

# Homeotic factor ATBF1 induces the cell cycle arrest associated with neuronal differentiation

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## Summary

The present study aimed to elucidate the function of AT motif-binding factor 1 (ATBF1) during neurogenesis in the developing brain and in primary cultures of neuroepithelial cells and cell lines (Neuro 2A and P19 cells). Here, we show that ATBF1 is expressed in the differentiating field in association with the neuronal differentiation markers  $\beta$ -tubulin and MAP2 in the day E14.5 embryo rat brain, suggesting that it promotes neuronal differentiation. In support of this, we show that ATBF1 suppresses nestin expression, a neural stem cell marker, and activates the promoter of *Neurod1* gene, a marker for neuronal differentiation. Furthermore, we show that in Neuro 2A cells, overexpressed ATBF1 localizes predominantly in the nucleus and causes cell cycle arrest. In P19 cells, which formed embryonic bodies in the floating condition, ATBF1 is mainly cytoplasmic and has no effect on the cell cycle. However, the cell cycle was arrested when ATBF1 became nuclear after transfer of P19 cells onto adhesive surfaces or in isolated single cells. The nuclear localization of ATBF1

was suppressed by treatment with caffeine, an inhibitor of PI(3)K-related kinase activity of ataxa-telangiectasia mutated (ATM) gene product. The cytoplasmic localization of ATBF1 in floating/nonadherent cells is due to CRM1-dependent nuclear export of ATBF1. Moreover, in the embryonic brain ATBF1 was expressed in the cytoplasm of proliferating stem cells on the ventricular zone, where cells are present at high density and interact through cell-to-cell contact. Conversely, in the differentiating field, where cell density is low and extracellular matrix is dense, the cell-to-matrix interaction triggered nuclear localization of ATBF1, resulting in the cell cycle arrest. We propose that ATBF1 plays an important role in the nucleus by organizing the neuronal differentiation associated with the cell cycle arrest.

Key words: Cell cycle, Neuron, Differentiation, ATBF1, ATM, Isthmus, Rat

## Introduction

AT motif-binding factor 1 (ATBF1) is a 404 kDa transcription factor that contains four homeodomains and 23 zinc-finger motifs (Miura et al., 1995). A small alternatively-spliced product from the *Atbfl* gene was first identified as one of the DNA-binding factors (Morinaga et al., 1991) that interact with an AT-rich element located upstream of  $\alpha$ -fetoprotein (*Afp*) promoter to suppress the gene transcription. ATBF1 also cooperates with p53 to activate the *p21*<sup>Waf1/Cip1</sup> promoter and trigger cell cycle arrest (Kataoka et al., 2003; Miura et al., 2004). Owing to its ability to suppress *Afp* gene transcription in hepatic cells, ATBF1 is believed to be a factor responsible for differentiation of the liver. The reduced/absent expression of ATBF1 is associated with *Afp* gene expression in gastric cancers and correlates with malignant nature of tumors (Kataoka et al., 2001). We recently provided evidence that

*Atbfl* gene expression can act as a marker for endocrine responsiveness and is a prognostic indicator for breast cancer progression (Zhang et al., 2005). Furthermore, ATBF1 has recently been identified as a candidate of tumor suppressor for prostate cancer and is frequently mutated or lost in prostate cancer (Sun et al., 2005). An ATBF1 ortholog in *Drosophila*, *Zfh2*, binds to an AT-rich element located upstream of the DOPA decarboxylase (*Ddc*) gene responsible for specific neuronal differentiation of the central nervous system (CNS) (Lundell and Hirsh, 1992) and to an opsin regulatory element responsible for differentiation of the lens (Fortini et al., 1991), suggesting that ATBF1 is involved in neuronal differentiation. Previously, we have characterized a full-length cDNA of *Atbfl* and showed that it is highly expressed in the CNS and dopaminergic neurons in a neuronal differentiation-dependent manner (Miura et al., 1995; Ishii et al., 2003).

Here, we present evidence that ATBF1 plays an essential role in cell cycle arrest and neural differentiation during embryogenesis. ATBF1 is highly expressed in the nucleus of postmitotic cells, resulting in suppression of the nestin gene and activation of the *Neurod1* gene (previously NeuroD) for neuronal differentiation. The subcellular localization of ATBF1 is closely correlated with cell proliferation and cell cycle arrest.

## Materials and methods

### Immunohistochemistry

Pregnant rats (Standard Wistar ST) were administered BrdU (50 mg/kg) for 3 hours to label E14.5 embryos. Dissected embryonal brain and cultured cells were fixed in 4% paraformaldehyde and embedded in paraffin. Glass-mounted 4  $\mu$ m sections were deparaffinized and rehydrated, and the antigens were reactivated in 0.01 M citrate buffer (pH 6.0) at 110°C for 5 minutes. Incubation with primary antibodies against ATBF1 (D1-120; MBL) (Ishii et al., 2003), BrdU (5-bromo-2'-deoxy-uridine labeling and detection kit I; Roche), nestin (MAB353; Chemicon),  $\beta$ -tubulin III (T8660; Sigma) or proliferating cell nuclear antigen (Clone PC10; Dako Cytomation) was generally performed in a solution of phosphate-buffered saline containing 1% goat serum and 0.25% Triton X-100 at room temperature for 1 hour. The secondary antibodies were Alexa 594-conjugated goat anti-rabbit IgG and Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes). TUNEL staining was performed using the Dead-End system (Promega). Some sections were counterstained with DAPI (Kirkegaard and Perry Laboratories) or 3,3'-dihexyloxycarbocyanine iodide [DiOC<sub>6</sub>(3); Molecular Probes], while others were developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and counter stained with Hematoxylin. Images were obtained using an AX70 fluorescence microscope (Olympus) and an LSM5 confocal laser-scanning microscope (Zeiss).

### SYBR Green real-time RT-PCR analysis

Total RNAs isolated from whole brains at E12.5, E18.5, P1, P3, P7 and P28 using Trizol (Invitrogen) were reverse-transcribed and a part of each complementary DNA template was used for SYBR Green real-time PCR. Amplification was performed with the following primers: *Atbf1*, 5'-TTC TTT TCC TCC TCT CTC CTC ATC-3' and 5'-CGG TCC GTC GGA CTT TTG-3';  $\beta$ -tubulin III, 5'-AAG GCC TTC CTG CAC TGG TA-3' and 5'-TCT CGG CCT CGG TGA ACT C-3'; *Gfap*, 5'-CGC TCA ATG CTG GCT TCA-3' and 5'-AAG CGG TCA TTG AGC TCC AT-3'; and *Gapdh*, 5'-TGT GTC CGT CGT GGA TCT GA-3' and 5'-CCT GCT TCA CCA CCT TCT TGA-3'. The expression of each gene was normalized by the corresponding amount of *Gapdh* mRNA. The relative amounts of each product were calculated by the comparative CT ( $2^{-\Delta\Delta CT}$ ) method described in User Bulletin #2 of the ABI Prism 7000 Sequence Detection System (Applied Biosystems).

### Cell cultures

Neuroepithelial cells from the ganglionic eminence (GE) of E14.5 rats were mechanically dispersed and cultured in serum-free DMEM/F12 (1:1) supplemented with N-2 supplements (GIBCO) and 10 ng/ml FGF2 (Pepro Tech EC) for the induction of neurospheres. Neuro 2A mouse neuroblastoma cells were maintained in minimum essential medium (MEM) containing 10% fetal bovine serum. P19 mouse embryonal carcinoma cells were maintained in  $\alpha$ -MEM containing 10% fetal bovine serum. To induce neuronal differentiation, P19 cells were aggregated on bacterial grade dishes for 4 days with 0.5  $\mu$ M all-trans RA (Sigma) and then transferred to tissue culture dishes without RA (Rudnicki and McBurney, 1987). Cells were replated on glass slides coated with combinations of poly-L-ornithine (Sigma), fibronectin

(Invitrogen), laminin (Invitrogen), poly-L-lysine (Sigma) and gelatin (Sigma) for immunostaining.

### Transfections and constructs

The *Atbf1* expression vectors consists of an 11 kb of full-length human cDNA (Miura et al., 1995) inserted in the pCI vector (Promega) with an HA-tag or Myc-tag sequence at the 5'-terminal of the inserted sequence (Nojiri et al., 2004). The mouse *Neurod1* (*BETA2*)-luciferase reporter plasmid was kindly provided by Prof. M.-J. Tsai (Huang et al., 2000). The nestin enhancer fragment was a gift from Prof. H. Okano (Kawaguchi et al., 2001). The promoter fragments were subcloned into the pGV-B basic luciferase reporter plasmid (Promega). An internal control vector, pRL-TK (Promega) was co-transfected to normalize the efficiency of transfection and the cells were analyzed by the dual-luciferase assay system (Promega). Typically, 1  $\mu$ g of DNA was transfected with TransIT (Mirus) in 24-well dishes for 3 hours in the presence of 10 ng/ml FGF2, after which the cells received fresh medium.

### FACS analysis

Cultured cells were washed three times with PBS, and then stained in 50  $\mu$ g/ml propidium iodide (Sigma-Aldrich), 0.1% sodium citrate, 20  $\mu$ g/ml ribonuclease A and 0.3% IGEPAL CA-630 (Sigma-Aldrich). In total,  $1 \times 10^4$  cells were analyzed using a flow cytometer (FACScan; Becton Dickinson) to determine the cell cycle phases.

### Pharmacological reagents

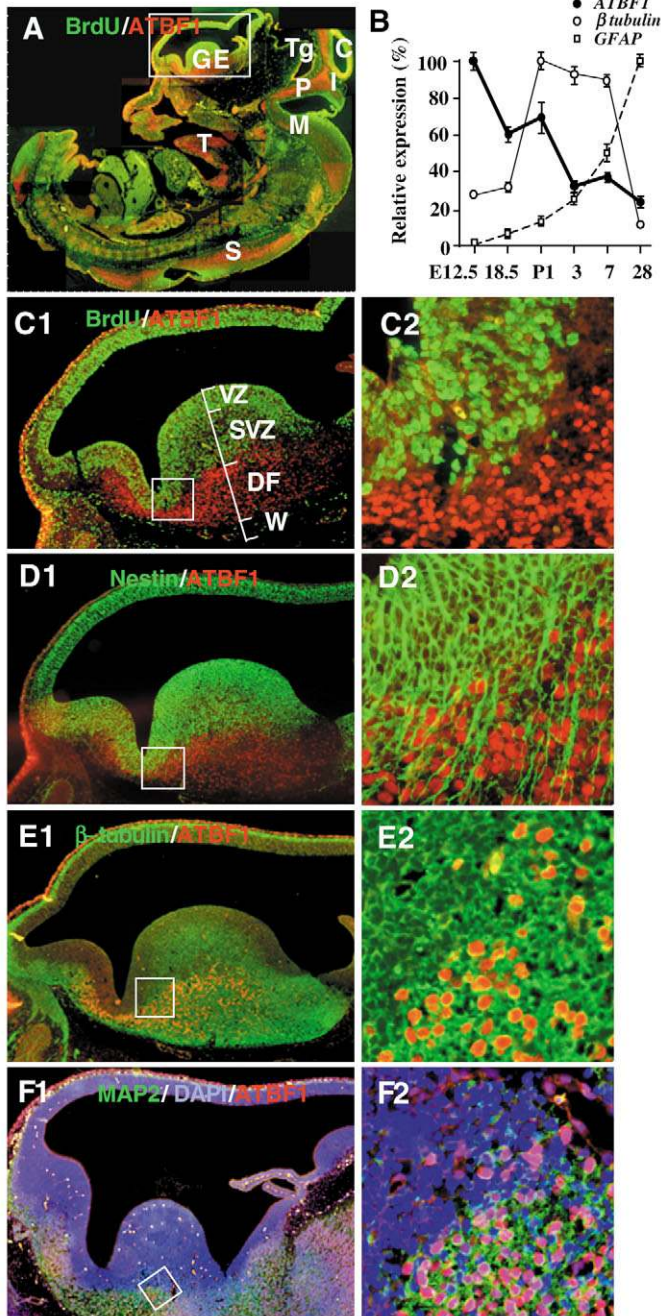
Leptomycin B (LMB, 20 nM; Sigma), wortmannin (100 nM; Calbiochem), ryanodine (10 nM-100  $\mu$ M; Sigma) and caffeine (1-20 mM; Sigma) were applied 30 minutes before changing the culture conditions during the treatment with RA (0.5  $\mu$ M; Sigma).

## Results

### ATBF1 expression is associated with cell cycle arrest

In E14.5 rats, ATBF1 was highly expressed in the ganglionic eminence (GE), medial tegmentum (Tg), colliculus (C), isthmus (I), pons (P), medulla (M), spinal cord (S) and tongue (T) (Fig. 1A). ATBF1-expressing cells and BrdU-labeled cells were distinctly separated (Fig. 1A,C1,C2). BrdU labeling in the proliferating zone and ATBF1 in the nucleus of postmitotic cells could be clearly demarcated without any overlap. ATBF1 expression was not detectable in the nucleus of proliferating cells, but was present in the cytoplasm at a low level. In the brain, *Atbf1* mRNA expression was highest at E12.5 and then decreased gradually with another transient peak at P1.  $\beta$ -Tubulin III mRNA increased at E18.5, reached a plateau until P7 and then decreased, while glial fibrillary acidic protein (*Gfap*) mRNA increased from P1-P3 (Fig. 1B).

The GE is an ideal region for investigating neuronal differentiation in the context of cell cycle regulation, because the process of neural differentiation can be clearly observed as separate layers composed of proliferating cells or fully differentiated neurons. BrdU-labeled cells were limited to the ventricular zone (VZ) and subventricular zone (SVZ), and absent from the differentiating field (DF) and white matter (W) (Fig. 1C1,C2). Nestin, a neural stem cell marker, was expressed in the VZ and SVZ (Fig. 1D1,D2). ATBF1 expression in the nucleus was mainly seen in the DF, and co-expressed with  $\beta$ -tubulin III (Fig. 1E1,E2). Most of the microtubule-associated protein 2 (MAP2)-positive cells also expressed ATBF1 (64 $\pm$ 9%,  $n=1505$ ) (Fig. 1F1,F2). ATBF1



**Fig. 1.** ATBF1 expression is associated with neuronal differentiation in E14.5 rats. (A) BrdU (green) and ATBF1 (red). ATBF1 is expressed in the ganglionic eminence (GE), medial tegmentum (Tg), isthmus (I), pons (P), colliculus (C), spinal cord (S), tongue (T) and elsewhere. (B) mRNA expression in embryonic and postnatal rat brains evaluated by real-time RT-PCR. (C1-F2) Magnification of the GE and further magnification of the framed area. (C1) BrdU labeling in the VZ and SVZ. ATBF1 is present in the differentiating field (DF) but not in the white matter (W). (C2) Higher magnification of the boxed area in C1. (D1) Nestin is present in the VZ and SVZ extending a fibrous network from the SVZ into the DF, but is segregated from ATBF1-positive cells. (D2) Higher magnification of the boxed area in D1. (E1)  $\beta$ -tubulin III is present in the DF with ATBF1. (E2) Higher magnification of the boxed area in E1. (F1) MAP2 is present in the DF with ATBF1. (F2) Higher magnification of the boxed area in F1.

expression in the GE was higher at earlier stages (E12.5 and E14.5) and decreased dramatically at a later stage (E18.5) (not shown).

### ATBF1 expression promotes neuronal differentiation

We found a distinct difference in nestin expression in cells overexpressing an HA-tagged mock vector (nestin/HA double positive cells  $79.4 \pm 2.1\%$ ,  $n=1525$ ) (Fig. 2A1-A5) and an HA-tagged ATBF1 cDNA ( $6.4 \pm 0.8\%$ ,  $n=1151$ ) (Fig. 2B1-B5) by histochemical analysis of cultured neuroepithelial cells. Furthermore, we observed suppression of the nestin-specific enhancer element with an ATBF1 expression vector as assessed by luciferase assay (Fig. 2E). These results were consistent with the observation that ATBF1 was only expressed in nestin-negative cells and vice versa in vivo (Fig. 1D1,D2). There was a notable increase in the number of  $\beta$ -tubulin/HA-double positive cells after transfection of the ATBF1 expression vector ( $36.7 \pm 3\%$ ,  $n=767$ ) (Fig. 2D1-D5), compared with a mock HA-tag vector ( $0 \pm 0\%$ ,  $n=653$ ) (Fig. 2C1-C5). This result was consistent with the luciferase assays demonstrating that the *Neurod1* promoter was activated (Fig. 2F) by ATBF1 to promote neuronal differentiation.

### Overexpression of ATBF1 induces cell cycle arrest

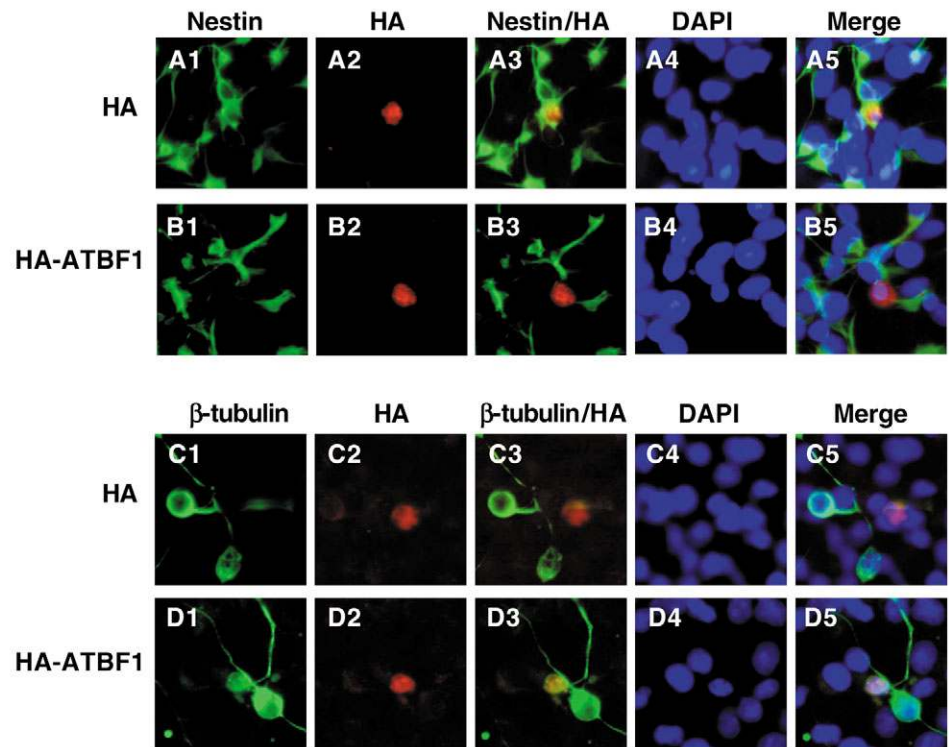
In a mouse neuroblastoma cell line, Neuro 2A cells, BrdU uptake was stopped once *Atbfl* was transfected (Fig. 3B1-B5). The amount of cells in the G1/G0 phase was 33% (M1 in Fig. 3E1,E3) after transfection of a mock vector and this increased to 77% (M1 in Fig. 3E2,E4) after the transfection of the ATBF1 expression vector. There was no remarkable change in the number of apoptotic cells after transfection of the ATBF1 expression vector ( $2.9 \pm 0.2\%$ ,  $n=1127$ ) (Fig. 3D1-D5) compared with that after transfection of a mock vector ( $3.0 \pm 0.8\%$ ,  $n=1310$ ) (Fig. 3C1-C5).

### Nuclear localization of ATBF1 is associated with cell cycle arrest

In a mouse embryonic carcinoma cell line, P19 cells, the cell cycle was maintained in the floating condition (Fig. 4A1) and embryonic bodies were formed at a high density of the cells (Fig. 4B1-B4), even though ATBF1 expression was elevated by 50-fold within 24 hours of RA treatment owing to induction of neuronal differentiation (Miura et al., 1995). ATBF1 was expressed in the cytoplasm of P19 cells in the floating condition, but became localized in the nucleus after the cells were separated from the embryonic bodies and became attached to the culture plate (Fig. 4C1-C4). FACS analysis revealed that the population in the G1/G0 phase was low when ATBF1 was present at a low level (Fig. 4A, M1=42%) or expressed in the cytoplasm in the embryonic bodies (Fig. 4B, M1=54%), and increased when ATBF1 was localized in the nucleus in adherent cultures (Fig. 4C, M1=83%), resulting in neuronal differentiation.

### Isolation from cell-to-cell interaction induces nuclear localization of ATBF1

Next, we examined the mechanism that controls the subcellular localization of ATBF1. Once the cells attached to coated surfaces ATBF1 became localized in the nucleus (Fig. 5A1-A5). We searched for the intracellular signaling pathway that induces the nuclear localization of ATBF1. As



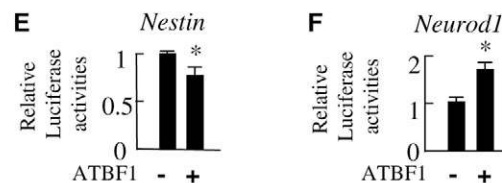
**Fig. 2.** Overexpression study of ATBF1 cDNA in primary cultured neuroepithelial cells.

(A1-5) Immunohistochemistry for nestin (green) overlaps with the HA-tag (red) after transfection of an HA-tagged mock expression vector. (B1-5) Nestin (green) expression is suppressed by HA-tagged ATBF1 (red) expression. (C1-5)  $\beta$ -Tubulin (green) does not overlap with an HA-tagged (red) mock expression vector. (D1-5)  $\beta$ -tubulin (green) expression overlaps with HA-tagged ATBF1 (red) expression. (E) A nestin-specific enhancer element is suppressed by ATBF1. (F) The *Neurod1* promoter is activated by ATBF1. \* $P < 0.01$ .

phosphatidylinositol-3-kinase [PI(3)K]-related pathways are involved in regulating the major trafficking processes of various nuclear factors for cell growth (Seoane et al., 2004) and neural differentiation (Hermanson et al., 2002; Peng et al., 2004), the effect of ATBF1 may also be related to PI(3)K-mediated signaling. To investigate such an involvement, we used inhibitors of PI(3)K family enzymes, namely LY294002 and caffeine (Sarkaria et al., 1999), during the neuronal differentiation process of P19 cells. After pre-treatment with LY294002 for 30 minutes, the nuclear localization of ATBF1 was partially inhibited and showed a mosaic appearance (Fig. 5A6). The effect of ryanodine treatment (100  $\mu$ M) was weak and showed partial inhibition of the nuclear localization of ATBF1 (Fig. 5A7). Pre-treatment with caffeine (5 mM) for 30 minutes induced distinct inhibition of the nuclear localization of ATBF1 (Fig. 5A8). Under the floating aggregated condition, ATBF1 was expressed in the cytoplasm (Fig. 5B1), but treatment with LMB (Nishi et al., 1994), an inhibitor of CRM1 (Kudo et al., 1997), increased the concentration of ATBF1 in the nucleus (Fig. 5B2). ATBF1 was immediately localized in the nucleus after treatment with EDTA (1 mM, 30 minutes) and a pipette that caused mechanical dissociation into single cells (Fig. 5B3).

#### ATBF1 expression in the midline part of the E14.5 rat brain

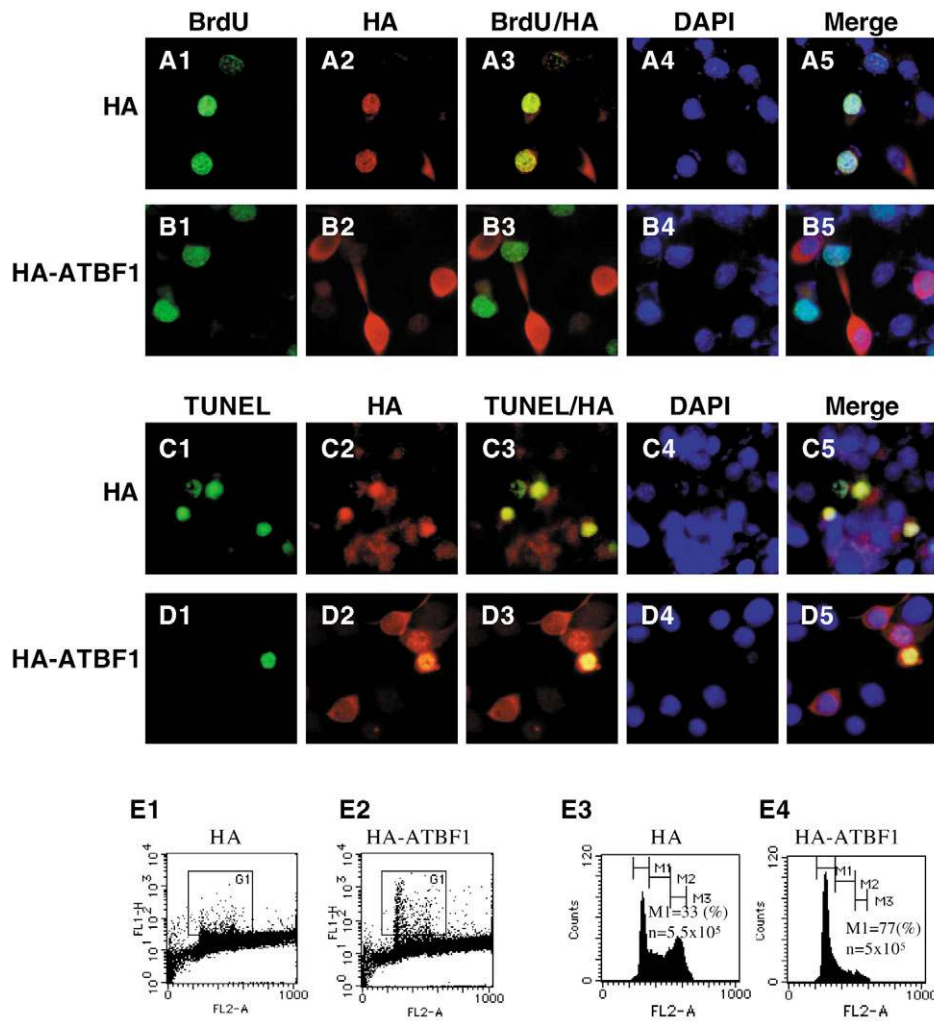
Although neuroepithelial cells divide rapidly during the early stages of neural development, the ratio of proliferation is not uniform in each region and the neuroepithelium expands at different rates to form its specialized morphology. ATBF1 is highly expressed in the midline part of the brain associated with curving structure at the tegmentum (Fig. 6B,B1) and thalamus (Fig. 6C,C1), and the narrowing structure at the isthmus (Fig. 6D,D1).



#### Nuclear localization of ATBF1 in the differentiating field of embryonic brain

The density of cells should alter the interaction of cell-to-cell and cell-to-extracellular matrix (ECM). Intracellular signaling is likely to respond differentially to cell-to-cell and cell-to-ECM interactions via adhesion molecules on cell surface. At the GE of the embryonic brain, ATBF1 was localized in the cytoplasm at the VZ (Fig. 7A,A1) and strongly expressed in the nucleus at the DF (Fig. 7A,A3). The expression of fibronectin (Fig. 7B), one of the constituent factors of the ECM, was sparse and cells were congested at a high density in the VZ (Fig. 7B,B1), while the ECM was dense with a lower density of cells in the DF (Fig. 8B,B3). We observed a similar phenomenon regarding the density of cells and the subcellular localization of ATBF1 at the isthmus (Fig. 7C,C1).

Pregnant rats were administered BrdU for 3 hours to label E14.5 embryos. The cells expressing ATBF1 in their cytoplasm (Fig. 7E,E1) were detected by BrdU incorporation indicating proliferation (Fig. 7D,D1) in the layer facing the ventricle with higher density of cells (Fig. 7C,C1, region I); by contrast, those expressing ATBF1 in their nucleus (Fig. 7E and E2) were arrested in the postmitotic phase without detection of BrdU (Fig. 7D,D2) in the outer layer facing the ECM with lower density of cells (Fig. 7C,C1, region II).



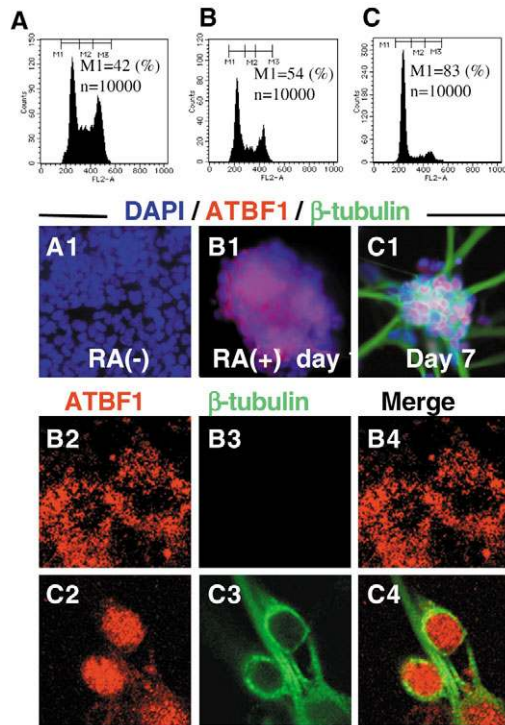
**Fig. 3.** ATBF1 induces cell cycle arrest in Neuro 2A cells without inducing apoptosis. (A1-5) HA-tag (red) and BrdU (green) overlap (yellow). (B1-5) BrdU (green) does not overlap with HA-tagged ATBF1 (red) expressions. The HA-tagged ATBF1 positive cells appear with neurite like projections. (C1-5) TUNEL staining (green) after transfection with an HA-tag (red) mock expression vector. (D1-5) TUNEL-positive cell (green) is small compared with HA-tagged ATBF1-positive cells that appear with larger cell bodies and neurite-like projections. (E1-4) FACS analysis after transfection with HA-tag or HA-tagged ATBF1 expression vector. The cells expressing HA-tag or HA-tagged ATBF1 were selected by gating the higher intensity of the green signal (gate: G1 indicated in E1 and E2). PI patterns of selected cells expressing HA-tag (E3) and HA-tagged ATBF1 (E4).

## Discussion

Ontogeny is a process by which immature cells differentiate into the specific cell phenotypes required to form the complicated structures of the body. Among various transcription factors, homeotic factors play important roles in embryonic morphogenesis. Most homeotic factors, which contain only one DNA-binding motif of 60 amino acids, are involved in orchestrating the development of a wide range of organisms. ATBF1 has four homeotic domains and 23 zinc-finger motifs in a single molecule that should control various target genes. Previously, the *Afp* (Morinaga et al., 1991; Yasuda et al., 1994) and aminopeptidase N (*APN*) (Kataoka et al., 2000) genes were identified as targets for ATBF1. The present study was designed based on the predicted roles for ATBF1 in neuronal differentiation both in vitro and in vivo.

ATBF1 was expressed in the postmitotic cells in association with  $\beta$ -tubulin III and MAP2, both markers of neuronal differentiation. ATBF1 expression was associated with the suppression of nestin during neuronal development. The nestin gene is regulated by interplay of SOX and POU factors in neural primordial cells (Tanaka et al., 2004). The suppressive effect of ATBF1 against the nestin-specific element may be due to competitive inhibition between POU factors and ATBF1, which also harbors a POU domain in its

structure (Morinaga et al., 1991). The function of ATBF1 in suppressing nestin and promoting neuronal differentiation appears to be opposite to that of the nuclear receptor co-repressor (N-CoR), which maintains the proliferation of nestin-positive neuronal stem cells (Hermanson et al., 2002). The translocation of N-CoR from the nucleus to the cytoplasm was observed as the 'on' switch of neuronal differentiation and the 'off' switch of nestin expression (Hermanson et al., 2002). Once ATBF1 localizes in the nucleus, it is likely to form complexes with nuclear receptors via its leucine-rich helix motif (2275, LSMLL), which may result in the dissociation of N-CoR (Perissi et al., 2004; Perissi et al., 1999). The function of ATBF1 in activating the *Neurod1* promoter in the nucleus is consistent with the function of Neurod1 as a key transcription factor that induces neuronal differentiation by activating *Trkb* and *p21<sup>Cip1/Kip1</sup>* promoters (Liu et al., 2004). ATBF1 might activate Neurod1 expression in cooperation with other co-factors known to regulate the promoter element of the *Neurod1* gene. The *Neurod1* gene promoter is regulated by complex formation with the bHLH transcription factor neurogenin (*Ngn1*), Smad1 and CBP/p300 (Sun et al., 2001). PIAS3 is a factor that forms co-activator complexes with Smads and p300/CBP for various target genes (Long et al., 2004). We have previously revealed a

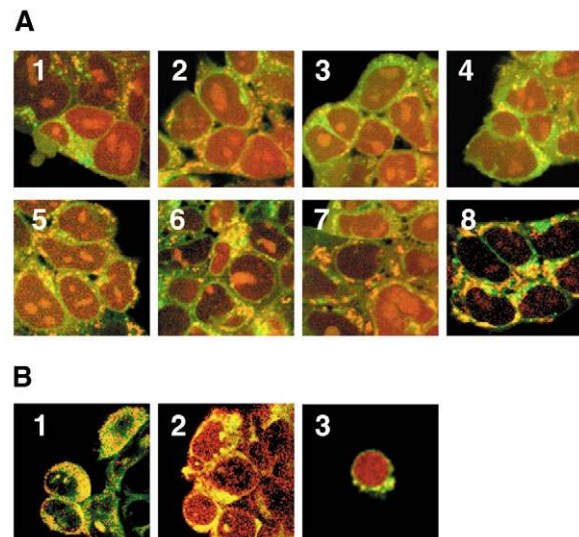


**Fig. 4.** PI patterns of P19 cells by FACS analysis corresponding to undifferentiated P19 cells (A), P19 cells after RA treatment for 24 hours in floating culture (B) and differentiated P19 cells on an adhesive dish after 7 days (C). Immunoreactivities of P19 cells for ATBF1 (red) and  $\beta$ -tubulin (green), overlaid with DAPI staining (blue). Undifferentiated P19 cells (A1). P19 cells after RA treatment for 24 hours in floating culture (B1). Differentiated P19 cells on an adhesive dish after 7 days (C1). ATBF1 (red) and  $\beta$ -tubulin (green) observed by confocal microscopy. P19 cells express ATBF1 in the cytoplasm after RA treatment for 24 hours in floating culture (B2-4). Differentiated P19 cells express ATBF1 in the nucleus on an adhesive dish after 7 days treatment with RA (C2-4).

strong interaction between ATBF1 and PIAS3 by co-immunoprecipitation experiments (Nojiri et al., 2004). Taken together, these studies suggest that ATBF1 in association with PIAS3, Smads and p300/CBP may play an important role in the activation of the *Neurod1* promoter.

Although overexpression of ATBF1 in Neuro 2A cells induced arrest of the cell cycle, elevation of ATBF1 in P19 cells during treatment with RA did not stop the progression of the cell cycle. This discrepancy may be due to the different subcellular localizations of ATBF1, namely, in the nucleus in Neuro 2A cells and in the cytoplasm in aggregated P19 cells. Thus, ATBF1 expression itself does not immediately induce cell cycle arrest. The expression of ATBF1 in the nucleus is required to arrest the cell cycle.

ATBF1 contains three leucine-rich domains, which are potential nuclear export signals (NESs) (Fornerod et al., 1997) (1267, LQLHLTHL; 2471, LPQLVSLPSL, 2504, LSHLPLKPL). Treatment of p19 cells with LMB resulted in nuclear localization of ATBF1, indicating that CRM1 (Nishi et al., 1994) may be involved in this process. The primary translocation of ATBF1 from the cytoplasm to the nucleus may be driven by the potential nuclear localization signals (NLSs)

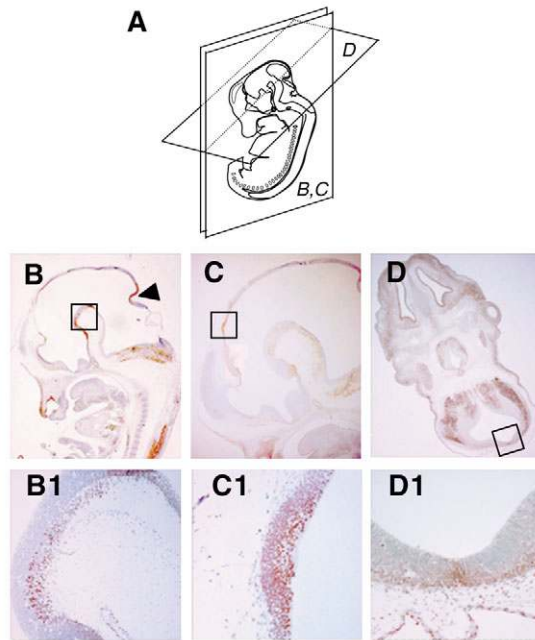


**Fig. 5.** Immunohistochemistry for ATBF1 (red, A,B) counterstained with the general membrane dye DiOC<sub>6</sub>(3) (green, A,B) in RA-treated P19 cells. (A) Subcellular localization of ATBF1 in the adhesive condition. Nuclear localization of ATBF1 on dishes coated with poly-L-onetime (A1), poly-L-ornithine+fibronectin (A2), poly-L-ornithine+laminin (A3), poly-L-lysine (A4) and gelatin (A5) at 3 hours after cell transfer to the dishes. Cells pre-treated with LY294002 (20  $\mu$ M, A6), ryanodine (100 nM, A7) and caffeine (5 mM, A8). (B) Subcellular localization of ATBF1 in floating conditions. ATBF1 is localized in the cytoplasm of cells in the aggregated and floating conditions (B1). Treatment with leptomycin B (10 nM, 1 hour, B2) induces ATBF1 accumulation in the nucleus in the floating condition. ATBF1 is localized in the nucleus of cells separated from embryonic bodies after treatment with EDTA (1 mM, 30 minutes) and pipette (B3).

(277, KRKPIILMCFLCK; 1387, KRPQLPVSDRHVYK; 2947, KRFRTQMTNLQLK; 2987, KRVVQVWFQNA-KEKSK) (Gorlich et al., 1994; Imamoto et al., 1995; Moroianu et al., 1995; Pollard et al., 1996) in ATBF1.

Among the ATBF1-expressing regions in the embryonic brain of rat, the isthmus displayed a distinct bending structure (Fig. 7C) that marked the border between the midbrain and the hindbrain, and was characterized by the origin of dopaminergic and serotonergic neurons. The higher density of cells on the ventricular side with cell-to-cell contacts contained ATBF1 in their cytoplasm and showed cell cycle progression (Fig. 7D1); by contrast, the lower density of cells on the dorsal side of the isthmal neuroepithelium had more intimate interactions with ECM molecules, such as fibronectin, and showed cell cycle suppression (Fig. 7D2). As a result of the different rates of cell growth in the adjacent layers, the tissue may form a bending structure. We observed ATBF1 expression in the nucleus on the concave faces of various curved (Fig. 6B1,C1) or narrowing structures in midline parts of the developing brain (Fig. 6D1). There are proliferating stem cells on the ventricular zone where cells exist at higher density with interaction of cell-to-cell contact before migration to other spacious regions of the brain.

To investigate whether cell-to-cell interaction or specific ECM stimulation is the primary determinant factor of the subcellular localization of ATBF1, we established an in vivo model to clarify this issue using P19 cells attached on flasks.

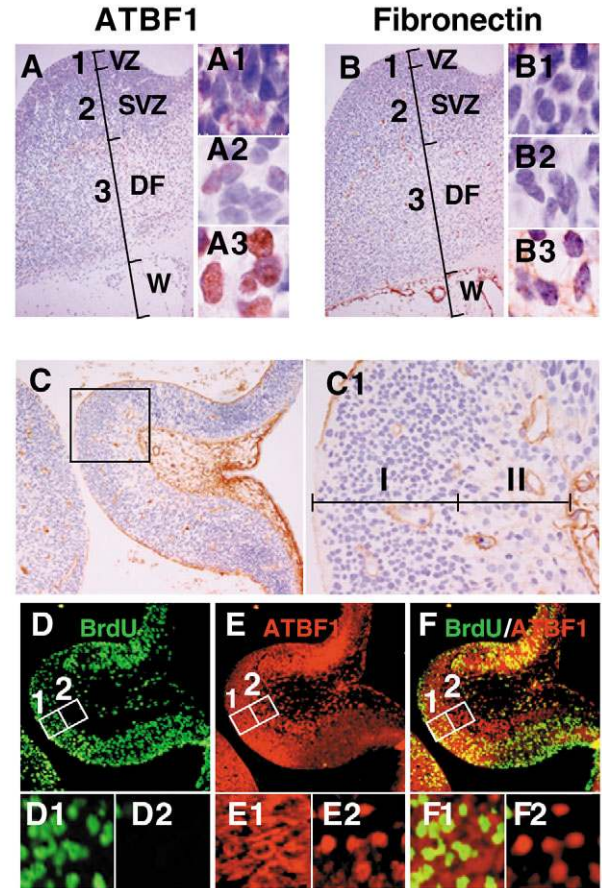


**Fig. 6.** ATBF1 expression in the midline region of the E14.5 rat brain. ATBF1 is expressed in the neuroepithelial cells at the curved regions and the narrowing part of the brain. (A) Plates showing sagittal (B,C) and horizontal (D) sections of the brain. (B1,C1,D1) Higher magnification of the boxed areas in B,C,D, respectively. ATBF1 is expressed at high levels in tegmentum (B1), thalamus (C1) and center of the isthmus (D1).

The higher density of cells in the DZ may be relevant to the microenvironment of the aggregated status of the P19 cell culture. The lower density of cells in the DF may correspond to the environment of the sparse P19 cells under adhesive conditions. A definite nuclear localization of ATBF1 was observed after attachment of P19 cells on coated glass slides, and there was no difference among coating materials in the pattern of ATBF1 expression (Fig. 5A1-A5). In addition, we observed distinct nuclear localization of ATBF1 in the isolated cells (Fig. 5B3). Therefore, the primary determinant factor for the cytosolic localization of ATBF1 would be direct cell-to-cell interaction.

We estimated the involvement of PI(3)K-related pathways for regulation of the subcellular localization of ATBF1, because various nuclear factors, including N-CoR linked to the cell cycle regulation as well as neuronal lineages determination are controlled by PI(3)K-related pathways (Hermanson et al., 2002). To investigate the involvement of PI(3)K pathways, we used LY294002 as a blocker of PI(3)K. A partial inhibition of the nuclear localization of ATBF1 was observed after treatment with LY294002. We considered different types of PI(3)K-related pathways that might play opposite roles in regulation of localization of ATBF1 namely an ataxia-telangiectasia mutated (ATM) pathway (Rotman and Shiloh, 1998; Sarkaria et al., 1999) that involves cytosolic signal in the nucleus, in contrast to an Akt/PKB pathway acting as a proliferating signal in the cytoplasm.

ATBF1 is specifically expressed until the postnatal stage in the nucleus of dopaminergic neurons (Ishii et al., 2003) that are selectively affected in ATM-deficient mice (Eilam et al.,



**Fig. 7.** ATBF1 expression at the GE and the isthmus in a sagittal section of E14.5 rat brain. (A) ATBF1 is expressed at the GE; in the cytoplasm of the VZ; weakly in the nucleus of the SVZ; strongly in the nucleus of the DF; absent from the white matter (W). (A1-A3) Higher magnification of the numbered areas in A. (B) Fibronectin is expressed on the surface of the ventricle, sparsely in the SVZ, in intercellular space of the DF and strongly in the white matter (W). (B1-B3) Higher magnification of the numbered areas in B. (C) Expression pattern of fibronectin at the isthmus. (C1) Higher magnification of the framed area of C. There are two regions: one has a high cell density (C1, region I), the other has a lower cell density (C1, region II). (A,B,C,C1) DAB and Hematoxylin counterstaining. BrdU (D,D1,D2), ATBF1 (E,E1,E2) and merged images of BrdU and ATBF1 (F,F1,F2). BrdU labeling indicates rapid proliferation on the ventricular side (D1) and suppression of the cell cycle on the dorsal side (D2). ATBF1 expression is present in the cytoplasm (E1) and the nucleus (E2).

1998). ATBF1 contains 28 potential Ser-Gln/Thr-Gln (SQ/TQ) cluster domains that may be a direct substrate for PI(3)K-related activity of ATM (Rotman and Shiloh, 1998). Absence or dysfunction of the ATM protein causes ataxia-telangiectasia (AT), a human disease that shows remarkable elevation of AFP expression (Chun and Gatti, 2004). The elevation of AFP may be explained by the dysfunction of ATBF1, as ATBF1 is the major suppressive factor for *Afp* gene transcription (Morinaga et al., 1991; Yasuda et al., 1994). These observations suggest that the expression of ATM, a member of the PI(3)K superfamily, is highly correlated with the function of ATBF1 as a gene regulatory factor in the nucleus.

Caffeine, which is known to be a blocker of the PI(3)K-related activity of ATM (Sarkaria et al., 1999), strongly inhibited ATBF1 translocation into the nucleus in P19 cells during neuronal differentiation (Fig. 6A8). This inhibition of the nuclear localization of ATBF1 should interfere with its function of stopping the cell cycle. These consequences are consistent with the observations of excessive proliferation of neuroepithelial cells and failure to induce neural tube closure after caffeine administration to mouse embryos (Marret et al., 1997).

Since caffeine has another effect through the activation of ryanodine receptors on the endoplasmic reticulum in the cytoplasm, we examined the possible involvement of this signaling pathway. In contrast to caffeine, ryanodine treatment weakly inhibited the translocation of ATBF1 from the cytoplasm to the nucleus. Thus, the results of the present study suggest partial involvement of ryanodine-induced  $Ca^{2+}$  signaling in regulating the subcellular localization of ATBF1, although the distinct effect of caffeine of keeping ATBF1 out of the nucleus is primarily associated with the PI(3)K activity of ATM in the nucleus. Thus, the signaling through the 28 SQ/TQ ATM target sites on ATBF1 that may regulate the nuclear localization of ATBF1 remains to be understood.

In this report, we have elucidated that the subcellular localization of ATBF1 has a remarkable functional meaning linking to cell proliferation versus cell cycle arrest. The alteration of signaling from the cell-to-cell to cell-to-matrix interactions triggered the nuclear localization of ATBF1. Overexpression studies supported the view that ATBF1 is one of the important factors in the nucleus that coordinates the cellular differentiation associated with the cell cycle arrest.

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