA microarray.

Recently, Ueno et al. have tried to replace the PA6 feeder layer with other materials with respect to differentiating ES cells to neural cells. The matrix components of the human amniotic membrane exhibited neural-inducing activity like PA6 cells [14]. Although this method is useful for the treatment of Parkinson's disease *via* cell transplantation, the direction of differentiation with these methods is uninvestigated. On the other hand, ES cells can be differentiated to various neurons by the SDIA method using normal or PFA-fixed PA6 cells. Therefore, the method we developed will be applicable to the development of a method for the treatment of other neurodegenerative diseases.

It was previously reported that the efficiency of human ES cells to differentiate to Tuj1-positive neurons on PFA-fixed PA6 cells was the same as that using normal PA6 cells, but tyrosine hydroxylase (TH)-positive neurons were decreased using fixed PA6 cells [15]. Although we did not assess the percentage of TH-positive neurons, expression of the TH-positive mesencephalic dopaminergic neuron marker *Nurr1* was detected in our experiment. In addition, various marker genes of the developing mouse brain were expressed in differentiated ES cells on PFA-fixed PA6 cells, so it is expected that the default state of neuroectoderm is induced by PFA-fixed PA6 cells. Importantly, the contamination of PA6 cells

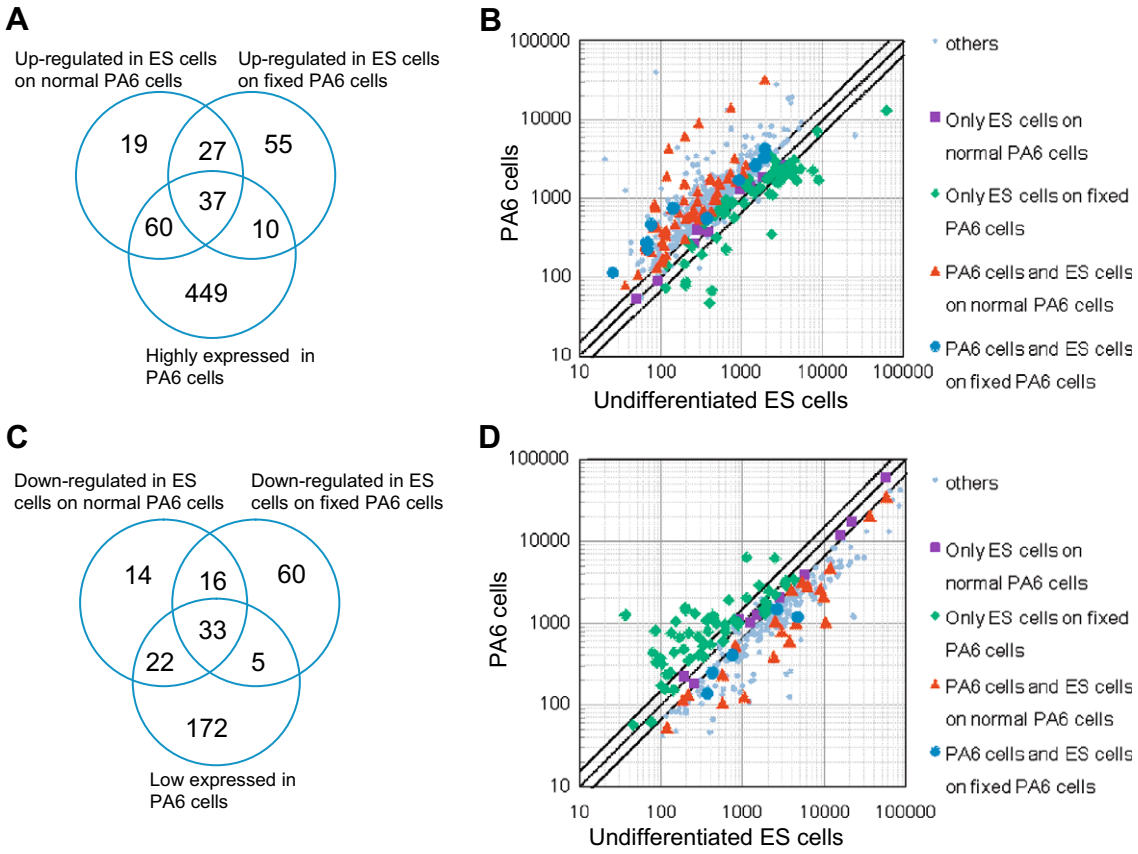

Fig. 2. Assessment of the effect of the contamination of PA6 cells on DNA microarray analysis. (A,C) The number of genes of which expression level was higher (A) or lower (C) in PA6 cells or ES cells on normal PA6 cells or fixed PA6 cells than in undifferentiated ES cells. (B,D) Comparisons of the expression profile of undifferentiated ES cells with that of PA6 cells. The genes selected in (A) are shown in (B) and those in (C) are shown in (D). Symbols defined in the figure represent the categories determined in (A) or (C).

will lead to indeterminate results such as the *Inhβa* expression that we detected by RT-PCR analysis. This will become a crucial problem when we search unknown genes associated with the differentiation of ES cells by the SDIA method. Thus, our developing method will be useful for conducting accurate analysis of transcriptome or proteome during differentiation of mouse ES cells to neuroectoderm using DNA microarray or mass spectrometry. Moreover, our developed method can be applied to differentiation induction systems using other feeder layers (*e.g.*, OP9 cells, which induce mouse ES cells to hematopoietic lineage cells [16]).

The expression of various marker genes changed during the differentiation of ES cells by the SDIA method

We attempted to analyze the gene expression profile during the differentiation of ES cells to neuroectoderm by the SDIA method using PFA-fixed PA6 cells. To analyze the early stage of neuroectoderm differentiation, we isolated differentiated ES cells at 6–96 h after differentiation induction and analyzed the gene expression profile with 32k oligo DNA microarray which contains the probes of mouse 32829 transcripts registered to the Ensembl data base. As a result, the expression levels of numerous genes were drastically changed. The lists of these genes were shown in Supplementary Tables 2 and 3. The expression levels of 377 transcripts decreased less than 0.5-fold at at least two time points (Fig. 3A). This group of genes contained the genes expressed in ES cells, specifically *STAT3*, *ERas*, *Zfp42*, *Zfp296*, *Nr0b1*, and *Klf4* [17,18] (Fig. 3B).

The expression levels of 205 transcripts increased more than twofold (Fig. 3C). We validated the expression of several genes, which were selected randomly from 205 up-regulated transcripts, by quantitative RT-PCR (qRT-PCR) (Fig. S2). This group of genes contained the marker genes, which are expressed in developing mouse neuroectoderm *in vivo* and are essential to head formation and neurogenesis (Fig. 3D). Crabp1 and Crabp2 are retinoic acid-binding proteins and are expressed in developing CNS [19]. Zic5 is a transcriptional factor with a zinc-finger motif that is expressed significantly in gastrulating mouse embryo [3]. *Otx2* is a paired-type homeobox gene and mouse homolog of *Drosophila orthdenticle*. *Otx2* is significantly expressed in the anterior part of epiblast at the pre–post gastrulating stage. The expression of *Otx2* was significantly increased at 24–48 h after differentiation induction and maintained at a high level in our experiment. This result suggests that differentiated ES cells at 24–48 h of differentiation by the SDIA method represent neuroectoderm in pre–post gastrulating embryo *in vivo*. SFRP2 is a secreted Wnt antagonist and is expressed in neuroepithelium at the neurulation stage [20]. ES cells express the neural stem cell marker *Sox1* with SFRP2 overexpression [20]. Thus, SFRP2 seems to have important roles in the differentiation of ES cells by the SDIA method. These results indicate that the differentiation of ES cells by the SDIA method represents neuroectoderm development *in vivo*.

In order to confirm this indication, we further analyzed the expression of several genes which are not known to be associated with neuroectoderm development *in vivo* by qRT-PCR. As a result, the expression levels of these genes were dynamically changed through the gastrulation to neural tube closure (Fig. 4), indicating that these genes are associated with neuroectoderm development *in vivo*. Therefore, the process of the differentiation of ES cells by the SDIA method seems to be very close to the neuroectoderm development *in vivo*.

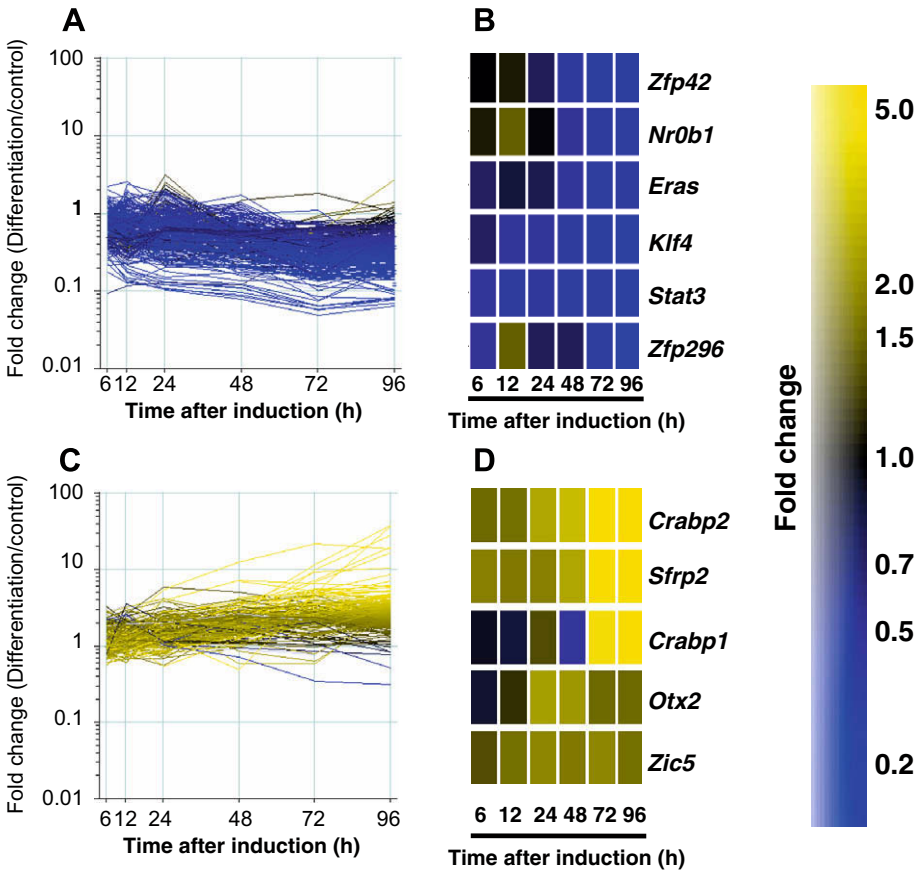

Fig. 3. DNA microarray analysis of the differentiation of ES cells by the SDIA method. After 6–96 h from differentiation induction on 4% PFA-fixed PA6 cells, differentiated ES cells were isolated. The total RNAs of differentiated ES cells at six time points were compared with that of undifferentiated ES cells by mouse 32k oligo DNA microarray. The genes whose fold changes were higher than 2.0 at at least two time points are shown in (A), and the genes whose fold changes were lower than 0.5 are shown in (C). The ES cells marker genes were down-regulated (B) and the neuroectoderm marker genes were up-regulated (D). The color used in the figures represents the levels of fold change shown in the right panel.

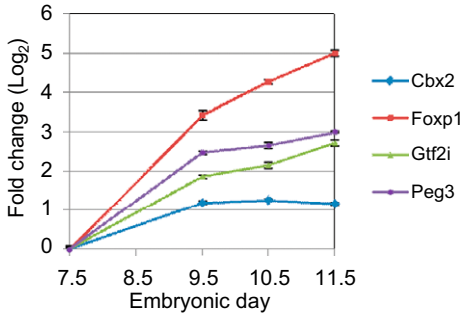

Fig. 4. The qRT-PCR analysis *in vivo* concerning the genes identified by DNA microarray. The expression of genes which were up-regulated during the differentiation of ES cells *in vivo* was analyzed by qRT-PCR. Mouse embryos were harvested at E7.5–11.5, and mRNA expression changes were analyzed. The raw data were normalized by each *Gapdh* expression level. The three normalized data at each developmental stage were averaged, and the log ratio compared with E7.5 was calculated.

Finally, our newly developed method in this report will be a powerful tool to analyze accurate gene expression during the differentiation of ES cells and can help to clarify the novel genes associated with mammalian neuroectoderm development. Therefore, the molecular mechanisms of this event will be revealed by further analysis of the identified genes by means of gain and loss of function analysis *in vitro* and spatial expression analysis during mouse embryogenesis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.06.122.

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