(One lambda) at Repro Cell[23]. Additional HLA typing was performed with PCR-rSSOP using WAKFlow (Wakunaga Pharmaceutical) at HLA Laboratory. We performed pedigree study of 4,743 Japanese families (17,325 members) and identified 2,117 haplotypes, including interlocus recombinant haplotypes, which were detected in family studies. The haplotype frequency was calculated by direct counting on the parents in the families. Sequence-based typing was performed with AlleleSEQR (Atria Genetics) at Mitsubishi Chemical Medience Corporation.

To estimate coverage of Japanese population by HLA homozygous donors, we first calculated the frequencies of all possible combinations of the 2,117 HLA haplotypes shown in **Supplementary Table 8**. Haplotype combinations that can be covered by a given homozygous donor were then identified and their frequencies were added to estimate coverage by the homozygous donor. When one *HLA-A, HLA-B, HLA-DRB1* heterozygous individual was covered by multiple homozygous donors, we counted only once to avoid overestimation.

The expected number (EN) of each homozygous haplotype at a given population size (PS) was first calculated as; EN = (haplotype frequency)^2 × PS. EN (if EN < 1) or 1 was then summed for each homozygous haplotype to estimate the expected numbers of unique HLA haplotype donors at the given PS.

Statistical analyses. Data are shown as the mean ± s.d. Statistical significance among multiple groups was evaluated with the Steel-Dwass test.

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Abstract	Recent studies have demonstrated that malignant tumors contain cancer-initiating cells (CICs, also known as cancer stem cells), which self-renew and are tumorigenic. Moreover, CICs are resistant to both irradiation and chemotherapy. These findings suggest that CICs are critical targets for successful cancer therapy. However, CICs have not been well characterized, due to a lack of specific markers for them. We recently established mouse glioma-initiating cell (GIC) lines, by overexpressing oncogenic $HRas^{L61}$ in p53-deficient neural cells. These cells form transplantable glioblastoma multiforme (GBM) with features of human GBM when as few as 10 cells are transplanted in vivo, suggesting that these GIC-like cells are enriched in CICs. Characterization of these GICs showed that they expressed little or no Sox11. The overexpression of exogenous Sox11 in GICs blocked their tumorigenesis by inducing their neuronal differentiation, which was accompanied by decreased levels of a novel oncogene, *plagl1*. These findings suggest that Sox11 and Plagl1 work as a tumor suppressor and oncogene, respectively, in GBM and are potential therapeutic targets.

Keywords (separated by '-')	Tumorigenesis - Glioma - Sox11 - Oncogene - Gliomagenesis - CIC

Chapter 11

Tumorigenesis of Glioma-Initiating Cells: Role of Sox11

Toru Kondo

Abstract Recent studies have demonstrated that malignant tumors contain cancer-initiating cells (CICs, also known as cancer stem cells), which self-renew and are tumorigenic. Moreover, CICs are resistant to both irradiation and chemotherapy. These findings suggest that CICs are critical targets for successful cancer therapy. However, CICs have not been well characterized, due to a lack of specific markers for them. We recently established mouse glioma-initiating cell (GIC) lines, by overexpressing oncogenic $HRas^{L61}$ in p53-deficient neural cells. These cells form transplantable glioblastoma multiforme (GBM) with features of human GBM when as few as 10 cells are transplanted in vivo, suggesting that these GIC-like cells are enriched in CICs. Characterization of these GICs showed that they expressed little or no Sox11. The overexpression of exogenous Sox11 in GICs blocked their tumorigenesis by inducing their neuronal differentiation, which was accompanied by decreased levels of a novel oncogene, *plagl1*. These findings suggest that Sox11 and Plagl1 work as a tumor suppressor and oncogene, respectively, in GBM and are potential therapeutic targets.

Keywords Tumorigenesis · Glioma · Sox11 · Oncogene · Gliomagenesis · CIC

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Introduction

During the last decade, tissue-specific stem cells were identified in almost all tissues. These cells self-renew and continuously generate the residential differentiated cells that are responsible for tissue functions and homeostasis (Weissman et al., 2001). Neural stem cells (NSCs) in the central nervous system (CNS), for example, self-renew and give rise to neurons, astrocytes, and oligodendrocytes throughout life (Gage, 2000).

The discovery of tissue-specific stem cells represented a big turning point in cancer research. The use of stem cell markers, for instance, revealed that malignant gliomas contain malignant cells that maintain the characteristics of tissue-specific stem cells (Kondo, 2006; Singh et al., 2004; Vescovi et al., 2006). Malignant gliomas also contain both proliferating cells that express NSC markers and cells that express either neuronal or glial markers, raising the possibility that these tumors contain NSC-like cancer cells. This idea is supported by the finding that malignant gliomas can arise from either NSCs or glial lineage cells (Bachoo et al., 2002; Dai et al., 2001; Uhrbom et al., 2002), such as oligodendrocyte precursor cells (OPCs) or astrocytes, which can behave like NSCs under certain conditions (Belachew et al., 2003; Doetsch et al., 1999; Kondo and Raff, 2000; Laywell et al., 2000; Nunes et al., 2003).

Additional evidence suggests that malignant tumors contain stem cell-like cancer-initiating cells (CICs, also known as cancer stem cells) (Globus and Kuhlenbeck, 1944; Hopewell and Wright, 1969; Copeland et al., 1975). Although many anti-cancer drugs are used to try to eliminate cancers, some cancer cells usually survive, and the cancer recurs, showing

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that the surviving cells are not only resistant to the anti-cancer drugs but also malignant. Various ATP binding cassette (ABC) transporters, such as the protein encoded by the multi-drug resistance gene (MDR), the multi-drug resistance protein (MRP), and the breast cancer resistance protein (BCRP1), contribute to the drug resistance in cancers (Gottesman et al., 2002; Wulf et al., 2001). Interestingly, some of these transporters are also expressed in many kinds of normal stem cells. BCRP1, for example, excludes the fluorescent dye Hoechst 33342, thereby identifying a side population (SP) that is enriched in stem cells (Goodell et al., 1996; Zhou et al., 2001). Together, these findings suggest that cancers might contain an SP rich in cells with the characteristics of CICs.

CIC-enriched populations can be obtained from cancers and cancer cell lines, by exploiting features common to tissue-specific stem cells, including cell-surface antigens such as CD133, their identification as side population (SP) cells, floating sphere formation assays, or a combination of these features (Kondo, 2006; Singh et al., 2004; Vescovi et al., 2006). However, CD133-negative cells and non-SP cells from tumors and cancer cell lines can also form tumors when transplanted in vivo (Mitsutake et al., 2007; Shmelkov et al., 2008). Therefore, it remains uncertain whether the existing isolation methods can identify bona fide CICs.

The overexpression of oncogenes can induce hematopoietic stem/precursor cells to transform into leukemic stem cell-like cells in culture, and the transplantation of small numbers of these cells can cause leukemias in vivo (Krivtsov and Armstrong, 2007). This observation suggests the existence of CIC-like cells in induced cancer models. Using a similar approach, we recently established an induced mouse glioma cell line, NSCL61 s. To do this, we transformed *p53*-deficient neural stem cells (NSCs) with oncogenic $HRas^{L61}$, because p53 is the most frequently mutated tumor-suppressor gene in human Glioblastoma multiforme (GBM), one of the most malignant brain tumors, and increased activation of the Ras signaling pathway is found in approximately 90% of GBM cases (Cancer Genome Atlas Research Network, 2008; Parsons et al., 2008). We analyzed NSCL61 s, human primary glioma sphere lines, and human glioma tissue samples using multiple approaches, and found that glioma-initiating cells (GICs) largely lost their *sox11* expression, but expressed *plagl1*, a transcriptional regulator of imprinting genes. In this review, I will give an overview of our recent results and discuss the future of GIC research.

Origin of Glioma Cells

The concept of CICs was first proposed several decades ago. For example, Globus and Kuhlenbeck suggested in 1944 that malignant brain tumors are generated from immature cells (NSCs or neural precursor cells) in the ventricular zone (VZ) (Globus and Kuhlenbeck, 1944); this hypothesis was subsequently proven through a number of experiments. For example, Hopewell and Wright (1969) discovered that brain tumors arose frequently from the VZ when carcinogenic pellets were randomly placed in the adult brain. Copeland and colleagues also showed that brain tumors were induced in the subventricular zone (SVZ) when mouse brains were infected with avian sarcoma viruses (Copeland et al., 1975). More recently, Doetsch et al. showed that astrocytes in the SVZ can behave like multipotent NSCs in the adult brain (Doetsch et al., 1999). Together with the knowledge that NSCs in the VZ/SVZ survive in adult animals and, unlike differentiated neural cells, proliferate throughout life, these findings suggest that NSCs in the VZ/SVZ have a higher probability of accumulating oncogenic mutations and transforming into CICs that retain the characteristics of NSCs and are malignant (Fig. 11.1). Consistent with this idea, malignant brain tumors, including GBM and medulloblastoma, are immunolabeled both for NSC markers, including Nestin, CD133, Bmi1, Sox2, Musashi1/2 and Olig2, and differentiation markers, including the neuronal marker MAP2, the astrocyte marker GFAP, and for the oligodendrocyte marker GC (Katsetos et al., 2001; Ligon et al., 2007; Toda et al., 2001).

OPCs may be the cells of origin for some gliomas. Although OPCs are committed to differentiating into oligodendrocytes in vivo, they can also differentiate into GFAP-positive astrocytes and acquire NSC characteristics, including the expression of NSC markers and multipotentiality, when cultured under specific conditions (Belachew et al., 2003; Kondo and Raff, 2000; Laywell et al., 2000; Nunes et al., 2003) (Fig. 11.1). Moreover, OPCs transformed with oncogenic HRas and c-Myc can form malignant glioma in vivo (Barnett et al., 1998).

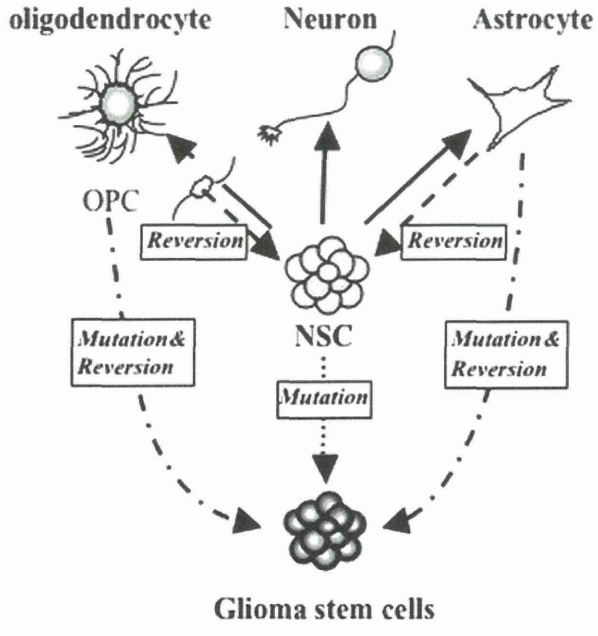

Fig. 11.1 Cell of origin for gliomagenesis. Many lines of evidence suggest that astrocytes and oligodendrocyte precursor cells, both of which can acquire multipotentiality, and NSCs are cells of origin for malignant glioma

By combining transgenic mouse technology and a retrovirus system, two groups have demonstrated that Nestin-positive NSCs and GFAP-positive astrocytes form malignant gliomas in vivo: Holland and his colleagues infected transgenic mice that expressed the avian leukosis virus (ALV) receptor under the regulation of either a *nestin* enhancer or a *gfap* promoter, with recombinant ALVs encoding oncogenic genes, such as platelet derived growth factor (PDGF) receptor beta, activated Akt, or activated Ras, and found that GBM developed in the brain (Dai et al., 2001; Uhrbom et al., 2002). De Pinho and colleagues overexpressed a constitutively active epidermal growth factor (EGF) receptor in NSCs or astrocytes with the loss of both p16/Ink4a and p19/ARF, transplanted them into the brain, and found that the cells formed high-grade gliomas (Bachoo et al., 2002). These findings suggested that NSCs and astrocytes are cells of origin for brain tumors.

GICs Induced in Mouse

It remains controversial whether GICs arise from NSCs, committed precursor cells, or differentiated neural cells. In addition, the relationship between the cell of origin for brain CICs and genetic alterations within these cells has not been elucidated, although a number of oncogenes and tumor suppressor genes are clearly associated with gliomagenesis. Now, however, it is possible to use neural lineage markers and flow cytometry to isolate neural lineage cells and examine which ones transform into CICs when overexpressing oncogenes and/or when tumor suppressor gene expression is reduced or absent. By using this strategy, we successfully generated two GIC lines, NSCL61 and OPCL61, from p53-deficient NSCs and OPCs respectively, but not from astrocytes, by overexpressing oncogenic $HRas^{L61}$ (Fig. 11.2). Both GIC lines self-renew, express NSC markers including Nestin and Sox2, and form transplantable GBM that shows hypercellularity, pleomorphism, multinuclear giant cells, mitosis, and necrosis within 2 months, even when as few as ten cells are transplanted in vivo (Hide et al., unpublished data).

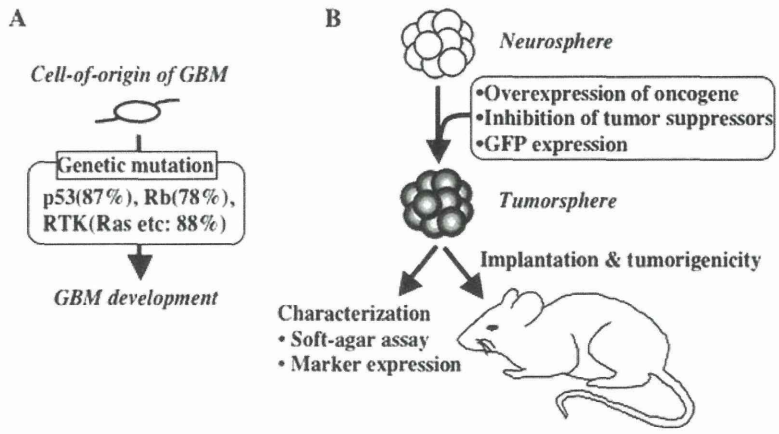

Fig. 11.2 Induced GIC model in mouse. (**a**) Glioma cells-of-origin transform into GICs by undergoing mutations for key factors involved in the p53, Rb, and/or receptor tyrosine kinase (RTK) pathways. (**b**) Induced GICs can be characterized in culture and in vivo

Sox11: A Novel Tumor Suppressor for Gliomagenesis

Given that our induced GIC lines contain both tumorigenic (GIC-like) and non-tumorigenic (non-GIC) cells, it should be possible to isolate the GIC-like cells, characterize them, and use them to identify novel therapeutic targets for GBM, by comparing their gene expression profiles with those of non-GICs. We were able to isolate both GIC and non-GIC clones from NSCL61 s by limiting dilution assays (Hide et al., 2009). We found that GIC clones proliferate much more slowly than non-GIC clones, although the mechanism is unknown. We also found that the GIC clones could be immunolabeled for NSC and glial cell markers but not for neuronal markers, whereas the non-GIC clones were largely positive for neuronal markers and lost the expression of NSC and glial markers. Moreover, a DNA microarray analysis revealed that the expression of a number of genes increased or decreased in the GIC clones compared with the non-GIC clones.

From among these genes, we chose to focus on the Sox11 transcription factor for the following reasons. First, Sox11 is not expressed in primary human glioma spheres, which are rich in GICs. Second, Sox11 induces the expression of the neuronal markers MAP2 and βIII tubulin in proliferating neural precursors cells (Bergsland et al., 2006). Third, the overexpression of a dominant-negative form of Sox11 blocks neuronal differentiation (Bergsland et al., 2006). Fourth, the National Cancer Institute's Repository for Molecular Brain Neoplasia Data (REMBRANDT) database (Madhavan et al., 2009) revealed that a downregulation of *sox11* mRNA is correlated with a significant decrease in the survival of glioma patients. Fifth, we found that human primary GBMs express *sox11* mRNA and Sox11 protein, whereas the recurrent GBMs lose Sox11. Together, these findings suggest that Sox11 may inhibit malignancy in GICs by inducing their neuronal differentiation (Fig. 11.3). In support of this idea, we found that the overexpression of Sox11 inhibits the tumorigenesis of GICs by inducing their neuronal differentiation and increases the susceptibility of GICs to chemotherapy. In contrast, the knockdown of *sox11* induces non-GIC clones to become tumorigenic (Hide et al., 2009).

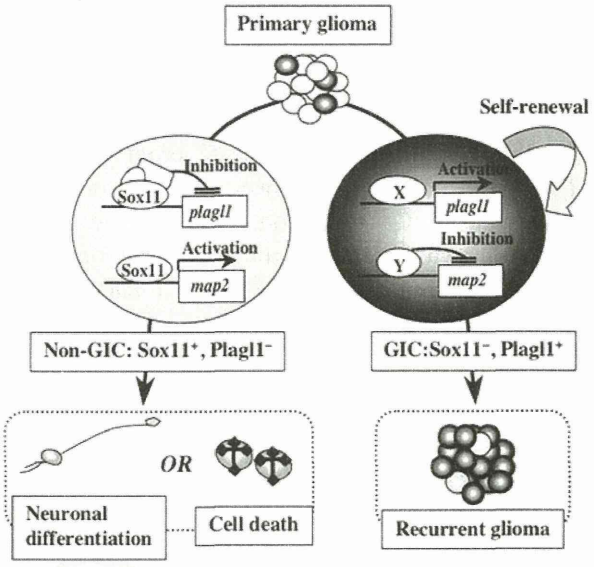

Fig. 11.3 Model of tumorigenesis by Sox11$^-$/Plagl1$^+$ GICs. Primary GBM contains both GICs, which are Sox11$^+$ and Plagl1$^-$, and non-GICs, which are Sox11$^-$ and Plagl1$^+$. Non-GICs probably differentiate into neurons and are sensitive to chemotherapy. Recurrent GBMs largely consist of GICs that are resistant to differentiation inducers and chemotherapy

Plagl1: A Novel Oncogene for Gliomagenesis

How does Sox11 inhibit the tumorigenesis of GICs? To identify targets of Sox11 in GICs, we analyzed the gene expression differences between non-Sox11-expressing and exogenous Sox11-expressing GICs using DNA microarray analysis and selected several genes whose expression was significantly affected by *sox11* overexpression. We focused on Pleiomorphic adenoma gene-like 1 (Plagl1) for the following reasons. First, *plagl1* is expressed in the neural stem/progenitor cells of developing neuroepithelial cells and decreases upon differentiation (Valente et al., 2005). Second, we found that *plagl1* is expressed in malignant human gliomas as well as in human GICs, although it was thought to be a tumor suppressor candidate (Van Dyck et al., 2007). Third, Plagl1 regulates several imprinted genes, including Insulin-like growth factor 2 (Igf2), H19, and Delta-like 1 (Dlk1), all of which are involved in tumorigenesis as well as early development (Varrault et al., 2006). Fourth, the REMBRANDT database revealed that glioma patients with downregulated *plagl1* mRNA show increased

survival rates compared to patients with intermediate levels of *plagl1* expression. Together, these data suggest that Plagl1 plays an important role in GICs.

We found that Sox11 can block the *plagl1* expression in GICs by binding to the 5' promoter region of the gene. Moreover, the overexpression of Plagl1 induces non-GICs to become tumorigenic, while its knockdown blocks the tumorigenicity of GICs. Collectively, these results suggest that Plagl1 plays an important role in the tumorigenicity of GICs (Fig. 11.3).

Discussion

Although tumors are the primary sources of CICs, induced CICs are a useful alternative source for their characterization, because CICs cannot presently be purified from tumors. In addition, induced CIC models can be used to identify experimentally the cell-of-origin of the cancer, the responsible oncogenic mutation, and the relationship between them. Indeed, we demonstrated that the overexpression of $HRas^{L61}$ induced p53-deficient NSCs to transform into GICs (Hide et al., 2009), consistent with recent findings that mutations in the p53 and RTK pathways as well as the retinoblastoma (Rb) pathway are critical for human GBM development (Cancer Genome Atlas Research Network, 2008; Parsons et al., 2008). Moreover, we successfully separated both tumorigenic and non-tumorigenic clones from induced GIC lines using limiting dilution methods and identified many genes, including Sox11 and Plagl1, that are up- or down-regulated in tumorigenic clones (Hide et al., 2009).

Sox11 was originally thought to be involved in tumor induction, because it is expressed in many malignant human gliomas. However, we showed that human primary glioma sphere cells and recurrent glioma as well as mouse tumorigenic clones are largely negative for Sox11, whereas primary gliomas express Sox11. On the other hand, Plagl1 was thought to be a tumor suppressor regulating both cell-cycle arrest and apoptosis. We found, however, that plagl1 is predominantly expressed in tumorigenic clones and human glioma sphere cells, that the overexpression of *plagl1* transforms non-GIC-like cells into GICs, and that its knock-down inhibits the tumorigenicity of NSCL61 s. Thus, it is important to establish a number of induced GIC models and examine their characteristics to identify therapeutic targets and develop related treatment methods.

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Research Report

Neural development of methyl-CpG-binding protein 2 null embryonic stem cells: A system for studying Rett syndrome

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Adenoviral vector

ABSTRACT

Mutations in methyl-CpG-binding protein 2 (MeCP2) gene cause the neurodevelopmental disorder Rett syndrome (RTT). Here, we describe a new experimental system that efficiently elucidates the role of MeCP2 in neural development. MeCP2-null and control ES cells were generated by adenoviral conditional targeting and examined for maintenance of the undifferentiated ES cell state, neurogenesis, and gliogenesis during *in vitro* differentiation. In addition, dopamine release and electrophysiological features of neurons differentiated from these ES cells were examined. Loss of MeCP2 did not affect undifferentiated ES cell colony morphology and growth, or the timing or efficiency of neural stem cell differentiation into Nestin-, TuJ- or TH-positive neurons. In contrast, gliogenesis was drastically accelerated by MeCP2 deficiency. Dopamine production and release in response to a depolarizing stimulus in MeCP2-null ES-derived dopaminergic neurons was intact. However, MeCP2-null differentiated neurons showed significantly smaller voltage-dependent Na^+ currents and A-type K^+ currents, suggesting incomplete maturation. Thus, MeCP2 is not essential for maintenance of the undifferentiated ES cell state, neurogenesis, or dopaminergic function; rather, it is principally involved in inhibiting gliogenesis. Altered neuronal maturity may indirectly result from abnormal glial development and may underlie the pathogenesis of RTT. These data contribute to a better understanding of the developmental roles of MeCP2 and the pathogenesis of RTT.

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Abbreviations: MeCP2, methyl-CpG-binding protein 2; RTT, Rett syndrome; BDNF, brain-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; ES cells, embryonic stem cells; DNs, dopaminergic neurons; TH, tyrosine hydroxylase; HPLC, high performance liquid chromatography; ACT, adenoviral conditional targeting; NSCs, neural stem cells; TTX, tetrodotoxin; TEA-Cl, tetraethylammonium-chloride; 4-AP, 4-aminopyridine; HBSS, Hanks' balanced salt solution

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1. Introduction

Rett syndrome (RTT) is a neurodevelopmental disorder that is the leading cause of mental retardation in females (Chahrour and Zoghbi, 2007). Up to 95% of classical RTT cases are caused by mutations in the methyl-CpG-binding protein 2 (MeCP2) gene, which is located at Xq28 (Amir et al., 1999). Knockout mouse models with disrupted MeCP2 function mimic many key clinical features of RTT, including normal early postnatal life followed by developmental regression resulting in motor impairment, irregular breathing and hindlimb clasping (Chen et al., 2001; Guy et al., 2001).

Although MeCP2 was previously thought to function solely as a transcriptional repressor, it was recently shown to act as both a repressor and an activator to regulate the expression of a wide range of genes (Chahrour et al., 2008). This suggests that using only genetic strategies to investigate MeCP2-related biology may be insufficient. Indeed, comparing the transcriptional profiles of whole MeCP2-deficient and wild-type mouse brains revealed subtle and non-specific transcriptional differences. In addition, although several MeCP2 target genes, including brain-derived neurotrophic factor (BDNF) and glial fibrillary acidic protein (GFAP), have been identified, the functional relevance of these genes remains largely unknown (Bienvenu and Chelly, 2006).

One of the most important but unanswered questions is the role of MeCP2 throughout various stages of neural development, particularly at early stages, including the period just prior to and at the onset of neural differentiation. However, the lack of a definitive experimental system has hampered efforts in this direction. Although anatomical studies have demonstrated that the timing of MeCP2 expression in brains is correlated with the maturation of the central nervous system, the fine spatiotemporal pattern of MeCP2 expression during early embryogenesis is largely unknown (Shahbazian et al., 2002). On the other hand, several recent studies that established and/or utilized effective cell culture systems provided valuable information on the roles of MeCP2 in the pathogenesis of RTT (Bienvenu and Chelly, 2006; Chahrour and Zoghbi, 2007). Studies on neural stem cells (NSCs) indicate that MeCP2 may play a role in neuronal maturation (Kishi and Macklis, 2004; Smrt et al., 2007). In another study, MeCP2 was shown to be involved in cell fate determination during neurogenesis (Setoguchi et al., 2006; Tsujimura et al., 2009). Moreover, two studies using an *in vitro* co-culture system have recently reported that MeCP2-deficient astroglia non-cell autonomously affected neuronal dendritic growth (Ballas et al., 2009; Maezawa et al., 2009). Whereas these studies provided important information, the role of MeCP2 throughout neural development is unknown, and in particular, the developmental abnormalities that eventually result in the onset of neurological symptoms in RTT have not yet been elucidated by conventional strategies.

RTT has been reported to be associated with abnormalities in the biogenic amine neurotransmitter/receptor systems, but these findings are controversial due to limitations of the experimental strategies used (Jellinger, 2003; Temudo et al., 2009). Some studies have demonstrated decreases in dopamine levels in the spinal fluid of human RTT patients and/or MeCP2-null animals, however, others have failed to find such changes (Ide et al., 2005; Perry et al., 1988; Samaco et al., 2009; Zoghbi et al., 1989, 1985). Pluripotent embryonic stem (ES) cells show great promise for uncovering the molecular mechanisms of development in various tissues by *in vitro* cell culture. The utility of the ES cell system for addressing a particular problem is largely determined by how efficiently the target cell type can be induced. A recent study described a co-culture system that efficiently induced mouse ES cells to differentiate into dopaminergic neurons (DNs) (Kawasaki et al., 2000). Taken together with both the present status of the controversy concerning dopaminergic abnormalities in RTT and the established experimental system that efficiently induces ES cells towards DNs, DNs may be a good candidate as a target cell type in initial studies that utilize pluripotent ES cells for analyzing functional abnormalities of MeCP2-null neurons.

Here, we develop an MeCP2-null ES cell system for analyzing developmental processes at the cellular level. We examine the role of MeCP2 in maintenance of the undifferentiated ES cell state, neuronal and glial differentiation, DN function, and neuronal maturation. We also show that MeCP2 is involved in the maturation of neurons and gliogenesis.

2. Results

2.1. *Generation of MeCP2-null ES cells by ACT*

We generated MeCP2-null and control ES cells by ACT as follows: parental ES cells (Me2loxIII6A), in which exons 3 and 4 of the Mecp2 gene on the X chromosome are flanked, were infected by Ad.Cre or control Ad.dE1.3, at a multiplicity of infection of 30 (Takahashi et al., 2006). Subsequently, the infected cells were detached and plated onto a 96-well plates at a very low concentration corresponding to less than one cell per well. Several colonies originating from isolated single cell clones were cultured and expanded in larger dishes. To generate control ES cell lines, the parental ES cells were infected with Ad.dE1.3, instead of Ad.Cre and were handled and expanded in the same way as mutant cells.

The original ES cells were derived from male E14 TG2a cell lines carrying both an X and Y chromosome (Guy et al., 2001). Genomic PCR with primer set 1 (oIMR1436 and oIMR1437) should produce a 400 bp band from hemizygous cells ($Mecp2^{-/y}$) when excision of Mecp2 exon 3 and part of exon 4 occurs, leading to the shortened and amplifiable size of this region (Fig. 1A). Non-excised DNA from parental or control ES cells is too long to be amplified by PCR using primer set 1. Primer set 2 (oIMR 1436 and oIMR 1438) can anneal and amplify only to unexcised DNA in parental or control wild-type ES cells ($Mecp2^{+/y}$) with a predicted band size of 416 bp, but cannot amplify the excised DNA due to the lack of the primer S1438 annealing site (Fig. 1A). The two selected Ad.Cre-infected clones were verified to be hemizygotes ($Mecp2^{-/y}$), in accordance with the results for Mecp2 hemizygous mice (Fig. 1B). In contrast, control ES clones infected by Ad.dE1.3 showed the wild-type band ($Mecp2^{+/y}$), in accordance with the results for wild-type mice (Fig. 1B).

We further confirmed the lack of MeCP2 mRNA expression by RT-PCR of these ES cell clones. Mecp2 has two splice isoforms, Mecp2 e1 and e2 (Kriaucionis and Bird, 2004; Mnatzakanian et al., 2004). The Ad.dE1.3-infected control ES