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Mecp2 deficiency leads to delayed maturation and altered gene expression in hippocampal neurons

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Abstract

It is well known that Rett Syndrome, a severe postnatal childhood neurological disorder is mostly caused by mutations in the *MECP2* gene. However, how deficiencies in MeCP2 contribute to the neurological dysfunction of Rett Syndrome is not clear. We aimed to resolve the role of MeCP2 epigenetic regulation in postnatal brain development in a *Mecp2*-deficient mouse model. We found that, while *Mecp2* was not critical for the production of immature neurons in the dentate gyrus (DG) of the hippocampus, the newly generated neurons exhibited pronounced deficits in neuronal maturation, including delayed transition into a more mature stage, altered expression of presynaptic proteins, and reduced dendritic spine density. Furthermore, analysis of gene expression profiles of isolated DG granule neurons revealed abnormal expression levels of a number of genes previously shown to be important for synaptogenesis. Our studies suggest that MeCP2 plays a central role in neuronal maturation, which might be mediated through epigenetic control of expression pathways that are instrumental in both dendritic development and synaptogenesis.

Keywords

neuronal maturation; *Mecp2*; synaptogenesis; dendritic spines; Rett Syndrome; hippocampus

Introduction

Rett Syndrome (RTT) is a neurodevelopmental disorder that affects one of every 15,000 female births. RTT patients develop normally until 6 to 18 months of age, but then regress rapidly experiencing a wide range of neurological defects, such as seizures, ataxia, and stereotypical hand movements. Individuals affected by RTT often survive into adulthood, and while some symptoms stabilize, others may worsen (Hagberg et al., 1983; Hagberg and Witt-Engerstrom, 1986; Kriaucionis and Bird, 2003). In most cases, RTT can be linked to loss-of-function mutations in the X-linked *MECP2* gene (Amir et al., 1999), which encodes a methylated-CpG binding protein that recruits additional factors such as histone deacetylase to repress transcription (Bird, 2002). Several lines of *Mecp2* mutant mice (KO) have been generated and these mice develop similar symptoms to those seen in RTT patients and have been widely used to study the etiology of human RTT (Chen et al., 2001; Guy et al., 2001; Shahbazian et al.,

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2002; Pelka et al., 2006). Nevertheless, the neurodevelopmental pathways and specific genes targeted by the disruption of this epigenetic regulatory control have not been determined.

Recent experimental evidence indicates that MeCP2 may play a vital role in neuronal maturation (Bienvenu and Chelly, 2006). A critical step in the process of neuronal maturation is synaptogenesis, which coincides with the increased expression of MeCP2 in developing neurons (Akbarian et al., 2001; Zoghbi, 2003) (Shahbazian and Zoghbi, 2002), suggesting that epigenetic modulation of gene regulation during this period might be critical for brain development. In fact, postmortem analysis has demonstrated reduced numbers of axonal and dendritic processes, decreased dendritic spine density, and lowered levels of the dendritic cytoskeletal protein MAP2 in RTT brains (Kaufmann and Moser, 2000; Armstrong, 2002). Consistent with human pathology, pyramidal neurons in the cortex of adult *Mecp2* null mutant (KO) mice were found to have smaller soma and less complex dendrites, though the morphology and density of dendritic spines were not determined in this study (Kishi and Macklis, 2004). Exogenous *Mecp2* expression could also lead to increased neurite complexity in cultured neurons (Jugloff et al., 2005), further suggest a role of MeCP2 in dendritic development. However, in another study, analyses of Golgi-stained cortical and subcortical neurons of *Mecp2* truncation mutant mice (*Mecp2*^{Y308}) did not reveal significant abnormalities in either dendritic arbor or spine density (Moretti et al., 2006). The discrepancy between these results could be due to differences in either the model systems analyzed or the methods used. Abnormalities in dendritic spines have been found in several developmental disorders [reviewed by (Fiala et al., 2002)]. Therefore it is critical to clarify whether *Mecp2* mutations affect spine development by monitoring the maturation of single neurons in a well defined cell population in order to understand the function of MeCP2 in neural development and the etiology of RTT.

Unlike most other brain regions, neurogenesis in the adult dentate gyrus (DG) persists throughout life. In adult mice, newborn DG neurons develop properties similar to mature granule neurons after approximately 4-8 weeks of differentiation. The properties of newborn neurons in the adult DG recapitulate embryonic hippocampal development (Song et al., 2005), providing a unique model system for studying the generation and maturation of neurons in postnatal brains (Gage, 2002). The hippocampus also provides a logical framework to study the pathogenesis of MeCP2 deficiency because the morphological maturation, functional properties, and molecular mechanisms of the hippocampus have been extensively characterized due to their potentially critical roles in learning and memory (Ziv and Garner, 2004; Nicoll and Schmitz, 2005), and because *Mecp2* KO mice have been shown to have impaired long-term potentiation and depression, impaired excitatory neurotransmission, and altered expression of neurotransmitter receptors in hippocampal neurons (Asaka et al., 2006; Moretti et al., 2006; Nelson et al., 2006).

Mecp2 has been found to be expressed in neural stem cells (NSCs) (Jung et al., 2003; Namiyama et al., 2004). While MeCP2 was shown to be involved in embryonic neurogenesis in *Xenopus*, studies have indicated that this is not the case in mice (Stancheva, 2003; Kishi and Macklis, 2004). Recent evidence has revealed that adult NSCs are different from embryonic NSCs in both the cellular environment they encounter and in their intrinsic genetic and epigenetic properties (Zhao et al., 2003; Cheng et al., 2005). Moreover, deletion of *Mecp2*-related Methyl-CpG binding protein 1 (Mbd1) specifically affects postnatal, but not embryonic, neurogenesis (Zhao et al., 2003), suggesting that postnatal neurogenesis may be particularly vulnerable to altered epigenetic regulation. Therefore, analyzing postnatal neurogenesis in the absence of *Mecp2* will provide critical information for understanding the function of this protein.

In this study, we have determined that *Mecp2* is not critical for the early stages of neurogenesis. In contrast, we show that immature neurons in the DG of KO mice exhibit deficits in their

ability to transition into later mature stages of development. This deficit results in adult *Mecp2* KO mice retaining characteristic features of immature brains, suggesting a stalled maturation. At a single neuronal level in the postnatal hippocampus, *Mecp2*-deficient neurons exhibited a reduced number of dendritic spines. By analyzing gene expression profiles of a homogeneous population of DG neurons isolated from KO brains, we have found that the expression levels of several genes encoding proteins that are likely to be involved in synaptogenesis were altered. Together, these data suggest that *Mecp2* is critical for the maturation of young neurons, possibly through regulating synaptogenic factors.

Materials and Methods

Animals

All animal procedures were performed according to protocols approved by the University of New Mexico Animal Care and Use Committee. The *Mecp2* KO mice (*Mecp2^{tm1.1Jae}*) used in this study were created by deleting exons 3 containing the MBD domain of *Mecp2* (Chen et al., 2001). These mice have been bred over 40 generations on to ICR background. They start to show neurological symptoms between 5 and 7 weeks of age and die before 10 weeks of age. For histological analyses, mice were euthanized by intraperitoneal injection of sodium pentobarbital. Mice were then perfused with saline followed by 4% PFA. Brains were dissected out, post-fixed overnight in 4% PFA, and then equilibrated in 30% sucrose. Forty- μ m brain sections were generated using a sliding microtome and were stored in -20°C freezer as floating sections in 96-well plates filled with cryoprotectant solution (glycerol, ethylene glycol, and 0.1M phosphate buffer, pH 7.4, 1:1:2 by volume).

Statistical analyses

All statistical analyses were performed using unpaired, two-tailed, Student's t-test and in all figures, the data bars and error bars indicate mean $\pm$ standard error (s.e.m).

Isolation and in vitro analyses of adult NSCs

Isolation of adult NSCs was performed based on the published method (Zhao et al., 2003). Briefly, forebrains without olfactory bulb and cerebellum (4 mice/ genotype, age- and sex-matched) were dissociated mechanically followed by enzymatic digestion using PPD (2.5 U/ml papain, 1U/ml DNaseI, and 200 mg/100 ml Dispase II) in DMEM high glucose (Cellgro, Herndon, VA). After filtering through a 70- μ m cell strainer (BD Falcon, San Jose, CA), a single cell suspension was loaded onto 50% percoll. The NSCs were separated from other cells by ultracentrifugation at 127 krpm for 30 min at 20°C using a SW41 rotor (Beckman, Fullerton, CA). The fraction containing NSCs (immediately above the red blood cell layer in the gradient) was collected, washed with PBS, and plated in N2 medium (DMEM/F12 1:1 containing N2 supplement (Invitrogen, Carlsbad, CA) supplemented with 20 ng/ml FGF-2 and 20 ng/ml EGF in a 5% CO_2 incubator. Cell proliferation analyses were performed as described (Lie et al., 2005). Briefly, BrdU was added to the culture medium at 5 μM for 16 hours, followed by fixation using 4% PFA. Cells were then stained with antibodies against BrdU (1:500, Accurate Chemicals, Westbury, NY) and Ki67 (1:1000, NovoCastra Laboratories, Newcastle upon Tyne, UK) and 10 $\mu\text{g/ml}$ DAPI. The percentage of BrdU⁺ cells or Ki67⁺ over total DAPI⁺ cells indicates the percentage of cells that are proliferating. For in vitro differentiation analysis, cells were incubated in N2 media containing 1 μM forskolin, 1 μM all-trans retinoic acid and 0.5% FBS for 7 days. Cells were then fixed by 4% PFA, followed by immunocytochemical analysis as described previously (Zhao et al., 2003). Primary antibodies used were: rabbit anti-type III β -tubulin (1:4000, Covance, Berkeley, CA), RIP (1:50, Hybridoma Bank, Iowa City, Iowa), s-100 β (1:1000; Sigma-Aldrich, St Louis, MO), and all secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used in 1:250 dilution. Cell phenotypes were analyzed using an Olympus BX51 Research microscope equipped with epifluorescence, an

optronics microfire digital color camera, and StereoInvestigator software (MicroBrightField). Cell counting was performed using an optical fractionator sampling design and formula (Gundersen et al., 1988). Four independent experiments (each had triplicates), using similar passages of cells, were performed for in vitro proliferation and differentiation assays.

In vivo neurogenesis analyses

In vivo neurogenesis analyses were performed essentially as described previously (Zhao et al., 2003). Briefly, in 8-week-old mice (11 WT and 8 KO), BrdU (50mg/kg) was injected daily for 7 consecutive days to increase the amount of labeling. In 4-week-old mice (6 WT and 9 KO), BrdU was injected once daily for 4 consecutive days. Mice were then euthanized 1 day post-injection to assess proliferation (and early survival) of labeled cells. For cell survival analysis, mice injected at 4 weeks of age (6 WT and 9 KO, 1 injection/day for 4 days) were euthanized 4 weeks post-injection. For immunohistological analysis, 1-in-6 serial floating brain sections (240 μ m apart) were performed based on the published method (Zhao et al., 2003). The primary antibodies used were: rat-anti-BrdU (1:500; Accurate Chemicals), mouse anti-NeuN (1:5000; Chemicon International, Temecula, CA), rabbit anti-S-100 β (1:500; Sigma), and chicken anti-Mecp2 (1:5000, a generous gift from Dr. Janine LaSalle University of California, Davis). Fluorescent secondary antibodies were used at 1:250 dilutions (donkey, Jackson ImmunoResearch). After staining, sections were mounted, coverslipped, and maintained at 4°C in the dark until analysis. BrdU-positive cells in the granule layer were counted using unbiased stereology (StereoInvestigator, MicroBrightField) with a 5- μ m guard zone as described elsewhere (Zhao et al., 2003). DG volume and cell density determinations were performed as described (Zhao et al., 2003). Phenotype analysis of BrdU⁺ cells was performed as described previously (Zhao et al., 2003). Briefly, 50 BrdU⁺ cells in the DG were randomly selected and their phenotypes (double labeling with either NeuN, S100 β , or neither) were determined using a Zeiss LSM510 laser scanning confocal microscope. The data were analyzed using a Student's t-test (Graphpad software, www.graphpad.com).

Quantification of mature, immature, and “transitioning neurons” in the DG

This procedure was performed based on the published method (Brown et al., 2003). Briefly, 40- μ m thick coronal tissue sections containing hippocampus were stained with antibodies against DCX (1:1000, goat, Santa Cruz), NeuN, and DAPI. The immunofluorescence signals were captured using a spinning disk confocal microscope (Nikon Eclipse TE2000-U, 40x oil, 1.2 NA). Quantification was done by a person who was blind to the genotypes of the mice. The numbers of NeuN⁻/Dcx⁺, NeuN⁺/DCX⁺, NeuN⁺/DCX⁻, and cells were quantified by examining z-stacks taken at 1- μ m intervals using MetaMorph imaging software (Molecular Devices Inc., Sunnyvale, CA). A total of 5 WT and 6 KO mice for the 4-week time point and 8 WT and 5 KO mice for the 8-week time point were used. Three z-stacks were taken from each animal and 6 image planes per optical stack were used for quantification.

Immunohistological analyses and quantification of synaptophysin

Staining and analyses of synaptophysin immunoreactivity were performed according to published method (Li et al., 2002). Briefly, 40- μ m thick brain sections were incubated in primary antibody against synaptophysin (rabbit, 1:100; Zymed, San Francisco, CA), followed by biotinylated secondary antibody (donkey anti rabbit IgG; 1:250; Jackson ImmunoResearch), then incubated in ABC reagent (VECTASTAIN ABC Kit, Vector Laboratories) and detected by diaminobenzidine (DAB Substrate Kit, Vector Laboratories, Burlingame, CA). The sections were then thoroughly washed, mounted, air dried, and coverslipped with Permount (Biomedica Corp., Foster City, CA). Sections incubated with normal rabbit IgG instead of a primary antibody (Sigma-Aldrich) were used as negative controls. Optical density analysis of synaptophysin staining was performed by placing 10 circles in each region using Image-J

software, as described elsewhere (Li et al., 2002). To categorize the clustered staining pattern, 63X (Zeiss Axioscope, NA = 1.4) images of brain sections were used. Images were captured at 1300 pixels x 1030 pixels using Slidebook software (Intelligent Imaging Innovations, Denver, CO). Using Image-J (NIH) image analysis software, a threshold value was determined for positive staining and remained the same throughout the data analysis. Large clusters were determined to be clusters greater than 300 pixels² and were quantified using the “Analyze Particles” function of Image-J. Sections with 0-5 “large clusters” in the molecular layer of the hippocampus were placed into category 1, and sections with greater than 5 “large clusters” were placed into category 2. The experimenter was blind to the genotypes of the sections. The number of large clusters in each category was counted for each genotype and age group and the data were used to create Figure 3e.

Retroviral grafting

Production of CAG-eGFP retrovirus and in vivo grafting into the DG of mice were performed as described elsewhere (Zhao et al., 2006). Briefly, CAG-eGFP plasmid was co-transfected with packaging plasmids pCMV-gag-pol and pCMV-Vsvg into HEK293T cells and the medium containing virus was collected, filtered, and concentrated using ultracentrifugation. For in vivo grafting, 4-week-old mice were anesthetized with isoflurane and virus (1.5 μ l with titer greater than 5×10^5 / μ l) was injected stereotactically into the DG using the following coordinates relative to bregma: anteroposterior, $-(1/2) \times d$ mm; lateral, -1.8 mm (if $d > 1.6$) or -1.7 mm; ventral, -1.9 mm (from dura). Four weeks after injection, mice were deeply anesthetized with pentobarbital and perfused with saline followed by 4% PFA.

Immunohistochemistry and dendritic spine density analyses

1-in-3 floating brain sections containing eGFP⁺ cells (120 μ m apart, approximately 8 brain sections) were used for immunohistological staining using a protocol described elsewhere (Zhao et al., 2003). The primary antibody used was rabbit anti-GFP (Invitrogen, Eugene, OR). Briefly, for spine quantification, a minimum of 12-15 images of dendritic fragments were taken at 25-100 μ m from the cell body of each eGFP⁺ neuron using a confocal microscope with an oil immersion objective (100x; NA = 1.3; Zeiss). Z-stacks at 1 μ m intervals were taken and merged for a maximum intensity projection. For quantification of dendritic spines, protrusions were counted along 10 μ m long dendrite segments measured using Image-J software (NIH Image). The “dendritic spine density” result was calculated as number of spines per 10 μ m length of dendrite. A minimum of 40 dendritic fragments (10- μ m each) from a minimum of 4 eGFP⁺ neurons were quantified from each animal. At least 3 animals from each genotype were analyzed and the final results were compared using a Student's t-test. The apposition of immunostained presynaptic boutons with eGFP-expressing postsynaptic spines was determined according to a published method (Belichenko et al., 2004). Briefly, the presynaptic terminal (synaptophysin⁺) and postsynaptic spine (eGFP⁺) were defined as apposed when there was an overlap between pre- and postsynaptic elements, or when these elements were separated by no more than one pixel (0.1 μ m).

Laser Capture Microdissection (LCM) and gene expression analyses

LCM, amplification by in vitro transcription, and probe labeling were performed using a highly reproducible protocol that has been adapted and optimized to analyze gene expression profiles using as little as 5ng total RNA (Phillips and Eberwine, 1996) (Dr. FH Gage, unpublished). Briefly, 4 KO and 4 WT mice (8 weeks of age) were used. Brains were rapidly removed from the cranium and flash-frozen in OCT mounting medium (TissueTek, Sakura Finetek, Torrance, CA) in a dry ice-isopentane slurry and stored at -80°C . The day before LCM, 12 μ m sections of brain were generated, stained with cresyl violet, and dehydrated. The granule cell layers of DG were captured using an Arcturus PixCelIII LCM microscope (Arcturus Bioscience Inc.,

Mountain View, CA). An example is shown in Figure 5. LCM was performed at a power level between 25 and 45 mV, between 2 and 20 ms duration, and at a median spot size setting. A minimum of 30 brain sections (less than half of the sections generated from each brain) were used to capture sufficient DG neurons for this study. Captured tissue was dissolved in cell lysis buffer in the MicroRNA kit (Stratagene, San Diego, CA) for 2 to 5 minutes immediately after capturing. Total RNA was isolated from cell lysate using a MicroRNA kit (Stratagene), and approximately 20 ng total RNA was obtained. Half of the sample was used to quantify RNA using a Ribo Green kit (Invitrogen). Finally, 10 ng total RNA was amplified using 3 rounds of a MessageAmp kit (Ambion, Austin, TX). During the last round, RNA was labeled with biotin (Enzo kit, Affymetrix, Santa Clara, CA), purified, and quantified. Biotin-labeled cRNA (30 µg) was fragmented and hybridized to Affymetrix U430 arrays (Affymetrix). Analysis of microarray data was performed using three distinct software programs as described in our previous publication (Barkho et al., 2006). Briefly, the data were pre-processed using the Affymetrix Microarray Analysis Suite (MAS) 5.0 and subsequently analyzed using dChip (Li and Wong, 2001), Drop Method (Aimone and Gage, 2004), and the algorithms of RMA (Tusher et al., 2001; Irizarry et al., 2003). The combination of these methods has been shown to reduce both false positives and false negatives (Aimone et al., 2004; Barkho et al., 2006). Specifically, for dChip, we selected genes that passed with 90% confidence and had a fold change greater than 1.2. For the Drop method (the PM-only, and PM-MM are considered as one method), we selected genes that passed with a confidence of 70%. For RMA, a fold change had to meet the criteria of being greater than 1.2 and have a false discovery rate (FDR) less than 30%. The FDR value used for RMA was based on the Significance for Analysis of Microarrays software (SAM) (Tusher et al., 2001). Only genes identified by all three software programs were included as differentially expressed genes and are listed in Table 1.

Real time quantitative PCR

Real time quantitative PCR was performed as described (Zhao et al., 2001)(Barkho et al., 2006). Briefly, the cDNA was synthesized using MessageAmp kit, (Ambion) for LCM samples. PCR primers were designed using PrimerExpress software (Applied Biosystems, Foster City, CA) and ordered from IDT Inc. (CA). The primer sets were first evaluated by standard PCR to determine that single PCR products of the predicted size were generated. A typical Real Time PCR reaction mix contained 1X SYBR Green Master Mix (Applied Biosystems), 100 nM of each oligonucleotide primer and 10 ng cDNA in a total volume of 25 µl. The reaction was carried out in an ABI 7700 System (Applied Biosystems). Each condition was acquired in at least triplicate, and data analysis was performed according to the protocol provided by Applied Biosystems. Standard curves were generated using a pre-made pool of mouse brain and spinal cord total RNA. The amount of mRNA for tested genes was calculated according to the standard curve for that particular primer set. Finally, the relative amount of the tested message was normalized to the level of an internal control message, hypoxanthine phosphoribosyl transferase (HPRT).

RESULTS

Early postnatal neurogenesis appears normal in Mecp2-deficient mice

We have previously found that mice deficient for Mbd1, a Mecp2-related protein, exhibited reduced adult hippocampal neurogenesis both in vivo and in vitro (Zhao et al., 2003). Because RTT manifests at 6-18 months of age in patients, well after primary neurogenesis, we asked whether a lack of functional Mecp2 causes deficits in postnatal neurogenesis that might be linked to neurological symptoms comparable to those seen in RTT patients. We therefore compared neural stem/progenitor cells (NSCs) isolated from 6-week-old male KO and wild type (WT) mice. At this age, the majority of KO mice have developed characteristic neurological signs of disease as previously reported (Chen et al., 2001; Guy et al., 2001). Using

bromodeoxyuridine (BrdU) incorporation as a measure of proliferation index, we found that KO NSCs proliferated at a rate that was indistinguishable from WT control cells (Fig 1A and 1B, $p=0.41$). Moreover, KO and WT cultured NSCs also differentiated into similar numbers of neurons (Fig 1C and 1D, $p=0.43$) and astrocytes (data not shown), indicating that there was no marked impairment in the proliferation or differentiation potential of *Mecp2*-deficient NSCs in culture. To examine postnatal neurogenesis *in vivo*, we assessed proliferation, survival, and differentiation of NSCs in the hippocampus of young mice. In the rodent hippocampus, granule cells of the DG develop postnatally, becoming morphologically mature at about 4 weeks of age, which corresponds roughly to the second year in humans (Seress and Mrzljak, 1992; Seress et al., 2001), when RTT symptoms first become apparent. Newborn cells were distinguished by incorporation of BrdU administered through intraperitoneal injections into either 4 week-old juvenile or 8-week-old young adult mice. Quantitative histological analysis at one day after the last BrdU injection showed no difference between KO and WT mice either at 4 or 8 weeks of age (Fig 1E-G). This finding suggested that, as found in cultured NSCs, the NSCs in KO mice proliferate normally in the DG of developing and mature mice. In a separate group of mice, long-term survival and differentiation of BrdU-labeled cells were examined by analyzing labeled cells 4 weeks after BrdU injections. We found that the numbers of BrdU-labeled cells that survived from 4 weeks to 8 weeks were also similar in both KO and WT mice (Fig 1H, $p=0.13$). Moreover, the percentage of BrdU-labeled cells co-labeled for either the neuronal marker NeuN (Fig 1I) or astrocyte marker GFAP (data not shown) did not differ significantly between KO and WT mice. Furthermore, neither the cell density nor the volume of the DG of KO mice was significantly different from WT mice (data not shown). Finally, to investigate whether there was a difference in the population of transient migrating neuroblasts or immature neurons, we quantified the number of cells stained with doublecortin (DCX), a microtubule associated protein (Brown et al., 2003) Francis et al., 1999; Gleeson et al., 1999; Magavi et al., 2000). Again, we found no difference between the number of DCX-positive cells in the DG of KO and WT mice (Fig 1J-S). Taken together, these *in vitro* and *in vivo* results strongly suggest that the lack of *Mecp2* does not impair the proliferation, survival, or differentiation of neural progenitors during this early stage of postnatal hippocampal neurogenesis.

Impaired maturation of *Mecp2*-deficient neurons

Despite the overall similar numbers of immature neurons in the DG of KO and WT mice, we noticed clear abnormalities in the neurite outgrowth from *Mecp2*-deficient immature neurons (Fig 1R and S). In the DG of WT mice, the cell bodies of the DCX⁺ neurons were typically located in the subgranular zone (SGZ) of the DG adjacent to the hilar region, with their processes perpendicularly extended through the granule cell layer (GCL) and exited into the opposite molecular layer (Fig 1N-P, arrowhead) (Brown et al., 2003; Rao and Shetty, 2004; Rao et al., 2005). In contrast, the processes of most DCX⁺ neurons in the DG of *Mecp2* KO mice were found to traverse nearly parallel along the hilar boundary of the DG (Fig 1Q-S, arrowhead). This morphological difference might reflect impaired maturation because since horizontal and short processes are characteristic features for new neurons at early stage of differentiation (Esposito et al., 2005; Ge et al., 2006; Zhao et al., 2006). Because the majority of the DG granule neurons are generated during postnatal development, we exploited the possibility that this deficit was widespread in the developing DG. We therefore compared the ratio of NeuN⁺ mature and DCX⁺ immature neurons in the DG of *Mecp2* KO and WT mice at two different ages: when the DG had just past the peak of primary cell genesis (4 weeks of age) and when DG had reached the adult level of maturity (8 weeks of age) (Fig 2A-G) (Mullen et al., 1992). Furthermore, we quantified the number of cells positive for both NeuN and DCX to distinguish the cells that were transitioning from an immature to a mature phenotype and to provide an additional index of this developmental maturation process (Fig 2E-G). Quantitative analysis of confocal images was used to determine the percentage of neurons in each maturation

stage (Fig 2A-G, DCX⁺/NeuN⁻; DCX⁺/NeuN⁺; DCX⁻/NeuN⁺) in both the KO and WT DG. The results summarized in Figure 2H show that whereas neither 4- nor 8-week-old *Mecp2* KO mice displayed significant differences in the percentage of mature or immature neurons compared to WT mice, the DG of 8-week-old *Mecp2* KO mice had a significantly higher percentage of “transitioning neurons” ($p < 0.001$). In fact, comparison of the number of “transitioning neurons” (DCX⁺/NeuN⁺) among total neurons showed that maturation from juvenile (4 weeks) to young adult (8 weeks) was accompanied by a 75 % decrease in the percentage of “transitioning neurons” in normal WT brain but only a 44% reduction in the KO brains (Fig 2I, $p < 0.0001$). In addition, comparison of the number of “transitioning neurons” (DCX⁺/NeuN⁺) and total immature neurons (both DCX⁺/NeuN⁻ and DCX⁺/NeuN⁺) showed that maturation from a juvenile (4 weeks) stage to a young adult (8 weeks) stage was accompanied, in the normal WT brain, by a 28.5% decrease in the percentage of “transitioning neurons” among the total population of DCX⁺ neurons (Fig 2J). However, the age-dependent reduction of this subpopulation of double positive “transitioning neurons” among total immature neurons did not occur in *Mecp2*-deficient mutants; but rather there was a 26.9% increase in the percentage of these neurons during this developmental time period (Fig 2J, $p < 0.0001$). This finding may indicate that the deficiency in *Mecp2* may have led to DCX⁺/NeuN⁺ double positive “transitioning neurons” being held up and failing to differentiate into more mature DCX⁻/NeuN⁺ neurons.

Impaired expression of developmentally regulated presynaptic proteins in *Mecp2*-deficient hippocampus

Because synaptogenesis is a crucial step for the maturation and integration of newborn neurons into the preexisting circuitry of the hippocampus, we investigated whether KO mice have deficits in synapse formation. For this purpose, we used an antibody against synaptophysin, a synaptic vesicle protein whose expression is known to reflect the distribution and density of presynaptic terminals (Li et al., 2002). We focused on the molecular layer of the hippocampus, where DG granule neuron dendrites receive input from perforant path axons from the entorhinal cortex (Henze et al., 2000). Overall, no consistent difference was found in optical density measurements of synaptophysin immunostaining between 4- and 8-week-old WT and KO mice suggesting that the level of expression, and thus the number of nerve terminals, was not affected by the lack of *Mecp2*. However, we did observe two patterns of synaptophysin immunoreactivity in both WT and KO mice: highly distributed small positive spots (< 300 pixels², Fig 3C, arrowheads), and “large clusters” (> 300 pixels², Fig 3C and 3D, arrows) that appeared to vary in density in different brain regions. To quantify the distribution of these large clusters and determine whether they might differentiate between the synaptic density of WT and KO hippocampus, we analyzed the number of “large clusters” (determined by Image-J quantitative software) found in the molecular layer, where dendrites of granule neurons form synapses. We found no significant difference in the number of large clusters in either 4-week-old or 8-week-old KO mice compared to their age-matched WT littermates (Fig 3E). However, when WT mice mature from 4 to 8 week of age, there was an 82.6% decrease in the number of large synaptic clusters in the molecular layer of the hippocampus (4-week, 18.29 ± 4.74 , $n = 14$ mice; 8-week, 3.19 ± 1.10 , $n = 13$ mice; $P < 0.01$), but such an age-dependent change was absent in KO mice ($P = 0.48$), suggesting a failure in dispersing these large clusters into a more uniform distribution of presynaptic terminals in the absence of functional *Mecp2*.

Altered dendritic spine distribution in *Mecp2*-deficient mutant neurons

As neurons mature the density of dendritic spines increases (Ge et al., 2006; Zhao et al., 2006). Consequently, reduced dendritic density is a common characteristic of the abnormal synaptic development seen in a variety of neurological disorders (Fiala et al., 2002). To clarify the current discrepancy in the literature and determine whether *Mecp2* mutations affect dendritic spine development, we decided to analyze the morphology of individual *Mecp2*-

deficient neurons in vivo. Because the persistent, albeit low level, of postnatal neurogenesis in the DG allowed us to trace the maturation of single new neurons, we performed detailed morphological analyses to investigate the maturation of these newly generated neurons in the *Mecp2*-deficient mice. Recombinant retroviruses, which are only capable of infecting dividing cells, have been previously used to label and follow the differentiation of NSCs in postnatal DG (van Praag et al., 2002; Ge et al., 2006; Zhao et al., 2006). We therefore injected recombinant retrovirus expressing enhanced green fluorescence protein (eGFP) under a chicken actin promoter (CAG-eGFP) (Zhao et al., 2006) into 4-week-old KO and WT mice and analyzed the morphology of new neurons after 4 weeks, a time when labeled new neurons would be expected to develop the dendritic morphology of fully mature neurons (van Praag et al., 2002; Ge et al., 2006; Zhao et al., 2006) (Fig 4A). As shown in Figure 4B-E, whereas most of the eGFP⁺ neurons expressed the mature neuronal marker NeuN, a few of them also expressed DCX, indicating NSCs that had not yet reached a fully matured state. To quantify the density of synapses, we counted the number of spines within each 10- μ m segment of dendrites imaged by high-resolution confocal microscopy. Quantitative analyses indicated that the spine density of eGFP⁺ neurons in the DG of *Mecp2* KO mice was significantly reduced compared to WT mice (Fig 4J, WT= 13.46 ± 0.31 , KO= 11.41 ± 0.11 ; $P < 0.005$). Because the formation of functional synapses requires apposition of presynaptic terminals and postsynaptic spines, we asked whether the postsynaptic spines of eGFP⁺ neurons in the DG of KO mice were adjacent to synaptophysin-positive presynaptic terminals. As shown in Figure 4K and L, similar percentages of eGFP⁺ spines in WT and *Mecp2* KO mice were apposed to synaptophysin-positive presynaptic terminals. These data indicate that, at the single neuron level, while newly matured neurons in *Mecp2*-deficient mice are able to form synaptic contacts, the number of these synapses is greatly diminished.

Altered gene expression of synaptic proteins in *Mecp2*-deficient DG granule neurons

To investigate the molecular mechanisms that might underlie the deficits in dendritic spine development in the DG of *Mecp2*-deficient mice, we investigated differential gene expression in the granule cells of the DG isolated by LCM from 8-week-old *Mecp2* KO and WT mice (Fig 5A-C). Relative levels of gene expression were determined by hybridization to mouse U430 gene array (Affymetrix) and the data were analyzed using three independent software packages based on distinct algorithms (see Materials and Methods). This bioinformatic strategy of using a combination of different analyses reduces the selection of both false positives and negatives as candidate genes (Barkho et al., 2006). Only genes that were identified by all three algorithms, as differentially expressed genes, were listed in Table 1. We found that the expression of 13 genes was significantly changed in the *Mecp2* KO DG granule neurons compared to WT controls. Consistent with the function of *Mecp2* as a transcription repressor, 12 of the 13 differentially expressed genes displayed increased expression in the KO neurons. The *Mecp2* gene was not on the list because a truncated mRNA corresponding to the 3' coding region of *Mecp2*, where Affymetrix probe sets hybridize, was expressed in KO neurons. Among the identified differentially expressed genes, Prefoldin 5 is involved in actin and tubulin folding and cytoskeleton formation (Hartl and Hayer-Hartl, 2002; Nolasco et al., 2005), and Syndecan 2 has been shown to be critical for synaptogenesis (Ethell and Yamaguchi, 1999; Ethell et al., 2001). We have confirmed that the expression levels of both Syndecan 2 and Prefoldin 5 mRNA were higher in KO neurons using Real time PCR analyses (Fig 5D-E). Alteration of either of these proteins could potentially affect dendritic development and neuronal morphology.

Discussion

With the identification of *MECP2* as the gene responsible for RTT, it becomes critically important to understand the role of *MECP2* in postnatal neural development. In this study, we

provide strong evidence that while the lack of *Mecp2* does not affect the production of NSCs, it does significantly impair subsequent steps in the maturation of neurons. First, we determined that the expression of specific markers defining the transition from immature to mature neurons was delayed in *Mecp2*-deficient NSCs. Second, we showed that the age-dependent shift in the expression pattern of synaptophysin in the molecular layer of the hippocampus did not occur in *Mecp2* KO brains. Third, using single neuronal labeling, we found that there were striking defects in the development of dendritic spines and synaptogenesis in DG of KO mice. Finally, the expression levels of several genes that are likely to be important for synaptogenesis, such as Prefoldin 5 and Syndecan 2, were found to be significantly altered in the DG of the hippocampus in *Mecp2* KO mice. This finding points toward a potential mechanism for the epigenetic effects mediated by ablating *Mecp2* control of gene expression.

The important role of *Mecp2* as an epigenetic regulator at later stages of neural differentiation, in contrast to that reported for *Xenopus* (Stancheva, 2003; Kishi and Macklis, 2004), is consistent with the normal development of the brain until birth both for RTT patients and *Mecp2* KO mice. While our results demonstrate clearly that the initial generation of new neurons in the DG was not altered, later steps in synapse formation were significantly perturbed. Although further efforts to examine the effects on early stages of neurogenesis in the SVZ could be useful, our studies, together with those of Kishi and Macklis (Kishi and Macklis, 2004), strongly support the view that *MeCP2* is not critical for the production of new, immature neurons in either the embryonic or postnatal mammalian brains.

The progressive acquisition of protein markers followed by the loss of their expression provides a well-defined system to measure the development and differentiation of newborn neural progenitor cells. During normal development, DCX is expressed transiently in immature neurons, and co-expression of DCX and NeuN (DCX⁺NeuN⁺) marks the end of the early immature neuronal stage. Subsequently, the expression of NeuN, but not DCX, is the hallmark of matured DG neurons (Brown et al., 2003). It seems likely that the age-dependent decrease in the DCX⁺NeuN⁻ immature population and increase in DCX⁻NeuN⁺ mature population that we observed in both control WT and *Mecp2* KO mice are due to both the decrease in cell proliferation and the increase in total number of granule neurons that occurs from 4 to 8 weeks of age. DCX⁺NeuN⁺ “transitioning neurons” can be detected as early as 12 days post-differentiation in the DG, and reach their highest level about three days later (Brown et al., 2003). The lower percentage of “transitioning neurons” observed in 4-week-old *Mecp2* KO mice compared to WT mice could result from delayed maturation of DCX⁺NeuN⁻ immature neurons into the “transitioning neuron” stage. [0] The WT adult DG had a lower percentage of “transitioning neurons” than young mice, indicating that the DG is more mature; however, this reduction is much attenuated in adult KO mice, suggesting that more immature neurons either become “stuck” or stay longer in the “transitioning neuron” stage and thus their transition into a mature stage is delayed. This hypothesis is consistent with previous reports indicating a greater percentage of immature neurons in the olfactory epithelium of *Mecp2* KO mice (Matarazzo et al., 2004), and with histone H3 lysine-9 acetylation and methylation patterns seen in *Mecp2*-deficient RTT human brains that correspond to an arrest at the transitioning stage of neuronal maturation (Kawasaki et al., 2005). Future studies will be needed to define how *Mecp2* regulates the expression of genes critical for this transitioning stage.

Synaptophysin staining has been widely used to indicate the numbers and distribution of neuronal synapses in mammalian brains (Li et al., 2002). However, while most studies have focused on determining the level of expression, we observed two distinct staining patterns (small positive spots and large clusters) in the developing mouse brain. These synaptophysin patterns change in a regulated manner in WT mice, with young brains exhibiting more large clusters than adult brain. The presence of large clusters could, therefore, correlate with immature stages of neuronal plasticity, when synaptophysin may not be well distributed to

synaptic sites. If this is the case, the persistence of clustered synaptophysin staining in adult *Mecp2* KO mice would be consistent with the notion that newborn neurons are “stalled” from further maturation in the absence of *Mecp2*. This stalling might be due to deficits in the neuronal transport mechanism or abnormalities in cytoskeleton structure in the absence of functional *Mecp2* protein. In fact, we have found that the expression levels of several cytoskeleton-related factors are altered in *Mecp2* mutant DG neurons (Table 1). Interestingly, we also observed that clustered synaptophysin staining was retained in the stratum lucidum of the hippocampus, which contains the terminals of CA3 neurons (data not shown). Further analyses of other brain regions should shed light on whether the lack of *Mecp2* as an epigenetic factor leads to a general impairment of synaptic development, as reflected by synaptophysin staining, and results in global deficits in neuronal maturation throughout the brain.

Dendritic spine density is a morphological indicator of neuronal maturation in hippocampal granule cells, and reduced spine density is correlated with impaired maturation of DG neurons (Zhao et al., 2006). Abnormalities in the morphology and density of dendritic spines have been found both in RTT patients and other neural developmental disorders, such as Down's syndrome, Fragile X syndrome, and Autism (Fiala et al., 2002), however whether *Mecp2* mutations affect dendritic development in animal models is currently not clear. To clarify the current controversy between human and mouse studies (Belichenko et al., 1997; Kishi and Macklis, 2004; Moretti et al., 2006), we used a retrovirus to mark single newborn neurons on the DG. Our findings of the reduced dendritic spine density in newly generated neurons in the DG are thus consistent with similar findings in human pathologies, suggesting that this abnormal dendritic development may be a common point of vulnerability that leads to the neurological deficits caused by these genetic disorders. Further experiments analyzing the dendritic morphology of eGFP⁺ neurons at earlier time points will help to define how *Mecp2* regulates the development of dendritic spines. The apposition of postsynaptic and presynaptic terminal components is a prerequisite for a functional synapse. Although we did not observe a significant difference in the percentage eGFP⁺ spines apposed to presynaptic terminals distinguished by synaptophysin, the evaluation of impairments in the number of functional synapses and potential abnormalities in synaptic transmission will require future electrophysiological analyses. Remarkably, the altered morphology seen in DCX⁺ immature neurons, including abnormal orientation of the processes (Fig. 1), was not found in GFP⁺ new neurons at 4-week post-labeling. This phenomenon might be due to the preferential death of morphologically altered immature neurons or, alternatively, the abnormal orientation might correspond to a characteristic of an immature stage of neuronal maturation within the first week of differentiation. New neurons display horizontal processes, or short processes similar to those seen in *Mecp2* KO brains and develop vertical processes during later stages of development (Esposito et al., 2005; Ge et al., 2006; Zhao et al., 2006). Further analyses of viral-labeled cells at early stages of neuronal differentiation will confirm this hypothesis.

Despite extensive effort, only a few genes, including Brain-Derived Neurotrophic Factor (Chen et al., 2003; Martinowich et al., 2003), DLX5 and 6 (Horike et al., 2005), and inhibitor of differentiation (ID1, 2, 3, and 4) genes (Peddada et al., 2006) have been shown to be regulated by *Mecp2*. Therefore, the identification of additional genes that are either directly or indirectly regulated by *Mecp2* in neurons is a critical step forward in delineating what role this epigenetic regulator of gene expression plays in the development of the brain, as well as other tissues. Several of the candidate genes we identified are involved in cytoskeleton structure formation, such as Prefoldin 5, Arpc3, Syndecan 2, etc (Table 1). Prefoldin 5 is involved in actin and tubulin folding. Mutations of prefoldins result in an abnormal cytoskeleton (Hartl and Hayer-Hartl, 2002). Syndecan 2 is a transmembrane heparin sulfate proteoglycoprotein that binds extracellular matrix components and growth factors and is expressed at the mature dendritic spines of hippocampal neurons. Like that of *Mecp2*, the expression of Syndecan 2 coincides with dendritic spine maturation (Ethell and Yamaguchi, 1999). Exogenous Syndecan 2

expression induces increased dendritic spine formation, whereas blocking Syndecan 2 phosphorylation by the EphB2 receptor results in reduced spine density (Ethell et al., 2001). Further mechanistic analyses will determine whether these genes are targets of *Mecp2* and functional consequences their altered expression in *Mecp2* KO neurons

In summary, our data indicate that *Mecp2* is not critical for the early stages of neurogenesis, but is important for neuronal maturation in the postnatal brain. The fact that adult *Mecp2* KO brains are more similar to immature WT brains than to mature WT brains suggests that *Mecp2* is critical for regulating the transition of neurons from immature to mature stages.

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Figure 1. *Mecp2* KO mice exhibit a normal early stage of postnatal neurogenesis even though immature neurons have an abnormal morphology

(A) For cell proliferation analyses, NSCs isolated from 6-week-old KO and WT brains were cultured in the presence of BrdU to label dividing cells. BrdU-labeled cells were detected by immunocytochemistry (red, BrdU; blue: DAPI nuclear staining; scale bar=10 μ m). (B) Quantitative analyses of BrdU-labeled cells indicated no significant difference in cell proliferation between KO and WT NSCs in vitro ($p=0.41$, $n=3$, t-test). (C) *Mecp2* KO NSCs can differentiate into neurons (TuJ1⁺, red) and astrocytes (S100b⁺, green) (blue: DAPI nuclear staining); scale bar=10 μ m. (D) There was no significant difference in neuronal differentiation between KO and WT NSCs in vitro ($p=0.43$, $n=3$ t-test). (E) Example of a brain section stained with antibodies to NeuN (green) and BrdU (red) for in vivo neurogenesis analyses. Neither 4-week-old (F) nor 8-week-old (G) KO mice exhibited significant deficits in the number of BrdU⁺ cells at either 1 day post-BrdU injection (F and G) or 4 weeks post-BrdU injection (H). At 4 weeks post-labeling, BrdU⁺ KO cells differentiated into similar numbers of new neurons (I) compared to WT mice. Low magnification (J-M, scale bar=100 μ m) and high magnification (N-S, scale bar=10 μ m) images of DG stained with antibodies against *Mecp2* (red nuclear staining) and DCX (green). Note that *Mecp2* staining is absent in KO brains (L, M, Q-S). (N-

S) DCX⁺ immature neurons in KO brains have disorganized morphologies compared to those in WT brains (arrowhead in P), with abnormal orientation of the processes of many DCX⁺ neurons (arrowhead in S). The dotted lines in N-S indicate the boundary of the granule cell layer. m, molecular layer, g, granule cell layer, and h, hilar region.

Figure 2. Immature neurons in the DG of *Mecp2* KO mice have delayed transitioning to mature stage

(A-D) Confocal images showing DG of the hippocampus labeled with antibodies against NeuN (A, green, mature neurons), DCX (B, red, immature neurons), and DAPI (C, blue, nuclear dye). Scale bars=100 μ m. (D) Merged image of A-C. (E-G) Higher magnification images of granule neurons and examples of neurons that are NeuN⁺DCX⁻ (asterisk) and NeuN⁺DCX⁺ (arrowhead). Scale bars=10 μ m. (H) Quantitative analyses indicate the percentage of mature neurons (NeuN⁺DCX⁻, white bar), immature neurons (DCX⁺NeuN⁻, light gray) and “transitioning neurons” (NeuN⁺DCX⁺, dark gray) in 4-week-old and 8-week-old mice. (I) Age-dependent changes in the proportion of “transitioning neurons” over total neurons are

significantly different between KO and WT mice ($p<0.0001$), (J) The percentage of “transitioning neurons” among total DCX⁺ neurons are also significantly different between WT and KO mice at both 4 and 8-weeks-old. While WT mice exhibited an age-dependent decrease in the proportion of “transitioning neurons” over total DCX⁺ neurons, KO mice displayed an increase ($p<0.0001$), suggesting more neurons are stalled at the transitioning stage in the KO brains.

Figure 3. *Mecp2* KO mice have altered presynaptic protein expression pattern

(A-C) Digitized bright-field micrographs show synaptophysin immunoreactivity in the adult and young mice. Scale bar=100 μ m. (A) The box indicates the region that is enlarged in C and D. (B) Sections were incubated with normal rabbit IgG, instead of synaptophysin antibody, as a negative control. (C) Example of a brain section with small synaptophysin positive spots (arrowheads) and two large clusters (arrows). scale bar=10 μ m. (D) The output of particle analysis of (C) produced by Image-J showing the two large clusters (arrows). (E) Number of large clusters in 4- and 8-week-old WT mice (** $P < 0.01$) compared to 4- and 8-week-old KO mice. Note that WT animals showed a clear age-dependent reduction in the density of large synapse clusters, whereas KO failed to show this developmental change.

Figure 4. Newly matured neurons in *Mecp2* KO hippocampus have reduced dendritic spine density and abnormal distribution

(A) Schematic diagram demonstrating stereotaxic grafting of CAG-eGFP retrovirus into the DG of 4-week-old KO and WT mice to label dividing neuroprogenitors in the germinal zone of the DG. At 4 weeks post grafting, many eGFP⁺ cells had differentiated into NeuN⁺ (B, C, D, arrowheads) and/or DCX⁺ (E) new neurons. (F, G) High resolution image of dendrites of eGFP⁺ neurons were used to quantify the density of dendritic spines (number of spines/10 μ m dendrites) to generate the data in (H-J). (G) High magnification view of the box in F. Scale bars in F and G=10 μ m. (J) New neurons in KO brains had reduced dendritic spine density ($P<0.005$, $n=3$ t-test). (H, I) Frequency distribution data indicate higher variation in spine

density in KO mice (I) than in WT mice (H), indicating an uneven distribution of spine density. (K) Z-stack confocal image showing apposition of presynaptic terminal marker synaptophysin (red, arrow) with eGFP⁺ spines of new neurons. (L) Quantitative analyses indicating that similar percentages of eGFP⁺ spines were apposed to presynaptic terminals in both WT and *Mecp2* KO mice (p=0.33, n=3, t-test)

Figure 5. Gene expression analyses of LCM-isolated DG neurons indicate altered expression of genes related to synaptogenesis

(A-C) Bright field images demonstrate the process of isolating DG granule neurons from cresyl violet-stained brain sections using LCM. (C) Isolated neurons (dark) were melted into the cap during the LCM procedure and used for RNA isolation. (D-E) Real time PCR analyses confirmed the differential expression of two of the candidate genes, Syndecan 2 and Prefoldin 5. The fold changes determined by real time PCR are consistent with those determined by microarray analyses ($p < 0.05$; $n = 3$ experiments with 4 mice/genotype).

Table1

Genes that are expressed at different levels in hippocampal granule neurons of *Mecp2* KO mice.

Gene Symbol	Gene Description	dChip Fold Change [*] (Range)	Drop Confidence [#] (Range)	Gene Bank IDs
Genes expressed at higher levels				
Pfdn5	Prefoldin 5	1.61	94.5 - 99.0%	NM_020031.1
1110013I11Rik	RIKEN cDNA 1110013I11 gene	2.58	99.80%	BF608615
Uchl1	Ubiquitin carboxy-terminal hydrolase L1	1.53	83.6 - 90.9%	NM_011670.1
Sfxn3	Sideroflexin 3	1.34	85.7 - 87.6%	NM_053197.1
AL024345	expressed sequence AL024345	1.42	76.2 - 85.7%	AV295157
5031401C21Rik	RIKEN cDNA 5031401C21 gene	1.42	98.0 - 98.4%	AW537061
Osbpl9	Oxysterol binding protein-like 9	1.36	87.6 - 89.3%	AI875733
Zipro1	Zinc finger proliferation 1	1.58	97.5 - 99.2%	AI326272
Arcp3	Actin related protein 2/3 complex, subunit 3	1.49	2.5 - 83.6%	BC013618.1
Sdc2	Syndecan 2	1.49	60.5 - 85.7%	NM_008304.1
1500011H22Rik	RIKEN cDNA 1500011H22 gene	1.37	26.7 - 83.6%	BC019498.1
1110020P15Rik	RIKEN cDNA 1110020P15 gene	1.44	63.9 - 83.6%	BF681728
Genes expressed at lower levels				
Hmgb1	High mobility group box 1	-1.46	83.6 - 87.6%	C78183

Note:

* Reference fold changes were obtained by dChip analysis;

Confidence values were obtained by Drop analysis; the ranges are the maximum and minimum if multiple probe sets were returned.