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Generation of Cloned Mice from Adult Neurons by Direct Nuclear Transfer[1]

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ABSTRACT

Whereas cloning mammals by direct somatic cell nuclear transfer has been successful using a wide range of donor cell types, neurons from adult brain remain "unclonable" for unknown reasons. Here, using a combination of two epigenetic approaches, we examined whether neurons from adult mice could be cloned. First, we used a specific antibody to discover cell types with reduced amounts of a repressive histone mark—dimethylated histone H3 lysine 9 (H3K9me2)—and identified CA1 pyramidal cells in the hippocampus and Purkinje cells in the cerebellum as candidates. Second, reconstructed embryos were treated with trichostatin A (TSA), a potent histone deacetylase inhibitor. Using CA1 cells, cloned offspring were obtained at high rates, reaching 10.2% and 4.6% (of embryos transferred) for male and female donors, respectively. Cerebellar Purkinje cell nuclei were too large to maintain their genetic integrity during nuclear transfer, leading to developmental arrest of embryos. However, gene expression analysis using cloned blastocysts corroborated a high rate of genomic reprogrammability of CA1 pyramidal and Purkinje cells. Neurons from the hippocampal dentate gyrus and cerebral cortex, which had higher amounts of H3K9me2, could also be used for producing cloned offspring, but the efficiencies were low. A more thorough analysis revealed that TSA treatment was essential for cloning adult neuronal cells. This study demonstrates, to our knowledge for the first time, that adult neurons can be cloned by nuclear transfer. Furthermore, our data imply that reduced amounts of H3K9me2 and increased histone acetylation appear to act synergistically to improve the development of cloned embryos

assisted reproductive technology, CA1 pyramidal cells, cloning, developmental biology, early development, hippocampus, mouse, neural cells, nuclear transfer, reprogramming

INTRODUCTION

Studies on animal cloning started in frogs [1, 2]. Then, a sheep was cloned by transplanting nuclei from adult mammary cells [3, 4]. Soon after this breakthrough, cloning was achieved by somatic cell nuclear transfer (SCNT) in mice [5] and in a bovine model [6], and it has now been achieved in many mammalian species, including the goat, pig, rabbit, cat, mule, horse, rat, and dog [7–9]. However, the success rates of animal cloning by SCNT are consistently low [8–10]. In the mouse, many types of differentiated somatic cells can be used as nuclear donors, such as cumulus cells [5], tail tip fibroblasts [11, 12], Sertoli cells [12], natural killer T cells [13], and granulocytes [14, 15]. As for undifferentiated or lineage-specific stem cells, embryonic stem (ES) cells [16], fetal neuronal cells [17, 18], hematopoietic stem cells [19], neuronal stem cells [19, 20], and keratinocyte stem cells [21] have been used successfully. However, some types of cells have proved difficult to use for producing cloned mice by direct SCNT. For example, although the production of clonal and cloned mice from olfactory sensory neurons and cerebral cortical neurons has been reported via the nuclear transfer ES (ntES) cell technique [22], live cloned mice produced by direct SCNT using adult brain neural cells have not been reported.

To clarify the causes of the low success rate of cloning by SCNT, studies have analyzed SCNT-derived embryos and found that many abnormalities seemed to have been caused by incomplete epigenetic reprogramming of the donor nucleus and were significant reasons for developmental failure. Thus, abnormal DNA methylation and histone modifications have been found in SCNT-derived embryos [8, 23–26]. Moreover, such embryos showed altered gene expression patterns resulting from epigenetic errors [27]. Boiani et al. [28] and Kishigami et al. [29] demonstrated abnormal Oct3/4 protein expression patterns in the inner cell mass (ICM) of SCNT-derived blastocysts.

The application of trichostatin A (TSA) and other histone deacetylase inhibitors has now increased the success rate of mouse cloning from differentiated somatic cells by 5-fold, probably by enhancing the accessibility of putative ooplasmic reprogramming factors [30–33]. We also found that the mean X:autosome (X:A) chromosome expression ratio in SCNT-derived embryos was consistently lower than in normally fertilized embryos irrespective of the donor cell type used [34]. At least one of the reasons for this X-linked gene repression appears to be upregulation of *Xist*, as this is a noncoding RNA gene responsible for the onset of X chromosome inactivation [35]. Indeed, after correction of the *Xist* expression pattern either by genetic knockout or via short interfering RNA-mediated knockdown, the expression patterns of X-linked

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genes as well as those of autosomal genes were largely normalized. Furthermore, the efficiency of cloning was increased by 9- to 10-fold, although the protocol for *Xist* knockdown in female embryos needs further refinement [34, 36, 37]. However, even after TSA treatment or repression of the ectopically expressed *Xist* gene, SCNT-derived embryos still have specific abnormal gene expressions. These include the tight repression of two discrete gene families, *Magea* and *Xlr*, located on large organized chromatin K9 modifications (LOCKs) of the X chromosome [34, 38].

The repressive histone mark, dimethylated histone H3 lysine 9 (H3K9me2), is observed not only in differentiated ES cells but also in undifferentiated ES cells [39]. It seems that ES cells accumulate somatic cell-type epigenetic characters, because the mice cloned from ES cells had typical cloned-type large placentas, which are about two to three times as large as those cloned from ICM cells. Moreover, RNA sequence profiling has revealed many differences in transcriptional programs between ES cells and ICM-derived outgrowths [40, 41]. Thus, these H3K9me2 LOCKs might be specific epigenetic marks of somatic cells that are strongly associated with tissue-specific gene silencing.

In the SCNT technique, the clone's genome is derived from a differentiated donor somatic cell nucleus. During reprogramming in the recipient ooplasm, the LOCKs of donor cells—at least in the *Magea* and *Xlr* clusters—were resistant to reprogramming by putative ooplasmic factor(s) and were consequently transmitted to the cloned embryos [34]. Thus, somatic-type LOCKs might affect the epigenetic status and gene expression patterns of SCNT-derived embryos and prevent proper genome reprogramming and term development. Indeed, microarray analysis of single SCNT-derived embryos revealed that they had a unique gene expression profile that was strictly dependent upon the type of donor cells [34, 38, 42]. In other words, it is expected that SCNT-derived embryos from differentiated somatic cells with low levels of H3K9me2 might have few LOCKs and enhance the efficiency of mouse cloning.

In the mouse, brain tissues show a lower LOCK ratio than other tissues [43], and it has also been reported that the mean X:A expression ratio in adult brain tissues is higher than in other tissues [44]. In addition, cloned mice derived from frozen cadavers could be generated only from brain cells using the ntES cell technique [45]. Based on the results of these reports, we hypothesized that the adult mouse brain might have some cells with low levels of H3K9me2 marks. If we could select such neural cells, their genome might be efficiently reprogrammed by SCNT, and we could expect the birth of cloned mice derived from differentiated adult neural cells. In the present study, we surveyed the adult mouse brain for neural cells having a low level of H3K9me2 marks and tried to generate cloned mice by direct nuclear transfer from such cells, with or without the application of TSA.

MATERIALS AND METHODS

Mice

Six- to 10-wk-old (C57BL/6 × DBA/2) F1 (BDF1) and 8- to 12-wk-old ICR female mice were used for the collection of recipient oocytes and as embryo transfer recipients, respectively. Adult female and male BDF1 mice (age, >6 wk) were used as nuclear donors. The animals were provided with unlimited water and commercial laboratory mouse chow and housed under controlled lighting conditions (lights-on, 0700–2100 h). Animals were maintained under specific-pathogen-free conditions. All animal experiments described here were approved by the Animal Experimentation Committee at the RIKEN Tsukuba Institute and were performed in accordance with the committee's guiding principles.

Histology

Brains from adult male and female mice were fixed overnight in 4% paraformaldehyde in PBS and embedded in paraffin wax. Embedded samples were sectioned (thickness, 4 μm) and subjected to hematoxylin-and-eosin staining and immunohistochemistry. For immunohistochemistry (see below), rehydrated sections were pretreated in a microwave for 10 min in the presence of 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval. Sections were then blocked for 1 h at room temperature in 0.5% blocking reagent (PerkinElmer Japan). Primary antibody incubations were carried out overnight at 4°C in blocking solution using a mouse anti-H3K9me2 antibody (1:500). The immunoreactions were visualized using biotin-labeled secondary antibodies and a Vectastain ELITE ABC kit (Vector Laboratories).

Donor Cell Preparation

Adult mice were euthanized by cervical dislocation. Their brains were exposed using fine scissors and forceps, and then the hippocampi were removed using fine forceps. Collected hippocampi were transferred into plastic dishes filled with calcium- and magnesium-free PBS (PBS(–)) and sliced into round portions. Then, small pieces of the CA1 or dentate gyrus regions were cut out from the cross-sectioned hippocampus and collected separately into 1.5-ml tubes. Next, 0.25% trypsin and 0.75 mM ethylenediaminetetra-acetic acid were added in each tube and incubated for 5 min at 37.5°C. After the addition of a half-volume of Dulbecco modified Eagle medium (DMEM; Sigma-Aldrich) containing 10% fetal calf serum, cell suspensions were obtained by gentle pipetting. The cell suspensions were washed twice with DMEM, then resuspended in DMEM with 0.2% collagenase IV (Sigma-Aldrich) and 0.01 mg/ml of deoxyribonuclease (Sigma-Aldrich) at 37°C for 20 min. Next, cell suspensions were washed three times with DMEM and kept at 4°C until injection. For the collection of cerebral cortex region cells, the cerebral cortex of the brain was dissected and minced with fine scissors in PBS(–), after which cells were collected in a manner similar to the hippocampal cells.

Purkinje cells were chosen as another candidate donor cell line with a low level of H3K9me2 marks. For the collection of such cells, the cerebellum was picked up using fine scissors and forceps and transferred into PBS(–). The cerebellum was then cut into small fragments using a scalpel. To collect Purkinje cells, we used Nerve-Cell Dissociation Solution (Sumitomo Bakelite Co., Ltd.). The pieces of cerebellum were transferred into a 15-ml tube with enzyme solution and incubated at 37°C for 15–20 min. After incubation, the tissue pieces were dissociated by gently pipetting and centrifuged at $300 \times g$ for 4 min. After discarding the supernatant, cells were resuspended in dispersion solution. Then, isolation solution was added to the bottom of the tube and centrifuged at $300 \times g$ for 5 min. After discarding the supernatant, cells were resuspended in DMEM and kept at 4°C until injection.

We also used cumulus cells and immature Sertoli cells to construct cloned embryos for embryo transfer or microarray analyses. Cumulus cells were obtained at the same time as oocytes were collected. Immature Sertoli cells were collected from 3- to 7-day-old BDF1 male neonatal mice as described previously [12]. Briefly, collected testicular cells were treated with 0.1 mg/ml of collagenase (Sigma-Aldrich) and 0.01 mg/ml of deoxyribonuclease (Sigma-Aldrich) for 30 min at 37°C, followed by 0.2 mg/ml of trypsin (Sigma-Aldrich) for 5 min at 37°C. The testicular cell suspension was washed with PBS(–) containing 4 mg/ml of bovine serum albumin and used for microinjection.

Oocyte Collection

Cumulus-oocyte complexes were collected from the oviducts of 8- to 12-wk-old BDF1 females that had been induced to superovulate by sequential injections of equine chorionic gonadotropin (Sankyo-Yell Yakuhin Co.) and human chorionic gonadotropin (Aska Pharmaceutical) and placed in potassium simplex optimized medium (KSOM) [46] containing 0.1% bovine testicular hyaluronidase. The dispersed cumulus cells were collected and used as nuclear donors. The denuded oocytes were placed in fresh KSOM and cultured until use.

Nuclear Transfer

Nuclear transfer was carried out as described previously [5]. Oocytes were transferred to a droplet of Hepes-buffered KSOM medium containing 5 μg/ml of cytochalasin B (CB) in a micromanipulation chamber, and the zona pellucida was perforated by applying several piezo pulses with the tip of an enucleation pipette. The metaphase II chromosome spindle complex was removed by drawing it into the pipette with a small amount of accompanying ooplasm. Before nuclear transfer, the donor nucleus was collected by being drawn in and out of an injection pipette until the cell's plasma membrane was disrupted. The

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TABLE 1. Development of cloned embryos from brain cells of adult male mice.

Type of donor cell	No. of embryos cultured	No. of 2-cell embryos (%)	No. of 4-cell embryos (%)*	No. of morulae/blastocysts (%)*	No. of embryos transferred (No. recipients)	No. of cloned offspring (%)†
CA1	194	150 (77.3)[ab]	121 (62.4)[a]	113 (58.2)[a]	98 (7)	10 (10.2)[a]
Purkinje	404	177 (43.8)[c]	134 (33.2)[b]	91 (22.5)[b]	91 (12)	0[b]
Dentate gyrus	138	99 (71.7)[a]	86 (62.3)[a]	80 (58.0)[a]	80 (5)	3 (3.8)[ab]
Cerebral cortex	124	107 (86.2)[b]	90 (72.5)[a]	81 (65.3)[a]	81 (5)	2 (2.4)[ab]
Sertoli‡	84	68 (81)[ab]	N.D.	N.D.	68	4 (6.0)[a]

* N.D. = not done.
† Percentage of embryos transferred.
‡ Data from a previous study [36]. Embryos were transferred at the 4-cell stage.
[a,b,c] Data with different superscripts are significantly different ($P < 0.05$, Fisher exact test).

zona pellucida and the membrane of the oocyte were perforated with a few piezo pulses, and then the donor nucleus was injected into the ooplasm. The reconstructed oocytes were transferred into a droplet of KSOM and incubated at 37.5°C under 5% CO_2 in humidified air for 1–3 h. The oocytes were activated by incubation for 1 h in Ca^{2+}-free KSOM containing 2.5 mM Sr^{2+}, 5 μg/ml of CB, and 50 nM TSA [30]. After activation, they were cultured in KSOM containing 5 μg/ml of CB and 50 nM TSA for 5 h. Then, the SCNT-derived embryos were transferred to KSOM containing 50 nM TSA and cultured for an additional 2 h. After TSA treatment, SCNT-derived embryos were transferred to KSOM and incubated at 37°C under 5% CO_2 in humidified air. Some of the SCNT-derived embryos were activated without TSA. In such cases, reconstructed oocytes were activated by incubation for 1 h in Ca^{2+}-free KSOM containing 2.5 mM Sr^{2+} and 5 μg/ml of CB. Then, they were cultured in KSOM containing 5 μg/ml of CB for 5 h, transferred to KSOM, and incubated at 37°C under 5% CO_2 in humidified air.

Embryo Transfer

After 72 h in culture, the SCNT-derived embryos from CA1, dentate gyrus, cerebral cortex cells, and cumulus cells that had developed to the morula/blastocyst stage were transferred into the uteri of pseudopregnant ICR female mice, which had been mated with a vasectomized ICR male mouse, at 2.5 days postcoitum (dpc). In the present study, Sertoli cell-derived embryos were used only for gene expression analysis. Therefore, the data on embryo transfer experiments using Sertoli cell SCNT are from our previous paper [36], in which 4-cell embryos were transferred into the oviducts of pseudopregnant mice at 0.5 dpc (see footnote to Table 1). All recipient females were euthanized at 19.5 dpc and examined for the presence of fetuses.

Immunostaining of Donor Cells and Reconstructed Oocytes

To confirm that the collected cells were CA1 cells or Purkinje cells, immunofluorescent staining using specific antibodies was performed. The primary antibodies were rabbit monoclonal anti-Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII; catalog no. ab52476; Abcam) as a CA1 cell marker and rabbit polyclonal anti-calbindin (catalog no. ab25085; Abcam), which recognizes vitamin D-dependent calcium-binding protein, as a marker of adult Purkinje cells. Alexa Fluor 488-labeled goat anti-rabbit immunoglobulin (Ig) G (Molecular Probes, Inc.) was used as the secondary antibody. CA1 and Purkinje cells collected as described above were washed three times in PBS(–) containing 0.5% polyvinylpyrrolidone (PVP) fixed in 4% paraformaldehyde in PBS for 30 min, then washed twice in PBS-PVP. Next, the cell suspensions were transferred into PBS with 1% bovine serum albumin and 0.1 % Triton X-100 for blocking for 1 h. Cell suspensions were then incubated with the primary antibodies dissolved in blocking buffer at room temperature for 2 h. After being washed three times in blocking buffer, the cells were further incubated with the secondary antibodies. Following three washes with PBS-PVP, DNA was stained with 4′,6-diamino-2-phenylindone (DAPI; 2 μg/ml; Molecular Probes, Inc.).

We also performed immunofluorescence staining of CA1 cells, Sertoli cells, and cumulus cells for detecting H3K9me2. Each cell type was fixed in 4% paraformaldehyde in PBS for 30 min and then washed twice in PBS-PVP. Next, the cell suspensions were transferred into PBS with 1% bovine serum albumin and 0.1 % Triton X-100 for blocking for 1 h. Cell suspensions were then incubated with a mouse anti-H3K9me2 antibody in the blocking buffer at 4°C for 16 h. After being washed three times in blocking buffer, the cells were further incubated with Alexa Fluor 488-labeled goat anti-mouse IgG (Molecular Probes, Inc.). Following three washes with PBS-PVP, DNA was stained with DAPI.

To investigate whether TSA treatment affected the epigenetic modification of SCNT-derived embryos, oocytes reconstructed with CA1 cell nuclei with or without TSA treatment were fixed at the pseudopronuclear stage (10 h after activation). Then, embryos were stained with a mouse anti-H3K9me2 or rabbit anti-acetylated H3K9 antibody (H3K9Ac; catalog no. 06–942; Upstate Cell Signaling Solutions). As the secondary antibodies, Alexa Fluor 488-labeled goat anti-mouse IgG or Alexa Fluor 488-labeled goat anti-rabbit IgG antibody were used, as appropriate. The immunostaining procedures were carried out as described above.

Live-Cell Imaging Analysis

To observe chromosomal dynamics in the SCNT-derived embryos, monomeric red fluorescent protein 1 fused with histone H2B (H2B-mRFP1) was prepared as described previously [47]. Collected metaphase II oocytes were injected with a few picoliters of mRNA solution before enucleation and nuclear transfer. After oocyte activation, the reconstructed oocytes were further cultured in KSOM containing 50 nM TSA for 2 h. The embryos forming pseudopronuclei were transferred to drops of CZB medium on a glass-bottomed dish, placed in an incubator (MI-IBC; Tokai Hit) on the microscope stage, and incubated at 37°C under 5% CO_2 in air. The devices used for imaging were as described previously [48]. Device control and image analysis were performed using MetaMorph software (Molecular Devices). For time-lapse observations, images were taken over 24 h at 15-min intervals. At each time point, 51 fluorescent images were taken 2 μm apart in the *z*-axis for optical sectioning.

Microarray Analysis

Total RNA was extracted with TRIzol (Invitrogen Life Sciences) from single SCNT-derived blastocysts cultured for 96 h. RNA from a single blastocyst was subjected to two rounds of linear amplification using the TargetAmp Two-Round Aminoallyl-aRNA Amplification Kit (Epicenter) according to the manufacturer's instructions. Amplified RNA was labeled with Cy3 dye (GE Healthcare) and hybridized to a whole-mouse genome oligo DNA microarray (4 × 44K; Agilent Technologies) for 16 h at 65°C according to the manufacturer's instructions. Scanning of microarray slides was performed with a DNA microarray scanner at 5-μm resolution (Agilent Technologies). Scanned image files were processed to measure signal intensities using Feature Extraction software (version 10.5; Agilent Technologies). The data sets of control in vitro fertilization (IVF)- and SCNT-derived blastocysts from cumulus cells treated with TSA are from our previous paper [34].

Microarray Data Analysis

Statistical analyses of microarray data were performed using Gene Spring GX 12.6 software (Agilent Technologies). All raw data were subjected to a 75% percentile shift normalization procedure to adjust for the differences in signal intensities between different chips. To reduce background noise, the genes of a microarray flagged with ''not detected,'' both in the SCNT and the IVF samples, were removed from the data set. Consequently, 33 988–35 180 out of 41 267 genes (probes) were used for statistical analysis. Normalized values were analyzed by moderated Student *t*-tests with a 5% significance level using the Westfall-Young procedure, and 2.0-, 5.0-, and 10.0-fold filters were applied to identify differentially expressed genes (DEGs) between SCNT- and IVF-derived embryos. The gene ontology (GO) enrichment analysis was performed using Gene Spring GX 12.6 software (Agilent Technologies) to detect any overrepresentation of functional categories of the selected genes.

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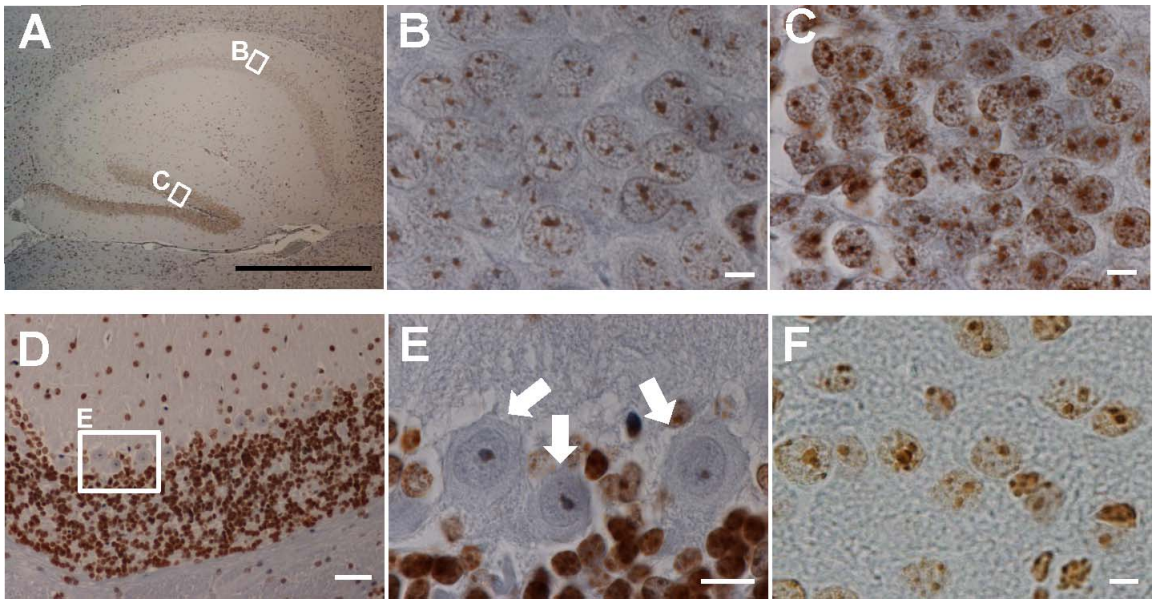


FIG. 1. Sections of the brain tissues from adult mice (age, 6–8 wk) immunostained for H3K9me2. **A**) Low-magnification micrograph of the hippocampal region. The rectangular areas are each shown at higher magnifications in **B** and **C**. **B**) Enlarged view of CA1 pyramidal cells. These cells showed relatively lower accumulation of the H3K9me2 repressive epigenetic marker, with only punctate positive areas in the nuclei. **C**) Enlarged view of neurons in the dentate gyrus. In these cells, strong signals of H3K9me2 were detected as punctate regions, and broadened H3K9me2 marks were also detected in their nuclei. **D**) Low-magnification micrograph of the cerebellum. **E**) Enlarged view of **D** indicating Purkinje cells (arrows). A very low accumulation of H3K9me2 was observed in the nuclei. **F**) Neurons in the cerebral cortex. These were densely stained for H3K9me2, as were the cells in the dentate gyrus in **C**. Bar = 1 mm (**A**), 8 μm (**B**, **C**, and **F**), 50 μm (**D**), and 30 μm (**E**).

Statistical Analysis

Developmental rates of SCNT-derived embryos were compared between groups using the Fisher exact test. Other statistical analysis methods are shown as appropriate in the text. A level of $P < 0.05$ was considered to be statistically significant.

RESULTS

Immunostaining of Adult Mouse Brain Sections, Cells, and Oocytes

First, we sought to identify neural donor cells with low levels of H3K9me2 in the brain tissue sections by immunostaining. Although no cells completely lacked the H3K9me2 mark, neural cells with relatively lower reactivity were found in hippocampal pyramidal cells located in the CA1 region (Fig. 1, A and B). Because strong H3K9me2 marks were only detected as small punctate areas in the nuclei of these cells, we choose them as candidate cells with a low H3K9me2 mark. On the other hand, cells with higher reactivity were found in the dentate gyrus (Fig. 1C) and the cerebral cortex (Fig. 1F). Purkinje cells in the cerebellum also showed low accumulations of H3K9me2 (Fig. 1, D and E). Similar patterns of H3K9me2 staining were observed in the brain sections of both sexes. Thus, we selected these as donor cells for the following nuclear transfer experiments.

The CA1 and dentate gyrus neurons exist as a cell layer in the hippocampus (Fig. 2A). Therefore, we could collect these cells by cutting off the regions with fine scissors. To confirm whether the brain cells we used were neuronal cells, collected donor cells were stained with an anti-CaMKII antibody (Fig. 2B), and the numbers of CaMKII-positive cells were counted (Fig. 2C). Almost all the cells we counted were positive for this antibody (727/737; 98.6%) (Fig. 2C), indicating that the donor cells we used for SCNT experiments were most likely hippocampal neuronal cells. Purkinje cells were identified easily because of their typical large size and morphology (Fig. 2D). Indeed, all the large cells collected were positive for calbindin, an established marker for Purkinje cells of the adult brain (Fig. 2E). Accordingly, we collected Purkinje cells depending on their size for the nuclear transfer experiments.

We also examined the H3K9me2 levels in the nuclei of immature Sertoli and cumulus cells used as controls and in the pseudopronuclei after nuclear transfer. As expected, the nuclei of Sertoli cells and cumulus cells were more intensely stained with the anti-H3K9me2 antibody than were the nuclei of CA1 cells (Supplemental Fig. S1A; Supplemental Data are available online at www.biolreprod.org). The levels of H3K9me2 signals in pseudopronuclei were similar in reconstructed oocytes with or without TSA treatment, indicating that the H3K9me2 repressive mark was not reduced by TSA after nuclear transfer, at least at the level of immunofluorescence (Supplemental Fig. S1B).

Development of Cloned Embryos Generated from Differentiated Neuronal Cells

After nuclear transfer, approximately 60% of embryos derived from CA1 pyramidal cells could develop to the morula/blastocyst stage, irrespective of the sex (male, 58.2%; female, 61.9%). Similar in vitro developmental rates were obtained with embryos derived from neurons of the dentate gyrus (male, 58.0%; female, 55.1%) or the cerebral cortex (male, 65.3%; female, 56.4%) (Tables 1 and 2). Although more than half (51.9%) of the embryos derived from female Purkinje cells could develop to the morula/blastocyst stage, those derived from male Purkinje cells showed a significantly lower developmental rate (22.5%) (Tables 1 and 2). We also investigated the developmental rates of SCNT-derived embryos from adult brain cells without TSA treatment. The blastocyst formation rates of these embryos were significantly decreased compared with TSA-treated, SCNT-derived embryos for all

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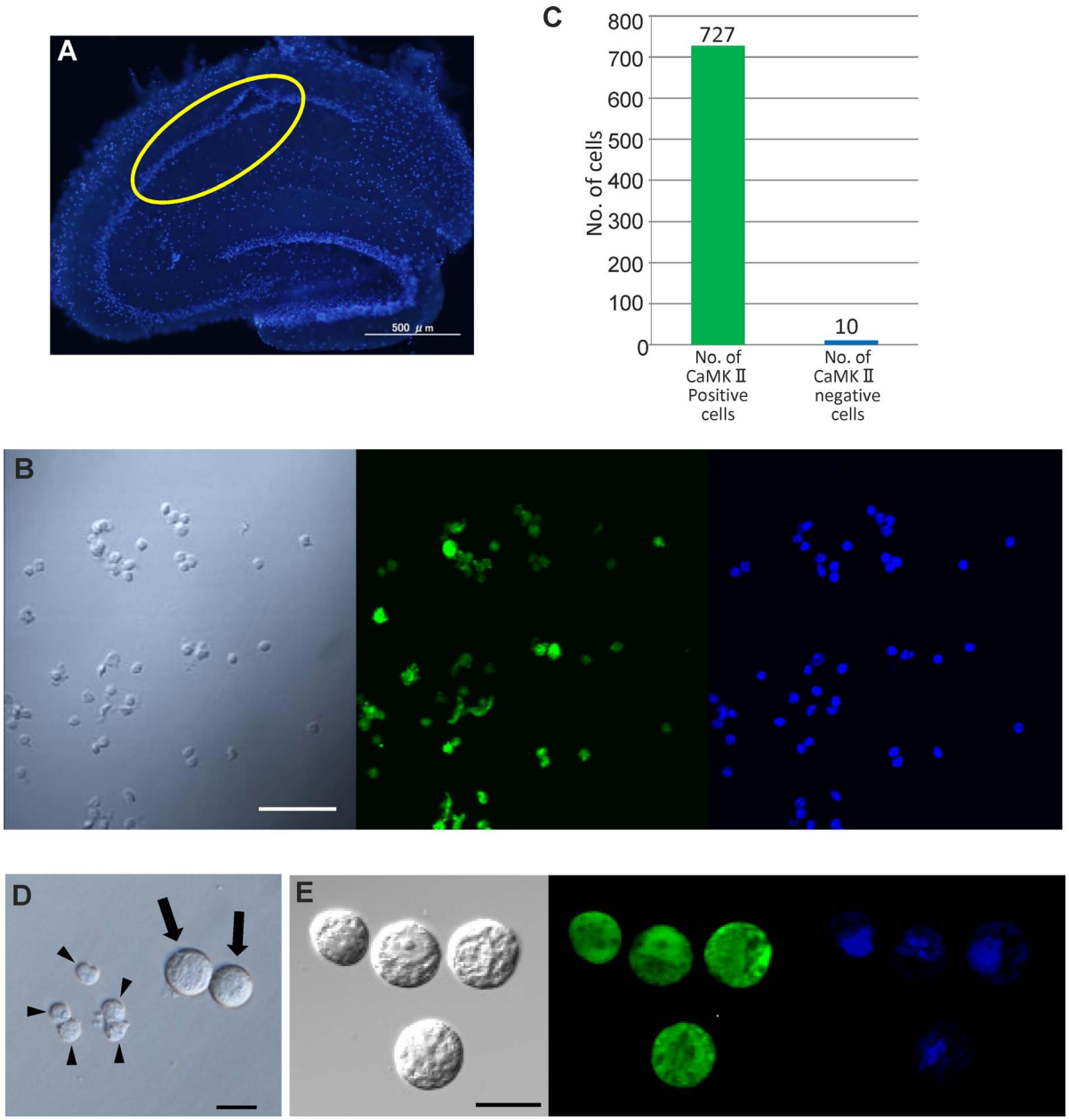


FIG. 2. Collection of neuronal cells for nuclear transfer. **A**) The hippocampus was stained with Hoechst 33342 dye (Sigma-Aldrich). The yellow circle indicates the CA1 region. **B**) CA1 pyramidal cells collected for nuclear transfer. Bright-field (**left**), anti-CaMKII staining (**middle**), and DAPI DNA staining (**right**) are shown. Almost all of the cells were positive for CaMKII. **C**) Numbers of CaMKII-positive and -negative cells among collected CA1 cells. Most (~99%) of the cells were positive for CaMKII, indicating that they were neurons. **D**) Collected Purkinje cells in a bright-field view (large arrows). These were 3- to 4-fold larger than cumulus cells, which are the donor cells most frequently used for mouse cloning by SCNT (arrowheads). **E**) Purkinje cells were immunostained for their specific surface marker, calbindin. Bright-field (**left**), anti-calbindin staining (**middle**), and DAPI staining (**right**) are shown. Bar = 500 μm (**A**), 80 μm (**B**), and 30 μm (**D** and **E**).

types of donor cells (Supplemental Table S1). For male donors, no significant differences were found between the blastocyst formation rates of SCNT-derived embryos from three types of brain cells (CA1, 23.6%; dentate gyrus, 20.3%; cerebral cortex, 24.1%) (Supplemental Table S1). For female donors, although SCNT embryos derived from the cerebral cortex showed a lower developmental rate, no significant differences were found between embryos derived from CA1 and dentate gyrus cells (CA1, 20.3%; dentate gyrus, 17.0%; cerebral cortex, 13.5%) (Supplemental Table S1). These results indicate that the presence of TSA, but not the degree of H3K9me2 marking of donor cells, influenced the preimplantation development of SCNT-derived embryos.

To investigate whether cloned mice could be produced efficiently from adult neurons, SCNT-derived embryos from each donor cell type were transferred into the uteri of recipient pseudopregnant females. As a result, we succeeded in generating cloned mice by direct nuclear transfer using adult neurons of different origins in combination with TSA treatment (Fig. 3). The SCNT-derived embryos from male CA1 pyramidal cells showed the highest full-term developmental rate after embryo transfer (10.2% of embryos transferred). Neurons from the dentate gyrus and the cerebral cortex also

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TABLE 2. Development of cloned embryos from brain cells of adult female mice.

Type of donor cell	No. of embryos cultured	No. of 2-cell embryos (%)	No. of 4-cell embryos (%)	No. of morulae/blastocysts (%)	No. of embryos transferred (No. recipients)	No. of cloned offspring (%)*
CA1	289	228 (78.9)[a]	190 (65.7)	179 (61.9)[a]	151 (13)	7 (4.6)
Purkinje	187	130 (69.5)[b]	116 (62.0)	97 (51.9)[b]	97 (6)	1 (1.0)
Dentate gyrus	225	172 (76.4)[b]	135 (60.0)	124 (55.1)[ab]	109 (8)	3 (2.8)
Cerebral cortex	181	134 (74.0)[b]	120 (66.3)	102 (56.4)[ab]	102 (6)	3 (2.9)
Cumulus	189	166 (87.8)[a]	126 (66.7)	106 (56.1)[ab]	106 (6)	6 (5.7)

* Percentage of embryos transferred.
[a,b] Data with different superscripts are significantly different ($P < 0.05$, Fisher exact test).

supported full-term development, but the efficiencies were lower (3.8% and 2.4%, respectively) (Table 1 and Fig. 3A). For female donors, SCNT-derived embryos from CA1 pyramidal cells showed slightly higher full-term developmental rates (4.6%) than those from dentate gyrus cells (2.8%) or cerebral cortex cells (2.9%), but no significant differences were found between these rates (Table 2 and Fig. 3B). These results indicate that the genomes of at least some of the differentiated neurons from adult brains can achieve full reprogramming after nuclear transfer to support full-term development. We also generated a cloned mouse from a female Purkinje cell but not from male cells (Table 2 and Fig. 3C). This low success rate with Purkinje cells might be a result of mechanical damage to the recipient oocytes and donor nuclei caused by the large size of the cells (Fig. 2D). Our live-cell imaging analysis for the chromosomal behavior supported this assumption. Approximately 50% of Purkinje-derived embryos showed abnormal chromosome segregation (ACS) at the first division (male, 52%; female, 49%), whereas CA1-derived embryos showed ACS at rates of 26.4% and 22.2% for male and female donors, respectively ($P < 0.05$ between Purkinje- and CA1-derived embryos in both sexes) (Supplemental Fig. S2).

Gene Expression Analysis of SCNT-Derived Blastocysts Using a Microarray

To gain further clues to understand how much the donor cell genomes were reprogrammed, we analyzed the gene expression profiles of single cloned blastocysts at 96 h after activation. First, we compared the number of DEGs of which expression levels exceeded 2-, 5-, and 10-fold changes from the levels of the control, IVF-derived blastocysts. For male donors, Purkinje cell-derived embryos exhibited significantly fewer DEGs than Sertoli cell-derived embryos at every fold-change category (Fig. 4A). CA1-derived embryos had smaller numbers of DEGs than Sertoli-derived embryos exceeding 5- and 10-fold changes (Fig. 4A). For female donors, the numbers of DEGs in CA1 cell-derived embryos were smaller at every fold-change category, and those in Purkinje cell-derived embryos were smaller at the 2-fold change level (Fig. 4B). These data were consistent with the H3K9me2 levels of the donor cells examined by fluorescence immunostaining (see above). Although there seemed to be a sex-related difference in the magnitude of differential expression, the data obtained cumulatively suggest that the genomes of CA1 pyramidal

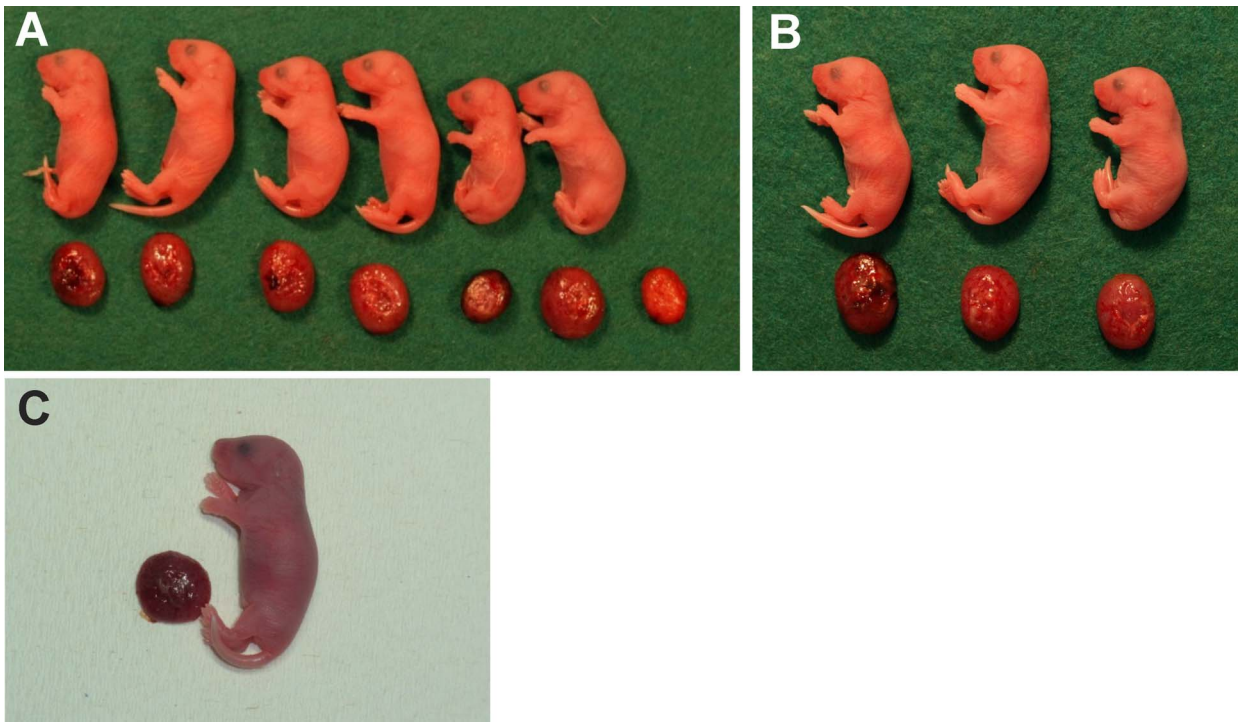


FIG. 3. Offspring cloned from neurons of adult mouse brains. **A**) Offspring cloned from male CA1 pyramidal cell nuclei. **B**) Offspring cloned from female CA1 pyramidal cell nuclei. **C**) A cloned offspring from a female Purkinje cell nucleus. All cloned mice look normal but all had SCNT-specific placentomegaly.

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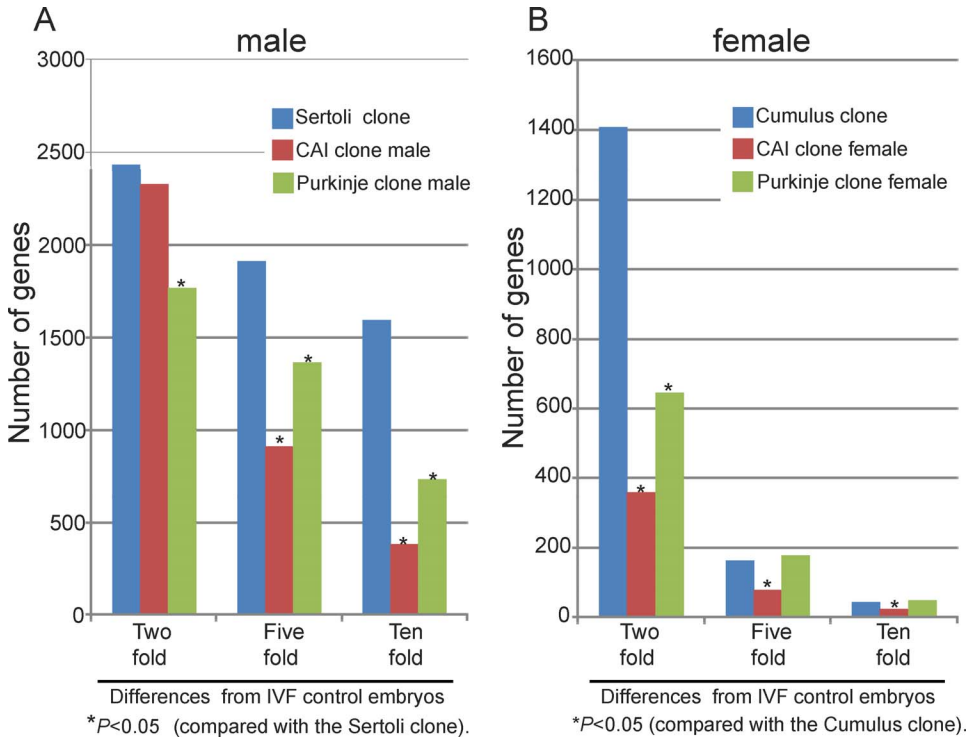


FIG. 4. Numbers of DEGs in cloned blastocysts from different donor cells (male [**A**] and female [**B**]) compared using moderated *t*-tests with the expression levels of IVF-derived blastocysts as controls. Blastocysts derived from CA1 pyramidal and Purkinje cells had significantly fewer DEGs than those derived from Sertoli cells or cumulus cells for at least some fold-change levels. *$P < 0.05$ vs. Sertoli cell (male)- or cumulus cell (female)-derived embryos (chi-square tests).

and Purkinje cells were more efficiently reprogrammed following SCNT than those of Sertoli or cumulus cells.

We then identified those genes that had restored their expression levels in CA1 cell- and Purkinje cell-derived cloned embryos from the levels observed in Sertoli cell (male)- or cumulus cell (female)-derived cloned embryos to those seen in IVF-derived embryos. By one-way ANOVA, 486 and 485 genes were identified as fulfilling this criterion for male and female donors, respectively. These genes were subjected to GO enrichment analysis to determine which functional categories were restored in CA1 cell- and Purkinje cell-derived cloned embryos. At the $P < 0.05$ significance level, 15 and 37 GO terms were identified to be overrepresented in male and female donors, respectively (Supplemental Figs. S3 and S4). Most of the GO terms were classified into ''biological process'' or ''cellular component'' (Fig. 5). Eleven GO terms were shared between male and female donors, comprising the majority (11/15) in male donors. For the female donors, the list of the GO terms was characterized by ribosome-related GO terms (top four and another six terms), although relatively few of the genes were categorized into these GO terms (35–564) compared with those of other major GO terms, such as ''metabolic process'' (8133) (Supplemental Fig. S4).

We examined specifically whether low levels of H3K9me2 marks of donor nuclei affected the expression patterns of genes located in LOCK regions. Contrary to our expectations, the *Xlr* and *Magea* family genes—typical LOCK genes on the X-chromosome—remained repressed in CA1 cell- and Purkinje

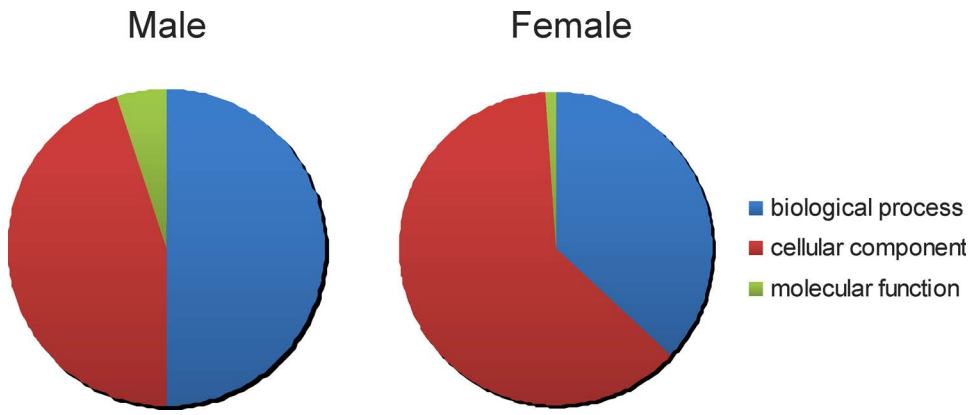


FIG. 5. GO categorization of the functions of DEGs in blastocysts derived from CA1 pyramidal cells or Purkinje cells compared with Sertoli cell (male)- or cumulus cell (female)-derived embryos but not with IVF-derived embryos. Most annotations were categorized as "biological process" or "cellular component" in both sexes. For details of the GO term enrichment, see Supplemental Figure S3 and S4.

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cell-derived embryos, similar to blastocysts derived from cumulus and Sertoli cells (Fig. 6). Thus, these LOCK genes were highly resistant to genomic reprogramming by nuclear transfer even in somatic cells with low levels of H3K9me2 marks.

Finally, we examined the expression levels of *Xist*, a gene responsible for X-inactivation in female cells, because ectopic expression of this gene from the active X chromosome critically affects the development of SCNT-derived embryos [34, 36]. When compared with ''standard'' cloned embryos (Sertoli cell- or cumulus cell-derived embryos), those from male CA1 and Purkinje cells showed significantly lower *Xist* expression levels (Fig. 6), indicating that the dysregulation of global gene expression caused by *Xist* expression could have been ameliorated in these cloned embryos.

DISCUSSION

Many types of somatic cells have been used as nuclear donors to generate cloned mice. In SCNT using neural cells other than neural stem cells, cloned mice have been obtained only when immature (fetal) neurons were used as nuclear donors [17]. Although several laboratories have succeeded in producing cloned (clonal) mice using differentiated neural cells from adult brains by a two-step cloning method combining ntES technology and tetraploid complementation [18, 22, 49–51], no reports of live mice produced from these donor cell types by direct SCNT have appeared. Those findings germinated the idea that the nuclei of differentiated neural cells have lost their potential for reproductive cloning [52, 53]. However, in the present study, we have demonstrated, to our knowledge for the first time, that the genome of terminally differentiated neural cells can be fully reprogrammed by direct SCNT.

It was our expectation that cloned offspring could be obtained using CA1 pyramidal cells, with a low content of nuclear H3K9me2. Indeed, embryos reconstructed with such cells developed to term at efficiencies similar to or even better than ''standard'' mouse cloning using immature Sertoli or cumulus cells. Additionally, neurons from the dentate gyrus and cerebral cortex could also generate cloned mice, although the efficiencies were relatively low. The latter finding was unexpected, because cerebral cortex neurons from adult mice have been considered to be ''unclonable'' donor cells by direct (one-step) nuclear transfer [17, 18]. In the present study, we used TSA in SCNT experiments, unlike previous cloning studies using adult neurons [22]. It is known that TSA treatment can enhance the pool of acetylated histones [54], increase DNA demethylation [55], and remarkably increase the success rates of cloning from somatic cells [30, 56, 57]. Indeed, the present study and previous reports indicated that TSA treatment dramatically increased the H3K9Ac levels in the pseudopronuclei of SCNT-derived embryos (Supplemental Fig. S2B) [58, 59], and no cloned offspring were born from adult brain cells without TSA treatment in the present study. Thus, epigenetic modifications induced by TSA in the reconstructed embryos were probably essential for our success in cloning from brain cells, such as cerebral cortex neurons. However, even after TSA treatment, H3K9me2 levels were not changed as far as we could examine by immunostaining (Supplemental Fig. S2B), indicating that TSA did not modify the H3K9me2 epigenetic marks (but possibly did modify others of donor cell origin). While we were revising this paper, an important study on mouse SCNT was published [60], which identified H3K9me3 in the somatic cell genome as one of the major epigenetic barriers to reprogramming by nuclear transfer. Those authors showed that reduction of H3K9me3 by introduction of mRNA for the lysine demethylase Kdm4d into oocytes significantly improved cloning success. Importantly, their data suggested that Kdm4d might exert its effects through a pathway similar to that of TSA and that it did not improve the expression levels of genes thought to be repressed by H3K9me2. Taken together, the high cloning efficiency of CA1 cells in the present study was very likely due to the synergistic effects of their inherently low H3K9me2 content and the increased histone acetylation level induced by TSA, which leads to reduction of H3K9me3.

The H3K9me2 epigenetic marks of somatic cell genomes are thought to be strongly associated with tissue-specific gene silencing, forming LOCK regions: large somatic cell-specific repressed chromatin blocks. The repression of gene expression in these LOCK regions in SCNT-derived embryos was not improved by TSA treatment [34]. According to the results of immunostaining in the present study, CA1 pyramidal cells and cerebellar Purkinje cells showed weak or very scanty H3K9me2 signals. Therefore, we expected that gene expression in LOCK regions could be improved by using these cells as the nuclear donors. However, our DNA microarray analysis revealed that gene expression of the typical LOCK genes—*Xlr* or *Magea* families—was not improved in the blastocysts derived from CA1 pyramidal or Purkinje cells. Thus, it is probable that these genes are within exceptionally strong LOCKs and that their repressed expressions are never relaxed until the genome enters the germline, where the entire set of genomic H3K9me2 marks is removed during primordial germ cell development [61]. Treatment of cloned embryos with a G9a histone methyltransferase inhibitor (BIX-01294) led to the expression of *Magea2*, but this was not associated with any improvement in cloning efficiency [62]. It would be interesting to determine the gene expression profiles in those cloned embryos at a genome-wide level. Nevertheless, the full-term developmental rates of embryos from CA1 pyramidal cells were better than those from the donor cells with high levels of H3K9me2 marks (dentate gyrus and cerebral cortex cells), especially for male donors. Therefore, we postulate an inverse association between cloning efficiency and the intensity of H3K9me2 marking of donor neural cells.

How could SCNT-derived embryos from male CA1 cells develop to term with such a high success rate? To answer this question, we observed the global gene profiles of single cloned blastocysts using microarrays. We found that the numbers of DEGs expressed at the 5- or 10-fold difference levels in male CA1 cell-derived blastocysts were significantly fewer than in Sertoli cell-derived blastocysts. GO analysis suggested that many of the genes with improved expression in male CA1 cell-derived embryos were metabolically important. This might have supported their better developmental efficiency to term. Unlike the male donor-derived embryos, female CA1 cell-derived embryos showed a modest improvement in gene expression patterns, which might explain why female CA1-derived clones did not show a higher birth rate compared with cumulus cell-derived clones. This sex-related difference in the developmental efficiency of CA1 cell-derived embryos might also be explained by differences in the *Xist* expression levels, as discussed below.

We also focused on the expression levels of *Xist*, because upregulation of this gene is one of the major causes of poor development of SCNT-derived embryos in the mouse [34, 36]. Intriguingly, the expression level of *Xist* in male CA1 cell-derived blastocysts was significantly lower than that in Sertoli cell-derived blastocysts. This might have accounted—at least in part—for the high rates of full-term development of male

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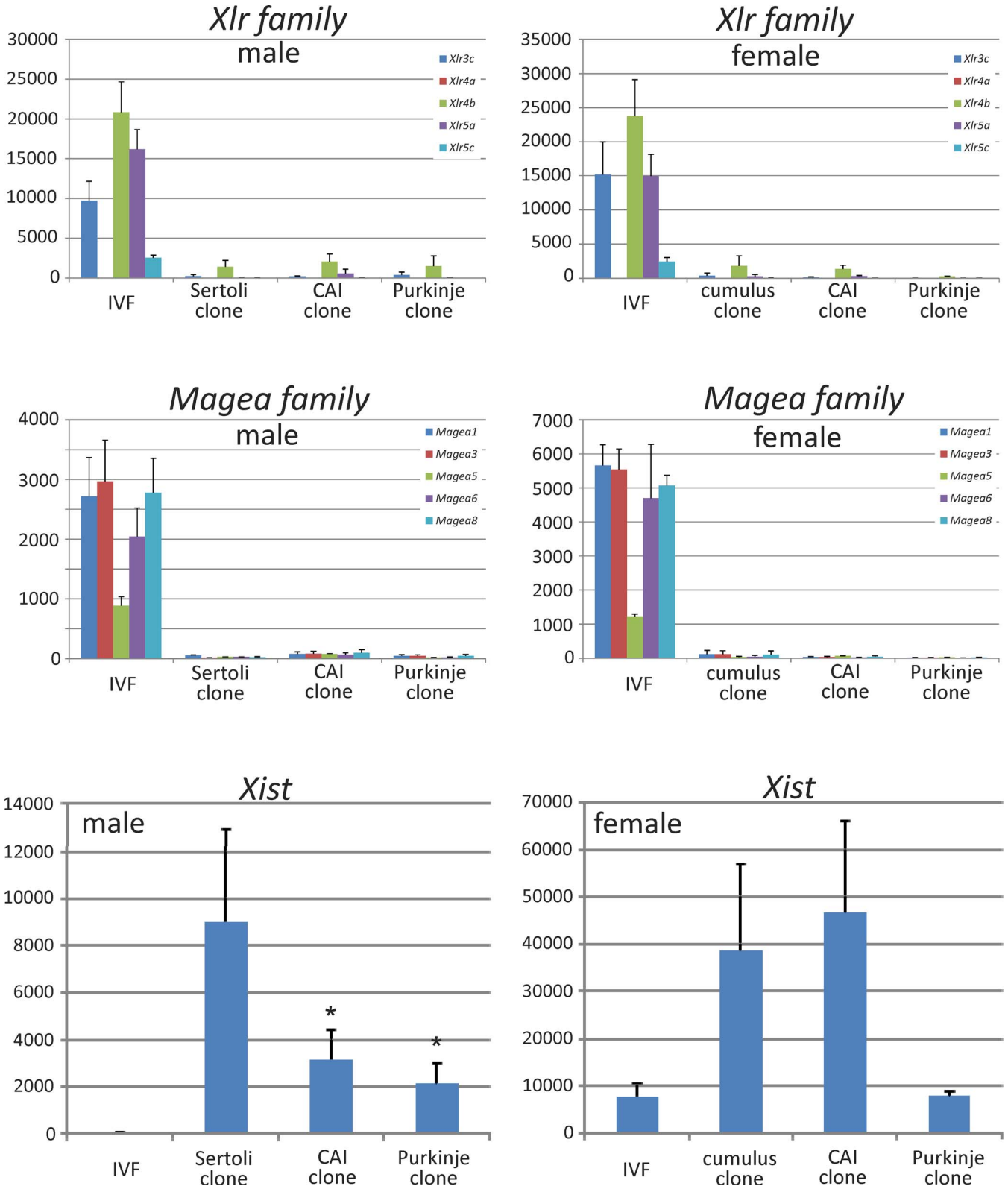


FIG. 6. Expression levels of genes prone to SCNT-specific aberrations. The *Xlr* and *Magea* families of genes located on LOCK regions of the X-chromosome remained repressed in embryos derived from CA1 pyramidal cells and Purkinje cells compared with embryos from Sertoli cells or cumulus cells. However, upregulation of the *Xist* gene typically shown in Sertoli cell- and cumulus cell-derived cloned embryos was improved in male CA1 cell- and Purkinje cell-derived embryos. $^{*}P < 0.05$ (Dunnett test with Sertoli cell- or cumulus cell-derived cloned embryos as controls). Error bars represent the mean ± SEM.

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CA1 cell-derived embryos, because in previous studies, the correction of ectopic *Xist* expression by gene knockout or knockdown could remarkably improve cloning efficiencies [34, 36, 37]. However, we did not expect a corrected expression level of *Xist* in male CA1 cell-derived cloned embryos, because we selected CA1 cells as donors based on low H3K9me2 contents in their nuclei, not for their *Xist* expression status. We have shown previously that *Xist* is activated after zygotic gene activation as a default, and only the imprinting imposed on the oocyte genome can repress it [63]. A certain proportion of brain cells might have similar repressing mechanisms to avoid leaky *Xist* expression, because X-linked genes in brain tissues normally maintain higher expression levels than autosomal genes [44]. It is also possible that the nearly normal functional genome in the CA1 cell-derived embryos might have served to correct the *Xist* expression level, as reported for androgenetic or parthenogenetic embryos [64, 65].

Unfortunately, we could produce only one female mouse and no male mouse from Purkinje cells by SCNT. This probably occurred as a result of physical damage to the recipient oocytes caused by the donor nuclei, which are too large to inject into oocytes or to collect from the brain tissue safely. Indeed, most of the collected Purkinje cells were ruptured in the droplets of PVP medium for donor cell manipulation. Furthermore, using live-cell imaging analysis, we confirmed that Purkinje cell-derived embryos at the first division were associated with ACS at rates as high as approximately 50% (Supplemental Fig. S2). Most embryos with ACS arrest their development after implantation, as revealed by live-cell imaging analysis for embryos derived from SCNT or round spermatid injection [66, 67]. Nevertheless, embryos cloned from Purkinje cells of both sexes showed better global gene expression profiles and relatively normal *Xist* expression levels, indicating that the genome of Purkinje cells can potentially support embryonic development after SCNT. This result might be consistent with our initial assumption that the genome of cells with a low H3K9me2 level in their nucleus can be reprogrammed more efficiently than that of cells with higher H3K9me2 contents.

In conclusion, we have succeeded, to our knowledge for the first time, in generating cloned mice from freshly isolated adult neurons of different origins using a histone deacetylase inhibitor, TSA. Although the SCNT-specific strong repression of genes on LOCK regions of the X chromosome could not be improved, our findings suggest the presence of an inverse association between the accumulation of H3K9me2 marks of donor cells and the normality of gene expression levels in the resultant cloned embryos. Thus, we expect that screening for low levels of repressive histone marks might be a practical approach when selecting donor cells for efficient cloning by SCNT in mammals. Even when the cells thus identified are difficult to prepare (as with CA1 cells in the present study), such information could lead to the targeted removal of specific repressive histones, which in turn might increase the cloning efficiency using easily accessible donor cells.

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